Application Note

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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.

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.

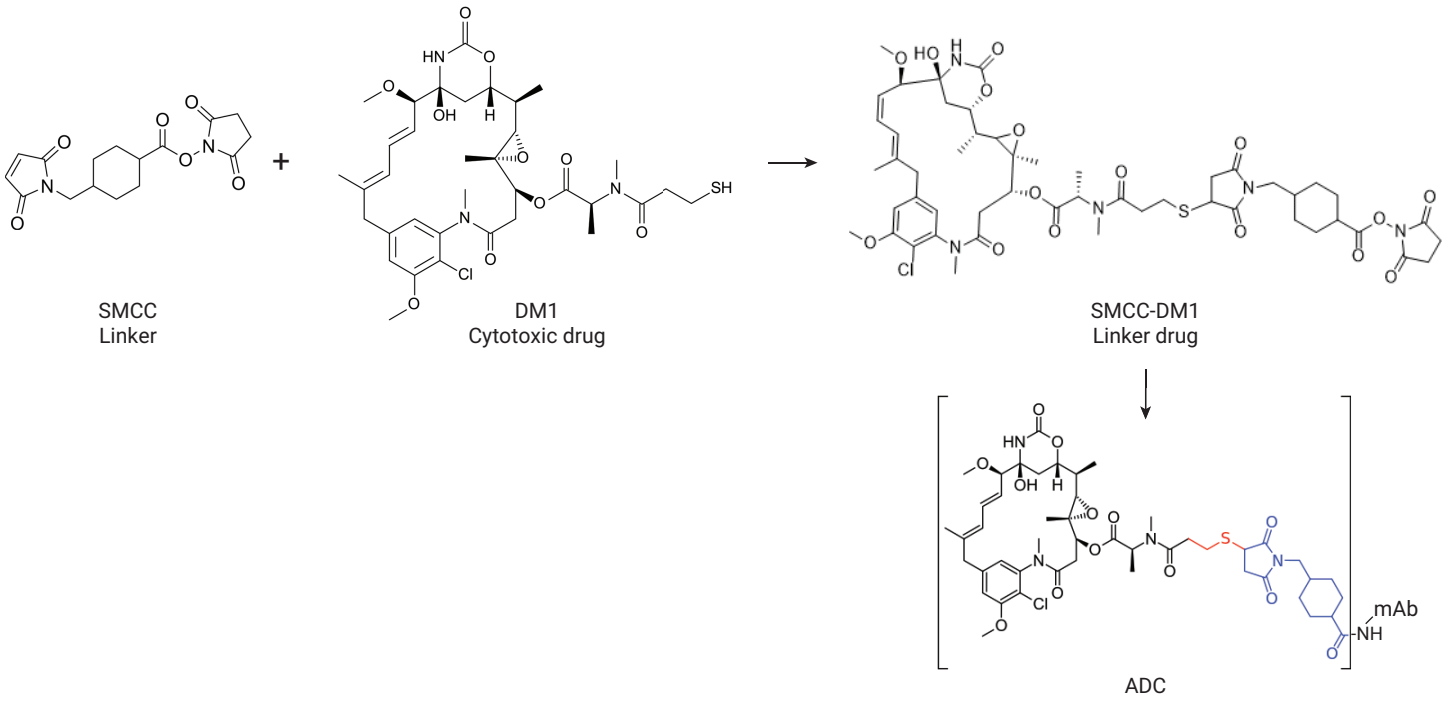

Figure 1. ADC conjugation process.

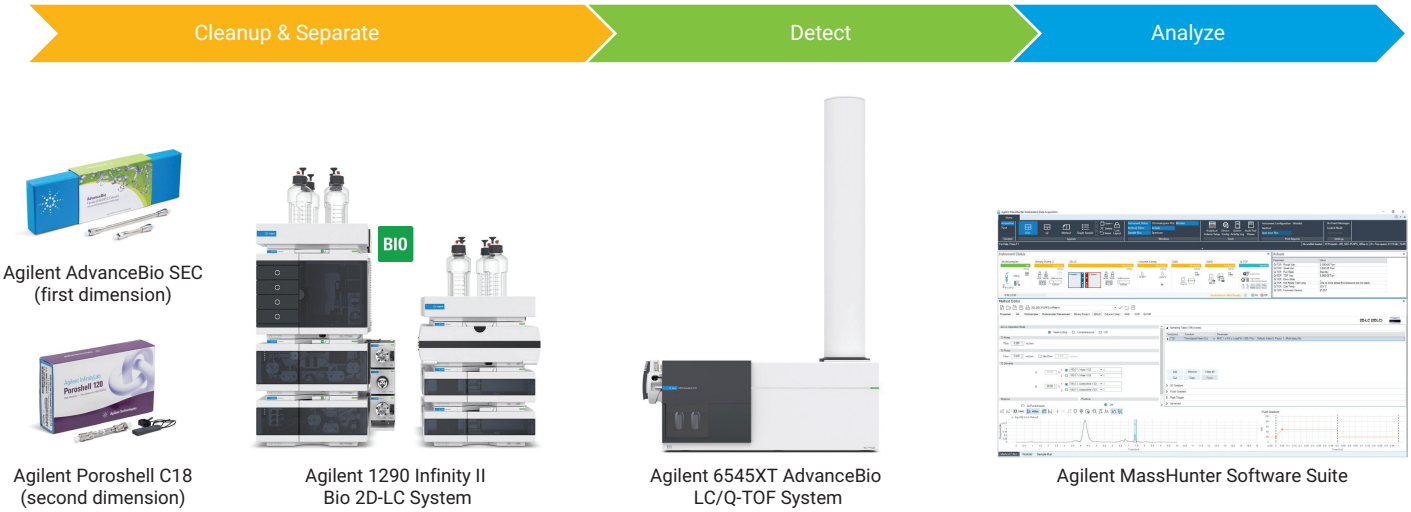

Figure 2. Analytical components of the Agilent Bio 2D-LC/Q-TOF analysis of ADC free drug content.

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.

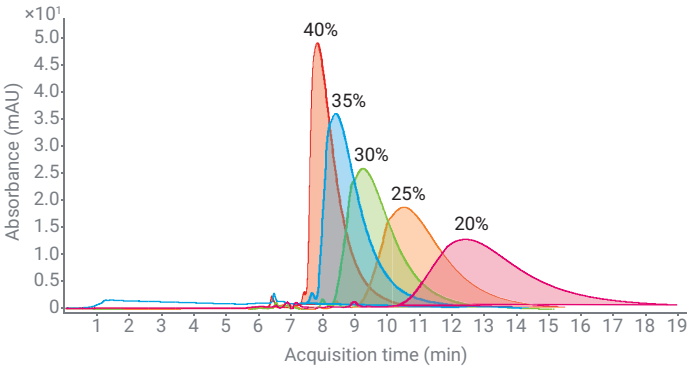

Figure 3. Scouting of ACN concentration from 20 to 40% in ^{1}D SEC for the DM1 standard.

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.

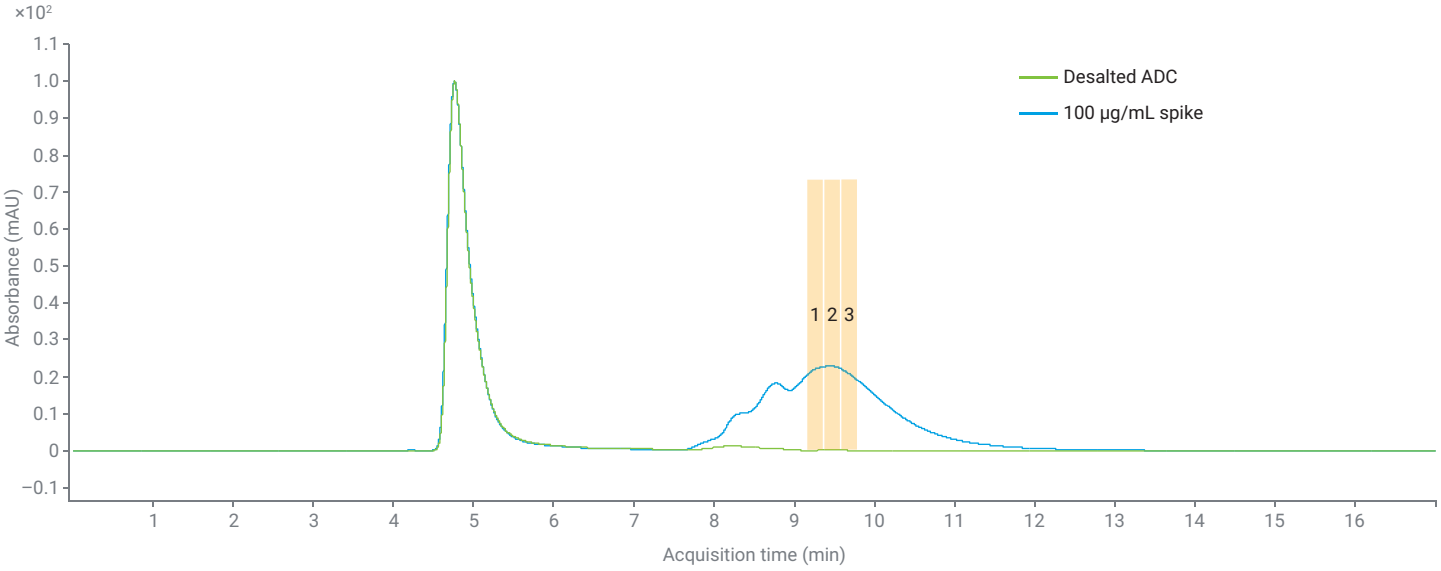

Figure 4. Overlaid UV chromatograms of first dimension SEC separation of desalted ADC (green) and 100 µg/mL spiked desalted ADC (blue). The shaded areas represent the three continuous cuts sampled into ^{2}D using multi-inject mode.

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.

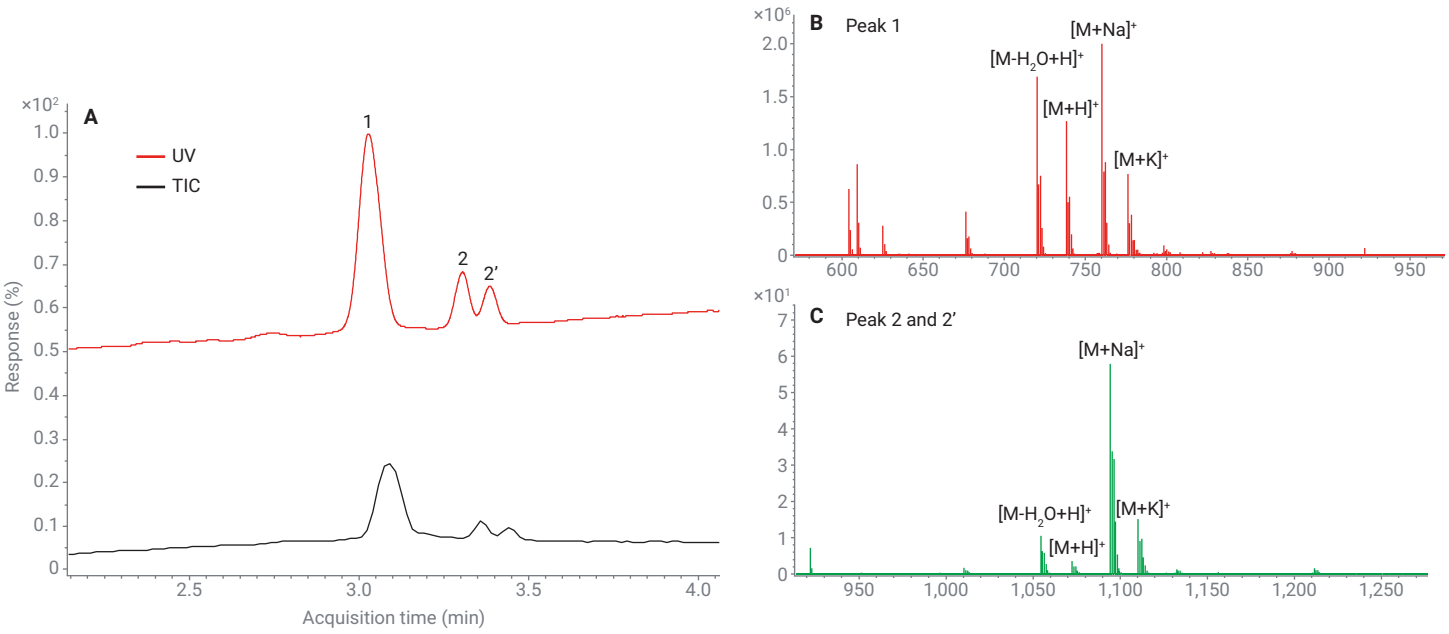

Figure 5. Identification of DM1 and SMCC-DM1 by second dimension LC/MS analysis. (A) UV and total ion chromatogram, (B) MS spectra of DM1, and (C) MS spectra of SMCC-DM1.

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.

References

1. Li, Y.; *et al.* Limiting Degradation of Reactive Antibody Drug Conjugate Intermediates in HPLC Method Development. *Journal of Pharmaceutical and Biomedical Analysis* **2014**, *92*, 114–118.
2. Hurwitz, E.; *et al.* The Covalent Binding of Daunomycin and Adriamycin to Antibodies With Retention of Both Drug And Antibody Activities. *Cancer Research* **1975**, *35*, 1175–1181.
3. Chari, R. V.; *et al.* Enhancement of the Selectivity and Antitumor Efficacy of a CC-1065 Analogue Through Immunoconjugate Formation. *Cancer Research* **1995**, *55*, 4079–4084.
4. Wong, D. Characterization of Antibody-Drug Conjugates Using 2D-LC and Native MS. *Agilent Technologies application note*, publication number 5994-4328EN, **2021**.
5. Stoll, D. R. Recent Advances in 2D-LC for Bioanalysis. *Bioanalysis* **2015**, *7(24)*, 3125–3142.
6. Singh, R.; *et al.* A New Triglycyl Peptide Linker for Antibody–Drug Conjugates (ADCs) with Improved Targeted Killing of Cancer Cells. *Molecular Cancer Therapeutics* **2016**, *15(6)*, 1311–1320.

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