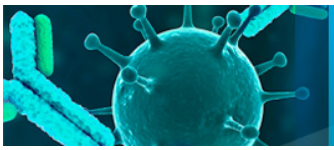


Benefits of Differential Ion Mobility Spectrometry for High-Sensitivity Quantification of Peptides

Using the SelexION® Ion Mobility device improves selectivity and sensitivity during biotherapeutic peptide analysis

Evgueni Fedorov
Biotrial Bioanalytical Services Inc., Laval, QC, Canada

Key Challenges of Peptide Quantification

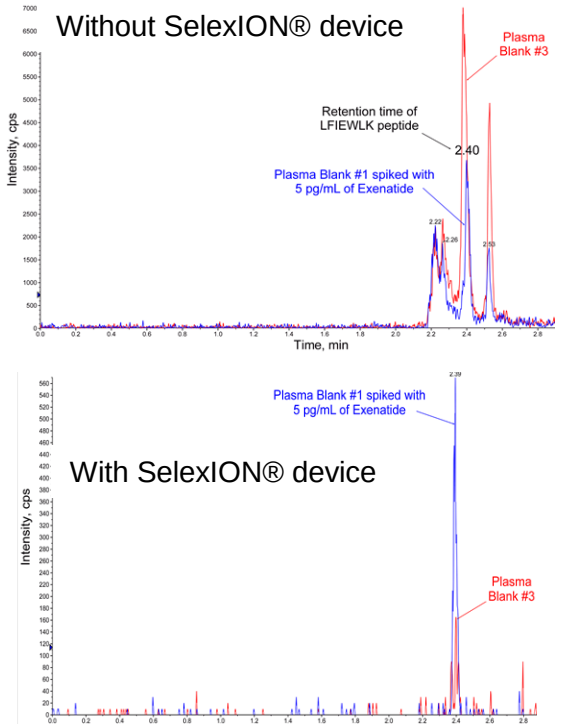
- **Poor MS/MS fragmentation**-Cyclic peptides or peptides with disulphide bond have either poor fragmentation or too much fragmentation
- **Lack of sensitivity**-Regular MRM which relies on daughter ion fragment has challenges in achieving highly sensitive assay specially in complex biological matrices
- **Selectivity challenges**-Parent ion to parent ion quantification, otherwise called Multiple Ion Monitoring is the best choice but will encounter very high noise background and matrix interference

Key Features of New Jet Injector SelexION®+ Technology

- **Novel Cell Design**- Addition of lens increases ion velocities into cell, reducing transit times through detrimental fringing fields
- **Increased Ion Transmission Efficiency** – Potential interferences originating from isobaric compounds present in complex plasma matrix
- **Shorter Ion Residence Time–** High background noise in MRM mode in processed plasma sample
- **Robustness**- Proven to meet regulatory guidelines for inter day and intraday precision and accuracy statistics

Key Benefits of SelexION®+ Multiple Ion Monitoring workflows

- **Enhanced Selectivity & Sensitivity**-With new cell design, SelexION+ DMS provides enhanced sensitivity and specificity without any compromise



MRM chromatograms of plasma samples analyzed with and without SelexION® device

- **Addressing isobaric interferences from complex matrix** – SelexION+ DMS reduces matrix interference and background noise originating from isobaric compounds present in complex plasma matrix
- **Improving Signal-to-Noise Ratio–** Reduces high background noise in MRM or MIM (multiple ion mode) in processed plasma sample
- **Ensuring better Data quality**- SelexION+ improves precision and accuracy statistics at lowest level of quantitation.

Some modern biopharmaceuticals (*e.g.,* exenatide, a glucagon-like peptide-1 agonist) are active at very low concentrations (down to several pg/mL). To detect these drugs in complex biological backgrounds at ultra-low levels, highly sensitive and selective assays are required for robust and accurate quantitative analysis. Using triple quadrupole mass-spectrometers for small molecule analysis by LC-MS/MS is a state-of-the-art technique, yet it is a highly reliable method, providing sensitive and robust detection for analytes in biofluids. For large peptides and proteins, however, sensitivity is reduced due to the formation of multiply charged ions during electrospray ionization, leading to a broad charge distribution and a dilution of the primary signal.

To improve the sensitivity issues related to the detection of relatively large biotherapeutic peptides (like exenatide), researchers can quantify signature peptides—small, unique peptides resulting from tryptic digests of the parent protein—rather than directly detect the intact protein itself. This technique can lead to a significant improvement in sensitivity, achieving LLOQs as low as 10 pg/mL [1]. However, during bioanalysis, overlapping peaks or impurities from biological matrices can negatively affect sensitivity and selectivity. In these cases, relying on MRM transitions alone for peptide detection may be inadequate for overcoming the isobaric interferences and high baseline noise that can negatively influence the signal-to-noise ratios during bioanalysis.

Other attempts to detect ultra-low levels of exenatide have relied on the use of more sensitive triple quadrupole instruments, but LLOQs from these studies did not show improvement. In these cases, the relatively large volumes of plasma that were needed for analysis produced high levels of chemical noise from the excessive levels of endogenous species still present after sample processing. Additionally, the selection of an exenatide signature peptide that was very small (only seven amino acids) increased the likelihood that the number of isobaric tryptic peptides with potential to overlap with the primary signal would be increased.

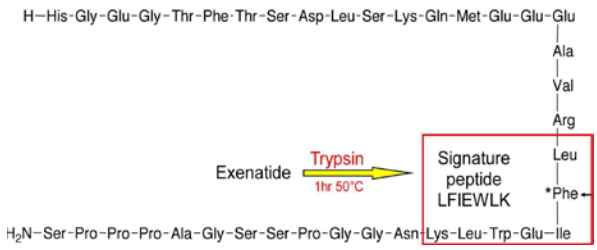
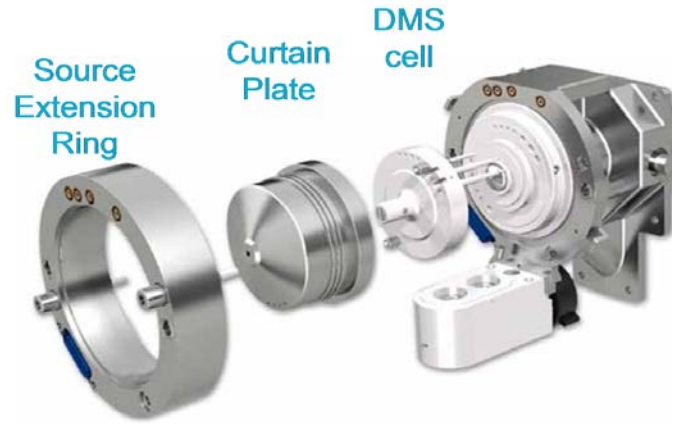


Figure 1. Sequence of exenatide, location of the deuterated amino acid in the internal standard (IS), and signature peptide sequence. Signature peptides for exenatide were generated using tryptic digestion, resulting in a seven-amino-acid-long peptide analyte. The internal standard was created by deuterating the Phe (*small black arrow*) of exenatide.

While the use of a high-resolution mass spectrometer may provide an alternative approach for resolving any near-isobaric interference affecting the LLOQ, there is a less-expensive alternative for detecting low-level signature peptides without compromising sensitivity or selectivity. In this work, the technique of differential ion mobility spectrometry (DMS) was used as an additional separation dimension, orthogonal to LC and MS/MS, during exenatide quantitation. DMS can be used to filter out co-eluting interferences by applying high and low energy fields from an asymmetric waveform (the separation voltage, SV) 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. By adding DMS-based separations to the LC/MS/MS workflow, we were able to successfully remove the overlapping interferences that were indiscriminately contributing to the exenatide peak area, reducing random co-eluting peaks and background noise to obtain a more accurate and selective exenatide signal.



SCIEX SelexION® Ion Mobility device for differential ion mobility spectrometry (DMS)

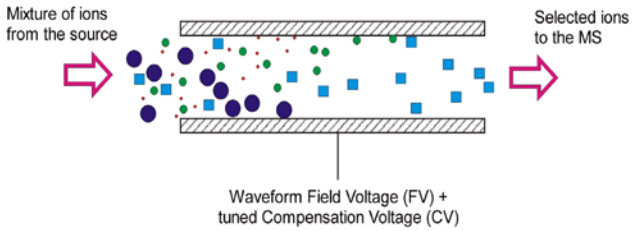


Figure 2. Principle of differential ion mobility spectroscopy (DMS). As the mixture of ions enters the drift tube, they are separated according to differences in each individual molecule's size, shape, and charge. An optimized combination of separation voltage (SV or waveform field voltage, FV) and compensation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

~~Methods~~

Methods

Sample Preparation:

Human plasma samples were thawed and aliquoted (0.5 mL). The exenatide-d5 IS (**Figure 1**, exenatide with the second phenylalanine labeled with deuterium at the phenyl ring) was added to the plasma samples, which were then subjected to solid phase extraction using Strata-XC (Phenomenex) cation-exchange cartridges. After evaporation, the samples were reconstituted in bicarbonate buffer and treated with trypsin. After incubation (1 hr at 50 °C), the samples were applied to a conditioned SOLAμ HRP microextraction plate (Thermo Scientifc). Samples were eluted with eluent (50 µL) and then were diluted with water (150 µL) for compatibility with HPLC conditions.

Chromatography:

LC System:	Shimadzu pumps (one of which had a built-in controller), a Shimadzu column oven and a PAL CTC autosampler
Analytical Column:	Supelco, Ascentis Express Peptide ES-C18 (50 x 2.1 mm), 2.7 µm with matching guard column, heated at 60° C
Trapping Column:	Supelco, Ascentis Express Peptide ES-CN (5 x 2.1 mm) 2.7 µm at ambient temperature
Pump A:	Shimadzu LC-20AD
Pumps B and C:	Shimadzu LC-10AD
Autosampler:	PAL CTC with DLW option
Injection volume:	40 µL
Flow rate:	0.5 mL/min (analytical column)
Mobile Phase:	A) 15/85/0.2 (v/v/v) acetonitrile/water/formic acid B) 95/5/0.1 (v/v/v) acetonitrile/water/formic acid

Mass Spectrometry:

Instrument:	SCIEX QTRAP® 6500 LC-MS/MS System with SelexION® Ion Mobility cell
Ionization source:	Electrospray ionization
Modifier:	None
Monitoring of ions:	Positive mode MRM
Q1 m/z (exenatide):	475.0@ Unit Resolution
Q1 m/z (IS: exenatide-d5):	477.5@ Unit Resolution
Q3 m/z (exenatide and IS):	688.4@ Unit Resolution
Dwell time:	50 ms (analyte and IS)
Curtain Gas (CUR):	30
Ion Source Gas 1 (GS1):	55
Ion Source Gas 2 (GS2):	70
Ion Spray Voltage: (IS):	5500
Temperature (TEM):	450 °C
Collision Activated Dissociation (CAD) gas:	12
Collision Energy (CE):	21
Declustering Potential (DP):	85
Entrance Potential (EP):	10
CXP:	12
DT:	High
DR:	Open
Compensation Voltage (COV):	28.80
DMO:	-3
Separation Voltage (SV):	3800

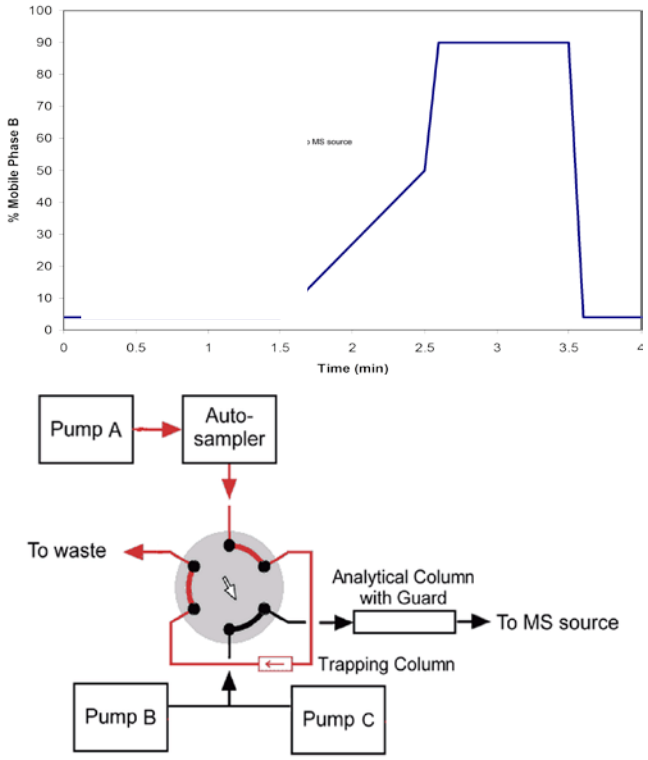


Figure 3. Mobile phase gradient and HPLC setup schematics. The mobile phase gradient used for HPLC analysis is outlined above (*top panel*), showing the percentage of mobile phase B to be mixed with mobile phase A. The blue arrows indicate when the valve was switched to elute from the trapping column and then switched back. The valve schematic (*lower panel*) shows how pumps are linked to the HPLC analytical and trapping column. Pumps A and C deliver Mobile Phase A, pump B delivers Mobile Phase B.

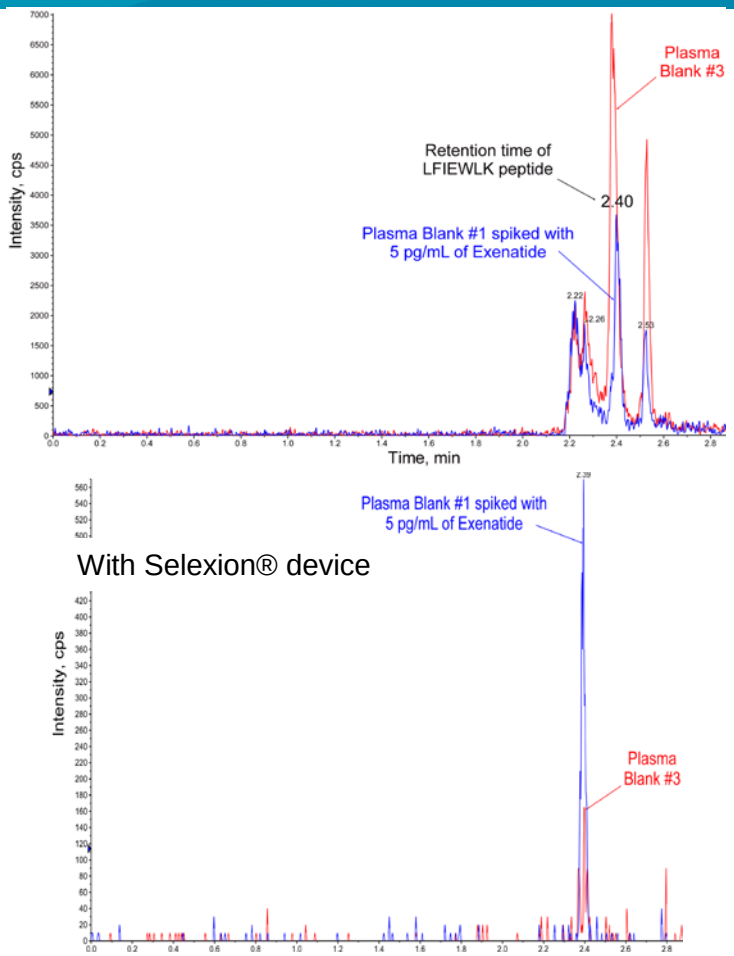


Figure 4.MRM Chromatograms of plasma samples analyzed with and without differential ion mobility spectrometry (DMS). Plasma samples spiked with exenatide (5 pg/mL) and blank plasma samples were analyzed for the presence of the exenatide signature peptide *without* the SelexION® Ion Mobility device (*top panel*) or *with* the SelexION device (*bottom panel*). Traces in *blue* indicate peaks due to exenatide-spiked plasma, and traces in *red* indicated peaks due to blank plasma samples.

Data Processing: Analyst v. 1.6 (IntelliQuan algorithm)

Results

A SelexION® Ion Mobility device was installed on the QTRAP LC/MS/MS system to augment the front-end separations of plasma samples spiked with low-levels of exenatide (5 pg/ml). Chromatograms of eluting plasma peaks were obtained either with or without DMS (**Figure 4**), and the differences in the profiles indicate that when DMS is used, there is a reduction in peaks overlapping with the exenatide signal. In **Figure 4**, a large peak from plasma blank #3 intersected the relatively smaller exenatide signature peptide peak at retention time 2.4 min (**Figure 4**, blue profile). When using the SelexION device during separations, interferences from the blank plasma were diminished significantly, and the exenatide signal predominated

Table 1. Contribution of overlapping plasma peaks to exenatide signal with and without differential ion mobility spectroscopy (using a SelexION® Ion Mobility device)

Plasma blank lot #	Peak manually integrated without SelexION	Contribution to LLOQ peak area	
		Without SelexION	With SelexION
1	Yes	37%	*15%*
2	Yes	*10%*	*2%*
3	*No*	*158%*	*14%*
4	*Yes*	*21%*	*9%*
5	*No*	*135%*	*11%*
6	*Yes*	*14%*	*5%*
7	*No*	*23%*	*4%*
8	*Yes*	*47%*	*5%*
9	*Yes*	*21%*	*5%*
10	*No*	*21%*	*4%*
11	*Yes*	*5%*	*16%*
12	*No*	*34%*	*2%*
13	*No*	*34%*	*7%*

the elution profile, indicating that DMS had been successfully applied to exclude background noise and overlapping signals.

To quantify DMS's impact on the prevalence of co-eluting background peaks, the contributions from blank plasma samples towards the total exenatide peak area were assessed (keeping all other settings the same). Plasma samples from 13 different lots (pre-treated with K2 and K3 EDTA) were added to plasma samples spiked with low levels of exenatide. The extent that co-eluting blank plasma peaks interfered with exenatide peak integration is indicated in **Table 1** and reported as the percent contribution to the LLOQ peak area. In most plasma lots, the use of DMS was able to reduce the percent contribution of blank plasma peak interferences to the exenatide signal (**Table 1**).

In some lots, the blank plasma peaks had a higher contribution to the signal than other exenatide-spiked plasma with low backgorund, a result which does not meet the established bioanalytical criteria for the detection and quantitation of non-endogenous analytes. Moreover, some samples could not be automatically integrated if a large component of the LLOQ peak area resulted from interfering peaks. However, DMS usage lowered the prevalence of blank sample interference to only 16% of the LLOQ, well within the acceptance criteria. Also, DMS-separated exenatide peaks exhibited consistent peak shapes

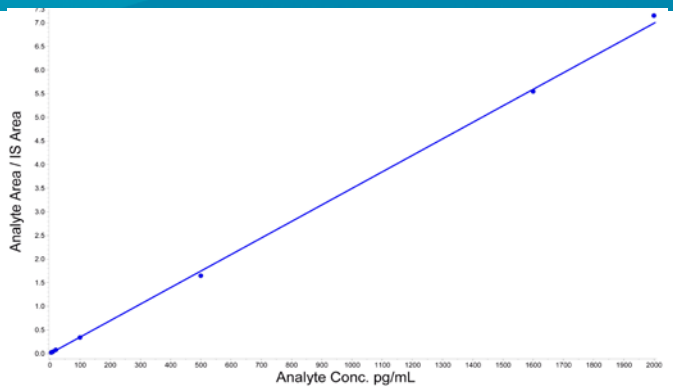


Figure 5. Calibration curve for exenatide with differential ion mobility spectroscopy (DMS) separations. Plasma samples with exenatide added over a range of 5-2000 pg/mL were analyzed by LC/MS/MS with the SelexION® Ion Mobility device in operation. Linear regression calculations (1/x weighting) gave an equation of y = 0.00349x + 0.00345 and an r value of 0.9996 for the resulting curve.

and interference-free peak areas, making automatic integration possible for every plasma lot.

To determine the best range of concentrations for the bioanalysis of exenatide in blood, plasma samples spiked with 5–2000 pg/mL of exenatide were evaluated using LC-MS/MS and DMS. An LLOQ of 5 pg/mL and excellent linearity (r = 0.9996) were achieved over three orders of magnitude (**Figure 5**), demonstrating that the detection of exenatide signature peptide can be carried out with the precision and accuracy needed to meet FDA guidance criteria for LC-MS/MS methods.

Conclusions

Using differential ion mobility spectroscopy (DMS) as an additional dimension during separations prior to LC-MS/MS quantification of exenatide signature peptides in regulated bioanalysis provided many advantages:

- Contributions to the signal from chemical noise and matrix impurities were significantly decreased.
- A lower LOQ was achieved in compliance with regulatory acceptance criteria.
- The need for manual integration of signature peptide peaks was eliminated.
- These benefits were obtained with less investment in instrumentation, compared to the costs of purchasing a high-resolution instrument.
- The DMS module is easy to tune and operate, is robust, and does not require calibration.

References

1. 1. J.-N. Mess, D. Villeneuve and F. Garofolo. Bioanalysis of Exenatide: Intact Versus Signature Peptide Approach to Reach Optimal Sensitivity in Large Molecule Quantification by LC-MS. 61th Conference of ASMS, 2013, Minneapolis, USA.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
www.sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
www.sciex.com/offices