Comparison of Two Different Methods for Measurement of Amyloid-β Peptides in Cerebrospinal Fluid after BACE1 Inhibition in a Dog Model

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Abstract. Beta-secretase is the first cleavage enzyme of amyloid-β protein precursor (AβPP) in the amyloidogenic pathway, leading to the formation of the plaque forming Amyloid-β (Aβ1-42) peptide. BACE (beta-site AβPP cleaving enzyme) 1 inhibition is therefore considered to be a promising disease modifying therapy for Alzheimer’s disease. An early assessment of the in vivo activity of BACE inhibitors was done in dogs since AβPP processing is the same as in humans and this species easily enables longitudinal cerebrospinal fluid (CSF) sampling. Aβ changes in CSF compared to baseline are used to evaluate target engagement of the compounds. Levels of Aβ1-37, Aβ1-38, Aβ1-40, and Aβ1-42 in CSF are measured with immunoassay (Mesoscale electrochemiluminescence technology) and with an ultra high-performance liquid chromatography mass spectrometry (UPLC-MS/MS). Two experimental BACE inhibitors were evaluated. With the immunoassay, a dose dependent decrease is observed for all four Aβ peptides. Measurements with the UPLC-MS/MS are in line with the immunoassay for Aβ1-37, Aβ1-38, and Aβ1-40, however, for Aβ1-42, differences are sometimes observed when comparing to changes seen in the other peptides with UPLC-MS/MS and with immunoassay results. Generally lower concentrations are measured with immunoassay. The reason for these differences is still unknown. Aβ1-42 is more prone to form aggregates compared to the other peptides. One hypothesis could be that while the immunoassay only measures free Aβ, bound and aggregated Aβ peptides are at least partially dissolved with the UPLC-MS/MS method, since acetonitrile is added to the CSF samples. This increases variability in the concentration of Aβ peptide measured with UPLC-MS/MS, especially for Aβ1-42, potentially masking the compound effect on Aβ1-42 levels.

Keywords: Alzheimer’s disease, amyloid-β peptides, β-secretase, cerebrospinal fluid, dog

INTRODUCTION

Amyloid plaques, amorphous and fibrillar deposits of amyloid-β (Aβ), are one of the neuropathological hallmarks of Alzheimer’s disease [1]. Mainly Aβ1-42 peptides have the tendency to form soluble oligomers, which precipitate and form plaques [2]. Aβ1-42 is a cleavage product of the transmembranous amyloid-β protein precursor (AβPP) via the β-secretase pathway. The cleavage by β-secretase (BACE1) liberates the Aβ N-terminus, together with sAβPPβ and a C-terminal fragment C99. C99 is subsequently cleaved by γ-secretase to yield Aβ and an AICD (Amyloid precursor protein Intracellular Domain) [3]. β- as well

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The effects of BACE inhibitors in vivo have a big impact on the outcome of the analysis. Sampling and processing procedures of CSF samples for peptide analysis (PAC-LC-MS/MS) are described. Controlled liquid chromatography-tandem mass spectrometry (LC-MS) and peptide adsorption-resin technology (UPLC-MS/MS) are described. As well as enzymes implicated in Aβ degradation [9]. The Aβ isoform pattern in CSF of dogs resembles that in humans [10] and the turnover of Aβ in the CSF is comparable between dog and humans [11].

Different assays are used to measure Aβ peptides in dog CSF. Immunoassays [6, 12], ultra high-performance liquid chromatography mass spectrometry (PAC-LC-MS/MS) [14], and peptide adsorption-resin technology (UPLC-MS/MS) [13] are described. Sampling and processing procedures of CSF samples have a big impact on the outcome of the analysis [15]. We use the dog as a preclinical species to evaluate the effect of BACE inhibitors in vivo [16]. The effects on Aβ1-37, Aβ1-38, Aβ1-40, and Aβ1-42 in CSF are measured with immunoassay (Mesoscale) and with UPLC-MS/MS and one aliquot of 100 µl was used for Mesoscale.

Plasma samples for drug exposure were taken at 0.5, 1, 2, 4, 8, and 24 h after dosing. Blood was sampled in EDTA tubes, protected from light, centrifuged and per sample 200 µl plasma was stored at -20°C until analysis. Baseline data of Aβ in CSF of the cisterna magna are used from other studies. CSF sampling from the cisterna magna was performed under short general intravenous anesthesia (0.2 ml medetomidine (Domitor®) and 2 ml propofol (Propovet®)). The studies were approved by the Ethical Committee on Laboratory Animal Testing (ECD, Janssen Beerse). UPLC-MS/MS method for drug concentrations in canine plasma.

Plasma levels of JNJ-A and JNJ-B were determined using a qualified research LC-MS/MS method. After protein precipitation (with acetonitrile) plasma samples were quantified on a reversed phase UPLC-column (Acquity BEH C18 1.7 µm, 2.1 × 50 mm; Waters). Mobile phases consisted of 0.1% formic acid (solvent A) and methanol (solvent B). Starting conditions were typically from 70% solvent A and 30% solvent B followed by a linear gradient to 5% solvent A and 95% solvent B over 1.0 min followed by an isocratic hold at a flow rate of 0.8 ml/min. For each compound, gradient conditions were optimized and differed slightly. LC-MS/MS analysis was carried out on an API-4000 MS/MS (Applied Biosystems, Toronto, Canada), which was coupled to an UPLC-system (Waters; Milford, US). The MRM, operated in the positive ion mode using the TurboIonSpray™ interface (electro-
spray ionization), was optimized for the quantification of each compound. The limit of quantification was at least 5.0 ng/ml for plasma samples for all analytes. The accuracy (intra batch accuracy from independent QC samples) was between 85% and 115% of the nominal value over the entire range for plasma samples.

**UPLC-MSMS method for amyloid peptides in canine CSF**

The UPLC-MSMS method for the analysis of amyloid peptides in CSF is described by Dillen et al. [13]. Dog CSF samples were provided in Micronic tubes. CSF sample preparation was performed by adding 1 volume of acetonitrile containing 10% NH4OH to 4 volumes CSF, resulting in final acetonitrile and NH4OH concentrations of 20% and 2%, respectively. UPLC analysis was performed on an Acquity 300 Å C18 1.7 µm 2.1 × 50 mm PST column (Peptide Separation Technology; pH range 1–12, Waters Inc., Milford, Canada) operated in the negative ion mode. The API4000 triple quadrupole instrument (Sciex, Toronto, Canada) was optimized for each individual peptide. SRM transitions were as follows: Aβ1-40 m/z 1017.6 (M-4H)4− > 1013.2, Aβ1-38 m/z 1032.1 (M-4H)4− > 1027.5, Aβ1-42 m/z 1081.6 (M-4H)4− > 1077 and Aβ1-12 m/z 1127.6 (M-4H)4− > 1123.2.

The limit of quantification was 100 pg/ml for all peptides. The accuracy (intra batch accuracy from independent QC samples) was between 80% and 120% of the nominal value over the entire range for the CSF samples. Study samples were analyzed in duplo and mean concentrations were reported.

The levels of each amyloid peptide in canine CSF study samples were expressed in absolute levels and relative to the start sample (predose) of the experiment. In each animal, peak areas obtained in the predose samples are defined as 100% and all other timepoints are expressed relative (in %) to the predose sample peak area.

**Multiplex immunoassay method for quantification of Aβ1-37, 1-40, and 42 levels in CSF**

Simultaneous specific quantification of human-type Aβ1-37, Aβ1-38, Aβ1-40, and Aβ1-42 in dog CSF was performed using MesoScale Discovery (MSD)’s electrochemiluminescence detection technology. Standards of human Aβ1-40, Aβ1-38, Aβ1-37, Aβ1-42 (Anaspec, San Jose, CA) were dissolved in dimethylsulphoxide (DMSO) at 0.1 mg/ml and stored at –80°C. For use in the assay, peptides were further diluted in casein buffer (0.1% casein in PBS) down to 10 pg/ml. Purified monoclonal antibodies specific for Aβ1-37 (JRD/Aβ37/3), Aβ1-38 (J&PRD/Aβ38/5), Aβ1-40 (JRF/Aβ40/28), and Aβ1-42 (JRF/Aβ42/26) were coated on MSD 4-plex 96-well plates. Plates were blocked with casein buffer (0.1% casein in PBS buffer) for 1–4 h at room temperature. After washing, standards and prediluted samples (1/2 dilution of the samples) were incubated in MSD 4-plex plates (MesoScale Discovery) overnight at 4°C together with SULFO-TAG-labeled human-specific detection antibody JRF/AβN25. The SULFO-TAG emits light upon electrochemical stimulation initiated at the electrode surface of the 4-plex plates. After overnight incubation, plates were washed and assays are developed using 2x Read Buffer (MesoScale Discovery) according to the manufacturer’s recommendations and plates were read on MSD Sector Imager 6000. CSF samples contaminated with blood were not used in the analysis.

All calibration standards and study samples were analyzed in duplicate. The results were averaged and treated as a single value for all further calculations. Only mean values with replicate well CVs of less than or equal to 20.0% were accepted. Any sample not meeting this criterion was excluded from any further calculation. The lowest limit of quantification, determined as the lowest calibrator concentration for which overall CV and bias are ≤25.0%, is 4.57 pg/ml for all Aβ-peptides measured.

**Statistics**

The amyloid levels at baseline in the cisterna magna and lateral ventricle were compared using a t-test. For comparison of the analytical methods, UPLC-MS/MS, and immunoassay, a paired t-test was used. Both comparisons were done under the assumption of a log-normal distribution.
RESULTS

Baseline Aβ levels in CSF

Before dosing, Aβ levels in CSF are measured to determine the baseline which is used as reference for evaluation of drug effects. Depending on the study, CSF is sampled from the lateral ventricle or cisterna magna. Over the past year, 99 and 48 baseline samples were taken from the lateral ventricle and cisterna magna, respectively.

Absolute concentrations for Aβ1-37, Aβ1-38, Aβ1-40, and Aβ1-42 are listed in Table 1. Absolute concentrations were the highest for Aβ1-40, followed by Aβ1-42 and Aβ1-37. Aβ1-38 was the least present. The concentrations measured with UPLC-MS/MS were consistently higher than with Mesoscale (p < 0.0001 for all peptides). The difference between both assays was positively correlated with the length of the peptide. The ratio UPLC-MS/MS/Mesoscale was highest for Aβ1-42. Levels in cisterna magna were approximately two times higher than in the lateral ventricle for both assays (p < 0.0001 for all peptides).

The correlation between the UPLC-MS/MS and Mesoscale values is shown in Fig. 1a and b. There was a good correlation between both assays for Aβ1-38 and Aβ1-40 (r² = 0.63 and 0.65 in the lateral ventricle and 0.78 and 0.57 in the cisterna magna for Aβ1-38 and Aβ1-40, respectively). Aβ1-37 showed a mixed picture since correlation was good in the cisterna magna, but not in the lateral ventricle. Correlation between UPLC-MS/MS and Mesoscale was low for Aβ1-42 (r² < 0.20).

Pharmacokinetics

Pharmacokinetic parameters are summarized in Table 2. Mean peak plasma levels ranged from 80 to 1460 ng/ml for JNJ-B (1.25 to 20 mg/kg) and from 13 to 1005 ng/ml for JNJ-A (0.08 to 10 mg/kg). The increase in plasma exposure was dose proportional for both compounds. T1/2 was approximately five times longer for JNJ-A (23.2–26 h) than for JNJ-B (4.5–6.6 h).

Aβ lowering was seen from a dose of 0.16 and 1.25 mg/kg onwards for JNJ-A and JNJ-B.

PK/PD modeling

The traditional analysis attempts to link drug plasma concentrations to the maximal decrease of Aβ peptides. However, a delay between plasma concentrations and Aβ peptide levels is typically observed. As such, any in vitro potency estimates obtained in this way are usually biased.

Therefore, a physiologically inspired analysis is proposed. The mechanism of action of the BACE inhibitors is a blockage of formation of the Aβ peptides. Therefore, the data is analyzed using a turnover model [19], which mimics the production and elimination of amyloid in the brain, where CSF Aβ levels are assumed to be a relevant biomarker for Aβ elimination in the brain, where CSF Aβ turnover model [19], which mimics the production and elimination of amyloid in the brain, where CSF Aβ levels are assumed to be a relevant biomarker for Aβ elimination in the brain.

\[ \frac{dR_{ijk}}{dt} = k_{in} \left( 1 - \frac{Imax C_{pijk}}{EC_{50k} + C_{pijk}} \right) - k_{out} R_{ijk} \]

where:
- \( R_{ijk} \): Aβ-peptide at time \( t \) for animal \( i \) with compound \( k \)
- \( k_{in} \): amyloid production rate
- \( Imax \): maximal inhibition (fixed to 100% for Aβ1-34, Aβ1-38, and Aβ1-42)
- \( C_{pijk} \): plasma concentration required for compound \( k \) to obtain 50% inhibition

The correlation between the UPLC-MS/MS and Mesoscale was low for Aβ1-42 (r² < 0.20).

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lateral ventricle</th>
<th>Cisterna magna</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UPLC-MS/MS</td>
<td>Mesoscale</td>
</tr>
<tr>
<td>Aβ1-37</td>
<td>493 ± 223</td>
<td>262 ± 99</td>
</tr>
<tr>
<td>Aβ1-38</td>
<td>2487 ± 767</td>
<td>924 ± 341</td>
</tr>
<tr>
<td>Aβ1-40</td>
<td>4869 ± 2436</td>
<td>2678 ± 811</td>
</tr>
<tr>
<td>Aβ1-42</td>
<td>1926 ± 732</td>
<td>550 ± 168</td>
</tr>
</tbody>
</table>
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Fig. 1. Correlation between absolute baseline concentrations for Aβ peptides in dog CSF measured with UPLC-MS/MS and Mesoscale immunoassay. n=99 for lateral ventricle (a) and 48 for cisterna magna (b).
were in line for both assays for A/H9252 values based on the UPLC-MS/MS for A/H9252 all four A
intra-individual variability. Both compounds lowered this often seen in vehicle groups and could be due to
at any time point were not considered relevant since
per group, mean changes within 20% from baseline
respectively. Especially with the small sample size
44
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discrepancy between UPLC-MS/MS and mesoscale
MS/MS based EC50 for compound A.

The plasma concentrations needed for a 50% low-
concentrations were in general smaller and within the range of the vehicle.

PK/PD analysis

The plasma concentrations needed for a 50% low-
r from the CSF by way of metabolism of A
levels reflect following therapeutic treatments
11]. The choroid plexus plays a major role in remov-
ing Aβ from the CSF by way of metabolism of Aβ
[22] and efflux from the CSF to the blood [23]. The
higher concentrations of the Aβ concentrations in the cisterna magna could therefore be a result of a higher
ercretion of brain Aβ at the subarachnoid side than at the
ventricular side and/or a fast uptake of Aβ from the
CSF via the choroid plexus in the lateral ventricle.
Free Aβ peptides in CSF and not self-aggregated
Aβ peptides and Aβ peptides bound to matrix proteins
are exposed to antibodies used for measurements in immunoassays and probably detected with immunoas-
says like the Mesoscale technology [15]. Acetominirile, an organic solvent, is added to the CSF sample for
UPLC-MS/MS [13]. Thus, bound Aβ peptides could be partially dissolved and free as well as bound Aβ
peptides after dissolution are measured. As a conse-
quence, consistently higher Aβ concentrations were
measured with the UPLC-MS/MS compared with the

respectively. Especially with the small sample size per group, mean changes within 20% from baseline at any time point were not considered relevant since this often seen in vehicle groups and could be due to intra-individual variability. Both compounds lowered all four Aβ isoforms in a dose related way. The data were in line for both assays for Aβ1-37, Aβ1-40 and Aβ1-42. There was a discrepancy in Aβ1-42 changes between the UPLC-MS/MS and Mesoscale for JNJ-B at the dose of 2.5, 5, and 10 mg/kg and for JNJ-A at the dose of 0.16, 0.31, and 2.5 mg/kg. With UPLC-MS/MS, differences with baseline were in general smaller and within the range of the vehicle.

**DISCUSSION**

Baseline Aβ concentrations in dog CSF were in general two times higher in CSF sampled from the cisterna magna than in CSF sampled from the lateral ventricle. CSF originates from a number of sites: the choroid
plexus of the lateral, third and fourth ventricles, directly from the brain by way of the ependymal lining of the ventricular system, and via the pial-glial membrane covering its external surface and from blood vessels in the pia-arachnoid. 35% of the CSF derives from the third and lateral ventricles, 23% from the fourth ventricle, and 42% from the subarachnoid space [20]. The CSF flows from the lateral ventricle into the third ventricle and then via the aqueduct into the fourth ventricle. From the fourth ventricle, CSF flows into the cisterna magna (cerebello-medullary cistern) of the subarachnoid space through the medial foramen and the two lateral foramina. The only functional communication between the cerebral ventricles and the subarachnoid spaces is in the fourth ventricle [21]. The sources of Aβ in CSF remain to be fully elicited; however, drainage from the brain seems to be the major origin since changes in CSF Aβ levels reflect changes in brain Aβ following therapeutic treatments [21]. The choroid plexus plays a major role in removing Aβ from the CSF, by way of metabolism of Aβ [22] and efflux from the CSF to the blood [23]. The higher concentrations of the Aβ concentrations in the cisterna magna could therefore be a result of a higher excretion of brain Aβ at the subarachnoid side than at the ventricular side and/or a fast uptake of Aβ from the CSF via the choroid plexus in the lateral ventricle.

**TABLE 2**

<table>
<thead>
<tr>
<th>Aβ1-42 changes in CSF (mean ± SDev, n = 4)</th>
<th>0.60</th>
<th>0.16</th>
<th>0.31</th>
<th>2.50</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_{1/2} h</td>
<td>4.5 ± 2.5</td>
<td>3.0 ± 1.1</td>
<td>4.5 ± 2.3</td>
<td>2.5 ± 1.0</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>C_{90} ng/ml</td>
<td>13.2 ± 4.3</td>
<td>31.6 ± 9.4</td>
<td>85 ± 4.0</td>
<td>571 ± 121</td>
<td>1905 ± 1057</td>
</tr>
<tr>
<td>T_{1/2} h</td>
<td>26 ± 8.8</td>
<td>26 ± 3.2</td>
<td>23 ± 3</td>
<td>25 ± 4</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>AUC_{90} ng/ml</td>
<td>540 ± 157</td>
<td>1301 ± 294</td>
<td>3020 ± 4530</td>
<td>72300 ± 38779</td>
<td></td>
</tr>
</tbody>
</table>

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**TABLE 2**

| PK/PD analysis |
|---|---|---|---|---|---|
| Dose mg/kg | 0.60 | 0.16 | 0.31 | 2.5 | 10 |
| T_{1/2} h | 4.5 ± 2.5 | 3.0 ± 1.1 | 4.5 ± 2.3 | 2.5 ± 1.0 | 2.0 ± 1.0 |
| C_{90} ng/ml | 13.2 ± 4.3 | 31.6 ± 9.4 | 85 ± 4.0 | 571 ± 121 | 1905 ± 1057 |
| T_{1/2} h | 26 ± 8.8 | 26 ± 3.2 | 23 ± 3 | 25 ± 4 | 26 ± 3 |
| AUC_{90} ng/ml | 540 ± 157 | 1301 ± 294 | 3020 ± 4530 | 72300 ± 38779 |

**DISCUSSION**

Baseline Aβ concentrations in dog CSF were in general two times higher in CSF sampled from the cisterna magna than in CSF sampled from the lateral ventricle. CSF originates from a number of sites: the choroid
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**FREE Aβ peptides in CSF and not self-aggregated**

Aβ peptides and Aβ peptides bound to matrix proteins are exposed to antibodies used for measurements in immunoassays and probably detected with immunoassays like the Mesoscale technology [15]. Acetominirile, an organic solvent, is added to the CSF sample for UPLC-MS/MS [13]. Thus, bound Aβ peptides could be partially dissolved and free as well as bound Aβ peptides after dissolution are measured. As a consequence, consistently higher Aβ concentrations were measured with the UPLC-MS/MS compared with the
Fig. 2. Changes (% from baseline) in Aβ levels in CSF (lateral ventricle) after dosing JNJ-3 at 0.08, 0.16, 0.31, 2.5, and 10 mg/kg, comparison of UPLC-MS/MS (blue line) and Mesoscale immunoassay (red line) for Aβ1-37 (a), Aβ1-38 (b), Aβ1-40 (c) and Aβ1-42 (d) (mean ± SEM, n = 4).
Fig. 3. Changes (% from baseline) in Aβ levels in CSF (lateral ventricle) after dosing JNJ-B at 0, 1.25, 2.5, 5, 10, and 20 mg/kg: comparison of UPLC-MS/MS (blue line) and Mesoscale immunoassay (red line) for Aβ4-37 (a), Aβ4-38 (b), Aβ4-40 (c) and Aβ4-42 (d) (mean ± SEM, n = 4 for 2.5 and 10 mg/kg and n = 6 for 1.25, 5 and 20 mg/kg).
Mesoscale. Aβ₁-42 has a higher affinity to aggregate than Aβ₁-40 [24] and can also bind to other proteins in the CSF [15]. This tendency could explain the higher UPLC-MS/MS/Mesoscale ratio for Aβ₁-42 compared to the smaller peptides. The higher tendency for Aβ₁-42 to bind and aggregate increases variability in measured concentrations [15, 25]. This is reflected in a low correlation between the concentrations measured with both assays for Aβ₁-42, while a higher correlation is seen for Aβ₁-38 and Aβ₁-40. The variable correlation between both assays for Aβ₁-42 could be coincidental and a consequence of the low concentrations for Aβ₁-37, which were around the lowest detection limit. However, whether aggregation is present in incurred samples or introducing post-sampling is also not clear. No additional data are currently available to support the aggregation hypothesis.

The aim of the model is to show acute target engagement of BACE inhibitors at an early stage in drug development and estimate an EC₅₀ for lowering Aβ peptides. Young to middle-aged animals are used for this purpose. To address the effect of BACE inhibitors in a more diseased state, older dogs, which show decreased Aβ₁-42 levels in CSF as in humans [6], could be used.

BACE inhibition results in lowering of Aβ peptides in CSF [26]. Both JNJ-B and JNJ-A dose-dependently lowered Aβ peptides in CSF. In the present studies, CSF was sampled from the lateral ventricle, however, similar drug effects can also be seen in CSF sampled from the cisterna magna. We prefer sampling from the lateral ventricle, however, because with Mesoscale. Aβ₁-42 can be missed with the UPLC-MS/MS assay. A hypothesis is that adding an organic solvent as acetonitrile in the UPLC-MS/MS assay results in a variable dissolution of aggregated and bound Aβ peptides, which could mask the effect of a drug. The importance of the dissolution is higher for the Aβ₁-42 peptide, which is more prone to aggregate and bind to proteins in CSF. However, additional studies are needed to support this hypothesis.

**DISCLOSURE STATEMENT**


**REFERENCES**


