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Application of the split-peak effect to study the adsorption kinetics of human serum albumin on a reversed-phase support

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ABSTRACT

The adsorption step of human serum albumin on a reversed-phase support was analyzed by studying the "split-peak" effect in mass-overload conditions. This behavior is characterized by the occurrence of a first non-retained fraction and is described by an analytical expression in the case of a Langmuirian adsorption isotherm. The method was applied to determine the column loading capacity, the number of mass-transfer units and the apparent adsorption rate constant measured at a given flow-rate.

The nature of the organic modifier influences the split-peak effect: it increases with the eluotropic strength of the organic solvent added to the buffer. Compared to the results with pure buffer, it is the association of two effects, the decrease of the column loading capacity and that of the apparent adsorption rate constant, which increases the split-peak effects observed when methanol and 2-propanol are added to the eluent. These results allow us to gain a better understanding of the role of the organic solvent in the elution behavior of proteins in reversed-phase high-performance liquid chromatography.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is widely used for separating proteins and peptides. A stoichiometric displacement model [1] was developed that accounts for protein elution and the importance of the role of the organic solvent was demonstrated.

However, some behaviors are not always well understood: for example, two or more peaks are sometimes observed from a single species [2], their occurrence and shape depending on mobile phase gradient conditions and column temperature. To describe the mechanism, two steps were suggested [3]: the first one is related to the adsorption kinetics of the protein, while the other one concerns all further conformational events occurring on the surface until elution.

Until now, kinetic studies have mainly focused on the conformational changes of the protein occurring after adsorption on the surface. However, examination of the first adsorption kinetic step is important for a better understanding of the elution behavior of proteins in RP-HPLC. Since slow adsorption kinetics are related to the

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split-peak effect [4,5], a possible approach is to investigate the occurrence of a non-retained peak in isocratic elution. Adsorption kinetics are usually analyzed from breakthrough curves or elution peak shapes [6], but the study of the split-peak phenomenon remains close to the conditions used in analytical chromatography.

The split-peak effect was first described theoretically many years ago by Giddings and Eyring [7], but was revealed experimentally for the HPLC of proteins due to their slow adsorption kinetics and because of the short columns and high flow-rates used. Nevertheless, the potentialities of the method were not fully exploited because kinetic measurements were limited to the linear range [4,5].

We described a kinetic model for irreversible adsorption [8], one which predicts the occurrence of the elution peak splitting in mass-overload conditions and irreversible adsorption: a solute fraction elutes at the void volume while the other one is irreversibly retained in the column. The unretained fraction increases with the sample size and an analytical expression relates this fraction to the number of mass-transfer units and to the column loading capacity.

On the basis of this model, the goal of the present work is to analyze the kinetic adsorption step of human serum albumin (HSA) on a reversed-phase support in the presence of various organic solvents in the mobile phase. In a preceeding paper [9] we have studied the influence of increasing concentrations of acetonitrile on the split-peak effect. An important decrease in the number of transfer units characterizing the adsorption kinetic process was observed when increasing amounts of acetonitrile were added to the eluent. A similar trend was observed when other organic modifiers were used. In this paper we shall study the influence of the nature of solvents with an hydroxyl group (methanol and 2-propanol). Since these solvents are often used to elute peptides or proteins on reversed-phase supports, this approach is useful in order to understand better the role of the organic modifier in the RP-HPLC of proteins.

THEORY

The adsorption kinetic model starts from the differential equation describing the solute migration through the column:

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

and the second order Langmuir 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 the mobile phase, z the abscissa in the column length, t the time and u the mobile phase velocity. Q is the amount of adsorbed solute, Q_x the maximum loading capacity and V_0 the mobile phase volume. D' is a global dispersion coefficient accounting for axial and eddy diffusion; k_a and k_d are the adsorption and desorption rate constants. The equilibrium constant is $K = k_a/k_d$.

Solutions of the above system of differential equations exist for the ideal case

($D' = 0$) and the analytical expressions depend on the boundary conditions: breakthrough curves in frontal elution [10] or peak profiles with a finite pulse injection [11] or a Dirac injection function [12].

As shown by Goldstein [11], the solution for a rectangular injection of time duration t_i and solute concentration C_i, is given by two separate expressions according to the time elapsed after the time t_0 necessary to elute a non-retained compound. The concentration at the column outlet is given by the ratio:

$$C = C_i P/E \tag{4}$$

For $t > t_0 + t_i$, P and E are given by:

$$P = J(nr, nT) - J(nr, nT - nT_i) \tag{5a}$$

$$E = J(nr, nT) - J(nr, nT - nT_i) + [1 - J(n, rnT)] \cdot \exp[(1 - r)(n - nT)] + \\ + J(n, rnT - rnT_i) \cdot \exp[(1 - r)(n - nT + nT_i)] \tag{5b}$$

For $t < t_0 + t_i$, P and E are given by

$$P = J(nr, \alpha T) \tag{6a}$$

$$E = J(nr, \alpha T) + [1 - J(n, r\alpha T)] \cdot \exp[(1 - r)(n - nT)] \tag{6b}$$

where the J function is given by:

$$J(x, y) = 1 - e^{-y} \int_0^x I_0(2\sqrt{\tau y}) \cdot e^{-\tau} d\tau \tag{7}$$

where I_0 is the Bessel function of zeroth order and

$$n = \frac{Q_x k_a}{\delta} \tag{8}$$

$$r = \frac{1}{1 + KC_i} \tag{9}$$

$$T = (t - t_0) \cdot \frac{\delta}{KQ_x r} \qquad T_i = t_i \cdot \frac{\delta}{KQ_x r} \tag{10}$$

Q_x is the column loading capacity and δ is the flow-rate. The parameter n is the number of transfer units characteristic of the adsorptive exchange. It is related to the plate height kinetic contribution [13] according to:

$$H_K = \frac{2Lk'^2}{n(1 + k')^2} \tag{11}$$

where L is the column length and k' is the solute capacity factor at infinite dilution ($k' = KQ_x/V_0$).

The split-peak effect is characterized from the ratio of the non-retained amount on the amount injected ($Q_i = C_i\delta$). The amount eluted as a first peak is given by integrating the elution peak expression for $t < t_i$ (eqns. 4 and 6).

In the case of irreversible adsorption ($k_d = 0$) and in the absence of dispersive effects ($D' = 0$), we have shown in a previous paper [8] that this ratio f is given by the expression:

$$f = \frac{Q_x}{nQ_i} Ln[1 + (e^{nQ_i/Q_x} - 1)e^{-n}] \tag{12}$$

When f is extrapolated to zero amount injected, the unretained fraction is f_0:

$$f_0 = e^{-n} = e^{-k_a Q_x/\delta} \tag{13}$$

The limit of eqn. 12 for zero flow-rate is the trivial expression of f: $f_\infty = 1 - Q_x/Q_i$, for $Q_i > Q_x$, and $f_\infty = 0$ for $Q_i < Q_x$.

A simulation algorithm was used [8] to solve numerically the set of differential eqns. 1 and 2. It is based on a numerical step procedure and describes the solute migration through the column accounting for adsorption kinetic effects in mass-overload conditions and for solute dispersion in the mobile phase. The simulations of the chromatographic process in non-linear elution have shown that the split-peak expression given by eqn. 12, can still be applied in the presence of dispersive effects and does not depend on the shape of the injection signal.

The parameters of eqn. 12, namely the number of mass-transfer units n and the maximum loading capacity Q_x, were determined by fitting the model to the variations of f as a function of sample size. Instead of desorbing the adsorbed protein by using another eluent after every injection, we determined the experimental variation of f as a function of the cumulated amounts injected: the validity of the method was previously demonstrated from numerical simulations [8]. The non-linear regression was performed with a Fortran program that uses the partial derivatives *versus* n and Q_x in eqn. 12 to converge to the best fit parameters.

MATERIALS AND METHODS

The chromatographic experiments were performed on an HPLC system: a pump (2150; LKB, Bromma, Sweden), a sample injector (7125; Rheodyne, Berkeley, CA, USA) with a 20-μl loop and a UV detector (Spectra-100; Spectra-Physics, San Jose, CA, USA), set at 280 nm.

The reversed-phase support (Spherisorb RP-C_6) of particle diameter 10 μm, pore size 80 Å and specific surface area 220 m^2/g, was packed into 50 × 4.6 mm stainless-steel columns, kindly supplied by S.F.C.C.-Shandon (Eragny sur Oise, France). The temperature of the column and that of the eluent was kept constant within 0.1°C using a thermostated–cryostated water-bath.

The mobile phase was a 0.067-M potassium phosphate buffer at pH 7.4 modified

with organic solvents, HPLC grade: acetonitrile, methanol and 2-propanol. The HSA samples (Sigma, A1887, St. Louis, MO, USA) were dissolved in the same eluent as that used for the mobile phase.

The data acquisition of the chromatographic signal was performed with a micro-computer (Apple IIe, Cupertino, CA, USA) equipped with a 12-bit analog-to-digital converter, and an instrumentation amplifier (Analog Devices, Norwood, MA, USA). The speed for data acquisition was selected so as to obtain a minimum of fifteen data per peak width and peak integrations were performed with the same computer. An integrator with a sampling rate high enough to define the width of the non-retained peak, can be used as well for peak area measurements.

RESULTS

Split-peak effect

Adsorption kinetics are revealed when a part of the solute injected is eluted as the first peak at the column void volume (0.4 ml) while the other part is irreversibly adsorbed on the support. As shown in Fig. 1, with an eluent containing 40% methanol, the successive injections of HSA (0.08 mg) lead to increasing amounts of the non-retained fraction. The saturation of the column is reached when the protein is totally eluted as the first peak.

The increase of the unretained fraction is related to the maximum loading capacity and to the rate of adsorption of the protein. The pattern of Fig. 1 changes with the nature of the eluent and more generally with the experimental conditions used. For example, Fig. 2 shows that the split-peak behavior increases when the same experiment is carried out at a lower temperature. With 20% acetonitrile [9] or 2-propanol in the buffer, the appearance of the first peak can already be noticed at the first injections. In

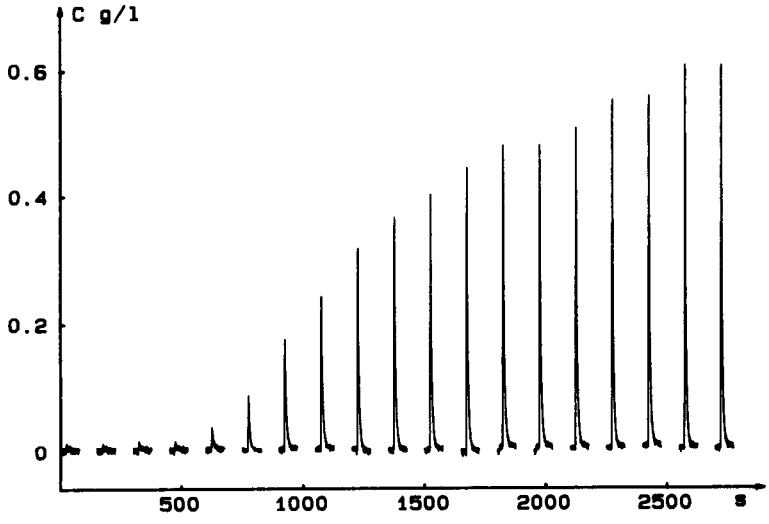

Fig. 1. Successive injections of HSA on a reversed-phase support at 20°C. Eluent: 0.067 *M* phosphate buffer pH 7.4 + 40% methanol. HSA sample size: 80 μg; L = 5 cm; V_0 = 0.4 ml; δ = 1 ml/min.

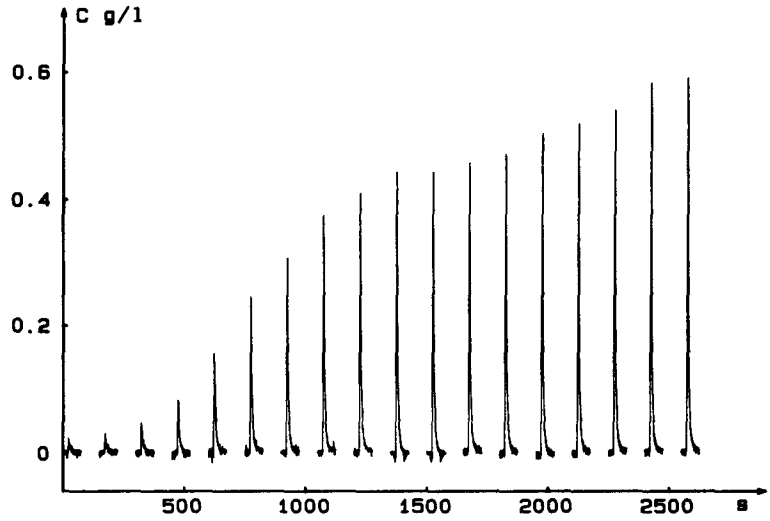

Fig. 2. Successive injections of HSA on a reversed-phase support at 10°C. Same experimental conditions as in Fig. 1.

contrast, with pure buffer or with solutions of methanol in the buffer, the occurrence of the first peak is observed only when the total amount injected is close to the saturation value.

HSA is desorbed with 40% acetonitrile after every experiment. The method permits the regeneration of the column for another split-peak study. However, reproducible experiments are only achieved when a first HSA adsorption had saturated the most active sites of the surface because the washing step with 40% acetonitrile does not permit the regeneration of high-affinity sites. The experiments are therefore performed on a surface which is not uniform, since some HSA still remains adsorbed on the surface. This is not a serious drawback with HSA, because protein–protein interactions are negligible: HSA is not retained on a column packed with a diol-support where the protein is covalently bound [14].

The amounts of non-retained solute are calculated from peak areas after calibration of the detector. The ratio of the cumulated unretained amounts to the total amounts injected gives the fraction f of unretained solute. The plot of $1/f$ as a function of the sample size provides a good display when split-peak effects are small, since important variations are observed at low f ratio before reaching the asymptotic value of 1 at large sample sizes.

The influence of the nature of the organic solvent on the split-peak effect is illustrated by the plot $1/f$ *versus* Q_i at 20°C (Fig. 3): at low sample sizes an important splitting of the HSA elution peak is observed with 20% 2-propanol (f_0 *ca.* 0.5). In contrast, with 40% methanol or with pure buffer, one can only observe the split-peak effect if the total amount injected is close to the column capacity. An important increase of the split-peak effect is observed at 10°C, by adding methanol to the eluent (Fig. 4).

As previously noticed when acetonitrile was used as a modifier [9], the split-peak

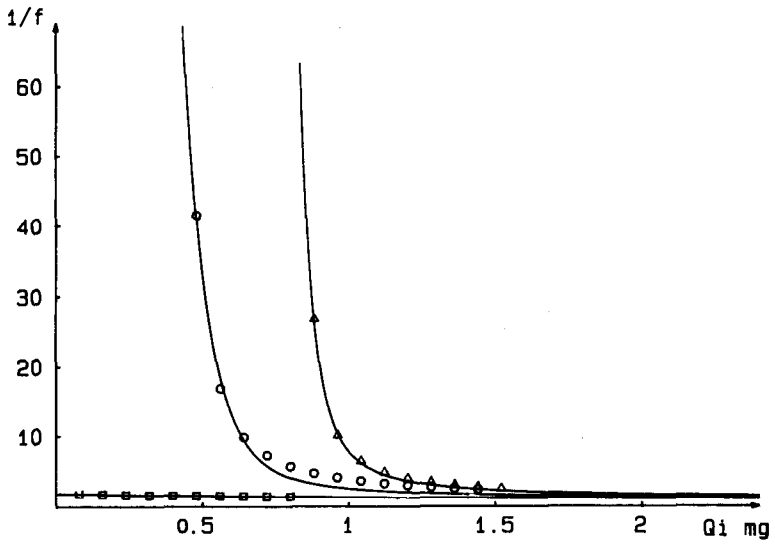

Fig. 3. Variation of the non-retained fraction f with the amount of HSA injected at 20°C. Eluent: 0.067 M phosphate buffer + organic solvent. ——— = Best fit of the theoretical model (eqn. 12). △ = Buffer; ○ = buffer + 40% methanol; □ = buffer + 20% isopropanol. HSA sample size: 80 μg; L = 5 cm; V_0 = 0.4 ml; δ = 1 ml/min.

effect increases with the amount of organic modifier added. This effect is more important with 2-propanol than with methanol. In the latter case 40% of organic modifier were necessary to observe a split-peak effect that differs significantly from that of the pure buffer. For this study we selected the concentration of the organic solvent added in a range which was convenient to study the split-peak effect.

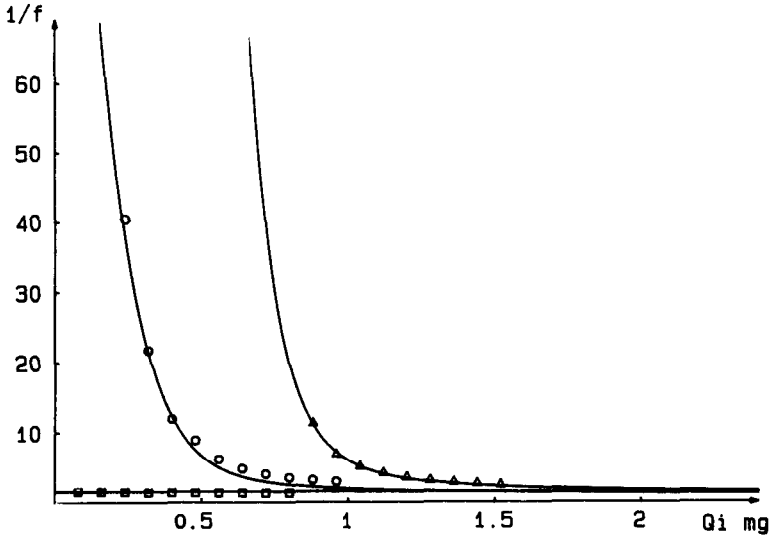

Fig. 4. Variation of the non-retained fraction f with the amount of HSA injected at 10°C. Same conditions as in Fig. 3.

TABLE I

KINETIC MEASUREMENTS FROM THE SPLIT-PEAK EFFECT

Eluent	Temperature (°C)	δ (ml/min)	n	Q_x/V_0 (g/l)	k_a ($l\,g^{-1}s^{-1}$)	t_0/n (s)	f_0 (%)
Buffer	10	1.0	10.0 ± 0.5	2.00 ± 0.05	0.20 ± 0.02	2.5	$4.5 \cdot 10^{-3}$
	20	0.5	14.0 ± 0.5	1.90 ± 0.05	0.15 ± 0.01	3.6	$8.3 \cdot 10^{-5}$
	20	1.0	21.0 ± 0.5	2.05 ± 0.05	0.41 ± 0.01	1.2	$7.6 \cdot 10^{-8}$
	20	1.5	14.0 ± 0.5	1.90 ± 0.02	0.43 ± 0.02	1.2	$8.3 \cdot 10^{-5}$
Methanol (40%) in buffer	10	1.0	5.2 ± 0.1	1.2 ± 0.1	0.17 ± 0.02	4.6	0.55
	20	0.5	5.8 ± 0.1	1.4 ± 0.05	0.08 ± 0.01	8.7	0.30
	20	1.0	8.3 ± 0.2	1.5 ± 0.05	0.22 ± 0.02	3.0	0.025
	20	1.5	6.6 ± 0.2	1.2 ± 0.05	0.33 ± 0.03	2.5	0.14
	20	1.75	6.4 ± 0.1	1.3 ± 0.05	0.34 ± 0.03	2.3	0.17
2-Propanol (20%) in buffer	10	1.0	0.47 ± 0.01	0.65 ± 0.05	0.030 ± 0.005	54	62.5
	20	0.5	0.85 ± 0.05	0.60 ± 0.05	0.030 ± 0.005	59	42.7
	20	1.0	0.65 ± 0.05	0.80 ± 0.1	0.035 ± 0.005	38	52.2
	20	1.5	0.66 ± 0.01	0.80 ± 0.1	0.049 ± 0.005	25	51.7

Kinetic measurements

Table I gives the values of the parameters n and Q_x determined from the least-square fit of eqn. 12 to experimental data. The errors on the estimated parameters for a 95%-confidence interval are given. The precision of the measurements depends on the value of the unretained fraction at infinite dilution (f_0). To visualize the time for protein adsorption the quantities $V_0/n \cdot \delta = t_0/n$ are listed in the same table: t_0/n is the simplified expression of the adsorption rate constant for a first-order kinetic mechanism. Fig. 3 and 4 show that good agreement is obtained between the theoretical model (full line) and the experimental data.

The precision on the Q_x determination is good (*ca.* 5%) when the kinetics of HSA adsorption are fast as with pure buffer of buffer plus methanol ($n > 3$): steep curves are observed, converging to the saturation value. The precision on the n determination is between 2 and 5%, but the optimal range for achieving kinetic measurements, is between 1 and 50%.

The variations of the unretained fraction with the sample size were studied at different flow-rates. Some dispersions in the maximum loading capacity determination are due to the poor reproducibility of the surface available for adsorption after every column regeneration. As with the theoretical plate height, the reciprocal of the number of transfer units increases with flow-rate. According to eqn. 8 a plot of $1/n$ *versus* δ should intercept the origin. This is not the case since a significant increase of k_a calculated from the n and Q_x values (eqn. 8) is observed with increasing flow-rates (Table I).

DISCUSSION

The split-peak model for mass-overload conditions is based on several assumptions: the irreversibility of the adsorption process, a Langmuir-type adsorption

isotherm, and no diffusion in the pores or in the stagnant mobile phase volume. It can therefore be applied to analyze experiments if these criteria are fulfilled.

The irreversibility of the adsorption process is shown from the absence of tailing in the shape of the non-retained peak: Giddings and Eyring [7] have shown that slow desorption originates important tailings: their expression is given by the product of a Bessel function and an exponential one decreasing with time. Moreover, no base-line drift was observed even at higher UV detector sensitivities.

The adsorption isotherm can not be determined in the case of irreversible adsorption (infinite Henry adsorption constant), but the adsorption of HSA was often found to be of the Langmuirian type and the maximum loading capacity was used to determine the surface available for protein adsorption.

The model does not account for the diffusion in the pores but the choice of an 80-Å support allows us to assume that HSA is mainly adsorbed on the external surface of the particles. Schmidt *et al.* [15] have shown that albumin is almost totally excluded from a 100-Å LiChrosorb diol-support. Moreover, as discussed in a previous paper [9], the maximum loading capacity in pure buffer corresponds to the adsorption of HSA as a monolayer on the external surface of the particles. The capacity of the support measured in the presence of methanol or 2-propanol in the buffer is even lower and this is in good agreement with the assumption that there is no diffusion of the protein into the pores.

The kinetic model accounts for the dispersion effects but not for the mass-transfer at the particle boundary or in the stagnant fluid between the particles [16]. Its importance is considerable since a variation of k_a calculated from n and Q_x values was observed with flow-rate (Table I). The experiments were performed only for a limited range of flow-rates but a general trend was observed within experimental errors: a decrease of k_a with increasing mobile phase velocities with an asymptotic value reached at the larger flow-rates studied. The important increase in k_a at low flow-rates reveals the contribution of diffusion in the stagnant fluid film. Therefore the adsorption rate constants measured in this work are not simply the rate constants for the adsorption chemical step, but must also be considered as apparent ones for the adsorption on the whole system particle plus the boundary layer.

The approach used in this work for measuring adsorption kinetics is similar to that used by Chase [6] to analyze frontal experiments, on the basis of Thomas' solution [10]: because of the difficulties in considering kinetic models based on a rigorous approach, the adsorption process is described by an apparent adsorption rate constant k_a, which is experimentally measurable and enables us to make a comparison between the various systems studied.

The split-peak effect is characterized by the f_0 value (Table I). When the splitting is important as with 2-propanol, the limiting value of f for infinite dilution, f_0, and therefore the number of mass-transfer units n, can be determined by extrapolation from the plot of f *versus* Q_i; but, in the case of fast adsorption kinetics or low split-peak effects, as with pure buffer or buffer modified with methanol ($f_0 < 0.01$), the model must be fitted to the experimental data in order to determine n.

The association of two effects, namely the lower adsorption rate constant and the lower loading capacity, is responsible for the increase in the split-peak observed in the presence of an organic solvent (Table I). Compared to the results with pure buffer, adsorption kinetics are roughly twice as slow with 40% methanol in the buffer and

twenty times as slow with 2-propanol. This may be related to a decrease of the interfacial energy in the presence of an organic modifier. Van Oss [17] has shown that the electron-acceptor parameter of the solvent and its decrease with additions of the organic modifier should explain the retention behaviors of proteins in reversed-phase liquid chromatography.

The slower kinetics observed with an organic solvent in the buffer may also be due to a restricted diffusion in the stationary-phase layer formed by the solvation of the alkyl chains of the reversed-phase support [18]. The lower capacities for HSA adsorption observed in the presence of organic modifiers can not be explained by the displacement retention model of proteins in RP-HPLC [1]. This could be due to an alteration of the native conformation of the protein in the presence of an organic solvent or to the restricted diffusion in the stationary-phase layer that prevents HSA from adsorption on the whole available surface.

With all the solvents studied and for a given flow-rate of 1 ml/min, the number of transfer units and therefore the k_a values measured at 10°C are smaller than those measured at 20°C (Table I). This is in good agreement with the usual kinetic variations with temperature.

The variations of the split-peak effect with sample size are related to the loading capacity of HSA. We did not study the split-peak effects with other proteins, but this variation will only be observed if the saturation of the support is achieved. This is not the case with all proteins because of possible self-association. Moreover, this type of study is easier with columns of low capacity.

CONCLUSIONS

The ability of the model to predict the occurrence of the split-peak phenomenon is satisfactory: its importance increases with increasing flow-rates, lower loading capacities or slower adsorption kinetics. HSA adsorption on the reversed-phase support is highly dependent of the nature of the eluent. The apparent adsorption rate constants are of the same order of magnitude with pure buffer as with a buffer modified with methanol. Slower adsorption kinetics are found with an eluent containing 2-propanol.

Because of the dispersion of the experimental measurements as a function of flow-rate, it is not possible to distinguish between kinetic mass transfers due to chemical adsorptive exchange and those due to diffusion in a stagnant fluid, but the determination of the apparent adsorption rate at a given flow-rate is useful in order to gain a better understanding of the role of the solvent in the first adsorption step of proteins.

Kinetic studies can be carried out by frontal elution but the split-peak method presents several advantages: experiments are quick, easy to perform, and are more precise because they are based on peak area measurements and not on the analysis of band broadenings, where dispersive effects may interfere. The amount of solute required is small, since the loading capacity can be determined from several injections and complete column saturation is not necessary. The method is however limited to adsorption studies on low capacity supports such as non-porous ones or those with pores small enough to exclude the protein. Moreover, as with the frontal technique, the analysis of the experimental data is based on a model assuming a Langmuir kinetic law

and this limits the applicability of the method to systems with a Langmuirian adsorption isotherm.

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