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# Chiral resolution of the enantiomers of new selective $CB_2$ receptor agonists by liquid chromatography on amylose stationary phases

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**Abstract**

Analytical HPLC methods using derivatized amylose chiral stationary phases, Chiralpak AD-H and Chiralpak AS, were developed for the direct enantioseparation of eight substituted 4-oxo-1,4-dihydroquinoline-3-carboxamide derivatives with one stereogenic center. Baseline separation ($R_s > 1.5$) was always achieved on amylose based Chiralpak AD-H column to the difference with Chiralpak AS. Using UV detection, a linear response was observed within a 180–420 μmol $L^{-1}$ concentration range ($r^2 > 0.991$) for three racemic compounds **1**, **3** and **4** with best pharmacological potentials; repeatability, limit of detection (LD) and quantification (LQ) were also determined: LD varied, for the solutes, from 0.36 to 2.56 μmol $L^{-1}$. Finally, the enantiopurity of these compounds was determined. Additionally, the effect of temperature variations upon isomer separations was investigated.


*Keywords:* Enantiomers separation; Chiral stationary phases; $CB_2$ receptor agonists; 4-Quinolone; Validation method

## 1. Introduction

Since the $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) characterization in the 60s, as the major psychoactive component of *Cannabis sativa L.*, many improvements have been made in cannabinoid pharmacology knowledge, particularly, in the 90s with the discovery of two G-protein coupled receptors: the $CB_1$ and $CB_2$ receptors. At present, researches are focused on the $CB_2$ receptor ligands to understand some of the physiological effects of cannabinoids, but actually, pharmacologist faces a lack of potent $CB_2$ selective agonists [1].

In this way, we synthesized a set of 3-carboxamido derivatives of 4-oxo-1,4-dihydroquinoline nucleus (4-quinolone) (Fig. 1). Some of these derivatives, especially compounds **1**, **3** and **4**, present a strong selectivity for the $CB_2$ subtype with agonistic properties [2].

Compounds **1**–**8** are characterized by a chiral center as shown in Fig. 1. Compounds **1**–**8** were obtained as racemates, and the enantiopure forms of **1**–**5** were also synthesized starting from the corresponding enantiopure amines. In all cases, the (*R*) enantiomers exhibited about 20-fold higher affinities than the (*S*) enantiomers, which highlighted the stereo-selective interaction to the $CB_2$ receptor [2].

The aim of this study was to develop a method for the resolution of all the racemic mixtures and for the enantiomeric purity determination of respective enantiomers of compounds **1**–**5**. Previous studies using liquid chromatography for the analytical resolution of 4-quinolone derivatives used derivatization procedures, or chiral mobile phase methods based on ligand exchange or chiral stationary phases (CSPs) methods including crown ether, protein-based CSPs [3]. Cellulose and amylose ester and carbamate derivatives coated onto a large-pore silica gel backbone have proved to be extremely useful CSPs for chiral resolution. To the best of our knowledge, only a study by Radhakrishna et al. [4] described the resolution of fluoroquinolone moxifloxacin enantiomers on a cellulose carbamate derivative (Chiralcel OD-H) and on cellulose ester derivatives (Chiralcel OJ and OB).

In the continuity of our work [5,6], in the separation of chiral compounds with cellulose CSPs we investigated,

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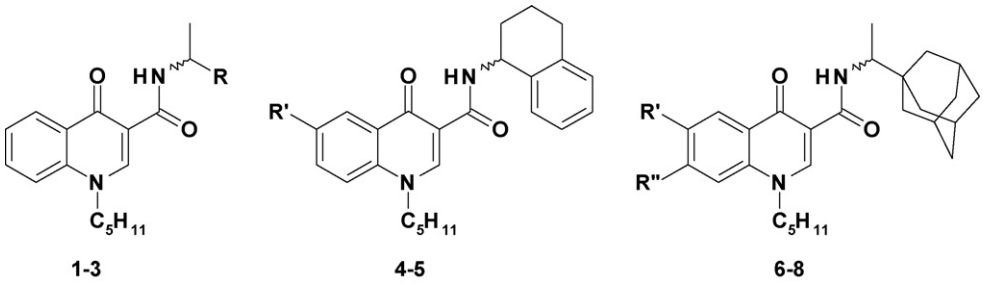


Fig. 1. $CB_2$ cannabinoid receptor ligands structures. (**1**) R: phenyl; (**2**) R: 1-naphthyl; (**3**) R: 2-naphthyl; (**4**) R′: H; (**5**) R′: Cl; (**6**) R′: H, R″: H; (**7**) R′: Cl, R″: H; (**8**) R′: H, R″: Cl.

in this paper, the direct separations of **1**–**8** (Fig. 1) on (tris-3,5-dimethylphenylcarbamate) amylose (Chiralpak AD-H) and (tris-(*S*)-1-phenylethylcarbamate) amylose (Chiralpak AS). Analytical methods were developed first to determine the stationary and mobile phases that permitted the best enantiomeric separations. Secondly, we have developed and validated analytical methods in order to quantify the enantiomeric purities of three compounds with best pharmacological potentials (**1**, **3** and **4**). Additionally, the effect of temperature variations upon isomer separations was investigated.

## 2. Experimental

### 2.1. Chromatography

Chiral chromatography was performed on a Chiralpak AD-H amylose (tris-3,5-dimethylphenylcarbamate; 250 mm × 4.6 mm i.d.; 5 μm), and on a Chiralpak AS amylose tris-(*S*)-1-phenylethylcarbamate; 250 mm × 4.6 mm i.d.; 10 μm) from Daicel (Chiral Technologies, Illkirch, France). A constant mobile phase flow of 1 mL $min^{-1}$ was provided by a gradient Waters 600E metering pump model equipped with a 7125 Rheodyne injector (20 μL loops). Detection was achieved with a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on a computer running with Millennium 2010 software. Mobile phase elution was made isocratically using *n*-hexane and a modifier (ethanol, 1-propanol or 2-propanol) at various percentages. Chromatography was performed at 25 °C unless noted otherwise to determine the temperature dependence of the enantiomeric resolution.

The peak of the solvent front was considered to be equal to the dead time ($t_0$) and was recorded for each particular run. For Chiralpak AD-H, it was about 3.60 min at 1 mL $min^{-1}$ (equal to the value obtained by injection of 1,3,5-tri-*tert*-butylbenzene used as a non-retained sample). For Chiralpak AS, it was about 3.70 min at 1 mL $min^{-1}$. Retention times were mean values of two replicate determinations.

### 2.2. Chemicals and materials

Compounds **1**–**8** were synthesized as previously described [2]. Ethanol, 1-propanol, 2-propanol and *n*-hexane were HPLC grade from Merck or Baker (Paris, France). All solutions were filtered (0.45 μm), degassed with a Waters in-line degasser apparatus. The mobile phases used were referenced as A, B or C for mixtures of hexane (v:v) and ethanol, 1-propanol or 2-propanol as alcohol modifiers, respectively.

Compounds were chromatographed by dissolving them in ethanol to a concentration of about 0.30 mM (which corresponds to 6 nmol injected) and passed through a 0.45 μm membrane filter prior to loading the column.

## 3. Results and discussion

The results of the chiral separation of **1**–**8** racemates chromatographed are summarized in Table 1 for Chiralpak AD-H and Table 2 for Chiralpak AS. The UV spectra of the enantiomers were identical and are, of course, very similar for compounds **1**–**8**. Detection was performed at the first maximum wavelength of absorption of each molecule (Table 2).

### 3.1. Influence of the mobile phase composition

Several kinds of mobile phase compositions were investigated by changing the nature and the percentage of the alcohol, ethanol, 1-propanol or 2-propanol. Baseline separation ($R_s > 1.5$) was obtained for all compounds on Chiralpak AD-H, and only for compound **3** on Chiralpak AS. Compound **1** was partially resolved ($R_s = 0.62$) on this last CSP. For both columns, the effects on these mobile phase variations on chromatographic parameters ($k, \alpha, R_s$) are those generally expected [7]. On AD-H, the change in the mobile phase modifier from ethanol to both propanols, which leads to decrease the mobile phase polarity, results in an increase in the retention factor, $k$ of both enantiomers (Table 1). Similar pattern are observed for selectivity and resolution for compounds **2**, **4**, **6**, **7** and **8** whereas compounds **1**, **3** and **5** show decrease variations of $\alpha$ and $R_s$ (Table 1). Additionally, replacement of 1-propanol by 2-propanol, whose polarity value is virtually the same, leads to a decrease in retention factors of the first eluted enantiomer, except for compounds **1** and **3** on Chiralpak AD-H (Table 1), and for all solutes, to an increase of both selectivity and resolution except for **8** whose $\alpha$ and $R_s$ decreased. In a general manner, on AD-H column, all compounds are resolved with the three alcohols tested and the enantiomeric resolution increase as the carbon number and the bulkiness of the alcohol increase from ethanol to 2-propanol except for **3** and **8** [8].

Table 1
Chromatographic parameters: retention factors ($k$), enantioselectivity factor ($\alpha$) and resolution ($R_s$) of **1–8** (Chirapak AD-H)

| Compound | Eluent | $k_1$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| **1** | A(80:20) | 2.44 | 1.14 | 2.29 |
| | A(70:30) | 1.61 | 1.14 | 1.98 |
| | A(60:40) | 1.22 | 1.13 | 1.69 |
| | B(90:10) | 7.48 | 1.04 | <0.50 |
| | B(80:20) | 3.15 | 1.06 | 0.91 |
| | C(80:20) | 3.28 | 1.28 | 4.01 |
| | C(70:30) | 2.07 | 1.23 | 2.87 |
| **2** | A(80:20) | 2.74 | 1.31 | 4.62 |
| | A(70:30) | 1.81 | 1.30 | 4.01 |
| | A(60:40) | 1.38 | 1.30 | 3.54 |
| | B(80:20) | 3.19 | 1.62 | 7.68 |
| | C(80:20) | 2.92 | 2.35 | 13.09 |
| | C(70:30) | 1.75 | 2.39 | 11.60 |
| **3** | A(80:20) | 3.20 | 1.67 | 9.30 |
| | A(70:30) | 2.13 | 1.67 | 8.48 |
| | A(60:40) | 1.60 | 1.67 | 7.67 |
| | B(80:20) | 4.62 | 1.37 | 5.21 |
| | C(80:20) | 5.11 | 1.66 | 8.22 |
| | C(70:30) | 3.14 | 1.57 | 6.59 |
| **4** | A(80:20) | 1.78 | 2.26 | 12.84 |
| | A(70:30) | 1.20 | 2.27 | 11.38 |
| | A(60:40) | 0.89 | 2.29 | 10.47 |
| | B(80:20) | 2.40 | 2.49 | 13.12 |
| | C(80:20) | 2.33 | 4.10 | 14.28 |
| | C(70:30) | 1.38 | 4.24 | 13.34 |
| **5** | A(90:10) | 4.11 | 3.33 | 20.13 |
| | A(80:20) | 2.04 | 3.01 | 15.80 |
| | A(70:30) | 1.40 | 2.92 | 13.58 |
| | B(80:20) | 1.65 | 2.30 | 11.47 |
| | C(80:20) | 1.59 | 3.36 | 16.83 |
| | C(70:30) | 1.13 | 2.96 | 12.94 |
| **6** | A(95:5) | 3.59 | 1.21 | 3.02 |
| | A(90:10) | 1.55 | 1.16 | 1.96 |
| | B(90:10) | 3.71 | 1.41 | 4.94 |
| | B(80:20) | 2.62 | 1.40 | 4.60 |
| | C(90:10) | 3.36 | 1.50 | 5.76 |
| | C(70:30) | 0.69 | 1.69 | 4.37 |
| **7** | A(95:5) | 3.52 | 1.00 | n.r. |
| | A(90:10) | 1.45 | 1.11 | <0.50 |
| | B(90:10) | 2.66 | 1.37 | 3.68 |
| | B(80:20) | 1.48 | 1.36 | 3.21 |
| | C(95:5) | 4.28 | 1.48 | 5.48 |
| | C(90:10) | 1.90 | 1.51 | 4.55 |
| | C(70:30) | 0.44 | 1.65 | 2.59 |
| **8** | A(95:5) | 4.06 | 1.07 | 1.31 |
| | A(90:10) | 2.21 | 1.00 | n.r. |
| | B(90:10) | 2.78 | 1.28 | 3.76 |
| | B(80:20) | 1.36 | 1.28 | 3.09 |
| | C(95:5) | 6.29 | 1.16 | 2.32 |
| | C(90:10) | 2.63 | 1.15 | 2.17 |
| | C(70:30) | 0.77 | 1.17 | 1.52 |

n.r: Not resolved; concentration ca. 0.30 mM. The flow-rate was 1 mL min$^{-1}$. The temperature was 25 °C. The mobile phases were referenced as A, B or C for mixtures of hexane (v:v) and ethanol, 1-propanol or 2-propanol as alcohol modifiers, respectively. Detection wavelengths are: 217 nm for **1**, **4** and **5**; 220 nm for **6**; 221 nm for **7**; 222 nm for **2**, **3** and **8**.

Table 2
Chromatographic parameters: retention factors ($k$), enantioselectivity factor ($\alpha$) and resolution ($R_s$) of **1–8** (Chiracel AS)

| Compound | Eluent | $k_1$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| **1** | A(95:5) | 3.39 | 1.00 | n.r. |
| | C(95:5) | 3.48 | 1.21 | 0.62 |
| **2** | A(95:5) | 3.79 | 1.00 | n.r. |
| | C(95:5) | 5.80 | 1.00 | n.r. |
| **3** | A(95:5) | 3.94 | 1.31 | 1.22 |
| | A(90:10) | 1.55 | 1.30 | 0.53 |
| | B(90:10) | 1.62 | 1.52 | 1.52 |
| | C(95:5) | 5.89 | 1.70 | 2.16 |
| | C(90:10) | 2.62 | 1.59 | 1.53 |
| **4** | A(95:5) | 3.66 | 1.00 | n.r. |
| | C(95:5) | 4.97 | 1.00 | n.r. |
| **5** | A(95:5) | 2.33 | 1.00 | n.r. |
| | C(95:5) | 4.48 | 1.00 | n.r. |
| **6** | A(95:5) | 1.15 | 1.00 | n.r. |
| | C(95:5) | 2.21 | 1.00 | n.r. |
| **7** | A(95:5) | 0.97 | 1.00 | n.r. |
| | C(95:5) | 1.50 | 1.10 | n.r. |
| **8** | A(95:5) | 0.61 | 1.00 | n.r. |
| | C(95:5) | 1.46 | 1.00 | n.r. |

n.r.: Not resolved; concentration ca. 0.30 mM. The flow-rate was 1 mL min$^{-1}$. The temperature was 25 °C. The mobile phases were referenced as A, B or C for mixtures of hexane (v:v) and ethanol, 1-propanol or 2-propanol as alcohol modifiers, respectively. Detection wavelengths are: 217 nm for **1**, **4** and **5**; 220 nm for **6**; 221 nm for **7**; 222 nm for **2**, **3** and **8**.

### 3.2. Influence of molecular structure

On Chiralcel AD-H, substitution of the chiral centre by a phenyl (compound **1**), a 1-naphthyl (**2**) or a 2-naphthyl group (**3**) results in an increase in retention factor and an increase in both selectivity and resolution (Fig. 2). The aromatic enrichment of **2** and **3** favours Π–Π interactions with the CSP aromatic residues and seems to be stereo-selective. Furthermore, partial hydrogenation, giving tetralin residue, in compound **4**, shows smaller retention factor of the first eluted enantiomer and both higher selectivity and resolution than **2** or **3**. The 6-chloro substitution on 4-quinolone ring (compound **5**) leads to smaller retention factor $k_1$ but higher values of $\alpha$ and $R_s$. In this case, halogen-substitution on 4-quinolone seems to enhance the stereo-selectivity from stronger or supplementary chiral interactions. Whereas the retention factors are in the same range for both compounds **1** and **4**, better selectivity and resolution for **4** are observed with all alcohol modifiers used. The reduction of aryl free rotation may promote stereo-selectivity of **4**.

Compounds **6**, **7** and **8** with adamantyl substituent, show in all mobile phase tested small retention factors (Fig. 2). In accordance with the normal phase mode used in this study, the large hydrophobic character of the adamantyl moiety contributes to weaken interaction with the CSP. Nevertheless, with 1-propanol or 2-propanol as organic modifiers, selectivity and resolution parameters of **6** are greater than values obtained with compound **1**. 6-Chloro or 7-chloro substitution in 4-quinolone of **6**, leading to compounds **7** or **8**, respectively, results in a decrease in

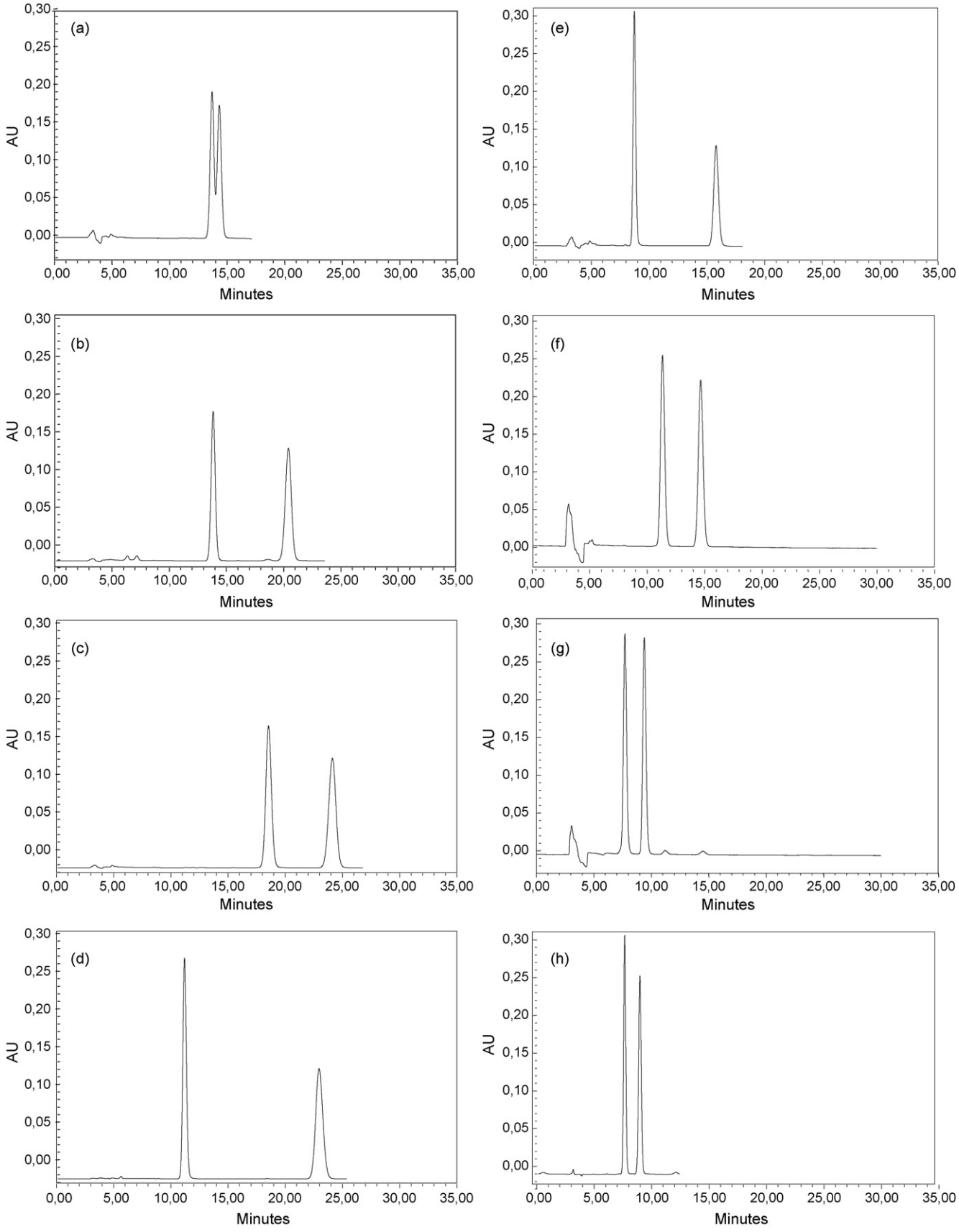


Fig. 2. Chromatograms for **1**–**8** (a–h), influence of the aromatic interaction and steric constraint of the solute (Eluent B(80:20); 1.0 mL $min^{-1}$; Chiralpak AD-H). Detection wavelengths are: 217 nm for **1**, **4**, **5**; 220 nm for **6**; 221 nm for **7**; 222 nm for **2**, **3** and **8**.

Table 3
Thermodynamic parameters of the enantiomer separations of **1** and **3** on both CSPs AD-H and AS

| Compound | CSP | $\Delta H_1^\circ$ (J mol$^{-1}$) | $\Delta H_2^\circ$ (J mol$^{-1}$) | $\Delta S_1^\circ + R \ln \Phi$ (J K$^{-1}$ mol$^{-1}$) | $\Delta S_2^\circ + R \ln \Phi$ (J K$^{-1}$ mol$^{-1}$) | $\Delta\Delta H^\circ$ (J mol$^{-1}$) | $\Delta\Delta S^\circ$ (J K$^{-1}$ mol$^{-1}$) |
|---|---|---|---|---|---|---|---|
| **1**[a] | AD | −15,197 | −15,079 | −42.0 | −39.9 | 118 | 2.1 |
| **3**[a] | AD | −17,707 | −15,568 | −47.0 | −36.0 | 2139 | 11 |
| **3**[b] | AS | −15,710 | −15,794 | −38.9 | −34.9 | 471 | 5.9 |

Temperature range: 15–40 °C with 5 °C intervals. Conditioning column time: 30 min. van't Hoff model: $\ln k = (-\Delta G^\circ/RT) + \ln \Phi = (-\Delta H^\circ/RT) + (\Delta S^\circ/R) + \ln \Phi$ and $\ln \alpha = (-\Delta\Delta G^\circ/RT) + \ln \Phi = (-\Delta\Delta H^\circ/RT) + (\Delta\Delta S^\circ/R) + \ln \Phi$ . $\Delta G^\circ$: molar Gibbs energy of the solute, $\Delta H^\circ$ and $\Delta S^\circ$: enthalpy and entropy of transfer of the solute from the mobile phase to the stationary phase, $T$: absolute temperature, $R$: gas constant, and $\Phi$: phase ratio.
[a] Eluent C(80:20).
[b] Eluent C(95:5).

retention factor, selectivity and resolution. These data are in opposition to the previous case of halogen substitution (compounds **4** and **5**) which seems to show the unfavourable steric hindrance due to adamantyl group for stereo-selective interactions. The more hydrophobic character of **6**, **7** and **8**, by comparison with **4** and **5**, could also contribute to decrease both retention factor ($k$) and stereo-selectivity ($\alpha$, $R_s$) with smaller chiral interactions with the CSP.

### 3.3. Influence of temperature

The influence of temperature has been partly investigated on AD-H for compounds **1** and **3** and on AS for **3**, as a potential factor affecting the enantioselectivity [9,10]. The variation in R ln $k$ (and R ln $\alpha$) versus 1/$T$ according to the van't Hoff model, shows linear relationships with $r^2 > 0.993$, showing no conformational changes on the stationary phase [9,10]. The stereo-selective interactions involved during the separation are unchanged in the studied temperature range [9]. The thermodynamic parameters (Table 3) are determined from slope and intercept of linear relationships obtained. For both compounds, whatever the CSP, negative $\Delta H^\circ$ indicates that it is energetically more favourable for the solute to be in the stationary phase. Negative $\Delta S^{\circ *}$ (with $\Delta S^{\circ *} = \Delta S^\circ + R \ln F$) also indicates an increase in the order of the chromatographic system as the solute is transferred from the mobile phase to the stationary phase. Both thermodynamic parameters are negative, which indicates that the transfer of the solutes from the mobile phase to the stationary phase is enthalpically governed. The $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ values are positive for **1** on Chiralpak AD-H and for **3** on both columns AD-H and AS, which indicates an entropy-driven separation in this temperature range.

### 3.4. Validation procedure

#### 3.4.1. Repeatability

The intra-day repeatability was assessed by seven ($n = 7$) injections of 0.3 mM solutions of compounds **1**, **3** and **4**. Repeatability expressed as R.S.D.s on retention and peak areas, were found to range between 0.2–0.3 and 0.6–0.9%, respectively.

#### 3.4.2. Limit of detection (LOD) and limit of quantification (LOQ)

LODs and LOQs of each enantiomer of **1**, **3** and **4**, presented in Table 4, were determined by serial dilutions of the solutions of these enantiomers to obtain an S/N of 3 and 10, respectively. The LODs were between 0.36 and 2.56 μmol L$^{-1}$ corresponding

Table 4
Linearity, LODs and LOQs of compounds **1**, **3** and **4**, enantiomer purity (Chiralpak AD-H, eluent C(90:10), 25 °C for **1** and eluent A(60:40), 40 °C for both **3** and **4**)

| Compound | Enantiomer [5] | Linear range (μmol L$^{-1}$) | Slope | Intercept | $r^2$ | LOD (μmol L$^{-1}$) | LOQ (μmol L$^{-1}$) | Enantiomeric purity (%) |
|---|---|---|---|---|---|---|---|---|
| **1** | | | | | | | | |
| $P_1$ | *R* (−) | 180–420 | 40105 ± 440 | 2101 ± 104 | 0.996 | 1.95 | 6.52 | 99.01 |
| | | 8.0–12.0 | 38249 ± 414 | 1822 ± 89 | 0.990 | | | |
| $P_2$ | *S* (+) | 180–420 | 40216 ± 396 | 2216 ± 99 | 0.998 | 2.56 | 8.54 | >99.35 |
| | | 9.0–12.0 | 38128 ± 295 | 1456 ± 82 | 0.992 | | | |
| **3** | | | | | | | | |
| $P_1$ | *R* (−) | 180-420 | 34605 ± 442 | −1351 ± 104 | 0.996 | 0.36 | 1.21 | >99.80 |
| | | 1.5–4.5 | 31953 ± 385 | 1116 ± 151 | 0.991 | | | |
| $P_2$ | *S* (+) | 180–420 | 34516 ± 394 | 1776 ± 99 | 0.998 | 0.61 | 2.02 | >99.88 |
| | | 3.0–6.0 | 32064 ± 426 | −1099 ± 140 | 0.995 | | | |
| **4** | | | | | | | | |
| $P_1$ | *R* (−) | 180–420 | 28567 ± 411 | −1012 ± 105 | 0.991 | 0.59 | 1.97 | 99.02 |
| | | 2.0–4.0 | 26133 ± 295 | 950 ± 88 | 0.988 | | | |
| $P_2$ | *S* (+) | 180–420 | 28659 ± 409 | −1252 ± 102 | 0.993 | 0.87 | 2.91 | 99.71 |
| | | 3.0–6.0 | 26102 ± 324 | 1025 ± 152 | 0.987 | | | |

to 0.12 and 0.85% minor enantiomer for a major enantiomer defined target concentration of 0.3 mM.

*3.4.3. Linearity and enantiomeric purity determination*

In order to determine enantiomeric purity, two linear ranges were studied: the first one covered the range 60–140% of the main enantiomer, the second beginning at the LOQ covered the range of enantiomeric impurity eventually detected for the minor enantiomer. The linearity of peak area versus concentration was subjected to statistical analysis using a linear-regression least-square method. The calibration curves were found to be linear with determination coefficient $r^2$ superior to 0.987 and the results are shown in Table 4. The difference of the slope obtained for both calibration ranges justifies the two calibration ranges.

Enantiomeric purities of compounds of interest **1**, **3** and **4** are found under the respective LOQ (Table 4). Minor enantiomers have been detected for (*R*)-**1**, (*R*)-**4** and (*S*)-**4** and do not exceed 1% corresponding to the enantiomeric purities of the chiral amino precursor [2].

## 4. Conclusion

The resolution results of compounds **1**–**8** described above, indicated that Chiralcel AD-H is well adapted for the enantioseparation. Baseline separation is only obtained for compound **3** on Chiralpak AS. The separation of **1** on Chiralpak AD-H and **3** on both AD-H and AS CSPs were found to be entropy driven processes. Methods were validated for three compounds of interest **1**, **3** and **4** with respect to repeatability, limit of detection, limit of quantification and linearity and make the chromatographic methods suitable to quantify enantiomeric purity. Further experimental applications of these methods would be performed to study the pharmacological distribution.

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