Analysis of Cysteine-Linked Antibody Drug Conjugates

Using Hydrophobic Interaction Chromatography on the Agilent 1260 Infinity II Bio-inert LC

Application Note

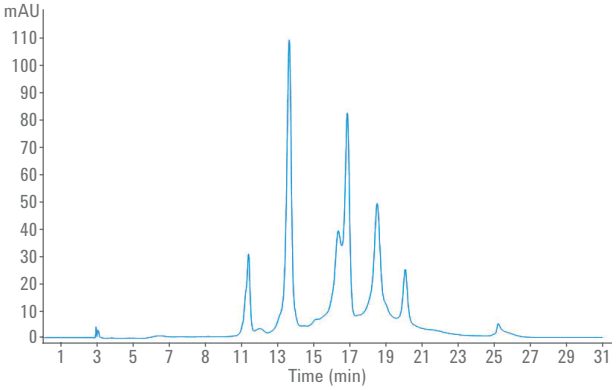
Biologics and Biosimilars

Author

Sonja Schneider
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

Hydrophobic interaction chromatography (HIC) is frequently used for the determination of drug-to-antibody ratio (DAR) of cysteine-linked antibody drug conjugates (ADCs). The eluents for this mild, nondenaturing analysis method contain high concentrations of salts (for example, 2 M ammonium sulfate). The Agilent 1260 Infinity II Bio-inert LC with completely inert sample flowpath was specially designed for conditions used in biochromatography. It is an optimal system for the analysis conditions used in HIC. This Application Note demonstrates the DAR determination of brentuximab vedotin using HIC. The DAR was found to be four drug molecules per antibody. In addition, excellent retention time and area precision were observed for the analysis of brentuximab vedotin using HIC.



Agilent Technologies

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 heterogenous.

Two types of ADCs are currently available on the market: lysine-linked ADCs (such as Trastuzumab emtansine and Kadcyla) and cysteine-linked ADCs[2] (such as brentuximab vedotin). Figure 1 shows the structure of the ADC brentuximab vedotin (SGN-035, Adcetris by Seattle Genetics Inc.). It consists of three components[3]:

- **mAb:** chimeric anti-CD30 monoclonal (cAC10)
- **A protease-cleavable dipeptide linker:** thiol-reactive maleimidocaproyl spacer, the dipeptide valine-citrulline linker, and *p*-amino-benzyloxycarbonyl spacer
- **Small molecule drug:** monomethyl auristatin E (MMAE)

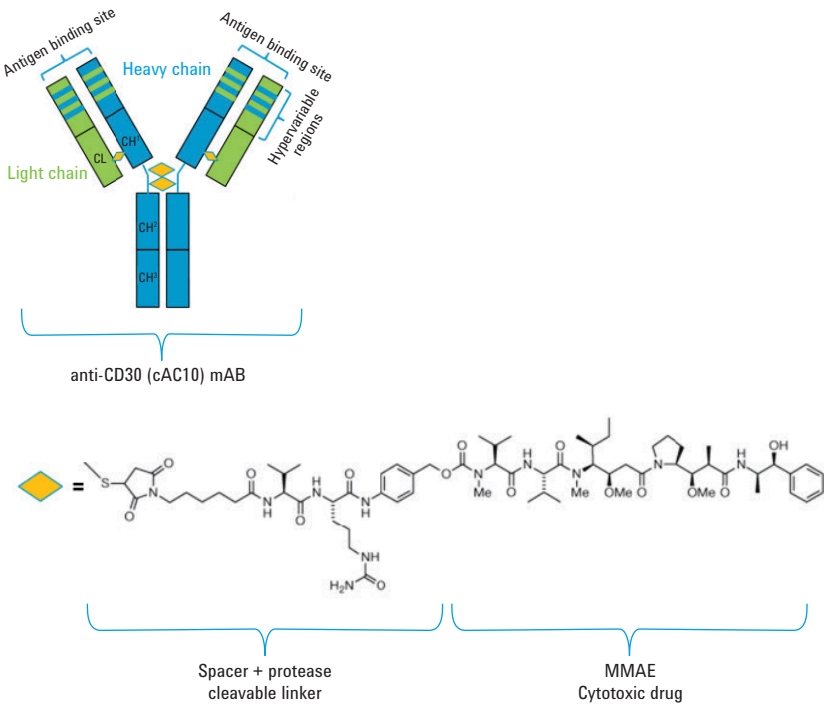


Figure 1. Structure of brentuximab vedotin.

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 drug-to-antibody ratio (DAR) determines the amount of payload that can be delivered to the tumor tissue[4]. The analysis used to determine DAR depends on the chemistry used for the linkage of the drug to the antibody. 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, two, four, six, and eight drugs per antibody. In contrast to lysine-conjugated ADCs, the heterogeneity is significantly reduced in cysteine-conjugated ADCs, enabling the analysis of DAR using hydrophobic interaction chromatography (HIC). HIC is the reference technique to separate the ADC molecules loaded with different numbers of drugs per antibody[5]. 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[5]. 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 be helpful to elute the proteins from the column.

The Agilent 1260 Infinity II Bio-inert LC is the next generation of Agilent Bio-inert LCs. It is specifically designed for conditions used in biochromatography, that is, high salt concentrations (2 M NaCl, up to 8 M urea), and high and low pH solvents (0.5 M NaOH, 0.5 M HCl), with a completely inert sample flowpath. All capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are completely metal-free so that biomolecules come in contact only with ceramics or PEEK[6]. The inert flowpath can prevent many of the typical issues with stainless steel systems such as corrosive effects, especially when working with high salt buffers, as often found in intact protein analysis methods such as HIC, ion-exchange chromatography (IEX), and size exclusion chromatography (SEC).

For HIC workflows, where high amounts of salt are used (ammonium sulfate in a concentration of up to 2 M in mobile phase A), the 1260 Infinity II Bio-inert LC is an excellent fit to prevent corrosion, which is often found in stainless steel systems.

This Application Note describes the HIC analysis of brentuximab vedotin using the 1260 Infinity II Bio-inert LC.

Experimental

Instrumentation

The Agilent 1260 Infinity II Bio-inert LC consisted of:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (Option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (Option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with Bio-inert Flow Cell (Option #028)

Columns

Generic HIC column

Software

Agilent OpenLAB CDS Version 2.1

Samples

Trastuzumab (Herceptin) dissolved in Buffer B at 10 mg/mL and brentuximab vedotin (Adcetris) dissolved in buffer B at 100 mg/mL.

Chemicals

Sodium phosphate monobasic and dibasic and ammonium sulfate were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Fresh ultrapure water was from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak). All solvents used were LC grade. Isopropyl alcohol was purchased from Merck, Germany.

Table 1. Chromatographic conditions.

Parameter	Value
Mobile phase	A) 2 M Ammonium sulfate in 100 mM sodium phosphate, pH7 B) 100 mM sodium phosphate, pH7 C) Isopropanol
Flow rate	0.4 mL/min
Gradient	Time (min) %A %B %C 0 55 45 0 15 0 80 20 20 0 80 20 21 55 45 0
Stop time	31 minutes
Needle wash mode	Standard wash
Injection volume	5 µL
Column temperature	30 °C
DAD	280 nm/4 nm, Ref. 300 nm/100 nm <0.025 minutes (0.5 seconds response time)(10 Hz)

Results and Discussion

Two samples were analyzed using HIC on the 1260 Infinity II Bio-inert LC with a ternary gradient including isopropyl alcohol that supports the elution of the proteins from the HIC column. Figure 2 shows the analysis of trastuzumab, demonstrating excellent peak shape. Figure 3 shows the analysis of brentuximab vedotin, revealing five main peaks that correspond to the mAb containing zero, two, four, six, and eight MMAE 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 with the conjugated drugs, the combination of covalent linkages and noncovalent forces between the antibody chains are sufficient to maintain the mAb in an intact form during the analysis. This is due to the mild, nondenaturing conditions of hydrophobic interaction chromatography, making it ideal for the analysis of ADCs. Each peak in Figure 3 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[5]. The average DAR was calculated based on the area percentage of each peak and its respective drug load. The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~4, meaning that approximately four molecules of MMAE are attached to each antibody molecule. This value is consistent with the literature[7].

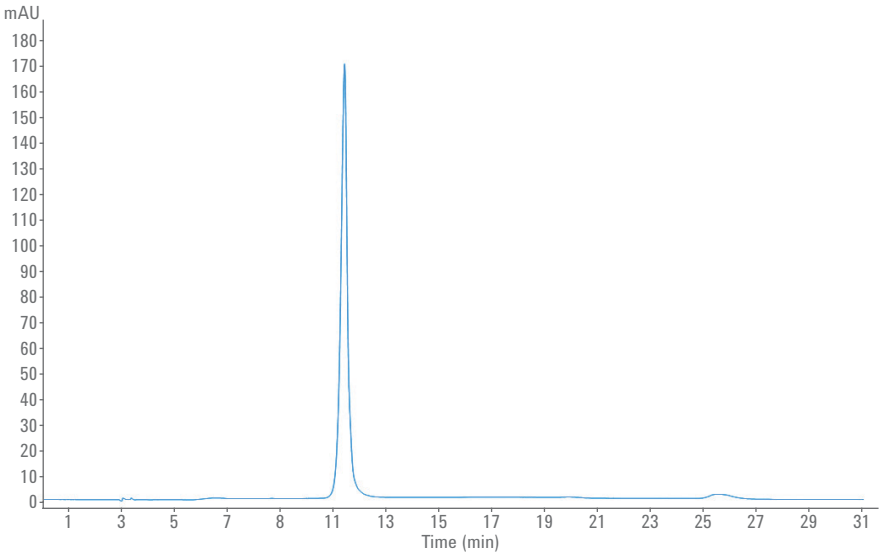


Figure 2. Analysis of trastuzumab on the Agilent 1260 Infinity II Bio-inert LC.

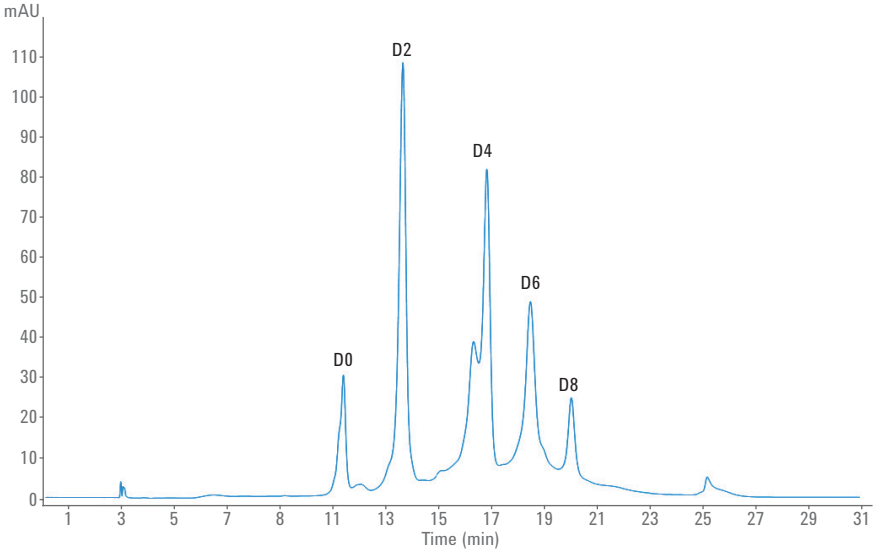
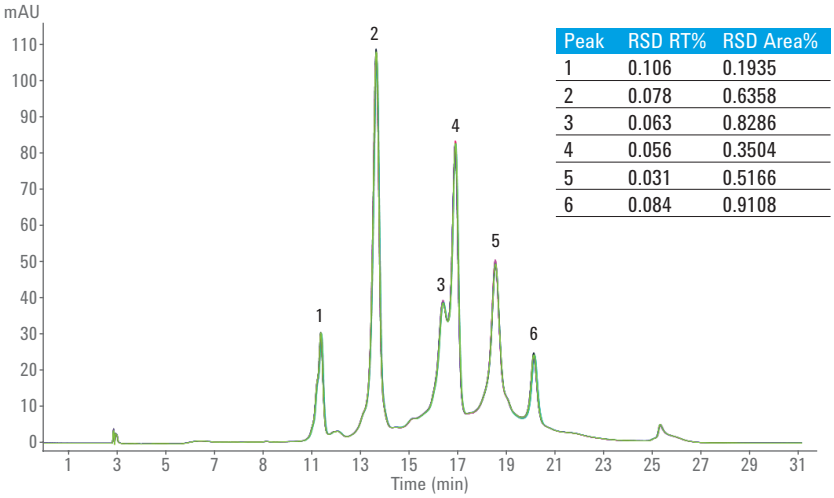


Figure 3. Analysis of brentuximab vedotin on the Agilent 1260 Infinity II Bio-inert LC. D0–D8 refers to different DAR species.

The analysis was also evaluated for precision of retention time (RT) and area (Figure 4). After seven subsequent runs, an excellent RT precision of lower than 0.085 % relative standard deviation (RSD) was found, except for the first peak, which had 0.1 % RSD. The area precision was also found to be high with RSDs lower than 0.92 %. The table in Figure 4 displays RT and area precision.

Conclusion

An Agilent 1260 Infinity II Bio-inert LC was used to show the DAR analysis of brentuximab vedotin using hydrophobic interaction chromatography (HIC). The determination of DAR is one of the most important quality attributes for ADCs. Cysteine-linked ADCs are frequently analyzed using HIC. The buffers used in this type of chromatography are highly corrosive due to the high amount of salt added to the mobile phase. These conditions are challenging for standard stainless steel instruments. The 1260 Infinity II Bio-inert LC has a completely inert sample flowpath, and all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are completely metal-free. With these materials, the 1260 Infinity II Bio-inert LC is optimally suited for the conditions used in biochromatography. The 1260 Infinity II Bio-inert LC showed optimal results for the DAR determination of brentuximab vedotin with stable and precise RTs and areas.



Peak	RSD RT%	RSD Area%
1	0.106	0.1935
2	0.078	0.6358
3	0.063	0.8286
4	0.056	0.3504
5	0.031	0.5166
6	0.084	0.9108

Figure 4. Separation of brentuximab vedotin on the Agilent 1260 Infinity II Bio-inert LC. Overlay of seven subsequent runs.

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. Marcoux, J.; *et al.* Native mass spectrometry and ion mobility characterization of Trastuzumab emtansine, a lysine-linked antibody drug conjugate. *Protein Science* **2015**, *24*, 1210–1223.

3. Younes, A.; *et al.* Brentuximab vedotin (SGN-35) for Relapsed CD30-Positive Lymphomas. *The New England Journal of Medicin*e **2010**, *363*, 1812–1821.

4. Wakankar, A.; *et al.* Analytical methods for physiochemical characterization of antibody drug conjugates. *mAbs* **2011**, *3:2*, 161–172.

5. 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.

6. Agilent InfinityLab Bio-inert LC Solution: True Bio-Inertness for Efficient Biomolecule Analysis. *Agilent Technologies Brochure*, publication number 5991-7361EN, **2017**.

7. van de Donk, N. W. C. J.; Dhimolea, E. Brentuximab vedotin. *mAbs* **2012**, *4,4*, 458-465.

www.agilent.com/chem

For Research Use Only.
Not for use in diagnostic procedures.

This information is subject to change without notice.

Published in the USA, October 1, 2017
5991-8493EN

Agilent Technologies