Displacement Study on a Vancomycin-Based Stationary Phase Using *N*-acetyl-D-Alanine as a Competing Agent

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Abstract

The analysis of the binding data of D,L-dansyl amino acids on a vancomycin stationary phase is investigated in relation to the addition of *N*-acetyl-D-alanine in the mobile phase. This eluent additive acts as a specific competing agent for the aglycone pocket of the immobilized chiral selector. A model taking into account both stereoselective and nonstereoselective interactions between the solutes and the stationary phase is used to fit the experimental data. From the results, the theoretical approach is considered to be adequate to describe the competing agent dependence on solute retention. To the best of our knowledge, this report constitutes the first example of a displacement study on a macrocyclic antibiotic stationary phase. This work shows that dansyl amino acids bind to the active aglycone pocket of the selector and that this interaction is enantioselective. The results also demonstrate that additional enantioselective sites at the vancomycin surface are involved in the chiral discrimination of these solutes.

Introduction

Various experimental approaches have been proposed to analyze the mechanisms of enantioseparation on chiral stationary phases (CSPs). For example, some studies have examined the temperature effects on retention and enantioselectivity (1–4). The changes in enthalpy and entropy associated with the transfer of the solute can be extracted from linear van't Hoff plots and analyzed in order to obtain information about the driving forces implied in the association process. Another approach for studying the interactions between an enantiomer and a selector involves the variation of the mobile phase composition (5–10). Such an investigation has been carried out for several solute–CSP associations by varying the proportion of the organic modifier (6), the pH (7,8), or the ionic strength (9,10) of the mobile phase.

The competitive (or displacement) approaches constitute another powerful tool to examine the retention behavior of enantiomers in the chiral selective environment. Classically, it may be carried out by injecting one compound as the solute while a fixed concentration of a possible competing agent is passed through the column in the mobile phase. Several examples of such studies have been reported with immobilized proteins. Wainer et al. (11–13) have studied thoroughly the competitive displacement of various drugs from a human serum albumin (HSA) stationary phase by different competing agents. Hage et al. (14–16) have also studied the effects of additives (such as digitoxin, acetyldigitoxin, or chiral compounds) on the solute retention for immobilized HSA. More recently, this approach has been applied with success to the study of enantiomer binding to the cellobiohydrolase stationary phase, using cellulose as a competing compound (17). Also, the investigation of the retention and the enantioselectivity of a new CSP (immobilized fatty-acid-binding protein) has been carried out for a large number of chiral compounds through displacement studies (18).

In this study, the displacement concept was applied specifically to the investigation of the enantioselective and nonselective binding of test solutes (D,L-dansyl amino acids) on a vancomycin stationary phase. In order to obtain information about the role of the vancomycin aglycone pocket in the enantioselectivity process, the solute retention factor was plotted against the concentration of the eluent *N*-acetyl-D-alanine (Ala). *N*-acetyl-D-Ala was used as a competing agent because it is able to bind specifically to this site (19). Using a general model describing the competing agent concentration dependence on the solute binding, the retention parameters as well as the association constant between *N*-acetyl-D-Ala and vancomycin were determined. The results will be discussed in relation to the variation of enantioselectivity in order to provide a precise picture of the enantiomer–selector association process.

For high-performance affinity chromatography in the zonal elution mode, the total retention factor (k) is a direct measure of the solute interactions within the column. The parameter k is related to the number of binding sites of the analyte to the stationary phase and the equilibrium constants for the solute at these sites (13):

$$k = \sum_{i=1}^{p} \beta_i j_i \left(\frac{m}{V_m} \right) = \sum_{i=1}^{p} k_i \qquad \text{Eq. 1}$$

where β_i is the equilibrium constants at the individual sites (i), j_i

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the fraction of each type of site, m the total moles of binding sites, V_m the void volume, and k_i the respective contributions to the total retention factor.

The binding of a chiral compound to a CSP can involve two kinds of sites at the surface of the selector: one class of nonselective binding sites with lower affinity and another class of enantioselective sites with higher affinity (17). Thus, k can be simplified as follows:

$$k = \sum_{q=1}^{l} k_{qs} + \sum_{r=1}^{t} k_{rns} \qquad \text{Eq. 2}$$

where the two terms are the sums of the retention factors corresponding with selective (s) and nonselective (ns) interactions, respectively. For a pair of enantiomers, it is expected that the k_{ns} terms are identical while the k_s contributions differ in relation to the stereoselectivity.

Williams et al. (18,19) have previously shown by nuclear magnetic resonance and modeling studies that a ligand such as *N*-acetyl-D-Ala is specifically bound in a 1:1 stoichiometry to the pocket of the aglycone of vancomycin (the structure of vancomycin is shown in Figure 1) via hydrophobic interactions and hydrogen bonds. This is explained by the fact that this antibiotic acts on bacteria by binding to cell wall mucopeptide precursors terminating in D-Ala. If *N*-acetyl-D-Ala is used as a mobile phase additive, it is expected that it will interfere on the retention of solutes interacting with the specific aglycone pocket. Thus, assuming that the pocket of the vancomycin aglycone constitutes one of the enantioselective sites of vancomycin for dansyl amino acid, equation 2 can be modified as follows:

$$k = \sum_{r=1}^{t} k_{rns} + \sum_{q=1}^{l-1} k_{qs} + \frac{k_{ls}}{1 + Kc} = k_{ns} + k_s + \frac{k_{ls}}{1 + Kc}$$

where k_{ls} is the part of the retention factor implying the solute enantioselective binding to the aglycone pocket, k_{ns} the part of the retention factor involved in the nonspecific binding, and k_s the part of the retention factor corresponding with enantioselective interaction unaffected by the competing agent. K is the association constant between *N*-acetyl-D-Ala and vancomycin and c the *N*-acetyl-D-Ala (competing agent) concentration. This simplified model allows for a simple estimate of the role of the aglycone pocket on the enantioseparation of dansyl amino acids on immobilized vancomycin. Also, it constitutes a valuable tool to explore the exact contributions of enantioselective interactions in the overall retention process of these solutes.

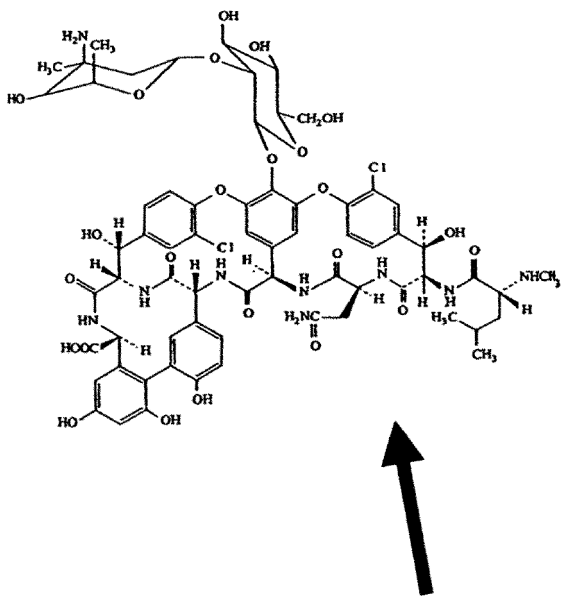

Figure 1. Vancomycin structure with the aglycone binding pocket (indicated by the arrow) (19).

Experimental

Apparatus

The HPLC system consisted of an LC 10AT Shimadzu pump (Touzart et Matignon, Courtaboeuf, France), a Rheodyne Model 7125 injection valve (Interchim, Montluçon, France) fitted with a 20-µL sample loop, and a Shimadzu SPD-10A UV–vis detector. An Astec 150- × 4.6-mm Chirobiotic V HPLC column (packed with a stationary phase produced by the chemical bonding of the macrocyclic glycopeptide vancomycin 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.

Reagents and operating conditions

D,L-amino acids 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 column temperature was maintained at 25°C for all the experiments. The mobile phase consisted in citrate buffer (pH 7.0)–methanol (90:10, v/v). The variation range of the *N*-acetyl-D-Ala concentration was 0 to 20mM. In order to examine the concentration dependencies of the solute retention corresponding with the binding capacity of the immobilized vancomycin, retention measurements were related to varying amounts of injected solute. Solute samples were prepared at different concentrations in the mobile phase from 0.125 to 10 µg/mL. The retention factor versus the sample amount plots exhibited a plateau at a sample concentration lower than 0.625 µg/mL, followed by a small decrease at higher solute concentrations. Thus, 20 µL of each solute at a concentration of 0.250 µg/mL was injected in triplicate (i.e., in linear conditions) (20).

Results and Discussion

The retention factor values for D,L-dansyl amino acids on immobilized vancomycin were determined in relation to the concentration of *N*-acetyl-D-Ala in the mobile phase (0 to 20mM). The coefficients of variation for the k values were < 0.5%, indicating a high reproducibility and a good stability for the chromatographic system. The k values were plotted against c for all the compounds. Figure 2 shows the k versus c plots of D,L-dansyl amino acids. In

all cases, the retention factors decreased when the competing agent concentration increased. Equation 3 was fitted to the experimental data using a nonlinear regression procedure. The values of the various parameters of equation 3 are shown in Table I. The nonlinear regression coefficients (R) were higher than 0.987. Thus, it appears clearly that the behavior of the solutes was well-described by the model taking into account the competition between *N*-acetyl-D-Ala and dansyl amino acids at the aglycone pocket of vancomycin. For all the dansyl amino acid enantiomers, the association constant between the competing agent and vancomycin varied between 820 and 1300 M^{-1}. This demonstrated that all the solutes studied (D- and L-enantiomers) interacted with the aglycone pocket. The *K* average value was similar to the association constant value reported for the *N*-acetyl-D-Ala binding to the macrocycle binding pocket (i.e., 1300 M^{-1} at 23°C) (18). From the magnitude of the retention factor k_{ls}, approximately 45–50% of the total binding observed for these compounds was dependent on the association with the aglycone pocket. The L-enantiomers exhibited a value of approximately 45%, and the D-enantiomers were bound at approximately 50% to this active site. This demonstrates that the aglycone pocket is implied in the chiral discrimination. Moreover, the apparent enantioselectivity (k_D/k_L) decreased when *c* increased (as shown in Figure 3). However, when the aglycone site was saturated by *N*-acetyl-D-Ala at a high displacer concentration (as shown in Figure 1, in which a roughly constant value of the solute retention factor is observed for the concentration range between 10 and 20mM), a substantial enantioselectivity remained for all the enantiomeric pairs. This suggests that other enantioselective sites are involved in the chiral discrimination of dansyl amino acids. The enantioselectivity values from the solute binding to the aglycone pocket ($\alpha_p = k_{lsD}/k_{lsL}$) as well as the "residual" enantioselectivity values ($\alpha_r = k_{nsD}+k_{sD}/k_{nsL}+k_{sL}$) were calculated (Table II). Such an observation is unusual in comparison with the results of other displacement studies carried out on protein CSPs. Using immobilized protein, only one enantioselective site is generally involved in the chiral discrimination process (11–17). This original behavior, observed for the chiral recognition on a vancomycin stationary phase, can be explained by the fact that this type of macrocycle contains several accessible chiral interaction sites. Moreover, Berthod et al. (21) have previously shown that carbohydrate moieties of the teicoplanin stationary phase offer enantioselective binding sites for various enantiomers. Thus, it is quite possible for dansyl amino acid enantiomers to interact with some chiral environments of the selector in addition to the active aglycone site. For the four pairs of dansyl amino acid enantiomers, a significant difference was observed for the α_p values (from 1.26 to 1.48). This demonstrates that the aglycone pocket is

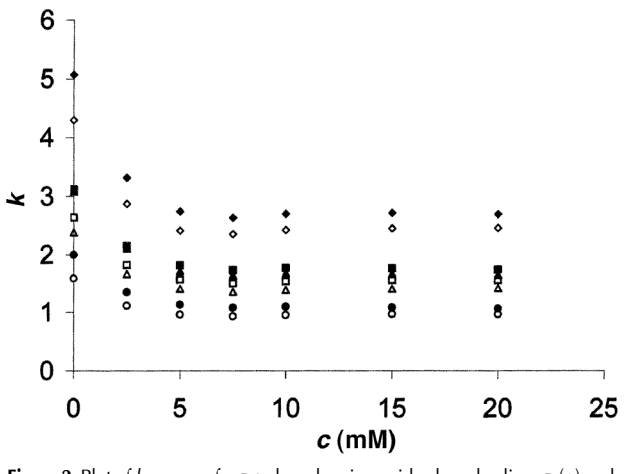

Figure 2. Plot of *k* versus *c* for D,L-dansyl amino acids: dansyl valines D (+) and L (△), dansyl serines D (●) and L (○), dansyl leucines D (■) and L (□), and dansyl phenylalanines D (◆) and L (◇).

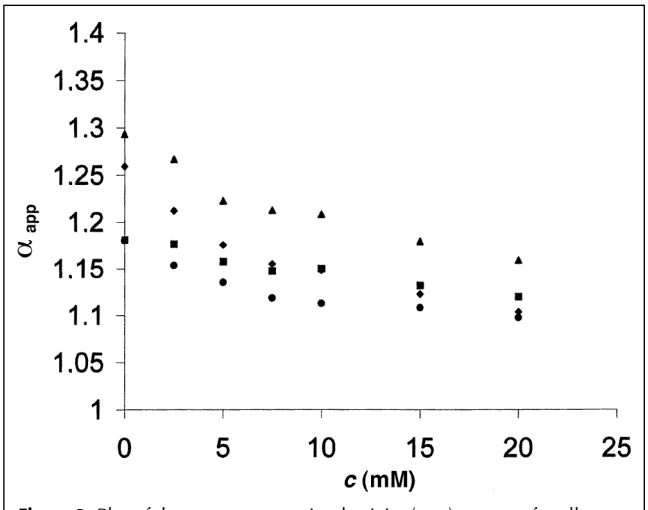

Figure 3. Plot of the apparent enantioselectivity (α_{app}) versus *c* for all enantiomeric pairs: D,L-dansyl valine (◆); D,L-dansyl serine (+); D,L-dansyl leucine (■); and D,L-dansyl phenylalanine (●).

Table I. Determination of the Model Parameters by Fitting Equation 3 to the D,L-Amino Acid Enantiomer Retention Factors on Immobilized Vancomycin

	$k_{ns} + k_s$	k_{ls}	*K* (M^{-1})
D-dansyl valine	1.51	1.58	820
L-dansyl valine	1.32	1.07	1118
D-dansyl serine	0.99	1.01	882
L-dansyl serine	0.91	0.68	1249
D-dansyl leucine	1.49	1.62	918
L-dansyl leucine	1.45	1.18	1144
D-dansyl phenylalanine	2.49	2.58	1080
L-dansyl phenylalanine	2.29	2.00	1305

Table II. Determination of the Enantioselectivity Values for All the Enantiomer Pairs

	α_{app}* (k_D/k_L)	α_p† (k_{lsD}/k_{lsL})	α_r‡ ($k_{nsD} + k_{sD}/k_{nsL} + k_{sL}$)
Dansyl valine	1.26	1.47	1.14
Dansyl serine	1.29	1.48	1.09
Dansyl leucine	1.18	1.26	1.11
Dansyl phenylalanine	1.18	1.28	1.08

* The apparent enantioselectivity.
† The enantioselectivity resulting from the compound binding to the aglycone pocket.
‡ The residual enantioselectivity.

responsible for different enantioselective interactions in relation to the structure of the compounds. Two solute groups can be distinguished: (*a*) D,L-dansyl leucine and dansyl phenylalanine with a lower α_p and (*b*) D,L-dansyl valine and dansyl serine with a higher α_p (Table II). Such a behavior can be explained by a steric hindrance phenomenon. The bulky dansyl amino acid (the first solute group mentioned) could limit access to the aglycone binding pocket, and thus the chiral recognition would be reduced. This is confirmed by the fact that D,L-dansyl tryptophan enantiomers (the most bulky of the dansyl amino acids) were not separated on this CSP (data not shown). Also, this result agrees well with the findings of the enantioseparation of amino acid derivatives on a teicoplanin stationary phase (21,22).

Conclusion

This work demonstrates the interest to use *N*-acetyl-D-Ala as a competing agent for the aglycone pocket of the macrocyclic antibiotic stationary phase. From the results, it is shown that the dansyl amino acids interact substantially with this active site (nearly 50% of the *k* value) of the vancomycin stationary phase. Furthermore, it is demonstrated that the aglycone pocket is an effective enantioselective site, but another class of sites is also involved in the chiral discrimination. This type of displacement study could be applied similarly to the investigation of the relative contribution of enantioselective and nonselective interactions for the solute binding on other types of commercially available macrocycles (such as teicoplanin or ristocetin A) that contain a similar aglycone pocket.

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