Sensitive Quantitation of Cyclic Peptide by Differential Mobility Separation Analysis

Featuring the SCIEX Triple Quad™/QTRAP® 6500+ LC-MS/MS System and SelexION® Differential Mobility Separation (DMS)

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Introduction

Large molecule bioanalysis has become a critical portion in biotherapeutic drug discovery and development. Multiple reaction monitoring (MRM) offered by the triple quadrupole and QTRAP® mass spectrometers is well known as the leading technology for large molecule quantification. Its sensitivity, specificity and speed allow scientists to quantify multiple target analytes at low levels in complex biological matrices. However, MRM analysis has its limitation when analyzing 1) complex matrix samples with significant isobaric interferences; 2) analytes, for example, cyclic peptides, with low fragmentation efficiency or lack of abundant fragment ions. In these scenarios, relying on MRM alone may be insufficient to provide desired sensitivity and/or selectivity. An orthogonal separation technology is required to improve quantification performance.

Differential Mobility Separation (DMS) using SelexION® Technology adds an additional level of selectivity providing gas phase separation of isobaric species based on their chemical properties and ion mobility (Figure 1). DMS applies high and low energy fields from an asymmetric waveform between two planes; ions from the peptide sample travel through the fields based on their size, shape and charge and differentiate based on their migration time (Figure 2). In addition to adding an additional level of ion separation prior to the instrument orifice, SelexION® Technology is also compatible with fast cycle times required for quantification workflows, including MRM or selective ion monitoring (SIM). Herein, a workflow combining DMS and SIM on SCIEX Triple Quad™/QTRAP® 6500+ LC-MS/MS System is demonstrated for cyclic peptide quantification in plasma.

Key Features of SelexION® DMS

- Adding an additional level of gas phase separation of isobaric species based on their chemical properties and ion mobility
- Highly compatible with fast cycle times required for quantification workflows
- Robust, reproducible, and stable for use in regulated bioanalysis.
- Easy to maintain, and can be Installed or removed in minutes with no need to break vacuum on the mass spectrometer.

Figure 1. High Selectivity Quantification using SelexION Technology on the QTRAP® System. The SelexION Technology is an easy to install differential mobility device used to provide additional selectivity to any quantitative experiment.

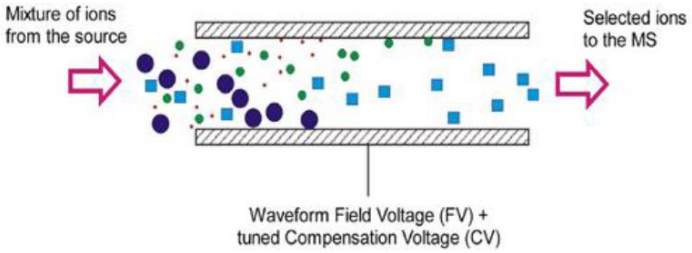

Figure 2. Principle of differential mobility separation (DMS). As the mixture of ions enters the drift tube, they are separated according to differences in each individual molecule's size, shape, and charge. An optimized combination of separation voltage (SV or waveform field voltage, FV) and compensation voltage (CV or COV) separates the analyte from background ions.

Methods

Sample Preparation: Plasma samples was deproteinized by adding acetonitrile and centrifuging at 15,000rpm for 10min. Oxytocin (Figure 3) standard was spiked into the supernatant to prepare calibration curve samples. After serial dilution, the oxytocin concentrations in samples were 0, 50, 100, 500, 1000, 5000, 10000, 50000 and 100000 pg/mL.

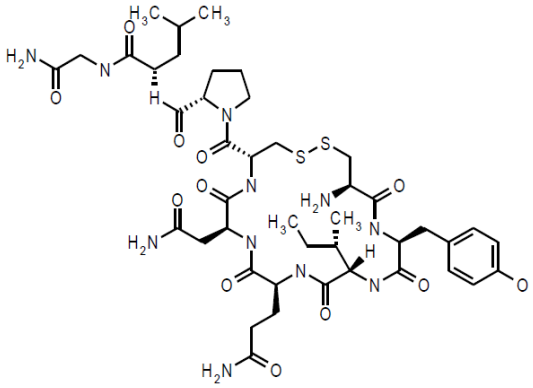

Figure 3. The structure of oxytocin.

LC-MS conditions: Each sample was analyzed in triplicate by a SCIEX QTRAP® 6500+ LC-MS/MS system with DMS activated, coupled with a Shimadzu 20ADXR HPLC system. Table 1 describes the chromatographic conditions. The mass spectrometer was operated in Selected Ion Monitoring (SIM) mode, in which only the precursor ion of the target analyte is transmitted to detector and used for quantitation. The SIM transition was selected as the singly charged molecular ions [M+H]+ at m/z 1007.6. Table 2 describes the MS conditions.

Table 1: Chromatographic conditions.

Parameters	Value
Stationary phase	*Restek Biphenyl column, 100 x 2.1 mm, 2.7µm*
Mobile phase A	*0.1% formic acid in water*
Mobile phase B	*0.1% formic acid in acetonitrile*
Flow rate	*400 µL/min*
Column temperature	*40 ºC*
Gradient	*linear separation gradient*
Run time	*15 min*
Injection volume	*1 µL*

Table 2: MS conditions for DMS-SIM acquisition mode.

Source/Gas Parameter	Value	DMS Parameter	Value
Curtain gas:	30	Modifier:	*2-propanol*
Ion source gas 1:	70	*Separation voltage:*	*3500*
Ion source gas 2:	*30*	*Compensation voltage:*	*0.6*
Source temperature:	*600*		
Ion spray voltage:	*5500*		
SIM transition		**Description**	
1007.6		$[M+H]^+$	

DMS-SIM method development

To analyze compounds with low CID fragmentation efficiency, e.g. cyclic peptides, scientists usually implement different quantitation workflows beside traditional MRM analysis. SIM is an ideal scan mode for cyclic peptides to improve quantitation sensitivity by only monitoring precursor ions. At the same time, combining DMS with SIM analysis successfully overcome the selectivity limitation of SIM mode and has been identified as effective strategy for cyclic peptide quantification in biological matrices.

To identify the most sensitive SIM transition for oxytocin quantification, the Q1 MS scan was performed with DMS off and on, respectively. In the DMS off condition (Figure 4a), the doubly charged ion $[M+2H]^{2+}$ at m/z 504.4 is the dominant precursor ion with significantly higher abundance than the singly charge ion $[M+H]^+$ at m/z 1007.6. However, the $[M+2H]^{2+}$ ion could not be used as the appropriate precursor ion for oxytocin quantification, due to the high background interference around m/z 504.4, especially in complex biological matrix. With DMS on and 2-propanol as DMS modifier (Figure 4b), the singly charge ion $[M+H]^+$ at m/z 1007.6 became the dominant ionization form, with ~20 folds S/N improvement comparing with DMS off mode, therefore was identified as the ideal precursor ion for oxytocin quantification at SIM mode.

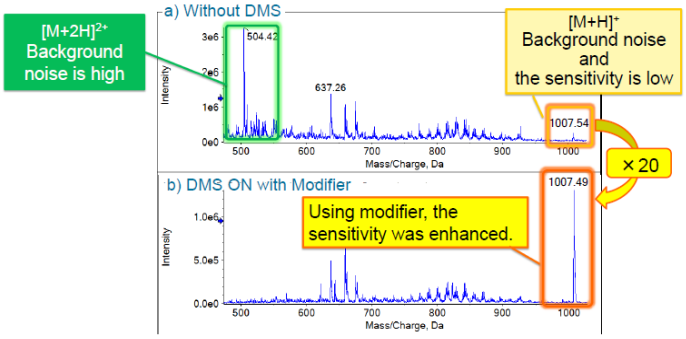

Figure 4. Q1 MS scan spectra for oxytocin with DMS off (a) and DMS on (b).

To further verify the SIM transition selection, the SIM acquisitions for $[M+2H]^{2+}$ and $[M+H]^{+}$ with DMS on or off were performed. As shown in Figure 5, the S/N for SIM acquisition at m/z 1007.6 with DMS on (Figure 5c) was significantly higher (~25 folds) than the S/Ns of SIM at m/z 1007.6 with DMS off (Figure 5a) or at m/z 504.4 with DMS off (Figure 5b). Therefore, m/z 1007.6 with DMS on was selected for oxytocin quantification in the following experiment.

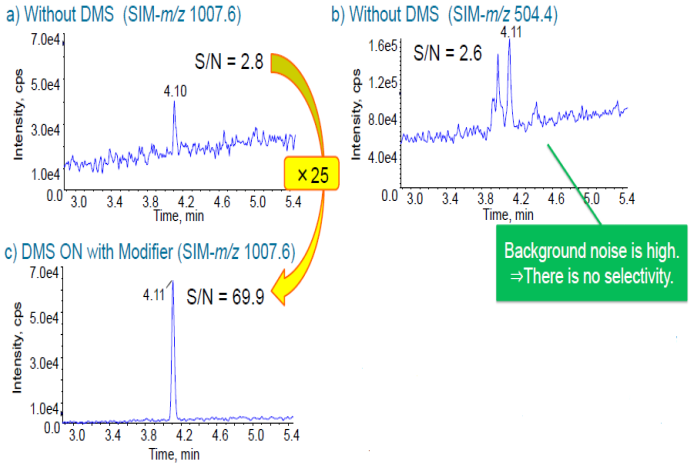

Figure 5. SIM spectra for oxytocin at m/z 1007.6 with DMS off (a), at m/z 504.4 with DMS off (b) and at m/z 1007.6 with DMS on (c).

Quantification results

With the optimized LC-MS conditions, the presented DMS-SIM assay achieved an LLOQ of 50 pg/mL for oxytocin quantification in plasma, with 1 µL on-column injection (Figure 6). As summarized in Table 3, the inter- and intra-day quantification reproducibility and accuracy were evaluated. The assay accuracy is 93-107%, and CV%s are below 5% for all tested samples during the two-day data acquisition. The calibration curves covered 3.5 orders of magnitude (50-100000 pg/mL) (Figure 7) and displayed a regression coefficient (r) of 0.998 using a weighting of $1/x^2$.

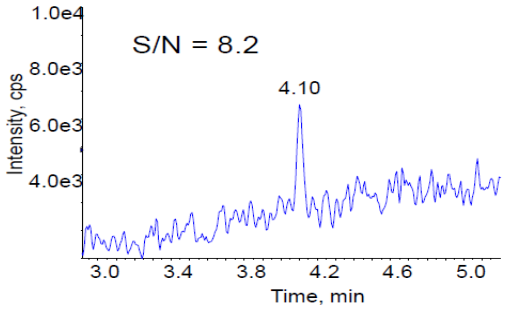

Figure 6. Extract ion chromatogram (XIC) of oxytocin at 50 pg/mL in plasma.

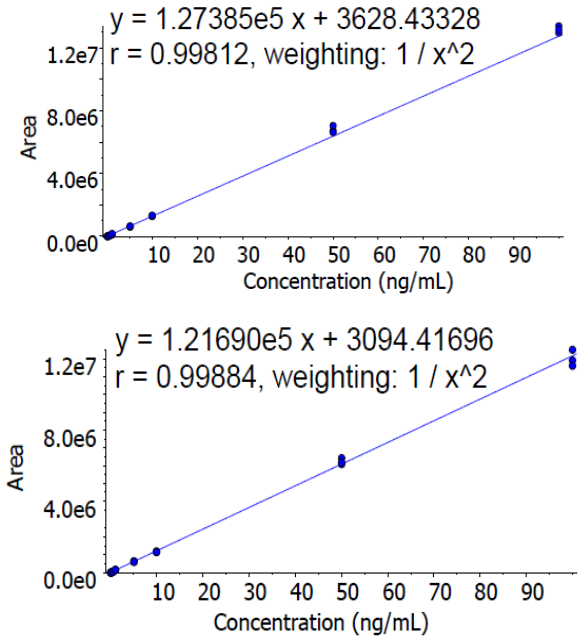

Figure 7. Calibration curves for oxytocin quantification in plasma (50-100000 pg/mL), generated in 2 days (top: Day 1; bottom: Day 2).

Table 3: Quantitation summary.

Actual Conc. (pg/mL)	Calculated Conc. (pg/mL)	Accuracy (%)	CV (%)
50	52	103.6	3.8
100	94	94.2	0.5
500	466	93.1	1.9
1000	992	99.2	4.2
5000	4881	97.6	2.6
10000	10217	102.2	2.4
50000	53322	106.6	3.2
100000	103439	103.4	1.6

Conclusion

A DMS-SIM workflow for cyclic peptide quantification on SCIEX 6500+ QTRAP® or Triple Quad™ 6500+ mass spectrometer was demonstrated. The utilization of DMS with modifier significantly reduced the background interference in SIM acquisition mode and changed the precursor ion charge state distribution. Oxytocin was quantified at 50 pg/mL level with high accuracy, reproducibility and 3.5 orders of dynamic range, showing a 25 folds sensitivity improvement over the DMS off mode. This workflow can be widely applied to quantify biotherapeutic molecules with low fragmentation efficiency in biological matrices as an alternative solution from traditional MRM analysis. It is worth noting that DMS can also be combined with high resolution accurate mass spectrometry (HRAMS) technique to quantify the similar types of biomolecules.

Document number: RUO-MKT-02-9353-A

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