

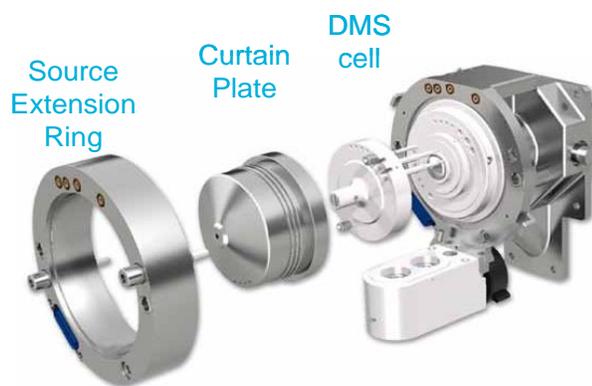
# Differential Mobility Separation Mass Spectrometry for Quantitation of Large Peptides in Biological Matrices

## Using SelexION® Technology Coupled with QTRAP® 5500 System

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The ability to quantify large peptides in biological matrix with adequate selectivity and sensitivity depends on several factors: 1) peptides are multiply charged, 2) peptides have varied fragmentation efficiency due size and structure, and 3) different matrices can cause varied background signal and interferences. Tryptic digestion can reduce the size of the peptide and improve fragmentation but introduces an additional step, which is undesirable in a high-throughput environment.

Detection limits for MRM acquisition can be heavily affected by the fragmentation characteristics of the peptide. Sensitivity is reduced if the peptide does not fragment or if it fragments too extensively (spreading ion current across many product ions). Cyclic peptides are especially difficult as they tend to have poor MS/MS efficiency. For these cases, high sensitivity can sometimes be achieved through monitoring the intact peptide in Single Ion Monitoring (SIM) mode. Methods based on SIM are not routinely utilized because they can have reduced selectivity and high background levels.



**Figure 1. High Selectivity Quantification using SelexION Technology on the QTRAP® 5500 System.** The SelexION Technology<sup>1</sup> is an easy to install differential mobility device that is available on a QTRAP® 5500 or 6500 system and is used to provide additional selectivity to any quantitative experiment. An asymmetric waveform alternates between high field and low field and ions will have a net drift towards one of the plates based on their high and low field mobility difference. A compensation voltage (CoV) is applied as the filtering voltage, which is tuned for the compound of interest. Other co-eluting isobaric or non-isobaric species that tune with different compensation voltages will be filtered away.



Differential mobility separation (DMS) mass spectrometry adds an additional level of selectivity to LC/MS/MS providing gas phase separation of isobaric species and co-eluting interferences to reduce background noise. Here, the utility of a SIM workflow combined with DMS for specificity was investigated for quantifying large therapeutic peptides in a protein precipitated plasma matrix.

## SelexION® Technology for Large Peptide Quantitation

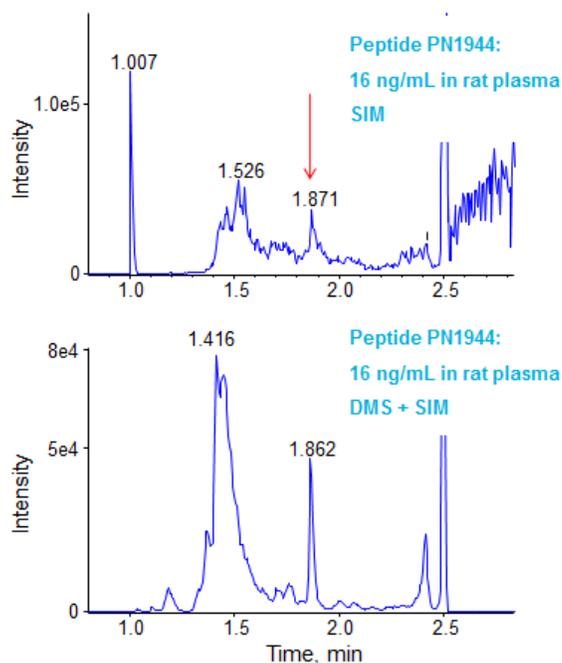
- SelexION Technology is a planar differential mobility separation device (DMS) that separates peptides based on difference in their chemical and structural properties.<sup>1</sup>
- SelexION Technology adds an orthogonal level of separation and selectivity prior to the instrument orifice (Figure 1).<sup>1</sup>
- SelexION Technology is compatible with fast cycle times required for monitoring multiple MRM transitions combined with narrow HPLC peaks.

## Methods

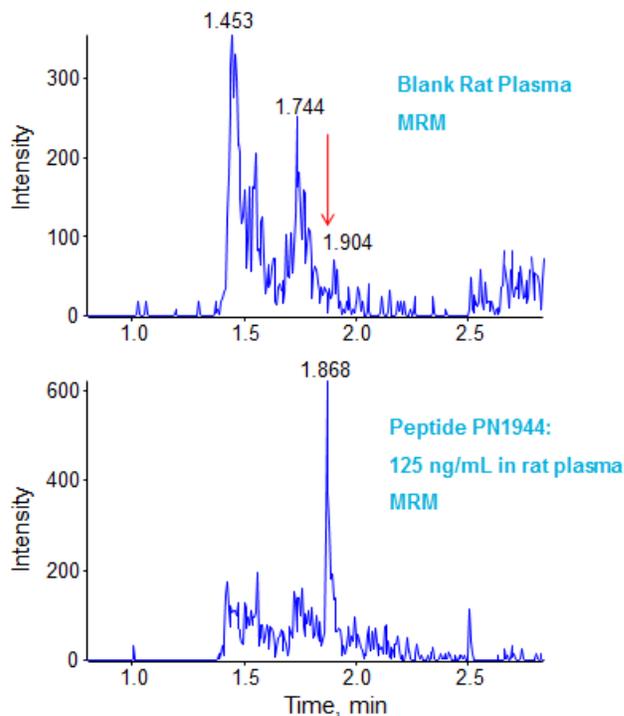
**Sample Preparation:** To extract the peptides from plasma, an aliquot of 100  $\mu\text{L}$  of each standard was mixed with 400  $\mu\text{L}$  of 5% formic acid in acetonitrile (with 150 ng/mL peptide). This was vortexed for 10 mins, then centrifuged at room temperature for 10 mins (21000 x g). The supernatant was transferred (425  $\mu\text{L}$ ) to a clean 96 well plate and evaporated to dryness at 50  $^{\circ}\text{C}$ . To reconstitute the sample, 30  $\mu\text{L}$  of acetonitrile was added and the sample was placed on the shaker for 15 mins. An additional 20  $\mu\text{L}$  of 2.5% formic acid in water was added and the sample was shaken for an additional 15 mins.

**HPLC Conditions:** The samples were analyzed using the Acquity UPLC system (Waters). The peptides were directly loaded onto a 2.1 x 50 mm column (Thermo Hypersil Gold, 1.9  $\mu\text{m}$ ). An elution gradient of 5-95% acetonitrile (0.1% formic acid) over 3 min was used with a flow rate of 800  $\mu\text{L}/\text{min}$ .

**MS/MS Conditions:** A QTRAP<sup>®</sup> 5500 system equipped with SelexION<sup>®</sup> Technology and a Turbo V<sup>™</sup> source was used. DMS parameters were optimized for each peptide of interest to maximize signal intensity, the following parameters were used (DMS temp  $\rightarrow$  low, Modifier  $\rightarrow$  none, Separation Voltage  $\rightarrow$  3800, Compensation Voltage  $\rightarrow$  15.5, DMO Offset  $\rightarrow$  -5.5, DMS Resolution  $\rightarrow$  off). Analysis is done using a single ion monitoring (SIM) acquisition strategy.



**Figure 3. Improved Signal/Noise using DMS.** Analysis of the peptide in SIM mode (monitoring precursor  $m/z$  in Q1 and Q3 with no collision energy for higher sensitivity) can yield poor selectivity. This is evident in the first sample (top panel) where the peptide signal at 16 ng/mL is largely obscured by background noise. Analysis of the same sample with DMS (bottom trace) shows greatly improved signal-to-noise for the target peptide.



**Figure 2. Multiple Reaction Monitoring (MRM) of Peptide PN1944 using QTRAP<sup>®</sup> 5500 System.** Multiple reaction monitoring (MRM) provides a high degree of selectivity for detecting peptides in complex matrices. However, with some large peptides, the nature of the fragmentation limits ultimate sensitivity in MRM mode, either because the peptide does not fragment well or fragments into many product ions. The LLOQ achieved in rat plasma using this approach was 125 ng/mL and was not sufficient to achieve the required detection limits for the experiment.

Single ion monitoring (SIM) is a variation of an MRM experiment where the parent ion is monitored in Q1 and Q3 without fragmentation in Q2. For large peptides, SIM methods can provide larger signal without the need for extensive compound optimization.

**Data Processing:** Results were analyzed using Analyst<sup>®</sup> Software 1.5 Quantification tools.

## High-Throughput Peptide Quantitation

It is undesirable in a high-throughput setting to optimize the extraction method and chromatographic conditions for every test compound being evaluated. A simple extraction method along with high throughput chromatographic conditions were developed and optimized for selectivity and sensitivity for a diverse set of peptides (~30 amino acids, 4 kDa) in multiple lots of rat plasma for analysis by LC-MS/MS.

In addition, generic MS acquisition methods are attractive but often limited by background noise and deliver reduced sensitivity, limiting the ability to generate a suitable pharmacokinetic (PK) profile for a peptide. MRM methods are

the gold standard for high sensitivity quantification but sometimes on large peptides, the fragmentation properties of the peptide can limit the ultimate sensitivity achieved (Figure 2).

Monitoring the intact form of the peptide using SIM can provide better sensitivity (Figure 3, top) but have much higher background levels which can also impact detection limits.

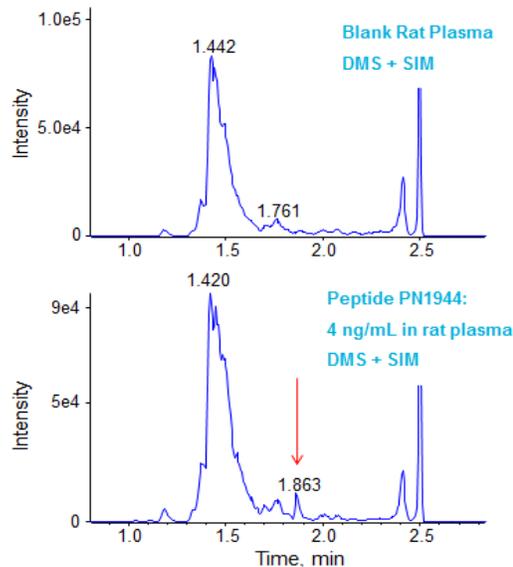
Combining the higher intensity MS method with differential mobility separation can provide the added selectivity needed to improve detection limits. Figure 3 (bottom) shows the improvement in S/N observed vs using SIM alone, a significant decrease in background was observed.

## Assay Performance using DMS with SIM

The lower limit of quantification (LLOQ) for the DMS + SIM acquisition strategy was 4 ng/mL (Figure 4, top) while conventional MRM was limited to 125 ng/mL (Figure 2), an ~30 fold improvement in detection limit. The statistics for the peaks observed at 4 ng/mL are shown in the table (Figure 4), good reproducibility and accuracy was achieved.

## Conclusions

- Differential Mobility Separation (DMS) using SelexION® Technology provides an orthogonal level of selectivity by separating components based on their chemical properties and ion mobility.
- Matrix interferences and background noise can be significantly reduced to improve selectivity and thus sensitivity
- An LLOQ of 4 ng/mL for a 30 amino acid peptide (~4 kDa) was achieved in protein precipitated rat plasma, an ~30x improvement over the MRM analysis strategy
- The DMS + SIM acquisition strategy combined with a high-throughput sample preparation and LC workflow achieves the desired lower limit of quantitation (LLOQ) for PK studies of large therapeutic peptides.
- DMS can also be used to improve sensitivity and selectivity in MRM based assays when high background or interferences are observed



Theoretical Concentration (ng/mL)	Replicate	Sample	Measured Concentration (ng/mL)	Relative Error (%)	Average Relative Error (%)	CV (%)
4	1	1	3.5	-12.5	2.2	13.5
		2	4.2	5		
		3	4.84	21		
		4	5.08	27		
	2	1	4.17	4.3		
		2	4.25	6.3		
		3	4.35	8.7		
		4	4.3	7.5		
	3	1	3.86	-3.5		
		2	3.15	-21.3		
		3	3.61	-9.8		
		4	3.75	-6.3		

**Figure 4. Lower Limit of Quantitation for a 4 kDa Peptide in Plasma using the DMS-SIM High-Throughput Quantification Workflow.** (Top) The chromatograms for the blank plasma and the LLOQ using the DMS + SIM approach are shown. The statistics for the measurements at the LLOQ are shown (bottom). Using the DMS+SIM strategy provides a ~30 fold improvement in detection limit over the conventional MRM approach.

## References

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2. Multiple Mass Spectrometric Strategies for High Selectivity Quantification of Proteins and Peptides: Solve the Most Challenging Quantitative Problems with QTRAP® 5500 System. SCIEX Technical Note RUO-MKT-02-3249-A.

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