Application Note

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Drug to Antibody Ratio Analysis of Brentuximab Vedotin in Monkey Plasma

Antibody drug conjugate analysis under native conditions using Agilent AdvanceBio 6545XT LC/Q-TOF systems

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Abstract

A complete workflow was developed to analyze the drug-to-antibody ratio (DAR) of brentuximab vedotin in monkey plasma under native conditions. This workflow used the Agilent AssayMAP Bravo automation platform to purify the drug molecule from biological matrix, which was then analyzed by an Agilent 1290 Infinity II Bio LC system and an Agilent AdvanceBio 6545XT LC/Q-TOF system to determine DAR. Understanding the stability of the antibody drug conjugate (ADC) over time is crucial for this type of drug's design and its clinical outcome. As a result, these data highlight the complete workflow to enable ADC research and development.

Introduction

By the end of 2022, 14 antibody drug conjugates were approved by the US Food and Drug Administration to treat a variety of cancers, and more than 100 ADC candidates have been investigated in clinical stages at present. The market trend has indicated that the ADC therapeutics market is set to grow from $6.5 billion in 2023 to $26.6 billion by 2032.[1] Unlike traditional drugs, ADCs are typically composed of three parts, a monoclonal antibody (mAb), a cytotoxic drug (payload), and a chemical linker that links the mAb and toxic drug. The traditional analytical approach of these large molecules is by use of a ligand binding assay (LBA) due to its sensitivity, high throughput, low cost, and ease-of-automation, but LBA cannot determine free payload concentration and DAR. Over the past two decades, liquid chromatography/mass spectrometry (LC/MS) has become an alternative method for analyzing these large molecules due to its high specificity, sensitivity, wide dynamic range, and fast method development. At the same time, LC/MS can avoid cross-reactivity, improve productivity, and reduce costs and delays related to reagent/antigen availability.[2]

Brentuximab vedotin, sold under the brand name Adcetris, is a cysteine conjugated ADC used to treat relapsed or refractory Hodgkin's lymphoma and systemic anaplastic cell lymphoma.[3,4] Traditionally, the plasma concentration of brentuximab was determined by LBA, but LBA cannot determine the average number of drug molecules conjugated to an antibody. In this application note, a hybrid LBA/LC/MS workflow is demonstrated, which combines LBA and LC/MS technologies to determine DAR in monkey plasma under native conditions using an AssayMAP Bravo, a 1290 Infinity II Bio LC, and an AdvanceBio 6545XT LC/Q-TOF system (Figure 1). The results show that this hybrid LBA/LC/MS workflow can be used for DAR analysis of ADCs without the need for a specific antibody, while providing enough sensitivity, high specificity, and fast method development. These factors will play an important role in drug discovery and development.

Figure 1. Agilent AssayMAP Bravo, Agilent 1290 Infinity II Bio LC, and Agilent AdvanceBio 6545XT LC/Q-TOF.

Experimental

Materials and methods

Formulated Adcetris (brentuximab vedotin) was obtained from Evidentic GmbH (Berlin, Germany). Goat Anti-Human IgG, Monkey ads-BIOT was obtained from SouthernBiotech (Birmingham, AL). Formic acid (FA), bovine serum albumin (BSA), and PBS buffer were purchased from Sigma-Aldrich (St. Louis, MO). LoBind plates (96-well) were purchased from Eppendorf USA (Hauppauge, NY), monkey plasma was purchased from BioIVT (Westbury, NY), and AssayMAP cartridges were obtained from Agilent Technologies (Santa Clara, CA).

Instrumentation

- Agilent AssayMAP Bravo Automation Platform (G5571AA)
- Agilent 1290 Infinity II Bio LC system including:
  - Agilent 1290 Infinity II Bio High Speed Pump (G7132A)
  - Agilent 1290 Infinity II Bio Multisampler (G7137A)
  - Agilent 1290 Infinity II thermostat column compartment (G7116A) equipped with a Standard Flow Quick Connect Bio Heat-Exchanger (G7116-60071)
- Agilent AdvanceBio 6545XT LC/Q-TOF system (G6549AA)

Software

- Agilent Protein Sample Prep Workbench 3.2
- Agilent MassHunter Acquisition software
- Agilent MassHunter BioConfirm software

Sample preparation

Stability sample preparation: Brentuximab was spiked into monkey plasma at 100 µg/mL and incubated at 37 °C for 0, 1, 3, 4, and 7 days. Stability samples were stored at –80 °C for future analysis.

Immunoaffinity purification of brentuximab: All steps were performed by the AssayMAP Bravo automation platform as shown in Figure 2 and Figure 3. Prior to immobilization, the 5 µL streptavidin cartridges were conditioned using 1% formic acid as both the priming buffer and equilibration buffer (Figure 2). Briefly, biotinylated antihuman Fc antibody was immobilized onto the conditioned streptavidin cartridges at 5 µL/min, then the cartridges were washed once with PBS +1 M NaCl buffer and once with PBS buffer (Figure 3).

Next, 100 µL of monkey plasma samples fortified with different concentrations of brentuximab were loaded onto the streptavidin cartridges at 5 µL/min, then washed once with PBS +1 M NaCl buffer and once with water. The final release step was carried out by eluting with 20 µL of 0.25% formic acid buffer in 10% acetonitrile at 5 µL/min and neutralized with 10 µL of 1 M ammonium bicarbonate buffer (Figure 4).

LC/MS analysis: Data acquisition was performed using a 1290 Infinity II Bio LC coupled to an AdvanceBio 6545XT LC/Q-TOF system with an Agilent Jet Stream source. Separation was obtained with an Agilent Bio SEC-3 size exclusion column (4.6 × 150 mm, 300 Å, 3 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization was used for brentuximab DAR analysis.

Figure 2. AssayMAP Immobilization application for conditioning SAW cartridges.

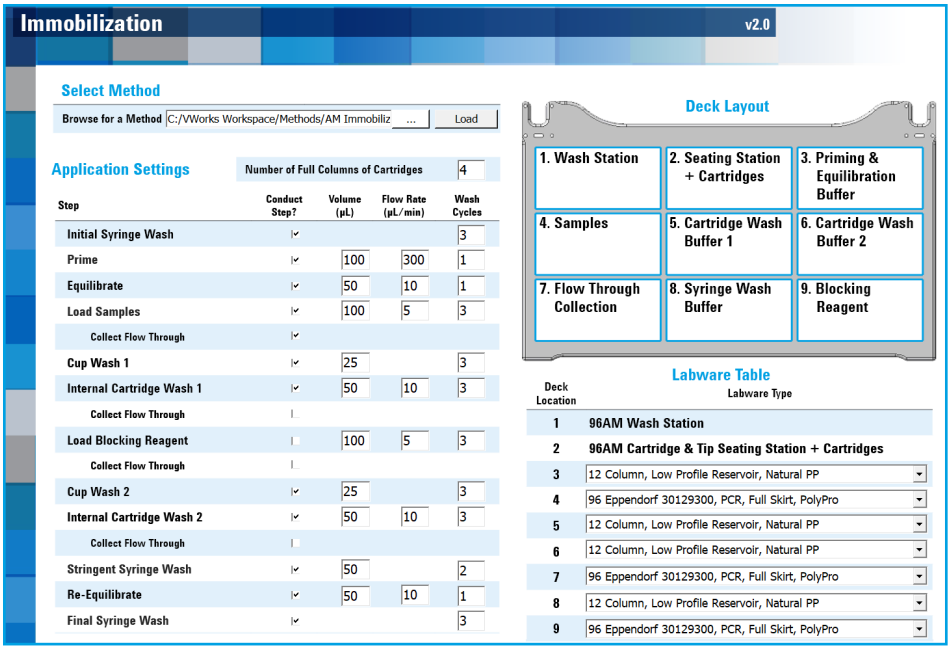

Figure 3. AssayMAP Immobilization application for capturing biotinylated antihuman Fc antibody.

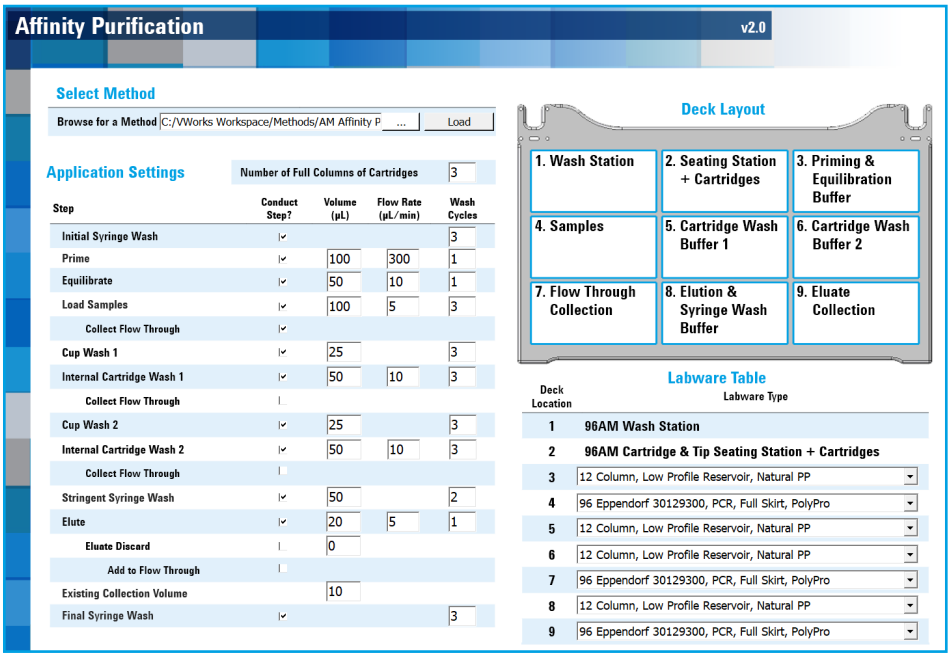

Figure 4. AssayMAP Affinity Purification application for intact ADC release.

Table 1. Liquid chromatography parameters.

LC Conditions	
Parameter	**Value**
Column	Agilent Bio SEC-3, 4.6 × 150 mm, 3 µm (p/n 5190-2514)
Column Temperature	25 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50:50 water:methanol)
Mobile Phase	A) 100 mM ammonium acetate buffer (pH 7)
Flow Rate	0.4 mL/min
Gradient Program	Isocratic
Stop Time	6.0 min

Table 2. MS acquisition parameters.

MS Conditions	
Parameter	**Value**
Ion Mode	Positive
Gas Temperature	365 °C
Drying Gas Flow	12 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary/Nozzle Voltage	5,500/2,000 V
Fragmentor/Skimmer	380/220 V

Data processing

All MS data were processed using MassHunter BioConfirm software.

Results and discussion

Method optimization for brentuximab DAR analysis

To improve the sensitivity and reproducibility of the ADC DAR analysis, sample preparation, LC, and MS conditions were all optimized, including AssayMAP application optimization, LC column selection, buffer selection, pH conditions, and mass spectrometry source conditions. The optimized LC and MS source parameters are listed in Tables 1 and 2.

DAR analysis of brentuximab stability in monkey plasma

The hybrid LBA/LC/MS workflow combines the advantages of two technologies for the analysis of large molecules in biological matrix, which decreased sample complexity and achieved excellent assay sensitivity. Figure 5 shows the raw spectrum of intact brentuximab under native conditions, in which the center of the ADC charge envelope was at 5,804 *m/z*. The intact brentuximab native raw spectrum is different from the denatured condition's raw spectrum because the ADCs are not unfolded and still in their native state. So, under native conditions, ADCs carry fewer charges compared to denatured conditions in which the ADC is unfolded, exposing more charged amino acids. Figure 6 shows the deconvoluted neutral spectrum of brentuximab; the DAR 2 species is the most abundant, followed by DAR 4 and DAR 0; the average DAR value is 2.53.

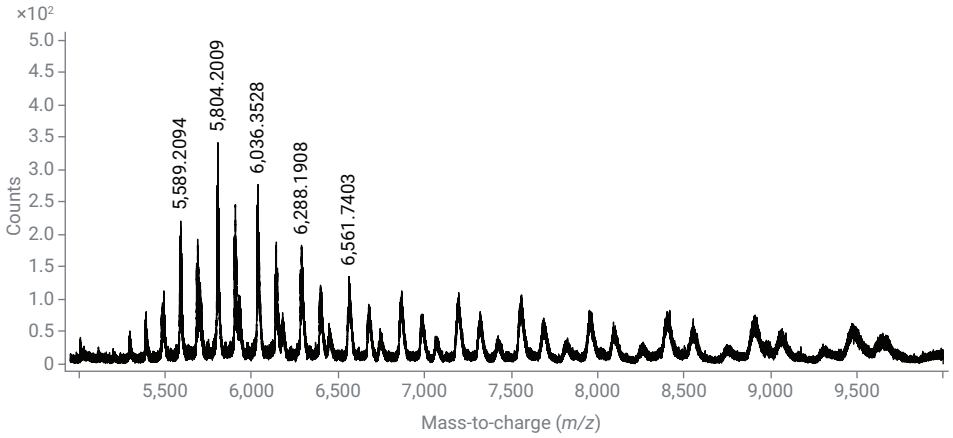

Figure 5. Brentuximab raw spectrum under native conditions.

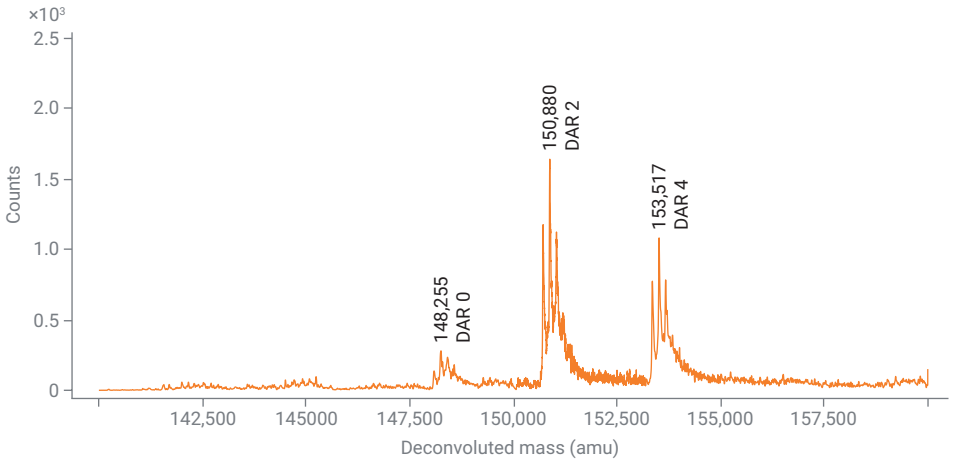

Figure 6. Brentuximab deconvoluted spectrum under native conditions.

MassHunter BioConfirm software was used to perform DAR data analysis. The stability samples were analyzed in duplicate, as shown in Figure 7 and Table 4. The average DAR value of brentuximab decreased over time; the average DAR dropped approximately 30% from day 0 to day 1, and the average DAR dropped another 25% from day 1 to day 3. The average DAR was then stable from day 3 to day 7. Over the seven-day incubation, the DAR 1 and DAR 3 species showed up in the samples, indicating that payloads were released from ADC during incubation. This result is in agreement with the payload release assay data (Agilent application note 5994-6802EN), demonstrating a good relationship between two different methodologies for ADC analysis.

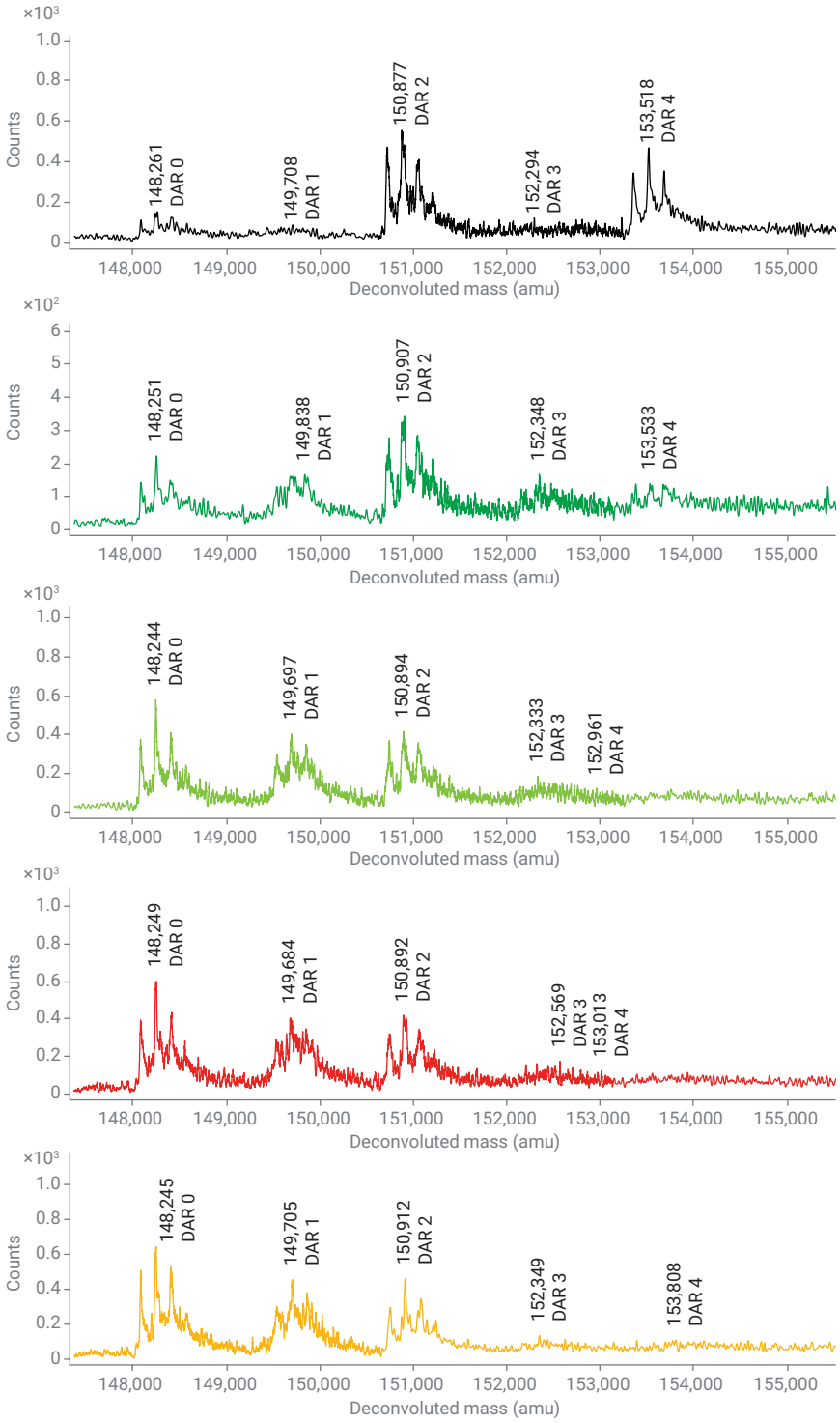

Figure 7. DAR analysis of stability samples.

Table 4. Brentuximab stability samples DAR results summary.

	Day 0	Day 1	Day 3	Day 4	Day 7
Average DAR	2.49 ± 0.01	1.8 ± 0.08	1.3 ± 0.04	1.31 ± 0.01	1.23 ± 0.04

Conclusion

DAR analysis is a critical aspect of ADCs because different DAR species contribute to very different pharmacokinetics and toxicological properties, which will directly affect that efficacy and safety of ADCs. The Agilent 1290 Infinity II Bio LC and Agilent AdvanceBio 6545XT LC/Q-TOF system are ideal platforms for intact ADC DAR analysis under native conditions. This automated hybrid LBA/LC/MS workflow combines the advantages of automation, LBA, and LC/MS technologies to provide adequate assay sensitivity and reproducibility for ADC DAR analysis in biological matrix under native conditions. This workflow requires minimum method development time, and can be applied to many other large molecules, such as monoclonal antibodies, chimeric antibodies, and humanized antibodies, which will greatly support ADC drug discovery and development.

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