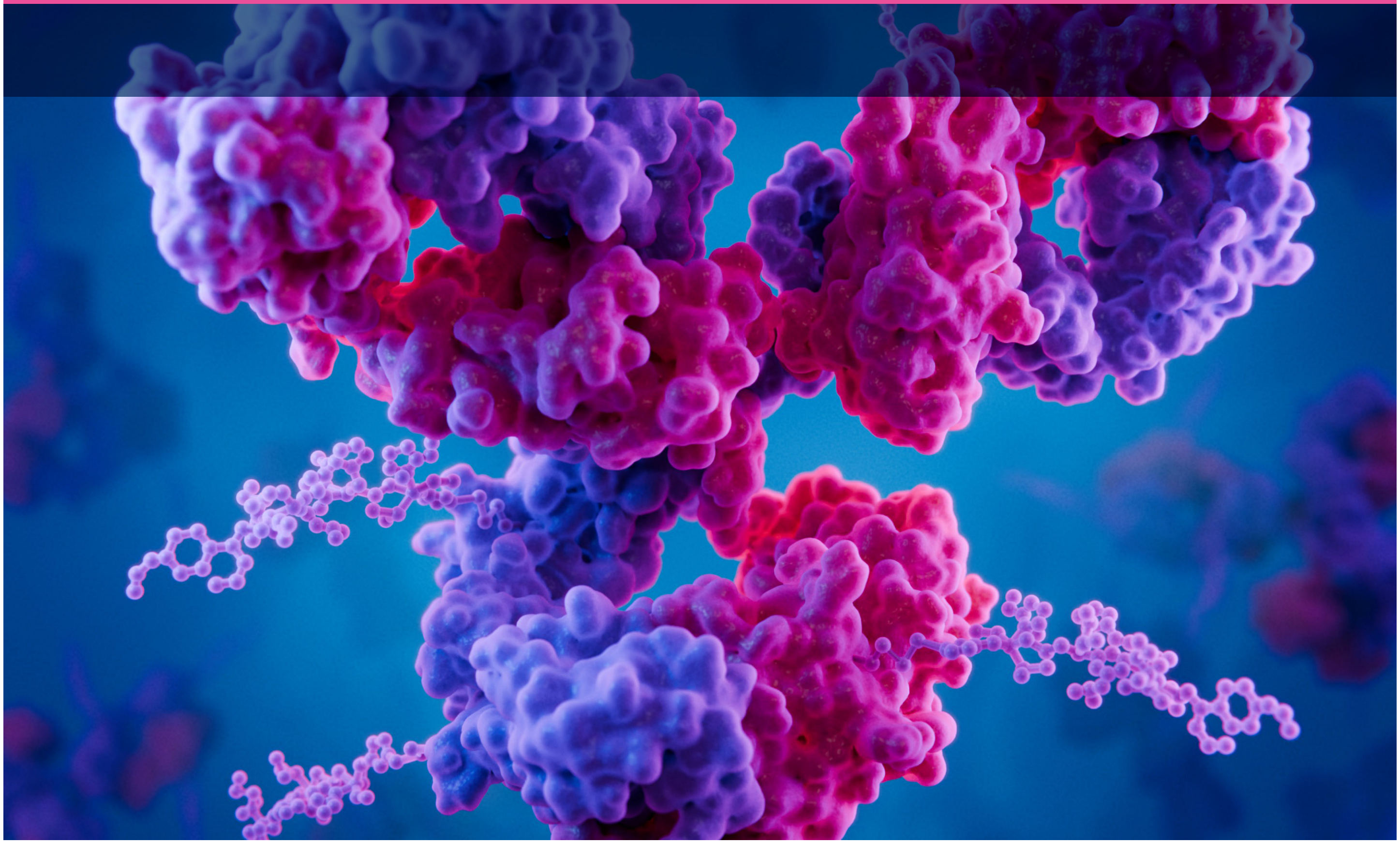
Agilent BioHPLC columns

Characterization of Antibody-Drug Conjugate Critical Quality Attributes

Application compendium



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Introduction

Background

Antibody-drug conjugates (ADCs) are powerful biologics that combine the high specificity of monoclonal antibodies (mAbs) with the potent cytotoxicity of small molecule drugs. In a traditional ADC, a small molecule drug is chemically linked to an mAb that selectively binds to target cells, such as cancer cells. This delivery system allows for a high local concentration of the drug at the target site, while minimizing exposure to healthy tissues.

Despite the elegance of this concept, ADCs are complex biomolecules that present signification analytical and manufacturing challenges. Developing an effective ADC requires:

- Engineering antibodies with affinity and specificity
- Selecting a potent drug and a suitable linker for stable conjugation
- Controlling the number of drug molecules attached to each antibody, known as the drug-to-antibody ratio (DAR)

In addition to the standard critical quality attributes (CQAs) typically assessed for biologics, ADC characterization introduces an additional key CQA, DAR. A low DAR may lead to low efficacy, while a high DAR can increase toxicity of the ADC and negatively impact pharmacokinetics. It is essential to have well-established analytical methods to fully characterize the biomolecule because ADCs are inherently heterogeneous, comprising mixtures with varying numbers of drug attachments.

Antibody-oligonucleotide conjugates (AOCs) along with oligonucleotide or peptide drug conjugates (ODCs or PDCs) represent the next evolution of traditional ADCs. AOCs replace the small molecule payload with an oligonucleotide such as an antisense oligonucleotide or siRNA. Oligonucleotides and peptides replace the mAb carrier for ODCs and PDCs, respectively, and can incorporate a wide range of molecule types as payloads. When choosing separation techniques for these next-generation conjugates, it is essential to consider the distinct chemical properties of both the carrier and the payload to ensure accurate characterization and performance.

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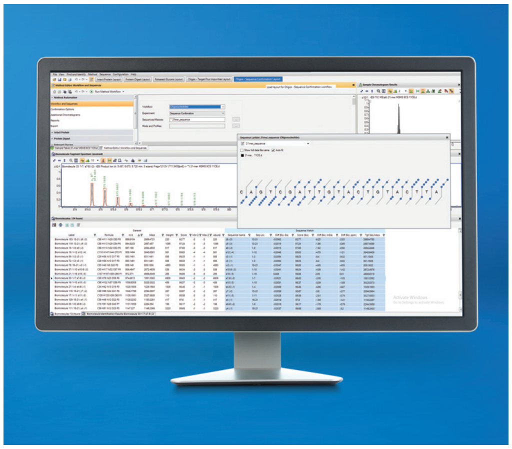
Getting started

Because the base of an ADC is an mAb, the same CQAs apply. Examples of these CQAs include purity analysis, intact/subunit analysis, aggregate/fragment analysis peptide mapping, charge variant analysis, and glycan analysis. Reversed-phase (RP) chromatography is typically used for purity analysis uses size exclusion chromatography. Charge variant and glycan analyses are typically conducted with ion exchange and hydrophilic interaction liquid chromatography, respectively.



As mentioned previously, the DAR is an attribute specific to antibody conjugate molecules. Because both the small molecule and linker are hydrophobic in an ADC, the available chromatographic techniques are RP chromatography coupled to mass spectrometry (MS) and hydrophobic interaction chromatography (HIC), which can be analyzed with UV. The RP-MS workflow favors the PLRP-S column as it has no silanols to cause secondary interactions or peak tailing. The MS data can be quite complex, even after deconvolution An integrated DAR calculator is available in Agilent MassHunter BioConfirm software, which calculates peak areas and height and automatically calculates the DAR value for the ADC. The BioConfirm report provides an easy electronic record where you can review, archive, share different analyses. Another common workflow to calculate DAR, particularly for Cys-linked ADCs, is HIC, detected by UV. The advantage of HIC is that the ADC stays in its native form and the DAR species are chromatographically separated. The column of choice for HIC is the Agilent AdvancedBio HIC column.

When performing peptide mapping for ADCs, an important additional attribue to assess is conjugate site mapping. This aspect helps determine where the drug and linker are attached to the mAb, which is crucial for understanding the ADC heterogeneity, stability, and efficacy. The recommended column for this workflow is the Agilent AdvanceBio Peptide Mapping column. By adding the linker and payload mass to the list of modifications, conjugate site mapping can be done with traditional peptide mapping in the same workflow.

This ADC compendium was designed to showcase complete end-to-end Agilent workflow solutions and to simplify method development for accelerating ADC innovation while ensuring analytical precision and efficiency.

Explore Agilent BioHPLC columns: www.agilent.com/columns/biohplc

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PLRP-S Polymeric Reversed-Phase Column for LC/MS Separation of mAbs and ADC

Analysis of Intact and Fragmented mAbs and ADC

Application Note

Biotherapeutics and Biologics

Author

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Abstract

This application note describes the application of polymeric-based reversed-phase columns for the characterization of large biomolecules such as monoclonal antibodies (mAbs) and antibody drug conjugate (ADC). Studies of both intact and fragment levels of mAbs were carried out to understand the performance of the polymeric column. The results demonstrate the superior separation performance of the column, and its suitability for routine LC/MS analysis of mAbs and ADC.

Introduction

Monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) are therapeutic molecules that are heterogeneous in nature [1]. These molecules can undergo various modifications during the drug development process requiring thorough characterization to ensure safety and efficacy. Reversed-phase liquid chromatography with mass spectrometry detection is most commonly used for primary characterization of mAbs and ADCs. The correct choice of LC column and method is critical to achieve reproducible high-resolution separations and high-quality MS data. Typically, using formic acid (FA), an MS-friendly ion-pairing agent, in the mobile phase leads to poor total ion chromatogram (TIC) peak shape with traditional silica-based reversed-phase columns. This affects LC/MS results (resolution, sensitivity, MS signal, accurate molecular weight information, and so forth). Hence, there is a critical requirement for an LC column compatible with formic acid conditions for enhanced LC/MS analysis of biomolecules.

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This study used a polymeric reversed-phase column (Agilent PLRP-S) to demonstrate the LC/MS analysis of mAbs and ADC. PLRP-S columns contain rigid, macroporous, spherical particles of polystyrene and divinylbenzene. They are physically and chemically stable across the complete pH range. The particles are inherently hydrophobic, therefore, neither bonded phases nor alkyl ligands are required for reversed-phase separations. This gives a highly reproducible material that is free from silanols and heavy metal ions. In this work, multiple therapeutic mAbs, including ADC, were analyzed using the PLRP-S column in an LC/MS method. This approach delivered better LC/MS results, and accurate mass determination of intact and fragmented mAbs and ADC.

Materials and methods

Samples

- Therapeutic mAb1, mAb2, and ADC (lysine-conjugated) were purchased from a local pharmacy, and stored according to the manufacturer's instructions.
- **Intact**: mAb1, mAb2, and ADC were diluted to a concentration of 2 µg/µL using 0.1% formic acid in 3% acetonitrile (ACN), and 1 µL was injected.
- **Reduction**: 20 µL of mAb (2 µg/µL) sample was mixed with 5 µL dithiothreitol (DTT) (1 M) followed by incubation at 37 °C for 1 hour.
- **Papain digestion**: 10 µL of mAb (2 µg/µL) sample was mixed with 5 µL digestion buffer (with cysteine), 5 µL of activated papain (Sigma). The mixture was incubated at 37 °C for 3 hours.
- **IdeS proteolytic digestion**: 20 µL of mAb (2 µg/µL) sample was mixed with 0.5 µL FabRICATOR (30 Units) (Sigma) and the mixture was incubated at 37 °C for 1 hour.

Instrumentation

- **LC**: Agilent 1290 Infinity LC system
- **MS**: Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) with an Agilent Jet Stream ion source

Conditions

Agilent 1290 Infinity LC system		
Column:	Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å (p/n PL 1912-1502)	
Injection volume:	1 µL	
Sample thermostat:	5 °C	
Mobile phase A:	0.1% FA in water	
Mobile phase B:	0.1% FA in ACN	
Gradient:	Intact	Fragments
	at 0 min → 20% B	at 0 min → 20% B
	at 4 min → 20% B	at 10 min → 50% B
	at 5 min → 40% B	at 10.1 min → 85% B
	at 10 min → 70% B	at 11 min → 85% B
	at 11 min → 90% B	at 11.1 min → 20% B
	at 11.1 min → 20% B	
Stop time:	11.1 min	
Post time:	4 min	
Column temperature:	80 °C	
Flow rate:	0.6 mL/min	

Agilent 6530 Accurate-Mass Q-TOF LC/MS	
Ion mode:	Positive ion mode, dual AJS ESI (profile)
Drying gas temperature:	350 °C
Drying gas flow:	8 L/min
Sheath gas temperature:	400 °C
Sheath gas flow:	11 L/min
Nebulizer:	35 psi
Capillary voltage:	5,500 V
Fragmentor voltage:	380 V
Skimmer voltage:	65 V
Oct RF Vpp:	750 V
Acquisition parameters MS mode:	Data acquired at 1 GHz, MS only mode, mass range 600-4,000 *m/z* (fragments), 2,000-6,000 *m/z* (intact)

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Results and Discussion

To achieve better chromatographic performance, trifluoroacetic acid (TFA) is typically added to the mobile phase as an ion-pairing agent to provide sharper chromatographic peaks. However, TFA is not suitable for MS analysis due to its signal suppression effect [2]. Formic acid is the preferred MS-friendly ion-pairing agent for biomolecule applications, but can cause poor peak shape on traditional silica-based columns. Achieving good chromatographic peak shape and a high signal-to-noise ratio (S/N) *m/z* mass spectrum is a tradeoff when working with high molecular weight proteins such as mAbs [3]. It is important to make the right LC column choice for MS compatibility and improved separation of mAbs and ADC. For guidance on using silica-based columns with LC/MS for mAb separations, refer to Agilent application notes: 5991-6296EN, 5991-4266EN, 5991-2116EN, and 5990-9631EN [3-6].

In this study, the polymeric-based PLRP-S column was used to analyze mAbs and ADC at both the intact and fragment level. PLRP-S is robust and mechanically stable, and available in a range of pore and particle sizes suited for large biomolecule applications.

Intact analysis

Figure 1 shows the LC/MS analysis of the intact mAbs and ADC using a PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. The column provided excellent TIC peak shapes with ≤0.1 minutes full width at half maximum (FWHM) mAbs and 0.25 minutes FWHM ADC. The narrow TIC peak width was obtained using a standard RP mobile phase system (ACN + FA). Similar peak widths were observed for two mAbs, demonstrating the suitability of the method for various mAb samples. Narrow peak width was also demonstrated with the ADC sample, which is highly heterogeneous. The same method conditions and column were also tested with UV detection, and the results were satisfactory with identical retention time profiles (data not shown).

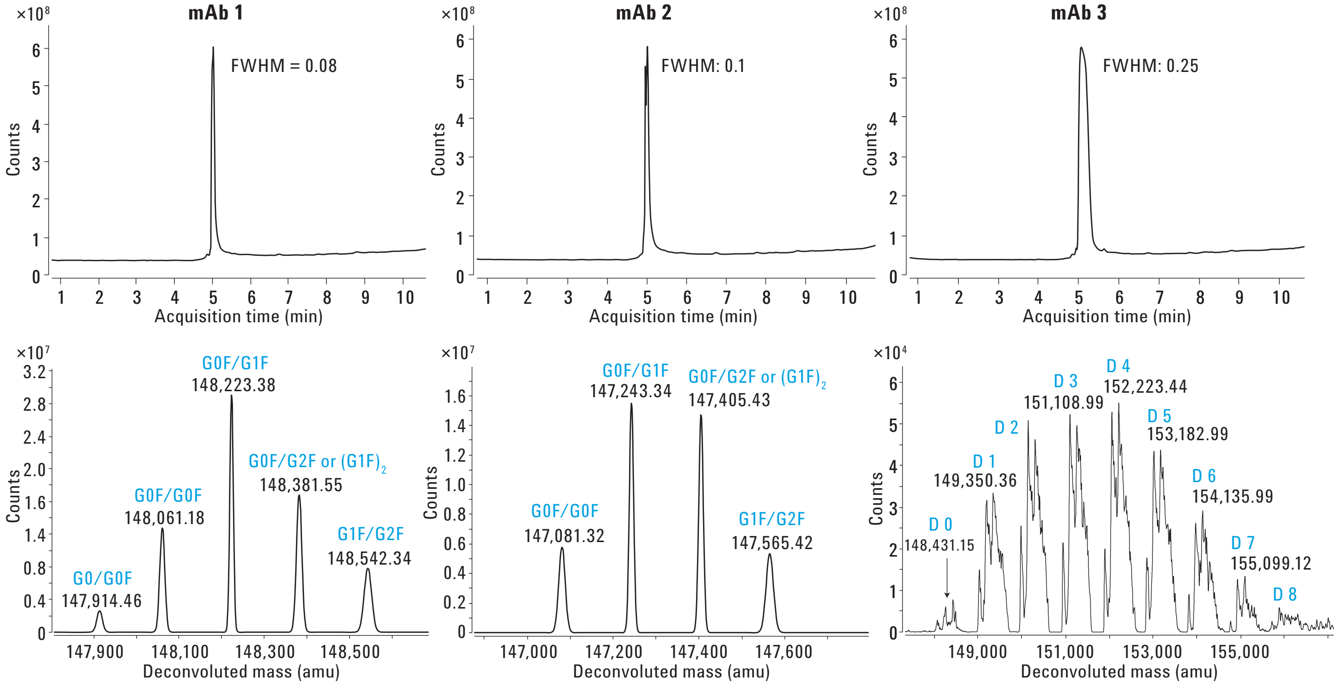

Figure 1. Intact mAb/ADC LC/MS analysis on an Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. Top: Total ion chromatogram; bottom: Deconvoluted spectrum. FWHM: full width at half maximum.

The raw mass spectra were converted to zero-charge mass spectra using Agilent MassHunter BioConfirm software. Figure 1 shows the deconvoluted spectra. Five major glycoforms are seen in the mAb1 deconvoluted spectrum, while four major glycoforms are evident in the mAb2 spectrum. The deconvoluted mass spectra for ADC showed increasing payload trend in steps of one drug load with eight major drug conjugations (D0 to D8).

Fragment analysis

To analyze the generated fragments, the samples were subjected to chemical and enzymatic reactions. Figure 2 shows the LC/MS separation of reduced, IdeS and papain-digested mAbs and ADC using a PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. The fragment peaks (LC, HC, ScFc, F(ab')2, Fc, 2 * Fab2, and ADC fragments with different drug conjugates species) are well separated on the PLRP-S column using the standard mobile phase system (ACN + FA). As expected, the two mAb samples resulted in two major fragments post digestion.

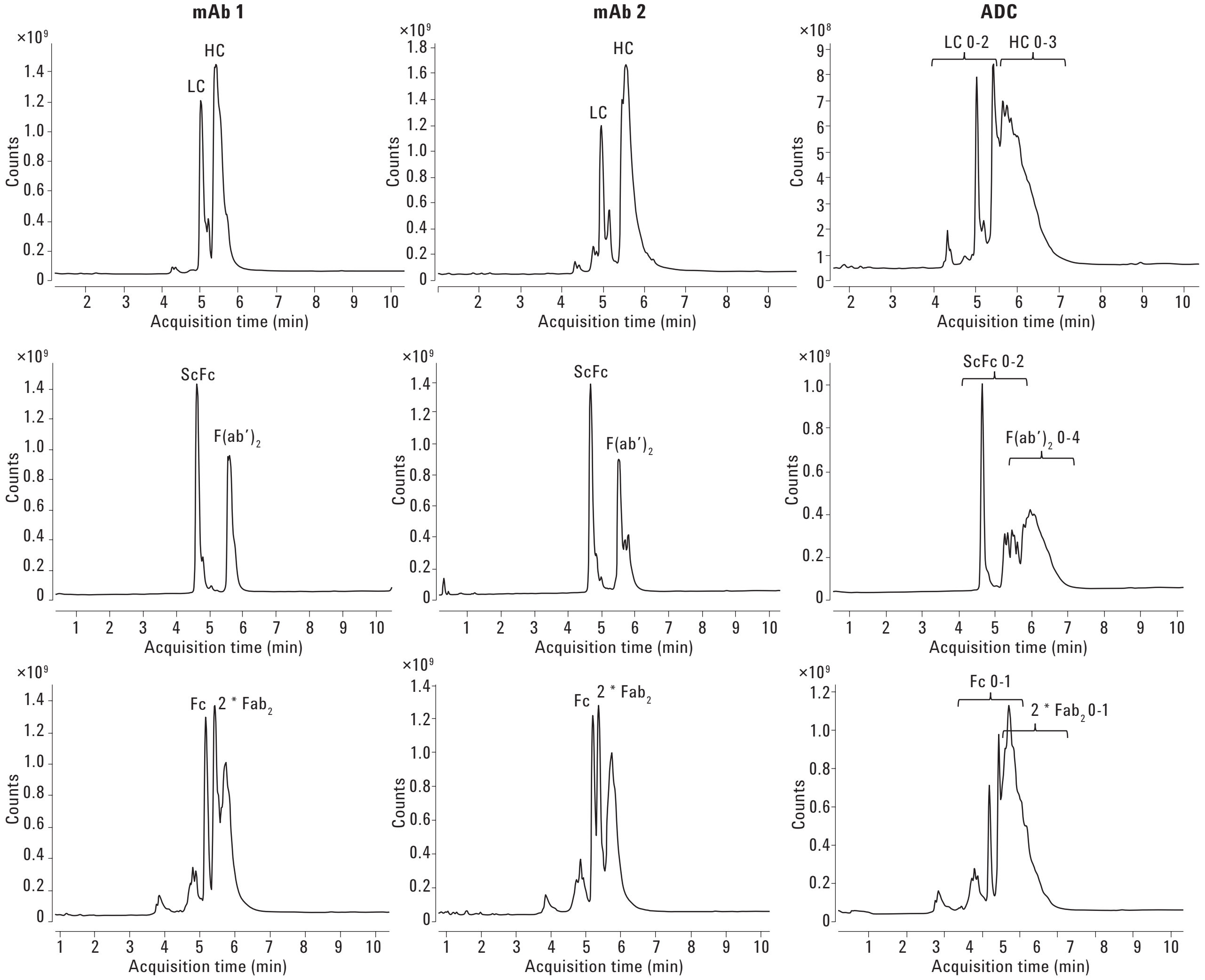

Figure 2. mAb fragment LC/MS analysis on an Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000Å column. Top: reduction; middle: IdeS digestion; bottom: papain digestion.

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The separations between these major fragment peaks are sufficient to enable better MS sensitivity and accurate molecular weight determination (Figure 3). Separation of the fragment species is very challenging with ADC samples, as conjugation at the various available lysine residues results in a high degree of heterogeneity. Figure 2 shows the separation of glycosylated ADC fragments on the PLRP-S column. It is evident that different drug conjugated species are reasonably separated under the discussed LC/MS conditions, which demonstrates the separation performance of the PLRP-S column. Figure 3 shows the representative deconvoluted spectra for mAb1 and ADC fragments.

Conclusion

- The Agilent PLRP-S column demonstrates superior separation performance for the analysis of mAbs and ADC at both intact and fragment levels.
- The Agilent PLRP-S provided better chromatographic performance and high-quality MS response with formic acid-containing mobile phases.
- Analyses of mAbs and ADC using Agilent PLRP-S columns coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS has been demonstrated.

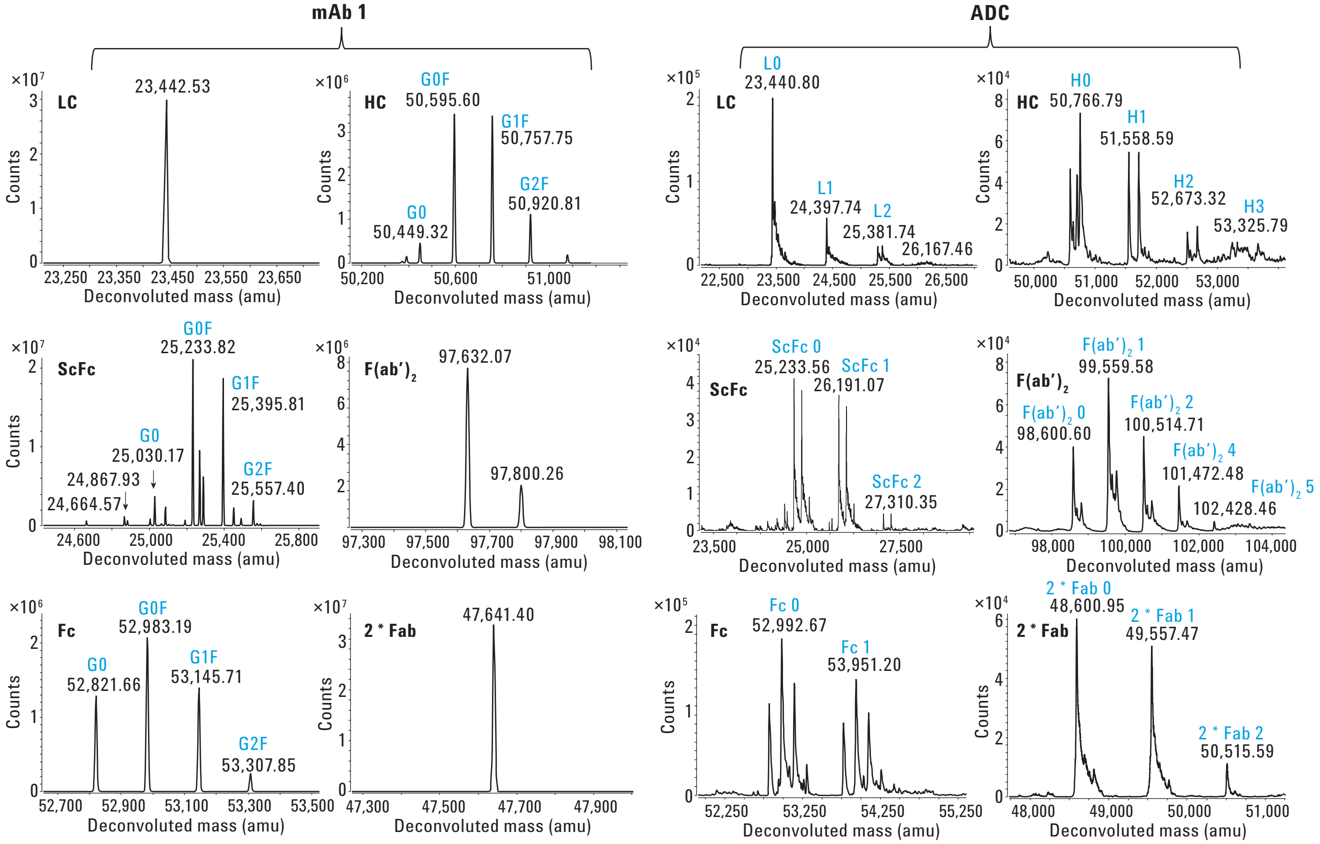

Figure 3. Representative deconvoluted mass spectrum. Top: reduction; middle: IdeS digestion; bottom: papain digestion.

References

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2. McCalley, D. V. Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. *Journal of Chromatography A* **2004**, *1038*, 77–84.
3. Gudihal, R.; Suresh Babu C. V.; Tang, N. *Analysis of Monoclonal Antibody(mAb) Using Agilent 1290 InfinityLC System Coupled to Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF)*; Application note, Agilent Technologies, Inc. Publication number 5991-4266EN, **2014**.
4. Suresh Babu, C. V. *LC/MS of Intact TherapeuticMonoclonal Antibodies Using Agilent AdvanceBio RP-mAb*. Application note, Agilent Technologies, Inc. Publication number 5991 6296EN, **2015**.
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Intact- and Peptide-Level Characterization of Trastuzumab Emtansine In Vitro Transformation

Using the Agilent AssayMAP Bravo and 6545XT AdvanceBio LC/Q-TOF

Suitable for Agilent
1290 Infinity III LC

Author

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Abstract

Antibody-drug conjugates (ADCs) are designed to facilitate the targeted delivery of cytotoxic drugs, improving antitumor effects while minimizing toxicity. Loss of the conjugated drug from the ADC leads to compromised efficacy and safety. In this application note, we demonstrate a highly specific, sensitive, and flexible workflow that combines ligand-binding affinity capture with high-resolution mass spectrometry (MS). Using this workflow, we successfully characterized the transformation of trastuzumab emtansine (T-DM1) after a four-day incubation in phosphate-buffered saline (PBS) and plasma at both intact and peptide levels.

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Introduction

Trastuzumab emtansine (T-DM1) is an ADC for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer.[1] Monoclonal antibody trastuzumab serves as antibody backbone of T-DM1. The lysine amines of trastuzumab are conjugated to the cytotoxic agent emtansine (DM1). The drug-to-antibody ratio (DAR) ranges from 0 to 8, with an average of approximately 3.5.[2] However, the conjugated drug may become detached from the ADC due to factors such as storage condition or stress over time. Drug loss leads to ADC compositional change and can affect its efficacy and safety. Therefore, characterization of the ADC transformation under certain conditions is crucial to elucidate the metabolic mechanism of the ADC.

In this application note, a ligand binding LC/MS (LB-LC/MS) hybrid assay was developed to evaluate the changes of T-DM1 at both intact and peptide levels after incubation in buffer and rat plasma for four days. The LB-LC/MS hybrid assay has advantages in specificity, sensitivity, and flexibility compared to traditional ELISA-based ligand binding assays. This hybrid assay consists of the Agilent AssayMAP Bravo protein sample prep platform, the Agilent 1290 Infinity II bio LC, and the Agilent 6545XT AdvanceBio LC/Q-TOF system. Data analysis was performed with Agilent MassHunter BioConfirm software, version 12.1. The workflow components are illustrated in Figure 1.

Experimental

Materials

Immunoaffinity and deglycosylation: Recombinant human HER2 extracellular domain (ECD) was purchased from ACROBiosystems (Newark, DE, U.S.). EZ-Link Sulfo-NHS-LC-Biotin and Zeba Spin Desalting columns, 7K MWCO, 0.5 mL, were from Thermo Fisher Scientific (Pittsburgh, PA, U.S.). Rat serum was from InVivos (Singapore). Rapid PNGase F was from New England Biolabs (Ipswich, MA, U.S.). AssayMAP Streptavidin cartridges (SA-W) were from Agilent. Trizma base, HEPES, ammonium hydroxide, and NaCl were from MilliporeSigma (Burlington, MA, U.S.).

Trypsin digestion: Urea, Trizma base, dithiothreitol (DTT), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma.

Generic: LC/MS-grade formic acid (FA) was purchased from Fisher Scientific (Pittsburgh, PA, U.S.).

The T-DM1 sample was purchased from Alliance Pharm (Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system.

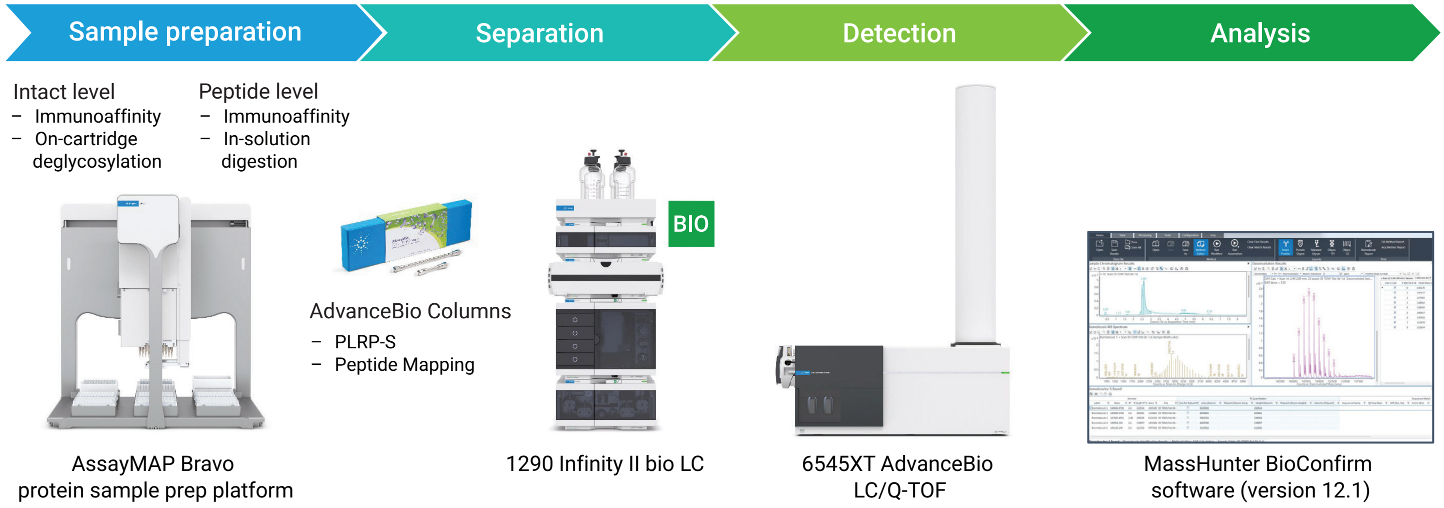

Figure 1. Agilent LB-LC/MS workflow components.

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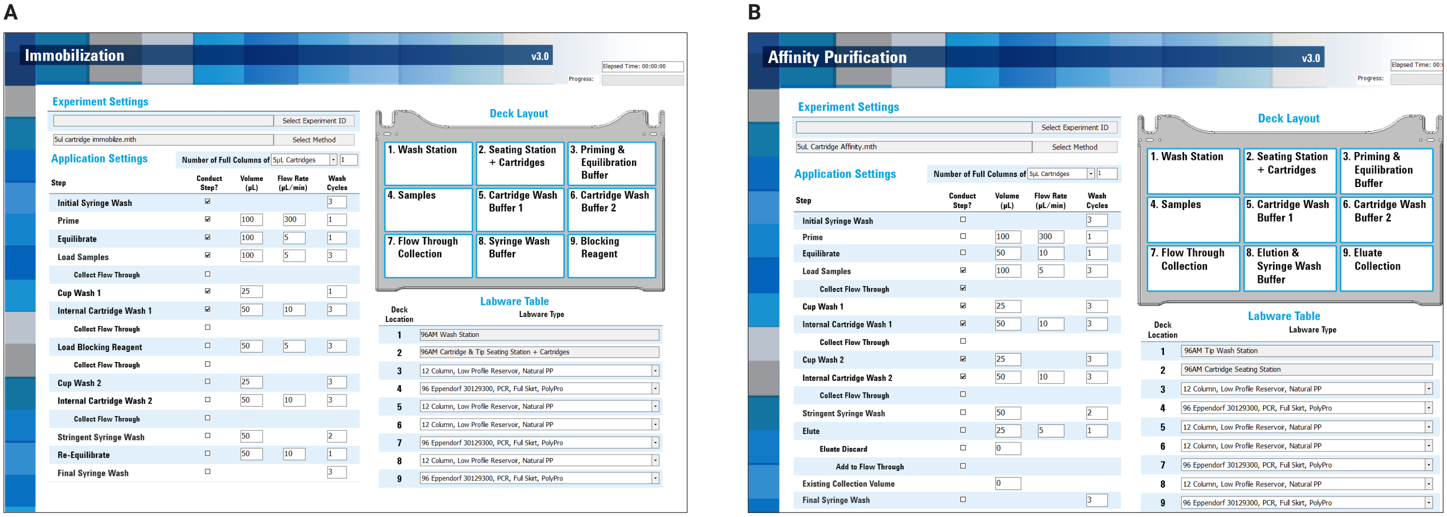
Sample preparation

Incubation: T-DM1 was spiked into rat plasma and PBS buffer separately at the concentration of 100 µg/mL. The spiked samples were incubated at 37 °C for four days. After the four-day incubation was completed, the control sample was prepared by spiking T-DM1 into plasma at 100 µg/mL concentration. All three samples were then subject to other procedures together.

Immunoaffinity purification: For this step, 0.2 mg HER2 ECD was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin followed by clean up with the Zeba Spin Desalting column, as per the manufacturer's instructions. The SA-W cartridges were conditioned with 1% FA. Then, the biotinylated HER2 ECD was immobilized onto the cartridges using the Immobilization app on the AssayMAP Bravo protein sample prep platform (Figure 2A). The cartridges were primed and equilibrated with 100 µL binding buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at the indicated flow rates in Figure 2B. The biotinylated HER2 ECD, in 100 µL binding buffer, was loaded onto the SA-W cartridges at 5 µL/min, then washed with binding buffer at 10 µL/min. Next, 50 µL sample was 1:1 diluted with binding buffer and loaded onto the ECD-SA-W cartridge at speed of 5 µL/min. After the sample was loaded, the cartridge was washed sequentially with 50 µL high-salt buffer (10 mM HEPES, 1 M NaCl, pH 7.4) then 50 µL of binding buffer, as shown in Figure 2B. For intact-level analysis, the cartridge was ready for deglycosylation.

On-cartridge deglycosylation: The T-DM1-bound cartridge was washed with 50 µL of 20 mM Tris (pH 8.0) and then reacted with heated PNGase F enzyme. The temperature of the Peltier deck was set at 45 °C to maintain an on-cartridge reaction temperature of approximately 37 °C. After 30 minutes, the reaction was completed. The cartridge was washed with 50 µL high-salt HEPES buffer and then with 0.003% FA. The deglycosylated T-DM1 was eluted with 15 µL of 1% FA into an elution plate containing 15 µL of 0.5% ammonium hydroxide. The application settings are shown in Figure 3A. A detailed description of each step is available in a previous application note.[3]

In-solution trypsin digestion: For peptide-level analysis, additional steps in affinity purification were carried out, including the stringent syringe wash and elution (Figure 2B). T-DM1 was eluted with 20 µL of 0.25% FA in 10% acetonitrile into 10 µL of 1 M ammonium bicarbonate. Next, using a single-plate in-solution digestion application (Figure 3B), DTT was added at 10 mM final concentration to the T-DM1 eluted from the affinity purification step. The plate was incubated, off deck, at 60 °C for 1 hour. After cooling down, IAA was added at 20 mM final concentration and the reaction took place, off deck, in darkness, for 30 minutes at room temperature. Additional DTT was added to neutralize excessive IAA. Lastly, 0.5 µg trypsin was added to each well and incubated at 37 °C overnight. Digestion was quenched with 10 µL of 10% TFA. The sample was subject to LC/MS analysis.



A

5ul cartridge immobilize.mth

Number of Full Columns of 5µL Cartridges 1

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☑	100	300	1
Equilibrate	☑	100	5	1
Load Samples	☑	100	5	3
Collect Flow Through	☐			
Cup Wash 1	☑	25		1
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	50	5	3
Collect Flow Through	☐			
Cup Wash 2	☐	25		3
Internal Cartridge Wash 2	☐	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		2
Re-Equilibrate	☐	50	10	1
Final Syringe Wash	☐			3

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

B

5uL Cartridge Affinity.mth

Number of Full Columns of 5µL Cartridges 1

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☐			3
Prime	☐	100	300	1
Equilibrate	☐	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		3
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		2
Elute	☐	25	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		0		
Final Syringe Wash	☐			3

Deck Location	Labware Type
1	96AM Tip Wash Station
2	96AM Cartridge Seating Station
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Figure 2. Agilent AssayMAP Bravo protein sample prep platform application settings for (A) immobilization and (B) affinity purification.

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Instrumentation

Automated sample preparation was performed using the Agilent AssayMAP Bravo protein sample prep platform (G5571AA).

For separation, the Agilent 1290 Infinity II bio LC system was used, including:

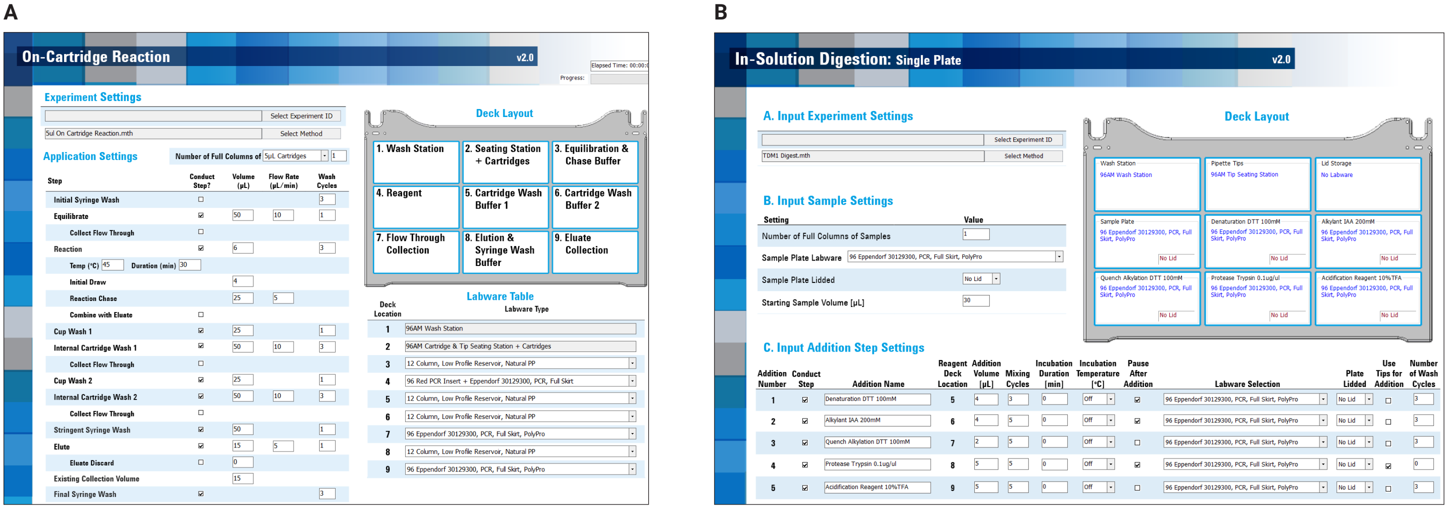
- Agilent 1290 Infinity II bio high-speed pumps (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bioinert Quick Connect heat exchanger, standard flow (option #065)

Samples were analyzed on the Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with the Agilent Dual Jet Stream ESI source.

Software

The following software was used in this study:

- Agilent VWorks Automation Control software, version 14.2
- Agilent AssayMAP Protein Sample Prep Workbench software, version 4.0
- Agilent MassHunter acquisition (TOF/Q-TOF) software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1



A

On-Cartridge Reaction

Elapsed Time: 00:00:0

Experiment Settings

5ul On Cartridge Reaction.mth

Application Settings

Number of Full Columns of 5µL Cartridges: 1

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☐			3
Equilibrate	☑	50	10	1
Collect Flow Through	☐			
Reaction	☑	6		3
Temp (°C) 45 Duration (min) 30				
Initial Draw		4		
Reaction Chase		25	5	
Combine with Eluate	☐			
Cup Wash 1	☑	25		1
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		1
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		1
Elute	☑	15	5	1
Eluate Discard	☐	0		
Existing Collection Volume		15		
Final Syringe Wash	☑			3

Deck Layout

1. Wash Station
2. Seating Station + Cartridges
3. Equilibration & Chase Buffer
4. Reagent
5. Cartridge Wash Buffer 1
6. Cartridge Wash Buffer 2
7. Flow Through Collection
8. Elution & Syringe Wash Buffer
9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Red PCR Insert + Eppendorf 30129300, PCR, Full Skirt
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

B

In-Solution Digestion: Single Plate

A. Input Experiment Settings

TDM1 Digest.mth

B. Input Sample Settings

Setting	Value
Number of Full Columns of Samples	1
Sample Plate Labware	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
Sample Plate Lidded	No Lid
Starting Sample Volume [µL]	30

Deck Layout

Wash Station 96AM Wash Station	Pipette Tips 96AM Tip Seating Station	Lid Storage No Labware
Sample Plate 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid	Denaturation DTT 100mM 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid	Alkylant IAA 200mM 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid
Quench Alkylation DTT 100mM 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid	Protease Trypsin 0.1ug/ul 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid	Acidification Reagent 10%TFA 96 Eppendorf 30129300, PCR, Full Skirt, PolyPro No Lid

C. Input Addition Step Settings

Addition Number	Conduct Step	Addition Name	Reagent Deck Location	Addition Volume [µL]	Mixing Cycles	Incubation Duration [min]	Incubation Temperature [°C]	Pause After Addition	Labware Selection	Plate Lidded	Use Tips for Addition	Number of Wash Cycles
1	☑	Denaturation DTT 100mM	5	4	3	0	Off	☑	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	No Lid	☐	3
2	☑	Alkylant IAA 200mM	6	4	5	0	Off	☑	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	No Lid	☐	3
3	☑	Quench Alkylation DTT 100mM	7	2	5	0	Off	☐	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	No Lid	☐	3
4	☑	Protease Trypsin 0.1ug/ul	8	5	5	0	Off	☑	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	No Lid	☑	0
5	☑	Acidification Reagent 10%TFA	9	5	5	0	Off	☐	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro	No Lid	☐	3

Figure 3. Agilent AssayMAP Bravo protein sample prep application settings for (A) on-cartridge deglycosylation and (B) in-solution digestion.

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for LC and MS. Table 3 displays the DAR calculation parameters in MassHunter BioConfirm software.

Table 1. LC parameters.

	Intact	Peptide
Column	Agilent PLRP-S 1000 Å column, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)	Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm, 120 Å (p/n 653750-902)
Thermostat	8 °C	8 °C
Solvent A	0.1% FA in H_2O	0.1% FA in H_2O
Solvent B	0.1% FA in acetonitrile	0.1% FA in 90% acetonitrile
Flow Rate	0.4 mL/min	0.4 mL/min
Gradient	Time (min) %B 0.0 10 1.0 10 2.0 37 4.0 37 4.5 50 5.5 50 6.0 10	Time (min) %B 0.0 3 1.0 3 70.0 45 71.0 90 73.0 90
Post-Time	2.5 min	5 min
Injection Volume	20 µL	20 µL
Column Temperature	60 °C	60 °C

Table 2. MS data acquisition parameters.

	Intact	Peptide
Source	Agilent Dual Jet Stream ESI	Agilent Dual Jet Stream ESI
Polarity	Positive	Positive
Drying Gas Temperature	350 °C	325 °C
Drying Gas Flow	12 L/min	13 L/min
Nebulizer	60 psi	35 psi
Sheath Gas Temperature	400 °C	275 °C
Sheath Gas Flow	11 L/min	12 L/min
Capillary Voltage	5,500 V	4,000 V
Nozzle Voltage	2,000 V	0 V
Fragmentor	380 V	175 V
Acquisition Mode	High mass range (30,000 *m/z*) Extended dynamic range (2 GHz)	Low mass range (1,700 *m/z*) Extended dynamic range (2 GHz)
Mass Range	*m/z* 900 to 5,000	*m/z* 300 to 1,700
Acquisition Rate	1 spectra/s	2 spectra/s
Reference Mass	*m/z* 922.0097, 1,821.9523	*m/z* 322.0481, 922.0097

Table 3. Agilent MassHunter BioConfirm software DAR calculation parameters.

	Parameters
DAR 0 Mass(es)	145,162
Linker Type	Lysine-linked (all)
Drug + Linker Mass (Da)	957.00
Peak Area	Use most abundant peak height
Max DAR Peak Number	8

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Results and discussion

Intact-level T-DM1 characterization

An automated sample preparation workflow was developed on the AssayMAP Bravo platform to address the challenging sample matrix of plasma. The workflow included immobilization of target antigen on the SA-W cartridge, immunoaffinity purification of T-DM1 from matrix, and deglycosylation of purified T-DM1 on-cartridge.

The deglycosylated T-DM1 was analyzed in high-mass-range mode on the LC/Q-TOF system. Figure 4 is the compilation of total ion chromatograms (A to C), raw spectra (D to F), and deconvoluted spectra (G to I) of the samples. All samples eluted within 2.4 to 3.4 minutes, with a mass envelope ranging from *m/z* 2,000 to 4,000, corresponding to charge states between +37 to +68. The clean total ion chromatogram and high-resolution raw spectra indicate the success of the automated sample preparation workflow in immunocapturing and deglycosylation of T-DM1 from matrix.

Nine drug-conjugated T-DM1 species were detected in the control and PBS-4d samples with mass matching DAR0 to DAR8; whereas, in the plasma-4d sample, only eight species were detected, missing DAR8. A profile shift toward lower DAR species was observed in both four-day samples, indicating the loss of drug in T-DM1 over the incubation period. The most abundant species shifted from DAR3 in the control to DAR2 in the incubated samples. The abundance of high DAR species, DAR7 and DAR8, either decreased significantly or totally disappeared in the incubated samples. All these changes attributed to the average DAR value decrease from original 3.5 to 2.7 in the incubated samples.

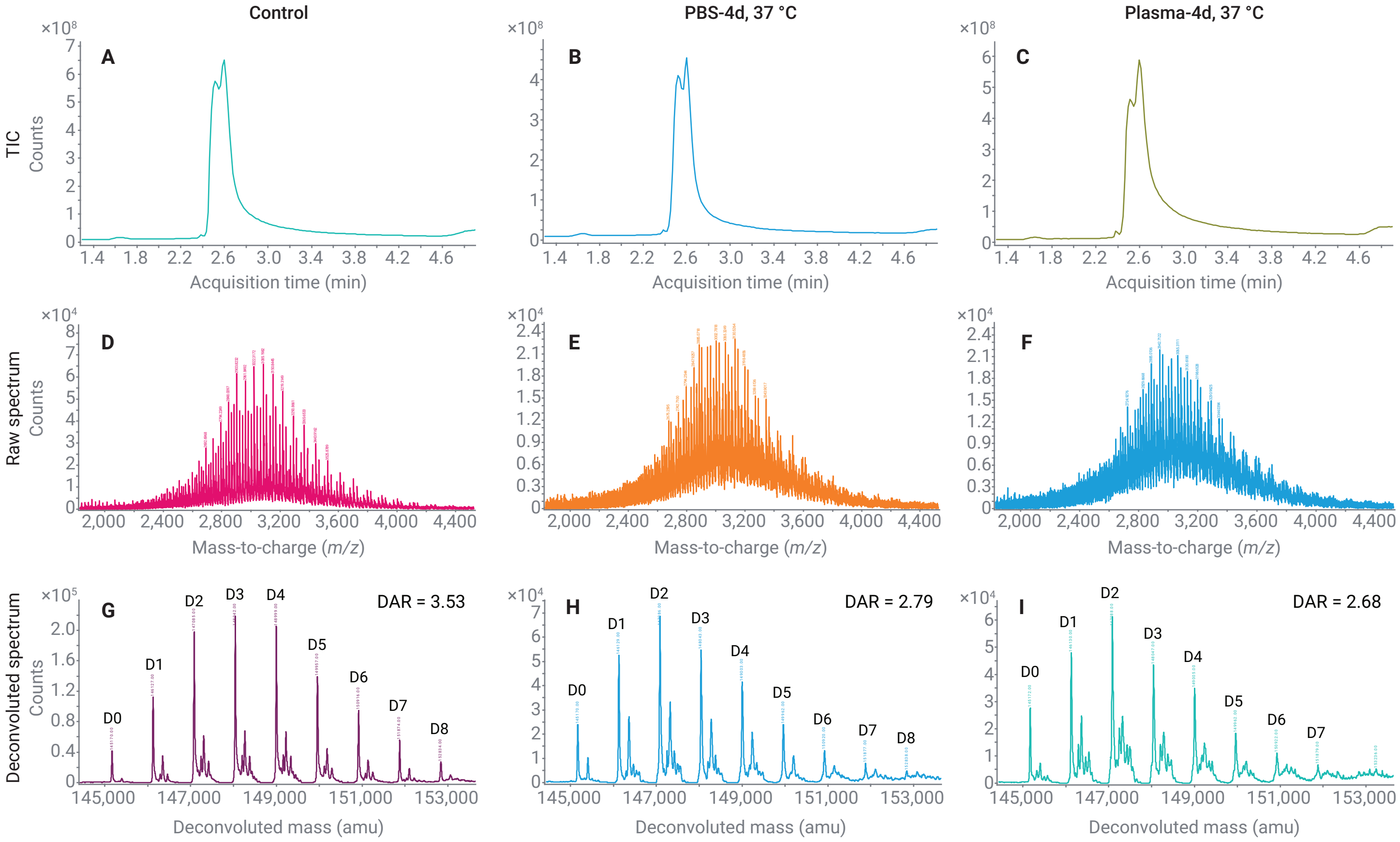

Figure 4. Total ion chromatograms, raw spectra, and deconvoluted spectra of control samples, and PBS-4d, and plasma-4d samples incubated at 37 °C.

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In addition to the major drug conjugated species (DAR0 to DAR8), there were also minor species detected and well resolved from main DAR peaks (Figure 5) thanks to the high resolution of the 6545XT AdvanceBio LC/Q-TOF system. Based on the chemical structure of T-DM1 (Figure 6) and the observed delta mass from major DAR species, those minor peaks were assigned to the best of our knowledge and listed in Table 4.

Figure 5 displays the zoomed-in view between DAR2 and DAR3 of the samples. It is a representation of the spectra profiles between other DARs. Peaks a, b, and c are present across all three samples. They represent glycation of antibody, DAR2 addition of free MCC linker[4], and DAR3 with loss of DM1 and addition of a cysteine residue.[5] Peaks d and e are only present in PBS-4d and plasma-4d samples.

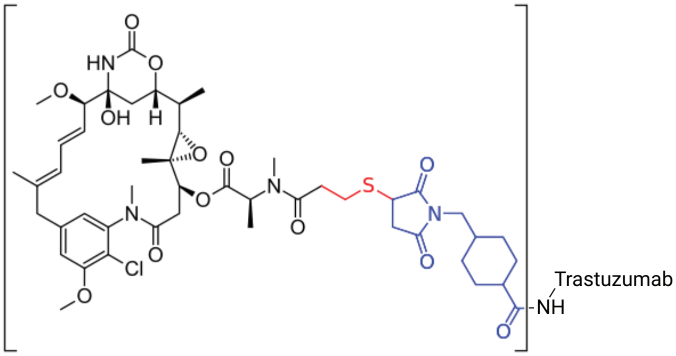

Figure 6. Chemical structure of T-DM1.

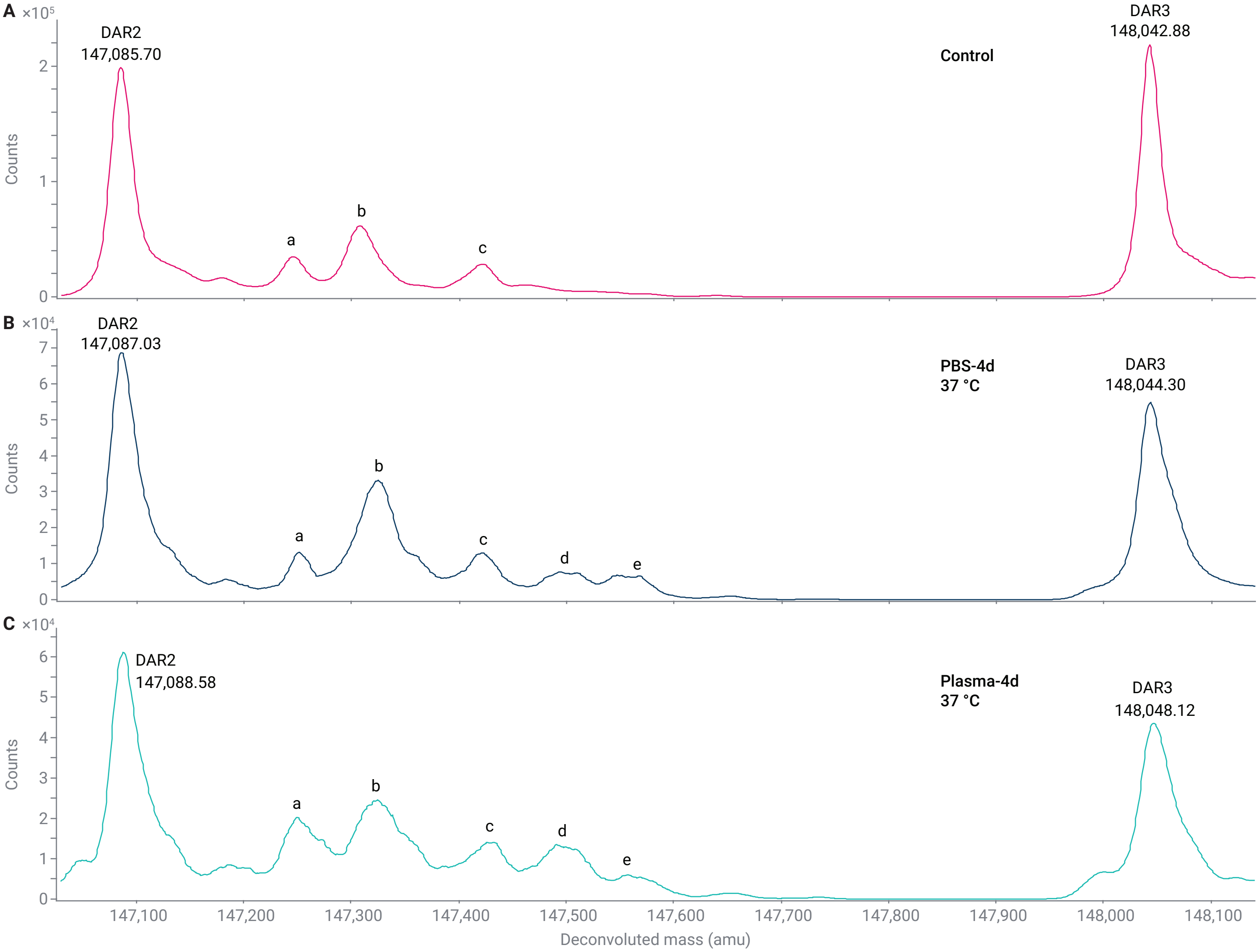

Figure 5. Zoomed-in view of deconvoluted spectra between DAR2 and DAR3 of (A) control, (B) PBS-4d, and (C) plasma-4d.

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Table 4. Assignment of minor peaks between DAR2 and DAR3.

Peak	Present In	Delta Mass from DAR2 (Da)	Delta Mass from DAR3 (Da)	Species
a	All	+161	–	Glycation of Trastuzumab
b	All	+220	–	DAR2+MCC
c	All	–	−622	DAR3-DM1+Cys
d	PBS-4d plasma-4d	–	−547	DAR3-maytansinol+OH
e	PBS-4d plasma-4d	–	−490	Unknown degradant

Peak d is DAR3 loss of maytansinol and addition of OH via ester hydrolysis. Peak e, with a delta mass of 490 Da from DAR3, is defined as an unknown species. However, it is certain that peaks d and e, absent from the control, were induced from incubation at 37 °C. Thus, they are related to T-DM1 degradation.

In addition to the above findings, a mass increase of each DAR was observed in the incubated samples compared to the control. For example, in the plasma-4d sample, the 3 and 6 Da mass increase on DAR2 and DAR3 respectively is displayed in Figure 5. The mass increase could be attributed to multiple site hydrolysis of MCC-DM1 over the course of incubation.[5]

The LB-LC/MS workflow, encompassing high-specificity immunoaffinity purification, a high-resolution and accurate mass LC/Q-TOF system, and a reliable deconvolution algorithm, enables the thorough characterization of T-DM1 at intact level.

Peptide-level T-DM1 characterization

In a previous application note[6], the drug-conjugated peptides were identified in neat T-DM1 using an in-solution digestion peptide mapping workflow. Our study further involved the immunoaffinity purification step to clean up T-DM1 from matrices before trypsin digestion. The resulting peptides were then analyzed using LC/Q-TOF in MS-only mode. To access the peptide-level difference between the samples, the precursor mass of those conjugated peptides was extracted and integrated in extracted ion chromatogram (EIC).

Three representative peptides are shown as examples in Figure 7. Generally, the abundance of the conjugated peptides decreases in both incubated samples compared to the control, indicating drug loss over the course of incubation. This observation aligns with the intact-level DAR value decrease in the incubated samples.

Comparing individual peptide of PBS-4d and plasma-4d reveals that the sites of drug loss varied between PBS and plasma condition. For K*VEPK and ICNVNHK*PSNTK peptides, they are either very scarce or completely undetected in plasma-4d. However, it is the reverse situation for ADYEK*HK peptide that the abundance of it in plasma-4d surpasses that in PBS-4d. These findings may suggest that the linked drug at these conjugation sites may be susceptible to loss in the matrices.

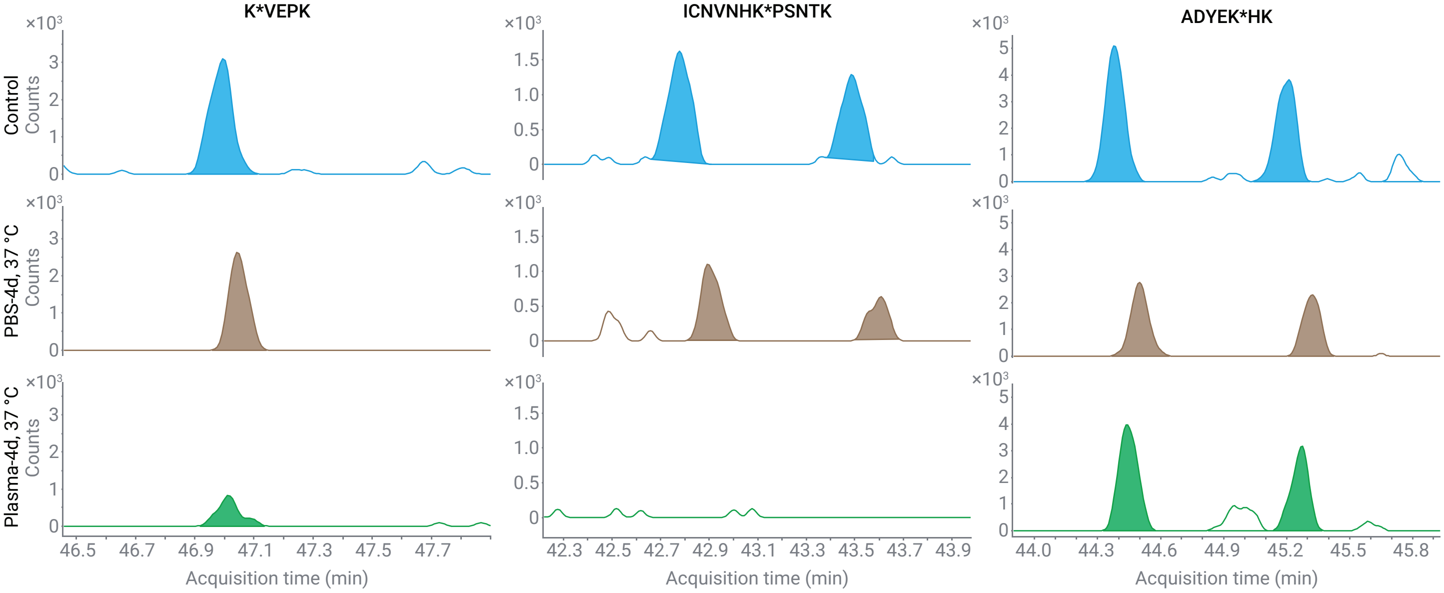

Figure 7. EICs of the exemplary drug conjugated peptides from control (top), PBS-4d (middle), and plasma-4d (bottom) samples. The asterisk marks the conjugation site.

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Conclusion

In this application note, we demonstrate the characterization of T-DM1 in vitro transformation at both intact and peptide levels using a ligand binding LC/MS (LB-LC/MS) workflow. This workflow is composed of the Agilent AssayMAP Bravo protein sample prep platform, 1290 Infinity II bio LC, and 6545XT AdvanceBio LC/Q-TOF system.

At the intact level, a series of minor species were well resolved from the major DAR peaks and successfully assigned to various degradation products, particularly in the treated samples. This result highlights the power of the high-resolution 6545XT AdvanceBio LC/Q-TOF in resolving the complex ADC MS spectra. At the peptide level, the overall abundance of conjugated peptides decreased in the treated samples, aligning with the result from the intact level. Peptide-level results also indicate that drug loss at the individual conjugation site may be susceptible to the matrix effect. This phenomenon warrants further investigation.

In summary, this LB-LC/MS workflow is a powerful tool for studying the in vitro transformation of T-DM1. It offers both specificity and sensitivity and holds great promise for future applications in drug discovery and development.

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Application Note

Biotherapeutics & Biosimilars

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.

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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.

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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

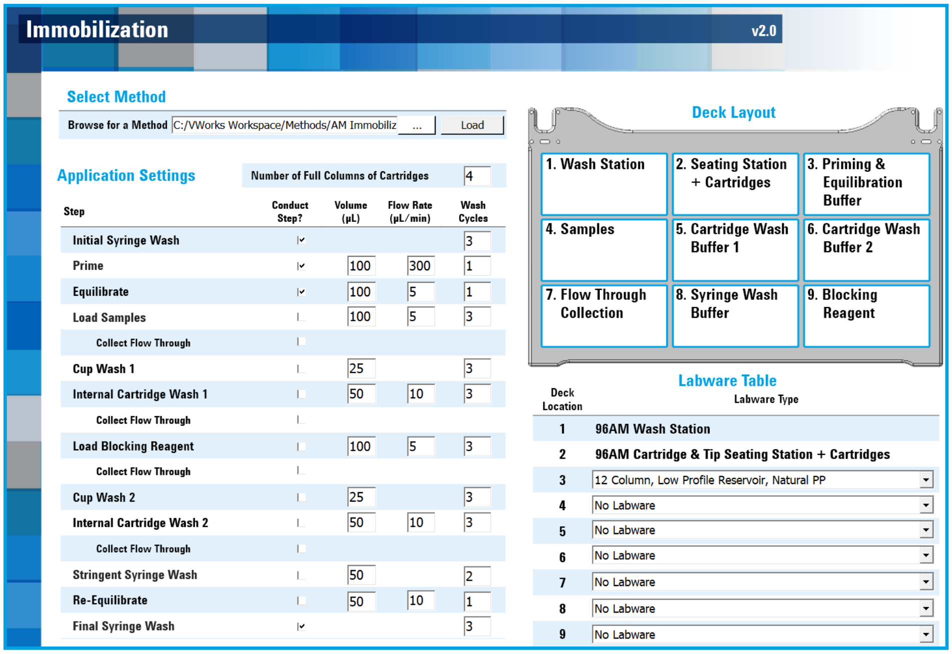
- Agilent Protein Sample Prep Workbench 3.2
- Agilent MassHunter Acquisition software
- Agilent MassHunter BioConfirm software

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.



Browse for a Method: C:/VWorks Workspace/Methods/AM Immobiliz

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☑	100	300	1
Equilibrate	☑	100	5	1
Load Samples	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 1	☐	25		3
Internal Cartridge Wash 1	☐	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☐	25		3
Internal Cartridge Wash 2	☐	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		2
Re-Equilibrate	☐	50	10	1
Final Syringe Wash	☑			3

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Syringe Wash Buffer	9. Blocking Reagent

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	No Labware
5	No Labware
6	No Labware
7	No Labware
8	No Labware
9	No Labware

Figure 2. AssayMAP Immobilization application for conditioning SAW cartridges.

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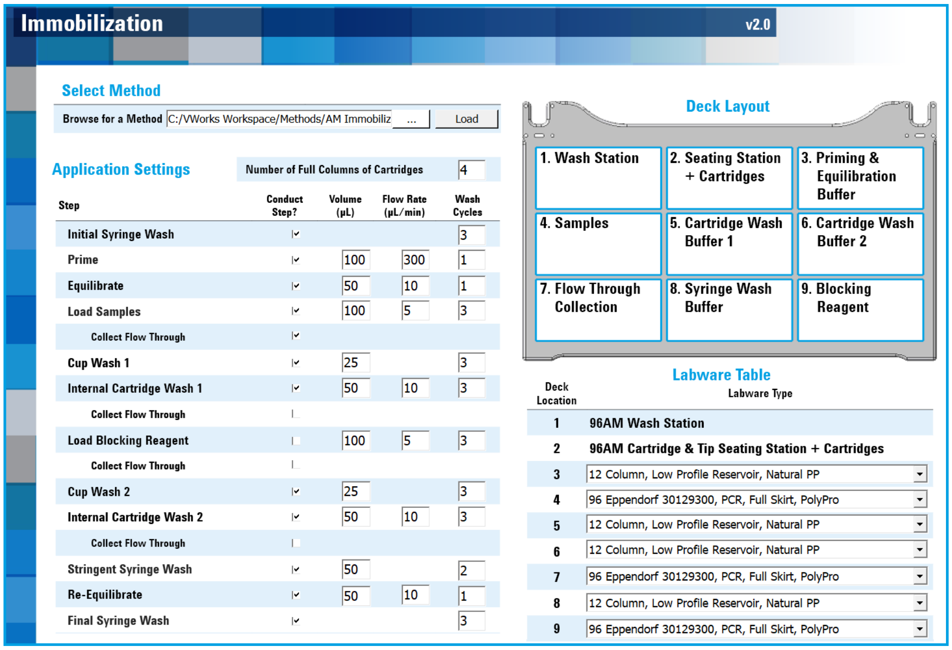

Select Method

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Application Settings

Number of Full Columns of Cartridges: 4

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Prime	☑	100	300	1
Equilibrate	☑	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		3
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Re-Equilibrate	☑	50	10	1
Final Syringe Wash	☑			3

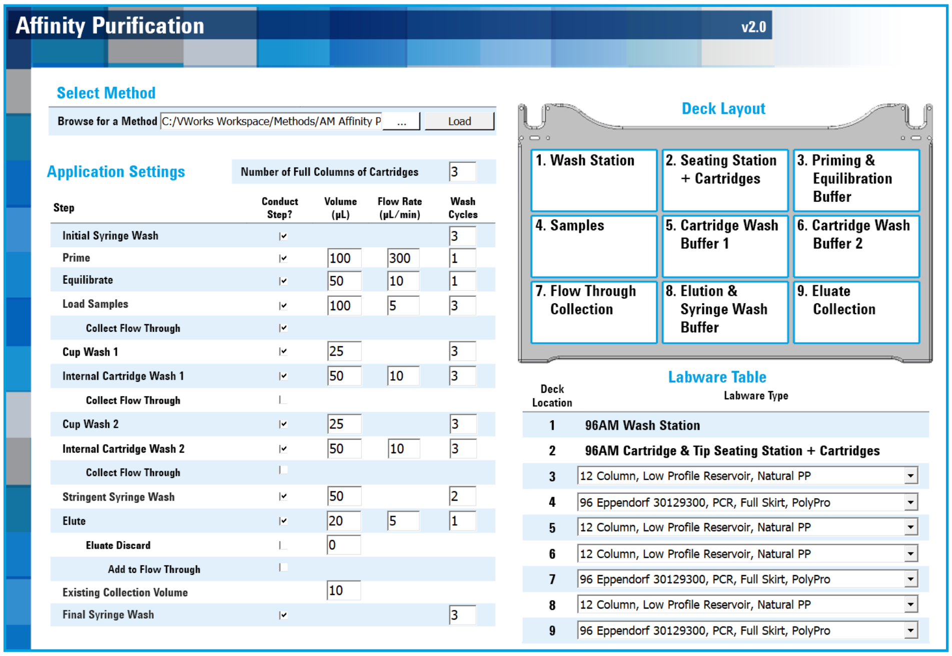
Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

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



Select Method

Browse for a Method: C:/VWorks Workspace/Methods/AM Affinity P

Application Settings

Number of Full Columns of Cartridges: 3

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Equilibrate	☑	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		3
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Elute	☑	20	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		10		
Final Syringe Wash	☑			3

Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Elution & Syringe Wash Buffer	9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

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.

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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.

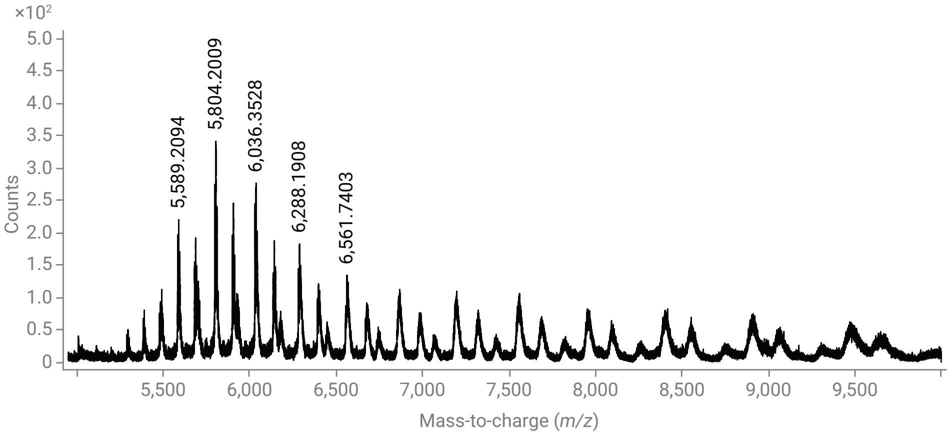

Figure 5. Brentuximab raw spectrum under native conditions.

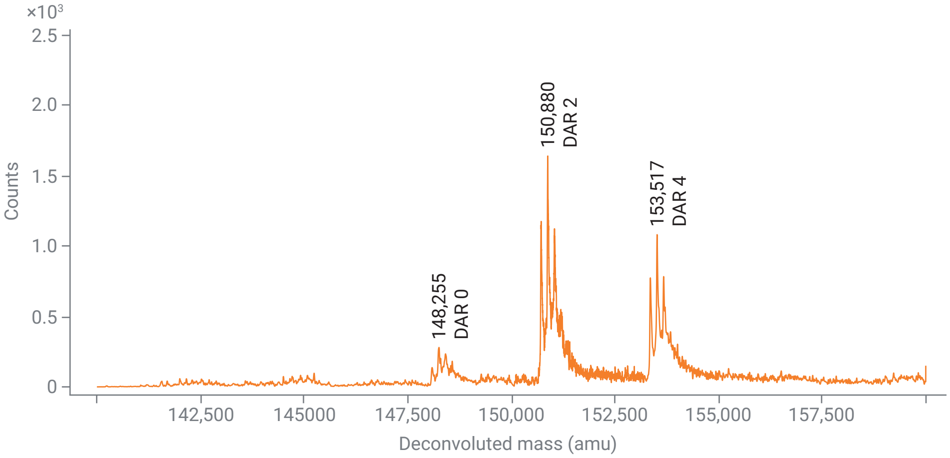

Figure 6. Brentuximab deconvoluted spectrum under native conditions.

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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.

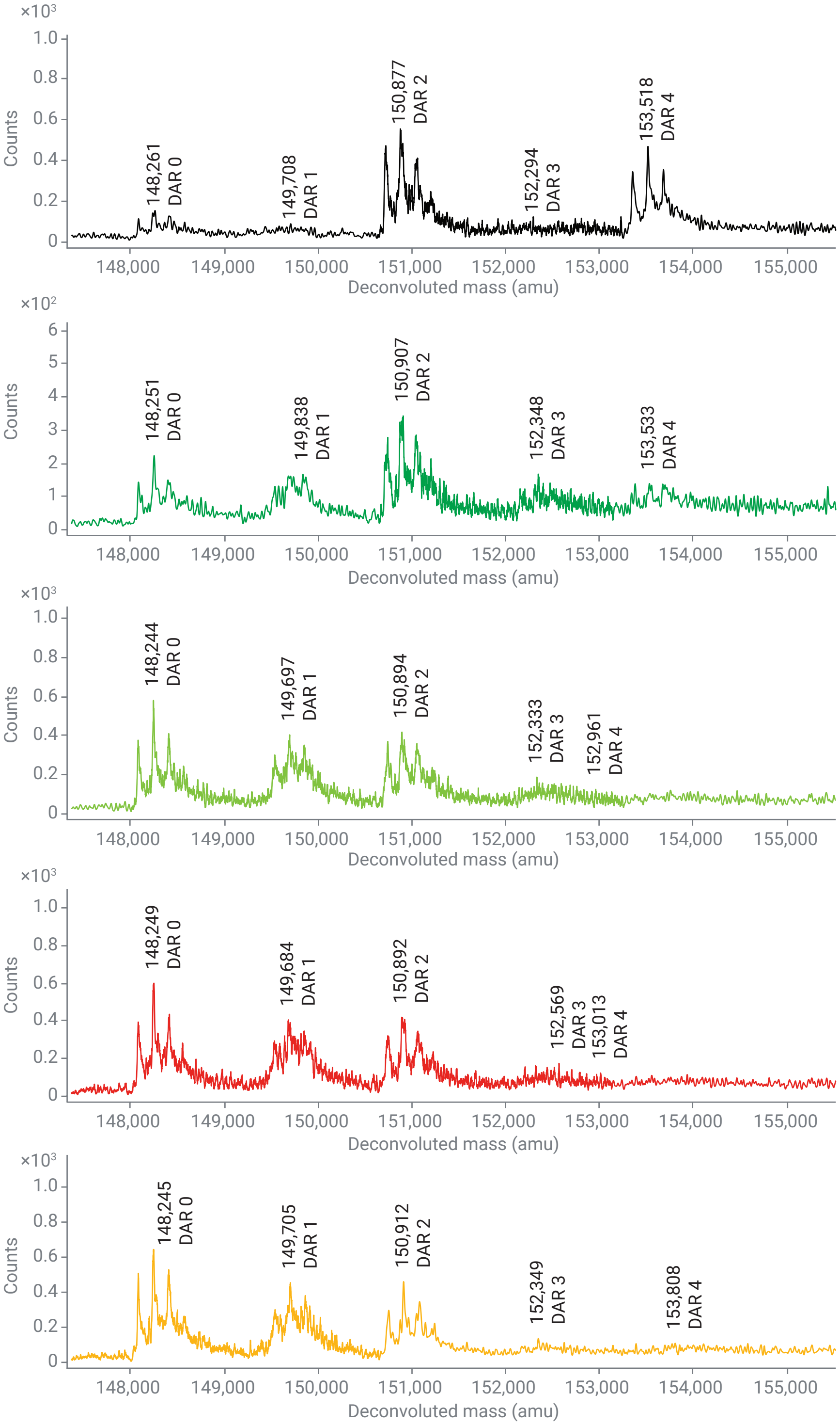

Figure 7. DAR analysis of stability samples.

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

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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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Application Note

Biopharma

Identification of Conjugation Sites in an Antibody Drug Conjugate

Suitable for Agilent
1290 Infinity III LC

Using the Agilent 6545XT AdvanceBio LC/Q-TOF system

Author

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Agilent Technologies, Inc.

Abstract

This application note highlights the workflow of drug conjugation site identification in an antibody drug conjugate (ADC). The workflow uses an Agilent AssayMAP Bravo protein sample prep platform, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm 12.1 software. Twenty-six conjugation sites were confidently identified in a lysine-linked ado-trastuzumab emtansine (T-DM1) sample. The results also showed exceptional mass accuracy and consistent reproducibility.

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Introduction

ADCs represent a cutting-edge class of biopharmaceuticals. ADCs are designed to deliver a cytotoxic payload specifically to a targeted site while minimizing off-target effects and enhancing therapeutic efficacy. ADCs are composed of a monoclonal antibody (mAb) linked to a potent cytotoxic agent using a chemical linker through a conjugation process. Since conjugation can occur at several available sites on lysine-based ADCs, multiple conjugation molecules can be present. Therefore, a lysine-conjugated ADC is a heterogenous mixture of conjugated biomolecules.[1]

Peptide mapping is a pivotal tool for the in-depth characterization of ADCs. Peptide mapping provides site-specific information about ADC conjugation sites. In this application note, the drug conjugation sites of a lysine-linked ADC, T-DM1, were characterized following the Agilent peptide mapping workflow. A 1290 Infinity II bio LC system and a 6545XT AdvanceBio LC/Q-TOF system were used in conjunction with an automated AssayMAP Bravo protein sample prep platform. Data analysis and mapping of conjugation sites were performed with MassHunter BioConfirm 12.1 software. The integrated workflow is illustrated in Figure 1.

Experimental

Materials

Urea, Trizma base, dithiothreitol (DTT), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma (Burlington, MA, USA). Formic acid (LC/MS grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The T-DM1 sample was purchased from Alliance Pharm (Singapore, Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Burlington, MA, USA).

Sample preparation

During sample preparation, 6.5 mg of TDM-1 were dissolved into 1.3 mL of denaturation buffer comprised of 8 M urea, 50 mM Tris (pH 8.0). Aliquots of 100 µL of the dissolved sample were transferred into the AssayMap sample plate wells. The digestion protocol was selected using the In-Solution Digestion: Single-Plate application. In this protocol, 10 µL of 100 mM DTT were added for sample reduction. The plate was incubated at 37 °C for two hours. For alkylation, 12 µL of 200 mM IAA were added, followed by incubation at room temperature in the dark for one hour. The sample was then diluted with 400 µL of water, and 20 µL of 0.5 µg/µL trypsin was added. After incubation at 37 °C overnight, the reaction was quenched by the addition of 60 µL of 10% TFA. The digested samples were subjected to LC/MS analysis.

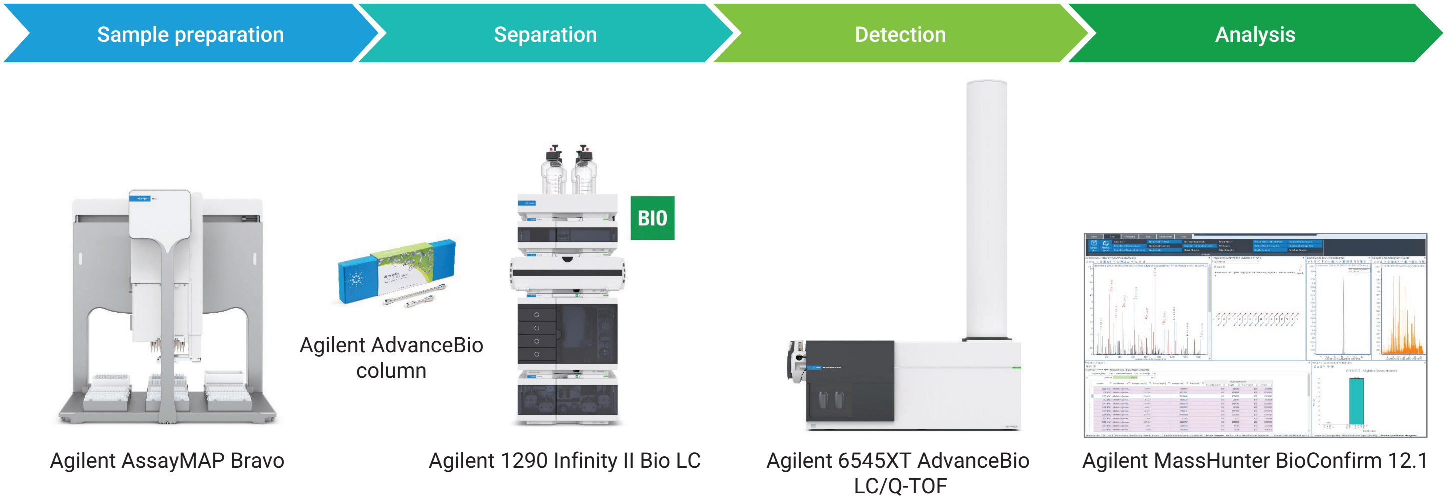

Figure 1. Agilent peptide mapping workflow.

Instrumentation

- Agilent AssayMAP Bravo protein sample prep platform (G5571AA)
- Agilent 1290 Infinity II bio LC system including:
  - Agilent 1290 Infinity II bio high-speed pumps (G7132A)
  - Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bioinert QuickConnect heat exchanger, standard flow (option #065)
  - Agilent 1290 Infinity II diode array detector (G7117B) with Agilent Max-Light cartridge cell, 10 mm
- Agilent 6545XT AdvanceBio LC/Q-TOF system with Agilent Dual Jet Stream ESI source

Software

- Agilent VWorks automation control software 14.1
- Agilent MassHunter data acquisition software 11.0
- Agilent MassHunter BioConfirm software 12.1

LC/MS analysis

Tables 1 and 2 list the parameters for LC and MS data acquisition used in the workflow.

Table 1. Liquid chromatography (LC) parameters.

LC Parameters	
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å (p/n 653750-902)
Thermostat	4 °C
Solvent A	0.1% Formic acid in H_2O
Solvent B	90% Acetonitrile and 0.1% formic acid in H_2O
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 3 1.0 3 70.0 45 71.0 90 73.0 90
Post Time	5 min
Injection Volume	20 µL
Column Temperature	60 °C

Table 2. Mass spectrometry (MS) data acquisition parameters.

Parameter	Value
Source	Agilent Dual Jet Stream ESI
Polarity	Positive
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 1,700
Acquisition Rate	8 Spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Minimum MS/MS Acquisition Rate	3 Spectra/sec
Isolation Width	Narrow (~*m/z* 1.3)
Max Precursor/Cycle	Top 10
Collision Energy	3.1 × (*m/z*)/100 + 1 for charge 2; 3.6 × (*m/z*)/100 – 4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 Counts and 0.001%
Dynamic Exclusion On	1 Repeat, then exclude for 0.2 min
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 Counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% Sstringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By charge state, then abundance; +2, +3, > +3

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Data analysis

Data were processed following the Protein Digest workflow in MassHunter BioConfirm 12.1 software. The conjugation was defined as a new type of modification, MCC-DM1, in the Chemical Data Dictionary Tool. The modification adds 956.3644 Da in mass specifically to the lysine residue. The processing method parameters are listed in Table 3.

Table 3. Protein digest processing method parameters.

Parameter	Value
Condition	Reduced
Mods and Profiles	Alkylation (iodoacetamide), MCC-DM1
Enzyme	Trypsin
Find Peptides	Display biomolecules containing MS/MS scans
Match Tolerances	MS match tolerance: ± 20 ppm MS/MS match tolerance: ± 50 ppm Warn if score is < 3.00 Do not match if score is < 3.00 Allow missed cleavages up to 2 Peptide length range: 4 to 70 Allow terminal truncation Max number of modifications: 4

Results and discussion

The antibody backbone of T-DM1 is trastuzumab. The lysine amines of trastuzumab and the cytotoxic agent DM1 (emtansine) are conjugated by a nonreducible thioether linker, N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC).[2] The structure of T-DM1 is shown in Figure 2.

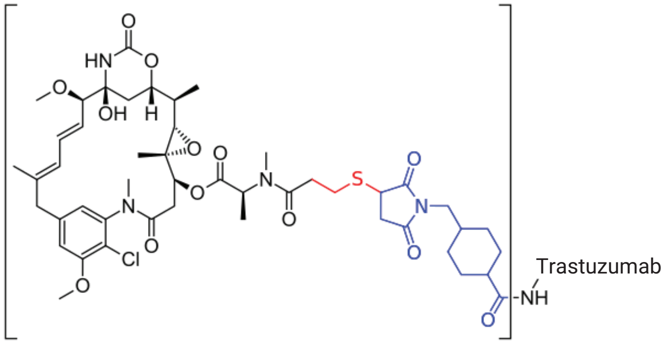

Figure 2. Molecular structure of T-DM1.

To locate the drug conjugation sites of T-DM1, the ADC was reduced, alkylated, and trypsin-digested using the In-Solution Digestion workflow on the AssayMAP Bravo platform. The digestion workflow was followed by LC/MS/MS analysis using the 6545XT LC/Q-TOF system coupled to the 1290 Infinity II bio LC system.

The DM1 payload has strong ultraviolet (UV) absorbance at 252 nm due to the presence of an aromatic ring and other chromophores in the structure. A comparison between UV chromatograms at 214 and 252 nm indicates that the main elution region of drug-conjugated peptides is from 36 minutes onwards (Figures 3A and 3B). This is further confirmed by the extracted ion chromatogram (EIC) of product ion *m/z* 547.22 (Figure 3C). This signature ion was produced from DM1 fragmentation during MS/MS. The conjugation of DM1 increases the overall hydrophobicity of the peptides, causing them to elute later in reversed-phase LC conditions.

An average sequence coverage of 94% was achieved for T-DM1. Figure 4 displays the overlaid biomolecule MS chromatograms of the peptides included in the coverage map from a single sample.

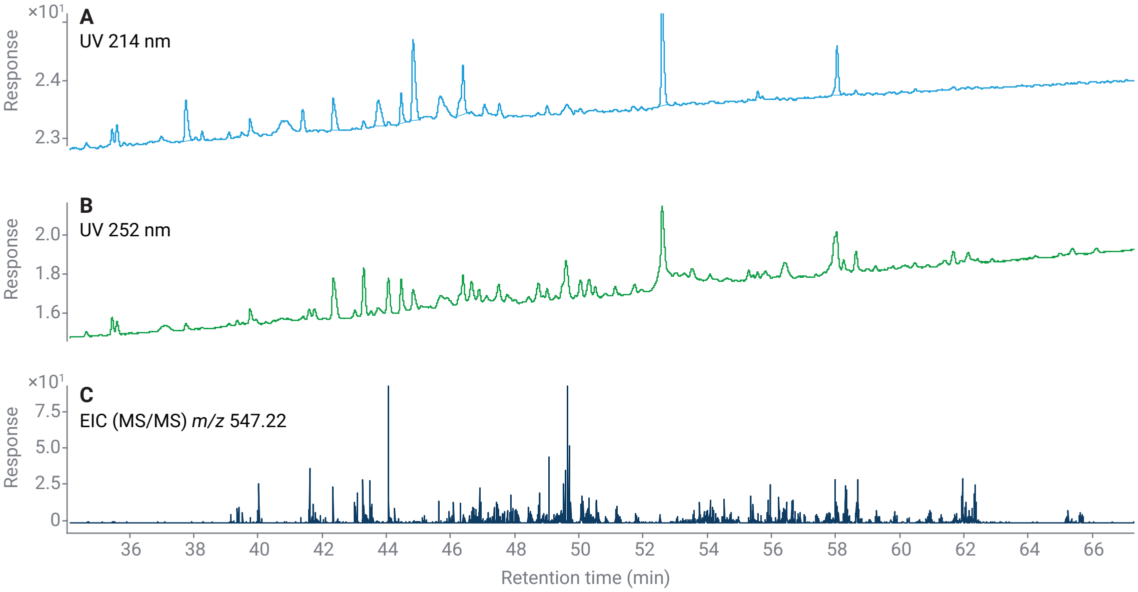

Figure 3. UV chromatograms of T-DM1 at (A) 214 nm and (B) 252 nm, and (C) the extracted ion chromatogram of T-DM1 product ion *m/z* 547.22.

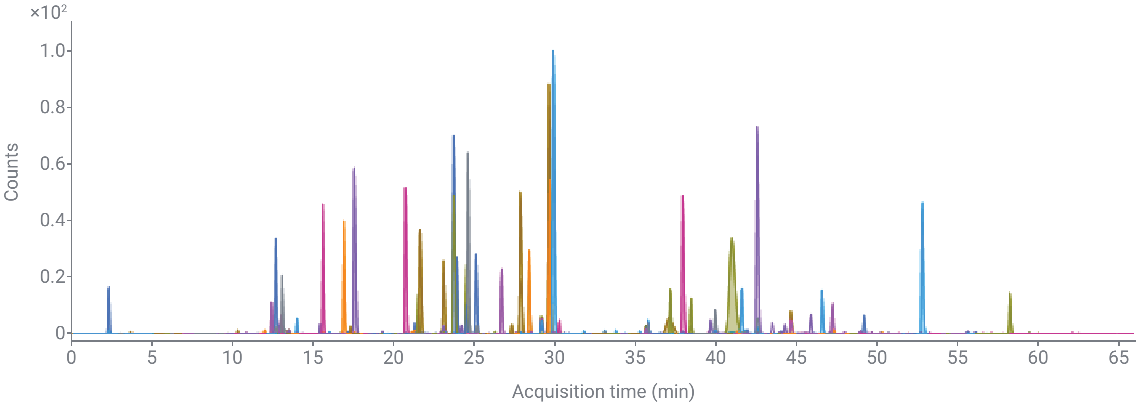

Figure 4. Overlaid biomolecule MS chromatograms of trypsin-digested T-DM1.

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Among these peptides, BioConfirm software automatically identified 26 out of 44 lysine sites with conjugated MCC-DM1. The mass accuracy of all identified drug-conjugated peptides was within 3 ppm. The site locations and peptide sequences are listed in Table 4.

Table 4. MCC-DM1 conjugated peptides identified in T-DM1. The conjugation sites are marked with an asterisk (*).

No.	Chain	Site	Sequence	RT (min)	Delta Mass (ppm)
1	Light	K42	YQQKPGK*APK	41.73	0.70
2	Light	K107	VEIK*R	50.37	1.03
3	Light	K145	EAK*VQWK	51.27	1.11
4	Light	K188	ADYEK*HK	43.47	1.74
5	Light	K190	HK*VYACEVTHQGLSSPVTK	43.30	2.15
6	Light	K207	VYACEVTHQGLSSPVTK*SFNR	50.23	0.58
7	Heavy	K30	LSCAASGFNIK*DTYIHWVR	58.42	1.05
8	Heavy	K43	QAPGK*GLEWVAR	55.43	0.28
9	Heavy	K65	YADSVK*GR	49.53	1.32
10	Heavy	K76	FTISADTSK*NTAYLQMNSLR	55.9	1.96
11	Heavy	K136	GPSVFPLAPSSK*STSGGTAALGCLVK	59.34	0.63
12	Heavy	K208	ICNVNHK*PSNTK	41.86	0.76
13	Heavy	K213	ICNVNHKPSNTK*VDK	39.39	1.77
14	Heavy	K216	VDK*K	48.28	0.86
15	Heavy	K217	K*VEPK	47.03	0.93
16	Heavy	K225	SCDK*THTCPPCPAPELLGGPSVFLFPPKPK	56.47	1.05
17	Heavy	K249	THTCPPCPAPELLGGPSVFLFPPK*PK	62.15	0.16
18	Heavy	K251	LFPPKPK*DTLMISR	54.10	0.69
19	Heavy	K291	FNWYVDGVEVHNAK*TKPR	49.09	0.18
20	Heavy	K293	TK*PR	46.72	0.26
21	Heavy	K323	EYK*CK	46.75	0.93
22	Heavy	K325	CK*VSNK	46.08	0.84
23	Heavy	K329	VSNK*ALPAPIEK	52.66	0.31
24	Heavy	K337	ALPAPIEK*TISK	55.56	0.26
25	Heavy	K343	AK*GQPR	46.47	0.00
26	Heavy	K417	LTVDK*SR	50.48	0.81

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Each identified sequence was verified through the following three criteria. LTVDKSR peptide with MCC-DM1 conjugated on lysine was used as an example in Figure 5 to illustrate the verification process.

- First, high quality MS/MS spectra with credible b and y ions were required to cover the peptide sequence. The fragment confirmation ladder feature in BioConfirm 12.1 software marks b and y ions based on their occurrence in the MS/MS spectra. This feature offers a rapid assessment of the quality of the MS/MS spectra for the biomolecule (Figure 5A). The enhanced peptide mapping algorithm in BioConfirm software also largely reduces artifacts for improved variable modification assignment, in this case MCC-DM1 modification.
- Second, the stereocenter in the DM1 molecule causes the drug-conjugated peptide to elute chromatographically as duplet peaks. The EIC of the peptide precursor ion confirms the presence of the stereoisomers (Figure 5B).
- Third, during peptide fragmentation, DM1 was also partially fragmented, generating DM1-associated ions. As shown in Figure 3C, *m/z* 547.22 is the most common and abundant fragment ion of DM1. Additionally, ions such as *m/z* 140.07, 453.19, and 485.22 are also present in the MS/MS spectra of the drug-conjugated peptides (Figure 5C). Furthermore, the loss of *m/z* 546.21 on y ions is commonly observed as partially fragmented DM1-linker-peptides.

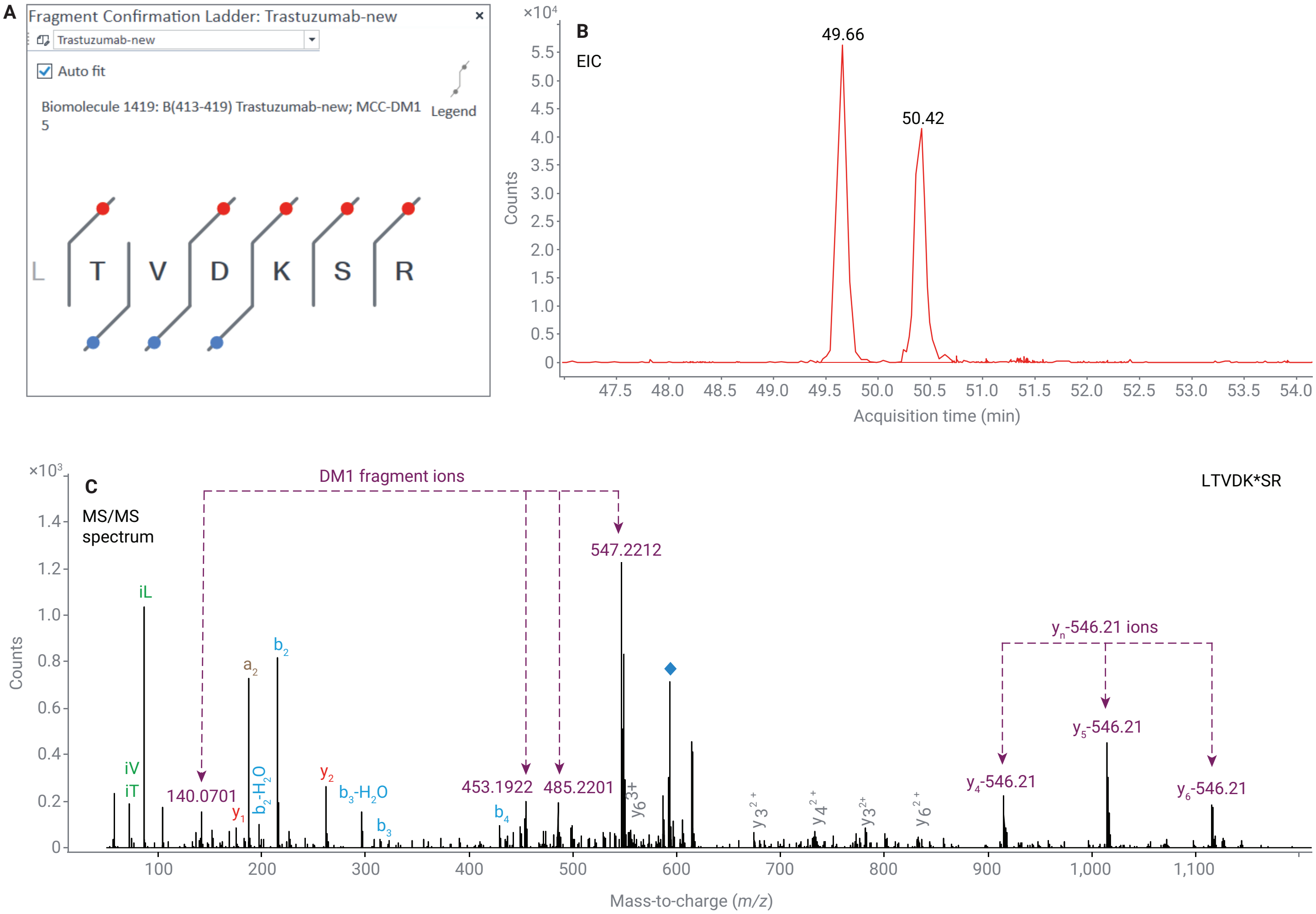

Figure 5. LTVDKSR peptide with MCC-DM1 modification on lysine. (A) The fragment confirmation ladder view in BioConfirm 12.1, (B) the extracted ion chromatogram (EIC) of precursor ion *m/z* 592.29^{3+}, and (C) the MS/MS spectra of LTVDKSR with MCC-DM1 modification.

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The results also showed excellent reproducibility between seven replicated samples. Figure 6 shows overlaid biomolecule MS chromatograms of drug-conjugated peptides, including YQQKPGKAPK from the light chain and ALPAPIEKTISK from the heavy chain. Retention time (RT) relative standard deviations (RSDs) for both peptides were 0.27 and 0.03%, respectively. RSDs of abundance, in terms of signal volume, were 4.80 and 5.01%, respectively. This superior reproducibility is attributed to the automated sample preparation, reliable acquisition engine, and advanced software algorithm.

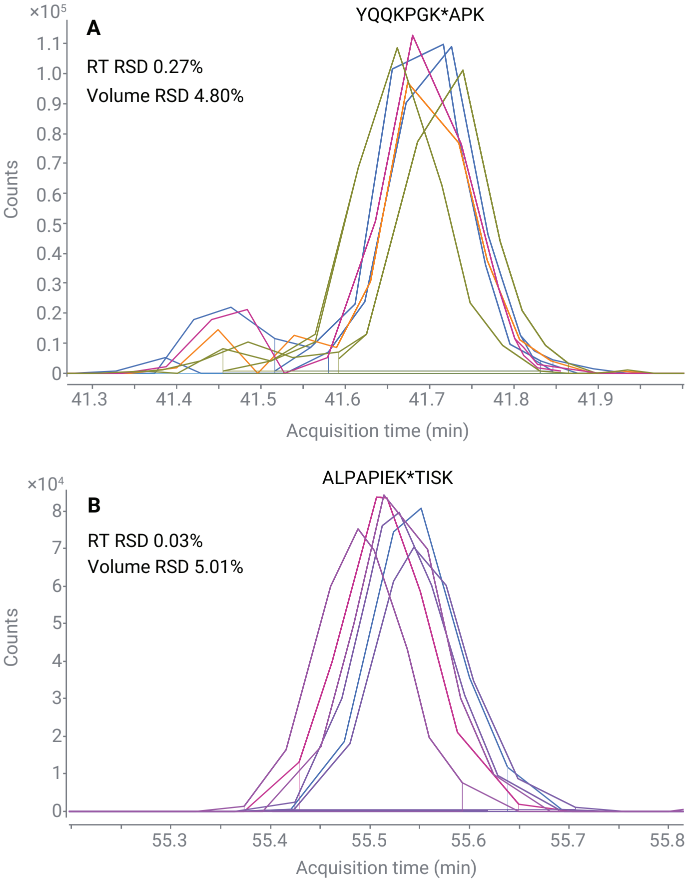

Figure 6. Overlaid biomolecule MS chromatograms of drug-conjugated peptides (A) YQQKPGK*APK and (B) ALPAPIEK*TISK.

Conclusion

In this application note, a lysine-linked ADC, T-DM1, was analyzed using the Agilent peptide mapping workflow to identify its drug conjugation sites. This workflow included an Agilent AssayMAP Bravo protein sample prep platform for automated sample preparation, an Agilent 1290 Infinity II bio LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF system, and Agilent MassHunter BioConfirm software. Using this workflow, 26 lysines were confirmed to be drug-conjugated. These results demonstrate that the Agilent peptide mapping workflow enables accurate and reproducible identification of drug conjugation sites in ADCs.

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Application Note

Biotherapeutics &
Biosimilars

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

Authors

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Muhammed Naqvi
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Pleasanton, CA

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.

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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.

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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.

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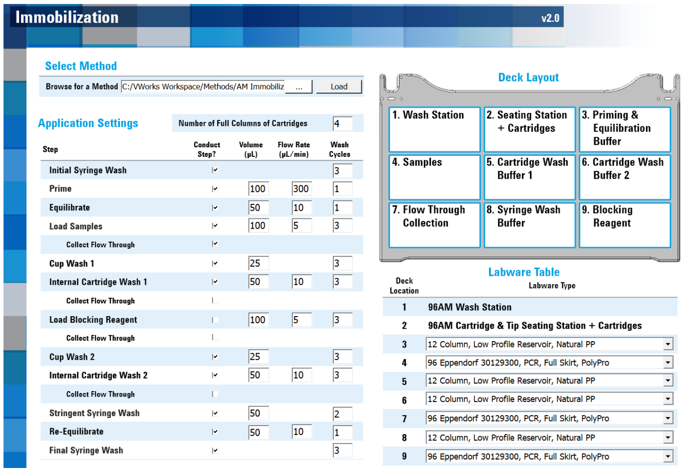
LC/MS analysis

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



Immobilization v2.0

Select Method

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Application Settings

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Prime	☑	100	300	1
Equilibrate	☑	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		3
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Re-Equilibrate	☑	50	10	1
Final Syringe Wash	☑			3

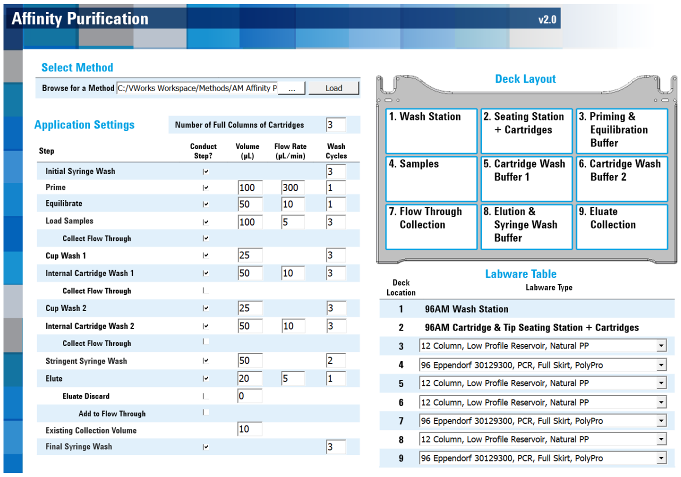
Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Figure 2. Agilent AssayMAP Immobilization Application settings.



Affinity Purification v2.0

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Equilibrate	☑	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☑	25		3
Internal Cartridge Wash 1	☑	50	10	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Elute	☑	20	5	1
Eluate Discard	☐	0		
Add to Flow Through	☐			
Existing Collection Volume		10		
Final Syringe Wash	☑			3

Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Elution & Syringe Wash Buffer	9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

Figure 3. Agilent AssayMAP Affinity Purification Application settings.

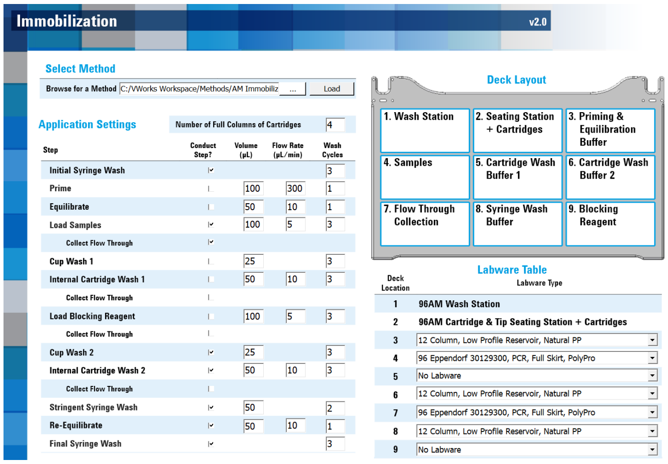
Table 2. MS acquisition parameters.

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



Select Method

Browse for a Method: C:/VWorks Workspace/Methods/AM Immobiliz

Application Settings

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☐	100	300	1
Equilibrate	☐	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☑			
Cup Wash 1	☐	25		3
Internal Cartridge Wash 1	☐	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☑	25		3
Internal Cartridge Wash 2	☑	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Re-Equilibrate	☑	50	10	1
Final Syringe Wash	☑			3

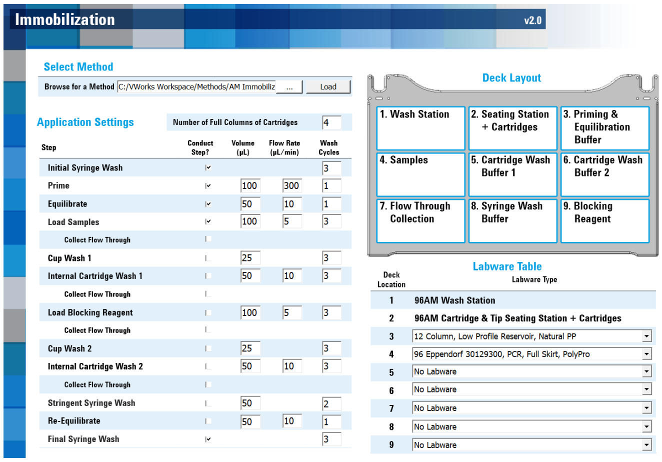
Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	No Labware
6	12 Column, Low Profile Reservoir, Natural PP
7	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
8	12 Column, Low Profile Reservoir, Natural PP
9	No Labware

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



Select Method

Browse for a Method: C:/VWorks Workspace/Methods/AM Immobiliz

Application Settings

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Prime	☑	100	300	1
Equilibrate	☑	50	10	1
Load Samples	☑	100	5	3
Collect Flow Through	☐			
Cup Wash 1	☐	25		3
Internal Cartridge Wash 1	☐	50	10	3
Collect Flow Through	☐			
Load Blocking Reagent	☐	100	5	3
Collect Flow Through	☐			
Cup Wash 2	☐	25		3
Internal Cartridge Wash 2	☐	50	10	3
Collect Flow Through	☐			
Stringent Syringe Wash	☐	50		2
Re-Equilibrate	☐	50	10	1
Final Syringe Wash	☑			3

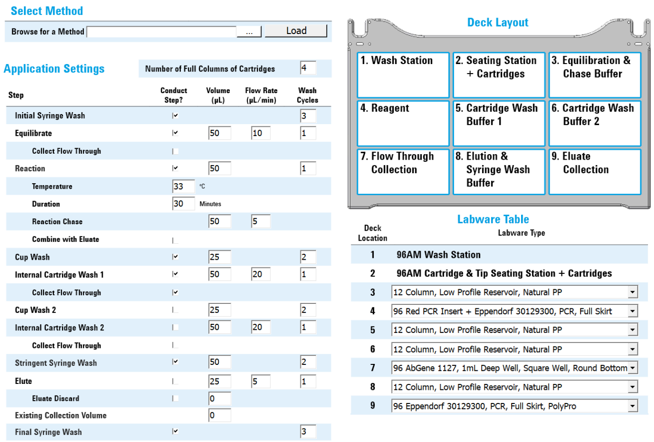
Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Priming & Equilibration Buffer
4. Samples	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Syringe Wash Buffer	9. Blocking Reagent

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro
5	No Labware
6	No Labware
7	No Labware
8	No Labware
9	No Labware

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


Select Method

Browse for a Method:

Application Settings

Number of Full Columns of Cartridges: 4

Step	Conduct Step?	Volume (µL)	Flow Rate (µL/min)	Wash Cycles
Initial Syringe Wash	☑			3
Equilibrate	☑	50	10	1
Collect Flow Through	☐			
Reaction	☑	50		1
Temperature	33 °C			
Duration	30 Minutes			
Reaction Chase		50	5	
Combine with Eluate	☐			
Cup Wash	☑	25		2
Internal Cartridge Wash 1	☑	50	20	1
Collect Flow Through	☑			
Cup Wash 2	☐	25		2
Internal Cartridge Wash 2	☐	50	20	1
Collect Flow Through	☐			
Stringent Syringe Wash	☑	50		2
Elute	☐	25	5	1
Eluate Discard	☐	0		
Existing Collection Volume		0		
Final Syringe Wash	☑			3

Deck Layout

1. Wash Station	2. Seating Station + Cartridges	3. Equilibration & Chase Buffer
4. Reagent	5. Cartridge Wash Buffer 1	6. Cartridge Wash Buffer 2
7. Flow Through Collection	8. Elution & Syringe Wash Buffer	9. Eluate Collection

Labware Table

Deck Location	Labware Type
1	96AM Wash Station
2	96AM Cartridge & Tip Seating Station + Cartridges
3	12 Column, Low Profile Reservoir, Natural PP
4	96 Red PCR Insert + Eppendorf 30129300, PCR, Full Skirt
5	12 Column, Low Profile Reservoir, Natural PP
6	12 Column, Low Profile Reservoir, Natural PP
7	96 AbGene 1127, 1mL Deep Well, Square Well, Round Bottom
8	12 Column, Low Profile Reservoir, Natural PP
9	96 Eppendorf 30129300, PCR, Full Skirt, PolyPro

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.

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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.

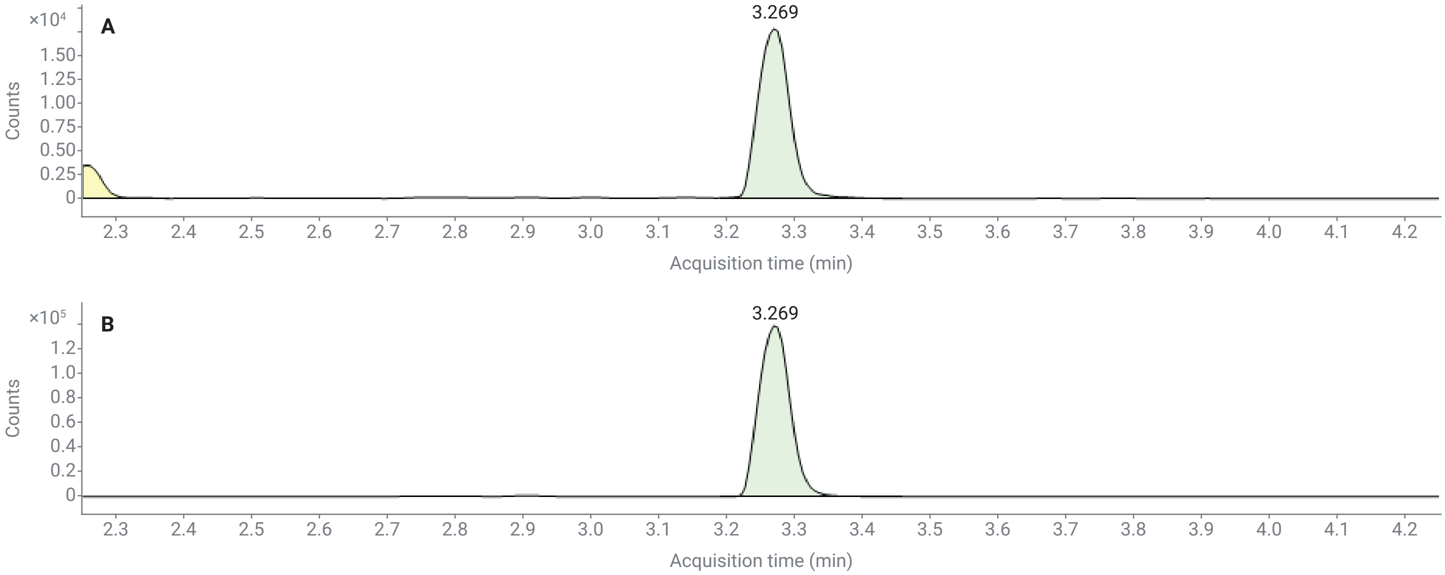

Figure 7. Brentuximab and trastuzumab surrogate peptides MRM chromatograms purified from monkey plasma. (A) VVSVLTVLHQDWLNGK quantifier (603.3 → 805.4) MRM chromatogram at 0.39 µg/mL; (B) VVSVLTVLHQDWLNGK* quantifier (606 → 808.4) MRM chromatogram.

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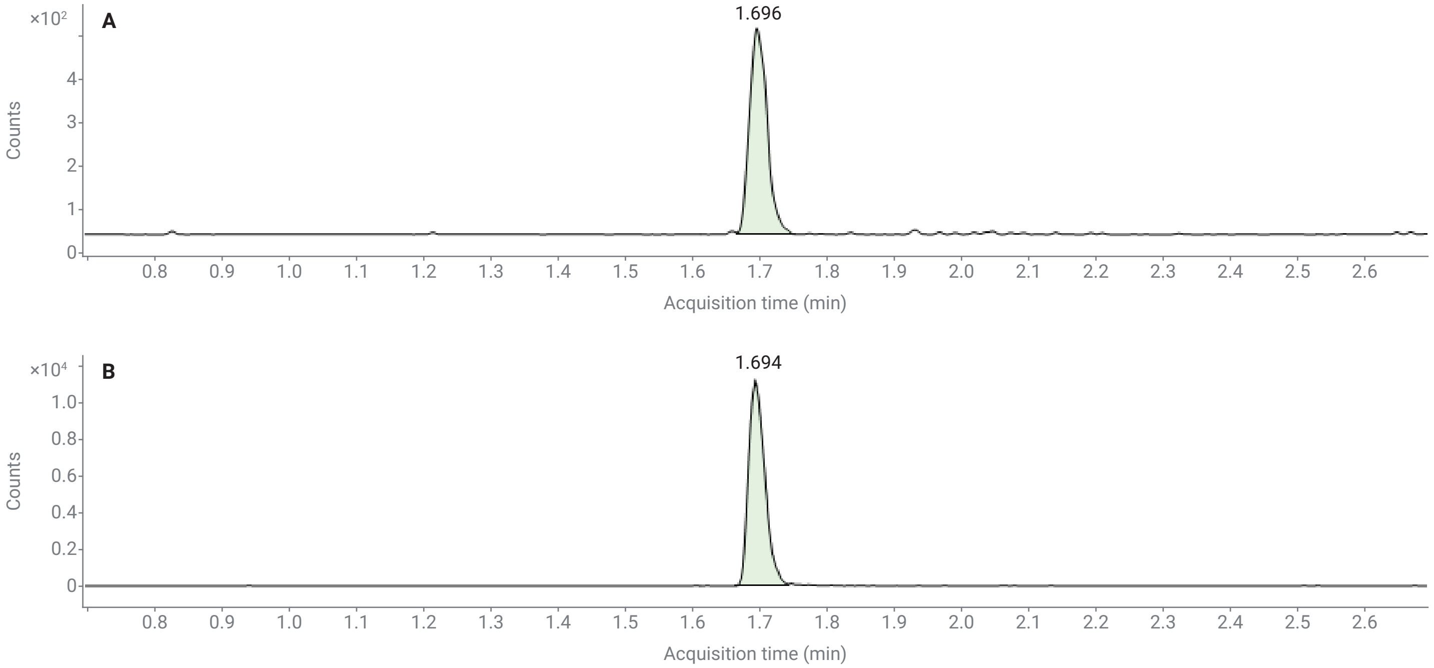

Figure 8. Brentuximab payload and internal standard MRM chromatograms purified from monkey plasma. (A) Payload quantifier (719.0 → 152.2) MRM chromatogram at 0.39 µg/mL; (B) payload internal standard quantifier (727.0 → 152.2) MRM chromatogram.

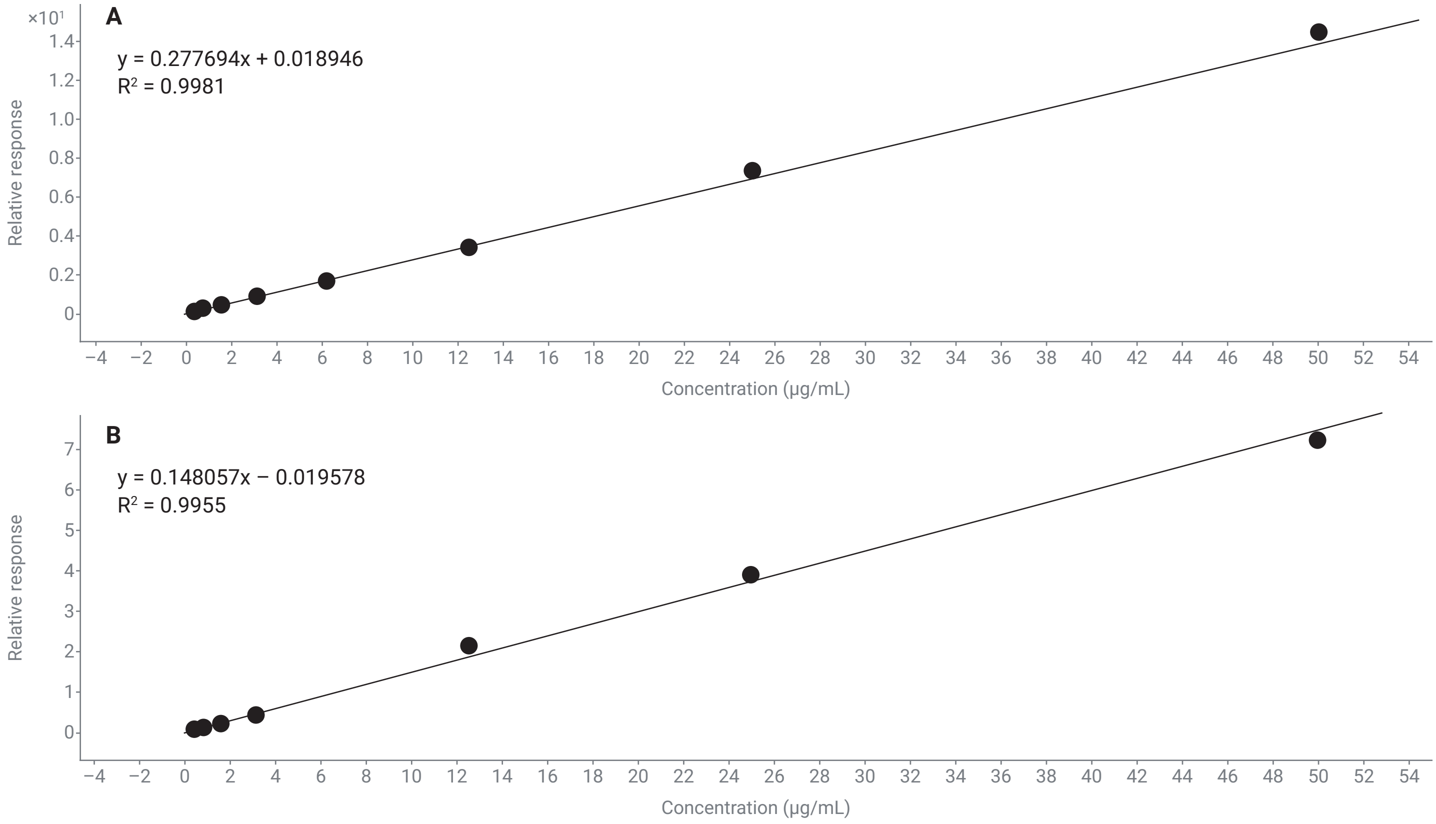

Figure 9. Calibration curve of brentuximab surrogate peptide and payload from 0.39 to 50 µg/mL in monkey plasma. (A) Surrogate peptide VVSVLTVLHQDWLNGK calibration curve; (B) payload calibration curve.

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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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Analysis of Free Drug Content in Antibody-Drug Conjugate Using 2D-LC/Q-TOF

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Abstract

Antibody-drug conjugates (ADCs) are complex therapeutic biomolecules composed of an antibody linked to a potent cytotoxic small molecule drug. ADCs are engineered to specifically target and eliminate cancerous cells while preserving healthy cells. One critical quality attribute (CQA) of ADCs is the free drug content, which is the unconjugated small molecule drug. This free drug content could be caused by incomplete conjugation or formation of a degradation product. The presence of free drug and related species could lead to compromised product efficacy and increased toxicity.

This application note demonstrates a two-dimensional liquid chromatography/quadrupole time-of-flight mass spectrometry (2D-LC/Q-TOF) approach to identify the free drug content of an ADC sample. The method combines size exclusion chromatography (SEC) in the first dimension (^{1}D) and reversed-phase (RP) separation in the second dimension (^{2}D). This method enables sensitive and straightforward analysis of free drug content in ADC without manual protein precipitation.

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Introduction

ADCs are complex therapeutic biomolecules composed of an antibody linked to a potent cytotoxic small molecule drug. The antibody enables targeted delivery of the cancer-killing drug to the tumor site while limiting the toxicity to healthy cells. There are three components within an ADC: a monoclonal antibody (mAb), a small molecule drug, and a linker. The linker reacts with the free drug first, transforming it into a linker-drug compound. This compound is subsequently conjugated to specific amino acid sites on the antibody. The process is illustrated in Figure 1.

Incomplete conjugation results in the presence of unbound drugs within the ADC, potentially leading to heightened toxicity. Therefore, measurement of free drug content is a unique CQA of ADCs. Reversed-phase liquid chromatography (RPLC) can be used for this CQA analysis.[1] However, ADC samples need to be pretreated to remove protein content before injection onto RP columns. Otherwise, the irreversible binding of antibodies to the stationary phase will damage the HPLC columns. Cleanup approaches include solid phase extraction (SPE)[2] and protein precipitation[3] with organic solvent. However, these manual and offline procedures are tedious and time consuming.

In the recent years, 2D-LC technology has been proven to be reliable and efficient in bioseparation of mAbs, ADCs, oligonucleotides, and related impurities. Different combinations of separation mechanisms have been reported.[4,5] Heart-cutting mode is the most commonly used 2D-LC mode whereby only the eluent of interest from ^{1}D is cut and transferred into ^{2}D for further separation. This mode largely reduces the complexity of the analysis.

In this study, we used a heart-cutting 2D-LC coupled to an LC/Q-TOF MS to identify free drug content in an ADC sample. The analytical components are depicted in Figure 2. This approach involved initially separating the ADC from small molecular species by SEC in the ^{1}D, and then achieved effective separation of free drug and associated impurities by RPLC in ^{2}D. This automated online protein removal procedure enhanced operational efficiency.

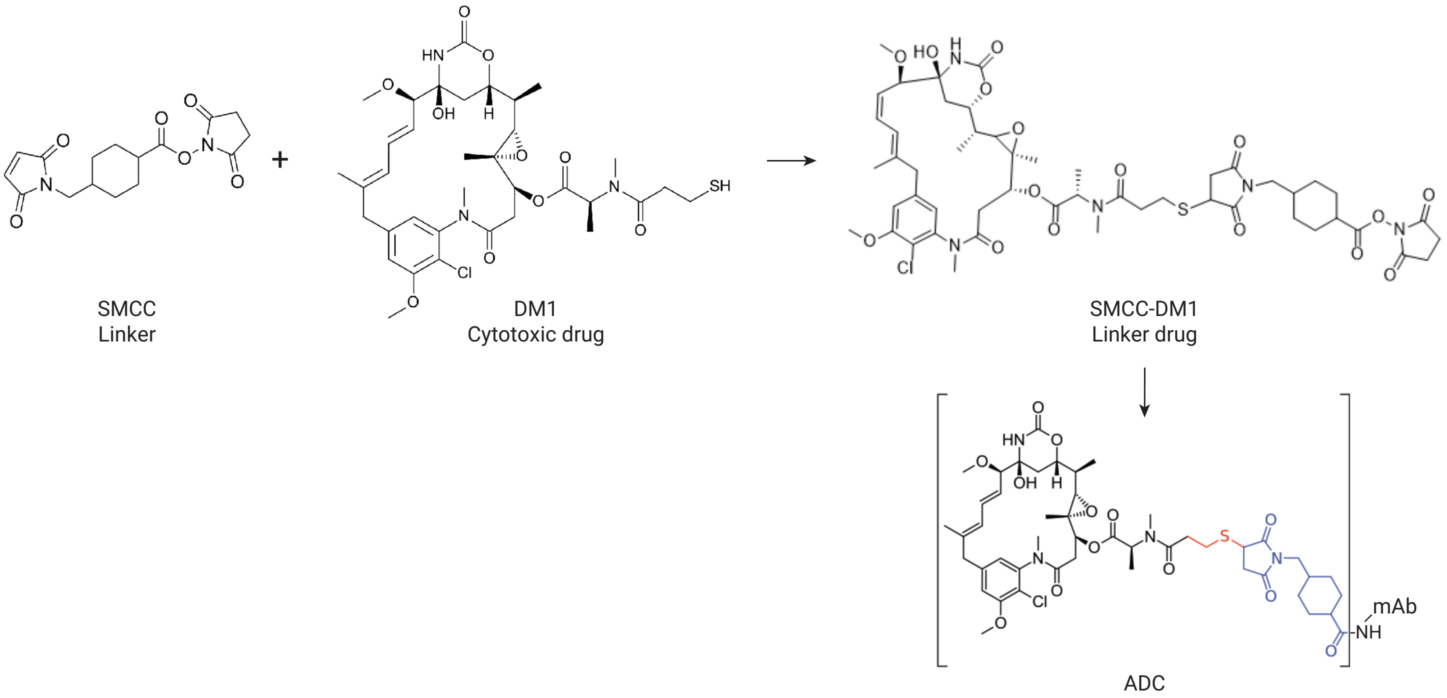

Figure 1. ADC conjugation process.

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Agilent AdvanceBio SEC (first dimension)

Agilent Poroshell C18 (second dimension)

Agilent 1290 Infinity II Bio 2D-LC System

Agilent 6545XT AdvanceBio LC/Q-TOF System

Agilent MassHunter Software Suite

Figure 2. Analytical components of the Agilent Bio 2D-LC/Q-TOF analysis of ADC free drug content.

Experimental

Materials and methods

Ammonium acetate and acetonitrile (ACN, LC/MS grade) were purchased from Merck Millipore (Burlington, MA, USA). Formic acid (FA, LC/MS grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was collected from an in-house Merck Millipore Milli-Q system (Burlington, MA, USA). The ADC sample was purchased from Alliance Pharm (Singapore, SG). The free drug (DM1) and linker drug (SMCC-DM1) standards were purchased from BroadPharm (San Diego, CA, USA).

Sample preparation

The ADC sample was desalted and dissolved in 100 mM ammonium acetate buffer (pH 7.0). The concentration was adjusted to 5 mg/mL before injection.

The DM1 and SMCC-DM1 were separately weighed and dissolved in 50% ACN, creating two individual stock solutions at a concentration of 5,000 µg/mL. Both compounds were then spiked to the ADC sample, resulting in a final spike concentration of 100 µg/mL for each compound.

Instrumentation

- Agilent 1290 Infinity II Bio 2D-LC including:
  - Two Agilent 1290 Infinity II Bio High Speed Pumps (G7132A) with Agilent Bio Jet Weaver mixer kit, 35 µL volume (G7132-68135)
  - Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (option #101, G4761A)
  - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with Agilent Quick-Connect Bio Heat-Exchanger, standard flow (option #065, G7116-60071)
  - Three Agilent 1290 Infinity Valve Drives (G1170A) equipped with 1x Agilent InfinityLab Bio 2D-LC ASM Valve (G5643B), 2x Agilent Multiple Heart-Cutting Valves with biocompatible 40 µL loops
  - Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) equipped with an Agilent Bio Standard Flow Cell for VWD (option #028, G1314-60188)
  - Agilent 1290 Infinity II Diode Array Detector FS (G7117A) equipped with biocompatible InfinityLab Max-Light Cartridge Cell (G7117-60020)
- Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent Dual Jet Stream ESI source

Software

- Agilent MassHunter Acquisition software 11.0
- Agilent MassHunter Qualitative Analysis software 11.0

2D-LC/MS analysis

Table 1. Liquid chromatography parameters.

Parameter	Value
First Dimension	
Column	Agilent AdvanceBio SEC 200 Å, 4.6 × 150 mm, 1.9 µm (PL1580-3201)
Thermostat	6 °C
Solvent A	100 mM ammonium acetate
Solvent B	Acetonitrile
Gradient	Isocratic, 40% B
Column Temperature	25 °C
Flow Rate	0.25 mL/min
Injection Volume	10 µL
UV Detection	252 nm at 20 Hz data rate
Second Dimension	
Column	Agilent Poroshell EC-C18, 3.0 × 50 mm, 1.9 µm (699675-302)
Solvent A	0.1% Formic acid
Solvent B	0.1% Formic acid + 95% acetonitrile/H_2O
LC Mode	Heart-cutting
Flow Rate	0.5 mL/min
Stop Time	17 min
Sampling Table	9.0 min, Time-based heart cut, HiRes 3 × 7.68 s, multi-inject: yes
Cycle Time	Analysis: 5 min Equilibration: 0.7 min
Gradient	38 to 65% B in 5 min
Flush Gradient	Time (min) %B 0 38 0.05 65 Duration 0.8 min, equilibration 0.7 min
Column Temperature	40 °C
UV Detection	252 nm at 20 Hz data rate

Table 2. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameters	**Value**
Source	Agilent Jet Stream ESI
Polarity	Positive
Drying Gas Temperature	300 °C
Drying Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Fragmentor	135 V
Skimmer	65 V
Quad amu	750 V
Mass Range	*m/z* 100 to 1,700
Acquisition Rate	1 spectra/s
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Reference Mass	922.009798

Results and discussion

First dimension SEC method development

SEC is a widely used technique to separate size variant molecules inside a given protein sample. A typical elution order is aggregates, followed by monomers, and then low molecular weight (Mw) species. Therefore, SEC is an ideal separation mechanism to be employed as the first dimension to set free drug content apart from the ADC.

Buffer solution at a neutral pH is usually adopted as an SEC mobile phase for protein analysis. Considering the compatibility with MS detection, ammonium acetate was selected over phosphate buffer as the SEC mobile phase in this study.

However, SEC separation of ADCs poses unique challenges because the conjugated small molecule drug increases the hydrophobicity of ADCs. This results in longer retention of ADC in the SEC column due to hydrophobic interactions with the column. What made it more difficult was that the drug and drug-linker compounds, which were naturally small and hydrophobic, would not elute with pure aqueous mobile phase. In fact, DM1 and SMCC-DM1 were fully retained on the column under aqueous conditions.

Introduction of a low percentage of organic solvent such as methanol, isopropanol, or acetonitrile can mitigate the hydrophobic interactions and facilitate the elution of both protein and small molecules. In our case, ACN was chosen as the organic modifier because of the low pressure generated in the column. The concentration of ACN was scouted from 20 to 40% in increments of 5% for the analysis of the DM1 standard. Figure 3 showed that 40% acetonitrile rendered the best peak shape of the DM1. This percentage of organic modifier falls within the organic solvent tolerance limit of 50% for the AdvanceBio SEC column. Therefore, it is safe to use without worry of column damage.

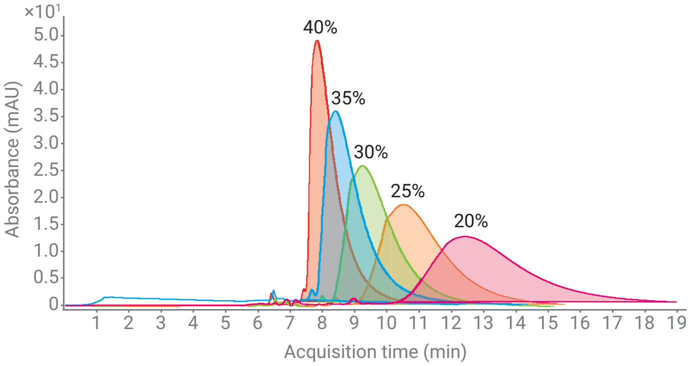

Figure 3. Scouting of ACN concentration from 20 to 40% in ^{1}D SEC for the DM1 standard.

Second dimension sampling

Using the 40% ACN in 100 mM ammonium acetate pH 7.0 as SEC mobile phase, the separation between ADC and free drug content was achieved in the 100 µg/mL spiked sample at the ^{1}D as illustrated in Figure 4. However, DM1 and SMCC-DM1 coeluted as a broad peak from 8 to 10 minutes in the spiked samples. This is partly because the Mw difference between the two molecules was not significant enough for separation under SEC. Moreover, the spiked DM1 and SMCC-DM1 could have had interactions with ADC at the column head during the initial stage of separation, which may have caused the broadening of the peaks.

Three fractions of the broad peak, as shaded in Figure 4, were sampled into three loops through multi-inject sampling mode. All three cuts were sequentially transferred to the ^{2}D in one shot and analyzed within one single ^{2}D gradient cycle. The multi-inject mode enabled sampling of a broad ^{1}D peak using standard 40 µL loops without hardware modification. It also reduced the run time by analyzing multiple cuts within one ^{2}D cycle. The total method time was only 17 minutes.

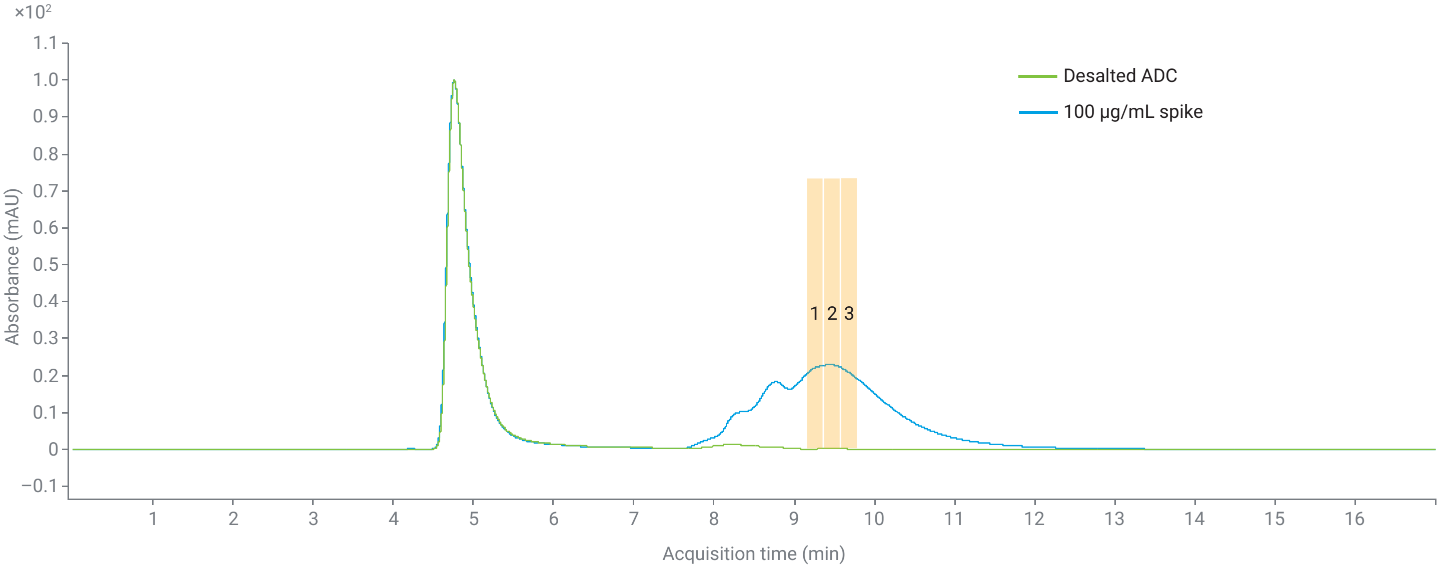

Figure 4. Overlaid UV chromatograms of first dimension SEC separation of desalted ADC (green) and 100 µg/mL spiked desalted ADC (blue). The shaded areas represent the three continuous cuts sampled into ^{2}D using multi-inject mode.

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Second dimension reversed-phase LC/MS analysis of DM1 and SMCC-DM1

Since RPLC has a proven track record of separating hydrophobic small molecules, it was chosen as the ^{2}D separation mechanism. A total of 96 µL of ^{1}D effluent was transferred onto a ^{2}D RP column. Three peaks were detected and separated in both UV and total ion chromatogram (TIC) as shown in Figure 5.

Peak 1 was confirmed as DM1 based on the measured mass of 738.2839 Da. The mass accuracy of it was 2.30 ppm from theoretical mass 738.2822 Da. Sodium and potassium adducts presented high abundance, which could be attributed to the use of buffer salt in ^{1}D SEC and its subsequent introduction to the ^{2}D during sampling. The neutral loss of water fragment was also detected due to in-source fragmentation.

Peak 2 and 2' have identical MS spectra. The mass of the $[M+H]^+$ ion was 1,072.3985 Da, which was confirmed as SMCC-DM1. The mass accuracy was 0.18 ppm from the theoretical mass of 1,072.3987 Da. This doublet peak phenomenon was caused by the presence of a stereocenter in the SMCC-DM1 molecule.[6] The two peaks are diastereomers that have identical mass.

Applying the 2D-LC/Q-TOF technique, the free drug content was separable from ADC in SEC, and then the individual components were successfully separated from each other in RP and confirmed by MS.

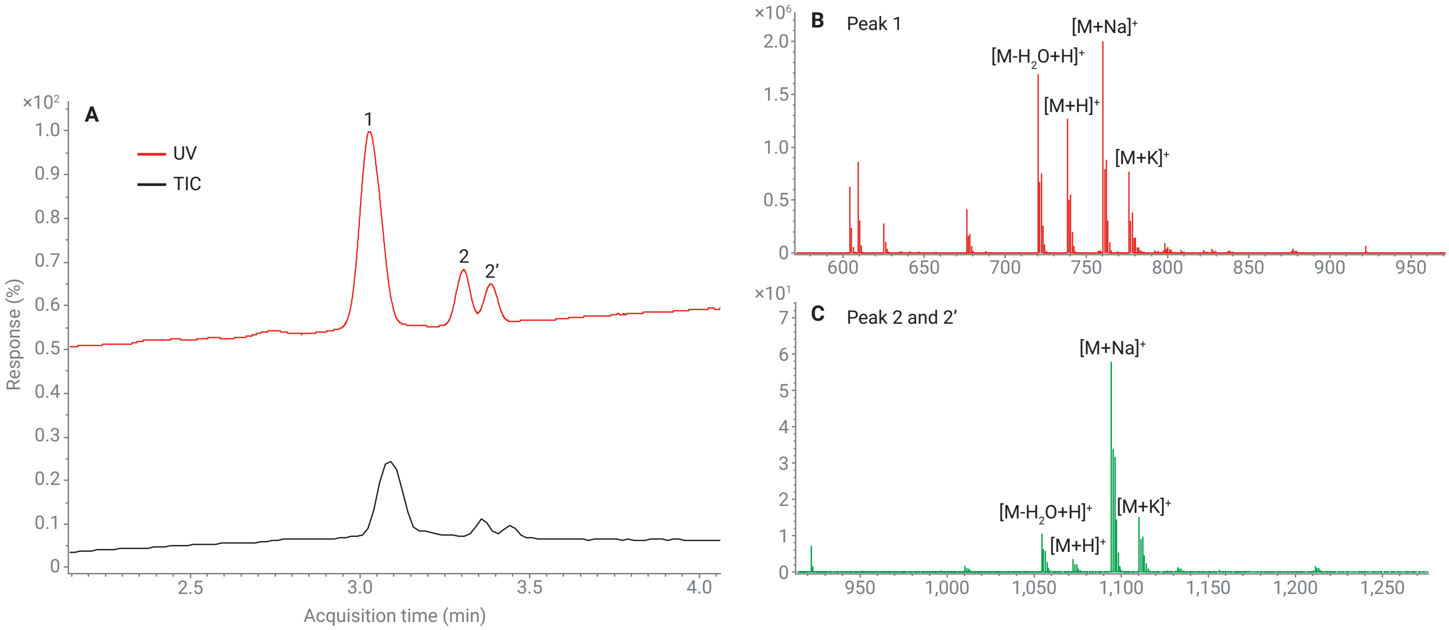

Figure 5. Identification of DM1 and SMCC-DM1 by second dimension LC/MS analysis. (A) UV and total ion chromatogram, (B) MS spectra of DM1, and (C) MS spectra of SMCC-DM1.

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Conclusion

Agilent has developed an innovative and effective two-dimensional liquid chromatography/quadrupole time-of-flight mass spectrometry (2D-LC/Q-TOF) method for the identification of free drug content in antibody-drug conjugates (ADCs). This solution uses the Agilent 1290 Infinity II Bio 2D-LC with the Agilent AdvanceBio SEC column, the Agilent Poroshell EC-C18 column, and the Agilent 6545XT AdvanceBio LC/Q-TOF. The Agilent MassHunter Workstation for LC/TOF and LC/Q-TOF 11.0 and Agilent MassHunter Qualitative 11.0 software were used for data acquisition and analysis.

This method integrates protein elimination and free drug identification into one analysis. Automated protein removal saves time and safeguards the RP column from deterioration. Moreover, the method achieves superior chromatographic separation between the drug and the linker-drug and enables reliable identification through accurate mass detection.

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Application Note

Biopharma

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Trusted Answers

Evaluation of SEC Columns for Analysis of ADC Aggregates and Fragments

Choosing the best stationary phase to overcome nonspecific interactions

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Abstract

Antibody drug conjugates (ADCs) use the specificity of a monoclonal antibody (mAb) to ensure that cytotoxic drugs are delivered to the right target. The use of ADCs is a highly efficient way of tackling various diseases. There are already ten ADC biotherapeutics that are approved and commercialized with many more in development. An example ADC is illustrated in Figure 1.

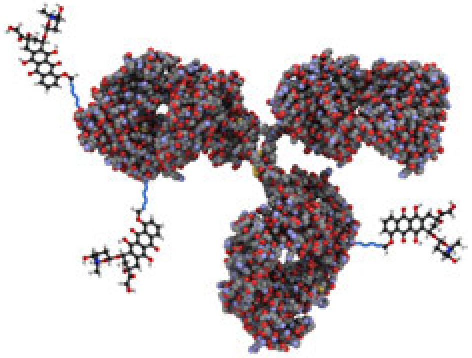

Figure 1. General structure of an antibody drug conjugate, courtesy of Quality Assistance S.A.

As with any biotherapeutic, the analysis of critical quality attributes is paramount. However, this analysis is complex due to the presence of the cytotoxic drugs attached to the antibody. Size exclusion chromatography (SEC) is effective, but still challenging, for the quantification of aggregates and fragments. ADCs are frequently more hydrophobic than mAbs alone and are therefore more susceptible to nonspecific interactions. This application note compares the performance of some of the commercially available SEC columns for the analysis of ADCs.

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Introduction

Currently there are ten approved commercially available ADCs. Brentuximab vedotin and trastuzumab emtansine belong to the two main classes of ADCs: Cys-linked and Lys-linked. Both employ an IgG1 monoclonal antibody as the vehicle. Brentuximab is an anti-CD30 chimeric mAb, and trastuzumab is an anti-HER2 humanized mAb. However, the approach taken to conjugate the small molecule drug differs in these ADCs.

In trastuzumab emtansine, the DM1 drug is conjugated to lysine (Lys) side chains using a noncleavable succinimidyl linker (SMCC). The resulting configuration is a random distribution of drug molecules across the surface of the protein. There are 90 Lys residues throughout the trastuzumab molecule, and each molecule may contain up to eight DM1 conjugates. In brentuximab vedotin, partial reduction of the intrachain disulfide bridges allows for up to eight monomethyl auristatin E (MMAE) drug molecules to be conjugated through cleavable valine-citrulline linkers.

Both approaches modify the hydrophobicity of the underlying mAb, but also present other problems. Trastuzumab emtansine is a heterogeneous mixture due to the random distribution of the drug molecules. Whereas brentuximab vedotin has a more specific distribution but at the expense of the loss of the stabilizing intrachain disulfide bonds.

These features make the analysis of such molecules more complex. Even size exclusion chromatography, the preferred method for quantification of aggregates, is more challenging due to more nonspecific interactions.

This application note explores the differences in stationary phase construction and particle size, both of which can aid the analysis of ADCs. It also determines which products exhibit fewer secondary interactions in the absence of any organic modifier. Using organic modifier to reduce some nonspecific interactions could also alter the level of aggregation observed.

For the purposes of this investigation, a range of commercially available SEC columns were chosen that have features designed to make them more suitable for analysis of complex molecules, like ADCs. Most contain a hydrophilic coating to mask interactions with the underlying silica particles. Some, such as column B from Vendor 1, have differences in the way this coating is attached, which alters the orientation on the surface.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Instrumentation

A UHPLC instrument with UV detection was used. Method parameters are listed in Table 1.

Table 1. Chromatographic conditions.

HPLC Conditions	
Columns	**A:** Agilent AdvanceBio SEC 300 Å, 4.6 × 300 mm, 2.7 µm **B:** Vendor 1 200 Å, 4.6 × 300 mm, 1.7 µm **C:** Vendor 2 300 Å, 4.6 × 300 mm, 1.8 µm
Mobile Phase	Phosphate buffered saline (PBS)
Flow Rate	0.35 mL/min
Column Temperature	30 °C
Injection Volume	5 µL Trastuzumab, 10 µL Brentuximab
Total Run Time	20 minutes

Results and discussion

It is immediately obvious from the shape of the peaks (tailing or even multiple components) where secondary interactions are causing issues. Column A, the Agilent AdvanceBio SEC 300 Å, 2.7 µm column, performs better than columns B and C, despite both having a smaller particle size. Figure 2 highlights how the peak shape and dimer/monomer resolution is superior with the AdvanceBio SEC 300 Å for analyzing the Lys-linked trastuzumab emtansine, compared to the competitor columns B and C. Column B exhibits increased secondary interactions, as shown by a loss of peak resolution. The loss of resolution is probably due to the different type of stationary phase chemistry on column B. Column C gives a slightly narrower peak shape, but the resolution is also inferior versus the AdvanceBio column (Table 2).

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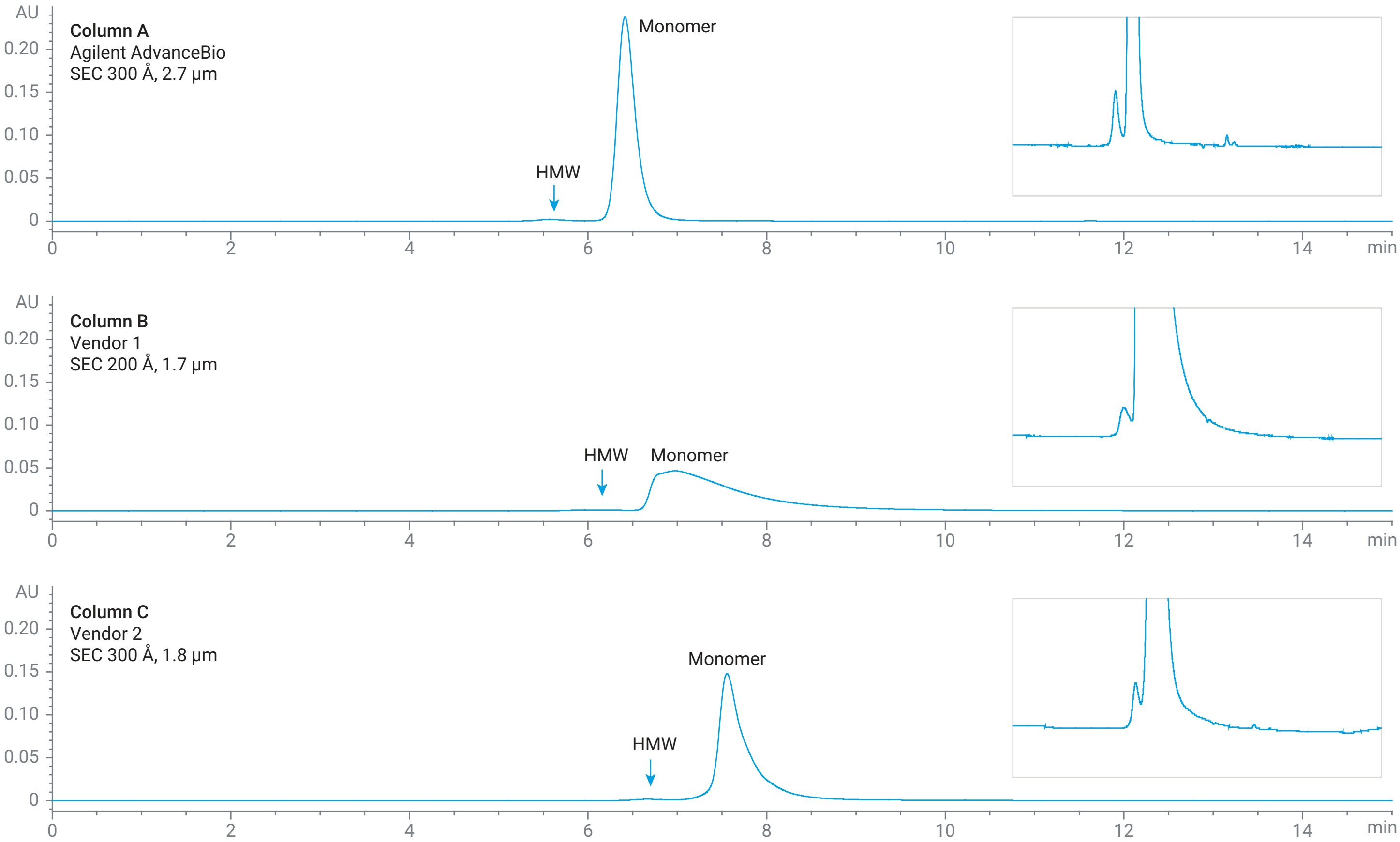

Figure 2. SEC of trastuzumab emtansine using 4.6 × 300 mm SEC columns with PBS, pH 7.4 at 0.35 mL/min. Inset shows full chromatogram zoomed into baseline region (0 to 0.02 mAU).

Table 2. SEC comparison values for trastuzumab emtansine: peak width at half height, tailing factor, percentage of aggregation and resolution.

Column	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
A: Agilent AdvanceBio SEC 300 Å, 2.7 µm	0.22	1.35	98.7	1.3	1.82
B: Vendor 1 200 Å, 1.7 µm	0.98	3.70	98.8	1.2	0.65
C: Vendor 2 300 Å, 1.8 µm	0.28	1.93	98.3	1.7	1.42

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Looking at the Cys-linked brentuximab vedotin in Figure 3, column B is divided into two peaks, which indicates that the stationary phase is interacting with the sample. For column C, a sharp peak for the monomer was obtained but the aggregate percentage confirms that the AdvanceBio SEC column has superior performance when evaluating the HMW species, almost absent in column C (Table 3).

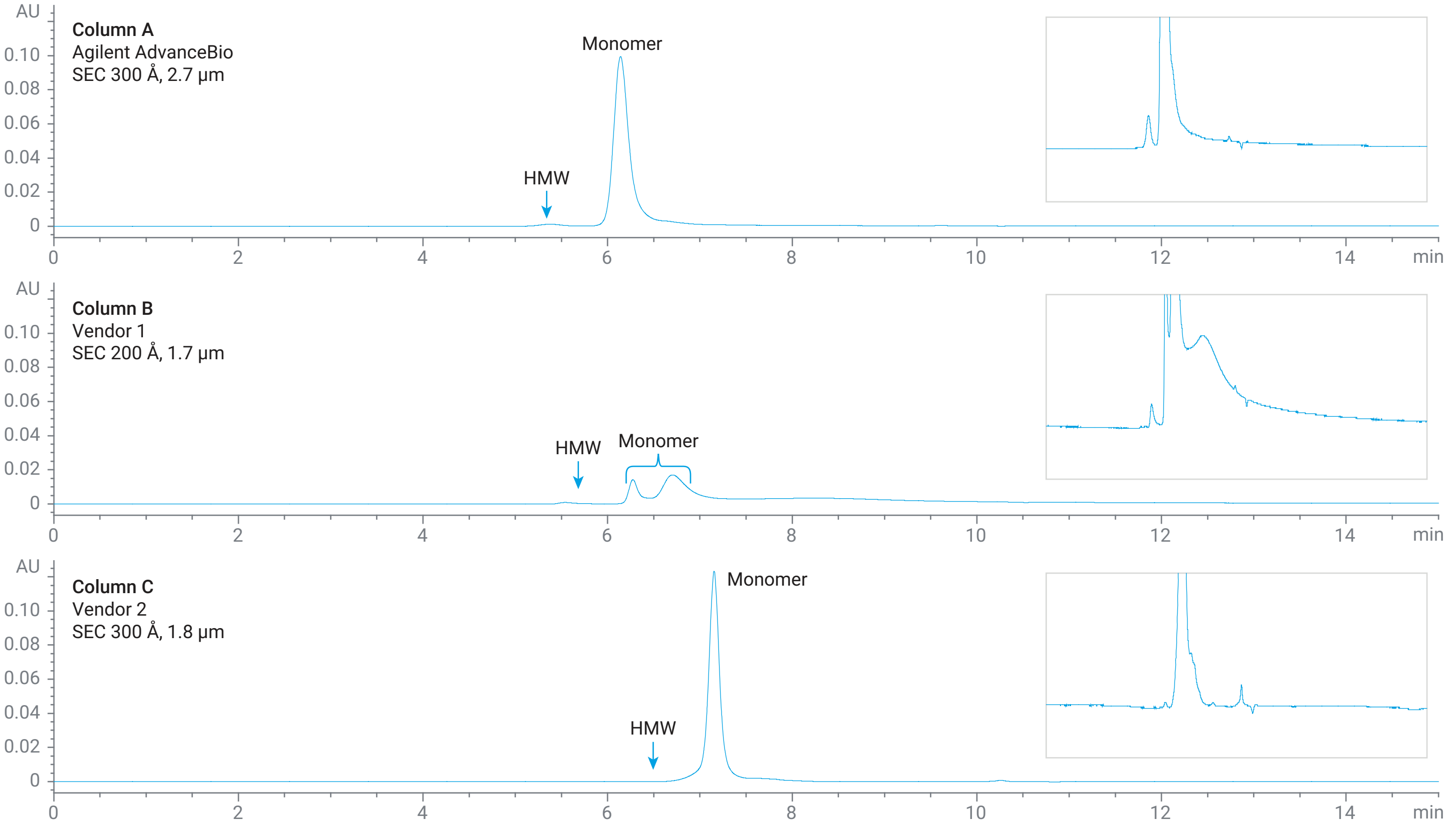

Figure 3. SEC of brentuximab vedotin using 4.6 × 300 mm SEC columns with PBS, pH 7.4 at 0.35 mL/min. Inset shows full chromatogram zoomed into baseline region (0 to 0.02 mAU).

Table 3. SEC comparison values for brentuximab vedotin: peak width at half height, tailing factor, percentage of aggregation and resolution.

Column	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
A: Agilent AdvanceBio SEC 300 Å, 2.7 µm	0.17	1.47	98.3	1.7	2.11
B: Vendor 1 200 Å, 1.7 µm	Not applicable	Not applicable	99.0	1.0	Not applicable
C: Vendor 2 300 Å, 1.8 µm	0.12	1.0	99.8	0.2	3.62

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The AdvanceBio SEC 300 Å, 2.7 µm column was also tested under several mobile phase conditions to better understand the chromatography, and to improve peak shape and resolution for both ADCs. This evaluation confirms the influence of pH ranging from 6.8 to 7.4, as shown in Figures 4 and 5. Higher resolution gives more accurate dimer/monomer detection and is achieved at pH 7.4 (Tables 4 and 5).

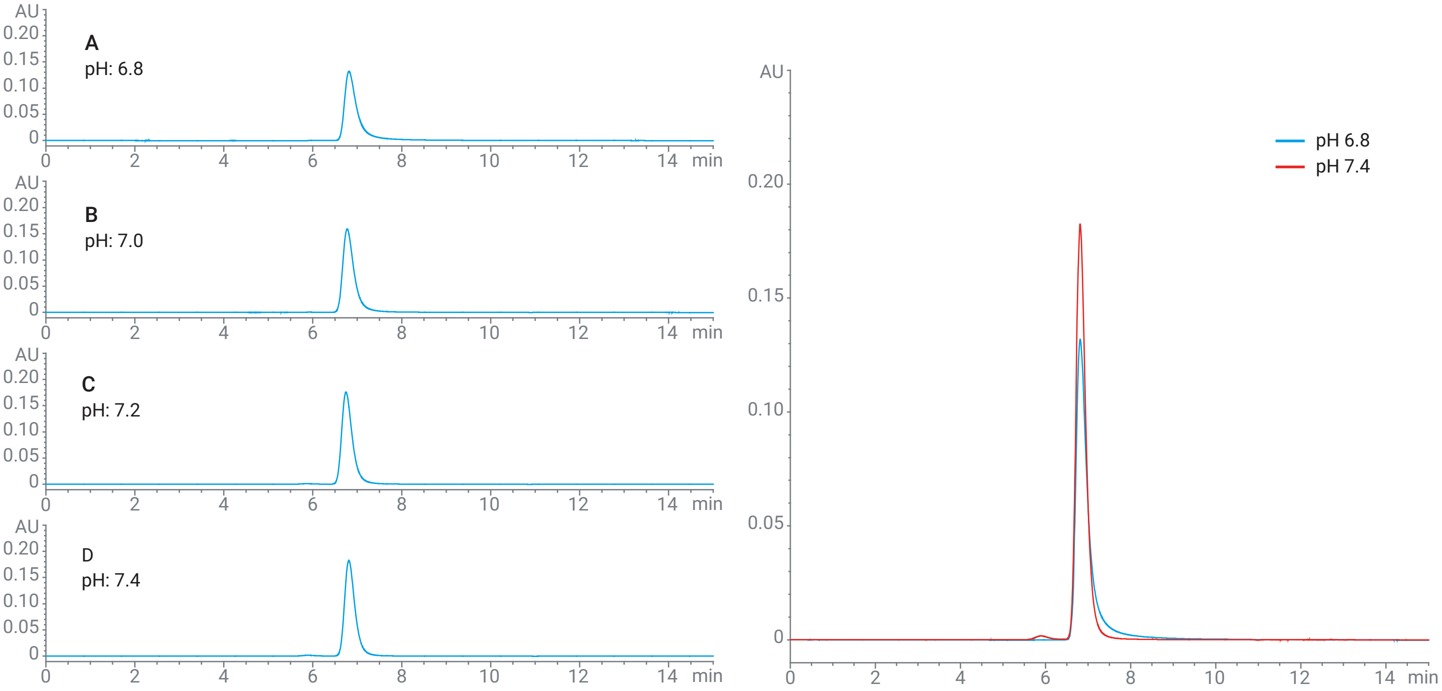

Figure 4. SEC of trastuzumab emtansine using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column running at 0.35 mL/min at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4.

Table 4. Effect of pH on chromatography for trastuzumab emtansine on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

pH	RT (min)	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
6.8	6.81	0.27	2.01	100	0	Not applicable
7.0	6.77	0.26	1.60	99.5	0.5	1.23
7.2	6.74	0.25	1.46	98.9	1.1	1.60
7.4	6.81	0.25	1.41	98.7	1.3	1.80

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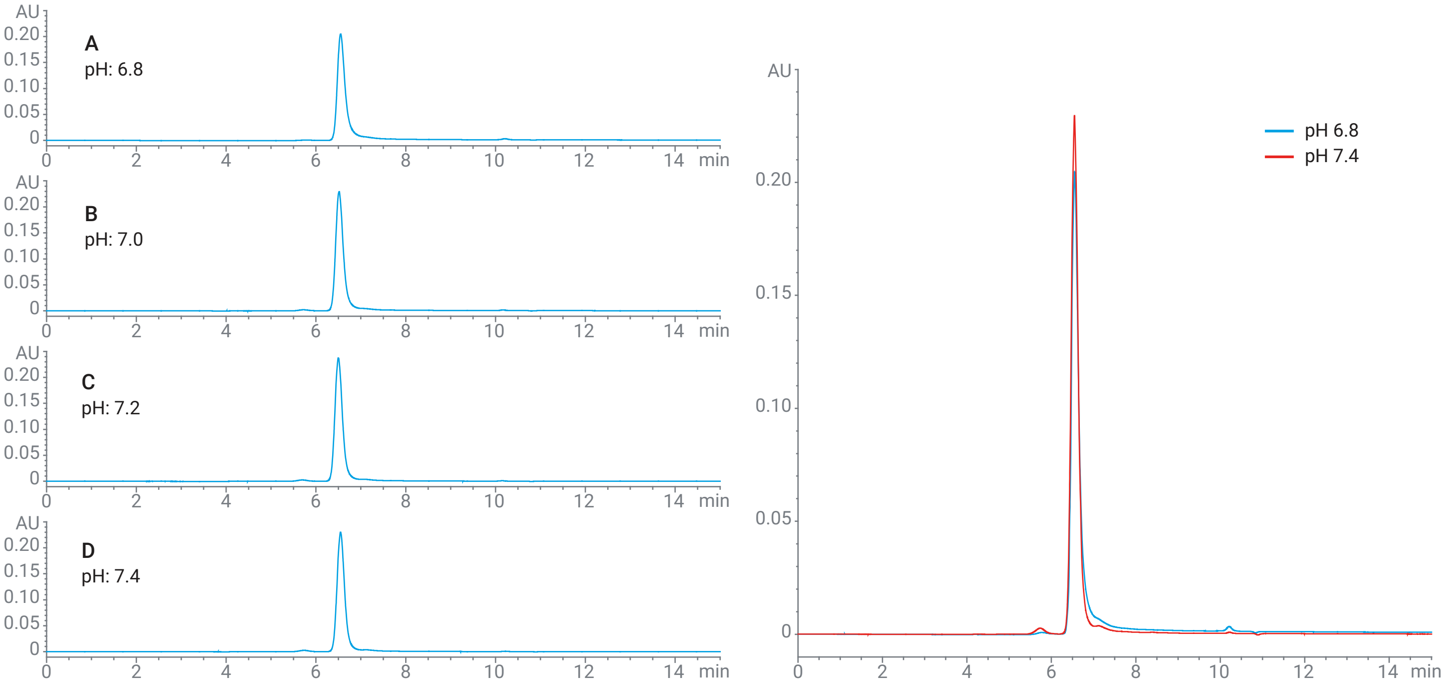

Figure 5. SEC of brentuximab vedotin using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column running at 0.35 mL/min at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4.

Table 5. Effect of pH on chromatography for brentuximab vedotin on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

pH	RT (min)	Width 50%	Tailing USP	Monomer %	Aggregate %	Resolution
6.8	6.55	0.18	1.70	99.5	0.5	1.92
7.0	6.51	0.18	1.37	98.7	1.3	2.18
7.2	6.50	0.18	1.28	98.5	1.5	2.20
7.4	6.54	0.18	1.24	98.4	1.6	2.25

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The effect of salt concentration was also evaluated with SEC chromatograms of both ADCs, trastuzumab emtansine (Figure 6) and brentuximab vedotin (Figure 7). 50 mM sodium phosphate pH 7.4 was used with the addition of 100, 200, and 300 mM of NaCl. For both ADCs, the use of high salt concentrations does not noticeably improve the performance, see Tables 6 and 7 respectively.

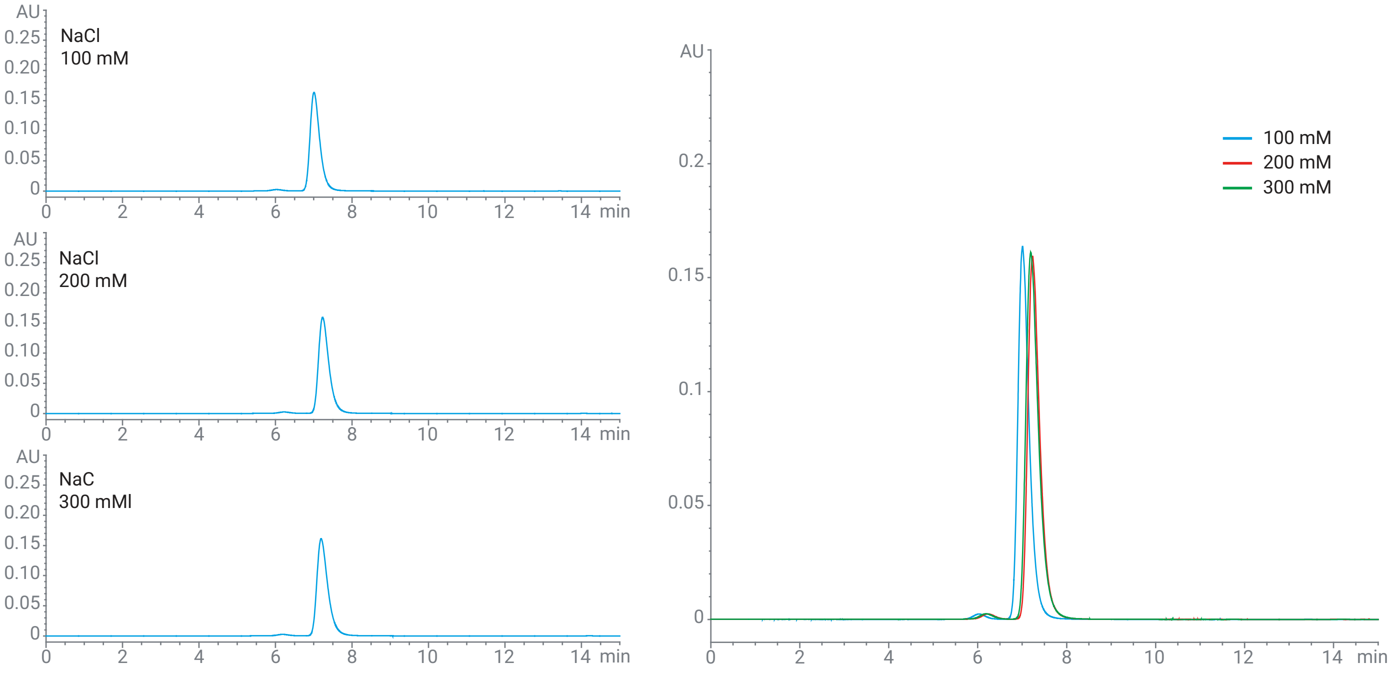

Figure 6. SEC chromatograms of trastuzumab emtansine using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column with PBS, pH 7.4 at 0.35 mL/min, showing the effect of NaCl concentration.

Table 6. Effect of NaCl concentration on chromatography for trastuzumab emtansine on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

NaCl Concentration	Monomer %	Aggregate %	Resolution
100 mM	98.3	1.7	1.98
200 mM	98.2	1.8	1.92
300 mM	98.2	1.8	1.85

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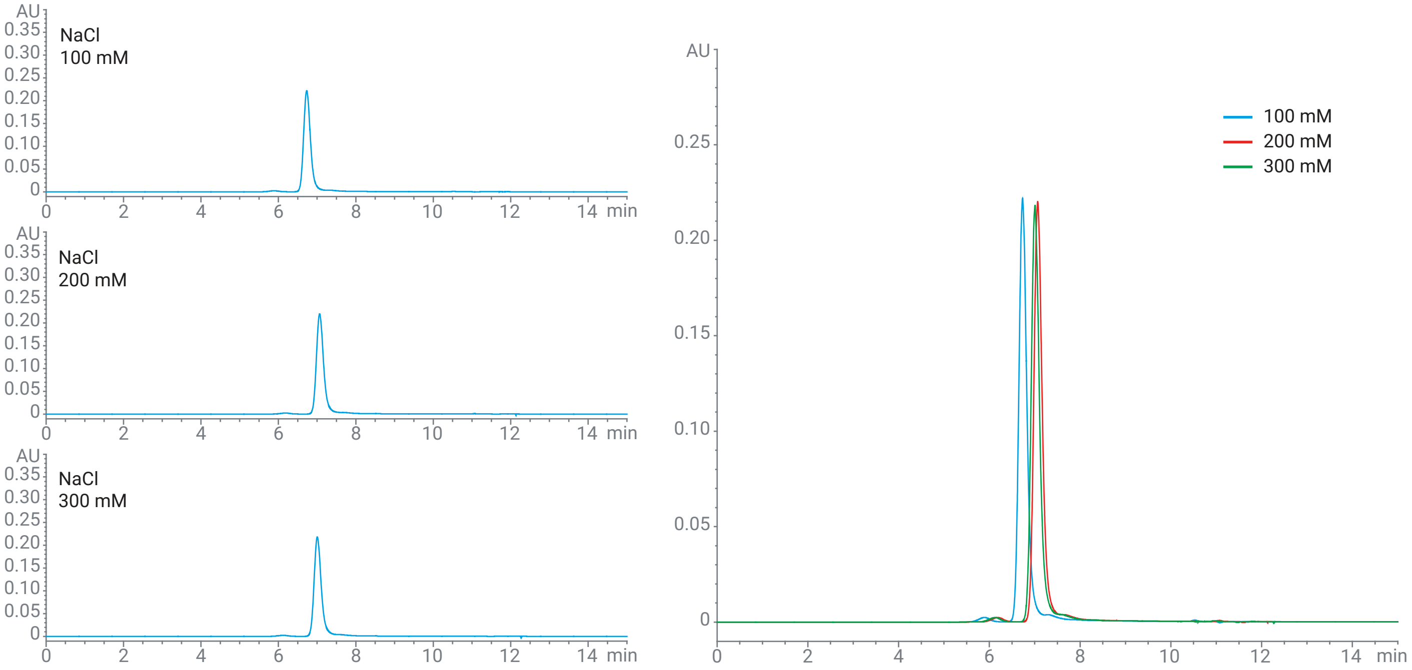

Figure 7. SEC of brentuximab vedotin using an Agilent AdvanceBio SEC 300 Å, 2.7 µm, 4.6 × 300 mm column with PBS, pH 7.4 at 0.35 mL/min, showing the effect of NaCl concentration.

Table 7. Effect of NaCl concentration on chromatography for brentuximab vedotin on an Agilent AdvanceBio SEC 300 Å, 2.7 µm column.

NaCl Concentration	Monomer %	Aggregate %	Resolution
100 mM	98.3	1.7	2.15
200 mM	98.2	1.8	2.15
300 mM	98.2	1.8	2.15

Conclusion

The choice of size exclusion stationary phase can have a marked impact on the efficacy for aggregate analysis of more challenging molecules such as ADCs. Choosing a sophisticated stationary phase with optimized hydrophilic polymer coating helps to overcome nonspecific interactions. Avoiding nonspecific interactions can lead to improved peak shape and resolution in the absence of organic modifiers in the mobile phase.

In addition, mobile phases having different pH values may have an important effect on the peak resolution, as shown with the Agilent AdvanceBio SEC 300 Å, 2.7 µm column. Moreover, it was shown that the concentration of NaCl does not particularly affect the separation, even when using 300 mM of salt.

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Application Note

Biopharma

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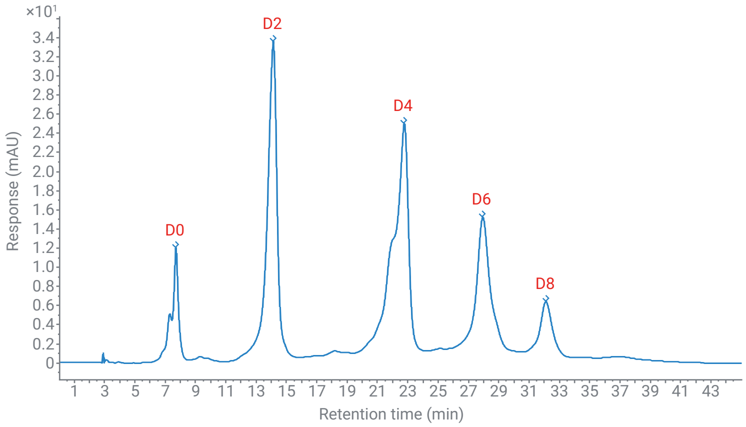
High Salt—High Reproducibility

Analysis of antibody drug conjugates using hydrophobic interaction chromatography with the Agilent 1290 Infinity II Bio LC System

Suitable for Agilent
1290 Infinity III LC



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Abstract

The determination of the drug-to-antibody ratio (DAR) is typically performed using hydrophobic interaction chromatography (HIC). The eluents for this mild, nondenaturing analysis method contain high concentrations of corrosive salts, which challenge the liquid chromatography (LC) system.

The Agilent 1290 Infinity II Bio LC System including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potential corrosive damage to the system. This application note demonstrates the DAR determination of brentuximab vedotin using HIC. The DAR was calculated to 3.7 drug molecules per antibody. Excellent reproducibility was found, demonstrating that the 1290 Infinity II Bio LC belongs to the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data. “Blank subtraction”, as a software feature of Agilent OpenLab CDS, removes drifting baselines due to less pure ammonium sulfate, enabling smooth integration.

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Introduction

Antibody drug conjugates (ADCs) are monoclonal antibodies (mAbs) to which a cytotoxic small molecule drug is chemically linked.[1] Compared to their corresponding antibodies, the structure is more complex and heterogeneous.

Cysteine-linked ADCs[2] (such as brentuximab vedotin, Adcetris by Takeda) has the small molecule attached to the free thiol groups of the partially reduced mAb.[3,4] The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero, two, four, six, and eight drugs per antibody. The average number of drugs conjugated to the mAb is one of the most important quality attributes of an ADC because it can directly affect safety and efficacy. The DAR determines the amount of payload that can be delivered to the desired tissue.[5]

HIC is the reference technique to separate cysteine-linked ADC molecules loaded with different numbers of drugs per antibody.[6] The relative hydrophobicity increases with the drug load of the ADC because the small molecules attached to the mAb are often relatively hydrophobic. Therefore, HIC is perfectly suited to monitor the DAR.

HIC is a nondenaturing analysis technique maintaining the native protein structure. It is typically performed at neutral pH, separating the proteins with a gradient from high to low salt concentration. The separation principle is the same as found in protein salting-out experiments.[6] In the high-concentration salt buffer used in mobile phase A, the proteins lose their hydration shell and are retained on the hydrophobic surface of the stationary phase. Mobile phase B is usually the same buffer (mostly phosphate) without added salt. With an increasing amount of mobile phase B in the gradient, the proteins re-assemble the water shell and are eluted from the column. The addition of a small amount of organic solvent such as isopropyl alcohol can also help to elute the proteins from the column.

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl,[7] up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g., oxidation, protein complex formation) can be avoided.

This application note presents the analysis of brentuximab vedotin with HIC for the determination of DAR, evaluating the precision of retention time and area. In addition, the advantages of the software feature "Blank Subtraction" in the processing method of OpenLab 2 are demonstrated to filter drifting baselines.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2 µL

Software

Agilent OpenLab CDS Version 2.5

Columns

Agilent AdvanceBio HIC column, 3.5 µm, 4.6 × 100 mm (p/n 685975-908)

Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

Samples

Brentuximab vedotin (Trade name Adcetris by Takeda, Tokyo, Japan) dissolved in half water, half solvent A (see below) at 100 mg/mL.

Note: As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

Buffer preparation

A: 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B: 50 mM phosphate buffer at pH 7 + 20% isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted,

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if necessary, to pH 7. Then, 198.21 g of ammonium sulfate for a total of 1.5 M was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer (→buffer A). The pH value was checked and adjusted, if necessary, back to pH 7 (the addition of high amounts of salt can change the pH). 200 mL of isopropanol and 800 mL of the prepared 50 mM phosphate buffer, pH 7 was mixed and added to an empty, amber-colored 1 L bottle (→buffer B). Both prepared buffers were filtered using a 0.2-µm membrane filter.

Note: The presence of small hydrophobic drug molecules conjugated to the mAb increases the overall hydrophobicity considerably. Consequently, it is necessary to include some organic modifier in the mobile phase (here: 20% isopropanol).

Method

Table 1. Salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 + 20% isopropanol
Gradient	0 minutes 30% B, 30 minutes 100% B Stop time: 45 minutes Post-time: 10 minutes
Flow rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 15 µL Sample temperature: 10 °C Needle wash: 3 s in water

Note: The high concentrations of salt used in HIC require a robust LC system, and the completely stainless steel (SST)/iron-free flow path of the 1290 Infinity II Bio LC prevents potential corrosion from high salt-containing buffers. In addition, washing features like seal wash and needle wash help to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in a concentrated salt solution for any length of time.

Note: When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for solvent A, including 1.5 M ammonium sulfate, use "Ammonium Sulfate 1.5 M" rather than *Generic Aqueous* or *Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

Results and discussion

Figure 1 shows the analysis of brentuximab vedotin, revealing five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR.

Although the interchain disulfide bridges are disrupted and occupied by the conjugated drugs, the combination of covalent linkages and noncovalent forces between the antibody chains is sufficient to maintain the mAb in an intact form during the analysis. This is due to the mild, nondenaturing conditions of HIC, making it ideal for the analysis of cysteine-linked ADCs. Each peak in Figure 1 corresponds to an intact mAb species with an increasing number of attached drugs molecules (zero to eight bound molecules, D0 to D8). The peaks were identified by comparing the HIC chromatogram to chromatograms found in literature for brentuximab vedotin.[6]

By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

Equation 1.

$$\mathrm{DAR} = \sum_{n=0}^{8} \frac{\text{LC peak area} \times n_{\text{drug}}}{\text{Total LC peak area}}$$

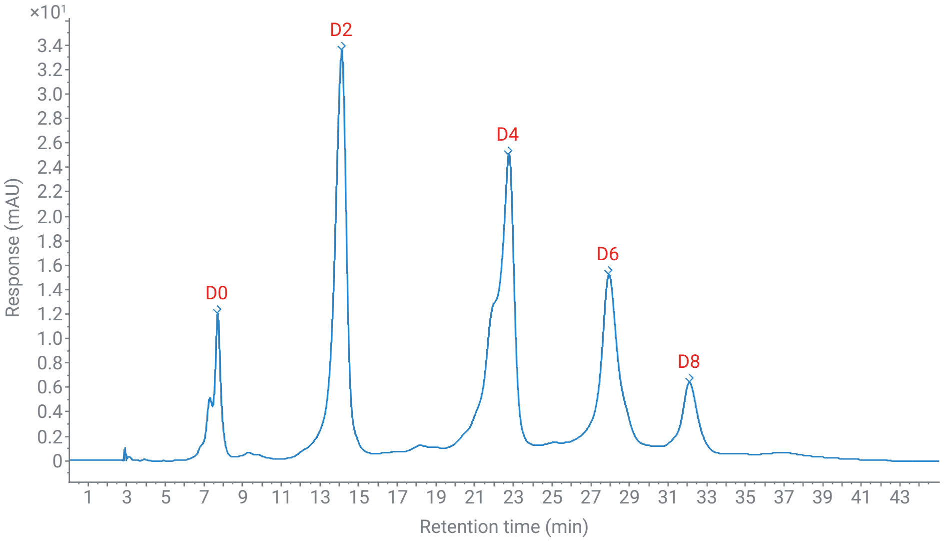

Figure 1. Analysis of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC. D0 to D8 refers to different DAR species.

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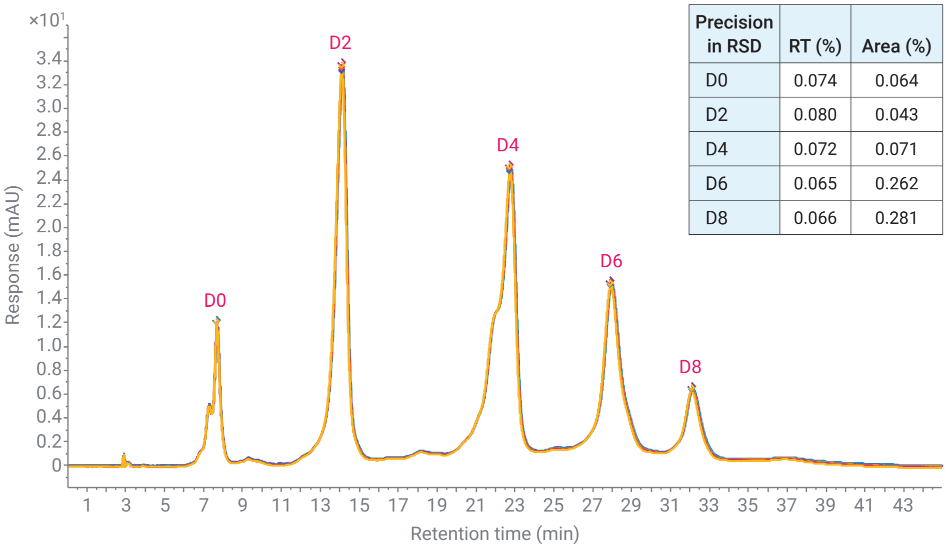
The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.7 (see Table 2). This value is consistent with the literature.[8]

The analysis was also evaluated for the precision of retention time (RT) and area (Figure 2). After seven subsequent runs, an excellent RT precision of lower than 0.081% relative standard deviation (RSD) was found. The area precision was also excellent, with RSDs lower than 0.282% (see table in Figure 2).

Ammonium sulfate is a very commonly used chaotropic salt in HIC analysis. The concentrations used typically range from 1 to 2 M salt, which is a considerable quantity. If a less pure salt is used in the analysis (which is sometimes even visible in the color of the salt crystals), the baseline of the chromatogram can drift significantly, resulting in potential integration errors. To approach this issue, a software feature called "Blank Subtraction" can be applied to filter out the baseline drift using the blank injection. This feature is found in the processing method of OpenLab 2 (see Figure 3). Figure 4 displays the chromatogram with different baseline behavior before and after the feature was applied.

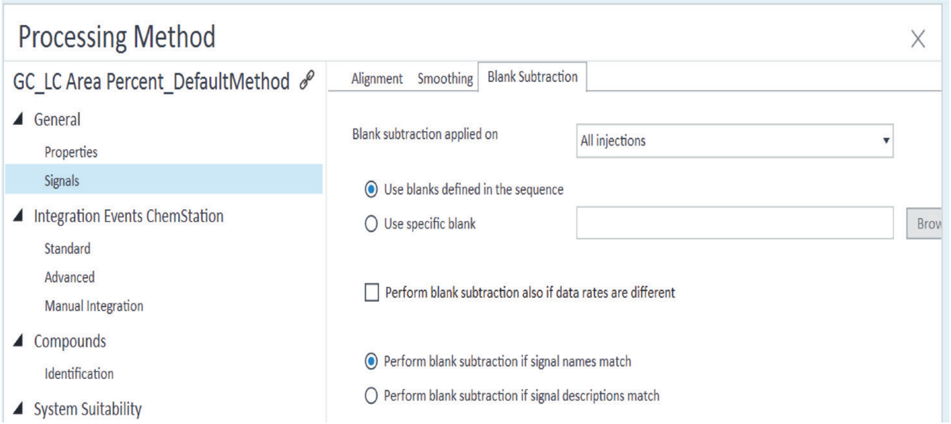
Table 2. DAR species results.

DAR Species	RT (min)	Area	Area%	DAR Calculated
D0	7.68	378.116	7.59	0
D2	14.12	1537.829	30.84	0.6196
D4	22.78	1756.026	35.22	1.415
D6	27.98	951.983	19.13	1.1506
D8	32.15	340.176	6.79	0.5482
			DAR	**3.733**



Precision in RSD	RT (%)	Area (%)
D0	0.074	0.064
D2	0.080	0.043
D4	0.072	0.071
D6	0.065	0.262
D8	0.066	0.281

Figure 2. Separation of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC (overlay of seven subsequent runs).



Blank subtraction applied on: All injections

● Use blanks defined in the sequence

○ Use specific blank

☐ Perform blank subtraction also if data rates are different

● Perform blank subtraction if signal names match

○ Perform blank subtraction if signal descriptions match

Figure 3. Screenshot of the "Blank Subtraction" feature in the processing method of Agilent OpenLab CDS 2.

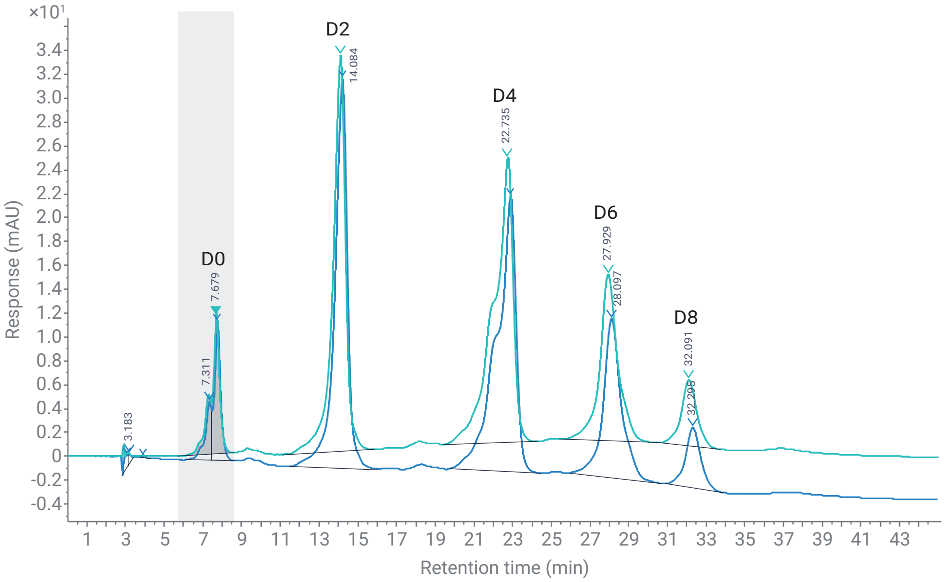


Figure 4. Comparison of HIC chromatogram before (blue) and after (green) blank subtraction.

Conclusion

Brentuximab vedotin was analyzed using HIC on the Infinity II 1290 Bio LC. All five expected DAR species were well separated corresponding to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species as well as the determination of the DAR, calculated to 3.7 drug molecules per antibody. The precision analysis of seven subsequent runs revealed excellent reproducibility for RT and area. The eluents used in HIC contain high concentrations of corrosive salts challenging the LC system. Due to its completely iron-free sample flow path, the 1290 Infinity II Bio LC is optimally suited for the conditions used in biochromatography, avoiding potential corrosive damage to the system.

Blank subtraction as a software feature of OpenLab 2 enables users to employ even less pure ammonium sulfate in their analysis without negatively affecting their results. Just by filtering out the blank runs, the baseline can be corrected to enable smooth integration calculation.

The combination of the biocompatible hardware of the 1290 Infinity II Bio LC with new software features of OpenLab 2 results in the highest confidence in generated data.

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Application Note

Biologics Development

Agilent

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An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs)

Using the Agilent 1260 Infinity II Bio-Inert LC

Author

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Abstract

This Application Note describes the characteristics of the AdvanceBio HIC column and its use in the analysis of antibody drug conjugates (ADCs). Hydrophobic interaction chromatography (HIC) is a method of performing separations of biomolecules with increasing hydrophobicity. This makes it a technique that is well suited to the analysis of ADCs, as these complex molecules are often more hydrophobic than the unmodified antibody. The technique can provide high resolution capable of separating species with minor differences in hydrophobicity, including those ADCs that have differing of drug-to-antibody ratio (DAR). A benefit of HIC comes from the mild conditions used for elution, which ensures that the biomolecule remains in an undenatured state. This can be advantageous for the analysis of cysteine-linked ADCs.

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Introduction

HIC uses mobile phases containing salts that reduce biomolecule solubility. This encourages absorption onto the HIC stationary phase. Reducing the level of salt through gradient elution allows the molecules to elute in order of increasing hydrophobicity. The separation may resemble reversed-phase chromatography, but without the ion pair reagents and high organic solvent composition that often lead to protein denaturation. This is important for the analysis of ADCs conjugated through cysteine links. The small molecule used as the payload is attached to free thiols of the partially reduced monoclonal antibody (mAb), but this results in a polydisperse distribution (Figure 1). It is essential to measure this distribution, known as the DAR, to determine the potency of the resulting ADC.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 water purification system (Millipore).

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option no. 100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option no. 019)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert flow cell (option no. 028)

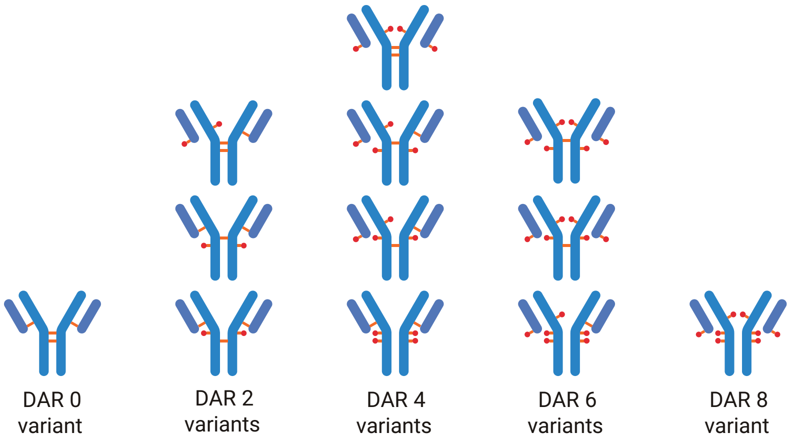

Figure 1. Polydisperse distribution of cysteine-linked ADCs with different quantities and positions of payload components.

Software

Agilent OpenLab 2.2 CDS

Method conditions

	HPLC conditions
Column	AdvanceBio HIC 4.6 × 100 mm (p/n 685975-908) AdvanceBio HIC 4.6 × 30 mm (p/n 681975-908)
Mobile phase	Eluent A) 50 mM Sodium phosphate, pH 7.0 Eluent B) 2 M Ammonium sulfate in 50 mM sodium phosphate, pH 7.0 Eluent C) Propan-2-ol Eluent D) HPLC grade water
Flow rate	0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Total run time	31 minutes

Typical gradient profile (see Figure 2)

Time	%A	%B	%C
0	50	45	5
15	75	0	25
20	75	0	25
21	50	45	5
31	50	45	5

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Results and Discussion

The high concentrations of salt used in HIC mean that a fully bio-inert LC will be beneficial. This is particularly the case if it is accompanied by additional features such as seal wash and needle wash to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in concentrated salt solution for any length of time. For that reason, using a quaternary LC system enables other channels to be used for organic modifiers and water or other flush solvents. The presence of small hydrophobic drug molecules conjugated to the mAb results in considerable changes to overall hydrophobicity of the mAb molecule. Consequently, it is necessary to include some organic modifier in the mobile phase (as seen in the method conditions and gradient profile sections).

Using HIC, it becomes possible to analyze cysteine-linked ADCs in their intact state (Figure 2). If reversed-phase techniques were used, the heavy and light chains that were not linked by a cysteine bond would become separated. It is possible to analyze cysteine-linked ADCs by reversed-phase chromatography, but only if the molecule is fully reduced[1].

By integrating the peak areas of the different DAR variants, it is possible to calculate the overall DAR (Equation 1).

$$\mathrm{DAR} = \sum_{n=0}^{8} \frac{\text{LC peak area} \times n_{drug}}{\text{Total LC peak area}}$$

Equation 1.

Table 1 shows the value calculated for this sample. DAR 4.04 is in agreement with the expected value, and also the value previously observed[2].

Table 1. Peak area to DAR results.

No.	RT (min)	Area	%Area	DAR	
1	8.03	763	6.9	0	0.00
2	9.77	2,759	25.1	2	0.50
3	13.31	3,936	35.8	4	1.43
4	15.91	2,565	23.3	6	1.40
5	18.26	978	8.9	8	0.71
				DAR	**4.04**

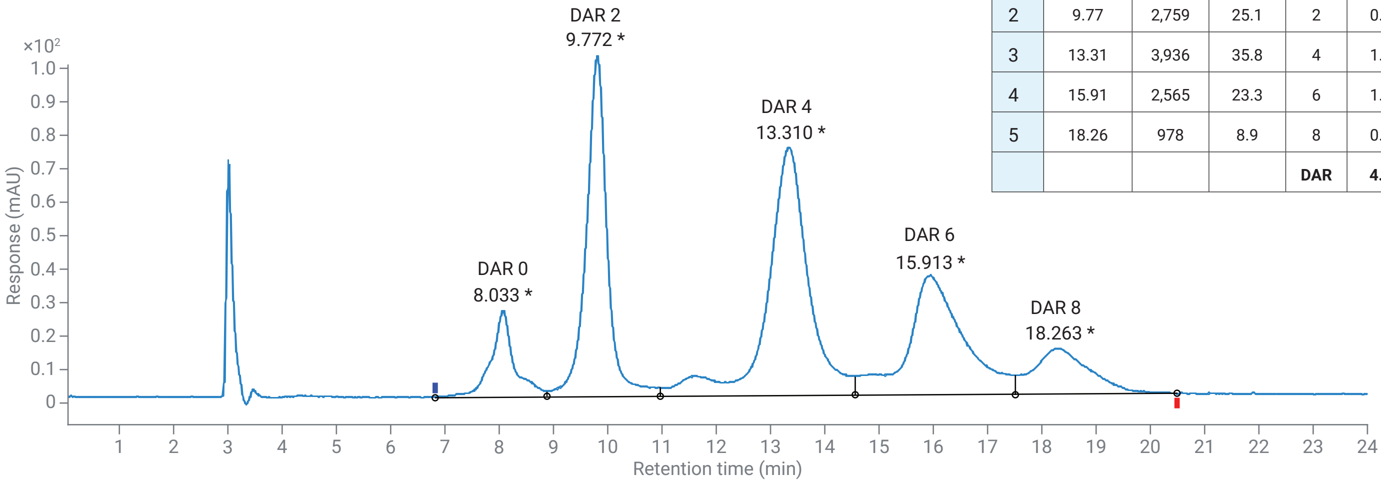

Figure 2. HIC separation of brentuximab vedotin (Adcetris).

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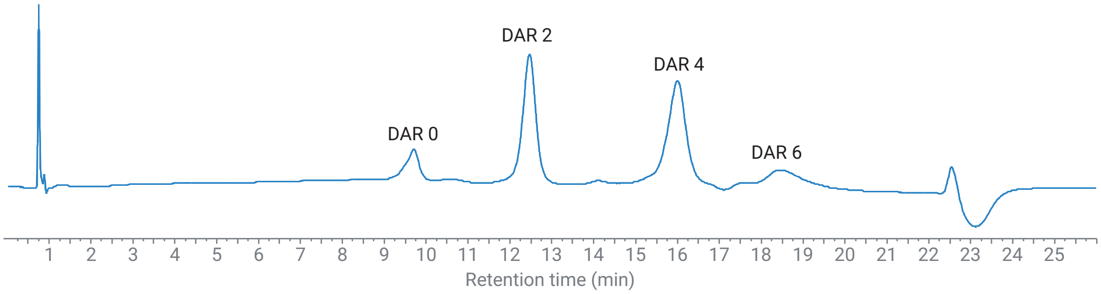
Take care with sample preparation to ensure that the sample is fully dissolved and that there is no discrimination of the DAR variants; DAR 0 is more hydrophilic than DAR 8, and can be selectively dissolved in the mobile phase. This will result in errors if all of the sample is not completely dissolved.

It is important to include the propan-2-ol gradient in the mobile phase conditions, as without this, it is possible to leave the DAR 6 and DAR 8 variants adsorbed to the column. Figures 3A, 3B, and 3C illustrate that, with insufficient propan-2-ol concentration, more hydrophobic DAR 6 and DAR 8 variants are retained.

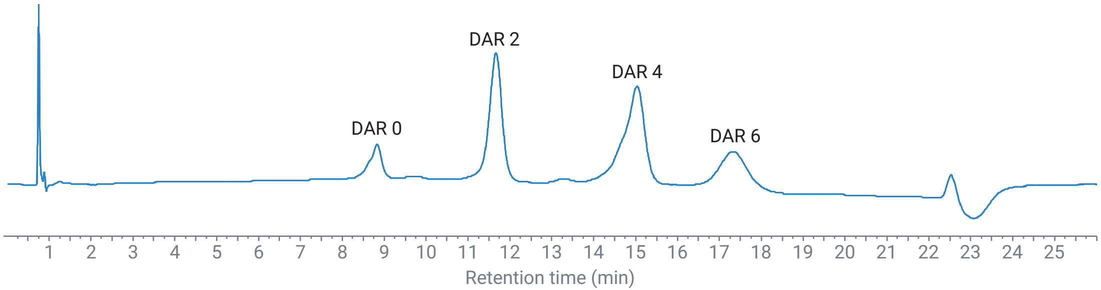
A Propan-2-ol gradient 5–10 %



A. Profile for 5–10 % gradient.

Time	%A	%B	%C
0	20	75	5
15	90	0	10
20	90	0	10
21	20	75	5
26	20	75	5

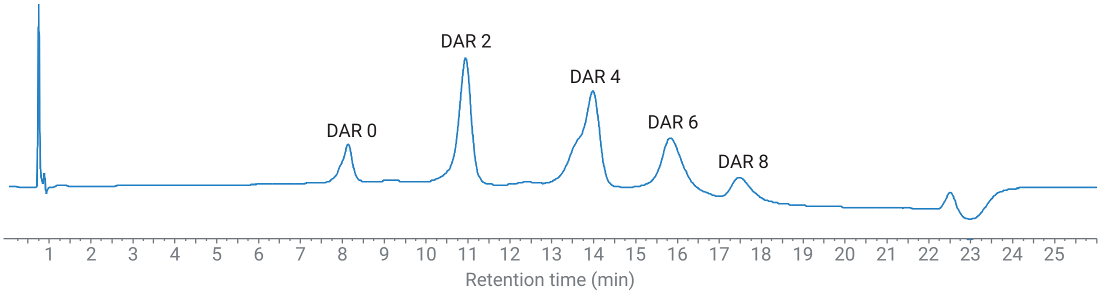
B Propan-2-ol gradient 5–15 %



B. Profile for 5–15 % gradient.

Time	%A	%B	%C
0	20	75	5
15	85	0	15
20	85	0	15
21	20	75	5
26	20	75	5

C Propan-2-ol gradient 5–20 %

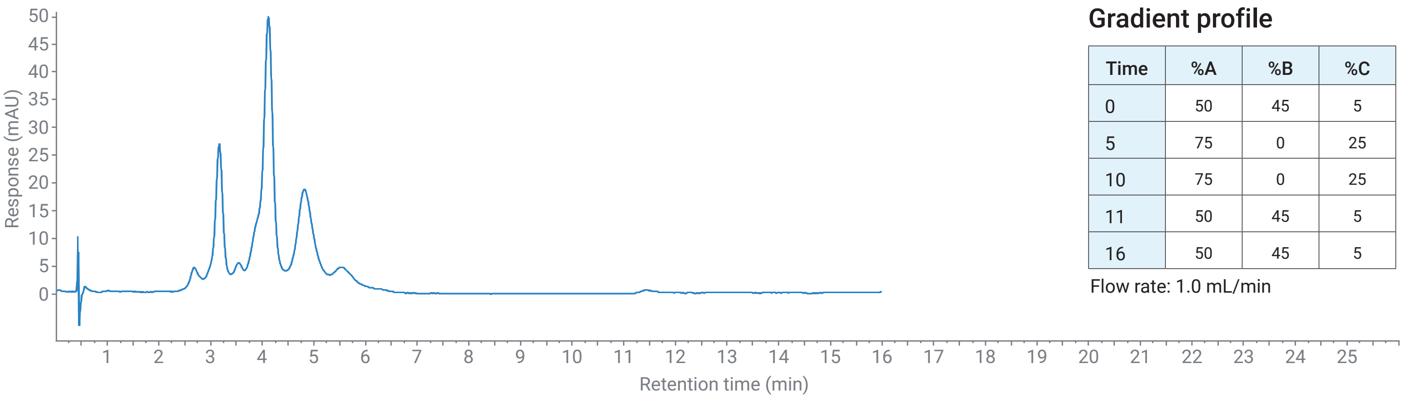


C. Profile for 5–20 % gradient.

Time	%A	%B	%C
0	20	75	5
15	80	0	20
20	80	0	20
21	20	75	5
26	20	75	5

Figure 3. Effect of propan-2-ol gradient in separation of ADC DAR variants.

With the appropriate use of flow rate and column length, together with the required amount of organic modifier in the gradient, it is possible to perform much faster separations on shorter 3 cm columns (Figure 4) compared to slower gradients on longer 10 cm columns (Figure 5). The results in Tables 2 and 3 show that the DAR values determined from these chromatograms are almost identical.

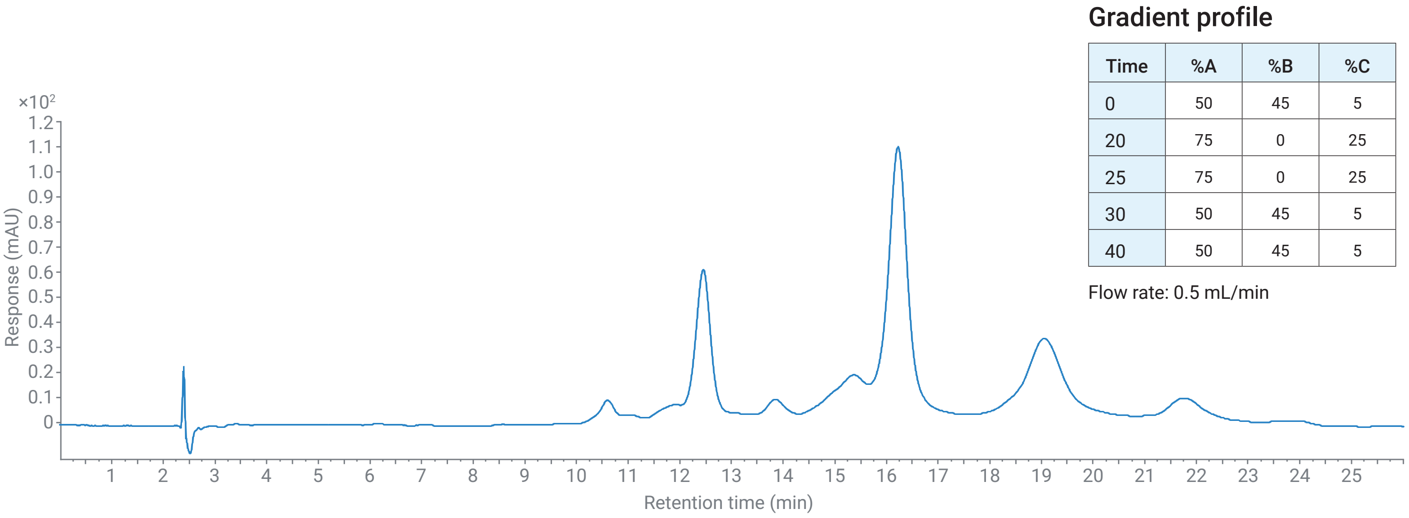


Gradient profile

Time	%A	%B	%C
0	50	45	5
5	75	0	25
10	75	0	25
11	50	45	5
16	50	45	5

Flow rate: 1.0 mL/min

Figure 4. Fast (8 minute) separation of an ADC mimic on AdvanceBio HIC 4.6 × 30 mm column.



Gradient profile

Time	%A	%B	%C
0	50	45	5
20	75	0	25
25	75	0	25
30	50	45	5
40	50	45	5

Flow rate: 0.5 mL/min

Figure 5. Slow (24 minute) separation of an ADC mimic on AdvanceBio HIC 4.6 × 100 mm column.

Table 2. DAR value from 8-minute separation (Figure 4).

No.	RT (min)	Area	%Area	DAR	
1	2.66	61.5	3.2	0	0.0
2	3.14	321.4	16.9	2	0.3
3	4.10	894.1	46.9	4	1.9
4	4.80	471.1	24.7	6	1.5
5	5.52	158.0	8.3	8	0.7
				DAR	**4.4**

Table 3. DAR value from 24-minute separation (Figure 5).

No.	RT (min)	Area	%Area	DAR	
1	10.61	304.3	3.0	0	0.0
2	12.46	1,768.9	17.2	2	0.3
3	16.22	4,905.3	47.7	4	1.9
4	19.04	2,420.5	23.5	6	1.4
5	21.76	879.1	8.6	8	0.7
				DAR	**4.4**

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Conclusions

We have demonstrated that by controlling the mobile phase composition, together with the choice of flow rate and column dimensions, it is possible to use the AdvanceBio HIC column for analysis of the DAR value of ADCs with both speed and accuracy.

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Application Note

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Mass Spectrometric Characterization of Antibody-siRNA Conjugates using the Agilent 6545XT AdvanceBio LC/Q-TOF

Author

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Agilent Technologies, Inc.

Abstract

Small interfering RNAs (siRNAs) are a promising type of RNA-based therapeutic drugs that demonstrate effectiveness on gene silencing through RNA interference (RNAi). However, due to the chemical nature of siRNA, a vehicle is often required to deliver siRNA into target cells, and a monoclonal antibody (mAb) is typically employed as the delivery vehicle. The characterization of covalently linked mAb-siRNA conjugates is important in drug development and manufacturing processes. Using traditional reversed-phase LC/MS methods presents an analytical challenge due to the unstable nature of the mAb-siRNA conjugates. This application note describes a novel LC/MS method for the characterization of mAb-siRNA conjugates under their native conditions. An Agilent 1290 Infinity II LC, AdvanceBio SEC column, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software were all used in this workflow.

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Introduction

With the discovery of RNA interference (RNAi) mechanism and the development of RNAi technology for silencing the target gene expression, small interfering RNAs (siRNAs) have demonstrated their effectiveness as a new class of therapeutic drugs.[1,2] However, one of the main challenges in the siRNA-based drugs development is the low efficiency of siRNA delivery to target cells. There are various proposed approaches to solving this problem, but the use of covalently linked siRNAs to biogenic molecules (human monoclonal antibodies) seems to be a viable solution. These biogenic molecules are specific to target cells to deliver siRNA loads, with minimized human immune reactions.

mAb-oligonucleotide conjugates have been used successfully for targeted delivery of oligonucleotide to specific types of cells and tissues.[3,4] In antibody-siRNA conjugates, the chemically stabilized siRNAs are covalently attached to the partially reduced antibody via its free thiol groups. When controlling the number of siRNA per antibody, antibody components of the conjugates still retain their normal functions and activities. Using this new delivery technology, the oligonucleotide payloads are now enabled efficient targeted treatment.

Biomedical researchers are improving the bioavailability, targeting of delivery, and biological activity (efficacy) of mAb-siRNA conjugates as therapeutic drugs. But there is also an urgent need to have accurate and more reliable analytical methods to characterize novel biomolecules, such as conjugates, that may exhibit different physical and chemical properties from siRNA and mAb.

In this study, an LC/MS-based analytical method for characterization of native intact mAb-siRNA conjugates was developed. This workflow features various AdvanceBio columns for sample separation, and the 6545XT AdvanceBio LC/Q-TOF system with the large molecule SWARM autotune feature and extended mass range of up to 30,000 *m/z* for sample analysis (Figure 1).

Experimental

Materials and methods

Unconjugated mAb, double strand siRNA (control sample), and anion exchange column-purified mAb-siRNA conjugates (DAR1, deglycosylated forms) were provided by an Agilent collaborator.

Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibody-siRNA conjugates sample preparation

The mAb was partially reduced with a commonly used reducing agent. The reduced mAb was then reacted with SMCC linker and siRNA (activated siRNA). The unreacted free thiol groups (–SH) of the mAb were modified with a chemical reagent. The reaction mixture was further purified by anion exchange column. Unreacted antibody elutes were separated, along with the solvent front and late eluting DAR1, DAR2, and unreacted siRNA. The purified DAR1 was then used for mass spectrometry analysis under denaturing and native conditions.

To perform native MS analysis, preserving the protein samples in pH-neutral and volatile aqueous solutions, such as ammonium acetate[5] is crucial. Therefore, sample desalting and buffer exchange are usually needed before the MS analysis. Briefly, the mAb-siRNA conjugates stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using a Bio-Rad Bio-Spin P-30 (40,000 mol wt limit) cartridge. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. A DAR1 sample was then pipetted to the top of the column and centrifuged for 5 minutes at 1,000 × g. The conjugate was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis.

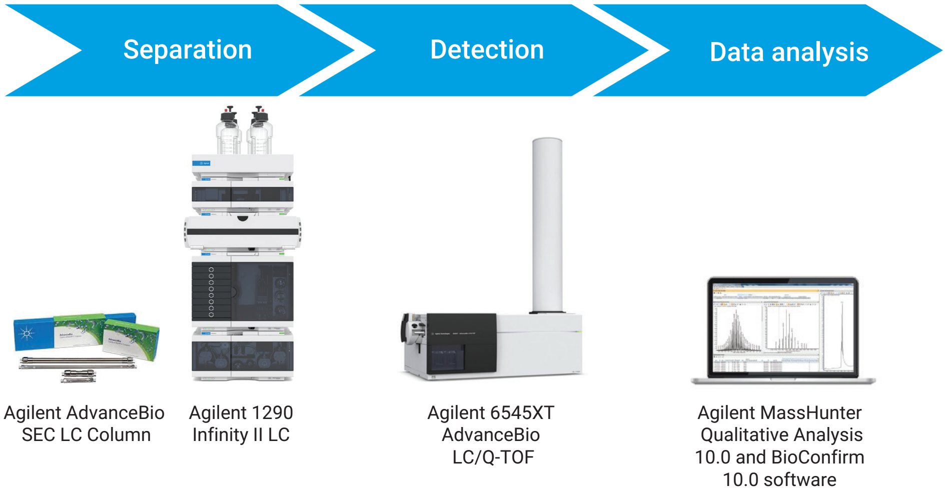

Figure 1. Analytical components of the native protein LC/MS analysis workflow.

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Instrumentation

- Agilent 1290 Infinity II LC, including:
  - Agilent 1290 Infinity II High-Speed Pump (G7120A)
  - Agilent 1290 Infinity II Multisampler (G7167B)
  - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/QTOF

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system, equipped with an Agilent Jet Stream source. Agilent MassHunter LC/MS data acquisition (version 10.1) workstation software was used with the large molecule SWARM autotune feature.

LC/MS analysis of the intact mAb (control) and intact deglycosylated mAb-siRNA conjugate (DAR1) under denaturing conditions was carried out using the traditional reversed-phase HPLC method.[6] An Agilent PLRP-S column (2.1 × 50 mm, 1000 Å, 5 µm, p/n PL1912-1502) was used with acetonitrile as an organic solvent and 0.1% formic acid.

Sample separations under the native LC/MS conditions were obtained with an Agilent AdvanceBio SEC LC column (4.6 × 30 mm) and a 5-minute isocratic run using 100 mM ammonium acetate solvent for the intact mAb (control sample). An AdvanceBio SEC LC column (4.6 × 300 mm, 200 Å, 1.9 µm, p/n PL1580-5201) was used in a 12 minutes isocratic run for the intact deglycosylated mAb-siRNA conjugate.

Tables 1 and 2 list the detail LC/MS parameters used.

Table 1. Liquid chromatography parameters.

	Agilent 1290 Infinity II LC System		
	Intact mAb and mAb-siRNA	**Native Intact mAb**	**Native Intact mAb-siRNA**
Column	Agilent PLRP-S (1000 Å, 2.1 × 50 mm, 5 µm) (p/n PL1912-1502)	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n PL1580-1201)	AdvanceBio SEC (200 Å, 4.6 × 300 mm, 1.9 µm) (p/n PL1580-5201)
Thermostat	4 °C	4 °C	4 °C
Solvent A	0.1% Formic acid in DI water	100 mM NH_4OAc (pH 7)	100 mM NH_4OAc (pH 7)
Solvent B	0.1% Formic acid in 100% acetonitrile		
Gradient	0 to 1 min, 0 to 20% B 1 to 7 min, 20 to 70% B	Isocratic: 0 to 5 min, 100% A	Isocratic: 0 to 12 min, 100% A
Column Temperature	60 °C	Room temperature	Room temperature
Flow Rate	0.5 mL/min	0.2 mL/min	0.3 mL/min
Injection Volume	1 to 5 µL	1 to 5 µL	1 to 5 µL

Table 2. Native MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System		
MS Condition	**Intact mAb-siRNA**	**Native Intact mAb-siRNA**
Source	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	350 °C	365 °C
Dry Gas Flow	12 L/min	12 L/min
Nebulizer	60 psig	35 psig
Sheath Gas Temperature	400 °C	300 °C
Sheath Gas Flow	11 L/min	12 L/min
VCap	5,500 V	5,500 V
Nozzle Voltage	2,000 V	2,000 V
Fragmentor	380 V	300 V
Skimmer	140 V	220 V
Quad AMU	500 *m/z*	3,000 *m/z*
Mass Range	300 to 8,000 *m/z*	3,000 to 10,000 *m/z*
Acquisition Rate	1 spectrum/sec	1 spectrum/sec
Acquisition Mode	Positive, extended (10,000 *m/z*) mass range	Positive, extended (10,000 *m/z*) mass range

Data processing

All MS data files of the native and intact mAbs or mAb-siRNA conjugates were processed using Agilent MassHunter qualitative analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Antibody-siRNA conjugates

mAb-siRNA conjugates were synthesized by site-specific conjugation between the free thiol residues of a human IgG antibody and the strand of the siRNA with a linker that can react with the free thiol group. The ratio of siRNA-to-antibody (DAR) is usually 1 or 2 (Figure 2). Synthesis involved the partial reduction reaction using a commonly used reducing agent. The partial reduction did not alter the antibody structure and retained its biological function as a delivery vehicle. After the conjugation reaction, the free thiol was then blocked with a modifier to prevent any further reactions.

HPLC separation of DAR1 on ion exchange column

After the conjugation reaction, all nonconjugated molecules (solvents, excess siRNA, and linker) should be removed. Various techniques exist for the purification of desired mAb-siRNA conjugates from synthetic mixture to remove reactants and by-products. These techniques include gel filtration/size exclusion chromatography, protein A or protein G affinity chromatography for antibodies, and ion-exchange chromatography.

Ion-exchange chromatography is an excellent technique for separating charged molecules and is commonly used to separate and purify oligonucleotides. In this study, mAb-siRNA conjugate target (DAR1) was purified using anion exchange column to remove other impurities.

Various LC/MS methods for mAb-siRNA conjugate characterization

LC/MS analysis of intact mAb and mAb-siRNA conjugates under denaturing conditions

To investigate and characterize the intact mAb and mAb-siRNA conjugates (glycosylated and deglycosylated), the reversed-phase HPLC approach to analyze the samples under denaturing conditions was used first. A PLRP-S column (2.1 × 50 mm, 1000 Å) was used with a 5-minute gradient for intact mAb and an 8-minute gradient for the conjugates at a flow rate of 0.5 mL/min.

Figure 3 shows the LC/MS analysis of intact unconjugated and deglycosylated mAb under denaturing conditions. The charge state distribution of denatured

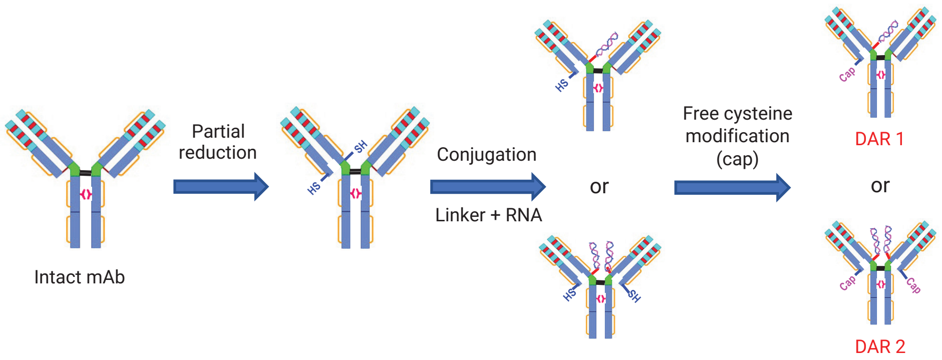

Figure 2. General scheme of antibody-siRNA (mAb-siRNA) conjugate chemical synthesis.

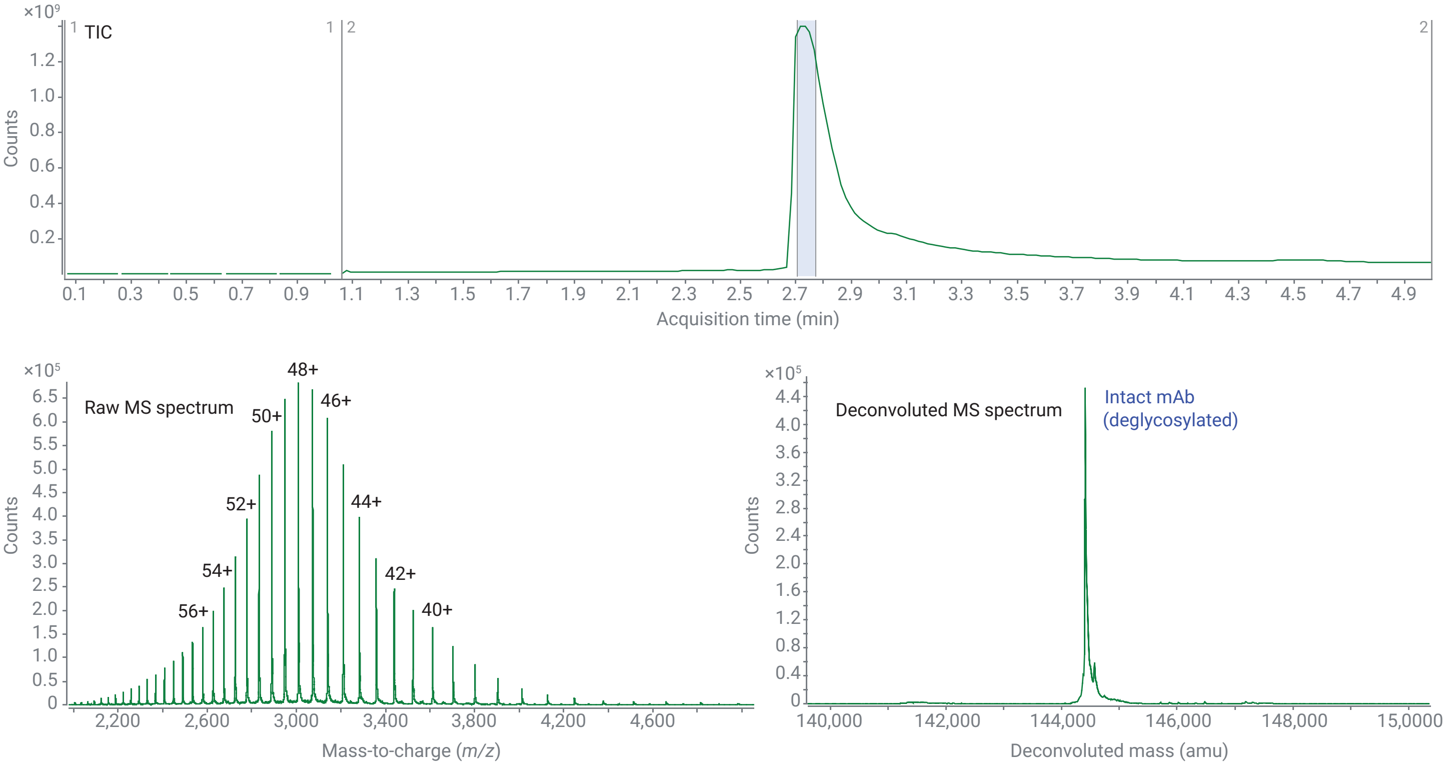

Figure 3. LC/MS analysis of intact deglycosylated mAb under denaturing MS conditions (acetonitrile and 0.1% formic acid).

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mAb spanned in the typical mass range of *m/z* 2,000 to 5,000 (30+ to 75+). Only one major protein was detected, and this was the fully deglycosylated antibody sample.

Figure 4 illustrates the LC/MS profile of the intact deglycosylated mAb-siRNA conjugate with a DAR value of 1. The extracted ion chromatogram (EIC, Figure 4B) also shows that there are several charge state distributions over the mass range *m/z* 1,000 to 4,000, indicating that many biomolecules existed in the sample.

Multiple species were expected to be present in this sample, so MS data deconvolution and mass matching to the protein or oligonucleotide sequences were performed on each of the four HPLC peaks over retention time period of 3.4 to 4.3 minutes (Figure 4C).

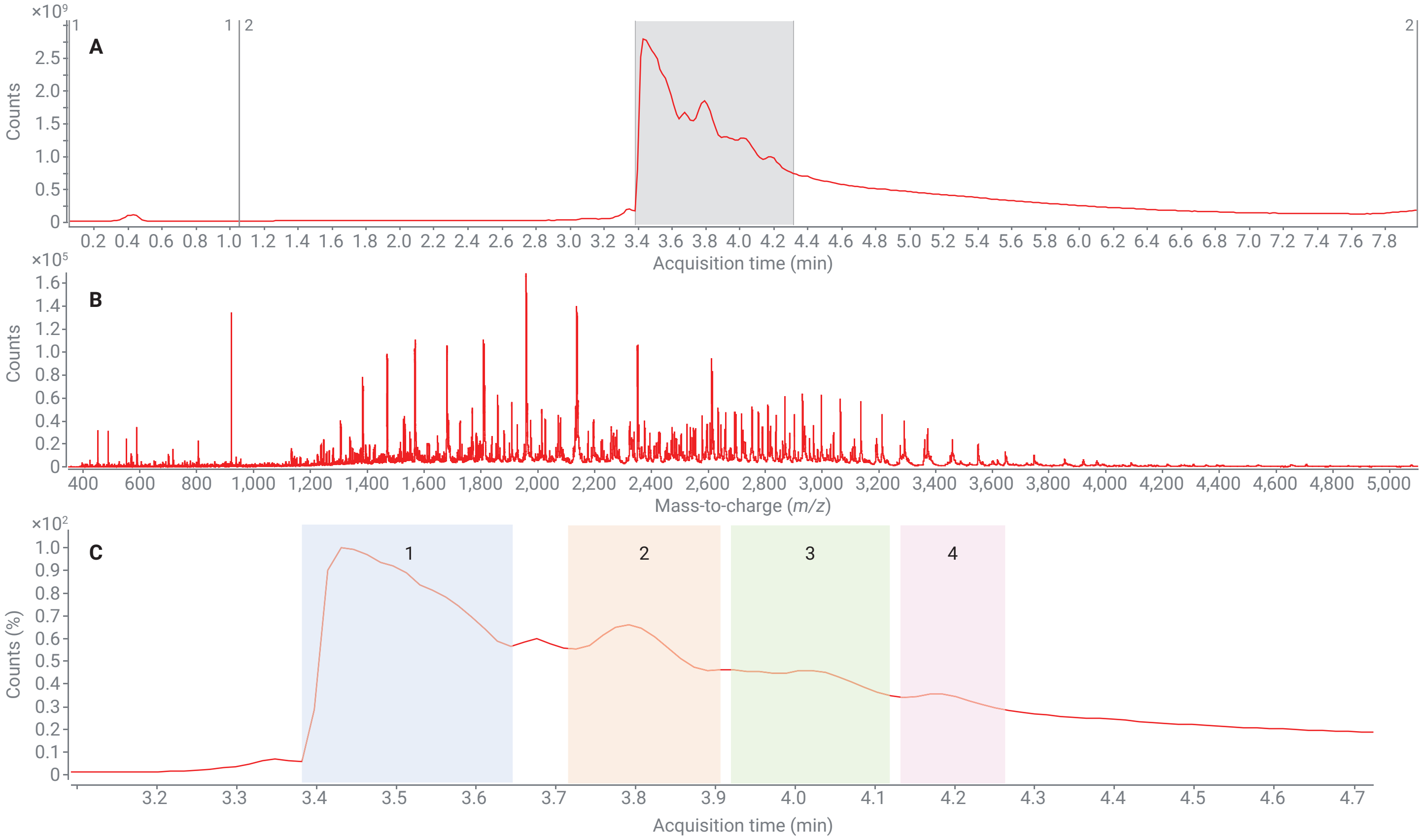

Figure 4. A) MS TIC of an intact deglycosylated mAb-siRNA conjugate on the reversed-phase (PLRP-S, 2.1 × 50 mm, 1000 Å) column. B) Extracted ion chromatogram (EIC) of the chromatographic separated peaks over retention time of 3.4 to 4.3 minutes. C) Zoomed-in chromatogram of the highlighted peaks (in gray box). The MS data from each of the HPLC peaks (1 to 4) were deconvoluted and analyzed. Results are shown in Figure 5.

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To obtain as much detailed information as possible, the MS deconvoluted mass range was set from 20 to 160 kDa for the raw MS data of all four LC peaks. This broad mass range should cover small proteins such as the mAb light chain, all the way up to mAb-siRNA conjugate. After careful calculation, many degraded/reduced antibody or antibody-oligo conjugate forms were identified and labeled, as shown in Figure 5 (peaks 1 to 4). These structural assignments are solely based on the mass matching and hypothesis of the conjugation reaction. Further investigation is needed to identify the actual conjugation sites of these molecules.

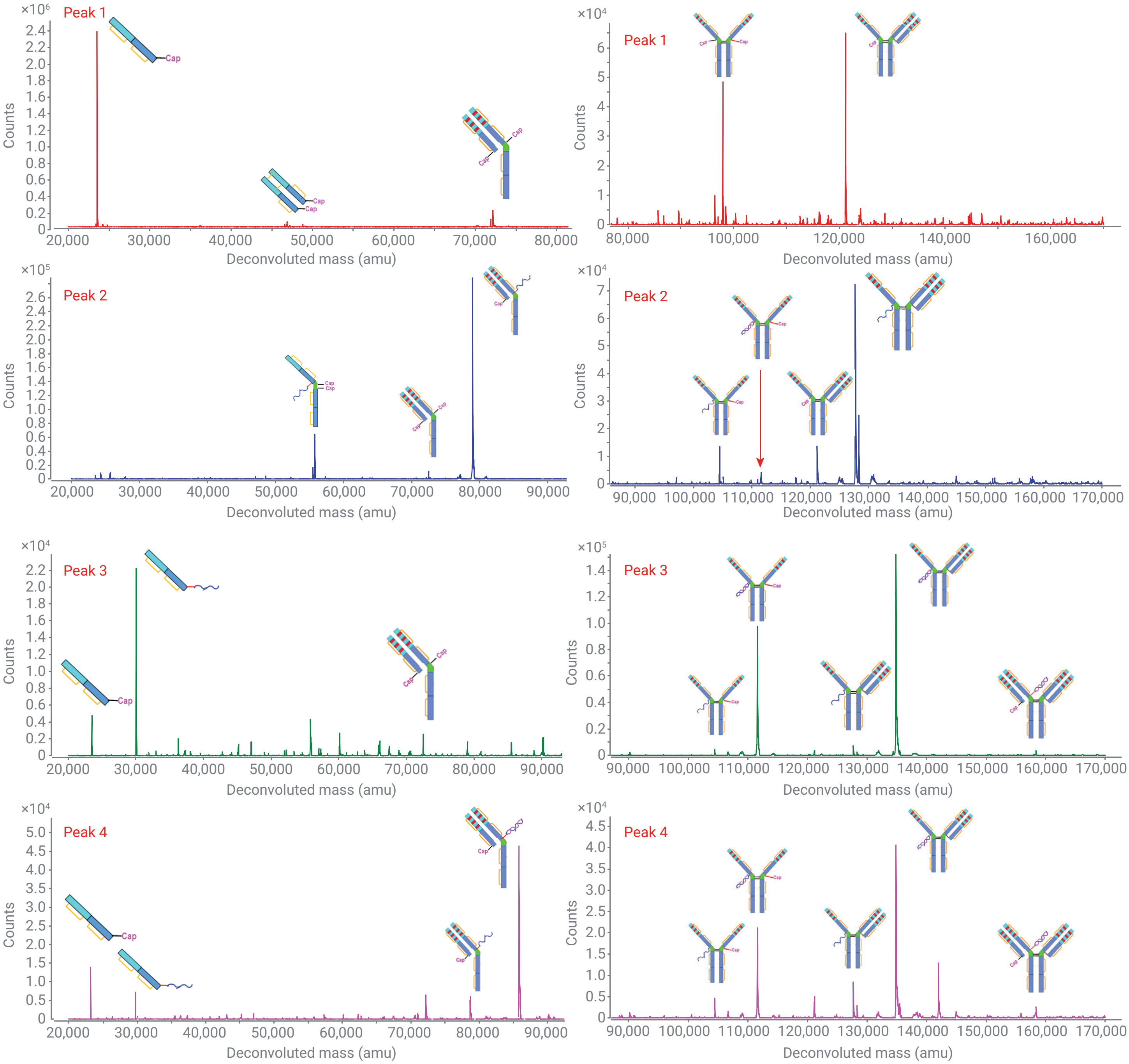

Figure 5. MS deconvoluted spectrum of deglycosylated mAb-siRNA sample (DAR1) (HPLC peak 1-4). LC/MS analysis was performed under denaturing MS conditions.

The results show that many dissociated molecules from mAb-siRNA conjugates were observed in all four LC peaks. They are: mAb light chain (with Cap or RNA), mAb heavy chain (with Cap or RNA), half of mAb-siRNA conjugates, conjugates without 1 or 2 LCs, and more. These results indicate that most of the conjugates were dissociated under the denaturing LC/MS condition. As the conjugation reaction likely occurred at the disulfide bonds between LC and HC, it broke the strong disulfide bond linkages and turned them into weak electrostatic interaction. It is, therefore, difficult to detect the intact conjugates under traditional LC/MS conditions using organic and acidic solvents.

Native LC/MS analysis of intact mAb and its mAb-siRNA conjugates

Native mass spectrometry has emerged as a widely used technique for characterizing intact proteins and noncovalent protein complexes. Native MS analysis enables probing of protein molecules while preserving their native structural conformation. This method minimizes the interferences from organic solvent and acid in the mobile phase, making it an ideal analytical tool for protein complexes with reduced stability or acid labile protein conjugates, such as some mAb-RNA conjugates.

Figure 6 demonstrates the LC/MS profile of intact unconjugated and deglycosylated mAb control under native conditions. Approximately 5 µg of mAb was injected onto an AdvanceBio SEC LC column using a 5-minute isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The charge envelope of the native mAb was in the mass range of *m/z* 5,000 to 10,000 (15+ to 29+). The Q-TOF source conditions were optimized, and excellent quality native MS spectra with low ppm (<5 ppm) in mass errors were obtained (data not shown).

The 6545XT system demonstrated excellent detection sensitivity for the mAb-siRNA conjugates under the native LC/MS conditions. Approximately 5 µg of the conjugate samples were injected onto an AdvanceBio SEC long column (300 mm) using a 12-minute isocratic flow at 0.3 mL/min. A longer chromatographic run method was developed to optimize the sample separation and maximize the sensitivity of detection.

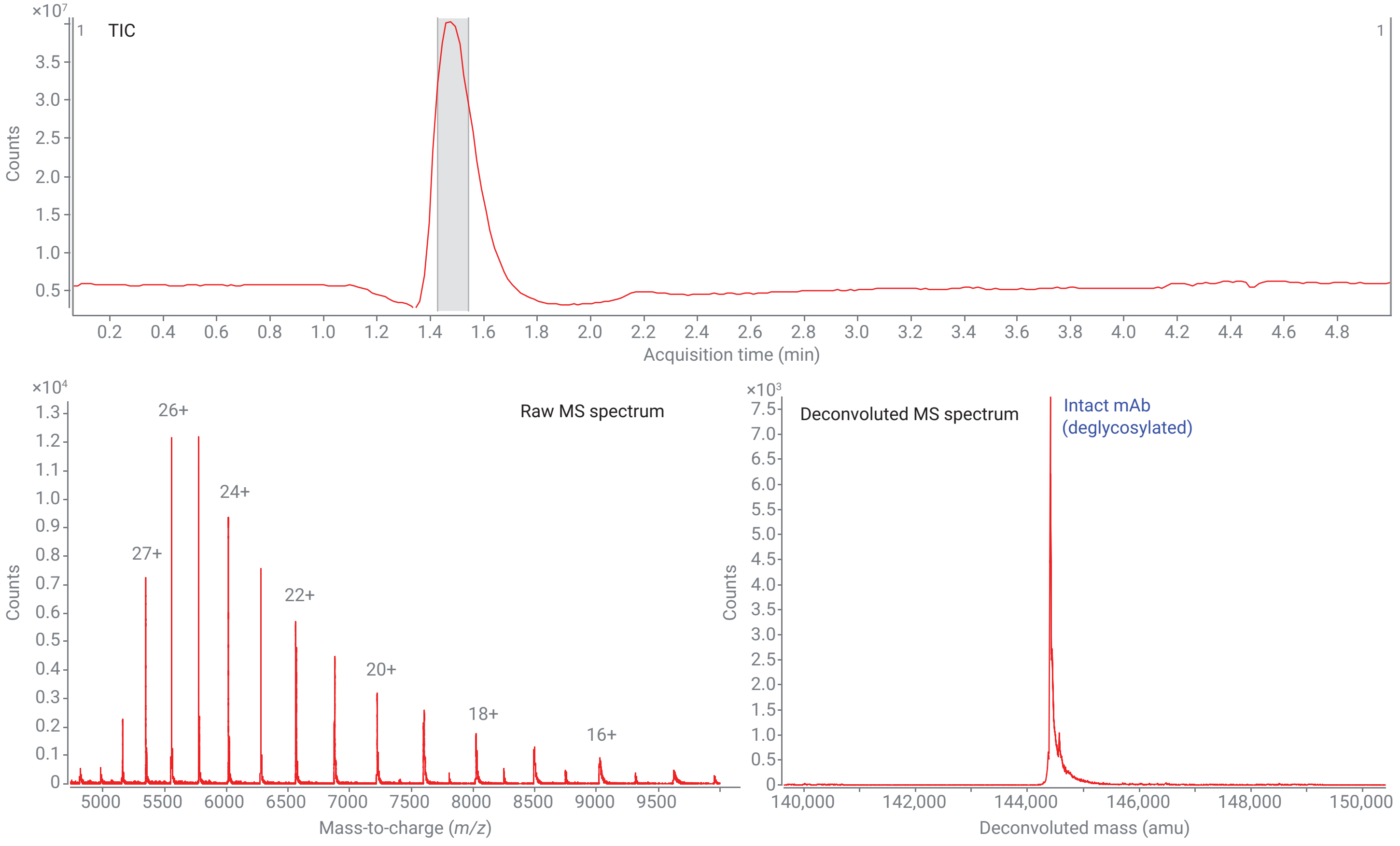

Figure 6. Native LC/MS analysis of intact deglycosylated mAb (in 100 mM ammonium acetate, pH 7).

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Figures 7 and 8 illustrate the native SEC column separated LC/MS analysis of an intact deglycosylated mAb-siRNA conjugate (DAR1) sample. Two major LC peaks with MS charge envelopes ranging from *m/z* 5,500 to 10,000 were detected. The deconvoluted spectrum revealed that there were two major forms of mAb-siRNA conjugate in each LC peak. Peak 1 contained DAR1 with one cap and DAR1 with three caps (see inset figures in 7C for structural illustration). Peak 2 included intact mAb + two caps (most abundant) and the tailing of DAR1 with one cap from peak 1 (Figure 8C, inset).

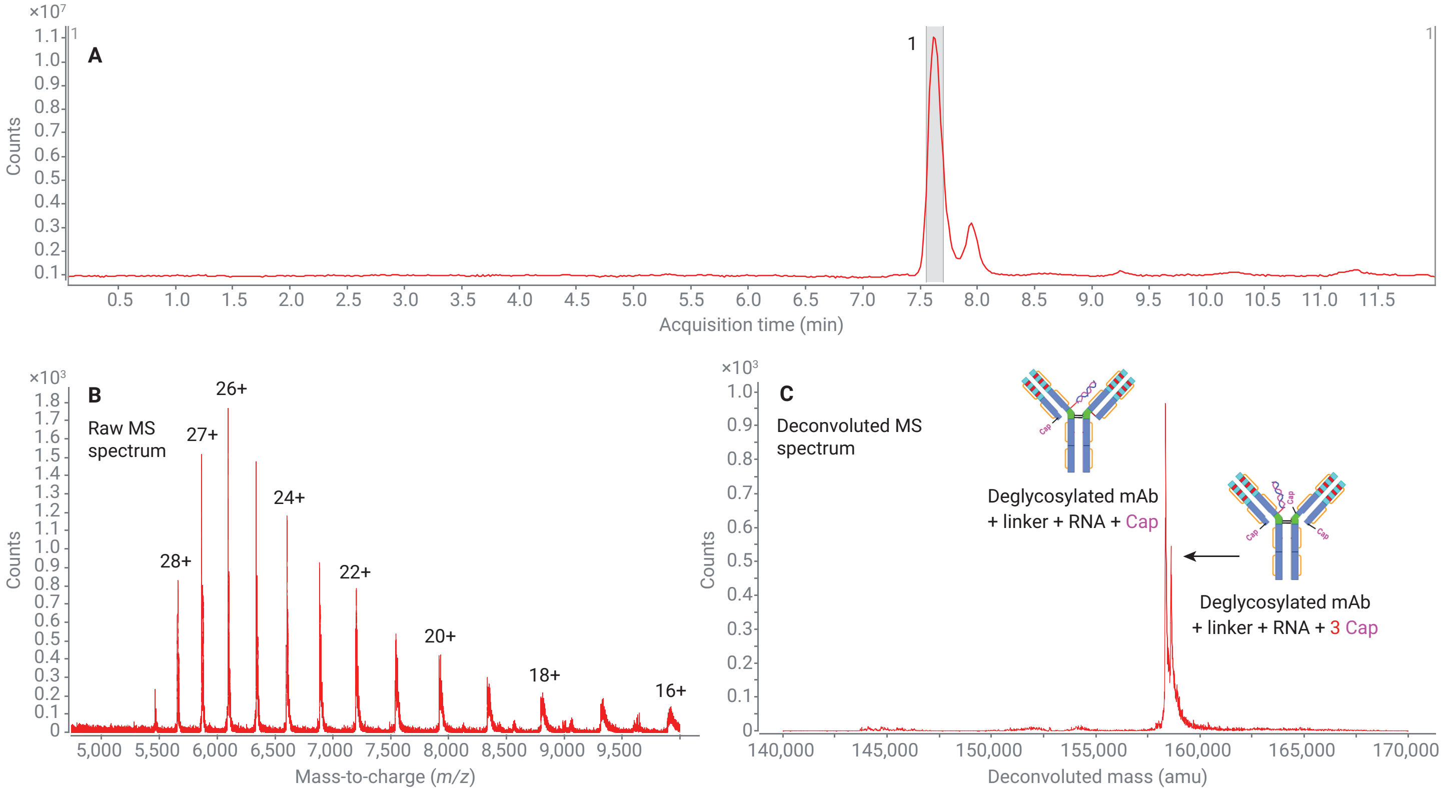

Figure 7. Native SEC LC/MS analysis of mAb-siRNA conjugate (DAR1): (A) HPLC chromatogram of SEC column separated DAR1. (B) Raw MS spectrum of intact DAR1 (peak 1). (C) The deconvoluted MS spectra of intact DAR1 (peak 1), indicating two forms of DAR1 were detected.

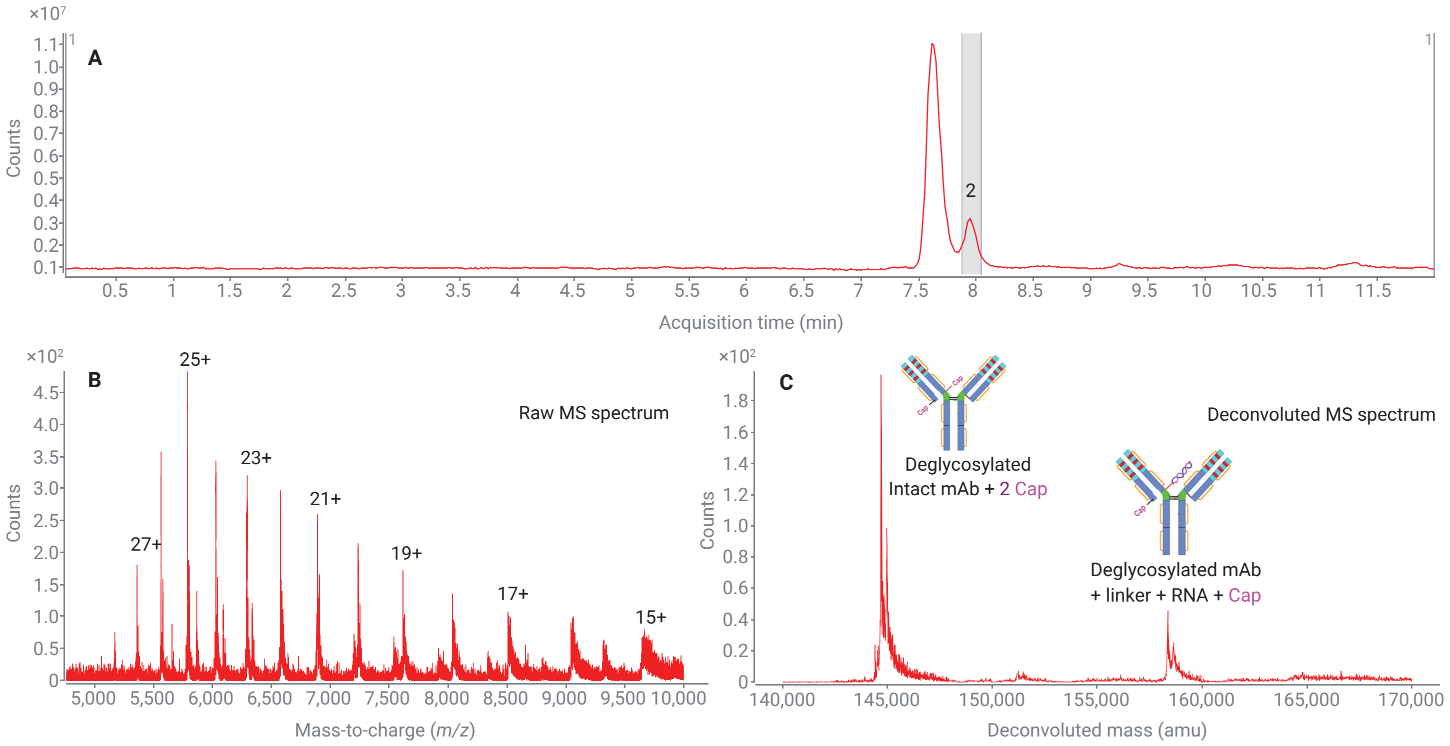

Figure 8. Native SEC LC/MS analysis of mAb-siRNA conjugate (DAR1): (A) HPLC chromatogram of SEC column separated DAR1. (B) Raw MS spectrum of intact DAR1 (peak 2). (C) The deconvoluted MS spectra of intact DAR1 (peak 2), indicating mAb + two caps was the most abundance molecule in peak 2.

Conclusion

A novel LC/MS method was developed for the characterization of mAb-siRNA conjugates under their native conditions. This optimized workflow uses the 1290 Infinity II LC with the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to *m/z* 30,000, and MassHunter BioConfirm software. This native MS analysis method not only can provide accurate mass information for mAb-siRNA conjugate structural assignments, but also enable chromatographic separation and relative quantitative analysis on various types of mAb-siRNA conjugates.

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DE-012919

Published in the USA, March 13, 2026
5994-9059EN

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