Comparison of different matrices as potential quality control samples for neurochemical dementia diagnostics

Corresponding author: Prof. Dr. med. Piotr Lewczuk, Lab for Clinical Neurochemistry and Neurochemical Dementia Diagnostics, Department of Psychiatry and Psychotherapy, Schwabachanlage 6, 91054 Erlangen, Germany; Phone: +49-9131-85 34324; Fax: +49-9131-85 34238; E-mail: Piotr.Lewczuk@uk-erlangen.de
Abstract

BACKGROUND: So far, assay-vendor independent quality control (QC) samples for neurochemical dementia diagnostics (NDD) biomarkers are not available on the market, which forces NDD laboratories to prepare their own QC samples, mostly based on cerebrospinal fluid (CSF) samples rests.

OBJECTIVE: To prepare and test alternative matrices for QC samples that could facilitate internal and external (inter-laboratory) QC of the NDD biomarkers.

METHODS: Three matrices were tested in this study: (A) human pooled CSF, (B) Aβ peptides spiked into human prediluted plasma, and (C) Aβ peptides spiked into solution of bovine serum albumin in PBS. All matrices were tested also after supplementation with an antibacterial agent (sodium azide). We tested short- and long-term stability of the biomarkers and performed an inter-center variability study.

RESULTS: NDD biomarkers turned out to be stable in almost all samples stored at different conditions for up to 14 days as well as in the samples stored deeply frozen up to one year. Sodium azide did not improve the biomarkers stability. Inter-center variability of the samples sent under room temperature (pooled CSF, lyophilized CSF, and four artificial matrices) was comparable to the results obtained on deeply frozen samples in other large-scale projects.

CONCLUSION: We believe that it is possible to replace self-made CSF-based QC samples with large-scale volumes of the QC samples prepared with artificial peptides and matrices, which would greatly facilitate intra- and inter-center QC of the NDD measurements.

Introduction

 Neurochemical dementia diagnostics (NDD), along with the amyloid β (Aβ) PET imaging, has become the most important modality for the early diagnosis of Alzheimer's disease (AD) [1, 2]. Cerebrospinal
fluid (CSF) biomarkers, particularly Aβ peptides, show alterations in very early preclinical stages of the disease, probably decades before the onset of the first clinical symptoms [3, 4]. Therefore it is not surprising that more and more laboratories are willing to establish NDD as their routine tools either for an early AD diagnosis or to increase probability of the underlying AD pathology in clinical trials [5, 6]. On the other hand, partially due to the physical-chemical properties of Aβ peptides and Tau proteins, quality control (QC) of the NDD analyses is very difficult; large-scale international scientific studies performed in the last years [7, 8] have shown that also the inter-laboratory precision of the NDD measurements requires further optimization. As a matter of fact, insufficient validation of the NDD measurements is considered one of the most important factor preventing CSF biomarkers from general acceptance as a routine AD diagnostic tool [9].

So far, an assay-independent QC sample for any of the NDD biomarkers has not been available on the market, which forces laboratories to develop their own sample preparation procedures and QC materials, most commonly based on pooling of available CSF sample rests. Such approach makes intra-laboratory QC difficult and inter-laboratory QC even impossible, for at least three reasons: (a) the concentrations of the biomarkers in samples prepared by different centers are of course different; (b) quality of samples rests used for the preparation of the QC material is not always optimal, and (c) operating procedures to prepare the QC material significantly differ among centers. Moreover, longitudinal QC, taken together with the growing frequency of the analyses, requires preparation of the QC material in very high amounts, which is not always possible in these laboratories that have limited access to large number and volumes of the CSF samples.

In our previous study [10], we addressed feasibility of the generation of the NDD QC material based on large-scale CSF pools. In the current study, we tested also other, partially artificial, matrices and sample preparation procedures for the development of potential QC samples.

Materials and Methods
1. Preparation of the samples; conditions for the short- and long-term stability testing; influence of the antibacterial factor (sodium azide)

The Ethical Committee of the University of Erlangen-Nuremberg approved use of human CSF samples for this study. Short- and long-term stability was evaluated in the following matrices:

(A) Human pooled CSF without (A0) and with (A+) addition of sodium azide (NaN3, final concentration 0.1%). Briefly, freshly collected human CSF samples were pooled (to assure anonymity and non-traceability), frozen and stored at -80°C. For this project, the samples were thawed and pooled again to obtain appropriate volumes of homogenous samples; aliquots were then prepared and stored at the conditions described below.

(B) Artificial Aβ peptides (Aβ1-42 and Aβ1-40; AnaSpec Inc., Fremont, USA) spiked into human plasma prediluted 1:200 with 0.97% phosphate-buffered saline (PBS, pH 7.4; Amresco, Solon, USA) and Tween 20 (Roth, Karlsruhe, Germany), without (B0) and with (B+) addition of NaN3 (final concentration 0.1%). Briefly, 5 mL of human plasma was diluted with 995 mL of PBS + 500 µL of Tween 20, and divided into two equal volumes. To one portion (500 mL), 5 mL of 10% NaN3-stock was added. Recombinant powdered Aβ peptides (0.5 mg) were reconstituted in 1 mL of dimethyl sulfoxide (DMSO; Sigma, St. Louis, USA) following further dilution 1:1000 (in two steps) in DMSO to the stock-concentration of 500 ng/mL. These stock solutions were then aliquoted and frozen at -80°C. Immediately before the preparation of the aliquots, 250 µL of a peptide/DMSO solution was added to 9.750 mL of prediluted plasma (with- and without NaN3).

Furthermore, material B was also tested as a mixture of Aβ1-42 and Aβ1-40 in one sample. Briefly, artificial peptides were added to the prediluted serum to achieve the same final concentrations as in the separate Aβ1-42 and Aβ1-40 samples.

(C) Artificial Aβ peptides spiked into 0.04% bovine serum albumin (BSA; Roth, Karlsruhe, Germany) prediluted in PBS/Tween 20, without (C0) and with (C+) addition of NaN3 (final concentration 0.1%). Briefly, the material C was prepared analogously to the material B with the exception that instead of prediluted plasma, 0.04% BSA/PBS+Tween was used as a diluent.
All six types of the samples (three matrices, each with- and without NaN3) were aliquoted immediately after the preparation and stored: (a) at room temperature (RT), (b) in a refrigerator (+4°C), and (c) frozen at -20°C. One aliquot from each of these six sets was deep frozen (-80°C) immediately after the preparation, and served as a reference sample. The remaining aliquots were kept for: 1-7, 10, and 14 days following transfer into -80°C. A summary of the samples preparation protocols for the STS testing is presented in table 1. After the completion of the storage time (i.e. 14 days), all aliquots of a given sample type were thawed and immediately tested in one analytical run (one ELISA or chemiluminescence plate) per biomarker. All measurements were performed in duplicates.

The influence of NaN3 (antibacterial agent) on the concentrations of the biomarkers was tested by the comparison of their concentrations after storage of the samples for five days at room temperature with and without NaN3.

For long-term stability testing, samples prepared analogously to those for the STS testing were deep-frozen and stored at -80°C for 6, 9, and 12 months, following measurements.

2. Assays

STS and LTS tests were performed with the following assays: Aβ1-40 (IBL International GmbH, Hamburg, Germany), Aβ1-42 (Innogenetics, Ghent, Belgium and Meso Scale Discovery, Rockville, USA), Tau (Innogenetics and Meso Scale Discovery), pTau181 (Innogenetics).

3. Preparation of the freeze-dried samples

1 mL of CSF aliquots was filled into 2-mL SCHOTT TopLyo® glass vials (SCHOTT AG, Germany), and partially stoppered with 13 mm lyophilization stoppers. Freeze-drying of CSF was performed using a commercial Martin Christ Epsilon 2+4 LSC freeze-dryer (CHRIST, Germany). Samples were placed on
a heating shelf of the freeze-dryer at a temperature of +4 °C and slowly pre-freezed (1 degree/min) down to -80 °C with additional frozen hold step for 3 hrs. Primary and secondary drying was achieved at a vacuum of 30 mTorr and 15 mTorr, respectively. The temperature of the samples was monitored during the freeze-drying process using sample vials with thermoprobes. Cycle time was 26 hrs. In the end of the cycle, freeze-dried samples were closed, removed from the freeze-dryer, sealed to inhibit the humidification, and kept in the dark at -80 °C until shipment. Shipment has been done at ambient temperatures. Freeze-dried samples were rehydrated with deionized water.

4. Inter-center variability testing (external quality control, EQC)

For the inter-center variability testing, the participating 25 laboratories obtained the following samples, all of them shipped by regular mail at ambient conditions:

EQC-1: human pooled CSF;

EQC-2: sample EQC-1 freeze-dried and send to the participating centers as powder to be reconstituted in 1 mL of distilled water;

EQC-3: Aβ1-42 + Aβ1-40 dissolved in prediluted plasma. Briefly, 50µL of a 500 ng/mL Aβ1-42 stock and 320µL of a 500 ng/mL stock Aβ1-40 was diluted in 17 mL of human plasma prediluted 1:200 in PBS/Tween.

EQC-4: Aβ1-42 + Aβ1-40 diluted in plasma, analogously to the material EQC-2, whereas the volumes of the spiked stocks were 35µL of Aβ1-42 and 160µL of Aβ1-40.

EQC-5: Aβ1-42 + Aβ1-40 diluted in BSA/PBS+Tween. Briefly, 50µL of a 500 ng/mL Aβ1-42 stock and 320µL of a 500 ng/mL Aβ1-40 stock was diluted in 17 mL of 0.04% BSA/PBS+Tween.

EQC-6: Aβ1-42 + Aβ1-40 diluted in 0.04% BSA in PBS+Tween, analogously to the material EQC-4, whereas the volumes of the spiked stocks were 35µL of Aβ1-42 and 160µL of Aβ1-40.

The summary of the samples for the EQC is presented in table 2.
5. Inter-assay imprecision

For inter-assay imprecision, a set of aliquots of human pooled CSF was prepared and promptly refrozen at -80°C. One aliquot was freshly thawed immediately before the analyses, which were performed by different operators and on different days in the time span of fourteen months.

Results

1. Short-term stability

The results of the short-term stability (STS) testing of the prediluted plasma-based samples (B0 and B+) are presented in figures 1 and 2, and the resulting normalized concentrations and their coefficients of variation (CV’s) are presented in tables 3 and 4. Taken together, both biomarkers turned out stable when stored under all three conditions, with only single cases of the concentrations exceeding 80% - 120% range of the starting concentrations, irrespectively if the two peptides were tested separately (fig. 1) or as a combination (fig. 2). Expectedly, the samples stored at -20°C were even more stable than those stored at +4°C or room temperature.

The results of the STS testing of the human pooled CSF samples (A0 and A+) are presented in the figure 3, and the normalized concentrations and CV’s are shown in table 5. Aβ1-40 was very stable in the CSF samples stored at the three tested conditions up to 14 days, whereas Aβ1-42, expectedly, was apparently less stable. Interestingly, addition of NaN3 resulted in an improved stability of Aβ1-42 in the CSF samples (compare fig. 3c and 3e for Aβ1-42 tested with Innogenetics assays, and 3d and 3e for MSD assays). Tau tested with Innogenetics assays (3g and 3h) showed stable concentrations over the whole tested period, whereas when tested with MSD assays (3i and 3j), its concentration obviously dropped after 2 - 3 days of storage. Phosphorylated Tau, expectedly, characterized with stable concentrations up to 14th day of storage (fig. 3k and 3l).

The results of the STS testing of the peptides diluted in BSA/PBS+Tween (C0 and C+) are presented in figure 4, and the corresponding normalized concentrations and CV’s are shown in table 6. Also in this matrix, Aβ1-40 turned out very stable at all three tested conditions, with apparent drop of
concentration in a sample stored for longer than 10 days at room temperature without NaN3 (fig. 4a). Interestingly, also Aβ1-42 was relatively stable in this matrix, however, with unexpected and unclear rise in its concentration in a RT sample at day 5 when tested with Innogenetics (fig. 4c) but not with MSD (fig. 4e) assay.

2. Influence of the antibacterial factor

The differences in the concentrations of the biomarkers possibly resulting from addition of sodium azide are presented in table 7. Application of 0.1% NaN3 apparently did not change starting concentrations of the two peptides nor did it influenced their concentrations after 5 days at room temperature.

3. Long-term stability

The results of the long-term stability (LTS) are presented in figure 5. The concentrations of Aβ1-40 in the samples stored deeply frozen for one year was apparently stable in all samples, with an exception of the sample based on prediluted serum not supplemented with NaN3 (fig. 5a, Mat. B0). Interestingly, Aβ1-42 was very stable in prediluted serum-based samples (fig. 5b, Mat. B0 and B+) as well as, to a lesser degree, CSF samples (fig. 5b, Mat. A0 and A+), but much less stable (in terms of rising and dropping concentrations) in a BSA/PBS samples (fig. 5b, Mat. C0 and C+). Tau (fig. 5c) and pTau181 (fig. 5d) showed stable concentrations in the CSF samples stored for up to one year.

4. Inter-assay variability

The results of the inter-assay variability of the biomarkers tested in a pooled human CSF sample are presented in fig. 6. Inter-assay imprecision (the number of the repetitions are in brackets) of the biomarkers was: 11.7% (25), 10.4% (30), 9.4% (17), and 6.8% (20) for Aβ1-42, Aβ1-40, Tau, and pTau181, respectively.
5. Inter-center variability

The results of the biomarkers measurements reported by the participants of the inter-center study are presented in figure 7 and table 8. The concentrations in the pooled CSF sample (EQC-1 on figures 7a, c, e, and f) showed moderate variability, expectedly slightly higher in case of Aβ1-42 (~25%) than in case of other biomarkers, with the lowest variability, as expected, in case of pTau181 (<10%). Comparable variability was obtained in case of lyophilized CSF (EQC-2 on figures 7a, c, e, and f), with the exception that one participant reported unexpectedly low Aβ1-40 concentration in EQC-2 (particularly when compared to his EQC-1 result), which increased overall variability of Aβ1-40 in the lyophilized material to 45%. Interestingly to note is that the concentrations of Aβ1-40, Aβ1-42, and pTau181 reported in the lyophilized material (EQC-2) paralleled very well, with two or three exceptions, the concentrations in the native CSF (EQC-1) but were consistently 20 - 30% lower. In case of Tau, however, a vice versa held true: the concentrations in lyophilized material were consistently 50 - 60% higher than in the native CSF.

Aβ1-40 in the artificial samples characterized with the variability around 20% (fig. 7b); moreover, Aβ1-40 concentrations reported by the participants were almost identical irrespectively of the material tested (compare EQC-3 vs. EQC-5 and EQC-4 vs. EQC-6 on fig. 7b), furthermore, the concentrations in EQC-4 and EQC-6 were, as expected, almost ideally halves of the concentrations in EQC-3 and EQC-5, respectively.

Similar holds true for Aβ1-42 (fig. 7d); with overall inter-center variability around 25%, the concentrations in EQC-3 and EQC-5 were very well comparable to each other (excepting the participants # 2 and 5), and the same was observed regarding the samples EQC-4 and EQC-6. Furthermore, also in case of Aβ1-42, the concentrations in EQC-4 and EQC-6 turned out, as expected, almost ideally 70% of the the concentrations in EQC-3 and EQC-5, respectively.

Discussion

In this study, we present the results of the validation of three matrices (one based on human CSF, and two based on artificial solutions) as potential QC samples for NDD biomarkers.
At the beginning of this study (Summer 2011), no commercial material was available for QC of the CSF AD biomarkers; meanwhile the situation has improved, as majority of the assay vendors include QC samples in their kits; however, it is obvious that such samples are specific-assay-tailored, and cannot be reliably used to control the quality of other manufacturers' assays.

In our study, three matrices were tested: (A) human pooled CSF, (B) $\text{A}\beta$ peptides spiked into prediluted human plasma, and (C) $\text{A}\beta$ peptides spiked into BSA/PBS solution. Currently most, if not all, centers use self-made pooled CSF QC samples for their purposes; this approach requires collection of large-scale CSF "rests", which should meet at least minimal quality criteria. Moreover, such CSF rests should be collected in a relatively short time, to avoid obsolescing of the CSF samples before required volume has been collected. Further limitation of such approach is that it is difficult to control the target concentrations of the biomarkers, and, unless a given center has an access to large scale number and volumes of pathologic (in sense of neurodegeneration diseases) samples, it is difficult to prepare samples with different levels of the biomarkers concentrations. To avoid all these limitations, an artificial sample could be prepared by spiking defined amounts of artificial peptides/proteins into a matrix mimicking CSF. Human plasma, prediluted 1:200, to achieve the CSF-level of the concentration of albumin, i. e. the most abundant CSF protein \cite{11}, or the solution of bovine albumin at the concentration of 0.4 g/L, seemed the most obvious candidates. Of course such artificial matrices have limitations, too; none of them would correspond ideally to the "real" CSF: in prediluted plasma, other blood-derived proteins (for example immunoglobulins) are overconcentrated compared to the CSF, whereas they are absent in BSA/PBS solution; on the other hand, in both solutions, brain-derived proteins (other than those spiked) are completely absent.

Following the recommendations by Lisinger et al \cite{12}, we tested stability of the biomarkers in question in two settings: short-term, which included three temperature conditions, and long-term, which was done on the samples kept at $-80^\circ\text{C}$ (usual long-term storage condition).

To test short-term stability, we applied the isochronous method \cite{13,14}, which means that the aliquots were stored at different conditions (room temperature, refrigerator, and $-20^\circ\text{C}$) for a defined time
following transfer to the reference condition (deep freezer) for the time remaining to the end of the study, and then simultaneously analyzed, together with a reference sample stored at the reference conditions from the beginning. For our study, -80°C was chosen as the reference condition, since convincing data are available that NDD biomarkers are sufficiently stable at this temperature \cite{15,16} and deeper freezing (on liquid nitrogen, for example) is probably not necessary. The greatest advantage of the isochronous method is that all aliquots can be analyzed in the same analytical run (on the same ELISA plate) irrespectively of the time they had been stored at a defined condition, which eliminates the influence of the inter-assay measurement imprecision.

In this study, we also tested if the stability of the biomarkers could be improved by application of an antibacterial agent, sodium azide (NaN₃), which was brought about by the suggestion of decreased Aβ concentrations in CSF samples due to bacterial growth \cite{17}. Our study did not confirm positive influence of the antibacterial agent on the stability of the NDD biomarkers, and we do not recommend supplementation of QC samples with NaN₃.

For the long-term stability testing, samples were stored deeply frozen and periodically analyzed on ELISA plates of different production charges and, in one case, even with vendor-introduced modifications of the assay format. Interpretation of these results must therefore take into consideration that the obtained variability is the superposition of the variability of the biomarkers concentrations and the inter-assay imprecision of the measurement methods. Long-term variation of measurements, usually large compared to the degree of degradation of biomolecules, is one the major problems in the determination of long-term stability and shelf-life \cite{18}; on the other hand, we believe that such approach is more reliable, compared to the extrapolations of the results of short-term stability with application of mathematical equations, as proposed by other investigators \cite{15}. Similarly, Lisinger et al do not recommend attempts to estimate long-term stability by extrapolating short-term stability data via the Arrhenius-equation \cite{12}. To our opinion, the observed maximal variability in the range of ±20% should be considered an acceptable result. As a matter of fact, the concentrations of Aβ1-42 and Aβ1-40 in
prediluted plasma without NaN3 supplementation (B0) deviated not more than ±10% in all measurement points, i.e. actually within expected inter-assay variability.

For the inter-center study, six samples were prepared and sent to the participants under room temperature. This approach differs from the protocols of other large-scale inter-center projects, where samples were sent frozen [7, 8, 19]. Inter-center variability of the biomarkers obtained in this study in pooled CSF was in the range comparable with the results of the studies coordinated by the group of K. Blennow, with the variation of Tau and pTau expectedly lower that this of Aβ1-42 [8]. Interestingly, comparable variation was obtained with lyophilized sample, with the exception of somehow higher variation of Aβ1-40, which can be explained by outlying result reported by one center (#4) and low number of the participants. To our best knowledge, no data has been published so far on the inter-center variability of Aβ peptides in artificial solutions. The results of this study show not only reasonably low variation obtained in these matrices (~20 - 25%) but also plausible distribution of the results across the centers: in majority of cases, the participants reported either higher or lower concentrations of a given biomarker in all samples, which might denote that the performance of a center, and not the samples, require some optimization. Noteworthy is also that the concentrations of the Aβ peptides in the samples containing their lower concentrations (70% and 50% of Aβ1-42 and Aβ1-40, respectively) turned out indeed correspondingly lower.

Our study has at least one limitation: due to dynamical processes of its phosphorylation/dephosphorylation, and resulting problems of the molecular instability, it was currently impossible to spike Tau and phosphorylated Tau (pTau) into the artificial matrices tested in this study. Certainly further work is warranted to facilitate the inclusion of these important biomarkers.

In conclusion, we believe that it is possible to replace self-made CSF-based QC samples for the NDD with large-scale volumes of the samples prepared with artificial peptides and matrices and at different concentrations, which would greatly facilitate intra- and inter-center QC of the NDD measurements.
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Figure legends

Fig. 1. Results of the short-term stability testing of the samples based on prediluted plasma, when single peptides were spiked, presented as normalized concentration (in percent) of the reference samples; horizontal axes show storage time (days) at the corresponding storage conditions: RT, room temperature; +4°C, refrigerator; -20°C, frozen at -20°C.

1a. Non-stabilized Aβ1–40;  
1b. Stabilized Aβ1–40;  
1c. Non-stabilized Aβ1–42 (Innotest);  
1d. Stabilized Aβ1–42 (Innotest);  
1e. Non-stabilized Aβ1–42 (MSD);  
1f. Stabilized Aβ1–42 (MSD);

Fig. 2. Results of the short-term stability testing of the samples based on prediluted plasma, when Aβ1–42 and Aβ1–40 were spiked into one sample.

2a. Non-stabilized Aβ1–40;  
2b. Stabilized Aβ1–40;  
2c. Non-stabilized Aβ1–42 (Innotest);  
2d. Stabilized Aβ1–42 (Innotest);

Fig. 3. Results of the short-term stability testing of the human pooled CSF samples, presented as normalized concentration (in percent) of the reference samples; horizontal axes show storage time (days) at the corresponding storage conditions: RT, room temperature; +4°C, refrigerator; -20°C, frozen at -20°C.

3a. Non-stabilized Aβ1–40;  
3b. Stabilized Aβ1–40;  
3c. Non-stabilized Aβ1–42 (Innotest);
3d. Stabilized Aβ1-42 (Innotest);
3e. Non-stabilized Aβ1-42 (MSD);
3f. Stabilized Aβ1-42 (MSD);
3g. Non-stabilized Tau (Innotest);
3h. Stabilized Tau (Innotest);
3i. Non-stabilized Tau (MSD);
3j. Stabilized Tau (MSD);
3k. Non-stabilized pTau181 (Innotest);
3l. Stabilized pTau181 (Innotest);

Fig. 4. Results of the short-term stability testing of the biomarkers diluted in BSA/PBS+Tween, presented as normalized concentration (in percent) of the reference samples; horizontal axes show storage time (days) at the corresponding storage conditions: RT, room temperature; +4°C, refrigerator; -20°C, frozen at -20°C.

4a. Non-stabilized Aβ1-40;
4b. Stabilized Aβ1-40;
4c. Non-stabilized Aβ1-42 (Innotest);
4d. Stabilized Aβ1-42 (Innotest);
4e. Non-stabilized Aβ1-42 (MSD);
4f. Stabilized Aβ1-42 (MSD);

Fig. 5. The results of the long-term stability testing.

5a. Aβ1-40;
5b. Aβ1-42;
5c. Tau;
5d. pTau181;
Fig. 6. Inter-assay imprecision of the biomarkers tested in human pooled CSF

6a. Aβ1-40;
6b. Aβ1-42;
6c. Tau;
6d. pTau181;

Fig. 7. Results of the inter-center variability testing. Horizontal axes represent the participants (in a random order); vertical axes present concentrations of the biomarkers (pg/mL).

7a. Aβ1-40 in liquid CSF (EQC-1) and in lyophilized CSF (EQC-2);
7b. Aβ1-40 in prediluted plasma (EQC-3 and EQC-4), and in BSA/PBS+Tween (EQC-5 and EQC-6);
7c. Aβ1-42 in liquid CSF (EQC-1) and in lyophilized CSF (EQC-2);
7d. Aβ1-42 in prediluted plasma (EQC-3 and EQC-4), and in BSA/PBS+Tween (EQC-5 and EQC-6);
7e. Tau in liquid CSF (EQC-1) and in lyophilized CSF (EQC-2);
7f. pTau181 in liquid CSF (EQC-1) and in lyophilized CSF (EQC-2);
References


