An Improved SPE/LC/MS/MS Platform for the Simultaneous Quantitation of Multiple Amyloid β Peptides in Cerebrospinal Fluid for Preclinical or Biomarker Discovery

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APPLICATION BENEFITS

- Improved sensitivity using Xevo TQ-S.
- Reduced sample size required using Xevo TQ-S.
- Fast, flexible platform for peptide quantitation.
- One LC/MS/MS method for quantitation of multiple peptides, reduced reliance on ligand-binding assays in discovery segment.
- Highly selective sample preparation using Oasis® mixed-mode sorbent in µElution format.
- Resolution, sensitivity of ACQUITY UPLC® PST columns for improved separation with shorter run times.

INTRODUCTION

A previous application note (720003682en) described in detail the development of a fast, flexible SPE/LC/MS/MS platform for the quantification of multiple amyloid beta (aβ) peptides from human or monkey CSF for use in a biomarker or preclinical discovery setting. In this work, the mass spectrometry platform has been updated from the Xevo® TQ MS to the Xevo TQ-S mass spectrometry system. This change facilitated both a 4X reduction in required sample size and a 4-5X increase in assay sensitivity.

Historically, quantification of aβ peptides in biological fluids has relied mainly on the use of immunoassays, such as ELISA. These assays are time consuming and expensive to develop, labor intensive, are subject to cross reactivity, and an individual assay is required for each peptide. In order to meet the throughput requirements and constant flow of demands for new peptide methods in a discovery setting, there is a need for a highly specific yet flexible methodology based on an LC/MS/MS platform. In this work, this platform is coupled with selective sample preparation for the simultaneous quantitation of multiple aβ peptides. This work focuses on methods for the 1-38, 1-40, and 1-42 aβ peptides, in support of preclinical and biomarker discovery studies. Sequence, pI and molecular weight (MW) information for these peptides is shown in Figure 1.

The solid-phase extraction (SPE) sample preparation protocol used to enrich the amyloid beta fraction in CSF is the protocol previously described. However, the sample size required is now only 50 µL instead of 200 µL. The SPE method concentrates the sample to improve detection limits while eliminating matrix interferences and optimizing solubility of the aβ peptides in the mass spectrometer injection solvent.

As strategies emerge for disease modification in Alzheimer’s Disease (AD), the quantification of other aβ species (in addition to aβ 38, 40, and 42) that may be linked to AD pathology may be required. The method described herein shows promise for adaptation to quantify those peptides as well.

Amyloid β 1-38
DAEFRHDSGYEVHHQKLVELFAEDVGSNKGAIIGLMVGG
MW 4132 pI  5.2

Amyloid β 1-40
DAEFRHDSGYEVHHQKLVELFAEDVGSNKAIIGLMVGGV
MW 4330 pI  5.2

Amyloid β 1-42
DAEFRHDSGYEVHHQKLVELFAEDVGSNKAIIGLMVGGVV
MW 4516 pI  5.2

Figure 1. Sequence, MW, and pI information for amyloid β peptides.
EXPERIMENTAL

ACQUITY UPLC Conditions

Column: ACQUITY UPLC BEH C18 300Å, 2.1 x 150 mm, 1.7 μm, Peptide Separation Technology

Part Number: 186003687

Column temp.: 50 °C
Sample temp.: 15 °C
Injection volume: 10.0 µL
Injection mode: Partial Loop
Flow rate: 0.2 mL/min.
Mobile phase A: 0.3% NH₄OH in H₂O
Mobile phase B: 90/10 ACN/mobile phase A

Strong needle wash: 60:40 ACN:IPA + 10% conc. NH₄OH (600 µL)
Weak needle wash: 90:10 0.3% NH₄OH in H₂O:ACN (400 µL)

Gradient:
Time Profile Curve (min) %A %B
0.0 90 10 6
1.0 90 10 6
6.5 55 45 6
6.7 55 45 6
7.0 90 10 6

Waters Xevo TQ-S MS Conditions, Electro spray Positive

Capillary Voltage: 2.5 V
Desolvation Temp: 450 °C
Cone Gas Flow: Not used
Desolvation Gas Flow: 800 L/Hr
Collision Cell Pressure: 2.6 x 10(-3) mbar

MRM transition monitored, ESI+: See Table 1

Table 1. MRM transitions and MS conditions for the amyloid β peptides and their N15 labeled internal standards.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Precursor Ion 4+</th>
<th>Product Ion 4+</th>
<th>Product Ion i.d.</th>
<th>Cone Voltage (V)</th>
<th>Collision Energy (eV)</th>
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</thead>
<tbody>
<tr>
<td>Amyloid β 1-38</td>
<td>1033.5</td>
<td>1000.3</td>
<td>b 36</td>
<td>33</td>
<td>23</td>
</tr>
<tr>
<td>Amyloid β 1-38 N15 IS</td>
<td>1046</td>
<td>1012.5</td>
<td></td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Amyloid β 1-40</td>
<td>1083</td>
<td>1053.6</td>
<td>b 39</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td>Amyloid β 1-40 N15 IS</td>
<td>1096</td>
<td>1066.5</td>
<td></td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td>Amyloid β 1-42</td>
<td>1129</td>
<td>1078.5</td>
<td>b 40</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Amyloid β 1-42 N15 IS</td>
<td>1142.5</td>
<td>1091.5</td>
<td></td>
<td>35</td>
<td>28</td>
</tr>
</tbody>
</table>

SPE Conditions

Sample Pre-treatment

50 µL human CSF or spiked artificial CSF + 5% rat plasma was diluted 1:1 with 5 M guanidine HCl and shaken at room temperature for 45 minutes. This was then diluted further with 50 µL 4% H₃PO₄ in H₂O and mixed.

Note: For spiked samples, samples were allowed to equilibrate at room temperature for 30 min after spiking and prior to dilution with guanidine HCl.

Sample Extraction with Oasis MCX

Samples were extracted according to the protocol in Figure 2 below. All solutions are made up by volume. All steps applied to wells of µElution plate containing samples

Oasis MCX µElution Protocol

Part Number: 186001830BA

Condition: 200 µL MeOH
Equilibrate: 200 µL 4% H₃PO₄ in H₂O
Load: 150 µL pretreated CSF
Wash 1: 200 µL 4% H₃PO₄ in H₂O
Wash 2: 200 µL 10% ACN
Elution: 2 x 25 µL 75/15/10 ACN/H₂O/conc. NH₄OH
Dilute: 25 µL H₂O

Figure 2. Oasis µElution MCX extraction protocol
RESULTS AND DISCUSSION

Mass Spectrometry

MS was performed in positive ion mode since CID of the 4+ precursor ion yielded several distinct product ions corresponding to specific b sequence ions (representative spectrum shown in Figure 3.)

The sensitivity increase provided by the Xevo TQ-S facilitated the use of 4X less sample whilst also improving detection limits by approximately 4-5X compared to the previous assay on the Xevo TQ. The lowest QC sample tested was 5X lower in concentration than the low QC when the standard Xevo TQ MS system was used. Earlier work by Rainville and Booth (application note 720003415en) describes the system improvement in more depth and demonstrates a similar sensitivity increase for the therapeutic peptide desmopressin.

Mass range of the instrument was also an important factor in obtaining specificity. The Xevo TQ-S MS has a mass range of 2048 on both quads, easily allowing us to choose a more specific 4+ rather than 5+ precursor and fragment pair.

UPLC Separation

Separation of the three amyloid β peptides is shown in Figure 4. While the exact amount of NH₄OH in the mobile phase was critical for negative ion sensitivity, the signal in ESI positive proved to be more robust to subtle changes in mobile phase composition, providing a minimum of >24 hour LC/autosampler stability. In contrast, 50% or more of the ESI negative signal was lost after 10-12 hours due to the natural change in NH₄OH concentration (volatility) in the mobile phase. This further reinforced the robustness of an ESI positive MS method.
Sample Preparation: SPE

SPE was performed using Oasis MCX, a mixed-mode sorbent, to enhance selectivity of the extraction. The sorbent relies on both reversed-phase and ion-exchange retention mechanisms to selectively separate the αβ fraction from other high abundance polypeptides in complex CSF samples. The Oasis µElution plate (96-well format) provided sample concentration, eliminating the need for evaporation and reconstitution. This has the benefit of saving time and eliminating peptide losses due to adsorption to the walls of the collection plate during dry down.

During initial method development, a high degree of non-specific binding (NSB) was observed when artificial CSF was extracted. Thus, 5% rat plasma (having a different amyloid β sequence) was added to bind to surfaces, eliminating NSB.

The SPE method was one of the more critical aspects of the overall methodology. Very selective isolation of the amyloid fraction coupled with the resolution of analytical-scale flow UPLC®, facilitates analysis of pre-clinical samples without the need for antibodies or time-consuming immuno-precipitation associated with ELISA methods. The increased sensitivity of the Xevo TQ-S enabled the sample volume to be reduced from 200 µL to 50 µL of CSF, making this method amenable to use in pre-clinical species.

![Image](image.png)

Figure 4. Representative UPLC/MS/MS analysis of amyloid β 1-38, 1-40, and 1-42 peptides extracted from artificial CSF + 5% rat plasma.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>200 µL Sample Xevo TQ</th>
<th>50 µL Sample Xevo TQ-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Curve Range</td>
<td>0.1-10 ng/mL</td>
<td>0.025 or 0.05-10 ng/mL</td>
</tr>
<tr>
<td>QC Range</td>
<td>0.2-6 ng/mL</td>
<td>0.04-6 ng/mL</td>
</tr>
</tbody>
</table>
Quantitation of Amyloid β Peptides in CSF

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Std. Conc.</th>
<th>RT</th>
<th>Area</th>
<th>IS Area</th>
<th>Response</th>
<th>Conc.</th>
<th>%Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank artificial CSF</td>
<td></td>
<td>5.73</td>
<td>19.7</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.05</td>
<td>5.71</td>
<td>230.4</td>
<td>3620.5</td>
<td>0.064</td>
<td>0.057</td>
<td>14</td>
</tr>
<tr>
<td>100 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.1</td>
<td>5.71</td>
<td>390.8</td>
<td>3585.1</td>
<td>0.109</td>
<td>0.108</td>
<td>8.1</td>
</tr>
<tr>
<td>250 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.25</td>
<td>5.71</td>
<td>778.3</td>
<td>3737.3</td>
<td>0.208</td>
<td>0.220</td>
<td>-12</td>
</tr>
<tr>
<td>350 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.35</td>
<td>5.71</td>
<td>1267.3</td>
<td>3693.8</td>
<td>0.343</td>
<td>0.372</td>
<td>6.2</td>
</tr>
<tr>
<td>500 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.5</td>
<td>5.71</td>
<td>1494.7</td>
<td>3566.8</td>
<td>0.419</td>
<td>0.457</td>
<td>-8.5</td>
</tr>
<tr>
<td>750 pg/mL artificial CSF</td>
<td>Standard</td>
<td>0.75</td>
<td>5.71</td>
<td>2733.5</td>
<td>4152.0</td>
<td>0.658</td>
<td>0.727</td>
<td>-3.1</td>
</tr>
<tr>
<td>1 ng/mL artificial CSF</td>
<td>Standard</td>
<td>1</td>
<td>5.71</td>
<td>3166.8</td>
<td>3792.5</td>
<td>0.835</td>
<td>0.926</td>
<td>-7.4</td>
</tr>
<tr>
<td>5 ng/mL artificial CSF</td>
<td>Standard</td>
<td>5</td>
<td>5.72</td>
<td>14773.9</td>
<td>3877.0</td>
<td>4.693</td>
<td>5.270</td>
<td>5.4</td>
</tr>
<tr>
<td>7.5 ng/mL artificial CSF</td>
<td>Standard</td>
<td>7.5</td>
<td>5.72</td>
<td>24763.9</td>
<td>3877.0</td>
<td>9.104</td>
<td>10.238</td>
<td>-5</td>
</tr>
<tr>
<td>10 ng/mL artificial CSF</td>
<td>Standard</td>
<td>10</td>
<td>5.72</td>
<td>33343.3</td>
<td>3662.5</td>
<td>9.104</td>
<td>10.238</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Figure 5. Representative standard curve and statistics from 0.05-10 ng/mL for amyloid β 1-42 extracted from artificial CSF + 5% rat plasma.

Table 3. Baseline levels of amyloid β peptides in 2 sources of pooled human CSF.

<table>
<thead>
<tr>
<th></th>
<th>Average Basal Level</th>
<th>% RSD of Basal Level</th>
<th>IS % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid β 1-38 Human CSF 1</td>
<td>1.396</td>
<td>5.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Amyloid β 1-38 Human CSF 2</td>
<td>0.702</td>
<td>1.7</td>
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</tr>
<tr>
<td>Amyloid β 1-40 Human CSF 1</td>
<td>5.429</td>
<td>3.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Amyloid β 1-40 Human CSF 2</td>
<td>2.611</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Amyloid β 1-42 Human CSF 1</td>
<td>0.458</td>
<td>5.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Amyloid β 1-42 Human CSF 2</td>
<td>0.226</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Average deviation values for all overspike QC samples.

<table>
<thead>
<tr>
<th></th>
<th>QC 0.04 ng/mL</th>
<th>QC 0.075 ng/mL</th>
<th>QC 0.15 ng/mL</th>
<th>QC 0.2 ng/mL</th>
<th>QC 0.8 ng/mL</th>
<th>QC 2 ng/mL</th>
<th>QC 6 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid β 1-38 Human CSF 1 and 2</td>
<td>2.3</td>
<td>5.8</td>
<td>-3.2</td>
<td>7.3</td>
<td>14.8</td>
<td>5.1</td>
<td>13.1</td>
</tr>
<tr>
<td>Amyloid β 1-40 Human CSF 1 and 2</td>
<td>-0.8</td>
<td>-3.2</td>
<td>-1.9</td>
<td>2.5</td>
<td>-2.6</td>
<td>-4.2</td>
<td>-3.8</td>
</tr>
<tr>
<td>Amyloid β 1-42 Human CSF 1 and 2</td>
<td>1.3</td>
<td>13.4</td>
<td>-3.6</td>
<td>5.6</td>
<td>2.0</td>
<td>-0.6</td>
<td>-0.2</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

- The increased sensitivity of the Xevo TQ-S triple quadrupole mass spectrometer facilitated the use of 4X less sample and a 4-5X improvement in quantification limits.

- An SPE-UPLC/MS/MS bioanalytical method was developed and validated for the simultaneous quantitation of multiple amyloid β peptides in human and monkey CSF.

- The combination of a highly selective extraction method based on mixed-mode SPE in μElution format and the resolution of UPLC chromatography was critical to achieving the accurate, precise and reliable quantitation of 3 major amyloid β peptides in human and monkey CSF.

- The use of positive ion MS/MS and b ion sequence fragments provided the MS specificity required for this application.

- 96 samples can be extracted and ready for injection in <30 minutes, providing the sample prep throughput required for pre-clinical and clinical studies.

- The method described herein eliminates time-consuming immunoassays or immunoprecipitation steps for pre-clinical work.

- This approach also allows one assay for the simultaneous measurement of several different amyloid β peptides from a single sample. This single assay provides a high degree of selectivity and specificity in a high-throughput format while still achieving the high sensitivity required for low level endogenous amyloid β peptides.

- The use of a single UPLC/MS/MS assay represents a significant advantage over an ELISA assay, which would require multiple assays with multiple antibodies to quantify each of the relevant peptides.

ORDERING INFORMATION

<table>
<thead>
<tr>
<th>Products Used in this Application</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oasis MCX μElution Plate</td>
<td>186001830BA</td>
</tr>
<tr>
<td>ACQUITY UPLC BEH C₁₈, 300Å, 2.1 x 150 mm, 1.7 μm, Peptide Separation Technology Column</td>
<td>186003687</td>
</tr>
</tbody>
</table>

References