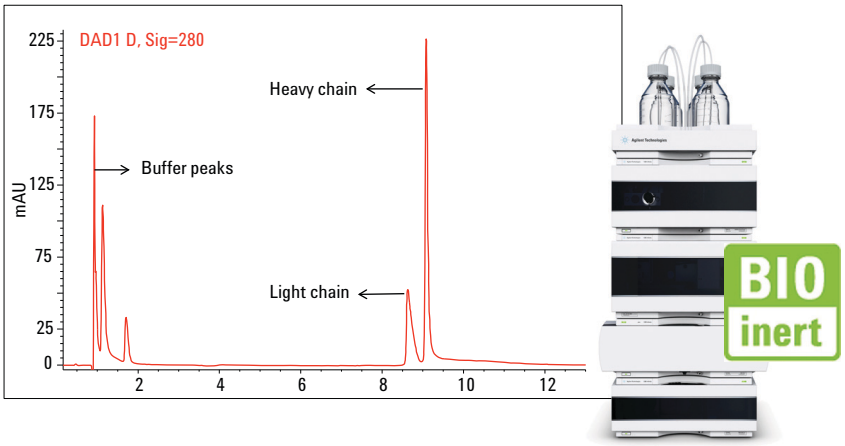
Disulfide Linkage Analysis of IgG1 using an Agilent 1260 Infinity Bio-inert LC System with an Agilent ZORBAX RRHD Diphenyl sub-2 µm Column

Application Note

Biotherapeutics & Biosimilars



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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 classes[1, 2]. However, atypical and significant reduction in the number of disulphide bonds has been observed in IgG1[3] 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/n 858750-944).

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

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	

Table 1
Chromatographic parameters used for RP HPLC.

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 purified 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 final 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 the1260 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.

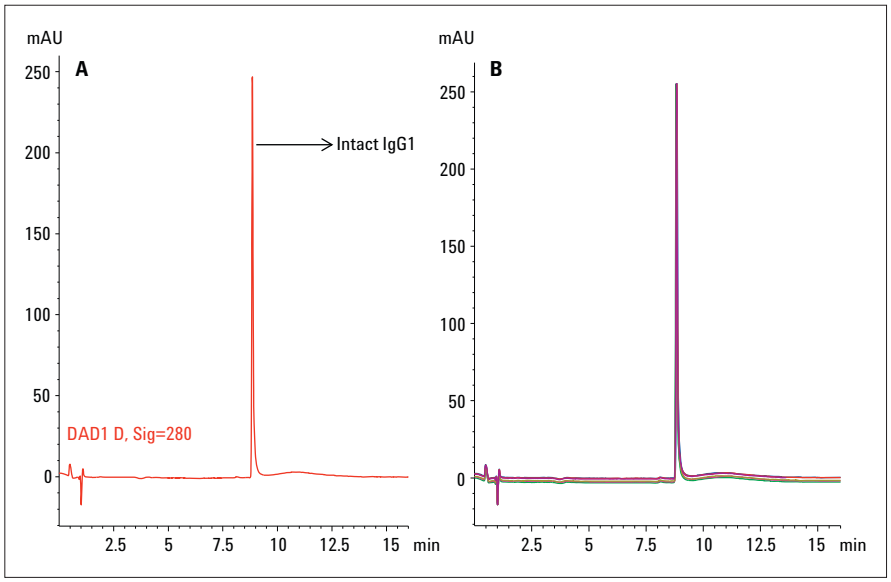

Figure 1
RP HPLC profile of intact IgG1 on an Agilent ZORBAX RRHD 300 Diphenyl, 2.1 × 100 mm, 1.8 µm column (A), and an overlay of six replicates (B).

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

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

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 confirmed by mass spectral analysis.

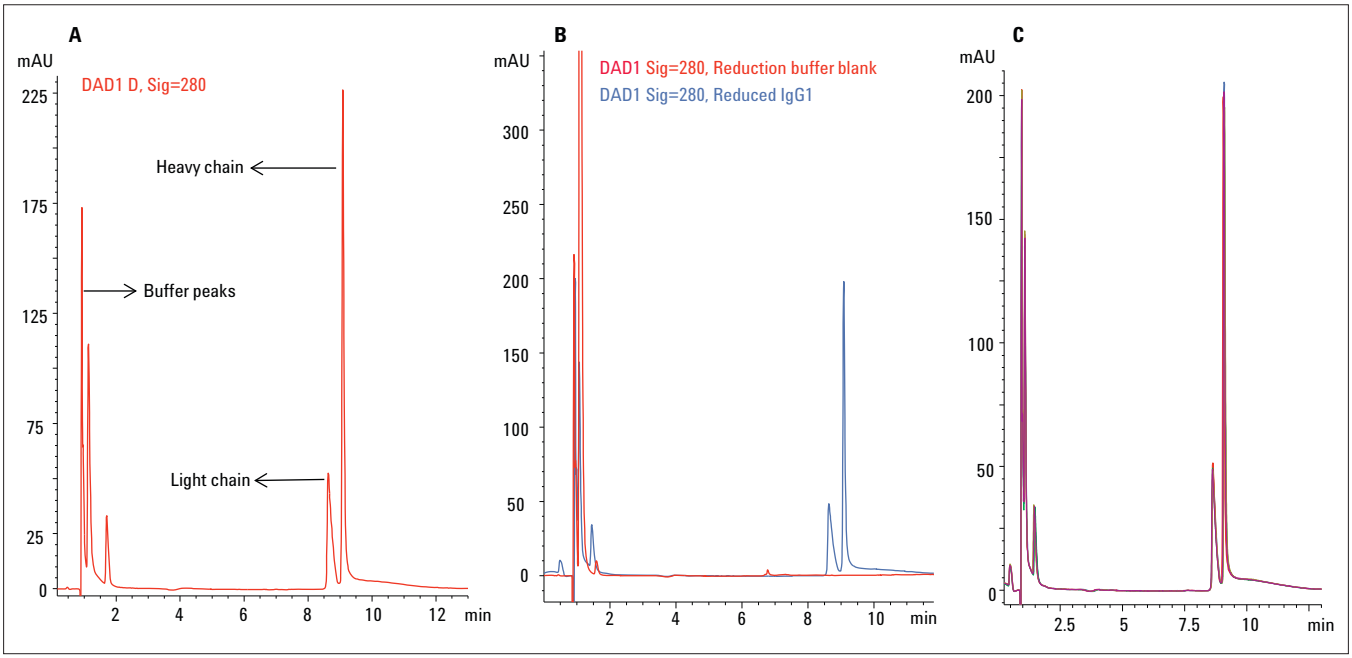

Figure 2
RP HPLC profile of (A) Reduced and alkylated IgG1, (B) overlaid with buffer blank, (C) overlay of six replicates.

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 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%)
9.084	0.152	1,520	0.390

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

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 reverse 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.

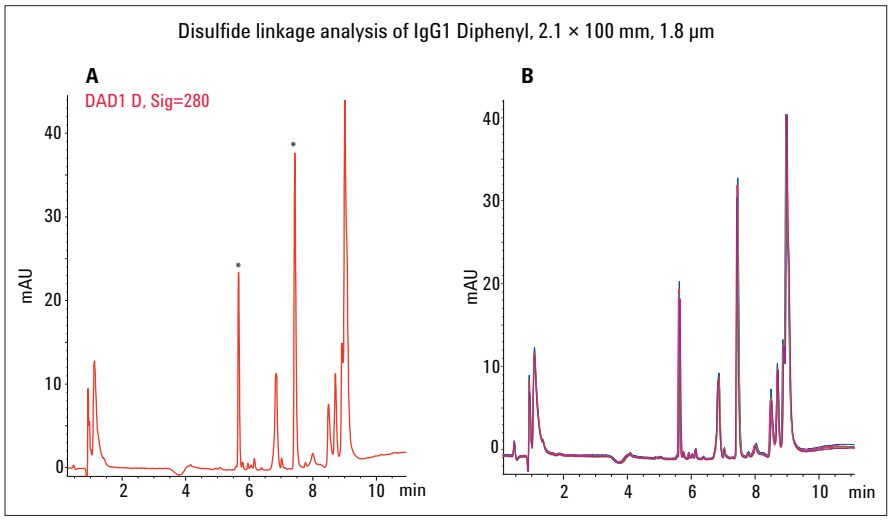

Figure 3
RP HPLC profile of (A) Lys C digested IgG1 and (B) overlay of six replicates. Peaks selected for RT and area RSDs are indicated by an asterix.

	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

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

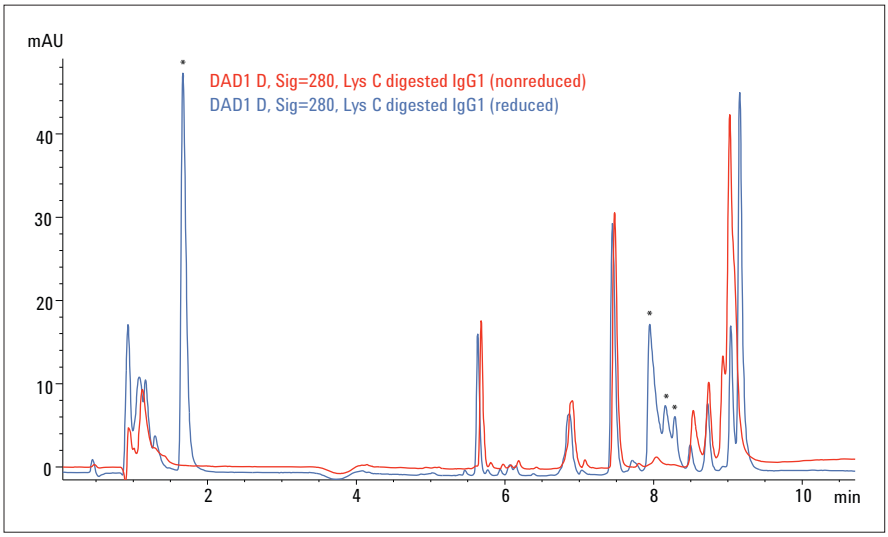

Figure 4
Comparison of peptide maps of Lys C digested IgG1 under nonreducing condition (red trace) followed by reduction (blue trace). Peptides bound through disulfide linkages are indicated by an asterix.

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

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© Agilent Technologies, Inc., 2013
Published in the USA, February 1, 2013
5991-1694EN

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