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Enantiomeric separation of some pharmaceutical intermediates and reversal of elution orders by high-performance liquid chromatography using cellulose and amylose tris(3,5-dimethylphenylcarbamate) derivatives as stationary phases

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Abstract

Several pairs of enantiomers of pharmaceutical intermediates were separated by HPLC directly on cellulose and amylose tris(3,5-dimethylphenylcarbamate) derivatives (Chiralcel OD and Chiralpak AD) using hexane as mobile phase with 2-propanol or ethanol as modifier. The separation and elution order of the enantiomers on the two columns using different alcohol modifiers were compared. Reversal of the elution order of some enantiomeric pairs associated with increased retention of many of these solutes upon changing the mobile phase modifier from 2-propanol to ethanol was observed. The effect of structural variation of two pairs of enantiomers on their k' and separation factor α was noted. Chiralcel OD and Chiralpak AD columns provided different retention, separation and elution order of some of the enantiomeric pairs.

Keywords: Enantiomer separation; Derivatization, LC; Chiral stationary phases, LC; Cellulose; Amylose tris(3,5-dimethylphenylcarbamate); Pharmaceutical intermediates

1. Introduction

The development of analytical methods that separate and quantify the enantiomers of pharmaceutical compounds continues to play an important role in the drug development process. In a previous paper [1], we reported the separation of four pairs of enantiomers on cellulose tris(3,5-dimethylphenylcarbamate) (Chiralcel OD) and amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) using hexane with alcohol modifiers as the mobile phase. It was noted that the Chiralcel OD and Chiralpak AD columns were complementary to each other, and separations of all the enantiomeric pairs were achieved on at least one of the columns. In a continuing effort to broaden the application of these cellulose- and amylose-based chiral stationary phases (CSPs) to pharmaceutical analysis, we have recently completed the study of enantiomeric separation of some additional pharmaceutical intermediates on Chiralcel OD and Chiralpak AD columns using hexane with alcohol modifiers as the mobile phase. This paper presents the results of our recent studies. All the enantiomeric pairs were successfully separated on at

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least one of the columns. It was found that, by changing the alcohol modifier in the mobile phase from 2-propanol to ethanol, elution orders of some enantiomeric pairs were reversed, and the retention of many of the solutes that were involved in the reversal of elution order was increased. In addition, Chiralcel OD and Chiralpak AD columns provided different retention, separation and elution order of some of the enantiomeric pairs.

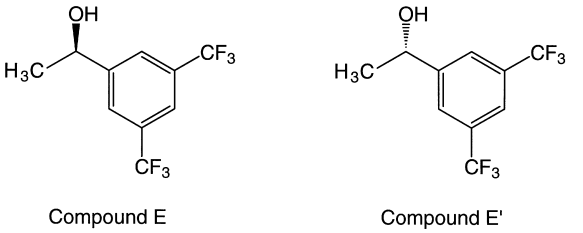

Fig. 1. Structures of compound E and its enantiomer compound E′.

2. Experimental

2.1. Instrumentation

The chromatography was performed on a Shimadzu high-performance liquid chromatography (HPLC) system equipped with a Model LC-10AS pump, a Model SIL-10A autosampler and a Model SPD-10AV UV detector (Kyoto, Japan). The stainless steel columns (25 cm×4.6 mm) packed with Chiralcel OD [cellulose tris(3,5-dimethylphenylcarbamate) coated on silica gel] and Chiralpak AD [amylose tris(3,5-dimethylphenylcarbamate) coated on silica gel] were purchased from Chiral Technologies (Exton, PA, USA). Chromatograms were acquired and processed by a PE Nelson data system equipped with Turbochrom software (version 6.1.1.1.0:K20) (PE Nelson, San Jose, CA, USA).

2.2. Materials

The HPLC-grade hexane and 2-propanol (IPA) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The 200 proof, dehydrated ethanol was purchased from Quantum (Newark, NJ, USA).

Authentic samples of the following groups of compounds (see Figs. 1–4 for their structures) were provided by Process Research Department of Merck Research Laboratories (Rahway, NJ, USA): (a) isolated 1-(*R*)-[3,5-bis(trifluoromethyl)phenyl]ethanol (compound E), and the racemic mixture of compound E and its enantiomer (compound E′); (b) isolated 4-benzyl-2-(*R*)-{1-(*R*)-[3,5-bis(trifluoromethyl)phenyl]ethoxy}morpholin-3-one (compound F), the mixture of compound F and its three stereoisomers (i.e., compounds F′, F″ and F‴), and isolated compound F‴; (c) isolated 2-(*R*)-{1-(*R*)-[3,5-bis(trifluoromethyl)phenyl]ethoxy}-3-(*S*)-(4-fluorophenyl)morpholine (compound G), and its enantiomer (compound G′) [both in the form of *p* toluenesulfonic acid (pTSA) salt]; (d) 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′); (e) 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

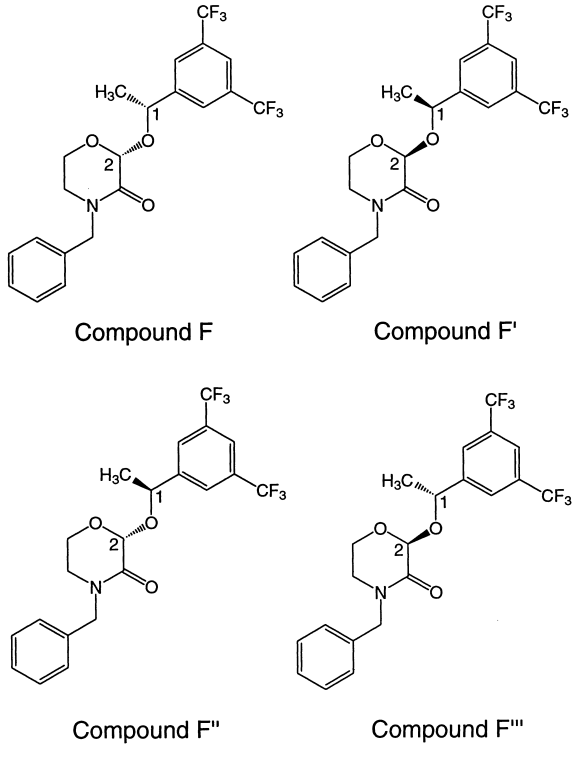

Fig. 2. Structures of compound F and its stereoisomers.

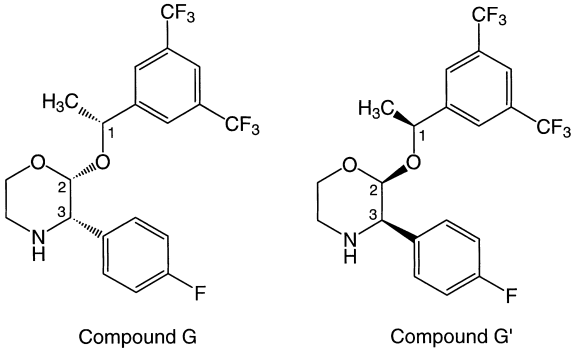

Fig. 3. Structures of compound G and its enantiomer compound G′.

compounds and mixtures are beyond the scope of this paper and will be published elsewhere.

2.3. Procedure of obtaining the free base form of compounds G and G′ from their pTSA salts

2-(*R*)-{1-(*R*)-[3,5-Bis(trifluoromethyl)phenyl]-ethoxy}-3-(*S*)-(4-fluorophenyl)morpholine (compound G) and its enantiomer (compound G′) were isolated pTSA salts. If the sample of the salt form was directly dissolved and injected into the chromatographic system, the pTSA would not be able to elute from the column under the selected chromatographic conditions due to the high polarity of pTSA. This would cause accumulation of pTSA 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 compounds G and G′, and remove the pTSA from the sample matrix for chromatographic injection: approximately 150 mg of compound G pTSA salt was weighed into a 50-ml centrifuge tube. Into the 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, each time with 5 ml deionized water. A 1-ml aliquot of the washed organic layer was transferred to 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 pTSA was used to analyze a sample treated with this procedure. The result indicated that this procedure could completely remove the pTSA from the sample for chromatographic injection.

2.4. Chromatographic conditions

The mobile phase consisted of HPLC-grade hexane and an alcohol modifier (IPA or ethanol) which were pre-mixed before use. The mobile phase flowrate was 0.5 ml/min. The column was at room

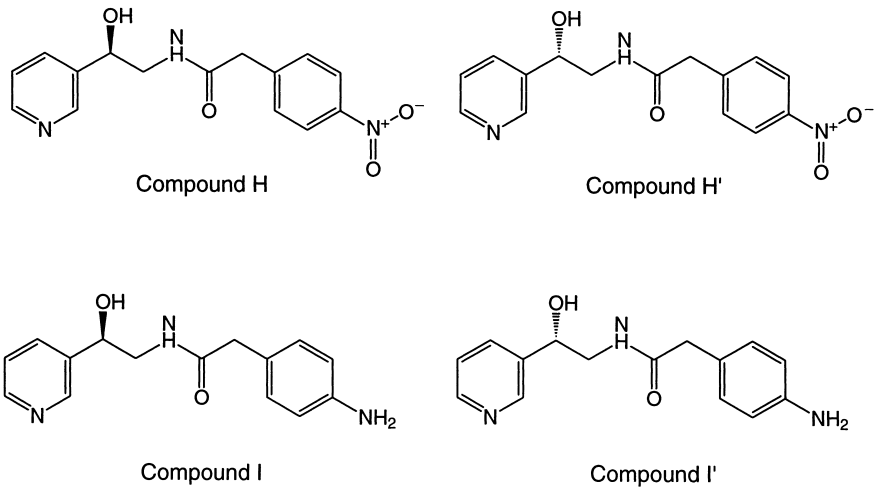

Fig. 4. Structures of compounds H and I as well as their enantiomers, compounds H′ and I′.

temperature (~22°C). UV detection was performed at 220 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 IPA–hexane or ethanol–hexane mixture, and noting the time of appearance of the peak due to hexane [2].

3. Results and discussion

Among the compounds involved in this study (Figs. 1–4), compounds E, F, G, H and I are the synthetically desired stereoisomer. Compounds E′, F′, G′, H′ and I′ are the enantiomers of compounds E, F, G, H and I, respectively. Two other stereoisomers of compound F were involved in this study. These stereoisomers, compounds F″ and F‴, are diastereomers of compounds F and F′, but are enantiomers to each other. The two CSPs used in our study were tris(3,5-dimethylphenylcarbamate) derivatives of cellulose or amylose coated on silica gel. Refs. [3–5] describe the structures of the derivatized subunits of the CSPs.

It has been assumed that the separation of enantiomers on these cellulose- and amylose-based CSPs was due to the formation of solute–CSP complexes through inclusion of the enantiomers into the chiral cavities in the higher order structures of the CSPs [3,5–7]. In the CSPs with carbamate derivatives, the binding of the solutes to the CSPs was achieved through interactions between the solutes and the polar carbamate groups on the CSPs [3,5,8]. The carbamate groups on the CSP can interact with solutes through hydrogen bonding using the C=O and NH groups, and through dipole–dipole interactions using the C=O moiety. In our study, the available functional groups on the solutes that can form hydrogen bonding with the C=O group on the CSPs include: (1) the OH group on compound E; (2) the NH group on compound G; (3) the OH and NH groups on compound H; (4) the OH, NH and NH_2 groups on compound I. Compounds F, H and I all possess a C=O group for hydrogen bonding with the NH group on the CSPs. In addition, the $N^+–O^-$ dipole on compound H can form hydrogen bonding with the NH group on the CSPs. Dipole–dipole interactions can occur between the C=O group on the CSPs and the following functional groups on the solutes: (1) the C=O group on compounds F, H and I; (2) the $N^+–O^-$ dipole on compound H. Wainer et al. [7] have reported that solute having aromatic functionalities could provide additional stabilizing effect to the solute–CSP complex by insertion of the aromatic portion of the solute into the chiral cavity. In our case, this type of stabilization effect may also exist due to the presence of the aromatic functionalities on all the solutes. Chiral discrimination between the enantiomers is due to the differences in their steric fit in the chiral cavities [3,5,7].

In the first five sections below, the separations of the five groups of stereoisomers on the two CSPs using hexane and an alcohol modifier (IPA or ethanol) as the mobile phase are presented. In the two subsequent sections, additional observations are discussed.

3.1. Separation of compounds E and E′

The chromatograms of the separation of compounds E and E′ on the Chiralcel OD and Chiralpak AD columns with hexane and an alcohol (IPA or ethanol) modifier as mobile phase are shown in Fig. 5. On the Chiralcel OD column, although baseline separation was achieved using either IPA or ethanol as the mobile phase modifier (Fig. 5a and b), the IPA modifier provided better separation. While the amylose-based Chiralpak AD had the same derivatization group (3,5-dimethylphenylcarbamate) as its cellulose-based counterpart (Chiralcel OD) did, it provided quite different chiral recognition compared to Chiralcel OD – the elution order of the enantiomers on the AD column was reversed using hexane–IPA mobile phase, while the two enantiomers co-eluted in hexane–ethanol mobile phase (Fig. 5c and d).

Okamoto's research group has reported numerous examples in which Chiralcel OD and Chiralpak AD columns showed different chiral recognition abilities, including the exhibition of different elution orders of many enantiomeric pairs on the two columns [3,5,8]. We also reported this kind of difference in a previous paper [1]. Okamoto and co-workers attributed the difference in chiral recognition ability between the two CSPs to the conformational difference between them [3,5]. Our observation on the difference in chiral recognition ability between the Chiralcel OD

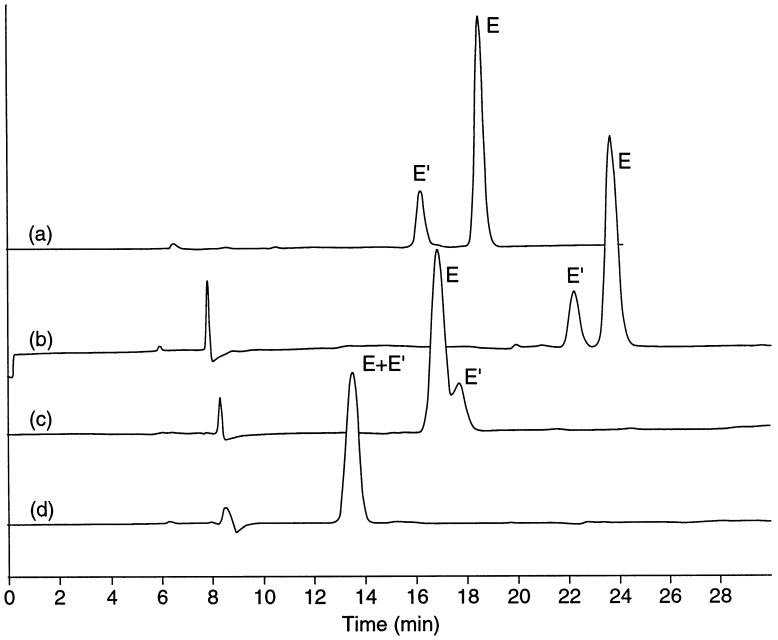

Fig. 5. Chromatograms of the separation of compounds E and E′. HPLC conditions: (a) Chiralcel OD column with hexane–IPA (97.5:2.5, v/v) as mobile phase; (b) Chiralcel OD column with hexane–ethanol (98.7:1.3, v/v) as mobile phase; (c) Chiralpak AD column with hexane–IPA (99:1, v/v) as mobile phase; (d) Chiralpak AD column with hexane–ethanol (99:1, v/v) as mobile phase.

and Chiralpak AD columns is believed to be due to the same reason.

3.2. Separation of compound F and its stereoisomers

Compound F has two chiral centers as shown in Fig. 2. The simultaneous reversal of both chiral centers gave the enantiomer, compound F′. Two other possible stereoisomers (compounds F″ and F‴) are enantiomers to each other, but are diastereomers to compounds F and F′.

Separation of the four stereoisomers was performed on the OD and AD columns with hexane–IPA or hexane–ethanol as mobile phase, respectively (Fig. 6). Under each of the four sets of conditions, separation of the four stereoisomers was achieved. On the Chiralcel OD column, only slight change of selectivity was noticed when the mobile phase changed from hexane–IPA to hexane–ethanol (Fig. 6a and b). The elution order obtained with hexane–ethanol mobile phase remained unchanged compared to that obtained using hexane–IPA mobile phase. On the AD column, however, the selectivity changed dramatically when the mobile phase changed from hexane–IPA to hexane–ethanol (Fig. 6c and d). The elution orders of the enantiomeric pairs F/F′ and F″/F‴ were reversed.

The reversals of elution orders of enantiomers on cellulose- and amylose-based CSPs upon changing the alcohol modifiers in the mobile phase have been reported by a number of research groups [9–11]. The authors attributed the reversals of elution orders to an alteration of the steric environment of the chiral cavities by the change of alcohol modifiers. In our case, the reversal of the elution orders of the enantiomeric pairs F/F′ and F″/F‴ upon changing the alcohol modifier from IPA to ethanol was probably due to the same reason.

3.3. Separation of compounds G and G′

Compound G has three chiral centers as shown in Fig. 3. Although theoretically seven other stereoisomers of compound G might exist, only one stereoisomer was possibly present in samples of

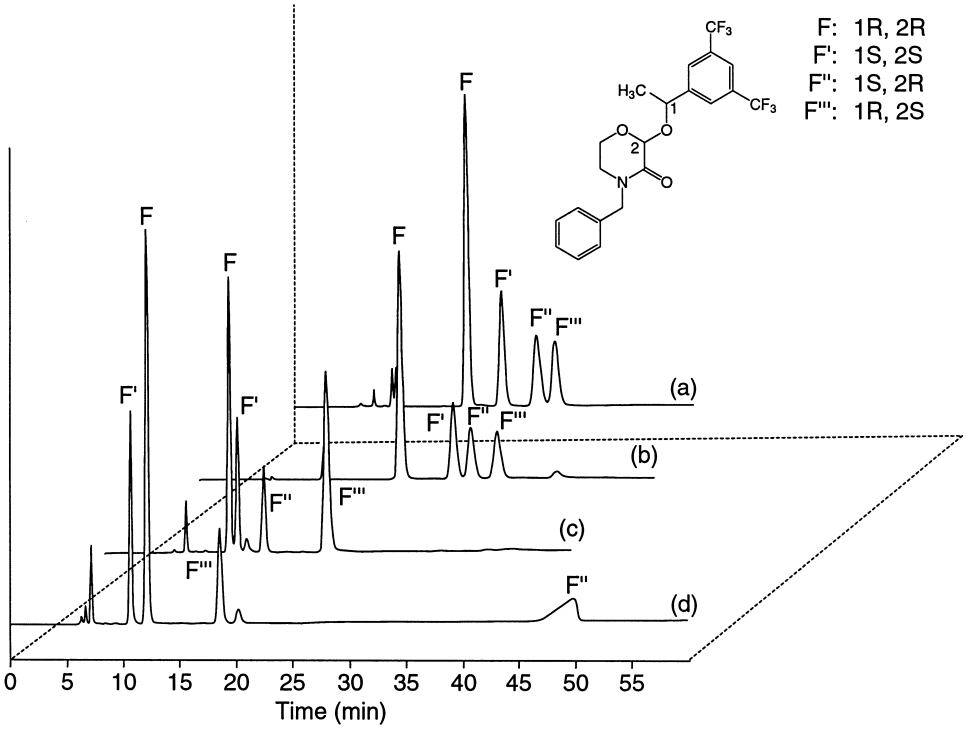

Fig. 6. Chromatograms of the separation of compound F and its stereoisomers. HPLC conditions: (a) Chiralcel OD column with hexane–IPA (92:8, v/v) as mobile phase; (b) Chiralcel OD column with hexane–ethanol (96:4, v/v) as mobile phase; (c) Chiralpak AD column with hexane–IPA (90:10, v/v) as mobile phase; (d) Chiralpak AD column with hexane–ethanol (92:8, v/v) as mobile phase.

compound G based on the synthetic route used to prepare compound G. Compound G was synthesized from compound F, partly by converting the C=O group on compound F into a 4-fluorophenyl group on compound G to form the chiral center 3. The configurations of chiral centers 1 and 2 of compound G were controlled at the stage of compound F. The typical chiral purity of compound F was 100.0%, with the trace amount of the other stereoisomers all left in the mother liquors during the isolation of compound F. Therefore, only one stereoisomer of compound G was possible to be generated from compound F during the formation of the chiral center 3. The configuration of the chiral center 3 on this stereoisomer was reversed from that on compound G, while the configurations of the other two chiral centers remained the same. This diastereomer of compound G could be monitored by a reversed-phase achiral HPLC method and it was typically not present in purified samples of compound G. Although compound G′ (whose three chiral centers were simultaneously reversed compared to those on compound G) was unlikely to be present in samples of compound G, as discussed above, it was still desirable to have a method to collect analytical data on this enantiomer of compound G during the drug development in order to address any regulatory concerns. Therefore, the development of a chiral separation method for compounds G and G′ was carried out.

Chromatograms of the separation of compounds G and G′ on the Chiralcel OD and Chiralpak AD columns using hexane as the mobile phase with different alcohol modifiers are shown in Fig. 7. Baseline separation was achieved on each column with either IPA or ethanol as mobile phase modifier.

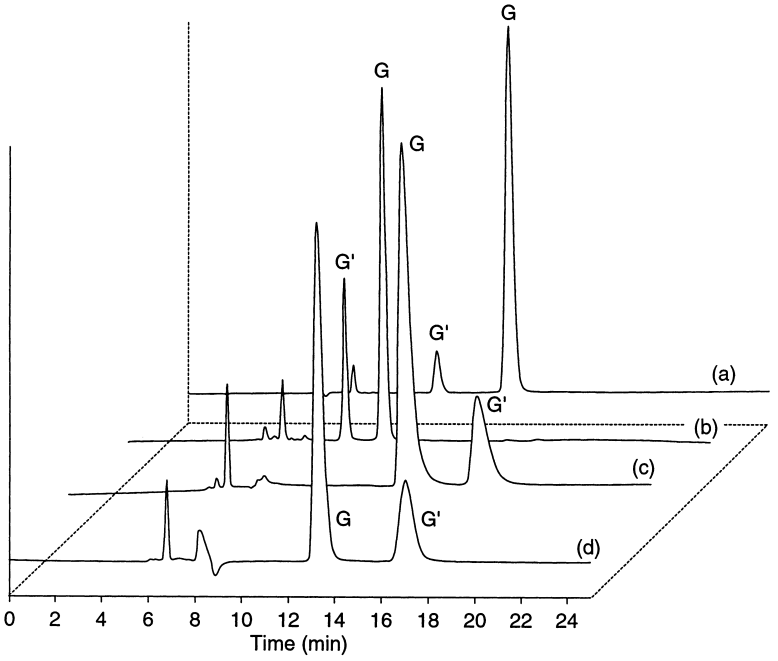

Fig. 7. Chromatograms of the separation of compounds G and G′. HPLC conditions: (a) Chiralcel OD column with hexane–IPA (92:8, v/v) as mobile phase; (b) Chiralcel OD column with hexane–ethanol (92:8, v/v) as mobile phase; (c) Chiralpak AD column with hexane–IPA (99:1, v/v) as mobile phase; (d) Chiralpak AD column with hexane–ethanol (99:1, v/v) as mobile phase.

The elution order of the enantiomers on the Chiralcel OD column was again reversed compared to that on the amylose-based AD column.

3.4. Separation of compounds H and H′

Chromatograms of the separation of compounds H and H′ on the OD and AD columns with hexane–IPA or hexane–ethanol as the mobile phase are shown in Fig. 8. The best separation was achieved with hexane–ethanol mobile phase on the Chiralcel OD column (Fig. 8b). On the AD column, only partial separation was achieved (Fig. 8c and d). However, the elution order of the enantiomers was reversed on the AD column when the mobile phase modifier was changed from IPA to ethanol.

3.5. Separation of compounds I and I′

Fig. 9 shows the chromatograms of the separation of compounds I and I′ on the OD and AD columns with hexane–IPA or hexane–ethanol as the mobile phase. On the OD column, no satisfactory separation was achieved, with the two enantiomers either partially separated (Fig. 9a) or co-eluted (Fig. 9b). On the AD column, partial separation was obtained with hexane–IPA mobile phase (Fig. 9c), while excellent separation was achieved with hexane–ethanol mobile phase in less than 15 min (Fig. 9d). On the AD column, we once again observed the reversal of elution order of the enantiomers when the mobile phase modifier was changed from IPA to ethanol.

3.6. Increased retention associated with the reversal of elution order when the mobile phase modifier was changed from IPA to ethanol

In the previous sections, we described the reversal of elution orders of the enantiomeric pairs F/F′, F″/F‴, H/H′ and I/I′ on the Chiralpak AD column

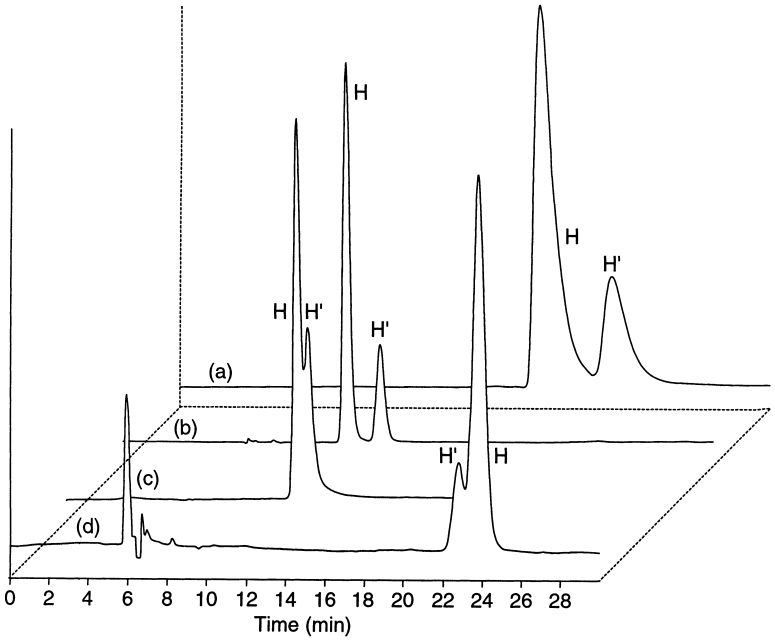

Fig. 8. Chromatograms of the separation of compounds H and H′. HPLC conditions: (a) Chiralcel OD column with hexane–IPA (70:30, v/v) as mobile phase; (b) Chiralcel OD column with hexane–ethanol (70:30, v/v) as mobile phase; (c) Chiralpak AD column with hexane–IPA (50:50, v/v) as mobile phase; (d) Chiralpak AD column with hexane–ethanol (50:50, v/v) as mobile phase.

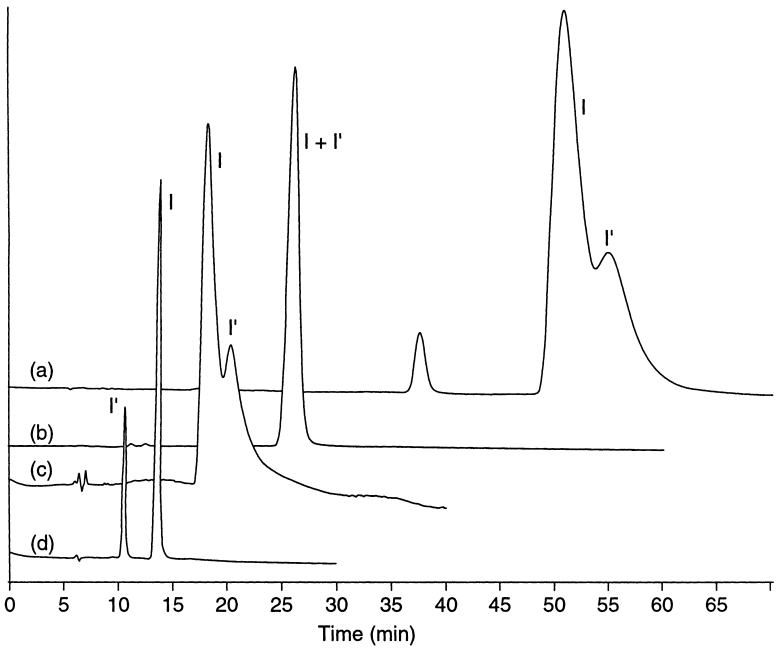

Fig. 9. Chromatograms of the separation of compounds I and I′. HPLC conditions: (a) Chiralcel OD column with hexane–IPA (70:30, v/v) as mobile phase; (b) Chiralcel OD column with hexane–ethanol (70:30, v/v) as mobile phase; (c) Chiralpak AD column with hexane–IPA (70:30, v/v) as mobile phase; (d) Chiralpak AD column with hexane–ethanol (35:65, v/v) as mobile phase.

Table 1
Effect of alcohol modifiers in the mobile phase on the retention factors of compound F and its stereoisomers on Chiralpak AD column

Mobile phase modifier	k'_F	$k'_{F'}$	$k'_{F''}$	$k'_{F'''}$
IPA (1.3 *M*)	0.80	0.92	1.30	2.20
Ethanol (1.4 *M*)	0.98	0.75	7.17	2.04

when the mobile phase modifier was changed from IPA to ethanol. In a previous paper [1], we reported a similar case in which the elution order of an enantiomeric pair was reversed on the AD column when the mobile phase modifier was changed from IPA to ethanol. In these cases, associated with the reversal of the elution orders, increased k' values of many of the solutes were observed.

Table 1 lists the k' values of compound F and its stereoisomers on the AD column using IPA and ethanol as modifiers, respectively, with the molar concentration of ethanol being slightly higher than that of IPA. Since the polarity of ethanol (P' value 4.3) is larger than that of IPA (P' value 3.9) [12], it is expected that the k' value obtained using ethanol modifier would be smaller than that obtained using IPA modifier at the same molar concentration, if solvent polarity is the only factor in determining the k' value. However, the k' values of F and F″ obtained using ethanol modifier, at even slightly higher molar concentration than that of IPA, were larger than those obtained using IPA modifier, suggesting that the polarity of the mobile phase modifier was not the dominating factor in determining the k' of these two solutes on the AD column. Table 2 lists the k' values of enantiomeric pairs A/A′ [1], H/H′ and I/I′ on the AD column using IPA and ethanol as modifiers, respectively, with the molar concentration of ethanol being higher than that of IPA. In all the cases in Table 2, the k' values obtained with ethanol modifier were larger than those obtained with IPA modifier at lower molar concentrations, again suggesting that the polarity of the mobile phase modifier was not the dominating factor in determining the k' of these solutes on the AD column.

In all the cases we studied on Chiralcel OD column, where no reversal of elution order was observed when the mobile phase modifier was switched from IPA to ethanol, we did not observe increased k' values with the use of ethanol modifier, compared to the k' values obtained with IPA modifier. On the Chiralpak AD column, however, there appeared to be an association between the reversal of elution order and the increase of retention of many of the solutes involved when the mobile phase modifier was changed from IPA to ethanol. The increased retention with the use of ethanol modifier, along with the reversal of elution order, could all be due to an alteration of the steric environment of the chiral cavities by the ethanol modifier.

3.7. Comparison of retention and separation of enantiomeric pairs H/H′ and I/I′ on OD and AD columns

Structurally, the only difference between compounds H and I is that the nitro group on compound H is replaced by an amino group on compound I (Fig. 4). However, this difference made the retention behaviors of the two compounds quite different on both the Chiralcel OD and Chiralpak AD columns. Table 3 compares the retention factors of the two

Table 2
Effect of alcohol modifiers in the mobile phase on the retention factors of compounds A, A′, H, H′, I and I′ on Chiralpak AD column

Mobile phase modifier	Compounds A and A′[a]	Compounds H and H′	Compounds I and I′
IPA	Modifier concentration: 1.3 *M* k': 2.24, 2.61	Modifier concentration: 6.5 *M* k': 0.91, 1.00	Modifier concentration: 6.5 *M* k': 0.58, 0.72
Ethanol	Modifier concentration: 1.7 *M* k': 3.30, 5.55	Modifier concentration: 8.6 *M* k': 2.74, 2.90	Modifier concentration: 8.6 *M* k': 1.30, 2.32

[a] Results from Ref. [1].

Table 3
Effect of structural variation of enantiomeric pairs H/H′ and I/I′ on their retention and chiral separation

Mobile phase	Enantiomeric pair	OD column	AD column
Hexane–IPA	H, H′	MP: 70/30 k': 2.11, 2.73; α=1.29	MP: 70/30 k': 2.58, 2.75; α=1.07
	I, I′	MP: 70/30 k': 7.57, 8.28; α=1.09	MP: 70/30 k': 1.90, 2.29; α=1.21
Hexane–ethanol	H, H′	MP: 70/30 k': 0.92, 1.21; α=1.32	MP: 50/50 k': 2.74, 2.90; α=1.06
	I, I′	MP: 70/30 k': 3.37 (no separation); α=1.00	MP: 50/50 k': 1.30, 2.32; α=1.78

MP: Mobile phase composition (hexane–IPA or hexane–ethanol, v/v).

compounds and their corresponding enantiomers on the OD and AD columns. On the OD column, with either hexane–IPA or hexane–ethanol as the mobile phase, compounds H and H′ had smaller k' values than those of compounds I and I′, under the same conditions. However, on the AD column, with either hexane–IPA or hexane–ethanol as the mobile phase, compounds H and H′ had larger k' values than those of compounds I and I′, under the same conditions. The different retention of compounds H and I on the *same* column was obviously caused by the two different groups (nitro and amino groups) on compounds H and I, respectively. However, the difference in retention *order* of compounds H and I exhibited on the two *different* CSPs must be related to the conformational difference between the two CSPs, since OD and AD columns have the same derivatization group.

In Table 3, differences in separation factor (α) for enantiomeric pairs H/H′ and I/I′ can also be noticed. Compared to the α for I/I′, the α for H/H′ is larger on the OD column and smaller on the AD column. There are two aspects of this observation: (1) the two enantiomeric pairs showed different chiral selectivity (α) on each of the two CSPs; (2) on the OD column, the magnitudes of the α values for the H/H′ and I/I′ pairs are in an order just opposite to that on the AD column. Regarding the first aspect, the difference in selectivity between the two enantiomeric pairs on the *same* CSP is apparently due to the difference between the nitro and amino groups on compounds H and I, respectively. With respect to the second aspect, since the Chiralcel OD and Chiralpak AD CSPs have different conformations while having the same derivatization group, it is apparent that the OD's conformation is more favorable to the chiral recognition of the H/H′ pair (compared to the chiral recognition of the I/I′ pair), and the AD's conformation is more favorable to the chiral recognition of the I/I′ pair (compared to the chiral recognition of the H/H′ pair). Therefore, the higher order structures of the CSPs played an important role in chiral recognition. The importance of the higher order structure of the polysaccharide-based CSPs in chiral recognition has been discussed by many others [3,5,6,13–16]. Our results on the difference in chiral recognition between the OD and AD columns on the separation of enantiomeric pairs H/H′ and I/I′, as well as E/E′ and G/G′ (discussed in Sections 3.1 and 3.3) support this point.

4. Conclusion

Baseline separations of enantiomeric pairs E/E′, G/G′, H/H′, I/I′ and the stereoisomers of compound F were successfully achieved using Chiralcel OD or Chiralpak AD column. In some cases, baseline separation could be achieved on more than one column. The results further demonstrated the effectiveness of the tris(3,5-dimethylphenylcarbamate) derivatives of cellulose (Chiralcel OD) and amylose (Chiralpak AD) CSPs for the separation of enantiomers.

Several cases of reversal of elution order of the enantiomers, associated with the increase of retention of many of these enantiomers, were observed on the Chiralpak AD column when the mobile phase modi-

fier was changed from IPA to ethanol. This is believed to be due to an alteration of the steric environment of the chiral cavities on the CSP by ethanol.

Chiralcel OD and Chiralpak AD columns provided different retention, separation and elution order of some of the enantiomeric pairs due to the conformational difference between the two CSPs.

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