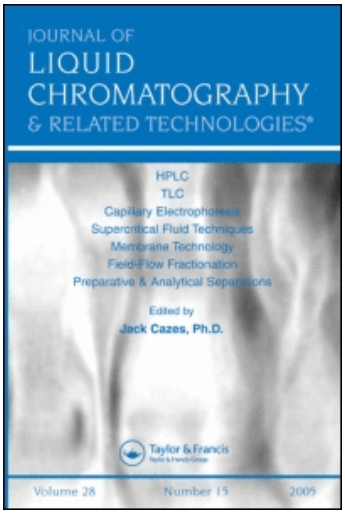

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Li, Xiaoping and McGuffin, Victoria L.(2007) 'Thermodynamics and Kinetics of Chiral Separations with β-Cyclodextrin Stationary Phase: I. Effect of Mobile Phase Composition', Journal of Liquid Chromatography & Related Technologies, 30: 5, 937 — 964
To link to this Article: DOI: 10.1080/10826070701191177
URL: http://dx.doi.org/10.1080/10826070701191177

Journal of Liquid Chromatography & Related Technologies®, 30: 937–964, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701191177

Thermodynamics and Kinetics of Chiral Separations with β-Cyclodextrin Stationary Phase: I. Effect of Mobile Phase Composition

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Abstract: A series of coumarin-based solutes, consisting of warfarin, coumachlor, coumafuryl, coumatetralyl, and 4-hydroxycoumarin, are utilized to elucidate the thermodynamic and kinetic aspects of chiral separations. These solutes are separated on a β-cyclodextrin stationary phase with a polar-organic mobile phase. Preliminary results demonstrate that the equilibration time is extremely long when the mobile phase consists of acetonitrile, methanol, or mixtures thereof. However, the equilibration time is substantially reduced when acetic acid or triethylamine modifiers are added. Linear isotherms are obtained when acid modifier is present, but nonlinear isotherms are obtained when no modifier or only amine modifier is present. The concentration of each component of the mobile phase is varied individually to observe the effects on the thermodynamic and kinetic behavior, including the retention factor, chiral selectivity, and kinetic mass transfer rate. In general, an increase in methanol or triethylamine concentration decreases retention, but an increase in acetic acid concentration slightly increases retention. However, it is noteworthy that triethylamine enhances chiral selectivity, whereas acetic acid diminishes it. Acetic acid has the opposite effect when amine is not present, indicating that the acid and amine interact with each other. With regard to the kinetic behavior, the mass transfer rate constant of the coumarin solutes generally decreases as the retention factor increases. However, the second enantiomer often has a surprisingly

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faster mass transfer rate than the first enantiomer, even though it is more retained. This difference indicates that different kinetic contributions arise at the chiral and achiral selective sites.

Keywords: β-Cyclodextrin, Chiral separation, Thermodynamics, Kinetics, Polar organic mode

INTRODUCTION

The separation of chiral compounds has been of great interest in academia and industry because of their prevalence and biological importance. The building blocks of the body, including biopolymers such as proteins, nucleic acids, and polysaccharides, are composed of chiral monomers. Naturally, the chiral centers in these monomers exist in only one of the two possible enantiomeric forms, demonstrating different responses to each enantiomeric form of drugs, foods, pesticides, and so on.[1] In previous years, the pharmaceutical industry suffered from indiscrimination of the two enantiomers. Whereas one isomer may produce the expected therapeutic effect, the other may be inactive or, even worse, may produce detrimental side effects. In 1992, the U.S. Food and Drug Administration (FDA) issued guidelines for the development of stereoisomeric drugs.[2] Not only the drugs but also their degradation products and metabolites must be separated and analyzed individually. Thus, the development and understanding of chiral separations is necessary and important.

Cyclodextrins are cyclic oligosaccharides containing six or more D-glucose units connected through α-1,4 glycosidic bonds. The structure of β-cyclodextrin contains seven glucose units with 35 chiral centers. As a result of the large number of chiral centers, cyclodextrins are ideal for chiral separations. Native and derivatized cyclodextrins have a long history of use as chiral stationary phases (CSPs). They were first immobilized to solid supports in 1978[3] and, since then, extensive work has been done to improve the stability and versatility of these CSPs.[4]

Once a specific stationary phase is chosen, mobile phase composition is the factor that will most significantly affect the separation. Reversed-phase mobile phases are the most popular and successful when native cyclodextrins are used as CSPs.[5,6] In aqueous media, the hydroxyl groups of the cyclodextrin are oriented toward the exterior surface, which makes the interior of the cyclic cavity relatively nonpolar and hydrophobic. Consequently, solutes may enter the cavity and be retained by dispersion interactions as well as by hydrogen bonding interactions with the rim. Many publications are available on the effect of mobile phase composition in the reversed-phase mode.[5–20] Systematic studies have been performed on the effect of organic modifier type, modifier

concentration, buffer type, buffer concentration and ionic strength. More recently, polar-organic phase separation has also become widely utilized.[21,22] In nonaqueous media, the nonpolar solvent will enter the cavity, while the solutes will interact with the rim of the cyclodextrin mainly by hydrogen bonding.[23] The polar-organic mode has many advantages over the reversed-phase mode. It increases the column stability and sample capacity, shortens the separation time, and improves the resolution. The mobile phase usually consists of four components: acetonitrile, alcohol, acid and amine modifiers. It is generally believed that acetonitrile and alcohol govern retention, whereas acid and amine modifiers govern selectivity. Armstrong et al.[24] first utilized polar-organic mobile phases with native β-cyclodextrin CSP to separate some β-blocker pharmaceuticals. Since then, many other enantiomers have been separated in the polar-organic mode.[23,25–27] However, limited information is available on the effect of polar-organic mobile phase composition, especially detailed and systematic characterization studies. Notably, there have been investigations of mobile phase effects on β-blockers[28] and amino acids.[29] In these studies, the thermodynamic effects on retention factor and chiral selectivity have been carefully investigated, however the kinetic effects on mass transfer rates are usually overlooked. Both thermodynamic and kinetic information are equally important for elucidation of the retention mechanism. Recently, Ching et al. investigated both thermodynamic and kinetic information on derivatized cyclodextrin CSP.[30,31] The equilibrium constants and kinetic rate constants for the chiral separation were obtained by moment analysis on the basis of the solid-film linear driving force model. The overall mass transfer coefficients were determined to be 0.97 and 1.21 s^{-1} for S- and R-fluoxetine, 1.77 and 14.1 s^{-1} for RSR- and RRS-nadolol, and 11.2 s^{-1} for the co-eluted SRS- and SSR-nadolol. These kinetic data suggest that mass transfer rates are relatively rapid for these chiral compounds on derivatized cyclodextrin CSPs.

In addition to the effect of mobile phase composition, the memory effect is another interesting issue that needs to be addressed. The existence of memory effects with acid and amine modifiers has been demonstrated with polysaccharide columns by Stringham et al.[32,33] In their work, acids and amines showed persistent effects after their removal, indicating strong interaction with the polysaccharide CSP. However, there has been no report on these effects with cyclodextrin CSPs. Considering that cyclodextrin has the same monomers (glucose units) as polysaccharides, the memory effect is worthy of investigation.

The goals of this research are as follows: 1) to test the memory effect in β-cyclodextrin CSP in the polar-organic mode, 2) to investigate equilibration time and isotherm behavior, and 3) to conduct a systematic study with varying composition of each individual mobile phase component and observe the trends in thermodynamic and kinetic behavior.

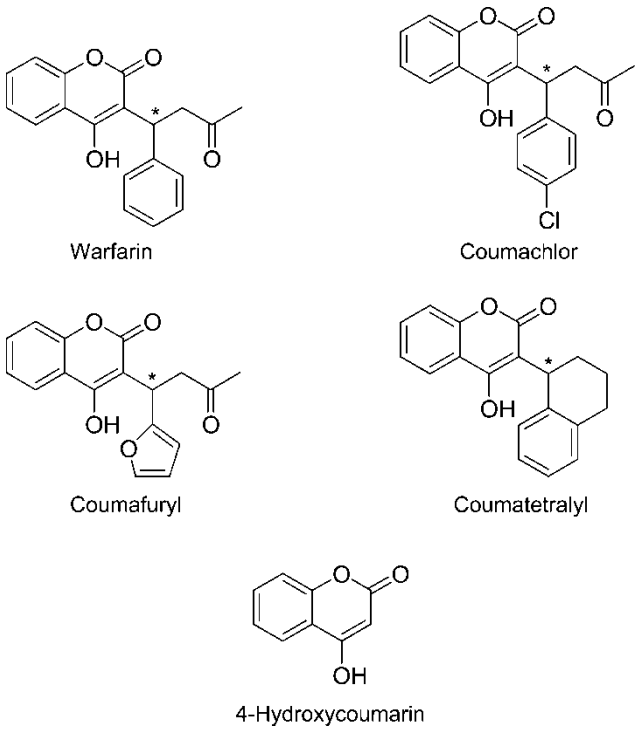

Figure 1. Structure of coumarin-based solutes.

EXPERIMENTAL

Chemicals

As depicted in Figure 1, five coumarin-based solutes are chosen based on their structural similarities. Warfarin, coumachlor, coumafuryl, and coumatetralyl are chiral, whereas 4-hydroxycoumarin is achiral. The solutes are obtained from Sigma-Aldrich as solids and are dissolved in high-purity acetonitrile (Burdick and Jackson, Baxter Healthcare) to yield standard solutions at 10^{-4} M. Nitromethane is used as non-retained marker and is added to each solution at a concentration of 15% (v/v). High-purity acetonitrile, methanol (Burdick and Jackson, Baxter Healthcare), acetic acid (Sigma-Aldrich), and triethylamine (Sigma-Aldrich) are used for mobile phase preparation.

Experimental System

The solutes are separated on a capillary liquid chromatography system. The column is a fused-silica capillary (200 μm i.d., 105 cm, Hewlett-Packard)

that is packed via the slurry method and terminated with a quartz wool frit.[34] Columns prepared by this method have very uniform packing across the diameter and along the length. The silica packing (Cyclobond I 2000, Astec) is characterized by a 5.2 μm particle size, 0.89 mL/g pore volume, and 305 m^2/g surface area, reacted with β-cyclodextrin at a bonding density of 0.39 $\mu mol/m^2$.

In the chromatographic system, the mobile phase is delivered by a single-piston reciprocating pump (Model 114 M, Beckman Instruments) operated in the constant-pressure mode at a nominal flow rate of 1.2 μL/min. After injection (Model ECI4W1, Valco Instruments), the samples are split between the column and a fused-silica capillary (50 μm i.d., Polymicro Technologies), resulting in an injection volume of 15 nL.

The polyimide coating is removed from the capillary column at 99 cm length to facilitate on-column detection by laser-induced fluorescence. A helium-cadmium laser (Model 3074-20M, Melles Griot) provides excitation at 325 nm. The fluorescence emission is isolated by a liquid filter (1% aqueous $NaNO_3$) and two interference filters (420 nm, S10-410-F, Corion), and is detected by a photomultiplier tube (Centronic Model Q4249BA, Bailey Instruments). The resulting photocurrent is amplified, converted to the digital domain (Model PCI-MIO-16XE-50, National Instruments), and stored by a user-defined program (Labview v5.1, National Instruments).

Data Analysis

After data collection, the zone profile for each solute is extracted from the chromatogram. Each profile is fit by nonlinear regression to the exponentially modified Gaussian (EMG) equation by using a commercially available program (Peakfit v4.14, SYSTAT Software). The EMG equation is chosen because the statistics of fit are better than those for any other model that has been demonstrated to have physical meaning. Specifically, for the zone profiles analyzed in this study, the square of the correlation coefficient (R^2) and the F-statistic from the EMG model are higher than those from the Thomas[35,36] or Giddings[37] models. The residuals from the EMG model are more random and smaller than those from the Thomas or Giddings models as well.

The EMG equation is the convolution of Gaussian and exponential functions, with the resulting form

$$C(t) = \frac{A}{2\tau}\exp\left[\frac{\sigma^2}{2\tau^2} + \frac{t_g - t}{\tau}\right]\left[\operatorname{erf}\left(\frac{t - t_g}{\sqrt{2}\sigma} - \frac{\sigma}{\sqrt{2}\tau}\right) + 1\right] \quad (1)$$

where C(t) is the concentration as a function of time, A is the peak area, t_g is the retention time of the Gaussian component, σ is the standard deviation of the Gaussian component, and τ is the standard deviation of the exponential

component. Symmetrical zone broadening, which arises from diffusion and mass transfer processes that are fast relative to the separation time, is quantified by σ. Asymmetrical broadening, which arises from volumetric sources (i.e., injectors, unions, etc.), electronic sources (i.e. amplifiers, etc.), nonlinear isotherms, and diffusion and mass transfer processes that are slow relative to the separation time, is quantified by τ. By judicious design of the experimental system, the asymmetrical broadening from volumetric and electronic sources can be minimized.[38,39] The contribution to asymmetric broadening from nonlinear isotherms can be eliminated by injecting solutes at sufficiently low concentrations. Thus, under these ideal conditions, slow mass transfer processes will dominate the asymmetrical broadening. The resultant regression coefficients of the EMG equation are used to calculate the thermodynamic and kinetic parameters.[40] The thermodynamic parameters of retention factor (k) and chiral selectivity (α) are given by

$$k = \frac{(t_r - t_0)}{t_0} \tag{2}$$

$$\alpha = \frac{k_2}{k_1} \tag{3}$$

where t_r and t_0 are the elution times of a retained and nonretained solute, respectively, and $t_r = t_g + \tau$. The kinetic parameters are given by

$$k_{ms} = \frac{2kt_0}{\tau^2} \tag{4}$$

$$k_{sm} = kk_{ms} = \frac{2k^2t_0}{\tau^2} \tag{5}$$

where k_{sm} is the adsorption rate constant for transfer from mobile to stationary phase and k_{ms} is the desorption rate constant for transfer from stationary to mobile phase.

RESULTS AND DISCUSSION

Memory Effect

In the first series of experiments, warfarin is utilized as the solute to investigate memory effects on the β-cyclodextrin CSP. Two polar-organic mobile phase compositions are used: the first mobile phase contains acetonitrile and methanol at a 90%:10% ratio by volume; the second mobile phase contains acetonitrile and methanol (90%:10%) together with acetic acid (0.30%) and triethylamine (0.20%) modifiers. Warfarin is initially injected at the first mobile phase composition. This result serves as the "original"

retention data (t_r) for comparison when acid and amine are not present in the mobile phase. Then the second mobile phase is introduced with another warfarin injection. Finally, the first mobile phase is switched back, and warfarin is injected over successive days to monitor the peak shape and retention time. Representative chromatograms are illustrated in Figure 2. The original peaks have a broad and fronting shape, with a retention time of approximately 35 min (Figure 2A). When acid and amine are added to the mobile phase, the elution time of warfarin is greatly reduced and the peak shape becomes narrow and Gaussian (Figure 2B). When acid and amine are subsequently removed from the mobile phase, the retention time of warfarin increases, with increased peak broadening as well (Figures 2C and 2D), until 3 days later when the retention time reaches a maximum (Figure 2E). Thereafter, the retention time begins to decrease and the peak shape becomes broader and more fronting (Figure 2F). But even after 10 days, the retention time does not return to the original value shown in Figure 2A. These data indicate two possibilities: either a memory effect of the acid and amine exists in the cyclodextrin CSP, or the equilibration time for the acetonitrile:methanol mobile phase is extremely long. The second possibility can also be considered as the existence of a memory effect of methanol.

In the approach used by Stringham et al.[32,33] to remove the memory effect of acid or amine on a polysaccharide CSP, the column was washed with alcohols or water. Since methanol is already a component of the

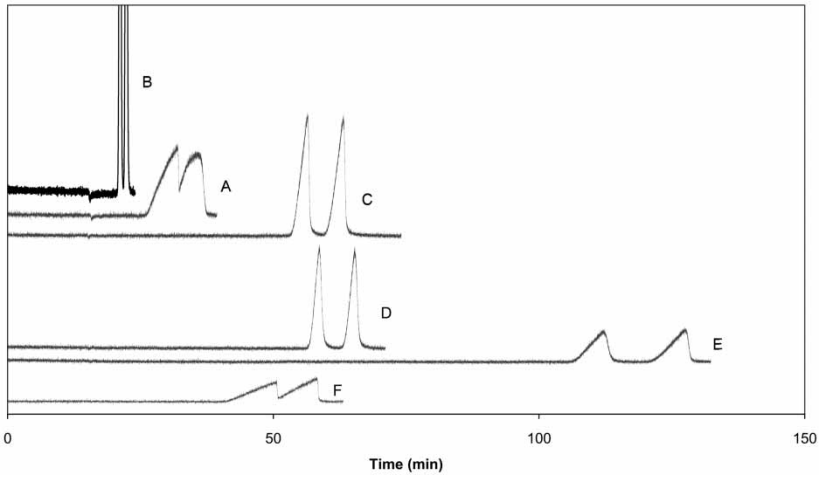

Figure 2. Representative chromatograms of memory effect from acetic acid and triethylamine. Column: β-cyclodextrin. Mobile phase composition: acetonitrile:methanol (90%:10%) (A) original data before acid and amine modifier added; (B) acetic acid: triethylamine (0.30%:0.20%) added; (C) removal of acid and amine modifier at hour 4; (D) day 1; (E) day 3; (F) day 10.

polar-organic mobile phase, methanol alone is used herein to remove any possible memory effect of acid or amine. The β-cyclodextrin column is washed with methanol for one day, then the mobile phase is switched back to acetonitrile:methanol (90%:10%), followed by the injection of warfarin over successive days. Representative chromatograms are shown in Figure 3. After one day, the retention time for warfarin is extremely long at around 150 min (Figure 3A). Moreover, the peak shape is tailing instead of fronting as seen previously in Figure 2. After two days, the retention time is reduced to around 65 min with a fronting peak shape (Figure 3B). The retention time of warfarin continues to decrease over several days, but does not return to the original value even after 10 days (Figure 3D). However, these retention times are closer to the original values than those obtained after removal of acid and amine (Figure 2). These data indicate that washing with methanol provides some improvement but cannot completely eliminate the memory effect of acid or amine. On the contrary, methanol itself exhibits an even stronger memory effect over short periods of time (e.g., less than 1 day). These results suggest that more detailed investigation of the effect of the individual components in the polar-organic mobile phase is warranted. Prior to these experiments, the β-cyclodextrin column is washed with pure acetonitrile for several weeks to remove any residual memory effect of the previous mobile phases.

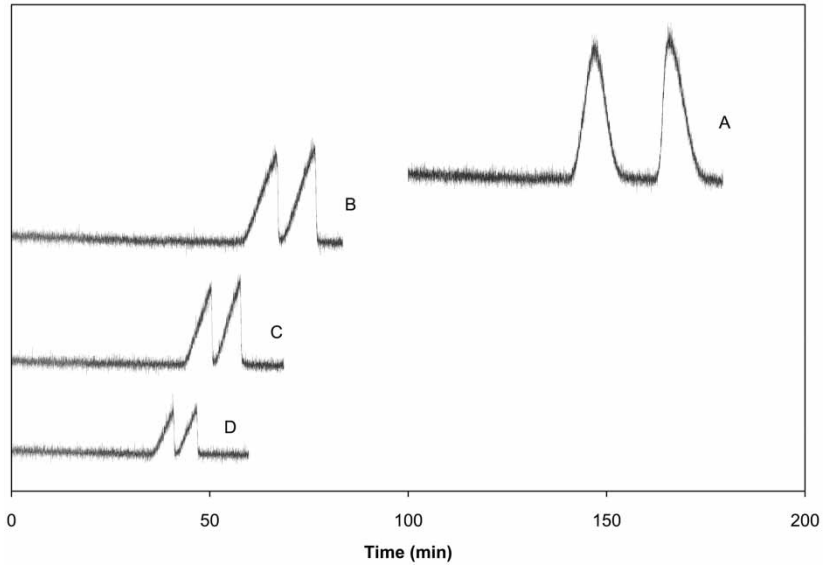

Figure 3. Representative chromatograms of memory effect from methanol. Column: β-cyclodextrin. Mobile phase composition: acetonitrile:methanol (90%:10%) after removal of pure methanol mobile phase at (A) day 1; (B) day 2; (C) day 3; (D) day 10.

Two-Component Mobile Phase

In these experiments, acetonitrile and methanol are the only components in the mobile phase; no acid or amine is added. The percent of methanol is varied at 3%, 5%, 10%, 15%, and 20%. Each composition of the mobile phase is maintained for several days in order to observe the rate of achievement of equilibrium conditions.

The retention time of warfarin over successive days is reported in Table 1. Using the second day as an example, the retention time decreases as the mobile phase composition changes from 3% to 10% methanol. From 10% to 20% methanol, however, the retention time shows an increasing trend. For each composition, the retention time is not stable even after several days. Interestingly, the trends in retention time over days are not the same for each concentration. At lower concentrations (3% and 5%), the retention time decreases over successive days. At higher concentrations (15% and 20%), the retention time increases over successive days. At an intermediate concentration of 10% methanol, the retention time shows an initial decrease followed by an increase. By careful observation, the trend over successive days is noted to be the same as the trend for the next higher concentration of methanol. Using 3% methanol mobile phase as an example, the retention time of warfarin decreases over successive days, and retention time also decreases when changing from 3% to 5% methanol. Using 15% methanol as an example, the retention time of warfarin increases over successive days, and retention time also increases when changing from 15% to 20% methanol. These observations suggest that methanol may accumulate on the β-cyclodextrin column. While a low concentration of methanol can compete with the solute warfarin and serve as a displacing agent to decrease its retention time, higher concentrations may build up a layer of methanol on the cyclodextrin surface. This layer can serve as a partitioning medium and can increase retention time because the effective volume of the stationary phase increases with increasing methanol concentration.

Table 1. Retention time of first eluted warfarin enantiomer over successive days in a two-component mobile phase

Mobile phase composition (acetonitrile:methanol) (%)	Retention time (min)			
	Day 1	Day 2	Day 3	Day 4
97:3	183.5	174.8	174.1	N/A[a]
95:5	93.4	81.3	76.1	N/A
90:10	28.1	22.9	27.8	31.6
85:15	N/A	48.0	50.9	55.3
80:20	100.3	96.1	105.4	108.3

[a]Not available (N/A).

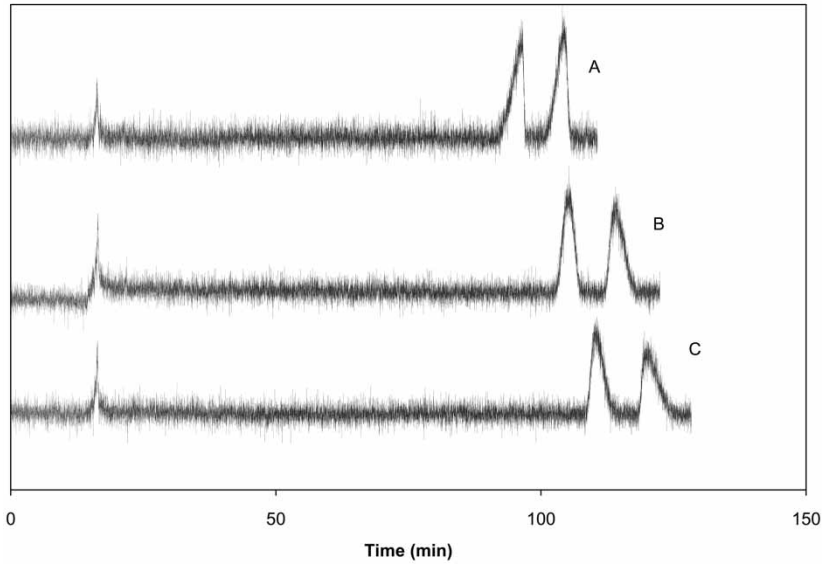

Figure 4. Comparison of chromatograms in two-component mobile phase. Column: β-cyclodextrin. Mobile phase composition: acetonitrile:methanol (80%:20%) (A) day 2; (B) day 3; (C) day 6.

Another interesting observation is the change in warfarin peak shape with concentration of methanol. At concentrations of 3% to 15% methanol, all the peaks are fronting. At 20% methanol, however, different peak shapes appear over successive days as shown in Figure 4. The peaks on the second day are fronting (Figure 4A), those on the third day are slightly tailing (Figure 4B), while those on the sixth day are strongly tailing (Figure 4C). This change in peak shape suggests a change in the interaction between warfarin and the cyclodextrin CSP. A tailing shape is usually considered as a sign of a Langmuir or BET type I isotherm, where there exists a limited number of interaction sites on the stationary phase. As the concentration of solute increases, these sites become completely occupied and the remaining solute molecules are not retained. A fronting shape usually suggests an anti-Langmuir or BET type III isotherm, where there is weak interaction between the solute and stationary phase. As the concentration of solute increases, it has stronger interactions with other solute molecules bonded to the stationary phase than with the stationary phase alone. It is certainly possible that the retention mechanism changes with increasing methanol concentration due to the formation of a methanol layer, as discussed above. However, it is also possible that a single mechanism exists with a more complicated isotherm, such as BET type V, which combines the features and behavior of the BET type I and III isotherms. More detailed studies are

needed to elucidate the underlying reason for the change in peak shape with methanol concentration.

Three-Component Mobile Phase

In these experiments, acetic acid or triethylamine is added to an acetonitrile: methanol (90%:10%) mobile phase to prepare a three-component mobile phase. The retention time, equilibration time, peak shape, and isotherm effect are compared for the different mobile phase conditions.

Retention Time and Equilibration Time Investigation

As in the previous study, warfarin is injected over successive days to observe the trends in retention time and equilibration time. Three different mobile phase compositions are reported. The first mobile phase is a two-component mobile phase containing acetonitrile and methanol (90%:10%). The second mobile phase added 0.30% acetic acid to the first mobile phase, while the third mobile phase added 0.20% triethylamine to the first mobile phase.

The retention time of warfarin measured over successive days at each mobile phase composition is reported in Table 2. The average retention time, standard deviation, and percent relative standard deviation (RSD) are reported as well. In the two-component mobile phase, equilibrium is not reached even after four days, which can be discerned from the large RSD of 13%. In fact, the retention time is still changing even after one week (results not shown). When acid or amine modifier is added to the mobile

Table 2. Retention time of first eluted warfarin enantiomer over successive days in two-component and three-component mobile phases

	Mobile phase composition (acetonitrile:methanol:acid:amine)		
	90%:10%: 0%:0%	90%:10%: 0.30%:0%	90%:10%: 0%:0.20%
Retention time (min)			
Day 1	28.1	28.6	12.3
Day 2	22.9	29.1	12.3
Day 3	27.8	28.9	11.9
Day 4	31.6	N/A[b]	N/A
Average	27.6	28.9	12.2
St. Dev.	3.7	0.3	0.3
% RSD[a]	13	1	2

[a]Relative standard deviation (% RSD) = (Average/St. Dev.) × 100%.
[b]Not available (N/A).

phase, equilibrium is reached much faster (one day or less) and the RSD is reduced to 1–2%. This indicates that acid or amine modifier can help greatly to shorten the equilibration time of the mobile phase.

Table 2 can also be used to compare the retention time for different mobile phase compositions. When compared to the two-component mobile phase, the addition of 0.30% acetic acid modifier does not have a significant effect upon retention time, which increases only slightly. On the contrary, the addition of 0.20% triethylamine modifier greatly shortens the retention time. The suspected reasons for this behavior will be discussed in the next section.

Peak Shape and Isotherm Investigation

As the mobile phase composition is changed, not only do the retention time and equilibration time change, but also the peak shape. Unusual fronting and tailing shapes are frequently encountered, as discussed in the previous sections. Since these peak shapes are usually associated with nonlinear isotherms, this possibility is examined by injecting warfarin at different concentrations ranging from 5×10^{-5} to 8×10^{-4} M.

As discussed previously, fronting peaks are observed in the two-component mobile phase composition with acetonitrile:methanol (90%:10%). But when both acid and amine modifiers are added, symmetric Gaussian peaks are observed. To determine which modifier is responsible for the Gaussian peak shape, two different mobile phases are investigated. One contains 0.30% acetic acid modifier and the other contains 0.05% triethylamine modifier. Representative chromatograms of warfarin are illustrated in Figures 5 and 6. With acid modifier alone, the retention time is virtually unchanged and the peak shape is Gaussian until the highest warfarin concentration (8×10^{-4} M), where the retention time decreases slightly and is accompanied by slight tailing. This suggests that a linear isotherm is obtained in the mobile phase with acid modifier over most of the solute concentration range in this study. However, with amine modifier alone, fronting peaks are observed even at the lowest warfarin concentration and the peaks become more retained and more asymmetric with increasing concentration. Although the maxima are shifted to longer retention time, all the peaks for the first enantiomer appear to arise from the same initial position. The peaks for the second enantiomer do not arise from the same initial position, but are shifted to longer retention time with increasing warfarin concentration. This behavior is indicative of a competitive nonlinear isotherm, where both enantiomers are competing for the same adsorption sites on the cyclodextrin CSP.

The change in peak shape with mobile phase composition is interesting and informative. Fronting peaks are observed when no modifier or only amine modifier is present in the mobile phase. As noted above, fronting shape is usually indicative of an anti-Langmuir or BET type III isotherm. When no acid or amine modifier is present, the methanol may accumulate on the cyclodextrin surface, as discussed above. This may be the reason why

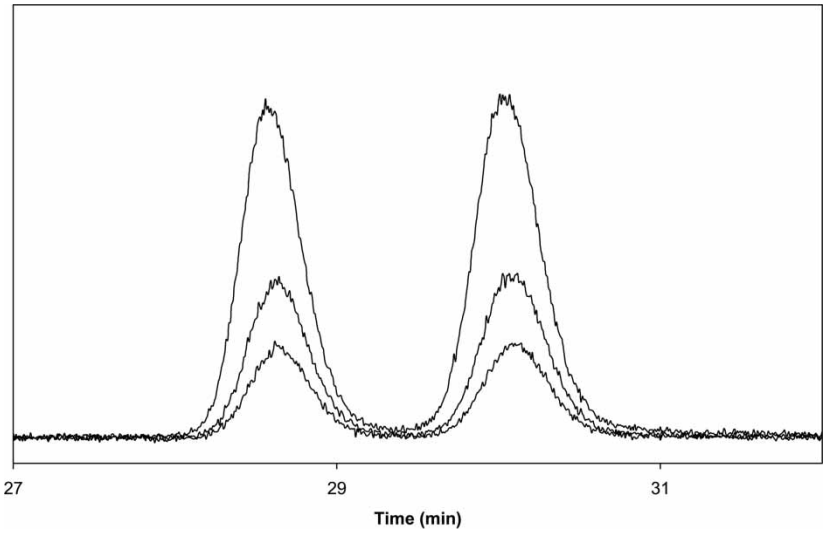

Figure 5. Warfarin peak shape in the presence of acetic acid. Column: β-cyclodextrin. Mobile phase composition: acetonitrile:methanol:acid (90%:10%:0.30%). Warfarin concentration from highest to lowest: 8×10^{-4} M; 4×10^{-4} M; 2×10^{-4} M.

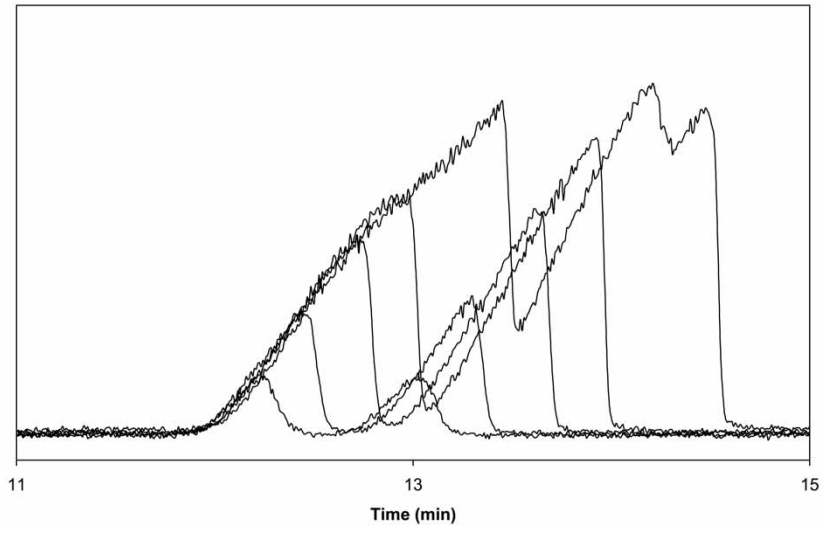

Figure 6. Warfarin peak shape in the presence of triethylamine. Column: β-cyclodextrin. Mobile phase composition: acetonitrile:methanol:amine (90%:10%:0.05%). Warfarin concentration from highest to lowest: 8×10^{-4} M; 4×10^{-4} M; 2×10^{-4} M; 1×10^{-4} M; 5×10^{-5} M.

warfarin cannot interact directly with cyclodextrin and tends to interact with other warfarin molecules instead. When triethylamine is present, it has a tendency to interact with the acidic hydroxyl group on warfarin ($pK_a = 4.5$) to facilitate its elution, which corresponds to the much shorter retention time. But it probably cannot compete and displace warfarin from the cyclodextrin sites or from the sites where the solute molecules interact with each other, thus it does not affect the fronting shape. In contrast, acetic acid ($pK_a = 4.76$) can effectively compete and replace warfarin and methanol from similar sites of cyclodextrin. In this manner, the linear range of the isotherm is increased and the fast acid-base kinetics improve the peak shape.

Four-Component Mobile Phase

When all four components are present in the mobile phase, the peak shapes are within the linear isotherm range to provide reasonable thermodynamic and kinetic information. Thus, as the concentration of each mobile phase component is changed, a detailed examination of its individual effect on the thermodynamic and kinetic behavior of the coumarin-based solutes is possible. The mobile phase composition of acetonitrile:methanol:acid:amine (99%:1%:0.15%:0.10%) is chosen as the standard composition, from which all variations are measured. Methanol is varied at 0%, 1%, 3%, and 5%; acetic acid is varied at 0.05%, 0.10%, 0.15%, 0.20%, and 0.30%; triethylamine is varied at 0.05%, 0.10%, 0.15%, and 0.20%.

Comparison of Solutes

At the standard mobile phase composition, the retention factor, chiral selectivity, and rate constants are reported in Table 3. The trend in retention factor has a close relationship with the solute structure in Figure 1. As generally believed, retention in the polar-organic mode with the β-cyclodextrin CSP is mainly due to hydrogen bonding. The achiral compound, 4-hydroxycoumarin, has the largest retention factor due to its strong intermolecular hydrogen bonding with the stationary phase. The interactions of two sites, the hydroxyl and carboxyl groups of 4-hydroxycoumarin, contribute predominantly to the retention. The relatively planar structure of 4-hydroxycoumarin facilitates the simultaneous interaction of both sites with the hydroxyl groups on the rim of cyclodextrin. All of the chiral solutes have smaller retention than 4-hydroxycoumarin because of the bulky groups inserted between the hydroxyl and carboxyl groups, which block their simultaneous interaction. Coumafuryl, warfarin, and coumachlor have a similar structure but differ slightly in one substituent on the chiral carbon. For coumafuryl, the oxygen of the furan ring has the ability to hydrogen bond with the hydroxyl group. Due to this intramolecular hydrogen bonding, the intermolecular hydrogen bonding of the hydroxyl group with the stationary phase is decreased. Thus,

Table 3. Comparison of thermodynamic and kinetic behavior of solutes at standard mobile phase composition[a]

Solute	k_1[b]	k_2[b]	α[c]	k_{ms1} (s^{-1})[d]	k_{ms2} (s^{-1})[d]	k_{sm1} (s^{-1})[e]	k_{sm2} (s^{-1})[e]
Warfarin	1.06	1.39	1.31	23	30	25	42
Coumachlor	1.11	1.41	1.26	20	24	22	34
Coumafuryl	0.82	0.98	1.19	33	46	27	45
Coumatetralyl	2.87	3.05	1.06	7	11	20	33
4-Hydroxy-coumarin	3.52	N/A[f]	N/A	0.5	N/A	1.7	N/A

[a]Mobile phase composition, acetonitrile:methanol:acetic acid:triethylamine (99%:1%:0.15%:0.10%).

[b]Retention factor for the first (k_1) and second (k_2) eluted enantiomers.

[c]Chiral selectivity (α).

[d]Desorption rate constants for transfer of the first and second eluted enantiomers from stationary to mobile phase (k_{ms1}, k_{ms2}).

[e]Adsorption rate constants for transfer of the first and second eluted enantiomers from mobile to stationary phase (k_{sm1}, k_{ms2}).

[f]Not available (N/A).

coumafuryl has the smallest retention factor. By replacement of the furan ring with a benzene ring, warfarin and coumachlor do not have the same intramolecular hydrogen bonding as coumafuryl. Thus, they have slightly larger but similar retention factors due to their similar structures. Coumatetralyl has a much larger retention factor than the other three chiral solutes. The hydroxyl group can also form intramolecular hydrogen bonds with the carbonyl group, which is present in coumafuryl, warfarin, and coumachlor, but not present in coumatetralyl. Thus, coumatetralyl has the least intramolecular hydrogen bonding and, hence, the largest intermolecular hydrogen bonding with the stationary phase. Moreover, the substituent group of coumatetralyl is relatively nonpolar, which enhances its interaction with the nonpolar cyclodextrin cavity. All of these effects contribute to its largest retention factor among the chiral compounds.

Chiral selectivity, also summarized in Table 3, can help to explain the difference between the two enantiomers and their interactions with the cyclodextrin CSP. Under all conditions, warfarin has the largest chiral selectivity, around 1.30. Coumachlor has a slightly smaller chiral selectivity, around 1.25, due to the chlorine group on the benzene ring. The aromatic substituents of warfarin and coumachlor have the ability to draw the molecule to hydrogen bond closer to the chiral center by interacting with the cyclodextrin cavity. In fact, the chlorine group on coumachlor further enhances this process and weakens the much stronger hydrogen bonding interaction of the carboxyl group in the cyclic ester. The presence of chlorine enhances

retention, but not the chirally selective interaction of hydrogen bonding. Thus, the chiral selectivity of coumachlor is actually less than that of warfarin. Coumafuryl has a chiral selectivity around 1.20. Again, this is related to the smaller chirally selective interaction of hydrogen bonding, as discussed above. Coumatetralyl has the least selectivity among all the chiral molecules at around 1.05. This is related to the different structure of coumatetralyl. Its chiral carbon is within a ring structure, which restricts motion and makes it more difficult to discriminate between the two enantiomers. Also, the substituents on the chiral carbon do not have hydrogen bonding ability. These substituents are relatively nonpolar, and have a better chance to interact with the cavity of cyclodextrin. In other words, the groups with stronger inclusion effects contribute more greatly to retention but are not chirally selective.

The kinetic rate constants also have a direct relationship with the solute structure. The rate constants for 4-hydroxycoumarin are one to two orders-of-magnitude smaller than those of the chiral molecules, as shown in Table 3. Because of the two strong and simultaneous hydrogen bonding sites, the adsorption and desorption processes for 4-hydroxycoumarin on the cyclodextrin surface are extremely slow. For the chiral solutes, the restricted accessibility of the hydrogen bonding sites also influences their rates of mass transfer. In general, the longer the solutes are retained, the smaller are their rate constants. However, within each chiral pair, the second enantiomer has a faster rate of mass transfer than the first enantiomer, even though it is more retained. This behavior suggests that the chiral and achiral selective sites have different kinetic contributions, with the chiral sites having surprisingly faster mass transfer rates. Warfarin, coumachlor, coumatetralyl, and 4-hydroxycoumarin all have a faster adsorption rate (k_{sm}) than desorption rate (k_{ms}). Hence, the limiting mass transfer process is from stationary to mobile phase for these solutes. However, for coumafuryl, the limiting mass transfer process is from mobile to stationary phase.

Effect of Methanol

The effect of methanol on thermodynamics is shown in Table 4. As mentioned previously, methanol is able to hydrogen bond with the β-cyclodextrin CSP. Thus, as the concentration of methanol is increased from 0% to 5%, the retention factor of all solutes is decreased. The decrease in retention factor for all chiral compounds is similar at 44% to 48%. However, the decrease in retention factor for 4-hydroxycoumarin is the largest at 55%, which suggests that methanol is affecting 4-hydroxycoumarin more than the other compounds. This behavior also supports the previous discussion of the strong hydrogen bonding interaction between 4-hydroxycoumarin and the stationary phase. The effect of methanol concentration on chiral selectivity is relatively small. Warfarin, coumachlor, and coumatetralyl show a slight

Table 4. Effect of methanol concentration on thermodynamics[a]

	Warfarin			Coumachlor			Coumafuryl			Coumatetralyl			4-Hydroxycoumarin
% Methanol	k_1[b]	k_2[b]	α[c]	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α	k
0	1.27	1.66	1.31	1.26	1.59	1.26	0.98	1.15	1.18	3.30	3.53	1.07	4.49
1	1.06	1.39	1.31	1.11	1.41	1.26	0.82	0.98	1.19	2.87	3.05	1.06	3.52
3	0.78	1.02	1.30	0.79	0.99	1.25	0.61	0.73	1.20	2.17	2.26	1.04	2.59
5	0.68	0.87	1.28	0.71	0.87	1.23	0.54	0.64	1.20	1.85	1.88	1.02	2.02

[a]Other mobile phase composition, acetic acid:triethylamine (0.15%:0.10%).
[b]Retention factor for the first (k_1) and second (k_2) eluted enantiomers.
[c]Chiral selectivity (α).

decrease, but coumafuryl shows a slight enhancement. The effect of methanol on kinetics is shown in Table 5. As the concentration of methanol is increased, all the solutes show an increase in the desorption rate constant for transfer from stationary to mobile phase (k_{ms}). But the adsorption rate constant for transfer from mobile to stationary phase is relatively constant. In other words, because methanol acts as a displacing agent, it influences the desorption rate rather than the adsorption rate.

Effect of Triethylamine

Tables 6 and 7 demonstrate the effect of triethylamine on thermodynamics and kinetics when the acetic acid concentration is held constant at 0.15%. As the concentration of triethylamine is increased from 0.05% to 0.20%, the retention factor of all solutes is decreased. However, the chiral selectivity is simultaneously increased for warfarin, coumachlor, and coumatetralyl. As noted above, it is most likely that triethylamine is interacting with the acidic hydroxyl group of the solute molecules. Because these interactions do not directly involve the chiral center, they are probably decreasing the achiral interactions such that the chiral selectivity is actually enhanced. As the concentration of triethylamine is increased, the rate constants for mass transfer (k_{ms} and k_{sm}) are increased as well. This indicates that triethylamine increases both adsorption and desorption rates for all solutes, but the desorption rate increases faster.

Effect of Acetic Acid

Tables 8 and 9 demonstrate the effect of acetic acid on thermodynamics and kinetics when the amine concentration is held constant at 0.10%. As the concentration of acetic acid is increased from 0.05% to 0.20%, the retention factor of all chiral solutes increases. However, a further increase from 0.20% to 0.30% actually decreases the retention factor for warfarin, coumachlor, and coumatetralyl. Coumafuryl shows a continual increase over the concentration range. On the contrary, 4-hydroxycoumarin shows a continual decrease with an increase in acid concentration. While the acid serves as a displacing agent for 4-hydroxycoumarin, it may have a different effect for the chiral solutes. It is noteworthy that the chiral selectivity decreases for the chiral solutes, which suggests that acetic acid may be interacting with chiral selective sites of the stationary phase. The rate constants for mass transfer (k_{ms} and k_{sm}) decrease for the chiral solutes, but are relatively constant for the achiral solute, 4-hydroxycoumarin.

To understand this behavior more fully, a three-component mobile phase is examined to determine whether acetic acid acts in the same manner without amine. Table 10 shows the effect of acetic acid on thermodynamics and kinetics of a single solute, warfarin, when no amine is present. The data in this table are used as a direct comparison to those in Tables 8 and 9.

Table 5. Effect of methanol concentration on kinetics[a]

	Warfarin				Coumachlor				Coumafuryl				Coumatetralyl				4-Hydroxy-coumarin	
% Methanol	k_{ms1} (s^{-1})[b]	k_{ms2} (s^{-1})[b]	k_{sm1} (s^{-1})[c]	k_{sm2} (s^{-1})[c]	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms} (s^{-1})	k_{sm} (s^{-1})
0	19	23	24	38	17	21	21	33	34	34	33	39	7	11	22	39	0.4	1.8
1	23	30	25	42	20	24	22	34	33	46	27	45	7	11	20	33	0.5	1.7
3	30	42	23	42	23	33	18	32	61	62	37	46	8	14	17	32	0.9	2.3
5	33	47	22	41	28	36	20	31	73	56	39	36	N/A[d]	N/A	N/A	N/A	1.2	2.4

[a]Other mobile phase composition, acetic acid:triethylamine (0.15%:0.10%).

[b]Desorption rate constants for transfer of the first and second eluted enantiomers from stationary to mobile phase (k_{ms1}, k_{ms2}).

[c]Adsorption rate constants for transfer of the first and second eluted enantiomers from mobile to stationary phase (k_{sm1}, k_{ms2}).

[d]Not available (N/A).

Table 6. Effect of triethylamine (TEA) concentration on thermodynamics[a]

	Warfarin			Coumachlor			Coumafuryl			Coumatetralyl			4-Hydroxy-coumarin
% TEA	k_1[b]	k_2[b]	α[c]	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α	k
0.05	1.26	1.62	1.28	1.40	1.74	1.24	1.09	1.30	1.20	3.77	3.92	1.04	4.41
0.10	1.06	1.39	1.31	1.11	1.41	1.26	0.82	0.98	1.19	2.87	3.05	1.06	3.52
0.15	0.95	1.26	1.34	0.95	1.21	1.28	0.71	0.84	1.19	2.51	2.68	1.07	2.79
0.20	0.81	1.09	1.35	0.80	1.03	1.29	0.58	0.68	1.18	2.17	2.33	1.07	2.43

[a]Other mobile phase composition, acetonitrile:methanol:acetic acid (99%:1%:0.15%).
[b]Retention factor for the first (k_1) and second (k_2) eluted enantiomers.
[c]Chiral selectivity (α).

Table 7. Effect of triethylamine (TEA) concentration on kinetics[a]

	Warfarin				Coumachlor				Coumafuryl				Coumatetralyl				4-Hydroxy-coumarin	
% TEA	k_{ms1} (s^{-1})[b]	k_{ms2} (s^{-1})[b]	k_{sm1} (s^{-1})[c]	k_{sm2} (s^{-1})[c]	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms} (s^{-1})	k_{sm} (s^{-1})
0.05	13	19	16	31	10	14	14	24	23	32	25	42	3	6	12	24	0.4	1.5
0.10	23	30	25	42	20	24	22	34	33	46	27	45	7	11	20	33	0.5	1.7
0.15	36	43	34	55	30	38	29	46	39	65	28	54	10	13	25	35	0.7	1.9
0.20	37	58	30	63	38	37	31	38	55	114	32	78	18	30	39	70	0.9	2.2

[a]Other mobile phase composition, acetonitrile:methanol:acetic acid (99%:1%:0.15%).

[b]Desorption rate constants for transfer of the first and second eluted enantiomers from stationary to mobile phase (k_{ms1}, k_{ms2}).

[c]Adsorption rate constants for transfer of the first and second eluted enantiomers from mobile to stationary phase (k_{sm1}, k_{ms2}).

Table 8. Effect of acetic acid concentration on thermodynamics when amine is present[a]

% Acetic acid	Warfarin			Coumachlor			Coumafuryl			Coumatetralyl			4-Hydroxy-coumarin
	k_1[b]	k_2[b]	α[c]	k_1	k_2	α	k_1	k_2	α	k_1	k_2	α	k
0.05	0.86	1.19	1.38	0.79	1.05	1.33	0.57	0.72	1.25	2.66	2.83	1.07	3.73
0.10	1.00	1.35	1.35	0.98	1.27	1.29	0.73	0.87	1.20	2.81	2.99	1.07	3.56
0.15	1.06	1.39	1.31	1.11	1.41	1.26	0.82	0.98	1.19	2.87	3.05	1.06	3.52
0.20	1.08	1.40	1.29	1.15	1.44	1.25	0.91	1.07	1.18	2.97	3.13	1.05	3.46
0.30	0.97	1.23	1.26	1.13	1.38	1.22	0.94	1.10	1.17	2.97	3.09	1.04	3.44

[a]Other mobile phase composition, acetonitrile:methanol:triethylamine (99%:1%:0.10%).
[b]Retention factor for the first (k_1) and second (k_2) eluted enantiomers.
[c]Chiral selectivity (α).

Table 9. Effect of acetic acid concentration on kinetics when amine is present[a]

	Warfarin				Coumachlor				Coumafuryl				Coumatetralyl				4-Hydroxy-coumarin	
% Acetic acid	k_{ms1} (s^{-1})[b]	k_{ms2} (s^{-1})[b]	k_{sm1} (s^{-1})[c]	k_{sm2} (s^{-1})[c]	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms1} (s^{-1})	k_{ms2} (s^{-1})	k_{sm1} (s^{-1})	k_{sm2} (s^{-1})	k_{ms} (s^{-1})	k_{sm} (s^{-1})
0.05	37	54	32	64	24	37	19	39	68	50	39	36	9	14	23	39	0.3	1.3
0.10	25	35	25	47	21	25	21	32	30	27	22	23	8	12	22	35	0.5	1.8
0.15	23	30	25	42	20	24	22	34	33	46	27	45	7	11	20	33	0.5	1.7
0.20	21	27	23	38	16	21	19	30	37	53	34	57	7	10	19	31	0.5	1.7
0.30	20	26	19	32	14	19	16	26	28	39	26	43	5	8	15	26	0.5	1.7

[a]Other mobile phase composition, acetonitrile:methanol:triethylamine (99%:1%:0.10%).

[b]Desorption rate constants for transfer of the first and second eluted enantiomers from stationary to mobile phase (k_{ms1}, k_{ms2}).

[c]Adsorption rate constants for transfer of the first and second eluted enantiomers from mobile to stationary phase (k_{sm1}, k_{ms2}).

Table 10. Effect of acetic acid concentration on thermodynamics and kinetics of warfarin without amine present[a]

% Acetic acid	k_1[b]	k_2[b]	α[c]	k_{ms1} (s^{-1})[d]	k_{ms2} (s^{-1})[d]	k_{sm1} (s^{-1})[e]	k_{sm2} (s^{-1})[e]
0.05	2.60	2.96	1.14	5	6	13	16
0.10	1.71	1.93	1.13	8	8	13	16
0.15	1.20	1.36	1.13	11	13	13	17
0.20	0.97	1.09	1.13	12	15	12	16
0.30	0.75	0.84	1.12	19	25	14	21

[a]Other mobile phase composition, acetonitrile:methanol (99%:1%).

[b]Retention factor for the first (k_1) and second (k_2) eluted enantiomers.

[c]Chiral selectivity (α).

[d]Desorption rate constants for transfer of the first and second eluted enantiomers from stationary to mobile phase (k_{ms1}, k_{ms2}).

[e]Adsorption rate constants for transfer of the first and second eluted enantiomers from mobile to stationary phase (k_{sm1}, k_{ms2}).

Interestingly, the trends in retention are completely opposite and suggest a difference in mechanism. When the amine is absent, an increase in the acetic acid concentration alone decreases the retention factor of warfarin. Under these conditions, acetic acid probably behaves as a displacing agent for the acidic solute. When triethylamine is present in the mobile phase, the acetic acid tends to interact with the amine first to minimize the amine effect. That is why the retention factor of the chiral solutes increases initially for 0.05% to 0.20% acid in Table 8. But for 0.20% to 0.30% acid, where the amine has been completely consumed, the system behaves in the same manner as acid present alone and the retention factor decreases. The chiral selectivity exhibits the same trend and decreases whether or not triethylamine is present. This behavior indicates that acetic acid is interacting with the chirally selective sites of the stationary phase. Thus, as the retention factor is decreased, the chiral selectivity is reduced as well. Although both acid and amine can affect retention, they are actually interacting with different positions of the stationary phase or with the solutes. The acid interacts with chiral selective sites, whereas the amine interacts with achiral selective sites. With regard to the kinetic behavior, in the absence of amine, acetic acid serves only to increase the desorption mass transfer rates (k_{ms}), but not the adsorption rates (k_{sm}).

SUMMARY

In this study, the effect of mobile phase composition on thermodynamics and kinetics of chiral separations is examined. Mobile phase composition affects

the equilibration time, retention time, as well as peak shape. Two-component mobile phases containing only acetonitrile and methanol cannot reach equilibrium even after several weeks. But both acid and amine modifier shorten the equilibration time greatly from weeks to one day or less. Whereas the acetic acid modifier can increase the linear isotherm range and ensure a Gaussian peak shape, the triethylamine modifier can dramatically decrease the retention time while still enhancing chiral selectivity. In four-component mobile phases, all of the coumarin-based solutes are within their linear isotherm range and give reasonable thermodynamic and kinetic information. The elution order is strongly dependent on the hydrogen bonding ability between the solutes and the stationary phase. 4-Hydroxycoumarin has the largest retention due to simultaneous interactions at the hydroxyl and carboxyl sites. The chiral solutes have smaller retention due to steric effects and intramolecular hydrogen bonding. The kinetic rate constants typically decrease with an increase in retention factor. The mass transfer kinetics for 4-hydroxycoumarin are much slower than those for the chiral molecules because of the simultaneous interactions. For the chiral solutes, the second enantiomer has a surprisingly faster mass transfer rate than the first enantiomer although it is more retained. This behavior suggests a difference in the kinetic contributions for chiral and achiral selective sites. The concentration of each component in the four-component mobile phase is varied to observe its individual effects. Methanol can hydrogen bond with the stationary phase, thus decreasing retention and increasing the kinetics for all solutes. Triethylamine decreases retention but enhances chiral selectivity by interacting with the hydroxyl group of the solutes and with achiral selective sites of the stationary phase. But acetic acid increases retention and reduces chiral selectivity by interacting with the amine and with chiral selective sites. Both acid and amine modifiers increase adsorption and desorption rate constants. When both acid and amine are present in the mobile phase, they tend to interact with each other and complicate the separation process.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Thomas E. Beesley (Astec) for providing the Cyclobond I 2000 stationary phase and for helpful discussions. Mr. Kahsay Gebre-Yohannes (Michigan State University) assisted with the experimental measurements.

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Received December 8, 2006
Accepted December 15, 2006
Manuscript 6980L