APPLICATION OF MICROFLUIDIC/TANDEM QUADRUPOLE LC-MS/MS FOR MRM BASED TRANSLATIONAL RESEARCH ANALYSIS OF PUTATIVE HEART FAILURE PEPTIDE BIOMARKERS IN HUMAN PLASMA

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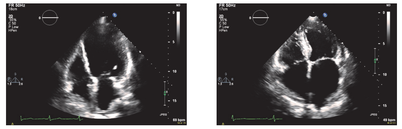
INTRODUCTION

The application of tandem quadrupole MS with microfluidic chromatography for the analysis of proteolytic peptides in human plasma is compared and contrasted with nanoscale LC and high-resolution oa-ToF MS configurations. A tandem quadrupole platform was considered for its performance in terms of sensitivity, selectivity, precision, and linearity. Microfluidic chromatography was selected as it afforded the optimum balance of sensitivity and throughput, whilst minimizing isobaric coelution, thereby providing an ideal LC-MS configuration for the application to large sample cohorts in translational studies. This LC-MS configuration was also utilized to demonstrate that proteolytically digested, non-depleted plasma samples from heart failure patients could be classified with good discriminative power using a subset of proteins previously suggested as candidate biomarkers for cardiovascular diseases. Heart failure (HF) represents a clinical population for which validated biomarkers are sparse. The aetiology of the disease also means that the phenotypic changes are likely to be multifactorial. As a research proof of principle and to test the potential sensitivity to classify this disease, healthy controls, HF patients with preserved ejection fraction (HFPEF) and HF patients with reduced ejection fraction (HFREF) were analyzed.



HFREF HFPEF

Figure 1. Echocardiogram (apical 4 chamber view) of the heart showing an example of HFREF (dilated left ventricle) and one of HFPEF (LV hypertrophy with dilated atria).

METHODS

Sample preparation

Various stable isotope labeled (SIL) peptides whose light analogues are putative biomarkers for cardiovascular disease (CVD) were spiked at various levels into un-fractionated, tryptically digested EDTA human serum.

The SIL peptides were simultaneously spiked into diluted digested 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. Samples were injected, separated and detected using a reversed phase gradient on various LC-MS platforms.

This analysis was replicated eight times with MRM acquisition modes using all possible combinations of the LC and MS /MS platforms detailed below and graphically summarized in Figure 2. The analysis was replicated another four times using the ion mobility (IM) functionality of SYNAPT G2-Si with both LC platforms.

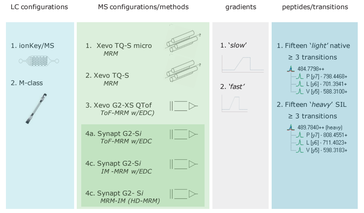


Figure 2. Experimental design LC-MS configuration comparison.

Human blood samples were collected from a cohort of twenty healthy donors, twenty HFPEF patients, and twenty HFREF patients, following informed consent. All HFPEF patients had an ejection fraction of ≥50% and HFREF patients had an ejection fraction ≤40%.

All sera were mixed with ammonium bicarbonate in the presence of RapiGest, reduced, alkylated and digested overnight using trypsin.

LC systems

IonKey/MS integrated microfluidics
- Gradient: 3-40% Mobile Phase B in 45 mins
- Chromatographic channel: 150 µm x 100 mm BEH C18 130 Å 1.7 µm
- Flowrate: 1.0 µl/min

Nanoscale LC system
- Gradient: 3-30% Mobile Phase B in 90 mins
- Column: 75 µm x 250 mm BEH C18 130Å 1.7µm
- Flow rate: 300 nl/min

Mobile phases (both IonKey and nanoscale LC)
- A: Water + 0.1% formic acid
- B: ACN + 0.1% formic acid

Tandem MS/MS platforms

Tandem Quadrupole MS
- Xevo TQ-S
- Xevo TQ-S micro

Quadrupole-Time of flight (Q-ToF) MS
- Xevo G2-XS QTof
- SYNAPT G2-*Si* (ion mobility enabled)

Informatics

The tandem quadrupole and high resolution Q-ToF LC-MS peptide MRM data were both quantified with either TargetLynx or Skyline. All statistical analyses were conducted with SIMCA and SPSS statistics.

RESULTS

Transition selection and evaluation

Experiment wide transition evaluation was conducted by normalizing transitions intensities to the most abundant transition for a given peptide. A summary is shown in Figure 3 for one of the oa-ToF instruments, contrasting the relative abundance of endogenous and SIL transitions for some selected peptides. Similar experiments were conducted for all possible configurations and only those transitions retained that illustrated good agreement.

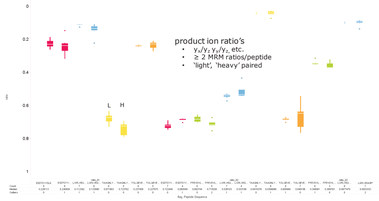


Figure 3. Experiment wide MRM (oaToF) transition evaluation (normalized transition values; $I_{transition\ x}/I_{most\ abundant\ transition}$). L = 'light' (endogenous), H = 'heavy' (SIL).

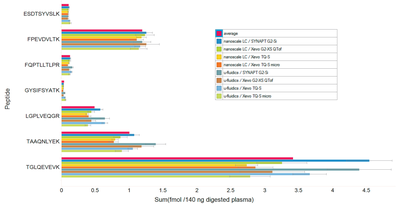


Figure 4. LC-MS configuration average multi-level single point concentration endogenous monitored plasma peptides.

Comparison LC-MS configurations and experimental variation

The concentration and coefficient of variation (CV) were calculated for each individual SIL spike-level, representing a multi-level single point average and error estimate , illustrated in Figure 4. The obtained CV values were compared against the average *S/N* (across all peptides), shown in Figure 5, providing combined performance metrics for precision and sensitivity.

The results shown in Figure 6 provide an estimate of MS to the experimental variation. uncorrected CV values range from 10 to 30%. Internal standard correction reduces this 5 to 8%. Retention reproducibility is typically better than 1%. An example of the throughput increase from the use of microfluidics is shown in Figure 7. On average, a 2-fold reduction in analysis time was observed without a substantial in increase in the number of detected isobaric interferences.

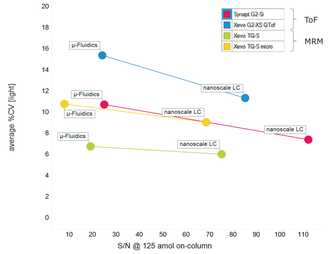


Figure 5. Average experiment wide endogenous multi-level single point peptide MRM CV (%) and S/N LC-MS configurations.

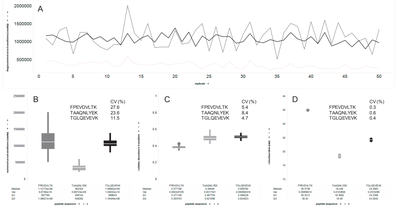


*Figure 7. Raw summed MRM transition abundances as a function of replicate experiments (n = 50) for SIL peptides FPEVDVLT[**K**], TAAQNLYE[**K**], and TGLQEVEV[**K**] spiked at a fixed level in different, independent undepleted plasma digest samples (A), raw summed transition intensity variability (B), intra normalized transition intensity variability (C), and retention time variability (D). [**K**] = $^{13}C_6{}^{15}N_2$ labeled.*

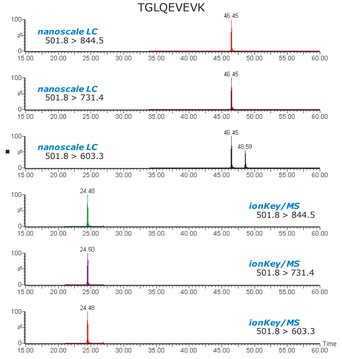


Figure 7. Throughput/speed of analysis comparison nanoscale LC (top) vs. micro-fluidics (bottom).

Heart Failure

Multivariate analysis of proteins showed that patient samples could be classified using OPLS-DA, using the data and results related to one of the SIL spike levels, as illustrated by the scores distribution in Figure 8. Partial separation (A) of healthy controls and HF (combined HFPEF and HFREF) can be observed. A partial separation model (B) could be developed for HFREF and HFPEF .

The proteins contributing mostly to the separation were ApoA1, CRP and plasma protease C1 inhibitor. Univariate analysis of these three proteins showed significant changes in levels between the groups, as summarized in Figure 9A-C. Good discriminant power was obtained by combining these protein surrogate peptides, with an area under the receiver operating characteristic curve of 0.937 obtained as illustrated in Figure 9D.

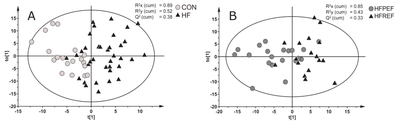


Figure 8. OPLS-DA analysis showing the classification of patient and control samples (circle = normal healthy patients; triangles = heart failure (HFPEF or HFREF) patients (A)) and the classification of HFPEF and HFREF samples (circles = HFPEF patient samples; triangles = HFREF patient samples (B)).

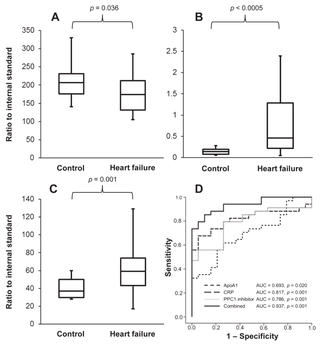


Figure 9. Univariate analysis of ApoA1 (A), CRP (B) and Plasma Protease C1 Inhibitor (C) in HFPEF and HFREF and receiver operating curve performance analysis of peptide surrogates for Apo1, CRP and plasma protease C1 (D).

CONCLUSION

- **IonKey/MS affords twice the throughput and nanoscale LC on average five times the sensitivity (same amounts loaded)**
- **Sensitivity in terms of *S/N* ratio was shown to be roughly comparable across the four MS platforms**
- **The best combination of throughput, sensitivity, linearity and reproducibility was afforded by the IonKey/MS - Xevo TQ-S platform**
- **Multivariate analysis showed that HF samples could be classified using OPLS-DA. Near complete separation of healthy controls and HF (combined HFPEF and HFREF) patients can be observed.**
- **The discriminating proteins primarily contributing to the MVA separation were ApoA1, CRP and plasma protease C1 inhibitor. Univariate analysis of these three proteins showed significant changes in levels between groups**
- **Good discriminating power was obtained by combining these protein surrogate peptides, with an AUC of 0.937 obtained for ROC curve analysis ($p < 0.001$**

References

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