Automatic Protein Disulfide Bond Mapping of a Monoclonal Antibody Using the Agilent Accurate-Mass Q-TOF LC/MS Platform and BioConfirm Software Algorithm

Application Note

Biotherapeutics and Biosimilars

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Introduction

Monoclonal antibodies (mAbs) are a very important class of biopharmaceutical molecules with a wide range of therapeutic and diagnostic applications. For mAbs, higher-order structure plays a critical role in efficacy, and is greatly influenced by disulfide bonds [1]. Drug quality assessment necessitates the determination of disulfides, including confirmation of expected linkages and perturbations. Knowledge of disulfide bonds may influence mAb production efforts from candidate selection to formulation. Typically, disulfide bond mapping presents serious analytical challenges, because many combinations of bonds can be created when the molecule is exposed to stress [2]. We have developed a LC/MS method using an Agilent 1290 Infinity II UHPLC, an Agilent 6545 Q-TOF/MS, and Agilent MassHunter BioConfirm B.08.00 software for accurate disulfide bond mapping. This method can readily be used to map the scrambling of disulfide bonds in a monoclonal antibody.

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Materials and Methods

Sample preparation

The formulated Herceptin (Trastuzumab) (Genentech, So. San Francisco, California, USA) was desalted using the Bio-Spin 6 cartridge (Bio-Rad), then denatured with 8 M urea. Prior to characterization studies, Herceptin was reconstituted and digested with trypsin/Lys-C mix with or without reduction with dithiothreitol (DTT) and alkylation using iodoacetamide (IAM).

Instrumentation

LC Parameters	
Instrument	Agilent 1290 Infinity II UHPLC
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm
Injector volume	5 µL
Mobile phase	A) DI water + 0.1 % formic acid B) Acetonitrile + 0.1 % formic acid
Flow rate	0.2 mL/min
Gradient	0 to 42 %B in 35 minutes, 45 to 100 %B in 5 minutes, hold 5 minutes at 100 %B
Stop time	50 minutes
Post time	10 minutes

MS Parameters	
Instrument	Agilent 6545 Accurate-Mass Q-TOF
Source	Dual AJS in positive mode
Drying gas temperature	325 °C
Drying gas flow	13 L/min
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
Nebulizer pressure	35 psi
Capillary voltage	4,000 V
Fragmentor voltage	175 V
Skimmer voltage	65 V
MS range	100 to 1,700 *m/z*
MS scan rate	5 spectra/s
Auto MS/MS range	50 to 1,700 *m/z*
MS/MS scan rate	3 spectra/s

Data analysis

An acquired data file of a protein digest of Herceptin was loaded into MassHunter BioConfirm B.08.00, and analyzed by a Molecular Feature Extractor (MFE), an untargeted feature finding algorithm. MFE finds features by removing ions that are persistent across the retention time (RT), then grouping ions into proposed features using evidence of isotopic clusters, adducts, neutral losses, and multiple charge states.

Each feature was given a Quality Score; a perfect score is 100. The following factors were considered when setting the Quality score:

- Signal-to-noise ratio (S/N)
- RT peak shape
- RT peak width
- Consistency of ion retention time
- Mass difference between ion species
- Whether it is a single-ion compound

The features found were compared to a theoretically digested Herceptin sequence with post-translational modifications, cysteine disulfide linkages, and sample preparation artifacts. In a typical run, approximately 2,000 putative peptides were found and assigned a Quality Score for the annotation of these sequence characteristics. In addition, the quality of disulfide bond linkages was assessed using the Bio Score value, which is determined by a formula that allows users to specify the weight given to the MS score and the MS/MS score (determined by various factors, such as peak intensity and matched ions).

Results and Discussion

Disulfide bond formation is a post-translational process that can affect the structure and function of therapeutic proteins. Incomplete or incorrect disulfide bond linkages can generate protein misfolding, which will eventually influence the drug efficacy. Therefore, it is critical to confirm that all disulfide bond linkages are correct.

The typical IgG-1 antibody Herceptin contains a total of 16 disulfide bonds (Figure 1), 12 of which are intra-chain linkages (four in the light chain and eight in the heavy chain). In addition, there are four inter-chain disulfide bonds (two links each light chain and heavy chain, and two disulfide bonds in the hinge regions link the two heavy chains).

The thorough mapping of all of disulfide bonds in an mAb is a critical but challenging task for the biopharmaceutical industry. With the Agilent 6545 LC/Q-TOF and MassHunter BioConfirm B.08 software, we demonstrated a straightforward LC/MS/MS-based method for accurate disulfide bond mapping.

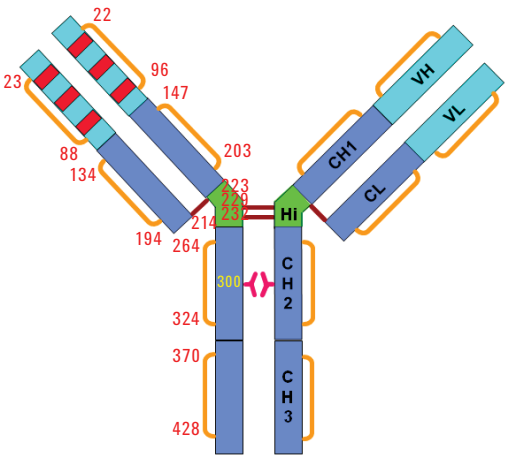

Figure 1. Humanized IgG-1 Herceptin disulfide bond linkage structure.

To compare and identify the disulfide-linked peptides unambiguously, two mAb samples (nonreduced versus reduced) were prepared prior to the proteolytic digestion. In brief, one mAb sample was treated with 8 M urea, 20 mM DTT, and 40 mM IAM to achieve full reduction. The other sample was only treated with 8 M urea to denature (nonreduced) the mAb molecule. Both mAb samples were digested with trypsin/Lys-C mix (1:20, w/w) for better digestion efficiency.

Reversed-phase LC/MS/MS data were then collected for each sample under the same LC/MS conditions. As expected, the disulfide bonds of the mAb were cleaved in the reduced sample, while the intact S-S bond linked peptides (larger mass) remained in the native sample. We then used the Comparative Analysis program in the MassHunter Qualitative Analysis software for LC/MS data comparison (Figure 2). The mirror plot (2B) clearly shows the major differences between these samples. There were many larger peptide molecules with longer HPLC retention times detected in the nonreduced trypsin/Lys-C digested Herceptin sample (arrows in 2A).

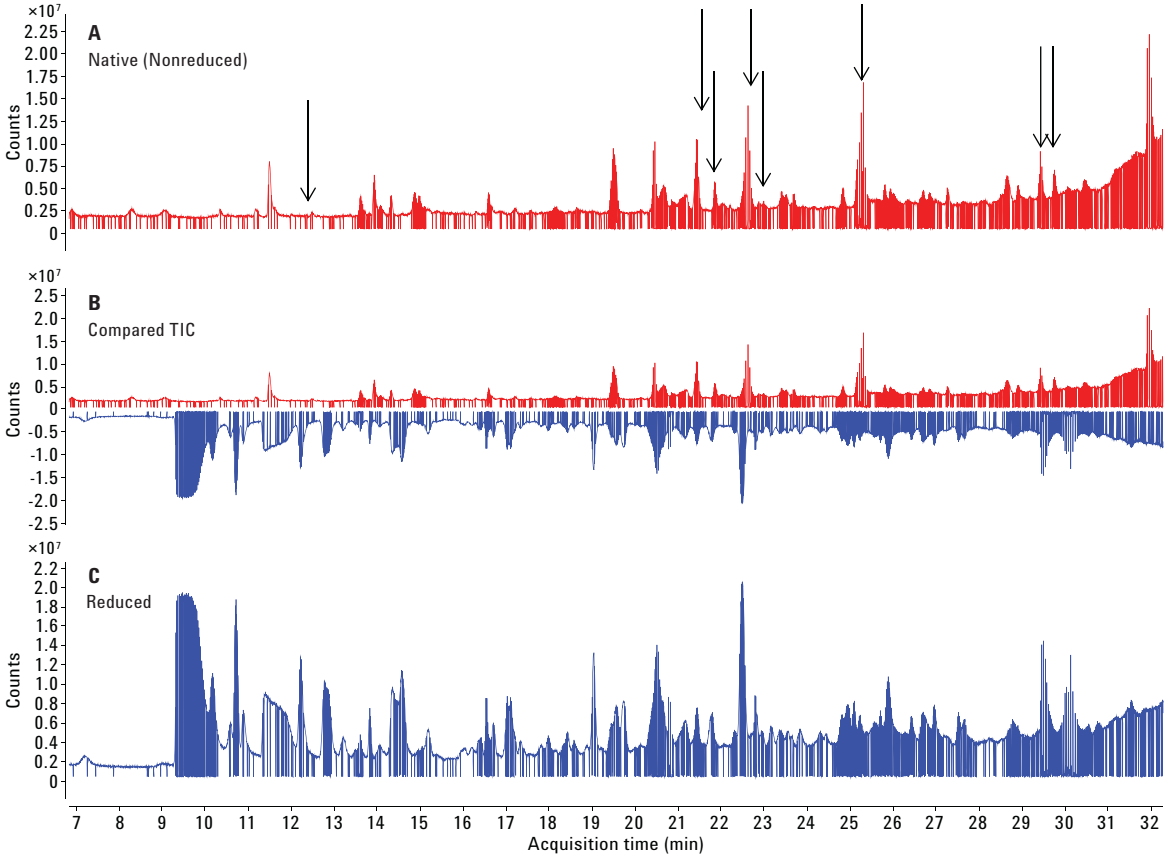

Figure 2. Comparison of nonreduced (native) vesus reduced samples.

The disulfide bond mapping algorithm (BioConfirm B.08.00) can overcome the difficulty in manually determining disulfide bonds present in proteins, in particular mAbs. Based on protein sequence, the algorithm generates all theoretically possible peptides based on experimental conditions (proteolytic enzymes, alkylating reagents) and PTMs (Figure 3). The algorithm then rapidly searches for these features in peptide MS and MS/MS data, and the results are generated with both the Quality Score and the Bio Score.

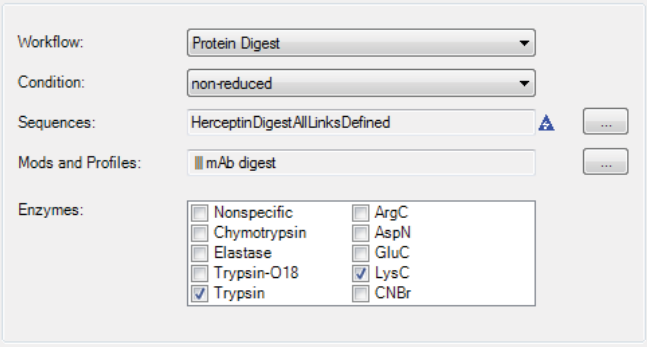

Figure 3. A screen capture showing the method setup of disulfide bonds analysis in Agilent BioConfirm B.08.00.

This algorithm for mapping disulfide bonds in MassHunter BioConfirm B.08.00 is also able to discern native state linkages. Scrambled linkages can be eliminated from consideration because they have much lower Quality and Bio scores. Table 1 shows the results where the B22–B96 disulfide linkage in the heavy chain has been defined in the Herceptin sequence with a Bio Score of 67.15. Other disulfide linkages that received lower Bio scores were likely due to their low MS/MS scores in the fragment ion matching of the large molecules and low abundances for missed cleavage peptides.

The analyst can examine these graphically for higher confidence in the mapping results as shown in Figure 4. The peptide sequences and the molecular mass of the disulfide-linked peptides from the heavy chain of the native Herceptin sample are shown in the top panel. Only the 3^+ (*m/z* 796.0351) and 4^+ (*m/z* 597.2808) ions of the disulfide-linked peptides were detected. MS/MS spectra are ranked by the number of matching fragments and explanations of the product ions using the peptide sequence. Product ion spectra of the 4^+ (*m/z* 597.2808) precursor ion (B) are labeled with b (blue) and y ions (red).

Table 1. Results of Disulfide Bond Mapping with all Linkages Defined in the Input Sequence

Sequence	Mass (Da)	RT	Score (MFE)	Score (Bio)	Enzyme	Missed	Links
NQVSLTCLVK + WQQGNVFSCSVMHEALHNHYTQK	3,844.8329	14.561	100	48.25	Trypsin + LysC	0 + 0, 0 + 0	Cysteine disulfide bond(B370-B428) Cysteine disulfide bond(D370-D428)
TPEVTCVVVDVSHEDPEVK + CK	2,328.1014	11.996	100	66.87	Trypsin + LysC	0 + 0, 0 + 0	Cysteine disulfide bond(B264-B324) Cysteine disulfide bond(D264-D324)
LSCAASGFNIK + AEDTAVYYCSR	2,384.081	12.06	100	67.15	Trypsin + LysC	0 + 0, 0 + 0	Cysteine disulfide bond(B22-B96) Cysteine disulfide bond(D22-D96)
THTCPPCPAPELLGGPSVFLFPPK + THTCPPCPAPELLGGPSVFLFPPK	5,004.4906	24.659	80	29.77	Trypsin + LysC	0 + 0	Cysteine disulfide bond(B229-D229) Cysteine disulfide bond(B232-D232)
VTITCR + SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	4,819.2476	20.08	100	28.96	Trypsin + LysC	0 + 0, 0 + 0	Cysteine disulfide bond(A23-A88) Cysteine disulfide bond(C23-C88)
SGTASVVCLLNNFYPR + HKVYACEVTHQGLSSPVTK	3,820.9116	15.65	100	56.64	Trypsin + LysC	0 + 2, 0 + 2	Cysteine disulfide bond(A134-A194) Cysteine disulfide bond(C134-C194)
SGTASVVCLLNNFYPR + VYACEVTHQGLSSPVTK	3,555.7594	17.559	100	57.18	Trypsin + LysC	0 + 0, 0 + 0	Cysteine disulfide bond(A134-A194) Cysteine disulfide bond(C134-C194)

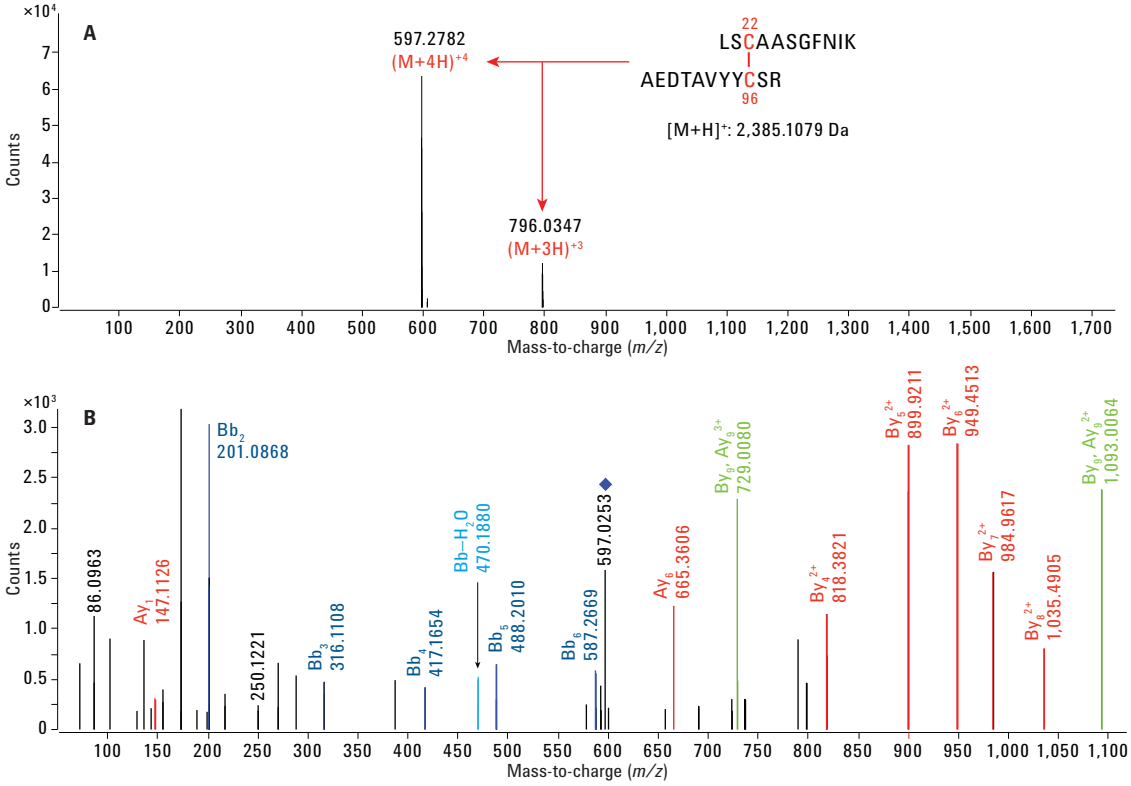

Figure 4. Mass spectra of the disulfide-containing peptides in the heavy chain of Herceptin. A) MS spectrum, B) MS/MS fragmentation spectrum.

All 16 pairs of disulfide bonds in the Herceptin sample (native) have been correctly detected and identified with the BioConfirm B.08.00 algorithm (Table 2). Conversely, none of these disulfide-containing peptides were detected in the reduced Herceptin digest sample.

Table 2. Summary of S-S Bonds Identified in the Herceptin Sample

S-S Bond location	S-S Links	Peptide sequence	Measured mass (Da)
LC	A23–A88/C23–C88	VTITC(23)R + SGTDFTLTISSLQPEDFATYYC(88)QQHYTTPPTFGQGTK	4,819.2467
LC	A134-A194/C134-C194	SGTASVVC(134)LLNNFYPR + VYAC(194)EVTHQGLSSPVTK	3,555.7588
HC	B22-B96/D22-D96	LSC(22)AASGFNIK + AEDTAVYYC(96)SR	2,384.0811
HC	B147-B203/D147-D203	STSGGTAALGC(147)LVK + DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC(203) NVNHKPSNTK	7,916.9289
HC	B264-B324/D264-D324	TPEVTC(264)VVVDVSHEDPEVK + C(324)K	2,328.1009
HC	B370-B428/D370-D428	NQVSLTC(370)LVK + WQQGNVFSC(428)SVMHEALHNHYTQK	3,844.8311
LC–HC	A214-B223/C214-D223	SFNRGEC(214) + SC(223)DK	1,260.4925
HC–HC	B229-D229/B232-D232	THTC(229)PPC(232)PAPELLGGPSVFLFPPKPK + THTC(229)PPC(232)PAPELLGGPSVFLFPPKPK	5,454.7975

Conclusions

We have developed and demonstrated an analytical workflow that uses an Agilent UHPLC, an Agilent 6545 LC/Q-TOF, and Agilent MassHunter BioConfirm B.08.00 software for the accurate disulfide bond mapping of a monoclonal antibody. This workflow can rapidly map disulfide bonds to their locations, and use the MS/MS fragmentation data from the disulfide-containing peptides to calculate the Quality Score for higher confidence results. This workflow can also be applied to the disulfide bond scrambling mapping of a monoclonal antibody.

References

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2. H. Liu, K. May. "Disulfide bond structures of IgG molecules: Structural variations, chemical modifications and possible impacts to stability and biological function" *MAbs* **4**, 17-23 (2012).

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