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SIMPLIFIED HPLC METHOD WITH SPECTROPHOTOMETRIC DETECTION FOR THE ASSAY OF CLOFIBRIC ACID IN RAT PLASMA

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ABSTRACT

A simple and rapid reversed-phase HPLC method for the assay of clofibric acid in rat plasma is presented. After a simultaneous deproteinization-extraction step of a 200 μL plasma sample with a solution of propyl paraben in acetonitrile, the clear extract was injected onto a Microsorb-MV C18 chromatographic column and eluted with 1% acetic acid in acetonitrile-water (45:55) at the rate of 0.8 mL/min. At the detection wavelength of 230 nm, clofibric acid and propyl paraben, the internal standard, eluted at 7.0 min and 5.5 min, respectively. Peak responses were linearly related to concentrations of clofibric acid in the range 1.5-30 μg/mL, with a minimum detectable concentration of analyte of 75 ng on-column. Recoveries of clofibric acid from plasma samples spiked at 1.5-30 μg/mL levels of analyte were >94% (range 94.6-99%). The proposed method was easily applied to the determination of the plasma plasma levels of clofibric acid derived from the oral and intraperitoneal administrations of clofibrate as a liquisolid compact and as the contents of a commercial soft gelatin capsule to rats.

INTRODUCTION

Liquisolid compacts are dry, nonadherent, free-flowing and readily compressible powder formulations of liquid and solid poorly water-soluble drugs, obtained by the simple admixture of

the drug or its solution in nonvolatile, high boiling point solvents with suitable powder excipients (1,2). Interest on liquisolid compacts has been fostered by reports indicating that use of this type of formulation will result in the improvement of the *in vitro* dissolution profile of certain liquid and solid lipophilic drugs (3,4).

As part of a study intended to evaluate the efficiency of liquisolid compacts as a means of systemically delivering lipophilic liquid drugs, this laboratory decided to compare the plasma levels of the antilipidemic agent clofibrate after its oral and intraperitoneal administration as a liquisolid compact formulation and as the liquid contents of a commercial soft gelatin capsule to rats. For this reason, an analytical method was required to determine the temporal changes in ensuing plasma drug levels.

Following its oral administration to humans and animals, clofibrate undergoes rapid and quantitative hydrolysis by intestinal, plasma and tissue esterases (5-9). As a result, analytical methods for clofibrate only measure the active metabolite p-chlorophenoxyisobutyric acid (clofibric acid), and not the parent molecule (9-27). In this regard, the analysis of clofibric acid in biological samples has been accomplished largely through the use of spectrophotometry (9-11), gas chromatography (GC) (12-18) and high-performance liquid chromatography (19-27). In addition to being nonspecific, spectrophotometric methods are found to yield low recovery values after extraction of the sample with a water-immiscible organic solvent (9,10) or to require a preliminary chemical modification of the analyte (5). In contrast, GC methods are specific and accurate, but they usually include a lengthy and often tedious sample extraction and purification (12-14) as well as a derivatization step (12-16,18).

In comparison to GC methods, the use of HPLC approaches circumvents the need for a preliminary sample derivatization and, in addition, offer the advantages of greater simplicity and flexibility of experimental conditions. However, some of the proposed HPLC methods are prone to potential sample losses in the course of the transferring of highly volatile solvents used for extracting clofibric acid from the biological matrix (20,24,27) or of a multistep sample purification procedure (19). In other cases, the method necessitates an ion-pair reagent (27), special detection conditions (26) or a very large volume of sample (20,27).

The purpose of this report is to describe a reversed-phase HPLC method with UV detection that will permit the analysis of clofibric acid in rat plasma in a rapid and straightforward manner, without the need for transfer steps or extraction with a highly volatile solvent, and using a small plasma sample. This method is well suited for studies evaluating the systemic delivery of drugs from solid dosage forms such as liquisolid compact formulations in animal models.

EXPERIMENTAL

Materials and Reagents

Clofibric acid, clofibrate, and propyl paraben were obtained from Sigma Chemical Company, St. Louis, MO, and were used as received. Clofibric acid was also prepared by saponification of clofibrate using previously described conditions (13) and recrystallization from diluted ethanol. Analytical reagent grade glacial acetic acid, and HPLC grade acetonitrile and water were from J.T. Baker Inc., Phillipsburg, NJ. The internal standard solution was prepared by dissolving propyl paraben in acetonitrile to a concentration of 1 μg/mL. This solution was stable indefinitely. A stock solution of clofibric acid was prepared by dissolving this compound in a few drops of ethanol, and diluting to volume with water to a concentration of 120 μg/mL.

Apparatus and Chromatographic Conditions

The chromatographic system consisted of LC-10 pump, LC-90 spectrophotometric detector (Perkin-Elmer), and ChromJet recording integrator (Spectra- Physics). Samples were introduced through a Model 7125 injector fitted with a 50 μL sample loop (Rheodyne).

Separations were performed on a Microsorb-MV C18, 5 μm, 15 cm x 4.6 mm i.d. column (Rainin), protected with an Adsorbosil C18 guard column (Alltech). Elutions were carried out at ambient temperature with 1% acetic acid in acetonitrile-water (45:55), filtered and degassed prior to use. The flow rate was 0.8 mL/min, and the detection wavelength was 230 nm and 0.05 AUFS.

Method Validation

The linearity of the method was investigated by diluting the stock solution of clofibric acid (120 μg/mL) either with water or drug-free rat plasma to concentrations in the range 1.5-30 μg/mL, and putting these solutions through the proposed assay method. A calibration curve was constructed by plotting the ratio of the peak heights of clofibric acid to internal standard against the concentration of clofibric acid added.

Analyte recovery was determined by comparing the peak heights of clofibric to internal standard for standard preparations in plasma against the same preparations in water. Within-day reproducibility was assessed by analyzing a set of samples containing various concentrations of clofibric acid in plasma at six different times. Day-to-day reproducibility was studied by analyzing the same sets of spiked plasma samples over three consecutive days.

Animal Studies

Sprague-Dawley rats, 225-250 g in weight and fasted overnight, were divided into groups of 4 rats each. To one group, a solution of clofibrate liquisolid compact in 50% ethanol

was administered by the oral route using a curved oral-feeding needle; to another group, the same solution was administered intraperitoneally. These experiments were repeated with the contents of a commercial soft gelatin capsule (Atromid-S®) previously diluted with 50% ethanol to the same drug concentration as the liquisolid compact formulation. All treatments were carried out using a 20 mg/kg dose. Blood samples were collected periodically by the orbital sinus technique into polypropylene test tubes that contained a small amount of disodium EDTA. After mixing with the aid of a vortex mixer, the blood samples were centrifuged at 5000 rpm for 10 min to separate the corresponding plasmas, which were immediately transferred with Pasteur pipets to clean test tubes and kept at 4°C pending their analysis.

Assay Method

To a 13 x 100 mm screw-cap culture tube, 200 μL of plasma sample and 200 μL of internal standard solution were added in succession (to samples estimated to contain between 15 and 60 μg/mL of clofibric acid, 25 μL of acetonitrile was also added). After gentle shaking, the mixture was vortexed for 30 seconds and next centrifuged at 5000 rpm for 10 min. An aliquot of the clear supernatant was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Because of its pKa of 3, clofibric acid is expected to be present at the physiological pH of the plasma almost entirely ionized (5). For this reason, this compound is generally eluted using mobile phases composed of various ratios of acenotrile-water or methanol-water and to which either a buffer or an organic acid has been added to lessen tailing and improve resolution from other sample peaks. In the proposed method, acetonitrile and acetic were selected over methanol and buffers to minimize column inlet backpressure. In addition, acetic acid was found more convenient to a buffer since it can be added to the mobile directly, thus simplifying the preparation of the mobile phase. The concentration of acetic acid added was selected after determining its effect on both resolution R (calculated from $2[t_1-t_2]/w_1+w_2$) between the internal standard peak and analyte peak and on peak shapes.

Chromatograms of a rat plasma sample free of clofibric acid and of a plasma sample containing clofibric acid and propyl paraben, the internal standard, are shown in Figures 1(a) and 1(b), respectively. A resolution >2.0 between the two compounds was obtained in < 8 minutes. Plasma components coextracted with the analyte did not interfere with the analysis. Using the recommended detection conditions, the limit of detection of clofibric acid was about 75 ng on column. A greater sensitivity has been achieved at a detection wavelength below 230 nm (22,23). However, the addition of acetic acid to the mobile phase lead to detector overload due to

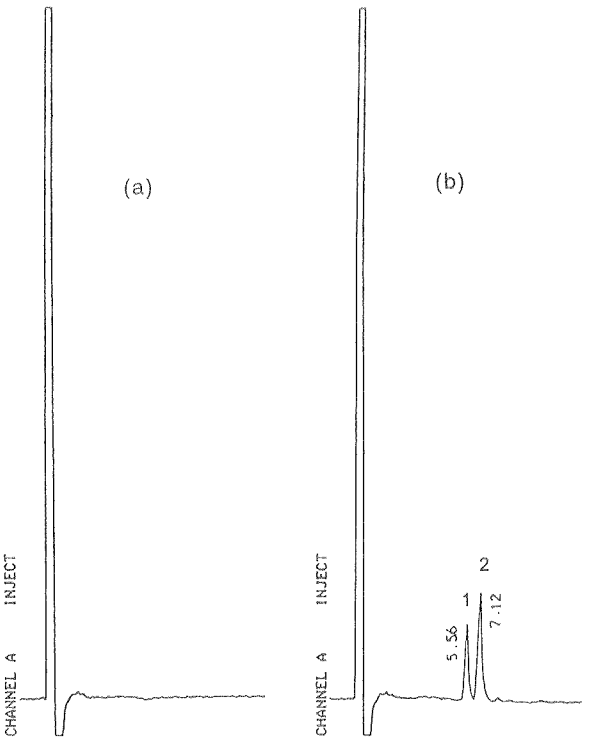

Fig. 1 Typical chromatograms of plasma samples obtained from: (a) a rat before clofibrate administration, and (b) a rat that had received a 20 mg/kg oral dose of clofibrate. Key: 1, propyl paraben, the internal standard; 2, clofibric acid.

significant solvent self-absorption at wavelengths below 230 nm. A way to obviate this problem may be to replace the acetic acid by a less absorbing acid such as phosphoric acid. Furthermore, acetonitrile deproteinization of plasma samples and centrifugation prior to injection of the supernatant has been criticized as giving lower sensitivity than methods employing liquid-liquid extraction (26); but this detail is contingent to the dilution ratio of the sample with deproteinizing solvent, which in the proposed method was kept sufficiently low (i.e., 1:1) so as to permit accurate assay of clofibric acid in rat plasma at concentrations as low as 1.5 μg/mL. Nevertheless, for samples suspected of containing clofibric acid in concentrations ranging from 15-60 μg/mL, it is advisable to increase the volume of the test sample by adding acetonitrile (ca.

Table 1

Data for recovery of clofibric acid from rat plasma by proposed HPLC method

		Recovery	
Amount added, μg/mL	No. of samples	Mean ± SD, %	RSD, %
1.5	6	98.2 ± 0.01	0.01
1.875	6	98.3 ± 0.01	0.01
3.75	6	95.2 ± 0.05	0.05
7.5	6	95.6 ± 0.05	0.05
15.0	6	94.9 ± 0.03	0.03
30.0	6	95.6 ± 0.05	0.05

Table 2

Interday and intraday reproducibility of peak height ratio data for proposed HPLC method[a]

		Intraday		Interday, 3 days	
Concentration, μg/mL	No. of samples	Mean ± SD	RSD, %	Mean ± SD	RSD, %
1.875	3	0.29 ± 0.01	3.45	0.28 ± 0.01	3.35
3.75	3	0.56 ± 0.03	3.35	0.58 ± 0.02	3.80
7.5	3	0.72 ± 0.01	1.39	0.73 ± 0.01	3.39
15.0	3	1.41 ± 0.03	2.13	1.48 ± 0.04	2.37
30.0	3	2.86 ± 0.08	2.80	2.93 ± 0.10	3.41

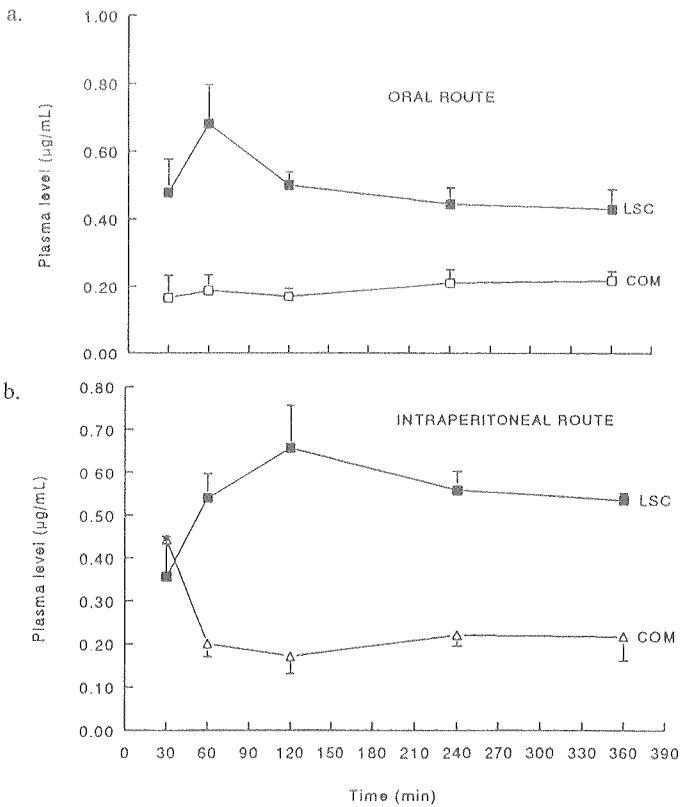

Figure 2. Temporal changes of the plasma levels of clofibric acid in rats receiving either a commercial soft gelatin capsule (COM) or a liquisolid compact (LSC) of clofibrate (20 mg/kg) by the oral and the intraperitoneal routes. Vertical lines represent the SEM for 5 rats.

25 µL) to ensure more quantitative recoveries of analyte. Alternatively, one could decrease the volume of internal standard solution; or evaporate the sample to dryness and reconstitute the residue in a as small as possible volume of mobile phase or of a solvent compatible with the mobile phase (20,22,24).

Results of a recovery study from spiked plasma, performed at levels of clofibric acid between 1.5 and 30 µg/mL are summarized in Table 1. In general recoveries at all levels were better than 94% of the amount added, and the pooled RSD was 0.03%.

The calibration curve was linear from 1.5 to 30 µg/mL (n = 4), with the line equation being $y = 0.17x + 0.03$ ($r = 0.999$), where y is the ratio of the peak height of clofibric acid to

Table 3

Retention times relative to clofibric acid of related arylphenoxyisobutyric acid compounds

Compound	Solvent A[a]	Solvent B[b]
Clofibric acid	1.00	1.00
Bezafibrate	1.03	1.25
Fenofibric acid	2.26	1.58
Gemfibrozil	4.16	2.46
Clofibrate	4.45	2.75
Fenofibrate	10.39	6.67

[a]Solvent A: 1% acetic acid in acetonitrile-water (45:55), 0.8 mL/min; retention time of clofibric acid = 7.52 min.
[b]Solvent B: 1% acetic acid in acetonitrile-water (55:45), 0.8 mL/min; retention time of clofibric acid = 4.10 min.

internal standard, x is the concentration of clofibric acid (μg/mL), and r is the correlation coefficient.

Results of intraday and interday precision studies for various levels of clofibric acid, in terms of mean ± SD and RSD of peak height ratios, are summarized in Table 2.

Figures 2(a) and 2(b) depict the time-related changes in plasma levels of clofibric acid that follow oral and intraperitoneal administrations, respectively, of a clofibrate liquisolid compact and the contents of a commercial soft-gelatin capsule. It is quite evident that the liquisolid compact formulation yields significantly higher plasma drug levels than the commercial product at all experimental intervals. These results parallel those observed *in vitro* and indicating that the release of clofibrate from a liquisolid compact is greater than from a commercial soft gelatin capsule (4).

As shown in Table 3, the recommended experimental conditions will also permit the differentiation of clofibric acid from clofibrate, the parent compound, and from other related aryloxyisobutyric acid compounds. In those instances where the metabolite is the compound present in the plasma, the precursor elutes much later, especially in the case of fenofibrate.

In summary, the proposed HPLC method should be useful for the assay of clofibric acid in the large number of plasma samples that one encounters in the course of pharmacokinetic studies of clofibrate in small animal models. It is simple in term of experimental steps, economic in terms of reagents and sample requirements, and valid in terms of linearity, accuracy, reproducibility and specificity.

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URINARY PROTEIN AND ALBUMIN DETERMINATIONS BY HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY#

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ABSTRACT

Sensitive high-performance gel-permeation chromatographic protein assay method was developed by using non-ionic detergent of Brij-58 which was UV transparent as compared to Nonidet P-40. A column (70 x 8.0 mm I.D.) packed with Develosil 100 Diol-5 (pore size 10 nm) was used for the protein determination. A commercially available column (300 x 8.0 mm I.D.) packed with Develosil 300 Diol-5 (pore size 30 nm) was used for the urinary albumin determination. Eluent used was a 0.1 M sodium phosphate buffer (pH 5.6) containing 0.3 M sodium chloride, 1% (v/v) Brij-

#A part of this work was presented in the 20th annual meeting of the international society for pediatric and adolescent diabetes, Atami-city, Shizuoka, Japan, November 1994.

58, 50% (v/v) glycerol. Flow-rates for protein assay and albumin assay were 1.0 and 0.5 ml/min, repectively. Proteins were eluted at the position of the exclusion limit in the case of protein assay (100 Diol-5). Albumin was eluted from 300 Diol-5 column as a symmetric peak. Bovine serum albumin (BSA) was used as an external standard for both protein and albumin assays. Analysis times were within four min for protein assay, and 32 min for albumin assay, respectively. Improved sensitive measurement of BSA at 5-20 ng level was achieved as compared to Nonidet P-40 by use of UV 210 nm. This method was successfully applied for various urine samples, such as healthy random urine of before and after sports, and the 24-h urines from insulin-dependent diabetes mellitus (IDDM) patients. Comparisons with clinical urinary protein and albumin tests were performed using IDDM urine. Three cases out of four tests for sports showed increased protein and albumin content after physical exercise. Thus, this HPLC method was proven to be applicable to the protein and albumin measurements in human urine.

INTRODUCTION

Previously, we developed a high-performance gel-permeation chromatographic (HPGPC) protein separation method using a non-ionic detergent of Nonidet P-40 in the eluent, and this method was successfully applied to the purification of human serum biotinidase in a high yield (1). However, strong UV absorbance at 280 nm by Nonidet P-40 decreased the sensitivity for the protein detection.

In this study, we further studied to improve the sensitivity of the HPGPC protein separation method by using more UV-transparent non-ionic detergent, Brij-58. It was found that Brij-35 (at 1% v/v) was not applicable to urine analysis, because column-inlet pressure increased during analysis. Further, the base-line separation between protein peak and smaller moleculer weight compounds was not obtainable when urine analyses by using Brij-35 were continued, which suggested the low recovery of proteins or lipids from the column. On the other hand, when Brij-58 (at 1% v/v) was used instead of Brij-35 as a component of the eluent, satisfactorily repeatable and applicable analysis of

urine was achieved. For protein analysis, a special column was devised; i.e., diol-type silica gel with pore size 10 nm was packed into a 70 x 8 mm I.D. stainless steel tube in order to elute the proteins at a retention time of exclusion limit. We firstly report in this paper about the protein and albumin content measurements in human urine.

MATERIALS AND METHODS

Chemicals and reagents: Bovine serum albumin (BSA) and gamma-globulins (bovine Cohn fraction II) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Brij-58, Brij-35, and detergent starter kit (containing MEGA-8, MEGA-9, n-octyl-beta-D-thioglucoside) were from Wako Pure Chemicals Co., Osaka, Japan. Nonidet P-40, Triton X-100, and glycerol were from Nacalai Tesque Co., Kyoto, Japan. Sodium dodecyl sulfate (SDS) was from Bio-Lad, Richmond, CA, USA. A BCA (bicinchoninic acid) protein assay kit was from Pierce Chemical Co., Rockford, IL, USA. Develosil specially-packed column of Develosil 100 Diol-5 (70 x 8 mm I.D.: 10 nm mean pore-size, 5 μ meter mean particle diameter) and Develosil column of Develosil 300 Diol-5 (300 x 8 mm I.D.: 30 nm mean pore-size, 5 μ meter mean particle diameter) were from Nomura Chemical Co., Seto-City, Aichi, Japan.
Specimens: Human urine (24-h urines) from patients of insulin-dependent diabetes mellitus (IDDM) was kindly donated from National Children's Hospital (Tokyo, Japan). Other 24-h and radomly sampled healthy urines were from volunteers in this institute.

Human urine samples were filtered through Ekicrodisc 13 (0.2 μm VERSAPOR; Gelman Sciences Japan, Ltd., Tokyo, Japan), and stored at -80 °C.

High-performance liquid chromatography: A model 655A-11 pump (Hitachi, Tokyo, Japan) was used. Injector was a model U-6K (diaphragm type; 2 ml sample loading loop, Waters, Milford, USA). Detection was carried out by a model lambda-Max spectrophotometer (model 481, Waters; detected at 210 nm or 280 nm). Recorder was Hitachi 561 (Hitachi), and Chromatopac C-R6A data processer (Shimadzu) was also used. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. Eluent (2 l) was made as follows; 1 l of 0.2 M sodium phosphate buffer (pH 5.6) containing 0.6 M sodium chloride was first made. To this buffer, 1 l of glycerol and 20 ml of Brij-58 was then added and mixed thoroughly (usually 30 min). Thus, final concentrations were as follows: sodium phosphate (0.1 M), sodium chloride (0.3 M), glycerol (50%, v/v), and Brij-58 (1%, v/v), respectively. Flow-rates were 1.0 ml/min for protein assay (7-cm long column) and 0.5 ml/min for albumin assay (30-cm long column), and column inlet pressures were 100 kg/cm^2 and 120 kg/cm^2, respectively, at the column temperature at 35°C. Detection for urine protein was performed at 210 nm.

HPLC urine protein assay: Filtered urine (0.04 ml) was dissolved into 0.36 ml of this eluent (10-fold diluted). This 10-fold diluted urine was further 5-fold diluted with the above eluent; i.e., finally 50-fold diluted urine was made. Standard BSA (2 mg/ml; BSA standard solution of Pierce BCA protein assay kit) was 1000-fold diluted by this eluent, and 0.02 ml (40ng of BSA) was injected into the HPGPC system with the 7-cm long column (external standard; Fig. 1, left panel). Usually, 0.01 ml of 50-fold diluted urine sample was injected into the HPLC system and analyzed.

HPLC urine albumin assay: The 10-fold diluted urine was further 10-fold diluted with the HPGPC eluent; i.e., 100-fold diluted

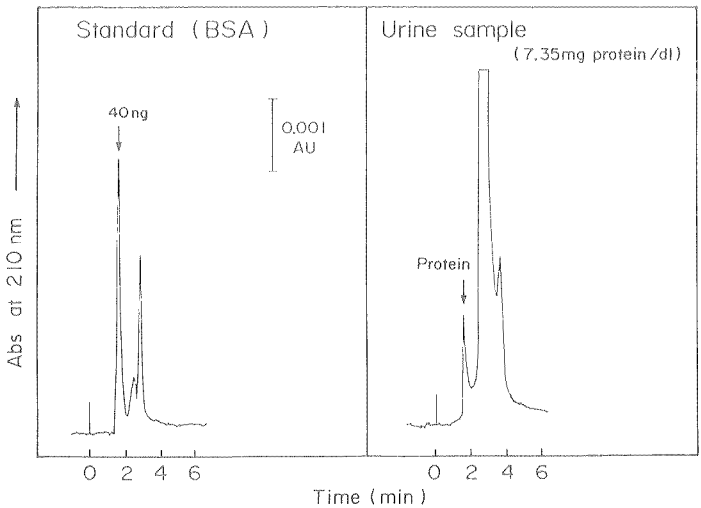

Fig. 1. Typical protein analysis of urine by HPGPC method with 10 nm pore-size and 70 x 8 mm I.D. column. Left panel; 0.02 ml of BSA solution (0.002 mg/ml) was injected into the HPLC system. Right panel; 0.01 ml of 50-fold diluted urine (IDDM patient's urine) was injected into the HPLC system. Other conditions are as described in Materials and Methods.

urine was made for albumin analysis. BSA was 100-fold diluted as above, and 0.01 ml (200 ng; Fig.3, left panel) was injected into the HPGPC system with the 30-cm long column as an external standard. Usually, 0.1 ml of 100-fold diluted urine sample was injected into the HPLC system and analyzed.

Another urine protein and albumin assays: In order to compare the values obtained by HPLC method, urine protein and albumin contents in IDDM urine samples were measured by photometric and immuno assay method, respectively, at the clinical laboratory of National Children's Hospital, Tokyo, Japan. Urine protein was

measured by a photometric kit (Micro TP-Test Wako, Pyrogallol-red Mo (VI) method, Wako Pure Chemical Co., Osaka, Japan). Urine albumin was measured by a turbidic immuno assay kit (ALB-TIA "Seiken", Nippon Roche Co., Tokyo, Japan).

Urine biotinidase assay: Biotinidase activity in human urine was assayed by using biotinyl-6-aminoquinoline as substrate as previously reported (3), and biocytin hydrolyzing activity was also determined by measuring the liberated L-lysine from biocytin by HPLC-amino acid analyzer (OPA method; Hitachi L-6200, Hitachi Co., Tokyo) (4).

RESULTS AND DISCUSSION

In a previous paper, we developed a high-recovery protein separation system by using diol-type silica gel column; i.e., high-performance gel-permeation chromatographic system (HPGPC system) with the eluent containing non-ionic detergent of Nonidet P-40 (1). Further, peak symmetry of carbonic anhydrase in the HPGPC system was considerably improved by increasing the glycerol concentration in the eluent from 2.5 % (v/v) to 30 % (v/v) (2).

Since Nonidet P-40 showed high UV absorbance and sensitivity for protein was not sufficient for urine analysis, we further tested another non-ionic detergent which showed low UV absorbance. We found only Brij-58 was satisfying for the urine protein analysis; i.e., 1) SDS solution of 2% in the phosphate buffer eluent of reference 2 did not dissolve gamma-globulin at all, 2) MEGA-8, MEGA-9, and Tween 20 gave no straight base-lines, 3) Tween 40 and 60 showed low solubility to phosphate buffer, 4) n-octyl-thio-D-glucoside showed high UV absorbance at 210 nm. Brij-58 was satisfactory and repeatable to urine analysis when glycerol

concentration of 50% (v/v) was used. On the other hand, Brij-35 showed an increase in column-inlet pressure even at 50% (v/v) glycerol eluent. Since high sensitivity was necessary for urine protein and albumin analysis, more stable base line was preferable; i.e., we found that a better and straight base line was obtainable at the pH of the eluent of 5.6 instead of 6.0, which was used in previous reports (1, 2).

In order to elute the proteins on the position of the exclusion limit, we chose 10 nm pore-size diol-type silica gel column (70 x 8.0 mm I.D.). As shown in Fig. 1 (left panel), BSA eluted as a triangle-shaped peak. This suggested that the elution of BSA occurred at the exclusion limit (5). Peak height of standard BSA was linearly correlated with injected amount (from 10 to 40 ng) of BSA (Fig. 2) intersecting the origin (0, 0).

Repeatability was tested by injecting 20 ng of BSA nine-times; retention time was 1.73 ± 0.0075 min (coefficients of variation; CV = 0.43 %), peak height (at 210 nm, 0.01 AUFS) was 39 ± 3.2 mm (CV = 0.82 %). Linear correlation curve was obtainable between 0 to 4 ug of BSA (data not shown). Thus, the wide range of linearlity from the origin for BSA was a unique feature of this chromatographic method as compared to the other photometric methods such as Lowry's method (6). This linear character of calibration curve is expected to be usefull for the application onto automated and convenient calculation with a data processor.

A typical example of urine protein analysis was shown in Fig. 1 (right panel). Urinary protein content was measurable within four min.

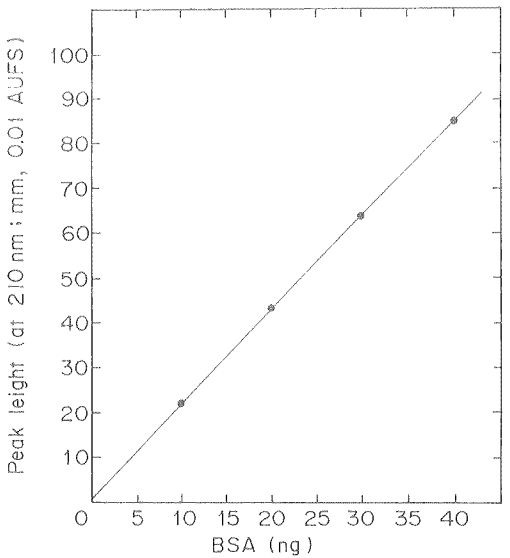

Fig. 2. Correlations between weight amount of BSA injected into HPLC system (70 x 8 mm I.D. column with 10 nm pore-size) and observed peak height (in mm) at 210 nm. 0.005, 0.01, 0.015, and 0.02 ml of diluted BSA (0.002 mg/ml) was injected into the HPLC system.

With 30 cm long commercial column packed with 30 nm pore-size diol-type silica gel, urinary albumin was measurable within 32 min as shown in Fig. 3. BSA was eluted at a retention time of 17 min.

In order to assess whether major urine proteins elute at the position of the exclusion limit of the protein assay column (10 nm pore-size, 70 mm long) or not, we collected the triangle-shaped peak of the exclusion limit (see Fig. 1, right panel) using one of the IDDM urine (protein concentration; 21.8 mg/dl). Then, a portion of the collected fraction was re-

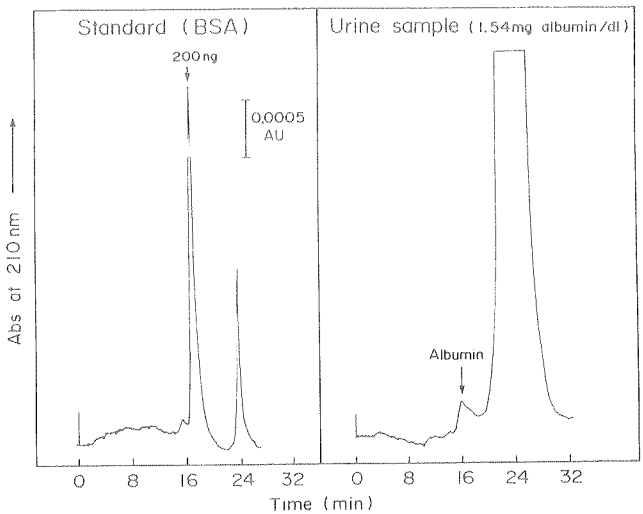

Fig. 3. Typical albumin analysis of urine by HPGPC method. Left panel; 0.01 ml of BSA solution (0.02 mg/ml) was injected into the HPLC system with 30 nm pore-size and 300 x 8 mm I.D. column. Right panel; 0.1 ml of 100-fold diluted urine (IDDM patient's urine) was injected into the HPGPC system. Other condition are as described in Materials and Methods.

analyzed by injecting into the above described albumin analyzing system with 30-cm column of 30 nm pore-size (Fig. 4, panel A), and compared to the elution pattern of the dialyzed urine of the patient (Fig. 4, panel B). As shown in Fig. 4, fractionated peak exhibited essentially the same elution pattern to the original dialyzed urine. This result indicates that most of the urine proteins are eluted at the exclusion limit of the 7-cm protein analyzing column as expectedly. Interestingly, small molecular size compounds of the urine, which eluted after the inclusion limit (about 20 min; e.g., Fig 3, right panel), was

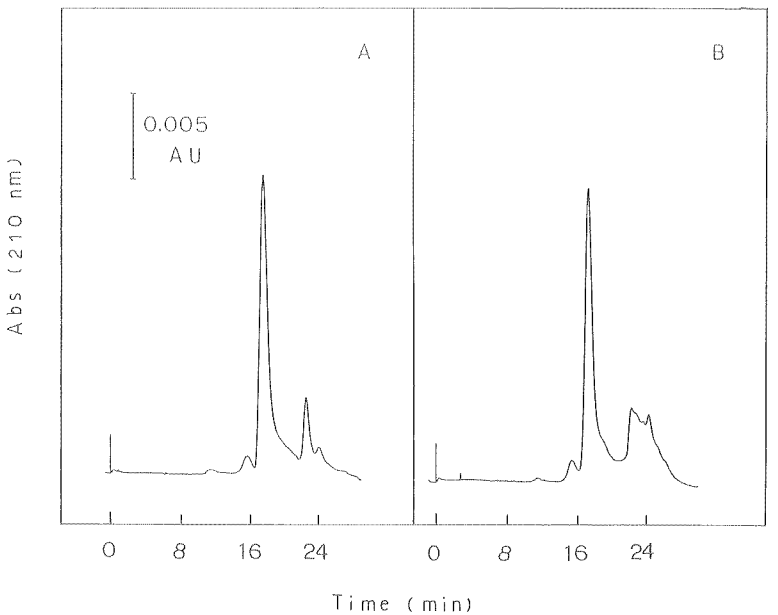

Fig. 4. Re-analysis of the collected peak fraction at the exclusion limit of protein analyzing system. An IDDM urine (protein concentration; 21.8 mg/dl) was 10 fold diluted with the HPGPC eluent. A 0.2 ml portion of the diluted urine was injected into the protein analyzing sytem with 7-cm column, and fractioned the triangle-shaped peak of the exclusion limit (1 ml). Then, a portion (0.18 ml) of the collected fraction was injected into the albumin analyzing system with 30 cm column (panel A). This re-analyzed chromatogram was compared with the chromatogram of original dialyzed urine (panel B). In panel B, 0.02 ml of the dialyzed urine was injected and analyzed as a reference. Dialysis of the urine (0.2 ml of urine) was performed at ambient for 24 h against 1000-fold volume (200 ml) of the HPGPC eluent.

decreased after a simple collection of the triangle-shaped peak at the exclusion limit of the protein analyzing column (7-cm long; 10 nm pore-size). Thus, this protein analyzing system with 7-cm long column is a useful tool to dialyze the urine sample within 4 min.

Then, the HPLC-protein and albumin analysis method was applied onto the 35 samples of IDDM 24-h urine, and compared to the results from clinical photometric protein and immuno turbidic albumin assay methods. Average protein excretions as measured by HPLC and photometric clinical methods were 17.2 ± 20.1 (mean ± SD) mg/day and 46.8 ± 56.5 mg/day, respectively. There was no significant correlation between them. Interestingly, we found a significant correlation ($r = 0.60$; $p < 0.01$) between protein excretion (HPLC value) per day and duration (years) of IDDM (submitted for publication), although clinical protein value showed no such correlation. This suggests that the HPLC protein assay method is preferable to analyze IDDM urines which may contain more complex interfering materials from diabetes. Average albumin excretions as measured HPLC and immuno clinical method were 16.9 ± 22.5 mg/day and 5.3 ± 10.3 mg/day, respectively. There was a correlation between HPLC and clinical immuno albumin measuring methods; i.e., $r = 0.705$ ($p < 0.01$). HPLC albumin excretion values were correlated with the duration of IDDM with $r = 0.63$ ($p < 0.01$), however clinical values at this time were not ($p < 0.05$, submitted for publication). HPLC method showed more reliable results to indicate the correlations which were present between duration of IDDM and excretion of albumin and protein (7) as compared to other clinical methods. This may be the result of the HPLC analysis method, which separates proteins from the possible interfering small molecular

TABLE I

Effect of sports (physical exercise) on the urinary protein and albumin excretions in the urine.

Sports / Excretion	Before	After	
	Cycling for 1h		
Protein	1.60 mg/dl	3.20 mg/dl	
Albumin	0.44 mg/dl	0.79 mg/dl	
Biotinidase *	ND	ND	
	Tennis for 75 min		
Ptrotein	2.00 mg/dl	3.75 mg/dl	
Albumin	0.50 mg/dl	2.30 mg/dl	
Biotinidase *	13.3	ND	
	Swimming for 2h	0h	2h
Protein	5.90 mg/dl	7.00 mg/dl	10.8 mg/dl
Albumin	0.36 mg/dl	0.72 mg/dl	1.44 mg/dl
Biotinidase *	N.D.	33.3	116.7
	Tennis for 45 min (7y child)		
Protein	2.06 mg/dl	1.59 mg/dl	
Albumin	0.29 mg/dl	0.15 mg/dl	
Biotinidase *	N.D.	30.9	

* Biotinidase; Specific activity was expressed as pmol biocytin hydrolyzed/min per mg of urine protein (4). N.D.; Not detectable. Protein and albumin content were measured by HPLC.

weight compounds in the IDDM urine. Thus, these HPLC protein and albumin measuring methods were shown to be applicable to disease urines of the IDDM patients.

Effects of sports on urinary excretion of protein and albumin were also tested, since such a test may be helpfull for estimation of physiological state of kidney (8). The results were shown in Table I. As a reference, the excretion of biotinidase activity was also shown (Table I). Although a child (7 y) seemed to show different physiology from adults, three adults exhibited the increased protein and albumin excretions after sports. Biotinidase excretion into urine seemed not to be directly related to physical exercise. Thus, this HPLC protein and albumin assay method was also expected to be applicable to the field of sports medicine.

ACKNOWLEDGEMENTS

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SIMULTANEOUS MEASUREMENT OF FLURBIPROFEN, IBUPROFEN, AND KETOPROFEN ENANTIOMER CONCENTRATIONS IN PLASMA USING L-LEUCINAMIDE AS THE CHIRAL COUPLING COMPONENT

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ABSTRACT

A high performance liquid chromatographic method was developed for the quantitation of the R- and S- enantiomers of 2-arylpropionic acid namely flurbiprofen, ibuprofen and ketoprofen, in plasma.
The procedure involved extraction of drugs and internal standard from acidified plasma into dichloromethane. After evaporation of the organic layer, the compounds were derivatized with L-leucinamide after addition of ethyl chloroformate as the coupling reagent. The former diastereoisomeric amides were chromatographied at ambient temperature on a reversed phase column using 0.06M KH_2PO_4 - acetonitrile - triethylamine (51:49:0.1) as the mobile phase pumped at a flow rate of 1.8 ml/min. This assay allowed determination of 0.1μg/ml of both R- and S- enantiomers with an acceptable precision (maximum coefficient of variation of 7.5%) using a 0.5-ml plasma sample.

INTRODUCTION

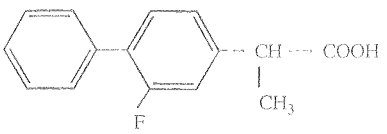
Flurbiprofen (FLU), ibuprofen (IBU) and ketoprofen (KT), potent non-steroidal anti-inflammatory drugs (NSAID) of the 2-arylpropionic acid class (Fig. 1) are currently available in their racemic form. It is well-known that the enantiomers may exert different pharmacodynamic effects. Pharmacokinetic differences have been reported as well (1). The prostaglandin synthetase inhibiting effect of these drugs is attributable to the S(+) enantiomer (2). Therefore, the stereoselective determination of drug enantiomers in plasma is of potential clinical importance (3,4).

Today, three methods are currently used to achieve chromatography enantioselective resolution of racemic compounds, especially 2-arylpropionic acids (5) :

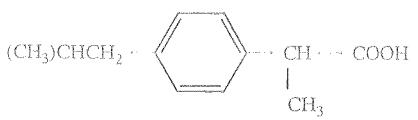
- using chiral HPLC columns
- using achiral HPLC columns with chiral mobile phase
- by derivatization with optical reagents and separation on achiral columns.

Few diastereoisomer derivatization methods were described to achieve the chromatographic separation of FLU, IBU and KETO enantiomers (Table 1). Some of these stereoselective assays entailed extensive sample preparation or lengthy chromatography times associated with a lack of sensitivity. Three of them (7, 8, 13) consist of extraction of the racemate from acidified plasma followed by conversion to a mixed anhydride with ethylchloroformate and derivatization with L-leucinamide. This procedure was first described by Björkman (15). These three methods suffer from a time-consuming extraction step (7, 13) or necessitate a cleanup extraction before injection (8).

We report a simultaneous analysis of the enantiomers of FLU, IBU and KETO after formation of diastereoisomers of chiral drugs with L-leucinamide hydrochloride. The major modifications of the previously reported assays include simple extraction of unresolved drugs from acidified plasma and rapid reversed-phase HPLC separation of the diastereoisomers within 13 minutes. There is no detectable racemization of either NSAID or leucinamide during the reactions, which are complete within 3 minutes.



Flurbiprofen



Ibuprofen

Ketoprofen

FIGURE 1 : Chemical structures of the studied 2-arylpropionic acid derivatives.

TABLE 1
Previous HPLC Enantiomeric Assays on Human Plasma

Authors	Derivatizing reagent	MQC[a] (µg/ml)	Sample volume [b](ml)
Ketoprofen			
Sallustio *(1986)* (6)	R-2-phenylethylamine	0.2	0.2
Björkman *(1987)* (7)	L-leucinamide	0.25	0.5 - 1
Foster *(1987)* (8)	L-leucinamide	0.1	0.5
Hayball *(1991)* (9)	S-1-phenylethylamine	0.15	1
Ibuprofen			
Mehvar *(1988)* (10)	S-(-)-1-(1-naphthyl)ethylamine	0.1	0.5
Wright *(1992)* (11)	R-(+)-α-phenylethylamine	0.25	0.5
Lemko *(1993)* (12)	S-(-)-1-(1-naphthyl)ethylamine	0.1	0.5
Flurbiprofen			
Berry *(1988)* (13)	L-leucinamide	0.1	0.5
Knadler *(1989)* (14)	S-(α)-methylbenzylamine	0.025	0.5

[a] MQC : minimum quantifiable concentration for each enantiomer
[b] sample volume : volume plasma required for assay

MATERIALS AND METHODS

Reagents and Chemicals

Racemic FLU, IBU, KETO and the two internal standards indomethacin and S (+) naproxen were purchased from Sigma (St Quentin Fallavier, France). Pure R- and S- enantiomers of each drug were kindly supplied by The Boots Company Ltd. (Nottingham, England) for FLU and IBU and by Rhône-Poulenc Rorer (Vitry-Alforville, France) for KETO, respectively. Acetonitrile, dichloromethane and triethylamine were supplied by Info-Labo (Sainte-Foy-la-Grande, France) and were of HPLC reagent grade. Methanol (HPLC) grade was supplied by Prolabo (Paris, France). Ethyl chloroformate and L-leucinamide were obtained from Sigma. Water was deionized and doubly-glass distilled. All others reagents were of analytical grade.

Apparatus and Chromatographic Conditions

The analysis were performed on a Waters Assoc. (Milford, MA, USA) chromatographic system, including a Model 45 constant-flow pump, a Wisp Model 710 B automatic injector and a Lambda Max Model 480 ultraviolet detector operated at 225 nm (IBU analysis) or 275 nm (FLU and KETO analysis), respectively. Samples were chromatographied on an Ultrabase C_{18} 5µ (250 mm x 4.6 mm i.d.) stainless steel column (Shandon HPLC, Cheshire, United Kingdom). Chromatograms were recorded on a 10-mV recorder Omniscribe (Houston Instruments) at a chart-speed of 0.5 cm/min. The mobile phase consisted of 0.06 M KH_2PO_4 - acetonitrile - triethylamine (51 : 49 : 0.1, v/v) pumped at a flow rate of 1.8 ml/min. Before use, the mobile phase was filtered through a 0.45 µm filter (Sartorius, Göttingen, FRG).

Standard Solutions

Stock solutions of (±) FLU, (±) IBU, (±) KETO and internal standars S(+)

naproxen and indomethacin (1 mg/ml) were prepared in methanol and stored at -20°C until use. Appropriate dilutions of each racemate were made in drug-free human plasma to give final enantiomer concentrations of 1, 2, 4, 6, 8 and 10 μg/ml. A solution of S(+) naproxen at 2.5 μg/ml was used for enantiomeric assay of IBU and solutions of indomethacin at 25 and 50 μg/ml were prepared for stereospecific analysis of FLU and KETO, respectively. Ethyl chloroformate (60mM) was prepared in acetonitrile. The derivatizing reagent, L-leucinamide (0.1 M) was prepared in triethylamine - methanol (0.14 : 1).

Work-up and Derivatization of Plasma Samples

To an aliquot of plasma (500 μl of standard or sample) were added 100μl of the appropriate internal standard solution, 500 μl of 0.6M sulfuric acid and 15 ml of dichloromethane. The constituents were then mixed for 20 minutes. After centrifugation at 2000g for 5 minutes, the organic layer was transferred to clean tubes and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The residue was reconstituted in 100 μl of triethylamine in acetonitrile (50mM), followed by the addition of 50μl of ethyl chloroformate solution (60mM in acetonitrile) and vortexed for 30s. A 50-μl aliquot of L-leucinamide solution (0.1M) was added, vortex-mixed briefly and allowed to stand. After 2 minutes, the reaction was terminated by the addition of 50 μl of bi-distilled water. Aliquots of 10-50 μl of the resulting solutions were injected into the HPLC system.

Extraction Efficiency

To assess the efficiency of the extraction step, standard solutions of each racemic compound (FLU , IBU, KETO) were extracted in the absence of internal standard using the above extraction procedure. The chromatographic conditions were identical to that described for the enantiospecific determinations. The peak heights after injection of racemic NSAID extracted from plasma were compared to those generated from direct injections of aqueous solutions of these compounds.

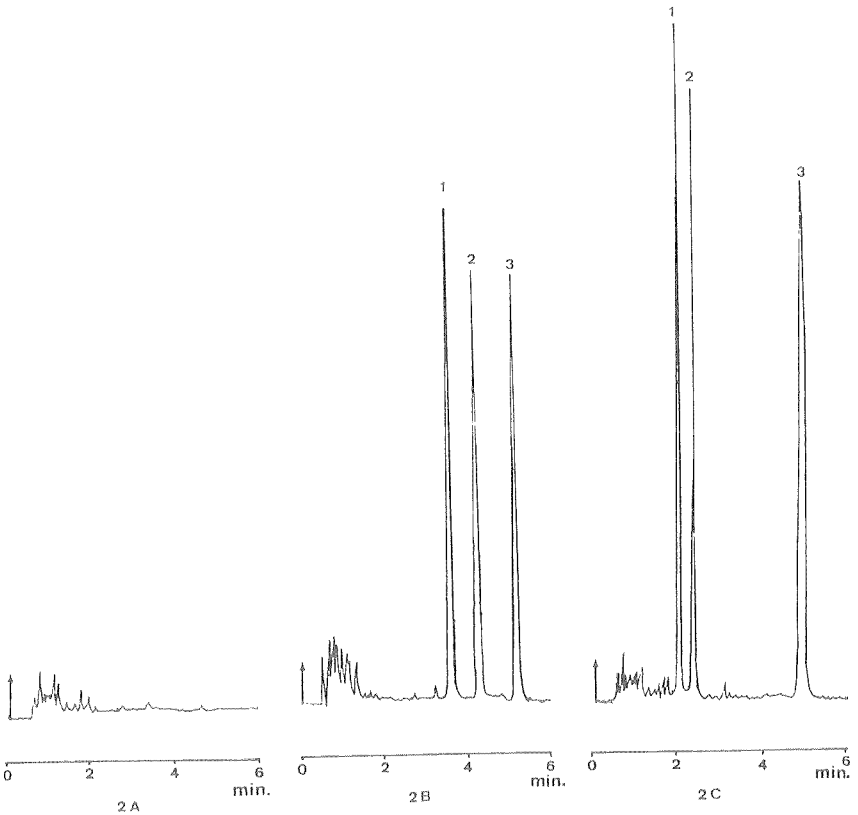


FIGURE 2 : Chromatograms of blank plasma (figure 2A) and drug-spiked human plasma with 6 µg/ml of FLU (figure 2B), KETO (figure 2C) as racemates.

Peak 1 : R(-) enantiomer ; peak 2 : S(+) enantiomer ; peak 3 : internal standard

λ : 275 nm ; AUFS : 0.05

RESULTS AND DISCUSSION

The diastereoisomers of chiral FLU, IBU and KETO were formed at ambient temperature in less than 3 minutes by utilizing L-leucinamide and ethylchloroformate. The amide derivatives formed by reaction of the carboxylic group of the NSAID with the chiral amine, L-leucinamide are separated on an achiral column in a reversed-phase system.

Representative chromatograms of blank and drug-spiked samples are shown in Figures 2 and 3. We did not observe additional peaks formed under the conditions used for derivatization. The elution order of the

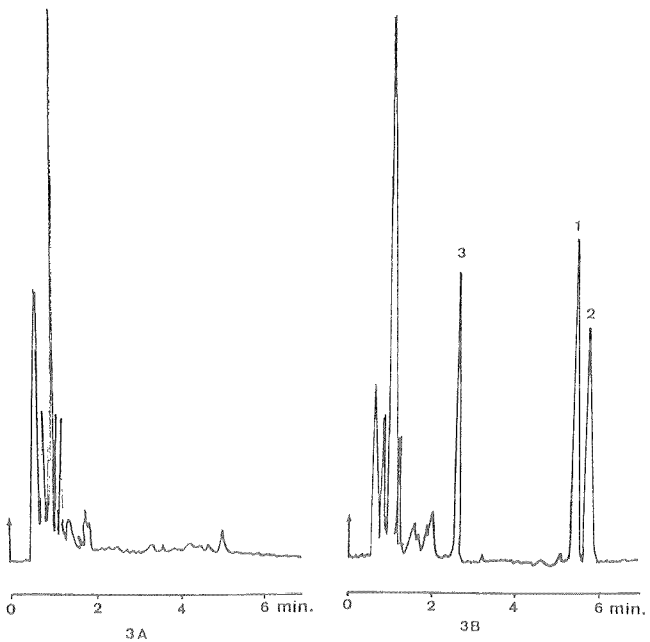


FIGURE 3 : Chromatograms of blank plasma (figure 3A) and drug-spiked human plasma with 6 µg/ml of IBU (figure 3B) as racemates.
Peak 1 : R(-) enantiomer ; peak 2 : S(+) enantiomer ; peak 3 : internal standard
λ : 225 nm ; AUFS : 0.05

TABLE 2
Precision of the FLU, IBU and KETO assays

Compound	Theorical concentration (µg/ml)	Experimental concentration mean (SD) (µg/ml)	CV (%)[a]
S(+) FLU	0.5	0.51 (0.037)	7.2
	2.5	2.69 (0.16)	5.9
	5	5.11 (0.28)	5.5
	7.5	7.67 (0.44)	5.7
R(-) FLU	0.5	0.53 (0.04)	7.5
	2.5	2.74 (0.2)	7.3
	5	5.2 (0.32)	6.1
	7.5	7.74 (0.5)	6.4
S(+) IBU	0.5	0.48 (0.046)	9.6
	2.5	2.57 (0.19)	7.3
	5	5.27 (0.41)	7.8
	7.5	7.65 (0.61)	7.9
R(-) IBU	0.5	0.55 (0.04)	7.3
	2.5	2.52 (0.21)	8.4
	5	5.37 (0.37)	6.5
	7.5	7.44 (0.52)	7.0
S(+) KETO	0.5	0.45 (0.037)	8.2
	2.5	2.10 (0.097)	4.6
	5	4.21 (0.23)	5.4
	7.5	6.33 (0.4)	6.3
R(-) KETO	0.5	0.45 (0.03)	6.7
	2.5	2.13 (0.12)	5.4
	5	4.2 (0.22)	5.3
	7.5	6.39 (0.39)	6.0

[a] : coefficient of variation

diastereoisomers was confirmed by derivatization and chromatography of optically pure R- and S- enantiomers. Using these samples, it was shown that L-leucinamide derivatives of R-FLU, R-IBU and R-KETO always eluted prior to their respective S-isomers.

The precision and repeatability of the method were assessed by triplicate analysis of four control plasma samples containing 1, 5, 10, 15 µ/ml of FLU, IBU and KETO, respectively. These analyses were repeated during three consecutive days (n=9). Thus the corresponding concentrations of each enantiomer S(+) and R(-), were 0.5, 2.5, 5 and 7.5 µg/ml, respectively. The results are presented in Table 2.

TABLE 3
Linear Least-Square Regression Analysis for Each Drug Enantiomer

Compound	Regression equation [a]	Regression coefficient	p-value
S (+) FLU	y = 0.201 (0.005) x - 0.027	0.988	p = 0.0001
R (-) FLU	y = 0.227 (0.0004) x - 0.019	0.992	p = 0.0001
S (+) IBU	y = 0.156 (0.005) x - 0.009	0.982	p = 0.0001
R (-) IBU	y = 0.182 (0.004) x + 0.009	0.988	p = 0.0001
S (+) KETO	y = 0.197 (0.003) x + 0.019	0.993	p = 0.0001
R (-) KETO	y = 0.226 (0.005) x + 0.005	0.989	p = 0.0001

[a] x = enantiomer concentration ;
y = peak-height ratio of compound / internal standard

The precision of the assay for FLU, IBU and KETO enantiomers at low and high concentrations were within acceptable limits for clinical studies. Over the entire concentration range studied, the coefficients of variation for determination of S- and R- FLU enantiomers were less than 7.5%. Similar coefficients of variation were obtained for IBU and KETO enantiomers, being less than 9.6% and ≤8.2%, respectively.

The calibration curves generated over the enantiomeric concentration range from 1 to 10 μg/ml (supplied as racemic drug) were linear for both S- and R- enantiomers of each drug. Linear least-square regression analysis for nine calibration curves of each enantiomeric drug is depicted in Table 3. The slopes of the lines describing both FLU, IBU and KETO enantiomers are not statistically different (Wilcoxon - test : NS, $p<0.01$) indicating non-stereoselectivity in the extraction and/or derivatization methods.

The extraction yields for the racemic mixture were 88.6 ± 8.4 % (FLU), 81.9 ± 9.5 %(IBU) and 80.8 ± 7.1 % (KETO), respectively.

The limit of detection (LOD, signal to noise ratio = 3) was found to be 0.1 μg/ml for each enantiomer.

CONCLUSION

The present method is common to the determinations of R- and S-enantiomers of FLU, IBU and KETO. It is simpler than most of the methods reported in the literature. This HPLC procedure only requires one extraction step of racemates from plasma and a rapid derivatization of less than 3 minutes. This analytical procedure employing relatively simple HPLC technology is sensitive enough for use in single-dose pharmacokinetic studies.

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A SIMPLE, SPECIFIC, AND RAPID HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR GLIBENCLAMIDE IN PLASMA

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ABSTRACT

A simple, rapid, sensitive and relatively specific reverse phase HPLC assay procedure for glibenclamide in rat and human plasma is described. The method employs considerably smaller sample volume (0.2 ml) and a single step extraction with dichloromethane. A relatively high degree of sensitivity (detection limit of the assay was 20 $ngml^{-1}$) was obtained by using fluorescence detection at excitation and emission wavelengths of 308 and 360 nm, respectively. The mean percentage recovery of the drug in the concentration range of 30-120 ng was 100.6% while the between-day coefficient of variation for the same concentration range was 7.29%.

The assay procedure offers a much better degree of resolution for the glibenclamide peak than was previously reported. This was achieved through the use of a chromatographic

column with a finer packing material (Novapak C_{18}, 4 μm) and by selecting the ideal composition, pH and elution rate of the mobile phase - methanol: 0.05 M potassium dihydrogen phosphate (61:39 v/v, pH 4 and pumped at 1.2 $ml.min^{-1}$).

It is hoped that this micro assay technique will find wider application in both clinical and subclinical pharmacokinetic studies involving glibenclamide.

INTRODUCTION

Glibenclamide is a second generation sulfonylurea derivative commonly prescribed for the control of hyperglycemia in Type II (non-insulin dependent, NIDDM) diabetes and thereby to prevent or retard the development of chronic complications of the disease. Glibenclamide produces its hypoglycemic effect by specific interactions with receptors in pancreatic ß-cells to stimulate insulin secretion and to enhance tissue response to endogenous insulin[1]. Glibenclamide is also believed to reduce the hepatic extraction of insulin, thereby increasing its systemic availability to regulate tissue glucose utilization[2].

The hypoglycemic effect of glibenclamide are achieved within a narrow therapeutic range of drug concentration. It has been postulated that there is a threshold plasma glibenclamide concentration which must be exceeded before its hypoglycemic activity can occur[3]. The absolute values for this drug concentration range would essentially depend on the specificity and relative sensitivity of assay method employed.

A number of analytical techniques have been described for glibenclamide in biological samples. These include UV spectrophotometry[4] flurometry[5], radioimmunoassay[6] and high performance liquid chromatographic methods[7-12]. Of these, the

latter appear to offer simplicity and improved sensitivity. All published HPLC methods do however, require substantial sample volume ($\geq$ 1 ml plasma) and cannot be readily adapted for application in situation involving limited sample volume as in investigational studies with smaller laboratory animals.

In the present study, we report a highly specific, relatively simple and rapid HPLC microassay technique for glibenclamide and discuss its potential for wider application in clinical and subclinical pharmacokinetic investigations.

MATERIALS AND METHODS

Chemicals and Reagents

Glibenclamide was a gift from Saudi Pharmaceutical Industries and Medical Appliances Corporation (SPIMACO, Saudi Arabia) while warfarin was obtained from Sigma Chemical Corporation (St. Louis, MO, USA). Methanol (HPLC-grade) was purchased from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and procured from various other sources.

Standard Solutions

For both glibenclamide and warfarin 100 ml of stock solutions of 1 g/L were initially prepared in methanol. Working standards were subsequently obtained by further dilution of the stock solutions to appropriate concentrations.

Instruments

A Waters High-Performance Liquid Chromatographic System (Waters Associates, Milford, MA, USA) was used in the assay. It consisted of a System Controller (M-720), a Data Module (M-730), an Autosampler (WISP-712), a Scanning Fluorescence Detector (M-470), a Novapak C_{18} precolumn and column (4 μm, 150 mm x 3.9 mm ID) and a Solvent Delivery System (M-501).

Sample Preparation

To 0.2 ml of rat or human plasma, 15 μl of internal standard (5 mg/L - warfarin) and 3 ml of dichloromethane were added. The sample was vortex-mixed for 1 min, shaken on a rotary mixer for 5 min and then centrifuged at 1000 g for 15 min. The organic layer was transferred to another glass centrifuge tube and evaporated to dryness under a stream of purified nitrogen gas. The residue was reconstituted with 0.1 ml of mobile phase and 80 μl was injected onto the HPLC column.

Chromatographic Conditions

The mobile phase consisted of 0.05 M ammonium dihydrogen phosphate and methanol (39:61%, v/v) with the pH adjusted to 4.0. The mobile phase was pumped at 1.2 $ml.min^{-1}$ and the fluorescence detector was set at excitation and emission wavelengths of 308 nm and 360 nm, respectively.

RESULTS AND DISCUSSION

The need to develop a new HPLC assay procedure for glibenclamide arose from our failure to adapt a number of the published methods for use in pharmacokinetic interaction studies of the drug in the rat model. The volume of blood samples that can serially collected from such a model without inducing serious haemodynamic changes is fairly limited ($\leq$ 0.5 ml). Despite appropriate modification of the published extraction procedures [8,11] to reflect the reduced sample volume glibenclamide was invariably co-eluting with an endogenous plasma component. As a result, quantitation of the drug was greatly compromised. This problem was also encountered by Abu-Nasif and co-workers (personal communication) in their attempts to adapt the methods described by previous authors [9,10], to assay glibenclamide in

rat plasma. It was apparent that most of the published methods had employed columns with similar packings (spherisorb 5 μm) and ultra violet (UV) detection in the region of 200-230 nm - a band where absorption by extracted endogenous plasma components is relatively high. These points were taken into consideration when we started to develop the present method. Extraction with dichloromethane and the use of finely packed column (Novapack C_{18}, 4 μm) together with fluorescence detection gave better resolution for glibenclamide. Furthermore, the sensitivity of the assay estimated on the basis of sample volume used was considerably improved. The detection limit of the assay was 20 ng/ml.

Figures 1 and 2 show typical chromatograms obtained from rat and human plasma. The chromatograms for the rat plasma were obtained from analysis of samples collected following *in vivo* administration of glibenclamide (0.9 mg kg^{-1}, p.o.) while the chromatogram for the human plasma were obtained after spiking plasma samples with the drug. The relative retention times for warfarin (the internal standard) and glibenclamide were 6.7 and 12.3 min., respectively. Blank human plasma gave a much cleaner chromatogram than rat plasma. The reportedly coeluted interfering plasma component is well resolved from glibenclamide, having a retention time of 11.4 min.

Quantitation

Plasma glibenclamide was quantitated by relating peak height ratios (glibenclamide/internal standard) to a calibration curve prepared from triplicate determination of fresh plasma (rat and human) spiked with known drug solutions (concentration range 25 - 200 ng/ml). There was very good correlation between drug concentration and peak height ratio as evidenced by the equation for the regression analysis.

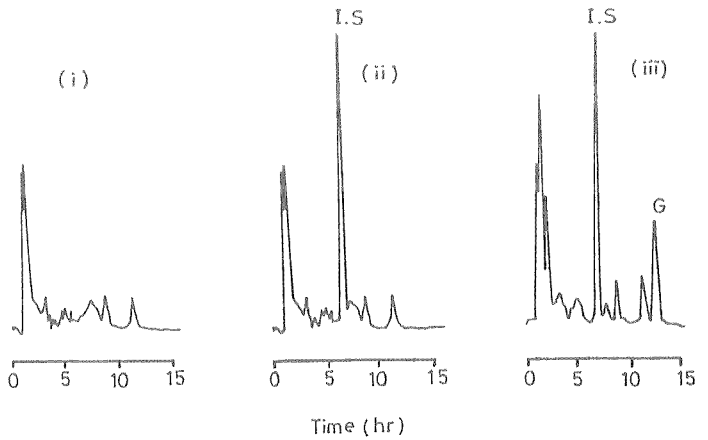

Fig. 1 Typical chromatograms obtained from extracts of (i) blank rat plasma and (ii) blank plasma spiked with internal standard (IS) and (iii) plasma collected 2 hr following an oral dose of (G) glibenclamide (0.9 $mg.kg^{-1}$).

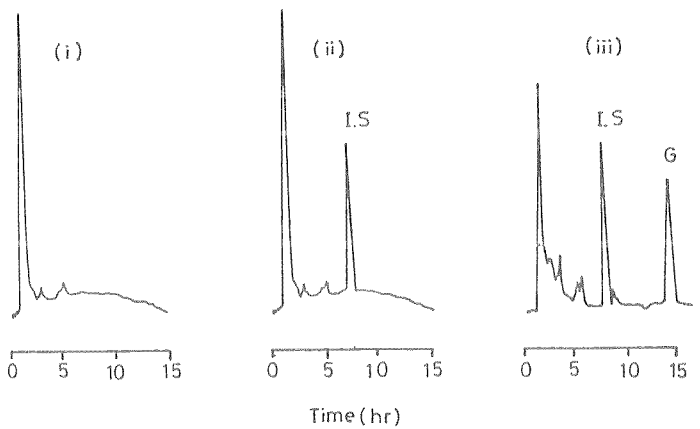

Fig. 2 Typical chromatograms of extracts of (i) blank human plasma (ii) blank plasma with internal standard (IS) and (iii) plasma containing internal standard and glibenclamide (G).

TABLE 1. Between-day precision for the determination of glibenclamide in rat plasma.

Drug Concentration (ng. ml^{-1})		
Added		Measured
30	Mean	42.30
	SD	3.80
	CV,%	8.98
60	Mean	72.20
	SD	4.94
	CV,%	6.84
120	Mean	134.00
	SD	8.11
	CV,%	6.05

$Y = 0.0595 + 0.0027\ x$

$r = 0.989$

Precision

The reproducibility of the assay was assessed by assaying known standards (three concentration points in the calibration curve) over a 3-week period. The corresponding values for the

TABLE 2. Absolute recovery of glibenclamide from rat plasma

Concentration (ng/ml)	Recovery % (mean ± SD, n = 6)
30	101.7 ± 4.86
60	99.18 ± 4.70
120	100.88 ± 6.23

coefficient of variation were 8.98, 6.8 and 6.05% for the 30, 60 and 120 μgml^{-1}, respectively. These relatively lower values for this paramter indicates a good reproducibility of the assay.

Percentage Recovery

This was assessed by adding known amounts of glibenclamide to drug-free plasma to make concentration of 30, 60 and 120 μgml^{-1}. The samples were subjected to the various steps described in the section on sample preparation before being injected into the chromatograph and subsequently peak heights obtained from the resulting chromatograms. Also obtained were the peak height ratios of absolute drug standards corresponding to the three concentrations (prepared in mobile phase instead of plasma). Absolute recoveries which are summarized in Table 2 were calculated by comparing the two sets of peak height ratios.

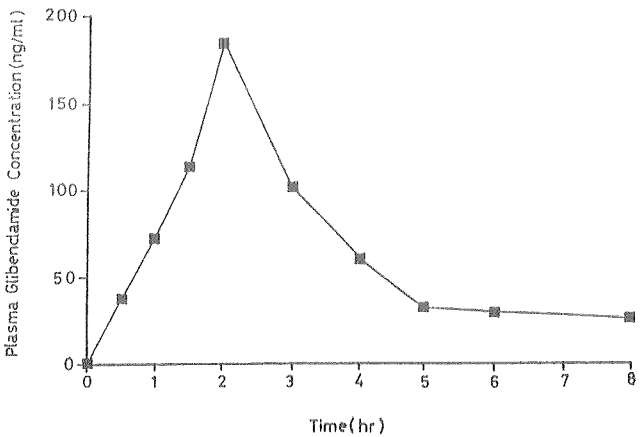

Fig. 3 Plasma glibenclamide concentration versus time profile in normal rats (n = 5).

Application

A representative pharmacokinetic profile for glibenclamide in rats treated with an oral dose of 0.9 $mgkg^{-1}$ is shown in figure 3. The concentration-time curve was constructed from serially collected plasma samples in femoral artery cannulated rats.

CONCLUSION

The HPLC method described in this report is simple, specific, sensitive and highly reproducible. It requires very small volume of plasma sample. This factor alone may be crucial in investigations involving small laboratory animals or in situations where there is a need for the simultaneous estimation of other related pharmacodynamic parameters such as plasma insulin and glucose levels. Overall, we believe that this method has the potential for a much wider application in both clinical and experimental studies.

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ANALYSIS OF AZATHIOPRINE AND 6-MERCAPTOPURINE IN PLASMA IN RENAL TRANSPLANT RECIPIENTS AFTER ADMINISTRATION WITH ORAL AZATHIOPRINE

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ABSTRACT

Studies on the pharmacokinetics of azathioprine (AZA) and its main metabolite, 6-mercaptopurine (6-MP) in patients treated with oral AZA, such as renal transplant recipients, require an analytical method with a high sensitivity. Since the substances differ substantially in their physicochemical properties, it is hardly feasible to develop an extraction procedure and select chromatographic conditions for the simultaneous determination of both substances. Therefore, we have developed two specific chromatographic assays for determination of AZA and 6-MP in plasma. A solid-phase extraction (C8 Isolute) procedure was used for AZA with guaneran as internal standard (IS), while 6-MP was purified on mercurial cellulose and 6-mercaptopurine arabinoside was used as IS. Chromatographic analyses were achieved using C8 and C18 reversed phase columns for AZA and 6-MP, respectively. The methods were reproducible with intra- and inter-assay coefficients of variations below 6 %. The average recoveries of AZA, 6-MP and their respective IS were higher than 79%. The limit of quantitation of AZA and 6-MP in plasma is 0.2 ng.

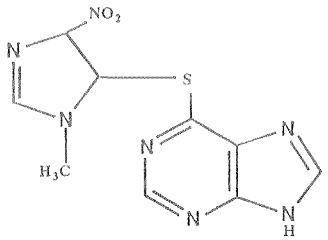
INTRODUCTION

Azathioprine (AZA), a pro-drug of 6-mercaptopurine (6-MP) (Fig. 1), has been widely used either alone or in combination with other immunosuppressants such as prednisolone and cyclosporin, to prevent the rejection of transplanted organs (1, 2). It has also been used in the treatment of autoimmune and inflammatory diseases (3-5). There is a marked intra- and inter-patient variability in the pharmacokinetics of AZA and its main metabolite, 6-MP (6-10). In clinical use, AZA is generally administered on a mg per kg body weight basis and dose adjustments are based on the occurrence of myelosuppression with leukopenia (11).

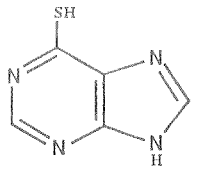
AZA is rapidly metabolized *in vivo* to 6-MP, the immediate metabolite; 6-thiouric acid (6-TU), the final end-product; and 6-thioguanine nucleotides (TGN), the active moiety intracellularly (12-14). Determination of AZA and 6-MP plasma concentrations have been suggested for therapeutic drug monitoring (TDM) (9, 15). Little is known about the relationship between the pharmacokinetics of the AZA and 6-MP and clinical effects, due to lack of assay suitable for large TDM studies.

Several methods for the quantitative determination of AZA and 6-MP in biological fluids have been proposed. These include liquid chromatography (LC) with UV (15-20) or fluorescence detection (21, 22) and gas chromatography-mass spectrometry (23). Some of these methods do not use an internal standard (IS) (18) or use 6-thioguanine as IS (15, 17, 21), which has been detected in plasma, urine (24) and erythrocytes (25) as a minor metabolite and it is therefore less suitable as an IS.

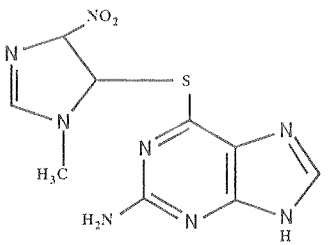
The extraction procedure, the chromatographic condition, and choice of IS in this study were modified from previous methods in order to obtain a better specificity and sensitivity with low intra- and inter-assay variability. With this optimized method in our hands we now have the opportunity to once more address the issues of correlation between AZA and 6-MP pharmacokinetics and clinical outcome.



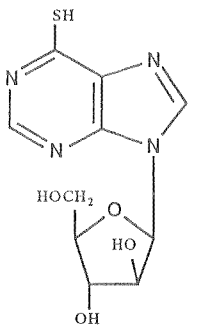
Azathioprine
Mw 277.29



6-Mercaptopurine
Mw 152.19



Guaneran
Mw 302.18



6-Mercaptopurine arabinoside
Mw 284.30

1. Structural formulae of AZA, 6-MP and the used IS.

EXPERIMENTAL

Chemicals

Guaneran (6-[(1-Methyl 4-nitro-5-imidazolyl)thio)-2-aminopurine], was a generous gift from Dr. Gertrude Elion (Wellcome Foundation, Research Triangle Park, N.C.). Azathioprine, 6-mercaptopurine and 6-mercaptopurine arabinoside were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Mercurial cellulose was prepared according to described procedure (26). Methanol and acetonitrile were HPLC grade (JT Baker, Deventer, Holland). Sodium dihydrogen

phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$ p.a.) was from Merck (Merck, Darmstadt, Germany).

Equipments

Reversed phase LC was performed at room temperature using a CM4000 multiple solvent delivery system pump (Milton Roy Co., Rochester, NY, USA), a CMA-240 autosampler (Carnegie Medicine, Inc., Stockholm, Sweden) and a variable wavelength UV detector (Milton Roy Co., Rochester, NY, USA). For the solid-phase extraction, a VacMaster (International Sorbent Technology, Mid-Glamorgan, UK) was used.

Determination of plasma AZA

Solid-phase extraction cartridges C8 (Isolute 100 mg, part Nr 290-0010-A, International Sorbent Technology, Mid-Glamorgan, UK) were conditioned before use with 2.5 ml of methanol and 3.5 ml of 10 mM phosphate buffer pH 7.0. Fifty µl of guaneran solution (1.5 µg/ml) was added as IS to 1 ml plasma and mixed with 2 ml 10 mM phosphate buffer pH 7.0. The mixture was applied on the cartridge with an approximate flow rate of 2.5 ml/min. The cartridge was then washed sequentially with 3 ml of 1% acetonitrile in phosphate buffer pH 7.0. The cartridge was then dried by aspirating air for 2 minutes. The compound was eluted with 2 ml of 5% methanol in ethyl acetate. The eluate was collected in a glass test-tube and evaporated to dryness under a stream of nitrogen in a water bath (40°C). The residue was finally dissolved in 50 µl of mobile phase and 20 µl was injected directly on an HSpecosphere 3CR C8 column, (80 x 4.6 mm, 3 µm, Perkin-Elmer, Norwalk, CT, USA). The mobile phase consisted of 9 % acetonitrile in 10 mM sodium phosphate buffer, pH 6.2 at a flow rate of 1.2 ml/min. AZA and guaneran were detected at 280 nm.

Determination of plasma 6-MP

To 1 ml plasma, 50 µl of 6-mercaptopurine arabinoside (IS) (2.8 µg/ml) solution and 2-3 mg mercurial cellulose were added. The mixture was vortexed for 30 seconds and centrifuged (550 g, 5 min.). The pellet

was resuspended in 2 ml phosphate buffered saline (PBS) and centrifuged. This was repeated twice. Finally, the pellet was resuspended in 250 µl of freshly prepared 20 mM 2-mercaptoethanol, centrifuged and 100 µl injected directly on an ODS column (250 x 4.6 mm, 5 µm, Beckman, Fullerton, CA, USA). The mobile phase consisted of 10 mM sodium phosphate buffer with 2% acetonitrile, pH 3.0 at flow rate of 1.2 ml/min. 6-MP was detected at 323 nm.

Assay validation

The intra- and inter-assay variability of AZA and 6-MP assays were examined at two concentration levels and in a pooled plasma from patients treated with AZA. Standard samples were prepared by spiking blank plasma with known amounts of AZA and 6-MP and used for calibration curves. The capacity factor of each compound was calculated as $(t_r-t_0)/t_0$, where t_r is the retention time of the compound and t_0 is the retention time of the first distortion of baseline.

Assay specificity

As AZA is frequently coadministered with other immunosupressive drugs and antiinflammatory drugs, we examined possible chromatographic interferences with AZA and 6-MP. The following substances were injected without a sample preparation onto the column: cyclosporin, prednisolone, chloroquine, acetylsalicylic acid, diltiazem and nifedipine.

Analysis of data

The chromatographic data was collected and processed on a Macintosh Classic computer (Apple Inc., Chicago, IL, USA) equipped with Chromac 3.1. software (Drew Ltd, London, UK). AZA and 6-MP concentrations in patient samples were determined by comparing the response factor (i.e. ratios of their respective peak areas to that of the IS) of samples with those of standard curve determined from at least seven data points.

RESULTS AND DISCUSSION

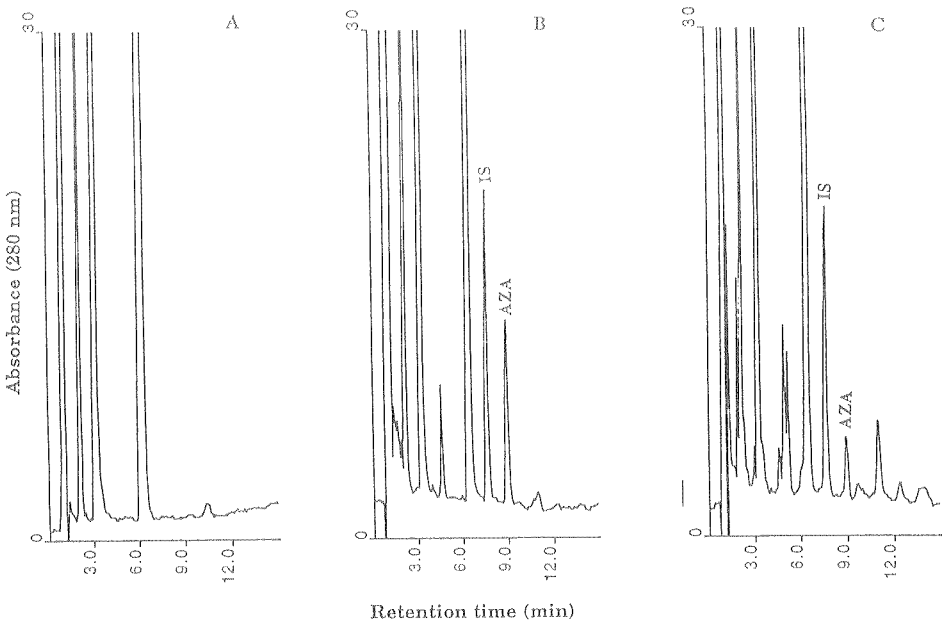
Specificity. Table 1 shows summary of the chromatographic conditions used in the present study. Figures 2A-C and 3A-C show chromatograms obtained from blank plasma, plasma spiked with AZA and 6-MP and their respective internal standards and plasma samples obtained from a patient receiving a single oral dose of AZA (150 mg). A satisfactory separation of substances and their respective IS from endogenous compounds was achieved in less than 20 minutes. Capacity factors (k') of AZA and IS were 10.3, and 8.5, respectively. The corresponding k' of 6-MP and IS were 2.9 and 5.8, respectively. Endogenous plasma components did not interfere with AZA, 6-MP and/or the used IS. Only a very small peak interfering with the IS of 6-MP was observed in plasma. However, the size of this peak was less than 3% of that IS and did not have any significant impact on the calculation of the drug concentration. None of the substances mentioned in Experimental was found to interfere with AZA and 6-MP and/or internal standards (not shown). It must be noted that coffeine elutes before guaneran (IS) and it may interfere with the measurement of IS at high concentrations. In such a case, a different mobile phase for the chromatographic column should be used (18 % methanol in 10 mM sodium phosphate buffer, pH 6.2).

Recovery. The drug recovery was determined by comparing spiked plasma samples with aqueous solutions of AZA, 6-MP and IS. Mean overall recoveries of AZA and 6-MP in the range 2-100 ng/ml were 79.0 ± 8.3% and 82 ± 6.9%, respectively. The IS guaneran and 6-mercaptopurine arabinoside had the recoveries of 92.0 ± 7.9% and 80.0 ± 7.4%, respectively.

Precision of the method. Table 2 shows the results of a precision study, both intra- and inter-assay variability for two levels of plasma AZA and 6-MP. The results show good precision (CV < 6 %) for both inter- and intra-assays. At lower concentrations of AZA and 6-MP (<2 ng/ml), the intra-assay variability was 15.8 and 7.5 %, respectively.

Table 1. Summary of conditions for HPLC analyses of azathioprine (AZA) and 6-mercaptopurine (6-MP).

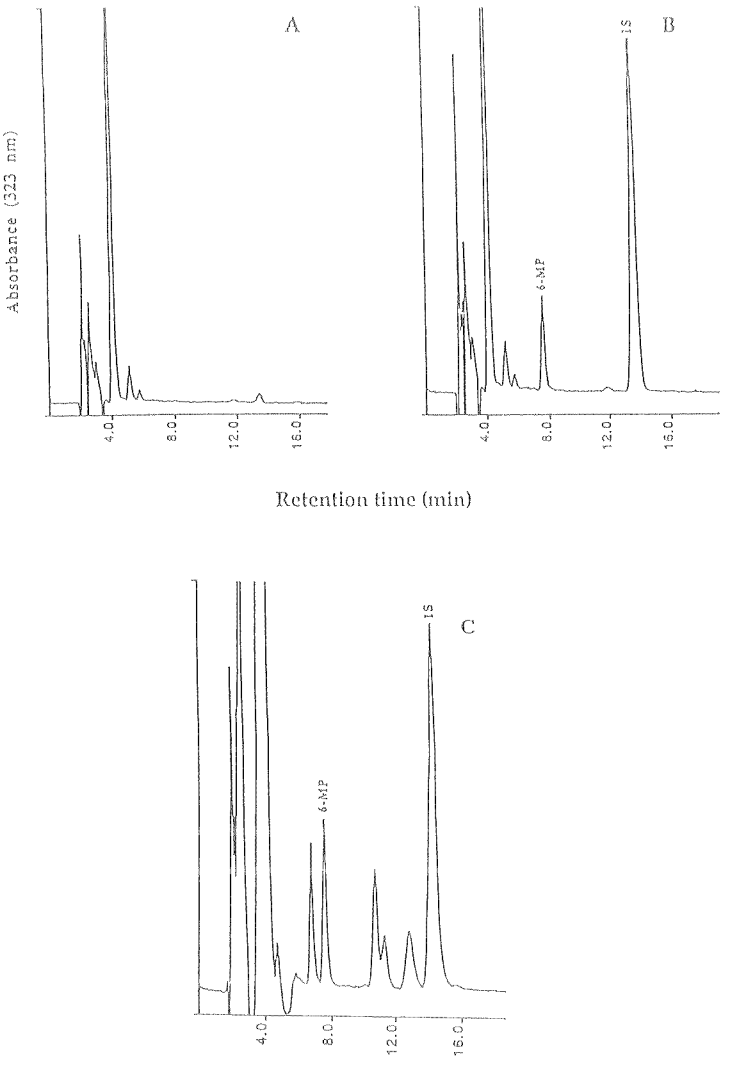
Parameter	Assay conditions for AZA	Assay conditions for 6-MP
Column	HSpecosphere 3CR C8 (80 x 4.6 mm, 3 µm)	ODS (250 x 4.6 mm, 5 µm)
Mobile phase	Sodium phosphate buffer (10 mM, pH 6.2), 9 % acetonitrile	Sodium phosphate buffer (10 mM, pH 3.0), 2 % acetonitrile
Temperature	Ambient	Ambient
Range (AUFS)	0.001	0.001
Flow rate	1.2 ml/min	1.2 ml/min
Wavelength	280 nm	323 nm
Internal standard	Guaneran	6-mercaptopurine arabinoside
Retention time	7.7 min for IS, 8.9 min for AZA	7.6 min for 6-MP, 13.6 min for IS



2. Representative chromatograms of (A) a blank plasma sample, (B) a blank plasma supplemented with 13.8 ng/ml of azathioprine (AZA) and 75 ng/ml of the internal standard (IS), and (C) a patient's plasma sample (3.9 ng/ml) collected 2 hours after a single oral dose of AZA to which 75 ng/ml of IS was added.

Limit of detection and quantitation. The smallest detectable quantity of AZA and 6-MP, defined as at least three times the baseline noise signal was about 0.2 ng for both substances injected onto the column. This amount corresponds to a concentration of 0.5 ng/ml of plasma. Lower concentrations could be quantitated by injecting larger sample volumes.

Linearity. The linearity of these methods was studied in spiked plasma solutions in the concentration range of 0.5-55 ng/ml for AZA and 1-200 ng/ml for 6-MP. The results from linear regression analysis were slope = 0.094, Y intercept = 0.015, and r = 0.999 for AZA (Figure 4A)



3. Representative chromatograms of (A) a blank plasma sample, (B) a blank plasma supplemented with 15.2 ng/ml of 6-mercaptopurine (6-MP) and 140 ng/ml of the internal standard (IS), and (C) a patient's plasma sample (26.5 ng/ml) collected 2 hours after a single oral dose of azathioprine (AZA) to which 140 ng/ml of IS was added.

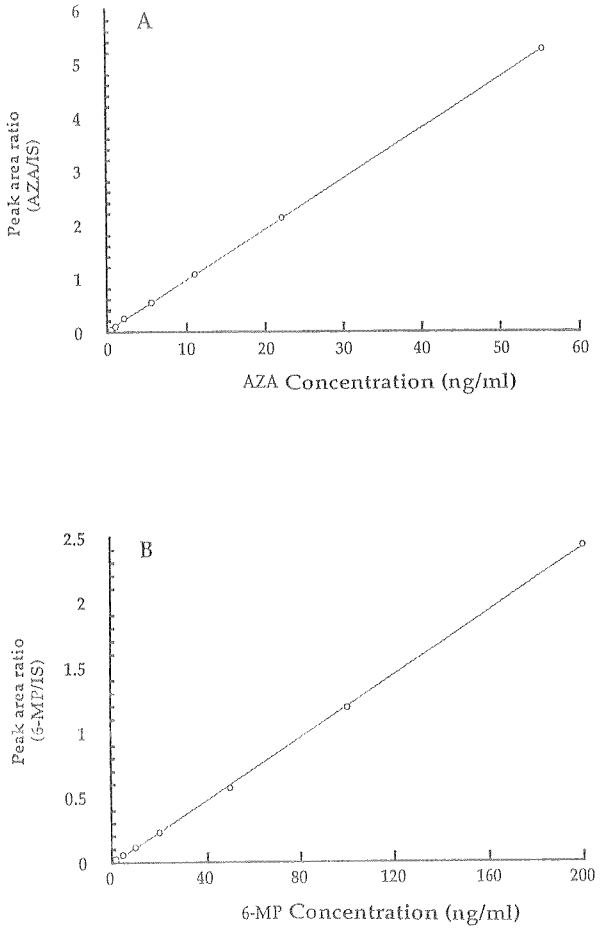
Table 2. Intra- and inter-assay variabilities of Azathioprine (AZA) and 6-mercaptopurine (6-MP) methods in blank human plasma and in plasma from patients treated with oral AZA.

	Spiked level ng/ml	Measured mean (ng/ml)	CV %	n
Intra-assay				
AZA	5.5	5.7	5.6	8
	55.0	58.9	2.7	8
	-	7.0*	5.8	10
6-MP	10.0	10.4	1.6	6
	100.0	109.5	2.3	6
	-	143.5*	3.6	8
Inter-assay				
AZA	5.5	5.6	1.9	5
	55.0	55.8	4.3	5
6-MP	10.0	10.21	5.0	5
	100.0	105.9	4.3	5

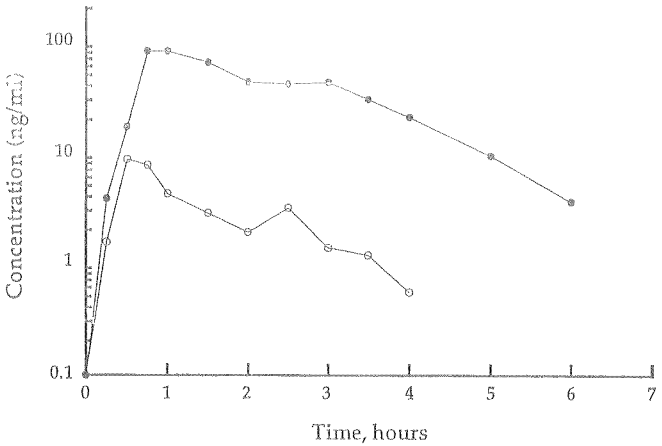
* pooled plasma from patients treated with AZA

and slope = 0.012, Y intercept = -0.010, and r = 0.999 for 6-MP (Figure 4B).

Clinical application of the method. An example of the disappearance of AZA and 6-MP in plasma studied over a period of 6 hours following an oral administration of 150 mg of AZA is shown in Figure 5. Plasma concentration versus time curve was fitted to a two compartment model using an iterative non-linear least-squares fitting program with a weighting factor 1. The absorption half-life of AZA was found to be 0.4 h. The elimination half-life and area under the plasma concentration versus time curve were 0.9 h and 16.8 $ng/ml^{-1}/h$, respectively. The corresponding values for 6-MP were 0.9 h and 218.9 $ng/ml^{-1}/h$, respectively.



4. Calibration curve for the assays of AZA (A) and 6-MP (B) in human plasma.



5. Semilogarithmic plot of plasma AZA (○) and 6-MP (●) concentration-time curve obtained in one patient with renal transplant after a 150 mg oral dose of AZA.

CONCLUSION

The plasma AZA and 6-MP extraction procedures used in the present study are simple and reproducible with low intra- and inter-assay variabilities. The choice of 6-mercaptopurine arabinoside as IS is superior to 6-thioguanine, since the latter has been found in plasma, urine and in erythrocytes as a minor metabolite. The limit of quantitation (0.2 ng) is sufficient for studying the pharmacokinetics and TDM of AZA in plasma during a period of 6 hours and that of 6-MP during an even longer period following the oral administration of a dose of AZA. Furthermore we suggest that this method may be evaluated for TDM in the clinical setting in a large scale.

ACKNOWLEDGEMENT

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ION-EXCHANGE CHROMATOGRAPHY AND ION-PAIR CHROMATOGRAPHY. COMPLEMENTATION OF HPLC ANALYSIS OF AMINO ACIDS IN BODY FLUIDS BY PRE-COLUMN DERIVATIZATION USING ORTHO-PHTHALDIALDEHYDE

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Abstract

The determination of free amino acids in some human body fluids using reversed phase high performance liquid chromatography and pre-column derivatization with ortho-phthaldialdehyde/mercaptoethanol is a highly sensitive and reproducible method. However not all amino acids can be determined using this easily automatizable method. Several special amino acids, as for example cystin which contains sulphur, can be determined using a separation procedure of ion-exchange and/or ion-pair chromatography. These separation procedures are described here.

Introduction

A variety of amino acids and different concentrations of these are to be found in various body fluids such as blood, urine, cerebrospinal fluid and in aminiotic fluid during pregnancy [1]. The concentration of the single amino acids depends upon sex, age and circadian rhythm and feeding and is therefore subjected to continual changes. In addition to these normal changes in the concentration of the amino acids, pathological changes can also occur, for example due to metabolic diseases and deficiency symptoms. Within the field of perinatal medicine the diagnostics can be supported by detecting pathological changes and these can be markers of certain insufficiencies [1].

High performance liquid chromatography (HPLC) is particularly well suited to quantitatively determine a number of amino acids and similar substances in biological samples parallel in a single analysis [1-3].

The separation procedure still used today, which has been superior over a longer period of time, is ion-exchange chromatography (IEC) [4]. Since direct detection is extremely difficult or very insensitive in many of these substances, a post-column derivatization with good chromophores or fluorescence-active substances was connected to the separation online. At the end of the 1970s experiments were also made with ion-pair chromatography (IPC) using post-column derivatization [4,5] even when this separation procedure was first described as a solvent-generated ion-exchange system or dynamic ion-exchange chromatography [4]. The separation procedure for amino acids used today is the reversed phase (RP) chromatography with pre-column derivatization [6,7]. Table 1 lists the pre-column and post-column derivatization methods.

Each of these derivatization or separation methods used has its advantages and disadvantages. A paper [23], which compares the four pre-column derivatization methods that are used most often, comes to the conclusion that favours the easily automatizable OPA procedure, despite its disadvantages, only primary amines and none containing sulphur. The two most important secondary amino acids, proline and hydroxyproline, can be determined through a combination of the derivatization with OPA and NBD in a second, 15 minute separation [26,27].

In this study two isocratic separation methods are described in detail, which were developed for the separation of arginine metabolites using ion-exchange [28,29] and ion-paired chromatography [30,31]. These methods are a good complement to the OPA and OPA/NBD procedure.

TABLE 1

Pre-Column and Post-Column Derivatization Methods for the Analysis of Amino Acids

Post-column derivatization	Pre-column derivatization
Fluorescamine [6]	6-Aminoquinolyl-N-hydroxysuccinimidylisocyanate, AQC [10-12]
Ninhydrin [6]	Diaminoazobenzeneisothiocyanate, DABTH [6]
ortho-Phthaldialdehyde [6,8]	Diethyl ethoxymethylenemalonate [13]
Sodium benzoxazole-2-sulfonate [6,9]	4-(Dimethylamino)azobenzene-4'-sulfonyl chloride, Dabsyl [6,7,11,14]
	1-Dimethylaminonaphthalene-5-sulfonyl chloride, Dansyl [6,7,11]
	Diphenylindonylisothiocyanate, ITH [6]
	9-Fluorenylmethyl chloroformat, FMOC [11,15]
	Fluorescamine [7]
	Fluorodinitrobenzene, DNP [6,7,16]
	7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole, NBD [6,11]
	Methylisocyanate, MTH [6]
	Naphthalene-2,3-dicarboxaldehyde, NDA [11,17]
	ortho-Phthaldialdehyde, OPA [6,7,11,18]
	Phenylisothiocyanate, PTH, PITC, PTC [6,10,11,19-21]
	Sodium benzoxazole-2-sulfonate [22]

Materials

Chemicals

The amino acid standards (listed in table 2) and the albumine from bovine were supplied by the firms Sigma Chemie GmbH, Deisenhofen, Merck, Darmstadt, and Fluka, Neu-Ulm, all from Germany. The derivatization reagents were supplied by Merck, Darmstadt, and Fluka, Neu-Ulm (both from FRG).

The buffer substances used (sodium dihydrogen phosphate, sodium acetate), acids (perchloric acid, boron acid, ortho-phosphoric acid, sulphuric acid and hydrochloric acid), salts (sodium chloride) and the basis used (sodium hydroxide) and also the ion-pair reagent (heptane

sulphonic acid sodium salt monohydrate) all came from the firms Merck, Darmstadt, and Fluka, Neu-Ulm, both FRG.

Methanol with purity grade pro analysis is a product of the firm Merck, Darmstadt, or the firm Fluka, Neu-Ulm, FRG. The "destilled" water used was tap water, since it has been found to be UV purer compared to the bought product. This water is only deionized.

Body Fluid Samples

The amniotic fluid was collected during labour. The blood samples were withdrawn from the umbilical cord after delivery. These samples were stored in a refrigerator over a short period of time or were placed uncentrifuged in a deep freeze at approx. -20°C.

HPLC Equipment

The apparatus for the RP chromatography with amino acids derivatized with OPA is made up from pieces of equipment acquired from the following producers: the ERC Gesellschaft für den Vertrieb wissenschaftlicher Geräte m.b.H., Alteglofsheim (degasser type 3510), "Gynkotek" Gesellschaft für den Bau wissenschaftlich-technischer Geräte mbH, Germering bei München (autosampler Gina 160, fluoresence detector type RF 1001 and a C-R3A integrator with keyboard, monitor and floppy disk drive) and Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin (programmer 50B, two pumps type 64.00, a 6-port automatic valve with selected use of either automatic or manual injection, an injection valve with built-in reed relay and a column oven with integrated control unit). The separation column used first was an OPA-special column with a coarseness of 5µm and dimensions of 250mm x 4.6mm from the firm Knauer and later a Nova-Pak C18 also with 5µm and 3.9mm x 300mm from Waters, Eschborn, all firms FRG. As a protection for these columns, pre-colums with dimensions of 10-20mm x 4mm filled with C18 resp. ODS were set up and were changed every week or every two weeks.

The modular apparatus for the ion-exchange chromatography consists of a pump model 300C from the firm Gynkotek, Germering, an injection valve, a column oven type 89.00 and a variable wavelength monitor type 87.00 from Knauer, Berlin, an integrator HP 3394 from the firm Hewlett Packard, Waldbronn, all FRG, and a flowmeter from Phase Separations Limited, Queensferry Clwyd, U.K. The columns used from the firm Knauer, Berlin, FRG, are filled with Nucleosil

100-5 SA. We either used two serially operated columns with dimensions of 30mm x 4.6mm and 250mm x 4mm (combination A) or two columns 250mm x 4mm (combination B).

The ion-pair chromatography apparatus contains a programmer 50B, a pump type 64.00, an injection valve and a column oven as column space from the firm Knauer, Berlin, a digital thermometer HP 2802A with PT 100 sensor from Hewlett Packard, Waldbronn, and a spectralphotometer UVD/160-2 and a C-R3A integrator with keyboard, monitor and floppy disk drive from the firm Gynkotek, Germering. We used either the separation column with a length of 250mm and an ID of 4mm filled with Eurospher 100-C8, 5µm supplied by Knauer, Berlin, or another column, 125mm in length, ID 4.6mm filled with spherisorb ODS II, 5µm supplied by Melz VDS GmbH, Berlin, all firms FRG.

Additionally "operation boxes" are included in the equipment for the RP and ion-pair chromatography, which were developed and constructed by the engineer from our working unit. An auto-zero function is built into these "operating boxes", which the detectors used do not have. Furthermore a pump control is integrated into the automatized apparatus, which switches the autosampler on hold when the column counter pressure sinks.

Other Equipment

A digital pH-meter from the firm Knick Elektronische Messgeräte GmbH & Co., Berlin, is used for measuring the pH with an electrode type 405 from Ingold Messtechnik GmbH, Steinbach im Taunus. The temperature controlling of the solutions is done with a thermostat type E3 from Haake, Berlin, and a Thermomix 1419 bath 4K from B. Braun, Melsungen AG, Melsungen.

The centrifuges used are a model Labofuge A with an angled rotor, 12 x 15ml glasses from the firm Heraeus-Christ, Osterode am Harz, and a model 3200 from Eppendorf Gerätebau Netheler+Hinz GmbH, Hamburg.

We used a spectrophotometer model 25 with matching recorder model 24-25ACC from Beckmann Instruments GmbH, München.

An ultrasonic bath Sonorex type RK 106 S from Bandelin electronic GmbH & Co. KG, Berlin, was used for homogenizing and degassing the eluents and solutions. For mixing the samples for preparing the injection solutions and for manual experiments of derivatization reactions, a mixer type 750.01 REAX 1 D from Heidolph-Elektro GmbH, & Co.,KG, Kelheim, likewise all FRG, was used.

Methods

Standard Solutions

All the standard solutions were deep frozen in portions to increase their durability and were stored at about -20°C. When there was a deposit volume of 10ml 400mg albumin as matrix and 90mg sodium chloride to improve the solubility later was added [27]. The concentrations of the single amino acids in solution 1 (table 2 shows the composition) - with the exception of ASN (250µmol/l), GLY (350µmol/l), MET (235µmol/l) and THR (210µmol/l) - lie within the range 185 to 200µmol/l. The composition of the concentrations in the three other solutions was selected according to the various UV activities at 268nm. The concentrations of the single amino acids are kept at almost the same in all the three solutions. The concentration of INO amounts to about 50µmol/l, ARG(N0) 40µmol/l, CYS 200µmol/l, HYT 80µmol/l, TRP 65µmol/l, L-NAME 50µmol/l, KYN 125µmol/l and ADE 20µmol/l.

OPA Method [24-27]

Before the samples can be derivatized they have to be deproteinized. As the derivatization takes place in the basic medium, deproteinizings with the addition of an acid [32] are unsuitable. The simple way is to add methanol, which can be added to HOS as "internal" standard [27]. 200µl of the sample, respectively 200µl of the dissolved, deep frozen standard solution is diluted with 800µl methanol, mixed and finally centrifuged with the Eppendorf 3200. Then 200µl are removed, either by manual or automatic derivatization, diluted with 80µl sodium borate solution pH 9.5 and 60µl OPA reagent (50mg OPA in 4.5ml methanol absolute with 0.5ml sodium borate solution pH 9.5 and 50µl 2-mercaptoethanol) and mixed. After a reaction time of about 3.5 minutes 25µl 0.5N hydrochloric acid is added as stop reagent and mixed together. Using the manual method an additional dilution of 1 to 4 is made with acetate buffer (eluent). It is not possible to do this with the autosampler Gina 160. The injection volume amounts to 20µl.

The column is heated to 40°C. The amino acids are separated by a flow of 1.0ml/min with a methanol gradient in sodium acetate buffer, 50mmol/l and pH 7.0. They start with 15% methanol. The gradient continues to increase linearly to 20% in 30 minutes, to 35% at 45 minutes and to 75% at 70 minutes. Then the methanol content remains constant for 5 minutes before it drops again to 15% within 5 minutes. The detector is regulated at 330nm excitation and 450nm emission. The duration of the chromatogramme lasts for 75 minutes.

OPA/NBD Method [26,27]

Preparation of the samples is the same as for the pure OPA method, that is, the same deproteinized solution can be used. Using a fresh deposit the methanol can contain the "internal" standard dehydroproline. 50µl of this deposit is mixed together with 250µl sodium borate solution and 50µl sulphur-free OPA solution (50mg in 4.5ml methanol and 0.5ml borate solution) and warmed at 60°C for 2 minutes. Then 25µl chlornitrobenzofurazan solution (50mg in 5ml methanol) is added, mixed and kept at a temperature of 60°C for 10 minutes. 120µl 0.5N hydrochloric acid are added to terminate the reaction. Then 20µl are injected.

The eluents used are the same as those used for the OPA method. The columns are also heated to 40°C. We start with 10% methanol at a flow of 1ml/min. The gradient increases linearly to 30% within 10 minutes, then a further 5% in the next 2 minutes, finally reaching 50% at 15 minutes. Then the methanol content is reduced to 10% within 5 minutes. A chromatogramme takes 20 minutes. The detector is regulated at 470nm excitation and 550nm emission.

Ion-Exchange Chromatography

This isocratic method was developed for the separation of arginine metabolites at room temperature with an eluent which contains 200mmol/l buffer and 10vol.% methanol with a pH of 2.3 [28,29].

The deep frozen standards are dissolved in 1ml water. For deproteinizing 250µl standard or sample solutions are added to 25µl approx. 4.5 molar perchloric acid, mixed and centifuged. 20µl are injected from the excess. The 30 minute separation is done isocratically with 25mmol/l, when not stated otherwise, sodium dihydrogen phosphate in water with 12.5vol.% methanol with a pH value of 2.3 at 35°C column temperature and 0.75 or 1.0ml/min according to the column combination. Detection was made at 268nm.

Ion-Pair Chromatography

This isocratic method was also developed for the separation of arginine metabolites [30,31]. The preparation of the sample is identical to that of the ion-exchange chromatography. The eluent is composed of water with 10 vol.% methanol and 18.5mmol/l heptane sulphonic acid sodium salt as ion-pair reagent. At room temperature a separation with a flow of 1.5ml/min lasts 30 (C8) or 45 to 60 (ODS) minutes. The UV detector is regulated at 268nm.

TABLE 2

Composition of the Standard Solutions of the Amino Acids

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
"Adenine"	ADE		X	X	
L(+)-Alanine	ALA	X			
L-2-Aminoadipic acid	AAD	X			
L-2-Aminobutyric acid	ABA	X			
3-Aminobutyric acid	b-ABA	X			
4-Aminobutyric acid	g-ABA	X			
L-Arginine hydrochloride	ARG	X			
L(-)-Asparagine	ASN	X			
L-Aspartic acid	ASP	X			
L-Citrulline	CIT	X			
L-Cystine	CYS	X	X	X	
3,4-Dehydro-DL-proline	DHP				
L-Glutamine	GLN	X			
L-Glutatic acid	GLU	X			
L-Glutathione	GSH	X			
L-Glycine	GLY	X			
L-Histidine hydrochloride	HIS	X			
L-Homoserine	HOS				
5-Hydroxy-L-lysine hydrochloride	HYL	X			
4-Hydroxy-L(-)-proline	HPR	X			
5-Hydroxy-L-tryptophan	HYT		X	X	X

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
Inosin	INO	X	X	X	
L(+)-Isoleucine	ILE	X			
L-Kynurenine	KYN		X	X	
L-Leucine	LEU	X			
L-Lysine monohydrocloride	LYS	X			
L-Methionine	MET	X			
N-Methyl-L-arginine acetate	ARG(ME)				
3-Methyl-L-histidine	HIS(ME)				
N-Nitro-L-arginine	ARG(NO)		X		X
N-Nitro-L-arginine methylester	L-NAME		X		X
L-Ornithine monohydrochloride	ORN	X			
L(-)-Phenylalanine	PHE	X			
ortho-Phosphoserine	P-SER				
L(-)-Proline	PRO	X			
L-Saccharopine	SAC	X			
L(-)-Serine	SER	X			
Taurine	TAU	X			
L(-)-Threonine	THR	X			
L-Tryptophan	TRP	X	X	X	
L(-)-Tyrosine	TYR	X			
L(+)-Valine	VAL	X			

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Composition of the Standard Solutions of the Amino Acids

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
"Adenine"	ADE		X	X	
L(+)-Alanine	ALA	X			
L-2-Aminoadipic acid	AAD	X			
L-2-Aminobutyric acid	ABA	X			
3-Aminobutyric acid	b-ABA	X			
4-Aminobutyric acid	g-ABA	X			
L-Arginine hydrochloride	ARG	X			
L(-)-Asparagine	ASN	X			
L-Aspartic acid	ASP	X			
L-Citrulline	CIT	X			
L-Cystine	CYS	X	X	X	
3,4-Dehydro-DL-proline	DHP				
L-Glutamine	GLN	X			
L-Glutatic acid	GLU	X			
L-Glutathione	GSH	X			
L-Glycine	GLY	X			
L-Histidine hydrochloride	HIS	X			
L-Homoserine	HOS				
5-Hydroxy-L-lysine hydrochloride	HYL	X			
4-Hydroxy-L(-)-proline	HPR	X			
5-Hydroxy-L-tryptophan	HYT		X	X	X

TABLE 2

Continuation

Amino acid	Abbreviation	Solution-No.			
		1	2	3	4
Inosin	INO	X	X	X	
L(+)-Isoleucine	ILE	X			
L-Kynurenine	KYN		X	X	
L-Leucine	LEU	X			
L-Lysine monohydrocloride	LYS	X			
L-Methionine	MET	X			
N-Methyl-L-arginine acetate	ARG(ME)				
3-Methyl-L-histidine	HIS(ME)				
N-Nitro-L-arginine	ARG(NO)		X		X
N-Nitro-L-arginine methylester	L-NAME		X		X
L-Ornithine monohydrochloride	ORN	X			
L(-)-Phenylalanine	PHE	X			
ortho-Phosphoserine	P-SER				
L(-)-Proline	PRO	X			
L-Saccharopine	SAC	X			
L(-)-Serine	SER	X			
Taurine	TAU	X			
L(-)-Threonine	THR	X			
L-Tryptophan	TRP	X	X	X	
L(-)-Tyrosine	TYR	X			
L(+)-Valine	VAL	X			

When the apparatus has not been in use the columns are previously washed with buffer and salt-free eluents, that is water or methanol/water.

Results and Discussion

OPA Method

The separation of standard solution 1 is illustrated in figure 1, as it is used for calibration. 28 of the 31 amino acids present can be detected. Additionally HOS was added as "internal" standard to the standard solution. Figure 2 shows a separation of the primary amino acids in amniotic fluid. Not all the substances present in the standard can be found. When the integrator is regulated to a higher sensitivity some small peaks are still visible, which could stem from AAD, g-ABA and HYL. Furthermore 7 peaks not assigned to the standard, can be found as unknown peaks. It cannot be ruled out that these are amino acids such as P-SER, ARG(ME) and HYT for example; but they could also be so-called biogenic amines or their derivates or other amino-functional substances. The concentrations of these substances, the 3 amino acids and the 7 unknown ones, must lie below 1µmol/l, since this is approximately - though it varies according to the specific substance - the threshold limit of the chromatogramme illustrated in figure 2.

OPA/NBD Method

Figure 3 shows the separation of the secondary amino acids according to the OPA/NBD method for the same samples illustrated in figures 1 and 2. Here an "internal" standard, dehydroproline, can also be included, although this is not absolutely necessary, since the reagent peak can be used as such. These chromatogrammes show several peaks which cannot be assigned to the standard and cannot be identified. Possibly a second proline peak and an arginine one can be seen.

Both these methods run automatically in so far as the sample apparatus performs the whole OPA derivatization and functions as automatic injector for the durable OPA/NBD derivates. Only when the GINA 160 apparatus is in action with the OPA method, there is the disadvantage that no further dilution can finally be performed, which not only shortens the life of the pre-columns but also that of the main column as well.

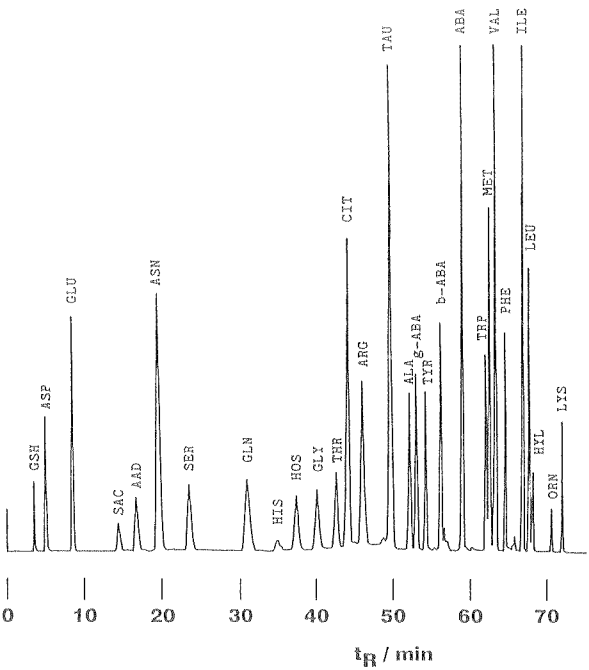

FIGURE 1: Chromatogramme of a manually OPA derivatized standard solution 1 on Nova-Pak C18, attenuation 512mV full scale, abbreviations see table 2 and t_R - retention time

With these two methods it is not possible to determine inosine or either of the amino acids cysteine and cystine which both contain sulphur.

Ion-Exchange Chromatography

The separation for arginine metabolites only, which was developed by Raberger and co-workers [28,29], is achieved with a buffer concentration of 200mmol/l and a pH of 2.3 at room temperature. The injection of an amniotic fluid sample produced several peaks which could not be assigned to the arginine metabolites. In addition to the arginine metabolites, ARG(NO) and L-NAME, 6 further amino acids could be detected. More detailed investigations and single injections of all of the 40 amino acids present, resulted in INO, CYS, HYT, TRP, KYN and ADE being found as well, whereby in actual fact adenine is not really an amino acid, but can occur in body fluids.

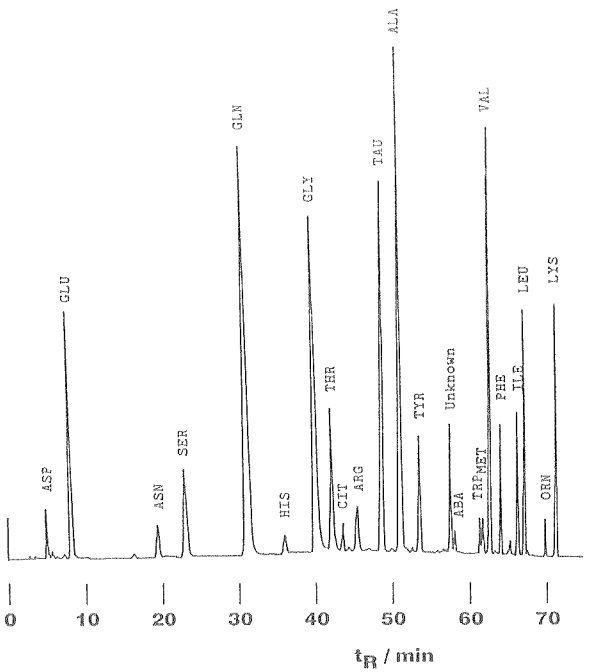

FIGURE 2: Separation of the primary amino acids of manually OPA derivatized, deproteinized amniotic fluid (No. 140) on Nova-Pak C18, attenuation 512mV full scale

On account of spectroscopic examinations in UV the concentrations in the standards were selected in such a way that the resulting peaks could possess approximately the same size peak surface, or that no single peak could be extremely dominant. For each of the 8 substances all of the same weight, extinctions were measured at 268nm - the detection wavelength - between 0.04AU (CYS) and 0.82AU (ADE). The molar extinction coefficients determined approximately from these measurements lie in the range of about 2000 to 25000 liter pro mol and centimeter.

Very soon it was apparent that the quality of the separation depends very much on the pH. A mere fluctuation of 0.05 to 0.1 in the pH level worsens the separation drastically. In our laboratory the room temperature changes quite considerably at times with a fluctuation of up to a maximum of 10°C, and therefore the pH level could not be held stable. The only way to compensate this and bearing in mind the apparatus at our disposal, was to keep the columns at a con-

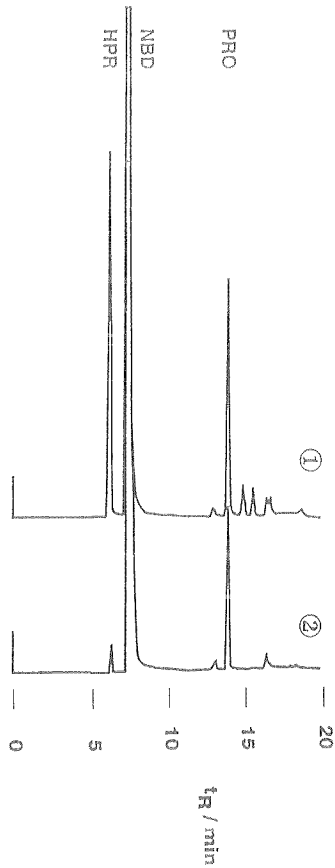

FIGURE 3: Separation of the secondary amino acids of an OPA/NBD derivatized standard solution 1 (①) and amniotic fluid (No. 140,②) on Nova-Pak C18, attenuation 16mV full scale, abbreviations see table 2 and t_R - retention time

stant temperature, in this case at 35°C. Figure 4 in three sets of measurements shows how the pH level for the eluent with 200mmol/l buffer and 10vol.% methanol is dependent on the temperature. As a result of this, the pH level of the eluent is regulated at the prevailing room temperature, which at 35°C gives a pH of 2.3.

The increase in temperature also caused a strong reduction in the retention times, so that the buffer concentration had to be decreased. Figure 5 shows the dependence of the retention

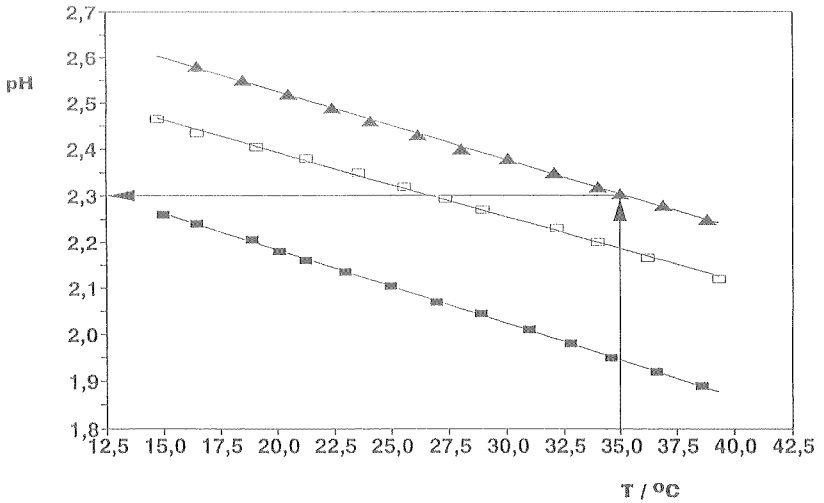

FIGURE 4: Temperature dependence of the pH level of the sodium dihydrogen phosphate solution, 200mmol/l in water with 10vol.-% methanol

times on the buffer concentration for column combination A. Next time and thereafter we used a buffer concentration of 25mmol/l. Furthermore we examined the influence of the methanol amount. Whilst in some of the substances the retention times are almost dependent on the amount of methanol, in others they decrease by up to 16% with an increase of the methanol content from 7 to 13%. In addition the width of the peak becomes smaller.

Figure 6 illustrates the chromatogrammes of the standard solutions 2 to 4. The chromatogrammes contain two intricate separation problems, as can also be seen in figure 5. These are the pairs of substances CYS/ARG(NO) and KYN/L-NAME which are not always separated. In this deposit used from standard solution 2, the results of the spectroscopic measurements were not yet included.

The columns used deactivate within the course of time. Figure 7 shows two chromatogrammes of standard solution 2, which were made under the same conditions at an interval of two months of each other. The retention times are reduced in all the substances and under three peaks in the second chromatogramme there are always 2 substances. We have not been successful in regenerating or reactivating the columns by rinsing with other fluids.

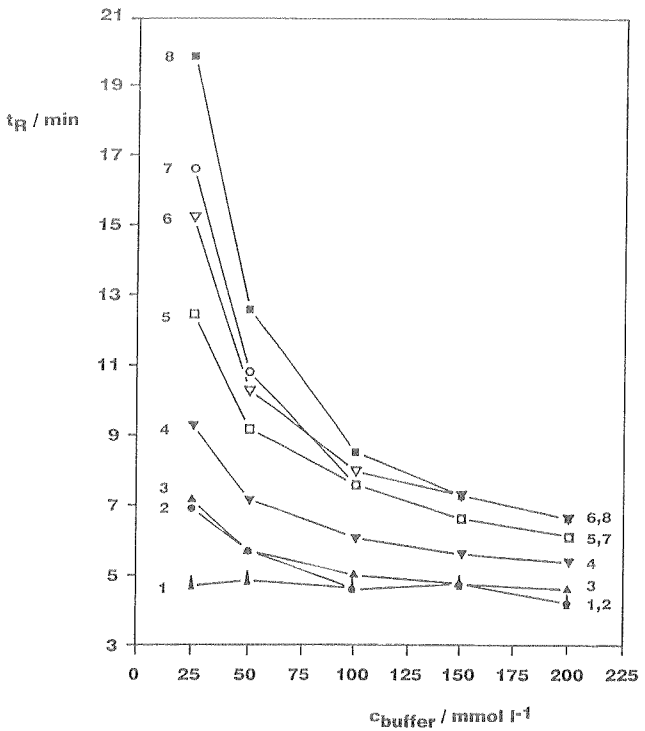

FIGURE 5: Dependence of the retention times t_R of the substances in standard solution 2 of the buffer concentration c_b of the eluent with 13vol.% methanol at 35°C;
1) INO, 2) CYS, 3) ARG(NO), 4) HYT, 5) TRP, 6) KYN, 7) L-NAME and 8) ADE

Figure 8 shows how the separation method is used on an amniotic fluid sample (No. 114) and an umbilical cord blood sample (No. 2). In the case of the amniotic fluid a clear assignment of TRP (11.45min) and CYS (6.25min) is possible. The peak at 4.3min could stem from INO without a sign whether only INO or other substances as well form this peaks. Similar situations occur with the blood sample. TRP (12.1min) and CYS (7.45min) can clearly be assigned over the retention time comparisons. All the other peaks cannot be assigned alone. These two chromatogrammes show characteristic peak distributions in the front retention time range for amniotic fluid - here 2 peaks up to 6 minutes - and umbilical cord blood - 5 peaks here up to 7

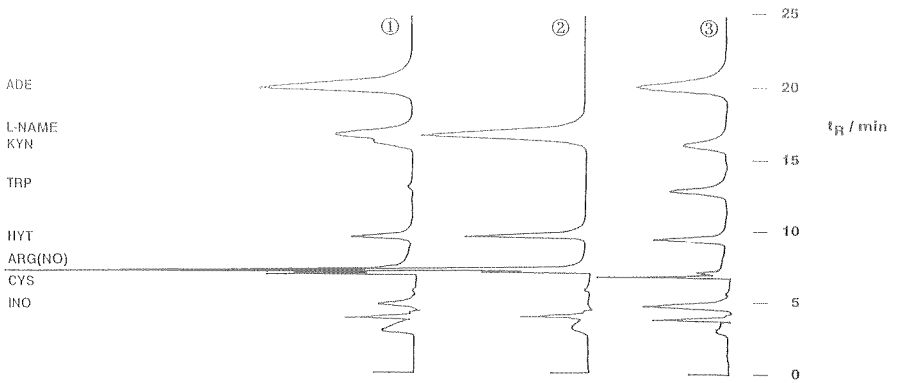

FIGURE 6: IEC: Chromatogrammes of the standard solution 2 (①), 4 (②) and 3 (③) on the column combination A, eluent 25mmol/l buffer in water with 11vol.% methanol, attenuation 64mV full scale, abbreviations see table 2 and t_R - retention time

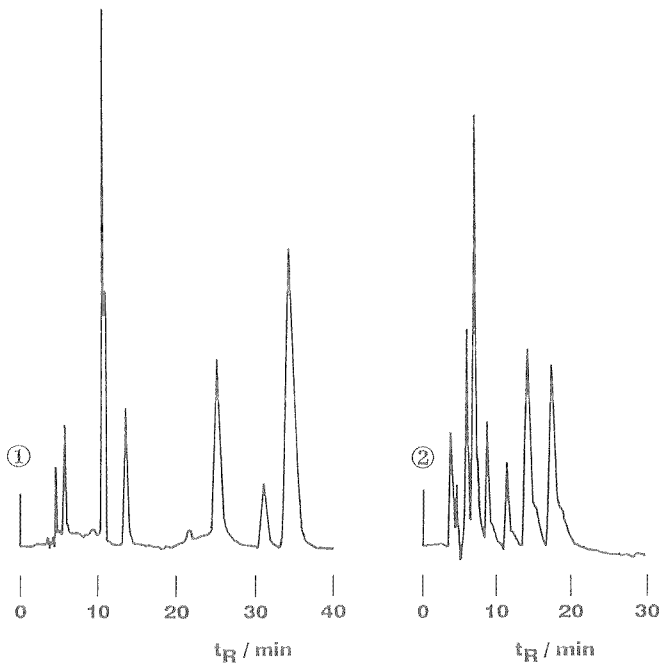

FIGURE 7: Deactivation of the ion exchange columns within 2 months (① ⇒ ②), with the example of the separation of standard solution 2 on combination B, eluent with 50mmol/l buffer and 12.5vol.% methanol, attenuation 64mV full scale

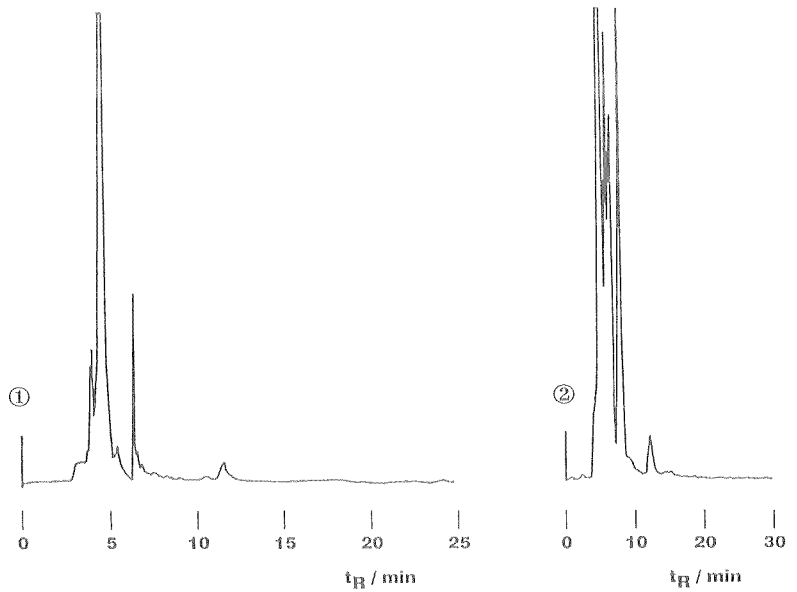

FIGURE 8: IEC: Chromatogramme of amniotic fluid (No. 114,①), combination A with 25mmol/l buffer with 12.5vol.% methanol and an umbilical cord blood sample (No. 2,②), combination B with 32.5mmol/l buffer and 12.5vol.% methanol, attenuation 64mV full scale

minutes. The above mentioned deactivating of the columns which is so fatal for proving the calibration hardly plays a part in this problem, for the substances are mostly present in such a very small concentration that nevertheless 2 peaks can be detected again. It is possible to make a quantitative evaluation according to the concentration when the peak is clearly identified. Difficulties only occur in the CYS peak as this represents two substances, dimeric cystine and monomeric cystein, which however produces an even smaller molar extinction coefficient. In the chromatogrammes of amniotic fluid and blood further peaks can occur which cannot be identified as amino acids, which is not otherwise to be expected when using mixtures containing so many different substances. In some of the amniotic fluid samples kynurenine was found in very small amounts. If the samples contain a lot of TRP then they can be compared with the quantitative determination using OPA.

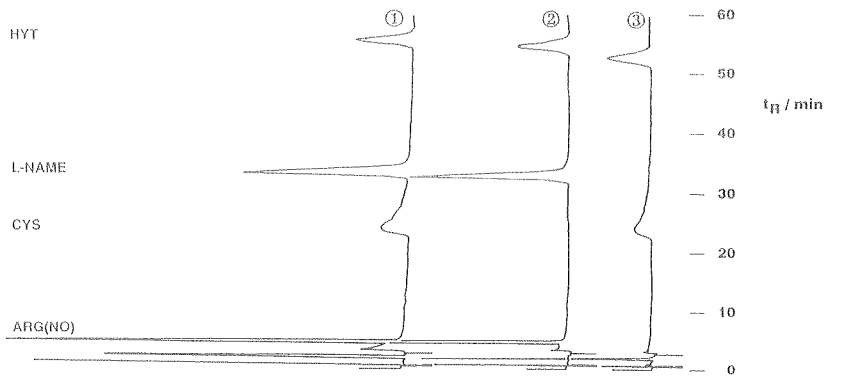

FIGURE 9: IPC: Chromatogrammes of standard solution 2 (①), 4 (②) and 3 (③) on the column Spherisorb ODS II, attenuation 16mV full scale

Ion-Pair Chromatography

The chromatogrammes of the 3 standard solutions from 2 to 4 on the Spherisorb ODS II column are shown in figure 9. With this method in addition to the arginine metabolites CYS and HYT can also be determined. The other substances achieve the peak at 2.1 minutes so that a quantitative evaluation is not possible. When the Eurospher C8 is in use there is no change in the succession of the retention, only the times are about half as long. With this method too, the columns are liable to deactivate after some time. In the case of the ODS columns the deactivation happens very much more quickly than in the RP8, as the three analyses performed after each other within three hours already show in figure 9. The retention time of HYT decreases by 2.2 minutes. In this case the deactivation is reversible. The columns can be reactivated again by rinsing with water, then methanol, then dichlormethane/tetrahydrofurane (1:1, v:v), methanol and water. Sometimes it is enough just to wash the equipment with water so that for example it can be switched off over night.

As in the ion-exchange chromatography, characteristic differences can be observed here too in the chromatogrammes for amniotic fluid and blood (see figure 10). These samples are identical with those in figure 8. A peak of cystine can clearly be seen in both samples. It is not possible to assign or identify the peak up to 5 minutes retention time. When the samples contain hydroxytryptophane it is then possible to check the concentrations determined with the IPC

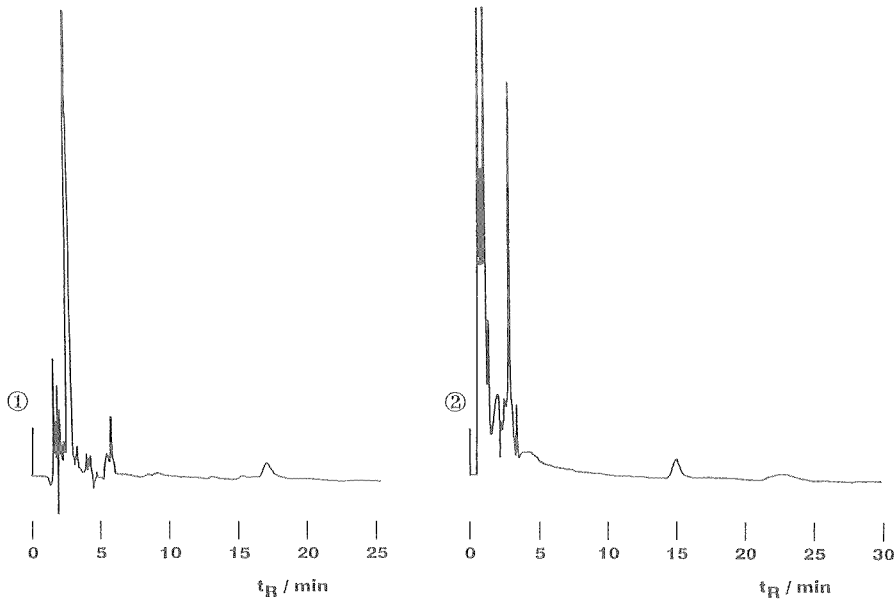

FIGURE 10: IPC: Chromatogramme of an amniotic fluid (No. 114,①), column Eurospher C8 and an umbilical cord blood sample (No. 2,②) on Spherisorb ODS II, attenuation 16mV full scale

method with the IEC method. In further measurements of amniotic fluid samples a discrepancy emerged in the concentrations determined for CYS using IEC and IPC. The concentrations determined with IPC were larger. A more detailed investigation showed that the peak assigned to cystine contains two substances. In addition to CYS, UV-activative tyrosine appears at this point as well. However, since TYR is determined using the OPA method, it is possible to calculate the concentration of cystine.

Both the methods, IEC and IPC, of determination of special amino acids are less sensitive due to UV detection at 268nm and have higher detection limits. Also peak assignment or identification is partially not possible. This aspect could be improved by using a diodenarray detector, since the few UV-active amino acids could then probably be identified through the spectra. If lower detection limits are required, then a post-column derivatization has to be used.

Conclusion

The two methods for determining special amino acids using IEC and IPC described here are suitable as an appropriate complement to the determination of amino acids with OPA and/or OPA/NBD. In this way in addition to the arginine metabolites, adenine and the other amino acids cystine, kynurenine, hydroxytryptophane and - to a limited extent - inosine and cystein can be determined. These methods are of interest when these amino acids do not have to be determined all the time, otherwise it is more appropriate to use another derivatization method for the determination of amino acids.

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THERAPEUTIC MONITORING OF TACROLIMUS (FK 506) USING LIQUID CHROMATOGRAPHY. A REVIEW

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Abstract

Tacrolimus (FK 506) is an macrolide immunosuppressive agent which is widely used for management of transplant patients. The drug is potent at concentrations well below the concentration of cyclosporine for patient management, but like cyclosporine, FK 506 also shows side effects such as nephrotoxicity and neurotoxicity. Because FK 506 exhibits wide variation in pharmacokinetics and a narrow therapeutic range, therapeutic monitoring of the drug is essential. Concentration of FK 506 can be monitored by high performance liquid chromatography (HPLC), Enzyme Linked Immunoassay (ELISA), HPLC-ELISA, HPLC-Mass Spectrometry and Fluorescence Polarization (FP) Immunoassay.

FK 506, The Discovery

The immunosuppressive agent Tacrolimus (FK 506, Prograf, Fujisawa Pharmaceutical Company, Osaka, Japan) was isolated from fermentation broth of Streptomyces tsukubaensis in 1984 (1,2). The stain was named as tsukubaensis because the stain was isolated from a soil sample of Tsukuba, Ibaraki, Prefecture, Japan. The drug was recently named as tacrolimus. The drug was demonstrated to posses

immunosuppressant properties by inhibiting interleukin-2 (IL-2) production in vitro and also inhibited the response of mixed lymphocyte culture at concentrations even 100 times lower than the widely used immunosuppressant, cyclosporine (3). In 1989, the drug was tested on a liver transplant recipient who did not respond to the immunosuppressant therapy using cyclosporine (4). The investigators of the University of Pittsburgh found FK 506, to be a very effective drug for immunosuppression with relatively few side effects, but the investigators in United Kingdom found the drug very toxic for management of transplant patients. The apparent discrepancy was found later due to lack of proper knowledge of pharmacokinetic parameters of the drug as well as lack of proper therapeutic drug monitoring. Large clinical trial of FK 506 was started in 1989 for patients undergoing chronic liver allograft rejection. Currently, the drug is approved by Federal Drug Administration (FDA) for management of patients receiving liver allografts.

Following the first international congress on FK-506, the Pittsburgh group reported 10 % improvement in patient survival, 16 % improvement of graft survival, and 21% lower rejection rate in liver transplant patients with replacement of cyclosporine by FK 506 (5). In addition, FK 506 can reverse a rejection episode where cyclosporine is ineffective (6). In kidney transplant patients, use of FK 506 as an immunosuppressant, eliminated the need of using prednisone or antihypertensive agents. Fk 506 also showed promises for use in heart transplant patients (7). The immunosuppressant activity was found to be effective as concentrations at least ten times lower than cyclosporine.

Chemical Structure and Pharmacology

FK 506 is a 23 membered macrolide lactone which is neutral, hydrophobic and crystallizes as colorless prisms. The molecular weight of the compound is 803. The structure includes a hemiketal function and an alpha, beta diketonamide group. Unlike cyclosporine, FK 506 is soluble in alcohol. The drug is also soluble in acetone, ethyl acetate, acetonitrile, dichloromethane, chloroform etc but insoluble in water. The drug is present in two conformational forms in organic solvent and the relative amounts of

each conformer depends on the temperature. The drug is quite stable at room temperature for many months in crystalline form, but is less stable in solution (1, 8,9). The structure of FK 506 is entirely different from the structure of cyclosporine which is cyclic peptide containing 11 amino acids. However, FK 506 has structural similarity with potential immunosuppressant rapamycin, and both contains pipecolic acid moiety. However, the mechanism of action of FK 506 is different from rapamycin.

FK 506 can be administered as oral dose intravenous injection. The oral bioavailability of the drug is variable (5-67%) due to poor and erratical absorption. However, unlike cyclosporine, absorption of FK 506 is less dependent on the availability of bile. After absorption, the drug is rapidly distributed outside plasma compartment and the volume of distribution ranges from 5 to 65 L/Kg (10). In plasma, the drug is largely bound to alpha-1 acid glycoprotein (11) and in whole blood FK 506 is mainly associated with erythrocytes. The half life of the drug can vary between 3.5 to 40 h with a mean of 11.3 h

The rapid clearance of FK 506 compared to cyclosporine is related to liver not renal function. In rat model, FK 506 was found to distribute more rapidly than cyclosporine. Coadministration of erythromycin causes accumulation of parent drug while administration of steroids lead to accumulation of inactive metabolites of FK 506.

FK 506 is metabolized by liver and small intestinal microsomes containing cytochrome P-450 3A and the fast pass metabolism by liver may be important in the disposition of the drug (12). Following metabolism, approximately 95 % of the drug is eliminated by biliary route mainly as metabolites and about 5% are excreted in urine unchanged. The drug is extensively metabolized to several metabolites including O-demethylated, hydroxylated, O-demethylated hydroxylated and dihydrodiol metabolites (13-14). Recently, the 15-demethylated, 13-demethylated and double demethylated metabolite from erythromycin-induced human liver microsome has been reported (15-16). Georges et al also recently reported the isolation of 15 desmethyl FK 506 and 15, 31-desmethyl FK 506 from human liver microsomes which retain in vitro immunosuppressive activity, using

fast atom bombardment mass spectrometry and nuclear magnetic resonance spectrometry (17). FK 506 should be used with caution in patients receiving microsomal enzyme inducer or inhibitors. FK 506 itself also inhibits the activity of cytochrome P-450 dependent drug metabolism.

Mechanism of action of FK 506

Although the precise molecular action is not completely understood, FK 506 has similar immunosuppressive property of cyclosporine. However, FK 506 can suppress murine or human mixed lymphocyte reactivity and the generation of cytotoxic T cells at concentrations 100 times lower than that of cyclosporine. FK 506 inhibits early T-cell activation events that are required for lymphokine gene expression (18). Like cyclosporine, FK 506 also selectively blocks calcium dependent intracellular signaling events in others, possibly related signal transduction pathways. FK 506 however, binds to cytosolic receptors which are different from cyclophilin A, the major receptor which binds cyclosporine. FK 506 binds to a 12 k-Da cytosolic FK 506 binding protein termed as FKBP 12. The FKBP 12.FK 506 complex binds specifically to the calcium and calmodulin dependent serine and threonine phosphatase, calcineurin and inhibits its phosphatase activity in vitro. Recently, Wiederrecht et al using gel filtration technique identified a novel FK 506 binding high molecular weight protein with 110 k-Da molecular weight (19).

Toxicity of FK 506

Since both FK 506 and cyclosporine inhibits a series of isomerase enzymes, there are some similarities in their side effects. However, FK 506 is free from some side effects of cyclosporine namely, gingival hyperplasia and hirsutism (20-21). However, FK 506 like cyclosporine can cause nephrotoxicity. FK 506 decreases glomerular filtration rate and renal blood flow but increases renal vascular resistance. The nephrotoxicity may be related to an increased production of thromboxane A 2 production in the renal parenchyma (22). FK 506 also can cause hypertension, but the incidence appears to be 50 % less than that experienced with cyclosporine. FK 506 also has a diabetogenic effect probably due to

a change in the islet cells's response to hyperglycemia and a change in peripheral sensitivity of insulin. The incidence of major neurological side effect is low (5%) with FK 506 and most of them occur during the first month following liver transplant (23). According to the Pittsburgh study, the patterns and timing of opportunistic infections after surgery is similar under FK 506 and cyclosporine therapy, occurring early in the post transplant stage (24).

Therapeutic Drug Monitoring of FK 506

Therapeutic drug monitoring of FK 506 presents an analytical challenge because the recommended trough plasma levels are 0.5-2.0 ng/mL for clinically stable liver and kidney transplant recipients. Another major problem in therapeutic monitoring of FK 506 is that like cyclosporine, erythrocytes serves as a large reservoir for FK 506. The exchange of drug between erythrocytes and plasma is rapid and temperature dependent. Therefore plasma assay requires prior to separation from red blood cells, the sample should be equilibrated at least for 1 h at 37°C (25). At 37°C, the plasma levels of FK 506 are 30-40% higher than those found at room temperature.

The therapeutic range of FK 506 is still under study. They depend on the temperature at which the plasma was separated from blood cells, the extraction protocol as well as the assay techniques. A therapeutic range of 0.2-0.8 ng/mL was suggested for plasma if the separation of plasma from blood cell was achieved at room temperature, but a therapeutic range of 0.5-2.0 ng/mL was also recommended if plasma was separated from blood cells at 37°C. The concentration of FK 506 measured in whole blood was substantially higher than the concentration measured in plasma. A therapeutic range of 2.5-14 ng/mL was recommended for whole blood FK 506 concentration using ELISA assay following liquid/liquid extraction while a therapeutic range of 4.0-20 ng/mL has been recommended for Abbot's IMx assay for FK 506 (26).

HPLC in monitoring FK 506 concentration

FK 506's absorption at 192 nm allows HPLC isolation and U.V. detection only for pharmaceutical application where sensitivities

around 50-100 ng/ mL is acceptable but not for monitoring FK 506 concentration in plasma or whole blood. Takada et al determined FK 506 concentrations in rat serum using HPLC with chemiluminescence determination. The dansyl hydrazine reagent reacts with carbonyl group of FK 506 to form a fluorescent product with an excitation maximum at 350 nm and an emission maximum at 510 nm (27). The authors extracted FK 506 from 100 μL of rat plasma using ethyl acetate and derivatized FK 506 with dansyl hydrazine reagent. The excess derivatizing reagent was destroyed with sodium pyruvate solution and the initial clean up of sample was achieved with Sep-Pak cartridge and using 70 % and 80% methanol as eluting solvent. The HPLC procedure involves column switching technique using two different mobile phases. The mobile phase one contains methanol/water (70:30 by vol) and mobile phase II contains methanol/water (90:10 by vol). Both pre column and HPLC column used were derivatized silica (C-18 reverse phase). The lower end of sensitivity for the assay was 5 ng/mL of FK 506 concentration in plasma.

Wong et al described a supercritical fluid chromatography and HPLC of cyclosporine and FK 506. Using a Bondpack C-18 column at 70°C and acetonitrile/water (80:20 by vol) as a mobile phase, the two more polar tautomers of FK 506 eluted at 4 min as one peak while FK 506 showed a retention time of 5 min. The detection wavelength was 202 nm. However, in supercritical fluid chromatography (biphenyl column and pressure program from 100 to 300 atmosphere at 10 atmosphere/min, FID detection), the tautomers of FK 506 eluted (17.75 min) after FK 506 (17.48 min) (28).

HPLC - ELISA in Monitoring FK 506 Concentration

Friob et al described a combined HPLC-ElISA assay for therapeutic monitoring of FK 506 in transplant patients. The authors used 200 μL of serum for solid phase extraction and after drying, reconstituted the residue with 30 μL of methanol for HPLC injection. The HPLC system used a reverse phase Micro-Pak MCH-5, 15 cm column (heated at 72°C) and an isocratic solvent system of acetonitrile/water (71:29 by vol). The elution of peaks were monitored at 212 nm. The FK 506 tautomers eluted at 4.6 and 5.2 min with a proportion of peak areas of 1:9. For the ELISA assay, C 18

solid phase Bond-Elute columns were used to extract FK 506. The sensitivity limit reached as low as 0.1 ng/mL (29).

Warty et compared solid phase extraction followed by ELISA assay with liquid-liquid extraction, HPLC separation and ELISA assay for determining concentrations of FK 506 in plasma (30). The solid phase extraction using C-18 Sep-Pak cartridge required 100 μL of plasma (30). After prewashing cartridge with 4 % acetic acid, plasma sample was applied and FK 506 was extracted with methanol and analyzed by ELISA. The authors also described a liquid-liquid extraction of FK 506 from 100 μL of plasma with dichloromethane after initial acidification of plasma with 1 mL of 0.1 N hydrochloric acid. After extraction, excess solvent was evaporated and the dry residue was reconstituted with methanol for injection into HPLC. The HPLC column was a 3.9 mm X 15.0 cm analytical column filled with μBond Pak C-18. The column temperature was set at 60°C and the mobile phase composition was methanol/water (80:20 by vol), acidified to pH 6.0 with hydrochloric acid. The flow rate was 0.8 mL/min. The detection was achieved with a photodiode array detector at 214 nm. The retention time of FK 506 was 4.8 min. Authors collected two different fractions (0-3.6 min and 3.6-6.0 min) which contained 99% of all FK 506. Both fractions were evaporated and residue analyzed for FK 506 using ELISA. Authors found no difference in FK 506 concentrations obtained by solid phase extraction and ELISA versus liquid-liquid extraction, HOLC and ELISA. However, solid phase extraction and ELISA showed higher FK 506 levels than liquid-liquid extraction and HPLC only in patients with abnormal liver function. This may be due to cross reactivity of FK 506 metabolites with ELISA.

HPLC-Mass Spectrometry for FK 506 Monitoring

Christians et al described a HPLC-mass spectrometry assay for FK 506 using a synthetic internal standard, 32-O-acetyl FK 506 (31). Internal standard was synthesized by incubating FK 506 with acetic anhydride for 2 h at 75°C. After solid phase extraction of FK 506 and internal standard (5 ng) from 1 mL of whole blood or plasma using solid phase extraction, the parent drug and the metabolites were separated by a HPLC system using a reverse phase C-8 column (100 X 4 mm, particle size 3 μm). The separation was achieved with

solvent gradient using analysis time 0 min: 60% acetonitrile, analysis time 8.1 min: 95% acetonitrile. The flow rate was 0.3 mL/min. The retention time of FK 506 was 8.5 min while that of the internal standard was 10.5 min. For mass spectral analysis, the authors used negative chemical ionization with methane or butane as reactant gas. The temperature of mass spectrum source was 250°C while the temperature of quadruple was 120°C. The mass spectrum was run in a selected ion mode monitoring m/z 776, 790, 792, 804, 808, 836 and 834. The authors reported a limit of detection of 25 pg at a signal to noise ratio of 1:8 and the assay was linear from 25 pg to 50 ng. The between run precision was 10.5% at 5 ng level while the within run precisions were 4.7% and 7.7% respectively at 3 and 10 ng levels. In blood sample, FK 506 and a metabolite at 790 amu was regularly found. However, in urine, FK 506, and other metabolites at 776 amu (double demethylated), 790 (double demethylated) and 792 (double demethylated and hydroxylated) were also found. In HPLC with U.V. detection, several FK 506 metabolites can not be separated. The authors by using LC/MS can quantitate individual metabolites.

Immunoassays for monitoring FK 506 concentrations

Fuijisawa has described an enzyme linked immunoassay (ELISA) methodology to measure FK 506 concentrations. The assay used ELISA plates coated with anti FK 506 antibody, an FK 506 horseradish peroxidase conjugate that competes with free FK 506 and an appropriate substrate for peroxidase. However, ELISA is a semiautomated technique. Recently, Grenier et al described an automated assay for measuring FK 506 concentrations in whole blood using IMx analyzer (Abbott Laboratories, Abbott Park, IL). The FK 506 assay for the IMx analyzer utilizes four reagents: a precipitation reagent to extract FK 506 from whole blood, a capture reagent consisting of latex microparticles to which FK 506 antibody was covalently immobilized, an FK 506 alkaline phosphatase conjugate reagent and an enzyme substrate reagent consisting of 4-methylumbelliferyl phosphate. The sensitivity of the assay is 3.3 ng/mL while precision analysis showed CVs of 11.8, 9.6 and 8.1% at FK 506 levels of 15, 25 and 65 ng/mL (32).

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GENERAL PAPERS

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COMPARISON OF SOLVENT MODULATION WITH PREMIXED MOBILE PHASES FOR THE SEPARATION OF CORTICOSTEROIDS BY LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of eight common corticosteroids is optimized by solvent modulation and by conventional premixed solvents. The separation is achieved on an octadecylsilica column using aqueous acetonitrile and methanol mobile phases. The optimized separations are compared with respect to accuracy, total analysis time, critical resolution, and overall quality of the separation. The solvent modulation approach compares favorably to premixed solvents in all of these respects and, hence, is demonstrated to be a very promising optimization strategy.

INTRODUCTION

In liquid chromatography, solute retention and selectivity are controlled primarily by varying the composition of the mobile phase. Various theoretical models have been developed to predict the effect of

mobile phase composition on retention. These include classical thermodynamic models, such as those based on regular solution theory (1-5) and solvophobic theory (6-9), as well as statistical thermodynamic models (10-14). While these models can elucidate trends and provide qualitative information, they have limited accuracy for predicting quantitative information about the chromatographic separation. This limitation is due to the complex nature of retention processes such that a complete and rigorous theoretical treatment is elusive.

Empirical and semi-empirical models are used extensively to predict and to optimize chromatographic separations. These models can be generally classified into three categories: simultaneous, sequential, and interpretive (15). In the simultaneous or grid search methods, all experiments are performed concurrently and the conditions that yield the most desirable separation are selected as the optimum. These methods typically require a large number of experiments in order to ensure that the global optimum is identified. In the sequential methods, a few initial experiments are performed and, based on their outcome, the conditions are chosen for subsequent experiments. These methods utilize an iterative search technique such as simplex (16,17), Hooke¬Jeeves directed search (18), or Box--Wilson steepest ascent path (19) to progressively approach the optimum conditions. Thus, the sequential methods may require fewer experiments to reach the optimum. An important advantage of both the simultaneous and sequential methods is that they do not rely on any predictive model to identify the optimum conditions. In the interpretive or regression methods, a few preliminary experiments are performed and the data are fitted to a predefined

mathematical function. After calculation of the regression coefficients, the separation can be predicted at intermediate conditions to construct a complete response surface, from which the optimum is identified.

Among these methods, the regression approach has proven to be the most widely used in liquid chromatography. This method is attractive because, by using a linear or quadratic model (20-23), only a few experiments are required to optimize the mobile phase composition. However, these models implicitly assume that molecular interactions are ideal, so that solutes interact independently with each component of the mobile phase. Unfortunately, molecular interactions are not completely independent of one another within a solvent mixture, especially for the polar solvents used in reversed-phase liquid chromatography. This non-ideal behavior limits the accuracy with which solute retention can be predicted by using these models (24,25).

Recently, solvent modulation was introduced as a practical alternative to premixed mobile phases for liquid chromatography (26-28). In this technique, individual solvent zones are introduced sequentially onto the chromatographic column. Because the zones are spatially and temporally separated, solutes undergo interaction independently within each solvent zone. Thus, the overall retention of the solute is a simple time-weighted average of its capacity factor in the individual solvent zones. Consequently, optimization of the chromatographic separation is more accurate and requires fewer preliminary experiments by using solvent modulation than by using premixed mobile phases (28).

In this work, solvent modulation and premixed mobile phases are used to optimize the separation of eight common corticosteroids. The

separation is achieved on an octadecylsilica column using aqueous methanol and acetonitrile mobile phases. Based on these results, the optimization methods are compared with respect to accuracy, total analysis time, critical resolution, and the overall quality of the separation.

THEORETICAL CONCEPTS AND OPTIMIZATION STRATEGIES

Premixed Solvents

In reversed-phase liquid chromatography, a mixture of aqueous and organic solvents is often employed to effect the separation of solutes. Various approaches have been utilized to optimize the composition of these mixed solvents (29-31), among which the commercially available optimization program known as DryLab I™ is one of the most popular and successful (32-37). By using the regression approach, this program combines semi-empirical models of chromatographic retention and dispersion with a few initial experiments for the solutes of interest in order to optimize their separation. In this approach, a linear model is employed to relate the solute factor (k) and the mobile phase composition (ϕ)

$$\log k = \log k_W - s\phi \qquad [1]$$

where the slope (s) is a constant that is characteristic of each solute within the chromatographic system, and the intercept ($\log k_W$) is the logarithm of the solute capacity factor using pure water as mobile phase. The solute capacity factor is calculated from the experimental data as

$$k = \frac{(t_r - t_o)}{t_o} \qquad [2]$$

where t_r and t_0 are the elution times of a retained and nonretained solute, respectively.

Practical application of this technique requires that solute retention be measured by using at least two solvent compositions. This enables the estimation of the coefficients s and k_w for each solute by linear regression. Based on the values of these coefficients, the solute capacity factor can be predicted at other mobile phase compositions by means of Equation [1]. From the predicted capacity factors, the resolution between adjacent solutes can be calculated as follows

$$R_S = \frac{N^{1/2}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right) \qquad [3]$$

where N is the number of theoretical plates, α is the selectivity factor, and k is the capacity factor. The quality of the separation is evaluated by means of the resolution between the least-resolved solute pair, called the critical resolution (R_{crit}). In order to optimize the separation, the critical resolution is mapped as a function of the solvent composition (20,36,38). From this resolution map, the mobile phase that yields the highest value of the critical resolution can be determined by visual inspection.

Solvent Modulation

The general concept and theory of solvent modulation have been discussed previously by Wahl *et al.* (26-28). In this technique, the overall retention of solute i is given by

$$k_i = \frac{\sum_{j=0}^{n} x_j \left(\frac{1+k_{ij}}{k_{ij}}\right)}{L} - 1 \qquad [4]$$

where k_{ij} is the capacity factor of solute i in solvent j, x_j is the solvent zone length, and the limit of summation n represents the number of solvent zones required to elute the solute from a column of length L. Thus, in solvent modulation, the overall retention is varied by means of the type, sequence, and length of the solvent zones applied to the column.

The strategy for optimization by this technique requires preliminary measurement of solute capacity factor in each solvent of interest. Based on these measurements, the overall capacity factor under the conditions of solvent modulation can be estimated by means of Equation [4]. Next, the resolution between adjacent solutes i and i+1 is calculated as

$$R_{i,i+1} = \frac{N^{1/2}}{2} \left(\frac{k_{i+1} - k_i}{2+k_i + k_{i+1}} \right) \qquad [5]$$

The quality of the separation is then assessed by using a modified form of the multivariate function known as the Chromatographic Resolution Statistic (CRS) developed by Schlabach and Excoffier (39)

$$CRS = \left(\sum_{i=1}^{m-1} \left(\frac{R_{i,i+1} - R_{opt}}{R_{i,i+1} - R_{min}} \right)^2 \frac{1}{R_{i,i+1}} + \sum_{i=1}^{m-1} \frac{(R_{i,i+1})^2}{(m-1)\, R_{avg}^2} \right) \frac{t_f}{m} \qquad [6]$$

where m is the total number of solutes, t_f is the elution time of the final solute, R_{opt} is the optimum or desired resolution, R_{min} is the minimum acceptable resolution, and R_{avg} is the average resolution which is given by

$$R_{avg} = \frac{1}{m} \sum_{i=1}^{m} R_{i,i+1} \qquad [7]$$

The first term of the CRS function is a measure of the extent of separation between each pair of adjacent solutes in the chromatogram. This term

approaches zero when the individual resolution elements approach the optimum value, and approaches infinity when the individual resolution elements approach the minimum value. The second term of the CRS function reflects the uniformity of spacing between solutes, and approaches a minimum value of unity when the individual resolution elements are equal to the average value. The final term of the CRS function is intended to minimize the analysis time, and may be neglected if this is not a primary goal of the optimization.

In order to optimize the separation, the solvent zone lengths which yield the minimum value of the CRS function must be determined. This minimum CRS value may be determined in two ways: 1) by varying the length of each solvent zone systematically to produce the complete response surface from which the optimum is determined by visual inspection, or 2) by using an sequential search routine such as the simplex method (17,40). The former method is time consuming, but provides a detailed view of the complete response surface. The latter method is more efficient but, because the surface may contain many local maxima and minima, care must be taken to ensure that the global optimum is identified. Consequently, a combination of these approaches is desirable.

EXPERIMENTAL METHODS

Chromatographic System

A chromatographic pump equipped with two 40-mL syringes (Model 140, Applied Biosystems, Foster City, CA, USA) is used to deliver the

mobile phase at 0.5 mL/min. Sample introduction is achieved by using a 10-μL injection valve (Model EQ 60, Valco Instruments Co., Houston, TX, USA). The chromatographic column (47 cm × 0.46 cm i.d.) is packed with octadecylsilica material (Spheri-5 RP-18, 5 μm, Applied Biosystems) to have a total plate number (N) of approximately 10,000 for the solutes of interest. Solute detection is accomplished by using a variable-wavelength UV-visible absorbance detector (240 nm, 0.005 AUFS, Model 166, Beckman Instruments, San Ramon, CA, USA).

Materials and Methods

The following corticosteroids are utilized in this investigation: cortisone (17α,21-dihydroxy-pregn-4-ene-3,11,20-trione), hydrocortisone (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione), tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione), tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one), prednisone (17α,21-dihydroxy-pregna-1,4-diene-3,11,20-trione), prednisolone (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione), methylprednisolone (11β,17α,21-trihydroxy-6α-methyl-pregna-1,4-diene-3,20-dione), and dehydrocorticosterone (21-hydroxy-pregn-4-ene-3,11,20-trione). These corticosteroids shown in Figure 1 are obtained from the Sigma Chemical Company (St. Louis, MO, USA) and are used without further purification. Standard solutions are prepared in methanol at 10^{-3} M concentration for tetrahydrocortisone and tetrahydrocortisol, and at 10^{-6} M concentration for all other steroids. Organic solvents are high-purity, distilled-in-glass grade (Baxter Healthcare, Burdick & Jackson Division, Muskegon, MI, USA); water is deionized and double distilled in glass (Model MP-3A, Corning Glass Works, Corning, NY, USA).

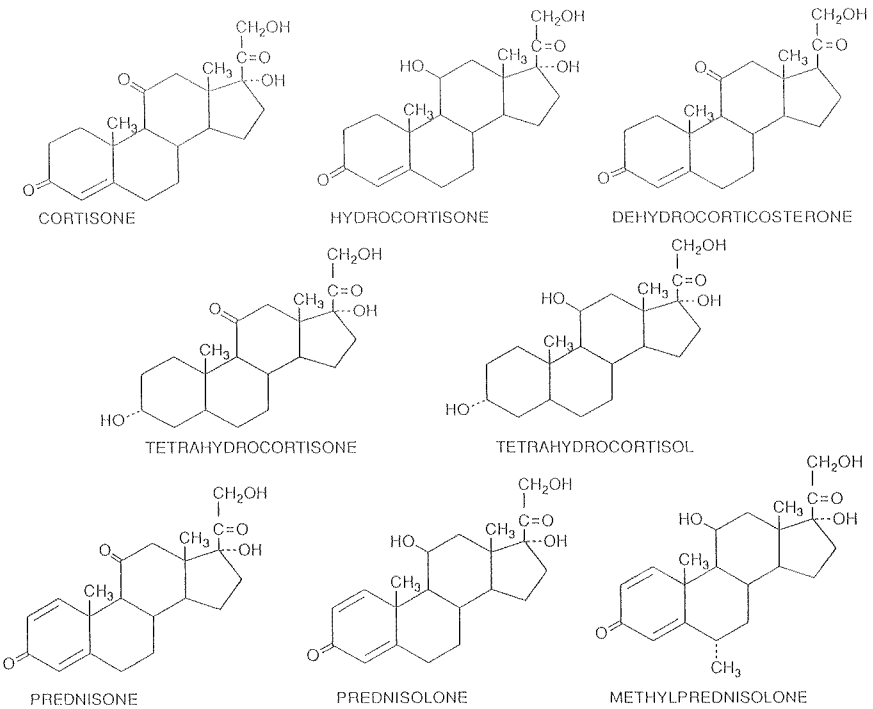

FIGURE 1: Structure of corticosteroids.

Computer-Assisted Optimization Programs

The optimization program for premixed mobile phases, DryLab I™ Isocratic HPLC Simulation/Optimization Program (LC Resources Inc., Lafayette, CA, USA), is executed on an IBM-compatible computer with 80486 microprocessor. From the initial measurement of solute retention times, this program uses linear regression to calculate the capacity factor as a function of the mobile phase composition. In addition, the total analysis time, selectivity factor, and critical resolution are calculated from Equations [1] to [3], assuming a plate number of 10,000. The optimum

conditions are identified from a graph of the critical resolution as a function of mobile phase composition (38).

The optimization program for solvent modulation is written in the Fortran 77 language and executed on a VAX Station 3200 computer (Digital Equipment, Maynard, MA, USA) (26-28). From the initial measurement of solute capacity factors, the overall capacity factor of each solute is calculated under the conditions of solvent modulation by using Equation [4]. The resolution of each solute pair is calculated by using Equation [5], and the overall quality of the separation is assessed by means of the CRS function in Equation [6], where the selected values for the optimum and minimum acceptable resolutions are 1.5 and 0.5, respectively. The optimum conditions are identified by two methods. In the topographic mapping method, these calculations are performed while systematically incrementing each solvent zone length within a prescribed range. By graphing the resulting CRS values as a function of the solvent zone length, a complete response surface is constructed. The minimum CRS value is then located by visual inspection of this response surface (28). In the sequential search method, the modified simplex algorithm of Nelder and Mead (40) is employed. This algorithm permits expansion and contraction of the simplex during the search and will converge at the optimum position. In order to ensure the identification of the global optimum, both the size and the location of the initial simplex are varied systematically in 200 independent searches (28). For each initial simplex, the calculations are performed according to Equations [4] to [6] at each successive vertex, and the best conditions are continuously updated and stored in a file. This file contains the solvent zone lengths, the solute

elution order, the predicted capacity factors, the predicted resolutions, and the corresponding CRS value for the separation.

RESULTS AND DISCUSSION

In this work, computer-assisted optimization programs using solvent modulation and premixed solvents are compared to optimize the separation of eight corticosteroids. The practical utility of both techniques requires that solute retention be measured in solvents of different compositions, from which the optimum conditions for the separation can be predicted. The capacity factor of each corticosteroid was measured on an octadecylsilica column using 35% and 50% acetonitrile as well as 60% and 75% methanol. Table I summarizes the retention measurements for the corticosteroids.

In the methanol mobile phases, all of the corticosteroids are well separated except for prednisolone and hydrocortisone. On the other hand, in the acetonitrile mobile phases, the corticosteroids are separated with the exceptions of prednisone and hydrocortisone as well as methylprednisolone and tetrahydrocortisone. Thus, the least-resolved solute pairs vary with the type of organic modifier.

To optimize the separation of corticosteroids using premixed solvents, the DryLab I™ program is utilized. Based on the preliminary measurements of the capacity factors in Table 1, this program calculates the retention of the corticosteroids at other solvent compositions according to Equation [1]. In order to determine the optimum solvent composition,

TABLE 1

Capacity Factors for Corticosteroids on Octadecylsilica Stationary Phase using Methanol and Acetonitrile Mobile Phases

CORTICOSTEROIDS	CAPACITY FACTOR (k)			
	Methanol–Water		Acetonitrile–Water	
	60%	75%	35%	50%
Prednisone	1.57	0.41	1.66	0.50
Cortisone	1.73	0.48	1.83	0.58
Prednisolone	2.14	0.55	1.40	0.41
Hydrocortisone	2.18	0.56	1.69	0.50
Dehydrocorticosterone	2.97	0.69	4.09	1.39
Methylprednisolone	3.79	0.86	2.53	0.70
Tetrahydrocortisol	4.29	0.96	1.94	0.50
Tetrahydrocortisone	5.17	1.08	2.53	0.63

the critical resolution of the least-resolved solute pair is mapped as a function of the mobile phase composition. The resolution maps for the aqueous acetonitrile and methanol mixtures are shown in Figures 2 and 3, respectively. For the acetonitrile mixtures, the composition of 30% acetonitrile is predicted to yield the highest value of the critical resolution. The least-resolved solutes are prednisone and hydrocortisone, with a predicted resolution of 0.3. Because this region of the resolution map is irregular, slight variations in the mobile phase composition may result in a large change in the critical resolution. For the methanol mixtures, however, a more rugged optimum region is observed between 55% and 60% methanol. The least-resolved solutes are prednisolone and hydrocortisone, with a predicted resolution of 0.3. Because this region is

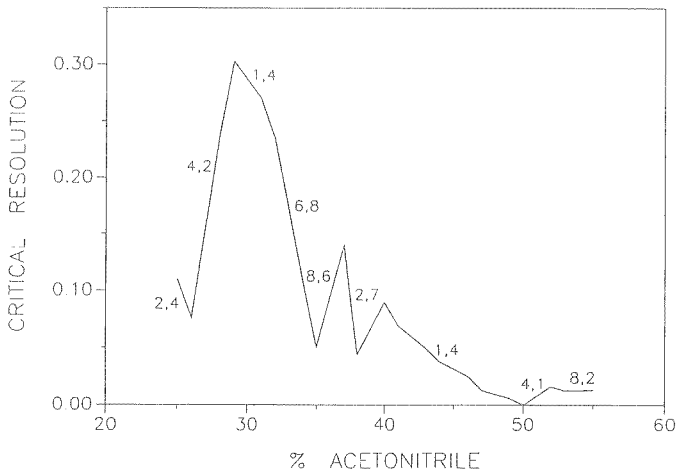

FIGURE 2: Critical resolution as a function of the mobile phase composition for aqueous acetonitrile mixtures. Column: 47 × 0.46 cm i.d., packed with octadecylsilica material. Solutes: (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) dehydrocorticosterone, (6) methylprednisolone, (7) tetrahydrocortisol, (8) tetrahydrocortisone.

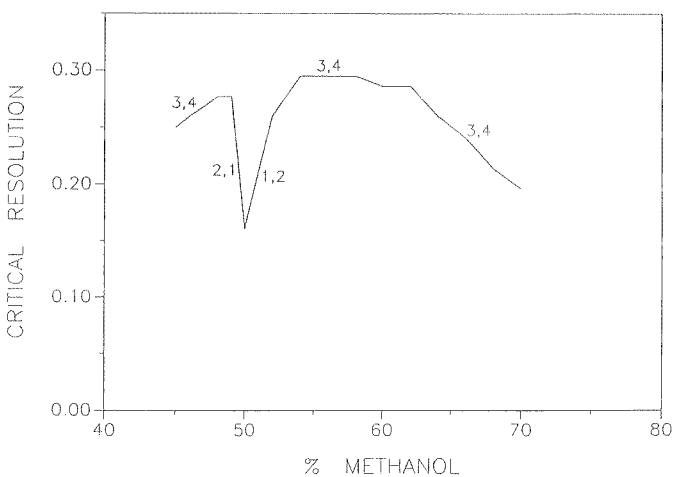

FIGURE 3: Critical resolution as a function of the mobile phase composition for aqueous methanol mixtures. Experimental conditions as given in Figure 2.

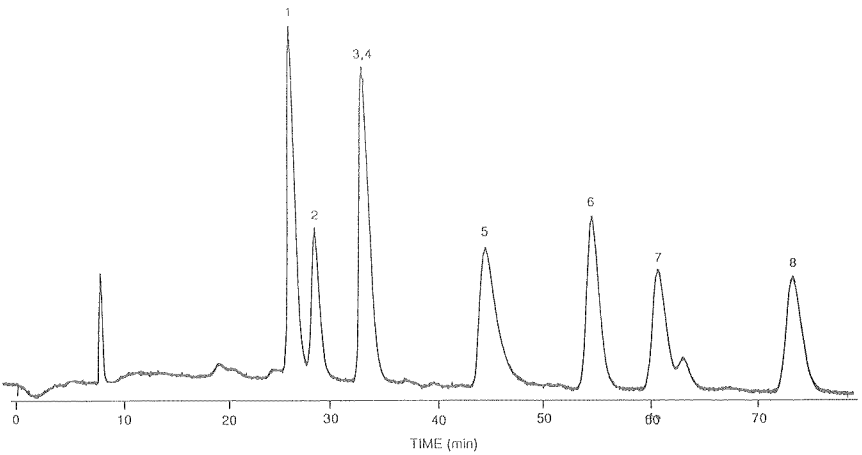

FIGURE 4: Experimental chromatogram of corticosteroids obtained under the predicted optimum conditions for premixed mobile phases. Column: 47 × 0.46 cm i.d., packed with octadecylsilica material. Mobile phase: 56% methanol, 0.5 mL/min. Detector: UV-visible absorbance detector, 240 nm, 0.005 AUFS. Solutes: (1) prednisone, (2) cortisone, (3) prednisolone, (4) hydrocortisone, (5) dehydrocorticosterone, (6) methylprednisolone, (7) tetrahydrocortisol, (8) tetrahydrocortisone.

relatively broad and flat, slight variations in the mobile phase composition will not be as detrimental. Thus, 56% methanol was chosen as the optimum mobile phase composition and was used to obtain the separation shown in Figure 4. From this chromatogram, it is apparent that prednisolone and hydrocortisone are completely overlapped ($R_S \approx 0.3$), whereas all other solutes are fully resolved. The experimentally measured capacity factors are in good agreement with the theoretically predicted values from Equation [1], as summarized in Table 2, with an average relative error of ±3.22%.

To optimize the separation of corticosteroids using solvent modulation, the four solvent systems shown in Table 1 are utilized.

TABLE 2

Comparison of Experimental and Theoretical Capacity Factors under the Predicted Optimum Conditions for Premixed Mobile Phases

CORTICOSTEROIDS	CAPACITY FACTOR (k)		
	Theory†	Experiment	Relative Error (%)‡
Prednisone	2.46	2.25	–8.54
Cortisone	2.60	2.54	–2.31
Prednisolone	3.27	3.11	–4.89
Hydrocortisone	3.32	3.11	–6.32
Dehydrocorticosterone	4.62	4.55	–1.52
Methylprednisolone	5.84	5.80	–0.68
Tetrahydrocortisol	6.61	6.60	–0.15
Tetrahydrocortisone	8.08	8.19	1.36
Average			±3.22

† Calculated by using Equation [1].
‡ Calculated as 100 x (Experiment – Theory)/Theory.

Although there are twelve possible permutations of a two-solvent modulation sequence, the computer-assisted search routines provide a rapid and effective means to identify the most promising permutation. For each permutation, the sequential simplex method is used to determine the minimum CRS value on the complete response surface. The results of this preliminary search are summarized in Table 3.

From these results, the most promising solvent modulation sequence is identified to be 50% acetonitrile followed by 60% methanol (CRS_{min} = 1.9). The least-resolved solutes are cortisone and hydrocortisone, with a predicted resolution of 1.30. Although this permutation initially appears to

TABLE 3

Evaluation of the Permutations for a Two-Solvent Modulation Sequence

Solvent 1	Solvent 2	t_f (min)	R_{crit}	CRS_{min}
60% Methanol	75% Methanol	29.3	0.20	95
75% Methanol	60% Methanol	28.8	0.19	96
35% Acetonitrile	50% Acetonitrile	32.3	0.15	113
50% Acetonitrile	35% Acetonitrile	33.9	0.15	104
60% Methanol	35% Acetonitrile	50.3	0.72	26
35% Acetonitrile	60% Methanol	51.9	0.99	3.6
60% Methanol	50% Acetonitrile	30.3	0.86	8.1
50% Acetonitrile	60% Methanol	31.1	1.30	1.9
75% Methanol	35% Acetonitrile	18.9	0.20	97
35% Acetonitrile	75% Methanol	19.3	0.18	98
75% Methanol	50% Acetonitrile	18.6	0.18	100
50% Acetonitrile	75% Methanol	18.6	0.18	101

be very promising, a more detailed inspection by the topographic mapping method reveals that the response surface is highly irregular. Variations in the solvent zone length as small as ±1.0 cm alter the identity of the least-resolved solute pair and cause a significant change in the critical resolution.

As a consequence of this limitation, the next most promising permutation of 35% acetonitrile followed by 60% methanol (CRS_{min} = 3.6) is selected for further study. The chromatograms corresponding to each of these solvents are shown in Figure 5. The least-resolved solutes in 35% acetonitrile are methylprednisolone and tetrahydrocortisone, whereas those in 60% methanol are prednisolone and hydrocortisone. Although

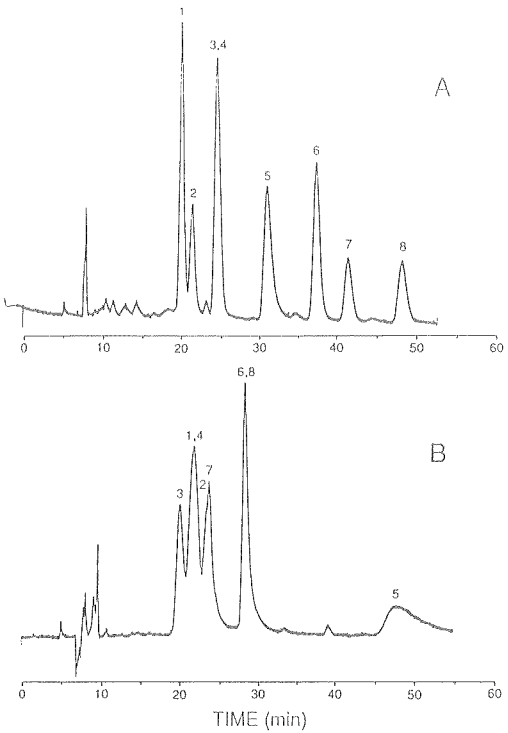

FIGURE 5: Separation of corticosteroids in individual mobile phases for solvent modulation. Mobile phase: (A) 60% methanol, (B) 35% acetonitrile, 0.5 mL/min. All other experimental conditions as given in Figure 4.

the separation of all solutes is not achievable in either of these solvents individually, the results in Table 3 suggest that the modulation of these solvents may provide a more beneficial separation. In order to determine the optimum conditions for solvent modulation, the complete response surface was constructed by the mapping method. The topographic and contour maps of the CRS response surface are shown in Figure 6 as a function of the solvent zone length. When expressed in terms of the

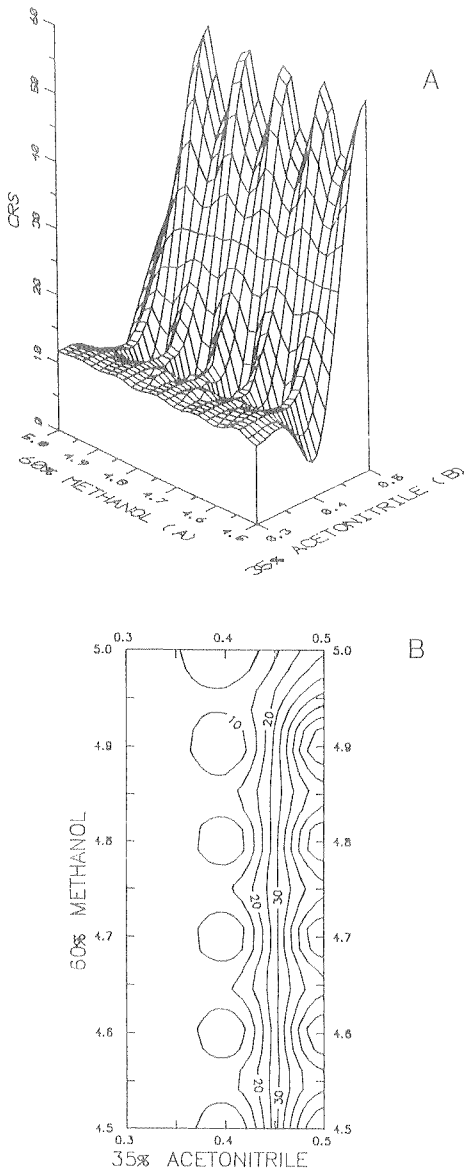

FIGURE 6: Topographic (A) and contour (B) maps of the CRS response surface as a function of the fractional zone lengths for 35% acetonitrile and 60% methanol.

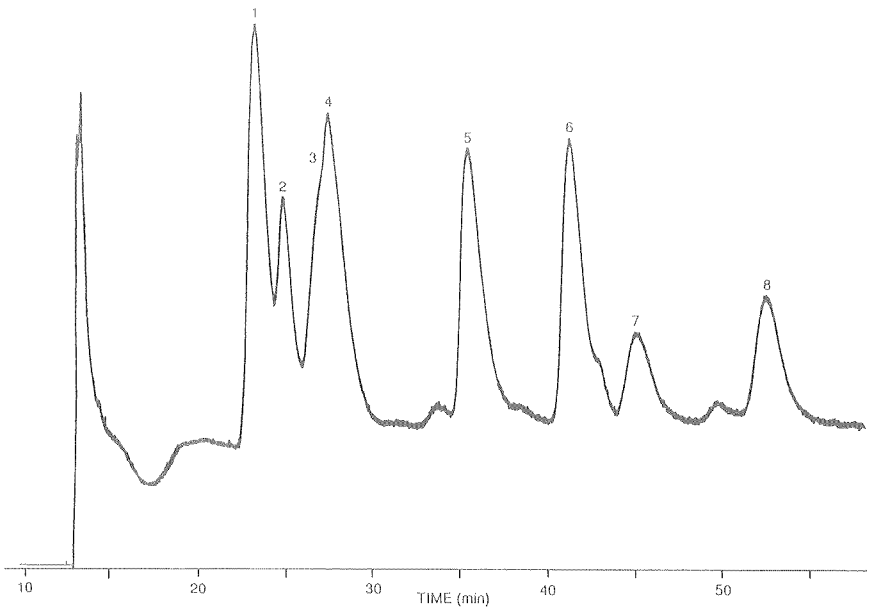

FIGURE 7: Experimental chromatogram of corticosteroids obtained under the predicted optimum conditions for solvent modulation. Mobile phase: solvent modulation sequence of 35% acetonitrile and 60% methanol in fractional zone lengths of 0.4 and 4.8, respectively, 0.5 mL/min. All other experimental conditions as given in Figure 4.

fractional length (x_j/L), these maps may be used to determine the optimum conditions independent of the column length. From these maps, the minimum CRS is predicted for zones of 35% acetonitrile and 60% methanol in fractional lengths of 0.4 and 4.8, respectively, which correspond to absolute lengths of 19 and 226 cm, respectively, for the 47 cm column utilized in this study.

The experimental chromatogram in Figure 7 shows good separation of all corticosteroids with the exception of the least-resolved solute pair, prednisolone and hydrocortisone ($R_{crit} \approx 0.7$). The experimentally measured capacity factors agree well with the theoretically predicted

TABLE 4

Comparison of Experimental and Theoretical Capacity Factors under the Predicted Optimum Conditions for Solvent Modulation

CORTICOSTEROIDS	CAPACITY FACTOR (k)		
	Theory†	Experiment	Relative Error (%)‡
Prednisone	1.59	1.58	–0.63
Cortisone	1.76	1.78	1.14
Prednisolone	1.93	2.08	7.77
Hydrocortisone	2.06	2.10	1.94
Dehydrocorticosterone	3.08	2.97	–3.57
Methylprednisolone	3.59	3.60	0.28
Tetrahydrocortisol	3.82	4.01	4.97
Tetrahydrocortisone	4.76	4.82	1.26
Average			±2.70

† Calculated by using Equation [4].
‡ Calculated as 100 x (Experiment – Theory)/Theory.

values from Equation [4], as summarized in Table 4, with an average relative error of ±2.70%.

The optimization methods used in this work may be compared on the basis of the following criteria: average relative error in predicted capacity factor, total analysis time, critical resolution, and overall quality of the separation assessed by the CRS function. The average relative error is ±2.70 for the solvent modulation technique, compared with ±3.22 for premixed solvents. When optimized with solvent modulation, the corticosteroid separation is achieved experimentally in 52 minutes with a critical resolution of 0.7, and a CRS value of approximately 26. When

optimized with premixed solvents by DryLab I™, the corticosteroid separation is achieved experimentally in 73 minutes with a critical resolution of 0.3, and a CRS value of approximately 146. On the basis of these criteria, the separation achieved by using solvent modulation is at least comparable to and, in some respects, significantly better than that achieved by using premixed solvents.

CONCLUSIONS

Solvent modulation is a practical alternative to premixed mobile phases in liquid chromatography. Because the solvent zones are spatially and temporally separated from one another, solute retention is a simple time-weighted average of the retention in each individual solvent. Consequently, optimization of the separation is more accurate and requires fewer preliminary experiments with solvent modulation than with premixed solvents. In this study, the separation of corticosteroids was optimized by each technique and compared with respect to accuracy, total analysis time, critical resolution, and overall quality of the separation. The solvent modulation approach compares favorably in all of these respects and, hence, is demonstrated to be a very promising optimization strategy.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF EQUILIN, ESTRONE, AND ESTRONE DERIVATIVES WITH CYCLODEXTRINS AS MOBILE PHASE ADDITIVES

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ABSTRACT

β-Cyclodextrin and two derivatized β-cyclodextrins were utilized as mobile phase additives for the liquid chromatographic resolution of equilin and estrone, as well as estrone from 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone on a C_{18} stationary phase. β-Cyclodextrin proved to be suitable for the separations of these steroids but the modified β-cyclodextrin provided better resolution. A comparison was made of the separation and retention obtained with β-cyclodextrin with that of the modified β-cyclodextrins. Apparent inclusion complex strengths for estrone and its derivatives with each cyclodextrin were calculated. The effects of mobile phase cyclodextrin concentration and methanol content on selectivity and capacity factor were examined.

INTRODUCTION

Cyclodextrins are cyclic oligosaccharides formed by the enzymatic degradation of starch. They are comprised of α-(1,4)-linked D(+)-glucopyranose units which form a toroidal-shaped molecule having a hydrophobic cavity and a hydrophillic exterior face. The most common form of cyclodextrin used in high performance liquid chromatography is β-cyclodextrin, which consists of seven glucose units and forms a cavity of diameter 7.8 Å.

Cyclodextrins are used in high performance liquid chromatography to provide inclusion complex interactions between the cyclodextrin cavity and solute. They are employed both as a bonded stationary phase and as a mobile phase additive. They are used predominantly in the separation of optical isomers as the chiral environment at the cavity rim can be used as a chiral selector [1,2]. Cyclodextrins, however, can also be used for non-chiral separations for eluents that are difficult to separate, such as similarly-structured molecules [3]. The differences in inclusion complex strengths between solutes and the cyclodextrin cavity, as well as differences in the interaction with the rim functional groups, can result in improved chromatographic separation. It has been found that steroids have a structure suitable for the formation of inclusion complexes with β-cyclodextrins [4-6]. The formation of these inclusion complexes has allowed for

β-cyclodextrins to be utilized in the separation and analysis of steroids by HPLC [7-10].

The work presented here focuses on the use of β-cyclodextrin and modified β-cyclodextrins as mobile phase additives in the separation of steroid molecules. For the modified cyclodextrins, the hydroxyl groups on the rim of the cyclodextrin cavity are replaced with either methyl or hydroxyethyl groups which increases the hydrophobic character of the cyclodextrin cavity relative to the hydrophillic exterior. These differences will change the inclusion complex strength which can lead to greater selectivity.

This paper describes and characterizes the separation of equilin and estrone, two very similarly structured estrogens of importance to the pharmaceutical industry. The direct analysis of equilin and estrone without cyclodextrin has been reported [11]; however the use of cyclodextrins as a mobile phase additive greatly improves the resolution of these two molecules. To date there has been no report on the use of cyclodextrins in the separation of equilin and estrone. Also presented in this work is the separation of estrone from three of its related derivatives/metabolites, specifically 16α-hydroxyestrone (16α-HE), 2-hydroxyestrone (2-HE), and 4-hydroxyestrone (4-HE). A comparison of the separation obtained with unmodified β-cyclodextrin, methyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin as mobile phase additives will be discussed along with the effects of varying cyclodextrin and methanol concentration.

EXPERIMENTAL

Apparatus

Chromatography was performed using a liquid chromatographic system consisting of a model M6000-A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injector containing a 10 µL loop (Rheodyne, Cotati, CA, U.S.A.), and a Model LC290 UV detector (Perkin Elmer, Norwalk, CN, U.S.A.). The chromatograms were recorded on a Model DE120 strip chart recorder (Goerz Electro, Austria). The column used with the mobile phase additives was a 5 µm Zorbax ODS (150 x 4.6mm I.D.), purchased from Chromatographic Specialties (Brockville, ON, Canada). The β-cyclodextrin column was a Cyclobond I (250 x 4.6 mm I.D.), purchased from Advanced Separation Technologies (Whippany, NJ, U.S.A.). When not in use, the columns were stored in 100% methanol.

Chemicals

Equilin, estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone were obtained from Sigma (St. Louis, MO, U.S.A.). β-Cyclodextrin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and hydroxyethyl-β-cyclodextrin (HE-β-CD) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade methanol was purchased from Fisher (Fair Lawn, NJ, U.S.A.). Monobasic potassium phosphate, dibasic

potassium phosphate, and phosphoric acid were obtained from A&C Chemicals (Montreal, QC, Canada). Water was doubly distilled and deionized.

Procedures

The mobile phase was prepared by mixing methanol with potassium phosphate buffer. Cyclodextrin was dissolved and the mixture was degassed and filtered through a 0.45 μm membrane filter. The pH was lowered with 10% phosphoric acid and raised with dibasic potassium phosphate. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 2 μl. The wavelength of detection was 200 nm.

Retention times were determined by averaging at least three separate determinations. A reproducibility study was conducted where six injections had an RSD of less than 1% for the capacity factor and of less than 2.5% for the resolution factor.

RESULTS AND DISCUSSION

In this investigation, the chromatographic separation of equilin and estrone, as well as estrone from three of its metabolites/derivatives, was

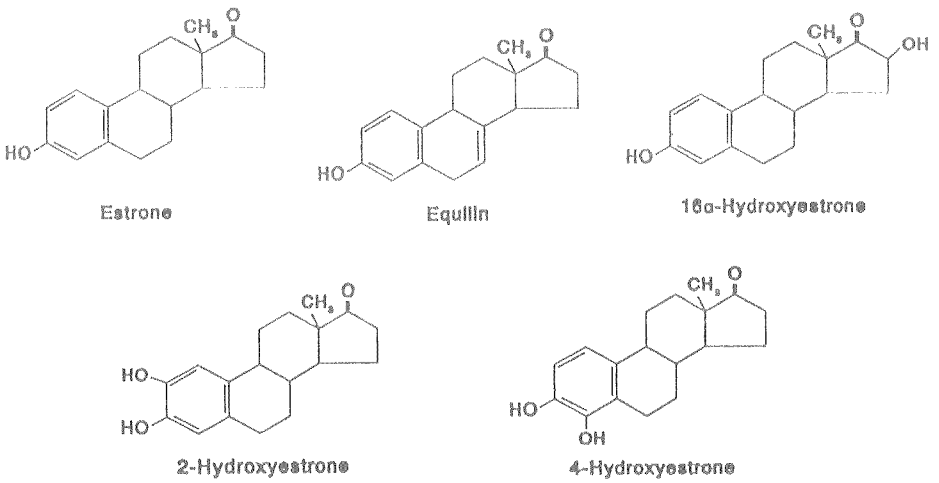

FIGURE 1 Steroids studied

examined using β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin as mobile phase additives with a C_{18} stationary phase. Figure 1 presents the five molecules under investigation.

Without cyclodextrins or other additives in the mobile phase, the resolution of equilin and estrone is poor. The eluents' peaks were wide with no baseline separation. However, the presence of cyclodextrins in the mobile phase improves the separation of the two estrogens. Figure 2 compares the separation of equilin and estrone when the mobile phase additive was β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin. β-Cyclodextrin provides baseline separation of the two steroids; however the resolution is greatly improved when DM- or HE-β-cyclodextrin is the mobile

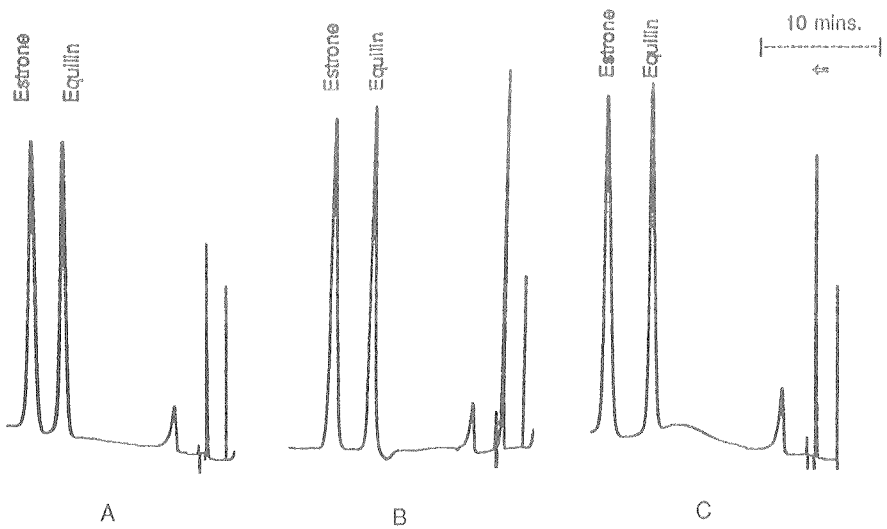

FIGURE 2 Comparison of cyclodextrin type in the mobile phase on separation of equilin and estrone. Mobile phase is 45:55 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) β-CD; (B) DM-β-CD; (C) HE-β-CD.

phase additive. Evidently, the inclusion complex strength of equilin over estrone was changed significantly enough to give an improvement in resolution.

Retention times decrease when some form of cyclodextrin is present in the mobile phase, an indication that inclusion complexes are formed. Table 1 summarizes the separation and retention of equilin and estrone with various cyclodextrins in the mobile phase. A comparison is made with a β-cyclodextrin stationary phase using a comparable mobile phase organic modifier composition. Analogous separation on a β-cyclodextrin column

TABLE 1

Retention and Separation of Equilin and Estrone Comparing Different Cyclodextrins

Mobile Phase Additive	$k'_{EQUILIN}$	$k'_{ESTRONE}$	α
No Cyclodextrin	15.8	17.6	1.11
β-Cyclodextrin	9.09	10.9	1.20
DM-β-Cyclodextrin	8.43	10.9	1.29
HE-β-Cyclodextrin	10.6	13.5	1.27
β-Cyclodextrin Stat. Phase	2.33	1.93	1.21

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin. In the case of the β-cyclodextrin stationary phase, the mobile phase is 60:40 0.05M KH_2PO_4:methanol.

required very short retention times. Increasing the aqueous ratio in the mobile phase to increase solution retention resulted in broad peaks. The elution order of equilin and estrone is reversed when comparing the cyclodextrin mobile phase to a cyclodextrin stationary phase, further evidence of the formation of inclusion complexes.

Figure 3 shows an example of how the various β-cyclodextrin forms affect the resolution of estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone. The unmodified β-cyclodextrin provides suitable separation of the 2- and 4-hydroxyestrone isomers however there is barely baseline separation of the 4- and 16α-isomers. Replacing the β-cyclodextrin with DM-β-cyclodextrin allows for better separation of the 4- and 16α-

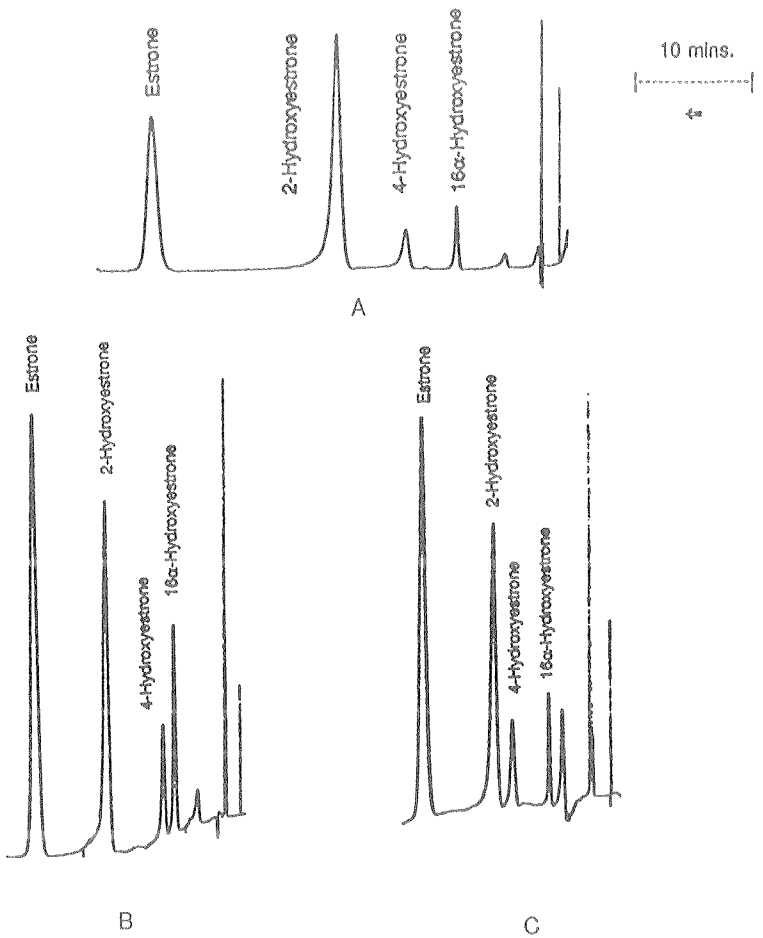

FIGURE 3 Comparison of cyclodextrin type in the mobile phase on separation of estrone from its derivatives. Mobile phase is 55:45 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) HE-β-CD; (B) β-CD; (C) DM-β-CD.

isomers but does not separate the 2- & 4-isomers as well as the unmodified β-cyclodextrin. HE-β-cyclodextrin as the mobile phase additive yields the best separation for estrone, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-hydroxyestrone. In all cases, estrone is well separated from its hydroxy

TABLE 2

Comparison of Cyclodextrins for Estrone and its Derivatives

Mobile Phase Additive	$k'_{ESTRONE}$	$k'_{2\text{-}HE}$	$k'_{4\text{-}HE}$	$k'_{16\alpha\text{-}HE}$	$\alpha_{2\text{-}\&4\text{-}HE}$
No Cyclodextrin	88.8	44.5	45.5	17.2	1.02
β-Cyclodextrin	16.3	10.3	5.49	4.63	1.88
DM-β-CD	13.0	7.92	6.63	3.92	1.19
HE-β-CD	29.4	15.6	10.6	6.92	1.47

* All mobile phases are 55:45 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin.

derivatives. Table 2 shows the relationship between capacity factor for estrone and its three derivatives and the type of cyclodextrin used in the mobile phase. Table II also lists how each type of cyclodextrin affects the separation factor for the similarly structured 2- and 4-hydroxyestrones. As was noted in Figure 3, β-cyclodextrin provides the best separation of the 2- and 4-isomers.

Effect of Cyclodextrin Concentration

Figure 4 shows the effect of mobile phase DM-β-cyclodextrin concentration on the separation of equilin and estrone. The addition of even a small amount of DM-β-CD results is an considerable increase in resolution.

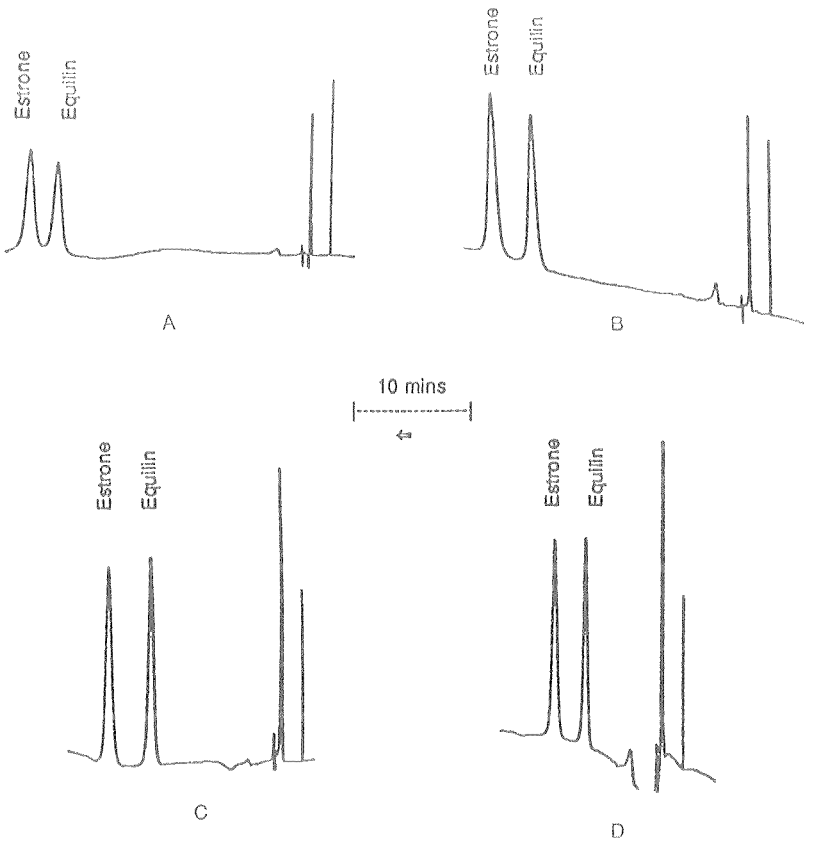

FIGURE 4 Effect of cyclodextrin concentration on the separation of equilin and estrone. (A) 0 mg/ml; (B) 1 mg/ml; (C) 5 mg/ml; (D) 13 mg/ml methyl-β-cyclodextrin. Mobile phase is 45:55 0.05M KH_2PO_4.

As the cyclodextrin concentration is increased, the capacity factors for equilin and estrone decrease dramatically indicating the formation of relatively strong inclusion complexes. The reduction in capacity factor is accompanied by an increase in separation factor. Table 3 lists the effect of

TABLE 3

Effect of DM-β-Cyclodextrin Concentration on Separation of Equilin and Estrone

[DM-β-CD] (mg/ml)	α	Rs
0.0	1.11	1.25
1.0	1.16	2.34
2.0	1.21	2.70
5.0	1.31	2.94
10.0	1.38	3.05
13.0	1.38	3.06

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with the indicated amount of cyclodextrin.

DM-β-cyclodextrin on the separation of equilin and estrone. The immediate increase in separation and resolution factors is followed by a levelling off area where increases in cyclodextrin concentration no longer affect resolution. Each steroid has a different affinity for the cyclodextrin cavity, thus varying the concentration will alter the elution rate of each solute along the column. The presence of the double bond on the B-ring in equilin, which is absent in estrone, will result in a more rigid structure compared to estrone. This is an indication that the steroid geometry plays a role in inclusion complex formation as the double bond is the only difference between the two compounds. The more rigid structure of equilin forms a stronger inclusion complex than the more flexible estrone.

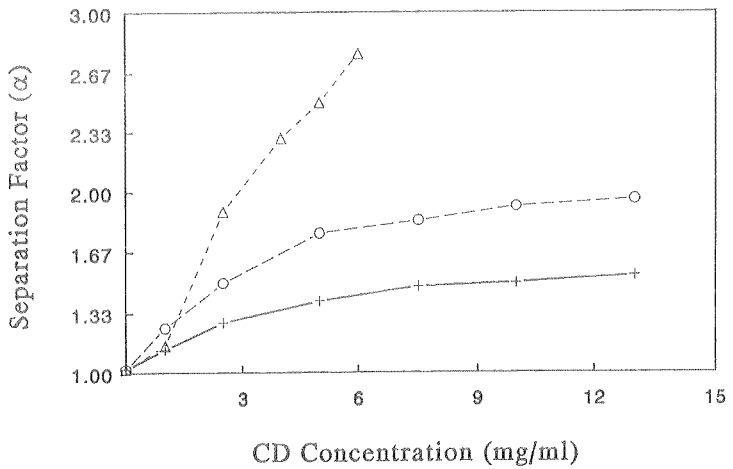

FIGURE 5 Effect of cyclodextrin concentration of the separation of 2- and 4-hydroxyestrone.Mobile phase is 60:40 0.05M KH_2PO_4:methanol plus indicated amount of cyclodextrin. + = DM-β-CD; ∆ = β-CD; O = HE-β-CD.

Figure 5 shows the effect of mobile phase concentration of the three different types of cyclodextrins on the resolution of 2- and 4-hydroxyestrone. β-Cyclodextrin had the greatest effect on resolution, achieving a dramatic increase in resolution. Due to its limited solubility, β-cyclodextrin has a maximum mobile phase concentration of 6 mg/ml at this methanol concentration. Increases in separation were also noted for DM-β-cyclodextrin and HE-β-cyclodextrin as mobile phase additive, but not of the same extent as β-cyclodextrin.

Using the relationship between capacity factor and cyclodextrin concentration developed by Fujimura *et al.* [12], we can calculate the

apparent formation constant (K_f) for the inclusion complex from the following equation:

$$\frac{1}{k'} = \frac{1}{k_0'} + \frac{[CD]\,K_f}{k_0'}$$

where [CD] is the cyclodextrin concentration in the mobile phase and k_0' is the capacity factor for a solute that does not form an inclusion complex. A plot of 1/k' *versus* cyclodextrin concentration gives a straight line from whose slope the formation constant can be determined. It should be noted that these apparent formation constants are dependant on the methanol concentration in the mobile phase as a change in the mobile phase polarity will affect the inclusion complex strength [7]. Table 4 summarizes the apparent formation constants of estrone and its derivatives with the three types of cyclodextrin studied. The methanol content of the mobile phase was 45% v/v. Three of the four steroids formed a stronger inclusion complex with the unmodified β-cyclodextrin while 2-HE formed a stronger complex with the DM-β-CD. In all cases, inclusion complex strength was weaker with the HE-β-cyclodextrin than with the other two forms. The difference in inclusion complex strength between 2- and 4-HE suggests that inclusion complex formation occurs with the A-B rings of the steroids. However, the differences in inclusion complex strength between estrone and

TABLE 4

Apparent Formation Constants for Estrone and it's Derivatives

	K_f (M)			
Mobile Phase Additive	Estrone	2-HE	4-HE	16αHE
β-Cyclodextrin	622	409	1138	482
DM-β-Cyclodextrin	713	481	849	466
HE-β-Cyclodextrin	376	318	714	278

* All mobile phases were composed of 55:45 0.05M KH_2PO_4:methanol

16α-hydroxyestrone implies that an inclusion complex also forms with the C-D rings, so selectivity can be based on differences in both ring systems.

Inclusion complex strength alone cannot be used to predict retention in this system as the solutes may also interacting distinctively with the stationary phase.

Effect of Methanol Concentration

The retention profiles of all five steroids followed the reverse-phase model where the capacity factor of all the steroids decreased with increasing mobile phase methanol concentration [13]. The resolution of equilin and estrone also decreased with increasing methanol concentration. Increasing the organic content of the solvent will weaken the strength of the inclusion

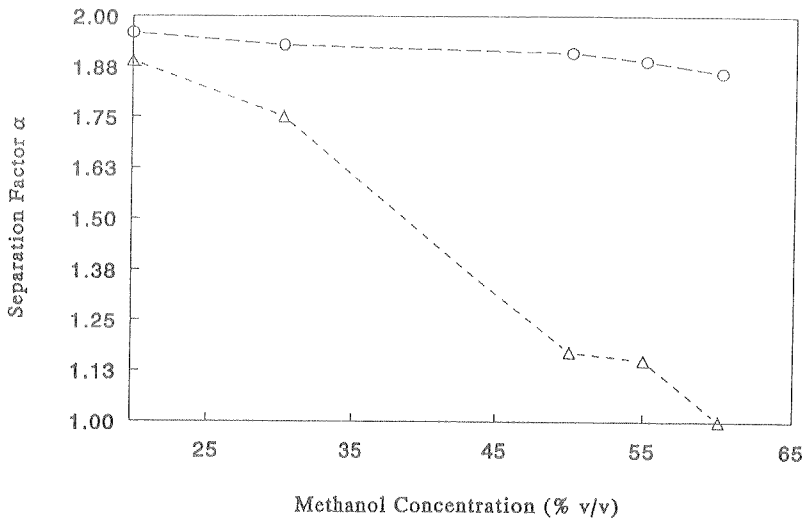

FIGURE 6 Effect of methanol concentration on the separation of 2- & 4-hydroxyestrone and 4- & 16α-hydroxyestrone. Mobile phase contains 4 mg/ml DM-β-cyclodextrin. O = $\alpha_{4\text{-},16\alpha\text{-HE}}$; Δ = $\alpha_{2\text{-},4\text{-HE}}$.

complex formed between the guest and the cyclodextrin [14]. Figure 6 shows the effect of methanol concentration on the separation of 2- & 4-HE and 4- & 16α-HE using DM-β-cyclodextrin as the mobile phase additive. The separation of the 2- and 4-hydroxy isomers declines with increasing methanol concentration until there is no separation at 60% methanol content. For the separation of 4- and 16α-HE, however, the reduction in retention is not accompanied by a loss in separation as the methanol concentration did not alter their relative inclusion complex strengths.

CONCLUSION

Cyclodextrins as a mobile phase additive have proven to be useful in the separation of two similarly structured steroids, equilin and estrone. There was an increase in resolution when β-cyclodextrin was used as a mobile phase additive. There was a further increase in resolution when the cyclodextrin was modified with methyl or hydroxyethyl groups. β-Cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin were also suitable as mobile phase additives in the separation of estrone, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-hydroxyestrone. Inclusion complexes can be formed with the A-B rings and/or with the C-D rings of the steroids.

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ION-PAIR REVERSED-PHASE HPLC IDENTIFICATION OF SUGAR NUCLEOTIDES IN CELL FREE EXTRACTS OF *STREPTOMYCES GRISEUS*

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ABSTRACT

2,6-Dideoxysugars, biosynthetically derived from glucose through sugar nucleotide intermediates, are important structural components of antibiotics. A HPLC method was developed for the detection of ADPG, UDPG, CDPG, GDPG and/or dTDPG in cell free extracts of *Streptomyces griseus*. The resolution of these sugar nucleotides and fourteen additional related mono-, di- and triphosphoribo-nucleotides was achieved by gradient elution, ion-pair reversed-phase chromatography (RP-IPC) over an ODS-C18 column. Cell free extracts contained UDPG, GDPG and UDP, thus implicating the two sugar nucleotides in chromomycin-A_3 biosynthesis.

INTRODUCTION

The Aureolic acids are a family of antitumor antibiotics produced by Streptomycetes. Chromomycin A_3 is an antineoplastic

member of the aureolic acids produced by strains of *Streptomyces griseus*. The structure of chromomycin A_3 is composed of chromomycinone, a highly functionalized aglycone which is substituted at position 2 and 6 with tri- and disaccharide moieties comprised of 2,6-dideoxysugars. Biosynthetic studies of chromomycin A_3 conducted in our laboratory have indicated that glucose is the major precursor in the biogenesis of all five dideoxysugars [1]. The mechanism of dideoxy-sugar formation in bacteria often involves sugar nucleotides such as UDPG, or related cytidine, adenosine, guanosine, or thymidine sugar nucleotides as key intermediates. In an investigation of the biosynthetic potential of our *Streptomyces griseus* culture producing chromomycin A_3 [1], we sought to determine whether sugar nucleotides such as these could be directly detected in cell-free extracts of the producing culture, as a means of guiding additional work concerned with the enzymology of sugar biotransformations.

Liquid chromatography (LC) (including ion-pairing reversed-phase HPLC) has been used in the analysis of nucleotides, nucleosides and their bases in biological samples, usually derived from mammalian preparations [2-8]. GDP-Mannose, GDP-fucose, UDPG and CDPG each have been resolved by strong-anion-exchange (SAX) chromatography [9-11]. UDPG and UDP-galactose have been separated by lectin affinity HPLC as well [12]. The separation of major purine bases, their nucleotides and nucleosides has also been achieved by glass column HPLC with reversed-phase particle packings [13]. However,

the simultaneous HPLC resolution of sugar nucleotides of relevance to deoxysugar biogenesis has not been reported. Since in our own experience, sugar nucleotides like TDPG, ADPG and GDPG were inseparable by SAX chromatography we established an ion-pair, reversed-phase, high-performance liquid chromatographic (RP-HPLC) method for the complete resolution of this family of biosynthetically related compounds in cell free extracts of *Streptomyces griseus*. The analytical separation was obtained using a 5-μm Whatman Partisil ODS-5 C18 column at room temperature under gradient conditions, with quantitation by UV detection at 262 nm.

EXPERIMENTAL

Reagents and Chemicals

All nucleotides were of the highest purity available and were purchased from Sigma. Tetrabutylammonium hydroxide (TBAH) (40% aqueous solution) was also purchased from Sigma (St.Louis, MO, U.S.A.). HPLC grade monopotassium hydrogen phosphate and orthophosphoric acid (85%) were both from Fisher Scientific (Fair Lawn, New Jersey, U.S.A.). Acetonitrile (HPLC grade) was from E. M. Science (Gibbstown, NJ, U.S.A.) and was filtered through type HV 0.45-μm Millipore membranes (Bedford, MA, U.S.A.) before use. Stock solutions of sugar nucleotides (1 mg/ml) were prepared in ultrapure water (E. M. Science, NJ, U.S.A.) and filtered using a 0.2 μm filter (Gelman Science, Ann Arbor, MI, U.S.A.), and kept frozen at -20°C. Standards were routinely used to calibrate HPLC analyses.

Instrumentation

The LC system consisted of a Rheodyne injector type 7125 with a 100-µl loop connected to a Model LC-6A HPLC pump, a SPD-6AV Module UV-VIS detector, a CR-501 Chromatopac recording integrator and a SCL-6B system controller, all from Shimadzu Co. (Osaka, Japan). The analytical column (250 mm x 4.6 mm I.D.) was packed with 5-µm partisil ODS-C18 (Whatman Inc., Clifton, New Jersey, U.S.A.), and preceded by a guard column of the same composition (Alltech Inc., Deerfield, IL, U.S.A.).

Chromatographic Conditions

For solvent system 1, the mobile phase consisted of two solvents: A, pH 5.3, 15 mM KH_2PO_4, containing 10 mM TBAH; and B, pH 5.3, 35 mM KH_2PO_4 with 10 mM TBAH in 30% (v/v) acetonitrile. The mobile phase was prepared in double distilled water, degassed by stirring under house vacuum for 2-3 h, filtered through a 0.22-µm Millipore filter membrane, then degassed again before use. The separation was obtained at a flow rate of 1 ml/min using a concave gradient ranging from 5% to 100% of solution B over a period of 58 min (TABLE 1) while eluting peaks were monitored at 262 nm. Alternatively for those nucleotides not well resolved, the separation was also obtained using solvent system 2 at a flow rate of 1.2 ml/min using a gradient ranging from 0% to 33.3% of solution B over a period of 30 min.

TABLE 1

HPLC Gradient Used in Nucleotide Resolution

Time min	% Solvent Composition A	B	Gradient Curve
Initial	95	5	-
12	95	5	04
20	69	31	04
28	52	48	06
35	45	55	04
45	40	60	04
48	15	85	06
52	0	100	-
58	95	5	-

A: 10 mM Tetrabutylammonium hydroxide, (TBAH), 15 mM KH_2PO_4, pH 5.3
B: 30% CH_3CN, 10 mM TBAH, 35 mM KH_2PO_4, pH 5.3.Flow rate: 1 ml /min.

Growth of *Streptomyces griseus*

A slant of *Streptomyces griseus* (ATCC-13273) was used to inoculate 25 ml of culture medium in 125 ml DeLong flasks [1]. The composition of the medium was (% w/v) dextrose 2.5% stage I, and 5% in stage II, 0.3% sodium chloride, 0.3% calcium carbonate, 1.5% soybean meal. Cultures were incubated at 27 °C while shaking at 250 RPM on

G25 Gyrotory shakers from New Brunswick Scientific Co. After 72 h, stage I cultures were used to inoculate (10 % by volume) stage II cultures which were incubated as before. Stage II cultures (24 h, 70 h, 120 h and 168 h) were harvested by filtration through cheese cloth and subsequent centrifugation of the filtrate in a Sorvall RC-5 Superspeed refrigerated centrifuge at 13,200 x g for 10 min. Cell pellets were washed twice with 30 ml of chilled pH 6, 0.12 M KH_2PO_4 buffer, and centrifuged at 13,200 x g for 10 min each time. Pellets were suspended in cold pH 6, 0.12 M phosphate buffer in H_2O and in 50% MeOH:H_2O to a final concentration of 0.5 g cells (wet weight) per ml. Cell free preparations were made by passing cell suspensions twice through a French Press at 17,000 psi, followed by centrifugation at 50,000 x g for 50 min. Supernatants were filtered through a 0.4-µm filter (Gelman, Acrodisc 13) and 20 µl samples were injected for HPLC nucleotide analyses.

UDPG-Pyrophosphorylase Activity

UDPG-Pyrophosphorylase activity in *Streptomyces griseus* was determined by the modified method of Franke and Sussman [14] in which UDPG synthesis from UTP and glucose-1-phosphate is measured. The reaction mixture contained in a volume of 910 ml: 1.4 mmoles of Glucose-1-phosphate; 0.7 mmole of UTP; 1 mmole of $MgCl_2$; 1.2 mmoles of NAD^+; 340 mmoles of Tris/HCl pH 8.0 buffer; 80 mg (25-50 munits) of UDPG dehydrogenase. Reactions were incubated at room temperature for 1 min while the absorption at 340 nm was

recorded. One unit of enzyme activity reduces 2.0 mmoles of NAD^+ per min under these conditions.

Calibration, Reproducibility and Sugar Nucleotide Recovery

Calibration graphs were prepared using standard solutions of sugar nucleotides, and HPLC-UV quantitation was based on peak areas. Linearity in detector response was observed between 0.1 μg-2.5 μg for each compound. Least squares regression lines intercepted near zero with correlation coefficients greater than 0.98. In general, the measurement range of this method is 0.04 aufs (absorbance unit full scale) with a signal-to-noise ratio no less than 2.5/1. Under these conditions, the detection limit of this assay is 0.1 μg (approximately 8 mM) for dTDPG, UDPG, ADPG, GDPG and CDPG. The retention volumes of analytes and the void volume (2.0 mL) are listed in TABLE 2. Recoveries of standards of dTDPG, UDPG, ADPG, GDPG and CDPG were determined by preparing 320 mM solutions of these sugar nucleotides in fresh, 72 h cell free extracts, and by injecting spiked extracts for HPLC analyses.

Confirmation of peaks in *Streptomyces griseus* cell free extracts was done by co-injections with standard samples.

RESULTS AND DISCUSSION

A reversed phase, concave gradient, ion-pairing HPLC method was designed to assay sugar nucleotides in *Streptomyces griseus* cell free extracts. Sugar nucleotides of interest were well resolved by this

TABLE 2

Retention Volumes of Nucleotides by Ion-Pairing RP-HPLC, Solvent System 1, (Void Volume= 2.0 ml)

Compound	Mean Retention Volume[a] (ml)
CMP	13.8±0.3
UMP	16.4±0.4
GMP	22.0±0.3
dTMP	27.0±0.2
AMP	30.2±0.1
CDP	32.0±0.1
UDP	34.3±0.1
GDP	35.0±0.2
dTDP	38.5±0.5
ADP	40.8±1.0
CTP	42.9±0.2
UTP	45.0±0.1
GTP	45.5±0.1
dTTP	54.1±1.0
ATP	58.2±1.2
CDPG	25.9±0.3
UDPG	28.2±0.1
GDPG	30.3±0.2
dTDPG	33.1±0.1
ADPG	35.8±0.1

[a]Mean of at least 3 replicates ± SD.

TABLE 3

Retention Volumes of Various Nucleotides Using Solvent System 2

Compound	Retention Volume (ml)
UTP	21.0
UDP	14.3
UDPG	10.5
CDPG	9.2
GDPG	12.8
GTP	20.0
dTDP	16.4
dTDPG	13.2
AMP	11.8
ADPG	15.2

method, and the system could be used to resolve twenty nucleotides (TABLE 2). A second system was established to resolve those nucleotides with very similar retention volumes (TABLE 3). HPLC resolution of a mixture of standards of CMP, UMP, GMP, CDPG, UDPG, GDPG, dTDPG, ADPG, dTDP, ADP and dTTP is shown, for example, in FIGURE 1.

Our strategy was to assay *Streptomyces griseus* cell free extracts for sugar nucleotides, to confirm their presence by co-injection with standards, and to confirm the existence of compounds by the presence

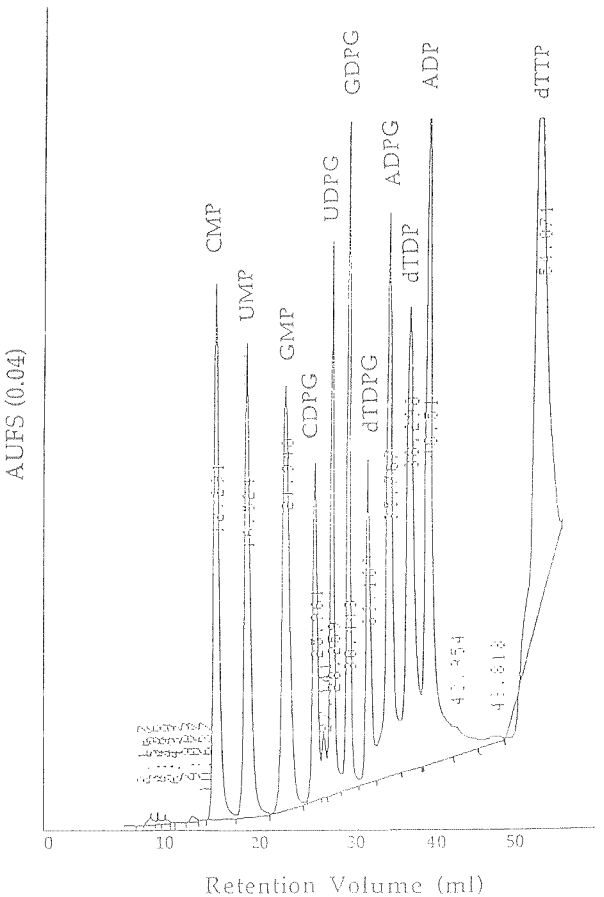

FIGURE 1.

RP-HPLC Chromatogram produced by injecting a mixture containing 1.5 mg of nucleotide standards using solvent system 1 (TABLE 1).

of enzyme activities that form them. While the use of enzyme assays alone has been used to detect the presence of sugar nucleotide biosynthetic enzymes, this combination LC and enzyme approach has apparently never been used. Recoveries of the five sugar nucleotides from spiked extract samples ranged between 97% and 100%. This result indicates that the sugar nucleotides are not subject to enzymatic or other decomposition in cell free extracts. Antibiotic biosynthesis typically begins at 48 h with this culture [1], and cell samples were taken periodically at 24 h intervals beginning at 24 h and ending at 168 h of growth. Representative chromatograms of cell-free-extracts of stage II *Streptomyces griseus* cultures harvested at 70 h and 120 h are shown in FIGURE 2. None of the peaks eluting in the 70 h extract were prominent, although relatively small peaks with *Rv* for UDPG and GDPG were observed. However, peaks for UDPG (90 mM), GDPG (40 mM) and UDP (37 mM) were observed in the 120 h cell extract. None of the other peaks observed in these extracts corresponded to known nucleotides evaluated in this study. Peaks, such as that at R_v 14.3 in the 120 h sample, (FIGURE 2) could be putative deoxysugar nucleotide intermediates important in chromomycin A_3 biogenesis, and for which no standards are available.

Using HPLC, the time-course for formation of UDPG was evaluated from 24 h to 168 h. Over this time period, UDPG begins to increase in concentration at 70 hours, and climaxes between 120 to 140 hours (FIGURE 3). Although nucleotide identities in extracts were confirmed by coinjections with authentic standards and with the

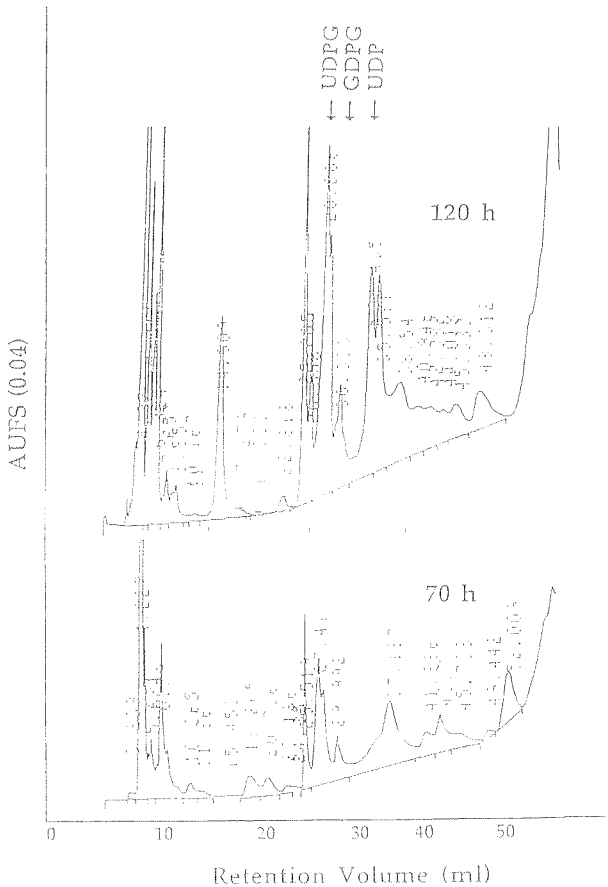

FIGURE 2.

Chromatograms of 70 h and 120 h *Streptomyces griseus* cell-free extracts.

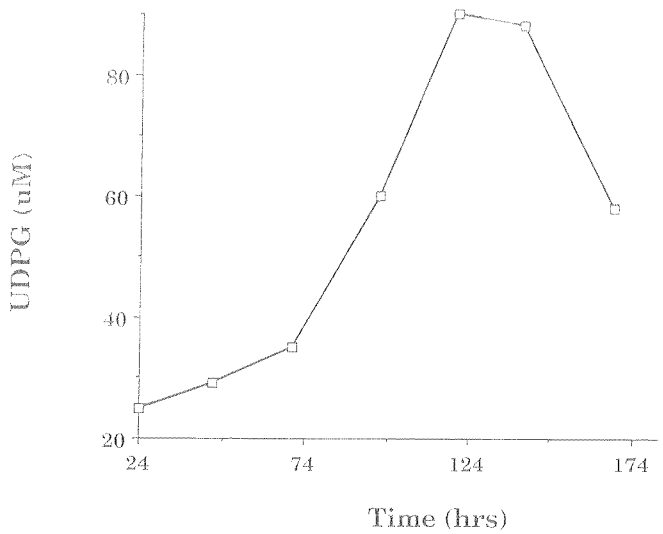

FIGURE 3.

Concentration of UDPG in cell free extracts *vs* fermentation time as determined by HPLC analysis.

second HPLC system (TABLE 3), the presence of imperfectly resolved unknown analytes appearubg as shoulders on peaks for UDPG, and GDPG and an overlapping peak with UDP (FIGURE 3) precluded accurate quantitative analyses of the nucleotides, or their ready characterization by isolation, chemical or spectral means.

The results suggested that UDPG was the active sugar nucleotide intermediate involved in the conversion of glucose to 2,6-dideoxy sugars in the biosynthesis of aureolic acid[1]. Cell free extracts of *S.griseus* were evaluated for UDPG pyrophosphorylase enzyme activities to confirm that the enzyme system necessary for the

formation of UDPG from UTP and glucose-1-phosphate was present. Enzyme activity *vs* time (0.022 units/mg protein at maximum) increased in essentially the same manner as UDPG by HPLC.

This HPLC ion-pairing, reversed-phase gradient approach affords a rapid means of assessing cell free extracts of antibiotic-producing Streptomycetes for the possible presence of sugar nucleotides relevant to antibiotic biosynthesis. Results from chromatographic analyses provide information crucial to the specific nature of enzymes such as UDPG pyrophosphorylase involved in sugar activation and metabolism.

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A SIMPLE HPLC ASSAY FOR PROGUANIL AND ITS ACTIVE METABOLITE CYCLOGUANIL: APPLICATION TO OXIDATION PHENOTYPING

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ABSTRACT

A simple high-performance liquid chromatographic (HPLC) method for simultaneous determination of proguanil and its active metabolite cycloguanil in human urine has been developed. Quinine sulphate was used as the internal standard. The assay uses a reversed phase C18 microbore column (2 mm I.D. x 10 cm) packed with 3 μm ODS Hypersil. The chromatographic separation was achieved by using an isocratic mobile phase comprising acetonitrile-aqueous phosphate buffer (10:90, v/v) containing 200 mM sodium dodecyl sulphate adjusted to pH 2. The mobile phase was pumped at 0.4 ml/min. The eluant was monitored by a UV detector operating at 254 nm. The assay was based on an organic extraction with 1-hexanol/ether (40: 60, % v/v) and then back-extracted into a small volume of acidic aqueous solution before injection onto the HPLC column. With this procedure coefficients of variation were less than 8%. The detection limit was 0.5 μg/ml of urine. The method is simple, sensitive, selective and allows for routine analysis of urine samples in the genetic drug oxidation phenotyping study in ethnic population.

INTRODUCTION

Proguanil (PG) is an antimalarial drug commonly used for malaria (i.e., *Plasmodium falciparum*) prophylaxis. Although it is not widely used in Southeast Asia regions, the metabolism of proguanil is important as the drug itself is not

pharmacologically active and must be metabolised by P450 enzyme to form the active metabolite, cycloguanil (1). The metabolism of proguanil, which is catalysed by a particular cytochrome P450 isoenzyme *(CYP2C19)*, has also been found to exhibit an evidence of polymorphism (1-4). The genetic drug oxidation polymorphism of proguanil has been studied in a few ethnic populations. Two oxidation phenotypes have been identified. In Caucasian population, most subjects are extensive metabolisers (EMs) of proguanil, whereas a minority of subjects are poor metabolisers (PMs). For instance approximately 3% of a Caucasian population were identified as PMs of proguanil, whose the ability to convert proguanil (PG) to its active metabolite, cycloguanil (CG) was markedly reduced (1). An increasing number of studies have demonstrated that genetic oxidation polymorphism of proguanil shows pronounced inter-ethnic variability. In contrast to the Caucasian population, the incidence of PMs of proguanil appears to be considerably higher in non-Caucasian populations. A study carried out in 100 healthy Kenyan adults showed that 35% were identified as PMs (5). A recent study in a Thai population reported the incidence of proguanil PMs to be 18% (6). The difference in ability of individuals to convert this prodrug to the active metabolite, cycloguanil, could have important clinical consequences with respect to the therapeutic success of this antimalarial prodrug.

A number of high-performance liquid chromatographic (HPLC) methods have been developed (7-11). These methods allow for the simultaneous determination of proguanil and its metabolite cycloguanil in biological fluids. However, most of these assays involved many extraction steps and some used solid phase extraction as sample preparations before HPLC analysis which is less economic and time consuming. The present report describes a simple and sensitive HPLC method for simultaneous quantitation of proguanil and its metabolite cycloguanil in human urine. The applicability of this procedure is demonstrated by the analysis of urine samples collected from volunteers who participated in a study of drug oxidation phenotyping which was aimed to determine the PM incidence of proguanil, in an ethnic population.

MATERIALS AND METHODS

Reagents and Chemicals

Proguanil hydrochloride and cycloguanil were gift from Major M D Edstein, Australian Army Malaria Research Unit, New South Wales, Australia. Quinine

sulphate (as an internal standard) was purchased from Sigma Chemical Co (St Louis, MO, USA). Sodium dodecyl sulphate (SDS), orthophosphoric acid, HPLC-grade acetonitrile, 1-hexanol and diethyl ether were purchased from BDH Chemicals Ltd (Poole, England). All chemicals used were analytical grade. Glassware was cleaned and silanized with 0.05% Aquasil® (Pierce Chemical Co., Rockford, IL, USA) before use. Water was double glass distilled and MilliQ® filtered.

Standard Solutions and Internal Standard

Stock solutions (100 μg/ml) of proguanil and cycloguanil were prepared in HPLC water and stored at -20°C until required. These solutions was found to be stable for at least 2 weeks at -20°C. Urine standard solutions of proguanil and cycloguanil for the calibration curves were prepared by proper dilution of the stock solutions with drug-free urine so that concentrations of 0.5, 1, 2.5, 5, 10, 20 and 50 μg/ml were obtained. The internal standard solution of quinine sulphate (40 μg/ml) was prepared in HPLC-grade water. This solution was protected from light and stored at -20°C until required; it was found to be stable for at least 2 month.

Sample Preparation

To 2 ml of urine sample in a silanized centrifuge tube, 150 μl of 4 M NaOH and 200 μl of internal standard solution (40 μg/ml quinine sulphate) were added. The contents were then extracted with 6 ml of 1-hexanol/ether (40: 60, % v/v) by mechanically shaking for 25 minutes. The samples were centrifuged for 10 minutes at 1500 g (at 4°C) to separate the phases. The organic layer was transferred to a clean tapered glass centrifuge tube containing 250 μl of 0.1% orthophosphoric acid. The mixture was shaken for 25 minutes and centrifuged (1500 g, 4°C) for 10 minutes. The organic layer was aspirated and discarded. The aqueous extract was transferred to the autosampler plastic vials and 50 μl was injected into the HPLC column.

Chromatographic Conditions

The HPLC system consisted of a Model 250 Perkin Elmer LC pump (Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a Jasco Model AS-950 autosampler (Jasco Corporation, Tokyo, Japan). A variable wavelength ultraviolet detector (Spectroflow 757, Kratos Analytical Instruments, Ramsey, NJ, USA) operating at 254 nm was used with a setting of 0.01 a.u.f.s. The chromatographic response was recorded by a Shimadzu R3A integrator (Shimadzu, Kyoto, Japan).

A microbore HPLC column (2 mm I.D. x 10 cm) packed with a reversed-phase C18 material, 3 μm ODS Hypersil (Phenomenex, CA, USA) was used. The column efficiency was over 3000 plates per 10 cm. Analysis of the samples of proguanil and cycloguanil was performed using a mobile phase consisting of an acetonitrile-aqueous phosphate buffer (10 mM Na_2HPO_4) mixture (10:90, v/v) containing 200 mM sodium dodecyl sulphate (SDS) and adjusted to pH 2 with orthophosphoric acid. The flow rate of the mobile phase was 0.4 ml/min (back pressure approximately 1800 psi). Chromatographic separations were performed at room temperature.

Recovery

The assay recovery of proguanil and cycloguanil from urine samples was determined at 0.5 and 20 μg/ml. Absolute recovery was calculated by comparing the peak heights from 5 extracted urine samples with those obtained by direct injection of the pure drug standards. The absolute recovery of the internal standard (quinine) was assessed using the same procedure.

Calibration Curve

Standards corresponding to 0, 0.5, 1, 2.5, 5, 10, 20 and 50 μg/ml of proguanil and cycloguanil were prepared in drug-free urine. The sample analysis was performed as described above and standard curves were run daily. Quantitation is based on peak height ratios (proguanil or cycloguanil/internal standard). An unweighted least squares regression line was fitted to each individual calibration curve.

Proguanil Oxidation Phenotyping Study

The study protocol was approved by the Southern Regional Health Authority Ethics Committee (Otago), Dunedin, New Zealand. The subjects were 43 unrelated, healthy Maoris who were all born in New Zealand, aged from 18 - 35 years. On the study day, urine was voided immediately before they receiving an oral dose of 200 mg proguanil hydrochloride (Paludrine® tablets, ICI New Zealand). Urine was collected for the following 8 hours. The total urine volume was recorded and an aliquot was kept frozen at -20°C until analysis. Proguanil (PG) and its active metabolite cycloguanil (CG), were measured by the HPLC procedure described above. Individuals with urinary concentration (PG/CG) ratios greater than 10 were classified as PMs of PG (1).

Statistical Analysis

Results given are mean ± S.D. Student *t-test* was used throughout the study unless otherwise stated.

RESULTS AND DISCUSSION

The liquid chromatographic separation of proguanil, its active metabolite cycloguanil, and the internal standard (quinine) from the endogenous urine peaks was achieved by using a reversed phase C18 microbore column with an acetonitrile-aqueous phosphate buffer mixture (10:90, v/v) containing 200 mM SDS adjusted to pH 2. Under these chromatographic conditions, cycloguanil, quinine (the internal standard) and proguanil were eluted with retention times of 9.7, 14.7, and 18.6 min, respectively (Figure 1). The present HPLC assay developed is selective for proguanil in the presence of its metabolite cycloguanil and the internal standard . The peaks of proguanil, its metabolites and the internal standard were well resolved.

Figure 1 also shows chromatograms of blank (drug-free) urine, and urine spiked with 0.5 μg/ml proguanil, and 0.5 μg/ml of cycloguanil. Under these chromatographic conditions, no endogenous sources of interference were observed. More than 100 human blank urine samples were analysed in the study and no endogenous peaks with retention time similar to proguanil, cycloguanil and the internal standard (quinine), were detected.

The mean recovery of proguanil from urine was 83.0 ± 8% (S.D.) at 0.5 μg/ml and 85.7 ± 7% at 20 μg/ml (n = 5). The mean recovery of its metabolite cycloguanil from urine was 88.0 ± 7% (S.D.) at 0.5 μg/ml and 90.5 ± 8% at 20 μg/ml (n = 5). Recovery of the internal standard was also satisfactory at the concentration used with a recovery of 80 ± 5% (n = 5).

The standard curve of both proguanil and cycloguanil was linear over the concentration range of 0.5 to 50 μg/ml with the square of the correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curves can be expressed by the equations: $y = 0.1343x$; and $y = 0.1186x$, for proguanil and cycloguanil respectively, where y is the peak ratio and x is the urinary drug concentration (μg/ml). The intercept (a) in all calibration curves were statistically insignificant ($p > 0.1$) and were thus not included for the calculations. The day-to-day coefficient of variations (C.V.) of the slope of the standard curves of both proguanil and cycloguanil were less than 7% (n = 5).

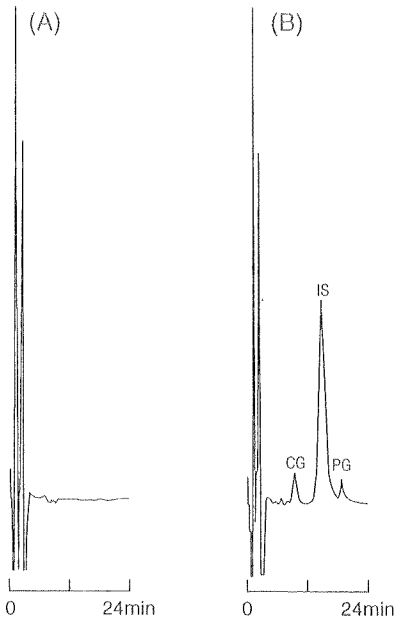

FIGURE 1

Typical chromatograms of extracts of human urine samples: (A) blank urine; and (B) urine spiked with 0.5 μg/ml cycloguanil and 0.5 μg/ml proguanil.
Peaks: CG = cycloguanil; PG = proguanil; IS = internal standard (quinine).

The within-day (within-run) reproducibility and accuracy of the proguanil and cycloguanil assay are presented in Table 1. At all concentrations studied the C.V. was less than 8%. These results indicate good precision of the assay. The C.V. of the assay at a concentration of 0.5 μg/ml, was 7.7% for proguanil and 6.4% for cycloguanil with good accuracy (Table 1). These C.V. values are lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). Thus, the MQC or the detection limit of sensitivity for this assay was assigned at 0.5 μg/ml.

Urine samples stored at -20°C for up to 2 months showed no signs of decomposition and there was no difference in the urinary concentrations of proguanil and cycloguanil between the fresh samples and the stored urine samples ($n = 6$, $p > 0.2$). This indicates that proguanil and cycloguanil are stable under these storage conditions for at least 2 months.

TABLE 1

Within-day Reproducibility and Accuracy of the Assay for Proguanil and Cycloguanil in Human Urine Samples

Spike Concentration (μg/ml)	Observed Concentration[1] (μg/ml)	C.V. (%)	Accuracy[2] (%)
Proguanil			
0.5	0.52 ± 0.04	7.7	104
20	21.0 ± 1.1	5.2	105
Cycloguanil			
0.5	0.47 ± 0.03	6.4	94
20	19.8 ± 0.9	4.5	99

[1] Results given are mean ± S.D. (n = 5)

[2] Accuracy (%) = $\frac{\text{observed concentration}}{\text{spiked concentration}} \times 100$

The present method was used to determine the proguanil and cycloguanil concentrations in urine samples collected from healthy volunteers who participated in a study of the proguanil oxidation phenotyping. Examples of chromatograms obtained from a poor metaboliser (PM) and an extensive metaboliser (EM) of proguanil are shown in Figure 2. Of 43 Maori volunteers phenotyped, 3 subjects were identified as PMs of proguanil. This represents 7% prevalence of the proguanil poor metaboliser phenotype in the Maori population. Full details of this study on the evidence of poor metabolisers of proguanil in the New Zealand Maori population were published elsewhere (12). Although the detection limit of sensitivity (0.5 μg/ml) for the present assay was not as low as the other previous reports, we found that it provided sufficient sensitivity for the purpose of phenotyping. None of the urine samples analysed had a concentration of proguanil and cycloguanil below the detection limit. Some urine samples in fact needed to be diluted before analysis as the concentrations were too high.

In summary, a sensitive HPLC method has been described for the quantitative analysis of proguanil and its active metabolite cycloguanil in human urine. The procedures are simple and less time-consuming as the assay does not use solid

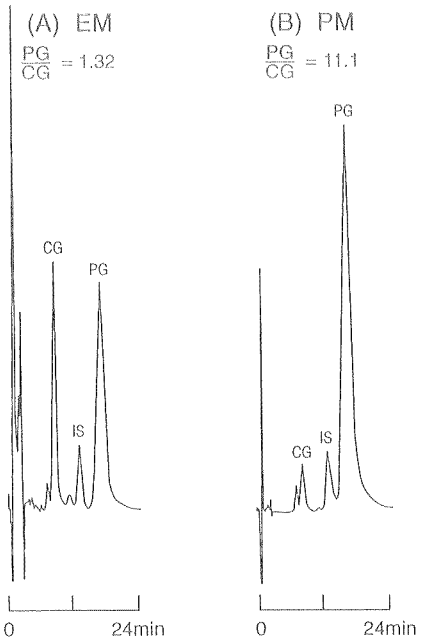

FIGURE 2

Examples of chromatograms of urine samples (0-8 hours) collected from an extensive metaboliser (A); and a poor metaboliser (B), after an oral dose of 200 mg proguanil hydrochloride (Paludrine® tablets, ICI New Zealand). Peak identification is as in Figure 1.

phase extraction. The method provides a sufficient sensitivity for the simultaneous determination of proguanil and cycloguanil. The assay has been shown to be suitable for use in studies of proguanil oxidation phenotyping in ethnic populations.

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DETERMINATION OF MINOR IMPURITIES OF (R)-1-ACETOXYMETHYL-3-(7-[(1-N,N-DIMETHYLCARBAMOYL)-6-(4-FLUOROPHENYL)-INDOL-3-OYL]-1H,3H-PYRROLO[1,2-c] THIAZOL-3-YL)PYRIDINIUM CHLORIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Minor impurities of (R)-1-acetoxymethyl-3-(7-[(1-N,N-dimethylcarbamoyl)-6-(4-fluorophenyl)-indol-3-oyl]-1H,3H-pyrrolo[1,2-c]thiazol-3-yl)pyridinium chloride (I), a pro-drug, were determined using high-performance liquid chromatography. Manufacturing impurities, degradation products, and active drug, (R)-7-[(1-N,N-dimethylcarbamoyl)-6-(4-fluorophenyl)-indol-3-oyl]-1H,3H-pyrrolo[1,2-c]thiazole (II) were separated using a reversed-phase system with gradient elution. Detector response was linear for II to approximately 470 μg/mL which represents 47% of the drug concentration. The procedure provides relative standard deviations of 3.0% to 13.2% in typical bulk drug lots. A variety of reversed-phased columns were evaluated for the assay method with the optimum resolution achieved using a 5-μm Zorbax Rx C-8 packing.

INTRODUCTION

The synthesis of (R)-1-acetoxymethyl-3-(7-[(1-N,N-dimethylcarbamoyl)-6-(4-fluorophenyl)-indol-3-oyl]-1H,3H-pyrrolo[1,2-c]thiazol-3-yl)pyridinium chloride (I, See Figure 1), a platelet activating factor antagonist and pro-drug has been previously reported [1,2]. As reported recently [2], this class of compound has in vitro and in vivo activity in assay models for inflammation and septic shock. The synthesis of I is necessitated due to the lack of water solubility of (R)-7-[(1-N,N-dimethylcarbamoyl)-6-(4-fluorophenyl)-indol-3-oyl]-1H,3H-pyrrolo[1,2-c]thiazole (II), the active drug. This paper describes the use of high performance liquid chromatography (HPLC) for the quantitation of minor impurities in I bulk drug substance. Potential manufacturing impurities and degradation products are determinable by the procedure in addition to the quantity of active drug, II, in the bulk drug.

Although not a fluoroquinolone, the structure was viewed as being similar enough to other fluoroquinolones previously reported by this laboratory [3-5], which had been separated by gradient elution. Using these gradients as a starting point, and with the goal of separating the active drug from the pro-drug, the separation described was developed. The separation was further complicated by the vast structural differences between intermediates III and IV, with IV being a very lipophilic molecule and III being a very hydrophilic molecule. In this work a separation was pursued to adequately resolve the pro-drug and active drug and in the same chromatographic run quantitate minor impurities and degradates which can show marked differences in retention times using reversed-phase systems. The stability of I sample preparations was also an issue. Being a pro-drug, designed to hydrolyze rapidly to the active drug in the body, sample preparations had a limited usable lifetime.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model SP-8800 ternary pump and chromjet data handling system (Spectra-Physics, Santa Clara, CA, USA). A

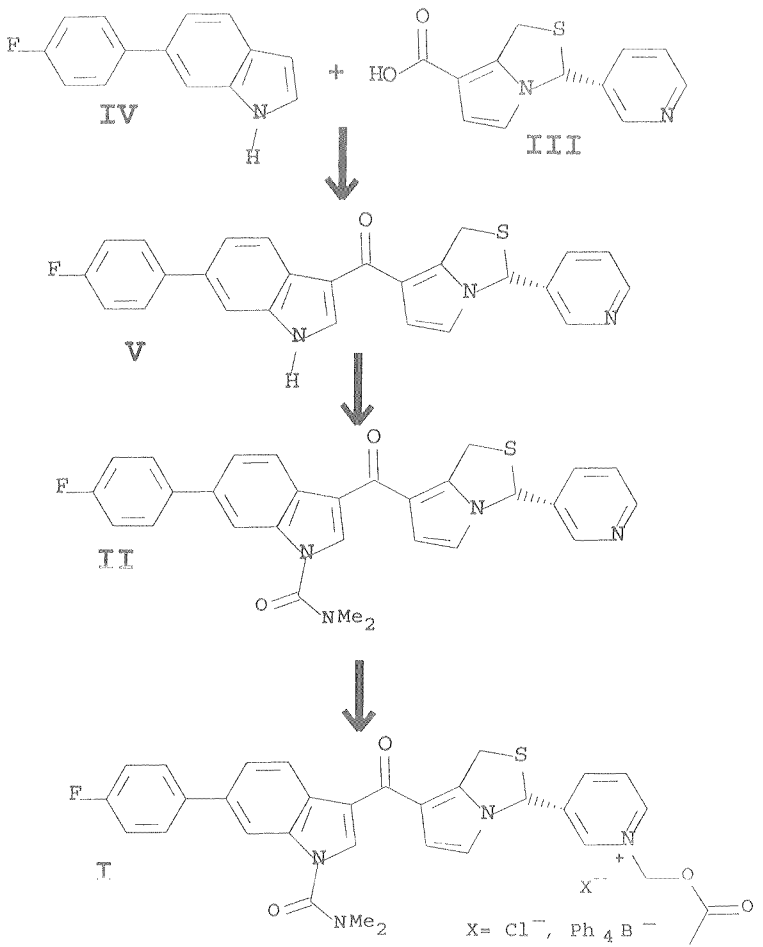

Figure 1. Synthetic Scheme for Preparation of I

Model 757 variable-wavelength UV detector (ABI Analytical Kratos Division, Ramsey, NJ, USA) and a Model SIL-10A autosampler with sample cooler at about 5°C (Shimadzu Scientific, Columbia, MD, USA) were used. Chromatographic separations described in this method were made using Zorbax Rx C-8 columns (5 μm, 80 Å) measuring 25 cm x 4.6 mm I.D. (Mac-Mod Analytical, Chadds Ford, PA). The following columns were also

evaluated for their suitability: Alltima C-18, 4.6 mm x 15 cm, 5 μm 100 Å and Nucelosil C-18, 5 μm, 4.6 mm x 15 cm (Alltech Associates, Deerfield, IL, USA); CSC-Nucelosil 5 μm, 4.6 mm x 15 cm, (Resolution Systems, Wilmette, IL, USA); Nucelosil C-18, 5 μm, 4.6 x 15 cm, (Column Resolution Inc., San Jose, CA, USA); Primesphere C-18HC, 4.6 mm x 25 cm, 5 μm (Phenomenex, Torrance, CA, USA); Spherisorb S10ODS C-18, 4.6 mm x 30 cm, 10 μm and Spherisorb S5ODS C-18, Hi-Chrom Rev, 5 μm, 4.6 mm x 25 cm (Regis, Morton Grove, IL, USA). Prior to use, the components of the eluent were filtered through 0.45 μm nylon membranes (Alltech).

Reagents

Acetonitrile (ACN) was Omni-Solv grade from EM Sciences (Cherry Hill, NJ, USA). The aqueous mobile phase was prepared by dissolving 2 mL of perchloric acid (Fisher Scientific) in 1 liter of water. Sodium pentane sulfonate was reagent grade from Aldrich Chemical Co. (Milwaukee, WI, USA). All bulk drug and related impurities were synthesized at Abbott Labs (North Chicago, IL, USA). Isolated compounds were characterized by ^{1}H, ^{13}C NMR and mass spectrometry. The diluent was prepared by mixing 1 liter of aqueous mobile phase and 1 liter of acetonitrile.

Chromatographic Conditions

A two step linear gradient was used, mixed with the ternary pump as shown in Table I. Other conditions were: flow-rate, 1.0 mL/min; pressure approximately 1800 psi; detector, 254 nm at 0.10 a.u.f.s., attenuation 128, and injection volume, 20 μL. All separations were performed at ambient temperature. The sample preparations were kept at about 5°C before injection.

Analytical Procedure

Bulk drug samples of I were prepared by dissolving approximately 100 mg of drug substance in 50 mL of diluent followed by dilution to 100.0 mL with diluent. A 1% standard of II was prepared by dissolving approximately

TABLE 1.

Linear Gradient for HPLC Eluent

Time (min)	Aqueous $HClO_4$ (%)	ACN (%)
0	90	10
5	90	10
15	60	40
65	05	95
66[a]	90	10
90[a]	90	10

[a] Used to re-equilibrate the column to the initial conditions.

100 mg in 20 mL of acetonitrile in a 100-mL volumetric flask, followed by dilution to volume with diluent. The above solution was serially diluted 5 mL to 50 mL then 5 mL to 50 mL with diluent. The amounts of impurities were estimated in the sample by comparing the corresponding peak areas in the samples and standard preparations. Impurity content was calculated on the anhydrous basis by correcting the sample concentration for the amount of water and residual solvents contained in the drug substance. The drug substance typically contains approximately 1% water, as measured by Karl Fisher titration and 1-5% isopropanol, as measured by gas chromatography.

Results and Discussion

Since compound I is a pyridium salt and II is the neutral base without the methylacetoxy portion, the chromatographic behavior of I is relatively independent of pH where as II is pH dependent as well as sensitive to the organic modifier of the eluent. The most symmetrical peak shapes for I and II were obtained in eluents containing aqueous solutions at pH values of approximately 2 to 4. The retention characteristics of the I, II, and related impurities did not vary significantly with the type of buffer. However, the order of elution of I and II could be reversed using trifluoroacetic acid (0.2%)

or sodium pentane sulfonate (0.2% w/v, pH=2.0 with H_2SO_4). The decision to use perchloric acid for the aqueous solution was based on the lack of interfering peaks in the blank solutions and lack of integration problems when II elutes before the drug I which were observed when II eluted after I. The separation of impurities and peak shapes for all three systems were not significantly different and the trifluoroacetic acid system and variations on it were in fact used for the LC-MS determination of minor impurities.

Single isocratic eluents failed to adequately resolve the drug and pro-drug and did not retain intermediate III at all or the retention of IV was unacceptably long (>60 min) and broad. For these reasons, a gradient elution system was developed. Single organic modifiers of tetrahydrofuran, methanol, and acetonitrile were used in this approach. Again, only acetonitrile modifier proved acceptable. Mixed organic systems also proved unacceptable. The gradient used provided the needed resolution between I and II and acceptable retention times for the other intermediates (Figures 2 and 3). If intermediate III could be eliminated, then the gradient could be varied in order to increase the resolution between I, II, and V. Because this was not the case for the synthetic scheme adopted, the system described proved to be the best.

A detection wavelength of 254 nm provides a very similar response for the impurities, the active drug (I), and pro-drug (II), providing an accurate estimation of unknowns quantitated versus II. The response of I versus II is approximately 0.8, which is the ratio of the molecular weights. This is not a surprising result considering the wavelength used and the functional groups in the back-bone of the drug. A response factor for V has not been determined at this time due the lack of an acceptably pure lot, but it would again not be expected to differ much from I and II. Chelating agents such as EDTA were evaluated because they have been used for other nitrogen containing drugs [3-5] due to the possible adsorption of the drug to metal surfaces or peak broadening in the presence of trace metals. The use of EDTA did not improve the separation and was not used further.

Several C-8 and C-18 reversed-phase packings were evaluated for the determination of pro-drug impurities. For this evaluation, identical gradient profiles were used as described in the text. The results are summarized in

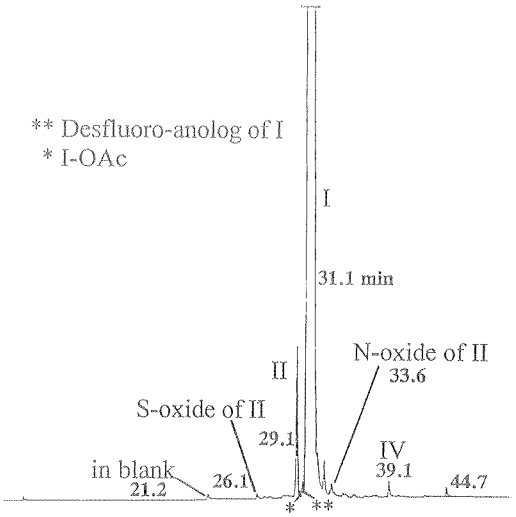

Figure 2. Typical Chromatogram of a Representative Lot of I

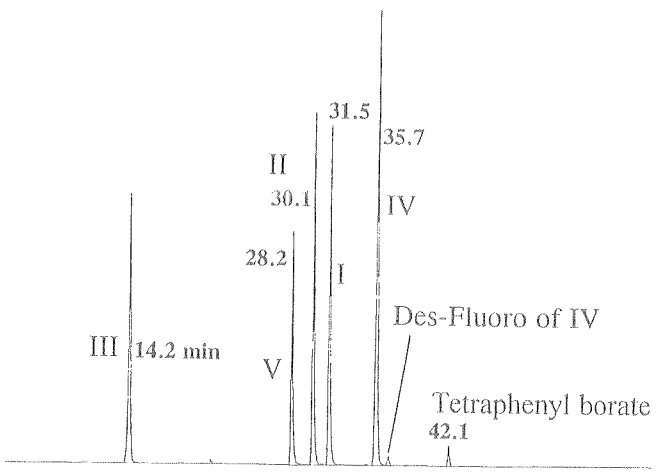

Figure 3. Synthetic Mixture of Intermediates

TABLE 2

Compariton of Column Packing on the Resolution of I and II and Tailing Factor of I

Packing	Resolution Factor[a]	Tailing Factor[a]
Nucelosil, Alltech 4.6 mm x 15 cm, 5 μm	2.0	1.4
Alltima C-18 4.6 mm x 15 cm, 5 μm	2.6	2.2
Nucelosil, CSC 4.6 mm x 15 cm, 5μm	4.0	1.0
Nucelosil, Column Resolution 4.6 mm x 15 cm, 5 μm	2.9	1.7
Primesphere C-18HC 4.6 mm x 25 cm, 5 μm	6.0	1.0
Spherisorb S5ODS 4.6 mm x 25 cm, 5 μm	7.3	2.5
Workhorse S10ODS 4.6 mm x 25 cm, 10 μm	7.3	1.5
μ-Bondapak C-18 3.9 mm x 30 cm, 5 μm	2.4	1.5
Zorbax Rx C-8[†] 4.6 mm x 250 cm, 5 μm	4.8	1.0

[a]U.S. Pharmacopia XXIII, p. 1777.
[†]System desribed in text.

Table II. The comparison of resolution factors calculated between I and II, which is a critical separation in our application, demonstrates that the separation is largely a function of the type of reversed-phase column used. Some of the other columns evaluated did produce greater resolution between I and II but sacrificed peak shape to obtain more resolution. The only other column that appeared acceptable was the Primesphere C-18 column, which had

a resolution of 6.0 (typical R=4-6). The choice of the Zorbax RxC-8 column over the Primesphere C-18 column was based on this laboratory's experience with the stability of the Zorbax column and its multiple uses in this laboratory. In our limited use the Primesphere performed equally as well and most likely could be substituted.

The system used in the procedure is also more useful than others evaluated in the resolving more impurities, the most important of which is the desfluoro-analog of the active drug. It's relative retention time was 0.99 and in the best scenario was a leading shoulder on the main drug peak. The peaks that can be seen in a typical lot of I are shown in Figure 2.

Detector response for II was linear from 0.5 μg/mL to 470 μg/mL and for I was linear from 1.1 μg/mL to 430 μg/mL (correlation coefficients >0.999). Linearity curves of concentration versus response essentially intersected the origin for both compounds, allowing the use of one-point calibration for quantitation of impurities. Assay precision was assessed by performing the procedure on one lot of I. Two analysts performed the determinations on different days. The assay precision (relative standard deviation values) ranged from 3.0% to 13.2% for impurities having mean values of 0.48% to 0.11%.

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PURIFICATION OF SYNTHETIC LIPOPEPTIDE CONJUGATES BY LIQUID CHROMATOGRAPHY

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ABSTRACT

Conventional normal phase liquid chromatography (LC) was implemented for the purification of a synthetic lipid-peptide conjugate and its electrophilic lipid precursor. N-bromoacetic distearoylphosphatidylethanolamine (DSPE-$COCH_2Br$) was extracted from the crude reaction mixture and purified in a single chromatographic step with a gradient of chloroform, methanol, and 50% aqueous acetic acid. This compound was covalently linked to Ser-Phe-Leu-Leu-Arg-Asn-$(\beta Ala)_3$-Tyr-$NHCH_2CH_2SH$, a hydrophilic decapeptide, and the conjugate was both extracted and purified in a single step by normal phase gradient LC using a hand-packed 3 mL Sep-Pak™ column. The eluent was a mixture of hexane, chloroform, 2-propanol, acetic acid, and water. Runs were monitored by thin layer chromatography and the plates developed with iodine and ninhydrin.

INTRODUCTION

Recent interest in liposome-based targeted drug delivery and membrane-mimetic approaches for advanced materials has produced a growing list of derivatized phospholipids. Recognition molecules (biotin, antigens, antibodies), drugs (methotrexate), and enzymes have all been conjugated to phospholipids (1). Purification of these compounds is often difficult, particularly as the amphiphilic

character of the molecule increases. However, successful purification has been achieved with normal phase thin layer chromatography (TLC) for phosphatidylethanolamine (PE)-biotin (2), dinitrophenylated PE (3), ω-carboxyalkyl PE (4), and methotrexate-conjugated PE (5, 6). Similarly, high-performance liquid chromatography (HPLC) and liquid-liquid extraction (LLE) have been implemented for the recovery of lipid-biotin conjugates and N-(4-(*p*-maleimidophenyl)butyryl) phosphatidylethanolamine (PE-MPB), respectively (7, 8).

Nevertheless, HPLC remains limited by its expense, and while TLC and LLE are much less costly, they are ill-suited for scaled-up preparative work. This report describes the application of micro liquid chromatography (LC) to the purification of both an electrophilic phospholipid derivative and its highly amphiphilic conjugate to a decapeptide. The advantage of this technique is its low cost and ease of implementation, since the compounds can be extracted from the crude reaction mixture and purified in a single LC step.

MATERIALS AND METHODS

Materials

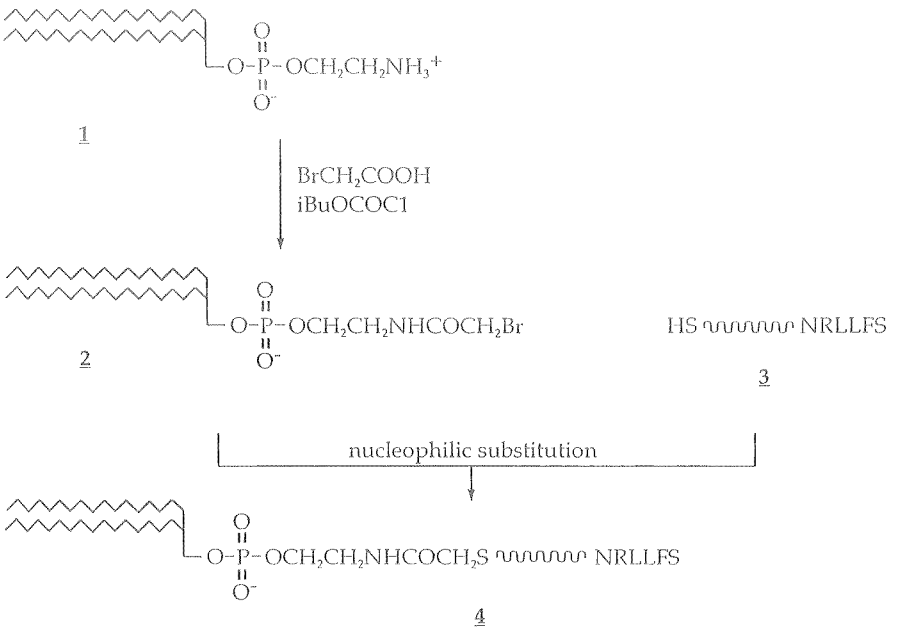
Routine TLC was performed using silica plates (20x20 cm, 250 μm) purchased from Whatman and cut to a 4x7 cm dimension. The plates were developed with iodine for the detection of all compounds. Free amines were specifically detected with a ninhydrin spray (0.2 M in absolute ethanol), and free thiols were detected by reaction with Ellman's reagent. Fine silica (silica gel Davisil™, grade 633, 200-425 mesh, 60 Å) was purchased from Aldrich. Filtration mini-columns (3-mL capacity) used for micro-LC were supplied by Baker (#7121-03). An 8-port Sep-Pak™ cartridge rack by Waters Associates was used to apply negative pressure onto the micro-LC columns. All solvents were HPLC grade from Fisher Scientific. The synthesis of N-bromoacetyl-distearoylphosphatidylethanolamine (DSPE-$COCH_2Br$) and lipopeptide conjugates has been described in greater detail elsewhere (9).

Purification of DSPE-$COCH_2Br$

Eighty-one and a half milligrams of crude DSPE-$COCH_2Br$ were dissolved in 0.6 mL $CHCl_3$/MeOH 95/5 v/v and loaded on a glass chromatography column which contained a 2x23 cm silica packing. Rinsing was performed with a total of 2x0.4 mL of the same solvent. Elution was conducted with the following gradient of $CHCl_3$/MeOH/$AcOH_{50\%aq}$ v/v/v: 10 mL, 95/5/0, fraction [1]; 10 mL, 93/7/0, [2]; 10 mL, 91/9/0, [3]; 10 mL, 90/10/0.5, [4]; 10 mL, 88.6/9.9/1.5, [5]; 30 mL, 88.7/9.8/2.5, [6-14]; 30 mL, 84.8/12.7/2.5, [15-23]; 30 mL, 82.6/14.5/2.9, [24-26]; 30 mL, 77.3/19.3/3.4, [27-28]; 30 mL, 73.3/21.9/4.8, [29-31]; 30 mL, 69.5/25.7/4.8, [32-34]; and 30 mL, 63.8/31.4/4.8, [35-40]. The fractions of interest were combined, concentrated on a rotating evaporator (40°C, 200 Torr), transferred with a minimum (2 - 4 mL) of $CHCl_3$/MeOH 2/1 v/v into two 10-mL tared glass tubes, and concentrated to 0.5 mL under a strong stream of nitrogen in a heat block at 45°C. The residue was subsequently emulsified by vortexing in 3 mL of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v and freeze-dried overnight under 0.1 mm Hg vacuum. A total of 52.6 mg (40%) of a white fluffy solid (DSPE-$COCH_2Br$) was obtained.

Synthesis and LC purification of the phospholipid-peptide conjugate

Briefly, 14.3 mg (12 μmol) of the solid phase synthesized decapeptide (Ser-Phe-Leu-Leu-Arg-Asn-(βAla)$_3$-Tyr-$NHCH_2CH_2SH$ (3)) were weighed into a 2-dram screw-cap glass vial previously flushed with nitrogen (Scheme 1). A well-mixed clear solution of 10.4 mg (12 μmol) of DSPE-$COCH_2Br$ (2), 5 mL $CHCl_3$/MeOH 1/1 v/v, 300 μl water, and 32.5 μl (10 eq) of triethylamine (Et_3N) was then added upon stirring. After sparging with nitrogen, the vial was capped. Reaction was allowed for 15 hours at room temperature and was followed by TLC in E3 (Table 1). The reaction mixture was subsequently concentrated under a strong stream of nitrogen with a heat block at 45°C, vortexed in five volumes (6 mL) of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v, frozen in liquid nitrogen, and evaporated under vacuum (0.1 mm Hg) overnight (lyophilized), thus yielding 20 mg of a crude white fluffy solid. Two 10-mL Vacu-Pak filtration columns (Baker) were hand-packed with fine silica (200-425 mesh), compressed, and the packing topped with a 20 μm pre-filter (provided with the commercial polypropylene



Scheme 1. Methodology for phospholipid-peptide conjugation.

TABLE 1

Chromatography Solvent Mixtures†

	Hexane	$CHCl_3$	iPrOH	$AcOH_{glacial}$	H_2O
E0	33	9.4	52	0.9	4.7
E1	28	10	55	3	10
E2	18	10	65	3	10
E3	15	8	60	1	16
E4	15	5	60	3	17

† volumic composition

columns). Each column was loaded with 10 mg of crude dissolved in 2 mL of E0. Elution was conducted at 1 mL/min with a gradient in the following manner: the initial 2-mL load was eluted in fractions [1,2]; 2 mL, E0, [3-4]; 3 mL, E0, [5-7]; 3 mL, E0/E1 1/1 v/v, [8-10]; 6 mL, E1, [11-16]; 3 mL, E1/E2 1/1 v/v, [17-19]; and 6 mL, E2, [20-25]. Fractions [13-17] of both columns were combined and concentrated to 0.5 mL under a strong stream of nitrogen in a heat block at 45°C. The white residue was subsequently emulsified upon vortexing in 3 mL of $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v and freeze-dried overnight under 0.1 mm Hg vacuum, yielding pure lipopeptide (4) as a white fluffy powder.

RESULTS

DSPE-$COCH_2Br$ was readily extracted from its crude reaction mixture and purified in a single step by normal phase LC. Elution was performed with a gradient of chloroform, methanol, and a 50% aqueous acetic acid solution. The fractions were analyzed by TLC in $CHCl_3$/MeOH/$AcOH_{50\%aq}$ 70/30/1 v/v/v and the compound (R_F=0.75) detected with iodine. Unlike PE (R_F=0.70), it gave a negative result upon treatment with ninhydrin. Little retention of compound on the silica column was noticed. Thus, a typical batch of 80-mg PE yielded about 50 mg of the bromoderivative after reaction and LC purification. The overall yield was 40%, conversion of DSPE into DSPE-$COCH_2Br$ being the limiting factor. ^{1}H-NMR and mass spectroscopy data have been reported (9).

Once purified, the bromoderivatized phospholipid was covalently coupled to the thiol-terminated decapeptide via a nucleophilic substitution in the presence of triethylamine. The reaction mixture was evaporated to dryness, and a single micro-scale LC sufficed to extract and purify the phospholipid-peptide conjugate. The small filtration columns were packed with fine wet silica (hexane), tightly compressed with a spatula, and the bed was topped with a pre-filter provided with the commercial plastic columns. A discrete series of eluents (Table 1) was designed by modification of a normal phase HPLC gradient of hexane, chloroform, tetrahydrofuran, 2-propanol, and water previously used for its high selectivity toward naturally occurring phospholipid classes (10). However, since the lipopeptide described in this report is markedly more hydrophilic than natural phospholipids, including phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylinositol, acetic acid was

TABLE 2

R_F Values in Given TLC Solvent Systems†

	E1	E2	E3	E4¶
DSPE-$COCH_2Br$ (2)	0.65	0.60	0.48	0.67
lipopeptide (4)	0.35	0.40	0.26	0.56
peptide (3)	0.15	0.15	0.12	0.51
peptide disulfide	‡	‡	‡	0.35

† for solvent systems, see Table 1
¶ triethylamine had an R_F value of 0.10 in this eluent
‡ compound not seen

added as an ion-pairing agent to minimize retention on the polar stationary phase. As noted in Table 2, the R_F values of each molecular species were distinct, indicative of the excellent selectivity of this quinternary mixture. Furthermore, the addition of acetic acid hindered spreading of the chromatographic peaks, thus improving resolution. Optimal gradient elution conditions for the given reaction mixture are as indicated in the Materials and Methods section above. A typical load of 10 mg per micro-column of dry crude reaction mixture (containing 20%-30% of lipopeptide) yielded approximately 4 mg of pure lipopeptide after lyophilization in $CHCl_3$/MeOH/H_2O 10/10/80 v/v/v. Confirmation of product chemical structure was verified by NMR.

DISCUSSION

Purification of amphiphilic lipid biomolecules is often demanding, and a variety of approaches have been described. HPLC has been used extensively for

the purification and quantitation of naturally occurring phospholipids. Silica phases and complex quinternary eluents were implemented by Redden (10) and Christie (11). Likewise, Rehman used an isocratic elution protocol with acetonitrile, methanol, and aqueous phosphoric acid (12), and Abidi eluted major lipid classes with an isocratic mixture of acetonitrile, methanol, and aqueous tetraalkylammonium phosphate on a C18 column (13). This work has been recently extended to the purification of monoalkyl- and dialkyl-surfactants, including surfactin, a cyclic heptapeptide possessing a long aliphatic moiety (14, 15), and a synthetic dialkyl peptide prepared by solid phase synthesis (16). In the latter report, two palmitoyl chains were covalently linked to the lysyl-terminus of a resin-bound peptide. Reverse phase C_{18} HPLC was feasible because of the dominant hydrophilic nature of the 23-residue peptide. Nonetheless, HPLC remains limited by its expense and complexity. Although the application of TLC is much less costly and has proven successful for both lipid separation and quantitation, it is ill-suited for scaled-up preparative work.

Conventional LC provides an alternative, inexpensive approach for amphiphile purification with preparative capabilities. LC has been used previously to successfully purify both naturally occurring phospholipids, as well as derivatives of phosphatidylethanolamine with N^{α}-iodoacetyl-(N^{ε}-dansyl)lysine and 3-(pyridyl-2-dithio) propionic acid (17-20). LC has also been applied to the purification of surfactin analogs (21). Typically, eluants have been isocratic or gradient mixtures of chloroform/methanol or chloroform/methanol/water.

Apart from its use as an isolated purification procedure, LC has also been used as a refining step in LLE procedures. Impure PE-MPB recovered after a chloroform/brine treatment required further purification by LC over silica in chloroform/methanol (20). Similarly, normal phase LC with chloroform/methanol was necessary to purify PE-COOH after initial liquid-liquid extraction (22). However, in contrast to LLE procedures, LC avoids the loss of amphiphile at the liquid-liquid interface and reduces the quantity of solvent required for product purification. For example, 25 mL was typically required to purify 10 mg of crude lipopeptide using the approach described in this report. Furthermore, expensive unreacted peptide can be recovered using an LC protocol.

In an effort to prepare receptor activating membrane-mimetic surfaces, we synthesized an amphiphilic conjugate of distearoylphosphatidylethanolamine and a

decapeptide which includes the minimal human thrombin-receptor peptide agonist Ser-Phe-Leu-Leu-Arg-Asn. Purification of this lipid-peptide conjugate and the phospholipid derivative, DSPE-$COCH_2Br$, was difficult due to the amphiphilic nature of these compounds. However, a successful result was achieved with gradient LC. In particular, a quinternary eluant in which acetic acid was added as an ion-pairing agent was effective for purification of the lipopeptide. This report provides the first description of normal phase LC for the purification of N-haloalkyl derivatives of PE and associated lipid-peptide conjugates.

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DETERMINATION OF DEXTROMETHORPHAN HYDROBROMIDE IN BULK FORM AND DOSAGE FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple LC-procedure for quantification of dextromethorphan in pure forms and pharmaceutical preparations (tablets, syrups, and drops) is described. The HPLC-separation of the active ingredient from the complex matrices of the dosage formulations is undertaken by dilution or extraction in the mobile phase [acetonitrile/acetate buffer (40 mM, pH 4.3) = 75:25, v/v] and elution on a reverse-phase μ-Bondapak™ C_{18} column (30 cm × 3.9 mm ϕ, 10 μm) isocratically (1.5 ml.min^{-1}) with UV-detection at 278 nm at ambient temperature. Good recovery testing of drug masses added to the dosage forms was obtained.

INTRODUCTION

Dextromethorphan [(+)-3-methoxy-17-methyl-9α,13α-14α-morphinan] hydrobromide is a commonly prescribed antitussive in many cough-cold preparations. The drug has gained a wide acceptance as a non-addictive agent as it is almost devoid of analgesic activities. The utility of high performance liquid chromatography (HPLC) has made a dramatic impact in the analysis of organic compounds, especially the therapeutic agents in pure forms, biological specimens and dosage formulations. Various HPLC-methods have been described for the analysis of dextromethorphan, particularly in cough-cold syrups, employing ion-pairing or buffering.[1-12] Most of the described HPLC-procedures focussed on the improvement of the separation ability of dextromethorphan antitussive from other drug substances, rather than on the drug resolution from additives in various dosage formulations containing the drug. The application of several of these HPLC-procedures for quantification of the drug in pharmaceutical preparations was not always successful in separating the active ingredient enoughly each time. The drug separation occurs at excessive retention times with peak tailing in some cases.

The main task of the present study was to develop a rapid and sensitive method for accurate determination of dextromethorphan hydrobromide in the presence of the drug degradation products, various excipients, dye's, diluents, lubricants and/or sugar bases. The established HPLC-procedure was to be applied for the determination of the drug content in tablets, syrups and drops.

EXPERIMENTAL

Instruments

Shimadzu LC-10 AD liquid chromatograph attached to SPD-10A tunable UV-detector, CTO-10A column oven controller, DGU-3A mechanical degasser, and C-R4A Chromatopac data unit, Shimadzu Corp., Analyt. Instrum. Div., Kyoto-Japan. Fixed loop injector (Rhydone, 20-μl) was utilized to carry the samples onto the column [Waters prepacked μ-Bondapak™ C_{18} column (10 μm, 30 cm $\times$ 3.9 mm ϕ), Waters Assoc., Milford-Mass., USA]. The mobile phase containing acetonitrile (HiPerSolv™ BDH Chemicals Ltd., Poole-UK) and 40 mM acetate buffer pH 4.3 (75:25, v/v) was prepared, filtered by aid of a suitable Millipore filter, then degassed using a Branson 1200 ultrasonic-bath. The separation was performed isocratically at a flow rate of 1.5 ml.min^{-1} by setting the UV-detector at 278 nm at ambient temperature.

Chemicals and Dosage Formulations

Reference dextromethorphan . HBr. [Lot. # 9211224, assigned content 100.1%] was kindly supplied by Saudi Pharm. Ind. & Med. Appl. Corp. (SPIMACO), Quassim-Saudi Arabia. The supplied material was utilized without further treatments.

Pharmaceutical formulations. Various dosage preparations, tablets, syrups and drops, were collected randomly from local pharmacies.

- Tussilar™ tablets (sugar coated, BN 313274) and drops (BN 360283) are products of Kahira Pharm. & Chem. Ind. Co, Cairo-Egypt, each tablet or 1 ml drops contains 15 mg dextromethorphan.

- Romilar™ Expectorants, syrup (BN B6312 MFD0892) contains 15 mg of the drug in a teaspoonful, in addition to ammonium chloride and dexpanthenol, F. Hoffmann-La Roche Ltd, Basel-Switzerland.

Internal standard (IS). Labetalol, 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenyl-propyl)amino]ethyl]benzamide, Glaxo Gp. Res. Ltd., Greenford, Middlesex-UK, was used as internal standard (2 $mg.ml^{-1}$, in the mobile phase).

Analytical Techniques

Standard solutions and graphs. The stock solution of dextromethorphan.HBr, 1 $mg.ml^{-1}$ in the mobile phase, was diluted to 200 $\mu g.ml^{-1}$ as a working solution. To prepare the standard curve, serial dilutions containing 50-200 $\mu g.ml^{-1}$ of the drug in the mobile phase were prepared by diluting the working solution. Triplicate injections of each dilution were made and the curve, concentration *vs* detector response ($A_{278\ nm}$), was plotted. The slope consistency of the prepared standard graphs was checked at different days.

Drug analysis. In case of tablets, at least 20 tablets were weighed to get the average weight of a tablet. An aliquot of the powdered tablet, syrup or drops, claimed to contain 300 mg of the drug was transferred into a 100-ml calibrated flask. About 75 ml of the mobile phase were then added and extraction was performed mechanically for ~7 minutes before completing the volume with the mobile phase. From the filtered extract were diluted 1-ml portions separately in 20-ml volumetric flasks by the mobile phase to give a final concentration of 150 $\mu g.ml^{-1}$ after adding 2 ml of the internal standard. Replicate injections of each solution were made. To

determine the drug content refer either to the prepared calibration curve or compute the drug mass by sample/equivalent standard direct matching.

Recovery testing. To 1 ml of the drug extract in the mobile phase claimed to contain 1.5 mg dextromethorphan.HBr, an equal mass of the reference drug substance was added from the stock solution in the mobile phase, followed by 2 ml of the internal standard in the mobile phase in 20-ml volumetric flask. The volume was completed with the mobile phase, then mixed well to homogenize. Triplicate injections were made to calculate the average ratio response, due to the added masses *i.e.* the area of each added drug compared with that of the internal standard.

RESULTS AND DISCUSSIONS

Different mobile phases and columns have been described for separation and determination of dextromethorphan usually admixed with other active drug substances. Recently, Thomas *et al.*[12] described a mixed/ion-pair liquid chromatographic method for quantification of the drug in admixtures with ascorbic acid, caffeine, chlorpheniramine maleate and paracetamol dispensed as sachets by utilizing a buffered aqueous acetonitrile mixture containing an ion-pairer. The separation was achieved on a Hypersil phenyl-2 column with multiwavelength UV-detection. The separation parameters for dextromethorphan were not the ideal. Preliminary investigations have been carried out to improve the peak asymmetry and reproducibility. Several mobile phase compositions have been tested to reach effective separation of the drug from the different formulations additives in the various pharmaceutical preparations containing it. Such C_{18}-columns have been widely used for separation of amines in many preparations.[7] Many microparticulate

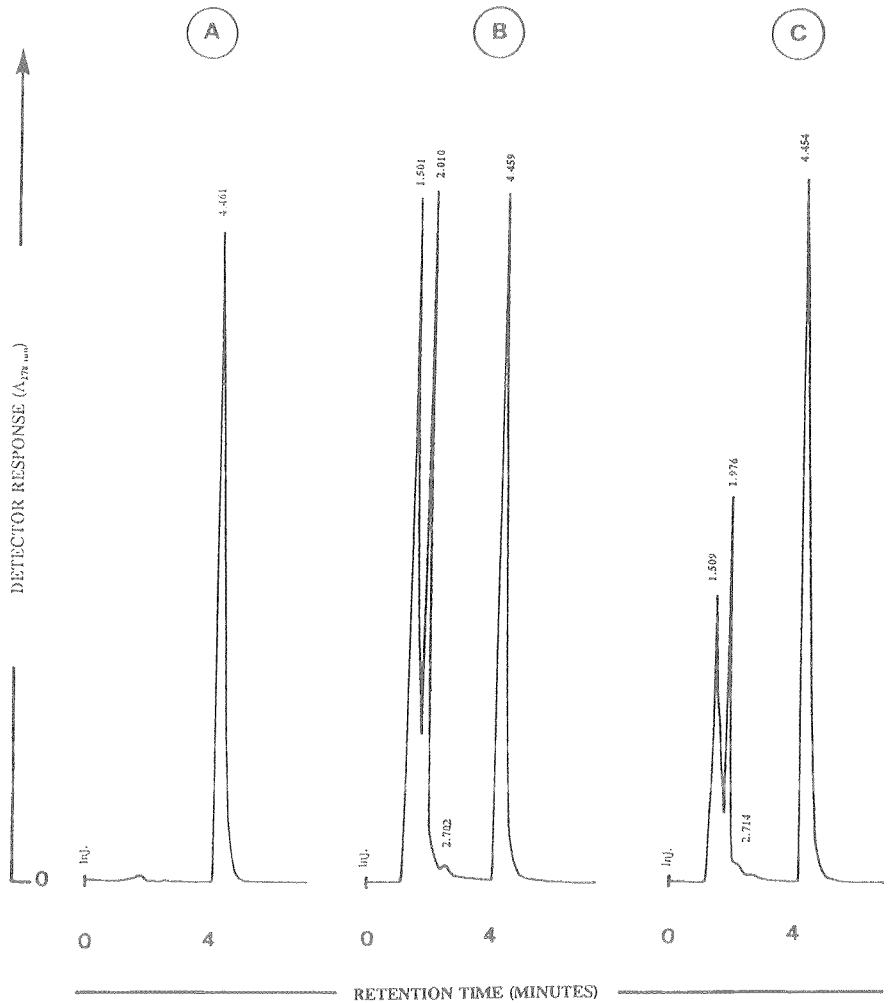

Fig. 1: HPLChromatographic separation of (A): pure dextromethorphan hydrobromide (150 $\mu g.ml^{-1}$), (B): same drug concentration from tablets extract, and (C): recovery testing of 75 μgml^{-1} drug added to the same amount from the tablets extract.

Table 1

Assay and recovery of dextromethorphan hydrobromide[x] in dosage formulations by adopting the proposed HPLC-procedure.

Formulation*	Assay[+]	Recovery[+]
Tablets	97.6±0.40(0.41, 6)	100.9±0.29(0.28, 4)
Syrup	101.2±0.13(0.12, 4)	100.1±0.53(0.53, 4)
Drops	108.9±0.18(0.16, 6)	100.4±0.81(0.81, 5)

[x]Purity 100.02±0.11% (n = 6).
[+]For more details, see experimental section.
[+]X±SD (CV, n), each run is the average of at least 3 experiments.

reverse-phase columns with octadecylsilane (ODS, C_{18}) packings from different manufacturers had been tried. Good match between the reverse-phase micro-Bondapak™ column (10 μm, 30 cm × 3.9 mm ϕ) and a mobile phase containing acetonitrile and acetate buffer (40 mM, pH 4.3) = 75:25, v/v, was found to be more efficient for the separation and quantification of the antitussive agent and, hence, provide excellent elution of the active drug substance from excipients and other formulations additives. Typical HPLC-separation of pure dextromethorphan and the drug from the tablets extract in the mobile phase is shown in figure 1. The drug was consistently eluted (t_R = ~4.46 min.) at different days with excellent peak symmetry (factor = 1.01). Table 1 collects the results of analysis of a pure drug sample and the drug in tablets, syrups, and drops, in addition to the results of recoveries of added 50% drug mass. The precision of the method is clearly reflected as the obtained low deviations and variations.

Although the HPLC-assay is an external standard method, it was believed that the addition of an internal standard would slightly improve the precision of the assay procedure. Labetalol, a α- and β-adrenergic blocker, was found useful for such a purpose. The relative retention time (rel-t_R) of dextromethorphan to the internal standard is 1.67.

CONCLUSION

Excellent resolution of the antitussive agent dextromethorphan hydrobromide was obtained by the investigated HPLC-procedure. The applicability of the method for routine drug analysis revealed that the proposed procedure is simple, rapid and precise enough for the quantification of the named drug in several commercially available cough-cold tablets, syrups, and drops.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NALIDIXIC ACID IN TABLETS

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ABSTRACT

A rapid, specific and reliable high performance liquid chromatographic assay of Nalidixic acid in tablets has been developed. Reversed-Phase chromatography was conducted using a mobile phase of 0.05 M Ammonium acetate Methanol and acetonitrile, (65, 5, 30% v/v) pH 5 and detection at λ 254 nm. The recovery and coefficient of variation from six placebo tablets containing 100 mg of Nalidixic acid were 100.2% and 0.56 respectively. Replicate regression analyses of three standard plots in the concentration range 1 - 20 mcg/ml obtained on three different

days gave a correlation coefficient (0.99996) and the coefficient of variation of the slopes 0.089%. The assay was precise within day and between days as indicated by ANOVA test. It is suggested that the proposed HPLC method should be used for routine quality control and dosage form assay of Nalidixic acid.

INTRODUCTION

Nalidixic acid is 1-ethyl-1,4dihydro7methyl4-oxo-1,8 naphthyridine 3 carboxylic acid, a urinary antimicrobial agent[1].

Various methods have been developed for the determination of Nalidixic acid in pharmaceutical dosage forms. Fluorometry[2], polarography[3], UV spectrophotometry[4], Gas chromatography[5] and PMR spectrometry[6].

The purpose of this study, was to develop a simple and direct HPLC assay for the quantitation of Nalidixic acid in tablet formulations.

EXPERIMENTAL

Chemical and Reagents:

Nalidixic acid[7] and methyl paraben[8] were used without further purifications. Acetonitrile[9], Methanol[9] and water were HPLC grade. All other chemicals were of U.S.P. or A.C.S. quality and were used as received.

Chromatography:

A waters HPLC systems[10] was used consisting of the following components: One Model 45 pump, the WISP Model 710B autosampler, the Model 481 UV detector set at 254 nm at 0.05 AUFS, the model 730 data system. Chromatographic separation was accomplished using C_{18} column, 3.9 mm x 300 mm μBonda pack C_{18} column with 10um packing.

Chromatographic Conditions:

The eluting medium consisting of 0.05 M Ammonium acetate, methanol and acetonitrile (65, 5, 30% v/v, pH 5) was prepared and degassed by bubbling helium gas for 5 min prior to use. Column equilibrium with the eluting solvent was established by pumping the mobile phase at a rate of 0.2 ml/min overnight. The flow rate was set at 1.5 ml/min during analysis. The chromatogram was recorded and integrated at a speed of 0.2 cm/min.

Internal Standard:

A stock solution of methyl parahydroxy benzoate 1 mg/ml was prepared weekly and stored at 4°C.

Preparation of Standard Solution of Nalidixic Acid:

A stock solution was prepared by dissolving 10 mg of Nalidixic acid in 10 ml water. Ten aliquots equivalent to 0.5, 1, 2,

4, 6, 9, 12, 15, 18, and 20 ug of Nalidixic acid were added to one ml volumetric flask. After the aliquot of the internal standard equivalent to 20 ug was added to each 1 ml flask, the flasks were brought to volume by acetonitrile and mixed thoroughly. Three 20 uL injections of each standard solution of Nalidixic acid containing the internal standard were made to prepare standard plots. The peak area ratios of Nalidixic acid : methyl paraben were plotted against Nalidixic acid concentrations. Least square linear regression analysis was used to determine the slope, Y-intercept, and the correlation coefficients of the standard plots.

Sample Preparation:

Individual tablets containing 500 mg Nalidixic acid were pulverized using a mortar and pestle, and completely transferred to 250 ml volumetric flask. Ten ml of deionized water was added and the flask was swirled for 2-3 min. The volume was adjusted to 250 ml with methanol and the flask was mechanically shaken for five min. Five ml of the solution was removed into a centrifuge tube and centrifuged at 3000 r.p.m. for 5 min. One hundred ul of the supernatant was transferred to a 10 ml volumetric flask containing two hundred ul of methyl paraben stock solution, and the volume completed with the mobile phase. Twenty ul was injected onto the column for quantitation. Ten replicate commercial tablets were analyzed for statistical evaluation of the assay.

Quantitation:

The amount of Nalidixic acid per dosage form was determined from the following equation.

$$Q = [R/A + B] \times \text{dilution factor}$$

were Q is the mg Nalidixic acid per dosage form, R is the peak area ratio (drug/internal standard), A is the slope of the calibration curve and B is the y-intercept.

Recovery of Nalidixic Acid from the fabricated placebo tablets:

The reference tablets containing 500 mg of Nalidixic and 50 mg each of starch and lactose were prepared and subjected to the described HPLC assay and B.P. 88 to measure the accuracy and precision.

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms obtained following analysis of Nalidixic acid in tablets. Using the chromatographic conditions described, Nalidixic acid and Methyl paraben were well separated and their retention times were 7.27 and 4.0 min, respectively. For both compounds sharp and symmetrical peaks were obtained with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of the peak area ratios. No interfering peaks were found in the chromatogram due to table t excipients. Figure 2 shows a calibration plot for the peak area ratios

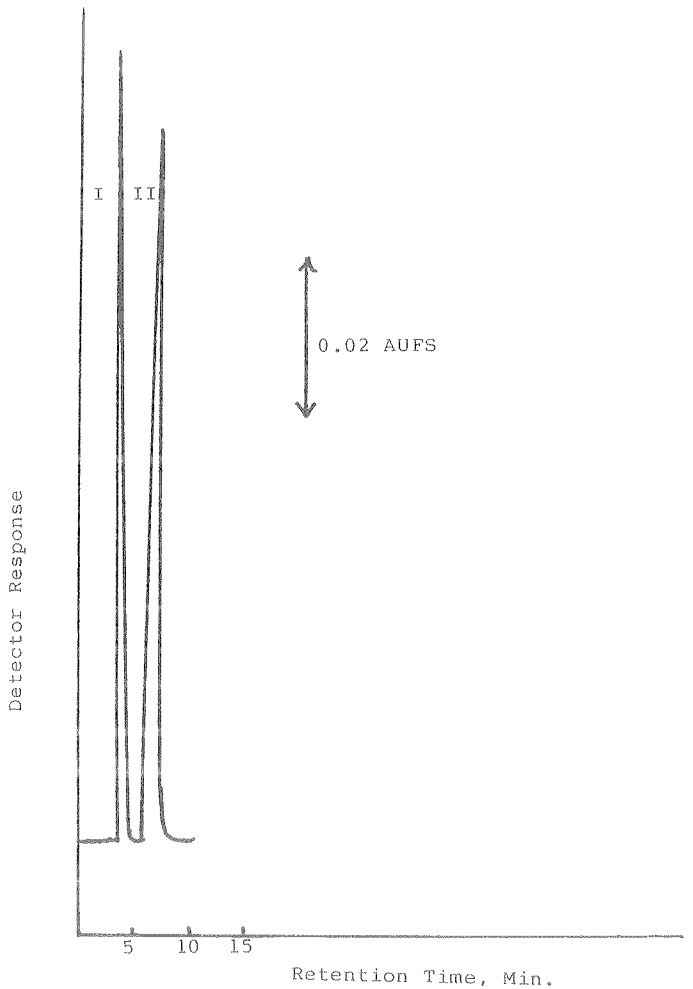

Figure 1: Chromatogram of Nalidixic Acid tablet.

Key 1. Methyl-paraben
2. Nalidixic acid

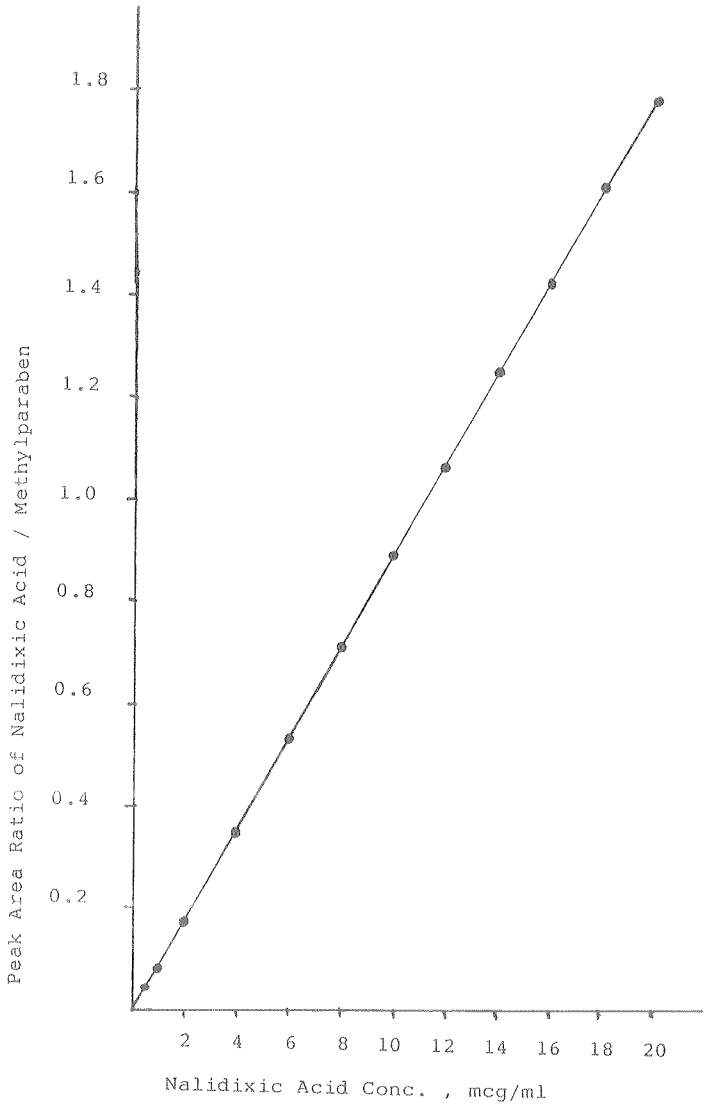

Figure 2: Standard calibration plot of Nalidixic acid

Table 1

Regression Analyses of the Three Standard Plots of Nalidixic Acid.

Standard[a]	Slope[b]	Intercept[b]	Correlation[b] Coefficient
1	0.09015	-0.0071	0.99986
2	0.09014	-0.0072	0.99979
3	0.09016	-0.0070	0.99989

a) obtained in 3 different days
b) The mean of 3 determinations at each drug concentration.

of varying amounts of Nalidixic acid (0.5-20 ug/ml) to a constant amount of methyl paraben (20 ug/ml). The plot was highly linear (r=0.99995) and the regression analysis of the data gave the slope and intercept as:

$$Y = 0.09015x - 0.0071$$

where y and x are the peak area ratio and Nalidixic acid concentration respectively. Three replicate analyses of Nalidixic acid at concentrations of 0.5 - 20 ug/ml were assayed at three different days over one week period. The results of this evaluation are summarized in Table I. The average correlation was 0.99984 and the coefficient of variation of the slopes of the three lines was 0.089%. Analysis of variance of the data showed no detectable difference in the slopes of the three standard plots ($F_{2.409}$, $P > 0.01$). The results, thus confirmed the excellent linearity of the calibration plots and high reproductivity of the assay.

Table II

Analysis of Variance for Intra- and Inter day Precision

Day/Assay	1	2	3	4	5	6
1	500.9	500.6	499.3	502.2	503.1	499.1
2	502.7	500.8	498.5	500.4	500.9	497.3
3	501.8	500.5	497..7	503.7	499.2	502.1
4	502.1	498.5	500.3	501.9	498.1	499.3

Mean = 500.458
SD = 1.7356
CV % = 0.3468

ANOVA TEST

Source of Variation	DF	Sum of Squares	Mean of Squares	F ratio	P
Within day	5	31.914	6.38	2.614	0.05
Between day	3	3.699	1.233	0.505	0.05
Error	15	36.685	2.44		
Total	23	72.298			

Precision and Accuracy: Six placebo tablets containing 50 mg each of lactose and starch and 500 mg Nalidixic acid were assayed for four consecutive days for intra and interday precision studies. The average recovery shown in Table II was (500.458) with the coefficient of variation 0.341%. Estimation of day to day and

Table III

Recoveries From Spiked Placebo Tablets

Method	n	Amount Added	Amount Received	CV%
B.P.	6	100	99.05	1.40
HPLC	6	100	100.2	0.56

n = number of replicates

within day precision were calculated by ANOVA test. The calculated F values, $F_{0.05}(5, 15) = 2.614$ and $F_{0.05}(3, 15) = 0.505$ were smaller than the table values $F_{0.05}(5, 15) = 2.9$ and $F_{0.05}(3, 15) = 3.9$ respectively. Thus it was concluded that there was no significant difference for the assay which was tested within day and between days.

Recovery :

Table III compares the average recovery by the B.P. and the proposed HPLC method for placebo samples containing 100 mg Nalidixic acid and 50 mg each of the lactose and starch. the average % recovery was 100.2 and 99.05 for the HPLC and the B.P. method, respectively and their % coefficient of variation were 0.56 and 1.4, respectively. The values obtained using the HPLC method compared favorably with those obtained using the B.P. method. The smaller recovery by the B.P. method may have been caused by the loss of the drug during several sample preparation steps.

Table IV

Recovery of Nalidixic acid from Commercial Tablets by HPLC Method.

Table I	Amount found mg	% of Label Claim
1	510	102.0
2	508	101.6
3	512	102.4
4	505	101.0
5	509	101.8
6	506	101.2
7	500	100.0
8	504	100.8
9	502	100.4
10	503	100.6
		Mean 101.18
		CV% 0.71516

Analysis of Nalidixic acid in Tablets:

Table IV presents the results obtained from the HPLC analysis of 10 Nalidixic acid tablets (500 mg) commercially available. The mean percent recovery was 101.18% with 95% confidence limits of 503 to 509. Each of the tablets analyzed showed highly uniform Nalidixic acid content between 100-102.4% of the label claim. the requirements for content uniformity of

Nalidixic acid tablets in the B.P. specify that the potency must fall within 95-105% of the label claim. Thus the tablets selected randomly in this determination met the B.P. requirements for the content uniformity.

CONCLUSION

The HPLC method developed in this study has the advantages of simplicity, precision and convenience. It also allows for the direct determination of Nalidixic acid by passing several tedious steps involved in other assay methods. Therefore, the methods should be useful for routine analytical and quality control assay of Nalidixic acid in dosage forms.

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COMPUTER-ASSISTED METHOD FOR THE THRESHOLD OPTIMIZATION OF FLUORESCENCE DETECTION CONDITIONS IN HPLC OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract

The computer assisted method of λ_{ex}, λ_{em} optimization in programmed fluorescence detection of PAHs included in EPA norm has been investigated. Optimization procedure utilises the preference of the threshold methods. Finding of optimum detection conditions was done in the reduced parametric space (λ_{ex}, λ_{em}). This space consisted of points with guaranteed minimal detection sensitivities of tested PAHs. The relative detection sensitivities of PAHs, elution characteristics, different natural fluorescences, chromatographic resolution of hardly separable pairs of peaks and also the reproducibilities of peak heights and

retention times under gradient conditions were taken into consideration. The results of optimization indicate that it is difficlut to find acceptable compromise for fluoranthene and chrysene. By shifting of emmision wavelength the sensitivities of chrysene and fluoranthene changed in the mutually opposite direction. Therefore developed program used five steps. Fluoranthene, pyrene and benzo(a)anthracene, chrysene were measured at different detection conditions. If there is a specific need to analyse most dangerous PAHs then the program can be adjusted accordingly.

Introduction

The sensitivity of chromatographic analysis (observed peak height in a chromatogram) is an important aspect of the chromatographic process. The detection limit is often defined as the concentration of the analyte that will provide a signal-to-noise ratio of 3 : 1 [1]. The minimum detectable amount of the analyte is determined by the detector noise, detector sensitivity towards the analyte of interest (defined as signal output per unit mass concentration of analyte in eluent) and also by the performance of the chromatographic system [2]. Analytical chemist is generally interested in the minimum detectable concentration. It can be increased by the injection of larger sample volume, preconcentration on a precolumn as well as the selection of optimal detection conditions [3].

Sixteen polycyclic aromatic hydrocarbons (PAHs, according to EPA 610 norm) are commonly detected in HPLC by UV and fluorescence detection. Because of rather different fluorescence detection characteristics (optimal λ_{exc}, λ_{emis}) of tested PAHs, the programmable fluorescence detectors are used to satisfy the acceptable signal intensity of all components [4]. Many combinations of λ_{exc}, λ_{emis} were published in the literature [5-7].

The aim of this paper is to discuss the optimization procedure which utilises the fluorescence spectra of individual PAHs for the optimization of λ_{exc}, λ_{emis} in programmed fluorescence detection. Procedure reflects the differences between natural fluorescences, elution characteristics and chromatographic separation of tested PAHs.

Experimental

Instruments

Reversed phase HPLC was performed on a Waters Assoc. model 510 pumps with a Supelco LC-PAH column (length = 150 mm, inner diameter = 4.6 mm) and a

Tab. 1. The Composition of the Gradient Mobile Phase

Time [min]	w(A) [%]	w(B) [%]
0	100	0
1	100	0
15	0	100
25	0	100

A = 50 % v/v acetonitrile, B = acetonitrile, flow rate = 1.3 ml/min

Waters Assoc. programmable fluorescence detector. Fluorescence spectra were measured by LS-50, Perkin Elmer luminiscence spectrophotometer (2% filter, 5 nm both slits, spectra measured 3 times and averaged) . The composition of the gradient mobile phase is listed in Tab. 1.

Chemicals

The standards of 16 polycyclic aromatic hydrocarbons naphthalene (1), acenaphthylene (2), acenaphthene (3), fluorene (4), phenanthrene (5), anthracene (6), fluoranthene (7), pyrene (8), benzo(a)anthracene (9), chrysene (10), benzo(b)fluoranthene (11), benzo(k)fluoranthene (12), benzo(a)pyrene (13), dibenz(a,h)anthracene (14), benzo(g,h,i)perylene (15), indeno(c,d)pyrene (16) ,all over 98% of purity, were purchased from Supelco, USA. The acetonitrile for gradient was from Merck, Germany. Standard solutions of individual polycyclic aromatic hydrocarbons were prepared by dissolving of the analytes in acetonitrile (1 $mg.dm^{-3}$). Ten microliters of standard solution (200 μg/ml of each PAH) was injected to the chromatograph.

Optimization program

Optimization program was written in Turbo Pascal 7.0 and runs on PC-AT, 486 DX, 33 MHz. Program works from the lowest wavelength to the highest (step 1 nm).

Theory

The goal of the optimization of detection conditions of a group of components with the different spectral characteristics is to obtain the areas of all peaks in a chromatogram as maximal as possible. The area of Gaussian peak A_i can be related to the peak height at the maximum $h_{max,i}$ by the following equation [3]:

$$A_i = h_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} \quad (1)$$

where σ_i is the standard deviation of *i-th* Gaussian peak i. If the fluorescence intensity ϕ is the quantity measured by a detector, then assuming small enough absorbances at the exciting wavelength, $h_{max,i}$ can be expressed in terms of the excitation beam intensity ϕ_0, quantum yield ξ, the proportionality factor *k* including the geometry of the fluorescence spectrometer, molar absorptivity ε, the cell path length *l* and a concentration of the analyte $c_{max,i}$.

$$h_{\max,i} = \phi_{\max,i} = \phi_0 \cdot \xi_i \cdot \left(2.3 \cdot \varepsilon_i \cdot l \cdot c_{\max,i}\right) \quad (2)$$

where $\phi_{max,i}$ is the fluorescence intensity at the peak maximum. Thus eq. (1) can be transformed to the following expression:

$$A_i = \phi_{\max,i} \cdot \sigma_i \cdot \sqrt{2 \cdot \pi} \quad (3)$$

It follows from eq (3), that the area of the Gaussian peak depends linearly on ϕ max,i .

Optimization criterion

To design the suitable optimization criterion, the following facts (concerning to the tested PAHs) should be taken into account.

- different fluorescence spectra,
- different natural fluorescences,
- retention order in chromatographic separation,
- chromatographic resolution of "critical" pairs of peaks,
- reproducibilities of retention times under gradient conditions.

The optimal combination of λ_{ex} and λ_{em} for the individual component can be easily found from it's excitation and emmision fluorescence spectra. However ,the same task applied to the set of components is much more complicated. If the natural fluorescence of component is high, detection can be easy at non-optimum wavelengths, whereas if the natural fluorescence is low, it may be desirable to approach the highest sensitivity possible.

Theoretically, each component could be measured at its own optimal detection conditions. However this is only the theoretical possibility. The feasible number of program steps is limited both by the reproducibility of retention times under gradient conditions and chromatographic resolution of "critical" pairs of peaks. Therefore the compromise between the sensitivity of detection and the number of program steps should be found.

The optimization criterion should reflect the above mentioned realities. In our work it was defined by the following way. At first, to evaluate the error of

experimental measurements, the linearity relationship between the fluorescence intensity $\phi_{i,j}$ corresponding to particular emission wavelengths $\lambda_{em,i}$ as read out from the emission spectra and the fluorescence intensities $\phi_{ex,j}$ read out from the excitation spectrum at the corresponding excitation wavelengths $\lambda_{ex,j}$ was verified for all tested PAHs.

$$\phi_{i,j} = f(\phi_{ex,j}) \tag{4}$$

The meanings of ϕ_i, $\phi_{ex,j}$, $\lambda_{em,i}$ and $\lambda_{ex,j}$ are shown on the excitation and emission spectra of benzo(b)fluoranthene in Fig. 1, 2.

As it was expected the function f in eq. (4) was linear for all tested compounds. The intercepts a_i of the equation

$$\phi_{i,j} = a_i + b_i \cdot \phi_{ex,j} \tag{5}$$

were negligible as expected, while the quality of this model expressed by the correlation coefficients (near 1.0 for all tested compounds) is considered as acceptable. Therefore, the eq. (5) can be written as

$$\phi_{i,j} = b_i \cdot \phi_{ex,j} = \frac{b_i'}{\phi_{ex,max}} \cdot \phi_{ex,j} \tag{6}$$

where $\phi_{ex,max}$ is the maximal fluorescence intensity in an excitation spectrum (from which also the $\phi_{ex,j}$ values are read out). The maximum possible fluorescence intensity ϕ_{max} can be obtained by the measurement at the main maxima of excitation and emission spectra. Dividing eq. (6) by ϕ_{max} writing in appropriate fashion

$$\frac{\phi_{i,j}}{\phi_{max}} = \frac{b_i'}{\phi_{max}} \cdot \frac{\phi_{ex,j}}{\phi_{ex,max}} \tag{7}$$

and introducing new symbols gives

$$\phi_{norm} = b_i'' \cdot \phi_{ex,norm} \tag{8}$$

According to eq. (8) the fluorescence intensity $\phi_{i,j}$ normalised with respect to ϕ_{max} i.e. ϕ_{norm}, is directly proportional to $\phi_{ex,norm}$ i.e. to the fluorescence intensity $\phi_{ex,j}$ normalised to $\phi_{ex,max}$. The proportionality factor - slope b_i'' depends on the choice of $\lambda_{em,i}$ (see Fig. 2). Investigation on the relationship between b_i'' and $\phi_{em,i}$ read out from the emission spectra at the different $\lambda_{em,i}$ values manifested that the slope b_i'' can be written as

$$b_i'' = \frac{\phi_{em,i}}{\phi_{em,max}} = \phi_{em,norm} \tag{9}$$

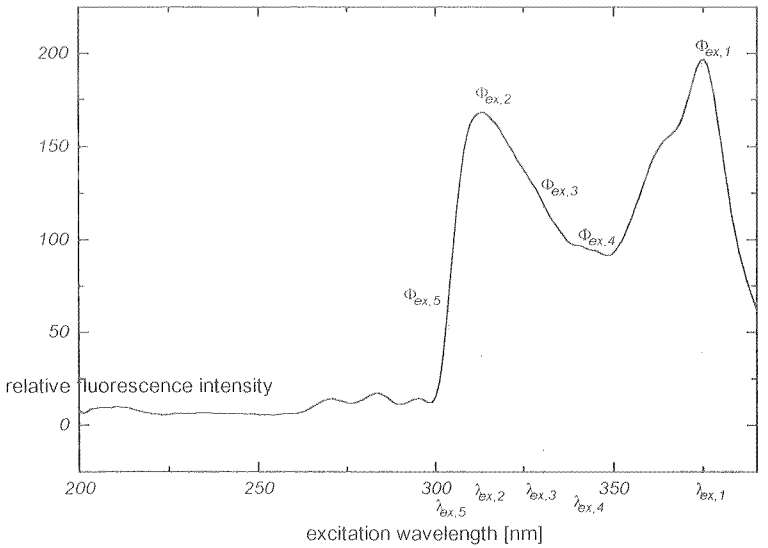

Fig. 1. Excitation spectrum of Benzo(b)fluoranthene measured at optimum emmision wavelength 445 nm..

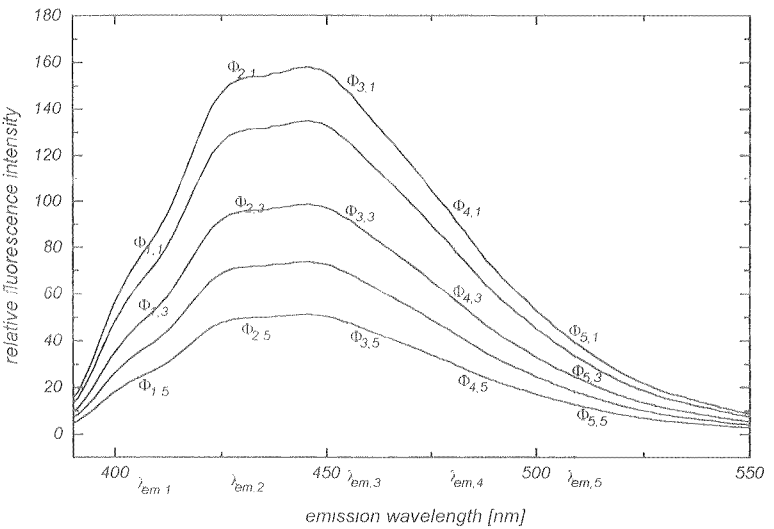

Fig. 2. Emission spectra of Benzo(b)fluoranthene (c = 0.0985 mg/ml) measured at λ_{ex1}, λ_{ex2}, λ_{ex3}, λ_{ex4}.

where $\phi_{em,max}$ is the maximal fluorescence intensity in a corresponding emission spectrum. Combining eq. (8) and (9) gives the following equation for ϕ_{norm}

$$\phi_{norm} = \phi_{ex,norm} \cdot \phi_{em,norm} \tag{10}$$

The values of all these quantities lie in the interval <0;1>. According to eq. (10), the optimization criterion was designed by the following expression:

$$K_k = 1 \qquad if \qquad \phi_{norm(\lambda_{exc},\lambda_{emis}),k} \geq P_k \tag{11}$$

$$K_k = 0 \qquad if \qquad \phi_{norm(\lambda_{exc},\lambda_{emis}),k} < P_k \tag{12}$$

$$OC = \sum_k K_k \tag{13}$$

where k designates the component (PAH) and P_k is a threshold value of fluorescence intensity over which the sensitivity of detection is acceptable. Optimization criterion OC is an integer (number of components which passed the threshold condition), defined as a sum of boolean expressions K_k.

Results and discussion

Before any optimization the goal of the process should be defined unambiguously. In this case several facts should be taken into consideration. The sensitivity of fluorescence detection can be increased by the programming of excitation and emission wavelengths. One possible way is to determine the minimum number of λ_{exc}, λ_{emis} combinations and satisfy the acceptable sensitivity of detection for all tested solutes. The choice of optimal λ_{exc}, λ_{emis} combinations should be based on finding conditions at which the largest number of compounds can be analysed at sufficiently low detection limits. Therefore the differences between natural fluorescences are considered. Threshold values P_k, over which the absolute fluorescence intensities are acceptable, were chosen with respect to the different natural fluorescences of tested PAHs (see Table 2).

As it can be seen from Table 2, acenaphthylene displays a very low natural fluorescence in comparison with other PAHs, then it was excluded from optimization process. It can be detected by UV absorption much more successfully. The optimal λ_{exc}, λ_{emis} indeno(c,d)pyrene was far from all other PAHs then it should be measured at its own maximum 300 / 500 nm. Table 2 shows the differences between natural fluorescences of tested PAHs. Benzo(k)fluoranthene displays highest natural fluorescence (approximately 15 x higher than naphthalene fluoranthene and indeno(c,d)pyrene which display the lowest fluorescence). The fluorescences of other PAHs (except anthracene,

Table 2. Comparison of natural fluorescences of tested PAHs measured at their own optimal λ_{exc}, λ_{emis}

No	compound	λ_{exc} / λ_{emis}	relative fluorescence intensity (normalized to compound 12)	concentration [mg/ml]
1	Naphthalene	282 / 340	0.06	0.0985
2	Acenaphthylene	246 / 332	0.0008	0.0990
3	Acenaphthene	258 / 336	0.35	0.0985
4	Fluorene	234 / 312	0.34	0.0990
5	Phenanthrene	246 / 383	0.21	0.0980
6	Anthracene	250 / 406	0.75	0.0985
7	Fluoranthene	282 / 456	0.05	0.0985
8	Pyrene	331 / 395	0.18	0.0985
9	Benzo(a)anthracene	284 / 392	0.29	0.0990
10	Chrysene	270 / 381	0.27	0.0985
11	Benzo(b)fluoranthene	301 / 445	0.14	0.0985
12	Benzo(k)fluoranthene	310 / 420	1.00	0.0985
13	Benzo(a)pyrene	373 / 406	0.59	0.0985
14	Dibenz(a,h)anthracene	298 / 402	0.24	0.0985
15	Benzo(g,h,i)perylene	385 / 413	0.15	0.0985
16	Indeno(c,d)pyrene	300 / 500	0.07	0.0990

benzo(a)pyrene) are approximately on the same level (natural fluorescences of benzo(b)fluoranthene, benzo(g,h,i)perylene and pyrene is slightly lower). The data from Table 2 were utilised in the choice of threshold P_k values. Low natural fluorescence of components were compensated by adequately high level of P_k in optimization. By this way the optimum was shifted closer to conditions which favoured compounds with lower natural fluorescence.

Optimization procedure

It follows from eq. (11), (12) and (13) that the sets of points with the different values of optimization criterion OC can be appointed in the three-dimensional space (λ_{exc}, λ_{emis}, OC). The value of OC (at the certain co-ordinates of parametric space) indicates the number of components which passed the threshold condition. The illustrative example of such "overlapping " plot (only of four components - because of the complexity of plot for all sixteen PAHs) is shown in Fig. 3.

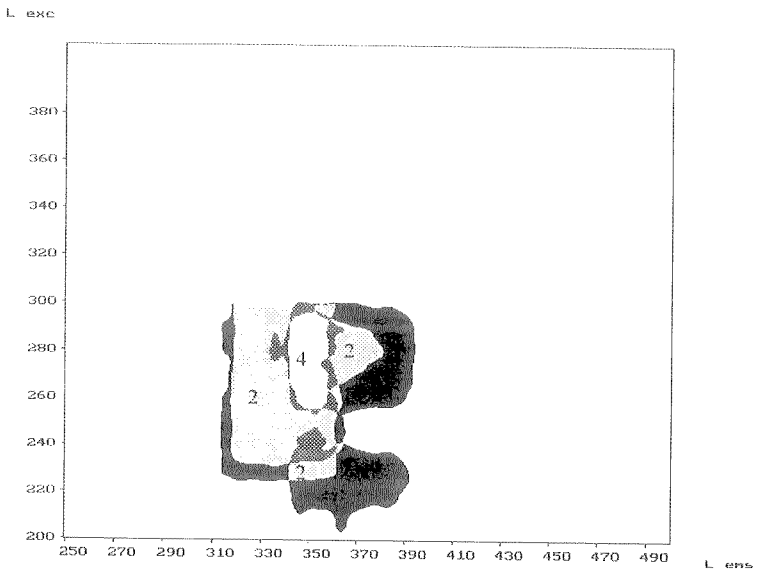

Fig. 3. " Overlapping" plot for naphthalene, acenaphthene, acenaphtylene and phenanthrene. Threshold was 0.25 for all components.

This kind of plot still contains a lot of redundant information and it's not lucid. Each component can generally be located in the points with the different value of optimization criterion *OC*. The "overlapping" plot can considerably be simplified in the next step of optimization . In this procedure each component is located to the points with the highest value of criterion *OC* (to the points with the highest number of other components which passed threshold condition together with this component) and deleted from the points with lower value of *OC*. After this procedure each component is situated in the set of points with one value of *OC* only. Optimal λ_{ex}, λ_{em} combinantion for the given set of components (included in the above set of points) then lies in the point with the highest minimum value of ϕ_{norm} for the given set of components.

The chromatogram of tested PAHs is shown in Fig. 4. Chromatographic resolution of "critical" groups of peaks (acenaphthene, fluorene), (benzo(a)anthracene, chrysene), (benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene) were not sufficient for the non-problematic switching of λ_{ex}, λ_{em}. The ability to quantitate the peaks will be affected by retention time variability.

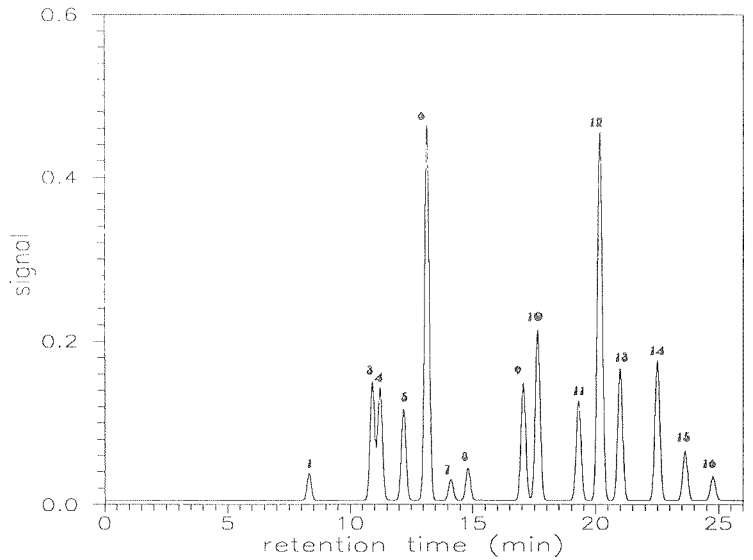

Fig. 4. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 1. For the conditions see experimental. For the numbering of peaks see Table 2.

According to this fact, above groups would be optimized together and maximum possible number of wavelengths switching is nine. The threshold values P_k were chosen with respect to the natural fluorescences of tested PAHs. Table 3 shows the results of optimization.

In program No. 1. the maximum possible number of λ_{ex}, λ_{em} switches were used. In other words program No. 1 represented maximal overall sensitivity of detection (optimized with above approach) of tested PAHs which could be achieved using separation system described in this work. Naphthalene, phenanthrene, anthracene and indeno(c,d)pyrene were measured at their ideal conditions. With fluoranthene having relatively low sensitivity some of the sensitivity of adjacent component in a chromatogram pyrene was sacrified by choosing compromise condition that favoured fluoranthene.

This approach was applied also for benzo(b)fluoranthene which displays also low natural fluorescence in comparison with benzo(k)fluoranthene and benzo(a)pyrene. The high sensitivity of benzo(k)fluoranthene results in a

Table 3. The Results of Optimization. For Numbering of PAHs see Table 2.

Program No.	component No.	λ_{ex} / λ_{em}	ϕ_{norm}
1	1	282 / 340	1.0
	3	237 / 323	0.65
	4		0.63
	5	246 / 383	1.0
	6	250 / 406	1.0
	7	271 / 410	0.89
	8		0.31
	9	266 / 384	0.75
	10		0.83
	11	281 / 428	0.91
	12		0.63
	13		0.68
	14	290 / 413	0.81
	15		0.79
	16	300 / 500	1.0
2	1	282 / 340	1.0
	3		0.62
	4		0.45
	5	252 / 376	0.91
	6		0.75
	7	268 / 406	0.73
	8		0.39
	9		0.66
	10		0.025
	11	285 / 424	0.90
	12		0.75
	13		0.66
	14		0.62
	15		0.73
	16	300 / 500	1.0
3	1	282 / 340	1.0
	3		0.62
	4		0.45
	5	252 / 376	0.91
	6		0.75
	7	268 / 390	0.11
	8		0.74
	9		0.69
	10		0.77
	11	285 / 424	0.90
	12		0.75
	13		0.66
	14		0.62
	15		0.73
	16	300 / 500	1.0

predominance of this peak in the group. Nine switches of excitation and emmision wavelenghts during analysis is relatively high number. In some cases (fluorescence detector does not allow the programming of such number of switches) it is desirable to reduce this number in spite of decreasing the sensitivity of detection. Therefore we examined this possibility. The initial groups of components used in program No. 1 (see Table 3) were gradually overlapped and detection of them were optimized. In program No. 2, naphthalene, acenaphthene and fluorene were optimized together. According to low natural fluorescence of naphthalene, this group was measured at naphthalene's ideal detection condition. The values of ϕ_{norm} for acenaphthene and fluorene listed in Table 3 were similar to the program No. 1. Detection conditions for phenanthrene and anthracene were shifted to the optimum of phenanthrene, because of high natural sensitivity of anthracene. Program No. 2 and No. 3 differed in the detection conditions for the group of fluoranthene, pyrene, benzo(a)anthracene and chrysene only. Fluoranthene displayed low natural fluorescence, therefore it should be favoured in compromise conditions. The results of optimization show that it is hard to find acceptable compromise for fluoranthene and chrysene. By shifting of emmision wavelength the sensitivities of chrysene and fluoranthene changed in the mutually opposite direction. Therefore, if there was a need to analyse both fluoranthene and chrysene, they would not be measured at the same detection condition. Finally, group, which consisted of benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene and benzo(g,h,i)perylene. Under the compromise conditions for this group benzo(b)fluoranthene response was 90 % its ideal conditions response, with benzo(k)fluoranthene 75 % of its ideal, for benzo(a)pyrene and dibenzo(a,h)anthracene around 65 % and or benzo(g,h,i)perylene almost 73 %. Indeno(c,d)pyrene was measured at their own ideal λ_{ex}, λ_{em}. Chromatograms of tested PAHs measured at above conditions are shown in Fig. 4 - 6. In order to establish the reproducibility of the method (peak heights), seven replicate injections of a standard PAH mixture were made. Results are listed in Table 4.

The toxicological characteristics of tested PAHs show that the health risks of individual PAHs are not at the same level. PAHs are known to be carcinogenic as a result of oxidative reactions in the body [8]. According to literature [9-11], PAHs can be classified into 4 groups. Naphthalene, acenaphthylene, acenaphthene, fluorene (group A) are weak carcinogenic resp. non-carcinogenic. Phenathrene, anthracene, fluoranthene, pyrene, chrysene and benzo(g,h,i)perylene (group B) are moderately active, benzo(b), benzo(k)fluoranthene, dibenzo(a,h)anthracene

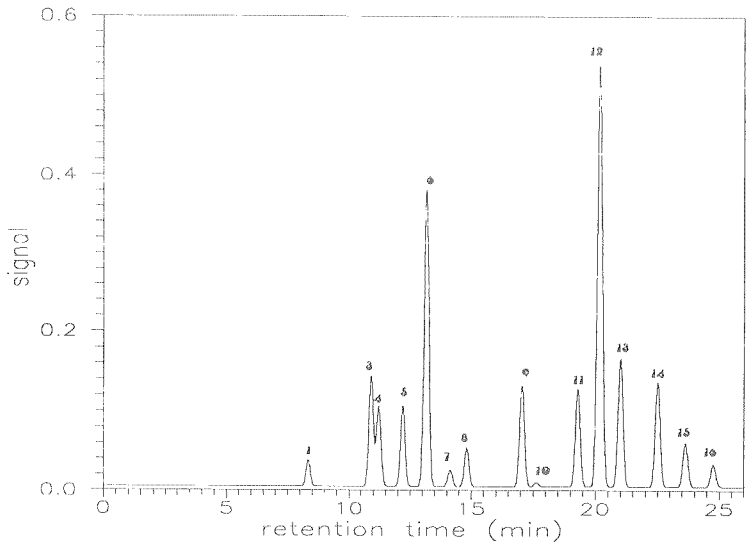

Fig. 5. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 2.

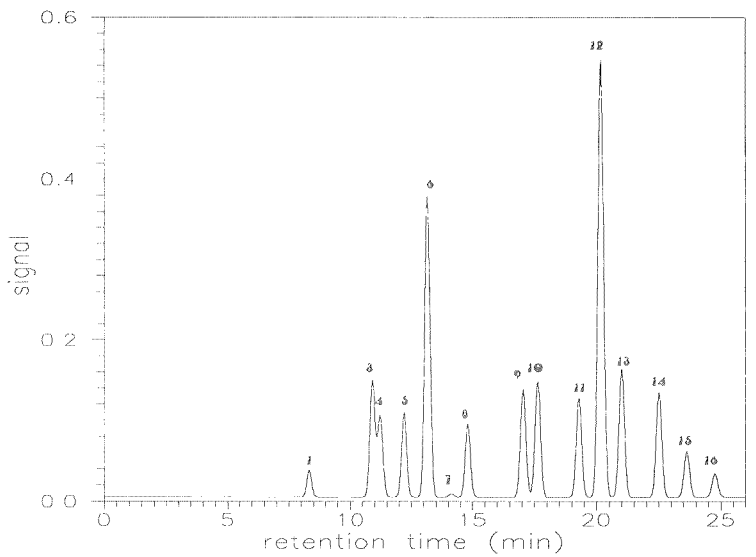

Fig. 6. The chromatogram of PAHs tested mixture (c = 200 μg/ml of each PAH) measured at detection conditions of program No. 3.

Table 4. Reproducibility (% RSD) of Detector Response (peak heights) and retention times for Seven Replicate Injections of PAH mixture (conc. of each PAH was 200 μg/ml).

compound	% *RSD* of retention time	% *RSD* of peak height
Naphthalene	1.1	2.2
Acenaphthylene	1.4	-
Acenaphthene	2.1	1.2
Fluorene	1.6	1.1
Phenanthrene	1.8	0.7
Anthracene	1.1	0.9
Fluoranthene	1.0	2.1
Pyrene	0.9	1.5
Benzo(a)anthracene	1.6	1.1
Chrysene	3.0	1.0
Benzo(b)fluoranthene	2.0	0.8
Benzo(k)fluoranthene	1.5	0.9
Benzo(a)pyrene	1.1	0.7
Dibenz(a,h)anthracene	0.7	1.2
Benzo(g,h,i)perylene	1.4	0.5
Indeno(c,d)pyrene	1.2	0.4

and indeno(c,d)pyrene are strong carcinogens and benzo(a)pyrene is the most dangerous carcinogen. If there was a specific need to analyse most dangerous PAHs from the tested mixture then the program could have been adjusted accordingly.

For example benzo(a)anthracene and chrysene have the similar natural fluorescences, but chrysene is only moderately active carcinogen and benzo(a)anthracene is strong carcinogen. This could be taken into consideration in optimization. Afterwards conditions were shifted closer to the ideal conditions of benzo(a)anthracene (275 / 386 nm). Under these conditions benzo(a)anthracene response was 92 % of its ideal conditions and chrysene 66 % of its ideal.

Conclusion

Presented work discusses the method of λ_{exc}, λ_{emis} optimization in programmed fluorescence detection. The procedure utilises the preference of the threshold optimization. Parametric space was reduced to the points with guaranteed minimal detection sensitivities of tested PAHs. The relative detection sensitivity of each PAH, elution characteristics, different natural fluorescences, chromatographic resolution of "critical" pairs of peaks and also the reproducibilities of retention times under gradient conditions were taken into account. The threshold values P_k were chosen with respect to the differences between the natural fluorescences of PAHs. By this way PAHs with lower natural fluorescence were measured at such compromise conditions that favoured them (naphthalene, fluoranthene, benzo(b)fluoranthene). If there is a specific need to analyse especially the most dangerous PAHs then the toxicological data should be taken into consideration.

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TRIACYLGLYCEROL ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ATMOSPHERIC PRESSURE CHEMICAL IONIZATION MASS SPECTROMETRY: *CREPIS ALPINA* AND *VERNONIA GALAMENSIS* SEED OILS

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ABSTRACT

Unusual seed oils having significance for chemical synthesis, *Crepis alpina*, or with fatty acids which contain functional groups important in the preparation of plastics, *Vernonia galamensis*, were analyzed by a new reversed-phase high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry technique. Using this method, we have identified 16 triacylglycerols in the *Crepis alpina* oil and 18 triacylglycerols in *Vernonia galamensis* oil and showed greater sensitivity for detection of and improved identification of triacylglycerols compared to previous analyses using the techniques of reversed-phase and silver ion high performance liquid chromatography with a flame ionization detector. The most abundant *Crepis alpina* triacylglycerols were: linoleoyldicrepenynoylglycerol (33.0%), tricrepenynoyl (32.3%), palmitoyldicrepenynoyl

(11.5%), dilinoleoylcrepenynoyl (6.7%) glycerols. The remaining triacylglycerols occurred at five or less mole percent abundance. The most abundant *Vernonia galamensis* triacylglycerols were: trivernoloyl (43.3%), linoleoyldivernoloyl (21.3%), oleoyldivernoloyl (7.9%), palmitoyldivernoloyl (8.2%) and stearoyldivernoloyl (6.4%) glycerols. The remaining triacylglycerols occurred at four or less mole percent abundance. These studies provided new knowledge concerning the triacylglycerol composition of these oils and show that the atmospheric pressure chemical ionization technique is suitable for mass spectral identification of neutral molecules which do not contain a chargeable functional group.

INTRODUCTION

In order to fully evaluate the utility of unusual seed oils having significance for chemical synthesis, such as *Crepis alpina* and *Vernonia galamensis*, knowledge of their triacylglycerol composition is important.

Crepis alpina seed oil (CAO) is a source (70-80%) of crepenynic acid [cis-9-octadecen-12-ynoic acid; (C)(1)]. This acid is a useful intermediate in the chemical synthesis of deuterium-labeled compounds for human metabolism studies (2,3). Likewise, *Vernonia galamensis* (VGO), a potential source of epoxy fatty acids, is also useful for preparation of deuterium-labeled fats for human metabolic studies (2).

In addition, because VGO contains 70-80% of an unsaturated epoxy fatty acid, vernolic (*cis*-12,13-epoxy-*cis*-9-octadecenoic) (V), there has been much interest in its applications for the manufacture of commercial products (4-10). VGO has potential industrial uses for coating formulations and production of epoxy resins (11,12). Also, VGO is a potential source of raw material for elastomers (13) and chemicals for plastics manufacturing (14,15).

TAG composition data for these oils has been obtained by reversed phase-high performance liquid chromatography (RP-

HPLC) (16) and silver ion-high performance liquid chromatography (Ag-HPLC) (17) with flame ionization detection (FID). While the FID proved satisfactory for quantitation of the eluted TAG, identification of the individual TAG species required the collection of fractions for characterization by proton and carbon nuclear magnetic resonance spectroscopy and conversion to methyl esters for GC analysis to identify the TAG constituent FA (16,17).

We recently reported the development of a RP-HPLC technique, coupled with a quadrupole mass spectrometry (MS) equipped with an atmospheric pressure chemical ionization (APCI) interface for qualitative analysis of standard TAG species eluted from an HPLC column (18). The resultant simple spectra contained only the protonated TAG molecular ion (M+1) and diacylglycerol fragments to conclusively identify TAG (18,19).

We report here the use of the coupled RP-HPLC/APCI-MS technique for qualitative and semi-quantitative analysis of TAG with unusual FA.

EXPERIMENTAL

Material

CAO and VGO were obtained from K. Carlson and R. Kleiman (USDA, ARS, NCAUR, Peoria, IL). Solid-phase extractive purification of TAG to avoid interference by non-TAG during RP-HPLC/APCI-MS was performed by a previously reported procedure (16).

Methods

RP-HPLC equipment consisted of a quaternary pump system with membrane degasser (LDC 4100 MS, Thermo Separation Products, Schaumburg, IL), and two columns in series: An Adsorbosphere C18 25 cm x 4.6 mm, 5 μm (12% carbon load)

(Alltech Assoc., Deerfield, IL)and an Adsorbosphere UHS C18 25 cm x 4.6 mm, 10 μm (30% carbon load).

A quadrupole mass spectrometer system (Finnigan MAT SSQ 710C, San Jose, CA) was used which was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature at 400°C, capillary heater temperature of 265°C, corona current of 6 μA, high purity nitrogen as sheath gas at 60 psi and auxiliary gases at 25 mL/min).

CAO and VGO TAG were separated using a gradient solvent program with propionitrile (PrCN), dichloromethane (DCM), and acetonitrile (ACN) as follows: 45% PrCN throughout, initially 20% DCM and 35% ACN, held for 15 minutes; DCM was increased to 25% and ACN decreased to 30% over 5 minutes, and held for 15 minutes; DCM was further increased to 30% and ACN decreased to 25% over 5 minutes, and held for 35 minutes; the composition was returned to the initial conditions over 5 minutes. The flow rate was 1 mL/min. The effluent was split so that ~600 μL/min went to an evaporative light scattering detector (ELSD) and ~400 μL/min went to the APCI interface. In both analyses the sample size injected was 5 μl of TAG mixture (50 mg solute per 2 mL hexane).

GC Analysis

The purified TAG were transmethylated and the methyl esters analyzed by GC by a previously reported procedure for SBO (20). GC reference standards for crepenynic acid and vernolic acid were obtained by transmethylation of tricrepenynoylglycerol and trivernoloylglycerol, respectively, previously collected by RP-HPLC (16).

RESULTS AND DISCUSSION

RP-HPLC/APCI-MS identification data (mass spectra results) for the TAG are presented in Table 1 (CAO) and

TABLE 1

Crepis Alpina Seed Oil Triacylglycerols Determined by Reversed-Phase HPLC Coupled with Quadrupole Mass Spectrometer Via Atmospheric Pressure Chemical Ionization[a]

TG Name[b,c]	Mol. Wt.	DG1 Mass[d]	DG1 Int. %[e]	DG2 Mass	DG2 Int. %	DG3 Mass	DG3 Int. %	TG+1 Mass[f]	TG Int. %	Ret. Time[g]	Mole %
CCCx	870	593(C,Cx)	4.2	595 (C,C)	0.7			871	100	11:44	0.5
CCC	872	595 (C,C)	15.2					873	100	13:01	32.3
CCL	874	595 (C,C)	7.0	597 (C,L)	13.2			875	100	16:29	33.0
CCM	822	595 (C,C)	N.U.	545 (C,M)	21.3			823	100	18:00	0.2
CCO	876	595 (C,C)	8.4	599 (S,O)	10.6			877	100	20:23	4.1
CLL	876	597 (C,L)	12.8	599 (L,L)	11.0			877	100	21:20	6.7
CCP	850	595 (C,C)	13.3	573 (C,P)	14.2			851	100	21:31	11.5
CC 20:1	904	595 (C,C)	9.1	627 (C,20:1)	3.7			905	100	24:41	0.5
CCS	878	595 (C,C)	9.0	601 (C,S)	10.2			879	100	26:37	4.0
LLL	878	599 (L,L)	9.0					879	100	27:52	1.7
PLC	852	597 (C,L)	12.1	573 (C,P)	4.9	575 (P,L)	11.0	853	100	28:23	2.4
CCA	906	629 (C,A)	11.6	595 (C,C)	9.3			907	100	31:03	0.6
SLC	880	597 (C,L)	5.3	601 (C,S)	12.3	603 (S,L)	9.6	881	100	32:33	1.4
LLP	854	575 (P,L)	21.3	599 (L,L)	31.3			855	100	34:25	0.5
CLA	908	597 (C,L)	5.4	629 (C,A)	12.3	631 (L,A)	14.0	909	100	37:36	0.2
LLS	882	599 (L,L)	44.9	603 (L,S)	50.0			883	100	40:36	0.4

[a]See Experimental Section for HPLC-Mass Spectrometry Conditions.

[b] See Fig. 1 for Reversed Phase-HPLC Coupled with Quadrupole Mass Spectrometer Via Atmospheric Pressure Chemical Ionization Chromatogram.

[c]TG=triacylglycerol. Triacylglycerol fatty acids: C, crepenynic; L, linoleic; O, oleic; S, stearic; M, myristic; A, arachidic; 20:1, twenty carbon fatty acid with one double bond unspecified location; and Cx, crepenynic with one additional double bond unspecified location.

[d]DG 1,2,3 are diacylglycerol fragments remaining after loss of one fatty acid residue from the triacylglycerol during mass spectrometry.

[e]Int.% is the abundance of a particular ion with respect to the most abundant ion

Table 2 (VGO). The RP-HPLC/APCI-MS total ionization curves for the mass spectral identified TAG, with respect to RP-HPLC retention time, are presented in Fig. 1 (CAO) and Fig. 2 (VGO).

Example mass spectra obtained for tricrepenynoyl (same FA), linoleoyldicrepenynoyl (2 same FA, 1 different FA) and palmitoyllinoleoylcrepenynoyl (all different FA) glycerols are presented in Fig. 3a, 3b, 3c, respectively, and for trivernoloyl (same FA), linoleoyldivernoloyl (2 same FA, 1 different FA) and oleoyllinoleoylvernoloyl (all different FA) glycerols are presented in Figs. 4a, 4b, 4c, respectively.

For each of the CAO TAG, the base peak is the protonated TAG molecular ion. For vernolic acid-containing VGO TAG, except trivernoloyl and linoloyl divernoloyl glycerol, the base peak is one of the diacylglycerol fragments (DG). Other mass spectral peaks are the distinctive diacylglycerol fragment masses which are presented in Tables 1 and 2. For TAG containing only one FA, one DG fragment is required for identification; for TAG with one different and two same FA, two DG are required; and for TAG with three different FA, three DG are required for identification. The diacylglycerol fragments conclusively identify TAG with the same molecular weight (19). Also, protonated molecular ions plus propionitrile (TG+1+55(Pr)) are observed in the CAO mass spectra (Fig. 3a,b,c). The origin of the [M+38]+ ions (Fig. 3a,b,c) is not known. Also, through selective ion monitoring, TAG like PLC and LLL, which eluted in the same RP-HPLC peak (Fig. 1), could be identified by the appropriate masses (Table 1, Fig. 3c).

It is important to note that trilinolenoylglycerol produced a mass spectrum in previous RP-HPLC/APCI-MS work (18) similar to the mass spectrum obtained here for

TABLE 2

Vernonia Galamensis Seed Oil Triacylglycerols Determined by Reversed-Phase HPLC Coupled with Quadrupole Mass Spectrometer Via Atmospheric Pressure Chemical Ionization[a]

TG Name[a,b]	Mol. Wt.	DG1 Mass[c]	DG1 Int.%[d]	DG2 Mass	DG2 Int.%	DG3 Mass	DG3 Int.%	(TG+1) Mass[e]	(TG+1) Int.%	(TG+1)-18[f] Mass	(TG+1)-18[f] Int.%	(TG+1)-36[f] Mass	(TG+1)-36[f] Int.%	(TG+1)-100[f] Mass	(TG+1)-100[f] Int.%	(TG+1)+102[f] Mass	(TG+1)+102[f] Int.%	HPLC Ret. Time[g]	Mole %
VVV[h]	926	631 (V,V)	87.7					927	100	909	62.4	891	22.8	827	11.9	1029	35.8	7:55	43.3
VVL	910	631 (V,V)	56.6	615 (V,L)	95.3			911	100	893	54.7	875	12.3	811	10.1	1013	40.3	10:07	21.3
VVO	912	631 (V,V)	76.0	617 (V,O)	100			913	96.3	895	46.2	877	14.8	813	6.3	1015	46.7	11:47	7.9
VVP	886	631 (V,V)	100	591 (V,P)	84.1			887	91.1	869	36.0	851	14.4	787	5.9	990	42.0	12:21	8.2
LLV	894	615 (L,V)	100	599 (L,L)	69.0			895	79.9	877	53.6			795	5.9	998	33.5	13:28	3.6
VVS	914	631 (V,V)	74.3	619 (V,S)	100			915	97.4	897	44.9	879	20.5	815	8.3	1017	45.6	14:25	6.4
OLV	896	601 (O,L)	97.3	615 (V,L)	100	617 (V,O)	85.0	897	80.8	879	70.3			797	6.2	1000	59.1	16:05	1.5
PLV	870	575 (P,L)	71.9	615 (V,L)	100	591 (V,P)	24.3	871	51.2	853	42.7			771	4.7	974	26.8	17:12	1.9
LLL	878	599 (L,L)	41.3					879	100									18:48	0.4
SLV	898	603 (S,L)	75.8	615 (V,L)	100	619 (V,S)	26.7	899	60.0	881	45.0			799	5.1	1001	34.8	20:14	1.4
POV	872	577 (P,O)	58.4	617 (V,O)	100	591 (V,P)	25.3	873	35.9	855	22.5			773	2.6	976	25.4	20:36	0.8
LLO	880	599 (L,L)	48.0	601 (L,O)	77.9			881	100									23:16	0.3
LAV	926	631 (L,A)	100	647 (A,V)	18.6	615 (V,L)	96.9	927	44.0	909	44.6			827	4.0	1029	27.8	24:02	0.5
LLP	854	599 (L,L)	69.9	575 (L,P)	60.0			855	100									24:43	0.4
SOV	900	605 (S,O)	87.8	617 (V,O)	100	619 (V,S)	22.5	901	51.4	883	35.7			801	2.1	1003	29.8	24:50	1.0
OOL+ LLS	882	601 (L,O)	53.7	603 (O,O)	57.2	599 (L,S)	67.1	883	100									28:53	0.5
OAV	928	633 (O,A)	69.9	647 (A,V)	15.9	617 (V,O)	100	929	29.9	911	28.6			829	4.3	1031	18.6	29:10	0.3
POL	856	575 (P,L)	100	577 (P,O)	21.7	601 (L,O)	33.7	857	5.8									30:53	0.2

[a]Mass spectrometer total ionization chromatogram peaks, see Fig. 2. For analysis conditions, see Experimental Section.
[b]TG=triacylglycerol. Triacylglycerol fatty acids: S, stearic; P, palmitic; O, oleic; L, linoleic; V, vernolic; and A, arachidic
[c]DG 1,2,3 are diacylglycerol fragments remaining after loss of one fatty acid residue from the triacylglycerol during mass spectrometry.
[d]Int.% is the abundance of a particular ion with respect to the most abundant ion formed during mass spectrometry.
[e]TG+1 is the protonated triacylglycerol molecular ion.
[f](TG+1)-18, (TG+1)-36, (TG+1)-100 and (TG+1)+102 is the loss of one water, two water molecules, hexanal or addition of protonated hexanal to the vernolic acid of the TAG during mass spectrometry.

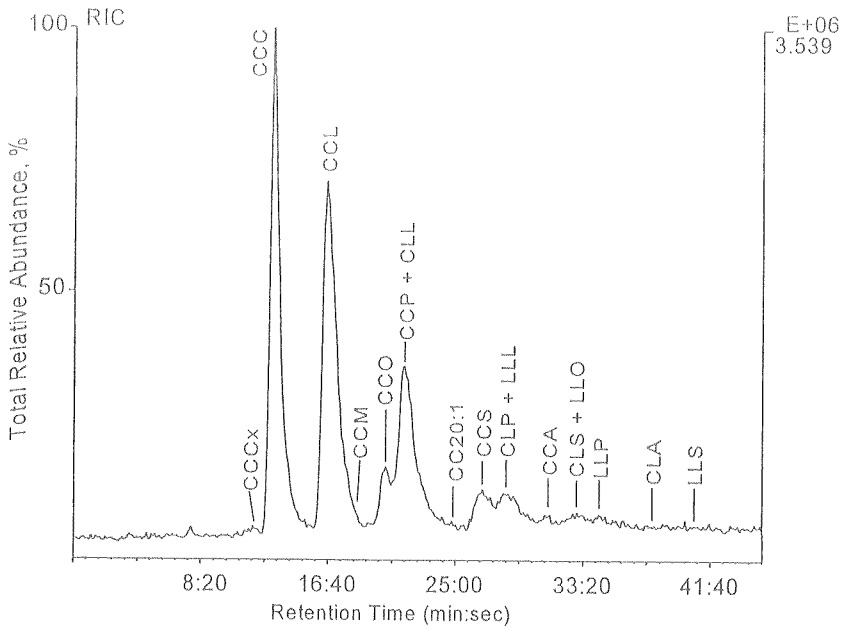

FIGURE 1.
Reconstructed ion chromatogram of *Crepis alpina* seed oil. Triacylglycerol fatty acids: C, crepenynic; Cx, crepenynic with one additional π bond; L, linoleic; O, oleic; M, myristic; P, palmitic; S, stearic; A, arachidic; 20:1, twenty carbon fatty acid with a double bond at an unspecified location.

tricrepenynoylglycerol. However, the CAO contained only 0.3% linolenic acid. Therefore, this potential problem is not of concern for RP-HPLC/APCI-MS analysis of CAO.

In addition to the diacylglycerol and parent ions observed in the APCI spectra for vernolic acid- containing TAG, protonated molecular ions and diglyceride fragments exhibiting loss of water are observed. The number of fragments exhibiting loss of water is dependent on the number of vernoloyl chains present. Also, there are protonated molecular ion fragments which have reduced mass due to loss

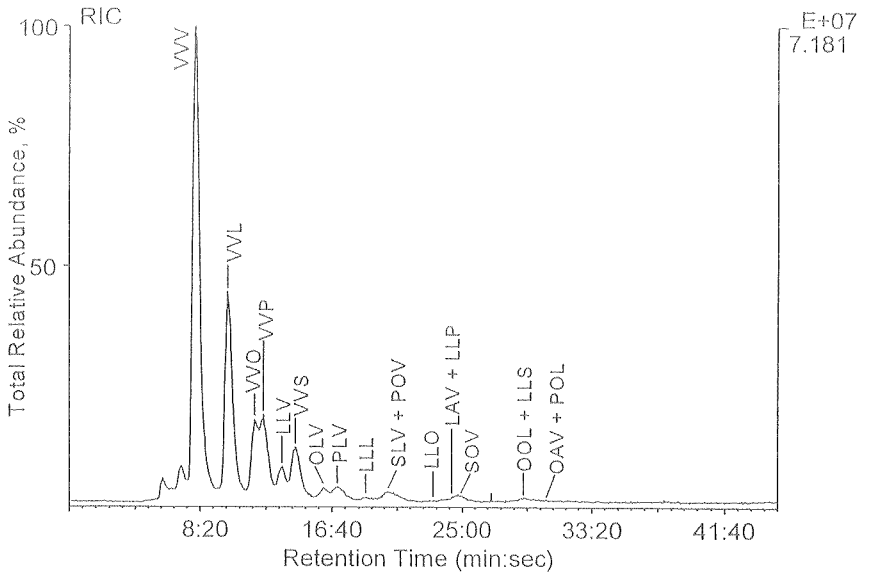

FIGURE 2.
Reconstructed ion chromatogram of *Vernonia galamensis* seed oil. Triacylglycerol fatty acids: V, vernolic; L, linoleic; O, oleic; P, palmitic; S, stearic; A, arachidic.

of hexanal from cleavage possibly due to the proposed fragmentation pattern depicted in Fig. 5 during APCI-MS of the TAG. The corresponding diacylglycerol fragment [V, 12:2], which would result from hexanal loss via the depicted pattern in Fig. 5 is observed in spectra for VVV (Fig. 4a) and VVL (Fig. 4b). In addition, the hexanal adduct of the protonated molecular ion may be observed in the spectra for the three vernolic acid TAG given in Fig. 4. The origin of masses 966 to 1015 for VVV (Fig. 4a), 950 to 1000 for VVL (Fig. 4b) and 927 to 986 for OLV (Fig. 4c) is not known. Also, through selective ion monitoring, TAG like SLV and POV, LLP and LAV, and OAV and POL, which eluted in the same RP-HPLC peaks (Fig. 2), could be identified by the appropriate

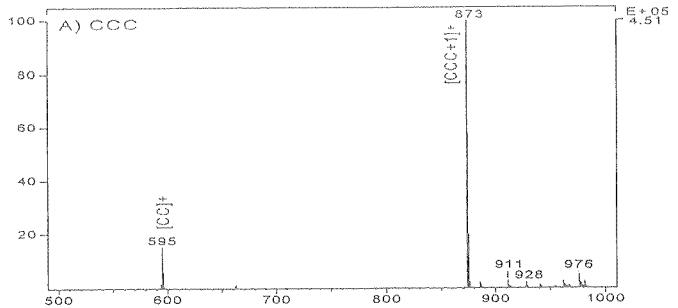

FIGURE 3a.
Mass spectrum of tricrepenynoylglycerol. Identities of fragment ions are shown in brackets. Triacylglycerol fatty acids: C, crepenynic.

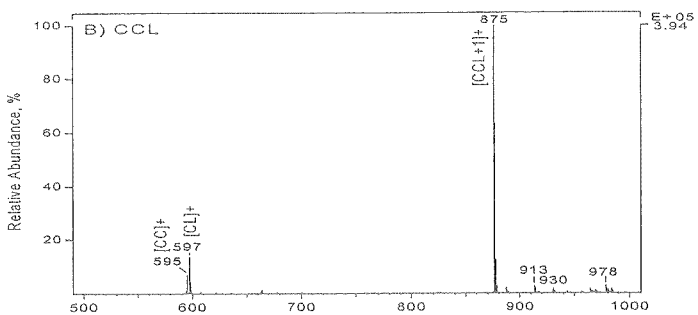

FIGURE 3b.
Mass Spectrum of linoleoyldicrepenynoylglycerol. Identities of fragment ions are shown in brackets. Triacylglycerol fatty acids: C, crepenynic; L, linoleic.

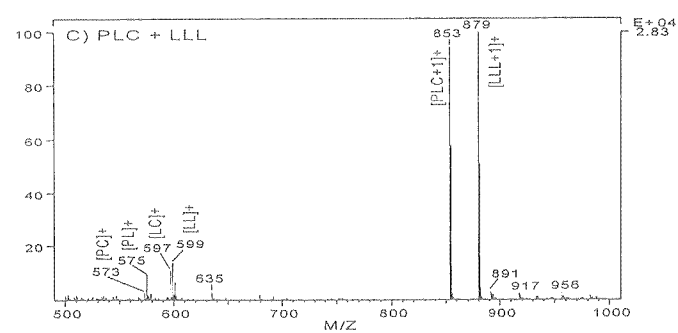

FIGURE 3c.
Mass spectrum of palmitoyllinoleoylcrepenynoylglycerol with minor trilinoleoylglycerol, which coelute during reversed phase-HPLC (Fig. 1). Triacylglycerol fatty acids: C, crepenynic; L, linoleic; P, palmitic.

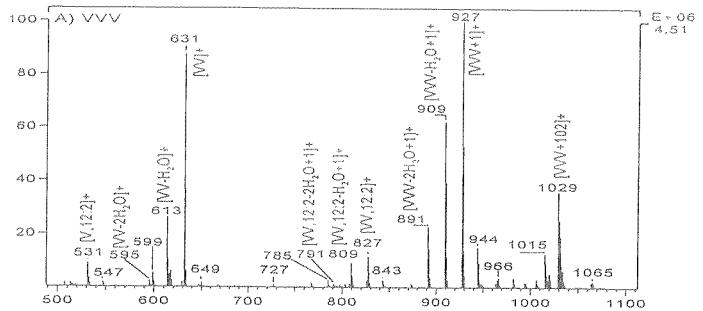

FIGURE 4a.
Mass spectrum of trivernoloylglycerol. Identities of fragment ions is shown in brackets. Triacylglycerol fatty acids: V, vernolic; 12:2, vernolic minus hexanal fragment (mass=100).

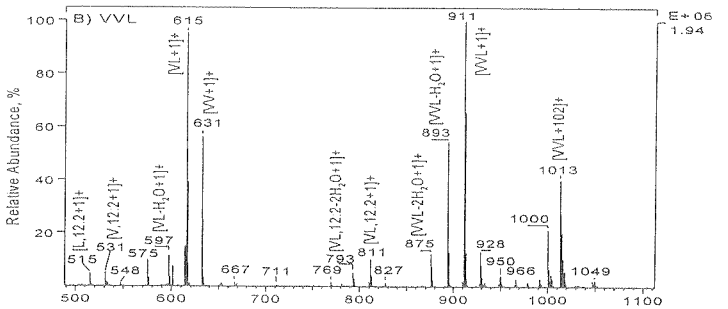

FIGURE 4b.
Mass Spectrum of linoleoyldivernoloylglycerol. Identities of fragment ions is shown in brackets. Triacylglycerol fatty acids: V, vernolic; L, linoleic; 12:2, vernolic minus hexanal fragment (mass=100).

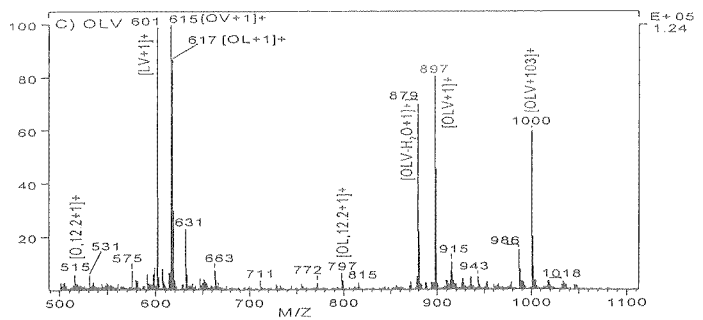

FIGURE 4c.
Mass spectrum of oleoyllinoleoylvernoloylglycerol.Identities of fragment ions is shown in brackets. Triacylglycerol fatty acids: V, vernolic; L, linoleic; O, oleic; 12:2, vernolic minus hexanal fragment (mass=100).

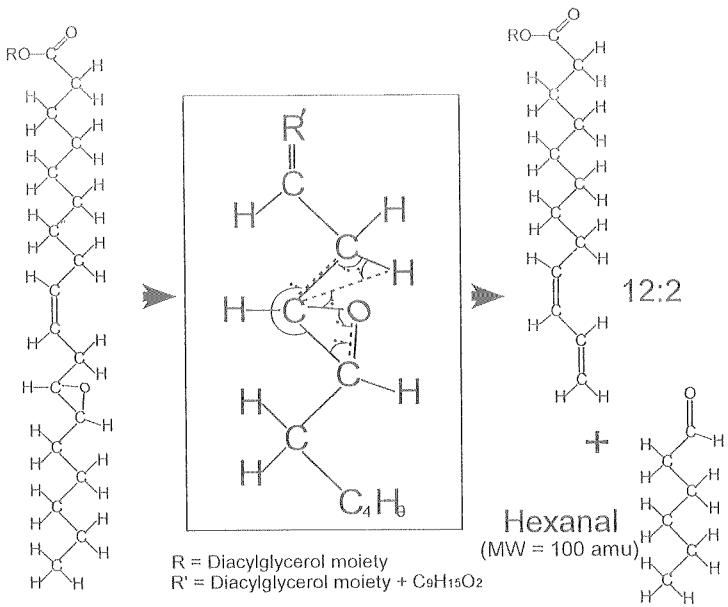

FIGURE 5.
Proposed mechanism for mass spectral cleavage of vernolic acid to yield hexanal during reversed phase-HPLC coupled with mass spectrometer with atmospheric pressure chemical ionization of vernolic acid containing triacylglycerols.

masses (Table 2). Thus, the spectra for VGO TAG (Fig. 4) are more complex and show loss of water and hexanal fragments from V compared to the spectra for *Crepis alpina* oil (Fig 3) and soybean oil (20). RP-HPLC/APCI-MS produced simple spectra for the TAG of CAO, including those TAG with the alkene-alkyne containing FA of crepenynic acid.

This RP-HPLC/APCI-MS technique identified more CAO TAG than the RP-HPLC-FID (16) and Ag-HPLC-FID techniques (17). Even coeluting TAG like CCP and CLL, CLP and LLL, or CLS and LLO (Fig. 1) were differentiated by producing extracted ion chromatograms of individual masses. One TAG identified,

CCCx, contained a crepenynic acid with two other π bonds (probably forming a second acetylene bond) and was not previously identified. In addition, our RP-HPLC/APCI-MS procedure conclusively identified 19 TAG in VGO which were not previously conclusively identified, including OLV, PLV, LLL, SLV, POV, LLO, LLP, LAV, SOV, LOO, OAV and POL (Fig. 2).

Mole percent of the TAG components was determined by summation of selected ion masses (protonated molecular ion and DG fragments) obtained by APCI-MS and listed in Table 1 (CAO) and Table 2 (VGO). By this MS method, the most abundant CAO TAG were: linoleoyldicrepenynoylglycerol (33.0%), tricrepenynoyl (32.3%), palmitoyldicrepenynoyl (11.5%), and dilinoleoylcrepenynoyl (6.7%). The remaining twelve CAO TAG were found at four or less mole percent abundance.

The most abundant VGO TAG were: trivernoloylglycerol (43.3%), linoleoyldivernoloyl (21.3%), oleoyldivernoloyl (7.9%), palmitoyldivernoloyl (8.2%) and stearoyldivernoloyl (6.4%) glycerols. The remaining 13 VGO TAG occurred at four or less mole percent. Trivernoloyl and linoleoyldivernoloyl-glycerols were also determined previously to be the most and second most abundant TAG in VGO by RP-HPLC-FID (16). The RP-HPLC/APCI-MS technique for qualitative and quantitative analysis of TAG showed greater sensitivity for detection of and improved identification of TAG compared to the previously reported techniques for the TAG analysis (16,17,22-26). Some TAG could be identified, but not quantitated. In CAO, three TAG were not quantitated: crepenynoyllinoleoyloleoyl gycerol (CLO), crepenynoyllinoleoyl-20:1 (CL,20:1), and dilinoleoyloleoyl glycerol (LLO). These TAG have the same equivalent carbon number as, coeluted chromatographically with, and share common masses with dicrepenynoylstearoyl

TABLE 3

Comparison of Fatty Acid Composition of Seed Oils as Calculated from Triacylglycerol Composition[a] and as Determined After Transmethylation of the Seed Oil[b]

Method	Fatty Acid Percent												
	C[c] or V[d]	14:0	16:0	18:0	18:1	18:2	20:0	18:3	20:1	20:2	22:0	24:0	UID
		Crepis Alpina Seed Oil											
LC/APCI-MS[e]	72.2	0.1	4.4	2.0	1.4	19.3	0.3	0.0	0.2	0.0	0.0	0.0	0.2
GC-FID[f]	75.0	0.5	3.9	1.3	2.5	15.9	0.3	0.3	0.3	0.1	0.1	0.0	0.0
		Vernonia galamensis Seed Oil											
LC/APCI-MS[e]	77.3	0.0	3.4	3.0	4.0	12.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0
GC-FID	73.7	0.0	3.7	3.1	4.7	14.0	0.4	0.1	0.3	0.0	0.1	0.1	0.0

[a]Triacylglycerol Composition Determined by Reversed Phase-High Performance Liquid Chromatography Coupled with Atmospheric Pressure Chemical Ionization Mass Spectrometry (RP-HPLC-APCI-MS).

[b]Gas Chromatography with Flame Ionization Detection (GC-FID).

[c]C, crepenynic acid

[d]V, vernolic acid

[e]The RP-HPLC/APCI-MS TAG mole percent composition, Tables 1 & 2, was converted to TAG weight percent composition for valid comparison of calculated fatty acid composition with experimental fatty acid which is related to weight percent obtained by GC-FID.

gycerol (CCS), dicrepenynoylarachidoyl gycerol (CCA), and crepenynoyllinoleoylstearoyl (CLS) gycerol, respectively.

Application of the APCI-MS technique for accurate quantitation of individual TAG species may require the use of response factors based on analysis of TAG standard mixtures of known weight. We evaluated the APCI-MS quantitation for CAO TAG (Table 1) and VGO TAG (Table 2) by comparing the FA composition, calculated from the TAG composition, with the experimental FA composition, obtained by GC-FID analysis of the transmethylated TAG mixtures, as presented in Table 3. The mole percent data was converted to weight percent data to make a more valid comparison with GC-FID data, which is related to number of TAG carbons or TAG weight. While the APCI-MS technique is much improved over other MS quantitative methods, there is some variation from the fatty acid composition determined by the GC-FID methods, as reported

previously (18). The disparity of response between TAG is largely due to differences in fragmentation patterns between TG and DG fragments, which depends on different levels of unsaturation within the TAG. The more similar the series of analytes, the less will be the disparity between responses of the TAG. Nevertheless, there is good agreement between the calculated FA composition from RP-HPLC/APCI-MS and the FA composition determined by GC-FID.

The RP-HPLC/APCI-MS technique has conclusively identified 16 TAG in the CAO and 18 TAG in VGO and shows greater sensitivity for detection of and improved qualitative analysis of TAG compared to the previously used RP-HPLC-FID and Ag-HPLC-FID (17,18).

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PHENOLIC ACIDS ISOLATED FROM PLANTS

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ABSTRACT

A simple and rapid method is described for the estimation by high-performance liquid chromatography of *cis* and *trans* isomers of the substituted cinnamic acids, p-coumaric (p-CA) and ferulic (FA), using p-anisic acid (p-AA) as internal standard. Chromatographic separation and quantification were performed on a reversed-phase Nova-Pak C_{18} column with isocratic elution water-n-butanol-acetic acid (98:1.5:0.5). A flow-rate of 1.5 ml/min, a column temperature of 35°C and detection at 270 nm were employed. As little as 175ng/ml for p-CA and 63 ng/ml for FA can be estimated by this procedure with a 20 μl injection. Application of this method are illustrated by estimation of these acids in extracts, prepared with 1 M sodium hydroxide of barley straw, mixed grass hay and alfalfa hay.

INTRODUCTION

The substituted cinnamic acids p-coumaric and ferulic are widely distributed in plants. These acids crosslink lignin to structural

carbohydrates of plant cell walls [1] and lignin is known to depress cell wall digestibility presumably by reducing access to the structural carbohydrates by anaerobic bacteria. Because ruminants consume vast quantities of forage and thus depend on microbial fermentation of cell walls for energy [2-3], the analysis of phenolic monomers content of forages may allow to increase the appreciation of the effects of these acids on animal nutrition.

Several workers have proposed methods for the determination of phenolic acids. Some procedures are based on the separation of the trimethylsilylether (TMS) derivatives of the substituted cinnamic acids by GLC [4-7]. Other authors have developed procedures for the determination of these acids in biological samples, plants and soil by HPLC [8-10]. In this work we describe a simple and rapid RP-HPLC method for the simultaneous determination of *cis* and *trans* isomers of p-coumaric and ferulic acids in forages for the study of ruminant metabolism.

EXPERIMENTAL

Chemicals and Reagents

p-*trans*-CA, *trans*-FA and p-AA were obtained from Fluka Chemie (Buchs, Switzerland), n-butanol of HPLC grade from Carlo Erba (Milan, Italy). Water was previously distilled and purified with a Milli-Q system purchased from Millipore (Bedford, MA, USA). Other chemicals were of the highest purity commercialy available.

Equipment

HPLC analyses were performed with a Waters Model 600E system equiped with a loop injector Waters U6K, a Waters Model 484 UV-detector and a Waters Model 745B integrator.

Standard Solutions

Stock solutions of p-*trans*-CA, *trans*-FA and p-AA (1 mg/ml) were prepared in methanol and kept at 4°C up to one month. All manipulations of *trans*-phenolic acids were carried out in "white" fluorescent light to prevent isomerization.

Calibration graphs were constructed and were linear over the concentration range investigated, 5 to 200 μg/ml.

The *cis*-isomers were prepared by leaving the *trans*-isomers solutions exposed to light.

The quantification were achieved by regression analysis of the peak areas of each compound against concentration .Triplicate injection were made. For calculations it is assumed that the *cis*-isomers have the same response factor as the *trans*-isomers.

Sample preparation

All forage samples were dried at 35°C for 48 h and ground through a 1mm screen. 100 mg of sample were accurately weighed and 5 ml 1 M NaOH were added to completely soak the sample. The mixture was shaken under nitrogen, leaved at room temperature overnight in the dark and filtered (No 1 porosity glass sinter). 0.5 ml of p-AA solution (1 mg/ml in methanol), the internal standard, were added and the solution was acidified with 6 M hydrochloric acid to pH $<$ 1. Solution was extracted with ethyl acetate (3 x 5 ml). The organic phase was then separated and dried in a rotary evaporator from Büchi (Flawil, Switzerland). The residue was dissolved in 5 ml of mobile phase. The solution was filtred through a 0.45 μm filter and 20 μl were injected on to the HPLC column.

Chromatographic Conditions

RP-HPLC separations were carried out on a Nova-Pak C_{18} column (150 mm x 3.9 I.D.; 4-μm particles) (Waters). The mobile phase was

water-n-butanol-acetic acid (98:1.5:0.5). The flow rate and column temperature were 1.5 ml/min and 35°C respectively. The detection wavelength was set at 270 nm.

RESULTS AND DISCUSSION

Method Performance

The nature and the percentage of organic modifier of the mobile phase, and column temperature were studied to determine their influence on system selectivity thus stablishing the best conditions for separation.

The solvents studied were methanol, ethanol, iso-propanol and n-butanol. Table 1 shows the variations of the retention time with the organic solvent.

On the basis of these results we decided to select n-butanol as the organic modifier. As Fig.1A shows the retention time of species in the chromatographic system decreased when the n-butanol content increased, as expected.

The effects of column temperature (25-50°C) on the capacity factors are shown in the Fig. 1B. In these experiments the n-butanol content of the mobile phase was kept constant and equal to 1.2% .

The results of this study allow us to select for the determination of phenolic acids the conditions specified under Experimental.

Chromatograms of standards and the barley straw sample are shown in Fig.2. Under these chromatographic conditions there were no other endogenous forage components that can interfere with the peaks of the analytes.

The retention times were ca. 4.5 min for p-*trans*-CA and 5.4 min for *trans*-FA. The retention time of I.S was ca. 12.0 min. The retention times of *cis*-CA and *cis*-FA were ca. 5.1 min and 6.8 min respectively.

TABLE 1

Effect of the Organic Modifier on the Retention Time

Organic Solvent	Retention Time (min) P-CA	FA
methanol	50.8	93.2
ethanol	36.7	54.9
iso-propanol	21.2	35.6
n-butanol	6.5	8.1

* Organic modifier porcentage = 1.2%

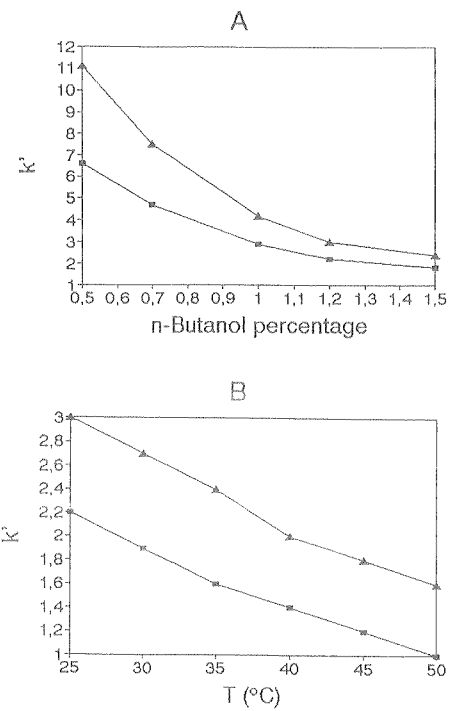

FIGURE 1. Optimization of the analitical conditions. (A) Relation between capacity factors (k') and n-butanol concentration, column temperature 35°C . (B) Effect of column temperature on k' values. Mobile phase contained 1.2% of n-butanol.
Symbols. ▲ = p-*trans*-CA; ■ = *trans*-FA.

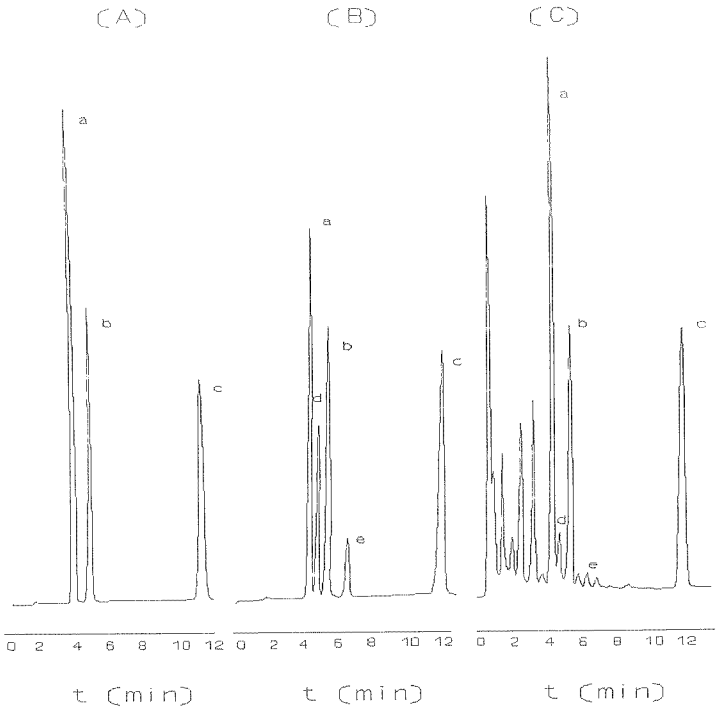

FIGURE 2. Chromatographic separation. (A)-Standard solution of p-*trans*-CA (a) and *trans*-FA (b). (B)- Standard solution of p-*trans*-CA (a), *trans*-FA (b), p-*cis*-CA (d) and *cis*-FA (e). (C) barley straw sample. I.S. was p-AA (c).

Linearity

Linearity was checked by measuring different concentrations in the range (5-200)μg/ml for p-*trans*-CA and *trans*-FA. In all cases the concentration of internal standard was 100 μg/ml. Linear relationships between the peak areas and the concentrations tested were found. The average slopes and y-intercepts of the calibration curve equations are shown in Table 2.

TABLE 2

Calibration Curves and Standard Addition Method.

Calibration Curves

Acid	Linear regression equation	R^2	N	Concentration Range(μg/ml)
CA	$y = 6.81\ 10^{-3}\ x + 3.35\ 10^{-2}$	0.9992	8	5-200
FA	$y = 4.69\ 10^{-3}\ x - 1.64\ 10^{-2}$	0.9995	8	5-200

Standard Addition Curves

Acid	Linear regression equation	R^2	N	Conc. Added Range(μg/ml)
CA	$y = 6.65\ 10^{-3}\ x + 3.14\ 10^{-1}$	0.9966	5	2.5-20
FA	$y = 4.75\ 10^{-3}\ x + 1.53\ 10^{-1}$	0.9999	5	2.5-20

N = number of concentrations

The standard addition method was used to check for chemical interferences in the quantitation of different acids. The equations calculated are shown in Table 2.

The slopes found of the calibration and standard addition curves were similar for each compound. Statistical analysis by the t-test showed that the slope values are not statistically significant ($t = 0.033$, $p = 0.97$ for p-CA and $t = 0.0027$, $p = 0.98$ for FA).

Analytical Recovery, Precision and Accuracy

Analytical recovery was evaluated by assaying forage samples spiked with different amounts of each acid ranging from (2.5-20 μg/ml)

TABLE 3

Inter-day Precision and Accuracy

Acid	Conc.Added (μg/ml)	Conc.Found (μg/ml;mean ± SD; n = 5)	CV(%)	RE(%)
CA	2.5	2.56 ± 0.10	3.9	2.4
	5	5.16 ± 0.08	1.6	3.2
	10	10.17 ± 0.15	1.5	1.7
	15	15.07 ± 0.07	0.4	0.5
	20	20.38 ± 0.12	0.6	1.9
FA	2.5	2.42 ± 0.02	0.8	3.2
	5	4.86 ± 0.11	2.3	2.8
	10	9.79 ± 0.16	1.6	2.1
	15	14.76 ± 0.08	0.5	1.6
	20	19.68 ± 0.13	0.7	1.6

for p-CA and FA. Replicate analyses (n = 5) at each concentration were made. The mean recoveries for p-CA and FA were: 101.96 ± 1.02 (CV = 1.0%) and 97.79 ± 0.67 (CV = 0.7%) in barley straw, 98.35 ± 1.12 (CV = 1.1%) and 96.87 ± 2.02 (CV = 2.1%) in alfalfa hay and 102.13 ± 2.12 (CV = 2.1%) and 97.76 ± 1.88 (CV = 1.9%) in mixed grass hay.

The inter-day precision and accuracy were assessed by analyzing forage samples containing different concentrations of each metabolite, five times per day during one week. The results for barley straw are shown in Table 3. Similar results were obtained for mixed grass hay and alfalfa hay.

The limit of detection was 175 ng/ml for p-CA and 63 ng/ml for FA with a 20 μl injection. The limit of detection was determined from the calibration curves acording to the method described by Miller and Miller [11].

CONCLUSION

An RP-HPLC system has been developed for the determination of *cis* and *trans* isomers of the p-coumaric and ferulic acids. The method is accurate, precise, rapid and easy to perform, and should be applicable to studies of forage digestibility in ruminants.

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THE BOOK CORNER

GAS CHROMATOGRAPHY: ANALYTICAL CHEMISTRY BY OPEN LEARNING, Ian A. Fowlis, John Wiley and Sons, New York, 1995; 258 pages; ISBN 0-471-95467-5 and in paperback as ISBN 0-471- 95468-3.

The text provides a working knowledge of gas chromatography and gas chromatographs. It is recommended for those who are just assuming duties for gas chromatographic determinations and for those who may have been taught how to perform a specific chromatographic determination and desire a fundamental understanding of the technique. Chemistry students who are ready for courses in analysis will find this text an excellent introduction to chromatographic practice.

The chapters cover the basic instrumentation, packed and capillary systems, injectors, detectors, data handling, quantitative and qualitative analyses. Additional sections provide introductory information on combined techniques, analysis of less-volatile substances, and environmental analysis. Specific topics generally are easy to locate from consulting the table of contents or index. Thus the student has a good reference text as well as a good learning guide.

The relationships among fundamental chromatographic parameters are dealt with concisely and in an easy-to-follow manner. Although the text disclaims coverage of chromatographic theory (the student is referred to another text in the *Analytical Chemistry by Open Learning* series for this information.), it does provide sufficient information for a basic understanding of the chromatographic process. In this respect it is weak only the the order in which a couple of topics are presented: The importance of carrier gas selection is mentioned a couple of times before an explanation is presented; and focusing or refocusing is discussed before it is defined (except perhaps in a 60-word sentence on

temperature programming). These are exceptions to the the generally clear and logical way concepts and practice are presented.

The repeated emphasis on the importance of the time spent in the mobile phase is especially welcomed for it has been my experience that students should learn that concept first.

The text stresses to the point of redundancy that no text can provide the understanding of practical experience in the laboratory. Nevertheless the reasons behind standard chromatographic technique are clearly explained. I found only one exception: the importance of the position of the needle tip during injection leaves the student without any guide or reference for knowing what should be done.

The "Fowlis Rule" for determining which compounds may be successfully handled by gas chromatography comes from the author's years of experience. If a solute does not dissolve in ethyl acetate, one had best look for another method for separation, according to the rule.

The figure on the split/splitless injector is confusing. Although the pathway for carrier to septum purge is clearly open, the pathways to the column and split outlet appear blocked. I found only one incidence of lack of clarity in the text: "... the programmed temperature vaporising injector sometimes called the split/splitless injector."

Two omissions are puzzling. The author gives considerable (and deserved) credit to Walter Jennings for work in capillary chromatography and uses the "DB" series in cataloguing chromatographic phases. Nevertheless, Jennings' 1987 text, *Analytical Gas Chromatography*, is not listed in the Bibliography. Finally, I am not sure how the author kept from drawing a lesson from the statement that "on-column injection ... is the method of choice for the *discriminating* chromatographer."

Reviewed by
Leonard H. Ponder, FAIC
Shimadzu Scientific Instruments, Inc.
Columbia, Maryland

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(20), 4195 (1995)

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Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(20), 4197-4201 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1996

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA.

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact:

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MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography & Extraction, Indianpolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

APRIL 17 - 19: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization," Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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Following are acceptable reference formats:

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2. N. H. Foda, "Quantitative Analysis of Fluvoxamine Maleate in Tablet Formulations by HPLC," J. Liquid Chrom., **18**, 1591-1601 (1995)

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

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