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Role of the vancomycin-ristocetin heterodimerization on the enantioselectivity of D,L-tryptophan and D,L-dansyl tryptophan

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Abstract

In this paper, a chromatographic system using immobilized ristocetin as chiral stationary phase and vancomycin as chiral mobile phase additive (CMPA) was described in order to investigate the role of the glycopeptide heterodimerization on the retention and enantioselectivity of D,L-tryptophan and D,L-dansyl tryptophan. A simplified interaction model was derived considering the formation of heterodimers between immobilized ristocetin and vancomycin. This theoretical approach was convenient to describe adequately the retention behavior. When the CMPA concentration increased, the solute retention factor increased for all the solute enantiomers studied indicating that the vancomycin adsorbed on the immobilized ristocetin played a preponderant role in the retention. The D,L-tryptophan enantioselectivity on the dynamically modified stationary phase was improved by a factor of 1.3, probably due to a glycopeptide conformational change upon heterodimerization. On the other hand, a decrease in the chiral discrimination of D,L-dansyl tryptophan was observed. Such a behavior seems to result from the antagonist enantioselective properties of the two glycopeptides for the dansyl amino acids.
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Keywords: Enantiomer separation; Heterodimerization; Vancomycin-ristocetin; Tryptophan; Dansyltryptophan

1. Introduction

The macrocyclic antibiotics have been widely used in high-performance liquid chromatography (HPLC) as chiral stationary phases (CSPs) [1–5]. Only a few studies have used glycopeptides as chiral mobile phase additives (CMPAs). Sharp and Risley [6] evaluated the macrocyclic antibiotic LY333328, used as a mobile phase additive, for the enantioseparation of a series of dansyl amino acids. They compared the influence of various stationary phases such as phenyl, cyano, silica or C_8 phases and investigated the role of the mobile phase composition. In addition, Sun and Olesik [7] studied the influence of the simultaneous use of vancomycin both as stationary phase and mobile phase additive on the chiral separation of 9-fluorenylmethyl chloroformate (Fmoc) amino acids and flurbiprofen. These authors demonstrated that the addition of the selector in the mobile phase allowed the enantiomer resolution. Furthermore, recent results have shown that the vancomycin dimerization affected the chiral recogni-

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tion of amino acid derivatives. It was demonstrated that the glycopeptide homodimer formation was responsible for an enhancement of the enantioselectivity by a factor varying from 1.5 to 3.7 in relation to the eluent pH and the compound type [8,9]. This was attributed to an indirect effect where the conformation of the vancomycin pocket is altered upon vancomycin dimerization [8,9]. Recently, Staroske et al. reported that two different glycopeptides were able to form heterodimers [10]. The heterodimerization constants were greatly higher than the homodimerization constants of each species. For example, the vancomycin-ristocetin association constant was around 10-fold higher than the vancomycin-vancomycin association constant [10].

The aim of this paper was to investigate the role of the vancomycin-ristocetin heterodimerization on a solute enantioselectivity. The retention and the chiral recognition of D,L-tryptophan and D,L-dansyl tryptophan were analyzed in a chromatographic system using a ristocetin CSP with vancomycin as CMPA. A theoretical model was derived taking into account the formation of heterodimers between the immobilized ristocetin and the ‘‘free’’ mobile phase vancomycin. Such an interaction model was fitted to the experimental data and the enantioselectivity values were analyzed in order to compare the chiral discrimination properties of the immobilized ristocetin and the CSP dynamically modified by the heterodimer formation.

2. Theoretical considerations

Theoretically, multiple vancomycin and ristocetin species are expected to be present in the chromatographic system using vancomycin as CMPA and ristocetin as immobilized chiral selector: the vancomycin monomer V and dimer V_2 in the mobile phase; the immobilized ristocetin R_s, the vancomycin monomer V_s and dimer V_{2s} adsorbed to the chromatographic support and the heterodimer between immobilized ristocetin and vancomycin $(RV)_s$. So, the solute retention behavior could be related to several equilibria and the retention factor of solute S is given by:

$$k = \frac{Q_{Ls}}{Q_M} = \phi\left[\frac{[SR_s] + [S(RV)_s] + [SV_s] + [SV_{2s}]}{[S] + [SV] + [SV_2]}\right] \quad (1)$$

where Q_{Ls} and Q_M are the total amount of solute in the stationary and mobile phase, respectively, and ϕ is the phase ratio of the column. Several assumptions can be made in this chromatographic system. As the heterodimerization between vancomycin and immobilized ristocetin is known to exhibit a high association constant [10], the concentration of vancomycin in the stationary phase is assumed to be several orders of magnitude higher than the vancomycin concentration in the mobile phase. Therefore, the mobile phase vancomycin contribution to the solute retention variation can be assumed to be negligible over the low concentration ranges [11]. In addition, it has been shown previously that the vancomycin affinity for a silica chromatographic support was relatively limited [8]. So, it is strongly expected that the amount of vancomycin (monomer and dimer) adsorbed to the chromatographic support is reduced compared to the vancomycin amount interacting with the immobilized ristocetin. Therefore, introducing the association constants between the solute and the glycopeptide species, the following simplified relation can be obtained from Eq. (1):

$$k = \phi[R_s](K + K_{SRV}K_{RV}[V]) \quad (2)$$

where K is the association constant between S and R_s, K_{SRV} the association constant between S and $(RV)_s$ and K_{RV} the association constant between V and R_s.

Since the amount of immobilized ristocetin bound to the solute represents only a small part of the total ristocetin concentration R_{ts}, the following relation can be described:

$$[R_{ts}] = [R_s] + [(RV)_s] \quad (3)$$

$$[R_s] = \frac{[R_{ts}]}{1 + K_{RV}[V]} \quad (4)$$

Thus, the combination of Eqs. (2) and (4) gives:

$$k = \frac{k_{c=0} + k_{RV}K_{RV}[V]}{1 + K_{RV}[V]} \tag{5}$$

where $k_{c=0}$ is the solute retention factor for an eluent vancomycin concentration equal to 0 and k_{RV} is the solute retention factor for the stationary phase dynamically modified by the heterodimer formation.

The apparent enantioselectivity (α) is classically described by the following relation:

$$\alpha = \frac{k_{(2)}}{k_{(1)}} \tag{6}$$

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

In addition, the apparent enantioselectivity for the stationary phase dynamically modified by the heterodimer formation (α_{RV}) can be obtained as follows:

$$\alpha_{RV} = \frac{k_{RV(2)}}{k_{RV(1)}} \tag{7}$$

3. Experimental

3.1. Apparatus

The HPLC system consisted of a LC Shimadzu pump 10AT (Sarreguemines, France), a Rheodyne injection valve Model 7125 (Interchim, Montluçon, France) fitted with a 20 μl sample loop, a Shimadzu SPD-10A UV–visible detector ($\lambda = 254$ or 300 nm). An Astec 150 mm×4.6 mm Chirobiotic R HPLC column (packed with a stationary phase produced by chemically bonding the macrocyclic glycopeptide ristocetin to a 5 μm silica gel) were used with controlled temperature (25 °C) in an oven Igloocil (Interchim).

3.2. Reagents and operating conditions

D,L-Tryptophan, D,L-dansyl tryptophan, D,L-dansyl valine enantiomers and vancomycin were obtained from Sigma–Aldrich (Saint-Quentin, France). Acetonitrile (HPLC grade) and ammonium acetate, acetic acid and sodium hydroxide 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 acetate buffer (50 m*M*, pH 6.0)–acetonitrile (85:15, v/v). The flow-rate was set at 0.8 ml/min. The vancomycin concentration in the mobile phase ranged from 0 to 5 m*M*. Samples were prepared in the mobile phase at a concentration of 3.0 μg/ml (retention was sample concentration-independent, i.e., under linear elution conditions). Vancomycin samples were prepared in the mobile phase at a concentration of 0.07 μg/ml. A 20-μl volume of each solute was injected in triplicate and the retention times were measured. The void time was determined using the mobile phase peak.

3.3. Non-linear regression analysis of retention data

The model equation was fitted to the retention factors of the solutes by a non-linear regression using the software Table curve 2D (SPSS Science Software, Erkrath, Germany).

4. Results and discussion

4.1. D,L-Tryptophan and D,L-dansyl tryptophan retention using immobilized ristocetin as CSP and vancomycin as CMPA

The retention factors for tryptophan and dansyl tryptophan enantiomers were determined at a column temperature equal to 25 °C for all the vancomycin concentrations (c, 0–5 m*M*). The relative standard deviations of the k values were less than 1.1%, indicating a high reproducibility and a good stability for the chromatographic system. At $c = 0$, the L-enantiomer of dansyl tryptophan was more retained on the ristocetin CSP than the D-enantiomer while the reverse was observed for the tryptophan enantiomers. This is in accordance with the previous data from Ekborg-Ott et al. [12] who had shown that the ristocetin stationary phase retained the L-dansyl amino acid more strongly than the D-dansyl amino acid.

The k values were plotted against c for the two solute pairs. As shown in Fig. 1, at the first, the

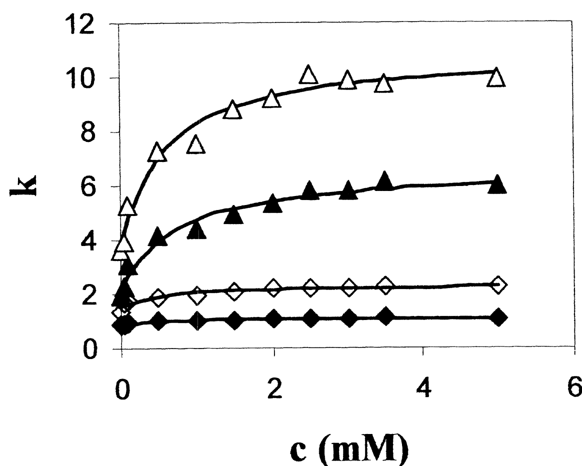

Fig. 1. Plots of k against c for the D,L-(▲, △) dansyl tryptophan and D,L-(◊, ♦) tryptophan enantiomers. The theoretical curves are recreated using equation parameters obtained by fitting Eq. (5) to the solute retention factors (——). See operating conditions in the Experimental section.

retention factors increased markedly with the CMPA concentration increasing followed by a gradual approach to a maximum. Such an observation is consistent with a solute retention mainly governed by interactions, between the solute and the stationary phase, modified by the dynamically adsorbed vancomycin. Moreover, a small amount of vancomycin was injected onto the column using the same mobile phase but without additive. The retention factor was higher than 125 indicating that the macrocyclic glycopeptide interacted very strongly with the stationary phase as expected (see the Theoretical considerations section). It was possible to fit theoretical binding curves to the experimental data using Eq. (5). The equation parameters and the R^2 and F coefficients are listed in Table 1. Fig. 1 also shows

Table 1
Determination of the model parameters for D,L-tryptophan and D,L-dansyl tryptophan by fitting Eq. (5) to the solute retention factors

	L-Datrp*	D-Datrp	L-Trp*	D-Trp
R^2	0.978	0.977	0.968	0.925
F	180	170	123	50
$k_{c=0}$	3.69	2.11	0.86	1.50
k_{RV}	10.85	6.63	1.12	2.34
K_{RV} (M^{-1})	1749	1372	1415	1762

* Datrp: Dansyl tryptophan; trp: tryptophan.

the theoretical curves obtained for the compound enantiomers. The solute retention factor for the dynamically modified stationary phase (k_{RV}) was higher than $k_{c=0}$ by a factor varying from ~1.3 to ~3 in relation to the compound type. It can be noted that k_{RV} increased more strongly for the dansyl tryptophan enantiomers (~3) than for the tryptophan enantiomers (from 1.3 to 1.5). In the linear conditions used in this study, the solute retention factor is directly function of the number of both the active sites in the column and the solute affinity constant for the stationary phase [13]. So, the increase in the k_{RV} value is consistent with an increase in the column saturation capacity (associated to a possible enhancement of the solute affinity) due to the presence of the vancomycin sites induced by the heterodimer formation. For all the solutes, the heterodimerization constant varied between 1372 to 1762 M^{-1} with an average value of ~1570 M^{-1}. This was lower than the value reported by Staroske et al. by a factor of ~5 [10]. It can be noted that the operating conditions, the technique used [nuclear magnetic resonance (NMR) spectroscopy and electrospray ionization mass spectrometry (ESI-MS)] as well as the constant calculation methodology used were quite different. Nevertheless, such a discrepancy could be also explained by the fact that the immobilization of ristocetin on the chromatographic support could affect the orientation of the glycopeptide and lead to a less efficient ability for the heterodimerization than in a solution.

4.2. *D,L-Tryptophan and D,L-dansyl tryptophan enantioselectivity using immobilized ristocetin as CSP and vancomycin as CMPA*

Apparent enantioselectivity α (Eq. (6)) was plotted against c. Fig. 2 shows the plots for the D,L-dansyl tryptophan enantiomers and D,L-tryptophan enantiomers. In order to establish a quantitative estimation of the enantioselectivity properties of both the ristocetin and the dynamically modified ristocetin CSPs, the apparent enantioselectivity α_{RV} (Eq. (7)) was calculated from the parameters determined previously by the curve-fitting method (Table 2). Two behaviors were observed. For the dansyl tryptophan enantiomers, a slight decrease in the chiral discrimination was obtained with an

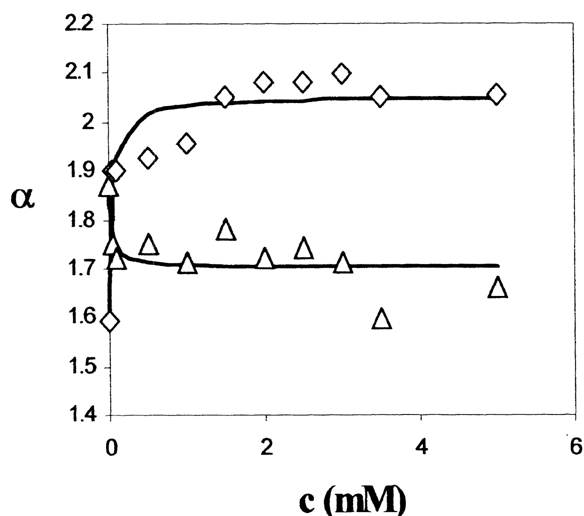

Fig. 2. Plot of apparent enantioselectivity α against c for the D,L-dansyl tryptophan (△) and D,L-tryptophan (◊) enantiomer pair. The theoretical curves are recreated using equation parameters obtained by fitting Eq. (5) to the solute retention factors (——). See operating conditions in the Experimental section.

enantioselectivity varying from 1.87 to 1.64. For the tryptophan enantiomers, an increase in the chiral discrimination by a factor of 1.3 was found, α reaching a maximum value at the CMPA concentrations higher than 1.5 mM. Chromatograms showing the improvement of the tryptophan enantiomer separation with CMPA concentration increasing are provided in Fig. 3. A mechanism was proposed to explain this difference in the separation factor variation for the two enantiomeric pairs.

As reported recently, the vancomycin selector is not able to discriminate the enantiomers of tryptophan under reversed-phase conditions [14]. So, the chiral recognition improvement dependent on the heterodimer formation is the result of an indirect effect of vancomycin on the stationary phase prop-

Table 2
Comparison between the enantioselectivity values obtained for the immobilized ristocetin (α) and the stationary phase modified by heterodimers (α_{RV}) for D,L-tryptophan and D,L-dansylated tryptophan

Apparent enantioselectivity	Datrp*	Trp*
α	1.87	1.59
α_{RV}	1.64	2.09

* Datrp: dansyl tryptophan; Trp: tryptophan.

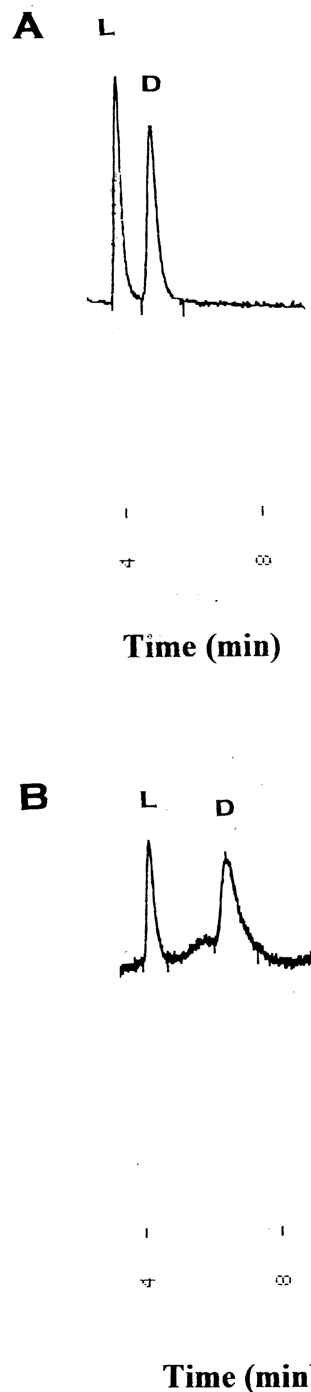

Fig. 3. Chromatogram at $c=0$ mM (A) and $c=2$ mM (B) for the D,L-tryptophan enantiomer pair. See operating conditions in the Experimental section.

erties. It is expected that a glycopeptide (vancomycin and/or ristocetin) conformational change upon dimerization occurs, as classically described for the glycopeptide homodimerization [8,9,15,16]. This would affect the interaction energy or the saturation capacity of the selectors for D,L-tryptophan. An increase in the energy of the stereoselective interactions and/or in the number of favorable chiral regions eventually associated to a reduction in the number of non-selective binding sites and/or in the energy of non-stereoselective interactions can be involved in this process.

In contrast, the vancomycin selector is able to discriminate weakly the enantiomers of dansyl tryptophan in aqueous medium, with a greater affinity for the D-enantiomer [17]. At the same time, it was shown that the ristocetin selector has a greater affinity for the L-enantiomer (Table 1). Therefore, it seems likely that the solute interactions with the enantioselective sites of adsorbed vancomycin act on the chiral recognition antagonistically to the stereoselective interactions of solute with the immobilized ristocetin sites. As described above for tryptophan, it can be noted that a change in the interaction energy or in the capacity saturation of glycopeptides upon heterodimerization is expected to occur. Such a phenomenon could enhance the antagonist effects of the two selectors on the enantioselectivity and participate to the chiral discrimination decrease.

Additional experiments were carried out in order to study thoroughly the enantioselectivity behavior for D,L-dansyl tryptophan. As Rundlett and Armstrong [17] showed that the vancomycin selector was able to discriminate more strongly the enantiomers of dansyl valine than the enantiomers of dansyl tryptophan ($\alpha_{dnsval} = 11$ vs. $\alpha_{dnstrp} = 1.1$), the retention and separation factors of D,L-dansyl valine were investigated in the chromatographic system. If antagonist enantioselective properties of the two glycopeptides govern mainly the chiral recognition behavior of dansyl tryptophan, a more significant decrease in the dansyl valine enantioselectivity or a reverse elution order of enantiomers should occur when the CMPA concentration increased. At $c = 0$, as classically expected for the ristocetin selector, the L-enantiomer was more retained than the D-enantiomer ($k_{c=0\ (L)} = 1.94$ vs. $k_{c=0\ (D)}$ 1.54). However, it

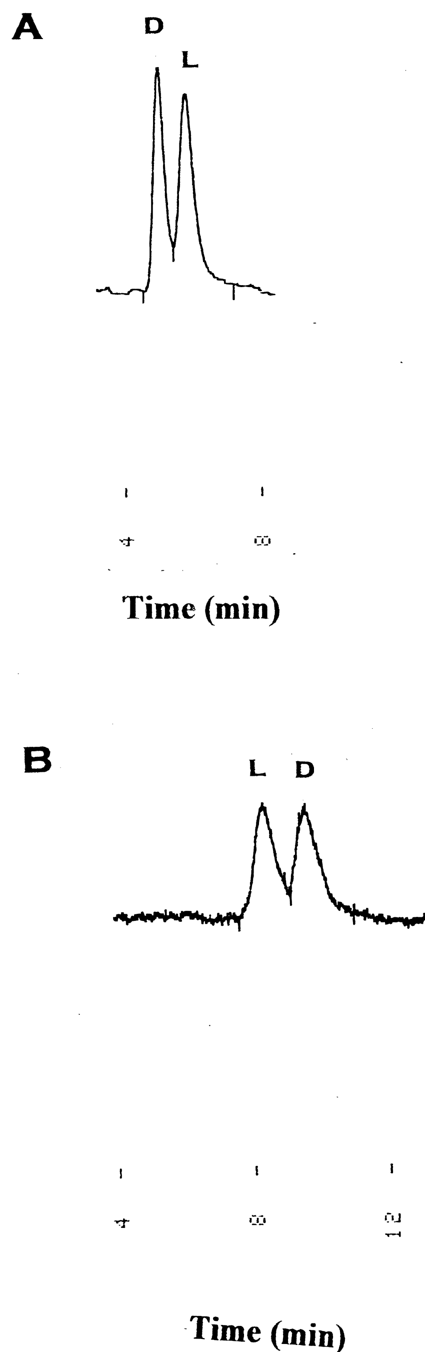

Fig. 4. Chromatogram at $c = 0$ mM (A) and $c = 0.5$ mM (B) for the D,L-dansyl valine enantiomer pair. See operating conditions in the Experimental section.

was found that adding vancomycin to the mobile phase reversed the elution order of the enantiomers, i.e., the D-enantiomer became more retained than the L-enantiomer for $c > 0.05$ mM ($k_{RV\ (D)} = 6.13$ vs. $k_{RV\ (L)} = 5.23$). Fig. 4 shows chromatograms representing this reverse elution order with CMPA concentration increasing.

This result is quite consistent with the mechanism proposed above. For dansyl amino acids, the presence of vancomycin adsorbed to the immobilized ristocetin creates a dual antagonist system in the stationary phase. One glycopeptide selector is assumed to bind more strongly one enantiomer while the other glycopeptide is predominantly associated with the second enantiomer. So, when the vancomycin selector is responsible for a weak inverse enantioselectivity, a slight decrease in α is observed with CMPA concentration increasing (case of dansyl tryptophan). If the inverse enantioselectivity governed by vancomycin is very significant, the addition of vancomycin in the mobile phase is able to reverse the elution order of the enantiomers (case of dansyl valine).

In summary, this study investigated the retention and the chiral recognition of D,L-tryptophan and D,L-dansyl tryptophan enantiomers on a ristocetin CSP using vancomycin as CMPA. It appears clearly that the solute retention increase with increasing additive concentration is dependent on the formation of heterodimers in the chromatographic system. It is shown that this phenomenon increases significantly the D,L-tryptophan chiral recognition, probably due to a glycopeptide conformational change upon heterodimerization. In contrast, a decrease in the enantioselectivity for D,L-dansyl tryptophan was observed. Such a behavior seems to be the result of the antagonist enantioselective properties of the two glycopeptides for the dansyl amino acids.

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