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THE INFLUENCE OF UREA ON THE RETENTION OF PROTEIN IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

The retention behaviour of eight proteins in high performance hydrophobic interaction chromatography (HPHIC) in a mobile phase consisting of ammonium sulphate-potassium dihydrogen phosphate containing urea was studied. By using the stoichiometric displacemant model for retention (SDM-R) of solute in liquid chromatography, and by measuring surface tention of the mobile phase in the presence of urea, also and UV-spectra of proteins, it was found that in the presence of urea in the mobile phase of HIC, even though the urea somewhat strong solvent in terms of result, the change of the conformation of protein molecular in hydrophobic interaction systems due to the presence of urea is considered as the major contribution to the retention of proteins.

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INTRODUCTION

Urea and guanidine hydrochloride are solubilizing and denaturing agents for proteis, They are often used for the extraction of proteins from bacterium body [1].For instance,7.0mol/L guanidine hydrochloride or 8.0mol/L urea solution is used for the extraction of very strongly hydrophobic proteins but which causes denaturation. Therefore refolding and purification are needed in order to obtain bioactive and pure proteins. It has been demmostrated that this could be renatured by High performance hydrophobic interaction chromatography[2,3]. Because of the small injection volume, the urea or guanidine hydrochloride in the sample has a negligible influence on the retention of proteins. The addition of urea or guanidine hydrochloride to the eluent to increace the solubility of the proteins has a significant influence on the retention characteristic of proteins. In our previous works[4,5] we found that urea in a concentration of 3.75mol/L influenced the retention behaviour of proteins in HIC significantly. Because the concentration of urea is constant during the separation process, the molecular conformation of the protein didn't change, When the concentration of urea is not certain, but changeable during separation, for example, in the preparative chromatography large volume of sample is necessory, how does the denaturing agent influence the retention behaviour of protein? Many studies on the retention mechanism of protein in hydrophobic chromatography[6,7] did not include molecular conformation changes during separation into their theory. In a previous paper[8] we have investigated the influence of guanidine hydrochloride on retention. In this continuation paper reports the results of the influence of the concentration of urea in the mobile phase on the retention of proteins under the aspect of the change of the molecular conformation.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of three isocratic solvent pumps (Model LC-6A, Shimadzu, kyoto, Japan) with a system controller (Model SCL-6B,Shimadzu). Detection was performed by a UV-VIS detector (Model SPD-6AV, Shimadzu, Kyoto, Japan). Silica (7μm, 500Å) was modified to having ether end-group and packed into a 40mm ×2mm stainless-steel column by means of a column packing apparatus (Model 1224, Chemico, Osaka, Japan). The UV-spectra of protein solution were recorded by a Spectrophotometer (Perkin-Elmen, Norwalk, Connecticut, USA).

Chemicals

Cytochrome C (Cyt-C), myoglobin (MYO), ribonuclease (RNase), bovine serum albumin (BSA), concanavalin A(Con A),Ferritin (FER), a-chymotrypsinogen A (a-CTY-A) were obtained from Sigma (St. Louis, MO, USA), Lysozyme (LYS) from Dongfeng Biochemical Factory (Shanghai, China). Other chemicals were purchaesd from Xi'an Chemical Co. (Xi'an, China) and were analytical grade.

Eluent Composition

The mobile phases were prepared with three pumps using following solutions: solution A, 3mol/L ammonium sulphate-20mmol/L potassium dihydrogen phosphate, pH 7.0; solution B, 8mol/L urea-20mmol/L potassium dihydrogen phosphate, pH 7.0; solution C, 20mmol/L potassium dihydrogen phosphate, pH 7.0.

Operation

By mixing of the three solutions various mobile phases with different concentration of urea were obtained. The mobile phase was filtrated and sonicated (5min.) before using. The column was equilibrited with at least 5 ml new mobile phase before injecting a sample solution. The flow-rate was set up at 0.2ml/min and eluent was monitored at 280nm.

The dead volume of chromatographic system was deteremined with solution C. The concentration of water in mobile phase was measured according to the following equation[6]

$$[H_2O] = \frac{d_A\varphi_A + d_B\varphi_B + d_C\varphi_C - W_S}{0.018}(mol/L)$$

where d_A. d_B and d_C are the densities of solution of A, B, and C, respectively, φ_A, φ_B and φ_C are the volume fractions (V/V) of solutions A, B and C in the mobile phase, respectively, W_S is the amount of salt in the mobile phase. The surface tention of mobile phase was determined according to literature[9].

RESULTS AND DISCUSSION

Generally, in order to purify a certain protein which has a strong hydrophobicity from inclussion body or other origins, firstly make it denatured with 8.0mol/L urea, or 7.0mol/L guanidine hydrochloride, then make it refolded and purified. HIC has been considered to be not only a separation method but also a tool for protein refolding[2]. In the condition of the gradient elution model in HIC, the weak eluent is an aqueous solution of 2.5-3.0mol/L sodium sulphate or ammonium sulphate and so on. The addition of 8.0mol/L urea would cause the precipation of proteins and/or salts. According to the different proteins we used

urea of a high concentration as possible, but without causing any precipatation in chromatographic systems.

The retention values of BSA for various concentration of urea in HIC are presented in Fig.1. It shows that the retention of BSA drop with increacing concentrations of urea. The same results were obtained at other proteins (Table 1). The results suggest that the elution strength of urea in HIC is comparable the water.

Influence of the Surface Tention on the Retention of Proteins

It would be helpful that we measure the relationship between k′ value and surface tention of the mobile phase of ammonium sulphate-potassium dihydrogen phosphate containing urea of different concentrations. As shown in Fig.2, it was found that the surface tention increased with the concentration of urea.This is similar to the system of guanidine hydrochloride[8].At low concentrations of urea. the surface tention of eluent increased more quickly than at high concentrations, Horvath found that[10] the retention of proteins increases proportionally to the surface tention of mobile phase. Our results are in contradiction to the one of Horvath. He and co-workes , which used only non-denaturating salts in their eluent, found that the retention of proteins increases proportionally to the surface tention of the mobile phase. The addition of the denaturating urea to the eluent changes the retention behaviour of proteins. A nonlinear drop of the retention with increacing concentrations of urea was observed.

The results above show that the urea existed in the mobile phase of HIC don't cause only the change of surface tention of the system similar to water.

Relationship between [H_2 O] in Mobile Phase and k′

One of the authors[6,11] pointed out that the most rational retention mechanism of proteins in hydrophobic interaction chromatography and in reversed-phase

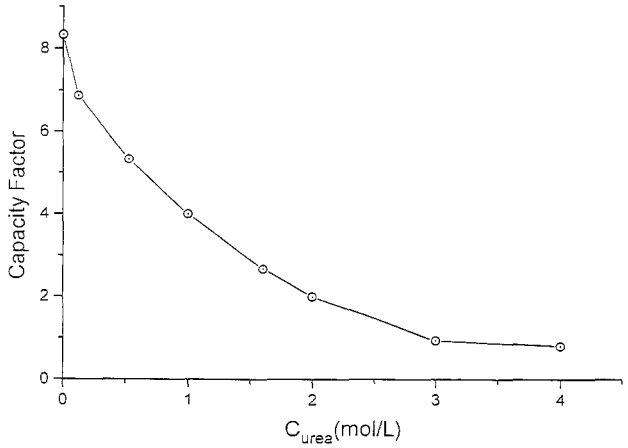

Fig. 1. Dependence of the retention of BSA on the concentration of urea. Condition: see experimental except eluent: 0.60mol/L $(NH_4)_2SO_4$ - 20mmol/L KH_2PO_4 -Urea.

TABLE 1 Capacity Factores of Eight Proteins in Different C_{urea}

C_{urea}	Cyt-t	MYO	RNase	LYS	BSA	Con.A	FER	α-CTY-A
0.00	10.67	14.00	11.33	28.93	8.33	11.67		
0.10				22.33				
0.12	10.33	13.33	11.33		6.87	8.00		
0.50				14.67				
0.52	8.33	9.33	11.33		5.33	5.33		35.67
1.0	6.00	6.80	9.67	8.33	4.00	3.33	6.00	17.33
1.5				4.67				
1.6	4.00	4.00	5.67		2.67	2.00	2.33	9.27
2.0	3.34	3.67	4.00	2.84	2.00	1.67	1.33	6.67
3.0	2.00	2.67	2.00	1.17	0.93	0.80	0.80	3.33
4.0	1.80	1.20	0.87	0.73	0.80	0.73	0.73	1.33
5.0	1.00	0.87	0.80					0.80
6.0	0.93	0.80						

Composition of the mobile phase: 20mol/L KH_2PO_4 -$(NH_4)_2SO_4$ 4-Urea. The used $C_{(NH_4)_2SO_4}$ for Cyt-C, Myo, RNase, Lys, BSA, Con A, Fer, α-CTY-A were separately 1.80, 0.75, 1.56, 1.05, 0.60, 1.12, 0.45, 0.75 mol/L

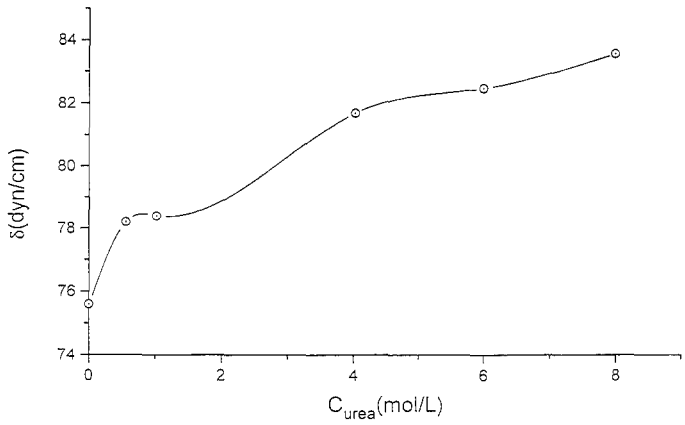

Fig. 2 The effect of urea on the surface tention.
Condition: aqueous solution of 1.40mol/L $(NH_4)_2SO_4$ -20mmol/L KH_2PO_4 -Urea. Others see experimental.

chromatography might be a stoichiometric displacement process between protein and solvent molecules, with water as displacing agent. Hence the plot of the logarithm of capacity factor of proteins *versus* the logarithm of concentration of water in the mobile phase should be linear. This was conformed for high concentration of water in the eluent. At low concentration a nonlinear relationship was found (Fig.3). This can be explainted by the fact that the concentration of urea is low when the concentration of water is high in the mobile phase[6]. The parameters of the plots of logk′-log[H_2O] for the eight proteins are shown in Table 2.

It is important that retention increaces with the increasing concentration of water in the eluent. This result is contradictory to the system of ammonium sulphate-water. In other words, when the eluent contains urea, water couldn't be seen as the displacing agent in the system of HIC. But from the fact that the k′ value of the proteins decrease with increacing concentrations of urea in high

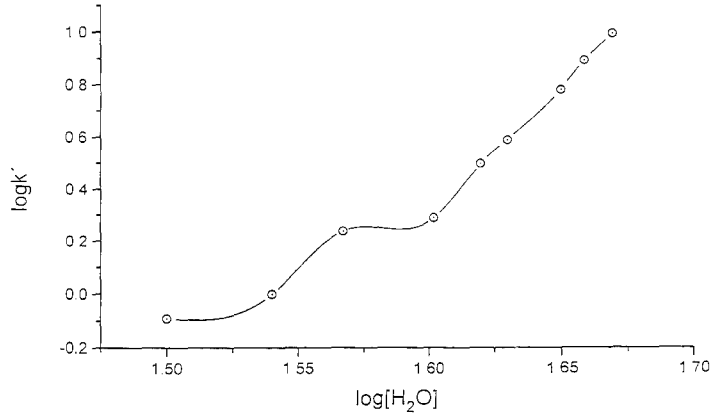

Fig. 3 Plot of logk′ of Cyt-C against log [H_2 O] in the mobile phase condition: see experimental except eluent: 0.40mol/L (NH_4)$_2$ SO_4 - 20mmol/L KH_2 PO_4 -Urea.

TABLE 2 The Parameters of the Plot of logk′-log[H_2 O]*

Protens	Cyt-c	MYO	RNase	LYS	BSA	Con. A	FER	α-CTY-A
C	0.9659	0.9685	0.9839	0.9847	0.9811	0.9644	0.9084	0.9803
Z	6.35	7.44	10.6	16.7	10.1	10.7	11.3	14.4
LogI	-9.71	-11.7	-16.8	-27.1	-16.3	-17.5	-18.5	-23.1

* The meanings of parameters C, Z, logI see ref.[6]

concentration, urea seems to be the displacing agent in HIC. However, when we ploted the logk′-logC_{urea} according to the stoichiometric displacement concept, the curve is not straight too. This means disagreement with the view of considering urea to be the displacing agent in HIC system.

The Change of Molecular Conformation of Proteins

It is well known that proteins change the three-dimesional structure in 8.0mol/L urea. But when the concentration of urea is relative low (0-6.0mol/L),

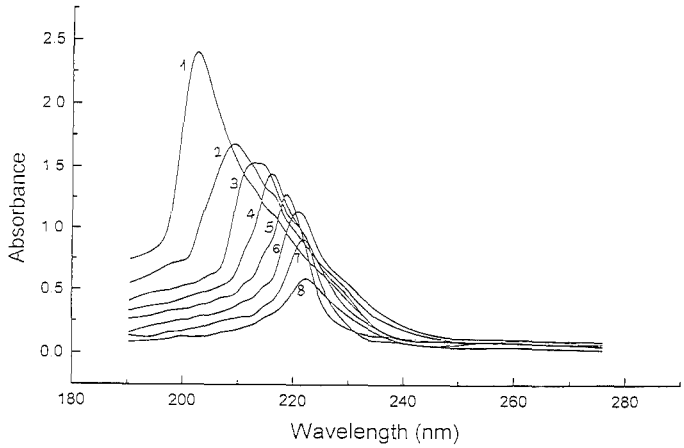

Fig. 4 UV-spectra of BSA in the solution with different C_{urea}.
1, 2, 3, 4, 5, 6, 7, 8 denote the different C_{urea}: 0.00, 0.52, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, respectively.

the question arises , if the molecular conformation of proteins really changes.

UV spectrophotometry is one of the most important tools for investigating the changes of the molecular conformation and refolding of proteins[1]. Aqueous protein solutions with and without urea were prepared, and the UV-spectra were recorded. The spectra were corrected by the blank solution without urea and proteins. They were compared with spectra of the undenatured proteins. Fig. 4 shows the UV-spectra of BSA in a solution consisting 0.62mol/L ammonium sulphate and different concentration of urea. It can be seen that the absorbance density of BSA is getting lower when the concentration of urea increaces. At the same time a shift of the maximum absorption towards longer wavelength is obeseved. From the results it can be concluded that besides solvophobic interaction between urea and protein others take place. Because of the change of molecular conformation of proteins in aqueous solution of urea, the stoichiometric

displacement model can not be used for the explanation of the retention behaviour of proteins in HIC.

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SIMULTANEOUS DETERMINATION OF BUZEPIDE, PHENYLPROPANOLAMINE, AND CLOCINIZINE IN PHARMACEUTICAL PREPARATIONS BY ION-PAIR REVERSED-PHASE HPLC

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ABSTRACT

A rapid, accurate and precise method for the determination of buzepide, phenylpropanolamine, and clocinizine in a cough-mixture is described. The method is based on reversed-phase ion-pair HPLC. The only sample preparation necessary for the analysis is its dilution with the mobile phase. The resulting solution is analyzed on a column packed with Lichrosorb RP-18 (10μm) with acetonitrile in aqueous 5 10-2M sodium perchlorate, pH 3.0 (15:85) as initial mobile phase (2 mL·min-1) and detection at 220 and 254 nm. Then a linear gradient up to 95% acetonitrile in 20 min, is applied. The detection limits are 500 ng injected for phenylpropanolamine, 40 and 5 ng for buzepide and clocinizine, respectively.

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INTRODUCTION

Reversed-phase ion-pair liquid chromatography (RP-IPC) is a well established method for the separation of ionic or ionizable organic compounds. With proper us of the technique, complex separations can be readily achieved, since both ionized and un-ionized components are analyzed under the same chromatographic conditions. Recently, most publications have discussed the application of RP-IPC for the simultaneous determination of the active components in pharmaceutical formulations. The analysis time is short, and usually only a simple dilution of the sample is required prior to injection.

Buzepide (2,2-diphenyl-4-examethyleneiminobutyramidemethiodide), phenyl-propanolamine hydrochloride, and clocinizine hydrochloride (1-(4-chlorobenzhydril)-4-cinnamylpiperazine-dihydrochloride) are active components employed in cold preparations. One commercial product (Denoral, Rhone-Poulenc, Milano, Italia) extensively used in Italy to relieve congestion of the nasal mucosa and sinuses in the treatment of colds, sinusitis, rhinitis, and hay fever, is a combination of the three drugs, which are present in other formulations sold on the European and U.S.A. markets. The dosage form (tablets) also contains excipients, some of which may interfere with the analysis of the active ingredients.

Phenylpropanolamine hydrochoride (I) is very often present as decongestant, in cough-cold formulations and many HPLC methods have been developed for its determination in pharmaceutical dosage forms and biological fluids (1-6). On the contrary, no information is appeared in literature on the analysis of buzepide (II) or clocinizine (III) alone or in combinations with (I) by HPLC.

This paper reports the simultaneous determination of (I), (II), and (III) by RP-IPC. Validation data are presented for the analysis of the drugs in commercial samples of cold formulations.

EXPERIMENTAL

Apparatus

The modular HPLC apparatus consisted of a single piston ternary gradient pump (Varian, Model 2510) equipped with a 10 µL sample loop, a Varian Polychrom 9065 photodiode-array detector, and a personal computer IBM PS/2. The analytical column was of stainless-steel (250 mm x 4.0 mm I.D.) packed with 10 µm Lichrosorb RP-18 (Merck,Darmstadt, Germany). The temperature of the column was maintained at 25 °C.

Standards and reagents

All reagents used were of analytical-reagent grade. The drug standards were supplied by Rhone-Poulenc (France) and were used as obtained. Acetonitrile was of HPLC grade. Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a Millipore filter (pore size 0.45 µm) and vacuum degassed by sonication before use.

HPLC conditions

The mobile phase was prepared by mixing acetonitrile with $5 \cdot 10^{-2}$M solution of sodium perchlorate in water, pH 3.0, adjusted with 70% perchloric acid. Flow-rate was 2.0mL·min^{-1} and detection was performed at 220 and 254 nm. A gradient elution was applied, consisting of a linear increase of acetonitrile from 15 to 95% (V/V)in 20 min.

At the end of the elution, the initial mobile phase was passed through the column for 10 min to allow a good re-equilibration of the chromatographic system.

Calibration standard solutions

Stock solutions of compounds I-III were prepared by dissolving weighed

amounts in the mobile phase containing 50% acetonitrile to make 100 mL. A set of standard solutions was prepared by diluting aliquots of the stock solutions to give concentrations ranging from 50 to 1000 $\mu g \cdot mL^{-1}$ for (I), from 1 to 15 $\mu g \cdot mL^{-1}$ for (II) and from 1 to 100 $\mu g \cdot mL^{-1}$a for (III). The calibration graphs were constructed by plotting the peak areas obtained at the wavelength of 254 nm for (I) and (III), and at 220 nm for (II), versus the amounts (μg) injected.

Sample preparation

25 tablets were weighed and ground to a fine powder. Enough powder to represent ten tablets was accurately weighed and mixed with 15 mL of the mobile phase containing 50% acetonitrile. The mixture was immersed in a ultrasonic bath for 5 min, brought to volume (20 mL) and filtered.

10 μL-aliquots were injected into the liquid chromatograph, setting the detector at 220 nm, for the analysis of buzepide. The solution was subsequently diluted ten times and injected with the detector settled at 254 nm, for the analysis of clocinizine and phenylpropanolamine.

RESULTS AND DISCUSSION

Figures 1A and 1B show the chromatograms of a standard solution of phenylpropanolamine, buzepide and clocinizine, recorded at 220 and 254 nm, respectively. The detection wavelength selected depended on the molar absorptivity of the compound concerned. In particular, 254 was chosen for I and III, whereas 220 was chosen for buzepide since this compound possesses insignificant UV absorption at wavelengths higher than 230 nm. When injecting standard solutions of buzepide a trace impurity was found to elute immediately after peak III. It could be observed only at 220 nm, and therefore the presence of

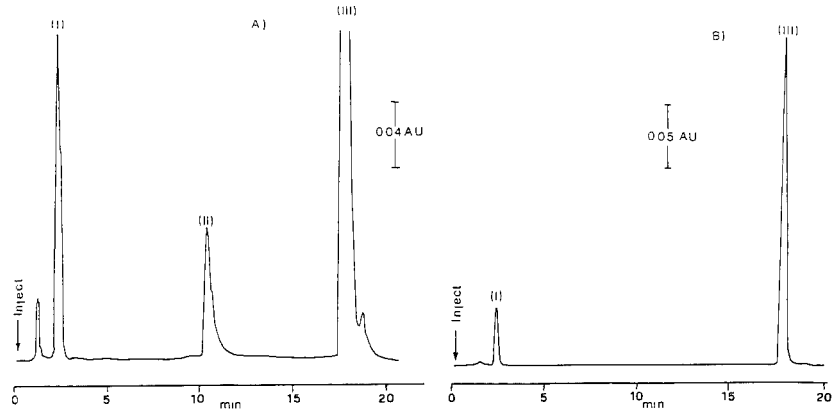

Figure 1 Typical chromatograms obtained at 220 nm (A) and 254 nm (B) for a standard solution containing 1 mg mL^{-1} of phenylpropanolamine (I) and buzepide (II), and 8 mg mL^{-1} of clocinizine (III).

this impurity did not interfere in the measurement of the peak area corresponding to clocinizine.

The photodiode-array detector allowed the estimation of the purity parameter format values (7) which are very useful in the analysis of a pharmaceutical preparation both in confirming peak purity and peak identification. The values were calculated over the range 220-367 nm and resulted 233.185 $\pm$ 0.011 for I, 223.075 $\pm$ 0.009 for II, and 241.171 $\pm$ 0.010 for III.

The capacity factors (reported in Table 1) were reproducible under the experimental conditions used, the coefficient of variation (C.V.) ranging from 1.2 to 1.9 for within-day and from 2.3 to 3.9% for between-day studies. The average analysis time was about 30 min.

The calibration graphs were constructed from five consecutive injections over the covered range of concentration, as indicated in the experimental section. The least square regression fit showed good linearity, passing through the origin. The

TABLE 1
Analytical Parameters for Compounds I-III

Compound	Capacity Factor	Detection Wavelength (nm)	Detection limit (ng injected)
I	1.6	254	500
II	8.3	220	40
III	15.4	254	5

TABLE 2
Calibration Curves for Compounds I-III: linear regression of the amount injected (x) versus the peak area (y); mean value ± standard deviation at 95% confidence interval (t=3.18; n=5)

Compound	Intercept	Slope	R^2
I	(-0.30 ± 0.02)E3	(0.205 ± 0.003)E3	0.9996
II	(-0.31 ± 0.02)E3	(2.54 ± 0.04)E3	0.9996
III	(-6.8 ± 0.2)E3	(20.3 ± 0.2)E3	0.9975

data obtained for the calibration lines are shown in Table 2. The detection limits, calculated as a signal-to-noise ratio of 2:1, are reported in Table 1.

Within-day precision was analyzed using standard solutions containing varying concentrations of each compound. The sample was then run on five separate occasions over the course of the day. The within-day variation of the determination was minimal, with a CV of 0.9%. Between-day precision involved the analysis of a particular standard solution each day for five consecutive days. Between-day precision was also good, with a CV of 1.5%.

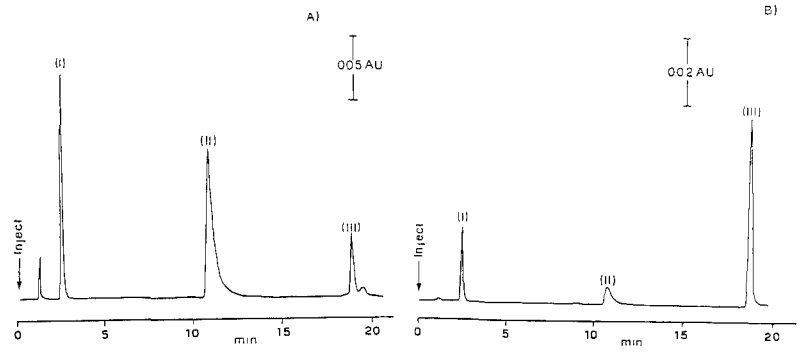

Figure 2 (A) Chromatogram recorded at 220 nm after injecting a sample of Denoral, (B) Chromatogram recorded at 254 nm, after a tenfold dilution.

TABLE 3
Analysis of a Pharmaceutical Formulation

Active principle	Label, mg/tablet	Assay results, mg/tablet	CV
Phenylpropanolamine	25.0	24.3	1.1
Buzepide	1.0	0.98	1.2
Clocinizine	5.0	4.92	0.9

*mean of five determinations.

The procedure was applied to the analysis of commercial samples of a pharmaceutical formulation containing all the three active principles, in the dosage form of tablets. Figure 2A and 2B show the chromatogram obtained from the analysis of the tablets. No problems were encountered with interfering compounds, since the impurity deriving from buzepide does not absorb at 254 nm, the wavelength at which chromatogram 2B was recorded.

The results obtained are shown in Table 3. The quantities found was in conformity with the values claimed by the manufacturer. Therefore, we can conclude that the developed method can be successfully adopted for the quantitation of I-III in pharmaceutical formulations.

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DIRECT HPLC MONITORING OF LIPASE ACTIVITY IN REVERSE MICELLAR MEDIA

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ABSTRACT

Given the profusion of biotechnological applications of the nonaqueous use of lipases, we have evaluated the possibilities of exploiting the inherent advantages of high performance liquid chromatography (HPLC) for a simple, rapid assay of lipase activity in reverse micellar media, as a convenient alternative to previously reported spectroscopic methods, using both a model system and esterification reaction, and different commercial lipases. The results obtained after a screening for optimized chromatographic conditions in the reverse-phase mode indicate that a satisfactory resolution of the reaction components can be obtained following a straightforward protocol, which permits an accurate, reliable quantitation of the reaction progress, regardless of the enzyme used.

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INTRODUCTION

Nonaqueous enzymology has consolidated during the last decade as a promising, exciting area of research (1-3) with important biotechnological implications, particularly those concerning organic syntheses and bioconversions in food, pharmaceutical, agricultural, petroleochemical or even military industries. Among the different experimental approaches proposed to place a functional enzyme in a water-restricted environment (each having its inherent advantages), one of the most popular is the solubilization of the protein in the so-called reverse micelles (4,5), where the enzyme is physically confined in an aqueous "micropool" separated from the bulk organic phase ("oil") by a monolayer of surfactant. This distribution contributes to a quite efficient biocatalysis by enabling a maximization of the water-oil interfacial area and a ready solubilization of many nonpolar substrates (and products) in the organic phase.

Although reverse micelles have been also employed as membrane mimetic systems for the study of membrane-bound or membrane-associated proteins (6,7) or hydrophobic peptides (8), or as microemulsion preparations for topic drug delivery (9), the attention in these systems has been mainly polarized towards applied enzymology facets, and in particular quite profusely towards the use of lipases in varied transformations of evident industrial interest, such as ester synthesis, triglyceride hydrolysis or interesterification, etc.

An overview of the literature during the last years evidences that whereas the monitoring of lipase activity in organic solvents using insoluble enzyme (powdered, entrapped or adsorbed to a support) has made use in some instances of liquid chromatographic procedures, in the case of reverse micellar solutions most of the work has been based on spectroscopic determinations. Thus, among the methods most often employed are that of Lowry & Tinsley (10) or modifications of it (11), that of phenol red cosolubilized in the micelle as an acid-base indicator (12), one based on Fourier-transfrom Infrared (FTIR) spectroscopy (13, 14) or the use

of chromogenic lipase substrates, such as p-nitrophenyl palmitate (15) or more sophisticated ones (16,17). To a lesser extent, gas chromatography (18), thin-layer chromatography (19) or alkalimetric titrations (20,21) have been also used occasionally.

Paradoxically, however, in spite of all the inherent advantages of high-performance liquid chromatography (HPLC), the use of this technique is scarcely found in the direct monitoring of lipase-mediated conversions occurring in reverse micellar media, which is somewhat understandable taking into account the number of components to be separated *a priori*: organic solvent, surfactant (plus sometimes cosurfactant), enzyme, water, substrate(s) and product(s).

In this preliminary work, we have made an initial evaluation of the possibilities of HPLC in its reverse-phase (RP) mode, as a versatile tool for the simple, rapid assay of lipase activity in reverse micelles, by selecting a reverse micellar model system [Aerosol OT (AOT) in isooctane], model lipases (from *Candida rugosa*, *Rhizopus delemar* and *Pseudomonas sp.*) and a model reaction (the esterification between n-decanoic acid and 1-butanol) (Fig. 1). A screening for chromatographic conditions has been carried out to obtain a satisfactory resolution of most of the reaction components and a fast, straightforward sample preparation procedure is proposed, valid for other lipases and model reactions assayed. It is shown that the lipase activity can be accurately and reliably followed by RP-HPLC, the results being comparable to those obtained by other techniques.

EXPERIMENTAL PROCEDURES

Materials.

Lipase (EC 3.1.1.3) from *Candida rugosa* (formerly named *cylindracea*), type VII, was purchased from Sigma (St. Louis, MO.) and used without further purification. Lipases from *Rhizopus delemar* and from *Pseudomonas sp.* were generously provided by Amano Intl. Enzyme Co. (Nagoya, Japan) and also used without additional purification. AOT was

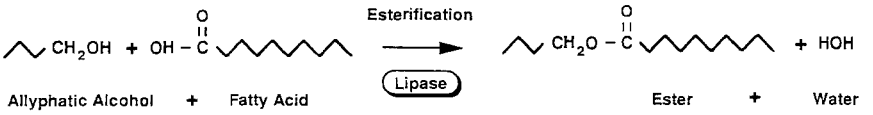

FIGURE 1. Lipase-catalyzed model esterification reaction in reverse micelles.

obtained from Serva (Heidelberg, Germany), purified by preparative RP-HPLC, and vacuum-dried overnight prior to use to ensure a minimal water content. Organic solvents were of analytical or HPLC grade, from Merck (Darmstadt, Germany). Bidistilled water was purified through a Millipore Milli-Q system (Millipore, Milford, MA). Fatty acids, alcohols and salts were obtained from commercial suppliers with a >99% purity.

Sample Preparation.

The reverse micellar solutions were prepared by adding appropriate quantities of alcohol (typically 250 mM) and fatty acid (typically 100 mM) to a solution of 100 mM AOT in isooctane. To start the reaction, a few microliters of lipase solution in 10 mM Tris-HCl (pH 7.5) were injected to the substrate(s)-containing reaction medium, and after a few seconds of gentle shaking a clear, transparent, enzyme-containing reverse micellar solution was obtained (zero time). Aqueous lipase concentrations and injected volumes were adjusted to obtain in all cases an overall enzyme concentration in the organic medium of 0.4 mg of lipase sample per mL. The water content of the micelles is defined by the parameter w_0 (4), which denotes the quotient of molar concentrations of water and surfactant.

Activity Measurements.

The reactions were carried out in screw-capped vials at 25 °C. Periodically, aliquots of 20 μL of the reaction medium were withdrawn and placed in an eppendorf and the solvent was rapidly evaporated under a N_2 stream. The residue was redissolved in 40 μL of chromatographic mobile phase, and after centrifugation at 13,000*g* for 3 min to separate the nonsolubilized enyme, 10 μL of the supernatant were injected onto a

LiChroCART 125-4 (LiChrospher 100 RP-18) cartridge, from Merck. The liquid chromatograph consisted of M-510 solvent-delivery systems, an automated gradient controller, a U6K universal injector and a 410 differential refractometry detector, all from Waters (Milford, MA).

Other details of experimental conditions can be found in the corresponding legends to Figures.

RESULTS AND DISCUSSION

Although *gradient conditions* for elution are not unusual in RP-HPLC separations and can undoubtedly afford both higher versatility and resolution, our purpose throughout this work was to design and optimize a simple, reliable protocol for a rapid, 'comfortable' monitoring of lipase activity using *isocratic elution* (preferable for differential refractometry detection), to make the chromatographic technique attractive when compared to the reported spectroscopic methods.

Our first attempts were aimed at the direct injection onto the reverse-phase column of the enzyme-containing reverse micellar solution and subsequent chromatographic separation of lipase, surfactant, isooctane, n-decanoic acid, 1-butanol and butyl decanoate. As a first trial, methanol-water and acetonitrile-water (containing 0.1% TFA) mixtures around 50 % (by volume) of organic component were tested as mobile phase as a compromise both to have a reasonable resolution of substrates and products and to dissolve (although denatured) the lipase in the eluent. Unfortunately, under these conditions the reaction product could not be eluted from the column (not shown), a requisite that we considered mandatory for reliable quantitation of enzyme activity (based on simultaneous *appearance of the ester* and *disappearance of the substrates*). Since even at 70% (by volume) of acetonitrile in the mobile phase, the reaction product was not eluted, we made an estimation of the approximate composition of organic component in the eluent necessary for

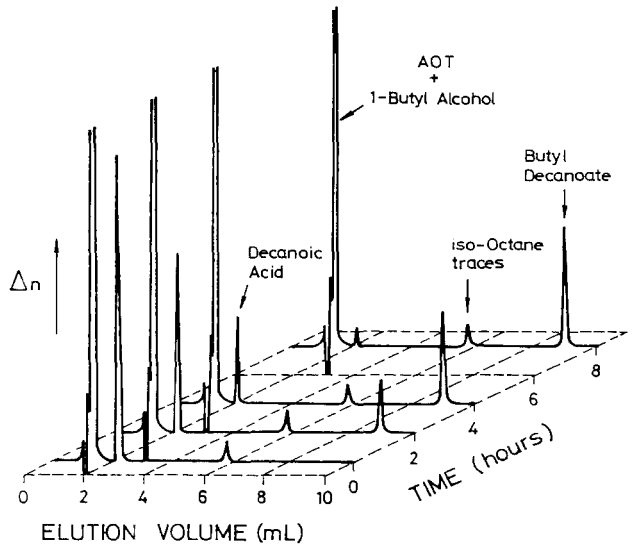

FIGURE 2. RP-HPLC monitoring of the *Rhizopus delemar* lipase-mediated esterification of n-decanoic acid and 1-butanol in an AOT/isooctane reverse micellar system (w_0=8.9). The elution profiles correspond to aliquots from the reaction medium taken at different incubation times and treated as described in the Experimental Procedures. The column was isocratically eluted with 0.1% TFA-containing acetonitrile-water (85:15, v/v) at a flow rate of 1.0 mL/min. Samples were monitored using differential refractometry detection.

isocratic elution of the ester, by performing a screening experiment under gradient conditions (using UV detection) from 50% to 90% acetonitrile (not shown). It was decided that an isocratic mobile phase (containing 0.1% TFA) composed of acetonitrile-water (85:15, v/v) should be adequate for the separation and visualization of both substrates and products. Since in this case the direct injection of the reaction mixture was not possible due to the high content or organic component of the eluent (in which the lipase was not soluble), a simple, rapid protocol to remove the enzyme before sample injection was therefore developed, which is described in the Experimental Procedures.

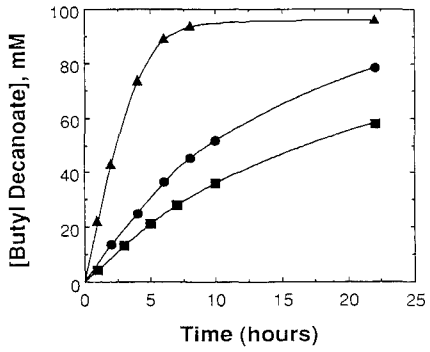

FIGURE 3. Time course of lipase-catalyzed formation of butyl decanoate in reverse micelles (w_0=8.9), as monitored by RP-HPLC, for different lipases assayed: (▲) *Rhizopus delemar*; (●) *Pseudomonas sp.*; and (■) *Candida rugosa*.

Fig. 2 depicts the gratifying results obtained upon implementation of the above conditions and illustrates, as an example, how the time course of *Rhizopus delemar* lipase-catalyzed ester formation in reverse micelles can be easily followed by RP-HPLC using a differential refractometry detector. Notice that elution of all the components in the reaction mixture (except the lipase) takes less than 10 min and that both one of the substrates (n-decanoic acid) and the ester product (butyl decanoate) are completely resolved from the other components.

Fig. 3 depicts, as an example, kinetics of the production of butyl decanoate in the reverse micellar medium by the different lipases assayed, as monitored by RP-HPLC. These results show that the method proposed can be conveniently used not only to monitor the progress of synthetic reactions and obtain accurate kinetic data, but to establish a comparison of the relative activities of enzymes from different sources.

On the other hand, when the influence of the water content of the reverse micelles (w_0) on the activity of the *Candida rugosa* lipase was analyzed, the profile obtained from HPLC data (Fig. 4) was found to be similar to that previously reported for the same enzyme and micellar system using spectroscopic determination of the reaction progress (22).

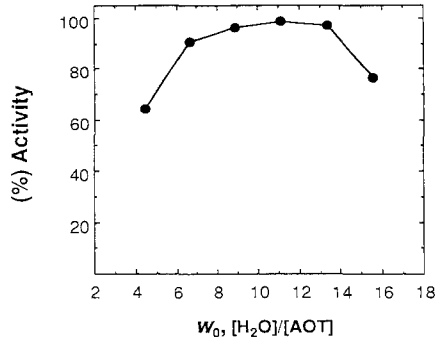

FIGURE 4. Profile of the dependence of the HPLC-determined activity of *Candida rugosa* lipase on the water content of the reverse micelles (w_0).

CONCLUSION

We provide here preliminary data which show that the widely acknowledged advantages of HPLC can be exploited as an alternative methodology for a rapid, direct monitoring of lipase activity in reverse micellar media, especially for comparative purposes which only require simple model reactions. In this case, the chromatographic approach can afford a higher reliability for quantitative determinations than some conventional spectroscopic assays and the possibility of automation and easy manipulation of a large number of samples.

On the other hand, optimization of conditions for the HPLC assay of model reactions involving longer fatty acids will require to explore other mobile phases to attain satisfactory separations in reasonable times. As an example, we have verified that isocratic elution on the above chromatographic system with a mobile phase as simple as pure methanol yields elution volumes for hexyl myristate, palmitate, stearate and oleate of 3.6, 4.9, 6.8 and 5.3 mL, and permits an easy monitoring of ester formation catalyzed by different lipases. Work is in progress in this direction.

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF ACETYLATED ANTHOCYANINS IN LOWBUSH BLUEBERRIES

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ABSTRACT

The anthocyanin pigments of blueberries (*Vaccinium angustifolium* Ait.), previously reported as the 3-glucosides, galactosides and arabinosides of delphinidin, cyanidin, petunidin, peonidin and malvidin have been shown to occur both as non-acylated and acetylated forms by chromatographic, chemical and spectral analyses. In 'Fundy' blueberries, acetylated anthoycanins accounted for over 32% of the anthocyanin content. The two major acetylated anthocyanins were characterized as the 3-acetylglucoside and 3-acetylgalactoside of malvidin. This is the first report of acylated anthoycanins in lowbush blueberries and the first report on the presence of acetylated 3-galactosides of the five commonly occurring anthocyanidins.

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INTRODUCTION

The lowbush blueberry, *Vaccinium angustifolium* Ait., is grown commercially in eastern Canada and northern United States, and used in frozen, canned and fresh markets. The color of the fruit, which is a primary quality attribute, is determined by the composition and concentration of the anthocyanins present (2). There are few reports on the anthocyanin composition of the lowbush blueberry (1,2). Francis *et al.* (1), using paper chromatography for the separation and characterization of the anthocyanins, reported the occurrence of the 3-glucosides and 3-galactosides of delphinidin, malvidin, petunidin, cyanidin, and peonidin, plus a small amount of 3-arabinosides of the same anthocyanidins in an unspecified variety of lowbush blueberry. However, no acylated pigments were identified (1,2).

In this article we report the isolation and characterization of simple and acetylated 3-galactosides, glucosides and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin in the lowbush blueberries, by reverse phase-high performance liquid chromatography (RP-HPLC) and capillary gas-liquid chromatography (GLC).

MATERIALS AND METHODS

Materials

The lowbush blueberries (*Vaccinium angustifolium* Ait., cv. 'Fundy'), were harvested in August 1993 from a planting of Agriculture Canada Research Station, Kentville, Nova Scotia. The berries were frozen and kept at -38°C until use.

The 'Cabernet Sauvignon' (*Vitis vinifera*) grape skin was obtained from the Summerland Research Centre, British Columbia, and the other standards were from Sarsynthèse, Merignac, France.

All other chemicals used were of reagent or higher grade.

Extraction

The dark colored blueberry tissue (skin and outer layer pulp) was separated from the less pigmented tissue by squeezing partially thawed berries between the thumb and forefinger. The pigmented tissue (40 g) was blended with 30 mL of MeOH/formic acid/water (70/1/29, MFW) for 10 min in a stainless steel blending bowl equipped with a water jacket for temperature control. The material in the bowl was maintained at 15 ± 1°C by passing refrigerated water through the water jacket throughout the extraction. The resulting slurry was filtered through a 0.45 µM Durapore filter (Millipore), and the filtrate was partially purified by open column chromatography before HPLC analysis.

Purification and HPLC analysis

The filtrate (20 mL) was applied on to an Amberlite CG-50 (Aldrich, Wilwaukee, WI) column (30 X 500 mm) which had been previously washed with 95% ethanol containing 1% formic acid, and equilibrated with 1% formic acid in water. The loaded column was then washed with 1% formic acid (500 mL) and the anthocyanin pigments were collected by washing the column with 1% formic acid in methanol. The dark colored band was collected (10 mL) and mixed with an equal amount of 1% formic acid in water. The mixture was

filtered, and the filtrate was repeatedly injected in the HPLC column for analysis and preparation of anthocyanin isolates.

The HPLC system (Waters Chromatography, Milford, MA) used consisted of a Waters 990 photodiode array detector and a SuperPac Pep-S column (5 μM, 4 x 250 mm; guard column, 4 x 10 mm; Pharmacia) which was placed in thermostated control system (Waters) and operated at 26.0 ± 0.1 °C throughout the analysis. The following solvents and elution profiles were used: solvent A, 5% formic acid in water; solvent B, methanol; elution profile: 0-4 min, 10-12% B (linear gradient); 4-10 min, 12-15% B; 10-20 min, 15-20% B; 20-23 min, 20% B; 23-32 min, 20-30% B; 32-40 min, 30-35% B; 40-48 min, 35-37% B; 48-50 min, 37-70% B; 50-53 min, 70% B; 53-55 min, 70-10% B. The flow rate was 1.2 mL/min. The same equipment and elution profile was used for the preparation of anthocyanin fractions, and the characterization of the anthocyanins.

Compositional analysis of anthocyanins

The chemical composition of anthocyanin fractions accumulated from the HPLC system was determined by HPLC and GC as described by Gao and Mazza (1994). A micro amount (0.1-0.2 mg) of anthocyanin accumulated from the analytical HPLC was hydrolyzed in MeOH-2N HCl at 100°C for 2 h. An aliquot (10 μL) of the hydrolyzate was injected on the HPLC for detecting aglycones and possible acylating phenolic acids, simultaneously monitored at 280 and 525 nm. An aliquot (0.5 μL) of a chloroform extract of the remaining hydrolyzate was analyzed by GC for aliphatic acylation in the anthocyanin.

The hydrolyzate was then used for the analysis of the sugar moieties in the anthocyanin molecule, as described previously (3).

Spectral analysis

Spectral data for purified individual anthocyanins and for pigments subjected to alkaline hydrolysis were recorded by the on-line photodiode array detector, and were compared with those of the anthocyanin standards. The anthocyanin standards were from a *Vitis vinifera*, cv. 'Cabernet Sauvignon', which is known to contain simple and acetylated 3-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (4). Alkaline hydrolysis was carried out according to Markham (5).

RESULTS AND DISCUSSION

Analytical RP-HPLC of the methanolic extracts from lowbush blueberries revealed the presence of 25 anthocyanin peaks (Fig. 1). Each peak was collected, concentrated under reduced pressure, subjected to acid hydrolysis in HCl-MeOH and analyzed by HPLC for anthocyanidins and acylating phenolic acids. The results of these analyses are presented in Table 1. Acylating phenolic acids were detected in none of the hydrolyzate from the fractions containing the 25 peaks, which is consistent with the spectral characteristics of the peaks shown in Table 2. The presence of cinnamic acid acylation in anthocyanin molecules would produce a pronounced shoulder at 300-320 nm beside the anthocyanin absorption peak at 280 nm. The fraction

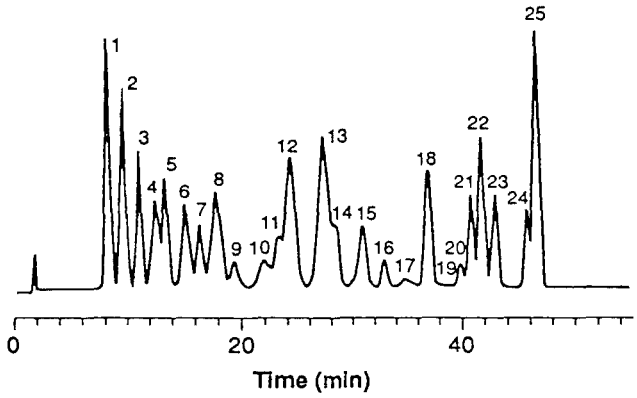

Fig. 1. RP-HPLC of anthocyanins in the methanolic extract of lowbush blueberries (*Vaccinium angustifolium* Ait. cv. 'Fundy'). Elution monitored at 525 nm. Pk numbers refer to anthocyanin fractions in Tables 1 through 4. Conditions for HPLC analysis are given in Materials and Methods.

which contained peaks 13 and 14 had two aglycones, which indicates that there is more than one anthocyanin present in the fraction although they eluted as almost a single peak on RP-HPLC (Fig. 1). In subsequent analyses for sugars and aliphatic acylating acids by capillary GC (3), acetic acid, in the form of methyl acetate, was detected in the fraction containing peaks 13 and 14, and each fraction containing peaks 16 through 25 (Table 3), indicating extensive acetylation of anthocyanins in blueberry anthocyanins. The HPLC fraction which was found to contain more than one aglycone also contained two types of sugars. There were a total of three types of sugars detected in all fractions, and these were identified as galactose, glucose, and arabinose (Table 3).

Table 1. Retention times on RP-HPLC of the aglycones from anthocyanins in lowbush blueberries cv. 'Fundy'

Anthocyanin peaks or standards		t_R on HPLC (min)	Anthocyanidin t_R(min)	Phenolic acid
Pk	1*	8.5	22.8	--‡
	2	10.0	22.8	--
	3	11.4	30.9	--
	4	13.0	22.8	--
	5	13.8	30.8	--
	6	15.4	35.5	--
	7	16.8	30.8	--
	8	18.2	35.5	--
	9	19.9	38.7	--
	10	22.4	35.5	--
	11	23.7	38.8	--
	12	24.6	41.8	--
	13/14	27.7/28.7†	41.7/22.7†	--
	15	31.2	41.7	--
	16	33.2	30.8	--
	17	35.2	30.8	--
	18	37.1	22.7	--
	19	38.8	35.4	--
	20	40.9	38.7	--
	21	41.0	30.8	--
	22	42.0	41.8	--
	23	43.1	35.5	--
	24	45.9	38.6	--
	25	46.7	41.7	--
Delphinidin			22.7	
Cyanidin			30.8	
Petunidin			35.5	
Peonidin			38.7	
Malvidin			41.8	

* The fraction number refers to the peaks in the HPLC chromatogram shown in Fig. 1.

† In the order of decreasing peak area on HPLC if more than one peak found in the acid hydrolyzate of the collected peak.

‡ -- denotes "not detected".

Table 2. Anthocyanins in lowbush blueberry and their spectral characteristics

Anthocyanin fraction	Peak area (%)	HPLC t_R (min)	Alkali Hydrolysis product†	λ_{max} (nm)	$E_{440}/E_{vis\ max}$ (%)	$E_{UVmax}/E_{vis\ max}$ (%)	$AlCl_3$
1*	7.12	8.53	--	278, 528	29.0	63.3	+
2	7.69	10.0	--	279, 528	26.4	58.1	+
3	4.47	11.4	--	280, 518	30.7	62.3	+
4	3.42	13.0	--	278, 526	26.6	56.0	+
5	4.42	13.8	--	280, 518	31.2	69.7	+
6	3.88	15.5	--	279, 528	26.6	55.1	+
7	2.69	16.8	--	280, 518	31.6	64.0	+
8	5.52	18.2	--	280, 528	25.2	57.1	+
9	1.19	19.9	--	280, 519	30.8	61.5	-
10	1.50	22.4	--	278, 540	15.8		‡
11	2.41	23.7	--	280, 520	30.5	78.5	-
12	8.19	24.6	--	279, 530	24.7	56.6	-
13	9.26	27.7	--	278, 529	25.0	56.2	-
14	2.84	28.7	1	280, 529	28.1	62.9	‡
15	3.24	31.2	--	278, 530	25.4	57.1	-
16	0.88	33.2	3	280, 520	28.2	47.6	+
17	0.45	35.2	7	280, 520	32.4	66.0	+

18	4.83	37.2	2	279, 530	25.2	59.7	+
19	0.08	38.8	6	278, 525	26.6	62.2	‡
20	0.77	40.9	9	280, 522	29.0	69.0	-
21	3.06	41.1	5	281, 508	30.6	64.5	+
22	5.57	42.0	12	280, 532	25.5	63.7	-
23	3.36	43.1	8	280, 530	24.4	58.1	+
24	2.17	45.9	11	280, 520	31.0	73.3	-
25	10.6	46.7	13	280, 530	25.4	61.8	-
Dp 3-Gl-Ac		37.3		280, 530	25.8	59.0	
Dp 3-Gl		10.0		279, 528	27.5	57.7	
Cn 3-Gl-Ac		41.1		280, 520	30.0	66.0	
Cn 3-Gl		13.9		281, 520	30.6	65.5	
Pt 3-Gl-Ac		43.2		280, 528	27.0	55.8	
Pt 3-Gl		18.1		279, 528	27.5	60.0	
Pn 3-Gl-Ac		46.1		281, 518	31.4	66.6	
Pn 3-Gl		23.6		280, 519	31.8	67.3	
Mv 3-Gl-Ac		46.7		280, 530	27.3	60.3	
Mv 3-Gl		27.5		280, 528	27.8	60.6	

* The fraction number refer to the peaks in the HPLC chromatogram shown in Fig. 1.
† Numbers refer to the corresponding retention time of the anthocyanin peak on HPLC (shown in Fig. 1).
‡ Data not available or no clear-cut response.

Table 3. Retention time on capillary gas chromatography of sugars and aliphatic acylating acids from anthocyanin peaks in the lowbush blueberry cv. 'Fundy'

Anthocyanin peaks or standards		Sugar(s) t_R (min)	Acylating acid t_R (min)
Pk	1*	10.42/11.65	--‡
	2	11.15/12.90	--
	3	10.43/11.66	--
	4	6.78/7.03/7.53/7.78	--
	5	11.15/12.89	--
	6	10.45/11.68	--
	7	6.78/7.03/7.54/7.77	--
	8	11.16/12.89	--
	9	10.43/11.66	--
	10	6.78/7.03/7.54/7.77	--
	11	11.15/12.89	--
	12	10.40/11.63	--
	13/14	11.12/12.85; 10.40/11.63†	3.50(small)
	15	6.77/7.02/7.53/7.76	--
	16	10.40/11.62	3.51
	17	6.76/7.01/7.51/7.75	3.51
	18	11.12/12.85	3.51
	19	10.40/11.62	3.51
	20	10.45/11.69	3.51
	21	11.15/12.89	3.51
	22	10.43/11.67	3.51
	23	11.14/12.87	3.51
	24	11.14/12.87	3.51
	25	11.14/12.88	3.51
Arabinose		6.78/7.04/7.55/7.78	
Rhamnose		6.94/8.12	
Xylose		7.14/8.37/9.04	
Galactose		10.42/11.66	
Glucose		11.14/12.88	
Acetic acid			3.51
Oxalic acid			8.26
Malonic acid			9.20
Succinic acid			10.28

* Fraction number refers to the peaks in HPLC chromatogram shown in Fig. 1.

† For fraction(s) that contained more than one sugar, the sugar displaying the larger total peak area is listed first.

‡ "--" denotes "not detected".

To further characterize the anthocyanin pigments corresponding to each of the 25 HPLC peaks, small amounts (0.05 mg) of each of the fractions collected from the HPLC column outlet were treated with NaOH (2N) according to Markham (5). The hydrolyzate from the alkaline treatment was re-injected onto the HPLC; the retention times and the spectral characteristics were recorded for the hydrolysis product(s), and compared to the anthocyanin standards from an extract of 'Cabernet Sauvignon ' grape skins (Table 2).

Fraction 13, containing peaks 13 and 14, produced, upon alkali hydrolysis, a small de-acylated fraction, which was eluted earlier than the original peak while a major peak having the original retention time reoccurred in the chromatogram. The results indicate that the fraction is a mixture of acylated and simple anthocyanins, which is consistent with the relatively small acetic acid peak in the GC analyses for these fractions.

Although the spectral characteristics were not readily determined for the acetylated anthocyanins in the fraction which contained peaks 13 and 14, the spectral data for the corresponding de-acylated anthocyanin were easily obtained from the HPLC peak(s) of the alkaline hydrolyzate, and the spectral data could easily be matched to that of the acetylated pigment in the sample and in the standard pigment (Table 2).

All the spectra for the individual anthocyanins showed a shoulder at around 440 nm of the visible maxima. This is characteristic of 3-glycosylation (6), and indicates that the anthocyanins of lowbush blueberries are 3-glycosylated.

Table 4. Identification of anthocyanins in lowbush blueberry cv. 'Fundy'

Anthocyanin peak		Alkali hydrolysis product†‡	Sugar	Aglycone	Anthocyanin standards position§	Anthocyanins identified[J]
Pk	1*	--	Ga	Dp	--	Dp 3-Ga
	2	--	Gl	Dp	Dp 3-Gl	Dp 3-Gl
	3	--	Ga	Cn	--	Cn 3-Ga
	4	--	Ar	Dp	--	Dp 3-Ar
	5	--	Gl	Cn	Cn 3-Gl	Cn 3-Gl
	6	--	Ga	Pt	--	Pt 3-Ga
	7	--	Ar	Cn	--	Cn 3-Ar
	8	--	Gl	Pt	Pt 3-Gl	Pt 3-Gl
	9	--	Ga	Pn	--	Pn 3-Ga
	10	--	Ar	Pt		Pt 3-Ar
	11	--	Gl	Pn	Pn 3-Gl	Pn 3-Gl
	12	--	Ga	Mv	--	Mv 3-Ga
	13/14	1	Gl;Ga‡	Mv; Dp‡	Mv 3-Gl	Mv 3-Gl; Dp 3-Ga-Ac
	15	--	Ar	Mv	--	Mv 3-Ar
	16	3	Ga	Cn	--	Cn 3-Ga-Ac

17	7	Ar	Cn	--	Cn 3-Ar-Ac
18	2	Gl	Dp	Dp 3-Gl-Ac	Dp 3-Gl-Ac
19	6	Ga	Pt	--	Pt 3-Ga-Ac
20	9	Ga	Pn	--	Pn 3-Ga-Ac
21	5	Gl	Cn	Cn 3-Gl-Ac	Cn 3-Gl-Ac
22	12	Ga	Mv	--	Mv 3-Ga-Ac
23	8	Gl	Pt	Pt 3-Gl-Ac	Pt 3-Gl-Ac
24	11	Gl	Pn	Pn 3-Gl-Ac	Pn 3-Gl-Ac
25	13	Gl	Mv	Mv 3-Gl-Ac	Mv 3-Gl-Ac

* The peak number refer to the peaks in the HPLC chromatogram shown in Fig. 1.
† Number refers to the retention time of the corresponding peak on HPLC chromatogram (Fig. 1).
‡ In the order of decreasing peak(s) areas for sugars and aglycones in fraction(s) which contained more than one anthocyanin.
§ The anthocyanin standards are positioned by their retention time which corresponds to that of the respective anthocyanin peaks.
[J] Abbreviation: Ga, Galactose; Gl, Glucose; Ar, Arabinose; Dp, Delphinidin; Cn, Cyanidin; Pt, Petunidin; Pn, Peonidin; Mv, Malvidin; Ac, Acetylated.

Based on the results of this study, lowbush blueberries contain at least 25 anthocyanins, including the acetylated glucosides and galactosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, as well as the arabinosides of delphinidin, cyanidin, petunidin and malvidin (Table 4). The identity of the majority of the anthocyanins listed in Table 4 was further confirmed by co-chromatography of authentic pigments obtained from commercial sources (Sarsynthèse, Merignac, France) and/or from 'Cabernet Sauvignon ' grape skins which are known to contain simple and acylated forms of delphinidin, cyanidin, petunidin, peonidin and malvidin (2,4). The results reported here are consistent with the finding of Francis *et al.* (1) that the lowbush blueberries contain a number of 3-glucosides and galactosides of the five commonly occurring anthocyanidins, plus a small amount of arabinosides. However, extensive acetylation of the anthocyanins was found in the present study. The discrepancy is probably due to the use of different blueberry varieties in the two studies, similar to the compositional differences which are common in grapes (e.g. 'Cabernet Sauvignon ' vs. 'Pinot Noir ', with the later variety containing no acylated pigments) (2). A follow-up study from this laboratory (7) has indicated that the anthocyanin composition of the lowbush blueberries are variety-dependent. It is also possible that the acetylated pigments were not detected by paper chromatography which was used in the study by Francis *et al.* (1).

This is also the first report documenting the presence of acetylated galactosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. A study on the compositional differences among various cultivars and hybrid varieties of lowbush blueberries is reported elsewhere (7).

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DETERMINATION OF CHLORINE AND CHLORINE DIOXIDE BY NON-SUPPRESSED ION CHROMATOGRAPHY AND APPLICATION TO EXPOSURE ASSESSMENT IN THE PAPER INDUSTRY

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ABSTRACT

A non-suppressed ion chromatography method for the determination of chlorine and chlorine dioxide by their representative ions, chloride and chlorite, has been developed. The method utilizes a phosphate buffered iodide solution for the absorption of chlorine and chlorine dioxide from air and conversion to their representative ions. Analysis is by non-suppressed ion chromatography with a 1-hexane sulfonic acid, sodium salt mobile phase and detection by conductivity. The analytical detection limit was found to be 0.16 μg/mL and 0.13 μg/mL for chloride and chlorite, respectively. The method was adapted for use in a passive sampler and used to assess exposure to chlorine and chlorine dioxide at four pulp / paper mills. A total of 336 personal exposure samples were collected of which 25.2% had detectable levels of chlorine and 3.9% had detectable levels of chlorine dioxide. For those samples with detectable exposures, the overall geometric means and standard deviations were GM = 38 ppb, σ_g = 2.5 and GM = 33 ppb, σ_g = 2.5, for chlorine and chlorine dioxide, respectively.

INTRODUCTION

The ability to simultaneously detect chlorine and chlorine dioxide is of concern in the workplace where there is concomitant exposure. Chlorine and chlorine dioxide are used extensively as bleaching agents in the production of pulp and paper, flour, leather, and textiles. Due to its ability to produce explosive mixtures in air and its chemical instability, chlorine dioxide is generally produced at the site of its use.[1] Both chlorine and chlorine dioxide are strong oxidizers and severe irritants, causing irritation and conjunctivitis at low exposure levels; higher exposure levels or extended exposure times may result in lower respiratory irritation, including pulmonary edema. The current Occupational Safety and Health Administration (OSHA) permissible exposure limits (PEL) for an eight-hour time-weighted-average (TWA) are 0.1 ppm and 1.0 ppm, with short-term exposure limits (STEL) of 0.3 ppm and 1.0 ppm, for chlorine dioxide and chlorine, respectively.[2]

An established method for the collection and quantitation of airborne chlorine and chlorine dioxide utilizes a neutral-buffered phosphate / iodide absorbing solution with spectrophotometric analysis of formed iodine at 350 nm. The sample is analyzed at neutral and acid pH's in order to separately quantitate both Cl_2 and ClO_2:[3]

At Neutral pH:

$$Cl_2 + 2\ I^- \longrightarrow I_2 + 2\ Cl^-$$

$$ClO_2 + I^- \longrightarrow \tfrac{1}{2}\ I_2 + ClO_2^-$$

At Acid pH:

$$ClO_2^- + 4\ H_3O^+ + 4\ I^- \longrightarrow 2\ I_2 + Cl^- + 6\ H_2O$$

It is seen that under neutral pH conditions, the ions, chloride and chlorite, are surrogate analytes for chlorine and chlorine dioxide, respectively. A suppressed ion chromatographic technique was developed which increased

the sensitivity, specificity, and simplicity of the neutral-buffered iodide method by simultaneous determination of the representative ions, Cl^- and ClO_2^-, at a single pH.[4-5] The purpose of this work was to adapt this method to a non-suppressed ion chromatography analytical system. The technique was subsequently utilized for exposure assessment of workers in four pulp/paper mills using sample collection by personal passive dosimeter[6-7] containing the neutral-buffered iodide absorbing solution.

MATERIALS AND METHODS

The analytical system consisted of a Waters M-6000A pump and an AllTech/Wescan ICM-300 ion chromatography module which included an oven set at a temperature of 30°C, a 100 μl PEEK sampling loop, and a model 315 conductivity detector set at 5 μS full scale. The output of the conductivity detector was recorded and integrated on a SpectraPhysics Datajet integrator.

Due to its wide range of tolerance to pH (2 to 12) and organic solvent percentage (0-100), the initial screening of mobile phase for chromatographic resolution of chloride and chlorite was done with an Alltech universal anion column, 10 μm particle size with column dimensions of 150 mm x 4.6 mm. Several common ion chromatography mobile phases, including potassium hydrogen phthalate (EM Science), sodium hydroxide (J.T. Baker), sodium bicarbonate (Fisher), and ethylenediaminetetraacetic acid, disodium salt (EDTA), (Aldrich), were screened. The alkanesulphonates, 1-octane sulfonic acid, sodium salt (Aldrich) and 1-hexane sulfonic acid, sodium salt (Aldrich), which have been utilized as ion association agents in HPLC[8] were also screened. All these mobile phases were tested over a range of concentration, pH, and flow rate.

After optimization of mobile phase, analyses were done on a Wescan conventional anion/s column, 10 μm particle size with column dimensions of 250 mm x 4.6 mm. Due to the high affinity of this column for iodide, it was

pretreated with 500 mLs of a 1% sodium iodide solution adjusted to a pH of 4.5 with acetic acid.

For collection of air samples, the absorbing medium consisted of 1 mM sodium phosphate - monobasic, 1 mM sodium phosphate - dibasic, and 10 mM sodium iodide. This solution was prepared in the following manner: 2 millimoles sodium phosphate, dibasic, 99.95% (Aldrich), and 2 millimoles sodium phosphate, monobasic, 99% (Aldrich) were diluted to 50.0 mL with distilled, deionized water. An aliquot of this solution was diluted 1:10 with distilled, deionized water and analyzed by ion chromatography to screen for interfering compounds and chloride contamination. To remove the interfering peaks, this solution was filtered through an Alltech Maxi-clean IC-OH cartridge. This procedure was repeated until the solution was clean, usually requiring the use of four cartridges. After this procedure, 25 mls of the cleaned phosphate solution were added to 10 millimoles of sodium iodide, 99.99% (Aldrich), and diluted to one liter with distilled, deionized water. The pH of this solution was adjusted to 8.5 with sodium hydroxide then filtered through an Alltech 0.2 μm, Nylon 66 filter and stored in the refrigerator until use.

All samples and standards were prepared in the absorbing media. Standards were prepared from sodium chloride, 99.999% pure (Aldrich), and sodium chlorite, 80% pure (Aldrich). Initially, the purity of a particular lot of sodium chlorite was determined by the single buffer dual pH 350 nm spectrophotometric method.[3] The amount of chloride in the chlorite was quantitated by ion chromatographic analysis of a stock solution in comparison to a chloride standard curve. In subsequent use, the chlorite content was confirmed by the ratio of the chlorite and chloride peaks in a chlorite stock solution: a reduction in the chlorite to chloride ratio indicated a decomposition of the sodium chlorite reagent.

Solutions of ions screened as potential interferants included acetate, carbonate, nitrate, nitrite, sulfate, and sulfide; all were prepared from their

respective sodium salts, and only high purity reagents were used to minimize chloride contamination.

Personal exposure monitoring for chlorine and chlorine dioxide using the optimized non-suppressed ion chromatographic method was conducted at four paper / pulp mills. Samples were collected using a modified Tulane passive sampler.[9] As seen in Figure 1, the modified sampler consisted of a 37 mm polystyrene filter cassette with teflon filters (Millipore FA) chemically welded to the exterior and interior faces using methylene chloride. The liquid sample chamber volume was reduced by the inclusion of eighteen 6 mm borosilicate glass balls. The balls were prewashed with chromic acid and rinsed with distilled deionized water. The exterior of the sampler was painted black to prevent photodegradation of the sample. In use, the sampler was filled with approximately 3 mL of absorbing solution and clipped onto the worker's collar. After the monitoring period (usually a full 8-hour shift), the sampler was retrieved, capped, and stored under refrigeration until analysis. In preparation for analysis, the absorbing solution was extracted from the sampler which was then washed with an aliquot of fresh absorbing medium. The sample and wash were combined and diluted to 4.0 or 5.0 ml with additional absorbing medium.

Due to the ubiquitous presence of chloride ion, caution was required in the preparation of samples to prevent contamination. All containers and approximately 10% of the dosimeters used were washed with absorbing solution and checked by ion chromatography for chloride contamination. In addition about 10% of the passive samplers brought to the field were randomly chosen and held as blanks which were subsequently checked for contamination.

Prior to use in the field, the passive sampler was calibrated in the laboratory using standard test atmospheres of Cl_2 and ClO_2 generated in a bench top exposure system. Chlorine test atmospheres were prepared by dilution of chlorine in nitrogen mixtures from a compressed gas cylinder with room air. Chlorine dioxide test atmospheres were prepared using the NCASI

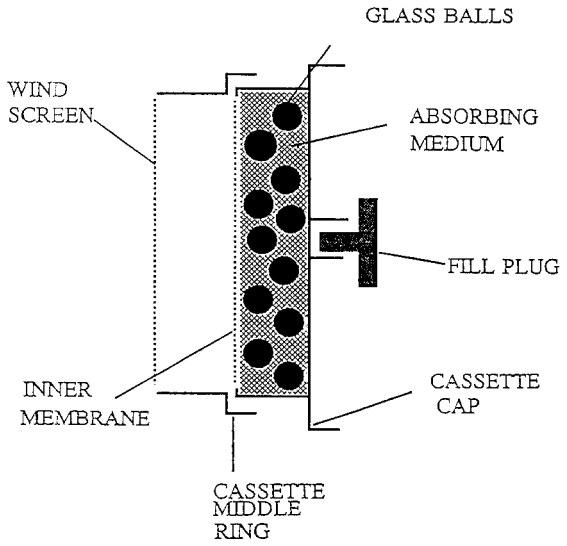

FIGURE 1 Schematic diagram of modified Tulane passive dosimeter.

generator which involves passing a stream of chlorine through a solution of $NaClO_2$, which quantitatively converts chlorine to chlorine dioxide.[10] The sampler was calibrated over a range of exposure concentrations and exposure times. The primary calibration was performed at 25°C; supplemental correction factors were developed at temperatures of 15°C and 35°C.

RESULTS AND DISCUSSION

Mobile phases prepared from sodium hydroxide, sodium bicarbonate, and EDTA all showed high background conductance and excessive system noise. Separation of chloride and chlorite was achieved with a potassium hydrogen phthalate mobile phase, but an inability to resolve the indicating ions

from other ions in the absorbing media limited its usefulness as a mobile phase. Separation was not achieved with 1-octane sulfonic acid. A 3.5 mM 1-hexane sulfonic acid, sodium salt mobile phase, adjusted to pH 4.4 with acetic acid, and pumped at a flow of 2.0 mLpm, achieved separation of the indicating ions, chloride and chlorite, from each other and from possible interfering peaks in the absorbing solution. A chromatogram of a standard solution of Cl^- and ClO_2^-, obtained with the optimized chromatographic conditions, is shown in Figure 2.

Different lot numbers of the column resulted in slight alterations of retention times of the ions but was correctable by adjusting the pH of the mobile phase over a range of 4.1- 4.7. Column ageing also required adjustment of the mobile phase pH to maintain separation of the ions.

To prevent microbial growth, the column was stored in a 50% acetonitrile / 50% water solution when not in use. All solutions, including the mobile phase, were susceptible as well and were filtered through a 0.2 μm filter and stored in the refrigerator when not in use to reduce the risk of microbial contamination and growth.

Peaks from unknown contaminants in the phosphates and sodium iodide used to make the absorbing solution required the testing of several batches of these chemicals of different purities from a variety of suppliers. The use of very high purity reagents was required to eliminate these unknown peaks. However, even with high purity reagents, the phosphates required further clean up with an anion exchange resin before the addition of the iodide.

Several ions were screened as possible interferences. The representative ions for carbon dioxide (carbonate) and nitrogen dioxide (nitrate and nitrite) were selected due to their relatively ubiquitous presence in air. Sulfate and sulfide were selected due to the presence of sulfur compounds in the paper industry environment. Acetate was considered due to its use in adjusting the pH of the mobile phase used in the analysis of the samples. Because airborne bromine would be absorbed by the sampling media, the possible interference of bromide ion was also examined. Of the ions screened,

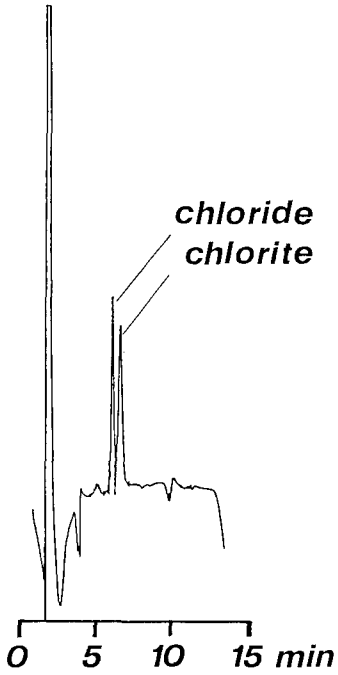

FIGURE 2 Chromatogram of mixed standard of chloride and chlorite, both 10 μg/ml. IC conditions: 250 mm x 4.6 mm Wescan conventional anion/s column; 3.5 mM 1-hexane sulfonic acid sodium salt, adjusted to pH 4.5 with acetic acid and pumped at 2.0 mLpm. Detection: conductivity at 5 μS full scale.

only nitrate had a retention time within one minute of either of the representative ions, eluting 0.7 minutes after the chlorite. Nitrite had the next closest retention time eluting almost two minutes after the chlorite. Bromide, sulfide and acetate all had retention times less than three minutes; where as sulfate and carbonate had significantly longer retention times than chloride or chlorite. The results of these screenings show that none of the ions considered would normally interfere with the chromatographic analysis of chloride and chlorite.

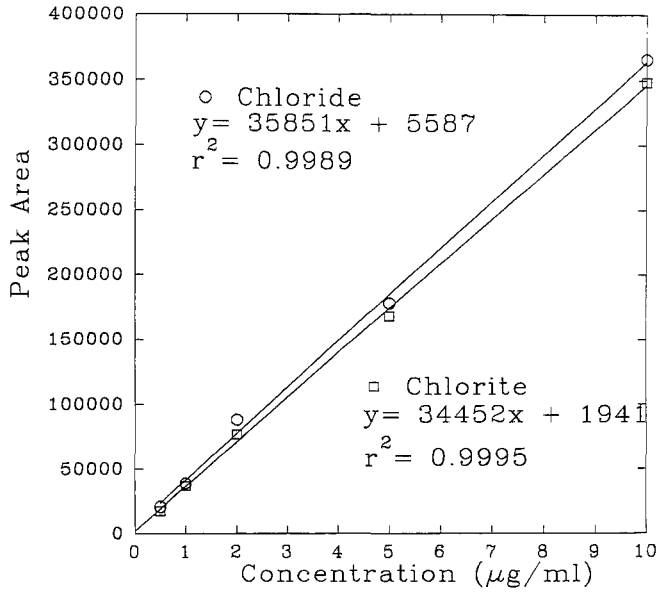

FIGURE 3 Standard curves for ion chromatographic analysis of chloride and chlorite.

Since chlorite ion is unstable in light and thermally degrades with time, standards and samples should be stored in opaque containers in the refrigerator. In general, samples should not be stored for more than two weeks and fresh standards should be made on a weekly basis.

A representative standard curve can be seen in Figure 3. Multiple injections of a 0.5 μg/mL standard resulted in coefficients of variation of 10.5 and 8.8 % for the analytical response of chloride and chlorite, respectively. Based on these results, analytical limits of detection for chloride and chlorite are estimated to be 0.16 μg/mL and 0.13 μg/mL, respectively. This would correspond to air concentrations of 19 ppb chlorine and 16 ppb chlorine dioxide in a 30 liter sample actively collected in 10 ml of absorbing media. Likewise, for the 37-mm modified Tulane passive sampler with a final

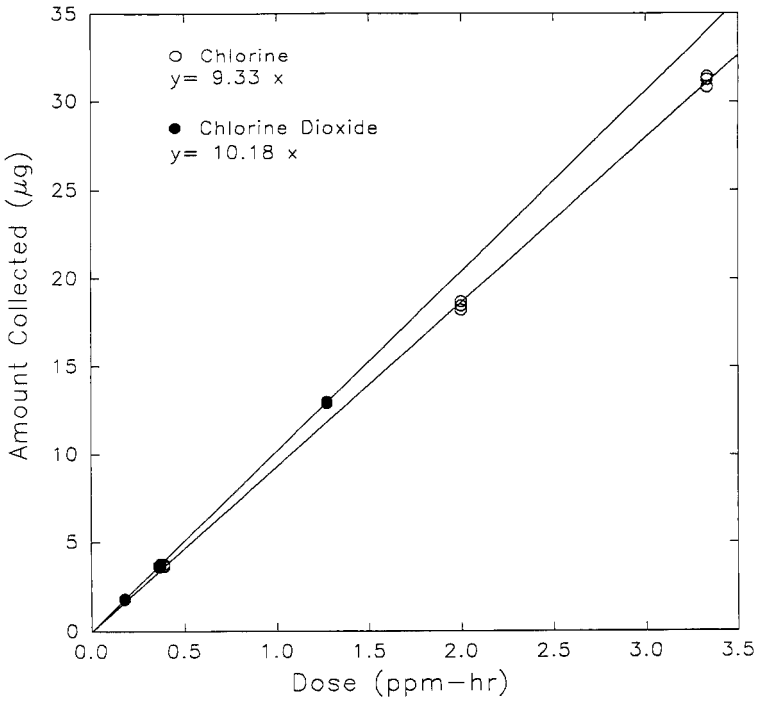

FIGURE 4 Calibration of passive sampler for chlorine and chlorine dioxide at 25°C and 43% RH.

absorbing media volume of 5.0 ml and 8 hours of exposure, the method detection limits would be approximately 10 ppb for both chlorine and chlorine dioxide.

Calibration of the modified Tulane passive sampler yielded mass transfer constants of 9.33 μg/ppm-hr and 10.18 μg/ppm-hr for chlorine and chlorine dioxide at 25°C as seen in Figure 4. Slight temperature corrections were necessary for use under conditions warmer or cooler than 25°C as seen in Figure 5.

The passive sampler was used to assess exposure to chlorine and chlorine dioxide at four paper / pulp mills, see Figure 6 for sample

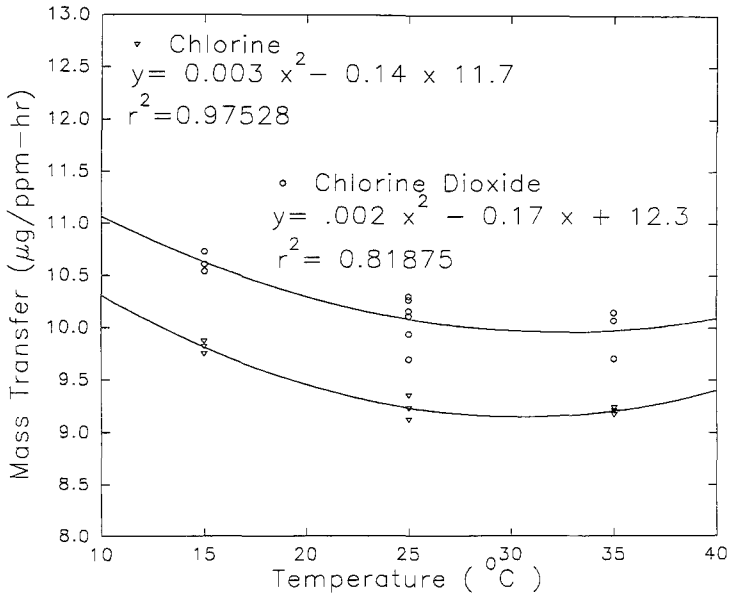

FIGURE 5 Effect of temperature on mass transfer rate of modified Tulane passive sampler for chlorine and chlorine dioxide.

chromatogram. These four mills have undergone conversion to the chlorine / chlorine dioxide bleaching process within the last five years. At the same time, modifications were made to process equipment and procedures to reduce the emission of the chlorine gases and thus the potential for exposure of the workers. At each of the four mills, a total of nine work shifts were monitored over a period of 5 work days. On each shift, approximately 10 workers from various areas of the plant were monitored for exposure. This resulted in a total of 336 personal exposure samples being collected at the mills. Of these, 85 (25.2%) had detectable levels of chlorine and only 13 (3.9%) showed any chlorine dioxide. For those samples with detectable exposures, the overall geometric means and standard deviations were GM = 38 ppb, σ_g = 2.5 and GM = 33 ppb, σ_g = 2.5, for chlorine and chlorine dioxide, respectively.

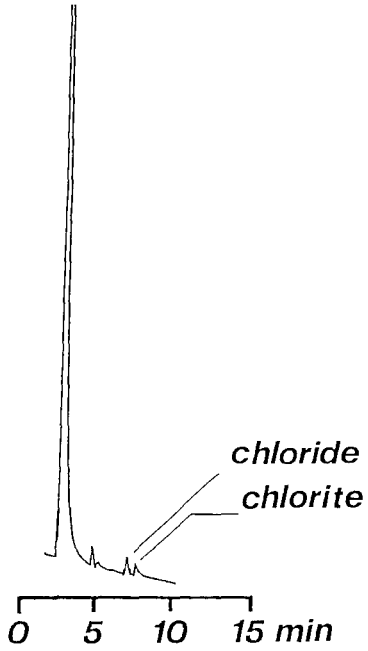

FIGURE 6 Chromatogram of sample from pulp / paper mill. IC conditions as in Figure 2.

A more detailed breakdown of the results of the exposure survey by workareas in the mills is shown in Table 1. In general, exposure to chlorine and especially chlorine dioxide occurs most frequently in the bleach plant areas of these mills. Operators and product testers in these areas may be exposed when entering the bleach washer and generator areas and when collecting samples for quality control. Inadequate ventilation in the bleach plant control room may also result in continuing low-level exposure. Maintenance personnel working on bleach plant equipment also may have frequent exposure to relatively high levels of chlorine and chlorine dioxide. Process upsets and fluctuations may allow residual chlorine and chlorine dioxide to be carried over with the bleached pulp resulting in low-level emissions in the paper

Table 1
Summary Breakdown of Personal Sample Results for Chlorine and Chlorine Dioxide in Four Pulp / Paper Mills*

Work Area	Mill No.	n	Cl_2			ClO_2		
			GM	σ_g	%>LOD	GM	σ_g	%>LOD
woodyard	25	8	18.2	2.6	88	---	---	0
	27	6	7.3	2.6	17	---	---	0
	30	6	10.0	2.8	50	---	---	0
	32	6	---	---	0	---	---	0
pulping		18	13.3	3.2	50	---	---	0
		6	---	---	0	---	---	0
		8	8.3	2.9	25	---	---	0
		4	---	---	0	---	---	0
bleaching		35	11.0	2.8	46	5.7	1.5	14
		42	7.0	2.4	14	5.4	1.7	2.3
		47	6.6	1.8	19	5.4	1.4	6.4
		39	11.9	4.2	28	6.1	2.1	7.7
maintenance		10	10.3	2.3	50	5.8	1.6	10
		24	---	---	0	---	---	0
		11	5.6	1.4	9.1	---	---	0
		7	---	---	0	---	---	0
power plant & chemical recovery		7	26.9	4.4	71	---	---	0
		6	7.4	2.0	33	---	---	0
		3	---	---	0	---	---	0
		9	7.4	3.2	11	---	---	0
paper machines & finishing		10	11.2	2.5	50	---	------	0
		6	---	---	0	---	---	0
		11	6.8	2.1	18	---	---	0
		7	---	---	0	---		0

* GM = geometric mean (ppb), σ_g = geometric standard deviation, %>LOD = percentage of samples with levels of chlorine or chlorine dioxide greater than the limit of detection (~10 ppb for either chlorine or chlorine dioxide). For calculation of geometric mean and standard deviation, samples less than the LOD were given a value of ½ the LOD.

machine and finishing areas. Chlorine may also be used in the power plant and boiler houses for water treatment and biocontrol. Other areas of the mill are subject to episodic exposures due to their proximity to the bleach areas and the dispersion of any offgassed chlorine and chlorine dioxide.

CONCLUSIONS

This method provides for simple, specific, and sensitive determinations of chlorine and chlorine dioxide with analysis by their representative ions, chloride and chlorite. The analytical limit of detection was found to be 0.16 μg/ml chloride and 0.13 μg/ml chlorite. Several possible interferants were considered and found not to affect the analysis. This was confirmed with a field test of the method utilizing passive dosimetry at paper mills.

It is recommended that only very high purity reagents be used in the preparation of solutions and that all solutions be filtered and stored in the refrigerator to reduce microbial growth. Care must be taken to prevent contamination of the solutions with chloride from external sources.

Calibration of the modified Tulane passive sampler yielded mass transfer constants of 9.33 μg/ppm-hr and 10.18 μg/ppm-hr for chlorine and chlorine dioxide at 25°C; however, a correction is required to adjust for temperature effects on the mass transfer rate.

Exposure assessment at four paper / pulp mills showed the utility of the method with the geometric mean of the exposures being approximately 1/25 and 1/3 of the PELs for chlorine and chlorine dioxide respectively.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF RANITIDINE IN HUMAN PLASMA

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Abstract

A rapid reversed phase HPLC method for determination of ranitidine in human plasma has been developed. The procedure involved extraction of the drug from alkalinized plasma spiked with the internal standard (procainamide) using 4% v/v isopropanol in ethylacetate. The extract was evaporated under nitrogen and the residue was reconstituted with methanol and injected onto U-Bondapak C_{18} column. The mobile phase is 8% v/v acetonitrile in 10 mM potassium phosphate buffer (pH 5.1); at a flow rate of 2.5 ml/min and UV detection at 330 nm. The efficiency of extraction was 90% with a detection limit of 20 ng/ml. The within-day coefficient of variations ranged from 4.09% to 5.81%, whereas those of between-day were from 7.5% to 9.51%.

Introduction

Ranitidine is a histamine H_2-receptor antagonist which is very effective in the treatment of gastric and duodenal ulcers [1].

Several reversed phase high-performance liquid chromatographic methods have been reported for determining ranitidine plasma concentrations [2-9]. These methods present various difficulties including, long and tedious extraction procedure [7], use of ion-pair chromatography [6, 9], high percentage of the organic modifier in the mobile phase [4, 5, 8], interferences of endogenous plasma substances [6, 3] and low sensitivity [3].

In contrast, the method described here is simple, less expensive, free of interferences, time-saving, sensitive and selective for the quantitation of ranitidine in human plasma, which makes its applicability in bioavailability studies valuable.

Materials and Methods

Apparatus:

A Waters HPLC Unit (Waters Associates, Milford, MA, USA) was used. It consisted of a system controller (M-720), a data module (M-730), an auto-injector (WISP-712), a solvent delivery system (M-501), UV detector (M-484), and a reverse phase U-Bondapak C_{18} column (10 um, 15 cm x 3.9 mm I.D.).

Chemicals and Reagents:

Ranitidine hydrochloride (Glaxo Ltd., Herts, U.K.) as well as procainamide hydrochloride (Smith Kline and French Laboratories Ltd., Herts, U.K.) were kindly gifted. Acetonitrile, methanol and ethylacetate (HPLC grade) were from E.

Merck (Darmstadt, Germany), potassium phosphate buffer (BDH Chemicals Ltd., Poole, U.K.) and isopropanol (Riedel-De Haen Ag, Seelze-Hannover, Germany) were used. All chemicals were of analytical grade.

Stock Solutions:

A stock solution of ranitidine in methanol was prepared at a concentration of 1 mg/ml. A stock solution of 1 mg/ml procainamide (internal standard) in methanol was prepared and diluted to a concentration of 10 mg/l in methanol for the assay.

Chromatographic Conditions:

A mobile phase containing 8% v/v acetonitrile in 10 mM potassium dihydrogen phosphate (pH 5.1) was used. The flow rate was 2.5 ml/min and the detector was set at a wavelength of 330 nm and a sensitivity of 0.005 AUFS. Experiments were conducted at ambient temperature.

Sample Preparation:

To 1.0 ml of human plasma, 25 ul of internal standard (10 mg/l) and 15 ul of 6N NaOH were added. Then 2.0 ml of 4% v/v isopropanol in ethylacetate was added. The tube was then mechanically shaken for 20 minutes, and then centrifuged at 3000 rpm for 10 minutes. The organic layer was transferred to a 5 ml centrifuge tube and evaporated under a stream of purified nitrogen to dryness at 40^{o}C. The residue was reconstituted in 100 ul methanol and 75 ul of the sample was injected onto the column.

Calibration and Recovery:

Calibration was performed by adding known amounts of ranitidine to blank human plasma to yield concentrations over a range of 0.05-1.0 mg/l, and these standards were then extracted according to the procedure described above.

Ranitidine recovery from plasma was determined by comparing the peak height ratios to those obtained from similar aqueous solutions.

The precision of analysis was assessed by 7 replicate analyses of human plasma spiked with ranitidine to give concentrations of 0.075, 0.35 and 0.8 mg/l, and then within-day variations were calculated. Day to day variations were also calculated for the above concentrations up to 10 days (Table 1).

Specificity

Other drugs (Cimetidine, metoclopramide, naproxen, diclofenac, ibuprofen, phenylbutazone, ketoprofen, lidocaine, verapamil, diazepam, glibenclamide, chlorpheniramine and brompheniramine) were tested for their possible interferences in the HPLC assay. All the investigated drugs were not detected within 15 minutes from the injection except lidocaine which eluted at 8.1 min.

Human Studies:

Pharmacokinetic studies on ranitidine involved its administration to a normal volunteer. Clinical investigations excluded cardiac, hepatic and renal diseases. The volunteer

Table 1: Within- and between-day precision of ranitidine in human plasma.

Within-day*			Between-day**		
Added conc. (mg/l)	Measured conc. (mg/l)	%Bias@	Added conc. (mg/l)	Measured conc. (mg/l)	%Bias@
0.075			0.075		
Mean	0.072	-4.00	Mean	0.061	-18.67
SD	0.0041		SD	0.0058	
CV%	5.64		CV%	9.51	
0.350			0.350		
Mean	0.344	-1.71	Mean	0.350	0.0
SD	0.020		SD	0.029	
CV%	5.81		CV%	8.29	
0.800			0.800		
Mean	0.881	10.13	Mean	0.841	5.13
SD	0.036		SD	0.063	
CV%	4.09		CV%	7.5	

* Mean values represent seven different plasma samples for each concentration.

** Mean values represent seven different plasma samples for each concentration assayed on different days over a period of 10 days.

@ Bias=100x(Measured concentration-Added concentration/added concentration

received 150 mg ranitidine tablet orally after fasting overnight, and 3 ml of blood samples were drawn in heparinized tubes prior to drug administration and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 hrs after administration. Heparinized blood samples were centri-

fuged at 3000 rpm for 10 min., and plasma samples were kept at -20°C until analysis.

Results and Discussion

Using the described procedure, the chromatograms of ranitidine and the internal standard are shown in Fig. 1. Ranitidine showed a peak (b) with a retention time of 5.83 minutes, internal standard showed a peak (a) and a retention time of 3.79 min.

Fig. 1 shows the chromatograms obtained from human plasma extract taken 4.0 hrs (B) and 7.0 hrs (C) following ranitidine administration.

Operation of the detector at 330 nm, rather than 229 nm [3, 6] resulted in a cleaner chromatogram since there was no interference from endogenous plasma constituents or concomitant drugs without loss of sensitivity.

The calibration curve for ranitidine was linear over the range investigated with a correlation coefficient of 0.99 (Fig. 2). The detection limit was 20 ng/ml.

The recovery of ranitidine from the human plasma using this method was 90% on the average, which compared favorably with those reported by other workers [2, 5, 6].

The overall precision of the method was measured on the basis of samples with three different concentrations (Table 1).

The effect of freezing and thawing of the plasma samples was studied over a period of two weeks at 0.075 and 0.35 mg/l. There was no significant differences in the

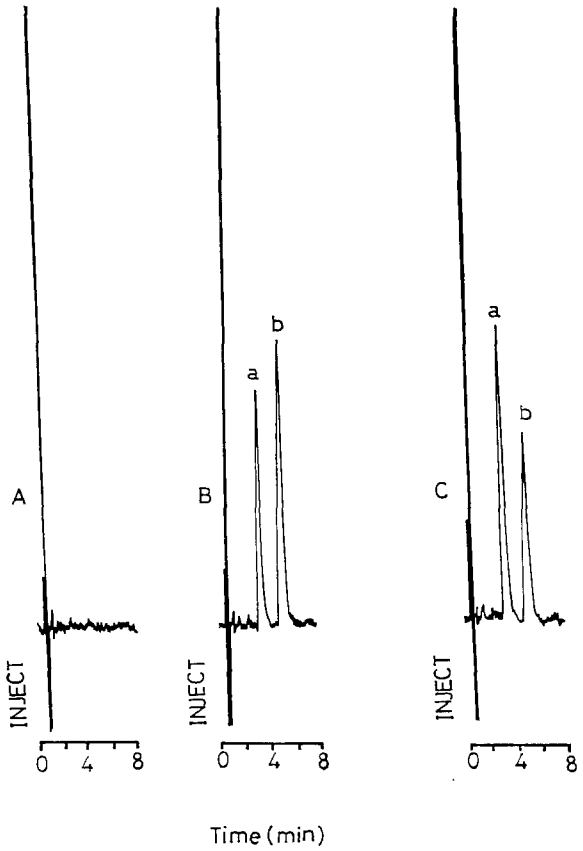

Fig. 1: Typical chromatograms of human plasma extract following oral administration of 150 mg ranitidine to a human volunteer. Blood samples were drawn prior to dose (A), 4 hrs (B) and 7 hrs (C) after administration of ranitidine. Peaks: (a) is internal standard and (b) is ranitidine. Chromatographic conditions are as in text.

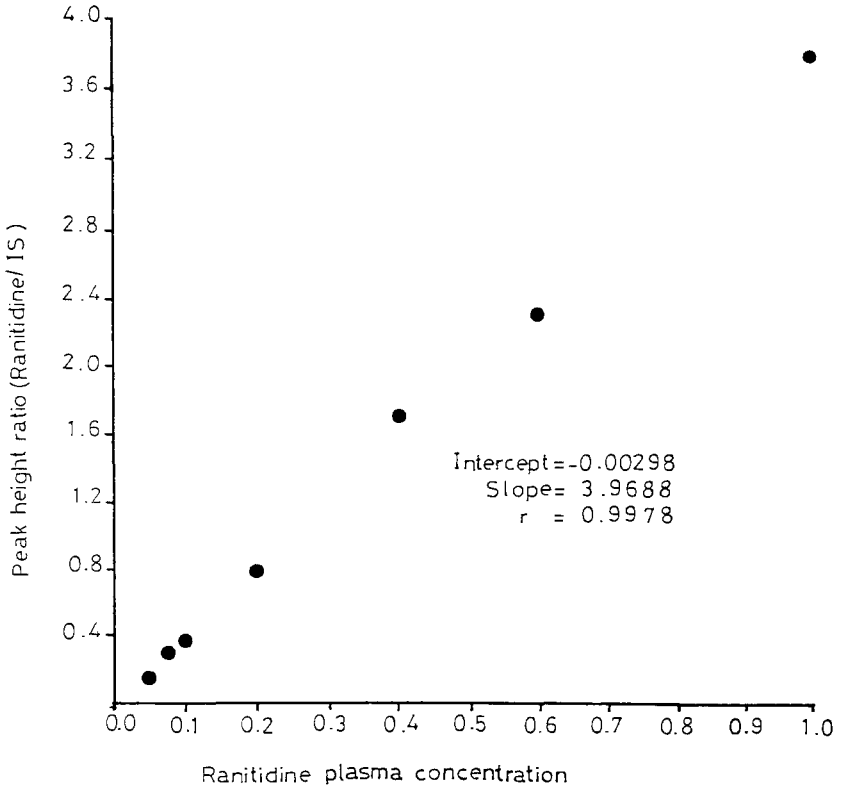

Fig. 2: Standard curve for ranitidine in human plasma. Least squares linear regression analysis resulted in: Y = -0.0029+3.9688x, r=0.9978.

results of the concentrations measured which assured that freezing and thawing of the sample had no effect on the estimation of ranitidine by the above procedure.

The applicability of the assay procedure is illustrated in Fig. 3 which shows the ranitidine plasma concentration-

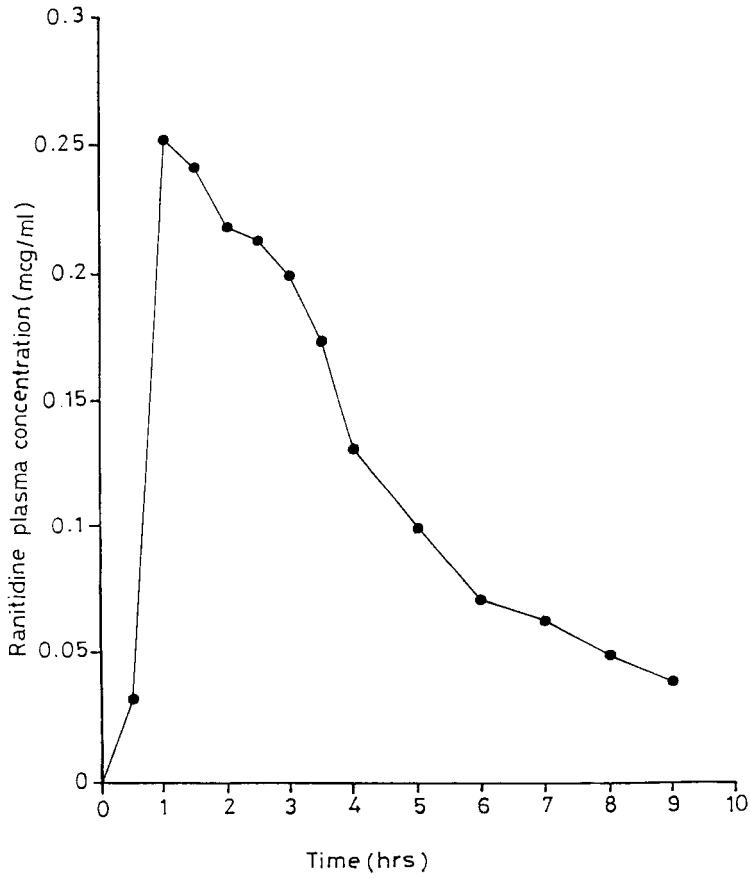

Fig. 3: Plasma level vs time following oral administration of 150 mg ranitidine to a human volunteer.

time profile following oral administration of ranitidine to a healthy subject.

The advantage of this assay is its simplicity, sensitivity, selectivity as well as its application in pharmacokinetic and bioequivalency studies of ranitidine.

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FURTHER INVESTIGATIONS IN HPLC SYSTEMS CONTAINING SILICA AND POLAR BONDED SILICA PACKING DYNAMICALLY MODIFIED BY CAMPHORSULFONIC ACID

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ABSTRACT

The investigation of chromatographic (HPLC) systems containing camphorsulfonic acid (CSA) has been continued. Similarly to the previous finding for silica, on polar bonded silica packings CSA forms double layer. The adsorbed amount of CSA was determined by the evaluation of break-through curves. It was found that the addition of triethylamine in a small excess to the CSA containing eluent results in the formation of a versatile chromatographic system. The adsorbed amount of CSA and TEA from such eluents was determined by the estimation of the break-through curves were gained by simultaneous refractometric and UV spectrophotometric detection. The migration of the compounds follows a normal phase mechanism evidenced by the behavior of the examined medicinal compounds.

INTRODUCTION

In a previous paper [1] the adsorption of camphorsulfonic acid (CSA) on bare silica was followed by the break-through method, using methanol-chloroform-CSA and acetonitril-chloroform-CSA mixture as mobile phase. In the same study the formation of CSA-double layers was suggested and the amount of CSA bonded in the 1. and 2. layer has been determined. The different

sorptional feature of the two CSA-layers was revealed. It seemed reasonable to extend the research to the study of CSA-bonding power of cyanopropylsilica and other polar bonded silica sorbents representing a polarity weaker than that of the bare silica and thereby the field of application of CSA-silica systems should be widened [2]. In trying to increase the versatility, i.e. selectivity of the system, as an organic modifier triethylamine was added in excess to the mobile phase. The separation power of a similar chromatographic system containing cyanopropylsilica, CSA as ion exchanger and diethylamine as a counter base, was carefully studied by Szepesi et al. [3,4]. Whether in our case, the retention bahavior of the substances tested should reflect a normal- or rather a reversed phase character of the silica/CSA-TEA, etc containing system, was a further question to be answered.

EXPERIMENTAL

Materials

All of the model stubstances, but not the fluoroquinolones, and the steroids were a quality of Ph.Hg.VII. (Pharmacopoeia Hungarica VII.).

Fluoroquinolones were synthetized at Chinoin Pharmaceutical Works (Budapest) and used without further purification.

The steroids were synthetized at Gedeon Richter Pharmaceutical Works (Budapest) and used without further purification.

Methanol HPLC grade (Chemolab, Budapest)

Chloroform Ph.Hg.VII., was used after purification by the Ph.Hg. method.

Triaethylamin, TEA (LOBA Chemie, Fishamend, Germany)

(+)10-Camphorsulfonic acid (CSA) monohydrate (Merck).

Chromatography

The HPLC apparatus was comprised in Waters (Millipore, USA) Mode 501 solvent delivery system, LABOR MIM (Budapest, Hungary) Model QE 308 variable wavelength UV photometer, Yokogava (Tokyo, Japan) Type 3051 recorder. For the determination of triethylamine adsorption the effluent was monitored with RI detection (Waters Differential Refractometer, Model R 401).

The packings were filled into stainless steel columns (250 x 4 mm I.D.). Adsorbents were purchased from BST (Bioseparation-Technique Ltd., Budapest, Hungary) in a particle size 5 μm of each: Silica Si-100-S, Diol Si-100-S, Cyanopropyl Si-100-S, Phenylsilica Si-100-S.

Break-through curves

The amount of adsorbed CSA and TEA was determined by the evaluation of the correspondent break-through curves. The equipment used for the break-through measurements is a modified version was described in a previous publication [1]. For the simultaneous measurement of CSA and TEA adsorption an apparatus working with two parallel switched pumps, columns and detectors (UV, RI) was applied [5].

Mobile phase, substance application, recording.

As eluent the mixture of chloroform and methanol 99:1 containing given concentration of CSA and/or TEA was used. The eluents were filtered and degassed prior to chromatography. The flow rate was 1.0 ml/min. A Model 7125 sampling valve (Rheodyne, Berkley, USA) was applied.

The column-temperature was controlled by recirculating water through an isolated stainless jacket from termostat (Ultrathermostate MLW Type U2C, Freital, Germany).

The model substances were solved in the correspondent eluents. The basic compounds, due to solubility problems, were applied in their base form.

The chromatograms were obtained and recorded, the retention data were collected by a Hewlett-Packard integrator Model 3396 Ser 2.

Each retention data was calculated as an average of three parallel runs. The mobile phase hold-up time was signalled by the solvent peak of methanol

RESULTS AND DISCUSSION

Table 1 involves the data of adsorption for CSA on silica and three polar bonded silica packings. The results were gained by the evaluation of the break-through curves. The main message of the table is, that in accordance with the earlier (1) and present finding for silica, CSA forms two distinct layers (i.e.

Table 1

The adsorption of CSA on silica and polar bonded silica columns (Mobile phase: chloroform - methanol 99:1).

Column	CSA in the eluent M	CSA adsorbed uM/g	
		Layer 1.	Layer 2.
SILICA	0.005	242.2	-
	0.010	240.0	115.0
	0.020	249.2	249.2
SILICA-DIOL	0.005	40.6	28.32
	0.010	65.6	37.32
	0.020	97.2	99.2
CYANOPROPYL-SILICA	0.005	49.2	21.0
	0.010	76.0	39.12
	0.020	110.8	119.52
PHENYL-SILICA	0.005	56.0	13.12
	0.010	85.2	52.8
	0.020	124.0	111.2

double layer) on the surface of each of the three polar bonded silica adsorbents. Another characteristic of the table is the increase of the adsorbance by increasing the CSA-concentration of the eluent. This is true for both of the CSA-layers. At a CSA concentration 0.02 M the amount of adsorbed CSA by the second layer equals with the one adsorbed by the first layer. This unexpectedly high value in the 2nd layer may come from the simultaneous adsorption of CSA by the empty 2nd layer and the continous occupation of the free sites of the first layer. The amount of CSA adsorbed by the polar bonded silica layers is about 1/3rd of that bonded by the bare silica. The gradual increase of CSA in the 1st. layer parallels with a steep rise of CSA-adsorption into the 2nd. layer.

Table 2 shows the amount of adsorbed CSA and TEA in the same chromatographic system, when the TEA was present in a minute excess. In the latter case double layer formation does not take place. The comparison of the

Table 2

Simultaneous adsorption of TEA and CSA by different silica packings

Column	Eluent $CHCl_3$ - MeOH 99:1			Ratio of adsorbed TEA:CSA
	+0.012 M TEA	+0.012M TEA + 0.010 M CSA		
	Adsorbed amount uM/g			
	TEA	TEA	CSA	
SILICA	352.4	329.2	261.2	1.26
SILICA-DIOL	74.88	74.4	46.4	1.62
CYANOPROPYL-SILICA	152.64	149.2	114.8	1.31
PHENYL-SILICA	160.0	144.8	108.8	1.33

corresponding data of the Tables 1 and 2 shows, that TEA, excepted cyanopropyl silica, strongly reduces the adsorbed amount of CSA by bare silica as well as polar bonded silica surfaces. On the other hand, CSA has small influence on the adsorption of TEA by the silicas. The TEA and CSA are adsorbed in an amount corresponding to a mol ratio approximately 1.3, excepted diol silica, where the ratio index is higher, 1.6. This fact allows to conclude, that TEA and CSA occupies the major part of the silica surface in the form of a (1:1) salt-like compound and in addition, a part of the superfluous TEA adheres to the free silanol groups of the surface. Considering the moderately polar character of the weakly alkaline (non adsorbed TEA) mobile phase the stationary phase in this chromatographic system must definitely exert a polar sorbent activity, since all the solutes migrate according to the rules of a normal phase mechanism.

Table 3 show the retention for 25 structurally different medicinal compounds, having neutral, acidic or basic character. It can be seen, that the mobile phase-C, containing CSA and in a small excess TEA, is suitable for the elution of the organic bases too. We met only one application of the latter eluent type, when certain steroids were separated [6]. It is worthy of note, that with

Table 3

The retention (t_R min) of drug compounds in different CSA (TEA) containing chromatographic systems
(SI: silica; Diol:silicadiol; Phe: Phenylsilica; CNP:cyanopropylsilica; NH_2:amonosilica; NE:no elution within 90 minutes.

COMPOUND	$CHCl_3$: MeOH 99:1 A)					$CHCl_3$:MeOH 99:1 +0.010 M CSA B)					$CHCl_3$:MeOH 99:1 +0.010 M CSA +0.012 M TEA C)				
	SORBENT					SORBENT					SORBENT				
	Si	Diol	Phe	CNP	NH_2	Si	Diol	Phe	CNP	NH_2	Si	Diol	Phe	CNP	NH_2
Norgestrel	10.5	3.5	4.5	4.5	1.2	3.5	2.9	3.3	3.4	1.5	2.9	3.0	2.9	3.3	1.3
Ethisterone	12.9	3.8	5.0	4.7	1.2	3.8	3.0	3.5	3.6	1.5	3.2	3.1	3.0	3.3	1.3
Norethisterone	15.0	3.9	5.5	4.5	1.2	4.1	3.1	3.8	3.7	1.5	3.3	3.2	3.1	3.5	1.3
Benzoic acid	11.1	7.7	6.3	NE	NE	6.4	6.7	5.6	5.2	2.3	NE	NE	55.6	NE	NE
Salicylic acid	NE	8.2	5.4	NE	NE	6.9	7.8	5.2	6.0	5.6	NE	NE	NE	NE	NE
Acetylsalicylic. acid	NE	8.3	7.5	NE	NE	7.3	6.1	6.4	6.9	1.9	NE	7.5	NE	NE	NE
Hexobarbital	7.6	3.7	4.2	3.9	1.3	3.4	3.0	3.0	3.2	1.2	3.0	3.2	3.0	3.4	1.4
Phenobarbital	22.4	10.2	8.0	7.8	3.4	8.7	7.6	5.4	6.4	2.4	8.2	8.3	5.9	11.8	4.8
Caffeine	42.0	5.2	14.0	7.4	1.2	NE	9.6	NE	23.3	1.2	4.1	3.3	5.2	3.7	1.3
Theobromine	NE	15.5	36.8	26.4	1.5	NE	38.6	NE	NE	1.6	9.1	6.6	9.3	7.0	1.7
Theophylline	NE	26.3	60.5	19.7	2.8	NE	56.7	NE	NE	4.7	24.8	NE	28.6	15.1	3.2
Benzocaine	45.3	14.1	14.1	4.4	1.3	40.6	6.2	3.4	5.5	2.3	3.9	3.5	3.6	3.1	NE
Lidocaine	NE	NE	NE	NE	1.1	NE	NE	NE	NE	1.5	2.7	2.6	2.8	3.1	1.2
Tetracaine	NE	NE	NE	NE	1.3	NE	NE	NE	NE	2.4	10.0	5.5	9.1	9.5	1.2
Procaine	NE	NE	NE	NE	1.6	NE	NE	NE	NE	4.6	22.6	7.4	13.5	15.4	1.5
Ethylmorphine	NE	NE	NE	NE	1.3	NE	12.9	NE	NE	1.3	23.0	8.3	18.1	13.9	1.3
Codeine	NE	NE	NE	NE	1.3	NE	15.1	NE	NE	1.3	25.7	9.2	19.3	15.0	1.4
Chlorpromazine	NE	NE	NE	NE	1.3	NE	12.1	NE	34.2	1.2	2.8	5.5	10.7	7.6	1.2
Levomepromazine	NE	NE	NE	NE	1.3	NE	7.7 8.0	NE	28.9	1.2	6.8	3.8	6.0	5.2	1.2
Promethazine	NE	NE	NE	NE	1.3	NE	8.5	NE	29.2	1.2	9.8	4.7	9.5	6.8	1.3
Trifluperazine	NE	NE	NE	NE	1.3	NE	44.3	NE	NE	1.2	11.2	6.0	14.4	9.1	1.2
Quinine	NE	NE	NE	NE	1.2	NE	30.6	NE	NE	1.3	58.3	14.8	30.8	18.0	1.5
Quinidine	NE	NE	NE	NE	1.2	NE	24.7	NE	NE	1.3	34.8	10.4	18.4	14.2	1.4
Cinchonine	NE	NE	NE	NE	1.2	NE	57.4	NE	NE	1.3	49.7	13.8	24.3	18.8	1.4
Cinchonidine	NE	NE	NE	NE	1.2	NE	60.3	NE	NE	1.3	72.4	19.7	35.9	25.6	1.6

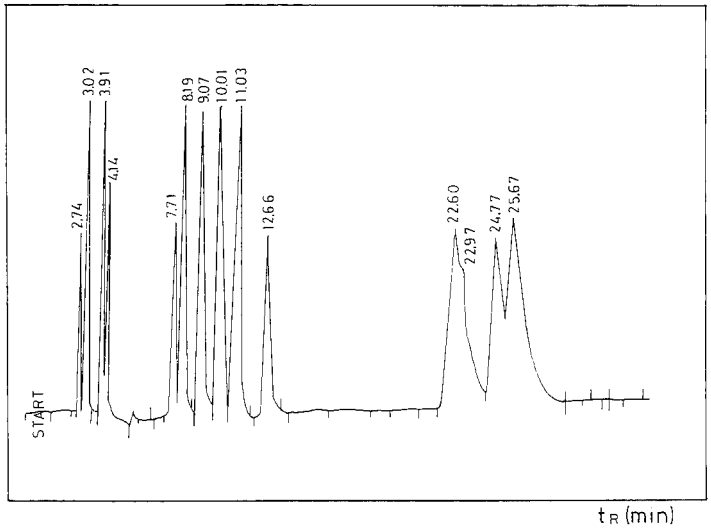

Figure 1: Representative chromatogram of 15 model compounds.

Sorbent: silica, eluent: C (see Table 3).
2.74 lidocaine, 3.02 hexobarbital, 3.91 benzocaine
4.14 caffeine , 7.71 levomepromazine,
8.19 phenobarbital, 9.07 theobromine, 10.01 tetracaine,
11.03 promathazine, 12.66 chlorpromazine, trifluperazine,
22.60 procaine, 22.97 ethylmorphine, 24.77 theophylline,
25.67 codeine.

this alkaline eluent the best separations were achieved just on silica gel surface, which, due to its too polar feature, could not be used at all when the mobile phase contained CSA in excess [2]. Another benefit using the TEA-excess containing (basic) eluent is, that it dissolves well also the salt forms of the amine pharmaceutics, which will migrate in their base form. These basic compounds could not be eluted at all with chloroform-methanol mixture with or without CSA content (Table 3, eluent A and B, compds. 12-25). In the system-C homologs (as codeine-ethylmorphine) or structurally closely related substances (see compds. 9→11, 18→21, 22→25) can be separated. The quality of separation is illustrated by Fig. 1. The chromatogram of five "floxacins", is

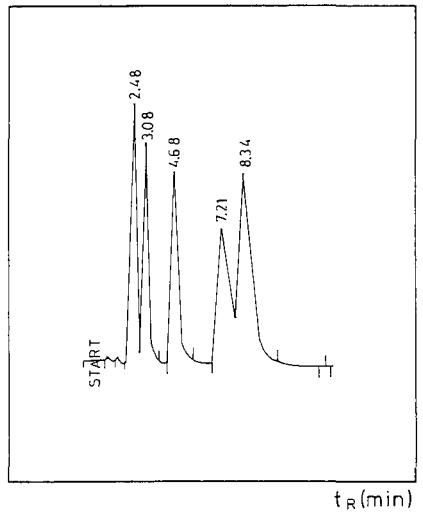

Figure 2: Chromatogram of five gyrase inhibitor fluoroquinolone derivatives.

2.48 pefloxacin, 3.08 lomefloxacin,
4.68 amifloxacin, 7.21 ciprofloxacin,
8.34 norfloxacin.

shown by Fig. 2. These gyrase inhibitor fluoroquinolone derivatives could be eluted from the aminosilica surface and separate well with the alkaline eluent (C). The strong retention of acids (benzoic-, salicylic acid, etc.) against the alkaline eluent may be explained by a substitution effect they exert on the bonded CSA.

The comparing of certain retentions evidences the normal phase character of the chromatographic system-C.: cf. the retentions caffeine-theobromine-theophylline, procaine-tetracaine, codeine-ethylmorphine, etc.

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RAPID AND SENSITIVE DETERMINATION OF THALIDOMIDE IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive and rapid High-performance liquid chromatographic method using U.V.detection, has been developed for the analysis of thalidomide in plasma. This involved a single liquid-solid extraction on Extra-Sep-C8 column in the presence of an internal standard (ciprofloxacin). Analysis was performed by isocratic elution with a mobile phase consisted of 0.01 M aqueous potassium dihydrogen phosphate containing 21 % (V/V) acetonitrile and 4.5 mM Heptane sulfonic acid, adjusted to pH 2.3, with U.V detection at 295 nm. The limit of sensitivity of the assay was 0.06 mg/l. The method was applied to a pharmacokinetic study (50 to 100 mg)in patients with erythema nodusum leprosum (ENL) with a good accuracy (96-111 %) and precision (less than 5.8 %).

INTRODUCTION

Thalidomide (α- phtalimidoglutarimide) was introduced as a non-barbiturate hypnotic in 1956, and was withdrawn from the market in 1961 because of teratogenic effects (1). Its immunosuppressive properties have been known and Thalidomide

has been used in different immunologically mediated diseases including lepromatus leprosy, aphtous stomatitis, and discoid lupus erythematosus for over twenty years (2,3).

Recently, this drug was proposed in the treatment of acute and chronic graft-versus-host disease (GVHD) in bone marrow transplant patients (4).

Several analytical methods using colorimetric detection (5), UV spectrometry (6), paper and thin layer chromatography (7,8) have been reported for thalidomide but these procedures suffer from a lack of selectivity and/or sensitivity. A Gas chromatography method was described but accuracy and precision were not indicated (9). Several H.P.L.C methods have been recently proposed. However two H.P.L.C methods have been developped (10,11) but they are characterized by rather poor sensitivity with achieving detection limit of only 1.0 mg/l.

Another H.P.L.C assay was proposed (12) with a reported sensitivity near 0.1 mg/l, but no data was available concerning accuracy and precision. Recently, a sensitive, specific, and accurate H.P.L.C procedure was described (13) but the sample work-up procedure using a liquid-liquid extraction was time-consuming thereby hampering extended pharmacokinetic study with large numbers of samples. In this paper, we describe a rapid, accurate and sensitive method for the quantitative determination of thalidomide in plasma by H.P.L.C using reversed-phase chromatography, solid phase extraction and U.V. detection.

MATERIALS AND METHOD

Chemicals

Thalidomide was kindly supplied by Dr Frankus from Chemie Grünenthal (Aachen, Germany) and ciprofloxacin (internal standard) by Bayer-Pharma Laboratories (Sens, France).
H.P.L.C grade acetonitrile, methanol and dimethylformamide, RPE-ACS grade potassium phosphate monobasic and sodium dihydrogen phosphate were obtained from Carlo-Erba (Milan,

Italy) and used for the preparation of an 0.066M aqueous phosphate buffer. 1-Heptane sulfonic acid (H.S.A) was from Sigma (Saint Louis, U.S.A).

Analytical grade orthophosphoric acid, hydrochloric acid and di-sodium hydrogen phosphate 2-hydrate were from Merck (Darmstadt , Germany). Extraction columns (Extra-Sep C_8, 200 mg , 3 ml, Lida Manufacturing Corp.,Kenosha, U.S.A) were from Touzart-Matignon (Paris, France). Ultrapure water was obtained before use, through a Milli Q- plus water purification system (Millipore Corp.,U.S.A).

Chromatography

H.P.L.C system consisted of a model 510 Waters pump, a model 231 Gilson autosampler unit with a 100 µl loop, models 484 Waters U.V. absorbance detector and 746 integrator-recorder.The column (150 x 4.6 mm i.d) was packed with Nucleosil C_8, 5 µm particule size (Touzart-Matignon, France). The mobile phase consisted of 0.01M aqueous potassium dihydrogen phosphate solution containing 21 % (V/V) acetonitrile and 4.5 mM H.S.A, adjusted to pH 2.3 with concentrated orthophosphoric acid.

Prior to use, the mobile phase was filtered through a H.V.L.P 04700 Durapore membrane (Millipore Corp.,U.S.A). This, was carried through the column at 0.8 ml/min and the detection wavelength was set at 295 nm. All separations were carried out at room temperature.

Standard solutions

The stock solution (1000 mg/l) of thalidomide was prepared weekly in dimethylformamide, and stored at + 4°C. The stock solution (100 mg/l) of ciprofloxacin (I.S.) was weekly prepared in methanol and stored in similar conditions.

The working solutions were prepared by dissolving the stock solution in 0.01N HCl, at final concentrations of 10.0- 5.0 - 2.5 - 1.25 - 0.625 mg/l for thalidomide, and 2.0 mg/l for I.S.

Calibration standards : (0 - 0.0625 - 0.125 - 0.25 - 0.5 - 1.0 mg/l) were made daily from pooled human plasma. These were prepared according to the sample preparation procedure.

Sample preparation

The solid-phase extraction column (Extra-Sep C_8) was conditioned by elution with 3 x 1 ml of 0.066M phosphate buffer. Plasma sample (1.0 ml) supplemented with 60 µl of I.S. methanolic solution (2.0 mg/l) and mixed with an equal volume of 0.066M pH 7.4 phosphate buffer, was loaded onto the conditioned column. This fraction was washed with 3 x 1 ml of 0.1M HCl. Both thalidomide and I.S. were recovered with 5 x 0.2 ml of dimethylformamide. The eluate was evaporated to dryness at ambient temperature under a gentle stream of nitrogen gas. The dry residue was reconstituted in 130 µl of mobile phase and an aliquot of 100 µl was injected into the chromatographic system.

RESULT AND DISCUSSION

Analytical conditions

Solid - phase extraction (SPE) provides fast and efficient sample preparation, it reduces sample handling and eliminates the risk of emulsification (13,14). The use of ciprofloxacin as I.S. improves the reproducibility of the method (Table 1).

Two U.V. detection peaks are available for the analytical determination of thalidomide in biological fluids. Several authors (11,12,15) have selected a wavelength below 254 nm for U.V. detection, which gives a good sensitivity but in contrast a poor specificity.

We have selected a wavelength at 295 nm as CZEJKA M.J et al (10), which optimizes the specificity of the assay (Figure 1).

Linearity

Standard curves were constructed by plotting the peak-area ratio of thalidomide to the internal standard versus the concentration of thalidomide.

The calibration curve was linear over the selected following range (0.0625 - 1.0 mg/l) in plasma, and characterized by the equation : $y = 1.294\ x - 0.010$, ($r = 0.9994$, $n = 6$).

TABLE 1

Within-day Variability an Accuracy in Measured Thalidomide Concentrations in Plasma.

Conc. spiked (mg / l)	Conc. found (mg / l) mean ± S.D.	Accuracy (%)	Coefficient of variation (%)
0.125 (n = 8)	0.125 ± 0.006	100.0	4.8
0.50 (n = 7)	0.494 ± 0.039	98.8	7.8
1.0 (n = 7)	0.953 ± 0.070	95.3	7.3

Recovery

The absolute recovery of the SPE was determined by comparing the peak areas of thalidomide obtained from freshly prepared spiked plasma sample extracts to those obtained from aqueous standard solutions with the same concentration. The recovery was 79.5 ± 4.2 % at 0.5 mg/l and 80.3 ± 4.6 % at 1.0 mg/l, n =3

Chromatograms

Figure 1 shows chromatograms obtained with extracts from plasma spiked with 0.5 mg/l thalidomide (Figure 1a), blank plasma (Figure 1b), and plasma sample obtained 4 hours after the administration of a 100 mg daily oral dose of thalidomide, to an erythema nodusum leprosum patient (Figure 1c).

Concentrations were calculated by comparing the ratio of peak areas of samples (major peak/ I.S), with these of the calibration curve made daily. Thalidomide and I.S. were eluted in 10.6 and 16.2 min., respectively.

Limit of quantitation

The limit of quantitation for thalidomide was 0.0625 mg/l (Figure 2), when 1.0 ml of plasma was used.

The limit was taken as a chromatographic peak four times higher than baseline noise; it was always the lowest point of the calibration curve, giving good accuracy (111 %) and precision (C.V = 5.8 % , n = 6). This limit of quantitation was sufficient for pharmacokinetic studies.

Accuracy and precision

The within-day variability of the method was characterized by coefficients of variation lower than 7.8 % (Table 1).

The inter-day variability in plasma was assessed over thirty days from four series of human plasma samples containing 0.0625, 0.125, 0.50 and 1.0 mg/l of thalidomide (Table 2). The C.V for inter-day analysis was less than 6 % and the accuracy inside a 99.2 - 111 % range.

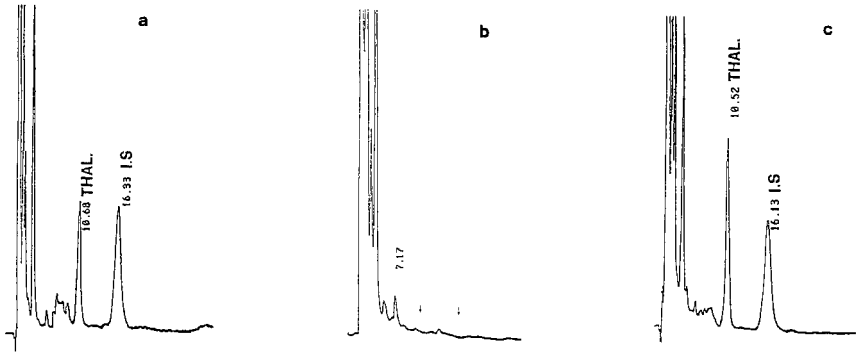

FIGURE 1 : Chromatograms obtained from extract of

(a) plasma spiked with thalidomide (THAL.)(0.5 mg/l) and internal standard (I.S)

(b) human blank plasma

(c) plasma sample obtained at the 4 th hour from a patient included in a pharmacokinetic study : THAL. : 0.8 mg/l

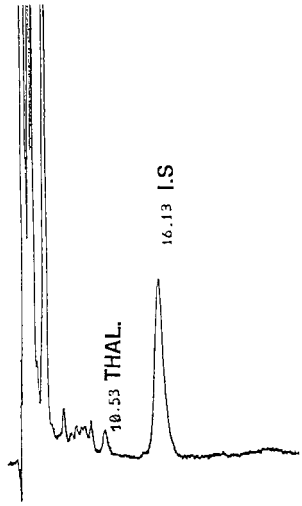

FIGURE 2 : Chromatogram obtained from extract of a blank plasma spiked at the limit of quantitation : 0.06 mg/l of THAL.

TABLE 2

Between-day Variability and Accuracy in Measured Thalidomide Concentrations in Plasma.

Conc. spiked (mg / l)	Conc.found (mg / l) mean ± S.D	Accuracy (%)	Coefficient of variation (%)
0 (n= 6)	N.D.	-	-
0.0625 (n = 6)	0.0697 ± 0.004	111.0	5.8
0.125 (n = 6)	0.120 ± 0.007	96.0	5.8
0.5 (n = 6)	0.528 ± 0.025	106.0	3.8
1.0 (n = 6)	0.993 ± 0.056	99.3	5.0

N.D. = Not Detected

TABLE 3

Retention Time for Different Drugs Administered in Patients Receiving Thalidomide Treatment.

Drug	Retention time (min)
ciclosporine	N.D
ciclophosphamide	N.D
prednisolone	N.D
hydroxyzine	N.D
nifédipine	N.D
diltiazem	N.D
amphotéricine B	N.D
clonazépam	N.D
clobazam	N.D
diazépam	N.D
desmethyl-diazépam	N.D
acyclovir	2.0
flucytosine	2.5
métronidazole	3.0
azothioprine	4.4
ceftazidime	4.5
céfotaxime	8.0
thalidomide	10.6
ciprofloxacine	16.3

N.D = Not Detected

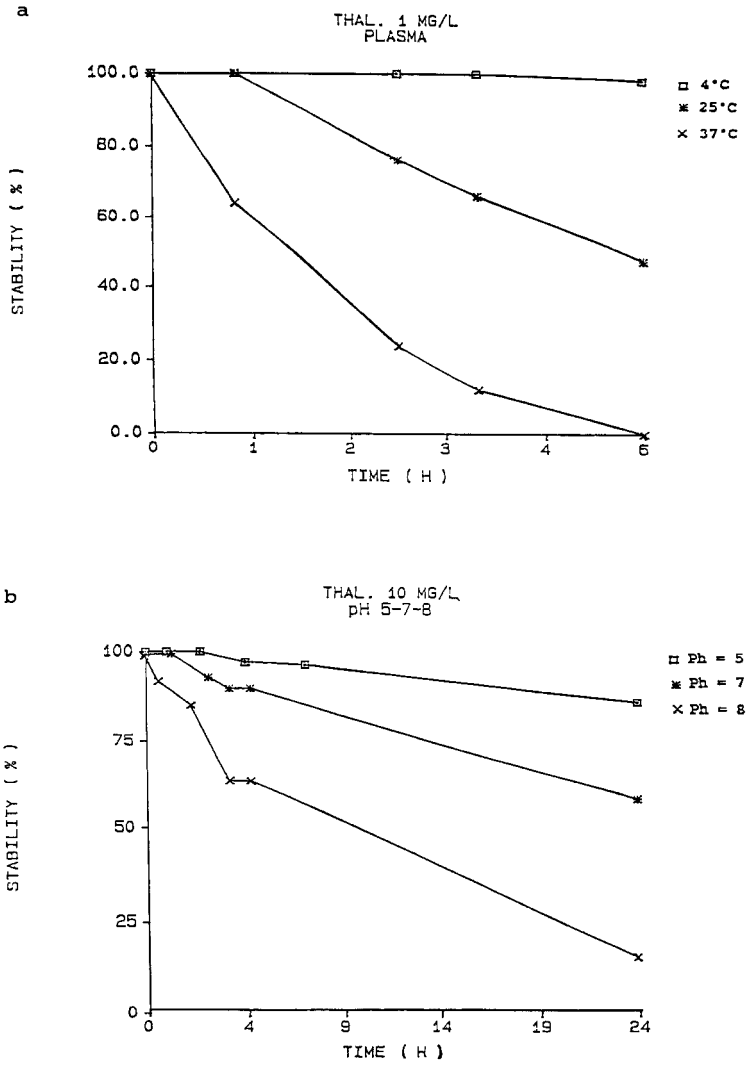

FIGURE 3 : Curves of thalidomide stability

(a) plasma extracts at different temperature conditions.

(b) in different pH conditions of aqueous buffer.

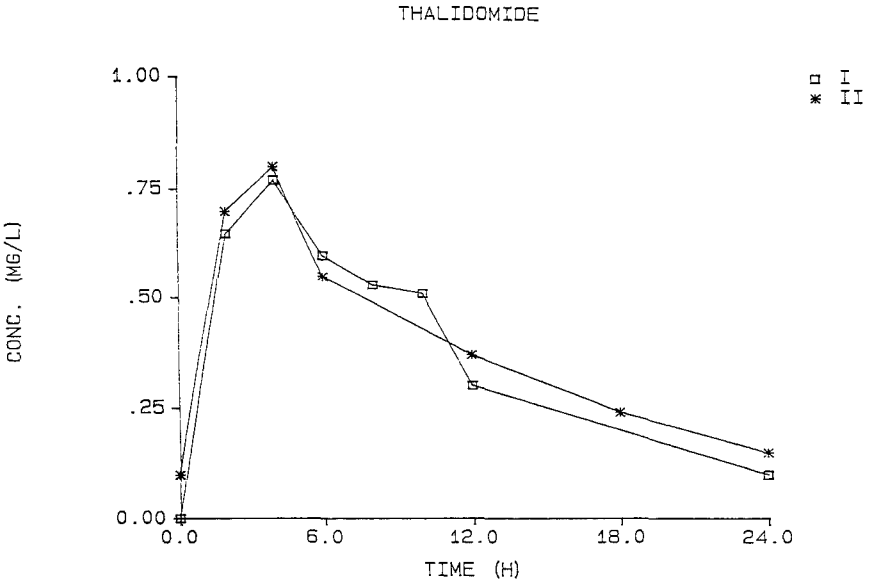

FIGURE 4 : Plasma levels of thalidomide with time following single dose oral administration (100 mg) in patient I, and at steady-state (100 mg/24 h) in patient II.

Specificity

Table 3 , lists the retention times of different drugs potentially administered to patients receiving thalidomide. Most were not detected, and no interferences appeared when peaks occurred on chromatograms. Their retention times were sufficiently different from those of thalidomide and I.S. to preclude any chromatographic interference.

Stability

Several authors reported an hydrolysis of thalidomide in aqueous medium at pH > 7 (10, 12, 14). We have investigated the influence of both temperature and pH on its stability.

Thalidomide and ciprofloxacin (I.S) were stable in stock solutions for at least a week in the refrigerator. A good stability was observed for at least 6 h at + 4°C and 1 h at 25°C in plasma samples (1.0 mg/l).(Figure 3 a).

No significant degradation was observed at 10 mg/l in aqueous buffer at pH 5 and kept at 25 °C. Instability appeared at pH 7 and increased in alcaline conditions (Figure 3 b).

Thalidomide stability was verified in both 0.066M phosphate buffer (pH 7.4) and H.P.L.C injection solvent for at least 25 min. and 12 h, respectively.

Pharmacokinetic application

The applicability of this assay was examined in a preliminary pharmacokinetic study in erythema nodosum leprosum patients.

The plasma concentration-time curves of thalidomide are shown in figure 4. From the results of this preliminary study, it appears that this method would be suitable in plasma drug monitoring of both E.N.L patients and bone marrow recipients suffering of graft-versus-host-disease (GVHD).

CONCLUSION

The H.P.L.C assay described here showed good reproductibility, accuracy and selectivity. It is sufficently sensitive and suitable to be used in pharmacokinetic studies, and therapeutic drug monitoring.

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RETENTION DEPENDENCE ON ORGANIC MODIFIER AND INTERACTION REAGENT CONCENTRATION IN REVERSED-PHASE ION-INTERACTION HPLC

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ABSTRACT

The effect played on ion-interaction chromatographic retention by the concentrations of the organic modifier and of the ion-interaction reagent as a function of the ionic strength is studied.

Experiments are performed for a series of analytes characterized by different chemical properties (nitrate, nitrite, iodide, ascorbic, orotic and p-aminobenzoic acids, aniline, benzylamine, p- and m-aminophenol) in the absence and in the presence of organic modifiers (acetonitrile and methanol) and of sodium perchlorate to control the ionic strength (I = 1.0 M).

Concentrations of the ion-interaction reagent (octylammonium o-phosphate) ranging between 1.0 and 60.0 mM and of the organic modifier (methanol and acetonitrile) ranging between 0 and 45% are considered.

Retention interdependence on the concentrations of ion-interaction reagent and of the methanol in the mobile phase is also studied.

The results are discussed and compared with literature data and the relevant role played on the retention by ionic strength is underlined.

INTRODUCTION

Many reversed-phase HPLC methods make use, in order to improve the chromatographic response, of ion-interaction reagents added to the mobile phase. The ion-interaction reagent forms with the analyte a ion-pair which, due to the increased lipophilic properties, is retained onto the reversed-phase surface; this process does not necessarily involve a modification of the stationary phase surface.

According to other hypotheses, the interaction reagent contained in the mobile phase is adsorbed onto the surface of the stationary phase through adsorption and electrostatic forces, giving rise to an electrical double-layer (1-4). The interaction properties of the originary reversed-phase packing material are therefore modified.

These techniques are generally refered as ion-interaction or ion-pair chromatography and they all make use of a reversed-phase stationary phase. As concerns the choice of the mobile phase, most of the methods utilize an organic-aqueous mixture which contains the interaction reagent, whilst a few others (5-8) make use of an aqueous solution of the interaction reagent.

Previous results obtained in this laboratory with mobile phases formed by aqueous solutions of different interaction reagents were in agreement with the hypothesis of a dynamic modification induced by

the flowing interaction reagent on the surface of the stationary phase. It can be anyway supposed that this modification does not involve the whole surface of the stationary phase (9-11).Two kinds of retention sites (12,13) therefore can simultaneously be present on the surface, namely conventional reversed-phase adsorption and ion-interaction sites produced in the dynamic modification.

The predominance in the retention process of the solute of ion-interaction or adsorption mechanism can be correlated (12) with the double-layer electrical potential and, through the Stahlberg equation (3), with the concentration of the ion-interaction reagent, in agreement with models recently proposed (14) by Zou and coworkers.Also the concentration of the organic modifier in the mobile phase plays an important role, due to the competition of the organic solvent for the column surface (15-18). Literature results, mostly obtained for aqueous-organic mobile phases, generally agree in observing a retention decrease when the concentration of the organic modifier in the mobile phase increases. Different behaviours were on the contrary observed as concerns the dependence of retention on the concentration of the interaction reagent.

In order to lead a further contribution in this study, in the present paper a systematic study is carried out about the retention dependence on organic modifier and ion-interaction reagent concentration. In order to separately investigate on the effects of the factors, the use of aqueous and hydro-organic mobile-phases is compared. Octylammonium o-phosphate at pH 6.4 is used as the interaction-reagent in a very wide concentration range (between 1.0 and 60.0 mM). The effect of ionic strength is also investigated, by comparing two series of experiments performed with and without the correction for constant ionic strength. Taking into account that also the chemical properties of the analytes can play a role, analytes characterized by different chemical properties were chosen and namely: nitrate, iodide, nitrite, ascorbic acid, orotic acid, aniline, benzylamine, phenylurea, ethylenethiourea, 1,4-aminobenzoic acid, 1,4-amino- and 1,3-amino-phenol.

MATERIALS

Apparatus

Analyses were carried out with a Merck-Hitachi Lichrograph chromatograph Model L-6200, equipped with a two-channel model D-2500 Chromato-integrator, interfaced with a UV-Vis detector model L-4200 and a L-3720 conductivity detector of the same firm.

A Metrohom 654 pH-meter equipped with a combined glass-calomel electrode was employed for pH measurements and a Hitachi mod.150-20 spectrophotometer for absorbance measurements.

Chemicals and Reagents.

Ultrapure water from Millipore Milli-Q was used for the preparation of solutions. Octylamine and orotic acid were Fluka analytical grade chemical and sodium nitrate, sodium nitrite, sodium iodide, ascorbic acid, aniline, benzylamine, phenylurea and ethylenthiourea were Merck analytical grade chemicals. Ortho-phosphoric acid was C.Erba chemical.

METHODS

Chromatographic conditions. Procedure.

A 5 μm ODS-2 Spherisorb Phase Separation column fully end-capped and with a carbon load of 12% (0.5 mM/g) was used, together with a guard pre-column Lichrospher RP-18 (5 μm).

The solutions to be used as the mobile phase were prepared by adding the amount of o-phosphoric acid required to obtain the desired pH value to the amount of octylamine and of organic solvent needed to

prepare the prefixed concentrations. The pH value so obtained in aqueous-organic solution is also reported as an "operational" pH value (19). The same procedure was followed for the preparations of solution at controlled ionic strength. A constant I =0.10 M ionic strength was realized, for addition of sodium perchlorate, which does not absorb at the used wavelengths.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary. The use of a premixed water-organic eluent flowing in isocratic conditions permitted to achieve shorter equilibration times. Between the uses of different mobile phases and after use, the stationary phase was washed by flowing water (1.0 ml/min for 15 min) and then a 50/50 v/v water/methanol or water/acetonitrile mixture (1.0 ml/min for 15 min).

Dead times were evaluated for each series of experiments through injections of $NaNO_3$ solutions (20 ppm) and conductometric detection of the unretained Na^+.

Spectrophotometric detection at 230 nm was employed.

RESULTS AND DISCUSSION

Effect of organic modifier concentration.

Table I reports the capacity factors k' obtained for the analytes investigated at different concentrations of methanol and acetonitrile. For both the solvents was observed a retention decrease which follows an exponential shape which could be fitted (correlation coefficient r^2 always >0.95) by the mathematical function $y = A\ e^{-kx}$. Figure 1 reports the plots of the natural logarithms of the capacity factors as a function of the per cent concentration of methanol (figure 1A) and acetonitrile (figure 1B), which were fitted by straight lines.

The observed decrease in retention can be ascribed, besides to the increasing solvent eluotropic strength with the increasing organic concentration, to the effect played by the organic modifier on the

TABLE I

Capacity factors as a function of organic modifier % concentration.
Stationary phase:Phase Separation ODS-2 Spherisorb 5 μm endcapped.
Ion-interaction reagent: octylammonium o-phosphate 5.0 mM pH 6.4
Spectrophotometric detection:230 nm.
Flow-rate 0.7 ml/min and 1.5 ml/min for phenylurea and aniline.

METHANOL

Analyte / MeOH conc.	0%	5%	10%	15%	25%	35%	45%
Iodide	6.29	4.75	3.36	2.67	1.85	1.28	0.79
Nitrate	5.12	3.53	2.67	2.21	1.62	1.32	0.72
Nitrite	3.24	2.61	2.10	1.84	1.44	1.02	0.69
Ascorbic Acid	3.84	3.29	1.93	1.73	1.22	0.84	0.59
Benzylamine	0.72	0.57	0.47	0.34	0.25	0.24	***
Ethylenethiourea	0.84	0.58	0.43	0.38	0.25	0.21	***
Phenylurea	14.88	10.75	6.24	4.62	2.40	1.38	***
Aniline	14.25	7.16	5.07	3.80	2.44	1.58	***

ACETONITRILE

Analyte / ACN conc.	0%	5%	10%	15%	25%
Iodide	6.29	3.65	2.80	1.76	0.68
Nitrate	5.12	2.60	1.92	1.28	0.44
Nitrite	3.24	1.93	1.57	1.04	0.37
Ascorbic Acid	3.84	1.65	1.14	0.79	0.32
Benzylamine	0.72	0.38	0.37	0.34	***
Ethylenethiourea	0.84	0.47	0.38	0.34	***
Phenylurea	14.88	6.58	3.85	2.30	0.89
Aniline	14.23	5.83	4.28	3.22	1.73

capacity of the modified surface of the stationary-phase, due to the competition which takes place for the surface itself between the organic modifier and the ion-interaction reagent.

Since the results of table I and figure 1 do not show any easy correlation between retention and chemical properties of the analytes investigated, the observed retention decrease could likely be ascribed more to the varied interaction properties of the moiety adsorbed onto the surface than to the analyte characteristic properties.

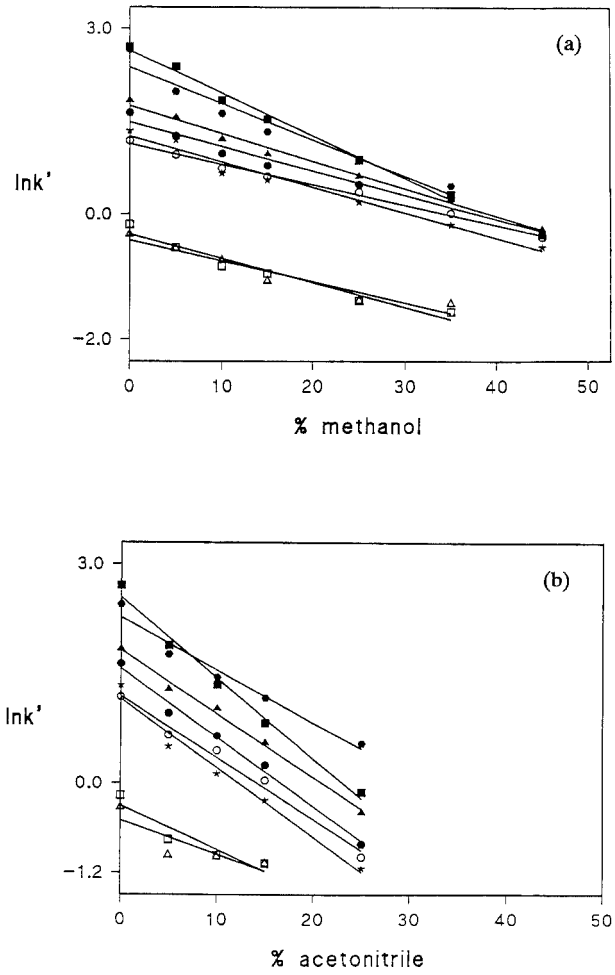


FIGURE 1 ln of capacity factor as a function of organic modifier % concentration: A-methanol, B-acetonitrile.
Stationary phase: Phase Separation ODS-2 Spherisorb 5μm endcapped.
Ion-interaction reagent: octylammonium o-phosphate 5.0 mM pH 6.4.
Spectrophotometric detection 230 nm. Flow-rate 0.7 ml/min and 1.5 ml/min for phenylurea and aniline.
Analyte: ▲-iodide; ● -nitrate; ○ -nitrite; ✦ -ascorbic acid; - △ benzylamine; ⬢ - aniline; □ -ethylenethiourea; ■ -phenylurea.

Effect of ion-interaction concentration

A general disagreement is reported in literature as concerns the retention dependence on ion-interaction concentration. The retention dependence on ion-interaction concentration was shown (9,20-22) to be a function of the charge of the analyte, which in turn can affect the double-layer electrical potential. It follows that with increasing ion-interaction reagent concentration, a retention decrease can be observed when the charges of the analyte and of the ion-pairing agent are of the same sign (2), no retention dependence for uncharged analytes (9,22) and a retention increase when the charges of ion-pairing and solutes are of different sign (9,22).

When retention increases with the ion-interaction reagent concentration, different behaviours were observed and namely: a linear increase (21,23), a shape which reaches a plateau (24,28) or a parabolic dependence (5,9,11,29-35).

In order to consider the effect of the concentration of the ion-interaction reagent independently on the concentration of the organic solvent, we carried out this study by using as the mobile phase an aqueous solution of the interaction reagent.Furthermore, to study the retention behaviour in the most general conditions, a wide concentration range (between 1.0 and 60.0 mM) of the ion-interaction reagent and analytes characterized by different chemical properties were considered.

The capacity factors obtained are plotted in figure 2 as a function of the ion-interaction reagent concentration. Generally (a part p-aminophenol and benzylamine which show a small retention decrease with the increase of the ion-interaction reagent concentration) the shape of the capacity factors vs. concentration shows a maximum, whose entity and position varies for the different analytes investigated.

The comparison of our results with literature data show that the behaviour here obtained with aqueous mobile phase is comparable with the data obtained with hydro-organic mobile phases and, more important point, that the literature disagreement is only apparent, because it depends on the range of concentration explored. The

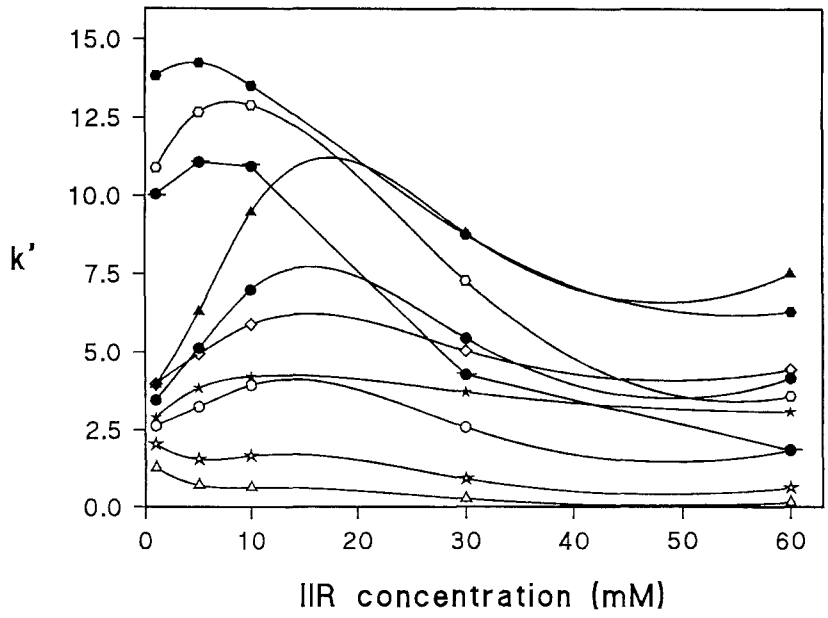


FIGURE 2 Capacity factor as a function of ion-interaction reagent (IIR) concentration.
Chromatographic conditions as in figure 1.
Analyte: ▲-iodide; ● -nitrate; ○ -nitrite; ✦ -ascorbic acid; - △ benzylamine; ⬣-aniline; ☆ p-aminophenol; ◇-m-aminophenol; ⬡p-aminobenzoic acid; —●— orotic acid.

complete behaviour follows in fact a parabolic shape and the other dependencies experimentally observed (21, 24-28) hold only for narrow concentration ranges.

The portion of the curve to the left of the maximum can be easily explained, in agreement with literature: the stationary phase surface is becoming more ionogenic due to the adsorption of hetaerons onto the surface and the overall energy increases due to ion-ion interactions. A maximum of retention is then reached in correspondence of the surface capacity (maximum adsorption capability) of the stationary phase. Since retention also depends on the chemical properties of analytes

and on their ability to form ion-pairs, the parabolic shapes are different for the different analytes.

The problem with all the existing theories seems to lie to the right of the curve maximum, also observed for bare-silica (35) and polymer-based (17,29) stationary-phases. Why the decrease in retention?

Decrease has been ascribed to the formation in the mobile phase of micelles and to the taking place of competitive adsorption reactions when the concentration of the interaction reagent exceeds the critical micelle concentration (11,30,31). But if retention depended on the critical micelle concentration, the retention dependence for the same chromatographic conditions would have to be similar for all the analytes (36) and the maximum of retention would correspond to the critical micellar concentration (CMC) value of the interaction reagent (40). These conditions are not verified for the data reported in figure 2. As concerns the CMC range, literature does not report the value for octylammonium ortho-phosphate but the CMC for octylammonium chloride (37) is available and equal to 90 mM, which is a very higher concentration than those at which we observe the maxima of retention (5-10 mM).

Another hypothesis ascribes the retention decrease to the increase of counter-ion concentration (29,34,36). Counter-ions can exert a shielding effect towards the accessibility for the analytes to the stationary-phase surface and can also remarkably affect the double-layer potential. In agreement with this suggestion, phosphate concentration, which in our experiments increases with the increasing octylammonium concentration, could be responsible for these effects.

It must be anyway taken into consideration that in the above experimental conditions, in which the ion-interaction reagent concentration varies from 1.0 to 60.0 mM, the total ionic strength of the mobile phase remarkably varies. In order to verify if the retention behaviour as a function of the ion-interaction reagent concentration and in particular if the retention decrease observed for the higher concentrations could depend on the ionic strength variations, a series of experiments (below reported) was also performed, in which the total

ionic strength in the mobile phase was kept constant (at concentration of 0.10 M) for addition of sodium perchlorate.

The effect of the organic modifier concentration on the retention dependence on the ion-interaction concentration.

In order to collect further information about the roles played on the retention by the ion-interaction reagent and the organic modifier concentrations, three new series of experiments were performed for concentrations of octylammonium phosphate ranging between 3.0 and 25.0 mM, at prefixed methanol concentrations of 5, 15 and 45%. The values obtained for the capacity factors are presented in table III and in figures 3 and 4. The shapes clearly show as the retention dependence on the ion-interaction concentration also depends on the methanol concentration, as on the other hand theorectically predicted by Horvath (5). The following considerations can be drawn out. A decrease in the curve maxima universally occurs upon the addition of organic modifier.Then, the shape of the curves about the maxima becomes sharper and more pronounced , even while the overall k' values decrease from neat aqueous solutions. Moreover, for some ions (nitrite, nitrate, iodide) the maxima of the curve shift to the left (lower ion-interaction concentration) with increasing organic, while for phenylurea and ascorbic acid the maxima remain approximately constant. Aniline shifts left but to an intermediate degree. In all the instances, the retention of ionic solutes with the increasing of hetaeron concentration, initially rises, reaches a maximum and then falls.

The decrease of the dielectric constant induced by the increasing of the organic content will reduce the surface concentration of hetaerons with the resulting loss in retention. In agreement, the hydrophobic portions of solute molecules will diminish in retention, resulting in more pronounced slopes for the hydrophobic ions with respect to the harder inorganic ions. The trend is evident in figure 3 and 4 where the iodide, nitrate and nitrite ions decrease by ca. 30-50% from

TABLE II

Capacity factor as a function of ion-interaction reagent concentration.
Stationary phase: Phase Separation ODS-2 Spherisorb 5 μm endcapped.
Ion-interaction reagent: 5.0 mM octylammonium o-phosphate pH 6.4.
Spectrophotometric detection: 230 nm. Flow-rate 0.7 ml/min.

	ion-interaction reagent concentration				
Analyte	**1.0 mM**	**5.0 mM**	**10.0 mM**	**30.0 mM**	**60.0 mM**
Iodide	3.95	6.29	9.47	8.79	7.50
Nitrate	3.46	5.12	6.98	5.43	4.16
Nitrite	2.63	3.24	3.93	2.58	1.86
Ascorbic acid	2.90	3.84	4.20	3.71	3.09
Benzylamine	1.28	0.72	0.64	0.26	0.14
Aniline	13.83	14.23	13.51	8.75	6.29
p-aminophenol	2.04	1.56	1.67	0.93	0.63
m-aminophenol	3.97	4.93	5.87	5.03	4.43
p-aminobenzoic acid	10.89	12.67	12.89	7.27	3.59
Orotic Acid	10.05	11.07	10.93	4.26	1.88

TABLE III

Capacity factor as a function of ion-interaction and methanol concentration.
Ion-interaction reagent: 5.0 mM octylammonium o-phosphate pH 6.4
Stationary phase: Phase Separation ODS-2 Spherisorb 5μm endcapped.
Spectrophotometric detection 230 nm
Flow-rate 0.7 ml/min, for phenylurea and aniline 1.5 ml/min.

Analyte	**5% 3 mM**	**15% 3mM**	**45% 3 mM**	**5% 5 mM**	**15% 5 mM**	**45% 5 mM**	**5% 10 mM**	**15% 10 mM**	**45% 10 mM**	**5% 25 mM**	**15% 25 mM**	**45% 25 mM**
Iodide	3.39	2.16	0.51	4.67	2.62	0.77	4.15	2.60	0.82	5.01	2.59	0.84
Nitrite	2.17	1.63	0.47	2.56	1.80	0.67	2.64	1.64	0.73	2.02	1.41	0.72
Nitrate	2.78	1.80	0.50	3.67	2.17	0.70	3.29	2.12	0.77	3.18	2.07	0.78
Ascorbic acid	2.20	1.51	0.44	2.61	1.70	0.57	2.68	1.46	0.64	1.77	1.21	0.64
Phenylurea	9.69	4.53	0.99	10.02	4.27	***	9.00	4.61	0.95	9.39	4.72	0.96
Aniline	7.81	4.29	1.21	7.16	3.80	***	6.46	3.78	1.15	5.48	3.31	1.08

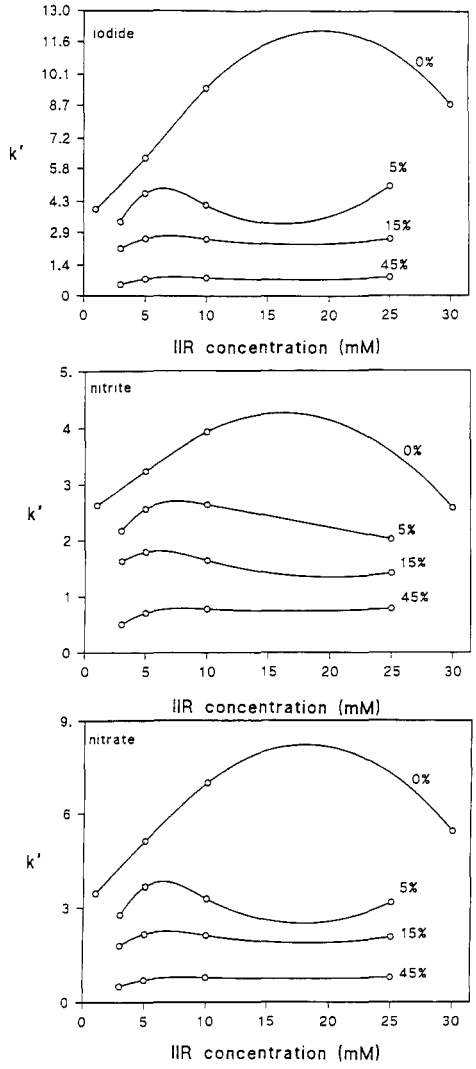


FIGURE 3 Capacity factor as a function of ion-interaction reagent (IIR) concentration for different methanol % concentrations. Chromatographic conditions as in Fig.1. Analytes: iodide, nitrate and nitrite.

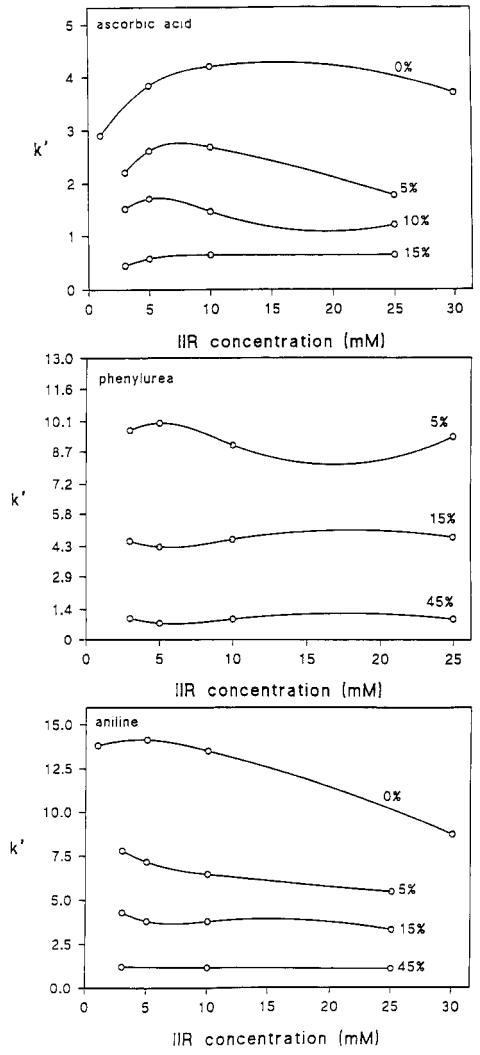


FIGURE 4 Capacity factor as a function of ion-interaction reagent (IIR) concentration for different methanol % concentrations. Chromatographic conditions as in Fig.1. Analytes: aniline, phenylurea and ascorbic acid.

0 to 55% of methanol, while the bulkier ions decrease by 50% or more from 5 to 10% of methanol.

Here again, a decreasing retention to the right of the maximum for the curves of figures 3 and 4 can be observed.

The curves are progressively flatting with the increasing methanol concentration and for the highest methanol concentrations investigated, the retention is practically independent on ion-interaction reagent concentration.

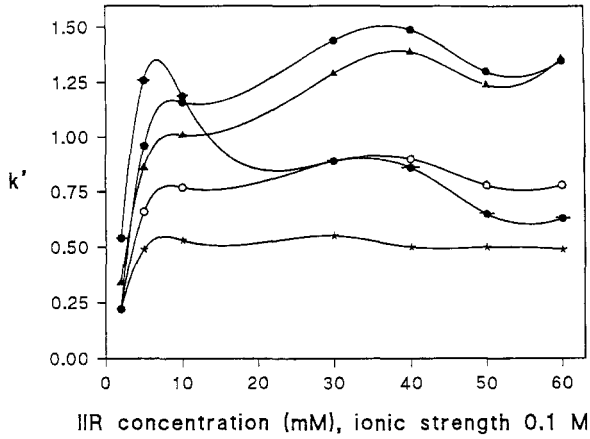
The effect of the ionic strength on the retention dependence on the ion-interaction concentration.

On the basis of the above considerations, a series of experiments were performed at constant ionic strength I (I= 0.10 M for sodium perchlorate). The results obtained for concentration of octylammonium o-phosphate ranging between 2 and 60 mM, are reported in table IV and in figure 5. It must be said that in general the presence of sodium perchlorate strongly decreases the retention and this can be explained through a shielding effect which the added electrolyte exerts towards the accessibility for the solute of the active sites present on the stationary-phase surface. The general decrease of retention did not

TABLE IV

Capacity factor as a function of ion-interaction reagent concentration, at constant ionic strength 0.10 M ($NaClO_4$).
Stationary phase: Phase Separation ODS-2 Spherisorb 5 μm, endcapped
Ion-interaction reagent: 5 0 mM Octylammonium o-phosphate, pH=6.4
Spectrophotometric detection 230 nm. Flow-rate: 0 7 ml/min.

	ion-interaction reagent concentration						
Analyte	2.0 mM	5.0 mM	10 mM	30 Mm	40 mM	50 mM	60 mM
Iodide	0.34	0.86	1.01	1.29	1.39	1.24	1.36
Nitrate	0 22	0 96	1.16	1.44	1.49	1 30	1 32
Nitrite	0.22	0 66	0.77	0.89	0 90	0 78	0.78
Ascorbic acid	0 22	0 49	0 53	0.55	0 50	0 50	0 49
Aniline	10 81	10.24	8 66	6 93	6 74	5 46	5 45
Orotic Acid	0 54	1.26	1 19	0 89	0 86	0 65	0 63



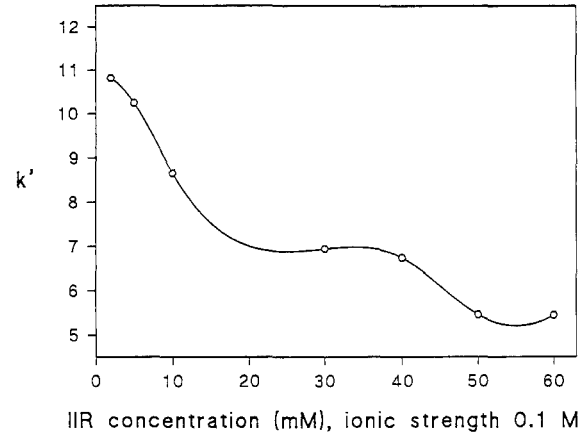


FIGURE 5 Capacity factor as a function of ion-interaction reagent (IIR) concentration at constant ionic strength I=0.10 M for $NaClO_4$. Chromatographic conditions and symbols as in figures 1 and 2.

allow us to investigate all the analytes before considered, because some of them, under the experimental conditions used, do not show appreciable retention times. The retention decrease due to the addition of the electrolyte ranges between 50% and 79% for nitrate, iodide, nitrite, orotic and ascorbic acids, while is very lower (maximum 20%) for aniline. This may be due to the more lipophilic properties of aniline and to the predominance in the retention process of partition mechanism with respect to ion-interaction mechanism.

As concerns the dependence of the retention on the ion-interaction concentration, nitrite, nitrate, iodide and ascorbic acid show an initial increase and then a plateau behaviour. On the contrary orotic acid and aniline show a characteristic and similar shape with the presence of two different maxima and a general decrease in the curve to the right of the major maximum.

As a result, the retention decrease observed for the higher ion-interaction reagent concentrations (table II, figure 2) can be ascribed to the uncontrolled ionic strength only as concerns the behaviour of the more hydrophilic ions. The addition of the electrolyte seems on the contrary not to significantly affect the behaviour of the more lipophilic species, such as aniline and orotic acid. A different retention mechanism, in which adsorption forces predominate on electrostatic ones, could perhaps be hypothesized for these species.

ACKNOWLEDGEMENTS

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VALIDATION OF AN HPLC METHOD FOR THE DETERMINATION OF SODIUM IN LY293111 SODIUM, A NOVEL LTB_4 RECEPTOR ANTAGONIST, USING EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

Analysis of inorganic ions such as sodium or chloride in pharmaceutical compounds has traditionally employed ion-chromatography (IC) with conductivity detection. A new quantitative method for the determination of sodium in LY293111 sodium, a novel LTB_4 receptor antagonist, using high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) is discussed. The separation of sodium from other ions and interferences was achieved using a Zorbax 300 SCX cation-exchange column suitable for use with organic solvents. Acceptable levels of precision, linearity, recovery, selectivity and limit of detection were achieved during the validation of the method. The results of this method were within 99.8% agreement when compared to the theoretical amount of sodium in LY293111 sodium. HPLC coupled with evaporative light scattering detection offers a practical alternative to IC using conductivity detection in pharmaceutical compounds.

INTRODUCTION

Pharmaceutical drug candidates are often synthesized as salts in order to increase the aqueous solubility or stability of the drug. The most common pharmaceutical salt forms are sodium salts of acids and hydrochloride salts of amines.[1] Quantitation of inorganic counter-ions is necessary to determine the overall purity of a drug substance. Traditionally, the analysis of pharmaceutical counter-ions has been performed on ion-chromatography (IC) systems which employ conductivity detection. IC systems are generally dedicated for the analysis of ions and are not practical for use with other reversed-phase or normal phase pharmaceutical applications. This paper focuses on a new approach for the quantitation of inorganic counter-ions which utilizes existing HPLC systems common in the pharmaceutical laboratory with ELSD.

Recently, ELSD has been introduced commercially and has gained acceptance as a sensitive universal detector.[2-3] ELSD operates by nebulizing the volatile effluent from the HPLC column into a fine mist. The mist is carried through a heated drift tube which evaporates the mobile phase and leaves behind non-volatile solute particles. The fine cloud of solute particles is carried at a high speed through a beam of light where the scattered light is detected by a photomultiplier. The amount of light scattered is dependent upon the size and number of particles and therefore proportional to the concentration. The mobile phase used with ELSD must be free of non-volatiles which would be detected by the ELSD thus limiting its use for some traditional applications of ion-exchange/ion-pairing chromatography. However, we have developed a new quantitative HPLC method for the determination of sodium using ELSD with ammonium acetate, a volatile buffer salt.

ELSD has been shown in the literature to successfully detect phospholipids,[4-9] triglycerides, fats and fatty acid esters,[10-13] carbohydrates,[14-15] synthetic polymers,[16] and steroids.[17] Surprisingly, no information has been published on ELSD for the determination of inoganic salts. The aim of this paper is to show the applicability of an evaporative light scattering detector to accurately detect sodium in a pharmacuetical compound. In this study LY293111 sodium, a novel LTB_4 receptor antagonist, will be used to validate a method for

the quantitation of sodium. Experimental results will be compared to the theoretical sodium amount to verify the accuracy of the method.

MATERIALS AND METHODS

Chemicals

Sodium chloride A.R. and acetic acid A.R. were purchased from EM Science (Gibbstown, New Jersey). LY293111 Sodium lot 309MH3 was supplied by Eli Lilly and Company (Indianapolis, IN). HPLC grade methanol and ammonium acetate A.R. were purchased from Mallinckrodt (Paris, Kentucky). Water used was deionized and filtered through a Milli-Q water purification system (Millipore, New Bedford, MA). NF grade nitrogen was used for the ELSD.

Apparatus

The HPLC system used for this study consisted of a Shimadzu SCL-10A controller, LC-10AS pump, SIL-10A auto injector and a DGU-3A membrane degasser (Shimadzu, Kyoto, Japan). A Sedex 45 evaporative light scattering detector was used (Richard Scientific, Novato, CA). Sodium was separated on a 15 cm x 4.6 mm I.D. Zorbax 300 SCX cation-exchange column (Mac Mod Analytical, Chads Ford, PA). A guard column was not used.

Chromatographic Conditions

Operating conditions for the Sedex ELSD were optimized prior to the onset of the study. The conditions were optimized to obtain the greatest signal-to-noise ratio. The optimum conditions were: nitrogen pressure in the ELSD nebulizing chamber set at 14 psi and the temperature of the drift tube set at 25°C. The gain control for the ELSD was set at 5.

The mobile phase comprised of 50% methanol /50% aqueous buffer. The buffer was a 0.05 M ammonium acetate solution adjusted to pH 6.0 with acetic acid. The mobile phase flow rate was set at 1.5 mL/min. The column temperature for the Zorbax 300 SCX was ambient. Injection volume was 100 uL and the run time was 500 seconds. All sample preparations were sonicated for two minutes to completely dissolve the material.

RESULTS

The method for sodium determination was validated for the parameters of linearity, precision, recovery, selectivity and limit of detection. The validation of this method follows the general USP guidelines suggested for an HPLC method.[18]

Linearity

It is usual practice to perform linearity determinations over a wide range of sample concentrations to fully assess the linear dynamic range of the detection system. The linearity of the method was determined by injecting 22 samples which were serial dilutions from a stock solution of sodium chloride to locate the working range. The samples represented a range of 0.0001-1.0 mg/mL sodium concentration. The linear range of sodium was determined to be 0.1-1.0 mg/mL. This range included ten standards and resulted in a correlation coefficient of 0.9997. The linear range represents 20-200% of the nominal target concentration of 0.5 mg/mL.

Precision

The precision of this method was evaluated in two ways. First, ten replicate injections of the same sample were injected to determine the reproducibility of the method apart from analyst error. Second, ten separate preparations were injected singly to determine the overall precision of the method. 12.5 mg/mL solutions of LY293111 sodium diluted with mobile phase were prepared. LY293111 sodium contains approximately 4% sodium making the actual sodium concentration near the target nominal concentration of 0.5 mg/mL. The samples were then sonicated for 2 minutes until all the material was in solution. The samples were run against a five point standard curve (0.3-0.8 mg/mL) made from sodium chloride. The results indicated 1.5% RSD for ten replicate injections and 1.3% RSD for ten separate preparations. See Figure 1 for a typical sample chromatogram.

Selectivity

As part of the USP guidelines for validation, a method must be proven to be selective for the analyte of interest. For this method selectivity was assessed

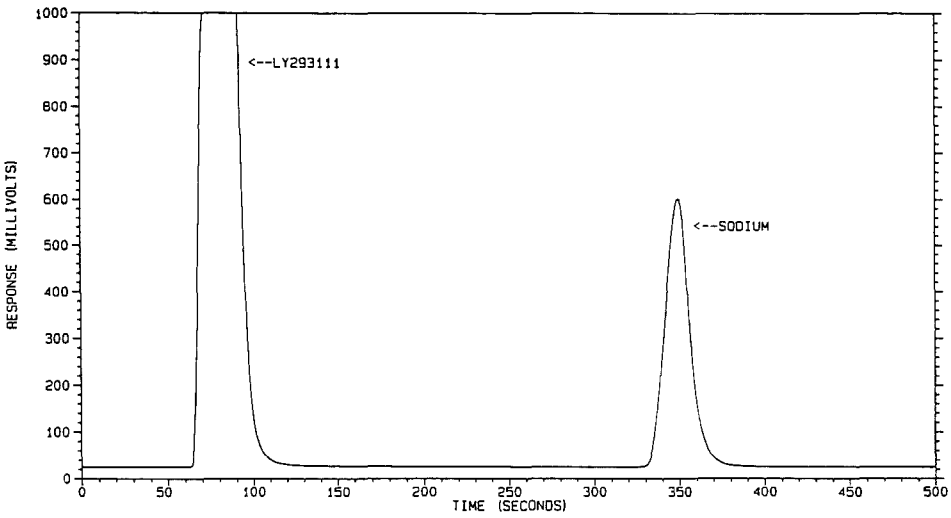

FIGURE 1. Typical sample chromatogram.

by separating sodium from all interfering peaks including LY293111 and similar monovalent ions such as potassium and lithium. The retention times of sodium, lithium, potassium, chloride and LY293111 are 350, 260, 440, 70, and 80 seconds respectively. Chromatograms of a blank, standard and sample are shown in Figure 2.

Recovery

Recovery was determined by a standard addition technique whereby ten separate preparations of LY293111 sodium containing approximately 0.4 mg/mL sodium were spiked with an additional 0.2 mg/mL sodium from a stock solution of sodium chloride. The ten samples were run against a standard curve consisting of five standard solutions in the range 0.3-0.8 mg/mL. The average percent recovery for the ten preparations was 104.5%.

Limit of Detection (LOD)

The LOD is defined as the lowest concentration of sample that can be clearly detected above the baseline noise. Typically this value is three times the

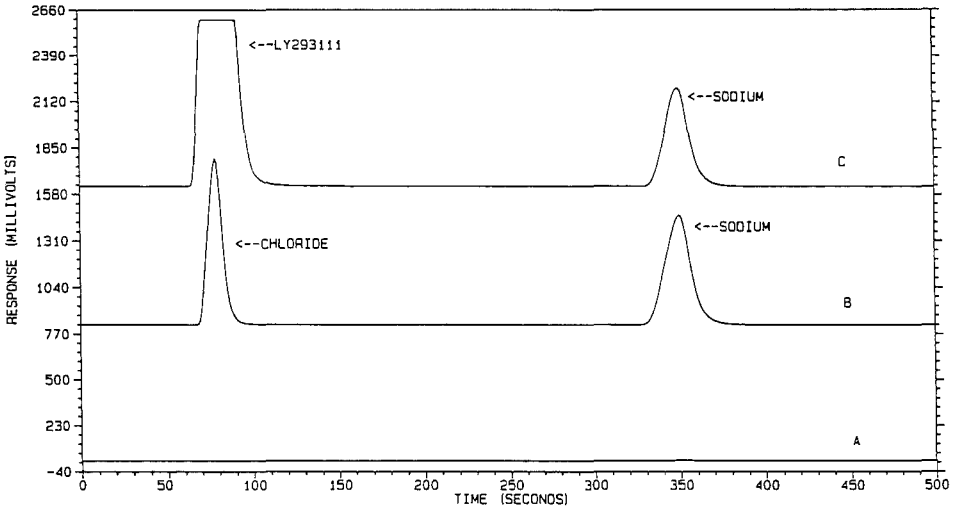

FIGURE 2. Chromatograms of a blank (A), standard (B) and a sample (C).

level of baseline noise. For this method the limit of detection was determined to be 0.0025 mg/mL. However, the limit of detection could be a much lower if a higher gain setting was used on the Sedex detector. The gain for this method is 5 and the gain can be adjusted to 12 for maximum sensitivity.

Theoretical Comparison

The results from ten separate preparations yielded a value of 4.05% sodium in LY293111 sodium. The theoretical amount of sodium in LY293111 sodium is 4.06%. Comparison of these results show the experimental results agree within 99.8% of the theoretical amount.

DISCUSSION

The applicability of a commercial evaporative light scattering detector for the analysis of sodium in a pharmaceutical compound has been demonstrated. The validation of this assay demonstrates that ELSD is an effective and practical

alternative to ion-chromatography with conductivity detection. The competitive nature of the pharmaceutical industry forces companies to analyze potential drug candidates in a quick, inexpensive manner in order to save time and conserve resources. ELSD provides a cost effective way of quickly determining the levels of ions in pharmaceutical compounds. Since ELSD is capable of detecting many types of solutes other than just ions, the versatility of an ELSD provides a practical tool for an analytical chemist . The cost savings of buying an ELSD and using existing HPLC equipment versus buying a dedicated IC system is substantial.

The practicality of this method should not be limited to only pharmaceutical compounds. This method has also been shown to determine sodium levels in various liquid forms such as beverages or other solutions. The intention of this paper is to propose the use of ELSD as an effective alternative for the determination of any inorganic salt for various applications. Our paper has shown the validity of ELSD for the determination of sodium in a pharmaceutical compound; however, other ions, such as chloride or other anions, may be quantitated with ELSD as well.

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SIMULTANEOUS MICROASSAY OF ALFENTANIL, FENTANIL, AND SUFENTANIL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A microassay for the simultaneous determination of alfentanil, fentanil and sufentanil by high performance liquid chromatography with ultraviolet detection in plasma and urine samples from newborn babies is reported. The HPLC analysis was performed with a commercially available 4 μm particle size reverse phase Waters Nova pak Cyano Column (8mm x 100 mm dimensions), guard pak cyano cartridges and a UV/VIS detector at 214 nm. Each run was completed within 10 min. The detection limits for the analysis were 0.15 ng, 0.12 ng and 0.25 ng for alfentanil, fentanil, and sufentanil, respectively with 50 μl injection. The respective retention times were 5.78 $\pm$ 0.29, 6.56 $\pm$ 0.32, and 7.08 $\pm$ 0.15 minutes for alfentanil, fentanil, and sufentanil. Applicability of this technique was evaluated in 6 newborn babies receiving constant I.V. infusion of fentanyl at 3.0 microgram/kg/h. Their plasma fentanyl concentrations were 3.04 $\pm$ 0.44 μg/L which correlated with adequate pain control. Microassay is relatively simple, rapid and precise and would be useful for therapeutic drug monitoring of these drugs in the newborn or in cases of limited sample volume.

INTRODUCTION

Fentanil, alfentanil, and sufentanil are semisynthetic opioid analgesics that are widely used as post-operative analgesics and as sedatives for patients requiring mechanical ventilation. They are also being used as premedications for surgery and other painful procedures. Clinically, these agents are well accepted as analgesics due to their high efficacy and safety margin. Alfentanil, fentanil, and sufentanil

provide adequate analgesia when administered intramuscularly or intravenously. These drugs exhibit important pharmacokinetic and pharmacodynamic differences in potency and rate of equilibrium between the plasma and the site of drug action.

Several chromatographic techniques, either for the single analysis of fentanil or the simultaneous analysis of fentanil and alfentanil or fentanil and sufentanil, have been previously reported (15,16). However, no studies have been performed, thus far, on the simultaneous analysis of these opiates in plasma and urine samples. Sensitive and selective assays for the determination of small concentrations of these analgesics in human plasma, urine and tissue samples utilized either radioassays (1,2), radioimmunoassay (3-6), gas liquid chromatography (7-11), thin layer chromatography (12), infrared and nuclear magnetic resonance (13,14) and high performance liquid chromatography with ultraviolet detection (15,16).

Radioassays, although very sensitive, are not suitable for routine monitoring of these drugs in patients because they require the use of radioactively labelled drugs. The RIA methods are very sensitive and sufficiently selective, but they are expensive and commercial anti-serum is not always available. Antisera have been prepared for each of the three drugs that show little cross-reactivity for the other fentanyl derivatives and known metabolites.

Among the chromatographic techniques commonly used, thin layer chromatography is simple but usually lacks sensitivity and selectivity, whereas gas chromatography, though more specific and reliable, is time-consuming because it usually requires derivatization of the opiates. Therefore, high performance liquid chromatography is the technique usually preferred due to its specificity, sensitivity and reliability.

These analgesics are increasingly used at present to provide relief from pain during anaesthesia in newborn infants although the methods available for pain measurement are limited. An analytical method with the sensitivity necessary to detect, quantitate and separate these drugs at the therapeutic concentrations are, therefore, extremely desirable. The widespread use of these potent drugs (alfentanil, fentanil and sufentanil) has created a need for chromatographic techniques to identify and quantitate low levels of these compounds in biological fluids. Due to the low levels being monitored, the method of detection must be free of endogenous interference or external contamination. This assay has been shown to be useful for therapeutic drug monitoring of these drugs in plasma and urine samples.

MATERIALS AND METHODS

Apparatus

The chromatographic separation was carried out at room temperature with a high-resolution on liquid chromatograph consisting of a Waters HPLC system model 510 HPLC pump, Waters 715 Ultrawisp autosampler, a Waters 994 UV-VIS detector with a variable wavelength. Peak height measurements of the drugs were integrated on a Waters model 820 integrator plotter. The separation column was a Nova pak reverse phase cyano column 8 mm x 100 mm (dimensions), with 4 μm particle size (Waters). PH-M-82 standard Ph meter, IEC centra-8R centrifuge, concentrator-Jouan RC 1010.

Reagents and Solutions

All chemicals used were of analytical grade unless otherwise stated. Sodium dihydrogen orthophosphate, sodium monobasic, orthophosphoric acid, sodium hydroxide, ammonium hydroxide (analytical grade), acetonitrile and N-Hexane (HPLC grade) were obtained from B.D.H. (Pooled, U.K.). Sigmacote (silanising reagent) and fentanil citrate were supplied by Sigma Chemical Company (U.S.A.). Alfentanil-Hydrochloride and sufentanil citrate were purchased from Janssen Pharmaceuticals, Belgium. Only HPLC-grade de-ionized water was used. The 15 ml screw cap culture tubes used were silanized prior to use to avoid drug adsorption on glass and all the glassware was rinsed prior to use with ethanol to remove substances interfering with chromatographic analysis.

The mobile phase consisted of a mixture of acetonitrile, phosphate buffer 5mM (Ph - 3.2) (70:30 V/V). The mobile phase was always filtered through a 0.45 μm filter and degassed under suction. Stock solutions of fentanil (50 μg/ml), alfentanil (500 μg/ml) and sufentanil (50 μg/ml) were prepared by dissolving appropriate amounts of the drugs in water. All stock solutions were stored at room temperature and were protected from prolonged exposure to light. These were used as a stock solution for the preparation of assay standards by serial dilution with plasma.

Plasma Samples and Patient Population

Drug free venous blood samples (1-2 ml) were collected from healthy human subjects receiving no medication. In addition, arterial blood samples (0.2 ml) taken from umbilical artery catheters were obtained from newborn infants receiving continuous intravenous infusion of fentanyl at 3.0 microgram/kg/h. Their birth weights (grams) and postnatal ages (days) were 3.8 $\pm$ 0.40 kg and 2-4

days, respectively. Blood was collected into plastic tubes containing lithium heparin and centrifuged to 10 minutes at 3000 rpm. Plasma was separated and stored at -80°C until the time of analysis.

Chromatographic Conditions

The chromatographic analysis was performed under isocratic conditions and at ambient temperatures, with the detector set at 214 nm with a sensitivity of 0.01 or 0.02 absorbance units (AUFS). The mobile phase was a mixture of acetonitrile, phosphate buffer 5mM, Ph - 3.2 (70:30 V/V). The flow rate was 2.5 ml/min with a pressure of 1200 psi and the run time less than 10 minutes.

Extraction Procedure

To 0.05 ml of standards or samples (plasma, urine) were pipetted into culture tubes containing 0.05 ml 4N NaoH, 0.1 ml acetonitrile and 500 μl of extraction solvent (n-Hexane). The mixture was vortexed for 30 seconds. All samples were centrifuged at 2000 rpm for 5 minutes. The organic phase was transferred into a glass tube and the solvent was evaporated under nitrogen at 30°C for about 10 minutes. The residue was reconstituted in 0.1 ml of the mobile phase and 50 μl injected into a column.

Calibration Curves for the Simultaneous Determination of Alfentanil, Fentanil, and Sufentanil in Standard Solutions

Ten working standards of alfentanil, fentanil and sufentanil were prepared in distilled water. The stock solutions were serially diluted with water by a factor of ten in 25 ml volumetric flasks for the preparation of the working standards. The resulting concentrations of the solutions were 3.0 - 500 ng/ml of the alfentanil free base, 2.5 - 150 ng/ml of the fentanil free base, and 5.0 - 200 ng/ml of the sufentanil free base. Five replicate injections of each of these standard mixtures were made. The peak height of alfentanil, fentanil and sufentanil were plotted separately as a function of the corresponding concentration of each compound.

Determination of Alfentanil, Fentanil and Sufentanil in Human Plasma and Urine

A set of calibration curves were constructed for the blood plasma and urine samples using the standard additions method. The samples were spiked with mixtures of alfentanil, fentanil and sufentanil at the different concentration levels (0,2.5,5.0,10.0,25,50,100,200 ng/ml). The extracted samples were used for HPLC analysis. Five replicate injections were made for each concentration level. The peak height of alfentanil, fentanil and sufentanil were plotted against the corresponding concentration of the drugs. The regression analysis shows good linear relationships between peak height and quantity of drug.

RESULTS AND DISCUSSION

The present paper describes an HPLC method for the simultaneous determination of alfentanil, fentanil and sufentanil in 50 μl blood plasma and urine samples. This method offers rapidity, very good separation of the drugs, satisfactory sensitivity with ultraviolet detection and good precision and accuracy. Using the described conditions, the analysis was completed within approximately 10 minutes with complete separation of these three drugs as shown in Fig. 1. The resolution factor between adjacent peaks was calculated and found to be 1.56 between alfentanil and fentanil and 1.04 between fentanil and sufentanil.

In the method described here, the Nova pak cyano columns were found to be suitable. Nova pak packing material has low surface activity (acidity) and is endcapped to minimize silanol interactions and

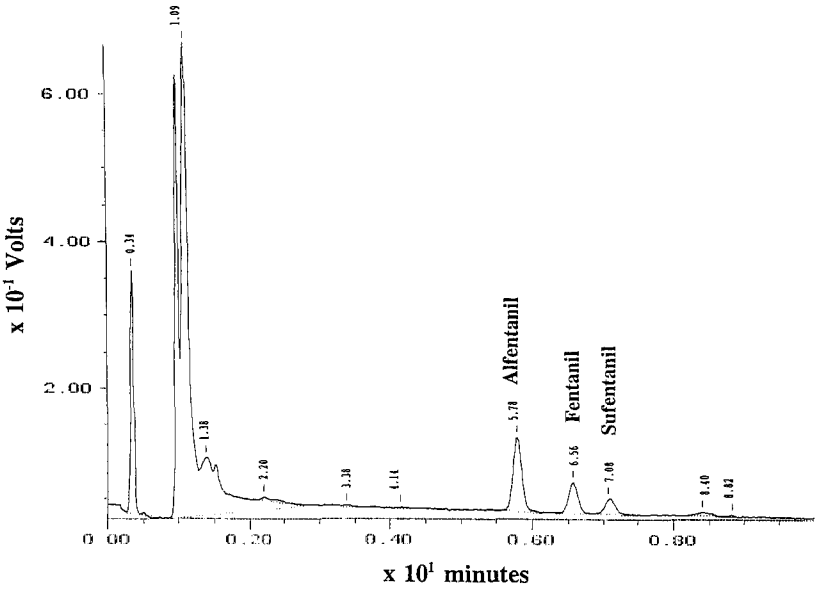

Figure 1

Chromatogram of a mixture of alfentanil, fentanil and sufentanil standard (10 ng/ml, 5 ng/ml and 5 ng/ml, respectively).

provide improved peak shape for basic molecules thereby reducing the need for mobile phase additives such as amine modifiers.

At the retention times of alfentanil, fentanil and sufentanil, no interferences from endogenous compounds were found in the chromatograms of extracted blood plasma and urine samples shown in Fig. 2 and 3. Since these opioids are usually used simultaneously with other drugs, the specificity for this assay has been tested in the presence of those medications that are commonly administered to patients. None of these medications - furosemide, morphine, calcium chloride, dobutamine, midazolam, ampicillin sodium, gentamicin, cefotaxime, diazepam, phenytoin, pavulon, and vitamin K - interfered with our method when they were added to blank plasma or urine samples.

Standard curves were linear and passed through the origin between the concentrations 3.0 - 500 ng/ml for alfentanil, 2.5 - 150 ng/ml for fentanil, and 5.0 - 200 ng/ml for sufentanil, respectively. The respective correlation coefficients were 0.996, 0.994 and 0.997 for these drugs, which indicate high precision. The lower limit of quantitation with a 50 μl sample size (injection) were 0.15 ng for alfentanil, 0.12 ng for fentanil, and 0.25 ng for sufentanil. The lower determination limits were 3.0 ng/ml, 2.5 ng/ml and 5.0 ng/ml for alfentanil, fentanil, and sufentanil, respectively. The retention times were 5.78 $\pm$ 0.29 minutes for alfentanil, 6.5 $\pm$ 0.32 minutes for fentanil, and 7.08 $\pm$ 0.15 minutes for sufentanil. The recovery determined at various concentrations for plasma and urine samples was in the range of 85.0 $\pm$ 4.08, 83.3 $\pm$ 3.84 and 78.2 $\pm$ 2.89%, for alfentanil, fentanil, and sufentanil, respectively.

Replicate analyses were conducted on four different concentrations (10,25,50, and 100 ng/ml) for each drug. Each concentration was replicated eight times under the same conditions in order to check the reproducibility and precision of the method. Inter- and intra-assay precision was determined by analysis of spiked samples on five consecutive days. Accuracy and reproducibility was calculated as the percentage difference between amount of drug added to drug-free plasma and amount of drug measured (Table 1). Coefficients of variation of the method were $<$ 10%. The experimentally determined concentrations agreed with the actual concentrations.

The proposed method is suitable for the simultaneous determination of alfentanil, fentanil and sufentanil in very small volumes of plasma and urine samples (50μl). The previously reported methods for the simultaneous determination of these drugs were based on 1 ml plasma samples and required longer

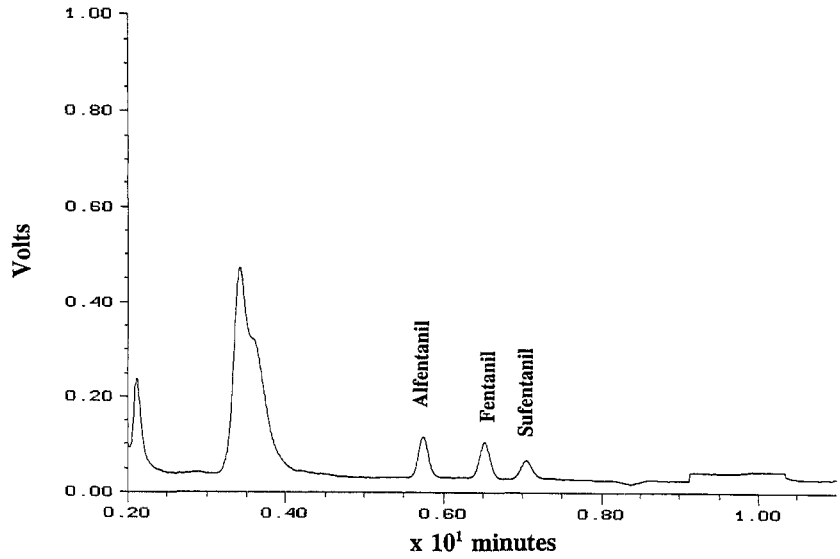

Figure 2

Chromatogram of extracted alfentanil, fentanil and sufentanil from blood plasma samples (5 ng/ml).

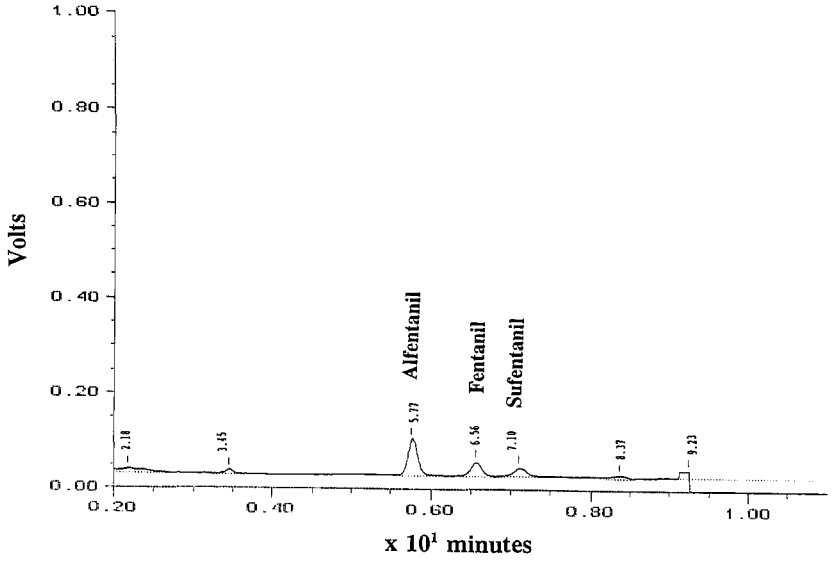

Figure 3

Chromatogram of extracted alfentanil, fentanil and sufentanil from urine sample (5 ng/ml, 2.5 ng/ml and 5.0 ng/ml, respectively).

TABLE 1

Reproducibility, Precision and Accuracy of the Method

Drug	Drug Concentration Injected (ng/ml)	Drug Concentration Found (ng/ml) *	Confidence Interval 95%	Coefficient of Variation (CV) %
Alfentanil	10.0	8.90 ± 0.39	8.70 - 9.10	4.50
	25.0	25.4 ± 0.55	25.16 - 25.65	2.17
	50.0	49.9 ± 1.30	49.91 - 50.05	2.60
	100.0	99.8 ± 1.50	99.68 - 99.83	1.50
Fentanil	10.0	9.75 ± 0.65	9.73 - 9.77	6.62
	25.0	25.9 ± 0.78	25.85 - 25.94	2.99
	50.0	48.4 ± 2.41	48.3 - 48.5	4.98
	100.0	100.3 ± 0.96	100.3 - 100.4	0.95
Sufentanil	10.0	9.41 ± 0.66	9.71 - 9.77	6.16
	25.0	25.5 ± 0.55	25.30 - 25.80	2.14
	50.0	50.5 ± 0.15	50.26 - 50.33	1.30
	100.0	100.2 ± 0.80	100.16 - 100.24	0.80

* Values are expressed as mean ± SD of 8 determinations. Volume injected was 50

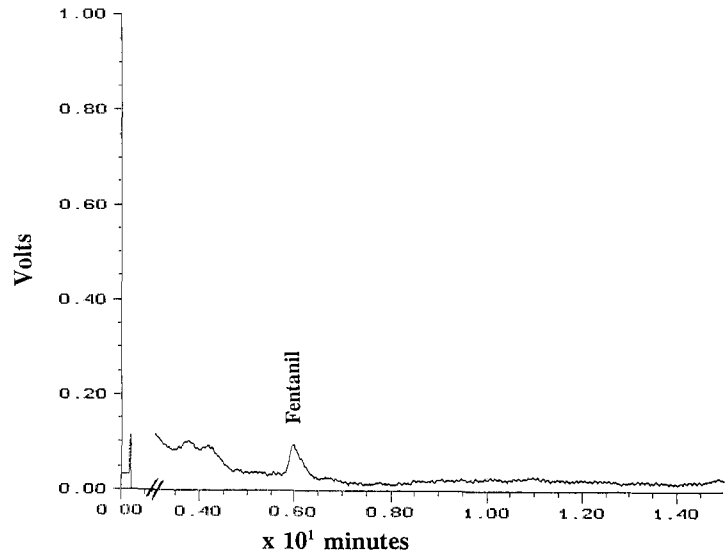

Figure 4

Chromatogram of fentanil from a newborn baby receiving a constant intravenous infusion of fentanil at 3.0 microgram/kg/hr. Fentanil concentration was 3.7 ng/ml.

extraction procedures (15,16). Kumar et al (16) have reported extraction of fentanil and alfentanil at Ph-3. However, as reported by Janicki et al (17) and supported by our own experiments, these drugs are hydrolyzed in acidic solutions by cleavage of propionic acid, thus reducing the sensitivity of the method.

We have used the proposed technique for the therapeutic drug monitoring of fentanyl in small, sick newborn babies receiving constant I.V. infusion of fentanyl 3.0 microgram/kg/h (Fig. 4). Their plasma fentanyl concentrations at these doses were 3.2 $\pm$ 0.22 ng/ml corresponding to an adequate pain control in these babies. We suggest that this microassay designed for the simultaneous determination of fentanyl, alfentanil and sufentanil be used in newborn and children or whenever there is limited sample volume.

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ISOLATION AND QUANTIFICATION OF IVERMECTIN IN BOVINE MILK BY MATRIX SOLID PHASE DISPERSION (MSPD) EXTRACTION AND LIQUID CHROMATOGRAPHIC DETERMINATION

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ABSTRACT

A technique for the extraction and liquid chromatographic determination of ivermectin residues in bovine milk is described. Avermectin was used as an internal standard. Fortified and blank milk samples (5.0 mL) were blended with 2.0 g C_{18} (octadecylsilyl derivatized silica) in a syringe barrel. After a 2 minute equilibration, the aqueous phase was removed from the column by vacuum aspiration. The ivermectin residues were eluted from the C_{18}/milk matrix with ethyl acetate. After further cleanup by silica solid phase extraction, ivermectin derivatives were formed and then quantified by liquid chromatography with fluorescence detection. The recoveries of fortified ivermectin residues (1.0 - 8.0 ppb) averaged 97.7%. The injected extracts are free from matrix interferences making it easy to calculate the amount of residue present.

INTRODUCTION

The avermectins are macrocyclic lactones derived from the mycelia of the actinomycete *Streptomyces avermitilis* (1). They are active against helminths and arthropods at very low doses (2). Two avermectins have been commercialized to date (3). Abamectin, a mixture consisting of >80% avermectin B_{1a} (B_{1a}) and <20% avermectin B_{1b} (B_{1b}), is widely used as an insecticide and miticide on agricultural crops. Ivermectin, a mixture consisting of >80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and <20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}) (Figure 1), is widely used as an antiparasitic agent in animals and man.

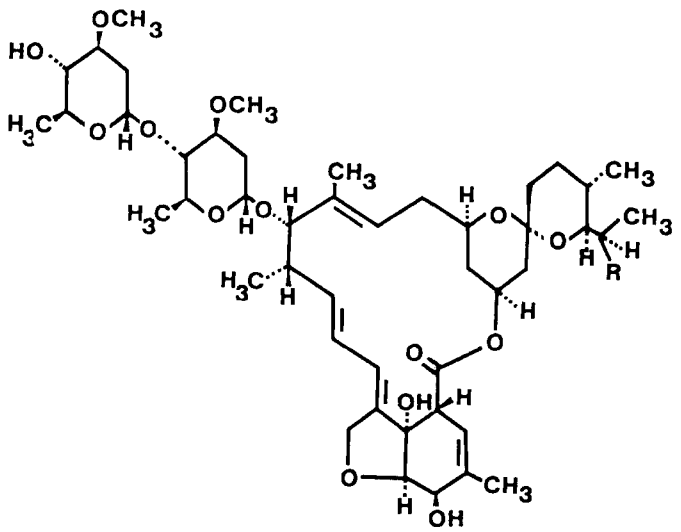

FIGURE 1. The structure of Ivermectin: R= $-C_2H_5$ for H_2B_{1a}, and R= $-CH_3$ for H_2B_{1b}.

Ivermectin residues are depleted slowly from the animal. Tway et al. (4) found the unaltered drug in cattle liver over a period of 28 days post administration. The level of mammary gland excretion of the administered dose is very high (5). Drug depletion studies have shown that dosed ivermectin is secreted through the mammary gland over many weeks (5,6), underscoring concern that residues may be found in retail milk. Ivermectin is currently not approved for use in lactating dairy cows (7). Extrapolation from tissue levels indicates that a regulatory analytical method should be capable of detecting 2 ng/mL (ppb) in milk (Dr. William White, Food and Drug Administration, Center for Veterinary Medicine, personal communication.)

Alvinerie et al.(6), Schenck et al.(8) and Kijak (9) have reported methods for the extraction and determination of ivermectin residues in milk. Unfortunately, these methods employ time consuming multiple extraction, centrifugation and liquid-liquid solvent partition cleanup steps. Recently, Barker et al (10) demonstrated that biological matrices can be homogeneously dispersed with C_{18} (40 μm octadecylsilyl derivatized silica), the resulting mixture packed into a column, and various residues selectively eluted from the column. This method, called matrix solid phase dispersion (MSPD), provides a rapid alternative to conventional methods for milk and tissue extractions. Long (11-14) has shown that MSPD can be used for the extraction of a wide variety of drugs from milk.

This paper describes a rapid, reliable method using a modified MSPD extraction with a solid phase extraction (SPE) cleanup for the determination of ivermectin from milk.

EXPERIMENTAL

Apparatus

(a) *Ultrasonic bath*- Branson Model 2200 (Branson Cleaning Equipment, Shelton, CT), or equivalent.

(b) *Micro pipettors*- Electronic Digital Pipette (Rainin Instruments, Woburn, MA) or equivalent.

(c) *Syringe barrels*.- Used as extraction columns; empty reservoirs, 15-mL and 25-mL sizes; 20 μm frits; and Bond Elut Column Adapters (Varian Sample Preparation Products, Harbor City, CA.)

(d) *SPE vacuum manifold*.- equipped with individual flow control valves so that the vacuum to each SPE column can be shut off (Supelco Corp., Bellefonte, PA) or equivalent.

(e) *Spatula*- Stainless steel.

(f) *Polypropylene volumetric flasks*- 50-mL size, for standard solutions (Nalge Co., Rochester, N.Y.) or equivalent.

(g) *Nitrogen evaporator*- N-Evap (Organomation Associates, Berlin, MA) or equivalent.

(h) *Oil bath*- 95-100°C.

(i) *Centrifuge tubes*.- 15 mL, conical, to be silylated once every two months. Fill each tube to the top with Sylon-CT and allow to stand 20 minutes. Rinse thoroughly with toluene followed by methanol, and fill with methanol. Let it stand 20 minutes and then rinse with acetone and allow to dry. These tubes are cleaned by hand immediately after use by soaking in methylene chloride followed by detergent for at least 3 hours, followed by thorough rinsing with hot water, distilled water and acetone.

(j) *Liquid chromatograph*- Model 650-15 fluorescence spectrophotometer (Perkin Elmer Corp., Norwalk CT).

Operating conditions: excitation wavelength 364 nm; emission wavelength 455 nm; time constant 3. Analytical column: Econosil C_{18}, 5 μm, 4.6 x 250 mm (Alltech Associates, Deerfield, IL); guard column, Brownlee Newguard RP-18, or equivalent (Applied Biosystems Inc., San Jose, CA), ambient temperature; series 410 LC pump with ISS-100 autosampler (Perkin Elmer). Injection volume 50 μL; solvent flow rate 1.0 mL/min.

Reagents and Materials

(a) *Solvents*.- *N,N*-Dimethylformamide (DMF), Photrex reagent grade (J.T. Baker, Phillipsburg, NJ); tetrahydrofuran (THF), HPLC grade (Fisher Scientific, Fair Lawn, NJ); all other organic solvents were distilled in glass, suitable for spectrophotometry, and LC grade (EM Science, Gibbstown, NJ).

(b) *Acetic anhydride*.- Analytical reagent grade (Malinckrodt Inc., Paris, KY).

(c) *N-Methylimidazole*- 99% (Aldrich Chemical, Milwaukee, WI)

(d) *Water*- Filtered and deionized, Milli-Q Plus Water Treatment System (Waters Corp., Milford, MA).

(e) *Sodium sulfate*- Anhydrous, granular (12-60 mesh), ACS reagent grade (JT Baker, Phillipsburg, NJ).

(f) *Analytical standard*- Ivermectin, 1.38% (w/w) H_2B_{1a}, 0.21% (w/w) H_2B_{1b}, in glycerol formal was obtained from Merck Sharpe and Dohme Research Laboratories, Rahway, NJ.

(g) *Internal standard*- Abamectin (87% B_{1a} and 9.4% B_{1b}) was obtained from Merck Sharpe and Dohme Research laboratories).

(h) *Stock standard solutions*- Weigh 0.36 g ivermectin standard into a 50 mL volumetric flask, and

dilute to volume with methanol (100 μg/mL H_2B_{1a}). Store at -20°C.

(i) *Fortification standard solutions*- Dilute 0.5 mL ivermectin stock standard solution to volume in a 100 mL volumetric flask with methanol. Store at -20°C.

(j) *Internal standard solutions*- Dissolve 1 mg abamectin standard in 10 mL methanol (100 μg/mL B_{1a}). Dilute 0.5 mL abamectin stock standard solution to volume in a 100 mL volumetric flask with methanol (500 ng/mL B_{1a}). Store at -20°C.

(k) *MSPD column material*- Bulk C_{18}, Bondesil, 40 μm, 18% load endcapped, octadecylsilyl-derivatized silica (Varian).

(l) *Solid phase extraction (SPE) columns*.- Bond Elut LRC silica, 500mg (Varian).

(m) *Sylon CT*- 5% dichlorodimethyl silane in toluene (Supelco, Bellefonte, PA)

(n) *Derivatizing reagent*- Sequentially mix 0.9 mL DMF, 0.3 mL acetic anhydride and 0.2 mL N-methylimidazole just before use.

(o) *LC mobile phase*- Methanol/THF/water (85+15+5, v/v/v)

(p) *Milk samples*- Raw bovine milk was obtained from FDA, Center for Veterinary Medicine, Beltsville, MD, and stored at -80°C.

Sample fortification

An appropriate volume of the 500 ng/mL (H_2B_{1a}) fortification standard (50 - 400 μL) and 200 μL of the B_{1a} internal standard solution were added to a 50 mL graduated cylinder. Control raw milk was added to the 25 mL mark. Standard and milk were mixed by inverting the cylinder 5 times. (Use of a graduated cylinder rather

than a volumetric flask will allow for more efficient mixing of the lipophilic ivermectin with milk lipids.) Five mL aliquots of this milk were taken through the extraction.

Working standards

Working standard solutions suitable for a 4 point standard curve were prepared daily. Appropriate volumes of fortification standard and 100 μL of internal standard solution were added to four 25-mL volumetric flasks. Methanol was added to volume.

Extraction and cleanup

Fit an empty 25-mL syringe barrel (reservoir) with a 20 μm frit. Add 2.0 g of C_{18} to the barrel. Wash the C_{18} sequentially with 5 mL petroleum ether, 5 mL acetone and two 5 mL aliquots of methanol. After the last methanol is eluted, aspirate the column with full vacuum for <5 seconds. (NOTE: Do not wash the C_{18} column with water after last methanol wash.) Close the valve on the vacuum manifold head.

Pipette 5.0 mL milk onto the prepared C_{18} column. Mix the milk with the C_{18} using a stainless steel spatula. Leave the spatula in the column and allow the milk/C_{18} mixture to equilibrate for 2 minutes, occasionally stirring with the spatula.

Carefully remove the spatula from the column, rinsing it with a stream of water from a wash bottle, collecting the water in the column. Open the valve on the vacuum manifold head, and apply sufficient vacuum to elute the milk from the column at a rate of $\leq$3 drops per

second. When all the milk is eluted from the column, rinse down the sides of the column with ca. 5 mL water from a wash bottle. Elute the water from the column using vacuum. Repeat the water wash. Dry the column by vacuum aspiration for 5 minutes.

Remove the C_{18} MSPD column from the vacuum manifold. Fit a 15-mL size empty syringe barrel with a frit and fill to ca. 5 cm. with sodium sulfate. Attach the sodium sulfate column below the C_{18} MSPD column with an adapter. Place the tandem columns over a 15 mL silylated centrifuge tube. Add 10 mL ethyl acetate to the top (C_{18}) column. Using a rubber pipette bulb, apply sufficient pressure to initiate flow of the solvent. Allow the solvent to drip by gravity flow, collecting the eluate in the silylated tube.

Remove and discard the columns and evaporate the ethyl acetate eluate to dryness at ≤50°C with the aid of a stream of nitrogen. A small amount of oily residue will remain. Add 2 mL of 40% ethyl acetate in hexane to the centrifuge tube, vortex mix and place in ultrasonic bath for 1 minute.

Place a silica SPE column on the vacuum manifold and wash with 3 mL of 40% ethyl acetate in hexane. Transfer the contents of the silylated centrifuge tube to the silica SPE column and elute at a rate of 1 drop per second. Rinse the silylated centrifuge tube two times with 1 mL 40% ethyl acetate in hexane, transferring the rinsings to the silica SPE column. Save the silylated tube for later collection of SPE column eluate. Wash the silica SPE column with an additional 5 mL 40% ethyl acetate in hexane. Discard all the 40% ethyl acetate in hexane washes.

Elute the silica SPE column with 5 mL of 50% ethyl acetate in methanol, collecting the eluate in the silylated centrifuge tube. Evaporate the eluate to

dryness at ≤60°C with the aid of a stream of nitrogen. Less than 0.05 mL of residue should remain in the silylated tube. Excess moisture, methanol or residue can lead to incomplete derivatization.

Derivatization of Ivermectin

For preparation of standards, add 5.0 mL of each of the working standard solutions to a silylated centrifuge tube. Evaporate to dryness at ≤60°C with the aid of a stream of nitrogen. Add 0.1 mL of freshly prepared derivatizing agent to the sample and standard silylated tubes. Stopper tubes, vortex briefly and centrifuge for a few seconds at low speed. Place the tubes in a 95°C oil bath. The solution should turn black.

After 1 h remove tubes, allow to cool. Add ca 1 mL chloroform to each tube and vortex mix. Elute chloroform on a silica SPE column which was prewashed with 4 mL chloroform. Wash the silylated centrifuge tube with three 1 mL portions of chloroform and elute each through the silica SPE column. Wash the silica SPE column with 2 mL chloroform, collecting all the eluates. Evaporate to dryness at ≤60°C with the aid of a stream of nitrogen. Dissolve residue in 0.5 mL methanol, vortex mix and centrifuge briefly. Inject 50 μL of each standard and each sample into the LC system.

Determine the peak heights of the H_2B_{1a} and B_{1a} fluorescent derivatives. Calculate the peak height ratios for each of the sample and standard injections as follows:

Peak height ratio =

(Peak Height H_2B_{1a}/Peak height B_{1a})

Construct a linear regression curve of the peak height ratio vs ng/mL H_2B_{1a} in the standards. Using the peak height ratios of the milk sample injections, calculate concentration (ng/mL) of H_2B_{1a} injected from the standard curve.

Calculate concentration of H_2B_{1a} in the milk sample as follows:

$$H_2B_{1a} \text{ ng/mL (sample)} = H_2B_{1a} \text{ ng/mL (injected)}/5$$

where 5 is the number of mL milk taken through the extraction.

RESULTS AND DISCUSSION

To test the performance of the method, raw milk samples were fortified initially with ivermectin only. Later samples were fortified with ivermectin and abamectin (internal standard). Milk samples were extracted, cleaned up, and derivatized. Derivatized residues were detected by liquid chromatography with fluorescence detection (Figure 2A). Representative chromatograms of control milk samples show no interfering matrix background peaks (Figure 2B). The recoveries of fortified ivermectin residues from the milk (1.0-8.0 ppb) were calculated both with and without the internal standard. The results are shown in table 1.

Ivermectin was rapidly extracted from milk using MSPD. When the milk was mixed with the C_{18}, ivermectin and the milk lipids were adsorbed in a thin layer onto the surface of the C_{18} particles. The polar milk matrix components and most of the milk proteins were eluted with the aqueous phase. Ivermectin was eluted from the column with ethyl acetate.

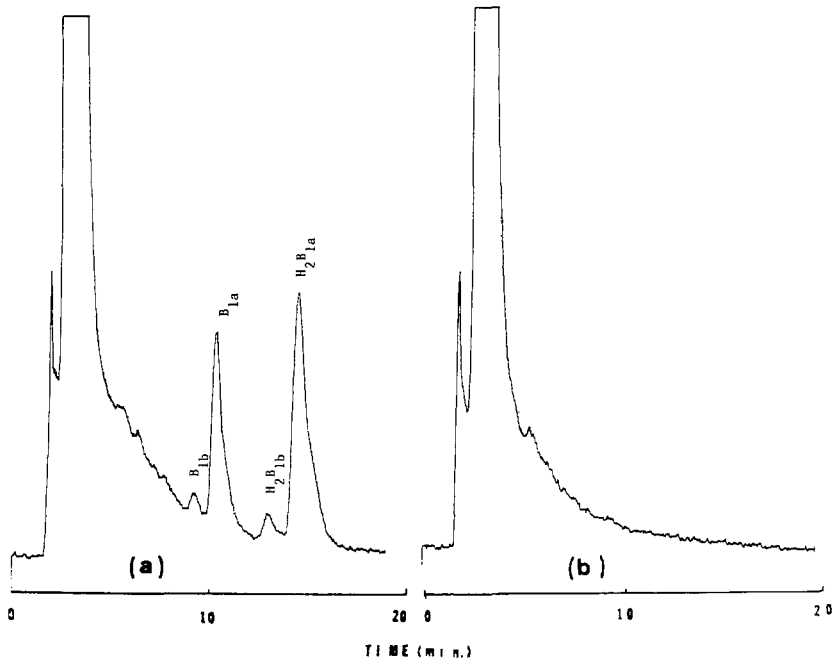

FIGURE 2. Typical chromatograms of (a) milk fortified with 4.0 ppb ivermectin (H_2B_1) and abamectin internal standard (B_1), and (b) control milk.

TABLE 1.

Recoveries of Ivermectin from Bovine Milk.

Fortification level (ppb)	% Recovery (% RSD)[a] calculated using internal standard	% Recovery (% RSD)[b] calculated without internal standard
1.0	97.0 (2.0)	85.7 (8.4)
2.0	104.1 (0.7)	83.5 (4.6)
4.0	97.7 (0.9)	91.5 (10.3)
8.0	99.9 (1.7)	81.0 (8.5)

[a] n=3
[b] n=7

The ethyl acetate MSPD eluate contained some milk co-extractants, which interfered with the derivatization of ivermectin. Further cleanup using commercially available SPE columns was explored. The ethyl acetate MSPD extracts from milk spiked with 50 ppb ivermectin were evaporated and the residue was cleaned up using an alumina, Florisil or silica SPE column, as per published procedures (15-18). The efficiency of the SPE column cleanup was evaluated using LC with UV detection (17). The silica SPE cleanup developed by Vuik (18), for the determination of avermectin in cucumbers, was far superior to both the alumina and Florisil SPE cleanups for these particular extracts. The silica extracts exhibited a minimal number of interfering coextractants when analyzed by LC-UV, and a minimal amount of residue remaining after the solvent was evaporated.

MSPD has been widely used for the extraction of drugs from milk. Typically, 2.0 g C_{18} is mixed with 0.5 g milk in a mortar and pestle. The resulting homogenate is transferred into a syringe barrel. Van Pouck et al. (19) have developed a modified MSPD method for the determination of sulfonamides in milk. Five g milk is mixed directly with 2.0 g C-18 in a syringe barrel. This eliminates the need to transfer the homogenate and allows larger volumes of milk to be extracted, thereby increasing the sensitivity of the method.

The MSPD method eliminates many of the problems associated with traditional isolation techniques. Traditional methods for the isolation of ivermectin from milk (6,8-9) employ multiple solvent extractions and centrifugation steps which extract the ivermectin along with the milk fat. Ivermectin is then separated from the fat by multiple liquid-liquid partition steps. Using the MSPD method, ivermectin is rapidly separated from the milk matrix coextractants without having to use tedious

liquid-liquid extraction and centrifugation steps. The savings in time make the MSPD method attractive when compared to traditional techniques.

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KINETICS OF AZADIRACHTIN HYDROLYSIS IN MODEL AQUATIC SYSTEMS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The hydrolysis of azadirachtin-A isomer (AZ-A) was studied at 20°C in the dark in buffered distilled water at pH 4, 7 and 10, and in unbuffered sterilized and unsterilized pond water. Individual solutions were fortified in triplicate with pure AZ-A and formulated AZ-A separately. Hydrolysis of AZ-A in pond water was studied by using pure AZ-A only. At pH 10, AZ-A fortified either in pure form or as formulation, was hydrolysed rapidly and the DT_{50} was only about 2 h. At pH 4, the DT_{50} values for the pure and formulated AZ-A were 19.2 and 38.3 d, respectively, indicating that the chemical is relatively stable in acidic medium. The stability was diminished at pH 7 and the corresponding DT_{50} values were 12.9 and 30.5 d. The data show that the hydrolysis of AZ-A is greatly influenced by pH in the order pH 10 >>> pH 7 > pH 4. The differences in DT_{50} values between pure AZ-A and formulated AZ-A, at pH 4 and 7, suggest that hydrolysis is considerably retarded by the surfactants in the formulation. The average pH and DT_{50} values for the sterilized and unsterilized pond water were 8.08 ± 0.49 and 7.36 ± 0.28, and 6.91 d and 11.94 d, respectively. The faster degradation of pure AZ-A in

sterilized water, compared to the unsterilized water, was likely due to chemical hydrolysis. Microbial action in the degradation of AZ-A in the unsterilized pond water appeared to be minimal.

INTRODUCTION

The use of conventional broad-spectrum synthetic insecticides, although impressively effective in controlling various insect pests in Canada, is in rapid decline due to public concern and regulatory demands for selective and environmentally safe pest control products. Consequently, research in recent years has been turning more and more towards natural insecticides originating from plants, which are presumed to be innocuous. Among all the phytochemical pesticides being studied at present, azadirachtins (AZ), a mixture of seven structurally related tetranortriterpenoids isolated from the seeds of the neem tree or Indian lilac [*Azadirachta indica* A. Juss (Meliaceae)], have properties useful for the management of pests and show considerable promise to function as effective insecticides [1]. The major isomer, azadirachtin-A (AZ-A) has relatively high insecticidal activity. It causes mortality, improper molting, impaired reproductive capacity and deters feeding in various insect species, yet is safer to natural enemies and nontarget organisms than most conventional insecticides [2]. Neem-based formulations containing AZ-A have been registered in U.S.A. for the use on nonfood crops and ornamentals [3].

Recent laboratory [4] and field studies [5] have shown that neem preparations containing AZ-A are effective in controlling various target pests including the spruce budworm (*Choristoneura fumiferana* Clem.), a destructive defoliator of conifer forests in eastern Canada and north-eastern U.S.A. During any broadcast

application the insecticide may accidently drift or run-off into bodies of water. The use of neem-based formulations in forestry therefore requires an understanding of the aquatic dissipation of the major toxic component present, AZ-A, to evaluate its stability and toxic potential to aquatic organisms.

So far, very little information is available on the persistence and degradation of AZ-A, either in a pure state or as a formulated material, in waters of different acidities. The little that is available is inconclusive. Larson [6] reported the influence of low pH (3.8 to 4.2) on the stability of AZ. On the contrary, Tewari [7] found rapid hydrolysis of the material under acidic and alkaline conditions. We have investigated the dissipation of pure and formulated AZ-A in buffered distilled water over the pH range of 4 to 10 (to cover the spectrum of water acidities found in forest environment), and in sterilized and unsterilized natural waters under controlled laboratory conditions. Studies were conducted in the dark to diminish photolytic effects. The data are presented in this paper.

MATERIALS AND METHODS

Analytical grade azadirachtin-A (> 95 % purity) was purchased from Sigma Chemical Co., St. Louis, MO and used without any purification. It gave a single spot (R_F 0.58, 2-propanol:n-hexane 11:9) on silica gel TLC plate and a distinct single peak (RT 20.6 min) in HPLC [8]. The commercial formulation used was Azatin® (AgriDyne Inc., 417 Wakara Way, Salt Lake City, Utah 84108) containing 3 % AZ (mainly AZ-A by weight), *ca.* 4 % naphthalene, *ca.* 2 % butanol [9], emulsifiers (to keep the AZ uniformly distributed in the spray mix) and probably a sunscreen ingredient. The contents of the formulation are proprietary information

of AgriDyne Inc. Glass distilled water was used in all dissipation studies. Pond water was collected from the Searchmont area in N. Ontario in amber bottles (4 L) and had the following properties: pH 7.36; turbidity (JTU) 18.8; sp. conductivity (μmhos/cm) 18.4; hardness (mg of $CaCO_3$/L) 13.9; organic matter 16 μg/g; total P 0.098 μg/g; total N 0.093 μg/g; and Fe as $Fe^{2\oplus}$ 0.16 μg/g.

Buffer solutions of pH 4, 7 and 10 were prepared using ACS grade chemicals and distilled and sterilized water according to the procedure of Dean [10]. For pH 4, 10.21 g of potassium hydrogen phthalate, [$KH(C_8H_4O_4)$] per L (0.05 M); for pH 7, 3.40 g of potassium dihydrogen phosphate (KH_2PO_4) and 3.55 g of disodium hydrogen phosphate (Na_2HPO_4) per L (0.025 M each); and for pH 10, 2.10 g of sodium hydrogen carbonate ($NaHCO_3$) and 2.65 g of sodium carbonate (Na_2CO_3) per L (0.025 M each) were used. The pH of each solution was checked periodically using a pH meter (Model SA720, Orion Research Inc., Boston, MA 02129, USA).

For dissipation studies, each buffer solution (500-mL) was placed in triplicate in 1-L stoppered Teflon bottles covered outside with aluminum foil. Exactly 0.5 mL of standard AZ-A in methanol containing 40.0 μg/mL was added to each bottle to give an initial concentration of 40 μg/L. Each solution was shaken well and incubated in an environmental chamber (Controlled Environments, Winnipeg, Canada) kept at 20 ± 1°C. The dissipation was allowed to continue for 25 d post-treatment.

To study the dissipation of AZ-A in the formulation, 10-mL aliquots of the stock solution in methanol containing 4 μg AZ-A per mL were added to 1.0 L of each of the buffer solutions in triplicate to give an initial concentration of 40 μg/L.

The Teflon bottles were sealed, covered completely with Al-foil, shaken well and incubated in the environmental chamber as above for the hydrolytic degradation to occur over a 60-d period.

The pond water was filtered under suction using Gelman 5-μm PTFE membrane filter. A portion of the filtered water was sterilized in an autoclave (Amsco 2022) for 20 min. Both the sterilized and unsterilized water samples (500 mL each in triplicate) were fortified with standard AZ-A, to give initial concentrations of 40 μg/L. Dissipation experiments were conducted as in the hydrolysis of pure AZ-A in buffer solutions.

The kinetics of AZ-A loss was followed by measuring the residual concentrations of the chemical at known intervals of time (*i.e.*, 15 min after fortification, until the termination of the study) and plotting AZ-A concentration *vs.* time. The data points in Figures 1 to 4 are the means of triplicate measurements and the error bars represent standard deviations (SD). Unfortified buffer solutions and filtered pond waters served as the controls. No pH adjustments were done for the pond waters fortified with AZ-A, however their pH values were monitored throughout the study period.

At specific intervals of time, aliquot samples (10-30 mL) were taken from each bottle after thorough shaking and extracted with dichloromethane after the addition of aqueous sodium chloride. The organic layer was dried with anhydrous sodium sulfate and flash evaporated to dryness. The residue was taken in ethyl acetate and cleaned by Florisil® minicolumn. The column was eluted with ethyl acetate and the eluate, after concentration, was quantified by HPLC for AZ-A content. The details of extraction, cleanup and analysis of AZ-A are given elsewhere [8]. The

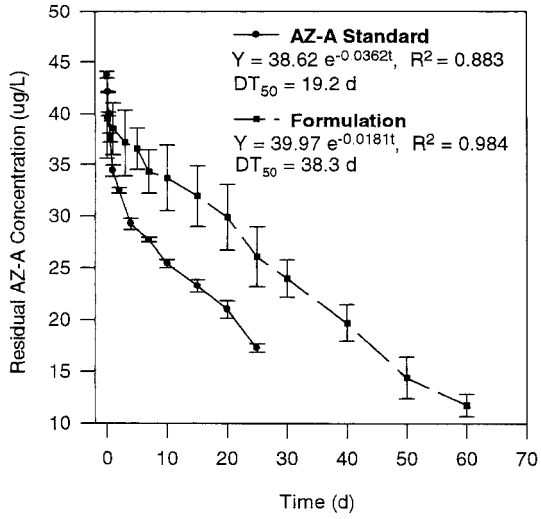


Figure 1. Degradation of AZ-A in distilled water buffered at pH 4 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

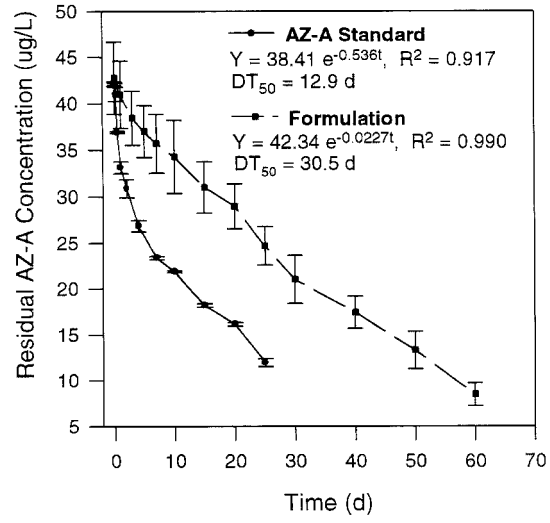


Figure 2. Degradation of AZ-A in distilled water buffered at pH 7 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

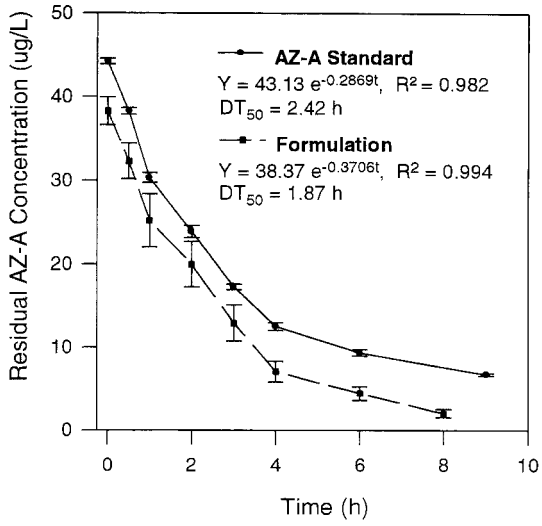


Figure 3. Degradation of AZ-A in distilled water buffered at pH 10 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

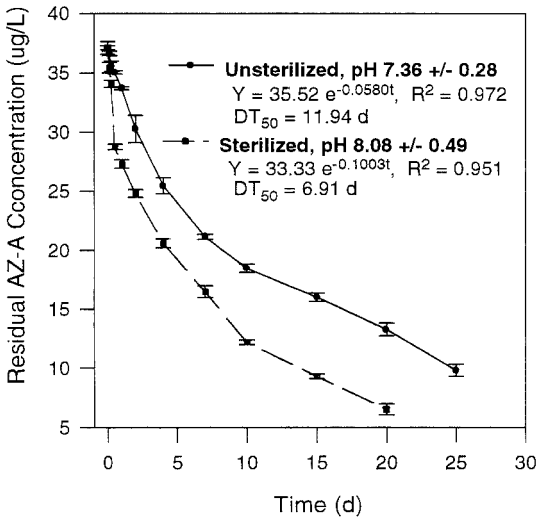


Figure 4. Degradation of AZ-A in sterilized and unsterilized pond water. Error bars represent +/- SD (n=3).

recovery of AZ-A from the buffer solutions and pond water at the 10 µg/L level was > 96 % and the limits of detection and quantification were, respectively, 1.0 and 3.0 µg/L.

RESULTS AND DISCUSSION

In the dark, at a constant incubation temperature and under sterile conditions, the degradation of AZ-A in the buffer systems was due to hydrolytic reaction in which the AZ-A molecule reacted with H_2O under the catalytic influence of $H^{\oplus}$ or $OH^{\ominus}$ ions, forming new products containing C– O bonds. The AZ-A degradation in the buffer solutions at pH 4, 7 and 10 and sterilized and unsterilized natural water was obtained using the first-order rate equation [11]

$$Y = Y_o e^{-kt} \quad (1)$$

where Y represents the AZ-A concentration at time t (d or h, depending on rate), Y_o represents the initial AZ-A concentration and k is the rate constant. Logarithmic transformation yielded the linear equation:

$$2.303 \log_{10} (Y/Y_o) = -kt \quad (2)$$

As the concentration dissipated to 50 % of the initial amount, the DT_{50} (time required for 50% of the initial concentration to dissipate) value could be determined from equation (3) for each experiment:

$$DT_{50} = (2.303 \log_{10} 2) / k \quad (3)$$

All the plots of residual concentration of AZ-A *vs.* time were curvilinear for the pH range 4 to 10 (Figs. 1 to 3) and for the natural water (Fig. 4). The degradation data obeyed the exponential equation (1), indicating that the reaction followed first-order kinetics. Regression analysis of the residues remaining at time t gave a good fit, with R^2 (coefficient of determination) ranging from 0.883 to 0.994. The

values of k, R^2 and DT_{50} for the acidic (pH 4), neutral (pH 7) and alkaline (pH 10) buffers, and for the sterilized and unsterilized natural water samples, are listed in the corresponding figures.

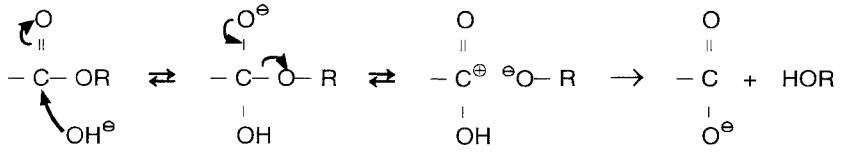
A comparison of the degradation of AZ-A under acidic, neutral and alkaline conditions show that the chemical, either in the pure or formulated form, remains relatively stable under acidic (pH 4) conditions (Fig. 1). Nearly 39 % of pure AZ-A and 66 % of AZ-A in the formulation remained in the buffer after 25 d. About 30 % of fortified AZ-A in the formulation remained in the buffer on the last day (60 d post-application) of sampling. The k and DT_{50} values for the pure AZ-A in the acidic buffer were, respectively, 0.0362 d^{-1} and 19.2 d. The corresponding values for the AZ-A in the formulated material were 0.0181 d^{-1} and 38.3 d, respectively.

The rate of degradation of pure AZ-A and formulated AZ-A in the buffer at pH 7 (Fig. 2) was higher compared to that in the pH 4 buffer. Only about 28 % of pure AZ-A and 58 % of AZ-A in the formulation remained on 25 d. About 20 % of AZ-A remained in the formulated material after 60 d. Correspondingly, the rate constants, k, were higher (0.0536 d^{-1} for pure AZ-A and 0.0227 d^{-1} for formulated AZ-A) and DT_{50} values (12.9 d for pure AZ-A and 30.5 d for formulated AZ-A) were lower, indicating greater degradation. Considering the k and DT_{50} values at pH 4 and 7, it is obvious that the rate of hydrolytic degradation of AZ-A increased with pH.

Under alkaline conditions (pH 10), the hydrolytic degradation of AZ-A either in the pure form or in formulation was extremely rapid (Fig. 3). The chemical was completely degraded in both the samples within 10 h, indicating the instability of AZ-A in strong alkaline solutions. The rate constants, k, were relatively high

(0.2869 h^{-1} for pure AZ-A and 0.3706 h^{-1} for formulated AZ-A) and the DT_{50} values found were hours, not days (2.42 h for pure AZ-A and 1.87 h for the formulated material). Despite the differences recorded, statistical treatment showed no difference between the two DT_{50} values (ANOVA $P > 0.05$). From the data in Figures 1 and 3, it is apparent that the effect of raising the pH from 4 to 10, reduced the DT_{50} values for the pure AZ-A and AZ-A in the formulation by a huge factor of nearly 190 and 492, respectively.

Azadirachtin is a complex molecule with a number of carboxylic ester groups and epoxide rings [12]. Its high instability in alkaline solutions is likely due to base-catalysed reactions involving aceyl-oxygen cleavages in the molecule through nucleophilic attack, thus altering the ester ($-\overset{\overset{O}{\|}}{C}-OR$) to carboxylate ($-\overset{\overset{O}{\|}}{C}-O^{\ominus}$) groups as shown below:



Such base-catalysed hydrolytic degradations are found to be common among organophosphorus insecticides, cleaving the C– O– P bonds [13, 14]. Nucleophilic attack by $OH^{\ominus}$ at the C atoms in the epoxide rings in the molecule, forcing them to open, followed by abstraction of protons from H_2O by alkoxide ions ($-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$) to form diols ($-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$), causing structural alterations, is also very likely.

The stability of AZ-A in the acidic medium (Fig.1) shows that acid-catalysed hydrolysis, *i.e.*, initial protonation of carbonyl oxygen as well as the O atom of

epoxide rings followed by the attack on the aceyl oxygen and/or the oxonium ion by the weak nucleophile, H_2O, is not a vigorous one. Also, unlike alkaline hydrolysis, the acid-catalysed reactions could be reversible. These factors could have contributed to the overall stability of AZ-A in the acidic medium.

Figures 1 and 2 show that AZ-A in the formulation degraded relatively slowly compared to pure AZ-A in the buffers at pH 4 and 7. This difference in degradation rates is attributable to the presence of surfactants (emulsifiers) in the formulation. Surfactants are large molecules and, by virtue of their combined polar (hydrophilic) and apolar (lipophilic) nature, form micelles in solution [15]. Azadirachtin molecules, being nonpolar and oleophilic, are likely to be partitioned and incorporated or solubilized inside the hydrophobic core of micelles, shielding them from the attack by the $H^{\oplus}$ ions present in the surrounding medium. There are no such micelles in the buffer solutions fortified with pure AZ-A, hence no such shielding mechanism was available for the molecules. It is likely that under high alkaline medium (pH 10), either micellization did not occur, or the micelles disintegrated rapidly, releasing the AZ-A molecules to $OH^{\ominus}$ ions for rapid interaction. Presently, there is sufficient evidence [16] for surfactant-facilitated stabilization of pesticide molecules in solution, however no such specific information is available yet on the stabilization of AZ-A molecules in solution. Additional research is necessary to study the influence of additives in stabilizing AZ-A molecules in spray formulations.

The AZ-A concentration (μg/L) in sterilized and unsterilized pond water as a function of time (d) is graphed in Figure 4. The initial concentrations in both samples were similar, 37.13 ± 0.54 μg/L (sterilized) and 37.14 ± 0.18 μg/L

(unsterilized). The average pH values for the sterilized and unsterilized water samples during the course of the study were 8.08 ± 0.49 and 7.36 ± 0.28, respectively. The values suggest that: (i) unsterilized water has some buffering capacity (low variability in pH) due to dissolved ions and humic compounds and (ii) the relatively high pH of sterilized water is due to the hydrolysis of the CO_3^{2e} and PO_4^{3e} ions in the solution.

The hydrolysis of AZ-A in sterile water was relatively rapid, probably due to the higher pH value (8.08 ± 0.49). No residues of the chemical were detected after 20 d, indicating its instability to hydrolysis. On the contrary, the AZ-A degraded rather slowly in the unsterilized water; about 26 % of the fortified amount remained after 25 d. Comparison of the rate constants (0.1003 d^{-1} for sterilized and 0.0580 d^{-1} for unsterilized water) and DT_{50} values (6.91 d for sterilized and 11.94 d for unsterilized water) definitely confirmed the slow loss of AZ-A in the unsterilized water. Contributing factors to this phenomenon could be the lower pH (7.36 ± 0.28) of the medium and absence of sufficient microbial population in the water. Azadirachtin is reported to have antiseptic and antimicrobial properties [17, 18]. Possibly, AZ-A used for the fortification of unsterilized water destroyed most of the microbial population, thus preventing their action on the degradation process. It is obvious that further research on the biological degradation of azadirachtin in natural waters, under controlled laboratory conditions, is necessary to validate this hypothesis. In addition, the role of sunlight and temperature, as well as the components in water such as humic compounds, metal ions, Bronsted acids and bases [19], on the degradation of AZ would be useful to get a holistic picture of its persistence in natural waters.

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SEPARATION OF YTTRIUM FROM LANTHANOID ELEMENTS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Separation of yttrium(III) and heavy lanthanoid elements(III) was performed by centrifugal partition chromatography (CPC) employing different types of stationary phase components, 2-ethylhexyl phosphonic acid mono-2-ethylhexyl ester (EHEHPA) and Versatic 10 (VA10). The retention volume (V_R) in CPC provided with the EHEHPA stationary phase increased with the atomic number of lanthanoid elements, and Y was eluted between Ho and Er. Yttrium can be completely separated from most of lanthanoids other than Dy, Ho and Er. As for the VA10 stationary phase, Y was separated from Dy, Ho and Er. Isolation of Y from a lanthanoid mixture was accomplished by means of two steps of CPC fractionation through the EHEHPA stationary phase, followed by treatment through the VA10 stationary phase; the content of each lanthanoid element in the final Y fraction was reduced to less than 0.3% against the content of Y.

INTRODUCTION

Yttrium occurs in natural resources along with lanthanoid elements, and separation and purification of Y require multi-stage processes due to chemical similarities of rare earth elements. Liquid-liquid extraction has provided effective separation methods for rare earth elements (1); industrial

separation of Y has been performed by means of series of mixer–settlers employing various types of extractants such as Versatic 911 and tributylphosphate (2). However, it has required a large number of stages and long separation time for complete isolation of high–purity elements.

Centrifugal partition chromatography (CPC) has been developed as an analytical and preparative separation method for chemically–similar materials (3). In this chromatography, an organic solution containing an extracting reagent can be employed as a stationary phase without any solid support, therefore, the stationary and mobile phases can be selected to optimum conditions on the basis of liquid–liquid extraction behavior.

In recent years, the mutual separation of trivalent lanthanoid elements was successfully performed on CPC employing stationary phases containing acidic organophosphorus extractants, such as di(2–ethylhexyl) phosphoric acid (DEHPA) (4–6), 2–ethylhexyl phosphonic acid mono–2–ethylhexyl ester (EHEHPA) (7,8) and di(2,4,4–trimethylpentyl)phosphinic acid (DTMPPA) (9,10). A series of lanthanoids were eluted in the increasing order of distribution ratios between the stationary and mobile phases, corresponding to the decreasing order of their ionic radii. The ionic radius of Y(III) is close to those of heavy lanthanoids, and the extractability of Y with DEHPA is placed between Ho and Er, exhibiting the low separation factors of 1.65 for Y and Ho (Y/Ho), and 1.37 for Er/Y (11). Since these separation factors are not sufficiently large, quantitative separation of Y from lanthanoid elements seems to be difficult even by means of CPC provided with acidic organophosphorus extractants. Other kinds of extractants having the desired selectivity are required for the separation of Y from accompanying heavy lanthanoids. Versatic 10 (VA10) is a bulky tertiary alkyl mono–carboxylic acid and has better selectivity for the separation of Y from heavy lanthanoids in contrast with acidic organophosphorus extractants, though the separation factors for neighboring lanthanoids are rather low (12,13).

This study deals with chromatographic separation of Y and some lanthanoid elements by CPC provided with the EHEHPA stationary phase and the VA10 stationary phase. Isolation of Y from lanthanoid elements was performed by fractionation from CPC with EHEHPA, followed by separation on CPC with VA10.

EXPERIMENTAL

Materials

An extractant, 2-ethylhexyl phosphonic acid mono-2-ethylhexyl ester (EHEHPA) (PC-88A; Daihachi Chemical Ind. Co. Ltd.), was washed with a sodium hydroxide solution to remove acidic impurities, and its purity was checked to be 96% by potentiometric titration in ethanol with a sodium hydroxide solution. Versatic 10 (VA10) was obtained from Shell Chemical Co., and its purity (97%) was also determined as above. A 50% (v/v) ethanol solution of 0.014% (w/v) Arsenazo III (Dojindo Lab.) was used for a post column reaction. Standard solutions of 1000 ppm of Y, Gd, Tb, Dy, Ho, Er and Tm in 1 M (M = mol dm^{-3}) HNO_3 (Wako Pure Chemical Ind.) were dried up, and diluted with deionized water or pH-buffered solution to the desired concentration.

Apparatus

Chromatographic separation was performed by means of centrifugal partition chromatograph (CPC, Model NMF; Sanki Engineering Ltd.) modified for use at higher temperature up to 70°C. Six partition cartridges (type 250W, total volume 125 cm^3) as separation columns were arranged in a CPC rotor, where one cartridge was composed of 400 microcells. A mobile phase was sent into CPC by a triple plunger pump (LBP-V; Sanki). A part of the eluted solution from CPC was continuously moved to a detection system by a double plunger pump (NP-FX-10U; Nihon Seimitsu Kagaku Co., Ltd). The detection system consisted of a post column-reactor (ICA-3041; Toa Electronics Ltd.), a photometric detector with a flow cell (ICA-3020; Toa) and a pen recorder (FBR-251A; Toa).

Extraction Equilibrium

A toluene solution of 0.02 M $(EHEHPA)_2$ was shaken over 30 min with an equal volume of an aqueous phase containing 2×10^{-5} M of each rare earth ion. The aqueous pH was adjusted to an appropriate value with 0.1 M $(H,Na)CHCl_2COO$. In the extraction with VA10, a kerosene solution of 0.25 M $(VA10)_2$ was contacted with a 0.1 M $NaNO_3$ aqueous solution containing 0.01 M $(H,Na)CH_3COO$ as a buffer. After equilibrium, the

concentrations of metals were measured by inductively coupled plasma atomic emission spectrometry (ICP–AES) (SPS 1200A, Seiko Instruments & Electrics Ltd.); the concentration of the extracted element in the organic phase was determined after stripping into 1 M HNO_3.

CPC Process

The toluene solution of 0.02 M $(EHEHPA)_2$ was held in microcells of CPC–cartridges as the stationary phase. The aqueous solution containing 0.1 M $(H,Na)CHCl_2COO$ was chosen for the mobile phase. Further, the kerosene solution of 0.25 M $(VA10)_2$ and the 0.1 M $NaNO_3$ solution buffered with 0.01 M $(H,Na)CH_3COO$ were respectively used as the stationary and mobile phases. The mobile phase was equilibrated with the corresponding organic phase before use. This mobile phase was pumped into the stationary phase through a rotary seal joint at a flow rate of 3 cm^3 min^{-1}. Under the rotation of CPC–rotor at 900 ~ 1100 rpm, the mobile phase flowed through the column as small droplets, replacing a part of the stationary phase in the microcells. At a constant volume ratio of two phases, the sample solution containing $5 \times 10^{-4} \sim 4 \times 10^{-3}$ M of each rare earth ion was charged into the column through a sample loop (1 cm^3). A part of the eluted stream was continuously monitored by measuring the absorbance of lanthanoid–Arsenazo III complexes at 650 nm.

Chromatographic Parameters

Chromatographic parameters were evaluated to clarify elution characteristics of rare earth elements in CPC. The number of theoretical plates (N) is related to the retention volume (V_R) and the peak width (W):

$$N = 16 \left(V_R / W \right)^2 \tag{1}$$

The separation factor (α_c) in chromatography is evaluated from the retention volumes of corresponding components 1 and 2:

$$\alpha_c = \frac{V_{R,2} - V_0}{V_{R,1} - V_0} \tag{2}$$

where V_0 is the elution volume for an unretained component.

The resolution (R_s) is evaluated by the distance between the two peaks and their peak widths.

$$R_s = \frac{2\ (V_{R,2} - V_{R,1})}{W_1 + W_2} \tag{3}$$

RESULTS AND DISCUSSION

Extraction with EHEHPA

Mutual separation of rare earth elements is based on the differences in the distribution ratios between the stationary and mobile phases. Extraction behavior of Y and some heavy lanthanoids with EHEHPA in toluene was examined in order to optimize separation conditions on CPC. Figure 1 represents the distribution ratios (D) of closely resemble rare earths as a function of pH, where the extractant concentration is formally expressed as a dimer $(EHEHPA)_2$, since the acidic organophosphorus extractants are commonly present as dimeric species in nonpolar diluents. The slopes of the log D–pH plots were of 2.7 to 2.9, indicating that the extraction proceeds in the following reaction (7);

$$M^{3+}_{aq} + 3(HA)_{2,org} \rightleftharpoons M(HA_2)_{3,org} + 3H^+_{aq} \tag{4}$$

where M denotes the rare earth element and $(HA)_2$ is the dimer of extractant. The extraction constants (K_{ex}) for Eq.(4) are listed in Table 1. The value of K_{ex} increased with atomic number of lanthanoids, and that for Y was situated between Ho and Er. These K_{ex} values for the toluene solution were lower about 2 order of magnitude than those for the kerosene solution (7); the K_{ex} values have been found to decrease in the order hexane > carbon tetrachloride > 1,2–dichloroethane > toluene > benzene for extraction with acidic organophosphorus compounds (14). This lower extractability in toluene may permit to employ the dilute acid as the mobile phase of CPC, and/or the higher concentration of extracting reagent in the stationary phase. The separation factors (α_D) are summarized in Table 1. The values of α_D for Y/Ho and Er/Y were low and the separation of Y from Ho and Er might be rather difficult by CPC with the EHEHPA stationary phase, since the mutual separation has been often insufficient for lanthanoids having low α_D values less than 2 (7).

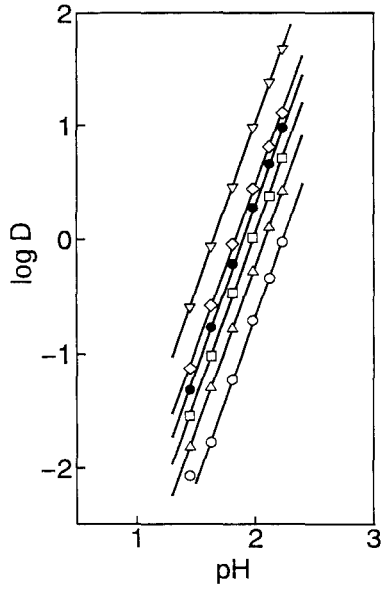

FIGURE 1 Extraction of rare earth elements with 0.02 M $(EHEHPA)_2$ in toluene. 0.1 M (H, Na)$CHCl_2COO$, 25°C, (●) Y, (○) Tb, (△) Dy, (□) Ho, (◇) Er, (▽) Tm.

TABLE 1
Extraction Constants and Separation Factors of Rare Earth Elements in EHEHPA System at 25°C

Element	log K_{ex}	α_D
Tb	−1.65	
		3.1
Dy	−1.15	
		2.0
Ho	−0.85	
		1.9
Y	−0.58	
		1.5
Er	−0.41	
		3.5
Tm	0.14	

Organic phase : 0.02 M $(EHEHPA)_2$ in toluene.
Aqueous phase : 0.1 M (H,Na)$CHCl_2COO$.

Application to CPC

The extraction system with EHEHPA was applied to the CPC procedures for rare earths, Y, Ho and Er, having low separation factors. The sample solution (1 cm^3) containing 1×10^{-3} M Y or a mixture of Ho and Er was introduced into the column containing 0.02 M $(EHEHPA)_2$–toluene. Resulted chromatographic peaks on CPC are presented in Fig. 2. The elution peak of Y was situated between the Ho and Er peaks, in the agreement with corresponding order in the distribution ratios. The retention volume became large with rising pH owing to increase in the distribution ratio. The retention volume can be related to the D value in the ordinary chromatograph as:

$$V_R = V_m + D V_s \quad (5)$$

where V_s and V_m are the volumes of the stationary and mobile phases, respectively. Plots of the V_R values against the D values determined by liquid–liquid extraction are shown in Fig. 3. The solid line indicates the calculated value from Eq.(5) by employing the experimental values of 78 cm^3 for V_m and 47 cm^3 for V_s. The experimental points were in fair agreement with the calculated ones; the retention volumes of rare earths can be predicted from individual extraction data.

As can be seen in Fig. 2, the chromatogram of Er and Ho was considerably overlapped at low pH as 1.98, while this chromatographic separation was improved with an increase in pH. Further, a rise in temperature improved the peak profile; e.g., the N values for Y increased from 17 at 25°C to 30 at 40°C. This enhancement may be attributable to the promotion of extraction rate with the rise of temperature. The separation factors between Ho and Er were evaluated as close values, 2.8 and 2.7 at 25°C and 40°C, respectively. The resolution of 0.77 at 25°C was somewhat enhanced to 0.83 at 40°C owing to the increasing N. At higher temperature of 50°C, no distinct chromatogram was obtained because of gradual losses of the stationary phase along with the mobile phase.

Separation of Y from Lanthanoids on CPC with EHEHPA

Chromatographic separation of Y and lanthanoid elements was examined by using EHEHPA as the CPC stationary phase at 40°C. The

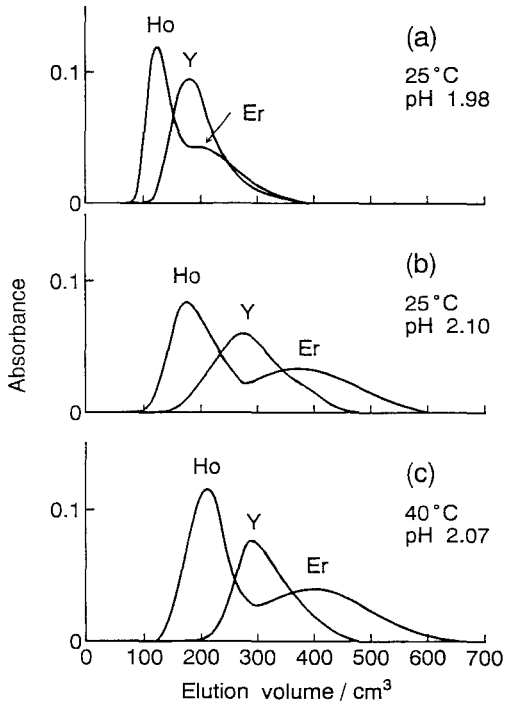

FIGURE 2 Chromatograms of Y and the binary mixture of Ho and Er by CPC with EHEHPA. CPC : 6 cartridges of 250W, 1100 rpm; stationary phase : 0.02 M $(EHEHPA)_2$ in toluene; mobile phase : 0.1 M (H, Na)$CHCl_2COO$; sample : 1×10^{-3} M Y, or a mixture containing 1×10^{-3} M Ho and Er, 1 cm^3.

chromatogram for a mixture of 4×10^{-3} M Y, Tb and Tm is shown in Fig. 4. Yttrium was completely separated from Tb and Tm. The other lanthanoids lighter than Tb will be less retained than Tb due to lower distribution ratios and heavier lanthanoids than Tm having higher distribution ratios will be more retained than Tm.

Under the same conditions, the chromatograms for desired lanthanoids, Dy, Ho and Er, together with Y are presented in Fig. 5. The separation factor between Dy and Y was relatively high as 3.0, and only a tailing of

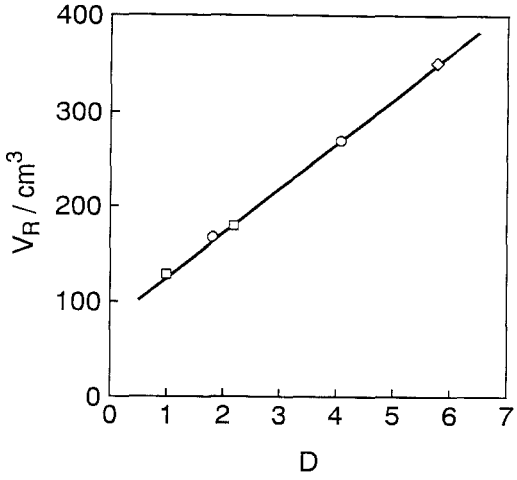

FIGURE 3 Relation between the retention volume and the distribution ratio in EHEHPA system at 25°C. (○) Y, (□) Ho, (◇) Er; the solid line indicates the calculated value from Eq.(5).

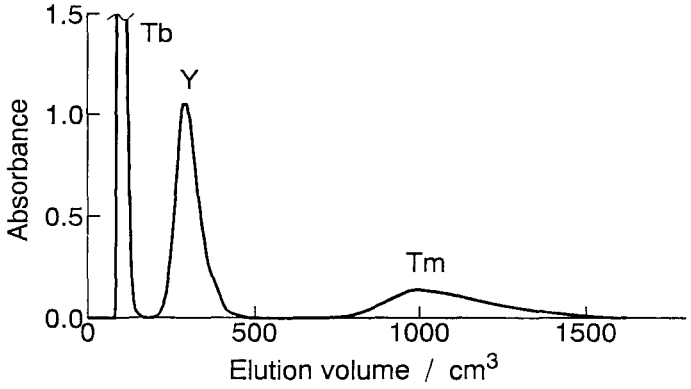

FIGURE 4 Separation of the ternary mixture of Y, Tb and Tm by CPC with EHEHPA; 40°C, pH 2.07; sample : 4×10^{-3} M Y, Tb and Tm, 1 cm^3.

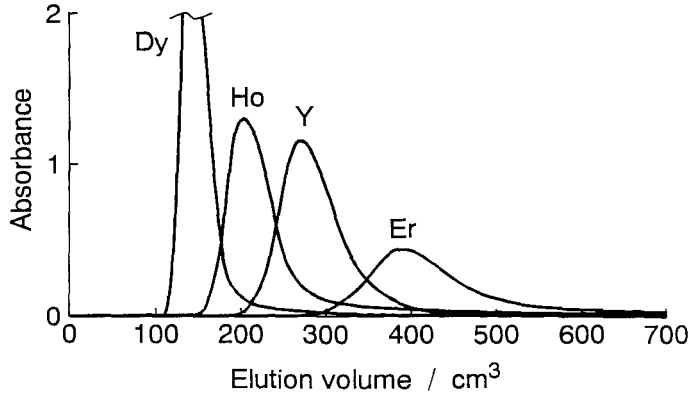

FIGURE 5 Chromatograms of Y, Dy, Ho and Er by CPC with EHEHPA. 40°C, pH 2.07; sample : 4×10^{-3} M each rare earth element, 1 cm^3.

the Dy peak reached the Y peak. Large parts of the chromatographic peaks of Ho and Er overlapped with that of Y, in which the separation factors were low such as 1.54 for Ho/Y and 1.62 for Y/Er, and the resolutions were still so low as 0.56 for Ho/Y and 0.72 for Y/Er. These findings suggest that the chromatographic separation of Y from Ho and Er is difficult by CPC with the EHEHPA stationary phase, and the Y fraction will accompany a little amount of Dy and considerable amounts of Ho and Er. Additional treatment is required for further purification of Y, that is, the removal of Dy, Ho and Er from the Y fraction.

Extraction with VA10

The extractability of lanthanoids by VA10 from 1 M $(H,Na)NO_3$ has been reported to increase with the atomic number and then Y is extracted very closely to middle lanthanoids rather than heavy lanthanoids (13). Therefore, the separation of Y from heavy lanthanoids may be performed by employing VA10 as the stationary phase.

The extraction of Y and related lanthanoids with VA10 in kerosene was examined. Figure 6 illustrates plots of the distribution ratios of Y and

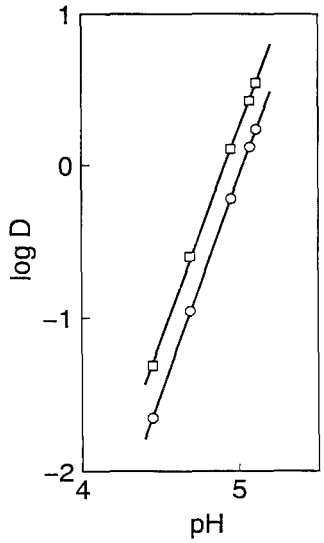

FIGURE 6 Extraction of Y and Ho with 0.25 M $(VA10)_2$ in kerosene. 0.1 M $NaNO_3$ and 0.01 M (H, Na)CH_3COO, 25°C, (○) Y, (□) Ho.

TABLE 2
Extraction Constants and Separation Factors between Lanthanoids and Y in VA10 System

M	25°C		40°C		55°C	
	log K_{ex}	α_D(M/Y)	log K_{ex}	α_D(M/Y)	log K_{ex}	α_D(M/Y)
Y	−13.27	–	−12.47	–	−11.87	–
Gd	−13.12	1.4				
Tb	−12.94	2.1				
Dy	−12.88	2.5	−12.14	2.1	−11.55	2.1
Ho	−12.93	2.2	−12.16	2.0	−11.58	1.9
Er	−12.92	2.2	−12.12	2.2	−11.54	2.1

Organic phase : 0.25 M $(VA10)_2$ in kerosene.
Aqueous phase : 0.1 M $NaNO_3$, 0.01 M (H,Na)CH_3COO.

Ho from 0.1 M $NaNO_3$ solution against pH at 25°C. Yttrium was less extracted than Ho, in the reverse order by EHEHPA. The distribution ratios of Dy and Er were very close to that of Ho. These plots gave straight lines with slopes of about 3, and the extraction reaction also follows Eq. (4). (13,15)

The extraction constants and the separation factors between lanthanoids and Y are summarized in Table 2. The value of log K_{ex} increased with rising temperature in a similar extent for each element. The extraction constants of heavy lanthanoids were very close to each other, and the value for Y was smaller than those for heavy lanthanoids. The separation factor of Y was found to be relatively large above 2 for Tb, Dy, Ho and Er. The α_D values tend to decrease only slightly with rising temperature from 25°C to 55°C, but they are still close to 2 even at 55°C.

Chromatographic Behavior on CPC with VA10

On the basis of extraction behavior, the kerosene solution of 0.25 M $(VA10)_2$ was employed as the stationary phase and the mobile phase of 0.1 M $NaNO_3$ solution was adjusted to an appropriate pH with 0.01 M (H, Na)CH_3COO for CPC separation of Y from heavy lanthanoids. Figure 7 illustrates the CPC chromatograms of a mixture of Y and Ho at different temperatures. Here, Ho was chosen as a typical accompanying heavy lanthanoid, because the separation factor for Ho/Y was the lowest among heavy lanthanoids. The pH of the mobile phase was adjusted to be lower at higher temperature, since the retention volume increased with the rise of temperature, due to the increase in the distribution ratios of rare earth elements.

Figure 8 shows the relationship between V_R and D evaluated from the liquid–liquid extraction for Y and Ho at different temperature. The V_R values increased linearly with increasing D values, indicating that the retention behavior of the metal ion corresponds to the distribution behavior. The V_R values were found to be a little larger than those calculated from Eq. (5) using experimental values of 65 cm^3 for V_m and 60 cm^3 for V_s. This deviation may be due to an increase in the distribution ratio, perhaps caused by a local rise in temperature in column cartridges near a heater.

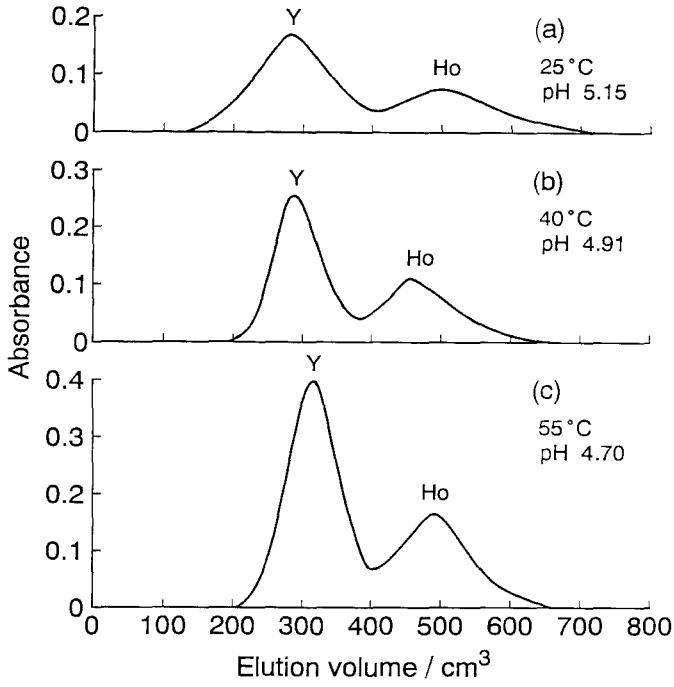

FIGURE 7 Chromatograms of the binary mixture of Y and Ho by CPC with VA10 at different temperature. CPC : 6 cartridges of 250W, 900 rpm; stationary phase : 0.25 M $(VA10)_2$ in kerosene; mobile phase : 0.1 M $NaNO_3$ and 0.01 M (H, Na)CH_3COO; sample : 1×10^{-3} M Y and Ho, 1 cm^3.

In Fig. 7, the values of N for both elements increased with rising temperature, while the separation factor slightly decreased. These opposite trends for separation efficiency were partially compensated, while the resolution for Y and Ho slightly increased, such as 0.89 at 25°C, 0.95 at 40°C and 1.0 at 55°C.

Figure 9 illustrates individual chromatographic peaks for Dy, Ho, Er and Y under the optimum conditions at 55°C. The retention volume for Ho was a little smaller than those for Dy and Er, as predicted from their extractabilities. The chromatogram of Y only slightly overlapped with those

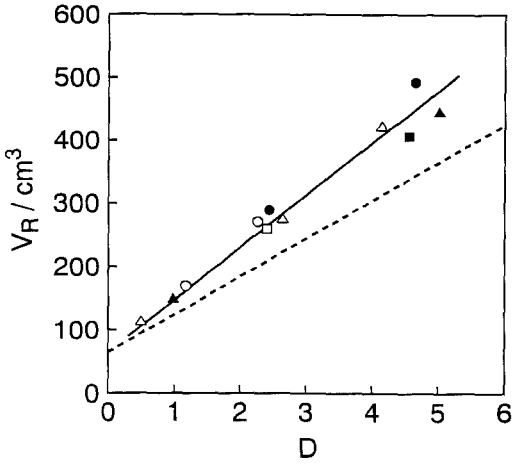

FIGURE 8 Relation between the retention volume and the distribution ratio in the VA10 system. open : Y, closed : Ho; (○,●) 25°C, (△,▲) 40°C, (□,■) 55°C; the broken line indicates the calculated value from Eq.(5).

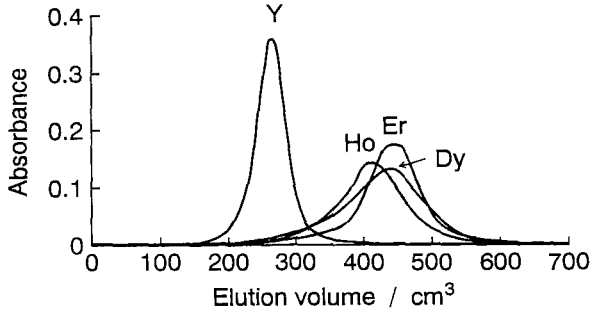

FIGURE 9 Chromatograms of Y, Dy, Ho and Er by CPC with VA10. 55°C, pH 4.70; sample : 1 × 10^{-3} M each rare earth element, 1 cm^3.

of lanthanoids, and good resolution between Y and each lanthanoid was accomplished as R_s = 1.1 (Ho/Y), 1.3 (Dy/Y) and 1.6 (Er/Y).

Separation of Y from Rare Earth Mixture

The separation and purification of Y from lanthanoid mixtures was performed through subsequent CPC separation steps with EHEHPA and VA10. A sample solution (1 cm^3) containing 4 × 10^{-3} M each of Y, Dy, Ho and Er was injected to the CPC system using the EHEHPA–toluene solution as the stationary phase, since other lanthanoids will be readily separated through the EHEHPA stationary phase. Figure 10 shows the chromatogram on CPC with 0.02 M $(EHEHPA)_2$–toluene. The chromatogram gave distinct peaks of Dy, Ho and Y, while the peak for Er was no longer distinguishable from the overlapped peak of Y. The effluent mobile phase was fractionated from around 230 cm^3 to 430 cm^3 corresponding to the Y elution.

The rare earth elements in the fractionated solution were concentrated by extraction into a 0.02 M $(EHEHPA)_2$–toluene solution at pH > 3, and then the extracted elements were stripped into 1 M HNO_3. The aqueous

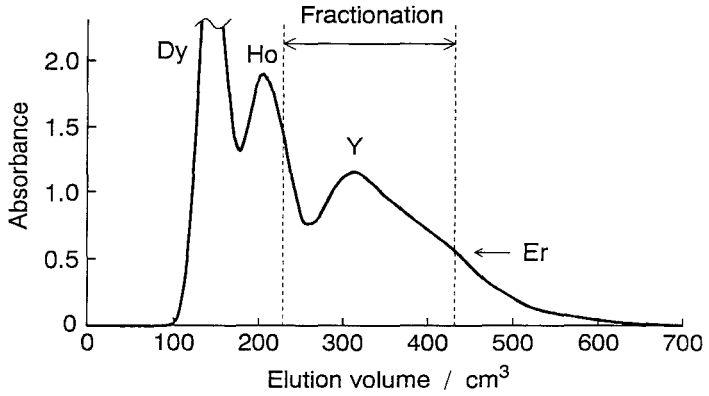

FIGURE 10 Fractionation of Y–rich effluent in the separation of the mixture of Y, Dy, Ho and Er by CPC with EHEHPA. 40°C, pH 2.07; sample : 4 × 10^{-3} M Y, Dy, Ho and Er, 1 cm^3.

solution was evaporated to dryness, and the residue was resolved into 8 cm^3 of water. In these procedures, the recovery of rare earths was confirmed to be quantitative.

Rare earth elements in the Y fraction through CPC with EHEHPA were further introduced into the VA10–CPC system. Figure 11 shows the chromatogram for 1 cm^3 of the solution on CPC with a 0.25 M $(VA10)_2$–kerosene solution at 55°C. Yttrium was eluted with a mobile phase of pH 4.70 yielding almost base-line separation from the accompanying lanthanoids. The Y fraction in the region of about 190 – 340 cm^3 was collected and analyzed by ICP–AES.

Isolation of Y from lanthanoid elements by subsequent CPC treatments is summarized in Table 3. In the CPC with EHEHPA, most of Dy was eliminated from the Y fraction, but considerable amounts of Ho and Er were remained in the Y fraction. By further separation through CPC with VA10, these residual lanthanoids were entirely removed to less than 0.003 against Y content.

In conclusion, the separation and isolation of Y from chemically similar lanthanoids was performed through two steps of CPC processes employing different kinds of stationary phases. Yttrium was fractionated

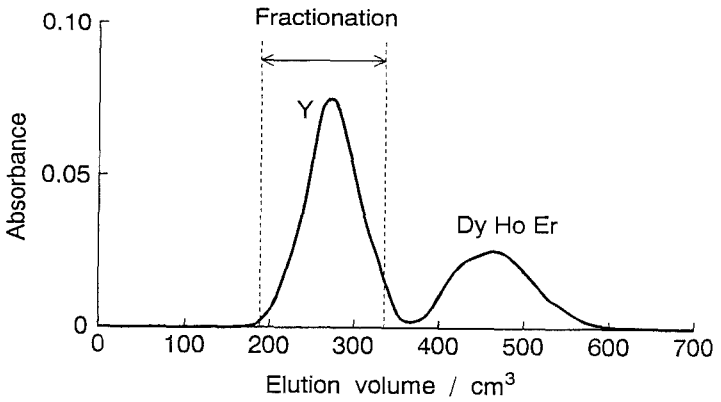

FIGURE 11 Separation of Y from Dy, Ho and Er by CPC with VA10. 55°C, pH 4.70.

TABLE 3
Separation of Y from Dy, Ho and Er by CPC

Element	EHEHPA		VA10	
	Yield	Relative content [M]/[Y]	Yield	Relative content [M]/[Y]
Y	0.89	–	0.91	–
Dy	0.0064	0.0071		0.0004
Ho	0.14	0.16		0.0019
Er	0.45	0.51		0.0026

accompanying only a few lanthanoids, Dy, Ho and Er, separated from other lanthanoid elements lighter than Dy and heavier than Er, by means of the EHEHPA stationary phase. The Y fraction was further purified by removing the accompanying lanthanoids through the stationary phase of VA10 having sufficient separation factor between Y and heavy lanthanoids.

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SEMI-PREPARATIVE SEPARATION OF TARAXERYL-ACETATE AND COUMARINS FROM *ARTEMISIA DALAILAMAE* KRASCHEN BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

High-speed countercurrent chromatography (HSCCC) has been successfully applied to the semi-preparative separation of taraxeryl-acetate, isofraxidin and scopoletin from *Artemisia dalailamae* Kraschen. The separations were performed with a two-phase solvent system composed of chloroform-methanol-water (2:2:1, v/v/v). The main three pure fractions were analyzed by MS, IR, NMR etc. for structure determination. The results indicate that the method is suitable for semi-preparative separations of these compounds.

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INTRODUCTION

Countercurrent chromatography (CCC) is a special liquid-liquid partition chromatography without using solid support matrix. As a result, CCC eliminates adsorptive loss entirely and denaturation of samples. Samples can be recovered quantitatively [1]. CCC is very suitable for preparative separation of natural products.

In the past thirty years, a great advance has been made in CCC. More than ten sets of CCC instrumentation have come out successively. In the early of 1980s, especially, an epochmaking development in CCC technique was brought forth by the discovery of a unique hydrodynamic phenomenon which led to the development of high-speed countercurrent chromatography (HSCCC)[2,3]. The new method is characterized by high partition efficiency and large retention capability of the stationary phase under a high flow-rate of the mobile phase, yielding efficient separation in a few hours or less than one hour. Now, HSCCC has developed into analytical and preparative instrumental approaches.

HSCCC is now finding increasing use in separation problems, especially in the field of natural products, such as alkaloids[4, 5], flavonoids [4, 6], flavonol glycosides[7] and plant hormones[8, 9].

This paper presents the results of semi-preparative separations of a crude chloroform extract from *Artemisia dalailamae* Kraschen and two semi-pure fractions of this extract by HSCCC.

EXPERIMENTAL

Apparatus

The multilayer coil planet centrifuge used in the present study was produced by the Beijing Institute of New Technology Application, Beijing, China. The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 8 cm from the central axis of the centrifuge. The separation column was prepared by winding a long piece of PTFE tubing, about 110 mm in length, 1.6 mm i.d. and 0.3 mm wall thickness , directly onto one of the holder hubs of 6 cm diameter to form a multilayer

coil. The β value, which is the ratio of the rotational radius to the revolutional radius, ranges from 0.4 at the internal terminal to 0.7 at the external terminal. The total capacity of the multilayer coil was measured as 250 ml approximately. The revolution speed of the apparatus is adjustable with a speed controller in the range from 0-1000 rpm; 800 rpm was used in the present separations.

The solvent was pumped into the column with a constant-flow pump (Model PB-1A, Beijing Orient Scientific Instrument Factory, Beijing, China). A UV detector (Model ZW-1, Beijing Institute of New Technology Application, Beijing, China) was used to monitor the effluent at 260 nm. A chromatographic data system (C-R2AX; Shimadzu Kyoto, Japan) was used to record the chromatogram.

Selection and Preparation of Two-phase Solvent System

The samples used in the present experiments were of medium polarity, so a chloroform-water system was selected as the basic dinary solvent system with methanol as assistant. A series of chloroform-methanol-water solvent systems in different proportions were prepared , and the respective settling time and two-phase volume ratio were determined. A two-phase solvent system composed of chloroform-methanol-water at a volume ratio of 2:1:1 was chosen and used in the present experiments. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

Preparation of Sample Solutions

A crude alcoholic extract from *A. dalailamae* Kraschen was dissolved in warm distilled water and the residue removed by filtration. The aqueous solution was then extracted successively with petroleum benzine, chloroform and n-butanol. The chloroform extract was evaporated to dryness and then dissolved in the lower chloroform phase of the two-phase solvent system at a concentration of about 500 mg/ml. Two semi-pure fractions of isofraxidin and scopoletin obtained from the separation of this chloroform extract by

classical silica gel column chromatography followed by crystallization, were also dissolved in the lower chloroform phase respectively. The three sample solutions were used in the subsequent experiments.

Separation Procedure

The coiled column was first entirely filled with the upper aqueous phase as stationary phase, and then the apparatus was rotated at 800 rpm, while the lower chloroform phase was pumped into the coiled column at a flow-rate of 1.5 ml/min as mobile phase. When no upper stationary phase appeared from the outlet of the column and all of the system was in a state of equilibrium, the sample solution was injected through the sampling port. The effluent was continuously monitored by a UV detector at 260 nm, and collected with test tubes according to chromatograms. Three separations were performed in turn without the renewal of stationary phase.

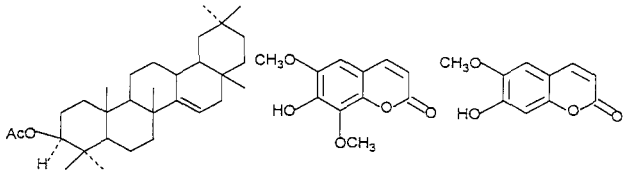
Structural Identification

The separations by HSCCC gave four pure compounds, and three of them were identified as taraxeryl-acetate, isofraxidin and scopoletin by MS, NMR, IR and element analysis. Their chemical structures were illustrated in Figure 1.

RESULTS AND DISCUSSION

Table 1 shows the systematic selection of the two-phase solvent system. A series of solvent systems composed of chloroform-methanol-water were prepared in different volume proportions. Their settling time and two-phase volume ratios were determined respectively.

Successful separation by HSCCC is mainly dependent on the selection of a suitable two-phase solvent system[10]. A two-phase solvent system with a settling time of less than 30 seconds is desirable for HSCCC apparatus[2,11,12]. At the same time, in order to avoid the excessive waste of solvent, the two-phase volume ratio should be close to 1[12]. According



(a) taraxeryl-acetate (b) isofraxidin (c) scopoletin

Figure 1. Structures of taraxeryl-acetate(a), isofraxidin(b) and scopoletin(c)

TABLE 1

Settling Time and Two-Phase Volume Ratios of a Series of Chloroform-Methanol-Water Solvent Systems.

No.	Volume proportion C-M-W[a]	Settling Time (seconds)	Volume Ratio UP/LP[b]
1	10:0:10	>30	1.0
2	10:1:9	>30	0.95
3	10:2:8	>30	0.94
4	10:3:7	>30	0.93
5	10:4:6	23	0.91
6	10:5:5	15	0.86
7	10:6:4	19	0.74
8	10:7:3	>30	0.52
9	10:8:2	--[c]	--[c]
10	11:5:5	21	0.82
11	9:5:5	15	0.98
12	8:5:5	15	1.0
13	7:5:5	>30	1.3

[a] C-M-W: Chloroform-Methanol-Water.
[b] UP/LP : Upper Phase/Lower Phase.
[c] -- : indicates solvent system exists in a single phase.

to these principles, the result of Table 1 shows that solvent systems of No. 5, 6, 7, 10, 11, 12 are more suitable to HSCCC and especially No.6,11,12, all with the shortest settling time of 15 seconds would produce much high and stable retention of the stationary phase in the coiled column of HSCCC. As the lower chloroform phase would be used as the mobile phase, the two-phase solvent system of No. 6, which is composed of chloroform-methanol-water at a 2:1:1 volume ratio, was selected for use in the present experiments.

Figure 2 shows the chromatogram of separation of the crude chloroform extract from *A. dalailamae* Kraschen by HSCCC. The sample size was 0.1 ml (or 50 mg). Three main peaks were eluted in one hour and are labeled as 1, 2, 3 in Figure 2. This separation gave 25.5 mg of 1, 4.4 mg of 2 and 5.0 mg of 3. Their chemical structures were then determined as taraxeryl-acetate, isofraxidin and scopoletin respectively.

Figure 3 and Figure 4 show the chromatograms of separations of the semi-pure fractions of isofraxidin and scopoletin by HSCCC.

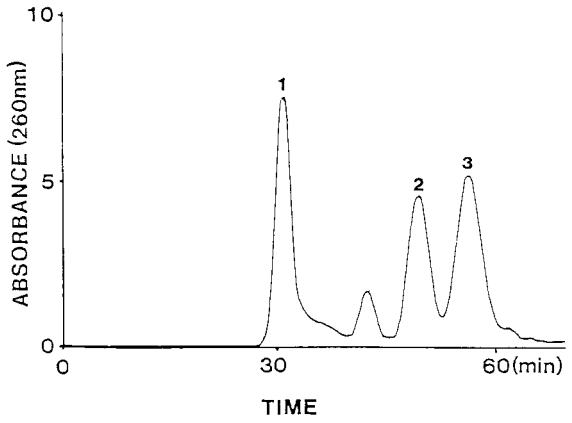


Figure 2. Chromatogram of separation of the chloroform extract from *A. dalailamae* Kraschen with chloroform-methanol-water (2:1:1), lower phase mobile, sample size 0.1ml (50 mg), flow-rate 1.5 ml/min. (1) taraxeryl-acetate, (2) isofraxidin and (3) scopoletin.

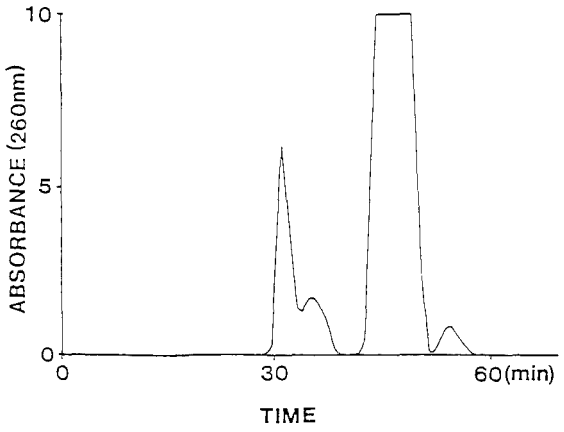


Figure 3. Chromatogram of separation of the semi-pure fraction of isofraxidin with chloroform-methanol-water (2:1:1), lower phase mobile, sample size 0.1ml, flow-rate 1.5 ml/min. The largest peak isofraxidin.

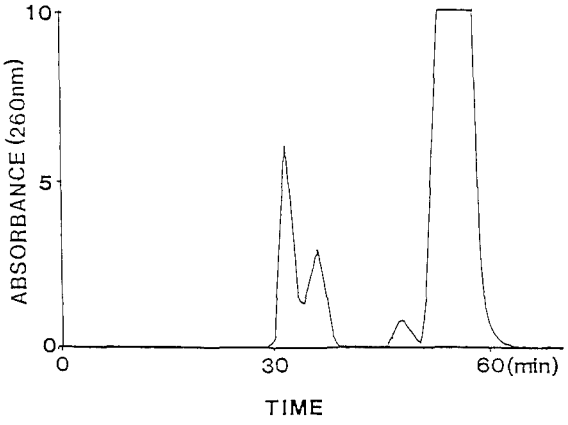


Figure 4. Chromatogram of separation of the semi-pure fraction of scopoletin with chloroform-methanol-water (2:1:1), lower phase mobile, sample size 0.1ml, flow-rate 1.5 ml/min. The largest peak is scopoletin.

The successful identifications of the three compounds show that both crude extract and semi-pure fractions have been successfully chromatographed. The results show that HSCCC has a good reproducibility and can be conduced without the renewal of stationary phase before every injection, when the range of polarity of a sample is not too wide.

ACKNOWLEDGMENT

We wish to thank Dr. Satish Kumaran for his revising this manuscript.

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MEETING ANNOUNCEMENT

PrepTech'95
INDUSTRIAL SEPARATION TECHNOLOGY CONFERENCE

February 13 - 15, 1995

Sheraton Meadowlands Hotel
East Rutherford, New Jersey

PrepTech'95 will present an intensive three-day program on industrial separation science. Technical sessions will provide detailed coverage of current and emerging technologies by international authorities.

Focusing on practical aspects of industrial separation technology, the program will include oral presentations, poster sessions, roundtable discussions, workshops, and an equpment exhibition.

The main focus of the oral presentations will be preparative chromatography. Topics covered in the poster sessions are intended to broaden the technical scope by including preparative chromatography; membrane separations; electro-separations; extraction; centrifugation.

Each day will be based on a theme:

> Monday, February 13th - Large Molecule Separations, including Applications of Affinity Chromatography; DNA Endotoxin Virus Separations; Protein Separations.
>
> Tuesday, February 14th - Process design, including Process Dsign Concepts; Economic Issues in Process Chromatography; Clean-in-Place; Sanitation.

Wednesday, February 15th - Separation Technology for the 90's and Beyond, including Separations of Industrial Importance; Small Molecule Separations; New and Innovative Separation Techniques.

Further information may be obtained from Ms. Joan Lantowski, ISC Technical Conferences, Inc., 30 Controls Drive, P. O. Box 559, Shelton, CT 06484-0559, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(2), 407-412 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

FEBRUARY 13 - 15: PrepTech '95, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. Brian Howard, ISC Technical Conferences, Inc., 30 Controls Drive, Shelton, CT 06484-0559, USA.

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

NOVEMBER 1 - 4: 31st Western Regional ACS Meeting, San Diego, California. Contact: T. Lobl, Tanabe Research Labs, 4450 Town Center Ct., San Diego, CA 92121, USA.

NOVEMBER 5 - 7: 30th Midwestern Regional Meeting, ACS, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr, Miami, OK 74354, USA.

NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

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

1996

FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

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

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

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

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

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

1997

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

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

1998

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

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

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

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

2000

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

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

2001

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

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