Optimization of a Rapid Chromatographic Method for a Multiplexed SISCAPA Assay

Christine Miller1, Matt Rope1, Monica Resetti2, Terry Pearson1, Leigh Anderson2
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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 aliquots (SISCAPA) has been the most popular method of target peptide and greatly reduce the complexity of the sample. The relative purity of the SISCAPA sample preparation also allows rapid LC/MS analysis to be used which increased 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.

SISCAPA Methodology

With the increased sensitivity from thermal gradient ion focusing electrospray ionization and increased ion sampling with the breakdown capillary and dual ion funnel standard flow LC/MS became 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 5.1 mm i.d. columns.

- Synthetic standards for the 11 target peptides (right) were used to optimize the chromatography for the thermospray ionization MRM method.
- The 7.1 mm column provides superior leading peak and peak capacity compared to nanoflow which results in excellent separation and retention time reproducibility.

Initial 25 min method

<table>
<thead>
<tr>
<th>Column</th>
<th>Fused silica 75 µm ID x 50 cm (n=3) column at 40 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.6 mL/min; A= 0.1% formic acid (FA) in water; B= 0.1% FA in 90% ACN/water</td>
</tr>
<tr>
<td>Gradient</td>
<td>10%B at 0 min, 14%B at 0.01 min, 16%B at 2 min, 22%B at 3 min, 25%B at 3.1 min, 40%B at 3.7 min, 70%B at 3.8 min, then 10%B at 3.9 min</td>
</tr>
<tr>
<td>Stop time</td>
<td>8 min; Post time 5 min</td>
</tr>
<tr>
<td>Quality</td>
<td>Excellent injection, baseline separation of last peak, no instrument drift</td>
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</tbody>
</table>

Results and Discussion

Effect of solvent choice of analyte

LC/MS methods typically use 1% formic acid (FA) as the mobile phase for improved sensitivity. Although the chromatographic performance is generally superior with trifluoroacetic acid (TFA) Several common modifiers were tested. As expected, TFA caused major signal suppression (near right). Using 0.5% formic acid as a modifier gave better results, even better than the 0.1%FA typically used on (far right). On average, the peak heights at 0.5% F2 were 14% of those at 0.1%FA (range 0.1-21%) while the peak widths were only marginally impacted. The final method with EISMA was then used on a set of SISCAPA samples. The averaged 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 enriched at a rapid standard flow LC/MS technique.
- Standard flow LC/MS using ion funnel technology on a QQQ mass spectrometer was equivalent to that achieved at lower flow rates.
- Method development was done using optimal ion detection to ensure signal stability.
- The optimized 3 min method was used to analyze a SISCAPA calibration set created from spiking standard peptides into a pooled plasma sample.

- Ion Funnel Technology

The Agilent 1200 IQR QTOF coupled to a high performance ion funnel technology on a D2000 Famos 5 i.d. column at 40 °C using an Agilent JMSJet interface.

- Proteotypic tryptic peptides (initially 5 peptides per protein) were selected representing known protein biomarkers: PAZ, P3CD, G cysteine, LAP, binding protein, transmembrane receptor, ferritin light chain, transferrin, alpha-1 antisemiphenin, and thyroglobulin. Prototypic peptides for thyroglobulin included those reported by Harlow and Walters.

Each sample was subjected to an additional tryptic digestion and peptide identification using LC/MS/MS. Peaks within four technical replicates were identified as tryptic peptides, chosen to be at least 50% relative intensity.


- Enrich target peptides and decrease sample complexity

The complexity of these sets is reduced significantly by enriching the peptide mixture with specific antibodies followed by on-column de-salting (off-column sub-flow) to have a focused attention on nonfetal chromatography and improved mass accuracy. The performance of an analytical platform despite its focused redemptions for high-throughput applications in clinical laboratories.

Enrich target peptides and decrease sample complexity

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

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