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DETERMINATION OF BINDING AFFINITY OF ENANTIOMERS TO ALBUMIN BY LIQUID CHROMATOGRAPHY

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SUMMARY

The principles of the determination of the binding affinity constants of small molecules to albumin by liquid chromatography, using albumin as a mobile phase additive, are outlined. Chromatographic conditions for determinations of constants are presented and applied to enantiomers of tryptophan and omeprazole. The influence of albumin on the retaining properties of LiChrosorb RP-8, Phenyl Hypersil and LiChrosorb Diol was studied.

INTRODUCTION

A knowledge of protein binding is of fundamental importance when studying the biological activities of drugs, *e.g.*, in pharmacodynamic and pharmacokinetic studies[1]. The main techniques for determining the interaction between proteins and small ligands are equilibrium dialysis and ultrafiltration, but liquid chromatography can also be used[2]. The chromatographic techniques are fast and generally easy to perform as ordinary high-performance liquid chromatographic (HPLC) equipment is sufficient. The chromatographic technique of Hummel and Dreyer[3] has been used to study the binding of L-tryptophan[4] and warfarin[5]. The affinities of warfarin, furosemide and phenylbutazone[6] and also L-tryptophan[7] to albumin have been studied by adding the protein to the cluent. An equilibrium saturation method[8] has been used to study warfarin–albumin binding and the influence of free fatty acids and sodium dodecyl sulphate on this interaction.

Chromatographic systems with proteins in the mobile or the stationary phase have been utilized for the separation of compounds with different degrees of protein binding. Albumin and α_1-acid glycoprotein are known to bind enantiomers to different extents and can be used as chiral selectors immobilized on a solid phase for the separation of racemates[9–11].

A previous study showed that enantiomeric carboxylic acids can be separated by addition of albumin to the mobile phase[12]. The retention of the enantiomers is regulated

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by the concentration of albumin, pH and type of solid phase. A retention model was presented. In this study the model was applied to the determination of the binding affinities of omeprazole and tryptophan to albumin. The principles for the choice of experimental conditions, *e.g.*, the solute and the albumin concentration, are presented.

Proteins interact strongly with certain silica-based solid phases[13] and it has been found that bovine serum albumin affects the retaining properties of an ODS column[14]. The adsorption of albumin to the solid phases (LiChrosorb Diol, LiChrosorb RP-8 and Phenyl Hypersil) used in the binding studies was investigated.

EXPERIMENTAL

Apparatus

The pump was an Altex Model 110 A solvent metering pump (Beckman, Berkeley, CA, U.S.A.) and the stainless-steel frit in the outlet check valve Kel-F washer was replaced with a PTFE supporting net (from a HibarLiChroCart unit; E. Merck, Darmstadt, F.R.G.). The pump was equipped with a pulse damper (Touzard et Matignon, Vitry, France). The UV detector was a SpectroMonitor D variable-wavelength detector (LDC, Riviera Beach, FL, U.S.A.). The injector was a Rheodyne Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop.

The separation columns (100 × 4.6 mm I.D.) were made of stainless-steel with a polished inner surface, equipped with modified Swagelok® connectors. A precolumn, identical with the separation column and packed with LiChroprep RP-18 (25–40 μm), was inserted before the injector to protect the analytical column from impurities in the mobile phase unless stated otherwise. The precolumn was equipped with stainless-steel frits (2-μm porosity) and the separation columns were equipped with stainless-steel sieve filters (2- or 3-μm porosity) with spreaders.

The chromatographic system was thermostated by a HETO water-bath (Birkerød, Denmark) Type 02 PT 923.

Chemicals

Phenyl Hypersil (5 μm, 120 Å pore diameter, basic silica) was obtained from Shandon (London, U.K.) and LiChrosorb Diol and LiChrosorb RP-8 (5- and 7-μm, respectively, 100 Å pore diameter, basic silica) were obtained from E. Merck.

Human serum albumin (HSA) fraction V, essentially fatty acid free (A-1887), and D- and L-tryptophan were obtained from Sigma (St. Louis, MO, U.S.A.). Racemic 2-(*p*-chlorophenoxy)propionic acid was obtained from Janssen (Beerse, Belgium) and (−)-2-(*p*-chlorophenoxy)propionic acid was kindly supplied by the Department of Organic Pharmaceutical Chemistry, University of Uppsala, Sweden. Racemic omeprazole was a gift from Hässle (Mölndal, Sweden) and N,N-dimethyl-N-octylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.).

All other substances and solvents were of analytical-reagent or reagent grade and used without further purification.

Chromatographic technique

Column packing and column testing were performed as described previously[12]. In the studies, the packings of the analytical columns were fixed with sieve filters, as frits sometimes give rise to sudden pressure increases. Eluents containing albumin gave

rise to slow pressure increases. During 1 week the back-pressure increased from 400–500 to 800 p.s.i. for the LiChrosorb Diol and the Phenyl Hypersil columns and from 450–600 to 1000 p.s.i. for the LiChrosorb RP-8 column.

The mobile phase, the columns and the injector were thermostated at 20.0 $\pm$ 0.1°C. No solvent inlet filter was used and the mobile phases were not recirculated. The flow-rate was 1 ml/min in the tryptophan study and 0.5 ml/min in the omeprazole study. The reference cuvette of the UV detector was filled with mobile phase when albumin was used at concentrations $\geqslant 30\ \mu M$.

The mobile phases were aqueous phosphate buffers with different additives such as albumin, DMOA or inorganic salts. The solutes were dissolved in the mobile phase. Sample solutions and stock solutions of albumin were stored in a refrigerator at 6°C for a maximum of 1 week.

The capacity factor, k', was calculated from the solute retention volume, V_R, and the elution volume of albumin, V_a, from $k' = (V_R - V_a)/V_a$. V_a represents the interparticle volume of the column once it has been equilibrated with an albumin-containing mobile phase. V_a was determined by injection of an excess of albumin or pure phosphate buffer. The total porosity, ε_m, was obtained by injection of sodium nitrate, which was assumed to be unretained.

RESULTS AND DISCUSSION

Secondary equilibria in the mobile phase, *e.g.*, acid–base reactions and complexations, are frequently used to regulate retention and selectivity in liquid chromatography[15]. The chromatographic technique can also be used to evaluate complexation constants[16]. Addition of albumin as a complexing agent to the mobile phase can affect the retention and selectivity of solutes due to protein binding:

$$X + P_i \overset{K_{XP(i)}}{\rightleftharpoons} XP_i \qquad (1)$$

where $K_{XP(i)}$ is the equilibrium constant for binding of X to a binding site (i) on the protein P. This protein–solute interaction has been used to obtain highly selective chromatographic separations, *e.g.*, the resolution of enantiomers[12,17]. An application of the technique with albumin as the chiral selector in the mobile phase is shown in Fig. 1. The two enantiomers of omeprazole are completely resolved ($R_s = 1.7$) within 18 min. The binding affinity of ligands to albumin can be determined from retention data in systems with different albumin concentrations in the mobile phase.

Retention model

The binding affinities of omeprazole ($pK_1 = 4.0$, $pK_2 = 8.7$) and tryptophan ($pK_1 = 2.4$, $pK_2 = 9.4$) were studied at pH 7.4, where omeprazole is mainly uncharged whereas tryptophan has a zero net charge. In the retention model previously derived[12] it was assumed that negatively charged solutes were retained as ion pairs with a counter ion to the stationary phase. Uncharged solutes are retained according to

$$X_m + A_s \overset{K_{XA}}{\rightleftharpoons} XA_s \qquad (2)$$

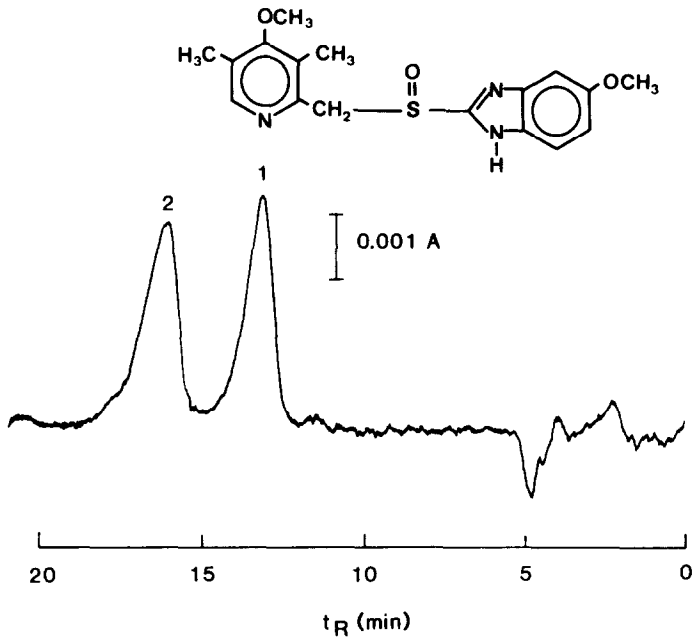

Fig. 1. Resolution of the enantiomers of omeprazole. Solid phase: LiChrosorb Diol. Mobile phase: 50 μM HSA and 0.010 M DMOA in phosphate buffer (pH 6.5, μ = 0.1). Analytical columns: two 100 × 4.6 mm I.D. UV detection: 302 nm. Flow-rate: 0.33 ml/min. The order between the two enantiomers has not been determined.

where K_{XA} is the equilibrium constant for the adsorption of the solute X to the adsorption site A on the solid phase. The monolayer capacity of the adsorbing stationary phase, K° (mol/g), is defined as

$$K^\circ = [A]_s + [XA]_s \tag{3}$$

and the binding of buffer components to the solid phase is assumed to be negligible. The assumption was also made[12] that binding to one site (P_1) on the protein is dominating and that the solute is applied in such a low concentration that the binding isotherms to both the solid phase and the protein are linear. The following equation for the capacity factor, k', was derived:

$$k'_X = \frac{W_s K^\circ K_{XA} + V_{is}}{V_a[1 + n_1 K_{XP(1)} C_p]} \tag{4}$$

where W_s is the weight of the solid phase (g), n_1 is the number of binding sites P_1 of 1 mol of protein, $K_{XP(1)}$ is the equilibrium constant of the binding of the solute X to binding site P_1 on the protein $(mol/l)^{-1}$ (see eqn. 1) and C_p is the total concentration of albumin (mol/l). The stationary phase is defined here as the sum of the phase in the pores not accessible to albumin, V_{is} (ml), and the solid phase. The aqueous phase outside the pores containing albumin, V_a (ml), is regarded as the mobile phase. In eqn.

4 it is assumed that albumin is not affecting the retaining properties of the solid phase. However, the model has to be modified if albumin interacts with the solid phase and thus changes its retaining properties.

Adsorption of albumin

The adsorption of albumin to LiChrosorb RP-8, Phenyl Hypersil and LiChrosorb Diol was studied by frontal analysis. The column was equilibrated with phosphate buffer (pH 6.5, $\mu = 0.1$). A mobile phase containing 30 μM of albumin was then applied and the breakthrough of the protein was registered, as demonstrated in Fig. 2, by the front boundary (1) of the respective solid phase. The amount of albumin adsorbed on the column was calculated by integration between V_m, obtained from the retention of sodium nitrate before the adsorption of albumin, and the breakthrough volume of albumin as shown by the shaded areas in Fig. 2. The amounts adsorbed were 0.86 μmol/g on LiChrosorb RP-8 and 0.24 μmol/g on Phenyl Hypersil. The adsorption to LiChrosorb Diol was too low to be measured with acceptable precision. The amount of albumin adsorbed corresponds to a coverage of about 12% of the total surface area

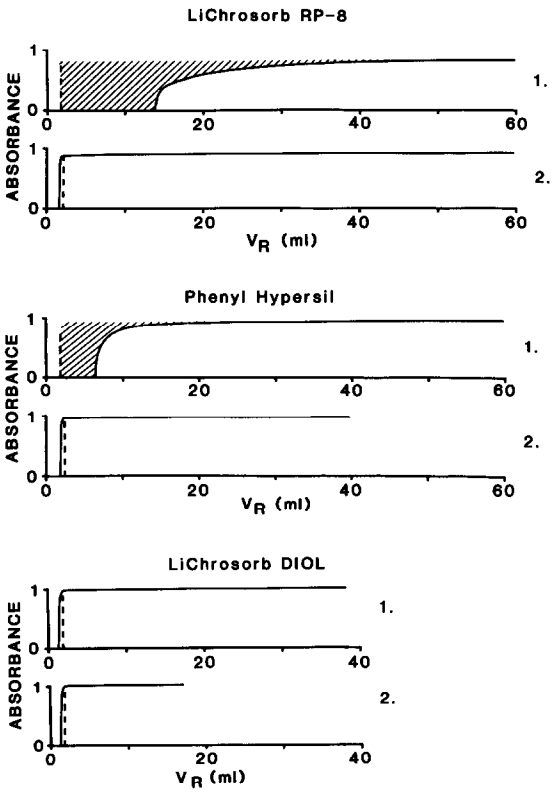

Fig. 2. Frontal analysis of albumin. Mobile phase: 30.5 μM HSA in phosphate buffer (pH 6.50, $\mu = 0.10$). Column: 100 × 4.6 mm I.D. (no precolumn used). UV detection: 280 nm (LiChrosorb RP-8), 276 nm (Phenyl Hypersil and LiChrosorb Diol). Broken lines, V_m. The mobile phase was applied twice, corresponding to front boundaries (1) and (2). Columns were washed with 400 ml of buffer between the applications. The amount of albumin adsorbed was calculated from the shaded areas.

of LiChrosorb RP-8 and 5% of Phenyl Hypersil, assuming a monolayer adsorption of albumin (elliptically shaped, 38 × 150 Å[18]) and using the specific surface areas of the solid phases given by the manufacturers.

The reversibility of the albumin binding was studied by passing 400 ml of the buffer through the column, followed by a new application of the albumin-containing mobile phase. The breakthrough of the albumin now occurred at a volume lower than the V_m obtained with sodium nitrate [see front boundary (2) of the respective solid phase in Fig. 2]. This indicates a high degree of irreversibility for the albumin adsorption, and is in agreement with the observations of Barford and Sliwinski[19] that bovine serum albumin is irreversibly bound to C_8 and C_{18} alkylsilicas from phosphate buffer at pH 2 and 7.

The changes in the binding properties of the columns after equilibration with an albumin containing phase are further illustrated by the retention studies presented in Tables I and II. These were made in connection with the frontal analysis studies. Samples of albumin, sodium nitrate and tryptophan were injected before the application of the albumin-containing phase and albumin and sodium nitrate were injected after the application. Injections of all three samples were made after washing the columns with buffer. After a new application of albumin, the protein and sodium nitrate were injected again. Finally, after washing with buffer, albumin, sodium nitrate and tryptophan were injected. The frontal analysis studies, and also the binding studies presented below, were performed over a 5–7 day period, with the exception of the frontal analysis study with the Diol phase. The decrease in retention volume due to the influence of pure buffer on the solid phase was checked using the Diol phase. Phosphate buffer was recirculated through the column during 19 days after the albumin-containing phase had been washed off, after the first application. After this period a 4% decrease in the retention volume of albumin, sodium nitrate and tryptophan were observed (Tables I and II).

TABLE I

RETENTION VOLUME OF ALBUMIN (V_a) AND SODIUM NITRATE (V_m) AND POROSITY, ε_a, CALCULATED FROM V_a

Mobile phase solvent: phosphate buffer (pH 6.5, μ = 0.1).

*Mobile phase**	*LiChrosorb RP-8*			*Phenyl Hypersil*			*LiChrosorb Diol*		
	V_a(ml)	ε_a	V_m(ml)	V_a(ml)	ε_a	V_m(ml)	V_a(ml)	ε_a	V_m(ml)
Solvent	1.04	0.62	1.21	0.99	0.60	1.11	0.73	0.44	1.10
30 μM HSA** in solvent	0.73	0.44	1.19	0.66	0.40	1.14	0.68	0.41	1.06
Solvent***	0.70	0.42	1.21	0.67	0.40	1.12	0.70 0.67§	0.42 0.40§	1.07 1.04§
30 μM HSA** in solvent	0.68	0.41	1.18	0.64	0.38	1.10	0.66	0.40	1.02
Solvent***	0.68	0.41	1.17	0.67	0.40	1.14	0.66	0.40	1.00

* Applied to an unused column in the order indicated.

** 200 ml passed before injection.

*** 400 ml passed before injection.

§ After 19 days of recirculating mobile phase.

TABLE II

RETENTION VOLUME OF D- AND L-TRYPTOPHAN BEFORE AND AFTER HSA HAS BEEN USED AS A MOBILE PHASE COMPONENT

Mobile phase: phosphate buffer (pH 6.5, μ = 0.1).

Treatment of column	*Enantiomer*	*LiChrosorb RP-8**		*Phenyl Hypersil**		*LiChrosorb Diol**	
		$V_R \pm s$(ml)	n	$V_R \pm s$(ml)	n	$V_R \pm s$(ml)	n
Before application of HSA		12.5 ± 0.03	3	5.17 ± 0.008	6	1.47 ± 0.02	3
After 1st application	D	9.73 ± 0.04	3	4.56 ± 0.005	4	1.42 ± 0.001	3
of 30 μM HSA	L	9.78 ± 0.02	3	4.62 ± 0.03	7	1.40 ± 0.005	3
	D					1.36 ± 0.004**	3
	L					1.36 ± 0.003**	4
After 2nd application	D	9.18 ± 0.06	3	4.41 ± 0.002	4	1.33 ± 0.004	3
of 30 μM HSA	L	9.23 ± 0.02	3	4.50 ± 0.01	4	1.30 ± 0.003	3

* s = standard deviation; n = number of repeated injections.

** After 19 days of recirculating mobile phase.

The adsorption of albumin to the LiChrosorb RP-8 and Phenyl Hypersil phases resulted in a significant decrease in the albumin retention volume (V_a) and of ε_a (Table I). The porosity was calculated from the retention volume of albumin, $\varepsilon_a = V_a/V_0$ (where V_0 is the volume of the empty column tube). ε_a of the phases is close to the value given for a totally excluded solute, $\varepsilon_0 = 0.4$ (the interparticle porosity)[20]. The retention of sodium nitrate (V_m) and, consequently, the total porosity ε_m was almost unchanged.

The results in Table I clearly indicate that part of the aqueous phase in the column is not available to albumin. A further decrease in the retention volume of albumin on LiChrosorb RP-8 and Phenyl Hypersil was obtained after the equilibration with an albumin-containing phase. This decrease remained after changing to an albumin-free mobile phase indicating that the retention change was due to an irreversible process.

On the LiChrosorb Diol phase the retention volume of albumin was almost the same before and after the introduction of albumin in the mobile phase. It is interesting that the adsorption of albumin on LiChrosorb RP-8 and Phenyl Hypersil has such an effect on the binding properties that the albumin retention volume became the same as on LiChrosorb Diol. The reason might be that hydrophobic parts of the albumin molecule are sorbed at the hydrophobic moieties on the surface of LiChrosorb RP-8 and Phenyl Hypersil and the remaining retention is mainly due to interactions with the hydrophilic parts of the three phases.

The effect of albumin adsorption on the retention of tryptophan on the three solid phases is shown in Table II. The adsorption of albumin gave rise to a decrease in the retention, which was 4% on LiChrosorb Diol but 21% and 11% on LiChrosorb RP-8 and Phenyl Hypersil, respectively. The subsequent application of albumin gave a minor decrease in the retention of about 4% on all the supports. As shown in Table II, there is a small difference in the retention volume for D- and L-tryptophan. This indicates that the adsorbed albumin has a low capacity and/or a change in the binding properties of albumin occurs when it is immobilized on the solid phase.

The retention model shown above (eqn. 4) assumes that albumin is excluded

from the pores and that the binding properties of the solid phase are independent of the albumin concentration in the mobile phase. The albumin adsorption studies presented above show that the model is valid when using the LiChrosorb Diol phase. However, when applying the more hydrophobic phases, *e.g.*, LiChrosorb RP-8, the effect of adsorbed albumin has to be taken into consideration. The solid phase can be regarded as a heterogeneous surface with two kinds of binding sites (A, A*) with limited binding capacities[21]. The unmodified solid phase (sites A) has the capacity $K^{\circ\prime}$; $K^{\circ\prime}$ is less than K° (eqn. 3) owing to adsorption of albumin. The capacity of the immobilized albumin, $K^{\circ *}$, is given by

$$K^{\circ *} = [A^*]_s + [XA^*]_s \tag{5}$$

where A* is an adsorption site of the immobilized albumin. Assuming linear binding isotherms to both sites gives the following expression for the capacity factor:

$$k'_x = \frac{W_s\,(K^{\circ\prime}K_{XA} + K^{\circ *}K^*_{XA}) + V_{is}}{V_a\,[1 + n_1 K_{XP(1)}C_P]} \tag{6}$$

where K^*_{XA} is the equilibrium constant of the adsorption of solute X to the site of adsorbed albumin. When determining the binding affinity for solutes it is important to use a column that previously has been exposed to albumin, as the retaining properties of the solid phase might be affected.

Influence of albumin binding on chromatographic behaviour

A protein, P, present in the mobile phase, can affect the retention of a solute, X, by formation of a complex, XP, in the mobile phase. If one binding site of the protein (P_1) is dominant, the stability constant of the complex is defined by

$$\frac{[XP_1]}{[X][P]} = K_{XP(1)} \tag{7}$$

The complexation has a constant influence on the chromatographic retention if the conditions are such that the binding ratio $[XP_1]/[X]$ is unchanged during the elution.

The binding ratio depends on the total concentration of the protein and the magnitude of the binding affinity, as shown by eqn. 8, and also on the total concentration of the solute[22]:

$$\frac{[XP_1]}{[X]} = \frac{n_1\ K_{XP(1)}C_P}{1 + K_{XP(1)}[X]} \tag{8}$$

where C_P is the protein concentration in the mobile phase, and the binding ratio will assume a constant value when $K_{XP(1)}[X] \ll 1$.

The influence on the binding ratio of the magnitude of the three parameters stability constant, protein concentration and solute concentration is illustrated in Figs. 3 and 4. The computations are based on eqn. 8 assuming $n_1 = 1$. Fig. 3 is valid for

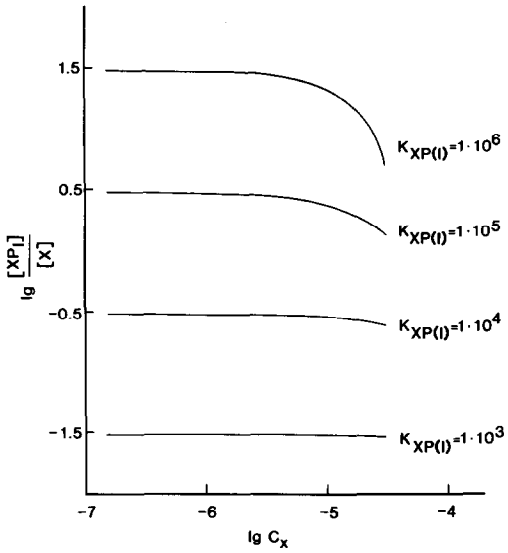

Fig. 3. Calculated solute–protein binding ratios at constant protein concentration (C_p = 30 μM).

a constant protein concentration of 30 μM. It shows that the maximum solute concentration giving a constant binding ratio decreases strongly with increasing $K_{XP(1)}$. Fig. 4 shows the influence of the protein concentration when $K_{XP(1)} = 10^5$. The limiting sample concentration for a constant binding ratio increases with increasing C_p.

When applying the results of the calculations to chromatographic conditions, it must be kept in mind that the calculations are valid for the solute concentrations in the mobile phase. The distribution of the solute to the stationary phase decreases its concentration in the mobile phase and the limiting concentration of the solute in the injected sample will increase with increasing retention. The relationship between the initial solute concentration, C_X°, and the actual concentration in the mobile phase in the

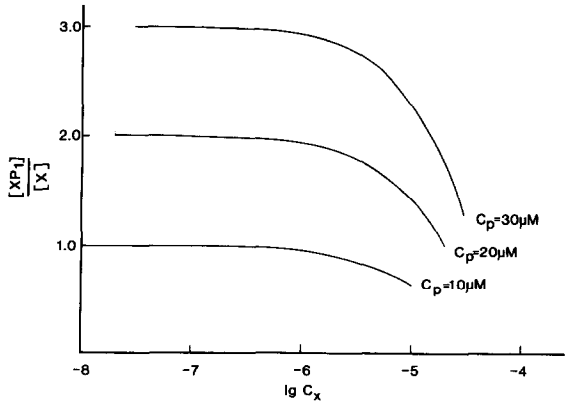

Fig. 4. Calculated solute–protein binding ratios with a binding constant $K_{XP(1)} = 1 \cdot 10^5$.

injected zone, C_X, is given by

$$C_X = \frac{C_X^\circ}{1 + k'_X} \tag{9}$$

where

$$C_X = [X] + [XP_1] \tag{10}$$

It is of vital importance for the chromatographic performance to work in a solute concentration range where the binding ratio $[XP_1]/[X]$ is constant. The maximum concentration of the solute decreases during the chromatographic migration and if the concentration change is accompanied by a change in the binding ratio, it will result in a peak deformation and a concentration-dependent capacity ratio[12].

Evaluation of binding affinity

When the retaining phase is an adsorbent, the capacity ratio can also be influenced by the competition of the solute molecules for its limited binding capacity. However, eqn. 6 is only valid for linear adsorption isotherms, *i.e.*, when the competition for the binding capacity of the adsorbent is negligible.

When the binding ratio is constant and the adsorption isotherm is linear, the binding affinity of the solute to the protein can be determined from a reciprocal plot based on eqn. 6. For an uncharged solute, X, it has the following form:

$$\frac{1}{k'_X} = \frac{1}{q(a + b)} + \frac{n_1 K_{XP(1)} C_P}{q(a + b)} \tag{11}$$

where $q = W_s/V_a$, $a = (K^{\circ\prime} K_{XA} + K^{\circ *} K^*_{XA})$, $b = V_{is}/W_s$, $K_{XA} = [XA]_s/[X]_m\,[A]_s$ and $K^*_{XA} = [XA^*]_s/[X]_m\,[A^*]_s$.

A plot of $1/k'_X$ *versus* C_p should be linear and the affinity constant, $n_1 K_{XP(1)}$, is obtained from the ratio of the slope to the intercept. Thus, modification of $K^\circ K_{XA}$ due to albumin adsorption has no importance in the evaluation of the binding affinity, since this term is eliminated in the calculation of $K_{XP(1)}$.

To obtain estimates of $n_1 K_{XP(1)}$ of acceptable accuracy, the intercept should preferably not be lower than 0.1, which means that the capacity ratio of the solute in the absence of protein should not exceed 10; k'_X lower than 1 should be avoided since it is usually determined with too low precision. The choice of the solid phase then has to be based on the properties of the solute, giving a suitable retention.

The protein concentration in the mobile phase should, if possible, be varied to such an extent that the ratio between the limiting k'_Xvalues is at least 2. The choice of albumin concentration is, however, limited by its UV absorbance. For this reason it is hardly possible to determine binding affinities below 10^3 with this technique. Using UV detection below 300 nm the concentration of albumin is limited to about 80 μM. A higher concentration can be used if the solute or an added UV-absorbing probe (indirect detection) has absorptivity above 300 nm[12].

Determination of high binding affinities requires low protein concentrations. To obtain a sufficient excess of the protein, the solute concentration must be infinitesimal and hence problems with the detectability will occur. In practice the highest binding affinity that can be determined is about 10^5.

The application of these principles to the determination of the binding affinities of omeprazole and tryptophan is demonstrated in Figs. 5 and 6. Both compounds can appear in two enantiomeric forms with different binding affinities.

Omeprazole is highly hydrophobic and a suitable retention was obtained with LiChrosorb Diol as solid phase. The peaks showed strong tailing, indicating non-linear binding to the adsorbent. However, it has been shown[21] that such effects can be eliminated by a competing ion pair, and good peak symmetry and concentration-independent retention were obtained when 0.01 *M* DMOA was present in the mobile phase.

The plotting of the results in accordance with eqn. 11 is shown in Fig. 5. Several injections were made at each albumin concentration and the $1/k'$ range is indicated by bars. The resolution of the enantiomers in the presence of albumin was never less than 0.8 and the overlapping of the two peaks did not affect the k' values. The experiments were performed with successively increasing albumin concentrations in the mobile phase. The resulting affinity constants are given in Table III; the standard deviations were obtained by combining the standard deviations of the slopes with that of the intercept. The constants found are of the same magnitude as those obtained by equilibrium dialysis[23]. The latter study did not give the different affinity constants of the enantiomer as a racemic solute mixture was used.

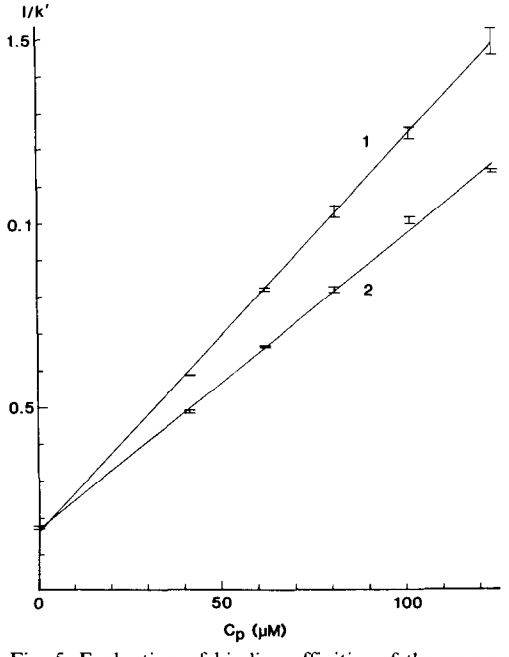

Fig. 5. Evaluation of binding affinities of the omeprazole enantiomers. Solid phase: LiChrosorb Diol. Mobile phase: HSA and 0.010 *M* DMOA in 0.066 *M* phosphate buffer (pH 7.35, μ = 0.17). Analytical columns: two 100 × 4.6 mm I.D. Solute concentration: 6.8 μM of racemate. UV detection: 302 nm.

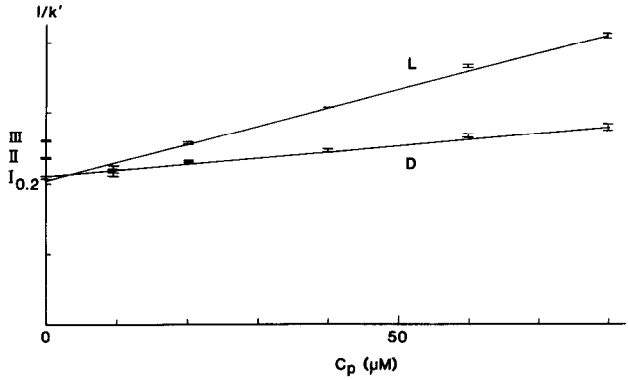

Fig. 6. Evaluation of binding affinities of L- and D-tryptophan. Solid phase: Phenyl Hypersil. Mobile phase: HSA, 0.151 *M* sodium chloride and 0.02% (w/w) sodium azide in 0.040 *M* phosphate buffer (pH 7.40, μ = 0.25). Analytical column: 100 × 4.6 mm I.D. Solute concentration: 10.2 μM of each enantiomer injected separately, except at C_p = 9.60 μM, where the concentration was 2.6 μM. UV detection: 280 nm. $1/k'$ at C_p = 0 obtained after equilibrating the column with albumin of concentration (I) 9.60, (II) 40.0 and (III) 80.0 μM.

Tryptophan is fairly hydrophilic and a suitable retention was obtained with Phenyl Hypersil as the adsorbent; 0.02% (w/w) sodium azide was added to the mobile phase as a bacteriostat. The runs were made with successively increasing concentrations of albumin and the results are plotted in Fig. 6. The $1/k'$ range obtained by repeated injections and the affinity constants found are given in Table III. The affinity constant found for the L-form is in very good agreement with results obtained by affinity chromatography, whereas there is larger difference in the constants for the D-form[24].

Experiments were also carried out to determine the binding affinities of the enantiomers of 2-(*p*-chlorophenoxy)propionic acid to albumin in phosphate buffer (pH 7.4, μ = 0.25). Owing to the high binding affinity of the enantiomers to albumin [$n_1 K_{XP(1)} > 10^5$], a strongly retaining solid phase (LiChrosorb RP-8) had to be used. Good resolution of the enantiomers was obtained but the resulting intercept was too small to enable the affinity constants to be measured with acceptable precision.

Repeated tests of the properties of the solid phases were made during the experimental series by runs at C_p = 0 after washing the columns with 400 ml of an albumin-free mobile phase. On the LiChrosorb Diol phase such test runs were made after the use of 41.4, 81.2 and 124 μM albumin. The k' values decreased successively but the total decrease after the use of 124 μM albumin was less than 2%. The test with the Phenyl column was made after the use of 9.60, 40.0 and 80.0 μM albumin. The k' values at C_p = 0 decreased when exposed to increasing albumin concentration, as indicated by the $1/k'$ values shown in Fig. 6. The first k' value fits the lines in Fig. 6 well. An explanation might be that there is a change in the retention characteristics of the solid phase due to the unfavourable high pH used and/or a rearrangement of adsorbed albumin with time exposing adsorbing sites with different adsorptive properties.

TABLE III
BINDING AFFINITY OF OMEPRAZOLE AND TRYPTOPHAN
Chromatographic conditions as in Figs. 5 and 6.

Compound	$n_1 K_{XP(1)} \pm s^*$	
	*Found***	*Literature*
Omeprazole:		
Enantiomer 1	$(6.6 \pm 0.4) \cdot 10^4$ $(n=15)$	$2 \cdot 10^{4}$****
Enantiomer 2	$(4.6 \pm 0.2) \cdot 10^4$ $(n=14)$	
L-Tryptophan	$(1.29 \pm 0.02) \cdot 10^4 (n=23)$	$1.1 \cdot 10^{4}$§
D-Tryptophan	$(4.4 \pm 0.1) \cdot 10^3$ $(n=23)$	$1.3 \cdot 10^{3}$§

* s = standard deviation; n = total number of injections.

** Evaluated from one experimental series of successively increasing HSA concentration in the mobile phase.

*** Equilibrium dialysis[23] using racemate. $n_1 K_{XP(1)}$ calculated from degree of binding assuming $n_1 = 1$. Buffer composition: phosphate buffer (pH 7.35, $\mu = 0.17$).

§ Affinity chromatography[24]. Mobile phase: phosphate buffer (0.04 *M*, pH 7.4), containing 0.15 *M* sodium chloride and 0.02% (w/w) sodium azide.

CONCLUSIONS

The binding affinity of small molecules to albumin can be determined using the protein as a complexing agent in the mobile phase. The technique is generally applicable to affinity constants $[n_1 K_{XP(1)}]$ in the range 10^3–10^5. Silica-based surface-derivatized solid phases with mean pore diameters of approximately 100 Å can be used, provided that the solid phases are exposed to an albumin solution prior to the binding study. The choice of the solid phase must be based on the solute properties in each instance. The albumin concentration range is limited by its inherent UV absorbance. The method has been applied to the determination of affinity constants of the enantiomers of tryptophan and omeprazole.

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REFERENCES

1 W. J. Jusko and M. Gretch, *Drug Metab. Rev.*, 5 (1976) 43.
2 B. Sebille and N. Thuaud, in W. S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. 2 CRC Press, Boca Raton, FL, 1982, p. 379.
3 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 63 (1962) 530.
4 S. F. Sun and F. Wong, *Chromatographia*, 20 (1985) 495.
5 S. F. Sun, S. W. Kuo and R. A. Nash, *J. Chromatogr.*, 288 (1984) 377.
6 B. Sebille, N. Thuaud and J. P. Tillement, *J. Chromatogr.*, 204 (1981) 285.

7 B. Sebille and N. Thuaud, *J. Liq. Chromatogr.*, 3 (1980) 299.
8 B. Sebille, N. Thuaud and J. P. Tillement, *J. Chromatogr.*, 180 (1979) 103.
9 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 316 (1984) 617.
10 P. Erlandsson, L. Hansson and R. Isaksson, *J. Chromatogr.*, 370 (1986) 475.
11 J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.
12 C. Pettersson, T. Arvidsson, A.-L. Karlsson and I. Marle, *J. Pharm. Biomed. Anal.*, 4 (1986) 229.
13 F. Lottspeich and A. Henschen, in A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Editors), *High Performance Liquid Chromatography in Biochemistry*, VCH, Weinheim, 1985, Ch. 4.3.
14 H. Yoshida, I. Morita, T. Masujima and H. Imai, *Chem. Pharm. Bull.*, 30 (1982) 2287.
15 B. L. Karger, J. N. LePage and N. Tanaka, in C. Horvath (Editor), *High-Performance Liquid Chromatography*, Vol. 1, Academic Press, New York, 1980, p. 113.
16 C. Hórvath, W. Melander and A. Nahum, *J. Chromatogr.*, 186 (1979) 371.
17 J. Hermansson, *J. Chromatogr.*, 316 (1984) 537.
18 T. Peters, Jr., in F. W. Putnam (Editor), *The Plasma Proteins*, Vol. 1, Academic Press, London, 2nd, ed., 1975, p. 147 and ref. 7 therein.
19 R. A. Barford and B. J. Sliwinski, *J. Dispersion Sci. Technol.*, 6 (1985) 1.
20 J. C. Giddings, *Dynamics of Chromatography, Part 1*, Marcel Dekker, New York, 1965, p. 198.
21 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
22 K.-G. Wahlund and T. Arvidsson, *J. Chromatogr.*, 282 (1983) 527.
23 C.-G. Regårdh, M. Gabrielsson, K.-J. Hoffman, I. Löfberg and I. Skånberg, *Scand. J. Gastroenterol.*, 20, Suppl. 108 (1985) 79.
24 C. Lagercrantz, T. Larsson and I. Denfors, *Comp. Biochem. Physiol. C*, 69 (1981) 375.