Quantitation of mAb and ADC Aggregation Using SEC and an Aqueous Mobile Phase

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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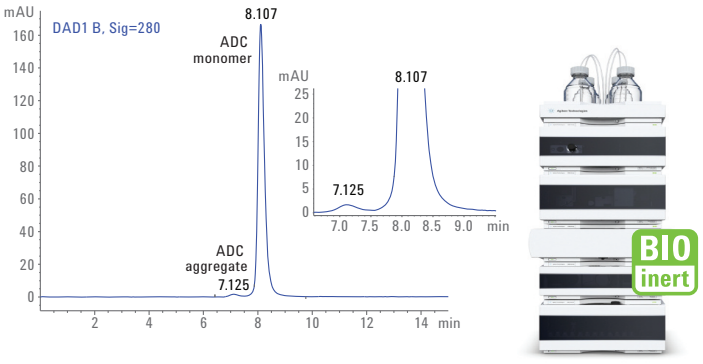
Abstract

Size exclusion chromatography (SEC) is an important tool for monitoring monomers, dimers, aggregates, and potential degradants in biotherapeutic protein samples, including monoclonal antibodies and their derivatives. As aggregation is considered a critical quality attribute, quantitation is required.

This application note describes a simple and sensitive method for the quantitation of aggregates in a biotherapeutic mAb and antibody drug conjugate (ADC) using an Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column and an Agilent 1260 Infinity Bio-inert Quaternary LC. The method uses the same aqueous mobile phase, without the addition of organic modifier, for the analysis of the mAb and the more hydrophobic ADC. The optimized method was also able to monitor aggregate and degradants created by pH/temperature stress. This simple and reproducible method, coupled with the corrosion resistance of the instrument, is suitable for routine QA/QC analysis of mAbs and ADC for the biopharma industry.



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Introduction

Therapeutic proteins are subjected to aggregation and degradation during all stages of development, such as expression, refolding, downstream processing, formulation, sterilization, and storage. In addition, the attachment of hydrophobic payload to form the ADC would also enhance hydrophobicity-driven aggregation. Although aggregates/degradants are present in low concentrations, they may have a big impact on the quality of biologics, leading to activity loss, decreased solubility, and increased immunogenicity. Size exclusion chromatography is the standard method used to characterize protein aggregation. Here, we show the benefits of using the Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column for separation, quantification, and monitoring the integrity of a therapeutic mAb and ADC. AdvanceBio SEC columns are a breakthrough technology for SEC analysis. The columns have been designed and manufactured by Agilent with an innovative silica particle and unique bonding chemistry to deliver resolution and size separations over a wide range of sample types, without the need to add organic modifier to the mobile phase. The analysis of the monoclonal antibody and the more hydrophobic ADC uses the same aqueous mobile phase.

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

Trastuzumab and antibody drug conjugate (T-DM1) 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 chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10, USA) was used.

Linearity and range

The calibration curve was constructed with eight standard concentrations of trastuzumab and ADC from 15.625 to 2,000 µg/mL.

Limit of quantitation (LOQ) and limit of detection (LOD)

Trastuzumab and ADC (T-DM1) were used for LOD and LOQ measurements. The biomolecule concentration that provided a signal-to-noise ratio (S/N) > 3 was considered as LOD and S/N > 10 was considered as LOQ.

Procedure

Mobile phase (10 µL) was injected as blank, followed by individual linearity levels in triplicate. Area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD %) values. LOD and LOQ were established from the lower linearity level injections. Average area of each linearity level was plotted against the concentration of the analyte to determine the calibration curve for the monomers.

Preparation of trastuzumab and ADC aggregates

Trastuzumab and ADC aggregates were prepared by diluting monoclonal antibody in mobile phase to a final concentration of 2 mg/mL. pH stress was carried out as described elsewhere 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 1 min waiting time between the pH shifts, with constant stirring at 500 rpm. The resulting solution was incubated at 60 °C for 60 min.

Results and Discussion

Separation and detection

For SEC quantitation of aggregation, monomer, dimer, and higher aggregates, it is vital that the mobile phase does not affect sample composition. As environmental conditions can change the level of aggregation, it is important that the SEC separation can be accomplished in aqueous mobile phases at neutral pH and with low levels of salt. Figure 1 demonstrates the excellent separation of intact therapeutic trastuzumab mAb in 15 minutes using the AdvanceBio SEC column under the chromatographic conditions frequently used for proteins, that is, phosphate buffered saline at pH 7.4. The peak was symmetrical and eluted at a retention time consistent with the molecular weight of an mAb, indicating that the separation was based on size, and that were no secondary interactions. Figure 1 also shows a magnification, revealing the presence of a small amount of aggregate. Absence of an early or late eluting peak suggested that the marketed mAb preparation was homogenous without any indication of aggregation or degradation.

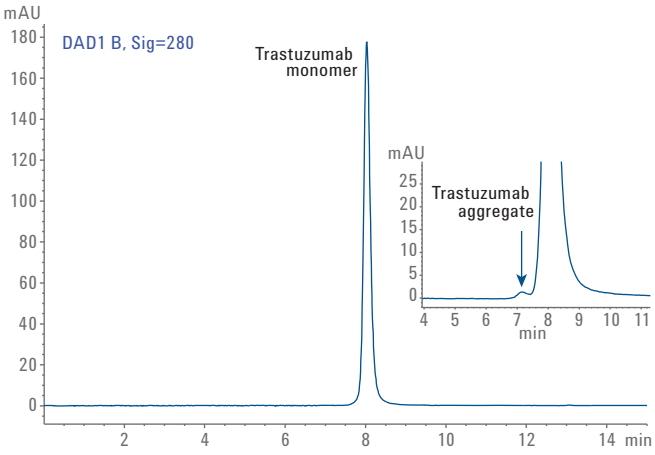

Figure 1. SEC profile of (A) intact trastuzumab (B) magnified region showing trastuzumab aggregates on an Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column.

SEC of ADC

Most published methods for SEC analysis of ADC performed on marketed SEC columns using aqueous phase led to poor peak shape and incomplete resolution of aggregate from the monomeric conjugate. This effect was due to nonspecific interaction of the hydrophobic payload with the stationary phase. Addition of 15% 2-propanol has been shown to overcome this effect [2]. When ADC T-DM1 was analyzed using the AdvanceBio SEC column with an aqueous mobile phase, the PBS led to symmetrical peaks and better resolution of monomer and aggregate, indicating no nonspecific interaction of the hydrophobic drug with the stationary phase (Figure 2).

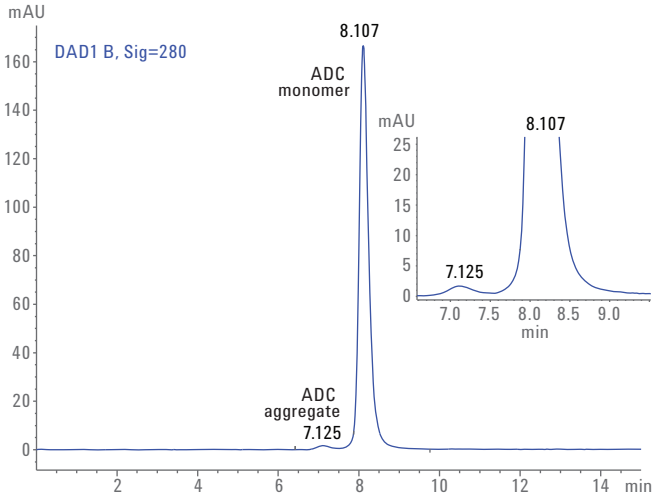

Figure 2. SEC profile of intact T-DM1 (ADC) on an Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column using PBS, pH 7.4, as mobile phase.

Precision of retention time and area

Table 1 shows the average retention times and area RSDs from six replicates of trastuzumab mAb and an ADC analysis. The retention time and peak area RSDs were less than 0.04% and 1%, respectively, which demonstrates excellent reproducibility of the method and, thus, the precision of the system.

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

	Retention time		Peak area	
Sample	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Trastuzumab innovator	8.034	0	100	0
ADC	8.106	0.005	98.91	0.33

Limit of detection and limit of quantitation

The LOD and LOQ were 15 µg/mL and 31 µg/mL, respectively, for trastuzumab and ADC, indicating that the method was sensitive. Observed LOD and LOQ values of trastuzumab and ADC are tabulated in Table 2, and the overlay of LOD and LOQ chromatograms with blank is shown in Figure 3.

Table 2. LOD, LOQ, and S/N results (n = 3).

Concentration (µg/mL)	S/N	Average area
Trastuzumab		
15.625 (LOD)	7.8	12.62
31.25 (LOQ)	21.4	29.16
62.5	32.7	60.74
ADC		
15.625 (LOD)	10.5	15.20
31.25 (LOQ)	15.5	37.89
62.5	37.9	80.24

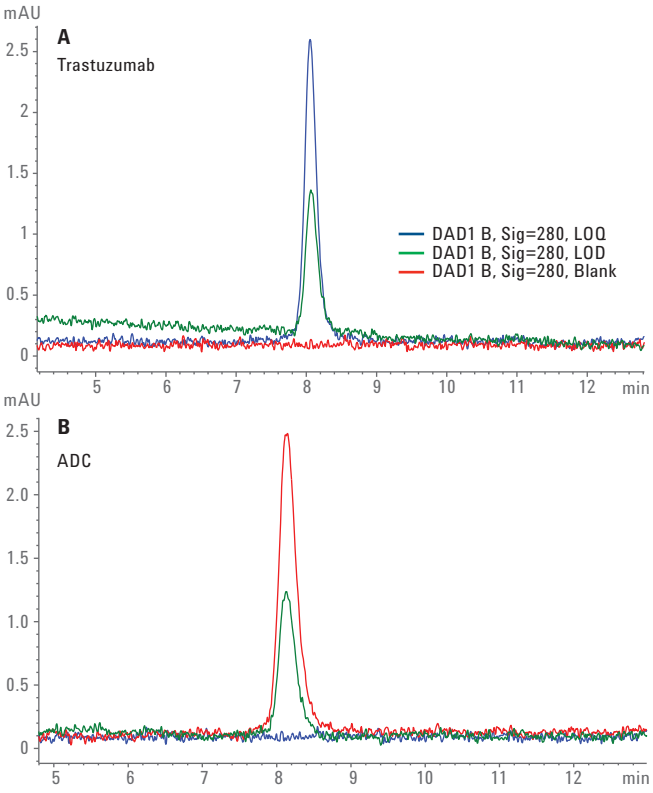

Figure 3. LOD and LOQ chromatograms of trastuzumab and ADC overlaid with blank.

Linearity

Linearity curves for trastuzumab and the ADC were constructed from the LOQ level to the highest concentration level in the study using area response and concentration of trastuzumab/ADC. The accuracy results are shown in Table 3. The linearity curve for trastuzumab/ADC in the concentration range 12.5 to 2,000 µg is shown in Figure 4.

Table 3. Summary of linearity range (n = 3) for trastuzumab and ADC.

Trastuzumab		ADC	
Concentration (µg/mL)	Avg area	Concentration (µg/mL)	Avg area
15.625	16.4	15.625	22.6
31.25	30.6	31.25	37.9
62.5	64.4	62.5	91.2
125	140.7	125	178.8
250	277	250	348.4
500	538.2	500	704.7
1000	1095	1000	1400
2000	2179	2000	2821

Aggregation/degradation analysis

We compared the native and forced-stress trastuzumab and ADC by 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, respectively [3].

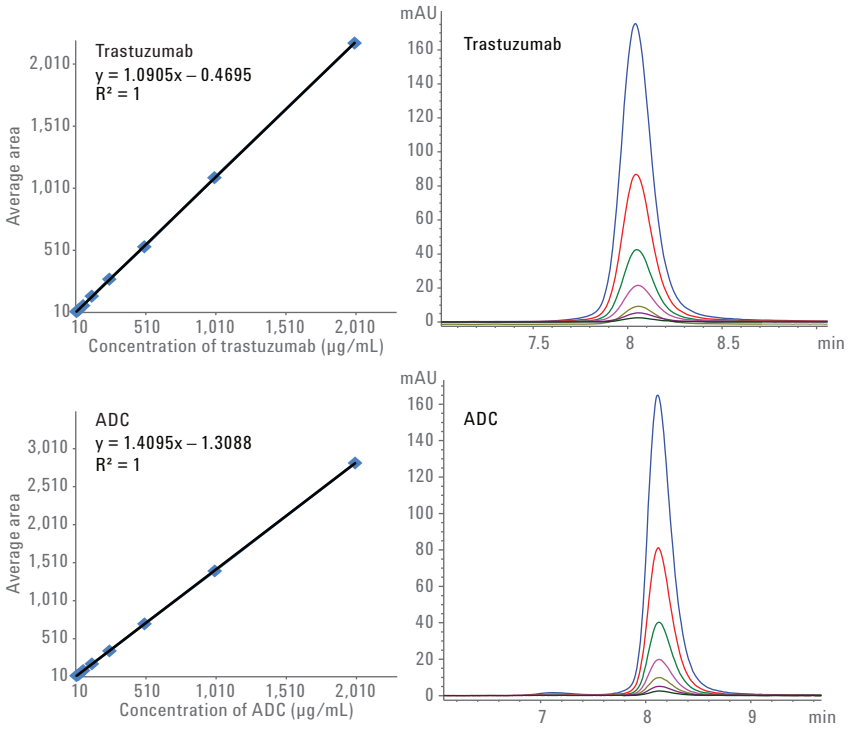

Figure 4. Linearity curve with eight standard concentrations of trastuzumab and ADC ranging from 15.62 to 2,000 µg/mL showing excellent coefficient values. Also shown are chromatogram overlays for the linearity ranges.

The chromatograms of pH/heat-induced aggregates shown in Figures 5 and 6 indicate that the AdvanceBio SEC column was able to separate and detect aggregates as well as degraded trastuzumab and ADC. Intact, aggregates, and degradants were distinctly separated from each other.

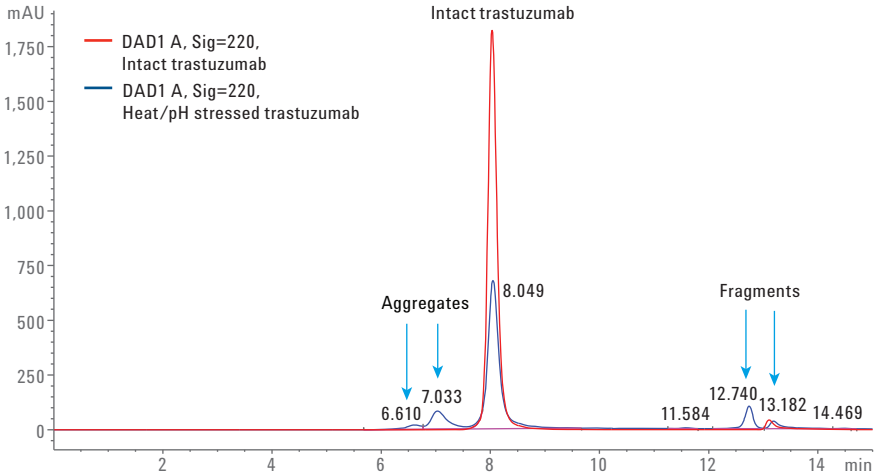

Figure 5. Chromatogram of native (control; red trace) trastuzumab overlaid with 2 mg/mL trastuzumab pH/heat stressed using an Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column.

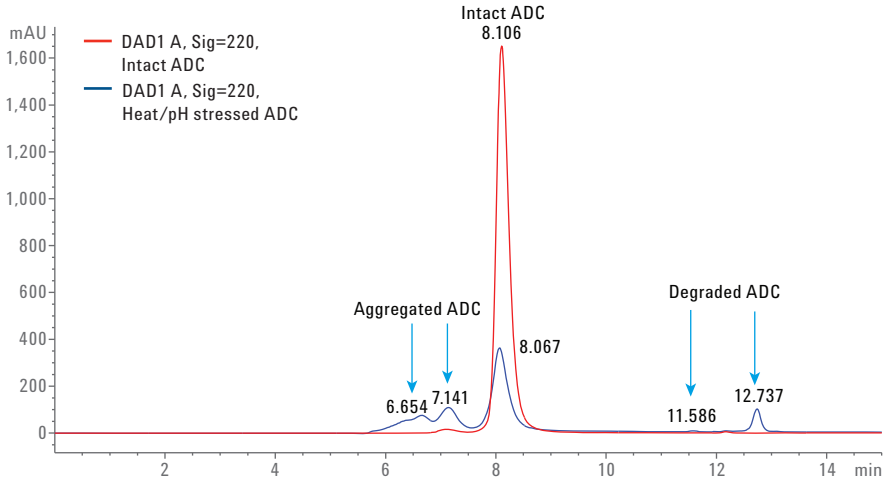

Figure 6. Chromatogram of native (control; red trace) ADC overlaid with 2 mg/mL ADC pH/heat stressed using an Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm column.

Quantitation of trastuzumab and ADC aggregation and degradation

Based on the area percent, the relative quantitation of aggregates and degradants in trastuzumab and ADC are summarized in Table 4.

It is evident from the data that there is a pronounced increase in the levels of aggregates and degradation of trastuzumab and ADC with relative decrease in the monomeric forms, which decreased to 71% and 54%, respectively. Although these results are encouraging, they must be supported with biological activity data to access the potency loss with respect to aggregation/degradation.

Table 4. Retention time and peak area of trastuzumab and ADC monomer, aggregates, and fragments.

Intact trastuzumab		**Stressed trastuzumab**	
Retention time (min)	**Area %**	**Retention time (min)**	**Area %**
7.14	0.140	6.61	2.8
8.034	96.8	7.033	13.26
13.10	3.0	8.03	71.83
		12.74	7.65
		13.18	4.0
Intact ADC		**Stressed ADC**	
7.115	2	6.654	19
8.106	97.8	7.141	17.8
		8.06	54.92
		11.58	0.2
		12.73	7.5
		14.46	0.2

Conclusions

We showcased several excellent tools for method development and monitoring the purity and stability of therapeutic mAb trastuzumab/ADC T-DM1. We first used the Agilent AdvanceBio SEC column to develop a simple, high-resolution separation of mAbs. Notably, the AdvanceBio SEC column was able to provide superior resolution of hydrophobic ADC without using organic modifiers in the mobile phase. Area and retention time precision of the method were excellent, and demonstrated the method's reliability. Linearity curves with eight standard concentrations of mAb and ADC, ranging from 15 to 2,000 µg/mL, had excellent coefficient of linearity values, indicating that the method was quantitative and accurate. The LOD and LOQ for mAb and ADC were found to be 15 µg/mL and 25 µg/mL, respectively, indicating that the method was sensitive. In addition, stress studies of mAb and ADC demonstrated that the AdvanceBio SEC column was able to separate, detect, and quantify aggregates and degradants based on area percent. Such a simple and reproducible method, coupled with the bioinertness and corrosion resistance of the Agilent 1260 Infinity Bio-inert Quarternary LC, make this solution suitable for QA/QC analysis of monoclonal antibody/ADC for the biopharma industry.

References

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3. 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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