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Effect of the mobile phase on the retention behaviour of optical isomers of carboxylic acids and amino acids in liquid chromatography on bonded Teicoplanin columns

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Abstract

Conditions for separation of enantiomers of underivatized amino acids phenyl glycine and tryptophan and of mandelic acid as test compounds were studied on a Chirobiotic T column packed with amphoteric glycopeptide Teicoplanin covalently bonded to the surface of silica gel. The effects of the mobile phase composition on the retention and selectivity under analytical conditions, on the profile of the adsorption isotherms of the enantiomers and on the overloaded separation were investigated. The concentration of ethanol or of methanol in aqueous–organic mobile phases and the pH of the mobile phase affect not only the retention and selectivity, the saturation capacity and the isotherm profile, but also the solubility of the acids, which should be taken into account in development of preparative separations. A compromise between the separation selectivity and the solubility should be made in selecting the mobile phase suitable to accomplish preparative separations at acceptable production rate and throughput of the operation.

Keywords: Mobile phase composition; Enantiomer separation; Retention behaviour; Carboxylic acids; Amino acids; Teicoplanin

1. Introduction

Chiral stationary phases based on macrocyclic antibiotics have attracted much attention since their introduction because of 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 mixtures 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 and have been successfully applied for separations of the enantiomers of underivatized amino acids, carboxylic acids

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and other compounds [2–5]. Their excellent chiral discrimination capability is attributed to simultaneous stereospecific polar and ionic interactions on multiple chiral centres and binding sites (more than 20), located in the cavities of a basket-like molecular structure of the bonded glycopeptide antibiotics.

The bonded Teicoplanin phase shows affinity to compounds with a carboxylic group. Remarkable is its excellent enantioselectivity for underivatized amino acids and peptides [4]. The L-enantiomers of naturally occurring and of unnatural amino acids are eluted before the D-enantiomers [5–8], but some cyclohexane carboxylic acids show reversed elution order [9]. The most important role in the retention is probably the interaction between the carboxylic group of the sample solutes and 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.

Owing to the increasing demand, the development of methods for obtaining pure enantiomers has become very important. The direct separation by preparative HPLC is now widely used [11]. Better stability, high enantioselectivity and improved loading capacity of Teicoplanin with respect to the traditional chiral protein phases make this stationary phase potential candidate for preparative HPLC chiral separations. The cost of the bonded Teicoplanin material is comparable to the cyclodextrin bonded chiral phases for HPLC, which generally show much lower enantioselectivity and loading capacity.

In preparative HPLC, the production costs should be kept as low as possible, which makes necessary working at a high selectivity and loading capacity as a pre-requisite for high production rate. Hence, it is usually necessary to work with overloaded separation columns, where the adsorption isotherms are non-linear. The equation of the isotherm describing adequately the experimental distribution data should be determined to allow optimization of separation conditions. 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 most simple 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 and c that in the mobile phases and a (dimensionless), b (in l/mol) 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, in mol/l, is the column saturation capacity). Sometimes the Langmuir model does not fit well the experimental data and more complex isotherms should be used. If a compound can be adsorbed on two different adsorption centres (1 and 2), the distribution can be often adequately described by bi-Langmuir model with different coefficients a_1, b_1 and a_2, b_2 characterising 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 Eq. (2) has been found useful for the description of distribution of enantiomers if the retention of the less retained enantiomer can be attributed only to non-specific interactions and the retention of the more strongly retained one to the simultaneous effects of the non-specific interactions with adsorption centres 1 and of specific interactions with chiral adsorption centres 2 [12].

The coefficients of the isotherms are affected by the properties of the stationary phase and by the mobile phase used for the separation. The main factors affecting the distribution isotherm are the concentration ratios of the less polar and the more polar solvent in a binary mobile phase and, for ionizable compounds, the pH and the ionic strength.

In addition to the sample distribution described by suitable isotherm equation, the volume of the feed, V_i, and the sample concentration in the feed, c_i, which determine the loading factor, L_f, as the part of the useful column saturation capacity, $q_s(V_S)$, used

per one separation operation, should be taken into account in designing a preparative HPLC separation. The loading factor is directly proportional to the product of the feed volume and of the compound concentration in the feed (Eq. (3A)):

$$L_f = \frac{V_i c_i}{q_s V_s} \quad (3A)$$

$$L_f\,(\text{opt}) = \frac{1}{6}\left(\frac{\alpha - 1}{\alpha}\right)^2 \quad (3B)$$

With some simplification, the loading factor for the product and limiting impurity with ''touching bands'' can be estimated from the separation factor, $\alpha = k_{0,2}/k_{0,1}$ of the two compounds under analytical conditions using Eq. (3B) [13].

The economics of the preparative separations is the best at the highest production rate, Pr, i.e., with the highest amount of purified product at the yield Y_i per cycle time, t_c, achieved at the highest loading factor allowed by the separation selectivity:

$$\text{Pr} = \frac{4V_i c_i Y_i}{\pi d_c^2 t_c} = \frac{L_f q_s Y_i}{(1-\epsilon)t_c} \quad (4)$$

Here, d_c is the inner diameter, q_s the saturation capacity and ϵ the porosity of the separation column. The cycle time for isocratic separation is in the most simple case the time between the injection and the end of the elution of the last band, but it can be reduced by using recycling technique.

Hence, appropriate loading factor is particularly important for separations of compounds with limited solubilities, where the sample feed per chromatographic operation often may be limited by sample solubility rather than by the band broadening due to non-linear distribution behaviour. This is the case, e.g., with unnatural racemates of amino acids the chiral separation of which was investigated in present work. We determined the effects of the mobile phase on retention, separation selectivity, distribution isotherms and solubility with the aim to find most adequate conditions for preparative chiral separations of compounds with carboxylic groups and of amino acids on the Teicoplanin stationary phase. For this purpose, simple test compounds were selected: mandelic acid as a carboxylic acid and phenyl glycine and tryptophan as amino acids.

2. Experimental

2.1. Sample test compounds

Compounds L-, D- and L,D-mandelic acid (99+% purity) were purchased from Fluka (Buchs, Switzerland), L-, D- and L,D-phenyl glycine, L-, D- and L,D-tryptophan (both 99+% purity) from Sigma–Aldrich (Steinheim, Germany) and were used as obtained.

2.2. Column

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

2.3. Instrumentation

To acquire the data necessary for the determination of the equilibrium isotherms, 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.

2.4. Mobile phases

Methanol and ethanol, HPLC grade (Lichrosolv), were obtained from Merck (Darmstadt, Germany), triethyl amine and acetic acid (both p.a. grade) from Sigma–Aldrich. 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 degassed 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.

2.5. Determination of the solubility of pure enantiomers and of racemates of amino acids

Saturated solutions of the pure isomers and of the racemates of phenyl glycine and of tryptophan were prepared by mixing exactly weighed excess amounts of the solutes in the mobile phases. The mixtures were agitated and kept overnight at 25°C. The concentrations of the amino acids dissolved in the saturated solutions were determined after appropriate dilution by HPLC of 5-μl samples on an X-Terra C_{18}, 5 μm, 150×4.6 I.D. column (Waters, Milford, MA) with methanol–0.03 mol/l KH_2PO_4 buffer (80:20), pH 3, at 35°C, using an external standard calibration curve method.

2.6. Determination of the distribution isotherms of enantiomers by frontal analysis

The equilibrium isotherms were measured using the frontal analysis method [14]. The mobile phase was stored in one of the solvent flasks of the solvent delivery system, 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 (40°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 break-through curve), corrected for the volume of the tubing between the mixing point of the liquids pumped in each channel and the column top [15] (0.35 ml). The solutions of the individual enantiomers and of the racemate of phenylglycine (0.025 mol/l), tryptophan (0.05 mol/l) and mandelic acid (0.25 mol/l) in the appropriate 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 10–20 subsequent steps of the frontal analysis curve were measured. The data obtained were used in the appropriate mass-balance equation (Eq. (5)) [15] 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:

$$Q_{i+1} = Q_i + \frac{(c_{i+1} - c_i)(V_{i+1} - V_0)}{V_S} \quad (5)$$

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 the steps i and $(i + 1)$, respectively, Q_i and Q_{i+1} are the concentrations of the adsorbed compound in the 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.

3. Results and discussion

3.1. Retention behaviour of amino acids under analytical conditions and their solubilities

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. For maximum production rate in chiral preparative separations, both high separation selectivities and high loading factors are essential. To investigate optimum conditions for preparative separation of underivatized amino acids on a Chirobiotic T column, we measured the effects of the mobile phase on the retention of phenyl glycine and tryptophan as the test compounds.

The retention of D-phenyl glycine 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. 1A). In agreement with earlier results obtained for other

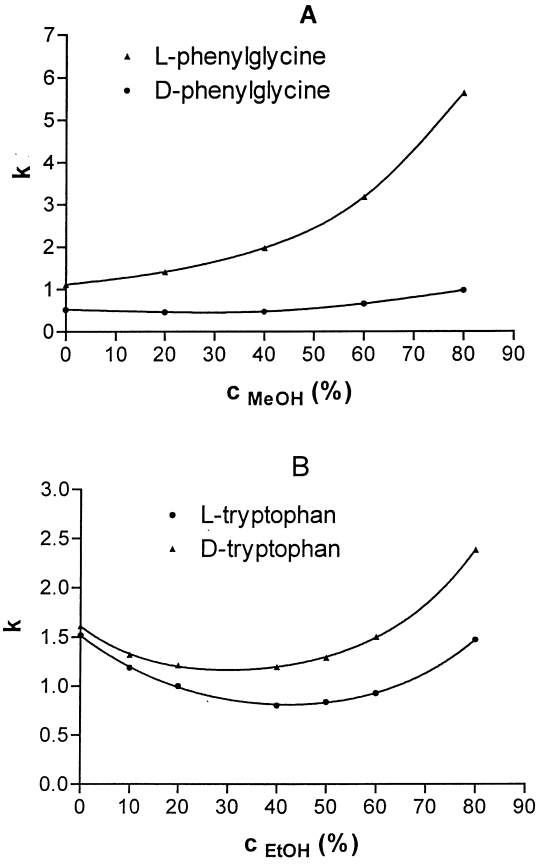

Fig. 1. Retention factors, *k*, of the enantiomers of phenyl glycine in aqueous methanol (A) and of tryptophan in aqueous ethanol (B) on a Chirobiotic T column at 35°C.

amino acids [2], the chiral selectivity (the separation factor α of the D- and of the L-enantiomers, Table 1) for phenylglycine significantly increases in mobile phases with higher concentrations of methanol and ethanol. The retention of both L- and D-tryptophan first decreases and then increases as the concentration of either methanol or ethanol increase and minimum retention is observed in mobile phases containing 40–50% methanol or ethanol in water (Fig. 1B), but the enantioselectivity for tryptophan improves as the concentration of ethanol or of methanol increase, like with the phenylglycine enantiomers (Table 1). Different retention behaviour of the two amino acids indicates that the separation mechanism probably involves different types and (or) numbers of active adsorption sites in each case.

To develop suitable procedures for preparative HPLC separations of amino acids on Chirobiotic T columns, the effects of the solubilities in the mobile phase should be taken into account. Unfortunately, we did not find solubility data for phenylglycine and tryptophan in mixed aqueous–organic solvents in the literature, but the solubilities of the racemates of other acids are significantly lower than corresponding solubilities of the pure enantiomers [16]. We determined experimentally the solubilities, $c_{i,max}$, of the racemates and of the L- and D-enantiomers of phenylglycine and of tryptophan in aqueous–organic solutions with various concentrations of methanol or ethanol (Table 1).

In all solvents, we found significantly lower solubilities of the racemates of both phenylglycine and tryptophan than the solubilities of the corresponding pure enantiomers (Fig. 2). This may limit the loading factor and the optimum use of the column saturation capacity for preparative separations. The reason for this behaviour may be in the interactions between the amino and the carboxylic groups in the molecules of the L- and D-enantiomers, which possibly form associates which are less polar and hence less soluble in aqueous–organic mobile phases than the pure enantiomers. The solubility of phenylglycine decreases with increasing concentration of methanol or ethanol in aqueous–organic mobile phases (Fig. 2B), but the solubility of tryptophan first impairs with increasing concentration of ethanol or methanol in the mobile phase up to 30%, improves in solvents with 30–60% alcohol and then again decreases as the concentration of the organic solvent increases (Fig. 2A).

3.2. Adsorption isotherms and optimization of overloaded separation conditions for phenyl glycine and tryptophan

The adsorption isotherms of the enantiomers of phenyl glycine and of tryptophan were measured in various mobile phases optimized with respect to the separation selectivity and sample solubility. The best-fit coefficients of the Langmuir and bi-Langmuir isotherms are listed in Table 2. The distribution of less retained L-phenylglycine and L-tryptophan between the stationary and the mobile phases is adequately described by the Langmuir model (Eq.

Table 1
Optimized ''touching band'' separation conditions on a Chirobiotic T column[a]

M. phase	$t_{R,D}$ (min)	α (D/L)	$c_{i,max}$ (g/l)	$L_{f,opt}$ (%)	$V_{i,opt}$ (ml)	Pr
Phenyl glycine, $q_s = 0.018$ g/ml						
0% MeOH	3.55	2.12	1.45	4.67	0.479	0.843
20% MeOH	4.05	3.04	0.86	7.50	1.288	0.442
40% MeOH	5.00	4.15	0.70	9.60	2.042	0.294
60% MeOH	7.01	4.80	0.54	10.45	2.841	0.165
80% MeOH	11.12	5.79	0.39	11.41	4.295	0.076
10% EtOH	3.70	2.81	1.13	6.90	0.901	0.690
20% EtOH	4.15	3.34	0.80	8.18	1.511	0.402
40% EtOH	5.99	4.90	0.45	10.56	3.445	0.160
50% EtOH	7.23	5.50	0.30	11.15	5.457	0.089
60% EtOH	8.65	5.71	0.23	11.34	7.416	0.056
80% EtOH	12.43	4.93	0.09	10.59	17.050	0.016
Tryptophan, $q_s = 0.042$ g/ml						
0% MeOH	4.39	1.06	2.06	0.06	0.010	0.039*
20% MeOH	3.62	1.15	1.53	0.28	0.0627	0.219*
40% MeOH	3.37	1.30	1.49	0.89	0.205	0.746*
60% MeOH	3.83	1.38	1.61	1.25	0.266	0.874
80% MeOH	4.94	1.47	1.39	1.73	0.427	0.591
10% EtOH	3.90	1.11	1.76	0.16	0.031	0.116*
20% EtOH	3.72	1.21	1.45	0.52	0.123	0.397*
40% EtOH	3.69	1.49	1.43	1.80	0.431	0.802
50% EtOH	3.85	1.54	1.57	2.04	0.445	0.848
60% EtOH	4.20	1.61	1.68	2.42	0.495	0.832
80% EtOH	5.68	1.62	1.06	2.44	0.788	0.396
Mandelic acid, $q_s = 0.0012$ g/ml in 96% ethanol, $q_s = 0.0014$ g/ml in 86% ethanol						
96% EtOH	3.35	4.34	1.5	9.87	0.064	0.249*
86% EtOH	3.20	1.82	1.5	3.38	0.026	0.107*
Buffer 1	2.74	1.59	15.2	2.28	0.095	4.50*
Buffer 2	2.80	2.60	38.0	6.33	0.246	27.1*
Buffer 3	2.63	1.82	38.0	3.40	0.113	13.8*

[a] Column: Chirobiotic T, 150×4.6 mm, 10 μm, $V_s = 0.813$ ml, $\epsilon = 67.4\%$, $N = 1000$, flow-rate 1 ml/min. MeOH, methanol; EtOH, ethanol. Buffer 1: methanol–water–triethyl amine (19:76:5, v/v), adjusted with acetic acid to pH 4.0 ($q_s = 0.078$ g/ml). Buffer 2: ethanol–water–triethyl amine (47.5:47.5:5, v/v/v), adjusted with acetic acid to pH 6.6 ($q_s = 0.182$ g/ml). Buffer 3 (polar organic phase, $q_s = 0.155$ g/ml), methanol–acetonitrile–triethyl amine–acetic acid (54.5:45.1:0.2:0.2, v/v/v/v). The meaning of the symbols is explained in the text.

(1)), but the bi-Langmuir isotherm (Eq. (2)) provides better description for more strongly retained D-isomer.

Both the loading factor and the production rate in preparative chromatography increase with increasing selectivity (separation factor, α) and feed concentration, c_i (Eqs. (3A,B) and (4)). Because the solubility decreases but α increases with increasing concentration of methanol or ethanol in aqueous–organic mobile phases (Table 1), the optimum composition of the mobile phase providing maximum production rate in preparative separation of the enantiomers of amino acids can be selected as a compromise between the separation selectivity and the solubility.

To estimate the optimum separation conditions, we used the separation factors, α, determined in various mobile phases to calculate optimum loading factors, $L_{f,opt}$, for ''touching band'' separations from Eq. (3B). These values were introduced into Eq. (3A) to calculate optimum feed volumes, V_i, for maximum feed concentrations, $c_{i,max}$, allowed by the solubilities of the racemates in each mobile phase, assuming a constant value of the column saturation capacity, q_s, approximately equal to the nonspecific saturation capacity for the D-enantiomers, determined

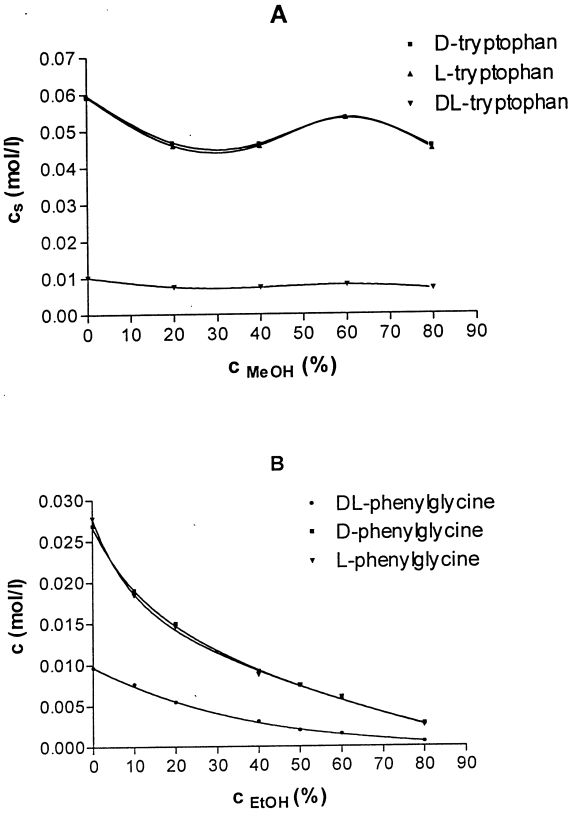

Fig. 2. Solubility c_S (mol/l) of phenyl glycine in aqueous methanol (B) and of tryptophan in aqueous ethanol (A) at 20°C.

from the Langmuir isotherms in Table 2. For practical isocratic ''touching band'' preparative separations, the optimum feed volumes should not exceed significantly 2σ of the band dispersion to avoid unfavourable effects on the separation [13], which corresponds to 250 μl for the column used (approx. 1000 theoretical plates for the more strongly retained D-enantiomers). The calculated optimum V_i values lower than 2σ at low loading factors of tryptophan ($<0.8\%$) are limited by too low a separation selectivity. If the feed volumes higher than 2σ are used to compensate for limited solubilities, additional band broadening and a loss of separation may occur. The results are given in Table 1.

The production rates per the cross-section area at a constant column length of 15 cm, *Pr* (in mg cm^{-2} min^{-1}), were calculated using Eq. (4) assuming the recovery yield $Y_i = 99\%$ using optimized feed volumes, $V_{i,opt}$ (or V_i corresponding to $2\sigma = 250$ μl if the calculated optimum $V_{i,opt}$ were higher than 2σ, marked with asterisks in Table 1) and the cycle time equal to the retention time of the more strongly retained D-enantiomer, $t_{R,D}$ plus the time of the injection pulse. The mobile phases providing the highest production rates for ''touching band'' chiral separation of the enantiomers of phenylglycine and tryptophan are pure water and 60% methanol or 50–60% ethanol, respectively. These calculations do not take full account of possible competitive effects on the equilibrium distribution of the enantiomers, but this simplification probably does not cause gross errors, as imperfect solubility of the amino acids limits their useful concentrations to the range with only minor deviations from the linear isotherm profile.

The analytical and the ''touching band'' preparative separations of the enantiomers of phenyl glycine in water on a Chirobiotic T column are compared in Fig. 3A,B. The actual loading factor for phenyl glycine, 2.3%, was approximately one-half of the optimum value, but this was the maximum allowed by limited solubility at the feed volume corresponding to the band broadening of 2σ. Under these conditions, ''touching band'' separation was achieved, as expected.

The chiral separation of tryptophan in 60% ethanol at the loading factor of 2.76% (approximately 10% higher than the optimum calculated value for the feed volume corresponding to the band dispersion of 2σ) was only slightly worse than the ''touching band'' separation (not shown). The production rates for both phenylglycine and tryptophan can be further increased using a higher loading factor and product fraction cutting.

The peak asymmetry of the more strongly retained D-phenyl glycine under analytical conditions apparent in Fig. 3A does not improve if the injected sample mass is decreased, so that it probably cannot be attributed to the column overload. The analysis of the band profiles based on the stochastic theory of chromatography reveals that there are at least two additional adsorption sites for the D-enantiomers with respect to the L-enantiomers. This can be probably explained by complex structure of Teicoplanin resulting in more adsorption centres interacting with the individual enantiomers than assumed by the three-point chiral recognition model. Hence, the

Table 2
Coefficients of Langmuir and bi-Langmuir isotherms of enantiomers of phenyl glycine, tryptophan and mandelic acid on a Chirobiotic T column at 35°C[a]

Mobile phase, isotherm, enantiomer	a_1	a_2	b_1 (l/mol)	b_2 (l/mol)	q_{NS} (mol/l)	q_{CH} (mol/l)	C. D.
Phenylglycine							
Water, Langmuir, L-	1.155	–	10.476	–	0.11		0.9998
D-	1.944	–	16.148	–	0.12		0.9993
Water, bi-Langmuir, D-	1.155	0.82	10.476	29.5	0.11	0.028	0.9993
Tryptophan							
Ethanol–water (6:4), Lang., L-	1.486	–	4.391	–	0.338		0.9997
D-	1.720	–	5.544	–	0.310		0.8695
Ethanol–water (6:4), bi-Lang., D-	1.486	1.02	4.391	84.8	0.338	0.012	0.9996
Mandelic acid							
Ethanol–water (96:4), Lang., L-	0.186	–	8.62	–	0.022		0.9992
D−	0.807	–	96.4	–	0.008		0.9998
Ethanol–water (86:14), Lang., L-	0.182	–	12.7	–	0.014		0.9973
D-	0.332	–	37.5	–	0.009		0.9999
Buffer 1, Langmuir, L-	1.15	–	0.67	–	1.72		0.9999
D-	1.61	–	3.15	–	0.51		0.9995
Buffer 1, bi-Langmuir, D-	1.15	0.45	0.67	2.51	1.72	0.18	0.9995
Buffer 2, Langmuir, L-	1.01	–	0.91	–	1.10		1.0000
D-	1.156	–	0.97	–	1.19		0.9996
Buffer 2, bi-Langmuir, D-	1.01	0.39	0.91	8.15	1.10	0.048	0.9992
Buffer 3, Langmuir, L-	1.079	–	0.594	–	1.82		0.9998
D-	1.328	–	1.303	–	1.02		0.9999
Buffer 3, bi-Langmuir, D-	1.079	0.37	0.594	16.67	1.82	0.022	0.9993

[a] Langmuir isotherm, Eq. (1); bi-Langmuir isotherm, Eq. (2); non-specific saturation capacity, $q_{NS} = a_1/b_1$; chiral saturation capacity, $q_{CH} = a_2/b_2$. Buffers 1–3 as in Table 1. C.D., coefficient of determination.

description of the distribution of the enantiomers on the basis of the bi-Langmuir model is probably too oversimplified, in spite of the formally good fit of the experimental distribution data to Eq. (2) [17].

3.3. Retention behaviour, adsorption isotherms and optimization of overloaded separation conditions for mandelic acid

The retention of the L-isomer of mandelic acid is not significantly affected by pH, but the retention of the D-isomer and the selectivity of the chiral separation of L- and D-mandelic acid increase with increasing pH up to pH 6.5, which limits the stability of the Chirobiotic T column (Fig. 4). The concentration of methanol or ethanol does not affect the retention of the two enantiomers of mandelic acid very significantly in the mobile phases containing up to 60–70% methanol or ethanol in water, but the retention of the D-enantiomer and the enantioselectivity increase in mobile phases containing 80–100% organic solvent (Table 1).

The isotherms of the enantiomers of mandelic acid are more complex than the isotherms of the amino acids, even though the experimental distribution data can be formally described by the Langmuir isotherm (Table 2). In non-buffered mobile phases, the L-enantiomer elutes earlier than the D-enantiomer at low mandelic acid concentrations, but the order of elution is reversed at the concentrations of mandelic acid higher than 0.05 mol/l (Fig. 5B). This behaviour cannot be adequately described by the bi-Langmuir model, which assumes that the enantiomer showing specific (chiral) interactions with the chiral

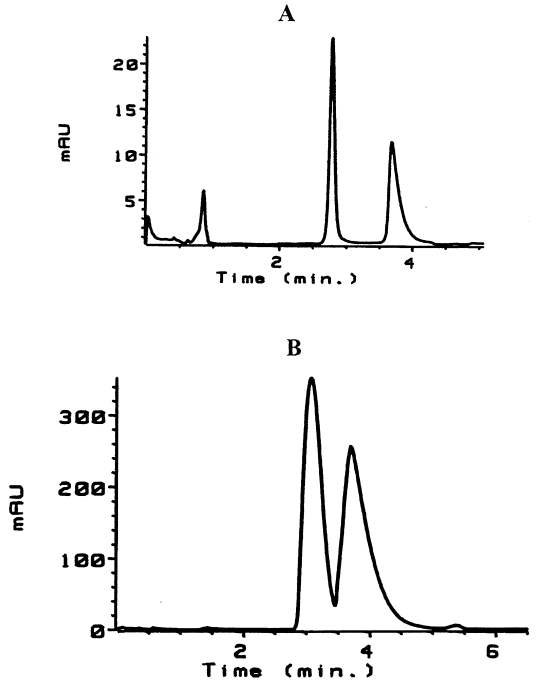

Fig. 3. Separation of (A) 5 μl of 0.009 mol/l and (B) 250 μl of 0.009 mol/l racemate of phenyl glycine on a Chirobiotic T, 5 μm, 150×4.6 mm column. Mobile phase: water, 35°C, 1 ml/min, detection UV, 254 nm. The L-enantiomer elutes before the D-enantiomer.

stationary phase is retained more strongly over the whole concentration range than the enantiomer with only non-specific retention interactions [12].

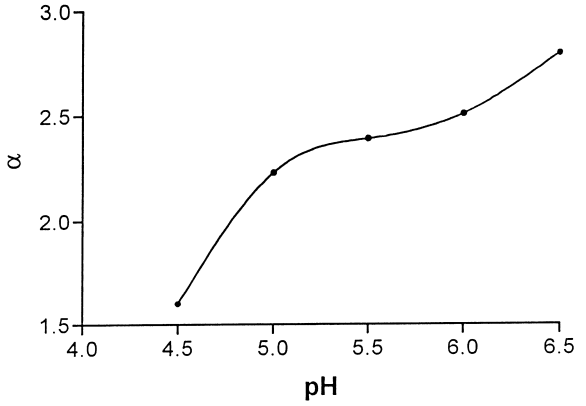

Fig. 4. Effect of the mobile phase pH on the separation factors, α, of the enantiomers of mandelic acid on a Chirobiotic T column in mobile phases ethanol–water–triethylamine 17.5:70.2:12.3 (v/v/v) with pH adjusted by the addition of acetic acid, at 35°C.

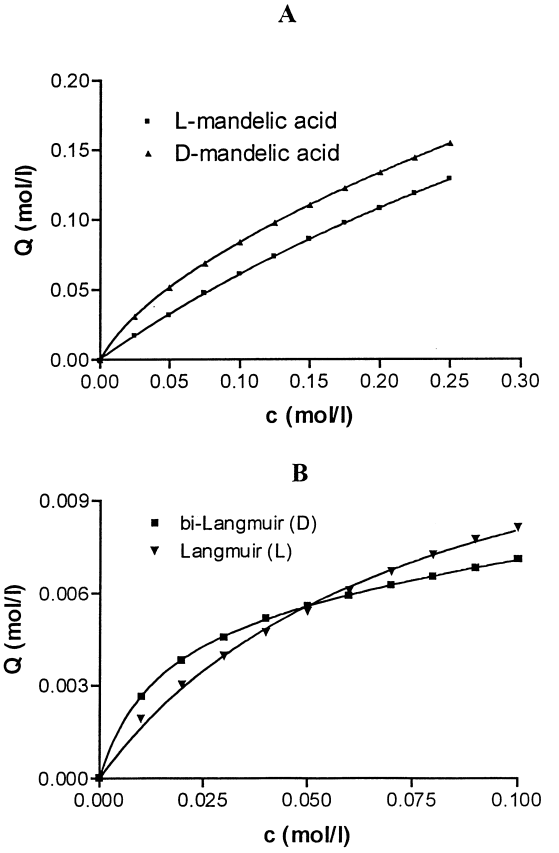

Fig. 5. Adsorption isotherms of the enantiomers of mandelic acid on a Chirobiotic T column at 35°C in (A) ethanol–water–triethylamine–acetic acid (17.5:70.3:8.7:3.5, v/v), pH 6.5, and (B) in ethanol–water (86:14, v/v). *c*, concentration in the mobile phase; *Q*, concentration in the stationary phase, both in mol/l. Points, experimental data; lines Langmuir model (L-enantiomer), bi-Langmuir model (D-enantiomer).

To avoid detrimental effect of changing acid–base equilibrium on the separation of high feed concentrations of mandelic acid, it is necessary to add a buffer to the feed and to the mobile phase. We used triethyl ammonium acetate (TEAA) buffer for this purpose, because it is volatile and can be removed from the product and because it was earlier recommended for the separations on macrocyclic chiral stationary phases [2]. We found that 5% or higher buffer concentrations are necessary to keep the pH and the dissociation degree of mandelic acid stable up to 0.25 mol/l in aqueous–organic solvents. Under these conditions, regular distribution between the

Chirobiotic T chiral stationary phase and the buffered mobile phase is observed (Fig. 5A) and can be described either by Langmuir or by bi-Langmuir isotherms with coefficients having real physical meaning (Table 2).

This behaviour reflects the complexity of the interactions of enantiomers with multiple adsorption centers in Teicoplanin. Only 88% mandelic acid is ionized at pH 4.5, but the ionization is almost complete (99.8%) at pH 6.5. The separation factor, α, for the two enantiomers improves at a higher pH (Fig. 4), which can be attributed to increasing effect of specific chiral interactions for the anionic form of more strongly retained D-mandelic acid with Teicoplanin. The retention of L-mandelic acid is almost independent of pH, probably because at a pH higher than 4.5 only a small non-ionized fraction of the acid shows stronger interactions with the Teicoplanin column (Fig. 4).

The experimental distribution data (Fig. 5A,B) suggest significantly higher column saturation capacities for both the D- and the L-enantiomers in buffered than in non-buffered solutions (Table 2). The pH of non-buffered solutions of mandelic acid can be considerably low (e.g., pH 2.2 in 0.25 mol/l solutions, approx. 98% non-ionized form). This indicates that the adsorption on the ionic adsorption centres of the Teicoplanin chiral stationary phase is probably more important than the adsorption on the non-ionic centres both for chiral and achiral contributions to the retention of the two enantiomers of mandelic acid. Fig. 5B indicates that the non-ionic interactions and the non-ionic contribution to the retention on Teicoplanin may be more significant for the L- than for the D-enantiomer.

The solubility of mandelic acid is high enough (more than 40 g/l) in all aqueous–organic solvents tested and does not limit the loading factor, which increases with increasing pH. On the other hand, the solubility of triethyl ammonium acetate (and hence the buffer capacity) is limited in mobile phases with high concentrations (90–100%) of methanol or ethanol, which provide best selectivity for the separation of the enantiomers of mandelic acid (Table 1). The pH controls the ionization of mandelic acid and the concentration of the TEAA buffer controls the buffering capacity and consequently the maximum loading factor available for preparative separations of the enantiomers. As the mandelic acid dissolved in the mobile phase itself decreases the pH of the solution and its dissociation degree, it is essential for a successful separation to adjust the pH of the racemate feed to the same value as that of the mobile phase before the application onto the column.

The optimum composition of the mobile phase for overloaded chiral separation of mandelic acid on a Chirobiotic T column has to be selected as a compromise between the separation selectivity and the buffering capacity controlling the feed loading factor. The limiting feed concentration of mandelic acid in non-buffered mobile phases should not exceed 1.5 g/l to avoid poor separation because of poor acid dissociation, which limits the maximum feed volume for ''touching band''separation and the production rate (Table 1). In 20% methanolic TEAA (buffer 1) at pH 4.0 the feed concentration can be increased 10 times, leading to the production rate increased eighteen times with respect to 96% ethanol providing greatest separation factor under analytical conditions (Table 1). Analytical and overloaded ''touching band'' separations of enantiomers in racemate samples of mandelic acid on a Chirobiotic T column in buffer 1 are similar to the separations of phenyl glycine in Fig. 3. Using mobile phase with 1:1 ethanol buffer at pH 6.6 (buffer 2), increased buffering capacity allowed to rise the feed concentration of mandelic acid to 38 g/l which, together with improved separation factor, resulted in a 5-times increase in the production rate with respect to buffer 1 (Table 1). At this pH, 99.8% of mandelic acid is in the anionic form.

In addition to the separation in the reversed-phase mode with buffered aqueous–organic mobile phases, we investigated possibilities for overloaded mandelic acid chiral separation in so-called ''polar/organic mode'' with a non-aqueous buffer 3 comprised of methanol, acetonitrile, triethyl amine and acetic acid (54.5:45.1:0.2:0.2, v/v/v/v) [2]. Here, the separation selectivity, the optimum loading factor and the production rate were higher than with buffer 1, but lower than in buffer 2. These separation conditions, in spite of the production rate approximately half of that in buffer 2, have the advantage of a significantly lower buffer concentration, which is sufficient to maintain regular distribution behaviour of mandelic acid.

4. Conclusions

The Chirobiotic T chiral stationary phase has better separation selectivity and sorption capacity than other chiral stationary phases for enantiomeric separations of some carboxylic acids and amino acids. These properties can be used for preparative chiral separations. The conditions for the preparative separation should be optimized to provide a compromise between the separation selectivity and the solubility of the acids in the mobile phase. For the separation of the enantiomers of mandelic acid, the mobile phase should be buffered to enhance the ionization, which provides better separation selectivity and column saturation capacity. The effects of the buffer solubility and buffering capacity in aqueous–organic solvents should be taken into account when optimizing the separation conditions. The ''polar/organic'' separation mode with non-aqueous buffers allows separations in mobile phases with lower buffer concentrations, but at a lower production rate.

Although the separation of the enantiomers of the mandelic acid and of the amino acids probably could be accomplished at a lower cost using classical (e.g., crystallization) techniques, we believe that the approach for the optimization of the mobile phase taking into account limited solubilities described in this work can be useful for HPLC chiral separations of ionizable compounds, not only on glycopeptide stationary phases.

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