**ABSTRACT**

Purpose: The novel capillary Thermo Scientific™ MAbPac™ RP column (4 μm, 0.15 mm x 150 mm) developed originally for intact protein analyses has proven to be an excellent tool for peptide mapping of tryptic digest of therapeutic antibodies. This research aims at demonstrating the utility of this column for mapping some Post Translational Modifications (PTMs) at peptide level.

**Methods:** Intact mAbs were digested using a Thermo Scientific™ SMART Digest™ Trypsin Kit. In disulfide bond mapping experiments, unreduced and reduced digests were compared. Using heavy (H215N150) water to enable glycosylation site occupancy and chemical deamination, Peptides were selected and analyzed in data dependent acquisition using a Thermo Scientific™ Orbitrap™ 3000 Q Exactive™ System coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer controlled by Thermo Scientific™ Xcalibur™ Chromatography Data System (CDS) and Thermo Scientific™ Xcalibur™ software.

Results: Highly reproducible peptide mapping method resulting in almost 100% sequence coverage using only 100 ng digest enabled to identify all the disulfide bonds and led to confident, reproducible characterization of site occupancy of mAbs.

**INTRODUCTION**

Peptide mapping is an important step during biotherapeutic characterization. From drug discovery to bioproduction, peptide mapping is one of the key analytical tools. A general peptide mapping workflow consists of four essential steps beginning with the digestion of proteins with sequence specific proteases such as trypsin, Glu-C or Glu-C, etc., followed by peptide separation by HPLC, mass spectrometric data acquisition of the separated peptides and finally data analyses using a peptide mapping software.

Monitoring of PTMs in therapeutic proteins to ensure batch to batch reproducibility, efficacy and safety of the drug materials is necessary and required by different regulatory agencies. Some of the PTMs such as disulfide bond formation or glycosylation have crucial roles in the folding of proteins into their final three dimensional, biologically active conformation. Disulfide bond conformation in light and heavy chains is a structural prerequisite to the correct folding of antibodies. The number of disulfide containing peptides is one of the limitations of the useful lifetime of therapeutics. Aside from the importance of determining site occupancy of mAbs, characterization of site occupancy of mAbs is also one of the requirements by biopharmaceutical industry.

**MATERIALS AND METHODS**

Sample Preparation

Trastuzumab (mAb) was used in this work. mAbs was digested applying Thermo Scientific™ SMART Digest™ Trypsin Kit, the digestion in general was followed by reduction with TCEP, (Thermo Scientific™). (Bond-Breaker™ TCEP solution, Neutral pH and alkylation with iodoacetamide (Acros Organics, Fisher Scientific™) except for unreduced digest at disulfide mapping. Labeling reaction of reduced and alkylated peptides with heavy (H215N150) water (Sigma-Aldrich) to discriminate masses was carried out according to [1]. PNGase F was a Rapid™ PNGase F kit (New England Biolabs Inc, Ipswich, MA).

Chromatography and mass spectrometry

In capillary chromatographic experiments, mobile phase "A" was water containing 0.1 % formic acid and mobile phase "B" was 80/20 % acetonitrile/water (v/v) containing 0.1 % formic acid (by volume). The flow rate applied was 2 μl/min. The gradient started at 5 % "B" and increased to 50 % "B" over 30 min. Analyses of peptides were performed at 45°C.

Tryptic peptides were analyzed in data dependent acquisition mode (Full MS-d3Top5) in positive mode. The spray voltage was 1.5 kV and the capillary temperature was set at 320°C. MS1 spectra were collected in the scan range of m/z 350-1200 at 70 000 resolution, the AGC target in MS1 data acquisition was set to 3x10⁶ at 60 ms maximum injection time. Data dependent MS² experiments for top 5 ions were carried out using 28 kV normalized collision energy at 17 500 resolution and 80 ms injection time. The minimum AGC target and threshold were set at 2x10⁵ and 2x10⁴ the quad isolation window was 1.9 T. The number of transients were 1 in both MS¹ and MS².

**RESULTS**

Figure 1 exhibits three separations of 100 ng of tryptic digest of Trastuzumab using different columns and injecting samples prepared at different times. Apart from the identical profiles and very similar retention times, sequence coverage obtained for the light chain was identical in the three experiments, resulting in 98.1 %, slightly lower but still acceptable values were achieved for the heavy chain. The XICs of the three peptides containing 7, 19 and 26 amino acids are depicted by Figure 2. Peak width measured at the half height slightly increased from 0.12 min to 0.15 min as the gradient progressed in 15 min. This indicates that no significant peak broadening was observed which makes this column a good tool for peptide mapping.

**DISCUSSION**

Peptide mapping is an important step during biotherapeutic characterization. From drug discovery to bioproduction, peptide mapping is one of the key analytical tools. A general peptide mapping workflow consists of four essential steps beginning with the digestion of proteins with sequence specific proteases such as trypsin, Glu-C or Glu-C, etc., followed by peptide separation by HPLC, mass spectrometric data acquisition of the separated peptides and finally data analyses using a peptide mapping software.

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Data Analysis

MS data were processed using Thermo Scientific™ BioPharma Finder™ 3.2 Software.

**RESULTS**

Table 1. Identified Disulfide Bond Containing Peptides in Unreduced Digest

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Theoretical m/z</th>
<th>Experimental m/z</th>
<th>Error, ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>QGHEALHNHYTQK</td>
<td>4087.9570</td>
<td>4087.9624</td>
<td>1.31</td>
</tr>
<tr>
<td>QQHYTTPPTFGQGTK</td>
<td>4819.2422</td>
<td>4819.2524</td>
<td>2.13</td>
</tr>
<tr>
<td>PAPELLGGPSVFLFPPK</td>
<td>5454.7832</td>
<td>5454.7925</td>
<td>0.72</td>
</tr>
<tr>
<td>LVK/SRWQQGNVF</td>
<td>3454.6079</td>
<td>3454.6106</td>
<td>0.78</td>
</tr>
<tr>
<td>LVK/DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYI</td>
<td>13</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Cysteine linkage Type of disulfide bond</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:C23/1:C88 Light-Light, 1ss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:~C229,~C232/2:~C229,~C232 Heavy-Heavy, 2ss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:C22/2:C96 Heavy-Heavy, 1ss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2:C370/2:C428 Heavy-Heavy, 1ss</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

1. Liu, Z., Cao, J., He, Y., Qiao, L., Xu, C., Lu, H., Yang, P. Tandem 18O Stable Isotope Labeling for Quantification of N-Glycome. J. Proteome Res. 2010, 9, 227-238.

**ACKNOWLEDGEMENTS**

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