Clinica Chimica Acta 466 (2017) 1–5

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Measurement of pyridoxal 5′-phosphate, pyridoxal, and 4-pyridoxic acid in the cerebrospinal fluid of children

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ARTICLE INFO

Article history:
Received 6 November 2016
Received in revised form 24 December 2016
Accepted 27 December 2016
Available online 28 December 2016

Keywords:
Pyridoxal phosphate
4-pyridoxic acid
Vitamin B6
Liquid chromatography
Fluorescence detection
Epilepsy

ABSTRACT

Background: We quantified pyridoxal 5′-phosphate (PLP), pyridoxal (PL), and 4-pyridoxic acid (PA) in the cerebrospinal fluid (CSF) of children and to investigate the effect of age, sex, epilepsy, and anti-epileptic drug (AED) therapy on these vitamers.
Methods: CSF samples prospectively collected from 116 pediatric patients were analyzed. PLP, PL, and PA were measured using high-performance liquid chromatography with fluorescence detection, using pre-column derivatization by semicarbazide. Effects of age, sex, epilepsy, and AEDs on these vitamers and the PLP/PL ratio were evaluated using multiple linear regression models.
Results: The PLP, PL, and PA concentrations were correlated negatively with age and the PLP/PL ratio was correlated positively with age. Multiple regression analysis revealed that the presence of epilepsy was associated with lower PLP concentrations and PLP/PL ratios but sex and AED therapy had no influence on these values. The observed ranges of these vitamers in epileptic and non-epileptic patients were demonstrated.
Conclusions: We showed the age dependence of PLP and PL in CSF from pediatric patients. Epileptic patients had lower PLP concentrations and PLP/PL ratios than non-epileptic patients, but it is unknown whether this is the cause, or a result, of epilepsy.

1. Introduction

Vitamin B6 consists of various vitamers, including pyridoxal (PL), pyridoxamine, pyridoxine, their phosphorylated forms (pyridoxal 5′-phosphate [PLP], pyridoxamine 5′-phosphate, and pyridoxine 5′-phosphate), and 4-pyridoxic acid (PA). PLP, a biologically active form of vitamin B6, is involved in numerous biochemical reactions as a cofactor [1]. PLP is an essential cofactor of glutamate decarboxylase, which converts glutamic acid, an excitatory neurotransmitter, to γ-aminobutyric acid, a main inhibitory neurotransmitter in the central nervous system. Lack of PLP results in intractable epileptic seizures, such as neonatal epileptic encephalopathy caused by pyridox[am]ine-phosphate oxidase (PNPO) deficiency [2]. Insufficient PLP also affects monoamine neurotransmitter metabolism because aromatic L-amino acid decarboxylase, an enzyme to synthesize dopamine and serotonin from their precursors, is dependent on PLP.

There are few reports on vitamin B6 in cerebrospinal fluid (CSF), especially for children. Reference values of PLP in children were reported using high-performance liquid chromatography (HPLC) with fluorescence detection [3,4]. Other vitamers have been measured using liquid chromatography-tandem mass spectrometry, which showed that PLP and PL are the main forms of vitamin B6 in the CSF [5,6]. Reference values for CSF PL and PA in children 1 year of age or older were reported in only one study [6]. The impact of some factors, such as sex, epilepsy, and anti-epileptic drugs (AEDs), on these vitamer concentrations needs to be taken into account to interpret the assay results correctly. The negative effect of AEDs on the CSF PLP and PL concentration, and lower PL concentrations in males have been reported in children [6]. However, another study reported no effect of AEDs or seizures on CSF PLP concentrations [4].

Abbreviations: AED, anti-epileptic drugs; PA, 4-pyridoxic acid; PDXK, pyridoxal kinase; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PNPO, pyridox[am]ine-phosphate oxidase.

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http://dx.doi.org/10.1016/j.cca.2016.12.027
0009-8981/

In this study, we quantified the PLP, PL, and PA concentrations in CSF obtained from pediatric patients to investigate the effect of age, sex, epilepsy, and AEDs on the concentrations of these vitamers and the PLP/PL ratio. We also compared these values with reference values in the literature.

2. Patients and methods

2.1. Patients

CSF samples were prospectively collected between October 2014 and November 2016 from pediatric patients (1 month to 17 y) who underwent lumbar puncture to investigate neurological symptoms (e.g. epileptic seizures, developmental delay) or suspected meningitis. We excluded patients with: 1) vitamin B6 supplementation (high-dose therapy or supplement intake other than regular food); 2) CSF samples not protected from light within 15 min; 3) metabolic diseases that may affect vitamin B6 metabolism; 4) acute neurological process such as acute encephalitis/encephalopathy, febrile seizures, and status epilepticus; 5) significant blood contamination; and 6) statistical outliers. This study was approved by the ethics committee of Okayama University Hospital. Written informed consent was obtained from the parents or guardians of all the patients before the procedure.

2.2. CSF collection protocol

Because no rostrocaudal gradient of B6 vitamers has been reported [5], we used any fraction of the CSF samples in this study. Collected CSF samples were protected from light within 15 min and frozen within 2 h. The stability of CSF B6 vitamers for 10 h and plasma PLP for 12 h at room temperature was reported when the samples were kept in the dark [5,7]. They were stored at −80 °C until analysis. When the samples were collected outside Okayama University Hospital, they were shipped on dry ice to our laboratory.

2.3. Measurement of PLP, PL, and PA

We obtained PLP monohydrate (82870), PL hydrochloride (P9130), PA (P9630), and ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) dihydrate (09-1420) from Sigma Aldrich (St. Louis, MO, USA), sodium dihydrogen phosphate (197-09705), disodium hydrogen phosphate (197-02865), semicarbazide hydrochloride (192-00372), and glycine (073-00732) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetonitrile (1.00030.2500) was from Merck Millipore (Billerica, MA, USA). Ultrapure water was prepared using a Direct-Q UV3 system (Merck Millipore).

Simultaneous measurement of PLP, PL, and PA was conducted according to Talwar et al. [7] with some modifications. Primary stock solutions of PLP, PL, and PA were prepared, mixed, and diluted with ultrapure water to achieve final concentrations of 250 μmol/l for each component. This secondary stock solution was further diluted with ultrapure water to 1024 nmol/l to make a single-point calibrator. Quality control samples at 64 nmol/l were also prepared.

Derivatization of samples was conducted as described below. Briefly, 200 μl of undiluted CSF samples or 100 μl of serum/plasma samples diluted two-fold with 100 μl ultrapure water were derivatized by adding 16 μl of derivatization agent (370 mg/ml of semicarbazide hydrochloride and 250 mg/ml of glycine dissolved in ultrapure water). After vortexing and incubating the samples at 40 °C in the dark for 30 min, 16 μl of 70% perchloric acid was added and vortexed for deproteinization. After centrifuging for 5 min at 16,000 × g, 170 μl of supernatant was transferred to a new microtube and 23 μl of 25% sodium hydroxide was added to adjust the pH to 3.0–5.0. The samples were then filtered through 0.45-μm Millex LH filters (Merck Millipore) and subjected to HPLC.

The HPLC system consisted of a Waters Alliance 2695 module with a Waters 2475 multi λ fluorescence detector (Waters). The mobile phase A was 60 mmol/l sodium phosphate buffer with EDTA-2Na at an approximate pH of 6.5 (18 mmol/l disodium hydrogen phosphate, 42 mmol/l sodium dihydrogen phosphate, and 0.4 g/l EDTA-2Na), the mobile phase B was 100% acetonitrile, and the mobile phase C was ultrapure water. A gradient mode was used (Table 1), and the injection volume was 30 μl. The separation was performed at a flow rate of 1.2 ml/min through a reverse-phase column (Atlantis T3, 3 μm, 3.0 × 50 mm, Waters Japan) at 35 °C with a guard column (Atlantis T3 VanGuard Cartridge, 3 μm, 3.9 × 5 mm, Waters). The PLP and PL derivatives were detected using excitation (Ex) at 370 nm and emission (Em) at 460 nm. PA was detected by its natural fluorescence (Ex 320 nm, Em 420 nm). Concentrations were calculated using area under the curve, with a single-point calibrator at 1024 nmol/l. The entire analysis time was 20 min.

2.4. Statistical analysis

Statistical analysis was performed using JMP 4.0 (SAS Institute). Correlation was evaluated using Spearman's correlation coefficient. The independent effects of age, sex, epilepsy, and AEDs were tested using multiple regression analysis. The significance level was set to 0.05.

3. Results

3.1. Patient characteristics

There were 178 patients who underwent lumbar puncture to measure PLP, PL, and PA concentrations in the CSF. Sixty-two patients were excluded for the following reasons: 1) 40 patients with CSF samples protected from light > 15 min after collection; 2) 9 patients with acute neurological process; 3) 8 patients with metabolic diseases; 4) 2 patients on high-dose PLP therapy; and 5) 3 patients considered to be statistical outliers, whose PLP, PL, and PA concentrations were all above the cut-off values (3rd quartile + 1.5 × interquartile range). The data from the remaining 116 patients (68 males and 48 females) were used for analysis in this study.

Patient age ranged from 1 to 199 months (median age, 48 months). There were 62 patients with a diagnosis of epilepsy (median age, 32.5 months), 50 of whom were taking AEDs. There were 54 patients without epilepsy (median age, 75.5 months), 6 of whom were taking AEDs. For these 6 patients, AEDs were administered for suspected epileptic seizures, or were used as muscle relaxants for dystonia or spasticity, or as mood stabilizers. In total, 56 patients were taking AEDs (median age, 45.5 months) and 60 were not taking AEDs (median age, 49.5 months). The administered AEDs were valproic acid in 29 patients, levetiracetam in 14 patients, zonisamide in 13 patients, phenobarbital in 9 patients, carbamazepine in 8 patients, topiramate in 8 patients, clonazepam in 8 patients, lamotrigine in 7 patients, clobazam in 7 patients, ethosuximide in 3 patients, potassium bromide in 2 patients, nitrazepam in 1 patient, and rufinamide in 1 patient either as monotherapy or in combination.

Table 1
High-performance liquid chromatography gradient settings.

Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve
Initial	1.2	98.6	1.4	0.0	
3.5	1.2	98.6	1.4	0.0	Linear
7.0	1.2	94.0	6.0	0.0	Linear
7.1	1.2	0.0	60.0	40.0	Step
10.0	1.2	98.6	1.4	0.0	Step
20.0	1.2	98.6	1.4	0.0	Linear