EVALUATION OF A NOVEL TANDEM QUADRUPOLE MASS SPECTROMETER FOR THE MULTI-POINT INTERNAL CALIBRATION ANALYSIS OF PEPTIDES

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INTRODUCTION

Translational and biomarker verification studies are challenged in that they not only require the analysis of large sample cohorts with high-throughput, but also demand high sensitivity, high resolution and selectivity over a large dynamic range. Targeted LC-MS/MS based assays afford analyte quantification with the reproducibility and throughput required in order to rapidly assess biomarker performance. Multiple Reaction Monitoring (MRM), using tandem quadrupole mass spectrometry, is an enabling technology that provides speed and selectivity, whilst miniaturized LC systems offer additional improved sensitivity. Here, the application of micro-fluidics (IonKey) coupled to a novel tandem quadrupole MS/MS system (Xevo TQ-XS), using a multi-point internal calibration methodology for the quantitation of peptides and proteins, is demonstrated.

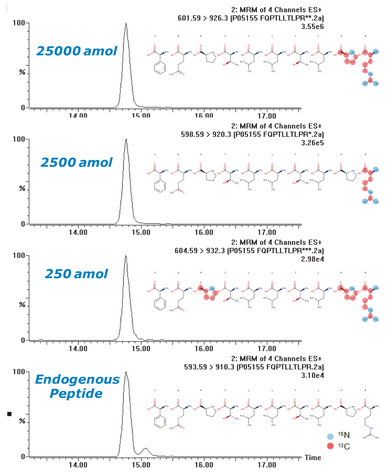

Figure 1. A three point Multi-Point Internal calibration curve is constructed from 3 differentially labeled versions of the analyte of interest, that are spiked into the sample at three different levels.

METHODS

Sample preparation

- Stable Isotope Labeled (SIL) peptides, whose analogues are putative biomarkers for cardiovascular disease (CVD) [*1*], were spiked at various levels into diluted, non-depleted, un-fractionated, tryptically digested human serum (20 - 1000 ng/µl of matrix).
- Blood samples were collected from a cohort of 20 healthy donors, 20 HFPEF patients, and 20 HFREF patients. All serum samples were mixed with NH_4HCO_3 in the presence of RapiGest, DDT reduced, IAA alkylated and trypsin digested overnight.

LC-MS system

IonKey integrated micro-fluidics

- Acquity UPLC M-Class System
- Mobile Phase A - Water + 0.1% Formic Acid
- Mobile Phase B - Acetonitrile + 0.1% Formic Acid
- Gradient: 3-25% Mobile Phase B in 11 min
- 150 µm x 100 mm BEH C18 130 Å 1.7 µm
- Flowrate: 1.0 µL/min
- IonKey Temperature - 35˚C
- Injection Volume - 1 µL

Mass spectrometry

- Xevo TQ-XS tandem quadrupole system
- Capillary Voltage - 3.5kV
- Source Temperature - 100˚C
- Resolution - Unit Mass Resolution

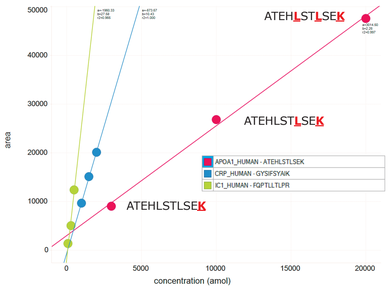

Figure 2. Multi-Point Internal Calibration can be used for multiple analytes simultaneously. Here, the quantification of 3 proteins in human serum that are present over 3 different ranges is shown.

Experimental design

SIL peptides were spiked into diluted, non-depleted, digested human serum matrix (200 ng/uL) at 12.5 fmol/µL and serially diluted in matrix to various levels over the range 0.00625 - 12.5 fmol/µL. This experiment had previously been replicated eight times using all possible combinations of LC and MS/MS platforms shown in Figure 3 using various MRM acquisition modes [*2*].

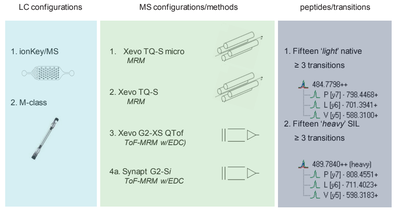

Figure 3. LC-MS configurations and MRM methods.

This analysis was replicated using the Xevo TQ-XS tandem quadrupole mass spectrometer combined with integrated micro -fluidics. The sensitivity achieved with this new instrument was compared to the other platforms previously utilized.

Calibration methods

A subset of these samples were then selected and re-analysed (20 ng/µL of matrix) using a new, novel multi-point internal calibration methodology as follows. Three different SIL variants of the same peptides were sourced (PepScan, Lelystad, The Netherlands) for three different peptides. These differentially labeled SIL peptides were then used to spike a calibration line within each of the samples selected from the previous study. This concept is demonstrated in Figures 1 and 2.

The endogenous level of two of the peptides, representing potential CVD biomarkers, were quantified by calibration against this internal calibration curve. The third peptide, not endogenously present, was used to perform a comparison of the internal calibration approach to the more widely used technique of quantifying against a separate external calibration line.

This comparison experiment was performed over a more limited linear range to replicate a "real world" experiment where the analyte of interest (biomarker) may be present over a much smaller reference range, and was performed using a higher level of matrix (1000 ng/µL) to replicate a high sensitivity biomarker assay.

RESULTS

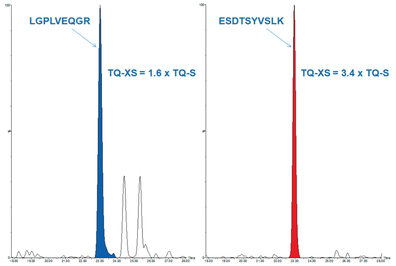

Figure 4 - Chromatograms showing response of two tryptic peptides on Xevo TQ-XS using IonKey for 125 amol of peptide injected on column, and how this compares to the Xevo TQ-S in terms of S/N.

Sensitivity

The sensitivity of the Xevo TQ-XS mass spectrometer was compared directly to the previously used mass spectrometry platforms in terms of signal to noise ratio (S/N) at 125 amol on column. Figure 5 shows the response for two of the SIL peptides analysed. The increase in sensitivity observed for the new MS platform compared to the Xevo TQ-S platform varied from no increase to a 3.4 times increase in S/N. When the results from all of the peptides analysed (nine) were averaged, the mean increase was 1.6 times. Figure 5 shows how the LC-MS platform compared overall in comparison to the platforms used in the previous study [*2*].

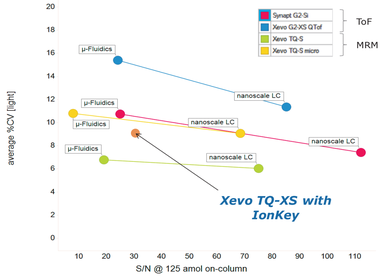

Figure 5. Average performance metrics for data acquired using the new TQ-XS mass spectrometer when compared to other MS platforms used in a previous study [2].

Multi-Point Internal Calibration

The multi-point internal calibration method was used to analyse eighteen HF patient samples, comprising six samples from each of the three groups. A faster gradient was used with an increased flow rate because of reduced matrix consumption, resulting in improved throughput and sensitivity (LoD < 10 amol). Figure 6 shows the chromatographic performance for the two endogenous peptides analysed using this methodology.

Figure 7 shows the two trends observed for one of these studied CVD biomarkers (Plasma Protease C1 Inhibitor, FQPTLLTLPR) studied in this and a previous study [2]. Even with the different mass spectrometer, calibration method, LC conditions, matrix level and number of samples used, the same trends were observed for both biomarkers.

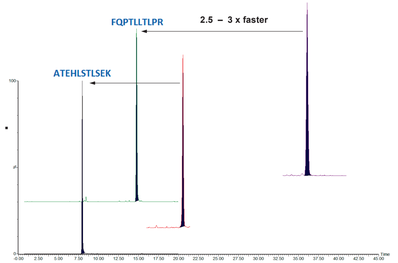

Figure 6. Response for the SIL form of the two endogenous peptides analysed in 20 ng/µL of matrix, using faster (2.5 - 3 times) IonKey LC conditions (20 min cycle time).

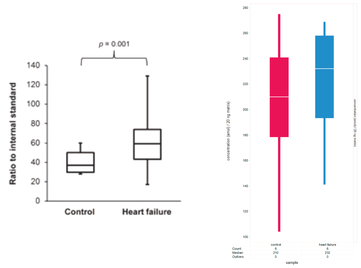

Figure 7. Comparison of data obtained in a previous study (left) [2], n=60), to data collected using the multi-point internal calibration methodology (right, n=18) for Plasma Protease C1 Inhibitor (FQPTLLTLPR).

Nine patient samples were randomly selected and spiked with 500 amol/µL of the exogenous GYSIFSYAIK, and analysed using the internal multi-point method (200 - 1000 amol/uL), as well as with an external calibration line. This experiment was performed at 1000 ng/µL of matrix to replicate a high sensitivity biomarker assay. Figure 8 shows the improvements in precision seen due to the improved compensation for matrix effects seen with multi-point methodology.

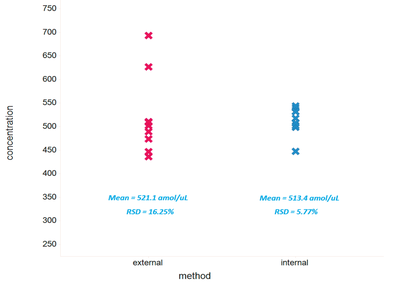

Figure 8. Analysis precision for nine matrix (1000 ng/µL) samples, spiked with an exogenous peptide (500 amol/µL), using external and internal multi-point calibration methods.

CONCLUSIONS

- **A new and novel tandem quadrupole mass spectrometer was successfully utilized to quantitatively analyse non-depleted, tryptically digested human serum samples for putative peptide biomarkers using a novel multi-point internal calibration methodology**
- **The Xevo TQ-XS was shown to be on average 1.6 times more sensitivity that the Xevo TQ-S MS platform when using IonKey integrated micro-fluidics for tryptic peptide analysis**
- **The increased sensitivity allowed for faster gradients to be used due to the reduced level of sample required for analysis, increasing throughput**
- **The multi-point internal calibration method also increased throughput further as no external calibrators are required to assess linearity**
- **The multi-point internal calibration methodology was also shown to be superior to an external calibration in terms of compensation for matrix effects**

References

1. MRM-based multiplexed quantitation of 67 putative cardiovascular disease biomarkers in human plasma. *Domanski et al.* Proteomics. 2012 Apr;12(8):1222-43.
2. Advances in Quadrupole and Time-of-Flight Mass Spectrometry for Peptide MRM based Translational Research Analysis. *Mbasu et al.* Proteomics. 2016 (in press)

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