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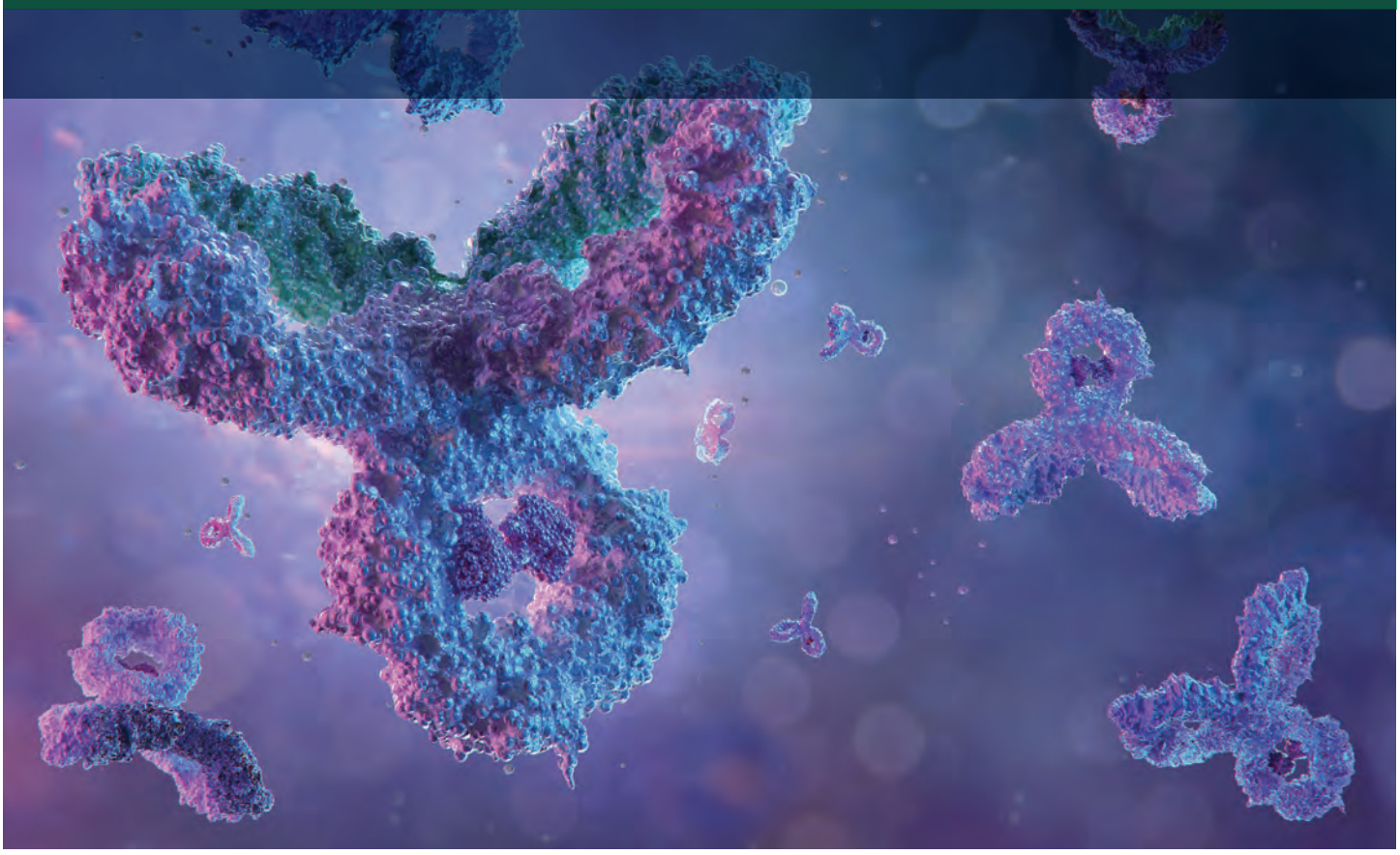
Agilent Biocolumns

Intact & Subunit Purity

Application Compendium



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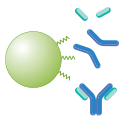
Intact & Subunit Purity

Background

Reversed-phase chromatography remains one of the most valuable tools in the chromatographer's armory. It is a well understood technique relying on hydrophobic interactions between the analyte and the stationary phase. For intact proteins, the technique uses gradients of organic solvents as mobile phase, typically with an ion pair reagent. Under these conditions, the molecule is likely to become denatured. It is a sensitive technique as the sample is concentrated as it is retained by the column and it is useful with mass spectrometry. It is therefore suitable for determining the accurate mass of an intact protein.

For large proteins, such as monoclonal antibodies, and even for smaller fragments such as heavy and light chains or Fab and Fc regions of an IgG molecule, wide pore columns are recommended. Agilent offers 300 and 450 Å products in many configurations including fully porous, Poroshell technology featuring superficially porous particles, and a selection of alkyl bonded phases. The bonded phase is typically shorter chain length (C8 or C4/C3) or more unique ligands such as diphenyl that may offer different selectivity.

With the accuracy of reversed-phase chromatography it is possible to use intact and fragment analysis to compare biosimilars with originator biotherapeutics. However, it is always necessary to perform extra tests to identify the specific location of the different variants that may be detected.



Intact and Subunit Purity

Large molecule chromatography (>150 Å)

Selectivity options for every separation need

AdvanceBio RP-mAb

Ideal for monoclonal antibodies

Attribute	Advantage
450 Å pore, superficially porous particles	Optimum design for high-resolution mAb separations
Extended column lifetime	Lower operating costs

ZORBAX RRHD 300 Å 1.8 µm

UHPLC separations

Attribute	Advantage
1200 bar maximum pressure	UHPLC-compatible
1.8 µm particles	Maximum resolution

PLRP-S*

Ideal formic acid performance for MS detection

Attribute	Advantage
Polymeric particle with no silanol interactions	Better peak shape, better recovery, and lower carryover
Durable, resilient particles	Reproducible results over longer lifetimes

*** Available in PEEK lined stainless hardware**

Getting Started

Selecting a reversed-phase column for intact protein analysis requires consideration of several interrelated factors: Sample molecular weight and the best suited particle pore size, column chemistry, the instrumentation to be used particularly the type of detector, mobile phase conditions, and speed or throughput requirements to name a few.

Larger analytes require larger pore sizes. With some exceptions, pore sizes for intact protein analysis are typically 300–500 Å. As a rule of thumb, the pore size should be at least three times the hydrodynamic radius of the protein. The AdvanceBio RP-mAb column has 450 Å pores ZORBAX RRHD 300 Å, ZORBAX 300SB, and Poroshell 300 all have 300 Å pores, and PLRP-S is available in many pore sizes. While substantially larger than what is commonly used for intact proteins, the 1000 Å, 5 µm PLRP-S columns give excellent results for intact protein and protein fragment analysis.

For reversed-phase columns, a general guideline for choosing a column chemistry is, the higher the molecular weight, the shorter the alkyl chain should be. Hence, C18 columns are commonly used for peptides while C8, C4, and C3 columns are commonly used for intact protein separations. In addition to linear alkyl chains, diphenyl phases are available for the AdvanceBio RP-mAb, ZORBAX RRHD 300, and ZORBAX 300SB columns. Sometimes, the alternate selectivity of the diphenyl phase can provide the separation needed. PLRP-S is a polymeric particle rather than a silica-based particle. It gives a typical reversed-phase separation, with somewhat different selectivity and the advantage of wide pH tolerance.

The instrumentation available determines what maximum pressure can be achieved. One can certainly use a column with a 600 bar pressure maximum on a UHPLC capable of 1200 bar. But care should be taken not to over pressure the column, which can lead to premature column failure. Within Agilent's reversed-phase portfolio, the ZORBAX RRHD column has a maximum backpressure of 1200 bar, and can thus be used for high speed, high-pressure separations.

When considering instrumentation and backpressure capabilities, it is worth considering whether the method under development will ever need to be transferred to another LC system with a different maximum backpressure. If so, it would be cost- and time-effective to develop a method that can be run on all platforms.

Detector selection and mobile phase conditions are often related. For protein separations, this is commonly a decision between using UV detection or mass spectrometry (MS). Traditionally trifluoroacetic acid (TFA) has been used as an ion pairing agent for separations with UV detection, while formic acid is preferred for MS detection. TFA is typically used for UV detection as it gives excellent peak shape on silica-based columns, however it leads to ion suppression in mass spectrometry. Formic acid preserves MS sensitivity, but gives less than ideal peak shape on silica-based columns, therefore polymeric PLRP-S column is recommended for formic acid mobile phases. With an understanding of the trade-offs, one can use formic acid mobile phase with silica-based columns, or TFA with mass spectrometry. There's also no disadvantage to using formic acid or PLRP-S with UV detection.

Water/acetonitrile gradients are commonly used for reversed-phase separations of intact proteins and monoclonal antibody fragments and are generally suitable for Agilent reverse-phase columns. A different organic solvent, such as methanol or isopropanol may produce a helpful change in selectivity in the case of some separations. The AdvanceBio RP-mAb columns give their best results with an organic mobile phase containing isopropanol, acetonitrile, and water. Application note number 5991-6274EN gives more detail on this column and mobile phase pairing.

The AdvanceBio Desalting-RP product offers a cartridge-format, PLRP-S based approach to online desalting. This cartridge is typically used before mass spectrometry analysis, but could be used with UV detection and fraction collection to desalt samples before subsequent analysis.

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Monitoring Product-Related mAb Fragments

Intact protein analysis with the Agilent 1290 Infinity II Bio LC System enables UV and MS detection of low molecular weight species

Author

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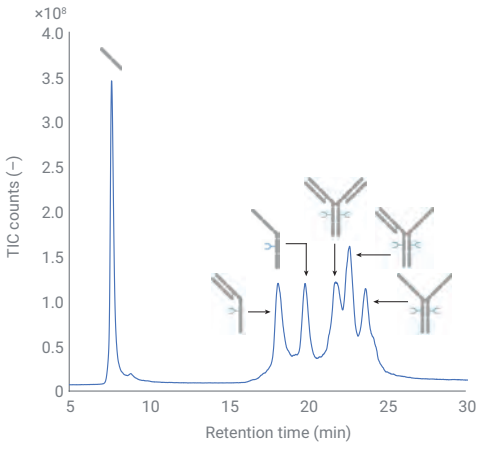
Abstract

Product-related impurities such as low molecular weight (LMW) or high molecular weight (HMW) species are considered critical quality attributes (CQAs) in therapeutic monoclonal antibody (mAb) products and need to be monitored across the drug production process. This application note developed an RPLC method based on the excellent performance of the Agilent 1290 Infinity II Bio LC System combined with the PEEK-lined Agilent PLRP-S column. By analyzing the reduced heavy and light chains of the NISTmAb, excellent relative retention time and area deviations were observed, even with extremely shallow gradient slopes. After method development, all relevant LMW fragments, such as two heavy chains (H2) or two heavy chains and one light chain (H2L), could be separated and detected. Due to the sequential coupling of the UV and MS detector, this method can be used in several areas of the biopharmaceutical production chain. The method also stands as an alternative to SDS-PAGE/CE-SDS with the possibility to analyze two CQAs – LMW species and post-translational modifications (PTMs) – in one run.



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Introduction

mAbs are a major product class of biopharmaceuticals and have been used successfully to tackle various diseases.[1] These biomolecules possess a conserved heterotetrameric structure, consisting of two heavy chains and two light chains connected by disulfide bonds. During manufacturing or improper storage, product-related impurities such as LMW species (see Figure 1) or HMW species (e.g., antibody dimers) can be formed. Those impurities can be present even after extensive purification steps, making it essential to monitor them as a CQA for a drug product. HMW species such as antibody dimers, trimers, or higher aggregates can routinely be analyzed and separated by size exclusion chromatography (SEC) with UV detection.[2] Coupling of SEC with MS detection can be performed to further characterize impurities regarding molecular weight and PTMs.[3] The analysis of LMW species such as heavy chain (H), light chain (L), or H2L fragments can be carried out by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS).[4] Unfortunately, CE-SDS cannot be coupled to MS detection due to high ion suppression caused by SDS, and therefore proposed identities of LMW species are often based on empirical knowledge. This application note shows an alternative analysis of LMW species of mAbs based on the excellent performance of the 1290 Infinity II Bio LC and the PEEK-lined PLRP-S column. Due to the reversed-phase liquid chromatography (RPLC) mode, all relevant reduction-induced LMW fragments of the NISTmAb can be detected with UV and MS for routine or in-depth analysis as needed.

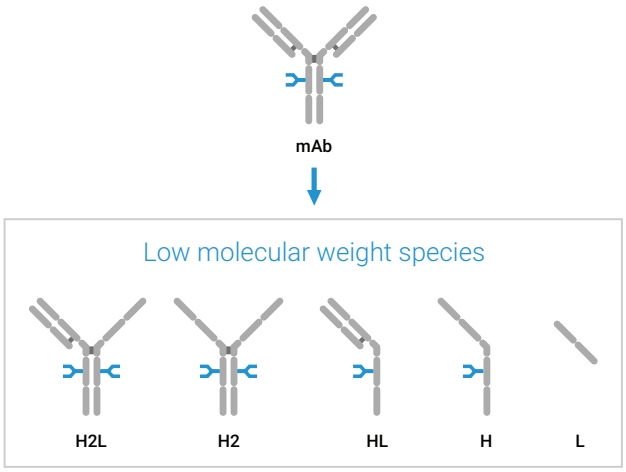

Figure 1. Schematic overview of reduction-induced LMW species of monoclonal antibodies (mAb). Abbreviations: H2L (two heavy chains and one light chain), H2 (two heavy chains), HL (one heavy chain and one light chain), H (heavy chain), and L (light chain).

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF 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) equipped with a Standard Flow Quick
- Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter Qualitative Analysis (10.0 or later)
- Agilent MassHunter BioConfirm (10.0 or later)

Columns

Agilent PLRP-S 5 µm 1000 Å, 2.1 × 100 mm PEEK-lined

(part number PL1912-2502PK)

Chemicals

Agilent InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the Agilent-NISTmAb (part number 5191-5744) were used. 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). DL-dithiothreitol (DTT) was purchased from Merck (Darmstadt, Germany).

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Sample preparation

To partially reduce the NISTmAb, 40 µg were incubated with 1 mM DTT in an amber glass vial directly in the 1290 Infinity II Bio Multisampler at 4 °C. Full reduction into heavy chain (H) and light chain (L) was achieved by incubating 40 µg of NISTmAb with 10 mM DTT at 60 °C for 30 minutes. Injection concentration was 1 mg/mL NISTmAb or reduced NISTmAb.

Method

Table 1. LC method for analyzing the intact NISTmAb and corresponding LMW species with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent PLRP-S 5 µm 1,000 Å, 2.1 × 100 mm PEEK-lined
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 25% B 9.00 min – 30% B 34.00 min – 38% B 34.01 min – 100% B 36.00 min – 100% B 36.01 min – 25% B 40.00 min – 25% B
Flow rate	0.400 mL/min
Temperature	60 °C with thermal equilibration devices installed
UV Detection	VWD: 280 nm, 10 Hz/MS: see Table 2
Injection	Injection volume: 0.3 µL Sample temperature: 4 °C Wash: 3 s with water (flush port)

Table 2. Source and MS parameters for the analysis of the intact NISTmAb and corresponding LMW species.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOFn
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	4,000 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended (m/z 10,000) mass range
Mass Range	*m/z* 100 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	*m/z* 922.0098

Results and discussion

The analysis of biopharmaceuticals throughout the production process from manufacturing to quality control demands the best performance possible from LC systems. To evaluate the 1290 Infinity II Bio LC's performance regarding the analysis of mAb fragments, the NISTmAb was entirely reduced with DTT, resulting in H and L fragments. Figure 2 shows the relative retention time and area standard deviations (RSD) based on seven consecutive injections. It shows that the retention time and area precision of the 1290 Infinity II Bio LC coupled to the 6545XT AdvanceBio LC/Q-TOF is excellent and perfectly suited for analyzing mAb fragments with shallow gradients. Even though the LC method consists of two linear gradient steps with slopes of 0.32 and 0.55 %B/min, the RSD values remain low with 0.190% (L) and 0.056% (H) for the retention time and 0.530% (L) and 0.744% (H) for the area precision.

One of the major challenges when analyzing LMW species with RPLC is the insufficient resolving power to separate antibody fragments such as H2 or H2L due to their similarity in hydrophobicity compared to the actual mAb. These fragments can occur in the fermentation process or by partial reduction in the final product. However, these fragments can also be generated artificially by partial reduction over time with a low amount of DTT and decreased temperature. With this technique, an RPLC method based on the PEEK-lined PLRP-S and the 1290 Infinity II Bio LC was developed.

The dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler can be seen in the chromatogram of Figure 3. All of the relevant mAb fragments depicted in Figure 1 can be nicely resolved and change over time due to the addition of DTT. In particular, the separation of the H2, H2L fragments, and the NISTmAb is exceptionally good for RPLC, rendering the combination of the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC the method of choice for the analysis of LMW. Thanks to the RPLC mode, the 1290 Infinity II Bio LC System can easily be coupled to the 6545XT AdvanceBio LC/Q-TOF, and MS data can be analyzed in Agilent MassHunter BioConfirm. After deconvolution, the spectra in Figure 3 depict the main glycoforms of the respective fragments. The characteristic glycosylation of the NISTmAb shows that it is possible to analyze PTMs of the different fragments easily with this method. Additionally, Figure 4B shows the extracted ion chromatograms (EIC) of representative ions for the fragments clustering around the mAb peak. These EICs also offer good peak shape owing to the resolving power of the PEEK-lined PLRP-S column.

Since the instrumentation setup comprises the 1290 Infinity II Variable Wavelength Detector and the 6545XT AdvanceBio LC/Q-TOF in sequence, UV and MS detection is possible in one run with little to no band broadening and convenient method transfer from process development to quality control (Figure 4A).

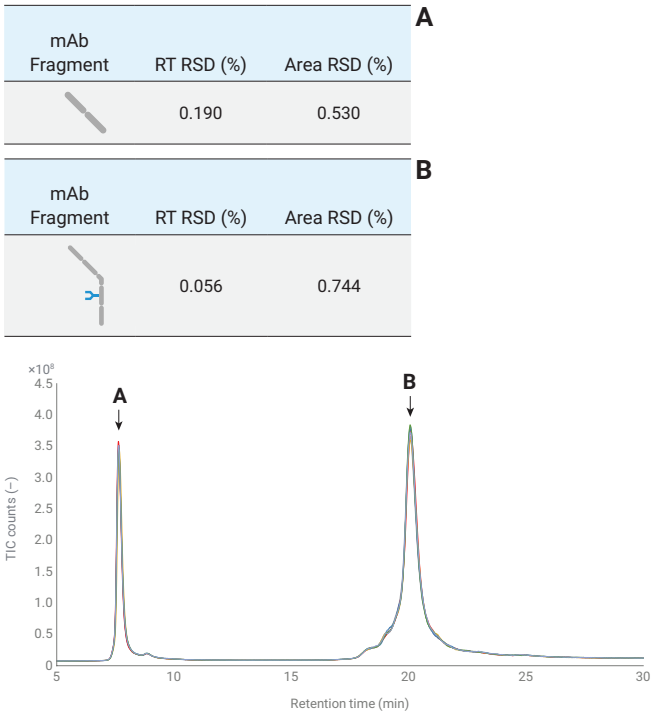

Figure 2. Relative retention time and area precision (RSD, n = 7) values for the Agilent 1290 Infinity II Bio LC analyzing heavy and light chain fragments derived by reduction of the NISTmAb.

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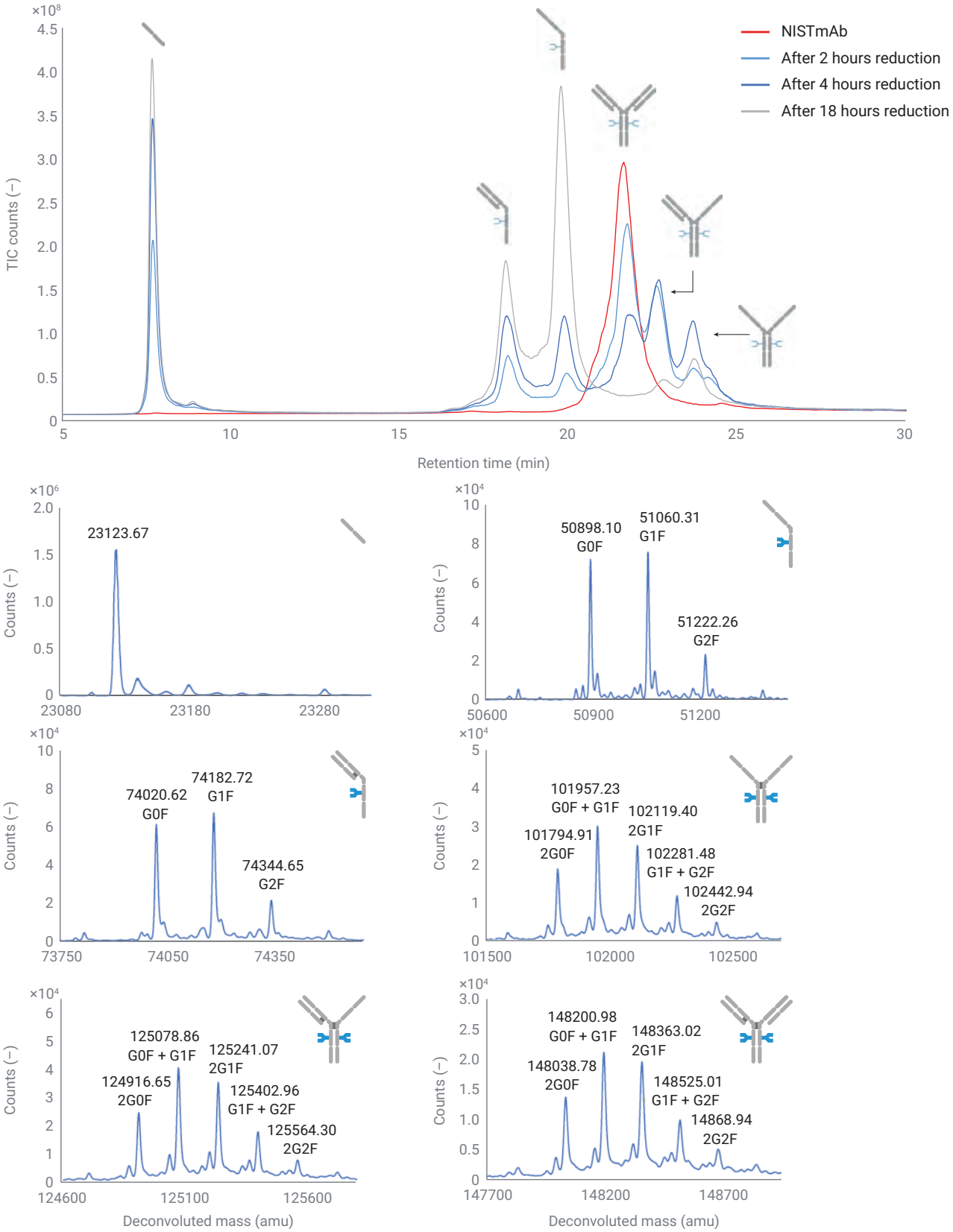

Figure 3. Chromatograms of the dynamic partial reduction of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and detected with the Agilent 6545XT AdvanceBio LC/Q-TOF. Corresponding extracted spectra of the respective fragments show the characteristic glycosylation of the NISTmAb.

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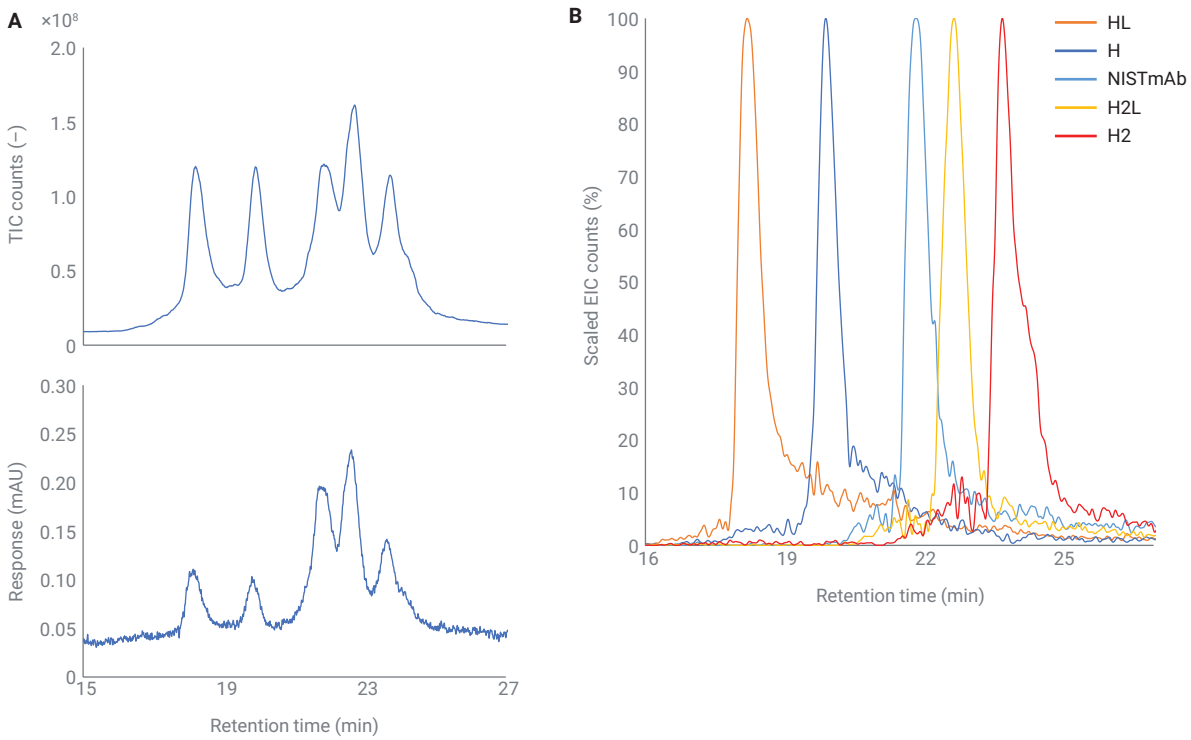

Figure 4. MS and UV chromatogram of the fragments clustering around the NISTmAb acquired in one run (A). Extracted ion chromatograms of NISTmAb fragments showing excellent peak shape (B).

Conclusion

Traditional SDS-PAGE and the modern equivalent CE-SDS are widely used to analyze product-related impurities like LMW and HMW species. However, structural identification of LMW species with these methods has been challenging and primarily based on empirical knowledge. This application note presents an RPLC method capable of separating all relevant reduction-induced LMW species of the NISTmAb. The 1290 Infinity II Bio LC showed excellent retention time and area precision values based on the heavy chain and light chain fragment analysis. Dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler and subsequent detection with the 6545XT AdvanceBio LC/Q-TOF showed the potential of the method to analyze post-translational modifications. When combined with fragment analysis, this capability can accelerate biopharmaceutical development. That is why the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC are a future-proof combination for the analysis of biopharmaceuticals across the production process up to final quality control.

References

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

An Analysis of ADCs of IgG1 and IgG2 Subclasses

Authors

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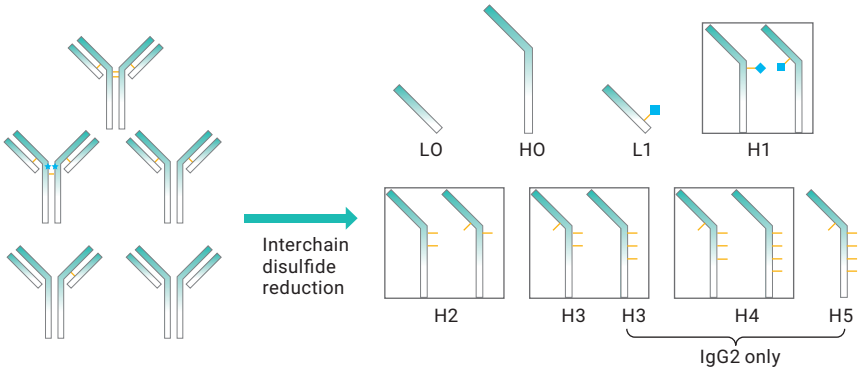
Abstract

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 IgG1 and IgG2 subclasses in this study.



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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 subclass1.

Hydrophobic interaction chromatography (HIC) is a common approach for determining conjugate distribution, and calculating DAR for ADCs manufactured from IgG1 mAbs. However, monitoring conjugate distribution and DAR for ADCs manufactured from IgG2 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 IgG2 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 IgG1 and IgG2 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 IgG1 and IgG2 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.

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 H2O B) 0.08 % TFA in 90 % ACN
Column temperature	80 °C (IgG1) 70 °C (IgG2)
Post-column cooler	35 °C
Injection volume	2 µL (IgG1) 3 µL (IgG2)
Flow rate	1 mL/min (IgG1) 0.8 mL/min (IgG2)
Detection	UV at 214 and 280 nm
Autosampler temperature	10 °C
IgG1 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
IgG2 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

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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

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.

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

Figure 1.

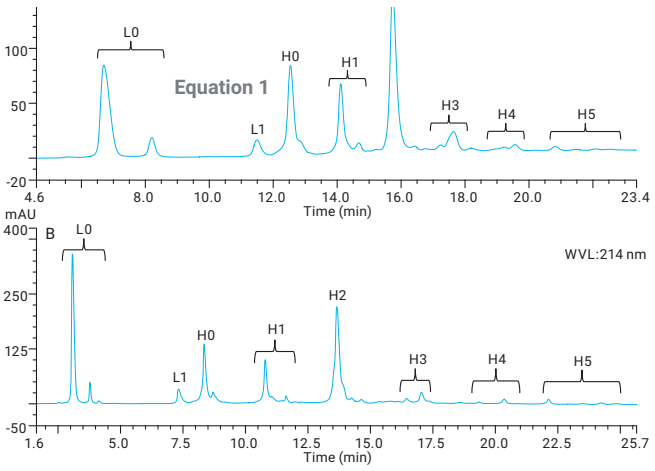

Figure 1. Comparison of reduced RP profiles obtained using a C4 HPLC column (A) and an Agilent ZORBAX RRHD SB300-C8 (B) for a cysteine-conjugated IgG2 ADC.

Results and Discussion

Figure 1 compares the reversed-phase chromatographic profiles of an ADC manufactured from an IgG2 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. 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.

Figure 2 shows RP chromatograms of a reduced ADC manufactured from an IgG1 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.

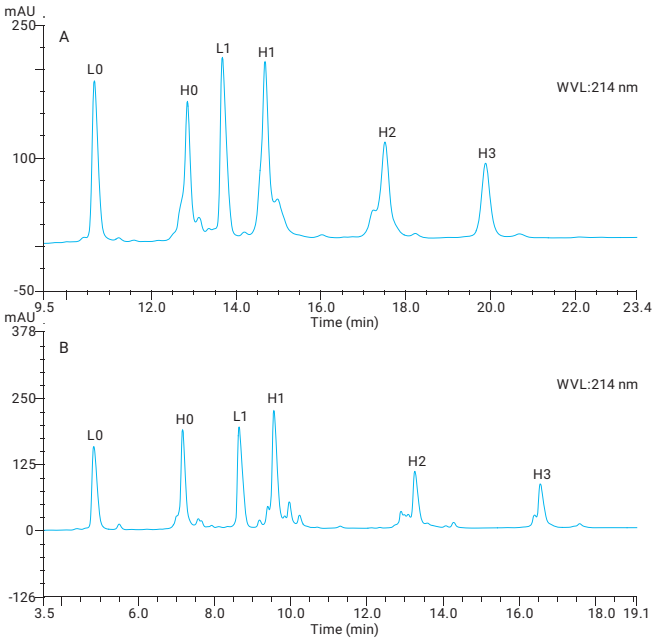

Figure 2. Comparison of PLRP-S HPLC (A) and Agilent ZORBAX RRHD SB300-C8. 1.8 µm (bottom) profiles for a cysteine-conjugated IgG2 ADC.

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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.

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 IgG1 and IgG2 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

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

	IgG2 ADC		IgG1 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

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Application Note

Intact & Subunit Purity

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Fast and High Resolution Analysis of Intact and Reduced Therapeutic Monoclonal Antibodies (mAbs)

The Agilent Bio-inert LC and AdvanceBio RP-mAb Columns

Authors

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Abstract

Therapeutic monoclonal antibodies (mAbs) have become the most rapidly growing class of therapeutics in development for many diseases. Novel mAbs are entering clinical trials at a rate of 40 per year. There is also an urgent need for an analytical method that can be used for high-throughput analysis of large number of samples to support the growing biopharma development. This Application Note describes a fast and high-resolution method for the analysis of intact and reduced therapeutic Innovator and Biosimilar mAbs by reverse phase HPLC. Separation was achieved using an Agilent 1260 Infinity Bio-inert LC system with Agilent AdvanceBio RP-mAb C4 and Diphenyl columns. RP-mAb columns give the advantage of superficially porous 3.5 µm particles with 450 Å wide pores for improved accuracy and short analysis time compared to fully porous particles of the same size. The bio-inertness of the system, together with high resolution, and short and reproducible methods makes it highly suitable for biopharma QA/QC analysis.

Introduction

Evaluating the molecular similarity of a biosimilar to the reference or the innovator molecule is crucial during biosimilar development. A number of physicochemical methods are required by regulatory agencies involving a wide range of comparability programs. The authorities want to see comparability data on platforms that the previous company or the innovator submitted, primarily high-performance liquid chromatography (HPLC), TOF, Q-TOF mass spectrometry, and capillary electrophoresis. HPLC is a well-established technique for the determination of intact protein by size exclusion or ion exchange. However, technological developments in the fi eld of reverse phase (RP) chromatographic stationary phases (a large pore size of 300 Å or fused core particles with short alkyl chains) have made them promising tools for analyzing intact proteins1.

Historically, mAbs and their fragments are analyzed with limited success using widepore, totally porous particle RP HPLC. Due to their large size and limited diversity, analysis times are often unacceptably long, and mAb peaks can elute as broad bands, compromising resolution. In contrast, high effi ciency superfi cially porous columns easily separate mAbs and their fragments in minutes with high effi ciency.

In this work, we have demonstrated the suitability of the Agilent 1260 Bio-inert Quaternary LC system and Agilent AdvanceBio RP-mAb columns to achieve high resolution and rapid separation of intact and fragmented therapeutic innovator and biosimilar rituximab. The unique design of the AdvanceBio RP-mAb column offers unique selectivity due to its superfi cially porous particles (3.5 µm) with wide pores (450 Å). The column delivers a significant speed and resolution advantage while maintaining compatibility with all instruments..

Experimental

Equipment

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60 mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm (p/n 799775-944)
- Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm (p/n 799775-904)

The complete sample flow path is free of any metal components, therefore, the sample never contacts metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Software

Agilent ChemStation B.04.03 (or higher)

Reversed-phase HPLC parameters

Chromatographic parameters for intact and reduced mAb analysis using AdvanceBio RP-mAb columns are shown in Table 1.

Table 1. Chromatographic parameters used for intact and reduced analysis.

Parameter	HPLC (intact and reduced mAbs)
Mobile phase	A) Water + 0.1 % TFA B) IPA:ACN:Water (70:20:10) + 0.09 % TFA
Columns	Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm
Gradient	Time (min) % B 0 15 0.5 25 1.5 35 1.51 35 3.0 60 4.0 60
Post time	2 minutes
Injection volume	1 µL
Flow rate	1.0 mL/min
TCC	80 °C
UV detection	220 and 280 nm

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Reagents, samples, and materials

AInnovator and biosimilar rituximab were purchased from a local pharmacy and stored according to the manufacturer's instruction. PBS and tris(2-carboxyethyl) phosphine (TCEP) were purchased from Sigma-Aldrich. All chemicals and solvents were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile and 2-propanol were purchased from Lab-Scan (Bangkok, Thailand). For intact and reduced analysis, rituximab samples were diluted to 2 mg/mL using PBS.

Sample preparation

Reduction of mAbs

For the separation of the light and heavy chains, an aliquot of 0.5 MTCEP stock was added to the mAb samples to obtain a final concentration of 10 mM. The mixture was held at 60 °C for 30 minutes.

Results and Discussion

The AdvanceBio RP-mAb column with superficially porous particles and wide pores delivers higher resolution and faster run times to provide accurate, reproducible results when analyzing monoclonal antibodies for biopharma discovery, development, and QA/QC applications. Combined with the Agilent 1260 Infinity Bio-inert Quaternary LC System with a power range up to 600 bar, it can be used for mAb separation. The mobile phase was a combination of isopropanol (IPA), acetonitrile (ACN), water, and trifluoroacetic acid (TFA). Figures 1 and 2 depict the optimized RP HPLC elution profile of intact innovator and biosimilar rituximab on an AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm and AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column, respectively, demonstrating excellent peak shape and fast separation in 4 minutes. Comparing Figures 1 and 2 demonstrates that different selectivity can be obtained through the use of different bonded phases using the same chromatographic conditions, with the diphenyl phase resolving in finer detail.

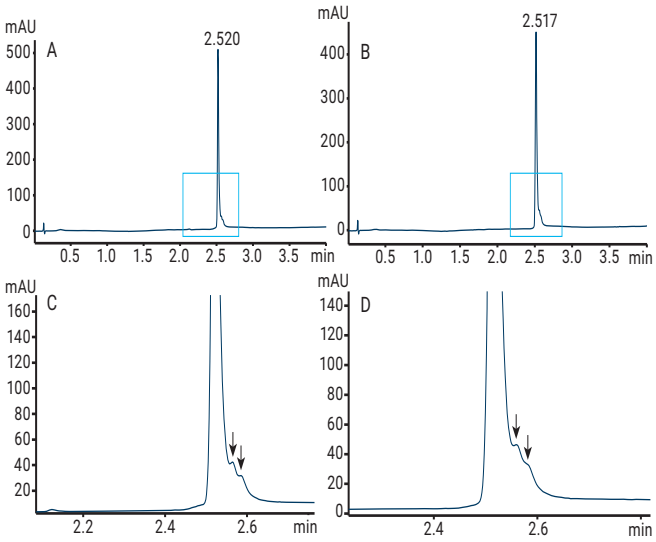

Figure 1. RP-HPLC analysis of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

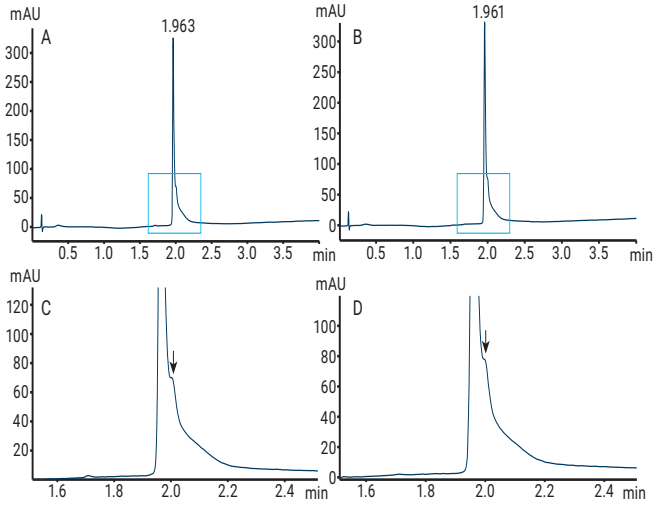

Figure 2. RP-HPLC profile of intact innovator rituximab (A) and biosimilar rituximab (B) on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. C and D show zooms.

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Reduced mAb analysis

TCEP was used to separate free antibody light and heavy chains. AdvanceBio RP-mAb columns are very effective in providing fast and high-resolution separations of antibody fragments. The profiles in Figures 3 and 4 show a rapid reversed-phase analysis optimized for the separation of antibody fragments in approximately 4 minutes using C4 and diphenyl phases, respectively.

In both cases, due to reduction of the disulfide bonds, mAbs eluted as distinct light chain (LC) and heavy chain (HC) separations with high effi ciency. The same gradient used for the intact analysis was able to resolve the LC and HC for the reduced samples. As we have seen with intact mAb analysis, the LC and HC show different selectivities with diphenyl and C4 columns. RP HPLC analysis of intact and reduced innovator and biosimilar using AdvanceBio RP-mAb diphenyl and C4 columns indicates that the mAb pair are highly similar.

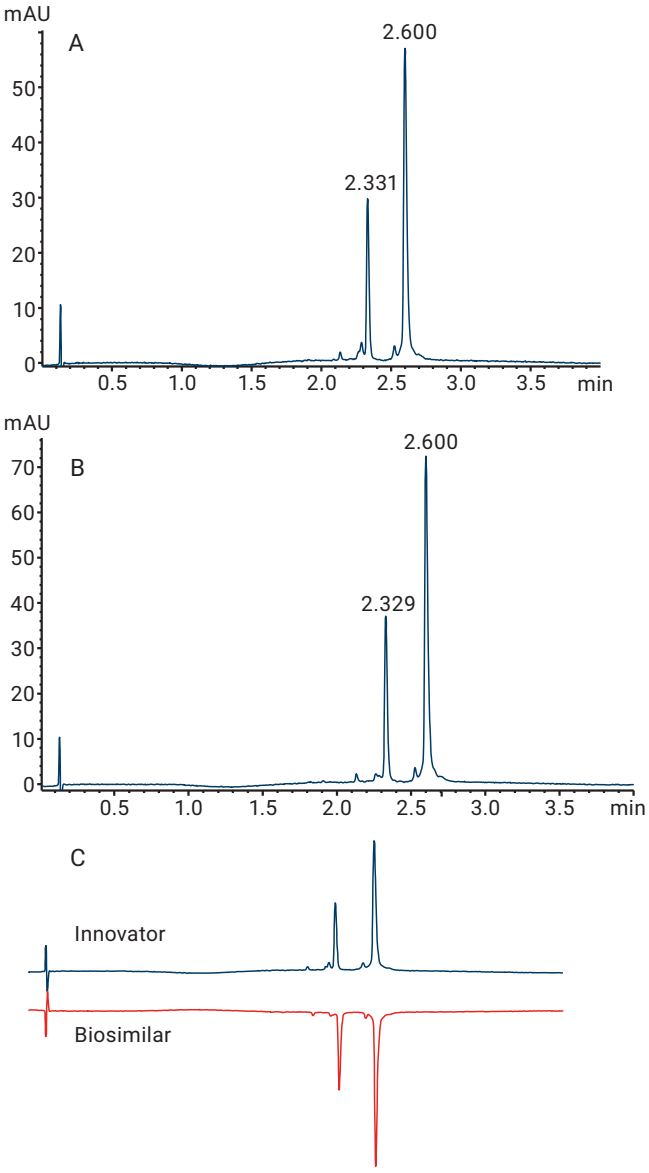

Figure 3. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

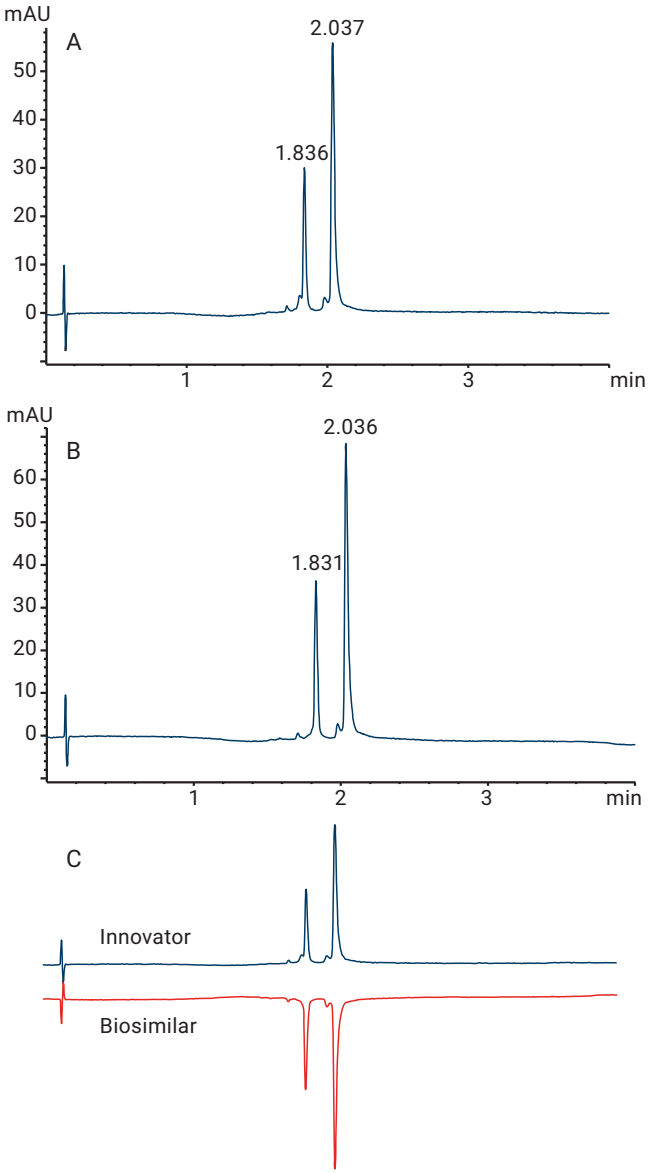

Figure 4. RP-HPLC profiles of innovator rituximab (A) and biosimilar rituximab (B) separated on an Agilent AdvanceBio RP-mAb C4, 2.1 × 50 mm, 3.5 µm column. Mirror plot image overlays (C).

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Precision of retention time and area

Tables 2 and 3 present the average retention times and area RSDs from six replicates of intact and TCEP reduced innovator, and biosimilar rituximab for the diphenyl and C4 phases. The results show that both columns provide precision of RT and area within the acceptable limit of ±3 % and ±5 %, respectively.

Conclusion

In this application note, we have demonstrated a simple LC-UV-based approach to define the molecular similarity between a biosimilar and its innovator reference. We first used the Agilent 1260 Bio-inert Quaternary LC system with Agilent AdvanceBio RP-mAb Diphenyl and C4 columns to develop a high-resolution and rapid separation of intact mAbs. Using the same method, we were also able to show the separation of light chain and heavy chain after TCEP reduction. Area and RT precision of intact and reduced analysis using AdvanceBio RP-mAb columns were excellent, and show the reliability of the method. Such fast, simple, and reproducible methods for intact and reduced analysis of mAbs, coupled with bio-inertness of the system makes this solution suitable for the comparability analysis of mAbs for the biopharma industry.

Reference

1. Navas, N; *et al.,* Anal. Bioanal. Chem. 2013, 405, pp 9351-9363.

Table 2. Retention time and peak area RSD (%), n = 6 for intact analysis

	Retention time		Peak area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	1.96	0	71.61	1.98
Biosimilar rituximab	1.95	0.26	77.3	0.47
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab	2.51	0.20	66.7	0.458
Biosimilar rituximab	2.51	0	73.3	1.86

Table 3. Retention time and peak area RSD (%), n = 6 for reduced analysis

	Retention time		Peak area	
Samples	**Mean (min)**	**RSD**	**Mean (mAU/min)**	**RSD**
Agilent AdvanceBio RP-mAb, Diphenyl, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	2.32	0.60	19.71	4.24
Innovator rituximab HC	2.58	1.52	57.33	1.57
Biosimilar rituximab LC	2.32	0.07	23.56	3.25
Biosimilar rituximab HC	2.60	0.05	58.40	5.61
Agilent AdvanceBio RP-mAb, C4, 2.1 × 50 mm, 3.5 µm				
Innovator rituximab LC	1.83	0	21.5	1.4
Innovator rituximab HC	2.03	0.04	51.2	2.25
Biosimilar rituximab LC	1.83	0.03	24.47	3.84
Biosimilar rituximab HC	2.03	0.06	52.66	0.84

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Disulfide Linkage Analysis of IgG1 using an Agilent 1260 Infinity Bio-inert LC System with an Agilent ZORBAX RRHD Diphenyl sub-2 µm Column

Authors

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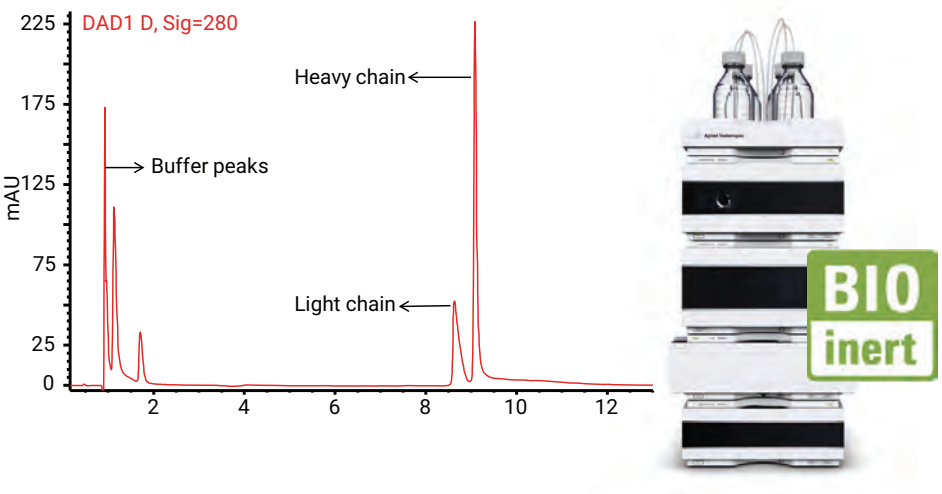
Abstract

This Application Note describes a simple method for the analysis of disulfide linkages in monoclonal antibodies (mAbs) by reversed-phase HPLC. Separation was achieved using an Agilent 1260 Infinity Bio-inert LC System and an Agilent ZORBAX RRHD 300 Diphenyl sub-2 µm particle column. Diphenyl 1.8 µm columns deliver UHPLC performance for reversed-phase separations of intact proteins and peptide digests. Together with UHPLC instruments, these versatile columns enable higher order characterization with shorter analysis times. The 1260 Infinity Bio-inert LC System has a power range up to 600 bar and is capable of handling the higher pressures demanded by emerging column technologies with smaller particles down to 1.7 µm.



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Introduction

Although recombinant mAb therapeutics have advanced enormously in recent years, little is known about their disulfide bond patterns. Complete disulfide bond assignment of IgG1 antibodies can be challenging due to their large size and substantial number of disulfide linkages. Disulphide bonding is critical to maintaining immunoglobulin (IgG) tertiary and quaternary structure for therapeutic monoclonal antibodies (mAb). Both inter- and intra-chain disulphide bonds are formed intracellularly in the expression host prior to secretion and purification during mAb production processes. Disulphide bond shuffling has previously been reported for IgG2 and disulphide-mediated arm-exchange for IgG4, reflecting innate behaviour of these IgG classes1, 2. However, a typical and significant reduction in the number of disulphide bonds has been observed in IgG13 that present significant issues for manufacturing of therapeutic mAbs. This Application Note demonstrates the suitability of the 1260 Bio-inert Quaternary LC System for separating and analyzing the disulfide linkages of IgG1 by reversed-phase HPLC on ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column. Ultrahigh performance liquid chromatography (UHPLC) separation using sub-2 µm particles improves resolution per time and sensitivity, shortens run times, and thus enables the analysis of IgG1, reduced IgG1, and the peptides resulting from digestion of IgG1.

Equipment

Instrumentation

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60 mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (p/n858750-944).

The complete sample flow path is free of any metal components such that the sample never gets in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

Software

Agilent OpenLAB CDS ChemStation Edition for LC & LC MS Systems, Rev. C.01.04

HPLC analysis

Table 1 Chromatographic parameters used for RP HPLC.

Parameter	Conditions	
Mobile phase A	Water + 0.1% TFA	
Mobile phase B	Acetonitrile + 0.09% TFA	
Gradient	Time (min)	Mobile phase B (%)
	0 minutes	25
	3 minutes	35
	4 minutes	40
	5 minutes	40
	15 minutes	90
	16 minutes	25
	Post time	5 minutes
Injection volume	3 µL (Needle with wash, flush port active for 7 seconds)	
Flow rate	0.3 mL/min	
Data acquisition	214 and 280 nm	
Acquisition rate	20 Hz	
Flow cell	60 mm path	
Column oven	50 °C	
Sample thermostat	5 °C	

Reagents, Samples and Materials

The human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. DL-Dithiothreitol (DTT), iodoacetamide, trizma base, and Endoproteinase Lys C were purchased from Sigma Aldrich. All chemicals and solvents used were HPLC grade and highly purifi ed water from Milli Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile was of gradient grade and purchased from Lab-Scan (Bangkok, Thailand).

Reduction and alkylation of Intact IgG1

IgG1 was diluted to 2 mg/mL using 100 mM Tris HCl and 4 M Gu HCl, pH 8.0. An aliquot of 10 µL of 0.5 M DTT stock was added to obtain a final concentration of 5 mM. The mixture was held at 37 °C for 30 minutes. The reaction mixture was cooled briefly to room temperature (RT). An aliquot of 26 µL of 0.5 M Iodoacetamide stock was added for a fi nal concentration of 13 mM. It was allowed to stand for 45 minutes. Once removed, the solution was quenched with 20 µL of DTT for a final concentration of 10 mM.

Lys C digestion of IgG1 and reduction

IgG1 was diluted to 1 mg/mL using 100 mM Tris HCl, pH 8.0. Endoproteinase Lys C in 100 mM Tris HCl, pH 8.0 was added at an enzyme protein ratio of 1:100 (w/w). The mixture was incubated overnight at 37 °C. The reaction was stopped by lowering the pH to 6.0 by adding 10 % TFA. Later, the reduction of Lys C digested IgG1 was carried out as described earlier in this Application Note.

Results and Discussion

Separation and Detection

A ZORBAX RRHD 300 Diphenyl 1.8 µm column has the advantage of low pH and temperature stability, and, combined with the 1260 Infinity Bio-inert Quaternary LC System with a power range up to 600 bar and capabilities of handling the higher pressures, can be used for protein separation. Figure 1 **A** depicts the optimized RP HPLC elution profile of intact IgG1 on a ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column demonstrating excellent retention of IgG1 in 15 minutes. The reproducibility of analysis was tested with six replicates. Figure 1 **B** shows the overlay of six replicates.

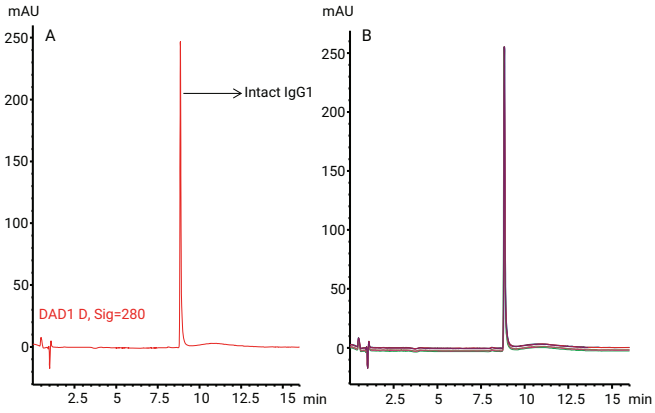

Figure 1. RP HPLC profile of intact IgG1 on an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (A), and an overlay of six replicates (B).

Table 2 Retention time and area RSD (%), n = 6 for intact IgG1

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
8.838	0.086	1,170	0.461

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The effect of reduction and alkylation of the disulfide bonds in intact IgG1 was tested. Figure 2 shows the reversed-phase chromatogram of A reduced and alkylated IgG1 B overlay with reduction/alkylation buffer blank and C overlay of six replicates showing separation reproducibility. Due to the reduction of the disulfide bonds, the IgG1 is separated into its light and heavy chains. The IgG1 eluted as distinct light chain (LC) and heavy chain (HC) as indicated in Figure 2; however, this was not confi rmed by mass spectral analysis.

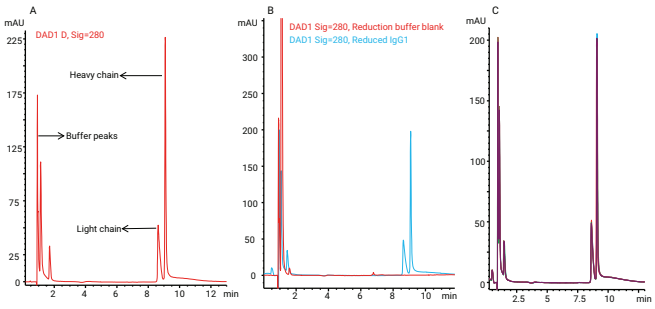

Figure 2. RP HPLC profile of (A) Reduced and alkylated IgG1, (B) overlaid with buffer blank, (C) overlay of six replicates.

Table 3. Retention time and area RSD (%), n = 6 for Light chain

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
8.638	0.091	504.33	2.780

Table 4. Retention time and area RSD (%), n = 6 for Heavy chain

Retention time		Peak area	
Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
9.084	0.152	1,520	0.390

Peptide maps resulting from Lys C digestion of intact IgG1 under nonreducing conditions resulted in a less intricate RP HPLC profile. A representative chromatogram of the IgG1 digest (Figure 3 A) displays the two (baseline separated) peaks that were selected for area and RT precision. The overlay results in sharp peaks with good resolution and excellent separation reproducibility (Figure 3 B).

Further, we wanted to compare the reversed-phase profile of IgG1 under nonreduced and reducing conditions to determine the peptides bound through disulfide linkages. The overlay of Lys C peptide maps of nonreduced IgG1 (red trace) and reduced IgG1 (blue trace) is depicted in Figure 4. The appearance of additional peaks (indicated by an asterix) after reduction of Lys C digested IgG1 confirms they are bound through disulfide linkages.

Precision of retention time and area

The precision of the retention time and area for intact IgG1, reduced IgG1 and endoproteinase Lys C digested IgG1 under nonreduced conditions are given in Tables 2, 3, and 4. The results show that the ZORBAX diphenyl sub-2 µm column shows precision of RT and area to be within 3% and 5 % respectively.

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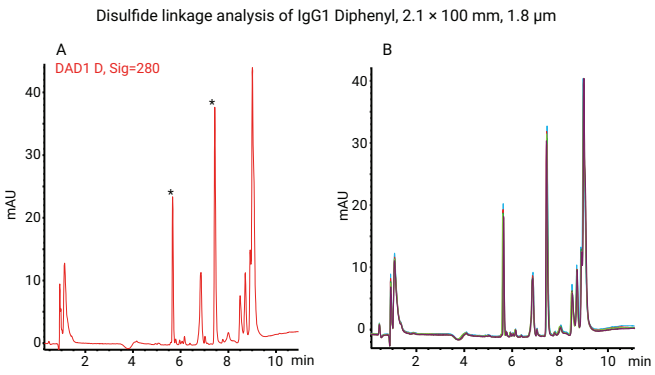

Figure 3. RP HPLC profile of (A) Lys C digested IgG1 and (B) overlay of six replicates. Peaks selected for RT and area RSDs are indicated by an asterix.

Table 5 Retention time and area RSD (%), n=6 for Lys C digested IgG1

	Retention time		Peak area	
	Mean (min)	RSD (limit: ±3.0 %)	Mean (mAU/min)	RSD (limit: ±5.0 %)
Peak 1	5.525	0.307	60	0.544
Peak 2	7.444	0.140	132.45	1.113

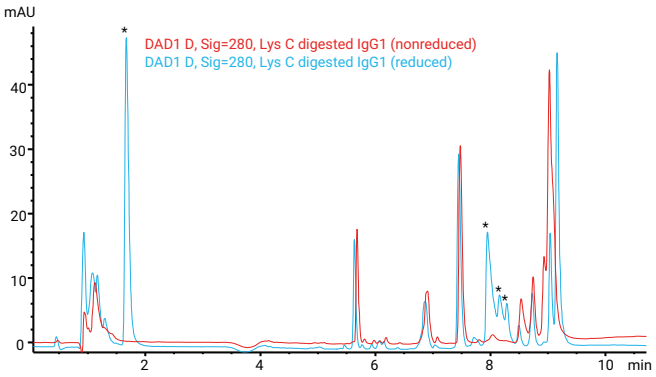

Figure 4. Comparison of peptide maps of Lys C digested IgG1 under nonreducing condition (red trace) followed by reduction (blue trace). Peptides bound through disulfide linkages are indicated by an asterix.

Conclusion

Disulfide linkage analysis is important to study some of the post-transitional modifications of proteins for biopharma process development and monitoring. We have shown the combination of an Agilent 1260 Infinity Bio-inert Quarternary LC System and an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column to perform reproducible and high resolution analysis of disulfide linkage analysis of monoclonal antibodies for biopharma process development and monitoring. Area and RT precision of the method were excellent and proved reliability. Further, the 1260 Infinity Bio-inert LC has a power range up to 600 bar and is capable of handling the higher pressures demanded by emerging column technologies with smaller particles down to 1.7 µm. The bio-inertness and corrosion resistance of the instrument coupled with a simple and reproducible method make this solution particularly suitable for the QA/QC analysis of monoclonal antibody for the biopharmaceutical industry.

References

1. R. Mhatre, J. Woodard, C. Zeng, Strategies for locating disulfide bonds in a monoclonal antibody via mass spectrometry spectrometry, Rapid Commun. Mass Spectrom. 13 (**1999**) 2503–2510.
2. T.-Y. Yen, H. Yan, B.A. Macher, Characterizing closely spaced, complex disulfide bond patterns in peptides and proteins by liquid chromatography/electrospray ionization tandem mass spectrometry, *J. Mass. Spectrom.* 37 (**2002**) 15–30.
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Application Note

Charge Variant Analysis

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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

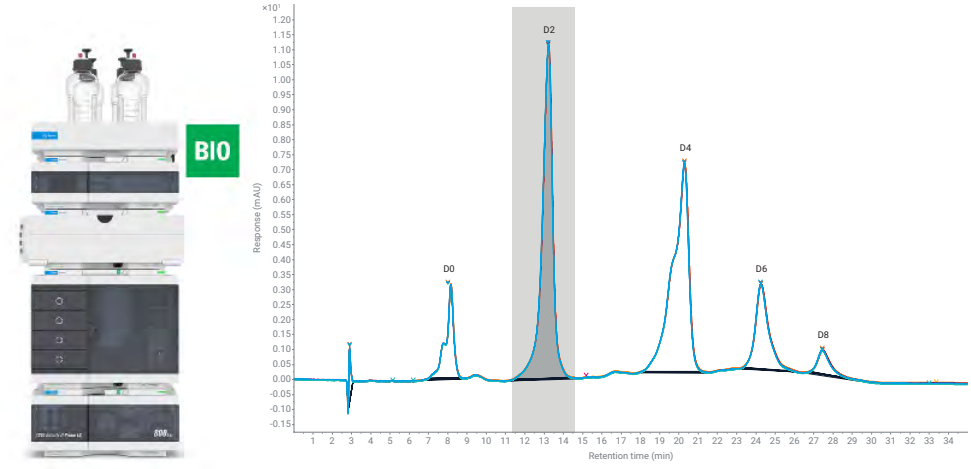
Author

Sonja Schneider
Agilent Technologies, Inc

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

Columns

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.

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Method

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: WThe 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).

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).

Equation 1.

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

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**

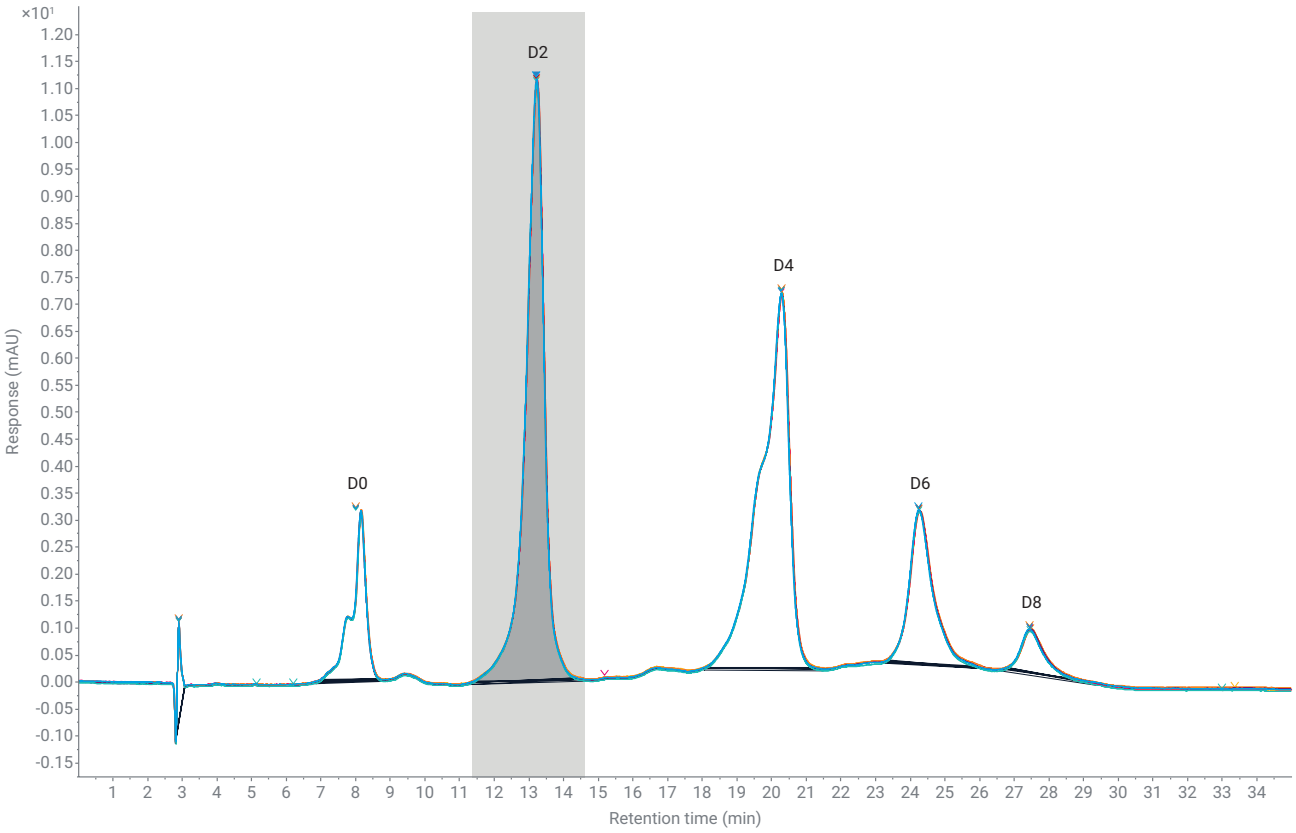

Figure 1. Separation of brentuximab vedotin on the Agilent 1260 Infinity II Prime Bio LC. D0–D8 refers to different DAR species. An overlay of seven subsequent runs is displayed. Blank Subtraction was applied to filter out the baseline drift caused by the ammonium sulfate salt in buffer A using blank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

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.

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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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High Salt—High Reproducibility

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

Author

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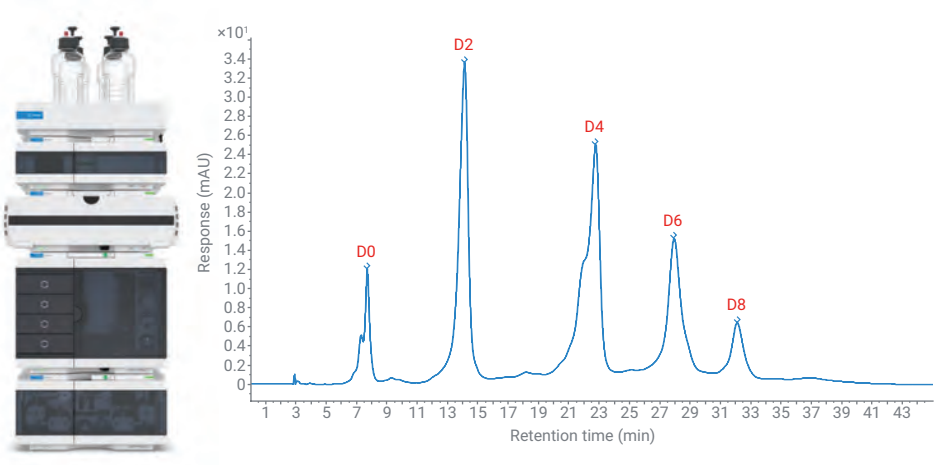
Agilent Technologies, Inc.

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.



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Introduction

Antibody-drug 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,

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	HPLC (intact and reduced mAbs)
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.

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

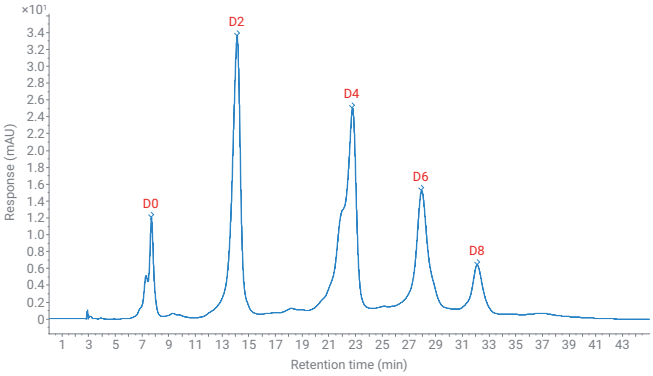

Figure 1. Analysis of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC. D0 to D8 refers to different DAR species.

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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

Peak ID.

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

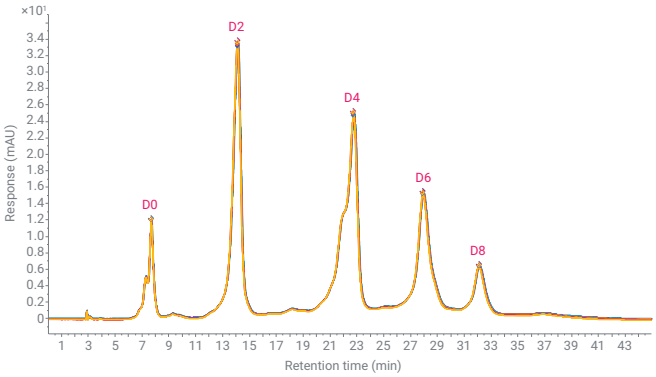

Figure 2. Separation of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC (overlay of seven subsequent runs).

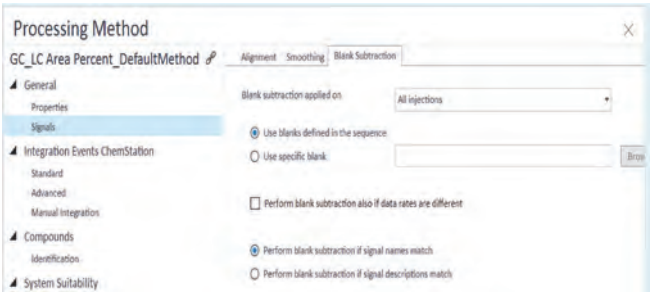

Figure 3. Screenshot of the "Blank Subtraction" feature in the processing method of Agilent OpenLab CDS 2.

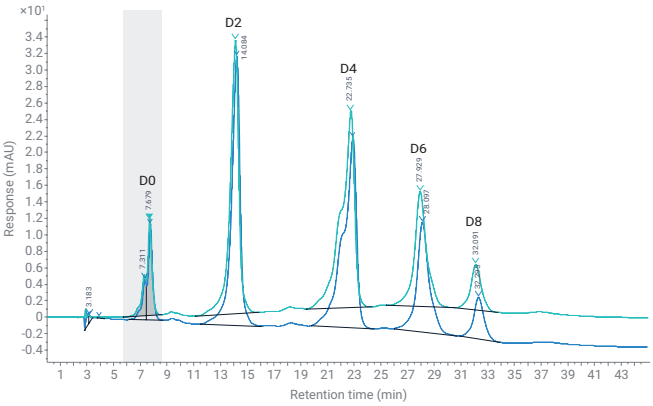

Figure 4. Comparison of HIC chromatogram before (blue) and after (green) blank subtraction.

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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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7. High Stakes. High Performance. High Confidence: Agilent 1290 Infinity II Bio LC. *Agilent Technologies brochure, publication number 5994-2376DE*, **2020**.
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Application Note

Intact & Subunit Purity

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AdvanceBio HIC: a Hydrophobic HPLC Column for Monoclonal Antibody (mAb) Variant Analysis

Using the Agilent 1260 Infinity II Bio-Inert LC

Authors

Andrew Coffey and

Sandeep Kondaveeti

Agilent Technologies, Inc.

Abstract

This Application Note describes the separation of oxidized monoclonal antibody (mAb) variants from their native form using the Agilent AdvanceBio HIC column. Oxidation of exposed amino acid side chain residues such as methionine, cysteine, and tryptophan is a common degradation pathway for monoclonal antibodies, and presents a major analytical challenge in biotechnology. Often, oxidized mAbs have decreased potency compared to their native form[1]. Therefore, to ensure the therapeutic efficacy of the mAb products, analysis of such degradation is critical. Oxidation of amino acid residues on an mAb can alter the hydrophobic nature of the mAb by the increase in polarity of the oxidized form, or also due to resulting conformational changes[2]. HPLC methods for separating biomolecules based on differences in hydrophobicity include reversed-phase and hydrophobic interaction chromatography (HIC). HIC can be applied to characterize mAb variants resulting from post-translational modifications (PTMs). The AdvanceBio HIC column provides excellent resolution of oxidized mAb variants from unmodified forms, and can resolve oxidized species without mAb digestion into subunits or other sample preparation methods.

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Introduction

mAbs and related products such as antibody drug conjugates (ADCs) and bispecific antibodies (bsAbs) are the fastest growing classes of biotherapeutics. Recombinant mAbs are subject to many PTMs during processing, delivery, and storage. Among these modifications, oxidation of exposed amino acid side chains such as methionine (Met) and tryptophan (Trp) is a common occurrence. Various researchers have reported that oxidation of mAbs has an adverse effect on product shelf life and bio-activity[1,2]. Therefore, developing analytical methods to detect oxidized mAb variants has gained interest. The sulfoxide and sulfone side chains of methionine-oxidized mAb products are larger and more polar compared to the native form, which may alter protein structure, stability, and biological function. Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and HIC, are often used to characterize mAb variants. Recently, several studies have indicated that HIC can be applied to monitor oxidation of recombinant mAbs with reasonable selectivity and ease, as an excellent alternative to RPLC[3].

HIC is similar to RPLC in that separation of analytes is based on hydrophobic interactions with the stationary phase. The elution order in HIC enables proteins to be ranked based on their relative hydrophobicity. Unlike RPLC, HIC employs nondenaturing conditions, does not require the use of organic solvents or high temperatures, and separations are carried out at physiological pH, allowing for the preservation of protein structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC[4].

AdvanceBio HIC is a silica-based HPLC column designed for the separation of mAbs and related products. Its unique proprietary bonded phase chemistry provides high resolution and desired selectivity for the analysis of mAbs and mAb variants. This Application Note describes the separation of oxidized NIST mAb variants using an AdvanceBio HIC column.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Humanized IgG1k mAb sample (product item no. 8671) was obtained from NIST SRM Standards. 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

mAb Oxidation with t-BHP treatment

A solution of 1 mL of NIST mAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 70 % tert-butyl hydroperoxide (t-BHP) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Reaction conditions used to obtain Figure 6 data: 2 % (v/v) of 70 % *t*-BHP solution was added to a 1-mL sample of NIST mAb (1 mg/mL), and the reaction mixture was injected onto the column. The sample vial was held at 7 °C, and multiple injections from the same vial were carried out.

mAb Oxidation with H_2O_2 treatment

A solution of 1 mL of NIST mAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 50 % hydrogen peroxide (H_2O_2) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Results and discussion

Protein oxidation is frequently monitored in stability studies or stressed samples during formulation development (for example, samples exposed to a chemical oxidant such as hydrogen peroxide (H_2O_2), UV light, or metal ions). In this study, *t*-BHP and H_2O_2 were used as chemical oxidants to promote oxidation of NIST mAb samples. It was previously reported that both of these reagents tend to specifically oxidize Met side chain residues of the mAb. H_2O_2 more readily oxidizes less accessible, buried residues, whereas *t*-BHP is known to target more surface-exposed Met residues[7]. Figure 1 illustrates the reaction scheme for Met oxidation induced by chemical oxidants.

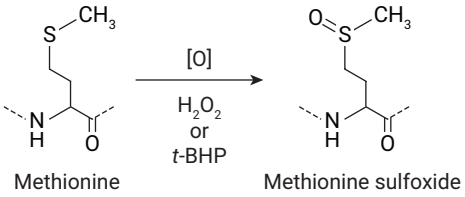

Figure 1. Methionine oxidation induced by chemical oxidant.

The NIST mAb (humanized IgG1k) amino acid sequence in Figure 2 shows that there are six possible surface-accessible Met residues located on both heavy chains of the mAb. Based on prior studies for most human IgG1-subclass antibodies, Met residues localized to the CH_2 and CH_3 domains of the antigen binding, or Fc, region are known to be highly susceptible to oxidation[5]. In the case of NIST mAb, Met 255 and Met 431 correspond to the amino acid residues prone to oxidation. This is depicted by the illustration in Figure 3.

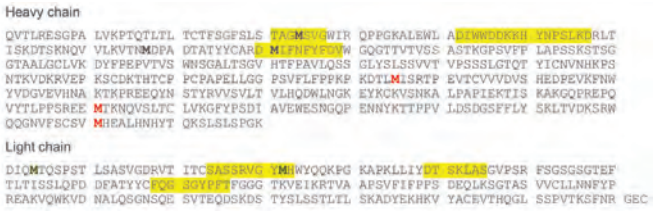

Figure 2. NIST mAb amino acid sequence.

Method conditions

Parameter	HPLC conditions
Column	AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
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
Flow rate	0.3 to 0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Final sample concentration	1 mg/mL
Detection	UV, 220 nm
Gradient profile	Flow rate: 0.5 mL/min Time %A %B 0 50 50 20 100 0 25 100 0 30 50 50 40 50 20
Shallower Gradient	Flow rate: 0.3 mL/min Time %A %B 0 40 60 40 90 10 45 90 10 50 40 60 60 40 60

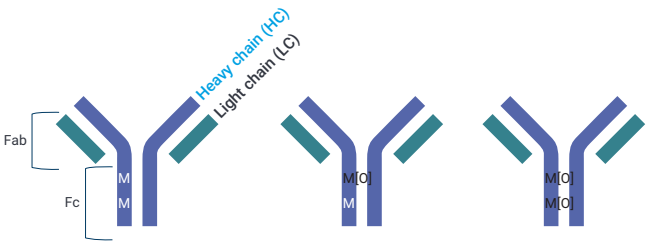

Figure 3. Methionine residues located in Fc region are most susceptible to oxidation in human IgG1 mAbs.

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An AdvanceBio HIC column was able to differentiate oxidized mAb variants from the untreated mAb sample under low salt starting conditions. Oxidation of the NIST mAb with t-BHP under reported experimental conditions resulted in multiple peaks with shorter retention times, presumably due to conformational change. The HIC chromatogram (Figure 4) showing earlier retained peaks labeled 1 to 6 likely indicates the result of oxidized Met residues on the mAb, and peak 7 with a retention time of approximately 12.6 minutes, corresponds to nonoxidized mAb. For the H_2O_2-treated mAb sample, complete oxidation occurred, with three peaks eluting in a shorter retention time, indicating more aggressive oxidation of Met residues. These differences in the chromatograms of the IgG1k mAb sample incubated with two different oxidation reagents suggest that reactivity is governed by solvent accessibility of the Met residues and steric limitations of the oxidizing agent, as previously reported[6].

To further improve the resolution, a slower and shallower gradient was used. Using a flow rate of 0.3 mL/min and a starting ammonium sulfate concentration of 1.2 M with a lower gradient rate of 25 mM/min, better resolution was achieved with a relatively short analysis time (Figure 5). In this chromatogram, multiple mAb-oxidized species are clearly observed from the untreated mAb sample.

Time	RT (%)	Area (%)
0	50	50
20	100	0
25	100	0
30	50	50
40	50	50

50 mM/min

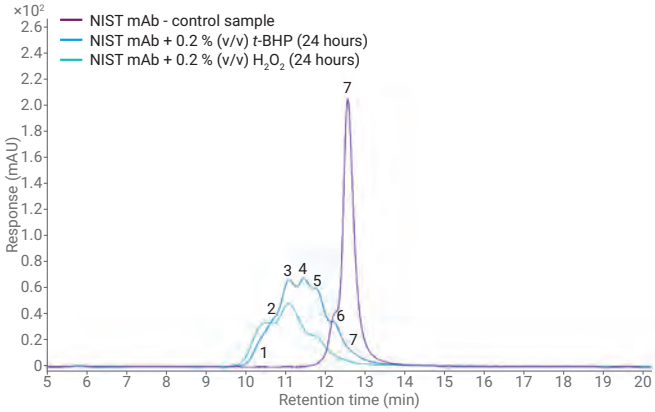

Figure 4. Separation of oxidized NIST mAb variants using lower starting salt concentration.

Time	RT (%)	Area (%)
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

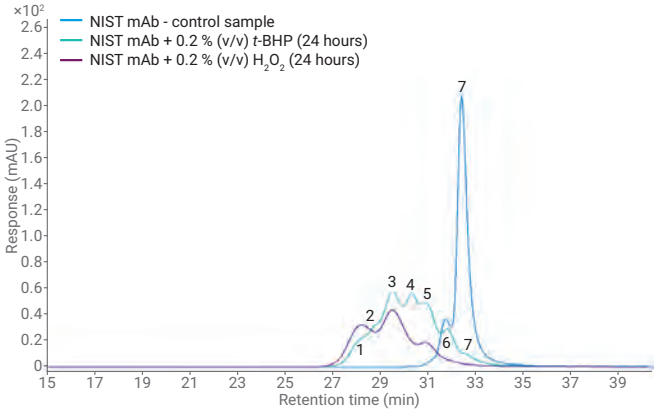

Figure 5. Separation of oxidized NIST mAb variants using a shallow gradient.

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In Figure 6, the NIST mAb sample was incubated with 2 % (v/v) *t*-BHP, and the oxidation reaction was monitored at various time points using shallower gradient conditions. As represented by an overlay of chromatograms, the mAb oxidation progressed with *t*-BHP incubation time. Multiple mAb oxidation species were observed within a few hours of the oxidation reaction. This suggested that surface-accessible Met residues in both heavy chains of the mAb sample might be oxidized randomly, which was previously reported[5]. Further oxidation of the mAb sample after 10 hours of reaction led to a broad peak, indicating forced oxidation. It has previously been speculated that oxidation of deeply buried Met residues can lead to a more dramatic structural change, which may cause the mAb to partially unfold[7]. Partially unfolded mAb is likely to have more conformational variation, resulting in a broader peak with a large retention time shift.

Time	% A	% B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

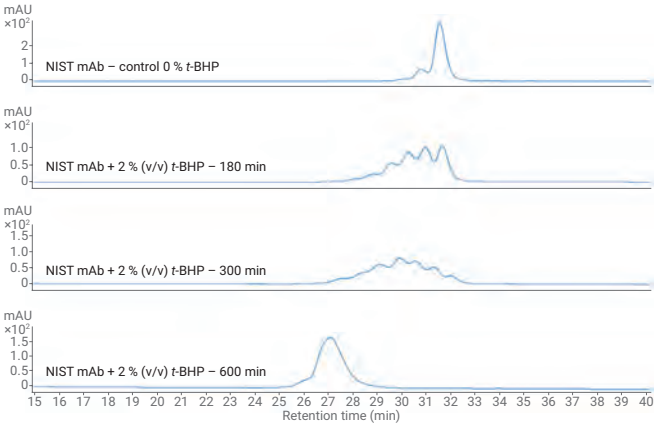

Figure 6. Monitoring the *t*-BHP oxidized mAb reaction.

Conclusion

The AdvanceBio HIC column demonstrated the separation of oxidized mAb variants from its native form. Using the AdvanceBio HIC column, optimal separation of oxidized mAb variants can be achieved using slower flow rates and shallower gradient conditions, while maintaining relatively short analysis times.

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Additional Application Notes

AdvanceBio RP mAb

Publication Number	Title
5991-6296EN	LC/MS Analysis of Intact Therapeutic Monoclonal Antibodies Using AdvanceBio RP-mAb
5991-4723EN	Reducing Cycle Time for Quantification of Human IgG Using the Agilent Bio-Monolith Protein A HPLC Column
5991-5125EN	Cell Culture Optimization Using an Agilent Bio-Monolith Protein A Column and LC/MS
5991-6094EN	Bio-Monolith Protein G Column - More Options for mAb Titer Determination

PLRP-S

Publication Number	Title
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5991-6559EN	Measuring Drug-to-Antibody Ratio (DAR) for Antibody-Drug Conjugates (ADCs) with UHPLC/Q-TOF

AdvanceBio HIC

Publication Number	Title
5994-0149EN	An AdvanceBio HIC Column for Drug-to-Antibody Ratio (DAR) Analysis of Antibody Drug Conjugates (ADCs)
5994-1869EN	Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

ZORBAX RRHD 300SB

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