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Circulating pyridoxal 5′-phosphate in serum and whole blood: implications for assessment of vitamin B6 status

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Abstract

Objectives: Concentrations of pyridoxal 5′-phosphate (PLP) in serum and whole blood are routinely measured. The suitability of these markers in capturing vitamin B6 insufficiency is not well studied.

Methods: In 212 subjects, concentrations of PLP and thiamine pyrophosphate (TPP) were simultaneously measured in EDTA-whole blood using Chromsystems® (52052) method on HPLC devices. The whole blood PLP concentrations were compared to serum PLP concentrations measured using reagents from Immundiagnostik® (KC 2100). The whole blood TPP concentrations measured with the Chromsystems® (52052) were compared to those measured by the Chromsystems® (35000) method. Concentrations of homocysteine (tHcy) and cystathionine (Cys) were measured and used to judge the PLP methods.

Results: Serum PLP concentrations were on average 41% lower than whole blood PLP [mean (SD)=55.4 (83.0) vs. 131 (217) nmol/L]. Serum and whole blood PLP showed a strong correlation [Pearson correlation coefficient=0.724, p<0.001, n=204]. Eighty-five samples showed discrepant results for PLP status (serum PLP ≤30 nmol/L, but whole blood PLP >51 nmol/L) while 102 samples showed coherent results (reference group). The discrepancy group showed higher odds ratio for elevated tHcy >12.0 µmol/L compared to the reference group [OR (95% confidence intervals, CI)=2.1 (1.2–4.0)]. The OR (95% CI) of elevated Cys >300 nmol/L was 1.9 (1.0–3.5) in the discrepancy group compared to the reference group. TPP concentrations were 6% lower when using the Chromsystems®, 52052 compared to levels measured with Chromsystems®, 35000.

Conclusions: Serum and whole blood PLP concentrations disagree in a substantial number of samples. Serum PLP was better in reflecting elevated tHcy and Cys compared to whole blood PLP. Whole blood PLP underestimates the prevalence of vitamin B6 insufficiency. Methods of measuring TPP concentrations in whole blood were exchangeable.

Keywords: cystathionine; deficiency; homocysteine; pyridoxal 5′-phosphate; thiamine pyrophosphate; vitamin B6.

Introduction

Vitamin B6 comprises pyridoxal, pyridoxine, pyridoxamine, and their corresponding 5′-phosphate derivatives. Insufficient plasma pyridoxal 5′-phosphate (PLP) and low vitamin B6 intake have shown associations with inflammation [1] and increased risk of cardiovascular diseases, cancer, and depression [2–5]. PLP is the active form of vitamin B6 which is involved in synthesis of neurotransmitters such as dopamine and serotonin. Moreover, PLP participates in metabolism of homocysteine (Hcy) through the transsulfuration pathway to cystathionine (Cys) and cysteine. Vitamin B6 is stored mainly in the liver and muscle tissues. The turnover time of vitamin B6 differs between different body compartments [6].

Several markers can reflect the response of vitamin B6 status to B6 intake and can thus be used as indicators for insufficiency of this vitamin. The direct measurements of circulating PLP concentrations in serum/plasma [7, 8] and red blood cells [9] are commonly used markers in clinical laboratories. Plasma circulating concentrations of functional metabolic markers such as total homocysteine (tHcy) [10] and Cys [11] have been used as B6 markers. Other indirect or functional markers include erythrocyte aminotransferase saturation by PLP and tryptophan metabolites via the kynurenine pathway [7, 12]. However, there is insufficient evidence to tell which marker can better show inadequate B6 status. Some investigators suggested that vitamin B6 status is best assessed by using a combination of multiple biomarkers [13].

Although PLP does not capture all biologically active vitamin B6, plasma PLP could be the best single indicator

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because it responds to increasing vitamin intake, reflects tissue stores of the vitamin [14] and correlates to liver PLP [15]. The US Institute of Medicine (IOM) of The National Academy of Sciences prioritized maintenance of sufficient plasma PLP concentration >20 nmol/L to determine sufficient dietary vitamin B6 intake [16]. Plasma PLP <20 nmol/L [17] or <30 nmol/L [18, 19] have been used to define the lower end of normal vitamin B6 status.

There are no studies comparing the relationship between serum/plasma and whole blood concentrations of PLP and the performance of these markers in capturing samples with possible vitamin B6 insufficiency. The present study aimed at comparing serum and whole blood PLP concentrations in samples referred to our laboratory with indication for vitamin B6 status assessment. We measured serum concentrations of tHcy and Cys as metabolic markers to judge the presence of lowered PLP in serum compared to whole blood. Commercially available methods for PLP measure simultaneously thiamine pyrophosphate (TPP, vitamin B1). A secondary aim of this study was to compare concentrations of TPP in whole blood measured using the simultaneous assay with PLP and levels of this markers measured using an independent HPLC assay for TPP in whole blood.

Materials and methods

Samples

Between March and June 2021, we collected residual serum and whole blood samples from 212 independent individuals. The samples were sent to our lab for determination of serum PLP and EDTA-whole blood vitamin B1 measured as TPP. The inclusion criteria were residual serum (at least 250 µL) and EDTA-whole blood (at least 500 µL) bio specimens carrying the same barcode, collected on the same day and time. Serum samples should not show any visible blood hemolysis (due to possible release of PLP from the damaged erythrocytes) and the samples should have normal serum creatinine (≤79.6 µmol/L for females and ≤106.1 µmol/L for males). Creatinine was measured using Creatinine Jaffé Gen.2 on Roche/Hitachi cobas c701 (Roche®) that corrects for unspecific reactions caused by pseudo-creatinine chromogens by reducing the results by −26 µmol/L. All samples were processed, protected from light, and frozen shortly after arrival to our lab. The serum and whole blood samples with identical barcodes were anonymously collected and recoded with a randomly assigned numbers. All person-specific data, except for sex were not available for us. No information was available on clinical conditions that lead to ordering vitamin concentrations. There was also no information on using supplemental or therapeutic doses of the vitamins. All samples were stored at −20 °C for 1 week up to 2 months until analysis of the vitamin markers. Since the samples were anonymously collected, no ethical approval was required. Figure 1 shows the study flow diagram and the order of measuring the biomarkers.

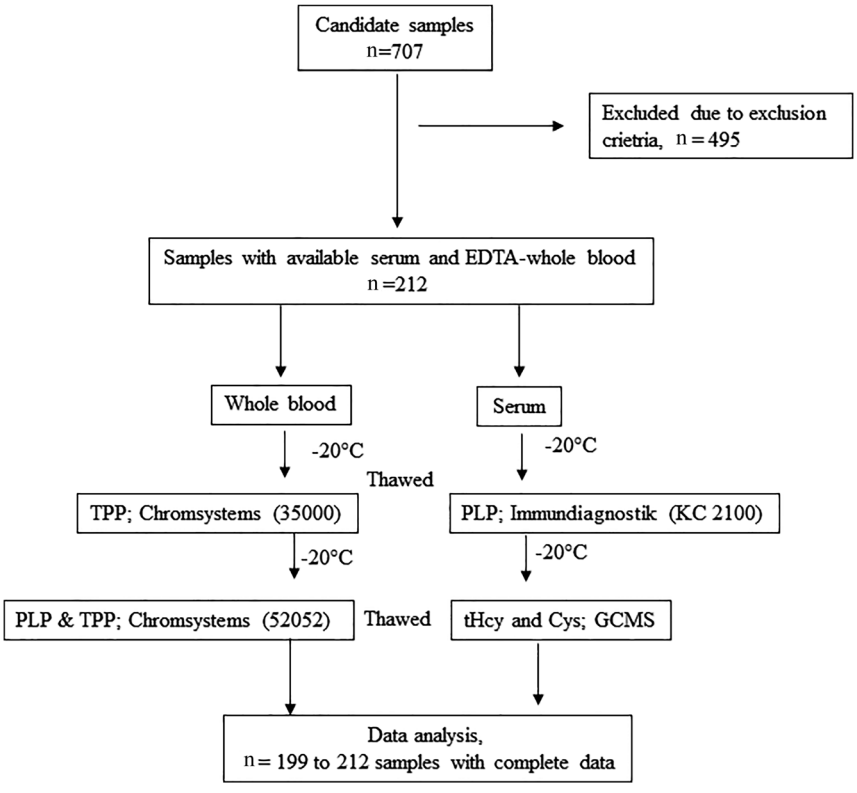

Figure 1: Study flow diagram.

Reagents and methods

PLP was measured in serum samples using reagents from Immundiagnostik® (KC 2100) and HPLC device connected to a fluorescence detector (1100-Agilent). Whole blood TPP was measured using an independent kit reagents from Chromsystems® (35000) using external standard on HPLC system connected to a fluorescence detector (1100 – Agilent). After measuring whole blood TPP, the whole blood samples were frozen at −20 °C for a maximum of 2 months and were used for measuring whole blood PLP using reagents from Chromsystems® (52052) on HPLC connected to a fluorescence detector (1260-Agilent). The Chromsystems® (52052) method measures simultaneously PLP and TPP in EDTA-whole blood and relies on using internal standard (Supplementary Table S1). For all three methods, we used commercially available quality control materials (low and high levels) to account for between-day variations. The between-day coefficient of variations (CV %) for serum PLP were <4% at 12.5 µg/L and 29.7 µg/L [with both Immundiagnostik® (KC 2100) and Chromsystems® (52052) methods]. The CVs% for the TPP assay were <4% at concentrations of 27.7 and 102.9 µg/L (with both Chromsystems® 35000 and 52052).

Serum tHcy and Cys were simultaneously measured using gas chromatography mass spectrometry method according to Stabler et al. [20]. The CVs% for the tHcy and Cys assay were 4.2 and 5.6% for tHcy assay at concentrations of 15.6 µmol/L and 29.8 µmol/L, respectively. The CV% was 9.8% for the Cys assay at concentration of 374 nmol/L. The CV% for creatinine was less than 5%.

Statistical analyses

The statistical analyses were performed using version 28 of IBM® SPSS® Statistics package (SPSS Inc., Chicago, IL, USA). p-values <0.05 were considered statistically significant.

One-Sample Kolmogorov-Smirnov Test and Lilliefors Significance Correction in addition to Q-Q plots were used to judge the distribution of the continuous variables. The concentrations of PLP, TPP, tHcy, Cys, and creatinine were not normally distributed. Also the log-transformed serum and whole blood PLP, serum tHcy, and whole blood TPP (Chromsystems®, 52052) were not normally distributed, but the distributions of the log-transformed variables were closer to normality. Thus, the log-transformed data was used for all tests that assume normal distribution of the data. The descriptive data are shown as mean (SD) or median [interquartile range, IQR] for continuous variables and absolute (n) and relative frequencies (%) for categorical variables.

The correlation between two continuous variables was studied using Pearson test applied on the log-transformed data. We studied the correlation between serum and whole blood concentrations of PLP. However, since these two parameters were principally not measuring the same PLP fraction, we mainly focused on explaining the discordance between these two parameters in classifying samples as having sufficient or insufficient vitamin B6 status. We used ANOVA and Post-hoc Thamhane-T2 tests to compare the log-transformed concentrations of the biomarkers between independent groups.

The prevalence of elevated tHcy (>12.0 µmol/L and >20.0 µmol/L) and elevated Cys (>300 nmol/L) were used to discriminate samples with insufficient PLP compared to those with sufficient PLP. Logistic regression analyses were used to calculate the odds ratio (OR) and 95% Confidence Intervals (CI) of elevated serum tHcy and Cys in the group with discrepant serum and whole blood PLP results compared to the group with sufficient serum and whole blood PLP levels (reference group). The logistic regression analyses were adjusted for sex and creatinine and the crude and adjusted OR (95% CI) are presented. PLP concentrations above 30 nmol/L were considered normal in line with previous epidemiological studies [18, 19]. The cut-off for insufficient whole blood PLP of ≤51 nmol/L was suggested by the reagent's manufacturer (Chromsystems®). In sensitivity analysis, we also used the medians of serum and whole blood PLP methods (30.4 nmol/L for serum PLP and 86.7 nmol/L for whole blood PLP) or alternatively a cut off of 20 nmol/L for serum PLP to define low PLP.

Scatter plots and Person correlation were used to judge a possible bias between the two TPP methods in whole blood (Chromsystems®, 35000 and 52052). In addition, we calculated the difference between TPP levels measured with Chromsystems®, 35000 (first thawing cycle) and those measured with Chromsystems®, 52052 (second thawing cycle). We studied the correlation between this difference and the concentrations measured using Chromsystems®, 35000 (i.e., the single TPP method used for TPP assay in samples not thawed before).

Results

Comparison of serum and whole blood PLP

Concentrations of PLP in whole blood [mean (SD)=131 (217) nmol/L] were higher than those in serum [mean (SD)=55.4 (83.0) nmol/L] (Table 1). The mean and (SD) of the difference between the two markers were [76 (176) nmol/L]. This difference corresponds to on average 41% higher whole blood PLP compared to serum PLP. The concentrations of PLP in serum and whole blood showed a strong direct correlation [Pearson correlation coefficient R=0.724, p<0.001, n=204] (Figure 2). Serum PLP showed inverse correlation with serum tHcy (R=−0.163, p=0.017, n=212) and Cys (R=−0.213, p=0.002, n=212). Similarly, whole blood PLP correlated with tHcy (R=−0.186, p=0.008, n=204) and Cys (R=−0.161, p=0.022, n=204).

We defined three distinct subgroups based on serum and whole blood PLP levels (Table 2); samples with serum PLP >30 nmol/L and whole blood PLP >51 nmol/L (reference group, n=102), samples with serum PLP ≤30 nmol/L and whole blood PLP ≤51 nmol/L (insufficiency group, n=16), and samples with lowered serum PLP but normal whole blood PLP (discrepancy group, n=85). There was one sample with low whole blood PLP of 43 nmol/L and high serum PLP of 522 nmol/L. Measurements of whole blood PLP were missing in 8 samples (4 of them with lowered serum PLP). The group with discrepant serum and whole blood PLP showed concentrations of tHcy similar to those in the reference group [mean (SD)=15.5 (9.3) vs. 13.1 (6.6) µmol/L; p=0.224]. Serum Cys levels were higher in the discrepancy group compared to the reference group [mean (SD)=554 (773) vs. 360 (441) nmol/L; p=0.016].

The sex- and creatinine-adjusted OR (95%) of elevated tHcy levels >12.0 µmol/L was 2.1 (1.2–4.0) in the discrepancy group compared to the reference group. The adjusted OR

Table 1: Descriptive results of concentrations of PLP in serum and whole blood, PLP metabolic markers, TPP, and creatinine.

Pyridoxal 5′-phosphate (PLP), nmol/L	n	Mean (SD)	Median [IQR]
Serum PLP (Immundiagnostik®, KC 2100)	212	55.4 (83.0)	30.4 [36.1]
Whole blood PLP (combined method with TPP, Chromsystems®, 52052)	204	131 (217)	87 [56]
Complementary PLP functional markers			
Homocysteine, µmol/L	212	15.1 (10.8)	12.2 [7.1]
Cystathionine, nmol/L	212	465 (638)	289 [303]
Whole blood TPP, nmol/L			
Single method (Chromsystems®, 35000), nmol/L	212	204 (73)	194 [78]
Combined method with PLP (Chromsystems®, 52052), nmol/L	199	182 (55)	175 [57]
Renal function marker			
Creatinine, µmol/L	212	73.8 (17.5)	72.5 [23.9]

IQR, interquartile range; SD, standard deviation; TPP, thiamine pyrophosphate.

(95%) of tHcy levels >20.0 µmol/L was 4.3 (1.7–10.9). Moreover, the adjusted OR (95% CI) for elevated Cys (>300 nmol/L) was 1.9 (1.0–3.5) in the discrepancy group compared to the reference group (Table 3).

When the median serum and whole blood PLP were used to define low PLP status (Supplementary Figure S1 and Supplementary Table S2), the concentrations of tHcy and Cys were highest when both serum and whole blood PLP were below the corresponding medians. The group with serum PLP below the median, but whole blood PLP above the median (n=20) appeared to have higher levels of tHcy and Cys, whereas the group with only whole blood PLP below the median had rather low mean tHcy and Cys (Supplementary Table S2). Similar results were obtained when the cut off value of 20 nmol/L was used to define lowered serum PLP (data not shown). However, multiple comparisons and low sample size in the subgroup analysis could have caused loss of statistical significance of some tests.

Comparison of whole blood TPP assays

The concentrations of whole blood TPP measured using the single TPP-method (Chromsystems®, 35000) were higher than those measured with combination method of TPP and PLP (Chromsystems®, 52052) [mean (SD)=204 (73) nmol/L vs. 182 (55) nmol/L; mean (SD) of the difference=20 (44) nmol/L; p <0.001]. The correlation between TPP concentrations with the two assay methods was strong; Pearson correlation coefficeint R=0.814, n=199, p<0.001 (Supplementary Figure S2). The TPP levels measured with the Chromsystems®, 52052 assay were on average 6% lower than those measured with the Chromsystems®, 35000. This difference appeared to be systematic because the concentrations of TPP with Chromsystems®, 35000 correlated with the difference between TPP levels between Chromsystems®, 35000 (first time thawed) and Chromsystems®, 52052 (second time thawed); R=0.611, n=199, p<0.001 (Supplementary Figure S3).

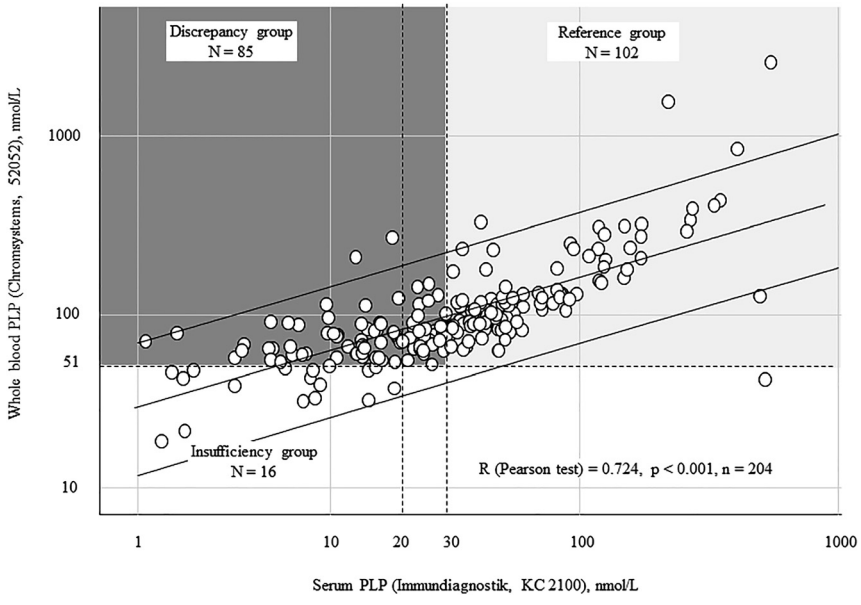

Figure 2: Scatter plot of serum PLP and whole blood PLP measured parallel in 204 samples. The cutoffs used to define B6 insufficiency are; serum PLP ≤30 nmol/L and whole blood PLP ≤51 nmol/L. Accordingly, 16 samples had B6 insufficiency using both markers; 85 samples had discrepant results (low serum PLP, but normal whole blood PLP), and 102 samples had normal serum and whole blood PLP concentrations.

Table 2: The concentrations of serum and whole blood PLP, metabolic PLP markers and creatinine in 204 samples according to subgroups of agreements between serum and whole blood PLP assays.

n	Reference group 102	B6 insufficiency 16	Discrepancy group 85	p-Value Insufficiency vs. reference group	p-Value Discrepancy vs. reference group
Serum PLP (Immundiagnostik, KC 2,00)	>30 nmol/L	≤30 nmol/L	≤30 nmol/L	/	/
Whole blood PLP (Chromsystems, 52052)	>51 nmol/L	≤51 nmol/L	>51 nmol/L	/	/
Serum PLP in nmol/L, mean (SD)	90.0 (95.0)	7.8 (5.5)	17.1 (7.9)	/	/
Whole blood PLP in nmol/L, mean (SD)	189 (295)	40.2 (9.9)	80.8 (32.4)	/	/
tHcy in µmol/L, mean (SD)	13.1 (6.6)	27.4 (25.3)	15.5 (9.3)	0.021	0.224
Cys in nmol/L, mean (SD)	360 (441).	410 (194)	554 (773)	0.102	0.016
Creatinine in µmol/L, mean (SD)	76 (17)	75 (20)	71 (18)	0.999	0.228

The groups were compared using ANOVA test applied on the log-transformed data. When ANOVA test was significant, post-hoc Thamhane-T2 test was used to compare the insufficiency and discrepancy subgroups with the reference group. The cutoffs used to define B6 insufficiency are; serum PLP ≤30 nmol/L and whole blood PLP ≤51 nmol/L.

Table 3: The frequency and probability of elevated total homocysteine and cystathionine concentrations in samples with concordant and discordant concentrations of PLP in serum and whole blood.

n	Reference group Serum and whole blood PLP showing sufficiency 102	Serum and whole blood PLP showing insufficiency 16	Discrepancy group Serum PLP showing insufficiency and whole blood PLP showing sufficiency 85
tHcy >12.0 µmol/L	n=45 of 102 (44.1%) OR=1.0	n=13 of 16 (81.3%) Crude OR=5.4 (1.5–20.4) adj. OR=6.9 (1.8-26.9)	n=49 of 85 (57.6%) Crude OR=1.7 (0.96–3.1) adj. OR=2.1 (1.2–4.0)
tHcy >20.0 µmol/L	n=7 of 102 (6.9%) OR=1.0	n=9 of 16 (56.3%) Crude OR=17.4 (5.0–61.0) adj. OR=18.7 (5.2-66.4)	n=20 of 85 (23.5%) Crude OR=4.2 (1.7–10.4) adj. OR=4.3 (1.7–10.9)
Cys >300 nmol/L	n=43 of 102 (42.2%) OR=1.0	n=10 of 16 (62.5%) Crude OR=2.3 (0.8–6.8) adj. OR=2.6 (0.8–7.9)	n=46 of 85 (54.1%) Crude OR=1.6 (0.9–2.9) adj. OR=1.9 (1.0–3.5)

Logistic regression analyses were used to calculate the odds ratio (OR) and the 95% confidence intervals (CI) of having an elevated tHcy and Cys concentration among samples with B6 insufficiency (i.e., both serum and whole blood PLP are lowered) or those with discrepant serum and whole blood PLP levels compared to the reference group (i.e., both serum and whole blood PLP are above the corresponding cutoffs). The regression analyses were adjusted for sex and serum creatinine. The cutoffs used to define B6 insufficiency are; serum PLP ≤30 nmol/L and whole blood PLP ≤51 nmol/L. Cys, cystathionine; PLP, pyridoxal 5′-phosphate; tHcy, total homocysteine.

Discussion

Methods that measure PLP concentrations in whole blood are widely used in clinical laboratories [21–23], but their performances in detecting B6 insufficiency compared to serum/plasma PLP is not established. We studied the implications of using serum versus whole blood PLP to evaluate vitamin B6 status. We found that serum and whole blood PLP concentrations disagree in a substantial number of samples that would be classified as having vitamin B6 insufficiency for instance in samples with serum PLP <30 nmol/L but whole blood PLP >51 nmol/L. Concentrations of tHcy and Cys were higher and elevated levels of tHcy and Cys were more prevalent in the discrepancy group than in the reference group where both serum and whole blood PLP were in the corresponding normal ranges. The results suggest that serum PLP rather than whole blood PLP could better reflect elevated concentrations of tHcy and Cys. With regard to TPP, the concentrations were 6% lower when using the Chromsystems®, 52052 compared to levels measured with Chromsystems®, 35000. The minor differences between the two TPP assays could be due to prenalaytical factors such as thawing

and refreezing or to the use of internal standard in the Chromsystems®, 52052 method, but not in the Chromsystems®, 35000.

Plasma PLP declined as a response to dietary vitamin B6 depletion within approximately 2 weeks [24], while a vitamin B6 rich diet or supplementation of vitamin B6 leads to higher plasma PLP [1]. Whole blood PLP was rarely measured in clinical studies despite its popular use in clinical laboratories. The hemoglobin in the erythrocytes is known to have a high binding capacity for PLP. Erythrocyte's PLP is derived from plasma pyridoxal that is converted to PLP by erythrocyte pyridoxal kinase. The kinetic of PLP in human erythrocytes is not well studied. Bhagavan et al. [25], have shown that erythrocyte and plasma PLP concentrations are similar in individuals on normal diets. In subjects taking large dose of vitamin B6, erythrocytes PLP level increases to a higher degree than plasma PLP [25]. The significance of directing supplemental vitamin B6 into the erythrocytes is not clear. However, Bhagavan et al. [25], suggested that erythrocytes PLP could have a slower turnover rate and this compartment could be involved in regulation of plasma PLP levels including repletion of plasma PLP upon short term insufficiency. We have shown that lowered serum PLP may better reflect elevated tHcy and Cys. Thus, it is unlikely that treatment with vitamin B6 may explain low serum PLP and normal whole blood PLP in the discrepancy group. Vitamin B6 binding proteins play a role in sequestering the vitamin in tissues (i.e., PROSC, hemoglobin) or in plasma (albumin) [26]. Our results suggest that serum PLP could be a better marker for B6 status than whole blood PLP. In line with this suggestion, Leung et al. have shown that plasma PLP, but not red blood cell PLP were related to inflammation scores in patients with colorectal cancer [27]. These results collectively suggest that lowered serum or plasma PLP reflect functional vitamin B6 insufficiency better than whole blood PLP.

Measurements of PLP to judge vitamin B6 status maybe influenced by recent intake [16], renal dysfunction, acute inflammation [28] and pre-analytical conditions such as blood hemolysis. Hemolysis may result in an increase in serum PLP because of leakage from the erythrocytes. There are several gaps in knowledge regarding optimal markers that indicate vitamin B6 status, especially in clinical conditions where PLP plays a functional role in the disease process (i.e., inflammation, neurotransmitters, pain syndrome). The significance of capturing high amount of PLP in red blood cells is not clear, but requires more investigations. In addition, systematic differences between PLP concentrations measured with HPLC (30–40% higher values) and LC-MS/MS methods have been reported [21]. However, the observed discrepancy between serum and whole blood PLP is not likely to be related to methodological issues.

The present study has some limitations. First, we did not measure markers of inflammation and have no access to clinical data to verify whether low serum PLP in the discrepancy group could be explained by age or inflammatory conditions. Second, due to limited samples size, we were not able to conduct subgroup analyses by age or sex. Third, compared to serum PLP, whole blood PLP may be affected by hematocrit or erythrocyte volume fraction. A low hematocrit such as in subjects with anemia or kidney dysfunction may result in a lower whole blood PLP, without affecting plasma PLP. The present results need to be confirmed and possible factors that may differentially affect plasma and whole blood PLP should be investigated.

Conclusions

Serum and whole blood PLP levels disagree in a substantial number of samples. Detection of vitamin B6 insufficiency using serum PLP was better in reflecting intracellular metabolic abnormalities such as elevated tHcy and Cys compared to whole blood PLP. Measurements of whole blood PLP is widely used in clinical laboratories, but it appears to underestimates the prevalence of vitamin B6 insufficiency. Serum PLP is likely to be a better marker than whole blood PLP in clinical settings and population studies. Future studies need to investigate the effect of inflammation on the kinetics of serum versus red blood cell PLP. The methods of measuring TPP concentrations in whole blood in this study showed only 6% deviation which was likely to be caused by thawing and refreezing, suggesting that the methods are exchangeable.

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Author contributions: RO, planning the study, data analyses and drafting the manuscript; CM, planning and conducting the analytical work; JG, planning, critical input to the study and overall supervision.
Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Not applicable since the samples were anonymously collected, no ethical approval was required.

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