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Investigation of the Heterogeneous Adsorption Behavior of Selected Enantiomers on Immobilized α_1-Acid Glycoprotein

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A complete census was made of the interactions between enantiomeric solutes and the chiral protein column CHIRAL-AGP with the theory of nonlinear LC as tool. The surface is heterogeneous, having a small number of strong enantioselective adsorption sites and a large number of weak nonselective ones. When the eluent pH was increased, the "linear" retention of (i) the amines increased strongly as a result of a strong increase in the enantioselective binding strength, whereas (ii) the retention of the aprot increased slightly as a result of an increase in both the enantioselective binding strength and its capacity. The retention of (iii) the acid has a maximum originating solely from the enantioselective binding energy, whereas the nonselective equilibria decreased steadily. For all compounds, the enantioselective equilibrium constants increase relatively more than the nonselective ones with increasing pH.

Many pharmaceuticals are chiral. Although most of their physicochemical properties are identical (except for their interactions with polarized light and with other enantiomers), the two enantiomers of the same chiral drug may have different pharmacological and pharmacokinetic behaviors. Therefore, it is important to separate the enantiomers of drugs, whether for analytical or for production purposes. HPLC is a most suitable separation method for this purpose, provided proper chiral stationary phases are available.

A large number of new chiral stationary phases (CSPs) for liquid chromatography have been developed and studied during the past decade.[1−7] Usually, they consist of an achiral matrix (e.g., porous silica) with bonded chiral ligands. These ligands can be small groups (e.g., Pirkle phases[1]) or macromolecules, such as cellulose derivatives[3] or even proteins.[5,6] Examples of protein ligands are cellobiohydrolase I (Cel7A, previously denoted CBH I),[7] bovine serum albumin,[5,8] ovomucoid,[9] and α-chymotrypsin.[10] The protein CSPs have generally much smaller loadabilities, as compared to the small-molecule selectors,[1] and should therefore not be used for preparative applications. The reason is that fewer large molecules (e.g., proteins) can be immobilized in the column, resulting in a lower density of the enantioselective sites, as compared to when smaller selectors are used. However, protein columns are advantageous for some complicated analytical applications. For example, the use of water-based eluents allows for direct injection, which is essential in applications such as microdialysis in which only microliters of samples are available, which makes extraction difficult.[11,12]

The human plasma protein α_1-acid glycoprotein (AGP) is classified as one of the positive acute-phase proteins[13,14] and is the most important of the plasma proteins for binding basic drugs, including β-adrenergic receptor blockers, antidepressants, and local anesthetics in the human plasma.[14,15] The amino acid sequence of the single polypeptide chain, containing 181 amino acids, was determined by Schmid et al.[16] The very low isoelectric point of AGP, 2.7 in phosphate buffer[17] is due to its high sialic acid content; the pI of the deglycosylated protein is 5.3.[15] Of the 12 tyrosine residues, 5 are located near the protein surface.[18,19] Schmid et al. reported that one of the three tryptophan residues is partly buried, whereas the other two are completely buried in the inner core of the protein.[19] The five carbohydrate units are located on the protein surface.[19] According to one hypothesis, the binding site of basic drugs can be modeled by a conical pocket

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that contains lipophilic regions at the base (on the internal surface) and an anionic region close to the spike of the cone.[20] Protonated aliphatic nitrogen guides drug molecules toward the anionic region. Hydrophobic hydrocarbon moieties of the interacting drugs provide anchoring in the lipophilic region(s) of the binding site. In this context, it is worth mentioning that the immobilization of AGP onto the silica surface influences the conformation of the protein.[21]

α_1-Acid glycoprotein as immobilized on porous silica is the most popular protein column today because of its unique ability to separate enantiomers of many different classes of chiral compounds. The retention and selectivity of any particular chiral compound can be adjusted easily by modifying the eluent. When the eluent pH is increased, the following general pattern has been observed: for bases and aprotic compounds, the retention increases, whereas it decreases for acids.[22−28] The enantioselectivity does not follow simple rules for either bases, aprotic compounds or acids, but depends on the compound.[22−28] Upon adding uncharged modifiers to the eluent, such as 2-propanol, both the enantioselectivity and the retention decreases for cationic compounds,[29] whereas for anionic compounds, the retention decreases, and for some cations, the enantioselectivity increases.[30] Upon adding positively charged modifiers, the retention and enantioselectivity increase somewhat for anionic compounds.[30]

The theory of nonlinear chromatography has previously been used successfully to characterize the two different protein columns bovine serum albumin (BSA) and cellobiohydrolase (Cel7A).[8,31−33] It was found that both columns contain two different groups of adsorption sites, enantioselective and nonselective. Both types of sites contribute to retention, but only the former kind of interaction contributes to resolution. In fact, the nonselective sites degrade the separation.[34] The relative effects of the interactions of the two sites on the linear "global" retention data can be calculated from the isotherm parameters. Since the nonselective interactions significantly contribute to the retention in the case of the protein columns studied so far,[34−37] those must always be taken into account when discussing chiral discrimination. Consequently, the apparent separation factor based on linear data is always lower than the true separation factor (the ratio of the enantioselective equilibrium constants obtained from isotherm data). The stoichiometry of the enantioselective enantiomer−protein interaction has been determined for BSA and Cel7A, both having only one enantioselective site.[34,35]

In this study, the theory of nonlinear chromatography was applied to the characterization of the interaction between some model compounds and the protein column CHIRAL-AGP. Eight enantiomers from four different enantiomeric pairs were used as model compounds: the basic compounds alprenolol and 1-(1-naphthyl)ethylamine, the acidic 2-phenylbutyric acid and the neutral compound methyl mandelate. More specifically, we wanted to (1) characterize the thermodynamics of the enantioselective and nonselective adsorption of the model compounds on CHIRAL-AGP, (2) determine its dependence on the eluent pH, and (3) determine the stoichiometry of the enantioselective interaction.

THEORY

For a more thorough theory, see ref 36 or 38.

An adsorption isotherm is the ratio between the equilibrium concentrations of a compound in the stationary phase and in the mobile phase at constant temperature. The Langmuir equation developed by Langmuir[39] is the simplest model for a nonlinear isotherm. It accounts well for the adsorption of single components onto homogeneous surfaces at low and moderate concentrations.[38] This model is expressed by the classical equation

$$\theta = \frac{q}{q_s} = \frac{bC}{1 + bC} = \frac{\Gamma}{1 + \Gamma} \qquad (1)$$

where θ is the fractional surface coverage, q is the stationary phase concentration at equilibrium with the mobile phase concentration C, q_s is the monolayer capacity or specific saturation capacity of the stationary phase, and b is a term (dimension of the reverse of a concentration) that depends on the adsorption energy. The product $\Gamma = bC$ characterizes the deviation of the isotherm from linear behavior. $a = bq_s$ is the equilibrium constant at infinite dilution and is identical with the initial slope of the isotherm.

Many chiral separation systems contain two different types of adsorption sites on the adsorbent surface. The bi-Langmuir equation has proved to be essential for describing chiral chromatographic adsorbents of the protein type. This equation contains the sum of two Langmuir expressions and is written

$$q = \frac{a_I C}{1 + b_I C} + \frac{a_{i,II} C}{1 + b_{i,II} C} \qquad (2)$$

where the coefficients b_I and b_{II} are related to the energy of adsorption and a_I and $a_{i,II}$ are the equilibrium constants at infinite dilution on these two types of sites, respectively. $q_{I,s}(= a_I/b_I)$ and $q_{i,II,s}(= a_{i,II}/b_{i,II})$ correspond to the monolayer capacities of the two adsorption sites, here denoted as site I and site II, respectively. In enantiomeric separations, the values of the coefficients $q_{I,s}$ and b_I are the same for the two enantiomers. The coefficients $q_{II,s}$ and b_{II} are different for the two enantiomers. Usually, b_{II} is much larger than b_I, and $q_{II,s}$ is much smaller than $q_{I,s}$.

The linear retention time, assuming the bi-Langmuir model, is given by the following expression, where t_0 is the hold-up time

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and F is the phase ratio (with $F = (1 - \epsilon)/\epsilon$, where ϵ is the total porosity of the column).

$$t_R = t_0(1 + k) = t_0\left(1 + F\frac{dq}{dC}\right) = t_0(1 + F(a_I + a_{II})) \quad (3)$$

When the isotherms are unavailable or cannot be modeled, the enantioselectivity is characterized empirically by the *apparent* separation factor for the two enantiomers, α_{app}. Using the initial slopes of the isotherms (see eq 2), this ratio is given by

$$\alpha_{app} = \frac{k_2}{k_1} = \frac{a_{2,II} + a_I}{a_{1,II} + a_I} \quad (4)$$

This factor characterizes the ability of a chiral stationary phase to perform an analytical chiral separation but cannot be used for meaningful thermodynamic investigations. When the isotherms are modeled using, for example, the bi-Langmuir model (eq 2), the contributions of type II sites for both enantiomers can be derived and the "true" chiral separation factor, α_{true}, can be determined.

$$\alpha_{true} = \frac{k_{2,II}}{k_{1,II}} = \frac{a_{2,II}}{a_{1,II}} \quad (5)$$

Only this separation factor is meaningful in a discussion of the mechanism of chiral discrimination. However, it should be mentioned that this is a dynamic approach; it does not describe the true enantioselective site from a mechanistic point of view. The question of whether the two enantiomers adsorb onto the same site(s) cannot be answered by using single component isotherms; for this, competitive isotherms must be performed.[38]

The relative enantioselectivity contribution to retention (REC) is derived from the equilibrium constants at infinite dilution and is an inverse measure of the influence of nonselective adsorption on retention under linear conditions.

$$REC_i = 100\frac{a_{i,II}}{a_{i,II} + a_I} \quad (6)$$

EXPERIMENTAL SECTION

Apparatus. The chromatographic system used consisted of two ESA 580 pumps (Chelmsford, MA), one of them mastering the other in the gradient programming mode. The outlets of these two pumps were connected directly by means of a low-dead-volume PEEK tee. All connections between the tee and the flow cell were made with 0.17 mm PEEK capillaries. A MIDAS automatic injector (Spark Holland, AJ Emmen, The Netherlands) and a Lambda 1010 UV detector (Bischoff, Leonberg, Germany) were used. The CHIRAL-AGP column (100 × 4.0 mm, 5 μm) consisted of α_1-acid glycoprotein immobilized on silica and was bought from ChromTech AB (Stockholm, Sweden). The column was placed in a water jacket, and its temperature was kept constant using an MN6 Lauda circulating water bath (Lauda, Königshofen, Germany). A computer data acquisition system using the software CSW 1.7 (DataApex Ltd., Praha, Czech Republic) was used to record the chromatograms. The pH was measured with a Mettler-Toledo MP 125 pH meter (Mettler-Toledo, Schwerzernbach,Switzerland) or a Metrohm 632 pH meter (Metrohm, Herisau, Switzerland).

Chemicals. (+)-Methyl L-mandelate and (−)-methyl D-mandelate (purum > 99%) were from Fluka (Fluka Chemikal, Buchs, Switzerland). (*R*)-(+)-1-(1-naphthyl)ethylamine, (*S*)-(−)-1-(1-Naphthyl)ethylamine (purum > 99%), (*R*)-(−)-2-phenylbutyric acid, and (*S*)-(+)-2-phenylbutyric acid (99%) were from Aldrich Chem. Co. (Milwaukee, WI). L-(−)-Alprenolol hydrogentartrate monohydrate and D-(+)-alprenolol hydrogentartrate monohydrate were from AstraZeneca R&D (Mölndal, Sweden). The acetate buffers were prepared from acetic acid (>99.8%) from Riedel-de Haën (Seelze, Germany), and anhydrous sodium acetate (>99%) and 2-propanol LiChrosolv were from Merck (Darmstadt, Germany). The phosphate buffer was prepared from phosphoric acid (99%) from Merck and 1.0 mol of sodium hydroxide Fixanal from Riedel-de-Haën. The water used was from Millipore, MilliQ grade. The buffer solutions and the stock solutions of enantiomers were filtered through 0.45 μm filters (Kebo, Spånga, Sweden).

Column and Quantification of the Stationary Phase. In the case of the commercially available column CHIRAL-AGP (ChromTech AB, Hägersten, Sweden), the protein human α_1-acid-glycoprotein (AGP) has been immobilized on silica particles packed into a 100 × 4.0 mm stainless steel column. To evaluate the concentration of the AGP immobilized on the silica support, an amino acid analysis was performed at the Department of Biochemistry, Uppsala University, Sweden, as follows: An ~5 mg portion of the dried column packing was hydrolyzed with 6 M HCl at 110 °C for 24 h to cleave all of the peptide bonds in the presence of a known amount of norleucine as internal standard. The hydrolysate was analyzed with an LKB ALPHA-PLUS amino acid analyzer using ninhydrin detection. From the weights of all of the amino acids except for cysteine and tryptophan, which were back-calculated from the known amino acid sequence,[15,40] a total weight of protein was obtained. The amount of protein (carbohydrates excluded) bonded to the silica was found to be 66 mg/g of packing. The amount of protein (carbohydrates excluded) in the column, 47.5 mg, was derived from the bonded protein/silica concentration and from the dry weight of packing material in the column (total weight, 720 mg).

In addition, elemental analysis of the column material (at Mikrokemi AB, Uppsala, Sweden) showed that it contained 1.26−1.29 wt % nitrogen. According to the literature, the total nitrogen content of AGP molecule is 10.7%.[17] Combining these two values and the total weight of the column material (720 mg) yields 84.8−86.8 mg AGP in the column, which is very close to the amino acid analysis results (87.1 mg of whole protein). Although the total carbohydrate content could not be measured by amino acid analysis, the results indicated that CHIRAL-AGP contains the glyconated protein.

Preparation of Mobile Phase. Solutions of acetate buffer of pH 4.0, 4.5, 5.0, 5.5, and 6.0 and a phosphoric buffer of pH 6.5 with ionic strength of $I = 0.050$ M were used as the mobile phase. All of these solutions were prepared by mixing 1.00 M sodium acetate and concentrated acetic acid (17.49 M) in different proportions or from 1.00 M sodium hydroxide and 1.00 M phosphoric acid solutions. The volumes of acetic acid and sodium

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acetate solutions required to achieve the desired pH were calculated using the Henderson−Hasselbalch equation together with the thermodynamic acid dissociation constants and the proper activity coefficients for the actual ionic strength. The exact pH value was measured for each solution. In Tables 1−4, the exact values of the pH of the solutions used are reported. The mobile phase contained buffer and 0.25% 2-propanol. The 2-propanol was added to the buffer by first lifting out exactly 12.50 mL of buffer from a filled 5000 mL glass volumetric flask and then adding exactly 12.50 mL 2-propanol and, if necessary, adjusting the liquid level with pure buffer to the proper position of the calibration mark. The flow rate was 0.80 mL/min.

Procedures. A 20-μL portion of a 0.025 mM solution of each enantiomer was injected twice at each mobile phase pH value. Single enantiomer adsorption isotherms of the two methyl mandelate, alprenolol, 1-(1-naphthyl)ethylamine, and 2-phenylbutyric acid enantiomers were determined using the frontal analysis method performed in the staircase mode. The solute concentration in the eluent was increased stepwise by programming the solvent delivery system in the high-pressure gradient mode to execute step gradients (5 or 10% of the total bulk concentration) at the appropriate times. Three different bulk concentrations of both methyl mandelate, 1-(1-naphthyl)ethylamine, and 2-phenylbutyric acid enantiomers were used: 5 μM, 0.1 mM, and 2 mM, respectively. Four solutions of each enantiomer were used for alprenolol at pH 4.97: 0.25 μM, 5 μM, 0.1 mM, and 2 mM. The dynamic concentration range was 8000, except for alprenolol at pH 4.97 (160 000) and 1-(1-naphthyl)ethylamine at pH 5.97 (5200). The column temperature was kept constant at 20.0 °C.

Five different wavelengths were used for UV detection, depending on the concentration and nature of the compound: 230, 254, 265, 270, 285, 300, and 320 nm.

The column hold-up volume, V_0, was determined to be 0.928 mL, corresponding to the elution time of the first buffer/water disturbance peak. All frontal analysis data were corrected for the dead volume contribution of the instrument and for the column hold-up volume. The total correction volume, V_T, was determined to be 0.952 mL (V_0 is included in V_T). The volume of the stationary phase, V_S, was determined by subtracting the column hold-up volume from the geometric volume of the column, that is, $V_S = V_G - V_0 = (1.257 - 0.928)$ mL $= 0.329$ mL.

The best values for the parameters of the bi-Langmuir isotherm were calculated using a nonlinear regression method, the Gauss−Newton algorithm with the Levenberg modification, as implemented in the software PCNONLIN 4.2 from Scientific Consulting (Apex, NC). In the regression, the experimental data were given a weight equal to $1/q_{pred}$, where q_{pred} is the stationary-phase concentration predicted by the model. This forces the program to tolerate the same relative error on each data point and avoids sacrificing the precision of the low-concentration data, which are important for linear chromatography.

RESULTS AND DISCUSSION

Linear Retention Data. Before performing nonlinear studies, it is always necessary to do some preliminary linear studies. The analytes selected were methyl mandelate, alprenolol, 1-(1-naphthyl)ethylamine and 2-phenylbutyric acid enantiomers (see structures Figure 1). Figure 2 shows the dependence of linear retention factors of the compounds on the mobile phase pH. It is evident

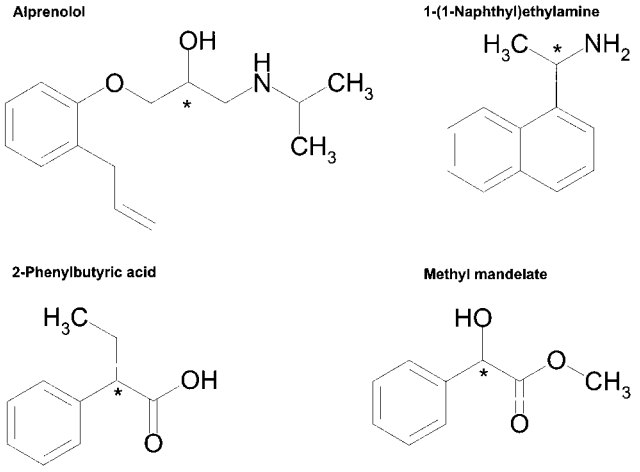

Figure 1. Structures of the chiral solutes investigated in this study. The chiral centers are marked with asterisks.

that the retention factors of all substances increase except those for the enantiomers of the acid (2-phenylbutyric acid). The strongest rise in retention is observed for the secondary amine alprenolol and the lowest for the aprotic compound, methyl mandelate, with the primary amine, 1-(1-naphthyl)ethylamine, somewhere in between. The acid has a maximal retention at pH 4.5 for both enantiomers. The enantioselectivities increase with increasing pH except for naphthylethylamine (see Table 5), although the retention of the acid decrease above pH 4.5 its "apparent" enantioselectivity (cf. Eqn. 4) increases. Excessive retentions or too low retentions must be avoided for accurate isotherm measurements,[38] which limits the choice of the pHs studied with nonlinear chromatography.

Equilibrium Adsorption Isotherms. Figures 3 to 6 show the isotherms of the four analytes included in this study.

Bases (Alprenolol and Naphthylethylamine) and the Neutral Compound (Methyl Mandelate). The top left insets of Figures 3, 4, and 5 show that all the adsorption isotherms of the *R* and *S* enantiomers of the two amines and the aprotic compound are linear in the low concentration range (i.e., below 5 μM), except for alprenolol at pH 5.0 where an additional lower concentration range was necessary in order to reach linearity. For both amines, the initial slopes of the isotherms increase with increasing pH, in accordance with the linear data (cf. Figure 2). The order of the increase of the initial slope depends mainly on the pH and secondarily on the type of enantiomer [$S > R$ for alprenolol and $R > S$ for naphthylethylamine and methyl mandelate] adsorbed on the column, in accordance with the linear data (cf. Figure 2). The main parts of Figures 3 and 4 show that the isotherms of the amines are no longer linear in the intermediate concentration range (i.e., between 0.005 and 0.1 mM), albeit for alprenolol at pH 4.0 and naphthylethylamine at pH 5.0 and 5.5, the isotherms seem to remain linear. The main part of Figure 5 shows that the isotherms for the aprot are nearly linear, even at relatively high concentration. The curvature is readily seen to increase with increasing pH. The order of the isotherms remains the same. The two isotherms for each pair of enantiomers at a given pH become close and tend to be parallel. The top right insets of Figures 3−5 show the isotherms for the high concentration range, where the characteristic parallel behavior becomes more pronounced. The resolution between the enantiomers of each pair decreases at high

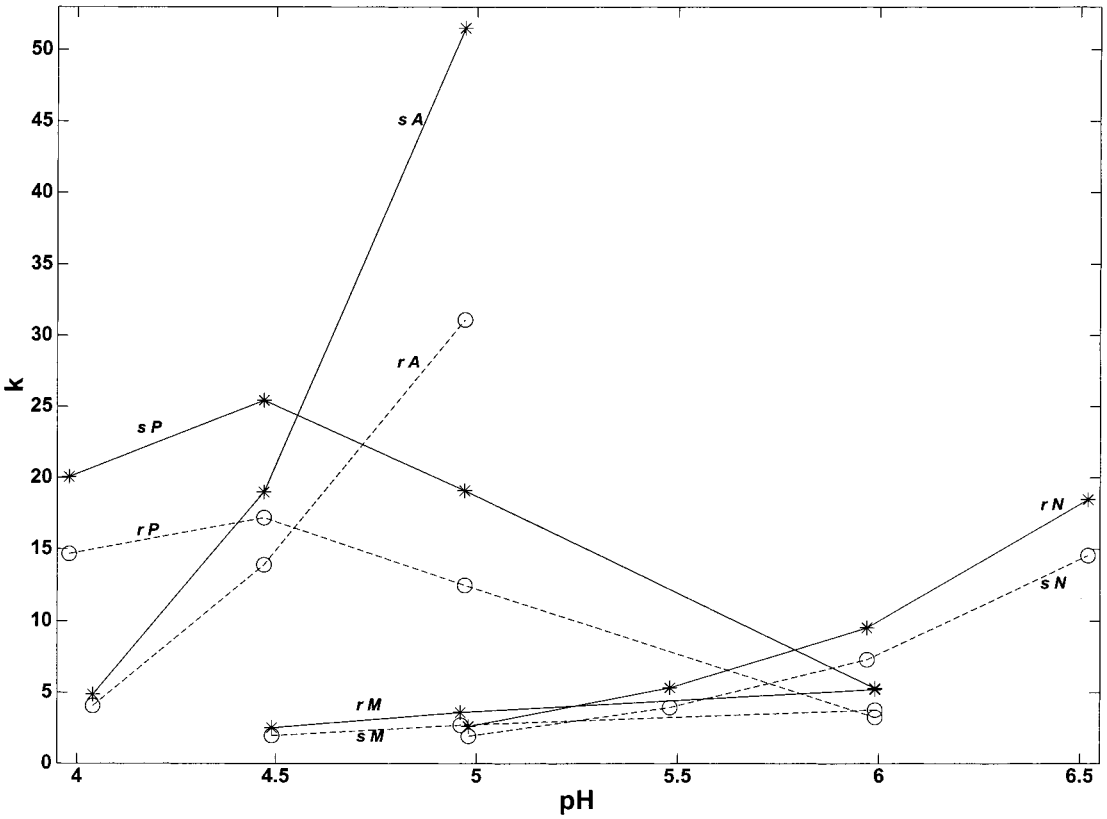

Figure 2. Retention factors of the first (circles, dashed lines) and second eluted (asterisks, solid lines) enantiomer of the analytes versus the eluent pH. Experimental conditions: column, 100 × 4.0 mm; stationary phase, immobilized α_1-acid glycoprotein on silica; eluent, acetic buffer at $I = 0.050$ M and 0.25% 2-propanol; mobile phase flow rate, 0.80 mL/min; temperature, 20.0 °C; 20 μL of 0.050 mM racemates dissolved in eluent were injected. Analytes: (rA) (*R*)-alprenolol, (sA) (*S*)-alprenolol, (rP) (*R*)-2-phenylbutyric acid, (sP) (*S*)-2-phenylbutyric acid, (sM) (*S*)-methyl mandelate, (rM) (*R*)-methyl mandelate, (sN) (*S*)-1-(1-naphthyl)ethylamine, (rN) (*R*)-1-(1-naphthyl)ethylamine.

Table 1. Bi-Langmuir Isotherm Parameters for Alprenolol[a]

type of sites	pH	*a*	RSD[b] (%)	*b* (mM^{-1})	RSD[b] (%)	q_s (mM)	q_s[c]
I	4.04	2.00	(4.5)	0.224	(8.5)	8.96	1.32
	4.47	3.47	(5.5)	0.444	(8.9)	7.82	1.15
	4.97	4.87	(4.0)	0.501	(7.2)	9.71	1.43
R,II	4.04	8.97	(0.8)	4.41	(2.8)	2.03	0.299
	4.47	32.5	(1.3)	12.1	(3.2)	2.70	0.397
	4.97	86.8	(1.5)	28.8	(2.7)	3.02	0.444
S,II	4.04	11.2	(0.7)	3.81	(1.9)	2.94	0.433
	4.47	46.7	(1.3)	17.2	(3.4)	2.72	0.400
	4.97	130.3	(1.4)	39.8	(2.4)	3.27	0.481

[a] Experimental conditions are given in Figure 3. [b] Relative standard deviation. [c] Monolayer capacity in number of adsorbed molecules per molecule of immobilized AGP.

Table 2. Bi-Langmuir Isotherm Parameters for 1-(1-Naphthyl)ethyl Amine[a]

type of sites	pH	a	RSD[b] (%)	*b* (mM^{-1})	RSD[b] (%)	q_s (mM)	q_s[a]
I	4.98	3.21	(1.3)	0.0634	(6.6)	50.6	7.44
	5.48	5.44	(2.0)	0.0991	(10)	54.9	8.08
	5.97	8.38	(3.0)	0.198	(11)	42.4	6.24
	6.52	13.1	(1.6)	0.292	(4.4)	44.9	6.61
S,II	4.98	2.44	(1.6)	2.22	(3.1)	1.10	0.162
	5.48	5.79	(1.7)	4.02	(4.0)	1.44	0.212
	5.97	13.7	(1.9)	8.62	(6.4)	1.58	0.232
	6.52	26.0	(1.6)	13.6	(4.4)	1.91	0.281
R,II	4.98	4.40	(0.8)	2.83	(2.3)	1.56	0.230
	5.48	9.37	(0.8)	5.64	(3.7)	1.66	0.244
	5.97	17.7	(1.5)	10.0	(5.9)	1.76	0.259
	6.52	36.3	(1.5)	17.9	(4.2)	2.03	0.299

[a] Experimental conditions are given in Figure 4. [b] Relative standard deviation. [c] Monolayer capacity in number of adsorbed molecules per molecule of immobilized AGP.

concentrations. These effects are due to complete saturation of the selective sites (eq 2).

The Acid (2-Phenylbutyric Acid). The top left inset in Figure 6 shows the low, linear part of the adsorption isotherms for the *R* and *S* enantiomers of the acid. The order of the isotherms is quite different, as compared to the other compounds. A first interesting observation is that pH 4.5 gives the highest initial slope, and below and above this pH, the slope decreases, which is in agreement with the linear experiments in which the retention factor had a maximum at pH 4.5. A second interesting feature is that all of the isotherms for the *R* enantiomer are below the lowest isotherm for the *S* enantiomer. This means that in this pH range, the order of the isotherms is determined primarily by type of enantiomer and secondarily by pH (where there is a maximum at pH 4.5), which is in agreement with the linear data (cf. Figure 2). The main part of Figure 6 shows the weakly nonlinear behavior of the isotherms in the intermediate concentration range. It shows that the isotherms for pH 4.0 become close to those for pH 4.5, that is, the curvature is considerable for the former as a result of the maximum of the enantioselective interaction energy (b_{II}) at this pH (see below); however, the order of the isotherms remains the same as in the low concentration range. The inset in the

Table 3. Bi-Langmuir Isotherm Parameters for Methyl Mandelate[a]

type of sites	pH	a	RSD[b] (%)	b (mM^{-1})	RSD[b] (%)	q_s (mM)	q_s[a]
I	4.49	1.56	(7.3)	0.0959	(23)	16.2	2.38
	4.96	1.76	(5.1)	0.139	(13)	12.6	1.85
	5.99	1.58	(6.4)	0.107	(22)	14.8	2.18
S,II	4.49	4.04	(2.6)	1.98	(4.5)	2.04	0.300
	4.96	6.14	(1.3)	2.89	(2.9)	2.13	0.313
	5.99	9.62	(1.0)	3.03	(2.3)	3.17	0.466
R,II	4.49	5.71	(1.7)	2.41	(4.1)	2.37	0.349
	4.96	8.81	(0.75)	4.10	(3.2)	2.15	0.316
	5.99	13.6	(0.57)	4.69	(2.8)	2.89	0.425

[a] Experimental conditions are given in Figure 5. [b] Relative standard deviation. [c] Monolayer capacity in number of adsorbed molecules per molecule of immobilized AGP.

Table 4. Bi-Langmuir Isotherm Parameters for 2-Phenylbutyric Acid

type of sites	pH	a	RSD[b] (%)	b (mM^{-1})	RSD[b] (%)	q_s (mM)	q_s[a]
I	3.98	6.29	(3.1)	0.120	(12)	52.5	7.72
	4.47	5.76	(3.3)	0.160	(11)	35.9	5.28
	4.97	4.11	(3.8)	0.140	(13)	29.4	4.33
R,II	3.98	33.3	(1.1)	9.06	(3.7)	3.68	0.541
	4.47	42.5	(1.3)	12.2	(3.6)	3.47	0.511
	4.97	28.5	(0.96)	7.97	(3.0)	3.58	0.527
S,II	3.98	45.9	(0.83)	8.46	(2.6)	5.42	0.797
	4.47	61.8	(1.1)	13.3	(2.7)	4.65	0.684
	4.97	47.3	(1.0)	11.9	(2.8)	3.97	0.584

[a] Experimental conditions are given in Figure 6. [b] Relative standard deviation. [c] Monolayer capacity in number of adsorbed molecules per molecule of immobilized AGP.

bottom right corner of Figure 6 shows that some shifts have occurred between the isotherms. At the high concentration, the isotherms are instead ordered primarily by pH (i.e., they increase with decreasing pH with no maximum adsorption at pH 4.5) and secondarily by type of enantiomer [$S > R$]. The intersections are a consequence of the behavior of the enantioselective sites having a maximum at pH 4.5 and the decrease of nonselective adsorption with increasing pH (see below).

Bi-Langmuir Parameters. The best values for the parameters of the bi-Langmuir model are listed in Tables 1−4. Each set of data was fitted to eq 2 using six independent parameters in these equations.

Bases (Alprenolol and Naphthylethylamine) and the Neutral Compound (Methyl mandelate). The nonselective equilibrium constants (a_I terms) of the amines increase with increasing pH, whereas for the aprot, it is unaffected. Comparing the lowest and highest pH, the coefficient a_I increases nearly 2.5 times and ∼4-fold for alprenolol and naphthylethylamine, respectively (Tables 1−2). For both compounds, this is due to an increase in the interaction energy coefficient strength b_I (since $a_I = b_I q_{I,s}$) with pH, whereas their respective nonselective monolayer capacities are unaffected (cf. Tables 1 and 2). In the case of the aprot, all parameters describing the nonselective interactions (a_I, b_I, and $q_{I,s}$) are not significantly affected by the increasing pH (cf. Table 3).

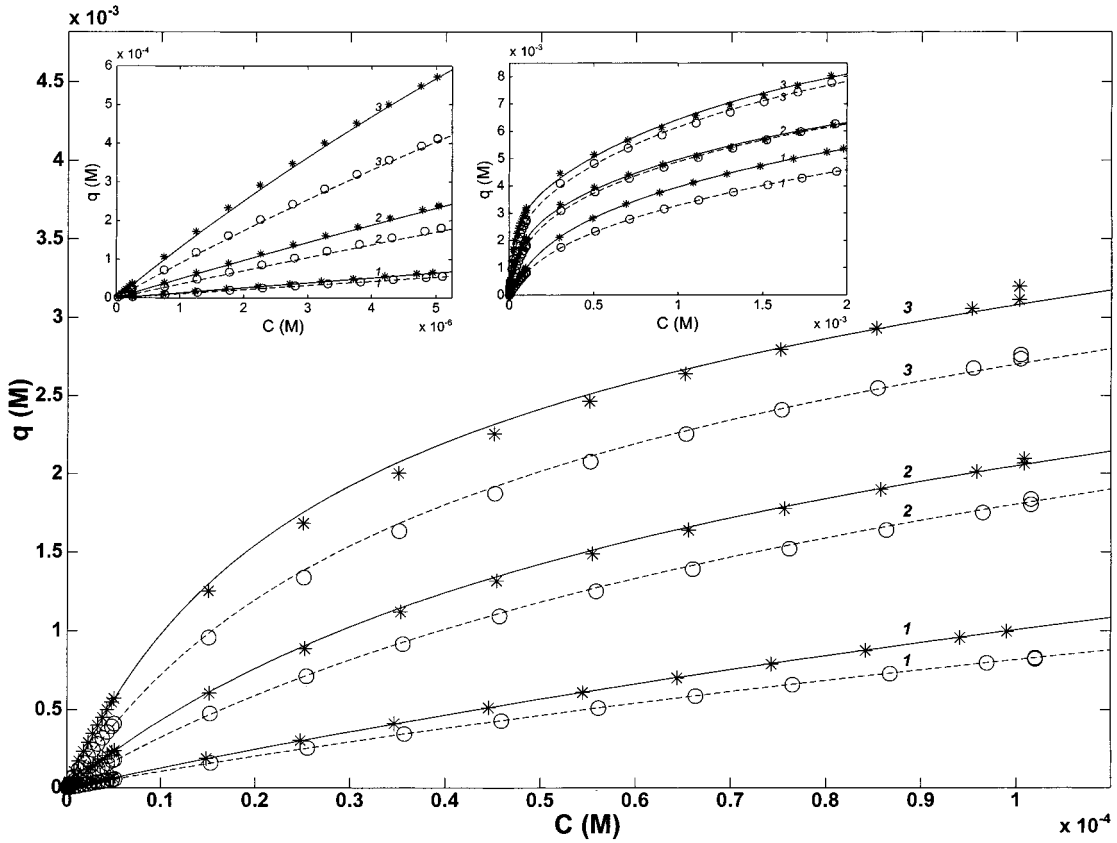

Figure 3. Single-component equilibrium isotherms for *R* and *S* enantiomers of alprenolol at increasing pH. Conditions are as in Figure 2. Symbols, experimental data: ○, *R* enantiomer; *, *S* enantiomer. Lines: best calculated bi-Langmuir isotherms (parameters in Table 1), dashed lines for the *R* enantiomer, solid lines for the *S* enantiomer. Eluent pH values are (1) 4.04, (2) 4.47, and (3) 4.97. The figure shows the medium concentration range where the highest mobile phase concentration is 0.11 mM. The top left inset shows the low concentration range (<5.25 μM), and the top right inset shows the high concentration range (up to 2.0 mM).

Table 5. Apparent and True Enantioselectivities and Relative Enantioselectivity Contribution to *Linear* Retention of the Analytes

analyte	pH	α_{app}	α_{true}	relative enantioselectivity contribution (%)[a] first isomer	second isomer
alprenolol	4.04	1.204	1.249	81.7	84.8
	4.47	1.395	1.437	90.3	93.1
	4.97	1.474	1.500	94.7	96.4
1-(1-naphthyl)ethylamine	4.98	1.348	1.805	43.2	57.8
	5.48	1.318	1.618	51.5	63.2
	5.97	1.182	1.294	62.0	67.9
	6.52	1.264	1.397	66.5	73.5
2-phenylbutyric acid	3.98	1.317	1.377	84.1	88.0
	4.47	1.400	1.454	88.1	91.5
	4.97	1.575	1.658	87.4	92.0
methyl mandelate	4.49	1.300	1.416	72.2	78.6
	4.96	1.338	1.434	77.8	83.4
	5.99	1.352	1.410	85.9	89.6

[a] See eq 6.

The enantioselective equilibrium constants for the enantiomers of the amines (the coefficients a_{II}) and the neutral compound increase rapidly with increasing pH, in particular for the more retained (*S*)-alprenolol. The a_{II} coefficients of the first and second eluted enantiomers of alprenolol increased 9.7 and 12 times, respectively, when increasing the pH from 4.0 to 5.0 (cf. Table 1). The corresponding a_{II} coefficients for naphthylethylamine increased 11 and 8.2 times, respectively, when increasing pH from 5.0 to 6.5 (cf. Table 2), and finally, both of the a_{II} coefficients of methyl mandelate increased 2.4 times when increasing the pH from 4.5 to 6.0 (cf. Table 3). For both amines, these trends originate mainly from the strong increases in the enantioselective interaction energies (b_{II}); the enantioselective saturation capacities increased relatively slightly with increasing pH (cf. Tables 1 and 2). This is in contrast to (*S*)-methyl mandelate, in which $b_{S,II}$ and $q_{S,II,s}$ have equal importance (cf. Table 3).

From the above, it can be concluded that when increasing the eluent pH, the retention of the bases (especially for (*S*)-alprenolol) and the aprot increases, mainly as a result of a much stronger enantioselective interaction energy but somewhat also because of a stronger nonselective interaction energy for the amines (especially for naphthylethylamine).

The Acid (Phenylbutyric Acid). The linear retention of the 2-phenylbutyric acid has a maximum at pH 4.5 (cf. Figure 2). This is in contrast to the nonlinear data for the acid in Table 4, for which the nonselective equilibrium constant (a_I coefficient) decreases steadily with increasing pH. It is 53% larger at pH 4.0 than at pH 5.0. For the acid, the decrease in the coefficient a_I with increasing pH is explained by a decrease in the saturation capacity; $q_{I,s}$ is 79% larger at pH 4.0 than at pH 5.0.

The explanation for the unusual linear behavior of the acid can be found in the influence of the pH on the enantioselective equilibrium constants (a_{II}) for both enantiomers. At pH 4.5, $a_{S,II}$

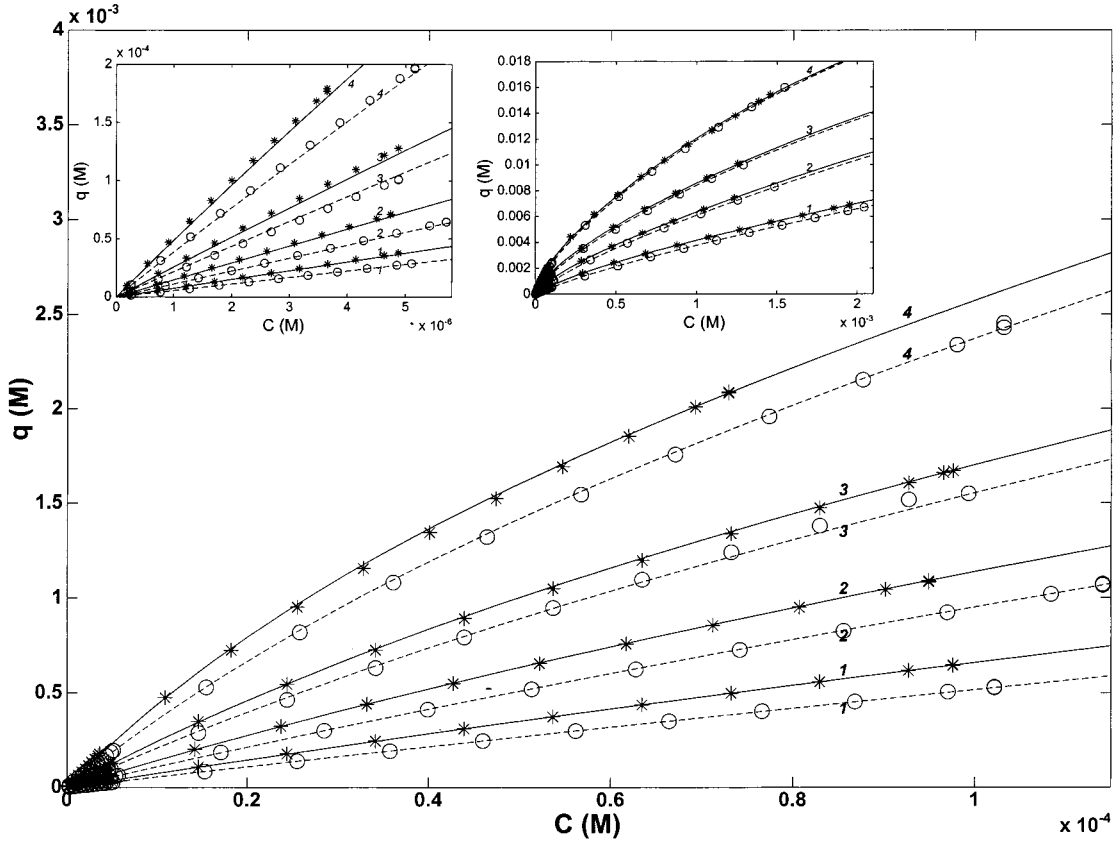

Figure 4. Single-component equilibrium isotherms for *R* and *S* enantiomers of 1-(1-naphthyl)ethylamine at increasing pH. Conditions are as in Figure 2. Symbols indicate experimental data: ○, *S* enantiomer; *, *R* enantiomer. Lines are the best calculated bi-Langmuir isotherms (parameters in Table 2): dashed lines, *S* enantiomer; solid lines, *R* enantiomer. Eluent pH values are (1) 4.98, (2) 5.48, (3) 5.97, and (4) 6.52. The figure shows the medium concentration range where the highest mobile phase concentration is 0.11 mM. The top left inset shows the low concentration range (<5.8 μM), and the top right inset shows the high concentration range (up to 2.1 mM).

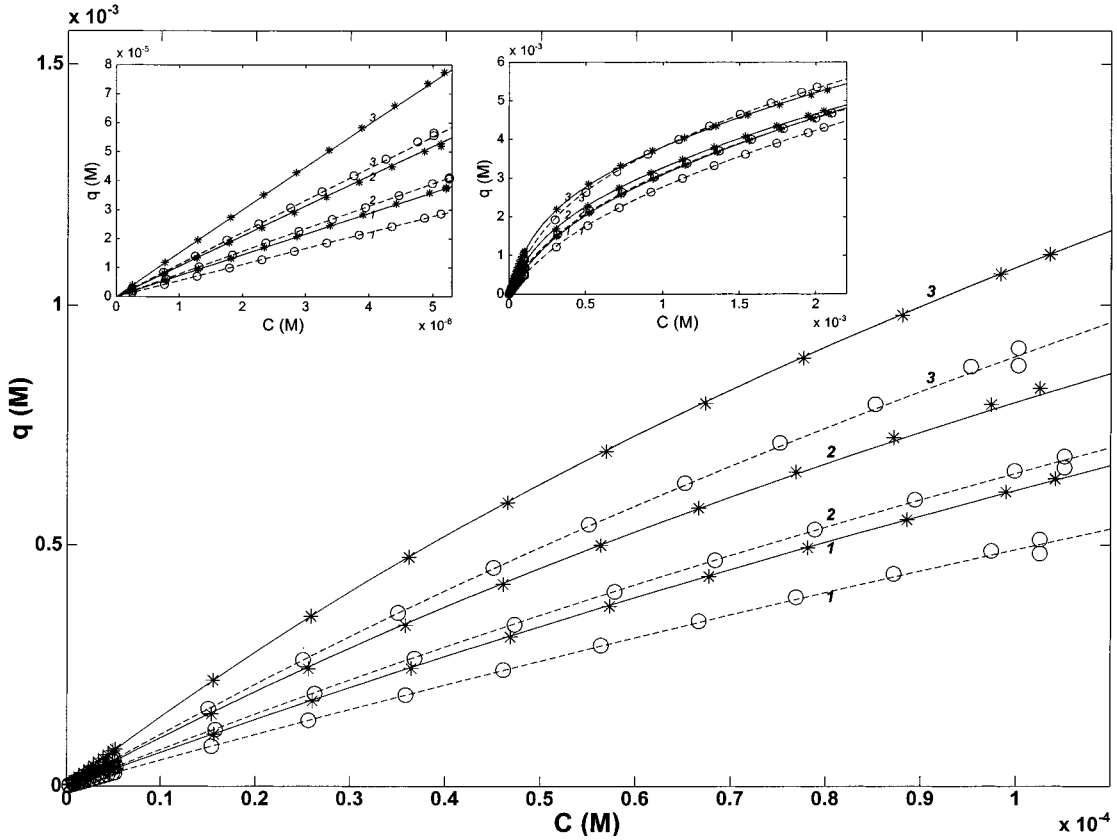

Figure 5. Single-component equilibrium isotherms for *R* and *S* enantiomers of methyl mandelate at increasing pH. Conditions are as in Figure 2. Symbols indicate experimental data: ○, *S* enantiomer; *, *R* enantiomer. Lines are the best calculated bi-Langmuir isotherms (parameters in Table 4): dashed lines, *S* enantiomer; solid lines, *R* enantiomer. Eluent pH values are (1) 4.49, (2) 4.96, and (3) 5.99. The figure shows the medium concentration range where the highest mobile phase concentration is 0.11 mM. The top left inset shows the low concentration range (<5.3 μM), and the top right inset the high concentration range (up to 2.2 mM).

is 35 and 31% larger than at pH 4.0 and 5.0, respectively. At pH 4.5, $a_{R,II}$ is 28 and 49% larger than at pH 4.0 and 5.0, respectively. This trend is mainly due to the interaction energy of the chiral sites (the b_{II} values). The saturation capacity of the first-eluted *R* enantiomer ($q_{R,II,s}$) is nearly independent of pH, and that of the *S* enantiomer ($q_{S,II,s}$) decreases only slightly with increasing pH.

It can be concluded from the above that the unusual retention behavior of the acid (see Figure 2) is entirely due to the enantioselective interaction energies for both enantiomers having a maximum value at pH 4.5, whereas the energies of the nonselective sites decrease steadily with pH (which is expected for an acid with a pK_a ~4).

True and Apparent Enantioselectivity. In Table 5 are presented the apparent (α_{app}) and true (α_{true}) enantioselectivities, together with the relative enantioselectivity contribution to retention (REC) for all compounds at infinite dilutions (i.e., "linear" chromatography). For naphthylethylamine, α_{app} decreases with increasing pH, whereas it increases for all others compounds (cf. Table 5). For naphthylethylamine, the enantioselective equilibrium constant (a_{II}) for the first-eluted enantiomer increases more than the more-retained enantiomer, whereas the converse is true for alprenolol (i.e., the true separation factor decreases for the former and increases for the latter). In the case of methyl mandelate, the term α_{true} is constant, which is logical, because both a_{II} coefficients of methyl mandelate increased 2.4 times (cf. Table 3). However, there is a certain increase in the apparent enantioselectivity with increasing eluent pH, because the relative importance of the nonselective interactions decreases, that is, REC increases (cf. Table 5). We can also see that the true enantioselectivity of naphthylethylamine decreases with increasing pH (Table 5), but in this case, the decrease was due to a stronger increase of the interaction energy for the first eluted enantiomer (cf. Table 2), as compared to the second eluted enantiomer.

For all compounds, the REC increases with increasing pH (Table 5); thus, the column generally becomes more chiral at high pH.

Comparison with Previous Results. The heterogeneous adsorption of enantiomers on AGP generally shows a pattern similar to the previous studies with Cel7A and BSA,[33−35] with a high interaction strength at the low-capacity enantioselective site and a low interaction strength at the nonselective site. However, one exception is alprenolol, which, when adsorbed on AGP, has only an approximately three times higher density of nonselective sites as compared to enantioselective ones. Moreover, with increasing eluent pH, the interaction of both enantiomers of the amines to both types of sites was increased on AGP, whereas in the case of Cel7A, only the interaction of the second eluted enantiomer increased.

The Stoichiometry at the Enantioselective Site. The values of the monolayer capacities are obviously a function of the amount of protein immobilized on the silica matrix. Therefore, evaluation of the stoichiometry of the enantioselective interaction requires determination of the protein concentration in the column combined with the capacity values obtained from the isotherms. The

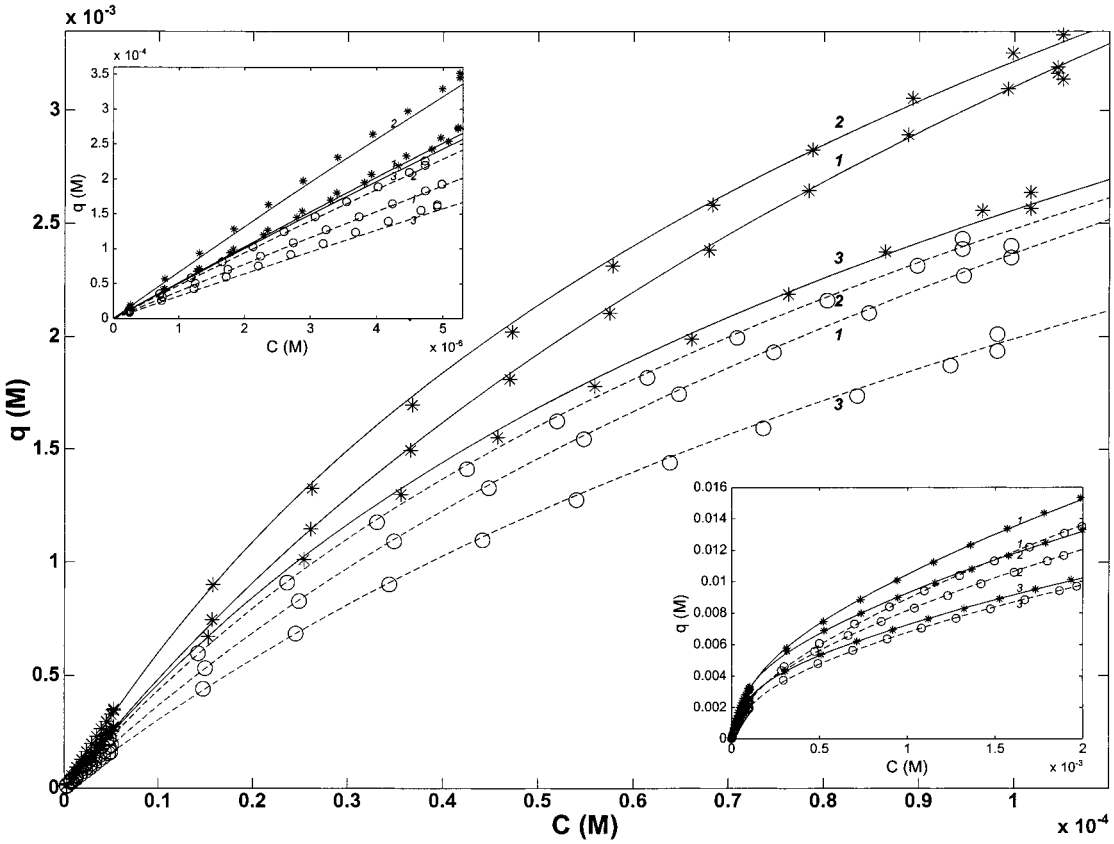

Figure 6. Single-component equilibrium isotherms for *R* and *S* enantiomers of 2-phenylbutyric acid at increasing pH. Conditions are as in Figure 2. Symbols indicate experimental data: ○, *R* enantiomer; *, *S* enantiomer. Lines are the best calculated bi-Langmuir isotherms (parameters in Table 3): dashed lines, *R* enantiomer; solid lines, *S* enantiomer. Eluent pH values are (1) 3.98, (2) 4.47, and (3) 4.97. The figure shows the medium concentration range where the highest mobile phase concentration is 0.11 mM. The inset in the upper left corner shows the low concentration range (<5.3 μM), and the inset in the bottom left corner shows the high concentration range (up to 2.0 mM).

protein concentration (C_{AGP}) is easily calculated from the amount of protein in the column as determined by amino acid analysis, 87.1 mg, and the volume of the stationary phase, 0.33 mL (for further details, see the Experimental Section). In Tables 1−4, the monolayer capacities corresponding to the number of solute molecules that interact with each bonded protein molecule are reported for either enantioselective or nonselective sites obtained from the ratio q_s/C_{AGP}, which for the latter ranges from 1.15 to 7.44. Those values could originate from adsorption on the protein, the matrix, or both. The ratios $q_{1,II,s}/C_{AGP}$ and $q_{2,II,s}/C_{AGP}$ are 0.33−0.44 and 0.40−0.48 for alprenolol, 0.16−0.28 and 0.23−0.30 for naphthylethylamine, 0.51−0.54 and 0.58−0.80 for the acid, and 0.30−0.47 and 0.32−0.43 for the aprot. A value less than unity was expected for all of these ratios because of steric hindrance of access to the enantioselective site and possible degenerative unfolding of some of the bonded protein molecules.

The results from the present study for alprenolol were compared with an earlier study with cellobiohydrolase I (Cel7A). The saturation capacities of the two columns in moles per liter are not easily comparable, because there are different amounts of selector in the columns. For example, $q_{I,s}$ and $q_{2,II,s}$ are 18 and 0.6 mM for Cel7A and 9 and 3 mM for AGP. An incorrect interpretation would be that more analyte is adsorbed at the enantioselective sites of AGP and that maybe even multiple sites exist in the column. The difference between the columns is not the enantioselectivity saturation capacity at the individual protein molecules, but rather the number of immobilized protein molecules in the columns.

Comparing the bi-Langmuir Parameters with Structural Studies. At present, more detailed structural data about the AGP protein and its sites for interaction with drugs (enantioselective and nonselective) are not available. This is probably due to the large amount of covalently bounded carbohydrate (45%),[15] which makes X-ray crystallography difficult. According to Kaliszan et al., the binding site of basic drugs should consist of a conical pocket containing hydrophobic regions at the base and an anionic region close to the spike of the cone.[20]

Our results show that the retentions of both enantiomers of both bases increase dramatically with increasing eluent pH (cf. Figure 2), mostly as a result of a tremendous increase in the enantioselective interaction strength (cf. Tables 1−2). At the same pH (pH 5), the equilibrium strength of the enantioselective interaction is much stronger for alprenolol, as compared to naphthylethylamine ($a_{I,II}$ is 36 times larger for the first eluted enantiomer and 30 times larger for the last eluted enantiomer. Calculations using the Hansch method to estimate the distribution constants of the amines in octanol/water showed that naphthylethylamine ($K_D = 2.69$) is more hydrophobic than alprenolol ($K_D = 2.10$).[41]

(41) Schill, G.; Ehrsson, H.; Vessman, J.; Westerlund, D. *Separation Methods for Drugs and Related Compounds*, 2nd ed.; Swedish Pharmaceutical Press: Stockholm, Sweden, 1983.

Therefore, in contrast to Kaliszan et al.,[20] our results demonstrate that hydrophobicity plays no essential role in the chiral interactions. The structure of the less hydrophobic alprenolol binds much more strongly to the enantioslective site, as compared to the more hydrophobic naphthylethylamine (see above and in Tables 1-2); however, the latter compound binds to a much greater extent to the nonselective interactions, as compared to alprenolol. The capacity of the nonselective site is 5.2 times larger for naphthylethylamine than for alprenolol at the same pH (cf. Tables 1−2, pH 5). In this context, it is worth mentioning that Kaliszan et al. used traditional linear chromatography in combination with chemometrics (thus, the enantioselective sites could not be distinguished from the nonselective ones). However, in line with Kaliszan et al., our results demonstrated that ionic binding between amines and an anionic region on the AGP protein is extremely important for the strength of the enantioselective interaction (however, it is not essential, see below).

The interactions of the neutral compound at the nonselective sites were unchanged with increasing pH, whereas the energy of the enantioselective interactions increased moderately (cf. Table 3), demonstrating that ionic bindings are not essential for the chiral recognition. For the acid, the equilibrium constants of the nonselective interactions decreased steadily with increasing pH, whereas the equilibrium constant of the enantioselective interactions had an maximum at pH 4.5.

CONCLUSIONS

Frontal analysis in the staircase mode was used for nonlinear adsorption studies of different protolytic classes of chiral drugs to α_1-acid glycoprotein. The compounds were retained at different kinds of sites, nonselective and enantioselective, and the adsorption was best fitted by the bi-Langmuir model.

With increasing eluent pH, the interaction of both enantiomers of both the amines to both types of sites was increased; however, the increase in the energy on the enantioselective interaction was much greater than on the nonselective one. At the same pH, the energy of the enantioselective interaction for the amine alprenolol was stronger than that of the more hydrophobic amine naphthylethylamine. For 2-phenylbutyric acid, a very interesting feature was observed: the acid has a maximum of retention at an intermediate pH. The nonlinear studies revealed that the maximum originates solely from enantioselective interactions. For all compounds used, there was a 1:1 stoichiometry at the enantioselective site.

Our results demonstrated that ionic binding is very important for the enhancement of the chiral interaction. On the other hand, the hydrophobicity of the molecule plays no significant role in the chiral interaction. Ionic interactions as well as hydrophobicity play a role in the nonselective interactions of basic drugs.

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SUPPORTING INFORMATION AVAILABLE

Staircase data of (*R*)- and (*S*)-alprenolol at pH 5.0 at the second-highest concentration range. This material is available free of charge via the Internet at http://pubs.acs.org.

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