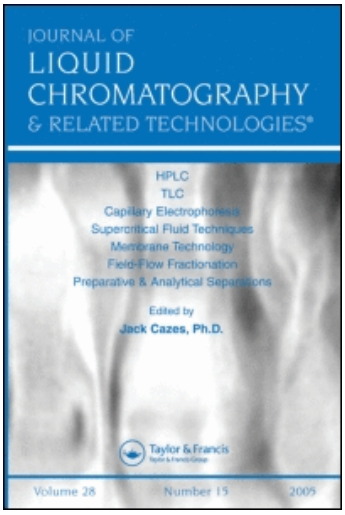

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INFLUENCE OF MOBILE PHASE COMPOSITION ON THERMODYNAMIC PROPERTIES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Tris(1,3-dimethyphenylcarbamate)-derivatized cellulose, bonded to silica, was used as a chiral stationary phase for the high performance liquid chromatographic separation of optically active potential drug substance and corresponding enantiomer over the temperature range of 12 to 40 in the reversed-phase mode. A decrease in temperature caused an increase in the retention for solutes. The van't Hoff plots generated using acetonitrile/acetate buffer mobile phase system were nicely linear, thus, we calculated enthalpies and entropies of solute transfer from the mobile to stationary phase. The influence of the mobile phase composition was studied in detail. The cavity formation effect was the major factor that governs the solute distribution between the mobile and stationary phases for acetonitrile-rich mobile phase, while the effect of solvation due to acid–base equilibrium became significant in highly aqueous mobile phase. Thermodynamic data also revealed that enantio-separation

was enthalpy-controlled separation on a chiral cellulose-derivatized column under iso-enantioselective temperature.

INTRODUCTION

Reversed phase liquid chromatography (RPLC) has been one of the most popular chromatographic tools since the 1980s. There have been numerous studies of temperature effect on solute retention in RPLC (1), extending the territory to a variety of systems including linear hydrocarbons (2), peptides (3), alkaloids (4), steroids upon addition of cyclodextrin to the mobile phase (5), enantiomers on chiral column (6,7), and polyaromatic hydrocarbons of various sizes and shapes (8,9).

Additionally, in the past two decades, a special emphasis has been placed on the organic synthesis of enantiomerically pure compounds in the pharmaceutical industry. This emphasis can be attributed in a large part, to the heightened awareness that pharmaceutical and toxicological differences can exist between enantiomers in the living system. Consequently, chromatographic enantio-separation has become a priority, and is still a challenge despite the exponential development in chiral technologies.

Separations performed with chiral stationary phases can be accomplished through a number of different mechanisms. The major mechanisms are ligand exchange, -interactions, and inclusion. Cellulose-derivatives exhibits excellent capability for enantio-separation as the chiral stationary phase (10). Many cellulose-derivative columns are commercially available. Although, the emphasis of most of the RPLC studies has focused on the application of immobilized cellulose as a chiral resolving phase, a few mechanistic questions have been considered.

R-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone (11), Figure 1, is a new compound

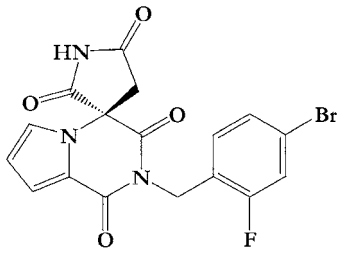

Figure 1. Chemical structure of *R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine)1,2′,3,5′-tetrone.

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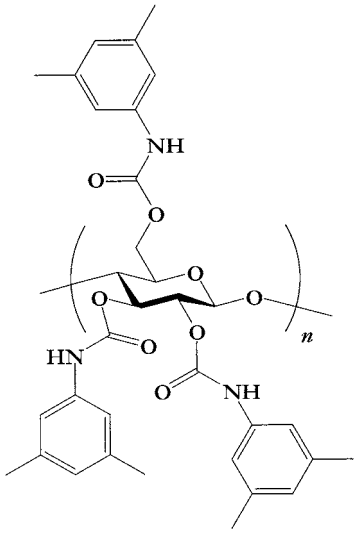

Figure 2. Chemical structure of tris(1,3-dimethylphenylcarbamate)-cellulose.

synthesized in our laboratory as a potential drug substance for the treatment of diabetes. We choose this compound, and corresponding enantiomer, as proof for investigation on the mechanism of retention and enantio-separation on the commercially available chiral column (Chiralcel OD-RH). As shown in Figure 2, the stationary phase of Chiralcel OD-RH consists of the repeating unit of chemically modified-cellulose.

Determination of thermodynamic parameters through van't Hoff plots for enantiomers, were successfully undertaken with Chiralcel OD-RH as a chiral resolving column. In this paper, we present the results of our investigation into the mechanism for enantio-separation on the Chiralcel OD-RH column.

EXPERIMENTAL

R-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone and corresponding *S*-enantiomer were synthesized in the laboratory of Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Ammonium acetate was of reagent grade from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HPLC grade acetonitrile was also purchased from Wako Pure Chemical Industries Ltd. The water was deionized prior to usage.

The chromatographic system we used was a Hitachi (Tokyo, Japan) HPLC D-7000 system. The wavelength of the detector was set at 297 nm. The column used in this experiment was Chiralcel OD-RH packed with chemically modified cellulose as the stationary phase (4.6 mm I.D. × 150 mm). The column was placed in the column oven equipped with a cooling unit, and column temperature was controlled with an accuracy of ±0.1°C over the temperature range of 12 to 40°C.

The mobile phases used were acetonitrile/0.01 *M* acetate buffer (pH 4.7) mixtures (50/50, 55/45, 57/43, 60/40, 65/35, and 70/30 v/v%). The solvent mixtures were prepared by measuring separately, and then mixing known volumes of acetonitrile and acetate buffer. Every mixture was degassed prior to its use for at least 10 min. The nominal flow rate was fixed at 0.5 mL/min throughout.

The sample solution was prepared by dissolving enantiomers in acetonitrile. With each change in column temperature and mobile phase composition, the column was permitted to reequilibrate by flushing with 15 void volumes of eluent. Duplicate injections were performed at each column temperature and mobile phase composition. Retention factors of enantiomers were measured, being employed to calculate the thermodynamic properties of solute transfer from the mobile to stationary phase.

RESULTS AND DISCUSSION

R-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine)1,2′,3,5′-tetrone and *S*-enantiomer were chromatographed at different column temperatures from 12 to 40°C, employing Chiralcel OD-RH column, with cellulose-derivative bonded to silica, as the stationary phase and acetate buffer/acetonitrile system as the mobile phase. The representative chromatogram is shown in Figure 3.

First, the effect of acetonitrile concentration on the chiral separation of enantiomers was investigated. The retention factors of each enantiomer were measured for mobile phases consisting of various acetonitrile volume fractions. The separation factors for enantiomers were unchanged at fixed column temperature.

Second, chromatographic retention data from variable-temperature runs were used to estimate thermodynamic properties, enthalpy (H) and entropy (S), according to the well-known van't Hoff relation:

$$\ln k = -\Delta H^{\circ}/RT + \Delta S^{\circ}/R + \ln \Phi \quad (1)$$

where Φ is phase ratio, and R represents gas constant. A linear van't Hoff plot is indicative of ΔH° that is invariant with temperature, whereas nonlinear van't Hoff plots suggest a change in the nature of the interaction between the solute and the stationary phase, or both. Thus, linear portions of these plots give enthalpies and entropies of the solute transfer from the slope and intercept, respectively.

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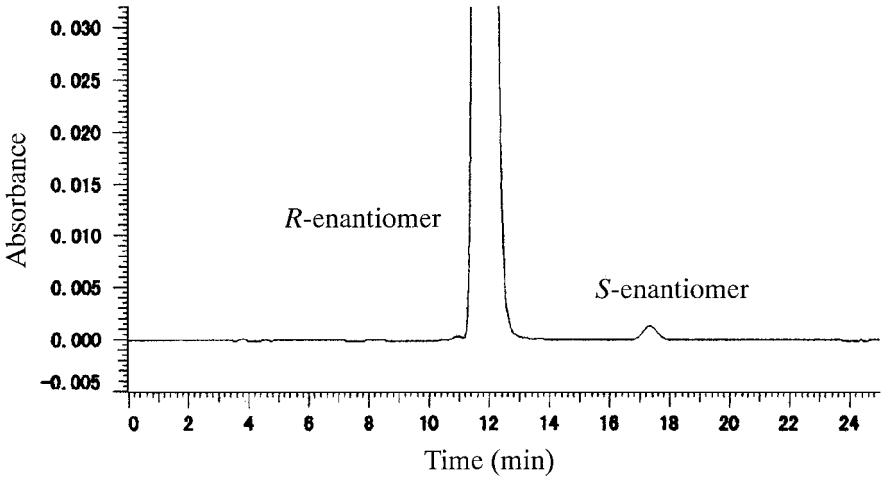

Figure 3. Separation of *R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo[1,2-a]pyrazine-4-spiro-3′-pyrrolidine)1,2′,3,5′-tetrone and corresponding enantiomer, at the level of 0.1%, on a cellulose-derivative column in the reversed-phase mode. HPLC operating conditions: column, Chiralcel OD-RH (4.6 mm I.D. × 150 mm); column temperature, 25°C; mobile phase, 0.01 *M* acetate buffer (pH 4.7)–acetonitrile (1:1); flow rate, 0.5 mL/min; detection, 297 nm.

As shown in Figure 4, the retention times of solutes increase with a decrease in column temperature. The resultant van't Hoff plot is nicely linear over the entire temperature range studied, and the regression correlation coefficients of the fits were in excess of 0.999. These plots can, therefore, be used to calculate enthalpies and entropies of transfer, although the latter can be difficult to determine owing to the nontrivial nature of calculating the phase ratio.

The enthalpies and entropies of solute transfer are plotted against mobile phase composition in Figures 5 and 6. (Note that the lines in Figures 5 and 6 are drawn to facilitate the understanding of changes of thermodynamic parameters as a function of acetonitrile content and have no statistical significance.) In our experiment, the observation is that ΔH° values get less negative with increasing acetonitrile content (60–75%) in the mobile phase. As acetonitrile is added to initially pure water, it first enters cavities in the water structure. Then, with increasing acetonitrile content, acetonitrile molecules can no longer be accommodated within these cavities and microheterogeneity eventually sets in, and at higher acetonitrile content the acetonitrile solution chemistry seems to be dominated by self-association or clustering of acetonitrile.

Therefore, in a higher acetonitrile content region, the nonpolar solute can seek out and be preferentially solvated in acetonitrile-rich mobile-phase environment,

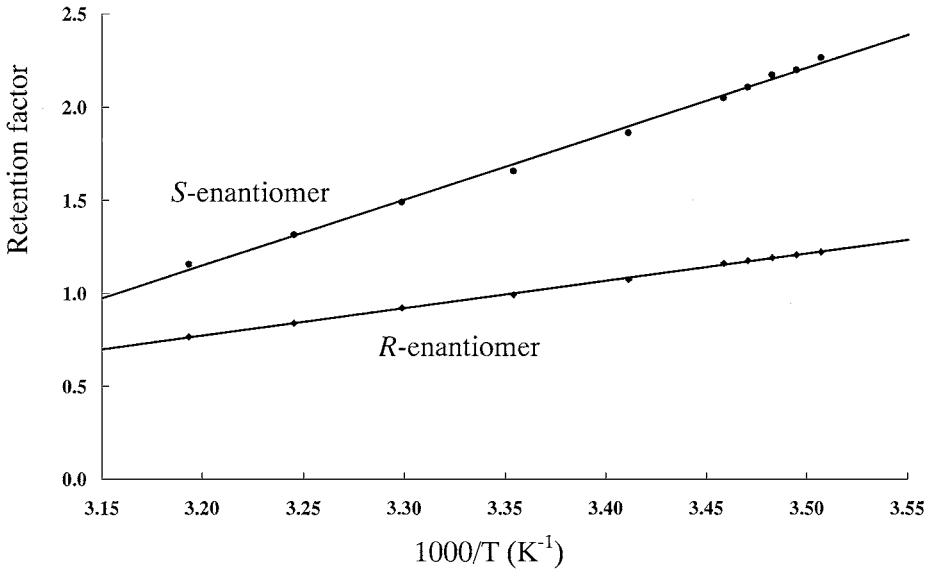

Figure 4. The representative van't Hoff plots for *R*-2-(4-bromo-2-fluorobenzyl)-(1,2,3,4-tetrahydropyrrolo [1,2-a] pyrazine-4-spiro-3′-pyrrolidine)1,2′,3,5′-tetrone and *S*-enantiomer. HPLC operating conditions: column, Chiralcel OD-RH; mobile phase, acetonitrile–acetate buffer (60:40, v/v); flow rate, 0.5 mL/min; detection UV at 297 nm.

whereas in a lower acetonitrile content region, the solute comes increasingly less in contact with acetonitrile molecules and, unavoidably, more in contact with water molecules. For nonpolar solutes, the difference in solute–solvent interactions between the mobile and stationary phase is negligible, and ΔH° is mainly dependent on the cavity formation effects.

Whenever a solute is introduced into a phase, a hole should be formed in the phase to accommodate the solute and the cavity formation is enthalpically endothermic. The cavity formation enthalpy in the mobile phase is much larger than that in the stationary phase because several interactions including dispersive interaction, dipole–dipole, hydrogen-bond interactions, are much weaker in the stationary phase than that in the mobile phase in the reversed phase mode.

Therefore, the solute prefers the stationary phase to the mobile phase in view of cavity formation enthalpy, which is in agreement with experimental data. The cavity formation enthalpy gets larger as the mobile phase gets more polar. This result strongly supports the argument that the cavity formation effect is the major factor that governs the solute distribution between the mobile and stationary phases for this system. However, in the composition range of 50–60% of acetonitrile, a

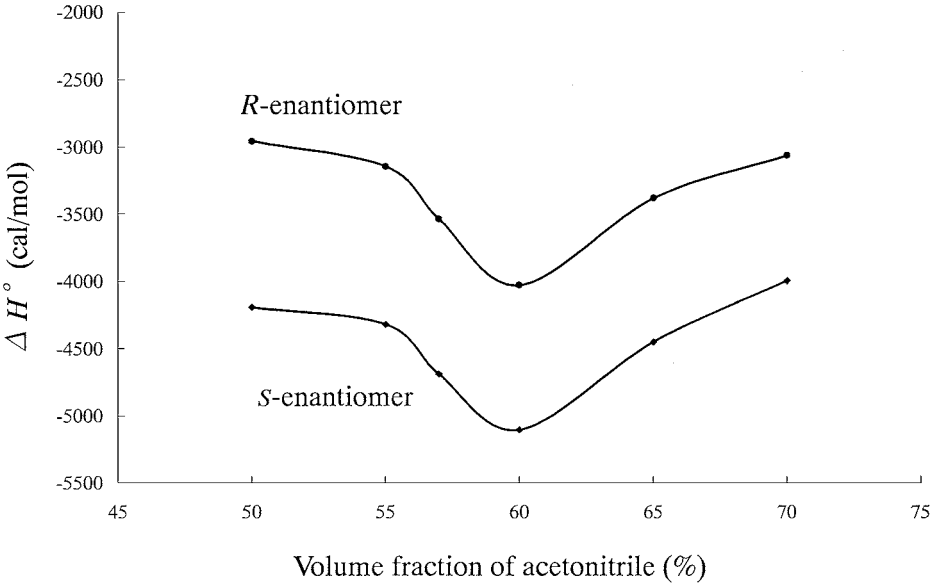

Figure 5. Relationship between enthalpies of transfer and acetonitrile content in the mobile phase.

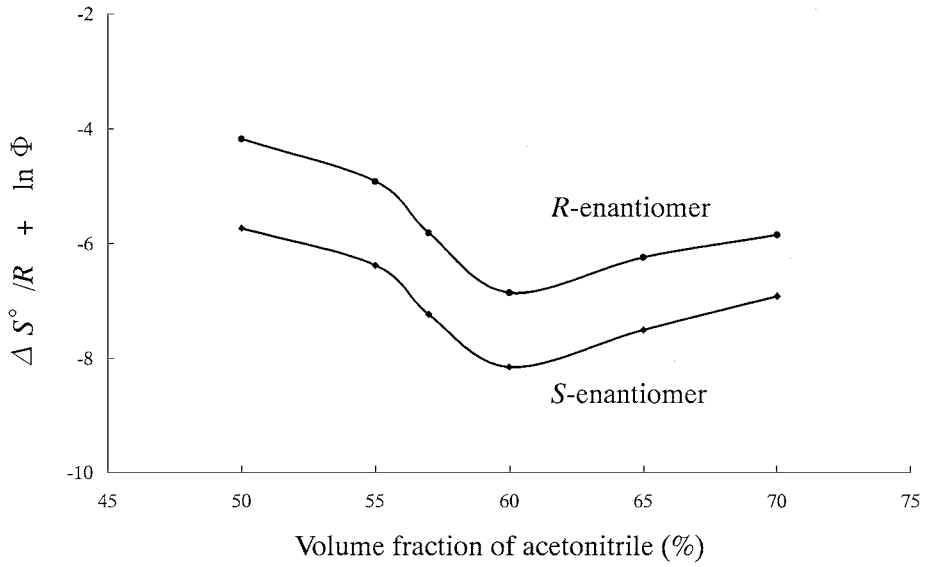

Figure 6. Relationship between entropic terms ($\Delta\Delta S^\circ/R + \ln \Phi$) of transfer and acetonitrile content in the mobile phase.

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different trend was observed. If the cavity formation effect was still dominant, ΔH° should have become more negative as acetonitrile composition in the mobile phase decreases.

Enthalpies obtained can rarely be ascribed to a single process but are rather a complex combination of various contributions such as solute-site solvation and desolvation, the unsolvated solute-site interaction, solvation of bound solute, conformational changes, multiple equilibria, and mixed retention mechanisms. In view of interaction enthalpy between a solute and solvent, the solute , if polar, will prefer the mobile phase to the stationary phase in RPLC.

To help us understand the unusual dependence of ΔH° on a function of acetonitrile content, especially at lower than 60% in the mobile phase, we invoke the following descriptive model. The solute has an acidic proton on the succinimide ring in its structure. As the volume fraction of acetate buffer (pH 4.7) increases in the mobile phase, dissociation of the solute occurs in the mobile phase. The specific solute–solvent interaction owing to the ionized solute functional group may play an important role. Since the ionized solute is quite polar, the specific interaction in the mobile phase should be somewhat strong, and will be stronger in a more polar solvent, thus ΔH° should progressively become less negative with increase of solvent polarity (decrease of acetonitrile content). Then, this hypothesis was also demonstrated by the observation of the entropic terms of solute transfer from the mobile to stationary phase, in which the pattern was similar to that of enthalpies at respective mobile phase compositions.

Generally the stationary phase is rather viscous, so a solute in this phase will lose a portion of freedom (entropy) compared to the solute in the mobile phase. Therefore, the entropies of solute transfer from the mobile to stationary phase would be negative in the reversed-phase mode. In the range of 60–70% acetonitrile, entropic term ($\Delta S^\circ/R + \ln \Phi$) gets less negative with increasing acetonitrile content in the mobile phase. As the volume fraction of acetonitrile in the mobile phase increases, clusters of acetonitrile was formed in this range, therefore entropies of solute transfer increase (12).

The reverse situation also appears in a system of relative highly aqueous mobile phase. This increase of entropy of solute transfer from the mobile to stationary phase reflects an increase in the order of the solute in the mobile phase, suggesting an increase of ionic form of solute surrounded by water molecules. Consequently, the hydration shield around the compound is more constructed in the mobile phase. The variation of trends of ΔH° and ΔS° are the results of the combination of solute–solvent interaction and cavity formation effect. The two factors exert the contrary effects each other.

To further investigate the chiral separation, van't Hoff plots were generated for enantiomers. By plotting $\ln \alpha$ (α, separation factor) versus the reciprocal of absolute temperature, all processes that do not contribute to the enantiomeric

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Table 1. The Enthalpic and Entropic Contributions to the Enantio-Separation on a Cellulose-Modified Column at 25 (unit: cal/mol)

Mobile Phase Composition (Acetonitrile %)	Thermodynamic Properties		
	$\Delta\Delta H^\circ$	$-T\Delta\Delta S^\circ$	$\Delta\Delta G^\circ$
50	−1236	924	−312
55	−1175	868	−307
57	−1155	840	−315
60	−1076	768	−308
65	−1070	749	−321
70	−932	635	−297

discrimination cancel out, and information about the thermodynamic parameters of the separation can be obtained from the following equation:

$$\ln \alpha = \Delta\Delta H^\circ/RT + \Delta\Delta S^\circ/R \quad (2)$$

The enthalpy and entropy differences for the interaction of enantiomers with the stationary phase can be obtained, according to Equation 2. On the basis of the three point model (13) of chiral recognition, one expects the more stable chiral stationary phase to solute complex, to be more rigidly structured by a greater number of simultaneous interactions, than the less stable complex ($\Delta\Delta H^\circ < 0$); on the other hand, due to steric interactions, the latter is less rigidly structured and more conformationally mobile ($\Delta\Delta S^\circ < 0$); enthalpic and entropic forces should have opposite effects on chiral recognition.

The enthalpic and entropic contributions to the enantio-separation are comparatively summarized in Table 1. The enthalpic contribution to chiral separation is greater than the entropic contribution, indicating that enantio-separation should be mainly accomplished by enthalpy-driven force over the temperature range investigated in this study. While enthalpy was increased with a increase of the volume fraction of acetonitrile, entropic term was decreased. The changes in these two factors were virtually canceling each other out, resulting in only small change in $\Delta\Delta G^\circ$ and, consequently, a small change in the separation factors. In fact, plots of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ for enantiomers are linear with correlation coefficient of 0.998.

The observed linearity is associated with a phenomenon called enthalpy–entropy compensation. This is the reason the separation factors between enantiomers were invariant against changes of mobile phase composition at a certain temperature.

While investigations of chromatographic retention mechanisms are both interesting and important, the goal of most analysts would be improved separations. It is intriguing to use van't Hoff plots to determine a temperature

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Table 2. Iso-Enantioselective Temperature Between *R*-2-(4-Bromo-2-fluorobenzyl)-(1,2,3,4-Tetrahydropyrrolo [1,2-a] Pyrazine-4-spiro-3′-pyrrolidine)-1,2′,3,5′-tetrone and Corresponding *S*-Enantiomer on a Cellulose-Derived Column Employing a Mixture of Acetonitrile and Acetate Buffer as a Mobile Phase

Acetonitrile Content (%)	Iso-Enantioselective Temperature (°C)
50	126
55	130
57	136
60	144
65	153
70	164

HPLC operating condition: column, Chiralcel OD-RH (4.6 mm I.D. × 150 mm); mobile phase, 0.01 *M* acetate buffer (pH 4.7)-acetonitrile; flow rate, 0.5 mL/min; detection, 297 nm.

at which a separation is optimized. Further, there exists a certain temperature (iso-enantioselective temperature) where Gibbs free energy difference equals zero due to enthalpy–entropy compensation. Therefore, at iso-enantioselective temperature enantio-separation will not occur. Because of enthalpy–entropy compensation, iso-enantioselective temperature was calculated at each mobile phase composition. The iso-enantioselective temperature fell around 140°C, as listed in Table 2. There will be an inversion of elution order above iso-enantioselective temperature because below iso-enantioselective enantioseparation is governed by enthalpy, whereas entropically separation of enantiomers will occur above iso-enantioselective.

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