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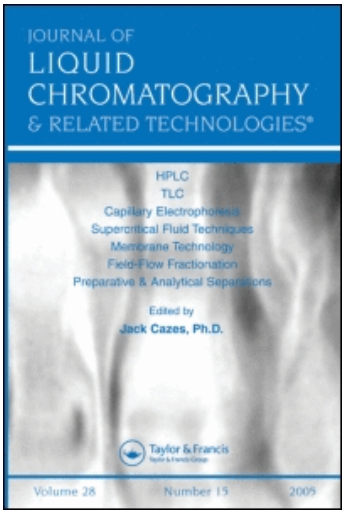

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Use of an Amino Stationary Phase to Study the Vancomycin Dimerization Dependence on Solute Enantioselectivity

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Use of an Amino Stationary Phase to Study the Vancomycin Dimerization Dependence on Solute Enantioselectivity

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ABSTRACT

Earlier chromatographic data (Slama et al. Anal. Chem. **2002**, *74*, 5205) showed that the vancomycin dimerization was responsible for an enhancement of the dansyl valine enantioselectivity. In this paper, a new chromatographic system was used in order to investigate more easily the effects of the vancomycin dimerization on the solute chiral recognition. The retention and separation of *D*, *L* dansyl serine and *D*, *L* dansyl valine enantiomers were examined using an amino stationary phase and vancomycin as chiral mobile phase additive. A simplified interaction model was derived considering only the formation of vancomycin dimers in the mobile phase. This theoretical approach was convenient to describe,

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adequately, the retention behavior of the enantiomers. At an eluent pH of 5.5, it was shown that the glycopeptide dimerization increased the dansyl amino acid enantioselectivity by a factor around 1.50. This study demonstrated interest in using an amino stationary phase to study the glycopeptide dimerization dependence on the solute enantioselectivity.

Key Words: Vancomycin dimerization; Chiral recognition; Dansyl amino acids; HPLC.

INTRODUCTION

During the last two decades, several research papers have been published on the separation of enantiomers by high-performance liquid chromatography. Various types of chiral selectors have been used involving amino acids, proteins, crown-ethers, polysaccharides, and recently, macrocyclic antibiotics. The macrocyclic antibiotics have been widely used in high-performance liquid chromatography as chiral stationary phases (CSPs).[1–6] However, only a few studies have investigated the enantioselectivity effects of these selectors when used as eluent additives. Sharp and Risley[7] 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 C8 and investigated the role of the mobile phase composition. In addition, Sun and Olesik[8] studied the influence of the simultaneous use of vancomycin, both as stationary phase and mobile phase additive on the chiral separation of F-moc amino acids and flurbiprofen. These authors showed that the addition of the selector in the mobile phase allowed the enantiomer resolution. It was hypothesised that this chiral recognition phenomenon was related to the formation of vancomycin dimers in the chromatographic system. Furthermore, our group, using silica gel as stationary phase and vancomycin as a mobile phase additive, recently demonstrated that the vancomycin dimerization affected the chiral recognition of *D*, *L* dansyl valine.[9] The dimer formation of this antibiotic was responsible for an enhancement of the enantioselectivity by a factor of $\approx$3.7 at the eluent pH equal to 6.7. However, in that previous study, the retention model developed to describe the interactions between solute and vancomycin was very complex.[9] It took into account the formation of vancomycin dimers both "free" in the mobile phase and adsorbed to the silica gel.[9] Thus, the number of variables in the model equation was large and led to some assumptions for the determination of the model parameters.

So, the aim of this paper was to investigate the role of vancomycin dimerization on the solute chiral recognition in a more simplified chromatographic system in which there was no interaction between vancomycin and

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stationary phase. The *D*, *L* dansyl serine and *D*, *L* dansyl valine retention was analyzed using an amino stationary phase with vancomycin as chiral mobile phase additive (CMPA). A theoretical model was derived and fitted to the experimental data. The enantioselectivity values were analyzed for both vancomycin monomer and dimer, in order to compare the chiral discrimination properties of these two species.

THEORY

The solute retention behavior using vancomycin as CMPA and amino as stationary phase is related to multiple equilibria (see Fig. 1). The equilibrium constant K between the solute S and the amino stationary phase L_s is described as follows

$$K = \frac{[S.L_s]}{[S][L_s]} \tag{1}$$

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 assuming a 1 : 1 stoichiometry as previously used[10]

$$K_{S.\mathrm{V}} = \frac{[S,V]}{[S][V]} \tag{2}$$

where $[V]$ and $[S,V]$ are the vancomycin and complex concentrations in the mobile phase, respectively.

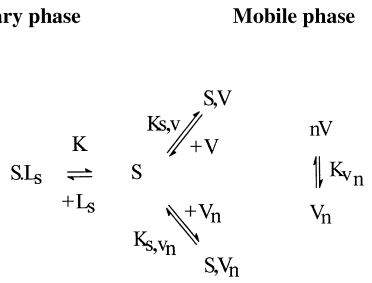

Figure 1. Chemical equilibria for model with vancomycin dimerization.

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It is well known, that vancomycin group antibiotics are able to self-associate in aqueous solution. These glycopeptides can form back-to-back dimers with four hydrogen bonds between two antiparallel polypeptide backbones.[11,12] Taking into account this phenomenon, the self-association equilibrium constant K_{V_n} for vancomycin in the mobile phase is equal to

$$K_{V_n} = \frac{[V_n]}{[V]^n} \tag{3}$$

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. In principle, the values of the association constants for the binding sites of V_n could be different. However, it has been previously suggested by Mackay et al.[13] that the binding pockets of the vancomycin dimer do not differ significantly in terms of affinity constants. Thus, an additional equilibrium is obtained taking into account the interaction of the solute with V_n in the mobile phase

$$K_{S,V_n} = \frac{[S,V_n]}{[S][V_n]} \tag{4}$$

In this model, it is assumed that vancomycin does not interact with the stationary phase. This is based on the fact that vancomycin is positively charged at the study pH of 5.5 (vancomycin pI $\approx$ 7.2). Consequently, it is expected to be repulsed by the cationic charges of the amino stationary phase (see below the results and discussion section). The model is also derived assuming that the adsorption of the solute–vancomycin complex at the stationary phase may be neglected. Therefore, the overall retention factor of the species S can be given by the following equation

$$k = \frac{Q_{L_s}}{Q_M} = \phi\left[\frac{[S.L_s]}{[S] + [S,V] + [S,V_n]}\right] \tag{5}$$

where Q_{L_s} and Q_M are the total amount of solute in the stationary and mobile phase, respectively. ϕ is the phase ratio of the column. The following equations are obtained by combination of Eqs. (1)–(5)

$$k = \phi\left[\frac{K[S]}{[S] + K_{S,V}[S][V] + K_{V_n}K_{S,V_n}[S][V]^n}\right] \tag{6}$$

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and

$$k = \frac{k_0}{1 + K_{S,V}[V] + K_{V_n} K_{S,V_n}[V]^n} \tag{7}$$

where k_0 is the solute retention factor for a vancomycin concentration equal to 0.

Transposing the equation, the following is obtained

$$\frac{1}{k} = \frac{1}{k_0}(1 + K_{S,V}[V] + K_{V_n} K_{S,V_n}[V]^n) \tag{8}$$

If the vancomycin self-association is neglected, i.e., $K_{V_n} = 0$, Eq. (7) becomes

$$k = \frac{k_0}{1 + K_{S,V}[V]} \tag{9}$$

and consequently

$$\frac{1}{k} = \frac{1}{k_0}(1 + K_{S,V}[V]) \tag{10}$$

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

$$\alpha = \frac{k_{(2)}}{k_{(1)}} \tag{11}$$

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

In the case of a model taking into account the vancomycin dimerization, α can be given by

$$\alpha = \frac{1 + K_{S,V_{(1)}}[V] + K_{V_n} K_{S,V_{n(1)}}[V]^n}{1 + K_{S,V_{(2)}}[V] + K_{V_n} K_{S,V_{n(2)}}[V]^n} \tag{12}$$

where $K_{S,V_{(2)}}$, $K_{S,V_{n(2)}}$ and $K_{S,V_{(1)}}$, $K_{S,V_{n(1)}}$ are the association constants between S and V or V_n for the more and the less retained enantiomer, respectively.

As well, true enantioselectivity can be obtained as follows

$$\alpha_{S.V} = \frac{K_{S,V_{(2)}}}{K_{S,V_{(1)}}} \tag{13}$$

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and

$$\alpha_{S,V_n} = \frac{K_{V_n} K_{S,V_{n(2)}}}{K_{V_n} K_{S,V_{n(1)}}} \tag{14}$$

EXPERIMENTAL

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 fluorimetric detector (Ex: 326 nm; Em: 533 nm) or a Shimadzu SPD-10A UV–visible detector. An amino Nucleosil 250 mm × 4.6 mm HPLC column packed with 5 µm particles (Macherey-Nagel, Hoerdt, France) was used with controlled temperature (30°C) in an oven Igloocil (Interchim).

Reagents and Operating Conditions

D, *L* dansyl serine and *D*, *L* dansyl valine enantiomers and vancomycin were obtained from Sigma Aldrich (Saint-Quentin, France). Methanol HPLC grade, hydrogenophosphate, and dihydrogenophosphate 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: 1.5 mL/min) consisted of phosphate buffer (50 mM; pH 5.5). The vancomycin concentration in the mobile phase ranged from 0 to 7.5 mM. *D*, *L* dansyl amino acid samples were prepared in phosphate buffer 50 mM; pH 5.5-methanol (90–10, v/v) at a concentration of 0.25 µg mL^{-1} (retention was sample concentration-independent, i.e., in linear elution conditions). Vancomycin samples were prepared in the mobile phase at a concentration of 0.0625 µg mL^{-1}. Twenty microliter of each solute were injected in triplicate and the retention times were measured. The void time was determined using thiourea.

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 GmbH, Erkrath, Germany).

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RESULTS AND DISCUSSION

Model Validation for the Solute Retention Using an Amino Stationary Phase and Vancomycin as Chiral Mobile Phase Additive

The retention factors for *D*, *L* dansyl serine and *D*, *L* dansyl valine enantiomers were determined at a column temperature equal to 30°C for all the vancomycin concentrations (c, 0–7.5 mM). The relative standard deviations of the k values were less than 0.3%, indicating a high reproducibility and a good stability for the chromatographic system. In addition, a small amount of vancomycin was injected into the column using the same mobile phase without additive. The retention factor was equal to 0.04. This indicates that the macrocyclic glycopeptide did not interact significantly with the stationary phase as expected, shown in the theoretical section. The $1/k$ values were plotted against c for the two solute pairs. For example, the $1/k$ vs. c plots for the dansyl serine enantiomers is shown in Fig. 2. At $c = 0$, dansyl valine and dansyl serine

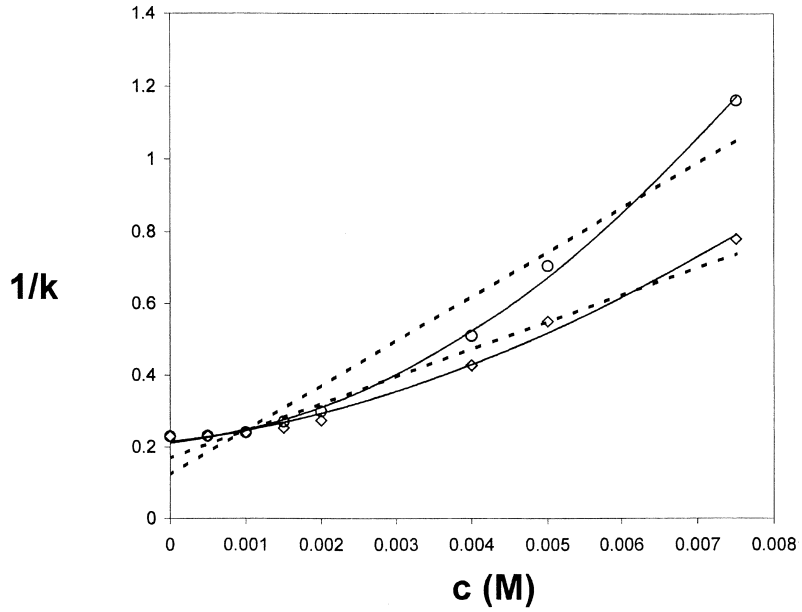

Figure 2. Plots of $1/k$ against c for *D* (○) and *L* (◇) dansyl serine at $T = 30$°C using an amino stationary phase. The theoretical curves are recreated from Eqs. (8) (——) and (10) (- - - -). See operating conditions in the experimental section. Error bars are within the experimental points.

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interact significantly with the amino stationary phase ($k = 16.97$ and 4.28, respectively). This is due, at least in part, to the favorable ionic interactions between cationic amino groups of the stationary phase and the solute carboxylate. For both enantiomers, the retention factor decreased when the vancomycin concentration increased. Such an observation is consistent with a solute retention governed partly by interactions between the solute and vancomycin in the eluent. The *D* enantiomer retention factor decreased more strongly than the *L* enantiomer retention factor when the eluent vancomycin concentration increased (Fig. 2). This means that the *D* enantiomer–vancomycin complex is more stable than the *L* enantiomer–vancomycin one. This is in accordance with previous studies which showed an inverse elution order for *D*, *L* dansyl amino acids ($D > L$) using a vancomycin column[14] or a chromatographic system with vancomycin dynamically adsorbed to the stationary phase.[9]

As shown in Fig. 2, an upward concave curvature was obtained for the $1/k$ vs. c plots. This means that a vancomycin self-association was involved in the solute retention as expected with the Eq. (8) model. It was possible to fit, for $n = 2$, theoretical binding curves to the experimental data using Eq. (8). The parameters and the R^2 and F coefficients obtained from the curve fitting procedure are listed in Table 1. The relative standard deviations of the data are lower than 10%. Figure 2 shows the theoretical curves for the dansyl serine enantiomers and, as a comparison, the best fits obtained using the model which neglects the vancomycin dimerization [Eq. (10)]. The model parameters are presented in Table 1. It is well known that the F values constitute more discriminating parameters than the R^2 value when assessing the significance of model equations.[15] These values obtained from the curve-fitting procedure according to Eq. (8) confirm that the interaction model, taking into account the vancomycin dimerization, is more adequate to describe the retention behavior of the solutes. Moreover, the calculated retention factor ($k_{0\text{calc}}$) is close to the experimental one ($k_{0\text{exp}}$) from the chromatographic experiments without vancomycin in the mobile phase (Table 1). This result confirms that vancomycin is able to self-associate in an aqueous mobile phase and that this dimerization affects the solute interaction with the selector. The use of a positively charged stationary phase allows elimination of an undesirable vancomycin adsorption to the stationary phase. Therefore, the interaction model is more simplified for studying the solute interaction with monomer and dimer of the selector.

Vancomycin Dimerization and Solute Enantioselectivity

In order to establish a quantitative estimation of the enantioselectivity properties of dimeric and monomeric vancomycin, true enantioselectivity values $\alpha_{S,V}$ [Eq. (13)] and $\alpha_{S,V_{n=2}}$ [Eq. (14)] were calculated from parameters determined previously by the curve-fitting method (Table 2). A significant

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Table 1. Determination of the model parameters by fitting Eqs. (8) (for $n = 2$) and (10) to the *D*, *L* dansyl serine and *D*, *L* dansyl valine retention factors.

	Model without dimerization, Eq. (10)				Model with dimerization, Eq. (8)			
	L daser[a]	*D* daser	*L* daval[a]	*D* daval	*L* daser	*D* daser	*L* daval	*D* daval
R^2	0.961	0.941	0.935	0.932	0.992	0.998	0.980	0.987
F	148	95	100	97	313	1,081	146	227
$k_{0\text{calc}}$[b]	5.93	8.26	9.17	14.53	4.76	4.64	7.32	7.63
$K_{S,V}$ (M^{-1})	450	1,023	363	1,210	78	85	88	147
$K_{V_{n=2}S,V_{n=2}}$ (M^{-2})	—	—	—	—	37,504	67,473	28,048	67,268

[a]daser, dansyl serine; daval, dansyl valine.
[b]$k_{0\text{exp}}$ dansyl serine, 4.28; $k_{0\text{exp}}$ dansyl valine, 6.42.

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Table 2. Estimation of the true enantioselectivity values from the model parameters for both vancomycin monomer ($\alpha_{S,V}$) and dimer ($\alpha_{S,V_{n=2}}$).

Enantioselectivity	daser[a]	daval[a]
$\alpha_{S,V}$	1.08	1.67
$\alpha_{S,V_{n=2}}$	1.79	2.40

[a]daser, dansyl serine; daval, dansyl valine.

increase in the enantioselectivity was observed for the glycopeptide dimer with $\alpha_{S,V_{n=2}}$ around 1.43 or 1.65 times higher than $\alpha_{S,V}$. This result is consistent with the previous work, which showed that the vancomycin dimerization increased the *D*, *L* dansyl valine enantioselectivity.[9] The difference with the increase observed previously ($\approx$1.4 vs. $\approx$3.7)[9] could be explained by the variation in the chromatographic operating conditions and the change in the interaction model. The previous work[9] was carried out with a mobile phase consisting of methanol–citrate buffer pH 6.7 (10–90), at a column temperature of 20°C. In addition, the interaction model was quite more complex because of the adsorption of positively charged vancomycin to the negatively charged silica surface. The model equation involved a large number of variables and some simplifying assumptions leading to more uncertainty in the determination of the model parameters.[9] Nuclear magnetic resonance experiments have demonstrated that glycopeptide dimerization increases the affinity for cell wall analogs by a factor of 1 up to 10. For example, Beauregard et al.[11] and Mackay et al.[13] have shown that the vancomycin affinity for the *D* alanine derivatives was enhanced by a factor from 1.4 to 3 by the antibiotic dimerization. The formation of back-to-back dimers affects the structural organization of the aglycone pocket, resulting in a more constrained conformation.[16] It is well established that amino acids bind to vancomycin via their carboxyl groups, which interact with the secondary amine of glycopeptide, and then fold into the aglycone pocket for additional interactions[17] Therefore, the chiral discrimination increase related to the dimer formation can be explained mainly by an indirect effect where the conformation of the vancomycin pocket is altered upon vancomycin dimerization.

As well, the apparent enantioselectivity [Eq. (11)] was determined. A chromatogram showing the separation of *D*, *L* dansyl serine enantiomers (at the eluent vancomycin concentration of 7.5 mM) is also provided in Fig. 3. α was plotted against c as shown in Fig. 4 for the *D*, *L* dansyl serine enantiomers. The experimental α value increased when c increased between 0 and 7.5 mM for the two enantiomeric pairs. The theoretical curve, recreated using model parameters of Table 1, indicates that α is expected to vary

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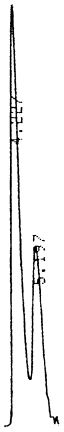

Time (min)

Figure 3. Chromatogram at $c = 7.5\,\text{mM}$ for the dansyl serine enantiomer pair (first peak: *D* enantiomer) at $T = 30^{\circ}\text{C}$ using an amino stationary phase. See operating conditions in the experimental section.

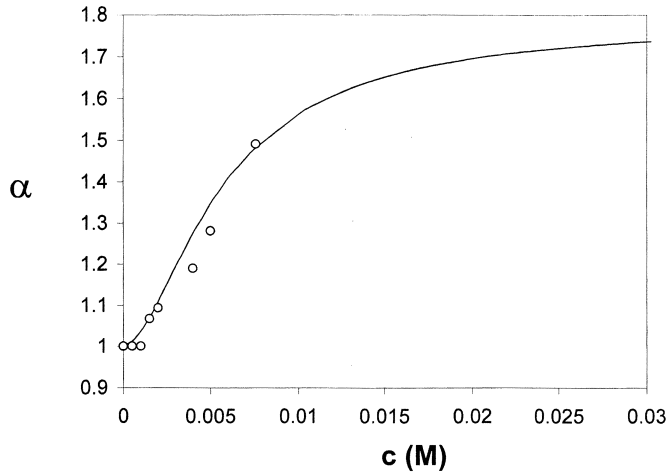

Figure 4. Plot of apparent enantioselectivity α against c for the *D*, *L* dansyl serine enantiomer pair at $T = 30^{\circ}\text{C}$ an amino stationary phase. The theoretical curve (——) is recreated from Eq. (12) using model parameters of Table 1. See operating conditions in the experimental section. Error bars are within the experimental points.

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asymptotically from 1 for $c = 0$ mM to around $K_{V_n} K_{S,V_{n(1)}} / K_{V_n} K_{S,V_{n(2)}}$ for high vancomycin concentrations (Fig. 4). At the low vancomycin concentrations, experimental and calculated values of α are close, showing the reliability of the theoretical approach. It can be noted that, at high c values (higher than 7.5 mM), it was not possible to observe, experimentally, the "quasi-saturation" of the apparent enantioselectivity because of the excessive noise, which did not allow for obtaining chromatographic data.

CONCLUSION

This study investigated the retention and the chiral recognition of *D*, *L* dansyl serine and *D*, *L* dansyl valine enantiomers on an amino stationary phase using vancomycin as CMPA. It appears, clearly, that the solute retention decrease with increasing additive concentration is dependent on the formation of vancomycin dimers in the chromatographic system. It is shown that this glycopeptide dimerization significantly increases the chiral recognition properties of the selector. This paper focuses on the interest to use an amino stationary phase to study, more easily, the glycopeptide dimerization dependence on the solute enantioselectivity.

ABBREVIATIONS

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_n	self-associated vancomycin in the mobile phase (n: number of self-associated vancomycin molecules)
S,V	solute–vancomycin monomer complex in the mobile phase
S,V_n	solute-self associated vancomycin complex in the mobile phase

Equilibrium constants

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

Chromatographic parameters

k	solute retention factor

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ϕ	phase ratio of the column
k_0	solute retention factor for vancomycin concentration equal to 0
α	apparent enantioselectivity
$\alpha_{S,V}$	true enantioselectivity for the interaction between S and V
α_{S,V_n}	true enantioselectivity for the interaction between S and V_n

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