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Vancomycin Dimerization and Chiral Recognition Studied by High-Performance Liquid Chromatography

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The retention and separation of D,L-dansylvaline enantiomers (used as test solutes) were investigated using silica gel as stationary phase and vancomycin as chiral mobile-phase additive. A retention model was developed to describe the mechanistic aspects of the interaction between solute and vancomycin in the chromatographic system. It considered the formation of vancomycin dimers both "free" in the mobile phase and adsorbed on silica. By fitting the model equation to experimental data, it appeared clearly that the approach taking into account the vancomycin dimerization described accurately the retention behavior of the compounds. The examination of the model equation parameters showed that the glycopeptide dimerization increased the enantioselectivity by a factor of ∼3.7. This study demonstrated the preponderant role of the vancomycin dimerization on the chiral recognition process of D,L-dansylvaline. Also, an additional analysis on a vancomycin chiral stationary phase indicated that the addition of vancomycin in the mobile phase promoted a greater enantioselectivity mediated by the formation of dimers in the stationary phase.

The use of glycopeptide antibiotics as chiral selectors was introduced in 1994 by Armstrong and co-workers.[1,2] Due to their structural characteristics, the macrocyclic compounds combine the chiral properties of different selectors such as proteins and cyclodextrins. For example, vancomycin contains 18 stereogenic centers, 3 macrocyclic rings linked by polar amide bonds, forming a hydrophobic cleft and ionizable groups. These glycopeptides can be used in liquid chromatography as chiral stationary phases (CSPs).[3−6] Alternatively, the macrocyclic antibiotics are used in capillary electrophoresis or in liquid chromatography as background electrolyte/eluent chiral additives.[7−11] In aqueous solution, macrocyclic antibiotics are able to self-associate. Two classes of compounds are described in relation to the self-association types: (i) teicoplanin group, which can form micellar aggregates due to the hydrophobic acyl side chain (hydrophobic tail); (ii) vancomycin group, which can form back-to-back dimers with four hydrogen bonds between two antiparallel polypeptide backbones.[12,13]

Only a few studies have examined the influence of these macrocyclic antibiotic structural organizations on enantioselectivity. The role of the micellar aggregates of teicoplanin on chiral recognition was initially analyzed by Gasper et al.[14] These authors have shown that this self-association can sometimes hinder or alter the chiral discrimination. To achieve the best enantioseparations, it is necessary to add a small amount of acetonitrile, which can inhibit the teicoplanin aggregation. However, to the best of our knowledge, the enantioselectivity dependence on the vancomycin group antibiotic dimerization has never been studied. Moreover, dimer formation of these antibiotics is responsible for a change in the ligand-binding affinity. Nuclear magnetic resonance experiments have demonstrated that glycopeptide dimerization increases the affinity for cell wall analogues by a factor of 1 up to 10.[15,16] Therefore, it is strongly expected that this dimerization could influence the enantioselectivity process mediated by macrocyclic chiral selectors.

The aim of this paper was to investigate the role of vancomycin dimerization on the chiral recognition of D,L-dansylvaline enantiomers used as test solutes. The solute retention was determined on a silica stationary phase using vancomycin as chiral mobile-phase additive (CMPA). A theoretical model, based on multiple equilibria between analytes, vancomycin, and silica stationary phase, was derived and fitted to the experimental data. In addition, D,L-dansylvaline retention was investigated on a vancomycin CSP using the same selector as a CMPA. The enantioselectivity values

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were analyzed for both vancomycin monomer and dimer as well as for immobilized vancomycin in order to compare the chiral discrimination properties of these three species.

THEORY

The solute retention behavior using vancomycin as CMPA and silica as stationary phase is related to multiple equilibria. The equilibrium constant K between the solute S and the silica stationary phase L_s is described as follows

$$K = [S.L_s]/[S][L_s] \quad (1)$$

where [S] and [$S.L_s$] are the solute concentrations in the mobile phase and the stationary phase, respectively. [L_s] is the concentration of the stationary-phase ligand.

The equilibrium constant $K_{S.V}$ between S and vancomycin in the mobile phase can be introduced by assuming a 1:1 stoichiometry as previously used[15,16]

$$K_{S.V} = [S.V]/[S][V] \quad (2)$$

where [V] and [S.V] are the vancomycin and complex concentrations in the mobile phase, respectively. It was previously shown that the macrocyclic glycopeptides can be adsorbed to silica.[14] It was demonstrated that vancomycin is able to associate strongly with the fused-silica capillary wall in a capillary electrophoresis system due to the presence of two amine groups.[14] Under the conditions of this study, vancomycin is positively charged (pI = 7.2) and is able to interact with the negatively charged silanol groups (pK_a of silica in pure water is ~5). Thus, the interaction between the silica stationary phase and vancomycin must be taken into account in the interaction model. The equilibrium constant $K_{V.Ls}$ between vancomycin and the stationary phase ligand L_s is as follows:

$$K_{V.L_s} = [V.L_s]/[V][L_s] \quad (3)$$

where [$V.L_s$] is the vancomycin concentration in the stationary phase. Additional interactions can be described between the adsorbed vancomycin and the solute

$$K_{S.V.L_s} = [S.V.L_s]/[S][V.L_s] \quad (4)$$

and between the solute−vancomycin complex formed in the mobile phase and the silica stationary phase

$$K'_{S.V.L_s} = [S.V.L_s]/[S.V][L_s] \quad (5)$$

where [$S.V.L_s$] is the solute−vancomycin complex concentration in the stationary phase. When the vancomycin self-association in both the mobile and stationary phases is taken into account in the equilibria, additional relations can be obtained.

The self-association equilibrium constant K_{V_n} in the mobile phase is equal to

$$K_{V_n} = [V_n]/[V]^n \quad (6)$$

where n corresponds to the number of self-associated vancomycin molecules and [V_n] is the concentration of self-associated vancomycin in the mobile phase.

V_n is also expected to interact with the silica stationary phase in such a way that the following equilibrium constant $K_{V_n.L_s}$ can be obtained

$$K_{V_n.L_s} = [V_n.L_s]/[V_n][L_s] \quad (7)$$

where [$V_n.L_s$] is the self-associated vancomycin concentration in the stationary phase. Three additionnal equilibria are obtained by taking into account the interactions of solute with V_n in both the mobile ($K_{S.V_n}$) and the stationary phases ($K_{S.V_n.L_s}$) and between the $S.V_n$ complex formed in the mobile phase and the silica stationary phase ($K'_{S.V_n.L_s}$)

$$K_{S.V_n} = [S.V_n]/[S][V_n] \quad (8)$$

$$K_{S.V_n.L_s} = [S.V_n.L_s]/[S][V_n.L_s] \quad (9)$$

$$K'_{S.V_n.L_s} = [S.V_n.L_s]/[S.V_n][L_s] \quad (10)$$

where [$S.V_n$] and [$S.V_n.L_s$] are the solute−self-associated vancomycin complex concentration in the mobile and the stationary phase, respectively. In principle, the values of the association constants for the binding sites of V_n could be different. However, it was previously suggested by Mackay et al.[15] that the binding pockets of the vancomycin dimer do not differ significantly in terms of affinity constants.

The retention factor of the species S is given by the following equation

$$k = \frac{Q_{L_s}}{Q_M} = \phi\left[\frac{[S.L_s] + [S.V.L_s] + [S.V_n.L_s]}{[S] + [S.V] + [S.V_n]}\right] \quad (11)$$

where Q_{L_s} and Q_M are the total amount of solute in the stationary and mobile phases, respectively. ϕ is the phase ratio of the column. The following equations are obtained by combination of eqs 1−11

$$k = \phi\left[\frac{K[S] + A[S][V] + B[S][V]^n}{[S] + K_{S.V}[S][V] + K_{V_n}K_{S.V_n}[S][V]^n}\right] \quad (12)$$

and

$$k = \frac{k_0 + \phi A[V] + \phi B[V]^n}{1 + K_{S.V}[V] + K_{V_n}K_{S.V_n}[V]^n} \quad (13)$$

where k_0 is the solute retention factor for a vancomycin concentration equal to 0, $A = (K'_{S.V.L_s})\,(K_{S.V}) = (K_{V.L_s})(K_{S.V.L_s})$, and $B = K_{V_n}(K'_{S.V_n.L_s})(K_{S.V_n}) = K_{V_n}(K_{V_n.L_s})(K_{S.V_n.L_s})$.

Thus, the overall solute retention factor can be indifferently described by the two following relations

$$k = \frac{k_0 + k_{\mathrm{S.V.L_s}} K_{\mathrm{S.V}} [\mathrm{V}] + k_{\mathrm{S.V}_n.\mathrm{L_s}} K_{\mathrm{V}_n} K_{\mathrm{S.V}_n} [\mathrm{V}]^n}{1 + K_{\mathrm{S.V}} [\mathrm{V}] + K_{\mathrm{V}_n} K_{\mathrm{S.V}_n} [\mathrm{V}]^n} \quad (14a)$$

or

$$k = \frac{k_0 + k_{\mathrm{V.L_s}} K_{\mathrm{S.V.L_s}} [\mathrm{V}] + k_{\mathrm{V}_n.\mathrm{L_s}} K_{\mathrm{V}_n} K_{\mathrm{S.V}_n.\mathrm{L_s}} [\mathrm{V}]^n}{1 + K_{\mathrm{S.V}} [\mathrm{V}] + K_{\mathrm{V}_n} K_{\mathrm{S.V}_n} [\mathrm{V}]^n} \quad (14b)$$

where $k_{\mathrm{S.V.L_s}}$, $k_{\mathrm{S.V}_n.\mathrm{L_s}}$, $k_{\mathrm{V.L_s}}$, and $k_{\mathrm{V}_n.\mathrm{L_s}}$ are the retention factors of S.V, S.V$_n$, V, and V$_n$, respectively. In a simplified model assuming similar interactions between solute and free or adsorbed vancomycin species (monomer and dimer), i. e., $k_{\mathrm{S.V.L_s}} \approx k_{\mathrm{V.L_s}}/K_{\mathrm{S.V}} \approx K_{\mathrm{S.V.L_s}}$ and $k_{\mathrm{S.V}_n.\mathrm{L_s}} \approx k_{\mathrm{V}_n.\mathrm{L_s}}/K_{\mathrm{S.Vn}} \approx K_{\mathrm{S.V}_n.\mathrm{L_s}}$, the following relation is obtained

$$k = \frac{k_0 + k_{\mathrm{V.L_s}} K_{\mathrm{S.V}} [\mathrm{V}] + k_{\mathrm{V}_n.\mathrm{L_s}} K_{\mathrm{V}_n} K_{\mathrm{S.V}_n} [\mathrm{V}]^n}{1 + K_{\mathrm{S.V}} [\mathrm{V}] + K_{\mathrm{V}_n} K_{\mathrm{S.V}_n} [\mathrm{V}]^n} \quad (15)$$

If the vancomycin self-association is neglected, i. e, $K_{\mathrm{V}_n} = 0$, eq 14b becomes

$$k = \frac{k_0 + k_{\mathrm{V.L_s}} K_{\mathrm{S.V.L_s}} [\mathrm{V}]}{1 + K_{\mathrm{S.V}} [\mathrm{V}]} \quad (16)$$

The apparent enantioselectivity (α_{sil}) is classically described by the following relation

$$\alpha_{\mathrm{sil}} = k_{(2)}/k_{(1)} \quad (17)$$

where $k_{(2)}$ and $k_{(1)}$ are the retention factors of the more and the less retained enantiomers, respectively.

As well, true enantioselectivity can be obtained as follows

$$\alpha_{\mathrm{S.V}} = K_{\mathrm{S.V}(2)}/K_{\mathrm{S.V}(1)} \quad (18)$$

and

$$\alpha_{\mathrm{S.V}_n} = \frac{K_{\mathrm{V}_n} K_{\mathrm{S.V}_n(2)}}{K_{\mathrm{V}_n} K_{\mathrm{S.V}_n(1)}} \quad (19)$$

EXPERIMENTAL SECTION

Apparatus. The HPLC system consisted of a 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 fluorometric detector (Ex, 326 nm; Em, 533 nm) or a Shimadzu SPD-10A UV−visible detector. A Macherey-Nagel 250 mm × 4.6 mm silica HPLC column (packed with 5-μm particles) and an Astec 150 mm × 4.6 mm Chirobiotic V HPLC column (packed with a stationary phase produced by chemically bonding the macrocyclic glycopeptide vancomycin to a 5-μm silica gel) were used with controlled temperature (20 °C) in an oven Igloocil (Interchim).

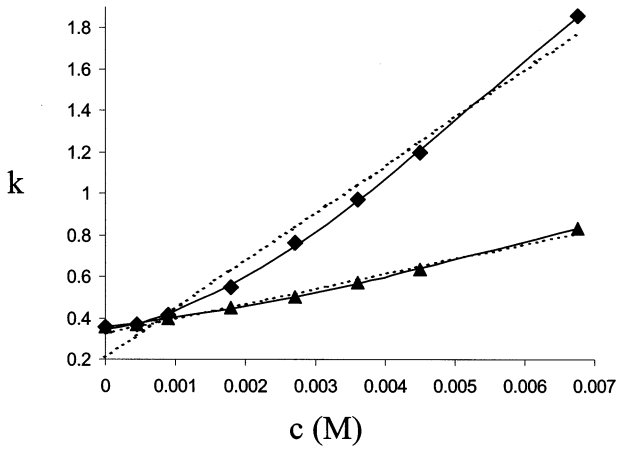

Figure 1. Plots of k against c for D- (◆) and L- (▲) dansylvaline at $T = 20$ °C using a silica column. The theoretical curves are recreated from eqs 15 (—) and 16 (- - -). See operating conditions in the Experimental Section. Error bars are within the experimental points.

Reagents and Operating Conditions. D,L-Dansylvaline enantiomers and vancomycin were obtained from Sigma Aldrich (Saint-Quentin, France). HPLC grade methanol , 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 (flow rate: 0.5 mL/min for the silica column and 0.8 mL/min for vancomycin column) consisted of citrate buffer (50 mM; pH 6.7)−methanol (90−10, v/v). The vancomycin concentration in the mobile phase ranged from 0 to 4.5 mM (for the vancomycin stationary phase) and 0 to 6.75 mM (for the silica stationary phase). D,L-Dansylvaline enantiomer samples were prepared in the mobile phase at a concentration of 0.25 μg·mL^{-1} (retention was sample concentration-independent, i.e., in linear elution conditions[6]). For the determination of $k_{\mathrm{V.L_s}}$ on the silica column, vancomycin samples were prepared in the mobile phase at a concentration of 0.0625 μg·mL^{-1}. A 20-μL aliquot of each solute was injected in triplicate, and the retention times were measured.

Nonlinear Regression Analysis of Retention Data. The model equation was fitted to the retention factors of the solutes by a nonlinear regression using the software Table curve 2D (SPSS Science Software GmbH, Erkrath, Germany).

RESULTS AND DISCUSSION

Model Validation for Solute Retention Using Silica as Stationary Phase and Vancomycin as CMPA. The retention factors for D,L-dansylvaline enantiomers on the silica stationary phase were determined at a column temperature equal to 20 °C for all the vancomycin concentrations (c, 0−6.75 mM). The coefficients of variation of the k values were less than 0.5%, indicating a high reproducibility and a good stability for the chromatographic system. The k values were plotted against c for the two enantiomers (Figure 1). At $c = 0$, dansylvaline interacts weakly with the silica stationary phase ($k = 0.36$). For both enantiomers, the retention factor increased when the vancomycin concentration increased. This behavior is consistent with the results obtained by Sharp and Risley.[10] Using a silica gel as stationary phase and the macrocyclic antibiotic LY333328 as chiral mobile-phase additive, these authors showed that dansyl amino acid retention increased when the additive concentration varied

Table 1. Determination of the Model Parameters by Fitting Eqs 15 and 16 to the D,L-Dansylvaline Retention Factors[a]

	L-dansylvaline		D-dansylvaline	
	eq 16[b] model without dimerization	eq 15[a] model with dimerization	eq 16[b] model without dimerization	eq 15[a] model with dimerization
R^2	0.9835	0.9997	0.9733	0.9994
RSS	3×10^{-3}	4×10^{-5}	5×10^{-2}	8×10^{-4}
F	149	5510	91	2442
$k_{0\ calc}$[c]	0.34	0.36 (<0.1)	0.21	0.35 (<0.1)
$k_{V_{n=2}L_s}$		4.55 (8.5)		5.91 (5.9)
$K_{S.V}$ (M^{-1})	1.3	26 (0.9)	2.1	32 (3.3)
$K_{S.VL_s}$ (M^{-1})	37		128	
$K_{V_{n=2}}K_{S.V_{n=2}}$ (M^{-2})		1800 (9.1)		8220 (9.0)

[a] RSS, residual sum of squares. Relative standard deviations (%) of the data are in parentheses for the eq 15 model parameters. [b] The $k_{V.L_s}$ equation parameter set to 1.80. [c] $k_{0\ exp} = 0.36$.

from 3 to 5 mM. Such an observation is consistent with a solute retention governed partly by an adsorption phenomenon of vancomycin on the silica stationary phase. Moreover, when a small amount of vancomycin was injected into the silica column using the same mobile phase but without additive, the retention factor was found to be equal to 1.80. This demonstrates that the macrocyclic glycopeptide monomer interacts significantly with the stationary phase as expected, shown in the theoretical section. As shown in Figure 1, an upward concave curvature is obtained for the k versus c plots. This suggests that a vancomycin self-association is involved in the solute retention as expected by the eq 14 model. It could be possible to fit for $n = 2$ theoretical binding curves to the experimental data using eqs 14a and 14b. However, the experimental uncertainty, the limited range of vancomycin concentration, and the large number of variables make it impossible to obtain valuable estimates of the equation parameters. The simplified model (eq 15), assuming that similar interactions occur between solute and adsorbed or free vancomycin, was tested. The vancomycin retention factor (1.80) was used as $k_{V.L_s}$ value. The curve-fitting method yielded reasonable values of equation parameters with moderate uncertainties varying from 2 to 20% relative. The parameters, the residual sum of squares (RSS) and the R^2 and F coefficients obtained from the curve fitting procedure, are listed in Table 1. The theoretical curves are presented in Figure 1. For comparison, the best fits obtained using the model, which neglects the vancomycin dimerization (eq 16), are shown in Figure 1 and the model parameters are presented in Table 1. It is well known that the F and RSS values constitute more discriminating parameters than the R^2 value when assessing the significance of model equations.[17−19] These values obtained from the curve-fitting procedure according to eq 15 confirm that the interaction model taking into account the vancomycin dimerization is more adequate to describe the retention behavior of the solutes. The calculated retention factor (k_{0calc}) is almost the same as the experimental one (k_{0exp}) from the chromatographic experiments without vancomycin in the mobile phase (Table 1). As well, the high values of $k_{V_{n=2}L_s}$ support the model involving the intervention of vancomycin dimer in the solute adsorption to silica stationary phase. It is not possible to obtain independent estimates of the $K_{S.V_{n=2}}$ values from the curve-fitting procedure (see eq 15). Therefore, $K_{V_{n=2}}$, calculated previously using capillary electrophoresis in similar operating conditions, were required to extract $K_{S.V_{n=2}}$ from the $K_{V_{n=2}}K_{S.V_{n=2}}$ product.[20] The dimerization constant values of 30 and 50 M^{-1} (determined at pH 6.7 and pH 7.0, respectively) were used.[20] The $K_{S.V_{n=2}}$ values obtained ranged from 274 to 164 M^{-1} for the D enantiomer and from 60 to 36 M^{-1} for L enantiomer. The interactions of the D enantiomer with vancomycin dimer are stronger that those observed with the vancomycin monomer by a factor varying from 8.6 to 5.1 (Table 1). This result is consistent with the previous data of Mackay et al.[15] and Beauregard et al.,[21] who showed that the vancomycin affinity for the D-alanine derivatives was enhanced by a factor from 1.4 to 3 by the antibiotic dimerization. In the case of L-enantiomer, a weaker difference is observed between $K_{S.V_{n=2}}$ and $K_{S.V}$.

Table 2. Estimation of the Enantioselectivity Values from the Model Parameters for Both Vancomycin Monomer ($\alpha_{S.V}$) and Dimer ($\alpha_{S.V_{n=2}}$). Comparison with the Apparent Enantioselectivity (α_{im}) Data Obtained on Immobilized Vancomycin[a]

	column	enantioselectivity
vancomycin monomer	silica	1.23 (3.9)
vancomycin dimer	silica	4.56 (12.7)
immobilized vancomycin	vancomycin	1.62 (0.1)

[a] Relative standard deviations (%) of the data are in parentheses.

Vancomycin Dimerization and Enantioselectivity. To establish a quantitative estimation of the enantioselectivity properties of dimeric and monomeric vancomycin, true enantioselectivity values $\alpha_{S.V}$ (eq 18) and $\alpha_{S.V_{n=2}}$ (eq 19) were calculated from parameters determined previously by the curve-fitting method. $\alpha_{S.V}$ and $\alpha_{S.V_{n=2}}$ are listed in Table 2. A significant increase in the enantioselectivity is observed for the glycopeptide dimer with $\alpha_{S.V_{n=2}} \approx 3.7$ times higher than $\alpha_{S.V}$. The apparent enantioselectivity on the silica column α_{sil} (eq 17) was studied as well. A chromatogram showing the separation of D,L-dansylvaline enantiomers (at the eluent vancomycin concentration of 6.75 mM) is also provided in Figure 2. α_{sil} was plotted against c (Figure 3). The α_{sil} value

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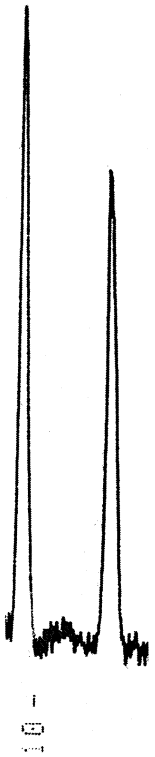

Figure 2. Chromatogramm at $c = 6.75$ mM for the dansylvaline enantiomer pair (retention times of L enantiomer 8.98 min and D enantiomer 14.06 min) at $T = 20$ °C using a silica column. See operating conditions in the Experimental Section.

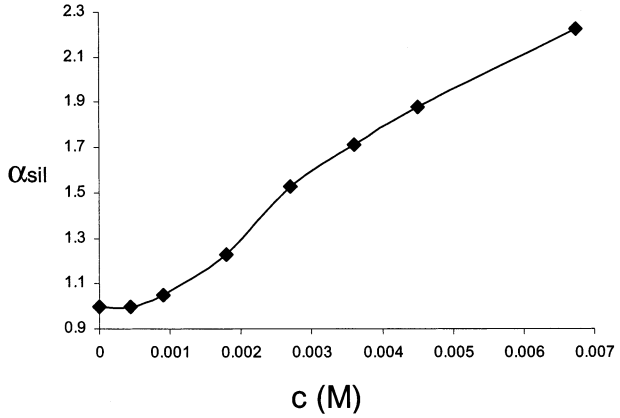

Figure 3. Apparent enantioselectivity α_{sil} vs c for the D,L-dansylvaline enantiomer pair at $T = 20$ °C using a silica column. See operating conditions in the Experimental Section. Error bars are within the experimental points.

increases roughly at low c value followed by a more stronger increase at higher vancomycin concentration. This observation can be analyzed by determining the relative proportions of each vancomycin species (λ) in the chromatographic system. Using the eq 15 parameters, the respective fractions λ of vancomycin monomer and dimer were estimated both in the mobile phase (λ_{VM} and $\lambda_{V_{n=2}M}$) and adsorbed to the stationary phase (λ_{VS} and $\lambda_{V_{n=2}S}$). The λ versus c plots are presented in Figure 4. It appears that the preponderant species in the chromatographic system, over the additive concentration range, are the two vancomycin monomers (V_M and V_S). When c increases, the two monomer relative populations decrease while the two dimer fractions increase. To

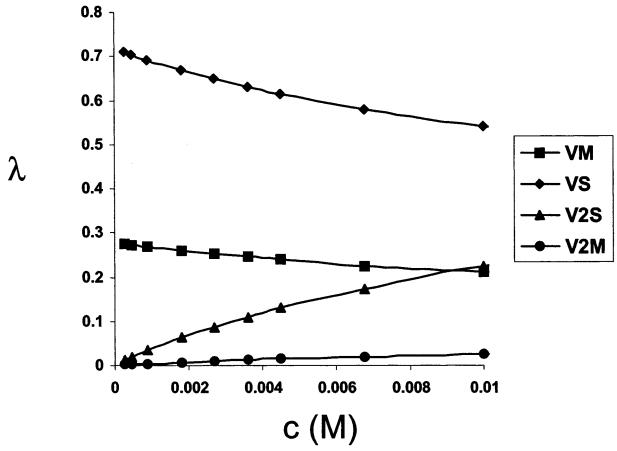

Figure 4. λ vs c created using eq 15 parameters. λ: fractions of vancomycin monomer and dimer in both the mobile phase and adsorbed to the stationary phase. λ_{VM} (VM); λ_{V2M} (V2M); λ_{VS} (VS); $\lambda_{V_{n=2}S}$ (V2S).

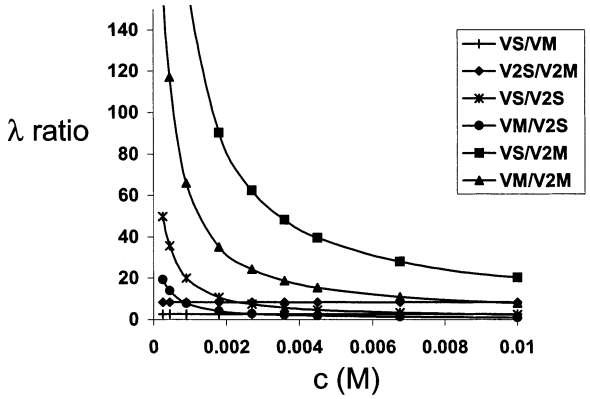

Figure 5. λ ratio vs c created using eq (15) parameters. λ: fractions of vancomycin monomer and dimer in both the mobile phase and adsorbed to the stationary phase. $\lambda_{VS}/\lambda_{VM}$ (VS/VM); $\lambda_{V_{n=2}S}/\lambda_{V_{n=2}M}$ (V2S/V2M); $\lambda_{VS}/\lambda_{V_{n=2}S}$ (VS/V2S); $\lambda_{VM}/\lambda_{V_{n=2}S}$ (VM/V2S); $\lambda_{VS}/\lambda_{V_{n=2}M}$ (VS/V2M); $\lambda_{VM}/\lambda_{V_{n=2}M}$ (VM/V2M).

complete this observation, the λ ratios were calculated and plotted versus c (Figure 5). Obviously, the $\lambda_{VS}/\lambda_{VM}$ and $\lambda_{V_{n=2}S}/\lambda_{V_{n=2}M}$ ratios (corresponding to the equilibrium constants between vancomycin species and silica stationary phase) are constant with $\lambda_{V_{n=2}S}/\lambda_{V_{n=2}M} > \lambda_{VS}/\lambda_{VM}$. As well, the various $\lambda_{V(S\text{ or }M)}/\lambda_{V_{n=2}(S\text{ or }M)}$ ratios decrease when c increases with a sharp decline at low additive concentrations. The dansylvaline retention and consequently the apparent enantioselectivity is the result of two antagonist effects: (i) the solute interaction with the silica-adsorbed vancomycin species governing the increase in the retention and (ii) the solute interaction with the eluent vancomycin species responsible for a decrease in the retention. In a simplified system where only one type of species is present, i.e., monomer or dimer, three possibilities can be evoked depending on the value of the $\lambda_{(V\text{ or }V_{n=2})S}/\lambda_{(V\text{ or }V_{n=2})M}$ ratio (assuming, as evoked above, identical interaction between solute and vancomycin free or adsorbed to silica): (a) If $\lambda_{(V\text{ or }V_{n=2})S}/\lambda_{(V\text{ or }V_{n=2})M} > 1 \rightarrow k$ increase/α_{sil} increase with ratio increasing; (b) if $\lambda_{(V\text{ or }V_{n=2})S}/\lambda_{(V\text{ or }V_{n=2})M} = 1 \rightarrow \alpha_{sil} = 1$; (c) if $\lambda_{(V\text{ or }V_{n=2})S}/\lambda_{(V\text{ or }V_{n=2})M} < 1 \rightarrow k$ decrease/α_{sil} increase with ratio decreasing (reverse elution order compared to (a)).

At low vancomycin concentrations, the enantioselectivity is governed principally by the monomeric vancomycin species as

shown in Figure 4. The relatively low $\lambda_{VS}/\lambda_{VM}$ value (Figure 5) and the reduced α_{SV} value are responsible for a weak possibility of chiral discrimination in this *c* range (Figure 3). When the additive concentration increases, the $\lambda_{V_{n=2}(S\text{ or }M)}$ fractions increase (Figure 4) associated with a strong diminution of the $\lambda_{V(S\text{ or }M)}/\lambda_{V_{n=2}(S\text{ or }M)}$ ratios (Figure 5). As $\lambda_{V_{n=2}S}/\lambda_{V_{n=2}M}$ is higher than $\lambda_{VS}/\lambda_{VM}$ and $\alpha_{S.V_{n=2}}$ is higher than $\alpha_{S.V}$, this modification of the monomeric and dimeric species fractions is responsible for the significant increase of the apparent enantioselectivity appearing in the high-CMPA concentration zone (Figure 3). Thus, the α_{sil} enhancement is mostly dependent on the high $\lambda_{V_{n=2}S}/\lambda_{V_{n=2}M}$ and $\alpha_{SV_{n=2}}$ values. It is demonstrated that the apparent enantioselectivity increase with the additive concentration is a complex phenomenon dependent on both the true enantioselectivity values ($\alpha_{S.V}$ and $\alpha_{S.V_{n=2}}$) and the relative populations of each vancomycin species in the chromatographic system. As well, it has been previously demonstrated that the formation of back-to-back dimers affects the structural organization of the aglycon pocket resulting in a more constrained conformation.[22] This suggests that the chiral discrimination increase related to the dimer formation is governed, at least in part, by the solute interactions with this modified aglycon pocket.

Vancomycin CSP versus Vancomycin CMPA: Comparison of the Chiral Discrimination Properties. It is well known that the immobilization of a selector can change its structural organization. For example, Enquist and Hermansson[23] reported that the immobilized form of α-1-glycoprotein has a more unfolded structure than the native chiral selector. In the case of the vancomycin stationary phase, it is strongly expected that the immobilization process could affect the possibilities of antibiotic dimerization. As the chiral discrimination is enhanced by the vancomycin dimerization, the chiral discrimination properties of the vancomycin stationary phase could be reduced in comparison to those of vancomycin CMPA. It should be noted also that a change in the vancomycin structure dependent on the immobilization could modify the enantioselective properties of the selector. To explore the chiral recognition properties of immobilized vancomycin, the retention factor values for D,L-dansylvaline enantiomers were determined on a vancomycin stationary phase using the same operating conditions as for the silica stationary phase. The apparent enantioselectivity ($\alpha_{im} = k_D/k_L$) is presented in Table 2. α_{im} and $\alpha_{S.V}$ exhibit similar values. Significant nonstereoselective interactions of the solute with the chromatographic support silica or the spacer arm of vancomycin could be responsible for a significant decrease in the enantioselectivity. However, it was previously shown by a displacement study, using *N*-acetyl-D-alanine as competing agent, that the enantioselectivity, resulting from the dansylvaline enantiomer binding to the stereospecific aglycon pocket, is only 16% higher than the apparent (global) enantioselectivity.[24] Thus, the more plausible explanation for the close values of α_{im} and $\alpha_{S.V}$ is that the chiral discrimination process on the vancomycin stationary phase is principally governed by the glycopeptide monomer. An additional analysis was carried out. Vancomycin was added in the mobile phase using the same immobilized macrocyclic selector. The vancomycin concentration range was between 0 and 4.5 mM. Figures 6 and 7 present respectively the *k* and the α_{im} versus *c* plots. The retention factors were enhanced when the additive concentration was varied from 0 to 4.5 mM (Figure 6) as obtained previously for the solute retention variation on the silica column (Figure 1). These results show that vancomycin adsorption to the stationary phase (silica support, spacer arm, and immobilized vancomycin) is an important phenomenon in the dansylvaline retention behavior. As shown in Figure 7, the separation factor increases strongly at the low-*c* range followed by a gradual rise up to a maximum at high additive concentrations. Chromatograms illustrating the CMPA effect on the separation of dansylvaline enantiomers at low concentration are provided in Figure 8. As α_{im} (at $c = 0$) and $\alpha_{S.V}$ exhibit similar values, this result demonstrates clearly that the addition of vancomycin in the mobile phase promotes a greater enantioselectivity mediated by the formation of dimers in the stationary phase. The α_{im} variation with *c* increase is quite different from the apparent enantioselectivity variation with CMPA concentration obtained previously (Figure 3) with the silica column. The sharp initial increase (Figure 7) could be explained by the fact that the mobile-phase vancomycin interacts favorably with the immobilized vancomycin monomer, due to the mass action law. This would

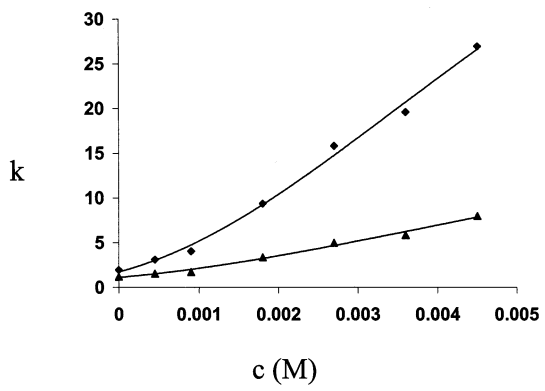

Figure 6. *k* vs *c* for D- (◆) and L- (▲) dansylvaline at $T = 20$ °C using a vancomycin column. See operating conditions in the Experimental Section. Error bars are within the experimental points.

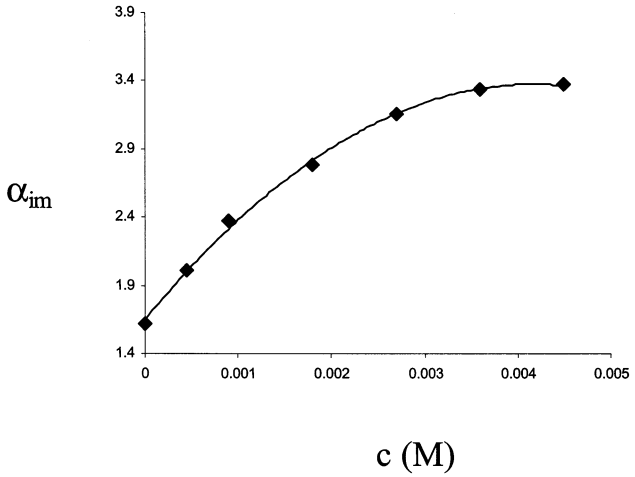

Figure 7. Apparent enantioselectivity α_{im} vs *c* for the D,L-dansylvaline enantiomer pair at $T = 20$ °C using a vancomycin column. See operating conditions in the Experimental Section. Error bars are within the experimental points.

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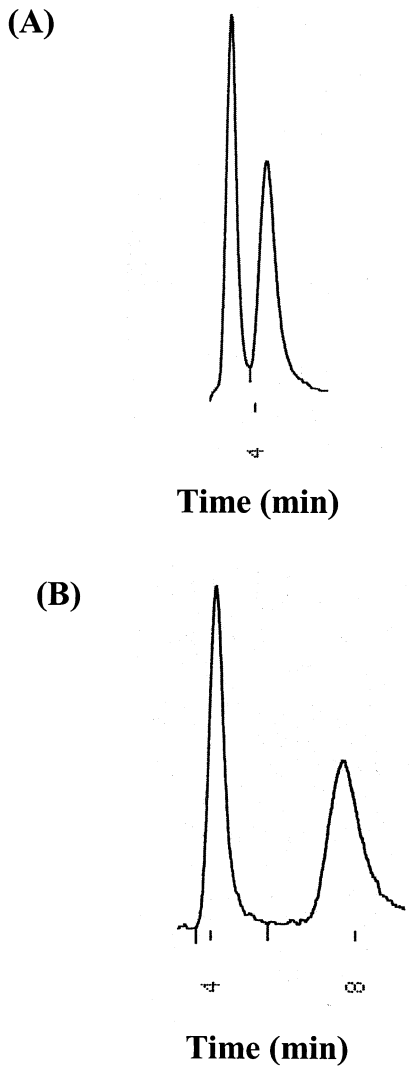

Figure 8. (A) Chromatograms at $c = 0$ mM (retention times of L enantiomer 3.30 min and D enantiomer 4.42 min) and (B) at $c = 0.9$ mM (retention times of L enantiomer 4.06 min and D enantiomer 7.58 min) for the dansylvaline enantiomer pair at $T = 20$ °C using a vancomycin column. See operating conditions in the Experimental Section.

allow the formation of back-to-back dimers, even at low CMPA concentrations and, concomitantly, the reduction of the number of immobilized vancomycin monomers available for the interaction with the solute. As well, the saturation at high concentrations is expected to result from the predominance of the dimer species both in the mobile phase and adsorbed to the stationary phase. Using the same chromatographic procedure, similar enantioselectivity variation with the CMPA concentration has been reported by Sun and Olesik[11] for the flurbiprofen and FMOC-amino acid enantiomers.

CONCLUSION

This study investigates the retention and the chiral recognition of D,L-dansylvaline enantiomers on silica and vancomycin stationary phases using vancomycin as CMPA. On the silica stationary phase, it appears clearly that the solute retention increase with increasing additive concentration is strongly dependent on the formation of vancomycin dimers in the chromatographic system. It is shown that this glycopeptide dimerization increases significantly the chiral recognition properties of the selector. Also, the analysis on the vancomycin stationary phase indicates that the addition of vancomycin in the mobile phase promotes a greater apparent enantioselectivity mediated by the formation of dimers adsorbed to the stationary phase.

GLOSSARY

Species

L_s	silica stationary phase
S	solute in the mobile phase
$S.L_s$	solute in the stationary phase
V	vancomycin monomer in the mobile phase
$V.L_s$	vancomycin monomer in the stationary phase
V_n	self-associated vancomycin in the mobile phase (n, number of self-associated vancomycin molecules)
$V_n.L_s$	self-associated vancomycin in the stationary phase
S.V	solute−vancomycin monomer complex in the mobile phase
$S.V_n$	solute−self-associated vancomycin complex in the mobile phase
$S.\ V.L_s$	solute−vancomycin monomer complex in the stationary phase
$S.V_n.L_s$	solute−self-associated vancomycin complex in the stationary phase

Equilibrium Constants

K	equilibrium constant between S and L_s
$K_{S.V}$	equilibrium constant between S and V
$K_{V.L_s}$	equilibrium constant between V and L_s
$K_{S.V.L_s}$	equilibrium constant between S and $V.L_s$
$K'_{S.V.L_s}$	equilibrium constant between S.V and L_s
K_{V_n}	vancomycin self-association equilibrium constant
$K_{V_n.L_s}$	equilibrium constant between V_n and L_s
$K_{S.V_n}$	equilibrium constant between S and V_n
$K_{S.V_n.L_s}$	equilibrium constant between S and $V_n.L_s$

Chromatographic Parameters

k	solute retention factor
ϕ	phase ratio of the column
k_0	solute retention factor for vancomycin concentration equal to 0
$k_{S.V.L_s}$	retention factor of S.V
$k_{S.V_n.L_s}$	retention factor of $S.V_n$
$k_{V.L_s}$	retention factor of V
$k_{V_n.L_s}$	retention factor of V_n
α_{sil}	apparent enantioselectivity on silica column
α_{im}	apparent enantioselectivity on vancomycin column
$\alpha_{S.V}$	true enantioselectivity for the interaction between S and V
$\alpha_{S.V_n}$	true enantioselectivity for the interaction between S and V_n

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