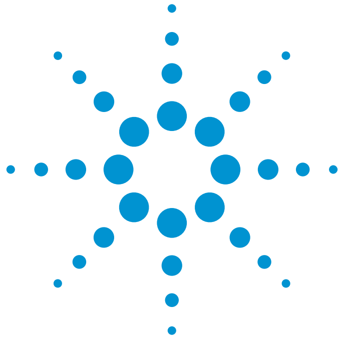


Determination of Drug-to-Antibody Distribution in Cysteine-Linked ADCs

An Analysis of ADCs of IgG_1 and IgG_2 Subclasses

Application Note

Biotherapeutics and Biosimilars

Authors

Lily Liu

Agensys, Inc.

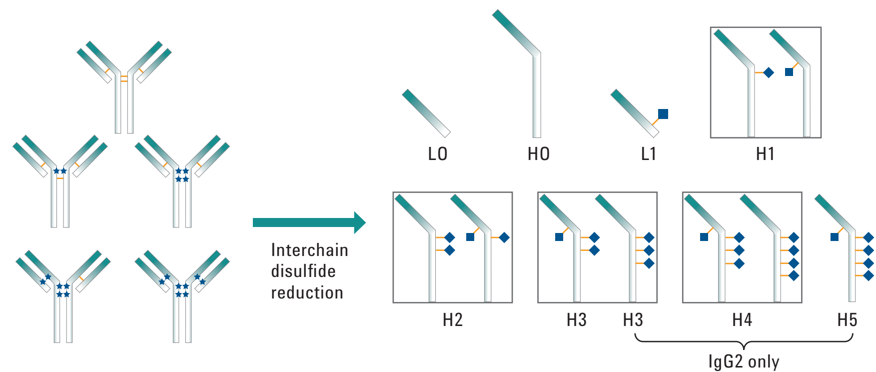
Santa Monica, CA, US

Andy Coffey

Agilent Technologies, Inc

Abstract

This Application Note shows the advantages of using sub-2 µm reversed-phase columns to gain extra resolution and accuracy in the determination of drug-to-antibody ratios. These ratios were determined in antibody-drug conjugates derived from antibody intermediates of the IgG_1 and IgG_2 subclasses in this study.



Agilent Technologies

Introduction

Antibody-drug conjugates (ADCs) represent a growing class of anticancer therapeutics that combine the specificity of an antibody with the potency of chemotherapeutic agents using covalent and chemically stable linkages. The ADC field is expanding with an increasing number of conjugation technologies being developed. One dominant class of ADCs includes conjugation to cysteine residues that are involved in the formation of interchain disulfide bonds through maleimide linkages. One of the principal critical quality attributes for ADCs that directly correlates with potency is the drug-to-antibody ratio (DAR). Up to eight or 12 drug-linkers may be conjugated per antibody, depending on the IgG antibody subclass[1].

Hydrophobic interaction chromatography (HIC) is a common approach for determining conjugate distribution, and calculating DAR for ADCs manufactured from IgG_1 mAbs. However, monitoring conjugate distribution and DAR for ADCs manufactured from IgG_2 mAbs by HIC is challenging, due to incomplete resolution between positional isomers and variably conjugated species. Reversed-phase (RP) chromatography can be used as an alternative or orthogonal technique for determining the DAR of ADCs following reduction of interchain disulfide bonds. Using this technique, the DAR may be calculated experimentally from the distribution of unconjugated and conjugated light and heavy chains. For ADCs manufactured from IgG_2 antibodies, RP is a more suitable method. This is because elution between unconjugated and variably conjugated light and heavy chains is dictated by the number of conjugated drug-linkers, regardless of the site of conjugation.

A limited variety of suitable HIC and RP columns are available for these applications. This Application Note describes the use of the Agilent ZORBAX RRHD SB300-C8 column for characterizing the distribution of unconjugated and variably conjugated light and heavy chains, and for determining the average DAR. Here, we describe RP UHPLC methods suitable for ADCs manufactured from both IgG_1 and IgG_2 antibodies. Compared to methods using common HPLC columns, the ZORBAX RRHD SB300-C8 column offers improved peak resolution, and yields similar distributions of unconjugated and conjugated light and heavy chains and DARs.

Materials and Methods

Reagents, samples, and materials

ADCs manufactured from fully human IgG_1 and IgG_2 antibody intermediates are proprietary. DL-dithiothreitol (DTT) was purchased from Thermo Scientific (Pierce NoWeigh DTT). All solvents used were HPLC grade, and were purchased from either VWR or Fisher Scientific.

Samples in their respective formulation buffers (pH 5–6) were diluted to 5 mg/mL, and the pH was adjusted to approximately pH 8 with 1 M *tris* pH 9. Partial reduction of the interchain disulfide bonds was achieved by incubation in 40 mM DTT at 37 °C for 15 minutes. After cooling to room temperature, reduced samples were diluted 1:1 with 2 % formic acid in 50 % acetonitrile to quench the reduction reaction.

Instruments

UHPLC with DAD detection system from an external vendor.

The equivalent Agilent instrument for UHPLC analysis is the Agilent 1290 Infinity II LC system, which is expected to delivery comparable, or better performance.

UHPLC method

Parameter	Value
Column	Agilent ZORBAX RRHD SB300-C8, 50 mm × 2.1, 1.8 µm
Other columns	Vydac 214MS, C4, 2.1 × 50 mm, 5 µm, 300 Å Agilent PLRP-S, 2.1 × 50 mm, 5 µm, 1,000 Å
Mobile phases	A) 0.1 % TFA in H_2O B) 0.08 % TFA in 90 % ACN
Column temperature	80 °C (IgG_1) 70 °C (IgG_2)
Post-column cooler	35 °C
Injection volume	2 µL (IgG_1) 3 µL (IgG_2)
Flow rate	1 mL/min (IgG_1) 0.8 mL/min (IgG_2)
Detection	UV at 214 and 280 nm
Autosampler temperature	10 °C
IgG_1 Gradient	Time (min) %B 0 34.5 3 38.0 5.5 38.5 25 55.0 25.1 75.0 26 75.0 26.1 34.5 Post time: 4 minutes
IgG_2 Gradient	Time (min) %B 0 30.0 3 30.0 21 45.0 21.1 75.0 22 75.0 22.1 30.0 Post time: 2 minutes

Peak assignments

Peak identities were confirmed by coupling the UHPLC with in-line mass spectrometry (data not shown). The major peaks corresponded to unconjugated and variably conjugated light and heavy chains. Peaks eluting as trailing shoulders from the major peaks were identified as having one or more intrachain disulfide bonds reduced. Multiple peaks corresponding to conjugated heavy chains were observed having the same mass, and were identified as being positional isomers, where the drug-linker was conjugated at different cysteine residues.

DAR calculation

The DAR value was calculated from the analysis of the UV chromatogram, using Equation 1.

Results and Discussion

Figure 1 compares the reversed-phase chromatographic profiles of an ADC manufactured from an IgG_2 mAb intermediate using a conventional C4 HPLC column and the ZORBAX RRHD SB300-C8 column. The peaks observed in the chromatogram corresponding to the UHPLC method using the ZORBAX RRHD SB300-C8 column are sharper, and show better resolution compared to the C4 HPLC column. Peak separation and resolution achieved using the ZORBAX RRHD SB300-C8 column enabled improved peak integration accuracy, and the DAR value was calculated to be 0.1 higher as a result.

$$DAR = 2\left(\sum_{n=0}^{1} \frac{\text{LC peak area} \times n_{drug}}{\text{Total LC peak area}} + \sum_{n=0}^{j} \frac{\text{HC peak area} \times n_{drug}}{\text{Total HC peak area}}\right)$$

Where: j = 3 for IgG_1 ADCs, and j = 5 for IgG_2 ADCs

Equation 1.

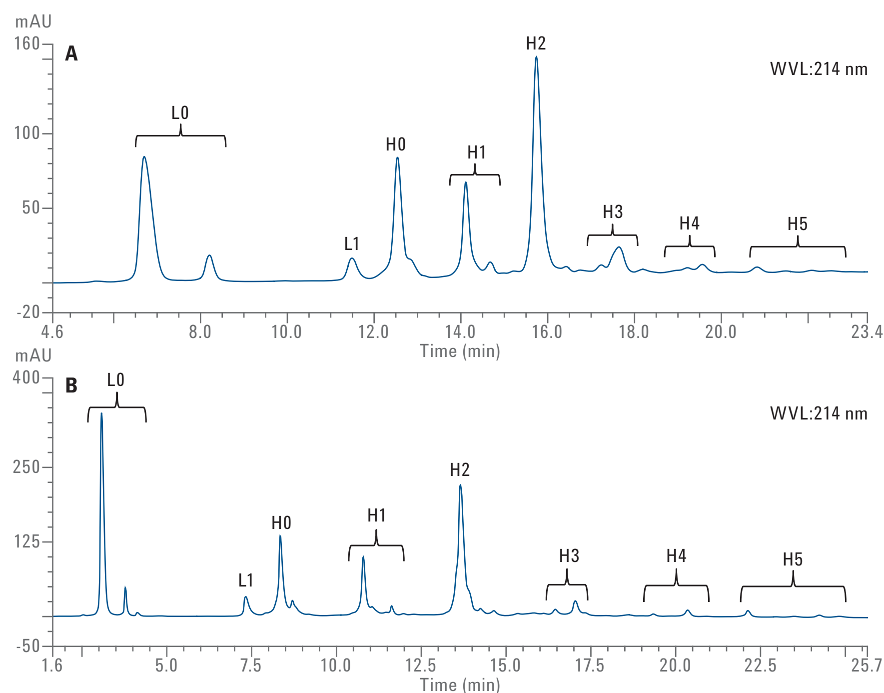


Figure 1. Comparison of reduced RP profiles obtained using a C4 HPLC column (A) and an Agilent ZORBAX RRHD SB300-C8 (B) for a cysteine-conjugated IgG_2 ADC.

Figure 2 shows RP chromatograms of a reduced ADC manufactured from an IgG_1 mAb resulting from analyses using both the Agilent PLRP-S column and the ZORBAX RRHD SB300-C8 column. Separation of the unconjugated and conjugated light and heavy chains on the ZORBAX RRHD 300SB-C8 column resulted in sharper peaks and improved resolution of the minor species in comparison to the PLRP-S HPLC column. The DAR value calculated from the results using the ZORBAX RRHD column was 0.1 higher than the result from the PLRP-S column. This difference can be attributed to more accurate peak integration.

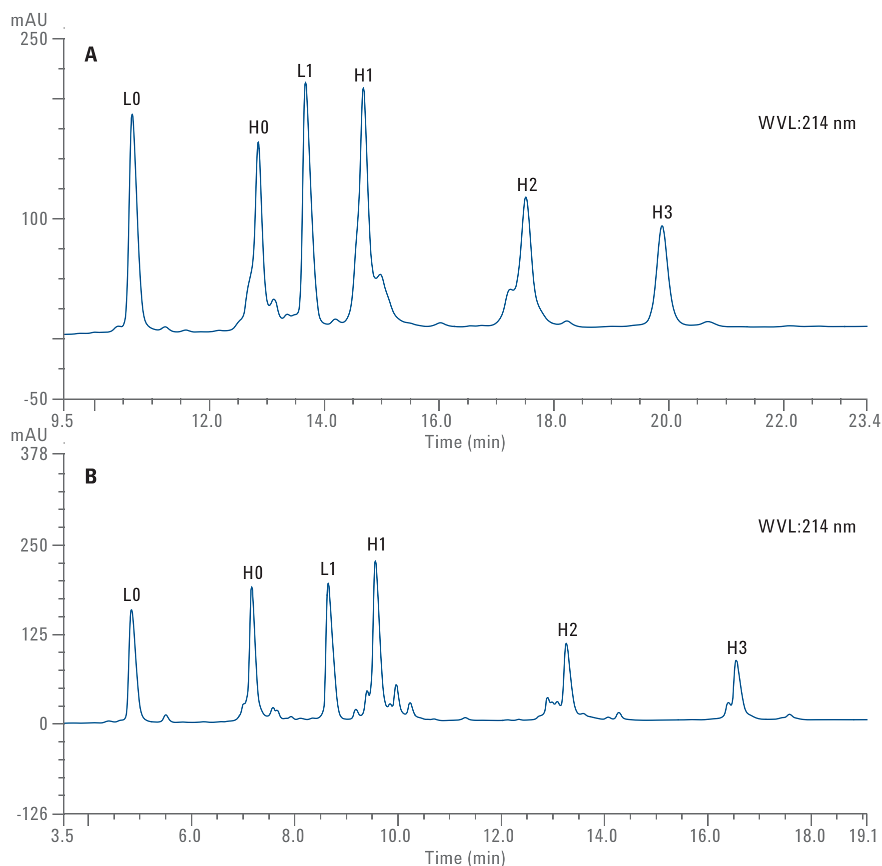


Figure 2. Comparison of PLRP-S HPLC (A) and Agilent ZORBAX RRHD SB300-C8, 1.8 µm (bottom) profiles for a cysteine-conjugated IgG_1 ADC.

Distributions and DAR value

Table 2 shows the distribution of unconjugated and conjugated light and heavy chains, and the calculated average DAR for the ZORBAX RRHD column and the two different HPLC columns. Relative peak areas and concomitant DAR values calculated using results obtained with the ZORBAX RRHD column are similar to those determined using the HPLC columns. The major advantage of using a sub-2 µm column is the improved peak resolution. This improved resolution results in better separation of minor species that were coeluting with the main peaks using the HPLC columns. The gradients presented in this Application Note can also be used to detect minor changes in sample stability and characterization of minor peaks by LC/MS. Also, the increased peak resolution and the higher pressure compatibility of the ZORBAX RRHD columns allow for development of shorter gradients for high-throughput testing.

Table 2. Relative peak areas of each species and average DAR value using an Agilent ZORBAX RRHD SB300-C8 compared with HPLC columns.

	IgG_2 ADC		IgG_1 ADC	
Peak ID	% by HPLC (C4)	% by UHPLC (Agilent ZORBAX RRHD)	% by HPLC (PLRP-S)	% by UHPLC (Agilent ZORBAX RRHD)
L0	22.6	23.7	14.3	13.3
L1	2.3	3.4	15.6	17.7
H0	17.3	15.6	16.8	15.4
H1	12.5	13.1	26.2	27.8
H2	32.4	30.9	16.8	15.8
H3	7.4	8.1	10.5	10.0
H4	2.1	3.2	–	–
H5	3.3	2.2	–	–
DAR	**3.5**	**3.6**	**3.6**	**3.7**

Conclusion

Reversed-phase methods can determine the distribution of unconjugated and conjugated light and heavy chains and for calculation of the DAR for ADCs. The Agilent ZORBAX RRHD SB300-C8 column has been shown to be suitable for these purposes to support characterization of ADCs derived from both IgG_1 and IgG_2 antibody intermediates. Peaks eluting from the ZORBAX RRHD SB300-C8 column were observed to be sharper and better resolved in comparison to the HPLC columns included in the comparison. This improvement enabled more accurate peak integrations and concomitant DAR values. The improvement in peak resolution from the ZORBAX RRHD SB300-C8 column also allowed detection of conjugation site positional isomers. RP methods using the ZORBAX RRHD SB300-C8 column have been shown to be suitable for characterizing the conjugate distribution of partially reduced ADCs in place of, or orthogonal to, hydrophobic interaction chromatography.

Reference

1. Wiggins, B.; *et al.* Characterization of Cysteine-Linked Conjugation Profiles of Immunoglobulin G1 and Immunoglobulin G2 Antibody–Drug Conjugates. *J. Pharm. Sci.* **2015**, *104(4)*, 1362-1372.

www.agilent.com/chem

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

This information is subject to change without notice.

© Agilent Technologies, Inc., 2017
Published in the USA, January 11, 2017
5991-7192EN

Agilent Technologies