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Featured Article

Round robin test on quantification of amyloid-β 1–42 in cerebrospinal fluid by mass spectrometry

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Abstract

Introduction: Cerebrospinal fluid (CSF) amyloid-β 1–42 ($A\beta_{42}$) is an important biomarker for Alzheimer's disease, both in diagnostics and to monitor disease-modifying therapies. However, there is a great need for standardization of methods used for quantification. To overcome problems associated with immunoassays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a critical orthogonal alternative.

Methods: We compared results for CSF $A\beta_{42}$ quantification in a round robin study performed in four laboratories using similar sample preparation methods and LC-MS instrumentation.

Results: The LC-MS results showed excellent correlation between laboratories (r^2 >0.98), high analytical precision, and good correlation with enzyme-linked immunosorbent assay (r^2 >0.85). The use of a common reference sample further decreased interlaboratory variation.

Discussion: Our results indicate that LC-MS is suitable for absolute quantification of $A\beta_{42}$ in CSF and highlight the importance of developing a certified reference material.

Keywords: Amyloid beta; Cerebrospinal fluid; Mass spectrometry; Interlaboratory study

1. Introduction

Amyloid-β 1-42 ($A\beta_{42}$) in cerebrospinal fluid (CSF) is a well-established biomarker for Alzheimer's disease (AD) [1]. Besides its use as a diagnostic tool, CSF $A\beta_{42}$ is also essential to monitor in clinical trials of anti–Aβ disease-modifying drugs, to verify target engagement and monitor the biochemical effect of the drug [2]. CSF $A\beta_{42}$ is routinely measured using different immunoassays [1]. However, broad-scale use of these antibody-based techniques in clinical practice is hampered by high variability. To identify sources of variability for $A\beta_{42}$ measurement, the Alzheimer's Association launched an external quality control program [3,4], which has pinpointed a number of problems associated with suboptimal assay standardization including harmonization across analytical platforms and assay kit batches [5]. The lack of assay standardization presents a problem for the use of $A\beta_{42}$ as a biomarker in routine clinical practice because it is not possible to establish generally applicable cutoff values for diagnosis and for research because results from studies performed in different laboratories cannot be readily compared [4,6]. Thus, there is a

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need for standardization of CSF AD biomarker assays having high analytical precision, selectivity, stability over long periods, and with low variability across centers.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS), using the selected reaction monitoring (SRM) mode, has been used for quantification of small molecules in the clinic for many years and is now also being investigated as an alternative to immunoassays for peptide and protein quantification [7] as well as being used as reference methods to standardize protein assays [8,9]. Recently, a mass spectrometric method for CSF $A\beta_{42}$ quantification in 200-μL CSF using a single solid-phase extraction (SPE) sample preparation step before LC-MS analysis was reported [10]. Using similar methods, mass spectrometric quantification of CSF $A\beta_{42}$ in clinical studies including AD and controls showed high precision, absence of matrix effects, and a similar separation of patient/control cohorts as enzyme-linked immunosorbent assay (ELISA) or xMAP (Luminex Corp, Austin, TX, USA) [11,12].

A key difference of mass spectrometric quantification compared with immunoassays is the ability to use a stable isotope–labeled analyte as an internal calibrant, or internal standard (IS), which is added to the crude samples before sample preparation. Because these molecules have identical physiochemical properties to the endogenous analyte, and are only distinguished from the analyte in the mass analyzer, they can be used to track and compensate for variations in the sample preparation and instrumental analysis procedures. Provided that sample preparation is performed under denaturing conditions so that both analytes and calibrants are dissociated from other sample components, the risk of matrix effects on quantification is eliminated. Thereby, mass spectrometry has the potential to provide unbiased quantification, decreasing interlaboratory measurement variation. In the present study, we test this hypothesis for CSF $A\beta_{42}$ measurements in a round robin study with four laboratories involved in the Global Biomarker Standardization Consortium of the Alzheimer's Association [5].

2. Materials and methods

Twelve pools of human CSF (from de-identified samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden) were aliquoted, frozen, and distributed to the participating laboratories, three vials for each laboratory and pool. Two vials were also analyzed at the Sahlgrenska University Hospital with ELISA (Innotest β-amyloid$_{(1–42)}$; Fujirebio, Gent, Belgium) for comparison.

SPE and MS quantification was performed as previously described [10] with some modifications. Although the general procedure was the same for all laboratories, it differed in some details which are listed in Table 1. Generally, unknown CSF samples were thawed at room temperature and spiked with uniformly ^{15}N labeled $A\beta_{42}$ (^{15}N-$A\beta_{42}$; rPeptide, Bogart, GA, USA) as IS, and external calibration was performed either in human or artificial CSF. For human CSF calibrants, endogenous $A\beta_{42}$ was used as IS, whereas ^{15}N-$A\beta_{42}$ was spiked in at varying concentrations. For the artificial CSF calibrants, ^{15}N-$A\beta_{42}$ was added as IS and native $A\beta_{42}$ was spiked in to construct the calibration curve. The concentration of endogenous $A\beta_{42}$ in unknown CSF samples was

Table 1
Procedures for participating laboratories

Procedure	Waters	PPD	U. Penn.	U. Got.
IS concentration, ng/mL	1	2	2	1.6
CSF volume, μL	200	100	250	200
Calbrator matrix	aCSF with 5% rat plasma	aCSF with 4 mg/mL HSA + IgG, glucose	aCSF with 4 mg/mL BSA	Human CSF
LC System	ACQUITY, 1D	ACQUITY; 2D trapping/ eluting	ACQUITY; 2D trapping/ eluting	Accela 1250
Dilution (injection)	50 μL + 25 μL H_2O (10 μL)	50 μL + 50 μL H_2O (30 μL)	50 μL + 50 μL H_2O (50 μL)	None. Dried eluate resuspended in 25 μL 79:20:1 H_2O/ACN/ NH_4OH (20 μL)
LC mobile phases	A: 0.3% NH_4OH B: 90:10 ACN/MP A	A: 0.3% NH_4OH B: 90:5:5 ACN/TFE/H_2O	A: 0.1% NH_4OH B: 75:25:5 ACN/MeOH/ TFE	A: 0.1% NH_4OH, 5% ACN B: 0.03% NH_4OH, 95% ACN
Column	Waters BEH 300 2.1 × 150 mm, 1.7 μm, 50°C	Waters BEH 300 2.1 × 150 mm, 1.7 μm, 50°C	Waters BEH 300 2.1 × 50 mm, 1.7 μm, 60°C	Thermo ProSwift RP-4H 1 × 250 mm, 50°C
Flow rate, μL/min	200	300	200	300
MS	Waters Xevo TQ-S	Waters Xevo TQ-S	ABSciex API 5000	Thermo TSQ Vantage
Transitions, m/z	1129.0→1078.5	1129.0→1078.5	1129.0→1078.5	1129.58→1054.03, 1078.79, 1107.06
Run time	8.5 minutes	8.5 minutes	12 minutes	14 minutes

Abbreviations: IS, Internal standard; CSF, cerebrospinal fluid; aCSF, artificial cerebrospinal fluid; HSA, human serum albumin; BSA, bovine serum albumin; LC, liquid chromatography; ACN, acetonitrile; MP A, mobile phase A; TFE, tetrafluoroethylene; MeOH, methanol; MS, mass spectrometer; m/z, mass-to-charge ratio.

determined by calculating the native $A\beta_{42}$/^{15}N-$A\beta_{42}$ ratio using the calibration curve. The spiked samples were denatured using 5-M guanidine hydrochloride (GdnHCl), mixed for 45 minutes, and acidified with 4% phosphoric acid (H_3PO_4) before SPE (CSF:GdnHCl:H_3PO_4 1:1:1 v/v).

Pretreated samples were loaded on a mixed-mode cation exchange SPE 96-well plate (Oasis MCX μElution; Waters, Milford, MA, USA). The wells were washed with 4% H_3PO_4 followed by 10% acetonitrile (ACN) before the samples were eluted with 75% ACN and 3% ammonium hydroxide (NH_4OH). Extracted samples were injected on a reversed phase LC column and eluted using a linear mobile phase gradient from 10% to 45% B over 5.5 minutes (A: 0.3% NH_4OH in water, B: 90:10 (v/v) ACN/mobile phase A). SRM was performed by isolating and monitoring the quadruply charged $(M+4H)^{4+}$ precursors and product ions (see Table 1 and Supplementary Material for a detailed description of methods).

3. Results

The twelve CSF pools used in this round robin study had $A\beta_{42}$ concentrations varying from 148 to 716 pg/mL as determined by ELISA. The $A\beta_{42}$ concentrations determined at the four different centers using LC-MS correlated significantly with the ELISA results (r^2 >0.85 for all centers).

The interlaboratory variation, calculated by comparing the LC-MS results from individual laboratories with the

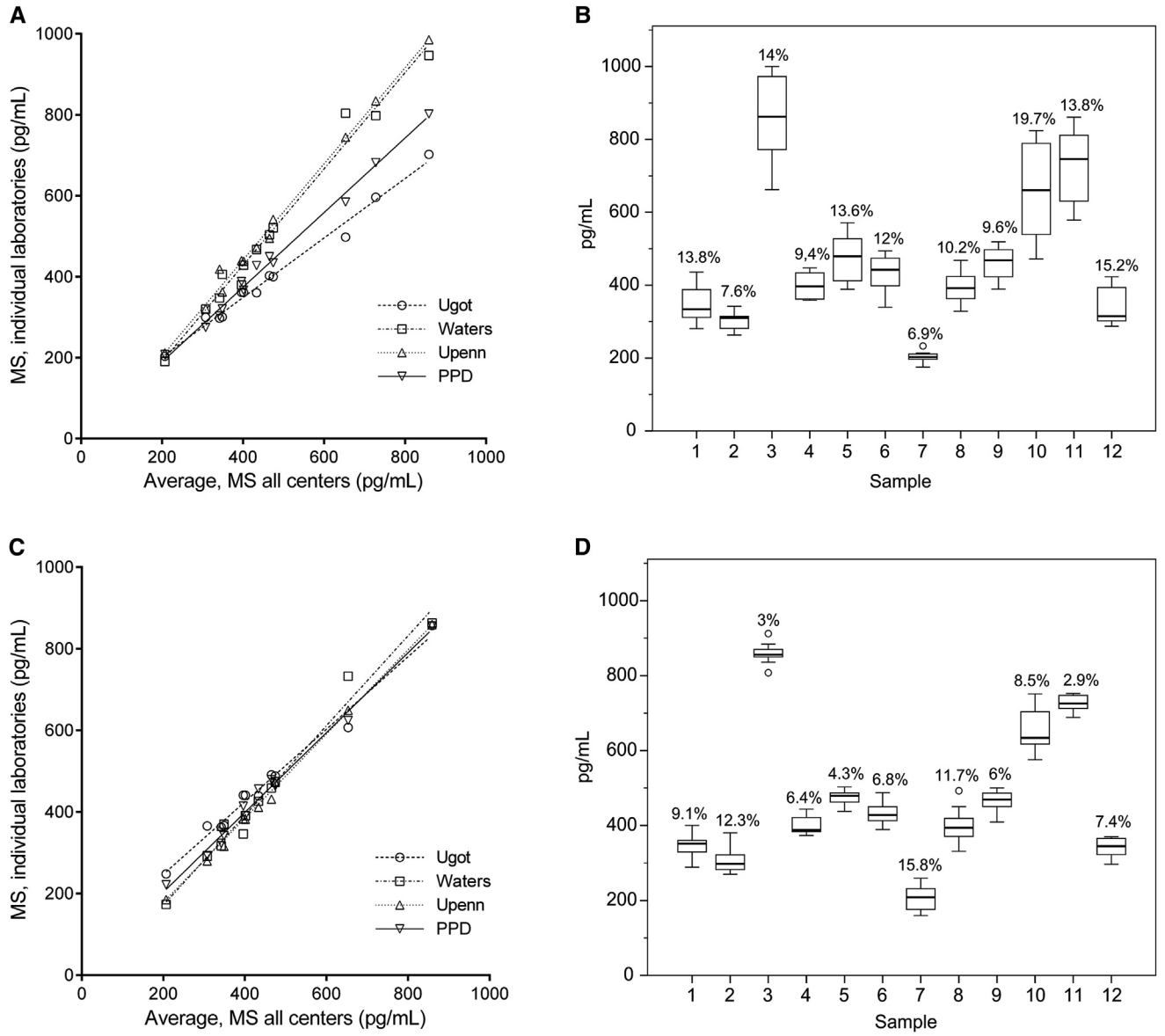

Fig. 1. Twelve pools of human CSF were analyzed at four different laboratories. (A) The interlaboratory variation, calculated by comparing the LC-MS, results from individual laboratories with the average results from all laboratories (r^2 >0.98). The average intralaboratory CVs was 4.7%. (B) The average interlaboratory CV was 12.2%. (C) Using sample 11 as a reference, the measurements for the other samples were adjusted. (D) After this correction, the average interlaboratory CV was 8.3%. Abbreviations: CSF, cerebrospinal fluid; LC-MS, liquid chromatography-tandem mass spectrometry.

average results from all laboratories, showed excellent correlation (r^2 >0.98; Fig. 1A). The average intralaboratory coefficient of variation (CV) was 4.7%, whereas the average interlaboratory CV was 12.2% (Fig. 1B).

It is likely that a large part of the interlaboratory variation can be attributed to the fact that each laboratory used different calibrant preparations. Thus, a common reference sample may decrease this variation. Since at this time no reference material for CSF $A\beta_{42}$ exists, we evaluated its potential benefit by designating one of the samples as reference. Sample 11 was chosen, having an $A\beta_{42}$ concentration above the average of the sample set [13] (758 pg/mL, average by all laboratories). Using the average concentration determined by the laboratories, a response factor was calculated for each laboratory [(sample 11 average pg/mL for all laboratories)/(sample 11 pg/mL for each laboratory)]. The results for the remaining samples were multiplied with the response factor obtained for each laboratory. After this correction, the average interlaboratory CV was 8.3% (Fig. 1C and D).

4. Discussion

We here report on a round robin study involving four laboratories using the same sample preparation procedure, but different LC-MS/MS instruments and calibration methods. The agreement of the methods was evaluated with aliquots from 12 CSF pools.

Using SPE under denaturing conditions in combination with stable isotope–labeled IS overcomes the immune affinity–based center-to-center variations possibly due to matrix-effects and lot-to-lot variations between assay kits.

Although the measurements of all four laboratories showed a strong linear correlation to the average interlaboratory results, there was a clear difference in the slope (Fig. 1A). This is most probably due to the different preparations of standards that were used, the concentrations of which have been determined at different sites and using different protocols. To overcome this problem, a reference material with an $A\beta_{42}$ concentration determined with a reference method is needed. In this study, the interlaboratory CV decreased from 12.2% to 8.3% when one sample was used to adjust the measurement values of the other samples, which clearly supports this concept. It should be noted that this improvement was obtained using a reference sample at a single concentration, thus it should be possible to improve method comparability further using a multiple point recalibration, e.g. using a series of dilutions.

Similar studies have been undertaken with ELISA analyses across centers using an external quality control program, launched by the Alzheimer's Association to identify sources of variability for measurement of $A\beta_{42}$. This study concluded an average interlaboratory CV of 23% [4].

5. Conclusions

In summary, our study shows that mass spectrometric $A\beta_{42}$ assays established in four laboratories using different instrumentation, calibration methods, and different calibrant preparations produce similar results. The use of a common reference sample further decreases interlaboratory variation, highlighting the importance of developing a standard reference material.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jalz.2015.06.1890.

RESEARCH IN CONTEXT

1. Systematic review: Cerebrospinal fluid amyloid-β 1–42 ($A\beta_{42}$) is a well-established biomarker for Alzheimer's disease and is increasingly used both in clinical diagnostics and in clinical drug trials. However, current Aβ immunoassays suffer from between-assay and between-laboratory variability, which hinders their general use in clinical diagnostics and introduces huge problems in clinical trials.
2. Interpretation: The results presented in the article suggest that mass spectrometric (MS) quantification of the $A\beta_{42}$ peptide relative to that of a stable-isotope labeled analog as internal standard is not subject to bias by matrix effects, as is quantification based on antibody-binding.
3. Future directions: An MS-based reference measurement procedure is needed to set the concentration of a certified reference material (CRM). The CRM may thus allow harmonization of commercially available assays for $A\beta_{42}$ and the establishment of globally accepted reference and decision limits that would be applicable across studies and laboratories.

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