Optimization of a Rapid Chromatographic Method for a Multiplexed SISCAPA Assay

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Introduction

Routine quantitation of proteotypic peptides in plasma offers significant analytical challenges in both sample preparation and chromatography. Using specific anti-peptide antibodies to enrich target peptides from the plasma digests (SISCAPA) can both enrich for the target peptides and greatly reduce the complexity of the sample. The relative purity of the SISCAPA sample preparation allows rapid LC/MS analysis to be used which increases the overall throughput of such studies. In this work, we show the optimization of both the chromatographic separation and the mass spectrometric parameters to yield an optimized method for a multiplexed assay.

Experimental

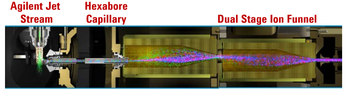
With the goal of developing a rapid (< 5minute) separation, different columns, column temperatures, mobile phases, flow rates and gradients were evaluated in order to select the best conditions for the multiplexed assay. SISCAPA samples were then analyzed using the optimized method.

All analyses were performed on an Agilent 1290 Infinity UHPLC coupled to a 6490 iFunnel QQQ using an Agilent JetStream interface.

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



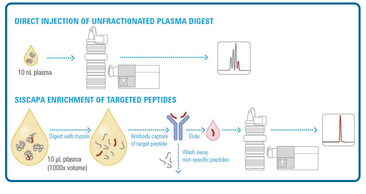
- Agilent JetStream is ESI with thermal gradient ion focusing confinement
- The standard heat sink was modified to provide additional shielding of the nebulizer to accommodate low flow rates

SISCAPA Methodology

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. Proteotypic peptides for thyroglobulin included those reported by Hoofnagle.

Each peptide was synthesized with an added N-terminal cysteine and coupled to KLH. Pools of five such immunogens were injected into two rabbits, whose titers to each peptide were followed by a "peptide ELISA". 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 mAb's were made against two peptides. RAbMAbs were selected for high affinity binding to peptides in solution by surface plasmon resonance analysis.

Each RAbMAb was independently covalently immobilized on magnetic beads and a pool of all 11 RabMAb reagents was used in an automated procedure to capture the 11 corresponding stable isotope labeled internal standards and cognate endogenous peptides from plasma digest matrix, after which bound peptides were eluted for LC-SRM analysis.



Enrich target peptides and decrease sample complexity

The near-purity of these analyte peptides, in contrast to the enormous matrix background of unfractionated plasma digests and their generally low absolute abundance (often sub-fmol) has to date focused attention on nanoflow chromatography and nanospray ESI as the preferred analytical platform despite its limited robustness for high-throughput applications in clinical laboratories.

Results and Discussion

With the increased sensitivity from thermal gradient ion focusing electrospray ionization and increased ion sampling with the hexabore capillary and dual ion funnel gain, standard flow LC becomes a workable, sensitive alternative to nanoflow LC-SRM for SISCAPA assays. For this work, optimized chromatography and MS conditions was developed for standard flow LC using 2.1 mm i.d. columns.

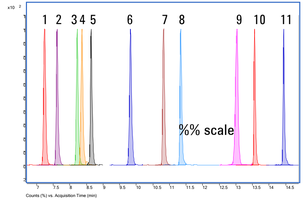
- Synthetic standards for the 11 target peptides (right) were used to optimize the chromatography for the development of a dynamic MRM method.
- The 2.1 mm column provides superior loading and peak capacity compared to nanoflow which results in excellent separation and retention time reproducibility.

#	Protein Name	Target Peptide
1	Mesothelin	LLGPHVEGLK
2	Alpha-fetoprotein	GYQELLEK
3	PAI3	EDGYHYLLDR
4	Mucin-16	ELGPYTLDR
5	Her-2	AVTSANIDEFAGCK
6	Transferrin Receptor	GFVEPDHYVVVGAQR
7	Thyroglobulin	FSPDDSAGASALLR
8	Thyroglobulin	VIFDANAPVAVR
9	Osteopontin	YPDAVATWLNPDPSQK
10	Ferritin light chain	LGGPEAGLGEYLFER
11	LPS binding protein	LAEGFPLPLLK

Initial 25 minute method

Column	**Eclipse Plus EC-C18 1.8 µm 2.1 x 150 mm column at 50°C**
Flow rate and solvents	0.4 mL/min; A= 0.1% formic acid (FA) in water; B= 0.1% FA in 90% acetonitrile (ACN)/water
Gradient	3%B at 0 min, 10%B at 1 min, 20%B at 10 min, 35%B at 14 min, 90%B at 15 min, hold to 19 min then 3%B at 20 min
Time	Stop time 21 min; Post time 5 min

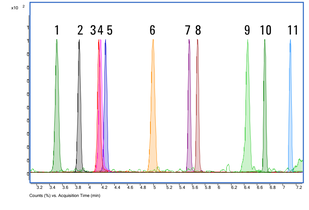
This initial method gave excellent separation (right) and generally good peak shapes. The osteopontin peptide, #9, showed some peak broadening. However, the injection-to-injection time was too long for a high-throughput assay.



11 minute method

Column	**Poroshell 120 EC-C18 2.7 µm 2.1 x 150 mm column at 50°C**
Flow rate and solvents	0.4 mL/min; A= 0.1% formic acid (FA) in water; B= 0.1% FA in 90% acetonitrile (ACN)/water
Gradient	3%B at 0 min, 14%B at 0.1 min, 20%B at 4 min, 35%B at 6 min, 90%B at 6.4 min, then 3%B at 6.6 min
Time	Stop time 7.8 min; Post time 3 min

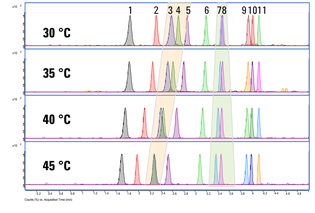
Using a superficially porous chromatographic phase allowed the development of a much faster separation while still using a 150 mm long column. The osteopontin peptide peak shape is still not good as good as the other peaks.



5 minute method with various column temperatures

Column	**Eclipse Plus EC-C18 1.8 µm 2.1 x 100 mm column**
Flow rate and solvents	0.4 mL/min; A= 0.1% formic acid (FA) in water; B= 0.1% FA in 90% acetonitrile (ACN)/water
Gradient	10%B at 0 min, 14%B at 0.01 min, 16%B at 2 min, 22%B at 3 min, 40%B at 3.7 min, 70%B at 3.8 min, then 10%B at 3.9 min
Time	Stop time 5 min; Post time none

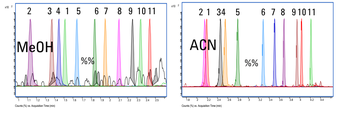
A faster chromatographic method was developed using a 50 mm column which is sufficient for the separation of this multiplexed assay. Column temperature was found to be an important parameter in the optimization of the shorter method



Results and Discussion

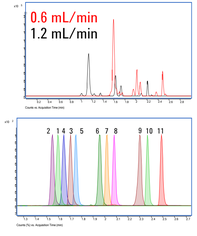
Effect of organic solvent choice

The choice of organic solvent influences not only the separation but also the ionization efficiency of the peptides. While acetonitrile (ACN), an aprotic solvent, is typically selected for peptide separations, methanol (MeOH) was found to yield better sensitivity for some of the peptides. The osteopontin peptide, which is the most problematic in the multiplex, showed poor results in MeOH (right) so ACN (far right) was used for future work.



3 min method: higher flow rates and larger pore columns

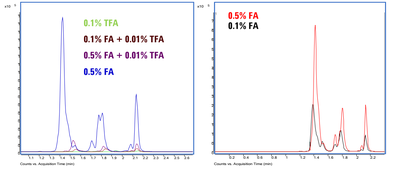
In order to develop the faster method, shorter columns were evaluated at higher flow rates than typically used (up to 1.2 mL/min). While the higher flow rates resulted in faster peak elution, there was a loss in sensitivity that was not acceptable. The column was also changed to a new wide pore sub-2 micron phase which improved the osteopontin peptide peak shape.



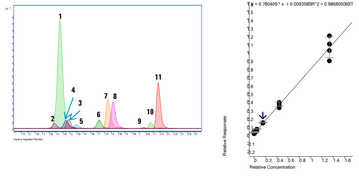
Column	**Zorbax 300SB-C18 RRHD 1.8 µm 2.1 x 50 mm column at 35°C**
Flow rate and solvents	0.6 mL/min; A= 0.1% formic acid (FA) in water; B= 0.1% FA in 90% ACN/water
Gradient	13%B at 0 min, 13%B at 0.5 min, 16%B at 1 min, 22%B at 1.6 min, 40%B at 2.1 min, 70%B at 2.2 min, then 13%B at 2.25 min
Time	Stop time 3 min; Post time none

3 min method: effect of acid modify

LC/MS methods typically use 0.1% formic acid (FA) as the modify for improved sensitivity, although the chromatographic performance is generally superior with trifluoroacetic acid (TFA). Several common modifier were tested (right). As expected, TFA caused major signal suppression (near right). Using 0.5% formic acid as a modifier gave the best results, even better than the 0.1%FA typically used (far right). On average, the peak heights at 0.5% FA were 140% of those at 0.1%FA (range 61 – 216%) while the peak widths were only marginally impacted.



The final method with 0.5%FA was then used on a set of SISCAPA samples. The overlaid MRM chromatograms (below left) and an example calibration curve for the stable-isotope labeled mesothelin peptide (below right) are shown.



Conclusions

- Multiplexed SISCAPA samples are sufficiently cleaned and enrich to allow rapid standard flow chromatography
- Standard flow LC/MS using ion funnel technology on a QQQ mass spectrometer was equivalent to that achieved previous using nanoflow LC/MS
- Method development was done to optimize injection-to-injection cycle time as well as sensitivity
- The optimized 3 minute method was used to analyze a SISCAPA calibration set created from spiking standard peptides into a pooled plasma sample