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Conformational and temperature effects on separation of stereoisomers of a C3,C4-substituted β-lactamic cholesterol absorption inhibitor on amylose-based chiral stationary phases

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Abstract

A direct liquid chromatography method was developed for the diastereo- and enantioselective analysis of a C3,C4-substituted β-lactamic hypolipodemic agent (SCH 48461) and its stereoisomers on two commercially available amylose-based chiral stationary phases (CSPs), namely, Chiralpak AS and Chiralpak AD. The mobile phase composition (type and content of alcoholic modifier) was considered to achieve baseline resolutions in a single chromatographic run. In order to investigate the influence of molecular flexibility on chiral recognition process, β-lactams were ring-opened and converted into β-amino esters derivatives. Thermodynamic parameters associated with adsorption equilibria between acyclic and cyclic stereoisomers and CSPs were calculated from chromatographic runs at various temperatures.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Conformational effects; Temperature effects; β-Lactams

1. Introduction

1,(4*S*) - Bis(4 - methoxyphenyl) - (3*R*) - (3 - phenylpropyl)-2-azetidinone (compound **1**, Fig. 1) is a selective and highly potent inhibitor of cholesterol absorption [1]. It has been extensively demonstrated that in vivo activity resided primarily in a single configuration (*S*-configuration) at the C4 carbon of the β-lactamic ring [2]. Both diastereomers *trans*-(3*R*,4*S*) and *cis*-(3*S*,4*S*) (compound **3**) exhibited remarkable reduction of total plasma cholesterol in man, whereas the corresponding enantiomers, *trans*-(3*S*,4*R*) (compound **2**) and *cis*-(3*R*,4*R*) (compound **4**), respectively, were inactive at a comparable dose [3].

Since the biological activity of C3,C4-substituted β-lactams strongly depends on their stereochemistry, there is a clear need for methods able to obtain compounds enantiomerically pure and analytical methods by which their enantiomeric purities can be determined.

High-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs), is an effective analytical tool for the resolution of chiral compounds, both at the analytical and preparative scale levels. HPLC can be successfully employed for pharmacological investigations on relative activities

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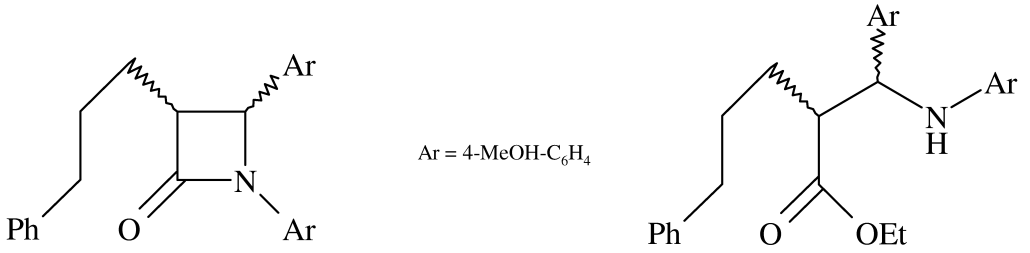

1: *trans*-(3*R*,4*S*) (SCH 48461)
2: *trans*-(3*S*,4*R*) (SCH 48462)
3: *cis*-(3*S*,4*S*) (SCH 48678)
4: *cis*-(3*R*,4*R*) (SCH 48679)

5: *threo*-(1*R*,2*S*)
6: *threo*-(1*S*,2*R*)
7: *erythro*-(1*S*,2*S*)
8: *erythro*-(1*R*,2*R*)

Fig. 1. Structures of β-lactams **1**, **4** and β-amino esters **5**, **8**.

of stereoisomers and to check the stereochemical course of asymmetric syntheses.

A few reports on the separation of the enantiomers of lactamic compounds have been previously published. The direct enantiomeric resolutions were carried out on a variety of CSPs, obtained by bonding small chiral moieties, synthetic or derivatized natural polymers and proteins on a silica gel support [4–9].

In this paper the chiral recognition ability of two commercially available amylose derivative CSPs, amylose (*S*)-methylbenzyl-carbamate (Chiralpak AS) and amylose 3,5-dimethylphenyl-carbamate (Chiralpak AD), towards our compounds was tested. Stereoselective separations were optimised by using different binary mixtures *n*-hexane–ethanol and *n*-hexane–2-propanol as eluents and gradually changing the column temperature. Additionally, in order to study the influence of conformational structure on chiral recognition process, β-lactams were ring-opened by hydrolysis reaction and the corresponding β-amino acids were converted into β-amino esters derivatives. The products synthesised by this route were analysed on the two aforementioned amylose-based CSPs, under different experimental conditions. Thermodynamic data associated with adsorption equilibria between acyclic and cyclic stereoisomers and CSPs were calculated from HPLC runs at various temperatures [10].

2. Experimental

2.1. Materials

Stainless steel Chiralpak AS and Chiralpak AD (250×4.0 mm I.D.) (Daicel Chemical Industries, Tokyo, Japan) columns were used. HPLC-grade solvents were furnished by Carlo Erba (Milan, Italy). β-Lactams **1**–**4** were obtained from Schering Plough (Milan, Italy).

Potassium *tert*.-butoxide was purchased from Fluka (Buchs, Switzerland) and lithium hydroxide monohydrate from Riedel-de Haën (Seelze, Germany). Thionyl chloride for synthesis was furnished by Merck (Darmstadt, Germany). Column chromatographic separations were accomplished on Merck silica gel 60. The reactions were monitored and purity of each compound was checked by thin-layer chromatography (TLC) on Merck Silicagel 60 F_{254} plates and spots were located by UV light. Anhydrous sodium sulfate (Carlo Erba) was used as drying agent.

2.2. Apparatus

Chromatography was performed using a Waters (Milford, MA, USA) 510 pump equipped with a Rheodyne (Cotati, CA, USA) injector and a Waters

Model 966 programmable multi-wavelength diode array detector.

Thermodynamic data were obtained from variable-temperature chromatography using a HPLC Perkin-Elmer (Norwalk, CT, USA) oven (range 10–40±0.5°C).

Melting points were taken on a Köfler (Reichert, Austria) hot stage apparatus and are uncorrected.

Electron ionization mass spectra were determined on a Hewlett-Packard HP59980B (Palo Alto, CA, USA) spectrometer, operating at 70 eV.

^{1}H-Nuclear magnetic resonance (NMR) spectra were obtained on a Gemini (Varian, Palo Alto, CA, USA) 200 MHz instrument in deuterochloroform solution; the chemical shift values were reported in ppm (δ) and standard abbreviations were used (b=broad, d=doublet, m=multiplet, q=quartet, s=singlet, t=triplet).

2.3. Synthetic procedures

2.3.1. Procedure for the preparation of the diastereoisomeric mixture of (3R,4S) and (3S,4S)-1,4-bis-(4-methoxyphenyl)-3-(3-phenylpropyl) 2-azetidinones ***1, 3***

trans-(3*R*,4*S*) 2-Azetidinone **1** (1.0 g, 2.5 mmol) was dissolved in tetrahydrofuran (20 ml) and to the solution cooled at 0°C, potassium *tert.*-butoxide (0.06 g, 0.5 mmol) was added. The mixture was stirred at 0°C for 1.5 h, then diluted with 1 *M* aqueous hydrochloric acid (25 ml) and extracted with diethyl ether (50 ml). The combined organic layers were concentrated under reduced pressure and the mixture of *trans* and *cis* 2-azetidinones **1**, **3** was purified by the silica gel column eluting with ethyl acetate–*n*-hexane (1:2, v/v) and crystallized from ethyl acetate–*n*-hexane, yield 98%, m.p. 94–96°C.

Mass spectrometry (MS) (*m*/*z*): 401 (M^+), 252, 242, 226, 161, 147, 134, 121.

The presence of the diastereomers **1** and **3** in the mixture was confirmed by a ^{1}H-NMR spectrum, which showed the characteristic patterns of the *trans* and *cis* isomers in the ratio of 3:1.

^{1}H-NMR ($CDCl_3$): δ 7.30–7.15 (m, **3** isomer 7H, **1** isomer 9H, aromatic protons), 6.99, 6.87 and 6.79 (doublets, 6H, **3** isomer aromatic protons), 6.90 and 6.78 (doublets, 4H, **1** isomer aromatic protons), 5.11 (d, 1H, **3** isomer 4-CH, J=6.0 Hz), 4.57 (d, 1H, **1** isomer 4-CH, J=2.2 Hz), 3.84 (s, 3H, **3** isomer OCH_3), 3.81 (s, 3H, **1** isomer OCH_3), 3.76 (s, 3H, **3** isomer OCH_3), 3.75 (s, 3H, **1** isomer OCH_3), 3.52 (dt, 1H, **3** isomer 3-CH), 3.10 (m, 1H, **1** isomer 3-CH), 2.65 (t, 2H, **1** isomer Ph-CH_2), 2.49 (m, 2H, **3** isomer Ph-CH_2), 1.84 (m, 4H, **1** isomer –CH_2–CH_2–), 1.42 (m, 4H, **3** isomer –CH_2–CH_2–).

2.3.2. Procedure for the preparation of ethyl 2-[α-{(4-methoxyphenyl)amino}-4-methoxybenzyl]-5-phenylpentanoates ***5–8***

2.3.2.1. (A) Preparation of 2-[α-{(4-methoxyphenyl)amino}-4-methoxybenzyl]-5-phenylpentanoic acids ***1a–4a***

A suspension of 2-azetidinonic stereoisomers **1**, **2**, racemate **3**, **4** and mixture **1**, **3** (0.1 g, 0.25 mmol) in absolute ethanol (10 ml) was added to a solution of lithium hydroxide (0.15 g, 3.6 mmol) in water (5 ml). The mixture was stirred at 70–80°C for 2 h, then cooled at room temperature. The solution was made slightly acidic by addition of concentrated hydrochloric acid, then neutralized to pH 7 by 10% aqueous sodium hydrogencarbonate solution. The mixture was extracted with ethyl acetate (3×20 ml) and the combined extracts were washed with brine (15 ml), dried over sodium sulfate and evaporated under reduced pressure. The obtained compounds were purified by crystallisation from ethyl acetate–*n*-hexane.

1a, **3a**: The diastereomeric mixture **1a**, **3a** was obtained from mixture **1**, **3** in 95% yield, m.p. 162–165°C.

MS (*m*/*z*) 419 (M^+), 252, 242, 178, 160, 121.

1a: This compound was obtained from stereoisomer **1** in 88% yield, m.p. 153–155°C.

MS (*m*/*z*): 419 (M^+), 252, 242, 226, 161, 147, 134, 121.

2a: This compound was obtained from stereoisomer **2** in 90% yield, m.p. 154–155°C.

MS (*m*/*z*): 419 (M^+), 373, 252, 242, 226, 147, 135, 121.

3a, **4a**: The racemic mixture **3a**, **4a** was obtained from mixture **3**, **4** in 92% yield.

MS (*m*/*z*): 419 (M^+), 373, 252, 242, 228, 160, 147, 121.

*2.3.2.2. (B) Preparation of the β-amino ethyl esters **5–8***

Diastereomeric mixture **1a**, **3a**, *threo* enantiomers **1a** and **2a**, racemic mixture **3a**, **4a** (0.08 g, 0.2 mmol) were separately dissolved in absolute ethanol (30 ml) and thionyl chloride (1 ml) was added dropwise under stirring. The mixture was stirred at room temperature and the reaction, monitored by TLC, was stopped when starting material had disappeared (about 24 h). After evaporation of the solvent under reduced pressure, the residue was dissolved in water, made alkaline by addition of aqueous ammonia and extracted with ethyl acetate (3×10 ml). The organic layer was washed with water (10 ml), dried over sodium sulfate and evaporated under reduced pressure. The obtained residue was purified by silica gel column eluting with an ethyl acetate–*n*-hexane (1:2 v/v) mixture.

5, **7**: The diastereomeric mixture **5**, **7** was obtained from mixture **1a**, **3a** as an oil in 50% yield.

MS (*m*/*z*): 447 (M^+), 252, 242, 226, 160, 121.

^{1}H-NMR ($CDCl_3$): δ 7.30–6.67 (m, 13H, aromatic protons), 4.43 (d, 1H, **7** isomer benzyl CH), 4.35, 4.14 (two q, 2H, **5** isomer OCH_2), 4.12, 4.00 (two q, 2H, **7** isomer OCH_2), 3.83 (d, 1H, **5** isomer benzyl CH), 3.79 (s, 6H, **5** isomer OCH_3), 3.78, 3.70 (two s, 6H, **7** isomer OCH_3), 3.68 (m, 1H, **7** isomer 2-CH), 3.00 (m, 1H, **5** isomer 2-CH), 2.70 (m, 2H, **7** isomer 5-CH_2), 2.54 (m, 2H, **5** isomer 5-CH_2), 1.60 (m, 4H, **5** and **7** isomers 3-CH_2 and 4-CH_2), 1.39 (t, 3H, **7** isomer CH_3), 1.18 (t, 3H, **5** isomer CH_3).

5: This compound was obtained from **1a** as an oil in 50% yield.

MS (*m*/*z*): 447 (M^+), 252, 242, 226, 160, 147, 121.

^{1}H-NMR ($CDCl_3$): δ 7.48–6.80 (m, 13H, aromatic protons), 6.54 (bs, 1H, NH), 3.85 (d, 1H, benzyl CH), 3.81 (q, 2H, OCH_2), 3.80 and 3.79 (two s, 6H, OCH_3), 2.74 (m, 1H, 2-CH), 2.60 (m, 4H, 5-CH_2 and 3-CH_2), 1.94 (m, 2H, 4-CH_2), 1.27 (t, 3H, CH_3).

6: This compound was obtained from **2a** as an oil in 60% yield.

MS (*m*/*z*):447 (M^+), 252, 242, 226, 160, 147, 121.

^{1}H-NMR ($CDCl_3$): δ 7.48–6.83 (m, 13H, aromatic protons), 6.52 (bs, 1H, NH), 3.85 (d, 1H, benzyl CH), 3.81 (q, 2H, OCH_2), 3.80 and 3.79 (two s, 6H, OCH_3), 2.74 (m, 1H, 2-CH), 2.60 (m, 4H, 5-CH_2 and 3-CH_2), 1.94 (m, 2H, 4-CH_2), 1.27 (t, 3H, CH_3).

7, **8**: The racemic mixture **7**, **8** was obtained from mixture **3a**, **4a** as an oil in 55% yield.

MS (*m*/*z*): 447 (M^+), 252, 242, 226, 161, 149, 121.

^{1}H-NMR ($CDCl_3$): δ 7.30–6.73 (m, 13H, aromatic protons), 5.00 (bs, 1H, NH), 4.18 (m, 1H, benzyl CH), 3.95 (q, 2H, OCH_2), 3.75, 3.70 (two s, 6H, OCH_3), 3.68 (m, 1H, 2-CH), 2.67 (t, 2H, 5-CH_2), 1.60 (m, 4H, 3-CH_2 and 4-CH_2), 1.38 (t, 3H, CH_3).

2.4. Chromatographic conditions

Mobile phases were mixtures of *n*-hexane and an alcohol modifier (ethanol or 2-propanol), degassed by sonication immediately before use. A flow-rate of 0.5 ml min^{-1} was used. The wavelength of detection was 260 nm. All analytical separations were performed at 25°C, except those used for the study of the thermodynamic data.

Standard solutions were prepared by dissolving 1–3 mg of each analyte in 10 ml of ethanol. The injection volume was 10 μl.

3. Results and discussion

*3.1. Optimisation of diastereo- and enantioselective separation of compounds **1–4***

The commercially available amylose derivative Chiralpak AS column was selected for its noticeable chiral recognition ability towards optically active compounds characterised by the presence of a β-lactam structural unit [11].

In order to achieve the diastereo- and enantioselective separation of all four stereoisomers **1**–**4** in a single run several parameters affecting the resolution, such as concentration and nature of the organic modifier, were taken into account during the optimisation of the method.

Enantioselectivity factor, α, and resolution factor, R_s, for two pairs of enantiomers **1**, **2** and **3**, **4** were not significantly affected by the use of different ethanol percentages, from 10 to 25%, in the mobile

phases. This indicates that, at constant temperature, the enantiorecognition process was unchanged over the explored alcohol concentration range. The influence of the nature of alcohol (ethanol or 2-propanol) was instead more pronounced. Analysis of experimental data, summarised in Table 1, reveals that only in presence of ethanol, as alcohol modifier, was the simultaneous separation of the four stereoisomers **1**–**4** performed.

Fig. 2 shows a typical chromatogram for resolution of compounds **1**–**4** on the Chiralpak AS column, using a mobile phase consisting of *n*-hexane–ethanol (90:10, v/v). When ethanol was replaced with 2-propanol better results in terms of enantioselectivity were observed, but, because of overlapping of peaks corresponding to diastereomers **1** and **4**, the complete separation of the four stereoisomers was not achieved.

For each enantiomeric pair the most retained stereoisomer had the *R* configuration at the C3 carbon of the 2-azetidinonic ring. The same elution order [*trans*-(3*S*,4*R*)<*trans*-(3*R*,4*S*) and *cis*-(3*S*,4*S*)<*cis*-(3*R*,4*R*)] was obtained using the Chiralpak AD column. The AD CSP was less effective and the resolutions were worse than those obtained with the AS CSP. The α values for the separation of *trans* enantiomers **1** and **2** dropped from 2.43 to 1.21 and from 2.66 to 1.70 employing *n*-hexane–ethanol (40:60, v/v) and *n*-hexane–2-propanol (70:30, v/v), respectively, as mobile phases. The selectivity for the *cis* enantiomeric pair **4**, **3** slightly increased (α=1.60 versus α=1.36) when ethanol organic modifier (30% in hexane) was used, while no resolution in presence of 2-propanol was obtained. All separations were carried out at a flow-rate of 0.5 ml min^{-1} and at temperature of 25°C.

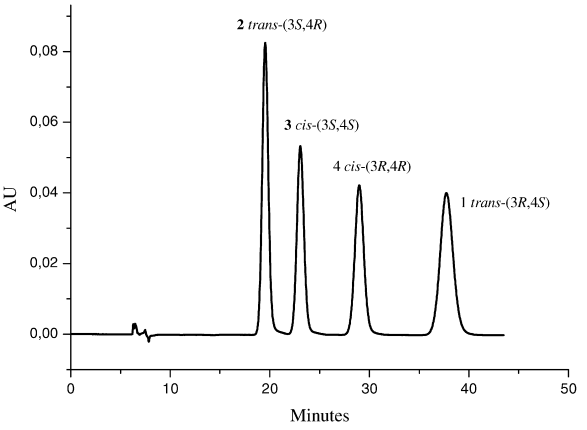

Fig. 2. Chromatographic separation of stereoisomers **1**, **4**. Column, Chiralpak AS (250×4.0 mm I.D.); eluent, *n*-hexane–ethanol (90:10, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm.

Although is rather difficult to interpret the chiral recognition mechanism on amylose-based CSPs, our experimental data allow us to formulate several hypothesis concerning the enantioseparation mechanism. Amylose backbone and polar carbamate groups (which interact with the analytes via hydrogen bonds [12]), common to both CSPs, probably control the elution order of the enantiomers, while

Table 1
Effect of eluent on separation of compounds **1**–**4**

Eluent	k[a]				Critical pair[b]	α[c]	R_s[d]
	1	**2**	**3**	**4**			
n-Hexane–2-propanol (85:15)	6.06	2.28	3.60	6.06	**1**, **2**	2.66	7.44
					4, **3**	1.69	4.31
					3, **2**	1.58	3.68
					1, **4**	1.00	–
n-Hexane–ethanol (90:10)	5.51	2.27	2.85	3.90	**1**, **2**	2.43	10.25
					4, **3**	1.36	4.12
					3, **2**	1.26	2.88
					1, **4**	1.41	4.41

Column, Chiralpak AS (250×4.0 mm I.D.); flow-rate, 0.5 ml min^{-1}; column temperature, 25°C; detection wavelength, 260 nm.
[a] The capacity factor.
[b] The analyzed pair of stereoisomers; the first number of the critical pair indicates the most retained stereoisomer.
[c] The separation factor of critical pair.
[d] The resolution factor of critical pair.

the 1-phenylethyl groups, peculiar of the AS CSP, gives rise to increasing stereoselectivity.

3.2. Effect of conformational flexibility

The presence of rigid 2-azetidinone nucleus controls analyte conformation and reduces, during the formation of reversible enantiomer–CSP diastereomeric complexes, the possible approaches of solutes towards achiral or chiral sites of the stationary phase. An augment of molecular flexibility may be either favourable or unfavourable with respect to the chiral recogniton mechanism [13].

In order to investigate this aspect, β-lactams were ring opened by hydrolysis reaction and the corresponding β-amino acids were converted into *β*-amino ethyl esters derivatives (see Experimental section).

Fig. 1 illustrates the structures of analytes **5**–**8**. Since some structural changes take place in the ring-opening of the β-lactams (an ester carbonyl in place of a β-lactam carbonyl and a H-bond forming NH which is absent in the β-lactam), a different chromatographic behaviour of cyclic and acyclic compounds on two amylose based CSPs cannot be ascribed exclusively to conformational differences.

trans stereoisomers **1**, **2** and *cis* racemates **3**, **4**, were separately submitted to the same procedure to give *threo* stereoisomers **5**, **6** and *erythro* racemates **7**, **8**, respectively. In the separation of each enantiomeric pair on chiral column, the elution order of enantiomers was established using mixtures in which the level of the desired enantiomer was arbitrarily elevated. The structures of products were confirmed from their spectral data. The peak corresponding to *erythro* isomer **7** was identified by chromatography of unequal mixture of diastereomers **5** and **7**, obtained after initial epimerisation at the C3 position of compound **1** *trans*-(3*R*,4*S*). The product obtained by such isomerisation and analysed by HPLC on the Chiralpak AS column, was found to be a 3:1 [*trans*-(3*R*,4*S*):*cis*-(3*S*,4*S*)] mixture of diastereomers, confirming data previously published [3].

The enantioselective analysis of β-amino esters **5**–**8** was carried out with both Chiralpak AD and AS columns. The analytical results, listed in Table 2, show that the ring-opening of the β-lactams was detrimental to chiral recognition with Chiralpak AS column. The enantiomeric pair **5**, **6** was not resolved, under any experimental conditions, and significative diminution of the enantioseparation factor for compounds **7**, **8** with respect to **3**, **4** was observed. The enantioselectivity factors obtained with Chiralpak AD column were influenced greatly by the alcoholic modifier used in the mobile phase [14]. Enantiomeric pairs **5**, **6** and **7**, **8** were not resolved by the Chiralpak AD column when a mixture of *n*-hexane–ethanol was employed. The use of *n*-hexane–2-propanol (70:30, v/v), as mobile phase, gave rise to a successful and rapid separation of enantiomers **7** and **8**; the enantioselectivity factor (α=2.17) observed for β-amino ester pair **7**, **8** was higher than that of corresponding β-lactam pair **3**, **4** (α=1.36). This difference in enantioselectivity suggests the participation of the alcohol modifier in altering the structure of the chiral cavities and, consequently, the

Table 2
HPLC of compounds **5**–**8**

Critical pair	Column	Eluent	k_1	α	R_s
5, **6**	AD	*n*-Hexane–2-propanol (70:30)	1.04	1.12	0.79
5, **6**	AD	*n*-Hexane–ethanol (40:60)	0.88	1.00	–
5, **6**	AS	*n*-Hexane–ethanol (95:5)	1.12	1.00	–
5, **6**	AS	*n*-Hexane–2-propanol (95:5)	1.54	1.00	–
7, **8**	AD	*n*-Hexane–2-propanol (70:30)	0.98	2.17	6.83
7, **8**	AD	*n*-Hexane–ethanol (40:60)	0.76	1.00	–
7, **8**	AS	*n*-Hexane–ethanol (95:5)	1.22	1.09	n.m.[a]
7, **8**	AS	*n*-Hexane–2-propanol (95:5)	1.96	1.15	n.m.

Columns, Chiralpak AS (250×4.0 mm I.D.) and Chiralpak AD (250×4.0 mm I.D.); flow-rate, 0.5 ml min^{-1}; column temperature, 25°C; detection wavelength, 260 nm.

[a] Not measured.

accessibility to the selective adsorption sites of the AD CSP, leading to a higher discrimination of chiral selector towards more flexible ring-opened enantiomers **7** and **8**. The enantiomeric discrimination of Chiralpak AD column became worse (α=1.12) for the *threo* isomers **5** and **6**.

3.3. Temperature effects

In order to investigate the thermodynamic parameters relative to the chiral resolution of compounds **1**–**8** by Chiralpak AS and AD columns, variable temperature study was carried out between 10 and 40°C. The differences between two enantiomers in enthalpy and entropy of adsorption onto stationary phases, $\Delta_{j,i}\Delta H^0$ and $\Delta_{j,i}\Delta S^0$, respectively, can be related to enantioselectivity factor, α, by the following equation [15,16]:

$$\ln \alpha = -\Delta_{j,i}\Delta H^0/RT + \Delta_{j,i}\Delta S^0/R$$

where the subscripts j and i refer to the more and less retained enantiomers, R is the gas constant and T the absolute temperature. The equation predicts that a plot of ln α vs. $1/T$ (Van 't Hoff plot) yields a straight line with a slope of $-\Delta_{j,i}\Delta H^0$ and intercept of $\Delta_{j,i}\Delta S^0/R$. However, non-linear trends can be occur when there is a significant change in chiral discrimination mechanism as a function of temperature.

The enantioselectivity values were calculated in 10°C increments over the temperature range studied, using binary mixtures *n*-hexane–ethanol and *n*-hexane–2-propanol as eluents. Experimental results are collected in Table 3.

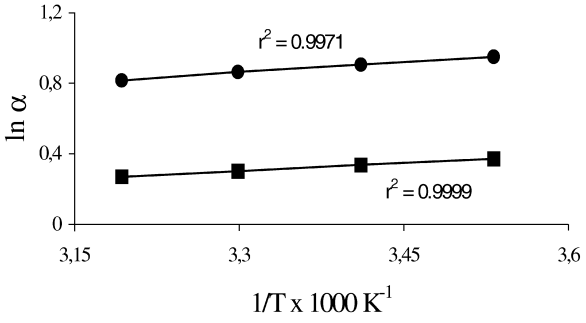

Fig. 3. Linear inverse relationship between ln α and temperature for enantiomeric pairs **1**, **2** and **3**, **4**. Column, Chiralpak AS (250×4.0 mm I. D.); eluent, *n*-hexane–ethanol (90:10, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm. ■=**3**, **4**; ●=**1**, **2**.

Inspection of thermodynamic parameters associated with adsorption of the enantiomeric pairs **1**, **2** and **3**, **4** with the Chiralpak AS column reveals that both enthalpic and entropic contributions were negative. This indicates that a more tightly structured enantiomer–CSP distereomeric transient complex results in lower molecular disorder [17]. The graphs ln α vs. $1/T$ obtained with the Chiralpak AS column (Fig. 3) clearly show that the temperature dependence of the enantioselectivity factor was highly linear (r^2>0.99). The same linear Van 't Hoff plots were observed for enantiomeric pairs **1**, **2** and **7**, **8** (Fig. 4, r^2>0.99) with the Chiralpak AD column, employing ethanol (10%) and 2-propanol (30%) in *n*-hexane as alcohol modifiers.

In particular, chiral separation of *erythro* enantio-

Table 3
Thermodynamic data

Enantiomeric pair	Column	Alcohol[a] (%)	$\Delta\Delta H^0$ (Kcal mol^{-1})	$\Delta\Delta S^0$ (cal mol^{-1} K^{-1})
1, **2**	AD	2-Propanol (30)	−0.08	0.78
1, **2**	AD	Ethanol (60)	−0.70	−2.00
4, **3**	AD	Ethanol (60)	0.13	1.06
5, **6**	AD	2-Propanol (30)	−0.01	0.17
7, **8**	AD	2-Propanol (30)	−3.48	−9.88
1, **2**	AS	Ethanol (10)	−0.77	−0.84
4, **3**	AS	Ethanol (10)	−0.60	−1.38

Columns, Chiralpak AS (250×4.0 mm I.D.) and Chiralpak AD (250×4.0 mm I.D.); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm.

[a] In *n*-hexane.

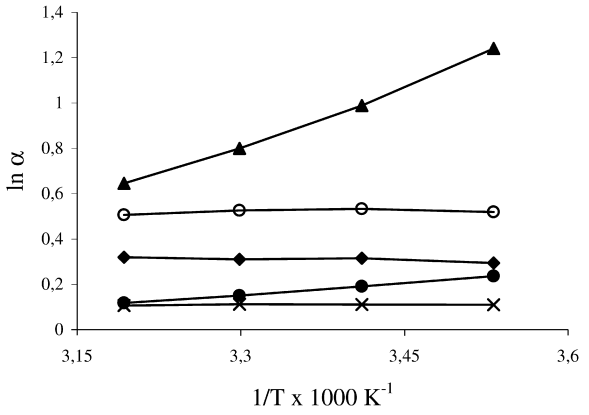

Fig. 4. The relationship between ln α and temperature on Chiralpak AD for enantiomeric pairs **1**, **2** (●) (ethanol), **1**, **2** (○) (2-propanol), **3**, **4** (♦) (ethanol), **5**, **6** (x) (2-propanol), **7**, **8** (▲) (2-propanol). Column, Chiralpak AD (250×4.0 mm I.D.); eluent, *n*-hexane–alcohol modifier (shown in parentheses); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm.

mers **7** and **8** was dominated by an enthalpic term ($\Delta\Delta H^0 = -3.48$ Kcal mol^{-1}) on an unfavourable negative entropic term ($\Delta\Delta S^0 = -9.88$ cal mol^{-1} K^{-1}) and thus the enantioselectivity dramatically improved with decreasing temperature. Variable temperature chromatograms reported in Fig. 5, show as, at lower temperatures, the decreasing in column efficiency, due to a slower kinetics of the adsorption–desorption process, was more than overcome by the increase in enantioselectivity. This trend caused an enhancement of chromatographic resolution of enantiomers **7** and **8** on the Chiralpk AD column at 10°C ($R_s = 14.02$) relative to the performance obtained at 40°C ($R_s = 3.71$).

Further analysis of graphs reported in Fig. 4 reveals the anomalous behaviour of the Van 't Hoff plots for three enantiomeric pairs *trans* **1**, **2**, *cis* **3**, **4** and *threo* **5**, **6**, using 2-propanol (for first and third pairs) and ethanol (for second pair), as alcohol modifiers. For these racemates α values were approximately constant when temperature was increased from 10 to 40°C. Under such circumstances, the enantioseparation can be conveniently carried out at higher investigated temperature. In fact, an increased chromatographic resolution and shorter analysis times, caused by enhancing in efficiency without decreasing in enantioselectivity, can be observed. The chromatograms relative to separation of enantio-

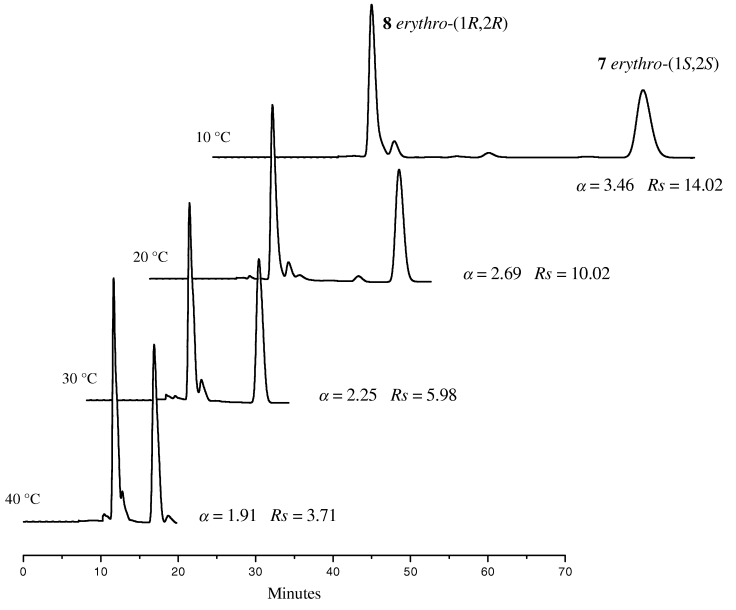

Fig. 5. Variable-temperature HPLC of enantiomers **7** and **8**. Column, Chiralpak AD (250×4.0 mm I.D.); eluent, *n*-hexane–2-propanol (70:30, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm; column temperature, from 10°C (top) to 40°C (bottom) in 10°C increments.

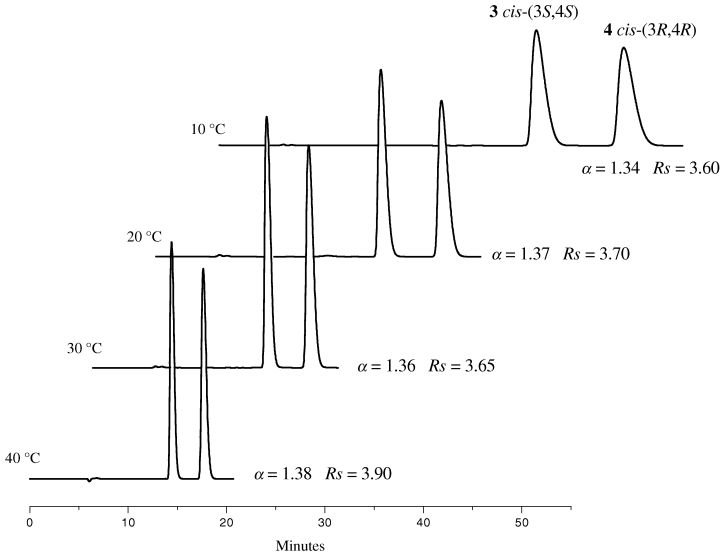

Fig. 6. Variable-temperature HPLC of enantiomers **3** and **4**. Column, Chiralpak AD (250×4.0 mm I.D.); eluent, *n*-hexane–ethanol (60:40, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 260 nm; column temperature, from 10°C (top) to 40°C (bottom) in 10°C increments.

mers **3** and **4** with Chiralpk AD column, depicted in Fig. 6, confirm this assumption.

To interpret the unusual effect of temperature on enantioselectivty, a different procedure to calculate both enthalpic and entropic quantities was applied. Since the α values did not change in a regular fashion with increasing temperature, we obtained thermodynamic data by plotting ln k vs. $1/T \cdot 10^3$ for each enantiomer of racemates *trans* **1**, **2**, *cis* **3**, **4** and *threo* **5**, **6** [18]. The relationships were linear with $r^2 > 0.98$. The positive $\Delta\Delta S^0$ values obtained (Table 3) indicated that the retention process of the longer retained enantiomer was followed by increased molecular disorder. The favourable entropic contribute to enantiorecognition probably arises from the sum of several processes that occur during the analyte–CSP diastereomeric transient complex formation, such as the desolvation of analytes and selector, solvation of complexes, conformational changes of selector and selectands.

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