Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1015 (2003) 99–110
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase

Tao Wang*, Robert M. Wenslow, Jr.

Analytical Research Department, Merck Research Laboratories, P.O. Box 2000, RY818-B225, Rahway, NJ 07065-0900, USA

Received 1 May 2003; received in revised form 10 July 2003; accepted 14 July 2003

Abstract

Following a previous publication, the present paper reports additional results on the effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) chiral stationary phase (CSP). Solid-state NMR ($^{1}H/^{13}C$ CPMAS) was utilized to identify and compare structural differences in Chiralpak AD caused by the various alcohol mobile-phase modifiers, many of which were not studied in the previous publication. The influences of the various alcohol modifiers (in hexane-based mobile phase) on the structure and chiral selectivity of the CSP were studied and compared. CPMAS spectra of Chiralpak AD flushed with the mobile phases displayed clear evidence of solvent incorporation into the CSP. When alcohol modifiers with varying size and bulkiness were used in the mobile phase, differences in structure and chiral selectivity were observed on Chiralpak AD based on solid-state NMR and chromatographic data. The change of *t*-butanol concentration in the *t*-butanol/hexane mobile phase caused changes of structure and chiral selectivity of the Chiralpak AD. These data further support our belief that the different chiral selectivities of the CSP associated with the use of different alcohol modifiers are due to different alterations of the steric environment of the chiral cavities in the CSP by the different mobile-phase modifiers.

Keywords: Mobile phase composition; Enantiomer separation; Chiral stationary phases, LC; Modifiers; Amylose; Polysaccharides; Alcohols

1. Introduction

Enantiomers of pharmaceutical compounds may display quite different pharmacological behaviors [1]. Therefore, the development of analytical methods that can identify, quantify and control the enantiomers plays a very important role in the drug development process. The US Food and Drug Administration (FDA) also issued guidelines for the development of stereoisomeric drugs [2], emphasizing the importance of identification and control of the stereoisomers. Currently, the use of high performance liquid chromatography (HPLC) to assess the chiral purity of drug substances, their synthetic intermediates and raw materials has become routine practice, owing to the commercial availability of a variety of chiral stationary

* Corresponding author. Tel.: +1-732-594-3736; fax: +1-732-594-3887.
E-mail address: tao_wang@merck.com (T. Wang).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0021-9673(03)01262-7

phases (CSP) for the direct separation of enantiomers. A recent product review of these CSPs has been published by Armstrong and Zhang [3]. Among these CSPs, polysaccharide-based CSPs form one of the most widely used classes of CSPs, due to their high versatility. Many review articles on this class of CSPs in general [4–7] and on the separation of drug enantiomers using this class of CSPs [8,9] have been published. Although the polysaccharide-based CSPs have been extensively used, detailed chiral recognition mechanisms at a molecular level have not been completely elucidated. One aspect of studying the chiral recognition mechanisms is to investigate and understand the effects of mobile-phase modifiers on the column selectivity in normal phase mode. Many research papers have been published to demonstrate the effects of mobile-phase modifiers (such as alcohols [10–19], water [20–23] and acid [22]) on column selectivity in the normal phase mode. In the studies on alcohol modifiers [10–19], the change of alcohol modifier resulted in changed chiral selectivity and, in some cases, even reversal of elution order of the enantiomers. These changes of selectivity were all rationalized by the authors as a result of alteration of the steric environment of the chiral cavities by the different alcohol modifiers. However, these arguments were all based on speculation and no direct spectroscopic evidence of structural changes of the CSPs was given.

Solid-state NMR has emerged as a key tool for structural analysis of HPLC stationary phases, especially for alkyl-bonded phases (C_8, C_{18}, C_{22}, C_{30}, etc.) [24,25]. Examples of the solid-state NMR applications in this field include determining the influence of alkyl chain length [26], temperature [27], solvent system [28–30], and column support material [31] on the stationary-phase morphology. The use of $^1H/^{13}C$ CPMAS solid-state NMR to monitor the structural change of a chiral CSP (Chiralpak AD) as a function of mobile phase composition was reported in a previous publication of ours [32]. Spectral evidence of incorporation of alcohol modifiers into the CSP and changes of CSP structure as a function of the type (ethanol versus 2-propanol) and concentration of the alcohol modifiers was clearly demonstrated. In this present paper, we report the study results on the effects of additional alcohol modifiers on the structure of Chiralpak AD using solid-state NMR ($^1H/^{13}C$ CPMAS). The effects of these alcohol modifiers on the chiral selectivity of the CSP are also reported in relation to the CSP structure in the presence of the particular alcohol modifier. These results should aid in the further understanding of chiral recognition mechanisms of the polysaccharide-based CSPs.

2. Experimental

2.1. Equipment

A Bruker DSX-400 NMR instrument (Billerica, MA, USA) was used throughout this study for obtaining solid-state NMR spectra of the CSP.

The chromatography was performed on a Shimadzu HPLC system consisting of a Model LC-10AS pump, a Model SIL-10A autosampler and a Model SPD-10AV UV detector (Kyoto, Japan). The stainless steel column (25 cm × 4.6 mm) packed with amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) coated on silica gel was purchased from Chiral Technologies (Exton, PA, USA). Chromatograms were acquired and processed by a PE Nelson data system equipped with Turbochrom software (version 6.1.2.0.1:D19) (PE Nelson, San Jose, CA, USA).

2.2. Material

The HPLC grade hexane and 2-propanol (IPA) as well as reagent grade *t*-butanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The 200-proof, dehydrated ethanol was purchased from Quantum Chemical Co. (Newark, NJ, USA). The HPLC grade 1-propanol was purchased from Aldrich (Milwaukee, WI, USA). The reagent grade 1-butanol was purchased from Sigma (St. Louis, MO, USA). Chiralpak AD column packing material coated on silica gel was a generous gift from Chiral Technologies (Exton, PA, USA).

Authentic samples of the following groups of compounds (see Fig. 1 for their structures) were provided by the Process Research Department of Merck Research Laboratories (Rahway, NJ, USA): (a) isolated 3-(*S*)-(4-fluoro)phenyl-4-benzyl-2-morpholinone (Compound A) in the form of [(1*S*)-(endo,anti)]-(−)-3-bromocamphor-8-sulfonic acid (BCSA) salt, and the racemic mixture of Compound A and its enantiomer (Compound A′) in the form of hydrochloride salt

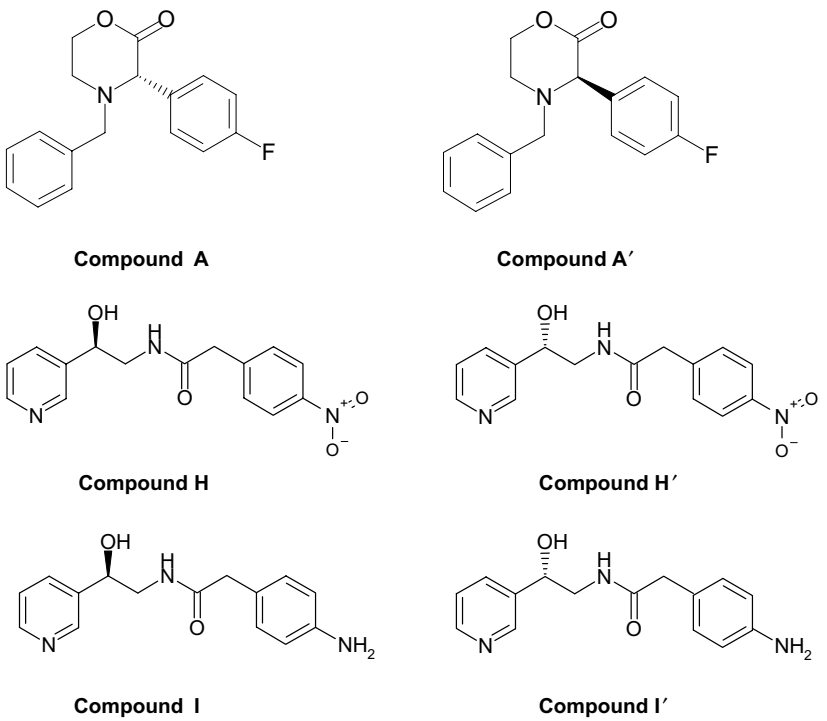

Fig. 1. Structures of compounds studied.

(HCl); (b) isolated *N*-[2-(*R*)-hydroxy-2-pyridin-3-yl-ethyl]-2-(4-nitrophenyl)acetamide (Compound H), and the racemic mixture of Compound H and its enantiomer (Compound H′); (c) isolated 2-(4-aminophenyl)-*N*-[2-(*R*)-hydroxy-2-pyridin-3-yl-ethyl]acetamide (Compound I), and the racemic mixture of Compound I and its enantiomer (Compound I′). The syntheses of these compounds are beyond the scope of this paper and will be published elsewhere.

2.3. Procedure of obtaining the free base form of Compound A from its BCSA salt

The Compound A was isolated as BCSA salt. If the sample of the salt formed was directly dissolved and injected into the chromatographic system, the BCSA would not be able to elute from the column under the selected chromatographic conditions due to the high polarity of BCSA. This would cause accumulation of BCSA on the column and deterioration of column performance over the long term. Therefore, the following procedure was developed to obtain the free base form of Compound A, and remove the BCSA from the sample matrix for chromatographic injection: Approximately, 150 mg of Compound A BCSA salt was weighed into a 50-ml centrifuge tube. 10 ml toluene and 6 ml ammonium hydroxide aqueous solution (6 wt.%) were added. The mixture was shaken for 2 min and the layers were allowed to settle by centrifuging. The organic layer was separated from the aqueous layer and washed twice with 5 ml deionized water. A 1-ml aliquot of the washed organic layer was transferred into a 50-ml volumetric flask. After evaporating the toluene solvent by nitrogen sweeping, the sample was dissolved and diluted to volume with the mobile phase for chromatography. A reversed-phase HPLC method (not described here) specific for BCSA was used to analyze a sample treated with this procedure. The result indicated that this procedure could completely remove the BCSA from the sample for chromatographic injection.

2.4. Chromatographic conditions

The mobile phase consisted of HPLC grade hexane and an alcohol modifier (ethanol, 2-propanol, 1-propanol, 1-butanol, or *t*-butanol). The flow rate was 0.5 ml/min. The column was at room temperature (~22 °C). The UV detection was performed at either 220 or 260 nm. The retention factor k' was determined as $k' = (t_R - t_0)/t_0$. The t_0 was determined by injecting hexane, which was a weaker solvent than the alcohol–hexane mixture, and noting the time of appearance of the peak due to hexane [33].

2.5. Preparation of CSP sample for solid-state NMR

Approximately 200 mg Chiralpak AD column packing was mixed with 10 ml of the corresponding mobile phase to be studied. The mixture was sealed in a vial and agitated on a laboratory rotator (Model 099A RD4512, Glas-Col, Terre Haute, IN, USA) for 6 h. The mixture was then settled on the bench and the solvent layer was removed by pipette. Nitrogen sweeping was used when necessary to remove excess solvent to obtained a wet paste of the column packing. The wet column packing was then analyzed using $^{1}H/^{13}C$ CPMAS solid-state NMR. Caution was taken to keep the column packing material wetted with the corresponding mobile phase, before and during the NMR experiment, by adding several drops of the mobile phase to the rotor containing the column packing.

2.6. Obtaining solid-state NMR spectra

All solid-state NMR spectra were obtained on the Bruker DSX-400 NMR spectrometer (9.4 T magnetic field strength) using a Bruker double-resonance CP-MAS probe and a standard CPMAS pulse sequence. The ^{13}C and ^{1}H resonance frequencies are 100.627 and 400.136 MHz, respectively, at this magnetic field strength. $^{1}H/^{13}C$ CPMAS NMR experiments were performed with 2.0 ms contact time, 4000 data points were acquired in 60 ms and then zero-filled to 8000 data points before transformation using 5.0 Hz of line broadening. Recycle delays for the $^{1}H/^{13}C$ CPMAS NMR experiments were 7.0 s. Rotor frequency was 6.0 kHz. All ^{13}C spectra were referenced to TMS using the carbonyl carbon of glycine (176.03 ppm) as a secondary reference.

3. Results and discussion

It is known that the alcohol modifiers used in the mobile phase in normal-phase mode have profound influence on chiral selectivity of polysaccharide-based CSPs [10–19]. Therefore, it would be interesting to gain structural information on this type of CSPs when they are in contact with mobile phases containing different alcohol modifiers. In our study, $^{1}H/^{13}C$ CP-MAS solid-state NMR spectra of Chiralpak AD in contact with a series of mobile phases consisting of hexane and an alcohol modifier (ethanol, 1-propanol, 2-propanol, 1-butanol or *t*-butanol) were obtained and the selected spectra compared in Fig. 2. The shift values of the resonance peaks in the spectra in Fig. 2 are tabulated in Table 1. The structure of Chiralpak AD described in [4–7] is shown in Fig. 3, with the carbon sites labeled for ease of discussion.

3.1. Effect of alcohol modifier concentration on CSP structure

For each alcohol/hexane mobile phase system, the concentration of the alcohol modifier was varied to investigate its effect on the structure of the CSP as reflected by the NMR spectra. As reported in a previous paper [32], solid-state NMR spectra were able to demonstrate that as the concentration of ethanol or 2-propanol increased in the mobile phase, the alcohol modifier gradually replaced the hexane, which was initially incorporated in the CSP, and the alcohol modifier itself became incorporated into the CSP. The NMR spectra of the CSP gradually changed with the increase of alcohol concentration until the concentration of ethanol or 2-propanol reached 20 or 5% in the respective mobile phase systems, and no further spectral changes were observed at higher alcohol concentrations. In this present study, the NMR spectra in Fig. 2(b,d,e) and peak shift data in Table 1 demonstrate that 1-propanol, 1-butanol and *t*-butanol were also incorporated into the CSP when they were in the mobile phases in contact with the CSP. Additional results indicate that in the 1-propanol/hexane, 2-propanol/hexane and 1-butanol/hexane mobile phase systems, no

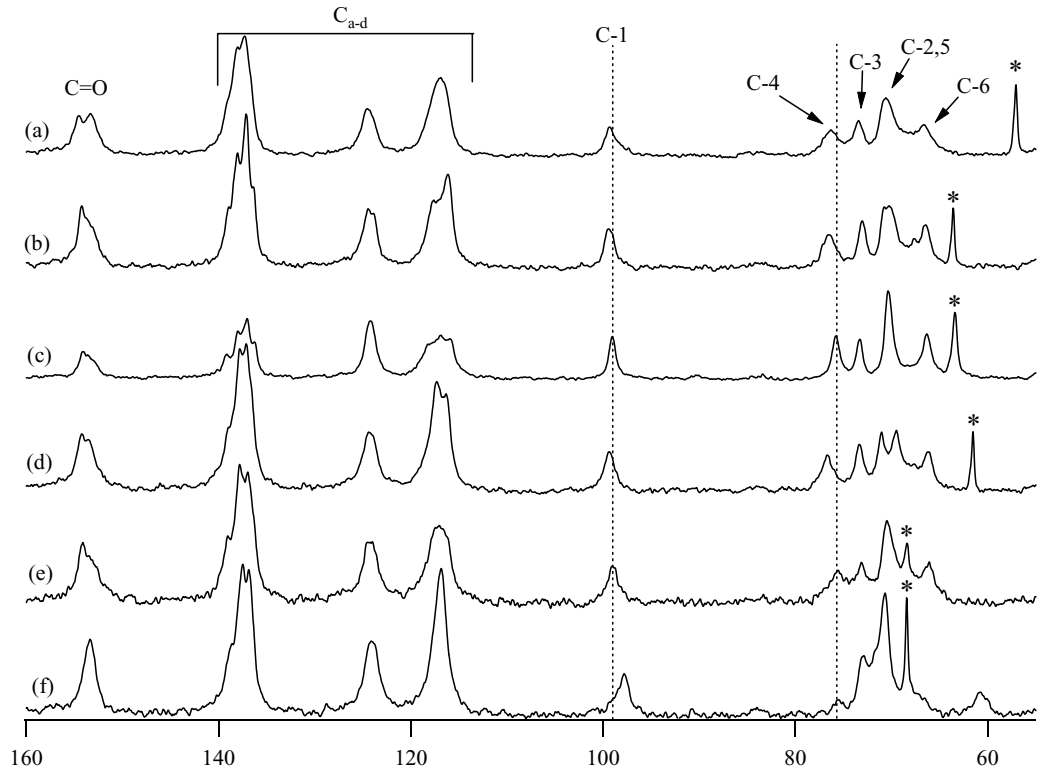

Fig. 2. $^{1}H/^{13}C$ CPMAS spectra of Chiralpak AD column packing in contact with mobile phases containing various alcohol modifiers. Mobile phase compositions: (a) hexane/ethanol, 60/40 (v/v); (b) hexane/1-propanol, 90/10 (v/v); (c) hexane/2-propanol, 90/10 (v/v); (d) hexane/1-butanol, 90/10 (v/v); (e) hexane/*t*-butanol, 90/10 (v/v); (f) hexane/*t*-butanol, 65/35 (v/v). All spectra are scaled to match the intensity of the peak at ∼124 ppm. Asterisks indicate peaks due to incorporated alcohols. Additional alcohol peaks present in the spectral region between 0 and 55 ppm (not displayed) are listed in Table 1.

changes of the CSP spectra were observed when the 1-propanol, 2-propanol or 1-butanol concentration was changed from 10 to 30%. This indicates that the CSP structure remained unchanged when the alcohol concentration in the mobile phase was above 10%. In the *t*-butanol/hexane mobile phase system, however, significant differences were noted among the spectra of the CSP when the *t*-butanol concentration was varied

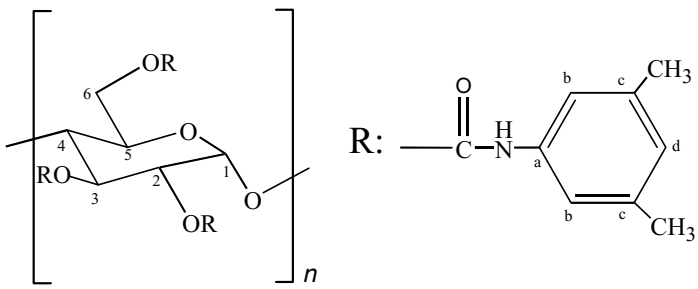

Fig. 3. Structure of Chiralpak AD.

Table 1
$^{1}H/^{13}C$ CPMAS chemical shift values for Chiralpak AD and incorporated alcohols in various mobile phase systems

Mobile phase system	Peaks due to alcohol modifier (ppm)	Peaks due to Chiralpak AD backbone[a] (ppm)					Peaks due to derivatization group on Chiralpak AD (ppm)
		C-1	C-4	C-3	C-2,5	C-6	
Hexane/ethanol (60/40, v/v)	Ethanol: 17.6 (C-2), 57.1 (C-1)	99.4	76.3	73.5	70.6	66.6	20.9 (CH_3); 116.9, 124.5, 137.2 (C_{a-d}); 153.4, 154.6 (C=O)
Hexane/1-propanol (90/10, v/v)	1-Propanol: 9.9 (C-3), 25.8 (C-2), 63.6 (C-1)	99.4	76.5	73.0	70.8, 70.2	66.6	20.9 (CH_3); 116.2, 117.6, 123.9, 124.5, 136.4, 137.2, 138.0, 139.0 (C_{a-d}); 153.2, 154.2 (C=O)
Hexane/2-propanol (90/10, v/v)	2-Propanol: 24.8 (C-1), 63.5 (C-2)	99.1	75.8	73.4	70.4	66.3	20.9 (CH_3); 116.0, 116.9, 118.2, 124.2, 136.3, 137.0, 138.0, 139.1 (C_{a-d}); 153.3, 154.1 (C=O)
Hexane/1-butanol (90/10, v/v)	1-Butanol: 13.5 (C-4), 19.0 (C-3), 35.1 (C-2), 61.6 (C-1)	99.4	76.7	73.4	71.1, 69.5	66.2	20.9 (CH_3); 116.2, 117.3, 124.3, 137.1, 137.8, 139.0 (C_{a-d}); 153.3, 154.1 (C=O)
Hexane/*t*-butanol (90/10, v/v)	*t*-Butanol: 30.9 (C-1), 68.4 (C-2)	99.1	75.7	73.1	70.6	66.1	20.9 (CH_3); 117.0, 124.2, 137.0, 137.8, 139.1 (C_{a-d}); 152.8, 154.2 (C=O)
Hexane/*t*-butanol (65/35, v/v)	*t*-Butanol: 31.0 (C-1), 68.4 (C-2)	97.8	75.6	72.9	70.7	60.8	20.8 (CH_3); 116.8, 124.1, 136.9, 137.5, 138.6 (C_{a-d}); 153.4 (C=O)

[a] Shift values for backbone carbons established in [32,36].

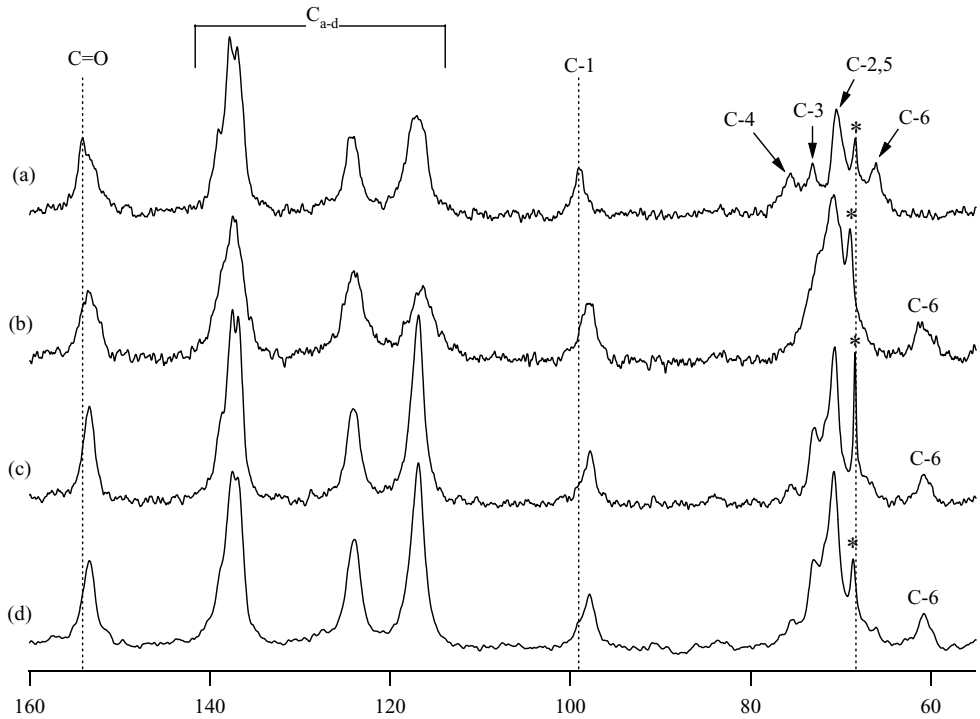

Fig. 4. $^{1}H/^{13}C$ CPMAS spectra of Chiralpak AD column packing in contact with (a) hexane/*t*-butanol, 90/10 (v/v); (b) hexane/*t*-butanol, 80/20 (v/v); (c) hexane/*t*-butanol, 65/35 (v/v); (d) hexane/1-butanol, 60/40 (v/v). All spectra are scaled to match the intensity of the peak at ∼124 ppm. Asterisks indicate peaks due to incorporated alcohols. Additional alcohol peaks are present in the spectral region between 0 and 50 ppm (not displayed).

from 10 to 40% in the mobile phase. As shown in Fig. 4 and Table 1, as the *t*-butanol concentration increased, the C-6 peak shifted up-field as much as 5.3 ppm and the C-1 peak shifted up-field 1.3 ppm. The peak for the C=O carbon also shifted up-field ∼1 ppm. More importantly, there appears to be a transition of the CSP structure at the *t*-butanol concentration of 20%, where the C-4, C-3 and C-2,5 peaks broadened and merged. This broadening of the peaks indicated that the CSP structure became less ordered. Interestingly, the *t*-butanol peak at ∼68 ppm was also affected by this structure transition as indicated by the down-field shift (∼0.5 ppm). When the *t*-butanol concentration reached 35%, the CSP became more ordered again as indicated by the narrowing of the peaks, while maintaining the shift changes at the C-1, C-6 and C=O positions. Further increase of the *t*-butanol concentration to 40% did not cause significant change of the CSP structure.

3.2. Effect of alcohol modifier shape (linear versus branched) on CSP structure

An examination of the spectra in Fig. 2 and the shift values in Table 1 revealed various differences among the spectra. Compared to the straight chain alcohols (1-propanol and 1-butanol), the branched alcohols (2-propanol and *t*-butanol) caused the C-1 and C-4 peaks to shift up-field. Changing 1-propanol to 2-propanol caused the C-1 and C-4 peaks to shift up-field by 0.3 and 0.7 ppm, respectively. Whereas changing 1-butanol to *t*-butanol cause the C-1 and C-4 peaks to shift up-field by 0.3–1.6 (depending on the *t*-butanol concentration) and 1 ppm, respectively. These data indicate that the structures of the CSP in contact with different alcohol modifiers are different. It has been reported that amylose tris(phenylcarbamate) possesses a four-fold left-handed helical structure based on X-ray analysis [34]. Our solid-state NMR

data [32] supported the assumption that Chiralpak AD possesses less than six-fold helical structure. Recently, using solution NMR and computer modeling Yamamoto et al. demonstrated that Chiralpak AD possesses a left-handed 4/3 helical structure [35]. Gidley and Bociek [36] proposed that structural features associated with the α-(1 → 4) glycosidic linkage on the helix play a dominant role in determining the C-1 and C-4 chemical shifts in the solid-state NMR spectra. As discussed earlier, spectral evidence demonstrated that the alcohol molecules were incorporated into the CSP structure. It is reasonable to expect that the C-1 and C-4 sites are subject to twisting caused by torsion force from the insertion of the alcohol molecules. The insertion of alcohol molecules of different size and shape may cause different degrees of twisting to the glucose units on the CSP helix. Since the branched alcohols have bulkier structures compared to the linear alcohols, the different degrees of twisting to the glucose units caused by bulkier molecules would generate different structural environments to the C-1 and C-4 sites, thus producing different C-1 and C-4 chemical shifts. Gidley and Bociek [36] observed ∼1 ppm up-field shifts for the C-1 and C-4 signals when amylose was complexed with *t*-butanol as opposed to 1-naphthol. Our data on Chiralpak AD, a derivatized amylose, are similar to those of Gidley and Bociek and therefore reasonable in terms of both magnitude and root cause.

Gidley and Bociek also reported that the C-1 chemical shift value is inversely proportional to either the sum of the moduli of torsion angles ϕ and ψ ($|\phi|+|\psi|$), or the modulus of torsion angle ψ ($|\psi|$) [36]. This means that a decrease of C-1 chemical shift value indicates an increase of factors $|\phi|+|\psi|$ and $|\psi|$, which translates to an increased degree of twisting of the glucose units away from a linear arrangement. Thus, in our case, the decreased C-1 shift values associated with the branched alcohols indicate increased twisting of the Chiralpak AD helical structure caused by the insertion of the branched alcohol molecules.

Between the spectra of Chiralpak AD in contact with linear and branched alcohol modifiers, in addition to the C-1 and C-4 shift differences, another difference can be noticed from Fig. 2. The linear alcohols caused the C-2,5 peak to split. 1-Propanol caused a noticeable split ($\Delta = 0.6$ ppm) of the C-2,5 peak, which appeared as one peak when the CSP was in contact with 2-propanol. Similarly, 1-butanol caused the C-2,5 peak to spilt into two clearly resolved peaks ($\Delta = 1.6$ ppm), which appeared as one merged peak when the CSP was in contact with *t*-butanol.

3.3. Effects of CSP structural differences on chiral selectivity (α)

Although the exact chiral recognition mechanism on the polysaccharide-based CSPs has not been fully elucidated, it has been generally thought that the chiral recognition of this class of CSPs is based on the formation of solute–CSP complexes through inclusion of the enantiomers into the chiral cavities in the higher order structure of the CSP [4,6,11,37]. The binding of the solutes to the CSPs is achieved through attractive forces such as hydrogen bonding, dipole–dipole and π–π interactions between the solute and the derivatization groups (such as benzoate and phenylcarbamate) on the CSPs [8,9,11,38]. Thus, the stereo environment of the chiral cavity plays a very important role in defining the chiral selectivity of the CSP. In our case, the different structural features of the CSP, caused by the alcohol modifiers of different sizes/shapes, combined with the incorporation of the alcohol modifier of different sizes/shapes into the CSP structure would ultimately result in different stereo environment of the chiral cavities in the CSP, yielding different chiral selectivities. Chromatographic separations of three pairs of enantiomers (see Fig. 1 for their structures) on Chiralpak AD using mobile phases containing hexane and various alcohol modifiers were performed. Some selected chromatograms are shown in Figs. 5–7. The complete set of chromatographic data obtained under all the conditions are tabulated in Table 2. The aforementioned structural differences of the CSP under the different mobile phase conditions were apparently reflected in these chromatographic data.

3.3.1. Effect of alcohol modifier shape (linear versus branched) on α

In most of the separation cases in Table 2, the use of branched alcohol modifiers on the separation of the same enantiomeric pair yielded elution orders different from those obtained using linear alcohols. Only two exceptions were observed: (1) for compound pair A and A′, the elution orders obtained using 1-propanol, is the same as that obtained using branched alcohols

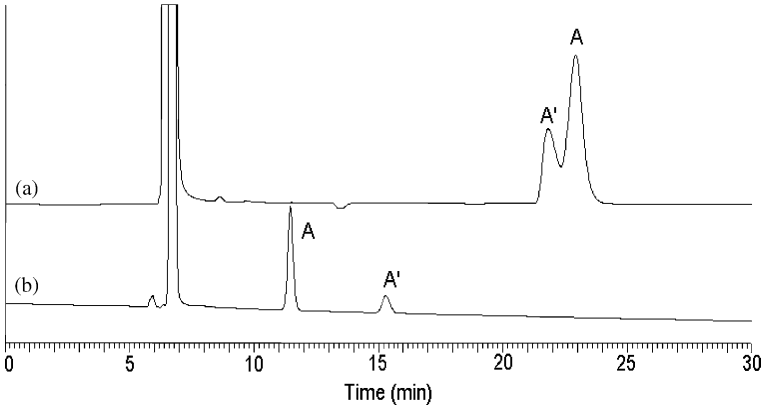

Fig. 5. Selected chromatograms for separation of Compounds A and A′ on Chiralpak AD using mobile phases (a) hexane/*t*-butanol, 90/10 (v/v) and (b) hexane/ethanol, 60/40 (v/v), respectively.

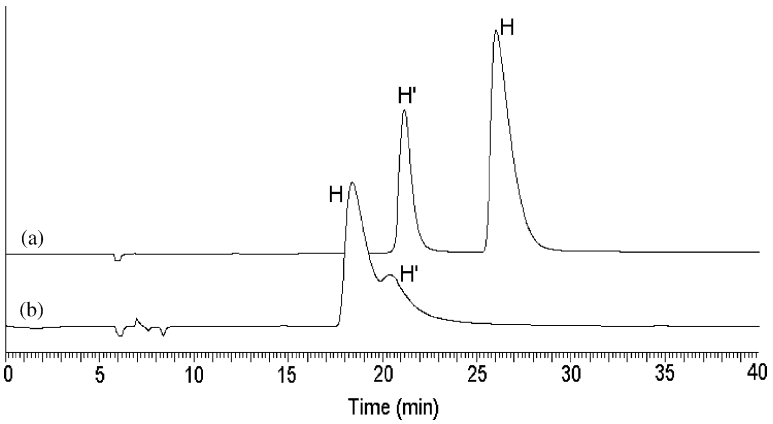

Fig. 6. Selected chromatograms for separation of Compounds H and H′ on Chiralpak AD using mobile phases (a) hexane/1-propanol, 70/30 (v/v) and (b) hexane/*t*-butanol, 60/40 (v/v), respectively.

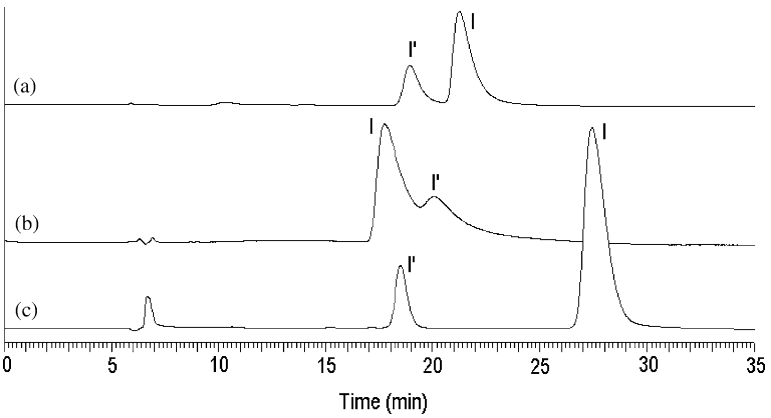

Fig. 7. Selected chromatograms for separation of Compounds I and I′ on Chiralpak AD using mobile phases (a) hexane/1-propanol, 70/30 (v/v); (b) hexane/2-propanol, 70/30 (v/v); and (c) hexane/ethanol, 60/40 (v/v), respectively.

Table 2
Chromatographic data on the separation of various enantiomeric pairs on Chiralpak AD column using different alcohol mobile-phase modifiers

Mobile phase solvents	Compounds A and A′				Compounds H and H′				Compounds I and I′			
	k'_1	α	Elution order	Mobile phase solvent ratio (v/v)	k'_1	α	Elution order	Mobile phase solvent ratio (v/v)	k'_1	α	Elution order	Mobile phase solvent ratio (v/v)
Hexane/ethanol	0.91	1.70	A/A′	60/40	4.32	1.06	H′/H	60/40	2.08	1.72	I′/I	60/40
Hexane/1-propanol	1.96	1.03	A′/A	90/10	2.59	1.32	H′/H	70/30	2.21	1.18	I′/I	70/30
Hexane/2-propanol	2.24	1.17	A′/A	90/10	2.58	1.07	H/H′	70/30	1.90	1.21	I/I′	70/30
Hexane/1-butanol	2.19	1.26	A/A′	90/10	3.63	1.00	NS[a]	70/30	2.86	1.06	I′/I	70/30
Hexane/*t*-butanol	2.70	1.07	A′/A	90/10	2.06	1.16	H/H′	60/40	3.27	1.08	I/I′	65/35

[a] NS: no separation.

(2-propanol and *t*-butanol); (2) for compound pair H and H′, co-elution was observed when 1-butanol was used. However, in both of these cases, the separation factor (α) obtained using branched and linear alcohols of the same molecular weight were noticeably different, therefore still supporting the argument that the CSPs structural differences (as observed by NMR) caused by branched versus linear alcohols are responsible for the differences in its chiral selectivity.

As mentioned earlier, significant structural changes of the CSP were observed when the *t*-butanol concentration in the *t*-butanol/hexane system was varied (Fig. 4). To investigate the effect of these structural changes on the chiral selectivity of the CSP, additional chromatographic data were obtained (Table 3). Very interestingly, when the *t*-butanol concentration was lowered from 40 to 20%, the elution order of the H/H′ pair reversed. Also, when the *t*-butanol concentration was lowered from 35 to 20%, the selectivity of the CSP on the I/I′ pair changed from 1.08 to 1.00 (no separation). However, for the A/A′ pair, the change of *t*-butanol from 30 to 10% did not change the chiral selectivity of the CSP at all. These data indicate that the structural change of the CSP caused by the change of alcohol concentration may or may not affect the chiral selectivity of the CSP, depending on the size and structure of the analytes.

3.3.2. Effect of alcohol modifier size (chain length) on α

The selectivity data in Table 2 indicate that the use of linear alcohols with different chain lengths (ethanol, 1-propanol and 1-butanol) yielded quite different chiral selectivities for the enantiomeric pair A/A′. While the α value was 1.70 when ethanol was used, it decreased to 1.26 when 1-butanol was used. When 1-propanol was used, the selectivity changed dramatically, with the elution order being reversed. For the enantiomeric pairs H/H′ and I/I′, some noticeable changes of α values were also observed (Table 2) when the three different linear alcohol modifiers were used. The structural differences of the CSP reflected by the different degrees of splitting of the C-2,5 peak associated with the use of linear alcohols of varying

Table 3
Chromatographic data on the separation of various enantiomeric pairs on Chiralpak AD column using *t*-butanol as mobile-phase modifier at different concentrations

Mobile phase solvent ratio (hexane/*t*-butanol, v/v)	Compounds A and A′			Compounds H and H′			Compounds I and I′		
	k'_1	α	Elution order	k'_1	α	Elution order	k'_1	α	Elution order
90/10	2.70	1.07	A′/A	–	–	–	–	–	–
80/20	–	–	–	18.3	1.09	H′/H	18.2	1.00	NS[a]
70/30	1.10	1.07	A′/A	–	–	–	–	–	–
65/35	–	–	–	–	–	–	3.27	1.08	I/I′
60/40	–	–	–	2.06	1.16	H/H′	–	–	–

[a] NS: no separation.

chain length (described earlier) may be a reason for these chiral selectivity differences described above.

3.3.3. Possible effect of CSP crystallinity on α

Fig. 2 shows that 1-propanol and 2-propanol caused the CSP to show more features (more resolved peaks) on the cluster of peaks at approximately 135–140 ppm, which were assigned to the phenyl group. These clusters showed four partially resolved peaks compared to three and two peaks when the CSP was in contact with *t*-butanol or *n*-butanol and ethanol, respectively. The increased resolution of the resonance peaks indicated increased crystallinity or ordering of the higher order structure of the CSP [32,36,39–41]. The importance of the higher order structure of the polysaccharide-based CSPs in chiral recognition has been discussed by many others [4,6,37,42–45]. Francotte and Zhang reported that the crystallinity of a meta-methylbenzoyl cellulose CSP played an important role in defining its chiral selectivity [45]. In our case, the structural differences of the CSP reflected by the different degrees of crystallinity could also contribute to the different chiral selectivities of the CSP (shown in Table 2) used with the various alcohol modifiers.

3.3.4. Other observations and discussions

Another observation is that it is not necessarily true that any structural variation of the CSP caused by the use of a different alcohol modifier will provide different chiral selectivity for any enantiomeric pair. Table 2 shows that the selectivities for enantiomers A and A′ were very similar when 1-propanol and *t*-butanol were used, even though the CSP showed structural differences with the use of these two different alcohol modifiers. On the other hand, these structural differences of the CSP caused enantiomeric pair H/H′ to exhibit different elution order. The same happened to enantiomeric pair I/I′. These data indicate that chiral recognition depends not only on the structure of the CSP, but also on the geometric characteristics (such as size, shape and location of the functional groups) of the solute. These geometric characteristics determine the degree of fit of the solute into the chiral cavities and the extents of attractive interactions between the various functional groups on the solute and the CSP [6,9,11,38].

Based on these data presented, it is clear that structural differences of Chiralpak AD exist when the CSP is used with different alcohol modifiers in the mobile phase. The structural differences evidenced by NMR data include (a) incorporation of different alcohol modifiers into the CSP, (b) difference in degree of twisting of the glucose units in the helical structure of the CSP, (c) difference in crystallinity or structural ordering, and (d) other unassigned structural differences reflected by the splitting of the C-2,5 peak. While the effects of (b), (c) and (d) are important in determining the higher order structure of the CSP and the steric environment of the chiral cavities, the effect of (a) is also important since the alcohol molecules of different shape and bulkiness incorporated into the CSP could be inside the chiral cavities and thus affect the geometric environment of the chiral cavities. We believed that it is the combination of all these structural factors that creates a net effect on the stereo environment of the chiral cavities, which controls the degree of fit of the solute into the cavities and determines the degree of interactions between the CSP and the solute through hydrogen bonding, dipole–dipole and π–π interactions. Another factor relating to the effect of alcohol modifier is the possibility of alcohol modifier forming a solvate with the solute. The solute solvated with different alcohol modifiers will have different size and shape, which will affect the fit of the solvated solute into the chiral cavity and in turn demonstrate different chiral selectivities. Our results could not shed light regarding this aspect, since the NMR results could only address the structure of the CSP, not the solute.

4. Conclusion

Alcohol mobile-phase modifiers of varying size and bulkiness had profound effects on the structure and chiral selectivity of Chiralpak AD. The various alcohol modifiers were all incorporated into the CSP. Chiralpak AD in contact with various alcohol modifiers possessed different structures as evidenced by $^{1}H/^{13}C$ CPMAS solid-state NMR. Compared to linear alcohols (ethanol, 1-propanol and 1-butanol), the branched alcohols (IPA and *t*-butanol) caused more twisting of the glucose units on the helical structure of the CSP. Other structural changes of the CSP were also observed when the various alcohol modifiers of different bulkiness were used. Significant differences in chiral selectivity of the CSP for three pairs of enantiomers

were observed when these various alcohol modifiers were used. The differences in chiral selectivity were attributed to CSPs observed structural changes caused by the alcohol modifiers of different bulkiness. It is believed that the combined effect of the various structural changes creates a specific stereo environment on the chiral cavities, which control the chiral selectivity through steric fit of the solute into the cavities along with CSP–solute interactions such as hydrogen bonding, dipole–dipole and π–π interactions. Therefore, the bulkiness of the alcohol modifier plays an important role in determining the chiral selectivity of the CSP. Finally, the change of concentration of an alcohol modifier (such as *t*-butanol) in the mobile phase can cause changes of structure and chiral selectivity of the Chiralpak AD.

Acknowledgements

The authors thank Chiral Technologies (Exton, PA, USA) for providing the Chiralpak AD column packing material used in this study.

References

[1] D.E. Drayer, Clin. Pharmacol. Ther. 40 (1986) 125.
[2] US Food and Drug Administration, Chirality 4 (1992) 338.
[3] D.W. Armstrong, B. Zhang, Anal. Chem. 73 (2001) 557A.
[4] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[5] E. Yashima, Y. Okamoto, Bull. Chem. Soc. Jpn. 68 (1995) 3289.
[6] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020.
[7] E. Yashima, C. Yamamoto, Y. Okamoto, SYNLETT (1998) 344.
[8] H.Y. Aboul-Enein, I. Ali, Il Farmaco 57 (2002) 513.
[9] H.Y. Aboul-Enein, J. Chromatogr. A 906 (2001) 185.
[10] I.W. Wainer, M.C. Alembik, E. Smith, J. Chromatogr. 388 (1987) 65.
[11] I.W. Wainer, R.M. Stiffin, T. Shibata, J. Chromatogr. 411 (1987) 139.
[12] K. Balmer, B.-A. Persson, P.-O. Lagerstrom, J. Chromatogr. A 660 (1994) 269.
[13] H.Y. Aboul-Enein, I. Ali, J. Biochem. Biophys. Methods 48 (2001) 175.
[14] A. Kunath, F. Theil, K. Jahnisch, J. Chromatogr. A 728 (1996) 249.
[15] A. Kunath, F. Theil, J. Wagner, J. Chromatogr. A 684 (1994) 162.
[16] T. Wang, Y.W. Chen, J. Chromatogr. A 855 (1999) 411.
[17] T. Wang, Y.W. Chen, A. Vailaya, J. Chromatogr. A 902 (2000) 345.
[18] Y. Tang, J.C. Reepmeyer, L.K. Revelle, J.A. Wilson, J. Chromatogr. A 752 (1996) 93.
[19] P.S. Bonato, L.R.P. de Abreu, C.M. de Gaitani, V.L. Lanchote, C. Bertucci, Biomed. Chromatogr. 14 (2000) 227.
[20] K. Balmer, A. Persson, P.-O. Lagerstrom, B.-A. Persson, G. Schill, J. Chromatogr. 553 (1991) 391.
[21] K. Balmer, P.-O. Lagerstrom, B.-A. Persson, G. Schill, J. Chromatogr. 592 (1992) 331.
[22] S. Svensson, J. Vessman, A. Karlsson, J. Chromatogr. A 839 (1999) 23.
[23] F. Wang, T. O'Brien, T. Dowling, G. Bicker, J. Wyvratt, J. Chromatogr. A 958 (2002) 69.
[24] M. Pursch, L.C. Sander, K. Albert, Anal. Chem. 71 (1999) 733A.
[25] K.B. Sentell, J. Chromatogr. A 656 (1993) 231.
[26] K. Albert, B. Pfleiderer, E. Bayer, in: D.E. Leyden, W.T. Collins (Eds.), Chemically Modified Surfaces, vol. 3, Gordon Breach, New York, NY, 1990, p. 233.
[27] M. Pursch, S. Strohschein, H. Handel, K. Albert, Anal. Chem. 68 (1996) 386.
[28] K. Albert, B. Evers, E. Bayer, J. Magn. Reson. 62 (1985) 428.
[29] R.C. Zeigler, G.E. Maciel, J. Am. Chem. Soc. 113 (1991) 6349.
[30] P. Shah, L.B. Rogers, J.C. Fetzer, J. Chromatogr. 388 (1987) 411.
[31] M. Pursch, D.L. Vanderhart, L.C. Sander, X. Gu, T. Nguyen, S.A. Wise, D.A. Gajewski, J. Am. Chem. Soc. 122 (2000) 6997.
[32] R.M. Wenslow, T. Wang, Anal. Chem. 73 (2001) 4190.
[33] L.R. Snyder, J.J. Kirkland. Introduction to Modern Liquid Chromatography, Wiley, New York, NY, 1974, p. 27.
[34] U. Vogt, P. Zugenmaier, Presented at the European Science Foundation Workshop on Scientific Interaction in Polysaccharide Systems, Uppsala, Sweden, 1983.
[35] C. Yamamoto, E. Yashima, Y. Okamoto, J. Am. Chem. Soc. 124 (2002) 12583.
[36] M.J. Gidley, S.M. Bociek, J. Am. Chem. Soc. 110 (1988) 3820.
[37] E. Francotte, R.M. Wolf, D. Lohmann, R. Mueller, J. Chromatogr. 347 (1985) 25.
[38] E. Yashima, Y. Okamoto, in: H.Y. Aboul-Enein, I.W. Wainer (Eds.), The Impact of Stereochemistry in Drug Development and Use, Wiley, New York, NY, 1997, Chapter 12, p. 345.
[39] R. Voelkel, Angew. Chem. Int. Ed. Engl. 27 (1988) 1468.
[40] R. Born, H.W. Spiess, Macromolecules 28 (1995) 7785.
[41] R.H. Marchessault, M.G. Taylor, C.A. Fyfe, R.P. Veregin, Carbohydr. Res. 144 (1985) C1.
[42] G. Hesse, R. Hagel, Chromatographia 6 (1973) 277.
[43] A. Ichida, T. Shibata, I. Okamoto, Y. Yuki, H. Namikoshi, Y. Toga, Chromatographia 19 (1984) 280.
[44] T. Shibata, T. Sei, H. Nishimura, K. Deguchi, Chromatographia 24 (1987) 552.
[45] E. Francotte, T. Zhang, J. Chromatogr. A 718 (1995) 257.