Quantification of vitamin B6 vitamers in human cerebrospinal fluid by ultra performance liquid chromatography–tandem mass spectrometry

M. van der Ham a,1, M. Albersen a,1, T.J. de Koning b, G. Visser b, A. Middendorp c, M. Bosma a, N.M. Verhoeven-Duif a, M.G.M. de Sain-van der Velden a,⁎

a Department of Metabolic Diseases and Netherlands Metabolomics Center, University Medical Center (UMC) Utrecht, Huispost KC02.069.1, Lundlaan 6, 3584 EA Utrecht, The Netherlands
b Department of Pediatric Metabolic Diseases, Wilhelmina Children's Hospital, University Medical Center (UMC) Utrecht, Huispost KC03.063.0, Lundlaan 6, 3584 EA Utrecht, The Netherlands
c Waters Chromatography B.V., Florijnstraat 19, Postbus 379, 4870 AJ Eten-Leur, The Netherlands

A R T I C L E  I N F O

Article history:
Received 25 August 2011
Received in revised form 17 October 2011
Accepted 8 November 2011
Available online 18 November 2011

Keywords:
B6 vitamers
Cerebrospinal fluid
Liquid chromatography
Tandem mass spectrometry

A B S T R A C T

Since vitamin B6 is essential for normal functioning of the central nervous system, there is growing need for sensitive analysis of B6 vitamers in cerebrospinal fluid (CSF). This manuscript describes the development and validation of a rapid, sensitive and accurate method for quantification of the vitamin B6 vitamers pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), pyridoxic acid (PA), pyridoxal 5′-phosphate (PLP), pyridoxamine 5′-phosphate (PMP) and pyridoxine 5′-phosphate (PNP) in human CSF.

The method is based on ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) with a simple sample preparation procedure of protein precipitation using 50 g L−1 trichloroacetic acid containing stable isotope labeled internal standards: PL-D1 for PL and PN, PN−13C4 for PN, PA-D1 for PA and PLP-D1 for the phosphorylated vitamers. B6 vitamers were separated (Acquity HSS-T3 UPLC column) with a buffer containing acetic acid, heptafluorobutyric acid and acetonitrile. Positive electrospray ionization was used to monitor transitions m/z 168.1 → 150.1 (PL), 169.1 → 134.1 (PM), 170.1 → 134.1 (PN), 184.1 → 148.1 (PA), 248.1 → 150.1 (PLP), 249.1 → 232.1 (PMP) and 250.1 → 134.1 (PNP).

The method was validated at three concentration levels for each B6 vitamer in CSF. Recoveries of the internal standards were between 93% and 96%. Intra- and inter-assay variations were below 20%. Accuracy tests showed deviations from 3% (PN) to 39% (PMP). Limits of quantification were in the range of 0.03–5.37 nM. Poor results were obtained for quantification of PNP.

The method was applied to CSF samples of 20 subjects and two patients on pyridoxine supplementation. Using minimal CSF volumes this method is suitable for implementation in a routine diagnostic setting.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Vitamin B6 is a water-soluble and for humans essential nutrient. It comprises different vitamers: the alcohol pyridoxine (PN), the aldehyde pyridoxal (PL), the amine pyridoxamine (PM), their phosphate esterified forms and pyridoxic acid (PA). Vitamin B6 metabolism includes several steps involving various enzymes [1], starting with carrier mediated uptake of the B6 vitamers from dietary resources in the small intestine [2].

Prior to transport across the cell membrane, phosphorylated B6 vitamers must be hydrolysed by a membrane-bound alkaline phosphatase. Intracellular (re-)phosphorylation by pyridoxal kinase is followed by a pyridox(am)ine phosphate oxidase (PNPO) mediated conversion of pyridoxine phosphate (PNP) and pyridoxamine phosphate (PMP) into pyridoxal phosphate (PLP), the active form [1] (Fig. 1). PA is the major degradation product of vitamin B6, which is excreted in urine [3].

PLP is well-known for its functions as co-factor in a large number of essential enzymatic reactions in the central nervous system, where it mainly catalyses amino acid and neurotransmitter metabolism. PLP plays an important role in the biosynthesis of dopamine, serotonin, glutamate, γ-aminobutyrate (GABA), ß-serine and histamine [1]. Although vitamin B6 deficiency is a rare condition, it gives rise to serious neurological symptoms, of which
Antiquitin seizures are the most distinctive. In addition, low plasma PLP levels have been associated with depression [4] and Alzheimer’s disease [5–7]. In 2009, Elstner et al. [8] reported a polymorphism in the pyridoxine kinase gene, which might be associated with the risk of Parkinson’s disease.

Vitamin B6 deficiency may arise from malnutrition, malabsorption of B6 vitamers from the diet, certain endogenous and exogenous nucleophiles (which inactivate PLP) or due to drugs which influence enzymes involved in PLP metabolism [1,9]. Inherited vitamin B6 deficiency is found in patients with antiquitin deficiency, an autosomal recessive disorder of cerebral lysine degradation caused by mutations in the ALDH7A1 gene [10], in PNPO deficiency, a disorder caused by mutations in the PNPO gene [11] and in hyperprolinaeemia type II [12]. Antiquitin deficiency is the major cause of pyridoxine dependent epilepsy (PDE) and together with PNPO deficiency it warrants vitamin B6 supplementation to overcome the intractable seizures seen in these conditions. Antiquitin deficiency can be effectively treated by administration of pyridoxine whereas in PNPO deficiency, PL or PLP needs to be administered.

Antiquitin or PNPO deficiency cannot be reliably diagnosed in plasma since biochemical abnormalities are not always present. In cerebrospinal fluid (CSF) of patients affected with antiquitin [13] and PNPO deficiencies [11], elevated levels of threonine, 3-methoxytyrosine (l-dopamine metabolite) and glycine have been found. These observations are explained by a decreased activity of the responsible PLP-dependent enzymes due to a lack of PLP (threonine dehydratase, aromatic L-amino acid decarboxylase (AADC) and enzymes involving the glycine cleavage system, respectively). In addition, concentrations of the dopamine metabolite homovanillic acid (HVA) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) are decreased in CSF of PNPO-deficient patients [11], probably the result of a decreased AADC activity. The abovementioned changes in CSF amino acid and neurotransmitter metabolite profiles are not observed in all PNPO-deficient patients [11,14,15]. In patients with antiquitin deficiency, CSF levels of piperolic acid [16] and α-amino adipic semialdehyde (α-AASA) [10] are increased. However, antiquitin deficiency is likely not the only cause of PDE [17,18]. In addition, Veerapandiyan et al. [19] describe a case of PLP dependent epilepsy in which no PNPO gene mutations were found. Thus, disturbances of vitamin B6 metabolism may be missed by biochemical profiling of the secondary effects of vitamin B6 deficiency. Direct analysis of vitamin B6 vitamers in CSF may overcome these diagnostic limitations.

In plasma, B6 vitamer concentrations have been determined using different high performance liquid chromatography (HPLC) based methods [20–26]. The number of methods for quantification of vitamin B6 in CSF is limited and methods are based on HPLC with fluorescence detection [9,27] or radioactive tyrosine decarboxylase assays [28]. To our knowledge, B6 vitamer reference values in CSF have been reported for PLP only whereas CSF concentrations of the B6 vitamers PL, PM(P), PN(P) and PA have not yet been described.
Information on all vitamin B6 vitamers is necessary to get more insight into its metabolism as has also been suggested by Mills et al. [11]. Insight in vitamin B6 metabolism might not only enhance our insight into the pathophysiology of disorders associated with vitamin B6 deficiency, but might eventually lead to therapeutic strategies.

We present the development and validation of a rapid, sensitive and accurate method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF by ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), using minimal CSF volumes and being suitable for implementation in a routine diagnostic setting. We established concentrations of CSF B6 vitamers in 20 subjects. In addition, CSF samples of two patients on pyridoxine supplementation were measured to demonstrate the applicability of our method.

2. Materials and methods

2.1. Reagents

PL-hydrochloride (>99%), PM-dihydrochloride (>98%), PN (>98%), PA (>99%), PLP-monohydrate (>97%), PMP (>98%) and heptafluorobutyric acid (HFBA, >99.5%) were obtained from Sigma–Aldrich (Steinheim, Germany). PNP was kindly provided by C.J. Argoudelis, Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign (Urbana, Illinois). PL-hydrochloride-D3 (99%), PN-hydrochloride-13C4 (99%), PA-D2 (98%) and methyl-D3-PLP (97%) were purchased from Buchem bv (Apeldoorn, The Netherlands). Trichloroacetic acid (TCA, >99%) and acetic acid (99–100%) were obtained from Merck (Darmstadt, Germany). Acetonitrile (UHPLC–MS) was purchased from Biosolve (Valkenswaard, The Netherlands).

2.2. Standard solutions (B6 vitamers and internal standards (IS’s)) and calibration curve

Stock solutions (µM) of vitamin B6 compounds were prepared for PL (6330), PM (523), PN (674), PA (1802), PLP (2048), PMP (2176) and PNP (1605). A ten-point calibration curve was prepared by diluting these stock solutions in a mixture (end concentration (nM) for PL (317), PM (26), PN (34), PA (90), PLP (102), PMP (54) and PNP (40)).

A ten point high-range calibration curve was prepared by diluting stock solutions of vitamin B6 compounds in a mixture (end concentration (nM) for PL (6330), PM (523), PN (674), PA (1802), PLP (2048), PMP (1088) and PNP (803)).

Stock solutions (µM) of stable isotope labeled B6 vitamers were prepared for PL-D3 (2372), 13C4-PN (2242), PA-D2 (2970) and PLP-D3 (1320). An internal standard (IS) working solution was prepared by diluting these stock solutions in a mixture (end concentrations (nM) for PL-D3 (47), 13C4-PN (9), PA-D2 (30) and PLP-D3 (132)).

All stock solutions and subsequent dilutions were prepared in TCA (50 g L−1 Milli-Q water) and stored at −80 °C.

2.3. Quality control samples (QC’s)

CSF material of at least 25 random subjects was pooled and B6 vitamers were spiked to achieve three different concentration levels (QC 1, QC 2 and QC 3). For QC 1, spiked levels (nM) were 8 (PLP) and 5 (PMP). PL, PM, PN and PA were not spiked to QC 1. For QC 2, spiked levels (nM) were 20 (PL), 8 (PM), 5 (PN), 9 (PA), 28 (PLP) and 10 (PMP). For QC 3, spiked levels (nM) were 190 (PL), 18 (PM), 10 (PN), 35 (PA), 73 (PLP) and 40 (PMP). QC samples were divided into small portions and stored at −80 °C until analysis.

2.4. Cerebrospinal fluid samples

CSF from lumbar puncture was obtained of 20 subjects (age eight months to 16.3 years; 14 boys and six girls) who were investigated for developmental delay (n = 14), movement disorder (n = 2) or both (n = 4). Samples were provided by the Sylvia Töth Center and archived at the Department of Metabolic Diseases (both Wilhelmina Children’s Hospital, University Medical Center (UMC) Utrecht, The Netherlands). CSF was collected by means of a routine standardized procedure in which six separate fractions (0.5, 1, 1.5, 2 and 2 mL, respectively) are immediately put on ice at the bedside. In this procedure, fraction IV is always protected from light and stored at −80 °C right after collection. This fraction was used to establish B6 vitamer concentrations. Exclusion criteria were CSF erythrocytes >100 mL−1, CSF leukocytes >10 mL−1 [29] and CSF protein >0.40 mg mL−1, preventing sample contamination through injury of the blood-CSF barrier. CSF samples of patients with acute infections and/or antibiotic treatment, human immunodeficiency virus, auto-immune diseases, neoplastic disorders, schizophrenia and/or anti-epileptic drug treatment, specific other drug treatment regimes (anti-tuberculosis drugs, corticosteroids, levo-dopa [9]), vitamin B6 supplementation, established (neuro)metabolic or other chronic disorders (such as malabsorption) and patients without sufficient information were all excluded.

From random patients who underwent lumbar puncture (n = 5), B6 vitamers were analyzed in CSF fractions II, IV and VI to study a possible concentration gradient as described for monoamine metabolites [30]. In addition, CSF of two patients (A and B) on pyridoxine supplementation was investigated to study the effects of vitamin B6 therapy on CSF vitamers (A: age 19 days, pyridoxine dosage 30 mg kg−1 day−1; B: age 6.25 years, pyridoxine dosage 27 mg kg−1 day−1). Signed informed consent was obtained for all CSF samples, which were blinded before analysis. Approval by the Medical Ethics Committee of the UMC Utrecht was obtained.

2.5. Sample and calibration curve preparation

For analysis of B6 vitamers in CSF, 60 µL of IS working solution was added to 60 µL of CSF in an Eppendorf tube. After mixing for 5 s, samples were protected from light and placed at room temperature for 15 min. After centrifugation (16 060 × g for 5 min), supernatants were transferred to a 96 wells plate and analyzed by UPLC–MS/MS. Samples were protected from light and were kept at 15 °C during analyses.

Calibration curve standards were diluted (1:1 v/v) with IS working solution and analyzed with each series of CSF samples for B6 vitamer quantification.

2.6. Instruments

A Xevo-TQ MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) source and an Acquity UPLC (Waters, Manchester, UK) were used. Masslynx software V4.1 (Waters, Manchester, UK) was used to control the instrument and for data acquisition.

2.7. Chromatographic and mass spectrometric conditions

Analytical UPLC columns with different chemistries (Acquity BEH C18, Acquity BEH C8, Acquity BEH Amide (all 1.7 µm, 2.1 mm × 100 mm) and Acquity HSS-T3 (1.8 µm, 2.1 mm × 100 mm) (Waters, Massachusetts, USA)) were tested. Selection parameters were retention stability, resolution and peak shape of the different B6 vitamers. Optimal results were obtained using an Acquity HSS-T3 guard column (1.8 µm, 2.1 mm × 5 mm) with an Acquity HSS-T3 analytical column (Waters, Massachusetts,
USA). Column temperature was kept at 25 °C and the injection volume was 10 μL. A two-step (linear) gradient of 3.5 minutes at a flow rate of 0.4 mL min⁻¹ between solvent A (650 mM acetic acid with 0.01% HFB) and solvent B (100% acetonitrile) was used. The gradient started with 100% solvent A. Between 0.5 min and 2.0 min it changed to 80% solvent A. In 0.1 min the gradient switched to 100% solvent B, which was maintained during the next 0.2 min. A direct switch to 100% solvent A was made and 1.2 min was used for column equilibration.

The MS was operated in the positive ESI mode. A capillary voltage of 0.5 kV and a cone voltage of 18 V were used. Source and desolvation temperatures were 150 °C and 550 °C, respectively. Ultra-high purity nitrogen was used for cone gas (18 L h⁻¹), desolvation gas (1150 L h⁻¹) and nebulising gas (100 L h⁻¹). For collision induced dissociation (CID), ultra-pure Argon (0.003 mbar) at a flow rate of 0.20 mL min⁻¹ was used. Multiple reaction monitoring (MRM) transition of each vitamin B6 compound was optimized by direct infusion. Unit resolution in the first and third quadrupole was used. Table 1 shows mass transitions, cone voltages and collision energies of individual vitamin B6 compounds and stable isotopes that were used as IS's. Since no analogue stable isotopes are available for PM, PMP and PNP, PL-D₃ and PLP-D₃ were used to calculate the area ratio for these B6 vitamers. See Fig. 2 for a MRM chromatogram (QC 2) of the different vitamin B6 compounds and isotope labeled internal standards.

2.8. Method validation

QC 1, QC 2 and QC 3 were used for method validation. Precision (intra- and inter-assay variation), accuracy, sensitivity (limit of detection (LOD) and limit of quantification (LOQ)), linearity, recovery, matrix effect, short-term stability, freeze-thaw stability and exposure to light were tested for all vitamin B6 compounds during assay validation.

Intra- and inter-assay (n = 10 and n = 20, respectively) variations were studied by analyzing QC 1, QC 2 and QC 3.

Standard addition experiments were performed to test assay accuracy. A random CSF pool was spiked with five different B6 vitamer levels (range of calibration curve) and the slope was compared with the slope obtained from the calibration curve.

QC 3 (n = 3) was used to determine LOD and LOQ for all vitamin B6 compounds (signal-to-noise ratios (S/N) 3 and 10, respectively). Linearity was evaluated using the high-range calibration curve. Standards of this curve were diluted (1:1 iv/iv) with IS working solution.

Recovery experiments were performed (n = 2) in QC 1, for both unlabeled and isotope labeled vitamin B6 compounds, by spiking before and after protein precipitation. For the unlabeled vitamin B6 compounds, 5 concentration levels (end concentration (nM) for PL (327), PM (129), PN (192), PA (137), PLP (153) and PMP (54)) were added. For the isotope labeled compounds, IS working solution was added. Recovery was tested by comparing peak areas of individual unlabeled and isotope labeled vitamin B6 compounds before and after protein precipitation.

Matrix effect was determined by comparing peak areas of individual isotope labeled compounds in standards of the calibration curve with peak areas of individual isotope labeled compounds in CSF samples (QC 1, QC 2 and QC 3 (n = 3)).

Short-term stability was tested by measuring vitamin B6 compounds in a prepared calibration curve together with 10 prepared random CSF samples at four time points during 11 days. During analyses and sample storage, samples were protected from light. Between analyses, calibration curve and samples were kept at 4 °C.

Two random CSF samples (n = 3) were used to test stability of the vitamin B6 compounds during 1, 3, 5 and 10 freeze-thaw cycles. Samples were thawed at room temperature and kept at room temperature for 1 h, protected from light.

QC 1, QC 2 and QC 3 (n = 2) and calibration curve standards were tested for the influence of daylight during 0, 4 and 10 h at room temperature before sample preparation. Slopes obtained from the calibration curves were compared. For QC 1, QC 2 and QC 3 the analyzed B6 vitamer concentrations were compared. Additional experiments to study the influence of light at different temperatures (exposure to light compared with darkness during 0, 4 and 10 h at room temperature versus ice) were performed.

Cross-talk was studied by spiking individual B6 vitamer compounds (n = 2) to randomly pooled CSF. Spiked concentrations (nM) were 295 (PL), 141 (PM), 195 (PN), 205 (PLP) and 97 (PMP). Concentrations of all B6 vitamers were compared in spiked and unspiked pooled CSF.

2.9. Statistical analysis

Analyse-it v2.23 (Analyse-it Software Ltd., Leeds, UK) was used to check for normal distribution (Anderson–Darling A²) and to determine calibration curve linearity for the vitamin B6 compounds (non-linear specification of 5%).

3. Results

3.1. Chromatographic and mass spectrometric conditions

During method development a contribution of PN (m/z 170.1 > 134.1) was observed in the MRM trace of PL-D₃ (m/z 171.1 > 153.1). Maximum resolution between PN and PL-D₃ was achieved using the Acquity HSS-T3 column. Total run time was 3.5 min. Retention times were 1.82 min for PL, 1.81 min for PM, 1.93 min for PN, 1.87 min for PA and 1.40 min for PLP, PMP and PNP (Fig. 2).

3.2. Method validation

Because spiked amounts of PNP were not detectable in QC 2 and QC 3, intra- and inter-assay CV's, LOD and LOQ could not be established. Accuracy deviation and ion-suppression were too high for PNP (data not shown). Given these poor results, PNP cannot be properly quantified with the described method.

Table 2 shows analyzed concentrations (nM) and intra- and inter-assay coefficients of variation (CV's) for all vitamin B6 compounds in QC 1, 2 and 3. Intra-assay CV's varied between 2.3% and 19.1%. Inter-assay CV's varied between 4.5% and 19.3%. To investigate precision of quantification of decreased PLP concentrations, we determined intra- and inter-assay CV's in a CSF sample with a PLP concentration of 1.9 nM. These were 17.7% and 16.9%, respectively.

Accuracy with a deviation of <10% was found for PL, PN, PA and PLP (Table 3). The B6 vitamers PM and PMP showed higher deviations (20% and 39%, respectively).

For each B6 vitamer, LOD and LOQ are shown in Table 3. LOD varied between 0.01 nM (PM and PN) and 1.61 nM (PMP). LOQ varied between 0.03 nM for PN and 5.37 nM for PMP.

Upper limits of linearity for all B6 vitamers are shown in Table 3. Recoveries were 103% for PL, 108% for PM, 96% for PN, 103% for PA, 104% for PLP and 99% for PMP. For the labeled vitamin B6 compounds, recoveries were comparable: 94% for PL-D₃ and 93% for 13C₄-PN and PA-D₂. PLP-D₃ showed a recovery of 96%.

While studying matrix effects, a signal reduction of 25% was found for PL-D₃ whereas signal reductions were 44%, 24% and 87% for 13C₄-PN, PA-D₂ and PLP-D₃, respectively.

Short-term stability was tested for PL, PA and PLP in prepared CSF samples. The other B6 vitamers showed a concentration <LOQ
Table 1
Mass transitions, cone voltages, collision energies and internal standards of the different B6 vitamers and stable isotopes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent ion [M+H]+</th>
<th>Daughter ion</th>
<th>Cone (V)</th>
<th>CE (eV)</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>168.1</td>
<td>150.1</td>
<td>15</td>
<td>12</td>
<td>PL-D3</td>
</tr>
<tr>
<td>PM</td>
<td>169.1</td>
<td>134.1</td>
<td>19</td>
<td>22</td>
<td>PL-D3</td>
</tr>
<tr>
<td>PN</td>
<td>170.1</td>
<td>134.1</td>
<td>18</td>
<td>20</td>
<td>PN-13C4</td>
</tr>
<tr>
<td>PA</td>
<td>184.1</td>
<td>148.1</td>
<td>25</td>
<td>14</td>
<td>PA-D2</td>
</tr>
<tr>
<td>PLP</td>
<td>248.1</td>
<td>232.1</td>
<td>24</td>
<td>14</td>
<td>PLP-D3</td>
</tr>
<tr>
<td>PMP</td>
<td>250.1</td>
<td>234.1</td>
<td>25</td>
<td>20</td>
<td>PLP-D3</td>
</tr>
<tr>
<td>PNP</td>
<td>251.1</td>
<td>153.1</td>
<td>15</td>
<td>12</td>
<td>PLP-D3</td>
</tr>
<tr>
<td>PL-13C4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA-D2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP-D3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Chromatogram of MRM transitions of vitamin B6 compounds (PL, PM, PN, PA, PLP, PMP and PNP) and isotope labeled internal standards (PL-D3, 13C4-PN, PA-D2 and PLP-D3) in CSF (QC 2).

Table 2
Precision of the described UPLC–MS/MS method (inter- and intra-assay variation) with mean B6 vitamer concentrations in QC 1, QC 2 and QC 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (nM)</th>
<th>Intra-assay CV (%), n = 10</th>
<th>Inter-assay CV (%), n = 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QC 1</td>
<td>QC 2</td>
<td>QC 3</td>
</tr>
<tr>
<td>PL</td>
<td>11.0</td>
<td>31.9</td>
<td>185.1</td>
</tr>
<tr>
<td>PM</td>
<td>1.8</td>
<td>11.9</td>
<td>23.6</td>
</tr>
<tr>
<td>PN</td>
<td>0.8</td>
<td>3.8</td>
<td>8.5</td>
</tr>
<tr>
<td>PA</td>
<td>1.8</td>
<td>12.7</td>
<td>47.6</td>
</tr>
<tr>
<td>PLP</td>
<td>8.7</td>
<td>26.2</td>
<td>64.7</td>
</tr>
<tr>
<td>PMP</td>
<td>7.4</td>
<td>13.4</td>
<td>51.6</td>
</tr>
</tbody>
</table>
in the analyzed CSF samples and could therefore not be included. After 11 days, no significant decrease was observed.

Stability tests during 10 freeze-thaw cycles showed no significant changes for PL, PN and PA compared to their respective inter-assay variations. Although PM showed no significant change after 3 cycles, a decrease of 18% and 34% was found after 5 and 10 cycles, respectively. PLP however already showed a decrease of 49% after 3 freeze-thaw cycles and a decrease of 86% was found after 10 cycles. For PMP stability could not be tested because of a concentration <LOQ in the used CSF samples.

Concentrations of all vitamin B6 compounds (PL, PM, PN, PA, PLP, PMP) in the calibration curve showed no significant change after 4 h of exposure to light at room temperature. After 10 h of exposure to light, only PA and PLP showed a significant decrease in concentration (31% and 50%, respectively).

CSF samples on the contrary (mean of QC 1, QC 2 and QC 3) already showed a significant decrease after 4 and 10 h of exposure to light at room temperature for PL, PM, PN, PLP and PMP (Fig. 3). PA showed no significant change. After 10 h at room temperature a decrease range of 42% for PN to 70% for PLP was found (Fig. 3). Additional experiments testing temperature-dependent stability (exposure to light at room temperature versus ice) showed light dependence and no influence of temperature. In line with this, exposure to darkness during 4 and 10 h at room temperature did not result in a significant change in any of the vitamin B6 compounds (data not shown).

Furthermore, no cross-talk was observed since spiking of individual B6 vitamers did not significantly change other B6 vitamer concentrations.

### 3.3. Cerebrospinal fluid samples

Concentrations ranges in human CSF (nM) were established for PL (14.8–42.5), PM (0.1–0.5), PA (0.09–4.1) and PLP (8.8–42.0) (Table 3). For the B6 vitamers PN and PMP, CSF concentrations were <LOQ. Vitamin B6 concentrations showed a normal distribution and no age and gender correlations were observed in this dataset.

No significant differences compared to inter-assay variations in B6 vitamer concentrations (PL, PM, PN, PA and PLP) were present between the different CSF fractions (II, IV and VI) of random lumbar punctures, indicating that there is no rostrocaudal gradient of B6 vitamers in human CSF. PMP concentration was <LOQ.

In CSF of two patients on pyridoxine supplementation (Table 3), highly increased concentrations of the B6 vitamers PL, PM, PN and PA were observed. PLP concentrations were at the upper limit of the established concentration range. In both patients, PMP concentrations remained <LOQ.

### 4. Discussion

In the present study we show for the first time the development of a rapid, sensitive and accurate UPLC–MS/MS method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF using minimal CSF volumes (60 µL). The described method is based on the HPLC method in plasma by Midttun et al. [23] with several modifications.

Information on vitamin B6 concentrations in CSF is limited in both healthy individuals and in patients with epilepsy, other neurological disease or disorders of vitamin B6 metabolism. Low levels of PL and PLP have been reported in CSF of only a few patients affected with PNPO deficiency (for PL n = 3 [11]; for PLP n = 4 [11,31]) and antiepileptic deficiency (for PLP, n = 2 [9]). However, no data on PN(P), PM(P) and PA concentrations in CSF have been published. Since our method shows intra- and inter-assay CV's of 17.2% and 16.9% for PLP at a concentration of 1.9 nM, levels below the concentration range of 8.8–42.0 nM can be quantified in human CSF. The same accounts for PL. Unfortunately, we did not have CSF samples of PNPO or antiepileptic deficient patients at our disposal.

Concentrations of the active co-factor PLP in CSF were comparable with those reported previously by Foon et al. [9] and Ormazabal et al. [27], who additionally showed an inverse correlation of CSF PLP with age. Our sample size was too small to allow for meaningful correlation studies.

No concentrations for PL, PM and PA have been reported in CSF before. Since we used a highly specific UPLC–MS/MS method with stable isotope labeled internal standards, our B6 vitamer measurements are very accurate.

The present study also shows that vitamin B6 concentrations are not affected by a rostrocaudal gradient, meaning that vitamers can be measured in a random CSF sample. However, it must be warranted that vitamin B6 is determined in CSF fractions, which were protected from light at the bedside and frozen immediately after collection to prevent degradation by light and multiple freeze-thaw cycles, respectively.

In plasma, vitamin B6 vitamer concentrations have been described by Midttun et al. [23]. In 94 healthy subjects aged 11–93 (median 56) years, plasma PL(P), PM, PN and PA were determined

### Table 3

Accuracy, sensitivity (LOD and LOQ) and linearity (upper limits) of B6 vitamers analyzed with the described UPLC–MS/MS method. B6 vitamer concentrations in CSF of 20 subjects (age eight months to 16.3 years) and in CSF of two patients (A and B) on pyridoxine supplementation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Accuracy (%)</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOD</td>
<td>LOQ</td>
</tr>
<tr>
<td>PL</td>
<td>105</td>
<td>0.46</td>
</tr>
<tr>
<td>PM</td>
<td>80</td>
<td>0.01</td>
</tr>
<tr>
<td>PN</td>
<td>97</td>
<td>0.01</td>
</tr>
<tr>
<td>PA</td>
<td>108</td>
<td>0.03</td>
</tr>
<tr>
<td>PLP</td>
<td>95</td>
<td>0.12</td>
</tr>
<tr>
<td>PMP</td>
<td>139</td>
<td>1.61</td>
</tr>
</tbody>
</table>

NA: not applicable.

* Determined LOQ of this vitamin B6 compound.
using an HPLC-MS/MS method. Whereas we found CSF B6 vitamer concentrations of PL > PLP > PA > PM, Midtun et al. [23] described plasma B6 vitamer concentrations of PLP > PA > PL. This is in agreement with the studies of Marszall et al. [21] and Talwar et al. [26] who also showed plasma PLP > PA > PL. Thus, clear differences in vitamin B6 profiles exist between plasma and CSF. In PL, PLP is the B6 vitamer with the highest concentration whereas in plasma PLP and PA are higher compared to PL. In addition, in CSF PM is present in quantifiable amounts whereas both Midtun et al. [23] and Marszall et al. [21] could not detect PM in plasma.

Interestingly, the described method allowed us to analyze vitamin B6 vitamers in CSF of two patients on pyridoxine supplementation. Highly elevated concentrations of PN (18.881 and 353 nM, respectively) and PL (up to approximately 90 times higher) were found. Surprisingly, PLP concentrations were at the upper limit of the established concentration range (41.2 and 39.9 nM, respectively). In addition, PA and PM were at least approximately 10 and 20 times higher. These findings clearly demonstrate that PN passes the blood-brain and/or blood-CSF barrier.

5. Conclusion

We present an innovative method for quantification of the vitamin B6 vitamers PL(P), PM(P), PN and PA in human CSF samples. Our method is suitable for implementation in a routine diagnostic setting since decreased PL and PLP concentrations can be detected. In addition, effects of vitamin B6 therapy on CSF vitamers can be monitored. Analyzing vitamin B6 vitamers will increase our knowledge of vitamin B6 metabolism and CSF vitamer concentrations in health and disease.

Acknowledgement

This research was supported by Metakids, The Netherlands.

References