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DETERMINATION OF TETRAHYDROBIOPTERIN AND ITS ANALOGUES IN BIOLOGICAL SAMPLES BY MICROBORE LIQUID CHROMATOGRAPHY

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ABSTRACT

Tetrahydrobiopterin and seven of its analogues (neopterin, xanthopterin, biopterin, pterin, tetrahydropterin, 6-methyltetrahydropterin, and 6-methylpterin) were separated on a 1 x 150 mm C_{18} microbore column. These analytes were detected by dual-electrode amperometry and UV absorption. Both a conventional glassy carbon electrode and an interdigitated array microelectrode were used. Low fmol amounts of xanthopterin, tetrahydropterin, tetrahydrobiopterin and 6-methyl-tetrahydropterin could be determined by electrochemical detection in the oxidative mode, but pmol amounts of the other analogues were determined by electrochemical detection in the reductive mode and with UV detection.

Catecholamines and their metabolites do not interfere with the determination of tetrahydrobiopterin and its analogues in biological samples. The developed method was explored for the determination of tetrahydrobiopterin in samples of human urine and rat tissue (brain, liver, and kidney).

INTRODUCTION

The pterins are a family of nitrogen heterocyclics that are currently of interest in medicine and biology. Among them, tetrahydrobiopterin (BH_4) has been drawing the most attention because it is a key cofactor essential for phenylalanine hydroxylase which catalyzes the synthesis of neurotransmitters including dopamine, epinephrine, norepinephrine, and serotonin in the CNS.[1] BH_4 is also a cofactor for nitric oxide synthase which catalyzes the production of nitric oxide, a candidate neurotransmitter.[2] In the brain of patients with either Parkinson's[3] or Alzheimer's disease,[4] a decreased concentration of BH_4 is thought to be associated with the decreased rate of neurotransmitter synthesis. Altered excretion patterns of pterins have also been observed in patients with cancer.[5,6] Therefore, a simple, sensitive and rapid analytical method for the simultaneous determination of BH_4 and its analogues in biological samples is desirable.

Pterins in biological samples have been determined by liquid chromatography with electrochemical detection (LCEC) and/or fluorimetric detection.[7-9] These methods are satisfactory for many purposes. In this study, a microbore column was used to lower the detection limit and to make the measurement of BH_4 and its analogues in biological samples somewhat simpler than for previous methods. An interdigitated array (IDA) microelectrode based on carbon film technology was also explored for determination of the reduced pterins.

MATERIALS AND METHODS

Chemicals and Standards

(6R)-5,6,7,8-Tetrahydro-L-biopterin (BH_4), 3,4-dihydroxyphenylacetic acid (DOPAC), epinephrine (EP), norepinephrine (NEP), 5-hydroxy-indoleacetic acid (5-HIAA), homovanillic acid (HVA), dopamine (DA), and serotonin (5-HT) were obtained from Research Biochemicals International, Natick, MA. 5,6,7,8-Tetrahydropterin (THP), D(+)-neopterin (NEP), and (1'S,

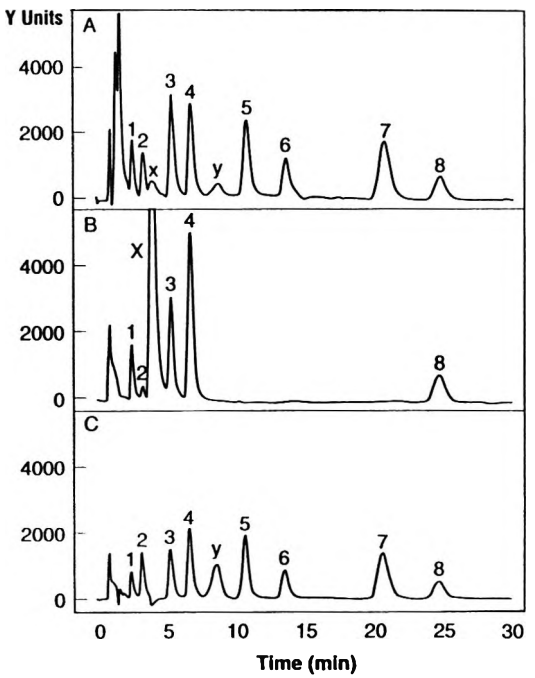

Figure 1. Chromatograms of BH_4 and its analogues using dual series glassy carbon electrodes for EC detection followed by UV detection. Microbore LC conditions: analytical column, UniJet C_{18}, 5 µm, 1 x 150 mm; mobile phase, 0.8% acetonitrile in 0.1 M phosphate buffer (pH 3.0), 4.5 mM sodium octylsulfonate, 54 µM disodium EDTA , which was maintained at 35°C; flow rate, 100 µL/min. The oven temperature containing the columns and detectors was maintained at 50°C (A) UV detection, 220 nm; (B) the downstream EC detection, applied potential of -0.7 V vs. Ag/AgCl; (C) the upstream EC detection, applied potential of +0.75 V vs. Ag/AgCl.
1 = NEP; 2 = XANP; 3 = BP; 4 = P; 5 = THP; 6 = BH_4; 7 = M-THP; 8 = M-P; X = oxygen; Y = system peak.

2'R)- biopterin (BP) were purchased from Fluka Chemical Corp., Ronkonkoma, NY, USA. Pterin (P), 6-methylpterin (M-P) and xanthopterin (XAP) were supplied by Sigma Chemical Co., St. Louis, MO, USA. DL-6-methyl-5,6,7,8-tetrahydropterin (M-THP) was obtained from Calbiochem-Novabiochem Corp., La Jolla, CA, USA. All other chemicals were of analytical-reagent grade and were used as received.

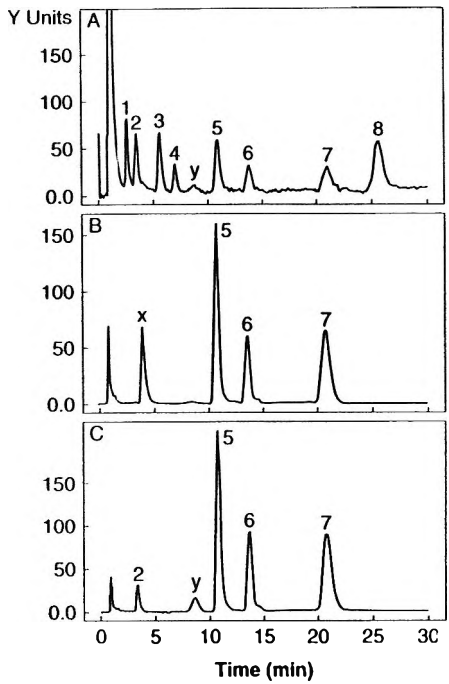

Figure 2. Chromatograms of BH_4 and its analogues using IDA dual electrodes for EC detection followed by UV detection.
Microbore LC conditions: column and mobile phase as in Figure 1.
(A) UV detection, 220 nm; (B) applied potential of -0.3 V vs. Ag/AgCl; (C) applied potential of +0.75 V vs. Ag/AgCl.
The peak assignments as in Figure 1.

Collection and Preparation of Human Urine Sample

Urine sample from a healthy volunteer was collected and immediately mixed with 10 N hydrochloric acid containing 1 mM disodium EDTA (10:1, v/v). The acidified urine sample was diluted to 1:5 (v/v) with distilled water and then filtered through a 0.2 μm membrane microfilter (MF-1 centrifugal microfilter, BAS, West Lafayette, IN, USA). An aliquot (5 μL) of the filtrate was subjected to LCEC analysis within 12 hours of storage at room temperature or within 2 weeks of storage in the dark at -20°C.

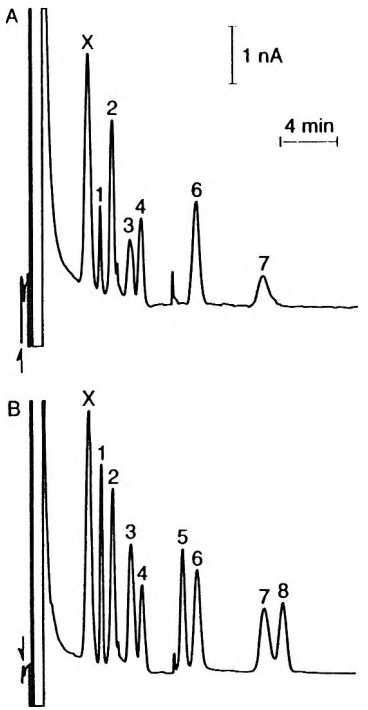

Figure 3. Chromatograms of catecholamines, their metabolites and reduced pterin derivatives. Microbore LC conditions as in Figure 1.
EC detection using dual parallel glassy carbon electrodes; (A) applied potential of +0.4 V vs. Ag/AgCl; (B) applied potential of +0.6 V vs. Ag/AgCl.
Peaks: 1 = DOPAC, 2 = THP, 3 = NE, 4 = BH_4, 5 = 5-HIAA, 6 = M-THP, 7 = EP, 8 = HVA, X = Decomposition peak from pterin standards.

Table 1

The Retention Factor (k) of Catecholamines, their Related Metabolites and BH_4 Derivatives

Cpd.:	DOPAC	THP	NEP	BH_4	5-HIAA	M-THP	EP	HVA	DA	5-HT
k value:*	5.7	6.7	8.2	9.2	12.4	13.8	19.4	21.1	60.0	>144

*The LCEC conditions as described for BH_4 and its analogues in the text.
t_0: 50 seconds.

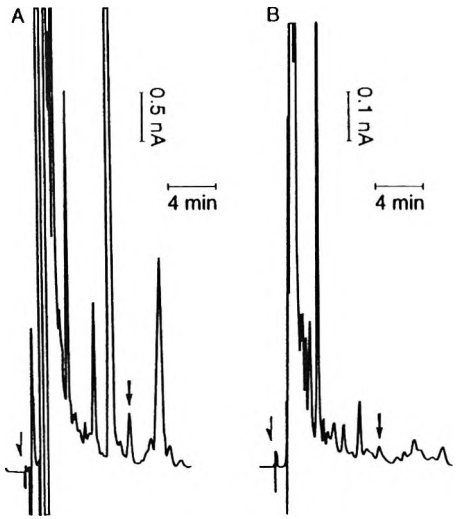

Figure 4. Chromatograms of BH_4 in urine of a healthy volunteer.
Microbore LC conditions as Figure 1.
(A) EC detection using a glassy carbon electrode, applied potential of +0.45 V vs. Ag/AgCl; (B) EC detection using an IDA electrode, applied potential of +0.4 V vs. Ag/AgCl. Arrow, BH_4

Preparation of Rat Tissue Samples

Rats were sacrificed by decapitation. The brain, liver, and kidney were quickly removed. Approximately 1 g each of the brain, liver and kidney were sliced and homogenized in 2 mL, 5 mL and 1 mL of 1 N hydrochloric acid containing 0.1 mM disodium EDTA, respectively. Following centrifugation at 1315 x g, each supernatant was filtered through a 0.2 μm membrane microfilter. A 5 μL aliquot of the filtrate was injected into the LCEC system. The samples were prepared and analyzed within 12 hours of storage at room temperature or within 2 weeks of storage in the dark at -20°C.

LCEC/LCUV

Determination of BH_4 and its analogues was performed on a BAS 200B chromatograph equipped with low dead volume micro-injector (5 μL loop, BAS), an EC detector using either a conventional glassy carbon electrode (3 mm) or a carbon IDA microelectrode (gap and width: 2 μm; cathode and

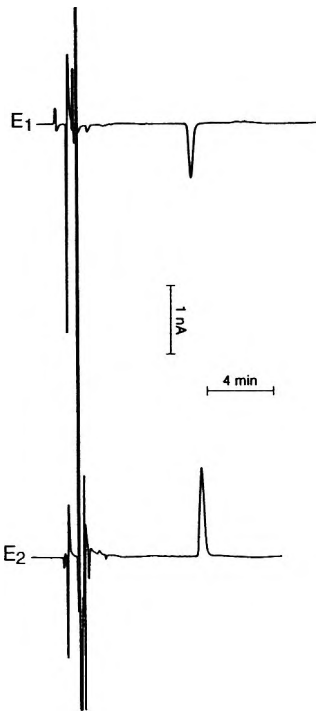

Figure 5. Chromatogram of standard BH_4.

Microbore LC conditions as Figure 1. EC detection using dual parallel glassy carbon electrodes; upper, applied potential of +0.45 V vs. Ag/AgCl; lower, applied potential of +0.6 V vs. Ag/AgCl.

anode: each 250 pairs; length: 2 mm; thickness of carbon film: 0.1 - 0.2 μ; NTT, Kanagawa, Japan) and a UV detector in series after the EC cell. The analytical column was a UniJet C_{18}, 5 μm, 1 x 150 mm (BAS); a UniJet C_{18} column, 5 μm, 1 x 100 mm (BAS) was installed before the injector to raise the overall system pressure for optimal pump performance. The oven temperature containing the columns and detectors was maintained at 50°C. The mobile phase was 0.8 % acetonitrile in 0.1 M monobasic sodium phosphate (pH 3.0), 4.5 mM sodium octylsulfonate, 54 μM disodium EDTA maintained at 35°C. The flow rate was 100 μL/min. EC detection using either dual glassy carbon or IDA electrodes (see reference 10 for the description of the IDA electrode) was at the indicated applied potentials vs. Ag/AgCl. UV detection in series after the EC cell was at 220 nm (1.2 μL flow cell with a 3 mm path length).

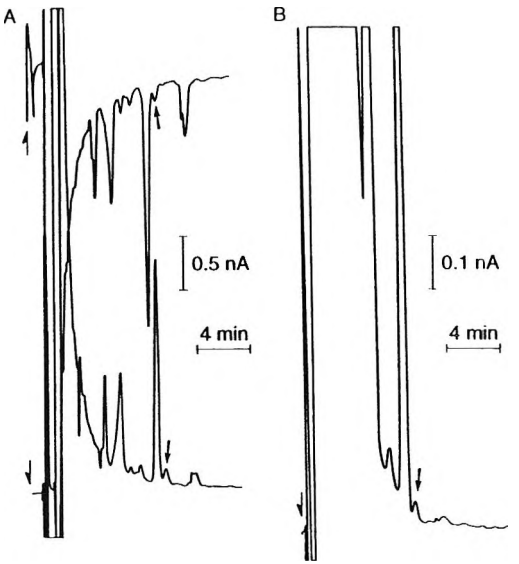

Figure 6. Chromatograms of BH_4 in rat brain sample.
Microbore LC conditions as in Figure 1.
(A) EC detection using dual parallel glassy carbon electrodes; upper, applied potential of +0.6 V vs. Ag/AgCl; lower, applied potential of +0.4 V vs. Ag/AgCl; (B) EC detection using an IDA electrode, applied potential of +0.4 V vs. Ag/AgCl. Arrow, BH_4.

RESULTS AND DISCUSSION

Initially, the separation of standards of BH_4 and its analogues was carried out on the microbore column. Various combinations of buffer solution, ion-pair and organic solvent were examined as a suitable mobile phase. The most satisfactory separation was obtained by using the mobile phase 0.8 % acetonitrile in 0.1 M monobasic sodium phosphate (pH 3.0), 4.5 mM sodium octylsulfonate, 54 μM disodium EDTA and maintaining the separation temperature at 50°C (Figure 1A). Both the oxidized and reduced forms of the pterins could be determined by EC detection corresponding to the reduction and oxidation process, respectively, at dual series glassy carbon electrodes (Figure 1B and 1C).

The IDA electrode, which was recently explored for LCEC via an joint research project by NTT, R&D Center, Kanagawa, Japan and BAS, USA,[10-13]

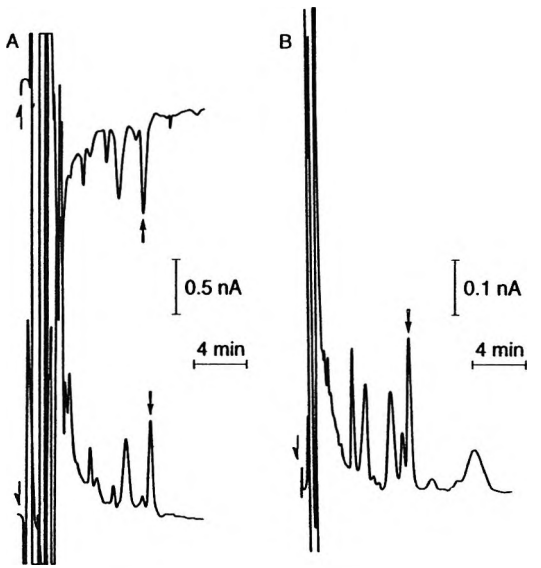

Figure 7. Chromatograms of BH_4 in rat liver sample.
Microbore LC conditions and EC conditions for A and B, respectively, as in Figure 6.

was also used for the detection of BH_4 and its analogues. BH_4 could not be detected by reductive mode downstream after it was oxidized upstream using the conventional dual series glassy carbon electrodes, but it could be detected when IDA electrode was used in the reductive mode (Figure 1B, 1C and Figure 2B, 2C).

In order to determine the pterins over a wide concentration range, we have used EC detection with dual series glassy carbon electrodes followed by UV detection. A linear response was observed for 0.1 - 100 pmoles on column for all 8 pterins using either oxidative or reductive EC detection and by UV absorbance, BH_4 exhibited a linear response from 32 fmoles on column only when using oxidative EC detection. The injected detection limit for BH_4 was 3.2 fmoles on column.

The stability of the pterins in various aqueous solutions was also examined. The reduced pterins are stable in either 1 N HCl or 1 N $HClO_4$ containing 0.1 mM disodium EDTA for 12 hours of storage at room temperature and for at least 2 weeks of storage in the dark at -20° C.

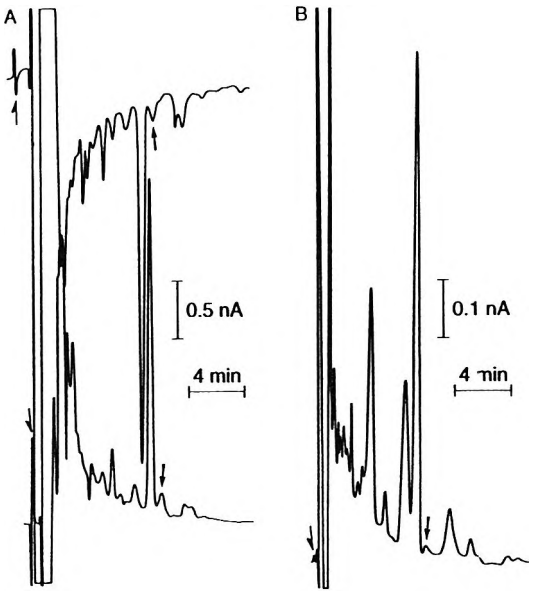

Figure 8. Chromatograms of BH_4 in rat kidney sample.
Microbore LC conditions and EC conditions for A and B, respectively, as in Figure 6.

Because the catecholamines and their metabolites are endogenous substances present *in vivo*, especially in brain, and can be detected by LCEC under the present conditions (oxidative), it is necessary to explore whether these compounds will interfere with the determination of BH_4 and its analogues. Mixtures of standard catecholamines and their metabolites were injected on this microbore LCEC system; the retention factor (k) of those compounds are shown in Table 1. All of the examined compounds possess a retention factor different from BH_4 and its analogues and can be baseline separated from them (Figure 3). The retention time of dopamine was longer than 50 minutes and serotonin was retained on column for more than 2 hours under the present conditions.

The developed method was then applied to the analysis of urine samples from a healthy volunteer. Collected urine was immediately acidified, diluted with distilled water and filtered using a centrifugal microfilter. An aliquot of the filtrate was directly injected on column. As shown in Figure 4, BH_4 in the urine sample is separated and detected without any interfering peaks. Previous studies have demonstrated that the effect of the different electrode potentials on electrochemical detection responses is characteristic of an analyte and therefore is an aid in its identification.[14-16] This approach was employed in this study for

the identification of BH_4 in the urine samples. When different applied potentials were used in a dual parallel mode for the EC detection of BH_4, different responses were obtained (Figure 5). The peak assignment of BH_4 in the urine samples was based on both its retention time and the response ratio. The identical EC detection responses of standard BH_4 and the corresponding peak in urine sample obtained by using the IDA electrode provided further support of this peak identity in human urine samples.

To further illustrate the potential utility of this method, rat brain, liver, and kidney were analyzed. After sacrifice, the tissue was sliced and homogenized in 1 N HCl containing 0.1 M disodium EDTA, centrifuged, and filtered. An aliquot of each filtrate was injected into the LCEC system without further clean-up. Typical chromatograms corresponding to the homogenized samples of rat brain, liver, and kidney are shown in Figure 6, 7, 8, respectively. Such a simple treatment of biological samples not only saves time but also decreases the risk of decomposition of the labile pterins and avoids interferences often introduced by more complicated sample preparation. Substances with longer retention times than BH_4 and its analogues (for example, dopamine and serotonin) in these biological samples made the analysis time longer but they can be easily switched out by using a column switching strategy. Normally they are too dilute to be a concern in most assays.

For the characterization of BH_4 in rat tissue, the same manner described for the identification of BH_4 in urine sample was used. Both the chromatographic behavior and the characteristic electrochemical responses obtained by using different electrode potentials compared well with those of standard BH_4.

CONCLUSION

An approach for the determination of BH_4 and its analogues using dual-electrode microbore LCEC provided excellent separation and favorable detection limits combined with a very simple treatment of biological samples.

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CHARACTERIZATION OF SULPHIDES BY GRAVITATIONAL FIELD-FLOW FRACTIONATION

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ABSTRACT

Various sulphides of the type $Cu_xZn_{(1-x)}S$ ($0<x<1$) were characterized in terms of particle size distribution by the gravitational field-flow fractionation technique. From the variation of the number average and weight average particle diameter with various parameters, such as solution pH and ionic strength, kind of detergent and time, in combination with microelectrophoretic measurements, useful conclusions about the stability and consequently the aggregation and deposition phenomena of these sulphides were deduced.

Comparison of the results obtained by gravitational field-flow fractionation with those found by laser particle size measurements confirmed the credibility of the method used.

INTRODUCTION

Chemical precipitation of heavy metals, which are of the most hazardous in the natural environment, is an effective process for the removal of these metals. This precipitation involves spontaneous precipitation of various salts, such as carbonates and sulphides.[1] The precipitation of heavy metals as sulphides, has the advantage of effectively removing heavy metals even at very low concentrations, owing to the low solubility of their sulphides.

Several stability studies have been performed on sulphides, stimulated by the needs of metal pollutant precipitation,[2] of mineral flotation,[3] and of sedimentation of sulphide phosphorus for cathode-ray tubes.[4] However the chemistry of the sulphide-electrolyte interface is much less known than the chemistry of the oxide-water interface. Hence there is a need not only to study the stability of the sulphides at various experimental conditions, but also to develop new methodologies for the characterization of metal sulphides.

One of the most important parameters for studying colloid's stability and deposition on solid surfaces, is the particle size distribution. In this work the relatively new technique of Gravitational Field-Flow Fractionation (GFFF) was used for the size determination of metal sulphide particles.

Field-flow fractionation (FFF) is a powerful, high-resolution separation method, which can be also used for the characterization of a wide variety of colloids, micelles, particulates and soluble macromolecules of biological interest.[5-9]

FFF is based on the action of an external field or gradient whose direction is perpendicular to the axis of flow in a thin channel. The field forces the sample species to accumulate in narrow zones so that each is intercepted by different flow laminae and thus displaced at different velocities down the flow channel. Different FFF methods are distinguished according to the nature of the external field used. In sedimentation FFF (SdFFF), a subtechnique of FFF, retention volume increases with particle diameter until steric effects dominate, at which transition point there is a foldback in elution order. The larger particles tend to move more quickly than the smaller ones in steric or gravitational FFF, and the separation mechanism is based more on inherent particle size than on the ability of a particle to undergo Brownian diffusion.[10] Gravitational FFF, which utilizes the Earth's gravitational field as an external field, has been applied to the rapid separation and/or characterization of various kinds of colloids, e.g. glass beads,[11] polystyrene latex beads[12] strengite and silica particles,[13,14] red blood cells,[15] etc.

In the present work GFFF is applied for the characterization of metal sulphide particles, as well as for studying the stability and consequently the aggregation and deposition phenomena of these sulphides.

EXPERIMENTAL

Materials

The samples used were polydisperse, irregular (as has been shown by TEM pictures and laser particle size measurements) colloidal particles of the mixed sulphides $Cu_xZn_{(1-x)}S$ (with x = 0.1, 0.2 and 0.5) provided by Dr. Dalas. FT-IR spectroscopy and X-ray diffraction methods gave the stoichiometry of the sulphides. The number average diameters of the $Cu_{0.1}Zn_{0.9}S$, $Cu_{0.2}Zn_{0.8}S$ and $Cu_{0.5}Zn_{0.5}S$ samples, as they were measured by a laser equipment, were found $(7.56 \pm 1.83)\mu m$, $(5.74 \pm 1.59)\mu m$ and $(6.02 \pm 1.91)\mu m$, respectively.

The suspending medium was triply distilled water containing various volumes of a low foaming, low alkalinity, phosphate, chromate and silicate-free detergent FL-70 (Fisher Scientific Co.) with a pH ranging between 9.0 and 10.5, and 0.02% by weight sodium azide (Fluka AG) as bacteriocide. The FL-70 solution is an uncharacterized mixture of ionic and non-ionic surfactants. For the study of the influence of the detergent nature on the sulphides' size two other surfactants were used: The non-ionic Triton X-100 (iso-octylphenoxypolyethoxyethanol) from BDH with a pH of 6.5 in 1% solution and the uncharacterized Decon-90 from Decon Laboratories Ltd with a pH of 10.1 in 0.05% solution.

For the pH adjustment various surfactants, as well as various concentrations of the same surfactant were added to the suspending medium, while for the variation of the solution ionic strength various amounts of the electrolyte $KClO_3$ were used.

Apparatus and Procedure

The dimensions of the GFFF system, which has been described in detail elsewhere,[9] were 50.3 cm x 2.0 cm x 0.028 cm. The channel void volume V_o (from the injection inlet to the flow cell), measured by the elution of the non retained sodium benzoate peak, was determined to be 2.80 cm^3.

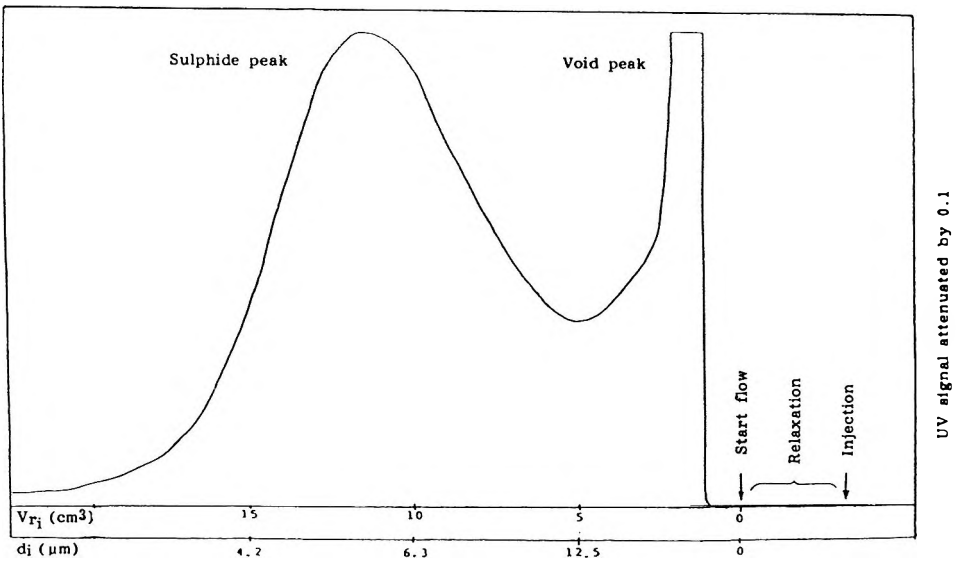

Figure 1. Fractogram and particle diameter scale of the sulphide $Cu_{0.2}Zn_{0.8}S$ obtained by the GFFF technique. Carrier solution: Detergent FL-70 (0.5% v/v)+0.02% (w/w) NaN_3 (pH = 9.4); sample = 25 μL; relaxation time = 10 min; flow rate = 40 cm^3h^{-1}.

The analysis was performed with a Gilson Minipuls 2 peristaltic pump coupled with a Gilson model 111 UV detector operated at 254 nm and a Goerz model RE 541 recorder. All experiments were performed at 25°C.

After 30 min of ultrasound stirring, 25 μL of the sample was injected with a syringe into the channel inlet. The relaxation time was 10 min, while the carrier flow rate varied between 35 and 43 cm^3h^{-1}. The obtained fractograms had the form of Fig. 1.

The electrophoretic mobilities of the sulfide particles were measured in a microelectrophoresis apparatus (Rank, Mark II) by using a four-electrode capillary cell.

The velocities of at least twenty particles in each direction of the electric field were measured at the two stationary layers with an accuracy of $\pm 10\%$. The pH of the colloidal suspensions was measured by using a combination glass-saturated calomel electrode (Metrohm).

For the identification of the particular sulphide prepared, a Phillips model PW 1130/00 X-ray diffractometer and a Perkin Elmer 16PC FT-IR spectrophotometer were used, while for the conversion of the measured in GFFF retention volume to particle size, the number average diameters of the three sulphides $Cu_{0.1}Zn_{0.9}S$, $Cu_{0.2}Zn_{0.8}S$ and $Cu_{0.5}Zn_{0.5}S$ were measured by the Spectrex ILI-1000 laser counter.

RESULTS AND DISCUSSION

Determination of Particle Size

The diameter of a spherical particle, d_i, in GFFF, assuming the absence of interactions between the sulphide particles, as well as between the sample particles and the glass column material, is given by the relation:[9]

$$d_i = \left(\frac{V_o}{V_{r_i}}\right).\frac{w}{3\gamma} = \frac{\lambda}{V_{r_i}} \tag{1}$$

where V_{ri} is the retention volume, w is the channel thickness, γ is a dimensionless factor which represents the complication of lift forces and related hydrodynamic effects,[11] and $\lambda = V_o w/3\gamma$ (cm^4).

For polydisperse samples, as it is the case here, the d_i expresses the Stokes diameter, from which the number average, d_N, and weight average, d_w, particle diameters can be determined by the known procedure described elsewhere:[13]

$$d_N = \frac{\sum_i d_i N_i}{\sum_i N_i} = \frac{\lambda\left[\sum_i N_i / V_{r_i}\right]}{\sum_i N_i} \tag{2}$$

$$d_w = \frac{\sum_i d_i S_i}{\sum_i S_i} = \frac{\lambda\left[\sum_i S_i / V_{r_i}\right]}{S} \tag{3}$$

where N_i is the number of particles with constant diameter d_i, S_i is the area under the elution curve of the N_i particles having constant diameter d_i, which is proportional to the weight of the N_i particles and S is the total area of the whole eluted particles.

The parameter λ is computed from Equation (2) using the mean value of d_N found from laser counter measurements for the three sulphides used, together with the values of V_n and N_i obtained from the fractogram. Thus with the aid of the GFFF technique the d_N and d_w values for the polydisperse sulphide samples $Cu_{0.1}Zn_{0.9}S$ (S1), $Cu_{0.2}Zn_{0.8}S$ (S2) and $Cu_{0.5}Zn_{0.5}S$ (S3) can be determined at various experimental conditions using Equations (2) and (3) with $\lambda_{S1} = 6.63 \times 10^{-3}$ cm^4, $\lambda_{S2} = 6.55 \times 10^{-3}$ cm^4 and $\lambda_{S3} = 8.43 \times 10^{-3}$ cm^4.

Variation of Particle Size with the Solution Ionic Strength

The net potential energy of interaction between the sulphide particles, as well as between the sulphide particles and the glass wall material depends on the effective Hamaker constant, the surface potential of the particles and the wall, and of the electrolyte concentration.[7] Variation of the electrolyte concentration, by adding to the suspending medium various amounts of the indifferent electrolyte $KClO_3$, resulted in a variation of the number average and the weight average particle diameter of the sulphide $Cu_{0.1}Zn_{0.9}S$ shown in Fig.2. Although it is well known that an increase in the solution ionic strength generally leads to an increase in particle diameter, our results show an inverse behaviour and the particle size decreases as the ionic strength increases, due to the fact that the interactions between the sulphide particles and the glass wall are stronger than those emerging between the sample particles, which could lead to particles' coagulation.

This is explained in terms of double-layer repulsion between the solute particles and the column material (glass). At low ionic strengths the thickness of the inaccessible region increases and the interactions between the colloidal particles and the column material are negligible. On the other hand, at high ionic strengths the particles are loosely adsorbed into a secondary minimum caused by the combined action of weak double-layer repulsion and strong van der Waals attraction. This explains why sulphide particles in a carrier of high ionic strength have a larger retention time than in a carrier of low ionic strength and consequently a smaller particle size, since, as Equation (1) shows, the particle diameter is inversely proportional to retention time.

Fig.2 shows that the obtained by GFFF particle size is close to that found by the laser counter at ionic strengths lower than 10^{-5} M, while at ionic strengths higher than 10^{-3}M the particle size is kept almost constant. The latter indicates that the attraction potential energy between the sulphide particles and the wall material increases with the electrolyte concentration in the range from

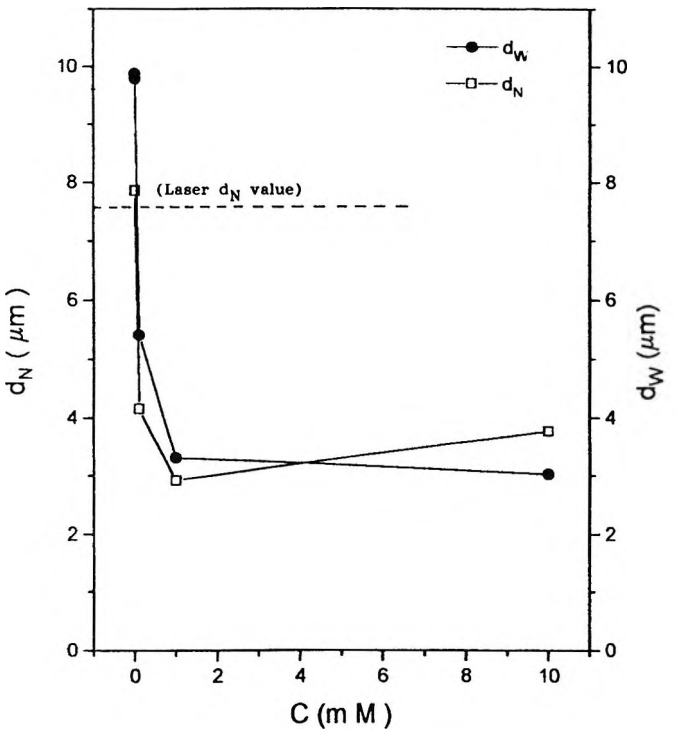

Figure 2. Variation of number and weight average particle diameter for the sulphide $Cu_{0.1}Zn_{0.9}S$ with the ionic strength of the carrier solution, which was adjusted by the electrolyte $KClO_3$. The rest conditions are the same as those in Fig.1.

10^{-5} M to 10^{-3} M, then it is being kept constant. A detailed study of this variation should lead to the determination of the attraction potential energy between the sulphide particles and the glass wall, and consequently between the sulphide particles and whichever solid surface, which is an object of high technological importance.

Variation of Particle Size with the Solution pH

The variation of the solution pH was succeeded either by changing the kind of surfactant (cf. Fig.3 for the sample $Cu_{0.5}Zn_{0.5}S$) or by changing the concentration of the detergent FL-70 (cf. Figs.4a and 4b for the samples $Cu_{0.1}Zn_{0.9}S$ and $Cu_{0.2}Zn_{0.8}S$, respectively). In the first case in which the variation of the pH suspension covers a relatively broad area (between 6.5 and 10.1), the number and weight average diameters of the $Cu_{0.5}Zn_{0.5}S$ particles

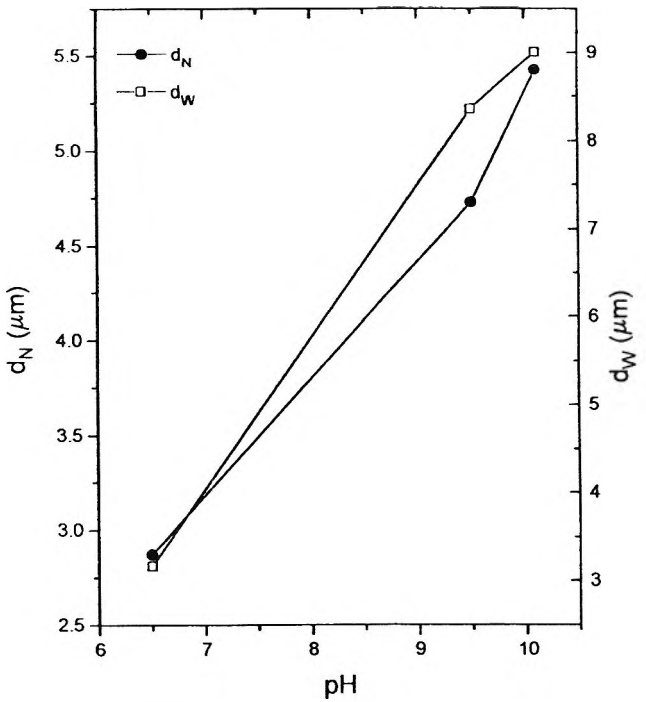

Figure 3. Variation of number average particle diameter for the sample $Cu_{0.5}Zn_{0.5}S$ with the pH solution, which was being changed by the nature of detergent.

increase with pH, although as Fig.5 shows, the particles' surface potential increases in the same pH range. The latter is attributed on the nature of the surfactant rather than on the pH solution.

Comparison of the d_N values determined by the GFFF technique, which are given in Table 1, with that found by laser counter measurements shows that the most suitable from the three used detergents are the alkaline Decon-90 and FL-70 minimizing the interactions between the sulphide particles, as well as those between the sulphide particles and the glass column material. But one must bear in mind that the conversion parameter λ was computed using carrier solution with the detergent FL-70, thus making the above comparison precarious. For this reason, we compare the polydispersity factors $\xi = d_w/d_N$ for the sample $Cu_{0.5}Zn_{0.5}S$ obtained by the GFFF technique using three different surfactants. Due to the fact that the sample is identical in all three surfactants the parameter ξ given in Table 1 is a measure also of the sample's degree of aggregation. Thus the aggregation rate increases from the Triton X-100 to

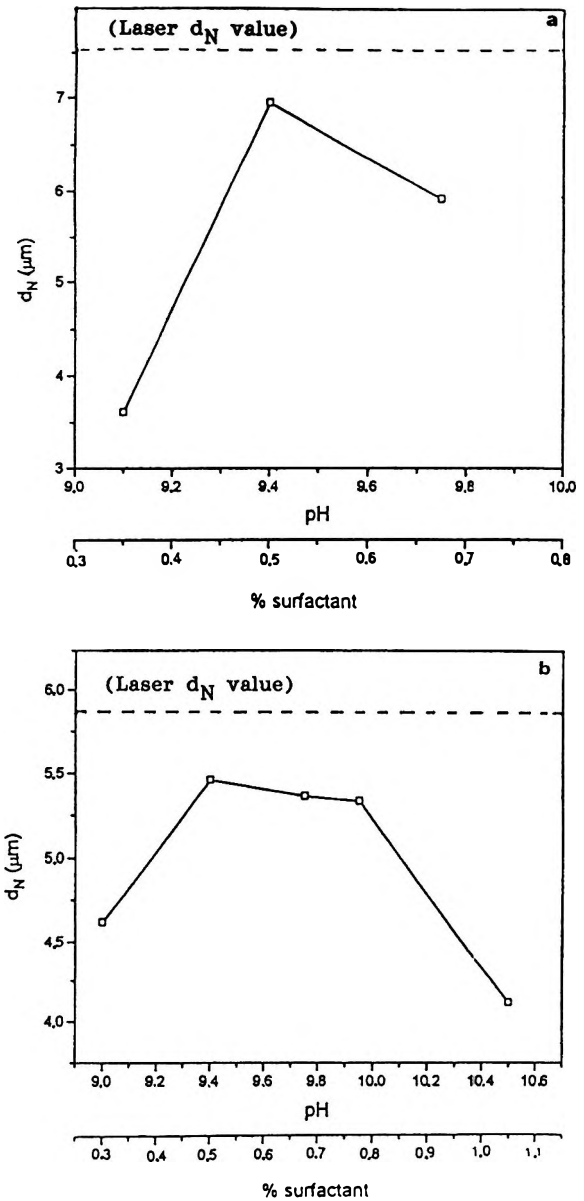

Figure 4. The dependence of number average particle diameter on the solution pH, which was being varied by the surfactant concentration. (a) Sample $Cu_{0.1}Zn_{0.9}S$; (b) Sample $Cu_{0.2}Zn_{0.8}S$.

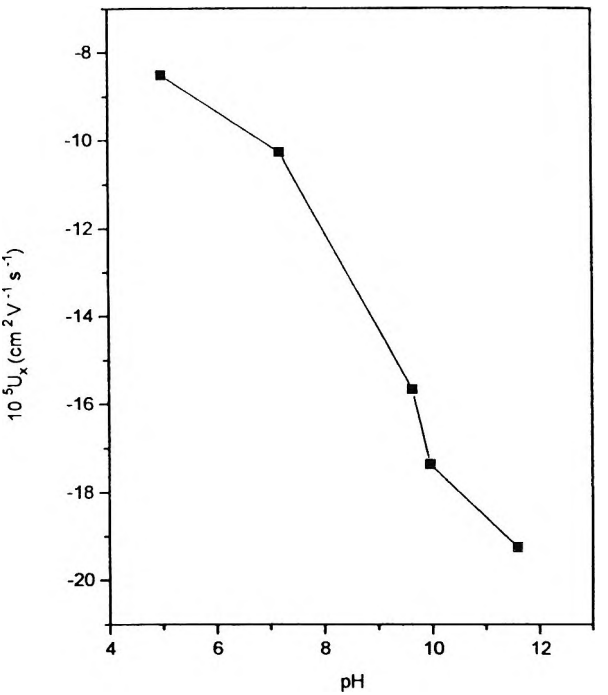

Figure 5. The dependence of the electrophoretic mobility, U_x, of the sample $Cu_{0.5}Zn_{0.5}S$ on the suspension pH.

Table 1

Number Average, d_N, and Weight Average, d_W, Particle Diameters for the $Cu_{0.5}Zn_{0.5}S$ Sulphide Sample Obtained by the GFFF Technique with Three Different Surfactants, as well as Corresponding Polydispersity Factors, ξ.

Detergent	pH	$d_N(\mu m)$	$d_W(\mu m)$	$\xi = d_W/d_N$
Triton X-100	6.5	2.81	3.30	1.17
FL-70	9.4	5.22	7.30	1.40
Decon-90	10.1	5.52	8.80	1.59
Laser Counter	---	6.02	---	---

FL-70 and then to Decon-90 indicating that the most suitable detergent minimizing the aggregation process for the sample $Cu_{0.5}Zn_{0.5}S$ is the non-ionic Triton X-100. The addition of the non-ionic Triton X-100 surfactant to the sulphide sample, whose particles carry a small negative charge, increased the stability of the dispersion to flocculation. The high stability of the sample is due to the adsorption of the non-ionic surfactant onto the sulphide particles.

In the second case, the variation of d_N with the solution pH in both samples appears a maximum at the pH value 9.4 corresponding to the detergent FL-70 concentration of about 0.5% v/v. The maximum d_N values for both samples are those approaching the d_N values found by laser counter measurements. The latter indicates that the ideal detergent concentration for minimizing the interactions between the sulphide particles and the glass column material and, consequently, that which is minimizing the deposition phenomena of the sulphide particles at the glass substrate, is that of about 0.5% by volume FL-70.

The decreased particle size at lower than 0.5% by volume concentrations of surfactant is attributed to the decrease of the surface potentials, as Figs.6a and 6b show, which leads to an increase in the attraction potential energy between the sulphide particles and the glass substrate. As a consequence an increase of the retention volume and a decrease of the particle diameter is observed.

The decreased particle size at higher than 0.5% by volume concentrations of surfactant, although the zeta potentials continued to become more negative, as Figs. 6a and 6b show, can be attributed either to the compression of the electrical double-layer by the increased surfactant concentration, which leads to an increase in the potential energy of attraction between the sulphide particles and the glass column or to the increase of the effective Hamaker constant A_{132}, which leads also to an increase of the attraction potential energy, U_A, between the particles and the wall.

The latter can be explained as follows: The surfactants, as it is well known, reduce the surface tension γ_{33} of the suspended medium, and consequently the Hamaker constant A_{33}, since:[16]

$$A_{33} \approx 2.1 \times 10^{-21} \gamma_{33} \qquad (4)$$

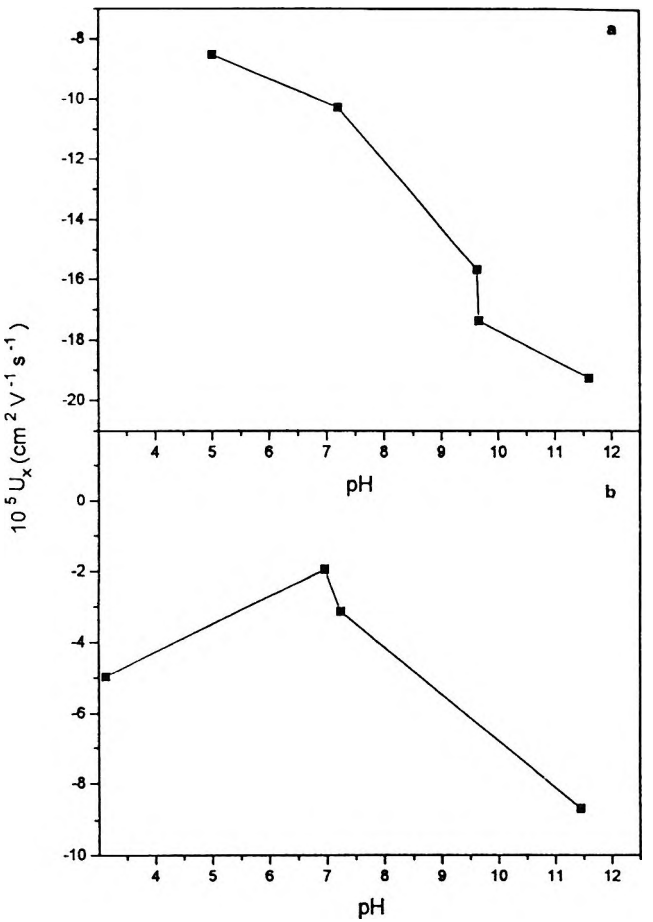

Figure 6. Variation of the electrophoretic mobility with the pH solution for the samples $Cu_{0.1}Zn_{0.9}S$ (a) and $Cu_{0.2}Zn_{0.8}S$ (b).

where γ_{33} is in $mJ.m^{-2}$ and A_{33} in J. The effective Hamaker constant, A_{132}, between the sulphide particles and the channel wall material in the presence of a medium is related with the Hamaker constants of the sample (A_{11}), the wall material (A_{22}) and the medium (A_{33}), via the equation:

$$A_{132} = \left(\sqrt{A_{11}} - \sqrt{A_{33}}\right)\left(\sqrt{A_{22}} - \sqrt{A_{33}}\right) \qquad (5)$$

Equation (5) shows that a decrease in the medium Hamaker constant A_{33} caused by an increase in the surfactant concentration leads to an increase in the effective Hamaker constant A_{132}-supposing that both A_{11} and A_{22} are independent of the surfactant concentration- and consequently to an increase in the attraction potential energy, since:[16]

$$U_A = -\frac{A_{132}}{12\pi} l^{-2} \qquad (6)$$

where l is the distance between the particles and the wall. In order to investigate whether the ionic strength or the effective Hamaker constant is the main factor affecting the interaction between the sulphide particles and the glass wall an experiment was performed with a carrier containing the surfactant FL-70 (0.5% v/v) and 10^{-3}M $KClO_3$ thereby varying its ionic strength (the A_{33} parameter is supposed to be kept constant in both carriers).

The obtained particle size is almost identical with that found with a suspending medium containing only the detergent FL-70 indicating that the variation of particle size with the surfactant concentration in that particular range should be attributed on the variation of the effective Hamaker constant rather than on the variation of the solution ionic strength.

It must be pointed out that the variation of the pH solution by adding different amounts of acids or bases, which presented in another work,[17] led (i) to the total adhesion of the $Cu_{0.1}Zn_{0.9}S$ particles at the glass channel wall at pH<7.5, (ii) to the partial adhesion of the same particles at the pH range between 7.5 and 9.0, and (iii) to the total elution of the suspended particles at pH higher than 9.0.

Variation of Particle Size with Time

The variation of number average and weight average diameter of the sulphide $Cu_{0.5}Zn_{0.5}S$ particles with time is shown in Fig.7. Contrary to the expected increase of the d_N and d_w values with time, due to possible aggregation, the particle diameters decrease with time, which means that the retention time increases with time. This should be attributed either to the increase of the interaction forces between the sulphide particles and the glass substrate, responsible for the slow movement of the particles along the column or to the "true" decrease of particle size with the agitation time. All the factors affecting the interaction forces between the particles and the wall are kept constant during the whole working time indicating that true particle's size

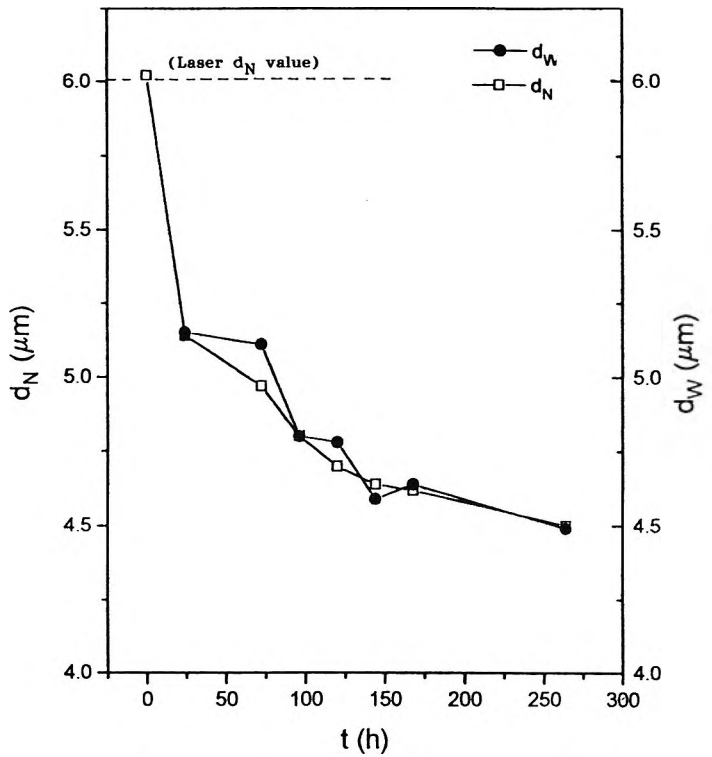

Figure 7. The dependence of number average particle diameter for the sample $Cu_{0.5}Zn_{0.5}S$ on the agitation time.

variation occurs with time. The stability of particle size at times greater than about 140 h (≈ 6 days) indicates that, before use, one should agitate these particular samples for a time interval of about six days. All of our experiments were performed with a constant agitation time of about 30 minfor all of the samples. thus making worthy all the presented comparisons.

CONCLUSION

From the data presented, it can be concluded that the ideal carrier solutions for minimizing coagulation and adhesion phenomena in studying sulphide samples by GFFF are those containing (i) between the surfactants FL-70, Decon-90 and Triton X-100 the non-ionic Triton X-100, (ii) between the concentrations of the FL-70 surfactant that of about 0.5% v/v, (iii) between the

concentrations of the indifferent electrolyte $KClO_3$ those which are lower than 10^{-5}M, and (iv) between the pH values of the suspended medium those which are higher than 9.0 and particularly that of 9.4 containing 0.5% v/v of the detergent FL-70.

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ENANTIOMERIC HPLC SEPARATION OF SELECTED CHIRAL DRUGS USING NATIVE AND DERIVATIZED β-CYCLODEXTRINS AS CHIRAL MOBILE PHASE ADDITIVES

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ABSTRACT

Native β-Cyclodextrin and three of its derivatives, hydroxypropyl, methyl and sulfated β-cyclodextrins were used as chiral mobile phase additives to investigate the enantiomeric separation of selected drugs by reversed phase HPLC. The chiral drugs investigated were oxazepam, temazepam, lorazepam, ketoprofen, fenoprofen, ibuprofen, chlorthalidone, terbutaline, trimeprazine and trimipramine. Different types of reversed phase columns were investigated including propylsilane, hexylsilane, octylsilane, octadecylsilane columns and a new nonporous octadecylsilane column. The new nonporous column gave separations for most of the drugs including short retention times. Terbutaline was not separated on the nonporous column, but did separate on hexylsilane and octylsilane columns. The effect of various mobile phases, organic modifier, type and concentration of the added cyclodextrin on peak shape, resolution and retention factors of the enantiomers were investigated.

INTRODUCTION

When a chiral selector is introduced into a mobile phase used with an achiral column, it offers advantages of flexibility, a wide range of possible additives, and often lower cost compared with an equivalent chiral stationary phase, in addition to avoiding the disadvantages associated with indirect chiral methods. Cyclodextrins (CDs) are widely used as chiral mobile phase additives.[1-6] They are cyclic, non-reducing oligosaccharides consisting of D-glucose units bonded through alpha- 1,4-linkages. According to the number of glucose units forming the cyclodextrin ring (six, seven or eight), one differentiates between alpha, beta and gamma cyclodextrin. The most commonly used CD is the β-CD. The outer surface of the CD molecule is hydrophilic due to the presence of primary and secondary hydroxyl groups, whereas the inner cavity is hydrophobic.[7]

When CDs are used as chiral mobile phase additives in reversed phase liquid chromatography, the separation mechanism is thought to be the result of formation of inclusion complexes in which the solute is included in the cavity of the CD. For enantiomers, chiral recognition occurs because of the selective complexation of the enantiomers with the CDs. Among the factors that control the enantioseparation process are, (i) differences in the stability/binding constants of the CD complexes, (ii) differences in the adsorption of CD complexes on the surface of the stationary phase and (iii) differences in the adsorption of free solute molecules on the CD layer that is adsorbed on the surface.[8] Because the enantioselective complexation is enhanced with stabilized inclusion of the hydrophobic part of the solute in the cavity of the CDs, the cavity size of CD is of great importance in inclusion complex formation. Only those guest molecules which can be fitted into the chiral cavity of the CD, resulting in intimate contact with the inner surface, can form stable inclusion complexes. If the size of the molecule is too small or too large, either a weak or no interaction is formed leading to little or no separation.

Furthermore, to obtain a chiral separation with CD, it is necessary for different interactions to take place between the enantiomers and the CD. These interactions include, dipole-dipole interactions, inductive, hydrogen bonding and hydrophobic (Van der Waals) interactions. If at least one of these interactions is stereochemically dependent, chiral separation of an enantiomeric solute is possible.[9]

Problems associated with the use of CDs are due to their poor solubility in aqueous organic solvents. The addition of urea to the mobile phase will enhance solubility, but it sometimes leads to problems with baseline stability

and a higher viscosity of the mobile phase.[10] An alternate way is to chemically modify one of the secondary hydroxyl groups of the CD. Chemical modification changes both the chiral selectivities and physical properties of the CD such as the strength and the nature of the polar intermolecular interactions between the host and the guest and the solubility of the materials.[11]

The differences in inclusion complex strengths between solutes and the CD cavity, as well as differences in the interaction with the rim functional groups can result in improved chromatographic separations. For chemically modified CDs, the hydroxyl groups on the rim of the cavity are replaced with either methyl, hydroxyethyl, hydroxypropyl, sulfate and acetyl groups to increase the hydrophobic character of the CD cavity relative to the hydrophilic exterior. These differences will change the inclusive complex strength which can lead to greater selectivity.[12]

In this study, the chiral chromatographic behavior of propylsilane, hexylsilane, octylsilane, octadecylsilane columns and a new nonporous octadecylsilane column were investigated with native and derivatized βCDs added to the mobile phase. The derivatized CDs were hydroxypropyl-β-CD, methyl-β-CD and sulfated-β-CDs. The chiral drugs investigated were chlorthalidone, terbutaline, oxazepam, lorazepam, temazepam, ketoprofen, ibuprofen, fenoprofen, trimeprazine and trimipramine. The most attractive features of the nonporous column are the use of low quantities of organic modifier in the mobile phase and very fast retention times.

EXPERIMENTAL

Materials

β-Cyclodextrin (β-CD) was purchased from TCI (Portland, Oregon, USA), hydroxypropyl β-Cyclodextrin (HP-β-CD-degree of substitution 4.0), methyl β-Cyclodextrin (M-β-CD-degree of substitution 12.7) and sulfated β-Cyclodextrins (S-β-CD-degree of substitution 14) were kindly supplied by American Maize Company (Hammond, IN, USA). Terbutaline hemisulfate, lorazepam, temazepam, ketoprofen, ibuprofen, trimeprazine hemi-(+)-tartrate and trimipramine maleate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chlorthalidone and fenoprofen reference standards were obtained from USP (Rockville, MD, USA). Acetonitrile and absolute methanol were purchased from J. T. Baker (Phillipburg, NJ, USA).

Triethylamine (TEA) was obtained from Fisher Scientific Co. (Orangeburg, NY USA) and trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Co (Milwaukee, WI, USA). All solvents were HPLC grade and mobile phases were filtered through a 0.45μm filter (Alltech Associates, Deerfield, IL. USA).

Instrumentation

Chromatography was performed on an HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, San Ramon, CA, USA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 10μL loop and a Waters Millipore Model 481 LC spectrophotometer (Milford, MA 01757, USA). A Spectra-Physics Model 4270 integrator (Spectra-Physics, San Jose, CA, USA) was used to record each chromatogram and peak area responses.

The following HPLC columns were purchased: Zorbax 300 SB-C3 (Mac-Mod Analytical Inc., Chadds Ford, PA, USA), Spherisorb 5C6 (150x4.6mm), Spherisorb S5C8 (150x4.6mm), Spherisorb S50DS1 (250x4.6mm, Phenomenex, Torrance, CA, USA) and a Micra Nonporous reversed phase (RP) C18 1.5μ HPLC column (33x4.6mm, Micra Scientific, Northbrook, IL, USA).

Chromatographic Conditions

All separations were performed at ambient temperature (23°C) with UV detection set at the UV maximum for each analyte. The maxima were 240nm for oxazepam, lorazepam and temazepam, 265nm for ketoprofen, fenoprofen and ibuprofen, 275nm for terbutaline and chlorthalidone and 250nm for trimipramine and trimeprazine.

The mobile phases consisted of 5 to 20mM concentrations of native or derivatized CDs dissolved in 0-10% v/v acetonitrile and 90-100% v/v of 0.1% aqueous trifluoroacetic acid (pH adjusted to 4.0 with triethylamine). The flow rate was 0.8mL/min. for the nonporous column and 1.0mVmin. for the other columns. Stock solutions of 1μg/mL of each individual analyte were prepared in the respective mobile phases.

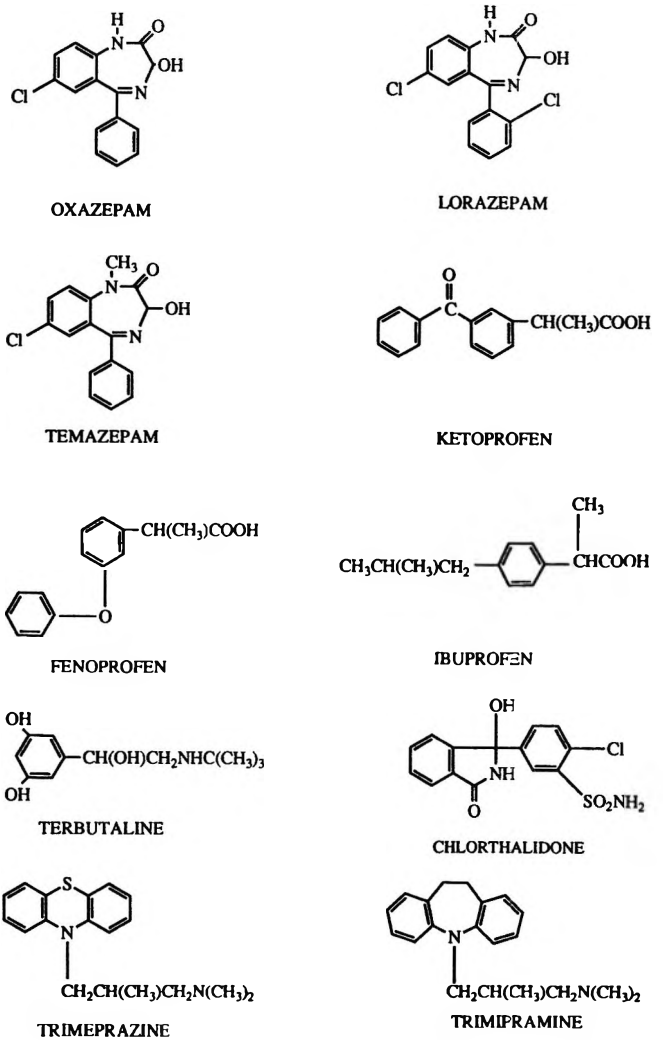

Figure 1. Chemical structures of analytes studied.

RESULTS AND DISCUSSION

The chemical structures of the analytes investigated are shown in Fig.1. Preliminary results in this laboratory showed that only chlorthalidone and terbutaline enantiomers were resolved on the hexylsilane and octylsilane columns with either β-CD or HP-β-CD added to the mobile phase. Mobile

phase conditions for chlorthalidone were 10mM β-CD or HP-β-CD dissolved in water:methanol (9: 1 v'v) pH4.0. The terbutaline enantiomers did not require an organic modifier for resolution and there was a complete loss of resolution upon the addition of any organic modifier to the mobile phase. The use of a 50mM ammonium acetate buffer gave sharper peaks for both chlorthalidone and terbutaline enantiomers on the hexylsilane and octylsilane columns. None of the analyses investigated in this study including chlorthalidone and terbutaline gave separations on the classical octadecylsilane or the stable bond propylsilane columns with β-CD, HP-β-CD, M-β-CD or S-β-CD added to the mobile phase.

The recently introduced nonporous reversed phase octadecylsilane column which had shown initial success for separation of chlorthalidone enantiomers was studied. Separations were achieved for oxazepam, temazepam, lorazepam, fenoprofen, ketoprofen, trimipramine, trimeprazine and chlorthalidone. Terbutaline and ibuprofen enantiomers could not be separated. Only trimipramine and trimeprazine were separated with native β-CD and ketoprofen and fenoprofen separated only with HP-β-CD added to the mobile phase. Oxazepam, temazopam and lorazepam separated with either HP-β-CD or M-β-CD and chlorthalidone was the only analyte where the enantiomers were separated with either β-CD or HP-β-CD. S-β-CD failed to resolve any of the analytes. In cases where enantioseparation was achieved, the mobile phase consisted of CDs added to solutions of 0.1% aqueous trifluoroacetic acid and acetonitrile and the presence of buffers in the mobile phase did not improve the separations.

Based on the initial success of the nonporous column, the chromatographic behavior of this column was further investigated. Tables 1 and 2 list the various mobile phase compositions and the retention factors of the analytes. In almost all cases, the resolution between enantiomeric pairs was greater than 1. In addition to the nonporous nature of the stationary phase, other unique features of the column are the requirement of a low concentration of organic modifier and the short retention times for analysis as shown in Figs 2 and 3.

Effect Of CD Type on Enantiomeric Separation

Chemical modification of the CD has been shown to 'stretch' the cavity mouth and therefore change the hydrophobicity of the molecules and the stereoselectivity of the inclusion process. The mouth of the CD hydrophobic cavity is surrounded by secondary hydroxyl groups which are locked into

Table 1

Resolution (Rs) of Lorazepam, Temazepam, Oxazepam, and Chlorthalidone Enantiomers on Nonporous Octadecylsilane Column

Analyte	Mobile Phase Composition (v/v)[a]		Retention Factors		
	A	B	k_1	k_2	Rs
Lorazepam	98	2	6.04	7.00	1.05
	96	4	6.60	7.56	1.05
	94	6	6.25	7.37	1.02
	92	8	6.54	7.33	1.00
	90	10	6.46	7.13	0.90
Temazepam	98	2	8.58	10.85	1.34
	96	4	8.25	10.10	1.25
	94	6	7.23	8.54	1.14
	92	8	7.42	8.50	1.10
	90	10	7.37	8.22	1.00
Oxazepam	98	2	3.79	5.60	1.97
	96	4	4.66	6.51	1.90
	94	6	4.19	6.00	1.74
	92	8	4.60	6.16	1.74
	90	10	4.11	5.50	1.65
Chlorthalidone	98	2	4.27	5.79	1.78
	96	4	--[b]	--	--
	94	6	--	--	--
	92	8	--	--	--
	90	10	--	--	--

[a] aqueous 0.1% TFA pH 4.0 (adjusted with TEA) - acetonitrile containing 15mM HP-β-CD. A = aqueous 0.1% TFA pH 4.0 (adjusted with TEA). B = Acetonitrile. [b] No resolution was achieved.

position and are considered to be important in chiral recognition.[13] In a derivatized CD, some hydroxyl groups are substituted with various functional groups, such as hydroxypropyl, methyl, sulfate and acetyl. The overall hydrophobic character of the CD will depend on the type of functional

Table 2

Resolution (Rs) of Trimeprazine, Timipramine, Ketoprofen, and Fenoprofen Enantiomers on Nonporous Octadecylsilane Column

Analyte	Mobile Phase Composition (v/v)[a]		Retention Factors		
	A	B	k_1	k_2	Rs
Trimeprazine	98	2	8.14	10.60	1.25
	96	4	7.90	10.44	1.10
	94	6	7.74	9.96	1.00
	92	8	--[b]	--	--
	90	10	--	--	--
Trimipramine	98	2	3.43	4.42	1.33
	96	4	4.53	7.26	1.10
	94	6	3.52	4.42	1.03
	92	8	--	--	--
	90	10	--	--	--
Ketoprofen	98	2	4.10	5.21	1.25
	96	4	4.15	5.06	1.00
	94	6	--	--	--
	92	8	--	--	--
	90	10	--	--	--
Fenoprofen	98	2	6.41	7.48	1.15
	96	4	6.19	7.19	1.00
	94	6	--	--	--
	92	8	--	--	--
	90	10	--	--	--

[a] aqueous 0.1% TFA pH 4.0 (adjusted with TEA) - acetonitrile containing 10mMβ-CD for trimiprazine and trimipramine and 15mM HP-β-CD for ketroprofen and fenoprofen. A = aqueous 0.1% TFA pH 4.00 (adjusted with TEA). B = acetonitrile. [b] No resolution was achieved.

groups present. In addition to the inclusion complex formed with a derivatized CD, modification of the CD rim is also critical for the type of interaction between the functional groups on the rim and the portion of the analyte outside the cavity that must take place for chiral recognition to occur. Included are

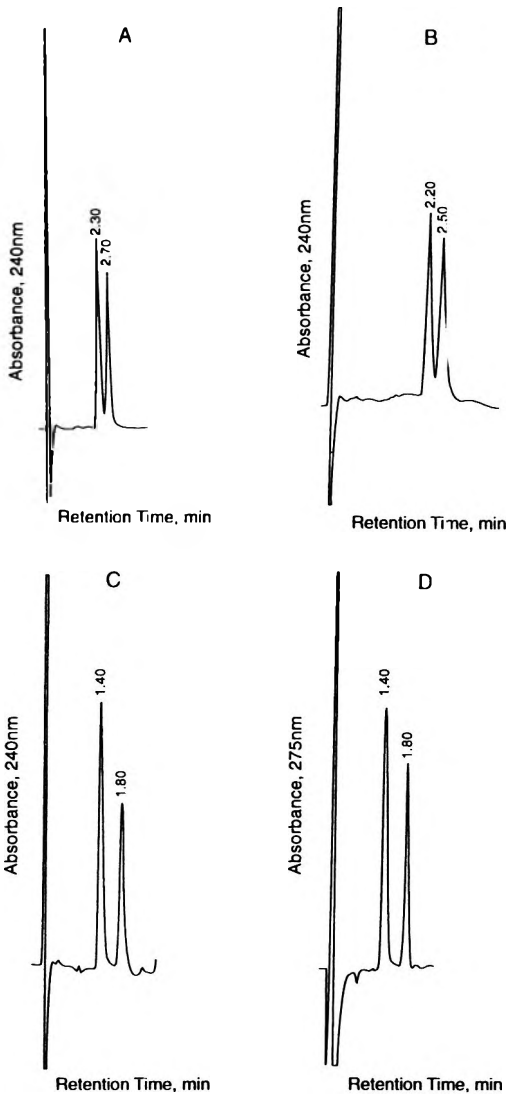

Figure 2. Enantiomeric separations of (A) lorazepam, (B) temazepam, (C) oxazepam and (D) chlorthalidone on the nonporous octadecylsilane column. Mobile phase consisted of 98:2 v/v aqueous 0.1% TFA pH 4.0 (adjusted with TEA) - acetonitrile containing 15mM HP-β-CD at a flow rate of 0.8mL/min.

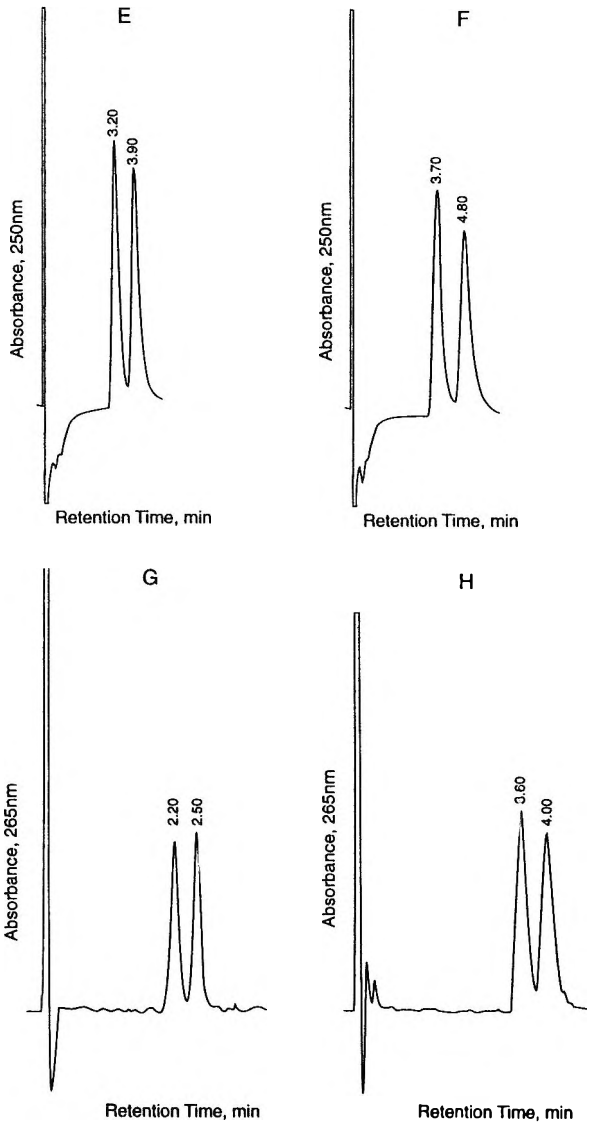

Figure 3. Enantiomeric separations of (E) trimeprazine, (F) trimipramine, (G) ketoprofen and (H) fenoprofen on nonporous octadecylsilane column. Mobile phase consisted of 98:2 v/v aqueous 0.1% TFA pH 4.0 (adjusted with TEA) - acetonitrile containing 10mM β-CD (for E and F) and 15mM HP-β-CD (for G and H) at a flow rate of 0.8mL/min.

hydrophobic (Van der Waals), hydrogen bonding and dipole-dipole interactions.[14] In contrast to the secondary hydroxyl groups which are locked into position on the native CD, the hydroxyl moiety of the hydroxypropyl group is free to rotate. This flexibility may allow for a closer approach between the hydroxyl groups and any hydrogen bonding moiety present in the solute leading to stronger or more stereospecific interactions than are possible with the native CD.

In the case of M-β-CD, Van der Waals interactions between the methyl groups on the CD rim and the hydrophobic groups on the solute chiral center can also provide enantioselectivity.[14]

Oxazepam, temazepam and lorazepam enantiomers were resolved with HP-β-CD but not with β-CD. The resolution occured because, in addition to the aromatic ring structures for inclusion in the cavity of the CD, all three analytes have a hydroxyl group at the chiral center which is available for specific hydrogen bonding interactions with the rim hydroxypropyl groups of HP-β-CD.

The same phenomenon explains the results we obtained for fenoprofen and ketoprofen. In addition to the two aromatic rings which form inclusion complexes with CD, both have carboxylic acid groups at the chiral center taking part in additional interactions with the functional groups on the rims of the derivatized CDs. Ibuprofen has only one aromatic ring compared to the other profens and is only partially resolved because of the small size of the molecule and hence its inability to form a strong or tight inclusion complex with the CD cavity.

It is interesting to note that trimipramine and trimeprazine enantiomers which failed to separate with HP-β-CD do not have any hydrogen bonding functional groups at or near the chiral center to interact with the hydroxypropyl or methyl groups on the rim of the CD.

The bulky nature of both analyses form strong inclusion complexes with native β-CD and the side chains interact favorably with the CD rim. Chlorthalidone was the only analyte to separate with either β-CD or HP-β-CD. This is because the bulky groups of chlorthalidone form an inclusion complex with β-CD and also the carbonyl and hydroxyl functional groups are available for hydrogen bonding interactions with the rim hydroxypropyl groups of the HP-β-CD.

Effect of Organic Modifier Concentration

One of the unique characteristics of the nonporous reversed phase column was the relatively low organic modifier concentration needed for chromatographic analysis. Compared to classic reversed phase columns, only about one-third of the organic modifier concentration used for a typical analysis is required. This is even more important in chiral mobile phase additive separations because the addition of organic modifier to the mobile phase is known to greatly decrease the solubility of CDs.[7] When CDs form complexes with analytes, it is assumed that the hydrophobic portion of the analyte sits inside the hydrophobic cavity of the CD and therefore the addition of organic modifier reduces the affinity of the analyte for the CD. The organic solvent competes with the solute for the preferred locations in the hydrophobic cavity resulting in various degrees of interactions of the compounds with the CD. Increasing the organic content of the mobile phase will weaken the strength of the inclusion complex formed between the analyte and the CD.[15]

The retention profiles of the analytes studied followed the general model where the capacity factor decreases with increasing organic modifier concentration, as in a classic reversed phase system consisting of an octadecylsilane stationary phase and a buffered aqueous-organic eluent.[16] In general, resolution decreased with increasing acetonitrile concentration for all the analyses in this study though the effect was less pronounced for temazepam, lorazepam and oxazepam.

For fenoprofen, ketoprofen, trimeprazine and trimipramine, enantioselectivity was lost when the acetonitrile concentration in the mobile phase was more than 6%. For chlorthalidone, enantioselectivity was lost when the acetonitrile concentration was only 2%, indicating an interference of the organic modifier with complexation of the analytes and CDs. Other organic modifiers such as methanol and ethanol were also investigated, but acetonitrile gave the best results.

Effect of Native β-CD and HP-β-CD Concentration

Table 3 lists the effect of β-CD or HP-β-CD concentrations in the mobile phase on the enantiomeric separation of the analytes. Enantioselectivity generally improved with increasing β-CD or HP-β-CD concentration in the mobile phase. It is thought that each enantiomer forms an inclusion complex upon the addition of at least 5mM of CD to the mobile phase and the two inclusion complexes have different capacity factors.

Table 3

Effect of CD Concentration on Resolution of Lorazepam, Temazepam, Oxazepam, Chlorthalidone, Trimeprazine, Trimipramine, Ketoprofen and Fenoprofen Enantiomers on Nonporous Octadecylsilane Column

Analyte	CD Concentration (mM)							
	5		10		15		20	
	HP-β-CD	β-CD	HP-β-CD	β-CD	HP-β-CD	β-CD	HP-β-CD	β-CD
Lorazepam	0.78	--[a]	1.05	--	1.05	--	1.00	--
Temazepam	1.00	--	1.08	--	1.89	--	1.22	--
Oxazepam	1.00	--	1.30	--	1.97	--	1.80	--
Chlorthalidone	1.10	1.00	1.35	1.85	2.50	1.20	1.72	0.90
Trimeprazine	--	0.84	--	1.25	--	1.10	--	0.93
Trimipramine	--	0.95	--	1.34	--	1.24	--	1.12
Ketoprofen	0.83	--	1.13	--	1.30	--	1.10	--
Fenoprofen	0.74	--	0.90	--	1.20	--	1.05	--

Mobile phase consisted of 98:2v/v aqueous 0.1% TFA pH 4.0 (adjusted with TEA) - acetonitrile containing the appropriate concentration of CD at a flow rate of 0.8 mL/min.
[a] No resolution was achieved.

When the concentration of CD falls below 5mM, the formation of the inclusion complex is incomplete and there is either partial or no resolution of the enantiomers.[17] Consequently, the concentration of CD is very important for resolution.

Increasing the β-CD or HP-β-CD concentrations in the mobile phase resulted in an increase in resolution of the enantiomers indicating the formation of relatively strong inclusion complexes. However, the increase in resolution reached a plateau region for all the analyses after which there was decreased resolution when the CD concentration was increased. This may be due to the increased bulk of the hydroxypropyl groups which would be expected to lead to a reduction of interactions with the secondary hydroxyl groups on the rim of the CD and result in reduction of enantiomeric resolution.

In conclusion, both the hexylsilane and octylsilane columns were successful in the enantiomeric separations of chlorthalidone and terbutaline among the analytes studied. The nonporous reversed phase column was shown to be applicable to enantiomeric separations of a group of diverse chiral drugs. In addition to short retention times, the use of low organic modifier concentration in the mobile phase offers a wide range of chromatographic applications for the column. In general, hydroxypropyl-β-CD as a chiral

mobile phase additive offers a wide variety of advantages over β-CD in terms of the ease of formation of inclusion complexes and the additional number of interactions possible with various functional groups present in drug molecules.

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STUDY OF RETENTION BEHAVIOR OF PESTICIDES FOR REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATION BY QUANTITATIVE STRUCTURE-RETENTION RELATIONSHIPS

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ABSTRACT

Regression models that are useful for the explanation of the reversed phase liquid chromatographic retention behavior of the pesticides ; triazines, phenoxyacetic esters and acetanilides were studied by quantitative structure - retention relationships (QSRRs). Descriptors were theoretically calculated by MM+ and AM1 method. The retention of triazines and phenoxyacetic esters in methanol-water was explained by solvation energy of specific site of solute, total solvation energy and polarizability which were chosen as nonspecific descriptors for acetonitrile-water. The retention of acetanilides was dependent not only on solvent-accessible surface area but on conformation of solute.

INTRODUCTION

The increasing use of HPLC for pesticide analysis is the result of its suitability for determining thermally labile and polar pesticides and its compatibility with on-line analysis.[1] There are many chromatographic methods for the determination of pesticide residues in crops, food and environmental samples.[2]

In order to develop the new chromatographic methods suitable for pesticide analysis, it is important to understand the retention phenomena of solutes.

Therefore, as the first step for elucidating the retention phenomena, we investigated the retention behavior of test solutes such as triazines, phenoxyacetic esters and acetanilides using quantitative structure - retention relationships (QSRRs).

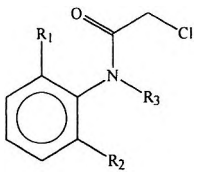
Quantitative structure-retention relationships (QSRRs) are well known methodologies of relating chemical structures of solute with the chromatographic retention parameters.[3] Through the statistical methods, retention data are characterized by various combinations of solute descriptors. QSRRs models obtained can be used to identify the most informative structural descriptors, describing the retention behavior of solutes and predicting the retention of solutes in a given chromatographic system.

When retention data are dependent on the molecular structure, QSRRs studies are suitable for testing new structurally related descriptors and they may be useful to discern new method for representing chemical structures.

Although there are many available descriptors commonly used in QSRRs studies, non-specific descriptors, shape descriptors, physicochemical descriptors, topological descriptors, and intermolecular descriptors[3,4] it has been difficult to describe the structural effect of solute on retention due to the lack of physical meaning of descriptors. But molecular mechanics and quantum chemical methods enables us to easily determine the structurally related descriptors.

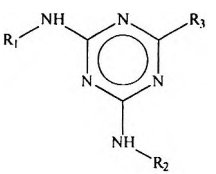
We have tried to find the structurally related descriptors that could be available to explain the retention behavior of the solutes by applying molecular mechanics and quantum chemical calculations to this QSRRs study.

Acetanilides



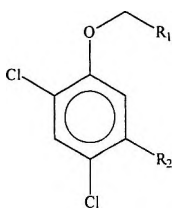
No.	1	2	3
R_1	C_2H_5	CH_3	H
R_2	C_2H_5	C_2H_5	H
R_3	CH_2OCH_3	$CHMeCH_2OCH_3$	i-C_3H_5

Triazines



No.	1	2	3	4	5
R_1	C_2H_5	i-C_3H_7	i-C_3H_7	i-C_3H_7	i-C_3H_7
R_2	C_2H_5	C_2H_5	i-C_3H_7	i-C_3H_7	i-C_3H_7
R_3	Cl	Cl	OCH_3	Cl	SCH_3

Phenoxyacetic esters



No.	1	2	3	4	5
R_1	COMe	COMe	$(CH_2)_2COMe$	$CO_2CMe_2C_5H_{11}$	$CO_2CMe_2C_5H_{11}$
R_2	H	Cl	H	H	Cl

Figure 1. Structures of solutes (continued)

Figure 1. Structures of solutes.

Table 1

Capacity Factors (log k') of Triazines, Phenoxyacetic Esters, and Acetanilides with MeOH-H_2O and MeCN-H_2O Eluents

Solute	No.	75%MeOH-H_2O	65% MeCN-H_2O
Triazine	1	-0.180	-0.017
	2	-0.009	0.135
	3	0.130	0.169
	4	0.171	0.290
	5	-0.350	0.426
Phenoxy-acetic ester	1	0.631	0.431
	2	0.492	0.609
	3	0.636	0.719
	4	1.469	1.504
	5	1.628	1.670
Acetanilide	1	0.399	0.552
	2	0.397	0.546
	3	-0.024	0.230

MATERIALS AND METHODS

Five triazines, five phenoxyacetic esters and three acetanilides (Fig. 1) were purchased from PolyScience (Niles, IL, USA) and their retention data are listed in Table 1. Methanol and acetonitrile (HPLC grades) were all from Baxter (Muskegon, MI, USA). Water was purified by using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Apparatus

All chromatograms were obtained using a Samsung Electron Devices SLC liquid chromatography (Suwon, Korea) system. Shodex C18 column (4.6 × 250mm, Showa Denko, Tokyo, Japan) for triazines and Shiseido Superiorex ODS column (4.6 × 250mm, Tokyo, Japan) for phenoxyacetic esters and acetanilides were used. Triazines were measured at 220nm and phenoxyacetic esters and acetanilides were measured at 230nm.

Table 2

Regression Model for Triazines Separated with 75% MeOH-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.224	0.011	0.996	1.000	NPSSE*
-2.036	0.105	0.000		Constant
R^2_{ADJ}=0.990	Std. Error=0.019		N=5	F=417.346

*NPSSE: nonpolar saturated water solvation surface energy in (kcal).

Table 3

Regression Model for Triazines Separated with 65% MeCN-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.108	0.011	0.985	1.000	TSE*
-5.333	0.077	0.000		Constant
R^2_{ADJ}=0.960	Std. Error=0.034		N=5	F=96.070

*TSSE: total water solvation surface energy in (kcal).

Isocratic elution was carried out with 75% (v/v) methanol-water and 65% (v/v) acetonitrile-water. The flow rate was 1.0mL/min. The retention times for all solutes were measured three times and their average values were used for further analysis.

Descriptor Generation

A IBM compatible PC using the HyperChem (Hypercube, Waterloo, Ontario, Canada) was used for getting the descriptors. The geometery optimization calculation of each solute was carried out by MM+ (5) in a periodic box.

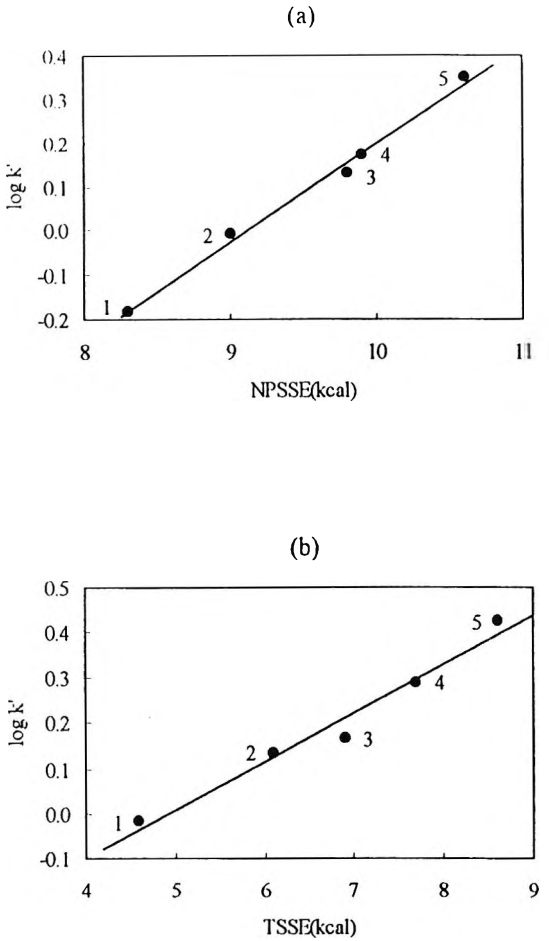


Figure 2. Plots of log k' vs. (a) nonpolar saturated water solvation surface energy (NPSSE) with 75% MeOH-H_2O (b) total water solvation surface energy (TSSE) with 65% MeCN-H_2O for triazines.

Total energy and the energy of the individual bonding component (bonds, angles, dihedrals, van der Waals interactions, hydrogen bonding, electrostatic energy) resulting from optimization calculation were taken as descriptors. Using optimized geometry, single point calculations were done with a semi-empirical method, AM1.[6,7] Then, total energy in semi-empirical calculation, the energy components, heat of formation, dipole moment, and components of dipole moment were considered as descriptors.

Table 4

Regression Model for Phenoxyacetic Esters Separated with 75% MeOH-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.727	0.045	0.994	1.000	PSSE*
3.110	0.136	0.000		Constant
R^2_{ADJ}=0.985	Std. Error=0.066		N=5	F=259.740

*PSSE: polar water solvation surface energy in (kcal).

Table 5

Regression Model for Phenoxyacetic Esters Separated with 65% MeCN-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.083	0.001	1.000	1.000	Polarizability
-1.333	0.032	0.000		Constant
R^2_{ADJ}=0.999	Std. Error=0.015		N=5 F=5671.190	

In addition, solvent-accessible surface area, solvent-accessible molecular volume, 1-octanol/water partition coefficient (log P), molar refractivity and polarizability were also calculated with ChemPlus (Hypercube, Waterloo, Ontario, Canada). On the other hand, total water solvation surface energy, nonpolar saturated water solvation surface energy, nonpolar unsaturated water solvation surface energy, and polar water solvation surface energy were calculated with PCMODEL (Vernon, USA), respectively.

Regression Analysis

Multiple linear regression analysis[8] for descriptor selection was carried out by a stepwise procedure, followed by model generation. Preliminary information on the interrelationships among the descriptors was obtained from the correlation matrix. Multicollinearity was also examined using tolerance.

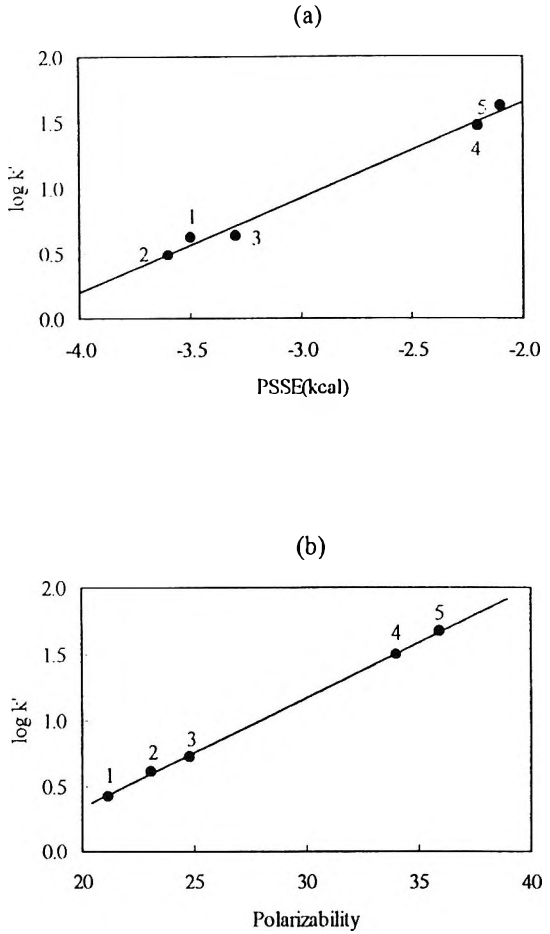


Figure 3. Plots of log k' vs. (a) polar water solvation surface energy (PSSE) with 75% MeOH-H_2O (b) polarizability ($Å^3$) with 65% MeCN-H_2O for phenoxyacetic esters.

The criteria for judging the best model were multiple correlation coefficient, standard error, and overall F-value for analysis of variance. Statistical calculations were performed with Macintosh Classic II using SYSTAT software (SYSTAT, Evanston, IL, USA).

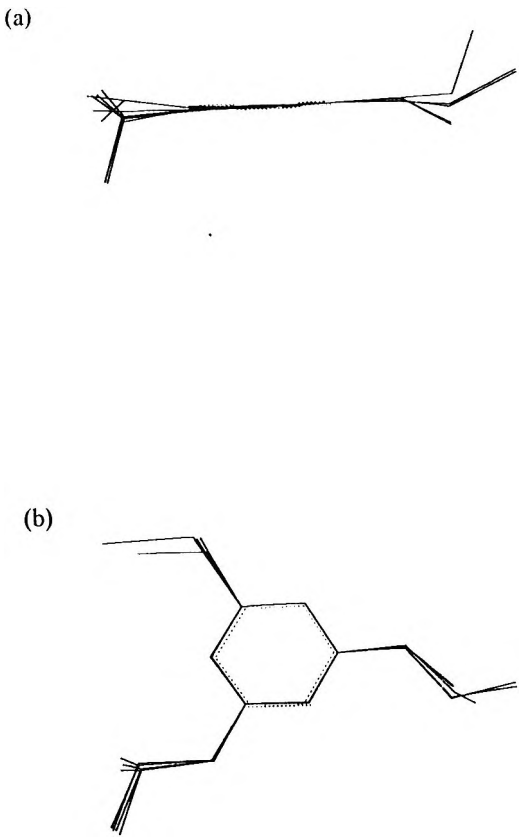


Figure 4. Optimized triazine structures by MM+. (a) orthogonal view (b) frontview.

RESULTS AND DISCUSSION

The most informative structural descriptors that are available to elucidate the retention mechanism in a given chromatographic system could be identified with QSRRs. Following MM+ and AM1 calculations, a number of descriptors were examined to characterize the molecular structure.[9,10] Stepwise regression method was applied to select the adequate descriptors that could be used to explain the retention behavior of solutes.

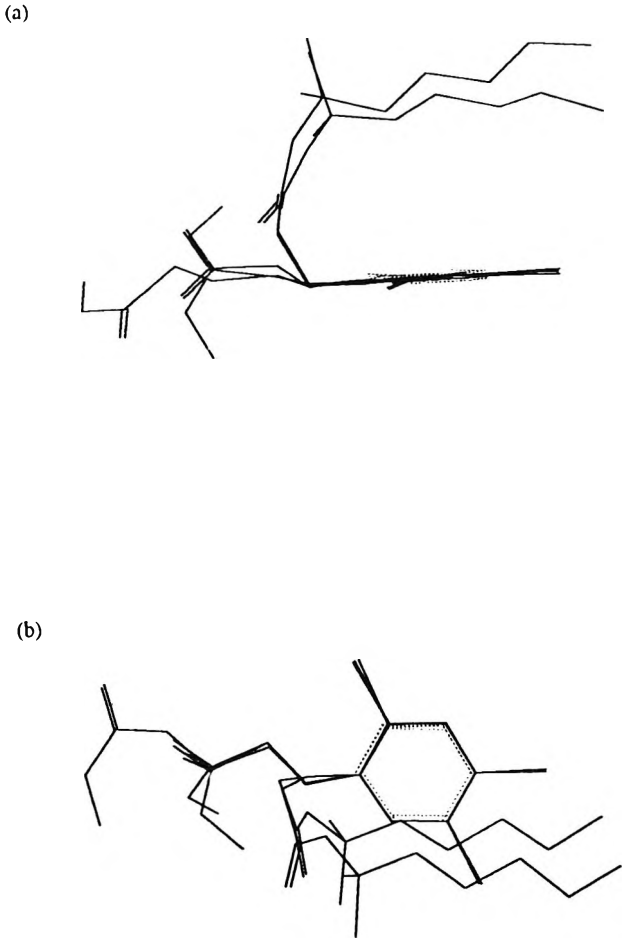


Figure 5. Optimized structures of phenoxyacetic esters by MM+. (a) orthogonal view (b) front view.

Among descriptors examined, nonpolar saturated water solvation surface energy (NPSSE) and total water solvation surface energy (TSSE) were selected as the descriptors for triazines with each mobile phase, respectively (Tables 2 and 3). Plots of the observed retention value (log k') vs. the value of descriptor for triazines with each mobile phase showed good correlations (Fig. 2).

Table 6

Regression Model for Acetanilides Separated with 75% MeOH-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.005	0.001	0.994	1.000	SASA*
-2.195	0.267	0.000		Constant
R^2_{ADJ}=0.977	Std. Error=0.037		N=3	F=85.004

*SASA: solvent-accessible surface area (Å^2).

Table 7

Regression Model for Acetanilides Separated with 65% MeCN-H_2O

Coefficient	Std. Error	Std Coefficient	Tolerene	Descriptor
0.004	0.000	0.994	1.000	SASA*
-1.427	0.204	0.000		Constant
R^2_{ADJ}=0.977	Std. Error=0.028		N=3	F=85.004

*SASA: solvent-accessible surface area (Å^2).

Total water solvation surface energy (TSSE) is the energy arising from hydration of solute surface and is the sum of nonpolar saturated water solvation surface energy (NPSSE), nonpolar unsaturated water solvation surface energy (NPUSSE), and polar water solvation surface energy (PSSE). Using methanol as organic modifier, the retention increased with increasing NPSSE of solute. It means that retention depended on the solvation of nonpolar groups of solute with methanol/water eluent.

In the case of acetonitrile as organic modifier, the retention increased with increasing TSSE of solute. From this relationship, it was assumed that retention of solute did not depend on solvation of specific nonpolar or polar group but on solvation of the whole molecule with acetonitrile/water eluent.

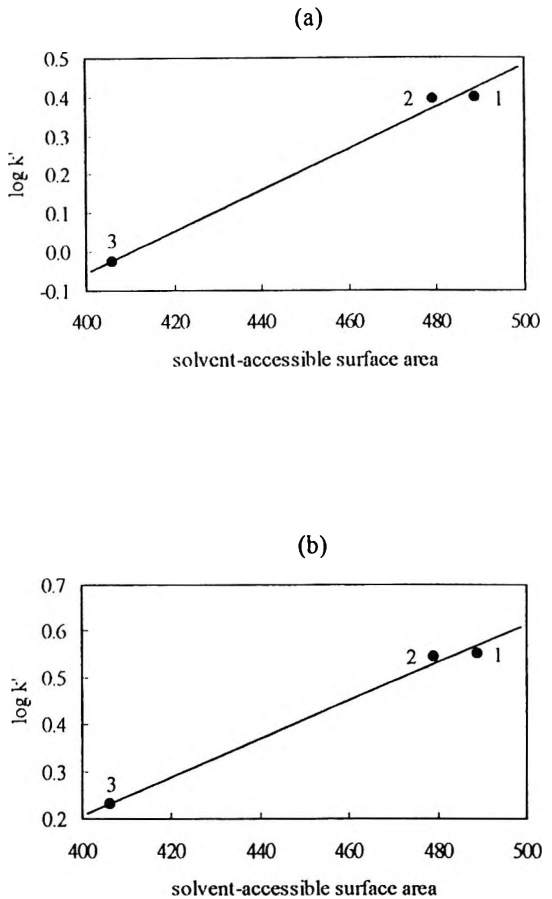


Figure 6. Plots of log k' vs. solvent-accessible surface area ($Å^2$) for acetanilides with (a) 75% MeOH-H_2O (b) 65% MeCN-H_2O.

For phenoxyacetic esters, polar water solvation surface energy (PSSE) arising from hydration of polar group of solute and polarizability were selected as the descriptors with each eluent, respectively (Table 4 and 5). Plots of the observed retention value (log k') vs. the value of descriptor for phenoxyacetic esters were shown in Fig. 3. Optimized molecular structures of triazines (see Fig. 4) were almost planar. On the other hand, phenoxyacetic esters had planar or C-shaped structures (see Fig. 5) according to substituents at R_1. If R_1 was isooctyl (phenoxyacetic ester #4 and #5), its structure was C-shaped.

From conformational considerations, phenoxyacetic ester #4 and #5 which have C-shaped structure had double binding site to the C_{18} ligates compared with phenoxyacetic ester #1, #2 and #3. On the other hand, the penetration of phenoxyacetic ester #4 and #5 into C_{18} ligates was sterically hindered.

Therefore, it would be needed another effect to describe the retention behavior of phenoxyacetic esters besides binding interaction between solute and C_{18} ligates. If organic modifier was methanol, it was suggested that the extent of solvation of carbonyl group of R_1 would have an effect on the retention. On the other hand, if organic modifier was acetonitrile, retention of solute was described by bulk property such as polarizability because acetonitrile had no specific functional group such as hydroxyl group in methanol which could interact with carbonyl group.

In comparison of descriptors included in retention model, there was a common feature in retention behavior of triazines and phenoxyacetic esters with each organic modifier. Major factor on the retention of the solutes was the extent of solvation of specific sites such as nonpolar or polar group with methanol/water eluent and was bulk property such as total water solvation energy and polarizability with acetonitrile/water eluent.

For acetanilides, solvent-accessible surface area (11) was selected as the descriptor regardless of organic modifier (Table 6 and 7). Plots of the observed retention value (log k') vs. the value of descriptor for each acetanilide were shown in Fig. 6.

Although solvent-accessible surface area of acetanilide #1 was different from that of acetanilide #2, their retention values were almost same. Since the phenyl group of acetanilides was bisected with the alkyl substituent on nitrogen atom (see Fig. 7), the penetration of solute into C_{18} ligates would be sterically hindered. If phenyl group would interact with C_{18} ligates, methylene group was the only difference between acetanilide #1 and #2. Therefore, it was supposed that acetanilide #1 and #2 would show similar retention value because the methylene group was very small part compared with the whole molecule.

In summary, for triazines and phenoxyacetic esters, it was suggested that there were two effects on the retention according to organic modifier, specific solvation effect and nonspecific effect. Specific solvation effect would occur if the organic modifier was methanol, which would solvate the nonpolar site of triazines and the polar site of phenoxyacetic esters.

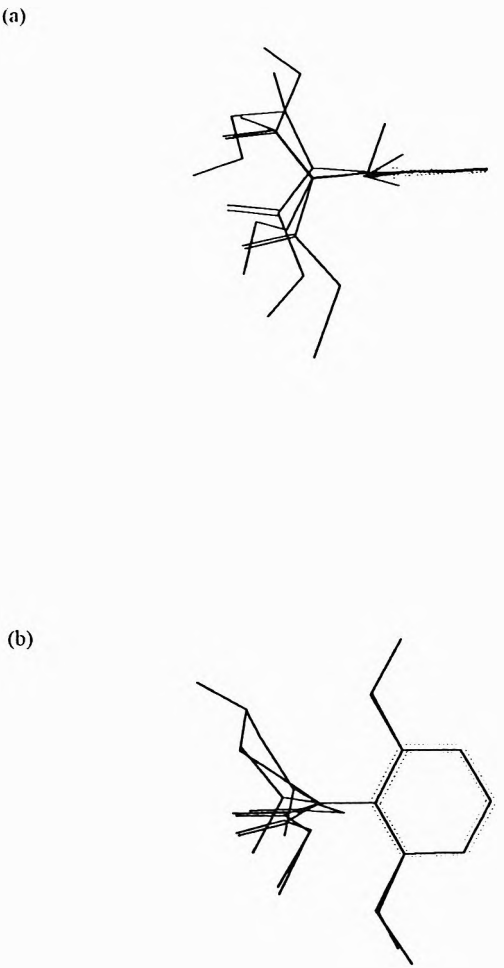


Figure 7. Optimized structures of acetanilides by MM+. (a) orthogonal view (b) front view.

On the other hand, if the organic modifier was acetonitrile, nonspecific effect depended on bulk properties of solute such as total water solvation energy and polarizability would be major factor on the retention. For acetanilides, although solvent-accessible surface area was selected as the descriptor regardless of organic modifier, conformation of solute had an additional effect on the retention behavior of solutes.

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EVALUATION OF A NEW MACROCYCLIC ANTIBIOTIC AS A CHIRAL SELECTOR FOR USE IN CAPILLARY ELECTROPHORESIS

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ABSTRACT

A new macrocyclic antibiotic, LY307599, has been evaluated as a chiral selector for the separation of the enantiomers of flurbiprofen using capillary electrophoresis (CE). The effect of varying separation buffer parameters such as buffer strength, pH, LY307599 concentration and methanol concentration were assessed. Using the optimized CE conditions, the separation of flurbiprofen enantiomers can be achieved using LY307599 as a chiral selector.

INTRODUCTION

Several approaches have been used to obtain chiral separations with capillary electrophoresis. These include the use of micelle-forming surfactants in micellar electrokinetic capillary chromatography (MECC),[1-7] immobilized chiral selectors in capillary gel electrophoresis[8-11] and various additives in free solution capillary electrophoresis (FSCE), also referred to as capillary zone electrophoresis (CZE).[12-39]

Free solution capillary electrophoresis is often the method of choice due to its capacity for the separation of a diverse array of analytes.[12] Within the realm of FSCE, specific additives have been successfully used to separate enantiomers including the use of native and modified cyclodextrins,[13-19] crown ethers,[20-23] carbohydrates,[24] proteins,[25-29] and enantioselective metal complexes.[30-31] More recently, macrocyclic antibiotics have been used as chiral selectors.[32-40] Within this group, the ansamycins rifamycin B and rifamycin SV have shown broad chiral selectivity for positive and negatively charged analytes respectively.[35] Rifamycin B has also been shown to be selective in the separation of racemic amino alcohols.[33] The glycopeptides ristocetin A, teicoplanin, vancomycin and A82846B have also shown promising chiral selectivity for many amino acid derivitives and carboxylic acids.[32,34, 36-40] Macrocyclic antibiotics such as A82846B provide multiple π-π electron interaction sites, hydrogen bonding locations and inclusion cavities for potential enantiospecific interactions. Despite similarity of these sites among macrocyclics, each displays varying success in the separation of different chiral analytes. In a recent report, a comparison of A82846B and vancomycin illustrated that a relatively small alteration in structure can significantly improve the resolution of flurbiprofen and dansyl valine enantiomers.[39] In this report we examined LY307599, a A82846B derivitive, for its applicability as a chiral selector using CE. Flurbiprofen, a non-steroidal antiinflammatory drug, was selected as the test analyte for evaluating LY307599 as a chiral selector in CE because the enantiomers have been successfully separated in other reports with vancomycin and A82846B.[32,39]

EXPERIMENTAL

The CE analysis was conducted using an automated Beckman P/ACE 2000 Instrument (Fullerton, CA) and the data collected on an in-house Hewlett Packard Model 1000 minicomputer (Palo Alto, CA). The 50 µm internal diameter 37 cm eCAP bare silica capillary (30 cm effective length) was obtained from Beckman Instruments, Inc. (Fullerton, CA). The absorbance wavelength was set at 254 nm. Samples were injected using a 5.0 second low pressure interval (0.5psi). The capillary temperature was controlled at 25 °C.

Sodium borate, 0.1 N and 5.0 N sodium hydroxide, 5.0 N hydrochloric acid and acetone were purchased from EM Science (Gibbstown, NJ). Potassium phosphate was obtained from Mallinckrodt (Paris, KY). Methanol was obtained from Curtin Matheson Scientific, Inc. (Houston, TX). The water used for buffer and sample preparation was deionized and filtered

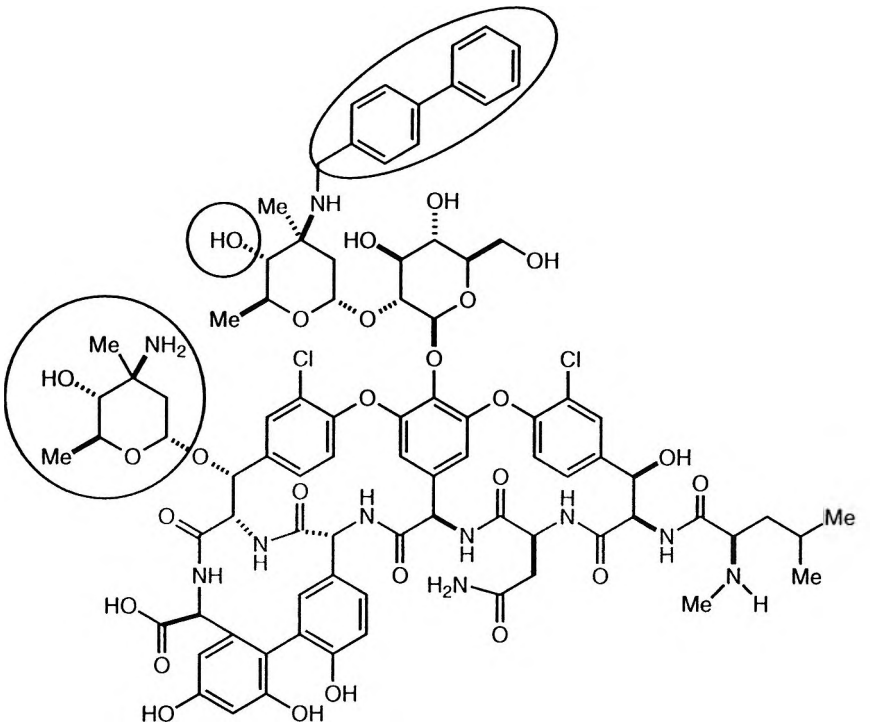

Figure 1. The structure of LY307599 (Molecular weight = 1731).

through a Millipore Milli-Q™ water purification system (New Bedford, MA). Run buffers and sample solutions were filtered through Acrodisc® Filters (0.2 μm) from Gelman Sciences (Ann Arbor, MI). Buffer pH was measured using a Brinkmann Model 691 pH meter (Westbury, NY). Flurbiprofen was obtained from Sigma-Aldrich Company (St. Louis, MO). Hydrochloride and trifluoroacetate salts of LY307599 were synthesized at Eli Lilly and Company (Indianapolis, IN).

Flurbiprofen samples were prepared at 1.0 mg/mL in 100 mM sodium borate buffer, unless otherwise noted. The 100 mM borate buffer was adjusted with 5.0 N NaOH or 5.0 N HCl to obtain the desired pH. The stability of the macrocyclic was not assessed, therefore all solutions of LY307599 were prepared fresh daily. LY307599 was first dissolved in methanol followed by the addition of the 100 mM borate to achieve the desired run buffer composition. Acetone was used as a neutral marker for electroosmotic flow (EOF).

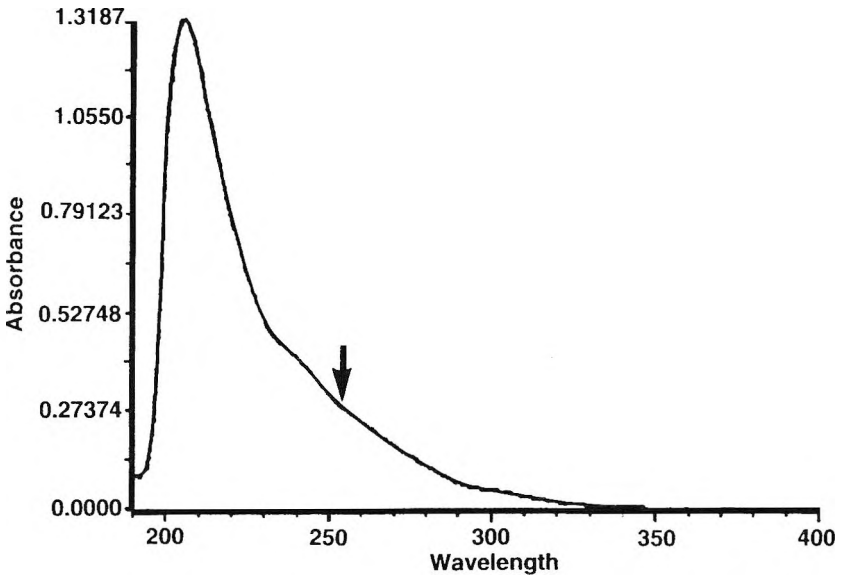

Figure 2. The UV spectrum of LY307599.

RESULTS AND DISCUSSION

The structure of LY307599 is shown in Figure 1. LY307599 is structurally similar to vancomycin and A82846B which have been previously reported.[32,39] The macrocyclic antibiotic LY307599 differs from vancomycin in three ways: The disaccharide amino sugar is epimeric at C-4 (epi-vancosamine), contains a biphenyl moiety on the amine at C5, and an additional epi-vancosamine at amino acid 6. The only difference between LY307599 and A82846B is the addition of the biphenyl moiety. The macrocyclic antibiotic LY307599 absorbs strongly in the lower UV range as shown in Figure 2.

Fortunately, the compound does not absorb greatly at 254 nm, allowing for the detection of flurbiprofen (Figure 3) at this wavelength. LY307599 presented unique challenges compared to A82846B and vancomycin due to its limited solubility in aqueous buffers, especially below pH 8.0, thus requiring lengthy development and parameter optimization to achieve enantioseparation.

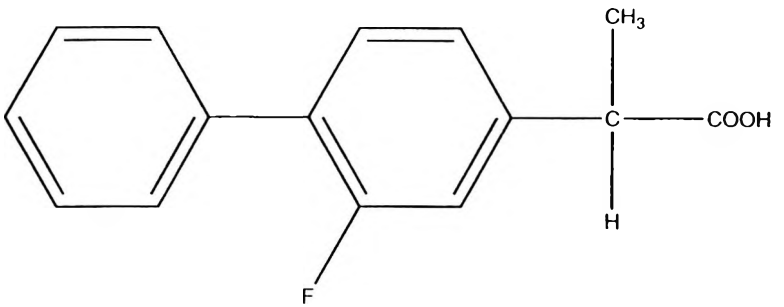

Figure 3. The structure of flurbiprofen.

Development

The hydrochloride and acetate salts were used in this study due to their improved solubility over other salt forms and the free base. Solutions of the macrocyclic antibiotic in 50 mM phosphate buffer were achieved at pH 3.0 (4.0 mM, LY307599) and in 100 mM borate buffer above pH 9.0 (7.0 mM, LY307599). In order to dissolve 1.0 mM of LY307599 at pH 7.0, the addition of 60% methanol was necessary, thus direct comparison of LY307599 with previous data using vancomycin and A82846B in aqueous neutral pH conditions was not possible. A 1.0 mM solution of LY307599 in the pH 7.0 phosphate/methanol buffer provided the enantiomeric separation of flurbiprofen yielding a resolution of 1.5, but the high concentration of methanol required for solubility of the macrocyclic inhibited a reproducible system and resulted in a noisy baseline.

Due to the lack of solubility of LY307599 in neutral pH buffers, the separation of flurbiprofen enantiomers was attempted in both acidic and basic conditions. Using 4.0 mM of LY307599 in 50 mM pH 3.0 phosphate buffer, flurbiprofen enantiomers could not be separated. The EOF of the system was determined by injecting a 2% acetone solution and was found to be 5.1×10^{-5} cm^2/V s using a voltage of 30 kv.

More successful results were observed in the basic pH range of 9.0 to 10.0 using borate buffer and 4.0 mM LY307599 concentration. To maximize analyte interaction time in the capillary under basic conditions, the voltage of the system was reduced to 10 kv for analysis in this pH range. This also reduced the baseline noise of the system. Borate buffer was used at 100 mM

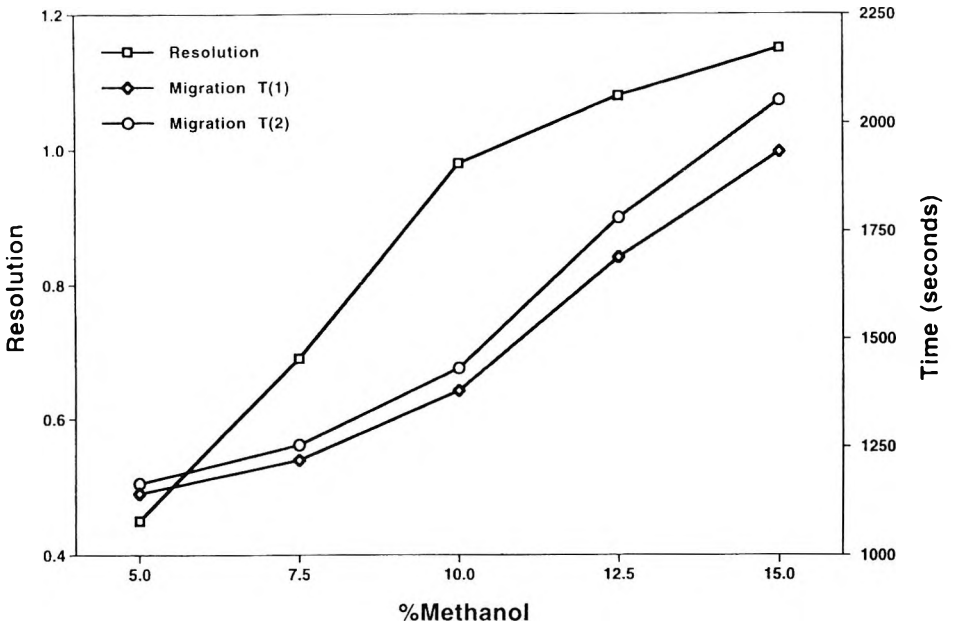

Figure 4. A plot of run buffer methanol content versus resolution and migration time. Run buffer is 3.0 mM LY307599 in methanol and 100mM borate at pH 9.2; Voltage is 10 kv; UV absorbance is 254 nm.

strength to further decrease EOF while maintaining a stable current of 60 μA. Early analysis revealed that the EOF was evidently still too fast to allow for sufficient interaction between flurbiprofen and 1.0 mM LY307599 in borate run buffer. Injections of flurbiprofen under these conditions resulted in a sharp analyte peak eluting in approximately 13.0 minutes.

The addition of methanol and increase in LY307599 concentration in the run buffer significantly slowed EOF and resulted in flurbiprofen enantiomer separations. The EOF of the system using 100 mM sodium borate buffer (pH 9.2) was measured to be 3.0×10^{-4} cm^2/V s. The addition of 15% methanol to the run buffer reduced the EOF to 2.2×10^{-4} cm^2/V s in 100 mM borate buffer, while the EOF further decreased to 1.4×10^{-4} cm^2/V s with the addition of 4.0 mM of LY307599 to the borate buffer.

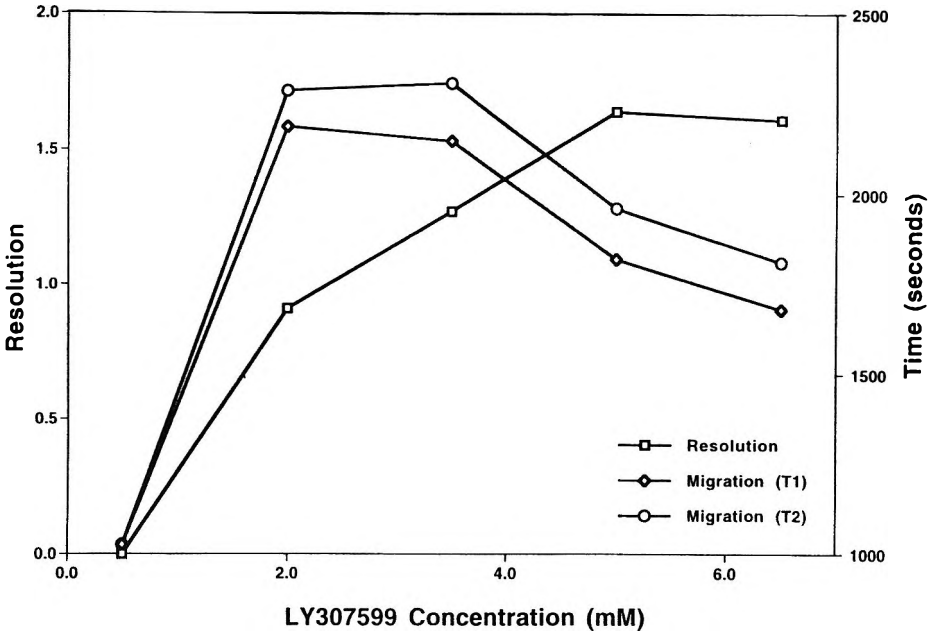

Figure 5. A plot of run buffer LY307599 concentration versus resolution and migration time. Run buffer is 15% methanol in 100mM borate at pH 9.2;. Voltage is 10 kv; UV absorbance is 254 nm.

Optimization

Using 10 mM borate buffer at pH 9.2 containing 3.0 mM LY307599, the effect of increasing methanol composition in the run buffer on flurbiprofen enantiomer separation was examined. Figure 4 shows the resulting effects on resolution and migration time. Resolution and migration both increased with increasing methanol content. Methanol content of 20% or greater in the run buffer resulted in lengthened migration times and degraded peak shapes. A run buffer containing 15% methanol was selected as optimum because of the satisfactory resolution of flurbiprofen enantiomers within a reasonable migration time.

Using 15% methanol, the concentration of LY307599 in the run buffer was increased from 0.5 mM to 6.5 mM. In general, resolution increased with increasing LY307599 concentration as shown in Figure 5. Migration time

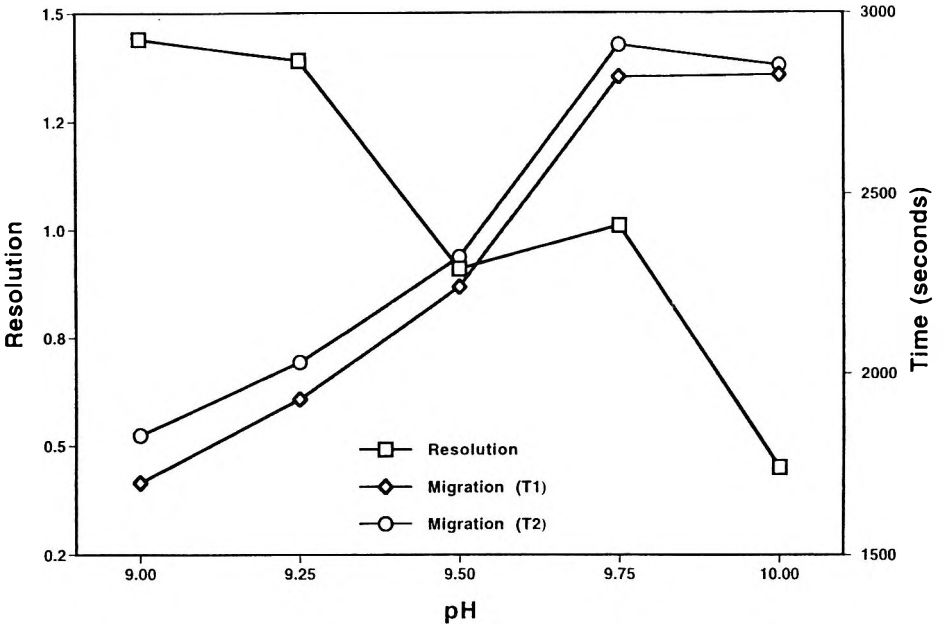

Figure 6. A plot of run buffer pH versus resolution and migration time. Run buffer is 15% methanol in 100 mM borate; Voltage is 10 kv; UV absorbance is 254 nm.

leveled off and began to decrease with increasing macrocyclic concentration. It was originally thought that this decrease in migration time might be due to methanol evaporation from sample vials during the lengthy analysis. The trial was repeated with each sample prepared immediately before analysis and the trend was reproduced. Further investigation is needed to study the effect of LY307599 interactions within this run buffer. Macrocyclic concentrations above 5.0 mM resulted in poor peak shapes and noisy baselines. An LY307599 concentration of 4.0 mM was chosen as optimum for resolution, peak shape and baseline noise.

Using 15% methanol and 4.0 mM LY307599, the pH of borate buffer was adjusted from 9.0 to 10.0 in increments of 0.25 pH units. Figure 6 shows the effect of increasing pH of the run buffer on the resolution and migration time. Resolution values decreased while migration times increased with increasing pH. It was originally thought that the higher pH run buffers would cause migration times to decrease as a result of a perceived higher EOF. However, a higher buffer ionic strengh at pH values greater than the pKa of borate may be

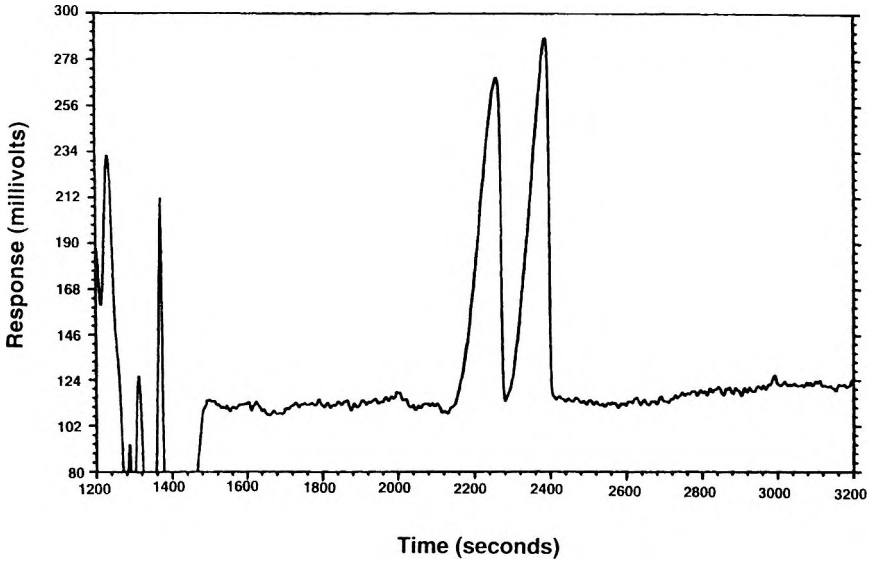

Figure 7. A sample electropherogram of the separation of flurbiprofen enantiomers Run buffer is 4.0 mM LY307599 in 15% methanol and 100 mM borate buffer at pH 9.2; Voltage is 10 kv; UV absorbance is 254 nm; Injection is 5 second low pressure interval (0.5 psi); Capillary temperature is 25°C; The sample is 1.0 mg/mL flurbiprofen.

the cause of the increased migration time. This effect would actually reduce EOF. Despite the increased time in the capillary at higher run buffer pH's, flurbiprofen is not as well resolved under these conditions. One plausible explanation for this is that at run buffer pH's approaching 10.0, the predominately negative character of both the analyte and LY307599 interfere with their stereospecific interaction.

An example electropherogram of the flurbiprofen enantiomer separation using the optimized run buffer conditions of 4.0 mM LY307599 dissolved in 15% methanol and 85% 100 mM borate is shown in Figure 7. A run buffer pH of 9.2 offered the best separation, without having to make a pH adjustment. It is worth noting that resolution values markedly increase with decreasing pH from 10.0 to 9.0. If it were not for the insolubility of LY307599 at and below pH 9.0, it is quite possible that this macrocyclic would serve as a much better chiral selector at a more neutral pH.

CONCLUSIONS

LY307599 has been shown to be another successful macrocyclic antibiotic chiral selector for the separation of enantiomers of flurbiprofen. The separation was shown to be affected by methanol content in the run buffer, run buffer pH and LY307599 concentration. The high EOF encountered at basic run buffer pH's was successfully countered with methanol. The parameters of run buffer pH, LY307599 concentration and methanol concentration were optimized to allow for adequate enantioseparation within a reasonable migration time.

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DETERMINATIONS OF TRILINOLEIN AND 1,2-DILINOLEOYL-3-OLEOYL-GLYCEROL IN VARIOUS PANAX GINSENG BY HPLC

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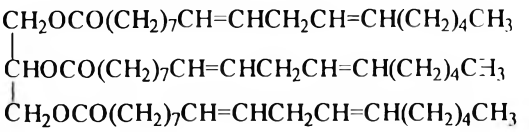
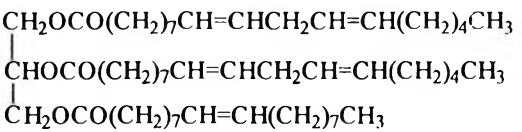
ABSTRACT

A simultaneous analysis of trilinolein and 1,2-dilinoleoyl-3-oleoyl-glycerol (DLO) in *Panax ginseng* C. A. Meyer (ginseng) by a high performance liquid chromatographic method was used in the analysis of various Ginseng Radix. Chromatographic analysis is achieved on an isocratic system consisting of a polymeric reversed phase C_{18} column with a mobile phase of acetonitrile-methanol (50:50, v/v) to elute the trilinolein and DLO. The system was detected at 205 nm. The results indicate that various ginseng extraction from Korean, Japanese and Chinese by n-hexane contained 0.37 ± 0.009, 0.39 ± 0.016, and 0.27 ± 0.009 mg/g, respectively for trilinolein; and 0.41 ± 0.009, 0.45 ± 0.01, and 0.22 ± 0.008 mg/g, respectively for DLO. Quantitative determination of the triacylglycerol content in different parts of ginseng showed that the contents were in the following order: rhizome head > main root > root hair.



Trilinolein



1,2-Dilinoleoyl-3-oleoyl-rac-glycerol

Figure 1. Chemical structures of trilinolein and 1,2-dilinoleoyl-3-oleoyl-*rac*-glycerol (DLO).

INTRODUCTION

Radix Ginseng, is the dry root of *Panax ginseng* C.A. Meyer (Araliaceae), a worldwide well-known traditional Chinese medicine with the popular name "ginseng"[1]. The root of ginseng contains about 4% of ginsenosides, which are generally considered as the pharmacologically active components of ginseng[2]. Except for ginsenosides, the other group of active components, triacylglycerol has been found in Panax pseudo-ginseng[3]. It has been recently reported that trilinolein of pseudo-ginseng inhibits adrenaline-induced human platelet aggregation[4]. This inhibition of trilinolein was accompanied by reduced ATP release and thromboxane B_2 formation[3]. During cardiopulmonary bypass, trilinolein also improves the erythrocyte deformability.[5,6] The typical triacylglycerol, trilinolein and 1,2-dilinoleoyl-3-oleoyl-*rac*-glycerol (DLO) (Figure 1) are triacylglycerol in all three esterified positions of glycerol with linoleic acid or oleic acid, respectively. In our unpublished data it was shown that DLO also possess pharmacological effects in the cardiovascular system. The content of triacylglycerol in ginseng may be one of the major active principles.[7]

In order to further study the actions of ginseng, it is important to investigate triacylglycerol content in original material ginseng. In this work, we used a polymeric reversed phase liquid chromatographic method for the determination of trilinolein and DLO in the content of various ginseng and different parts of ginseng.

MATERIALS AND METHODS

Chemicals and Reagents

Ginseng was purchased from a traditional oriental herbal drug store in Taipei. The origin of material ginseng was identified by the botanist at the National Research Institute of Chinese Medicine. Authentic compounds, trilinolein and DLO, were obtained from Sigma Chem. (St. Louis, MO, USA). Acetonitrile (HPLC far UV grade), n-hexane and methanol (HPLC grade) were obtained from LabScan Chem. (Dublin, Ireland). The stock solutions of trilinolein and DLO were dissolved in n-hexane at a concentration of 1 mg/mL.

Apparatus and Chromatography

The HPLC system consisted of an injector (Rheodyne 7125, Cotati, CA, USA), a variable wavelength UV-VIS detector (Soma, Tokyo, Japan) and a chromatographic pump (ICI model 1110, Australia). Separation was achieved on a HEMA polymeric reversed-phased C_{18} column, 250 x 4 mm, particle size 10 μm (Tessek A/S, The Science Park, Aarhus C, Denmark). The mobile phase was acetonitrile-methanol (50:50, v/v), and the flow rate was 1.0 mL/min. Triacylglycerols were monitored at a wavelength of 205 nm throughout the experiments. The system was operated at room temperature (25°C)..

Extraction

Ginseng powder (0.5 g) was boiled with 50 mL of n-hexane for 10 min. This procedure was repeated twice. The two filtrates were combined and diluted to 100 mL in a volumetric flask.

Precision

To determine the intra-assay variance of trilinolein and DLO, quadruplicate assays were carried out at the same concentrations (1, 5 or 20 μg/mL), at different times during the day.

Inter-assay variance of trilinolein and DLO were determined by assaying in quadruplicate, on days one, two, four and six. Coefficients of variation (C.V.s) were calculated from these values.

Determination of Trilinolein and DLO

Calibration graphs for trilinolein and DLO dissolved in n-hexane were constructed by HPLC of various known amounts of these compounds (0.5, 1, 5, 10 and 20 μg/mL). The contents of trilinolein and DLO in the crude extract of ginseng were determined by the regression equation for the peak area under the curve versus concentrations of these two compounds.

RESULTS AND DISCUSSION

Identification of trilinolein and DLO in the crude extract of ginseng was substantiated by liquid chromatography in the following four modes: (1) on the correct retention times of trilinolein and DLO, compare the retention times of trilinolein and DLO with crude extract of ginseng and authentic standards, respectively; (2) on the correct concentrations of trilinolein and DLO, after spiking with authentic compounds (trilinolein or DLO), in the crude extract of ginseng and measured their peak areas; the peak areas of trilinolein and DLO should be additive; (3) on the modification of mobile phase; by changing the ratio of acetonitrile:methanol from 5:5 to 3:7 (v/v), the retention times of trilinolein and DLO should be delayed owing to the ratio of acetonitrile bring decreased; (4) on the alteration of UV absorbance wavelength; by changing the wavelength from 205 nm to 230 nm, the peaks of trilinolein and DLO should disappear because peaks of trilinolein and DLO both do not show absorbance at 230 nm.

Under the conditions described above, the retention times of trilinolein and DLO were found to be 9.23 and 12.27 min, respectively (Figure 2). The detection limit for trilinolein and DLO, at signal-to-noise ratio of 3, were 0.1 and 0.5 μg/mL, respectively.

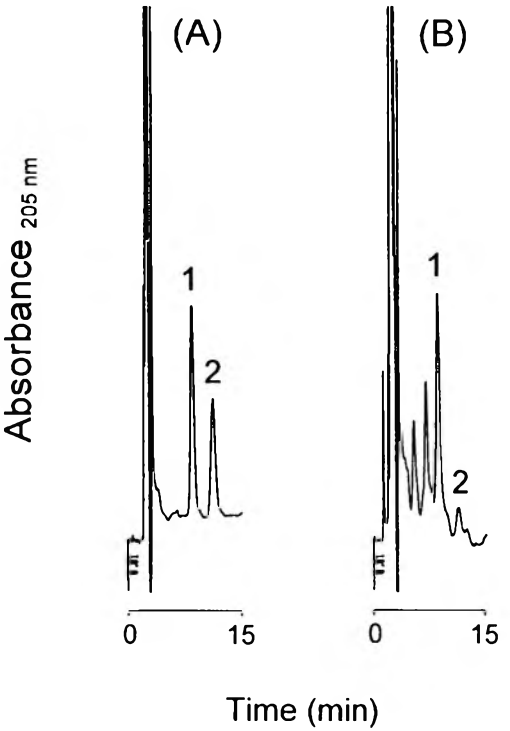


Figure 2. Elution profiles of the injection 20 μL of (A) mixture of trilinolein and 1,2-dilinoleoyl-3-oleoyl-glycerol (DLO), (B) extract of *Panax ginseng* C. A. Meyer was separated by a polymeric reversed phase C_{18} column with 205 nm. 1: trilinolein; 2: DLO.

The contents of trilinolein and DLO in the crude ginseng extract were determined from the linear regression equation of the calibration graphs for these compounds. The calibration curves of trilinolein and DLO were Y = 5.30E-5 X + 0.037 (r^2 = 0.999) and Y = 4.39E-5 X + 0.65 (r^2 = 0.998), respectively; here X is peak-area response and Y is amount of compound. The linearity ranges of trilinolein and DLO were 0.5-20 μg/mL.

The intra-assay C.V.s for the determination of trilinolein and DLO at concentrations of 1, 5, and 20 μg/mL were acceptable with C.V.s of less than 10 %. The inter-assay C.V.s for trilinolein and DLO at the same concentrations were less than 10 %.

Table 1

Trilinolein and 1,2-Dilinoleoyl-3-Oleoyl-Glycerol (DLO) Contents (Mg/G) in Various Ginseng Growth in Different Areas

Origin	Trilinolein	DLO
Korea	0.37 ± 0.009	0.41± 0.009
Japan	0.39 ± 0.016	0.45 ± 0.012
China	0.27 ± 0.009	0.22 ± 0.008

Data are expressed as mean ± SEM (n=6).

Table 2

Trilinolein and 1,2-Dilinoleoyl-3-Oleoyl-Glycerol (DLO) Contents (Mg/G) in Different Parts of China Ginseng

Different parts	Trilinolein	DLO
Rhizome head	0.34 ± 0.005	0.33 ± 0.015
Main root	0.27 ± 0.009	0.22 ± 0.008
Root Hair	0.25 ± 0.006	0.33 ± 0.024

Data are expressed as mean ± SEM (n=6).

Table 1 summarized the contents of trilinolein and DLO in n-hexane extracts of various ginseng. The highest yields of trilinolein and DLO were found in Korean and Japanese ginseng, respectively.

Table 2 shows the contents of different parts of ginseng. The data indicated that the contents were in the following order: rhizome head > main root > root hair for trilinolein; rhizome head = root hair > main root for DLO.

In conclusion, the proposed technique should be useful for the quality control, stability or pharmacokinetics of trilinolein and DLO in the traditional drug of ginseng.

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COMPARISON OF A STYRENE-DIVINYLBENZENE COPOLYMERIC ANION EXCHANGER AND A POLYMETHACRYLATE COPOLYMERIC ANION EXCHANGER FOR GLUCOSINOLATE SEPARATION

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ABSTRACT

Studies on gradient elution were undertaken on a Hamilton PRP-X100 column. Satisfactory analysis was produced by using a concentration gradient in which the eluent ion concentration was increased over the run. To conclude the overall investigation of glucosinolate separation by anion exchange chromatography, a polymethacrylate copolymeric anion exchanger was evaluated and compared with a silica anion exchanger and a polystyrene-divinylbenzene copolymeric anion exchanger for an isocratic analysis of glucosinolates. Different commonly used inorganic and organic anions as well as alkanesulfonate anions and borate-gluconate mixtures were tested as aqueous mobile phase.

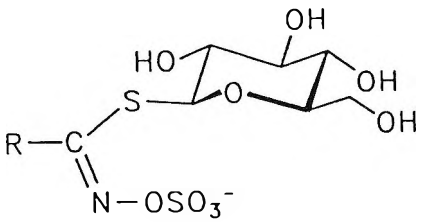

Figure 1. General structure of glucosinolates.

INTRODUCTION

Glucosinolates are thioglucosides which are naturally occurring constituents of Brassica vegetables. These compounds are important because of their potential toxicity and because epidemiology and other evidence indicate that some of them may inhibit some carcinogenic processes when consumed as part of the normal diet.[1-3] Accordingly, on the one hand, the identification and quantization of these compounds and on the other hand, obtaining purified glucosinolates in relatively large amounts as materials for nutrition and physiological investigations have become important. Glucosinolates are natural anionic compounds frequently isolated from plants as potassium salts or sometimes, as salts of sinapine (the choline ester of sinapic acid). Their general structure is shown in Figure 1.

Recently, we have proved that the glucosinolates can be analyzed by anion exchange chromatography.[4,5] All glucosinolates carry the same anionic sulfate group, but the great diversity of the R group leads to wide variations in the polarity and the hydrophilic and lipophilic characters of these natural compounds. In anion exchange chromatography, glucosinolates were eluted principally in order of decreasing polarity (alkenyl, arylalkyl then indolylglucosinolates), as in ion-pair chromatography.[6-8] On a silica-based ion exchanger, the ion exchange mechanism is predominant[4] and consequently no sufficient selectivities have been observed on this type of support between glucosinolates carrying the aglycone R group which have similar hydrophobicities. Contrariwise, the low capacity polymeric ion exchanger provides both adsorption and ion exchange sites with which the organic analyte ions may interact.[9-11] On a styrene-divinylbenzene (PS-DVB) copolymeric anion exchanger very strong reverse phase interactions between the glucosinolates, specially the indolylglucosinolates, and the polymeric backbone are noticed[5] and so, it was necessary to use an alkanesulfonate with a chain length of at least six carbons as the eluent anion in the mobile phase to elute

glucosinolates on this type of support. A good resolution for the analysis of many natural mixtures can be obtained with this type of anion exchanger, but with a long and unsatisfactory time of analysis for indolylglucosinolates in an isocratic elution mode.

In this paper to complete our previous study, a PS-DVB polymer-based column was evaluated under a gradient elution mode to optimize the conditions, specially the analysis time, for the separation of various glucosinolates greatly differing in substituent nature. The main aim of our overall study on the chromatographic behavior of glucosinolates as organic anions was the search for high performance liquid-solid extraction systems to obtain very pure individual intact glucosinolates from different natural products. Owing to the wide range of polarities of these compounds to be analyzed and/ or purified, there is a real need for several simple, reliable, complementary and alternative LC methods. So, in this paper the third type of support commonly used in anion exchange chromatography, a polymethacrylate copolymeric anion exchanger, which was a relatively more hydrophilic type polymer backbone than PS-DVB was also investigated, as a possible additional column material for the isocratic analysis of glucosinolates by anion exchange chromatography.

MATERIALS

Table 1 lists the glucosinolates studied. Sinigrin and glucotropaeolin were obtained from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) respectively. Gluconapin was purchased from Dr. A. Quinsac (Centre Technique Interprofessionnel des Oléagineux Métropolitains, Ardon, France). Progoitrin was extracted and isolated from rapeseed in our laboratory. Glucobrassicin was synthesized in our laboratory.[12] Eluent constituents were purchased as follows: distilled water from the Cooperative Pharmaceutique Française (Melun, France); acetonitrile (ACN), RS for preparative HPLC, from Carlo Erba (Milan, Italy); sodium acetate, potassium sulfate, sodium citrate, boric acid, sodium tetraborate decahydrate, sodium gluconate and glycerol from Prolabo (Paris, France); 1-butanesulfonic acid sodium salt, 1-hexanesulfonic acid sodium salt and 1-octanesulfonic acid sodium salt from Eastman Kodak (Rochester, NY, USA). All reagents were of analytical grade. Two compositions of borate-gluconate eluents were tested and prepared from a concentrate A containing 18 g/L boric acid, 25 g/L sodium tetraborate decahydrate, 16 g/L sodium gluconate and 250 mL/L glycerol. The borate-gluconate 1 was prepared by diluting 10 mL of concentrate A with 60 mL of acetonitrile and 430 mL of water and the borate-gluconate 2 was prepared by diluting 1 mL of concentrate A with 499 mL of water.

Table 1

Natural Glucosinolates Studies

Systematic Name of R Group	Trivial Name	Symbol
2-Propenyl	Sinigrin	SIN
3-Butenyl	Gluconapin	GNA
(2R)-2-Hydroxy-3-butenyl	Progoitrin	PRO
Benzyl	Glucotropaeolin	GTL
Indol-3-ylmethyl	Glucobrassicin	GBS

The liquid chromatographic apparatus consisted of a Varian (Palo Alto, CA, USA) Model 2010 solvent-delivery pump or a Bruker gradient pump (Merck, Darmstadt, Germany), a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20 µL sample loop and a Varian Model 2550 UV spectrophotometric detector or a Vydac (Wescan Instruments, Santa Clara, CA, USA) Model 213 A conductivity detector.

Separations were performed on a 100 x 4.1 mm I.D. column packed with 10 µm Hamilton PRP-X100 (Bonaduz, Switzerland) and on a 100 x 4.6 mm column packed with 10 µm Shimadzu Shim-pack IC-A1 (Kyoto, Japan).

Data were processed with a Shimadzu (Kyoto, Japan) CR 6A integrator/recorder. Experiments were carried out at room temperature and the detection wavelength was 229 nm.

To determine the system void volume, V_0, 20 µL of water were injected in each system. Each glucosinolate was injected alone and in admixture in the chromatographic mixtures.

The retention time of each compound was determined in each eluent by three consecutive determinations. The concentration of the standards were ca. 100 ppm in aqueous solution.

RESULTS AND DISCUSSION

Linear Gradient Elution on a PS-DVB Copolymeric Anion Exchanger

As we have demonstrated,[5] the isocratic analysis of glucosinolates on a Hamilton PRP-X100 column with hexylsulfonate eluent ion in water-acetonitrile mixture as mobile phase could be used as a complement to the current ion pairing method. However, when the natural mixture is composed of various glucosinolates greatly differing in substituent nature, a long and unsactifactory analysis time was observed (about 30 min at flow rate 2 mL/min to elute GBS) with this method. The judicious choice of a mobile phase gradient should avoid this disadvantage.

The strong elution strength of alkanesulfonate eluent ion can be explained by reverse phase interactions between the hydrophobic alkylchain and the polystyrene divinylbenzene polymeric backbone. So, with a view to developing, as far as possible, a gradient elution mode with this type of mobile phase, for a given concentration of hexylsulfonate in the eluent, we have investigated the amount of adsorbed hexylsulfonate on the stationary phase and the rate of attainment of equilibrium for the chromatographic system. Using a conductivity detector it was possible to monitor the effluent conductivity versus time. Whatever the nature of the solvent (water, acetonitrile or water/acetonitrile mixture) used to pre-wet the polymeric support, the amount of alkanesulfonate adsorbed was very low (10 µmole for a hexylsulfonate solution 5 mM in water/acetonitrile) and after eluent percolation of 2 mL the value of the effluent conductivity was equal to that of the eluent before percolation. Increasing the hexylsulfonate concentration or increasing the acetonitrile percentage in the mobile phase lead to no significant variation in the adsorbed amount. The total desorption was also very fast. Consequently, when the eluent composition was modified, returning to the inital conditions was easy and very quick.

These results are different from those classically observed in ion pair chromatography. The amount of adsorbed alkanesulfonate on n-alkyl bonded silica is noticeably more important than on the Hamilton PRP-X100 column and increases with an increase in the alkanesulfonate concentration or a decrease in the acetonitrile percentage in the mobile phase.[15] The strong reverse phase interactions between the alkanesulfonate eluent ion and the polymeric matrix were not a problem to carry out an elution gradient. The re-equilibration time needed between gradient runs should be short.

To optimize the conditions for the gradient mode analysis, it was necessary to seek the best column efficiency, to decrease the strong adsorption of the indolylglucosinolates onto the polymeric backbone without losing a good selectivity between each alkenylglucosinolate. The choice of acetonitrile rather than methanol or tetrahydrofuran as organic modifier in the mobile phase provided the best efficiency for all glucosinolates. Two approaches to gradient separation were investigated.

The first approach involved increasing the amount of the organic modifier (acetonitrile), thereby decreasing the retention time of glucosinolates that are retained predominantly by reverse phase such as arylglucosinolate GTL or indolylglucosinolate GBS. This method proved to be of limited utility. UV detection at 229 nm was used for the glucosinolate analysis. At this wavelength, all acetonitrile variation during the gradient run leads to variations in UV absorbance consequently increasing noticeably the background drift. Moreover system peaks and poor system reproducibility were observed.

The second approach was to utilize a concentration gradient in which the concentration of the hexylsulfonate eluent ion in water-acetonitrile (90:10) solution was increased over the run and satisfactory gradients were produced by this method. Figure 2 depicts the elution pattern of five glucosinolates (PRO, SIN, GNA, GTL, GBS) with an hexylsulfonate concentration gradient. The mobile phase gradient was obtained as following: hexylsulfonate 5 mM in water/acetonitrile (90:10) to hexylsulfonate 50 mM in water/acetonitrile (90:10) in 5 min then this composition was maintained for a further 10 min. A satisfactory analysis time for GBS (about 12 min at flow rate 1.5 mL/min) and a good separation of PRO, SIN, GNA are observed. Excellent retention time reproducibility was noted.

Isocratic Elution on a Polymethacrylate Copolymeric Anion Exchanger

The third type of support commonly used in anion exchange chromatography, a polymethacrylate copolymeric anion exchanger was then evaluated as a possible additional column material for the isocratic analysis of glucosinolates. For this study, five natural glucosinolates were selected on the basis of the large diversity of their aglycone part, which can be alkenyl: SIN and GNA, hydroxyalkenyl: PRO, arylalkyl: GTL or indolylalkyl: GBS. The great diversity of the R groups leads to wide variations in the polarity and the hydrophilic and lipophilic characters of these natural compounds. A chromatographic study of GTL and GBS compared to that of GNA should display the possible separation of glucosinolates in accordance with the three

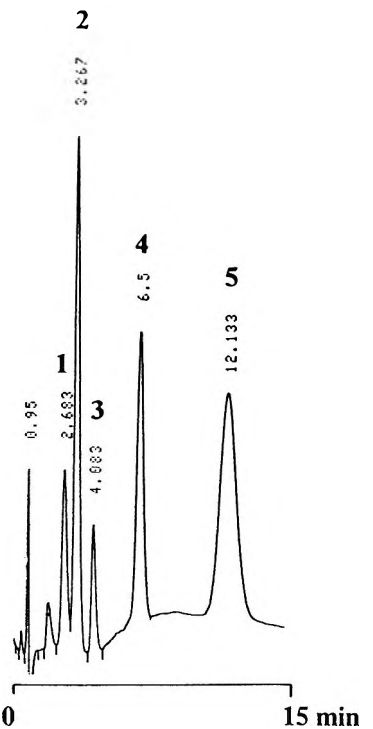

Figure 2. Chromatogram of five glucosinolates with an eluent ion concentration gradient. Column Hamilton PRP-X100 (100 x 4.1 mm I.D.), flow rate 1.5 mL/min and detection UV at 229 nm. Mobile phase gradient: hexylsulfonate 5mM in water/acetonitrile (90:10) to hexylsulfonate 50 mM in water/acetonitrile (90:10) in 5 min then standing this eluent composition to 15 min and returning to hexylsulfonate 5 mM to 17 min.
Elution order: (1) Progoitrin, (2) Sinigrin, (3) Gluconapin, (4) Glucotropaeolin, (5) Glucobrassicin

important subclasses: alkenyl, arylalkyl or indolylalkyl. SIN and GNA are two organic anions which differ only by one methylene group on their aglycone side chain R. By comparing the behaviour of SIN and GNA in different chromatographic systems, it was possible to evaluate and compare the ability of these systems to separate two compounds with a similar polarity. For the rapeseed glucosinolate analysis, the resolution of the PRO-GNA pair is particularly interesting owing to the importance of the ratio of these two major glucosinolates in this cruciferae family.

Table 2

Capacity Factors (k') and Relative Retention (α^a) of Some Glucosinolates on a Shim-pack IC-A1 (100 x 4.6 mm I.D.) Column With Different Anion Eluents at the same Concentration

	Capacity Factor					Relative Retention[a]	
Eluent Ion 2.5mM	k'_{PRO}	k'_{SIN}	k'_{GNA}	k'_{GTL}	k'_{GBS}	$\alpha_{PRO,GNA}$	$\alpha_{PRO,GBS}$
CH_3COO^-	8.11	10.40	*	*	*		
SO_4^{2-}	1.72	2.53	3.87	12.52	36.8	2.25	21.4
citrate, pH=8	0.79	1.08	1.40	3.81	11.21	1.8	14.2
$C_4H_9SO_3^-$	3.37	4.60	5.69	14.66	48.95	1.7	14.5
$C_6H_{13}SO_3^-$	2.67	3.39	4.12	9.54	28.11	1.5	10.5
$C_8H_{17}SO_3^-$	0.75	0.92	1.07	2.26	6.4	1.4	8.5

* compound with k' > 20.

[a] $\alpha_{1,2} = \frac{k'_2}{k'_1}$

Our study was mainly carried out on a Shimadzu Shim-pack IC-A1 polymethacrylate support incorporating a quaternary ammonium base as a functional group. Table 2 gives the capacity factors, k', for the five selected natural glucosinolates using different eluent anions. All eluent ionic strengths were obtained with the same 2.5 mM concentration of salt.

The choice of acetonitrile rather than methanol or tetrahydrofuran provided the best efficiency for all glucosinolates. As in anion exchange chromatography with a silica trialkylammonium exchanger[4] or a PS-DVB copolymeric anion exchanger,[5] the glucosinolates were eluted principally in order of decreasing polarity: alkenyl (PRO, SIN, GNA), arylalkyl (GTL) then indolylglucosinolates (GBS).

More retention and more selectivity have been observed on a Shim-pack IC-A1 column than on a silica anion exchanger for the glucosinolate analysis: Table 2 shows that increasing the length of the aglycone R part by one methylene unit in the case of the two alkenylglucosinolates SIN and GNA is sufficient to bring about a retention variation between these compounds.

Whatever the nature of the eluent ion, this separation has not been observed on a silica anion exchanger.[4] With commonly used monovalent eluent anion (as CH_3COO^- or NO_3^-), it is possible to elute arylalkyl or indolylglucosinolates on a silica anion exchanger[4] but not on a polymethacrylate anion exchanger (Table 2).

Less retention and less selectivity have been observed on a Shim-pack IC-A1 column than on a PS-DVB copolymeric anion exchanger: the elution strength of a divalent eluent anion such as SO_4^{2-} is sufficient to elute the more lipophilic compounds, GTL and GBS on a Shim-pack IC-A1 column but on a Hamilton PRP-X100 column, only the more hydrophilic compound PRO can be eluted under these conditions.

As expected in anion exchange chromatography, Table 2 shows that the elution strength of the different eluent ions is increased in the following order: monovalent eluent ion (CH_3COO^-) then divalent eluent ion (SO_4^{2-}) and then trivalent eluent ion (citrate pH 8). These results are in good agreement with those obtained on a Partisil SAX column and this supports the idea that normal ion exchange was occurring predominantly between the eluent ion and the organic eluted anion in spite of the very low ion exchange capacity of the Shim-pack IC-A1 column (50µeq/g). By increasing the concentration of the sulfate eluent (2.5 mM to 5 mM), the elution strength increases. The sulfate eluent ion at 5 mM concentration enables elution of all glucosinolates within a reasonable analysis time, typically twenty one minutes for GBS with a flow of 1 mL/min, but under these conditions the alkenylglucosinolates are rapidly eluted and are thus poorly separated out.

Since the ion exchange capacity of the Shim-pack IC-A1 column is very low, increasing the concentration of the eluent ion increases rapidly the background level and involves a loss of efficiency. Changing the nature of the eluent anion rather than increasing the concentration of the eluent anion would be better to decrease the glucosinolate retention on a polymethacrylate anion exchanger.

With the eluent anions commonly used in anion exchange chromatography (CH_3COO^-, SO_4^{2-}, citrate) the Shim-pack IC-A1 column produces poor chromatographic performance for glucosinolate analysis. Peaks are badly tailed and the number of theoretical plates are low even for solutes which are not much retained as PRO, SIN and GNA. This is illustrated in Figure 3 which shows the separation resulting on the Shim-pack IC-A1 column when sulfate was used as eluent. However it is interesting to note that the relative retention between PRO and GNA, the two major glucosinolates in rapeseed, is sufficient to consider that the simple chromatographic system

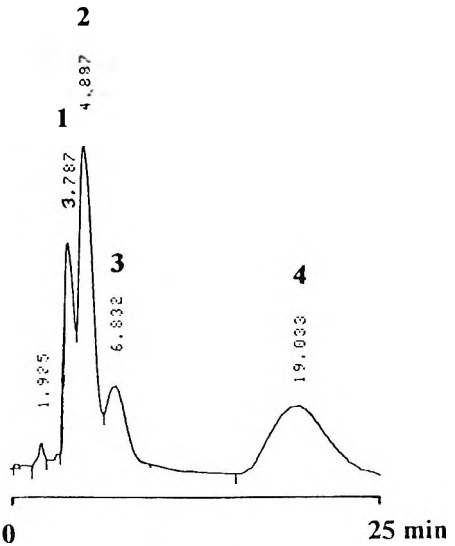

Figure 3. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1 mL/min and detection UV at 229 nm. Eluent: aqueous sulfate solution at 2.5 mM concentration.
Elution order : see Figure 2.

consisting of a Shim-pack IC-A1 column and an aqueous mobile phase containing SO_4^{2-} at low concentration (2.5 mM) is suitable for isolating pure individual progoitrin and gluconapin from natural mixtures. With this system, the concentration of the inorganic eluent anion is about 100-fold lower than the one used with a Hamilton PRP-X100 column.

We then tested the elution strength of borate-gluconate 1 as eluent. This eluent containing boric acid, sodium tetraborate and sodium gluconate acid with acetonitrile and glycerol is the standard eluent composition used with most polymethacrylate-based columns because of its low background conductance and relatively strong elution power.[13,14] Some researchers have suggested that the addition of acetonitrile in this eluent facilitates phase transfer and produces sharper peaks and shorter retention times for the analysis of polarizable anions such as nitrate and bromide.[13,14] With the chromatographic system made up of a Shim-pack IC-A1 column and the borate-gluconate 1 as mobile phase, the alkenylglucosinolates were eluted in the void volume and a very low retention for GTL and GBS was observed. Borate-gluconate 2 was then prepared by

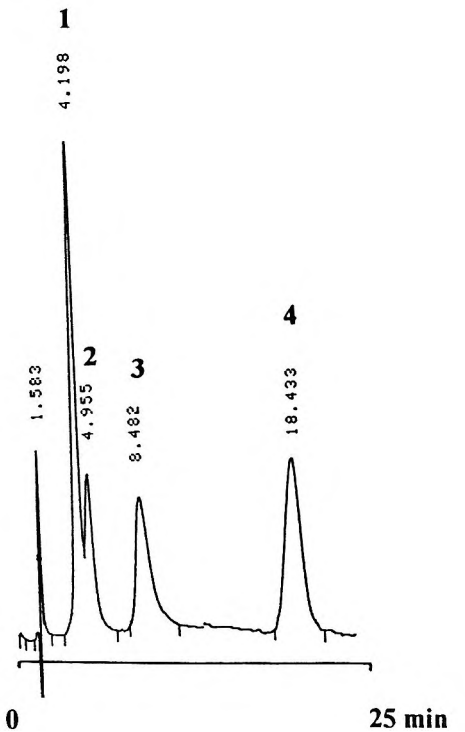

Figure 4. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1 mL/min and detection UV at 229 nm. Eluent borate-gluconate 2 prepared by diluting 1 mL of a concentrate containing 18 g/L boric acid, 25 g/L sodium tetraborate decahydrate, 16 g/L sodium gluconate and 250 mL/L glycerol with 499 mL of water.
Elution order: (1) Progoitrin, (2) Gluconapin, (3) Glucotropaeolin, (4) Glucobrassicin.

diluting concentrate A 500-fold with water and was then tested as eluent in the column. As can be seen in Figure 4, it is possible to separate a mixture of four glucosinolates (PRO, GNA, GTL, GBS) with this eluent and with a better efficiency than with a sulfate eluent ion (Fig. 3). However, the elution strength of borate-gluconate 2 was too strong and consequently a poor separation of alkenylglucosinolates was observed. Lower concentrations of borate-gluconate mixture should permit greater retention of alkenylglucosinolates but the concentration required was so low that deformation of the peak shape was observed as a result of column overloading when glucosinolates were injected.

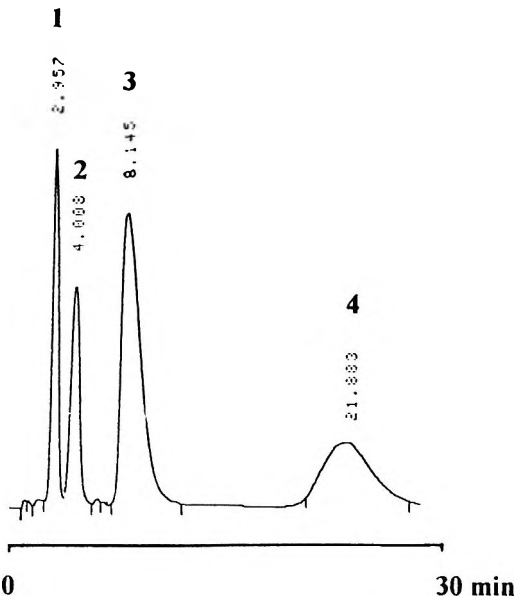

Figure 5. Chromatogram of standard mixture of glucosinolates. Column Shim-pack IC-A1 (100 x 4.6 mm I.D.), flow rate 1.5 ml.min^{-1} and detection UV at 229 nm. Eluent: aqueous hexylsulfonate solution at 2.5 mM concentration.
Elution order: see Figure 4.

In our previous paper,[5] we showed that the use of alkanesulfonate anions with a chain length of at least six carbons, as eluent anions, decreases noticeably the strong reverse phase interactions between the more lipophilic glucosinolates and the PS-DVB polymeric backbone. So, the elution strength of some alkanesulfonate anions on the polymethacrylate support was then tested.

Table 2 gives the capacity factors k' for the five glucosinolates using butylsulfonate, hexylsulfonate or octylsulfonate as eluent anion and Figure 5 depicts the elution pattern of a standard mixture of four glucosinolates (PRO, GNA, GTL, GBS) with an aqueous hexylsulfonate eluent ion at 2.5 mM concentration. It is now possible with these monovalent organic eluent anions in aqueous medium to elute all the glucosinolate subclasses. The separation efficiency was similar to that observed with sulfate anion eluent (Fig.3 and 5).

For a given eluent concentration, as the carbon number, n, of the alkanesulfonate eluent ion increases (n = 4, 6 or 8), the glucosinolate retention decreases (Table 2). The same results were observed for glucosinolate analysis on a Hamilton PRP-X100 column.[5] As expected,[11] solvophobic interaction increased with the carbon number, so the octylsulfonate anion was held more strongly at the ion exchange site than the butylsulfonate anion and thus the ion exchange equilibrium constant of glucosinolates was reduced with increasing carbon number in the eluent ion. The greater elution strength of these alkanesulfonate homologues compared with that of a monovalent organic eluent anion such as acetate can be explained by interactions between the hydrophobic alkyl chain and the polymethacrylate matrix. These interactions are not possible with acetate anions or inorganic anions.

The alkenylglucosinolates are eluted more rapidly with the divalent sulfate eluent than with the monovalent hexylsulfonate eluent whilst a shorter analysis time was observed for the indolylglucosinolates with the hexylsulfonate eluent than with the divalent eluent. These results confirm that the adsorption of the organic analyte ions onto the polymeric backbone was all the stronger as the compound includes a much more apolar aglycone component. On a polymethacrylate anion exchanger, the contribution of the reverse phase interactions with respect to the ion exchange interactions was more reinforced in the indolylglucosinolate than in the alkenylglucosinolate retention mechanism.

Table 2 clearly shows that the separation of the PRO/GNA and PRO/GBS pairs were related to the eluent ion used. The values of the relative retention α for these two pairs decreased as the carbon number n of the alkanesulfonate increased, whereas minor variations for these α values were observed on a PS-DVB polymer-based column. On a polymethacrylate-based column, the nature of the alkane residue of alkanesulfonate is an important parameter not only for decreasing the capacity factors as shown on a PS-DVB polymer-based column but also for increasing the selectivity between two glucosinolates.

For the qualitative analysis of glucosinolate mixtures including different subclasses, the chromatographic system made up of a Shim-pack IC-A1 column and an aqueous mobile phase containing 2.5 mM hexylsulfonate as eluent ion, under isocratic elution mode (Fig. 5) was a convenient chromatographic system which can be used in place of the chromatographic system made up of a column Partisil SAX and an aqueous mobile phase containing dihydrogenocitrate 0.5 mM as eluent ion.[4] The analysis time of the more retained compound (GBS) was similar in these two systems (about twenty minutes) and a better separation for the PRO/GNA and PRO/GBS pairs was observed on the polymethacrylate-based column.

CONCLUSION

For glucosinolate analysis, more retention and better selectivities were observed on a polymethacrylate anion exchanger than on a silica anion exchanger. The importance of the reverse phase interactions with respect to the ion exchange interactions in the glucosinolate retention mechanism was reinforced on a polymethacrylate-based column in comparison with a silica-based column and lessened in comparison with a PS-DVB copolymeric anion exchanger. Satisfactory separation between each glucosinolate subclass was observed in about 20 min on a Shim-pack IC-A1 column with an aqueous hexylsulfonate solution or an aqueous borate-gluconate mixture as mobile phase in an isocratic mode. The chromatographic system, made up of a column Shim-pack IC-A1 and an aqueous mobile phase containing SO_4^{2-} is suitable to isolating pure individual alkenylglucosinolates. To decrease the stong adsorption of the indolylglucosinolates onto the PS-DVB polymeric backbone without losing a good selectivity between each alkenylglucosinolate, an eluent ion concentration gradient on a Hamilton PRP-X100 column could be used. A good separation and a satisfactory analysis time for natural mixtures composed of various glucosinolates greatly differing in substituent nature could be obtained under these conditions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CONJUGATED AND UNCONJUGATED 3-OXO-Δ^4- AND 3-OXO-$\Delta^{4,6}$-BILE ACIDS IN HUMAN URINE

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ABSTRACT

A method for simultaneous determination of conjugated and unconjugated C_{24}-bile acids having 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-structures in human urine has been developed by high performance liquid chromatography coupled with dual wavelength UV detection. Satisfactory resolution was attained on a C_{18} reversed phase column by gradient elution with acetonitrile - sodium acetate within 50 min. The linearity of the calibration curve for each bile acid ranged from 10 pmol to 500 pmol per injection, and the detection limit was 2 - 4 pmol (SN = 5).

This method was used for direct analysis of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids present in the urine of healthy infants and patients with liver disease, after only a preliminary clean-up procedure using Bond Elut C18.

INTRODUCTION

Recently, a novel disorder in bile acid biosynthesis, named '3-oxo-Δ^4-steroid 5β-reductase deficiency, was described by Setchell et al.[1] and Clayton et al.[2] It is characterized by markedly elevated urinary levels of the amidates of 3-oxo-Δ^4-bile acids, 7α-hydroxy-3-oxo-4-cholenoic (CDCA-Δ^4-3-one) and 7α, 12α-dihydroxy-3-oxo-4-cholenoic (CA-Δ^4-3-one) acids (Fig. 1), and has been identified in more than twelve infants with liver diseases such as neonatal hepatitis and cholestasis.[1-5]

As an abnormal presence of these bile acids in urine reflects this disorder, their measurement in urine is important from a clinical point of view. We have also identified and determined these unusual bile acids in the urine from patients with severe cholestasis.[6,7]

The separation and quantification of these bile acids is carried out mainly by gas chromatography - mass spectrometry (GC-MS).[1-7] These procedures, however, have some disadvantages, such as tedious clean-up and insufficient information concerning the conjugation mode of the bile acids. Moreover, 3-oxo-Δ^4-bile acids are considered to be thermally unstable, and to be easily converted into their dehydrated products, 3-oxo-$\Delta^{4,6}$-bile acids, or into unknown degradation products, under the alkaline or acidic conditions usually used for deconjugation in the GC-MS method.

Therefore, high performance liquid chromatography (HPLC), which makes prior deconjugation unnecessary, and enables direct analysis of polar and thermally unstable biological substances, appears to be suitable for the separation and determination of bile acid conjugates.

This paper describes a simple method developed for the simultaneous quantitation of conjugated and unconjugated 3-oxo-Δ^4-bile acids and their dehydrated products, 3-oxo-$\Delta^{4,6}$-bile acids. The application of this method to the determination of these bile acids in urine from both healthy infants and patients with liver diseases is also discussed.

MATERIALS AND METHODS

Materials

CDCA-Δ^4-3-one, CA-Δ^4-3-one, 3-oxo-4,6-choladienoic (CDCA-$\Delta^{4,6}$-3-one) and 12α-hydroxy-3-oxo-4,6-choladienoic (CA-$\Delta^{4,6}$-3-one) acids were chemically synthesized as reported previously.[8-10] Glycine-conjugated bile acids and internal standards (IS) were synthesized according to the known methods using 2,2,2-trichloroethyl esters of the corresponding amino acids.[11-13]. Taurine-conjugated bile acids were prepared in the manner previously reported.[11] Bond Elut C18 cartridges were obtained from Varian (Harbor City, CA, U.S.A). All other reagents were of analytical grade.

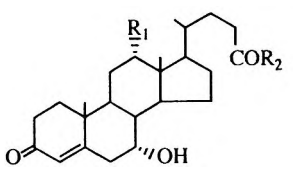
Urine Samples

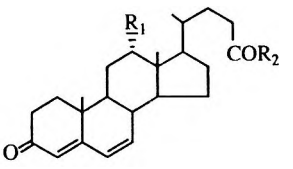
Urine samples were collected without preservatives from four patients (1 - 2 months old) with liver disease, who showed an abnormality in 3-oxo-Δ^4-steroid 5β-reductase by GC-MS analysis, and from ten healthy infants (1 - 2 months old) as a control. All specimens were stored at -25°C until analysis.

Apparatus and Chromatographic Conditions

The HPLC apparatus consisted of a Shimadzu LC-10A system equipped with an SPD-10A spectrophotometer, which was set at a dual wavelength mode (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on an Inertsil ODS2 (250 x 4.6 mm i.d., 5 μm, GL Sciences, Tokyo, Japan) column using a gradient elution mode at a flow rate of 1.0 mL / min. The column temperature was ambient. Mobile phase A was 30 mM sodium acetate - acetonitrile (80 : 20, v/v) adjusted to an apparent pH 4.2 with phosphoric acid, and mobile phase B was acetonitrile.

The gradient program was as follows: isocratic elution with mobile phase A for 4 min, then a linear gradient to 50 % of mobile phase B over a period of 31 min, and a discontinuous gradient to 65 % of mobile phase B. The absorbance of the eluent was monitored with dual wavelengths at 245 nm for 3-oxo-Δ^4-bile acids and at 280 nm for 3-oxo-$\Delta^{4,6}$-bile acids. The void volume (t_0) was measured with sodium nitrate (UV 210 nm).[14]





CDCA-Δ^4-3-one	: R_1 = H,	R_2 = OH	CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = OH
CA-Δ^4-3-one	: R_1 = OH,	R_2 = OH	CA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = OH
G-CDCA-Δ^4-3-one	: R_1 = H,	R_2 = $NHCH_2CO_2H$	G-CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = $NHCH_2CO_2H$
G-CA-Δ^4-3-one	: R_1 = OH,	R_2 = $NHCH_2CO_2H$	G-CA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = $NHCH_2CO_2H$
T-CDCA-Δ^4-3-one	: R_1 = H,	R_2 = $NH(CH_2)_2SO_3K$	T-CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = $NH(CH_2)_2SO_3K$
T-CDCA-Δ^4-3-one	: R_1 = OH,	R_2 = $NH(CH_2)_2SO_3K$	T-CDCA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = $NH(CH_2)_2SO_3K$
IS1	: R_1 = OH,	R_2 = $NH(CH_2)_2CO_2H$	IS2	: R_1 = OH,	R_2 = $NH(CH_2)_2CO_2H$

Figure 1. Structures of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids.

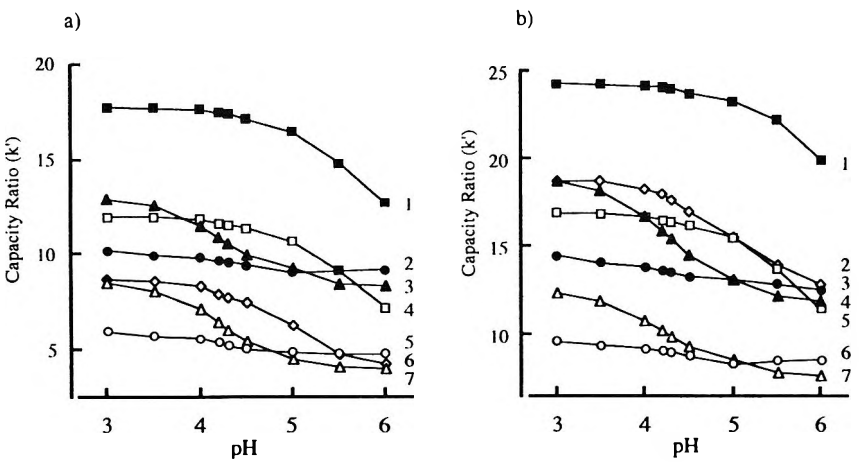


Figure 2. Effect of pH of mobile phase on capacity ratios (k') of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids. (a) 1 : CDCA-Δ^4-3-one, 2 : T-CDCA-Δ^4-3-one, 3 : G-CDCA-Δ^4- 3-one, 4 : CA-Δ^4-3-one, 5 : T-CA-Δ^4-3-one, 6 : IS1, 7 : G-CA-Δ^4-3-one, (b) 1 : CDCA-$\Delta^{4,6}$-3-one, 2 : IS2, 3 : T-CDCA-$\Delta^{4,6}$-3-one, 4 : G-CDCA-$\Delta^{4,6}$-3-one, 5 : CA-$\Delta^{4,6}$-3-one, 6 : T-CA-$\Delta^{4,6}$-3-one, 7 : G-CA-$\Delta^{4,6}$-3-one. IS1 : N-(7α, 12α-Dihydroxy-3-oxo-4-cholen-24-oyl)-3-aminopropionic acid. IS2 : N-(3-Oxo-4,6-choladien-24-oyl)-3-aminopropionic acid.

Analysis of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$- Bile Acids

To a urine sample (0.1 - 4.0 mL), appropriate amounts of the internal standards were added, and the solution was loaded on a Bond Elut C18 cartridge. The cartridge was washed with 4 mL of water, and the bile acids were eluted with 4 mL of methanol. After evaporation of the solvent under reduced pressure at less than 30°C, the residue was dissolved in methanol, and subjected to HPLC analysis.

RESULTS AND DISCUSSION

Separation of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$-Bile Acids

The chemical structures of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids examined and their abbreviations are shown in Fig. 1. 3-Aminopropionic acid amidates of CA-Δ^4-3-one and CDCA-$\Delta^{4,6}$-3-one, which were not present in human biological fluids and exhibited the same absorption maxima and molar absorptivity, were used as internal standards.

Exact control of pH in the mobile phase is essential for high resolution. Therefore, the effect of the mobile phase pH on the capacity ratio (k') values of unconjugated and glycine- and taurine-conjugated bile acids was initially examined on a reversed phase C_{18} column with a mixture of acetonitrile and sodium acetate. Acetonitrile gradient elution was employed in order to obtain sharp and symmetrical peaks, and complete separation of the fourteen 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acid conjugates including internal standards within a shorter period of time. Changes in the k' values in response to changes in the apparent pH of the initial mobile phase are shown in Fig. 2. As suggested by previous findings,[15-17] pH greatly influences the k' values of unconjugated and glycine-conjugated bile acids. In the lower pH region, a better separation of all the bile acids can be achieved, although the bile acids except for taurine conjugates, exhibit long retention times. The result of various combinations of pH and the acetonitrile gradient program showed that an apparent pH of 4.2 for the initial mobile phase provided good resolution of all the bile acids. The concentration of sodium acetate also affected on the k' values of glycine and taurine conjugates as was shown in previous reports,[15,17,18] although it did not affect the retention time of unconjugates or the peak shape of each bile acid. A higher resolution of all the bile acids was obtained with a salt concentration of over 30 mM.

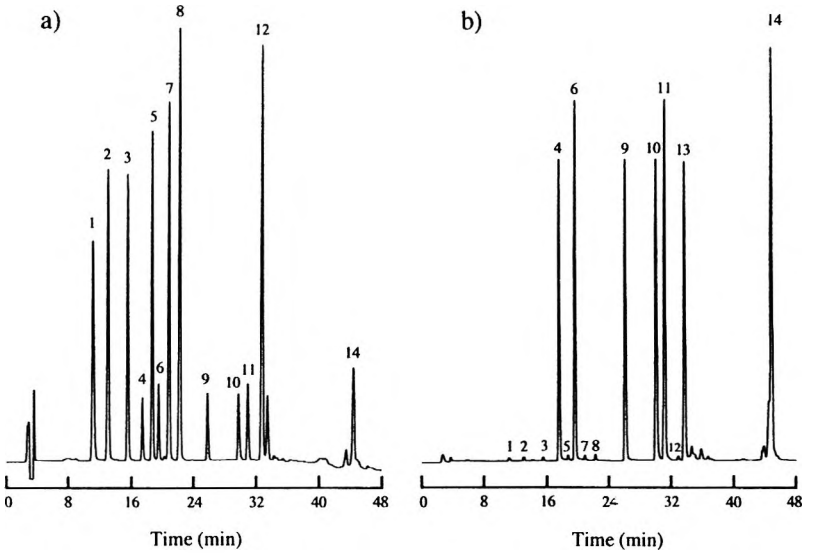


Figure 3. Typical high-performance liquid chromatograms of the standard 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids. (a) 1 : T-CA-Δ^4-3-one, 2 : G-CA-Δ^4-3-one, 3 : IS1, 4 : T-CA-$\Delta^{4,6}$-3-one, 5 : T-CDCA-Δ^4-3-one, 6 : G-CA-$\Delta^{4,6}$-3-one, 7 : G-CDCA-Δ^4-3-one, 8 : CA-Δ^4-3-one, 9 : T-CDCA-$\Delta^{4,6}$-3-one, 10 : G-CDCA-$\Delta^{4,6}$-3-one, 11 : CA-$\Delta^{4,6}$-3-one, 12 : CDCA-Δ^4-3-one, 13 : IS2, 14 : CDCA-$\Delta^{4,6}$-3-one.

From these results, a gradient of acetonitrile in the starting eluent consisting of 30 mM sodium acetate - acetonitrile (80 : 20) adjusted to apparent pH 4.2 was used in the present study. The optimal analytical conditions are as described in **MATERIALS AND METHODS**.

Typical chromatograms obtained using authentic specimens of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids were recorded simultaneously at wavelengths of 245 nm and 280 nm (Fig. 3). All the bile acids were completely separated from each other without noticeable peak asymmetry within 50 min. The retention of the bile acid species increased in the following order : CA-Δ^4-3-one < CA-$\Delta^{4,6}$-3-one < CDCA-Δ^4-3-one < CDCA-$\Delta^{4,6}$-3-one regardless of whether the bile acids were conjugated or not. When the bile acids were conjugated, the ordering of the k' values was taurine conjugates < glycine conjugates < unconjugates. Reproducible retention times were obtained for all the bile acids under the present HPLC conditions. The calibration curves were constructed by plotting the relative peak area of each bile acid to the internal standard against the amounts of the corresponding bile acid. A good linear relationship to each

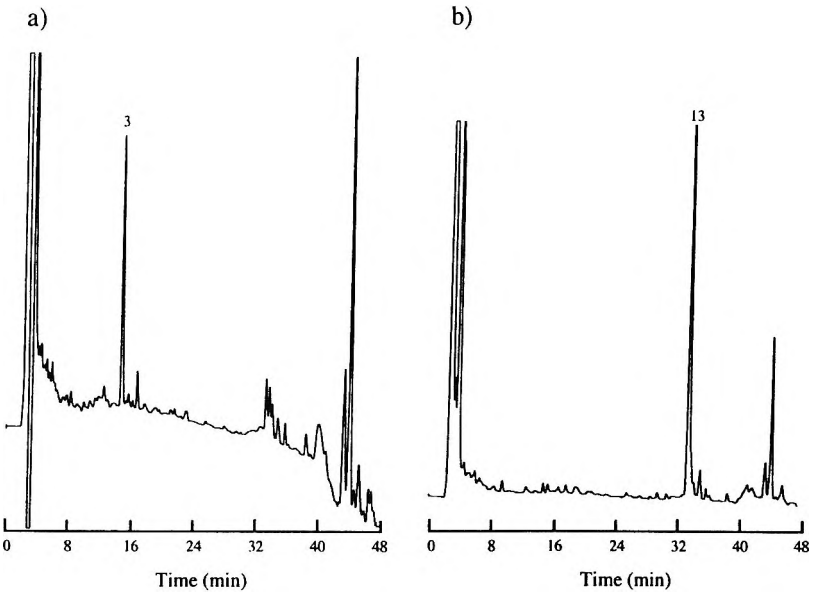


Figure 4. High performance liquid chromatograms of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids in urine from a healthy infant. Peak identity is the same as in Fig. 3.

Table 1

Recoveries of Conjugated Bile Acids Added to Urine

Bile Acid[a]	Relative Recoveries (%, n=5)		
	Unconjugate	**Glycine**	**Taurine**
CA-Δ^4-3-one	97.5 ± 2.4[b]	103.4 ± 1.3	99.0 ± 1.8
CDCA-Δ^4-3-one	102.2 ± 0.9	98.8 ± 1.3	100.2 ± 1.6
CA-$\Delta^{4,6}$-3-one	98.2 ± 2.5	101.3 ± 1.8	99.5 ± 2.8
CDCA-$\Delta^{4,6}$-3-one	101.3 ± 3.4	97.2 ± 3.2	98.3 ± 3.3

[a] See Fig. 1 for abbreviations.

[b] Each value represents Mean ± S.D. of the recovery to that of IS.

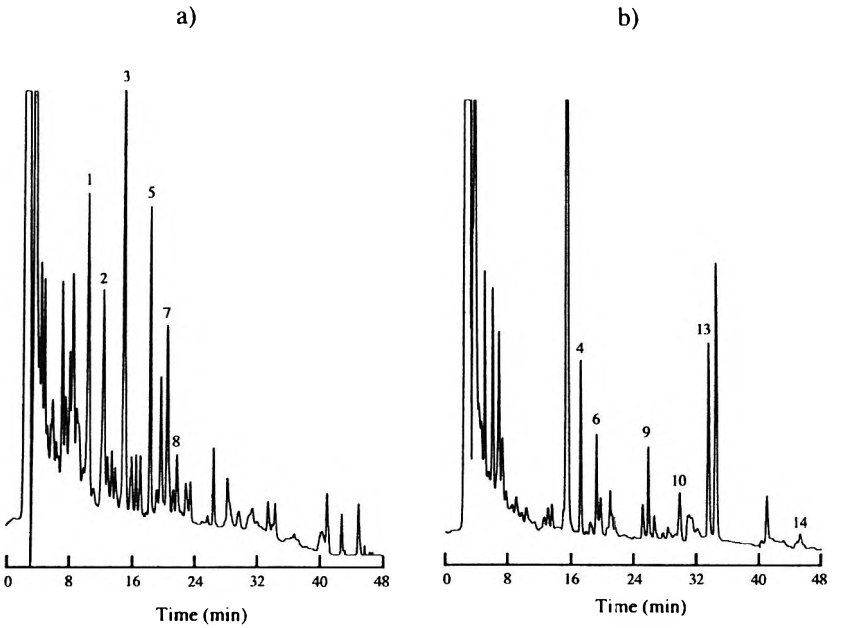


Figure 5. High performance liquid chromatograms of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids in urine from a patient with liver disease. Peak identity is the same as in Fig. 3.

bile acid was obtained over the range of 10 - 500 pmol with linear correlation coefficients of more than 0.999 for all the bile acids. The coefficients of variation for the measurement of 20 pmol of each standard bile acid were less than 3 %. The detection limits of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids were estimated to be *ca.*4 and 2 pmol (signal-to-noise ratio = 5), respectively.

Determination of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$ Bile Acids in Infant Urine

The HPLC method was applied to the determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in urine samples from healthy infants and from patients with liver disease, in which an abnormality in 3-oxo-Δ^4-steroid 5β-reductase was suggested.[6,7] Urine samples were submitted to a preliminary clean-up procedure using conventional C_{18} reversed phase extraction prior to HPLC analysis. The recovery rates through the clean-up procedure described in **MATERIALS AND METHODS** were tested by adding pre-determined

Table 2

Concentration of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$-Bile Acids in Urine from Normal Children and Patients with Liver Disease

	Concentration (nmol/mL)				
Bile Acid[a]	**Pl**[b]	**P2**	**P3**	**P4**	**Normal**[c]
CA-Δ^4-3-one	8.0	3.14	4.47	1.54	0.03 ± 0.03
G-CA-Δ^4-3-one	29.0	6.39	7.42	4.16	0.08 ± 0.12
T-CA-Δ^4-3-one	31.4	7.17	1.58	0.34	0.05 ± 0.03
CDCA-Δ^4-3-one	n.d.	0.51	1.18	0.08	n.d.[d]
G-CDCA-Δ^4-3-one	25.1	6.85	15.10	0.47	n.d.
T-CDCA-Δ^4-3-one	23.0	13.50	6.23	0.53	n.d.
CA-$\Delta^{4,6}$-3-one	2.61	0.08	0.20	0.20	n.d.
G-CA-$\Delta^{4,6}$-3-one	5.03	0.54	0.50	0.23	0.04 ± 0.02
T-CA-$\Delta^{4,6}$-3-one	7.56	1.18	0.31	0.24	0.02 ± 0.02
CDCA-$\Delta^{4,6}$-3-one	n.d.	0.18	0.15	0.13	n.d.
G-CDCA-$\Delta^{4,6}$-3-one	3.47	0.46	0.71	0.16	n.d.
T-CDCA-$\Delta^{4,6}$-3-one	4.04	3.15	1.02	0.07	n.d.
Total	139.2	43.15	38.87	8.15	0.22

[a] See Fig. 1 for abbreviations.
[b] Number of patients, P: Patient.
[c] Each value represents Mean ± S.D. (n=10).
[d] Not detected.

amounts of conjugated and unconjugated 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids (each 200 μg) to steroid-free urine (1.0 mL) prepared by charcoal extraction.[19] As shown in Table 1, all the bile acids were recovered at a rate of more than 97 %, and the coefficients of variation were less than 4 % (n=5). Decomposition or dehydration of the 7α-hydroxy group were not observed in the clean-up procedure.

Figures 4 and 5 show typical chromatograms obtained from the urine of a normal infant and a patient with liver disease, respectively. A marked difference in the bile acid profiles was observed between the normal infant and

liver disease patient. The peaks of almost all of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in Fig. 5 were identified on the basis of their retention times. The results obtained from four liver disease patients and ten normal infants are summarized in Table 2. The means of the total concentrations of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in urine of the normal 1 - 2 month-old infants were estimated to be 0.16 and 0.06 nmol / mL, respectively.

On the other hand, all the bile acid levels in urine from the liver disease patients were significantly higher than those in normal subjects, accounting for 7.12 - 116.5 nmol / mL for 3-oxo-Δ^4-bile acids and 1.03 - 22.71 nmol / mL for 3-oxo-$\Delta^{4,6}$-bile acids. The glycine and taurine amidates of CA-Δ^4-3-one and CDCA-Δ^4-3-one were the predominant components in liver disease patients, accounting for more than *ca.* 70 % of the total amounts of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids. The proportion of 3-oxo-$\Delta^{4,6}$-bile acids to total bile acids ranged from 7 to 16 %.

The proposed method, coupled with dual wavelength UV detection, is suitable for the simultaneous determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in routine clinical analysis. Further studies on the urinary excretion of these bile acids in patients with hepatobiliary disease using this method are in progress.

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SOLID-PHASE EXTRACTION OF PERPHENAZINE FROM BLOOD SERUM FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

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ABSTRACT

A solid-phase extraction (SPE) process has been developed with the aim of replacing multistage liquid-liquid extraction that is currently used for sample clean-up before the chromatographic determination of perphenazine (PPZ) in serum.

The method comprises the trapping of PPZ from 2 mL of serum by 100 mg of silica gel with polymeric C_{18} phase, followed by rinsing of the endogenous compounds with water and acetonitrile. The drug is eluted with 0.7 mL of methanol and the eluate is evaporated to dryness under stream of air. The residue is dissolved in 100 μL of methanol and 20-μL aliquot is injected onto an HPLC column packed with silica gel modified with cyanopropyl groups. To control the retention and separation of PPZ from the other compounds, ammonium acetate concentration in the mobile phase consisting of 90% v/v methanol has been varied from 10 to 40 mmol/L.

The SPE method enabled 86.2+5.0% of PPZ to be recovered which is 12% to 22% more than by liquid-liquid extraction procedure. The repeatability of 5.7% (rel.) and the limit of detection (S/N=3) of 1.3 ng/mL have been attained. The method has been verified on serum samples from patients medicated with PPZ.

INTRODUCTION

The currently used neuroleptics show undesirable side effects if their doses are too high. However, their clinical efficacy seems to be related to sufficiently high plasma concentrations. Optimal therapeutic range may differ for each patient and, besides, varies in the course of the disease.[1] Plasma monitoring of the antipsychotic drugs is required for their more effective and safer use.

Perphenazine, 4-[3-(2-chloro-phenothiazine-10-yl)propyl]-1-piperazine-ethanol, (PPZ) belongs to the most potent phenothiazine antipsychotic compounds. These drugs are typically analyzed by chromatographic methods, i.e., by HPLC or, to a lesser extent, by GC.

At present, separation of fourteen, mostly phenothiazine drugs by packed (cyanopropyl) column supercritical fluid chromatography has been studied.[2] Sample clean-up prior to chromatographic determination of PPZ in blood serum is inevitable.

Perphenazine and its dealkylated metabolite were separated on an HPLC column packed with octadecylsilanized silica gel by using a mobile phase consisting of methanol, water, dichloromethane, and ammonia.[3] For the determination of PPZ in serum, a cyanopropyl column and the mobile phase containing 90% v/v of methanol and 10% v/v of 10 mmol/l ammonium acetate were employed.[4]

Sample clean-up was performed by multistep liquid-liquid extraction, a cumbersome and time consuming process with recoveries of 70%,[3] or 64.3+6.8 to 74.3+5.7%.[4]

In this work, a method for simple and rapid analysis of PPZ in human plasma is presented. The method consists in a solid phase extraction of PPZ followed by an HPLC determination with UV detection.

EXPERIMENTAL

Reagents and Solutions

Stock solution of perphenazine (a pharmaceutical preparation with minimum content of 98.5% PPZ, Léčiva Prague, Czech Republic), 1 mg/mL, and its working solutions (0.1 and 0.01 mg/mL) were prepared in methanol and stored under refrigeration.

Aqueous solution of EXAPAT, a stabilized lyophilized human serum (Imuna Šarišské Michalany, Slovakia) was used in SPE method development. Lyophilized serum and its solutions were stored under refrigeration. The solutions were not older than 10 days. Patient sera were stored in a freezer not longer than 12 days. Two mL of the EXAPAT solution (as a blank or spiked with PPZ) or patient serum were applied onto SPE cartridge as a sample.

Isothermally distilled ammonia, redistilled acetonitrile, and triethylamine distilled under reduced pressure were used. All other reagents were of analytical grade (Lachema, Czech Republic).

HPLC mobile phases were prepared in a volume flask by diluting the volume of an aqueous solution of ammonium acetate with methanol. To prepare the solution of ammonium acetate, 0.1 mol/L acetic acid was neutralized with ammonia to pH 7.25 and diluted with water.

Apparatus

The HPLC equipment comprised of a model 8500 syringe pump (Varian, USA), a model RH 7125 injection valve (Rheodyne, USA) with 5-, 10- or 20-μL sample loop, a multiple wavelength detector, HP 1050, equipped with 1-μL cell (Hewlett-Packard, Japan) and a TZ 4520 recorder (Laboratory Instruments Prague, Czech Republic). CGC glass columns 150x3 mm i.d. and 30x3 mm i.d., packed with Separon SGX CN silica gel with cyanopropyl groups with particle size of 5 and 7μm, respectively, were used as the analytical column and guard column (all Tessek Prague, Czech Republic). The mobile phase was delivered at the flow rate of 0.5 mL/min.

The absorption curve of PPZ solution in 90% v/v methanol possessed maxima at 203 nm and 257 nm, the latter with slightly lower (91% rel.) absorbance value. HPLC detection of PPZ was performed at 257 nm.

Acidity was measured with a model OP-208 pH-meter equipped with an OP-0808-P glass-Ag/AgCI combined electrode adjusted by means of standard buffers of pH 2.1 and 7.0 (Radelkis, Hungary).

Silica-cart cartridges (Tessek Prague, Czech Republic) packed with 0.6 g of 60-μm oktadecylsilanized spherical silica gel and 1-mL Bakerbond-spe columns (Baker, USA) dry repacked with 100 mg of the sorbent were used in SPE method development. A Separon SGX RPS sorbent of polymeric character with the carbon content of 24% was utilized except some preliminary experiments where the Silica-cart cartridges were packed with Separon SGX C_{18} with the carbon content of 18% (Tessek Prague, Czech Republic). The Silica-cart cartridges were processed by means of a syringe, the Bakerbond-spe columns by using a model Baker spe-12G System vacuum manifold (Baker, USA), both at the flow rate of 1 mL/min.

RESULTS AND DISCUSSION

HPLC Determination

The concentration of PPZ was determined by a modification of previously published HPLC method.[4] The HPLC column was packed with silica gel with bonded cyanopropyl phase. Due to the basic character of PPZ molecules, their interactions with residual silanols played an important role in the retention mechanism of PPZ. As a consequence, the variation of ammonium acetate concentration in the mobile phase between 10 and 40 mmol/L enabled the retention of PPZ and, also, its separation from the other compounds to be optimized.

The effect of ammonium acetate concentration on PPZ retention is illustrated in Fig. 1. Because of a low solubility of PPZ in aqueous media, the content of methanol in the mobile phase was kept constant at 90% v/v.

To determine the concentration of PPZ in SPE eluents, calibration curves based on peak heights were used. The curves measured for five standard solutions, four injections of each, were linear in the range from 0,001 to 10 μg/mL PPZ with correlation coefficient ranging typically from 0,9997 to 0,9999 and with a negligible value of y-intercept. In the analysis of a patient sera, PPZ standard was injected before and after the sample and the results were evaluated by external standard method.

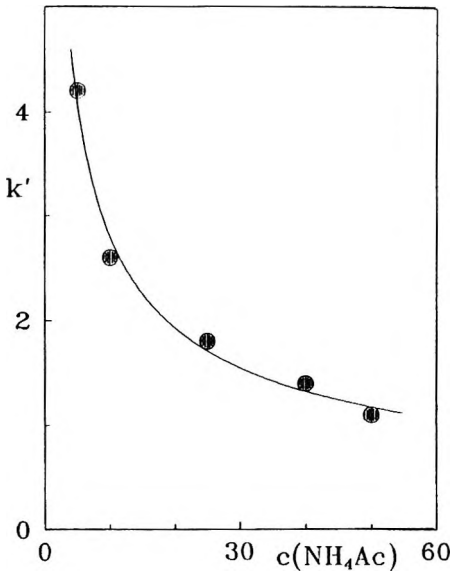

Figure 1. Perphenazine capacity factor as a function of ammonium acetate concentration, mmol/L, in the mobile phase. Column 150x3 mm i.d. and guard column 30x3 mm i.d., packed with 5-μm and 7-μm silica gel with cyanopropyl groups. Mobile phase: ammonium acetate in 90% v/v methanol, flow 0.5 mL/min. Samples: 5 μL of 0.4 μg/mL perhenazine in methanol.

SPE Method Development

Silica-cart cartridges were conditioned with 2 mL of methanol and, consecutively, with 1 mL of water. Two mL of standard serum as a blank or spiked with PPZ were passed through the cartridge and this was rinsed with 2 mL of water and, in some experiments, also with 2 mL of acetonitrile. In the next step, elution of matrix components or that of PPZ were studied by using various solvents or solutions. PPZ elution profile was generated by passing 1-mL volumes of the solvent through a single cartridge. To evaluate the elution of the sample matrix, chromatograms of blank samples were compared mutually.

The application of the cartridges packed with Separon SGX C_{18} was unsuccessful because of high retention of PPZ and band broadening. Good results were attained when the sorbent was replaced by Separon SGX RPS with polymeric C_{18} phase and higher carbon content and, thus, with lower concentration of residual silanols that could interact with PPZ molecule. After

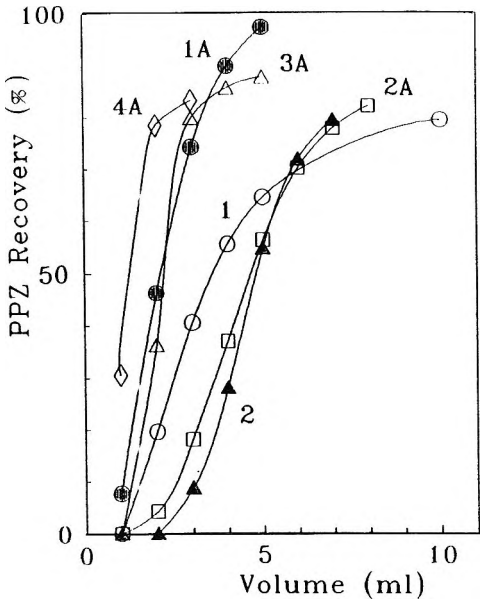

Figure 2. Elution profiles of perhenazine. Silica-cart cartridges packed with 0.6 g of Separon SGX RPS. Conditioning: 2 mL of methanol, 1 mL of water. Sample application: 2 mL of standard serum spiked with 1 μg/mL PPZ. Rinsing: 2 mL of water and, at the curves indicated with A, also 2 mL of acetonitrile. Elution: methanol (curves 1 and 1A); 1 mmol/L triethylamine in 90% v/v methanol (2, 2A); 10 mmol/L triethylamine in 90% v/v methanol (3A) and in 100% methanol (4A).

rinsing the cartridge with water, the matrix components were efficiently washed out with 2 mL of acetonitrile. It was verified that PPZ remains retained even when the cartridge is rinsed with 15 mL of acetonitrile. After rinsing step with acetonitrile, methanol was found as the most effective PPZ eluent as can be shown on the curves of cumulative % recovery vs. solvent volume in Fig. 2.

Aiming at the reduction of the excessive amount of sorbent used in the Silica-cart cartridges, these were replaced by 1-mL Bakerbond-spe columns repacked with 100 mg of Separon SGX RPS. The solid phase was conditioned with 1 mL of methanol and 0.5 mL of water. Endogenous compounds were washed out with 1 mL of water and 1 mL of acetonitrile after sample application. At these conditions, 2 mL of the standard serum spiked with 0.2 μg/mL PPZ was applied onto the SPE column and the drug recovery was estimated after elution with methanol, three measurements at each volume.

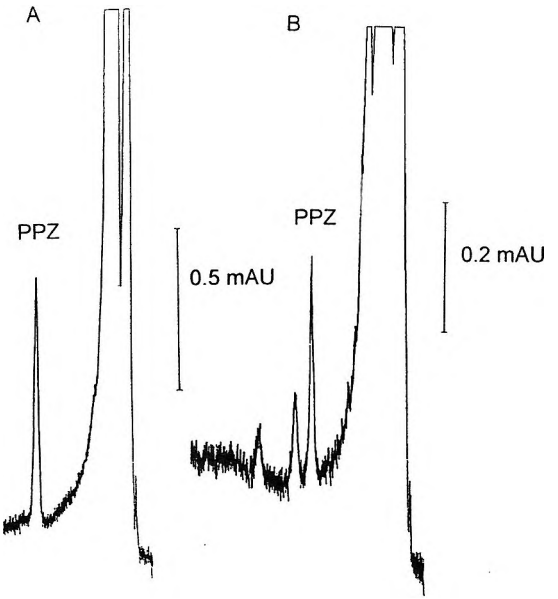

Figure 3. Chromatograms of standard serum spiked with PPZ and patient serum. Chromatogram A: 2 mL of standard human serum spiked with 10 ng/mL PPZ; concentration of ammonium acetate in the mobile phase 10 mmol/L; PPZ retention time 7.7 min. Chromatogram B: 2 mL of patient serum taken 4 hours after medication with a single dose of 16 mg of PPZ (0.35 mg/kg) and a daily dose of 25 mg of thioridazine. Other conditions as in A.

With 0.5 mL of methanol, 95.1% of PPZ was recovered. Further, practically 100% recovery was achieved by elution with 0.6 mL (99.3%), 0.7 (99.7%), and 0.8 mL (100.8%). The volume of 0.7 mL of methanol was found as sufficient to elute PPZ from the column. The recommended therapeutic range is 0.8 to 2.4 ng/mL PPZ in serum however, values as much as 12 ng/mL PPZ can be found because patients are frequently medicated with high doses of the drug.[5] In following SPE experiments, the PPZ concentration in serum was lowered to 10 ng/mL. The methanolic eluate was evaporated to dryness under stream of air and reconstituted with 100 μL of methanol. The volume of the solution injected onto HPLC column was increased to 20 μL, i.e. to a maximum sample volume that still did not cause band broadening and peak shape deformation. For five determinations of PPZ, the recovery of 86.2 ± 5.0% and the repeatability of 5.7%, expressed as relative standard deviation, were found. The observed decrease of the recovery from 99.7% to 86.2% is partly due to the preconcentration step (ca. 5% of the total difference of 13.5% as verified by

experiments with evaporation of 10 ng/mL PPZ methanolic solutions and dissolution of their residues), partly it could be explained by losses in the sample processing because these can have greater significance when working with PPZ concentrations in low nanogram range. The limit of detection, 1.3 ng/mL, based on a signal to noise ratio S/N=3 was comparable with the value of 0.75 ng/mL given in literature[4] and could be improved by using better quality HPLC column.

The method was verified on serum samples from patients medicated with perphenazine and, simultaneously, with some other pharmaceutics. No interferences caused by the presence of the other pharmaceutics were observed. In Fig. 3, chromatogram of standard serum sample spiked with PPZ and that of patient serum are introduced. The SPE method developed represents rapid and more efficient alternative to recently used liquid-liquid extraction of perphenazine from serum samples.

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SEPARATION AND DETERMINATION OF ORGANOGERMANIUM COMPOUNDS BY ION CHROMATOGRAPHY

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ABSTRACT

For nearly three decades, organogermanium compounds have become increasingly of interest owing to their extensive physiological and pharmaceutical activity. In this paper, two new high performance ion chromatographic methods for separation and determination of three kinds of organogermanium compounds β-carboxyethylgermanium sesquioxide (I), β-(α-methyl)-carboxyethylgermanium sesquioxide (II) and d-(β-carboxyethyl)germanium hydroxide (III) were proposed. A Dionex DX-300 ion chromatograph equipped with a Dionex PED- II pulsed electrochemical detector (conductivity mode), and a Dionex AI-450 chromatography workstation was employed. The separation was achieved by using ion-exchange or ion-exclusion mechanism.

The detection limits(S/N=3, expressed as germanium) for the three compounds were all below sub- μg/mL level. The methods have been applied to the analysis of tonic oral drinks, and the average recoveries for the three compounds range from 95 - 108%. The results obtained were in agreement with those of hydride generation atomic fluorescence spectrometry (HG-AFS).

INTRODUCTION

Since the late 1960s, the study on organogermanium compounds has been receiving considerable attention. It has been proved that many types of organogermanium compounds possess various kinds of biological activity: analgesic, hypotensive, fungistatic, bactericidal, antiviral, antimalarial, radioprotective, antitumor, interferon-inducing, and immunomodulating.[1] Among them, β-carboxyethylgermanium sesquioxide (Ge-32) was probably the most important compound that has been used as antitumor agent and immune adjuvant for clinical application. It was also approved to add in some kinds of tonic oral drinks in Japan and China due to its very low toxicity. In addition, its analogs such as β-(α-methyl)carboxyethylgermanium sesquioxide have similar bioactivity.[2] Further study revealed that the effects of various organogermanium compounds including β-carboxyethylgermanium sesquioxide and β-(α-methyl)carboxyethylgermanium sesquioxide on some enzymes were somewhat different.[3] In recent years, Chinese scholars have synthesized many kinds of β-carboxyethylgermanium sesquioxide analogs,[4] among which di-(β-carboxyethyl)germanium hydroxide is one of the latest species.[5] It probably has more potential than β-carboxyethylgermanium sesquioxide in clinical application owing to its better solubility in water as well as in organic solvents such as ethanol and acetone.

So far, the majority of the methods for determining organogermanium compounds were in relation to β-carboxyethylgermanium sesquioxide. The indirect method was based on determination of Ge^{4+} after acid digestion.[6] A thin layer chromatographic method coupled with fluorodensitometry detection was proposed for determining the purity of β-carboxyethylgermanium sesquioxide raw material,[7] but it is not suitable for real sample analysis. For the analysis of real drinks samples containing β-carboxyethylgermanium sesquioxide, four methods were reported , one was based on solvent extraction[8] and another on separation by using Dowex 1-X2 resin;[9] the quantifications were all carried out by atomic absorption spectrometry after acid digestion. In another study, we have also found that β-carboxyethylgermanium sesquioxide can not react with tetrahydroborate in acid media. On the basis of this,

inorganic germanium was determined directly and total germanium was determined after digestion by using hydride generation atomic absorption spectrometry (HG-AAS) or hydride generation atomic fluorescence spectrometry (HG-AFS); the difference of the two values was the amount of β-carboxyethylgermanium sesquioxide.[10,11] The HG-AFS method has been verified through inter-laboratory collaboration tests and applied to the investigation on β-carboxyethylgermanium sesquioxide content in tonic oral drinks held by Ministry of Public Health, P. R. China.[12] Recently, Zhang et al.[13] reported a simple differential pulse polarographic method for determination of β-carboxyethylgermanium sesquioxide based on its forming complex with 3,4-dihydroxy benzaldehyde. Considering the similar syntheses' method, possible transformation in organisms and different toxicity of β-carboxyethylgermanium sesquioxide analogs,[14] it is necessary to establish a method for the separation and determination of β-carboxyethylgermanium sesquioxide analogs. In this paper, two ion chromatographic methods for separation and determination of β-carboxyethylgermanium sesquioxide (Ⅰ) and its two analogs - β-(α-methyl)carboxyethylgermanium sesquioxide (Ⅱ) and di-(β-carboxyethyl)germanium hydroxide(Ⅲ) were proposed, and applied to the analysis of tonic oral drinks. The results obtained were in good agreement with those of HG-AFS.[12] These methods will possibly be used to quality control in manufacture process and study of metabolism in organisms for these three analogs.

MATERIALS AND METHODS

Apparatus

All experiments were performed on a Dionex Model DX-300 ion chromatograph equipped with an 110-μL sample loop and a Dionex Model PED-Ⅱ pulsed electrochemical detector in the conductivity detection mode. A Dionex AI-450 chromatography workstation was employed for data acquisition, data reduction and control of the ion chromatograph.

The ion-exclusion separation was achieved by using a Dionex IonPac ICE -AS6 column as separation column and 0.075 mmol/L heptafluorobutyric acid as eluent. A Dionex HPICE-AS1 column was used for comparison. The flow rates of eluents were 1.0 mL/min for IonPac ICE-AS6 column and 0.8 mL/min for HPICE-AS1 column, respectively. Chemical suppression was effected by a Dionex AMMS-ICE suppressor with 8.0 mmol/L potassium hydroxide as regenerant flowing at a rate of 1.8 mL/min. For the ion-exchange method, the

gradient program separation was carried out by using a Dionex IonPac AG4A-SC guard column and a Dionex IonPac AS4A-SC separation column. A Dionex AMMS-1 anion micromembrane suppressor was employed, 1.5 mmol/L and 10 mmol/L sodium tetraborate solutions were chosen as eluents, 50 mmol/L sulfuric acid solution as regenerant. The flow rates of eluent and regenerant were set at 1.0 mL/min and 3.0 mL/min, respectively.

Reagents

β-carboxyethylgermanium sesquioxide (purity: 99.4%) was synthesized by Guangzhou Institute of Military Medicine, China. β-(α-methyl) carboxyethylgermanium sesquioxide (purity > 99%) and di-(β-carboxyethyl)germanium hydroxide (purity > 99%) were both synthesized by Changchun Institute of Applied Chemistry of Academia Sinica. The stock solutions (1 mg/mL, expressed as germanium) of organogermanium compounds were separately prepared by dissolving appropriate amounts in hot water. Working solutions were prepared by diluting the stock solutions with water for ion-exchange separation and relevant eluent for ion-exclusion separation. The other reagents were of analytical reagent grade or higher purity except for heptafluorobutyric acid (Pfaltz & Bauer, Inc.) for which purity was 97%. Distilled deionized water was used throughout.

Procedure

A solution containing ^{I}, ^{II}, and ^{III} was injected into the ion chromatograph via a syringe. All calculations were based on peak area measurements. The amount of organogermanium compounds was expressed as germanium.

RESULTS AND DISCUSSION

The molecular structures of the three organogermanium compounds in this study are illustrated in Fig.1a,[5,15] and they can all dissolve in aqueous solution that exist as the form of organic acids[5,16] shown in Fig.1b. The pKa value of ^{I} was reported as 4.26.[17] Though the pKa value of ^{II} was not clear, the existence of α-methyl in ^{II} makes a slight difference of pKa values between ^{I} and ^{II}; to be exact, the acidity of ^{II} is relatively weaker since methyl has repulsive force against electron.

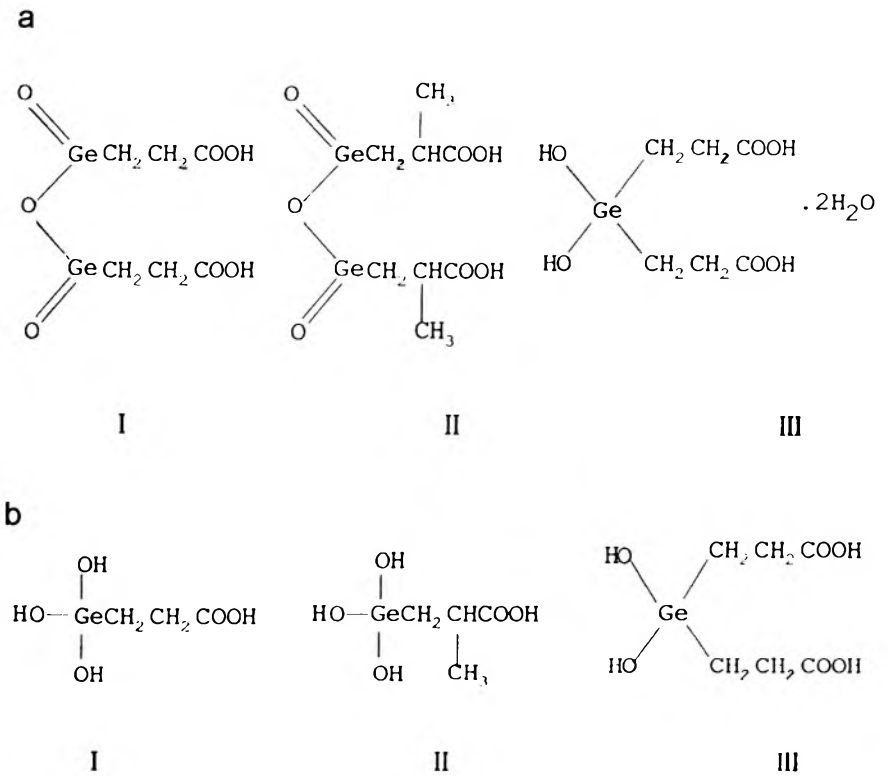

Figure 1. The molecular structures (a) and existing forms in aqueous solution (b) of three organogermanium compounds.

Preliminary study showed that the pKa1value and pKa2 value of [III] are 2.52 and 4.30, respectively.[5] So it was possible to separate one another by either ion-exchange method or ion-exclusion method. The detailed experimental conditions will be discussed separately in the following sections.

Ion-Exclusion Method

In general, ion-exclusion method is commonly used for separation of weakly ionized anions including organic acids and inorganic weak acids. For practical work, it is the most important to choose appropriate separation column and eluent. In this study, two separation columns (HPICE-AS1 and IonPac ICE-AS6) were used for comparison to decide which one can give better performance. The relatively slower flow rate of eluents for HPICE-AS1 is to

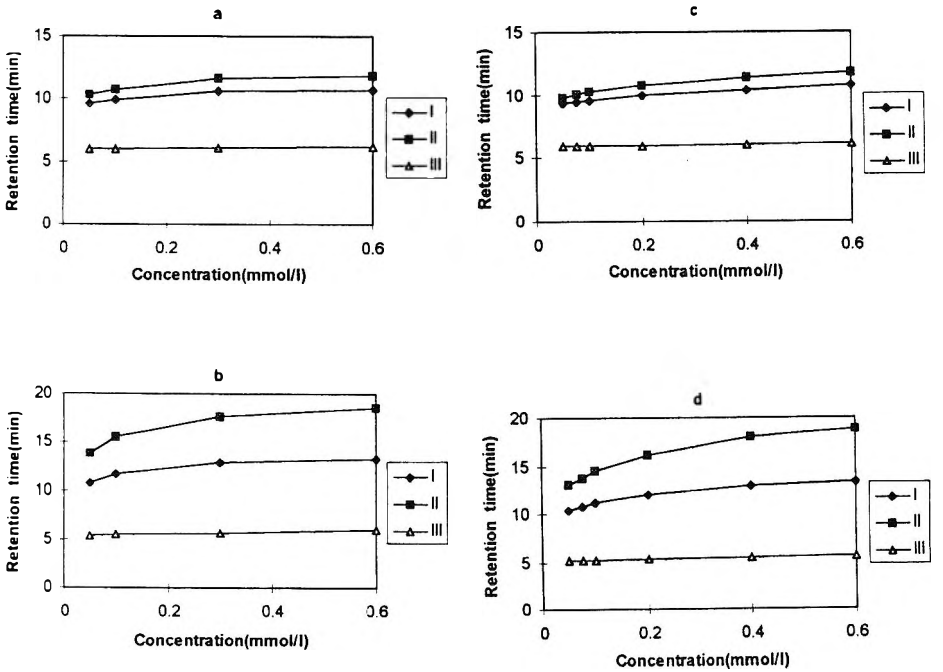

Figure 2. Effect of eluent concentration on retention time. (a) HPICE-AS1 column, sulfuric acid eluent; (b) IonPac ICE-AS6 column, sulfuric acid eluent; (c) HPICE-AS1 column, heptafluorobutyric acid eluent; (d) IonPac ICE-AS6 column, heptafluorobutyric acid eluent.

maintain not-too-high column pressure. In addition, different concentrations of sulfuric acid and heptafluorobutyric acid were employed for eluent selection. The relationships between retention time and eluent concentration by using various columns are shown in Fig.2.

It is well known that in ion-exclusion chromatography, the higher the pKa value of analyte the greater the retention. As expected, the three compounds were eluted out in sequence of ^{III}, ^{I}, ^{II}, and the retention time of all compounds increased by increasing the eluent concentration (Fig.2). It can be also observed from Fig. 3 that the baseline separation of ^{I} and ^{II} was not achieved on HPICE-AS1 column by using different concentrations of eluents although the relatively optimum eluent condition was found to be 0.1 mmol/L sulfuric acid or 0.2 mmol/L heptafluorobutyric acid. The cases of poor resolution for ^{I} and ^{II} may be the slight difference of acidity and poor efficiency of ion-exclusion stationary phase containing only sulfonic acid groups.

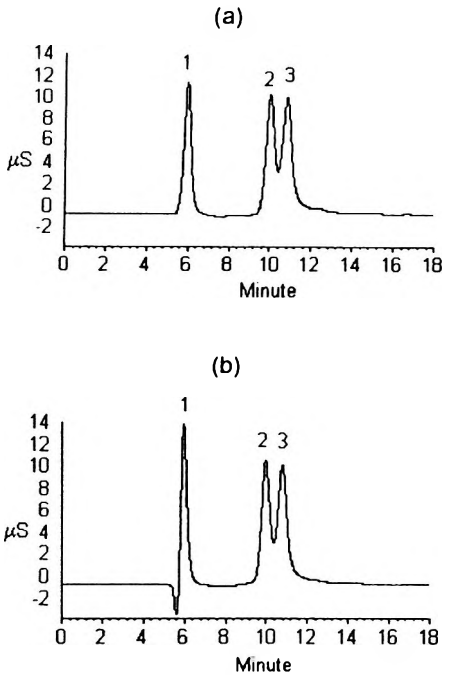

Figure 3. Separation of organogermanium compounds on HPICE-AS1 column. Peaks: 1 = ^{III} (20 μg/mL); 2= ^{I} (50 μg/mL); 3= ^{II} (50 μg/mL). Eluents: (a) 0.2 mmol/L heptafluorobutyric acid; (b) 0.1 mmol/L sulfuric acid.

Compared with HPICE-AS1 column, the IonPac ICE-AS6 column has hydrophobic functional groups within the resin structure which promote adsorption and hydrogen bonding besides sulfonic acid groups. These two additional retention mechanisms allow resolution of organic acids which are poorly resolved by ion-exclusion alone. In fact, as shown in Fig. 4, it was utilization of this separation method that reach the baseline resolution of the three compounds. Hence, the further study was carried out on the IonPac ICE-AS6 column.

In view of eluent, heptafluorobutyric acid is the typical choice in ion-exclusion chromatography due to the low conductivity of the product after chemical suppression which resulted in low detection limit, but the higher price restricts its intensive application. The cheaper sulfuric acid was used for comparison with heptafluorobutyric acid in this study, and the baseline separation of three compounds can be achieved with both of the acids as shown in Fig. 4.

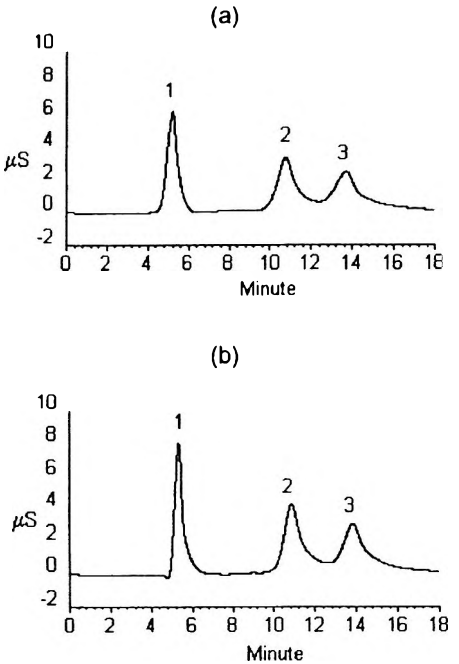

Figure 4. Separation of organogermanium compounds on IonPac ICE-AS6 column. Peaks as in Fig. 3. Eluents: (a) 0.075 mmol/L heptafluorobutyric acid; (b) 0.05 mmol/L sulfuric acid.

The further study on eluents concentration indicated that ^{I} and ^{II} would be partially overlapping (Rs<1.5) if the acid concentrations were too low, that is to say, the concentrations of heptafluorobutyric acid and sulfuric acid were below 0.07 mmol/L and 0.04 mmol/L, respectively. When the acid concentrations were too large, the peaks of ^{I} and ^{II} would become tailed peaks. Though the addition of organic modifier could improve the peak shapes of ^{I} and ^{II}, the resolution of ^{I} and ^{II} became poor because the decrease of retention time of ^{II} was greater than that of ^{I}, which can be explained by the stronger hydrophobicity of ^{II}. The optimum experiment conditions should meet two aspects of demands, baseline separation (Rs>1.5) and run time as short as possible. So, the eluent concentrations adopted were 0.075 mmol/L for heptafluorobutyric acid and 0.05 mmol/L for sulfuric acid, respectively, and heptafluorobutyric acid was preferred for real sample analysis because an apparent negative water peak would appear very near to the peak of ^{III} resulting in an inaccurate result by using sulfuric acid.

Ion-Exchange Method

It is also possible to analyze many organic acids by using anion-exchange method. Under the typical basic anion-exchange eluent conditions, the three organogermanium compounds existed as monovalent anions for ^{I}, ^{II}, divalent anion for ^{III}, respectively. It was proved by experiments that the commonly used eluents such as sodium carbonate, sodium carbonate/sodium bicarbonate and sodium bicarbonate could not give good separation for ^{I} and ^{II} which could be explained by weak acidity, identical charge and structure similarity of ^{I} and ^{II}, as well as high ionic strength of these eluents. The separation of ^{I} and ^{II} was improved by using sodium tetraborate eluent, and their baseline resolution was achieved when the concentration of eluent was as low as 1.5 mmol/L. Contrary to ion-exclusion method, the elution order was ^{II}, ^{I} and ^{III}. Since ^{III} exists as divalent anion in basic solution, the retention would be greater on stationary phase. Therefore, though three organogermanium compounds were separated by 1.5 mmol/L sodium tetraborate, the whole run time would last 70 min and the peak of ^{III} would be too flat to be identified and quantitatively measured. A better solution to this problem was utilization of a gradient program which consisted of two eluents: 1.5 mmol/L sodium tetraborate (E1) and 10 mmol/L sodium tetraborate (E2). The separation was completed within 40 min.

Because the maximum flow rate of regenerant used for AMMS-1 suppressor must not be higher than 3.0 mL/min, the suppression ability was limited. To avoid severe change of baseline, 10 mmol/L sodium tetraborate was adopted as the higher ionic strength eluent. The detailed gradient program was listed in Table 1, and the chromatogram was shown in Fig. 5. It needed about 10 min for equilibration prior to another injection.

Linearity, Precision, and Detection Limits

Under the optimum experimental conditions of both methods, the three compounds all showed good linearity. The detection limits, which are defined as the concentrations that give the peak intensity three-fold the baseline noise, were also calculated. The results are summarized in Table 2. It can be found that the detection limits obtained by using ion-exchange method are lower than those by ion-exclusion method though its run time is much longer.

For ion-exclusion method, the relative standard deviations for seven replicated analyses of a mixed standard solution containing 50 μg/mL ^{I}, 50 μg/mL ^{II} and 10 μg/mL ^{III} were 1.74 % for ^{I}, 1.97% for ^{II} and 2.45 % for ^{III}, respectively.

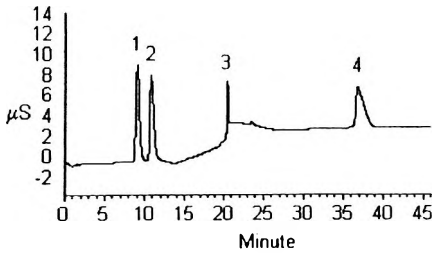

Figure 5. Separation of organogermanium compounds on IonPac AG4A -SC and IonPac AS4A-SC columns. Peaks: 1= ^{II} (20 μg/mL); 2= ^{I} (20 μg/mL); 3= system peak; 4= ^{III} (5 μg/mL).

Table 1

Gradient Program for Ion-Exchange Method

Time (Min)	E1 (%)	E2 (%)
0	100	0
11.0	100	0
20.5	0	100
45.0	0	100
45.1	100	0

For ion-exchange method, the relative standard deviations for seven replicated analyses of another mixed standard solution which contained 20 μg/mL ^{I}, 20 μg/mL ^{II} and 10 μg/mL ^{III} were 1.27 % for ^{I}, 2.87% for ^{II} and 1.22% for ^{III}, respectively.

Sample Analysis

Two kinds of tonic oral drinks which contained ^{I} (declared on labels by manufacturers) were centrifuged at 4000 rpm for 10 min to remove the suspension; 0.25 mL supernatant of sample 1 or 0.50 mL supernatant of sample 2 was transferred accurately into a 25 mL volumetric flask, and diluted to volume with 0.075 mmol/L heptafluorobutyric acid for ion-exclusion or water for ion-exchange separation. The diluted solution was injected after filtration through a 0.2 μm Gelman filter.

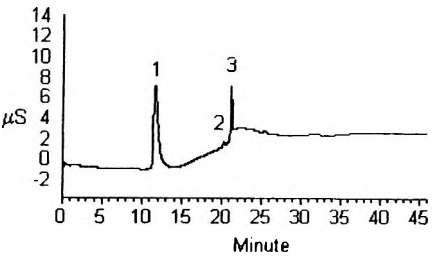

Figure 6. Chromatogram of sample 1 diluted solution on IonPac AG4A -SC and IonPac AS4A-SC columns. Peaks: 1= [I]; 2= chloride; 3= system peak.

Table 2

Linearity and Detection Limits for Organogermanium Compounds

Method	Analyte	Concentration Range (μg/mL)	Correlation Coefficient	Detection Limit (μg/mL)
Ion-exclusion	I	4 - 100	0.9994	0.14
	II	6 -100	0.9984	0.24
	III	1- 20	0.9999	0.053
Ion-exchange	I	2-50	0.9992	0.038
	II	2-50	0.9979	0.035
	III	1-20	0.9997	0.025

Since the most important synthesis path of [I] and its analogs was hydrolysis of relevant chlorides,[14,16] the real samples may contain chloride ion. The peak of chloride appeared in a chromatogram of real samples when using ion-exchange method (shown in Fig.6). When using ion-exclusion method, the chloride eluted out very near void volume, which interfered with the determination of [III].

So in this method, the real samples should be pretreated by using Dionex OnGuard-Ag Cartridge to remove chloride prior to dilution. As shown in Fig.7, the unknown peak which appeared in the chromatogram did not interfere with the determination of [III] in spiked sample.

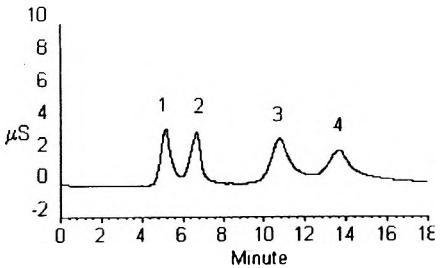

Figure 7. Chromatogram of spiked sample 1 diluted solution on IonPac ICE-AS6 column. Peaks: 1= [III]; 2= unknown; 3= [I]; 4= [II]. The spiked concentrations: [I] 20 μg/mL; [II]40 μg/mL; [III]10μg/mL.

Table 3

Analysis of Organogermanium Compounds in Tonic Oral Drinks

Sample	Analyte	Concentration (μg/mL)[a]		Added (μg/mL)	Recovery (%)[b]		Result of HG-AFS (μg/mL)[c]	Label Claim (μg/mL)
		Exclusion	Exchange		Exclusion	Exchange		
1#	I	21.59±0.19	21.29±0.44	20	107.8±3.69	95.35±1.20	20.38±0.39	21.40±1.07
	II	ND	ND	40	97.92±2.31	101.0±1.54	----	----
	III	ND	ND	10	98.36±1.08	96.79±0.61	----	----
2#	I	24.42±0.48	25.74±0.14				24.86±0.46	----
	II	ND	ND				----	----
	III	ND	ND				----	----

[a] Average of five determinations ± standard deviation.
[b] Average of four determinations ± standard deviation.
[c] Average of three determinations ± standard deviation.

The recoveries of the methods were determined in quadruplicate by spiking standard solutions in 1:100 diluted sample 1 solutions using both methods.

The results obtained are shown in Table 3; they were consistent with those of HG-AFS[12] and the manufacturer's label claim.

CONCLUSIONS

Two procedures have been developed for the separation and determination of three kinds of organogermanium compounds with ion chromatography. Either method is suitable for the analysis of tonic oral drinks and will probably be applied for metabolism study in future.

ACKNOWLEDGMENT

The authors thank the Dionex Corporation for support.

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A SIMPLE METHOD TO DETERMINE FREE AND GLYCOSYLATED VITAMIN B_6 IN LEGUMES

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ABSTRACT

A simple high performance liquid chromatography (HPLC) method for the determination of pyridoxamine, pyridoxal, pyridoxine and glycosylated pyridoxine in legumes was described. Samples were extracted with trichloroacetic acid and incubated with acid phosphatase and β-glucosidase enzymes. The analytical separation was achieved by isocratic elution (methanol:potassium phosphate buffer) in an octadecylsilica column within 13 min. High recoveries and precision were obtained. This procedure provides a good alternative to previous methods for the determination of vitamin B_6 in legumes.

INTRODUCTION

Vitamin B_6 is the name given to 3-hydroxy-2-methylpyridine derivatives that exhibit biological activity of pyridoxine in rats.[1] It occurs naturally in foods as pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their corresponding 5'-phosphates.

In certain plant foods, besides these biologically active vitamers, a remarkable content of glycosylated forms of PN (G-PN), including 5'-O-(β-D-glucopyranosyl) pyridoxine as the principal fraction, have been found.[2,3] The nutritional significance of these forms is related to their low bioavailability compared to B_6 vitamers.[4,5,6]

The measurement of vitamin B_6 in foods, constitutes a complicated analytical problem due to the existence, at relatively low levels, of multiple forms of this vitamin. The validity of methods depends mostly, on their particular application.[7,8] Some procedures developed for the quantification of B_6 vitamers exhibit several disadvantages for their use in routine work: lengthy procedure time in microbiological methods[9] or sophisticated gradient elution[10] and long retention times [11] in HPLC methods. In the case of quantification of glycosylated forms of PN, some HPLC methods have been reported for the direct analysis of 5'-O-(β-D-glucopyranosyl) pyridoxine,[12,13] however due to the existence of different glycosylated forms and the absence of readily obtainable standards for them, the indirect quantification of these forms as free PN constitute a better approach to measurement of the glycosylated species.[14]

Despite all the existing methods, there is very little information on the quantification of vitamin B_6 in certain complex food matrices such as legumes, which are known to be one of the best sources of this vitamin.[15] Most vitamin B_6 content data of legumes found in the literature, have been obtained by microbiological assay after thermal extraction in acidic media.[16,17] These conditions cause hydrolysis of glycosylated derivates of the vitamin, resulting in an overestimation of the nutritionally available vitamin B_6 content in legumes.[7] The effectiveness of many of the existing HPLC methods for determining the B_6 vitamers,[18,19] when they are applied to complex matrix food like legumes, have not been fully assessed, and many problems can appear, especially due to interferences with the matrix compounds.

The objective of this study was to evaluate each vitamer and the glycosylated forms of vitamin B_6 in legumes, developing a simple method which could be used in routine work. For this purpose the extraction procedure and the HPLC conditions were optimized. The extraction of vitamers B_6 from lentils, chick peas, and haricot beans was carried out using a chemical deproteinating agent, and enzymatic hydrolysis with acid phosphatase enzyme in order to determine the sum of the free and phosphorylated forms of each B_6 vitamer. Glycosylated pyridoxine was quantified indirectly as free PN released by enzymatic treatment of legume extracts with β-glucosidase. The quantification of B_6 vitamers were carried by HPLC in isocratic mode after the adequate pH adjustment of the eluate, in a post-column reaction coil, in order to avoid fluorescence interferences from impurities present in legume extracts.

MATERIAL AND METHODS

Chemicals and Reagents

Methanol HPLC grade was obtained from Lab-Scant Ltd. (Dublin, Ireland). Acid phosphatase from potato (0.4 U/mg specific activity), β-glucosidase from almonds (14 U/mg specific activity), pyridoxamine dihydrochloride (PM-2HCl), pyridoxal hydrochloride (PL-HCl), pyridoxine hydrochloride (PN-HCl) and pyridoxal 5'-phosphate (PLP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pyridoxamine 5'-phosphate hydrochloride (PMP-HCl) were obtained from Merck (Darmstadt, Germany).

Water was purified for chromatographic use with a Milli-Q system (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA) and for other procedures with a Milli-Ro system (Waters Chromatography Division). All other chemicals were of analytical grade.

Samples

Lentils (*Lens culinaris*, c.v. "castellana"), chick peas (*Cicer arietinum*, c.v. "blanco lechoso"), and haricot beans (*Phaseolus vulgaris*, c.v. "riñon") were purchased from a local market. The seeds were ground to pass 0.18 mm sieve and stored in the dark until analysis. Five replicates of each sample were analyzed to provide B_6 vitamer concentration data.

Extraction Procedure

0.4 g of lentils, chick peas, or haricot beans flour were homogenized with 40 mL 5% (w/v) trichloroacetic acid (TCA) for 30 min, filtrated through No. 40 Whatman filter paper and levelled with Milli-Ro water to 50 mL. An aliquot of the filtrate (5 mL) was transferred to a test tube and the pH was adjusted to 4.8 with 4M sodium acetate. 0.4 mL of 6.0 mg/mL aqueous solution of potato acid phosphatase was added and samples were incubated with shaking for 5 h at 37°C in a water bath. The reaction was quenched by the addition of 1.2 mL of 20% (w/v) TCA.

Upon cooling to ambient temperature, an aliquot was filtered through a 0.22 μm pore size nylon filter membrane and analyzed by HPLC. The extract was stored at 6°C for 2 days.

To hydrolyse the glycosylated forms of PN, 1.5 mL of a 1.25 mg/mL aqueous solution of β glucosidase were added to the previously described extract, prior pH adjustment to 5.0. Samples were incubated at 37 °C for 5 h in a shaking water bath. The reaction was quenched with 1.2 mL of 20% (w/v) TCA. An aliquot was filtered through a 0.22 μm pore size nylon filter membrane and analyzed by HPLC. The extract was stored at 6°C for 2 days.

Chromatographic Conditions

Determination of vitamin B_6 by HPLC was carried out using a modular chromatograph (Waters Associates, Milford, MA, USA), equipped with a Model M 510 pump, two Model M 45 pumps, a Rheodyne sample injector with a 50 μL loop and a Waters 470 scanning fluorescence detector (λ_{Ex} 328 nm, λ_{Em} 390 nm). Data were processed on a PC (NEC Corporation, Boxborough, MA, U.S.A).

The analytical column was a ODS2 Spherisorb, 10 μm, 300 mm x 3.9 mm i.d. (Sugelabor S.A, Madrid, Spain) with a guard column containing C_{18} Porasil B, 20 mm x 3.9 mm i.d. (Waters Associates).

The mobile phase consisted in a mixture of methanol and 0.033M potassium phosphate buffer pH 2.2 (2:98, v/v). The flow rate was 1.2 mL/min and the column temperature was 17 °C.

The acidic pH of the eluate was adjusted to pH 7 ± 0.5 in a post-column reaction coil with 0.3 M dipotassium hydrogen phosphate solution pumped at a flow rate of 0.7 mL/min from a second pump.

A mixture methanol:water (30:70), delivered from a third pump, was used to clean retained substances in the chromatographic column between injections. The cleaning step lasted 5 min and the conditioning of the column with mobile phase another 5 min.

Standard Solutions

Individual stock standard solutions were prepared by dissolving 4.2 mg of PM-2HCl, 14.1 mg of PL-HCl, 9.7 mg of PN-HCl, 5.0 mg of PMP-HCl and 7.0 mg of PLP in 100mL Milli-Q water and adding 0.5 mL of orthophosphoric acid. These stock solutions were stable for 2 months stored in the dark at 4°C.

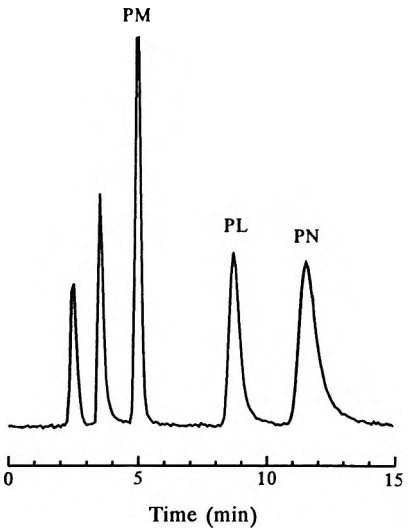

Figure 1. HPLC chromatogram of processed working standard mixture containing pyridoxamine (PM = 12 ng/ml), pyridoxal (PL = 24 ng/ml) and pyridoxine (PN = 34 ng/ml).

Mixed working standard solutions were prepared daily by transferring appropriate volumes of PM, PL and PN stock solutions to 100 mL conical flasks, diluting with 40 mL 5% (w/v) trichloroacetic acid in Milli-Ro water and subjected to the same extraction procedure used with samples.

The overall concentrations ranges for each vitamer were 0.8-12 ng/mL for PM, 0.8-24 ng/mL for PL, and 1-34 ng/mL for PN. Calibration curves were obtained by plotting areas versus concentration of processed working standard solutions.

Recovery Experiments

Vitamer recoveries were determined by spiking the legume samples with standard vitamers (0.69 μg/g PM, 1.35μg/g PL, 0.59 μg/g PN, 0.52 μg/g PMP, 0.82 μg/g PLP) before extraction. Recoveries were calculated by comparing the difference between the measured concentration in the spiked and non spiked samples respect to amount of standard B_6 vitamers added. Phosphorylated vitamers were recovered as their respective unphosphorylated forms.

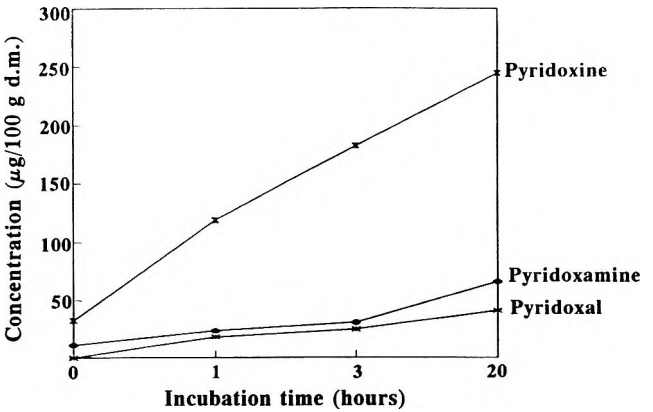

Figure 2. Effect of Takadiastase enzyme on B_6 vitamers content of lentils.

RESULTS AND DISCUSSION

In Figure 1, a chromatogram of processed PM, PL and PN standards is shown. Good resolution of the three B_6 vitamers in a relatively short time (13 min) was obtained. The retention times (min) were 4.98 ± 0.01 for PM, 8.69 ± 0.01 for PL and 11.52 ± 0.02 for PN. The limit detection of vitamers ranges between 0.2-0.4 ng/mL.

Peak areas were linearly related to the amount injected over the range investigated for each vitamer (0.04-0.60 ng/injection for PM, 0.04-1.20 ng/injection for PL and 0.06-1.70 ng/injection for PN). Correlation coefficients obtained for these calibration curves were always > 0.990.

Preliminary studies to hydrolyze the phosphorylated B_6 vitamers in legumes were carried out using Takadiastase enzyme.[20] As indicated in Figure 2, very high increases of PN was obtained after the enzymatic procedure (212 μg/100g), and since phosphorylated PN in foods occurs in very low amounts,[20] it was clear that in legumes some others forms of PN were hydrolyzed by the Takadiastase enzyme.

Figure 3 shows the results obtained with variable amounts of acid phosphatase enzyme to hydrolyse the phosphorylated B_6 vitamers of legumes and the plateau that was reached with 2.4 mg of enzyme. The increases of PN after the enzymatic hydrolysis ranged between 0-27 μg/100g.

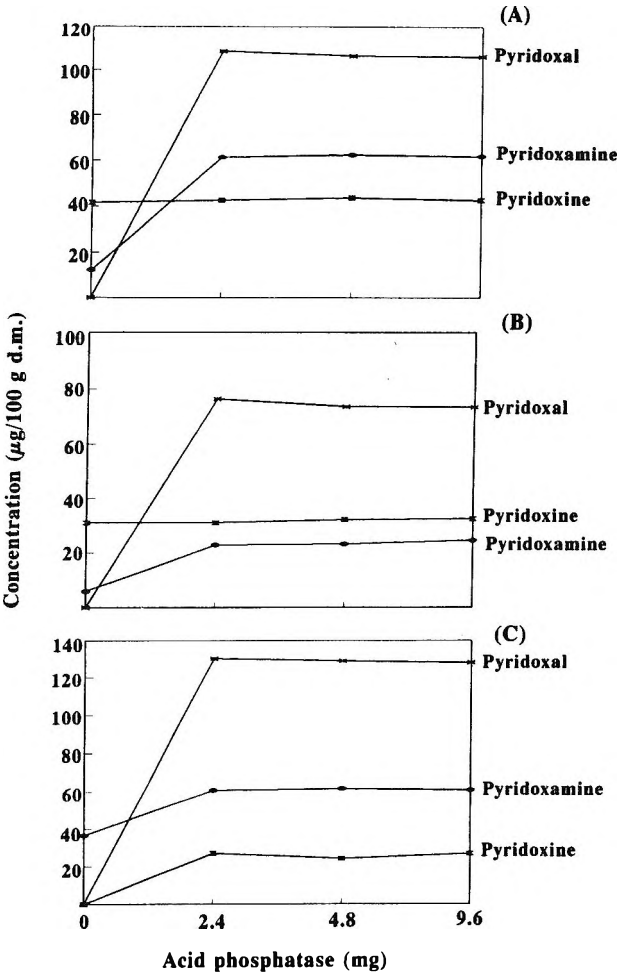

Figure 3. Content of B_6 vitamers in legumes, extracted with variable amounts of acid phosphatase enzyme. a) lentils, b) chick peas, c) haricot beans.

A peak at a retention time of 14.5 min present at the chromatogram of an non-enzymatic extract (Figure 4) could be related with G-PN forms. This chromatographic peak still remained when the acid phosphatase enzyme was used to hydrolyse the phosphorylated B_6 vitamers, and disappeared when extracts where incubated with β-glucosidase. However, when the enzymatic procedure was carried out with Takadiastase enzyme, this chromatographic peak did not appear, and a very

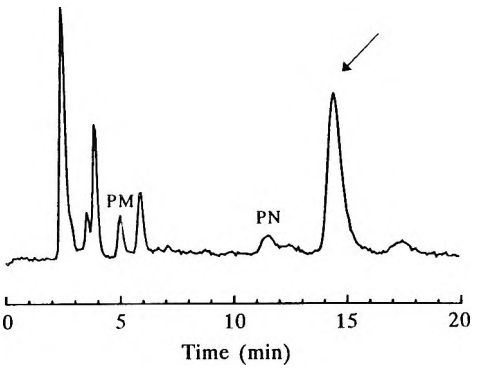

Figure 4. HPLC chromatogram of non-enzymatic lentil extract of B_6 vitamers.

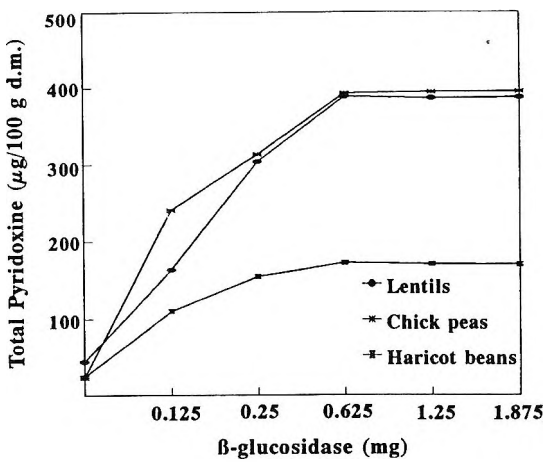

Figure 5. Total pyridoxine content in legumes obtained with variable amounts of β-glucosidase enzyme.

high amount of PN content was obtained. These results suggested that the peak with retention time of 14.5 min corresponds to glycosylated forms of pyridoxine and that the use of Takadiastase enzyme can produce an overestimation of the PN (sum of free and phosphorilated forms) of legumes including the G-PN as PN, while these

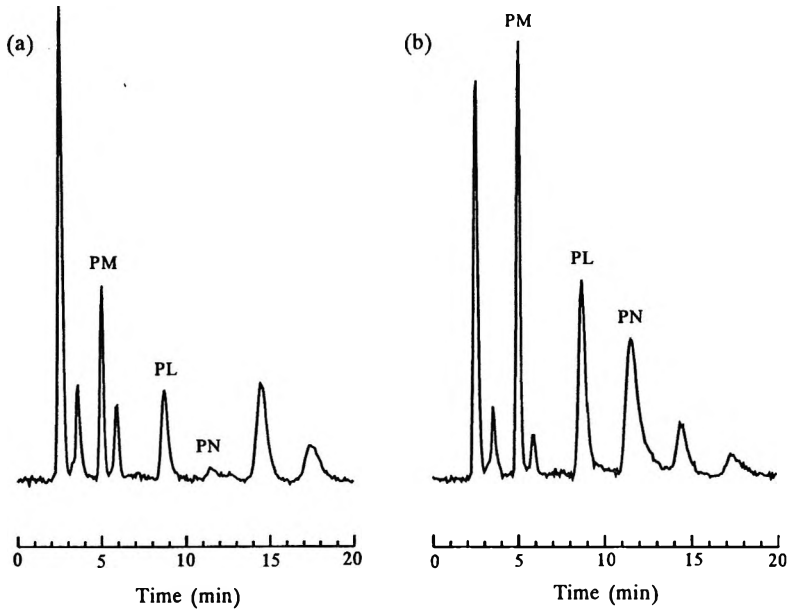

Figure 6. (a) HPLC chromatogram of pyridoxamine (PM), pyridoxal (PL) and pyridoxine (PN) in a haricot bean sample, (b) HPLC chromatogram of a haricot bean extract enriched with PM, PL and PN.

forms have lower biological activity. The G-PN in legumes was quantified indirectly as free PN. The legume extracts, obtained after acid extraction and enzymatic hydrolysis with acid phosphatase, were submitted to the action of β-glucosidase enzyme. Figure 5 indicates the results obtained with different amounts of β-glucosidase enzyme to hydrolyse the G-PN of legumes and it can be observed that a plateau was reached with 0.625 mg of enzyme. The difference between the PN data obtained with and without β-glucosidase enzyme (Figures 3 and 4) gave the G-PN content of legumes.

Quantitative analysis of vitamin B_6 in legumes by HPLC requires effective separations because of the complex legume matrix. The separation of non-phosphorilated B_6 vitamers with reverse phase on octadecylsilica columns was first reported by other authors.[18,19,20] The method of Gregory and Kirk[18] gives rapid separations of the three vitamers, but their application to complex samples is not sucessfull. A very similar system was used by Lim et al.[19] to separate PM, PL and PN, but when was applied to milk samples good results were not obtained.

Table 1

Recovery of Pyridoxamine (PM), Pyridoxal (PL), Pyridoxine (PN), Pyridoxamine Phosphate (PMP), and Pyridoxal Phosphate (PMP) Added to Legume Samples[a]

B_6Vitamers	Lentils	Chick Peas	Haricot Beans
PM	96 ± 0	96 ± 4	93 ± 3
PL	98 ± 2	98 ± 1	95 ± 2
PN	96 ± 1	95 ± 2	93 ± 1
PMP	106 ± 0	107 ± 2	100 ± 6
PLP	96 ± 1	98 ± 2	94 ± 4

[a] (%) Mean values ± SD of three replicates.

Table 2

Pyridoxamine (PM), Pyridoxal (PL), Pyridoxine (PN), and Glycosylated PN (G-PN) Content in Legumes (μg/100 g d.m.)[a]

Legumes	PM	PL	PN	G-PN[b]	Total B_6[c]
Lentils	63.3 ± 2.6	94.2 ± 2.8	44.2 ± 2.5	343.3 ± 3.4	546.5
Chick Peas	26.9 ± 0.8	74.8 ± 2.3	23.5 ± 1.3	371.5 ± 3.8	496.7
Haricot Beans	64.5 ± 1.2	128.8 ± 1.6	24.0 ± 0.2	146.2 ± 3.0	365.0

[a] Mean values ± SD of five replicates.
[b] G-PN = Difference of PN before and after hydrolysis with β-glucosidase.
[c] Total B_6 = Sum of all vitamers and G-PN calculated as PN.

Speek[20] obtains a good resolution of the three vitamers in some foods (banana, potato chips and corn-flour) however, long analysis time is required (40 min). Figure 6a shows a typical chromatogram of legume sample (haricot bean). The chromatographic procedure used enabled good separations of the three vitamers in a short time (23 min including the cleaning step).

PM, PL and PN peaks of extracts were identified by spiking and comparing their retention times with standards (Figure 6b). Recoveries for exogenous vitamers added to the samples are reported in Table 1. The results obtained provide evidence of a reasonable extraction efficiency comparable to other extraction methods.

Table 2 shows the concentration of PM, PL, PN and glycosylated PN in selected legumes. Regarding the total content of vitamin B_6, it appears that our own results, calculated as PN from the sum of all these forms, are in agreement with the total value of B_6 reported in Food Composition Tables.[21,22]

In conclusion, we propose a simple method that provides a valid approach to the measurement of free and glycosylated forms of vitamin B_6 from legume foods. Differentiation between total and glycosylated vitamin B_6 is of value for an accurate assessment of bioavailable vitamin B_6 from plant foods.

ACKNOWLEDGEMENTS

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PICOGRAM DETERMINATION OF AN AVERMECTIN ANALOG IN DOG PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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In the above paper, which was published in Journal of Liquid Chromatography and Related Technologies, **20(3)**, 443-458 (1997), an author was inadvertently omitted from the list of authors. The author list should read:

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* Author to whom correspondence should be addressed.

J. LIQ. CHROM. & REL. TECHNOL., 20(6), 973–978 (1997)

THE BOOK CORNER

LIQUID CHROMATOGRAPHY OF OLIGOMERS, C. V. Uglea, Chromatographic Science Series, Jack Cazes, editor, Volume 72, Marcel Dekker, Inc., New York, 1996, 344 pp., Price: $150.00

This book details the principles and mechanisms of, and the equipment and optimal working conditions for, the liquid chromatographic separation of well defined oligomeric species and fractions with narrow molecular weight distributions.

Providing a complete description of the applications and possible performance of liquid chromatography in the field of oligomer separation, *Liquid Chromatography of Oligomers* elucidates theoretical concepts such as the mechanism of retention and the thermodynamic explanation of oligomer separation, discusses the definition of chromatography, covers both simple and complex forms of chromatographic instrumentation, delineates procedures used for the fractionation of oligomeric mixtures, lists the main producers of chromatographic devices, describes the chemical non-homogeneity of polymers and oligomers, presents the nomenclature of oligomers, examines gel-permeation chromatography, and contains over 1475 pertinent citations to literature sources.

The book focuses on two major objectives: (1) the description of the procedures used by liquid chromatography for the characterization of oligomeric mixtures, and (2) the presentation of the equipment and optimal working conditions used to perform the separation of both well-defined oligomeric species and of fractions with narrow molecular weight distributions.

Chapter 1 deals with the definition and history of chromatography and the nomenclature of oligomers. Chapter 2 discusses molecular non-homogeneity of synthetic oligomers. Oligomers, as well as high polymers, are characterized by both molecular weight distribution and nonhomogeneity of chemical composition. Functionality distribution is also included in the molecular heterogeneity of oligomer mixtures.

Liquid chromatography of oligomers is presented in Chapter 3. Although liquid chromatography may be performed in various modes (thin-layer chromatography, partition chromatography, adsorption chromatography, etc.), liquid-solid chromatography represents the most important means for the study of the molecular weight distributions of oligomers.

Gel permeation chromatography became a part of liquid chromatography primarily because of its equipment characteristics. Its mechanism and performance make gel permeation chromatography a unique method with valuable applications in the chemistry of oligomers and polymers. Chapter 4 is dedicated to this method, covering the theoretical basis and mechanisms, column packings (gels), calibration procedures, and applications and equipment.

Liquid Chromatography of Oligomers is intended both for experts with experience in the field of oligomers and polymer synthesis and characterization and for students interested in becoming acquainted with the fascinating world of oligomers and macromolecules.

CHROMATOGRAPHIC DETECTORS - DESIGN, FUNCTION, AND OPERATION, By R. P. W Scott, Chromatographic Science Series, Jack Cazes, editor, Volume 73, Marcel Dekker, Inc., New York, 1996, 536 pp., Price: $150.00

Chromatographic Detectors is a well written book which is divided into three parts. The first part deals with detector properties and specifications. This is a short part, containing three chapters totalling 82 pages. The second part (4 chapters totaling 93 pages) discusses gas chromatography detectors, from their initial evolution till modern times. Part 3, which is the bulk of the book, is made up of 11 chapters totaling 325 pages and dealing with all types of liquid chromatography detectors. (See Table of Contents for details).

The book emphasizes the essential use of common specifications to describe all detectors, allowing easy comparison of their attributes, this practical guide . . . .

> Discusses the properties of chromatography detectors and the best way to measure their efficacy.
>
> Reviews factors that impair the column resolution before solutes reach the detector.

Describes and explains the relatives merits of the more popular detectors, including the most recent commercially available types, as well as lesser-known devices.

Explores the extensive number of TLC detectors available, including automatic scanning devices.

Surveys those chromatograph/spectrometer tandem systems that have been satisfactorily developed and details their interfacing, function, and areas of application.

The book is highly recommended to all those interested in chromatography.

CAPILLARY GAS CHROMATOGRAPHY, by D. W. Grant, Separation Science Series, John Wiley & Sons, Inc., New York, 1995, 295 pp.

This concise book is divided into nine chapters. The first chapter is an introduction. Chapter two deals with theory of open tubular columns, effects of gas compressibility, separation process, plate theory, kinetic or rate theory, and resolution of chromatography.

Chapter three covers capillary instrumentation, which includes the characterization of modern gas chromatograph, gas supply system, column coupling and connections, injector, chromatographic oven, detectors for capillary GC, peak measurement and data processing.

Chapter four discusses the use of fused silica open tubular (FSOT) columns, stainless steel open tubular columns, parameters effect on wall-coated open tubular (WCOT) columns, quantitative parameters and resolution, stationary phase in capillary GC, effect of temperature on retention, and measurement of retention time.

Chapter five deals with porous layer open tubular (PLOT) columns, which includes the theory, development, and types of columns, while, in chapter six, the emphasis is on sample introduction, including the theoretical basis of sample introduction, and sample introduction methods.

In chapter seven, the focus is on the preparation of samples using distillation, solvent extraction, supercritical fluid extraction (SFE), solid phase extraction, HPLC-CGC combination, headspace analysis, and sample derivatization.

Chapter eight concentrates on analysis and optimization, which includes quantitative analysis in capillary GC, principles and practice of trace analysis, theory and practice of programmed temperature operation, practical optimization and troubleshooting.

Chapter nine is an interesting one which deals with multidimensional GC and column switching, columns connected in series without switching, backflushing techniques, heart cutting techniques, and multicolumn analysis.

In general, the book is well written and illustrated, and incorporates several new practices and techniques which are supported with appropriate theoretical discussion. The new GC improvements presented here make capillary gas chromatography a useful and competitive analytical technique. The book is recommended to all interested chromatographers and other scientists interested in separation as an analytical tool.

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Chapter 8. **Analysis and Optimization** (235).

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Reviewed by
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J. LIQ. CHROM. & REL. TECHNOL., 20(6), 979–985 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium. Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd. Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 1 - 4: 1997 International Symposium, Exhibit & Workshops on Preparative Chromatography: Ion Exchange, Adsorption/Desorption Processes and Related Separation Techniques, Washington, DC. Contact: J. Cunningham. Barr Enterprises. 10120 Kelly Road, Box 279, Walkersville, MD 21793, USA. (301) 898-3772; FAX: (301) 898-5596.

JUNE 16 - 19: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach. L'Electrophorese Capillaire, Methode de Routine pour le Contrôle Qualité des Medicaments: Approche Pratique, Monpellier, France. (Training course given in two languages) Contact: Prof. H. Fabre, Tel: (33) 04 67 54 45 20; FAX: (33) 04 67 52 89 15.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce. Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

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

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (703) 231-8222.

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

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

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

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

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

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

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

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

1998

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

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

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

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

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

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

1999

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

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

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

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

2000

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

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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