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Technical Report

Method Optimization for the Analysis of Monoclonal Antibodies by Size-Exclusion Chromatography

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Abstract:

Antibody drugs using monoclonal antibodies pose concerns over aggregates formed during production and storage and their impact on safety and efficacy. During monoclonal antibody production, aggregates formation is monitored, and size-exclusion chromatography is one of the most widely used techniques. However, size-exclusion chromatography is performed at relatively low flow rates and requires long analysis times. Analyses of monoclonal antibody drugs must also take into account interaction between the monoclonal antibodies and column packing materials. This Technical Report provides an example of using a column packed with small particle material to optimize an analytical method for analysis of monoclonal antibody aggregates. This article investigates the effect of mobile phase salt concentration, flow rate, and pH on chromatographic separation and peak shape. Moreover we describe an example of method optimization by using a dedicated software for improving separation, sensitivity and reducing analytical time.

Keywords: size-exclusion chromatography, antibody drug, Nexera™ XS inert, method scouting system

1. Background

Pharmaceuticals have recently diversified away from low-molecular-weight compounds also to include macromolecular drugs. Biopharmaceuticals developed and produced with biotechnology have been particularly effective in treating wide range of diseases.

The most common biopharmaceuticals are antibody drugs that utilize monoclonal antibodies (mAbs). Due to their high level of specificity and affinity for target molecules, they offer the benefits of excellent therapeutic efficacy and mild side effects, and are used to treat a variety of diseases including autoimmune diseases and cancer. However, unlike pharmaceuticals that can be controlled artificially in manufacturing processes such as chemical synthesis, biopharmaceuticals are manufactured using living cells. Therefore, appropriate quality controls must be established at every production step to ensure uniformity and quality of the final product.

There are numerous steps from development to production of antibody drugs. The first step is to find an antibody effective against disease based on its affinity and specificity to target molecules. The next step introduces genetic information coding for the selected antibody into cells and determines what culture conditions ensure efficient protein expression. These culture conditions are then scaled up for mass production and the target protein is isolated from cultured cells and purified to produce the bulk drug. In order to produce high-quality antibody drugs, consistent and robust manufacturing methods are essential. The quality, efficacy, and safety of antibody drugs are assured by performing purity tests in conformance with ICH-Q6B[1], including purity analysis and structural analysis of aggregates and isomers, and other assessments as shown in Table 1. These assessments are extremely important at every step of pharmaceutical development, from early stages to product shipment.

mAbs form dimeric or multimeric aggregates depending on production and storage conditions. Aggregates in antibody drugs not only cause a decrease in pharmacological action but also elicit an immune response, thus affecting the efficacy and safety. For this reason, ICH-Q6B requires the separation of impurities such as monomers and aggregates in antibody drugs and determines their content. This article introduces an analysis of mAb impurities and fragments by size-exclusion chromatography (SEC).

Table 1 Examples of Quality Assessment Tests for Antibody Drugs

Item Tested	Purpose	Analysis Technique
Aggregates/ Fragments	Determine levels of aggregates and fragments	Size-exclusion chromatography, Micro flow imaging etc.
Charge variants	Characterization and monitoring of charge variants	Ion-exchange chromatography, Imaged capillary isoelectric focusing etc.
Sugar chain structures	Evaluate consistency of sugar chain structures	Hydrophilic chromatography, Reversed-phase chromatography, Mass spectrometry etc.
Structure	Evaluate molecular structure and specificity characteristics of bulk drug	Reversed-phase chromatography, Mass spectrometry etc.
Antibody-drug conjugates	Calculate coupling ratio of antibody-drug conjugate	Hydrophobic chromatography, Mass spectrometry etc.
Potency	Quantify biological activity	Affinity chromatography, Enzyme-linked immunosorbent assay etc.

1 Analytical & Measuring Instruments Division

2. Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) is a technique that separates molecules based on their size (Fig. 1). The column packing material contains numerous pores. Smaller molecules permeate deeper into these pores and take longer to pass through the column, on the other hand, larger molecules are unable to permeate the pores. Consequently, molecules are eluted from the column in order from largest to smallest and effectively sorted according to size. Conventional SEC analysis uses long columns 300 mm in length and low flow rates with long elution times from several tens of minutes up to an hour to ensure full separation between components. Recent column development has reduced the sizes of packing materials to achieve excellent separation with shorter elution times.

However, further improvements in separation and sensitivity require not just smaller column packing materials but optimization of the analytical conditions. In an ideal SEC separation, there is no chemical interaction between the molecules, packing material surfaces, and mobile phase. But in the SEC columns containing silica-based packing materials with chemically-bound common diol groups, electrostatic interaction may arise between proteins and residual silanol groups on the packing material surface causing proteins to adsorb to the silica gel. This results in peak tailing, delayed elution times, and other phenomena. Electrostatic interactions can be suppressed by adding sodium chloride to the mobile phase to negate these negatively charged silanol groups. The strength of these interactions with column packing material varies with the type of protein; it is necessary to determine the appropriate analytical methods for each protein.

This article presents an optimization of an analytical method for mAb aggregates and fragments that investigates the effects of mobile phase salt concentration, flow rate, and pH on separation with a 150 mm long Shim-pack™ Bio Diol-300 column with 2 µm diameter packing material.

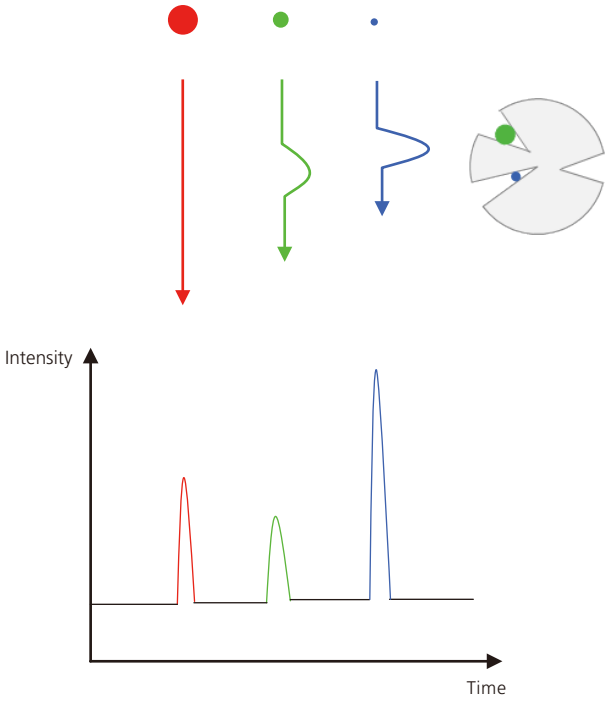

Fig. 1 Principle of Separation in SEC Analysis

3. Analysis

3-1. Analytical Conditions

Table 2 shows the analytical conditions that are common to all analyses. The mobile phase compositions are described in each section.

Table 2 Analytical Conditions

System	: Nexera XS inert
Column	: Shim-pack Bio Diol-300 *1 (150 mm×4.6 mm I.D., 2 µm)
Flow rate	: 0.2 mL/min (Fig. 2, Fig. 3, Fig. 9) 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4 mL/min (Fig. 5) 0.25 mL/min (Fig. 7)
Column Temp.	: 25 °C
Sample	: Monoclonal Antibody Standard (Conc. 500 µg/mL)
Vial	: TORAST™-H Glass Vial *2 (Shimadzu GLC Ltd.)
Injection vol.	: 5 µL
Detection	: 280 nm (SPD-M40 inert cell)

*1 P/N : 227-31010-01 *2 P/N : 370-04301-01

3-2. Impact of Mobile Phase Salt Concentration

Sequential analysis was performed to evaluate the impact of mobile phase salt concentration while automatically adjusting salt concentration by the mobile phase blending function, which is the feature of the method scouting system. Fig. 2 shows a chromatogram obtained using a 100 mmol/L phosphate buffer (pH 7.0) without sodium chloride and the same chromatogram expanded to show the area around the monomer peak in more detail. Fig. 3 also shows the same expanded area in chromatograms obtained using a 100 mmol/L phosphate buffer (pH 7.0) containing between 50 and 250 mmol/L of sodium chloride.

Fig. 4 shows the relation between sodium chloride concentration in the mobile phase and the peak symmetry factor and resolution. At 0 mmol/L sodium chloride in the mobile phase (Fig. 2), electrostatic interaction between the column packing material and mAb caused tailing of the monomer peak; but the symmetry factor of this monomer peak improved as the sodium chloride concentration was increased from 50 to 250 mmol/L. Peak resolution was also affected; it showed the best value around 50 and 100 mmol/L sodium chloride (Fig. 3, 4), which contributed to accurate peak integration. As a conclusions in this case, the better monomer peak shape and appropriate separation from aggregates were achieved with a sodium chloride concentration of 100 mmol/L. Hence a mobile phase sodium chloride concentration of 100 mmol/L was chosen for this mAb analytical method.

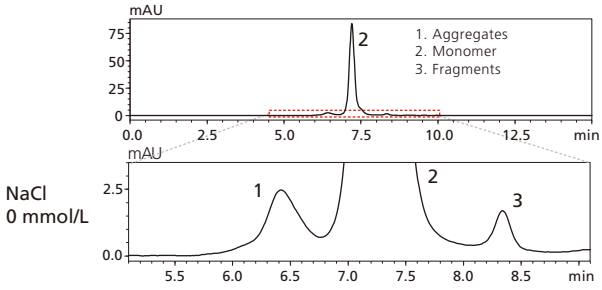

Fig. 2 Chromatograms without Sodium Chloride in Mobile Phase

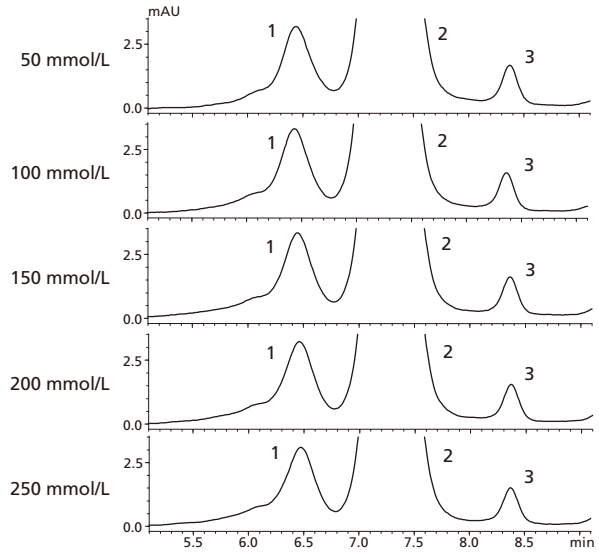

Fig. 3 Chromatograms with Sodium Chloride in Mobile Phase

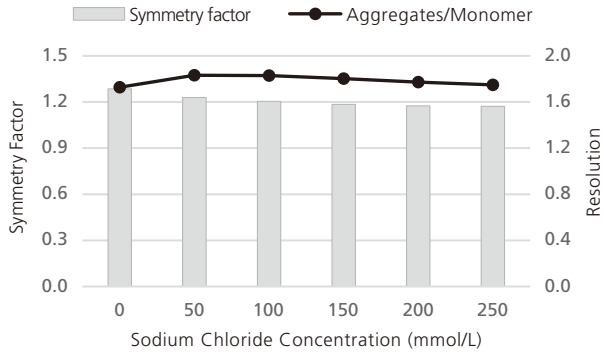

Fig. 4 Relationship between Chromatographic Performance and Sodium Chloride Concentration

3-3. Impact of Flow Rate

It's known that the flow rate should be optimized based on mobile phase composition, packing material particle size, physical and chemical properties of molecules, and other factors [2]. Fig. 5 and Fig. 6 show the impact of mobile phase flow rate on separation for this analysis.

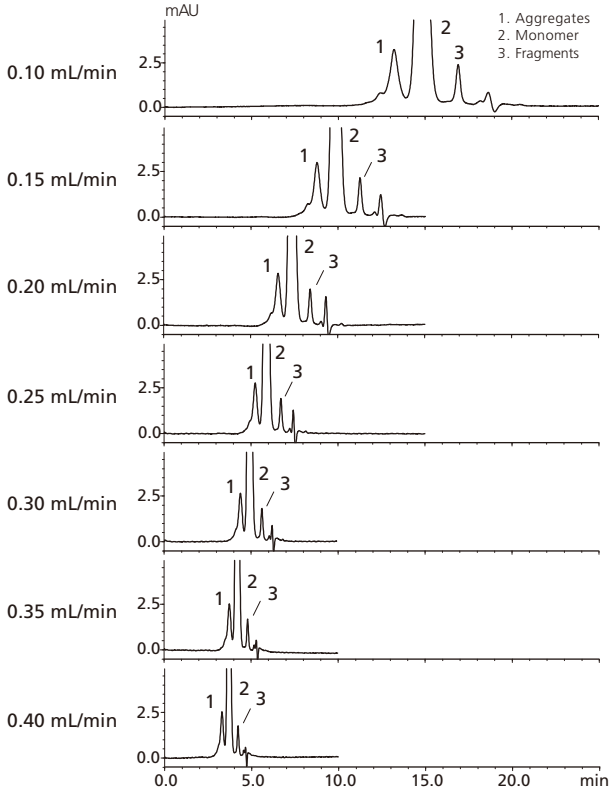

Fig. 5 Chromatograms and Flow Rate

As shown in Fig. 6, the resolution between aggregates and monomer, and between monomer and fragments improved at lower flow rate. Based on these results, a flow rate of 0.25 mL/min was chosen as it provides shorter analytical time and appropriate chromatographic separation (over 1.5 resolution).

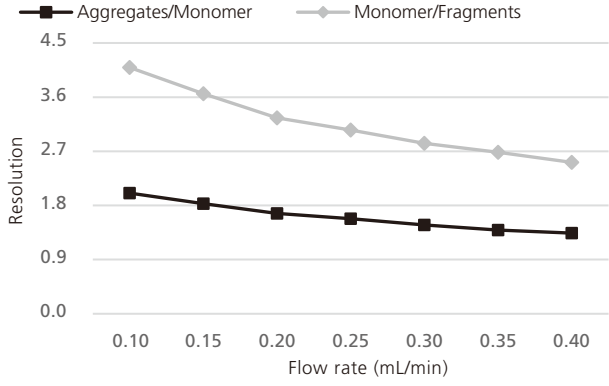

Fig. 6 Relationship between Flow Rate and Peak Resolution

3-4. Impact of Mobile Phase pH

Due to a large number of dissociable groups on protein molecule, their charge state and steric structure change depending on pH. Such changes are known to contribute to protein size and the strength of interaction with the column and thereby affect chromatogram peak shapes.

Fig. 7 shows chromatograms of mAb obtained with 100 mmol/L phosphate buffer containing 100 mmol/L sodium chloride adjusted to different pH. Each mobile phase was automatically prepared with the blending function and provided to the system. The pH 5.7 showed no aggregate and a huge unknown peak after the fragments; meanwhile, the pH 6.0 gave the smaller peaks related to aggregates and unknown components. Since the isoelectric point (pI) value of target mAb is larger than the pH level of these two mobile phases, the impact on the retention deriving from the electrical-charge interaction could be more significant in these two conditions.

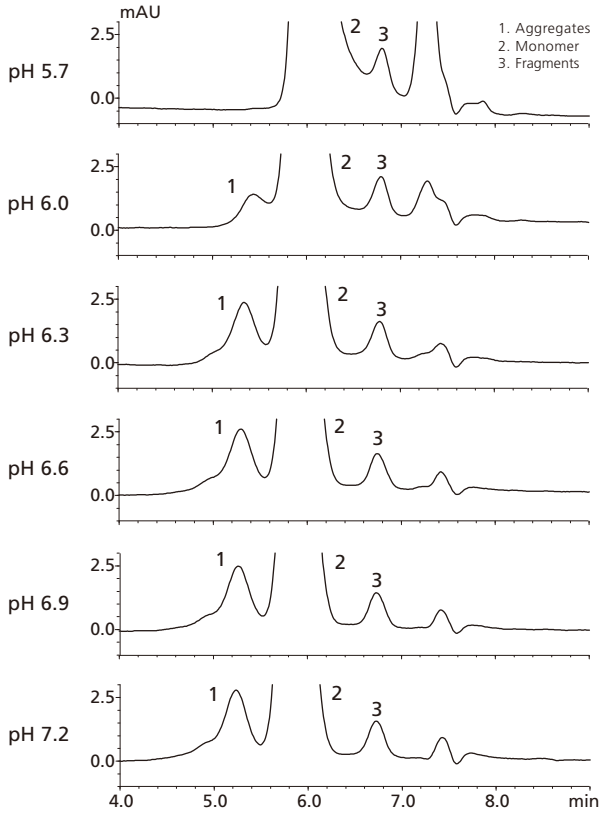

Fig. 7 Chromatograms and pH

As shown in Fig. 8, mobile phase pH did not significantly affected the symmetry factor at pH 6.0 and above, but the resolution between aggregates and monomer and between monomer and fragments improved with raising pH. The best resolution for the mAb in this study was obtained at pH 7.2. Proteins normally denature and recieve damages at highly acidic or alkaline solution. Hence the effect of pH should be investigated at neutral pH close to the antibody pI.

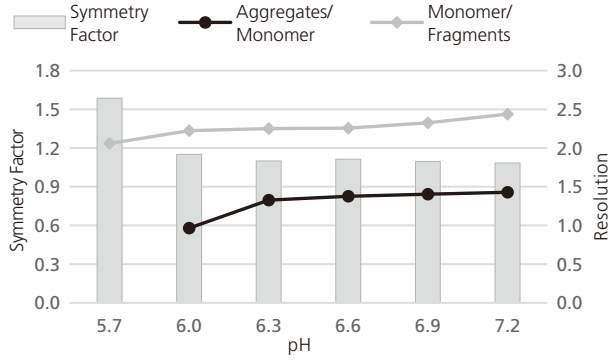

Fig. 8 Relationship between Chroamtographic Performance and pH

4. Column

Shim-pack Bio Diol improves the accuracy of analysis of biopharmaceuticals and medium-molecular-weight compounds. Shim-pack Bio Diol comes in four pore sizes to accommodate a wide range of molecular weights and analysis time can be shortened by the lineup of multiple particle sizes. Refer to the Shimadzu website for details [3]. Fig. 9 compares a Shim-pack Bio Diol with another commercially available SEC column installed in the same system (300 mm × 4.6 mm I.D., 3 µm, 300 Å). Better separation between monomers and fragments was achieved using Shim-pack Bio Diol-300.

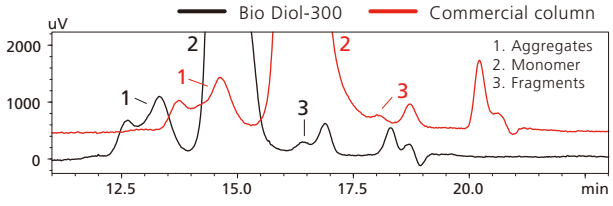

Fig. 9 Comparison of Bio Diol and Another Commercial Column (Chromatogram baselines shifted for comparison)

5. System Configuration

Biopharmaceutical analysis sometimes requires intense separation conditions, such as high salt concentrations and low pH, potentially harmful to HPLC systems. Additionally, molecule adsorption onto the equipment due to the interaction with the metal ion is one of the most serious issues, making it difficult to obtain highly reliable data with a standard HPLC system. Nexera XS inert (Fig. 10) is the specific UHPLC system with excellent reliability, robustness, and flexibility for such kind of analysis. It contributes to determining the characteristics of proteins, antibodies, and formulations produced with biotechnology, such as genetic engineering, cell fusion, and cell culture.

6. LabSolutions MD

This article indicates that screening analytical conditions with a variety of parameters is critical. On the other hand, manual operation for preparing tremendous mobile phases and methods is extremely time-consuming. The Shimadzu method development software, LabSolutions MD, allows automated creation of the combination of columns and mobile phases and the analytical sequences. The mobile phase blending function is also helpful for adjusting each mobile phase component, dramatically reducing the time to prepare numerous solvents and to optimize separation conditions. In this article, the mobile phase blending function was used to investigate salt concentration and pH.

7. Summary

Since aggregates of antibody drugs are known to negatively affect the pharmacological action and elicit an immune response, ICH-Q6B guides that the amounts of aggregates should be determined. This article investigated the impact of several SEC analysis parameters on mAb separation. SEC analyses are often performed at relatively low flow rates, but with Shim-pack Bio Diol-300 with 2 µm packing particle, superior separation was obtained even on a shorter column length for analysis times of faster than 15 minutes. By optimizing mobile phase salt concentration, flow rate, and pH, the chemical interaction between the mAb and packing materials was successfully controlled. Finally, aggregates, monomer, and fragments were separated with reasonable peak shapes. To correctly determine impurities in antibody drugs, it is essential to investigate these parameters and determine the optimum analytical conditions for each protein.

Reference

[1] Specifications : Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (PMSB/ELD Notification No. 571 dated May 1, 2001)

[2] Niki E. and Watanabe N. (1980). Introduction to High Performance Liquid Chromatography. Journal of Japan Oil Chemists' Society, 29 (2), 127-134.

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It inhibits metal-adsorption of biomolecules.

Fig. 10 Nexera XS inert

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