Application Note

Biopharma/Pharma

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Determining Antibody-Drug Conjugate Positional Isomer Distribution with Nonreduced CE-SDS and HIC

Using the Agilent ProteoAnalyzer system and the Agilent 1290 Infinity III Bio LC

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Abstract

Antibody-drug conjugates (ADCs) require characterization of both drug-to-antibody ratio (DAR) and drug conjugation location, because each influences drug efficacy. Hydrophobic interaction chromatography (HIC) robustly resolves hydrophobic payloads to quantify DAR distributions, but does not provide positional information. Alternately, nonreduced capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) reports fragmentation patterns that reflect payload placement at interchain disulfides but cannot directly assign DAR. Here, we combine HIC and nonreduced CE-SDS data, applying a previously described system-of-equations approach to estimate the relative abundance of positional isomers in a cysteine-conjugated ADC population.

The SigmaMAb ADC mimic was analyzed by CE-SDS using the Agilent ProteoAnalyzer system to determine the relative levels of the light chain (L), heavy chain (H), HL, HH, HHL, and intact mAb species. HIC analysis of the ADC using the Agilent 1290 Infinity III Bio LC with an Agilent AdvanceBio HIC column was used to determine the relative amounts of the DAR0, DAR2, DAR4, DAR6, and DAR8 species. Analysis of the coupled dataset revealed that the dominant species in the sample was a DAR4, with drugs located at both the F_{ab} disulfides, accounting for 41.5%. Additional major species included $DAR2_f$ (19.3%) and $DAR6_{fhh}$ (22.9%), while $DAR2_h$ and $DAR6_{ffh}$ were not observed. This integrated workflow using Agilent CE-SDS and Agilent 1290 Infinity III Bio LC systems enables the rapid estimation of ADC positional isomer composition using widely adopted methods, providing actionable structural insight without additional specialized assays.

Introduction

Antibody-drug conjugates (ADCs) have emerged as potent biopharmaceuticals that allow the delivery of payloads to specific tissues. ADCs have been widely adopted for the targeted delivery of cytotoxic cancer biopharmaceuticals directly to cancerous cells, reducing systemic side effects. The drug payloads are conjugated to antibodies with a stable linker attached to an amino acid. Generally, the linkers target reactive amino acids such as lysine and cysteine. The conjugation strategy used influences the drug-to-antibody ratio (DAR) and the potential locations of the drug. Since both DAR and drug location impact therapeutic efficacy, full characterization of an ADC requires determination of both parameters.

Canonical IgG1 monoclonal antibodies (mAbs) have four interchain reducible disulfides: one at each of the light chain (L) and heavy chain (H) junctions, referred to as the Fab (f) location, and two at the H:H junction, referred to as the hinge (h) location (Figure 1). Conjugation of the therapeutic to these cysteine residues by disulfide reduction allows for greater control of the DAR than conjugation to lysine. Each reduced disulfide bond allows for the addition of two drugs. While this allows for consistent DAR values, the exact location of the drug cannot be controlled. The addition of the drug breaks the disulfides that covalently link the light and heavy chains, allowing SDS to denature the antibody into individual light and heavy chains. Depending on the number and location of the drugs, different antibody fragments are formed in the presence of SDS (Figure 1).

Most therapeutic payloads are hydrophobic in nature, which allows hydrophobic interaction chromatography (HIC) to separate the DAR0, DAR2, DAR4, DAR6, and DAR8 species. This has resulted in the wide adoption of HIC for DAR determination.[1] However, positional information is not captured by HIC. In contrast, CE-SDS allows for quantitation of the fragments produced by addition of the drug payload, providing insights into drug location,[2] but does not allow for determination of the DAR. Previous work has demonstrated that, by combining HIC DAR data with the positional information from nonreduced CE-SDS, it is possible to mathematically determine the amounts of each positional isomer in the sample using a system-of-equations approach.[3]

The Agilent 1290 Infinity III Bio LC is specifically designed for conditions used in bio chromatography, such as the high-concentration buffers used for HIC analysis, to avoid potential corrosive damage to the system. The Agilent ProteoAnalyzer system automates CE-SDS separations of reduced and nonreduced samples using a 12-channel parallel capillary array, enabling efficient workflows. In this application note, the data from nonreduced CE-SDS using the ProteoAnalyzer system and HIC using the 1290 Infinity III Bio LC were applied to a previously described system of equations (Equations 1.1–1.9) to determine the amount of each positional isomer in the SigmaMAb ADC mimic.

Drug-to-Antibody Ratio	Positional Isomer		Fragments					
0	DAR0		mAb					
2	$DAR2_f$			HHL				L
	$DAR2_h$		mAb					
4	$DAR4_{ff}$				HH			2L
	$DAR4_{fh}$			HHL				L
	$DAR4_{hh}$					2HL		
6	$DAR6_{ffh}$				HH			2L
	$DAR6_{fhh}$					HL	H	L
8	DAR8						2H	2L

Figure 1. Antibody fragments produced under nonreduced denaturing CE-SDS analysis of cysteine-conjugated ADC.

Experimental

The SigmaMAb Antibody Drug Conjugate Mimic (Sigma, part number MSQC8-0.5MG) was used for all analyses.

CE-SDS using the Agilent ProteoAnalyzer system

The SigmaMAb ADC mimic was reconstituted to 10 mg/mL and further diluted to 1.5 mg/mL in nuclease-free water. Sample concentration was confirmed by NanoDrop (settings: Protein A280; Curve type: other E1%; Extinction coefficient: 14.3). The sample was then prepared under nonreducing conditions according to the Agilent Protein Broad Range P240 kit (part number 5191-6640) manual.[4] The samples were covalently labeled by incubating with the supplied reagents at 70 °C for 10 minutes[5] and analyzed across multiple capillaries on an Agilent ProteoAnalyzer system with the ProteoAnalyzer Broad Range kit LM Only method. For optimal results, the sample injection was decreased to 7 kV 6 seconds.[5] Sizing analysis was performed using NISTmAb as the ladder.[6]

Agilent ProSize data analysis software was used to determine the percent total of the mAb, HHL, HH, HL, H, and L chains.

Hydrophobic interaction chromatography

Software

Agilent OpenLab version 2.8 or later was used to determine peak areas for calculating the average DAR, as well as the relative percentages of the DAR0, DAR2, DAR4, DAR6, and DAR8 species.

The Agilent 1290 Infinity III Bio LC system comprised the following modules:

- Agilent 1290 Infinity III Bio Flexible Pump (G7131A)
- Agilent 1290 Infinity III Variable Wavelength Detector (G7114B) equipped with a biocompatible micro flow cell, 3 mm, 2 µL
- Agilent 1290 Infinity III Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity III Bio Multisampler (G7137A) with option no. 101

Column

Agilent AdvanceBio HIC column, 3.5 µm, 4.6 × 100 mm (part number 685975-908)

Buffer and solvent preparation

A. 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7
B. 50 mM phosphate buffer at pH 7
C. 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 to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7 (Buffer B). Ammonium sulfate (198.21 g; total concentration 1.5 M) was added to an empty amber-colored 1 L bottle and filled to 1 L using the prepared phosphate buffer (Buffer A). The pH value was checked and adjusted, if necessary, to pH 7 (the addition of high amounts of salt can alter pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

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).

Table 1. Chromatographic conditions* used in this study.

Parameter	Value
Buffers and Solvents	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 C) Isopropanol
Gradient	Gradient: 0 min 55% A, 40% B, 5% C 25 min 0% A, 75% B, 25% C Stop time: 35 min Post-time: 10 min
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

*When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method, as high amounts of salt can change the compressibility of the solvent. Using the preconfigured solvent tables enables the best pump performance.

Samples

The previously mentioned reconstituted unmodified SigmaMAb control and SigmaMAb ADC mimic, each at a concentration of 10 mg/L, were used as samples and diluted with 50% Buffer A (Table 1).

System-of-equations analysis

SciPy's lsq_linear python was used to solve the system of equations (Equations 1.1–1.9) for $DAR2_f$, $DAR2_h$, $DAR4_{ff}$, $DAR4_{fh}$, $DAR4_{hh}$, $DAR6_{ffh}$, and $DAR6_{fhh}$ (variables in bold) using least-squares minimization, with the variable values constrained to be greater than or equal to zero. The relative percentages for the individual antibody fragments (as determined by CE-SDS) and the DAR (as determined by HIC) were used as the input values. Because the CE-SDS analysis uses a dye that covalently labels primary amines, equations were corrected by determining the relative number of lysine residues in the specific antibody fragment in relation to the intact mAb.

Equations 1.1-1.9

$$\text{mAb} = (DARO + \boldsymbol{DAR2_h}) \times \frac{2 \times LysH + 2 \times LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{HHL} = (\boldsymbol{DAR2_f} + \boldsymbol{DAR4_{hh}}) \times \frac{2 \times LysH + LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{HH} = (\boldsymbol{DAR4_{ff}} + \boldsymbol{DAR6_{fhh}}) \times \frac{2 \times LysH}{2 \times LysH + 2 \times LysL}$$

$$\text{HL} = (2 \times \boldsymbol{DAR4_{ff}} + \boldsymbol{DAR6_{fhh}}) \times \frac{LysH + LysL}{2 \times LysH + 2 \times LysL}$$

$$\text{H} = (\boldsymbol{DAR6_{ffh}} + 2 \times DAR8) \times \frac{LysH}{2 \times LysH + 2 \times LysL}$$

$$\text{L} = (\boldsymbol{DAR2_f} + 2 \times \boldsymbol{DAR4_{ff}} + \boldsymbol{DAR4_{fh}} + 2 \times \boldsymbol{DAR6_{ffh}} + \boldsymbol{DAR6_{fhh}} + 2 \times \boldsymbol{DAR8}) \times \frac{LysH}{2 \times LysH + 2 \times LysL}$$

$$1 = \frac{\boldsymbol{DAR2_f} + \boldsymbol{DAR2_h}}{DAR2}$$

$$1 = \frac{\boldsymbol{DAR4_{ff}} + \boldsymbol{DAR4_{fh}} + \boldsymbol{DAR4_{hh}}}{DAR4}$$

$$1 = \frac{\boldsymbol{DAR6_{ffh}} + \boldsymbol{DAR6_{fhh}}}{DAR6}$$

Results and discussion

Reduced CE-SDS analysis of cysteine-conjugated ADCs

Nonreduced CE-SDS analysis of the SigmaMAb ADC mimic produces a fragmentation pattern consistent with L, H, HL, HH, HHL, and intact HHLL antibody species. The relative concentration of each peak is used to determine the percentage of each fragment relative to the total (Table 2, Figure 2).

Table 2. Relative percentage of each antibody fragment as determined by CE-SDS analysis using the Agilent ProteoAnalyzer system.

Antibody Fragment	Relative Percent
L	18.0%
H	14.7%
HL	14.8%
HH	29.9%
HHL	19.2%
HHLL (intact mAb)	3.6%

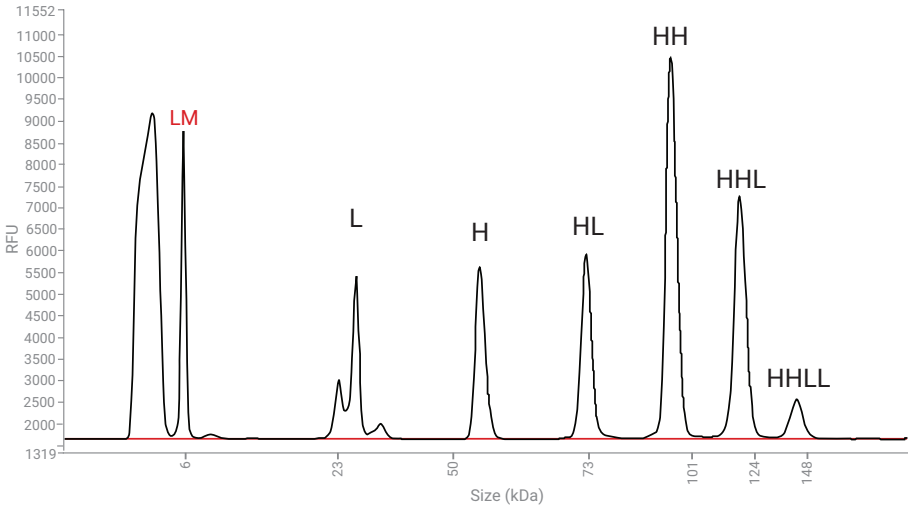

Figure 2. CE-SDS analysis of the SigmaMAb ADC mimic using the Agilent ProteoAnalyzer system under nonreducing conditions.

HIC was used to determine the overall DAR value according to Equation 2. The SigmaMAb ADC mimic has an average DAR of 4.04, consistent with the value of 4.4 reported in the certificate of analysis (CoA). Additionally, peak areas are used to determine the percentage of each DAR species (Table 3, Figure 3).

Equation 2

$$\text{Average DAR} = \sum \frac{\text{LC peak area x Number of drugs}}{\text{Total LC peak area}}$$

The values from Tables 2 and 3, along with the number of lysines in the L and H chains as reported by Sigma in the SigmaMAb data sheet, were used to solve the system of equations for the different positional DAR isomers (Table 4). Replicate computations with the same inputs return the same solutions, indicating that the algorithm only identifies a single best solution (Table 4).

Using the system of equations, 41.5% of the ADC population is composed of DAR4, with the payloads located at the two Fab locations ($DAR4_{ff}$ species). Summation of $DAR4_{ff}$, $DAR4_{fh}$, and $DAR4_{hh}$ values determined by the system of equations indicates that 48.1% of the ADC is composed of DAR4 species. This is consistent with the HIC data, which indicate that 48.3% of the ADC has a DAR of 4. The next major species determined by the system of equations are $DAR2_f$ and $DAR6f_{hh}$ at 19.3% and 22.9%, respectively. Notably, the $DAR2_h$ and $DAR6_{ffh}$ species are consistently absent. The total amounts of DAR2 and DAR6 calculated by the system of equations is consistent with the HIC values of 19.5% and 22.2%, respectively.

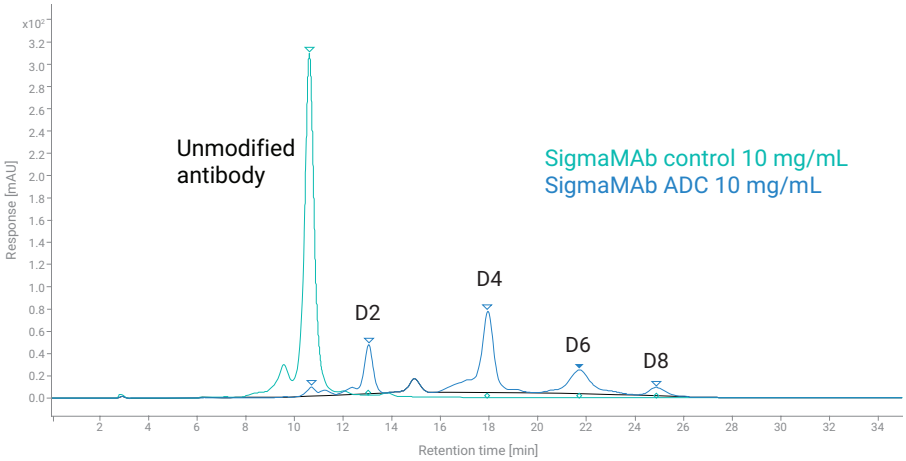

Figure 3. HIC analysis of the unmodified SigmaMAb control and the SigmaMAb ADC mimic using the Agilent 1290 Infinity III Bio LC. The DAR0, DAR2, DAR4, DAR6, and DAR8 peak areas are used to determine the relative amount of each individual species, as well as the average DAR.

Table 3. Relative percentage of each DAR species as determined by HIC using the Agilent 1290 Infinity III Bio LC.

Drug-to-Antibody Ratio (DAR)	Relative Percent
0	5.2%
2	19.5%
4	48.3%
6	22.2%
8	4.8%

Table 4. Relative percentages of each ADC species as determined by solving the system of equations using CE-SDS and HIC data.

ADC Species	Relative Percent
DAR0	5.2%
$DAR2_f$	19.3%
$DAR2_h$	0.0%
$DAR4_{ff}$	41.5%
$DAR4_{fh}$	3.1%
$DAR4_{hh}$	3.6%
$DAR6_{ffh}$	0.0%
$DAR6_{fhh}$	22.9%
DAR8	4.8%

Conclusions

The method presented here combines analytical techniques commonly used during ADC analysis to computationally determine the positional isomers of the ADC population. This allows for quick estimation of the relative ADC isomer abundance without the need for additional techniques. The Agilent ProteoAnalyzer system and the Agilent 1290 Infinity III Bio LC provide highly reproducible CE-SDS and HIC analyses for characterization of ADCs.

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PR7004-1560

Published in the USA, April 29, 2026
5994-9181EN

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