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應用手冊

A Flexible SPE-LC-MS/MS Platform for the Simultaneous Quantitation of Multiple Amyloid Peptides in Cerebrospinal Fluid

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Abstract

This work focuses on the development of UPLC, MS, and selective SPE sample preparation methods for the 1-38, 1-40, and 1-42 fragments of APP, in support of preclinical studies.

Benefits

- 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.
- High sensitivity and mass range achieved with Xevo TQ MS.
- Resolution, sensitivity of ACQUITY UPLC Peptide Separation Technology columns for improved separation with shorter run times.

Introduction

Fast, flexible platforms for peptide quantification are needed, particularly for a discovery setting. This type of methodology would be especially advantageous in the case of amyloid beta (aβ) peptides. The deposition/formation of insoluble aggregates, or plaques, of aβ peptides in the brain is considered to be a critical event in the progression of Alzheimer's Disease (AD) and thus has the attention of many researchers.

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 studies.

Development of a bioanalytical method for these peptides is further complicated by their propensity for aggregation, formation of oligomers, poor solubility, nonspecific binding, and hydrophobicity.

As aβ peptides may be present at very low concentrations, we developed a solid-phase extraction (SPE) sample preparation protocol to enrich the amyloid beta fraction in CSF. 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 solution. A high throughput, high resolution UPLC-MS/MS quantitation method was also developed.

This work focuses on the development of UPLC, MS, and selective SPE sample preparation methods for the 1-38, 1-40, and 1-42 fragments of APP, in support of preclinical studies. Sequence, pI and molecular weight (MW) information for these peptides is shown in Figure 1. The use of a single, high throughput assay for multiple aβ peptides- without time consuming immunoprecipitation steps was developed and validated. The speed, selectivity, and specificity of this technique for simultaneously quantitating multiple aβ peptides in CSF are demonstrated.

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

As strategies emerge for disease modification in AD, the quantification of multiple aβ species (in addition to aβ 38, 40 and 42) that may be linked to AD pathology may help to offer more insight into this disease and its progression. The method described herein shows promise for adaptation to quantitate those peptides as well.

Experimental

ACQUITY UPLC Conditions

Column:	ACQUITY UPLC BEH C_{18} 300Å, 2.1 x 150 mm, 1.7 µn Peptide Separation Technology
Column Temp.:	50 °C
Sample Temp.:	15 °C
Injection Volume:	10 µL
Injection Mode:	Partial Loop
Flow Rate:	0.2 mL/min.
Mobile Phase A:	0.3% NH_4OH in H_2O
Mobile Phase B:	90/10 ACN/mobile phase A
Strong Needle Wash:	60:40 ACN:IPA + 10% conc. NH_4OH (600 µL)
Weak Needle Wash:	90:10 0.3% NH_4OH in H_2O: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 MS Conditions, Electrospray 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

Peptide Name	Percursor 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

SPE conditions

Sample Pre-treatment

200 µL human CSF, monkey 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 200 µL 4% H_3PO_4 in H_2

O.

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. All solutions are made up by volume. All steps applied to wells of µElution plate containing samples.

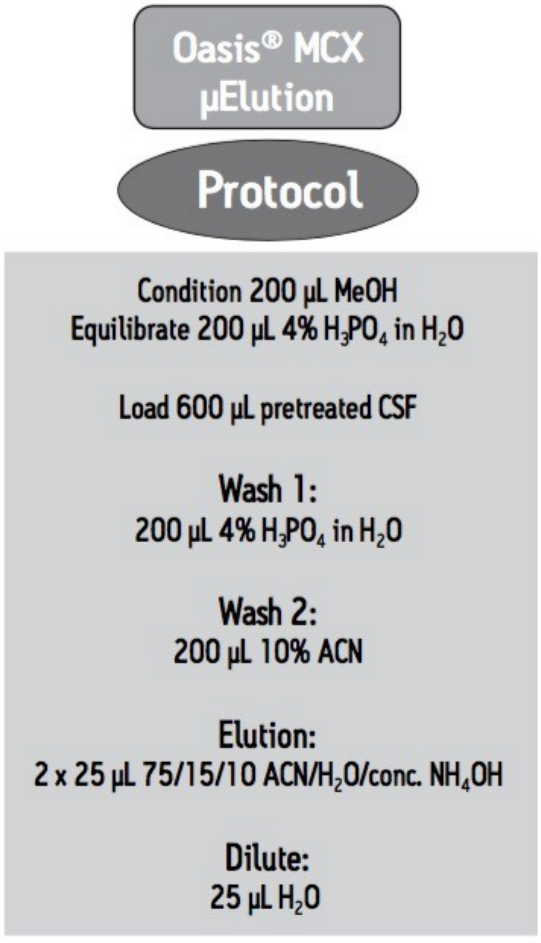

Figure 2. Oasis µElution MCX extraction protocol.

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.) MS/MS in negative ion mode yielded a dominant water loss. Figure 4 demonstrates one example of the specificity difference between both methods in CSF. Although overall sensitivity was higher in solvent standards using the negative ion method, the sensitivity difference was mitigated in the presence of matrix. The improved specificity and signal-to-noise in positive ion mode proved critical for accurate quantitation in CSF samples.

Mass range of the instrument was also an important factor in obtaining specificity. The Xevo TQ 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.

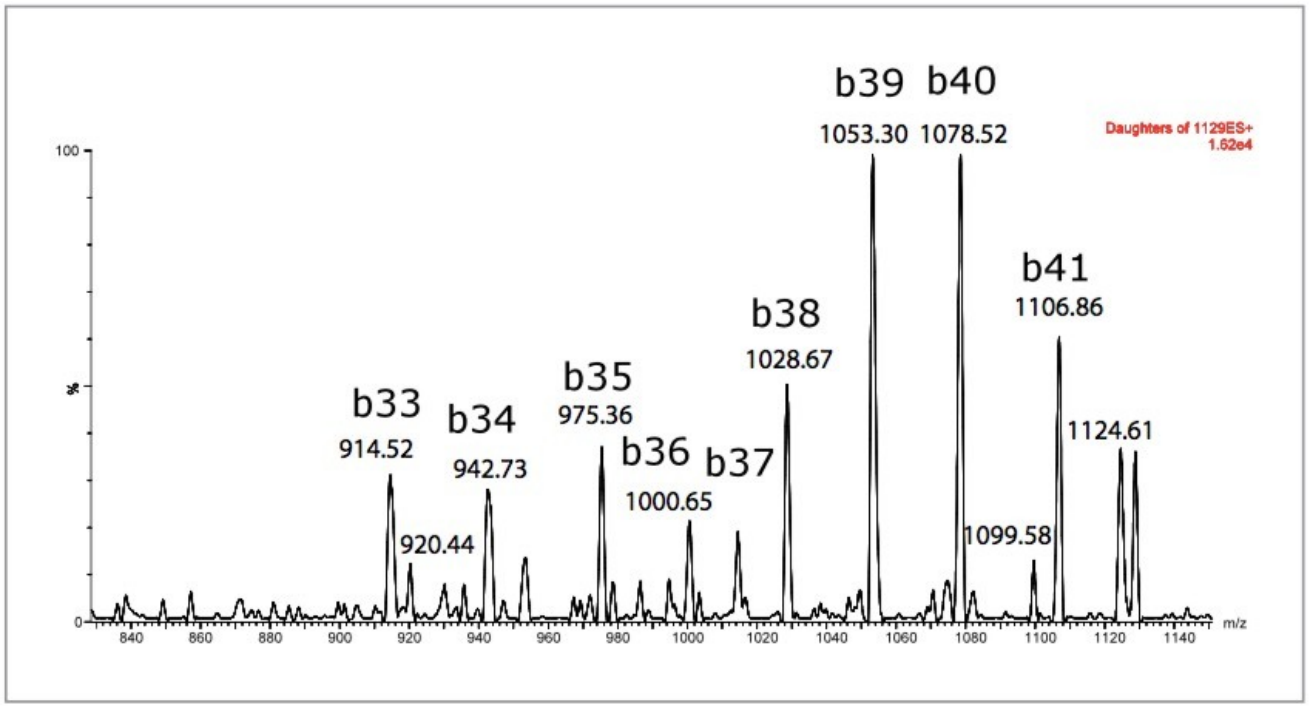

Figure 3. Representative ESI+ MS/MS spectrum for amyloid β 1-42 with fragment sequence ions labeled.

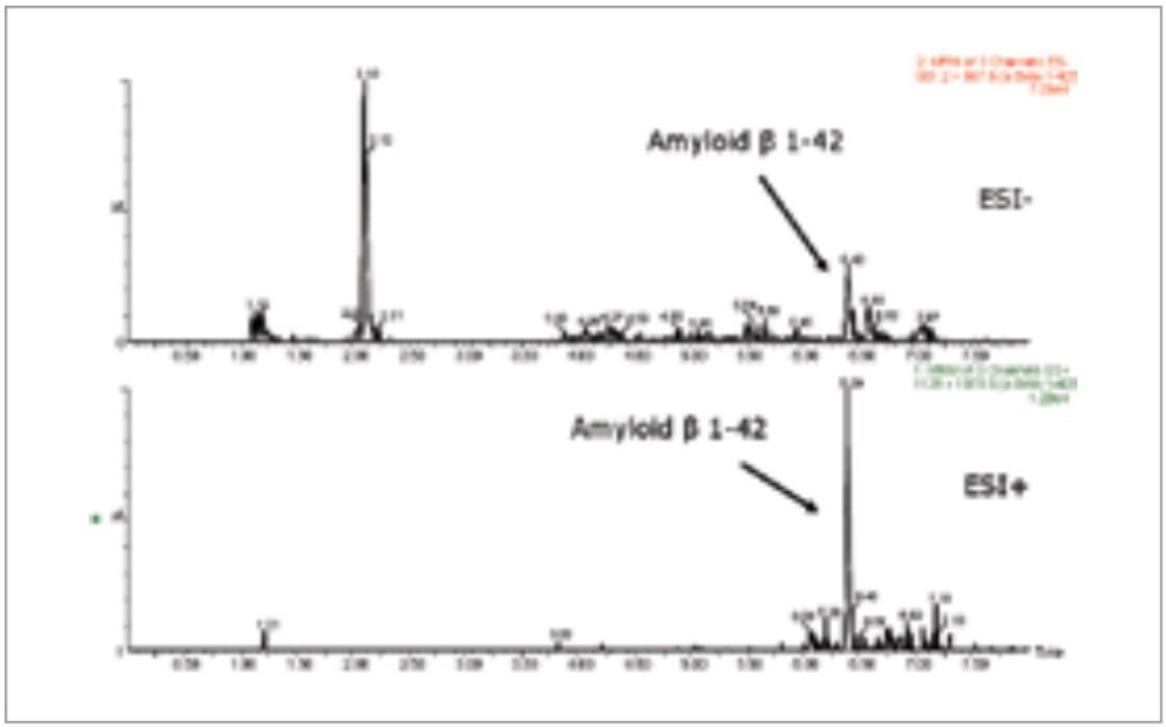

Figure 4. Comparison of MS specificity using either negative ion (water loss product ion) or positive ion (b sequence ion product ion) electrospray in human CSF.

UPLC Separation

Separation of the three amyloid β peptides is shown in Figure 5. While the exact amount of NH_4OH in the mobile phase was critical for negative ion sensitivity, the signal in ESI+ 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– 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.

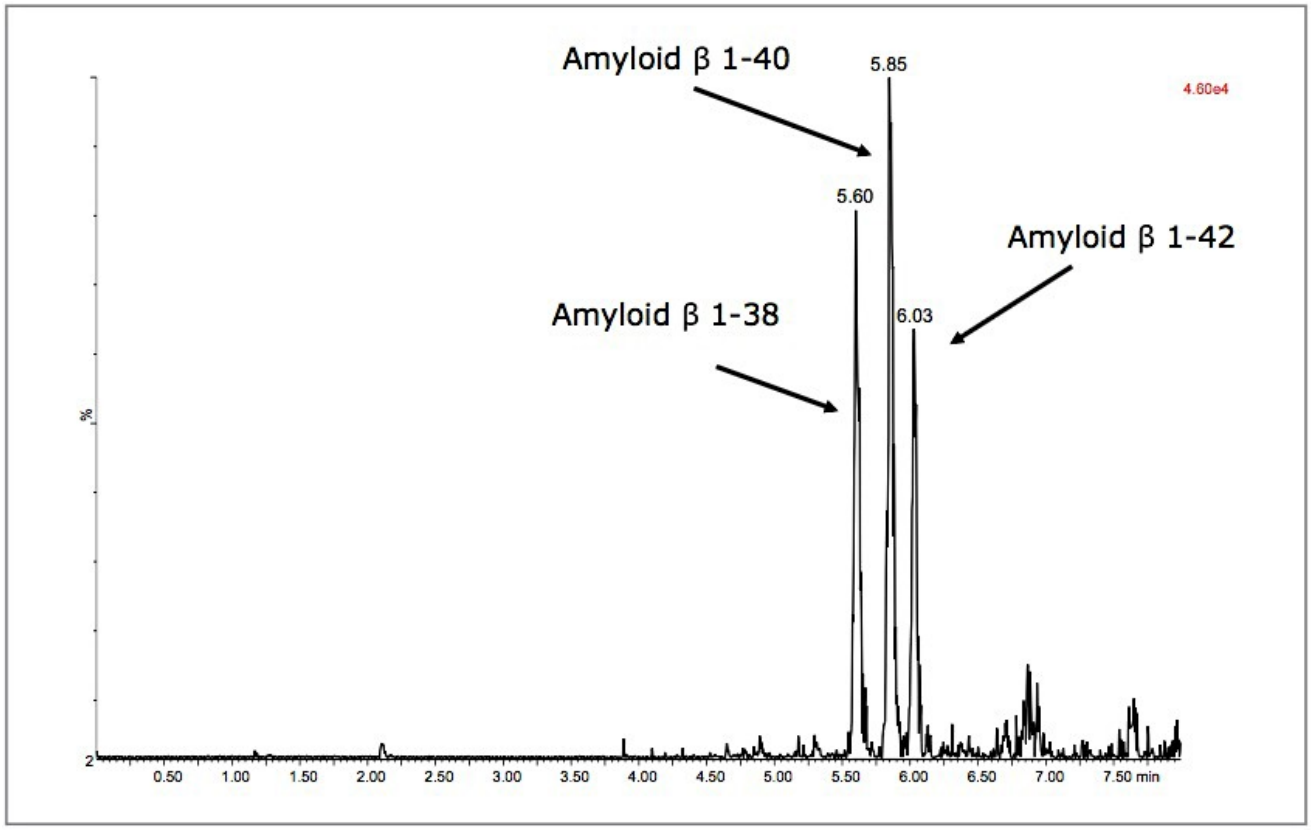

Figure 5. UPLC-MS/MS analysis of amyloid β 1-38, 1-40 and 1-42 peptides extracted from artificial CSF + 5% rat plasma.

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 aβ fraction from other high abundance polypeptides in complex CSF samples. The Oasis µElution plate (96-well format) provided significant concentration while eliminating evaporation and reconstitution, thus minimizing peptide losses. In addition, binding of the peptides by ion-exchange imparted orthongonality into the overall method as the UPLC separation is performed in the reversed-phase dimension.

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 eliminate the 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 rapid analysis of pre-clinical samples.

Linearity, Accuracy and Precision

N15-labeled internal standards were used for each peptide. Standard curves for all 3 aβ peptides were linear (1/x weighting) from 0.1 to 10 ng/ mL in artificial CSF + 5% rat plasma. A representative standard curve for amyloid β 1-38 is shown in Figure 6. Basal levels of the aβ peptides were quantitated using both standard curves prepared from over-spiked human CSF and from artificial CSF + 5% rat plasma. 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 β 1-42 extracted from 3 sources of human and 1 source of monkey CSF are shown in Figure 7. Statistics for the determination of basal levels of all 3 aβ peptides are shown in Table 2.

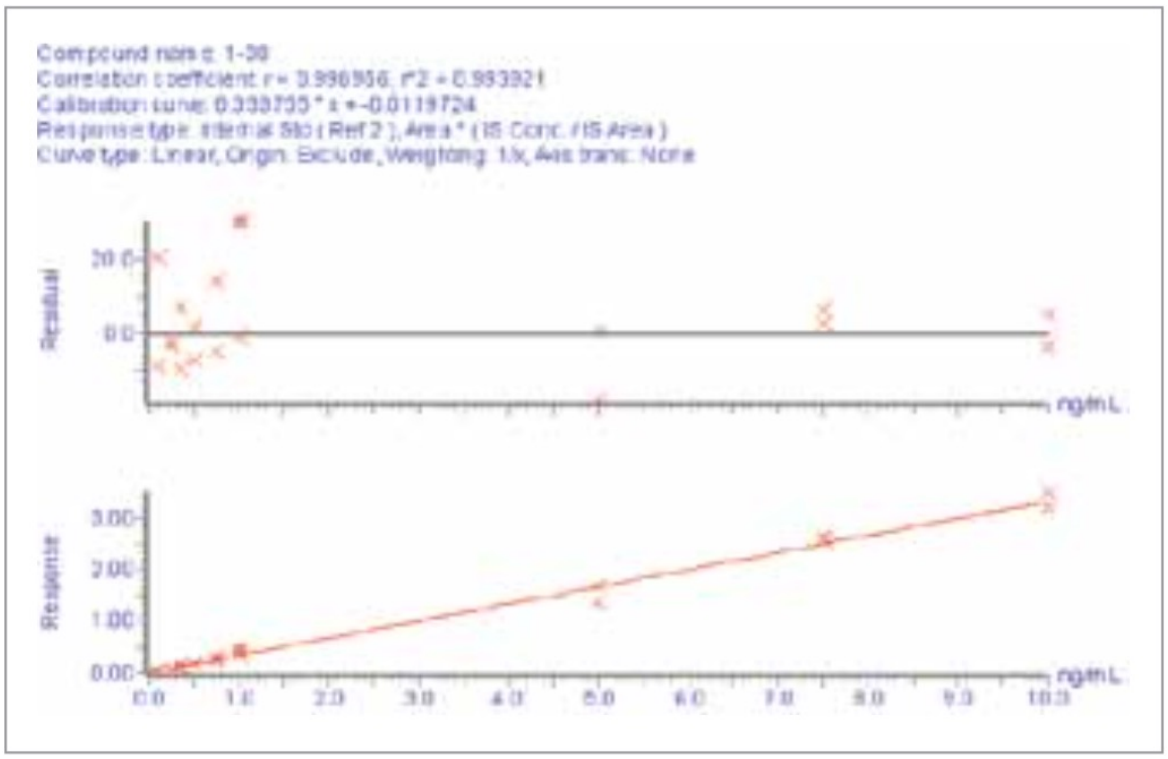

Figure 6. Representative standard curve for amyloid β 1-38 extracted from artificial CSF + 5% rat plasma.

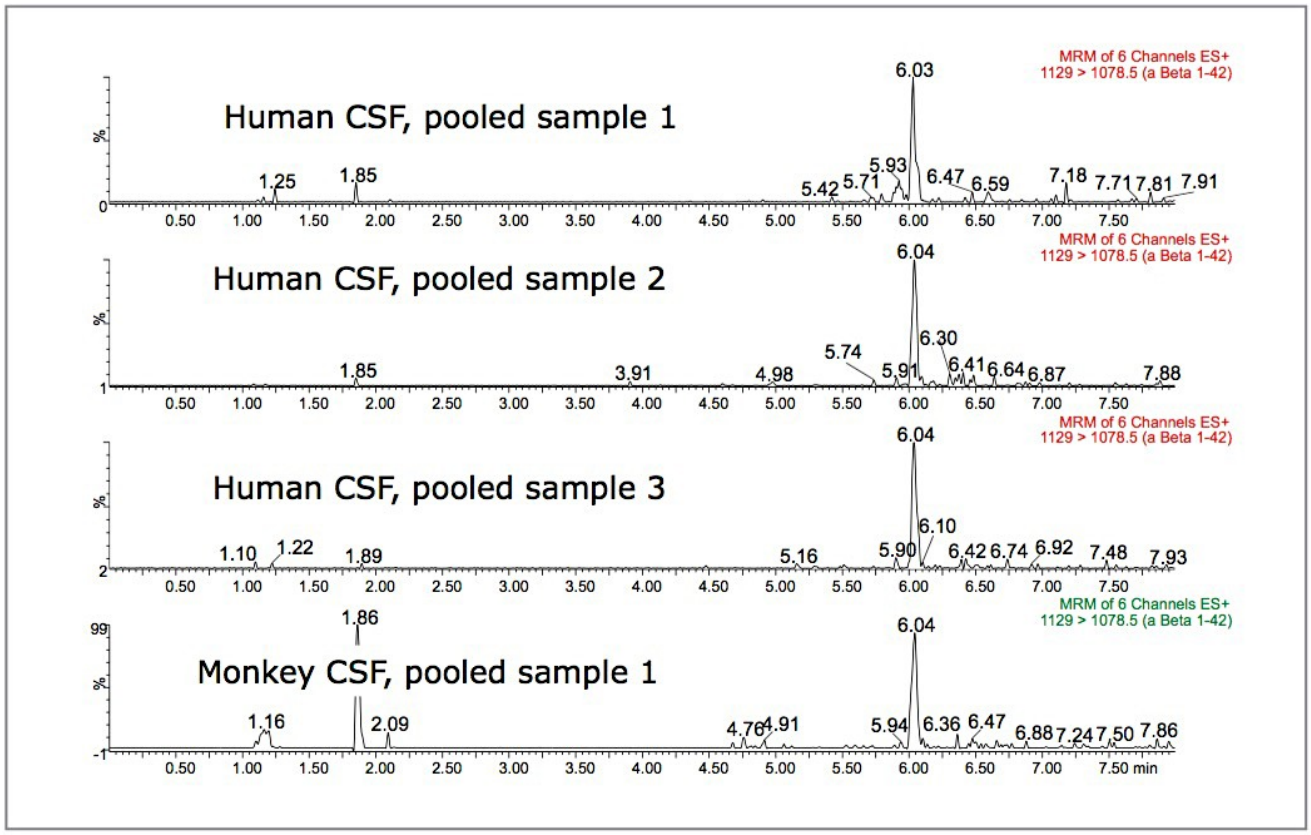

Figure 7. Representative chromatogram showing basal levels of amyloid β1-42 in 3 sources of human and 1 source of monkey CSF.

TABLE 2. Baseline levels of amyloid β peptides in 3 sources of pooled human CSF and 1 source of pooled monkey 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	83

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

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

Overspiked QC samples were prepared in 3 sources of pooled human 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.

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

Amyloid β 1-40

Overspike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
-	-	-	-	-	-	-
0.8	2.090	1.843	0.118	6	3/3	88.2
2	3.290	3.287	0.319	10	3/3	99.9
6	7.290	7.701	0.478	6	3/3	105.6

Amyloid β 1-38

Overspike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
-	-	-	-	-	-	-
0.8	3.350	3.054	0.016	1	2/3	91.2
2	4.550	3.929	0.011	0	3/3	99.9
6	8.550	8.209	0.500	0	3/3	105.6

Amyloid β 1-42

Overspike Conc. ng/mL	QC Conc.	Mean Calculated Conc.	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
-	-	-	-	-	-	-
0.8	1.263	1.145	0.058	5	3/3	90.7
2	2.463	2.403	0.121	5	3/3	99.9
6	6.463	5.986	0.701	12	3/3	105.6

Conclusion

- 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.
- The mass range and sensitivity of the Xevo TQ MS enabled the selection of higher m/z precursors for fragmentation and the choice of highly specific b ion fragments, resulting in increased signal-to- noise and overall improved specificity for the assay.
- 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-thoughput format while still achieving the high sensitivity required for low level endogeneous 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 quantitate each of the relevant peptides.

References

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