Application Note

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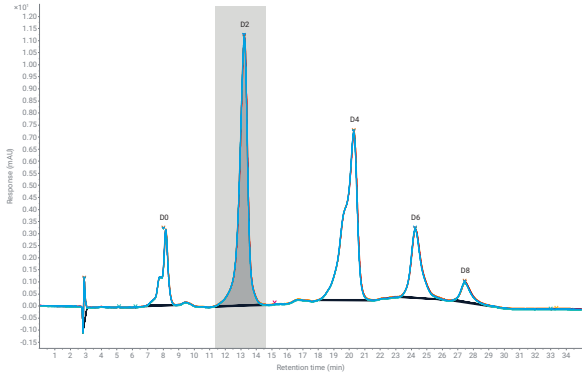
Convenient and Reliable Analysis of Antibody Drug Conjugates

Drug-to-antibody determination with ternary gradients on the Agilent 1260 Infinity II Prime Bio LC

Suitable for Agilent
1260 Infinity III LC



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Abstract

The addition of organic modifiers like isopropanol in hydrophobic interaction chromatography (HIC) can be an important parameter to decrease the retention of hydrophobic antibody drug conjugates (ADCs) as well as to adjust selectivity. This application note demonstrates the drug-to-antibody (DAR) determination of brentuximab vedotin using a ternary gradient with isopropanol as organic modifier in the third channel. Excellent reproducibility was found for this challenging combination of high-salt-containing buffer and organic solvent, making the Agilent 1260 Infinity II Prime Bio LC the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data.

Introduction

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 such as brentuximab vedotin are generated after the reduction of the interchain disulfides, resulting in free sulfhydryl groups that can be conjugated to specific maleimide linkers. The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero (D0), two (D2), four (D4), six (D6), and eight (D8) drugs per antibody.

Due to the hydrophobicity of the high DAR species in particular, the addition of an organic modifier such as isopropanol is helpful to enable full elution from the HIC column. Typically, in binary gradients, the modifier is added to the mobile phase used for elution (usually a buffer containing little or no salt). The Agilent 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of a third channel to add the organic modifier solvent. The combination of high-salt-containing buffers with organic mobile phases can be critical due to potential formation of salt crystals when the two solvents mix in the pump.

The 1260 Infinity II Prime Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: 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 made of MP35N, a nickel-cobalt alloy. For this reason, the 1260 Infinity II Prime Bio LC is optimally suited to the conditions used in bio chromatography, with the high concentrations of corrosive salts typically used in HIC, to avoid potential corrosive damage to the system.

Experimental

Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- 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 or later versions

Column

Agilent AdvanceBio HIC column 3.5 µm, 4.6 × 100 mm (part number 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 50% water: 50% 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

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 up to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7 (buffer B). 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, to pH 7 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

Method

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 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

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 1260 Infinity II Prime 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, which features 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 with five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. 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.[2]

The analysis was evaluated for the precision of retention time (RT) and area. With seven subsequent runs, an excellent RT precision of lower than 0.055% relative standard deviation (RSD) was found. This proves the excellence of the quaternary pump to run ternary gradients even with very challenging combinations of high-salt-containing buffers (such as the 1.5 M ammonium sulfate buffer used here) and isopropanol as organic modifier. The area precision was also excellent, with RSDs lower than 0.46%, except for the last peak (see the table in Figure 1).

Peak	%RSD RT	%RSD Area
DO	0.006	0.893
D2	0.030	0.321
D4	0.042	0.458
D6	0.046	0.344
D8	0.054	4.536

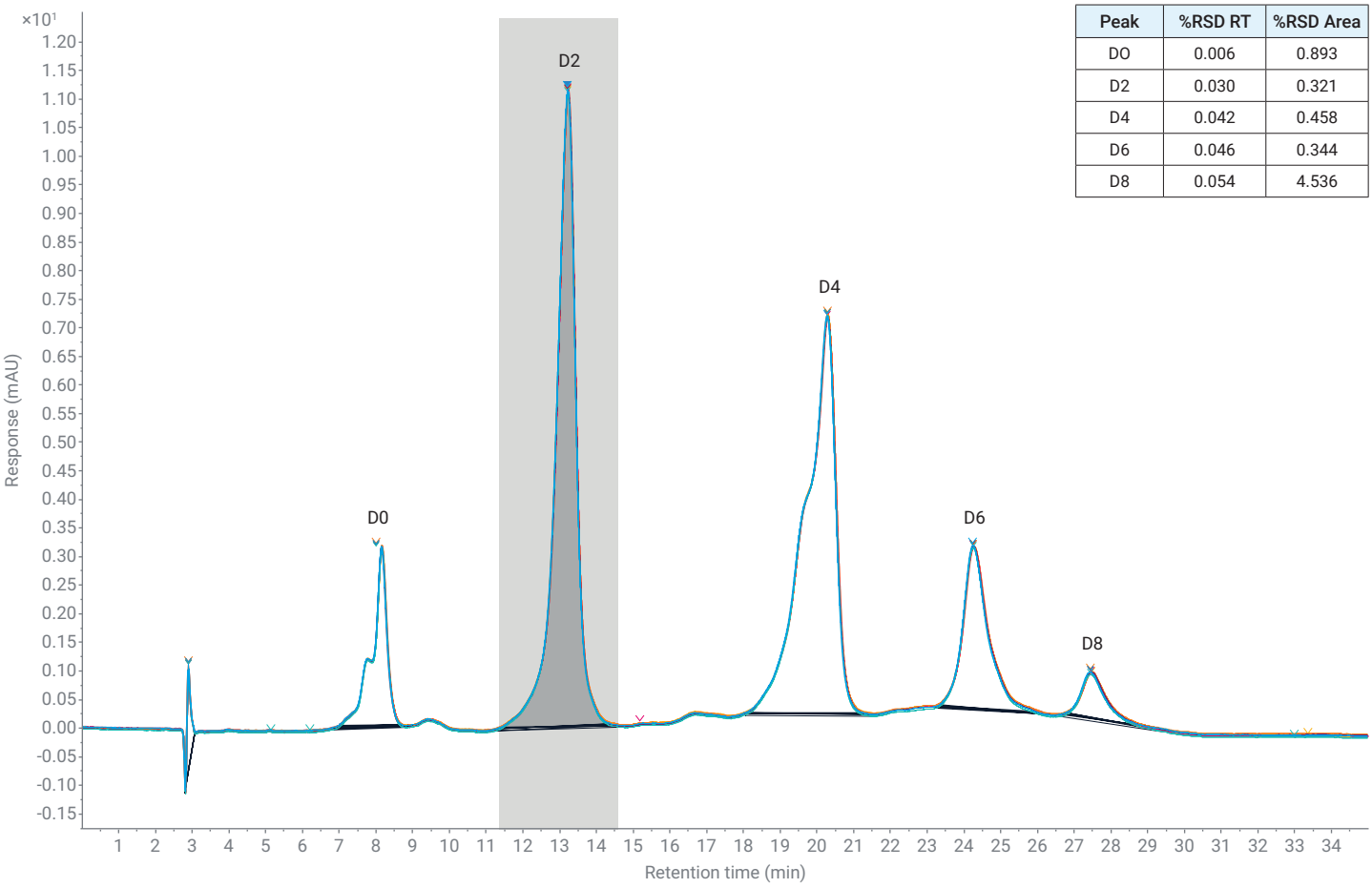

Figure 1. Separation of brentuximab vedotin on the Agilent 1260 Infinity II Prime Bio LC. D0–D8 refers to different DAR species. An overlay of seven subsequent runs is displayed. Blank Subtraction was applied to filter out the baseline drift caused by the ammonium sulfate salt in buffer A using blank injections run in the same sequence.

The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR. By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

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

Equation 1.

The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.3 (see Table 1).

Table 1. DAR species results.

DAR Species	RT (min)	Area	Area%	DAR Calculated
D0	8.00	89.18	8.11	0
D2	13.22	427.04	38.83	0.78
D4	20.29	405.58	36.88	1.48
D6	24.27	140.51	12.78	0.77
D8	27.48	37.38	3.4	0.27
			DAR	**3.3**

Conclusion

Brentuximab vedotin was analyzed using HIC in a ternary gradient with isopropanol in a third channel as organic modifier. All five expected ADC 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 3.3 drug molecules per antibody. The challenging solvent combination was managed outstandingly by the Agilent 1260 Infinity II Prime Bio LC including the Agilent 1260 Infinity II Bio Flexible Pump. The reproducibility of retention times was excellent, with relative standard deviations below 0.055%, allowing binary like performance for highest confidence in generated data.

References

1. McCombs, J. R.; Owen, S. C. Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry. *J. Amer. Assoc. Pharm. Sci.* **2015**, *17(2)*, 339–51.
2. Rodriguez-Aller, M. *et al.* Practical Method Development for the Separation of Monoclonal Antibodies and Antibody-Drug-Conjugate Species in Hydrophobic Interaction Chromatography, Part 1: Optimization of the Mobile Phase. *J. Pharm. Biomed. Anal.* **2016**, *118*, 393–403.

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