Comparative Analysis of Partial and Selective Reduction of Monoclonal Antibodies using the X500B QTOF System

Generally applicable method for selective reduction of inter-chain disulfide bonds of therapeutic monoclonal antibodies

Todd Stawicki, Kristi Kong, Sean McCarthy
SCIEX Framingham, Massachusetts (USA)

Introduction

Monoclonal antibodies (mAbs) are the largest class of therapeutic biologics. Complete characterization of a mAb often involves LC-MS analysis at the intact, subunit and peptide levels. Reduced subunit analyses are frequently confounded by artifacts of the chemical reduction procedure. Typical chemical reduction procedures for mAbs typically involve vast molar excesses of both denaturant, such as urea or guanidine hydrochloride, and reductant, such as dithiothreitol or tris(2-carboxyethyl)phosphine) (TCEP). The molar excesses of these non-volatile reagents often lead to premature dirtying of the LC-MS system if a divert valve is not used prior to electrospray ionization. More importantly, these reaction mixtures tend to be unstable over time or over freeze-thaw cycles and during analysis intramolecular disulfide bonds may reform which can complicate spectral interpretation.

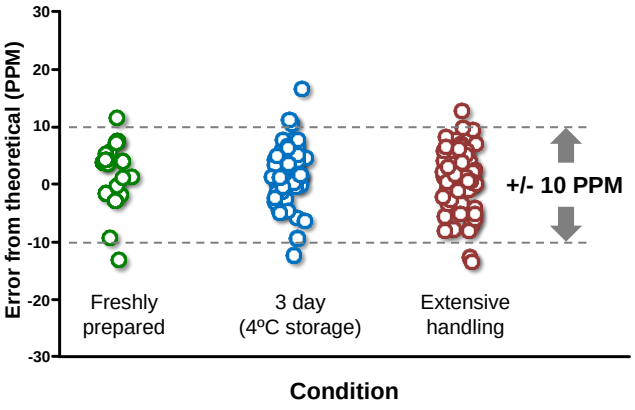

Figure 1: Scatter plot representing the PPM error from theoretical for each identified protein form in each condition. Each point represents one protein form. More technical replicates were tested for the more "stressed" conditions hence more data points. The data are jittered on the X axis for clarity.

This technical note seeks to address the above challenges. The following note describes a method for maintaining the intramolecular disulfide linkages in antibody subunits (heavy and light chain) and selectively reducing only the inter-molecular disulfides. This method has been applied to a range of therapeutic monoclonal antibodies. The described protocol in combination with the X500B QTOF Mass Spec, ExionLC™ system and BioPharmaView™ software enables a complete workflow.

Materials and Methods

For the selective reduction reaction 200 nM therapeutic monoclonal antibody was incubated with 32 µM tris(2-carboxyethyl)phosphine) (TCEP) in 20 mM Tris buffer pH 7.2 at 37ºC for 1 hour at 500 RPM. The key parameter for the reduction reaction is a 160x molar ratio of reductant to protein. Four different antibodies were tested: NIST mAb (National Institutes of Standards and Technology, P/N RM 8671), Adalimumab, Rituximab, and Trastuzumab were all purchased from Myoderm. Following incubation, the samples were split into three aliquots:

Aliquot Name	Description
Freshly prepared	Analyzed within 4 hours of reaction
3 day	Held at 4ºC for 3 days and then analyzed
Extensive handling	Subjected to a -80ºC freeze/thaw cycle, held at 4ºC for 3 days and then analyzed

Complete reduction was accomplished on 200 nM of a therapeutic monoclonal antibody added to a solution containing 5M guanidine HCl, 50 mM TRIS, and 10 mM DTT. The mixture with added antibody was incubated at 80°C for 10 minutes. The sample was split into two aliquots, one for immediate analysis and one subjected to a freeze thaw cycle in a -80°C freezer.

Each aliquot was analyzed at least 3 times using a benchtop X500B QTOF mass spectrometer using SCIEX OS equipped with an ExionLC™ system. Table 1 describes the liquid

chromatography conditions used in these analyses. Table 2 describes the mass spectrometry parameters used.

The data were processed using BioPharmaView™ 2.1 software for reconstruction of the intact proteins and for batch-to-batch comparisons.

Table 1: Chromatographic Conditions

Parameter	Value
Stationary phase	*Waters MassPREP™ Micro Desalting Column*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*0.3 mL/min*
Column temperature	*80ºC*

Table 2: Gradient Profile

Time	Flow Rate (ml/min)	%A	%B
0.0	0.3	85	*15*
2.0	0.3	*85*	*15*
10	*0.3*	*55*	*45*
12	*0.3*	*10*	*90*
13	*0.3*	*10*	*90*
13.5	*0.3*	*85*	*15*

Table 3: Mass Spectrometer Conditions

Parameter	Value	Parameter	Value
Curtain gas:	35	Time bins to sum:	*40*
Ion source gas 1:	50	*TOF start mass (Da):*	*400*
Ion source gas 2:	*50*	*TOF stop mass (Da):*	*5000*
Temperature:	*400*	*Accumulation time:*	*0.5 sec*
Scan type:	*TOF MS*	*Declustering potential (V):*	*120*
Polarity:	*Positive*	*Collision energy (V):*	*10*
Ionspray voltage:	*5500*	*CAD gas:*	*7*
Large Proteins(>70kDa):	*On*	*Decrease Detector Voltage:*	*Off*

Results and Discussion

The selective reduction reaction cleaves **inter**molecular disulfides while leaving **intra**molecular disulfides intact. The raw MS spectra of both the light chain (Figure 2) and heavy chain (Figure 3) illustrate this point. The selectively reduced NIST mAb (Figure 2&3, B) yields a lower overall charge state envelope (thus higher m/z) compared to the fully reduced and denatured NIST mAb (Figure 2&3, A). This is consistent with a more natively folded form of the proteins. A side benefit of the more natively folded form is a more clearly interpretable spectrum because of less low m/z interference.

The analysis of selectively reduced NIST mAb exhibited excellent mass accuracies against theoretical values. The identification and annotation for several of the heavy chain forms are shown (Figure 4).

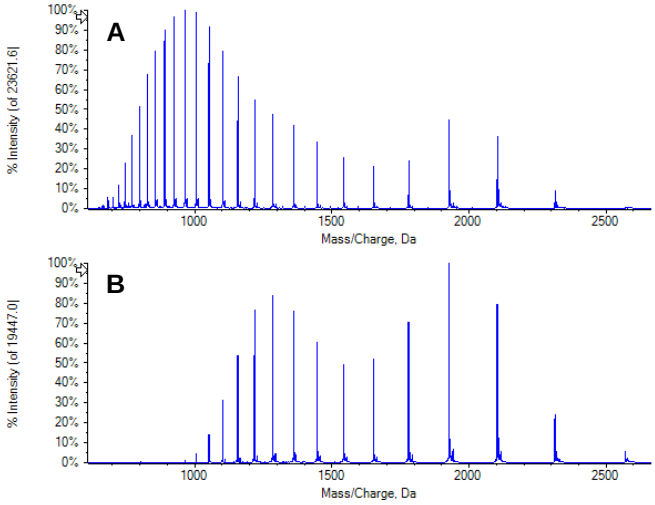

Figure 2: Raw MS spectrum of denatured and completely reduced NIST mAb light chain (A). Raw MS spectrum of selectively reduced NIST mAb light chain (B).

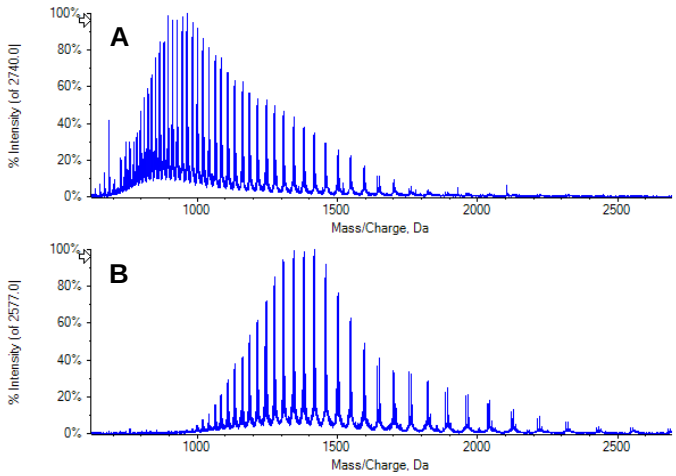

Figure 3: Raw MS spectrum of denatured and completely reduced NIST mAb heavy chain (A). Raw MS spectrum of selectively reduced NIST mAb heavy chain (B).

In addition to the results shown in Figure 4, all of the identified masses for the light chain and heavy chain glycoforms were consistent with only interchain reduction. Furthermore, LC-MS analysis demonstrates the reduced heavy and light chains are very stable in the final reaction mixture (Figure 1). Even in the "extensive handling" condition the mass accuracies for the majority of identified protein forms were within 10 PPM of theoretical.

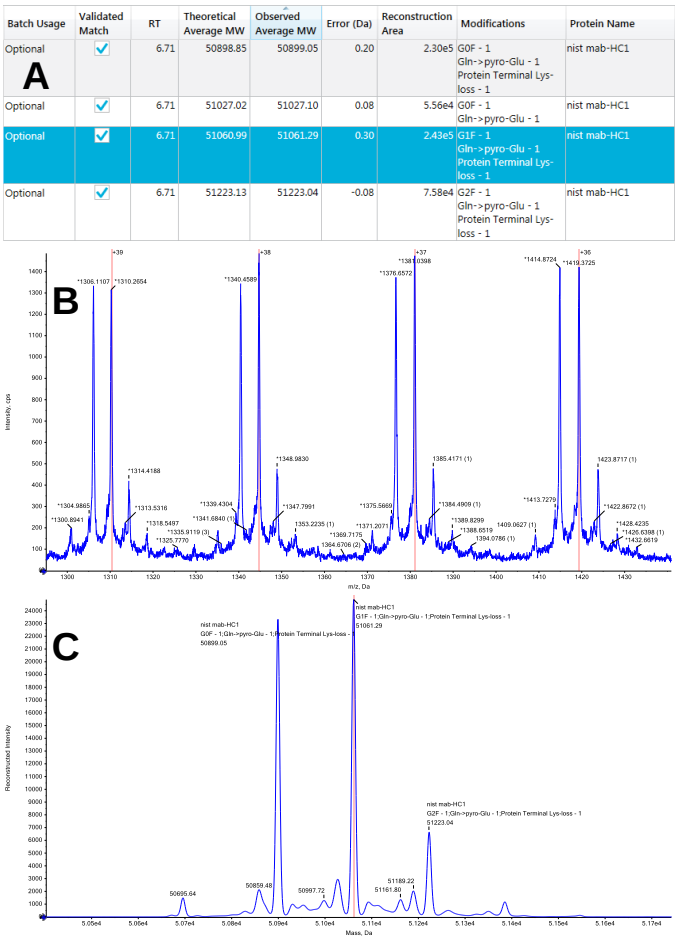

Figure 4: Several heavy chain glycoforms were identified by BioPharmaView™ with excellent accuracy against the theoretical values (A). The MS ions (B) and zero charge reconstruction (C) for the heavy chain G1F glycoform are highlighted.

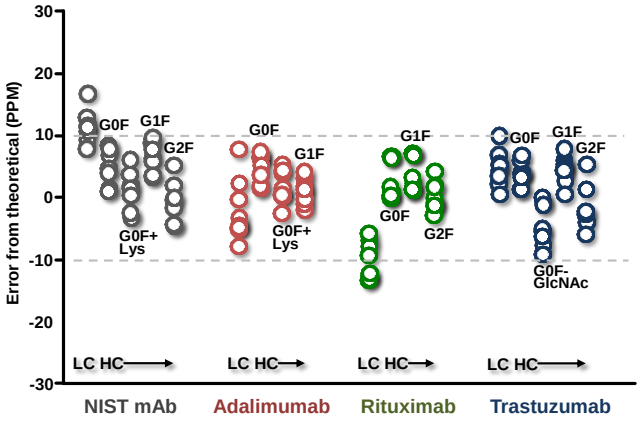

Figure 1. Scatter plot representing the PPM error from theoretical for each identified form for each therapeutic mAb. Each point represents one protein form. The points are colored and grouped on the X axis according to therapeutic. The data are jittered on the X axis for clarity.

Three other therapeutic monoclonal antibodies were analyzed alongside NIST mAb. The majority of the protein forms for all of the antibodies analyzed exhibited mass errors from theoretical under 10 PPM (Figure 5). All of the masses were consistent with only interchain disulfide reduction. Many other glycoforms were identified, but only the major ones are listed in Figure 5.

Conclusion

The results acquired on the X500B benchtop QTOF mass spec system and analyzed with BioPharmaView™ software suggest this workflow is capable of consistently and stably producing free light chain and heavy chain forms with only the interchain disulfide bonds reduced. Overall performance of the assay provides mass accuracy and reproducibility. Furthermore, the data illustrate the method is generally applicable over a range of commercially available monoclonal antibodies.

Document number: RUO-MKT-02-7675-A

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices