マイクロフロー / タンデム四重極LC-MS / MSのMRM測定による心不全バイオマーカーのトランスレーショナル・リサーチへの応用

Application of Micofluidics / Tandem Quadrupole LC-MS / MS for MRM Based Translational Research Analysis of Putative Heart Failure Peptide Biomarkers in Human Plasma

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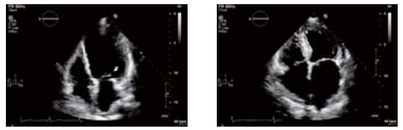
INTRODUCTION

タンデム四重極MSは、感度、選択性、精度及び直線性が高い装置として幅広く応用されている。本研究では、マイクロフローLCと組み合わせたタンデム四重極MSによるヒト血漿中の消化ペプチドの測定を行い、ナノフローLC及び高分解能oa-TOF MSの装置構成と比較を行った。マイクロフローLCは、質量が同一の妨害成分との分離を維持しつつ、感度と測定サイクルの適切なバランスが得られるために、トランスレーショナル・リサーチにおける大規模コホートに適した装置構成と考えられる。

このLC-MS構成を用いて、主要タンパク質の除去を行わずに消化した心不全患者からの血漿試料の測定を行い、先行研究により心血管疾患のバイオマーカー候補と考えられている複数のタンパク質を用いて、心不全患者の分別力を評価した。心不全は検証されたバイオマーカーが、まだ多くなく、複数の表現形の変化が病因と考えられている。この疾患の分別における潜在的な感度と手法有効性の評価を行うために、健常者、左室収縮能が保たれた心不全(HFPEF)患者、左室収縮能が低下した心不全(HFREF)患者の試料の測定を行った。



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.

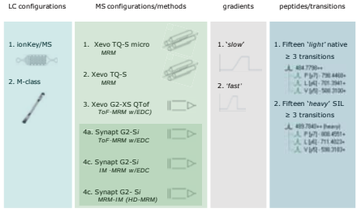


Figure 2. Experimental design LC-MS configuration comparison.

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.

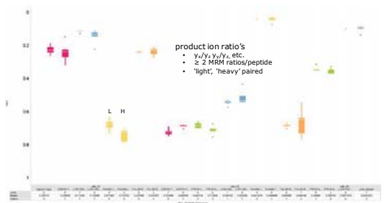


Figure 3. Experiment wide MRM (oaToF) transition evaluation (normalized transition values; $I_{transition\ x}/I_{most\ abundant\ transition}$). L = 'light' (endogenous), H = 'heavy' (SIL).

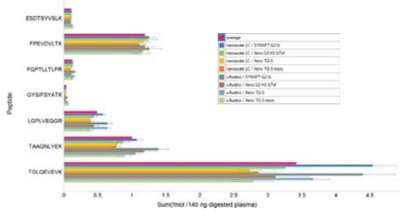


Figure 4. LC-MS configuration average multi-level single point concentration endogenous monitored plasma peptides.

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.

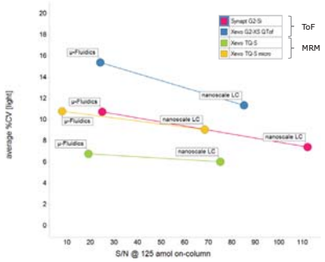


Figure 5. Average experiment wide endogenous multi-level single point peptide MRM CV (%) and S/N LC-MS configurations.

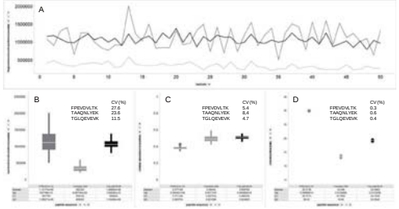


Figure 7. Raw summed MRM transition abundances as a function of replicate experiments (n = 50) for SIL peptides FPEVDVLT[K], TAAQNLYE[K], and TGLQEVEV[K] spiked at a fixed level in different, independent undepleted plasma digest samples (A), raw summed transition intensity variability (B), intra normalized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

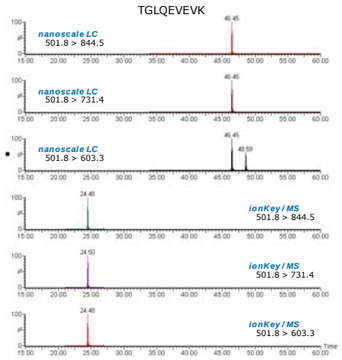


Figure 7. Throughput/speed of analysis comparison nanoscale LC (top) vs. micro-fluidics (bottom).

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.

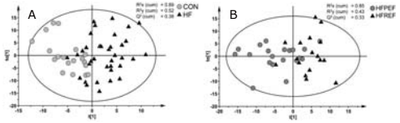


Figure 8. OPLS-DA analysis showing the classification of patient and control samples (circle = normal healthy patients; triangles = heart failure (HFPEF or HFREF) patients (A)) and the classification of HFPEF and HFREF samples (circles = HFPEF patient samples; triangles = HFREF patient samples (B)).

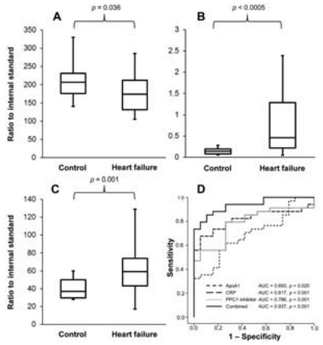


Figure 9. Univariate analysis of ApoA1 (A), CRP (B) and Plasma Protease C1 Inhibitor (C) in HFPEF and HFREF and receiver operating curve performance analysis of peptide surrogates for Apo1, CRP and plasma protease C1 (D).

CONCLUSION

- IonKey / MSは、ナノフローの条件と比べて2倍のスループットが得られた。一方ナノフロー条件の方が5倍の感度が得られた（同じ試料負荷量で）
- S / N比で比較すると、四重極タンデムMSMSと四重極TOFMSで、同程度の結果が得られた
- スループット、感度、直線性、及び再現性で評価すると、マイクロフロー / 四重極タンデムMSMSが最良だった。
- 多変量解析の結果、OPLS-DAにより、心不全の試料を分類できることが示された。HFPEFとHFREFの両方を含む心不全と健常者をほぼ分離できた。
- 多変量解析の第一主成分に最も寄与していたタンパク質は、ApoA1、CRP、及びプラズマプロテアーゼC1インヒビターだった。これらの3つのタンパク質の単変量解析はグループ間の優位な差を示していた。
- これらのタンパク質由来のペプチドを組み合わせることにより、良好な分別力が得られ、ROC曲線分析（p<0.001）において0.937のAUCが得られた。

References

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