Original Article

Pyridoxal 5′-phosphate and related metabolites in hypophosphatasia: effects of enzyme replacement therapy

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Abstract

Objective: To investigate the utility of serum pyridoxal 5′-phosphate (PLP), pyridoxal (PL) and, 4-pyridoxic acid (PA) as a diagnostic marker of hypophosphatasia (HPP) and an indicator of the effect of, and patient compliance with, enzyme replacement therapy (ERT), we measured PLP, PL, and PA concentrations in serum samples from HPP patients with and without ERT.

Methods: Blood samples were collected from HPP patients and serum was frozen as soon as possible (mostly within one hour). PLP, PL, and PA concentrations were analyzed using high-performance liquid chromatography with fluorescence detection after pre-column derivatization by semicarbazide. We investigated which metabolites are associated with clinical phenotypes and how these metabolites change with ERT.

Results: Serum samples from 20 HPP patients were analyzed. The PLP-to-PL ratio and PLP concentration were elevated in all HPP patients. They correlated negatively with serum alkaline phosphatase (ALP) activity and showed higher values in more severe phenotypes (perinatal severe and infantile HPP) compared with other phenotypes. PL concentration was reduced only in perinatal severe HPP. ERT reduced the PLP-to-PL ratio to mildly reduced or low-normal levels and the PLP concentration was reduced to normal or mildly elevated levels. Urine phosphoethanolamine (PEA) concentration did not return to normal levels with ERT in most patients.

Conclusions: The serum PLP-to-PL ratio is a better indicator of the effect of ERT for HPP than serum PLP and urine PEA concentrations, and a PLP-to-PL ratio of <4.0 is a good indicator of the effect of, and patient compliance with, ERT.

Key Words

asfotase alfa; liquid chromatography; vitamin B6; diagnostic marker; therapeutic monitoring
Conflict of Interest
T. Akiyama received a research grant and fees for lectures and advisory consultation from Alexion Pharmaceuticals, the manufacturer of asfotase alfa. T. Kubota received fees for lectures and advisory consultation from Alexion Pharmaceuticals. K. Ozono received fees for lectures and advisory consultation from Alexion Pharmaceuticals. T. Michigami received fees for lectures and advisory consultation from Alexion Pharmaceuticals. D. Kobayashi received a research grant from Alexion Pharmaceuticals. M. Noda received fees for lectures from Alexion Pharmaceuticals. N. Namba received fees for lectures and advisory consultation from Alexion Pharmaceuticals. Y. Mitani received a research grant from Alexion Pharmaceuticals.

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Ethics approval
This study was performed in accordance with the Declaration of Helsinki and approved by the Research Ethics Board at Okayama University Hospital. Written informed consent was obtained from the patients’ parents or guardians.
1. Introduction

Hypophosphatasia (HPP; MIM #241500, #241510, and #146300) is an inborn error of metabolism that is characterized mainly by defective mineralization of bone and/or teeth and low serum alkaline phosphatase (ALP) activity [1]. HPP is caused by abnormalities in the *ALPL* gene that encodes tissue-nonspecific ALP (TNSALP; EC 3.1.3.1). The onset age varies among patients, ranging from the prenatal period to adulthood. Based on the onset age and severity, six clinical forms are recognized: perinatal severe, perinatal benign, infantile, childhood, adult, and odonto-HPP.

In HPP, three metabolites are known to be elevated: urine phosphoethanolamine (PEA), plasma/serum pyridoxal 5′-phosphate (PLP), and inorganic pyrophosphate (PPi) [1]. TNSALP, which has reduced or absent activity in HPP, uses these metabolites as its substrate. Elevated PPi inhibits deposition of hydroxyapatite in bone, causing defective skeletal mineralization [2]. Alteration of vitamin B6 (VB6) metabolism reflected by abnormal PLP (active form of VB6) is thought to be associated with epileptic seizures [3].

In recent years, recombinant TNSALP enzyme replacement therapy (ERT) has become available by approval of asfotase alfa, which contains the catalytic TNSALP domain, the human immunoglobulin G1 Fc region, and L-aspartate residues [4]. This therapy improved skeletal radiographic findings and pulmonary and physical function in young patients with life-threatening HPP [4, 5].

Monitoring the effect of, and patient compliance with, ERT is important in clinical practice for optimization of treatment. A monitoring guidance was recently published and the role of assays for PLP, PEA, and PPi was described [6]. However, the target concentrations of these
metabolites were not clearly mentioned and there was no description about other PLP-related metabolites, such as pyridoxal (PL). Because PLP is a substrate and PL is a product of the enzymatic reaction of ALP, the simultaneous assay of PLP and PL may provide a better understanding of in vivo ALP activity in the blood during ERT than the PLP assay alone. Additionally, 4-pyridoxic acid (PA, a metabolite of PL) is a good biomarker for the VB6 status as well as PLP and PL [7].

In this study, we aimed to determine PLP, PL, and PA concentrations in serum samples obtained from HPP patients with and without ERT. We investigated which metabolites are associated with clinical phenotypes and how these metabolites are changed by ERT. We hypothesized that the PLP-to-PL ratio is a better indicator of the effect of, and patient compliance with, ERT than serum PLP and urine PEA concentrations.

2. Material and methods

2.1. Subjects

Subjects consisted of HPP patients who underwent blood and urine tests to diagnose HPP or to check adverse effects during ERT with asfotase alfa. A diagnosis of HPP was made based on the following: 1) low serum ALP activity compared with age- and sex-matched reference values; and either 2) clinical symptoms suggestive of HPP (skeletal symptoms such as defective mineralization, deformities, rickets, and/or premature loss of primary teeth); or 3) confirmation of ALPL gene abnormality, or both. Blood samples obtained during VB6 therapy were excluded. We also collected clinical data including phenotype, use of asfotase alfa, epileptic seizures, disability in daily activities measured using a modified Rankin Scale, serum ALP activity, urine PEA concentration, and ALPL gene abnormalities. This study was performed in accordance with the Declaration of Helsinki and approved by the Research
Ethics Board at Okayama University Hospital. Written informed consent was obtained from the patients’ parents or guardians.

2.2. Collection of serum samples

Blood samples were protected from light and serum was separated by centrifugation and frozen at either −20°C or −80°C. We recommended that the samples should be frozen within 1 hour after blood collection, particularly for serum samples with ERT. When this protocol could not be followed, the time between blood collection and protection from light, and the time between blood collection and storage in a freezer were documented. The frozen samples were shipped on dry ice to our laboratory at Okayama University Hospital and kept frozen at −80°C until analysis.

2.3. Assay of PLP, PL and PA

The frozen serum samples were thawed in a refrigerator at 4°C. Assay of PLP, PL and PA concentrations was conducted using high-performance liquid chromatography after derivatization of PLP and PL by semicarbazide hydrochloride under mildly acidic conditions, as previously reported [8, 9]. The PLP-to-PL ratio (PLP/PL), which is considered to reflect the activity of ALP (conversion of PLP to PL) and pyridoxal kinase (conversion of PL to PLP), was calculated.

2.4. Stability of PLP and PL in the serum samples with ERT

Keeping serum samples with ERT for a long time at room temperature may significantly affect the assay results of PLP and PL [6]. Because no test tubes containing an ALP inhibitor are commercially available in Japan, we investigated the influence of asfotase alfa on PLP and PL concentrations in serum samples collected using regular serum collection tubes. Serum
samples with ERT were divided into four aliquots and pre-treated under four different conditions: 1) serum was diluted two-fold with levamisole (20 mmol/L), an ALP inhibitor, in ultrapure water, and immediately derivatized by semicarbazide hydrochloride; 2) serum was diluted two-fold with ultrapure water (no levamisole) and immediately derivatized; 3) serum was diluted the same as in 2), kept at 4°C in the dark for 1 hour, then derivatized; and 4) serum was diluted the same as in 2), kept at the room temperature (24°C, air-conditioned) for 1 hour, then derivatized. PLP and PL were determined and their concentrations were compared among these four conditions.

2.5. Statistical analysis
To increase reliability of statistical analysis, we excluded the following serum samples: 1) samples without ERT protected from light more than 1 hour or frozen more than 6 hours after blood collection; 2) samples with ERT frozen more than 1 hour after blood collection; 3) samples with no documentation about the timing of protection from light and freezing; and 4) samples under the possible influence of a mother’s VB6 supplementation (described in the Results). Spearman’s correlation analysis and multiple regression analysis were performed using R 3.4.2 (https://cran.r-project.org/). The significance level was set to 0.05.

3. Results
3.1. Subject characteristics
There were 20 patients with HPP (13 females) enrolled into this study. Their clinical characteristics are presented in Table 1. Case 4 was reported elsewhere [10]. Eight patients had serum samples collected during ERT, and 18 patients had serum samples collected without ERT. There was one patient (Case 17) who temporarily discontinued ERT because of a possible allergic reaction. There was a 19-year-old patient (Case 16) with no skeletal or
dental symptoms but who had an epileptic seizure, for which VB6 was not administered. Because this patient had low serum ALP activity and an *ALPL* gene abnormality, we included this patient as an adult HPP patient.

Epileptic seizures were observed in five patients (Cases 1, 4, 6, 11, and 16). There were two patients (Cases 1 and 4) with VB6-responsive seizures, one patient (Case 11) with VB6 non-responsive seizures, and two patients (Cases 6 and 16) who had seizures but VB6 was not administered.

Modified Rankin Scale scores were high (severe disability in daily activities) in perinatal severe HPP but low with other phenotypes, except one patient (Case 11) who had a complication of cerebral palsy and VB6 non-responsive epilepsy. Patients with perinatal severe HPP who continued ERT for more than 6 months (Cases 2, 3, and 4) had stable or improved disability in daily activities.

Serum ALP activities were low in all patients and urine PEA concentrations were elevated except for one patient (Case 5) without ERT. Two patients (Cases 1 and 4) with VB6-responsive seizures showed the lowest serum ALP activities (14 and 11 U/L). After ERT started, there was a dramatic increase in serum ALP activity but PEA concentration decreased to within the normal range in only one patient (Case 3). The most common *ALPL* gene abnormality in our patients was c.1559delT, p.L520fs, which is characteristic in Japanese HPP patients [11].

3.2. Stability of PLP and PL with ERT

The assay results of serum samples (n = 4) collected during ERT with different pre-treatment
conditions are shown in Figure 1. After thawing at 4°C in a refrigerator, samples with no added levamisole derivatized immediately or after 60 minutes at 4°C showed no or a slight change in the PLP concentration (−5.0 to +5.9%) and no or a slight increase in the PL concentration (±0.0 to +4.9%) compared with serum samples with levamisole. Samples without levamisole derivatized after 60 minutes at 24°C showed no or a mild decrease in the PLP concentration (−5.7 to ±0.0%) and a mild increase in the PL concentration (+1.9 to +7.7%). Addition of levamisole impaired chromatographic separation of the PA peak. Therefore, we chose to derivatize serum samples after thawing at 4°C without levamisole for subsequent analyses.

3.3. PLP, PL, and PA concentrations without ERT

The results in samples without ERT are shown by closed symbols in Figure 2. There was a sample protected from light more than 1 hour after blood collection (Case 11), a sample with no information about the time between blood collection and protection from light/storage in a freezer (Case 6), and a sample from a patient whose mother had taken vitamin B6 supplements until delivery (Case 7).

Compared with the control ranges [9], the serum samples from all patients without ERT showed abnormally high PLP concentrations. PL concentrations were elevated in most of the samples but there was a sample with a reduced PL concentration from a patient with perinatal severe HPP with VB6-responsive seizures (Case 1). PA concentrations were mostly within normal limits but there were four older patients who had elevated PA. PLP-to-PL ratios were elevated in all patients.

Scatter plots of serum PLP, PL, and PA concentrations, the PLP-to-PL ratio, and the urine
PEA concentration against serum ALP activity are presented in Supplementary Figure 1. After excluding the three serum samples that were inappropriate for statistical analysis, correlation analysis revealed that the PLP concentration ($\rho = -0.554, p = 0.0349$) and the PLP-to-PL ratio ($\rho = -0.721, p = 0.0033$) correlated negatively with the ALP activity, but that the PL ($\rho = 0.291, p = 0.292$), PA ($\rho = 0.182, p = 0.516$), and PEA ($\rho = -0.268, p = 0.315$) concentrations did not correlate with ALP activity.

Serum ALP activity, serum PLP, PL, and PA concentrations, the PLP-to-PL ratio, and the urine PEA concentration are plotted against clinical phenotype in Supplementary Figure 2. Serum ALP activities were lower in more severe phenotypes (perinatal severe and infantile HPP). Serum PLP concentrations and PLP-to-PL ratios were higher in more severe phenotypes. Serum PL concentrations were low in perinatal severe HPP. However, other phenotypes showed higher but relatively constant PL concentrations. Serum PA concentrations did not show a clear difference among phenotypes. Urine PEA concentrations were higher in more severe phenotypes.

Because serum PLP, PL, and PA concentrations, and the PLP-to-PL ratio are age-related [9], we investigated the effect of age and phenotype severity (severe [perinatal severe HPP and infantile HPP] vs. mild [other phenotypes], analyzed as a categorical variable) on these values using multiple regression analysis. The PLP concentration was associated with phenotype severity ($p < 0.0001$) but not with age ($p = 0.388$). The PLP-to-PL ratio was also associated with phenotype severity ($p = 0.0133$) but not with age ($p = 0.734$). The PL concentration was associated with age ($p = 0.0442$) but not with the phenotype severity ($p = 0.213$). PA concentration was not associated with the phenotype severity ($p = 0.381$) or age ($p = 0.500$).
3.4. PLP, PL, and PA concentrations during ERT

The results in samples with ERT are shown by open symbols in Figure 2. There were three serum samples with ERT stored immediately at 4°C but frozen 1–5 hours after blood collection (Cases 2, 8, and 17). With ERT, PLP concentrations showed normal or mildly elevated values. All cases in which pretreatment serum samples were also obtained had a remarkable reduction in the PLP concentration by ERT. PL concentrations were mildly elevated or within the normal limits. A patient with perinatal severe HPP with a remarkably low PL concentration (Case 1) showed normalization of PL concentration. Serum PA concentrations were mildly low or within normal limits. The PLP-to-PL ratios were reduced or low-normal in all patients. When PLP concentrations are plotted against PL concentrations, samples with and without ERT were separated well by the dashed line (y = 4x). This indicates that a PLP-to-PL ratio of approximately 4.0 is a good cut-off value to evaluate the effect of ERT.

Two patients deserve additional attention. Case 7 had a rapid normalization of PLP and PL concentrations and a rapid reduction of the PLP-to-PL ratio in only 5 days (two injections of asfotase alfa). Case 17 had temporary discontinuation of ERT. This caused elevation of the PLP concentration and the PLP-to-PL ratio, which were reduced again by restarting ERT.

3.5. PLP, PL, and PA concentrations plotted against serum ALP activity in all samples

The assay results in all serum samples with and without ERT are plotted together in Figure 3. Correlation analysis revealed that the serum PLP concentration (ρ = −0.855, p < 0.0001) and PLP-to-PL ratio (ρ = −0.914, p < 0.0001) had strong negative correlations with serum ALP activity. Serum PL concentration showed a moderate positive correlation with ALP activity (ρ = 0.558, p = 0.0046) but serum PA (ρ = −0.136, p = 0.526) and urine PEA (ρ = −0.407, p =
0.0834) did not show a correlation with ALP activity.

4. Discussion

We conducted the assay for serum PLP, PL, and PA concentrations in HPP patients with and without ERT to confirm the usefulness of these metabolites as a diagnostic marker of HPP and to show the potential use of this assay to monitor the effect of ERT. We confirmed that an elevated serum PLP-to-PL ratio is a good diagnostic marker for HPP, along with other markers such as low serum ALP activity, elevated serum PLP concentration, and elevated urine PEA concentration [12]. We also demonstrated that the serum PLP-to-PL ratio reflected the effect of ERT more clearly than serum PLP and urine PEA concentrations. The PLP-to-PL ratio may be useful to evaluate the early effect of ERT before actual improvement in skeletal symptoms occurs. Because ERT is expensive, the PLP-to-PL ratio could be used to optimize asfotase alfa dosing and to monitor compliance in the subcutaneously administered ERT. We demonstrated that a PLP-to-PL ratio of approximately 4.0 is a good cut-off value to evaluate the effect of ERT. This cut-off value corresponds well to the upper limit of the serum PLP-to-PL ratio that was shown in previous studies [9, 13].

Elevated PL concentrations in most cases could be explained by highly excessive amount of PLP and residual TNSALP activity, which allows production of PL. Two patients with perinatal severe HPP and VB6-responsive seizures had the lowest PL concentrations among those at 0–1 months of age. PLP is essential to synthesize gamma-aminobutyric acid, a major inhibitory neurotransmitter in the brain. Because PLP cannot cross the blood-brain barrier, PLP must be dephosphorylated to PL by membrane-bound TNSALP to reach the brain [3]. Low serum PL concentrations suggest an insufficient supply of PLP to the brain and this may be a good indicator to consider ERT to prevent epileptic seizures, especially in neonatal
patients. Changes in the PL concentration with ERT were not as remarkable as those of the PLP concentration, particularly with older patients. Because PL is membrane-permeable, extra PL produced by a high ALP activity probably diffuses rapidly to the body tissues, keeping the serum PL concentration relatively constant.

Many commercial laboratories measure PLP alone as a VB6 assay. Our findings indicate the importance of measuring both PLP and PL. Having laboratories specifically report PLP and PL concentrations, if possible, will be helpful for clinicians to calculate the PLP-to-PL ratio and estimate the risk of VB6-responsive seizures based on the PL concentration.

When investigating the effect of ERT on serum PLP and PL concentrations, we checked the reliability of the PLP and PL assay for serum samples with asfotase alfa. Because derivatization of serum samples by semicarbazide takes 30 minutes, we first confirmed that the derivatization procedure did not significantly alter the PLP and PL concentrations by comparing between serum samples with and without levamisole. We then demonstrated that keeping the samples at 24°C for 1 hour without levamisole changed PLP and PL concentrations to a mild degree (<10%). The changes were less evident with the samples kept at 4°C for 1 hour. Therefore, when using regular serum collection tubes, we suggest that the blood samples should be protected from light to prevent photodegradation of PLP and cooled after collection, and that serum should be separated using a refrigerated centrifuge and frozen as soon as possible to obtain precise results. However, even though this is not possible in a clinical setting, if a blood sample is protected from light immediately after collection and serum is separated at room temperature and frozen within 1 hour, changes in PLP and PL concentrations will not be great enough to cause clinical misjudgment.
This study is limited by the small sample size. The sample collection procedure was not strictly controlled, although we recommended to separate and freeze serum within 1 hour after blood collection. Future directions include elucidating how concentrations of PLP and other related metabolites are associated with neurological and developmental symptoms, and how the assay of these metabolites can be used to determine the optimal dosing of asfotase alfa.

5. Conclusions

We confirmed the usefulness of the serum PLP-to-PL ratio and PLP concentration as a diagnostic marker of HPP. These measures are associated with serum ALP activity and clinical phenotypes. The PL concentration was low only in perinatal severe HPP. ERT with asfotase alfa decreased the PLP-to-PL ratio to mildly reduced or a low-normal level, and decreased the PLP concentration to normal or a mildly elevated level. ERT also normalized the low PL concentration in perinatal severe HPP. The PLP-to-PL ratio is a better indicator of the effect of, and patient compliance with, ERT than serum PLP and urine PEA concentrations, and a PLP-to-PL ratio of <4.0 is a good indicator of the effect of, and patient compliance with, ERT. The assay of these metabolites is considered to be helpful in the assessment of in vivo ALP activity in the blood during ERT.

Acknowledgements

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References


Figure 1

Stability of PLP and PL in different pre-treatment conditions

PLP and PL concentrations were measured after four pre-treatment conditions: addition of levamisole; no levamisole; no levamisole and storage for 60 min at 4°C; and no levamisole and storage for 60 min at 24°C. Although pre-treatment with no levamisole and storage for 60 min at 24°C showed the greatest changes in PLP and PL concentrations, the degree of the changes were within ±10%. PLP, pyridoxal 5′-phosphate; PL, pyridoxal

Figure 2

Concentrations of PLP, PL, and PA, and the PLP-to-PL ratio (PLP/PL) in all serum samples PLP, PL, and PA concentrations and the PLP-to-PL ratio are plotted against age (top two rows). Closed and open symbols represent serum samples without and with enzyme replacement therapy, respectively. Samples from the same patients are connected by lines in the same symbol shape. Samples that were inappropriate for statistical analysis are indicated by oblique lines over the symbols. Dashed lines represent reference ranges [9]. The scatter plot of PL vs. PLP (bottom row) demonstrates that serum samples with and without asfotase alfa are separated well by the dashed line (y = 4x). This indicates that a PLP-to-PL ratio of approximately 4.0 is a good cut-off value to evaluate the effect of asfotase alfa.

PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid
Figure 3

Serum ALP activity compared with serum PLP, PL, and PA concentrations, the PLP-to-PL ratio (PLP/PL), and the urine PEA concentration with and without ERT. Circles indicate samples without ERT and squares indicate samples with ERT. Open symbols indicate samples that were inappropriate for statistical analysis. Serum PLP concentration and the PLP-to-PL ratio correlated negatively with serum ALP activity. The serum PL concentration correlated positively with serum ALP activity. The serum PA concentration and the urine PEA concentration did not correlate with serum ALP activity.

ALP, alkaline phosphatase; PLP, pyridoxal 5’-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid; PEA, phosphoethanolamine; ERT, enzyme replacement therapy
Supplementary Figure 1

Serum ALP activity vs. serum concentrations of PLP, PL, and PA, the PLP-to-PL ratio (PLP/PL), and the urine PEA concentration without enzyme replacement therapy

The shape of symbols represents the clinical phenotype. Open symbols indicate samples that were inappropriate for statistical analysis. Serum PLP concentration and the PLP-to-PL ratio correlated negatively with ALP activity. Serum PL and PA concentrations and the urine PEA concentration did not correlate with ALP activity.

ALP, alkaline phosphatase; PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid; PEA, phosphoethanolamine

Supplementary Figure 2

Clinical phenotype vs. serum ALP activity, serum PLP, PL, and PA concentrations, the PLP-to-PL ratio (PLP/PL), and the urine PEA concentration

In each clinical phenotype, the same symbol shape in the six graphs represents the same individual patient. Open symbols indicate serum samples with inappropriate storage conditions for PLP, PL, and PA assays. Serum ALP activities showed lower values in more severe phenotypes (perinatal severe and infantile hypophosphatasia). Serum PLP concentrations and PLP-to-PL ratios were higher in more severe phenotypes. Serum PL concentrations were low in perinatal severe HPP but other phenotypes showed higher and relatively constant values. Serum PA concentrations did not show a difference among phenotypes. Urine PEA concentrations were higher in more severe phenotypes.

ALP, alkaline phosphatase; PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PA, 4-pyridoxic acid; PEA, phosphoethanolamine
Table 1

Clinical characteristics of subjects

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<th>Serum PLP (nmol/L)</th>
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<td>2 mg/kg × 3</td>
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<td>1</td>
<td>0y 1m</td>
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<td>216 (4–38)</td>
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<td>0y 0m 0d</td>
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<td>1y 8m</td>
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<td>1</td>
<td>12y 2m</td>
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<td>0</td>
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Infantile hypophosphatasia
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<th>Age</th>
<th>Days</th>
<th>Months</th>
<th>Years</th>
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<th>PLP</th>
<th>PEA</th>
<th>Phosphate</th>
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<td>22**</td>
<td>14**</td>
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<td>18</td>
<td>31</td>
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<td>c.550C&gt;T, p.R184W (heterozygous)</td>
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mRS, modified Rankin scale; ALP, alkaline phosphatase; PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PEA, phosphoethanolamine;

VB6, vitamin B6; d, days; m, months; y, years

† Time between blood collection and protection from light/storage in a freezer was not documented.

‡ Mother took vitamin B6 supplements until delivery.
* Samples were stored at 4°C immediately but frozen 1–5 hours after blood collection.

** Sample was protected from light 1–5 hours after blood collection.

Reference values: serum ALP from Tanaka et al. [14]; serum PLP, PL, and PA from Akiyama et al. [9]; urine PEA from Imbard et al. [15]. For serum ALP, reference values for 20 years old are shown for patients over 20 years old. For serum PLP, PL, and PA, reference values for 4–11 months old are shown for patients under 4 months old, and those for 7–17 years old are shown for patients over 17 years old. For urine PEA, reference values for 18–19 years old are shown for patients over 19 years old.