Application Note

Biopharmaceuticals

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Surmounting the Challenges of Bispecific Antibody Characterization

Using new optimized column chemistries to detect product impurities during manufacturing or storage

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Abstract

Bispecific antibodies, as their name implies, can simultaneously bind to two separate targets or antigens. This unique behavior creates opportunities for a wide range of applications.[1] Several different types of molecules are described as bispecific monoclonal antibodies (Bs-MAbs). In this application note, we focus on the immunoglobulin (IgG)-like Bs-MAb with asymmetric binding regions, Emicizumab.

Emicizumab[2] is an anti-factor IXa/factor X Bs-MAb used for treating hemophilia A1. It comprises two nonidentical heavy chains (HCs) and two identical light chains (LCs) (Figure 1). This asymmetric configuration can make characterizing the molecule more challenging. However, as for all monoclonal antibody biotherapeutics, analysis of its primary structure and identification of the range of variants that potentially arise during manufacturing and storage is essential.

Considering the rapid growth and development efforts in the field of bispecifics, the purpose of this application note is to present the characterization of critical quality attributes of such complex engineered antibodies. We employ a suite of analytical techniques, which are commonly used in release testing, stability testing, drug substance and drug product characterization, including LC/UV, and LC/MS.

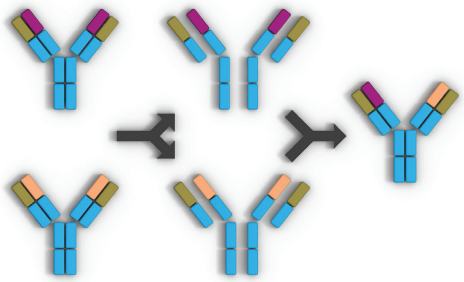

Figure 1. A schematic overview showing the formation of a typical bispecific Ab. As in the case for Emicizumab, two different heavy chains are shown.

Introduction

As of August 2020, two Bs-MAbs are currently approved worldwide, blinatumomab and emicizumab. These Bs-MAbs are representative of new engineered antibodies. They have increasingly attracted interest in the field of new drug development studies. Different Bs-MAbs are been studied containing different molecular architectures, for this reason the biomanufacturing of such molecules is a critical step. The isolation and purification of recombinant IgG-like Bs-MAbs is often associated with an increased level of product-related impurities (aggregates, by-products) and other impurities, which arise from degradation. To overcome these problems, Agilent has developed new HPLC column chemistries, optimized for the separation of such impurities.

Keeping in mind critical parameters such as safety and efficacy, we provide an overview of the separation and characterization of Emicizumab using products from the Agilent biocolumns portfolio. Aggregation is a challenging problem during biotherapeutic protein manufacture and can affect the efficacy and half-life of the product. Size exclusion chromatography (SEC) is an ideal technique for the separation of protein aggregates. Following SEC, another mild, nondenaturing technique often used for characterization and purification of proteins in their native form is ion-exchange chromatography (IEX), which separates protein variants based on their accessible surface charge. These charge variants can result from post-translational modifications (PTMs) such as glycosylation, lysine truncation, etc.

Hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC), despite having a similar basic retention process, are both important techniques for characterizing intact Bs-MAbs. HIC mobile phases are nondenaturing and a native protein structure is more likely to be maintained. In RPC however, separation is achieved using organic solvents and ion-pair reagents, which often result in protein denaturation. RPC does have the advantage however of being more easily interfaced to mass spectrometry.

Experimental

Materials

Emicizumab (mg/mL) was a gift from the customer. Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from Millipore Sigma. All chemicals used were ≥99.5% pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Mobile phases were prepared fresh daily and filtered through a 0.22 µm membrane filter before use.

Instrumentation

An Agilent 1260 Infinity II bio-inert LC system with the following configuration was used for SEC, IEX, and HIC:

- Agilent 1260 Infinity II bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

An Agilent 1290 Infinity LC with the following configuration was used for intact and subunit analysis by UV:

- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1260 Infinity II diode array detector (DAD) (G7115A)

LC/MS analysis

LC/MS analysis were performed on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system. Tables 1 and 2 detail LC/MS parameters used.

Columns

- Agilent AdvanceBio SEC 200 Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)
- Bio MAb, NP5, PEEK, 4.6 × 250 mm (part number 5190-2407)
- Agilent AdvanceBio HIC, 4.6 × 100 mm
- Agilent PLRP-S 1000 Á, 2.1 × 50 mm, 5 µm (part number PL1912-1502)

Software

- Agilent OpenLab CDS 2.2 software
- Agilent Buffer Advisor software
- Agilent MassHunter LC/MS (version 10.1)

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC		
Sample Type	Intact mAb	mAb Subunits (HC and LC)
Column	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 µm (p/n PL1912-1502)	Agilent PLRP-S, 2.1 × 50 mm, 1000 Å, 5 µm (p/n PL1912-1502)
Thermostat	4 °C	4 °C
Solvent A	0.1% Formic acid in DI water	0.1% Formic acid in DI water
Solvent B	0.1% Formic acid in 100% acetonitrile	0.1% Formic acid in 100% acetonitrile
Gradient	0 to 1 min, 0 to 20% B 1 to 3 min, 20 to 50% B 3 to 4 min, 50 to 70% B	0 min, 25% B 5 min, 55% B 6 min, 70% B 6 to 7 min, 70% B
Column Temperature	80 °C	60 °C
Flow Rate	0.5 mL/min	0.8 mL/min

Table 2. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF		
Sample Type	Intact mAb	mAb Subunits (HC and LC)
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas Temperature	350 °C	350 °C
Gas Flow	12 L/min	12 L/min
Nebulizer	60 psig	35 psig
Sheath Gas Temperature	400 °C	350 °C
Sheath Gas Flow	11 L/min	11 L/min
VCap	5,500 V	4,000 V
Nozzle Voltage	2,000 V	500 V
Fragmentor	380 V	180 V
Skimmer	140 V	65 V
Quad amu	1,000 *m/z*	300 *m/z*
Mass Range	500 to 8,000 *m/z*	100 to 3,200 *m/z*
Acquisition Rate	1.0 spectra/s	1.0 spectra/s
Reference Mass	922.0098	922.0098
Acquisition Mode	Positive, Extended (10,000 *m/z*) Mass Range	Positive, Standard (3,200 *m/z*) Mass Range, HiRes (4 GHz)

Results and discussion

Intact and fragment analysis by reversed-phase chromatography

LC/MS was performed on both the intact and reduced sample using a PLRP-S reversed-phase column, the deconvoluted intact mass clearly shows the different glycoforms corresponding to the different combinations of G0F, G1F, and G2F on both heavy chains (Figure 2). Analysis of the reduced sample gave a single light chain, and two nonidentical heavy chains corresponding to the expected 1 to 444 and 1 to 448 sequences (Figures 3 and 4).

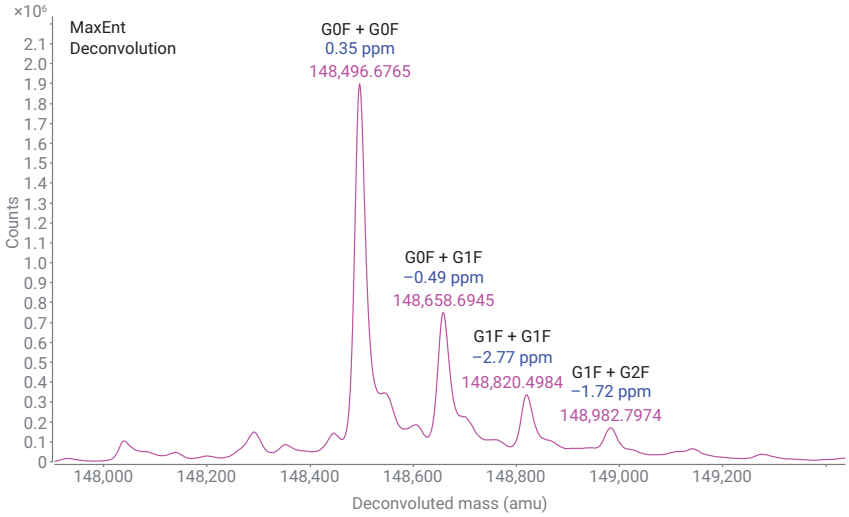

Figure 2. MS deconvolution spectra of intact Emicizumab (0.25 µg injection).

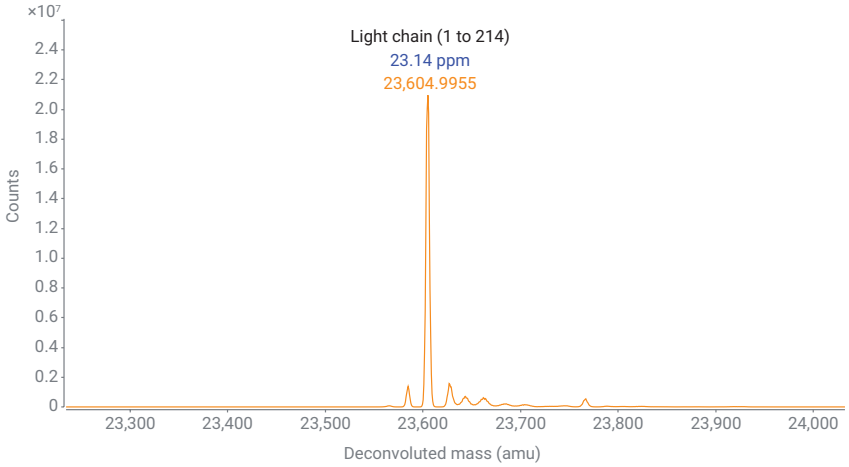

Figure 3. Reversed-phase chromatography of Emicizumab subunit – light chain (0.5 µg injection).

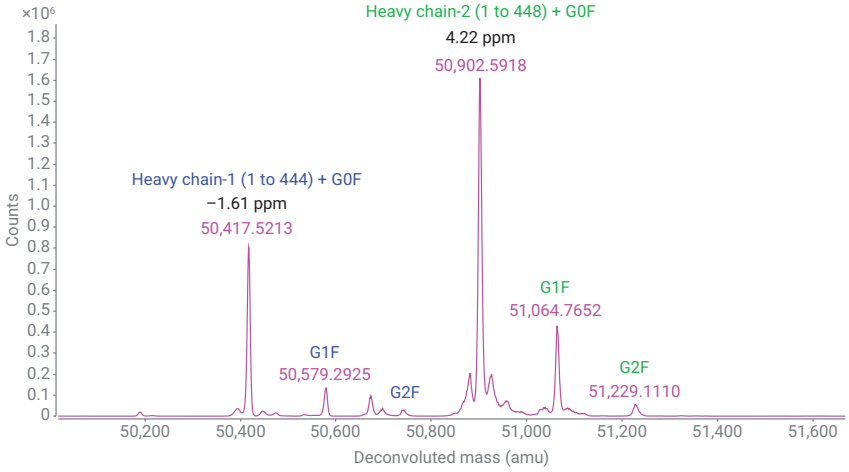

Figure 4. Reversed phase chromatography of Emicizumab subunit – heavy chains (0.5 µg injection).

Aggregate analysis by size exclusion chromatography

SEC was used to characterize size variants. The separation was performed with an AdvanceBio SEC 200 Å 1.9 µm column. Two mobile phases, 150 mM phosphate buffer at pH 6.8 and 50 mM phosphate buffer with 200 mM NaCl at pH 6.8 were investigated for SEC analysis, and the chromatograms are shown in Figure 5. Table 3 summarizes the results using the two mobile phases in terms of monomer peak symmetry, peak width at half-height, and percentage of aggregation. The addition of salt results in a more symmetric and narrower monomer peak. Liquid chromatography parameters are shown in Table 4.

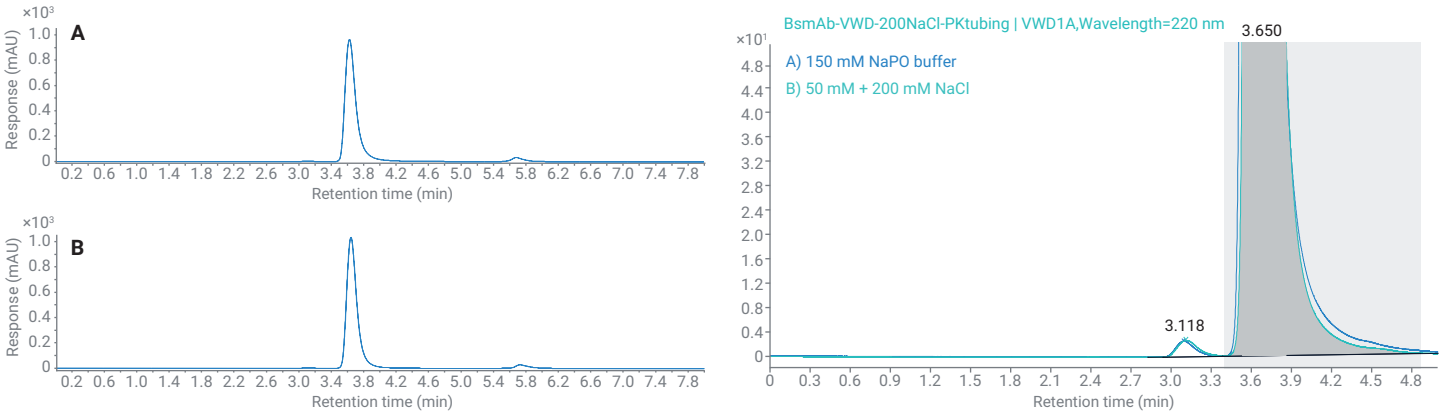

Figure 5. SEC chromatograms of Emicizumab with mobile phases: (A) 150 mM phosphate buffer at pH 6.8; (B) 50 mM phosphate buffer + 200 mM NaCl at pH 6.8.

Table 3. SEC monomer peak symmetry, peak width at half height and percentage of aggregation using mobile phases: 150 mM phosphate buffer at pH 6.8 and 50 mM phosphate buffer with 200 mM NaCl at pH 6.8.

	Width 50%	Asymmetry (As)	Monomer %	Aggregate %
150 mM Phosphate, pH 6.8	0.137	1.45	99.7	0.25%
50 mM Phosphate + 200 mM NaCl, pH 6.8	0.132	1.35	99.7	0.30%

Table 4. LC parameters for SEC analysis.

Parameter	Setting				
Column Temperature	25 °C				
Mobile Phase A) Water B) 1 M NaCl C) 245 mM NaH_2PO_4 D) 420 mM Na_2HPO_4	Eluent: NaPO, pH 7.0				
		A	B	C	D
	150 mM	55.3	0.0	21.5	23.3
	50 mM + 200 mM NaCl	65.5	20.0	6.2	8.3
Flow Rate	0.35 mL/min				
Injection Volume	3 µL				
Detection	UV at 280 nm				

Charge variants analysis by ion exchange chromatography

Cation-exchange chromatography was used to characterize charge variants. When running separations with salt gradients, Buffer Advisor software is very helpful in streamlining the mobile phase preparation and screening process and defining salt gradients. The charge variant profile is shown in Figure 6 under optimized mobile phase and gradient conditions. Peaks eluted before the main peak are considered to be acidic variants, and peaks eluted after the main peaks correspond to basic variants. The integrations of the peak areas of these species and the LC conditions are shown in Tables 5 and 6. As shown, the sample contained more acidic variants than basic variants. The charge heterogeneity could indicate different PTMs including glycosylation and loss of C-terminal lysine among others, which alter the surface charge of the protein. These modifications need to be monitored and quantified throughout drug manufacturing.

Table 5. Area percentage of acidic variants, main peak, and basic variants by IEX.

	Area%
Acidic Variants	13.6
Main Peak	84.9
Basic Variants	1.5

Table 6. LC parameters for IEX analysis.

Parameter	Setting
Column Temperature	45 °C
Mobile Phase A) Water B) 1.5 M NaCl C) 20 mM NaH_2PO_4 D) 20 mM Na_2HPO_4	Gradient: 80 to 140 mM NaCl, 10 mM NaPO, pH 6.0 Time (min) A B C D 0 44.7 5.3 43.5 6.5 40 40.0 10.0 42.1 7.9 41 16.7 33.3 37.1 12.9
Flow Rate	0.8 mL/min
Injection Volume	3 µL
Detection	UV at 280 nm

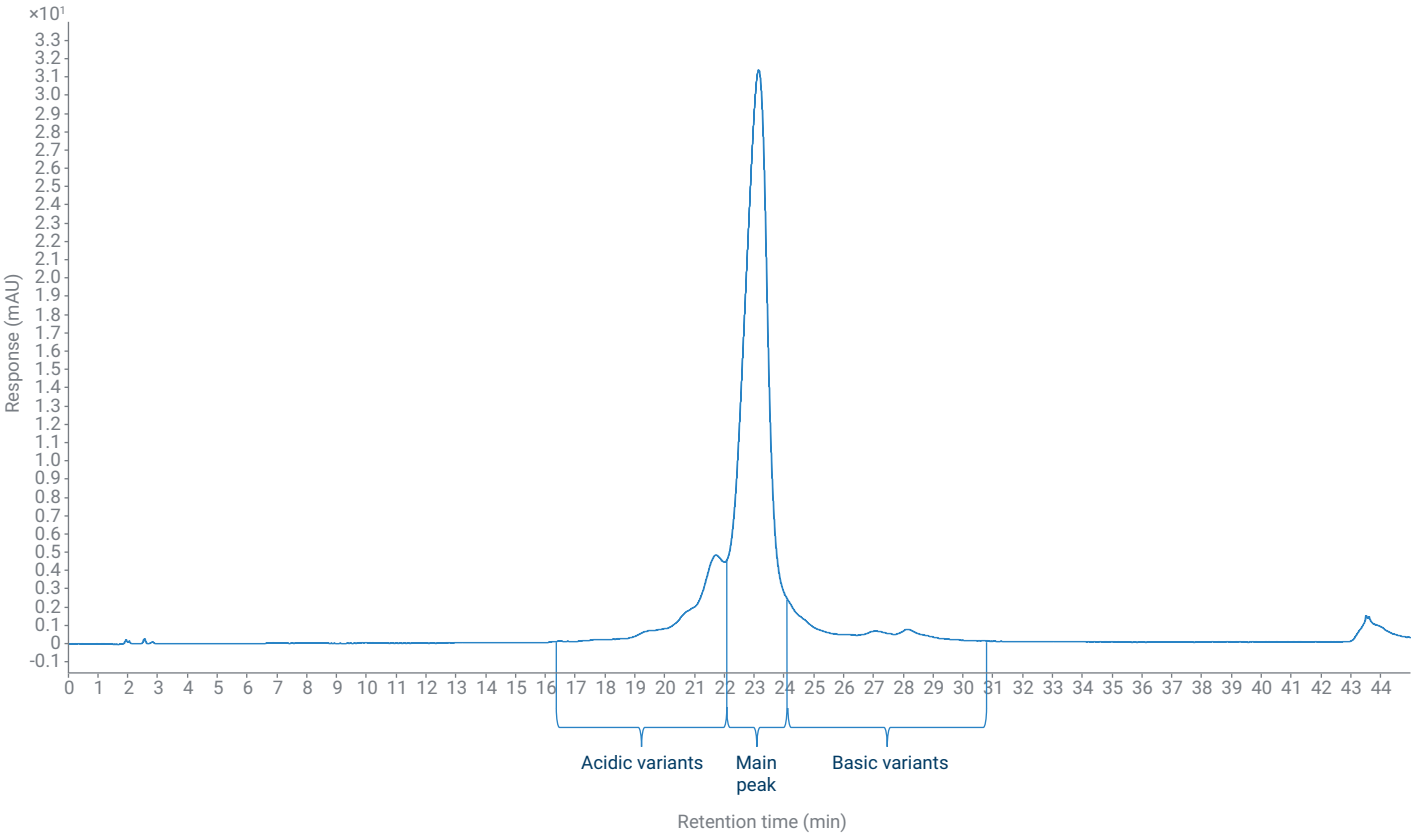

Figure 6. IEX chromatogram of Emicizumab showing the presence of the acidic and basic variants.

Intact analysis by hydrophobic interaction chromatography

Besides its use in analyzing antibody-drug-conjugates, HIC has also become a useful method to characterize mAb variants resulting from PTMs, with enhanced resolution compared to some other techniques. The AdvanceBio HIC columns with finely tuned hydrophobicity are suitable for a wide range of modalities including IgG like Bs-MAbs. As shown in Figure 7, some variants were eluted before the main peak, which could be possibly attributed to PTMs such as oxidation (further experiments are needed for confirmation), and the variants eluted after the main peak could correspond to some more hydrophobic aggregates. Other PTMs including deamidation and isomerization could also be separated with HIC. LC conditions are listed in Table 7.

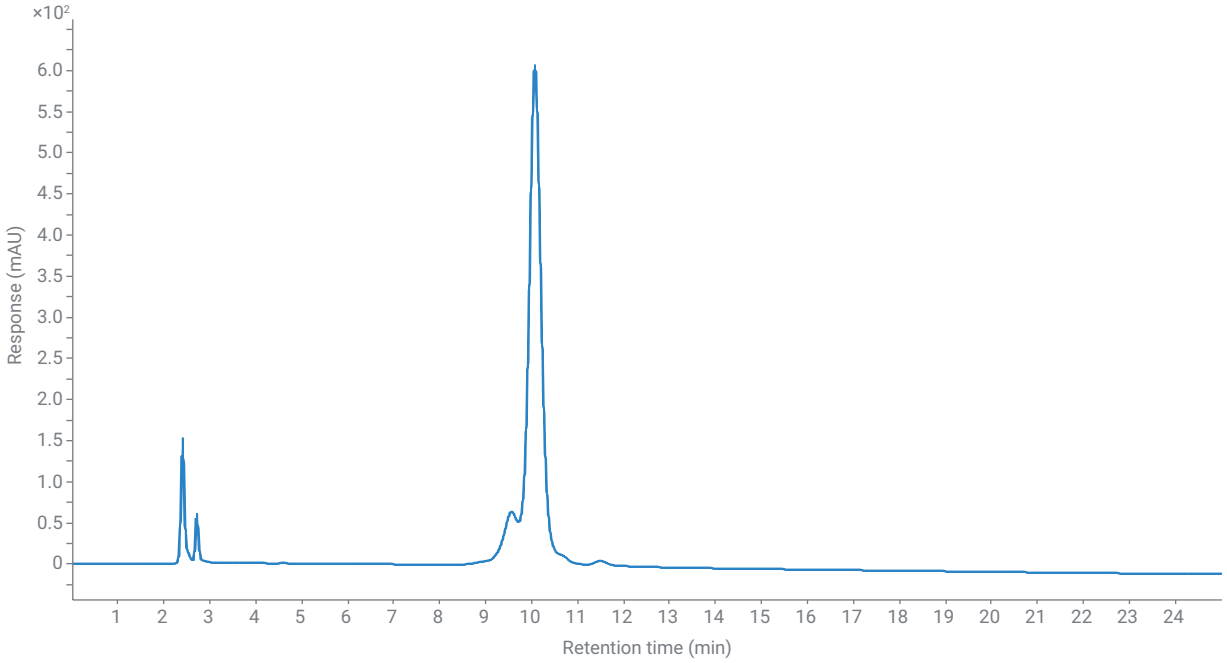

Figure 7. HIC chromatogram of Emicizumab.

Table 7. LC parameters for HIC analysis.

Parameter	Setting
Column Temperature	25 °C
Mobile Phase A) 50 mM NaPO + 2 M $(NH_4)_2SO_4$, pH: 7.0 B) 50 mM NaPO pH: 7.0	Gradient: Time (min) B% 0 50 20 100 25 100 26 50 40 (stop time)
Flow Rate	0.5 mL/min
Injection Volume	5 µL
Detection	UV at 280 nm

Conclusion

This study demonstrates that different chromatographic techniques can be used to overcome the drawbacks associated with the analysis of difficult molecules such as bispecific antibodies. RPC was used to confirm the correct structure with both intact mass and verification of identical light chains and nonidentical heavy chains through fragment analysis of the reduced molecule. SEC, IEX, and HIC were also successfully used to determine size variants, charge variants, and PTMs, respectively, supporting the typical CQAs of such complex molecules.

References

1. Fan, G. *et al.* Bispecific Antibodies and Their Applications *J. Hematol. Oncol.* **2015**, *8*, 130.
2. Scott, L. J. *et al.* Emicizumab-Kxwh: First Global Approval. *Drugs* **2018**, *78*, 269–274.

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