giving net

$$O_2^- + L + 2H^+ \rightarrow H_2O + L' \quad (21)$$

This last mechanism is completely different from that suggested by reactions 1 and 2, 6 and 7, or 17 and 18, for which the net reaction is the dismutation reaction 22. The sequence of reactions

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (22)$$

17, 19, and 20 describes the catalysis of the oxidation of the ligand by O_2^- by the various Cu(II) complexes. If this mechanism occurs in vivo, and Cu(II) is bound to a biological target, then degradation of the target may take place. In such a case the Cu(II) will enhance the damage caused by O_2^-, rather than protect against the toxicity of this radical in biological systems.

Conclusions

The observations in the present study suggest that O_2^- oxidizes some Cu(II)-peptides, in which Cu(II) is bound to the peptide nitrogen atoms. This is a probable alternative mechanism for the catalysis of O_2^- destruction by copper in biological systems. In many cases the catalysis proceeds via reactions 1 and 2, especially in the case of the native enzyme as well as in the case of Cu(II)-phenanthroline and Cu(II)-bipyridine, where the formation of Cu(I) as an intermediate has been demonstrated.[16] In the latter complexes the ligands are good π acceptors, and therefore, they stabilize the low-valent oxidation state of the transition-metal complexes.

However, it should be noted that in many biological systems Cu(II) is complexed to the peptide nitrogen atoms, and in these systems O_2^- can oxidize the metal ion as indicated in this study. A similar mechanism is expected for Mn(II) and Fe(III) complexes. Thus, if O_2^- is able to oxidize transition-metal complexes, it is expected that at least in some of the systems biological damage will occur via reactions 17 and 19 or via direct oxidation of biological targets by Cu(III) complexes in addition to the Fenton reaction.

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Chromatographic Band Profiles and Band Separation of Enantiomers at High Concentration

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Abstract: Experimental band profiles for large sample sizes of both single compounds and various binary mixtures of the optical antipodes of *N*-benzoylalanine are obtained on immobilized bovine serum albumin (BSA). The two enantiomers are resolved on this stationary phase. In the case of mixtures, the individual elution profiles are acquired by continuous fraction collection, followed by chromatographic analysis of these fractions. The equilibrium isotherms of each of the two chiral isomers between the two phases of the chromatographic system are determined by frontal analysis on the same column. These isotherms can be accounted for with great precision by a model assuming the existence of two kinds of sites on the stationary phase. For each isomer, the adsorption on each type of site is well described by a Langmuir equation. The first type of site is selective and interacts more fervently with the D isomer. The second type of site is not chiral selective, and the corresponding Langmuir isotherms are identical for the two isomers. The experimental profiles are nearly identical with the profiles calculated by the semi-ideal model of nonlinear chromatography, using the competitive Langmuir isotherms derived from the measured single-component isotherms.

Introduction

Chromatography has become the most general and versatile method for the separation of enantiomers.[1,2] Most work in this area has dealt with analytical separations that require the nearly complete resolution between the bands of the analyte components and their proper detection but not the collection of any purified material. There is a rapidly growing interest in the scaling up of the chromatographic procedures in order to prepare and recover significant amounts of very pure chiral isomers for various applications in pure chemistry or in the pharmaceutical industry.[3] In such preparations, the injection of large-size samples becomes necessary. This permits the collection of more concentrated fractions from which the recovery of the purified isomers is easier. Under such experimental conditions, however, the band profiles become broader and unsymmetrical, the bands overlap, and the separation seems to degrade.

These phenomena are due to the nonlinear behavior of the phase equilibrium isotherms at high concentrations and to the competition between the molecules of the different mixture components for interaction with the stationary phase. In previous papers, a theoretical investigation of the band profiles of pure compounds[4] and of the components of binary mixtures[5,6] has been discussed. This work permits a description of the progressive separation of the sample.[7] The two primary phenomena observed for a binary mixture at high concentrations are the displacement and the

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tag-along effects.[5,7,8] Both result from the competition between the molecules of the two components for access to the retention mechanism (e.g., adsorption sites, ions or ligands bonded to the surface of the stationary phase).

The displacement effect occurs when the second component is present in larger quantity and/or when it saturates the column more easily than the first one. Then, the second-component molecules crowd out those of the first component. The band of the first component is eluted earlier and is narrower than the band of the same amount of that compound injected pure. It has a characteristic L-shape profile. Under opposite experimental conditions, when the first component is present in larger amount and/or saturates the column more easily than the second one, the tag-along effect takes place. The second component is carried downstream with the first one and spread out over a wide elution range.[8]

The validity of these theoretical results has been confirmed by a number of experimental results regarding pure compounds[9,10] as well as binary mixtures.[11-14] These results have shown the importance of the dependence of the individual band profiles on the isotherm equation used and on the value of the parameters. For several reasons, it was interesting to apply these results to the investigation of the separation of enantiomers. Besides the practical advantage of being able to optimize the experimental conditions of such separations, there are several theoretical reasons.

First, it is possible to separate the contributions of the nonselective molecular interactions from those that contribute to the chiral recognition. The former (e.g., the activities in the mobile phase, the solute interactions with the nonselective sites in the column, etc.) should be identical for both isomers. It is rare in solution thermodynamics that one single contribution can easily be identified. Second, we have repeatedly observed that the classical Langmuir isotherm accounts imperfectly for the adsorption isotherm of a pure compound on a conventional stationary phase (silica or chemically bonded C18).[15] Better results are obtained with bi-Langmuir isotherms, assuming the existence of two noncooperative sites on the adsorbent surface.[16,17] The extension of these results to binary mixtures raises some difficult questions that would be greatly simplified with enantiomeric mixtures because the contributions of nonselective interactions to the isotherm could be easily separated from the contribution of the chiral selective interactions.

Several general enantioselective phase systems have been developed. Chemically bonded chiral groups,[2] cyclodextrins,[1] and immobilized proteins bound to the surface of porous silica particles are the three main approaches. One of the phases of the last group, using bovine serum albumin (BSA),[18] was available and easy to use. It provided an attractive test to the validity of these speculations. This phase has been developed by Allenmark et al.[19-23] Under analytical conditions it separates the *N*-benzoyl derivatives of the enantiomers of a number of amino acids, including those of D- and L-alanine.[19] The effects of the concentration of 1-propanol on the retention behavior and resolution of these compounds have been studied.[20] BSA is a globular protein consisting of 581 amino acids with 17 intrachain disulfide bridges.[24] It includes two cavities where the selectivity and the binding energy are quite different. The presence of multiple binding sites with varying mechanisms for the formation of diastereoisomer complexes has been demonstrated.[25]

The purpose of this paper is to demonstrate the accuracy with which the theory of nonlinear chromatography permits the calculation of individual band profiles and a two-site adsorption model can account for competitive adsorption.

Theory

There are two theoretical problems to solve here. First, we have to derive the relationship between the individual elution profiles of the two isomers in overloaded chromatography and their equilibrium isotherms in the phase system used. Second, we need a proper equation for the representation of the phase equilibrium isotherm data.

I. Elution Profiles in Nonlinear Chromatography. The elution profile of a compound on a chromatographic column can be obtained as the solution of the mass balance for this compound with a proper set of initial and boundary conditions.[4-6] The differential mass balance equation for one compound is written

$$\frac{\partial C}{\partial t} + F\frac{\partial Q}{\partial t} + \frac{\partial (uC)}{\partial z} = D\frac{\partial^2 C}{\partial z^2} \tag{1}$$

where C and Q are the concentrations of the compound studied in the mobile and stationary phase, respectively, at time t and column length z, F is the phase ratio of the column packing, with $F = (1 - \epsilon)/\epsilon$, where ϵ is the column packing porosity, u is the mobile-phase velocity, and D is the coefficient of axial dispersion.

Further discussion of this equation requires a relationship between the two functions C and Q. Normally, a kinetic equation relating the rate of variation of the concentration in the stationary phase, $\partial Q/\partial t$, to the concentrations in the mobile and stationary phases would be needed. This equation accounts for the kinetics of mass transfers between the two phases of a chromatographic system. We know that these transfers are fast in liquid chromatography.

Giddings[26] and Haarhof and Van der Linde[27] have shown that when the mass-transfer kinetics in the column is fast, but not infinitely fast, eq 1 and the kinetic equation can be replaced by

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a\frac{\partial^2 C}{\partial z^2} \tag{2}$$

where q is the equilibrium concentration of the compound in the stationary phase when the mobile phase concentration is C. Then q is given by the equilibrium isotherm

$$q = f(C) \tag{3}$$

D_a is the apparent diffusion coefficient, related to the column HETP (height equivalent to a theoretical plate) by the equation

$$D_a = Hu/2 \tag{4}$$

H is the column HETP for the compound considered.

The set of eqs 2–4 constitutes the semi-ideal model of chromatography. In the case when $D_a = 0$, hence, $H = 0$ (eq 4), we have the ideal model.[28-32] The corresponding equation can be solved in closed form for one compound and any isotherm.[15] A system of two such equations for the two components of a binary

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Table I. Adsorption Isotherm Equations[a]

Simple Langmuir Isotherm for a Single Component

$$q_i = \frac{a_i C}{1 + b_i C} \tag{a}$$

Two-Site Langmuir Isotherm for a Single Component

$$q_i = \frac{a_{i,1} C_i}{1 + b_{i,1} C_i} + \frac{a_{i,2} C_i}{1 + b_{i,2} C_i} \tag{b}$$

Binary Competitive Langmuir Isotherm

$$q_i = \frac{a_i C_i}{1 + b_1 C_1 + b_2 C_2} \tag{c}$$

Two-Site Binary Competitive Langmuir Isotherm Model

$$q_i = \frac{a_{i,1} C_i}{1 + b_{1,1} C_1 + b_{2,1} C_2} + \frac{a_{i,2} C_i}{1 + b_{1,2} C_1 + b_{2,2} C_2} \tag{d}$$

[a] In these equations the coefficients a and b are the isotherm parameters; the first subscript (i) indicates the component considered (i = 1 or 2). Component 1 is eluted first; the second subscript (1 or 2) indicates the type of adsorption sites considered; and by convention, site 1 is the selective site, site 2 is nonselective.

mixture can be solved in closed form too, but only for competitive Langmuir isotherms.[7]

Numerical solutions of eq 2 (or of a set of several such equations for a multicomponent problem) can be calculated by computer integration.[4,5] We have used a finite difference method.[4,33] In these methods, each term in the left-hand side of eq 2 is replaced by a finite difference. It can be shown that, if we neglect the higher order error terms, the left-hand side of eq 2 becomes equal to

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = \frac{\Delta z u}{2}(1 - a_c)\frac{\partial^2 C}{\partial z^2} \tag{5}$$

where Δt and Δz are the time and space increments in the numerical integration, respectively, and a_c is the Courant Friedrichs Lewy (CFL) number of the numerical solution,[34,35] $a_c = u\Delta t/(1 + k'_0)\Delta z$. In the method we have used here, a_c must be between 0 and 1 to assure convergence of the calculations.

If we equate the right-hand side of eqs 2 and 5, we see that the numerical integration gives us the solution of eq 2 that is the actual elution profile for a real column provided the integration increments are properly chosen. We have selected a space increment equal to $2H$ and a time increment corresponding to a CFL number equal to $^1/_2$, that is

$$\Delta t = H(1 + k')/u \tag{6}$$

There are other numerical procedures than the one discussed here that permit the calculation of solutions of eq 5.[36,37] They lead to essentially the same results. A detailed investigation of the numerical analysis shows that the best results are given by a calculation procedure that duplicates the behavior of the Craig machine.[37]

II. Representation of Isotherm Data. In most cases, the Langmuir isotherm equation (Table I, eq a) accounts well for the adsorption data measured for a pure compound in liquid chromatography.[9,10] The agreement is poor in some cases, however; and better results can be obtained with an isotherm model assuming that there are two types of noncooperative independent adsorption sites (Table I, eq b).[15-17] Experimental data regarding small polar organic molecules on alkyl-bonded silica[15] or proteins on ion exchangers[38] are well accounted for by such a model. In the former case, one of the two sites is certainly made of the alkyl chains chemically bonded to the surface of porous silica; the other one is probably the bare silica surface and, more specifically, the unreacted silanol groups.

With a Langmuir isotherm, a monolayer coverage is observed at high solute concentrations in the mobile phase and the stationary-phase concentration tends toward a saturation value given by

$$q_{s,i,j} = a_{i,j}/b_{i,j} \tag{7}$$

The product of this concentration by the amount of stationary phase in the column is the column saturation capacity. It is convenient to report the sample size to the column saturation capacity. This ratio is the loading factor. It indicates the degree of column overloading experienced. Usually, linear chromatography behavior is observed for loading factors below 0.05%, moderate overloading below a few percent, and severe overloading above 5–10%. When a bi-Langmuir isotherm is used, there is a column saturation capacity for each type of site. Although the physical meaning of this loading factor becomes questionable, it is still convenient to report the sample size as the fraction of the total column saturation capacity.

For binary mixtures, the competition for access to the adsorbent is accounted for by using the competitive Langmuir isotherms (Table I, eq c). In the case of a surface with two types of noncooperative independent adsorption sites, the isotherm is the sum of a binary Langmuir isotherm for each site (Table I, eq d). Although straightforward, this extension does not seem to have been considered previously.

The considerable practical advantage of eq a or b is that the numerical values of the coefficients $a_{i,j}$ and $b_{i,j}$ are the same in eqs a and c or b and d, respectively. Accordingly, the experimental determination of the coefficients of a set of binary isotherms is no more difficult than the measurement of the pure component isotherms, easily carried out by frontal analysis.

Experimental results show that the individual elution profiles of the components of a binary mixture exhibit significant differences from the profiles predicted by the theory of nonlinear chromatography on the basis of binary Langmuir adsorption isotherms.[14] The competitive Langmuir isotherm model is expected to fail because several basic assumptions made in its derivation are not satisfied in most practical cases. The model assumes that the two components have the same column saturation capacity and that the mobile- and the stationary-phase solutions are ideal. Some of these assumptions are expected to hold much better in the case of enantiomeric mixtures than for any other case. The two chiral isomers have exactly the same thermodynamical properties as long as the chiral recognition mechanism is not involved. The activity coefficients of the two enantiomers in the mobile phase, the column saturation capacity for the nonspecific sites, and all the contributions to the free energy associated with the retention mechanism that do not involve chiral recognition are the same for both enantiomers. Since the competitive Langmuir isotherm model gives fairly good results with homologous compounds,[14] it should be expected to account very well for the properties of enantiomers.

III. Calculations. The single-component adsorption data were fit to the Langmuir (Table I, eq a) and the bi-Langmuir equations (Table I, eq b) by using a minimization program developed in the laboratory. Linearization of the Langmuir equation and least-squares fit of the data to derive the parameters a and b is trivial. One practical difficulty in applying a direct least-squares fit method to determining the bi-Langmuir isotherm parameters is that there are four parameters. The determinations of these

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parameters are strongly correlated for real data. Perturbations in one parameter may be compensated for almost entirely by one or more of the remaining three parameters. On the other hand, success in simulating individual band profiles through numerical calculation of the solution of eq 1 requires an accurate estimate of the parameters of the isotherm equation used. Thus, effective use of the bi-Langmuir isotherm requires a carefully planned approach.

The two-site isotherm equation (Table I, eqs b and d) was used because of the failure of a single-site model to describe adequately the curvature of the isotherm. Previous experience showed that the bi-Langmuir isotherm gave excellent results in a number of experimental cases[15,38] and suggested a practical strategy to achieve accurate representation of adsorption data in a wide range of concentrations. As we see later, adsorption takes place preferentially on the first type of sites (chiral selective) at low concentrations. In part because of the low surface coverage of BSA on the silica, however, these sites are easily saturated at high concentrations. We carried out measurements of single-component adsorption at concentrations 3 times as high as the concentration of each component in a binary mixture for which there is no visible separation (i.e., no valley) between the two bands. Under these conditions, adsorption on the second type of site (nonselective for chirality) predominates.

The coefficients of the isotherms of each isomer are determined by first applying a best fit scheme using a Simplex algorithm. This calculation allows a good fit for the isotherm contribution corresponding to the nonselective sites since the weight of the data points corresponding to the high concentrations at which the sites of this first type are saturated contributes most significantly. This procedure permits an accurate determination of the coefficients for the second term (Table I, eq b). The corresponding contribution is then calculated and subtracted from each data point, leaving the contribution of the first site. The corresponding points are then fit on the Langmuir isotherm, which provides a much more accurate estimate of the second set of coefficients than the Simplex algorithm.

The individual band profiles computations were carried out with the bi-Langmuir isotherm model and the parameters just determined. Several different implementations of the finite difference method can be used to calculate these profiles.[36] The results obtained differ slightly with the method used because of small numerical errors introduced in calculations that require more than 10 million loops. Excellent agreement between experimental results and the elution profiles predicted by one of these programs has been demonstrated previously.[4,9,10,13,14] The band profiles reported here have been obtained with an algorithm similar to the Craig model although more general and flexible in its principle.[36] A detailed discussion of the properties of these different algorithms and of the control of the amount of numerical diffusion they introduce will be published elsewhere.[37]

Experimental Section

I. Equipment. All the chromatographic experiments (frontal analysis, elution of high concentration bands, fraction collection, and analysis of collected fractions) were performed on an HP 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a diode-array UV detector, an automatic sampling system, a computer data acquisition system, and a multisolvent delivery system. Also, a Gilson 203 fraction collector (Middleton, WI) was used to complement the HP system.

II. **Chemicals. Column.** A Resolvosil-BSA-7 (Alltech, Deerfield, IL) column was used for the entire work (frontal analysis, profiles of high concentration bands, fraction analysis).

Materials. *N*-Benzoyl-(DL-, L-, and D-)alanine (Sigma, St. Louis, MO) and 1-propanol were used as received, without further purification.

Mobile Phase. Throughout all chromatographic runs, the mobile phase was a 10 mM phosphate buffer aqueous solution, with a constant concentration of 3% 1-propanol (v/v) and a pH between 6.65 and 6.70. The flow rate of the mobile phase was 1 mL/min. For the UV detector wavelengths of 240 and 254 nm were employed, depending on the concentration range under consideration.

III. **Procedures.** The elution profiles for the pure enantiomers were obtained by direct calibration of the detector. In the concentration range investigated, the calibration curve of the detector (plot of absorbance versus concentration) was linear. The response factors of the two isomers are identical, making the transformation of the detector signal (profile of the eluent optical density versus time) into a real chromatogram (profile of the eluate concentration versus time) a simple task.

In the case of the binary mixtures, fractions were collected at 0.1-min intervals during the elution of high-concentration binary bands. Aliquots of these fractions were subsequently injected on the same column, under analytical conditions, in order to determine the concentration of each component at specified times of the band profiles. A properly selective detector (e.g., recording polarimeter) being unavailable to us, this is the only practical method permitting the determination of the individual elution profiles of the two enantiomers in the mixed zone. The nonselective UV detector gives the total concentration profile of the two isomers and permits a test of the accuracy of the individual profiles derived from the analysis of the collected fractions (Figure 1). There is an excellent agreement between the two total chromatograms on Figure 1, demonstrating the validity of the experimental procedure. The lack of any additional band broadening due to the fraction collector results from the large number of fractions collected (standard deviation of an analytical band of L isomer, 71 s; number of fractions collected per standard deviation, 12).

The adsorption isotherm coefficients of the two isomers were obtained by frontal analysis. The classical procedure was followed.[9,10,39]

Also, to perform the simulations, additional parameters were required. The dead time of the column was 92.4 s, the column length was 15 cm, and the HETP was 0.0165 cm for the L isomer and 0.0212 cm for the more retained D isomer. The HETP was averaged for the simulation of the binary mixtures.

Table II. Isotherm Coefficients[a]

		N-benzoyl-L-alanine	*N*-benzoyl-D-alanine
site 1	a	14.16	35.09
	b	7570	17236
site 2	a	4.41	4.25
	b	222	214

[a] *a* is dimensionless; *b* has units of liters per mole.

Table III. Column Saturation Capacity, q_s[a]

	N-benzoyl-L-alanine	*N*-benzoyl-D-alanine
site 1	0.001875	0.002036
site 2	0.01995	0.01986
total	0.02183	0.02190

[a] q_s has units of moles per liter.

Table IV. Loading Factors[a]

	N-benzoyl-L-alanine	*N*-benzoyl-S-alanine
1/1	11.93	11.89
1/3	5.84	17.54
3/1	16.57	5.71

[a] Sample amount in percent of total column saturation capacity.

Results and Discussion

We have studied successively the adsorption of the single components on the immobilized BSA stationary phase, the elution profiles of high-concentration bands of the pure enantiomers, and the individual elution profiles of binary mixtures of various compositions.

I. Single-Component Adsorption Data. Single-component isotherms were measured by frontal analysis.[9,39] Attempts at fitting the adsorption data to a Langmuir isotherm failed. The same data were then fitted successfully on the bi-Langmuir isotherm (see Figure 2). The values of the coefficients of the isotherms giving the best fit of the data are reported in Table II. The values of the column saturation capacities are given in Table III. A bi-Langmuir model is reasonable in the case of the competitive adsorption of two enantiomers, since it is easy to explain the coexistence of two noncooperative, independent types of adsorption sites on the surface of immobilized BSA.

Most intermolecular interactions are nonchiral specific and result in identical contributions to the retention of the two isomers. All the solute–solvent interactions, the adsorption on the bare silica and on most of the side groups on the protein surface, and all the

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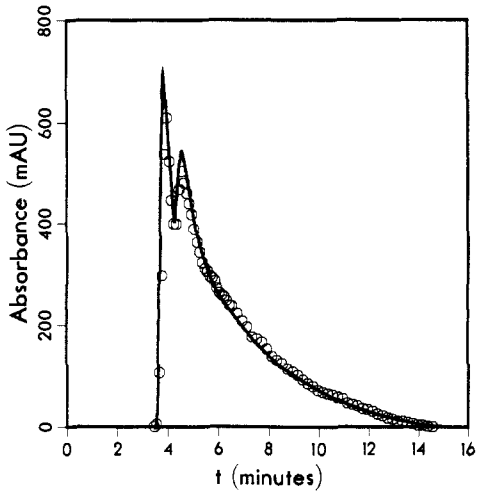

Figure 1. Total chromatogram derived from the analysis of collected fractions compared to the chromatogram recorded by the detector. Experimental conditions: column length, 15-cm i.d. 4 mm; stationary phase, immobilized BSA on silica; mobile phase, 3% 1-propanol in a 10 mM phosphate buffer (pH 6.7); mobile-phase flow rate, 1 mL/min; mixture composition, 1/3.

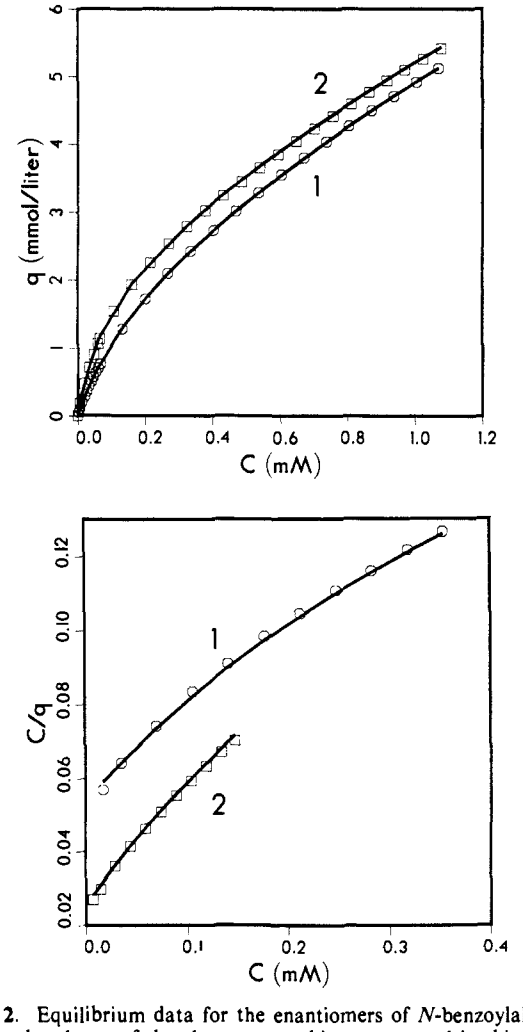

Figure 2. Equilibrium data for the enantiomers of *N*-benzoylalanine between the phases of the chromatographic system used in this work. Experimental data (markers) and best bi-Langmuir isotherm (solid lines). See eq d, Table I, and coefficients in Table II. Same experimental conditions as for Figure 1. Isotherm representation: top, q vs C (high concentration); bottom, C/q vs C (low concentration).

interactions involving only the bulky benzoyl group on the solute molecule are nonchiral specific. Of the two terms in the bi-Langmuir isotherms, one should account for these nonspecific

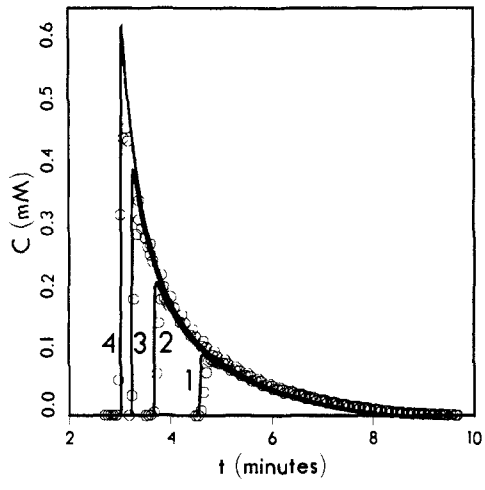

Figure 3. Comparison between experimental chromatograms (markers) and calculated band profiles (solid lines) for *N*-benzoyl-L-alanine at increasing sample sizes. Sample size: (1) 0.145, (2) 0.290, (3) 0.435, (4) 0.580 μmol. For isotherms and isotherm coefficients used in the simulations, see Table I, eq d, and Table II, respectively. Same experimental conditions as for Figure 1.

interactions. If this assumption is correct, this term must be identical in the isotherms of the two enantiomers, which is exactly what we observe (Table II, data for site 2). The relative differences between the two coefficients of the second term of the bi-Langmuir isotherm (nonselective sites) for the two isomers are both 3.6%, within the range of experimental errors. The contribution of these nonselective sites to the column saturation capacity is the same for both isomers, within 0.5%. All these results are in agreement with the assumption of this term accounting for nonselective interactions.

Since immobilized BSA separates the enantiomeric *N*-benzoyl-DL-alanine, chiral selective interactions must take place, and there are enough amino acid residues on the external surface or on the walls of the inner cavities of BSA to account for selective interactions. The first term of the bi-Langmuir isotherm is very different for the two isomers. The "*a*" coefficient is 2.5 times larger for the D isomer than for the L isomer and the "*b*" coefficient 2.25 times larger for the D than for the L isomer. However, the column saturation capacity is only 8.5% larger for the D than for the L isomer. This much higher affinity of the first type of sites for the D isomer confirms the interpretation.

Finally, although the affinity of the first type of site for *both* isomers is much larger than the affinity of the second type of site for either, the column saturation capacity is 10 times larger for the second type of site than for the first. At high concentrations, the first type of site will be saturated and the chiral selectivity lost. The production rate of an immobilized BSA column for the separation of enantiomers is 1 order of magnitude lower than for the separation of other compounds with more different structures and the same relative retention. In this latter case, the nonselective sites that have a much higher saturation capacity would contribute. Even then, the production rate would still be 2 orders of magnitude less than with a more conventional chemically bonded phase. Typical sample sizes on a C18 silica column are of the order of 200–400 μmol.[9,10,13,14]

II. Elution Profiles of High-Concentration Bands of Pure Enantiomers. Figures 3 and 4 compare the elution profiles of large samples of pure *N*-benzoylalanine, the L isomer in Figure 3 and the D isomer in Figure 4. The solid lines are the elution profiles calculated by using the isomer data in Table II. The points on Figures 3 and 4 are experimental data. They were obtained by selecting some of the points in the file acquired by the chromatograph data station for each experiment and by calculating the corresponding concentration from the known detector response factor. Much larger sample sizes could be used with the L isomer than with the D isomer, because the sample of the latter that was available to us contained small amounts (ca. 4%, w/w) of the other

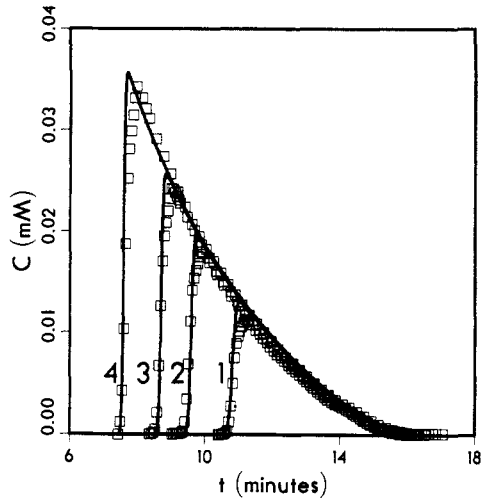

Figure 4. Comparison between experimental chromatograms (markers) and calculated band profiles (solid lines) for *N*-benzoyl-D-alanine at increasing sample sizes. Sample size: (1) 0.0298, (2) 0.0521, (3) 0.0745, (4) 0.112 μmol. For isotherms and isotherm coefficients used in the simulations, see Table I, eq d, and Table II, respectively. Same column and experimental conditions as for Figure 1.

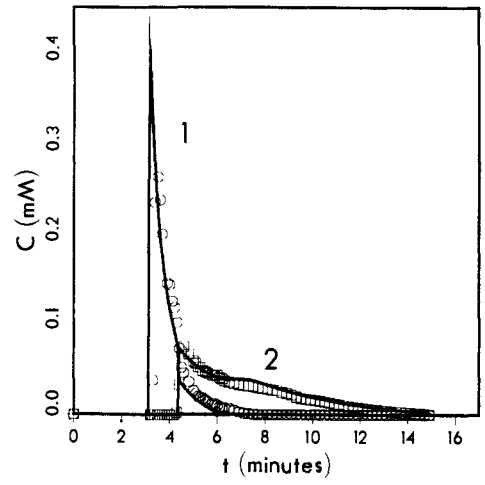

Figure 5. Comparison between experimental chromatograms (markers) and calculated band profiles (solid lines) for a 1/1 mixture of the D and L enantiomers of *N*-benzoylalanine. Individual band profiles: (1) L isomer, (2) D isomer. Sample size: 0.260 μmol for each isomer. For isotherms, isotherm coefficients, and percent total loading used in the simulations, see eq d Table I, Table II, and Table III, respectively. Same experimental conditions as for Figure 1.

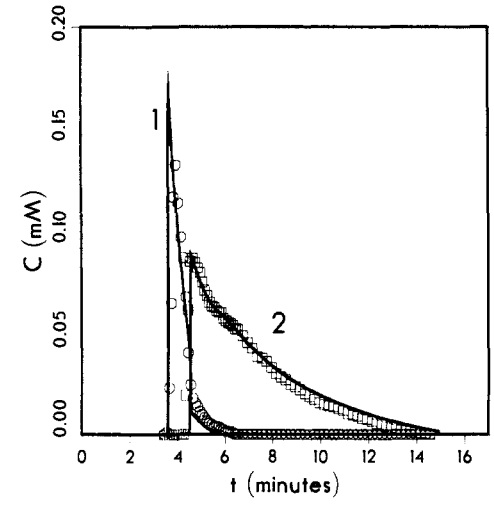

Figure 6. Comparison between experimental chromatograms (markers) and calculated band profiles (solid lines) for a 1/3 mixture of the D and L enantiomers of *N*-benzoylalanine. Individual band profiles: (1) L isomer, (2) D isomer. Sample size: 0.105 μmol of the L isomer, 0.392 μmol of the D isomer. For isotherms, isotherm coefficients, and percent total loading used in the simulations, see eq d Table I, Table II, and Table III, respectively. Same experimental conditions as for Figure 1.

enantiomer. Interference between the two bands would have altered the single-component profiles, due to the onset of competitive effects.

In Figure 3, the band profiles on the L isomer exhibit the characteristic shape of a bi-Langmuir isotherm with a long, tailing rear part and a very sharp, nearly vertical narrow front. Compared to the profiles corresponding to a simple Langmuir isotherm, the band profile differs much more from a right triangle and the rear part is more strongly bowed. There is an excellent agreement between the calculated and the experimental profiles. Especially noteworthy is the agreement between experimental and simulated results regarding the position of the front shock layers, their slopes, and their heights. Also, the profiles, both experimental and simulated, have tails that coincide as predicted.[4]

In Figure 4, the band profiles of *N*-benzoyl-D-alanine are reported. As explained above, the range of sample size investigated is approximately 5 times lower because of the aforementioned impurity. Because of the lower concentrations achieved, the profiles observed are more nearly triangular and the effect of the two-site isotherm is less obvious. The tailing still occurs but the profiles are much less bowed than for the L isomer. Similarly, the front of the profile is less steep and the peak apex less sharp. The agreement between experimental and theoretical profiles, as in Figure 3, is very good. Some slight deviations take place, however, especially for the low sample sizes where the thermodynamic effects are less dominant compared to the kinetics of mass transfers. The fronts of the experimental profiles demonstrate a greater amount of diffusion than predicted by the simulated profiles, which may be related to the lower efficiency of the column for this isomer. This effect decreases with increasing sample size when the front gets sharper. A much better agreement can be obtained with simulated profiles corresponding to a lower efficiency, to account for this more rounded shape. Then, the peak height decreases a little, the retention time of the peak maximum increases, and the upper part of the rear profile bows slightly, fitting exactly the experimental results.

The isotherms determined by frontal analysis permit the prediction of band profiles that fit exactly the experimental results.

III. Individual Elution Profiles of High-Concentration Bands of Mixtures of Enantiomers. The experimental determination of the coefficients of binary isotherms is extremely difficult and tedious.[40] A great advantage of the Langmuir isotherms is that the same numerical coefficients are used in the single-component and in the competitive isotherms. This reduces considerably the amount of experimental work required. The binary isotherms given by eq d, Table I, and the coefficients derived from the single-component isotherms have been used for the calculation of the individual elution profiles of the components of three mixtures of the enantiomers of *N*-benzoylalanine. Their relative compositions are 1/1 (Figure 5), 1/3 (Figure 6), and 3/1 (Figure 7), respectively. The total sample size accounts for approximately 24% of the column saturation capacity in all three cases. As above, the solid lines on the figures are the results of the calculations simulating the individual elution band profiles and the symbols are experimental data points. In this case, however, experimental data points are obtained from the analysis of the collected fractions.

The profiles obtained with an equimolar mixture (Figure 5) exhibit features that are characteristic of the displacement and the tag-along effects.[5-8] The rear of the first component band profile has a steep inflection tangent at the moment when the sharp front of the second component elution band appears. This is due to the displacement of the L isomer by the D isomer. The tail of the first component behind the second shock layer ends when a short, nearly horizontal plateau on the profile of the second-

(40) Ma, Z.; Katti, A. M.; Guiochon, G. *J. Phys. Chem.*, in press.

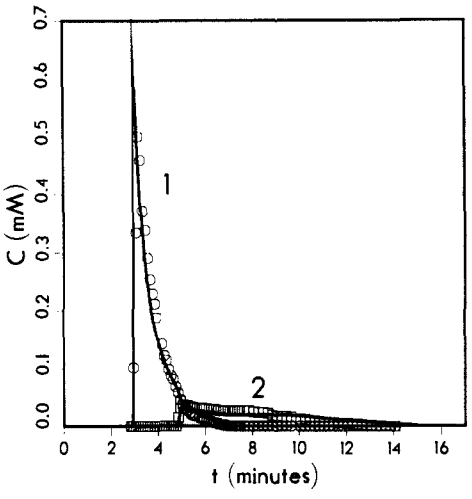

Figure 7. Comparison between experimental chromatograms (markers) and calculated band profiles (solid lines) for a 3/1 mixture of the D and L enantiomers of *N*-benzoylalanine. Individual band profiles: (1) L isomer, (2) D isomer. Sample size: 0.491 μmol of the L isomer and 0.165 μmol of the D isomer. For isotherms, isotherm coefficients, and percent total loading used in the simulations, see eq d Table I, Table II, and Table III, respectively. Same experimental conditions as for Figure 1.

component band begins. This plateau is due to the tag-along effect. The presence of the L isomer in the mixed zone, between the zones of pure L and pure D isomers, accelerates the migration of the D isomer in this region.

There is an excellent general agreement between the individual band profiles calculated by the computer and the experimental profiles. Only a few minor, local differences between the two sets of profiles can be found. For the first component, the calculations predict a retention time that is slightly too short, a rear profile that is slightly too steep when the front of the second component is eluted, and a tail, behind the rear shock layer, that is somewhat too short and not quite concentrated enough. On the other hand, the rear of the profile when the first component is pure and the inflection at the elution of the front of the second component are accurately predicted. For the second component, the agreement is even better. The retention time of the front, its height, the elution profile in the mixed zone, and the tail are all accurately predicted. The plateau of the second component (tag-along effect) is more strongly marked and slightly higher on the calculated profile than is observed experimentally.

The elution bands for a 1/3 mixture (Figure 6) exhibit almost only those features characteristic of an intense displacement effect, while the elution bands for a 3/1 mixture (Figure 7) show only those characterizing the tag-along effect.[5-8] In Figure 6, the band of the first isomer is narrow; its rear has a nearly vertical part, dropping almost instantaneously from 40 to 15% of the maximum height, quite an unusual feature for a chromatographic band. The extent of the tail of the first band is less than in Figure 4 (the tail is shorter and approximately half as concentrated). Again, there is an excellent agreement between the calculated profiles and the experimental results. The calculations predict exactly the first component band profile (Figure 6), except for the tail of this band, which is predicted to be slightly shorter and less concentrated than is observed. Similarly, they give the exact retention time and height of the second band and an excellent rendition of the inflection point on the rear of the second band, at the time when the first band ends. This inflection point or shoulder, which looks like the start of the separation of a third component, is the only visible feature left by the tag-along effect under these experimental conditions (1/3 mixture).

The individual elution band for a 3/1 mixture (Figure 7) is typical of what happens under conditions where the tag-along effect predominates. The band of the first isomer is strongly overloaded; its retention time is shorter than on any previous chromatograms. A very slight displacement effect is made visible by the short concentration drop on the rear of the first band profile, which is very well rendered by the predicted profile. The second-component band is spread over a very long period of time, a characteristic feature of the tag-along effect. When the ratio of the concentrations of the two components in the feed (C_2^0/C_1^0) tends toward zero, the length of the plateau tends toward the difference, $t_{R,2}^0 - t_{R,1}^0$, between the analytical retention times of the two components.[8] The calculated profile for the second component accounts accurately for the retention time of the front, its height, the length and height of the plateau, and the tail at the end of the band profile.

The individual band profiles derived from our experimental results are in excellent agreement with the profiles calculated by using the theory of nonlinear chromatography. This result is made possibly only because the kinetics of mass transfers between phases in the column used is fast and the equilibrium isotherm is accurately accounted for by the equations we have adopted with the numerical coefficients we have determined. As such, this agreement is a confirmation of the validity of eq d (Table I) to represent the competitive interaction behavior of the enantiomers studied with immobilized BSA.

Conclusion

The determination of the equilibrium isotherms of isomers between the two phases of a chromatographic system on which they are resolved casts new light on the retention mechanism(s) involved in the separation. In the specific case of BSA immobilized on silica, we have shown the existence of two different retention mechanisms, contributing differently to the separation. One of these mechanisms is chiral selective; the other is not. However, the chiral-selective mechanism involved in the separation of highly hydrophobic enantiomers on BSA has nearly equal saturation capacities for the two optical isomers. This saturation capacity is much lower (10 times) than that of the nonselective mechanism.

Both of these mechanisms are most complex. They include all kinds of molecular interactions, between the nonchiral groups of the amino acid residues of the protein as well as the silica surface and the molecules of enantiomers in the case of the nonselective mechanism and between each amino acid of the protein and the enantiomeric molecules in the case of the chiral-selective mechanism. It is remarkable that the overall result can be accounted for correctly by as simple a model as the bi-Langmuir isotherm.

When the sample size is increased, the selective retention mechanism is rapidly overloaded. Its separation power toward the enantiomers disappears for sample sizes with which many stationary phases as well as the nonselective mechanism are only slightly overloaded. In part, this effect is due to the low surface coverage of the silica by BSA. An increase in the surface density of the protein would result in a proportional increase in the column saturation capacity, up to a point at least. This increase would be accompanied by a proportional rise in the column capacity factor, i.e., the retention times. The combination would have little effect on the production rate in preparative liquid chromatography unless a stronger mobile phase with a higher organic solvent concentration is used, permitting the decrease of the retention times and the cycle time. The effect of a change of the mobile-phase composition on the column saturation capacity is still uncertain.

Finally, the usefulness of computer simulation in preparative chromatography is demonstrated by our results. Since there is a nearly exact agreement between the individual band profiles generated by the computer and those determined experimentally, it is possible to investigate the influence of a variety of experimental parameters on the separation, production rate, and recovery yield. Classical techniques of optimization are easily applicable.

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