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Theoretical background of short chromatographic layers Optimization of gradient elution in short columns

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Abstract

Although linear salt gradient elution ion-exchange chromatography (IEC) of proteins is commonly carried out with relatively short columns, it is still not clear how the column length affects the separation performance and the economics of the process. The separation performance can be adjusted by changing a combination of the column length, the gradient slope and the flow velocity. The same resolution can be obtained with a given column length with different combinations of the gradient slope and the flow velocity. This results in different separation time and elution volume at the same resolution. Based on our previous model, a method for determining the separation time and the elution volume relationship for the same resolution (iso-resolution curve) was developed. The effect of the column length and the mass transfer rate on the iso-resolution curve was examined. A long column and/or high mass transfer rate results in lesser elution volume. The resolution data with porous bead packed columns and monolithic columns were in good agreement with the calculated iso-resolution curves. Although the elution volume can be reduced with increasing column length, the pressure drop limits govern the optimum conditions.

Keywords: Short column; Ion-exchange chromatography; Iso-resolution curve; Gradient elution; Monolithic column; Optimization

1. Introduction

In many biotech companies recombinant protein drugs are purified by several chromatography processes. However, in many cases these processes are so complicated and delicate that the operation must be carefully carried out, and sometimes additional care must be taken in order to maintain very high purity and low variability of the product [1–6]. These difficulties arise mainly from the fact that a target recombinant protein drug must be purified from very similar protein variants or isoforms.

Chromatography using electrostatic interaction known as ion-exchange chromatography (IEC) is most commonly used for such protein drug separations. Although various elution methods are available for IEC, linear gradient elution (LGE), in which salt concentration is increased linearly, is the most efficient method for difficult protein separations [2,6,7]. However, since LGE has many variables such as gradient slope, flow velocity and column dimensions as well as mobile phase pH, it is difficult to optimize the separation.

It is well known that low mass transfer rates in the stationary phase govern the performance of protein chromatography [2,6,7]. Therefore, numerous efforts have been devoted to analyze mass transfer mechanism in protein chromatography and to develop chromatographic supports (media) that possess less mass transfer resistance. Even when porous supports having relatively large pores (ca. 100 nm) pore diffusion of large proteins is still very low. One of the methods that can enhance the mass transfer rate is to use a convection-aided support. Perfusion chromatography, membrane-based chromatography, fabric-support chromatography and monolithic-support chromatography are such convection-aided chromatography which can permit high separation performance at high flow velocities. Good review papers are available for the convection-aided chromatography [8–13].

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We have developed mathematical models for LGE-IEC [6,14–16]. These models can be applied to predict the separation performance and also to obtain important information on molecular recognition [17].

It is empirically well known that the impact of the column length on the separation performance in LGE-IEC is not so significant compared with the isocratic elution. We have shown that a very short column (1 cm) can be employed for fine separation of protein variants based on the dimensionless group derived from the mathematical model [6,15]. We have then demonstrated that the same resolution (separation performance) can be obtained with a given column when the gradient slope and the flow-velocity are properly adjusted [16]. However, it is also shown that the separation time and the elution volume (buffer consumption) change with the gradient slope and the flow velocity values at the same resolution. During our investigation on the resolution with short columns [15], we found that it was not easy to pack a very short column with conventional porous beads. However, very short (thin) columns are now commercially available as monolithic columns and membrane-based columns. Therefore, we have decided to re-investigate the separation performance of very short columns based on the extended version of our model [6,15,16].

In this paper, the effects of the column length and the mass transfer resistance on the separation performance in LGE-IEC were investigated on the basis of the model developed for LGE-IEC. The experimental data with monolithic stationary phase IEC as well as conventional porous bead IEC were analyzed with the model. The optimization strategies in terms of the separation time and the elution volume (buffer consumption) were developed.

2. Experimental

2.1. Chromatography column

SP Sepharose HP gels (6% cross-linked agarose, sulfopropyl group, particle diameter ca. 37 μm) were packed into a plastic column (50 mm × 9.0 mm i.d., total bed volume V_t = 3.18 mL,) according to the procedure recommended by the supplier (Amersham Biosciences, Uppsala, Sweden). Poly(glycidyl methacrylate-co-ethylene dimethacrylate) disks (3 mm × 12 mm i.d.) with a weak anion-exchange group were contained in a specially designed disk holder column from BIA Separations (Ljubljana, Slovenia). This disk is called hereafter CIM-QA.

2.2. Materials

Model proteins employed in this study are ribonuclease A (RNase A, product no.R4875) and Bovine milk β-lactoglobulin (Lg, product no.L0130) from Sigma (St. Louis, MO, 45A). β-Lg contains lactoglobulins A (LgA) and B (LgB). Other reagents were of analytical grade.

2.3. Chromatography apparatus

Column experiments were carried out on a fully automated liquid chromatography system ÄKTA Explorer 100 (Amersham Biosciences, Uppsala, Sweden).

2.4. Linear gradient elution experiment

The SP Sepharose HP column was equilibrated with a starting buffer (buffer A) containing 0.03 M NaCl. The same buffer solution containing 0.5–1.0 M NaCl was used as final elution buffer (buffer B). The linear gradient elution was performed by changing the buffer composition linearly from buffer A to buffer B with time. Namely, the NaCl concentration was increased with time at a fixed pH and buffer compositions. The gradient slope g is shown in M/mL. The linear mobile phase velocity u was calculated with the cross-sectional area A_c and the column bed void fraction ε as: $u = F/(A_c\,\varepsilon)$ where F is the volumetric flow rate. The column bed void fraction ε was determined from the peak retention volume of Dextran T 2000 pulses. The experiments were performed at 298 ± 1 K.

3. Theoretical

3.1. Peak retention volume as a function of gradient slope

The outline of our model [5,6,14–17] is briefly explained below. The peak retention volume is a function of gradient slope in LGE-IEC. The peak salt concentration I_R increases with increasing gradient slope, $g = (I_f - I_0)/V_g = (I_f - I_0)/(F\,t_g)$ [M/mL] (I_f: final salt concentration, I_0: initial salt concentration, V_g: gradient volume, t_g: gradient time). The I_R values can be correlated with the following normalized gradient slope,

$$GH = (gV_o)\left[\frac{(V_t - V_o)}{V_o}\right] = g(V_t - V_o) \quad (1)$$

V_t is the total bed volume, V_o is the column void volume, and g is the gradient slope of the salt. $H = (V_t - V_o)/V_o = (1 - \varepsilon)/\varepsilon$ is the phase ratio. $\varepsilon = V_o/V_t$ is the bed void fraction (interstitial volume of the bed). g is defined by the following equation

$$g = \frac{(I_f - I_0)}{V_g} \quad (2)$$

I_f is the final salt concentration, I_0 is the initial salt concentration, and V_g is the gradient volume. Linear gradient elution experiments are performed at different gradient slopes (GH values) at a fixed pH. The salt concentration at the peak position I_R is determined as a function of GH. The GH–I_R curves thus constructed do not depend on the flow velocity, the column dimension, the sample loading (if the overloading condition is not used), or the initial salt concentration I_0

[6,14–17]. The experimental GH–I_R data can commonly be expressed by the following equation [6,14–17]

$$GH = \frac{I_R{}^{(B+1)}}{[A(B+1)]} \tag{3}$$

From the law of mass action (ion exchange equilibrium) [3,5,6,17–21], the following relationship can be derived.

$$A = K_e \Lambda^B \tag{4}$$

Here, B is the number of sites (charges) involved in protein adsorption, which is basically the same as the Z number [19] and the characteristic charge [21], K_e is the equilibrium association constant, and Λ is the total ion exchange capacity. From the ion-exchange equilibrium model [3,5,6,17–21] and Eq. (4), the following equation is derived [16,17].

$$K - K' = K_e \Lambda^B I^{-B} \tag{5}$$

Here K is the protein distribution coefficient, K' is the distribution coefficient of salt, and I is the ionic strength (salt concentration). The SMA model equation [21] is reduced to Eq. (5) when the sample loading is low (Λ is not influenced by protein).

4. Results

Based on the rigorous mathematical model for LGE-IEC, the following dimensionless group was derived, which can be employed for optimization of LGE with a few experimental data [6,15,17].

$$Y = \frac{(D_m I_a Z)}{(GHud_p^2)^{1/2}} \tag{6}$$

D_m is the molecular diffusion coefficient and d_p is the particle diameter of IEC media. I_a represents a dummy variable having a numerical value of 1 so that Y becomes dimensionless. This dimensionless group is based on the assumption that the height equivalent to a theoretical plate (HETP) (or variance of the peak width) is proportional u, which is usually valid for high flow velocities with rather big particles (>100 μm). On the basis of this dimensionless group experimental data over a wide range of chromatography conditions were successfully correlated [6,15,17]. One of the interesting findings is that the same resolution can be obtained with a very short column provided that a very shallow gradient slope is employed [6,15].

However, it is also desirable to develop another parameter, which is not based on the above-mentioned assumption (HETP is proportional to u). This is especially important for small particle and/or long column systems, and also for above-mentioned convection-aided chromatography of very short columns.

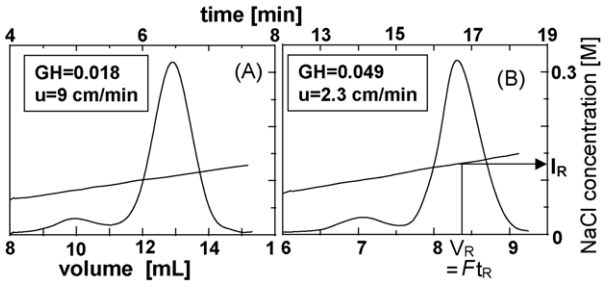

Fig. 1. Linear gradient elution curves of ribonuclease A at pH 8 on SP Sepharose HP (0.9 cm i.d. × 5 cm bed-height) with the same O value (O = 4800).

For this purpose the following parameter was proposed where $(HETP)_{LGE}$ is HETP for LGE [16].

$$O = \frac{(ZI_a)}{(G(HETP)_{LGE})} \tag{7}$$

The elution curves obtained for various combinations of experimental conditions such as Z, G and u are very similar when the O values are set to be equal [16]. Although the same resolution can be obtained with various combinations of operating/column variables (Fig. 1), there may be some optimum condition because retention time t_R and retention volume V_R is a function of G and u,When HETP is not dependent on the flow velocity such as the monolithic column, Eq. (7) can be simplified as Milavec et al. [22] derived

$$t_{g2} = t_{g1}\left(\frac{V_{o2}}{V_{o1}}\right)\left(\frac{F_1}{F_2}\right)\left(\frac{Z_1}{Z_2}\right) \tag{8}$$

Although this equation is very simple and easy to use, unfortunately it does not give information on the buffer consumptions and the separation time.

For conventional porous bead chromatography the peak broadens with increasing flow velocity due to the diffusion mass transfer in the pores. Therefore, when the flow velocity u is increased, the gradient slope g must become shallower in order to obtain the same O value. Similarly when u is decreased, g must be increased. With the aid of the $(HETP)_{LGE}$–u curve this calculation can be done. The method for determining $(HETP)_{LGE}$–u relationships was described in a previous paper [16].

Once the u and the g values are determined, the separation time t_S and the buffer consumption BC are calculated as follows:

$$t_S \approx t_R \approx \frac{(I_R - I_0)}{g} + V' \tag{9}$$

$$BC \approx \frac{V_R}{V_t} = \frac{Ft_R}{V_t} = \frac{t_R u \varepsilon}{Z} \tag{10}$$

where, V' is the elution volume for the salt.We call the $t_R - V_R/V_t$ curve at a constant O value the "iso-resolution curve" as the same resolution with different separation time and buffer consumption can be obtained on this curve.

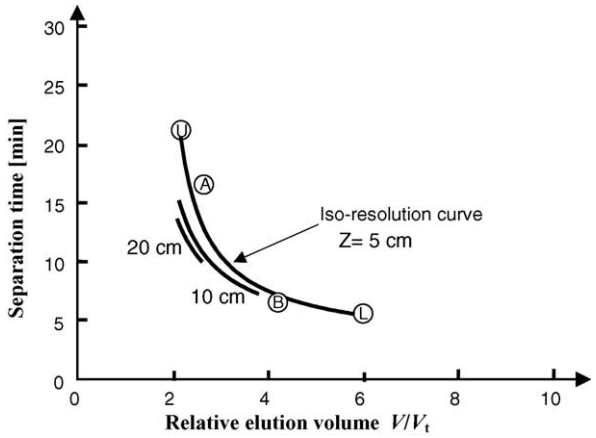

Fig. 2. Separation time and relative elution volume relationships as a function of column length. The same bed volume is assumed. The relative elution volume is often referred to as column volume (CV), and implies the relative buffer consumption. For the column length Z =5 cm, the upper limit (symbol U) and lower limit (symbol L) are shown. The symbols A and B are the data from the elution curves shown in Fig. 1B and Fig. 1A, respectively.

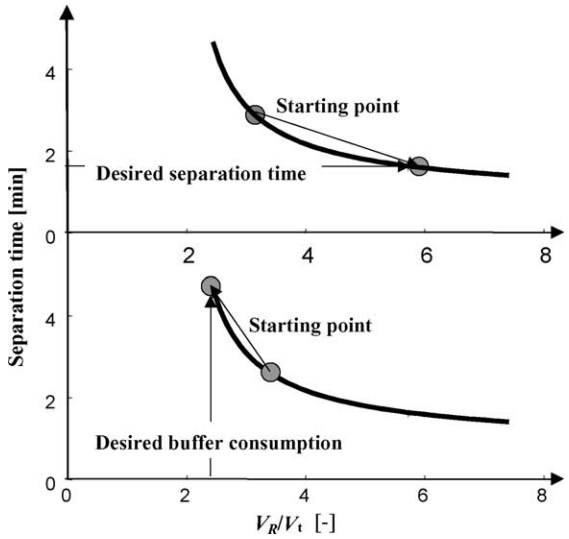

Fig. 3. Illustration on how to adjust the flow velocity and the gradient slope for reducing separation time (upper figure) and buffer consumption (lower).

In Fig. 2, the calculated iso-resolution curves are shown. The open circles A and B are the data from Fig. 1(B and A), respectively. These data are in good agreement with the calculated iso-resolution curve. As shown in the figure, the separation time becomes longer as the elution volume decreases. On the contrary, large elution volume is needed for rapid separation. It is especially important to know where your separation conditions are located. For example, if your separation is carried out at point A in Fig. 2, it is not wise to decrease the flow velocity for reducing the buffer consumption. Similarly, if your separation is performed at point B it is not advantageous to reduce the separation time as very large buffer consumption is needed.

For the calculation of the iso-resolution curves, there are the upper and lower limits shown in the symbol U and L in the figures. The upper limit is determined by the gradient slope. The gradient slope of two CV may be the limit for a very steep gradient. The lower limit L is due to the flow velocity or the pressure drop. When the column length is increased, the curve shifts to the left and the region between the limit U and L becomes smaller. This means that the buffer consumption decreases with increasing column length. But at the same time short-time separation becomes difficult. This is especially true for large-scale or process-scale separation where the maximum operating flow velocity due to the pressure drop limit is significantly affected by the column bed height [5,22–25]. Fig. 3 shows how to adjust the operating conditions according to the iso-resolution curve.

The effect of mass transfer rate on the iso-resolution curve is shown in Fig. 4. The mass transfer (diffusion) term in the HETP equation was changed as shown in the figure. When the mass transfer is slow, namely large C^0, the curve shifts to larger V/V_t values. Also as shown in the region b in the figure, the separation time does not change with V/V_t. The curve is almost parallel to V/V_t axis. On the contrary, the iso-resolution curve shifts to lower V/V_t values with increasing mass transfer rates (smaller C_0 values). In the region a, the curve is almost parallel to the y-axis. The elution volume does not change with separation time.

The separation performance of monolithic columns like other convection-aided chromatography does not depend on the flow velocity. Typical results are shown in Fig. 5, where the data at different flow-rates are almost superimposable. Based on the O value, the conditions are chosen for different column heights of monolithic columns. The results are shown in Fig. 6. Once again, the curves at different column heights are almost the same.

As the normalized gradient slope is the governing parameter, the resolution was correlated with GH as sug-

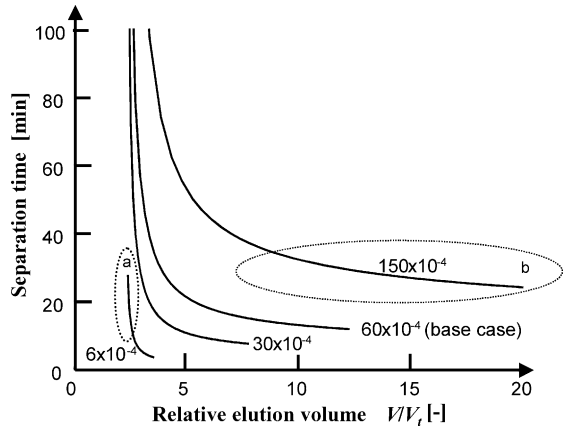

Fig. 4. Effect of mass transfer on separation time and relative elution volume relationships. HETP = $A^0 + C^0 u$ where $A^0 = 7.2 \times 10^{-3}$ and the C^0 values are shown in the figure. O = 9000, Z = 5 cm, the other parameter values are the same in Fig. 2. Note that for very high mass transfer case (region a), V/V_t remains constant whereas for very low mass transfer case (region b) the separation time does not change significantly.

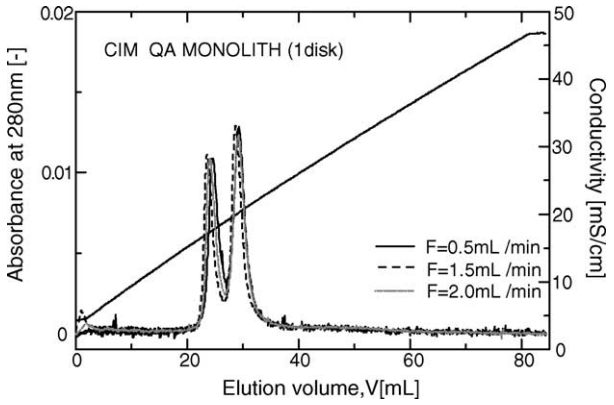

Fig. 5. Effect of flow-velocity on the resolution for a thin monolithic column. Gradient slope $g = 6 \times 10^{-3}$ M/mL. Sample, lactoglobulin; pH 7.

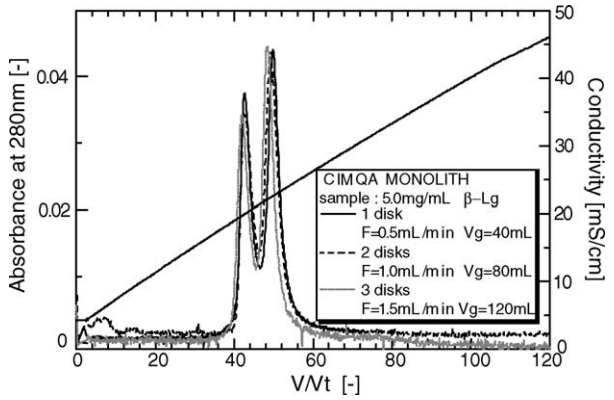

Fig. 6. Effect of column length (thickness) on the resolution. $GH = 0.00239$. The residence time is the same.

gested by Eq. (6). A good correlation was found as shown in Fig. 7.

Finally, the relationships between the separation time and the separation volume as a function of monolithic column height are shown in Fig. 8. As there is no significant mass

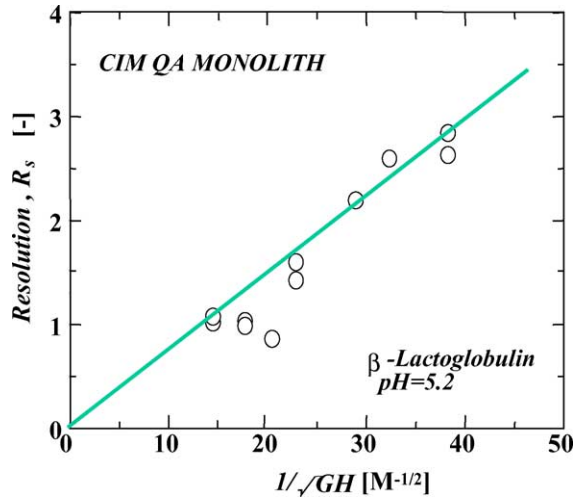

Fig. 7. Resolution as a function of gradient slope for a CIM-QA disk (3 mm thickness).

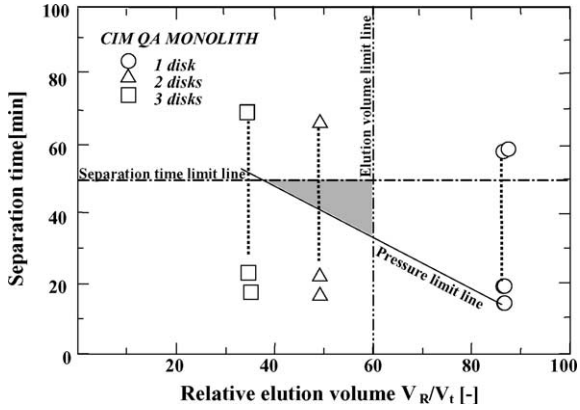

Fig. 8. Separation time and relative elution volume relationships as a function of column length for monolithic columns. Sample: Lg, $g = 0.006$ M/mL, pH 7 fictitious limits for pressure, separation time and elution volume are drawn in the figure.

transfer resistance, V_R/V_t does not change, which is similar to region a in Fig. 4. Although increasing thickness can reduce the elution volume, the accompanying pressure drop increase becomes a critical variable. If for example the operating conditions must be chosen according to a fictitious pressure limit line drawn in the figure, high speed separation is difficult for thick columns. Other important constraints are separation time limit and elution volume (buffer consumption) limit as shown in Fig. 8. There is an operating window shown by a shaded triangle, which is determined by these three limits, pressure, separation time and elution volume.

5. Discussion

As mentioned in the text, various convection-aided or small-bead HPLC have been developed not only for analytical but also for preparative separations. However, especially for preparative and process separations it is difficult to choose proper conditions for required purification specifications.

In isocratic elution once the column bed height Z is fixed, there is only one flow velocity u that can give the required resolution. On the contrary, various combinations of the flow velocity u and the gradient slope g are possible to obtain the same resolution. Compared with Z and u, both of which affect the pressure drop, g is easy to change to great extent. For small scale-separations, the buffer consumption is not a major issue where as it is critical for the economics of process chromatography.

It is usually not easy to pack a very short, and wide diameter column with conventional porous beads. On the other hand, large scale short monolithic columns of various configurations are available. Therefore, it is interesting to investigate the opportunities of short columns for process chromatography.

Even with such short monolithic columns, the maximum pressure drop limit is critical for process chromatography like conventional packed beds. Further investigation on this aspect

is needed for process development of monolithic column bio-chromatography.

6. Nomenclature

A	$=K_e \Lambda^B$
A_c	cross-sectional area (cm^2)
B	the number of binding sites
BC	buffer consumption (V_R/V_t)
C_0	initial concentration (mg/mL)
d_p	particle diameter (cm)
D_m	molecular diffusion coefficient (cm^2/s)
F	volumetric flow rate (mL/min)
g	gradient slope (M/mL)
GH	normalized gradient slope ($(g V_o)H = g(V_t - V_o)$) (M)
H	phase ratio ($(V_t - V_o)/V_o$)
$(HETP)_{LGE}$	plate height for linear gradient elution (cm)
I	ionic strength (salt concentration) (M)
I_a	dimensional constant having a numerical value of 1 (M)
I_f	final salt concentration (M)
I_0	initial salt concentration (M)
I_R	peak salt concentration (M)
K	distribution coefficient
K'	distribution coefficient of salt
K_e	equilibrium association constant
O	dimensionless parameter $((Z I_a)/(G(HETP)_{LGE})$
t_g	gradient time (min)
t_R	retention time (min)
t_S	separation time (min)
u	linear mobile phase velocity (cm/min)
V	elution volume (mL)
V'	elution volume for the salt (mL)
V_g	gradient volume (mL)
V_o	column void volume (interstitial volume) (mL)
V_R	retention volume (mL)
V_t	column volume (mL)
Y	dimensionless parameter $= [(D_m I_a Z)/(GH\, u\, d_p{}^2)]^{1/2}$
Z	column length (cm)
ε	void fraction of column $= V_o/V_t$
Λ	total ion-exchange capacity (meq./mL)

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