APPLICATION OF DIFFERENTIAL ION MOBILITY MASS SPECTROMETRY TO PEPTIDE QUANTITATION.

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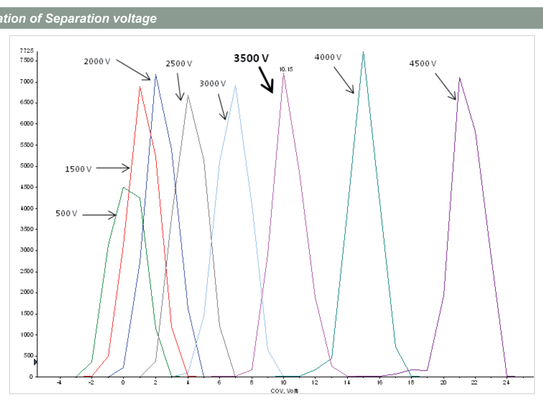
INTRODUCTION

The quantitative determination of therapeutic peptides to support PK and TK studies can sometimes be challenging. Poor MS/MS sensitivity combined with often poor selectivity fragments can compromise the desired lower limits of quantitation (LLOQ). In addition the adsorptive properties and/or polarity of peptides can compromise recovery and extract cleanliness from biological matrices with further impact on sensitivity and selectivity. In such cases MRM approaches even when combined with efficient UPLC separations may not be sufficient to provide adequate signal to noise ratios at LLOQ in the presence of isobaric interferences or high baseline noise.
Differential ion mobility spectrometry (DMS) may provide a useful tool in such instances by providing an additional and orthogonal degree of selectivity. Whilst often accompanied by a loss in absolute sensitivity if the gains in selectivity are sufficient then real gains in LLOQ will be realized. Alternatively, selectivity gains from DMS may allow a less selective sample preparation to be used allowing for overall improvements in sensitivity due to an increase of extraction recovery.
This poster presents two case studies were DMS using the Selexion device is evaluated for the quantitation of therapeutic peptides. Selectivity, sensitivity and precision are compared with and without DMS. The potential benefits and limitations of the technique are discussed.

OPTIMISATION

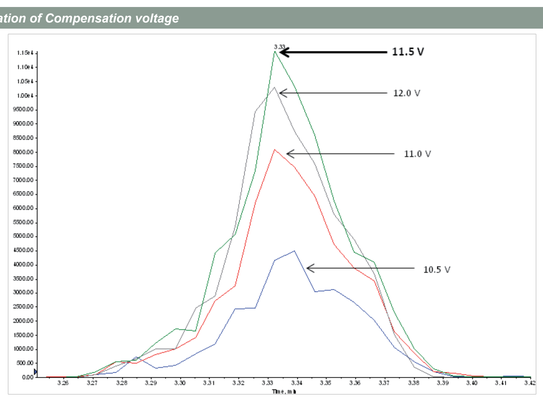
The SelexIon device needs only a few minutes for installation onto the API 5500 and can be accomplished without breaking the MS vacuum. For best performance, 20-30 minutes are required to equilibrate the electrode at the desired temperature (low/med/high).
Within the device, ions are separated by differential mobility due to size and shape. A combination of separation voltage (SV) and compensation voltage (COV) is optimized to give potential separation of analyte ions from background ions. Optimization of these parameters is very simple and can be performed as part of instrument tuning.
The optimization of SV is performed by constant infusion at low flow of analyte in solution whilst ramping COV (Figure 1). The optimal combination of separation and compensation voltages is the one that give the most separation whilst maintaining maximum peak intensity. Although optimal SV is usually obtained at around 4500V it is usual to choose a lower value to ensure system robustness and stability.

Figure 1 Optimisation of Separation voltage



As COV is influenced by mobile phase and source conditions, on column COV optimization is performed by injection of analyte at a flow rate and mobile phase composition comparable to the intended LC conditions (Figure 2). In some instances additional selectivity may be achieved by use of modifier (eg Methanol, acetonitrile, isopropanol, acetone) introduced into the Selexion device at low flow. No modifier was used in the case studies presented.

Figure 2 Optimisation of Compensation voltage



CASE STUDY 1

In this case study a proprietary peptide (Peptide A) MW 4113.7 g/mol was evaluated. This peptide is known to exhibit adsorptive characteristics and SPE clean up (reverse phase or mixed mode) from human plasma resulted in very low recoveries. As a consequence protein precipitation with methanol is the only feasible extraction approach from human plasma. Additionally due to poor fragmentation, selective fragments are not available for quantitation and fragment m/z 136 must be used to obtain adequate sensitivity. Under these conditions the resulting LLOQ is severely compromised by this lack of selectivity despite a 6 minute gradient LC elution (Figure 3). Without DMS, using only MRM with +ESI (Table 1) an LLOQ of only 50 ng/mL is achievable from human plasma. Elevated baseline and a number of closely eluting peaks requiring careful setup of peak integration parameters.

Table 1 MS parameters for Peptide A

	AB Sciex 5500 Q-Trap
Ion source / polarity	ESI / positive
CAD	High
CUR	30
TEM	700°C
Gas 1	70
Gas 2	50
Ion spray voltage	5500 V
	MRM
Transitions	1029.3 / 136.0 (Peptide A) 1106.7 / 123.0 (analog IS)
Dwell time	150 msec for Peptide A 100 msec for analog IS
Resolution Q1 / Q3	unit / unit
	SelexION
DT (temperature)	Low
DR (throttle gas)	Off
COV (Compensation voltage)	11.5
DMO	- 3
SV (Separation voltage)	3500

Using the same protein precipitation procedure and LC gradient conditions DMS (optimized parameters Table 1) was evaluated for improvement in selectivity. A significant improvement was observed. Despite a loss of absolute signal of approximately factor 5 a reduction of background interference of approximately factor 20 was observed. This resulted in an overall gain in S/N of about factor 4-5 (Figure 4). This facilitated a lowering of the feasible LLOQ from 50 to 8 ng/mL (Table 2) without changing extraction or gradient LC conditions.

Figure 3 Peptide A at 20 ng/mL from a protein precipitation extract from human plasma without DMS

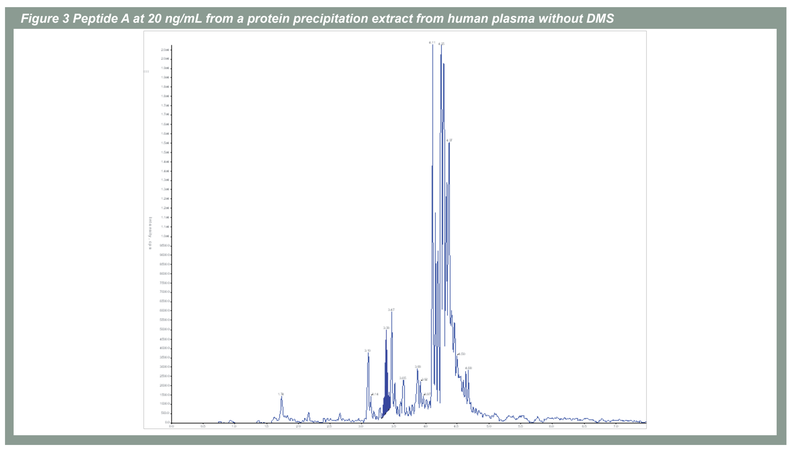


Figure 4 Peptide A at 20 ng/mL from a protein extract from human plasma with DMS

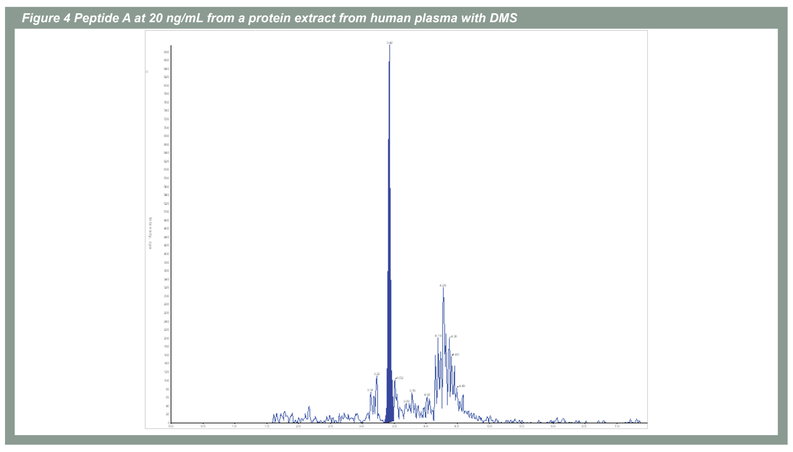


Table 2 Lower limit of quantitation (LLOQ) data for Peptide A

	Spiked level Peptide A (ng/mL)			
	8	**20**	**50**	**100**
Precision (CV%) without SelexION	N/AP	25.0	13.7	6.0
Precision (CV%) with SelexION	6.3	7.8	8.1	6.3
n	6	6	6	6

CASE STUDY 2

In this case study the quantitation of a therapeutic peptide (Peptide B) MW 1311 g/mol in rat plasma was evaluated. This peptide was extracted from rat plasma using polymer based reverse phase SPE. A recovery of 65% was achieved. Samples were chromatographed on a fused core peptide column using a methanol/water gradient with Formic acid as acid modifier. Using +ESI MRM (Table 3) a range of 0.04 – 10 ng/mL could be routinely achieved. At LLOQ a S/N of 10 (analyte intensity 1000 counts background 100 counts) resulted in a precision of 7.4% (Figure 5).

Table 3 MS parameters for Peptide B

MS/MS	**AB Sciex 5500 Q-Trap**
Ion source / polarity	ESI / positive
CAD	High
CUR	30
TEM	700°C
Gas 1	50
Gas 2	60
Ion spray voltage	5000 V
	MRM
MRM Transitions	656.4 / 249.0 (Peptide B) 661.4 / 249.0 (labelled IS)
Dwell time	100 msec for Peptide B
Resolution Q1 / Q3	unit / unit
	SelexION
DT (temperature)	High
DR (throttle gas)	Off
COV (Compensation voltage)	15.0
DMO	- 3
SV (Separation voltage)	3500

Applying DMS to this method resulted in a 10 fold decrease in background with absolute analyte sensitivity exibiting a 6 fold decrease. Whilst S/N improved to 16 at LLOQ (0.04 ng/mL) there was no marked improvement in precision at this level (Table 4). A lowering of LLOQ could also not be facilitated under these conditions. Background, however was almost completely eliminated allowing easier and more consistent peak integration (Figure 6).

Table 4 Linearity and precision for Peptide B with and without DMS

SPE Extracts without DMS	**Linear Regression $1/X^2$ y=0.243x + 0.000808 (r=0.9987)**		
Intra run (n=6)	Concentration (ng/mL)	Precision (%)	Accuracy (%)
	0.04	7.4	100
	0.08	4.8	101
	0.2	2.2	97.6
	0.8	0.9	97.2
	1.6	**0.6**	**102**
	10	1.2	103
SPE Extracts with DMS	**Linear Regression $1/X^2$ y=0.237x + 0.000257 (r=0.9976)**		
Intra run (n=6)	Concentration (ng/mL)	Precision (%)	Accuracy (%)
	0.04	10.2	100
	0.08	6.0	101
	0.2	4.7	97.7
	0.8	2.4	97.0
	1.6	2.2	102
	10	1.3	103

Figure 5 Peptide B at 0.04 ng/mL extracted with solid phase extraction without DMS

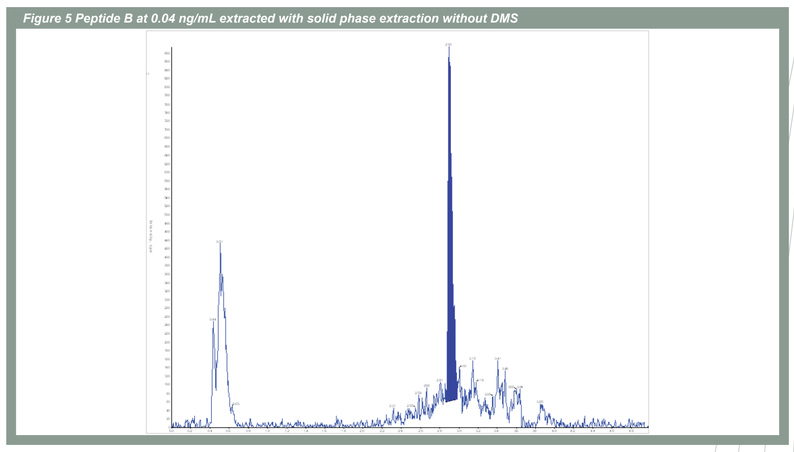
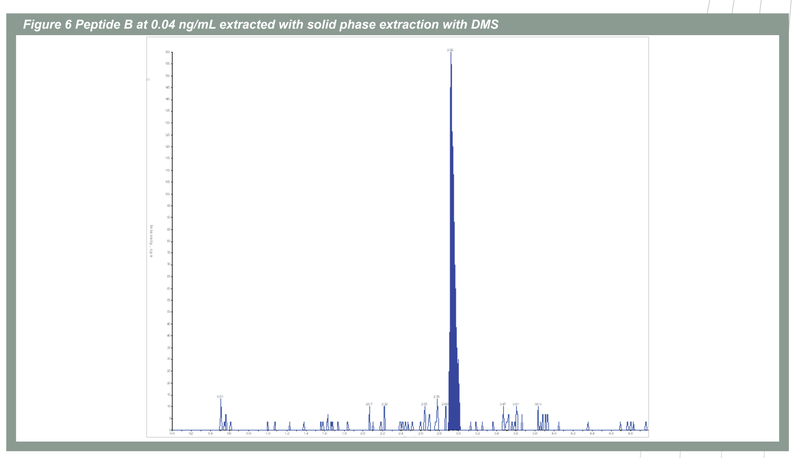


Figure 6 Peptide B at 0.04 ng/mL extracted with solid phase extraction with DMS



A protein precipitation approach was also evaluated for Peptide B. As absolute recovery was compromised using SPE it was anticipated that a lowering of LLOQ by factor of 2 could be achieved by combining recovery gains of protein precipitation with selectivity gains from DMS.
Without the benefits of SPE cleanup in this instance only an LLOQ of 0.08 ng/mL could be achieved without DMS due to high background and closely eluting isobaric interferences (Figure 7). With DMS all background interferences were removed (Figure 8). However, due to the inherent loss of absolute signal associated with DMS a similar LLOQ to that demonstrated for SPE extraction could be achieved as absolute signal was the limiting factor.

Figure 7 Peptide B at 0.08 ng/mL extracted with protein precipitation without DMS

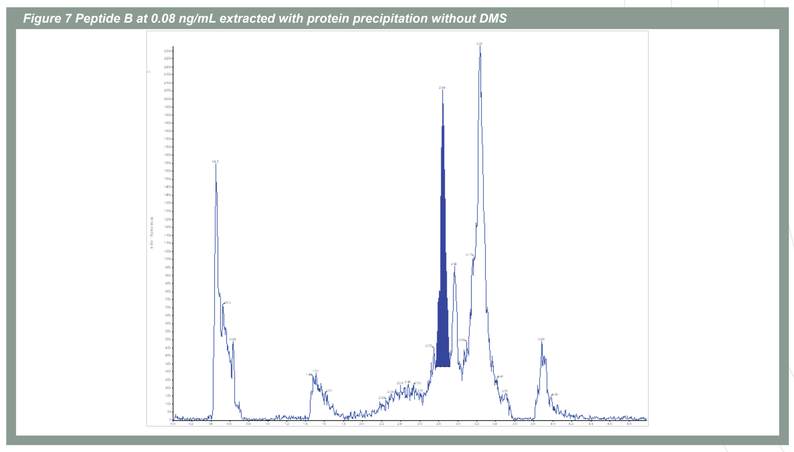
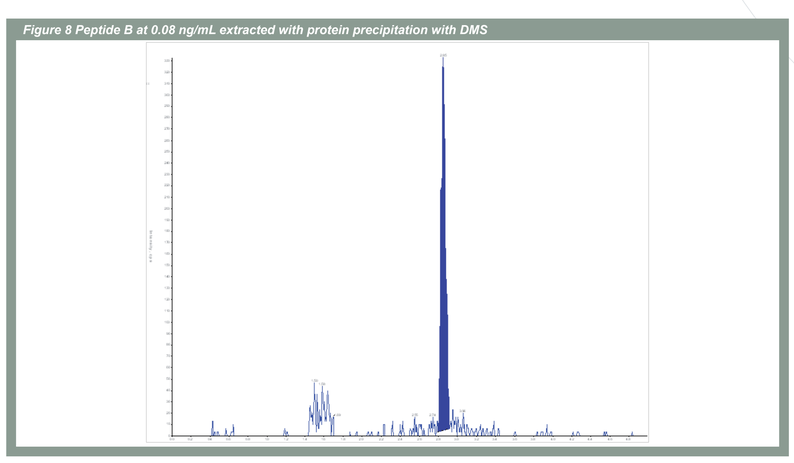


Figure 8 Peptide B at 0.08 ng/mL extracted with protein precipitation with DMS



CONCLUSION

Differential ion mobility spectrometry may provide a useful additional or orthogonal selectivity during the quantitation of peptides (and of course conventional small molecules). In application to peptide quantitation in particular selectivity gains may be significant as separation of multiply charged analyte precursors from singley charged background interference is likely. The true gain in sensitivity as a function of absolute sensitivity and selectivity will be analyte dependant and will also be influenced by choice of MRM transition, chromatographic separation and extract cleanliness. Often for peptides sensitivity is already compromised by a number of factors including low bioavailability, adsorption to surfaces, formation of multiple charge states and poor or non selective fragmentation. In these instances additional tools to aid lowering of LLOQs are to be welcomed. In some cases true gains in sensitivity may not be realised or be required but improvements in selectivity may bring other benefits namely, simpler extraction methods, shorter chromatographic runs or improved peak integration.

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Poster presentation at EBF 5th Open Symposium Barcelona, November 14-16 2012