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EXPERIMENTAL AND COMPUTER SIMULATION STUDIES OF SOLUTE-SOLUTE INTERACTIONS IN LIQUID CHROMATOGRAPHY

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ABSTRACT

Solute-solute interactions, due to self-association as well as mixed association, are shown to arise for the steroids cortisone, tetrahydrocortisol, tetrahydrocortisone, and methylprednisolone. From molecular mechanics and dynamics simulations, these interactions appear to be driven by strong electrostatic and hydrogen bonding forces. These interactions have a significant effect on solute retention and dispersion behavior under routine operating conditions in reverse-phase liquid chromatography.

INTRODUCTION

Solute-solute interactions may be broadly defined as an intimate contact or short-range association between molecules that persists as the concentration of solute is decreased. Solute-solute interactions have been studied extensively in bulk solution by measurement of colligative properties such as vapor pressure, melting point, freezing point, conductance, etc.[1-3] In addition, infrared, NMR, and other spectroscopic methods have been used to examine solute-solute interactions at the molecular level in order to identify the bonding sites and to determine the aggregation number.[4-6]

By combining the information obtained from these two types of experimental measurements, the equilibrium constant(s) for solute aggregation, as well as the activity coefficients and excess thermodynamic functions for the solution, may be calculated for comparison with theoretical models.

From a theoretical perspective, solute-solute interactions represent a deviation from ideal solution behavior by violation of the assumption of random molecular distribution. A variety of theoretical models have been developed to account for these deviations. In the classical paper by McMillan and Mayer,[7] the grand canonical ensemble method was applied to the generalized case for multicomponent gas or liquid systems. This statistical thermodynamic approach enabled the prediction of the radial distribution function and, henceforth, the thermodynamic properties of the solution. Stigter[8] combined the McMillan-Mayer theory together with simple models of van der Waals and hydrogen bonding forces to interpret the thermodynamics of aqueous solutions of sucrose and glucose. Kozak, Knight, and Kauzmann[1] similarly combined the McMillan-Mayer theory with several lattice models for aqueous solutions of hydrophobic solutes. Nemethy and Scheraga,[9,10] as well as Pratt and Chandler,[11,12] subsequently developed models of solute-solute interactions based on the hydrophobic theory.[13-15] Although the thermodynamic consequences of solute-solute interactions are reasonably well understood in bulk solution, this understanding has not been widely applied to multiple phase systems such as extraction and chromatography.

At the present time, very few studies have documented the effect of solute-solute interactions in chromatography. Amaya and Sasaki[16] investigated the gas chromatographic retention behavior of a binary mixture of chloroform and methyl ethyl ketone on a nonpolar stationary phase (Apiezon J). In addition, they examined binary mixtures of chloroform with carbon tetrachloride and with toluene on a polar stationary phase (polyethylene glycol). In every case, they observed an increase in the retention time of both solutes in the binary mixture, which was attributed to solute-solute interactions in the stationary phase. These effects were qualitatively explained by using a theoretical model in which the mobile phase was treated as an ideal gas and the stationary phase as a regular solution.[16] More recently, solute-solute interactions have been implicated in supercritical fluid and liquid chromatography.[17-19] However, because of the innate complexity of condensed phases, a rigorous and comprehensive theoretical model has yet to be developed.

Classical thermodynamic models based on regular solution theory[20-24] and the hydrophobic theory,[25,26] as well as statistical thermodynamic models,[27-29] have been developed for the prediction of solute retention. In practical application of such models, solute-mobile phase and solute-stationary phase interactions are considered predominant, whereas solute-solute interactions are invariably neglected. This neglect is often justified by an argument of statistical

probability, since the concentration of solute molecules is small with respect to that of the phases. However, if the energy of interactions is sufficiently large, solute-solute interactions may become important despite the low concentration.

In this study, the self-association and mixed association of corticosteroids are demonstrated to arise under routine operating conditions in reverse-phase liquid chromatography. In order to facilitate understanding of the origin and nature of these strong solute-solute interactions, molecular mechanics and dynamics calculations are employed. Finally, these results are used to explain the observed deviations in chromatographic retention and dispersion behavior.

EXPERIMENTAL

Materials and Methods

The following corticosteroids are utilized in this investigation: cortisone (17α,21-dihydroxy-pregn-4-ene-3,11,20-trione), tetrahydrocortisol (3α,11β, 17α,21-tetrahydroxy-5β-pregnane-20-one), tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione), and methylprednisolone (11β,17α,21-trihydroxy-6α-methyl-pregna-1,4-diene-3,20-dione). These corticosteroids, shown in Figure 1, are obtained from the Sigma Chemical Company (St. Louis, MO, USA). Standard solutions are prepared in acetonitrile at 10^{-6} M concentration for cortisone and methylprednisolone and at 10^{-3} M concentration for tetrahydrocortisol and tetrahydrocortisone. Organic solvents are high-purity, distilled-in-glass grade (Baxter Healthcare, Burdick & Jackson Division, Muskegon, MI, USA); water is deionized and double distilled in glass (Model MP-3A, Corning Glass Works, Corning, NY, USA).

Experimental System

A chromatographic pump equipped with two 40-mL syringes (Model 140, Applied Biosystems, Foster City, CA, USA) is used to deliver the mobile phase, 35% aqueous acetonitrile, at 0.5 mL/min. The solutes are introduced by means of a 10-μL injection valve (Model EQ 60, Valco Instrument Co., Houston, TX, USA) to the reverse-phase liquid chromatography column (47 cm × 0.46 cm i.d., 5-μm octadecylsilica, Spheri-5 RP-18, Applied Biosystems). Solute detection is accomplished by using a variable-wavelength UV-VIS absorbance detector (240 nm, 0.005 AUFS, Model 166, Beckman Instruments, San Ramon, CA, USA).

The chromatographic data are evaluated by manual calculation according to the method of Foley and Dorsey[30] for exponentially modified Gaussian peak profiles. The figures of merit, such as area, capacity factor, plate number, skew, etc., are determined from these calculations.

Molecular Mechanics and Dynamics Simulations

Simulations of the interaction between corticosteroid molecules are performed using classical molecular mechanics and dynamics methods (BioGraf version 3.0, Biodesign Inc., Pasadena, CA, USA) on a Silicon Graphics Indigo computer (Model CMNB003, Mountain View, CA, USA). A generic force field, Dreiding,[31] is employed to calculate the total energy of the molecule as the sum of the bonding and non-bonding interactions. The bonding interactions include contributions from stretching (E_s), bending (E_b), and torsional (E_ω) energy between atoms that are covalently bonded. The non-bonding interactions consist of contributions from van der Waals (E_{vdw}), electrostatic (E_Q), and hydrogen bond (E_{hb}) energy between atoms that are not covalently bonded. The van der Waals energy is expressed by a standard Lennard-Jones equation

$$E_{vdw} = AR_{ij}^{-12} - BR_{ij}^{-6} \quad (1)$$

where R_{ij} is the distance between atoms i and j, and A and B are empirically derived constants. The electrostatic energy (E_Q) is calculated by using Coulomb's law

$$E_Q = 332.0637\, Q_i\, Q_j / \varepsilon\, R_{ij} \quad (2)$$

where Q_i and Q_j are the net charge on atoms i and j, respectively. The dielectric constant is assumed to be that of a vacuum environment ($\varepsilon = 1$). The hydrogen bonding energy is expressed as:

$$E_{hb} = D_{hb} [5 (R_{hb} / R_{DA})^{12} - 6 (R_{hb} / R_{DA})^{10}] \cos^4 (\theta_{DHA}) \quad (3)$$

where θ_{DHA} is the bond angle between the hydrogen donor (D), the hydrogen atom (H), and the hydrogen acceptor (A), while R_{DA} is the distance between the donor and acceptor. D_{hb} and R_{hb} are the energy and the maximum distance used to define the hydrogen bond, the magnitude of which depend on the convention used for assigning charges in the force field model.[31]

To simulate the solute-solute interactions between the corticosteroids, a systematic three-step approach is used. First, the bonding energies are minimized in order to determine the optimum three-dimensional structure and charge distribution for each corticosteroid. These individually optimized

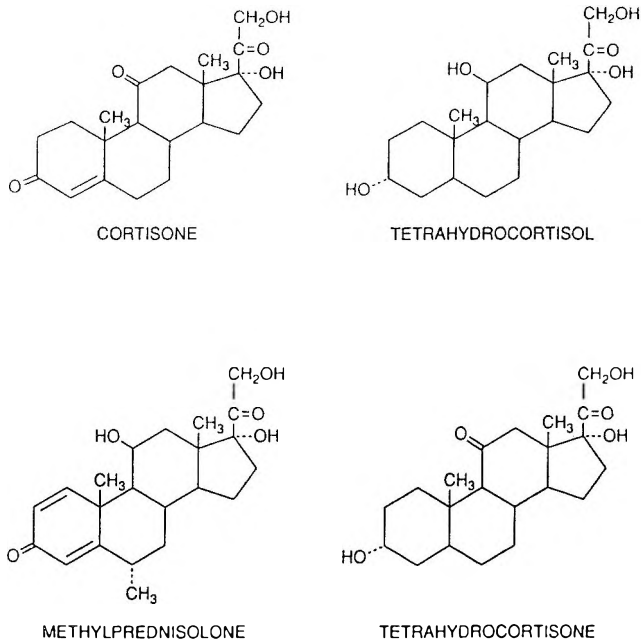

Figure 1. Structures of corticosteroids.

structures are then arranged in pairwise combinations. Next, a Monte-Carlo search is performed to examine all possible spatial orientations and to identify those with lowest energy.[32] For this study, the steroid pairs are randomly varied in 200 different relative spatial positions and the non-bonding energy is minimized in 30 incremental steps at each of these positions. The final stage of the optimization involves a more refined energy minimization of the most promising orientations (typically 50) identified from the Monte-Carlo search. The annealed dynamics method simulates the exchange of thermal energy between the environment and the steroid pair, thereby allowing translational, vibrational, and rotational motion to minimize the total energy. For this study, the steroid pairs are simulated to be annealed in the temperature range from 300 to 600 K in 20 incremental steps. From among all of these conformations, the one with the lowest total energy is identified as the optimum structure for the steroid pair.

The total interaction energy (ΔE) is calculated by subtracting the energy at infinite separation distance from that at the optimum distance.

$$\Delta E = E_{opt} - E_{\infty} \qquad (4)$$

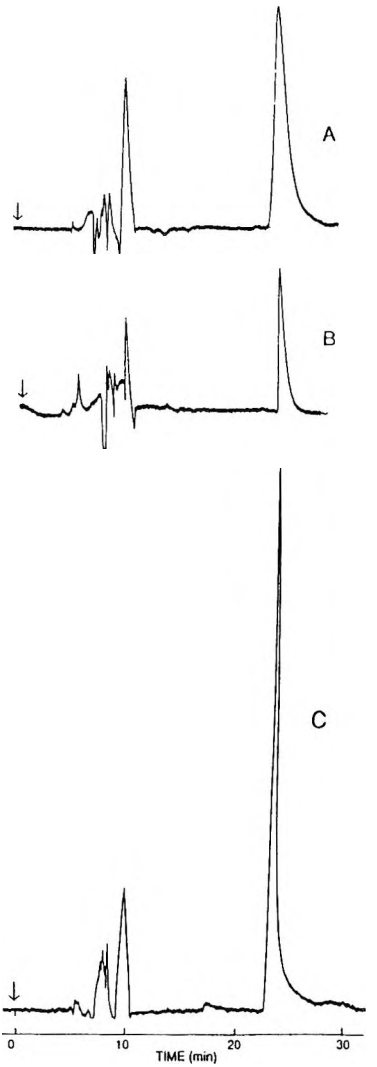

Figure 2. Chromatograms of corticosteroids analyzed individually and in mixtures. Column: 47 x 0.46 cm i.d., packed with 5-μm octadecylsilica material. Mobile phase: 35% aqueous acetonitrile; 0.5 mL/min. Detector: UV-VIS absorbance detector (240 nm, 0.005 AUFS). Solutes: (A) cortisone (10^{-6} M), (B) tetrahydrocortisol (10^{-3} M), (C) mixture of cortisone and tetrahydrocortisol.

Table 1

Comparison of the Chromatographic Figures of Merit for Corticosteroids Analyzed Individually and in Mixtures*

	Cortisone	Tetrahydro-cortisol	Methyl-prednisolone	Tetrahydro-cortisone
Area				
Individual	0.580	0.164	0.517	0.209
Mixture	0.727	0.727	0.677	0.677
Capacity Factor				
Individual	1.56	1.48	2.36	2.26
Mixture	1.47	1.47	2.02	2.02
Plate Number				
Individual	900	4000	1200	3200
Mixture	4700	4700	8900	8900
Skew				
Individual	1.49	1.56	1.65	1.62
Mixture	1.33	1.33	0.87	0.87

* Experimental conditions as given in Figure 2.

In the same manner, the van der Waals (ΔE_{vdw}), electrostatic (ΔE_Q), and hydrogen bonding (ΔE_{hb}) components of the interaction energy can be calculated:

$$\Delta E_{vdw} = E_{opt,vdw} - E_{\infty,vdw} \quad (5)$$

$$\Delta E_Q = E_{opt,Q} - E_{\infty,Q} \quad (6)$$

$$\Delta E_{hb} = E_{opt,hb} - E_{\infty,hb} \quad (7)$$

When defined in this manner, the most stable solute-solute pair will have the greatest negative interaction energy.

RESULTS

Experimental Studies

In a previous study,[33] we performed the routine analytical separation of eight corticosteroids by reverse-phase liquid chromatography, using an octadecylsilica stationary phase and aqueous methanol and acetonitrile mobile phases. During the course of this study, we observed that specific pairs of steroids exhibited different retention and dispersion behavior when they were analyzed individually and in mixtures. Because of the theoretical and practical significance, it was desirable to evaluate this anomalous behavior in greater depth and detail.

The chromatograms of the steroids cortisone and tetrahydrocortisol are shown individually in Figures 2A and 2B, respectively, and their mixture is shown in Figure 2C. The chromatographic figures of merit derived from these chromatograms are summarized in Table 1. Within the error of the manual measurements, the sum of the areas for the individual steroid peaks is approximately equal to the area for the composite peak, which confirms that the steroids are co-eluting. However, the capacity factor for the composite peak (1.47) is less than that for the individual peaks (1.56 and 1.48). The standard deviation of replicate measurements is ± 0.02 ($n = 6$), thus the difference in capacity factor is statistically significant at the 99% confidence level.[34] Furthermore, the number of theoretical plates is significantly higher and the skew is significantly lower for the composite peak than for the individual steroid peaks.

The chromatograms of the steroids methylprednisolone and tetrahydrocortisone are shown individually in Figures 3A and 3B, respectively, and their mixture is shown in Figure 3C. The chromatographic figures of merit derived from these chromatograms are summarized in Table 1. As in the previous case, the capacity factor for the composite peak is significantly lower than that for the individual steroids. The plate number is significantly higher and the skew is significantly lower for the composite peak than for the individual steroid peaks.

These results clearly demonstrate that the retention and dispersion of these specific pairs of corticosteroids differ when they are analyzed individually and in mixtures. Thus, contrary to traditional theoretical models, the chromatographic behavior of one solute is influenced by the presence of another solute. Strong solute-solute interactions have been reported previously by Bennet et al.[35] for bile acids, which have similar skeletal structure to the steroids examined herein. These authors observed little association at the 10^{-3} M

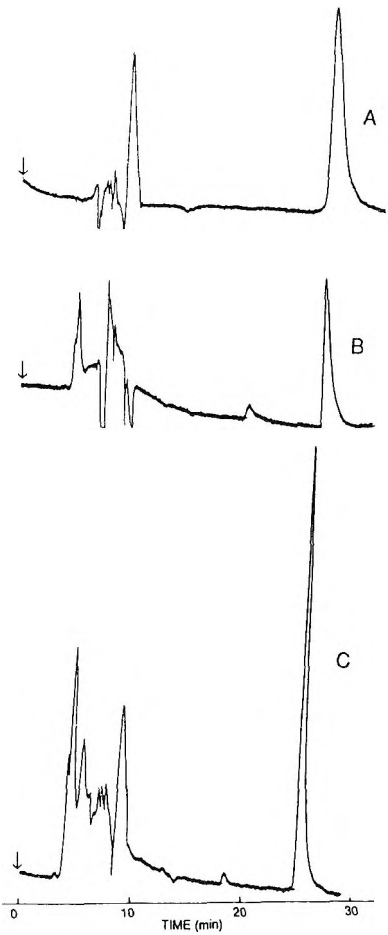

Figure 3. Chromatograms of corticosteroids analyzed individually and in mixtures. Solute: (A) methylprednisolone (10^{-6} M), (B) tetrahydrocortisone (10^{-3} M), (C) mixture of methylprednisolone and tetrahydrocortisone. Experimental conditions as given in Figure 2.

concentration level for monohydroxy bile acids, but increasingly stronger interaction for two or more hydroxyl substituents. Hydroxyl groups on the flexible side chain at C-17 showed less interaction than those on the more rigid skeleton, particularly the α face. In addition, carbonyl groups on the side chain played a relatively minor role. These associations were attributed to hydrogen bonding between dimers, however tetramers and higher oligomers were implicated at higher concentrations. Although other workers have suggested

that hydrophobic interactions play an important role,[36] the predominance of hydrogen bonding effects has been confirmed for the bile salts,[37-39] as well as for cholesterol.[35,40,41]

In order to investigate the nature and strength of solute-solute interactions between the corticosteroids, computer simulations are performed by molecular mechanics and dynamics methods. This approach has been used successfully by Hanai et al.[42-44] to examine solute-stationary phase interactions in chromatography.

Computer Simulation Studies

The two cases of solute-solute interactions to be examined are cortisone with tetrahydrocortisol and methylprednisolone with tetrahydrocortisone. For each case, there are three possible pairwise combinations, two of which are homogeneous and the other heterogeneous. By examining each of these pairwise combinations, we gain an appreciation for the nature and strength of interactions that exist for self-association as well as for mixed association. Molecular mechanics and dynamics calculations have been performed in order to determine the optimum conformation and to estimate the interaction energy for each of these pairwise combinations.

Figure 4 shows the optimized structures for each of the pairwise combinations of cortisone with tetrahydrocortisol. Each pair of steroids is held together by van der Waals, electrostatic, and hydrogen bonding forces. The magnitude of these forces varies with the structure and orientation of the functional groups. The carbonyl and hydroxyl groups interact predominantly by electrostatic and hydrogen bonding forces, whereas the hydrocarbon skeleton interacts via van der Waals forces. The optimum conformation for the cortisone-cortisone pair (Figure 4A) is a head-to-head orientation that permits intermolecular hydrogen bonding between the carbonyl and hydroxyl groups on the side chain at C-17. In contrast, the tetrahydrocortisol-tetrahydrocortisol pair (Figure 4B) prefers a head-to-tail orientation that allows interaction between the hydroxyl group at C-11 of one molecule with the carbonyl and hydroxyl groups on the side chain of the other molecule. Finally, the cortisone-tetrahydrocortisol pair (Figure 4C) prefers a head-to-head orientation that allows interaction between the carbonyl group at C-11 of cortisone with the hydroxyl group at C-11 of tetrahydrocortisol, as well as between the carbonyl group on the side chain of cortisone with the hydroxyl group on the side chain of tetrahydrocortisol.

The total energy for each pairwise combination of cortisone with tetrahydrocortisol at infinite and at optimum separation distance is summarized in Table 2. The total energy is comprised of the bonding energy from stretching, bending, and torsional forces, as well as the non-bonding energy

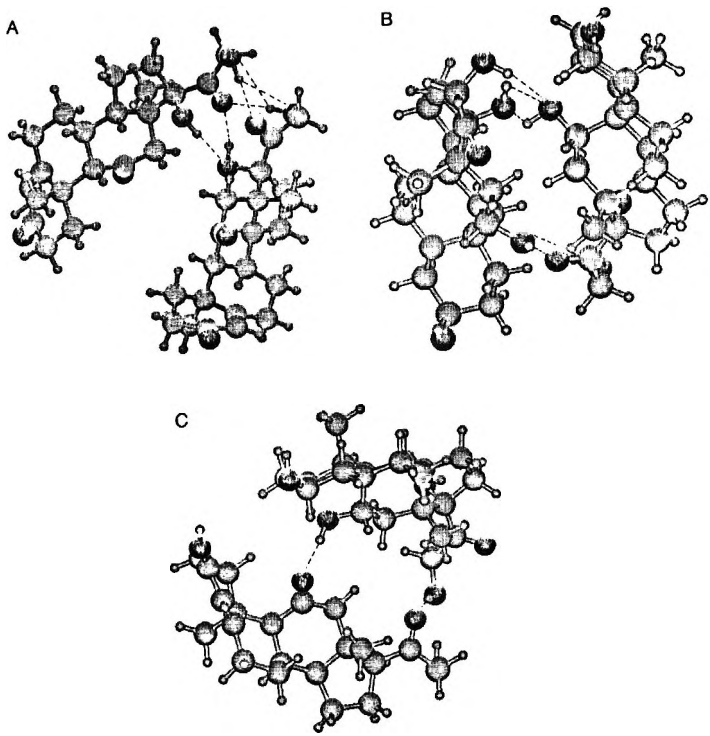

Figure 4. The optimum configuration for interaction between (A) two cortisone molecules, (B) two tetrahydrocortisol molecules, and (C) cortisone and tetrahydrocortisol molecules. (O) carbon, (°) hydrogen, (●) oxygen, (- - -) nonbonding atoms that meet the defined energy and distance constraints for hydrogen bonds according to Equation (3).

from van der Waals, electrostatic, and hydrogen bonding forces. Because the bonding energy at infinite separation distance is nearly identical to that at the optimum distance, the interaction energy is dependent primarily upon the non-bonding interactions. From Table 2, it is apparent that the van der Waals component is large, but remains relatively constant as the molecules approach from infinite to optimum distance. In contrast, the electrostatic and hydrogen bonding components vary considerably. The electrostatic energy decreases for the cortisone-cortisone pair and the cortisone-tetrahydrocortisol pair, but increases for the tetrahydrocortisol-tetrahydrocortisol pair. The hydrogen bonding energy decreases notably for all pairwise combinations, but especially so for the tetrahydrocortisol-tetrahydrocortisol pair. These results suggest that the molecular interactions are controlled predominantly by electrostatic and hydrogen bonding forces. The total interaction energy of the cortisone-

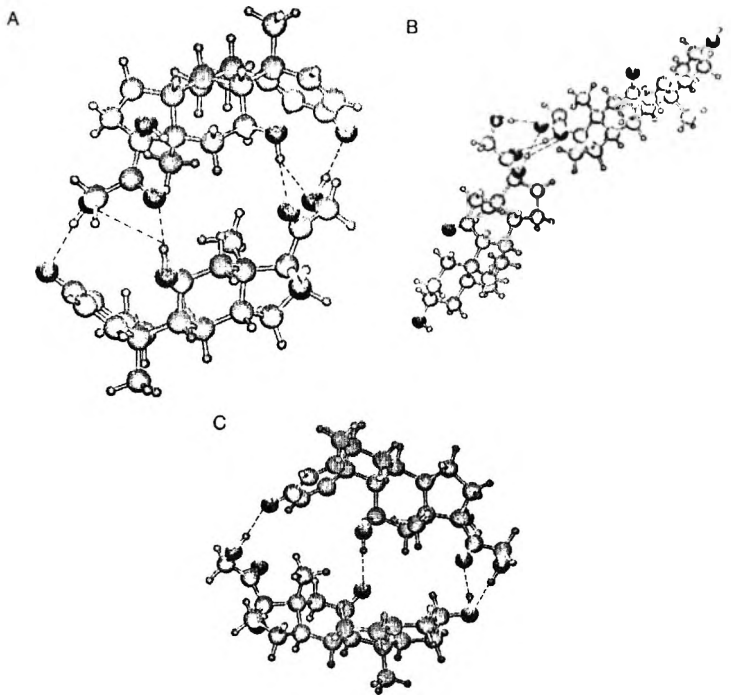

Figure 5. The optimum configuration for interaction between (A) two methylprednisolone molecules, (B) two tetrahydrocortisone molecules, and (C) methylprednisolone and tetrahydrocortisone molecules. (O) carbon, (°) hydrogen, (●) oxygen. (- - -) nonbonding atoms that meet the defined energy and distance constraints for hydrogen bonds according to Equation (3).

cortisone and tetrahydrocortisol-tetrahydrocortisol pairs is less negative than that of the cortisone-tetrahydrocortisol pair, which indicates that formation of the latter pair is more energetically favorable.

In a similar manner, Figure 5 shows the optimized structures for each of the pairwise combinations of methylprednisolone with tetrahydrocortisone. The optimum conformation for the methylprednisolone-methylprednisolone pair (Figure 5A) is a head-to-tail orientation that permits hydrogen bonding between the carbonyl group at C-3 and the hydroxyl group at C-11 of one molecule with the hydroxyl and carbonyl groups on the side chain of the other molecule. The tetrahydrocortisone-tetrahydrocortisone pair (Figure 5B) prefers a head-to-head orientation that allows interaction between the carbonyl and hydroxyl groups on

Table 2

Total Energy (E) and the Components of van der Waals Energy (E_{vdw}), Electrostatic Energy (E_Q), and Hydrogen Bonding Energy (E_{hb}) at Infinite Separation Distance (∞) and at the Optimum Separation Distance (opt) for the Pairwise Configurations of Corticosteroids Shown in Figures 4A to 4C

Corticosteroid Pair	Energy (kcal/mol)											
	E_∞	E_{opt}	ΔE	$E_{\infty,vdw}$	$E_{opt,vdw}$	ΔE_{vdw}	$E_{\infty,Q}$	$E_{opt,Q}$	ΔE_Q	$E_{\infty,hb}$	$E_{opt,hb}$	ΔE_{hb}
Cortisone-Cortisone	196.1	168.2	-27.9	101.0	101.0	0.0	8.3	4.5	-3.8	-11.2	-31.0	-19.8
Tetrahydrocortisol-Tetrahydrocortisol	192.0	160.1	-31.9	100.2	101.1	0.9	1.3	5.4	4.1	-11.1	-39.0	-27.9
Cortisone-Tetrahydrocortisol	198.0	165.4	-32.6	98.1	101.1	3.0	10.2	5.3	-4.9	-11.0	-31.0	-20.0

Table 3

Total Energy (E) and the Components of van der Waals Energy (E_{vdw}), Electrostatic Energy (E_Q), and Hydrogen Bonding Energy (E_{hb}) at Infinite Separation Distance (∞) and at the Optimum Separation Distance (opt) for the Pairwise Configurations of Corticosteroids Shown in Figures 5A to 5C

Corticosteroid Pair	Energy (kcal/mol)											
	E_∞	E_{opt}	ΔE	$E_{\infty,vdw}$	$E_{opt,vdw}$	ΔE_{vdw}	$E_{\infty,Q}$	$E_{opt,Q}$	ΔE_Q	$E_{\infty,hb}$	$E_{opt,hb}$	ΔE_{hb}
Methylprednisolone-Methylprednisolone	197.4	159.3	-38.1	99.1	99.3	0.2	11.2	1.8	-9.4	-11.1	-31.0	-19.9
Tetrahydrocortisone-Tetrahydrocortisone	194.2	168.2	-26.0	100.4	102.1	18.7	10.3	4.1	-6.2	-11.1	-30.1	-19.0
Methylprednisolone-Tetrahydrocortisone	194.1	161.4	-32.7	101.2	101.3	0.1	10.1	2.4	-7.7	-11.4	-36.3	-24.9

the side chains. Finally, the methylprednisolone-tetrahydrocortisone pair (Figure 5C) prefers a head-to-tail orientation that allows interaction between the hydroxyl group at C-11 of methylprednisolone with the carbonyl group at C-11 of tetrahydrocortisone, between the carbonyl group at C-3 of methylprednisolone with the side chain of tetrahydrocortisone, as well as between the side chain of methylprednisolone with the hydroxyl group at C-3 of tetrahydrocortisone.

The total energy for each pairwise combination of methylprednisolone with tetrahydrocortisone at infinite and at optimum separation distance is summarized in Table 3. As in the previous case, the van der Waals component remains relatively constant, whereas the electrostatic and hydrogen bonding components decrease significantly as the molecules approach from infinite to optimum distance. The methylprednisolone-methylprednisolone and methylprednisolone-tetrahydrocortisone pairs have the most negative interaction energy and, hence, are the most energetically favorable combinations.

From these molecular mechanics and dynamics simulations, we may conclude that significant non-bonding interactions exist between cortisone and tetrahydrocortisol and between methylprednisolone and tetrahydrocortisone. For both cases, the total interaction energy is of comparable magnitude (approximately -33 kcal/mole) and arises predominantly from electrostatic and hydrogen bonding interactions.

DISCUSSION

In order to understand the effect of these solute-solute interactions, it is helpful to discuss briefly the structure of octadecylsilica and the associated mechanism(s) of solute retention.[45-49] Octadecylsilica is comprised of alkyl chains covalently bonded to the silica surface, with residual silanol and siloxane groups. Nonpolar solute molecules may interact predominantly with the alkyl chains, whereas polar functional groups may interact with the weakly acidic silanol groups or weakly basic siloxane groups. The former interactions arise from relatively weak van der Waals forces and, thus, tend to be rapidly reversible. In contrast, the latter interactions arise from stronger electrostatic and hydrogen bonding forces, which are characterized by slow mass transfer kinetics.[50] These interactions often result in lower plate number and higher asymmetry or skew.

For solutes such as the corticosteroids, which possess a hydrocarbon skeleton together with varying numbers of carbonyl and hydroxyl groups, a dual retention mechanism is likely to occur.[51] In the aqueous acetonitrile mobile phases utilized for the present study, steroids with readily accessible carbonyl groups exhibit the lowest plate number and highest asymmetry. For example,

the plate number for cortisone, which has carbonyl groups at C-3 and C-11, is significantly less than that for tetrahydrocortisol, which has hydroxyl groups at these positions (Table 1). However, in aqueous methanol mobile phases, which can form hydrogen bonds with the carbonyl groups, the plate number for cortisone is significantly increased and the asymmetry is reduced. These observations suggest that interaction of the carbonyl group, which is weakly basic, with silanol groups or other Lewis-acid impurities in the silica is largely responsible for the observed peak profiles.

The retention and dispersion of the individual steroids and their mixtures can, therefore, be rationalized in terms of the number of carbonyl groups that are available to adsorb at the silica surface. For the case of cortisone with tetrahydrocortisol, the cortisone-tetrahydrocortisol pair is more energetically favorable than either of the homogeneous pairwise combinations. Because of the extensive intermolecular and intramolecular hydrogen bonding in the cortisone-tetrahydrocortisol pair, there are fewer free carbonyl groups than in the individual steroids.

Similarly, for the case of methylprednisolone and tetrahydrocortisone, the methylprednisolone-tetrahydrocortisone pair is energetically favorable and provides extensive hydrogen bonding to mask the carbonyl groups. In each case, the solute-solute interactions serve to reduce adsorption at the silanol groups, hence the composite peak is less retained and has higher plate number and lower skew than the individual steroids.

Based on the interaction energy in Table 3, we would expect the homogeneous methylprednisolone-methylprednisolone pair to be at least as prevalent as the heterogeneous pairs discussed above. To test this hypothesis, the chromatographic peak profile was analyzed as the concentration of methylprednisolone was increased from 10^{-6} M to 10^{-3} M, comparable to tetrahydrocortisone. The plate number correspondingly increased from 1200 to 2100, and the skew decreased from 1.65 to 1.40. Thus, methylprednisolone appears to undergo self-association in addition to mixed association with tetrahydrocortisone. The molecular mechanics and dynamics simulations may prove to be useful in predicting other cases of solute-solute interactions.

CONCLUSIONS

By a combination of experimental and computer simulation techniques, corticosteroids are shown to undergo both self-association and mixed association at the 10^{-3} M concentration level. Like the structurally similar bile acids, the steroids interact primarily by electrostatic and hydrogen bonding forces. These interactions have a significant influence upon solute retention and dispersion under routine operating conditions for reverse-phase liquid

chromatography. Because solute-solute interactions are invariably neglected in theoretical and semi-empirical models, they may have a detrimental effect on the prediction and optimization of chromatographic separations.

ACKNOWLEDGEMENTS

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THE EFFECT OF MOBILE PHASE ON PROTEIN RETENTION AND RECOVERY USING CARBOXYMETHYL DEXTRAN-COATED ZIRCONIA STATIONARY PHASES

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ABSTRACT

We have examined the effect of mobile phase conditions on the elution and recovery of proteins from a carboxymethyl dextran coated zirconia stationary phase. Mobile phases containing the Lewis bases phosphate and fluoride were examined as a function of concentration. We found that proteins only eluted from the stationary phase when some minimum concentration of Lewis base was present in the mobile phase. The effect of ionic strength was also examined. We found that a fairly high ionic strength is needed to elute the proteins with good recovery. The concentrations of the Lewis bases and the ionic strength needed to elute the proteins depended on the identity of the protein and the Lewis base. The pH of the mobile phase also plays a role; when the pH of the system is below the isoelectric point of a protein, it is difficult to elute that protein.

INTRODUCTION

The use of zirconia as a stationary phase support for HPLC has been of great interest in our laboratory.[1] The advantages of zirconia include excellent chemical stability (stable in solution from pH 1 to 14), excellent thermal stability (up to at least 100 °C),[2] and excellent mechanical stability. One particular area of research is the use of polymer coated zirconia materials for bioseparations. The advantages of zirconia can be coupled with a polymer coating that imparts useful surface chemistry to the material. Polymer gels are widely used for biochemical separations due to their excellent biocompatability. However, these gels are not mechanically strong and cannot be used at high flow rates and/or in organic solvents. This problem can be overcome by coating a useful polymer on a rigid support material, such as silica or zirconia. Many examples of polymer-coated silicas have been reported.[3,4,5] However, silica is inherently unstable at high pHs. Bonded phase and coated silicas also have problems with irreversible binding of proteins due to silanol-protein amine interactions, coulombic interactions, or hydrophobic interactions.[6] Zirconia is stable under these conditions, but is problematic in other ways. The surface of zirconia contains several types of sites, the most important chromatographically being the strong, hard Lewis acid sites.[7] These sites arise from the unsatisfied coordination of the surface zirconium atoms. The hard Lewis acid sites will adsorb any hard Lewis bases present in the mobile phase, including, but not limited to, phosphate, fluoride, hydroxide and carboxylic acids.[8,9] As proteins have many carboxylate groups, adsorption of a protein onto a zirconia surface is likely. Thus, we hoped that the polymer coating would shield the Lewis acid sites from the proteins. We have successfully used poly(ethyleneimine) coated zirconia to separate proteins[10] and nucleic acids[11] via ion exchange chromatography. Poly(butadiene) coated zirconia used in the reverse phase mode, has proven to be only marginally successful in the separation of proteins, due to the multiplicity of interactions encountered.[12] The polymer coating imparts the desired hydrophobic character to the phase; however, the zirconia surface also has Lewis acid sites that will adsorb proteins and other Lewis bases. In order to block these surface sites, a strong Lewis base (phosphate) is included in the mobile phase. This in turn creates a negatively charged surface which will retain proteins by coulombic interactions. Counteracting these interactions requires a high concentration of a non-interacting salt to screen the ion exchange sites. Thus, a complicated mobile phase is needed in order to counteract all of these interactions with the proteins. In this paper we examine a hydrophilic polymer (dextran) for use in the chromatography of proteins.

Several reports have examined the coating of dextran on silica for use in protein chromatography.[13,14,15,16,17] These materials include both size exclusion and affinity phases. We have recently reported the synthesis of carboxymethylated dextran (CMD) coated zirconia.[18] The dextran coating is

highly hydrophilic and easily derivatized, owing to its many pendant hydroxyl groups. These alcohol groups can be derivatized with a variety of ligands to create a useful chromatographic phase. We are currently working on an affinity phase based on the CMD-zirconia that has Cibicron Blue groups attached to the CMD polymer.

Before further derivatizing the coated dextran, the behavior of proteins on the CMD-zirconia was explored. In particular, we were interested in identifying any non-specific interactions that might subsequently interfere in the later use of the CMD-zirconia material as a support for affinity chromatography. We had hoped that the carboxymethyl-dextran coating would shield the Lewis acid sites from the proteins. However, previous studies using different probes have shown that this is not the case and the Lewis acid sites are still available to the solutes.[18] Blackwell[19] has shown that proteins can be eluted with fluoride ion (a hard Lewis base) in the mobile phase. We believe that the Lewis acid/base and ion exchange interactions are the dominant interactions on the CMD coated zirconia, and we will investigate these retention mechanisms by measuring the effects of Lewis base concentration, the mobile phase ionic strength, and pH on protein retention and recovery.

MATERIALS

Zirconia colloid (1000 Å in nitric acid, nominal pH 2, 20% solids by weight, Lot IV-40) was obtained from Nyacol Corp. (Ashland, MA). The dextran (9300 MW), the piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES), 2-[N-morpholiono]ethanesulfonic acid (MES), iodoacetic acid and all proteins were obtained from Sigma Chemical Co. (St. Louis, MO).

The relevant physical characteristics of the proteins are listed in Table 1. Dibasic potassium phosphate (reagent grade), sodium fluoride (reagent grade), sodium sulfate (reagent grade) and sodium chloride (reagent grade) were obtained from Mallinckrodt (Paris, Kentucky). Urea (ACS grade) and the 50% (w/w) solution of sodium hydroxide were obtained from Fisher Chemical (Fair Lawn, NJ). Boron trifluoride etherate was obtained from Aldrich (Milwaukee, WI). Sephadex G-10 SEC material was obtained from Pharmacia (Piscataway, NJ).

All water was deionized and then passed through Barnstead ion exchange and Organic Free cartridges followed by a 0.45 μm filter. All water was also boiled for 15 minutes to remove dissolved carbon dioxide prior to use.

Table 1

Protein Solute Sources and Physical Characteristics

Protein	Source	Mw[a]	pI[b]	Symbol
Cytochrome c	horse heart	11,700	9.4	■
Ovalbumin	chicken egg	45,000	4.9	X
Albumin	bovine serum	66,300	4.7	▼
Peroxidase	horseradish	34,000	9	▲
Laccase	pyricularia oryzae	62,000	3.2	●
Lysozyme	chicken egg white	14,310	11.0	◆
Transferrin	human	78,000	5.9	$
Hexokinase	bakers yeast	104,000	5	#

[a] molecular weight
[b] isoelectric point

METHODS

Preparation of Zirconia Particles

Zirconia particles for chromatography were prepared from colloidal zirconia by the polymerization induced colloidal aggregation (PICA) method (batch Coac-15).[20] The colloid had been centrifuged to remove fines and then resuspended in a 1% nitric acid solution. Particles were sintered at 750 °C for 6 hours, followed by treatment at 900 °C for 3 hours in a muffle furnace.[20] The particles were then pre-treated in a series of chemical steps. The particles were placed in a glass Erlenmeyer flask and freshly boiled water was added to just cover the surface of the particles. The slurry was then sonicated under vacuum for 10 minutes to remove air from the pores of the particles. This slurry was transferred to a plastic bottle. The water was decanted. Freshly made 0.5 M sodium hydroxide (made from 50% by weight solution) was then added so that the final volume was five volumes the volume of the particles. The slurry was placed on a shaker bath for 24 hours. The particles were allowed to settle and the supernatant was decanted. The particles were then rinsed with water. Freshly made 0.5 M nitric acid was then added in the same proportion as the sodium hydroxide. The slurry was then placed on a shaker bath for 24 hours. The supernatant was then decanted and the particles were rinsed twice with water. They were then dried under vacuum at 110 °C overnight.

The surface area and pore volume of the particles were measured from the nitrogen adsorption isotherm using a Micromeritics ASAP 2000 porosimeter. Pore diameters were estimated using the BJH equation, which assumes

cylindrical pores.[21] Surface areas were estimated using the BET method from the nitrogen sorptometry data.[22] The physical characteristics of these particles are the same as in Reference 18.

Preparation of Carboxymethyl-dextran Coated Zirconia

Carboxymethylated dextran was prepared and coated onto zirconia as described previously.[18] A 0.1 g sample of the CMD was dissolved in 50 mL of 100 mM PIPES, pH 6.5 to make a 5 g/L solution of CMD. To 40 mL of this solution, 4 g of zirconia particles were added. This suspension was sonicated under vacuum for five minutes and then capped. The bottle was then placed on a shaker bath for 2 days, with periodic manual shaking.

After the allotted time, the particles were allowed to settle and the supernatant was decanted. Ethanol (40 mL) was then added and the slurry was shaken for ten minutes. The particles were allowed to settle for thirty minutes and the ethanol was decanted. This procedure was repeated for 50:50 ethanol:chloroform (v:v) and chloroform. The particles were then allowed to air dry at room temperature.

The coated particles were placed in a 30 mL septum flask and 10 mL of chloroform were added. The flask was capped and sonicated for 5 minutes. 1,4-butanediol diglycidyl ether (BUDGE) (17 μL) was added via syringe and the flask was sealed while flushing with nitrogen. A boron trifluoride etherate solution in chloroform was prepared using 0.5 mL of boron trifluoride etherate and 7 mL of chloroform. 0.5 mL of this solution was then added to the flask containing the coated particles. The particle suspension was swirled and allowed to sit for 30-40 minutes. After this time, the solution was removed and the particles were rinsed with ethanol and allowed to dry at room temperature.

Chromatography

Carboxymethyl-dextran-coated zirconia was packed in 5.0 cm x 0.46 cm stainless steel columns by the stirred upwards slurry technique in HPLC grade isopropanol.[23] The Sephadex G- 10 column was packed by gravity filtration in a 5.0 cm x 0.46 cm stainless steel column. All columns used 0.45 μm screens (Chromtech, Apple Valley, MN) in the end fittings instead of the thicker frits to reduce protein adsorption.[24] Experiments were run on a HP 1090L Liquid Chromatograph with diode array detection and a Chemstation data handling system. Protein recovery was calculated by Equation 1.

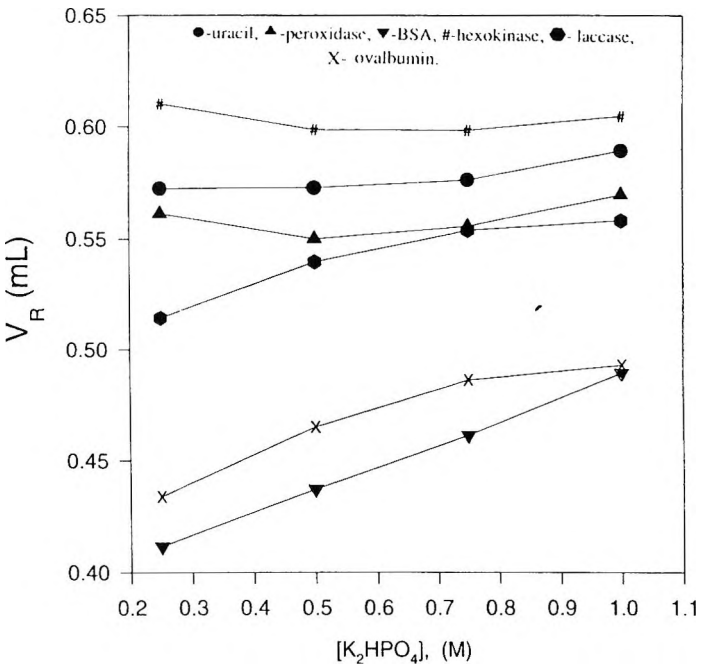

Figure 1. Effect of phosphate concentration on protein elution volume. The mobile phase was potassium phosphate at various concentrations, pH 7. Conditions: The flow rate was 0.5 mL/min. Proteins were 1% by weight in the mobile phase buffer. Detection was at 280nm.

$$\% \text{ Recovery} = \frac{\text{Area}_{\text{CMD-ZrO}_2}}{\text{Area}_{\text{Seph}}} \text{X}(100\%) \qquad (1)$$

where $\text{Area}_{\text{CMD-ZrO}_2}$ is the area count of the peak (as measured by the data system) from the CMD-ZrO_2 column and $\text{Area}_{\text{Seph}}$ is the area count from a Sephadex G-10 column which, we assume, will have no interactions with the solutes used. Area counts are obtained from experiments using protein injections from the same solution and are performed under the same conditions. The area counts used for the calculation are averages of two or more measurements. All other parameters are listed in the figure captions. Retention volumes were calculated from the retention times, as measured by the integrator or integration program (i.e. the peak maximum), multiplied by the flow rate. All retention volumes are averages of two or more separate experiments.

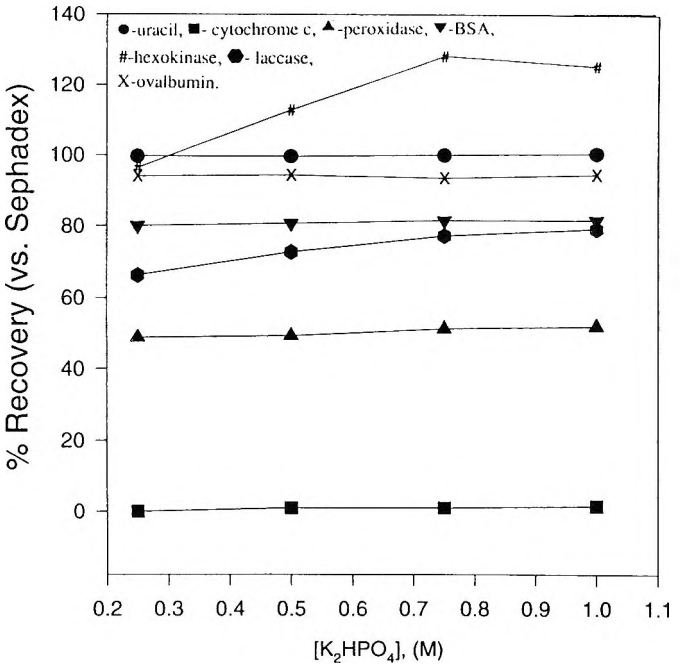

Figure 2. Effect of phosphate concentration on protein recovery. Protein recovery is expressed as the percentage of the area measured compared to that on a column of identical size packed with Sephadex G-10 under the same conditions. All other conditions the same as in Figure 1.

RESULTS AND DISCUSSION

Effect of Phosphate and Fluoride Concentration

In order to investigate the Lewis acid/base interactions, the concentrations of two strong, hard Lewis bases (phosphate and fluoride) were varied and the effect on protein retention and recovery was observed. If Lewis acid/base interactions were important, we would expect that the protein retention volume would decrease and protein recovery would increase as the Lewis base concentration in the mobile phase was increased. Figures 1 and 2 show the effect of phosphate concentration on the retention (Figure 1) and recovery (Figure 2) of various proteins. Cytochrome c (see ■ in Figure 2) does not elute at the lowest concentration of phosphate and its recovery is very poor in all cases. We attribute this to the high charge on this protein at pH 7. At this pH, cytochrome c will have a positive charge and will be strongly retained on the

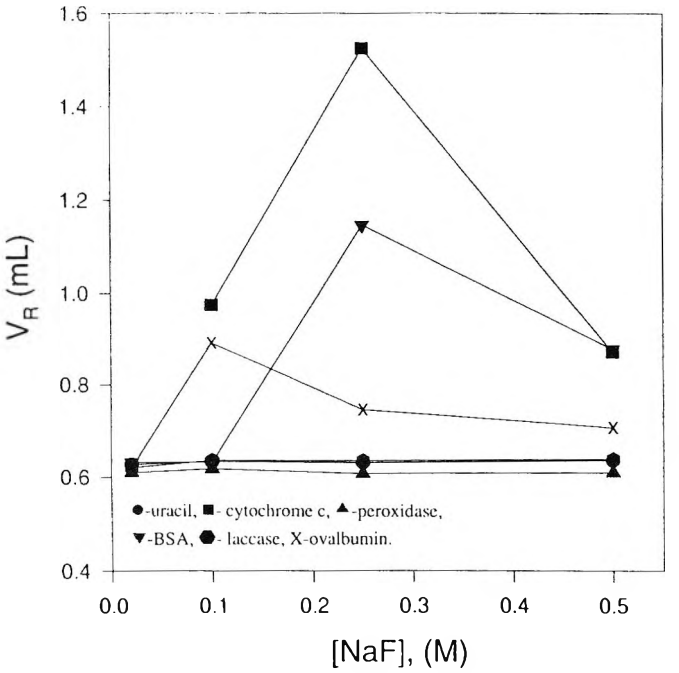

Figure 3. Effect of fluoride concentration on protein elution volume. The mobile phase was 20 mM MES + 250 mM Na_2SO_4 + the fluoride concentration shown at pH 5.5. Other conditions are the same as in Figure 1

negatively charged phosphate adsorbed on the zirconia surface. The small size of the protein also allows it to penetrate the dextran layer and interact strongly with the surface. Peroxidase, which also has a high pI but is larger (about twice the molecular weight), has a higher recovery, although it is still only about 50% recovered. The other proteins show little, if any, change in retention or recovery as the phosphate concentration is changed. Ovalbumin, BSA and laccase all show a slight increase in retention volume as the phosphate concentration is increased. However, they are all eluted before the totally included volume, as measured by uracil. At pH 7, these proteins are negatively charged, as is the zirconia surface. Thus, they are kept from entering the pore space by an ion exclusion mechanism. As the phosphate salt concentration (as well as the ionic strength) is increased, the surface charge is screened from the protein's charge, allowing the protein access to more of the pore space and thus increasing the elution volume. The recoveries of these proteins, however, does not change and are all near 100%.

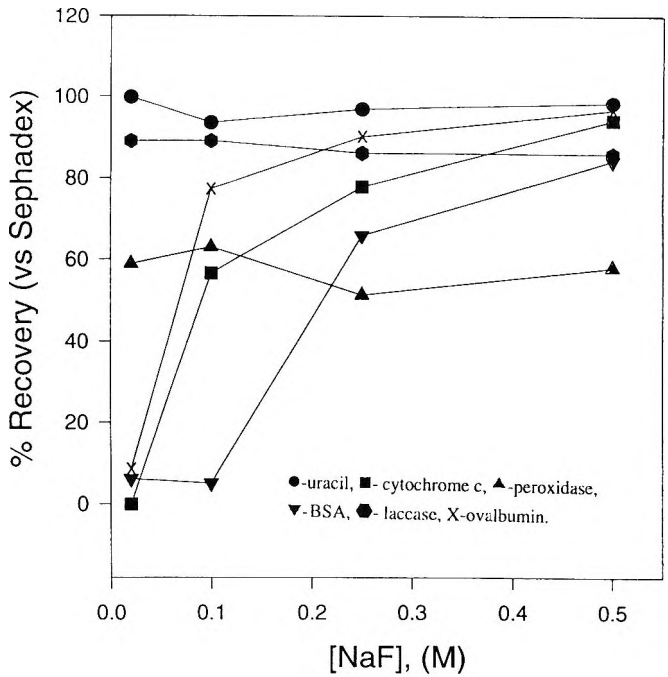

Figure 4. Effect of fluoride concentration on protein recovery. Protein recovery is expressed as the percentage of the area measured compared to that on a column of identical size packed with Sephadex G-10 under the same conditions. All other conditions are the same as in Figure 3.

The effect of the fluoride concentration on protein retention and recovery (Figures 3 and 4) is quite different from that of the phosphate mobile phases. The recoveries of several of the proteins (cytochrome c, BSA and ovalbumin) are very low at the lowest fluoride concentration (20 mM). However, the recovery improves remarkably as the concentration of sodium fluoride is increased. In sharp contrast to phosphate systems, even cytochrome c is eluted with close to 100% recovery when the fluoride concentration is increased to above 0.25 M. The retention volumes of three of the proteins change over the concentration range examined. Cytochrome c is very sensitive to the amount of fluoride present, as are ovalbumin and BSA. The retention behavior of ovalbumin and BSA (hydrophobic proteins)[25] is possibly due to the increased contribution of hydrophobic interactions as the ionic strength increases. The behavior of the cytochrome c can be fully rationalized as follows. At 20 mM sodium fluoride, there is not enough fluoride to block the Lewis acid surface sites and the cytochrome c is irreversibly retained. As the fluoride concentration is increased, the Lewis acid sites are blocked by fluoride adsorption, but a

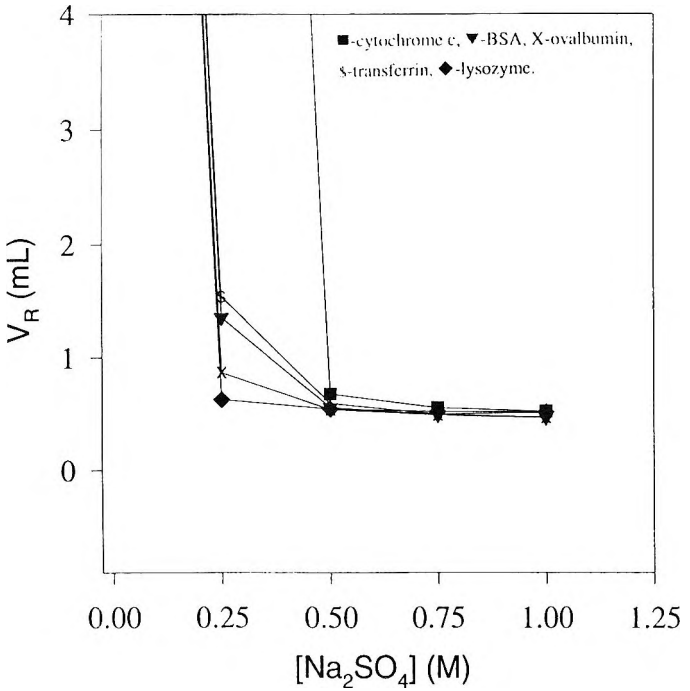

Figure 5. Effect of ionic strength on the retention time of proteins in fluoride containing mobile phases. All mobile phases contain 20mM MES + 100mM NaF + the sodium sulfate at the concentration shown. All other conditions are the same as in Figure 1.

negative charge is established on the surface due to the adsorbed fluoride. This negative charge retains the positively charged cytochrome c. Finally, between 0.25 and 0.50 M fluoride, the ionic strength becomes high enough to screen the surface charge from the cytochrome c, which results in the observed decrease in retention. Thus, it appears that Lewis base concentration is not the sole controlling factor in retention of proteins. Ionic strength is also an important factor.

Effect of Ionic Strength

The effect of the ionic strength of the mobile phase on the retention and recovery of proteins was examined by varying the concentration of sodium sulfate, a non-interacting salt, in the presence of a fixed concentration of the two Lewis bases (phosphate and fluoride). The two Lewis bases were examined

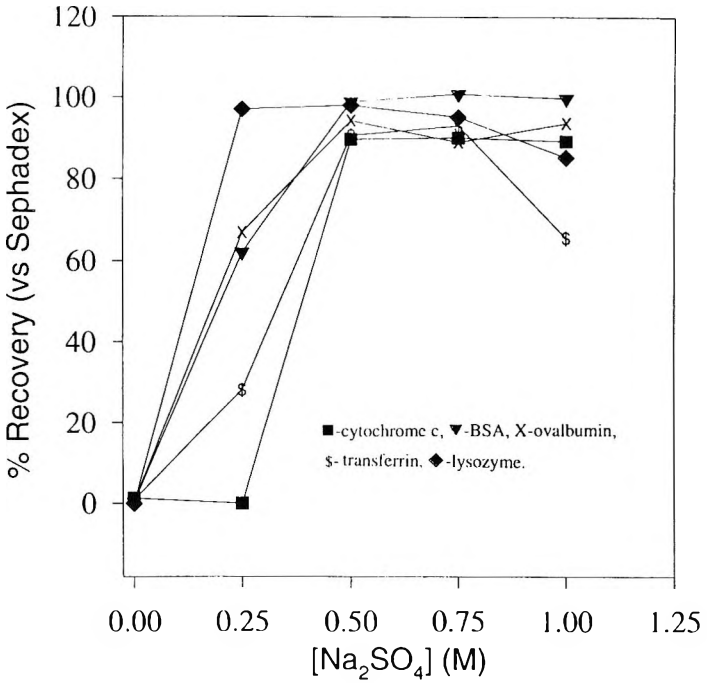

Figure 6. Effect of ionic strength on the recovery of proteins in fluoride containing mobile phases. Protein recovery is expressed as the percentage of the area measured compared to that on a column packed with Sephadex G-10 under the same conditions. All other conditions are as in Figure 1.

separately at a concentration of 100 mM each. It is important to note here, that the two different Lewis base buffers do not have the same ionic strength.

Figures 5 and 6 show the effect of the ionic strength of the mobile phase on the retention (Figure 5) and recovery (Figure 6) of proteins in fluoride mobile phases. These mobile phases contained 100 mM sodium fluoride and 20 mM MES buffer at pH 5.5 and a variable concentration of sodium sulfate. Figure 5 shows that at sodium sulfate concentrations below 0.25 M, no protein eluted from the CMD-coated zirconia. When the ionic strength was increased by adding 0.250 M sodium sulfate, all proteins except cytochrome c were eluted with some recovery. There is some selectivity in the retention volumes at this sodium sulfate concentration, which disappears when the ionic strength is increased. When the sodium sulfate concentration is 0.5 M or greater, the proteins all elute at about the same volume, and there is very little resolution between them.

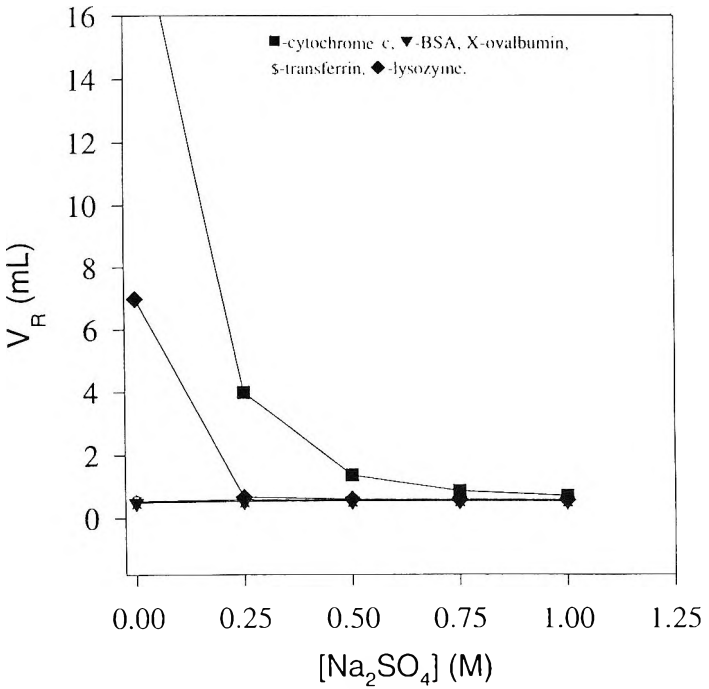

Figure 7. Effect of ionic strength on the retention of proteins in phosphate containing mobile phases. All mobile phases contain 100mM K_2HPO_4 at pH 7 + sodium sulfate at the concentration shown. All other conditions are the same as in Figure 1.

The effect of ionic strength on protein recovery is shown in Figure 6. When the sodium sulfate concentration is 0.5 M or above, the proteins all elute with 80% or better recovery. It appears that a high ionic strength is required to screen the ion exchange sites originating from the surface adsorbed fluoride.

Phosphate containing mobile phases show different behavior than that of the fluoride mobile phases. These experiments were all run with mobile phases containing 100 mM potassium phosphate at pH 7.0. Unlike the fluoride case, all the proteins except cytochrome c do elute when there is no sodium sulfate present in the mobile phase (Figure 7). The trend in protein recovery (Figure 8) is also different from the fluoride case. To be completely recovered, Cytochrome c requires a sodium sulfate concentration of at least 0.75 M to be recovered completely. All other proteins are eluted with some recovery (lysozyme is the lowest at 38%), even in the sulfate free mobile phase. Lysozyme does not behave as expected, since it has a higher pI than cytochrome c, and thus, should have a higher negative charge and be retained more than the

cytochrome c. This may be due to the moderately larger size of lysozyme in comparison to cytochrome c. Lysozyme also has fewer[18] basic residues (positively charged) than does cytochrome c.[23] Since cytochrome c is smaller, the average surface charges (charge per unit surface area) of the two proteins is different, with cytochrome c having a higher average positive surface charge.[25] This will make it more attractive to the negative charges on the surface of the zirconia than the lysozyme.

We should note, here, that this does not appear to be a good size exclusion chromatographic phase for proteins. Under mobile phase conditions which give us good recovery of all the proteins, the elution volume differences between the proteins are very small. However, the fact that most of the proteins elute at approximately the void volume (as measured by uracil), implies that the pore space is accessible to the proteins. This is very important for later studies on derivatized supports. If the proteins can not access the pore space, where most of the ligands are located, the capacity of the column will be very poor, and the phase will not be very useful. It appears that we do not have to worry about this problem for proteins that are as large as the ones tested here.

Effect of pH

Since it is apparent that the zirconia surface charge resulting from adsorbed Lewis bases is important, a study of the effect of pH on retention and recovery was undertaken. We chose mobile phase conditions (100 mM K_2HPO_4 + 0.75 M Na_2SO_4) under which the proteins all eluted at pH 7. Figures 9 and 10 show the effect of changing the pH of a phosphate mobile phase on protein retention and recovery. We can see that at pH 3, most proteins do not elute, and those that do (lysozyme) have a higher retention volume than at the other pHs. As the pH is increased, the proteins become less retained. This can be explained as the combination of two effects. The first, is the charge on the protein itself. At pH 3, all of the proteins used as solutes have a positive charge and will be retained (in these cases, irreversibly) by the negatively charged phosphate/zirconia surface. As the pH is raised, some of the proteins (BSA and ovalbumin) will become negatively charged, and will no longer be retained by the zirconia surface. The other mechanism accounts for the behavior of the high pI proteins (cytochrome c, peroxidase and lysozyme). As the pH is lowered, the concentration of hydroxide present in the solution will decrease. This shifts the equilibrium between phosphate and hydroxide for the surface Lewis acid sites in favor of phosphate. This will increase the amount of phosphate adsorbed and change the surface charge.[26] Thus, the proteins that are negatively charged will be affected by the pH due to the increased charge on the surface. Of course, the protonation state of the adsorbed phosphate will also change, decreasing the surface charge. However, it appears from these results, especially the behavior of lysozyme and cytochrome c, that the surface charge increases as the pH is decreased.

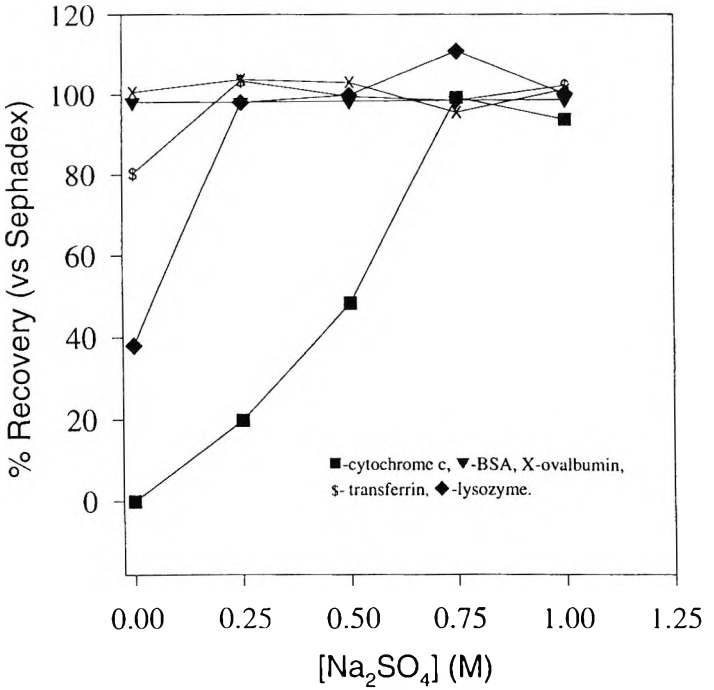

Figure 8. Effect of ionic strength on the recovery of proteins in phosphate containing mobile phases. Protein recovery is expressed as the percentage of the area measured compared to that on a column packed with Sephadex G-10 under the same conditions. All other conditions are as in Figure 5.

Effect of the Type Of Lewis Base

As discussed above, a Lewis base is necessary to effect elution of proteins. As can be seen in Figures 1 and 3, protein retention is not strongly influenced by the concentration of either phosphate or fluoride, once a minimum concentration has been reached. This is most likely due to the fact that both of these anions are strongly adsorbed by zirconia and the equilibrium will strongly favor the adsorbed state. Thus, changing the concentration will only affect those proteins that have a very strong affinity for zirconia. However, when these Lewis bases adsorb to the surface of the zirconia, a negative charge is formed on the surface. At the mobile phase pHs used, the adsorbed Lewis bases will impart a cation exchange character to the zirconia support material. Proteins with a positive charge (i.e. proteins whose pI is higher than the pH of the mobile phase), will be retained by the surface. Increasing the ionic strength of the mobile phase by adding a neutral salt will screen the ion exchange sites and the proteins are eluted from the material.

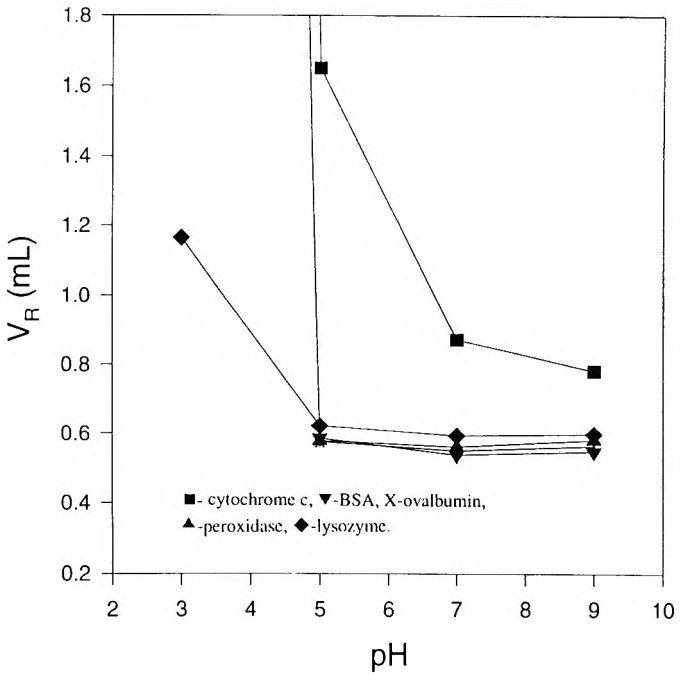

Figure 9. Effect of mobile phase pH on the retention volume of proteins. All mobile phases contain 100 mM K_2HPO_4 + 0.75 M Na_2SO_4 adjusted to the pH reported either by concentrated HCl or NaOH. All other conditions are the same as in Figure 1.

Although the two different mobile phases both contain strong Lewis bases, they do exhibit some differences. When there is no sodium sulfate in the mobile phase, the proteins are not eluted at all by the fluoride mobile phase, yet all proteins, except cytochrome c, are eluted from the phosphate mobile phase. This can be explained by examining the ionic strength of the two mobile phases. When there is no sodium sulfate in the mobile phase, the ionic strength of the 100 mM potassium phosphate mobile phase is 0.6 M, while the ionic strength of the sodium fluoride/MES mobile phase is only 0.24 M. This difference in mobile phase ionic strength is sufficient to explain the differences in the eluting strength of the two mobile phases. Another difference between the two mobile phases is the ionic strength at which cytochrome c is eluted. The phosphate mobile phase requires a higher concentration of sodium sulfate (0.75 M) to fully elute the cytochrome c than the fluoride mobile phase (0.5 M). This is can be explained by examination of the surface charge imparted to the surface by the two mobile phases. Fluoride will only have one negative charge per adsorbed molecule, while phosphate will have between one and two at the pHs used for the two mobile phases. This difference appears in the behavior of

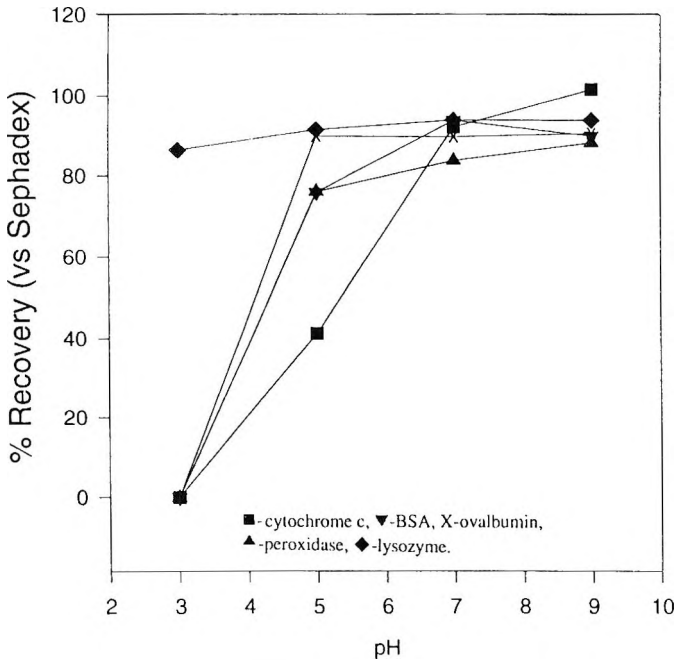

Figure 10. Effect of mobile phase pH on the recovery of proteins. All mobile phases contain 100 mM K_2HPO_4 + 0.75 M Na_2SO_4 adjusted to the pH reported either by addition of concentrated HCl or NaOH. All other conditions are the same as in Figure 1.

the highly charged protein cytochrome c. It will take a higher ionic strength to screen the charge on the phosphate-adsorbed surface than that on the fluoride-adsorbed surface. Thus, the ability of a Lewis base to elute a protein will depend on how highly charged the protein is.

CONCLUSIONS

The elution behavior of a set of highly varied (pI, MW) proteins was examined on CMD-coated zirconia. We found that both Lewis acid/base interactions and coulombic (ion exchange) interactions are taking place. The Lewis acid/base interactions are eliminated by adding a hard Lewis base (e.g. phosphate or fluoride) to the mobile phase. The ion exchange effects can be minimized by increasing the ionic strength of the mobile phase. We also found that both phosphate and fluoride buffers work well in eluting proteins. However, there are differences between the two mobile phase buffers in the surface charge they produce on zirconia.

Although, this does not appear to be a suitable phase for size exclusion chromatography, the proteins can access all the pores in the material, which is important for affinity chromatography.

We have also shown that, by choosing the correct mobile phase conditions, non-specific interactions can be eliminated and the CMD-zirconia phase can make a suitable support for affinity ligands.

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PROBLEMS OF BASELINE CONSTRUCTION FOR CALCULATION OF MOLECULAR WEIGHT AVERAGES IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

Problems associated wtih poor baseline recovery between the end of the polymer chromatogram and the beginning of the solvent impurity peaks are discussed, and an approach for accurate calculation of molecular weight averages of polymers is described. Polystyrene SRM 706, where the presence of low molecular weight materials is suspected, was used as a sample polymer by way of example. Tetrahydrofuran and a refractometer were used as mobile phase and detector, respectively. Although a stable baseline was obtained under the appropriate conditions, baseline resolution was not obtained at the end of the polymer chromatogram, and the peak-end response was flat and parallel to the extrapolated baseline. The reason for no baseline resolution was found to be due to the existence of low molecular weight materials, by measuring the chromatogram of the polymer from which low molecular weight materials were removed. Inclusion of the response below molecular weight (MW) 2000 resulted in extremely low values of number-average MW.

Cutting of MW below 8000 approached the certified value. To avoid arbitrary selection of cutoff MW (or retention volume), several selection rules are proposed.

INTRODUCTION

Size exclusion chromatography (SEC) is one of the techniques that can measure both weight-average and number-average molecular weights (MW) simultaneously, and has a long history of over three decades. Although it took a couple of hours to get results at the beginning, developments of high-performance columns and accurate pumping systems made possible the calculation of MW averages of polymers by SEC in a half hour. The development of molecular weight-sensitive detectors, such as a light scattering photometer and a viscometer, enabled the calculation of MW averages without prior construction of a calibration curve. However, several reports of round-robin tests for the comparison of MW averages of polystyrenes (PS) calculated by SEC showed us that values of MW averages were scattered, and obtaining accurate and precise data of MW averages was not easily accomplished.[1-3] Especially the relative standard deviation of number-average MW was about 13%.[2]

One of the possible reasons considered for this scattering of data is concerned with the baseline construction. Before an SEC chromatogram is successfully interpreted, a suitable, accurate baseline must be constructed. Normally, retention volumes, V_a and V_b, corresponding to the beginning and the end of the chromatogram traced on a strip chart recorder or on a computer display must be chosen properly; then, a linear baseline is drawn between them. The choice of V_a, the low-retention volume or high-MW-end of the chromatogram, is usually straightforward. Here, the baseline before the chromatographic band is usually stable and not influenced by low MW impurities. The selection of V_b, the high-retention volume or low-MW-end of the chromatogram, however, is rather difficult and depends on the separation of the polymer chromatogram from peaks of solvent impurities and on the recovery of a stable baseline. With baseline resolution of all peaks, the choice of V_b is obvious.

If the baseline is unstable, or there is significant noise, this can be a difficult problem. However, even though the baseline is stable, that is, the baseline between the sample injection and the beginning of the polymer chromatogram and the baseline after the solvent impurity peaks are stable and the extrapolation from the former baseline superimposes the latter baseline, poor baseline recovery after the polymer chromatogram is often encountered with polymers. This presents a serious problem in establishing the correct baseline and the end-point limit for the chromatogram. Oligomers included in the

polymers may disturb the baseline recovery. In some instances the detector response from oligomeric materials of the polymers may coincide with the onset of the response for the impurity peaks. For some systems, where the viscosity of the sample solution is much higher than that of the solvent used as the mobile phase, pre-peak or post-peak "undershoot" of the baseline may occur. In these cases, it has been found that better experimental agreement occurs when the pre-peak baseline is taken as the true one.[4]

The purpose of this investigation is to study the baseline construction, and aims to clarify whether the poor baseline recovery between the end of the polymer chromatogram and the beginning of the solvent impurity peaks is caused by the existence of oligomeric materials in the polymer or by the viscosity difference between the polymer solution injected to the SEC system and the mobile phase. The influence of the cutoff limit to number-average MW (designated as M_n) and the possibility of connecting an additional column, packed with gels of narrou pore sizes, are also discussed.

EXPERIMENTAL

SEC measurements were performed with a Jasco Trirotar high performance liquid chromatograph (Jasco Corp., Tokyo, Japan) with a Model SE-31 differential refractometer (RI) and a Model KT-15 solvent degasser (both from Showa Denko Co., Tokyo, Japan). Two SEC column systems were used in this study: column system A consisted of two Shodex KF 806M (300-mm x 8-mm i.d.)(Showa Denko), packed with a mixture of polystyrene (PS) gels of nominal exclusion limits of 10^3, 10^4, 10^5, and 10^6 Å, and column system B was two Shodex KF 806M + one Shodex KF 800D (100-mm x 8-mm i.d.), packed with PS gel of narrow pore size (comparable to PS gel packed in Shodex KF 801). The number of theoretical plates of column sytem A was 22,000 by injecting 0.25 mL of a solution of 0.3% benzene in tetrahydrofuran (THF).

Sample used in this experiment, by way of example, was SRM 706 PS (NIST, Washington, DC), which was used directly without any treatment and, also, was used after purification by precipitation (designated as purified SRM 706 PS). The purification of SRM 706 PS was performed as follows: one gram of the polymer was dissolved in 100 mL chloroform and the solution was poured into 500 mL methanol drop by drop, followed by filtration of the precipitate and by drying the precipitate to constant weight at 40 °C under reduced pressure for 24 h. The calibration curve of the SEC system was constructed by determing the peak retention volumes of PS standards of narrow MW distributions. MW's of the standards were $1.8x10^6$, $6.7x10^5$, $4.11x10^5$, $2.0x10^5$, 97,200, 20,400, and 2,100, purchased from Pressure Chemical Co. (Pittsburgh, PA) and 1,000 and 500 from Toso (Tokyo, Japan).

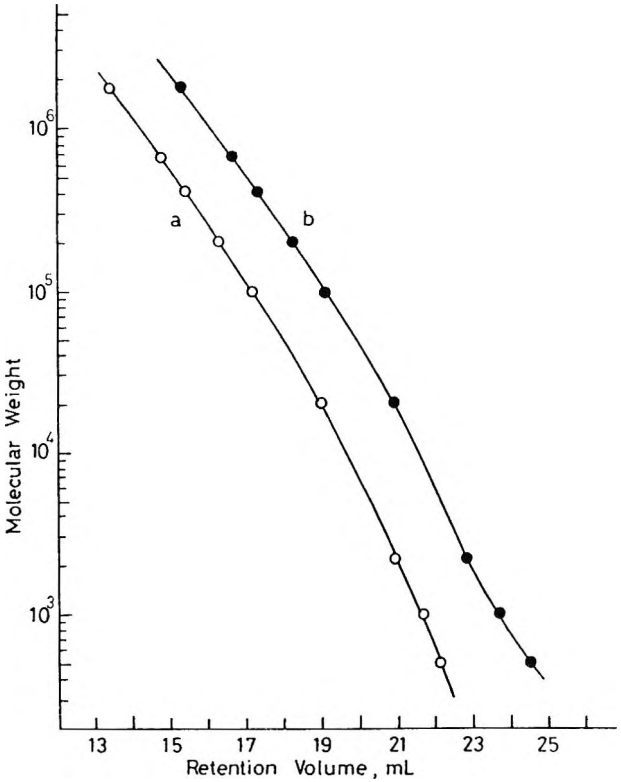

Figure 1. Calibration plots of column system A (two Shodex KF 806M columns) (a) and column system B (two Shodex KF 806M columns + one Shodex KF 800D column) (b).

THF was used as the mobile phase. The antioxidant, BHT (2,6-di-t-butyl-p-cresol), was added at a concentration of 0.05%. The flow rate was 1.0 mL/min and the sample injection volume was 0.25 mL. Detector attenuation was x8. Sample concentrations were 0.2% (w/v) for SRM 706 PS into column system A, 0.12% for SRM 706 PS into column system B, and 0.1% for PS standards of narrow MW distributions, except those of MW 6.7×10^5 and 1.8×10^6 (both 0.05%).

RESULTS AND DISCUSSION

Calibration curves for the two column systems, A and B, are shown in Figure 1. Shodex KF 800D is packed with PS gel corresponding to that packed in Shodex KF 801 column; it has a exclusion limit of PS MW 1,000.

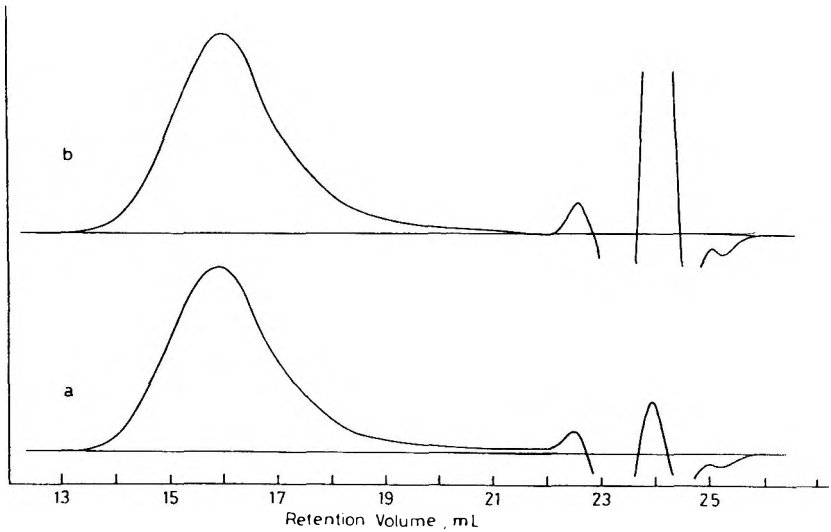

Figure 2. Chromatograms of polystyrene SRM 706 PS (a) and purified SRM 706 PS (b) obtained with column system A.

The column is commercially available under the name of "Solvent-Peak Separation" column. The calibration curve of column system B, in Figure 1, is shifted 1.9 mL to the right of that of column system A, and the slope of two calibration curves was the same down to MW 2,000 from the higher MW region. The shift of retention volume increased below MW 1,000. As the two calibration curves have the same slope above MW 2,000, it can be said that the connection of Shodex KF 800D column to Shodex KF 806M columns does not influence the measurement of MW averages for polymers (see Tables 1 and 2).

Chromatograms of SRM 706 PS and purified SRM 706 PS measured with column system A are shown in Figure 2. Chromatograms were recorded with a strip chart recorder. A linear baseline was manually drawn between the beginning of the polymer chromatogram and the end of the final solvent impurity peaks for the chromatogram of SRM 706 PS (Figure 2 (a)). The extrapolation of the baseline from the injection point of the sample solution to the end of the final solvent impurity peak agreed well with the baseline drawn between the beginning of the polymer chromatogram and the end of the solvent impurity peaks. These phenomena are often encountered when a stable baseline is obtained. This stable baseline was usually obtained within a couple of hours after the startup of the SEC system.

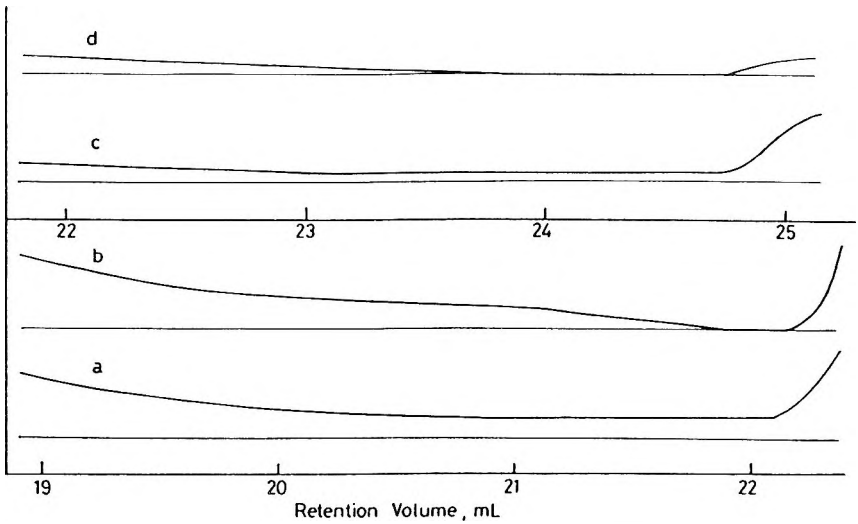

Figure 3. Magnified chromatograms of the low MW ends of polymer chromatograms. (a) SRM 706 PS in column system A; (b) purified SRM 706 PS in column system A; (c) SRM 706 PS in column system B; (d) purified SRM 706 PS in column system B.

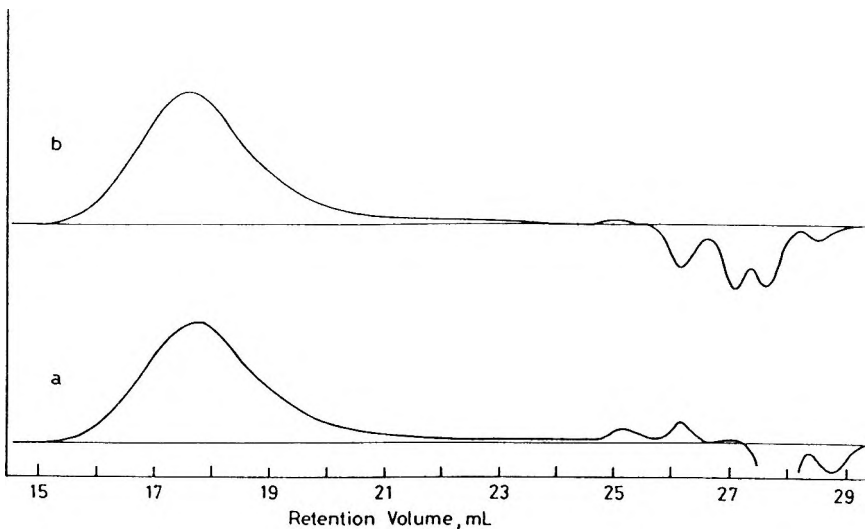

Figure 4. Chromatograms of polystyrene SRM 706 PS (a) and purified SRM 706 PS (b) obtained with column system B.

Table 1

Molecular Weight Averages of PS SRM 706 Measured with Column System A at Different Cutoff Molecular Weights (Retention Volumes)

	Molecular Weight			
	SRM 706		Purified SRM 706	
Cutoff MW (V_R, mL)	Weight-Average	Number-Average	Weight-Average	Number-Average
580 (22.0)	2.73×10^5	0.476×10^5	---	---
700 (21.85)	---	---	2.84×10^5	0.678×10^5
2,000 (21.0)	2.76×10^5	0.958×10^5	2.86×10^5	0.931×10^5
5,000 (20.3)	2.78×10^5	1.18×10^5	2.87×10^5	1.15×10^5
8,000 (19.75)	2.80×10^5	1.33×10^5	2.89×10^5	1.30×10^5

In the chromatogram of SRM 706 PS (Figure 2 (a)), the peak response after retention volume (V_R) 21.0 mL (MW 2,000) was flat and parallel with the baseline. Part of the chromatogram between V_R = 19 and 22 mL is magnified and shown in Figure 3 (a) to display the flatness of the response clearly. This flat response is also often experienced. There seems to be no elution of polymer (or oligomer) molecules below MW 2,000 and it may be possible to establish the high retention volume (low MW) end of the chromatogram V_b to be 21.0 mL and to draw a baseline between V_b and V_a, which is the low retention volume (high MW) end of the chromatogram (= 13.3 mL). Using the baseline thus constructed, or using the baseline extrapolated from the beginning of the polymer chromatogram, MW averages are usually calculated, cutting off retention volume over 21.0 mL (MW less than 2,000). However, there is no guarantee that PS oligomers below MW 2,000 are not included in this sample.

Now, in order to verify whether the reason of the flat response is due to the existence of low MW material below MW 2,000 or the viscosity difference between the sample solution and the mobile phase, SRM 706 PS was purified to remove low MW materials. The chromatogram of the purified SRM 706 PS is shown in Figure 2 (b) and the magnified one in Figure 3 (b). Obviously, the

elution of oligomeric materials below MW 2,000 was observed and the baseline resolution was obtained at V_R = 21.85 mL (MW 700). The polymer chromatogram was adequately separated from the solvent impurity peaks. Low MW materials below MW 700 were removed by purification and good baseline recovery was obtained. The flat response between the polymer chromatogram and the solvent impurity peaks, as shown in Figures 2 (a) and 3 (a), can be concluded to be due to the elution of low MW materials, not to a viscosity difference.

The arbitrary selection of curve limits is one of the most significant and restrictive aspects of accurate MW analysis by SEC. Although the assignment of the low MW limit intuitively causes the dominant error in the value of M_n,[5] the oligomer response may be incorporated into the calculation of MW averages of polymers. In this instance, the end points for the calculation of SRM 706 PS and purified SRM 706 PS are 22.0 mL (MW 580) and 21.85 mL (MW 700), respectively. The influence of cutoff MW (or retention volume) on the values of MW averages is shown in Table 1.

Inclusion of the response above V_R 21.0 mL (below MW 2,000) resulted in extremely low values of M_n. The effect upon the values of weight-average MW (M_w) was small.[5] Cutting off MW below 8,000 (above V_R = 19.75 mL) approaches the value of M_n of 1.30 - 1.33 x 10^5, which is close to the certified value from NIST (1.35 x 10^5) where the certified value was measured by membrane osmometry. It is thought that the value of M_n obtained by membrane osmometry is usually higher than the true value because of the permeation of polymer molecules less than MW 10^4 through the membrane used for the membrane osmometry experiment. This concept coincides with our result that the value of M_n, calculated excluding the response below MW 8,000, was almost the same as the NIST certified value.

No one can avoid the inclusion of the all response before the solvent impurity peaks into the calculation of MW averages, from the standpoint of the SEC calculation procedure. On the other hand, when the value obtained by membrane osmometry has to compare with the values obtained by SEC, or is used as a certified one, the response related to MW less than 10,000, or at least less than 8,000, should not be included into the calculation of MW averages. In order to estimate polydispersity, for example, the choice of cutoff MW 8,000 for SRM 706 PS (cutoff V_R 19.75 mL) is reasonable. However, this selection is too intuitive and, if a chromatogram shifts to the low MW region, then cutoff MW must be less than 8000. In the chromatogram of SRM 706 PS (Figure 2 (a)), the area between V_R 19.75 mL (MW 8,000) and 22.0 mL (MW 580) is 2.2% of the total area and the ratio of the response at V_R 19.75 mL to that at the maximum one is 3.7%.

Table 2

Molecular Weight Averages of PS SRM 706 Measured with Column System B at Different Cutoff Molecular Weight (Retention Volume)

	Molecular Weight			
	SRM 706		Purified SRM 706	
Cutoff MW (V_R, mL)	Weight-Average	Number-Average	Weight-Average	Number-Average
480 (24.6)	2.68×10^5	0.463×10^5	---	---
820 (23.85)	---	---	2.78×10^5	0.742×10^5
1,800 (23.0)	2.71×10^5	0.880×10^5	2.75×10^5	0.907×10^5
5,000 (22.1)	2.73×10^5	1.21×10^5	2.81×10^5	1.20×10^5
8,000	2.74×10^5	1.32×10^5	2.82×10^5	1.30×10^5

Therefore, one possible procedure for selecting the cutoff MW (or retention volume) is that, when a chromatogram is tailing, the height near the end of the chromatogram where the height is less than 3.5% of the maximum one of the polymer chromatogram can be excluded from the calculation of MW averages. Or, the portion of the area less than 2% of the total from the end of the polymer chromatogram can be neglected during the calculation. This discussion can also be applied to the chromatogram for which baseline resolution is attained, as in the case of purified SRM 706 PS (Figure 2 (b) and Table 1), but exhibits tailing. Inclusion of the all response into the calculation resulted in very low values for M_n.

In order to further separate the polymer chromatogram from the solvent impurity peaks, a column of Shodex KF 800D was connected to two Shodex KF 806M columns; the resultant chromatograms are shown in Figure 4. Flat baseline of the chromatogram of SRM 706 PS (Figure 4 (a)) was obtained at V_R = 23.0 mL (MW 1,800) up to V_R=24.7 mL (MW 440) where the front of the solvent impurity peaks appeared. The purpose of the baseline resolution was not attained with this sample because of the existence of low MW materials in the

polymer. Similarly to the case of purified SRM 706 PS with column system A, the baseline resolution for purified SRM 706 PS with column system B was attained at V_R = 23.85 mL (MW 820).

Although baseline resolution was not attained for SRM 706 PS, the connection of Shodex KF 800D was effective to draw apart (increase) the distance between MW 800 and 500 from 0.25 mL to 0.9 mL as retention volume, which makes it easy to estimate V_b. Thus, the connection of Shodex KF 800D is valuable for eliminating or for minimizing the cutpoint problems. The magnified chromatograms of these two samples are shown in Figure 3 (c) and (d).

MW averages at different cutoff MW were calculated with column system B and the results are listed in Table 2. Similarly to Table 1, it is obvious that in order to get a comparable value of M_n obtained by membrane osmometry, cutting off MW less than 8,000 is necessary.

In conclusion, the procedure for constructing the baseline is to draw a linear baseline from the beginning of the polymer chromatogram to the end of the solvent impurity peaks, or to extrapolate the baseline at the beginning of the polymer chromatogram. The flat response at the end of polymer chromatogram is due to the existence of low MW materials. Several selection rules for the cutoff-point limit are proposed. When the SEC results are to be compared with the certified values obtained by membrane osmometry, MW less than 10,000, or at least less than 8,000, should not be included in the calculation of MW averages. In order to estimate polydispersity, the response (height) less than 3.5% of the maximum one at both sides (or at the end of the polymer chromatogram) should be excluded. The portion of the area less than 2% of the total from the end of the polymer chromatogram can be neglected from the calculation. The influence on the M_w value by this procedure is insignificant (the decrease of 0.7% from the original value). Except for these two cases, no excuse to cutoff MW into the calculation of MW averages is considered. All response up to the beginning of the solvent impurity peaks should be included into the calculation.

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TEMPERATURE PROGRAMMING IN OPEN TUBULAR LIQUID CHROMATOGRAPHY

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ABSTRACT

We have examined the separation of test mixtures by linear and step temperature gradients in open tubular capillary liquid chromatography, in reverse and normal-phase modes. Separations using temperature gradients with the upper temperature limit of 433 K were compared to isothermal runs in terms of chromatographic efficiency, analysis time and selectivity. When a temperature gradient (363 K to 433 K in 12 min) was utilized in reverse phase separation of chlorobenzenes, a 40 % reduction in analysis time, compared to an isothermal separation at 363 K, was accomplished.

INTRODUCTION

Liquid chromatography in capillary columns is a fast developing technique, offering the potential of increased performance in comparison to the well established wide-bore columns. In order to reach high column efficiency (one million theoretical plates) needed for the resolution of complex mixtures, open tubular columns are being developed for liquid chromatography.[1-8] While isocratic and isothermal separations are limited in their application range, the mobile phase gradient elution mode offers the tremendous advantage of tuning selectivity by eluent composition in the same separation.

This elution mode also offers easy access to sample enrichment.[9] Control of chromatographic separation selectivity in HPLC by physical parameters such as temperature, is particularly interesting. The use of elevated temperatures for reverse phase HPLC has been advocated, as a means to increase column efficiency, shorten analysis time and alter separation selectivity.[3,4,10-16] The effect of temperature changes on separation may be compared with the effect of changing the composition of mobile phases. A change in column temperature can lead to significant changes in band position (selectivity) whenever the shape and the size of two compounds differs significantly. It can also affect peak shape, especially when adsorption effects are profound at low temperatures.

Temperature programming can be a practical alternative to gradient elution. Lawrence and Scott[15] applied temperature programming with conventional size LC columns for a normal phase separation. From the temperature dependence of k', the heats of solute transfer from the stationary to mobile phase were determined by Knox and Vasvari.[17] They have indicated the potential benefits of temperature gradient liquid chromatography(TG-LC). The effect of increasing temperature on analyte retention times, peak symmetry, and chromatographic efficiency in isothermal separation was investigated by Grushka.[18] Snyder[19] compared mobile phase gradient elution and temperature programming, while Snyder and Kirkland[20] elaborated on temperature programming. The use of temperature gradients to achieve reverse phase liquid chromatographic separations for systems detected by plasma techniques was demonstrated by W. R. Biggs,[21] while Renn and Sinovec[22] examined the effect of temperature on separation efficiency in size exclusion chromatography. McNair[23] evaluated the potential of temperature programming in conjunction with microbore HPLC columns, and concluded that this produces faster analyses and increased efficiency.

The effects of temperature changes for achieving separation are complex, involving changes in thermodynamic parameters, efficiency and separation time. The relationship between the capacity factor, k', and the column temperature can be estimated by the equation:[13]

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \phi \qquad (1)$$

where R is the universal gas constant, T is the temperature in kelvin, while ΔH and ΔS denote enthalpy and entropy of a solute molecule transfer from the stationary phase to the mobile phase, respectively. The phase ratio of the column, ϕ, represents the stationary to mobile phase volume ratio. Differences in sorption behavior in HPLC are characterized by enthalpy changes. The change in capacity factor with temperature can be put in a form which represents the capacity factor ratio k'_1/k'_2 at two different temperatures T_1 and T_2:[24]

$$\frac{k'_1}{k'_2} = \exp\left[\frac{\Delta H(T_2 - T_1)}{RT_1T_2}\right] \quad (2)$$

As predicted by the above equation, the effect of temperature programming on the capacity factor ratio will strongly depend on the solute ΔH value. For the same temperature change, the solutes with large ΔH will be strongly affected, whereas for solutes with small ΔH the temperature change will not produce significant changes in the capacity ratio. When reduction in retention times of the late eluting solutes is desirable, changing the column temperature can provide performance similar to that of solvent gradient systems.

Using a temperature gradient, the retention of the peaks could be selectively influenced. This fact might contribute to the solution of some common chromatographic problems. Early eluted substances usually show a sharp peak-form, but often an insufficient resolution. On the other hand, the longer compounds are retained the broader the resulting peaks are. These peaks are difficult to integrate and in some cases, they may even be undetectable, due to their fusion with the base-line.

The aim of this work is to study the effect of temperature programming on retention and efficiency in open tubular liquid chromatography (OTLC).

EXPERIMENTAL SECTION

The instrumental set-up utilized for step temperature gradients does not differ from the instrumental set-up used for isothermal high temperature separation, which has been described elsewhere.[9] When linear temperature gradients were performed, a laboratory-constructed column oven was replaced with a column oven (forced air heating) equipped with an HPLC microprocessor programmer 50 A/B from Knauer (Berlin, Germany). This set-up, depicted in Fig. 1, enabled control of the temperature gradient with a maximum heating rate of 6°/min and a maximum column temperature of 433 K. To prevent possible solute precipitation in the detector cell, the cell had to be heated. One problem encountered with the experimental set-up for step temperature gradients used in this study, was strong base-line sloping (UV detection) caused by changes of refractive index with temperature.[25,26] In order to avoid this baseline sloping, the detector cell was taken from the column oven and placed in a separate heating unit. The cell was kept at a constant temperature, which corresponded to the midpoint temperature of the linear gradient. The use of a restrictor to generate high pressure allows one to work well above the normal boiling point of the solvent, thus increasing the effective useful temperature programming range. A SB-methyl-100 column ($l = 5$ m, 50 μm i.d., film thickness $d_f = 0.25$ μm) was purchased from Lee Scientific (Salt Lake City, UT, U.S.A.), while an OV-1 7 column ($l = 20$ m, 50 μm i.d.,

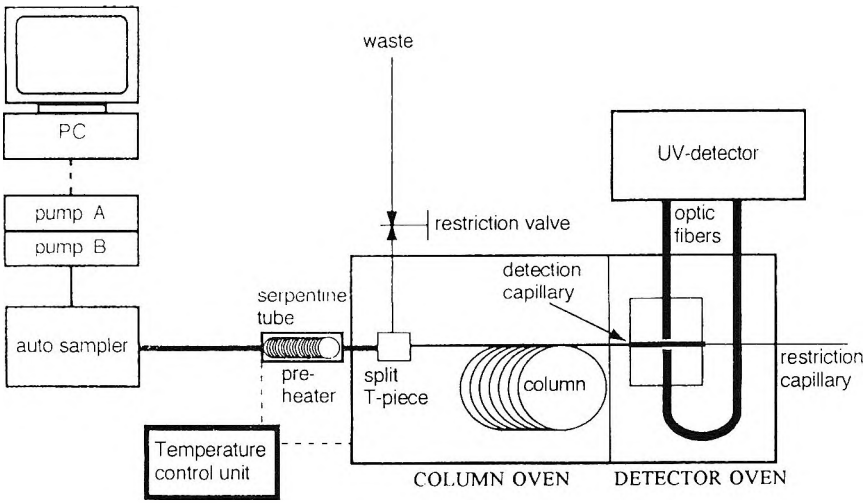

Figure 1. Experimental set-up for linear temperature programming.

d_f = 0.1 μm) and an OV-1701 column (l = 5 m, 50 μm i.d., d_f = 0.4 μm) were obtained from Macherey-Nagel, Oensingen, Switzerland. Bare fused silica capillaries used either as restrictors, or as analytical columns (14.4 m, 50 μm i.d.) for normal-phase LC separation, were from Polymicro Technologies (Phoenix, AZ, U.S.A.). The testosterone-esters (acetate, cypionate, propionate, enanthate, and benzoate) were obtained from Sigma (Buchs, Switzerland). The chlorobenzenes were obtained from Aldrich-Chemie (Steiheim, Germany) or Fluka (Buchs, Switzerland). Methanol and n-hexane were of HPLC grade from Rathburn (Welkerburn, UK). Molecular sieve beads 0.3 nm , 10 mesh, from Merck (Darmstad, Germany) were used to dry n-hexane.

RESULTS AND DISCUSSION

Reversed-Phase Separation

OTLC is ideal for temperature programming in LC, due to a rapid heat transfer through thin silica walls (~ 100 μm) and low volumetric flow rates which promote fast heating and equilibration of the eluent. Different shapes of temperature programs in chromatography can be classified as: step, linear, convex, concave, and multisegment. The simplest program is a single step in which the column temperature is changed instantaneously at a certain time. In OTLC, the volume of mobile phase is much larger than the stationary phase volume. Because

Table 1

Column Plate Number at 373 K and 423 K and Apparent Plate Number for the Step Gradient (373-423 K). The Other Conditions as in Figure 1

Solute	373 K	423 K	Step Gradient
Testosterone-benzoate	6434	18767	16515
Testosterone-enanthate	6089	16530	14856

of the low flow rates of OTLC (the optimum flow rate for a 50 μm i.d. column is below 1 μL/min) the elution time of the unretained compound (t_o) is quite large. Therefore, the start of the temperature gradient should be delayed for the value of t_o. If the gradient was started immediately after injection, the system would reach the final temperature even before an unretained compound eluted from the column. In this case, the effect of the step temperature gradient would be as if the whole run was performed isothermally at the final step temperature. This should be taken into consideration when separation procedures using temperature gradients are optimized. All step gradients performed with the OV-17 column were started 40 min after sample injection. An increase in column temperature during the separation greatly decreases the retention time and increases the relative peak sensitivity. There are several mechanisms responsible for sample retention in reverse phase HPLC (hydrophobic interactions, adsorption on residual silanols and solute solubility in the mobile phase and stationary phase).[27] The relative contribution of these processes are altered when a temperature gradient run is performed. Furthermore, solute mass transfer in the mobile and stationary phases, a kinetic process, is also changed in the temperature gradient mode. Combined thermodynamic and kinetic effects in the temperature gradient mode lead to peak compression. In Table 1, the plate numbers obtained for testosterone-benzoateand testosterone enanthate in isothermal runs (373 K and 423 K) were compared with the apparent plate numbers for the same solutes, obtained by the step gradient 373-423 K. Clearly, only minimal efficiency (ten percent) has been sacrificed by the temperature programming when compared with the isothermal run at 423 K. The commercial capillary columns utilized in this work, were designed for application in supercritical fluid chromatography and have limited stability at elevated temperature when aqueous mobile-phases are used.[4] By running temperature programming, the analytical columns are exposed to the elevated temperatures for shorter periods of time then when isothermal runs are performed. A ten percent loss of efficiency is than negligible in comparison to the benefit of prolonging column lifetime. The separations of five testosterone-esters obtained under isothermal conditions at 373 K (chromatogram I) and with step gradients from 373 K to 383 K, and from 373 K to 393 K (chromatograms II and III) are shown in Fig. 2.

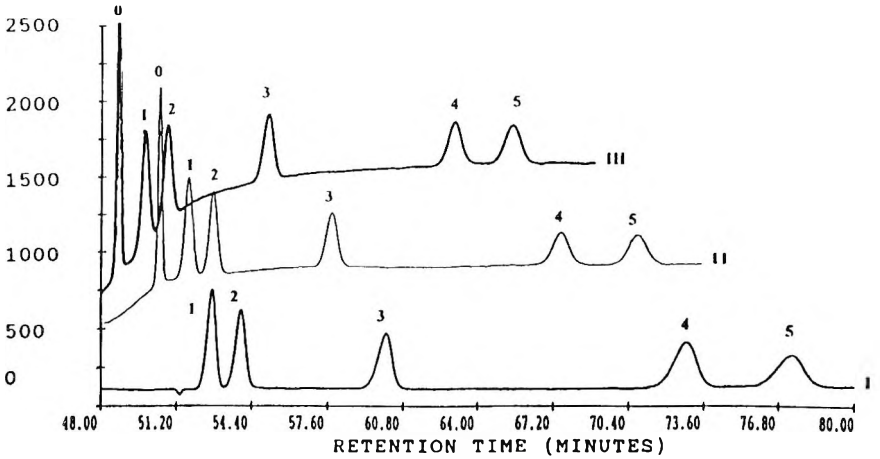

Figure 2 . Effects of step-temperature gradients on the separation of a mixture of testosterones. Column OV-17, 20 m x 50 μm i.d., d_f = 0.1 μm; MeOH/H_2O 30:70 (v/v), flow rate = 1.0 μL/min; UV detection at 241 nm. Peaks: 0) potassium iodide, 1) testosterone-acetate, 2) testosterone-propionate, 3) testosterone-benzoate, 4) testosterone-enanthate, 5) testosterone-17β cypionate. I = 373 K, II = isothermal 41 min at 373 K then step gradient to 383 K, III = isothermal 41 min at 373 K then step gradient to 393 K.

The separation of a test mixture of chlorobenzenes using linear temperature gradients in OTLC in reverse phase mode were examined. In Fig. 3, a comparison was made between an isothermal separation at 363 K (chromatogram I) and two linear step gradients from 363 K to 433 K at 2°/min and 6°/min, chromatograms II and III, respectively. Tropolone was used as unretained compound to determine t_o.

The linear temperature gradients were started 7 min after sample injection. As expected, early eluting solutes were not significantly affected by the temperature gradients. Shorter analysis times for the solutes with larger k' (peaks labeled 4, 5 and 6) and increased peak sensitivity (inversely proportional to peak width), were observed. There was a significant improvement in the relative peak sensitivity in the gradient mode compared with isothermal elution.

The excellent efficiency of the temperature-programmed separation can be attributed to initial concentration of analytes on the column due to the weak mobile phase, particularly for strongly retained components. As the temperature is raised, the analytes desorb from the stationary phase and migrate down the column with more favorable phase transfer kinetics.

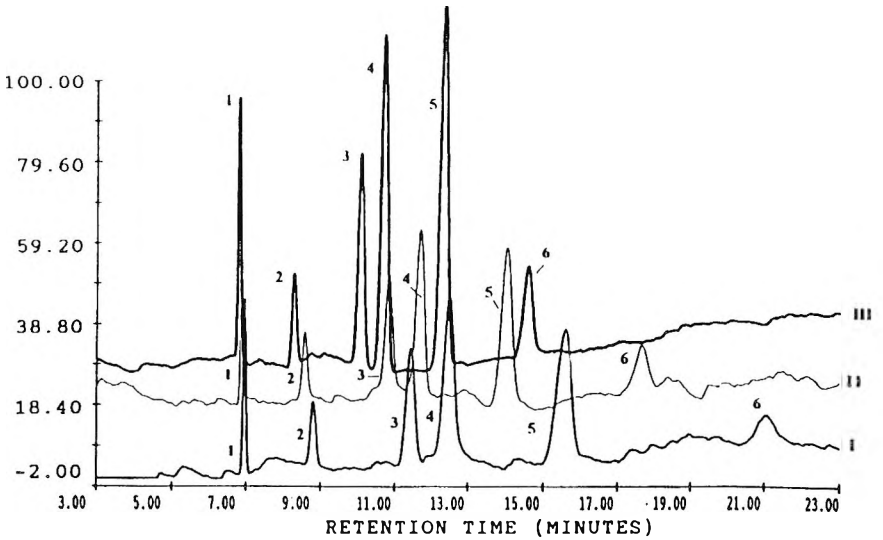

Figure 3. Separation of a mixture of chlorobenzenes. Column SB-methyl-100, 5 m x 50 μm i.d., d_f = 0.25 μm; MeOH/H_2O 25:75 (v/v), flow rate = 0.75 μL/min; UV detection at 210 nm. Peaks: 1) tropolone, 2) 1,4-dichlorobenzene, 3) 1,2,4 tri-chlorobenzene, 4) 1,2,4,5-tetrachlorobenzene, 5) pentachlorobenzene, 6) hexachlorobenzene. I = 363 K, II = isothermal 7 min at 363 K then gradient 2 °/min to 433 K, III = isothermal 7 min at 363 K then gradient 6 °/min to 433 K.

Normal-Phase Separation

As expected, a pure untreated fused-silica column shows selectivity towards aromatic hydroxy compounds. The separations of the test compounds obtained on a 14.4 m x 50 μm fused-silica column, with n-hexane as mobile phase, are displayed in Fig. 4. The separations obtained at 301 K, 333 K, and by temperature programming: isothermal at 301 K for 20 min than to 373 K with a heating rate of 6°/min, are labeled as chromatograms I-III, respectively. A qualitative comparison of these separations shows several apparent changes in band spacing and band shape, due to this difference in temperature. For example, the two peaks, labeled 2 and 3, displayed a profound peak tailing at 301 K, caused by a strong solute adsorption on the silanol groups. At elevated temperatures (at 333 K and in the temperature gradient runs), the peak symmetry significantly improved. In normal-phase liquid chromatography, sample retention is governed by solute adsorption to the stationary phase. For retention to occur, a sample molecule must displace one or more solvent molecules from the stationary phase. In addition to this displacement effect, polar solute molecules can exhibit very strong interaction with particular sites on the stationary phase (localization effect).

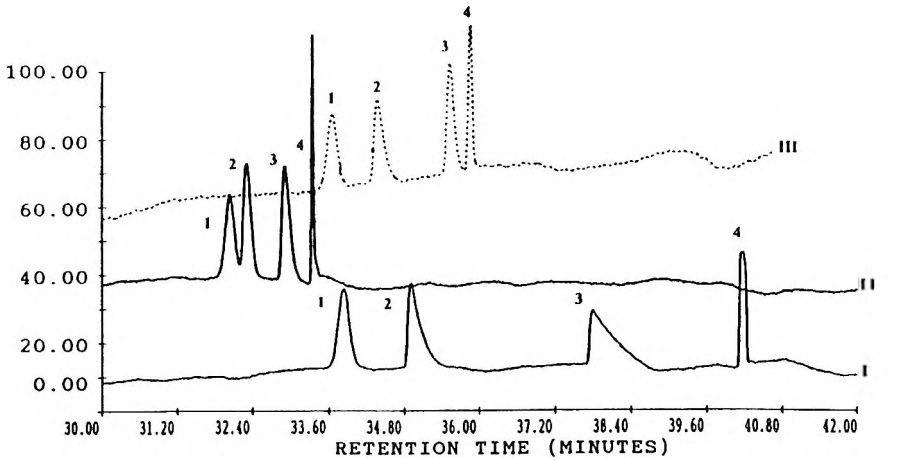

Figure 4. Fused silica capillary column, 14.4 m x 50 μm i.d., n-hexane, flow rate = 0.92 μL/min: detection UV at 210 nm. Peaks: 1) 2,6-di-tert-butyl-4-methylphenol,2) 2,6-di-methylphenol,3) 1-naphthol,4) 2-naphthol. I = 301 K, II = 333 K, III = isothermal 20 min at 301 K then gradient 6 °/min to 373 K.

These two effects, displacement and localization, are the primary sources of mobile phase selectivity in normal-phase HPLC. To alter these affects, thus changing selectivity, temperature gradient can be utilized. In Fig. 5, a comparison between two gradient rates is depicted. Selectivity obtained with a heating rate of 3 °/min is displayed in chromatogram I, while the chromatogram labeled II corresponds to a separation accomplished with a temperature gradient of 6 °/min.

CONCLUSION

Temperature programming is a useful mode in liquid chromatography for extending the applicability of the isocratic mode, optimizing resolution, shortening analysis times and at the same time achieving a higher relative peak sensitivity. The concept of the temperature gradient separation in OTLC parallels the strategy for temperature programming in column liquid chromatography or microbore column liquid chromatography. When applying temperature programming, care must be taken to avoid a large temperature difference between the column wall and the mobile phase. The small radius and mass of open tubular capillary columns makes them more suitable than micro-bore or conventional bore columns for temperature programming in liquid chromatography. Temperature programming in HPLC may be a useful supplement to gradient elution. Instrument requirements are somewhat simpler for temperature programming than for gradient elution. It is much simpler to control the temperature of a mobile phase

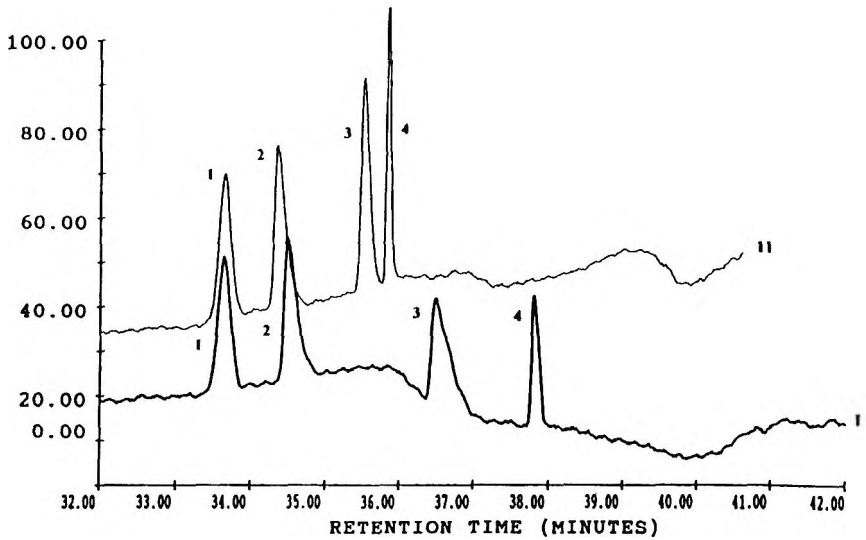

Figure 5. Temperature programming at 3 °/min (I) and 6 °/min (II). Condition as in Fig. 4.

than to uniformly mix two mobile phases with the precision necessary for reproducible separation in open tubular capillary LC. Temperature programming in combination with gradient elution may be an interesting approach for future separations in micro column HPLC.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF TWO LICHEN LECTINS WITH ARGINASE ACTIVITY DIFFERING IN THEIR GLYCOSYL MOIETY

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ABSTRACT

Two isolectins from the lichen *Xanthoria parietina*, one of them retained by the thallus and another secreted from the thallus to the medium, both showing arginase activity, have been purified to homogeneity. Ethanol-soluble products obtained after acidic hydrolysis of both proteins have been analyzed by HPLC under isocratic conditions using acetonitrile-water (80:20, v/v) as mobile phase. Detection is performed by measurement of UV absorbance at 195 nm, using a highly sensitive detector. Purification of standard sugars by filtering aqueous solutions through an activated alumina column is absolutely required. The glycosyl moiety of secreted arginase is composed by galactose and glucose whereas that of thalline enzyme contains N-acetyl-D-glucosamine and glucose.

INTRODUCTION

Plants and animals are able to synthesize lectins responsible for several cell surface interactions.[1,2] Many of these glycoproteins are enzymes that act like lectins[2,3] and even several isoforms of only one protein, differing in their glycosyl moiety, are able to bind to the same receptor in the cell surface. The identification and classification of animal proteoglycans is based on the structure of the glycosaminoglycan moiety,[2] which can be studied by analyzing the unsaturated disaccharide units that are produced by controlled, partial hydrolysis with a particular enzyme, such as chondroitinases ABC and AC[4,5] or *Streptomyces* hyaluronidase.[6] Disaccharides obtained after enzymatic digestion are usually separated by HPLC in isocratic mode on NH_2 columns, monitoring the analytes by absorbance at 232 nm.[4-6]

These separations have been improved by Numura et al.[7] by using a TSK Gel Amide 80 column which avoids the overlapping of several peaks corresponding to different disaccharides. Recently, the analysis of these oligosaccharides has been performed by capillary electrophoresis as pyridylaminated isomaltooligosaccharide[8] or 1-phenyl-3-methyl-5-pyrazolone derivatives.[9] The sodium borate buffer used in these analyses is proposed to play a key role in the separation by preferentially complexing with the diols of specific carbohydrate moieties[10] on the corresponding glycoprotein.

Lichenized fungi produce lectins which have been related to the recognition of the compatible algal partner.[11,12] Some of these lectins act as specific enzymes, such as glycosylated arginase from *Xanthoria parietina*.[13] However, lichen arginase can be produced as several different isoforms differing in both amino acid composition or glycan moiety and, in addition, they can be retained by the thallus or secreted to the medium.[14]

In this paper, we study two different isolectins of the lichen *X. parietina* by analyzing their sugar composition by HPLC after total hydrolysis of their glycosyl residues.

MATERIALS

Plant Material

Xanthoria parietina (L) Th. Fr., growing on *Robinia pseudoacacia* L. was collected in Montejo de la Sierra (Madrid). Thalli were air-dried and stored at 4°C in the dark, no longer than two weeks.

Reagents

Sugars (D-fructose, D-fucose, D-glucose, D-galactose and D-mannose), amino sugars (D-glucosamine, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine) and ribitol were provided from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade, Carlo Erba, Milan, Italy) was used as received and doubly distilled water was filtered through Millipore GS filters (0.22 μm pore diameter) before use.

A Micropack SP NH_2-5 column (Varian, Palo Alto, CA, USA), packed with 10 μm spherical silica which has a chemically bounded phase containing aminopropyl groups and supplied in hexane for use in normal phase, was prepared for sugar analysis in reversed-phase mode.[15]

Acrylamide 2x from Serva (Heidelberg, Germany) and N,N'-methylene-*bis*-acrylamide from Sigma were used for PAGE, employing N,N,N',N'-tetramethylethylenediamine (Sigma) and ammonium persulfate (E. Merck) as polymerizing agents. Size-exclusion chromatography on column was achieved by using Sephadex G-150 from Pharmacia (Uppsala, Sweden).

Glucose oxidase, galactose oxidase, and horseradish peroxidase from Sigma were used to identify glucose and galactose. Fetuin was also obtained from Sigma. Other chemicals were obtained from E. Merck (Darmstadt, Germany).

METHODS

Purification of Glycosylated Arginases

Samples of 15 g of *X. parietina* thalli were floated on 150 mL 10 mM Tris-HCl, pH 9.1, for 1 h at 26°C in the dark. Secreted arginase (SA) was purified from the incubation media according to Planelles and Legaz[16] by precipitation with ammonium sulfate at 50% saturation, adsorption on calcium phosphate gel (SA was desorbed with 220 mM Tris-HCl) and filtration through a Sephadex G-150 column (30 x 3 cm I.D.). The algal-binding protein (ABP) was pre-purified from recently collected thalli according to Bubrick et al.[11] by two successive precipitations with ammonium sulfate (20% and 40% saturation, respectively) and later purified as above(16). ABP was desorbed from hydroxyapatite with 180 mM Tris-HCl. To test the homogeneity of both proteins, 150 μL of the corresponding solution, containing about 6.0 μg protein, were mixed with 75

μL of aqueous glycerol (v/v) and applied onto 12% polyacrylamide gels. The running buffer was 50 mM Tris-glycine, pH 8.3 and, at this pH, the current generated at 180 mV was about 25 mA at 4°C after equilibration.

Acidic Hydrolysis and Sugar Extraction

Samples of 4.0 μg of purified lectins were hydrolyzed with 0.5 mL 6 N HCl for 2 h at room temperature.[16] Mixtures were dried in air flow and the residues were dispersed in 1.0 mL of cold 80% (v/v) ethanol and stored at -13°C for 14 h. The precipitates were then discarded and the supernatants heated at 60°C for 20 min. To these supernatants, 1.0 mL of 80% cold ethanol was added and then heated again to dryness.

This procedure was repeated three times under the same conditions as above. The last residues were reconstituted with 1.0 mL of cold 80% ethanol and centrifuged at 3000*g* for 15 min.[17] The supernatants were loaded into the chromatographic column.

Samples of 40 μg fetuin (a glycoprotein of well defined monosaccharide composition) were hydrolyzed in the same way with 5.0 mL 6 N HCl in order to confirm that the sugars from lectins have been quantitatively released without destruction by HCl. Glycidic moiety of fetuin is composed of galactose, mannose, glucosamine, galactosamine and sialic acids,[18] such as N-acetylneuraminic acid to which a residue of N-acetylgalactosaminitol is linked.[19]

Purification of Standards

Samples of 5.0 mL of solutions standards in doubly distilled, filtered water, containing 40 mg of sugar, were applied onto a column of activated alumina (11 cm x 1.0 cm I.D.). The samples were eluted with doubly distilled, filtered water. Fractions of 5.0 mL volume were monitored for the sugar content according to Dubois et al.[20] The fraction containing the highest amount of the corresponding sugar was dried in air flow, redisolved in 1.0 mL 80% ethanol and analyzed by HPLC.

Purified fractions of both glucose and galactose were identified by action of specific enzymes. Aliquots of 0.5 mL of the fraction containing the highest amount of the corresponding monosaccharide were incubated for 30 min at 30°C with 1.0 mg of glucose oxidase[21] or 1.0 mg of galactose oxidase,[22] 0.5 mg peroxidase and guaiacol. Increase of absorbance was measured at 475 nm.

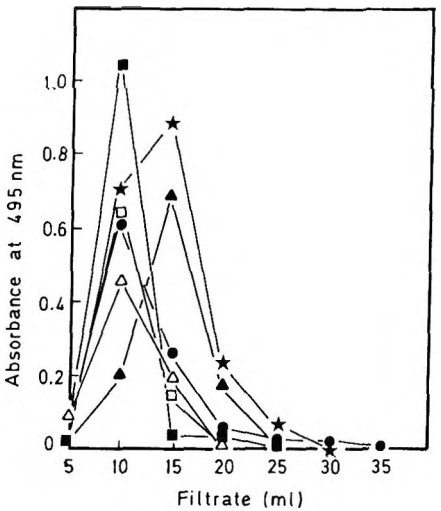

Figure 1. Elution profiles of standard monosaccharides, glucose (filled circles), fructose (empty squares), glucosamine (filled squares), mannose (empty triangles), galactose (filled triangles), and fucose (filled stars) filtered through an alumina column.

Table 1

Retention Times of Standards Filtered Through an Alumina Column

Standard	Retention Time (min)
N-Acetylgalactosamine	3.24
N-Acetylglucosamine	3.04
Glucosamine	3.65
Galactose	2.86
Glucose	4.93
Fructose	3.26
Fucose	2.70
Mannose	3.49
Glucuronic Acid	2.03

HPLC Separation of Sugars

HPLC was performed with a Varian Model 5060 liquid chromatograph equipped with a SpectraSystem UV2000 detector (SpectraPhysics, Fremont, CA, USA) and a Vista CDS 401 (Varian) computer. The chromatographic conditions were as follows: column, MicroPack NH_2 10P/N (30 cm x 3 mm I.D.) from Varian: sample loading, 10 μL; mobile phase, acetonitrile-water (80:20, v/v) isocratically; flow rate, 1.0 mL min^{-1}; temperature, 20°C; detector, UV (195 nm), 0.005 a.u.f.s.; attenuation 64; internal standard, 2.0 mg mL^{-1} ribitol (retention time = 6.42 min).

Quantitation was performed by injecting different amounts of standards after filtration through the alumina column, estimated according to Dubois et al.[20] Indirect calibration was achieved by using ribitol as internal standard.

RESULTS

ABP was purified about 110-fold with an overall yield of 27.8%. SA was purified about 67-fold with an overall yield of 31.7%. Each purified protein gave only one anodic band in PAGE. These proteins, purified at homogeneity, were subjected to complete acidic hydrolysis and ethanol-soluble fractions were extracted.

Elution of some standards from alumina column was carried out by using twice distilled, Millipore-filtered water. Results are shown in Fig. 1, and summarized in Table 1. Fructose, glucose, mannose and glucosamine eluted at 10 mL filtrate (fraction 2) whereas galactose and fucose eluted at 15 mL filtrate (fraction 3). Fractions containing the highest amount of the corresponding monosaccharide were used to be chromatographed by HPLC. Figs. 2 and 3 show two examples of the purity of commercial standards.

Standard galactose (Fig. 2A) was resolved into two main peaks with retention time values of 2.82 min and 3.25 min., respectively. However, after filtration through the alumina column, fraction 3, eluted at 15 mL filtrate, yielded only one peak with a retention time of 2.86 min (Fig. 2B). The contaminating substance could be fructose (retention time 3.26 min) or N-acetyl-D-galactosamine (retention time 3.24 min). An aliquot of this fraction 3 was incubated with 1.0 mg of galactose oxidase for 30 min at 30°C and the formation of the corresponding D-galacto-hexodialdose was confirmed. Standard glucose was resolved into three main peaks at 2.05 min, 3.10 min and 4.87 min, respectively (Fig. 3A). After filtration through the alumina column, fraction 2, filtered at 10 mL, was resolved as only one peak at 4.93 min (Fig. 3B).

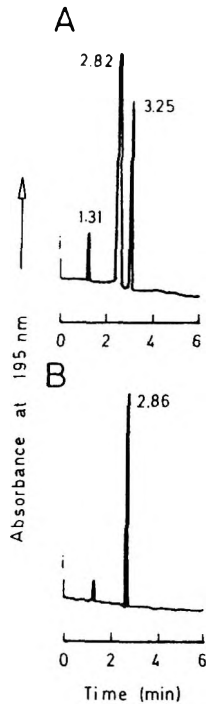

Figure 2. HPLC elution profiles of standard galactose (A) before and (B) after filtration through an activated alumina column. Number near the peak indicates retention time in min.

The contaminating substances were identified as N-acetyl-D-glucosamine (retention time 3.04 min) and glucuronic acid (retention time 2.03 min). This fraction 2 behaved as a very good substrate for glucose oxidase, since formation of gluconic acid was confirmed. Standards of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were revealed as pure substances in HPLC.

Table 1 shows the retention time values for the different standards after their filtration through the alumina column. The peak with a retention time of about 1.3 min was always identified as acetonitrile which individualized from that contained in the mobile phase after sugar dilution. Figure 4 shows the chromatographic traces of an acidic hydrolysate of pure ABP.

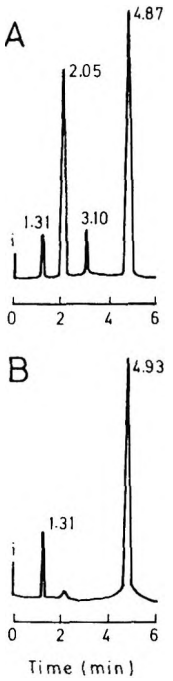

Figure 3. HPLC elution profiles of standard glucose (A) before and (B) after filtration through alumina.

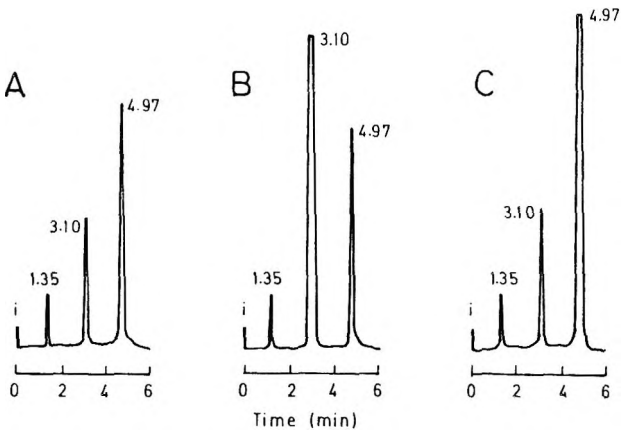

Figure 4. HPLC elution profiles of (A) ethanol-soluble fraction obtained from ABP lectin hydrolysate, (B) the same sample loaded with N-acetyl-D-glucosamine and (C) loaded with glucose.

Two main peaks were detected, with retention time values of 3.10 min and 4.97 min, respectively (Fig. 4A). After loading with the different filtered standards, the peak at 3.10 min only quantitatively increased after loading the sample with standard N-acetyl-D-glucosamine (Fig. 4B) whereas that at 4.97 min only increased after loading the sample with glucose (Fig. 4C).

Figure 5 shows the chromatographic traces of an acidic hydrolysate of secreted arginase. Two main peaks were revealed, with retention time values of 2.94 min and 4.98 min, respectively (Fig. 5A). This last peak quantitatively increased after loading the sample with standard glucose (Fig. 5B). However, two well defined peaks appeared after loading the sample with standard N-acetyl-D-glucosamine (t_R = 3.10 min), one of them at 2.92 min and another at 3.14 min (Fig. 5C). The quantitative increase of the original peak at 2.94 min was only achieved by loading the sample with standard galactose (Fig. 5D). Peaks of galactose and N-acetyl-D-glucosamine were also revealed as different after chromatographying an equimolar mixture of both standards (Fig. 5E).

Baseline correction was always applied. This was constructed from the start of the first peak to the lowest valley point at the end of the same peak and, in this way, after a baseline segment was constructed, the area of the peak was corrected. The result was stored in a time and area file and then, the next baseline segment was calculated for the included peak. Each successive baseline segment started at the end of the preceding one, to include all the peaks.[15]

The response of the detector appeared to be almost linear in the range from 0.01 μg to 2.5 μg of mass injected (Fig. 6), although sensitivity for N-acetyl-D-glucosamine was about 50 times higher than that found for glucose. Error inherent to direct calibration was estimated as standard error in six repeated injections for each one concentration of standard solutions. Equation for the calibration straight line was obtained by linear regression, r^2 of which measured the goodness of fit.

Quantitative analysis of the glycosyl rest of both lectins revealed that ABP contained 6 residues of N-acetyl-D-glucosamine and 6 residues of glucose per molecule of enzyme whereas SA contained 4 residues of galactose and 8 of glucose per enzyme molecule. The number of residues was calculated over a molecular weight of 21.6 kDa for both arginase isoforms (Molina et al., in press). Quantitative analysis of the glycosyl residue of fetuin revealed that the numbers of residues per mole of protein were 12.88 for galactose, 9.07 for mannose and 18.6 for glucosamine, calculated on the basis of a molecular weight of 48.4 kDa, whereas those found by Spiro[18] were 12.4, 8.1 and 13.2, respectively (data are not shown).

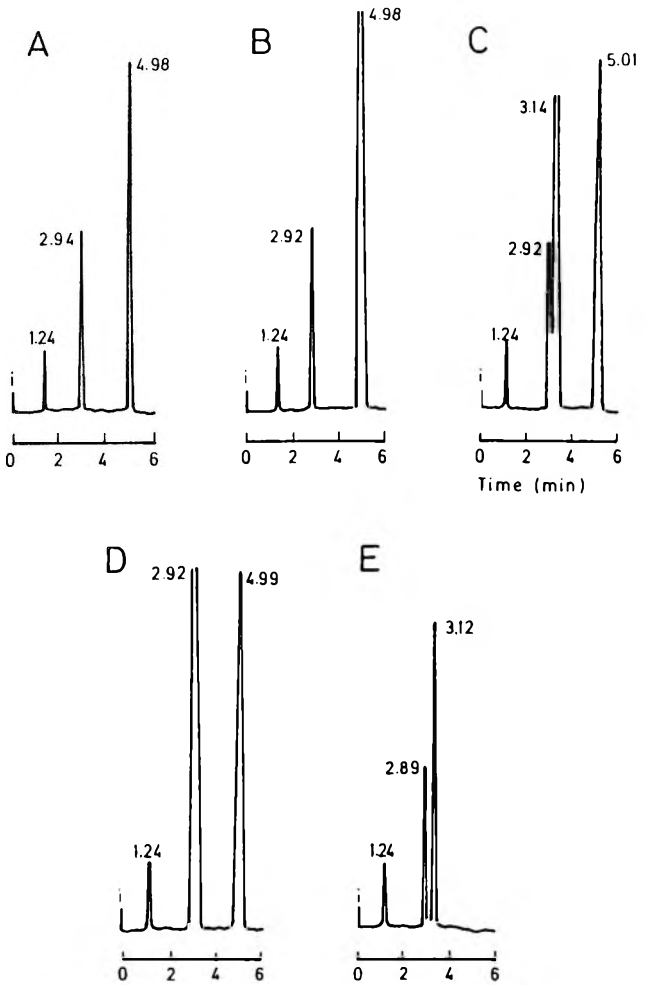

Figure 5. HPLC elution profiles of (A) ethanol-soluble fraction obtained from SA lectin hydrolysate, B) the same sample loaded with glucose, (C) with N-acetyl-D-glucosamine, (D) with galactose and (E) an equimolar mixture of standards galactose and N-acetyl-D-glucosamine.

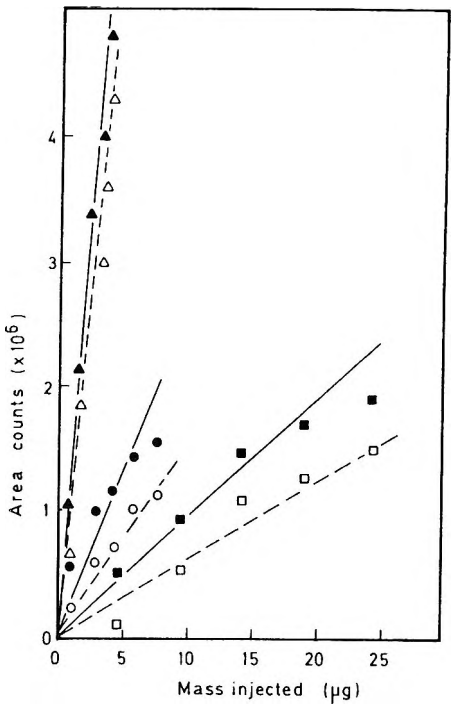

Figure 6. Calibration lines of sugar standards by HPLC. Direct calibration of galactose, $y = 246351x + 24796$; $r^2 = 0.97$ (filled circles); glucose: $y = 89367x + 32987$; $r^2 = 0.96$; (filled squares), and N-acetylglucosamine: $y = 9253970x + 343023$; $r^2 = 0.99$ (filled triangles), and indirect calibration with respect to an internal standard (2.0 mg mL^{-1} ribitol) of galactose: $y = 124297x - 11322$; $r^2 = 0.97$ (empty circles); glucose, $y = 55466x + 7654$; $r^2 = 0.99$ (empty squares), and N-acetylglucosamine, $y = 9253967x - 70878$; $r^2 = 0.99$ (empty triangles). Data are the mean of six replicates. The standard error was never larger than the symbols.

DISCUSSION

Results obtained here concern the rapid identification of lichen isolectins by analyzing the composition of their polysaccharide moiety by HPLC after acidic hydrolysis. The use of a UV detector, such as the SpectraSystem UV2000, 6 to 10 times more sensitive that other previously used (for example, VariChrom TM VUV/10),[15] reveals the appearance of contaminating substances in standard solutions in spite of their analyticial purity degree (Figs. 2A and 3A). However, other standards, such as N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, remain uncontaminated for long time periods. Thus, a re-purification step of standards is required to remove contaminating products

before loading the sugar solution onto the chromatographic column (Fig. 1). When possible, the fraction from the alumina column, containing the highest amount of sugar, has been analyzed by specific enzymatic reaction. The identity of glucose and galactose, eluted from alumina, has been confirmed by reaction with glucose oxidase[21] and galactose oxidase.[22] Anyway, the HPLC peaks of standards, after filtration through alumina, coincide with the main peak obtained from non-filtered substances.

The method for analyzing sugars used here has been revealed as highly sensitive, accurate and repetitive.[15] Both isolectins clearly differ in one of their sugar components (Figs. 4 and 5), although previous reports on *Xanthoria* lectin do not describe protein heterogeneity[11] because only thalline lectin has been isolated. In spite of this, it has recently been described the occurrence of, at least, two thalline arginases, one of which does not contain the glycosyl moiety.[13] In addition, SA from *X. parietina* is quite different from that purified from another lichen species, *Evernia prunastri*, the glycosyl residue of which is composed of glucose, fructose and mannose.[16]

The presence of galactose in *Xanthoria* SA is very interesting in order to explain the binding capabilities of this secreted lectin[13] and sufficiently differs in its chromatographic behaviour from N-acetyl-D-glucosamine, since the α coefficient between standards gives a value of 1.07. N-acetyl-D-glucosamine is not deacylated since no glucosamine appears in the chromatographic analysis. On the other hand, acidic hydrolysis of the glycoproteins with 6 N HCl seems to be a technique that does not produce chemical modification of sugars, since the recovering of sugars from fetuin, used as a control, after hydrolysis is coincident with the well known monosaccharide composition of this glycoprotein.[18,19]

Although most of papers concerning lectin structure and activity are devoted to the identification of the polysaccharide sequence of the lectin receptor,[23,24] even those related to non-glycosylated lectins,[25] sometimes the sugar composition of their glycosyl rest has been analyzed by affinity chromatography[24] or specific enzyme digestion.[27] In this way, it has been described that lectin from *Datura stramonium* seeds contains 37% carbohydrate by weight of which 93% is arabinose and 7% galactose.[28-30] Other plant oligosaccharides which do not bind to protein have been analyzed by GLC[31] or ion-exchange chromatography.[32] Although good resolutions were obtained, procedures and analyses are complicated and tedious. In addition, more than 1.0 mg pure protein is required for analysis.

The rapid and sensitive HPLC procedure described here can be developed by using 4-5 μg of pure lectin and offers highly repetitive results for time analysis no longer than 9 min, including the elution time of the internal standard.

ACKNOWLEDGEMENTS

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RETENTION BEHAVIOUR OF TRIPHENYLETHYLENE DERIVATIVES IN REVERSE PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

A series of triphenylethylene derivatives was studied with different reverse phase liquid chromatographic columns. Octyl and octadecyl silica stationary phases were compared, as well as, one type of polymeric reverse phase. Because these triphenylethylene derivatives appear highly lipophilic, the polymeric column (PRP-1) did not produce satisfactory peaks. Also, the basic nature of these molecules requires such column properties that were most satisfactorily met by well end capped octyl silane phase columns, like Kromasil C_8 or deactivated Supelco LC-8-DB. These columns will be suitable for evaluating lipophilicity data of triphenylethylene derivatives, in order to use them in quantitative structure-activity relationship studies.

INTRODUCTION

Triphenylethylene compounds represent a new source of important drugs for cancer chemotherapy. Tamoxifen, (Z)-4-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]N,N-dimethylethylamine, and toremifene, (Z)-2-[4-(4-chloro-1,2-diphenylbut-1enyl)phenoxy]ethyidimethylamine, are well

known anticancer agents used in the case of breast tumours.[1-2] From the analytical point of view, there is not much data available concerning triphenylethylene derivatives.

For tamoxifen, some analytical methods have been published, including high performance liquid chromatography (HPLC),[3-4] GC-MS[5] and LC-MS.[6] Only a few analytical methods have been described for toremifene: HPLC with fluorescence,[7] ultraviolet light[8] and mass spectrometric detection.[9]

Tamoxifen and toremifene are highly lipophilic compounds, logarithms of apparent partition coefficients being 6.64 and 6.35, respectively.[10] Moreover, they are weakly basic organic molecules, e.g. pKa-value is 7.82 for tamoxifen.[11] For these reasons, the reverse phase liquid chromatography of these molecules and their analoques is not simple.[12-13]

In this investigation, reverse phase liquid chromatographic properties of twelve triphenylethylene derivatives and related compounds have been studied. Most of them are new ones, thus analytical data concerning these compounds has not been published earlier. This study is a preliminary outline about the RPLC properties of these molecules, the main aim being to investigate the lipophilicity of them later on.

Lipophilicity data shall be pronounced by log k values and, therefore, studied by RPLC, because conventional shake-flask method is not a suitable approach for these highly lipophilic compounds. Further, log k values are used in quantitative structure-activity relationships (QSAR) studies performed by comparative molecular field analysis (CoMFA) methods,[14] in order to develop new drug molecules.

EXPERIMENTAL

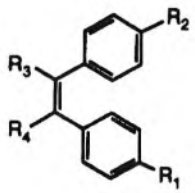
Materials and Chemicals

The triphenylethylene derivatives (Table 1) were obtained from Farmos Laboratories, Orion-Farmos Ltd (Turku, Finland). Standard compounds for lipophilicity correlation studies were of analytical grade (Table 2).

HPLC grade acetonitrile and methanol were purchased from Labscan Ltd (Dublin, Ireland). Ammonium acetate (Merck, Darmstadt, Germany) and triethylamine (Aldrich, Steinheim, Germany) were of analytical grade.

Table 1

Structures of Triphenylethylene Derivatives



	R_1	R_2	R_3	R_4
1. Toremifene E-isomer cirate	H	$OCH_2CH_2N(CH_3)_2$	CH_2CH_2Cl	H
2. Deaminocarboxyltorefene	OCH_2COOH	H	CH_2CH_2Cl	C_6H_5
3. Deaminohydroxytoremifene	OCH_2CH_2OH	H	CH_2CH_2Cl	C_6H_5
4. Toremifene citrate b. 16213P	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_2Br	C_6H_5
5. Fc-1158a citrate	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_2Br	C_6H_5
6. Demethyltoremifene citrate	$OCH_2CH_2NHCH_3$	H	CH_2CH_2Cl	C_6H_5
7. Fc-1530	H	OH	CH_2CH_3	cyclopentyl
8. Didemethyltoremifene hydrochloride	$OCH_2CH_2NH_2$	H	CH_2CH_2Cl	C_6H_5
9. 4-hydroxytoremifene	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_2Cl	C_6H_5OH
10. Fc-1530 b citrate	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_3	cyclopentyl
11. Fc-1159a citrate	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_3I	C_6H_5
12. Toremifene citrate	$OCH_2CH_2N(CH_3)_2$	H	CH_2CH_2Cl	C_6H_5

Table 2

Chemicals Used as References for log K-log P Correlation Studies

Compound	Source	log P*
Caffeine	Merck, Darmstadt, F.R.G.	-0.07
Diphenydramine	Sigma, St. Louis, MO	3.27
Isoniazide	Orion Corp., Espoo, Finland	-1.14
Quinine	Sigma	1.73
Salicylamide	University, Pharmacy, Finland	0.89
Sulfanilamide	Tamro Ltd, Helsinki, Finland	-0.72
Thioridazine	Star Ltd, Tampere, Finland	5.79
Toremifene	Orion-Farmos Corp., Finland	6.35
Vanillin	Merck	1.31

* From reference 16.

HPLC System

RPLC studies were performed with the following columns: Polymeric reverse phase (Hamilton PRP-1) 150x4.1 mm, 5 μm, (Hamilton, Reno, U.S.A.), deactivated octyl silica (Supelco LC-8-DB) 150x4.6 mm, 5 μm (Supelco, Bellefonte, U.S.A) , octyl silica (Kromasil C-8) 250x4.6 mm, 5 μm, (Eka Nobel, Sweden), deactivated octadecyl silica (Supelco LC-18-DB) 150x4.6 mm, 5 μm, (Supelco, Bellefonte, U.S.A.). The mobile phase contained typically acetonitrile:ammonium acetate 100 mM): triethylamine (TEA) (65: 35: 0.05), and pH of the aqueous phase was adjusted to 6.4 with concentrated acetic acid. In one experiment, methanol:ammonium acetate:TEA (80: 20: 0.05) was used as a mobile phase.

The HPLC apparatus consisted of Beckman (Altex) 210 A injector, Beckman 116 M solvent delivery system, Beckman 165 variable wavelength detector (Beckman Instruments Inc., Berkeley CA U.S.A.). Chromatograms were recorded with a Scintag 3122 (Scintag, Switzerland) strip and chart recorder. The flow rate was 1.0 mL/min, and the wavelenght of the UV detector varied between 230 and 255 nm depending on the compound chromatographed.

RPTLC System

As an aid in method development, reverse phase thin layer chromatography (RPTLC) was performed with 100x100 mm octyl bonded plates (Merck, Darmstadt, Germany). Samples were applied with Camag Linomat IV sample application system (Camag, Muttenz, Switzerland). Acetonitrile or methanol were tried as organic modifiers with a same kind of buffer as in HPLC, supplemented with TEA. The spots were visualized in UV light at 254 nm.

Calculation of Retention Parameters

Retention volumes (V_R) of compounds examined were measured from chromatograms. The samples were run as triplicates. The column dead volume (Vm) was determined by using sodium nitroprusside. The capacity factors of the compounds were calculated from the equation $k = V_R\text{-}Vm/Vm$.[15] Logarithms of capacity factors (log k) were plotted against log P - values of the reference compounds, the latter values being obtained from literature.[16] The correlations and graphs were produced with a Cricket Graph program of a Macintosh Plus PC. The R_m-values in RPTLC were calculated from the equation $R_m = \log (1/R_f\text{-}1)$.[17]

RESULTS AND DISCUSSION

Stationary Phase Effects

The earlier HPLC methods for toremifene and its metabolites have utilized reverse phase C_{18} columns[7,8] or a cyano column.[9] Because of the quite high pH values of the mobile phases used in those investigations that have been described, e.g. pH 6.4 - 8, we first tried a polymeric reverse phase (PRP-1) column. The reason for this was that polymeric phases stand a high pH (2-13)[18] and also, that a PRP-1 phase has been recently used succesfully for lipophilic compounds.[19] However, for triphenylethylenes the PRP-1 column, length being 150 mm and particle size 5 µm, proved not to be satisfactory at all, even though using a high portion of acetonitrile in the mobile phase. Most of the peaks were not sharp enough, although they were symmetric.

The next column evaluated was Supelco LC-18-DB, 150x4.6 mm, particle size 5 µm. As expected, the octadecyl silane phase causes much retention for the most lipophilic triphenyethylenes, like toremifene. This is also an advantage in separations from biological samples, if good peak shapes are obtained, and the speed of analysis can be improved by higher flow rates.[7,8]

Capacity factors obtained with this C_{18} column are presented in Table 3. For an unknown reason, one compound, FC-1530 B (10, Table 1) could not be chromatographed with this column at all.

A deactivated octyl silane column, Supelco LC-8-DB, 150x4.6 mm, 5 µm particle size, produced the best retention behavior for the whole series of triphenylethylenes studied. The capacity factors were markedly smaller than for the corresponding C_{18} column (Table 1) and therefore, this C_8 column seems to better suit our studies on log k values describing lipophilicity, and also for studies of synthetic product purity. Peak shapes were also more satisfactory when using Supelco C_8 compared with the C_{18} one.

Kromasil C_8 250x4.6 mm, 5 µm particle size, was included in this study being a cheap column, but producing good results in other HPLC studies . This column is not pronounced to be deactivated, but it seems to be tightly packed or properly end-capped, because the peaks had good shapes. This column was quite near the quality of Supelco LC-8-DB when concerning the aims of our studies. It is true, that this column is longer than the corresponding Supelco. The capacity factors of triphenylethylenes are presented in Table 3.

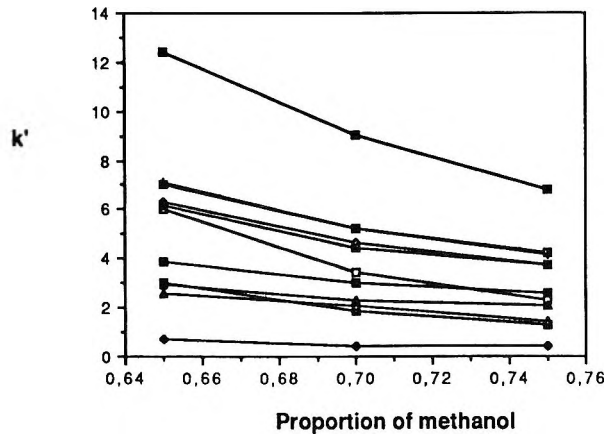


Figure 1. Capacity Factors of twelve thriphenylethylene derivatives as a function of percentage of acetonitrile in the mobile phase. Column: Supelco C_8.

Table 3

Capacity Factors (k) for Triphenylethylene Derivatives (1-12) for Different Silica Based RP Columns

	Column		
Compound	**Supelco C_{18}**	**Supelco C_8**	**Kromasil C_8**
1.	9.31	5.55	8.58
2.	0.64	0.69	0.79
3.	4.42	2.48	5.37
4.	9.23	5.02	7.16
5.	10.92	5.52	8.53
6.	5.04	3.09	3.52
7.	11.00	4.83	7.26
8.	3.55	2.41	2.79
9.	2.31	2.11	2.32
10.	*)	9.64	*)
11.	11.08	5.51	8.63
12	8.84	4.91	7.49

*) Could not be chromatographed in given conditions.

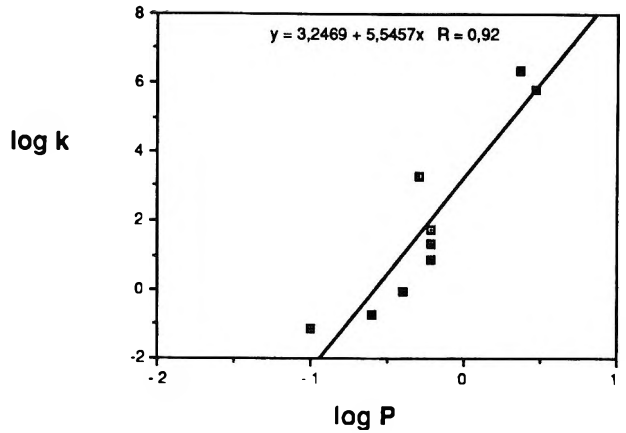


Figure 2. Correlation of log k and log P for reference compounds. Column: Hamilton PRP-1.

Mobile Phase Effects

Because of the highly lipophilic character of toremifene (log P= 6.35), the composition of mobile phase was constructed to contain a high portion of organic modifier. This approach has been used for lipophilic compounds.[19] Toremifene and some of its main metabolites, some of them included in this study, have been analysed by HPLC from biological samples using acetonitrile:ammonium acetate: triethylamine (TEA) as mobile phase.[8]

Other mobile phases that have been used in HPLC of toremifene and its metabolites have been: methanol:water (93:7) with 0.1 % TEA[7] and methanol: 0.1 M ammonium acetate (pH 8) (70:30). The mobile phase composition containing 65 % acetonitrile,[8] was first tested with RPTLC and it was then moved to HPLC. When trying RPTLC with MeOH:buffer with lower portions than 70 % of MeOH, toremifene and its analogues had far too much retention.

The mobile phase composition, according to Webster et al.,[8] was chosen to for this work because the main aims were to test columns and capacity factors for a series of triphenylethylenes. When varying the percentage of acetonitrile in the mobile phase (deactivated octyl silane column), the capacity factors were clearly affected (Fig 1). The PRP-1 column showed the kind of tendency as mentioned before, e.g. no satisfactory conditions were found for that polymeric phase. The diversity was found in lipophilicity studies as seen later.

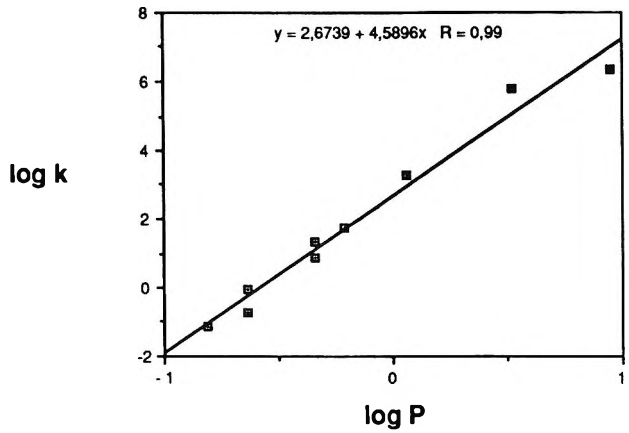


Figure 3. Correlation of log k and log P for reference compounds. Column: Supelco C_8.

Evaluation of Lipophilicity

In addition to method development, RPTLC was aimed to be used as a method for studying the lipophilicity of triphenylethylenes. RPTLC is a simple, rapid and low-cost method for such purposes, but accuracy is poor, especially when a UV scanner is not available. Even then, its applicability is not always satisfactory, owing to the unaccurate determination of the solvent front.[20]

Polymeric reverse phase (PRP-1) column is stabile in extreme pH conditions, and also applicable to highly lipophilic compounds when evaluating lipophilicity,[19,21] but in this case the peak shapes were unacceptable for some important compounds. Also, correlations between log k and log P values when using reference compounds were markedly worse than those when using silica based stationary phases (Fig 2). These correlations could not be improved by changing the percentage of acetonitrile in the mobile phase.

Silica based stationary phases gave satisfactory correlations for reference compounds, when log k values from HPLC and corresponding log P values from literature were subjected to linear regression (Table 2). Figures 3-5 present regression plots of those correlations obtained with Supelco C_8, Supelco C_{18} and Kromasil C_8.

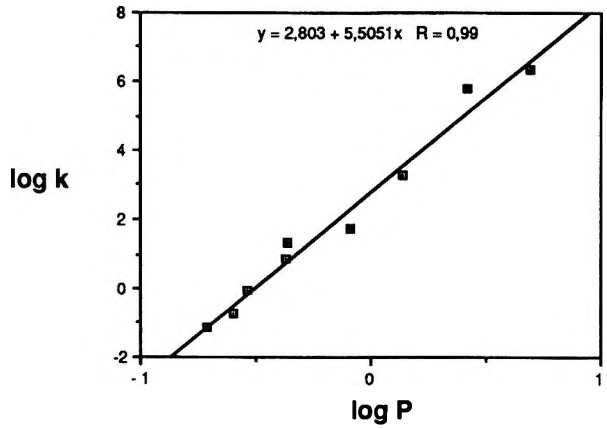


Figure 4. Correlation of log k and log P for reference compounds. Column: Supelco C_{18}.

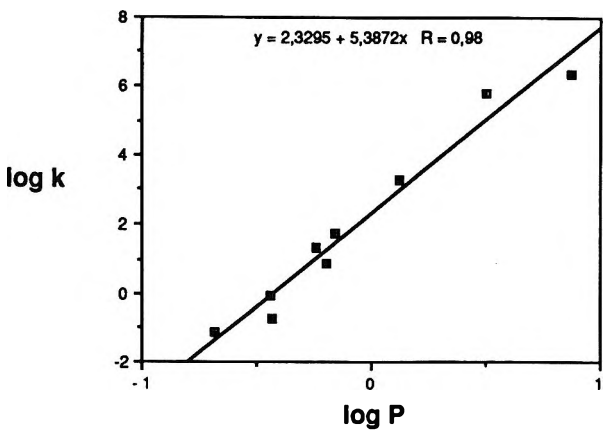


Figure 5. Correlation of log k and log P for reference compounds. Column: Kromasil C_8.

The results indicate, that these columns, preferably the C_8 coated ones, are suitable for lipophilicity studies for triphenylethylenes and related compounds even when a high portion of acetonitrile is used. The definitive evidence

should be obtained from comparison of triphenylethylenes themselves, by using a shake flask method and HPLC, but the very high lipophilicities of those compounds render this approach impossible.

One alternative to get parameters for a comparative study should be the use of calculation of log P by a computer, but we do not have such systems available. It seems obvious that lipophilicity parameters can be used for QSAR studies according to Braumann,[12] e.g. reverse phase columns simulate biological bilayers well, although some doubts have been presented and also new stationary phases have been introduced.[22]

In addition, mobile phase used here, containing acetonitrile as organic modifier, according to preliminary data seems to be suitable also for lipophilicity studies. This tendency has been noticed in studies performed with another classes of compounds,[19,23] in spite of doubtful findings for some series of compounds.[24] It is obvious, that mobile phase containing acetonitrile:ammonium acetate (65:35) with triethylamine as the amine modifier at pH 6.4, is suitable for RPLC purity studies of new triphylethylenes when using UV detector. Accordingly, it is possible to use this combination of mobile phase without the amine as an eluent for thermospray LC-MS of these compounds, at least, when using a deactivated RP column like Supelco LC-8-DB.[25,26]

CONCLUSIONS

Octyl silane (C_8) stationary phases are preferred for RPLC of triphenylethylenes, which are highly lipophilic compounds. Octadecyl chains cause too long a retention and also peak shapes are not satisfactory. In most cases, polymeric reverse phase column does is suitable for these molecules. A deactivated Supelco C_8 and Kromasil C_8 phase have almost equally good properties in retention of derivatives in question. A 150 mm long Supelco produces slightly better peak shapes than a 250 mm long Kromasil, but the latter one is markedly cheaper. The mobile phase should contain a large portion of organic modifier, and in this case acetonitrile (65 %) is the solvent of choice. With ammonium acetate (0.1 M) as a buffering salt, this system meets the requirements of thermospray LC-MS solvent, and in forthcoming studies that methodology will be applied. These RPLC conditions qive the possibility to go on to lipophilicity studies of these and new triphenylethylenes, in order to make use of retention data for quantitative structure relationship studies performed with CoMFA (Comparative Molecular Field Analysis). The shake flask method is not applicable because of the fairly high lipophilicities of these compounds, and mostly, for the same reason, the more conventional methanol/water mobile phase of RPLC combination is not preferable for these studies.

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MULTICOMPONENT ANALYSIS OF HIGHLY OVERLAPPED CAPILLARY ELECTROPHORETIC PEAKS USING MULTIWAVELENGTH CHARGE-COUPLED DEVICES DETECTION

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ABSTRACT

A chemometric method, Kalman filtering, was applied to deal with three-dimensional electropherograms, obtained by capillary electrophoresis connected to a charge-coupled device multiwavelength fluorescence detector. The capillary electrophoretic peaks of a mixture, which were not effectively separated, were resolved into electrophoretic peaks of individual components to obtain qualitative and quantitative information. The approach has been applied to analyze a mixture of rhodamine fluorescent dyes with excellent results.

INTRODUCTION

Capillary electrophoresis (CE) is an emerging, important technique in analytical chemistry because of its high efficiency, rapid analytical speed, small sample amount, etc.[1-3] In most reports to date, single channel detection is used, and a two-dimensionl electropherogram is produced.

However, multichannel detection, with high speed scanning UV/Vis detectors,[4-8] photodiode array detectors[9-12] and NMR[13,14] is more attractive because it gives a three-dimensional electropherogram with wavelength and retention information from which the separated compounds may be identified.

Charge-coupled devices (CCD) form a new class of multi-channel solid-state detectors which are applied to molecular spectroscopy, atomic spectroscopy, Raman spectroscopy, separation science, image analysis and some analytical chemical aspects.[15-18] It is also used as a sensitive detector for CE. Cheng et al.[19] first used CCD as fluorescence detector for CE and obtained a three-dimensional electropherogram with wavelength information. CCD was used by Chen et al.[20] for the on-line Raman spectroscopic detection, in CE, of a mixture of methyl red and methyl orange.

Sweedler et al.[21] utilized the time-delayed integration mode to detect fluorescein isothiocyanate-labeled amino acids in the 10^{-20} mole range, and quantitatively determined a sub-attomole quantity of bag cell neuropeptides collected from the giant mollusk Aplysia californica.[22] With a linear regression methodology, the multi-wavelength fluorescence electropherogram detected with CCD was used by Karger et al.[23] for the determination of base fragments attached with four kinds of fluorophores and rapid DNA sequencing.

Yueng et al.[24] and Takahashi et al.[25] performed an array capillary electrophoresis for DNA sequencing with CCD.

Another advantage of multi-channel detection is that, with a chemometric method, the three-dimensional data can be processed for the multicomponent analysis of highly overlapped electrophoretic peaks. Based on the differences of in spectra, individual constituents can be accurately identified and quantified from the unresolved electropherogram. In some other cases, this is more important and necessary. For instance, in four color fluorescent labeled DNA sequencing by CE, different length fragments may overlap with each other, owing to the migration speed differences of fluorophores.[26] If one did not exploit the merit of three-dimensional electropherograms from which the positions of different fluorophore-labeled fragments are located, it would be impossible to accurately obtain the DNA sequence.

In liquid chromatography (LC), the chemometric methods, least-squares multiple linear regression,[27,28] Kalman filtering,[29] factor analysis,[30] generalized rank annihilation,[31] multivariate curve resolution,[32-33] etc., have been used for the processing of multichannel data and multicomponent analysis of highly overlapped LC peaks. Similarly, these methods are able to be used for the multicomponent analysis of overlapped capillary electrophoretic peaks. However, there are only a few reports about this.

In this paper, a chemometric method, Kalman filtering,[34] was utilized for the processing of multiwavelength electrophoretic data and multicomponent analysis of highly overlapped electrophoretic peaks. The model compounds, three kinds of rhodamine fluorescent dyes whose spectra are overlapped, were separated by CE with CCD multiwavelength fluorescence detection. In the three-dimensional electropherogram two components' peaks were highly overlapped but, by the use of Kalman filtering, the mixture peak was divided into the peaks of the individual components. The qualitative and quantitative analyses of individual components were further accomplished. It may be concluded that CE with CCD multiwavelength detection is more powerful than with single-channel detection.

EXPERIMENTAL

Separation and Detection System

A CE system with a CCD multi-wavelength fluorescence detector, previously described[35] was used. The excitation beam emitted from a tungsten-bromine source (Shanghai Third Analytical Instrument Factory, China) was focused with a lens (10X magnification) onto the fused-silica capillary (100μm i.d., 370 μm o.d., (Yongnian Optical Fiber Factory, China) at a position located 5.5 cm from the cathodic end of the tube. From the capillary with a length of 51 cm a small section of protective coating was burned off with a gentle flame to form a detection region. The effective length was 45 cm.

The fluorescence emission was collected at right angles to the excitation source, and focused by the two lenses, onto the entrance slit of a polychromator (HR 320, Instruments, SA, Inc., USA), dispersed by a 1200 grooves/mm grating and irradiated on a 512 by 512 element CCD (Model 1530-P, EG & G, Princeton Applied Research, USA). Through an optical fiber, the data of photogenerated charge were transported into a computer for storage, calculation and output of results.

A high-voltage power supply, built in our laboratory, was used to provide a 20 kV potential for electrophoretic separation. The electrical connections were made at both ends of the capillary with platinum wires immersed in 5 mL reservoirs. Samples were introduced by siphoning at a height difference of 10 cm for 5 sec. The CCD chip was cooled with a Peltier device to -40°C, which reduced dark current and background noise. The monitoring of experiments and selection of operation parameters, e.g., exposure time, charge transferring pattern, data collection mode, were controlled by the use of OMA SPEC 4000 software installed in a Gateway 2000 computer.

Reagents

All reagents were of analytical grade; doubly distilled water was used for dilution. Stock solution of rhodamine 590 (R 90), rhodamine 610 (R610), rhodamine 640 (R640), purchased from Exciton Chemical (Dayton, Ohio, USA), were all prepared in 1×10^{-4} mol/L. Working solutions were produced by appropriate dilution. The run buffers for all the experiments were 10 mmol/L borates, prepared from sodium borate decahydrate and sodium hydroxide.

Procedure

The applied voltage and operating parameters of the CCD for fluorescence detection were selected and adjusted. Then the background spectrum was recorded and stored. After the sample was introduced by siphoning, the electrophoresis was initiated by switching on the high-voltage power supply and timer. At an appropriate time, the CCD detection was started and the fluorescence spectra were detected, in succession, using a data gathering mode of automatic background subtraction (Accume-B). Finally, a multi-wavelength fluorescence electropherogram was produced.

The observation vector needed for filtering was composed of spectra chosen from the electropherograms of individual components. The mixture electropherograms were treated with the proposed method, and the qualitative and quantitative information of every constituent were obtained.

Program

Based on the principle of Kalman filtering,[34] a program was compiled with MACRO language provided by the application software OMA SPEC 4000. The observation vectors, made up from standard spectra of the pure compounds, were first input. Then, each spectrum of the mixture electropherogram was processed by the filtering program, and the contributions of each component obtained. If a component is not present in the spectrum, the proportional parameter to its standard spectrum will be less than 10^{-4}. If several components contribute to a spectrum, the proportional parameters of each component to their standard spectra will be obtained, which demonstrates that the electrophoretic peaks are overlapped.

Using the proportional parameters obtained in filtering, the mixture spectra in overlapped electrophoretic peaks are resolved into spectra of individual components. Thus, the overlapping mixture three-dimensional electropherogram is divided into several three-dimensional electropherograms in

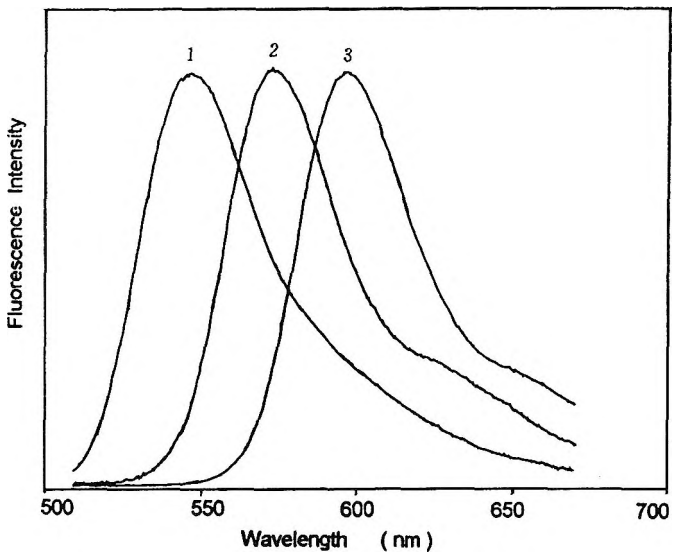

Figure 1. Fluorescence spectra of rhodamine 590 (1), rhodamine 610 (2), rhodamine 640 (3).

Table 1

Quantitative Results of Sample Mixtures

No.	C_{added} x 10^{-4} mol/L R590	R610	R640	C_{found} x 10^{-4} mol/L R590	R610	R640	RSD (%) R590	R610	R640
1	2.50	2.50	2.50	2.61	2.38	2.43	+4.40	-4.60	-2.76
2	2.50	2.50	5.00	2.41	2.39	5.13	-3.60	-4.32	+2.68
3	5.00	5.00	2.50	4.87	5.20	2.40	-2.46	+4.02	-3.80
4	1.25	1.25	2.50	1.32	1.18	2.62	+5.84	-5.20	+4.84

which the electrophoretic characteristics of individual components are shown. Finally, a calculation subprogram described previously[36] was used to obtain the qualitative and quantitative results for components.

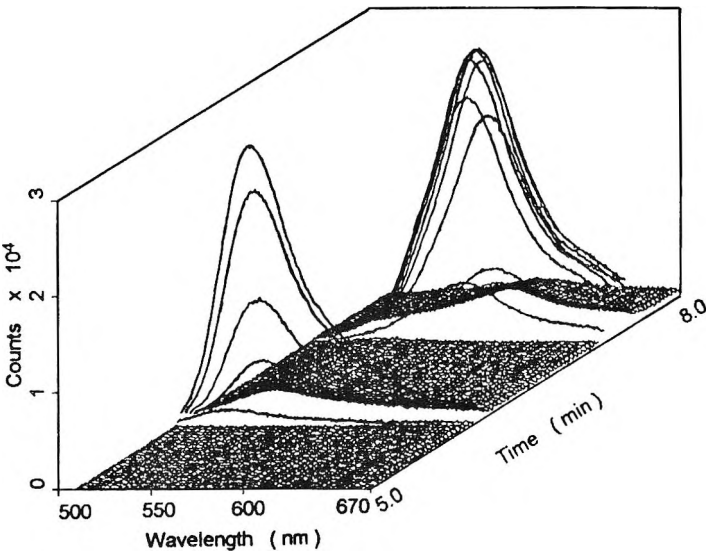

Figure 2. Three-dimensional electropherograms of a mixture. Experimental conditions: Buffer; 0.01 mol/L sodium tetraborate solution, pH 11. Sample: each at 10^{-4} mol/L, introduced by siphoning at a height difference of 10 cm for 5 sec. Applied voltage, 10 kV. The peaks were identified as R590 (1), mixture of R610 and R640 (2).

RESULTS

Fluorescence Spectra

A fluorescence spectrum with a wavelength window of 270 nm is able to be detected by CCD. If the fluorescence spectra from analyses do not overlap with each other, they will be independent peaks in a three-dimensional electropherogram, even though their migration times are equal. This allows their respective qualitative identification and quantification. However, if the fluorescence spectra from analyses are overlapped with each other, and there is little difference in their migration times, the peaks from the analyses overlap in the threedimensional electropherogram. In this case, it is impossible to acquire the qualitative and quantitative information of components unless the mixture peak is mathematically resolved. The fluorescence spectra of R590, R610, R640 are shown in Figure 1. It may be observed that the fluorescence maxima (nm) of RS90, R610 and R640 are 547.5, 577, 601, respectively, and their fluorescence spectra are seriously overlapped. If in electrophoresis several component coelute, mixture spectra are measured.

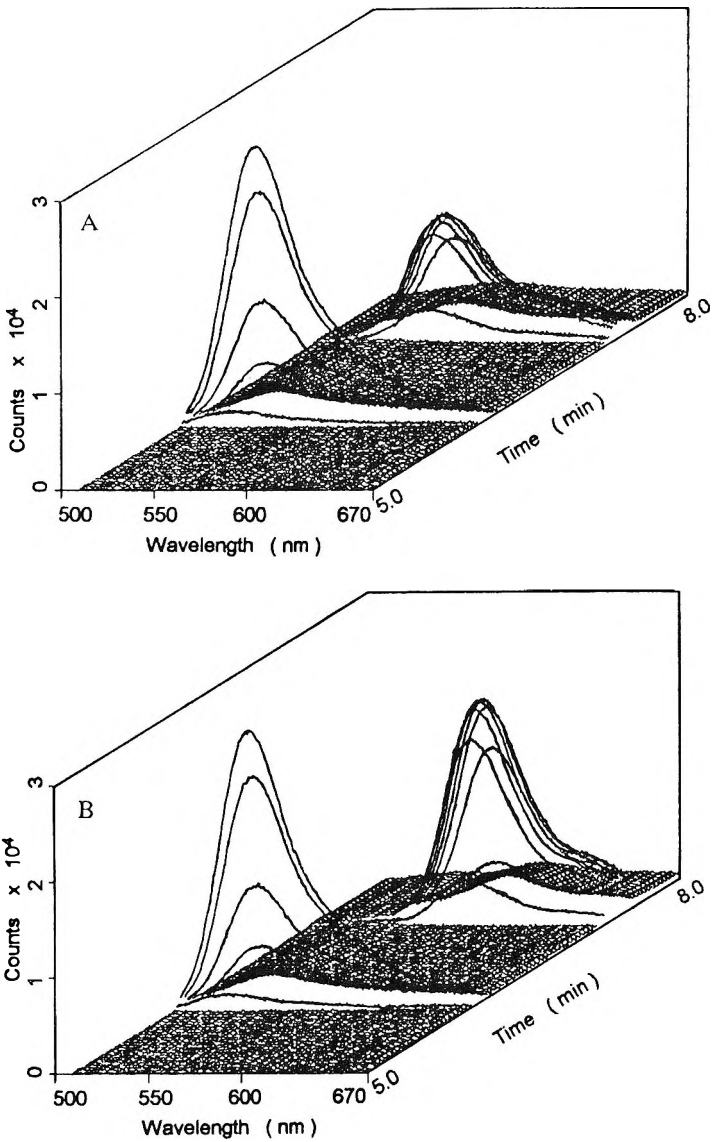

Figure 3. Three-dimensional electropherograms of R590, R610(A) and R590, R640(B) yielded by the proposed method.

Electrophoretic Characteristics of Samples

The experimental results at different pH's and applied voltages show that the migration speed of R590 is the fastest; its retention time is the shortest, hence, it always appears first in the detection window and is baseline separated from the other components. However, the migration speeds of R610 and R640 were slow and their retention times were similar. Therefore, they coelute in the detection window. As a result, two electrophoretic peaks are detected in the three-dimensional electropherogram. The first one is R590, and the last is the mixture peak of R610 and R640 (Figure 2).

This demonstrates that, under the experimental conditions used in this paper, the electrophoretic peaks of R610 and R640 were highly overlapped.

Electropherograms of Individual Components

The three-dimensional electropherograms of individual components, obtained by applying Kalman filtering to data given in Figure 2, are shown in Figure 3. The first peak represents R590, which is same as in Figure 2. The second peak is R610 (Figure 3A) and R640 (Figure 3B). By comparison, one can see that the spectra and sizes of these two peaks are different, which show the respective spectrometric and electrophoretic characteristics of R610 and R640 after the mixture peak was resolved.

The conventional two-dimensional electropherograms, derived from Figure 2 and Figure 3 by applying a calculation subprogram,[36] are shown in Figure 4. It may be observed that the peaks of R610 and R640 are highly overlapped. Without CCD for multi-wavelength detection, followed by a chemometric method, no individual electropherograms and quantitative information would have been obtained.

Results of Quantitative Analyses

The quantitative results of some mixture samples are listed in Table 1. one can see that the proposed method yielded equivalent accuracies for the components R610 and R640, whose peaks are highly overlapped in electropherograms, compared to the component R590 which is completely resolved in the electropherograms.

This demonstrates the performance of Kalman filtering for the multicomponent analysis of highly overlapped multi-wavelength CE peaks detected by CCD.

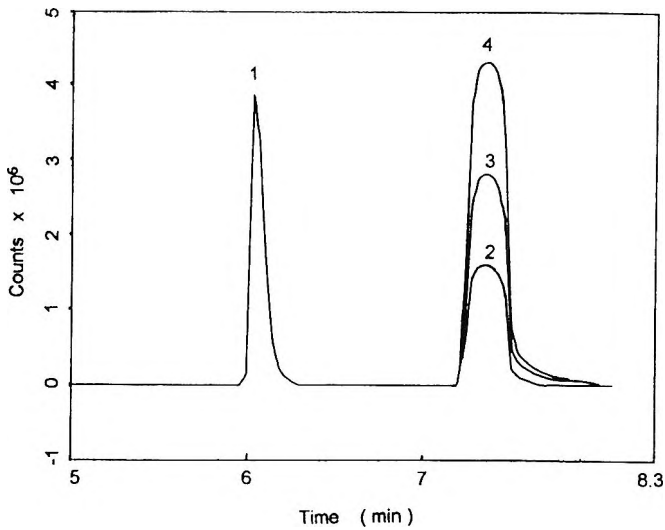

Figure 4. Two-dimensional electropherograms of a sample. The peaks were identified as R590 (1), R610 (2), R640 (3), the mixture of R610 and R640 (4).

DISCUSSION

The experimental results demonstrate that multi-wavelength CE data can be treated by the proposed method. The electropherograms and quantitative results of individual components are obtained, even though their electrophoretic peaks are highly overlapped. Even though the three component system used is simple, the conclusion is general.

In multi-component analysis, two situations may occur. First, although the peak count in the electropherogram increases, all peaks can be sequentially treated and resolved. Second, if the component count in a peak increases, this is not problematic, because Kalman filtering is a rigorous computational method for multi-component analysis; it will not be hard to resolve a mixture peak into the individual component peaks. Nevertheless, owing to the high separation effciency of CE, it is not likely that there are more than two components in a peak.

Other chemometric methods may similarly be used for the treatment of multi-wavelength CE data. That work is in progress. Even though the R610 and R640 are likely to be readily separated from each other by means of other kinds of buffers or by adding selectors, they were selected for the present work as an illustrative example of the CCD, Kalman filtering technique.

In multicomponent analysis by Kalman filtering, the accuracy of the standard spectra which make up the observation vector should be good. If there are errors in standard spectra, the accuracies of analytical results will be seriously affected.[37] In general, spectra at various concentrations of standard solutions are measured, the specific parameters at each wavelength are calculated by a least-squares linear regression and used as the observation vector in multicomponent analysis.

In multi-wavelength spectrometric detection in CE, accurate standard spectra of known concentrations can not be obtained. Therefore, for the resolution of overlapped peaks by Kalman filtering, the strategy applied was to select pure spectra from the electropherograms of individual component, which form the observation vector; this yields the proportional parameter to corresponding component. The procedure is simple and convenient to apply.

In multi-component analysis of overlapped LC peaks,[27-33, 38-40] the assumption is usually made that the peak shapes of each component in single and mixture LC should be identical, i.e., there is a good peak reproducibility under the experimental conditions. Therefore, a procedure is utilized that, at fixed wavelengths, the peak responses at different times are processed. In CE, however, due to the influences of various factors, it is difficult to keep a good reproducibility in peak shape and migration time. Therefore, there is no report about multi-component analysis of overlapped peaks obtained by a single-channel detector.

The procedure used in this paper, i.e., that at fixed migration times, spectra of mixtures are subjected to a multi-component analysis, is able to overcome the disadvantage of poor reproducibility in peak shapes. Under the experimental conditions, the spectra of the common components in the different runs do not vary with small fluctuations in experimental conditions.

Even if the peak shapes change, due to changing of experimental conditions, the proposed method, based on the spectra of individual components, separates the mixture electrophoretic peaks into the electrophoretic peaks of individual components, allowing an accurate qualitative and quantitative analysis.

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DETERMINATION OF IBUPROFEN ENANTIOMERS IN HUMAN PLASMA BY DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A sensitive and selective reverse phase high performance liquid chromatographic (HPLC) method was developed for the determination of the enantiomers of ibuprofen in human plasma. Ibuprofen and fenoprofen (internal standard, ISTD) are extracted from human plasma by n-butyl chloride, following acidification of plasma. The method is based on the separation of the diasteromers formed on reacting ibuprofen enantiomers and ISTD with S-(-)-1-(1-naphthyl)ethylamine. Separation is achieved by HPLC on an Inertsil ODS-2 column,with a mobile phase composed of water (pH 3.0):ACN (33.5:66.5). Detection is by fluorescence detection with excitation and emission at 280 and 320 nm, respectively. The mean retention times of S-(+)-ibuprofen, R-(-) -ibuprofen, S-fenoprofen and R-fenoprofen (ISTD) are approximately 11.3, 12.3, 7.7 and 8.5 minutes, respectively.

The assay is linear in concentration ranges of 100 to 20,000 ng/mL. The analysis of pooled quality controls (400, 2,000, and 16,000 ng/mL) demonstrates excellent precision with relative standard deviations (RSD) (n=18) range from 3.6% to 8.8%. The method is accurate with all intraday (n=6) and overall (n=18) mean values for the quality control samples being less than 7.0% from theoretical.

INTRODUCTION

Ibuprofen (IB, (±)-2-(4-isobutylphenyl)propionic acid), is an effective and well tolerated 2-arylpropionic acid non-steroidal anti-inflammatory drug. It contains a chiral center and is marketed as a 50:50 mixture of the S(+)- and R-(-)-enantiomers, although the pharmacological activities of ibuprofen are mainly associated with the S-(+)-enantiomer.[1-3] It is necessary to develop methods to quantitate the enantiomers of ibuprofen.

To date, there have been numerous analytical methods developed using GC or HPLC for the separation and quantitation of IB enantiomers in biological specimens.[4-22] However, these methods suffered lengthy sample preparation, presence of endogenous interferences, poor sensitivity, expensive chiral stationary phases and extensive column flushing procedure which, result in lengthy analysis time.

In this manuscript, a simple, sensitive and specific method is described for the determination of ibuprofen enantiomers in human plasma. The assay is based on the separation of the diasteromers formed on reacting ibuprofen enantiomers and ISTD with S-(-)-1-(1-naphthyl)ethylamine and chloroformate as the coupling reagent.

EXPERIMENTAL

Materials

R-(-)- and S-(+)-ibuprofen were obtained from RBI (Natick, MA, USA). Fenoprofen calcium (FENO, internal standard) was obtained from Sigma (St. Louis, MO, USA). Heparinized human plasma was obtained from Valley Biomedical. Acetonitrile, and n-Butyl chloride, HPLC grade were obtained from Burdick & Jackson (Muskegon, MI, USA). Acetic acid, and Sulfuric acid, GR grade, were obtained from EM Science (Gibbstown, NJ, USA). Ethyl chloroformate, ethanolamine and S-(-)-1-(1-naphthyl)ethylamine were obtained from Aldrich (Milwaukee, WI, USA).

Triethylamine was obtained from Sigma (St. Louis, MO, USA). Deionized water was processed through a Milli-Q water purification system, Millipore Corporation.

Chromatographic Systems

The HPLC system consisted of a Perkin-Elmer 200 LC pump (Norwalk, CT, USA), a Waters 717 autoinjector (Milford, MA, USA), and Jasco FP-920 fluorescence detector (Tokyo, Japan), with excitation and emission wavelength at 280 and 320 nm, respectively. The analytical column was an Alltech Inertsil ODS-2, 150 mm x 4.6 mm, 5-μm particle size (Deerfield, IL, USA), protected by a Brownlee Newguard RP-18 pre-column (15 mm x 3.2 mm, 7-μm particle size, ABI, San Jose, CA, USA). Data collection and calculations were conducted with an HP1000 Model A990 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, PA, USA). The mobile phase was water (pH 3.0)/acetonitrile (33.5:66.5) with a flow rate of 1.2 mL/min. The column was maintained at 27°C with an Eppendorf CH-30 column heater (Madison, WI, USA).

Preparation of Standard Solutions

Stock standard solutions of R-(-)- and S-(+)-ibuprofen (1 mg/mL) were prepared by dissolving 25 mg of R-(-)- or S-(+)-ibuprofen in 25 mL of methanol. A stock solution of internal standard (100 μg/mL) was prepared by dissolving 2.5 mg of fenoprofen in 25 mL of methanol. Working solutions of R-(-)- and S-(+)-ibuprofen (1 to 200 μg/mL) were prepared by diluting the stock solution with 25:75 methanol/water. The internal standard working solution (50 μg/mL) was prepared by diluting the stock solution with water. The R-(-)- and S-(+)-ibuprofen and the internal standard solutions were stored at 4°C. All solutions were stable for at least 1 month.

Quality Control Samples

Pooled quality control samples (QC samples) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. A control pool was also prepared at a concentration above the curve range (over-cure control) to evaluate precision and accuracy when specimens required analysis at partial volume.

Plasma control pools (400, 2000 and 16000 ng/mL) were prepared by diluting 100 μL of 100 μg/mL, 500 μL of 100 μg/mL, and 400 μL of 1 mg/mL R-(-)- and S-(+)-ibuprofen, respectively, to a 25-mL volume, using blank human

plasma. An over-curve control (30000 ng/mL) was prepared by diluting 300 µL of 1 mg/mL to a 10-mL volume with blank human plasma. All control pools were aliquoted into 4-mL polypropylene-vials and stored at approximately -20°C.

Sample Preparation

Calibration standards were prepared by adding 50 µL of R-(-)- and S-(+)-ibuprofen working solutions to 0.5 mL of blank human plasma. Clinical specimens and controls were prepared by aliquoting 0.5 mL of plasma into glass tubes.

Calibration standards, clinical specimens and controls were processed by adding 50 µL of internal standard, 200 µL of 20% sulfuric acid and 6 mL of n-butyl chloride. The samples were mixed on a vortex mixer for 5 minutes, and centrifuged at approximately 950 g for 5 minutes. The aqueous layer was frozen in a dry ice-acetone bath, the organic layer was transferred to a clean tube. The n-butyl chloride was evaporated to dryness under nitrogen.

Derivatization Procedures

The samples were reconstituted in 300 µL of 50 mM TEA, sonicated for 1 minute and mixed on a Vortex mixer for 30 s. Fifty microliters of 6 mM ethyl chloroformate was added to each tube and after 30 s, 25 µL of 10 mM S-(-)-1-(1-naphthyl)ethylamine was added. Three minutes later 25 µL of 1:40 ethanolamine/acetonitrile was added. The solvent was evaporated under nitrogen in TurboVap at 40°C and reconstituted in 250 µL of mobile phase. Twenty five microliters aliquots were injected into the HPLC system.

Validation

Duplicate calibration curves were analyzed on each of the three days of validation. One reagent blank (water substituted for plasma), blank plasma, control zero (blank plasma spiked with internal standard) and triplicate controls at each concentration (400, 2000 and 16000 ng/mL of R-(-)- and S-(+)-ibuprofen in plasma) were analyzed with each calibration curve. The calibration curves were obtained by weighted (1/C) least-squares linear regression analysis of the peak height ratios of R-(-)- or S-(+)-ibuprofen/internal standard vs. the concentration of R-(-)- or S-(+)-ibuprofen, respectively. The equations of the calibration curves were then used to calculate the concentration of R-(-)- or S-(+)-ibuprofen in the samples and controls from their peak height ratios.

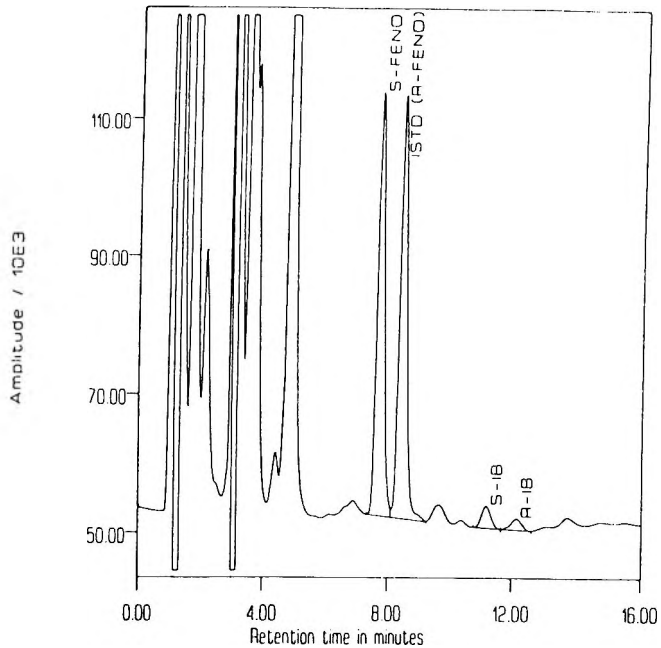

Figure 1. Chromatogram of a 100-ng/mL Calibration Standard.

RESULTS AND DISCUSSION

The diasteriomers of ibuprofen and fenoprofen were well separated from each other, as shown in representative chromatograms (Figures 1 - 3). The retention times of S-(+)-ibuprofen, R-(-)-ibuprofen, S-fenoprofen and R-fenoprofen (ISTD) were approximately 11.3, 12.3, 7.7 and 8.5 minutes, respectively.

Blank human plasma from twelve pools was tested for endogenous interferences. No endogenous interferences were found in the ibuprofen and ISTD regions.

Precision, and Accuracy of S-(+)-ibuprofen, and R-(-)-ibuprofen standards are in Tables 1 and 2. The standards show low values in deviation (<6.1%) and relative standard deviation (<5.9%). Calibration curves for S-(+)-ibuprofen, and R-(-)-ibuprofen in plasma were linear over the concentration range of 100 to 20000 ng/mL, with correlation coefficients greater than 0.9997 for both enantiomers.

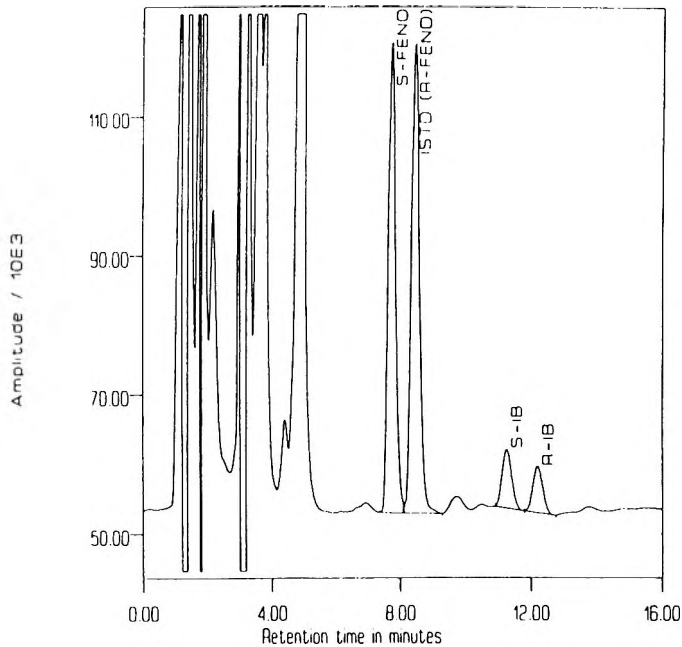

Figure 2. Chromatogram of an 400-ng/mL Quality Control Sample.

Data from the quality control samples are shown in Table 3 and 4. The within-day precision of the method as measured by the RSD of the daily mean (n = 6), ranged from 2.1% to 11.3% at the three control concentrations in human plasma. The overall precision ranged from 3.6% to 8.8% RSD (n = 18) for the 400-, 2000- and 16000-ng/mL of the S-(+)- and R-(-)-ibuprofen controls.

The accuracy of the method was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations of S-(+)-and R-(-)-ibuprofen in the plasma controls. All of the daily mean (n = 6) and overall mean (n = 18) values for the controls were within 7.0% of their expected values.

A QC sample pool containing 30000 ng/mL of S-(+)-ibuprofen and R-(-)-ibuprofen was prepared and analyzed with the high QC sample (16000 ng/mL) at the partial volumes of 50 μL. These aliquots were diluted to a final volume of 500 μL with blank plasma. The mean (n = 6) values for all partial volumes were within 6.9% of their expected values. The precision was better than 4.7% RSD (n = 6) at all partial volumes.

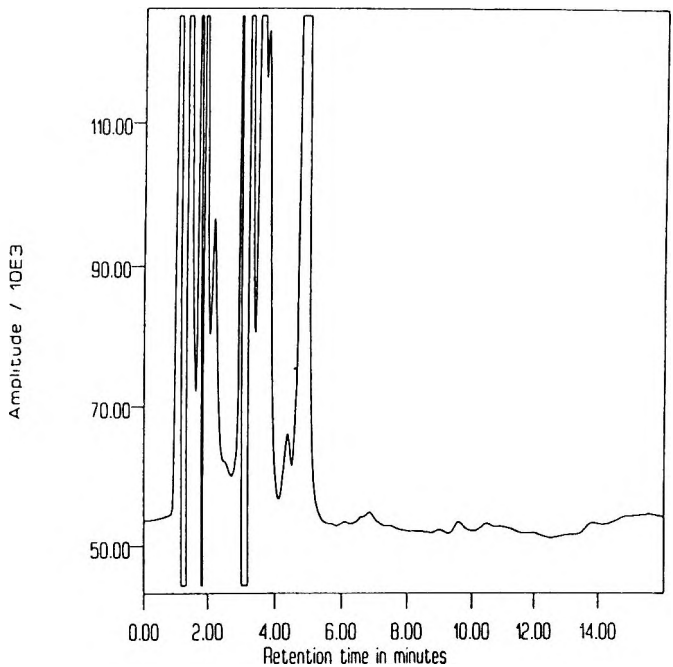

Figure 3. Chromatogram of Blank Human Plasma.

Table 1

Precision and Accuracy of S-(+)-Ibuprefen Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean ± S.D., n=6) (ng/mL)	R.S.D. (%)	Deviation (%)
100	101 ± 2.3	2.3	1.4
200	202 ± 6.96	3.4	1.1
500	502 ± 23.9	4.8	0.5
1000	987 ± 20.9	2.1	-1.4
5000	4910 ± 97.5	2.0	-1.8
10000	9950 ± 134	1.3	-0.5
20000	20200 ± 140	0.7	1.0

Table 2

Precision and Accuracy of R-(+)-Ibuprefen Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean ± S.D., n=6) (ng/mL)	R.S.D. (%)	Deviation (%)
100	106 ± 4.6	4.4	6.1
200	199 ± 11.7	5.9	-0.3
500	489 ± 14.5	3.0	-2.1
1000	980 ± 35.0	3.6	-2.1
5000	4910 ± 115	2.4	-1.8
10000	9950 ± 138	1.4	-0.5
20000	20200 ± 190	0.9	1.0

Table 3

Precision and Accuracy of S-(+)-Ibuprofen Quality Controls

Control Concentration (ng/mL)	Calculated Concentration (Overall mean ± S.D., n-=18) (ng/mL)	R.S.D (%)	Deviation (%)
400	407 ± 17.3	4.3	1.6
2000	1960 ± 166	8.5	-1.9
16000	15600 ± 580	3.7	-2.6

Table 4

Precision and Accuracy of R-(+)-Ibuprofen Quality Controls

Control Concentration (ng/mL)	Calculated Concentration (Overall mean ± S.D., n-=18) (ng/mL)	R.S.D (%)	Deviation (%)
400	395 ± 14.2	3.6	-1.6
2000	1950 ± 173	8.8	-2.5
16000	15800 ± 585	3.7	-1.2

The lower limit of quantitation (LLOQ) was set at 100 ng/mL of S-(+)-, and R-(-)-ibuprofen in human plasma. Six replicates of the lowest standard (100 ng/mL) were analyzed to evaluate the accuracy and precision at the LLOQ. At the LLOQ, the RSD (n = 6) of the peak height ratios was 10.2%, the RSD (n = 6) of the measured concentrations was 14.0%, and the deviation of the mean (n=6) of the measured concentrations from their nominal value was 7.0%.

Extraction recoveries were determined by comparing the peak heights of extracted calibration standards with the peak heights of pure recovery standards at the same nominal concentrations. The mean recoveries for S-(+)-ibuprofen, R-(-)-ibuprofen and the internal standard were 76.6%, 76.1%, and 89.6%, respectively.

Stability was tested by subjecting the QC samples to three freeze/thaw cycles, and storage for 24 hours at room temperature. The thawing and refreezing of QC samples and the storage of QC samples at room temperature, had little effect on the precision or accuracy of the results. The mean (n = 3) value was within 7.5% of the expected values.

Process stability was tested by extracting one set of calibration standards with duplicate QC samples and stored overnight at room temperature before analyzing. The storage of extracted samples at room temperature had little effect on the accuracy and precision of the results. The mean (n = 2) values of the controls was within 9.0% of the expected values.

In conclusion, the method presented here for the determination of S-(+)-ibuprofen and R-(-)-ibuprofen enantiomers in human plasma is precise, and accurate. The ruggedness of the procedure has been demonstrated by applying the methods for the analysis of calibration standards and quality controls.

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A PAIRED ION LIQUID CHROMATOGRAPHIC METHOD FOR THIAMINE DETERMINATION IN SELECTED FOODS

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ABSTRACT

A precise and selective method for determining the content of thiamine in dietetic and baby foods by reverse phase liquid chromatography with UV detection is proposed. Enzymatic extracts of food samples were subjeted to purification and preconcentration with a weak ionic exchange column (CBA) and 0.1 M $BaCl_2$ solution as eluent. Ion-pair chromatography using a C_{18} column and a mixture of 5 mM sodium hexanesulphonate as counter-ion, 10^{-2} M potassium dihydrogen orthophosphate/ phosphoric acid buffer solution, pH 2.8, and 0.1% triethylamine was employed. The thiamine was detected at 254 nm.

This method was used to determine the content of thiamine in baby meals, cereals and dietetic cookies. Recovery studies showed good results and the relative standard deviation (n=10) was 2.9%.

INTRODUCTION

An increasing awareness of the nutritional quality of food has arisen over the last few decades in industry, regulatory agencies and the public in general. This interest has led to an increased demand for rapid and accurate analytical methods for all nutrients, and among these, vitamins.

High Performance Liquid Chromatography has been increasingly used for the separation and determination of vitamins in foods, as, HPLC methods offer an attractive alternative to the more time-consuming chemical and microbiological assays for vitamins, due to their increased specificity, sensitivity and reduced analysis time.[1-3]

The sample treatment for thiamine analysis usually starts with an acid hydrolysis to liberate the thiamine from the food. Either hydrochloric or sulphuric acid is used for this purpose.[4-6] The determination of total thiamine in food products, requires a treatment of the acid extract with an enzyme mixture containing phosphatase activity, such as clarase,[7] β-amylase,[8,9] or takadiastase[4,10] at a pH of about 4-5. Papain has also been used, in addition to takadiastase, for the extraction of thiamine from meat products.[11] The enzyme must hydrolize the starch present, in order to improve sample filtration and release thiamine from its phosphate esters.

Some methods have described thiamine analysis using ultraviolet detection in food samples that contain sufficient amounts of thiamine, such as multivitamin pharmaceutical preparations[12] and enriched cereal products,[10,13,14] but methods using fluorescence detection appear to be more successful for the detection in unsupplemented food[2,4] that have microgram amounts of thiamine at natural levels, because of the low sensitivity of the UV detector . However, some authors[3,9,12] have pointed out several shortcomings of the fluorimetric method that would affect quantification, for example, the feasible presence of ultraviolet-absorbing compounds can seriously interfere by inducing the quenching of fluorescence.

The low levels of vitamins and high amounts of other interfering materials in many food products, often make chromatographic determination of the direct acid extracts unfeasible. In this case, a solid-liquid extraction step is adequate for the purification and preconcentration of the extracts.

This paper, describes a simple analytical method for thiamine in dietetic and baby foods which, involves extraction with HCl and takadiastase solution, clean-up and preconcentration with a weak exchange-ion column, packed with methylcarboxylate in acid form, followed by HPLC determination.

EXPERIMENTAL

Apparatus and Conditions

The HPLC system consisted of a Hewlett-Packard HP 1090 liquid chromatograph (Waldbronn, Germany), a Rheodyne 7010 injection valve with a 20 μL loop, a Hewlett-Packard(HP) 79881A filter photometric detector, a HP 85B personal computer and a HP 3390A integrator. Column effluent was monitored at 254 nm for thiamine.

The HPLC column used was a Lichrospher 100 RP-18 (125 x 4 mm i.d., 5 μm).

A Visiprep Vacuum Manifold (Supelco, Bellefonte, PA, USA) was used for solid-liquid extraction together with a Vacuum Brand GMBH membrane pump (Wertheim, Germany).

A PW 9422 Philips pH-meter equipped with a combined glass-Ag/AgCl electrode was employed for pH measurements.

The water bath used was from Grant Instruments (Cambridge, England).

Materials and Reagents

All solvents used were HPLC grade and were employed as supplied by manufacturers. High purity water was obtained through a Millipore Milli-Q system (Milford, MA, USA).

Analytical grade thiamine standard supplied by Sigma (St. Louis, MO, USA) was used. Individual stock solutions of this vitamin were prepared every third day in water to provide a concentration of 1 mg/mL. This solution was degassed with helium and stored in dark glass flasks in order to protect it from light, under -18°C refrigeration.

The working standard was prepared by adding aliquots of individual stock solution and diluting with water.

For acid hidrolysis, HCl (Merck, Darmstadt, Germany) was used and for enzymatic hidrolysis, takadiastase and clarase, both obtained from Fluka (Buchs, Switzerland). Sodium hexanesulphonate (Sigma, St. Louis, MO, USA), methanol HPLC grade (Romil Chemicals, Sps), phosphoric acid, potassium dihydrogen ortophosphate, triethylamine, barium chloride and sodium acetate anhydre (Merck, Darmstadt, Germany) were also employed.

Sample Preparation

Three types of foods were analyzed: baby meal, cereals and dietetic cookies. Finely ground samples containing some micrograms of thiamine, were weighed into a 50 mL volume erlenmeyer flask and 20 mL of HCl was added. The flask was put in a water bath at 100°C for 30 minutes. After cooling, the solution was adjusted to pH 4-4.5 with sodium acetate. Several types and amounts of enzymes were added. The solution was incubated in a water bath, previously heated to 47°C, during 3 h. The cooled samples were filtered through cellulose acetate filter (0.45 μm) and diluted with water in a 50 mL volumetric flask.

Purification of the Extract

An aliquot of this extract (2 mL) was passed through a CBA column, (previously conditioned by passing 1 mL MeOH and 1 mL of phosphate buffer 0.01 M at pH 4) packed with methylcarboxylate in acid form. The interfering substances were removed by washing with 2x500 μL phosphate buffer at pH 4. Thiamine was eluted from the column using 3x200 μL barium chloride 0.1 M.

Chromatographic Determination of Thiamine

The mobile phase was composed of 5 mM sodium hexanesulphonate (HSA) by adjusting the pH value of the solution to 2.8 through addition of 10^{-2} M potassium dihydrogen orthophosphate/phosphoricacid.

Methanol was selected as organic modifier.[15] The optimum concentration of methanol was selected, in order to get a retention time for the thiamine that avoided its overlap with the substances eluted in the elution front, and so that the run time was shorter in order to avoid the broadening of sample bands that make accurace quantification difficult. As a consequence of the experiences carried out, we arrived at the conclusion that the most adequate percentage of organic modifier was that of 15%.

0.1% of triethylamine was added to mobile phase in order to reduce band tailing, a consequence of the tendency of thiamine to interact with residual silanol groups.

This mobile phase was vacuum-filtered through a 0.45 μm nylon filter and degassed with helium before being used. It was pumped at a flow rate of 1 mL/min. Figure 1 shows the chromatogram obtained with a standard solution of 2.5 μg/mL of thiamine.

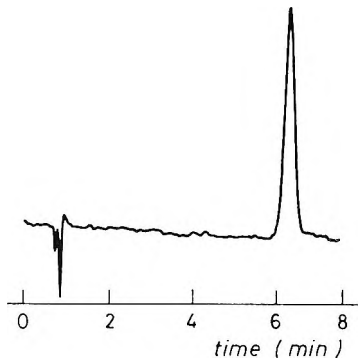

Figure 1. Chromatogram obtained from a standard solution of thiamine by using a Lichrospher 100 RP-18 (125x4 mm i.d., 5 μm). Mobile phase: H_3PO_4/KH_2PO_4 10^{-2}M, pH 2.8; hexanesulphonic acid 5 mM; methanol (15%); triethylamine (0.1%). Flow rate 1 mL/min. Thiamine concentration 2.5 μg/mL.

Table 1

Recoveries of Thiamine Obtained Using $BaCl_2$ as Eluent at Different Concentrations

Barium chloride (mol/L)	Recovery (%)
0.025	66.4
0.050	70.9
0.10	97.2
1.2	88.3

Table 2

Recovery of Thiamine Depending on Type and Concentration of Enzyme

Enzyme	Amount Added (mg/mL)	Recovery (%)
Clarase	3	79.0
	6	74.7
Takadiastase	3	90.0
	6	95.1

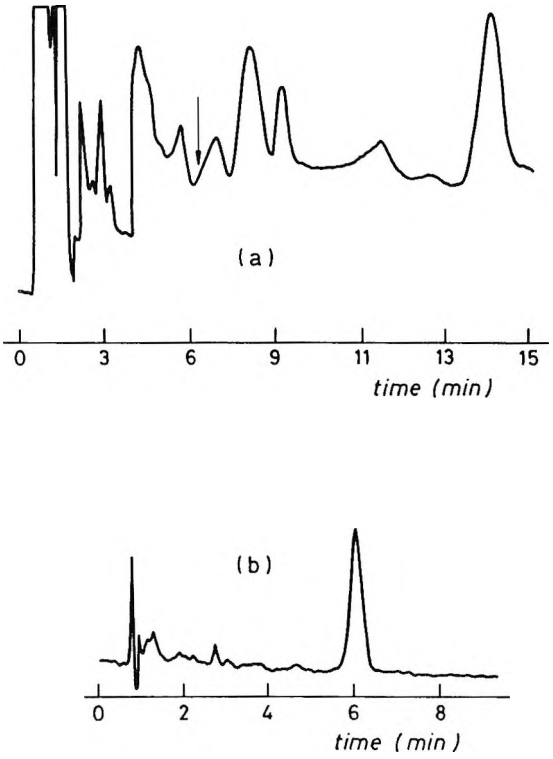

Figure 2. Chromatograms of enzymatic extract of baby meal: a) Sample directly injected; b) Sample subjected to a solid-liquid extraction step with a weak cation exchanger (CBA) and 10^{-1}M $BaCl_2$ as eluent. Chromatographic conditions as Figure 1.

Table 3

Recovery of Studies of Thiamine in Baby Meals

Amount In Baby Meal (μg/mL(	Amount Added (μg/mL)	Amount Found (μg/mL)	Recovery (%) ± RSD
	0.50	4.34	92.1 ± 5.1
3.88	1.00	4.84	96.0 ± 2.5
	1.50	5.31	95.3 ± 2.8

The quantification of thiamine was achieved by using the external standard method. The calibration curve constructed from the peak area versus thiamine concentration was linear (r=0.9995) from the quantification limit to at least 7 μg/mL of thiamine. Recalibration was performed regulary.

For the recovery test, known amounts of thiamine were added to the samples before the hydrolysis steps and resulting spiked samples were subjected to the entire analytical sequence. The thiamine was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate.

RESULTS AND DISCUSSION

Thiamine determination in food products requires a thiamine extraction step. This vitamin occurs in foods in three forms: thiamine mono-, di- and triphosphate. Extraction consists of an acidic hydrolysis step, so as to break the bonds with proteins, followed by enzymatic hydrolysis to convert thiamine phosphates to free thiamine.

As can be seen in Figure 2, HPLC food analysis without a previous purification step is not advisable. This figure shows a chromatogram corresponding to a sample of baby meal directly injected after enzyme digestion (Fig. 2a) and another corresponding to the same sample after being subjected to a solid-liquid extraction step (Fig. 2b). The great quantity of interfering substances present in the sample, as well as the lower concentration of thiamine, did not allow direct injection of the sample into the chromatographic system.

For the pre-treatment of the samples, in order to isolate and preconcentrate the thiamine, we tested a variety of sorbents and elution solvents, some of which had been assayed previously in our laboratory.[15] In practice, the hydrophobic sorbents such as C_{18} or C_8 bonded silica were not appropiate for thiamine isolation because the vitamin co-eluted with other interfering substances. The strong ion-exchanger with the sulphonic acid functional group (SCX), effectively retain the vitamin but give lower recoveries in the elution process. The best results were obtained with the weak cation exchanger CBA using $BaCl_2$ as eluent.

In order to optimize the $BaCl_2$ concentration, we tested the concentrations over a range of 0.025 to 1.2 M. The results, shown in Table 1, indicate that the best recoveries are obtained with a 10^{-1} M $BaCl_2$ concentration.

The thiamine extraction method was optimised using several samples of baby meal. The recoveries obtained to modify the acid concentration and the type and concentration of the enzyme were studied.

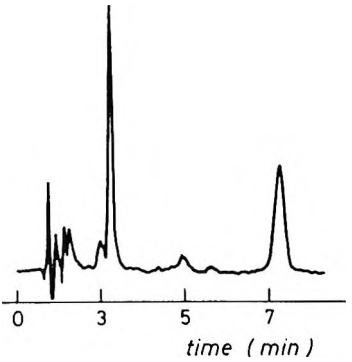

Figure 3. Chromatograms of the thiamine extracted from cereals. Chromatographic conditions as Figure 1. For extraction and preconcentration conditions see text.

HCl concentrations in the range of 0.1 to 1 M were tested to carry out the acid hydrolysis. The best results were obtained with a 0.1 M HCl solution, as found by other authors[4,16]

Different concentrations of clarase and takadiastase were tested in order to carry out the enzyme digestion. The recoveries obtained are given in Table 2. As can be seen, the best results were obtained employing 6 mg/mL of takadiastase.

In order to determine the accuracy of the method, recovery experiments were performed and the results obtained for baby meals are given in Table 3.

The precision of the method was investigated using different samples. The relative standard deviation (n=10) was always less than 3%. The detection limit, based on a signal-to-noise of 3:1, is 0.408 μg/g.

Several commercial samples (baby meals, cereals and dietetic cookies) were analyzed with this method. Figure 3 shows the typical chromatogram of the thiamine extracted from a cereal sample and the thiamine concentrations determined in these samples are given in Table 4.

CONCLUSIONS

The method described here was applied to the determination of thiamine in three different foods: baby meals, cereals and dietetic cookies. This method includes a clean-up procedure that allows the removal of the major interfering substances present in the samples and thiamine preconcentration which, makes the

Table 4

Thiamine Contents in Baby Meal, Cereals and Dietetic Cookies

Sample	Thiamine (μg/g)
Baby Meal	10.3
Cereals	2.3
Dietetic cookies	8.5

method sufficiently sensitive for these food samples, as indicated by the detection limit achieved. Therefore, this method may be applied to the analysis of thiamine in unfortified foods using an ultraviolet detector.

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ANALYSIS OF POLYMERASE CHAIN REACTION-AMPLIFIED DNA FRAGMENTS OF CLOSTRIDIUM BOTULINUM TYPE E NEUROTOXIN GENE BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

Detection of *Clostridium botulinum* neurotoxin-producing strains is primarily accomplished using the mouse bioassay. The polymerase chain reaction (PCR) method has proven to be a rapid, sensitive technique for amplifying target DNA sequences of pathogenic microorganisms. Four PCR-amplified gene fragments derived from the *Clostridium botulinum* Type E neurotoxin gene, ranging from 410-630 bp, were analyzed by capillary gel electrophoresis (CGE). Sample preparation of PCR fragments required membrane dialysis to remove salt ions. PCR fragments were analyzed by CGE using both linear and covalently cross-linked polymers. Conditions for low-viscosity entangled polymer solutions were optimized to achieve the desired separation efficiency. Assessment of both types of polymer systems included resolution, reproducibility, and sizing accuracy of the PCR fragments. Advantages and limitations of each polymer system are discussed. Electropherograms were compared to results obtained from the agarose slab gel method. CGE afforded more rapid analytical times, automation, higher resolution, and increased DNA sizing accuracy in comparison to the agarose slab gels.

INTRODUCTION

Analysis of the neurotoxin-producing strain, *Clostridium botulinum* Type E, in fish, is usually accomplished by the mouse bioassay.[1] Alternative tests include the enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and reverse phase haemagglutination. Recently, the polymerase chain reaction (PCR) has gained significant importance in detecting pathogenic microorganisms including *Vibrio cholerae,*[2] *Listeria monocytogenes,*[3] *Salmonella,*[4] *and enterotoxigenic Escherichia coli.*[5] Traditionallly, the amplification products are resolved and detected on an agarose or polyacrylamide gel. Although gel electrophoresis has become the workhorse in molecular biology, it has its disadvantages. The slab gel method is time consuming, labor intensive, and requires a hazardous chemical, ethidium bromide, for DNA detection. Capillary gel electrophoresis (CGE) is an alternative approach to the slab gel method, offering automation, higher speed of separation, increased sizing accuracy, and enhanced resolution and sensitivity.[6,7] Size separation of PCR products is obtained by electrophoresis of the double-stranded DNA product through a suitable polymer which serves as a molecular sieve. Several types of polymers can be used, of which the mechanism of separation is identical. These include covalently cross-linked (bis-acrylamide. polyacrylamide), hydrogen bonded (agarose), and linear (hydroxyethyl methylcellulose) polymers. The use of polyacrylamide[8] and hydroxyalkyl cellulose polymers[9,10] for separation of DNA fragments have been previously reported.

This paper reports the analysis of four *C. botulinum* Type E neurotoxin-gene PCR products ranging from 410-630 base pairs by CGE using both polyacrylamide and low viscosity entangled polymer solutions. Resolution, sizing accuracy, and sensitivity were determined with respect to each type of polymer system.

MATERIALS AND METHODS

Instrumentation

A commercially available Capillary Electrophoresis Unit (270A-HT, Applied Biosystems, Inc., Foster City, CA) was used. Data acquisition was controlled by a Macintosh IIci (Apple, Cupertino, CA). Polyacrylamide gel columns (3%T, 3%C), 75μm I.D. x 75cm, 50cm effective length, were purchased from J&W Scientific, Folsom, CA. Capillaries used with low-viscosity entagled polymer solutions, 75μm I.D. x 60cm, (40cm effective length) were purchased from Applied Biosystems, Inc.

Analyses were carried out using Model 600 software (Applied Biosystems, Inc). Data integration was carried out using a Laser Jet III (Hewlett-Packard, Boise, ID).

Reagents and Materials

Buffer used in conjunction with polyarylamide gels (100mM Tris-borate, 7M urea, pH 8.3), was purchased from Applied Biosystems, Inc. DNA Fragment Analysis Reagent and Buffer (low viscosity entangled polymer) was also purchased from Applied Biosystems, Inc. DNA molecular weight markers (pUC18 HaeIII digest, ϕX174 HaeIII digest) were purchased from Sigma.

DNA Sample Preparation

PCR templates were prepared by boiling *C. botulinum* Type E cultures for 10 minutes followed by DNA purification using Instagene DNA Purification Matrix (Biorad Laboratories, Inc.). PCR conditions were as described previously.[13] Primers used in the PCR were derived from the nucleic acid sequence data of *Clostridium botulinum* Type E.[11,12] PCR products analyzed by capillary gel elctrophoresis required membrane dialysis using a 0.025μm filter (Millipore) to remove salt ions. Removal of spuriuos PCR products including primer-dimers, single-stranded primers, and dNTPs was accomplished using a PCR Prep DNA Purification System (Promega). PCR fragments were quantified using a DU 650 UV/VIS spectrophotometer (Beckman).

Capillary Gel Electrophoresis Conditions

Polyacrylamide gel system

Applied voltage: -15kV
Temperature: 25°C
Electrokinetic Injection: -5kV, 10 sec
UV Detection: 260nm

Low-viscosity entangled polymer system

Applied Voltage: -13kV
Temperature: 30°C
Electrokinetic Injection: -5kV, 10 sec
UV Detection: 260nm

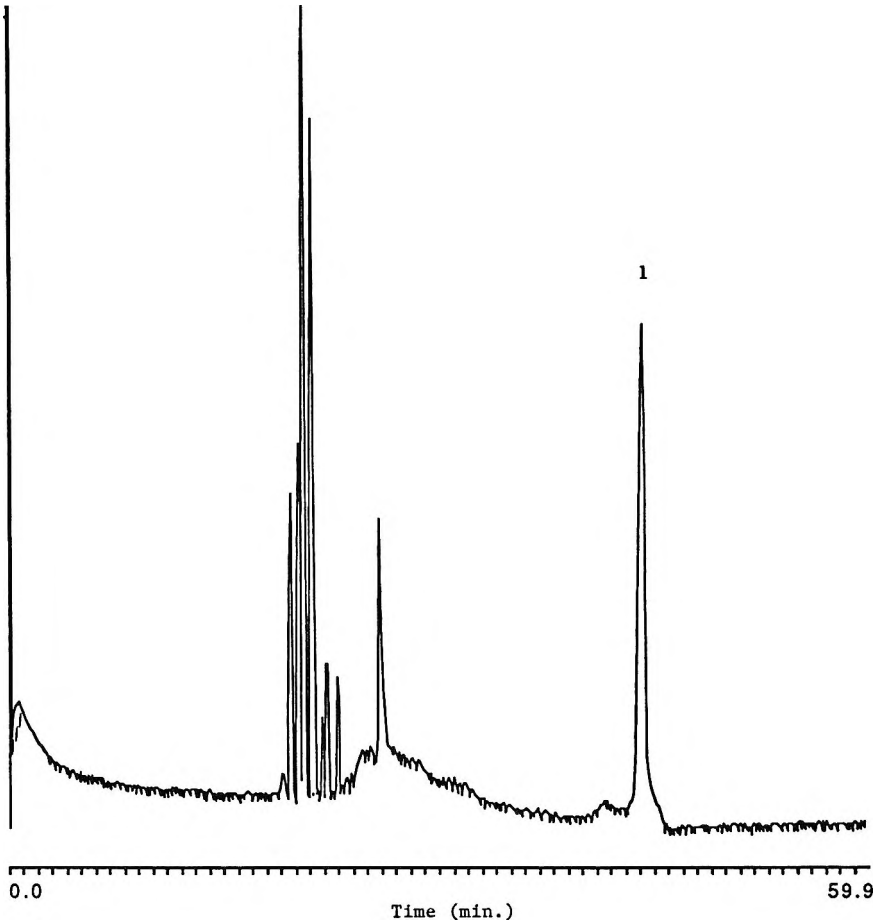

Figure 1. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp (1), using the polyacrylamide polymer system. CGE conditons: 100mM Tris-borate, 7M urea, pH 8.3, electrokinetic injection, -5kV/10sec., -15kV, 25^{0}C, and UV detection at 260nm.

DNA Fragment Size Determination

Migration times of four pUC18 HaeIII digest fragments (102, 298, 434, 587 bp) were used to produce a size calibration curve. Migration times of *C. botulinum* Type E PCR amplified DNA fragments were used to calculate the size of each fragment resolved by either polymer system. DNA fragment size accuracy using low-viscosity entangled polymer solutions was also determined

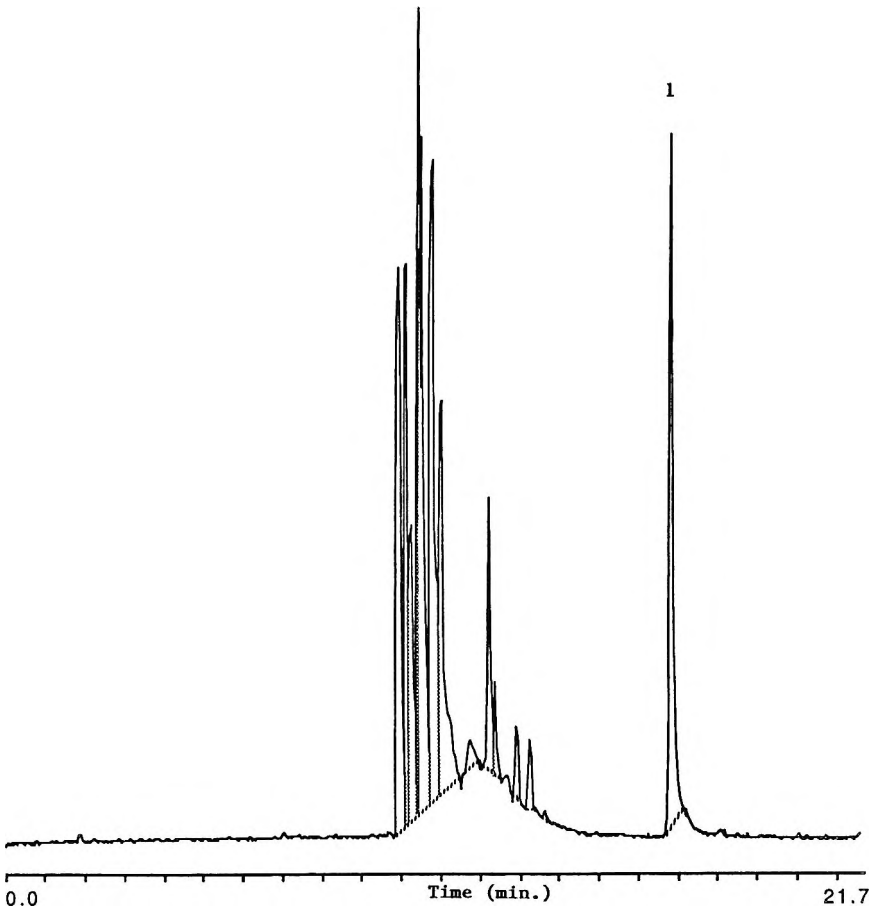

Figure 2. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp (1). using the low-viscosity entangled polymer system. CGE conditions: DNA Fragment Analysis Reagent and Buffer, electrokinetic injection, -5kV/10 sec., -13kV, 30^{0}C, and UV detection at 260nm.

by the following equation as per manufacturer's instructions (Applied Biosystems , Inc.):

slope = (Sz DNA2-Sz DNA 1)/(DNA 2 - DNA 1)

where DNA 1 and DNA 2 are migration times of two DNA standard (STD) peaks and Sz DNA 1 and Sz DNA 2 are the sizes of two DNA STD peaks in base pairs.

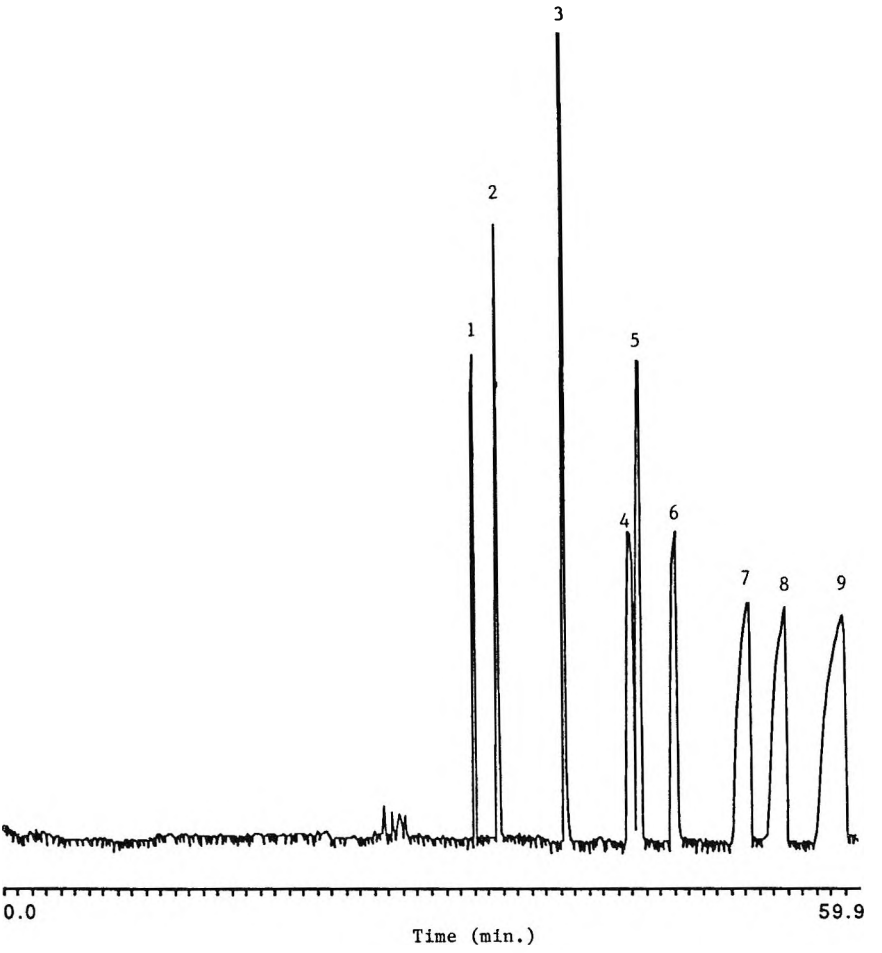

Figure 3. Separation of plasmid pUC18 HaeIII restriction digest using the polyacrylamide polymer system. The fragments identified are: (1) 80, (2) 102, (3) 174, (4) 257, (5) 267, (6) 298, (7) 434, (8) 458, and (9) 587 base pairs. CGE conditions: see Figure 1.

DNA Unknown Size = X +(DNA Unknown - DNA 1)*Slope

where DNA 1 and DNA Unknown are migration times of the smaller DNA STD peak used in the slope calculation and of unknown DNA, and X is the size of the DNA 1 STD peak in base pairs.

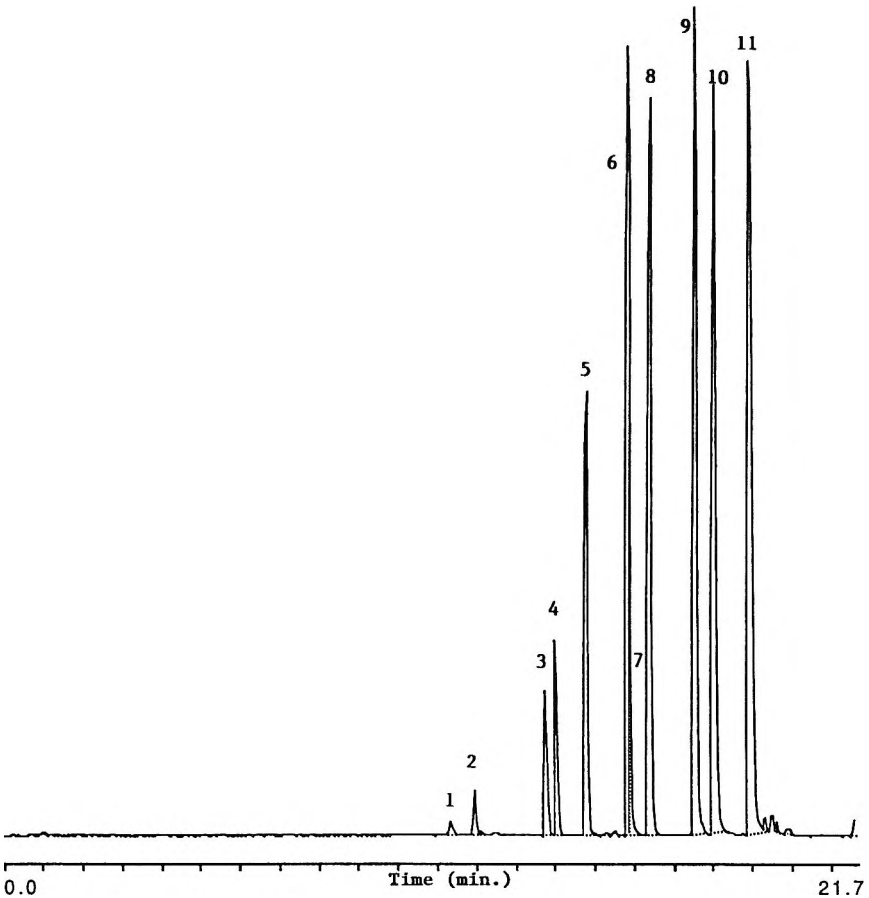

Figure 4. Separation of plasmid pUC18 HaeIII restriction digest using the low viscosity entangled polymer system. The fragments identified are: (1) 11, (2) 18, (3) 80, (4) 102, (5) 174, (6) 257, (7) 267, (8) 298, (9) 434, (10) 458, and (11) 587 base pairs. CGE conditions: see Figure 2.

Gel Electrophoresis

A 20μl aliquot of each PCR product was resolved on a 1.8% agarose slab gel in 1X TBE (Tris-borate, EDTA), electrophoresed at 1V/cm, and visualized by UV-induced fluorescence after staining with 1μg/mL ethidium bromide.

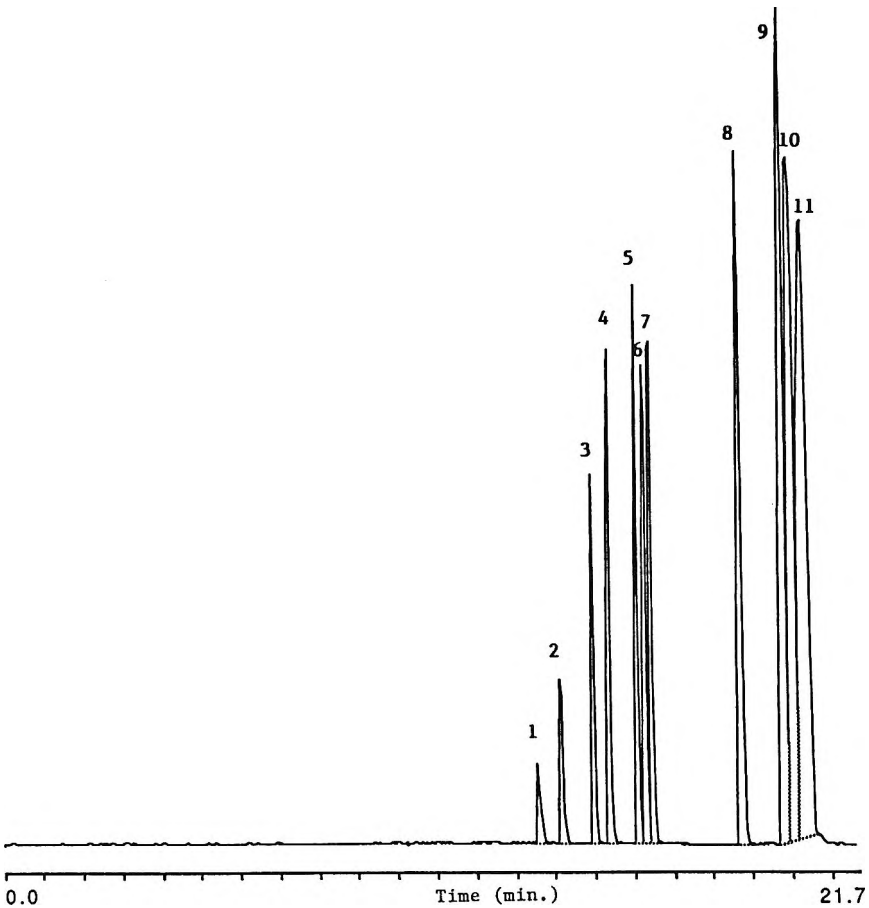

Figure 5. Separation of fX174 HaeIII restriction digest using the low viscosity entangled polymer system. The fragments detected are:(1) 72, (2) 118, (3) 194, (4) 234, (5) 271, (6) 281, (7) 310, (8) 603, (9) 872, (10) 1078, and (11) 1353 base pairs. CGE conditons: see Figure 2.

RESULTS AND DISCUSSION

Analysis of the PCR products by CGE required membrane dialysis to remove salt ions incurred in the PCR preparations. Salt ions compete with the PCR products resulting in less DNA electrophoresed onto either the polyacrylamide or coated capillaries. Specifically, membrane dialysis requires floating a Millipore 0.025μm filter on top of deionized water and pipeting

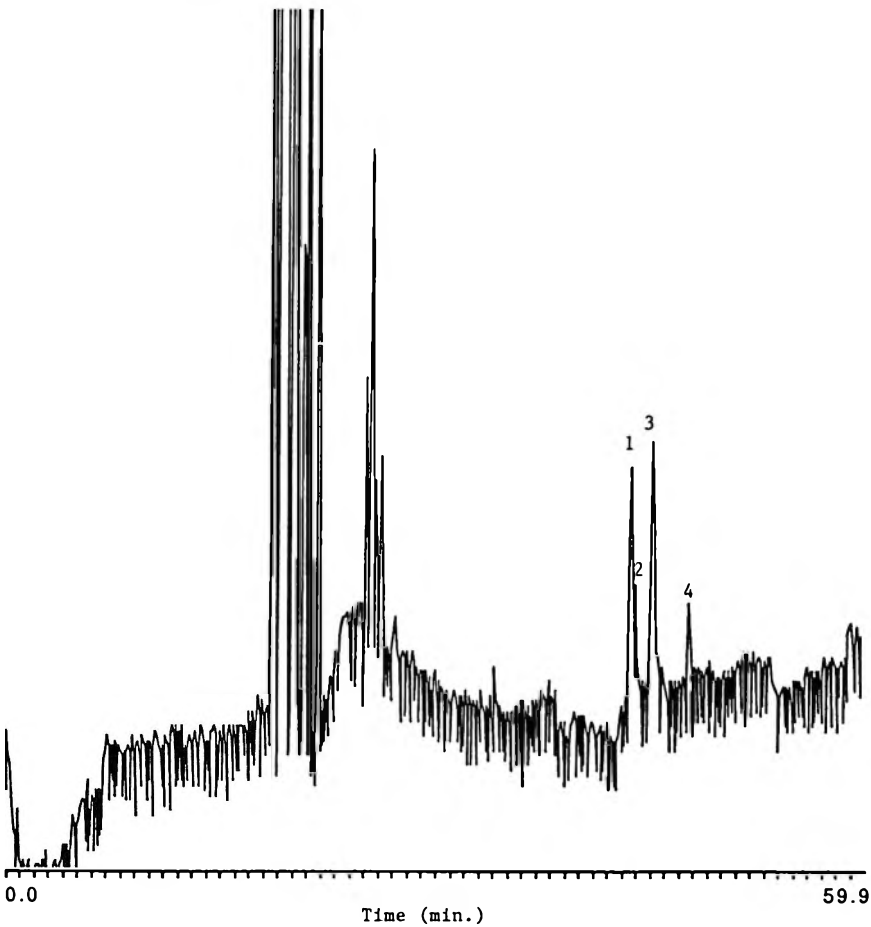

Figure 6. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using the polyacrylamide polymer system. The amplified DNA fragments detected are: (1) 410, (2) 471, (3) 513, and (4) 630 base pairs. CGE conditions: see Figure 1.

approximately 25μL of the PCR preparation onto the hydrophobic side of the filter. Removal of salt ions significantly increases the sensitivity of CGE (data not shown).

Figure 1 and 2 represent typical electropherograms demonstrating the separation of PCR products using both polymer systems. Identification of the 410 bp fragment using the low-viscosity polymer solution required approximately half the analytical time (30 minutes) needed for the

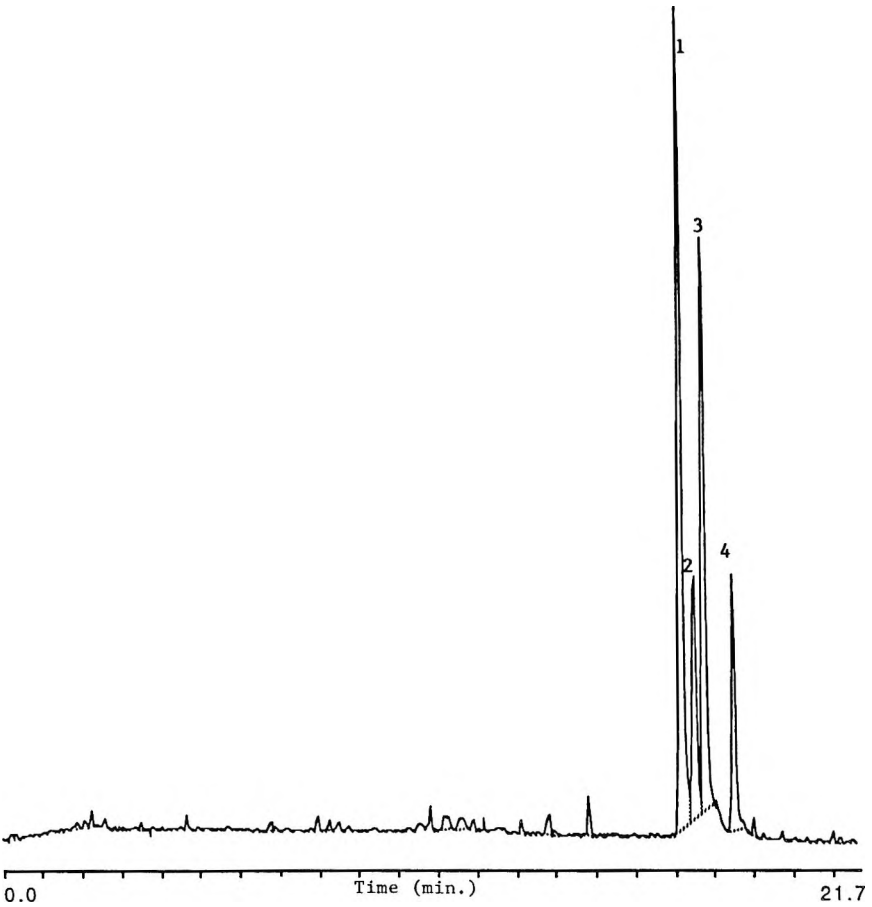

Figure 7. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using the low-viscosity entangled polymer system. The amplified DNA fragments are: (1) 410, (2) 471, (3) 513, and (4) 630 base pairs. CGE conditions: see Figure 2.

polyacrylamide polymer system. DNA molecular weight markers, pUC18 HaeIII and ϕX174 HaeIII restriction digests, were used to determine DNA size range capabilities with respect to both polymer systems.

DNA fragments in the size range of 434 - 587 base pairs appear to be the upper limit for 3% T, 3% C polyacrylamide gel caplillaries (Fig. 3). The low-viscosity entangled polymer system produced sharp peaks in the same DNA size range, and could even separate DNA fragments as large as 1000 base pairs (Fig. 4, 5).

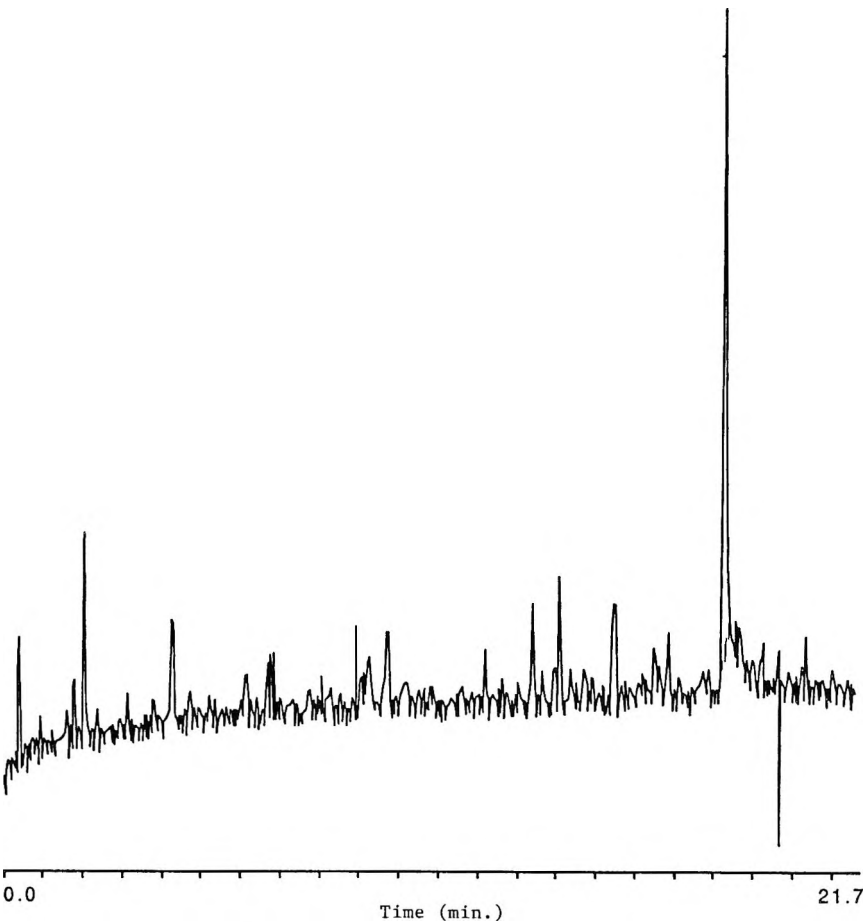

Figure 8. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragment, 410 bp, 35ng/mL, using the low-viscosity entangled polymer system. Primers and primer-dimers were eliminated using the PCR Prep DNA Purification System. CGE conditions: see Figure 2.

Resolution of four PCR products ranging from 410 - 630 base pairs by the polyacylamide and the low-viscosity entangled polymers is demonstrated in Fig. 6 and 7, respectively. The low-viscosity entangled polymer system resolved the 410 and 471 bp fragments, whereas the polyacylamide polymer could not. Base-line separation was not achieved in the polyacrylamide polymer system. The sensitivity of CGE using the low-viscosity entangled polymer solution approached picogram levels (20μL of a 35ng/mL 410 bp PCR product analyzed) (Fig. 8).

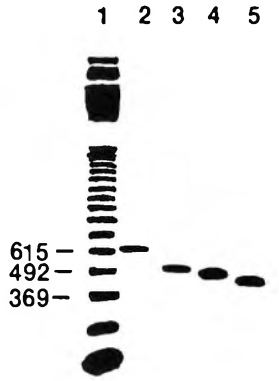

Figure 9. Separation of PCR amplified *C. botulinum* Type E neurotoxin gene fragments using 1.8% agarose slab gel. Lane (1) 123 bp DNA ladder, (2) 630, (3) 513, (4) 471, (5) 410 bp. Electrophoretic conditions: see text.

The polyacrylamide polymer system also provided sensitivities in the picogram range (data not shown). Sensitivity may further be enhanced by increasing the time of electrokinetic injection or voltage using both polymer systems. However, it appears that longer electrokinetic injection times can be achieved with the low-viscosity entangled polymer systems. DNA sizing accuracy was also determined using both polymer systems. For a 513 base pair PCR product, the percent error in accurately sizing the DNA fragment in the polyacrylamide and low-viscosity entagled polymers was 1.6 (505 bp) and 4.3 (535 bp) percent, respectively. However, if the DNA sizing equation was used in lieu of the linearity, the sizing accuracy was increased (1.8 percent, 522 bp).

Determining the DNA size of PCR products resolved on a agarose slab gel required a visual comparison of band migration to respective molecular weight markers (Fig. 9). Gel preparation, loading samples, and electrophoresis, required approximately three hours to complete.

PCR product analysis was less cumbersome using the low-viscosity entangled polymer system. The polymer solution can be flushed out of the capillary after every run, ensuring no DNA frament carryover into the next analysis. This is in contrast to the polyacrylamide gel capillaries which cannot be flushed and require each analytical run to go to completion.

CONCLUSION

Analysis of PCR products by CGE using polyacrylamide or low-viscosity entangled polymers provides an alternative molecular detection method to

agarose slab gels. CGE affords rapid analytical times, reproducibility, and greater DNA sizing accuracy and sensitivity in comparison to the slab gel method. It appears that low-viscosity entangled polymers offer greater flexibility in the analysis of PCR products in comparison to polyacylamide; this includes faster analytical times, greater range in DNA size separation, and convenience. CGE, used in conjunction with the PCR technique, may prove to be a novel method for identifying pathogenic bacteria including *C. botulinum* neurotoxin-producing strains.

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DETERMINATION OF CYCLOSPORIN-A IN HUMAN WHOLE BLOOD BY REVERSED PHASE LIQUID CHROMATOGRAPHY WITH SINGLE-STEP EXTRACTION

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ABSTRACT

A simple, rapid and accurate reversed-phase liquid chromatographic method, utilizing a single-step extraction procedure, was developed for the determination of cyclosporin-A (CyA) in human whole blood. In this study, the drug was extracted from whole blood with diethyl ether and separated isocratically within 7 min using an octyl-bonded silica column and a mobile phase of acetonitrile, methanol, deionized water and isopropanol (57:18:25:1.5, v/v). The column temperature was maintained at 65 °C. Cyclosporin-A was quantified by absorbance at 208 nm, with cyclosporin-D as internal standard. A linear relationship between the ratio of peak area and the concentration of CyA from 40 ng/mL to 1200 ng/mL was obtained. The lower limit of detection of CyA was 20 ng/mL. Intra-day and inter-day coefficients of variation of assay for CyA at the 200 ng/ml level were 4.8% (n=7) and 7.8% (n=6), respectively.

The recoveries of CyA were 98.6% - 99.5% for whole blood. The method has been used to determine CyA in whole blood samples from ten volunteers and provided data on the pharmacokinetics of the drug.

INTRODUCTION

Cyclosporin-A (CyA) is a selective immunosuppressive agent. It has been found to be extremely effective in prolonging the survival of patients who received kidney, pancreas, lung, liver, or bone marrow transplants.[1,2]

Although CyA has remarkably improved the survival rate of organ transplant patients, it causes various adverse effects on the kidneys and liver. For most patients, these side effects have been found to be dose-dependent, and the therapeutic index of CyA is very narrow. Therefore, the determination of CyA concentrations in a patient's whole blood is necessary to optimize the dosage of CyA for optimum therapeutic action with minimum toxicity.

Consequently, a further point of interest was determination of CyA concentrations in whole blood. It would be necessary for study of the pharmacokinetics and bioavailability of CyA in humans and for improvement of formulation of the drug.

Since the affinity of cyclosporin with erythrocytes is highly temperature dependent, the CyA concentration in whole blood, serum, or plasma may vary due to changes in the temperature, storage, and processing conditions.[3,4] The CyA concentration must be determined in whole blood instead of in serum or plasma to obtain meaningful data.

Two procedures have evolved, to allow the estimation of CyA concentration: monoclonal radioimmunoassay (RIA)[5,6] and high performance liquid chromatography (HPLC).[7-12] Although RIA is more sensitive than the UV detection in HPLC methods, the reliability of the assay by RIA is very poor because of the cross-reaction of the metabolites of the parent drug.[13,14]

Therefore, HPLC has been extensively used to determine CyA in biological samples. Nevertheless, it is difficult to process the samples because of the complexity of constituents in whole blood.

Improved chromatography of CyA has been introduced, but some HPLC methods, based on a classical liquid-liquid extraction, or application of expensive solid-phase cartridges, still retain the major disadvantages, such as

laboriousness, multistep and time-consuming for sample preparation. These hardly meet the needs of simplicity and rapidity for clinical drug monitoring and pharmacokinic studies.

This report describes a simple, rapid and sensitive HPLC procedure for the determination of CyA in whole blood by using a rapid, single-step, diethyl ether extraction and a reversed-phase isocratic separation on a C_8 analytical column. It has been used to determine CyA in whole blood samples from ten volunteers who had taken CyA capsules and provided data on the pharmacokinetics of the drug.

EXPERIMENTAL

Apparatus

The HPLC system used was an HP 109-IM liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a Model K501 high pressure sample injector (20-μL loop; Shanghai Scientific Instruments Factory, Shanghai, China), thermostated column compartment and a diode array detector (Hewlett-Packard, Waldbronn, Germany) operated at 208 nm. Chromatographic separations were carried out on a Spherisorb octylsilane (C_8) column (200 x 4.6 mm I.D.; particle size 7 μm; Dalian, China) operated at 65 °C. Control of the instrument, data storage, evaluation, integration and reporting were performed with an HP Series 300 computer (Hewlett-Packard, Boeblingen, Germany).

Standards and Reagents

Cyclosporin-A and cyclosporin-D were gifts from Fujian Institute of Microbiology. HPLC-grade acetonitrile and methanol (Linhai Chemicals Factory, Zhejiang, P. R. China) were used to prepare the mobile phase. All chemicals, except where otherwise stated, were of analytical grade, and water used in this assay was doubly distilled. Cyclosporin-A capsules were provided by Sandoz Pharmaceutical Ltd. (Basel, Switzerland).

Mobile Phase

The mobile phase consisted of acetonitrile, methanol, deionized water and isopropanol (57:18:25:1.5, v/v). This solution was passed through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) and was then degassed before use. The flow-rate of mobile phase was 1.40 mL/min with typical back pressure of 60 bar.

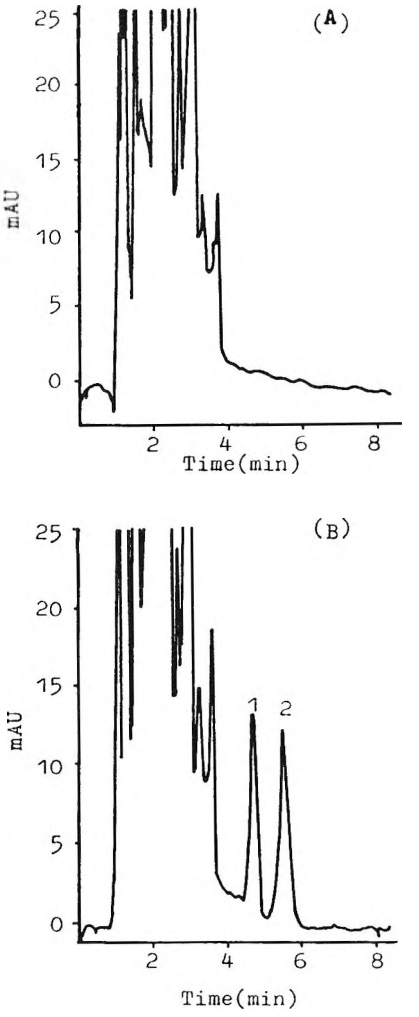

Figure 1. Chromatograms of cycosporin A in human whole blood. A. Blank whole blood; B. A whole blood sample collected 4h after oral administration of 200 mg of cyclosporin A. 1: Cyclosporin A; 2: Cyclosporin D (internal standard).

Preparation of Solutions

Cyclosporin-A and cyclosporin-D stock standard solutions (1.00 mg/mL) were prepared by dissolving 100 mg of cyclosporin-A and cyclosporin-D, respectively, in 100 mL of methanol and kept in a refrigerator.

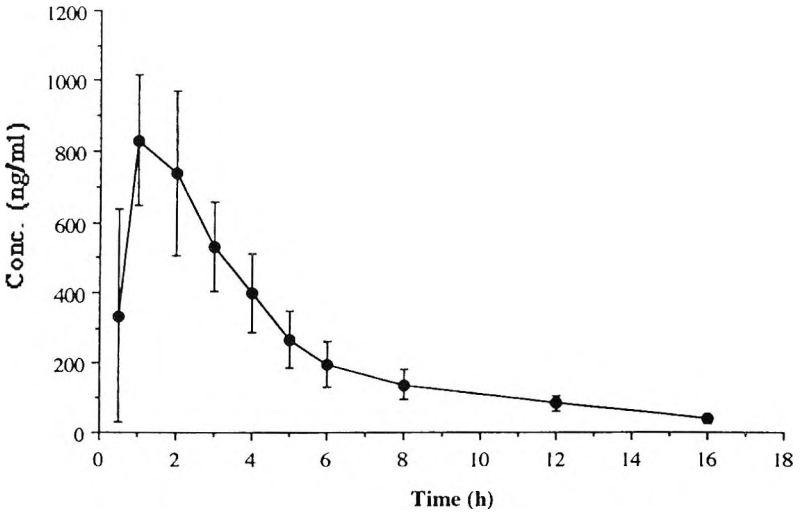

Figure 2. Mean whole blood concentration - time curve after oral administration of 200 mg of cyclosporin A to 10 healthy volunteers. (n=10, mean ± s).
•: Observed; —: Calculated; Concentration unit: ng/mL.

Table 1

Within-Day and Between-Day Precision of the Method

Concentration Added (ng/mL)	RSD (%) Within-Day (n = 7)	Between-Day (n = 6)
200	4.8	7.8
500	2.0	5.0
800	2.9	3.4

Analytical Procedure

Two milliliters of whole blood was pipetted into a chemically clean screw capped glass tube and 16 μL of internal standard (CyD) solution (100 μg/mL) and 100 mg sodium fluoride were added. The mixture was homogenized on a vortex mixer for 15 sec, and 5 mL of diethyl ether was added and mixed for 2 min. Then, 4 mL of the organic layer was collected and evaporated to dryness with air at 50 °C.

The residue was reconstituted with 150 μL of the HPLC mobile phase and 400 μl of hexane. After vortex mixing, 20 μL of the sample solution were injected into the HPLC system.

RESULTS

Chromatographic Separation

Figure 1 shows typical chromatograms of whole blood samples. Under the chromatographic conditions described, CyA and CyD had retention times of approximately 4.6 min and 5.5 min, respectively. It can be seen, from Figure 1, that good separation and detectability of CyA in whole blood were obtained with minimal interference from whole blood components. Hence, it is relatively easy to estimate the peak area with accuracy.

Precision

The data for studies of within-day reproducibility, evaluated by assaying 7 whole blood samples containing different concentrations of CyA, and between-day reproducibility, evaluated by assaying the same concentration, 7 times over a 6-day period, are summarized in Table 1. The range of percentage of relative standard deviation (%RSD) was from 2.0% to 4.8% for within-day analyses and from 3.4% to 7.8% for between-day analyses, respectively.

Linearity and Detection Limit of the Method

A series of whole blood samples containing 40, 100, 200, 400, 600, 800, 1000 and 1200 ng/mL of CyA was prepared to study the relationship between the ratio of peak area of CyA to CyD and the concentrations of CyA under selected conditions. The results showed that the peak area ratio was linearly related to the CyA concentration for the range of 40 - 1200 ng/mL. The linear equation for the concentration versus the ratio of peak area was

$$Y=1.10 \times 10^{-3}X - 7.65 \times 10^{-3}$$

with a correlation coefficient of 0.9994. The detection limit was 20 ng/mL.

Table 2

Recovery of Cyclosporin A from Spiked Whole Blood (n = 9, mean ± s)

Concentration Added (ng/mL)	Concentration Measured (ng/mL)	Recovery (%)	RSD (%)
200	199.0 ± 16.8	99.5 ± 8.4	8.4
500	493.1 ± 24.8	98.6 ± 5.0	5.1
800	794.0 ± 36.6	99.3 ± 4.6	4.6

Extraction Efficiency

Extraction efficiencies of CyA and the internal standard were determined by comparing peak areas of the analyses from extracted standards from whole blood to those from a chromatographic standard solution prepared in mobile phase at the equivalent concentration and chromatographed directly. The data obtained for different concentrations of CyA are summarized in Table 2. The extraction efficiency of the internal standard (800 ng/mL) from whole blood was 97.7 ± 5.7% (mean ± S.D.; n=10).

Application

Ten healthy male Chinese volunteers, aged 22.1 ± 1.0 and weighing 63.3 ± 3.6 kg, entered the study. All volunteers gave their written consent and underwent physical examinations. There were no abnormal findings in liver and kidney functions, specifically. After 12 h of overnight fasting, the volunteers received an oral dose of single 200 mg CyA. Blood samples (2.0 mL) were taken before medication and after 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0 and 16.0 hours, and were then kept at 4 °C. Figure 2 illustrates the profile of whole blood concentration versus time for CyA in the 10 volunteers.

DISCUSSION

Investigations of analytical columns that were packed with different stationary phases were conducted to determine which one would give optimum analytical conditions for CyA. The results showed that all of the cyano-, octyl-, and octadecyl-bonded silica can be used as the stationary phase for analysis of CyA. Among them, cyanosilane required the lowest temperature (about 45 °C) as the analytical column was packed with it. Nevertheless, CyA could not be separated from CyD because the retentions were very much alike. When the

Table 3

Pharmacokinetic Parameters of Cyclosporin A after Administering a Single 200 mg Oral Dose to Ten Healthy Chinese Volunteers

Patient	$T_{a1/2}$ (h)	$T_{\alpha 1/2}$ (h)	$T_{\beta 1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0\rightarrow\infty}$ (ng•h/mL)
1	0.80	0.55	4.44	1.62	571.4	2828.6
2	0.92	0.35	4.82	1.26	825.5	3102.3
3	0.96	0.12	4.73	0.90	833.3	4966.0
4	0.73	0.62	5.19	1.44	993.2	4682.0
5	0.96	0.64	5.45	1.62	1118.5	4804.1
6	0.88	0.17	4.89	0.72	1103.9	3945.8
7	1.87	0.07	2.91	1.08	806.2	4265.2
8	1.26	0.11	6.80	0.90	733.0	3232.5
9	0.69	0.55	6.16	1.44	726.4	3416.8
10	0.69	0.48	4.43	1.44	1088.0	4694.4
Mean	0.98	0.37	4.98	1.24	879.9	3993.8
± SD	0.35	0.23	1.05	0.33	186.8	796.3

analytical column was packed with octadecylsilane, the column temperature required was the highest. It was necessary to keep the temperature above 65 °C, or even higher than 72 °C, during the analysis.[10] It has been found that some problems occur with this column temperature condition, such as limited useful life of column and stability of the determination. One possible reason may be the formation of bubbles in the mobile phase. The best result for CyA was obtained with Spherisorb octylsilane (C_8) stationary phase. In this cause, the column temperature intervened cyanosilane and octadecylsilane stationary phase, and CyA and CyD can be fully baseline separated.

The effect of the composition of mobile phase on the chromatographic separation was investigated in this study. The results indicated that the optimum resolution for CyA, CyD and endogenous substances in whole blood was obtained when the mobile phase was composed of acetonitrile, methanol, water and isopropanol (57:18:25:1.5, v/v). The retention times of the CyA and CyD visibly increased with decreasing acetonitrile or increasing water content. Moreover, the responses of peak area tended to decrease, too. The retention time changed only slightly with varying methanol content.

Because of the cutoff wavelength of methanol, it is undesirable to increase methanol content. Additionally, the experimental results showed that addition

of isopropanol to the mobile phase increased the selectivity and stability of CyA since isopropanol changed the retention time of the endogenous peaks and had only a slight effect on the CyA peak.

The pharmacokinetics of CyA were studied in 10 healthy Chinese volunteers. After single oral administration of 200 mg CyA, the data obtained was fitted with a computer program: PKBP-N1.[15] Table 3 shows the pharmacokinetic parameters of ten volunteers to whom CyA was orally administered. The results suggest that the disposition of CyA was conformable to a two-compartment model. Peak concentration in whole blood occurred 1.24 h after ingestion and the mean peak concentration achieved was 879.9 ng/mL. This implied that CyA is absorbed rapidly in healthy individuals. Moreover, it was found that the standard deviations of the peak concentration and area under the curve (AUC) were large. This indicates that the individual variation is obvious after oral administration of CyA.

CONCLUSION

The method provides excellent recovery and good precision, and is simple and reliable in both chromatographic conditions and sample preparation. Furthermore, the analytical procedure is easy to handle and is very suitable for routine determination of a large number of samples because of the short time between sample injections. It has been successfully applied to the study of pharmacokinetics of CyA in whole blood samples obtained from 10 healthy volunteers during their participation in a clinical trial of CyA single oral dose.

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ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC AND HPLC SYSTEM TROUBLESHOOTING

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Details of this course may be obtained from Dr. Jack Cazes, P. O. Box 2180, Cherry Hill, NJ 08034-0162, USA. Tel: (609) 424-3505; FAX: (609) 751-8724; E-Mail: jcazes@voicenet.com.

J. LIQ. CHROM. & REL. TECHNOL., 19(13), 2191–2201 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

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

JULY 27 - 31: 37th Annual Meeting of the Aqmerican Society of Pharmacognosy, University of California, Santa Cruz, California. Contact: Dr. Roy Okuda, Chem Dept, San Jose State University, One Washington Square, San Jose, CA 95192-0101, USA. Tel: (408) 924-5000; FAX: (408) 924-4945.

AUGUST 7 - 9: 28th Canadian High Polymer Forum, Sarnia, Ontario, Canada. Contact: Kar Lok, BASF Corp, 11501 Steele Creek Rd, Charlotte, NC, 28273, USA. Tel: (704) 587-8240; FAX: (704) 587-8115.

AUGUST 8 - 10: 3rd Annual Symposium on Biomedical, Biopharmaceutical and Clinical Applications of Capillary Electrophoresis, Mayo Clinic, Rochester, Minnesota. Contact: Dr. S. Naylor, Mayo Foundation, Section of Continuing Education, Rochester, MN 55905, USA.

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

AUGUST 11 - 15: 26th ACS Northeast Regional Meeting, Western Conn State Univ, Danbury, CT. Contact: A. Alder, 11 Long Ridge Rd, Redding, CT 06896, USA; (203) 938-2920; Email: reglmtgs@acs.org.

AUGUST 11 - 16: 3rd International Hydrocolloids Conference, Sydney, Australia. Contact: Gail Hawke, P. O. Box N-399, Grosvenor Place, Sydney, NSW 2000, Australia. Tel: 61 02 241 3388; FAX: 61 02 241 5282.

AUGUST 11 - 16: ICORS '96: 15th International Conference on Raman Spectroscopy, Pittsburg, Pennsylvania. Contact: Sanford Asher, Chem Dept, University of Pittsburgh, PA 15260, USA. Tel: (412) 624-8570.

AUGUST 12 - 16: 11th Internationa Congress on Thermal Analysis & Calorimetry, Philadelphia. Contact: The Complete Conference, 1540 River Pk Dr, Sacramento, CA 95815, USA. Tel: (916) 922-7032.

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

AUGUST 18 - 23: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

AUGUST 21 - 23: 4th International Symposium on Capillary Electrophoresis, York, UK. Contact: Dr. T. Threlfall, Industrial Liaison Executive, Dept of Chem, University of York, Heslington, York, YO1 5DD, UK.

AUGUST 25 - 29: 212th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; Email: natlmtgs@acs,org.

AUGUST 25 - 30: International Symposium on Metal Hydrogen Systems: Fundamentals and Applications, Les Diablerets, Switzerland. Contact: MH-96, Inst of Physics, Univ of Fribourg, Perolles, CH-1700 Fribourg, Switzerland. Tel: 41 37 299 113; FAX: 41 37 299 772.

AUGUST 25 - 30: 12th International Congress on Chemical & Process Engineering, Praha, Czech Republic. Contact: Organizing Committe, CHISA'96, P. O. Box 857, 111 21 Praha, Czech Republic. Tel: 42 2 353287; FAX: 42 2 3116138.

SEPTEMBER 1 - 4: 4th Inetrnational Symposium on Preparative & Industrial Chromatography & Related Techniques, Basel, Switzerland. Contact: Secretariat Prep'96, Messeplatz 25, CH-4021 Basel, Switzerland. Tel: 41 61 686 28 28; FAX: 41 61 686 21 85.

SEPTEMBER 1 - 6: IUPAC Chemrawn IX, Seoul, Korea. Contact: IUPAC Chemrawn IX, Secretariat, Tongwon B/D 6th Floor, 128-27 Tangjudong, Chongro-ku, Seoul 110-071, Korea. FAX: 82 2 739-6187.

SEPTEMBER 1 - 6: 11th International Symposium on Organosilicon Chemistry, Monpellier, France. Contact: R. Corriu, University of Montpellier II, Place Eugene Batallon, cc007, 34095 Montpellier cedex 05, France. Tel: 67 14 38 01.

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

SEPTEMBER 4 - 9: International "Thermophiles '96" Symposium: Biology, Ecology & Biotechnology of Thermophilic Organisms, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

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

SEPTEMBER 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd International Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 17 - 20: 10th International Symposium on Cap|illary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. 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 17 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

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

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000; FAX: (703) 684-7349.

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090; FAX: (708) 634-4595.

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200. 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

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

NOVEMBER 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

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

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

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

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: 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; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

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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 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824.

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 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.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

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 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.ord.

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; Email: miscmtgs@acs.org.

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; FAX: (302) 738-5275.

1998

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.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw 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 21 - 25: 217th ACS National Meeting, Anaheim, Calif. 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 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 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. 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 20 - 24: 220th 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.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 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. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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; Email: natlmtgs@acs.org.

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; Email: natlmtgs@acs.org.

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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