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Study of protein–ligand binding effects by direct chromatographic on-column injection

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ABSTRACT

The direct zonal on-column injection method was applied to a high-performance liquid chromatographic study of pollutant–protein binding interactions in solution. The protein and the protein–ligand complex are excluded on the basis of the size from the diol support, and the free ligand penetrates into the pores and is more retained. The pattern of the ligand elution profile depends on the protein–ligand dissociation constant. This effect was quantitatively analysed by developing a numerical simulation algorithm in which the column is divided into slices of given thickness. The column length, flow-rate and shape of the injection signal are given as input parameters. A global dispersion coefficient accounts for peak broadening. A rapid equilibrium is assumed with the hypothesis that a monovalent ligand interacts with a single binding site on the protein. The interaction of bovine serum albumin with pentachlorophenol was studied, and an apparent dissociation constant for the protein–ligand complex was determined by fitting the theoretical profile to the experimental one. The effect of the acetonitrile content in the solvent was studied. An important decrease of the dissociation constant is observed that affects the chromatographic elution pattern.

INTRODUCTION

Chromatographic methods are now widely applied to study ligand–protein binding in solution [1]. The most popular technique for determining drug–protein interactions is the Hummel and Dreyer method [2], where the protein is added to an eluent containing the ligand. However, it is limited to measurements of systems with relatively low affinity constants, because the ligand has to be totally separated from the complex.

Another zonal elution method, experimentally easier to carry out, consists of directly injecting a mixture of the protein and the ligand complex into a size-exclusion column and eluting with the same solvent as that used for dissolving the sample. The protein and its complex are eluted first, followed by the free ligand. The elution pattern depends on the degree of the complex association, as shown by Soltes *et al.* [3] who studied the binding of bilirubin to human serum albumin by high-performance liquid chromatography (HPLC).

Nimmo and Bauermeister [4] were the first to analyse theoretically the method and to propose semi-quantitative laws for studying protein–ligand complexes in gel permeation studies. Stevens [5] incorporated the effects of association–dissociation kinetics in the elution behaviour.

Until now, the method has been limited to

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binding studies from peak-area determinations and therefore to systems in which the complex dissociates very slowly. This approach was applied to evaluate the binding affinity of prednisolone with corticosteroid-binding globulin [6] and to study protein–protein interactions [7,8]. The equilibrium is assumed to remain unchanged during the elution through the column.

The aim of the present work is to apply the direct injection method to protein–ligand binding studies under the conditions of rapid equilibration. The association constant is determined by fitting the simulated elution profiles to the experimental ones. We shall illustrate the usefulness of the method to study pollutant–protein interactions and to determine the experimental conditions under which the binding component is separated from the protein. To illustrate this approach, we shall study the interaction of bovine serum albumin (BSA) with pentachlorophenol, a system involving a strongly ligand-bound protein in water solutions, and investigate the effect of the acetonitrile content in the buffer.

THEORY

Simulation algorithm

A numerical simulation program was developed to calculate the concentrations of the protein P and the ligand L in equilibrium with the complex PL according to:

$$\frac{[P][L]}{[PL]} = K_D \tag{1}$$

where K_D is the dissociation constant.

The column was divided into equally spaced cross-slices at successive instants corresponding to multiples of a time increment [9]. This increment is the time needed for the mobile phase to flow from one slice to the next. The slice thickness is chosen arbitrarily to ensure an approach as close as possible to the ideal process. The boundary conditions assume an arbitrary function for the injection signal. In the numerical simulations a finite rectangular pulse injection was taken.

At any time and for every slice, the algorithm obeys the strict mass conservation, the mass action laws as well as the Henry law. This set of laws leads to solve a second-degree equation, whose positive root yields the desired results: on one hand, the concentrations of each species in the mobile phase and, on the other, the corresponding amounts in the adsorbed state.

The mobile phase progression in the column is simulated through the proper use of the slice indices. Axial dispersion of the solutes P or L between neighbouring slices is calculated according to the simplified Fick law. The axial dispersion is combined with the mole balances and equilibrium laws in every slice.

The simplified Fick law for any solute S (P or L) was simulated according to the scheme, using the total concentrations.

One can evaluate the concentration increments resulting from the exchange between neighbouring slices:

$$\underset{k,k+1}{\Delta}[S] = R([S]_k - [S]_{k+1}) \tag{2}$$

where $R = \dfrac{D'V_0}{\delta L \Delta z}$, and L is the column length, V_0 the volume of mobile phase, Δz the size thickness, δ the mobile phase flow-rate, and D' the global apparent dispersion coefficient. The subscripts k, $k + 1$ etc., are the slice indices.

Thus for the extreme slices, one obtains:

$$\begin{aligned} \Delta[S]_1 &= -R([S]_1 - [S]_2) \\ \Delta[S]_N &= -R([S]_{N-1} - [S]_N) \end{aligned} \tag{3}$$

and for the median ones:

$$\Delta[S]_k = -R([S]_{k-1} + [S]_{k+1} - 2[S]_k) \tag{4}$$

The simulations were performed in Fortran language using a personal computer (Compaq Deskpro Model 386/20e) equipped with an arithmetic coprocessor.

EXPERIMENTAL

Reagents and solutions

All chemicals used were of analytical grade. The salts NaH_2PO_4 and Na_2HPO_4 were from Merck (Darmstadt, Germany), BSA essentially

fatty acid-free albumin and pentachlorophenol were from Sigma (St. Louis, MO, USA).

The acetonitrile used for the preparation of mobile phases was of HPLC quality (Scharlau, Spain). Ultrapure Milli-Q water (Millipore, Bedford, MA, USA) was also used.

Acetonitrile–water mixtures containing 0.064 *M* phosphate buffer (pH 7.4) were used as mobile phases. The preparation of such solutions was performed by weighing acetonitrile and aqueous solutions and then calculating the volume percentage. The mobile phases were filtered through 0.47-μm filters using a Millipore (Bedford, MA, USA) filtration system and degassed in an ultrasonic bath.

The mobile phase was used to prepare the sample by dissolving a known amount of protein and ligand. The samples were eluted under isocratic conditions.

Equipment

The HPLC system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a Model series 1050 pump, a UV–visible variable-wavelength detector, a Model 023 recorder and a 7125 Rheodyne injection valve with a 20-μl sample loop. The column temperature was set at 20°C with a thermostated water-bath.

The column (150 mm × 4.6 mm I.D.) was slurry-packed with LiChrosorb 100 Diol (Merck) of 100-Å pore size and 10-μm particle diameter.

The ligand and the protein were detected at 329 nm near the isobestic point. At this wavelength, the pentachlorophenol–albumin complex response is equal to the sum of BSA plus pentachlorophenol responses. In preliminary experiments the detector was calibrated by injecting increasing amounts of pentachlorophenol, and the linearity of its response was checked.

The absorption spectra of pentachlorophenol, albumin and the pentachlorophenol–albumin complex were obtained with a Model Lambda 2 UV–VIS spectrophotometer (Perkin-Elmer, Beaconsfield, UK).

The analog outputs of the detectors were connected to a Model ADDA interface card on an Epson PC-286 microcomputer (Torrance, CA, USA) that controlled the data acquisition rate. The data, collected with the precision of four digits, were stored on a floppy disk.

RESULTS AND DISCUSSION

Simulation results

The simulations were performed by assuming that the protein and its complex are eluted at the the same retention volume V_0 and that the ligand injected as a single component is eluted later with a retention volume of $5V_0$. The results are in agreement with previous simulation results [4]. For a given C_L/C_P ratio of the total concentrations of protein and ligand injected, the elution pattern depends on the ratio C_p/K_D.

Fig. 1 illustrates typical simulation results obtained when the total concentrations for protein and ligand injected are equal. The figure shows that, for a high dissociation constant, the ligand is totally eluted as a symmetrical peak. The association with the complex is noticeable when $K_D = C_P/100 = C_L/100$. Lower ligand global concentrations enhance the complex formation (Figs. 2 and 3): the ligand is totally eluted as a complex at the protein retention volume if the dissociation constant is lower than $C_P/10^4$. It is still markedly associated for a value ten times

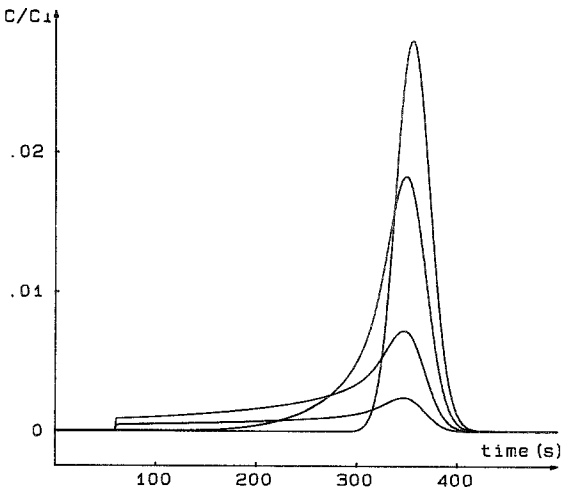

Fig. 1. Simulated elution profiles with $C_L/C_P = 1$. Flow-rate, 10 ml/min; $V_0 = 0.5$ ml; sample loop, 0.02 ml. $K_D = C_p/10$, $C_p/100$, $C_p/1000$, $C_p/10000$.

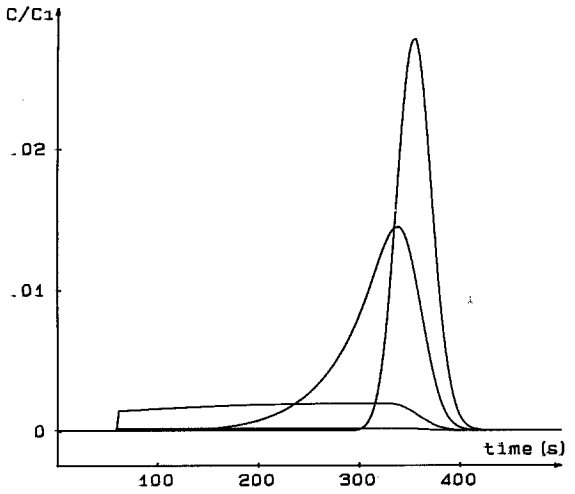

Fig. 2. Simulated elution profiles with $C_L/C_P = 0.5$. Parameters as in Fig. 1.

as large even with $C_L = C_P/2$ (Fig. 2). These elution patterns reveal a strong association of the ligand with the protein when the ligand concentration decreases.

The simulation results show that the peak distortion due to the binding of the ligand to the protein is very sensitive to the amounts of protein and ligand injected, and also to the magnitude of the dissociation constant. We shall exploit this effect to measure the dissociation constant from the chromatograms. This approach is useful to determine the experimental conditions for ensuring the total extraction of the ligand from its protein–ligand complex.

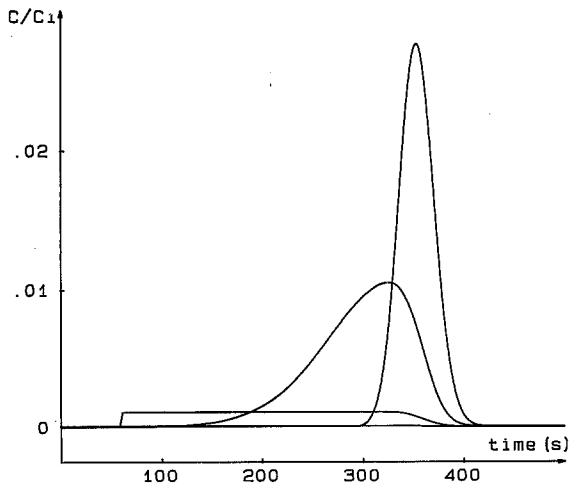

Fig. 3. Simulated elution profiles with $C_L/C_P = 0.1$. Parameters as in Fig. 1.

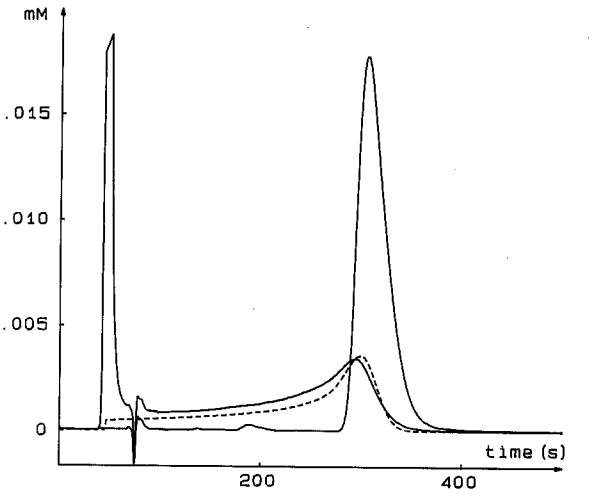

Fig. 4. Chromatographic profile of a mixture of pentachlorophenol and BSA on a Diol column: comparison with the pure elution peak. $C_P = 0.76 \cdot 10^{-3}$ *M*; $C_L = 0.78 \cdot 10^{-3}$ *M*; flow-rate, 1.5 ml/min; sample loop, 0.020 ml; mobile phase, 0.064 *M* phosphate buffer solution (pH 7.4)–acetonitrile (90:10, v/v).

Experimental study of the ligand–BSA interaction

The separation of the pentachlophenol from its complex with BSA was studied as a function of the acetonitrile content in the mobile phase. When the eluent is a pure buffer and the global concentration of ligand lower than that of BSA, the complex is totally associated and elutes at the retention volume of the protein.

In the presence of 10% (v/v) acetonitrile, one can compare (Fig. 4) the elution pattern obtained with a sample containing a mixture of the ligand and the protein and that with pure ligand at the same concentration. Fig. 4 illustrates the importance of the association effect, which reduces markedly the amount and the shape of the ligand elution peak. By reducing ten-fold the concentration of the ligand, the complex is totally associated and the amount of free ligand eluted is so small that it almost coincides with the baseline.

The numerical simulations were performed by

introducing the various experimental parameters: column length, injection volume, flow-rate and the amounts of protein and ligand injected. The theoretical result that best fits the experimental one is obtained for $K_D = 3 \cdot 10^{-7}$ M. The main drawback of the theoretical approach is the hypothesis of rapid equilibrium relative to the time of analysis. Stevens [5] discussed this point and showed that kinetic effects are negligible if the dissociation rate constant is lower than 0.02 s^{-1} when the retention time is *ca.* 600 s. It is difficult to evaluate experimentally the contribution of the kinetic rate for dissociation. Nevertheless, experiments performed at 0.5 ml/min give the same elution pattern, and the same K_D, as those at 1.5 ml/min.

The other limitation of the direct on-column injection method is the assumption that a monovalent ligand associates with only one class of binding sites on the protein. Binding data are generally represented in a Scatchard diagram [10] in which the moles of ligand per mole of macromolecular acceptor are plotted as a function of free ligand species. These plots are often non-linear, revealing ligand multivalency or the presence of several classes of acceptor binding sites.

The simulation algorithm could have been modified to account for complex interactions between the protein and the ligand, and chromatograms that agree well with the experimental ones could have been generated. However, in more sophisticated binding expressions the number of parameters to determine would increase and their values would be meaningless because of the inaccuracy of the fitting method.

The on-line injection method does not permit direct determination of the amount of ligand adsorbed as a function of its concentration as with the Hummel and Dreyer method [2]. The present work is based on simplified assumptions, and the dissociation constant determined by fitting the simulated profile to the experimental one has to be considered as an apparent one. The advantage of the method is to permit measurements of low dissociation constants in a domain where the Hummel and Dreyer method is not valid.

Increasing the amount of acetonitrile markedly reduces the association of the ligand with its complex, as shown in Fig. 5. The dissociation constant obtained from the best fit of the theoretical curve to the experimental one (Fig. 5a) is now $K_D = 4 \cdot 10^{-6}$ M. Fig. 5b shows the pattern obtained when the concentration of the ligand is reduced ten-fold. As predicted from the simulations, a pattern with a small baseline deviation is obtained.

With 30% (v/v) acetonitrile in the eluent, the ligand is totally dissociated and the area of the second peak is equal to that of the pure component. In these experiments, it was not possible to determine the dissociation constant: with the diol column, increasing the acetonitrile content re-

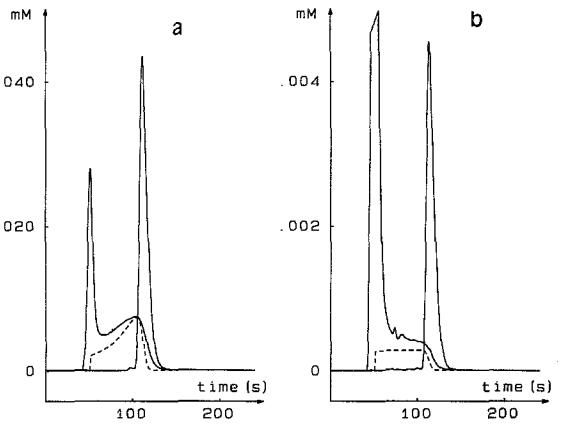

Fig. 5. Chromatographic profile of a mixture of pentachlorophenol and BSA on a Diol column: comparison with the pure elution peak. $C_P = 0.75 \cdot 10^{-3}$ M, (a) $C_L = 0.5 \cdot 10^{-3}$ M, (b) $C_L = 0.05 \cdot 10^{-3}$ M; flow-rate, 1.5 ml/min; sample loop, 0.020 ml; mobile phase, 0.064 M phosphate buffer solution (pH 7.4)–acetonitrile (90:20, v/v).

TABLE I

INFLUENCE OF THE ACETONITRILE CONTENT ON THE PENTACHLOROPHENOL–BSA DISSOCIATION CONSTANT

Acetonitrile content (%, v/v)	K_D (M)
0	$<10^{-9}$
10	$3 \cdot 10^{-7}$
20	$4 \cdot 10^{-6}$
30	$>10^{-5}$

duces the ligand retention time and therefore the resolution between the first and the second peak.

Table I summarizes the values of the dissociation constant of the complex pentachlorophenol–BSA as a function of the acetonitrile content. This table illustrates the domain where the direct on-column injection method can be applied to determine the dissociation constant. This range is complementary of that of the Hummel and Dreyer method [2]. This work shows that the limit of validity for applying the Hummel and Dreyer method with a diol column is for dissociation constants larger than 10^{-6} *M*.

CONCLUSION

The results presented here show that acetonitrile markedly decreases the association between the ligand and the protein. It is this interaction that determines the separation on a chromatographic column. The direct on-column injection method is useful to determine the experimental conditions, such as the solvent composition, that lead to the separation of the pollutant from its complex with proteins. This approach is especially promising to determine the conditions of complete pollutant recovery in biological samples when using clean-up procedures and cartridge extraction at trace level.

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