Analytical Selectivity Enhancements That Deliver Improved Resolution To Enable Identification Of Critical Quality Attributes
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Introduction
Peptide mapping by LC/MS is a preferred technique used for confirming the primary structure of protein biopharmaceuticals. High efficiency and high resolution peptide separations are critical for improved peptide identification. Formic acid isooxalate-pairing agent often used in the LC/MS runs yields broader peak shapes, resulting in co-elution of peptides. Additionally, poor peak capacity, peak broadening with higher mass loading, and poor resolution of closely related peptides are limitations with current LC/MS methods. In this presentation, we demonstrate the excellent performance of a unique charge hybrid/C18 bonded phase column using formic acid modifier for peptide map separation. Different LC/MS conditions were evaluated resulting in superior peptide separation with significantly improved peak shapes. Peptide mapping of heavy and light chains of a monoclonal antibody (mAb) resulted in 99% sequence coverage with mass accuracy of 5 ppm. The charged C18 column generated well-resolved peptide peaks enabling significant improvements in analyzing low abundance post-translationally modified peptides. Comprehensive analysis of post-translational modifications (PTMs) such as oxidation, deamidation, and glycosylation by LC/MS/MS will be discussed.

Experimental
Materials
Therapeutic mAb protein was purchased from a local pharmacy and stored according to the manufacturers’ instructions. All chemicals and solvents were LC/MS grade.

Peptide map
Before the digestion of the mAb with trypsin, the disulfide bonds were reduced (DTT) and alkylated (IAA) under denaturing conditions (guanidine-HCl). This pretreatment ensured complete mAb denaturation and solubilization, allowing efficient access of the protease to the target substrate. Following reduction and alkylation, the pH of the solution was adjusted to pH 7–8, and trypsin digestion (20 μL protein to protease w/w) was performed overnight at 37 °C. The samples were then immediately analyzed using LC/MS or were stored at −80 °C until use.

Instrumentation
LC System
- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (G7110B)
- Agilent 1290 Infinity II multi sampler (G7167B)

MS System
- Agilent 6450QX AdvanceBio LC/Q-TOF

LC/MS conditions
Columns: AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 μm, 120 Å; Column temperature: 55 °C; Mobile phase: A = 0.1% formic acid, B = 0.1% formic acid in ACN; Flow rate: 0.5 mL/min; Gradient (%B): 3% at 0 min, 3% at 1 min, 40% at 31 min, 95% at 33 min, 95% at 24 min, 3% at 34.1 min, stop time: 34.1 minutes
MS = Ionization mode: positive; Capillary voltage: 4000 V; Drying gas flow: 13 L/min; Drying gas temperature: 325 °C; Nebulizer: 35 psi; AUS sheath gas temperature: 175 °C; AUS sheath gas Flow: 12 L/min; Fragmentor: 175 V; Skinner: 65 V; Acq. Mode: MS and MS/MS; Scan range (MS): 400-1700 m/z, Scan range (MS/MS): 50-1700 m/z; MS scan rate (spectra/second): 10; MS/MS scan rate (spectra/second): 3
Data analysis: Data from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software.

Results and Discussion

Effect of gradient time and flow rate on peak capacity

Figure 1: Effects of flow rate (A) and gradient length (B) on peak capacity. Twenty extracted compound chromatograms peaks were selected across whole chromatogram to calculate peak capacity. Chromatographic parameters: (A) Gradient time 80 min, (B) Flow rate 0.5 mL/min. All other LC/MS conditions are as described in method section.

Extracted compound chromatogram for the identified peptides

Figure 2: Agilent AdvanceBio Peptide Plus column identified peptide chromatogram. Overlay extracted compound chromatograms for the matched peptides.

Separation of deamidated and nondeamidated peptides

Figure 3: Overlay extracted compound chromatograms showing the separation of nondeamidated and deamidated peptides using AdvanceBio Peptide Plus column. Modified sites are marked in red.

Separation of glycosylated and nonglycosylated peptides

Figure 4: MS/MS spectra of nondeamidated peptide and deamidated NTAYLQMNSLR peptide.

Figure 5: Overlay extracted compound chromatograms showing the separation of nonoxidized and oxidized DLTMSR peptide using AdvanceBio Peptide Plus column.

Figure 6: MS/MS spectra between nonoxidized (upper panel) and oxidized (bottom panel) DLTMSR peptide.

Conclusions
- The AdvanceBio Peptide Plus column delivers high peak capacity with sharp and narrow peaks using MS friendly formic and modifier mobile phase conditions.
- High resolution and high-efficiency separation of mAb peptide map provided > 95% sequence coverage.
- AdvanceBio Peptide Plus column generated well-resolved peptide peaks enabling significant improvement for peptide mapping separation.
- Precise characterization of PTMs using AdvanceBio Peptide Plus column and the Accurate Mass 6540QX AdvanceBio LC/Q-TOF.