Ultra-Rapid Analysis of Proteins by Cation Exchange Chromatography

Introduction

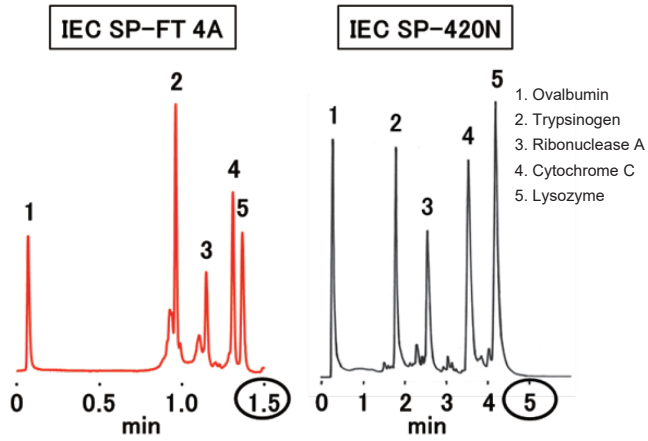
High performance liquid chromatography (HPLC) takes an important role in the pharmaceutical and biotech industry for the analysis of biological components such as proteins, peptides, and nucleic acids. Especially when analyzing a large number of samples at once, the performance of the HPLC column becomes the key for the accurate and rapid analysis required for the situation. Since biological components often carry ionic charges, ion-exchange mode chromatography is an effective method for separating those components. In addition, use of gradient method by changing the eluent's pH or salt concentration helps controlling the retention time and this leads to improved separation with higher resolution.

The Shodex IEC SP-FT 4A, a cation exchange chromatography column, is a "ultra-rapid" analysis column. 2.7-µm polyhydroxymethacrylate nonporous monodisperse particles modified with sulfopropyl functional group filled in the column also contributes to the high resolution. The IEC SP-FT 4A is an improved version of Shodex IEC SP-420N (discontinued), which was a nonporous rapid analysis column. The analysis time of IEC SP-FT 4A is one-third of that of IEC SP-420N, while the column remains to provide sharp peak shapes. Dimensions of the IEC SP-FT 4A column housing, made of PEEK, are 4.6-mm inner diameter and 10-mm length. It has an ion exchange capacity of 0.2 meq/g.

Results and Discussion

1. Separation of Protein Standards

Figure 1 shows the comparison of IEC SP-FT 4A and IEC SP-420N for the separation of protein standards. The resulting chromatograms demonstrated that even faster analysis was achieved by the IEC SP-FT 4A compared to IEC SP-420N. The five protein standards (ovalbumin, trypsinogen, ribonuclease A, cytochrom C, and lysozyme) were separated within 1.5 minutes.

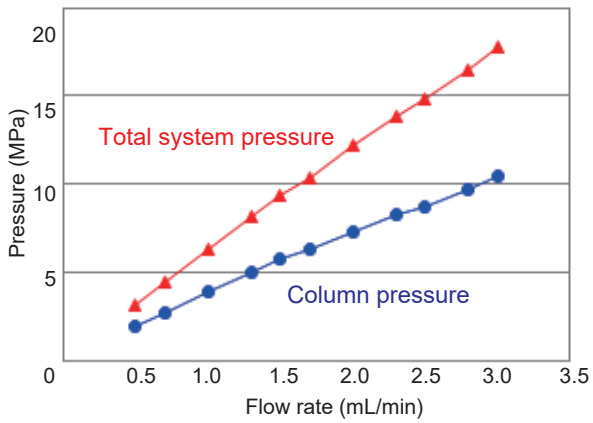


Column : IEC SP-FT 4A (4.6 mm I.D. x 10 mm)
IEC SP-420N (4.6 mm I.D. x 35 mm)
Eluent : (A) 20 mM MES buffer (pH 6.0)
(B) (A) + 0.5 M Na_2SO_4
Linear gradient
SP-FT 4A : (A) to (B) in 2 min
SP-420N : (A) to (B) in 15 min
Flow rate : 1.5 mL/min
Detector : UV (280 nm)
Column temp. : Room temp.

Fig.1. Comparison of IEC SP-FT 4A and IEC SP-420N

2. Column Pressure

Figure 2 shows the relationships between the flow rate and the column pressure of IEC SP-FT 4A. Because of the advanced particle-size controlling technique, the column back pressure can be kept at low despite of the 2.7-µm fine particles. This can be seen in its low system pressure of under 20 MPa at the fast, 3.0 mL/min, flow rate. This enabled the ultra-rapid analysis which is comparable to the analysis speed of the ultra-high performance liquid chromatography (UHPLC), but with a regular HPLC system.

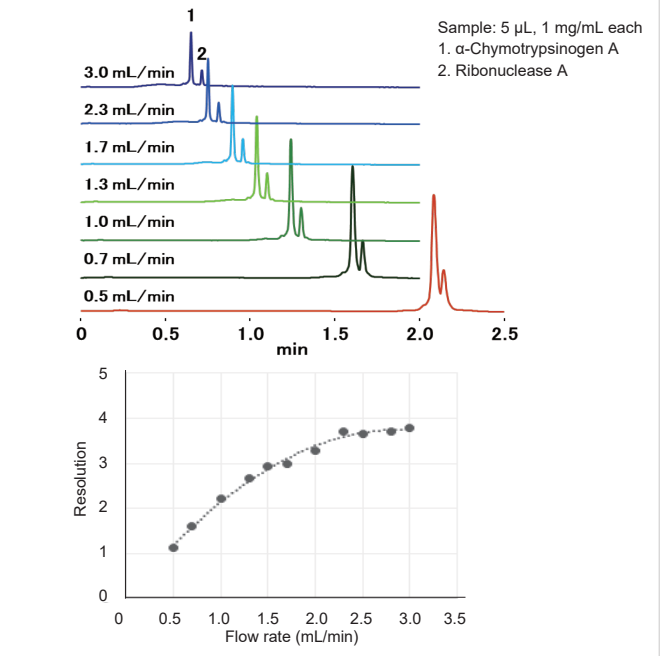


Column : IEC SP-FT 4A (4.6 mm I.D. x 10 mm)
Eluent : (A) 20 mM MES buffer (pH 6.0)
(B) (A) + 0.5 M Na_2SO_4
Linear gradient
(A) to (B) in 2 min
Flow rate : 0.5 - 3.0 mL/min
Detector : UV (280 nm)
Column temp.: 30 °C

Fig. 2. The relationships between the flow rate and the column pressure

3. Effects of the flow rate on the protein separation

Figure 3 shows the effects of flow rate on the separation of α-chymotrypsinogen A and ribonuclease A using the IEC SP-FT 4A. The resolution improved as the flow rate increased, and it achieved a constant value around the flow rate at 2.3 mL/min. Using the faster flow rate shortens the analysis time with a drawback of weaker peak intensity. The optimal flow rate recommended for the general analysis is around 2.0 mL/min.

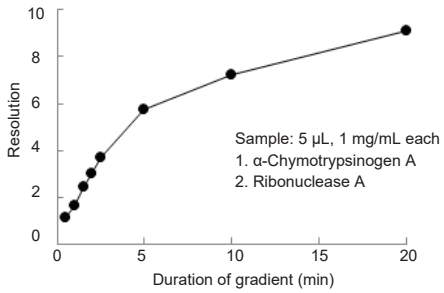


Refer to Fig. 2 for the analytical conditions used.

Fig. 3. The relationships between the flow rate and the resolution of protein standards

4. The effects of gradient settings on the protein separation

Figure 4 shows the effects of gradient settings for the separation of α-chymotrypsinogen A and ribonuclease A using the IEC SP-FT 4A. By using the slower increments for the gradient showed the increased resolution. The optimal gradient timing for the separation of α-chymotrypsinogen A and ribonuclease A was found to be 2 - 5 minutes. This ensures the good resolution and short analysis time.

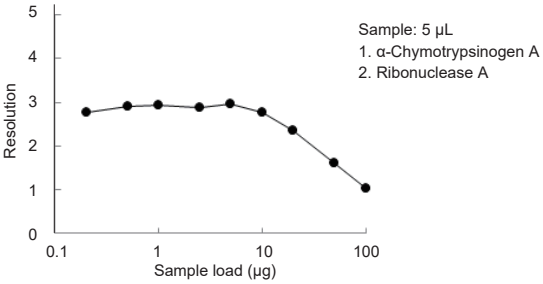


Column : IEC SP-FT 4A (4.6 mm I.D. x 10 mm)
Eluent : (A) 20 mM MES buffer (pH 6.0)
(B) (A) + 0.5 M Na_2SO_4
Linear gradient
(A) to (B) in different timings of 0.5 - 20 min
Flow rate : 1.7 mL/min
Detector : UV (280 nm)
Column temp.: 30 °C

Fig. 4. The relationships between the rate of eluent change and the resolution of protein standards

5. Sample Load

Figure 5 shows the effects of sample load on the separation of α-chymotrypsinogen A and ribonuclease A using the IEC SP-FT 4A. A consistent resolution was obtained when the total sample load was less than 5 µg (2.5 µg each). However, injection volume larger than 5 µg showed lower resolution. Therefore, the maximum sample load recommended for proteins analysis is 5 µg.



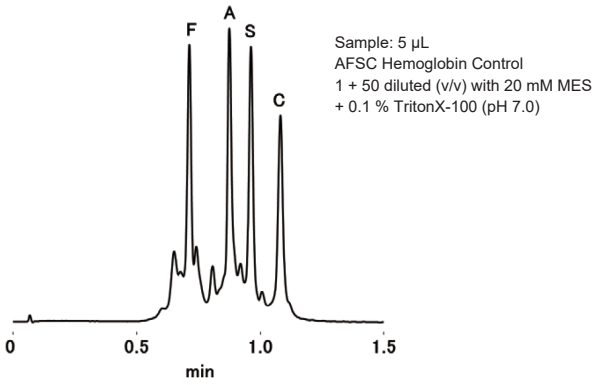
Column : IEC SP-FT 4A (4.6 mm I.D. x 10 mm)
Eluent : (A) 20 mM MES buffer (pH 6.0)
(B) (A) + 0.5 M Na_2SO_4
Linear gradient
(A) to (B) in 2 min
Flow rate : 1.7 mL/min
Detector : UV (280 nm)
Column temp.: 30 °C

Fig. 5. The relationships between the sample load and the resolution of protein standards

Applications

1. Separation of Hemoglobins

Figure 6 shows an example of hemoglobin separation using the IEC SP-FT 4A. The chromatogram demonstrates good separations of normal hemoglobins, HbF(F) and HbA (A), and abnormal hemoglobins, HbS(S) and HbC(C), in a control sample. The analysis was completed in a short time period (within 1.5 minute). The feasibility of the cation-exchange mode for the separation of hemoglobin variants that have deviated forms of amino acid sequences was demonstrated.

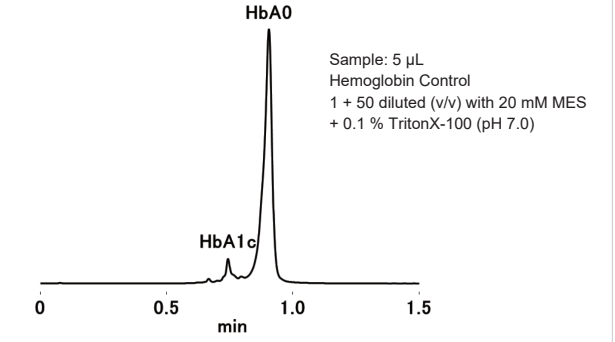


Column : IEC SP-FT 4A (4.6 mm I.D. x 10 mm)
Eluent : (A) 20 mM MES buffer (pH 6.0)
(B) (A) + 0.5 M Na_2SO_4
Linear gradient
(A) to (B) in 2 min
Flow rate : 1.7 mL/min
Detector : VIS (415 nm)
Column temp.: 30 °C

Fig.6. Separation of AFSC hemoglobin

2. Separation of Glycohemoglobin Control

Figure 7 shows the chromatogram of glycohemoglobin analysis using the IEC SP-FT 4A. HbA1c or glycohemoglobin test is a blood test that checks the amount of glucose bound to the hemoglobin and used as a diagnosis index for diabetics. Using the conditions described below demonstrated a good separation of HbA0, the main component of the hemoglobin in blood, and HbA1c within 1 minute.



Refer to Fig. 6 for the analytical conditions used.

Fig. 7. Separation of glycohemoglobins

Conclusions

The IEC SP-FT 4A is a cation exchange column filled with 2.7-µm polyhydroxymethacrylate nonporous monodisperse particles, which suppress the diffusion effect inside the column. This enabled the ultra-rapid analysis while keeping its high separation capability. The analysis time of the IEC SP-FT 4A was shortened to one-third of that of IEC SP-420N. The IEC SP-FT 4A offers an advantage in shortening the work-operation time by its ultra-rapid analysis. This would be especially beneficial for the quality control whereas a large number of samples need to be analyzed at once. Moreover, the highly-uniformed particles help suppressing the system pressure at around 20 MPa even at the fast flow rate of 3.0 mL/min. The column provides comparable analytical capabilities of the high-pressure tolerance UHPLC system, but with a use of a regular HPLC system.

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