Separate and Quantify Rituximab Aggregates and Fragments with High-Resolution SEC

The Agilent 1260 Infinity Bio-Inert Quaternary LC System and the AdvanceBio SEC 300Å, 2.7 µm Column

Application Note

Biologics and Biosimilars

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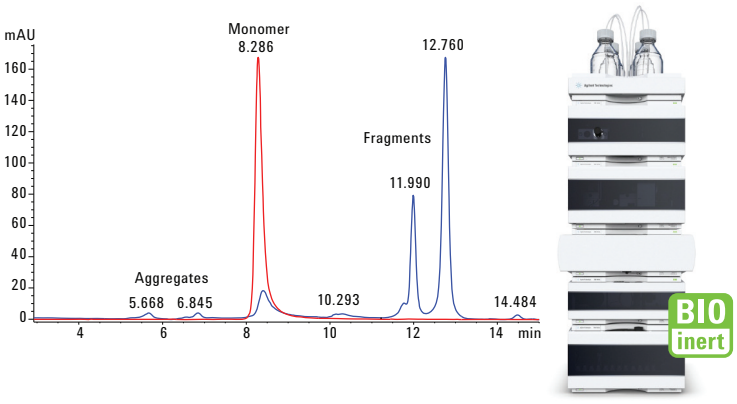
Abstract

Monoclonal antibody aggregation can arise from multiple mechanisms, including product and environmental factors. Size exclusion chromatography (SEC) is a standard method to determine and quantify aggregation and fragment levels of monoclonal based therapeutics. The focus of this application note is to showcase high-resolution separation and quantification of intact, aggregates, and fragments of biosimilar and innovator rituximab obtained from forced-stress studies. Separation and quantification was achieved using an Agilent 1260 Infinity Quaternary Bio-inert LC and an Agilent AdvanceBio SEC column. Calibration of the column was performed using molecular weight markers. The AdvanceBio SEC column provided sensitive and high-resolution separation for monitoring aggregates/degradants, and is ideal for applications where high resolution and sensitivity are essential.



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Introduction

Size exclusion chromatography (SEC) is a widely acceptable method to separate monomer, low molecular weight (LMW), and high molecular weight (HMW) variants in therapeutic mAbs. SEC under native conditions separates monomers and their variants based on their size by differential diffusion into the pores of the packing material. Successful development of a mAb-based pharmaceutical also requires assessment of aggregation and fragmentation resulting from forced degradation studies, which include physical and chemical degradation pathways. Size matters in the development of biotherapeutics since aggregates have been implicated in enhancing immunogenicity, and affect safety and efficacy. A significant challenge is identifying an SEC method that is capable of separating and monitoring such variants. Here, we show the benefits of one such method using an Agilent AdvanceBio SEC column, which is a breakthrough technology for SEC analysis. The column uses unique 2.7 µm silica particles and bonding chemistry to deliver high-resolution separation of mAbs and their mass variants. Retention time and area precision of the method are excellent, demonstrating the suitability of the column and bioinert LC system employed.

Materials and Methods

Instrument

We used a completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC system with a maximum pressure of 600 bar, consisting of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) containing bioinert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity DAD VL (G1315D with Bio-inert standard flow cell, 10 mm)

Software

Agilent ChemStation Rev. B.04.03 (or higher).

Conditions

Column:	Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301)
Mobile phase:	Phosphate buffered saline (PBS), 50 mM sodium phosphate containing 150 mM sodium chloride, pH 7.4
TCC temp:	Ambient
Inj vol:	10 µL
Flow rate:	0.8 mL/min
Detection:	UV, 220 and 280 nm

Reagents, samples, and materials

Innovator and biosimilar rituximab, cetuximab, trastuzumab, and ADC were purchased from a local pharmacy and stored according to the manufacturer's instruction. PBS, hydrochloric acid, and sodium hydroxide were purchased from Sigma-Aldrich, Corp. All the chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10, USA) was used

Calibration of the AdvanceBio SEC column

The AdvanceBio SEC column was calibrated by measuring the elution volumes of several standards (protein aggregates, thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa)). The log molecular weight values of the standards were plotted against the elution volume to determine the equivalent molecular weight of the sample.

Procedure

Mobile phase (10 µL) was injected as blank, followed by six replicates of intact and stressed mAbs to calculate area and retention time (RT) deviation.

Preparation of rituximab aggregates

Aggregates of mAbs were prepared by diluting monoclonal antibody in mobile phase to a final concentration of 2 mg/mL. pH stress testing was carried out as described earlier with slight modification [1]. Briefly, 1 M HCl was slowly added drop-wise to the sample solutions to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was approximately one minute waiting time between the pH shifts, while constant stirring at 500 rpm. The resulting solution was incubated at 60 °C for 60 minutes.

Results and Discussion

Separation and detection

The AdvanceBio SEC 300Å column was calibrated using a series of standard proteins with known molecular weights (Figure 1). Standard protein aggregates (void peak) in the protein marker were used to calculate the void volume, which eluted at 5.748 minutes on the column, corresponding to V_0 = 4.59 (mL). The calibration curve for proteins separated on the column shows a linear relationship, and defines the exclusion limit (670 k Da) for the protein range (1.3 to 670 kDa) analyzed (Figure 2). The molecular weight of an unknown protein can then be determined from its elution volume using this plot.

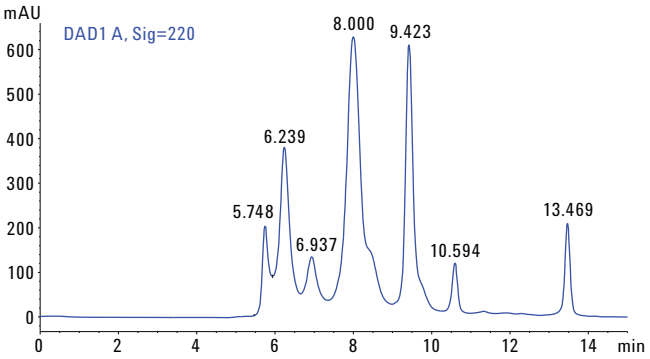

Figure 1. Separation of protein standards on the Agilent AdvanceBio SEC, 300Å, 7.8 × 300 mm, 2.7 µm column.

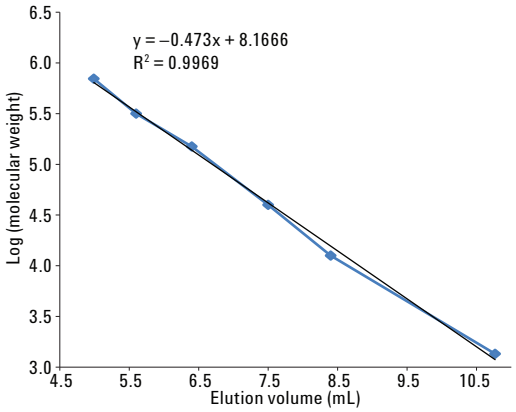

Figure 2. Calibration curve for standards on the Agilent AdvanceBio SEC-3, 300Å, 7.8 × 300 mm, 2.7 µm column.

Figure 3 demonstrates the excellent separation of intact mAbs in eight minutes using the AdvanceBio SEC 300Å. The absence of early or late eluting peaks suggests that the marketed mAb preparations were homogenous, with no indication of aggregation or degradation.

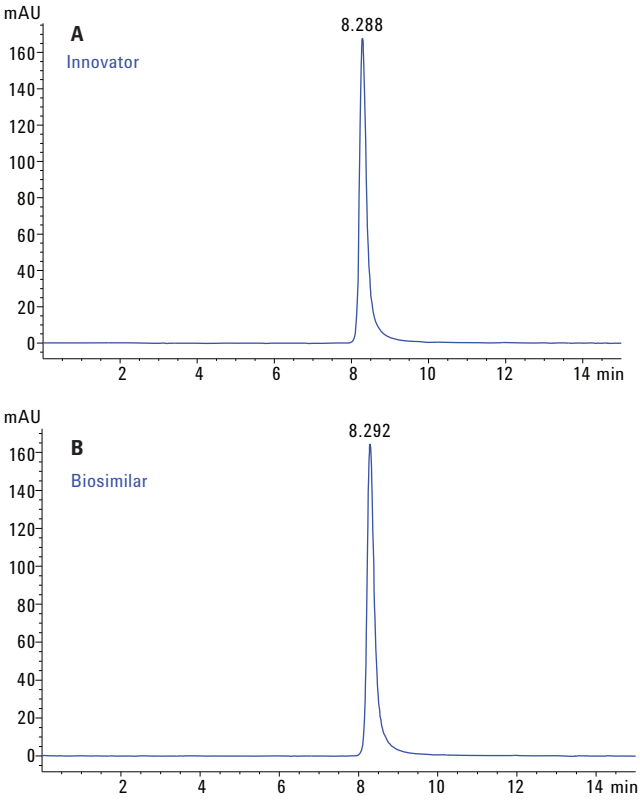

Figure 3. SEC profile of intact therapeutic mAbs on the Agilent AdvanceBio SEC, 300Å, 7.8 × 300 mm, 2.7 µm column: A: rituximab innovator; B: rituximab biosimilar.

Figure 4 demonstrates the overlays of biosimilar and innovator rituximab, exhibiting a sharp and symmetrical peak with no nonspecific interactions. Table 1 shows the average retention times and area RSDs from six replicates of intact mAbs. The retention time and peak area RSDs were less than 0.04 and 1%, respectively, which demonstrate excellent reproducibility of the method and, thus, the precision of the system. The RTs of the biosimilar and the innovator were comparable. Also, the purity by area percent was >99% for the innovator and the biosimilar, indicating that they were highly similar.

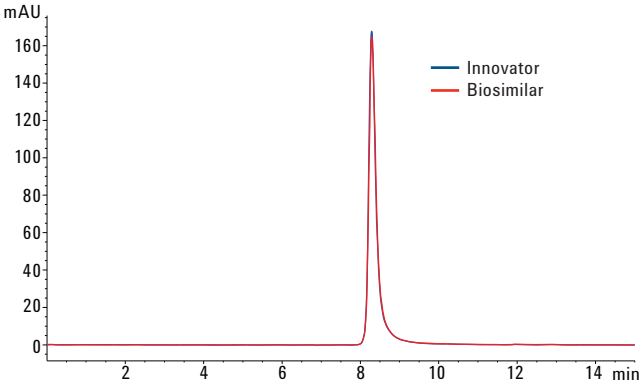

Figure 4. Overlay of rituximab innovator and biosimilar separated on the Agilent AdvanceBio SEC, 300Å, 7.8 × 300 mm, 2.7 µm column.

Table 1. Retention time and peak area precision of rituximab (n = 6).

	Retention time		Peak area	
Sample	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Rituximab innovator	8.28	0.04	99.33	1.21
Rituximab biosimilar	8.29	0	100	0

Aggregation/degradation analysis and quantification

We compared the intact and stress biosimilar and innovator mAbs using SEC for monitoring aggregates and degradants. Any peaks from the chromatographic run eluting before the monomeric form were considered as aggregates and any eluting later as degradants [2].

Figure 5 shows the SEC profiles of pH/heat-stressed innovator and biosimilar rituximab. The profiles indicate that the AdvanceBio SEC column, using the same intact analysis method, separated and detected aggregates and degraded mAbs. Based on the area percent, the relative quantitation of aggregates and fragments in innovator and biosimilar are summarized in Table 2.

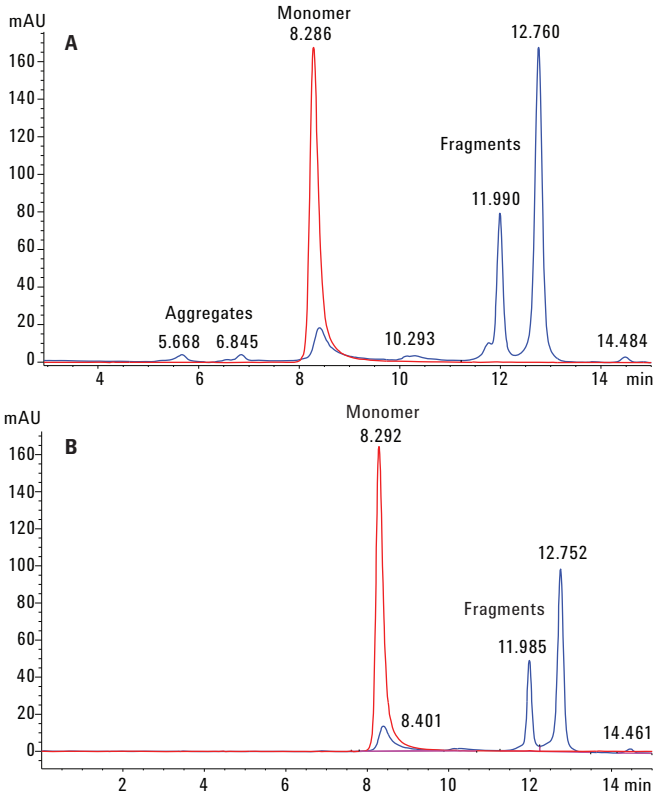

Figure 5. Agilent AdvanceBio SEC chromatogram of (A) intact rituximab innovator (red trace) overlaid with pH/heat-stressed sample (blue trace) and (B) intact rituximab biosimilar (red trace) overlaid with stressed sample (blue trace).

Table 2. Relative area percent of intact and stressed innovator and biosimilar rituximab.

Intact innovator		Stressed innovator	
Time	**Area %**	**Time**	**Area %**
8.288 (monomer)	100	1.289	4.24
		5.668	2.26
		6.845	2
		8.40 (monomer)	13.6
		10.29	2.81
		11.99	23.22
		12.76	51.01
		14.48	1.11
Intact biosimilar		**Stressed biosimilar**	
Time	**Area %**	**Time**	**Area %**
8.292 (monomer)	100	8.40 (monomer)	16.832
		11.985	24.24
		12.75	57.36
		14.46	1.5

It is interesting to note that the relative peak areas of the aggregate species of innovator mAb increased due to stress compared to the biosimilar. However, the fragmentation pattern and the peak areas of fragments for both mAbs were highly comparable. The monomeric form in innovator and biosimilar after stress was found to be 13 and 16%, while the predominant form in both the mAbs were fragments eluting at about 12.75 minutes, which was greater than 50%.

Lastly, to determine the ability of the AdvanceBio SEC column to resolve commercial monoclonals and ADC that differ closely in their molecular weight, single injection analysis of the samples were carried out. Figure 6 shows the overlays. The AdvanceBio SEC column resolved therapeutic mAbs and ADC according to their molecular mass, as we see clear differences in their retention times, demonstrating the suitability of this column for the analysis of these molecules.

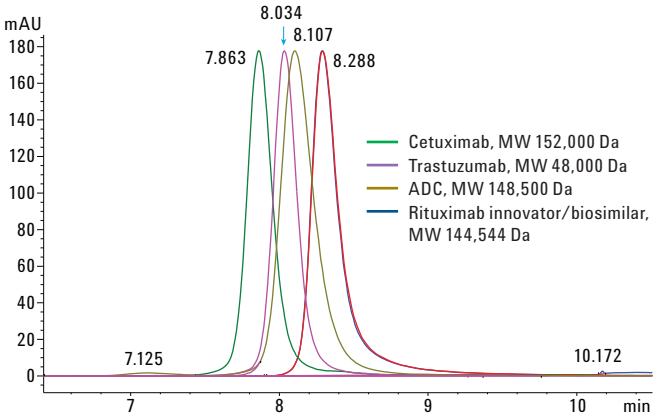

Figure 6. Overlay of rituximab innovator/biosimilar, trastuzumab, cetuximab, and ADC separated on an Agilent AdvanceBio SEC, 300Å, 7.8 × 300 mm, 2.7 µm column.

Conclusions

The process for mAb-based product development is complex, and involves myriad physicochemical characterizations for determining purity, aggregation, and other product-related variants. Size exclusion chromatography has been widely used for monitoring and quantitation of monomer, HMW, and LMW species under native conditions. In this work, the Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column was used to develop a simple method for separation of intact and stressed rituximab samples under native conditions. The optimized method resolved the monomer as a single symmetrical peak with a purity level of 100% and no sign of aggregates or fragments. The same method resolved and quantitated aggregates and fragments obtained from stress studies. This is only possible with a column such as AdvanceBio SEC that is capable of delivering high-resolution and sensitive separation of mAbs and their mass variants. In addition, the Agilent 1260 Infinity Bio-inert Quaternary LC provides certainty regarding bioinertness and corrosion resistance.

References

1. Başak Kükrer, B.; Filipe, V.; van Duijn, E.; Kasper, P. T.; Vreeken, R. J.; Heck, A. J. R.; Jiskoot, W. Mass Spectrometric Analysis of Intact Human Monoclonal Antibody Aggregates Fractionated by Size-Exclusion Chromatography. *Pharm. Res.* **2010**, *27*, 2197-2204.
2. Rodriquez-Diaz, R.; Wehr, T. Use of Size Exclusion Chromatography in Biopharmaceutical Development. In *Analytical Techniques for Biopharmaceutical Development*; Rodriquez-Diaz, R., Wehr, T., Tuck, S., Eds.; CRC Press: New York, **2005**.

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Printed in the USA
November 2, 2017
5991-6304EN

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