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Analysis of the band profiles of the enantiomers of phenylglycine in liquid chromatography on bonded teicoplanin columns using the stochastic theory of chromatography

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Abstract

The retention behaviour of the enantiomers of underivatized phenylglycine was studied on a Chirobiotic T column packed with amphoteric glycopeptide teicoplanin covalently bonded to the surface of silica gel. The retention and the selectivity of separation of the enantiomers increase with rising concentration of ethanol or of methanol in aqueous–organic mobile phases. The band profiles of the less retained L-phenylglycine are symmetrical, but the band profiles of the more strongly retained D-phenylglycine are tailing in all mobile phases tested. The band broadening does not diminish even at very low concentrations of phenylglycine, so that it cannot be attributed to possible column overload. The analysis of the band profile using the stochastic theory of chromatography suggests that the broadening can be attributed to at least two additional chiral centres of adsorption in the stationary phase contributing to the retention of the more strongly retained enantiomer in addition to the adsorption of the less retained one. This behaviour can be explained by the complex structure of the teicoplanin chiral stationary phase.

Keywords: Enantiomer separation; Stochastic theory; Band profiles; Chiral stationary phases, LC; Phenylglycine; Teicoplanin

1. Introduction

Chiral stationary phases based on macrocyclic antibiotics have attracted much attention since their introduction because of their broad applicability and ability to separate compounds in multiple modes of operation, which often show complementary enantioselectivity by promoting or suppressing specific non-covalent binding forces [1]: (1) reversed-phase mode with buffered or non-buffered aqueous–organic mobile phases, (2) normal-phase mode with mixed organic solvents as the mobile phases, or (3) so-called polar–organic mode with mobile phases usually comprised of acetonitrile, methanol or their mixture buffered with less than 1% triethylamine and acetic acid to suit the particular separation. Macrocyclic glycopeptides vancomycin, teicoplanin, avoparcin and ristocetin chemically bonded on silica gel are now available commercially [2] and have been successfully applied for separations of the

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enantiomers of underivatized amino acids, carboxylic acids and other compounds [3,4]. Their excellent chiral discrimination capability is attributed to simultaneous stereospecific polar and ionic interactions on multiple chiral centres and binding sites, located in the cavities of a basket-like structure of the bonded glycopeptide antibiotics.

Amphoteric glycopeptide teicoplanin (M.W. 1885) covalently bonded to the surface of silica gel as a column packing material enables chiral separation of compounds containing amino and carboxylic groups [5,6]. The bonded chiral phase contains seven benzene rings, six amidic groups, three ether groups connecting aromatic rings, one primary amino group and one carboxylic group. The minimum energy is assumed for a basket-like structure of the teicoplanin molecule formed by four aglycone connected macrocycles, to which three sugar moieties are connected: one D-mannose and two D-glucosamines. One glucosamine moiety carries a nonyl substituent, responsible for the surface-active properties of the molecule. This structure of the molecule possesses 23 chiral centres surrounding four pockets and cavities. This provides a relatively high probability of specific interactions of various solutes with a chiral centre.

The bonded teicoplanin phase shows affinity to compounds with a carboxylic group. Remarkable is its excellent enantioselectivity for underivatized amino acids and peptides [5]. The L-enantiomers of naturally occurring amino acids are eluted before the D-enantiomers [7,8], but some cyclohexane carboxylic acids show reversed elution order [9]. The carboxylic group of teicoplanin is sterically hindered by two bulky carbohydrate moieties, which, together with its distance of 12 Å from the amino group, makes impossible simultaneous interactions of the amino group of an amino acid with the carboxylic group of teicoplanin and of the carboxylic group of the amino acid with the amino group of teicoplanin. If the amino group of the amino acid is sterically hindered, its electrostatic interaction is substituted by hydrogen bond or by hydrophobic interactions [10]. The enantioselectivity impairs for more hydrophobic amino acids.

The selectivity of enantiomeric separation differs from one mode to another. Dipole–dipole, steric, $\pi-\pi$, dispersive, hydrogen-bonding interactions and formation of inclusion complexes all may affect the retention of the optical isomers. Because of the complex character of the stationary phase with multiple adsorption centres, unique chiral separations are possible.

Owing to the increasing demand for optically active compounds, the development of methods for obtaining pure optically active compounds has become very important. The direct separation of enantiomers by HPLC is now widely used and a large number of chiral columns are commercially available for this purpose, including cellulose-based [11], cyclodextrin [12], protein [13], π-acid and π-base [14] phases, many of them prepared by appropriate modification of silica gel support material [15]. The better stability, high enantiomeric selectivity and higher loading capacity of teicoplanin with respect to the traditional chiral protein phases make this stationary phase a potential candidate for preparative HPLC chiral separations of optical isomers of various compounds containing carboxylic groups or amino and carboxylic groups, without the necessity for previous derivatization.

Recently, we have studied the effects of the mobile phase on the retention, separation selectivity and band profiles of the enantiomers of phenylglycine and other carboxylic acids and amino acids with the aim to determine the most adequate conditions for preparative chiral separations of compounds with carboxylic groups and of amino acids on the teicoplanin stationary phase [16]. During these studies, we found that the band of the more strongly retained D-phenylglycine was tailing even at very low concentrations, whilst the band profile of the earlier eluted L-phenylglycine was symmetrical. Hence, the band tailing of the D-enantiomer is probably caused by other factors than by column overload.

To further elucidate this behaviour, we applied the stochastic theory of chromatography to the analysis of the band profiles of the two enantiomers. The application of the stochastic model of chromatography in enantiomer separation is not entirely new. Trapp and Schurig [17] developed software on the basis of the stochastic model of Keller and Giddings [18] to determine the enantiomerization barriers in dynamic chromatography. Other models of chromatography, for instance the equilibrium-dispersive

mass balance model, has also been used to investigate the effect of both the amount and the mass transfer kinetics of the chiral selective and non-selective sites on the band shapes and selectivity in enantiomer separation [19].

2. Theory

2.1. Isotherm model

To obtain an appropriate production rate in preparative HPLC, it is usually necessary to work with overloaded separation columns, where the adsorption isotherms are non-linear. Several models have been suggested to fit non-linear isotherms to single-component data describing the distribution of one sample component between the stationary and the mobile phases. The most common and the simplest is the two-parameter Langmuir isotherm:

$$Q = \frac{ac}{1 + bc} \tag{1}$$

Here, Q is the concentration of the sample compound in the stationary phase and c that in the mobile phases, and a and b are the coefficients of the isotherm ($a = k_0/\Phi$), where k_0 is the retention factor of the sample compound at infinite dilution, i.e. in analytical chromatography, $\Phi = V_S/V_M$ is the ratio of the volumes of the stationary, V_S, and the mobile, V_M, phases in the column and $b = a/q_s$, where q_s is the column saturation capacity.

If a compound can be adsorbed on two different adsorption centres (1 and 2), the distribution can often be described more adequately than by the Langmuir isotherm by using the bi-Langmuir model with different coefficients a_1, b_1 and a_2, b_2 characterising the different adsorption energies and saturation capacities of the two adsorption centres 1 and 2, $q_{s1} = a_1/b_1$, $q_{s2} = a_2/b_2$:

$$Q = \frac{a_1 c}{1 + b_1 c} + \frac{a_2 c}{1 + b_2 c} \tag{2}$$

The bi-Langmuir isotherm equation (2) has been found useful for the description of the distribution of enantiomers, if the retention of the less retained isomer can be attributed only to non-specific interactions and the retention of the more strongly retained enantiomer to the simultaneous effects of the non-specific interactions with adsorption centres 1 and of specific interactions with chiral adsorption centres 2 [20].

2.2. The stochastic model of chromatography

The stochastic model of chromatography was originally suggested by Giddings and Eyring [21]. This model describes the random migration of a solute molecule along the chromatographic column and depicts the chromatogram as the probability density function of the individual retention times of the molecules. Several attempts have been made to extend the stochastic model toward heterogeneous sorption kinetics [22] but so far the characteristic function approach seems to be the only efficient means to tackle this problem.

The characteristic function of a random variable X is defined by the expected value $E\{e^{i\omega X}\}$, where ω is an auxiliary variable. For a continuous random variable, the characteristic function can be calculated as:

$$\varphi(\omega) = \int \exp(i\omega t) f(t)\, dt \tag{3}$$

where $f(t)$ is the probability density function of the random variable. The fundamental properties of the characteristic function can be found in recent reviews [23,24]. Dondi and Remelli analyzed several chromatographic models and scenarios by means of the characteristic function approach [25]. The generalization of the stochastic model by Cavazzini et al. showed that virtually any type of heterogeneous kinetics can simply be considered via the characteristic function approach [26,27]. It has been demonstrated that the stochastic model of chromatography gives results identical to the lumped kinetic model [28]. The major advantage of the former model is its flexibility to handle various types of heterogeneous kinetics and that, in most cases, the only numerical step involved is the calculation of the inverse Fourier transform of the characteristic function in order to obtain the band profile.

When one considers the random migration of a molecule along the column, there are two fundamental assumptions regarding the random nature of the process:

(i) in one single adsorption step, the sojourn time of the molecule in the immobile state is described by an exponential distribution with τ as the average sojourn time;
(ii) the number of adsorption–desorption steps is governed by a Poissonian distribution and n is the average number of sorption steps.

The average residence time is the inverse of the desorption rate constant and is related to the sorption enthalpy via the equation [29]:

$$\tau = \tau_0 e^{E_a/RT} \quad (4)$$

where τ_0 is a constant typically $\approx 1.6 \cdot 10^{-13}$ s, E_a is the enthalpy of adsorption, R the universal gas constant and T the temperature.

These assumptions lead to the following characteristic function when one considers a homogeneous surface [27]:

$$\varphi(\omega) = \exp\left(n \left[\frac{1}{1 - i\omega\tau} - 1 \right] \right) \quad (5)$$

where i is the imaginary unit. This particular model ignores the contribution of the mobile phase to the band profile, only the stationary phase processes are taken into account. Therefore, the first moment of the above characteristic function gives the corrected retention time: $t'_R = n\tau$.

When the surface of the stationary phase is composed of m different sites, the following characteristic function is obtained for the residence time in the stationary phase:

$$\varphi(\omega) = \exp\left(n \left[-1 + \sum_{j=1}^{m} p_j \frac{1}{1 - i\omega\tau_j} \right] \right) \quad (6)$$

where p_j is the relative amount of site j and τ_j is the average residence time on site j. Formally, the characteristic function can be regarded as the Fourier transform of a signal. Accordingly, the band profile itself can simply be determined by a numerical inverse Fourier transformation step.

3. Experimental

3.1. Sample compound

L-, D- And D,L-phenylglycine (99+% purity) were purchased from Sigma–Aldrich (Steinheim, Germany) and were used as obtained.

3.2. Column

Chirobiotic T, 5 μm, 150×4.6 mm I.D., stainless steel was obtained from ASTEC (Whippany, NJ, USA).

3.3. Instrumentation

An HP 1090M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) was used, equipped with a 3 DR solvent delivery system and solvent reservoirs continuously stripped with helium to degas the mobile phase and the sample solution, an automatic sample injector, a column switching valve, a temperature-controlled column compartment, a diode-array UV detector and a data workstation.

3.4. Mobile phases

Methanol and ethanol, HPLC grade (LiChrosolv), were obtained from Merck (Darmstadt, Germany). Water was doubly distilled in glass with addition of potassium permanganate. The solvents were filtered using a Millipore 0.45-μm filter and the mobile phases were prepared by mixing the components in the required ratios and degassing by ultrasonication before the use. The sample solutions used for the chromatographic measurements and for the determination of the distribution data were prepared by weighing the required amounts of the sample solutes and dissolving in the mobile phase.

3.5. Determination of the distribution isotherms of optical isomers of phenylglycine by frontal analysis

The equilibrium isotherms were measured using the frontal analysis method [30]. The mobile phase was stored in one of the solvent flasks of the solvent delivery system, and the solution of sample solutes in a solvent of the same composition as the mobile phase in another flask. The gradient-delivery system was used to pump and mix the solutions needed for the frontal analysis experiments.

The ratio of the flow-rates of the two solutions controls the concentration of the solute delivered continuously to the column. It was adjusted from 0 to 100% in successive 5–10% steps. Time was allowed for the stabilization of the detector signal after each concentration change. The flow-rate (1 ml/min) and the column temperature (35°C) were kept constant during all the experiments.

In each experiment the solute concentration in the stationary phase was determined from the integral mass balance equation, using the experimental retention volume (inflection point of the breakthrough curve), corrected for the volume of the tubing between the mixing point of the liquids pumped in each channel and the column top [22] (0.35 ml). The solutions of the individual enantiomers of phenylglycine (0.025 mol/l) in the mobile phase were used for measuring the isotherms, where the breakthrough volumes on the frontal-analysis curve and the concentrations of the individual sample compounds corresponding to the plateau on the detector response record in 10 to 20 subsequent steps of the frontal analysis curve were measured. The data obtained were used in the appropriate mass-balance equation (Eq. (7)) [31] using a spread-sheet program run on a Pentium personal computer. The ADSTAT software (Trilobyte, Prague, Czech Republic) was used to fit the isotherm data by non-linear regression analysis:

$$Q_{i+1} = Q_i + \frac{(c_{i+1} - c_i)(V_{i+1} - V_0)}{V_S} \quad (7)$$

Here, c_i and c_{i+1} are the concentrations of the compound in the mobile phase in equilibrium with the stationary phase in the column in steps i and $(i + 1)$, respectively, Q_i and Q_{i+1} are the concentrations of the adsorbed compound in steps i and $(i + 1)$, respectively, V_{i+1} is the retention volume at the inflex point of the $(i + 1)$th step on the frontal analysis curve, V_0 is the column hold-up volume and V_S is the volume of the stationary phase in the column.

For the stochastic model, the calculation of the characteristic functions, the parameter estimation by non-linear least-squares curve fitting was performed on a Pentium PC using an in-house Fortran program containing the routines for the deconvolution of the two band profiles, Fourier transformation, etc.

4. Results and discussion

4.1. The effect of the mobile phase on the retention behaviour of phenylglycine

Amino acids are insoluble in non-polar solvents, hence the normal-phase mode is not suitable for their separation. Even in the reversed-phase mode the solubility of amino acids is limited and depends on the composition of the mobile phase. To investigate the optimum separation selectivity of underivatized phenylglycine on a Chirobiotic T column, we measured the effects of the concentration of methanol and of ethanol on the retention of the L- and D-enantiomers.

The retention of D-phenylglycine rapidly increases with rising concentration of the organic solvent in aqueous–organic mobile phases, whereas the retention of the L-enantiomer is only little affected by the composition of the mobile phase (Fig. 1) up to 60% ethanol and 80% methanol, i.e. decreasing polarity of the mobile phase enhances the specific adsorption on chiral adsorption centres much more significantly than the non-specific adsorption on the Chirobiotic T chiral stationary phase. In agreement with earlier results obtained for other amino acids [2], the chiral selectivity (the separation factor α of the D- and L-enantiomers, Table 1) for phenylglycine significantly increases in mobile phases with higher concentrations of methanol and ethanol. The retention of the L-enantiomer increases more significantly in mobile phases containing 80% or more ethanol than in mobile phases with higher concentrations of water, which is the reason for the decreased separation factor in ethanol-rich mobile phases. However, the solubilities of the underivatized phenylglycine enantiomers also decrease with increasing concentration of the organic solvent in the mobile phase [20].

4.2. Adsorption isotherms of the enantiomers of phenylglycine on a Chirobiotic T column

In aqueous–organic mobile phases the solubility of the enantiomers of phenylglycine is too low and the distribution isotherms are almost linear up to the saturation concentrations of phenylglycine in the mobile phases used. Both the Langmuir and the

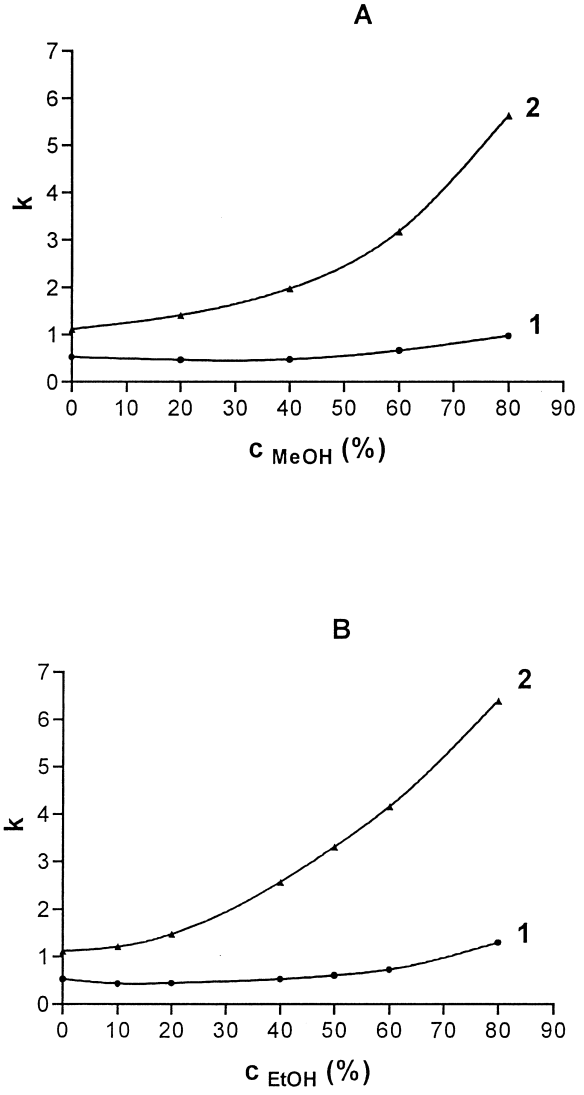

Fig. 1. Retention factors, *k*, of the enantiomers of phenylglycine on a Chirobiotic T column in aqueous methanol (A) and in aqueous ethanol (B) at 35°C. 1 = L-enantiomer, 2 = D-enantiomer.

bi-Langmuir model of adsorption were applied to the experimental distribution data of the pure enantiomers of phenylglycine in water. The best-fit coefficients of the Langmuir and bi-Langmuir isotherms are listed in Table 2. The distribution of both the L- and the D-enantiomers of phenylglycine between the stationary and the mobile phases is adequately described by the Langmuir model (Eq. (1)). The bi-Langmuir isotherm (Eq. (2)) provides

Table 1
Retention factors, *k*, and separation factors, $\alpha = k_D/k_L$, of the enantiomers of phenylglycine on a Teicoplanin chiral stationary phase in aqueous–organic mobile phases. Column: Chirobiotic T, 150×4.6 mm, 10 μm, $V_s = 0.813$ ml, $\varepsilon = 67.4\%$. MeOH, methanol; EtOH, ethanol

Mobile phase	k_L	k_D	α (D/L)
0% MeOH	0.52	1.11	2.12
20% MeOH	0.46	1.41	3.04
40% MeOH	0.48	1.98	4.15
60% MeOH	0.66	3.17	4.8
80% MeOH	0.97	5.62	5.79
10% EtOH	0.43	1.2	2.81
20% EtOH	0.44	1.47	3.34
40% EtOH	0.52	2.56	4.9
50% EtOH	0.6	3.3	5.5
60% EtOH	0.73	4.15	5.71
80% EtOH	1.3	6.4	4.93

four times lower values for the specific chiral saturation capacity of the Chirobiotic T column for the more strongly retained D-enantiomer with respect to the non-specific saturation capacity for the less retained L-enantiomer (Fig. 2). However, the macroscopic model cannot provide detailed information about the adsorption of the enantiomers on different adsorption sites of chemically bonded Teicoplanin.

4.3. Analysis of the band profiles using the stochastic theory of chromatography

The band profiles of the two enantiomers of phenylglycine are strikingly different. The narrow, symmetrical peaks of the less retained enantiomer indicate that it adsorbs on the stationary phase with dominantly one sorption energy. Heterogeneous sorption kinetics usually result in peak broadening and considerable asymmetry, particularly when the relative amount of the slow sites is much less than that of the quick sites [27,28]. Therefore, in our calculations we assumed that the L-enantiomer is retained on the stationary phase only by non-selective forces. The much broader and asymmetrical band profiles of the D-enantiomer are due to heterogeneous sorption kinetics: it is retained on the non-selective site in a similar manner as the L-enantiomer, furthermore several chiral selective sorption processes are possible on the various chiral centres. As a consequence, we assumed that the band profile of the less retained enantiomer is affected by

Table 2
Coefficients (±RSD) of Langmuir and bi-Langmuir isotherms of enantiomers of phenylglycine on a Chirobiotic T column in water at 35°C. Langmuir isotherm, Eq. (1); bi-Langmuir isotherm, Eq. (2); non-specific saturation capacity, $q_{NS} = a_1/b_1$; chiral saturation capacity, $q_{CH} = a_2/b_2$. C.D., coefficient of determination. The coefficients of the bi-Langmuir isotherm for the D-enantiomer were determined using fixed values of the Langmuir isotherm for the L-enantiomer

Isotherm, enantiomer	a_1	a_2	b_1 (l/mol)	b_2 (l/mol)	q_{NS} (mol/l)	q_{CH} (mol/l)	C.D.
Langmuir, L-	1.155±0.006	–	10.48±0.33	–	0.11		1
Langmuir, D-	1.944±0.051	–	16.15±1.91	–	0.12		0.999
Bi-Langmuir, D-	1.155±0.006	0.82±0.02	10.48±0.33	29.5±2.5	0.11	0.028	0.999

the non-selective sites and furthermore by the mobile phase dispersion and by extra-column effects. The band profile of the more retained enantiomer is affected by the same aforementioned phenomena, but the selective sites also contribute to its peak shape. The non-selective sites, the mobile phase dispersion, and the extra-column effects alter the band profiles of the two enantiomers in an identical manner.

Only the chiral selective sites are responsible for the dissimilarity of the two band profiles. The characteristic function of the *m*-site heterogeneous process (given in Eq. (6)) can be rewritten as:

$$\varphi(\omega) = \prod_{j=1}^{m} \exp\left(\frac{n_j}{1 - i\omega\tau_j} - n_j\right) \quad (8)$$

where $n_j = np_j$. The above equation demonstrates that the sorption processes that take place on the individual sites are independent, and the whole band profile is built up as the convolution of the band profiles developed due to the particular sites.

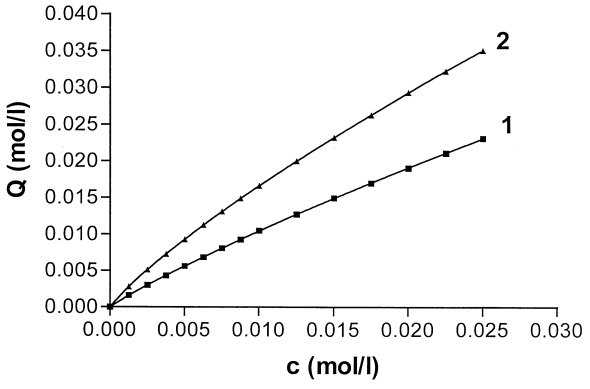

Fig. 2. Adsorption isotherms of the enantiomers of phenylglycine in water on a Chirobiotic T column at 35°C. *c*, concentration in the mobile phase; *Q*, concentration in the stationary phase, both in mol/l. Points, experimental data; lines, Langmuir model (1=L-enantiomer), bi-Langmuir model (2=D-enantiomer).

Accordingly, the band profile of the more retained enantiomer is the convolution of that of the less retained enantiomer with the band profiles due to the chiral selective sites.

Therefore, the following procedure was used to fit the band profile of the more retained component:

(i) the convolution of the band profile of the less retained enantiomer with a band profile calculated by the 1-site stochastic model is computed;

(ii) the parameters of the model are optimized with a non-linear least-squares method and the best-fit chromatogram is compared to the measured chromatogram of the more retained enantiomer;

(iii) if the fit is defective, the number of sites in the model is increased by one and the whole procedure (convolution and curve fitting) is repeated. Otherwise the calculation is stopped.

We implemented the above procedure for three chromatograms with different mobile-phase composition; the numerical result is summarized in Table 3. The best-fit chromatograms together with the measured ones are plotted in Figs. 3–5.

In each case, we found that the model that contains one chiral selective site, besides the non-selective site, did not give satisfactory result. In the insert of Fig. 3, one can see that when we assume that the band shape of the more retained enantiomer is the result of one selective and one non-selective site, as well as of mobile phase dispersion and extra-column effects (dashed line), the best-fit model is nearly symmetrical. Its shape is very far from the actual band shape of that enantiomer. When this model is fitted to the front of the peak only, we can

Table 3
Results obtained by fitting the two-site model to the band profiles of D-phenylglycine. n_i is the average number of adsorption–desorption steps on site i; τ_i is the average sojourn time on site i; $E_{a,i}$ is the enthalpy of adsorption on site i

	Water	RSD (%)	80:20	RSD (%)	60:40	RSD (%)
n_1	7745	6.26	7554	5.31	7637	7.40
τ_1 (s)	0.00744	5.87	0.01204	5.27	0.01928	7.39
$E_{a,1}$ (kJ/mol)	59.8	–	61.0	–	62.2	–
n_2	1.307	0.43	1.529	14.8	1.640	1.98
τ_2 (s)	5.24	0.33	4.95	3.06	4.92	1.24
$E_{a,2}$ (kJ/mol)	75.8	–	75.7	–	75.7	–

obtain a rough estimation of the band profile we would observe without the slowest chiral selective sites. When the model is extended to incorporate two selective sites — besides the non-selective site, mobile phase and extra-column effects — an excellent fit can be achieved for all mobile-phase compositions (see Figs. 3–5).

These results indicate that, on the Chirobiotic T stationary phase, D-phenylglycine is retained on at least two different chiral selective sites, besides the non-selective site. There is one dominant chiral selective site (type 1 in Table 3) that is present in a much larger concentration (roughly 5000-fold) than the other type of selective site. On the other hand, the desorption on the low-concentration selective sites is much slower than on the fast selective site. This configuration leads to substantial band broadening and tailing. Eq. (4) also allows us to calculate the enthalpy of adsorption. We can see that the enthalpy of adsorption is practically independent of the mobile-phase composition. It is around 61 and 76 kJ/mol on the two selective sites, respectively.

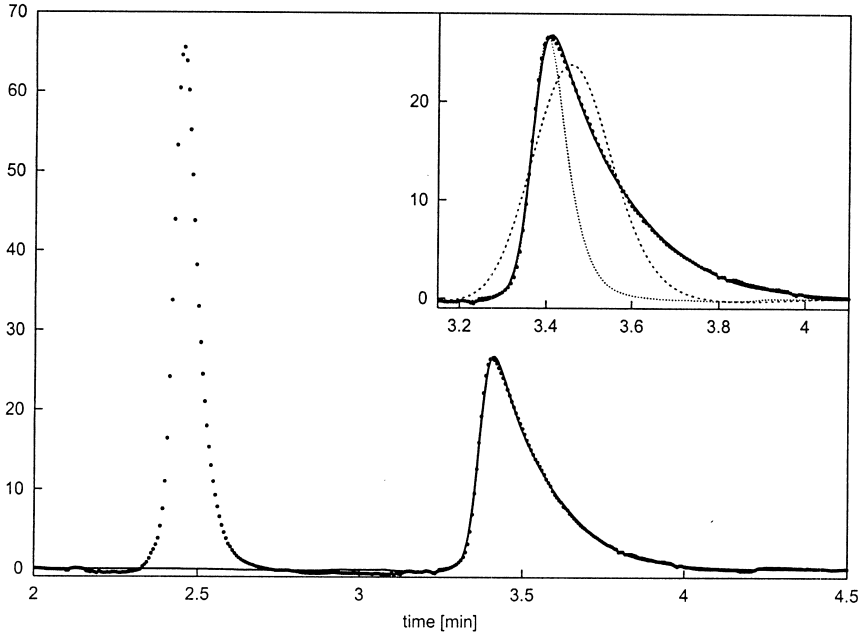

Fig. 3. Separation of 10 μl of 0.005 mol/l racemate of phenylglycine on a Chirobiotic T, 5 μm, 150×4.6 mm column. Mobile phase, water, 35°C, 1 ml/min; detection, UV, 254 nm. The L-enantiomer elutes before the D-enantiomer. Dots, experimental chromatogram; solid line, chromatogram recalculated on the basis of stochastic theory with one non-selective and two selective sites. The narrow dashed line in the insert shows the best-fit model based on one non-selective and one selective site fitted to the whole peak; the narrow dotted line shows the same model fitted to the front of the peak.

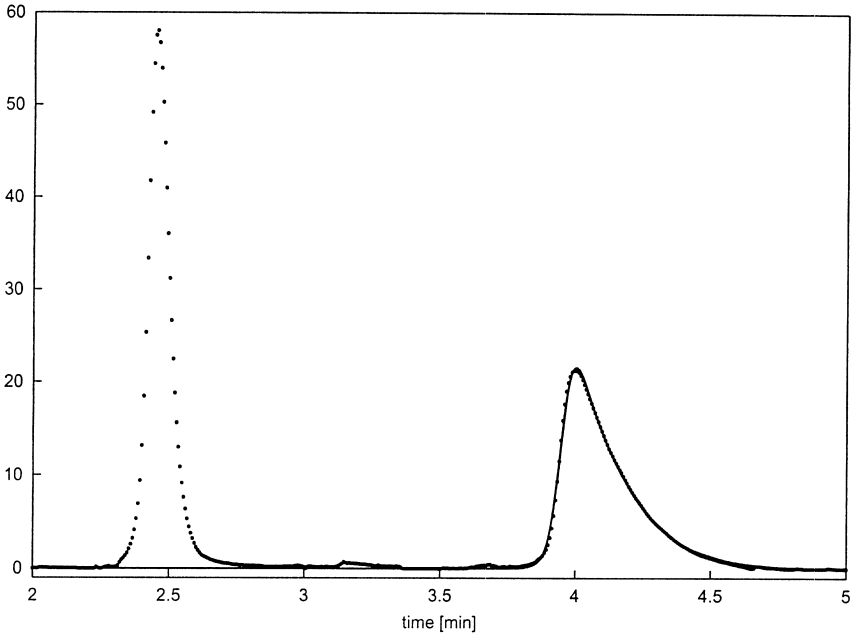

Fig. 4. Separation of 10 μl of 0.005 mol/l racemate of phenylglycine on a Chirobiotic T, 5 μm, 150×4.6 mm column. Mobile phase, water–methanol (80:20), 35°C, 1 ml/min; detection, UV, 254 nm. The L-enantiomer elutes before the D-enantiomer. Dots, experimental chromatogram, solid line, chromatogram recalculated on the basis of stochastic theory with one non-selective and two selective sites.

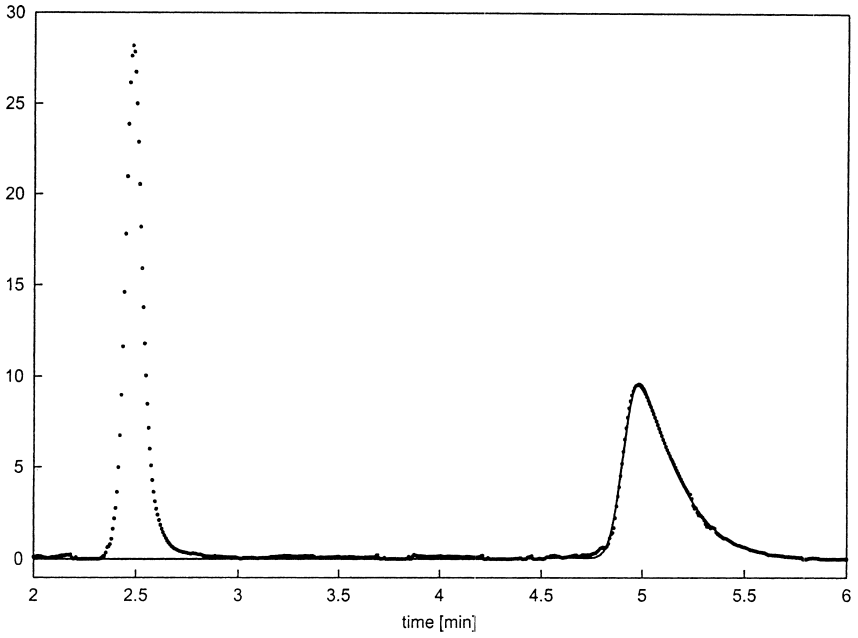

Fig. 5. Separation of 10 μl of 0.005 mol/l racemate of phenylglycine on a Chirobiotic T, 5 μm, 150×4.6 mm column. Mobile phase, water–methanol (60:40), 35°C, 1 ml/min; detection, UV, 254 nm. The L-enantiomer elutes before the D-enantiomer. Dots, experimental chromatogram; solid line, chromatogram recalculated on the basis of stochastic theory with one non-selective and two selective sites.

5. Conclusions

Excellent separation selectivity and sorption capacity of the Chirobiotic T stationary phase for enantiomeric separations of amino acids was the reason for the present study, in which the retention of the enantiomers was characterized by a macroscopic and a microscopic model. The results of the macroscopic approach, i.e. the isotherm determination (see Table 2), indicate that, on the basis of breakthrough curves, a Langmuir model can be fitted to the isotherm of the D-enantiomer. Note that this isotherm corresponds to a one-site model. Of course, a bi-Langmuir model (which is equivalent to a two-site model) is a more logical choice, but it does not really give a better fit than the Langmuir model. The reason for this is that column overload suppresses the contribution of the selective sites that are present much less than the non-selective sites. Due to the much fewer number of selective sites (q_{CH} versus q_{NS} in Table 2), they are quickly saturated as the column is overloaded and the non-selective site will dominate the chromatographic behaviour. The increase of the retention factor of the D-enantiomer in mobile phases with higher concentrations of organic solvents in the mobile phase suggests that the selective (chiral) interactions are polar ones, as the experimental behaviour observed in Fig. 1 is characteristic for normal-phase systems.

The microscopic approach, by means of the stochastic model, recognizes the presence of several chiral selective sites not because it is a molecular approach but because the sample concentration is very small and the slow selective sites influence the band profile through a convolution process. The fact that our calculations indicated the presence of three different sites (two chiral selective and one non-selective) does not necessarily mean that the D-enantiomer is indeed retained on exactly three types of sites.

We can state that *at least two selective sites* take part in the retention of the D-enantiomer. Three-site, four-site, etc., models would have given an excellent fit as they contain more parameters, and they are more flexible models. Models that contain fewer than three selective sites failed to fit the band profiles, therefore from the application of the stochastic model we can conclude that two is the minimum number of chiral selective sites responsible for the shape of the D-enantiomer.

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