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Technology Brief

Biopharma – Antibody Drug Conjugate (ADC) Characterization

Drug-to-Antibody Ratio (DAR) Calculation Using LCMS-9030 Q-TOF

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Highlights

- End to end workflow for Drug-to Antibody (DAR) calculation
- High Resolution Mass Spectrometry (HRMS) for Antibody-Drug Conjugate (ADC) Characterization

Technologies Featured

LCMS-9030 Q-TOF

Nexera XS inert LC

ShimOMNIA Bio HIC

Introduction

Antibody-drug conjugates (ADCs) are a class of biopharmaceutical drugs designed for targeted cancer therapy. It combines the specificity of monoclonal antibodies (mAbs) with a cytotoxic payload linked through a linker. The payload is typically a highly potent small-molecule toxin, which is crucial for their therapeutic effectiveness. Calculating the Drug-to-Antibody Ratio (DAR) is an essential parameter to describe the number of payloads conjugated to the antibody. A low DAR may diminish drug efficacy, while a high DAR can result in increased toxicity of the treatment. High-Resolution Mass Spectrometry (HRMS) has emerged as an accurate analytical method for measuring DAR effectively. Hydrophobic Interaction Chromatography (HIC) and Reverse Phase-High Liquid Chromatography Mass Spectrometry (RP-LCMS) analysis could provide precise DAR characterization on ADC sample. The commercial SigmaMAb Antibody Drug Conjugate (ADC) mimic is conjugated to dansyl fluorophores through an LC-SMCC crosslinker, with each dansyl-LC-SMCC attachment contributing a total mass of 668 Da. The average Drug-to-Antibody Ratio (DAR) for the SigmaMAb is reported as 4.5 on the Certificate of Analysis (CoA). The SigmaMAb ADC mimic sample was reduced into heavy and light chains, where the heavy chain may contain 0, 1, 2, or 3 fluorophores per molecule, and the light chain may contain 0 or 1 fluorophore, with all species differing by 668 Da.

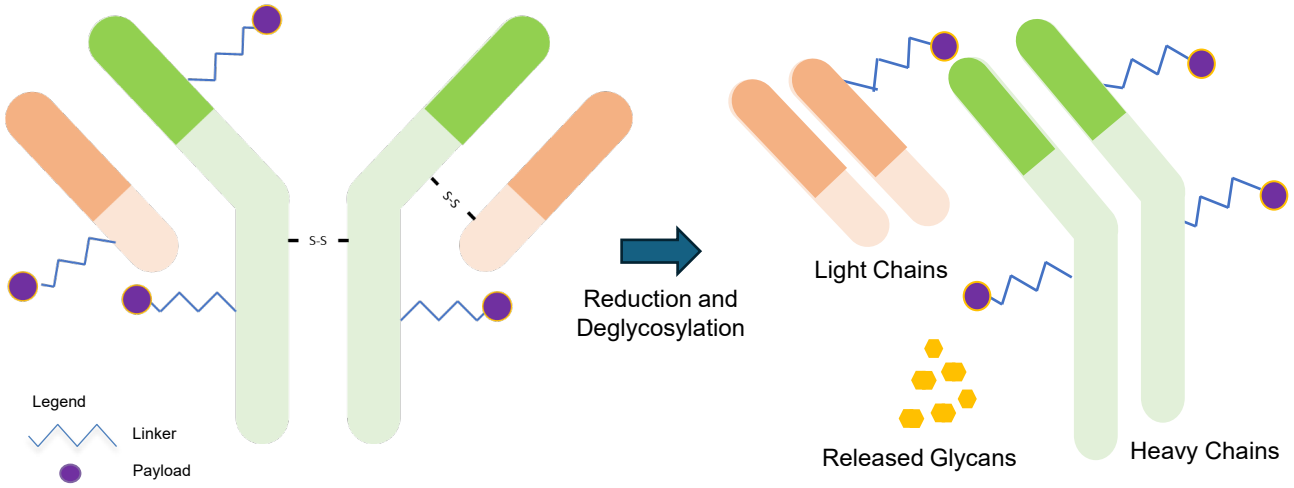


Fig. 1 Structure of Antibody Drug Conjugate (ADC) with a Drug-to-Antibody Ratio (DAR) 4 reduced to heavy and light chains

The samples were then injected into the LCMS-9030 Q-TOF, and data processing was conducted using Protein Metrics to calculate the DAR.

Native intact ADC mimic was injected onto the Nexera XS inert LC for HIC analysis and the chromatogram was compared against the LCMS-9030 Q-TOF subunit data to determine the fluorophore profile.

Experiment Design

Reagents and Chemicals

SigmaMAb Antibody Drug Conjugate (ADC) mimic was obtained from Merck Pte Ltd. Rapid™ PNGase F (non-reducing format) (P0711S) was purchased from New England BioLabs. TORAST-H Bio Vial (P/N: 370-04350-00) was obtained from Shimadzu.

Sample preparation

Subunit Q-TOF Analysis

10 µg of ADC mimic was denatured and reduced using 25 mM tris(2-carboxyethyl)phosphine (TCEP) by heating at 95 ˚C for 5 mins. Rapid™ PNGase F was added to the sample and incubated at 50 ºC for 10 minutes to remove glycosylation on the heavy chain region. Samples were diluted to a final concentration of 0.1 mg/ml using 0.1% formic acid in water and transferred to a Shimadzu TORAST-H Bio Vial. Samples were injected and analyzed by Shimadzu LCMS-9030 Q-TOF mass spectrometer. The analytical conditions are shown in Table 1.

Table 1. Analytical conditions on LCMS-9030 Q-TOF

LC Conditions	
Column	Shim-pack Scepter™ C4-300 2.1 × 150 mm, 1.9 µm (P/N: 227-31175-06)
Flow Rate	0.3 mL/min
Mobile Phase	A: 0.1% Formic acid in Water B: 0.1% Formic Acid in Acetonitrile
Elution mode	Gradient elution, 18 mins
Gradient Program	%B conc.: 0-2.0 min., 30%; 2.0-12.0 min.; 30% to 50%;12-14 min.,100%; 14 -18 min., 30%
Oven Temp.	80 ˚C
Injection Vol.	5 µL
Interface Conditions (LCMS-9030 Q-TOF)	
Interface	Heated ESI at 4.50 kV
Interface Temp.	300 ˚C
DL Temp.	200 ˚C
Heat Block Temp.	400 ˚C
Nebulizing Gas	3 L/min
Heating Gas Flow	10 L/min
Drying Gas Flow	10 L/min
Data acquisition (Q-TOF)	
MS Mode	MS positive
TOF m/z range	500-4000 m/z

Data files were uploaded onto Protein Metrics ADC workflow with the following workflow parameters as per Table 2. Protein Metrics software automatically deconvoluted the mass spectrum from the m/z acquired and identified various ADC variants. The final DAR value was determined based on the spectrum intensity calculated by Protein Metrics software.

DAR = 2X [Σ (intensity of drug-loaded subunits) / total intensity of antibody subunits]

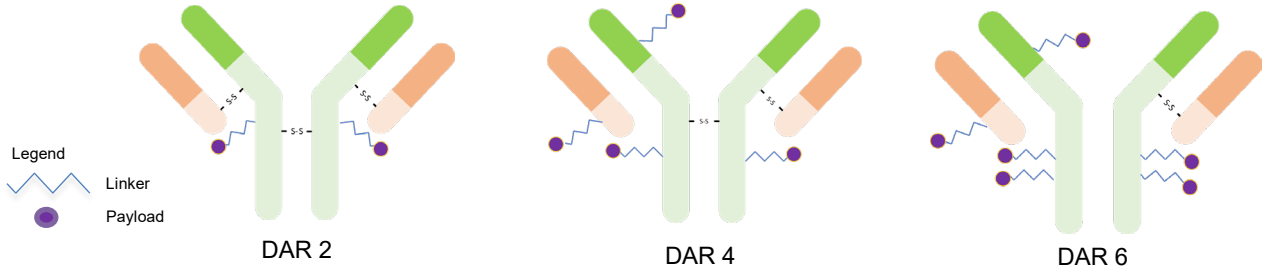


Fig. 2 Structure of Antibody Drug Conjugate (ADC) with different DAR variants

Table 2. Protein Metrics workflow parameters

Sequence and masses	
Changed N-terminal Q to pyro Glu	☑
Clip off C-terminal k	☑
N-glycans removed by PNGase F	☑
Mass Range	20,000 to 52,000
m/z range	500 to 4,000

Native HIC Analysis

ADC mimic was diluted to a concentration of 2.5 mg/mL with aqueous solution of 1.0 M ammonium sulphate in 50 mM sodium phosphate buffer (pH 7). Sample were transferred to Shimadzu TORAST-H Bio Vial and injected onto the Nexera XS inert LC with the conditions as shown in Table 3.

Table 3. Analytical conditions on Nexera XS inert LC

LC Conditions	
Column	ShimOMNIA Bio HIC Butyl 4.6 × 100 mm, 1.7 µm (P/N: 226-55963-03)
Flow Rate	0.3 mL/min
Mobile Phase	A: 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$/2-propanol (95/5) B: 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (80/20)
Elution mode	Gradient elution, 27 mins
Gradient Program	%B conc.: 0-1 min., 10%; 1-17 min.; 10% to 100%; 17-22 min.,100%; 22 -23 min., 100 to 10%; 23-27 min., 10%
Oven Temp.	25 °C
Injection Vol.	5 µL
PDA UV	
Absorbance	UV 210 nm

Results and Discussion

The SigmaMAb ADC mimic sample consists of a mixture of various DAR variants, as illustrated in Fig 2, with the average DAR reported as 4.5 on the CoA. The ADC samples were reduced into Light Chain (LC) and Heavy Chain (HC) and analyzed using RP-LCMS coupled with Protein Metrics software for data processing. Mass spectrometry was employed to identify the various DAR variants, as shown in the chromatography results in Fig 3. The analysis revealed various DAR variants from 0 drug molecules to 3 drug molecules conjugated to both the LC and HC. Protein Metrics software was utilized to deconvolute the spectra and identify the different DAR variants as showed in Fig 4. The weighted average of DAR for ADC mimic samples was calculated to be 4.3 as show in Table 4 which is comparable to the CoA DAR value of 4.5. The results indicated a low level of HC with 2 drug molecules conjugated on it. This is likely due to the reduction of intrachain disulfides on the light chain, which creates a second conjugation site.

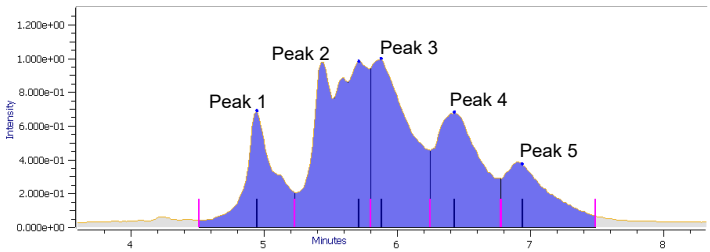
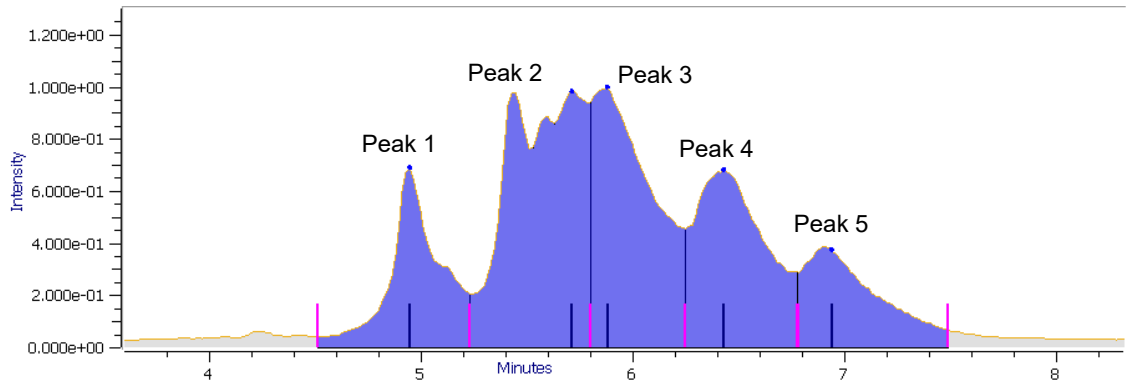


Fig. 3 Total Ion Chromatography (TIC) of Subunit Analysis



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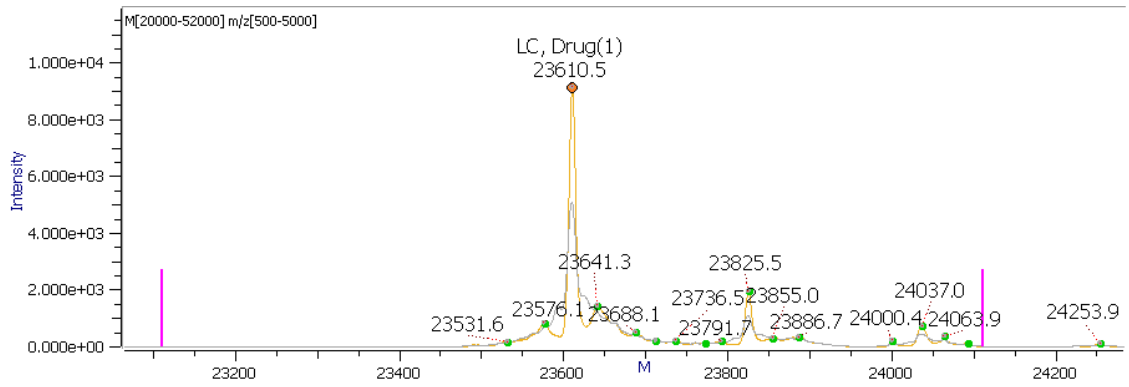

Fig. 4 Deconvoluted Spectrum of Peak 2, LC Drug 1

Table 4 . Average DAR Calculation

Protein name	Drug Count	Peak #	1		2		3		4		5	
			Intensity	Weighted Intensity	Intensity	Weighted Intensity	Intensity	Weighted Intensity	Intensity	Weighted Intensity	Intensity	Weighted Intensity
Heavy Chain (HC)	1						1022.715	1.32				
	2								361.965	0.94		
	3										162.948	0.63
Light Chain (LC)	0		5185.701	0								
	1				9150.992	1.21						
	2						62.801	0.02	664.705	0.18		
Average DAR value				**4.3**								

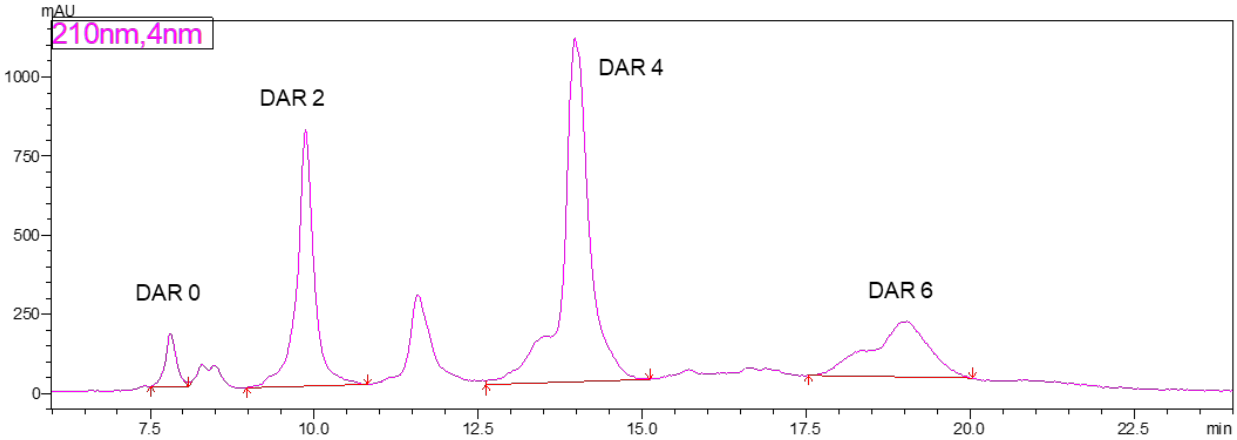

Fig. 5 HIC PDA Chromatogram

Results and Discussion (Continued)

HIC analysis was conducted on the native intact ADC mimic sample. The results showed various DAR variants ranging from DAR 0 to DAR 6, which aligns with the RP-LCMS data. The RP-LCMS analysis indicated that the LC had either 0 or 1 drug conjugated, while the HC exhibited 1, 2, or 3 drugs conjugated. The highest relative intensity observed on the HIC chromatogram, as illustrated in Fig 6, corresponds to DAR 4, which is consistent with the CoA DAR of 4.5 and the RP-LCMS result of DAR 4.3.

Conclusion

The end-to-end workflow utilizing the LCMS-9030 Q-TOF, Nexera XS inert LC, and Shim-pack column enables comprehensive characterization of ADCs. The analysis clearly illustrates that the LCMS-9030 Q-TOF is a highly effective tool for calculating DAR across various ADC variants with excellent sensitivity. It allows for the accurate identification of low-abundance DAR variants. Additionally, the results from HIC analysis complement the findings from LCMS-9030 Q-TOF, providing greater confidence in the accuracy of the data, which is crucial for drug development and regulatory submissions.

References

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