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The Separation Efficiency of Biopolymers with Short Column in Liquid Chromatography

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Abstract: The separation efficiency of biopolymers with a short column in liquid chromatography has been investigated in this paper. It was found that the column length has slight effect on the resolution of biopolymers under gradient elution. The reasons have been explained by stoichiometric displacement model for retention of solute. The column 1.0 cm long was also used in the separation and purification of recombinant human granulocyte colony-simulating factor (rhG-CSF). It only took 40 min and the purity by one step was found to be almost 100%.

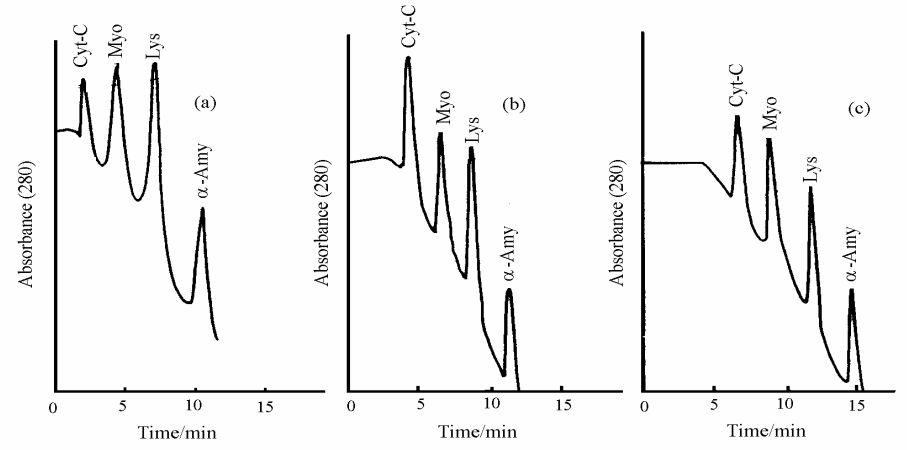
Keywords: Short column, biopolymer, stoichiometric displacement model for retention, Z value.

As an effective tool for the separation, purification, and preparation of biopolymers, high performance liquid chromatography has been widely used in the downstream of biotechnology and of active proteins from the tissue fruit in animals and plants. Scientists have paid much attention to explore new packings and improve column techniques in LC. The ratio of diameter to length of usual column is often less than 1 to 10. According to the separation of small solutes, the separation efficiency should be improved as the column length is increased. However, it is known that the retention behavior of biopolymers in HPLC is much different from that of small solutes. As a result, isocratic elution could be used for small solutes, while gradient elution has to be employed for biopolymer separations[1]. The separation efficiency of biopolymers on reverse-phase and ion-exchange columns less than 2.0 cm in length was reported by Moore and Eksteen[2,3]. The resolution of proteins in this kind of short column was shown to be almost comparable to that of 25-45 cm in length. To explain this phenomenon, the separation efficiency of biopolymers on short columns has been studied in this paper theoretically and experimentally. The separation of recombinant human granulocyte colony-simulating factor (rhG-CSF) produced in biotechnology with a short column, is as efficiency as that with a longer column.

1. Separation of proteins in HPHIC column with different lengths

Figure 1 shows the comparison of chromatograms of four kinds of protein, cytochrome-C (Cyt-C), myoglobin (Myo), lysozyme (Lys), and α-amylase (α -Amy) by using 5.0×4.0 mm I.D., 25.0×4.0 mm I.D. and 150.0×4.0 mm I.D. HPHIC columns, respectively under a linear gradient elution mode. From **Figure 1**, there is no significant difference in terms of the separation efficiency, though the columns have different lengths. The length of the shortest column is only one thirtieth of the longest one. This is in agreement with the results reported by Moore and Eksteen in RPLC and IEC[2,3].

Figure 1. Comparison of chromatograms for the separation of four proteins with different column lengths



All of chromatographic separations were performed at ambient temperature. The mobile phase consisted of solution A: 3.0 mol/L ammonium sulfate in 0.050 mol/L potassium dihydrogen phosphate (pH7.0), solution B: 0.050 mol/L potassium dihydrogen phosphate (pH7.0). Flow rate was 1.0ml/min and detection wavelength was 280nm. Linear gradient was 15.0 mins. Column length: (a) 5.0 mm, (b) 25.0 mm, (c) 150.0 mm

2. Z value of biopolymers

Two fundamental expressions of the stoichiometric displacement model for retention of solute (SDM-R) in liquid chromatography[4,5] can be expressed as:

$$lg\ k' = lg\ I - Z\ lg\ a_D \qquad (1)$$

$$lg\ I = Z\ j + lg \qquad (2)$$

Where k' is the capacity factor of the solute, a_D is the molar concentration of displacing agent in the mobile phase. Z is the total moles of the displacing agent released from the interface between the protein and stationary phase when one mole protein is adsorbed. lgI is a constant relating to the affinity of the protein for the stationary phase. j

is a constant relating to the affinity of the solvent for the stationary phase, and ϕ is the column phase ratio defined thermodynamically[6].

Combining **eqs.** (**1**) and (**2**), eq. (**3**) can be obtained as follows:

$$lg\ k' = Z\ (\ j - lg\ a_D\ ) + lg\ \phi \qquad (3)$$

In a certain chromatographic system, j and ϕ are constants[6]. So at a fixed concentration of mobile phase k' would be proportional to Z value. In the same chromatographic condition, the ratio of k' of two kinds of protein could be calculated by **eqns** (**4**):

$$\lg \frac{k_2'}{k_1'} = (Z_2 - Z_1) \times (j - \lg a_D) \qquad (4)$$

According to **eq**. (**4**), whether two kinds of protein could be separately eluted or not would be decided by the difference between Z values. In other words, as the difference of the Z values among the solutes is small, the difference of their k' values would be small also. Therefore, it is possible to use isocratic elution mode to separate and to wash them out, respectively. On the contrary, a gradient elution must be used when the difference of Z values is very large among the solutes. **Table 1** shows the Z values of four kinds of proteins in HPHIC and RPLC, respectively. The mobile phase in HPHIC was $(NH_4)_2SO_4$ in 0.01 mol/L KH_2PO_4 – H_2O (pH=7.0), while in RPLC it was 2-propanol in 0.05 mol/L KH_2PO_4-H_2O (pH=2.5)[7].

Table 1. *Z* values of standard proteins in HPHIC and RPLC[7]

Protein	Cyt-C	RNase	Lys	α -Chy
Z values in HPHIC	60.1	74.2	94.9	160
Protein	Ins	Cyt-C	Cab	BSA
Z values in HPRPC	15.7	30.5	45.8	117

From **Table 1**, it could be seen that the difference of Z values is often more than 10. It means that a given concentration of the mobile phase suitable for the elution of one kind of protein would not be suitable for the other. Thus, gradient elution is often used for the separation of biopolymers. During the process of gradient elution, k' values decrease quickly as the concentration of the displacer increases gradually. When its concentration changes to a suitable range, the protein would be desorbed from the stationary phase and passes through the column together with the mobile phase. Therefore, besides the character of proteins, the separation efficiency would largely depend on the concentration of the displacer, rather than the column length.

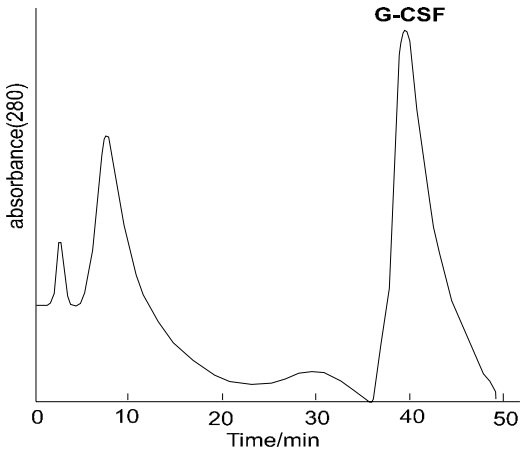
3. Purification of rhG-CSF in biotechnology

Granulocyte colony-stimulating factor (G-CSF) is an important therapeutic protein for helping cancer patient to resist secondary infections and the patient undergoing bone marrow transplantation following intense chemotherapy. It could also resist the suppression of bone marrow function after using myelotoxic chemotherapy[8].

We used the short HPHIC column (10.0 × 50.0 I. D.) for the separation and purification of the rhG-CSF in its *E.coil* extracting solution with only one step and good result was obtained. **Figure 2** shows that rhG-CSF could be separated from other

impurities completely. The purity of rhG-CSF was tested to be almost 100% by SDS-PAGE.

Figure 2. Separation and purification of rhG-CSF by using short HPHIC column



It could be concluded that the short column could give similar separation efficiency as the longer one in the separation of biopolymers. And it also has some other advantages, such as: (1) less irreversible adsorption of biopolymers on the stationary phase and higher mass recovery, (2) less amount of packing material and lower cost of column, (3) lower back pressure and simpler equipment to match with it.

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