Application Note

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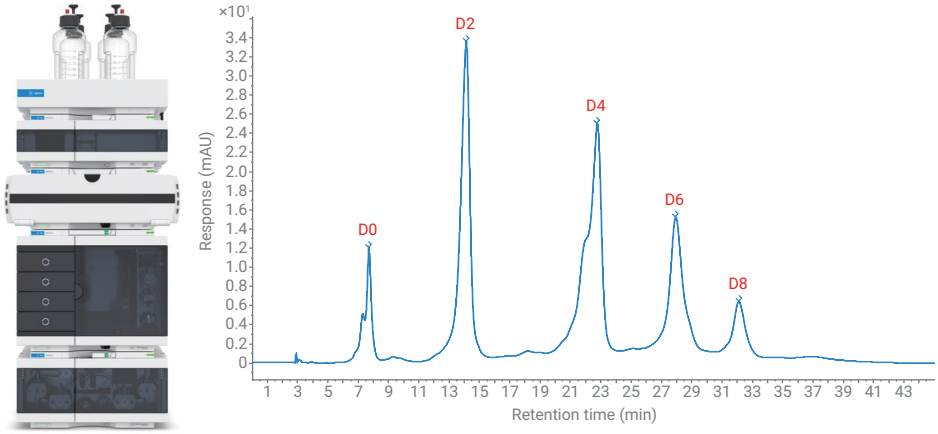
High Salt—High Reproducibility

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



Author

Sonja Schneider
Agilent Technologies, Inc.

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.

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,

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.

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

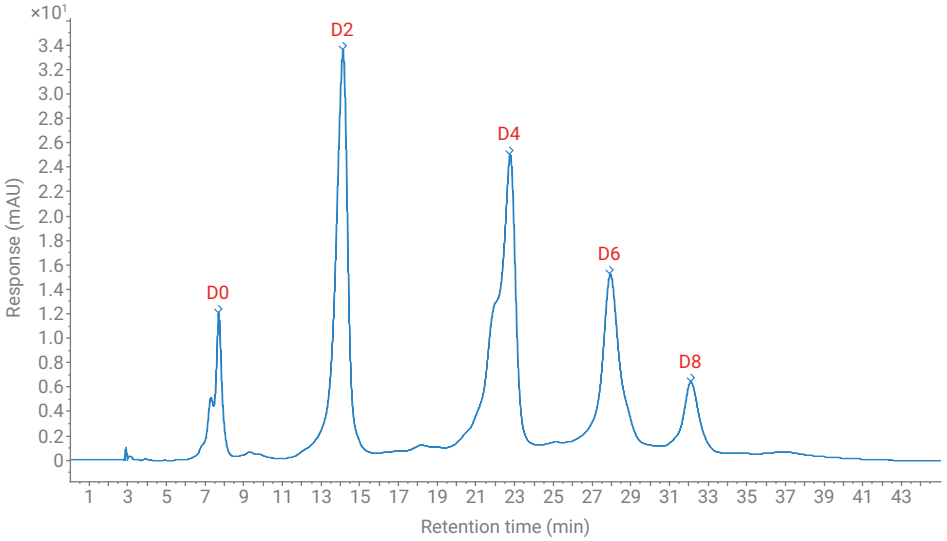


Figure 1. Analysis of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC. D0 to D8 refers to different DAR species.

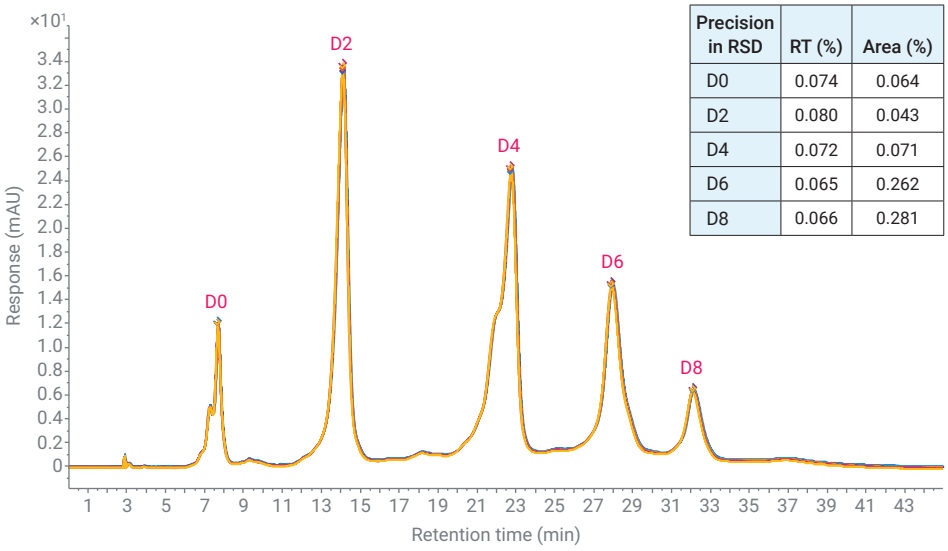
The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.7 (see Table 2). This value is consistent with the literature.[8]

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).

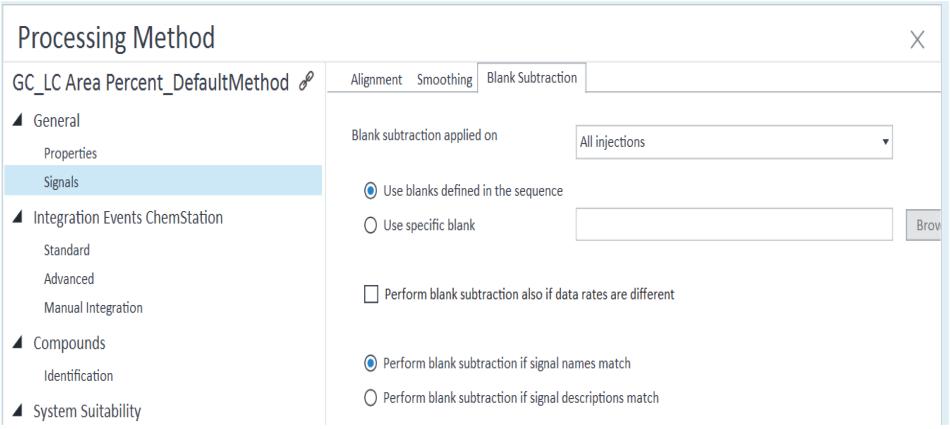


Figure 3. Screenshot of the "Blank Subtraction" feature in the processing method of Agilent OpenLab CDS 2.

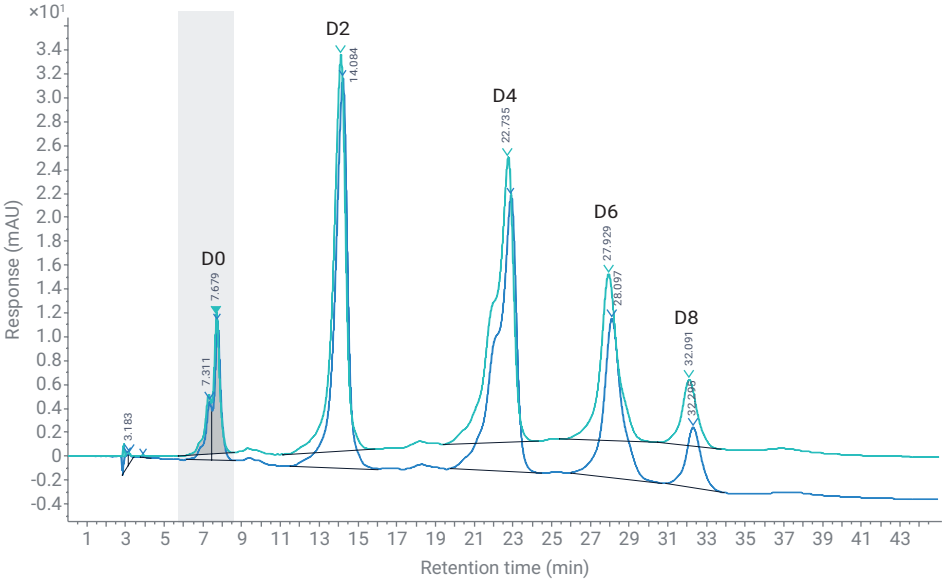


Figure 4. Comparison of HIC chromatogram before (blue) and after (green) blank subtraction.

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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