Application Note

Biologics Development

An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs)

Using the Agilent 1260 Infinity II Bio-Inert LC

Author

Andrew Coffey and
Sandeep Kondaveeti
Agilent Technologies, Inc.

Abstract

This Application Note describes the characteristics of the AdvanceBio HIC column and its use in the analysis of antibody drug conjugates (ADCs). Hydrophobic interaction chromatography (HIC) is a method of performing separations of biomolecules with increasing hydrophobicity. This makes it a technique that is well suited to the analysis of ADCs, as these complex molecules are often more hydrophobic than the unmodified antibody. The technique can provide high resolution capable of separating species with minor differences in hydrophobicity, including those ADCs that have differing of drug-to-antibody ratio (DAR). A benefit of HIC comes from the mild conditions used for elution, which ensures that the biomolecule remains in an undenatured state. This can be advantageous for the analysis of cysteine-linked ADCs.

Introduction

HIC uses mobile phases containing salts that reduce biomolecule solubility. This encourages absorption onto the HIC stationary phase. Reducing the level of salt through gradient elution allows the molecules to elute in order of increasing hydrophobicity. The separation may resemble reversed-phase chromatography, but without the ion pair reagents and high organic solvent composition that often lead to protein denaturation. This is important for the analysis of ADCs conjugated through cysteine links. The small molecule used as the payload is attached to free thiols of the partially reduced monoclonal antibody (mAb), but this results in a polydisperse distribution (Figure 1). It is essential to measure this distribution, known as the DAR, to determine the potency of the resulting ADC.

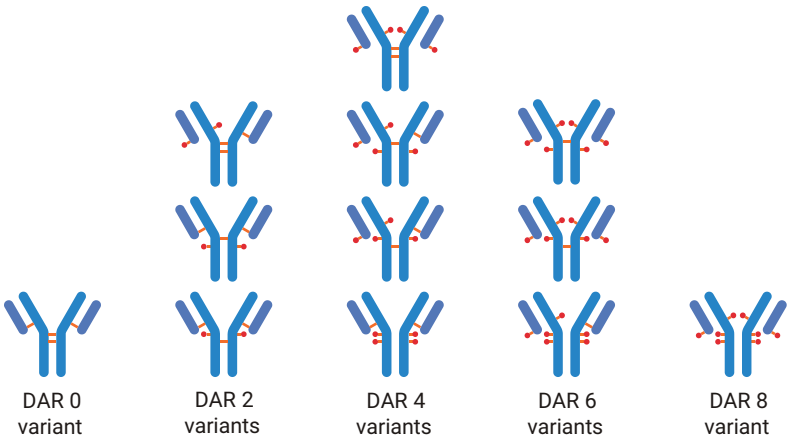


Figure 1. Polydisperse distribution of cysteine-linked ADCs with different quantities and positions of payload components.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 water purification system (Millipore).

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option no. 100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option no. 019)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert flow cell (option no. 028)

Software

Agilent OpenLab 2.2 CDS

Method conditions

	HPLC conditions
Column	AdvanceBio HIC 4.6 × 100 mm (p/n 685975-908) AdvanceBio HIC 4.6 × 30 mm (p/n 681975-908)
Mobile phase	Eluent A) 50 mM Sodium phosphate, pH 7.0 Eluent B) 2 M Ammonium sulfate in 50 mM sodium phosphate, pH 7.0 Eluent C) Propan-2-ol Eluent D) HPLC grade water
Flow rate	0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Total run time	31 minutes

Typical gradient profile (see Figure 2)

Time	%A	%B	%C
0	50	45	5
15	75	0	25
20	75	0	25
21	50	45	5
31	50	45	5

Results and Discussion

The high concentrations of salt used in HIC mean that a fully bio-inert LC will be beneficial. This is particularly the case if it is accompanied by additional features such as seal wash and needle wash to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in concentrated salt solution for any length of time. For that reason, using a quaternary LC system enables other channels to be used for organic modifiers and water or other flush solvents. The presence of small hydrophobic drug molecules conjugated to the mAb results in considerable changes to overall hydrophobicity of the mAb molecule. Consequently, it is necessary to include some organic modifier in the mobile phase (as seen in the method conditions and gradient profile sections).

Using HIC, it becomes possible to analyze cysteine-linked ADCs in their intact state (Figure 2). If reversed-phase techniques were used, the heavy and light chains that were not linked by a cysteine bond would become separated. It is possible to analyze cysteine-linked ADCs by reversed-phase chromatography, but only if the molecule is fully reduced[1].

By integrating the peak areas of the different DAR variants, 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.

Table 1 shows the value calculated for this sample. DAR 4.04 is in agreement with the expected value, and also the value previously observed[2].

Table 1. Peak area to DAR results.

No.	RT (min)	Area	%Area	DAR	
1	8.03	763	6.9	0	0.00
2	9.77	2,759	25.1	2	0.50
3	13.31	3,936	35.8	4	1.43
4	15.91	2,565	23.3	6	1.40
5	18.26	978	8.9	8	0.71
				DAR	**4.04**

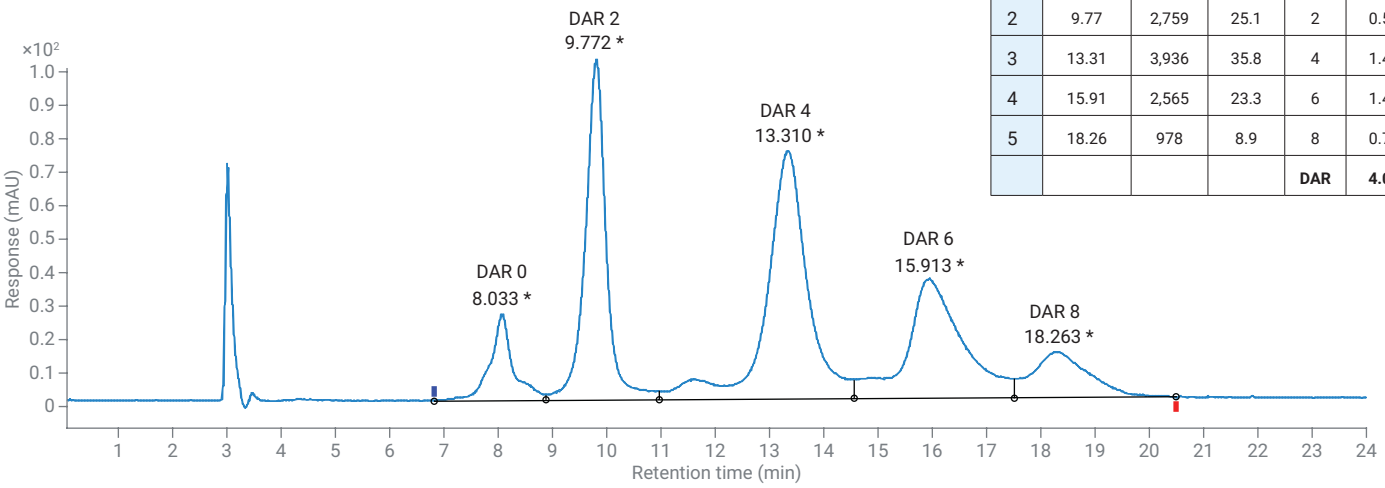
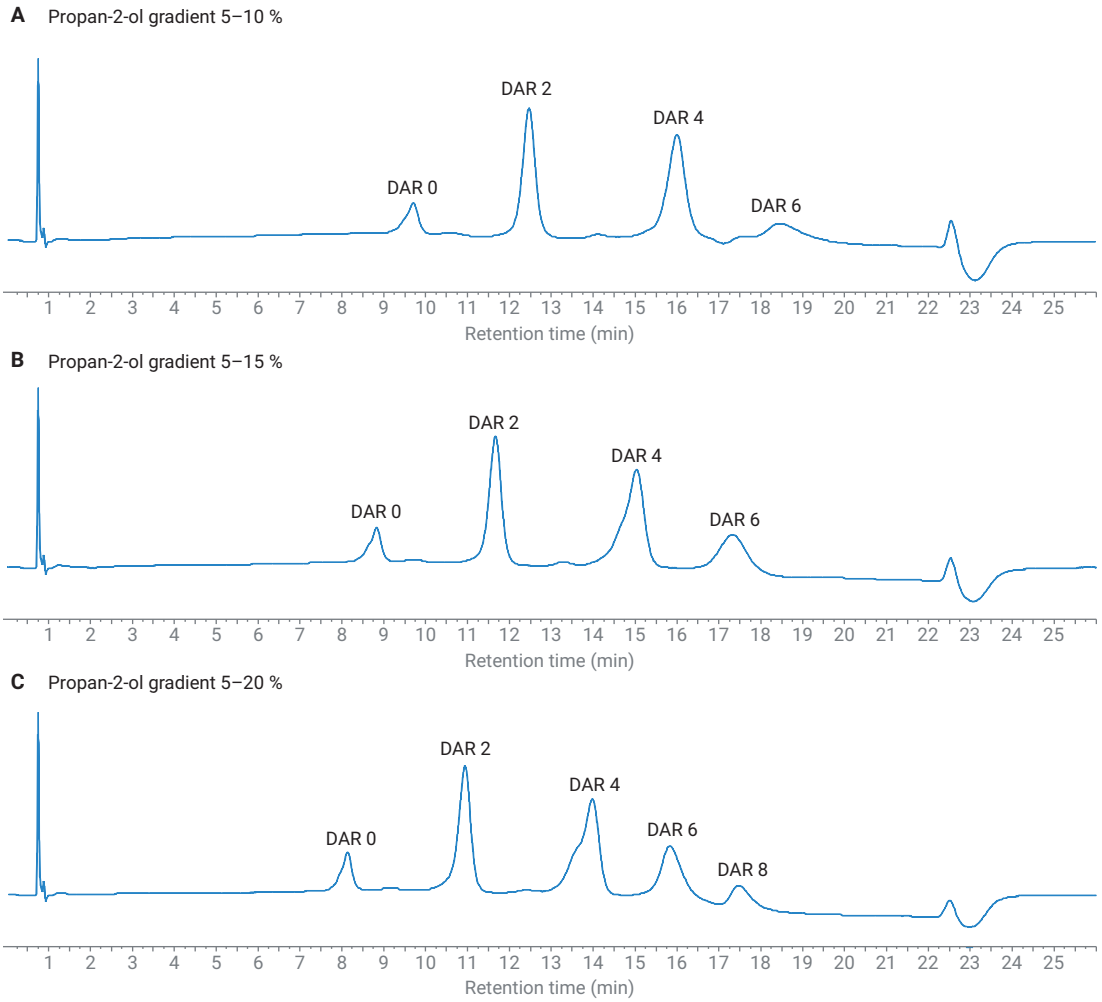


Figure 2. HIC separation of brentuximab vedotin (Adcetris).

Take care with sample preparation to ensure that the sample is fully dissolved and that there is no discrimination of the DAR variants; DAR 0 is more hydrophilic than DAR 8, and can be selectively dissolved in the mobile phase. This will result in errors if all of the sample is not completely dissolved.

It is important to include the propan-2-ol gradient in the mobile phase conditions, as without this, it is possible to leave the DAR 6 and DAR 8 variants adsorbed to the column. Figures 3A, 3B, and 3C illustrate that, with insufficient propan-2-ol concentration, more hydrophobic DAR 6 and DAR 8 variants are retained.



A. Profile for 5–10 % gradient.

Time	%A	%B	%C
0	20	75	5
15	90	0	10
20	90	0	10
21	20	75	5
26	20	75	5

B. Profile for 5–15 % gradient.

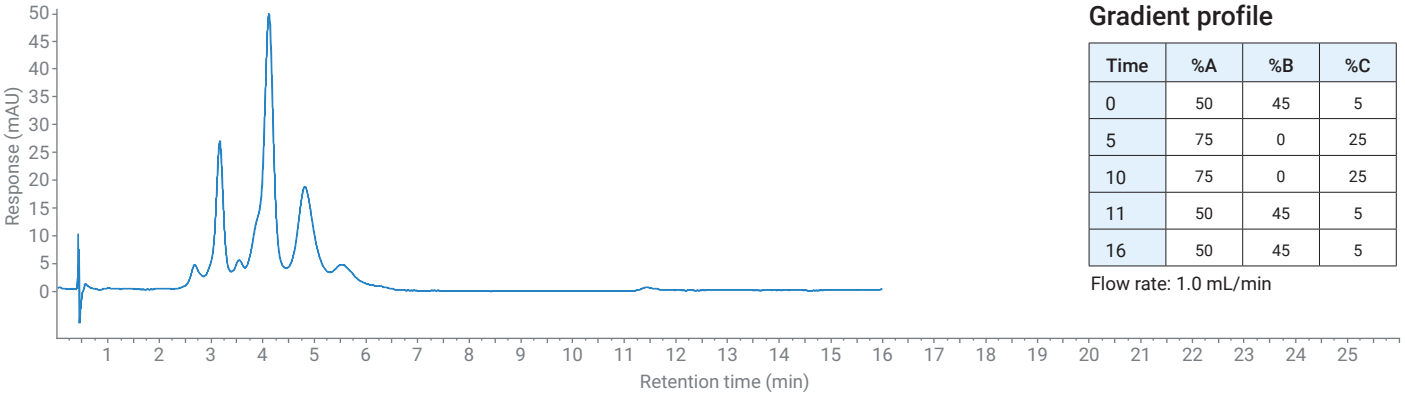
Time	%A	%B	%C
0	20	75	5
15	85	0	15
20	85	0	15
21	20	75	5
26	20	75	5

C. Profile for 5–20 % gradient.

Time	%A	%B	%C
0	20	75	5
15	80	0	20
20	80	0	20
21	20	75	5
26	20	75	5

Figure 3. Effect of propan-2-ol gradient in separation of ADC DAR variants.

With the appropriate use of flow rate and column length, together with the required amount of organic modifier in the gradient, it is possible to perform much faster separations on shorter 3 cm columns (Figure 4) compared to slower gradients on longer 10 cm columns (Figure 5). The results in Tables 2 and 3 show that the DAR values determined from these chromatograms are almost identical.

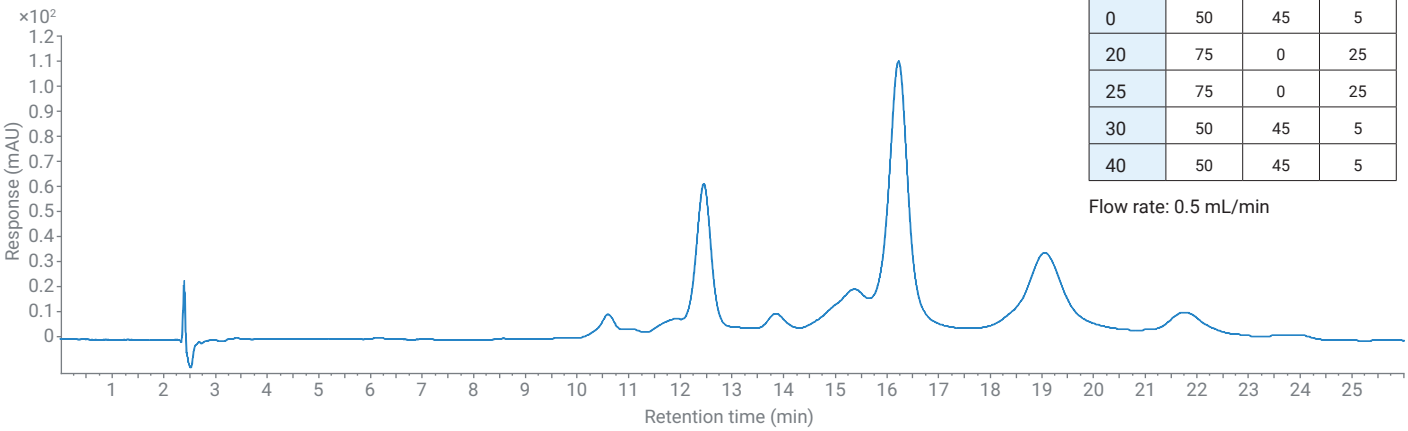


Gradient profile

Time	%A	%B	%C
0	50	45	5
5	75	0	25
10	75	0	25
11	50	45	5
16	50	45	5

Flow rate: 1.0 mL/min

Figure 4. Fast (8 minute) separation of an ADC mimic on AdvanceBio HIC 4.6 × 30 mm column.



Gradient profile

Time	%A	%B	%C
0	50	45	5
20	75	0	25
25	75	0	25
30	50	45	5
40	50	45	5

Flow rate: 0.5 mL/min

Figure 5. Slow (24 minute) separation of an ADC mimic on AdvanceBio HIC 4.6 × 100 mm column.

Table 2. DAR value from 8-minute separation (Figure 4).

No.	RT (min)	Area	%Area	DAR	
1	2.66	61.5	3.2	0	0.0
2	3.14	321.4	16.9	2	0.3
3	4.10	894.1	46.9	4	1.9
4	4.80	471.1	24.7	6	1.5
5	5.52	158.0	8.3	8	0.7
				DAR	**4.4**

Table 3. DAR value from 24-minute separation (Figure 5).

No.	RT (min)	Area	%Area	DAR	
1	10.61	304.3	3.0	0	0.0
2	12.46	1,768.9	17.2	2	0.3
3	16.22	4,905.3	47.7	4	1.9
4	19.04	2,420.5	23.5	6	1.4
5	21.76	879.1	8.6	8	0.7
				DAR	**4.4**

Conclusions

We have demonstrated that by controlling the mobile phase composition, together with the choice of flow rate and column dimensions, it is possible to use the AdvanceBio HIC column for analysis of the DAR value of ADCs with both speed and accuracy.

References

1. S. Zuo, Measuring Drug-to-Antibody Ratio (DAR) for Antibody Drug Conjugates (ADCs) with UHPLC/Q-TOF, *Agilent Technologies Application Note*, publication number 5991-6559EN, **2016**.
2. S. Schneider, Analysis of Cysteine-Linked Antibody Drug Conjugates, *Agilent Technologies Application Note*, publication number 5991-8493EN, **2017**.

www.agilent.com/chem

Printed in the USA, July 26, 2018
5994-0149EN

Agilent
Trusted Answers