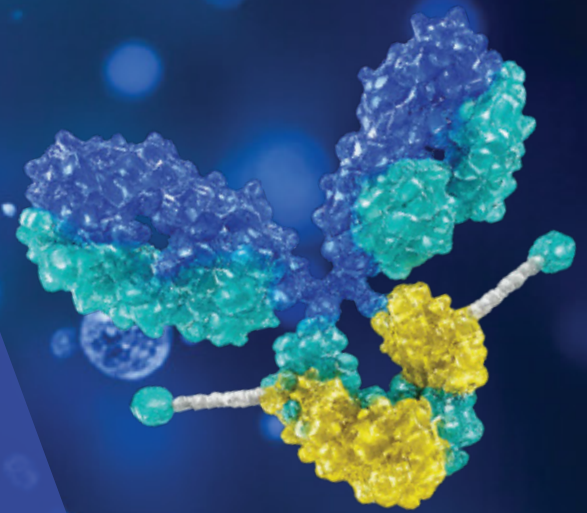


Robust Determination of Drug-to-Antibody Ratio (DAR) Using Orthogonal Analytical Techniques



Summary

Drug-to-Antibody Ratio (DAR) is a critical quality attribute influencing the efficacy, safety, and pharmacokinetics of antibody-drug conjugates (ADCs). Accurate DAR determination is essential across discovery, development, and regulatory stages.

In this whitepaper, we present a comprehensive analytical strategy integrating RP-HPLC, LC-MS, and HIC to ensure reliable and reproducible DAR determination. The complementary nature of these techniques provides quantitative accuracy, structural confirmation, and native-state characterization, enabling high-confidence decision-making for ADC development programs.

Introduction

ADCs have emerged as a promising strategy for cancer treatment, combining the specificity of monoclonal antibodies with the cytotoxic potency of small molecule drugs. The success of ADCs hinges on their ability to selectively deliver cytotoxic payloads to cancer cells while minimizing off-target effects. Critical to the design and optimization of ADCs is the accurate determination of the DAR, which quantifies the number of drug molecules conjugated to each antibody molecule. This study aims to explore the methodologies, principles, and comparative analysis of DAR determination techniques.

Traditional Methods vs. RP-HPLC, Mass Spectrometry, and Hydrophobic interaction chromatography:

Traditional methods for DAR calculation, such as UV-visible spectroscopy and peptide mapping, have inherent limitations in sensitivity, specificity, and accuracy. In recent years, RP-HPLC, mass spectrometry (MS), and hydrophobic interaction chromatography has emerged as a powerful technique for DAR analysis, offering high sensitivity, resolution, and structural information. The direct identification of drug molecules conjugated to antibodies by MS provides unambiguous confirmation of conjugation, enhancing the accuracy and reliability of DAR determination.

Comparative Analysis of DAR Determination Techniques:

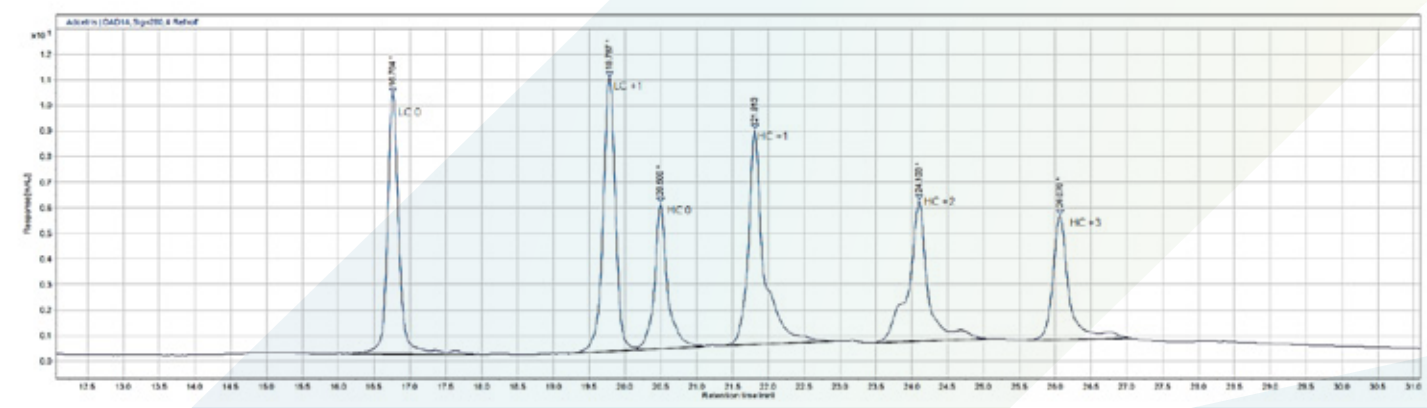
This section explores the principles and methodologies of three key techniques for DAR determination: RP-HPLC, LC-MS and HIC. RP-HPLC can effectively resolve different species of ADCs, including those with varying drug conjugation states. The use of RP-HPLC coupled with UV detection or mass spectrometry allows for precise quantification of individual species, enabling accurate calculation of DAR values. While MS provides structural insights by identifying mass peaks corresponding to drug payloads. The advantage of HIC lies in its ability to analyze ADCs under native conditions, providing a more accurate and physiologically relevant assessment of drug loading on the intact antibody molecule.

Methodologies

1. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

Reverse Phase High-Performance Liquid Chromatography holds significant importance in determining the DAR primarily due to its ability to precisely quantify both unconjugated light and Heavy chains with attached drug payloads, offering crucial insights into the potency and efficacy of ADCs. Additionally, it characterizes conjugation by revealing distribution and homogeneity, aids in process optimization by monitoring efficiency and guiding adjustments, and ensures regulatory compliance by providing robust and reliable data essential for approval, collectively making it an indispensable analytical tool in the development and characterization of ADCs.

DAR analysis by RP-HPLC



Chain	RT [min]	DAR Peak	Width [min]	Area	Area%	DAR
Light Chain	16.76	0	1.66	114.52	18.38	0
	19.78	1	1.01	126.81	20.36	0.52
				241.34		0.52
Heavy Chain	20.50	0	0.97	70.43	11.30	0
	21.81	1	1.52	127.82	20.52	0.33
	24.10	2	1.54	103.68	16.64	0.54
	26.07	3	1.64	79.51	12.76	0.62
				381.44		1.50
						4.04

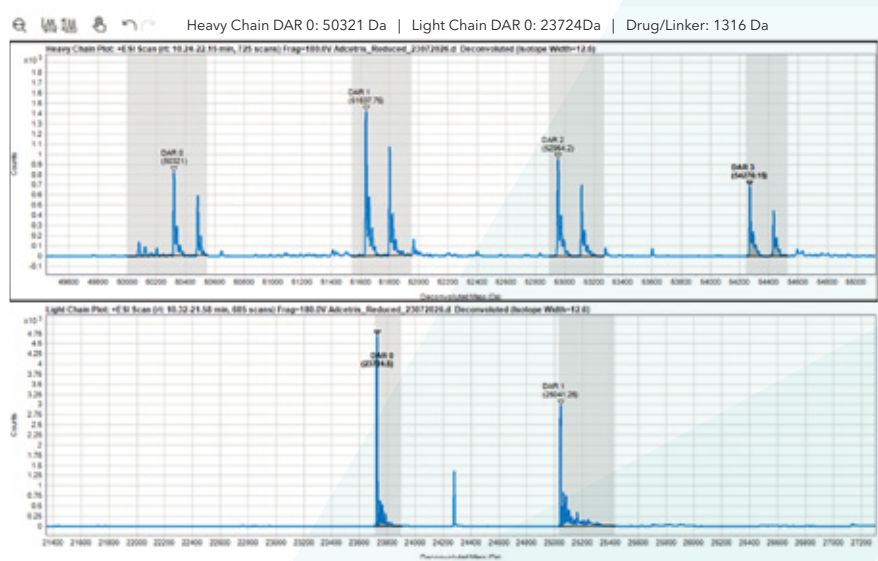
In this study, we investigated the DAR of a ADC by breaking four interchain disulfide bonds between two heavy and two light chains. The reduction process was executed utilizing 100mM Dithiothreitol (DTT) at 37°C for 10 minutes. Following sample preparation, RP-HPLC analysis was conducted using standard mobile phases containing 0.1% trifluoroacetic acid (TFA) and elevated column temperature to ensure optimal peak resolution and distinct separation between light chain (LC) and heavy chain (HC) species with varying drug loading. Through this analysis, we achieved the separation of LC, LC+1, HC, HC+1, HC+2, and HC+3 species. DAR calculation was performed by assessing the area under the curve for each peak, revealing a DAR value of 4.04

2. Liquid chromatography Mass Spectrometry (MS)

Reduced mass analysis serves as a fundamental technique in the determination of the drug-to-antibody ratio (DAR) for antibody-drug conjugates (ADCs). This method involves the reduction of disulfide bonds within the ADC structure, leading to the separation of the antibody chains and the associated drug payloads. Through the use of reducing agents such as dithiothreitol (DTT), the interchain disulfide bonds are cleaved, resulting in the liberation of the antibody heavy and light chains along with their respective drug conjugates.

Following reduction, the liberated antibody chains and drug payloads can be separated by reverse phase analyzed using mass spectrometry (MS) techniques. This approach allows for the quantification of both the antibody chains and the attached drug molecules, providing critical information for DAR determination. By comparing the mass spectra of the light chain and heavy chain with those of the light and heavy chain with payload attached on it, the relative abundance of drug-conjugated antibody chains can be assessed, enabling the calculation of the DAR.

DAR Deconvoluted Spectrum



Heavy Chain

DAR Peak	Start mass (Da)	End Mass (Da)	Area	%Area
0	50001.75	50550	2.33E+004	20.48
1	51540.4	51952.85	4.15E+004	36.49
2	52892.85	53270.85	2.88E+004	25.27
3	54244.9	54528	2.02E+004	17.76
DAR			1.4	

Light Chain

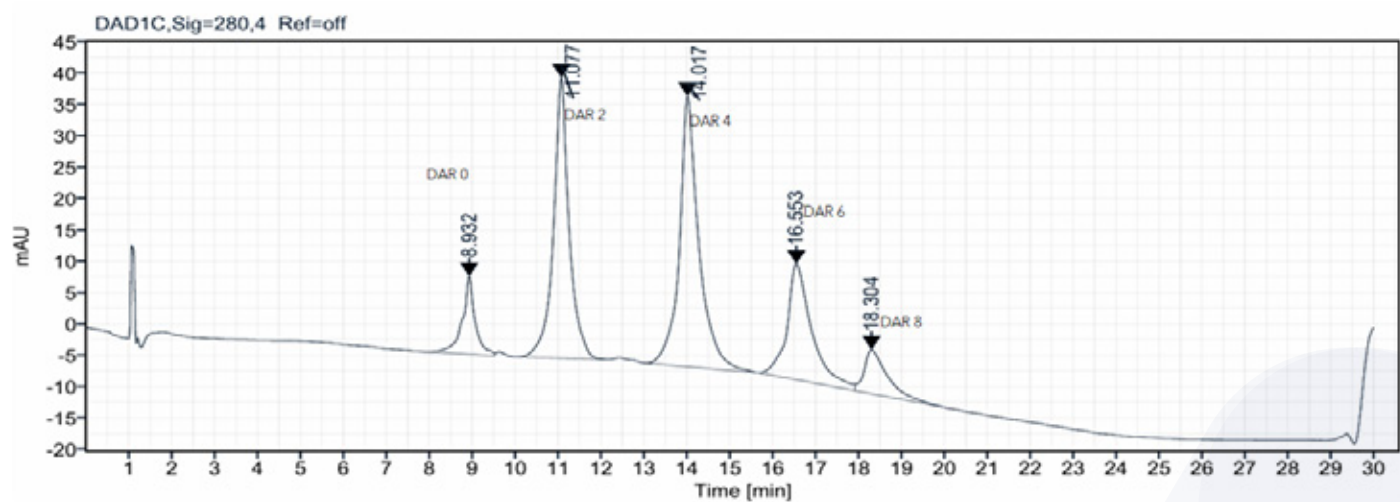
DAR Peak	Theoretical Mass (Da)	Observed Mass (Da)	Area	%Area
0	23711.95	23903.4	4.11E+004	45.29
1	25028.9	25435.3	4.97E+004	54.71
DAR			0.5	

Combined DAR: 3.8

In this study, we conducted reduced mass analysis of an antibody-drug conjugate (ADC) using the Agilent 6545 XT BIO-QTOF mass spectrometer to determine the drug-to-antibody ratio (DAR). The ADC sample underwent initial separation of its constituent chains via reversed-phase high-performance liquid chromatography (RP-HPLC), followed by mass analysis using the mass spectrometer. The resulting mass spectra were deconvoluted to determine the accurate mass of the antibody chains and their associated drug payloads. Subsequently, the acquired spectra were inputted into the DAR calculator software provided by Agilent, which utilized the area under the curve for each peak to calculate the DAR. Notably, the software computed DAR values separately for the light chain (LC) and heavy chain (HC) due to their distinct ionization properties, with the final DAR being a combination of both LC and HC values. Our findings revealed a DAR of 3.8 for the generated ADC, closely aligning with the DAR value obtained via RP-HPLC analysis.

3. Hydrophobic Interaction Chromatography (HIC)

One significant advantage of HIC over other methods, such as RP-HPLC and LC-MS, is its compatibility with native conditions for ADCs. Unlike RP-HPLC and MS, which often require the reduction of disulfide bonds within the ADC structure to separate its constituent components, HIC allows for the analysis of intact ADCs under native conditions. This means that HIC preserves the native conformation and structural integrity of the ADC, providing a more accurate representation of the drug loading on the intact antibody molecule. By analysing ADCs under native conditions, HIC offers a more physiologically relevant environment for studying drug conjugation and distribution.



RT [min]	DAR Peak	Width [min]	Area	Area%	DAR
8.932	0	1.56	256.60	6.82	0
11.077	2	2.10	1136.06	30.19	0.6
14.017	4	2.56	1365.20	36.28	1.45
16.553	6	2.24	739.15	19.64	1.17
18.304	8	2.01	265.57	7.05	0.56
			3,762.58	DAR	3.78

Employing a Tosoh HIC column with buffers containing ammonium sulfate and elution buffer devoid of ammonium sulfate, our analysis required no pretreatment steps such as reduction or augmentation of hydrophobicity. A 30-minute gradient containing 25% isopropanol (IPA) in mobile phase B facilitated optimal separation of ADC species with varying drug loadings. Notably, predominant peaks corresponding to DAR values of 2, 4, 6, and 8 were observed, with DAR 4 being the most abundant. The calculated average DAR of 3.78 aligns closely with values obtained from RP-HPLC and Mass Spectrometry analyses.

Conclusion

Determining the DAR of ADCs is critical for their development and quality control. According to our comprehensive analysis employing RP-HPLC, LC-MS, and HIC has provided valuable insights into the DAR of ADC under investigation. RP-HPLC analysis yielded a DAR value of 4.04, while MS analysis determined a slightly lower value of 3.8, and HIC-HPLC analysis revealed a DAR of 3.78.

The close agreement between the RP-HPLC and MS results suggests the robustness and consistency of these techniques in DAR determination. Importantly, the HIC-HPLC analysis provided additional information on the intact ADC structure without the need for reduction or digestion, offering insights into the native conformation and hydrophobic interactions governing drug conjugation. The obtained DAR values collectively contribute to a comprehensive understanding of the drug loading profile of the ADC, guiding its optimization and development as a targeted therapeutic agent.