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Unusual effect of column temperature on chromatographic enantioseparation of dihydropyrimidinone acid and methyl ester on amylose chiral stationary phase

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Abstract

This paper reports an unusual effect of column temperature on the separation of the enantiomers of dihydropyrimidinone (DHP) acid and its methyl ester on a derivatized amylose stationary phase by normal-phase liquid chromatography. The separation of the DHP acid enantiomers was investigated using both carbamate-derivatized amylose and cellulose stationary phases (Chiralpak AD and Chiralcel OD) with an ethanol–*n*-hexane (EtOH–*n*-Hex) mobile phase. On the amylose phase, the van 't Hoff plot of the retention factor of the *S*-(+)-DHP acid was observed to be non-linear while that of *R*-(−)-DHP acid was linear. Likewise, the van 't Hoff plot for DHP acid enantioselectivity was non-linear with a transition occurring at approximately 30 °C. Furthermore, the van 't Hoff plot for the DHP acid enantioselectivity factor for data taken when heating the column from 5 to 50 °C was not superimposable with the same plot prepared with data from the cooling process from 50 to 5 °C. This observation suggested that the stationary phase was undergoing a thermally induced irreversible conformational change that altered the separation mechanism between the heating and cooling cycles. Similar phenomena were observed for the separation of the enantiomers of the DHP ester probe compound. The conformational change of the AD phase was shown to depend on the polar component of the mobile phase. When 2-propanol (2-PrOH) was used as the modifier instead of EtOH, the van 't Hoff plots for DHP acid were linear and thermally reversible, suggesting that no such irreversible conformational change occurs with this modifier. Conversely, when the AD phase was pre-conditioned with a more polar methanol (MeOH) or water containing mobile phase, thermal irreversibility of DHP acid enantioselectivity was once again observed. Interestingly, when the stationary phase was changed to its cellulose analogue, the Chiralcel OD, all van 't Hoff plots for the retention and selectivity of DHP acid were thermally reversible for both EtOH–*n*-Hex and 2-PrOH–*n*-Hex mobile phases.

Keywords: Column temperature; Enantiomer separation; Chiral stationary phases, LC; Temperature effects; Conformational transition; Van 't Hoff plot, non-linear; Dihydropyrimidinone acid

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1. Introduction

Temperature is a critical parameter in chromatography and studying its effect on a separation is key to understanding the mechanism governing the chromatographic process. The effect of temperature on the retention and selectivity factors for a set of analytes is often interpreted using van 't Hoff plots of the chromatographic data. A van 't Hoff plot is a plot of either the logarithm of the retention factors of an analyte (i.e., van 't Hoff plot of k') or the selectivity factors for two analytes (i.e., van 't Hoff plot of α) versus the inverse of absolute temperature. For most separations, these plots are linear, indicating that the retention and/or selective processes governing the separation are unchanged over the temperature range studied [1,2]. Furthermore, it is typical that a separation is thermodynamically reversible. That is, both analyte retention and selectivity at a particular temperature are independent of thermal pathway taken to reach that temperature (i.e., heating or cooling).

There are cases where non-linear van 't Hoff plots for a separation have been observed [2–9]. For enantioseparations on chiral stationary phases, this non-linearity has been attributed to a change in the separation mechanism due to stationary phase ''desolvation'' [4], or a change in the conformation of the stationary phases over the temperature range studied [2,5,6]. However, to the best of our knowledge, there has not been a report of a thermally irreversible chiral separation as evidenced by non-superimposable van 't Hoff plots for the heating and cooling of the chromatographic system.

In this paper, we explore several unusual effects of temperature on the enantioseparation of dihydropyrimidinone (DHP) acid and methyl ester on a Chiralpak AD stationary phase under normal-phase conditions. Non-linear van 't Hoff plots of k' and α were obtained for the DHP acid which was attributed to an irreversible conformational change of the stationary phase. It is argued that this irreversible conformational change is influenced by the polar component of the mobile phase. For comparison, the separation of the DHP acid enantiomers was also studied using the cellulose analogue of the stationary phase (Chiralcel OD). This phase did not display the same irreversible effects seen for the AD column.

2. Experimental

2.1. Apparatus

All experiments were performed on an Agilent G1100 system with a column oven (which was used for the temperature control of the separation) and a photodiode array detector (Santa Clarita, CA, USA). The column temperature was measured by a calibrated Ertco High-Precision Thermometer (accuracy ±0.015 °C) (West Paterson, NJ, USA). The chromatographic data were acquired and analyzed by P.E. Nelson Turbochrom software (Cupertino, CA, USA). Chiralcel OD and Chiralpak AD columns (4.6×250 mm, 10 μm) were purchased from Chiral Technologies (Exton, PA, USA). Their structures are shown in Fig. 1. The recommended operating temperature by the vendor for these columns is between 0 and 40 °C. The water content of the mobile phases was measured by a 756 Brinkmann Coulometric KF instrument (Westbury, NY, USA).

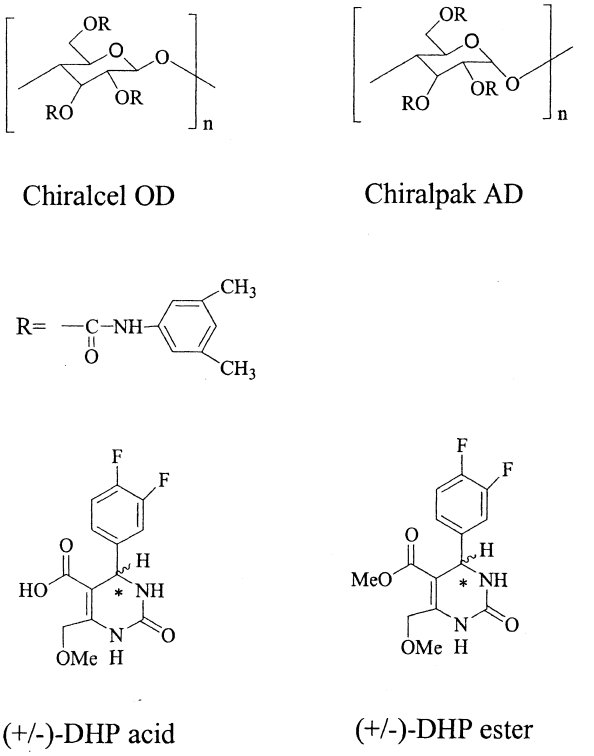

Fig. 1. Structures of Chiralcel OD and Chiralpak AD phases and the DHP acid and methyl ester analytes.

2.2. Chemicals

DHP acid and methyl ester racemates, and the pure enantiomers were prepared by the Process Chemistry Department, Merck Research Labs. (Rahway, NJ, USA). The structures of these compounds are shown in Fig. 1. 1,3,5-Tri-*tert*.-butyl-benzene was used as the void volume (t_0') marker and was purchased from Aldrich (Milwaukee, WI, USA). Trifluoroacetic acid (TFA) was purchased from Fisher Scientific (Springfield, NJ, USA). *n*-Hexane (*n*-Hex, greater than 85% *n*-Hex, greater than 99.8% total C6 isomers), 2-propanol (2-PrOH), ethanol (EtOH), and methanol (MeOH) were HPLC grade and purchased from EM Sciences (Gibbstown, NJ, USA).

2.3. Chromatographic conditions

The mobile phases were prepared at room temperature by diluting the alcohol modifiers in *n*-hexane to obtain the desired volume percent concentration for the study. Samples and the void volume marker were prepared in mobile phase at a concentration of ~0.5 mg/ml. The flow-rate was 1.0 ml/min. The chromatographic system was equilibrated with the mobile phases for 1 h whenever the experimental conditions (i.e., temperature, mobile phase, or column) were changed. The detector was operated at 220 nm with a 4 nm bandwidth. A 10-μl volume of each solution was injected. Elution orders were determined by spiking the pure enantiomeric standards with the racemic samples.

3. Results and discussion

3.1. Method development

DHP acid and ester (Fig. 1) are key intermediates for drugs of several therapeutic classes. Since these drugs are prepared as single enantiomers, control of the enantiomeric purity of these key synthetic intermediates is critical. As a result, enantioseparation methods were needed to monitor the enantiomeric purity of the DHP acid and ester. Since cellulose and amylose derivatives have been the most commonly used chiral stationary phases for LC enantioseparations [10–13], the initial screening experiments were performed with these phases. The separations were conducted by using a Chiralpak AD column under normal-phase conditions. Chromatograms of the separation of the enantiomers of the DHP acid and ester using EtOH–*n*-Hex and 2-PrOH–*n*-Hex mobile phases are given in Figs. 2 and 3, respectively. TFA was used as mobile phase modifier to improve peak shape of DHP acid. For comparison purposes, TFA was also used for the DHP ester separation. These initial experiments suggested that EtOH modified mobile phase was to be preferred. This mobile phase provided the best resolution of the DHP acid and ester enantiomers. Since temperature can have a dramatic effect on both enantioselectivity and res-

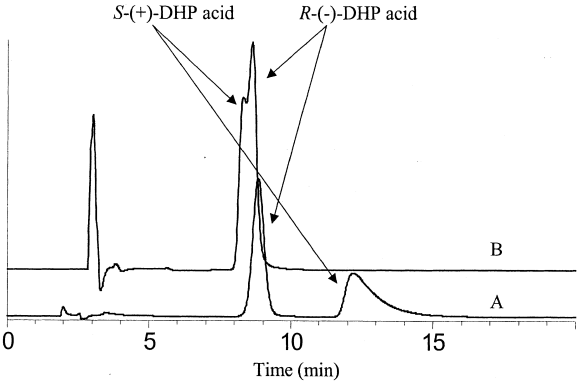

Fig. 2. Enantioseparation of DHP acid on the AD column. Mobile phase: (A) 15% EtOH in Hex with 0.1% TFA, (B) 15% 2-PrOH in Hex with 0.1% TFA. Column temperature: 25 °C.

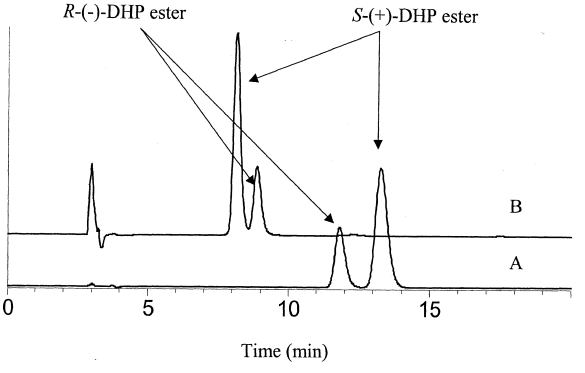

Fig. 3. Enantioseparation of DHP ester on the AD column. Mobile phase: (A) 15% EtOH in Hex with 0.1% TFA, (B) 15% 2-PrOH in Hex with 0.1% TFA. Column temperature: 25 °C.

olution, a thorough temperature study was then performed to optimize the separation and to gain insight into the separation mechanism.

3.2. Temperature studies

The relationship between the retention factor (k') for an analyte in a chromatographic system can be expressed in terms of the van 't Hoff equation:

$$\ln k' = -\left(\frac{\Delta H^0}{RT}\right) + \left(\frac{\Delta S^0}{R}\right) + \ln \Phi \quad (1)$$

In this equation, T is the absolute temperature of the chromatographic system, ΔH^0 and ΔS^0 are the changes in enthalpy and entropy of the analyte between the mobile and stationary phases, and Φ is the phase ratio.

If ΔH^0, ΔS^0 and Φ are independent of the column temperature, ln k' vs. $1/T$ should be linear. Furthermore:

$$\ln \alpha = -\left(\frac{\Delta\Delta H^0}{RT}\right) + \left(\frac{\Delta\Delta S^0}{R}\right) \quad (2)$$

ln α vs. $1/T$ (van 't Hoff plot of α) should also be linear if $\Delta\Delta H^0$ and $\Delta\Delta S^0$ are independent of the temperature. However, a non-linear plot will be observed if the stationary phase undergoes a conformational change in the temperature range [2–9].

Fig. 4A shows plots of the logarithm of the retention factor of DHP acid versus inverse temperature on the Chiralpak AD column using a 15% EtOH in *n*-Hex mobile phase. Two plots are shown for each enantiomer. One uses the retention factors observed at each temperature when heating the column upon its initial usage from the lowest to highest temperature of the range studied (filled symbols). The other plot (hollow symbols) uses the retention factors at the same temperatures during the cooling process. It was observed that the plots were not superimposable for the heating and cooling processes suggesting an irreversible separation process. Furthermore, the ''heating'' van 't Hoff plot for the *S*-enantiomer is clearly non-linear while the ''cooling'' plot for this enantiomer is nearly linear ($r^2 = 0.975$).

The van 't Hoff plots for the selectivity factor for the DHP acid enantiomers under the ''heating'' and ''cooling'' scenarios are shown in Fig. 4B. Non-linear plots for α were also obtained for both the ''heating'' and ''cooling'' processes. For the ''heating'' van 't Hoff plot (filled circles), the plot could be divided into two approximate linear regions. The first region is between 5 and 15 °C ($r^2 = 0.936$) and the second region is from approximately 40 to 50 °C ($r^2 = 0.999$) with a transition centered at approximately 30 °C, which is below the suggested maximum operating temperature for the column. In the first region, the selectivity factor slightly increases with the increase in the temperature. Beyond the

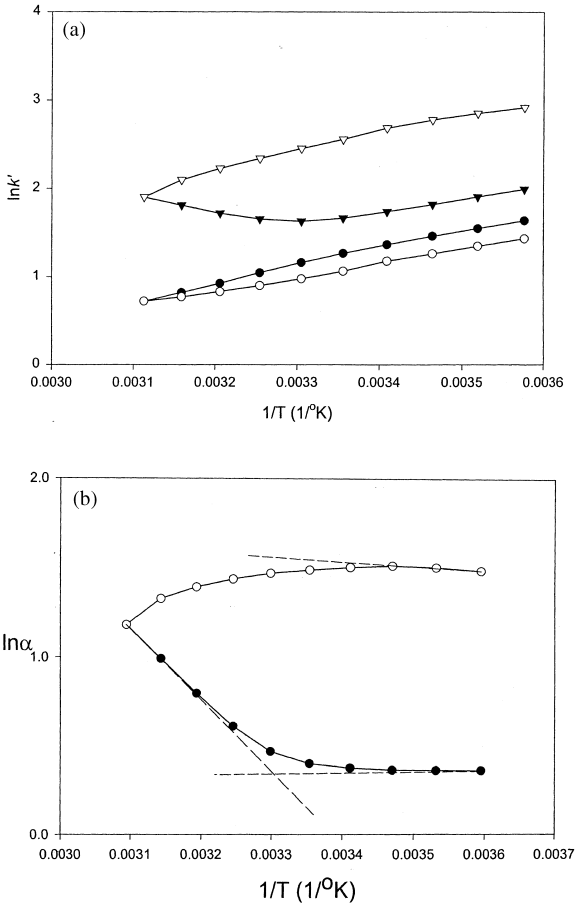

Fig. 4. van 't Hoff plots of k' and α of DHP acid on the AD column. Mobile phase: 15% EtOH in Hex with 0.1% TFA. (A) van 't Hoff plots of k'. Procedures: *R*-(−)-DHP acid, heating (filled circle), cooling (hollow circle); *S*-(+)-DHP acid, heating (filled triangle), cooling (hollow triangle). (B) van 't Hoff plots of α. Procedures: heating (filled circle), cooling (hollow circle).

transition temperature, there is then a dramatic increase in selectivity with the increase in temperature up to 50 °C. Upon cooling the chromatographic system (hollow circles), the selectivity further increases slightly from 50 °C down to approximately 20 °C. Then, it slightly decreases with a further decrease in temperature back down to 5 °C. While enantioselectivity increases slightly with the increase in temperature between 5 and 20 °C for both the heating and cooling processes, the magnitude of the enantioselectivity is clearly much greater upon cooling the column. Likewise, the resolution of the enantiomers is substantially improved after heating and then cooling the column. This is displayed in Fig. 5, which gives corresponding chromatograms at 5 and 45 °C for the heating and cooling processes.

The temperature dependence of the separation of the DHP ester enantiomers was investigated in the same manner. Fig. 6 shows the van 't Hoff plots of the selectivity factor for the ester enantiomers for the same heating and cooling cycles. As is seen for the acid enantiomers, the plots are non-superimposable. The van 't Hoff plot for the heating cycle shows a decrease in selectivity from approximately 5 °C to 35 °C up to a similar transition temperature of approximately 35 °C after which the selectivity increases with temperature from 40 to 50 °C. The cooling van 't Hoff plot for the ester enantiomers was similar in shape to that of the acid enantiomers. That is, upon cooling from 50 °C down to approximately 30 °C, an increase in selectivity is observed. The selectivity then levels off and only modest decreases are seen for further reductions in temperature back down to 5 °C. Unlike the acid enantiomers, the selectivity for the ester enantiomers at 5 °C is less than it was prior to heating the column because the heating and cooling plots cross each other. However, it is important to note here, that the magnitude of the selectivity for the ester enantiomers is very small compared to that of the acid enantiomers so that the changes seen in the van 't Hoff plots for the ester are less significant.

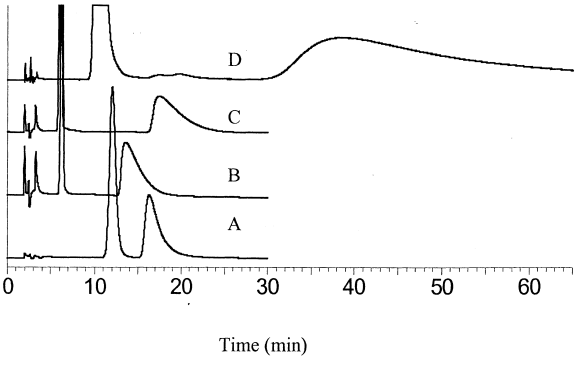

Fig. 5. Chromatograms of DHP acid with ethanol at 5 °C and 45 °C for heating/cooling processes on the AD column. Mobile phase: 15% EtOH in Hex with 0.1% TFA. (A) 5 °C, heating. (B) 45 °C, heating. (C) 45 °C, cooling. (D) 5 °C, cooling, absorbance enlarged 10-fold.

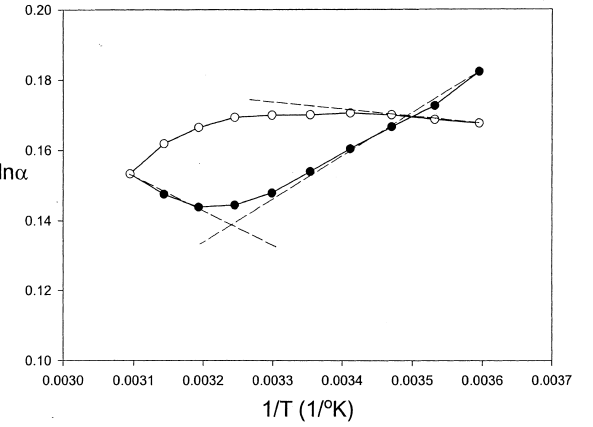

Fig. 6. van 't Hoff plots of α for DHP ester on the AD column. Mobile phase: 15% EtOH in *n*-Hex with 0.1% TFA. Procedures: heating (filled circle), cooling (empty circle).

The above observations suggest that the stationary phase could be undergoing an irreversible change in conformation at approximately 30 °C. The effect that this conformational change has on the separation of the acid and ester enantiomers is different. For the acid enantiomers, the selectivity is dramatically improved after heating the column. This indicates that the new conformation of the phase leads to stronger enantioselective interactions with the analyte, an increased number of accessible enantioselective sites, and/or a decrease in the number of accessible non-selective sites. Conformational changes for derivatized amylose or cellulose phases have previously been reported [2,6,10–17]. However, to the best of our knowledge, the reversibility of these changes was not investigated.

Recently, solid-state nuclear magnetic resonance (NMR) studies on the Chiralpak AD phase have

shown that the conformation of the phase is dependent on the polar mobile modifier under normal-phase conditions [18]. It was observed that the Chiralpak AD becomes more crystalline in character in the presence of 2-PrOH or EtOH as compared to pure *n*-Hex. Furthermore, the solvated conformation of the AD phase was observed to be different in the presence EtOH and 2-PrOH. The results of the NMR study were used to explain the reversal of elution order seen for enantiomers on an AD phase when the mobile phase modifier was changed from EtOH to 2-PrOH [19,20]. We also observed changes in elution order for both DHP acid and ester enantiomers when the modifiers changed from EtOH to 2-PrOH, see Figs. 2 and 3. Changes in solvation of the AD phase could alter the steric environment of the chiral cavities thereby effecting the enantioselectivity of the phase.

For our study, it was hypothesized that temperature may also have a dramatic effect on the solvated conformation of the AD phase and therefore the selectivity of the phase for the DHP acid and ester enantiomers. Upon heating the phase in the presence of EtOH, it is possible that the phase transitions to a more stable solvated conformation that is then maintained upon cooling the column. The significant changes made to the steric environment of the chiral cavities of the phase upon this conformational change would then explain the dramatic selectivity differences seen for the DHP acid enantiomers upon heating and then cooling the column.

Assuming that the above hypothesis is reasonable and that the phase is not permanently altered upon heating, efforts were made to regenerate the original separation. It was noted that the Chiralpak AD column was purchased from the manufacturer containing a 2-PrOH–*n*-Hex mobile phase. Thus, the same column used in the above experiments was equilibrated with 2-PrOH to see if the original conformation of the phase could be obtained. After exposing the phase to 50% 2-PrOH–*n*-Hex at 25 °C for 1 h, the column was re-equilibrated with the EtOH–*n*-Hex mobile phase and the DHP enantiomers were injected. Indeed, the original separation prior to heating the column was obtained suggesting that the original conformation of the AD phase was regenerated. Once this was done, the identical thermal transition was again observed upon re-heating and cooling the column across the temperature range studied in Fig. 4A and B.

To further explore the effects of the polar modifier on the separation of the DHP acid enantiomers, the same temperature experiments were performed using 2-PrOH as the mobile phase modifier instead of EtOH. In this case, the chromatograms for the heating and cooling cycles (Fig. 7) are essentially superimposable, indicating that solvation effects of 2-PrOH on the conformation of the AD phase are reversible with temperature.

3.3. Comparison of Chiracel OD

To investigate as to whether the same thermal behavior occurs with the Chiralcel OD phase, the cellulose analogue of the Chiralpak AD, similar experiments were conducted with this stationary phase. It was observed that van 't Hoff plots in the selectivity factor for both the DHP acid and ester enantiomers on the OD column were linear and superimposable for the heating and cooling cycles using a 12% EtOH in *n*-Hex mobile phase (data not shown here). This indicates that no conformational change occurs for the OD over the same temperature range used for the Chiralpak AD column. Likewise, using 2-PrOH modified mobile phase (12% 2-PrOH in *n*-Hex), the van 't Hoff plots in the selectivity factor for both the DHP acid and ester enantiomers were also superimposable for heating and cooling cycles. For the acid enantiomers, the plot was linear

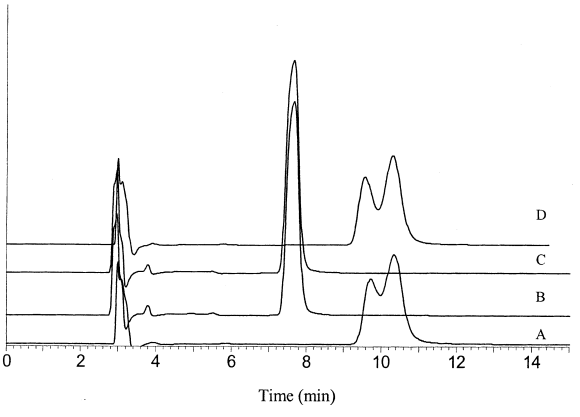

Fig. 7. Chromatograms of DHP acid with 2-propanol at 5 and 45 °C in heating/cooling processes on the AD column. Mobile phase: 15% 2-PrOH in Hex with 0.1% TFA. (A) 5 °C, heating. (B) 45 °C, heating. (C) 45 °C, cooling. (D) 5 °C, cooling.

with a positive slope. For the ester, the plot was slightly curved concave upward with a trend toward increasing selectivity with increasing temperature (Fig. 8). Thus, the irreversible conformational changes seen with the Chiralpak AD phase do not occur with the Chiralcel OD phase under similar mobile phase conditions.

3.4. Effect of column history on selectivity

The effect of polar mobile phase additives on the performance of the AD column was further investigated. The above observations suggest that strong solvation of polar mobile phase components may have an irreversible effect on the conformation and therefore the selectivity of the stationary phase. This should be considered during method development because some solvated conformations may be irreversible upon conditioning a column with a particular mobile phase. As a result, dramatically different results can be obtained using two identical columns that were previously exposed to different mobile phase modifiers.

The effect of pre-conditioning the Chiralpak AD column with MeOH prior to the separation of the DHP acid enantiomers was investigated. A new Chiralpak AD column was used to separate the DHP acid enantiomers over a temperature range from 5 to 45 °C (5 °C increments) with the same EtOH modified mobile phase (15% EtOH in *n*-Hex) used in the above experiments. The column was then conditioned with a mobile phase in which half of the EtOH was replaced with the more polar modifier MeOH to give a 15% (v/v) MeOH–EtOH (1:1, v/v) in *n*-Hex mobile phase. The column was then re-equilibrated with 15% EtOH in *n*-Hex mobile phase and the enantiomers were injected over the same temperature range. Finally, the column was conditioned with ~20 column volumes of 2-PrOH and the separation of the DHP enantiomers was repeated a third time over the same temperature range. The van 't Hoff plots for selectivity factors for the DHP acid enantiomers for the initial, post MeOH/EtOH wash, and post 2-PrOH wash runs are presented in Fig. 9.

Fig. 9 shows that the selectivity factors increase after washing the column with the MeOH–EtOH mobile phase. This suggests that the phase underwent an irreversible conformation change in the presence of MeOH that increased its selectivity for the DHP acid enantiomers. Once again, re-generation of the original separation was attempted by washing the column with 2-PrOH for an extended period of time. However, after such treatment, the selectivity of the enantiomers still remained higher than the original case. These results further indicate that the polar modifier solvation effects the conformation of

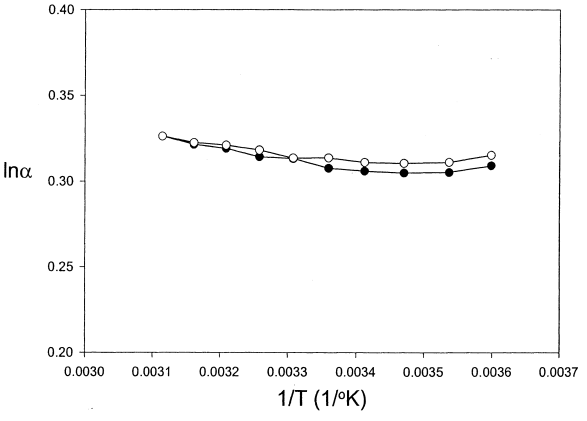

Fig. 8. van 't Hoff plots of α of DHP ester on the Chiralcel OD column. Mobile phase: 12% 2-PrOH in *n*-Hex with 0.1% TFA. Procedures: heating (filled circle), cooling (hollow circle).

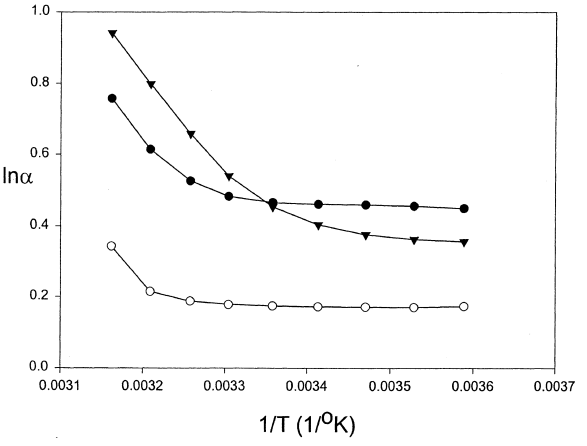

Fig. 9. Effect of column history on enantioseparation of DHP acid. Mobile phases: 15% EtOH in Hex with 0.1% TFA. Column: Chiralpak AD. Column history: injections before 15% MeOH–EtOH (1:1) in Hex with 0.1% TFA (hollow circle); injections after 15% MeOH–EtOH (1:1) in Hex with 0.1% TFA (filled circle); injections post 3 h of 2-PrOH wash (filled triangle).

the stationary phase. Therefore the observed selectivity of the stationary phase strongly depends on the column history.

Finally, the effect of water contamination of the mobile phase was explored to see if such contamination effects the performance of the column. On a Chiralcel OD column, the effect of water content on the separation of amino alcohol enantiomers has been studied [21–24]. A change in water content can also change elution order of a racemate [23]. Figs. 10A and B show the effect of mobile phase water at concentrations of 40, 140, and 320 ppm in the 15% EtOH in *n*-Hex mobile phase on the separation of the DHP acid at 5 and 50 °C, respectively. No significant effect of the increasing water concentration was seen at 5 °C. However, at 50 °C, a temperature greater than the previously observed transition temperature for this mobile phase, the selectivity of the enantiomers were observed to increase with the increase in the water content of the mobile phase. This increase in selectivity is due to the combination of a reduction in retention of the earlier eluting enantiomer and an increase in retention of the longer retained enantiomer. As such, it appears that water causes a thermally induced change in conformation of the phase that enhances selective interactions with the phase.

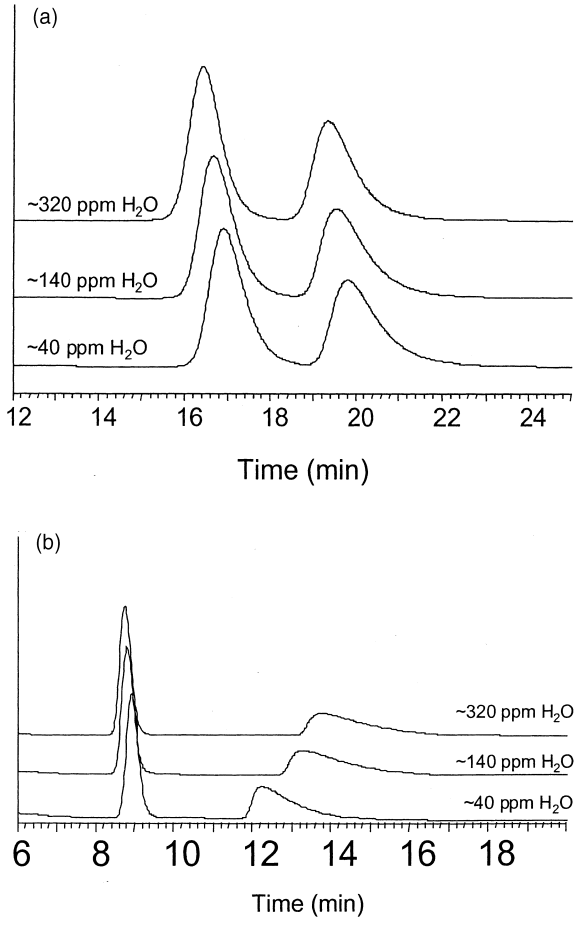

Fig. 10. Effect of water content on enantioseparation of DHP acid at different temperature on AD column. Mobile phases: 15% EtOH in Hex with 0.1% TFA. Column temperature: (A) 5 °C, (B) 50 °C.

4. Conclusion

An irreversible change in the conformation of a Chiralpak AD stationary phase was observed for the normal-phase separation of the enantiomers of a DHP acid and methyl ester. The apparent conformational change was thermally induced and depended on the polar component of the mobile phase. No such conformational changes were observed for the Chiralcel OD stationary phase, the cellulose analogue of the Chiralpak AD. Such conformational changes have implications for the reproducibility of enantiomeric recognition and should be considered during chiral method development. The procedure of changing column temperature has a pronounced effect on the enantioseparation of DHP acid and ester on the AD column. The history of the AD column also has a pronounced effect on the enantioseparation of DHP acid and ester. Moreover, the irreversible change in the conformation of these chiral stationary phases caused by modifiers and temperature can also play an important role in projecting large-scale enantioseparations, such as simulated moving bed (SMB) chromatography. Such conformational changes could be beneficial to SMB technology in terms of reproducibility and productivity.

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