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A new application of stopped-flow chiral HPLC: inversion of enantiomer elution order

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Abstract

A newly developed procedure to reverse the enantiomer elution order of compounds resolved on chiral stationary phases (CSPs) for HPLC is presented. The optimized analytical protocol is based on the effect of temperature on enantioselectivity and does not involve any changing in mobile phase composition or type of CSP. In essence, the approach entails variable temperature chromatography at two temperatures. The enantiomer separation is performed at a low column temperature, with stopping the flow prior to elution of the less retained enantiomer. Then, the column temperature is changed with the peaks trapped inside the column, followed by elution with the same mobile phase in reverse direction. Under these conditions, the more pronounced loss in free energy of binding for the more strongly bound enantiomer results in an inversion of the elution order. This procedure may be applied to each enantiomer pair that is separated by chiral HPLC under an appreciable enthalpy-control.

Keywords: Enantiomer separation; Polymeric chiral stationary phases; Stopped-flow chiral HPLC; Elution order reversal; CD detection; Temperature effects

1. Introduction

The elution order is one of the most important topics in the field of chiral HPLC analysis of enantiomers [1]. In the development of an enantioselective analytical method, it is desirable to elute the minor enantiomer impurity before the major isomer. The control of elution order allows for the avoidance possible interferences in determining the enantiomer purity, caused by the tail of the main enantiomer, especially when the enantioseparation is minimal [2]. In this context, the accurate determination of enantiomer composition represents an essential issue in quality control analysis, in organic synthesis and in pharmaceutical chemistry.

The enantiomers can elute easily in the opposite order by using totally synthetic CSPs based on low molecular mass selectors. A distinct advantage of such selectors is that they are usually available in both the configurations. Thus, by simply switching their chirality, a change of the elution order of separated enantiomer pair may be obtained [3]. Differently, the reversal of the enantioselectivity of a separation on CSPs based on natural chiral molecules, such as proteins, glycopeptides, polysaccharides and cyclodextrins, is a difficult result to obtain, and it would be considered unusual. The unavailability of the non-synthetic selectors in both the enantiomer forms does not permit an easy control of the enantiomer elution order. Furthermore, lack of detailed knowledge about the chiral discrimination process operating at a molecular level on most of these stationary phases does not enable to predict the interactions occurring between the enantiomers and the binding sites of the CSP. The reversal of the enantiomer elution order on CSPs based on proteins, carbohydrates and glycopeptides has been achieved by altering the mobile phase composition or column temperature [4–7].

In the present study, we report a newly developed procedure to reverse the enantiomer elution order of commercially available chiral drugs (compounds **2**, **3**, **5**; Fig. 1) and novel biologically active substances (compounds **1** and **4**; Fig. 1) on polymeric-based CSPs, without any change in properties of the eluent or type of the CSP. The novel analytical protocol is

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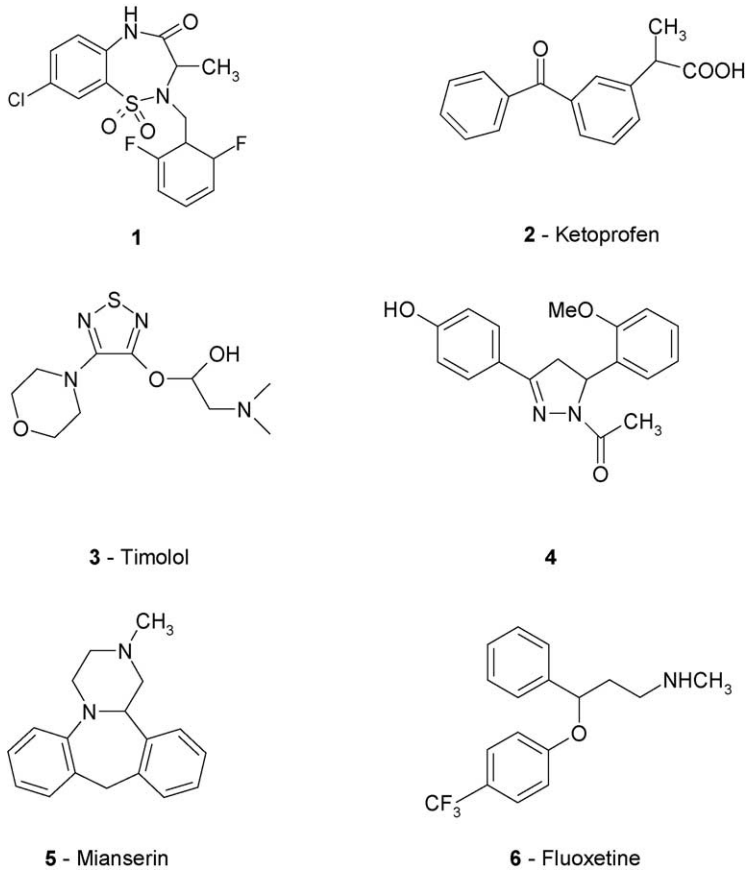

Fig. 1. Structures of the chiral analytes **1**–**6**.

based on the dependence of enantioselectivity on temperature and may be applied to each enantiomer pair that is separated by chiral HPLC under an appreciable enthalpy-control.

2. Experimental

Racemates **1** and **4** (Fig. 1) were synthesized by a chemical pathway reported elsewhere [8,9]. Compounds **2**, **3**, **5** and **6** were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy). Chiral HPLC of the analytes was performed by using stainless-steel Chiralcel OD (250 mm × 4.6 mm i.d.), Chiralpak AD (250 mm × 4.6 mm i.d.; Daicel Chemical Industries, Tokyo, Japan) and Chiraspher (Merck, Darmstadt, Germany) columns. Chiral HPLC was performed by using a Perkin-Elmer (Norwalk, CT, USA) 200 lc pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 100-μl sample loop, a HPLC Dionex STH 585 (Dionex Corporation, Sunnyvale, CA, USA) oven and a Jasco (Jasco, Ishikawa-cho, Hachioji City, Tokyo, Japan) Model CD 2095 Plus UV/CD detector. Low-temperature chromatography was performed by placing the column in a proper guard of a MGW Lauda Cryostat (Messgerate-Werk Lauda, Germany) and employing a 1-m connecting capillary placed in the ethylene glycol cooling bath, to ensure thermal equilibration of the mobile phase.

The mobile phases for chromatographic separations were filtered and degassed by sonication immediately before using.

Standard solutions, prepared by dissolving 1–3 mg of each analyte with 25 ml of chromatographic eluent, were employed. The injection volume was 10–20 μl.

The column hold-up times were estimated at each temperature by using 1,3,5-tri-*tert*-butylbenzene as non-retained marker.

To determine the enantiomer elution order of the compound **3**, a sample enriched in the (*R*)-enantiomer was chromatographed.

3. Result and discussion

The separation of enantiomers by HPLC on chiral stationary phase (CSP) is a thermodynamically controlled process. When an enantiomer resolution is observed, the enantioselectivity factor (α) may be related, at first approximation, to the column temperature by the following equation:

$$\ln \alpha = -\frac{\Delta_{j,i}\Delta H^{\circ}}{RT} + \frac{\Delta_{j,i}\Delta S^{\circ}}{R} \quad (1)$$

where the subscripts j and i refer to the more and less retained enantiomer, $\Delta_{j,i}\Delta H^{\circ}$ and $\Delta_{j,i}\Delta S^{\circ}$ are the differences between two enantiomers in enthalpy and entropy of adsorp-

tion, respectively, onto stationary phase, R is the gas constant and T the absolute temperature.

It must be pointed out, however, that the retention and enantioselectivity factors include the contributions coming from both enantioselective and non-enantioselective interactions [10]. Because the non-enantioselective and enantioselective contributions cannot be easily isolated [11], the α term's dependence on temperature is usually described by Eq. (1). In the following discussion, the term enantioselectivity refers to apparent experimental data estimated under usual chromatographic conditions.

The events leading to enantiomer separation are the time-averaged sum of all energy-allowed intermolecular selector-analyte recognition processes, each of which may have different geometric requirements and energetic contributions. In cases of the free energy contributions of analyte binding are linearly related to the temperature change, the plot of $\ln\alpha$ versus $1/T$ (van't Hoff plot) yields a straight line with a slope of $-\Delta_{j,i}\Delta H^{\circ}/R$ and an intercept of $\Delta_{j,i}\Delta S^{\circ}/R$. For the most chiral compounds resolved on monomeric- or polymeric-based CSPs, $\Delta_{j,i}\Delta H^{\circ}$ and $\Delta_{j,i}\Delta S^{\circ}$ terms have the same negative sign and the enantioseparation process is enthalpy-controlled [12]. This indicates that: (a) a more tightly structured enantiomer–CSP diastereomeric transient complex results in a lower molecular disorder; (b) the enantiorecognition results as a compromise between the favorable enthalpic contribution and unfavorable entropic contribution; (c) the enantioselectivity decreases with the increase of the temperature. In addition, under enthalpic control, the $\Delta_{j,i}\Delta H^{\circ}$ term is considered crucial in the determining the slope of the $\ln\alpha$ versus $1/T$ graph. Highly negative $\Delta_{j,i}\Delta H^{\circ}$ values are indicative of remarkably temperature-dependent chiral resolutions.

According to Eq. (1), when $\alpha = 1$, the isoenantioselective temperature (T_{iso}) can be calculated as follows:

$$T_{\mathrm{iso}} = \frac{\Delta_{j,i}\Delta H^{\circ}}{\Delta_{j,i}\Delta S^{\circ}} \quad (2)$$

Above the T_{iso}, enantiomer separation is entropy-controlled and a reversal of the elution order should be observed. Such phenomenon has sometimes been observed in gas chromatography (GC) at relatively high temperature [10], and rarely in HPLC [4,5]. HPLC chiral column vendors suggest caution against polymeric-based stationary phase instability at a temperature greater than around 45 °C, especially for polysaccharide-based stationary phases. Recently, Wang et al. have reported on temperature-induced conformational transitions of the amylose tris(3,5-dimethylphenylcarbamate; Chiralpak AD) CSP, in the normal-phase separation of the enantiomers of a dihydropyrimidinone (DHP) acid, beginning at about 30 °C. Such irreversible transitions have caused unpredictable effects on enantioselectivity [13].

Due to the comparatively low column temperatures, separation of enantiomers by chiral HPLC is generally enthalpy-governed and thus enantioselectivity improves as temperature decreases.

The first set of experiments was carried out to investigate the thermodynamic parameters relative to the chiral resolution of compounds **1**–**6** by using two coated-type CSPs, the polysaccharide-based Chiralcel OD and Chiralpak AD CSPs [14], and a synthetic polymer (poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester) covalently bonded to silica gel, the Chiraspher CSP [15]. Table 1 shows the chromatographic conditions used for the enantioseparations of the investigated analytes. On the Chiralcel OD CSP, the enantiomers of the compounds **3**–**6** were separated by using normal-phase eluents (compounds **3**, **4** and **6**) or a polar organic eluent (compound **5**). The Chiraspher and Chiralpak AD CSPs, in combination with normal-phase eluents, were successfully employed to resolve the racemic **1** and **2**, respectively.

Each racemic compound was submitted to temperature-dependent study between −5 and 45 °C with the exception of the compound **1,** which was resolved on the Chiraspher CSP in the temperature range between −5 and 55 °C. The enantioselectivity values were calculated in 10 °C increments over the investigated temperature range.

The van't Hoff plots resulting from this temperature study are depicted in Fig. 2. The effect of column temperature on enantioselectivity of the compounds **1**–**5** was unidirectional. The enantioselectivity factor values increased as the column temperature decreased and the five pairs of enantiomers were separated with a maximum α value at a temperature of −5 °C. Moreover, the van't Hoff plots for the same compounds showed a linear behavior with a regression coef-

Table 1
Thermodynamic data

Compound	Col/MP/TR/FR[a]	α (25 °C)	$\Delta\Delta H^{\circ}$ (kcal mol^{-1})	$\Delta\Delta S^{\circ}$ (cal mol^{-1} K^{-1})	T_{iso} (K)
1	Chiraspher/A/-5–55/1.0	1.17	−0.29	−0.67	432.8
2	AD/B/-5–45/1.0	1.36	−1.73	−5.18	334.0
3	OD/C/-5–45/1.0	1.52	−0.95	−2.38	399.2
4	OD/D/-5–45/1.0	1.63	−1.05	−2.57	408.6
5	OD/E/-5–45/0.5	1.59	−3.58	−9.33	383.7
6	OD/F/-5–45/1.0	∼1.00	−1.02	3.48	293.1

[a] Col/MP/TR/FR refers to the combination of experimental conditions used for enantioseparations. Col: column (Chiralcel OD 250 × 4.6 mm i.d.; Chiralpak AD 250 mm × 4.6 mm i.d.; Chiraspher 250 × 4 mm i.d.). MP: mobile phase (A: *n*-hexane–2-propanol, 80–20; B: *n*-hexane–ethanol, 80–20 + 0.15% TFA; C: *n*-hexane–2-propanol, 95–5 + 0.2% DEA; D: *n*-hexane–ethanol, 70–30; E: ethanol + 0.1% DEA; F: *n*-hexane–2-propanol, 98.5–1.5 + 0.2% DEA). TR: temperature range (°C). FR: flow rate (ml min^{-1}).

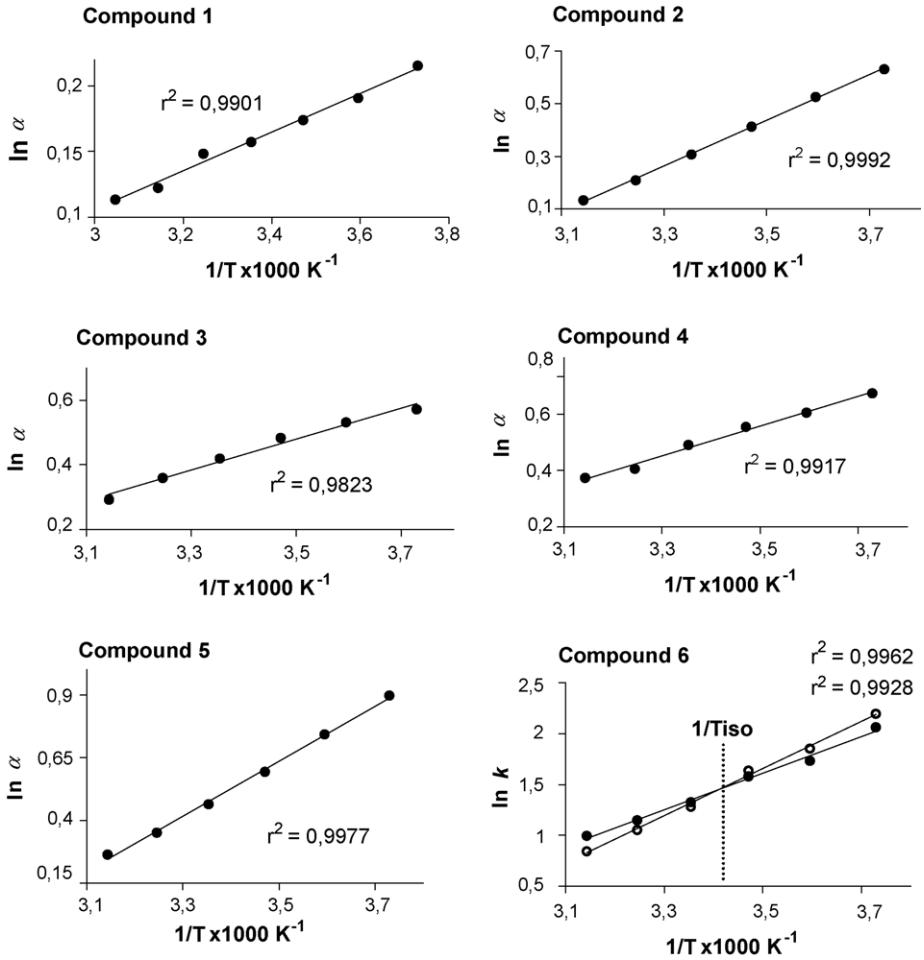

Fig. 2. Influence of temperature on enantioselectivity (compounds **1**–**5**) and retention (compound **6**). See Table 1 for experimental conditions.

ficient (r^2) value greater than 0.98. Therefore, the observed negative $\Delta_{j,i}\Delta H^\circ$ and $\Delta_{j,i}\Delta S^\circ$ were temperature independent.

Table 1 presents the thermodynamic data extracted from the chiral separations of the compounds **1**–**6**. For the compounds **1**–**5**, enthalpic and entropic terms had both negative sign, and hence, they exerted an opposite effect on the enantioselectivity. The $T\Delta_{j,i}\Delta S^\circ$ terms were always smaller than $\Delta_{j,i}\Delta H^\circ$ ones (enthalpic control), as usually observed in chiral HPLC. The enantioselectivity factor values increased at subambient temperatures, indicating that enthalpic contribution is the dominating thermodynamic driving force for chiral recognition. The α terms were highly temperature-dependent for the compounds **2** (Ketoprofen) and **5** (Mianserin). As evident from the data listed in Table 1, for these compounds the highest absolute $\Delta_{j,i}\Delta H^\circ$ values were obtained. Fig. 3 shows the typical variable temperature chromatograms for the enantioseparations of **5**. Ranging from −5 to 45 °C, a consistent decreasing of the enantioselectivity factor from 2.45 to 1.30 was observed.

Above the crossover temperatures, the enantiomers of the compounds **1**–**5** should elute in the opposite order [10,12]. However, the experimentally determined coalescence temperatures (Table 1) were much higher than the working temperature range of the CSPs employed.

The chiral resolution of the analyte **6** (Fluoxetine; Fig. 1) on the Chiralcel OD CSP represents an instance of entropically driven enantioseparation. Fig. 4 shows the chromatograms obtained at different temperatures when the compound **6** was resolved on the OD CSP using a mixture *n*-hexane–2-propanol–DEA, 98.5–1.5–0.2 (v/v/v), as a mobile phase. Elution orders were detected by monitoring the sign of the CD at 260 nm during chromatography. A progressive reduction of the column temperature from 45 to −5 °C produced a lowering in the enantioselectivity to a minimum resulting in a coelution of the enantiomers around the T_{iso}. Below this crossover temperature, the enantioseparation was again clear ($\alpha = 1.16$ at −5 °C) but the sign of enantioselectivity changed. The thermodynamic parameters associated with adsorption of the enantiomers with the CSP were calculated by plotting the ln *k* versus 1000/*T* [16]. The *k*-temperature relationships were highly linear ($r^2 > 0.99$) over the entire examined temperature range. The obtained positive $\Delta_{j,i}\Delta S^\circ$ value (Table 1) indicated that the entropic contribution was favorable to the enantiorecognition process. The van't Hoff plots of two enantiomers of Fluoxetine

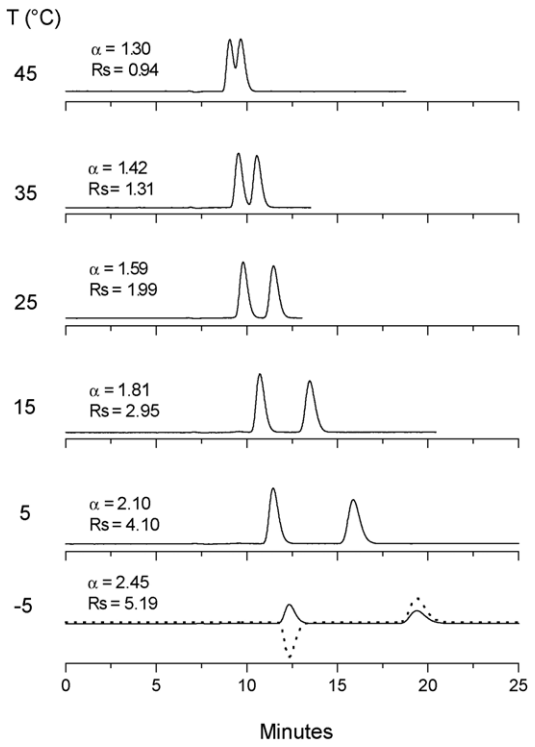

Fig. 3. UV (260 nm; solid traces) and CD (260 nm; dotted trace) detected variable-temperature HPLC of racemic **5** on the Chiralcel OD CSP. See Table 1 for experimental conditions.

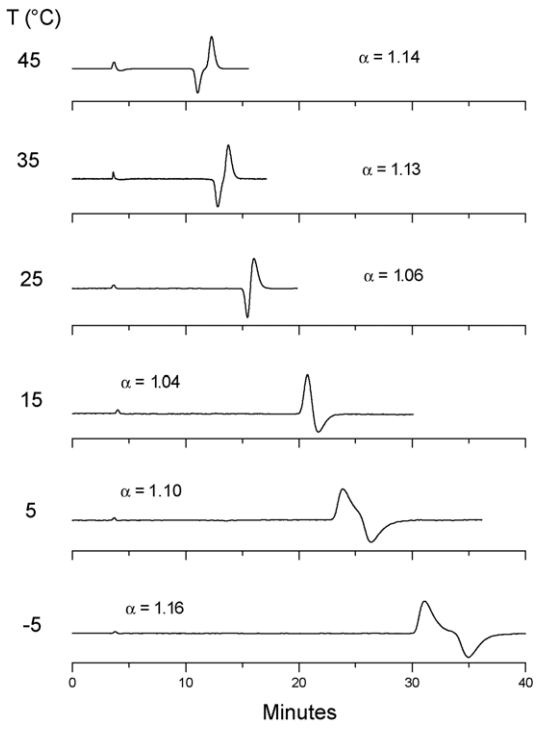

Fig. 4. CD (260 nm) detected variable-temperature HPLC of racemic **6** on the Chiralcel OD CSP. See Table 1 for experimental conditions.

(compound **6**) intersected at the $1/T_{iso} \times 100$ value. At the coalescence temperature (∼20 °C), the enthalpic and entropic contributions to chiral recognition were balanced and the enantiomers coeluted. Beyond T_{iso}, the enantiomers of the Fluoxetine (compound **6**) were separated either by decreasing or by increasing the temperature. It is interesting to note that the calculated coalescence temperature T_{iso} ∼20 °C falls in the isoenantioselectivity temperature region (between 15 and 20 °C) experimentally observed.

The second set of experiments was based on the following thermodynamic consideration: for a particular chiral compound which presents an appreciable temperature-dependent enantioselectivity, it should be possible to perform the inver-

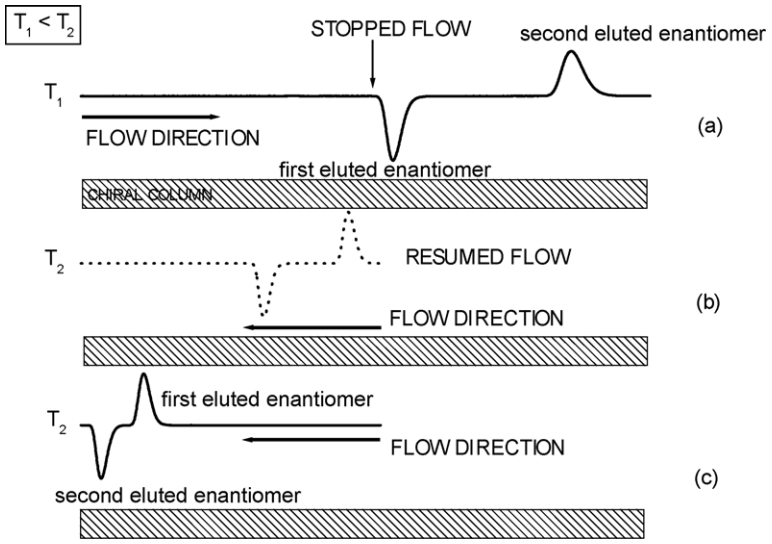

Fig. 5. Schematic illustration showing the experimental procedure to reverse the enantiomer elution order of **1**–**5**. (a) Racemic compound was injected onto the column at T_1 temperature. The mobile phase flow was stopped before the less retained enantiomer eluted. (b) The column was turned around and brought at a set higher T_2 temperature for a set time. The switched enantiomer pair still remained onto the column. (c) The eluent flow was resumed, leading to elution of the switched enantiomer pair.

sion of enantiomer elution order by running it two times over the same section of chiral column, but in reversed directions and at different temperatures.

Fig. 5 shows a schematic representation of the order of events in reversal enantiomer elution order experiments followed for the compounds **1**–**5**. The racemic sample was injected onto the chiral column placed in a cryostat at the lowest temperature (T_1) of the range-studied. After a specific time, the eluent flow was turned off. The turn-off eluent time strictly came before the retention time of the less retained enantiomer. Therefore, the enantiomers were baseline separated at T_1 but still remained on the CSP.

The column was then brought in a thermostat set at higher temperature T_2 and turned around. Thus, the direction flow was reversed. After a defined period (20 min), the flow was resumed. The outcome of this protocol resulted in a reversal of enantiomer elution order, and the enantioselectivity factor of reversed enantiomer pair (α_{REV}) was calculated according to the following equation:

$$\alpha_{REV} = \alpha_{T_1} a_{T_1} - \alpha_{T_2} b_{T_2} + 1 \quad (3)$$

where α_{T_1} and α_{T_2} are the enantioselectivity factors measured at the lowest temperature T_1 and the highest temperature T_2, respectively, a_{T_1} and b_{T_2} are fractional numbers that take into account the section of chiral column run by enantiomers at T_1 and T_2 temperatures. But since:

$$a_{T_1} = b_{T_2} = \tfrac{1}{2}$$

it follows that:

$$\alpha_{REV} = \frac{\alpha_{T_1}}{2} - \frac{\alpha_{T_2}}{2} + 1 \quad (4)$$

Without any significative difference in enantioselectivity at the T_1 and T_2 temperatures, the enantiomers clearly coeluete ($\alpha_{REV} = 1$).

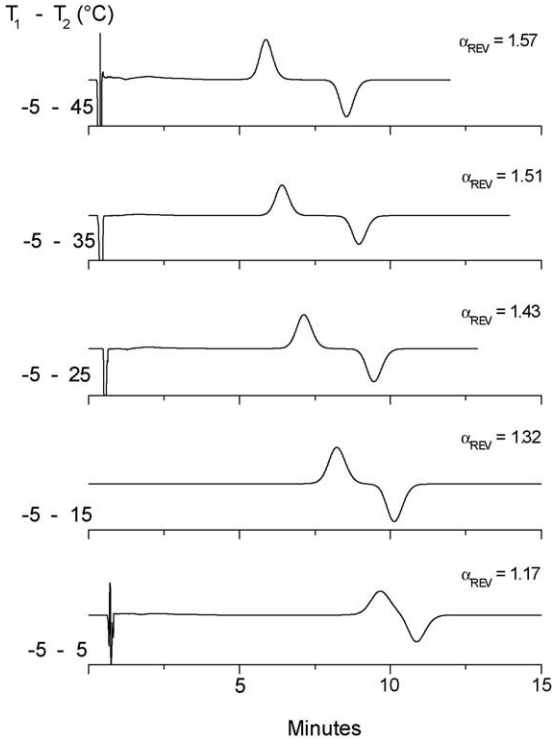

Fig. 6. CD (260 nm) detected variable-temperature HPLC of the switched enantiomer pair **5** on Chiralcel OD CSP. Flow rate at −5 °C (T_1) and 45 °C (T_2): 1.0 ml min^{-1}. See Table 1 for the other experimental conditions.

Nice baseline separations of the reversed enantiomer pairs **2**–**5** were obtained by using the above-described chromatographic system.

In Fig. 6, it is presented a typical series of temperature-dependent chromatograms recorded during the separation-

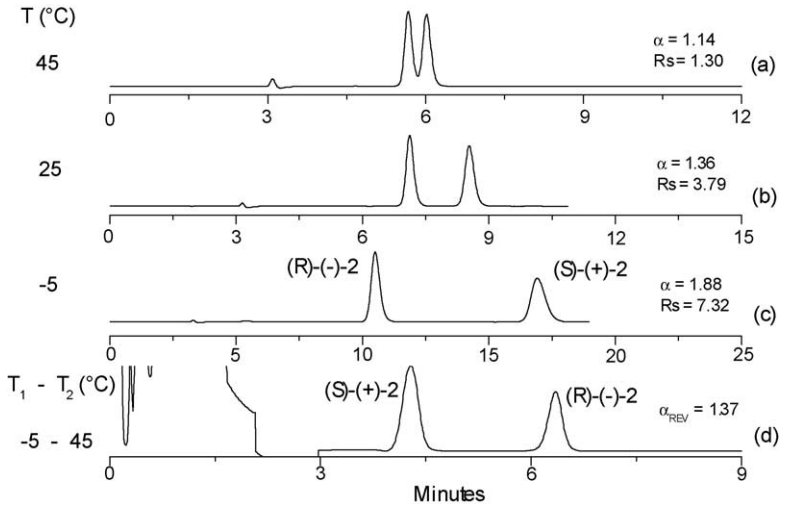

Fig. 7. Traces (a–c): variable-temperature HPLC of the enantiomers of **2** on the AD CSP. Trace (d): chromatogram showing the inversion of the enantiomer elution order of **2**. Flow rate at −5 °C (T_1): 1.0 ml min^{-1}; flow rate at 45 °C (T_2): 0.5 ml min^{-1}. See Table 1 for the other experimental conditions.

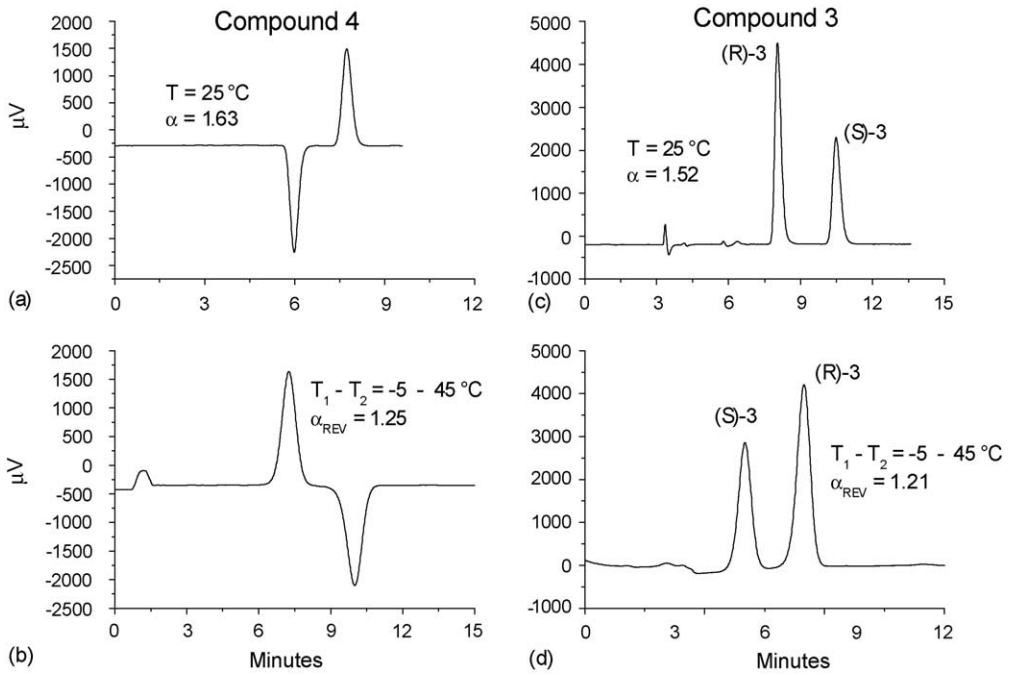

Fig. 8. Trace (a): CD (300 nm) detected HPLC of the enantiomers of **4** on the OD CSP. Trace (c): UV (254 nm) detected HPLC of the enantiomers of **3** on the OD CSP. Traces (b and d): chromatograms showing the inversion of the enantiomer elution order of **4** and **3**, respectively. Flow rate at −5 °C (T_1) and 45 °C (T_2): 1.0 ml min^{-1}. The other experimental conditions are the same as given in Table 1.

inversion process of the enantiomers of **5** on the OD CSP. The enantioseparation increased as the column temperature range T_1–T_2 was larger, and at the temperature range from −5 to 45 °C, enantioselectivity factor (α_{REV}) value of 1.57 was observed. By comparing the CD traces depicted in Figs. 3 and 6 (dotted lines), it is possible to note that the enantiomer elution order switched and the CD trace at 260 nm for the first eluted enantiomer became positive.

As shown in Fig. 7, also the resolution of Ketoprofen (compound **2**) on Chiralpak AD appeared to be highly temperature-dependent. A 10 °C column temperature increasing led to a decrease of the α-value by a factor of

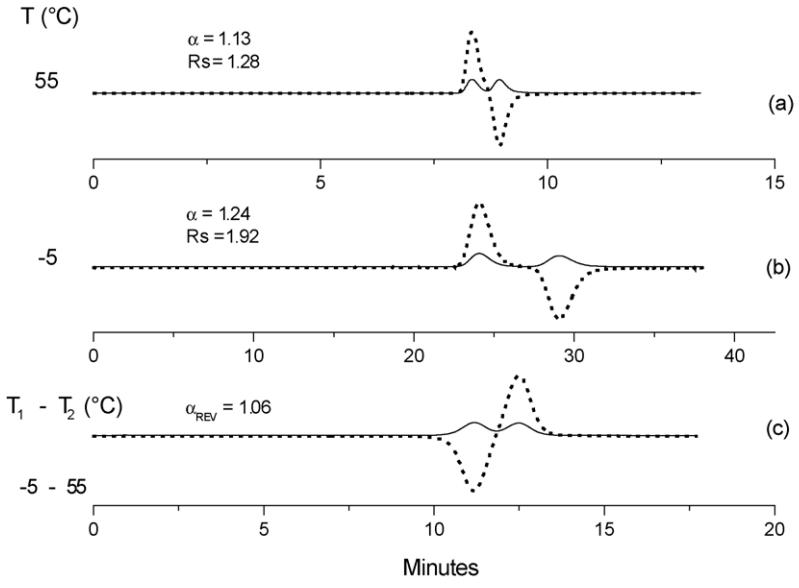

Fig. 9. Traces (a and b): UV (265 nm; solid traces) and CD (265 nm; dotted traces) detected variable-temperature HPLC of the enantiomers of **1** on the Chiraspher CSP trace (c): UV and CD chromatograms showing the inversion of the enantiomer elution order of **1**. Flow rate at −5 °C (T_1) and 55 °C (T_2): 1.0 ml min^{-1}. The other experimental conditions are the same as given in Table 1.

around 20%. At temperature of 25 °C and by using a mixture *n*-hexane–ethanol–TFA, 80/20/0.15 (v/v/v), as eluent, the (*R*)-(−)-**2** enantiomer eluted first and the enantiomer pair was resolved with a resolution factor of 3.79. The effect of temperature range T_1–T_2 on the resolution of the switched enantiomer pair was crucial. By fixing the temperature T_1 at −5 °C and progressively increasing the temperature T_2 up to 45 °C, an increasing of the resolution of the switched enantiomer pair **2** was achieved. At the T_1 and T_2 temperatures of −5 and 45 °C, respectively, the enantiomers eluted in the opposite order with the (*S*)-(+)-**2** enantiomer eluted first. An enantioseparation factor value of 1.37 was observed.

Fig. 8 reports the UV and CD HPLC traces for the inversion of the elution order of the compounds **3** (Timolol) and **4**, respectively. In both cases, although a peak broadening took place, the baseline resolution of the reversed enantiomer pairs was achieved.

The chromatographic separation of the compound **1** shows how the utilized approach could be profitably applied for switching the enantiomer elution order even when the enantioselectivity factor is only slightly temperature-dependent. By decreasing the temperature from 55 to −5 °C the enantiomers of **1** were separated on the Chiraspher CSP with enantioseparation factors of 1.13 and 1.24, respectively. From the sign of the CD traces (dotted lines) recorded at 254 nm (Fig. 9), it was possible to establish the elution order of the enantiomers. In Fig. 9c, the resolution of the reversed enantiomer pair is reported. Two switched enantiomers were again separated and gave rise to distinct peaks.

4. Conclusion

In conclusion, a newly developed procedure to reverse the enantiomer elution order of compounds resolved on chiral stationary phases (CSPs) for HPLC is shown. The chromatographic approach is based on the following operations: stopping-flow during the HPLC enantioseparation at T_1; reversing the flow direction; eluting the switched enantiomers at T_2 temperature ($T_2 > T_1$). The applicability of this procedure was demonstrated for five enantiomer pairs separated by chiral HPLC on polymeric-based CSPs.

Practically, analytical methods utilizing the variable temperature-flow inversion concept may nevertheless suffer from some limitations. For example, in bioanalytics, more peaks than those of the enantiomer may appear in the chromatogram; and most probably, these additional peaks also change their retention behaviour in course of elution order reversal. Consequently, overlapping and obscuring of the crucial enantiomer peaks may occur. Moreover, the observed slight peak broadening can diminish the sensitivity of method.

However, the presented temperature-based protocol may be improved in future and successfully extended to each enantiomer pair separated on natural as well as synthetic CSPs under an appreciable enthalpy-control.

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