A Multiplex 2D-UPLC-MS/MS-based Assay for Amyloid β and Related Biomarker Peptides in Human Cerebrospinal Fluid

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia among older populations. Most proposed AD models postulate that abnormal accumulation of amyloid β peptides (Aβ), fragments of the transmembrane amyloid precursor protein (APP), is closely associated with the disease process. Analysis of Aβ peptides in cerebrospinal fluid (CSF) has found increasing utility in clinical AD diagnosis, with Aβ1-42 being the most studied isoform implicated in amyloid pathology. Many studies have clearly demonstrated that Aβ1-42 CSF levels are decreased in AD patients compared to normal individuals. However, because of the inherently complex and heterogeneous population of endogenous Aβ isoforms, produced either through normal or abnormal secretase enzyme activities, there is on-going interest in having a "general-purpose" assay that can simultaneously quantify a variety of selected target peptides to obtain a broader biomarker picture. While immunoassays are most frequently applied to specific isoforms, particularly Aβ1-40 and Aβ1-42, mass spectrometric techniques are better suited to selectively measure multiple analytes in the same analysis without concern of cross-reactivity. Here, we describe a solid-phase extraction (SPE)-based 2-Dimensional-Ultra Performance Liquid Chromatographic®-tandem mass spectrometric (2D-UPLC®-MS/MS) assay, which provides high sensitivity and targeted analysis of multiple Aβ and related biomarker peptides in CSF.

Analytes

Analyte	Peptide Sequence	MW	pI	Net charge (pH10)	Hydro-phillicity
Aβ1-14	DAEFRHDSGYEVHH	1699	5.1	-4	0.5
Aβ1-15	DAEFRHDSGYEVHHQ	1827	5.1	-4	0.5
Aβ1-17	DAEFRHDSGYEVHHQKL	2068	5.7	-3.3	0.5
Aβ1-34	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL	3787	5.2	-4.5	0.1
Aβ1-38	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGG	4132	5.2	-4.5	0
Aβ1-40	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	4330	5.2	-4.5	-0.1
Aβ1-42	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	4514	5.2	-4.5	-0.1
APL1β28	DELAPAGTGVSREAVSGLLIMGAGGGSL	2586	3.8	-2.6	-0.1
*†**Aβ1-43***	*DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIAT*	*4615*	*5.2*	*-4.5*	*-0.1*

While the longer length Aβ isoforms may be biomarkers of disease-related changes, the shorter C-terminal truncated isoforms, Aβ1-14 and Aβ1-15, are of interest as potential indicators of alternative degradation pathways for CNS APP during treatments with γ-secretase modulators or inhibitors. The assay also includes APL1β28, a 28-amino acid nonamyloidogenic Aβ-like peptide derived from the APP-like protein APLP1 by the same secretase induced cleavages that produce Aβ1-42. The relative level of APL1β28 has been proposed as a more sensitive surrogate biomarker for Aβ1-42 production in the brain, as it may rise in parallel to Aβ1-42 from secretase-mediated overproduction without subsequently falling as may be occurring with Aβ1-42 due to plaque formation in AD brains.

The current assay utilizes five [^{13}C,^{15}N] stable isotope-labeled Aβ1-15*, Aβ1-17*, Aβ1-38*, Aβ1-40*, and Aβ1-42* as internal standards for the eight analytes.

†Update: Aβ1-43, recently described by T. Saito et al in Nature Neuroscience, 3 July 2011 (on-line), as "an overlooked species that is potently amyloidogenic, neurotoxic, and abundant in vivo," is currently being evaluated for potential inclusion in the assay.

Methodology

SAMPLE PRE-TREATMENT

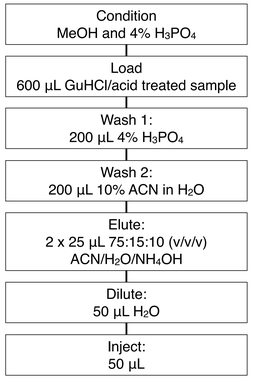
A 200-µL aliquot of human CSF (hCSF) or spiked artificial CSF (aCSF), containing 4 mg/mL bovine serum albumin (BSA) to eliminate non-specific binding, is fortified with the stable isotope-labeled internal standard Aβ peptides and treated with 200 µL 5M guanidine HCl. The sample is mixed for 45 min at room temperature and diluted with 200 µL 4% H_3PO_4.

It should be noted that analytical results for Aβ and other biomarker peptides may be impacted by preanalytical variables, such as collection and shipping container types. Eppendorf LoBind or equivalent tubes are recommended, and the potential value of using additives at the time of collection to minimize binding or aggregation is under investigation.

Oasis® MCX µElution

SOLID PHASE EXTRACTION (SPE)

The analytes are isolated from a pretreated 200-µL CSF sample using a Waters Oasis® MCX (strong cation-exchange mixed-mode) µElution 96-well plate. After sequential washes with 4% H_3PO_4 and 10% acetonitrile, the analyte peptides are eluted in a small volume of ACN/water/conc. NH4OH (75:15:10, v/v/v), and the extract is diluted with additional water to reduce the organic solvent content while still maintaining analyte solubility in the extract.



2D-CHROMATOGRAPHY

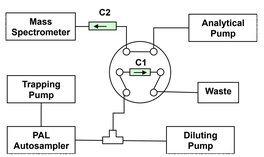
The analytes are separated under high pH, NH_4OH-based, reversed-phase chromatographic conditions, intended to minimize non-specific binding and aggregation. Because the analytes significantly vary in hydrophobicity, dramatically increasing over the range from Aβ1-14 through Aβ1-42, on-line aqueous dilution of the large sample extract band is required to ensure that the analyte peaks are efficiently focused on the precolumn. Following a brief desalting period with the precolumn effluent directed to waste, the valve is switched and the analytes are transferred to a UPLC® analytical column and separated using an appropriate gradient (Figure 1).

LC-MS/MS System:
CTC Analytics LC-PAL Autosampler
Agilent 1200 Series LC Binary Pumps
AB Sciex API 5000, Triple Quadrupole LC-MS/MS
Electrospray positive ionization (+ESI) multiple reaction monitoring (MRM) mode

Columns:
Trapping (C1): Waters Xbridge BEH C18, 2.1 x 30 mm, 3.5 µm
Analytical (C2): Waters ACQUITY UPLC® BEH300 C18, 2.1 x 150 mm, 1.7 µm
Temperature: 50 °C

Mobile phases:
A1: 98:2:0.1 H_2O/ACN/NH_4OH, v/v/v
B1: 65:25:10:5 ACN/MeOH/IPA/H_2O, v/v/v/v
A2: 0.1% NH_4OH in H_2O
B2: 70:25:5 ACN/MeOH/TFE, v/v/v

Flow Rates:
Loading: 0.2 mL/min
Diluting: 0.8 mL/min
Analysis: 0.3 mL/min



2D-UPLC Configuration - injection position

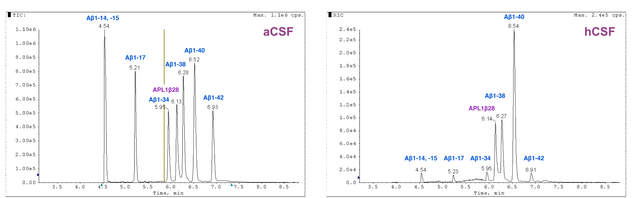


Figure 1: Total ion chromatograms of an extracted aCSF standard and an hCSF unknown

MASS SPECTROMETRY

The eluting analyte peaks are detected by multiple-reaction-monitoring (MRM) in positive electrospray ionization mode. Data are acquired in two sequential acquisition periods over the 12-minute run time due to the large number of MRM transitions required.

Analyte	MW	Precursor ion, m/z	Charge	Product ion, m/z	Charge	Ion type
Aβ1-14	1699	567.1	+3	156.1	+1	y1
Aβ1-15	1827	609.8	+3	284.0	+1	y2
Aβ1-17	2068	690.3	+3	646.4	+3	b2
Aβ1-34	3787	947.8	+4	1162.9	+3	b31
APL1β28	2586	862.8	+3	245.1	+1	b2
Aβ1-38	4132	1033.9	+4	1000.8	+4	b36
Aβ1-40	4330	1083.6	+4	1054.2	+4	b39
Aβ1-42	4514	1129.4	+4	1078.8	+4	b40
*†**Aβ1-43***	*4615*	*1154.8*	+4	*1124.9*	+4	*b42*

Results and Discussion

Representative aCSF Accuracy and Precision

Aβ 1-14

Calibrator	Theoretical	Measured	Back-calc
	(pg/mL)	(pg/mL)	Accuracy
1	25.0	24.6	98.2%
2	50.0	50.6	101%
3	80.0	81.2	101%
4	300	325	108%
5	1000	1020	102%
6	3000	3054	102%
7	8000	7715	96.4%
8	10000	9053	90.5%

QC Level	LLOQ	Low	Mid	High
	(pg/mL)	(pg/mL)	(pg/mL)	(pg/mL)
Theoretical	25.0	80.0	1000	8000
Mean (n=6)	24.5	81.7	1046	7315
C.V.	7.27%	1.78%	4.50%	8.64%
Accuracy	97.8%	102%	105%	91.4%

Aβ 1-42

Calibrator	Theoretical	Measured	Back-calc
	(pg/mL)	(pg/mL)	Accuracy
1	25.0	22.8	91.3%
2	50.0	54.1	108%
3	80.0	90.5	113%
4	300	319	106%
5	1000	997	100%
6	3000	2882	96.1%
7	8000	7462	93.3%
8	10000	9176	91.8%

QC Level	LLOQ	Low	Mid	High
	(pg/mL)	(pg/mL)	(pg/mL)	(pg/mL)
Theoretical	25.0	80.0	1000	8000
Mean (n=6)	25.3	79.1	983	7513
C.V.	13.9%	5.47%	5.30%	2.19%
Accuracy	101%	98.8%	98.3%	93.9%

APL1β28

Calibrator	Theoretical	Measured	Back-calc
	(pg/mL)	(pg/mL)	Accuracy
1	25.0	27.3	109%
2	50.0	45.3	90.5%
3	80.0	67.7	84.7%
4	300	304	101%
5	1000	1082	108%
6	3000	3079	103%
7	8000	8111	101%
8	10000	10206	102%

QC Level	LLOQ	Low	Mid	High
	(pg/mL)	(pg/mL)	(pg/mL)	(pg/mL)
Theoretical	25.0	80.0	1000	8000
Mean (n=6)	18.0	81.5	1037	7823
C.V.	29.2%	10.4%	4.27%	8.21%
Accuracy	72.1%	102%	104%	97.8%

The major goal of this work was to develop sensitive and efficient generic methodology to simultaneously extract and analyze a broad range of Aβ isoforms and other potential peptides of interest. Well-known issues with analyte solubility, adsorption, and aggregation were addressed by judicious choice of sample handling and SPE processing conditions, which were extensively investigated by Erin Chambers of Waters and adapted for this method.

Representative Human CSF Standard Addition

	Aβ1-14			Aβ1-42			APL1β28		
Replicate	Lot 1 (pg/mL)	Lot 2 (pg/mL)	Lot 3 (pg/mL)	Lot 1 (pg/mL)	Lot 2 (pg/mL)	Lot 3 (pg/mL)	Lot 4 (pg/mL)	Lot 5 (pg/mL)	Lot 6 (pg/mL)
1	48.0	46.2	59.8	211	293	429	1,312	869	1,327
2	56.4	48.7	70.7	212	295	409	1,253	931	1,383
3	44.7	46.7	62.7	208	308	439	1,311	1,015	1,263
Basal Mean	**49.7**	**47.2**	**64.4**	**210**	**299**	**426**	**1,292**	**938**	**1,325**
S.D.	6.02	1.34	5.62	2.27	8.10	15.2	33.8	73.1	60.0
C.V.	12.1%	2.84%	8.73%	1.08%	2.71%	3.56%	2.61%	7.79%	4.53%
1	135	127	131	290	375	469	3,182	2,924	3,417
2	130	125	135	277	366	476	3,372	3,055	3,439
3	133	124	152	282	382	483	3,410	2,964	3,243
Fortified Mean	**132**	**125**	**139**	**283**	**374**	**476**	**3,321**	**2,981**	**3,366**
S.D.	2.58	1.56	11.4	6.38	7.98	7.07	122	67.3	108
C.V.	1.95%	1.25%	8.15%	2.26%	2.13%	1.49%	3.68%	2.26%	3.20%
Basal Mean	49.7	47.2	64.4	210	299	426	1,292	938	1,325
+ Fortified Level	80.0	80.0	80.0	80.0	80.0	80.0	2,000	2,000	2,000
= Theoretical	130	127	144	290	379	506	3,292	2,938	3,325
Bias	**2.09%**	**-1.69%**	**-3.42%**	**-2.52%**	**-1.22%**	**-5.94%**	**0.89%**	**1.46%**	**1.25%**

SPE using pH-stable mixed-mode Oasis MCX sorbent was found to provide general extraction selectivity, via reversed-phase/cation-exchange retention mechanisms, with >60% recovery for a wide range of peptide biomarkers in CSF. The 96-well µElution format also provides extract concentration without requiring evaporation and reconstitution that often cause peptide losses. During method development, significant non-specific binding (NSB) issues were observed using artificial CSF as a surrogate matrix. Waters has described addition of 5% rat plasma (containing different Aβ sequences) to control NSB. While we found this to be effective, unacceptable chromatographic background was observed for some of the peptides in different lots of rat plasma. Substitution of 4 mg/mL BSA eliminated this issue while controlling NSB.

Chromatography of the smaller Aβ isoforms presented a significant challenge because their high relative hydrophilicity and low pI resulted in weak retention under high pH conditions. The large injection volume of a relatively "strong" extract also required on-line aqueous dilution for efficient precolumn trapping, while back-flush transfer onto the UPLC analytical column ensured high resolution separation with sharp peaks for sensitive analysis.

MS/MS detection was performed in positive ion mode, where CID for all but the smallest peptides yields more specific b sequence product ions than the predominant water loss ions observed in negative ion mode.

With the current methodology and instrumentation, the potential dynamic range extends from 25/50 pg/mL up to 10 ng/mL for all analytes. Accuracy and precision limits of 4-6-20/25% can be readily achieved for artificial CSF standards and QCs. Selectivity and standard addition accuracy have also been confirmed in multiple lots of human CSF. A fit-for-purpose method validation is being conducted with the following assay ranges: Aβ1-14, Aβ1-15, and Aβ1-17 (25.0 to 2500 pg/mL); Aβ1-34, Aβ1-38, and Aβ1-42 (50.0 to 5000 pg/mL); Aβ1-40 and APL1β28 (100 to 10000 pg/mL). *Aβ1-43 may be added pending successful evaluation.*

Conclusion

A multiplex SPE-based UPLC-MS/MS assay has been developed, which enables both high sensitivity and broad-spectrum targeted detection of multiple Aβ and related biomarker peptides in CSF. Analyzing such a wide range of peptides in one sample is extremely challenging due to their hydrophobicity differences, strong self-aggregation and non-specific binding tendencies. The assay has been optimized for ruggedness and reproducibility and has successfully undergone preliminary evaluations with blinded sponsor-provided test samples. A fit-for-purpose method validation is underway to support study sample analysis.

Acknowledgement

The authors wish to acknowledge Erin Chambers of Waters Corporation for sharing her extensive experience in developing the Oasis mixed-mode solid phase extraction procedure for Aβ peptides and valuable suggestions for assay optimization.