ANALYTICAL CHALLENGES IN THE DEVELOPMENT OF A QUANTITATIVE SPE/LC/MS/MS ASSAY FOR AMYLOID BETA PEPTIDES IN CEREBROSPINAL FLUID

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INTRODUCTION

The deposition/formation of insoluble aggregates of amyloid βpeptides (Aβ) in the brain is considered to be a critical event in Alzheimer's Disease (AD). Therapeutic strategies have focused on small molecule inhibitors or immunotherapy in lowering amyloid beta production or enhancing its clearance. Therefore, the need for highly sensitive and robust methods for quantitation of amyloid peptides in cerebrospinal fluid and their relationship to AD is of critical interest to many researchers. However, the analysis of these Aβpeptides is extremely challenging due not only to the relatively low abundance in biological fluids but also because they have a propensity for aggregation, 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 peptides, and have a limited linear dynamic range relative to LC/MS/MS. Immunoassays are subject to cross reactivity and non-specific binding, require expensive antibodies and rely on the selectivity of the antibody to enrich the sample/species. They are labor intensive and assay imprecision and matrix interferences are common issues. Therefore, there exists a need for a high throughput selective bioanalytical method based on LC/MS/MS with sample preparation capable of recovering pg/mL levels of amyloid peptides in the presence of high concentrations of interfering proteins and peptides.

While the time required developing immunoassays may be acceptable in later stages of the drug development process, it is hardly practical for the earlier stages, where a high throughput, reliable method capable of quantitating 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 precursor protein (APP), in support of preclinical studies. The use of a single, high throughput assay for multiple Aβ peptides, without time consuming immunoprecipitation steps, was developed and validated. The Aβ class of peptides, in particular, presents many unique analytical challenges including non-specific binding, poor solubility, aggregation, and lower MS sensitivity. Steps were taken at all stages of method development to minimize or eliminate the impact of these issues.

As strategies emerge for disease modification in AD, the quantification of multiple Aβspecies, in addition to Aβ38, 40 and 42, that may be linked to AD pathology may help to offer more insight to this disease and its progression. The method described herein shows promise for adaptation to quantitate those peptides as well.

Amyloid β 1-38, MW 4132 PI 5.2
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG
Amyloid β 1-40, MW 4330 PI 5.2
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Amyloid β 1-42, MW 4516 PI 5.2
DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

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

EXPERIMENTAL

UPLC® Method Conditions

Column: ACQUITY UPLC® BEH C_{18} 300Å 2.1 × 150 mm, 1.7 µm
Mobile Phases: A: 0.3% NH_4OH (by volume) in H_2O
B: 90% Acetonitrile, 10% mobile phase A
Gradient: hold 90% A for 1 min., ramp to 55% A in 5.5 min, hold 0.2 min, return to initial
Flow Rate: 0.2 mL/min
Injection: 10 µL
Temperature: 50 °C

MS Conditions

System: Waters Xevo™ TQ triple quadrupole mass spectrometer operated in ESI+ MRM mode
Desolvation Gas Flow: 800 L/hr
Source Temperature: 120 °C
Desolvation Temperature: 450 °C
Collision Cell Pressure: 2.6 X $10^{(-3)}$ mbar
MRM Transitions and conditions: see Table 1

Sample Pre-treatment

200 µL CSF (human, monkey, or spiked artificial CSF + 5% rat plasma) was diluted 1:1 with 5M guanidine HCL and shaken at room temperature for 45 minutes. This was then diluted further with 200 µL 4% H_3PO_4 in H_2O.
Note: for spiked samples, samples were allowed to equilibrate at room temperature for 30 min. after spiking, prior to dilution with guanidine HCl.

Solid Phase Extraction (SPE)
Oasis® MCX in µElution 96-well format

Condition: 200 µL methanol
Equilibrate: 200 µL 4% H_3PO_4 in H_2O
Load: 600 µL pre-treated sample
Wash 1: 200 µL 4% H_3PO_4 in H_2O
Wash 2: 10% ACN in H_2O
Elute: 2 X 25 µL 75:15:10 ACN:H_2O:conc. NH_4OH
Dilute: 25 µL H_2O
Inject 10 µL

Peptide Name	Precursor Ion	Product Ion	Product Ion ID	Cone voltage (V)	Collision energy (eV)
Amyloid β 1-38	1033.5	1000.3	b 36	33	23
Amyloid β 1-38 N15 IS	1046	1012.5		30	22
Amyloid β 1-40	1083	1053.6	b 39	33	25
Amyloid β 1-40 N15 IS	1096	1066.5		35	22
Amyloid β 1-42	1129	1078.5	b 40	28	30
Amyloid β 1-42 N15 IS	1142.5	1091.5		35	28

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

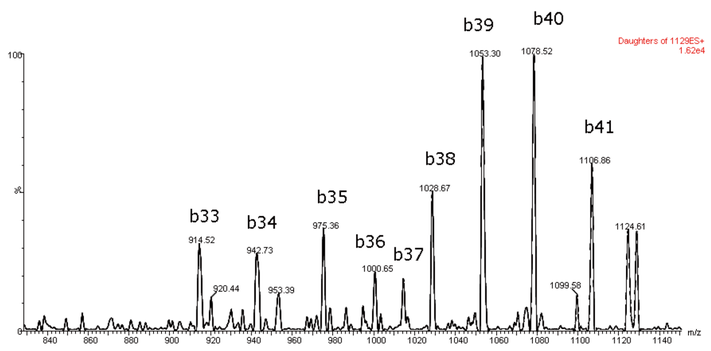

Figure 2: Representative ESI+ MSMS spectrum for amyloid β 1-42

RESULTS AND DISCUSSI ON

The greatest challenges encountered in the development of these methodologies were overcoming solubility, adsorption, 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 solvent are just a few of the factors that were key to addressing these problems.

MASS SPECTROMETRY

MS was performed in positive ion mode as CID of the 4+ precursor yielded several distinct product ions (representative spectrum shown in Figure 2) corresponding to inherently specific b sequence ions. MSMS in negative ion mode yielded a dominant water loss. Figure 3 demonstrates one example of the specificity difference be-tween both methods. Although overall sensitivity was higher in sol-vent standards using the negative ion method, the sensitivity dif-ference was mitigated in the presence of matrix and the improved specificity and signal to noise in positive ion mode proved critical for accurate quantitation in CSF samples.

UPLC

Separation of the three amyloid βpeptides is shown in Figure 4. While exact % NH_4OH in the mobile phase was critical for negative ion sensitivity, the signal in ESI+ proved to be more robust to sub-tle 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 natural change in NH_4OH 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® MCX, a mixed-mode sorbent, to enhance selectivity of the extraction. The sorbent relies on both reversed-phase and ion exchange retention mechanisms to selec-tively separate the amyloid βfraction from other high abundance polypeptides in complex CSF samples. The specific 96-well format used, Oasis® µElution, provided significant concentration while eliminating evaporation and reconstitution, minimizing peptide losses. In addition, binding of the peptides by ion-exchange im-parted orthongonality 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 (having a different amyoid βsequence) was added to eliminate the NSB.
The SPE was one of the more critical aspects of the overall methodology. Very selective isolation of the amyloid fraction coupled to the resolution of standard flow UPLC enabled very rapid analysis of preclinical samples.

LINEARITY, ACCURACY AND PRECISION

N15 labeled internal standards were used for each peptide. Stan-dard curves for all 3 amyloid βpeptides were linear, with 1/x weighting from 0.1 to 10 ng/mL in artificial CSF + 5% rat plasma. A representative standard curve for amyloid β1-38 is shown in Fig-ure 5. Basal levels of the amyloid peptides were quantitated using both standard curves prepared from over-spiked human CSF and from artificial CSF + 5% rat plasma, and calculated basal levels were not statistically different. The artificial CSF was chosen as it is a less expensive and more readily 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 βpeptides are shown in Table 2.Overspike QC samples were prepared in 3 sources of pooled hu-man CSF and 1 source of pooled monkey CSF at 0.2, 0.8, 2, and 6 ng/mL. Accuracy and precision values met the regulatory criteria for LC/MS/MS assays. Representative results from QC sample analysis are shown in Table 3.

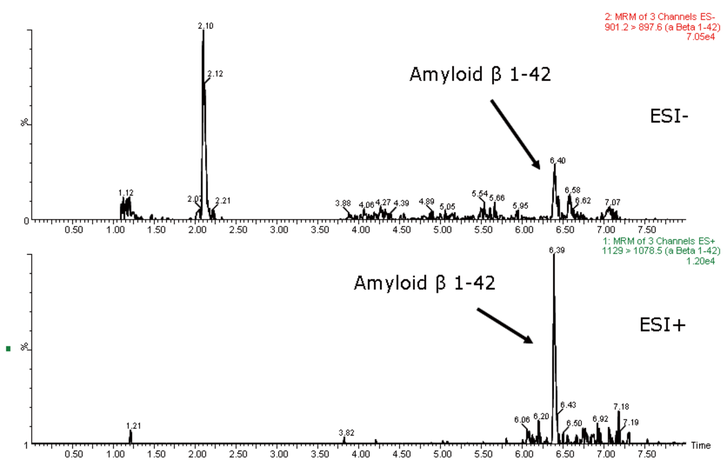

Figure 3: Comparison of MS specificity using either negative ion(water loss product ion) or positive ion (b sequence ion product ion) electrospray

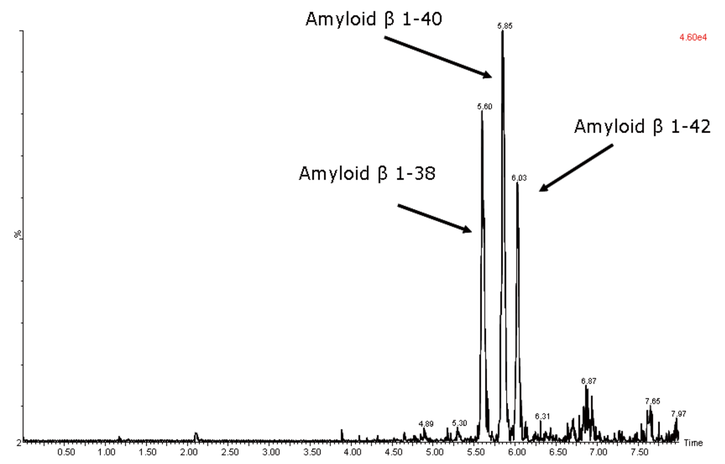

Figure 4: UPLC/MS/MS analysis of amyloid β1-38, 1-40 and 1-42 peptides extracted from artificial CSF + 5% rat plasma

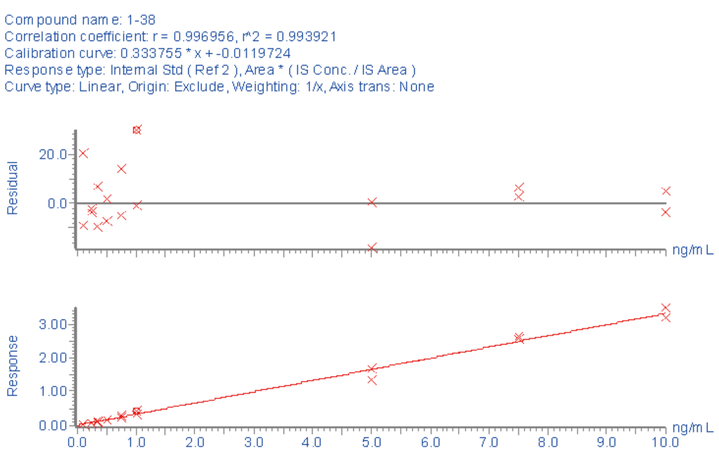

Figure 5: Representative standard curve for amyloid β1-38 extracted from artificial CSF + 5% rat plasma.

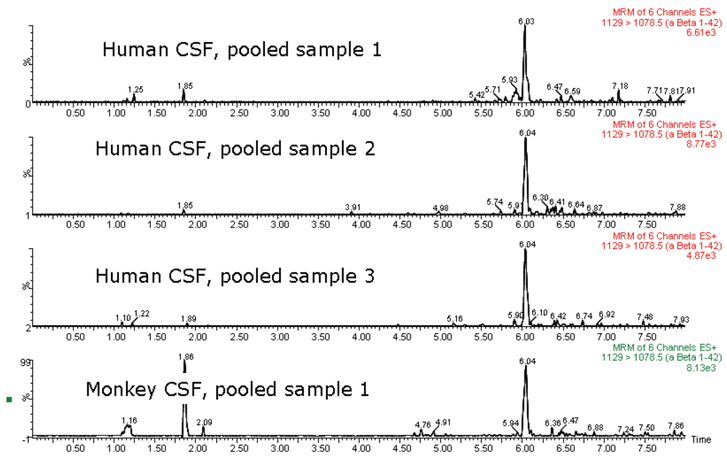

Figure 6: Representative chromatogram showing basal levels of amyloid β1-42 in 3 sources of human and 1 source of mon-key CSF.

Amyloid Beta 1-38

Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	1.585	2.354	1.014	1.713
2	1.650	2.103	1.371	1.605
3	1.614	2.464	0.950	1.947
4	1.657	1.939	1.608	1.541
5	1.820	2.158	1.471	1.675
6	1.486	1.995	1.167	1.644
Mean	**1.635**	**2.169**	**1.264**	**1.688**
Std. Deviation	0.110	0.204	0.262	0.140
% CV	**6.7**	**9.4**	**20.7**	**8.3**

Amyloid Beta 1-42

Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	0.519	0.616	0.421	0.675
2	0.421	0.656	0.481	0.621
3	0.542	0.644	0.534	0.623
4	0.471	0.567	0.348	0.659
5	0.476	0.573	0.487	0.700
6	0.561	0.713	0.510	0.688
Mean	**0.498**	**0.628**	**0.463**	**0.661**
Std. Deviation	0.052	0.055	0.068	0.033
% CV	**10.4**	**8.7**	**14.7**	**5.1**

Amyloid Beta 1-40

Replicate #	Human CSF Pool 1 ng/mL	Human CSF Pool 2 ng/mL	Human CSF Pool 3 ng/mL	Cyno CSF Pool 1 ng/mL
1	3.083	4.031	2.541	3.699
2	3.391	3.776	2.593	3.989
3	3.292	3.598	2.580	3.525
4	2.884	3.533	2.612	3.956
5	3.131	3.230	2.508	3.284
6	3.656	3.619	2.490	3.595
Mean	**3.240**	**3.631**	**2.554**	**3.675**
Std. Deviation	0.269	0.266	0.049	0.268
% CV	**8.3**	**7.3**	**1.9**	**7.3**

Table 2: Baseline levels of amyloid βpeptides in 3 sources of pooled human CSF and 1 source of pooled monkey CSF.

Amyloid β 1-38

Over-spike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	% CV	Number of Replicates Passed	Mean Accuracy
0.2	1.49	1.355	0.071	5	3/3	90.9
0.8	2.09	1.843	0.118	6	3/3	88.2
2	3.29	3.287	0.319	10	3/3	99.9
6	7.29	7.701	0.478	6	3/3	105.6

Amyloid β 1-40

Over-spike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	% CV	Number of Replicates Passed	Mean Accuracy
0.2	2.75	2.359	0.015	1	2/3	85.8
0.8	3.35	3.054	0.016	1	2/3	91.2
2	4.55	3.929	0.011	0	3/3	86.4
6	8.55	8.209	0.5	6	3/3	96

Amyloid β 1-42

Over-spike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	% CV	Number of Replicates Passed	Mean Accuracy
0.2	0.663	0.655	0.07	11	3/3	98.8
0.8	1.263	1.145	0.058	5	3/3	90.7
2	2.463	2.403	0.121	5	3/3	97.5
6	6.463	5.986	0.701	12	3/3	92.6

Table 3: Representative results from analysis of QC sam-ples prepared in pooled human CSF, source 3

CONCLUSIONS

1. An SPE-LC/MS/MS bioanalytical method was developed and validated for the simultaneous quantitation of mul-tiple amyloid βpeptides in human and monkey CSF.
2. The combination of a highly selective extraction method based on mixed-mode SPE in µElution format and the resolution of UPLC chromatography was key to the accurate, precise and reliable quantitation of 3 ma-jor amyloid βpeptides in human and monkey CSF.
3. The use of positive ion MSMS and b ion sequence frag-ments provided the MS specificity required for this ap-plication.
4. 96 samples can be extracted and ready for injection in < 30 minutes, providing the sample prep throughput required for pre-clinical studies.
5. The method described herein eliminates time-consuming immunoassays or immunoprecipitation steps for pre-clinical work.
6. The mass range and sensitivity of the Xevo TQ MS en-abled the selection of higher m/z precursors for frag-mentation and the choice of highly specific b ion frag-ments, resulting in increased signal to noise and over-all improved specificity for the assay.
7. This approach also allows one assay for the measure-ment of several different amyloid βpeptides simultane-ously in one sample with selectivity, specificity and in a high-thoughput format while still achieving the high sensitivity required for low level endogeneous amyloid βpeptides. This represents a significant benefit as an ELISA assay would require multiple assays with multi-ple antibodies.

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ACKNOWLEDGEMENT

The authors wish to acknowledge Wenlin Li (PDM, Pfizer) for her early work on amyloid βpeptides using immunoaffinity LC/MS/MS.

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