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Kinetic study of the adsorption of human serum albumin on immobilized antibody using the split-peak effect in immunochromatography

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ABSTRACT

The split-peak effect was used to determine the association rate constant of the antigen–immobilized antibody reaction. The amount of immobilized human serum albumin antibody on the chromatographic support was varied in order to find the optimal conditions to reduce the mass transfer contribution in the stagnant mobile phase fluid and measure the effective association rate constant of human serum albumin with the immobilized antibody. Kinetic studies as a function of flow-rate demonstrate the validity of the method consisting in determining the association rate constant from measurements performed on columns of various capacities. These experiments show that limitations due to mass transfer to the surface of the adsorbent are minimized at high flow-rates and for a low density of immobilized ligand.

INTRODUCTION

Studies of the reaction between antigens and antibodies are becoming increasingly important because of the development of immunoreactions. This type of interaction is utilized in various techniques, such as enzyme-linked immunosorbent assay (ELISA) or immunochromatography [1]. These techniques, based upon biospecific recognition, measure the interaction of antibody with immobilized antigen (or *vice versa*). However, it is not enough to know if an antigen–antibody reaction occurs, it is also important to analyse the kinetic steps of this reaction in order to optimize the separation methods based upon this mode of interaction. Moreover, a comprehensive approach to the kinetic process is necessary for the quantitative analysis of kinetic chromatographic immunoassays [2,3].

Reliable and fast methods of carrying out such immunochemical reaction studies are needed. An instrument based upon surface plasma resonance detection of the adsorbed amount was recently introduced for this type of study [4,5], but its sophistication makes its use difficult for routine investigations. Immunochromatography, which uses standard high-performance liquid chromatographic (HPLC) instruments, is well suited for this kind of measurement. Until now the technique has mainly been used as a powerful tool to isolate and purify a single substance from a complex mixture [1] or for rapid immunoassay tests in quantitative analysis [2,3].

Kinetic measurements generally use frontal elution chromatography. Analytical solutions based on the Thomas model [6] are often applied to interpret the chromatographic kinetic experiments [7–9]. The main difficulty with the chromatographic method for kinetic measurements is that several mass transfer processes are at work in non-linear conditions: the mass transport phenomena and the sorption processes. Numeri-

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cal procedures have also been applied to analyse the frontal elution curves and estimate the kinetics of biospecific adsorption of a protein with an immobilized monoclonal antibody [10]. The intraparticle mass transfer diffusion mechanisms could be neglected because non-porous particles were used.

The applications of high-performance immunochromatography for kinetic studies of the antigen–antibody interaction were first described by Sportsman and co-workers [11,12], but the unusual behaviour observed was not well understood. Experiments based upon the "split-peak" effect in linear elution conditions were presented by Walters and co-workers [13,14] to measure the adsorption kinetics on immunoadsorbents. In this method, part of the solute injected elutes from the column as a non-retained peak, while the rest is irreversibly adsorbed and does not elute from the column on the time scale of the experiment. The amount injected was kept as small as possible in order to avoid column overloading effects.

In previous papers, we have shown that the split-peak method may be used in non-linear elution conditions. A model based upon second-order Langmuir kinetics relates the amount injected to the column capacity and to the apparent adsorption rate constant [15]. The method was applied to a study of the adsorption kinetics of human serum albumin (HSA) on a C_6 reversed-phase support [16,17]. In spite of a packing with pores of small size from which the protein is excluded, the column capacity was still too large and the measurements did not allow us to distinguish between diffusion- and adsorption-limited processes. In the present work, we shall show that kinetic studies on adsorbents of low capacity enable reduction of the contributions for mass transfer into the mobile phase fluid and measurement of the effective adsorption rate constant of an antigenic protein (HSA) onto the immobilized antibody.

THEORY

We assume that the immobilized ligand X interacts with the adsorbate according to the chemical equilibrium:

$$A + X \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AX \tag{1}$$

where k_a and k_d are the association and dissociation rate constants. The binding rate equation is a Langmuir second-order kinetic law:

$$\frac{\partial q}{\partial t} = k_a c(q_x - q) - k_d q \tag{2}$$

where c is the concentration of solute in solution and q and q_x are, respectively, the amount adsorbed and the maximum capacity per unit volume of adsorbent.

Thomas [6] has shown that an analytical expression predicts the breakthrough curve on the basis of the following assumptions: adsorption isotherm of the Langmuir type, negligible axial dispersion and no rate-limiting steps due to transport to the adsorbent surface. Several analytical models were further developed to account for this last effect [7–9]. Starting from Goldstein solution [18] giving the equation for the zonal elution peak in mass-overload conditions, we have shown [15] that the split-peak effect is described by an analytical expression relating the adsorption yield to the amount injected, to the experimental parameters (flow-rate F, column length L) and to the kinetic constants (k_a, k_d and q_x). The model assumes negligible axial dispersion of the solute, a non-porous adsorbent or an adsorbent with small pores into which the protein molecule cannot penetrate.

If irreversible adsorption occurs on the time scale of the experiment ($k_d = 0$), the fraction of non-retained compound f is given by [15]:

$$f = \frac{Q_x}{nQ_i} \cdot \ln[1 + (e^{nQ_i/Q_x} - 1)\, e^{-n}] \tag{3}$$

where Q_i is the sample size, Q_x the maximum column loading capacity and n the number of transfer units characterizing the rate-limiting mass transfer step and the adsorption step.

If the rate for the sorption step is similar to the rate for slow mass transfer, it can be shown [8,9] that $1/n$ may be expressed as a sum of different increments:

$$\frac{1}{n} = \frac{1}{n_{mt}} + \frac{1}{n_k} \tag{4}$$

where n_k represents the contribution of binding kinetics and n_{mt} represents the contribution of the mass exchange into the stagnant fluid. n_k is a function of the effective second-order rate constant for the binding process and of the maximum loading capacity:

$$n_k = \frac{Q_x k_a}{F} = q_x k_a \cdot \frac{(1-\varepsilon)}{\varepsilon} \cdot \frac{L}{u} \quad (5)$$

where u is the interstitial mobile phase velocity and ε is the column void fraction.

The n_{mt} contribution depends upon the particle size, the pore structure of the packing and the column geometry. It is a complicated function of the flow-rate. For a given mobile phase velocity and a given chromatographic system (instrument, column geometry, nature of the packing), the n_{mt} parameter is independent of the density of immobilized ligand, and the effective adsorption rate constant, k_a, may be determined from the variation of n with q_x. The aim of the present work is to show that the split-peak method may be applied to determine the binding rate constant from the kinetic studies performed on supports on which various amounts of antibody are immobilized.

EXPERIMENTAL

Materials

Human serum albumin (ref. A 1887), rabbit polyclonal antibodies to human albumin (ref. A 0659) and tresyl chloride (ref. T 7907) were purchased from Sigma (St. Louis, MO, USA). LiChrospher SI 60 (10 μm diameter, 60 Å pore size) was from Merck (Darmstadt, Germany).

Apparatus

The chromatographic experiments were carried out using an HPLC system consisting of a dual-pump system (LC-9A; Shimadzu, Kyoto, Japan), a sample injector (7125; Rheodyne, Berkeley, CA, USA) equipped with a 20-μl loop and an optical scanning UV detector set at 280 nm (Spectra Focus; Spectra-Physics, San Jose, CA, USA). Spectra Focus software on an IBM PS2 personal computer controls the detector, performs on-line data acquisition via an OS/2 interface and reprocesses peak integrations.

Procedure

Immunoadsorbent. The rabbit polyclonal antibody was immobilized on the LiChrospher 60-Å silica support using the tresyl activation method [19]. The tresylated silica was synthesized according to the procedure described by Cabrera and Wilchek [19]. The antibody was immobilized on the support as follows: 1 g of activated silica was added to 4 ml of a solution of antibody in 1 *M* phosphate buffer and the mixture was shaken at ambient temperature for 5 h. The support was further washed with 0.1 *M* Tris–HCl buffer, pH 8.5, to remove unreacted tresyl groups. Four different supports were prepared by varying the concentration of antibody in the phosphate buffer solution used for immobilization (Table I).

Chromatography. The supports were vacuum slurry packed into polyether ether ketone (PEEK) columns (30 mm × 4.6 mm I.D.). The column temperature was maintained at 20 ± 0.1°C using a thermostated bath (CB 11C; Heto, Birkerod, Denmark). The mobile phase was phosphate-buffered saline (PBS; 10 m*M* phosphate buffer, pH 7.4, 150 m*M* NaCl); the HSA samples were dissolved in the eluent. After column saturation with HSA injections, protein desorption was achieved using 0.1 *M* citric acid buffer at pH 1.6.

RESULTS

Successive injections of HSA on the polyclonal anti-HSA columns were performed until saturation of the support was achieved. Fig. 1 illustrates the progressive overloading of the column after repeated injections of 2 μg of HSA. With the first injections an impurity is eluted while HSA is totally adsorbed onto the column. Then, after several injections, because of column saturation and kinetic effects, the amount of the first peak increases until it reaches a plateau. The percentage of the impurity corrective factor, given in relative detector response units, is calculated from the ratio of the area of the first peak observed on the antibody column to that measured when the same HSA solution is injected on a diol column. It is about 10% of the total HSA signal. The method does not give the actual impurity level in the sample, but this

TABLE I

VARIATION IN THE KINETIC PARAMETERS WITH THE DENSITY OF IMMOBILIZED ANTIBODY

Column	Antibody in reacting solution (g/l)	Q_x (μg)	F (ml/min)	$1/n$	$1/n_k$	k_a ($l\ g^{-1}\ s^{-1}$)
A	0.16	9	0.5	0.13 ± 0.01	0.08	12 ± 2
			1.0	0.23 ± 0.02	0.18	10 ± 2
			1.5	0.30 ± 0.03	0.25	11 ± 2
B	0.42	27	0.5	0.08 ± 0.01	0.03	10 ± 4
			1.0	0.11 ± 0.01	0.06	10 ± 3
			1.5	0.13 ± 0.01	0.08	12 ± 2
C	2.30	39	0.5	0.08 ± 0.01	0.03	7 ± 4
			1.0	0.09 ± 0.01	0.04	11 ± 4
			1.5	0.11 ± 0.01	0.06	11 ± 3
D	2.50	41	0.5	0.07 ± 0.01	0.02	10 ± 5
			1.0	0.09 ± 0.01	0.04	10 ± 4
			1.5	0.10 ± 0.01	0.05	12 ± 4

information is enough to correct for the unretained fraction: at each injection, the unretained HSA amount is calculated by subtracting the area corresponding to the impurity response (area of the first peak).

Fig. 1 shows, for comparison, the diagrams observed at two different flow-rates: 0.5 and 1.5 ml/min. The first signal corresponds to a non-retained impurity response, and it is only the increase in the HSA response that is analysed. At the higher flow-rate, the increase in the first peak occurs for lower amounts injected. To determine the adsorption rate constant, the split-peak behaviour is quantitatively analysed according to eqn. 3, by studying the variation in the unretained fraction as a function of the amount injected.

Fig. 2 shows the plot of $1/f$ *vs.* Q_i, where f is the ratio of the cumulative amounts injected to the cumulative amounts which are not irreversibly adsorbed and Q_i is equal to the cumulative amounts injected. This figure also shows the results of the experiments performed at a given flow-rate on columns of various binding capacities. A non-linear least-squares fit was used to adjust eqn. 3 to the experimental data. The method gives the two parameters necessary to describe the adsorption model: the column capacity for HSA, Q_x, and the number of transfer units, n.

The four columns studied differ in the density of the polyclonal antibody immobilized on the silica support and therefore in the column capacity, Q_x, for HSA (Table I). In order to check the possibility of non-specific adsorption, a column was packed with a silica support prepared exactly as described for coupling the antibody, but in this case no protein was added to the reacting solution. No peak area increase was noticed when repeated injections of HSA were performed on this column. Moreover, under similar elution conditions, the area of the non-retained HSA peak is equal to that observed with a diol column or with an antibody column at saturation. Therefore the non-specific HSA adsorption on the matrix used for the antibody immobilization may be considered to be negligible.

For each column, the number of transfer units determined at different flow-rates is listed in Table I. The reported errors on the parameter determination are given for a 95% confidence interval. Fig. 3 illustrates the variation of L/n as a function of the interstitial mobile phase velocity. This diagram may be analysed as classically where the theoretical plate height is plotted as

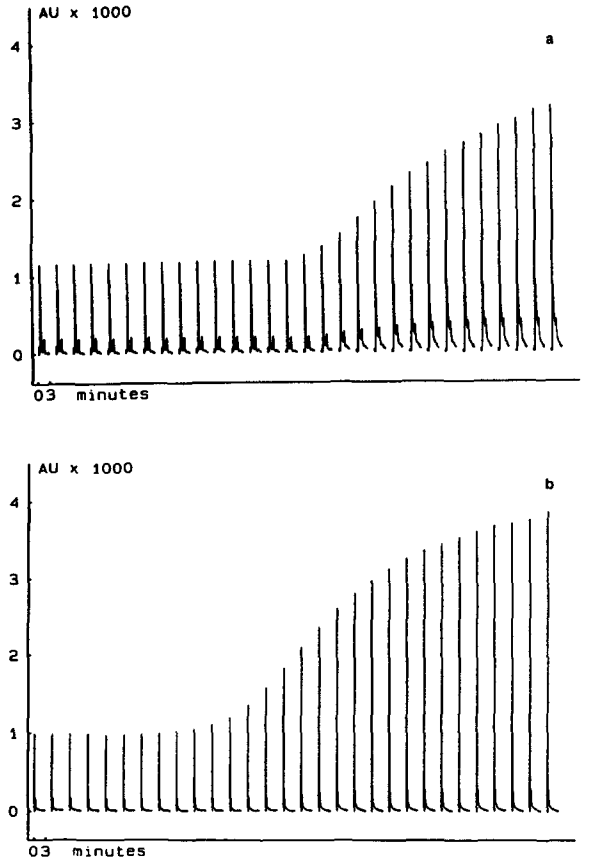

Fig. 1. Successive injections of HSA on a polyclonal HSA antibody support. Eluent: 10 m*M* phosphate buffer pH 7.4–150 m*M* NaCl. Injected volume: 20 μl. HSA concentration: 0.1 g l^{-1}. Data acquisition rate: 24 readings per s. $T = 20°C$. Column: D (30 × 4.6 mm, dead volume: 0.3 ml). (a) $F = 0.5$ ml min^{-1}; (b) $F = 1.5$ ml min^{-1}.

a function of the mobile phase velocity. L/n increases with increasing flow-rate, and this effect may be explained by mass transfer phenomena due to slow adsorption and/or to slow diffusion into the stagnant mobile phase fluid.

DISCUSSION

Fig. 4 shows that for each flow-rate studied the reciprocal of the global mass transfer unit, n, varies linearly with the reciprocal of q_x, the column capacity per volume of adsorbent. This result agrees well with eqns. 3 and 4 describing $1/n$ as a sum of various contributions. At a given

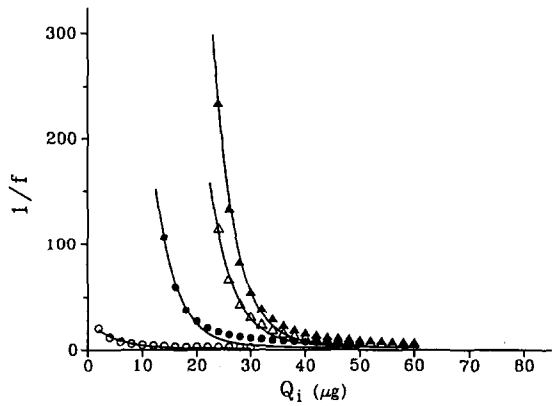

Fig. 2. Variation in the non-retained fraction f with the amount of HSA injected. Same experimental conditions as in Fig. 1 and $F = 1.5$ ml min^{-1}. Columns: ○ = A; ● = B; △ = C; ▲ = D.

mobile phase velocity, the model predicts a linear variation of $1/n$ *vs.* $1/q_x$, with the same n_{mt} contribution for the columns used. In this case, similar values for the contribution of the mass transfer in the stagnant mobile phase are to be expected, since the columns tested on a single instrument are of identical geometrical design and packing: they differ only in the density of immobilized antibody on the silica support. For each flow-rate, the value of $1/n_{mt}$ may be determined by extrapolating the straight lines to $1/q_x = 0$. Since the extrapolated values are similar for the three flow-rates studied (Fig. 4), the

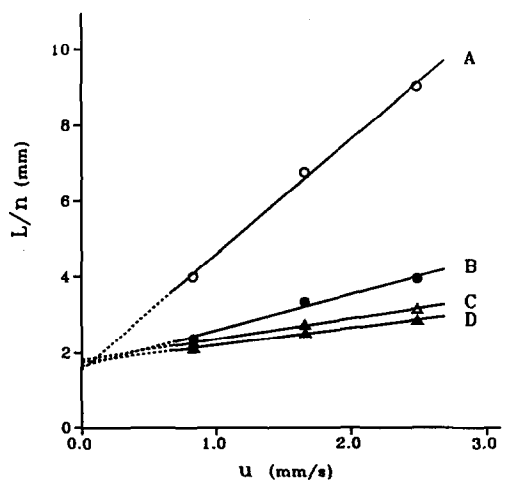

Fig. 3. Variation in the number of transfer units with the interstitial mobile phase velocity. Same experimental conditions as in Fig. 1. Columns: ○ = A; ● = B; △ = C; ▲ = D.

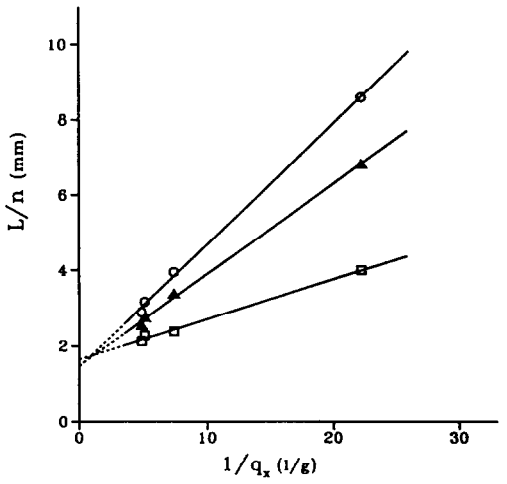

Fig. 4. Variation in the number of transfer units with the column capacity. Same experimental conditions as in Fig. 1. Interstitial mobile phase velocity: ○ = 0.5 ml min^{-1}; ▲ = 1.0 ml min^{-1}; □ = 1.5 ml min^{-1}.

variation of n_{mt} with the mobile phase velocity, u, may be considered negligible.

Similarly, a linear variation of L/n with u is found (Fig. 3). An important variation in the number of unit transfers, n, is observed for the column of lower HSA capacity. On the other hand, L/n is almost independent of flow-rate with the column packed with the adsorbent of higher density of immobilized antibody. The higher the straight-line slope, the lower is the density of immobilized antibody. The values of L/n extrapolated at $u = 0$ are similar for all the columns studied and correspond to a constant contribution to the overall mass transfer, independent of the density of the immobilized ligand.

Since n_{mt} is proportional to the theoretical plate height of an unadsorbed tracer [13], such behaviour may be independently examined by studying, as a function of the mobile phase velocity, the band broadening of the non-retained HSA elution peak. Fig. 5 shows that the variation in the theoretical plate height H with u is negligible. Also, as expected for all the columns studied, the efficiencies of the unretained HSA peak are close, within the range of experimental errors. They are about ten times higher than the optimal value for a 10 μm particle size support. The poor efficiencies generally observed in the HPLC of proteins [20] are the result of several complex mechanisms involving slow mass transfers due to diffusion into the stagnant fluid of the small pores of the packing, that between the silica particles, or the extra column contributions.

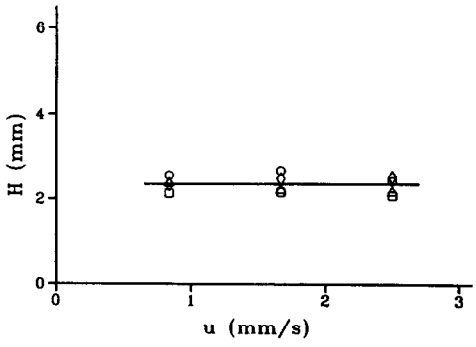

Fig. 5. Variation in the theoretical plate height of the unretained HSA peak with the interstitial mobile phase velocity. Same experimental conditions as in Fig. 1. Columns: ○ = A; ◇ = B; □ = C; △ = D.

These results demonstrate that, within experimental errors, n_{mt} may be considered a constant for all the columns and in the range of flow-rates studied. This contribution may be determined by extrapolating the straight lines L/n *vs.* u (Fig. 3) or L/n *vs.* $1/q_x$ (Fig. 4) to zero abscissa. In agreement with the assumption of a constant n_{mt} contribution, the intercepts with the ordinate axis are similar for the straight lines of both figures. The mean n_{mt} value is 20 ± 3.

Subtracting n_{mt} from the global mass transfer unit term, n, leads to the contribution for binding kinetics, n_k. Table I lists the n_k values determined for each column and each flow-rate studied. These results show that the contribution of mass transfer in the stagnant fluid film to the global mass transfer process is minimized when the kinetic experiments are performed at high flow-rates and with low amounts of immobilized antibody. The error made in neglecting the n_{mt} contribution with the column of lower capacity is about 20% at the higher flow-rates. The mass transfer in the stagnant fluid is a large part of the total $1/n$ value with the columns of larger capacity (columns C and D).

The effective adsorption rate constant was

calculated for each column and each flow-rate studied (Table I). Within experimental errors the results are in good agreement, and the mean value obtained for the effective adsorption rate constant is $k_a = 10.5$ l g^{-1} s^{-1} ($7.2 \cdot 10^5$ M^{-1} s^{-1}). It is of similar magnitude as that found in other kinetic studies of protein–protein interactions [4,5]. However, because of the heterogeneous nature of the polyclonal antibody used, the association rate constant characterizes an average adsorption process.

The mass transfer into the stagnant mobile phase fluid has several causes (nature of the column packing or extra-column effects), and this contribution could be reduced by a systematic study of the experimental conditions leading to this effect. Under the present HPLC experimental conditions, the transport process to the adsorbent can be faster than the rate constant of the immunoreaction. In some cases, with a high flow-rate and a low column capacity, direct measurement of the kinetic contribution is possible if this effect can be neglected or accounted for. The measurements are then performed in conditions in which non-linear effects are important considerations in the theoretical models.

CONCLUSIONS

The present work describes an easy immunochromatographic method based upon peak-area measurements to determine the association kinetic rate constant of the antigen–immobilized antibody system. The reverse interaction could be determined just as easily by immobilizing the antigen and studying the split-peak effect by injecting the antibody sample. So far very few kinetic measurements have been determined as a function of the density of immobilized ligand. Our results clearly demonstrate that this type of study enables determination of the sorption rate constant for the antigen–immobilized antibody interaction. Experiments performed at various flow-rates confirm the validity of this type of approach.

Selecting the appropriate experimental chromatographic conditions enables characterization of the kinetics of the antigen–antibody reaction at the solid–liquid interface. The rate-controlling step may not be diffusion into the stagnant fluid if the experiments are carried out at a high flow-rate on a support on which low amounts of antibody (or antigen) are immobilized. The characteristics of the packing material are also important in order to reduce the extra mass transfer contribution: a regular packing of beds with uniform and small size distribution must be achieved, and non-porous supports or supports with pores small enough to exclude the antigen or the antibody must be selected.

In the present work, we studied the interaction of HSA with an immobilized polyclonal antibody in order to define the optimal conditions for kinetic measurements. The advantage of immobilizing the antibody is that several systematic studies on the same column may be performed in order to demonstrate the feasibility of the method with chromatographic columns having capacities as low as a few micrograms. The amount of antibody consumed is minimal and equal to the amount immobilized. This problem is an important consideration when using expensive and rare monoclonal antibody samples.

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