Determining Disulfide Bond Position by Peptide Mapping with LC-MS/MS

Byung-Hee Shin,[1] Eric Johansen,[2] and St John Skilton
[1]*AB SCIEX, South Korea,* [2]*AB SCIEX, USA*

As biologics are increasingly well understood and their characterization has advanced, so have expectations about the ability to map higher order structure. Disulfide bonding in protein therapeutics may have an important effect on the integrity of the final product. Effective mapping of disulfide bonds in the proteins of interest contributes significantly to process efficiency. Mapping disulfide bonds may provide insight into the integrity of a biotherapeutic and allow an organization to identify sources of error early on, such as during cell culture or purification processes. Inappropriate disulfide bond arrangements contribute to aggregation and must be monitored as an aspect of patient safety. In recent Biosimilars guidelines from the USFDA [ref 4,5], and within the ICH framework [ref 1,2,3], disulfide bond mapping is seen as critical. Mass spectrometry is specifically referred to by EMA guidance documents discussing ICH guideline 6B: *"e) Sulfhydryl group(s) and disulfide bridges. If cysteine residues are expected, the number and positions of any free sulfhydryl groups and/or disulfide bridges should be determined, to the extent possible. Peptide mapping (under reducing and non-reducing conditions), mass spectrometry, or other appropriate techniques may be useful for this evaluation"* [ref 6]

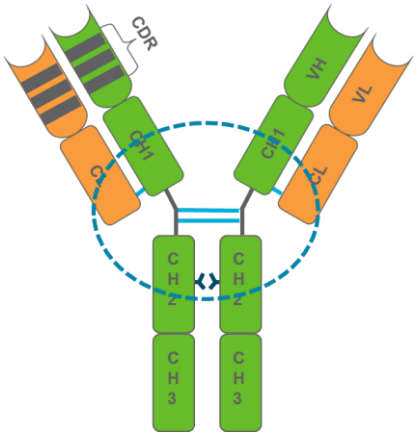

Figure 1: A typical IgG monoclonal Antibody. The representation of the disulfide bonds which hold the Heavy and Light Chains together are highlighted by a dotted circle

The ability to map disulfide bonding in monoclonal antibodies is a major advantage of fast LCMS. TOF systems with high resolution confer specific advantages in disulfide bond mapping because they can differentiate closely related species, provide confirmation of structure using rapid MS/MS, and perform this at fast chromatographic speeds. Dynamic Range is especially useful for coping with the large difference in ionization efficiencies for different disulfide bonded species, and the AB SCIEX Triple TOF® 5600+ system has the fidelity to monitor such variations. Additionally, disulfide bond mapping can be done as part of a peptide map, enabling efficiency gains such as obtaining multiple different strands of information simultaneously. AB SCIEX is able to provide tools that help organizations maintain their business and technological advantages in the production and marketing of a biotherapeutic product.

Experimental Design

Materials and Methods: In this technical brief an AB SCIEX Triple TOF® 5600+ system was used. Of particular utility was the ability to rapidly switch between MS and MS/MS, dynamically excluding ions that have already been monitored. While there are many techniques where MS and MS/MS can be obtained rapidly, the 5600+ system operates at speeds that facilitate structural analysis for small and large molecules alike, even for low abundance species.

Sample Preparation: For the purposes of examining the disulfide bonds, a reduced sample was prepared and compared to the non-reduced sample to look for differences in disulfide bonding patterns. 1 mg of monoclonal antibody of IgG1 isotype was denatured with urea, then half of the resulting sample was reduced with DTT and alkylated with iodoacetamide. The resulting denatured protein was then digested with trypsin (37 °C, four hours).

Chromatography: Rapid HPLC analysis of trypsin-digested monoclonal antibodies was performed using a Shimadzu HPLC system on a Jupiter C18 column (Phenomenex, 2.1 x 150 mm, 3 µm). Solvent A consisted of 2% acetonitrile and 0.1% formic acid, and solvent B consisted of 98% acetonitrile with 0.1% formic acid.

Mass Spectrometry: High flow LC/MS/MS analysis was performed on the AB SCIEX Triple TOF® 5600+ system coupled with the DuoSpray™ Source. Information dependent acquisition (IDA) was performed as follows: For each cycle of 1.3 sec, 0.25 sec MS scan was performed followed by 20 MS/MS scans of 50 ms each. All ions selected for MS/MS had a 2+ or greater charge state. Each selected ion was then placed in an exclusion list for 15 sec.

Data Processing: IDA data files were searched using ProteinPilot™ Software [3] against a FASTA database containing the sequences of the antibody and ~100 known protein contaminants. ProteinPilot™ Software can search for hundreds f biological modifications, sequence variants, and unexpected cleavages simultaneously without exponentially increasing the number of false positives. The data, which contains both MS and MS/MS information, was then brought into PeakView® software and used to generate MS extracted ion chromatograms (XICs) for each identified peptide. The relative quantitation of each peptide and any modified form(s) can be obtained using this approach and these XIC peak areas can be mined extensively for quantitative analysis, and to track changes in a protein product over time.

Results

The workflow showed where disulfide bond arrangements differed between the reduced and non-reduced samples. For the IgG examined, excellent spectral quality was obtained to demonstrate the unequivocal presence of disulfide bonds in the non-reduced sample. Figure 1 shows a stacked chromatogram display from PeakView® of all of the relevant species from two peptide maps. This display can be used to rapidly review where peaks are different between the reduced and non-reduced samples. This gives an indication of where disulfide bonds have been formed.

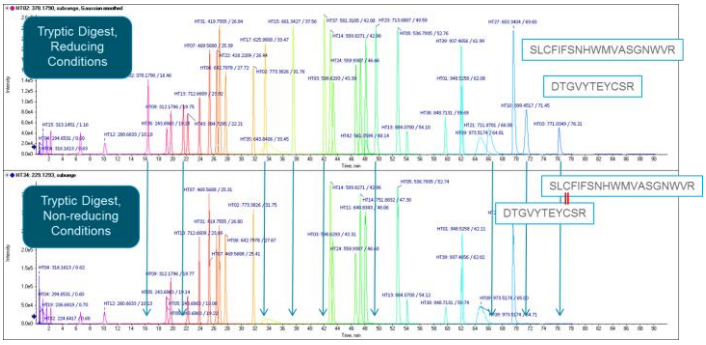

Figure 2. A stacked chromatogram view of the peaks in a peptide map. The Upper pane shows the peptide map for reducing conditions, the lower trace shows the non-reducing conditions. The arrows highlight where peaks have disappeared

Figure 3 illustrates the example of one of the species examined, the combined peptide HT03-HT12. The extracted chromatograms are shown for just those species that are relevant by choosing narrow exact mass chromatograms for three items: HT03, HT12, and the disulfide bonded species, HT03-HT12.

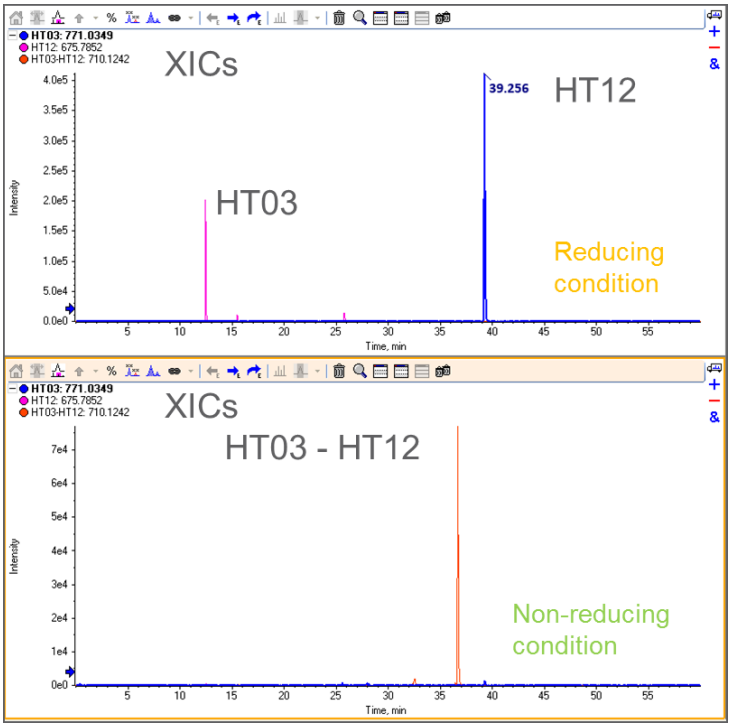

Figure 3. Comparison of extracted ion chromatograms for the individual peptides under reducing (upper pane) and non-reducing condition showing the disulfide bonded species (lower pane)

Under reducing conditions, it is very clear that the peptides exist as individual species, shown by the accurate mass extracted ion chromatograms (XICs) for each of the tryptic peptides. However, in the non-reduced condition (lower trace), the XIC of the species corresponding to bonded peptide is very clearly revealed.

The Need for Orthogonal Information

While this methodology is adequate proof of the existence of different species, additional, orthogonal information to corroborate the assignments may be needed. By using a hybrid, QTof instrument, MS/MS information can be obtained simultaneously. Historically, the user would have to design a second, subsequent experiment, targeting species that had been identified in the first pass, to obtain MS/MS information. However, in a direct response to customer need, AB SCIEX has developed the capability to simultaneously provide additional information in one

LC/MS/MS workflow by using information dependent acquisition (IDA). This single IDA experiment provides precursor information and MS/MS fragment information without any up-front method development or foreknowledge of the analytes. In the same rapid analysis, quantitative MS and MS/MS validation are provide simultaneously, increasing the depth of structural information available. The experiment itself is performed extremely fast, so that there is no loss of information – due to the ability to switch both rapidly (0.26 millisecons acquisition time for MS, followed by 50 ms each of 20 MS/MS). To avoid repeatedly sampling the same precursor ion, each selected ion was placed in an exclusion list for 15 sec, thereby ensuring the maximum amount of coverage. Although it is technological advances that have achieved this capability, this automation means that the user can move more swiftly to comparing their biologic to the reference standard rather than having to manage an instrument.

The data output of the experiment is processed in PeakView®. In Figure 4, the fragment evidence for each of the two peptides involved in a disulfide-bonded dipeptide are highlighted. The top pane shows the unlabeled, deconvoluted MS/MS spectrum, and fragment evidence supporting Heavy Chain Peptide 3 (HT03) is shown on the same spectrum in the middle pane. In the bottom pane fragment evidence for HT12 is highlighted. In each of the middle and bottom panes, a delta of the other peptide's mass is labeled which identifies the location of the disulfide bond.

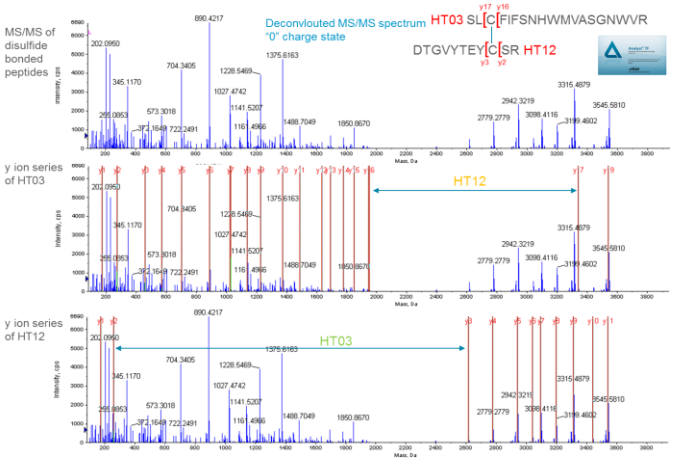

Figure 4: Fragment ion spectrum for the disulfide bonded species

and individual peptides. This is a particular strength of systems that provide accurate mass on precursors and fragment ions

Conclusions

The demands on organizations involved in the manufacture of biologics are increasingly geared towards providing efficient, cost-effective and rapid tools. Manufacturers also need to prove to the regulators that they can maintain manufacturing consistency, ensure patient safety, and demonstrate comparability . If an organization is able to demonstrate that the disulfide-bonding pattern of their biologic is consistent and meets expectations, then they are also more likely to meet regulatory guidelines, and maintain profitability in an increasingly competitive marketplace.

AB SCIEX has developed tools that help organizations maintain their business and technological advantages by rapidly and efficiently helping to map the disulfide bonds of a biotherapeutic.

References

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AB SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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