An HS-MRM Assay for the Quantification of Host-cell Proteins in Protein Biopharmaceuticals by Liquid Chromatography Ion Mobility QTOF Mass Spectrometry

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Abstract

The analysis of low-level (1-100 ppm) protein impurities (e.g., host-cell proteins (HCPs)) in protein biotherapeutics is a challenging assay requiring high sensitivity and a wide dynamic range. Mass spectrometry-based quantification assays for proteins typically involve protein digestion followed by the selective reaction monitoring/multiple reaction monitoring (SRM/MRM) quantification of peptides using a low-resolution (Rs ~1,000) 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 a 1-Da window). To avoid this phenomenon, we propose an alternative mass spectrometric approach, a high selectivity (HS) MRM assay that combines the ion mobility separation of peptide precursors with the high-resolution (Rs ~30,000) MS detection of peptide fragments. We explored the capabilities of this approach to quantify low-abundance peptide standards spiked in a monoclonal antibody (mAb) digest and demonstrated that it has the sensitivity and dynamic range (at least 3 orders of magnitude) typically achieved in 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). When considering the MW of rabbit phosphorylase (97.2 kDa), from which the spiked peptides were derived, the LOQ of this assay is lower than 50 ppm. Relative standard deviations (RSD) of peak areas (n = 4 replicates) were less than 15% across the entire concentration range investigated (0.1-100 nM or 1-1,000 ppm) in this study.

Video Link

The video component of this article can be found at https://www.jove.com/video/55325/

Introduction

Quantification of large biomolecules (proteins) in industrial settings is currently based on immunoassays (e.g., ELISAs), mainly due to several advantages: sensitivity, high-throughput, ease-of-use, and low cost per sample. When applied to analyze the low-abundance protein impurities (1-100 ppm of host-cell proteins (HCPs)) present in protein therapeutics, these biological assays typically provide the total HCP concentration (usually expressed in ppm or ng HCP/mg mAb), but they cannot identify and measure individual HCP contaminants. Several MS-based assays have recently been developed to complement ELISAs or to provide information that ELISAs fail to offer1,2,3,4,5,6,7,8. Because of sample complexity and the requirement to detect HCP peptides across a wide dynamic range in concentration (at least 3 orders of magnitude), multidimensional chromatographic methods tendering extensive sample fractionation have traditionally been employed to help identify low-abundance HCPs1,2,3,4,5,6,7.

A natural step following HCP identification and validation is HCP tracking (monitoring) across multiple batches of biopharmaceuticals. In this situation, single-dimension LC/MS methods have been proposed to improve sample throughput8,9. However, the accuracy and dynamic range of HCP measurements might be affected in a 1D LC/MS assay by the overwhelming presence of biopharmaceutical peptides. Compared to a multidimensional separation, the potential for signal interference19,20,21,22 is increased in a single-dimension chromatographic separation because the probability for more peptide precursors to be co-eluting is increased. The incorporation of orthogonal means for separating peptide precursors without extending the chromatographic separation time would clearly be advantageous. Travelling wave ion mobility (TWIM)10 has the capability to resolve congested MS spectra in milliseconds. Approximately 500 mobility separations can be performed during the elution of a single peptide, assuming a full chromatographic peak width of 10 s and considering that the runtime of an IM separation on the ion mobility instrument is 20 ms.
Mass spectrometric assays for protein quantification have been successfully developed over the past decade using the well-accepted selected (multiple) reaction monitoring approach (SRM/MRM method) implemented on tandem mass spectrometers. One of the limitations of this low-resolution mass spectrometric assay is the interference phenomenon observed when the peptide of interest has the “same” precursor and fragment mass as other co-eluting peptides present in the sample (within a 1-Da window). There are two ways to improve the accuracy of the SRM/MRM methods: one option involves an extra separation step at the precursor level to remove interfering precursor ions, while the other option is to increase the MS resolution of the precursor/fragment detection to avoid overlapping MS signals. The high-selectivity (HS) MRM acquisition mode described here takes advantage of both of these approaches by coupling the ion mobility separation of peptide precursors with the high-resolution (Rs ~30,000) MS detection of peptide fragments. The assay described here covers at least three orders of magnitude, which is the dynamic range typically observed in SRM/MRM proteomics experiments.

The utility of the HS-MRM assay for HCP quantification was demonstrated by monitoring the linearity of the signal produced by six peptide standards spiked at different concentrations (0.1- to 100-nM range) in a monoclonal antibody digest.

### Protocol

#### 1. Preparation of the infliximab digest (~24 h procedure)

1. Prepare fresh solutions of 50 mM ammonium bicarbonate, 1% anionic surfactant in 50 mM NH₄HCO₃, 500 mM dithiothreitol (DTT) in 50 mM NH₄HCO₃, and 500 mM iodoacetamide (IAM) in 50 mM NH₄HCO₃.
2. To 750 µL of 50 mM NH₄HCO₃ add 200 µL of infliximab mAb (10 mg/mL) and 50 µL of 1% anionic surfactant solution and denature the protein for 15 min at 60 °C in the presence of 0.05% anionic surfactant.
3. Add 40 µL of 500 mM DTT and reduce the sample for 60 min at 60 °C in the presence of 20 mM DTT.
4. Add 20 µL of 500 mM IAM and alkylate the sample for 30 min at room temperature in the dark in the presence of ~10 mM IAM.
5. Add the contents of the microcentrifuge tube to a glass vial containing 20 µg of MS sequencing-grade Lys C/trypsin and digest the sample for 3 h at 37 °C.
6. Add a second enzyme aliquot (20 µg) by transferring the digested sample to another glass vial containing 20 µg of MS sequencing-grade Lys C/Trypsin and digest the sample overnight (12-15 h) at 37 °C.
7. After the overnight proteolysis, add 5 µL of 100% formic acid (FA) and incubate the digest for 30 min at 37 °C to decompose the acid labile anionic surfactant.
8. Spin down the digest for 15 min at 4,000 x g in a centrifuge to precipitate the insoluble component of the anionic surfactant.
9. Recover ~1,000 µL of the infliximab digest and place it in an LC auto-sampler vial; the digest concentration should be ~2 mg/mL. Store the digest in a freezer at -20 °C until the LC/MS system is ready for analysis.

#### 2. Spiking of peptide standards (~30 min)

1. Thaw the infliximab digest by placing the frozen sample in the glass vial on a bench at room temperature.
2. Add 1 mL of 0.1% FA in H₂O to one rabbit phosphorylase b (PHO) digest vial to prepare 1-µM stock solutions for all six peptide standards from the PHO contained in the vial (the vial contains 1 nmol of each peptide).
3. Prepare 4 x 1-mL dilutions of the PHO stock solution to obtain solutions containing 0.1, 1, 10, and 100 nM peptides, using 0.1% FA as the dilution solvent. Prepare all dilutions in glass vials (LC auto-sampler vials) and add 100 µL of infliximab digest as the background matrix to all vials. Load 2 µg of digest background on-column with each 10-µL injection. Prepare a blank sample that contains the diluted infliximab digest (the same 1:10 dilution), with no spiked peptides.

#### 3. Setup of the LC/HDMS data acquisition method

NOTE: The workflow summarizing the steps required to set up an HS-MRM acquisition is depicted in Figure 1 and is described in detail in Sections 3-6. The acquisition of a data-independent HDMS database is required to establish the retention time of each monitored peptide, the parent m/z of the most abundant peptide ion following electrospray ionization, and the corresponding CCS (collisional cross section) derived from the ion mobility separation. In addition, the data-independent database provides information regarding the m/z of the three most abundant fragment ions for each peptide precursor. In the second step of the workflow (CE optimization), the sensitivity of the assay is increased by tuning the collision cell energy to obtain the highest ion intensity for each fragment ion. Finally, in the final step, all parameters described above are introduced to the HS-MRM method editor for each peptide monitored.

1. Perform LC/MS experiments on a quadrupole time-of-flight (QTOF) mass spectrometer coupled to an ultra-performance liquid chromatography system using a data-independent acquisition method (HDMS). The utility of the HS-MRM assay for HCP quantification was demonstrated by monitoring the linearity of the signal produced by six peptide standards spiked at different concentrations (0.1- to 100-nM range) in a monoclonal antibody digest.

2. Prepare two mobile phases containing 0.1% FA in water (Solvent A) and 0.1% FA in acetonitrile (Solvent B). Prepare two mobile phases containing 0.1% FA in water (Solvent A) and 0.1% FA in acetonitrile (Solvent B).

3. Prepare 4 x 1-mL dilutions of the PHO stock solution to obtain solutions containing 0.1, 1, 10, and 100 nM peptides, using 0.1% FA as the dilution solvent. Prepare all dilutions in glass vials (LC auto-sampler vials) and add 100 µL of infliximab digest as the background matrix to all vials. Load 2 µg of digest background on-column with each 10-µL injection. Prepare a blank sample that contains the diluted infliximab digest (the same 1:10 dilution), with no spiked peptides.

4. Use a charged surface hybrid (CSH) C18 column (2.1 x 150 mm, packed with 1.7-µm particles) for the separation of the spiked mAb digests.

5. Use a charged surface hybrid (CSH) C18 column (2.1 x 150 mm, packed with 1.7-µm particles) for the separation of the spiked mAb digests.

6. In the data acquisition software, click on "Create/Analysis Method" and choose "Generate an acquisition and processing method." Type the method name, browse to the method folder (directory), and click "Next."

7. For "Analysis Type," choose "Peptide Map (IMS)," and in the "Instrument System" tab, select the "Quaternary Solvent Manager" instrument. Edit the gradient settings to achieve peptide separation at a flow rate of 200 µL/min. Use a gradient elution from 1 to 40% Solvent B in 30min followed by a 2-min column wash and a 9-min equilibration, with a total runtime of 50 min. Introduce these experimental parameters in the LC method editor.
5. In the "Instrument System" tab, select the "Sample Manager" instrument. Set the sample temperature to 10 °C and the column temperature to 60 °C.
6. Operate the QTOF mass spectrometer in positive ion ESI Sensitivity mode, with a typical resolving power of 30,000 FWHM.

NOTE: LC/HDMS\textsuperscript{E} is a data-independent acquisition (DIA) mode that operates by rapidly collecting scans with alternating collision energies (in the collision cell) between a low energy (6 V for MS channel 1 scans) and an elevated energy (15- to 40-V ramp to produce the fragmentation spectra without precursor selection in MS channel 2).

1. Enter the default source-related MS parameters in the data acquisition software: from "My work/Instrument System," select the "Vion IMS QTof" tab, and in the "Tools" menu, enter these parameters: 3.0 kV capillary voltage, 100 °C source temperature, 100 V source offset, 50 L/h cone gas flow, and 40 V cone voltage. Set the desolvation temperature to 250 °C; the desolvation gas flow rate to 500 L/h, and the reference capillary voltage to 3.0 kV.
2. In the data acquisition software, click on "Create/Analysis Method" and choose "Generate an acquisition and processing method." Type the method name and browse to the method folder (directory) and then click "Next."
3. For "Analysis Type," choose "Peptide Map (IMS)," and in the "Instrument System" tab, select the "Vion IMS QToF" instrument. Click "Finish."

NOTE: The newly created LC/HDMS\textsuperscript{E} method opens automatically in the "Instrument Method Configuration" screen.
4. In the "Settings" tab, enter these parameters: 3.0 kV capillary voltage, 100 °C source temperature, 250 °C desolvation temperature, 50 L/h cone gas flow, and 500 L/h desolvation gas flow.
5. In the "Experiment" tab settings, select "High Definition MS\textsuperscript{E}" and choose "Analysis method runtime." In the "Scan settings" tab, enter these parameters: low mass, 100; high mass, 2,000; and scan time, 400 ms. In the "CE" tab, type "6 V" for the low-energy setting and choose a high-energy ramp from 15 to 40 V. Make sure to have "Disable data reduction" checked.
6. Infuse a solution of 50 ng/mL leucine enkephalin (LE) prepared in 50% acetonitrile with 0.1% FA at a flow rate of 10 μL/min for lock-mass calibration during data acquisition.

NOTE: The lock-mass data is acquired every 5 min using the same acquisition rate over the same mass range.
7. Analyze the 10-nM, PHO-spiked sample using the LC/HDMS\textsuperscript{E} assay described above by injecting 10 μL of sample (the amount loaded on-column is 100 fmol for each peptide standard).

### 4. Setup of the Tof-MRM method for collision energy (CE) optimization

1. From the LC/HDMS\textsuperscript{E} dataset, obtain the retention time, the precursor, and the fragment ion m/z for each PHO peptide. Retain the m/z and the charge state for the most abundant precursor ion and the corresponding three most abundant fragment ions.

NOTE: An example of low-energy and high-energy spectra typically recorded by the LC/HDMS\textsuperscript{E} assay is presented in Figure 2.
2. Use collision energy (CE) optimization in an SRM/MRM experiment to obtain the best signal for each peptide.\textsuperscript{23}
   1. In the data acquisition software, click on "Create/Analysis Method" and choose "Generate an acquisition and processing method." Type the method name, browse to the method folder (directory), and click "Next."
   2. For "Analysis Type," choose "Quantify." Pick "Quantify Assay Tof 2D chromatographic" and click "Next." In the "Instrument System" tab, select "Vion IMS QToF" and click "Finish."

NOTE: The newly created Tof-MRM method opens automatically in the "Instrument Method Configuration" screen.
3. In the "Settings" tab, enter these parameters: 3.0 kV capillary voltage, 100 °C source temperature, 250 °C desolvation temperature, 50 L/h cone gas flow, and 500 L/h desolvation gas flow.
4. In the "Experiment" tab settings, select "Function Table" and choose "One or more MS, MSMS or MRM functions." Using the down arrows, set up a "Tof-MRM" function for each peptide by entering three "transitions" (combinations of the most abundant precursor and three of its most abundant fragment ions).

NOTE: In Tof-MRM mode, the highest sensitivity is achieved using a scheduled MRM approach.
5. In the "Tof-MRM" function editor, choose one retention time window for each peptide (at least 1 min long), arranged according to the peptide elution order. In the "Tof-MRM" function editor, insert the peptide precursor m/z and the product m/z; select the quadrupole window as "Low" (4-Da window) for the peptide precursor; select a fixed scan time of 100 ms; and choose twelve different collision energy values in the range of 14-36 V, as follows: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, and 36, as shown in the example from Figure 3.

NOTE: With the Tof-MRM method described above, at least 10 data points per chromatographic peak are collected for each transition at each CE specified. To reduce the data file size, select the "Wide" option (6-Da window) for the signal of the fragment ion. Examples of the CE-optimized values are shown in Table 2.
6. Analyze the 10-nM, PHO-spiked sample using the Tof-MRM assay described above by injecting 10 μL of sample (the amount loaded on-column is 100 fmol for each peptide standard).

### 5. Setup of the final HS-MRM acquisition method for peptide quantification using ion mobility separation of peptide precursors

1. In the "Investigate" menu of the data acquisition software, display all 11 chromatographic traces generated for each CE for every peptide and integrate the peaks using the "Integrate" button from the "Processing Options/Operations" toolbar; the highest peak area will indicate the best PE.
2. Visually compare the best peak areas for the 3 fragments of each peptide and retain only the best "transition" (the fragment ion that produces the most intense signal); Table 2 displays the best "transitions" obtained from four PHO peptides.
3. Setup an HS-MRM method containing only one fragment ion per peptide. Use the CCS values of each peptide precursor from the LC/HDMS\textsuperscript{E} dataset.
   1. Modify the Tof-MRM method created in the previous section (Section 4) by selecting the "HS-MRM" function instead of the "Tof-MRM" function.
2. In the "HS-MRM" function editor, enter the following precursor-related parameters: m/z, quadrupole resolution: Low (4 Da), charge state, precursor CCS value and precursor CCS resolution: Low. For the product ion, enter its m/z, the optimum collision energy, choose a scan time of 0.4 s and select a "Wide" spectrum setting (6 Da) to include all of its isotopes. An example of the final HS-MRM acquisition method for all four peptides is presented in Figure 4.

4. Analyze all the samples using the HS-MRM method, starting with the mAb digest blank (non-spiked sample), followed by 4 replicate injections (10 µL each) of the following concentrations: 0.1, 1, 10, and 100 nM PHO peptides.

6. Creation of a processing method for the analysis of the HS-MRM dataset

1. Create a processing method for the analysis of the HS-MRM dataset.
   1. In analysis method created for the "HS-MRM" data acquisition method, click on the "Purpose" tab and choose "Manage Components."
   2. From the dropdown menu under "Experiment Type," select the "HS MS/MS / HS-MRM" option.
   3. Click on "Create/New Entry" and introduce the following parameters in the processing method editor: peptide retention time (RT), precursor charge state, precursor m/z, and precursor drift time (DT). For the fragment ion, enter its m/z and charge state and specify the "XIC" trace as the extraction mode.
   4. In the "Purpose" tab of the same "HS-MRM" acquisition method, click on "Default Amounts" and enter the following concentrations: 0.1, 1, 10, and 100 nM PHO peptides.
   5. Enter the following parameters in the "Processing/Extraction Settings": default mass tolerance, 10 ppm (for the m/z of all fragment ions) and default drift time, 5%; an example of the PHO peptide processing method is shown in Figure 5.
   6. For the calibration type, choose the linear curve fitting with $1/X^2$ weighting.

2. Process the HS-MRM data to integrate all chromatograms and to construct the calibration curves of each PHO peptide.

**Representative Results**

The individual sequences of six phosphorylase b peptide standards contained in the PHO peptide mixture are shown in Table 1, along with their retention times and their most abundant precursors observed in the HDMS<sup>E</sup> experiment. The first step in the development of a high-selectivity (HS) MRM assay is the acquisition of an HDMS<sup>E</sup> dataset to establish the elution times of each PHO peptide, along with its corresponding most abundant precursor and the three most abundant fragments. Figure 2 displays the HDMS<sup>E</sup> spectra acquired for one of the PHO peptides (Pep 6) spiked in the infliximab digest. After establishing the 3 best "transitions" (combinations of precursor and fragment masses) for each peptide, a Tof-MRM experiment is performed to find the optimum collision energy to maximize the signals generated for each peptide. The results of the CE optimization experiment are summarized in Table 2. The final HS-MRM assay (see Figure 4) retains only the best "transition" for each peptide and is used for analyzing all spiked samples. Examples of HS-MRM chromatograms generated for 4 PHO peptides across all the concentrations investigated are presented in Figure 7. Four calibration curves obtained for each peptide following the integration of the HS-MRM peaks highlighted in Figure 7 are displayed in Figure 8. In addition, the peak area relative standard deviation, calculated based on 4 replicate injections, is summarized in 4 tables shown in Table 3.

| Peptide ID | Peptide Sequence | Retention (min) | Charge states (+ 1, + 2, + 3, + 4) |
|------------|------------------|-----------------|-----------------------------------|
| Pep 1      | VLYPNDFNFEFGK    | 19.4            | 1442.6951, 721.8512, 481.5699, 361.4292 |
| Pep 2      | TCAYTNHTVPEALER | 16.0            | 1874.9065, 937.9569, 625.6404, 469.4821 |
| Pep 3      | IGEYISDLLQLRK    | 18.9            | 1678.8646, 839.9360, 560.2931, 420.4716 |
| Pep 4      | LLSYVDDEAFIR     | 21.1            | 1440.7369, 720.8721, 480.9172, 360.9397 |
| Pep 5      | LTAIGDVHNHPVVGDR | 19.7            | 1890.0080, 945.5076, 630.6742, 473.2574 |
| Pep 6      | VFADYEEYVK       | 17.7            | 1262.5939, 631.8006, 421.5362, 316.4039 |

*Table 1. PHO peptide standards contained in the Mass PREP mix spiked in the infliximab digest. Peptide retention times and their most abundant precursors (highlighted in bold) are displayed in tabular format.*
Table 2. Results of the ToF-MRM optimization experiment: the three most abundant fragments of each PHO peptide quantified in this study are indicated, along with the corresponding optimized collision energy.

| Peptide ID | Sequence          | Retention (min) | Peptide precursor | m/z & charge | Optimum Drift time (ms) | CE (V) |
|------------|-------------------|-----------------|-------------------|--------------|-------------------------|--------|
| Pep 2      | TCAYTNHTVLPEALER  | 16.0            | 625.6404 (+3)     | 6.2          | 714.3781 (+1)           | 827.4621 (+1) |
| Pep 4      | LLSYVDDEAFIR      | 21.1            | 720.8721 (+2)     | 7.5          | 865.4050 (+1)           | 964.4734 (+1) |
| Pep 5      | LITAIGDVNHPVVGDR  | 19.7            | 630.6742 (+3)     | 6.3          | 642.3570 (+1)           | 714.5688 (+1) |
| Pep 6      | VFADYEEYVK        | 17.7            | 631.8006 (+2)     | 7.0          | 830.3931 (+1)           | 832.4236 (+2) |

Table 3. Table containing the peak areas of HS-MRM chromatograms recorded for 4 PHO peptides (Pep 2, 4, 5, and 6) for each LC/MS injection (16 LC/MS runs and 4 concentrations tested).

The relative standard deviation was better than 15% for all peptides over the entire concentration range investigated.
Figure 1. Workflow diagram summarizing the three steps required for setting up an HS-MRM acquisition method. Please click here to view a larger version of this figure.

Figure 2. Example of HDMSE data:
(A) Low-energy spectrum showing the Pep 6 precursor ion. (B) High-energy fragmentation spectrum of the same peptide, displaying the top 3 most abundant fragment ions (circled) selected for Tof-MRM collision energy optimization. Please click here to view a larger version of this figure.
Figure 3. Parameters used for setting up a Tof-MRM optimization experiment. For each transition, eleven collision energies (in the range of 16 to 36 V) were tested. Please click here to view a larger version of this figure.

Figure 4. Example of the final HS-MRM method. Several parameters are required for each "transition," including the peptide precursor m/z, its charge state and ion mobility drift time, the m/z of the most abundant fragment ion, the optimum collision energy, and the MS acquisition time. Please click here to view a larger version of this figure.

Figure 5. Settings used by the processing method for analyzing the HS-MRM dataset. Each peptide is monitored by a single "transition" described by the peptide precursor m/z, charge state, and expected retention time, along with the m/z of the most abundant fragment and its charge state. Please click here to view a larger version of this figure.
Figure 6. Diagram of the ion mobility mass spectrometer.
In the HS-MRM acquisition mode, the precursors of the peptide that is being quantified are separated from other co-eluting (interfering) peptide precursors in the ion mobility cell, isolated by the quadrupole, and fragmented with a fixed collision energy in the collision cell. The signal produced by the peptide fragment ions is enhanced by adjusting the pusher frequency, and peptide quantification is performed using the high-MS-resolution (>30,000) signals produced by the most intense fragment ion of each peptide. Please click here to view a larger version of this figure.

Figure 7. HS-MRM chromatograms recorded for 4 PHO peptides at 4 different concentrations spanning 3 orders of magnitude (0.1, 1, 10, and 100 nM).
(A) Pep 2 chromatograms, (B) Pep 4 chromatograms, (C) Pep 5 chromatograms, and (D) Pep 6 chromatograms. Please click here to view a larger version of this figure.
Figure 8. Calibration curves for 4 PHO peptides across 4 different concentrations (0.1, 1, 10, and 100 nM). The tables under each curve display the individual peak areas (Y-values) recorded for each injection, while the second column from each table shows the percent deviation from the expected linear response. (A) Pep 2 calibration, (B) Pep 4 calibration, (C) Pep 5 calibration, and (D) Pep 6 calibration. Please click here to view a larger version of this figure.

Discussion

High-resolution (Rs >20,000) mass spectrometry is routinely used for the structural characterization of therapeutic proteins on a variety of instrument platforms. In contrast, MS-based protein quantification is typically performed by SRM/MRM on low-resolution (Rs ~1,000) tandem quadrupole mass spectrometers using signature peptides generated by the enzymatic cleavage of proteins. As single-dimension chromatographic separations cannot fully resolve complex peptide mixtures produced by enzymatic digestion, peptide co-elution is a common occurrence, even in the case of a single-protein digest. For very complex protein digests (e.g., for the quantification of 1-100 ppms of HCPs in the presence of a peptide-rich background produced by the therapeutic protein), the sensitivity, accuracy, or linearity of the SRM/MRM assay can be affected by interference.

The SRM/MRM assays have unidimensional selectivity, relying only on a "unique" combination of precursor/fragment masses. For this reason, these assays fail in situations when the peptide background changes unexpectedly (e.g., for biopharmaceutical samples obtained from different purification procedures). To overcome these limitations, we propose here a high-selectivity (HS) MRM assay implemented on an ion mobility-enabled high-resolution quadrupole time-of-flight (QTOF) hybrid mass spectrometer (for the instrument diagram, see Figure 6).

The instrument separates the precursors of the peptide of interest from other co-eluting (interfering) peptide precursors in the ion mobility cell, isolates the full isotopic envelope of the precursor in the quadrupole, and fragments it with a fixed CE in the collision cell. The signal produced by its most abundant peptide fragment is further enhanced by adjusting the pusher frequency (Target Enhancement), which selectively pushes mass regions of interest into the flight tube, rather than all ions, as with a full scan. Peptide quantification is performed using the high-MS-resolution (Rs ~30,000) signals produced by this fragment ion. Compared with the SRM/MRM assays, the HS-MRM assay offers two additional levels of selectivity: one is provided by the precursor-level ion mobility separation, while the second is offered by the increased mass resolution of the TOF analyzer. The results brought by these selectivity improvements are visible in the HS-MRM chromatograms displayed in Figure 7, which are free of interferences across three orders of magnitude.

Unlike the SRM/MRM assays, there are several parameters that can be adjusted to optimize the HS-MRM assays: the RT window around the peptide precursor (typically set at 0.2 min), the quadrupole isolation window (4 Da), the drift-time window surrounding the precursor (± FWHM of the precursor peak from the corresponding ion mobilogram), and the MS resolution of the fragment ion (20,000-40,000). The HS-MRM assays are very sensitive: the lowest detected amount for each PHO peptides is 1 femtomole on-column (or 0.1 nM in terms of peptide concentration).
When considering peptide MW (see Table 1 for accurate MWs), the amount detected on-column is on the order of 1-2 pg, while the column is loaded with a significantly higher amount (2 µg) of background peptides from the mAb digest.

By considering the molecular weight of the full-length PHO protein (97.2 kDa) from which the spiked peptides were derived, the assay is able to detect 50 ppm of a protein impurity in the presence of high-abundance background ions. Lower limits of detection (5-10 ppm) are achievable for lower molecular weight HCPs (10-20 kDa). The assay covers three orders of magnitude (as shown in the calibration curves from Figure 8), which means that it can measure HCPs in the 1-1,000 ppm range. Also, the reproducibility of the HS-MRM assays, illustrated in Table 3, matches very well with the reproducibility of small-molecule SRM/MRM assays, with peak area RSDs better than 15%.

We explored the capabilities of a novel assay for the quantification of spiked peptide standards in a monoclonal antibody (mAb) digest and demonstrated its sensitivity and utility to cover the wide dynamic range (at least three orders of magnitude) typically encountered in 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). By incorporating both targeted HRMS and ion mobility precursor separation, the HS-MRM assay has great potential for becoming a fast, high-throughput monitoring assay for multiple HCPs across multiple batches of biopharmaceuticals.

**Disclosures**

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