TITLE:
Absolute Quantification of $A\beta_{1-42}$ in CSF using a Mass Spectrometric Reference Measurement Procedure

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SHORT ABSTRACT:
A reference measurement procedure for absolute quantification of $A\beta_{1-42}$ in human CSF based on solid-phase extraction and liquid chromatography tandem mass spectrometry is described.

LONG ABSTRACT:
Alzheimer's disease (AD) is the most common neurodegenerative disease among the elderly and accounts for 60-80% of all cases of dementia. Currently, the diagnosis of AD is based on cognitive tests and mental state exams, but the peptide amyloid-beta (Aβ) in cerebrospinal fluid (CSF) is increasingly used in clinical trials and settings. As for most protein and peptide biomarkers, quantification is performed using antibody-based techniques such as enzyme-linked immunosorbent assay (ELISA). However, intra- and inter-laboratory variability of these assays hamper its use as a diagnostic marker in clinical routine.

An antibody independent Reference Measurement Procedure (RMP) was developed based on solid-phase extraction (SPE) and liquid chromatography (LC)-tandem mass spectrometry (MS/MS), where stable isotope labeled Aβ peptides were used as internal standards, enabling absolute quantification. A high-resolution quadrupole-orbitrap hybrid instrument was used for measurements. The method allows quantification of CSF $Aβ_{1-42}$ between 150-4000 pg/mL.

INTRODUCTION:

Alzheimer's disease (AD) is the most common form of dementia and affects about 35 million people worldwide[1]. Neuropathological hallmarks of the disease widely believed to lie at the core of AD pathogenesis are intracellular neurofibrillary tangles of hyperphosphorylated tau protein[2], and extracellular plaques consisting of aggregated amyloid-beta (Aβ) peptides[3]. In line with this, assessment of plaque pathology *in vivo* by biomarkers has recently been included in the research diagnostic criteria for AD[4]. For CSF measurements of $Aβ_{1-42}$, several immunoassays are available and used in many clinical laboratories[5]. The concentration of $Aβ_{1-42}$ in CSF is approximately 50% lower in AD patients than in cognitively normal elderly, reflecting the deposition of the peptide in plaques in the brain[6,7].

These biomarkers are mainly analyzed using immunoassays, *i.e.,* antibody based techniques, but these assays may be influenced by matrix effects[8]. The use of immunoassays on different technology platforms and lack of assay standardization[9,10] makes the introduction of global cut-off concentrations difficult[11,12]. An analytically validated RMP would permit uniform calibration of different assay platforms, ideally resulting in better comparability across analytical platforms, and better control of factors contributing to the overall measurement variability.

Absolute quantification of $Aβ_{1-42}$ using the developed LC-MS/MS method overcomes many of the issues associated with antibody-based methods. The method, listed as an RMP by the Joint Committee for Traceability in Laboratory medicine (JCTLM database identification number C11RMP9), will be used determine the absolute concentration of $Aβ_{1-42}$ in a Certified Reference Material (CRM) to harmonize CSF $Aβ_{1-42}$ measurements across techniques and analytical platforms. The described workflow should be of relevance for the development of candidate reference methods for peptides and proteins within other areas of medicine.

PROTOCOL:

Note: This protocol requires aliquots of at least 50 µL with a concentration of 50 µg/mL for each Aβ peptide as starting material. The Aβ peptides should be dissolved in 20% acetonitrile (ACN) and 4% concentrated ammonia solution in deionized water (v/v) and stored at -80 °C.

Caution: See Table 1 for safety information.

1. Preparation of solutions

1.1) Prepare 100 mL of 20% ACN and 4% concentrated ammonia solution in deionized water (v/v) by diluting 20 mL ACN and 4 mL concentrated ammonia (~25%) in deionized water. Adjust the final volume to 100 mL with deionized water. Make fresh daily.

1.2) Prepare 50 mL of 5 M guanidine-hydrochloride by dissolving 26.08 g guanidine-hydrochloride in deionized water to a final volume of 50 mL. Store at 20 °C and make fresh monthly.

1.3) Prepare 200 mL of 4% phosphoric acid in deionized water (v/v) by diluting 9.4 mL concentrated phosphoric acid (~85%) in deionized water. Adjust the final volume to 200 mL with deionized water. Store in the refrigerator and make fresh weekly.

1.4) Prepare 50 mL of 75% ACN and 10% concentrated ammonia solution (v/v) in deionized water by diluting 37.5 mL ACN and 5 mL concentrated ammonia (~25%) in deionized water. Adjust the final volume to 50 mL with deionized water. Make fresh daily.

1.5) Thaw at least 2.5 mL human CSF for the calibrators,.obtained from de-identified leftover samples from clinical routine analysis.

1.6) Prepare artificial CSF containing 150 mM Na, 3.0 mM K, 1.4 mM Ca, 0.8 mM Mg, 1.0 mM P and 155 mM Cl in deionized water and add bovine serum albumin to a final concentration of 4 mg/mL. Only 1 mL is needed per analysis but prepare a large volume, aliquot and store for future use.

2. Preparation of calibrators

2.1) Prepare 0.5 mL 4 µg/mL ^{15}N-Aβ_{1-42} peptide by adding 40 µL of 50 µg/mL ^{15}N-Aβ_{1-42} to 0.46 mL 20% ACN and 4% concentrated ammonia in a 0.5 mL microcentrifuge tube. Mix on vortex mixer for one minute.

2.2) Prepare 2 mL 100 ng/mL ^{15}N-Aβ_{1-42} peptide by adding 50 µL of the 4 µg/mL ^{15}N-Aβ_{1-42} to 1.95 mL 20% ACN and 4% concentrated ammonia in a 2 mL microcentrifuge tube. Mix on vortex mixer for one minute.

2.3) Prepare six calibrator solutions (A-F) by mixing the volumes of each solution indicated in Table 2. Use 0.5, 1.5 and 2 mL microcentrifuge tubes. Mix on vortex mixer for one minute.

2.4) Prepare the final calibrators (in duplicate) in 0.5 mL microcentrifuge tubes by adding corresponding calibration solutions and human CSF according to Table 3. Mix on vortex mixer for one minute.

3. Preparation of internal standard

3.1) Prepare 2 mL 0.8 µg/mL ^{13}C-Aβ_{1-42} peptide by adding 32 µL of 50 µg/mL ^{13}C-Aβ_{1-42} to 1.968 mL 20% ACN and 4% concentrated ammonia in a 2 mL microcentrifuge tube. Mix on vortex mixer for one minute.

3.2) Prepare 5 mL 16 ng/mL ^{13}C-Aβ_{1-42} peptide by adding 0.1 mL of 0.8 µg/mL to 4.9 mL 20% ACN and 4% concentrated ammonia in a 5 mL microcentrifuge tube. Mix on vortex mixer for one minute.

4. Preparation of response factor sample

Note: The response factor (RF) determination is performed to determine the concentration of the labeled peptide used for calibration (^{15}N-Aβ_{1-42}). This requires that the concentration of the native Aβ_{1-42} peptide has been determined using amino acid analysis (AAA). Thus, the volume and concentration of native Aβ_{1-42} peptid aliquots need to fulfill the requirements of the AAA.

4.1) Prepare 0.5 mL 4 µg/mL native (unlabeled) Aβ_{1-42} by adding 40 µL of 50 µg/mL native Aβ_{1-42} to 0.46 mL 20% ACN and 4% concentrated ammonia in a 0.5 mL microcentrifuge tube. Mix on vortex mixer for one minute.

4.2) Prepare a 2 mL 40 ng/mL mix of native and ^{15}N-Aβ_{1-42} by adding 20 µL of 4 µg/mL native Aβ_{1-42} and 20 µL of 4µg/mL ^{15}N-Aβ_{1-42} to 1.96 mL of 20% ACN and 4% concentrated ammonia in a 2 mL microcentrifuge tube. Mix on vortex mixer for one minute.

4.3) Add 20 µL of the 40 ng/mL mix to 0.38 mL artificial CSF in a 0.5 mL microcentrifuge tube. Prepare duplicates and mix on vortex mixer for one minute.

5. Sample preparation

Note: Thaw samples to be measured at room temperature on a roller.

5.1) Add 0.18 mL of each calibrator, response factor and unknown sample (including quality control [QC] samples if used) to a 1 mL protein 96 deep-well plate according to Figure 1 (assuming a full plate is used). Make sure to add the samples in, or close to the bottom of the wells.

5.2) Add 20 µL of internal standard to each well (*i.e.,* calibrators, response factors, QCs and unknowns). It is crucial to release the drop on the side of the well close to the surface of the sample without submerging the pipette tip.

5.3) Add 0.2 mL 5 M guanidine-hydrochloride to each well.

5.4) Place the sample plate on a microplate shaker and mix the samples for 45 minutes at 1100 rpm. The optimal frequencey might differ depending on instrumentation. Set the frequency and amplitude of the mixer so that the solutions are thoroughly mixed and no drops of internal standard or CSF are left unmixed on the side of the wells.

5.5) Add 0.2 mL of 4% phosphoric acid to each well. Vortex mix briefly.

6. Solid phase extraction

Note: In all washing, loading and elution steps, apply lowest possible vacuum after adding the solution and gradually increase as needed to load or elute the solution.

Disable the vacuum between each loading and elution step.

6.1) Put a reservoir tray for waste under a mixed-mode cation exchange 96-well solid phase extraction (SPE) plate in the extraction plate manifold chamber.

6.2) Condition the SPE sorbent by adding 0.2 mL methanol to each well.

6.3) Equilibrate the sorbent by adding 0.2 mL 4% phosphoric acid to each well.

6.4) Transfer all samples (about 0.62 mL in each well) from the deep-well plate to the SPE plate. It is highly recommended to use an eight channel pipette when transferring the samples from the deep well-plate to the SPE-plate. It is not crucial to transfer the entire or equal volumes of all samples since the samples contain an internal standard which will compensate for variations.

6.5) Wash the sorbent after the samples have passed through by adding 0.2 mL 4% phosphoric acid to each well.

6.6) After the washing solvent has eluted from the sorbent, replace the reservoir tray with a collection plate or tubes.

6.7) Elute the sample from the sorbent by adding 50 µL 75% ACN/10% concentrated ammonia twice. Note that this solution requires very low vacuum to pass through the sorbent. Remember to disable the vacuum between each addition.

6.8.1) OPTIONAL. Seal the collection plate or tubes and freeze at -80 °C. Remove the seal from the collection plate or tubes before proceeding to 6.8.2.

6.8.2) Dry the eluates by using vacuum centrifugation (without applying heat). This can take from one to several hours depending on the vacuum centrifuge.

6.8.3) Seal the containers and freeze at -80 °C.

7. Liquid chromatography

7.1) Prepare mobile phase A (5% ACN and 0.3% concentrated ammonia in deionized water [v/v]), B (4% deionized water and 0.1% concentrated ammonia in ACN [v/v]) and needle wash (50% ACN and 4% concentrated ammonia in deionized water [v/v]).

7.1.1) For 500 mL mobile phase A, add 25 mL ACN and 1.5 mL concentrated ammonia to deionized water. Adjust the final volume to 500 mL with deionized water.

7.1.2) For 500 mL mobile phase B, add 500 µL concentrated ammonia and 25 mL deionized water to ACN. Adjust the final volume to 500 mL with ACN.

7.1.3) Prepare 250 mL needle wash by adding 120 mL ACN and 10 mL to deionized water. Adjust the final volume to 250 mL with deionized water.

7.1.4) Put mobile phase A, B and needle wash bottles open in sonication bath for 20 minutes before use with the LC system

7.2) Dissolve each sample with 25 µL 20% ACN and 4% concentrated ammonia solution and place on shaker for 20 minutes. Centrifuge down the sample and place in the autosampler (keep at 7 °C).

7.3) Inject 20 µL sample on a 1×250 mm polystyrene-divinylbenzene (reversed-phase) monolithic column maintained at 50 °C.

7.4.1) Use the LC gradient shown in the Table 4 with a flow rate of 0.3 mL/min. Divert the first two and last five minutes to waste (post column) using a divert valve to reduce contamination of the mass spectrometer.

8. Mass spectrometric analysis

Note: These parameters were used for a quadrupole-orbitrap hybrid mass spectrometer equipped with a heated electrospray ionization source.

8.1) Set the parameters for the ion source according to Table 5.

8.2) Set the MS instrument to isolate the 4+ charge states of native $A\beta_{1-42}$ (1129.48 mass-to-charge ratio [*m/z]*), ^{15}N-$A\beta_{1-42}$ (1143.00 *m/z*) and ^{13}C-$A\beta_{1-42}$ (1179.50 *m/z*) in the quadrupole mass analyzer with an isolation width of 2.5 *m/z*.

8.2) Fragment the isolated peptides in the collision cell with a normalized collision energy (NCE) of 17.0. This might need to be tuned for each instrument even of the same type (and especially if using other types of instrument, *e.g.* a triple quadrupole MS).

8.3) Record fragment spectra with a resolution of 17.500 with an automatic gain control target of 2×10^5 charges and a maximum injection time of 250 ms.

9. Data processing

9.1) Use the sum of the product ions (with a mass tolerance of ±250 milli mass units [mmu]) in Table 6 to calculate the chromatographic areas for each peptide. Note that the ion types and numbers are only shown for native $A\beta_{1-42}$ product ions since they are the same for both ^{15}N-$A\beta_{1-42}$ and ^{13}C-$A\beta_{1-42}$.

9.2) Determine the average response factor of the two response factor samples by dividing the area under the curve (chromatographic peak) of ^{15}N-$A\beta_{1-42}$ with the area under the curve of native $A\beta_{1-42}$.

9.3) Adjust the concentration of the ^{15}N-$A\beta_{1-42}$ used for calibration by multiplying it with the response factor calculated in 9.2.

9.4) Construct a calibration curve by plotting the area ratios of ^{15}N-$A\beta_{1-42}$ to ^{13}C-$A\beta_{1-42}$ from the two sets of calibrators against the concentration.

9.5) Calculate the slope and intercept of the calibration curve suing linear regression.

9.6) Calculate the area ratio of native $A\beta_{1-42}$ to the internal standard (^{13}C-$A\beta_{1-42}$) for unknown samples.

9.7) Extrapolate the concentration of unknown samples from the calibration curve using the slope and intercept obtained in 9.5.

REPRESENTATIVE RESULTS:

The plate setup in Figure 1 is used for a full plate of samples. If fewer unknown samples are to be analyzed, the second calibrator, RF and QC set should be placed after the first half of the unknown samples.

As seen in Figure 2, the calibrators are close to the regression line with low standard deviations. The method has a lower level of quantification of 150 pg/mL with an upper level of quantification of 4000 pg/mL. The residual standard deviation of the calibration should of course be as low as possible. If calibration is non-linear the run should be discarded (depending on severity) and is most likely due to incorrect pipetting technique and/or errors in the dilution of calibrators. The coefficient of variation (CV) of replicates should be below 20% but preferably below 10%.

The native, ^{15}N- and ^{13}C-$A\beta_{1-42}$ elute from the LC column simultaneously (since they only differ on the isotopic level) with close to symmetrical peaks without significant tailing (Figure 3). At least ten measurements should be performed for each chromatographic peak and can be adjusted with the maximum injection time in the instrument method. All three peptides can be measured simultaneously for all measurements (calibration, RF and unknowns) during the MS analysis. However if the sensitivity of the method is suboptimal, only peptides of interest should be measured for each injection, *i.e*., only measure ^{15}N- and ^{13}C-$A\beta_{1-42}$ for calibrators, native and ^{15}N-$A\beta_{1-42}$ for RF samples and native and ^{13}C-$A\beta_{1-42}$ for unknown samples.

Figure 1: SPE & deep-well plates layout

Typical layout of calibrators (A-F), response factor sample (RF), quality control samples (QC) and unknowns.

Figure 2: Calibration curve.

Calibration curve constructed using ^{15}N-$A\beta_{1-42}$ at 172, 572, 1144, 2287, 3431 and 4574 pg/mL (adjusted using the response factor) and ^{13}C-$A\beta_{1-42}$ as internal standard in human CSF (n = 2). The area ratio of ^{15}N-$A\beta_{1-42}$/^{13}C-$A\beta_{1-42}$ is plotted (Y-axis) against the concentration (X-axis).

Figure 3: Chromatogram.

Chromatogram of 0.500 ng/mL native (endogenous) $A\beta_{1-42}$ (top panel) and 1.6 ng/mL ^{13}C-$A\beta_{1-42}$ (bottom panel) in human CSF.

Figure 4: Quantification of unknown $A\beta_{1-42}$ in unknown samples

The peak area ratio is calculated by dividing the native $A\beta_{1-42}$ chromatographic peak

area with the internal standard (^{13}C-Aβ$_{1-42}$) chromatographic peak area. The concentration of native Aβ$_{1-42}$ in the sample is extrapolate from the calibration curve.

Table 1: Safety information
Safety information for chemicals used for this protocol.

Table 2: Calibrator solutions
Calibrator solutions prepared in 20% ACN and 4% concentrated ammonia used for spiking CSF calibrators.

Table 3: Calibrators
Calibrators prepared in human CSF.

Table 4: LC gradient
The LC gradient used with a constant flow-rate of 300 µL/min.

Table 5: Ion source settings.
Parameters for the ion source to be set in the instrument tune software.

Table 6: Ions used for quantification
The 4+ charge states of the precursor ions are isolated in the quadrupole mass analyzer with an isolation width of 2.5 *m/z*. The product ions (with a mass tolerance of ±250 mmu) are used to calculate the chromatographic areas for each peptide. Ion types and numbers are only shown for native Aβ$_{1-42}$ product ions since they are the same for both ^{15}N-Aβ$_{1-42}$ and ^{13}C-Aβ$_{1-42}$.

DISCUSSION:
For the described method, instead of using a surrogate matrix we used the surrogate analyte approach[13-16], which enables calibration in human CSF. The surrogate analyte approach involves two different isotopically labeled standards. One (^{15}N-Aβ$_{1-42}$) is used to generate the calibration curve in human CSF while another (^{13}C-Aβ$_{1-42}$) is used as internal standard. Unknown endogenous Aβ$_{1-42}$ concentrations are then extrapolated from the calibration curve constructed using the ^{15}N-Aβ$_{1-42}$/^{13}C-Aβ$_{1-42}$ ratio by the calculated endogenous Aβ$_{1-42}$/^{13}C-Aβ$_{1-42}$ ratio. The surrogate analyte approach was used since there is no analyte-free CSF availible, and low Aβ$_{1-42}$ recovery was observed when using native Aβ$_{1-42}$ in a surrogate matrix during method development.

Since ^{15}N-Aβ$_{1-42}$ and native Aβ$_{1-42}$ may give different responses in the MS, the concentration of ^{15}N-Aβ$_{1-42}$ is adjusted by measuring a RF sample – an artificial CSF sample containing equal concentrations of ^{15}N-Aβ$_{1-42}$ and native Aβ$_{1-42}$ with a known concentration determined by AAA. The response factor might differ between different mass spectrometers as well as due to possible variations in the isotopic purity of the ^{15}N-labeled peptide between batches. Thus the response factor should be determined for each measurement day.

The most critical steps in this protocol are the preparation of calibrators and RF samples. Aβ peptides, and especially the Aβ$_{1-42}$, are very hydrophobic and easily stick to

pipette tips and surfaces of tubes[8,17,18]. To minimize loss of Aβ peptides during pipetting it is extremely important to saturate the pipette tips prior to delivery. Preferably, three volumes of peptide solution should be discarded prior to delivery to a new tube containing solution. Depending on the volume and concentration of the stock solution this is not always possible. The second best approach is of course to pipette the peptide solution up and down three times prior to delivery. For the same reason it is important to use appropriate sizes for tubes, avoiding large void volumes.

Previously published data for the method shows that recovery was within 100% (15%)[15,] The relative errors for the back-calculated calibrators were below 15% of the whole range defined by the calibrator curve[19].

One obvious limitation of this technique is its low throughput compared to automated immunoassays. However, the purpose of the described method is high accuracy and not throughput. This method can also be expanded to include the two shorter $A\beta_{1-38}$ and $A\beta_{1-40}$[19]. Another limitation of this method is that the operator will need extensive mass spectrometry training before running the analysis on the instrument.

Quantification using immunoassays is dependent on the interaction between the antibody and the antigen. This interaction could be affected by the presence of sample components that may interfere or compete with the interaction. In addition, the interaction may also be affected by the conformation of the antigen. These effects are difficult to control and are believed to be the main reason why it has been difficult to harmonize results between immunoassay platforms and between laboratories. Because quantification with MS is based on directly counting the target molecules relative to a stable-isotope labeled standard, quantification is absolute and generally unaffected by such matrix effects. In addition, diagnostic protein measurements by immunoassays should be supported by an unbroken chain of higher-order of measurement procedures and material, from validated LC-MS/MS and stable isotope-labeled internal standards to RMPs and a CRM, thus improving results comparability and reliability[20,21].

In conclusion, the described RMP for value assignment of $A\beta_{1-42}$ in CSF is an important step in developing a CRM that will help establish general cut-off concentrations for $A\beta_{1-42}$ in CSF. Exact cut-offs are highly important to make an accurate early diagnosis of AD, and of utmost importance when the new type of disease-modifying drugs will reach the clinic.

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JP and EP reports no disclosures. HZ has served on advisory boards of Roche Diagnostics, Eli Lilly and Pharmasum Therapeutics. KB has served as consultant or at advisory boards for Fujirebio Europe, IBL International and Roche Diagnostics. HZ and KB are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg.

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