INTRODUCTION

The deposition/formation of insoluble aggregates of amyloid peptides (Aβ) in the brain is considered to be a critical event in Alzheimer's Disease (AD). Therapeutic strategies have focused on small molecule inhibitors of amyloid beta production or enhancing its clearance. Therefore, the need for highly sensitive and robust methods for quantification of amyloid peptides in cerebrospinal fluid and their relationship to AD is of critical interest to many researchers. However, analysis of these Aβ peptides is extremely challenging due not only to the relatively low abundance in biological fluids but also because of a propensity for aggregation, which they are potentially bound by other proteins and have the tendency to form oligomers.

Measurement of these peptides routinely employs immunoassays (for their selectivity and sensitivity), or tedious immunoprecipitation followed by SPE. Immunoassays require more time to develop than LC/MS/MS assays, they require multiple assays for multiple Aβ species, and suffer from interferences from non-specific binding, poor solubility, aggregation and lower MS sensitivity. SPE is practical, however, for the earlier stages of the development process, it is hardly feasible for the earlier stages of the development process, where a high throughput, reliable method that can handle quantifying multiple peptides in a class is desirable.

This work focuses on the development of LC-MS, and selective SPE sample preparation methods for the 1-38, 1-40, and 1-42 fragments of amyloid beta peptides (Aβ). This is important in selecting appropriate methods for these issues. Therefore, there exists a need for a high throughput selective biomedical method to assess LC/MS/MS with sample preparation capable of recovering pg/mL levels of amyloid peptides in the presence of high concentrations of human and monkey CSF.

While the time required developing immunoassays may be acceptable in later stages of drug development, it is hardly feasible for the earlier stages, where a high throughput, reliable method capable of quantifying multiple peptides in a class is desirable.

RESULTS AND DISCUSSION

The greatest challenges encountered in the development of these methodologies were overcoming solubility, adhesion, and aggregation issues and obtaining adequate selectivity and sensitivity to meet the application requirements. Proper mobile phase and injection solvent composition, as well as judicious choice of SPE elution solvents, meet the application requirements. Proper mobile phase and injection solvent composition, as well as judicious choice of SPE elution solvents, meet the application requirements.

Mass Spectrometry

MS was performed in positive ion mode as CID of the 4+ precursor yielded several distinct product ions (Figure 2). A representative spectrum showing in Figure 3 corresponds to MS/MS sequence ions in human negative ion mode yielded a dominant water loss. Figure 3 demonstrates one example of the specificity difference between both methods. Although overall sensitivity was higher in ion-vent standards using the negative ion mode, the sensitivity difference was mitigated in the presence of matrix and the improved specificity and signal to noise ratio. Positive ion mode proved critical for accurate quantification in CSF samples.

UPLC

Separation of the amyloid β peptides is shown in Figure 4. While exact N15/HNOH in the mobile phase critical for negative ion sensitivity, the signal in ESI+ proved to be more robust to sub-flux changes in mobile phase composition, providing a minimum of +24 hour LC/autosampler stability. In contrast, 50% or more of the ESI-signal was lost after 10-12 hours due to the change in N15/HNOH concentration (volatility) in the mobile phase. This further reinforced the robustness of an ESI+ MS method.

Solid Phase Extraction (SPE)

SPE was performed using Oasis® HLB, 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 amyloid fraction from other high abundance polypeptides in complex CSF samples. The specific 96-well format used, Oasis® HLB, provided significant concentration while eliminating evaporation and reconstitution, minimizing peptide losses. In addition, binding of the peptides by ion-exchange imparted orthogonality into the overall method.

During initial method development a high degree of non-specific binding (NSB) was observed when artificial CSF was extracted. 5% rat plasma pre-treatment resulted in a 98% reduction of NSB. The ESI+ was one of the more critical aspects of the overall method. Very selective isolation of the amyloid fraction coupled to the resolution of standard flow UPLC enabled very rapid analysis of pre-clinical samples.

Linearity, Accuracy and Precision

N15 labeled internal standards were used for each peptide. Standard curves for all amyloid β peptides were linear, with R2 exceeding 0.99. RSQC samples were analyzed with a base level of 0.1 to 10 ng/mL in artificial CSF + 5% rat plasma. A representative standard curve for 1-38 is shown in Figure 5. Baseline levels of the amyloid peptides were quantitated using both standard curves prepared before and after SPE sample preparation. The artificial CSF was used to obtain baseline levels for each of the 1-38, 42 available matrix. Basal levels of amyloid β1-42 extracted from 3 sources of human and 1 source of monkey CSF are shown in Figure 6. Statistics for the determination of basal levels of all 3 amyloid β peptides are shown in Table 2. QC samples were prepared in 3 sources of pooled hu-man CSF and 1 source of pooled monkey CSF at 0, 2, 8, 42 and 56 ng/mL. Accuracy and precision data are available upon request.

LC/MS/MS Conditions

1. Acquisition: ESI+ in positive ion mode or ESI- in negative ion mode. For peptides of interest, the precursor ion was fragmented using CID in both positive and negative ion modes.
2. Collision energy (V): 20 for all precursors.
3. M/z selection: Selected precursor m/z values for fragmentations were 74, 76, 82, 92, and 94. The product ions were selected for fragmentation.
4. MS was performed in positive ion mode as CID of the 4+ precursor yielded several distinct product ions (Figure 3) corresponding to MS/MS sequence ions. NSRB in negative ion mode yielded a dominant water loss. Figure 3 demonstrates one example of the specificity difference between both methods. Although overall sensitivity was higher in ion-vent standards using the negative ion mode, the sensitivity difference was mitigated in the presence of matrix and the improved specificity and signal to noise ratio. Positive ion mode proved critical for accurate quantification in CSF samples.

Figure 1: Amino acid sequence and pI data for amyloid peptides 1-38, 1-40, and 1-42.

Table 1: Representative MS/MS analysis of amyloid β1-38, 1-40 and 1-42 peptides extracted from artificial CSF + 5% rat plasma.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>m/z (Da)</th>
<th>Charge</th>
<th>Product ions</th>
<th>Intensity %</th>
</tr>
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<tbody>
<tr>
<td>Amyloid β1-38</td>
<td>1000.5</td>
<td>4+</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>Amyloid β1-40</td>
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<td>4+</td>
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<td>80</td>
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<tr>
<td>Amyloid β1-42</td>
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<td>5</td>
<td>70</td>
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</tbody>
</table>

EXPERIMENTAL

UPLC Method Conditions

Column: ACQUITY UPLC® BEH C18 2.1 x 100 mm, 1.7 μm Mobile Phase: A: 0.1% NH4OH (by volume) in H2O, B: 0.1% NH4OH (by volume) in H2O, formic acid, 1% methanol Gradient: hold 90% A for 1 min, raise to 55% A in 5 min, hold 0 min return to initial Flow Rate: 0.2 mL/min Injection Volume: 5 μL

LC/MS/MS Conditions

Temperature: 50°C System: Waters Xevo™ TQ triple quadrupole mass spectrometer equipped with ESI+ MM mode Desolvation Gas Flow: 500 L/hr Desolvation Temperature: 350°C Cone Voltage: 50 V Collision Energy: 10 mV MRM Transitions and conditions: see Table 1 Sample Pre-treatment

200 μL CSF (human, monkey, or spiked artificial CSF) was diluted 1:1 with 5M guanidine HCL and diluted: 25 μL H2O 200 μL CSF (human, monkey, or spiked artificial CSF + 5% rat plasma) was diluted 1:1 with 5M guanidine HCL and 250 μL of the diluted sample was injected into the UPLC® Method Conditions. The specific 96-well format used, Oasis® HLB, provided significant concentration while eliminating evaporation and reconstitution, minimizing peptide losses. In addition, binding of the peptides by ion-exchange imparted orthogonality into the overall method.

ACKNOWLEDGEMENT

The authors wish to acknowledge Wenlin Li (Pfizer) for her early work on amyloid β peptides using immunofinity LC/MS/MS.


SELECTED REFERENCES


Table 1: Representative results from analysis of QC samples prepared in pooled human CSF, source 3

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Table 2: Representative results from analysis of QC samples prepared in pooled human CSF, source 3

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