Development of Automated SISCAPA Assays for High-Throughput Quantitation of Protein Biomarkers

Christine Miller[3], Leigh Anderson[1], Matt Pope[2], Morteza Razavi[2], Terry Pearson[2]

US HUPO 2012
Poster # 134

Agilent Technologies

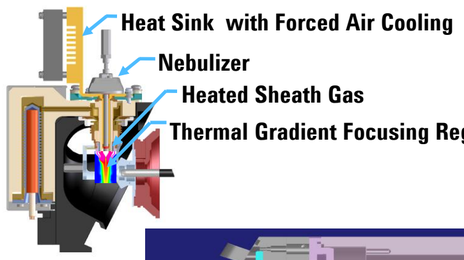
Introduction

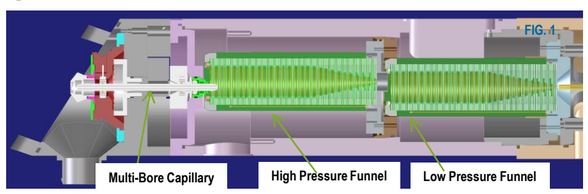
Quantitation of proteotypic peptides in digests of plasma by SRM-MS allows specific, internally-standardized measurement of protein biomarkers and can achieve sub-nanogram/mL detection levels when specific anti-peptide antibodies are used to enrich target peptides from the plasma digests (SISCAPA). We have developed an automated protocol for implementing this immunoaffinity enrichment of biomarker peptides. The effectiveness of the protocol was evaluated using a multiplex SISCAPA panel based on 11 rabbit monoclonal antibodies to specific peptides from 10 proteins spanning a wide plasma concentration range. Parameters for each of the 11 target peptides and cognate labeled standards have been optimized, permitting use of retention-time scheduled MRM data collection and rapid (3 min) analysis times. Results will be presented demonstrating the performance of the workflow for high-throughput quantitation of protein biomarkers.

Ion Funnel Technology

The Agilent 6490 QQQ incorporates iFunnel technology which is a combination of three fundamental innovations:

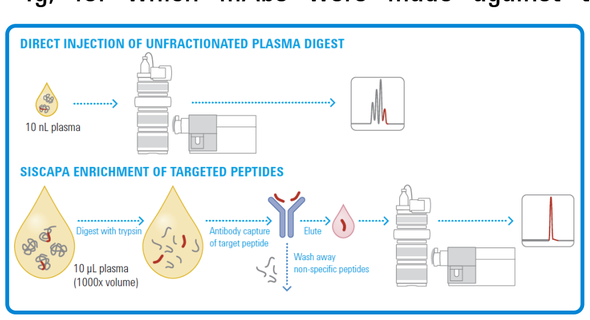
- Agilent Jet Stream technology – ESI with thermal gradient ion focusing confinement
- Hexabore sampling capillary with 6 independent parallel bores to enable sampling a much larger fraction of the ions
- Dual-stage ion funnel for efficient removal of large gas volumes and ion transfer to Q1 optics



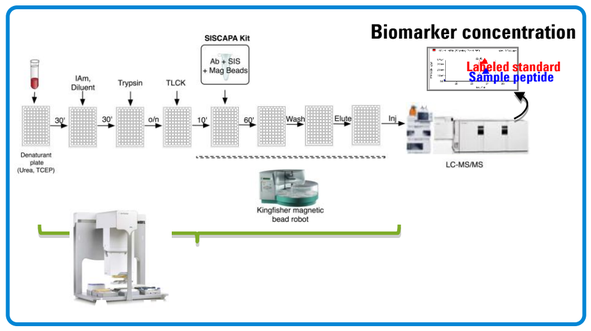


Ion Funnel Technology

For this study, proteotypic tryptic peptides (initially 5 peptides per protein) were selected representing known protein biomarkers: PAI3 (protein C inhibitor), LPS binding protein, transferrin receptor, osteopontin, ferritin light chain, mesothelin, alpha-fetoprotein, HER2/neu, CA-125 and thyroglobulin. Affinity-purified polyclonal antibodies against the two peptides for each protein showing highest titers were characterized in SISCAPA assays, after which rabbit monoclonal antibodies (RabMAbs) were prepared (Epitomics, Inc.) against the best performing peptide for each target, except for Tg, for which mAbs were made against two peptides.



SISCAPA Automation



The multiplexed capture reaction setup, bead wash and analyte elution steps for a SISCAPA assay have been automated on a Bravo Automated Liquid Handling Platform (Agilent) to allow processing of 96 samples in less than 30 minutes. The eluted peptides are delivered in a volume (20 µL) and solvent (5% acetic acid) suitable for subsequent injection into a reversed-phase LC system.

LC/MS Method Development

With the increased sensitivity on the QQQ with iFunnel technology, standard flow LC becomes a workable, sensitive alternative to nanoflow LC-MRM for SISCAPA assays. Standard flow LC with a 2.1 mm column provides superior loading and peak capacity compared to nanoflow which results in excellent separation and retention time reproducibility.

- The initial method had a 20 min runtime (figure A below). Retention time reproducibility was generally less than 0.2% RSD.
- A faster chromatographic method was developed using a 5 minute runtime (Figure B, below) using a 2.1 x 50 mm column. Column temperature was found to be an important parameter in the optimization of the shorter method.
- Using a Poroshell 120 EC-C18 2.1 x 50 mm column, an even faster 3 min method was developed (Figure C, below).

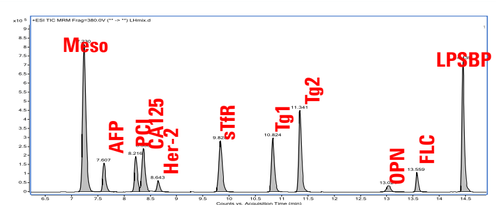


Figure A: Initial 20 min method.

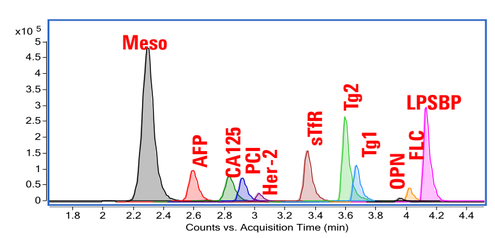


Figure B: Optimized 5 min method.

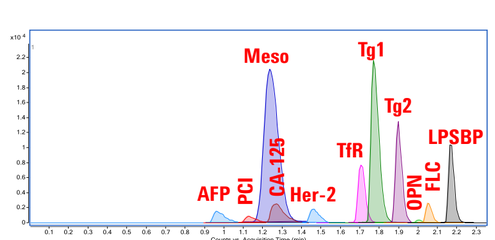
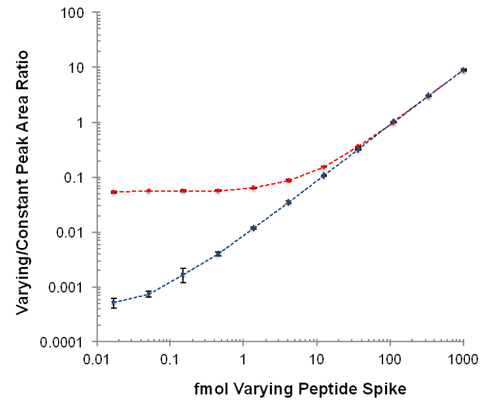


Figure C: Optimized 3 min method.

Results in Pooled Plasma

Using twelve-point dilution curves of both labeled internal standard peptides (i.e., reverse curves) and of unlabeled synthetic peptide (i.e., standard addition curves), we have characterized the response of these 11 assays in pooled human plasma digest matrix. The data clearly demonstrate that a majority of the analytes can be measured in the digest of 10 µl plasma using the optimized standard chromatography-ion-funnel MS method.

Soluble Mesothelin Peptide in Plasma.



The figure above show example standard curves for mesothelin peptide obtained by adding varying amounts of either the heavy internal standard peptide ("Reverse" curve: 3-fold dilutions down from 400 fmol per peptide) or a synthetic version of the endogenous (unlabeled, light) peptide ("Forward" or standard addition curve). The difference between the curves is the presence of endogenous light peptide derived from the endogenous protein in the plasma sample digested to provide the analytical matrix.

- The standard addition curve (Fwd) indicates an endogenous level of 5 fmol/10 µL plasma (18 ng/mL protein) with a 2% average within-run CV.
- The IS dilution curve (Rev) indicates an LOQ of ~50 amol/10 µL plasma (0.2 ng/mL protein) with an 8% average within-run CV.

Ultra High-Throughput

- RapidFire technology includes an ultrafast sampler and SPE-MS analyzer
- Substitutes for LC in high-throughput applications
- Has 7 sec cycle using flow injection analysis
- SISCAPA enrichment may provide some peptides at sufficient purity to allow MRM quantitation without conventional LC separation

Conclusions

- An integrated, automated, high-throughput MS-based protein assay platform is now feasible using Agilent components
- Multiplexed SISCAPA sample preparation can be automated for high-throughput LC/MS assays
- Standard flow LC/MS using ion funnel technology on a QQQ mass spectrometer was equivalent to that achieved previously using nanoflow LC/MS
- Future development will focus on further assessment of the robustness of the rapid LC/MS analysis