Rapid Quantitation of Substance P in Plasma Using Differential Mobility Spectrometry and Micro flow Liquid Chromatography

An exceptionally selective and sensitive strategy for high throughput peptide quantification is presented by coupling DMS (Differential Mobility Spectrometry) technology with µLC/MS/MS analysis.

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Key challenges of multiple-reaction monitoring for peptide quantification

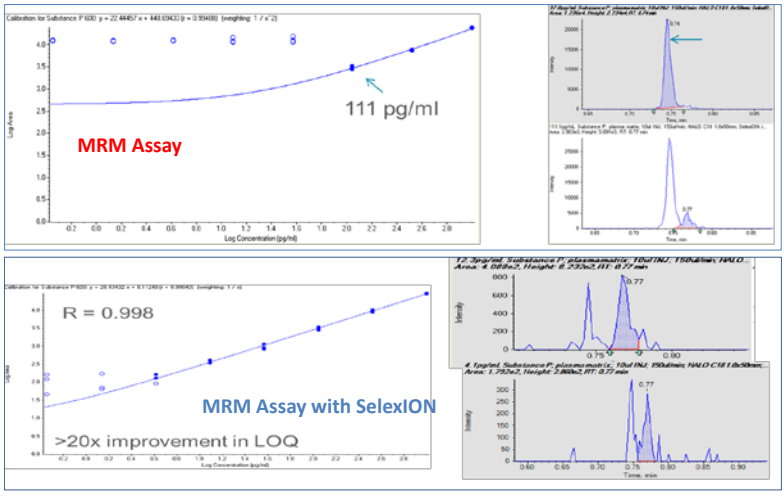
- **Impaired sensitivity in complex matrices** – Very low-level peptide detection (sub-pg/mL) can be suppressed by high background and competing ions in biological samples.
- **Lack of throughput** – Complex biological matrices hamper data resolution and require sophisticated sample preparation and/or advanced instrumentation.
- **Limited quantitation range** – Poor MS/MS sensitivity combined with often poor selectivity can compromise the ability to achieve lower limits of quantitation (LLOQ) at relevant physiological concentrations.

Key benefits of microflow based MRM DMS peptide analysis

- **Maximized sensitivity** – Enhanced ionization efficiencies promoted by lower flow rates and highly efficient µUHPLC chromatography contribute to improved limits of detection.
- **Increased selectivity** – Utilization of DMS allows reduction of background noise and isobaric interferences which enhances S/N ratios and reproducibility.
- **Accelerated throughput** – Higher selectivity and instant microfluidic gradient delivery permits less labor intensive sample preparation and shorter analysis times.

Key features of the eksigent® Ekspert™ microLC 200 and SelexION™ enabled AB SCIEX QTRAP® 6500 system

- **Dedicated µUHPLC system** – Designed from the ground up specifically for precise microfluidic flow control and instant gradient delivery.
- **Unique Differential Ion Mobility technology** – SelexION separates isobaric species along with single and multiple charge state interferences to reduce background levels and achieve better selectivity and LLOQs while retaining compatibility with commonly used UHPLC MRM speeds.



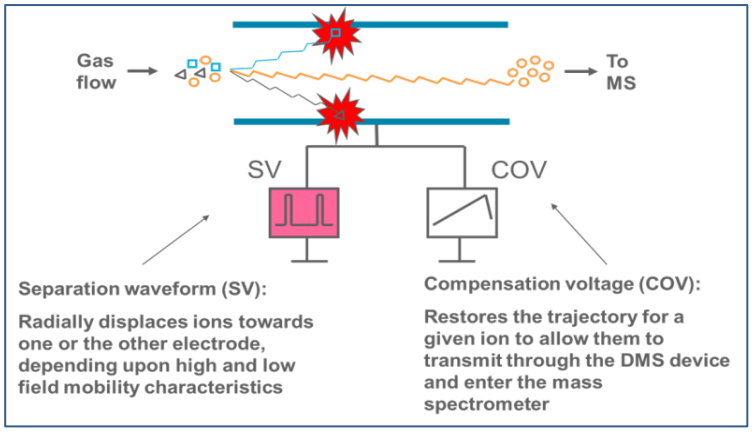
Shown above: A) Instrumentation deployed for the development of an MRM based assay for the quantification of substance P. SelexION enabled AB Sciex QTRAP 6500 System and eksigent Ekspert microLC 200 system. B) Showing increase in LOQ and calibration range and chromatograms with and without interference

Introduction

Substance P is an 11 amino acid neuropeptide that is known to modulate neural responses primarily associated with pain perception. Recent studies have shown that this peptide also plays a significant role in regulating the immune system, and that its increased production is part of the pathology of several autoimmune/inflammatory disorders including Inflammatory Bowel Disease and Rheumatoid Arthritis. Consequently, there is significant interest in analytical strategies that enable detection of Substance P at physiologically relevant concentrations. Here we describe a fast and robust method to detect and quantitate Substance P in protein precipitated plasma. We demonstrate that sub-femtomole limits of quantitation (LOQs) are obtained by combining traditional Multiple-Reaction Monitoring with micro-flow liquid chromatography and Differential Mobility Spectrometry (DMS). One of the main challenges of peptide quantitation using MRM based LC/MS strategies in plasma and other complex matrices has been interference from isobaric, non-target transitions. This results in a significant burden on the chromatographic separation to resolve targets from interfering species, leading to longer run times and increased method development. While ion mobility has previously been explored as a means to improve the selectivity of these assays, it does not always result in an improvement in LOQ. In this particular case, a rapid method for the quantitation of substance P was developed with a run time of less than 2 minutes; however the quality of quantification was compromised by an isobaric interference peak. By combining the sensitivity afforded by microflow liquid chromatography with the selectivity of DMS, a greater than 10 fold improvement in LOQ was observed enabling accurate quantitation at relevant low pg/mL concentrations.

Materials and Methods

Substance P (Sigma-Aldrich) was spiked into protein precipitated human plasma (1:1 to achieve final concentrations of 450 fg/mL – 1 ng/mL. 10 µL of each dilution was injected onto a 1.0 x 50mm HALO C18 column (eksigent®) and separated using an eksigent Ekspert microLC 200 system (Figure 1). Separation was achieved with a one minute linear gradient (5-90% B) delivered at 150 µL/minute. Mobile Phase A: Water, 0.1% formic acid and Mobile Phase B: Acetonitrile, 0.1% formic acid. The eluting peptide was analyzed using Multiple Reaction Monitoring (MRM) on a SelexION enabled AB Sciex QTRAP 6500. A 65µm ID electrode was used for electrospray ionization in order to prevent extra column band broadening. MRM transitions for Substance P (Figure 2) and DMS parameters (Figure 4) were optimized by infusion of Substance P.



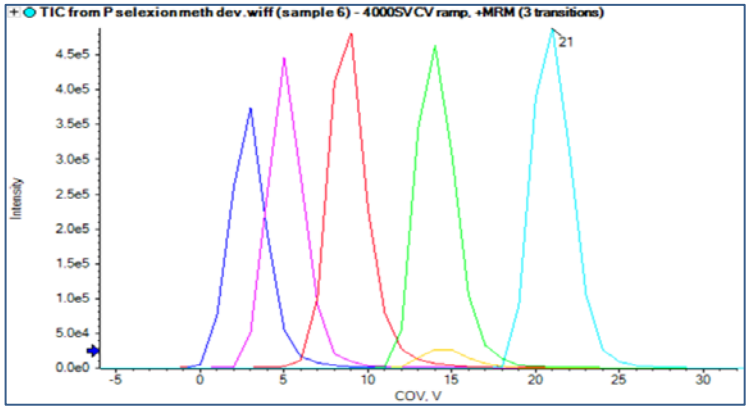
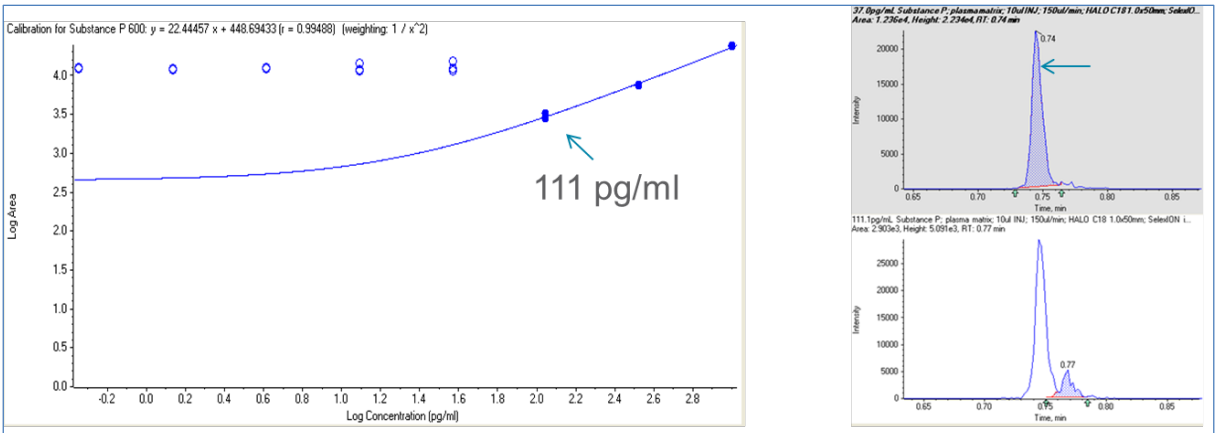


Figure 4: Optimization of SV and CoV for Substance P. Effect of SV and CoV on ion trajectory within DMS cell, each peak corresponds to a different SV, maximum mobility was observed at an SV = 4000 V.

Results

Three transitions for Substance P were optimized using infusion (Figure 2). The optimized transitions were utilized to build a 2 minute MRM method that was used to analyze a standard curve ranging 450fg/mL – 1 ng/mL concentrations of Substance P (Figure 3). The LOQ was determined to be 111pg/mL although upon further examination of the data, it was observed that the quantitative result was compromised by an interfering peak that precluded accurate quantitation below 111pg/mL (Figure 4).



Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	Substance P 600	0.45	0 of 3	N/A	N/A	N/A	N/A
2	Substance P 600	1.37	0 of 3	N/A	N/A	N/A	N/A
3	Substance P 600	4.10	0 of 3	N/A	N/A	N/A	N/A
4	Substance P 600	12.30	0 of 3	N/A	N/A	N/A	N/A
5	Substance P 600	37.00	0 of 3	N/A	N/A	N/A	N/A
6	Substance P 600	111.10	3 of 3	1.125e2	1.151e1	10.24	101.24
7	Substance P 600	333.30	3 of 3	3.167e2	5.462e0	1.72	95.03
8	Substance P 600	1000.00	3 of 3	1.037e3	2.572e1	2.48	103.73

Figure 3: A) Log/Log plot of peak area versus actual concentration. B) Peak integration pane showing the effect of interference on quantitation. C) Accuracy statistics table for the quantitation experiment.

It has been demonstrated that differential ion mobility with SelexION™ can be employed for the separation of analytes by exploiting differences in their mobility within alternating electric fields. As analyte ions move through the DMS cell, they are drawn toward two planar electrodes as a result of the applied Separation Voltage (SV). The ion of interest can then be steered back by applying a Compensation Voltage (CoV), which is often specific to an analyte ion. This mechanism can be used to filter out interferences (Figure 4). To determine whether DMS implemented using SelexION could be utilized to remove the interference in the Substance P MRM assay we used an infusion experiment to identify a Separation Voltage that imparts maximal mobility to the Substance P ion in CoV space. We found that an SV of 4000V required a CoV of 21V to restore the trajectory of the Substance P ion (Figure 4). The optimized SV and CoV parameters were incorporated into the MRM method and the Substance P calibration curve was reanalyzed with DMS enabled. The calibration curve demonstrates a significant enhancement in the selectivity of the MRM assay with >20-fold improvement in the measured LOQ. (Figure 4) The optimized SV and CoV parameters were incorporated into the MRM method and the Substance P calibration curve was reanalyzed with DMS enabled. The calibration curve demonstrates a significant enhancement in the selectivity of the MRM assay with >20-fold improvement in the measured LOQ. (Figure 4).

Symbol	Res. Mass	# (N)	b	y	# (C)
R	156.10111	1	79.05783	674.86336	11
P	97.05276	2	127.58421	596.81280	10
K	128.09496	3	191.63170	548.28642	9
P	97.05276	4	240.15808	484.23894	8
Q	128.05858	5	304.18737	435.71256	7
Q	128.05858	6	368.21666	371.68327	6
F	147.06841	7	441.75086	307.65398	5
F	147.06841	8	515.28507	234.11977	4
G	57.02146	9	543.79580	160.58557	3
L	113.08406	10	600.33783	132.07483	2
M	131.04049	11	665.85808	75.53280	1

Figure 1: Transitions available for the quantitation and qualification of Substance P. The +3 precursor ion (Q1) and b10 +2 product ion (Q3) were used as the primary quantitative transition.

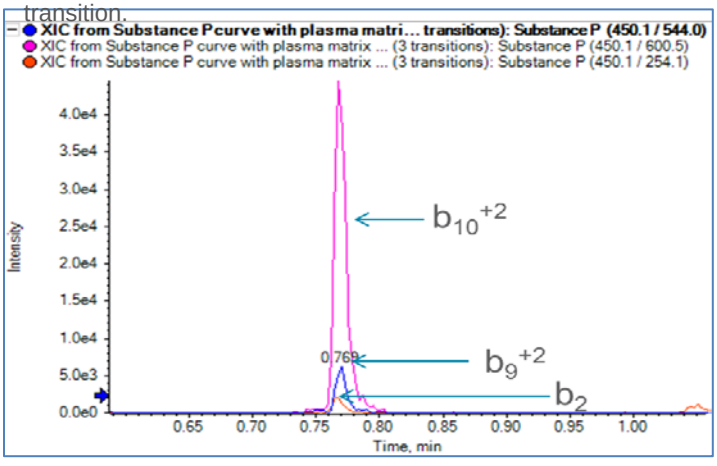
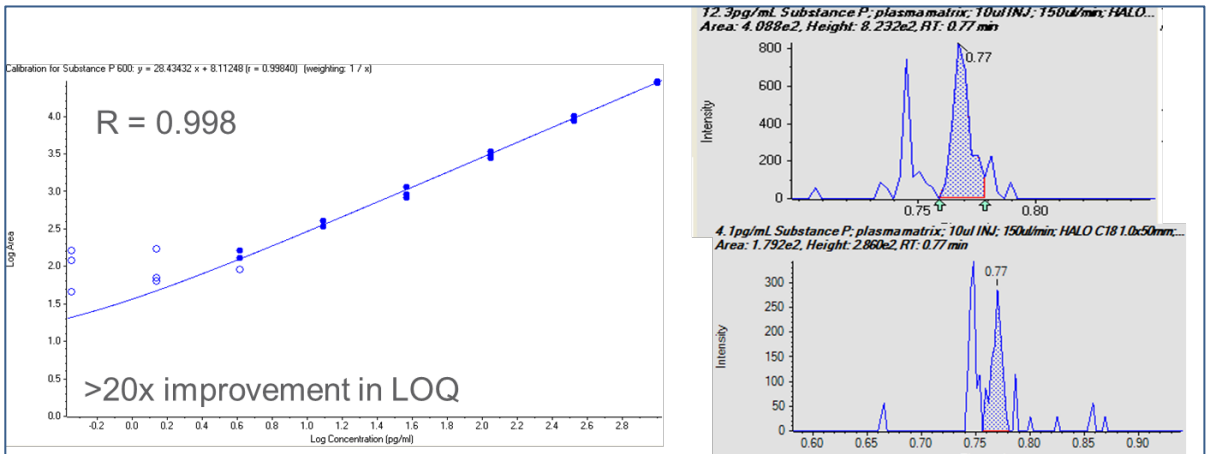


Figure 2: Chromatographically resolved peak of Substasnce P transitions during MRM method development.



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3	Substance P 600	4.10	2 of 3	4.887e0	8.938e-1	18.29	119.18
4	Substance P 600	12.30	3 of 3	1.242e1	1.450e0	11.68	100.95
5	Substance P 600	37.00	3 of 3	3.357e1	5.995e0	17.86	90.74
6	Substance P 600	111.10	3 of 3	1.071e2	1.058e1	9.88	96.39
7	Substance P 600	333.30	3 of 3	3.256e2	2.884e1	8.86	97.69
8	Substance P 600	1000.00	3 of 3	1.014e3	2.180e1	2.15	101.45

Figure 5: A) Log/Log plot of peak area versus actual concentration. B) Peak integration pane showing the effect of interference on quantitation. C) Accuracy statistics table for the quantitation experiment with DMS.

Conclusions

- An LOQ in the 5-10 pg/mL range can be achieved for Substance P with a two minute LC/MS acquisition method.
- Microflow liquid chromatography provides an alternative to higher flow LC/MS providing enhanced sensitivity without compromising quantitative quality or throughput.
- DMS with SelexION™ technology can be utilized as an orthogonal separation strategy to improve selectivity of an MRM assay without significantly increasing the analysis time.

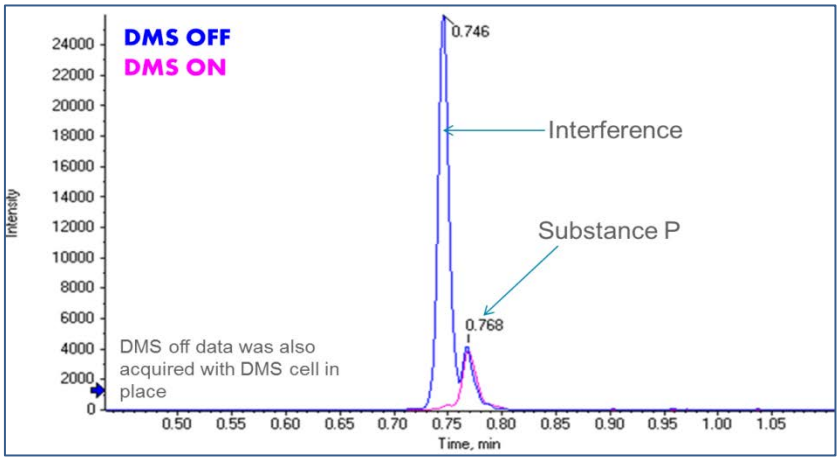

Figure 6: Comparison of Substance P peak with and without DMS. The peak at 0.746 minutes interferes with the quantitation of the Substance P peak at 0.768 minutes.

References

1. **Planar differential mobility spectrometer as a pre-filter for atmospheric pressure ionization mass spectrometry. Schneider BB, Covey TR, Coy SL, Krylov EV, Nazarov EG (.Int J Mass Spectrom. 2010 Dec 1;298(1-3):45-54)**
2. **Measurement of plasma-derived substance P: biological, methodological, and statistical considerations.** Campbell DE, Raftery N, Tustin R 3rd, Tustin NB, Desilvio ML, Cnaan A, Aye PP, Lackner AA, Douglas SD. Iin Vaccine Immunol. 2006 Nov;13(11):1197-203.

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