Chromatographic Behavior of Ion Pair Enantiomers of Dansyl Leucine Cyclohexylammonium Salt on a β-Cyclodextrin Stationary Phase and the Effect of a Competitive-Binding Mobile Phase Additive

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Abstract

The separation of dansyl leucine enantiomers on a β-cyclodextrin stationary phase is significantly complicated by the association of the amino acid with its cyclohexylammonium counter ion, in a mobile phase of 80:20 (v/v) methanol–water. This produces very unusual chromatography, with two partially superimposed peaks observed for each enantiomer at lower column temperatures. The peak shape is attributed to the irreversible, oncolumn conversion of the ion pair (I) to the free, protonated (neutral) dansyl amino acid (II+H). Increasing the ionic strength of the mobile phase greatly improves the chromatography by transforming the solute species to enantiomers of II (the anionic, free amino acid). Van't Hoff plots are constructed for both species I and II (under different mobile phase conditions) to provide thermodynamic insight into the major enantioselective driving forces of separation. The chiral discrimination of the stationary phase is found to be primarily enthalpically driven for both solutes. Finally, 1-adamantanecarboxylic acid (ACA) is investigated as a solute-competitive mobile phase additive to intentionally block the hydrophobic cyclodextrin cavities on the stationary phase. By varying the concentration of ACA additive in the mobile phase, control over the retention and chiral recognition of the stationary phase is demonstrated.

Introduction

Cyclodextrins (CDs) have been widely used in liquid chromatography (LC) for many years (1–5). In the separation of chiral compounds by high-performance LC (HPLC), CDs can be used as mobile phase modifiers in conjunction with achiral stationary phases (6–8), or they may be incorporated into a chiral stationary phase (CSP) and used with an achiral mobile phase (9–14). The mobile phase additive method is attractive because it is simple and allows the use of achiral stationary phases. However, because of the limited solubility of CDs, achieving an adequate mobile phase concentration to effect a particular separation can be challenging.

The retention mechanisms of model racemate compounds (such as derivatized D, L-amino acids) on β-CD CSP columns have been studied extensively, often with the aid of various mobile phase additives. It has been found, for instance, that salting-out agents (such as sucrose) can increase the retention and resolution of dansyl amino acid enantiomers (15). Furthermore, in methanol–water mobile phase systems, increasing the methanol content generally reduces the retention and resolution of the enantiomeric pairs by weakening the strength of inclusion complexation between the hydrophobic cavity of the host and guest molecules (16). Additionally, increasing the ionic strength typically decreases column residence time, which, in turn, reduces peak resolution. Li et al. (16) have also shown that pH changes can affect the retention, but not necessarily the enantioselectivity, which suggested that interactions of polar moieties on the solute with hydroxyl groups on the CD do not play a major role in chiral recognition. Finally, the effect on enantioselectivity of varying the concentration of other additives (such as triethylammonium acetate) has been observed to be much more complicated (17). Although these examples illustrate how various interactions available on the CSP may affect a separation, observed differences in retention or chiral resolution may also reflect changes in the chemical or physical properties (or both) of the solute.

To our knowledge, enantioselectivity in amino acid ion pairs has not been investigated. Typically, mobile phase conditions (i.e., amount of organic modifier, choice of buffer additive, and pH adjustment) are selected to yield the free, deprotonated dansyl amino acids, whose chromatography is well-characterized on β-CD stationary phases (9). However, because the counter ions of some commercially available dansyl amino

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acid salts can form ion pairs with the solute under certain chromatographic conditions, these species may also warrant study. Ion pairs (such as **I**) have the ability to form inclusion complexes with CDs, yet can be readily ionized to give the free dansyl amino acid (**II**, in equilibrium with its protonated form, **II+H**) and counter ion (**III+H**, or its deprotonated form, **III**) simply by adjusting the ionic strength, pH, or polarity of the mobile phase (refer to Figure 1). This property can allow differences in the retention mechanisms responsible for enantioselectivity in racemic mixtures of **I** and **II** to be compared on the β-CD CSP.

To demonstrate control over the enantioselectivity of the β-CD CSP (using **I** as the target analyte), 1-adamantanecarboxylic acid (ACA) was investigated as a mobile phase additive. It is known that unusually large binding constants exist between β-CD cavities and "space-filling" adamantane moieties, based on the fact that the diameter of these hydrophobic groups (7 Å) is such that it enables them to fit exactly into the β-CD cavity (18). Adamantyl-modified silica has been reported as a reversed-phase LC (RPLC) packing (19). In addition, the interaction of an adamantane end-capped polymer with a β-CD polymer has been investigated (20). To our knowledge, adamantane or functionalized adamantane species have not been previously utilized as mobile phase additives in β-CD CSP systems. In this work, ACA was used for its large binding affinity and high solubility in the mobile phase to effectively "block" the hydrophobic cavities of the CSP surface from access by solute molecules. Varying the concentration of this additive in the mobile phase could prove useful in tailoring the retention and chiral selectivity of the β-CD CSP toward enantiomers of various chiral compounds.

Experimental

Chromatographic methods

All experiments were performed on an HP-1100 liquid chromatographic system (Hewlett-Packard, Waldbronn, Germany) equipped with a quaternary pump, autosampler with variable volume injection capability, temperature-controlled column compartment, and UV detector (set to 254 nm). A circular dichroism detector (CD-1595, Jasco, Easton, MD), placed in parallel with the UV detector, was used to verify the enantiomeric composition of eluting peaks (analytical wavelength of 254 nm). Chromatograms were acquired using Turbochrom software (PerkinElmer, San Jose, CA), at a rate of 1 data point/s. The same software was used to obtain *N* [the number of theoretical plates calculated using the Foley-Dorsey approximation (21)] for each enantiomer peak in each separation. A new β-OH (a β-CD-containing CSP) column (Nucleodex, 200- × 4-mm, 5-µm particle size, Phenomenex, Torrance, CA) was used for all experiments, with a mobile phase of 80:20 (v/v) methanol–water (isocratic) and a flow rate of 0.90 mL/min. The column was flushed with 50:50 (v/v) methanol–water for 36 h prior to initiating the experimental work.

The dead time in the chromatograms was estimated using the refractive index change in the baseline caused by injection. Retention factors for the enantiomers of **I** and **II** were determined at column temperatures in the range 10–45°C, the chromatographic system was allowed to equilibrate for 60 min (15) prior to collecting data at each temperature (consecutive 5°C changes). Retention factors were also determined as a function of ACA concentration in the mobile phase (column temperature maintained at 15ºC), allowing a 60-min equilibration period at each concentration prior to the collection of data.

Chemicals

HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA) was used without further purification. HPLC-grade deionized water was obtained from a purification system (Picotech, Hydro Systems and Supplies, Garfield, NJ). Dansyl-D,L-leucine cyclohexylammonium salt was obtained from Sigma-Aldrich (Milwaukee, WI). Sample solutions [1.0 mg/mL in 80:20 (v/v) methanol–water] were freshly prepared each week and stored in a refrigerator (at 4°C) when not in use. The mobile phase additives, sodium sulfate (99+%) and ACA (99%), were obtained from Sigma-Aldrich. The Na_2SO_4 was used in excess

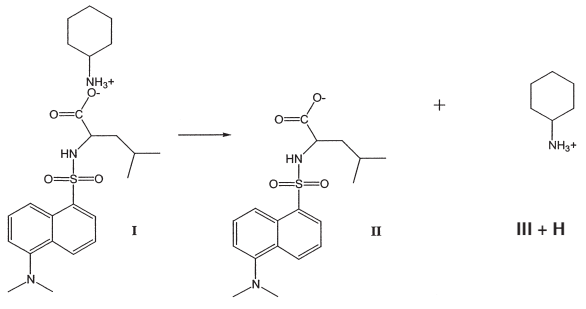

Figure 1. Schematic of the oncolumn dissociation of the dansyl leucine cyclohexylammonium ion pair (**I**) to give the free dansyl amino acid anion (**II**). Species **III + H** is the free cyclohexylammonium ion. Proton exchange equilibria between species **II** and **III + H** are omitted for clarity.

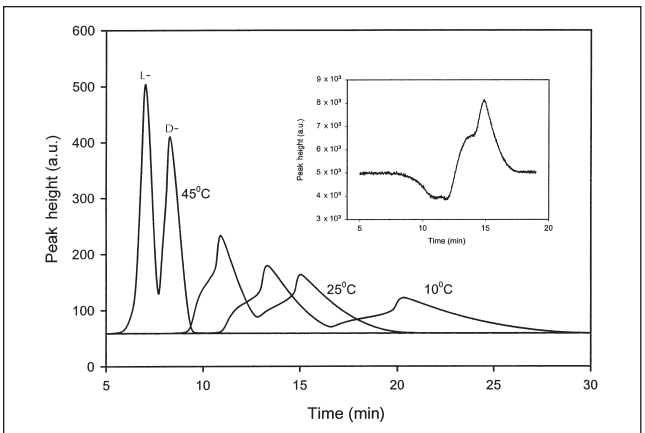

Figure 2. Overlaid chromatograms of L- and D-enantiomers of the solute showing the transformation of **I** to **II + H** (predominantly) on a β-CD CSP column as a function of temperature [the mobile phase was 80:20 (v/v) methanol–water, isocratic]. Inset: Typical chromatogram obtained using a circular dichroism detector showing the peaks of each enantiomer as distinct positive or negative waves.

to make a saturated mobile phase solution. The undissolved solids were removed by filtration through a glass microfiber filter (Whatman, Clifton, NJ) prior to use.

Solubility measurements of Na_2SO_4 mobile phase additive

The concentrations of saturated solutions of sodium sulfate in 80:20 (v/v) methanol–water were determined at 10°C, 25°C, and 45°C. Flame-sealed test tubes containing an excess amount (~ 250 mg) of Na_2SO_4 dissolved in 2 mL of solution were mechanically agitated at each temperature for a period of 3–5 h. Following this equilibration period, the test tubes were broken and the mother liquors removed and filtered. The mother liquors were subsequently placed in preweighed evaporation flasks and subjected to heating (35ºC) in a vacuum oven for a period of 15 h. After cooling to ambient temperature, the residue remaining in each flask was weighed. The solubility was calculated as milligrams of solid per gram of solvent.

Results and Discussion

Chromatographic peak shapes

Chiral separations on β-CD HPLC stationary phases are typically performed with buffer additives in the mobile phase (e.g., 22). The buffer serves not only to fix the pH of a given separation (this is usually less important when the mobile phase contains significant quantities of organic modifier), but also imparts ionic character to the mobile phase. Although both factors can play important roles in optimizing the efficiency of a separation, in this work the buffer component was omitted to facilitate ion pair formation. In addition, a low dielectric constant (i.e., high methanol content) mobile phase was used.

Figure 2 shows that at lower column temperatures distinct "bimodal" peak shapes can be observed for each enantiomer (enantiomer assignments verified with the aid of circular dichroism data, see Figure 2 inset) in the separation of a racemate of dansyl leucine cyclohexylammonium salt on the β-CD CSP. At higher temperatures, this peak shape is not observed because of coalescence. The bimodal peak shape is indicative of two chiral species, each with different retention properties. Each dansyl leucine enantiomer can exist as either an ion pair with its cylclohexylammonium counter ion (**I**) or as the free amino acid (**II+H**, in rapid equilibrium with its conjugate base, **II**). (Note: compounds **III/III+H** do not absorb at 254 nm.) Because the dissociation of **I** can be considered to be fast on a chromatographic time scale and neutral (uncharged) species are favored in a low-dielectric-strength medium, the broadness of the "secondary" enantiomeric peaks likely originates from the continuous, irreversible formation of, predominantly, **II+H** in the zone of **I** (i.e., the neutral, "free" dansyl amino acid travels down the column with a retention factor different from that of both **I** and **III**, unable to reassociate with the cyclohexylamine). At the lower column temperatures, this process is sufficiently slow to impart the distortions to the enantiomer peaks.

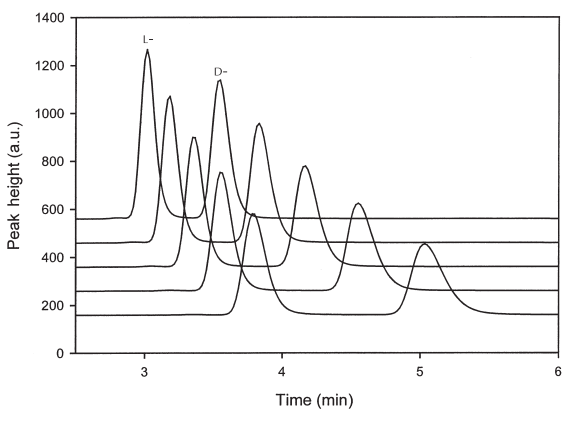

Figure 3. Overlaid chromatograms (offset for clarity) of L- and D-enantiomers of **II** on the β-CD CSP column at different temperatures (25°C, 30°C, 35°C, 40°C, and 45°C, in order from bottom to top). The mobile phase contains saturated sodium sulfate.

Sodium sulfate (note: the saturated concentration of Na_2SO_4 ranges from 0.4 mg/g mobile phase at 10°C to 0.5 mg/g at 45°C) was used as a mobile phase additive to determine the effect of increased ionic strength on the separation of **I**. This salt was selected because it mimics the ionizable groups on sulfated CDs, which are often used as chiral mobile phase additives (especially in capillary electrophoretic applications). From Figure 3, it can be seen that the resulting enantiomer peaks are much more Gaussian-shaped (indicative of a single solute species) and significantly less retained than those in Figure 2. This data is consistent with a chiral separation of the free, deprotonated (charged) dansyl amino acid, **II**. Even though significantly less retention is obtained for **II** relative to the uncharged species **I** and **II+H** (see Figure 2), the enantiomer separation remains very favorable. This suggests that the predominant enantioselective interactions (resulting from inclusion into the CD cavity) may not be adversely affected by the charge on the analyte or the increased ionic strength of the mobile phase. The chromatography of **II** on β-CD stationary phases has been reported in various literature (e.g., reference 9).

Thermodynamic treatment of the chiral separations

The retention mechanisms in many chromatographic systems have been analyzed with the aid of van't Hoff plots and enthalpy-entropy compensation studies (23). Because the phase ratio (ϕ) is difficult to quantitate experimentally, if it is assumed constant and independent of temperature, the differences between the enthalpic and entropic changes (ΔH and ΔS, respectively) associated with the partitioning of each enantiomer into and out of the stationary phase, as a function of temperature, can be compared using the simple equation:

$$\ln(\alpha) = -\Delta\Delta H/RT + \Delta\Delta S/R \qquad \text{Eq. 1}$$

where α is the selectivity coefficient ($= k_2'/k_1'$, where k_2' and k_1' are the retention factors for D- and L-dansyl leucine species, respectively), T is the temperature (°K), and R is the gas constant (8.314 $JK^{-1}mol^{-1}$). Figure 4A shows van't Hoff plots for each enantiomer of **I**. The plots for this chromatographic system and for the system in which sodium sulfate was added to the mobile phase (which produced **II**, plots not shown) were

linear (R > 0.998), which indicates that the major retention mechanisms in each case did not change as a function of temperature over the range studied. For the selectivity between enantiomers of **I** (Figure 4B), $\Delta\Delta H$ = –5.52 kJ/mol and $\Delta\Delta S$ = –15.6 $JK^{-1}mol^{-1}$. For the selectivity between enantiomers of **II**, $\Delta\Delta H$ = –4.88 kJ/mol and $\Delta\Delta S$ = –11.9 $JK^{-1}mol^{-1}$. The compensation temperatures were 354°K and 409°K for the two systems, respectively. Although these numbers imply more entropic character for the chiral separation of **I** relative to **II**, enantioselectivity was achieved predominantly through enthalpically driven mechanisms in both cases (attributed mainly to dansyl group complexation). In near-neutral pH solutions, negative ΔH and ΔS values have been reported in the literature for the interactions of similar solutes with β-CD stationary phases (23).

Dependence of enantioselectivity on ACA concentration: the effect of a solute-competitive mobile phase additive

Many investigations into chiral recognition using various CSPs have dealt with the variation of chromatographic parameters with the goal of improving enantiomer separation. The authors believe that it should also be possible to study chiral recognition by performing the converse: intentionally reducing the enantioselectivity of a CSP toward a given solute or solutes. In this work, ACA was introduced into the system of Figure 2, and the effect of the additive on the chromatography was observed. ACA is known to bind to β–CD very strongly [estimated association constant of ~ 9×10^4 at neutral pH (18)]. The compound also has a much higher solubility in polar solutions than adamantane, making it potentially useful in many chromatographic applications. An "inclusion site-blocker", this molecule tends to occupy the chiral hydrophobic pocket of β-CD (18), thus competing with solute molecules for these sites. Figure 5 shows the effect of various concentrations of this mobile phase additive on the separation of the enantiomers of **I**. The effect of the additive is twofold. First, increasing its concentration results in a decrease in resolution between the two dansyl leucine enantiomers. Second, the peaks sharpen and elute earlier. Although the first effect can be attributed to the blocking of the hydrophobic cavities on the CSP by the adamantyl moieties, the second is mainly the result of an increase in mobile phase ionic strength caused by the addition of the organic acid. The presence of ACA in the mobile phase (at least partially ionized) can enhance the conversion of **I** to the earlier-eluting species **II**, as described previously for the system with the sodium sulfate additive. Note that ACA has the ability to H-bond with surface CDs, however this mode of interaction is believed to minimally impact the enantioselectivity of the CSP (24).

Using arguments similar to Peyrin et al. (25), from the general form of an adsorption isotherm, an equation relating the change in the change in free energy (ΔG_S, where S = specific interaction) associated with a CD cavity that binds one enantiomer, caused by the introduction of ACA to the mobile phase, can be written as:

$$\{\delta[\Delta(\Delta G_S)]/\delta \ln c\}_T = -RT\{\delta \Delta \ln k'/\delta \ln c\}_T \qquad \text{Eq. 2}$$

where c is the concentration of ACA and R, k', and T are defined as before. Because we know that the ACA concentration influ-

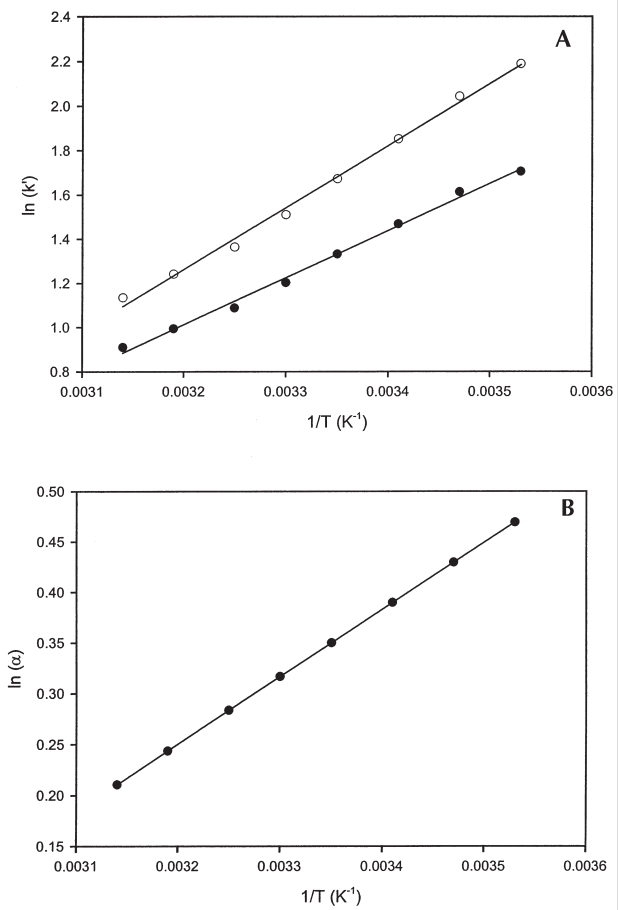

Figure 4. Retentive van't Hoff plots for the chromatographic system of Figure 2, showing trends for both the L- (filled circles) and D- (open circles) enantiomers of **I**, individually (A). Van't Hoff plot showing the chiral selectivity in the same system (B). The lines represent linear regression fits of the data points.

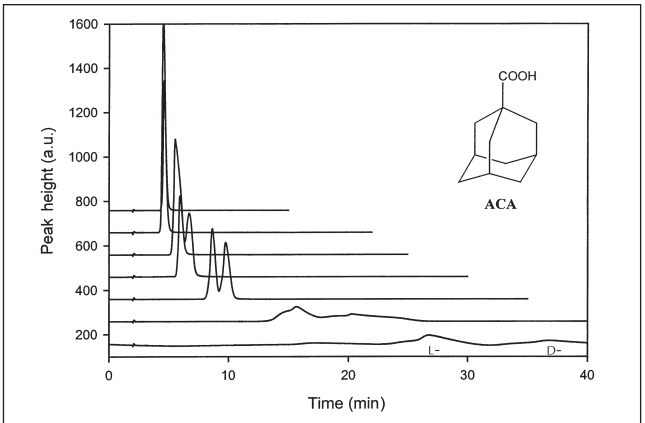

Figure 5. Overlaid chromatograms (offset for clarity) of L- and D-enantiomers of **I** on the β-CD CSP column obtained using various concentrations of the mobile phase additive ACA (see inset). From bottom to top the concentration of the additive is 0, 0.3, 0.6, 1.4, 2.7, 5.7, and 20mM. Note the increased conversion of **I** to **II** at higher ACA concentrations (fixed column temperature).

ences retention in more than one way (i.e., simultaneously blocks the CD cavities and promotes conversion of **I** to **II**), we consider only the effect of the additive on the selectivity factor (α). If the concentration dependence of the mobile phase additive on the selectivity factor is desired, equation 2 can be written as:

$$\{\delta[(\Delta\Delta G_{S,D}/\Delta\Delta G_{S,L})]/\delta \ln c\}_T = -RT\{\delta \Delta \ln \alpha/\delta \ln c\}_T \qquad \text{Eq. 3}$$

where the subscripts D and L indicate the enantiomer of dansyl leucine. Thus, a plot of the change in ln α versus ln c, at constant temperature, should relate the ratio of the energetic parameters $\Delta\Delta G_{S,D}$ and $\Delta\Delta G_{S,L}$ as a function of the concentration of ACA. For the chromatographic system in Figure 5, this plot is shown in Figure 6A. For comparison, Figure 6B shows overlaid plots of the change in ln k' versus ln c for each enantiomer. From Figure 6A it is apparent that the enantioselectivity decreases with increasing concentration of ACA up to a point at which enantioselectivity is no longer achieved (i.e., $\alpha = 1$). (Note: the two data points in Figure 6A not falling on the curve may be explained by a combination of the progressive change in the solute species from **I** to **II** with increasing amounts of ACA in the mobile phase and an accompanying increase in resolution that is brought about by higher separation efficiencies at the intermediate concentrations of ACA, as shown in Figure 7). Extrapolating from the low- and high-concentration regions of the plot, the critical additive concentration needed to achieve $\alpha = 1$ (i.e., "isoenantioselectivity") was estimated at approximately 4mM. This mobile phase concentration, the phase ratio, the estimated binding constant between the additive and cyclodextrin, and the surface area of the stationary phase should make it possible to obtain an estimate for the surface coverage of accessible CD sites on the CSP (assuming a 1:1 stoichiometry), provided that only chiral specific interactions with the CSP take place. Unfortunately, based on the relatively large value obtained for the "critical concentration" under the given set of chromatographic conditions, it can be concluded that ACA also exhibits nonspecific interactions with the stationary phase surface (and likely with itself as well) that cause it to accumulate at the CSP surface. Future work in this area may include the use of adamantol species, which do not possess ionizable groups and can be expected to aggregate to a lesser extent than ACA.

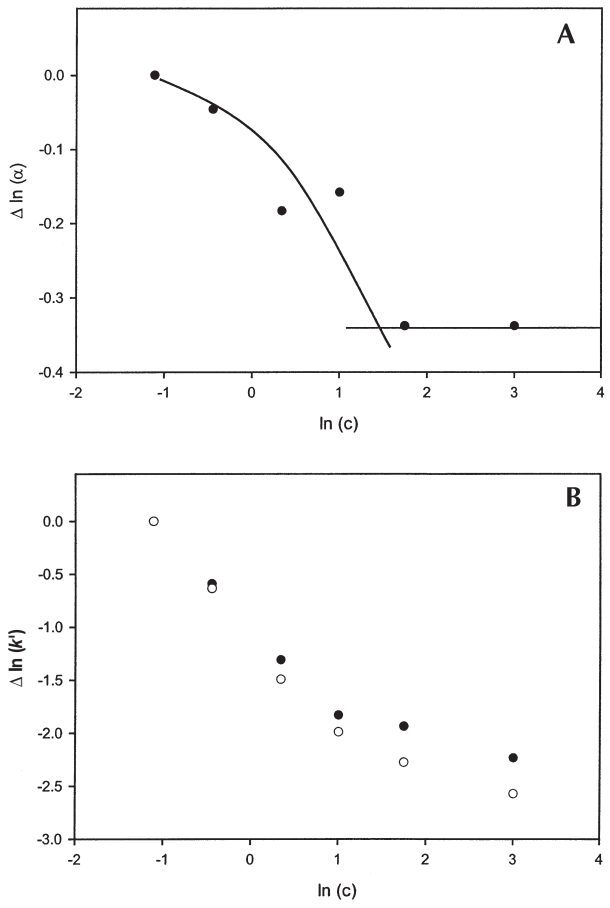

Figure 6. (A) Plot of Δ ln α versus ln c for the chromatograms in Figure 5 (c is the concentration of ACA in the mobile phase and α is the enantiomer selectivity factor). The lines represent extrapolations from high and low concentrations of the additive. (B) Plots of Δ ln k' vs. ln c for the L- (filled circles) and D- (open circles) solute enantiomers, individually.

Conclusion

Chiral separations of ion pairs may be investigated with the aid of a low ionic strength, low dielectric constant mobile phase. Clues to the complex mechanisms underlying the separation of dansyl leucine cyclohexylammonium ion pair enantiomers on a β-CD stationary phase can be inferred from the peak shape, separation factor, and retention under various chromatographic conditions. Careful selection of mobile phase additives can provide information on the chemical origins of predominant retentive or chiral interactions and can serve to both simplify the chromatographic system and improve separation efficiency. In addition, performing the separation as

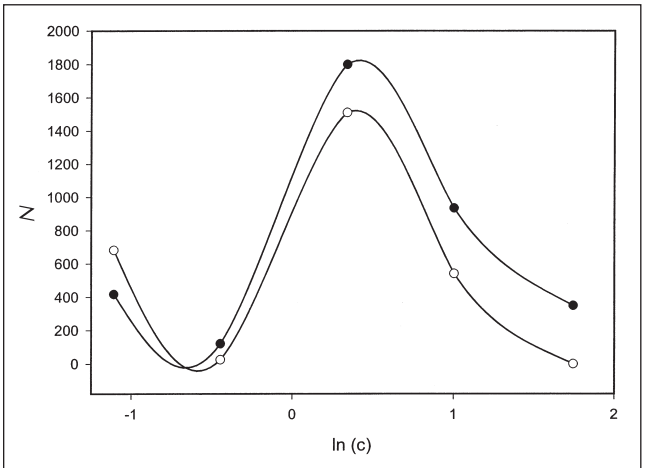

Figure 7. Plots of the number of theoretical plates (N) versus ln c for the chromatographic peaks in Figure 5 corresponding to the L- (open circles) and D-(filled circles) enantiomers of the solute species. The curves represent spline fits of the data.

a function of temperature can allow thermodynamic characterization of the system.

The separations of both the dansyl leucine cyclohexylammonium ion pair (**I**) enantiomers and free dansyl leucine anion (**II**) enantiomers were found to have predominantly enthalpic driving forces under the chromatographic conditions described in this work. The use of a solute-competitive mobile phase additive (ACA) provided supporting evidence for the β-CD cavity being the primary site of chiral recognition on this type of CSP. This site is known to be the origin of the predominant hydrophobic interactions that assist in the chiral recognition of an analyte; the interactions being strengthened by multiplicity within the space-confined cavity (complexation). The concentration of ACA mobile phase additive, while affecting the solute distribution by promoting the conversion of **I** to **II**, was also found to regulate the enantioselectivity of the CSP.

Although it serves to be aware of the potential for ion-pair formation during chiral chromatography, from a practical standpoint the chiral separation of dansyl amino acids should be performed under conditions of controlled ionic strength (and pH) to immediately break any ion pairs that could form. In this manner, the chromatography is greatly improved through shorter elution times and better enantiomer resolution.

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