Application Note

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Assessing Protein and Payload Stability of Antibody Drug Conjugate Brentuximab Vedotin in Monkey Plasma

Using Agilent AssayMAP Bravo and Agilent 6495 Triple Quadrupole LC/MS Systems

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Abstract

A complete workflow to quantitatively determine brentuximab vedotin's protein and payload stability in monkey plasma was developed. This workflow uses the Agilent AssayMAP Bravo automated liquid handling platform to purify drug molecules from a biological matrix. This was then analyzed with the Agilent 1290 Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems to determine protein and payload concentration. Understanding the stability of different parts of the drug molecules over time is crucial for drug design and its clinical outcome. As a result, these data highlight the complete quantification workflow to support antibody drug conjugate research and development.

Introduction

As of the end of 2022, 14 antibody drug conjugates (ADC) have been approved by the US Food and Drug Administration to treat a variety of cancers, and over 100 ADC candidates have been investigated in various 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, ADC is typically composed of three parts, a monoclonal antibody (mAb), a cytotoxic drug (payload), and a chemical linker, which links the mAb and toxic drug. The traditional analytical approach to monitor these large molecules is via the ligand binding assay (LBA), due to its sensitivity, high throughput, low cost, and ease-of-automation. However, LBA cannot determine payload concentration and/or if payloads are still attached to the mAbs. Over the past two decades, liquid chromatography/mass spectrometry (LC/MS) has become an alternative method for analyzing these large molecules due to their 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 delay related to reagent/antigen availability.[2]

Brentuximab vedotin, sold under the brand name Adcetris, is an ADC used to treat relapsed or refractory Hodgkin lymphoma and systemic anaplastic cell lymphoma.[3,4] Traditionally, the plasma concentration of brentuximab was determined by the ligand binding assay. In this application note, a hybrid LBA/LC/MS workflow is demonstrated, which combines LBA and LC/MS technologies to quantify the mAb and payload in monkey plasma; the method uses an Agilent AssayMAP Bravo, Agilent 1290 Infinity II Bio LC, and an Agilent 6495 triple quadrupole system (Figure 1). The results of this study show that this hybrid LBA/LC/MS workflow can be used for quantitative analysis of ADC without the need for a specific antibody, while providing excellent sensitivity, high specificity, and fast method development. These factors will play an important role in drug discovery and development.

Figure 1. Agilent AssayMAP Bravo automated liquid handling platform, Agilent 1290 Infinity II Bio LC, and Agilent 6495C triple quadrupole LC/MS.

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), PBS buffer, and stable isotope-labeled trastuzumab were purchased from MilliporeSigma (St. Louis, MO). Sequencing-grade trypsin was purchased from Promega (Madison, WI). LoBind plates (96-well) were purchased from Eppendorf USA (Hauppauge, NY). Monkey plasma was purchased from BioIVT (Westbury, NY), and Agilent AssayMAP cartridges were obtained from Agilent Technologies (Santa Clara, CA).

Instrumentation

- Agilent AssayMAP Bravo Protein and Peptide Sample Prep System automation system (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 Heat-Exchanger (G7116-60071)
- Agilent 6495 triple quadrupole system (G6495CA)

Software

- Agilent AssayMAP Bravo Protein Workbench
- Agilent MassHunter Acquisition software
- Agilent MassHunter Quantitative Analysis software

Sample preparation

Stability sample preparation: Brentuximab was spiked into monkey plasma at 50 µg/mL and incubated at 37 °C for 0, 1, 3, 4, and 7 days. The stability samples were also stored at –80 °C for future analysis.

Immunoaffinity purification of brentuximab: All steps were performed by the AssayMAP Bravo automation platform as shown in Figures 2 and 3.[5] Prior to beginning the application, streptavidin cartridges were conditioned using 1% formic acid. First, biotinylated antihuman Fc antibody was immobilized onto streptavidin cartridges at 5 µL/min. The cartridges were then washed once with PBS + 1 M NaCl, and once with PBS buffer (Figure 2). Next, 100 µL of sample, consisting of 30 µL of monkey plasma fortified with different concentrations of brentuximab and 1 µg/mL stable isotope-labeled trastuzumab in 80 µL PBS, was loaded onto the cartridge at 5 µL/min, then washed once with PBS + 1 M NaCl buffer, and once with distilled 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 neutralizing with 10 µL of 1 M ammonium bicarbonate buffer (Figure 3).

Trypsin digestion of brentuximab: Using in-solution digestion, a single-plate application on the AssayMAP Protein Workbench, dithiothreitol solution was then added to each sample at 10 mM final concentration and incubated at 60 °C for 1 hour. After cooling down, iodoacetamide was added to each sample at 10 mM final concentration and incubated at room temperature in darkness for 30 minutes. Lastly, 0.5 µg of trypsin was added to each sample and incubated at 37 °C overnight with shaking offline. Digestion was stopped by adding 10 µL 1% formic acid solution. Then, 20 µL were injected into LC/MS for peptide analysis.

Payload release of brentuximab: All steps were performed using the AssayMAP Bravo automation platform, as shown in Figures 4 and 5. Briefly, the 5 µL Protein A cartridges were blocked by 100 µL of 1% bovine serum albumin in PBS buffer. Next, 100 µL of sample (30 µL of monkey plasma diluted with 80 µL of PBS), were loaded onto the cartridge at 5 µL/min, then washed with 50 µL of 20 mM ammonium acetate buffer. Payload release was also carried out on the AssayMAP Bravo using the On-Cartridge Reaction Application as shown in Figure 6. Cartridges were conditioned with 50 µL of L-cysteine buffer (12.1 mg of L-cysteine in 50 mL of 20 mM ammonium acetate), then the payload was released by incubating the cartridge with 50 µL of papain buffer (100 mg of papain in 50 mL of L-cysteine buffer) at 33 °C for 30 minutes. Papain buffer was eluted into the collection plate with 15 ng/mL internal standard (MMAE-d_8). Lastly, cartridges were washed with pure acetonitrile, and the wash solution was combined with the papain buffer reaction products. 500 µL of acetonitrile was added to the final elution buffer, which was vortexed briefly and spun down at 4,200 rpm for 3 minutes. Finally, 100 µL of supernatant was transferred to a new LoBind plate and combined with 300 µL of water with 0.1% FA. For payload analysis, 20 µL were injected into the LC/MS.

LC/MS analysis

Data acquisition was performed using a 1290 Infinity II Bio LC coupled to a 6495 triple quadrupole system with a Jet Stream source. Separation was obtained with an Agilent Poroshell 120 EC-C18 column (2.1 × 50 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow. Positive electrospray ionization was used for brentuximab surrogate peptides and payload analysis. MRM transitions of peptides and payload are listed in Table 3 with optimal collision energy. Peptide VVSVLTVLHQDWLNGK is a conserved peptide from human IgG, which is shared between different human IgG isoforms. The stable-labeled trastuzumab will carry the same conserved peptide after trypsin digestion, with lysine having been stable isotope-labeled. In this case, the peptide from trastuzumab will be 8 Da larger than the native peptide digested from brentuximab with the exact amino acid sequence.

Table 1. Liquid chromatography parameters.

LC Conditions	
Column	Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm
Column Temperature	60 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Needle Wash	3 seconds in wash port (50:50 water:methanol)
Mobile Phase	A) Water +0.1% formic acid B) Acetonitrile +0.1% formic acid
Flow Rate	0.4 mL/min
Gradient Program	Surrogate peptide Time %B 0 5 1.0 5 4.0 50 4.1 90 5.0 90 5.1 5 Payload Time %B 0 20 1.0 20 1.1 70 3.0 80 3.1 90 4.0 90 4.1 20 5.0 20
Stop Time	6.0 min

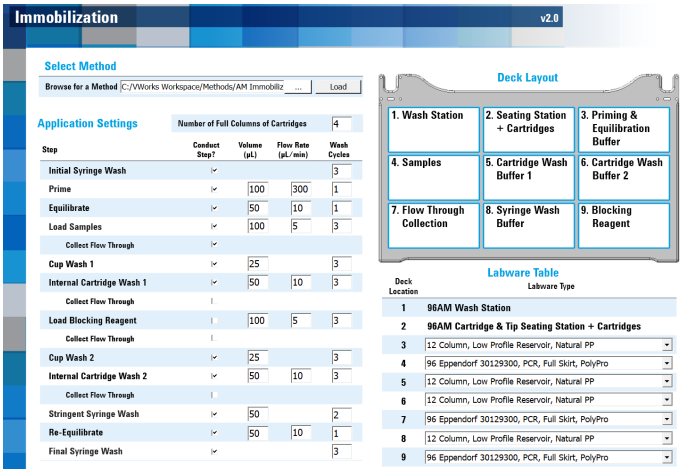

Figure 2. Agilent AssayMAP Immobilization Application settings.

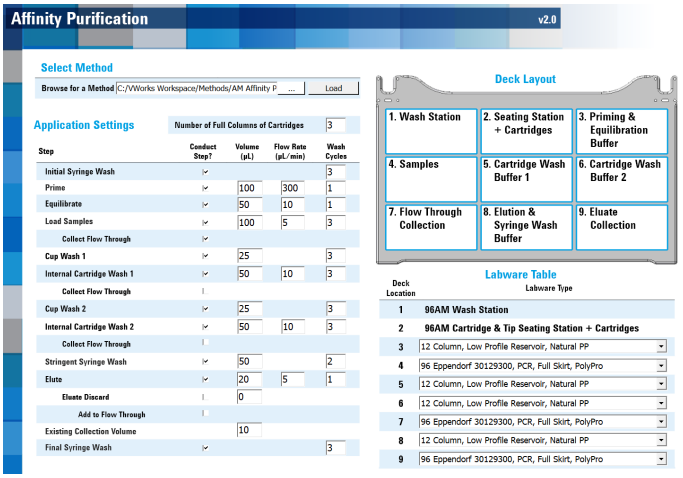

Figure 3. Agilent AssayMAP Affinity Purification Application settings.

Table 2. MS acquisition parameters.

MS Conditions	Surrogate Peptide	Payload
Ion Mode	Positive	Positive
Gas Temperature	250 °C	270 °C
Drying Gas Flow	17 L/min	15 L/min
Nebulizer Gas	40 psi	20 psi
Sheath Gas Temperature	280 °C	380 °C
Sheath Gas Flow	12 L/min	12 L/min
Capillary Voltage	4,000 V	3,500 V
Ion Funnel	200/110 V	200/100 V

Table 3. Surrogate peptides and payload MRM transitions.

Peptide	Precursor Ion	Product Ion	Collision Energy
VVSVLTVLHQDWLNGK	603.3	805.4	20
VVSVLTVLHQDWLNGK	603.3	712.4	20
VVSVLTVLHQDWLNGK	603.3	655.8	20
VVSVLTVLHQDWLNGK*	606.0	808.4	20
VVSVLTVLHQDWLNGK*	606.0	716.4	20
VVSVLTVLHQDWLNGK*	606.0	659.8	20
MMAE	718.6	152.2	46
MMAE-d_8	726.6	152.2	46

* Indicates stable isotope label

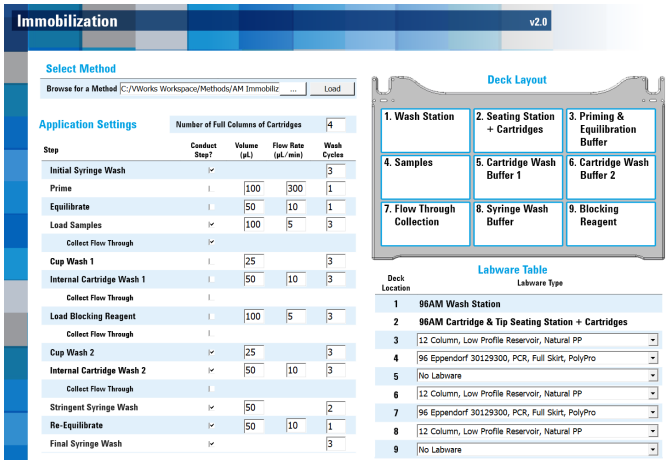

Figure 4. Payload Release Assay Protein A Cartridge Blocking step using the Immobilization Application settings.

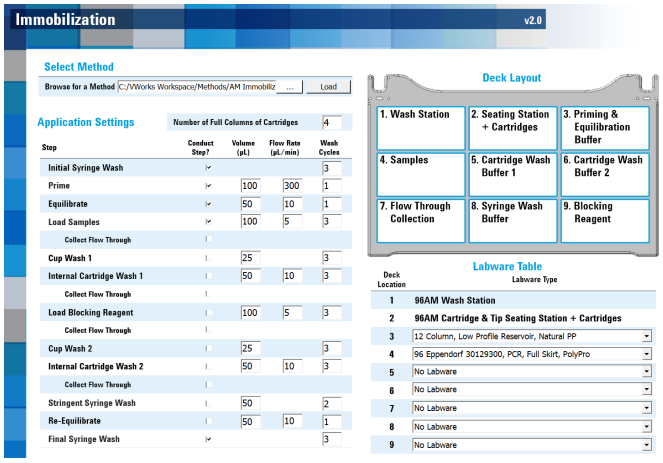

Figure 5. Payload Release Assay Sample Loading step using the Immobilization Application settings.

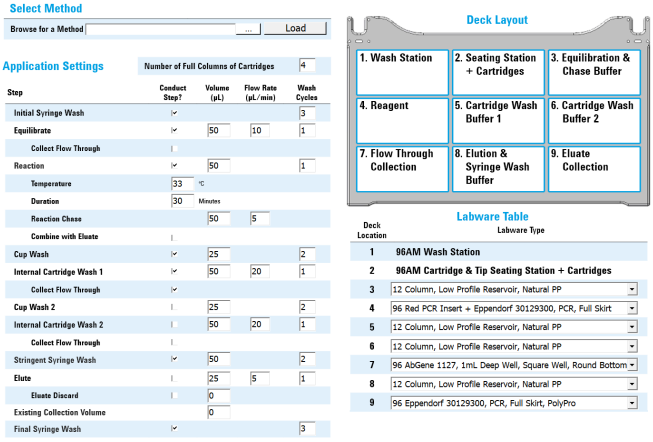

Figure 6. Payload Release Assay Using the On-Cartridge Reaction Application Settings for the papain reaction.

Data processing

All MS data were processed using MassHunter Quantitative Analysis software.

Results and discussion

Method optimization for surrogate peptide quantitative analysis

To improve the sensitivity and reproducibility for peptide and payload quantitative analysis, sample preparation, LC, and MS conditions were all optimized. Peptide and payload MRM transitions and collision energy were also optimized to achieve the best MS sensitivity for peptide and payload quantitative analysis. The optimized source parameters are listed in Table 2.

Quantitative analysis of brentuximab surrogate peptide and payload in monkey plasma

The hybrid LBA/LC/MS workflow combined the advantages of two technologies for bioanalysis of large molecules in biological matrix, which decreased sample complexity and achieved great assay sensitivity. Figure 7 shows the MRM chromatograms for the surrogate peptides from brentuximab and labeled trastuzumab purified from monkey plasma at 0.39 µg/mL. Figure 8 shows the MRM chromatograms of payload and its stable isotope-labeled internal standard.

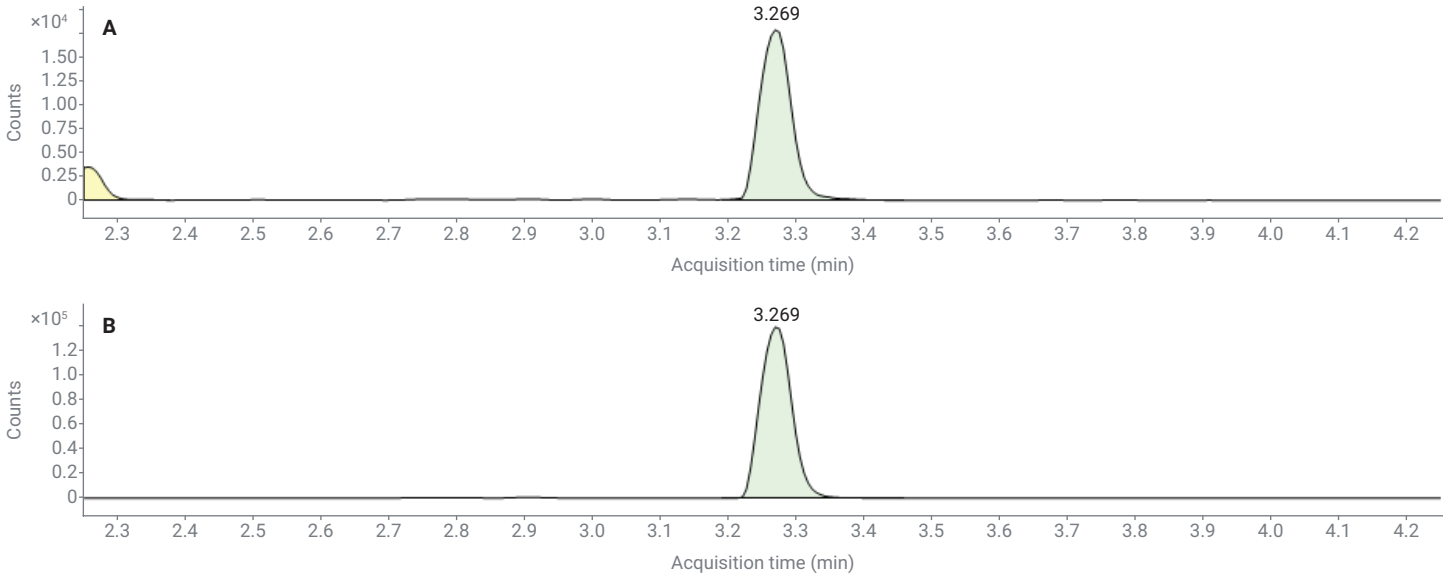

Figure 7. Brentuximab and trastuzumab surrogate peptides MRM chromatograms purified from monkey plasma. (A) VVSVLTVLHQDWLNGK quantifier (603.3 → 805.4) MRM chromatogram at 0.39 µg/mL; (B) VVSVLTVLHQDWLNGK* quantifier (606 → 808.4) MRM chromatogram.

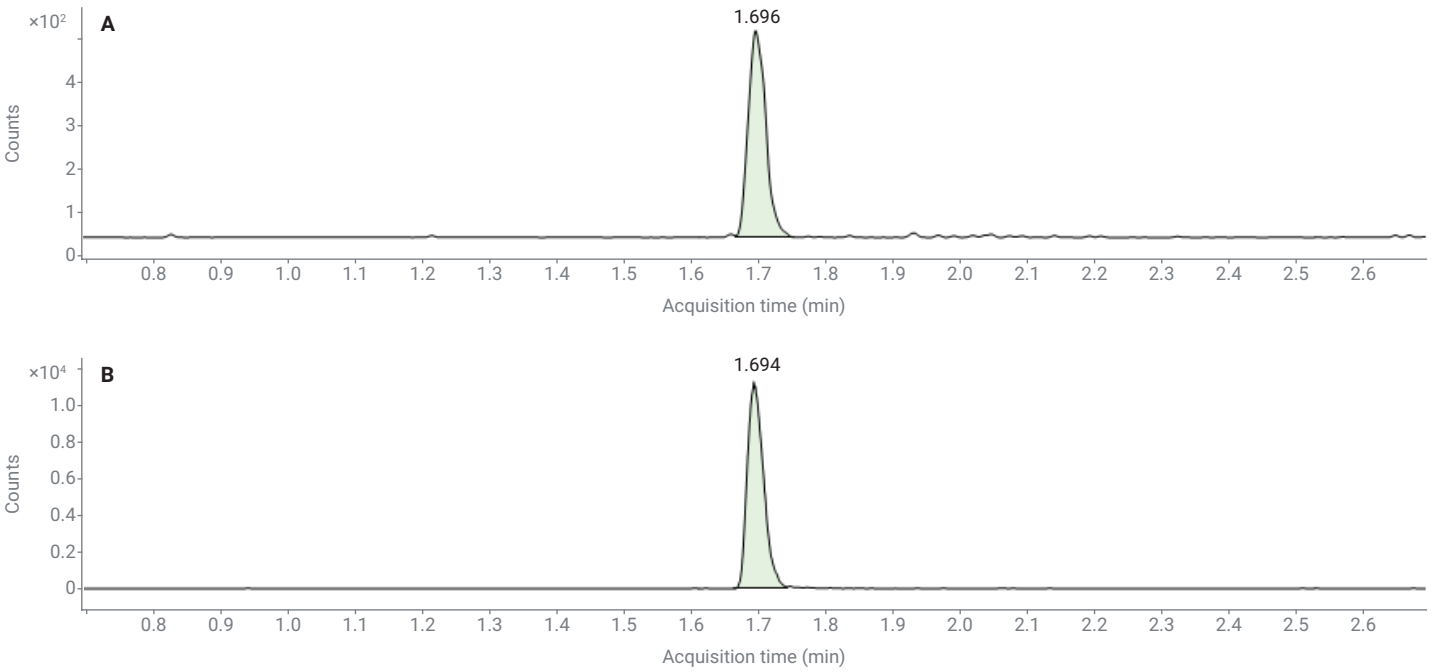

Figure 8. Brentuximab payload and internal standard MRM chromatograms purified from monkey plasma. (A) Payload quantifier (719.0 → 152.2) MRM chromatogram at 0.39 µg/mL; (B) payload internal standard quantifier (727.0 → 152.2) MRM chromatogram.

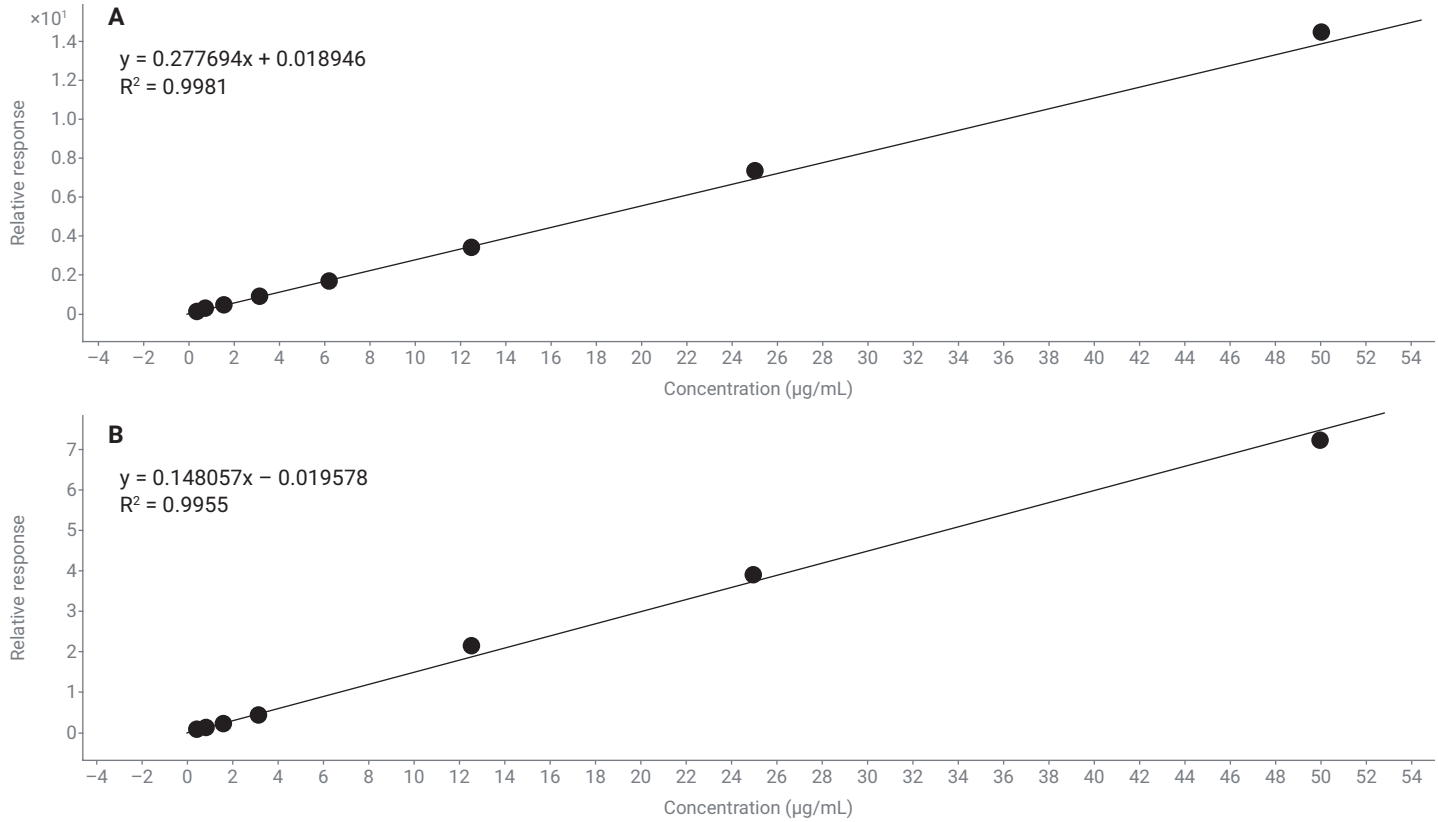

Figure 9. Calibration curve of brentuximab surrogate peptide and payload from 0.39 to 50 µg/mL in monkey plasma. (A) Surrogate peptide VVSVLTVLHQDWLNGK calibration curve; (B) payload calibration curve.

MassHunter Quantitative Analysis software was used to perform brentuximab surrogate peptide and payload quantitative analysis. As shown in Figure 9, both surrogate peptide and payload calibration curves are linear from 0.39 to 50 µg/mL with R^2 over 0.99, demonstrating excellent assay performance. The stability samples were analyzed in triplicate, as shown in Table 4. The surrogate peptide was very stable after 7 days, with values being close to the original concentration of 50 µg/mL. While the payload concentration decreased over time, concentration dropped approximately 40% from day 0 to day 1, and dropped another 25% from days 1 to 3. It was then stable from days 3 to 7.

Table 4. Brentuximab stability samples surrogate peptide and payload result summary.

	Day 0	Day 1	Day 3	Day 4	Day 7
Surrogate Peptide	46.7	49.4	53.4	55.8	56.2
%RSD	4.2	1.8	7.8	3.4	4.6
Payload	51.2	31.6	23.0	24.2	22.7
%RSD	7.8	2.9	6.9	4.0	3.6

Conclusion

The Agilent 1290 Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS system are ideal platforms for quantitative analysis. This automated hybrid LBA/LC/MS workflow combines the advantages of automation, LBA, and LC/MS technologies to provide excellent assay sensitivity and reproducibility for the quantitative analysis of ADC surrogate peptides and payload from biological matrix. Another advantage of this workflow is that the surrogate peptide is universal, meaning that it can be applied to many other human IgG-based mAb or ADC therapeutics. This workflow requires minimum method development time, which will greatly support drug discovery and development projects.

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