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Dansyl amino acid enantiomer separation on a teicoplanin chiral stationary phase: effect of eluent pH

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Abstract

The retention and separation of a series of D,L dansyl amino acids (used as test solutes) on a teicoplanin stationary phase were investigated over a wide range of mobile phase (citrate buffer–methanol, 90:10, v/v) pH. An approach based on the development of various equilibria was carried out in order to describe the retention behavior of the solute in the chromatographic system. The equilibrium constants corresponding to the transfer of the anionic and zwitterionic forms of the dansyl amino acids from the mobile to the stationary phase were determined. These values allowed one to explain the decrease in the retention factor and the associated increase in the separation factor as the eluent pH was increased. Thermodynamic parameter variations were calculated so that the driving forces of the solute association with the teicoplanin phase were derived. This approach indicated that the chiral discrimination was principally controlled by the interaction between the anionic form of the solute and the stationary phase.

Keywords: Enantiomer separation; Mobile phase composition; pH effects; Thermodynamic parameters; Chiral stationary phases, LC; Amino acids, Dns derivatives; Teicoplanin

1. Introduction

The use of macrocyclic antibiotics as chiral selectors has been introduced in 1994 by Armstrong and co-workers [1,2]. Due to their structural characteristics, the macrocyclic compounds combine the chiral properties of different chiral stationary phases (CSPs) such as proteins and cyclodextrins. For example, vancomycin or teicoplanin contains from 18 to 20 stereogenic centers, three or four inclusion cavities and ionizable groups. The complexity of the selector structure is responsible for several mechanisms in the chiral recognition. For the teicoplanin stationary phase, some studies have been carried out on the aspects of the enantiomer–macrocycle interaction. Both Berthod et al. [3] and Péter and co-workers [4,5] have expected that the selector ammonium group is implied in the enantioseparation of various amino acid derivatives via ionic interactions with the carboxylate group of the solutes. As well, it has been evoked that additional interactions must be implied such as hydrophobic effect, steric repulsion and hydrogen-bonding [3–6]. However, earlier studies have not specifically modeled the influence of

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the eluent pH on the solute enantiomer separation in the reversed-phase mode. In order to further explore the mechanistic aspects in the chiral discrimination on teicoplanin stationary phase, the effect of the respective ionizations of the test solutes (D,L dansyl amino acids) on the enantiomer retention behavior was investigated by varying the mobile phase pH. Using a treatment based on the development of various equilibria between the solute and both the mobile and stationary phases, the association constants of the solute anionic and zwitterionic forms with teicoplanin were calculated. Thermodynamic variations were obtained from Van 't Hoff plots and were discussed relative to this interaction model.

2. Theory

2.1. Solute retention equations

Teicoplanin contains a single primary amine and a single carboxylic acid group. The respective pK_a values are around 9.2 and 2.5 as reported previously [3]. As well, dansyl amino acids contain two ionizable groups, a carboxylic group with a pK_a around 2.5 and a tertiary amino group from the dimethylaminonaphtyl moiety of a pK_a equal to 4.6 [7]. Thus, the variation of the pH of the mobile phase aqueous fraction between 3.5 and 7.0 (as used in this study) is expected to strongly influence the solute retention. As described in the schematic representation of the different solute equilibria implied both in the mobile and the stationary phase (Fig. 1), three ionization constants can be introduced:

$$K_a = \frac{[L_{S2}^+]}{[L_{S1}^{-/+}][H^+]} \quad (1)$$

$$K_{a1} = \frac{[S^{-/+}]}{[S^-][H^+]} \quad (2)$$

$$K_{a2} = \frac{[S^+]}{[S^{-/+}][H^+]} \quad (3)$$

where $Ls_1^{-/+}$ and Ls_2^+ are, respectively, the fully ionized (–COO^- and –NH_3^+) and the protonated/ionized (–COOH and –NH_3^+) forms of the teicoplanin, S^-, $S^{-/+}$ and S^+ the various ionized forms of the dansyl amino acid solute. K_a, K_{a1} and K_{a2} are the protonation constants of the teicoplanin carboxylic group, the tertiary amino group and the carboxylic group of the dansyl amino acid, respectively. As well, the equilibrium constants of the solute form transfer from the mobile to the stationary phase are the following (Fig. 1):

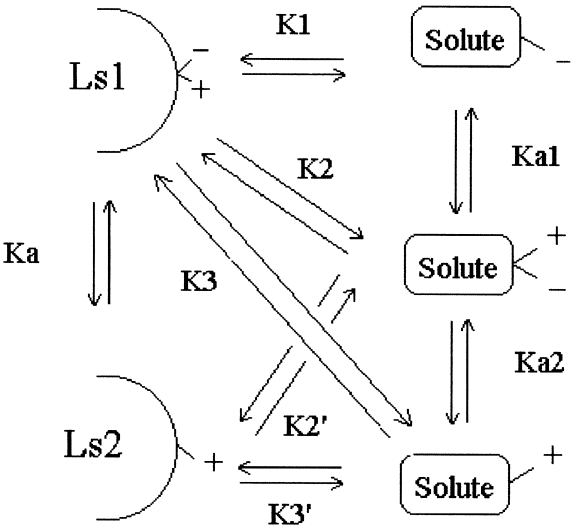

Fig. 1. Model for the various solute species interaction (S^-, $S^{-/+}$ and S^+) with the two $L_{S1}^{-/+}$ and L_{S2}^+ CSP forms.

$$K_1 = \frac{[S^-L_{S1}^{-/+}]}{[S^-][L_{S1}^{-/+}]} \quad (4)$$

$$K_2 = \frac{[S^{-/+}L_{S1}^{-/+}]}{[S^{-/+}][L_{S1}^{-/+}]} \quad (5)$$

$$K_3 = \frac{[S^+L_{S1}^{-/+}]}{[S^+][L_{S1}^{-/+}]} \quad (6)$$

$$K_2' = \frac{[S^{-/+}L_{S2}^+]}{[S^{-/+}][L_{S2}^+]} \quad (7)$$

Of course, the interaction between S^- and Ls_2^+ is not taken into account because the pK_a of the teicoplanin carboxylic group is around 2.5. As well, the association between S^+ and Ls_2^+ is expected to be negligible due to the ionic repulsion between the two species.

The retention factor is described as follows:

$$k' = \phi \frac{[S^- L_{S1}^{-/+}] + [S^{-/+} L_{S1}^{-/+}] + [S^+ L_{S1}^{-/+}] + [S^{-/+} L_{S2}^{+}]}{[S^-] + [S^{-/+}] + [S^+]} \quad (8)$$

where ϕ is the phase ratio. The total teicoplanin stationary phase concentration is equal to:

$$[L_S] = [L_{S1}^{-/+}] + [L_{S2}^{+}] \quad (9)$$

By combining all the Eqs. (1)–(9), the retention factor can be linked to the different ionization and transfer constants as follows:

$$k' = \frac{\phi K_1 + \phi K_2 K_{a1}[H^+] + \phi K_3 K_{a1} K_{a2}[H^+]^2 + \phi K'_2 K_a K_{a1}[H^+]^2}{1 + K_a[H^+] + K_{a1}[H^+] + K_{a1}K_{a2}[H^+]^2 + K_a K_{a1}[H^+]^2 + K_a K_{a1} K_{a2}[H^+]^3} \quad (10)$$

If the protonation of the carboxylic groups of both the teicoplanin and the dansyl amino acids is neglected (i.e., 10% at pH 3.5), a simplified equation is obtained using the same method:

$$k' = \frac{\phi K_1 + \phi K_2 K_{a1}[H^+]}{1 + K_{a1}[H^+]} \quad (11)$$

ϕK_1 corresponds to the solute retention factor at an eluent pH equal to 7.0 (pH>4.6+2). Eqs. (10) and (11) can be used to derive the relative contributions of the different chemical species on the retention and separation of the solute on the chiral stationary phase. Calculation of various association constants needs to know the phase ratio ϕ. In this study, the value determined by Péter et al. [4] from the technical data of the Astec Chirobiotic T column was used.

2.2. Solute–teicoplanin interaction energies

ΔG, ΔH and ΔS are the Gibbs free energy, enthalpy and entropy for the species transfer from the mobile to the stationary phase, respectively. These energies can be calculated using the following thermodynamic relationship:

$$\ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (12)$$

where K is a given equilibrium constant, i.e., K_1, K_2 or other constants.

3. Experimental

3.1. Apparatus

The high-performance liquid chromatography (HPLC) system consisted of an LC Shimadzu pump 10AT (Touzart et Matignon, Courtaboeuf, France), a Rheodyne injection valve Model 7125 (Interchim, Montluçon, France) fitted with a 20-μl sample loop, a Shimadzu SPD-10A UV–visible detector. An Astec 150 mm×4.6 mm Chirobiotic T HPLC column (packed with a stationary phase produced by chemically bonding the macrocyclic glycopeptide teicoplanin to a 5 μm silica gel) was used with controlled temperature in an Igloocil oven (Interchim). The mobile phase flow-rate was 0.8 ml/min.

3.2. Reagents and operating conditions

All the D,L dansyl amino acids (dansyl valine, dansyl serine, dansyl tryptophan, dansyl phenylalanine and dansyl leucine) were obtained from Sigma–Aldrich (Saint-Quentin, France). Methanol (HPLC grade), trisodium citrate and citric acid were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The mobile phase consisted of 0.01 *M* citrate buffer–methanol (90:10, v/v). The variation range of the citrate buffer pH was 7.0 to 3.5. To examine the concentration dependencies of solute retention corresponding to the binding capacity of the CSP, retention measurements were related to varying amounts of injected solute. Solute samples were prepared at different concentrations in the mobile phase from 1 to 10 μg/ml. A 20-μl volume of each solute was injected in triplicate and the retention times were measured. The retention factor versus sample amount plots exhibited a plateau at a sample concentration lower than 5 μg/ml followed by a small decrease at higher solute concentrations. Therefore, each solute was injected at a concentration of 2.5 μg/ml where the retention was sample

concentration-independent, i.e., under linear elution conditions [8].

3.3. Temperature studies

Compound retention factors were determined over the temperature range 5 to 35°C. The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to experiment.

4. Results and discussion

4.1. Determination of the equilibrium constants for the solute transfer from the mobile to the stationary phase

The retention factor values were determined at a column temperature equal to 20°C. The relative standard deviations (RSDs) of the k' values were $<0.5\%$ in most cases, showing a high reproducibility and a good stability for the chromatographic system. The k' values were plotted against pH for each D and L enantiomer and exhibited a similar shape. For example, Fig. 2 shows these plots for the D,L dansyl serine enantiomers. Using the K_a, K_{a1} and K_{a2} values, the data were fitted to Eq. (10) by a non linear regression procedure as described previously [9,10]. After the regression procedure, the calculated constants were used to estimate the retention factors for each solute. The correlation between the predicted and experimental k' values exhibited slopes higher than to 0.99 with $r^2>0.999$. When the same procedure was used with the simplified Eq. (11), a similar slope was observed with a good correlation, i.e., $r^2>0.990$ (Fig. 3). The difference between K_2 values calculated from Eq. (11) and Eq. (10) was lower than 8%. This means that the equilibria involving the L^+_{S2} and the S^+ forms can be neglected over the pH range without a significant loss of information. Table 1 reports the K_1, K_2, α_1 (K_{1D}/K_{1L}) and α_2 (K_{2D}/K_{2L}) values calculated from Eq. (11) for all the dansyl amino acid at a temperature of 20°C. The separation factor α (k'_D/k'_L) for D,L dansyl serine was also plotted against the pH values at $T=20$°C as shown in Fig. 4 in which the theoretical curve was obtained using Eq. (11).

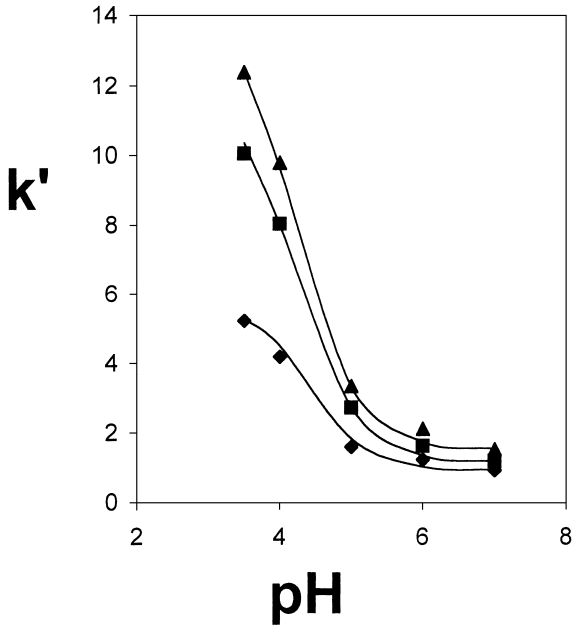

Fig. 2. Plots of k' against eluent pH for various D dansyl amino acids (▲: dansyl leucine; ■: dansyl valine; ♦: dansyl serine) at $T=20$°C with the theoretical curve recreated using Eq. (11) (———).

From these results, several observations can be made. Both K_1 and K_2 values increased with the hydrophobicity of the dansyl amino acids (Table 1).

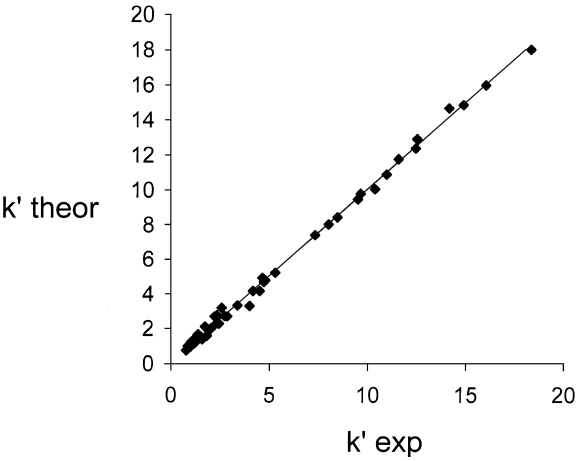

Fig. 3. Correlation between the theoretical (Eq. (11)) and experimental retention factors for all the solutes at $T=20$°C.

Table 1
Transfer equilibrium constants K_i (with standard deviations in parentheses) and selectivity α_i of anionic ($i=1$) and zwitterionic ($i=2$) species for the D and L enantiomers of the dansyl amino acids at a column temperature equal to 20°C

Compound	K_1	K_2	α_1	α_2
L Dansyl valine	11.4 (<0.1)	129.0 (<0.1)	1.20	1.09
D Dansyl valine	13.6 (<0.1)	140.7 (0.1)		
L Dansyl leucine	13.8 (<0.1)	148.8 (<0.1)	1.27	1.13
D Dansyl leucine	17.7 (<0.1)	168.6 (0.1)		
L Dansyl phenylalanine	22.9 (0.1)	201.2 (0.2)	1.17	1.07
D Dansyl phenylalanine	26.9 (0.2)	216.3 (0.3)		
L Dansyl serine	8.8 (<0.1)	66.3 (<0.1)	1.21	1.12
D Dansyl serine	10.7 (<0.1)	74.4 (<0.1)		
D/L Dansyl tryptophan	23.4 (<0.1)	248.8 (0.1)	1	1

This implies that the hydrophobic effect is an important parameter in the solute retention on the teicoplanin stationary phase. Armstrong et al. [1] have also indicated that hydrophobic interactions are engaged between the analyte and the macrocycle. However, the exact nature of this interaction (true hydrophobic inclusion or association with an hydrophobic cleft) has not be clearly determined. The fact that the enantiomers of dansyl tryptophan, i.e., the most bulky molecule, were not resolved over the eluent pH range on this column (see α values in Table 1) seems to indicate that a steric repulsion occurred in the retention process. As well, the solute retention increase as the pH decreased was governed by the interaction between the zwitterionic form of the solute and the $L_{S1}^{-/+}$ stationary phase form. The K_2 values were about tenfold higher than the K_1 values for all the solutes as shown in Table 1. It seems to be due to the strong interaction between the tertiary amino group of the solute and the carboxylate of the teicoplanin. Moreover, the inflexion point, observed for all the k' versus pH plots (Fig. 2) near the pK_a value of the solute amino group, confirmed this hypothesis. An additional silanophilic effect could be responsible for the increase in the k' values via the interaction between the cationic charge of the solute and the unreacted negatively charged silanol groups of the support. However, it has been demonstrated previously that the pK_a of silica in pure water is approximately 5 [11]. This implies that the protonated silanols had became preponderant when the solute was in the zwitterionic form. As a consequence, the silanophilic effect had been probably reduced in relation to the ionic interaction between the solute and the teicoplanin selector. Fig. 4 shows that the chiral discrimination decreased

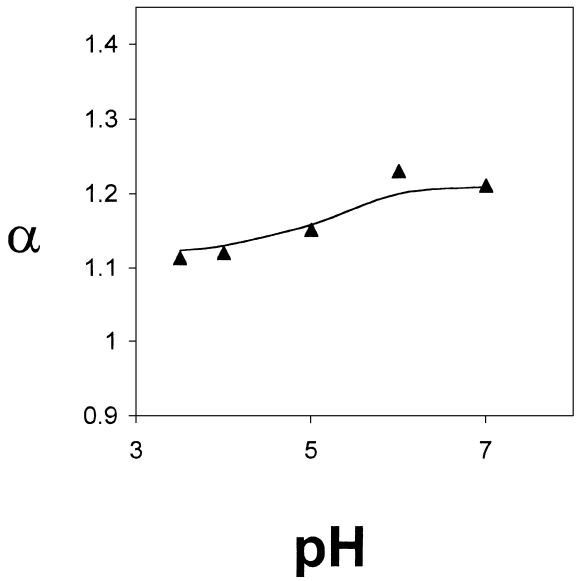

Fig. 4. Plot of α against eluent pH for the dansyl serine enantiomers with the theoretical curve recreated using Eq. (11) (———) at $T=20$°C.

when the pH decreased. An inflexion point was also observed around 4.5. This is in accordance with the α_1 and α_2 values reported in Table 1. The α_1 values were greater than the α_2 values for all the dansyl amino acids (except for dansyl tryptophan with α values equal to 1) demonstrating that the chiral discrimination was principally governed by the interaction between the anionic form of the solute and the $L_{S1}^{-/+}$ stationary phase. This confirms the crucial role of the carboxylate group of the solute in the chiral recognition. The weak α_2 values indicate that the interaction between the tertiary amino group of the solute and the carboxylate of the teicoplanin selector was not discriminating. It is well known that the Dalgliesh simplified model for the chiral recognition consists in a three-point interaction between the chiral selector and the three distinct groups of the solute near the chirality center. The fact that the tertiary amino group of the solute is far away from the stereogenic center can explained the non discriminating nature of this interaction.

4.2. Thermodynamic parameter variations

To study the influence of temperature on the K_i values, the same experiments were carried out at temperatures equal to 5, 10, 15, 25, 30 and 35°C. If there is no change in the solute interactions with the stationary phase in relation to temperature, then a plot of ln K_i versus $1/T$ would be linear with a slope of $(-\Delta H)/R$ and an intercept of $(\Delta S)/R$. The Van 't Hoff plots were all linear for the anionic and zwitterionic species of L and D dansyl amino acids. The correlation coefficients for the linear fits were higher than 0.97. Table 2 reports the ΔH and ΔS values corresponding to the K_1 and K_2 constants for the D enantiomers of the dansyl amino acid compounds. All these values were negative indicating that the various processes were controlled enthalpically. This is consistent with the results reported in the literature from various chromatographic data using CSPs [12–14]. The ΔH values for the anionic and zwitterionic forms of solute were similar while stronger differences existed between the ΔS values. ΔS values were less negative for the transfer of the zwitterionic form from the mobile phase to the teicoplanin. This is the reflect of a greater degree of freedom of the molecule when the solute was associated with the selector. It is well known that the chiral recognition mechanism is based on the short-range interactions [4,8]. Following the nonspecific long-range interactions (ionic or hydrophobic forces) which allow the approach of solute to the selector, the solute engages specific interactions such as Van der Waals interactions, steric repulsion or hydrogen bonds. These interactions are responsible for a great immobilization effect of the more retained solute [4,8]. Therefore, the less negative value of ΔS for the $S^{-/+}$–$L_{S1}^{-/+}$ association confirms that this type of interaction did not discriminate in the same manner than the S^-–$L_{S1}^{-/+}$. The interaction between the teicoplanin carboxylate and the cationic charge of the solute was probably not followed by the second step implying short-range forces.

An example illustrating the eluent pH effect on the separation of dansyl amino acid is provided in Fig. 5. Fig. 5A shows the separation of the enantiomers of dansyl serine at a pH equal to 3.5 for a column temperature of 5°C. In this case, the analysis time was long (17 min) with a low separation factor (1.15) due to the undesirable interaction of the zwitterionic form with the CSP. On the other hand, when the eluent pH was adjusted to 7.0, the solute was in the anionic form so that the analysis time was

Table 2
Thermodynamic parameters ΔH (kJ/mol) and ΔS (J/mol K) of the anionic (S^-) and zwitterionic ($S^{-/+}$) species transfer from the mobile to the $L_{S1}^{-/+}$ stationary phase for the D enantiomers of the dansyl amino acids (standard deviations in parentheses)

Compound	S^-–$L_{S1}^{-/+}$		$S^{-/+}$–$L_{S1}^{-/+}$	
	ΔH	ΔS	ΔH	ΔS
D Dansyl valine	−22.9 (0.2)	−56.7 (0.6)	−19.3 (1.9)	−24.7 (2.2)
D Dansyl leucine	−22.7 (0.1)	−53.8 (0.3)	−20.8 (1.5)	−28.3 (1.6)
D Dansyl phenylalanine	−26.9 (0.3)	−64.4 (1.1)	−24.4 (2.2)	−38.6 (3.0)
D Dansyl serine	−23.3 (<0.1)	−59.6 (<0.1)	−17.6 (<0.1)	−24.4 (<0.1)

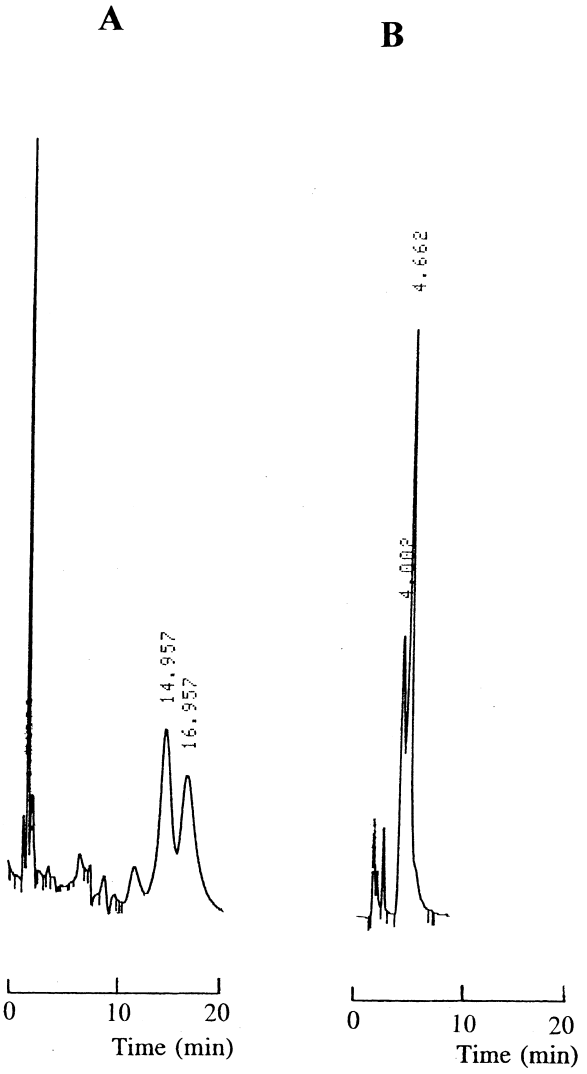

Fig. 5. Chromatograms representing the separation of the dansyl serine enantiomers (first peak: L enantiomer) at $T=5°C$ (A) for an eluent pH equal to 3.5; (B) for an eluent pH equal to 7.0.

short (5 min) and the separation factor equal to 1.29 (Fig. 5B)[1].

[1]A deviation of the 1:1 ratio (as expected for a racemate) was sometimes obtained whatever the operating condition and the enantiomer pair as can be seen in Fig. 5B. At the present moment, we are not able to provide a satisfactory explanation for this phenomenon.

5. Conclusion

This report shows how the two ionized species of the dansyl amino acid enantiomers interact with the teicoplanin chiral stationary phase. The equilibrium constants calculated from the chromatographic data allowed to demonstrate that the anionic form of the solute governed the chiral recognition while the zwitterionic form was implicated principally in the increase in the retention via an additional non discriminating interaction. Thermodynamic parameters indicated that this undesirable interaction implied long-range rather than short-range forces, probably represented by ionic interactions between the teicoplanin carboxylate and the solute tertiary amino groups.

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