Rate Parameters and Gradient Correlations for Gradient-Elution Chromatography

Robert G. Luo
Dept. of Chemical Engineering, Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, NJ 07102

James T. Hsu
Dept. of Chemical Engineering, Lehigh University, Bethlehem, PA 18015

Rate parameters and gradient correlations of proteins are very important to engineering investigations of gradient-elution chromatography. Before carrying out computer simulations for gradient process, these rate parameters and gradient correlations must be determined from experimental data. This work presents a systematic method for estimating and determining these parameters and correlations using experimental data and computer-simulation results based on isocratic runs. The values determined were then employed in gradient-elution studies by putting them into the rate model to perform computer simulations. Experimental runs under gradient conditions using the parameters and correlations just referred to were carried out in a DEAE Sepharose CL-6B ion-exchange chromatography. Comparisons between the experimental data and the simulation results show good agreement.

Introduction

Gradient elution, first developed by Alm et al. (1952) as a liquid chromatographic technique, has found applications in almost every form of chromatography that uses a liquid mobile phase (Lederer and Lederer, 1957). Gradient elution chromatography plays a very important role in separation of biomolecules in both analytical studies and large-scale processes (Frey, 1990). The recent, rapid development of the biotechnology industry demands downstream recovery and purification techniques with high selectivities. Consequently, their proper applications require fundamental engineering studies on these separation processes.

While the use of gradient elution for analytical-scale chromatographic separation is well established (Freiling, 1955, 1957; Jandera and Churacek, 1974, 1978, 1979, 1985; Jandera, 1989; Snyder, 1961, 1964; Snyder et al., 1979, 1989a,b; Snyder and Glajch, 1990; Tomellini and Hartuich, 1985; Tomellini et al., 1986), few studies have addressed the basic features of adsorption kinetics and mass-transfer characteristics of industrial uses of gradient-elution chromatography. The major problem with gradient-elution bioseparations is that the development, scale-up, and optimization of the processes depend on trial and error. This is usually time-consuming and expensive, especially for large-scale processes.

In order to carry out a complete engineering study of gradient-elution chromatography, a mathematical model is needed, which will more realistically simulate the chromatographic process than conventionally employed models. The model must take into account the combined effects of axial dispersion, film mass-transfer resistance, intraparticle diffusion, and surface adsorption kinetics. By using available numerical methods and high-speed computers, the mathematical model can be solved and then used to guide industrial scale-up, design, and optimization of the gradient-elution chromatography systems. Before 1985, the mathematical models used in most studies of this area were relatively simple—plate theory, for instance—and were not adequate to describe the actual gradient-elution process. In recent years, a more realistic approach, the rate model, which is based on the mass balance in the mobile phase and the stationary phase, was adopted by Gibbs and Lightfoot (1986), and Kang and McCoy (1989). To solve the models, Kang and McCoy

Correspondence concerning this article should be addressed to J. T. Hsu.

used the method of moments together with the numerical method of Runge-Kutta, while Gibbs and Lightfoot applied a special method developed by Reis et al. (1979) together with the method of finite difference.

Yamamoto and coworkers (1983a,b) have investigated, theoretically and experimentally, gradient elution in ion-exchange chromatography of proteins. By applying a continuous-flow plate model, they demonstrated how a simple analysis of small-scale experiments can guide the design and operation of large-scale separations of proteins by ion-exchange chromatography. However, the plate model required some simplifying assumptions, which limits its effectiveness. Kang and McCoy (1989) improved the studies just mentioned by a fundamental treatment of the transport phenomena in an ion-exchange column. A rate model of material balance was applied to the mobile phase and the method of moments was used to solve the model. While the rate model for the mobile phase is more accurate in describing the gradient-elution process than the plate model, the effects of intraparticle diffusion and surface adsorption kinetics have been ignored. Furthermore, although the method of moments (Kucera, 1965; Schneider and Smith, 1968) has long been popular with chromatographers, primarily because of its simplicity and possibility of working in the frequency domain where solutions are easily obtained, it suffers from a number of inherent drawbacks. One of them is that too much weight is placed on the tail part of the output peak corresponding to larger values of time, where signal noise and eluting impurities may contribute to inaccurate measurements (Anderssen and White, 1971).

Gibbs and Lightfoot (1986) have employed a more sophisticated rate model, which takes into account the mass transport of both mobile phase and stationary phase, and the surface adsorption kinetics. An assumption was made that the protein adsorption equilibrium constants were not position dependent, which is different from the real situation in which the protein adsorption equilibrium constants are functions of both time and position. Frey (1990) presented a set of equations for gradient-elution chromatography based on the exact solution of the isocratic model plus the effect of the "solvent modifier." Tsao and coworkers (1992) also adopted a rate model (Gu et al., 1992) in their studies on strategies for large-scale gradient-elution chromatography. The model was used to predict the protein peak positions and profiles of gradient concentrations. An empirical gradient correlation was used for proteins eluted in an ion-exchange system. Luo and Hsu (1993) applied the rate model in the investigation of intraparticle protein diffusion effect on gradient-elution chromatography. The individual and combined effects of three important factors—intraparticle diffusivity, adsorbent particle diameter, and linear gradient slope on the separation of proteins—were studied. A general strategy for design and optimization of gradient-elution process for biomolecule separations was suggested based on the investigation.

The purpose of this study was to establish a general procedure to estimate rate parameters and to determine gradient correlations based on experimental runs and computer simulations. The ultimate goal was to develop a methodology and an engineering tool for process development, scale-up, and optimization of gradient-elution chromatography for biomolecule separations.

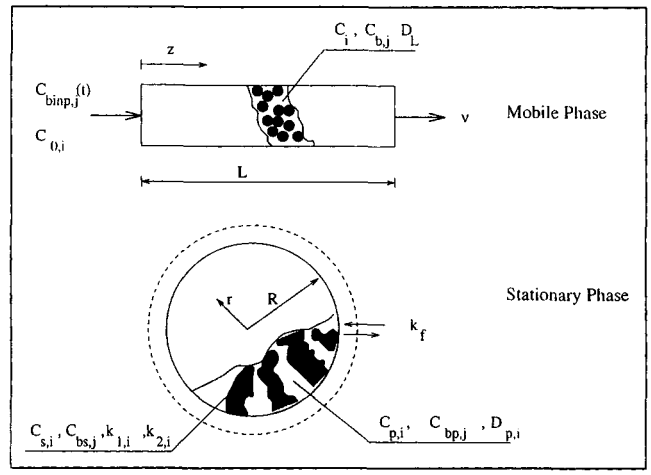

Figure 1. Chromatography column.

Model for Gradient-Elution Chromatography

Generally speaking, liquid chromatography for biomolecules is a combined process of fluid mechanics, mass transfer, and adsorption. Figure 1 is a schematic of a chromatographic system. The upper part of the figure is a column packed with adsorbent particles. Sample component i, with an initial concentration of $C_{o,i}$, flows through the column with eluting buffer in the z direction at an interstitial velocity, v; C_i is the concentration of sample component i in the mobile phase; $C_{b,j}$ is the concentration of eluting buffer component j in the mobile phase; ϵ is the column void fraction; D_L is axial dispersion coefficient. The lower part of the figure is an enlarged spherical adsorbent particle. $C_{p,i}$ is the concentration of sample component i in the liquid part (pore) of the particle; $C_{bp,j}$ is the concentration of eluting buffer component j in the liquid part (pore) of the particle; $C_{s,i}$ is the concentration of sample component i on the solid surface of the particle; $C_{bs,j}$ is the concentration of eluting buffer component j in the solid surface of the particle; $D_{p,i}$ is intraparticle diffusivity; $k_{f,i}$ is film mass-transfer coefficient; and $k_{1,i}$ and $k_{2,i}$ are kinetic constants.

In gradient elution processes the ionic strength, pH, concentration and composition of the mobile phase (i.e., eluting buffer) are changed with time according to a preset program. Since the retention strengths of the feed components depend on the conditions of the eluting buffer, the kinetic constants and distribution coefficients of these feed components are functions of mobile phase ionic strength, pH, or composition. This fact links the sample and the eluting buffer to form a complete mathematical model.

Using the assumptions below, the following mathematical model, which takes into account the combined effects of axial dispersion, film mass-transfer resistance, intraparticle diffusion, and surface adsorption kinetics, can be used to describe the gradient-elution process in an ion-exchange column:

1. The flow pattern is described by an axially dispersed plug-flow model.
2. The film mass-transfer rate is represented by a linear driving-force expression.
3. All particles packed in the column are spherical.
4. The adsorption kinetics are first order.

Mass balances for sample components

Mobile-phase mass balances are

$$\frac{\partial C_i}{\partial t} + v\frac{\partial C_i}{\partial z} - D_L\frac{\partial^2 C_i}{\partial z^2} = -\left(\frac{1-\epsilon}{\epsilon}\right)\left[\frac{3k_f}{R}(C_i - C_{p,i}|_{r=R})\right] \tag{1}$$

with initial and boundary conditions

$$C_i(z>0,t=0) = C_{p,i}(z>0,t=0) = 0 \tag{2}$$

$$D_L\frac{\partial C_i}{\partial z}\bigg|_{z=0} = -v(C_i|_{z=0^-} - C_i|_{z=0^+}) \tag{3}$$

$$\frac{\partial C_i}{\partial z}\bigg|_{z=L} = 0. \tag{4}$$

Stationary-phase mass balances are

$$\epsilon_p\frac{\partial C_{p,i}}{\partial t} + \frac{\partial C_{s,i}}{\partial t} = \epsilon_p D_{p,i}\left(\frac{\partial^2 C_{p,i}}{\partial r^2} + \frac{2}{r}\frac{\partial C_{p,i}}{\partial r}\right) \tag{5}$$

with initial and boundary conditions

$$C_{p,i}(r,t=0) = C_{s,i}(r,t=0) = 0 \tag{6}$$

$$\frac{\partial C_{p,i}}{\partial r}\bigg|_{r=0} = 0 \tag{7}$$

$$D_{p,i}\frac{\partial C_{p,i}}{\partial r}\bigg|_{r=R} = k_f(C_i - C_{p,i}|_{r=R}). \tag{8}$$

Particle surface adsorption rates are

$$\frac{\partial C_{s,i}(r,t)}{\partial t} = k_{1,i}C_{p,i} - k_{2,i}C_{s,i}. \tag{9}$$

Note that in Eq. 3

$$C_i|_{z=0^-} = C_{0,i}(t_{0,i} \geq t \geq 0) \tag{10}$$

$$C_i|_{z=0^-} = 0(t > t_{0,i}), \tag{11}$$

which means a pulse input. And in Eq. 9

$$k_{1,i} = f_{1,i}(C_{bp,j}) \tag{12}$$

$$k_{2,i} = f_{2,i}(C_{bp,j}), \tag{13}$$

where $i = 1, 2, \ldots, n$, where n is the number of sample components; $j = 1, 2, \ldots, h$, where h is the number of eluting buffer components. In these equations L is the length of the adsorbent bed; $t_{o,i}$ is the loading time, and $C_{bp,j}$ refers to mobile-phase buffer concentrations. It can be seen from Eq. 12 and Eq. 13 that the kinetic constants, $k_{1,i}$ and $k_{2,i}$, of sample components are functions of mobile-phase buffer concentrations, $C_{bp,j}$; thus the concentration of the eluting buffer needs to be considered in order to solve the sample's equations. When an equilibrium is reached for a first-order kinetics described by Eq. 9, the adsorption equilibrium constant of a sample component, $K_{a,i}$, can be expressed as the ratio of the forward kinetic constant and the backward kinetic constant,

$$K_{a,i} = \frac{k_{1,i}}{k_{2,i}}. \tag{14}$$

Unlike the situation in isocratic elution where $K_{a,i}$ is constant, in gradient-elution chromatography $K_{a,i}$ is a function of mobile-phase parameters such as pH, ionic strength, and composition. For example, protein retention in an anion-exchange chromatography is affected by the difference between the pH of the mobile phase and the pI of the protein, the larger the difference, the larger the $K_{a,i}$, thus the stronger the protein binding to the stationary phase. Protein retention is also affected by the ionic strength of the mobile phase, the lower the ionic strength, the larger the $K_{a,i}$, thus the stronger the protein binding to the stationary phase. Therefore, in order to solve the preceding equations for sample components, a set of equations for the mobile-phase eluting buffer is needed.

Mass balances for the eluting buffer

Mobile-phase mass balances are

$$\frac{\partial C_{b,j}}{\partial t} + v\frac{\partial C_{b,j}}{\partial z} - D_L\frac{\partial^2 C_{b,j}}{\partial z^2} = -\left(\frac{1-\epsilon}{\epsilon}\right)\left[\frac{3k_f}{R}(C_{b,j} - C_{bp,j}|_{r=R})\right], \tag{15}$$

with initial and boundary conditions

$$C_{b,j}(z>0,t=0) = C_{bp,j}(z>0,t=0) = C_{b0,j} \tag{16}$$

$$D_L\frac{\partial C_{b,j}}{\partial z}\bigg|_{z=0} = -v(C_{b,j}|_{z=0^-} - C_{b,j}|_{z=0^+}) \tag{17}$$

$$\frac{\partial C_{b,j}}{\partial z}\bigg|_{z=L} = 0. \tag{18}$$

Stationary-phase mass balances are

$$\epsilon_p\frac{\partial C_{bp,j}}{\partial t} + \frac{\partial C_{bs,j}}{\partial t} = \epsilon_p D_{bp,i}\left(\frac{\partial^2 C_{bp,j}}{\partial r^2} + \frac{2}{r}\frac{\partial C_{bp,j}}{\partial r}\right), \tag{19}$$

with initial and boundary conditions

$$C_{bp,j}(r,t=0) = C_{b0,j} \tag{20}$$

$$\frac{\partial C_{bp,j}}{\partial r}\bigg|_{r=0} = 0 \tag{21}$$

$$D_{p,j}\frac{\partial C_{bp,j}}{\partial r}\bigg|_{r=R} = k_f(C_{b,j} - C_{bp,j}|_{r=R}). \tag{22}$$

Particle surface adsorption rates are

$$\frac{\partial C_{bs,j}}{\partial t} = k_{b1,j}C_{bp,j} - k_{b2,j}C_{bs,j}, \tag{23}$$

where $j = 1, 2, \ldots, h$, where h is the number of components in the eluting buffer. Notice that in Eq. 17

$$C_{b,j}|_{z=0^-} = C_{binp,j}(t), \tag{24}$$

which means that the input buffer profile is a function of time. Notice that the eluting buffer concentration, $C_{b,j}$, is a function of both time and position. Consequently, the adsorption equilibrium constant of a sample component, $K_{a,i}$, is also a function of time and position.

In many bioseparations, the sample concentrations are dilute. This results in first-order adsorption kinetics. In agarose-based ion-exchange chromatographic processes adsorption equilibrium can be reached because usually the diffusion of biomolecules is the rate-limiting step (Skidmore et al., 1990). At equilibrium, the first-order adsorption rate equation, Eq. 9, becomes a linear isotherm:

$$C_{s,i} = K_{a,i}C_{p,i}, \tag{25}$$

where $K_{a,i}$ is the adsorption equilibrium constant of sample component i. Here one needs to be aware that although the isotherm is linear, the model is a *nonlinear* system because $K_{a,i}$ is a nonlinear function of mobile-phase parameters.

For kinetics with higher orders, Eq. 9 will be altered. In the case of a second-order reversible interaction, the equation can be written as

$$\frac{\partial C_{s,i}}{\partial t} = k_{1,i}C_{p,i}\left[C_T - \sum_{i=1}^{n} C_{s,i}\right] - k_{2,i}C_{s,i}. \tag{26}$$

Here $i = 1, 2, \ldots, n$, where n is the number of components in the sample; and C_T represents the maximum adsorption capacity of the adsorbent. At equilibrium, Eq. 26 reduces to the familiar Langmuir isotherm:

$$C_{s,i} = \frac{C_{p,i}C_T - C_{p,i}\sum_{j=1}^{n-1} C_{s,j}}{C_{p,i} + K_{d,i}}. \tag{27}$$

Here $K_{d,i}$ is the dissociation constant given by the following relation:

$$K_{d,i} = \frac{k_{2,i}}{k_{1,i}}. \tag{28}$$

There are $n + h$ sets of partial differential equations in this model. Here n represents the number of biomolecule components in the loading sample solution, and h represents the number of eluting buffer components. By adopting the dimensionless groups in Table 1, the equations in the model can be written in dimensionless forms. The dimensionless forms of Eqs. 1 to 13 for sample component are

$$\frac{\partial U_i}{\partial \tau} = \frac{1}{Pe}\sigma_{p,i}\psi_i\beta_i\frac{\partial^2 U_i}{\partial X^2} - \sigma_{p,i}\psi_i\beta_i\frac{\partial U_i}{\partial X} - 3\sigma_{p,i}\psi_i\xi_i[U_i - Q_i|_{\eta=1}] \tag{29}$$

$$U_i(X > 0, \tau = 0) = Q_i(X > 0, \tau = 0) = 0 \tag{30}$$

$$\left.\frac{\partial U_i}{\partial X}\right|_{X=0} = -Pe(U_i|_{X=0^-} - U_i|_{X=0^+}) \tag{31}$$

$$\left.\frac{\partial U_i}{\partial X}\right|_{X=1} = 0 \tag{32}$$

$$\epsilon_p\frac{\partial Q_i}{\partial \tau} + \frac{\partial S_i}{\partial \tau} = \epsilon_p\sigma_{p,i}\nabla^2 Q_i \tag{33}$$

$$Q_i(\eta, \tau = 0) = S_i(\eta, \tau = 0) = 0 \tag{34}$$

Table 1. Dimensionless Groups

$Q_i = C_{p,i}/C_{o,i}$	Dimensionless fluid concentration of sample component i in particle
$S_i = C_{s,i}/C_{o,i}$	Dimensionless surface concentration of sample component i in particle
$U_i = C_i/C_{o,i}$	Dimensionless mobile-phase concentration of sample component i
$Q_{b,j} = C_{bp,j}/C_{bo,j}$	Dimensionless fluid concentration of buffer component j in particle
$S_{b,j} = C_{bs,j}/C_{bo,j}$	Dimensionless surface concentration of buffer component j in particle
$U_{b,j} = C_{b,j}/C_{bo,j}$	Dimensionless mobile-phase concentration of buffer component j
$\sigma_{p,i} = D_{p,1}/D_{p,i}$	Dimensionless diffusivity parameter for sample component i
$\sigma_{bp,j} = D_{p,1}/D_{bp,j}$	Dimensionless diffusivity parameter for buffer component j
$\omega_{1,i} = k_{1,i}R^2/(\epsilon_p D_{p,1})$	Dimensionless kinetic parameter for sample component i
$\omega_{2,i} = k_{2,i}R^2/(\epsilon_p D_{p,1})$	Dimensionless kinetic parameter for sample component i
$\omega_{b1,j} = k_{b1,j}R^2/(\epsilon_p D_{p,1})$	Dimensionless kinetic parameter for buffer component j
$\omega_{b2,j} = k_{b2,j}R^2/(\epsilon_p D_{p,1})$	Dimensionless kinetic parameter for buffer component j
$X = z/L$	Dimensionless axial distance
$\eta = r/R$	Dimensionless radial distance in particle
$Pe = vL/D_L$	Peclet number
$\psi_i = K_{a,i}(1-\epsilon)/\epsilon$	Distribution ratio for sample component i
$\psi_{b,j} = K_{ba,j}(1-\epsilon)/\epsilon$	Distribution ratio for buffer component j
$\beta_i = vR^2\epsilon/(LD_{p,i}K_{a,i}(1-\epsilon))$	Bed-length parameters for sample component i
$\beta_{b,j} = VR^2\epsilon/(LD_{bp,j}K_{ba,j}(1-\epsilon))$	Bed-length parameters for buffer component j
$\xi_i = k_f R/(D_{p,i}K_{a,i})$	Film-resistance parameter for sample component i
$\xi_{b,j} = k_f R/(D_{bp,j}K_{ba,j})$	Film-resistance parameter for buffer component j
$\tau = D_{p,1}t/R^2$	Contact time parameter

$$\left.\frac{\partial Q_i}{\partial \eta}\right|_{\eta=0} = 0 \tag{35}$$

$$\left.\frac{1}{K_{a,i}}\frac{\partial Q_i}{\partial \eta}\right|_{\eta=1} = \xi_i(U_i - Q_i|_{\eta=1}) \tag{36}$$

$$\frac{\partial S_i}{\partial \tau} = \epsilon_p \omega_{1,i} Q_i - \epsilon_p \omega_{2,i} S_i \tag{37}$$

$$U_i|_{X=0^-} = 1 \qquad (\tau_{0,i} \geq \tau \geq 0) \tag{38}$$

$$U_i|_{X=0^-} = 0 \qquad (\tau > \tau_{0,i}) \tag{39}$$

$$\omega_{1,i} = F_{1,i}(Q_{b,j}) \tag{40}$$

$$\omega_{2,i} = F_{2,i}(Q_{b,j}). \tag{41}$$

Equations 15 to 24 can be written in dimensionless form:

$$\frac{\partial U_{b,j}}{\partial \tau} = \frac{1}{Pe}\sigma_{bp,j}\psi_{b,j}\beta_{b,j}\frac{\partial^2 U_{b,j}}{\partial X^2} - \sigma_{bp,j}\psi_{b,j}\beta_{b,j}\frac{\partial U_{b,j}}{\partial X} - 3\sigma_{bp,j}\psi_{b,j}\xi_{b,j}[U_{b,j} - Q_{b,j}|_{\eta=1}] \tag{42}$$

$$U_{b,j}(X > 0, \tau = 0) = Q_{b,j}(X > 0, \tau = 0) = 1 \tag{43}$$

$$\left.\frac{\partial U_{b,j}}{\partial X}\right|_{X=0} = -Pe[U_{b,j}|_{X=0^-} - U_{b,j}|_{X=0^+}] \tag{44}$$

$$\left.\frac{\partial U_{b,j}}{\partial X}\right|_{X=1} = 0 \tag{45}$$

$$\epsilon_p\frac{\partial Q_{b,j}}{\partial \tau} + \frac{\partial S_{b,j}}{\partial \tau} = \epsilon_p \sigma_{bp,j}\nabla^2 Q_{b,j} \tag{46}$$

$$Q_{b,j}(\eta, \tau = 0) = 1 \tag{47}$$

$$\left.\frac{\partial Q_{b,j}}{\partial \eta}\right|_{\eta=0} = 0 \tag{48}$$

$$\left.\frac{1}{K_{ba,j}}\frac{\partial Q_{b,j}}{\partial \eta}\right|_{\eta=1} = \xi_{b,j}[U_{b,j} - Q_{b,j}|_{\eta=1}] \tag{49}$$

$$\frac{\partial S_{b,j}}{\partial \tau} = \epsilon_p \omega_{b1,j} Q_{b,j} - \epsilon_p \omega_{b2,j} S_{b,j} \tag{50}$$

$$U_{b,j}|_{X=0^-} = U_{binp,j}(\tau). \tag{51}$$

For a linear adsorption isotherm, the equilibrium case of Eq. 37 can be written as

$$S_i = K_{a,i} Q_i. \tag{52}$$

In many cases the molecular sizes of the desorbents are much smaller than the biomolecules and the adsorbent pores. Thus the governing equations of the eluting buffer, Eqs. 42 to 51 can be reduced in dimensionless form as follows:

$$\frac{\partial U_{b,j}}{\partial \tau} = \frac{1}{1 + m/K_{ba,j}}\left[\frac{1}{Pe}\psi_{b,j}\beta_{b,j}\frac{\partial^2 U_{b,j}}{\partial X^2} - \psi_{b,j}\beta_{b,j}\frac{\partial U_{b,j}}{\partial X}\right] \tag{53}$$

$$U_{b,j}(X > 0, \tau = 0) = 1 \tag{54}$$

$$\left.\frac{\partial U_{b,j}}{\partial X}\right|_{X=0} = -Pe[U_{b,j}|_{X=0^-} - U_{b,j}|_{X=0^+}] \tag{55}$$

$$\left.\frac{\partial U_{b,j}}{\partial X}\right|_{X=1} = 0 \tag{56}$$

$$U_{b,j}|_{X=0^-} = U_{binp,j}(\tau), \tag{57}$$

where $m = \epsilon/(1-\epsilon)$.

The preceding set of partial differential equations (PDEs) was then transformed into a set of ordinary differential equations (ODEs) by applying the method of orthogonal collocation (Finlayson, 1972, 1980; Raghavan and Ruthven, 1983; Villadsen and Stewart, 1967; Villadsen and Michelsen, 1978). FORTRAN programs were written to solve these ODEs by calling an ODE solver IVPAG with Gear's method (Gear, 1971) in IMSL software library (IMSL User's Manual, 1989) on an IBM RS/6000 Model 950 mainframe computer. The effects of various parameters on sample elution peaks were investigated (Luo and Hsu, 1992, 1993). Rate parameters and gradient correlations were estimated and determined using experimental data and computer-simulation results based on isocratic runs.

Experimental Study

The experiments in this study can be classified into two categories. The first category contains experiments using isocratic elution runs. They are used to estimate rate parameters and determine adsorption equilibrium-constant correlations. In the simulations in previous studies the rate parameters required by the rate model and adsorption equilibrium-constant correlations were taken from the literature (Luo and Hsu, 1993). Because these parameters and correlations are affected by the column adsorbent and packing, they should be determined for each experimental system under isocratic conditions. This same experimental system can then be used for later gradient-elution studies. The second category contains experiments using gradient-elution runs. The experimental data were collected by a PC data-acquisition system.

Experimental system

Experimental Apparatus. The experiments were conducted in a 1.5- ×40.0-cm glass column (Spectrum Medical Industries, Inc., Los Angeles, CA). The packed adsorbent bed height was 22.0 cm. An adjustable plunger was used to minimize the dead space over the top of the bed to assure accurate buffer gradients. The buffer gradients were generated by an SSI 220 B HPLC pump and a model 231 gradient controller with a keypad (Scientific Systems, Inc., State College, PA). The ultraviolet absorbance of the eluent from the column was measured by a LKB 2238 Uvicord SII monitor (LKB-Produkter AB, Bromma, Sweden). The operating wavelength was 280 nm. The flow cell used in the monitor was a standard cell with an optical path length of 0.25 cm.

The experimental results were recorded by two means. The first one was a LKB 2210 1-channel potentiometric recorder (LKB-Produkter AB, Bromma, Sweden), which was used to show chromatograms directly during the experiments. The

second one was a computer data-acquisition system (Zenith Data Systems Corporation, St. Joseph, MI), which was used to record the chromatograms in digital forms so that they could be transferred later to an IBM RS/6000 Model 950 mainframe computer for data processing and analysis. When necessary, a fraction collector RediFrac (Pharmacia Biotech, Piscataway, NJ) was used to collect fractions.

Model Proteins. β-Lactoglobulin from bovine milk was used as the model protein in the experimental study. β-Lactoglobulin was chosen because it has unique characteristics. β-lactoglobulin A (LGA) has aspartic acid and valine, while β-lactoglobulin B (LGB) has glycine and alanine at residues 64 and 118, respectively (Piez et al., 1961). Their molecular weights are about the same, 35,500 (Townend et al., 1960). The slight differences in amino acid sequences results in very close isoelectric points of these two proteins. The pI value is 5.21 for β-lactoglobulin A and 5.34 for β-lactoglobulin B (Kaplan and Foster, 1971). Proteins with little difference in pI values are difficult to separate by isocratic elution in ion exchange chromatography. These two proteins have been separated over an anion exchanger by gradient elution (Piez et al., 1961), therefore they make a good "model" binary pair for this work. The proteins were purchased from SIGMA Chemical Company (St. Louis, MO). Three times crystallized and lyophilized β-lactoglobulin (Lot. 0130), which contained approximately equal amounts of β-lactoglobulin A and β-lactoglobulin B, was used for parameter estimation and separation studies. β-Lactoglobulin A (Lot. 7880) and β-lactoglobulin B (Lot. 8005) were used for determination of adsorption equilibrium constant correlations.

Adsorbent. In this work, a weak anion exchange resin DEAE (diethylaminoethyl) Sepharose CL-6B (Pharmacia Biotech, Piscataway, NJ) was chosen as the adsorbent. Sepharose CL-6B is a porous, agarose-based matrix in a beaded form, which is cross-linked for high stability. The diameter of the adsorbent particle is 0.011 cm. It was chosen for the present work because it was used before for β-lactoglobulin intraparticle diffusion studies (Boyer and Hsu, 1992) and gradient-elution studies (Yamamoto et al., 1983b), where adsorption isotherms and intraparticle diffusivity correlation were determined. Adsorption for both proteins is linear as long as the protein concentrations are less than 0.03% (weight to volume, Yamamoto et al., 1983b).

Buffer System. In order to generate ionic-strength (salt) gradient, two buffer reservoirs were used. The starting buffer, buffer A, was 18 mM TRIS/HCl, pH 7.9. The ionic-strength-altering buffer, buffer B, was 0.5 M sodium chloride. For a gradient-elution process, buffer A and buffer B were mixed by an HPLC pump and a gradient of ionic strength of this mixture was generated by altering the relative flow rates of buffers A and B. The flow rates were changed according to a preset program so that the ionic strength of the mixed buffer was a function of time.

Rate Parameter Estimations by Isocratic Elution Runs

The dispersion coefficient, D_L, bed void fraction, ϵ, intraparticle diffusivity, D_p, and film mass-transfer coefficient, k_f, are important process parameters in the rate model. For a gradient-elution process, the effect of eluting buffer conditions on the strength of protein adsorption is critical to process modeling. Therefore, before carrying out computer simulations for gradient elution, these rate parameters and gradient correlations must be found from experimental data. In the case of the current experimental system, the gradient correlations are expressed by the relationships between the protein adsorption equilibrium constants and the ionic strength of the eluting buffer.

Estimation of dispersion coefficient, D_L, and bed void fraction, ϵ

To estimate dispersion coefficient, D_L, and bed void fraction, ϵ, pulse experiments were conducted in the column using 0.5% Dextran 2000 (SIGMA, Lot. D-5376) as an indicator. The reason for using high molecule weight Dextran is to eliminate intraparticle diffusion so that an accurate column void fraction can be obtained. In order to prevent any adsorption effect, 0.5 M NaCl was used to elute the Dextran at a flow rate of 1.5 mL/min. This process can be described by a "dispersion model," which is discussed in detail by Levenspiel (1972). In this model, if column diameter, column length, and flow rate are all fixed, dispersion coefficient and void fraction will be the only parameters affecting the Dextran peak. The dispersion coefficient and void fraction were estimated by the following procedures. The experimental peak was recorded by a PC data-acquisition system and then transferred to a mainframe computer. On the mainframe, the fixed system parameters such as column diameter, column length, and flow rate were put into the "dispersion model." Computer simulations were conducted by gradually changing the values of the dispersion coefficient, D_L, and bed void fraction, ϵ, to curve fit the experimental data. While ϵ affects the position of the peak, D_L affects the shape of the peak. The larger ϵ, the longer the elution time. The larger D_L, the wider the peak. By matching the position of the simulated peak with the position of the experimental peak, it was found that the bed void fraction, ϵ, is 0.28. For the purpose of comparison, we also determined the void fraction using the empirical method, that is, taking the ratio of elution volume of the Dextran, V, and the column void volume, V_o. The value of V/V_o is 0.29. The two results are very close and the difference between them is only 3%. Using this bed void fraction (0.28), the simulation was repeated to estimate the dispersion coefficient. D_L was estimated as 0.235 cm^2/min by comparing the shape of the simulation peak with the shape of the experimental peak.

Estimation of film mass-transfer coefficient, k_f

The film mass-transfer coefficient, k_f, was determined from the correlation of Kataoka et al. (1973)

$$\frac{k_f}{v} = 1.85 m^{1/3} Re^{-2/3} Sc^{-2/3}, \tag{58}$$

where $m = \epsilon/(1-\epsilon)$, Re is the Reynolds number, and Sc is the Schmidt number. For this system at a flow rate, F, of 1.5 mL/min, k_f was calculated to be 0.22 cm/min. Since the rate-limiting step of this chromatographic process is intraparticle diffusion, variation of k_f over a range of ±50% from the correlated value resulted in no significant change in the simulation curve.

Estimation of initial adsorption equilibrium constants, $K_{0,i}$, and intraparticle diffusivity, $D_{p,i}$

In this case, 0.5% β-lactoglobulin was used as an indicator to conduct pulse experiments on the column with 0.5 M NaCl as the eluting buffer. The adsorption equilibrium constants obtained using this high-power eluting buffer are called initial adsorption equilibrium constants, $K_{0,i}$. Here $i = 1$ indicates β-lactoglobulin A and $i = 2$ indicates β-lactoglobulin B in this experimental system. For ion-exchange chromatography with adsorption equilibrium, the relationship between equilibrium constants, $K_{a,i}$, and initial equilibrium constants, $K_{0,i}$, is expressed by

$$K_{a,i} = K_{0,i} + A_{a,i} I^{B_{a,i}} \tag{59}$$

based on the studies of Gibbs and Lightfoot (1986), and Yamamoto et al. (1983a), where $A_{a,i}$ and $B_{a,i}$ are constants. The adsorption equilibrium constant, $K_{a,i}$, is an important parameter in gradient elution chromatography. It is slightly different from the distribution coefficient, K_i, which is defined as the ratio of the concentrations of the sample in the stationary phase and the mobile phase, while $K_{a,i}$ is defined as the ratio of the stationary-phase-sample surface concentration to the stationary-phase-sample liquid concentration in an adsorbent particle.

The estimation procedure was similar to that used for the dispersion coefficient and bed void fraction. The fixed system parameters such as column diameter, adsorbent particle diameter, and flow rate, together with the dispersion coefficient and bed-void-fraction values previously obtained, were put into the model presented earlier in this article, which is also identified as the "diffusion model." Computer simulations of these isocratic elution runs were conducted by gradually changing the values of the initial adsorption equilibrium constants, $K_{0,i}$, and intraparticle diffusivity, $D_{p,i}$ to curve fit the experimental data, as shown in Figure 2. While $K_{0,i}$ affects the position of the peak, $D_{p,i}$ affects the shape of the peak. The larger the $K_{0,i}$, the longer the elution time. The larger the $D_{p,i}$, the narrower the peak. By matching the position of the simulation peak with the positin of the experimental peak, it was found that the initial equilibrium constants, $K_{0,i} = 0.6$ for both β-lactoglobulin A and β-lactoglobulin B. With this initial equilibrium constant the simulation was conducted again. The intraparticle diffusivity was estimated as $D_{p,i} = 1.74 \times 10^{-5}$ cm²/min by matching the shape of the simulation peak with the shape of the experimental peak. The protein effective intraparticle diffusivity, D_e, can be calculated using the relationship $D_p = D_e/\epsilon_p$, here ϵ_p is the porosity of the adsorbent particle (Pharmacia, 1987). Note that the effective intraparticle diffusivity, D_e, is based on the total volume of the adsorbent particle. The intraparticle diffusivity or "pore diffusivity," D_p, is based on the accessible or pore volume of the adsorbent particle.

Determination of gradient correlations of adsorption equilibrium constants by isocratic elution runs

In this study, a new method was developed to determine the values of the constants $K_{0,i}$, $A_{a,i}$, and $B_{a,i}$. This method is simpler and less time-consuming than the isotherm method used by Yamamoto et al. (1983b). β-Lactoglobulin A (0.025%)

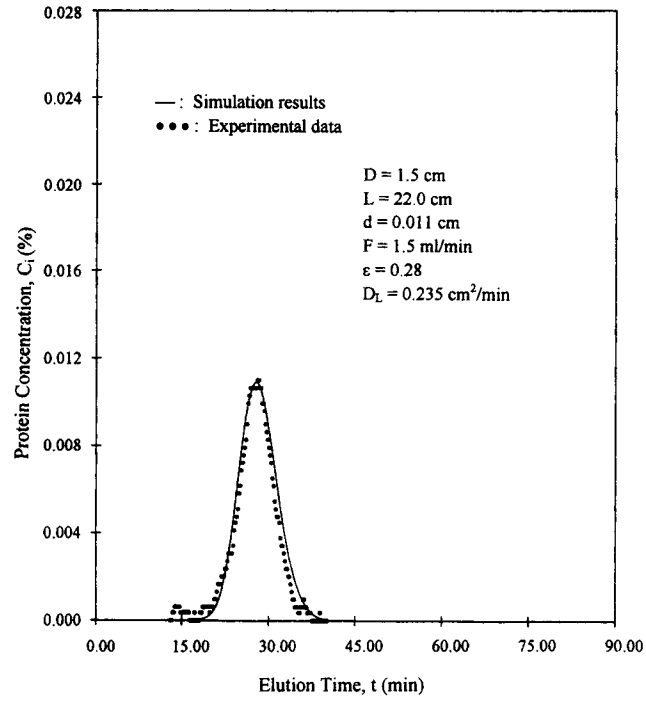

Figure 2. Estimated initial equilibrium constants, $K_{0,i}$, and intraparticle diffusivity, $D_{p,i}$.

Results: $K_{0,i} = 0.6$, $D_{p,i} = 1.74 \times 10^{-5}$ cm²/min, for both LGA and LGB.

and β-lactoglobulin B (0.025%) were studied individually in the DEAE Sepharose CL-6B chromatographic column. The eluting buffer was a mixture of 0.018 M TRIS/HCl at pH 7.9 and 0.5 M NaCl. The buffer's ionic strength was varied from 0.18 M to 0.30 M for different isocratic runs. For each ionic strength, an equilibrium constant value was found, respectively, for β-lactoglobulin A and β-lactoglobulin B by comparing the elution times of the simulation and the experimental data. From this, correlations of the equilibrium constants of β-lactoglobulin A and β-lactoglobulin B as functions of ionic strength of the mobile phase were found.

Determinations of constants $A_{a,i}$ and $B_{a,i}$ for β-lactoglobulin A and β-lactoglobulin B

Using the method mentioned earlier, the corresponding values of equilibrium constants, $K_{a,i}$ for β-lactoglobulin A (LGA) or β-lactoglobulin B (LGB) could be determined for a selected ionic strength. Figure 3 shows a data point of $K_{a,LGB}$ as an example of the estimation procedure. In this figure, the experimental peak was obtained by an isocratic elution of β-lactoglobulin B in the DEAE column. The eluting buffer used was a mixture of 50% buffer A (0.018 M TRIS/HCl, pH 7.9) and 50% of buffer B (0.5 M NaCl), which had an ionic strength of 0.25 M. The experimental peak was recorded by a PC data-acquisition system and then transferred to a mainframe computer. On the mainframe, the fixed-system parameters such as column diameter, adsorbent particle diameter, and flow rate, together with the dispersion coefficient, bed void fraction, intraparticle diffusivity, film mass-transfer coefficient obtained from the preceding studies, were put into the model. Computer simulations of isocratic elutions were con-

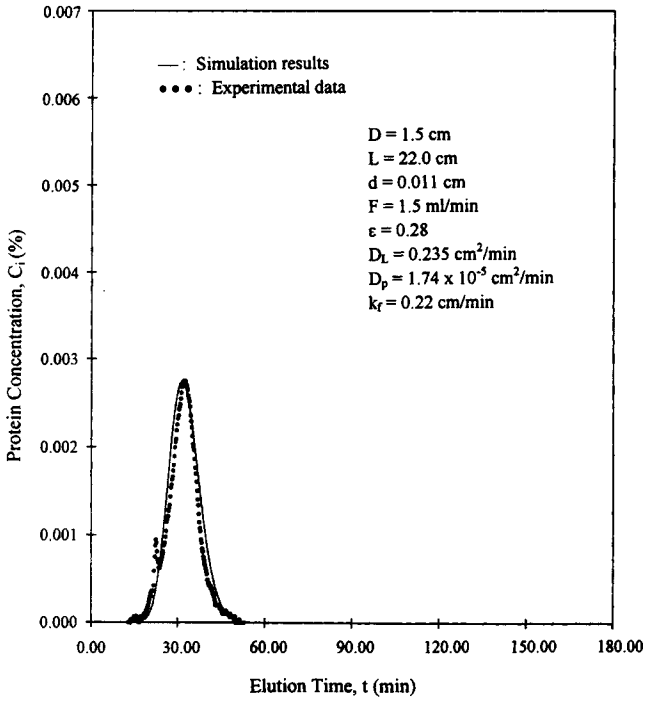

Figure 3. Equilibrium constant of β-lactoglobulin B (LGB) when $I = 0.25$ M; results: $K_{a,LGB} = 1.27$.

ducted by gradually changing the value of the adsorption equilibrium constant of LGB, $K_{a,LGB}$, to curve fit the experimental data. The larger $K_{0,i}$ is, the longer the elution time will be. By comparing the position of the simulation peak with the position of the experimental peak, it was found that the equilibrium constant of LGB, $K_{a,LGB} = 1.27$, for ionic strength, $I = 0.25$ M. Using this procedure, the values of $K_{a,LGA}$ and $K_{a,LGB}$ were determined at various ionic strengths from 0.18 M (65% buffer A) to 0.30 M (40% buffer A), and the data are listed in Table 2. Notice here the ionic strength, I, is defined by Debye-Huckel theory as follows:

$$I = \frac{1}{2} \sum C_k Z_k^2,$$

where C_k is the concentration of the kth type of ion, and Z_k is the charge on the kth type of ion (Pimentel and Spratley, 1971).

For the purpose of data analysis, Eq. 59 was rearranged by moving $K_{0,i}$ from the righthand side to the lefthand side. After taking logarithms on both sides of this equation, it becomes

$$\log(K_{a,i} - K_{0,i}) = \log A_{a,i} + B_{a,i} \log I. \qquad (61)$$

This equation provides a linear relationship between log $(K_{a,i} - K_{0,i})$ and log I for both β-lactoglobulin A and β-lactoglobulin B.

Table 2. Values of $K_{a,i}$ at Various Ionic Strengths

Ionic strength, I (M)	0.18	0.20	0.25	0.30
K_a, β-lactoglobulin A	8.12	5.21	1.78	1.07
K_a, β-lactoglobulin B	4.82	3.10	1.27	0.92

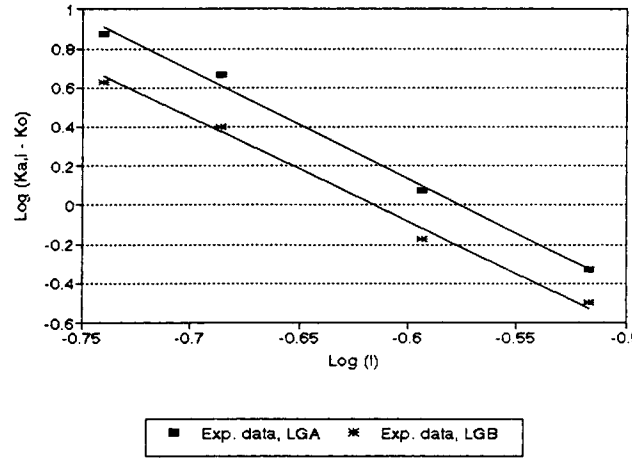

Figure 4. Constants $A_{a,i}$ and $B_{a,i}$ for β-lactoglobulin A (LGA) and β-lactoglobulin B (LGB) by linear regression.

Experimental results: $A_{a,LGA} = 6.20 \times 10^{-4}$, $B_{a,LGA} = -5.56$; $A_{a,LGB} = 5.35 \times 10^{-4}$, $B_{a,LGB} = -5.31$.

By putting the data in Table 2 into Eq. 61, linear regressions were performed as shown in Figure 4 for both LGA and LGB. The values of $A_{a,i}$ were determined from the intercepts of the Y axis, and $B_{a,i}$ were determined from the coefficients (i.e., slopes) of the X axis. It was found that for β-lactoglobulin A, $A_{a,LGA} = 6.20 \times 10^{-4}$, $B_{a,LGA} = -5.56$; for β-Lactoglobulin B, $A_{a,LGB} = 5.35 \times 10^{-4}$, $B_{a,LGB} = -5.31$. Thus, the equilibrium constant correlations for these two proteins can be written as:

$$K_{a,LGA} = 0.6 + 6.20 \times 10^{-4}\, I^{-5.56} \qquad (62)$$

$$K_{a,LGB} = 0.6 + 5.35 \times 10^{-4}\, I^{-5.31}. \qquad (63)$$

These two equations were used to plot $K_{a,LGA}$ and $K_{a,LGB}$ as functions of ionic strength, I, as shown in Figure 5 for both LGA and LGB. For comparison, the experimental data

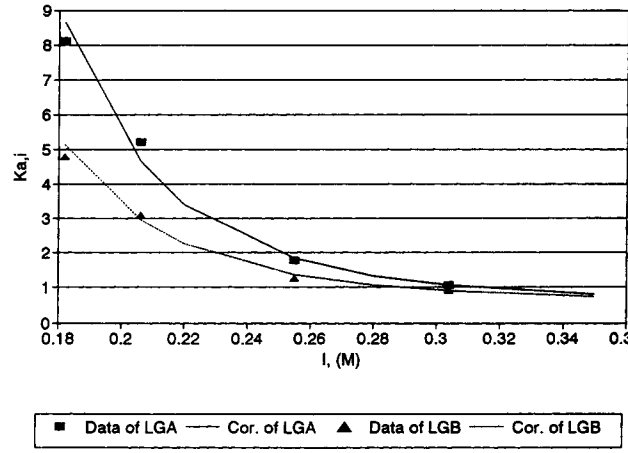

Figure 5. Equilibrium constant from correlations of β-lactoglobulin A (LGA) and β-lactoglobulin B (LGB) vs. experimental data.

Cor.: correlation.

Table 3. System Parameters of a DEAE Sepharose CL-6B Column

Column diameter, D, cm	1.5
Column length, L, cm	22.0
Adsorbent particle diameter, d_p, cm	0.011
Initial ionic strength of the buffer, I_o, M	0.196
Final ionic strength of the buffer, I_f, M	0.255
Sample loaded, S_L, mL	5.0

were plotted in the figure as well. It can be seen that the $K_{a,LGA}$ and $K_{a,LGB}$ curves plotted according to the correlations are very close to the experimental data points.

Gradient Elution of β-Lactoglobulin A and β-Lactoglobulin B and Comparison of Experimental Data and Simulation Results

Once the parameters and correlations had been found, computer simulations were carried out with gradient-elution runs by putting these experimentally determined parameters and correlations into the model. The system parameters are listed in Table 3. The rate and gradient parameters are summarized in Table 4.

In order to verify the simulation results, 5 mL of 0.025% β-lactoglobulin was separated in the same DEAE Sepharose CL-6B column by gradient elution. The gradient profiles were formed by altering the eluting buffer composition with 0.018 M TRIS/HCl, pH 7.9 as buffer A, and 0.5 M NaCl as buffer B. The experimental data were plotted together with the simulation results, as shown in Figure 6. Comparisons were made between the experimental data and the simulation results. The solid curves are the simulated peaks and the dotted curves are the experimental peaks. Good agreement was found between them.

General Systematic Procedure for Estimating Rate Parameters and Protein Gradient Correlations

This study provides a systematic method for estimating rate parameters and gradient correlations of β-lactoglobulin A and β-lactoglobulin B in an ion-exchange column using experiments and computer simulations. This method can be applied to general situations of gradient-elution chromatography of biomolecules. Figure 7 shows a summary of this method. System parameters such as column diameter, adsorbent particle diameter, and bed height are known from

Table 4. Protein Rate and Gradient Parameters on a DEAE Sepharose CL-6B Column

Protein	β-Lactoglobulin A	β-Lactoglobulin B
Bed void fraction, ϵ	0.28	0.28
Dispersion coefficient, D_L, cm²/min	0.235	0.235
Film mass-transfer coefficient, k_f, cm/min	0.22	0.22
Initial adsorption equilibrium constant, $K_{0,i}$	0.6	0.6
Intraparticle diffusivity, D_p, cm²/min	1.74×10^{-5}	1.74×10^{-5}
Constants $A_{a,i}$	6.20×10^{-4}	5.35×10^{-4}
Constants $B_{a,i}$	−5.56	−5.31

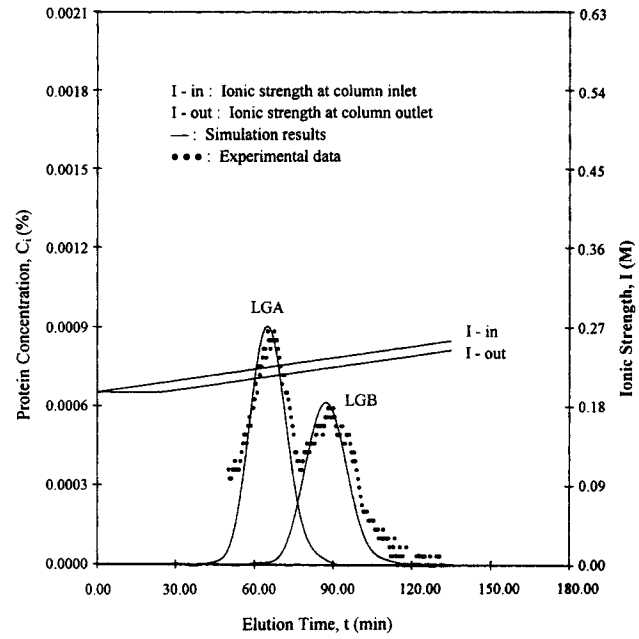

Figure 6. Simulation results vs. experimental data for gradient elution of β-lactoglobulin on DEAE Sepharose CI-6B with a linear gradient.

Initial ionic strength: 0.196 M; final ionic strength: 0.255 M; running time: 150 min.

the design. To estimate the bed void fraction, ϵ, and dispersion coefficient, D_L, pulse experiments are conducted on the column using Dextran 200, or other large molecules, as an indicator. Then computer simulations with a "dispersion

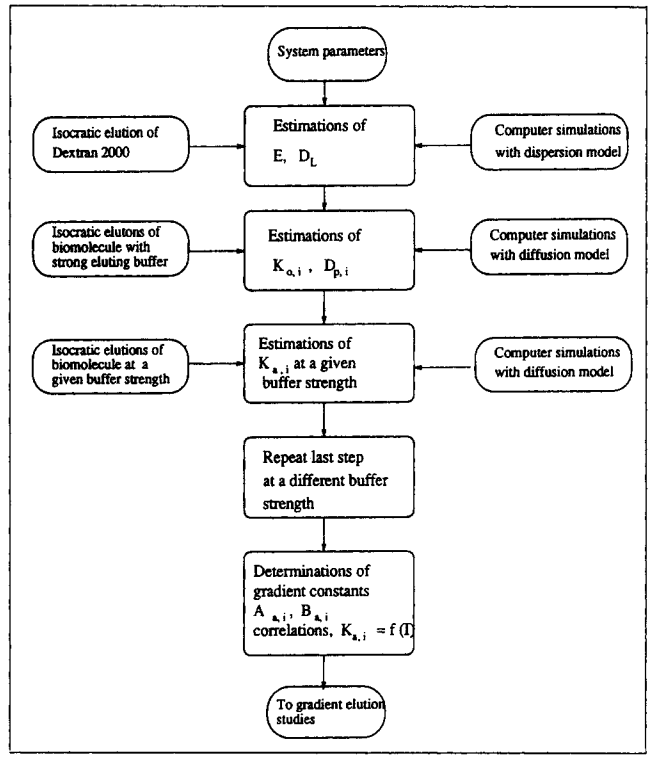

Figure 7. General procedure for estimating rate parameters and gradient correlations.

model" are carried out to obtain ϵ and D_L by curve fitting the experimental data. Once D_L and ϵ are determined, they are used to estimate the initial adsorption equilibrium constants, $K_{0,i}$, and intraparticle diffusivity, $D_{p,i}$. Pulse experiments are conducted with isocratic elution using high-power eluting buffer. Computer simulations of these isocratic elutions are carried out with the model presented in this article to determine the values of $K_{0,i}$ $D_{p,i}$ by matching them to experimental data.

Next, the corresponding value of equilibrium constant, $K_{a,i}$ of component i can be determined for a selected mobile-phase condition (ionic strength in this study). Experiments are conducted using isocratic elutions to collect data under different mobile-phase conditions (various ionic strengths from 0.18 M to 0.30 M in this study). System parameters together with rate parameters obtained from steps given earlier are put into the model to carry out computer simulations. $K_{a,i}$ can then be determined by matching the simulation peak with the experimental peak. Finally, the gradient constants of component i (for example, constants $A_{a,i}$ and $B_{a,i}$ for both β-lactoglobulin A and β-lactoglobulin B in this study) are determined by data analysis to establish the gradient correlations in which $K_{a,i}$ are expressed as functions of the parameters of the eluting buffer. These correlations and the rate parameters estimated earlier can then be used for gradient elution studies. The same approach can be extended to multicomponent gradient elution chromatography.

Conclusions

In this work, a new method was developed to determine the values of constants $K_{0,i}$, $A_{a,i}$, and $B_{a,i}$ in the protein gradient correlations based on experimental data and simulation results of a rate model. This method is simpler and less time-consuming than the isotherm method used by Yamamoto et al. (1983b). The protein adsorption isotherm in the model could be either linear or nonlinear, depending on the concentration of the protein solution. In both cases, the overall model is a nonlinear system due to the nonlinear feature of the gradient-elution process, that is, protein adsorption equilibrium constants are nonlinear functions of mobile-phase salt (NaCl) concentration.

Experimental studies were carried out to estimate rate parameters and protein gradient correlations using Gibbs and Lightfoot's (1986) equations and the authors' data for chromatographic profiles of β-lactoglobulin A and β-lactoglobulin B. These correlations, and the rate parameters estimated were then employed in gradient-elution studies by putting them in the model. Comparisons were made between the experimental data and the simulation results. Good agreement was found between them.

This systematic method was illustrated step by step in Figure 7. It can be applied to general situations of gradient-elution chromatography of biomolecules. The rate model used in this study can handle both linear and nonlinear adsorption isotherms by employing either Eq. 25 or 27. Although the dilute sample solutions used in this study have linear isotherms, the method can be readily extended to the situation of more concentrated protein solutions with nonlinear adsorption isotherms. In addition to salt gradient, the principle of this method can also be applied to other types of gradients such as pH or organic solvent composition.

Notation

$C_{bo,j}$ = mobile-phase initial concentration of buffer component j, kg/cm^3
$C_{binp,j}$ = mobile-phase input concentration of buffer component j, kg/cm^3
d = adsorbent particle diameter, cm
$I_{inp}(t)$ = input ionic strength profile
I_o = initial ionic strength
ΔI = change of ionic strength, M
$K_{b,j}$ = distribution coefficient of buffer component j, cm^3/cm^3
$Pe = V1/D_L$, Peclet number
$Q_{b,j} = C_{bp,j}/C_{bo,j}$, dimensionless concentration of buffer component j in particles
$Q_i = C_{p,i}/C_{o,i}$, dimensionless fluid concentration of sample component i in particles
R = adsorbent particle radius, cm
r = radial distance from center of spherical particle, cm
$S_{b,j} = C_{bs,j}/C_{bo,j}$, dimensionless surface concentration of buffer component j in particles
$S_i = C_{s,i}/C_{o,i}$, dimensionless surface concentration of sample component i in particles
S_L = sample loaded, mL
$s = \Delta I/\Delta V$, linear gradient slope, M/mL
$T = tF/V_0$, dimensionless time, equivalent to θ used by Yamamoto et al. (1983b)
t = time, min
$t_{r,i}$ = retention time of sample component i, min
$U_{b,j} = C_{b,j}/C_{bo,j}$, dimensionless mobile-phase concentration of buffer component j
$U_{binp,j} = C_{binp,j}/C_{bo,j}$, dimensionless mobile-phase input concentration of buffer component j
$U_i = C_i/C_{o,i}$, dimensionless concentration of sample component i in mobile phase
u = superficial velocity, cm/min
ΔV = change of elution volume, mL
$X = z/L$, dimensionless axial distance
z = axial distance coordinate, cm
∇^2 = Laplacian operator
μ = viscosity of fluid, g/cm-min
$\beta = Vr^2\epsilon/(LD_pK(1-\epsilon))$, bed-length parameter
ρ = density of the fluid, g/cm^3
$\eta = r/R$, dimensionless radial distance in particle
$\xi = k_f R/(D_p K)$, film-resistance parameter
$\psi = K(1-\epsilon)/\epsilon$, distribution ratio
$\tau = D_p t/R^2$, contact-time parameter
$\tau_{o,i} = D_p t_{o,i}/R^2$, input contact-time parameter of sample component i
θ = dimensionless time, used by Yamamoto et al. (1983b)
λ = intraparticle Peclet number

Literature Cited

Alm, R. S., R. J. P. Williams, and A. Tiselius, "Gradient Elution Analysis," *Acta Chem. Scand.*, **6**, 826 (1952).

Anderssen, A. S., and E. T. White, "Parameter Estimation by the Weighted Moments Method," *Chem. Eng. Sci.*, **26**, 1203 (1971).

Boyer, P. M., and J. T. Hsu, "Experimental Studies of Restricted Protein Diffusion in an Agarose Matrix," *AIChE J.*, **38**, 259 (1992).

Finlayson, B. A., *The Method of Weighted Residuals and Variational Principles*, Academic Press, New York and London (1972).

Finlayson, B. A., *Nonlinear Analysis in Chemical Engineering*, McGraw-Hill, New York (1980).

Freiling, E. C., "Ion Exchange as A Separation Method: IX. Gradient Elution Theory," *J. Amer. Chem. Soc.*, **77**, 2067 (1955).

Freiling, E. C., "Gradient Elution Theory," *J. Phys. Chem.*, **61**, 543 (1957).

Frey, D. D., "Asymptotic Relations for Preparative Gradient Elution Chromatography of Biomolecules," *Biotechnol. Bioeng.*, **35**, 1055 (1990).

Gear, C. W., *Numerical Initial-Value Problems in Ordinary Differential Equations*, Prentice Hall, Englewood Cliffs, NJ (1971).

Gibbs, S. J., and E. N. Lightfoot, "Scaling Up Gradient Elution Chromatography," *Ind. Eng. Chem. Fundam.*, **25**, 490 (1986).

Gu, T., Y.-H. Truei, G.-J. Tsai, and G. T. Tsao, "Modeling of Gradient Elution on Multicomponent Nonlinear Chromatography," *Chem. Eng. Sci.*, **47**, 253 (1992).

IMSL User's Manual, IMSL Math Library, Fortran Subroutines for Mathematical Applications, Houston, TX (1989).

Jandera, P., and J. Churacek, "Gradient Elution in Liquid Chromatography," *J. Chromatog.*, **91**, 207 (1974).

Jandera, P., and J. Churacek, "Gradient Elution in Liquid Chromatography," *J. Chromatog.*, **153**, 309 (1978).

Jandera, P., and J. Churacek, "Gradient Elution in Liquid Chromatography," *J. Chromatog.*, **170**, 1 (1979).

Jandera, P., and J. Churacek, *Gradient Elution in Column Liquid Chromatography*, Elsevier, Amsterdam (1985).

Jandera, P., "Predictive Calculation Methods for Optimization of Gradient Elution using Binary and Ternary Solvent Gradients," *J. Chromatog.*, **485**, 113 (1989).

Kang, K., and B. J. McCoy, "Protein Separation by Ion Exchange Chromatography: A Model for Gradient Elution," *Biotechnol. Bioeng.*, **33**, 786 (1989).

Kaplan, L. J., and J. F. Foster, "Isoelectric Focusing Behavior of Bovine Plasma Albumin, Mercaptalbumin, and β-Lactoglobulins A and B," *Biochemistry*, **10**, 630 (1971).

Kataoka, T., H. Yoshida, and T. Yamada, "Liquid Phase Mass Transfer in Ion Exchange Based on the Hydraulic Radius Model," *J. Chem. Eng. Japan*, **6**, 172 (1973).

Kucera, E., "Contribution to the Theory of Chromatography: Linear Non-Equilibrium Elution Chromatography," *J. Chromatog.*, **237**, 19 (1965).

Lederer, E., and M. Lederer, *Chromatography*, 2nd ed., Elsevier, Amsterdam (1957).

Levenspiel, O., *Chemical Reaction Engineering*, 2nd ed., Wiley, New York (1972).

Luo, R. G., and J. T. Hsu, "Simulation of Gradient Elution Chromatography by the Method of Orthogonal Collocation," *Proc. Computer Simulation Conf.*, Reno, NV, p. 668 (1992).

Luo, R. G., and J. T. Hsu, "Intraparticle Protein Diffusion Effect on Gradient Elution Chromatography," *Sep. Technol.*, **3**, 221 (1993).

Pharmacia Laboratory Separation Division Publications, *Gel Filtration Theory and Practice*, Uppsala, Sweden (1987).

Pharmacia LKB, *Biotechnology Product Catalog*, Piscataway, NJ (1992).

Piez, A. D., E. W. Davie, J. E. Folk, and J. A. Gladner, "β-Lactoglobulins A and B. I. Chromatographic Separation and Amino Acid Composition," *J. Biol. Chem.*, **236**, 922 (1961).

Pimentel, G. C., and R. D. Spratley, *Understanding Chemistry*, Holden-Day, San Francisco (1971).

Raghavan, N. S., and D. M. Ruthven, "Numerical Simulation of a Fixed-Bed Adsorption Column by the Method of Orthogonal Collocation," *AIChE J.*, **29**, 922 (1983).

Reis, J. F. G., E. N. Lightfoot, P. T. Noble, and A. S. Chiang, "Chromatography in a Bed of Spheres," *Sep. Sci. Technol.*, **14**, 367 (1979).

Schneider, P., and J. M. Smith, "Adsorption Rate Constants from Chromatography," *AIChE J.*, **14**, 762 (1968a).

Schneider, P., and J. M. Smith, "Chromatographic Study of Surface Diffusion," *AIChE J.*, **14**, 886 (1968b).

Skidmore, G. L., B. J. Horstmann, and H. A. Chase, "Modelling Single-Component Protein Adsorption to the Cation Exchanger S-Sepharose FF," *J. Chromatog.*, **498**, 113 (1990).

Snyder, L. R., "Prediction of Precise Isocratic Retention Data from Two or More Gradient Elution Runs," *J. Chromatog.*, **6**, 22 (1961).

Snyder, L. R., "Linear Elution Adsorption Chromatography," *J. Chromatog.*, **13**, 415 (1964).

Snyder, L. R., J. W. Dolan, and J. R. Gant, "Gradient Elution in High-Performance Liquid Chromatography," *J. Chromatog.*, **165**, 3 (1979).

Snyder, L. R., J. W. Dolan, and D. C. Lommen, "DRYLAB Computer Simulation for High-Performance Liquid Chromatographic Method Development," *J. Chromatog.*, **485**, 65 (1989a).

Snyder, L. R., J. D. Stuart, and D. D. Lisi, "Separation of Mixture of o-Phthalaldehyde-Derivatized Amino Acids by Reversed-Phase Gradient Elution/Accuracy of Computer Simulation for Predicting Retention and Band Width," *J. Chromatog.*, **485**, 657 (1989b).

Snyder, L. R., and J. L. Glajch, *Computer-Assisted Method Development for High Performance Liquid Chromatography*, Elsevier, Amsterdam (1990).

Tomellini, S. A., and R. A. Hartwich, "Computer-Based Numerical Integration for the Calculation of Retention Times in Gradient High-Performance Liquid Chromatography," *Anal. Chem.*, **57**, 811 (1985).

Tomellini, S. A., S.-H. Hsu, and R. A. Hartwich, "Prediction of Retention for Coupled Column Gradient Elution High-Performance Liquid Chromatography," *Anal. Chem.*, **58**, 904 (1986).

Townend, R., L. Weinberger, and S. N. Timasheff, "Molecular Interactions in β-lactoglobulin. IV. The Dissociation of β-lactoglobulin below pH 3.5," *J. Amer. Chem. Soc.*, **82**, 3157 (1960).

Villadsen, J. V., and M. L. Michelsen, *Solution of Differential Equation Models by Polynomial Approximation*, Prentice Hall, Englewood Cliffs, NJ (1978).

Villadsen, J. V., and W. E. Stewart, "Solution of Boundary-Value Problems by Orthogonal Collocation," *Chem. Eng. Sci.*, **22**, 1483 (1967).

Yamamoto, S., K. Nakanishi, R. Matsuno, and T. Kamikubo, "Ion Exchange Chromatography of Proteins-Prediction of Elution Curves and Operating Conditions. I. Theoretical Consideration," *Biotechnol. Bioeng.*, **25**, 1465 (1983a).

Yamamoto, S., K. R. Nakanishi, R. Matsuno, and T. Kamikubo, "Ion Exchange Chromatography of Proteins-Prediction of Elution Curves and Operating Conditions. II. Experimental Verification," *Biotechnol. Bioeng.*, **25**, 1373 (1983b).

Manuscript received Feb. 29, 1996, and revision received July 17, 1996.