Therapeutic peptides are highly potent, selective, relatively safe and well tolerated by humans which differentiates them from traditional small molecule drugs. Based on recent data, more than 60 peptide drugs have reached the market for treatment of a wide range of diseases including bone and prostate cancer, type 2 diabetes, retinopathy, heart failure and several others with increasing market potential evidenced by ~140 peptide development1. Therapeutic peptides are susceptible to proteolytic cleavages by serum and tissue proteases and potential biotransformation of their amino acids. Hence, there is a growing need for identifying every detectable metabolite or peptide catabolite during drug development and to monitor peptide biotransformations across the experimental sampling interval. Advancements in Liquid Chromatography (LC) coupled to Tandem Mass Spectrometry (MS/MS) have enabled comprehensive identification of peptide catabolites in a complex system such as serum of plasma, which is critical during drug discovery and development. The purpose of this study was to develop an integrated microflow LC-MS/MS solution coupled to the SCIEX M3 MicroLC system to take advantage of the gains in sensitivity from microflow LC and to identify peptide catabolites at low levels. The increased sensitivity is critical when the sample volume is limited as in small animal studies.

Materials and Methods

Sample Preparation: Three therapeutic peptides (human parathyroid hormone fragment 1-34 (PTH), human calcitonin gene related peptide (CGRP) and human angiotensin II receptor antagonist [1-17] (AII-Ra), obtained from Sigma-Aldrich were spiked in 50 µl of rat plasma (0.1 mg/ml) and were a control in HPLC grade water. Each incubation at 37 °C for 5, 10, 30 and 60 minutes was performed in cold acetonitrile (30 min at 4 °C) and centrifuged at 14000 RPM for 15 minutes. Aliquots of 10 µl of supernatant were transferred to 96 well plates and dried using a TurboVap expression using no heat. Pellet was reconstituted in 1000 µl of 2% formic acid in water prior to LC-MS/MS analysis. 50-μl mix plasma spiked with water was prepared under same condition, as control plasma sample.

Analytical Liquid Chromatography: A Shimadzu Prominence HPLC system with two LC-20AD pumps, CTO-20S OA column oven and a 5-μL OA autosampler was used for analytical-flow LC-MS/MS analysis. The column was 100 x 2.1 mm i.d. Inertsil C18 5 μm (Phenomenex). Mobile phase A, 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 350 µl/min. UV detection for the autosampler was 204/200 nm methanol/acetonitrile, injection volume was 5 μl, and the column was kept at 35 °C. The LC gradient method used for both analytical and microflow LC is listed in Table 1.

Microflow Liquid Chromatography: A SCIEX M3 MicroLC system, with an integrated autosampler, was used in direct injection mode in combination with a source mounted sub-column (SCIEX). A 50 x 0.2 mm i.d. MALDI PEEK C18 3.7 μm 160 A column was used with SCIEX. Mobile phase A water with 0.1% formic acid, and mobile phase B, acetonitrile with 0.1% formic acid was used at a flow rate of 10 µl/min. The column temperature was set to 35 °C. Injection volume was 5 µl, and the autosampler needle and needle connected of 1 µl/s using mobile phase B, followed by two times using mobile phase A.

MS/MS Conditions: For the microflow LC experiments, the standard electrode (100 µm i.d) was replaced in a 25 µm i.d nozzle with Duplex™ Source and TripleTOF® 6600 (SCIEX). LC-MS/MS data was collected with GDA (m/z 350-1600, Top 10, CE 40±5) and a SCIEX QTRAP®6500 with fixed window of 2500. The collision gas source parameter were used for each channel LC experiments.

Data Analysis: MetabolitePilot™ 2.0 was used for peptide catabolite identification and PeakView™ 2.2 software was used to compare the sensitivity of the microflow and analytical-flow LC-MS/MS data for the peptides top three catabolites. The improved S/N ratio with microflow LC resulted in detection of a greater number of (low concentration) peptide catabolites and often lack required sensitivity to identify low level metabolites. The microflow LC at 10 μl injection on average 15X improvement in signal-to-noise (S/N) ratio when compared to analytical flow LC for the top three major peptide catabolites detected after 50 min of incubation in rat plasma. This improvement in S/N ratio was critical when the sample volume is limited as in small animal studies, data shown in Figure 4-6.

Results

Most LC-MS/MS methods for bioanalytical studies use analytical flow rates of 350-500 µl/min to profile peptide catabolites and often lack the required sensitivity to identify low level metabolites. The microflow LC method at 10 μl injection on average 15X improvement in signal-to-noise (S/N) ratio when compared to analytical flow LC for the top three major peptide catabolites detected after 50 min of incubation in rat plasma. This improvement in S/N ratio was critical when the sample volume is limited as in small animal studies, data shown in Figure 4-6.

The PeakView® extracted Ion chromatogram (XIC) for three therapeutic peptides shows narrow peak widths and separation of these peptides with both analytical and microflow LC-MS/MS (Figure 3).

The therapeutic peptides and their top three catabolism products identified. The XIC of these three peptides were monitored to check sensitivity gain by microflow LC-MS/MS. The increased sensitivity is critical when the sample volume is limited as in small animal studies. The LC-MS/MS XIC profile from the SWATH acquisition of the control samples of rat plasma substrates and peptides show baseline chromatographic separation for these therapeutic peptides.

Table 1. LC gradient used for traditional and microflow LC-MS.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>5 - 10</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>10 - 20</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>20 - 35</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>35 - 37</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 2. The therapeutic peptide and their top three catabolism products identified. The XIC of these three peptides were monitored to check sensitivity gain by microflow LC-MS/MS.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Catabolite</th>
<th>Signal-to-Noise Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH</td>
<td>PTH(1-34)-1-33</td>
<td>45X</td>
</tr>
<tr>
<td>CGRP</td>
<td>CGRP(1-37)-36</td>
<td>15X</td>
</tr>
<tr>
<td>AII-Ra</td>
<td>AII-Ra(1-17)-16</td>
<td>20X</td>
</tr>
</tbody>
</table>

The improved S/N ratio with microflow LC resulted in detection of a greater number of low concentration peptide catabolites when compared to traditional analytical flow LC-MS method (Figure 6). The improved S/N ratio with microflow LC resulted in detection of a greater number of low concentration peptide catabolites when compared to traditional analytical flow LC-MS method (Figure 6).

Peptide Catabolites Coverage

The top three catabolites for each of the therapeutic peptides identified were monitored by both analytical and microflow LC-MS/MS. The list of the intact peptides and the catabolites monitored and detected are listed in Table 2.

Improved Sensitivity

The Improved S/N ratio with microflow LC resulted in detection of a greater number of low concentration peptide catabolites when compared to traditional analytical flow LC-MS method (Figure 6).

Improved Catabolite Coverage

The Fastest, Robust, and Reliable Method for Monitoring Peptide Catabolism in Plasma. Signal-to-noise ratio improvement of up to 46X enables detection of catabolism products at low levels. Microflow LC method coupled to the high resolution TripleTOF® 6600 system provides optimal sensitivity and confidence to identify the low-level metabolites in complex samples when sample volume is limiting factor.

References


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