An HS-MRM assay was developed for quantification of low abundance peptides present in a monoclonal antibody (mAb) digested to demonstrate that it has the required sensitivity and dynamic range (at least 3 orders of magnitude) for a typical HCP monitoring assay.

**INTRODUCTION**

- Analysis of low-levels (1-100 ppm) protein impurities (e.g. host cell protein-HCP) in protein biotherapeutics is a challenging assay requiring high sensitivity and a wide dynamic range.

- Massspectrometry based quantification assays for proteins typically involve protein digestion followed by selective reaction monitoring/multiple reaction monitoring (MRM)/MRM quantification of peptides using a low-resolution (m/z ~10-100) tandem quadrupole mass spectrometer. One of the limitations of this approach is the interference phenomenon observed when the peptide of interest has the “same” precursor and fragment mass (in terms of m/z values) as other co-eluting peptides present in the sample (within 1 Da).

- To avoid this phenomenon we propose an alternative mass spectrometric approach, a high selectivity (HS) MRM assay which combines ion mobility separation of peptide precursors with high-resolution (R~40,000) MS-detection of peptide fragments.

- We explored the capabilities of this approach for quantification of low abundance peptide standards spiked in a monoclonal antibody (mAb) digested and demonstrated that it has the sensitivity and dynamic range (at least 3 orders of magnitude) required for HCP analysis. All six peptide standards were detected at concentrations as low as 0.1 nM (1 femtomole loaded on a 2.1 mm ID chromatographic column) in the presence of a high-abundance peptide background (2 µg of a mAb digest loaded on-column).

**METHODS**

Sample Preparation

- A licensed mAb biosimilar (Inflectra, 10 mg/mL) was denatured with 0.04% CHAPS and 1 mM DTT, alkylated with 10 mM IAM (RT, 30 min) and digested with a mixture of Lys-C and porcine trypsin (Promega) overnight. A MassPREP digest standard containing six rabbit phosphorylase (PHO) peptides (Waters P/N 186006011) was spiked into the mAb digest at the following concentrations: 0.1, 1, 10, 100, and 1000 nM, while keeping the amount of mAb digest constant. Two MassPREP digest standards (1 µM and 100 nM) were prepared in 6 HCP-free stock solutions in 60 µL acetonitrile.

LC Conditions

- An LTQ Orbitrap Velos class system equipped with a 1CrN (charged surface) trap/LTQ linear ion trap (LTI)/100 micro-LTQ linear ion trap (LTQ) was used. The LTQ Velos was interfaced with an electrospray ion source. The source parameters were: sheath gas (N2) 8, auxiliary gas (N2) 5, spray voltage 1.5 kV, capillary temperature 275°C. The LTQ Velos was operated with a scan range of m/z 300-1800 and a resolution of 60,000 at m/z 400. The mobile phase composition was 97% solvent A (1% formic acid in ultrapure water) and 3% solvent B (1% formic acid in ultrapure acetonitrile) at a flow rate of 200 µL/min. The 1CrN (charged surface) trap/LTQ linear ion trap (LTI)/100 micro-LTQ linear ion trap (LTQ) was operated with a scan range of m/z 300-1800 and a resolution of 40,000 at m/z 400. The mobile phase composition was 97% solvent A (1% formic acid in ultrapure water) and 3% solvent B (1% formic acid in ultrapure acetonitrile) at a flow rate of 200 µL/min. The 1CrN (charged surface) trap/LTQ linear ion trap (LTI)/100 micro-LTQ linear ion trap (LTQ) was operated with a scan range of m/z 300-1800 and a resolution of 40,000 at m/z 400. The mobile phase composition was 97% solvent A (1% formic acid in ultrapure water) and 3% solvent B (1% formic acid in ultrapure acetonitrile) at a flow rate of 200 µL/min.

**RESULTS**

- All six peptide standards were detected at concentrations as low as 0.1 nM (1 femtomole loaded on a 2.1 mm ID chromatographic column) in the presence of a high-abundance peptide background (2 µg of a mAb digest loaded on-column).

- When considering the MRM of spiked-in peptides, the LOD of this assay is at the 0.5 ppm level.

- Relative standard deviations (RSD) of peak areas (n=4 replicates) were less than 15% across the entire concentration range investigated.

**CONCLUSIONS**

- The HS-MRM assay has great potential for becoming a fast, high-sensitivity monitoring assay for multiple HCPs across multiple batches of biotherapeutics.

- The HS-MRM assay holds promise for improving biopharmaceutical characterization as well as quantification of low-level protein impurities.