CHROMSYMP. 1438

DETERMINATION OF BINDING EQUILIBRIUM CONSTANTS BY NUMERICAL SIMULATION IN ZONAL HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY

CLAIRE VIDAL-MADJAR*, ALAIN JAULMES, MICHELLE RACINE and BERNARD SÉBILLE

Laboratoire de Physico-Chimie des Biopolymères, C.N.R.S. U.M. 27, 2 rue Henry Dunant, 94 320 Thiais (France)

SUMMARY

The equation of propagation of a signal of finite concentration through an affinity column is obtained by solving the mass conservation equations of liquid chromatography. Its numerical integration allows peak simulation, curve fitting and thence the determination of the equilibrium isotherm function. This method was applied to the measurement of ligand–protein interactions in zonal elution chromatography. In the range of concentrations studied, a three-parameter isotherm equation is convenient for characterizing the binding of phenylbutazone with human serum albumin on diol-silica. The data were analyzed with two different isotherm models: the two-independent-site model with specific and non-specific interaction and the stepwise-multiple-interaction model. The effects of stationary-phase coverage and temperature were studied. From these results were determined the amount of active immobilized protein and the equilibrium constant characterizing the affinity interaction (2.6 l/μmol), which is about twice as large as the value measured in solution.

INTRODUCTION

Affinity chromatography, a powerful technique for purifying biological materials, is also an important physicochemical method for the direct evaluation of the molecular interactions between soluble and immobilized biomolecules[1]. Since its combination with high-performance liquid chromatography (HPLC), the resulting analytical method, high-performance affinity chromatography (HPAC), has gained in speed and reproducibility[2].

Two classical elution techniques are used to measure molecular interactions: the frontal and the zonal elution methods. They differ by the input injection signal. In frontal elution, the solute concentration is suddenly raised and maintained at a constant value (Dirac step function). The binding isotherm is obtained directly from the breakthrough curves when the system is at quasi-equilibrium[3]. In zonal elution, the input signal is a short rectangular pulse. This technique is generally preferred to frontal elution because of its simplicity and the small amounts of sample required. However, because of difficulties in the mathematical treatment, its exploitation was limited to the

linear case for retention volumes extrapolated to zero sample size[1], but recently it was extended to a Langmuir-isotherm retention and an analytical expression of the elution zone was given[4,5].

In zonal elution, the retention volume and the shape of the output signal contain all the information needed to determine kinetic and equilibrium constants. It is the mass balance equations of chromatography which describe the propagation of signals through a chromatographic column[6]. They depend on the chemical equilibrium function and can be integrated by using the numerical procedures based on Godunov's method[7-9] or on Craig distribution computer modelling[10-12], without any limitations concerning the equilibrium isotherm model. These computer simulation methods were essentially developed to predict the elution behaviour under the overload conditions of preparative chromatography[13,14].

The aim of the present study is to show that the same numerical simulation approach can be used in HPAC to determine, by zonal elution, the partition isotherm characterizing the interaction of the solute and the immobilized molecule. The method is applied to a study of the interaction of a small molecule (phenylbutazone) with human serum albumin (HSA) immobilized on an HPLC support. The binding of drugs and small molecules to immobilized albumins has previously been studied by frontal elution[15,16], and bovine serum albumin (BSA) is mainly used as a chiral stationary phase in HPAC[17-22].

The characterization of the ligand interaction with the immobilized protein is useful for a better understanding of the mechanisms of separation of a small molecule on affinity columns. Moreover, the physiological importance of albumin has led to a great number of *in vitro* binding studies of drugs with albumin. It is interesting to compare the equilibrium constants, measured at the liquid–solid interface, with those of the ligand protein interacting in solutions and measured by different techniques[23], including HPLC[24], frontal analysis[24] and equilibrium/saturation methods[25,26].

THEORETICAL

The mass conservation equation of solute A during the propagation process is

$$\frac{\partial \bar{C}}{\partial t} + u \cdot \frac{\partial \bar{C}}{\partial z} - D \cdot \frac{\partial^2 \bar{C}}{\partial z^2} = - \frac{1}{V_0} \cdot \frac{\partial(\bar{Q}_A + \bar{Q}'_A)}{\partial t} \tag{1}$$

where $\bar{C}$ = the total concentration of solute A in the mobile phase, z = the abscissa of the slice along the column length ($z = 0$ corresponds to the inlet, $z = L$ to the outlet of the column), t = the time elapsed from the moment of injection, u = the average velocity of the liquid phase, D = the dispersive coefficient or global diffusion parameter, V_0 = the liquid external volume, $\bar{Q}_A$ = the total amount of solute A immobilized on the solid surface by affinity, $\bar{Q}'_A$ = the total amount of solute A inside the pores, $\bar{Q}^*_A$ and $\bar{Q}'_A{}^*$ = the values of $\bar{Q}_A$ and $\bar{Q}'_A$ at equilibrium.

The kinetic effects may be written in the global expression (where α is the rate constant):

$$\frac{\partial(\bar{Q}_A + \bar{Q}'_A)}{\partial t} = \alpha \left[(\bar{Q}_A + \bar{Q}'_A) - \bar{Q}^*_A + \bar{Q}'_A{}^*) \right] \tag{2}$$

When equilibrium is achieved

$$\bar{Q}_A'^* = \bar{Q}_A' = V_i \sigma_A \bar{C} \tag{3}$$

$$\bar{Q}_A^* = \bar{Q}_A = f(\bar{C}) \tag{4}$$

where $f(\bar{C})$ = the equilibrium isotherm characterizing the interaction of solute A with the affinity matrix X, V_i = the liquid internal pore volume, σ_A = the permeation coefficient of solute A and $V^* = V_0 + \sigma_A V_i$.

In the absence of kinetic effects, the mass balance equation of chromatography reduces to:

$$\frac{V^*}{V_0} \cdot \frac{\partial \bar{C}}{\partial t} + u \cdot \frac{\partial \bar{C}}{\partial z} + \frac{1}{V_0} \cdot \frac{\partial \bar{Q}_A}{\partial t} = D \cdot \frac{\partial^2 \bar{C}}{\partial z^2} \tag{5}$$

The propagation of the concentration profile depends on the equilibrium between the amount of solute, $\bar{Q}_A$, and the concentration in the liquid phase (eqn. 4).
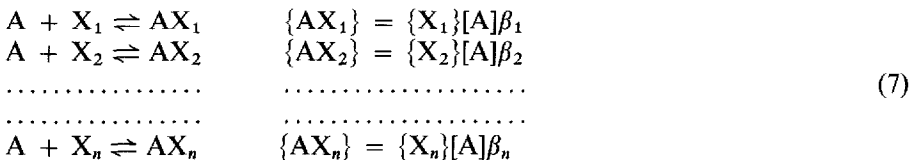
In previous papers[7,8] we have shown that it is possible to perform an approximate integration of eqn. 5 with any model of isotherm by using a numerical procedure where discrete length steps, Δz, and time steps, Δt, are used in the integration method. The dispersive effect corresponding to the second-order term is well approximated when Δz is equal to H, the height equivalent to a theoretical plate (HETP):

$$\Delta z = H = 2Dt_R/L \tag{6}$$

The chromatographic peak is then defined by the shape of the initial injection signal, the amount injected (or peak area), the global dispersive coefficient, D, and the equilibrium isotherm, $\bar{Q}_A = f(\bar{C})$; D is an apparent diffusion coefficient, accounting for the contributions to band broadening at zero concentration, including diffusion-limited kinetic effects[14].

In most cases, the ligand/albumin equilibria have been analysed according to the Scatchard model[27], assuming that the ligand is bound to classes of identical, independent binding sites. The alternative way of analysing the data is the use of a multiple-stepwise-equilibrium model[28].

In the case of the Scatchard model, the chemical equilibria may be written in the form (where the brackets [ ] represent concentrations, in mol/cm^3, in the liquid phase, and the braces { } surface concentrations, in mol/m^2):

$$
\begin{aligned}
A + X_1 &\rightleftharpoons AX_1 & \{AX_1\} &= \{X_1\}[A]\beta_1 \\
A + X_2 &\rightleftharpoons AX_2 & \{AX_2\} &= \{X_2\}[A]\beta_2 \\
\cdots\cdots\cdots & & \cdots\cdots\cdots\cdots \\
\cdots\cdots\cdots & & \cdots\cdots\cdots\cdots \\
A + X_n &\rightleftharpoons AX_n & \{AX_n\} &= \{X_n\}[A]\beta_n
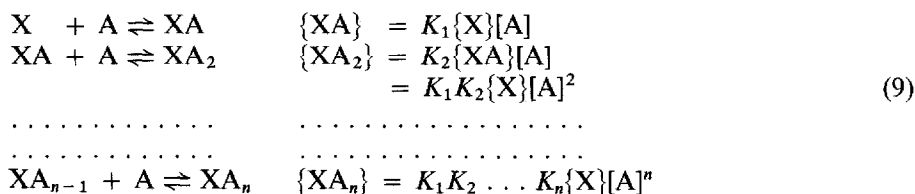\end{aligned}
\tag{7}
$$

The corresponding isotherm is given by the equation

$$\bar{Q}_A = \sum_{i=1}^{n} \{AX_i\}S = \sum_{i=1}^{n} \bar{Q}_{X_i} \cdot \frac{[A]\beta_i}{1 + [A]\beta_i} \tag{8}$$

where β_i is the association constant between A and X_i (class i sites) $\bar{Q}_{X_i}$ is the amount in moles of class i sites, and S is the surface area of the affinity sorbent. The parameter $\bar{Q}_X$ is defined as follows:

$$\bar{Q}_X = \sum_{i=1}^{n} \bar{Q}_{X_i}$$

The second system has been used by Lagercrantz *et al.*[15,16] to analyse the binding of a small molecule to immobilized serum albumin. It involves an immobilized protein, X, carrying several sites and a low-molecular-weight ligand, A, in solution. The equilibria are as follows:

$$
\begin{aligned}
X + A &\rightleftharpoons XA & \{XA\} &= K_1\{X\}[A] \\
XA + A &\rightleftharpoons XA_2 & \{XA_2\} &= K_2\{XA\}[A] \\
& & &= K_1 K_2\{X\}[A]^2 \\
& \cdots\cdots\cdots & & \cdots\cdots\cdots\cdots \\
& \cdots\cdots\cdots & & \cdots\cdots\cdots\cdots \\
XA_{n-1} + A &\rightleftharpoons XA_n & \{XA_n\} &= K_1 K_2 \ldots K_n\{X\}[A]^n
\end{aligned} \tag{9}
$$

where $K_1, K_2, \ldots K_n$ are the successive association constants and n the number of sites.
The expression of the equilibrium isotherm, deduced from the above equilibria, is

$$\bar{Q}_A = \bar{Q}_X[A] \cdot \frac{K_1 + 2K_1 K_2[A] + \ldots + nK_1 K_2 \ldots K_n[A]^{n-1}}{1 + K_1[A] + K_1 K_2[A]^2 + K_1 K_2 \ldots K_n[A]^n} = \bar{Q}_X y \tag{10}$$

where $\bar{Q}_A$ is the total amount of ligand bound to the matrix and y is the molar ratio between the bound ligand and the immobilized protein.
The physical meaning of both models has been discussed by Klotz and Hunston[28]. The identification of the constants of eqn. 10 with those of the Scatchard model is difficult with an analytical expression, except for K_1:

$$K_1 = \sum_{i=1}^{n} \bar{Q}_{X_i}\beta_i/\bar{Q}_X \tag{11}$$

In this work, both models will be considered as satisfactory approximations of the experimental isotherms.

EXPERIMENTAL

The reagents used were HSA (A1887, fatty-acid free) and phenylbutazone from

Sigma (St. Louis, MO, U.S.A.), diol-bonded Lichrospher Si-100 (diameter 10 μm, pore size 100 Å) from Merck (Darmstadt, F.R.G.).

The Schiff-base method was used to immobilize HSA on the diol support by the same procedure as that described previously for protein immobilization[2]. Supports of variable HSA coverage were prepared by changing the concentration of the protein solution in contact with the aldehyde support. The medium-coverage HSA sorbent was prepared from a 2-ml solution (4 mg/ml HSA) per g of diol-silica, and for the high-coverage sorbent a 10 mg/ml HSA solution was used. After protein immobilization, the excess of aldehyde groups were reduced by addition of sodium tetrahydroborate[2]. The amount of protein immobilized was determined by the Lowry method.

The HPLC system consisted of a pump (Model 2150; LKB, Bromma, Sweden), a 7125 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop and an UV detector (Model SPD 6A; Shimadzu, Kyoto, Japan), operated at 268 nm. Its response curve was linear within the whole range of solute concentrations studied.

The analogue output of the detector was connected to a digital voltmeter (Model 3497; Hewlett-Packard, Palo Alto, CA, U.S.A.), which displays the data in four digit precision. The data were stored on floppy disks. The theoretical treatments were carried out in Fortran language, using a microcomputer (Model AT2; IBM, Greenock, U.K.).

The stainless-steel column (50 mm × 4.1 mm I.D.) was vacuum-slurry-packed. It was placed in a water-bath, and its temperature was controlled within $\pm 0.1°$C. The eluent was 0.067 M (pH 7.4) potassium phosphate buffer. The liquid volume of the column was determined from the retention time of ^{2}H$_2$O. A weak, non-specific retention was observed on the diol-silica ($k' = 0.6$), but it was larger on aldehyde-silica ($k'_0 = 2.2$ at 37°C), the activated support used for protein immobilization.

RESULTS AND DISCUSSION

Curve fitting procedure
The changes in the elution profiles obtained were studied by injecting increasing amounts of phenylbutazone into the affinity column. The partition isotherm indicates a saturation effect wich causes tailing peaks for large sample sizes (Fig. 1).

The multisite-equilibrium model. The theoretical model first assumed for the partition isotherm is that of multisite adsorption. The equation for this was introduced into the numerical integration program, and its parameters were determined from best fitting between experimental and theoretical results. For the range of concentration studied, a three-parameter isotherm is sufficient to describe the whole set of elution peaks obtained at various concentrations on the medium-coverage HSA column (Fig. 1). The isotherm equation (eqn. 8) reduces to

$$Q_A = Q_{x_1} \cdot \frac{[A]\beta_1}{1 + [A]\beta_1} + k'_2[A]V^* \tag{12}$$

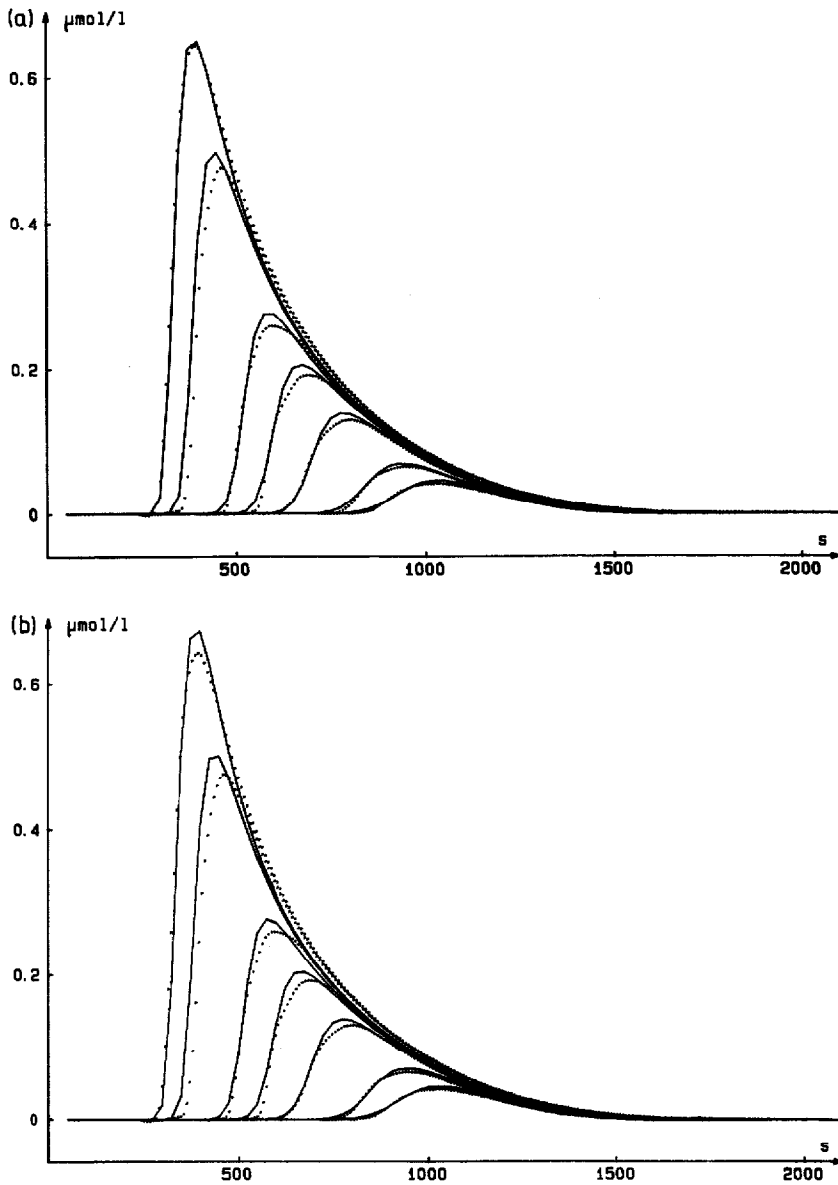where $k'_2 = Q_{x_2}\beta_2/V^*$.

Fig. 1. Elution peak of phenylbutazone for medium-coverage immobilized HSA. Column length: 5 cm. Temperature: 37°C. Flow-rate; 1 ml/min. Eluent: 0.067 M phosphate buffer (pH 7.4). ...., Experimental values stored by computer data acquisition; ———, best fit of the simulated elution peak. (a) Two-independent-site isotherm model (one specific, the other non-specific); $\beta_1 = 2.6$ l/μmol; $Q_{X_1} = 6.5$ nmol; $k'_2 = 4.4$; $H = 0.55$ mm. (b) Stepwise-multiple-equilibrium-isotherm model; $K_1 = 2.7$ l/μmol; $K_2 \ldots K_5 = 0.25$ l/μmol; $Q_X = 7.5$ nmol; $H = 0.55$ mm.

The limiting capacity factor, k_0', is then given by:

$$k_0' = \beta_1 Q_{x_1}/V^* + k_2' \tag{13}$$

This model describes a solute-immobilized ligand equilibrium with two groups of sites: one of high affinity and a second one of low affinity with non-specific interaction.

For the low-concentration peak a two-parameter equation fits the experimental elution peak as well as the three-parameter model, with a binding constant of $2.9 \cdot 10^{-3}$ μmol/l in the first case and $2.85 \cdot 10^{-3}$ μmol/l in the second one. The two-parameter isotherm equation is then of the Langmuir type

$$Q_A = k_0' V^* \cdot \frac{[A]}{1 + \beta[A]} \tag{14}$$

where $\beta = \beta_1(1 - k_2'/k_0')$. Both isotherms (eqns. 12 and 14) have equal second derivatives at the origin.

In Fig. 2, one can compare the elution peaks simulated from the Langmuir-type model (two-parameter isotherm) with those obtained from the two-site model having the same first two derivatives at the origin. The elution profiles coincide only in the low-concentration range (0–1 μmol/m^2), but marked differences between both models are observed at higher concentrations. Furthermore, the isotherm curves for both models are identical near the origin (Fig. 3). The isotherm function with two types of sites (specific and non-specific) increases linearly at high solute concentration.
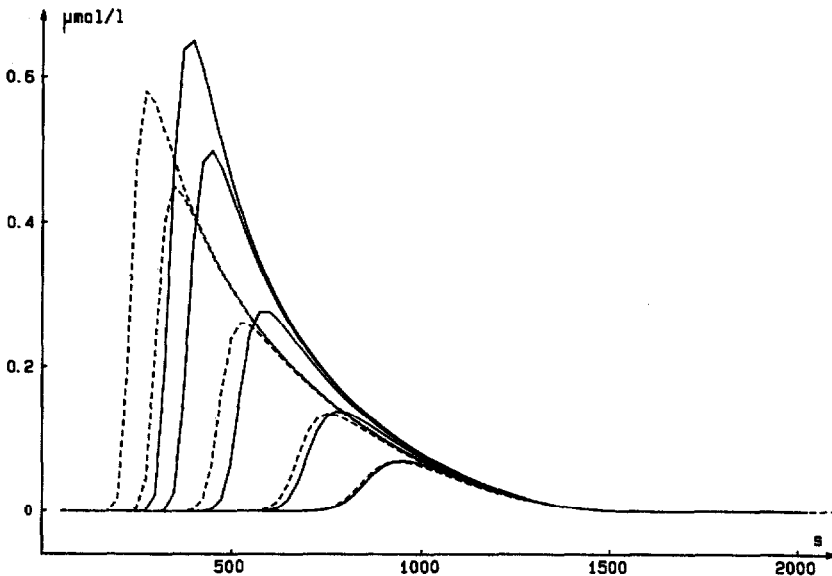

Fig. 2. Simulated elution peaks with the same first and second derivatives of the isotherm at the origin. ...., Two-parameter isotherm (eqn. 14); ————, three-parameter isotherm (eqn. 12).

Although the solute concentrations are ten times larger in the injection signal than at the column outlet, the simulation results show that the shape of the higher-concentration portion of the isotherm has only a weak influence on the low-concentration elution peaks.

The strategy of adjusting the theoretical model to the experimental one is to fit the low-concentration peak with a Langmuir isotherm. For the largest elution peaks, when important deviations between theory and experiment are observed, the three-parameter isotherm is used with the same first and second derivatives as for the Langmuir-type model.

The error of the parameters was determined according to the mathematical scheme of Phillips and Eyring[29]. The relative error at a 90% confidence level of the first derivative at zero concentration, k'_0, is 0.5%, that of the second derivative at zero concentration, k''_0, is 7% and that of k'_2 is 20%. Hence the corresponding relative errors of β_1 and Q_X are 10%.

The stepwise-multiple-equilibrium model. The data were analysed with the stepwise-multiple-equilibrium isotherm (eqn. 10). As with the previous model, good convergence of the curve fitting procedure is observed only with a three-parameter isotherm. Here, we assumed that the successive association constants, except the first one $(K_2, K_3, \ldots, K_n)$ are equal. As in the previous case, the first and second derivatives at the origin must be equal to those of the Langmuir-type isotherm, determined from the low-concentration elution peaks. In this case, $Q_X K_1 = k'_0/V^*$ and $\beta = K_1 - 2K_2$. As shown in Fig. 1b, with this model, the adjustment is as good as that in Fig. 1a, where the two-independent-site model was considered.
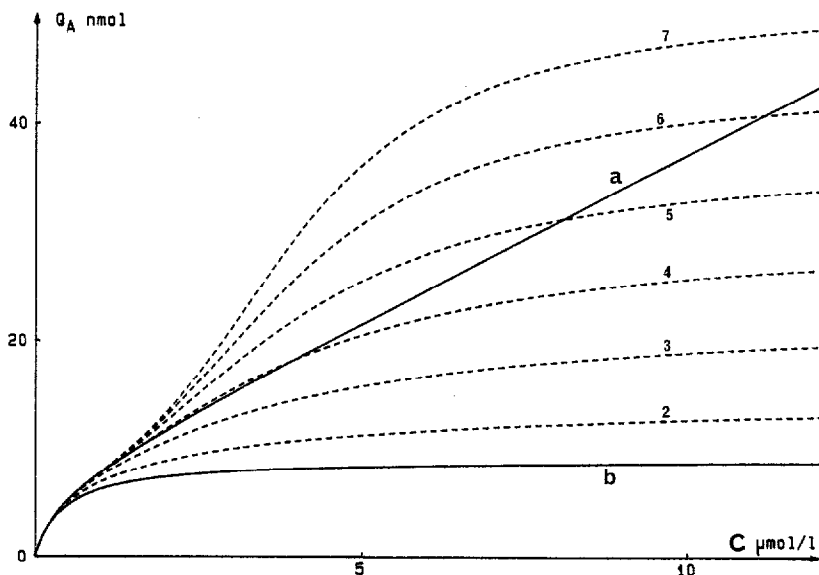

Fig. 3. Equilibrium isotherm of phenylbutazone on medium-coverage immobilized HSA. ———, (a) Independent-site-isotherm model (eqn. 12); ———, (b) Langmuir-type isotherm (eqn. 14). Same experimental conditions as in Fig. 1. ...., Stepwise multiple-equilibrium isotherm (eqn. 10). 2—7: number of steps or order of the polynomial expansion.

As in the previous case, the relative errors of K_1 and Q_X estimated at the 90% confidence level are 10%, but those of K_2 (relative error 20%) are larger.

The isotherm for this model was compared with that of the two-independent-site model used previously (Fig. 3). The best fit of the theoretical peak to the experimental one is obtained with a five-term polynomial expansion. When the order of expansion is increased, the isotherm shows two inflection points, starting with the fifth order; the presence of inflection points should generate shocks, *i.e.*, vertical parts in the calculated elution peaks, which are not observed in the experimental ones. The assumption of equal higher-order constants is unrealistic; it would be more likely for them to decrease in the order $K_2, K_3, \ldots, K_n$. However, since we could not obtain any precision in their determination, we limited the model to a three-parameter isotherm, which allows a good convergence in the determination of parameters Q_X and K_1. The value of K_2 can be taken only as a rough approximation, and only the order of magnitude is significant.

Effect of the amount of HSA immobilized

The effect of stationary-phase coverage on the binding properties of immobilized HSA was determined from the elution profiles of phenylbutazone injected at various concentrations. The equilibrium isotherm was determined on two columns with HSA immobilized in different amounts: Fig. 1a and b illustrate the peak adjustment for experiments made on the medium-coverage silica and Fig. 4 for that with high stationary-phase coverage. The results of the best fit by numerical simulation obtained with the isotherm models discussed above are summarized in Table I.

The retention volume of phenylbutazone increases in proportion to the amount of HSA immobilized within the errors of measurement of the effective amount of HSA immobilized; but the characteristics of the equilibrium isotherm are quite similar for both the medium- and high-coverage HSA–silica. The values (measured at 37°C) of the binding constant, β_1, characterizing specific interaction in the independent-site model, are equal for both HSA coverages. The capacity factor, k'_2, for non-specific interactions is roughly proportional to the amount of HSA immobilized. This shows that the non-specific sites are most probably those of the protein. Moreover, a weak retention is observed on diol-silica with a much lower capacity factor, $k'_0 = 0.6$.

The amount (in nmoles) of active protein can also be determined by this method. Its value is about half of the amount of protein effectively immobilized on the support. This is not surprising, since during the immobilization process some active groups of the protein will be bound to aldehyde-silica.

Table I also displays the constants when the experimental data are analysed with the stepwise-multiple-equilibrium model. The adjustment of the theoretical peaks obtained with this model for the high-coverage silica is almost identical to that obtained with the two-independent-site model, considered in Fig. 4 and is not given here. The binding constant, K_1, measured with the high-coverage sorbent is equal to that measured with the medium-coverage sorbent. Its value ($K_1 = 2.7 \, l/\mu mol$) is close to the binding constant ($\beta_1 = 2.6 \, l/\mu mol$) characteristic of the specific interaction between the solute and immobilized protein in the two-independent-site model (Table I).

The above results demonstrate the validity of the numerical method of determining the isotherm equilibrium from the elution peak analysis, since the equilibrium constants measured at high and medium stationary-phase coverage are in excellent agreement.
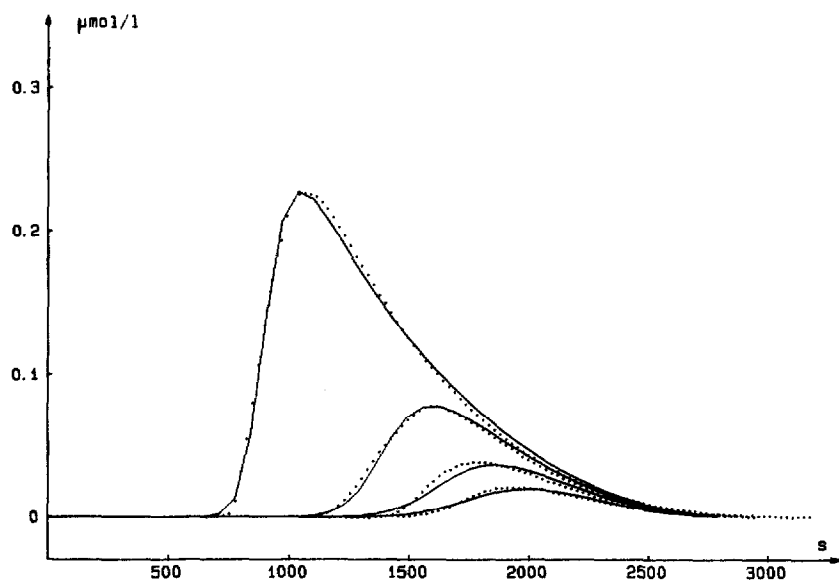
Fig. 4. Elution peak of phenylbutazone on high-coverage immobilized HSA. Column length: 5 cm. Temperature: 37°C. Flow-rate: 1.5 ml/min. Eluent: 0.067 M phosphate buffer (pH 7.4). ...., Experimental values stored by computer data acquisition; ———, best fit of the simulated elution peak with the two-independent-site isotherm model; $\beta_1 = 2.6$ l/μmol; $Q_{X_1} = 19$ nmol; $k'_1 = 9.1$.

Effect of temperature

The chromatographic behaviour of phenylbutazone, eluted in finite concentration in the presence of medium-coverage HSA, was studied as a function of temperature. For every temperature, the solute/immobilized HSA equilibrium isotherm was determined from the best fit of the simulated peak to the experimental one. The results are summarized in Table I. The equilibrium constants, characterizing the specific interaction of phenylbutazone with immobilized HSA, are similar for both

TABLE I

PARAMETERS OF THE EQUILIBRIUM ISOTHERM MODELS USED FOR NUMERICAL SIMULATION OF ELUTION PEAKS

Temperature and stationary phase coverage effects.

Immobilized HSA	t (°C)	H (mm)	Two classes of independent sites			Stepwise multiple equilibrium		
			Q_{X_1} (nmol)	β_1 (l/μmol)	k'_2	Q_X (nmol)	K_1 (l/μmol)	$K_2 \ldots K_5$ (l/μmol)
High coverage (40 nmol)	37	0.70	19.0	2.6	9.1	20.5	2.7	0.22
Medium coverage (15 nmol)	37	0.55	6.5	2.6	4.4	7.5	2.7	0.25
	30	0.64	7.9	2.9	4.4	8.8	3.0	0.25
	25	0.76	6.7	3.3	3.1	7.0	3.4	0.22
	20	1.00	5.7	4.3	5.7	6.4	4.4	0.35

isotherm models considered in this work. Their variations with temperature correspond to the Van 't Hoff plot. The molar enthalpy change, $\Delta \bar{H}$, measured from this plot, is -17 kJ/mol (standard deviation 20%).

The amount of active, immobilized protein should be independent of temperature. The calculated values are within a $\pm 20\%$ interval. This rather wide range, somewhat larger than the confidence level of 10%, is partly due to the curve fitting method, which leads to a $\approx 10\%$ error of the parameter determination. However, the main reason for this apparent discrepancy lies in the fact that the retention volumes measured on immobilized HSA are not constant and decrease perceptibly day by day. The reasons for this lack of stability are not clear. It may be due to protein degradation or to contamination by organic substances or microbial agents. The problem of the long-term stability of immobilized serum albumin is well known to column manufacturers and was carefully studied by Aubel and Rogers[22]. The advantage of the method we have developed in this work is that it allows peak shape analysis to be used to determine, at any stage, the amount of protein immobilized that is effectively reactive towards the drug.

Comparison with equilibrium measurements in solution

It is interesting to compare the association constant for phenylbutazone and HSA, measured with an immobilized protein, with the association constant measured in solution. However, prior to discussing this point, it is necessary to outline that, although the association constants of the equilibrium models are measured with the same units as those in solution, they do not refer to the same species, since some will refer to soluble HSA, others to HSA immobilized on the sorbent (eqn. 7 or 9). In affinity chromatography, we deal with heterogeneous equilibria involving surface concentrations; therefore, it is questionable to compare the data measured in affinity chromatography for solute/immobilized ligand association with those measured in the liquid phase, where one deals only with homogeneous equilibria and plain volumetric concentrations.

Moreover, the equilibrium constant, K_1, characterizing the solute/affinity sorbent interaction reflects an overall phenomenon, the interaction of the solute with the immobilized protein and with the sorbent. The contribution of the diol-silica to the total interaction is difficult to evaluate, but it seems to be small, since phenylbutazone is eluted with a weak retention and a symmetrical peak from the bare support.

The affinity constant, K_1, measured in this work (2.6 or 2.7 l/μmol, Table I), which characterizes the affinity interaction, is somewhat larger for the phenylbutazone–immobilized HSA interaction than the value measured in solution (Table II). The data in the literature were compiled from measurements made in the same solvent as in this work, and are scattered over a wide range, from 0.16 to 2.3 l/μmol. Boobis and Chignel[35] have shown that, as the concentration of albumin decreases, the binding of phenylbutazone increases. This phenomenon, which does not seem to be due to molecular aggregation at high protein concentrations, may explain the great scatter of the values for the equilibrium constants, reported in the literature, and the large value for the affinity constant characterizing the interaction of phenylbutazone with immoblized HSA.

TABLE II

COMPARISON OF ASSOCIATION CONSTANTS OF PHENYLBUTAZONE AND HSA, MEASURED BY DIFFERENT TECHNIQUES

t (°C)	$K_1 = \Sigma n_i \beta_i$ (l/μmol)	HSA concentration range (%)	Method
37	2.6	Immobilized	HPAC (this work)
37	0.85	0.01–0.2	Equilibrium-saturation HPLC[26]
36	2.3	Not given	Ultrafiltration[30]
37	0.16	0.2	Ultrafiltration[31]
37	0.35	0.2	Equilibrium dialysis[32]
37	0.75	0.2	Dynamic dialysis[33]
37	0.8	0.1	Fluorescence[34]
22	0.93	0.1	⎱ Ultrafiltration and
	0.20	1.0	⎰ equilibrium dialysis[35]

CONCLUSIONS

The curve fitting of the simulated theoretical profile to the experimental peak allows the determination of the equilibrium isotherm mainly for concentrations ranging from the detection threshold to the output concentration. A three-parameter-isotherm equation was found suitable to account for the peak distortions observed for phenylbutazone, when eluted from HSA, immobilized on a diol-support at a finite concentration. Two different isotherm equilibrium models were tested, but it was impossible to tell which one would fit the experimental data more closely.

The method offers a convenient way of revealing heterogeneities in affinity sites and permits the determination of the amount of immobilized protein that is active towards the solute. This parameter is an important factor for characterizing an affinity packing, especially when one considers that a decrease in the number of active protein sites occurs during the immobilization process, since some of them are bound to the support. Therefore, the amount of active protein is lower than the amount that is effectively immobilized. Moreover, the amount of active protein can readily be measured after a few solute injections, and this is useful when dealing with immobilized proteins of poor long-term stability.

Zonal elution and numerical simulation of the elution peaks also permit determination of the association constant for binding of the solute with the immobilized protein. These parameters are useful for comparing the various methods of protein immobilization and for evaluating the contributions of the support to the retention.

REFERENCES

1 G. Fassina and I. M. Chaiken, *Adv. Chromatogr. (N.Y.)*, 27 (1987) 247.
2 P. O. Larsson, M. Glad, L. Hanson, M. O. Månsson, S. Ohlson and K. Mosbach, *Adv. Chromatogr. (N.Y.)*, 21 (1983) 41.
3 L. W. Nichol, A. G. Ogston, D. J. Winzor and W. H. Sawyer, *Biochem. J.*, 143 (1974) 435.
4 F. H. Arnold and H. W. Blanch, *J. Chromatogr.*, 355 (1986) 13.
5 J. L. Wade, A. F. Bergold and P. W. Carr, *Anal. Chem.*, 59 (1987) 1286.

6 J. N. De Vault, *J. Am. Chem. Soc.*, 65 (1943) 532.
7 P. Rouchon, M. Schoenauer, P. Valentin, C. Vidal-Madjar and G. Guiochon, *J. Phys. Chem.*, 89 (1985) 2076.
8 M. J. González, A. Jaulmes, P. Valentin and C. Vidal-Madjar, *J. Chromatogr.*, 386 (1987) 333.
9 P. Rouchon, M. Schoenauer, P. Valentin and G. Guiochon, *Sep. Sci.*, 22 (1987) 1793.
10 J. E. Eble, R. L. Grob, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 384 (1987) 25.
11 J. E. Eble, R. L. Grob, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 384 (1987) 45.
12 J. E. Eble, R. L. Grob, P. E. Antle, G. B. Cox and L. R. Snyder, *J. Chromatogr.*, 405 (1987) 31.
13 L. R. Snyder, G. B. Cox and P. E. Antle, *Chromatographia*, 24 (1987) 82.
14 G. Guiochon and A. Katti, *Chromatographia*, 24 (1987) 165.
15 C. Lagercrantz, T. Larsson and H. Karlsson, *Anal. Biochem.*, 99 (1979) 352.
16 C. Lagercrantz and T. Larsson, *Biochem. J.*, 213 (1983) 387.
17 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 264 (1983) 63.
18 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 316 (1984) 617.
19 S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 425.
20 B. Bomgren and S. Allenmark, *J. Liq. Chromatogr.*, 9 (1986) 667.
21 S. Allenmark, S. Andersson and J. Bojarski, *J. Chromatogr.*, 436 (1988) 479.
22 M. Aubel and L. B. Rogers, *J. Chromatogr.*, 392 (1987) 415.
23 U. Kragh-Hansen, *Pharmacol. Rev.*, 33 (1981) 17.
24 B. Sébille, N. Thuaud and J. Tillement, *J. Chromatogr.*, 167 (1978) 159.
25 B. Sébille, N. Thuaud and J. Tillement, *J. Chromatogr.*, 180 (1979) 103.
26 B. Sébille, N. Thuaud and J. Tillement, *J. Chromatogr.*, 204 (1981) 285.
27 G. Scatchard, *Ann. NY Acad. Sci.*, 51 (1949) 660.
28 I. M. Klotz and D. L. Hunston, *Arch. Biochem.*, 193 (1979) 314.
29 G. H. Philips and E. M. Eyring, *Anal. Chem.*, 60 (1988) 738.
30 K. Rehse and K. Ehlert, *Arch. Pharm. (Weinheim, Ger.)*, 318 (1985) 667.
31 J. C. McElnay and P. F. d'Arcy, *J. Pharm. Pharmacol. Suppl.*, 29 (1977) 1P.
32 J. P. Tillement, R. Zini, P. d'Athis and G. Vassent, *J. Clin. Pharmacol.*, 7 (1974) 307.
33 K. F. Brown and M. J. Crooks, *Biochem. Pharmacol.*, 25 (1976) 1175.
34 V. Maes, J. Hoebeke, A. Vercruysse and L. Kanarek, *Mol. Pharmacol.*, 16 (1979) 147.
35 S. W. Boobis and C. F. Chignell, *Biochem. Pharmacol.*, 28 (1979) 751.