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QUANTITATION OF NEUTRAL LIPID MIXTURES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH LIGHT SCATTERING DETECTION†

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for quantifying reaction mixtures obtained from the lipase-catalyzed transesterification of fats and oils. The reaction mixtures of interest were composed of neutral lipid classes that include alkyl esters, free fatty acids, triglycerides, 1,2- and 1,3-diglycerides, and 1 (2)-monoglycerides. The method, a modification of a literature procedure, uses a binary mobile phase of hexane (A) and methyl-t-butyl ether (B) each modified with acetic acid (0.4%) and a gradient elution profile that reduced analyses' times by 25%. Lipid classes were measured by use of an evaporative light scattering detector (ELSD). Precision of injection and linearity of response of the ELSD over the range of sample amounts of interest were established for the lipid classes measured by use of standards. The method was applied to the compositional analysis of prospective biofuel alkyl esters prepared from fats, oils, and recycled greases.

INTRODUCTION

Currently, considerable effort is being expended in studies directed to the chemistry and biocatalytic transformations of fats and oils. Among the various transformations studied, lipase catalyzed acyl exchange reactions have been investigated extensively as a way of either altering the physical properties or improving the nutritional quality of fats or oils.[1]

The enzymatic transesterification of fat or oil triglycerides (TG) in alcoholic medium produces the corresponding fatty acid alkyl esters (AE), along with free fatty acids (FFA), diglycerides (DG), monoglycerides (MG) and unreacted TG. Accordingly, a facile method of separation and quantitation for these lipid materials would be useful for the optimization of reaction conditions and or product yields.[2]

Neutral lipid classes are more amenable to separation by high performance liquid chromatography (HPLC) than gas chromatography (GC) because GC analysis requires a pre-analysis fractionation step with subsequent derivatization, whereas normal phase HPLC is able to separate all neutral lipid classes without prior derivatization.[3,4] Previous investigators have found that silica-based normal phase HPLC separation of neutral lipid classes are sometimes irreproducible due to variable amounts of water bound to the silica surface. This problem, however, has been alleviated with the advent of bonded polar phases, such as cyanopropyl.[4,5]

Refractive Index detection is often used for isocratic HPLC quantitation of lipids. The UV detector is also widely used, but a major disadvantage of this detection method is the weak absorbance of fatty acyl groups.[6] The advent of the evaporative light scattering detector (ELSD), a mass sensitive detector, has greatly improved the usage of HPLC for lipid analysis.[7] This work describes a reproducible method for the separation of alcoholysis products of fats and oils using a cyanopropyl column along with subsequent quantitation of the separated species by ELSD after adequate optimization and calibration of the detector.

MATERIALS AND METHODS

Materials

The following lipid mixtures, purity >99%, were obtained from NuChek Prep, Inc. (Elysian, MN): i) methyl palmitate (MeP), tripalmitin (TP),

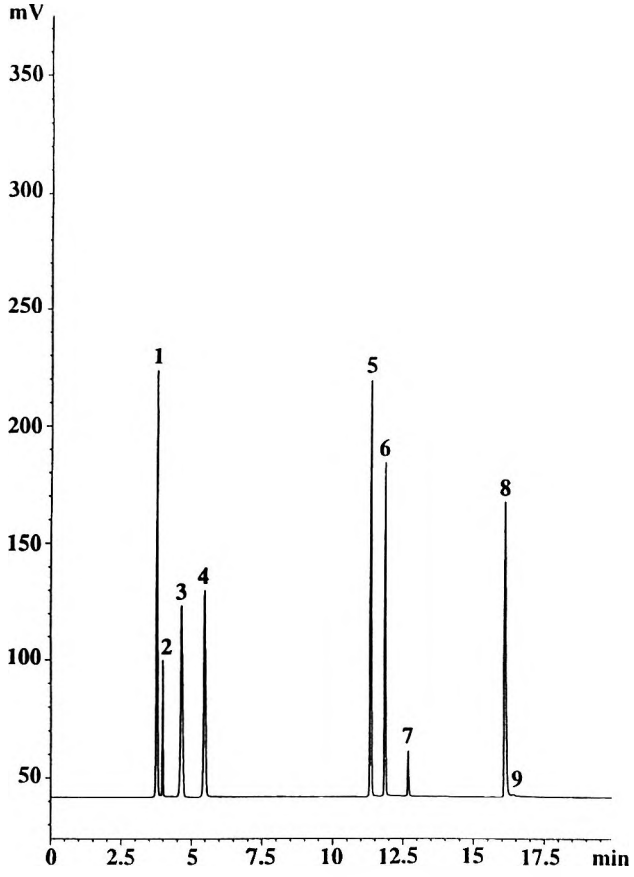

Figure 1. HPLC of neutral lipid standards. Peak Number 1: cholesterol oleate; 2: methyl oleate; 3: oleic acid; 4: triolein; 5: cholesterol; 6: 1,3-diolein; 7: 1,2-diolein; 8: 1-monoolein; 9: 2-monoolein.

dipalmitin (DP), monopalmitin (MP); ii) methyl stearate (MeS), tristearin (TS), distearin (DS), monostearin (MS); iii) methyl oleate (MeO), triolein (TO), diolein (DO), monoolein (MO); iv) methyl linoleate (MeL), trilinolein (TL), dilinolein (DL), monolinolein (ML). Each lipid component was 25 wt% of the mixture. Stearic acid (SA), palmitic acid (PA), triolein (TO), and methyl oleate (MeO) were obtained from Sigma Chemical Co. (St. Louis, MO). Oleic acid was obtained from Applied Science (State College, PA). Linoleic acid was obtained from Nippon Oil and Fats Co. (Amagasaki, Japan). All solvents used were HPLC grade; methyl t-butyl ether (MTBE) was obtained from J.T. Baker

Table 1

HPLC Solvent Gradient Program

Time (min)	%A[a]	%B[b]
0	100	0
5	100	0
15	20	80
17	20	80
17.1	100	0
27	100	0

[a] Solvent A = hexane + 0.4% acetic acid.
[b] Solvent B = methyl t-butyl ether + 0.4% acetic acid.

Inc. (Phillipsburg, NJ), and hexane was obtained from Burdick and Jackson (Muskegon, MI). Glacial acetic acid was analytical reagent grade obtained from Mallinkrodt (Paris, KY). Solvents used to make up HPLC gradient were degassed by helium sparge prior to chromatography.

HPLC

Separations were made on a Phenomenex (Torrance, CA) cyanopropyl (CN) column (250 x 4.6 mm i.d.) with an accompanying guard column (30x4.6 mm i.d.) of the same phase and a flow rate of 1.0 mL/min. A detailed elution scheme is given in Table 1. HPLC was conducted using a Hewlett Packard (HP) (Wilmington, DE) 1050 series liquid chromatograph with solvent cabinet, autosampler, and quaternary pump modules. A Varex (Burtonsville, MD) model IIA ELSD was used for detection. Program control, data acquisition, and analysis were carried out using H/P Chem Station software.

RESULTS AND DISCUSSION

The separation of free fatty acid (FFA) and neutral lipid standards obtained on the CN column using a mobile phase gradient of MTBE, hexane, and acetic acid is shown in Fig. 1. Separation of these compounds was initially attempted using the solvent gradient program reported by Al-Hamdy,[4] but there was inadequate resolution of peaks 1 through 4 under these conditions. We found that by decreasing the initial solvent gradient polarity profile and by

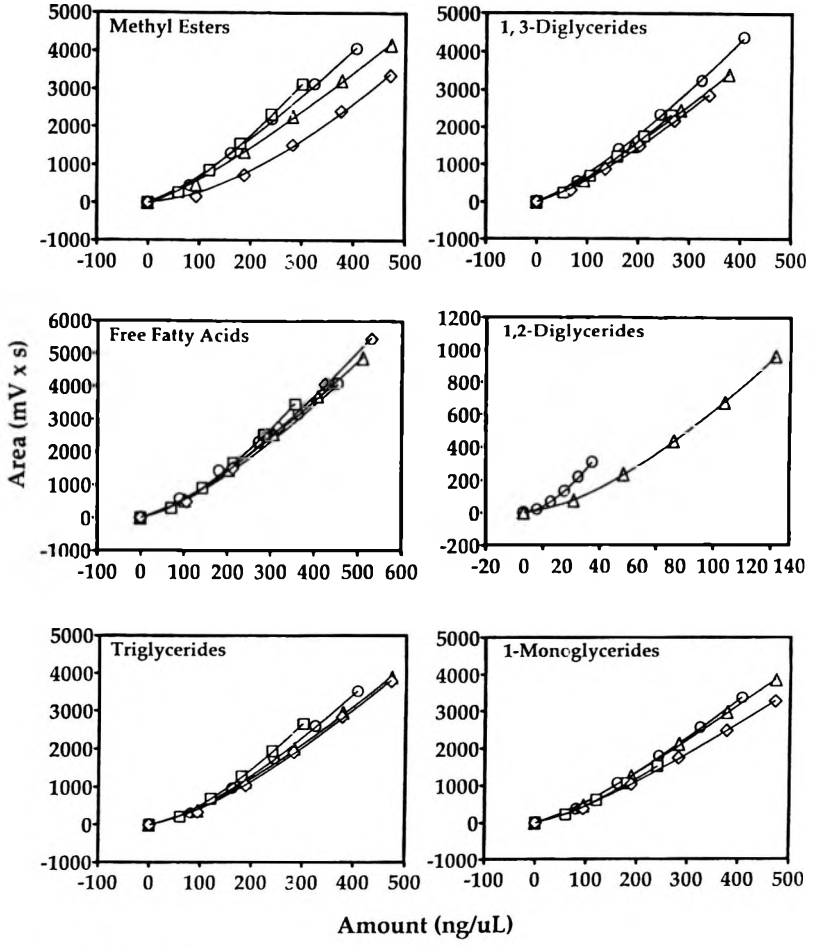

Figure 2. HPLC calibration curves of neutral lipids.

addition of acid modifier (acetic acid, 0.4%) to the mobile phase that a complete baseline separation was obtained that allowed for improved opportunities for sample analysis, automation, and quantitation (Table 1). Complete separation of all lipid classes studied was obtained within 17 minutes with an additional 10 minutes required for reequilibration of the column. Moreover, under the conditions used, there also appeared to be resolution of the 1 and 2-monoglyceride isomers.

Table 2

HPLC Retention Times of Neutral Lipid Standards[a]

	Lipid Class					
Acyl Group	**Methyl Ester**	**Free Fatty Acid**	**Triglyceride**	**1,3 Diglyceride**	**1,2 Diglyceride**	**Monoglyceride**
C16:0	3.91;[0.12]	4.54;[0.16]	5.44;[0.34]	11.88;[0.19]	12.69;[0.11]	15.76;[0.20]
C18:0	3.87;[0.04]	4.49;[0.11]	5.32;[0.10]	11.84;[0.07]	12.67;[0.04]	16.04;[0.13]
C18:1	4.02;[0.04]	4.77;[0.58]	5.70;[0.16]	11.96;[0.07]	----	15.75;[0.14]
C18:2	4.11;[0.06]	4.87;[0.23]	6.42;[0.36]	12.28;[0.12]	----	16.25;[0.30]

[a] Values shown are average retention times (n=15) in minutes, bracketed values relative standard deviation.

The sensitivity of an ELSD is controlled by a number of factors. Accordingly, optimization of the detector conditions was determined by repeatedly chromatographing a solution of methyl palmitate, palmitic acid, and tripalmitin at different evaporator tube temperatures (37-60°C) and nebulizer gas flows (0.8-2.35 L/min). The CN column was used with an isocratic solvent system of hexane + 0.4% acetic acid at a flow of 1.0 mL/min. From these runs the optimum detector conditions were determined to be 40°C at a nitrogen flow of 1.5 L/min.

Several papers have appeared on the principles, operation, and factors which significantly control detector response.[8,9] Previous work has shown detector response to be exponential.[10,11] In this work, linearity of detector response versus acyl moiety was determined by constructing calibration curves for all neutral lipid and FFA standards (see Figure 2). Five levels of dilution were prepared in hexane [95:5;hexane:MTBE + 0.4% acetic acid was used to facilitate dissolution of C16:0 and C18:0 series] for each set of lipid standards. Each level was chromatographed three times, sample size 20 μL per injection. The retention time variations for each lipid class studied are listed in Table 2. The data were analyzed using the HPLC data system. In all cases a power function relationship was confirmed with regression coefficients between 0.997 to 1.000.

The HPLC conditions developed for separation of lipid standards were used for the analyses of a series of transesterified triglycerides. The series of fatty acid alkyl esters was prepared by the lipase-catalyzed transesterification of rapeseed and soybean oils, tallow, and recycled greases for their prospective use

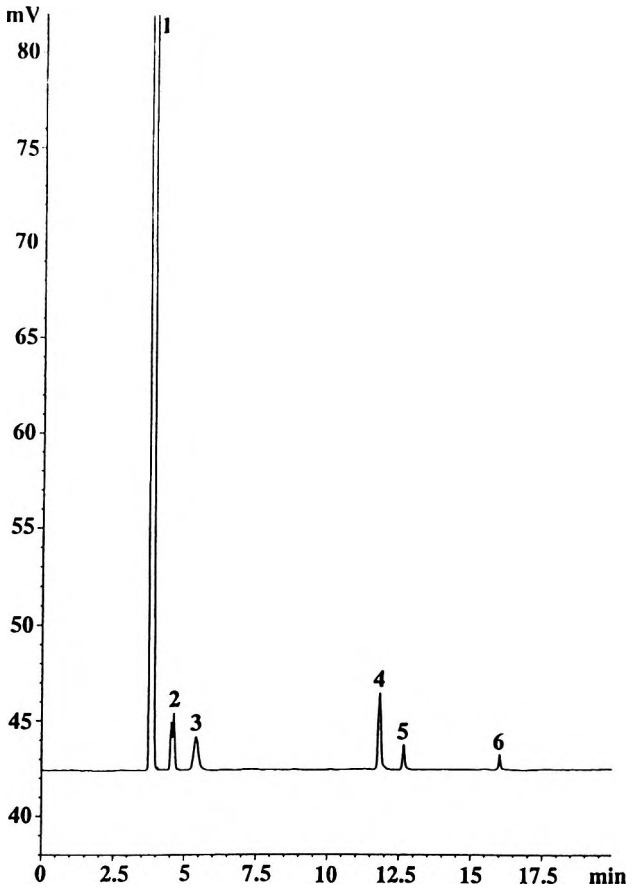

Figure 3. HPLC of tallow transesterified with isopropanol. Peak Number 1: isopropyl esters; 2: free fatty acids; 3: triglycerides; 4: 1,3-diglycerides; 5: 1,2-diglycerides; 6: 1-monoglycerides.

as biofuel additives.[12] A typical separation obtained for an alkyl ester mixture is shown in Figure 3. Because natural oils and fats are mixed triglycerides, their alkyl ester derivatives are obtained as a mixture of fatty acid esters that vary in chain length and degree of unsaturation. This can result in the splitting of an HPLC peak for a lipid class as exemplified in the free fatty acids, peak 2, in Figure 3. The quantitative results obtained in the analyses of this ester series are listed in Table 3. The first four entries in Table 3 show that the method is suitable for detecting low levels of free fatty acids and diglycerides in intact oils

Table 3

HPLC Composition of Transesterified Fats and Oils

Lipid Sample	Lipid Class[a] Alkyl Ester	Free Fatty Acid	Triglyceride	1,3 Diglyceride	1,2 Diglyceride	Monoglyceride
rapeseed oil	0.41	0.16	98.64	1.39	---	---
soybean oil	---	---	98.34	---	1.66	---
tallow	---	---	100	---	---	---
grease	---	42.24	47.6	6.99	2.52	0.65
ethyl rapeseed	97.04	2.96	---	---	---	---
ethyl soyate	95.48	3.06	---	0.89	0.34	0.22
ethyl tallowate	100	---	---	---	---	---
isopropyl tallowate	93.52	2.46	1.8	1.36	0.47	0.38
2-butyl tallowate	81.15	1.51	17.34	---	---	---
2-butyl grease	97.32	1.39	---	0.44	---	0.85
isobutyl grease	73.17	5.87	20.29	---	---	0.67

[a] Values given are expressed as wt% of lipid class in sample.

and fats, as well as the broad range of lipid classes that may be encountered in the analyses of recycled greases. The remaining entries in Table 3 give the results obtained for their ethyl, isopropyl and butyl ester derivatives. The data show that this HPLC method was effective for monitoring these lipase-catalyzed transesterifications in that it was effective in detecting minor amounts of unreacted glycerides and fatty acids in the transesterified mixtures. This is important if these esters are to meet the specifications outlined for prospective biofuels. The method enabled us to optimize the conditions for conversion of oils, fats, and greases to alkyl esters using the lipase-catalyzed procedure.

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[†] Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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DETERMINATION OF N^G-MONOMETHYL-L-ARGININE IN HUMAN AND DOG SERUM USING PRE-COLUMN O-PHTHALDIALDEHYDE DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

N^G-monomethyl-L-arginine (NMA) is a selective inhibitor of the enzyme nitric oxide synthase (NOS), which converts arginine to nitric oxide and citrulline. NMA may prove useful in the treatment of autoimmune and inflammatory disorders, as well as in preventing the cardiovascular effects associated with endotoxins and cytokines. An automated high performance liquid chromatographic method for determination of NMA in human serum samples was developed using pre-column derivitization with o-phthaldialdehyde and mercaptoethanol. NMA was extracted from serum using 5-sulfosalicylic acid and methanol, then injected into the HPLC system for on-line derivitization. A two mobile phase gradient (A: 70:30 methanol-water containing 0.01M K_2HPO_4, pH 7.9; B: water containing 0.01M K_2HPO_4, pH 6.9) was employed with 70:30 (A:B) for 15 min, 10:90 through 21 min, and 70:30 through 25 min. A Beckman ODS 3 μm 75 x 3.6 mm column was used with a

Hitachi L-6200A pump set at a flow rate of 1.8 mL/min and fluorescence detection at 340 nm excitation and 450 nm emission. The sensitivity of the assay was <50 ng/mL of plasma and the assay was linear over the range 0.05-500 μg/mL. The method was sensitive, reproducible, and specific for NMA.

INTRODUCTION

N^G-monomethyl-L-arginine (NMA) is a selective inhibitor of the enzyme nitric oxide synthase (NOS), which converts arginine to nitric oxide (NO) and citrulline.[1,2] NO formation is involved in the cardiovascular effects associated with endotoxins and cytokines.[3,4] Septic shock, caused by microbial endotoxins, is characterized by cardiovascular collapse and multiple metabolic derangements. A number of cytokines appear to be involved as intermediates in these endotoxin effects.[3] NO formation appears to be involved in the mechanism, as NO is a potent hypotensive, i.e., an endothelium-derived relaxing factor.[3]

NMA has been studied in experimental animals and humans to determine whether it can be used to prevent the hypotensive effects of cytokines and endotoxins.[3,5-7] NMA, administered to patients with septic shock, largely refractory to epinephrine administration, increased blood pressure.[7] Similarly, NMA increased blood pressure in renal cell carcinoma patients treated with the cytokine interleukin-2. Interleukin-2 treatment, which is among the only effective treatments for this form of carcinoma,[8] is limited by hypotensive effects,[9] which NMA may reverse.[3] Thus, NMA and other NOS inhibitors may have a role in the treatment of autoimmune and inflammatory disorders, as well as in preventing the cardiovascular effects associated with endotoxins and cytokines.

Chromatographic methods were described for isolation of NMA in biological substrates.[10,11] Precolumn derivitization with o-phthaldialdehyde and mercaptans and reverse phase HPLC methods were described for separation of methylated and nonmethylated amino acids, but these methods were not specific for NMA.[12,13]

To date, no sensitive method for the quantitative determination of NMA in human serum samples was available. An automated HPLC method involving precolumn derivatization was developed for determination of NMA in human and canine serum.

MATERIALS

N^G-monomethyl-L-arginine (NMA) standard, 5-sulfosalicylic acid, o-phthaldialdehyde, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO). Methanol, HPLC Grade, was purchased from Baxter Scientific (Deerfield, IL) and potassium phosphate, dibasic, ACS Grade, from Aldrich Chemical Company (Milwaukee, WI). Drug-free human serum was purchased from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD) and drug-free canine serum from Cocalico Biolcgicals, Inc. (Reamstown, PA).

The HPLC system consisted of a Hitachi AS 4000 Intelligent Autosampler, a Hitachi L-6200A Intelligent Pump, and a Waters Fluorescence Model 470 detector. All amino acids were separated using a Beckman Ultrasphere C18, 75 x 4.6 mm stainless steel column containing 3 μm ODS packing material.

METHODS

Standards

A stock NMA 1000 μg/mL solution was prepared by weighing 10.0 mg of NMA into a 10 mL volumetric flask and adding methanol to volume. A 100 μg/mL solution was prepared by transferring 1 mL of the first stock solution into a 10 mL volumetric flask and diluting to volume with methanol. Serum standards were prepared by adding stock NMA solutions to 5 mL of human or canine serum to give concentrations of 0.05 to 500 μg/mL.

Serum (standards, quality controls, and samples from dosed subjects) was vortexed vigorously before use. A 50 μL sample was aliquotted into a 1.5 mL polypropylene microcentrifuge tube to which 20 μL of 3% 5-sulfosalicylic acid solution and 250 μL methanol were added. The mixture was vortexed vigorously and then centrifuged at 4000 rpm for 10 min. The resulting supernatant was transferred into 200 μL limited-insert HPLC vials for injection into the system.

The derivitization solution was made by aliquoting 5 mL of o-phthaldialdehyde and 200 μL mercaptoethanol into a 100 mL volumetric flask and diluting to volume with 0.01M dibasic potassium phosphate buffer at pH 9.2. A 100 μL sample of the derivatization solution and a 20 μL sample of

serum supernatant were aspirated and dispensed into a reaction vial. The resulting solution was mixed by repetitive aspiration and dispensing for a reaction time of 3 min. Then 25 μL of the resulting solution was injected into the HPLC system and chromatographed according to the conditions below.

Mobile Phase

Mobile phase A was made of 70% methanol and 30% 0.01M dibasic potassium phosphate buffer, with the pH adjusted to 7.9 with phosphoric acid. Mobile phase B was 0.01M dibasic potassium phosphate buffer, with the pH adjusted to 6.9 with phosphoric acid. The mobile phase was passed through a 0.45 μm nylon membrane filter and degassed prior to use.

HPLC

The flow rate was maintained at 1.8 mL/min and the mobile phase gradient was 70:30 A:B from time 0 through 15 minutes, 10:90 from 16 through 21 minutes, and 70:30 from 22 through 25 minutes. The fluorescence detector was set at 340 nm excitation and 450 nm emission.

The correlation coefficient from least-squares regression calibration curves was constructed by plotting the standard concentration versus the corresponding peak area. The NMA concentrations of quality controls and unknown serum samples were calculated using the regression equation. Intraday accuracy (observed concentration/nominal concentration x 100) and precision (coefficient of variation) were determined by analyzing five samples of three different concentrations on the same day. Interday precision and accuracy were determined by analyzing duplicate samples of three different concentrations over five days.

RESULTS

Under the chromatographic conditions specified, the retention time of NMA in serum was approximately 9 min and there were no interfering peaks. Typical chromatograms in drug-free human serum, spiked human serum (25 μg/mL), and serum obtained from a patient 15 min after intravenous administration of NMA at 4 mg/kg, are shown in Figures 1A, 1B, and 1C, respectively. The same NMA peak is seen in the latter two figures, with no corresponding peak in the untreated sample.

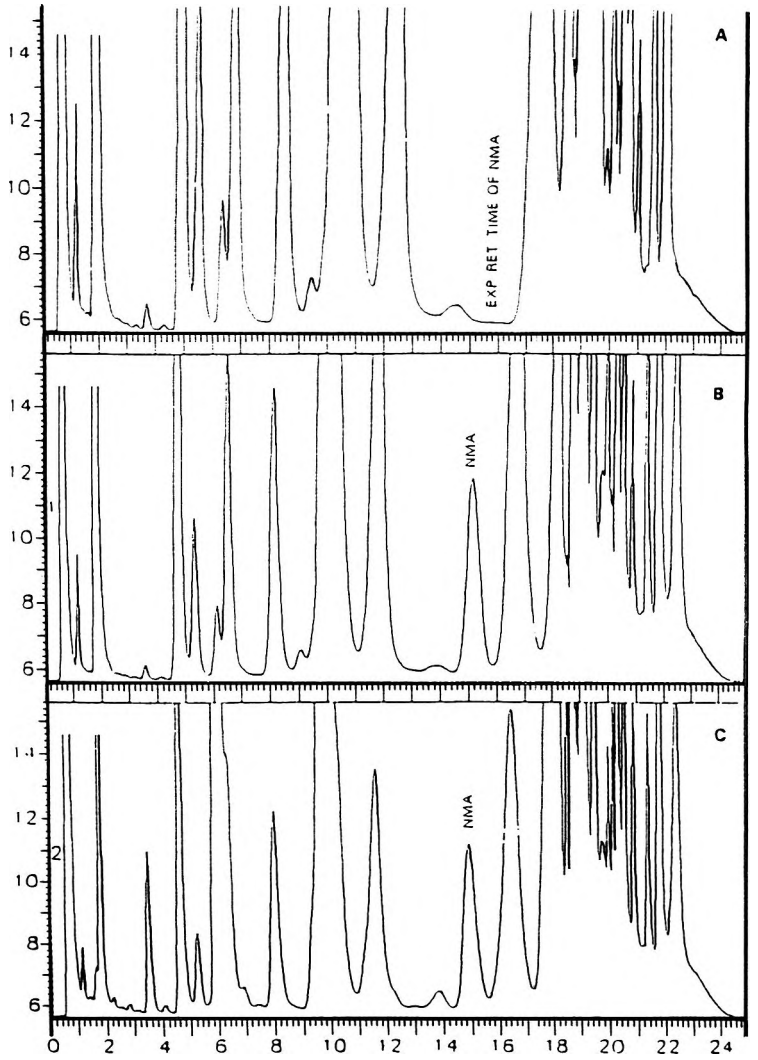

Figure 1. Representative chromatograms in (A) control human serum, (B) human serum spiked with NMA at 25 μg/ml, and (C) human serum obtained 15 min after intravenous administration of 4 mg/kg.

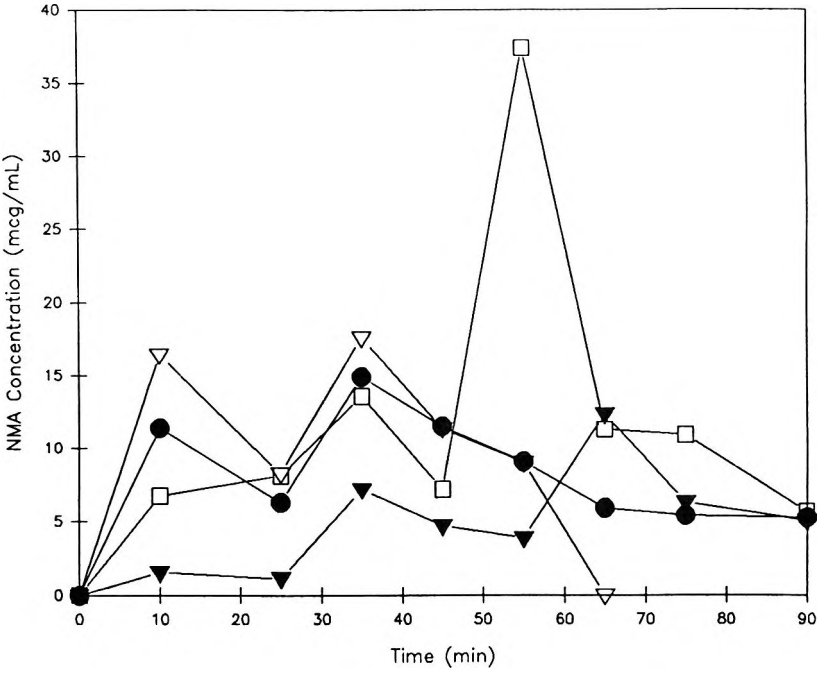

Figure 2. Concentrations of NMA determined over time in human serum samples from four subjects treated intravenously with 4 mg/kg.

Table 1

Intraday and Interday Variability in the NMA Assay

Nominal Concentration (μg/mL)	Intraday		Interday	
	Accuracy	Precision	Accuracy	Precision
1.0	95.4%	1.6%	100.0%	7.1%
10.0	110.1%	14.9%	106.0%	9.1%
100.0	109.9%	5.6%	111.8%	4.7%

In addition, no interfering peaks were seen in any other patient samples examined in this study. Canine serum showed the same pattern, with no interfering peaks and a retention time of approximately 9 min for NMA (data not shown).

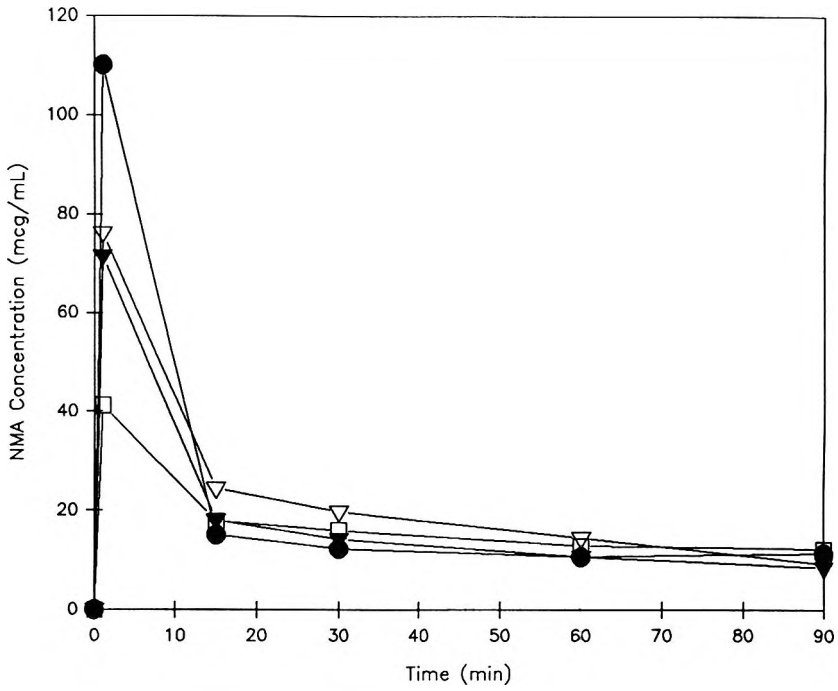

Figure 3. Concentrations of NMA determined over time in canine serum samples from four subjects administered 20 mg/kg by intravenous bolus.

The quantitative NMA concentration range was 0.05-500 μg/mL in serum. The correlation coefficient from least-squares regression calibration curves were typically >0.985. The accuracy and precision of the method were determined for intraday and interday variability (Table 1). The method was used to analyze serum samples from human and canine subjects enrolled in clinical and preclinical studies of NMA. Data from human subjects who received NMA intravenously at 4 mg/kg over 30 min for three doses are shown in Figure 2. Data from canine subjects who were administered a 20 mg/kg intravenous bolus dose of NMA over 30 seconds are shown in Figure 3.

DISCUSSION

Reverse phase HPLC is a powerful method for assaying amino acids in biological fluids. Pre-column derivitization methods were previously developed

for determination of amino acids, with o-phthaldialdehyde offering the highest sensitivity.[12,13] Reaction of amino acids with o-phthaldialdehyde and mercaptans produces thio-substituted isoindoles, which are highly fluorescent products.[12,13]

The adducts formed are unstable, which can limit the reproducibility and quantitative ability of assays based on such derivitization.

In this study, online automated derivitization was used to optimize the derivitization time and enhance the capacity of the method. The method could be used to analyze 25-50 samples in a single run.

The manual serum extraction procedure applied prior to automated HPLC was very quick. The method was sensitive, accurate, and specific for NMA in serum.

The limit of quantitation (0.05 μg/mL) was sufficient for the determination of serum NMA levels in humans and dogs up to 24 hours after dosing. This method is suitable for use in therapeutic drug monitoring of NMA in clinical trials.

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ASSAY OF FLURBIPROFEN IN RAT PLASMA USING HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the determination of flurbiprofen in rat plasma. The method employs a smaller sample volume (0.05 mL) and involved deproteinization of the biological sample with 2.5 volumes of acetonitrile for the determination of flurbiprofen. Ten microliters of the supernatant was injected onto a C_{18} reverse phase column. The mobile phase employed was acetonitrile-water-phosphoric acid (600:400:0.5, v/v/v). The flow rate was 1.5 mL/min. The column effluent was monitored by fluorescence detection at excitation wavelength of 250 nm and emission wavelength of 285 nm. The retention time was 3.4 min. The detection limit in rat plasma was 50 ng/mL. The mean percentage recovery of the drug in the concentration range of 0.05-5 μg/mL was 95.14% while the mean of the inter-day coefficient of variation of the same concentration range was 1.37%. The method was simple, rapid and accurate for quantitation of flurbiprofen in rat plasma.

INTRODUCTION

Flurbiprofen, dl-2-(2-fluoro-4-biphenylyl) propionic acid, is a potent nonsteroidal anti-inflammatory drug used for the treatment of rheumatoid arthritis[1] and its related conditions.

Gas chromatographic[2] and high performance liquid chromatographic[3-7] methods have been developed for the quantitation of flurbiprofen. The previously developed gas chromatographic method required time-consuming TLC separation followed by derivatization prior to analysis.[2] In the reported HPLC methods, flurbiprofen in human plasma, dog serum, and urine was determined. However, one of the previous methods required special apparatus for sample preparation[3] and in most of the previous work, a tedious extraction procedure[4-6] and more than 0.5 mL of serum[4] were employed. This paper describes HPLC methods with simple preparation procedures for the determination of flurbiprofen in plasma of small volume (0.05 mL) to study pharmacokinetics for flurbiprofen in rats.

MATERIALS AND METHODS

Materials

Flurbiprofen was kindly supplied by Samil Pharm. Co. (Seoul, Korea). HPLC grade acetonitrile was purchased from Merck Co.(Darmstadt, Germany) Phosphoric acid was received from Kokusan Chemical Works Ltd. (Tokyo, Japan). Water was distilled, deionized and filtered in house.

Preparation of Standard Solutions

Stock solution of flurbiprofen was dissolved in methanol(1 mg/mL). Standard solutions of flurbiprofen in water or rat plasma were prepared by spiking the appropriate volume(less than 10 μL per mL) of variously diluted stock solutions giving final concentrations of 0.05, 0.1, 0.5, 1, 2 or 5 μg/mL.

Recoveries from plasma were calculated by dividing the peak heights of the drug in rat plasma by those in water. Response factors were calculated by dividing the peak height of the drug by their concentrations (μg/mL).

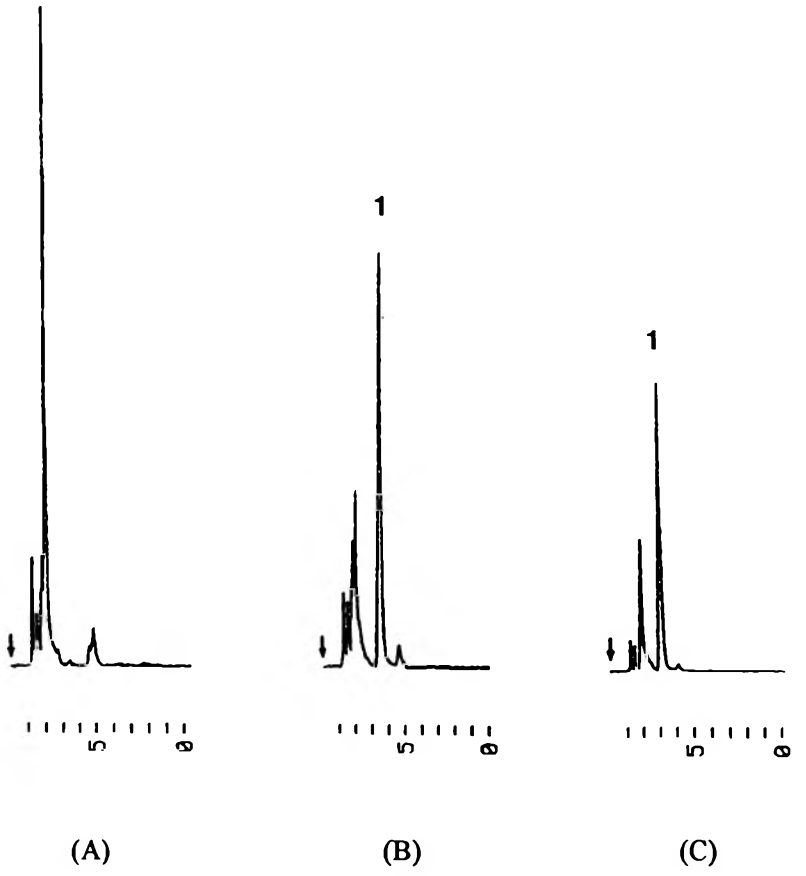

Figure 1. Chromatograms of (A) drug-free rat plasma, (B) rat plasma spiked with 0.5 μg/mL of flurbiprofen, (C) plasma obtained from a rat 480 min after intravenous administration of flurbiprofen at 2.5 mg/kg. Peak 1, flurbiprofen (3.4 min).

Sample Preparation

To 50 μL of rat plasma, 125 μL of acetonitrile were added for the deproteinization of the samples. After vortex mixing and centrifugation at 9000 *g* for 10 min, 10 μL of the supernatant were injected directly onto the HPLC column.

Table 1

Recoveries at Various Concentrations of Flurbiprofen in Rat Plasma

Concentration (μg/mL)	Response Factor[a] Mean ± S.D. (n=5)	Recovery (%)
0.05	1.359 ± 0.0092	98.39 ± 2.38
0.1	1.335 ± 0.0087	96.88 ± 3.38
0.5	1.205 ± 0.0100	91.63 ± 1.87
1	1.208 ± 0.0528	96.90 ± 3.78
2	1.187 ± 0.0168	94.08 ± 2.58
5	1.165 ± 0.0028	92.97 ± 2.87
Mean	1.243 ± 0.0822	95.14 ± 2.64

*peak height (10^{-1} mV)/concentration (μg/mL)

Table 2

Intra- and Inter-day C.V.s at Various Concentrations of Flurbiprofen in Rat Plasma

Concentration (μg/mL)	Intra-day C.V. (%)	Inter-day C.V. (%)
0.05	1.226	0.679
0.1	3.953	0.654
0.5	3.383	0.827
1	3.994	4.372
2	4.318	1.417
5	1.920	0.243
Mean	3.132	1.365

HPLC Apparatus

The HPLC system consisted of a Model 7725i injector (Rheodyne, Cotati, CA, USA), a Model 6200 intelligent pump (Hitachi, Tokyo, Japan), a guard column (C_{18}, 5 μm, Brownlee™, Alltech, Deerfield, IL, USA), a reverse phase

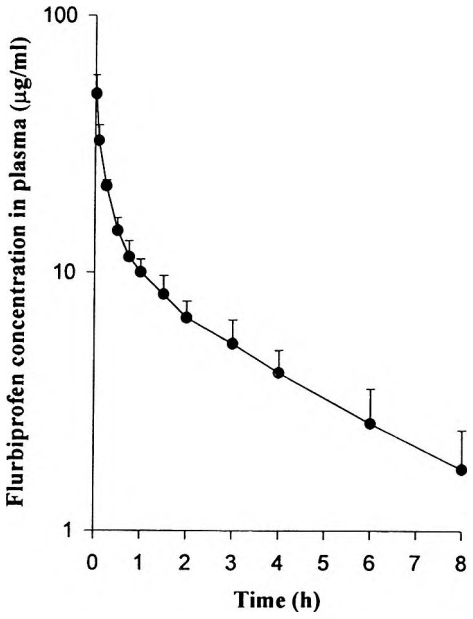

Figure 2. Plasma concentration-time profile of flurbiprofen after intravenous administration of flurbiprofen at 2.5 mg/kg to Sprague-Dawley rats. Bars represent standard deviations. Each point represents the mean S.D. (n=5).

column (C_{18}, 25 cm 4.6 cm I.D., particle size 5 μm, Hibar, Merck, Germany), a fluorescence spectrophotometer (F-1050, Hitachi, Tokyo, Japan) and a Model D-2520 integrator (Hitachi, Tokyo, Japan). The mobile phase, acetonitrile-water-phosphoric acid (600:400:0.5, v/v/v), was run at a flow rate of 1.5 mL/min and the column effluent was monitored by fluorescence detection at excitation wavelength of 250 nm and emission wavelength of 285 nm. The column temperature was ambient and the column back pressure was 120 kg/cm^2.

RESULTS AND DISCUSSION

Figure 1 shows typical chromatograms of drug-free rat plasma(A), drug standards in rat plasma(B) and plasma(C) collected at 8 h after intravenous administration of 2.5 mg/kg of flurbiprofen to a rat. No interferences from endogenous substances were observed in any of the biological samples. The

retention time for flurbiprofen was 3.4 min. The peaks were sharp and symmetrical, thus making the peak quantitation highly reliable. The detection limit for flurbiprofen in rat plasma was 50 ng/mL (Table 1), based on a signal-to-noise ratio of 3.0. The mean intraday coefficient of variation (C.V.s) of flurbiprofen in rat plasma was 3.13% (Table 2). The mean interday C.V.s for the analysis of the same samples on three days was 1.37% (Table 2). Mean percent recoveries of spiked flurbiprofen from plasma was 95.14% (Table 1).

Flurbiprofen was administered intravenously to 5 rats at the dosage of 2.5 mg/kg. Blood samples were collected from the femoral artery after 0.0167, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 8 hr. Fifty microliters of plasma sample were stored in a freezer prior to the HPLC assay. The mean plasma concentration-time profile of flurbiprofen is shown in Figure 2. The mean terminal half-life, total body clearance, apparent volume of distribution at the steady state and mean residence time of flurbiprofen was 2.78 hr, 47.94 mL/hr/kg, 156.78 mL/kg and 3.40 hr, respectively.

It can be seen that this simple and reproducible HPLC method has enough sensitivity for in vivo studies to evaluate flurbiprofen pharmacokinetics.

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IMPROVED ASSAY FOR SERUM AMPHOTERICIN-B BY FAST HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A selective and sensitive method for the determination of amphotericin B in dog serum, after deproteinization, using high performance liquid chromatography HPLC with UV detection at 382 nm was developed. After protein precipitation with methanol, 5 µl of the supernatant was injected onto a reversed phase C_{18} column (1.5µm, 33 x 4.6 mm ID), with a mobile phase composed of 0.05 M sodium acetate - acetonitrile - methanol (40:30:30, v/v). The analytical recovery of amphotericin B in serum was 92.30%. The linearity of the assay method was verified up to 15 µg/mL of amphotericin B. The assay was reproducible with satisfactory intra-day and inter-day coefficients of variations (CV < 3%). The sensitivity of the method was ≤0.625 ng of amphotericin B for serum. The method was successfully employed for the pharmacokinetic analysis of amphotericin B in dogs following intravenous administration of liposomal amphotericin B (AmBisome).

INTRODUCTION

Amphotericin B, a macrocyclic polyene antibiotic, has a broad spectrum of action against fungal (*Aspergillus, Candida, Cryptococcus, Blastomyces, Histoplasma*) and protozoan (*Leishmania*) pathogens.[1] This drug binds to ergosterol, the principal sterol in the membrane of susceptible cells, causing impairment of membrane barrier function. Administration of conventional colloidal dispersion in sodium deoxycholate (Fungizone) is associated with severe side effects in man and animals. Liposomal preparations of Amphotericin B (AmBisome) are much better tolerated and much higher doses can be administered without toxic reactions.[2]

High efficacy of liposomal amphotericin B against *Leishmania infantum*, the agent of human and canine visceral leishmanians, was shown in experimental[3] and clinical[4] studies. Recently, the role of this drug for the treatment of the disease in dogs is being investigated by dose-searching clinical trials.[5] In this animal, however, the pharmacokinetic behaviour of liposomal amphotericin B needs to be elucidated.

Several high performance liquid chromatographic (HPLC) assays have been reported recently, which, offer faster and more accurate and reproducible alternatives to bioassays for both pharmacokinetic studies and routine clinical use of amphotericin B.[6-16] HPLC offers improved sensitivity and specificity and is easier to standardise than bioassays are.[1] The chromatographic methods reported recently in the literature use conventional and short columns (30 - 60 mm length),[11,13] several of these also demonstrate a good sensitivity,[14] but use large volume of sample (50 - 150 µL). Repeated sampling of blood from small animals requires small amounts of plasma to avoid volume depletion. A convenient, sensitive method for measurement of AmB in small amounts of serum was needed.

Recently, little columns packed with non porous silica microspheres with diameters of 1.5 µm became available. These columns achieve better resolution and faster separation than that of conventional columns and only a small volume of sample is needed.

In this study, we have developed a sensitive, specific, accurate, and reproducible analytical method for the determination of AmB concentrations in serum samples. Using isocratic reversed phase method with 33 mm column (1.5 µm particle size) and adjustment of the mobile phase composition, it has been possible to separate and determine AmB in serum using a small volume of sample. This method has been successfully employed in pharmacokinetic studies using dog serum treated with liposomal AmB (AmBisome).

EXPERIMENTAL

Chemical and Reagents

Amphotericin B, reference standard was a gift from Bristol-Myers Squibb. (Sermoneta LT, Italy), sodium acetate, analytical reagent, was obtained from Carlo Erba (Milan, Italy), methanol and acetonitrile, HPLC-grade were obtained from Eurobase (Milan, Italy).

Chromatographic System

The HPLC system used for the analysis of all the samples and standards consisted of a Waters 600E HPLC pump (Waters, Milford, MA, USA), connected to autosampler system Waters 717 plus . Detection of amphotericin B was accomplished by Waters 994 photodiode array detector at a wavelength of 382 nm. Chromatograms, peak area, peak spectra, and peak purity parameters were obtained via Waters Millenium 2010 software.

Isocratic method of separation was achieved using MICRA NPS HPLC column, RP-C18 (1.5 µm, 33 x 4.6 mm ID), Micra Scientific (Northbrook, IL, USA). The mobile phase was 0.05 M sodium acetate - acetonitrile - methanol (40:30:30, v/v). The run time was 5 min with flow - rate of 0.7 mL/min.

Standard Solutions

Stock solutions of amphotericin B were prepared by dissolving amphotericin B authentic standard in DMSO (0.1 mg/mL). Serial dilutions of the stock solution were freshly prepared into appropriate concentrations using methanol. Standard curve samples of the sera were freshly prepared by spiking aliquots of the standard solutions of amphotericin B into the blank samples (200 µL of each serum sample). The concentration range of the final standard samples was 0.125 - 15.000 µg/mL of the supernatant.

Animal Treatment

Liposomal amphotericin B (AmBisome) was reconstituted in 5% dextrose and infused over 60 minutes via a peripheral vein of 4 dogs at the dose of 5 mg/kg bw. Blood was collected from a brachial vein at different time intervals

from 5 min. to 72 h after infusion. The blood was allowed to clot and the serum was separated by centrifugation at 1500 r.p.m. The samples were stored at -20°C pending assay.

Sample Preparation

Samples were deproteinized by adding 800μL of cold methanol to 200 μL of serum, were mixed by vortex-mixing and centrifuged at 5°C, 7000 r.p.m. for five minutes. The supernatant was filtrated through 0,45 μm Millipore filters. The 5 μL aliquots were injected into the column. The standards were assayed in the same manner.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, AmB was eluted approximately 2,5-3,0 min. Typical chromatograms of dog serum samples spiked with AmB and dog samples obtained after intravenous administration of AmBisome, are presented in Fig. 1. By minor adjustment of the composition of the mobile phase, it has been possible to determine amphotericin B with short retention time, which, permitted the analysis of a large number of samples obtained from pharmacokinetic studies.

The specificity of the method was assayed by comparing chromatograms of blank serum from dog. All chromatograms were free of interfering peaks. The Millenium PDA software determined the spectral homogeneity of the peak by comparing the peak apex spectrum against a spectrum of standard. Spectral analysis performed on the proposed AmB peak of methanolic standards, spiked sera and dog serum samples, obtained after AmBisome infusion, were essentially identical and exhibited spectral peaks normally associated with heptaenes (405, 382, 363, 344nm). This demonstrated that there was no interference from metabolites or endogenous substances. It also excluded the presence of the tetraene impurities of amphotericin B.

Recovery, Reproducibility, and Detection Limit

AmB was added to drug - free serum to provide concentrations of 0.25, 2.50 and 15.00 μg/mL. Recovery was determinated by replicate analysis (n=6) of the each of these serum pools. The peak areas obtained for AmB were compared with peak areas obtained by direct injection of working standard solution.

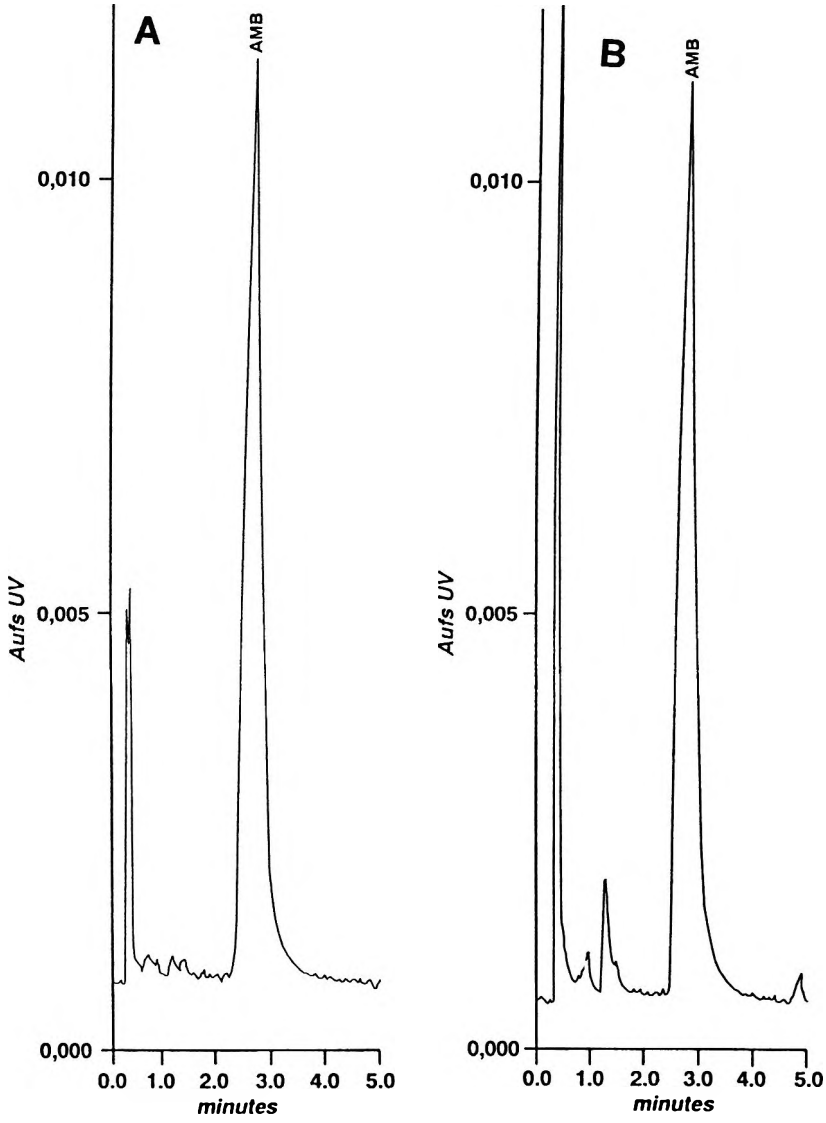

Figure 1. Typical chromatogram of amphotericin B in dog spiked serum at the final concentration of 10 µg/mL (A), and dog serum sample obtained after 60 min post infusion of AmBisome 5 mg/kg (B)

Analytical recoveries of added AmB from serum by the procedure of deproteinization (mean ± SD, n=6) were found to be 92.84% ± 2.37, 92.97% ± 1.79 and 91.11% ± 2.54.

The method was validated by performing replicate analysis (n=5) of pooled dog serum spiked with AmB in concentrations all over the studied range, on four separate days. Concentrations were determined using the respective standard curves prepared and checked on the day of the analysis. The results of the linear regression analysis showed that assay method had a verified linearity from 0.125 to 15.000 µg/mL of AMB with correlation coefficients of 0.9999 and errors of estimation ≤ 0.035. These levels were sufficiently high for the determination of AmB in serum of dog following intravenous administration of liposomal AmB. Y - intercept (0.029 ± 0.004) of calibration curves were calculated by standard statistical methods. Linear regression analysis was used to define accuracy by comparing the experimental values obtained for the AmB reference samples (y) with their theoretical values (x).

The precision and the overall accuracy of the method are presented in Table 1. The accuracy of the assay was defined as the mean of the absolute values of the percent difference of the determined concentrations from the nominal value and expressed as %RE. The deviations from theoretical values were ≤ 3% at all concentration levels assayed for each standard sample. The within - day precision, defined as the mean of the daily coefficients of variations at each concentration (n=5), ranged from 0.39% to 3.03%. The between - day precision, expressed as the coefficient of variations of the pooled four day data at each concentration (n=20), was in the range of 1.06% to 2.90%.

The detection limit of HPLC system was established by injecting decreasing amounts of spiked deproteinized serum onto the column. Limit of detection (LOD)was estimated directly by analysis of the method's peak to peak baseline noise and calculated using equation based on IUPAC model, proposed by Foley and Dorsey,[17] including standard deviation of blank signal and slope sensitivity. At a signal to noise ratio 3 : 1 it was 0.200 ng on column. Limit of quantitation (LOQ) was estabilished to be 0.625 ng injected onto the column, determined with acceptable accuracy and precision. This value, corresponded to a concentration of 0.125 µg/mL of the standard sample, included in the calibration curve as a lowest concentration level. The limit of detection can be affected by many variables, including the age and condition of the HPLC column and the variability associated with the sample preparation and dilution.

Table 1

Precision and Accuracy of the Assay Method for Determination of AmB

		Nominal Concentrations (μg/mL)						
		0.125	**0.250**	**0.500**	**1,000**	**5,000**	**10,000**	**15,000**
Day 1[1]	Mean	0.1287	0.2455	0.5116	1.0278	4.9487	9.9890	15.0219
	S D	0.0039	0.0048	0.0039	0.0040	0.1123	0.1408	0.1517
	C V (%)	3.03	2.00	1 00	0.39	2.27	1.41	1.01
	R E (%)	2.95	-1.80	2.32	2.78	-1.03	0.54	-0.15
Day 2[1]	Mean	0.1257	0.2472	0.5118	1.0240	4.9476	10.0106	15.0082
	S D	0.0027	0.0018	0.0029	0.0044	0.0831	0.1886	0.1591
	C V (%)	2.17	0.74	1.00	1.95	1.68	1.90	1.06
	R E (%)	0.56	-1.12	2.36	2.40	-1.05	0.11	0.05
Day 3[1]	Mean	0.1280	0.2456	0.5099	0.9940	5.0102	9.9438	15.0337
	S D	0.0028	0.0060	0.0029	0.0190	0.0912	0.1939	0.1308
	C V (%)	2.22	2.44	0.74	1.95	1.82	1.95	0.87
	R E (%)	2.40	-1.76	1.98	-0.90	0.20	-0.56	0.22
Day 4[1]	Mean	0.1277	0.2480	0.5126	1.0161	4.9401	9.9440	15.0210
	S D	0.0026	0.0032	0.0101	0.0040	0.0588	0.0967	0.1697
	C V (%)	2.07	2.49	2.59	0.39	1.19	0.98	1.14
	R E (%)	2.16	-0.80	2.52	1.61	-1.80	-0.56	0.14
Overall[2]	Mean	0.1275	0.2466	0.5115	1.0155	4.9616	9.9718	15.0212
	S D	0.0037	0.0055	0.0088	0.0115	0.0834	0.1881	0.1598
	C V (%)	2.90	2.23	1.72	1.62	1.72	1.90	1.06
	R E (%)	2.00	-1.36	-2.34	1.55	-0.77	-0.28	0.14

[1]n = 5 determinations.
[2]n = 20 determinations.
Precision expressed as %CV.
Accuracy expressed as %RE.

Application of the Assay Method

The analytical procedures described above have been successfully applied to determine AmB concentrations in samples collected over the period of 72 h from four dogs treated intravenously with liposomal amphotericin B (5 mg/kg

Table 2

Serum Amphotericin-B Concentrations of the Four Dogs Treated by Infusion of AmBisome (5 mg/kg)

Sample Time Post-Dose (Hours)	Concentration (μg/mL) Mean ± SD	Range
0.08	12.20 ± 15.40	3-22 - 29.99
0.33	17.58 ± 6.00	13.80 - 24.50
0.66	24.56 ± 6.43	17.24 - 29.34
1	37.81 ± 16.30	22.10 - 54.65
2	24.13 ± 13.01	9.69 - 34.95
4	19.14 ± 10.69	7.27 - 27.99
8	13.02 ± 7.09	5.23 - 19.10
12	9.79 ± 5.10	3.91 - 13.05
24	5.14 ± 2.71	2.10 - 7.28
48	1.52 ± 0.33	1.14 - 1.76
72	1.09 ± 0.16	1.07 - 1.10

body weight). Serum analysis (Table 2) showed that the concentration of the drug reached maximum value of 54.65 μg/mL 1h after the infusion, thereafter the concentration of drug decreased to the minimum values, but still present at a concentration above 1 μg/mL three days after the infusion.

CONCLUSION

Results, that have been obtained using reversed phase liquid chromatography, provides a fast method of determination and plasma-level monitoring of amphotericin B. High efficiency, short column containing small particle size (1.5μm), reduced analysis time, and solvent consumption and increased sensitivity of the method described, provided the high resolution separation and determination of amphotericin B, using little volume of sample. The lower detection limit was 0.125 μg/mL with 5 μL of sample, which, represents an improvement over the detection limits by previous methods. The sensitivity, specificity, accuracy, and reproducibility of this assay method permits fast analysis of large number of samples obtained from the pharmacokinetic studies.

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ANALYSIS OF DIURETICS IN URINE BY COLUMN-SWITCHING CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT

The potential of column-switching chromatography and fluorescence detection for the analysis of diuretics in urine is evaluated. Sample cleanup and chromatographic parameters have been optimized to achieve maximum sensitivity for the detection and quantification of some relevant diuretics. On the basis of these studies, an on-line procedure for the simultaneous determination of amiloride, furosemide, bumetanide and triamterene is presented. The linearity, precision, accuracy and sensitivity of the method are discussed. The utility of the described approach has been tested by analysing urine samples obtained after administration of bumetanide.

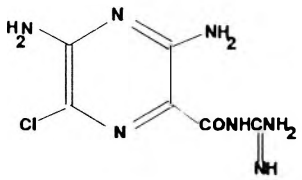
INTRODUCTION

The rapid and sensitive identification and quantification of diuretics in biological fluids are often required in therapeutic drug monitoring and in doping control tests. In this respect, Gas Chromatography (GC) coupled to Mass-Spectroscopy (MS) is the most reliable technique for diuretics caracterization.[1,2] However, owing to the time and cost involved when using the GC-MS approach, Liquid Chromatography (LC) is the method of choice for the analysis of diuretics.[3] Moreover, recent technological advances in LC have resulted in sophisticated systems which allow the detection of these drugs at ng/ml levels.[4] In contrast, the advances in the area of sample preparation (including cleanup and preconcentration of the analytes) have been more modest, and many analytical procedures still involve time-consuming manual methods. Whereas separation and identification of the interesting compounds can require a few minutes, sample preparation time can be one or even two orders of magnitude longer.[5] Therefore, the development of sample conditioning procedures that can reduce analysis times and improve sample throughput is an area of major interest.

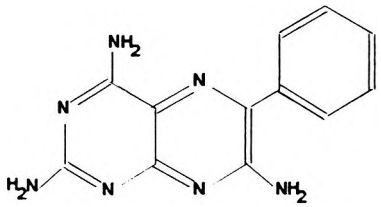
In the last years, column-switching chromatography has gained popularity in the context of sample preparation,[6] because switching devices allow the selective retention of the analytes in a primary column (or precolumn), whereas, matrix components are flushed-out; the enriched analytes are subsequently transferred to an analytical column, where they are separated and detected. Although, different configurations may be required depending on the polarity of the analyte, satisfactory recoveries can be obtained for most diuretics by using precolumns packed with apolar stationary phases (usually C_{18}).[7] Moreover, by selecting an appropriate switching device, additional dispersion of the analytes into the chromatographic system is minimized, so the sensitivity achieved is comparable to that obtained under a conventional approach. Successful applications of column-switching chromatography and UV detection to the analysis of diuretics have already been reported, the limits of detection being typically in the 0.002 - 2.0 μg/ml range.[7,8] Unfortunately, the analysis of these compounds by switching techniques is limited by the presence of apolar matrix components, which are also trapped in the precolumn. Therefore, more selective and sensitive detection may be required for the determination of some diuretics at therapeutical levels.[9]

This work was aimed to the evaluation of column-switching, in combination with fluorescence detection, for improving the selectivity and sensitivity in the analysis of diuretics. Experimental conditions have been optimized for the detection and identification of some native fluorescent diuretics (Figure 1).

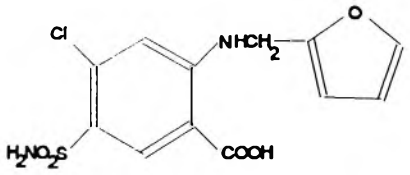
Amiloride



Triamterene



Furosemide



Bumetanide

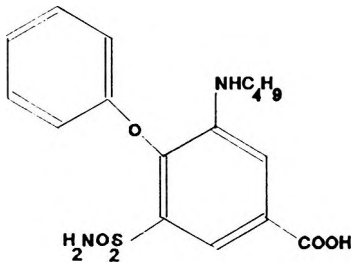


Figure 1. Chemical structures of the diuretics assayed.

A simple and rapid method for the simultaneous determination of amiloride, triamterene, furosemide and bumetanide is described. The usefulness of the described approach has been tested by determining bumetanide in real samples.

MATERIALS

Apparatus

The chromatographic system used consisted of two quaternary pumps (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 Series) with a sample loop injector of 100 μL, and a high pressure six-port valve (Rheodyne model 7000). For detection a fluorescence detector (Hewlett-Packard, 1046 Series) and a diode-array detector (Hewlett-Packard, 1040 series) were coupled in series. The detectors were linked to a data system (Hewlett-Packard, HPLC Chem. Station) for data acquisition and storage. The precolumn and the analytical column have been combined by means of a switching arrangement in a back-flush configuration. A schematic set-up has been published previously.[7] All assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical grade. Acetonitrile and methanol (Scharlau, Barcelona, Spain) were of HPLC grade. Water was distilled, deionized and filtered in 0.45 μm nylon membranes (Teknokroma, Barcelona, Spain). Amiloride hydrochloride was obtained from ICI-Pharma (Pontevedra, Spain), triamterene from Sigma (St. Louis, MO, USA), furosemide from Lasa (Barcelona, Spain) and bumetanide from Boehringer Ingelheim (Barcelona, Spain). Sodium dihydrogen phosphate monohydrate (Merk, Darmstadt, Germany), propylamine hydrochloride (Fluka, Busch, Switzerland) and phosphoric acid (Probus, Barcelona, Spain) were also used.

METHODS

Preparation of Solutions

Stock standard solutions of the diuretics (at a concentration of 2000 μg/mL for amiloride, furosemide and bumetanide, and at a concentration of 400 μg/mL for triamterene) were prepared by dissolving the pure compounds in methanol. Working solutions were prepared by dilution of the stock solutions with water. All solutions were stored in the dark at 2°C.

The phosphate buffer solutions were prepared by dissolving the appropriate amount of sodium dihydrogen phosphate monohydrate in 500 mL of water containing 0.7 mL of propylamine hydrochloride. The pH was then adjusted by adding the minimum amount of concentrated phosphoric acid.

Columns and Mobile-Phases

The precolumn (20 mm x 2.1 mm I. D.) was dry-packed with a Hypersil ODS-C_{18}, 30 μm stationary phase. A LiChrospher 100 RP 18, 125 mm x 4 mm I. D., 5 μm column (Merck, Darmstadt, Germany) or a Hypersil ODS-C_{18}, 250 mm x 4 mm I. D., 5 μm column (Hewlett-Packard) were used as an analytical column. A 0.05 M phosphate buffer of pH 3 was used as washing solvent to eliminate matrix components from the precolumn. Different phosphate buffer-acetonitrile mixtures (at a flow rate of 1.0 mL/min) were used as a mobile-phase; the ionic strength and pH of the buffers were varied from 0.001 to 0.1 M, and from 3.0 to 5.0, respectively. Solvents were filtered with 0.45 μm nylon membranes (Teknokroma) and degassed with helium before use.

Column-Switching Operation

At the beginning of each assay 5 μL of the samples were injected into the precolumn. Matrix components were flushed-out of the precolumn by pumping 0.05 M phosphate buffer (pH = 3) at a flow rate of 1.0 mL/min. Simultaneously, the analytical column was being reequilibrated with the mobile-phase. At t = 1.0 min, the switching valve was rotated, so the precolumn was incorporated into the flow-scheme of the analytical column. At t = 9.0 min, the valve was turned back to the original position to regenerate and requilibrate both, the precolumn and the analytical column. Rotation of the valve was manually performed.

Recovery Studies

Blank urine samples were spiked with the stock solutions of the diuretics, reproducing different concentrations in their respective therapeutical intervals.[10] The percentage of drug recovered was calculated by comparing the peak areas obtained for each compound in the spiked samples, with those obtained for direct injections of 5.0 μL of standards containing the same concentration of diuretic. Each concentration was assayed in triplicate.

Preparation of Standards for Calibration

Untreated urine (plasma in some instances) samples were spiked with the appropriate volumes of the stock solutions of the diuretics, placed in injection glass vials and processed as described above. Peak areas obtained for each compound were plotted versus analyte concentration, and the resulting calibration curves were used to calculate the concentration of the diuretics in unknown samples. Each concentration was assayed in triplicate.

Human Studies

Urinary excretion studies were performed with a human volunteer after a single dose administration of bumetanide (5.0 mg). Urine samples were collected at appropriate time intervals post-dose, and analyzed as described above. Each sample was assayed in triplicate.

RESULTS AND DISCUSSION

Chromatographic Separation and Detection of Diuretics

Initially, we investigated the chromatographic conditions necessary to achieve a satisfactory resolution of the compounds of interest by direct injection of aqueous standard solutions of the diuretics into the analytical column. Two different columns (LiChrospher 100 RP C_{18}, 125 mm x 4 mm I. D., 5 μm, and Hypersil ODS-C_{18}, 250 mm x 4 mm I. D., 5μm) in combination with different phosphate buffer/acetonitrile mixtures were tested. Best resolution of the diuretics assayed in a short time was obtained when using the LiChrospher column and a 0.05 M phosphate buffer of pH 3.0 as the aqueous component of the mobile-phase.

According to previous studies,[7] the precolumn was packed with a Hypersil C_{18}, 30 μm stationary phase for trapping the diuretics. In order to minimize base line drifts in the chromatograms, a 0.05 M phosphate buffer of pH 3 was also selected as washing solvent for flushing the precolumn during the cleanup stage. The fluorescence detector was programmed as listed in Table 1 (the UV signal was monitored at 254 nm). Table 1 summarizes the conditions finally selected for analysis of diuretics in urine.

Table 1

Time Schedule and Conditions Used in the Determination of Diuretics in Urine

Sample injection (t = 0 min)	5 μL of untreated urine			
Sample cleanup	precolumn: 20 mm x 2.1 mm I.D., packed with a Hypersil, ODS-C18, 30 μm phase washing solvent: 0.05 M phosphate buffer (pH = 3) at a flow-rate of 1.0 mL/min duration of the flushing: 1 min			
(valve rotation) (t = 1 min)				
Analytical separation	column: 125 mm x 4 mm I.D. Lichrospher RP 18, 5 μm mobile phase: 0.05 M phosphate buffer pH = 3)/acetonitrile, at a low-rate of 1.0 mL/min at 1 min: 100% phosphate buffer at 8 -m 9 min: 40% phosphate buffer/60% acetonitrile			
Detection wavelengths		$\lambda_{excitation}$ (nm)	$\lambda_{emission}$(nm)	
	amiloride:	286	418	(t = 0 - 4.9 min)
	triamterene:	365	440	(t = 4..9 - 6.0 min)
	furosemide:	233	389	(t = 6.0 - 8.0 min)
	bumetanide:	228	418	(t = 8.0 - 9.0 min)

(continued)

Table 1 (continued)

Time Schedule and Conditions Used in the Determination of Diuretics in Urine

End of the run (valve rotation) (t = 9.0 min)	
Recquilibration (t = 9.0 - 11.0 min)	precolumn and analytical column: 0.05 M phosphate buffer (pH = 3) at a flow-rate of 1.0 mL/min

Table 2

Analytical Data for Diuretics in Urine

Diuretic	Conc'n. Interval	Recovery* (n = 3)	Linearity (n = 10)		Intra-day Precision* (n = 8)	Inter-day Precision* (n = 6)
	(μg/mL)	(%)	A = a + bC	r	(%)	(%)
Amiloride	2.5 - 25.0	91 ± 1	a = 1.5 ± 0.3 b - 1.24 ± 0.01	0.9996	4	7
Triamterene	1.0 - 20.0	97 ± 3	a = -5 ± 5 b = 11.7 ± 0.2	0.998	4	5
Furosemide	5 - 100	93 ± 2	a = -2 ± 5 b = 1.66 ± 0.05	0.997	3	10
Bumetanide	0.10 - 3.50	106 ± 5	a = 0 ± 2 b = 12.5 ± 0.5	0.996	3	5

*Determined at half of concentration in tested range.

Analysis of Urine Samples

Selectivity

UV chromatograms showed that a vast majority of the urinary endogenous compounds were flushed-out from the precolumn with the first 1.0 mL fraction eluting from the precolumn. However, several apolar components can not be eliminated, even when flushing the precolumn with up to 10.0 mL of washing solvent. Phosphate buffers of different pH and water were also tested as washing solvents, but no significant improvements in selectivity were observed. In most instances, interferences due to the apolar matrix components can be avoided by optimizing the gradient elution profile. However, matrix components significantly increase background noise; in addition, recoveries for some diuretics are overestimated due to the contribution of the matrix to the analytical signal.[6]

As expected, fluorescence provided much better selectivity, even when flushing the precolumn with a volume of washing solvent as small as 0.25 mL. No significant differences in the fluorescence chromatograms were observed when flushing the precolumn with volumes of buffer in the 0.25 - 5.0 interval. However, UV signals showed that an important amount of matrix components was transferred to the analytical column when using volumes of phosphate buffer lower than 0.5 mL in the cleanup stage. In order to ensure an adequate performance of the analytical column, a volume of 1.0 mL was chosen as the best option for flushing the precolumn. In Figure 2 are shown the UV and fluorescence chromatograms obtained for blank urine under the conditions selected. This figure also shows a chromatogram obtained for urine spiked with a mixture of the diuretics assayed. As can be deduced from this figure, the analysis of interesting compounds can be performed with excellent selectivity when using fluorescence detection. On the other hand, no significant improvement in the signal-to-noise ratios was observed when volumes of urine in the 5 - 25 μL interval were processed. Since the injection of larger volumes would require longer cleanup periods to prevent deterioration of the analytical column, a sample volume of 5.0 μL was chosen as the best option for analysing urine samples.

Figure 2. **(right)** Chromatograms obtained for urine samples: (a) UV detection and blank urine, (b) fluorescence detection and blank urine and (c) fluorescence detection and urine spiked with a mixture of the diuretics assayed: amiloride (AML), 10.0 μg/mL; triamterene (TRN), 8.0 μg/mL; furosemide (FRS), 40 μg/mL; bumetanide (BMD), 0.4 μg/mL. For experimental details, see text.

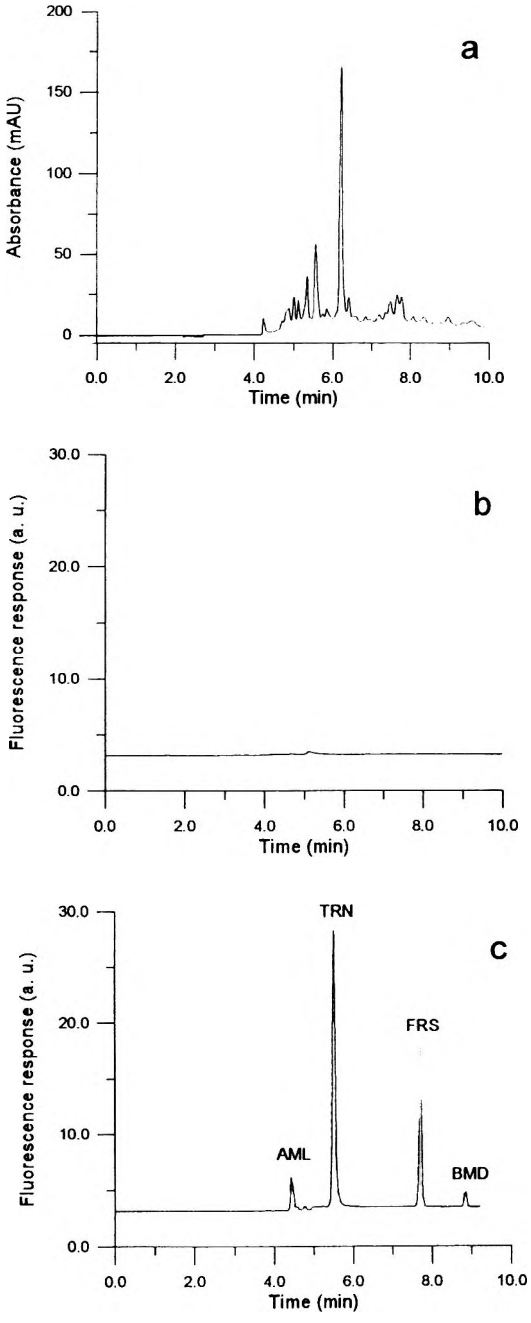

a
Absorbance (mAU)
200
150
100
50
0
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)
b
Fluorescence response (a. u.)
30.0
20.0
10.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)
c
Fluorescence response (a. u.)
TRN
FRS
AML
BMD
30.0
20.0
10.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Time (min)

Table 3

Accuracy for Diuretics in Urine

Diuretic	Sample Number	Added Concentration (μg/mL)	Determined Concentration (μg/mL)	E_r (%)
Amiloride	1	5.0	4.81 ± 0.05	- 4
		10.0	10 ± 1	0
		17.5	17 ± 2	- 3
	2	5.0	4.7 ± 0.4	- 6
		10.0	8.9 ± 0.3	- 11
		17.5	17 ± 2	- 3
	3	5.0	5.0 ± 0.4	0
		10.0	9.8 ± 0.1	- 2
		17.5	17 ± 2	+ 3
Triamterene	1	4.0	3.7 ± 0.3	- 8
		8.0	7.5 ± 0.5	- 6
		14.0	13 ± 1	- 7
	2	4.0	3.86 ± 0.12	- 3
		8.0	8.0 ± 0.1	0
		14.0	13.49 ± 0.06	- 4
	3	4.0	4.2 ± 0.2	+ 5
		8.0	8.5 ± 0.5	+ 6
		14.0	15.1 ± 0.5	+ 8
Furosemide	1	20.0	21.5 ± 0.2	+ 8
		40.0	41 ± 4	+ 3
		70.0	69.9 ± 0.4	- 0.1
	2	20.0	21 ± 2	+ 5
		40.0	41 ± 2	+ 3
		70.0	70 ± 5	0
	3	20.0	19.4 ± 0.1	- 3
		40.0	38.8 ± 0.8	- 3
		70.0	68 ± 3	- 3

Table 3 (continued)

Accuracy for Diuretics in Urine

Diuretic	Sample Number	Added Concentration (μg/mL)	Determined Concentration (μg/mL)	E_r (%)
		0.2	0.218 ± 0.012	+ 9
	1	0.4	0.41 ± 0.02	+ 3
		0.7	0.66 ± 0.02	- 6
		0.2	0.198 ± 0.001	- 1
Bumetanide	2	0.4	0.37 ± 0.01	- 8
		0.7	0.6466 ± 0.0005	- 8
		0.2	0.20 ± 0.01	0
	3	0.4	0.36 ± 0.02	- 10
		0.7	0.67 ± 0.02	- 4

n = 3

Analytical Parameters

The reliability of the described assay was tested by determining diuretics in urine in their respective therapeutical intervals. Table 2 summarizes relevant analytical data of the method. Satisfactory recoveries of the analytes were observed, which is in agreement with previously published results.[7] Linearity was also suitable over the studied intervals. In all instances, the intra-day reproducibility was of about 3 - 4%, whereas the inter-day precision ranged from 5% to 10%. These values can be considered acceptable and comparable to those of most reported LC assays.[11,12]

Accuracy of the method was tested by determining the concentration of the analytes in different spiked urine samples. The results obtained are shown in Table 3. In most instances, the method provided concentrations close to the actual concentrations, the relative errors ranging from -11% (for amiloride in sample 2 at a concentration of 10.0 μg/mL) to +9% (for bumetanide in sample 1 at the lowest concentration tested).

Table 4 compares the limits of detection obtained with the described assay (calculated as the concentration required to generate a signal-to-noise ratio of 3) with those reported by other LC assays. Analyte detectability is comparable

Table 4

Limits of Detection of the Different Methods Proposed for Analysis of Diuretics in Biological Samples

Detection	Sample			Limit of Detection		Reference
	Type	Volume	Cleanup	(ng/mL)		
Fluorescence	urine & plasma	0.2mL	precipit'n. of proteins followed by solid-phase extraction	bumetanide:	5	11
Fluorescence	urine & plams	1 mL	liquid-liquid extraction	furosemide: amiloride:	0.3 0.03	12
Fluorescence	urine & plasma	150 μL	precipit'n. of proteins	triamterene:	1	13
Fluroescence	urine	300 μL	liquid-liquid extraction	furosemide:	5	14
Fluorescence	urine	0.2 mL	precipit'n. of proteins	amiloride:	0.5	15
Fluorescence	urine & plasma	100 μL	precipit'n of proteins	furosemide:	5	16
UV	urine	50 μL	column-switching chromatography	amiloride: triamterene: furosemide: bumetanide:	20 7 5 4	7
Fluorescence	urine	5 μL	column-switching chromatography	amiloride: triamterene: furosemide: bumetanide:	50 0.01 5 0.1	this work

to that reported by other LC assays based in fluorescence detection, and in some instances, significantly better.[11,13] For amiloride, the limits of detection reported in references 12 and 15 are lower than that obtained in the present study. This is most probably due to the fact that in those methods, larger volumes of the (purified) samples were injected in the analytical column, and also due to the analyte preconcentration achieved during the sample preparation. Compared with the previously reported method based in column-switching, and UV detection previously reported,[7] the present assay provides comparable sensitivity for amiloride and furosemide; in the latter instance the volume of sample processed is ten times lower and, clearly, the described assay is more sensitive for triamterene and bumetanide.

Human Studies

The proposed method has been applied to the determination of bumetanide after a single dose administration of the lowest recommended dose (5.0 mg).[10] Figure 3 shows the chromatogram obtained 6 hours after dose, corresponding to a bumetanide concentration of (1.83 ± 0.07) μg/mL. No interferences from possible metabolites were observed.

The difficulties in detecting or quantifying bumetanide in biological samples arise because this diuretic is rapidly metabolized and eliminated (the lifetime of elimination is 4 - 6 hours). Therefore, most proposed procedures could not be suitable for the analysis of this drug in real samples and, specially, for doping control tests.[9] Shown in Figure 4, is the urinary excretion profile obtained for this diuretic, with the described approach. The assay allows the quantification of bumetanide at least 30 hours after drug administration, and it can be detected for a longer time. Therefore, the sensitivity of the described approach can be considered satisfactory for most applications concerning to the determination of bumetanide in urine, as well as, for doping control practice.

Utility

The combined administration of diuretics has been proved to be effective in the treatment of some diseases such as congestive heart failure and essential hypertension.[10] In this sense, amiloride-furosemide and triamterene-furosemide are the most commonly used combinations. Self-evidently, the proposed method is valid for analysing samples obtained from subjects treated with such mixtures. Also, hydrochlorothiazide and atenolol, which are commonly coadministered with the diuretics assayed, have been tested for possible interferences. Under the present conditions, atenolol is completely resolved

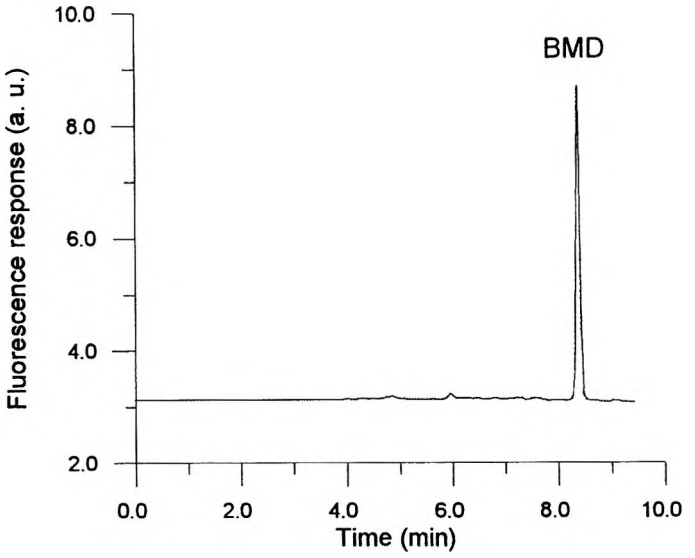


Figure 3. Chromatogram of a urine sample collected 6 h after administration of a single dose of 5.0 mg of bumetanide. For experimental details, see text.

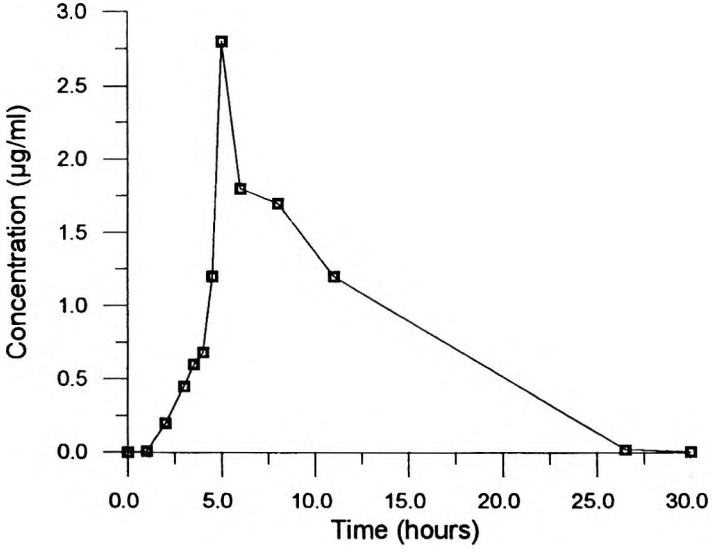


Figure 4. Urinary excretion time-profile of bumetanide. Dose administered, 5.0 mg/mL. For experimental details, see text.

from diuretics of interest. UV chromatograms showed that hydrochlorothiazide (which is also a diuretic) eluted at 6.0 min, thus, overlapping with triamterene. However, since hydrochlorothiazide does not exhibit native fluorescence, it does not interfere with the assay.

With the proposed procedure, the total analysis time takes about 11 min, and several hundred samples can be processed without replacement of the precolumn. Although, in principle, any biological fluid can be processed by switching-chromatography, samples containing a large fraction of proteins (e. g. blood, plasma or serum) are problematic, because irreversible adsorption of proteins results in an increase in back-pressure; moreover, the precolumn has to be frequently replaced in order to ensure an adequate analytical column performance.

With the present procedure, we observed good stability after the repetitive injection of plasma samples, which is most probably due to the small volume of sample injected. Experiments with plasma samples have confirmed the excellent selectivity of the described assay. Therefore, it can be easily adapted to the analysis of diuretics in this biofluid.

CONCLUSIONS

The combination of column-switching chromatography and fluorescence detection is a useful tool for the analysis of diuretics in biological fluids. Quantification and detection at therapeutical levels can be easily achieved in less than 11 min, with satisfactory accuracy and reproducibility. The main advantage over other LC methods using fluorescence detection is the considerable simplification of the cleanup process. Compared with column-switching methods based on UV detection, the described assay provides better selectivity and, in some instances, significantly improves the sensitivity. Since only 5 μL of sample are required, the method performs properly during several injections of urine. Moreover, the system shows excellent compatibility with plasma samples.

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EVALUATION OF PURE COUMARINS USING TLC-DENSITOMETER, SPECTRO-PHOTOMETER, AND HPLC WITH PHOTODIODE ARRAY DETECTOR

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ABSTRACT

A comparison of the UV spectra of twenty seven pure coumarins was carried out using TLC-densitometer, spectrophotometer and high performance liquid chromatography (HPLC) with photodiode array detector (DAD). The data obtained from TLC-densitometer and HPLC in reference to those of the spectrophotometer showed that the dependencies were statistically highly significant. Regardless of the structure of the coumarin, a common λ_{max} and/or λ_{min} can be detected by the different UV off/on-lines. A shift in the

absorption bands measured by TLC-UV and HPLC was noticed when compared to those measured by the spectrophotometer and the intensities of the absorption bands were not the same among the different UV off/on-lines.

INTRODUCTION

Coumarins are natural compounds widely distributed in plants and possessing a variety of biological activities[1-3] The most obvious physical property of most natural coumarins is the fluorescence they display in the UV light. This feature has been employed widely for their detection by using the different UV off/on-lines.

The basic planar chromatographic technique, thin layer chromatography (TLC), is a useful technique for analytical and preparative work, as well as for mobile phase optimization.[4-6] The "PRISMA" system developed by Nyiredy et al., simplifies the optimization process in planar and column chromatography.[7-8] Quantitative evaluation by TLC using direct photometric scanning, is a method which has not yet made much progress, even though it has been carried out for nearly two decades.[9] There are many optically interfering hazards present in the TLC technique, which for ordinary chromatographic purposes are not usually noticed *e.g.* air bubbles, specks of dust, quality of the sorbent, light absorbing or fluorescing impurities in the substrate, or chromatographic solvent, *etc.* The densitometric application of absorptiometry theory of Kubelka and Munk, based on a one dimensional approximation to transfer of radiation in scattering media, has been studied earlier.[10-11] It is possible to calculate the concentrations in TLC when the data are processed by computer.[12] However, it has been established from previous studies[13] that the Kubelka-Munk theory is valid only in a particular region of low concentration.

High performance liquid chromatography (HPLC) proved to be a rapid and sensitive method for the detection of phototoxic psoralens in citrus oil.[14-16] The application of HPLC to the determination of the strong photosensitizer bergapten in perfumes and suntan cosmetics has also been reported.[17-18] Of special interest is the HPLC determination of 8-methoxypsoralen plasma levels in conjunction with photochemotherapy in the treatment of psoriasis.[19-21]

Over the last few years, HPLC has proven useful for the investigation of furanocoumarins in plant materials and has been used, increasingly, for coumarin separations and determinations.[2,5,22-23]

Spectrophotometric methods of analysis have been worked out for many types of organic compounds. Psoberan is a mixture of the two furanocoumarins, psoralen and bergapten, isolated from the common fig tree. It is an active photosensitizer, recomended for treating vitiligo.[24] Analysis of Psoberan in a powder or medicinal forms, in production stages and in plant raw material, were performed using spectrophotometric methods. The levels of the photoactive furanocoumarin in the milk sap of the fig plant, were also determined using UV spectrophotometer.[25]

In the course of current strategy used for the isolation of coumarins from the plant material, quality control tests for the extracted fractions and the isolated pure compounds are always needed. To date, the UV spectra of coumarins could be obtained by using different chromatographic and spectroscopic UV off/on-lines *e.g.* TLC-densitometer, spectrophotometer and high performance liquid chromatography (HPLC), with reference to photodiode array detector (DAD). However, not much work has been carried out on the spectra obtained from the various UV off/on lines and a comparison for such UV off/on lines among each other is needed. The aim of the present study was to compare the UV spectra of standard coumarins, belonging to the main structural types of coumarins, using TLC-densitometer, and HPLC with DAD in reference to the spectrophotometer, in order to evaluate the different spectroscopic UV off/on lines commonly used for the detection of coumarins.

EXPERIMENTAL

Chemicals

The coumarins, imperatorin, isoimperatorin, osthol, oxypeucedanin, phellopterin, and psoralen were isolated and identified from *Angelica archangelica* L. at the Pharmacognosy Division, Department of Pharmacy, University of Helsinki. Angelicin, herniarin, isopimpinellin, methyl umbelliferone, umbelliferone, and xanthotoxin were obtained from Roth (Karlsruhe, Germany), ostruthol from Serva (Heidelberg, Germany), scopoletin from Sigma (St. Louis, U.S.A.) and 5-methoxypsoralen from Fluka (Switzerland).

Isobergapten, pimpinellin, and sphondin were isolated and identified from *Heracleum sphondylium* L. at the Department of Pharmacy, ETH Zürich, Switzerland. Anomalin, athamantin, cis-epoxypteryxin, ostruthin, peucedanin, pteryxin, peuarenarine, peuarenine, and xanthalin were isolated and identified

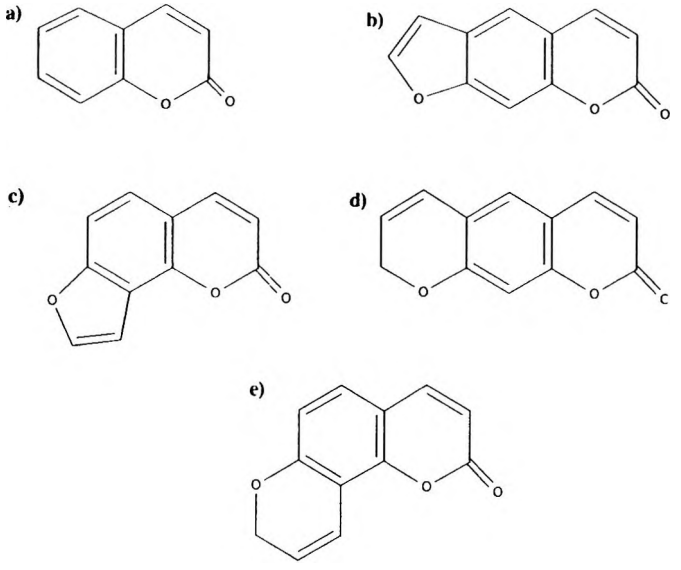


Figure 1. The main structural types of coumarins. a) simple coumarin; b) linear furanocoumarin; c) angular furanocoumarin; d) linear pyranocoumarin; e) angular pyranocoumarin

from *Peucedanum arenarium* W. & K. at the Department of Comparative Phytochemistry, University of Vienna. The *n*-hexane was of technical grade (Oy Exxon Chemicals Ab; Espoo, Finland) and was filtered before use. All other solvents were of HPLC grade (Rathburn, UK). Water was obtained through Alpha-Q (Millipore).

Volumetric flasks 25, 20, and 10 mL were used to prepare the coumarins in chloroform solutions. The concentration of the twenty-seven pure compounds, belonging to the main structural types of coumarins (Figure 1), used for TLC, HPLC, and spectrophotometer evaluations are listed in Table 1.

The coumarins were applied in the form of spots (30 µL) on 10 cm x 20 cm Kieselgel F_{254} TLC plates (Merck, Germany) using a Linomat IV TLC spotter (Camag, Switzerland). The TLC separations were performed in ascending, one-dimensional mode, in 21 cm x 22 cm unsaturated chambers (Camag; Muttenz, Switzerland) at ambient temperature. The solvent volume was 10 mL and the migration distance of the solvents was 8.5 cm. Visual inspection of the TLC plates was done under a UV lamp (Camag, Switzerland) at 254 nm and 366 nm.

Table 1

Concentration of the Standard Coumarins, Belonging to the Main Structural Types, used for TLC, HPLC, and Spectrophotometer Evaluations

Coumarin	Conc. in mg/mL		
	TLC	HPLC	Spectrophotometer
Simple coumarins			
1. Umbelliferone	$2.5x10^{-3}$	1	$2.5x10^{-3}$
2. Herniarin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
3. Scopoletin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
4. Ostruthin	$2.5x10^{-5}$	1	$2.5x10^{-4}$
5. Methyl umbelliferone	$2.5x10^{-3}$	1	$2.5x10^{-4}$
6. Osthol	$4.2x10^{-3}$	1	$4.2x10^{-3}$
Linear furanocoumarins			
7. 5-methoxypsoralen	$2.5x10^{-3}$	1	$2.5x10^{-4}$
8. Isopimpinellin	$3.0x10^{-3}$	1	$2.5x10^{-3}$
9. Imperatorin	$3.2x10^{-3}$	1	$2.5x10^{-3}$
10. Peucedanin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
11. Phellopterin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
12. Oxypeucedanin	$2.8x10^{-3}$	0.056	$2.8x10^{-3}$
13. Isoimperatorin	$3.5x10^{-3}$		$3.5x10^{-3}$
14. Psoralen	$2.5x10^{-3}$	1	$2.5x10^{-3}$
15. Ostruthol	$2.5x10^{-3}$		$2.5x10^{-3}$
16. Xanthotoxin	$<2.5x10^{-3}$	1	$<2.5x10^{-3}$
Angular furanocoumarins			
17. Angelicin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
18. Athamantin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
19. Pimpinellin	$4.0x10^{-3}$		$4.0x10^{-3}$
20. Sphondin	$<2.5x10^{-3}$		$<2.5x10^{-3}$
21. Isobergapten	$<2.5x10^{-3}$		$<2.5x10^{-3}$
Linear pyranocoumarins			
22. Xanthalin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
23. Peuarenarine	$2.5x10^{-3}$	1	$2.5x10^{-3}$
24. Peuarenine	$2.5x10^{-3}$	1	$2.5x10^{-3}$
Angular pyranocoumarins			
25. Cis-epoxypteryxin	$2.5x10^{-3}$		$2.5x10^{-3}$
26. Anomalin	$2.5.x10^{-3}$		$2.5x10^{-3}$
27. Pteryxin	$8.0x10^{-3}$		$8.0x10^{-3}$

The ultraviolet spectra were obtained using a dual wavelength flying-spot scanner CS-9000 (Shimadzu Corporation, Japan). The resolution window was 0.10 nm and the wavelength ranged between 200 nm and 370 nm.

A Waters Assoc. (Milford, Mass. U.S.A.) HPLC system equipped with 600E Multi-Solvent Delivery System was used for this study. The column for HPLC separation was an ELSIsphere 80 C (RP-18; 150 mm x 4 mm; Ø 7 µm, Estonia). The separation was performed at ambient temperature. A mobile phase which consisted of tetrahydrofuran (THF), acetonitrile, methanol, and water was selected (see Results and Discussion section). For all of the investigated coumarins, 10 µL were injected into the column, except in case of oxypeucedanin, 200 µl were injected. Isocratic elution was carried out at a flow rate of 1 mL / min. For UV spectra measurements, a DAD detector (Waters Assoc., Milford, Mass. U.S.A.) was used. The resolution window was kept at 1.3 nm. Wavelength ranged between 200 nm and 380 nm.

The UV spectra of coumarins were also recorded with a Philips PU 8740 UV-vis spectrophotometer of 2 mm resolution window, using one centimeter quartz sample cell at ambient temperature. Wavelength ranged between 200 nm and 400 nm.

Statistical Analysis

Calculation of the correlations and the regression analysis were performed with Stat/View SE+ Graphics™ software on a Macintosh SE Computer.

RESULTS AND DISCUSSION

The coumarin character is strongly indicated by its UV absorption. This phenomena is usually utilized for quality control tests of the extracted fractions from the plant material and for chromatographic analysis of a complex mixture of coumarins. Peak purity has long been a validation problem for the analyst. It often requires at least two analyses, if not more, under various chromatographic conditions. A wide range of chromatographic and spectroscopic UV off/on-lines are now available and used for recording the UV spectra of coumarins.

Thin layer chromatography (TLC), is a useful technique for both analytical and preparative work. TLC is also suitable for the development of the mobile phase of a preparative and analytical column chromatography.[4-6,26-27]

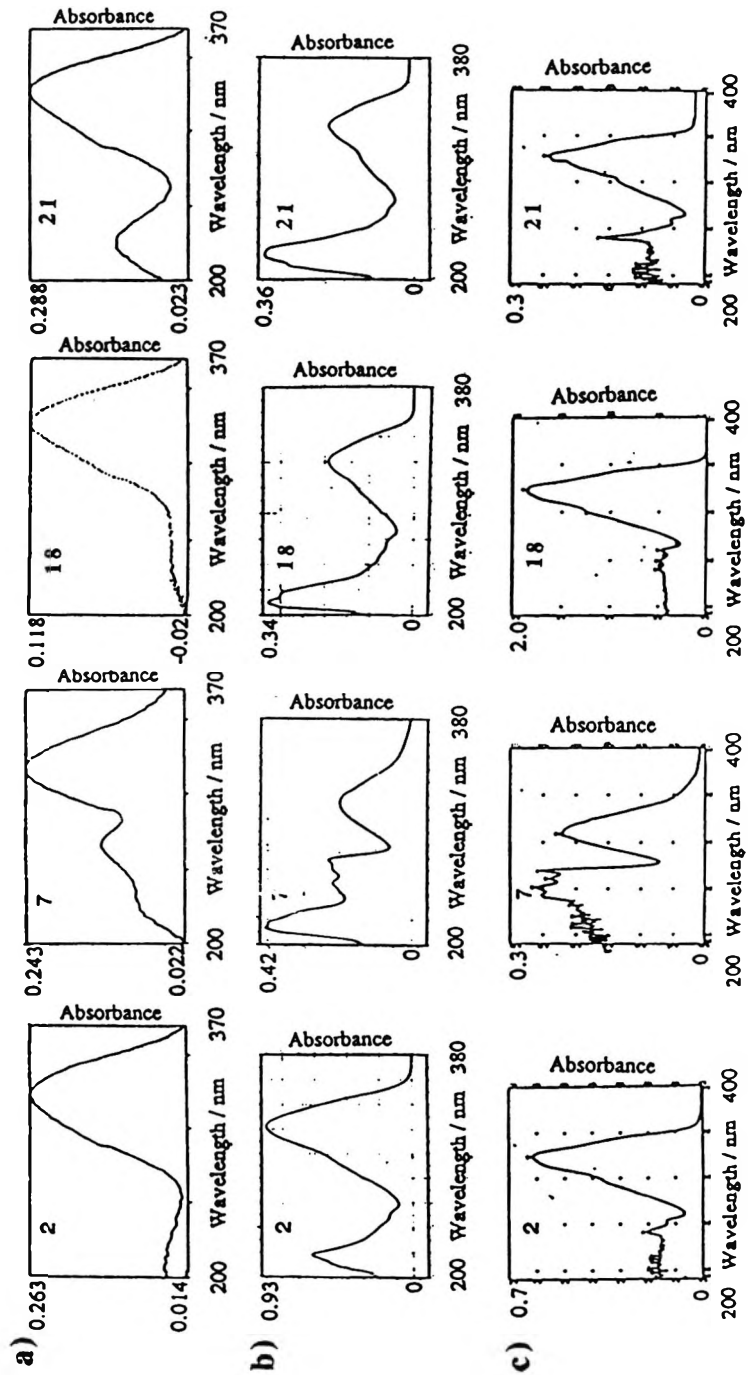
Silica is the most widely used stationary phase in planar chromatography and has an excellent separation power. The "PRISMA" optimization process always starts with this stationary phase.[7-8] Normal phase thin layer chromatographic (NP-TLC) plates were, therefore, used in this study to evaluate the coumarin spectra obtained by the densitometer. Dioxan (S_i=4.8), ethanol (S_i=4.3), and diethylether (S_i=2.8) in n-hexane (S_i=0) were selected, according to the "PRISMA" model, to give the best separation of twenty seven coumarins in unsaturated chambers with normal TLC plates. The selectivity point (P_S) of 333 and the solvent strength (S_i) was adjusted with *n*-hexane to 0.8, resulting in a mobile phase consisting of 5.6% dioxan, 6.3% ethancl, 9.7% diethyl ether, and 78.4% *n*-hexane.

Modern HPLC with DAD was used in this study to determine the UV spectra of coumarins. The handling of the numerous samples for analysis was facilitated by an automatic injection system. For the evaluation of a mobile phase with optimal selectivity to the pure coumarins, the optimisation design " PRISMA" was applied.[28] Following the usual optimization systems for modern HPLC[29-30], an optimal selectivity was reached by a four solvent mixture of THF (S_i=4.5), acetonitrile (S_i=3.2), methanol (S_i=2.6), and water (S_i=0). The mobile phase characterised by S_i=2.6 and P_S of 181 (6% THF, 65% acetonitrile, 10% methanol and 19% water) showed the best elution for the investigated coumarins.

Spectrophotometers are widely applicable to record the UV spectra of many compounds and numerous UV absorption spectra for distinguishing coumarins are existing.[31]

In this study, the UV spectra of the investigated coumarins obtained by spectrophotometer were used as references to compare with the corresponding spectra obtained by the TLC-densitometer and HPLC. The repeatability of the analysis varied between 0.00-0.18% and 0.00-0.10% for umbelliferone (compound 1), and between 0.02-0.25% and 0.04-0.16% for 5-methoxypsoralen (compound 7) with spectrophotometric and HPLC-DAD studies respectively.

Examples of the UV spectra of some compounds, belonging to the main structural types of coumarins, recorded by the different UV off/on-lines are illustrated in Figure 2. Generally, the UV spectra of each coumarin are characterized by certain absorption bands, some of which are shown in Figure 3. The spectra of each compound demonstrated a common λ_{max} and/or λ_{min}, regardless of its structural type. The different spectra showed at least one absorption band at the region of 300-330 nm. In some cases, the intensities of the absorption bands were changed among the different UV off/on-lines.


a)
b)
c)
Absorbance
Wavelength / nm
2
7
18
21
0.263
0.014
0.243
0.022
0.118
-0.02
0.288
0.023
0.93
0
0.42
0.34
0.36
0.7
0.3
2.0
200
370
380
400

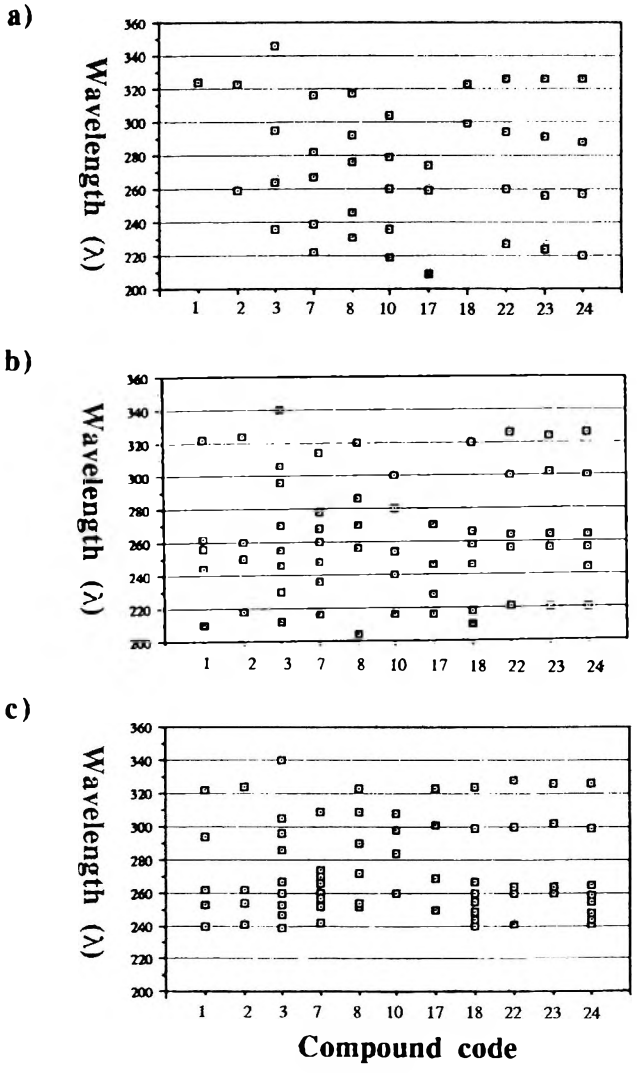


Figure 3. Comparison of the absorption bands of eleven compounds representing the main structural types of coumarins recorded by: a) TLC-densitometer; b) HPLC-DAD; c) spectrophotometer. Compounds coded as in Table 1.

Figure 2. (**left**) Examples of the UV spectra of some compounds belonging to the main structural types of coumarins, recorded by: a) TLC- densitometer; b) HPLC-DAD; c) spectrophotometer. Compounds coded as in Table 1.

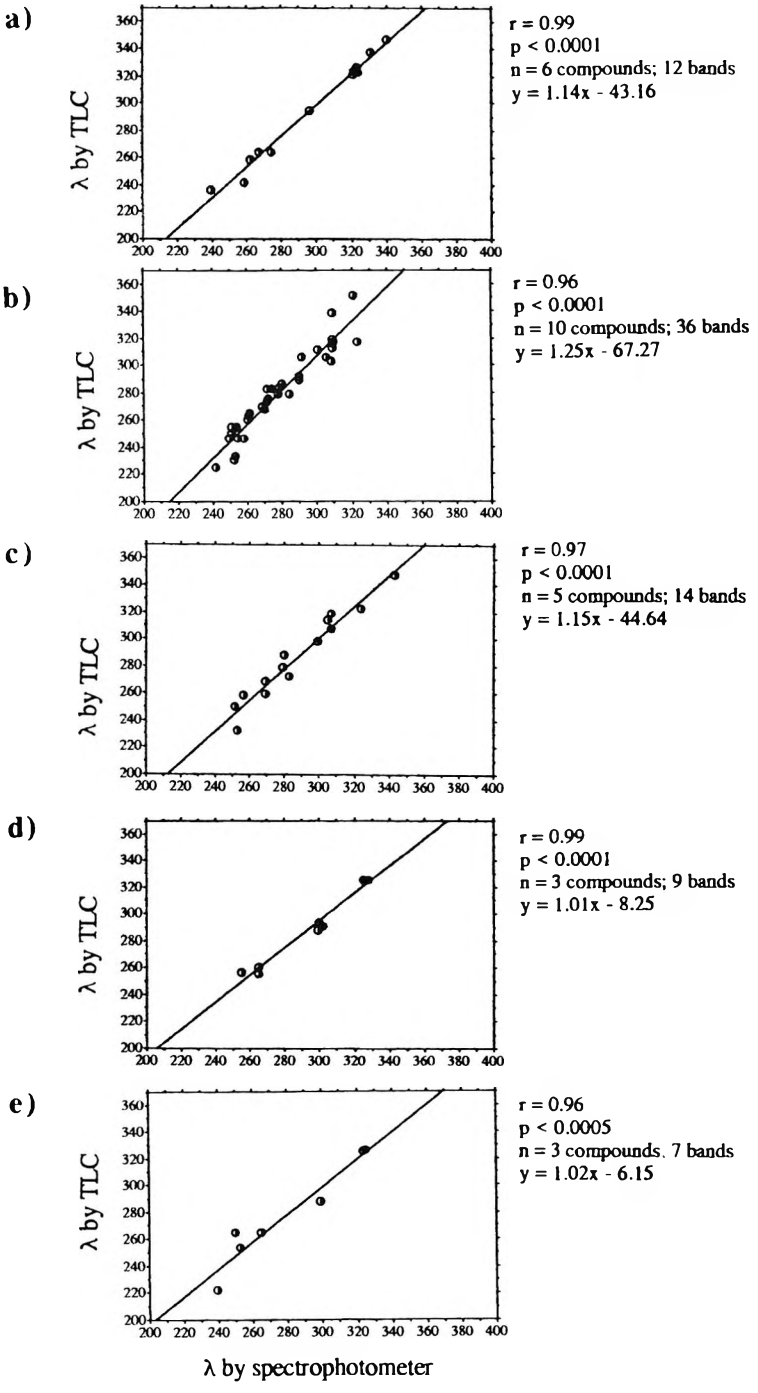

a)
λ by TLC
r = 0.99
p < 0.0001
n = 6 compounds; 12 bands
y = 1.14x - 43.16
b)
λ by TLC
r = 0.96
p < 0.0001
n = 10 compounds; 36 bands
y = 1.25x - 67.27
c)
λ by TLC
r = 0.97
p < 0.0001
n = 5 compounds; 14 bands
y = 1.15x - 44.64
d)
λ by TLC
r = 0.99
p < 0.0001
n = 3 compounds; 9 bands
y = 1.01x - 8.25
e)
λ by TLC
r = 0.96
p < 0.0005
n = 3 compounds, 7 bands
y = 1.02x - 6.15
λ by spectrophotometer

Table 2

Shifts in the Absorption Bands ± SD of Coumarins Measured by TLC-Densitometer and HPLC with DAD in Comparison to the UV Spectra Recorded by Spectrophotometer

Coumarin Type	Shift in the Absorption Bands ± SD	
	TLC Densitometer	HPLC
Simple coumarins (n=6)*	1.7 ± 6.7	1.4 ± 3.5
Linear furanocoumarins (n=10)	-2.2 ± 10.5	3.5 ± 9.6
Angular furanocoumarins (n=5)	0.4 ± 8.5	6.2 ± 7.8
Linear pyranocoumarins (n=3)	4.5 ± 4.8	1.4 ± 1.7
Angular pyranocoumarins (n=3)	1.2 ± 10.3	

*n = number of coumarins; number of bands see Figure 4.

For instance, 5-methoxypsoralen (compound 7) showed a λ_{max} at 308.8 nm of 84.6%, a λ_{max} at 316.2 nm of 100% and a λ_{max} at 314.0 nm of 48.8% responses by the spectrophotometer, the TLC-densitometer and the HPLC-DAD respectively. The UV spectra recorded by the spectrophotometer and HPLC-DAD were rather similar and demonstrated higher spectral resolution when compared to the spectra measured by TLC-densitometer (Figure 3).

HPLC with DAD can provide spectral resolution in addition to temporal resolution. It can detect UV light in discrete increments and present it to the analyst as a three-dimensional chromatogram. Such a chromatogram allows for simultaneous detection at all the wavelengths throughout the UV spectrum.[32]

Regression analysis of the data of TLC-densitometer and HPLC-DAD in reference to the spectrophotometer are shown in Figure 4 and Figure 5. The measurements by TLC showed more deviation from the regression line than those of the HPLC-DAD. However, the results obtained by each of HPLC-DAD and TLC-densitometer showed that the dependencies were statistically highly significant.

Figure 4. (**left**) Scattergram of wavelength measurements (λ_{max} and λ_{min}) by TLC-densitometer in reference to the spectrophotometer for the different structural classes of the investigated coumarin: a) simple coumarins; b) linear furanocoumarins; c) angular furanocoumarins; d) pyranocoumarins. Compounds coded as in Table 1.

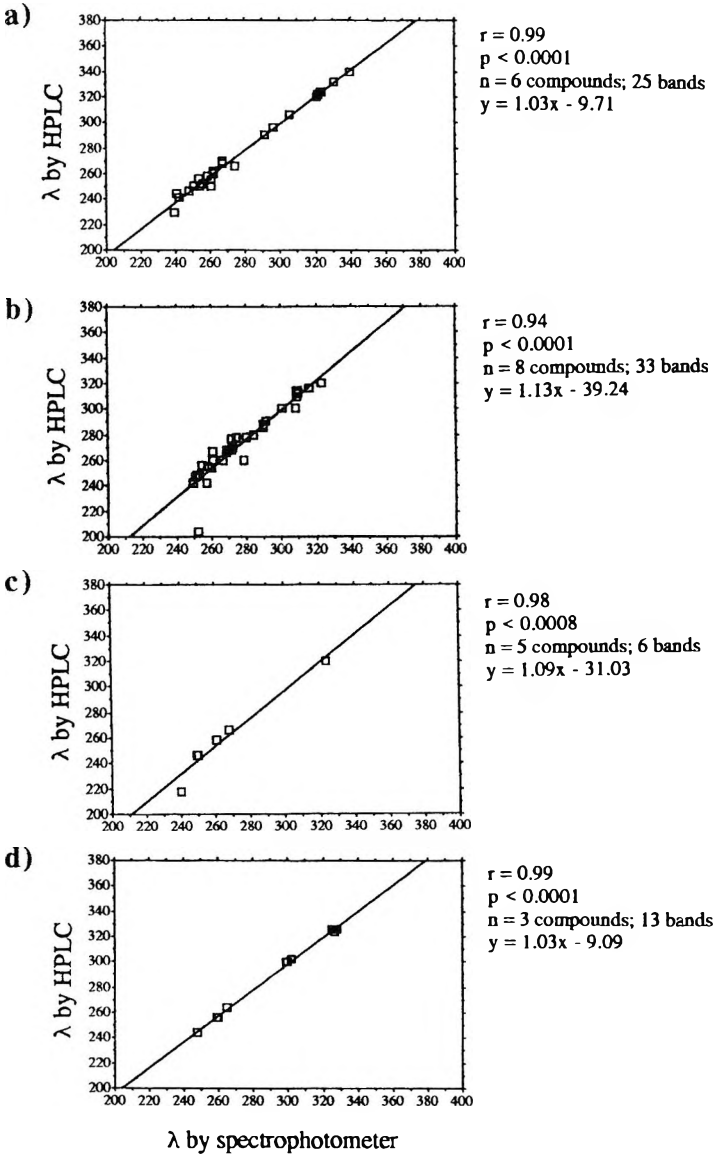


Figure 5. Scattergram of wavelength measurements (λ_{max} and λ_{min}) by HPLC-DAD in reference to the spectrophotometer for the different structural classes of the investigated coumarin: a) simple coumarins; b) linear furanocoumarins; c) angular furanocoumarins; d) pyranocoumarins. Compounds coded as in Table 1.

For each structural category of the investigated coumarins, there was a shift in the absorption bands measured by the TLC-densitometer and HPLC when compared to those obtained by the spectrophotometer (Table 2).

The difference in the UV spectra obtained by the various UV off/on-lines for a single compound may be attributed to the photomode whether it is reflection e.g. in TLC-densitometer or absorbance e.g. in spectrophotometer and HPLC-DAD. Another reason for the difference can be the interaction between the analyte and the mobile phase or the stationary phase. In addition, the individual sensitivity of the detector is subjected to pH, ionic strength, and solvent effect which can be a reason for the presence or absence of certain bands, e.g. in case of the spectra recorded by HPLC, a λ_{max} was observed at 200-220 nm.

The data obtained from the different UV off/on-line techniques is very useful for both quantification and tentative semiidentification. However, special attention should be paid to the shifts which occur for the maxima and the minima of the UV spectra in order to avoid false conclusions about the results.

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A NOVEL ION CHROMATOGRAPHIC METHOD USING ZWITTERIONIC SURFACTANTS AS THE STATIONARY PHASE AND WATER AS THE MOBILE PHASE

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ABSTRACT

Zwitterionic surfactants immobilized on the surfaces of octadecylsilica (ODS) are used for the stationary phase and water as the mobile phase for the ion chromatography (IC) of target analytes. The creation of an electrical double layer (EDL), when a zwitterionic stationary phase is in contact with the analyte ions,

is proposed to explain the separation mechanism. When an EDL is created using a zwitterionic stationary phase (ZWEDL), its properties differ considerably to those of a single charge-fixed stationary phase created EDL. For a ZWEDL, (i) the electrostatic field is increased, resulting in the simultaneous retention and separation of both cations and anions; (ii) the electrostatic affinity between the analytes in the ZWEDL and the stationary phase is extremely weak. This results in the "effective" distribution of the analytes between the stationary phase and the mobile phase without the need for ion-exchange. Since only water is used for the mobile phase, the sensitivity of detection by conductivity is vastly improved and the direct determination (without pre-concentration) of inorganic ions at ultra low levels is possible. Furthermore, since both positive and negative electrostatic fields are produced simultaneously, both analyte cations and anions are retained and separated in a single stage of the stationary phase. This provides the basis for a simple and rapid chromatographic method for the simultaneous analysis of cations and anions.

INTRODUCTION

For the analysis of inorganic ions by ion chromatography (IC), separation and detection are normally achieved in series. Usually, ion exchangers are used in the stationary phase to achieve the separation while conductometry is used for detection. The development of "suppression"[1] and "non-suppression"[2,3] ion exchange chromatographic techniques has enabled the successful separation and detection of a large number of ionic species. However, although these techniques provide high precision and good detection limits, increased resolution and sensitivity are difficult to obtain for a number of reasons:

1. The residual (in suppression IC) or the original (in non-suppression IC) background electrical conductance of the mobile phase contributes to the noise level and reduces the signal to noise ratio (S/N). This adversely affects the overall detection limits of IC, and restricts the minimum detection limits of most ionic species to the ppb region.

2. The overlapping of analyte species is common resulting in information loss and a decrease in resolution. Under certain conditions, with respect to the column and mobile phase, the retention intervals between the analyte species may be constant and can result in elution-

overlapping. For example, when two analyte species are eluted in series, one being higher in concentration than the other, the elution containing the lower concentration of analytes are often partly or completely overlapped by the elution containing the higher concentration of analytes.

3. For ion-exchange chromatography two separation procedures are necessary. One each for the separation of cations and anions; this reduces the number of samples that can be analyzed as a function of time.

Since these problems are largely associated with the use of ion-exchanges, they will never be effectively overcome unless alternative and suitable methods are developed. One solution is to use zwitterionic surfactants for the stationary phase. When a small amount of aqueous solution containing analyte cations and anions, is passed through a zwitterionic surfactant-immobilized stationary phase, the cations and anions receive an electrostatic attraction and repulsion from the stationary phase, simultaneously. As a result, the "net" electrostatic affinity between the analyte ions and the stationary phase is extremely low compared to the electrostatic affinity observed when using a single charged fixed stationary phase. The appropriate distribution of the analyte ions between the stationary phase and the mobile phase, which is critical for separation, is achieved by using water as the mobile phase. Furthermore, since negative and positive electrostatic fields are produced simultaneously, if a zwitterionic stationary phase is used, both the analyte cations and anions are retained and/or separated with a single stationary phase. This method of separating ions based on simultaneous electrostatic attraction and repulsion interactions has been termed "electrostatic ion chromatography (EIC)".[4,5]

In EIC, water is used as the mobile phase; therefore, the problem of low sensitivity, normally associated with conventional IC, is completely removed. EIC is less time consuming since the analyte cations and anions are separated within a single stage. Furthermore, the intervals between the retention time of the analyte ions can be extended by the adjustment of sample conditions.[6] Hence, the problems of overlapping eluents and incomplete separation of the target analytes are easily overcome.

To further elucidate the separation mechanism involved in EIC, an electrical double layer (EDL) model, proposed in previous study,[7] has been further investigated in this research. The initial results of identical samples which were analyzed in duplicate, using EIC and conventional IC methodologies, are also presented to enable comparison. The advantages of EIC over conventional IC are discussed.

EXPERIMENTAL

Apparatus

The HPLC system used in this study is the same as described in previous research[7] and consisted of the following. A Shimadzu (Kyoto, Japan) LC-6A system equipped with a pump (LC-7A), an auto-injector (SIL-6A) with a sample injection volume of 100 μL. A system controller (SCL 6A), and a conductivity detector (CDD-6A) interfaced with a photodiode array UV visible detector (SPD-M6A). The HPLC system was coupled to an inductively coupled plasma atomic emission spectrometry (Model 075 Plasma Atomcomp MKII, Thermo Jarrell-Ash, Franklin, MA) for the identification of the analyte cations. Three ODS-packed columns (L-Column, 250 x 4.6 mm I.D.; Chemical Inspection and Testing Institute, Tokyo, Japan), the first coated with CHAPS micelles, the second with Zwittergent-3-14 micelles, and the third coated with ammonium sulfobetaine-3 micelles, were used as the separation columns (zwitterionic columns). A commercial anion-exchange column (Shim-pack IC-A3; 150 x 4.6 mm I.D., Shimadzu, Kyoto, Japan) was also used for conventional IC measurements.

Reagents

The zwitterionic surfactants, CHAPS, Zwittergent-3-14 and ammonium sulfobetaine-3 were used to produce the zwitterionic stationary phases. These were obtained from Dojin (Kumamoto, Japan), Calbiochem (La Jolla, CA, USA), and Janssen Chimica (2340 Beerse, Belgium), respectively. The inorganic salts, which were used as the standard analytes, were purchased from Wako (Osaka, Japan) and all reagents were used as received. Pure water (conductivity, 0.01 - 0.03 μS/cm) obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used throughout.

RESULTS AND DISCUSSION

The Separation Mechanism in EIC

As with all types of chromatography, ion chromatography requires an "effective" distribution of the analyte ions between the stationary phase and the mobile phase to achieve separation of the analytes. In conventional IC, when a single charge-fixed stationary phase is used the separation of anions requires

the use of a positive charge-immobilized stationary phase, while for cations, a negative charge-immobilized stationary phase is necessary. The effective distribution of the analyte ions between the stationary phase and the mobile phase is achieved by ion-exchange. The ion exchange process can be better understood by the use of a stoichiometric model:

$$jA^{i+}_{(s)} + iR^{j+}_{(m)} \Leftrightarrow jA^{i+}_{(m)} + iR^{j+}_{(s)} \quad (1)$$

$$jA'^{i-}_{(s)} + iR'^{j-}_{(m)} \Leftrightarrow jA'^{i-}_{(m)} + iR'^{j-}_{(s)} \quad (2)$$

Equation's 1 and 2 represent the cation and anion-exchange, respectively. Where i and j denote the ion charge, $A^{i+}(s)$ and $A'^{i-}(s)$ denote the analyte ions, which are attracted by the charged stationary phase, and $R^{j+}(m)$ and $R'^{j-}(m)$ represent the ions present in the mobile phase, which are used for ion-exchange, i.e., the replacing ions. Further understanding of the stoichiometric model can be gained from the analysis of two monographs contributed by Helfferich[8] and Haddad and Jackson.[9] Since the stoichiometric model only allows for a quantitative investigation of the ion-exchange process, further illustration of the ion-exchange concept can be made via the introduction of the Stern model. For a single charge-fixed surface (the stationary phase in IC) in contact with an electrolytic aqueous solution, the ions of the electrolytes, having the opposite charge to the stationary phase, are surrounded by the charged stationary phase, creating an electrical double layer (EDL). The Stern model is shown in Figure 1. The analyte ions in the double layer receive their electrostatic attraction from the stationary phase. Therefore, to achieve an "effective" distribution of the analyte ions between the stationary phase (electrical double layer) and the mobile phase (bulk solution), namely, to release the analyte ions from the electrical double layer to the bulk solution, a procedure of ion exchange is required. To achieve ion separation when using a single charge-fixed stationary phase, the mobile phase must contain replacing ions in order to facilitate ion-exchange. Further clarification of the electrical double layer model, as applied to IC, is given in previous literature.[10-22]

It was initially proposed in previous research, that for a zwitterionic stationary phase in contact with ions, an "electrical double layer (ZWEDL)" is also created.[7] However, the properties of a ZWEDL differ to those of an electrical double layer created with a single charge-fixed stationary phase. These are illustrated in Figure 1 and are briefly described below.

(i) For a zwitterionic stationary phase, both positive and negative electrostatic fields are produced simultaneously, introducing both the analyte cations and anions in to the EDL. As a result, both of the analyte cations and anions are retained/separated with a single

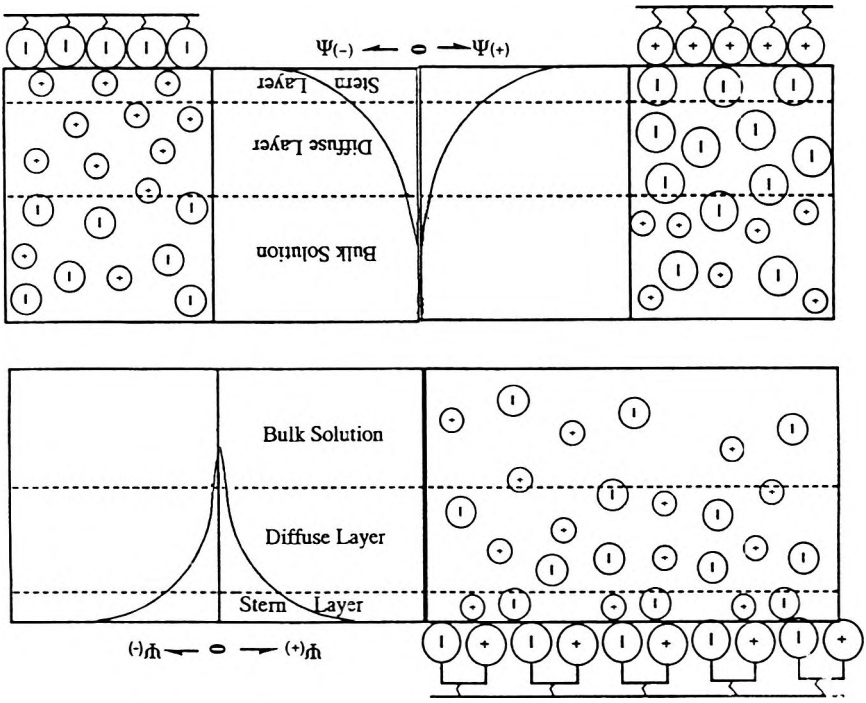


Figure 1. Electrical double layers created with a positive charge-fixed and a negative charge-fixed stationary phase (left side) and a positive/negative charge-fixed (zwitterionic) stationary phase (right side). The potential (ψ) of the electrostatic field produced by the charged stationary phase decreases with increasing the distance from the charged stationary surface to the solution. Ψ decreases up to zero in the bulk solution. Analyte ions are retained by the electrostatic field, creating an electrical double layer.

stationary phase. However, when a single charge-fixed stationary phase is used, the electrical double layer involves either the analyte cations, for a negative-charge-fixed stationary phase, or the anions, for a positive charge-fixed stationary phase. Therefore, for the separation of cations and anions, two stages (stationary phases), one for retaining/separating cations, one for retaining/separating anions, are required.

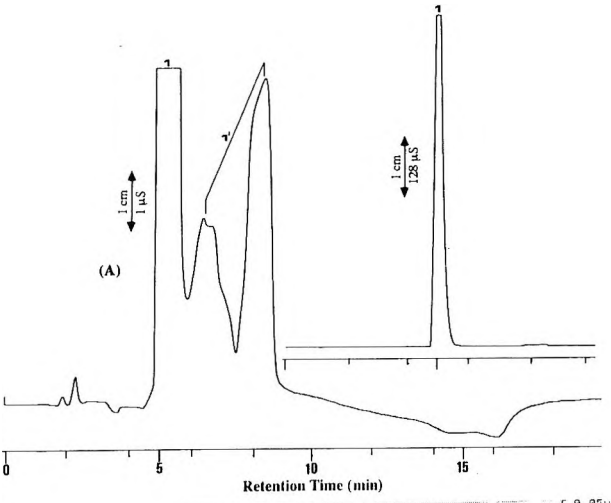
(ii) For a zwitterionic stationary phase, the distribution of the analyte ions between the electrical double layer and the bulk solution is achieved without the need for ion-exchange. This is because the positive and the negative charges are fixed in close proximity to each other in the

stationary phase. Therefore the analyte ions are receiving the effect of electrostatic attraction and repulsion simultaneously. Hence, the "net" electrostatic affinity between the stationary phase and the analyte ions in the electrical double layer is extremely weak. Adversely, for a single charge fixed stationary phase, the electrostatic affinity between the stationary phase and the analyte ions in the electrical double layer is 'strong' since the analyte ions are only subjected to electrostatic attraction. Therefore, to release the analyte ions from the electrical double layer into the bulk solution, ion-exchange is necessary.

The Elution of Identical Analyte Ions from the Stern Layer and the Diffuse Layer

When water is used as the mobile phase in EIC, identical analyte ions from the Stern and the diffuse layers elute at slightly different retention times. Although the analyte ions from the diffuse layer constitute the main detectable peak, identical analyte ions from the Stern layer are eluted as two small secondary peaks which appear as shoulders on the main peak. This is typified in Figure 2 (A, B and C) which shows the chromatogram of an aqueous solution containing 1.0 mM $Ba(NO_3)_2$. The chromatogram shown in Figure 2A was obtained using conductometric detection. While, the chromatogram shown in Figure's 2B and C were obtained using a photodiode array UV-Vis detection system and an ICP-AES detection system respectively. These three detection systems were used in series and the ratio of the peak areas, i.e. secondary peak: main peak, in each case, was found to be approximately 3:1000.

For each chemical species (salt) examined in this study, the peak areas of both the main peak, Am, and the secondary peaks, As, are proportional to the concentration of the analytes in the sample solutions. When the concentration of the analytes in the sample solution were relatively high, (mM levels), most of the analytes were eluted from the diffuse layer, i.e., the ratio of As/Am is very small. On the contrary, when the concentration of the analytes in the original sample solution were relatively low (μM levels), most of the analytes were eluted from the Stern layer, i.e., the ratio of As/Am is very large. The secondary peaks become critical when the concentration of the analytes in the original solution is sufficiently low. The actual critical concentration depends on the chemical species of the analytes and the conditions of the zwitterionic stationary phase. The hydrophobic analytes and/or stationary phase give larger critical concentrations than the hydrophilic analytes and/or the stationary phase.


1
1'
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1 μS
(A)
1 cm
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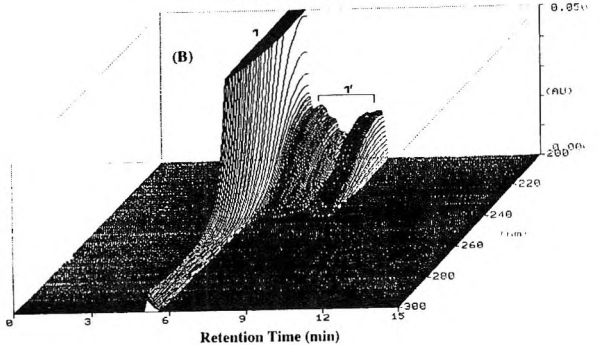
5
10
15
Retention Time (min)


(B)
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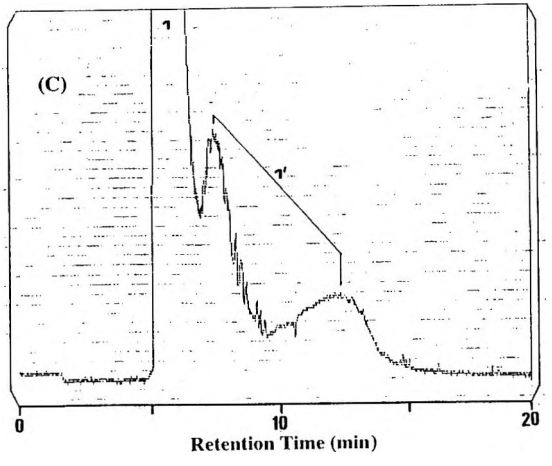
Retention Time (min)


(C)
1
1'
0
10
20
Retention Time (min)

In conventional IC, no differences are observed in the elution retention time for the analyte ions from the Stern layer and the diffuse layer when using a mobile phase which contains the replacing ions. The reason for this being that the analyte ions involved in the Stern layer and in the diffuse layer are replaced, almost simultaneously, when high levels of the replacing ions are present in the mobile phase. Naturally, when only water is used as the mobile phase, the analyte ions involved in the Stern layer exhibit stronger attraction properties than identical analyte ions in the diffuse layer, by means of the stationary phase. Therefore, the analyte ions involved in the Stern layer are eluted with a longer retention time. Furthermore, the analyte ions in the Stern layer may also be affected if both the analyte ions and the zwitterionic stationary phase possess strong hydrophobic properties. However, with the addition of a "sacrifice" species to the original sample solution, the analyte ions in the Stern layer can be persuaded to migrate to the diffuse layer.[7] Eventually, a single elution, and therefore a single peak, equal to the total number of target analyte ions is obtained.

Comparison of EIC and Conventional IC Detection Abilities

The background conductance and the noise level of the mobile phase are the two major factors which determine the detection limits when using conductometry for detection . Water is considered to be an ideal mobile phase for acquiring the lowest detection limits since its conductance and noise levels are low.[23] Previous research dealing with the separation of ions using water as the sole component of the mobile phase was published in 1968 by Saunders and Pecsok.[24] There are also recent literatures dealing with ion separation (using water as the mobile phase) using different types of stationary phases.[25,26] However, no literature has been found regarding the analysis of samples containing very low levels of analyte ions. Furthermore, the separation abilities of these methods were found to be poor compared to conventional IC. In our experience, when aqueous samples containing inorganic ions at ultra-low levels (low-μM) are injected into either a tightly cross linked polyacrylamide gel packed column[24] or a crown ether immobilized stationary phase,[25] analytes are retained in the column, i.e. are unable to elute out from the column.

Figure 2. **(left)** Chromatograms of an aqueous solution containing 1.0 mM $Ba(NO_3)_2$ obtained using a conductivity detection (A), a photodiode array UV-vis detection (B) and an ICP-AES detection (C). Column: ODS-packed column (250 x 4.6 I.D. mm) coated with Zwittergent-3-14; mobile phase: water; flow rate: 1.0 mL/min. The main peak (1) is corresponding to the ion pair Ba^{2+}-$2NO_3^-$ eluted from diffuse layer; the secondary peak (1') is corresponding to the identical ion pair eluted from the Stern layer.

This is probably due to the residual silanols and/or the hydrophobic properties of the stationary phase. The inability of the analyte ions to elute from the column, as experienced in trace analysis, is not observed with the EIC technique. Therefore, the separation abilities obtained using EIC are comparable to those of conventional IC, furthermore, a superior detection ability using conductometry with EIC over conventional IC is expected.

Four aqueous stock solutions containing (i) 0.02 μM each of NaCl, $NaNO_2$, NaBr $NaNO_3$ and NaI, (ii) 0.1 μM each of NaCl, $NaNO_2$, NaBr, $NaNO_3$ and NaI, (iii) 0.01 μM each of $CaCl_2$, $CaBr_2$, $Ca(NO_3)_2$, CaI_2 and (iv) 0.1 μM each of $CaCl_2$, $CaBr_2$, $Ca(NO_3)_2$ and CaI_2 were prepared and a sample volume of 100 mL was injected into the EIC system. Identical experimental conditions were used throughout. To test for reproducibility and stability of EIC during trace analysis, each of these solution mixtures were analyzed ten times. The sensitivity for conductometric detection was 0.1 μS/cm. Typical chromatograms for the stock solutions (ii and iv) are shown in Figures 3 and 4, respectively.

For stock solution (i), the standard deviation (%) was found to be 1.8 and 5.2 for the concentration (peak area) of the ion pairs Na^+ - Br^- and Na^+ - NO_3^-; and 0.8, 0.6, 0.6 in the retention time for ion pairs of Na^+ - Cl^-, Na^+ - Br^- and Na^+ - NO_3^-, respectively. A peak, corresponding to the Na^+ - I^- ion pair, is not observed probably because the conductance of the Na^+ - I^- involved in this elution is ultra-low, and beyond the detection limit. The NaCl has been contaminated during preparation and previous experience has shown that uncontaminated aqueous solutions containing ultra-low NaCl or $CaCl_2$ are very difficult to obtain. The analytes Na^+ - NO_2^-, were obscured by the negative "water-dip" and the peak corresponding to the ion pair Na^+ - NO_3^- was partly overlapped by a peak caused by dissolved carbon dioxide (CO_2). The "water-dip" observed in conventional IC, including the suppression type and non-suppression type),[27,28] is also observed in EIC. To further investigate the occurrence of the water-dip and the CO_2 peak, the water used as the mobile phase was injected into the EIC system. The chromatogram shown in Figure 5 shows a negative peak caused by the water-dip and a positive peak caused by dissolved CO_2. It is proposed that the dissolved CO_2 in the sample is separated from the water and that the negative peak corresponds to water without CO_2. The positive peak is thought to be caused by the presence of carbonate and these assumptions were confirmed when an aqueous solution containing $NaHCO_3/Na_2CO_3$ was injected into the EIC system. A peak, corresponding to carbonate, appeared with an identical retention time as CO_2.

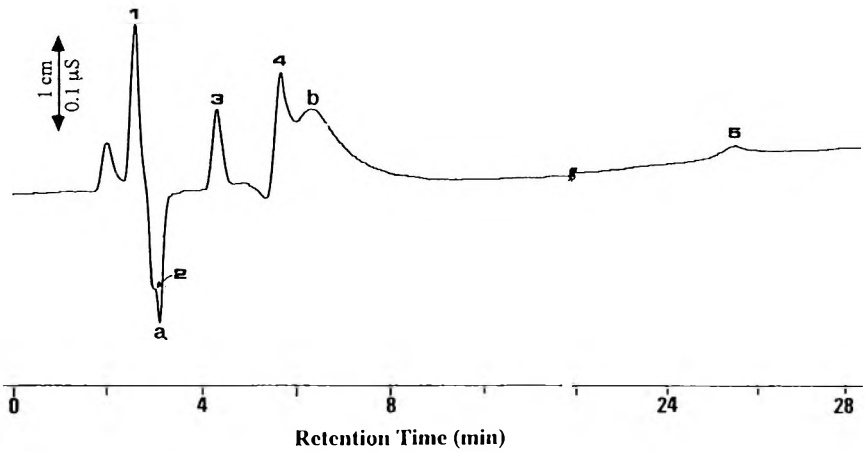


Figure 3. Chromatogram of an aqueous solution containing 0.1 µM each of NaCl, $NaNO_2$, NaBr, $NaNO_3$, and NaI obtained using EIC. Detection: conductivity. Other conditions are the same as described in Fig. 2. Peaks: 1, Na^+-Cl^-; 2, Na^+-NO_2^- (partly obscured by the water-dip); 3, Na^+-Br^-; 4, Na^+-NO_3^-; 5, Na^+-I^-. a, water-dip; b, CO_2.

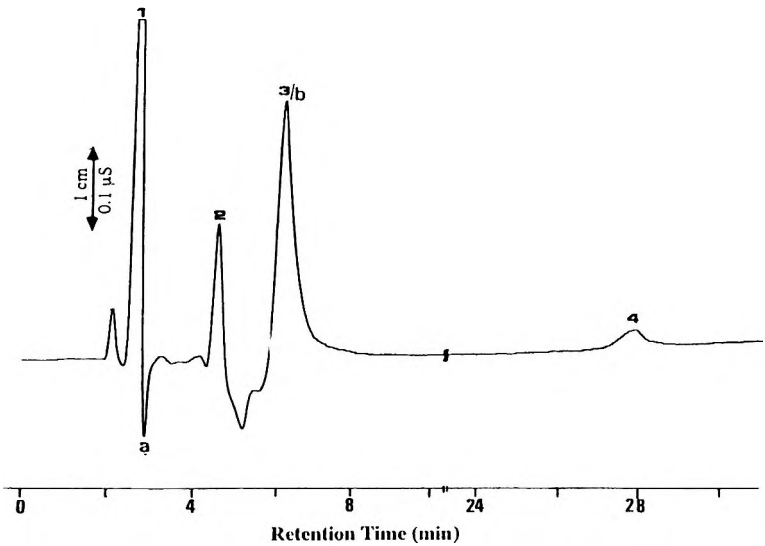


Figure 4. Chromatogram of an aqueous solution containing 0.1 µM each of $CaCl_2$*, $CaBr_2$, $Ca(NO_3)_2$ and CaI_2 obtained using EIC. Detection: conductivity. Other conditions are the same as described in Fig. 2. Peaks: 1, Ca^{2+}-$2Cl^-$; 2, Ca^{2+}-$2Br^-$; 3, Ca^{2+}-$2NO_3^-$; 4, Ca^{2+}-$2I^-$; a, water-dip; b, CO_2. *$CaCl_2$ has been contaminated.

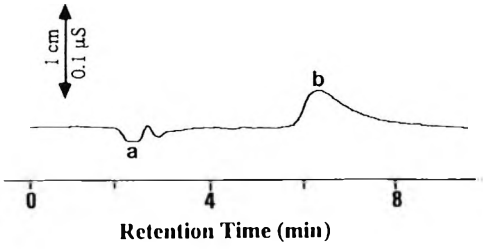


Figure 5. Chromatogram of 100 μL water used as the mobile phase. Other conditions are the same as described in Fig. 3. Peaks: a, water-dip; b, CO_2.

For solution (ii), the standard deviation (%) is 6.8, 0.6, 4.2 and 4.3 for the peak area and 0.8, 0.6, 0.6 and 0.7 for the retention times for ion pairs, Na^+-Cl^-, Na^+ - Br^-, Na^+ - NO_3^- and Na^+ - I^-, respectively. For solution (iii), the standard deviation (%) was found to be 3.4 and 8.5 for the peak area for ion pairs, Ca^{2+} - $2Br^-$ and Ca^{2+} -$2NO_3^-$. For the retention times, 0.8, 0.5 and 1.2 for ion pairs of Ca^{2+} - $2Cl^-$, Ca^{2+} -$2Br^-$ and Ca^{2+} - $2NO_3^-$, respectively. A peak corresponding to Ca^{2+} - $2I^-$ was not observed. The peak corresponding to Ca^{2+} - $2NO_3^-$ was completely overlapped by the CO_2 peak. Therefore, the peak area for Ca^{2+} - $2NO_3^-$ was calculated as the difference between the area of the peak due to Ca^{2+} - $2NO_3^-$/CO_2 and the area of the CO_2 peak. However, the ion pair Ca^{2+} - $2NO_3^-$ was completely separated from CO_2 when an ammonium sulfobetaine-3 stationary phase was used. For solution (iv), the standard deviation (%) for the peak areas were 2.1 and 5.6 and 3.5 for ion pairs of Ca^{2-} - $2Br^-$, Ca^{2+} - $2NO_3^-$ and Ca^{2+} - $2I^-$ respectively. The retention times for ion pairs Ca^{2+} -$2Cl^-$, Ca^{2+} - $2Br^-$, Ca^{2+} -$2NO_3^-$ and Ca^{2+} - $2I^-$, are 0.8, 0.5, 0.9 and 0.7 respectively.

A commercial column (Shim-pack IC-A3) was coupled to the HPLC system. An aqueous solution containing 8.0 mM p-hydroxybenzoic acid and 3.2 mM Bis-tris were used for the mobile phase (as recommended by the manufacturer). Stock solutions (ii) and (iv) were then analyzed using conventional IC. The conditions for conductometric detection were identical as used previously for EIC. Chromatograms in Figure 6 and 7 show that the level of base-line noise is very high and is almost the same intensity as the peak signal intensity for 0.1 μM of Br^- and NO_2^-. Furthermore, the stability of the base-line was very poor. In order to analyze all of the samples it was necessary to re-position the base line many times. After injection an inherent systematic peak appeared around a retention time of 14 minutes which was attributed to the CO_2. The peak corresponding to I^- was not observed under these conditions.

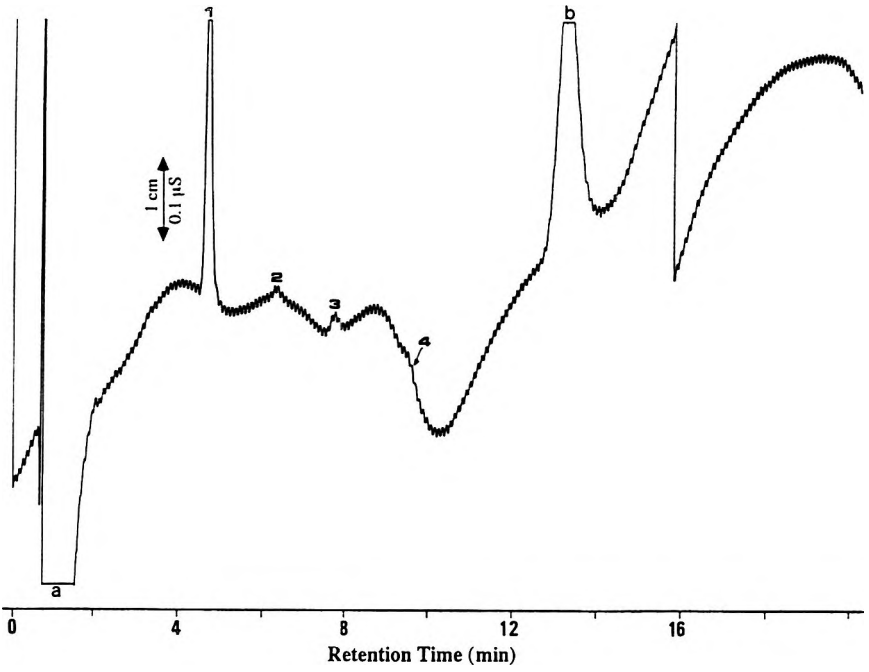


Figure 6. Chromatogram of an aqueous solution containing 0.1 μM each of NaCl*, $NaNO_2$, NaBr, $NaNO_3$ and NaI obtained using a conventional IC. Column: Shim-pack IC A3 (150 x 4.6 I.D mm); mobile phase: an aqueous solution conatining 8.0 mM p-hydroxybenzoic acid and 3.2 mM Bis-tris; flow rate: 1.0 mL/min. Other conditions are the same as described in Fig. 3. Peaks: 1, Cl^-; 2, NO_2^-; 3, Br^-; 4, NO_3^-; a, water-dip; b, CO_2. A peak due to I^- was not observed. *NaCl has been contaminated.

By direct comparison of the detection abilities of both EIC and conventional IC methodologies, the advantages of a water mobile phase over a mobile phase containing replacing ions have been demonstrated. Furthermore, while using water as the mobile phase, the detection ability of a UV-visible detection system is vastly improved.

Ultra-low levels of inorganic ions which have strong UV-absorption, for example, NO_2^-, Br^-, NO_3^- etc., is more easily detected. More so, even inorganic ions with much lower UV-absorption such as Cl^- could also be detected with a UV-visible detection when water was used as the mobile phase for IC.

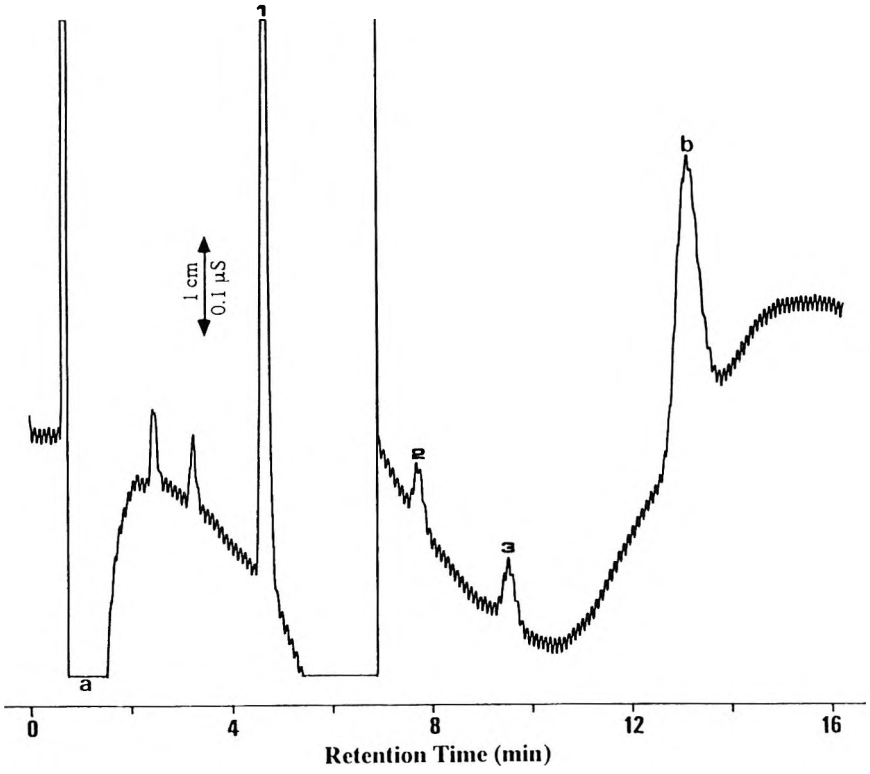


Figure 7. Chromatogram of an aqueous solution containing 0.1 μM each of $CaCl_2$*, $CaBr_2$, $Ca(NO_3)_2$ and CaI_2 obtained using a conventional IC (conditions are the same as described in Fig. 6). Other conditions are the same as described in Fig. 4. Peaks: 1, Cl^-; 2, Br^-; 3, NO_3^-; a, water-dip; b, CO_2. *$CaCl_2$ has been contaminated. A peak due to I^- was not observed.

CONCLUSION

The use of EIC rather than conventional IC offers several advantages. The EIC methodology is particularly useful for the simultaneous detection of inorganic cations and anions and the purification of inorganic salts, etc. However, EIC is to be strongly recommended for the analysis of trace levels of inorganic ions, since the detection limits obtained using EIC are extremely low. Furthermore, for the analysis of trace levels of inorganic ions, the analytes ions eluted from the Stern layer were equal to the sum of all the analytes and appeared as a single sharp peak. When samples containing inorganic ions at relatively high (> low-μM) levels are analyzed, identical analyte species are

involved in both the Stern layer and the diffuse layer. This can cause a secondary peak to be eluted together with the main peak. In this case a 'sacrifice' chemical is suggested which, when introduced into the original sample solution, 'encourages' the release of the analyte ions from the Stern layer to the diffuse layer, resulting in a single elution. The zwitterionic stationary phases, investigated in this and previous research, fail for the base-line separation of inorganic cations when they have the same charge. By designing the zwitterionic stationary phase, such that, the strength of the positive and the negative charges and the distance between the positive and the negative charges are appropriate, it is expected that the resulting zwitterionic stationary phase will result in the base-line separation of inorganic cations having the same charge. Pure water used in this and previous studies was the CO_2 -free one. When the conductivity detector was used with the ultra hight sensitivities, the very small amount of H^+/HCO_3^- due to the dissolved atmospheric CO_2 caused a small water-dip. This problem could be overcome by removing the dissolved CO_2 from the water mobile phase, but this will diminish the simplicity of EIC, which is its most significant advantage. Finally, it should be noted here that the conditions of the supporting column (used to obtain a zwitterionic stationary phase) are very important factors for determining the separation-abilities of EIC. When an ODS-packed column, initially coated with CHAPS, is used as the supporting column (for Zwittergent-3-14 or other zwitterionic surfactant-immobilized stationary phase formation) superior separation properties than those of a newly packed ODS column are obtained.

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ION EXCHANGE HPLC DETERMINATION OF PYRIDINIUM CROSSLINKS IN URINE AS MARKERS OF BONE RESORPTION

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ABSTRACT

The aim of this study was to improve the published method[1] for rapid and sensitive determination of the urinary pyridinoline as well as deoxypyridinoline, i.e. the crosslinking elements of bone and cartilage collagen. This method is based on the HPLC analysis of previously prepared urinary hydrolysates. Urine hydrolysate (6 N HCl, 110°C, 16 hours) was purified by selective fractionation using washing with a mixture of water -acetic acid-butanol (1-1-4 by volume) on small disposable columns filled with microgranular cellulose. Liquid chromatography was based on ion-exchange counteractions and was properly performed with the use of a strong cation exchanger as a stationary phase and with a buffered solution at pH=3.35 as the mobile phase, by keeping the oven temperature at 48°C and with the injection of a volume of 10 µl. The time desired for HPLC determination alone didn't exceed 15 min and by using a fluorescence detector (Shimadzu RF 535) set at 297 and 400 nm for excitation and emmision wavelengths, respectively, it reached a sensitivity of about 200 femtomoles for both the crosslinks.

The method developed was experimentally tried in testing excretion levels of both markers of collagen tissue breakdown in different groups of subjects. The results obtained are in high correlation with the assumed conditions in the investigated groups and, therefore, this procedure seems to be very useful and effective as a sensitive indication of bone mass resorption processes.

INTRODUCTION

Collagen is the major structural protein in the human body and the crucial protein of bone. Its molecular chains are formed intracellularly in osteoblasts, where, among others, triple helical structure originates and partial hydroxylation of lysine and proline occurs. Procollagen molecules subsequently leave intracellular space, their N- and C- terminal propeptides are cleaved and fibril formation can be initiated. It may be noted, that non-collagenous structure of collagen propeptides prevent arranging in fibriles inside osteoblasts on the one hand, while on the other hand, the intracellular enzymatic hydroxylation of some lysine residues is an important step for covalent binding of mature collagen molecules in the extracellular matrix. Nevertheless, once synthetized and excreted from osteoblasts, procollagen molecules are, after propeptide cleavage, aggregated into fibrils, later covalently bound, and thus an extracellular matrix is organized.[2] The inherent strength and structural stability of bone tissue produced in such a manner[3] is stabilised e.g. by hydrogen bonds, hydrophobic interactions, and, above all, by lysinonorleucine and pyridinoline (nonreducible) covalent inter- and intra-molecular crosslinks between adjacent α-chains.

Hydroxylysylpyridinoline (pyridinoline, PD) and lysylpyridinoline (deoxypyridinoline, DPD) are derived from three hydroxylysine or two hydroxylysine and one lysine residue, respectively, following a cascade of enzymic and nonenzymic reactions (Fig. 1)[4,5] and both originate exclusively in mature collagen tissues.[6] It is believed that, whereas, the major crosslink PD is widely distributed in almost all collagen tissues,[3,7] its minor analogue DPD is found practically only in bone and dentin. Due to the slow metabolism of dentin, the latter is considered as bone specific, where the ratio of PD to DPD is about 3.5 to 1.[8,9] In the course of life there is a continual connective tissue turnover, so that osteoblasts activity is responsible for bone formation, whereas, osteoclasts affect bone matrix resorption. If one of these processes predominates, some disorder in bone turnover may occur. In such a case, it is very important to monitor concentrations of bone breakdown markers.

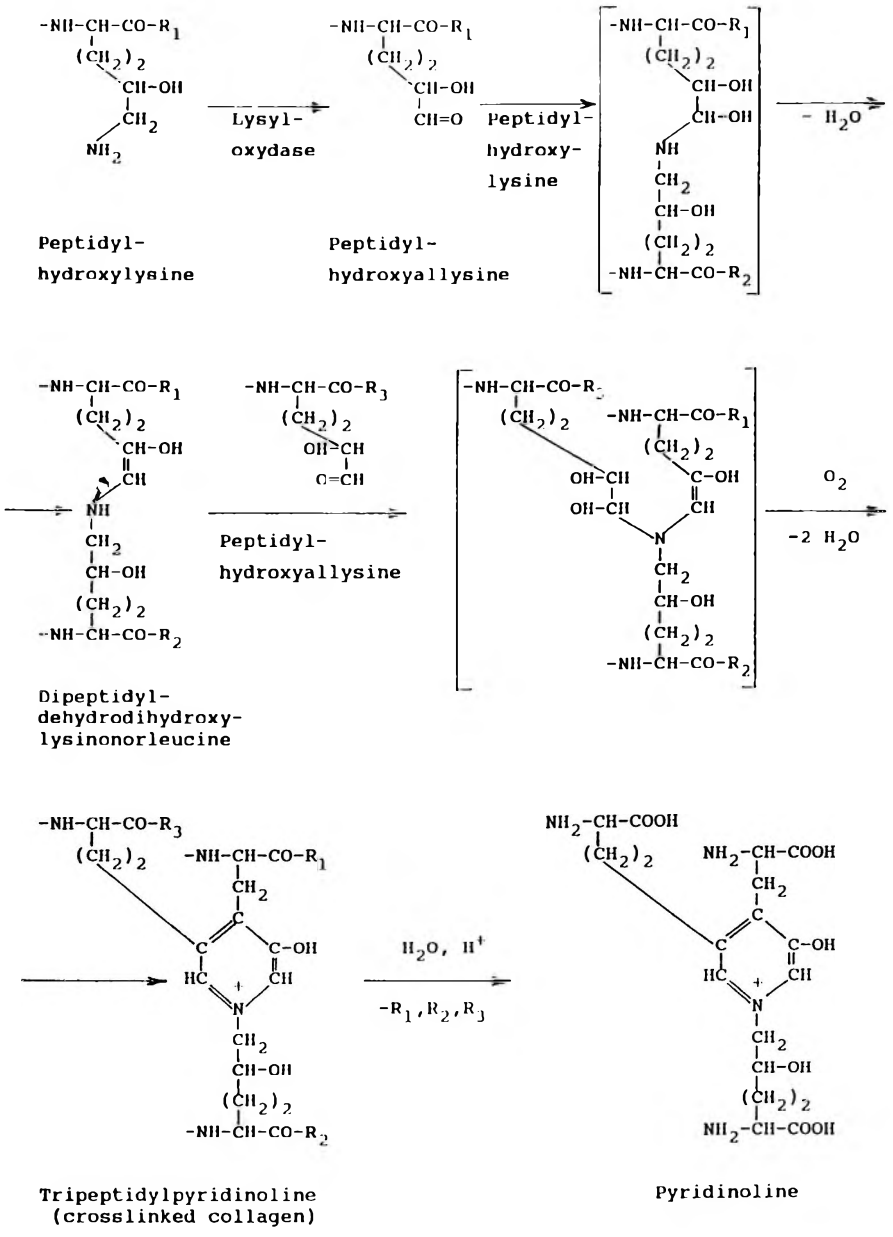

Figure 1. Possible mechanism of pyridinoline formation (schematically).

For such purposes urinary hydroxyproline determination is often used, but this determination is not specific enough as a bone collagen catabolism marker. Moreover, its urinary level depends on the diet and the presence of C1q and it is of a relatively lower sensitivity, especially in "slow losers" (only a slightly increased bone breakdown). This, as well as the possibility of its catabolism,[10-12] are the reasons why pyridinium crosslinks PD and DPD became still more useful in bone resorption monitoring, particularly in connection with some bone disorders such as osteoporosis, osteoarthrosis, Paget's disease, bone malignancies, etc. Both the markers are present in urine, partially in free form, partially bonded in various molecular weigh peptides.[13] Both are naturally fluorescent, enabling their sensitive detection, are unaffected by diet[14] and are not further catabolised.[15] However, due to their low levels in serum, clinical analyses are practically limited only to urine, i.e. UPD and UDPD determination.

Contemporarily, the most utilised methods for measurement of the pyridinium crosslinks concentration are based on ELISA, e.g.[13] as well as on HPLC methods. The ELISA method is very rapid and sensitive, but its selectivity is probably not sufficient each time.[16] HPLC is mostly employed in the well known, reverse phase modification, in the presence of ion-pairing agents, both with gradient[17] or (predominantly) with isocratic elution[18-20] as well. It seems, that ELISA can be very useful in screening the potentially at risk patient groups. But, for serious treatment of the selected subjects it is better to apply determination by the HPLC (which actually happens, as can be observed from the literature). The aim of this study is to improve the HPLC method for UPD determination,[1] previously published by our lab. It was based on ion-exchange chromatography (both crosslinks are special aminoacids). We wish to extend it for simultaneous measurement of both UPD and UDPD, apply it to selected groups of patients and to compare these results with the results of healthy subjects.

MATERIALS AND METHODS

Chemicals

Due to the fact, that both PD and DPD are not commercially available on the market, very often their isolation and purification is necessary, e.g. from cartilage.[3] In our case, both species are the kind gift of S.P. Robins, which enabled us to compare purity and verified the identity of our own prepared standards. n-butanol was partially obtained in HPLC grade from Fluka (Buchs, Switzerland), and partially together with other chemicals and solvents

from Lachema (Brno, Czech Republic). All were analytical reagent grade. Microgranular cellulose CC31 was purchased from Sigma-Aldrich (Prague, Czech Republic).

Patients and Samples

The utility of the modified and later described method was tested with fast urine samples in five different groups of subjects: 1) 28 controls (mean age 42), 2) 94 postmenopausal osteoporotic women (mean age 55), whose basal UPD and UDPD values were obtained before calcitonin therapy (Calsynar, Rhone Poulenc-Rorer). First control measurements were performed after 6 months of therapy and the second after 3 months, following the end of therapy. 3) 32 adolescents of both sexes (mean age 18), 4) 52 hemodialysed patients with chronic renal failure (mean age 51) and 5) 28 patients with blood malignancy (mean age 52). Urine samples were stored at -20°C in darkness until their later treatment (usually about 1 week), and it was found,[21] that under these conditions the tested stability of both crosslinks was 6 weeks and the extrapolated stability one can expect was about 25 years, respectively.

Sample Preparation

Aliquots of 2 mL urinary samples (Fig.2) were hydrolysed with an equal volume of the concentrated HCl (36% by weigh) at 110°C for 16 hours under N_2 atmosphere. 0.5 mL of each, thus obtained hydrolysate was mixed with 2.5 mL of n-butanol - acetic acid mixture (4:1 by volume) and loaded into a disposable column filled with 1 mL (volume of the sediment) of CC31 microgranular cellulose, which was fixed in the equipment with individually controled solvent flow (SPE Vacuum Manifold, Supelco SA, Gland, Switzerland).

Each of of the columns were then slowly rinsed with 12 mL of the mixture of n-butanol - water - acetic acid (4-1-1 by volume) to remove most of balast substances (especially hydrochlorides of amino acids, which are in these conditions reversibly sorbed), after which partially purified pyridinium crosslinks (irreversibly absorbed at these conditions) were at the very end eluted from the cellulose by 3 mL of deionised water (see Fig.2). The later (water) fractions were than desiccated in the vacuum rotatory evaporator SpeedVac (Philadelphia, USA) to dryness and reconstituted in 0.2 mL of the HPLC mobile phase before loading into the HPLC column.

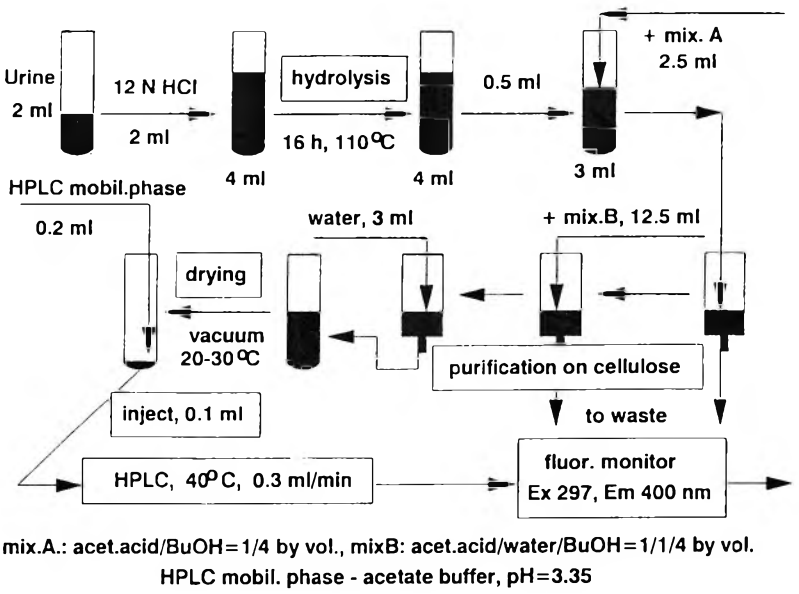

Figure 2. Overview of urine sample preparation.

Apparatus

A Spectra-Physics HPLC liquid chromatograph (SP 8100) with autosampler (SP 8110) was equipped with a Spectra-Physics computing integrator Chrom-Jet (SP 4400) and connected with a fluorescence monitor (Shimadzu RF 535).

HPLC

Before each chromatography series (mostly at the beginning of a day) a standard sample (pooled and well stored sample) was applied on the HPLC column for verifying of the column performance as well as the retention times of both the relavant crosslinks, i.e. UPD and UDPD. The columns used were cartridge type glass columns (CGC, 150x3 mm) filled with SEPARON HEMA-BIO 1000 SB (poly hydroxyethyl methacrylate modified with sulphobutyl groups). As stationary phase, spherical particle size 10 μm, (TESSEK, Prague, Czech Republic) was used. Isocratic mobile phase consisted of part A and B in a ratio of 70:30 by volume. Part A was 0.3M acetic acid with pH adjusted to 3.35 with 4M NaOH, part B was 0.45 M Na_2SO_4. A flow of 0.3 mL/min

generated a pressure gradient of about 2.4 MPa along the column. The column oven was thermostated at 48°C and the injected sample volume loop was 10 µl. The fluorescence detector setup was 297nm and 400nm excitation and emission wavelengths, respectively.

RESULTS AND DISCUSSION

Due to the fact that the principal aim of this study was to elaborate a HPLC method suitable for simultaneous determination of both the crosslinks and to verify its usefulness in some groups of previously selected subjects, we shall only briefly comment on the results obtained. The clinical aspects will be considered in our future intended study.

From the point of view of the time consumed and of the accuracy of the whole UPD and UDPD determination, it seems to be evident that the crucial step is foregoing separation and partial purification of hydrolysates (containing both crosslinks) on the cellulose-filled columns. Concerning this treatment, it is practically an independent chromatographic run, whose conditions should be adhered to. Even cellulose, which is used as the stationary phase, does not have adequate properties. However, it is usually employed (in our case as well). Unfortunately, under such conditions other naturally fluorescent substances eventually present in hydrolysate (e.g. desmosine and isodesmosine, the relevant crosslinks from mature elastin), can be adsorbed irreversibly on the cellulose surface. They can be the cause for the finding of a lot of undesirable peaks in the course of HPLC chromatography run. This could probably be the reason, why, when we work in commonly used ion-paired reverse phase HPLC mode, there is a greater occurence of interferring peaks close to the peaks which interest us. Therefore, we decided on an ion exchange principle, analogous to HPLC amino acid determination. Using fibrous cellulose, it was found[22] that this material was not ideal because of variations in packing density, which caused inconsistent elution of samples and large increases in pressure drop due to the elution of fine cellulose material after processing of several samples. This was the reason that often used fibrous cellulose (CF1) was substituted with microgranular cellulose CC31.[22]

The HPLC (alone) intra assay coefficient of variation (CV%) was 1.2% (obtained largely by use of the autosampler), estimated by 8 times multiple injecting of the stock standard urine sample previously elaborated on the only cellulose column in one step. The whole intra assay CV% was found to be 6.45%, determined by 5 times independent prefractionation of the same urine sample on 3 various cellulose columns, which can be considered as total uncertainty of the method elaborated. The inter assay CV% values differ

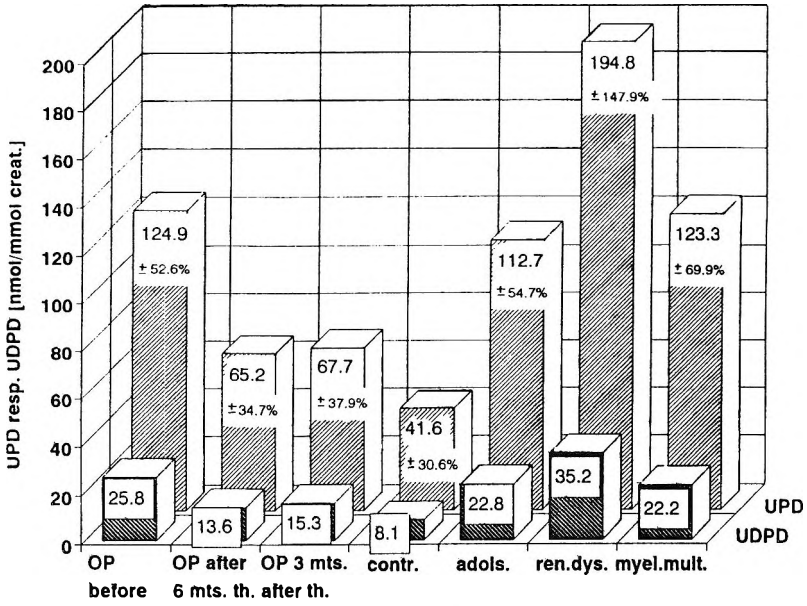

Figure 3. Results in some of the groups under study: UPD - mean values and coefficients of variation (CV%), UDPD - mean values only. Values are related to creatinine concentrations.

significantly depending on the subjects in the groups under study, and are summarized in Fig.3, together with mean values of UPD and UDPD, both related to urinary creatinine. In this figure, one can see that, in the group of osteoporotic women (OP), there were significantly elevated values of the crosslinks before therapy which, after 6 months of calcitonin therapy (Calsynar, Rhone Poulenc Rorer), markedly decreased to almost half of their basal values and, even during the following 3 months this tendency remained practically the same. Sex dependence was not found in our study. Relevant values in the control groups (i.e. healthy, premenopausal younger women) are slightly lower. Nevertheless, one would of course take into consideration, that, menopause can frequently start very rapid osteoporosis, so called "fast losers", when every retardation may be very beneficial.

Elevated values in the group of adolescents (Fig. 3) are in high agreement with the presumption of higher bone turnover, i.e. that acceleration of newly formed collagen matrix, as a consequence of osteoblasts function on one hand, is balanced by raising the rate of bone resorption by osteoclast action on the

other hand. Significantly higher concentrations of both the crosslinks in the group of chronically dialysed patients (next to last column in Fig. 3) give evidence that PD levels were influenced by serious system disease secondary altering bone metabolism, and for similar reasons, UPD and UDPD values in group with blood malignancy were probably elevated as well (myeloma multiplex, last column in Fig. 3).

In conclusion, it can be stated that, the described modified method yields relatively rapid, sensitive (limit of detection is about 200 femtomoles), and reproducible results, suitable enough for monitoring of kinetic and intensity of bone resorption in various groups of patients. It should be noted, that very good correlation was found (not shown) between both markers (corr. coef. R about 0.95). Therefore, when the ELISA method is used for the crosslinks determination, only one of them is sufficient.

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AN ISOCRATIC CONCURRENT ASSAY OF FREE METABOLITES, 4-HYDROXY-3-METHOXY MANDELIC ACID, 3-METHOXY-4-HYDROXY-PHENYLGLYCOL, NORMETANEPHRINE, METANEPHRINE, AND 5-HYDROXY-INDOLEACETIC ACID IN SAME SAMPLE OF URINE EXTRACT USING HPLC-ECD

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ABSTRACT

This paper describes a method for simultaneous extraction and concurrent quantification of metabolites of norepinepohrine (NE) and 5-hydroxytryptamine (5-HT) in human urine using an isocratic HPLC-ECD technique. These metabolites include 4-hydroxy-3-methoxy mandelic acid (VMA), 3-methoxy-4-hydroxyphenylglycol (MHPG), normetanephrine (NMTN), metanephrine (MTN), and 5-hydroxyindoleacetic acid (5-HIAA). The samples of urine were passed through preconditioned alumina-B column and washed with diethylether to remove interfering substances. Metabolites were extracted in ethylacetate.

The extracts were evaporated under nitrogen, reconstituted in water and injected into a 5μ spherical, C_{18}, reverse phase column for HPLC-ECD analysis. The analytes were eluted with mobile phase containing citric acid, sodium acetate, sodium octyl sulfate, Na_2EDTA, methanol 10%, and isopropanol 0.5%. Isoproterenol was used as an internal standard. Electrochemical detection was carried out at a potential of +0.60 V and a flow rate of 0.5 mL/minute. Peaks were characterized by their retention times. Concentrations were determined by the method using ratio of the peak areas of metabolites to that of internal standard.

A linear relationship between the ratio of the areas and concentrations was obtained between 5.0-90 ng/mL of each VMA, MHPG, NMTN, and MTN. The sensitivity for 5-HIAA was found to be ten times higher with a linear relationship between 1.0-8.0 ng/mL. Total elution time for all the metabolites was less than 20 minutes.

INTRODUCTION

The role of biogenic amines including catecholamines and serotonin have been implicated in a number of physiological and psychiatric dysfunctions including stress, depression, autonomic reactivity, eating disorders, mania and neurogenic tumors.[1-3] Concentration of monoamines and their metabolites in the cerebrospinal fluid (CSF) represents, most likely, the approximate central activity of monoamines, however, obtaining CSF is an invasive procedure and is not performed routinely. On the other hand, plasma measurement of monoamines and their metabolites is not considered to be reliable since they have a very rapid turnover and are found in extremely low concentrations. The major metabolites of norepinephrine (NE), epinephrine (E) and serotonin (5-hydroxytryptamine, 5-HT) are 4-hydroxy-3-methoxy mandelic acid (VMA), 3-methoxy-4-hydroxy phenylglycol (MHPG), nor-metanephrine (NMTN), metanephrine (MTN), and 5-hydroxyindoleacetic acid (5-HIAA) respectively, and are excreted in urine in high concentrations. These metabolites reflect activity of the brain as well as that of the peripheral noradrenergic, adrenergic, and serotonergic systems. Recently, quantification of normetanephrine (NMTN) and metanephrine (MTN) in 24-hour urine has been used for the diagnosis of hypertension,[4,5] myocardial infarction,[6] and muscular dystrophy[7] whereas, urinary excretion of 3-methoxy-4-hydroxymethylglycol (MHPG) and 4-hydroxy-3-methoxymandelic acid (VMA) has been used for the diagnosis of carcinoid syndrome, neuroblastoma, and pheochromocytoma as well as neurological and psychiatric disorders.[8-11] 5-HIAA, the major metabolite of 5-HT, has been associated with the diagnosis of behavioral problems[12] and

excessive urinary excretion has been used to diagnose carcinoid syndrome.[13,14] Analysis of these biogenic amines and their metabolites is also, increasingly, being used as markers of reactivity to field and laboratory challenges.[15] Studying the changes in the concentration of these metabolites of catecholamine as well as serotonin in 24 hours urine is therefore, of great importance and biologically more reliable not only for the diagnosis of diseases and behavioral problems but also for monitoring therapy.

In view of their importance, there is a constant search for developing simple, convenient, reliable, sensitive, and precise methods for quantification of these metabolites in a single sample. Earlier investigators had used separate methods for determination of each metabolite of catecholamines. VMA and MHPG were measured separately using gas chromatography.[16-18] Similarly, NMTN and MTN were each measured by separate methods. These procedures were quite reliable and specific but were complicated and time consuming since they involved derivatization of compounds and more than two metabolites could not be measured by one analytical method.[19] Recently, capillary gas chromatography has been used for measuring, simultaneously, a number of catecholamine metabolites in urine.[20] However, this method involves time consuming derivatization steps and is not convenient to use for monitoring changes in the activity of monoamines as a result of therapy. At our laboratories, we have earlier established HPLC-ECD methodology for quantification of 5-HT and 5-HIAA in platelets, plasma, and cerebrospinal fluid,[21] urinary free catecholamines,[22] catecholamine metabolites (MHPG and VMA) in urine,[23] as well as urinary normetanephrine and metanephrine, the methylated metabolites of norepinephrine and epinephrine respectively.[24] Although the techniques described in the above methods are very sensitive and reliable, they require separate HPLC-ECD analysis of the samples for different metabolites and, moreover, 5-HIAA could not be measured simultaneously with metabolites of NE and E. In the present report, we describe a simplified isocratic HPLC-ECD method for assaying, simultaneously, VMA, MHPG, NMTN, MTN, and 5-HIAA in a single sample of urine after purification on alumina B column, washing with diethylether and extraction with ethylacetate.

MATERIAL AND METHODS

Chemicals and Reagents

3-methoxy-4-hydroxy-mandelic acid, 4-hydroxy-3-methoxy phenylglycol, normetanephrine hydrochloride, metanephrine hydrochloride, 5-hydroxyindoleacetic acid, and isoproterenol hydrochloride, were purchased

from Sigma Chemical Co. (St Louis, MO). Octyl sulfate was obtained from Aldrich Chemical Co. (Milwaukee, WI). Alumina-B columns were purchased from BioRad Laboratories (Richmond, CA). Sodium acetate, citric acid, ethylenediaminetetraacetic acid (Na_2EDTA), and dibutylamine were obtained from Across Organics (New Jersey). All other HPLC grade chemicals, including diethylether and ethylacetate were obtained commercially. Alumina B columns were purchased from Waters Chromatography Division of Millipore (Millford, MA).

Mobile Phase

The solution used for eluting all the metabolites of our interest in the urine contained the following ingredients: citric acid 0.05M, sodium acetate 0.05M, sodium octyl sulfate 0.5mM, Na_2EDTA 0.075M, dibutylamine 0.5mM, methanol 10%, and isopropanol 0.5%. The pH of the solution was adjusted to 3.5 and solution was filtered through 0.45μm filter and degassed before use.

Standard Solutions

Standard solutions (stock) of VMA, MHPG, NMTN, MTN, 5-HIAA, and isoproterenol (ISOP) were prepared separately in HPLC grade water and each contained 1mg/mL of the metabolite. Each working standard solution was prepared just before use and contained 25ng VMA, 25ng MHPG, 50ng NMTN, 50ng MTN, 10ng ISOP (internal standard), and 3ng 5-HIAA in 50 μL of water.

The mixture of standard solutions was added for spiking either to water equivalent of urine volume or metabolite-free urine. Internal standard was added to the urine sample before extraction through Alumina-B column.

HPLC Equipment

HPLC-ECD system by Waters (Milford, MA) was used. The system consisted of electrochemical detector (ECD, Model 460) with a glassy carbon and auxillary electrodes and a Ag/AgCl reference electrode. Other parts of the equipment included an injector (U6K), a dual pump solvent delivery system (Model 590), and Waters data module, model 740. The chromatography column used for separation of metabolites was 5μ spherical, C_{18}, reverse phase, 3.9x150mm.

Metabolite-Free Urine (MFU)

A sample of urine (20mL) adjusted to pH 10.6 was exposed to light and air for a few days for metabolites to be degraded. Urine was then centrifuged at 3500 rpm for 15 minutes and the supernate adjusted to pH 6.5. Aliquots, 1.0 mL portions, were stored at -80°C until used.

Before calibration, MFU was spiked with known concentration of standards and extraction was carried out by the same procedure used for extraction of urine sample described below.

Identification of Individual Peaks

After equilibration of the system with elution buffer for a few hours at a flow rate of 0.5mL/minute, a 20 μL of working standard solution of the individual metabolites was injected sequentially to identify the peaks according to their respective retention times. A mixture of the working standards of all five metabolites and the internal standard was then injected for separating the peaks, and the HPLC system was calibrated with the aqueous standards.

Calibration of HPLC: Spiking of Standards

Water, 0.5 mL, was spiked with 50 μL solution containing 25 ng VMA, 25 ng MHPG, 50 ng NMTN, 50 ng MTN 10 ng ISOP, and 3 ng 5HIAA. The mixture was extracted from alumina B column by the procedure described below for urine sample. The extract was diluted appropriately and 20 μL was injected into the HPLC system.

Ratio of the areas under the individual peaks, with respect to internal standard, was calculated by the data module and concentrations were obtained as per mL urine. Similar procedure was used for calibration with MFU spiked with standards.

Standard curves of each metabolite were prepared with concentrations ranging as follows: VMA 2.5-80 ng, MHPG 10-80 ng, NMTN 10-90 ng, MTN 10-90 ng, 5-HIAA 1-10 ng, and a constant concentration, 10 ng of ISOP (internal standard) per milliliter. A 20 μL mixture of standard solutions was injected to validate the assay conditions, as well as to obtain coefficient of variations.

Extraction Procedure and HPLC Analysis

Preparation of alumina B columns

4.0 mL of distilled water was allowed to flow through the column followed by 4.0 mL of ethylacetate and 4.0 mL of diethylether. The column was allowed to be completely dry (20 minutes).

Extraction procedure

Urine, 0.5 mL containing internal standard or MFU, or water (pH 6.5, spiked with standards) was loaded on column and allowed to flow slowly. The fluid was not allowed to reach to the bottom of the column. The column was washed with 2.0 mL diethyl ether and metabolites were eluted with 3.0 mL ethylacetate into a glass tube. The eluate was dried at 37°C under N_2 and the residue was reconstituted in distilled water. After filtering through 0.2 μm filter appropriate dilution was made and 20 μL was injected into the HPLC system. Peaks of metabolites were separated at a flow rate of 0.5 mL/min and at sensitivity set at 5nAFs and potential at +0.60 V. Total elution time for all six eluents was set at twenty five minutes however, all the analytes were eluted within eighteen minutes.

RESULTS AND DISCUSSION

Chromatographic elution profiles of standards of VMA, MHPG, NMTN, MTN, ISOP (ISTD), and 5-HIAA are shown in Figure 1. A typical chromatogram obtained with aqueous standard mixture is shown in Figure 1A, with retention times of each metabolite. VMA was eluted at 3.680 minutes, MHPG at 5.220 minutes, NMTN at 7.325 minutes followed by MTN at 9.892 minutes. Internal standard ISOP was eluted at 13.532 minutes and the last eluent was 5-HIAA at 17.274 minutes. Although within 18 minutes all the analytes were eluted, running time of the chromatogram was kept at 25 minutes in order to allow for any electrical or room temperature fluctuations which may cause changes in retention times. The chromatogram in Figure 1B represents that of an extract of a spiked standard mixture with some changes in retention times of each eluent but, there was no disturbance in the order of elution of each metabolite. Figure 1C shows the profile obtained with a urine extract.

Standard curves were generated for each metabolite using a different range of concentrations. The area under the curve (AUC) obtained for the same concentration of different metabolites at the same sensitivity (5nAFS) was found to be a characteristic of each metabolite.

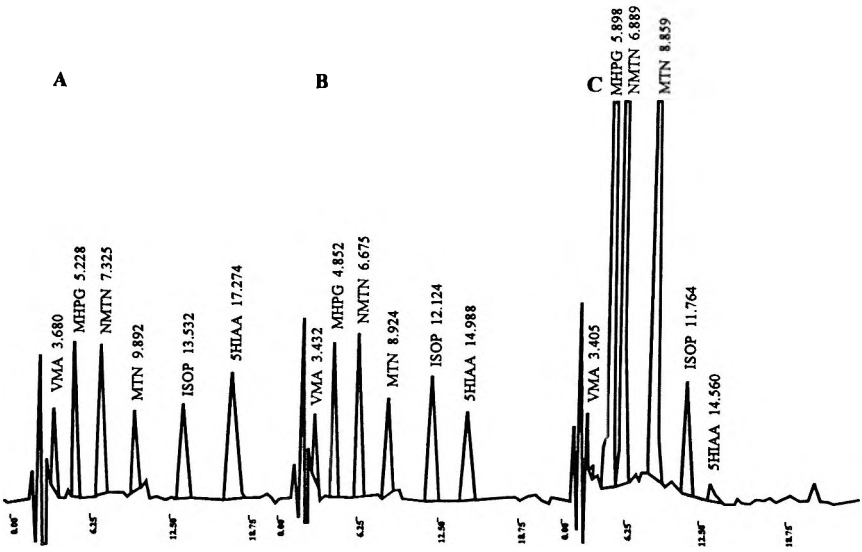

Figure 1. Chromatographic profile of standards of VMA, MHPG, NMTN, MTN, ISOP and 5-HIAA are shown in order of their retention times in 1A. Spiked standards of metabolites are shown in fig, 1B. Chromatogram 1C shows profile of metabolites in a urine extract.

For instance, AUC obtained for 5-HIAA was much greater than those of NMTN or MTN at the same concentration. Similarly, AUC for VMA and MHPG was greater than that of NMTN or MTN. Thus, the concentration required for 5-HIAA was much lower to achieve peak size similar to that of VMA, MHPG, NMTN, or MTN.

A standard curve, showing concentration as a function of ratio of AUC of the analytes to that of internal standard, for each metabolite is given in Figures 2 and 3.

The intra-assay coefficient of variation (CV) was determined from the ratio of the areas of curves of samples to that of ISOP (std) by injecting, repeatedly, seven times the same extract prepared from a single sample containing all five metabolites. CV for VMA was 6.92 %, for both MHPG and NMTN it was 4.5 %, for MTN it was 5.9 %, and for 5-HIAA the CV was 5.3 %.

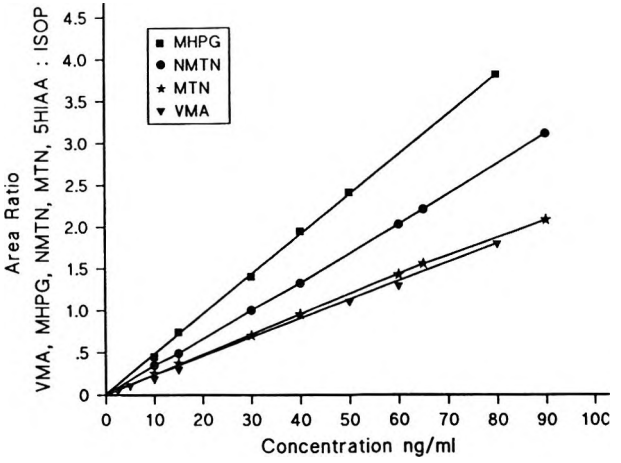

Figure 2. Standard curves of VMA, MHPG, NMTN and MTN show linear relationship between various concentrations and ratios of the areas of peaks of individual metabolites to that of the internal standard (ISOP).

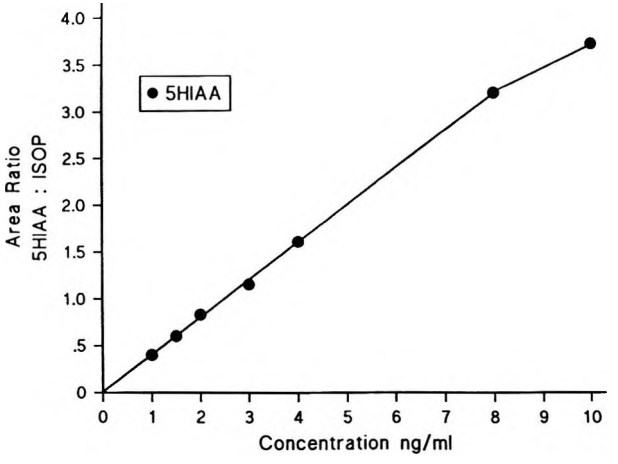

Figure 3. A standard curve showing a linear relationship between various concentrations of 5-HIAA and the ratio of the area of 5-HIAA peaks to that of ISOP.

The inter-assay coefficient of variation was determined from the percentage recovery after spiking seven different concentrations of each metabolite. The inter-assay coefficient of variation for VMA, MHPG, NMTN, MTN, and 5-HIAA was found to be 7.5 %, , 11.9 %, 8.69 %, 13.9 % and 16.17 %, respectively.

The percentage recoveries of all the metabolites were carried out using seven replicates and were found to range between 83 to 91. For VMA the recovery was 91.5±6.86 %, for MHPG it was 83.4±9.9 %, NMTN had a recovery of 88.3±7.7 %, MTN had 90.8±12.7 %, and 5-HIAA had 88.4±14.3 %. Validation of the procedure for urine samples was achieved by analysis of spot samples of urine obtained from normal subjects, which gave the values of free metabolites (ng/mL) VMA=22.7, MHPG=470.5, NMTN=293.3, MTN= 795.1, and 5-HIAA= 34.8.

Alumina B ion exchange columns used in this study for urine sample cleanup was found to be an important step for eliminating many interfering compounds which would otherwise interfere during the extraction procedure, as well as during HPLC analysis. Care was taken to condition the columns before loading the sample and to maintain a constant flow rate for proper elution of the metabolites of our interest. Extraction with two different solvents (diethylether and ethylacetate) sequentially improved recovery of metabolites. To the best of our knowledge, Alumina B columns have not been used earlier for urine sample clean up before solvent extraction and HPLC analysis of catecholamine and serotonin metabolites.

Addition of 0.5% isopropanol in mobilphase was found to increase the ion-pairing property, as well as decrease air bubble formation and stabilize the baseline. Isoproterenol was found to be a better internal standard compared with dihydroxybenzylamine (DHBA) and monohydroxybenzylamine (MHBA), with respect to its higher sensitivity and well separated peak from the analytes of our interest. There was no overlap of peak of ISOP with peaks of MTN or 5-HIAA.

Earlier studies have attempted to quantify simultaneously, VMA, MHPG, 5-HIAA, HVA, and DOPAC in urine using HPLC-ECD[25] but the recovery of all these metabolites was lower (56-80 %) compared to that achieved by the present method (83-91%) and the metabolites NMTN and MTN were not included in the analysis. The purification steps with Alumina B columns and diehylether wash in order to eliminate the interfering compounds, as well as an isocratic HPLC analysis in the present method were helpful for better recovery, as well as high sensitivity. Since the role of dopamine and its metabolites (DOPAC and HVA) in behavioral problems and in reactivity to challenges is

not clear, we attempted to include all major metabolites of NE, E, and 5-HT, that have been implicated in behavioral problems, so that total activity of these monoamines can be assessed in response to field stressors, or for diagnosing behavioral problems, as well as for follow-up for successful intervention in *different groups of patients. NMTN and MTN, together with VMA and MHPG analyzed simultaneously, should provide full profiles of NE, and E activity whereas, 5-HIAA levels reflect the activity of 5-HT system.

Analysis of these major and important five metabolites in a single sample of urine by one relatively simple method, is economical with respect to time, as well as expense since the method described can be used for the analysis of several urine samples in one day.

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HPLC/UV DETERMINATION OF SODIUM ACIFLUORFEN IN TROPICAL FISH

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ABSTRACT

This work reports the determination of sodium acifluorfen residue in fish species living in tropical rivers and lakes (*Piaractus mesopotamicus*). This determination was carried out through studies of LC_{50} - 96 hours and BCF- 192 hours (BCF = 1/100 LC_{50}). Controlled conditions in the static mode, with constant atmospheric air flow and dilution water at 25°C, were used in the laboratory tests. The fish samples were collected and submitted to appropriate analytical procedure. A method was developed that allows the residue quantitation of fish by HPLC/UV after soxhlet extraction and clean-up on silica gel and Florisil. The aquarium water samples were analyzed directly by HPLC/UV. The developed method proved to be adequate for LC_{50} and BCF determinations presenting recoveries above 75 %, with a relative standard deviation below 5 %.

INTRODUCTION

Pesticides have been widely used to eliminate forms of vegetable or animal life undesirable in agricultural cultivation and cattle raising, as well as in houses and gardens, and in public health projects, for combating the vectors which transmit illness.

These compounds, even when used correctly, can cause ecological and public health problems, favoring the appearance of new pests, eliminating pollinating insects and resulting in the slaughter of fish and birds. Another inconvenience caused by pesticides is the occurrence of toxic residues in foodstuffs, besides their persistence in the environment, especially in natural resources, so that they are transferred to other forms of life.[1]

The direct application of pesticides in lakes and rivers surfaces has been made to combat mosquitoes and other undesirable organisms, but their dispersion through the water bodies has been due especially, to the drainage water from soils contaminated by the systematic use of these pesticides in agriculture. Rain water transports these chemical compounds to the streams, rivers, lakes, estuaries and eventually to the oceans.[2]

Among the aquatic organisms, fish belong to the trophic levels in the aquatic food chain, which is used to evaluate water contamination. This evaluation of toxicity can be made by using such acute laboratory tests as Lethal Concentration (LC_{50}), as well as chronic tests called Bioconcentration Factor (BCF)[3]. BCF in aquatic organisms is primarily caused by passive partitioning of the chemicals between an aqueous (environment) and an organic (organism) compartment. BCF is also referred as the ratio of the concentration of a chemical in an organism to that in the environment, at steady-state equilibrium. It also correlates well with the chemical's octanol-water partition coefficient.[4-9]

Studies of the Bioconcentration Factor of these chemicals (accumulation directly from the aqueous environment), with representatives of various trophic levels such as algae, invertebrates and fishes, have demonstrated that increased bioconcentration occurs with increasing trophic level.[10]

Samples with high amounts of lipids, such as fish, are mainly extracted by soxhlet[11] or solid liquid extraction,[12, 13] and cleaned-up by adsorption columns containing adsorbents such as silica gel,[14] alumina[15] or Florisil,[11, 16-18] before being analyzed by techniques such as HRGC-ECD,[11] HRGC-MS,[13, 16] and HPLC-UV.[14]

This work determinated sodium acifluorfen residue in *Piaractus mesopotamicus*, a characteristic fish species in tropical rivers and lakes, through studies of LC_{50} - 96 hours and BCF - 192 hours (BCF=1/100 LC_{50}). These tests were carried out in laboratory controlled conditions in the static mode, with constant atmospheric air flow and dilution water (Standard Methods), at 25°C.[19] The fish samples were collected and submitted to an appropriate analytical procedure. A method was developed that allows the residue quantitation of fish by HPLC/UV after soxhlet extraction and clean-up on silica gel and Florisil. The aquarium water samples were analyzed directly by HPLC/UV.

EXPERIMENTAL

Fishes

Specimens of *Piaractus mesopotamicus*, in the juvenile period, were collected from the natural tank of CEPTA (Centro de Pesquisa e Treinamento em Aquicultura / Instituto Brasileiro do Meio Ambiente - IBAMA) Pirassununga - SP, Brasil.

The fish were acclimated in the laboratory for 30 days, in an asbestos box coated with epoxy resin layer. After this period of time, the fishes were transferred to a glass aquarium (100 L capacity) containing soft water, with concentrations of cations and anions controlled to be similar to the natural conditions. The population density was 1 g of fish per liter of water and the temperature was maintained at 25°C ± 1°C.

Acute Toxicity Test

The experimental procedure was based on EPA method 660/3-75-009.[20] The static system was selected as a model in a glass aquarium coated with a thin film of PVC to avoid contamination of the aquarium glass walls during 96 hours test.

Different concentrations of sodium acifluorfen, 69.28, 83.34, 90.71, 98.09 and 119.09 mg L^{-1} in water, were used during the experiment.

The minimum amount of sodium acifluorfen was determinated when the fish showed slight changes in their behavior and the maximum concentration, by 100 % death.

The LC_{50} calculation was carried out by computer using the JSPear Test method.[21] The dead organisms were collected and stored in a freezer (-18°C), until processed.

Bioconcentration Factor

BCF determination was realized in the same conditions as the acute toxicity tests (LC_{50}), with the organisms submitted to 1/100 of LC_{50} of sodium acifluorfen. The fish did not present a statistically measurable harmful effect during 192 hours of exposition.[22]

The experimental tests were performed in triplicate as the following:

- *blank*: soft water plus sodium acifluorfen (0.9184 mg L^{-1}); to evaluate chemicals and physical-chemical changes of the analyte during the test period.
- *control*: soft water plus organisms; to evaluate physiological conditions of organisms.
- *test*: soft water plus sodium acifluorfen (0.9184 mg L^{-1}) plus organisms.

Experimental protocol during tests: removing two fishes and 50 mL of aquarium water daily, which were stored in freezer (-18°C) or a refrigerator (10°C), respectively, for later analytical processing.

Sample

About 20 g of Pacu fish (*Piaractus mesopotamicus*) sample, taken as recommended by López et al.,[11] was cut and blended. Subsequently, the sample was freeze dried and submitted to extraction.

Extraction

A 1 g amount of sample was weighed and extracted in a Soxhlet extractor with 180 mL of methanol for 5 hours. The extract was then dried in a rotary evaporator under vacuum.

Clean-up

The residue was redissolved in 5 mL of methanol and transferred to a preparative column containing 10 g of silica, activated at 140°C for 4 hours and

conditioned with 30 mL of hexane. The column was sequentially eluted with 50 mL of hexane, 50 mL of dichloromethane, and 100 mL of methanol for the sodium acifluorfen. The methanol fraction was dried in a rotary evaporator and the residue was redissolved in 1 mL of methanol and then submitted to clean-up with Florisil, using a Florisil SPE cartridge conditioned with 10 mL of hexane. Elution involved 30 mL of hexane, that was discarded, 30 mL of dichloromethane and 40 mL of methanol. The fractions of dichloromethane and methanol were combined, dried under vacuum and dissolved in 1 mL of methanol, to be analyzed by HPLC/UV.

Analytical Conditions

The extracts obtained were submitted to HPLC analysis on a Shimadzu SPD-10 A liquid chromatograph, with a UV detector operated at 300 nm and a 20 μL injection loop. Analytical RP-HPLC separations were performed on a Supelco RP-18 (5 μm) column (250 mm x 4 mm I.D.) at 30°C. The initial elutions were performed with the mobile phase acetonitrile/water/acetic acid (60 : 40 : 15, v/v/v) at a flow rate of 1 $mL.min^{-1}$.

Recovery Study

This study was conducted through spiking an untreated sample of 1 g of fish or 20 mL of aquarium water with 1 mL of a standard solution of sodium acifluorfen in three different levels: 0.1, 0.5 and 1.0 mg L^{-1}. The fish sample was submitted to the extraction and clean-up processes. Water sample was analyzed directly without any preparative procedure. The tests were carried out five times to calculate standard deviation.

Quantitation

Sodium acifluorfen quantitation, in fish or water samples, was done by the external standard method. The sodium acifluorfen residue values for fish (R) in the samples were calculated according to the following equation:

$$R(mg/kg) = \frac{C \times Vf \times 100}{m \times r} \quad (1)$$

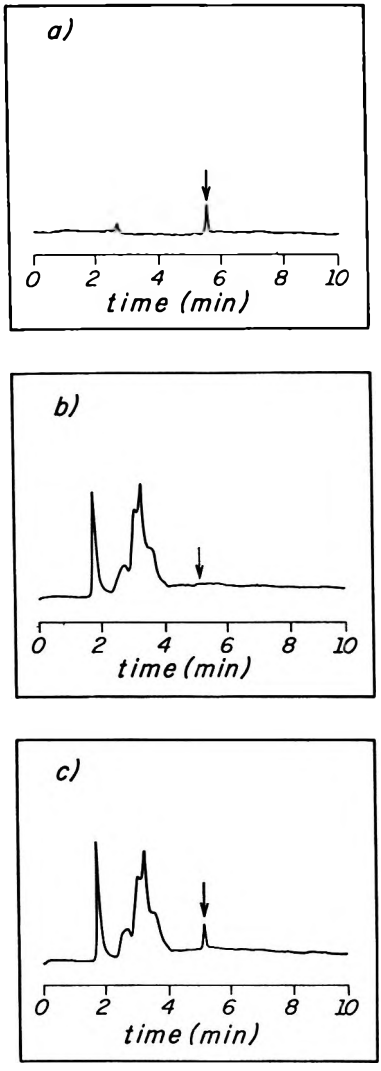

Figure 1. Chromatograms obtained by RP - HPLC with UV detection at 300 nm of: 1 mg L^{-1} of sodium acifluorfen (a), an untreated fish sample (b) and an untreated fish sample fortified with 1 mg L^{-1} of sodium acifluorfen. The arrows indicate the retention time of sodium acifluorfen.

Table 1

Recovery Study Using Fish and Aquarium Water Fortified with Sodium Acifluorfen in Three Levels and their Standard Deviations (S.D.) and Their Relative Deviations (R.S.D.)

Fortification Level (mg L^{-1})	Recovery (%)	
	Fish	**Water**
0.1	75.8 ± 5.2 (5.4)	94.1 ± 4.8 (5.0)
0.5	81.3 ± 4.9 (5.3)	97.2 ± 4.9 (5.5)
1.0	87.9 ± 5.2 (5.3)	99.3 ± 3.7 (4.5)

Recovery ± S.D. (R.S.D. %)

where:

C = concentration obtained from the analytical curve;
Vf = final dilution volume;
m = mass of fish;
r (%) = recovery.

RESULTS AND DISCUSSION

Figure 1, shows the chromatograms obtained with 1 mg L^{-1} of sodium acifluorfen standard (a), an untreated fish sample (b), and untreated fish sample fortified with 1 mg L^{-1} of sodium acifluorfen (c). A total absence of peaks at t_R = 5.7 minutes (retention time of sodium acifluorfen), in the chromatogram of an untreated fish sample (b), suggests no co-elution from matrix compounds.

Table 1, presents the results obtained in the recovery study of fish and aquarium water samples for three levels of sodium acifluorfen concentration.

The results obtained show that this method presented good recoveries for fish and water (higher than 75 %), presenting high efficiency, low standard deviation and good repeatability.

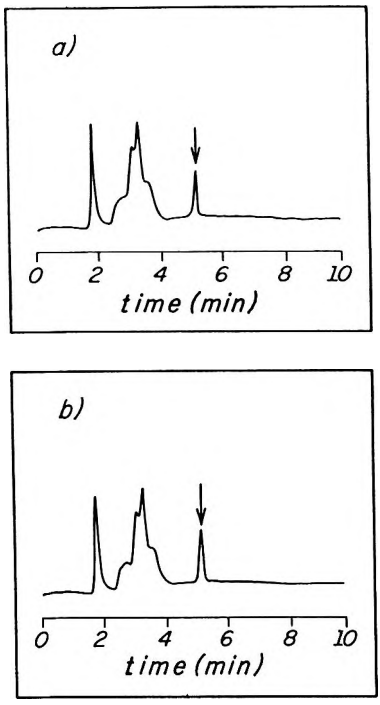

Figure 2. Chromatograms obtained by RP - HPLC with UV detection at 300 nm of: fish samples from the LC_{50}-96 hours test with sodium acifluorfen concentration in the aquarium of 69.28 mg L^{-1} (a) and 119.04 mg L^{-1} (b). The arrows indicate the retention time of sodium acifluorfen.

Table 2

Fish (*Piaractus Mesopotamicus*) Deaths Obtained in the LC_{50}-96 Hours Test Using 8 Test Organisms

Sodium Acifluorfen (mg L^{-1})	Mortality	LC_{50} (mg L^{-1})
69.28	0	
83.33	1	
90.71	5	91.84
98.09	7	
119.04	8	

Table 3

Residue (mg kg^{-1}) Determinated in Fish in the LC_{50}-96 Hours Test

Acifluorfen Sodium (mgL^{-1})	Residue (mg kg^{-1})
69.28	6.24
83.33	8.09
90.71	10.80
98.09	6.85
119.04	4.35

Acute Toxicity (LC_{50})

Figure 2, presents the chromatograms obtained with an extract of fish samples from the LC_{50} test at two sodium acifluorfen concentrations: 69.28 mg L^{-1} (a) and 119.04 mg L^{-1} (b).

Table 2 presents the deaths during the LC_{50}-96 hours test with different sodium acifluorfen concentrations with *Piaratus mesopotamicus* using 8 organisms in the juvenile period, with a 1 g of fish/L of water ratio. At the lowest concentration (69.28 mg L^{-1}) there were no deaths, but, in the subsequent experiments, the mortality increased proportionally with the increase in sodium acifluorfen concentration. The value obtained for LC_{50}-96 hours according to the JSPear program[21] was 91.84 mg L^{-1}, with a confidence range of 87.12-96.81 mg L^{-1}; where this concentration killed 50 % of the fish (*Piaractus mesopotamicus*).

In Table 3, the residue values (mg kg^{-1}) determinated for each sodium acifluorfen concentration in the LC_{50}-96 hours test can be observed.

The highest residue value determinated was 10.80 mg kg^{-1} when 90.71 mg L^{-1} of sodium acifluorfen was applied. This concentration approaches the determined LC_{50} value. After this point, increased sodium acifluorfen concentration decreased the residue value in fishes (Table 3).

Bioconcentration Factor

Figure 3 shows the chromatograms obtained with the aquarium water in the 8^{th} day of BCF test of water : *test* sample (a), *blank* sample (b) and *control* sample (c).

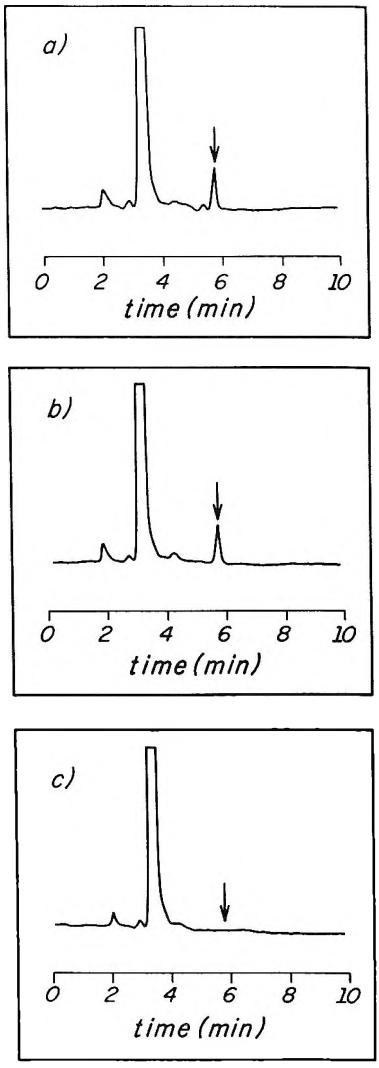

Figure 3. Chromatograms obtained by RP - HPLC with UV detection at 300 nm of water samples from the BCF test in the 8^{th} day using 0.9184 mg L^{-1} of Sodium Acifluorfen (1/100 LC_{50}) in *test* water (a) *blank* water (b) and *control* water (c). The arrows indicate the retention time of Sodium Acifluorfen.

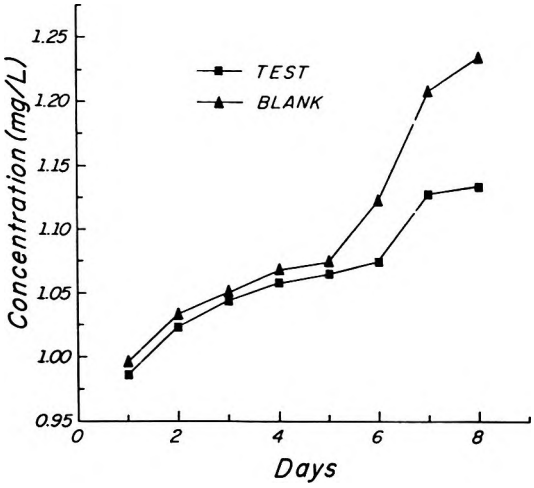

Figure 4. Study of the variation of the Sodium Acifluorfen concentration in the BCF test in aquarium water *test* and *blank*.

The chromatograms show the presence of sodiumacifluorfen at t_R = 5.7 minutes in the *blank* and *test* samples; note the absence of interfering peaks in the control sample. Figure 4 presents a study of sodium acifluorfen concentration during the BCF test. *Blank* aquarium water presented a slightly increased concentration during the time of test, which suggests evaporation of water. Concerning the test water aquarium, a decrease in the concentration of sodium acifluorfen occurred, which suggests the uptake of this compound by the fishes. No residue of sodium acifluorfen was detected present in fishes up to the limit of determination of the developed method (50 mg L^{-1}). It is likely that this compound is metabolized and eliminated by the organism, thus preventing the BCF determination.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ALKYL-CHAINED DIPHOSPHINES AND GROUP 6 AND 7 BINARY CARBONYLS

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ABSTRACT

This paper reports on the reversed phase high performance liquid chromatography (HPLC) of Group 6 carbonyl complexes [$M(CO)_6$ where M = Cr, Mo, W] and their Group 7 homonuclear [$M_2(CO)_{10}$ (M = Mn, Re)] and heteronuclear [$MnRe(CO)_{10}$] counterparts together with PPh_3 and its analogous alkyl-chained diphosphine ligands [$Ph_2P(CH_2)_nPPh_2$ where Ph = C_6H_5, n = 1 to 6]. Complete separations are achieved among the congeneric carbonyls and homologous phosphines on a reversed phase C_{18} column, using acetonitrile and water as the mobile phase. Separation between the Mn-Re bonded decacarbonyl from its parent, Mn_2 and Re_2 carbonyls, is also attained. The elution pattern is governed by the molecular size and mass and influenced by the nature of the central metal.

INTRODUCTION

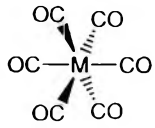
The use of HPLC as a separatory and synthetic tool in organometallic chemistry has been reviewed.[1] Recent developments have shown its value in the study of carbenes[2] and di,[3] tri,[4] and polynuclear[5] carbonyl complexes. We have also extended this technique to the separation of structurally similar homo-[6] and heterometallic[7] phosphine bridged complexes and co-ordination isomers.[8]

Surprisingly, however, little is known of the use of HPLC in the separation of the parent carbonyls and the free diphosphine ligands. Although, separation of these compounds by GC has met with some success,[9] the use of the technique for organometallics has its limitations because of the thermal sensitivity of many of the M-C bonded complexes. The separation of Gp 6 carbonyls has been reported by reversed phase HPLC on a C_{18} column, but complete separation could not be achieved.[10] In our previous work on Gp 7 binary carbonyls, a phenyl-column gave incomplete separation[8] under normal phase conditions. We were, also, not completely successful in separating the carbonyls on a silica column by normal phase chromatography, although, Coville *et al.*[11] had reported satisfactory separation on a preparative silica-based column (under normal-phase conditions). Little information is available on separation of the free diphosphines. As these materials are common precursors to a host of organometallic complexes,[12] it is imperative that a reliable method be developed, such that, the separatory conditions of these substances can be identified. This would alleviate the common problems encountered in the purification of inorganic reaction mixtures. It would also give a pointer to the study of more complex mixtures containing phosphine-substituted carbonyl complexes. In this paper, we report the use of a reversed phase C_{18} column in the separation of Group 6 & 7 carbonyls and all the common alkyl-chained diphosphines.

EXPERIMENTAL

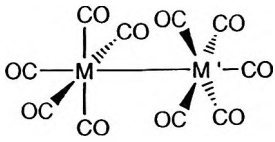
HPLC was performed on a Perkin-Elmer (Norwalk, CT, USA) Binary LC model 250 pump, equipped with a Perkin-Elmer model LC 290 variable wavelength UV spectrophotometric detector. A fully-endcapped Metaphase Crestpak (JASCO, Tokyo, Japan) $C_{18}S$ column (150 mm x 4.6 mm I.D.; 5 μm particle size; claimed 50,000 theoretical plates per metre) was used for separations. Chromatographic data were collected and analyzed on a Shimadzu (Tokyo, Japan) Chromatopac C-R6A data processor. UV detection was at 254 nm. The mobile phase used was acetonitrile-water (70:30, v/v; 80:20, v/v; 90:10, v/v) at flow rates of 0.8 mL min^{-1} and 0.6 mL min^{-1}.

Group 6 metal carbonyl compounds



	M
Complex **1**	Cr
Complex **2**	W
Complex **3**	Mo

Group 7 metal carbonyl compounds



	M, M'
Complex **4**	Mn, Mn
Complex **5**	Mn, Re
Complex **6**	Re, Re

free bis(diphenylphosphine) ligands

$Ph_2P—(CH_2)_n—PPh_2$

Complex **7**	dppm,	n = 1
Complex **8**	dppe,	n = 2
Complex **9**	dpppr,	n = 3
Complex **10**	dppb,	n = 4
Complex **11**	dpppe,	n = 5
Complex **12**	dpph,	n = 6

Figure 1. Structures of carbonyl complexes **1** - **6** and free diphosphine ligands **7** - **12**.

All solvents were of HPLC-grade from various suppliers. Sample solutions (conc. ~ 50 ppm) were prepared in pure acetonitrile, and were filtered through a 0.45 μm PTFE membrane filter before being introduced into the column by a Rheodyne Model 7125 injection valve. Typically, 5-10 μl aliquots were injected. HPLC runs were carried out, at least, in triplicate. The reproducibility of retention times between runs was ±2% or better.

With the exception of $MnRe(CO)_{10}$, all the compounds studied in this work were used as purchased. $MnRe(CO)_{10}$ was prepared according to literature methods.[9a]

RESULTS AND DISCUSSION

The structures of Gp 6 carbonyls, *viz.* $Cr(CO)_5$, **1**, $W(CO)_6$, **2** and $Mo(CO)_6$, **3**; Gp 7 carbonyls, *viz.* $Mn_2(CO)_{10}$, **4**, $MnRe(CO)_{10}$, **5** and $Re_2(CO)_6$, **6**, and the diphosphine ligands, *viz.* bis(diphenylphosphino)methane (dppm), **7**, 1,2-bis(diphenylphosphino)ethane (dppe), **8**, 1,3-bis(diphenylphosphino) propane (dpppr), **9**, 1,4-bis(diphenylphosphino)butane (dppb), 10, 1,5-bis(diphenylphosphino)pentane (dpppe), **11**, and 1,6-(diphenylphosphino) hexane (dpph), **12**, are shown in Fig. 1.

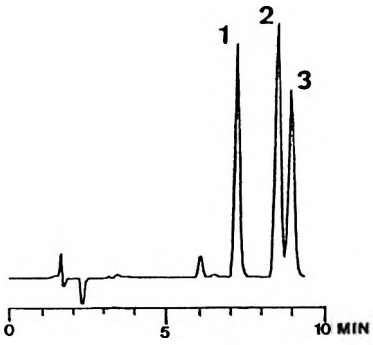


Figure 2. Reversed phase liquid chromatogram of Gp 6 metal carbonyl compounds on a Metaphase Crestpak C_{18} column (150 mm x 4.6 mm I.D.; 5 μm particle size). Mobile phase: acetonitrile-water (70:30). Flow rate: 0.8 mL min^{-1}. Detection wavelength: 254 nm. Peak identity: **1** = $Cr(CO)_6$ (k' = 2.55); **2** = $W(CO)_6$ (k' = 3.19); **3** = $Mo(CO)_6$ (k' = 3.39).

To arrive at a compromise between resolution and reasonable analysis times, different compositions of the mobile phase (acetonitrile-water) and flow rates were tested. The most satisfactory results for different groups of compounds were obtained under different conditions, as listed below:

a) Gp 6 metal carbonyl compounds: acetonitrile-water (70:30); 0.8 mL min^{-1}

b) Gp 7 metal carbonyl compounds: acetonitrile-water (80:20); 0.8 mL min^{-1}

c) Free diphosphine ligands: acetonitrile-water (90:10); 0.6 mL min^{-1}

The Gp 6 carbonyls can be completely separated by using the above conditions. $Cr(CO)_6$ was the most rapidly eluted, followed in turn, by $W(CO)_6$ and $Mo(CO)_6$. (Fig. 2). A similar trend was observed by Casoli *et al.*[10] on a reversed phase C_{18} column, although, the separation of the heavier congeners was incomplete. Our earlier use of a polar amino-cyano (PAC) column under normal phase conditions had failed to achieve any separations amongst this family.[6b,7] The superior performance of the Metaphase Crestpak C_{18} column (fully-endcapped spherical silica particles, with 18% carbon loading), in this case, is hence notable.

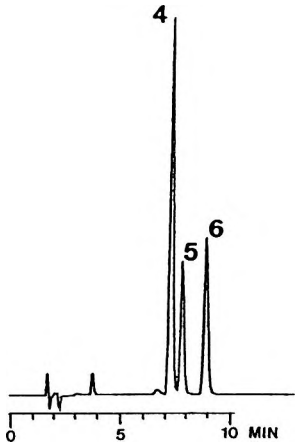


Figure 3. Reversed phase liquid chromatogram of Gp 7 metal carbonyl compounds on a Metaphase Crestpak C_{18} column (150 mm x 4.6 mm I.D.; 5 μm particle size). Mobile phase: acetonitrile-water (80:20). Flow rate: 0.8 mL min^{-1}. Detection wavelength: 254 nm. Peak identity: **4** = $Mn_2(CO)_{10}$ (k' = 2.58); **5** = $MnRe(CO)_{10}$ (k' = 2.84); **6** = $Re_2(CO)_{10}$ (k' = 3.39).

The elution behaviour has been rationalized based on the electronegativities of the central metal atoms (χ_{Cr} 1.56, χ_W 1.40, χ_{Mo} 1.30; Allred-Rochow scale), *viz.* elution time increases with decreasing electronegativities. Based on this principle, $Re_2(CO)_{10}$ (χ_{Re} 1.43) was expected to elute later than $Mn_2(CO)_{10}$ (χ_{Mn} 1.60). This is indeed observed with the heterobimetallic $MnRe(CO)_{10}$ sandwiching between the two homometallic dimers, all of which are segregated (Fig. 3). Although the $Re^{\delta+}$ - $Mn^{\delta-}$ bond is polarized, this does not appear to have any significant effect on the elution behaviour. The electronegativity factor is also manifested by the size effect. Large molecules are known to elute on HPLC after their smaller counterparts for solutes of similar molecular configurations and polarities. In fact, the proximity of the $Mo(CO)_6$ and $W(CO)_6$ peaks and their significantly larger capacity factors (k') compared to that of $Cr(CO)_6$ demonstrate the importance of the size influence.

The ability of the $C_{18}S$ column to facilitate the separation of $MnRe(CO)_{10}$ from its homonuclear parents is remarkable. Not only does it exhibit its effectiveness in dealing with congeneric mixtures, but also illustrates its value as a tool of purification in organometallic synthesis.

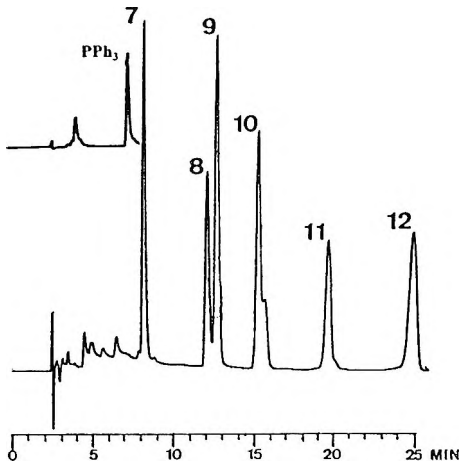


Figure 4. Reversed phase liquid chromatogram of triphenylphosphine (insert, on the same scale) and free diphosphine ligands on a Metaphase Crestpak C_{18} column (150 mm x 4.6 mm I.D.; 5 μm particle size). Mobile phase: acetonitrile-water (90:10). Flow rate: 0.6 mL min^{-1}. Detection wavelength: 254 nm. Peak identity: **7** = dppm (k' = 2.20); **8** = dppe (k' = 3.72); **9** = dpppr (k' = 3.95); **10** = dppb (k' = 4.97); **11** = dpppe (k' = 6.69); **12** = dpph (k' = 8.74) and **PPh_3** (k' = 1.75).

The syntheses of $MnRe(CO)_{10}$ from $Mn_2(CO)_{10}$ and $Re_2(CO)_{10}$ under photo- or thermolytic conditions or nucleophilic displacement of $ReBr(CO)_5$ by $Na[Mn(CO)_5]$ have been plagued with problems arising from the contamination of the homonuclear carbonyl parents[9a,13] The use of HPLC offers an effective purification means.

Our earlier success in the use of a PAC column in the separation of a series of Gp 6 phosphine-bridged dimers prompted us to use similar conditions for the free phosphine ligands. However the ligands appear to be strongly retained on the column based on a strong polar interaction between the amino-cyano bonded phase and the free phosphine sites in the ligands.[14] Under the present reverse phase conditions, PPh_3 and all the diphosphines tested are eluted according to the following order of increasing retention times: PPh_3 < dppm < dppe < dpppr < dppb < dpppe < dpph (Fig. 4). The full separation of this list of similar phosphines demonstrates the effectiveness of the $C_{18}S$ column in the use towards organometallic ligands. As expected, the difunctional phosphines are eluted after the monofunctional phosphine, PPh_3. The stepwise increase in the alkyl chain-length by adding a methylene ($-CH_2-$) unit is sufficient to influence and govern the elution behaviour of the diphosphines. in terms of size and mass effects. This is in contrast to our

finding earlier on the separation by normal phase HPLC on a PAC column, of a series of diphosphine-bridged complexes $M_2(CO)_{10}(\mu$-P-P) (M = Gp 6 metal; P-P = diphosphine) whereby the polarity of complex overshadows the mass effect induced by the ligand.[6] This difference is however not surprising considering the much larger size of the complexes than the ligands and the added influence of the metal centers with the associated metal-ligand bonds on the complex. It also shows that minor changes in metal and ligand environment are sufficient to alter the retention characteristics of these complexes.

The success experienced in this study of isostructural, congeneric and homologous compounds has encouraged us to examine other complex isomers which could not be separated by conventional chemical or chromatographic means. Current work is in progress in this direction on the same multimetallic clusters and aggregates.

ACKNOWLEDGMENTS

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DERIVATIZATION–HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF METHANOL IN HUMAN PLASMA

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ABSTRACT

A simple and sensitive high performance liquid chromatographic method is described for the determination of methanol in human plasma, as a highly sensitive derivative. The methanol, spiked in plasma, after simple ultrafiltration treatment, was derivatized with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one in a heterogeneous system, using benzalkonium chloride as phase transfer catalyst. The resulting derivative was chromatographed on a LiChrospher diol column with n-hexane:dichloromethane (9:1, v/v) as the mobile phase and 1-nitronaphthalene as the internal standard. The HPLC system showed good selectivity for methanol determination. Several parameters affecting the derivatization of methanol extracted from spiked plasma were investigated. The linear range for the determination of methanol in spiked human plasma was over 1-10 μmol/mL; the detection limit (signal to noise = 5, sample size 10 μL) of methanol was about 0.06 ± 0.02 μmol/mL.

The intraday relative standard deviation (n = 6) and the interday relative standard deviation (n = 8) were all below 5%. Recovery of methanol in spiked human plasma was greater than 98%.

INTRODUCTION

Methanol is one of the most popular solvents and finds extensive application in various industries. Consequently, the potential hazard of human exposure to methanol should not be neglected, especially that by inhalation[1-3] or consuming the methanol-contaminated alcoholic drinks. Methanol is rapidly and well absorbed by inhalation, oral and topical exposure routes. It is primarily metabolized in humans by an alcohol dehydrogenase system to formic acid.

Although formic acid accumulation is now reputed to be the cause of metabolic acidosis and ocular toxicity of methanol poisoning in humans, intraretinal metabolism of methanol, rather than elevated blood formic acid level, was suggested to be responsible for the retinal damage.[4] An accurate, sensitive method for determination of methanol in human plasma is very essential in clinical and forensic toxicology.

Many methods, including gas chromatographic (GC),[5-13] enzymatic,[14,15] spectrophotometric,[16,17] and high performance liquid chromatographic (HPLC)[18-21] methods have been described for the determination of methanol. Among these methods, a direct GC method, coupled with flame ionization detection and a capillary column, is the most widely used technique for determination of methanol in various matrices. The procedure of the direct GC method is simple, but its sensitivity is limited. Comparing with GC, relatively few HPLC methods have been described for the determination of methanol in plasma. Methanol is very simple in structure and transparent to UV detection; thus, the direct HPLC method usually has to be coupled with refractive index detection.

Therefore, with an attempt to increase the detection sensitivity, an analytical derivatization HPLC method, coupled with a UV detector is devised. The method is based on the chemical derivatization of methanol extracted from spiked plasma with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) in a heterogeneous system, using benzalkonium chloride (BAC) as phase transfer catalyst.

EXPERIMENTAL

Chemicals and Reagents

3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) and benzalkonium chloride (benzyl dimethyl n-tetradecylammonium chloride, BAC) (TCI, Tokyo, Japan), potassium hydroxide, 1-nitronaphthalene, methanol and ethanol (E. Merck, Darmstadt, Germany), dichloromethane, n-hexane and other reagents were of analytical reagent grade. Solutions of 1-nitronaphthalene and Br-MBX were prepared in dichloromethane as internal standard and derivatizing agent, respectively. Solutions of methanol and BAC, at various concentrations, were prepared by dissolving a suitable amount of methanol or BAC, respectively, in deionized water.

HPLC Conditions

A Waters-Millipore LC system with Model 510 LC pump, U6K injector, 746 integrator and a model 484 UV-VIS detector was used (Waters Chromatography Div., Millipore Corp., Milford, MA, USA). A LiChrospher diol column (250 x 4 mm I.D., 5 μm) and a mixed solvent of n-hexane:dichloromethane (9:1, v/v) as a mobile phase at a flow-rate of 1.2 mL/min were used.

The column eluate was monitored at 350 nm. The mobile phase was degassed with a vacuum filter before use.

Sample Preparation

A 270 μL volume of plasma was pipetted into a 10 mL glass-stoppered test tube, and 30 μL of aqueous solutions containing various amounts of methanol were added to each tube. The tubes were mixed for 10s. Then, 0.3 mL of 0.05 M potassium hydroxide solution was added and mixed by vortexing for 30s.

A 0.4 mL aliquot of spiked plasma sample was transferred into a Ultrafree-MC filter unit (30,000 NWML polysulfone PTTK membrane, Millipore, Bedford, MA, USA), then centrifuged at 2900g for 1h. A 0.2 mL aliquot of the ultrafiltrate was directly used for the derivatization as described under Derivatization Procedure.

Derivatization Procedure

A 0.2 mL portion of the plasma sample solution (after ultrafiltration) was pipetted into a 10 mL glass-stoppered test tube containing 0.1 mL of 0.5 M BAC solution. Then, 0.3 mL of 1-nitronaphthalene (80 μM) dichloromethane solution and 0.2 mL of Br-MBX dichloromethane solution (20 mM) were added. The reaction mixture was mechanically shaken for 2 h at 30°C in a thermostated water bath. At the end of the reaction, 3.0 mL of water was added to the reaction vessel, with slight shaking, to stop the reaction. After the separation of the organic phase, a 10 μL aliquot of the dichloromethane layer was injected for HPLC determination.

RESULTS AND DISCUSSION

The analytical derivatization of the extracted methanol (after simple ultrafiltration) with Br-MBX in a heterogeneous reaction system, using BAC as phase transfer catalyst, was studied. For the optimization of the extraction conditions of methanol from spiked plasma (2μmol methanol), and conditions for the derivatization of the extracted methanol, several related parameters, including the concentration of KOH, amount of phase transfer catalyst, derivatizing agent, and reaction time, were investigated. The effects of the tested parameters on the extraction/derivatization of methanol were evaluated by the peak-area ratio of resulting derivative to the 1-nitronaphthalene. Because of the high volatility of methanol, the possible loss of methanol in a plasma sample under ultrafiltration conditions should be considered. Before ultrafiltration, 0.3 mL of KOH at various concentrations were added to spiked plasma to study the effect on the extraction/derivatization of methanol. The yield of methanol derivative, evaluated by peak-area ratio, obviously increased at higher concentration of base, but the interference peak of the chromatogram was observed when the concentration of the added KOH was beyond 0.05 M. Therefore, 0.3 mL of 0.05 M KOH was selected as optimum alkali for determination of methanol extracted from plasma. Among the commonly used organic solvents, dichloromethane was found to be the optimum one for the derivatization of methanol in the heterogeneous system.

Effect of the Amount of Phase Transfer Catalyst

The effect of the amount of BAC on the formation of methanol derivative in the heterogeneous system was briefly examined. The results are shown in Figure 1. In the absence of BAC, no derivative was detected.

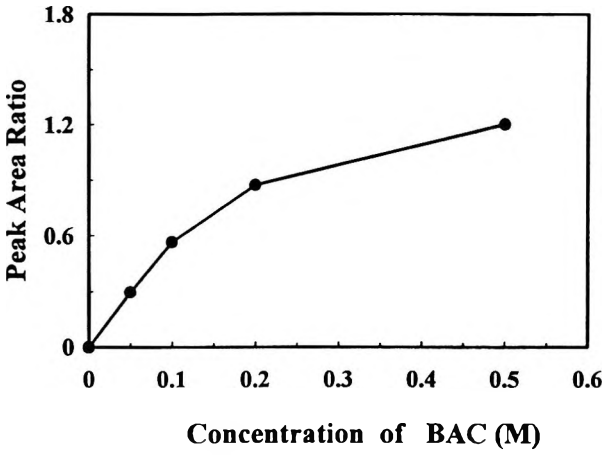

Figure 1. Effect of amount of phase transfer catalyst on the formation of the derivative of methanol extracted from human plasma.

As the concentration of BAC increased, the yield of derivatization of methanol increased. The solubility of BAC in water is limited to 0.5 M. So, 0.5 M BAC was selected as optimum phase transfer catalyst for determination of methanol.

Effect of the Amount of Derivatizing Agent

More than 4 μmol of Br-MBX was needed for derivatizing the methanol isolated from spiked plasma (2 μmol methanol) to a plateau formation of the derivative as shown in Figure 2. In this study, 4 μmol of Br-MBX was used for derivatization of methanol extracted from plasma.

Effect of Reaction Time

The effect of reaction time, at 30°C, on the derivatization of methanol extracted from plasma is shown in Figure 3. The results indicate that plateau formation of the derivative is not attainable in 3 h. That a long reaction time was required for the derivatization of methanol to reach the steady state is probably due, in part, to the high water solvation of methanol and ensuing poor phase partition.

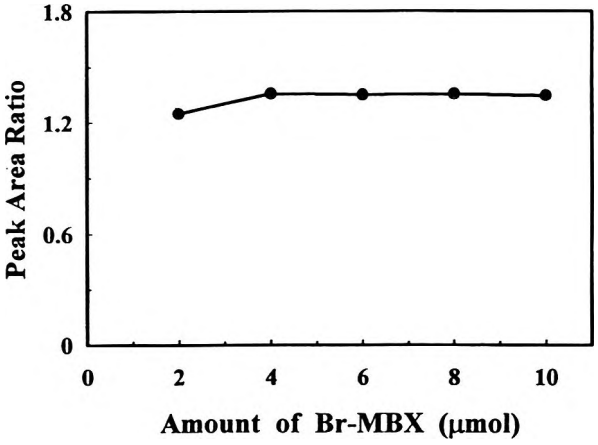

Figure 2. Effect of the amount of Br-MBX on the formation of the derivative of methanol extracted from human plasma.

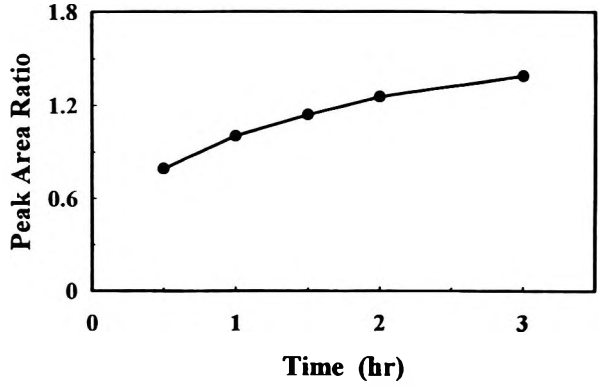

Figure 3. Effect of reaction time on the formation of the derivative of methanol extracted from human plasma.

In the light of the fact that in the reaction time of 2 h, the derivatization yield of methanol, which was evaluated by peak-area ratio, can reach more than 90% of that obtained from 3 h of reaction. The reaction time for determination of methanol in this study was set at 2 h.

Analytical Calibration

On the basis of the optimum extraction/derivatization conditions, we formulated the analytical procedure for methanol extracted from spiked plasma as described under the Methods section. The quantitative application of the method to the determination of methanol was evaluated at five different levels of methanol in the range of 1-10 μmol/mL spiked in plasma. The calibration graph was established with the peak-area ratio of the derivative to 1-nitronaphthalene as ordinate (y) vs. the amount of methanol in μmol/mL as abscissa (x). The linear regression equations obtained were

$y = (0.042 \pm 0.013) + (0.124 \pm 0.002)x$ for intraday assay (n = 6, r = 0.999);

$y = (0.038 \pm 0.006) + (0.124 \pm 0.001)x$ for interday assay (n = 8, r = 0.999).

The data indicate good linearity of the method. The detection limit (signal- to-noise ratio = 5) of methanol extracted from plasma is 0.06 ± 0.02 μmol/mL. The concentration of methanol in the range of 6.2-25 μmol/mL have been reported lethal to human beings.[22] So, the proposed method can be used in the analysis of biological samples of forensic interest.

Mass Spectral Analysis of the Derivative

A typical HPLC chromatogram for the analysis of methanol extracted from plasma is presented in Figure 4. Peaks 1 and 3 of the internal standard and the methanol derivative, respectively, in Fig. 4 did not receive interference from the reagent blank. The structure of the methanol derivative of peak 3 in Fig. 4 was briefly identified as 3-methoxymethyl-7-methoxy-1,4-benzoxazin-2-one by comparing the retention time with that of an authentic sample, which was synthesized and confirmed by electron ionization-mass spectrometry and a molecular ion at m/z = 221 corresponding to the methanol derivative was given. The stability of the methanol derivative, after derivatization, was studied over a period of 7 h; no significant change of the peak-area ratio was found. This indicates the favorable stability of the derivative for methanol analysis. The specificity of the method was studied by similar extraction /derivatization of ethanol, which usually coexists with methanol, from plasma spiked with 8 μmol of ethanol and 2 μmol of methanol, simultaneously.

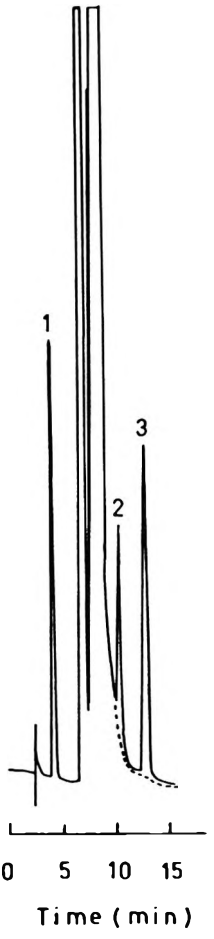

Figure 4. Composite HPLC chromatogram for the determination of methanol (solid line) in human plasma and reagent blank (dotted line). Peaks: 1, 1-nitronaphthalene (I.S.); 2, the derivative of ethanol; 3, the derivative of methanol. HPLC conditions: LiChrospher diol column (250 x 4 mm I.D.; 5 μm); mobile phase, n-hexane:dichloromethane (9: 1, v/v); flow rate, 1.2 mL/min; UV detection, 350 nm.

Under the present HPLC conditions, a composite chromatogram was obtained as shown in Fig. 4, indicating good separation of methanol derivative from the ethanol derivative (peak 2). The structure of the ethanol derivative was also tentatively identified as 3-ethyoxymethyl-7-methoxy-1,4-benzoxazin-2-one by electron ionization-mass spectrometry. The proposed method also revealed the potential for the analysis of ethanol in human plasma.

Table 1

Precision and Accuracy for the Analysis of Methanol from Spiked Plasma

Concentration Spiked (μmol/mL)	Concentration Found (μmol/mL)	R.S.D. (%)	Recovery (%)
Intraday* (n = 6)			
2.00	1.94 ± 0.08	4.12	97.0
5.00	5.13 ± 0.15	2.92	102.6
10.00	9.98 ± 0.11	1.10	99.8
Interday* (n = 8)			
2.00	1.95 ± 0.08	4.10	97.5
5.00	5.09 ± 0.14	2.75	101.8
10.00	9.92 ± 0.18	1.81	99.2

* Intraday data was based on six replicate analyses and interday data was from eight consecutive days.

Reproducibility and Recovery

The precision (relative standard deviation, R.S.D.) of the proposed method for analysis of methanol in human plasma at 10.0, 5.0 and 2.0 μmol/mL was studied. The results, presented in Table 1, show that the intraday and interday variances at the three concentrations were all below 3.0%, indicating satisfactory precision of the method. The relative recovery of the methanol, as shown in Table 2, is more than 97%, which was obtained from the calibration graph constructed from plasma spiked with different amounts of methanol over the range of 1-10 μmol/mL.

Stability of Methanol in Spiked Plasma

Methanol is a volatile substance and its stability in plasma under storage has to be considered. Two different concentrations of methanol at 5 and 10 μmol/mL of spiked plasma were studied to evaluate the stability of the methanol stored at -40 ± 5°C.

Table 2

Recoveries of the Analyses of Methanol from Spiked Human Plasma

Sample No.	Concentration Spiked (μmol/mL)	Concentration Found* (μmol/mL)	R.S.D. (%)	Recovery (%)
1	-	-	-	-
	5.00	5.18 ± 0.20	3.86	103.60
	10.00	10.02 ± 0.17	1.70	100.20
2	-	-	-	-
	5.00	5.08 ± 0.11	2.16	101.60
	10.00	9.93 ± 0.29	2.92	99.30
3	-	-	-	-
	5.00	5.17 ± 0.13	2.51	103.40
	10.00	9.93 ± 0.16	1.61	99.30
4	-	-	-	-
	5.00	5.04 ± 0.11	2.18	100.80
	10.00	9.89 ± 0.24	2.43	98.90
5	-	-	-	-
	5.00	5.02 ± 0.10	1.99	100.40
	10.00	9.88 ± 0.11	1.11	98.80
6	-	-	-	-
	5.00	5.07 ± 0.18	3.55	101.40
	10.00	9.92 ± 0.24	2.42	99.20
7	-	-	-	-
	5.00	5.04 ± 0.16	3.17	100.80
	10.00	9.86 ± 0.17	1.72	98.60
8	-	-	-	-
	5.00	5.08 ± 0.20	3.94	101.60
	10.00	9.90 ± 0.17	1.72	99.00

* Mean ± S.D. of three replicate analyses.

For each sample, determination of plasma methanol was performed on day 0, 3, 7, 14, 21, and 28. Statistical analysis of the results did not show a significant difference; therefore, methanol is stable in plasma samples stored at -40 ± 5°C for periods up to 28 days.

In conclusion, a simple and sensitive method based on the derivatization of methanol extracted from plasma with Br-MBX is developed. Separation of methanol from the spiked plasma by ultrafiltration, for the analysis, is very simple. The proposed method is expected to be feasible for the analysis of methanol in plasma related to the case of methanol poisoning.

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J. LIQ. CHROM. & REL. TECHNOL., 20(12), 1979 (1997)

ANNOUNCEMENT

AMERICAN MICROCHEMICAL SOCIETY 1998 A. A. BENEDETTI-PICHLER AWARD

The American Microchemical Society seeks nominations for the 1998 A. A. Benedetti-Pichler award in microchemistry. Nominations and eligibility are not restricted to members of the Society. Nominees from past years are eligible for renomination, although all information, including letters of nomination, should be updated and resubmitted.

The selection of the Award recipient is based on service to analytical microchemistry in its broadest sense, which includes research achievements in a wide variety of analytical chemistry disciplines and chemical measurements. The award also recognizes administration, teaching, or other means that promote the advance of microchemistry.

The nomination documents should include at least three letters of nomination, and a professional summary with a bibliography of publications. The letters of nomination should emphasize the candidate's accomplishments.

The deadline for nominations is Friday, October 24, 1997. The award consists of a plaque and travel expenses to the Fall, 1998 Eastern Analytical Symposium & Exposition, at which a symposium will be held in the awardee's honor. All documents should be sent to: Prof. R. G. Michel, Chemistry Dept., University of Connecticut, 215 Glenbrook Rd., Storrs, CT 06269-4060.

J. LIQ. CHROM. & REL. TECHNOL., 20(12), 1981 (1997)

ANNOUNCEMENT

1997 PREPTECH CONFERENCE

Industrial Separation Technology Conference
Hyatt Regency Hotel
Orlando International Airport, Florida

September 8 - 10, 1997

The 1997 PrepTech Conference will cover a range of practical topics in industrial separation science. The primary focus of the meeting is process-scale liquid chromatography applications in the pharmaceutical and biotechnology industries. Other topics of special interest to be included are process economics; regulatory compliance; integration of unit processes; sterilization/cleanup in place; reduction of solvent costs; applications of membranes; industrial-scale electrophoresis; simulated moving bed and continuous displacement chromatography; new column packings; and chiral separations.

The conference will present a 3-day program of technical lectures and poster sessions by international authorities and an exhibition supported by leading manufacturers of preparative chromatography and industrial membrane products.

Process development chemists and engineers with a professional interest in these and related areas are invited to submit abstracts for oral and poster presentations. Information regarding the technical program may be obtained from Robert Stevenson: Email rlsteven@crl.com; Tel: (510) 283-7619; FAX: (510) 283-5621.

Registration and exhibit information may be obtained from S. Galla, ISC Technical Conferences, Inc., 30 Controls Dr., Box 559, Shelton, CT 06484-0559, USA. Tel: (203) 926-9300; FAX: (203) 926-9722.

J. LIQ. CHROM. & REL. TECHNOL., 20(12), 1983 (1997)

EDUCATION ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

A Two-Day In-House Training Course

The course, which is offered for presentation at corporate laboratories, is aimed at chemists, engineers and technicians who use, or plan to use, high performance liquid chromatography in their work. The training covers HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC hardware module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection and Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration and Quantitation
- Logical HPLC System Troubleshooting

The instructor, Dr. Jack Cazes, is founder and Editor-in-Chief of the Journal of Liquid Chromatography & Related Technologies, Editor of Instrumentation Science & Technology, and Series Editor of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 35 years; he pioneered the development of modern HPLC technology. Dr. Cazes was Professor-in-Charge of the ACS Short Course and the ACS Audio Course on GPC, and has taught at Rutgers University. He is currently Visiting Scholar at Florida Atlantic University.

Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097. Tel.: (954) 570-9446; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(12), 1985–1994 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

AUGUST 2 - 9: 39th Rocky Mountain Conference on Analytical Chemistry, Hyatt Regency Denver, Denver, Colorado. Contact: Patricia Sulik, Rocky Mountain Instrumental Labs, 456 S. Link Lane, Ft. Colleins, CO, USA. (303) 530-1169; Email: gebrown@usgs.gov.

AUGUST 11 - 13: 10th International Symposium on Polymer Analysis and Characterization (ISPAC-10), University of Toronto, Canada. Contact: Prof. S. T. Balke, Dept. of Chem. Engg & Appl. Chem., Univ. of Toronto, Toronto, Ont., Canada, M5S 1A4. Tel/FAX: (416) 978-7495; Email: balke@ecf.toronto.edu.

SEPTEMBER 2 - 5: 12th International Bioanalytical Forum, Univ. of Surrey, Guildford, UK, sponsored by the Chromatographic Society (U.K.). Contact: Dr. E. Reid, 72 The Chase, Guildford GU2 5UL, U.K. Tel/FAX: (0) 1483-565324; Email: D.Stevenson@surrey.ac.uk.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

SEPTEMBER 8 - 10: 1997 PrepTech Conference, Hyatt Regency Hotel, Orlando International Airport, Florida. Contact: S. Galla, ISC Technical Conferences, Inc., 30 Controls Dr., Box 559, Shelton, CT 06484-0559, USA. Tel: (203) 926-9300; FAX: (203) 926-9722.

SEPTEMBER 14 - 17: International Ion Chromatography Symposium, Westin Hotel, Santa Clara, California. Contact: Janet Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052-0493, USA. Tel: (508) 359-8777; FAX: (508) 359-8778; Email: century@ixl.net.

SEPTEMBER 14 - 19: ACS Int'l Symposium on Systems Approach to Service Life Prediction of Organic Coatings, Breckenridge, Colorado. Contact: J. Martin, NIST, Bldg. 226, Rm B-350, Gaithersburg, MD 20899, USA. Email: jmartin@nist.gov.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

SEPTEMBER 22 - 25: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Fairmont Hotel, San Francisco, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 30 - OCTOBER 2: 53rd Southwest ACS Regional Meeting, Tulsa, Oklahoma. Contact: F. B. Growcock, Amoco Corp., E&PT, P. O. Box 3385, Tulsa, OK 74012, USA. Tel: (918) 660-4224; Email: fgrowcock@amoco.com.

OCTOBER 5 - 8: Conference on Formulations & Drug Delivery, La Jolla, California, sponsored by the ACS Div. of Biochem. Technol. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036. Tel: (202) 872-6286; FAX: (202) 872- 6013; Email: miscmtgs@acs.org.

OCTOBER 6 - 10: Validation d'une Procedure d'Analyse, Qulification de l'Appareillage; Application a la Chromatographie Liquide, Montpellier, France. (Training course given in French) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15; Email: hfabre@pharma.univ-montpl.fr

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 23: Sensors Expo: Conference on Exposition of Sensors, Detroit, Michigan. Contact: Expocon Mgmt. Assoc., 3363 Reef Rd, P. O. Box 915, Fairfield, CT 06430-0915, USA. Tel: (203) 256-4700; Email: sensors@expo.com.

OCTOBER 21 - 23: Biotechnica Hannover '97: Int'l. Trade Fair for Biotechnology, Hannover, Germany. Contact: D. Hyland, Hannover Fairs USA, Inc., 103 Carnegie Center, Princeton, NJ 08540, USA.

OCTOBER 21 - 24: 152nd Fall Technical Meeting & Rubber Expo'97, Cleveland, Ohio, sponsored by ACS Div. of Rubber Chem. Contact: ACS Meetings, 1155 16th St. NW, Washington, DC 20036 Tel: (202) 872-6286.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 25 - 30: 24th Annual Conference of the Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Providence, Rhode Island. Contact: ACS Div. of Anal. Chem., Tel: (301) 846-4797; FAX: (301) 694-6860; Internet: http://FACSS.org/info.html.

OCTOBER 26 - 29: ISPPP'97 – 17th International Symposium on the Separation of Proteins, Peptides, and Polynucleotides, Boubletree Hotel, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. FAX: (301) 898-5596.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 2 - 6: AAPS Annual Meeting & Expo, Boston, Mass. Contact: AAPS, 1650 King Street, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000.

NOVEMBER 2 - 6: 11th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Klyczynski, Electrosysthesis Co., 72 Ward Rd., Lancaster, NY 14086, USA.

NOVEMBER 6 - 8: 2nd North American Research Conference on Emulsion Polymers/Polymer Colloids, Hilton Head Is., SC. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561, USA.

NOVEMBER 7 - 8: 6th Conference on Current Trends in Computational Chemistry, Jackson, Miss. Contact: J. Leszcynski, Jackson State Univ., Chem. Dept., 1400 J. R. Lynch St., Jackson, MS 39217, USA. Tel: 601) 973-3482; Email: jersy@iris5.jusms.edu.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: AIChE Annual Meeting, Westin Bonaventure/Omni Los Angeles, Los Angeles, California. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

NOVEMBER 20 - 21: New Horizons in Chemistry Symposium, Los Angeles. Contact: J. May, Tel: (213) 740-5962; Email: jessy@methyl.usc.edu.

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FEBRUARY 28 - MARCH 1: 28th Annual Spring Prog. in Polymers: Degradation & Stabilization of Polymers, Hilton Head Is., South Carolina. Contact: A. V. Patsis, SUNY New Paltz, Inst. of Mater. Sci., New Paltz, NY 12561. Email: ims@mhv.net.

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 8 - 12: AIChE Spring National Meeting, Sheraton, New Orleans. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA.

MARCH 15 - 18: 14th Rocky Mountain Regional Meeting, Tucson, Arizona. Contact: R. Pott, Univ of Arizona, Tucson, AZ 85721.

MARCH 23 - 25: 5th Meeting on Supercritical Fluids: Materials and Natural Products Processing, Nice, France. Contact: F. Brionne, Secretariat, ISASF, E.N.S.I.C., 1 rue Grandville, B.P. 451, F-54001 Nancy Cedex, France. Email: brionne@ensic.u-nancy.fr.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036, USA.

MAY 3 - 8: HPLC'98 – 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques, Regal Waterfront Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; EMail: Janetbarr@aol.com.

MAY 20 - 22: 32nd Middle Atlantic ACS Regional Meeting, Wilmington, Delaware. Contact: G. Trainor, DuPont Merck, P. O. Box 80353, Wilmington, DE 19880-0353 Email: reglmtgs@acs.org.

MAY 26 - 29: VIIIth International Symposium on Luminescence Spectrometry in Biomedical and Environmental Analysis: Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Las Palmas de Gran Canaria (Canary Islands), Spain. Contact: Dr. Jose Juan Dantana Rodriguez, University of Las Palmas of G. C., Chem. Dept., Faculty of Marine Sci., 35017 Las Palmas de G. C., Spain. Tel: 34 (9) 28 45.29.15 / 45.29.00; FAX: 34 (9) 28 45. 29.22; Email: josejuan.santana@quimica.ulpgc.es.

MAY 27 - 29: 30th Central Regional ACS Meeting, Ann Arbor, Michigan. Contact: D. W. Ewing, J. Carroll Univ., Chem. Dept., Cleveland, OH 44118. Tel: (216) 397-4241. Email: ewing@jevaxa.jeu.edu.

JUNE 1 - 3: 31st Great Lakes Regional ACS Meeting, Milwaukee, Wisconsin. Contact: A. Hill, Univ. of Wisc., Chem. Dept., Milwaukee, WI 53201, USA. Tel: (414) 229-4256; Email: reglmtgs@acs.org.

JUNE 2 - 5: Third International Symposium on Hormone and Veterinary Drug Residue Anbalysis, Congres Centre Oud Sint-Jan, Bruges, Belgium. Contact: C. Van Peteghem, Dept. of Food Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel: (32) 9/264.81.34;

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483;

JULY 12 - 16: SFC/SFE'98: 8th International Symposium & Exhibit on Supercritical Fluid Chromatography & Extraction, Adams Mark Hotel, St. Louis, Missouri. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596; Email: Janetbarr@aol.com.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

AUGUST 31 - SEPTEMBER 3: AIChE Conference on Safety in Ammonia Plants & Related Facilities, Charleston Place, Charleston, South Carolina. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 13 - 18: 22nd International Symposium on Chromatography, ISC'98, Rome, Italy. Contact: F. Dondi, Chem. Dept., Universita di Ferrara, Via L. Borsari, 46, I-44100 Ferrara, Italy. Tel: 39 (532) 291154; FAX: 39 (532) 240707; Email: mo5@dns.Unife.it.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu, S.C.A.R., Faculty of Chemistry, University of Bucharest, 13 Blvd. Reepublicii, 70346 Bucharest - III, Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Res. Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

NOVEMBER 15 - 20: AIChE Annual Meeting, Fontainbleu/Eden Roc, Miami Beach, Florida. Contact: AIChE, 345 East 47th St., New York, NY 10017, USA. Tel: 1-800-242-4363.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA.

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

JUNE 25 - 30: HPLC'2000 – 24th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Seattle, Washington. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA. Tel: (301) 898-3772; FAX: (301) 898-5596.

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

DECEMBER 14 - 19: 2000 Int'l Chemical Congress of the Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th St., NW, Washington, DC 20036, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: pacific@acs.org.

2001

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

AUGUST 25 - 31: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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.

2003

MARCH 23 - 28: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 12: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 2: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC.

AUGUST 22 - 27: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 18: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 2: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2006

MARCH 26 - 31: 231st ACS National Meeting, Atlanta, GA. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 10 - 15: 232nd ACS National Meeting, San Francisco, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2007

MARCH 25 - 30: 233rd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 24: 234th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs @acs.org.

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1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

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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