

Homogeneous, Nonradioactive, Enzymatic Assay for Plasma Pyridoxal 5-Phosphate

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Background: Pyridoxal 5'-phosphate (PLP) is the biologically active form of vitamin B₆. Clinical studies suggest that low PLP concentrations are an independent risk factor for cardiovascular and other diseases. However, PLP concentrations are not routinely diagnosed because of the lack of a homogeneous, nonradioactive assay. We describe a homogeneous, nonradioactive, enzymatic PLP assay that uses the apo form of the PLP-dependent recombinant enzyme, homocysteine- α,γ -lyase (rHCYase).

Methods: PLP was removed from holoenzyme rHCYase by incubation with hydroxylamine to obtain apo-rHCYase. The restoration of enzymatic activity by reconstitution of the holoenzyme was linearly related to the amount of PLP bound to the enzyme. The amplification principle of the assay allowed nanomolar concentrations of PLP to be measured by the conversion (by reconstituted holo-rHCYase) of millimolar concentrations of homocysteine to H₂S. *N,N*-Dibutylphenylenediamine (DBPDA) was used for determination of H₂S, the combination of which forms a chromophore with high absorbance. The assay was initiated by incubation of 5 μ L of plasma with apo-rHCYase in a binding buffer for 60 min at 37 °C. Homocysteine (2.5 mmol/L) was added to the assay buffer and incubated at 37 °C for 20 min. The DBPDA reaction was allowed to progress for 10 min and then read at 675 nm.

Results: The PLP enzymatic assay has a lower limit of detection of 5 nmol/L and is linear to 200 nmol/L. The recovery of PLP was 98%. The mean within- and between-run CVs were 9.6% and 12%, respectively. Correlation of 45 samples in the PLP enzymatic assay and the B₆ ³H radioenzymatic assay (American Laboratory Products Co., Ltd.) yielded: $y = 0.9367x + 10.569$ ($R^2 = 0.9201$).

Conclusions: This new PLP assay is the first homogeneous, nonradioactive, vitamin B₆ diagnostic method. The assay is applicable to chemistry automated analyzers and may have wide clinical use.

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Pyridoxal 5'-phosphate (PLP)¹ is the biologically active form of vitamin B₆ and is involved in numerous metabolic pathways as an enzyme cofactor (1–3). Homocysteine (HCY), a risk factor for cardiovascular and other diseases, including Alzheimer disease (4, 5), is converted to cysteine by PLP-dependent transsulfuration enzymes, which condense HCY with serine to form cystathionine and subsequently convert it to cysteine (6). A major cause of homocysteinemia is insufficient intake of vitamin B₆, vitamin B₁₂, and folic acid, which are also necessary for HCY metabolism (7). Clinical studies suggest that vitamin B₆ is independently associated with an increased risk for cardiovascular disease (8–10), and recent studies have shown that plasma PLP concentrations are significantly decreased in other pathologic conditions, including rheumatoid arthritis (11). High total HCY (tHCY) and low vitamin B₆ plasma concentrations are associated with an increased risk for deep venous thrombosis independent of established risk factors for deep venous thrombosis. The association of low vitamin B₆ concentrations with deep venous thrombosis is also independent of the tHCY concentration (12).

Several methods have been developed for vitamin B₆ measurement. The most common assay for PLP uses activated apo-tyrosine decarboxylase, which requires PLP to convert [1-¹⁴C]tyrosine to ¹⁴CO₂, which is then trapped and counted for radioactivity. Other methods include the vitamin B₆ ³H radioenzymatic assay (REA), which is also based on PLP-dependent tyrosine decarboxylase (13–15) and HPLC (16, 17). These methods are relatively complex

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¹ Nonstandard abbreviations: PLP, pyridoxal 5'-phosphate; tHCY, total homocysteine; REA, radioenzymatic assay; rHCYase, recombinant homocysteine α,γ -lyase; and DBPDA, *N,N*-dibutylphenylenediamine.

and nonhomogeneous and require specialized equipment and materials, including radioactive reagents.

We describe in this report a single-enzyme, nonradioactive, homogeneous assay for PLP that uses the apo form of PLP-dependent, recombinant homocysteine α,γ -lyase (rHCYase) cloned from *Trichomonas vaginalis* (18, 19). The PLP enzymatic assay is simple, rapid, sensitive, and specific and requires only a small volume of plasma.

Materials and Methods

The following materials were obtained from Sigma-Aldrich: pyridoxal phosphate, pyridoxal, pyridoxamine, pyridoxine, dithiothreitol, sodium bisulfate, D,L-HCY, potassium ferricyanide, and Triton X-100. N,N-Dibutylphenylenediamine (DBPDA) was synthesized in our laboratory. rHCYase was produced in our laboratory (19). The vitamin B₆ ³H REA reagent set was purchased from American Laboratory Products Co., Ltd.

PREPARATION OF APO-rHCYase

Hydroxylamine is a carbonyl-specific reagent that reversibly dissociates PLP from the holoenzyme. Holo-rHCYase was treated with 10 mmol/L hydroxylamine in 1.8 mL containing 3.75 g/L protein, 40 μ L of 100 mmol/L dithiothreitol, and 200 μ L of 100 mmol/L hydroxylamine phosphate and incubated overnight at 4 °C. The PLP-depleted apoenzyme was precipitated with 450 g/L ammonium sulfate and centrifuged at 10 000g for 10 min. The supernatant was discarded, and the pellet was dissolved in 1.0 mL of 20 mmol/L potassium phosphate buffer (pH 7.6) containing 1 mmol/L EDTA and 1 mmol/L dithiothreitol. Gel filtration was then performed with 1.0 mL of the aforementioned solution loaded onto a 1.0 \times 10 cm G-25 column. The apoenzyme was eluted with 20 mmol/L potassium phosphate buffer (pH 7.6), and the protein peak was collected as measured at the absorbance at 280 nm. This protein peak contained PLP-depleted rHCYase at 0.1 g/L protein. Trehalose at a final concentration of 1.0 g/L was added as a preservative to the apoenzyme solution, which was stored at -80 °C.

DETERMINATION OF PLP CONTENT IN HOLO- AND APO-rHCYase

The PLP content of holo- and apo-rHCYase was determined fluorometrically with a modification of a published procedure (20). In brief, 0.2 mL each of holoenzyme (3.75 g/L) and apoenzyme (1.0 g/L) was added to 0.2 mL of 0.8 mol/L perchloric acid. The samples were then centrifuged at 10 000g for 5 min. The pellet was discarded, and the supernatant was used for HPLC analysis. The mobile phase, consisting of 0.1 mol/L sodium perchlorate and 0.5 g/L sodium bisulfite, was adjusted to pH 7.0 by addition of phosphoric acid and pumped at a flow rate of 1.0 mL/min through a LC-18-DB column. The eluate was assessed for PLP content with a spectrofluorometer

at an excitation wavelength of 300 nm and an emission wavelength of 400 nm. The detection limit for PLP was 0.5 pmol (20).

RECONSTITUTION OF HOLO-rHCYase

Apo-rHCYase (0.1 g/L) was preincubated at 37 °C for 60 min in phosphate buffer (pH 7.6) with 50 μ mol/L PLP (final concentration). Ten microliters of the sample was diluted in 290 μ L of phosphate buffer (pH 7.6), and 10 μ L was used for the enzyme activity assay. Ten microliters of the holo-rHCYase (1.0 g/L) was diluted in 290 μ L of phosphate buffer (pH 7.6), and 10 μ L was used for the enzyme activity assay. Enzyme activity was measured with a 3-methyl-2-benzothiazoline hydrazone assay for the keto product of the α,γ -elimination reaction (19). The assay was performed in 1 mL of 50 mmol/L phosphate buffer (pH 8.0) and 5 mmol/L D,L-HCY for 10 min at 37 °C with various amounts of enzyme. The reaction was stopped by addition of 0.5 mL of 45 mL/L trichloroacetic acid. Supernatant (0.5 mL) was added to 0.5 mL of 0.5 mL/L 3-methyl-2-benzothiazoline hydrazone in 1.0 mL of 1.0 mol/L sodium acetate (pH 5.2) and incubated for 30 min at 50 °C. The product was determined spectrophotometrically by the absorbance at 335 nm.

PLASMA SAMPLE PREPARATION

Blood was collected by venipuncture into a Vacutainer Tube (Becton-Dickinson) containing EDTA. After centrifugation, the plasma sample was stored at -80 °C until the time of assay.

PLP ENZYMATIC ASSAY PROTOCOL

Binding step. Five microliters of plasma or calibrators and 20 μ L of apo-rHCYase (0.1 g/L) were added to 475 μ L of binding buffer [10 mmol/L citrate-phosphate buffer (pH 5.3) containing 1 mmol/L EDTA] and mixed well. The binding step was performed for 60 min at 37 °C. All samples were assayed in duplicate. An apoenzyme blank was also included in each assay to correct for endogenous PLP that might be present in the apoenzyme extract.

Enzymatic reaction step. Five hundred microliters of assay buffer containing 200 mmol/L potassium phosphate buffer (pH 8.3), 2.5 mmol/L D,L-HCY, 1 mmol/L EDTA, 0.2 mmol/L dithiothreitol, and 2 g/L Triton X-100 were added to each tube. The reaction was carried out at 37 °C for 20 min.

Chromogenic step. The enzymatic reaction was stopped by addition of 50 μ L of chromophore reagent (40 mmol/L DBPDA in 6 mol/L HCl), followed by addition of 50 μ L of oxidizing agent (40 mmol/L potassium ferricyanide in 20 mmol/L potassium phosphate buffer, pH 8.3). The chromogenic reaction was allowed to progress for 10 min at 37 °C. The resulting absorbance was measured at 675 nm.

VITAMIN B₆ ³H REA METHOD

PLP was also determined using the vitamin B₆ ³H REA (American Laboratory Products Co., Ltd.). In this method, [³H]tyrosine is decarboxylated to [³H]tyramine by the PLP-dependent enzyme, tyrosine decarboxylase (13, 14). The activity of the apo form of tyrosine decarboxylase is linearly dependent on PLP concentration. The [³H]tyramine product of this reaction is selectively extracted in a scintillation mixture, with the unreacted [³H]tyrosine remaining in the aqueous phase.

SPECIFICITY FOR PLP

To determine the specificity of the PLP enzymatic assay, apo-rHCYase was first incubated with pyridoxal phosphate, pyridoxal, pyridoxamine, and pyridoxine, each at a concentration of 100 nmol/L. The PLP enzymatic assay was then performed with HCY as described above.

RECONSTITUTION STABILITY OF APO-rHCYase

Twenty microliters of apo-rHCYase (0.1 g/L), stored at 4 °C, and 5 μL of a 50 nmol/L PLP calibrator were added to 475 μL of binding buffer (pH 5.3). After a 60-min preincubation at 37 °C, 500 μL of assay buffer (pH 8.3) was added to each tube, vortex-mixed, and incubated for 20 min at 37 °C. The reconstituted holoenzyme was then used in the PLP enzymatic assay with HCY as described above.

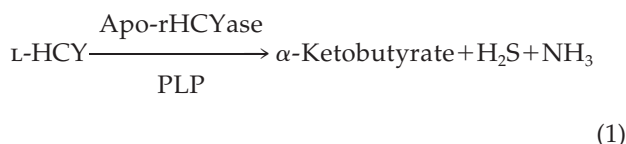
INTERFERENCE

Interference in the PLP enzymatic assay was determined by addition of the following substances: bilirubin (ICN), to achieve plasma concentrations of 125 and 250 μmol/L; hemoglobin (Sigma) at 2500 and 5000 mg/L; and triglycerides (Sigma) at 3.32 and 6.65 mmol/L. All plasma samples were obtained from three healthy individuals (Table 3).

Results

PRINCIPLE OF THE ASSAY

PLP is removed from the rHCYase holoenzyme by incubation with hydroxylamine to obtain apo-rHCYase. The restoration of enzymatic activity by reconstitution of the holoenzyme depends on the amount of PLP bound to rHCYase (Reaction 1):



H₂S combines with DBPDA to form an absorbance compound (Reaction 2). The absorbance is read at 675 nm:

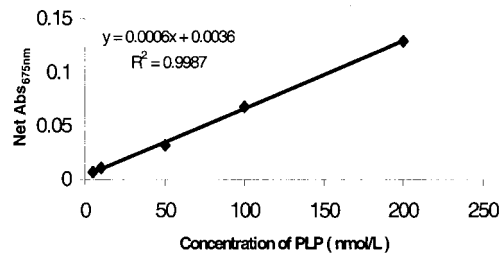
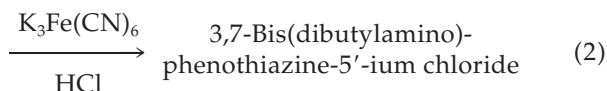


Fig. 1. Linearity of the calibration curve for the PLP enzymatic assay. Abs, absorbance. Equation for the line: $y = 0.0006x + 0.0036$ ($R^2 = 0.9987$).

CHARACTERIZATION OF apo-rHCYase

PLP was removed from holo-rHCYase by reaction with hydroxylamine phosphate for 12 h at 4 °C. The PLP was derivatized with sodium bisulfite. The PLP content of holo- and of apo-rHCYase was 3.8 ± 0.4 and 0.072 ± 0.008 per tetramer, respectively. The hydroxylamine-treated apo-rHCYase retained residual enzyme activity (0.46 U/mg of protein). Addition of 50 μmol/L (final concentration) PLP to apo-rHCYase produced a reconstituted activity of 16.1 U/mg of protein.

SPECIFICITY FOR PLP RECONSTITUTION

We confirmed the absolute dependence of HCYase activity on PLP. Other vitamin B₆ compounds, including pyridoxal, pyridoxamine, and pyridoxine, did not restore any detectable activity to apo-rHCYase.

STABILITY OF apo-rHCYase

The apo-rHCYase reconstituted with PLP in 20 mmol/L potassium phosphate buffer (pH 7.6) was stable at 4 °C for at least 1 month. The apoenzyme was stable at -80 °C for 1 year.

EVALUATION OF THE ASSAY

Linear response. To determine the linear response of the assay, we assayed five different PLP concentrations, 5, 10, 50, 100, and 200 nmol/L, by the present method. As shown in Fig. 1, the calibration was linear between 5 and 200 nmol/L.

Analytic recovery. The analytic recoveries for PLP added to human pooled serum are shown in Table 1. Recoveries

Table 1. Recovery of PLP added to plasma.^a

PLP added to plasma, nmol/L	Calculated, nmol/L	Observed, nmol/L	Recovery, %
0		58.4	
10	68.4	70.4	103
50	108.4	104.5	96
100	158.4	149.4	94
Average			98

^a PLP (13.6 mg) was dissolved in 10 mL of binding buffer, then diluted to 10, 50, and 100 nmol/L; 5 μL was used in the PLP enzyme assay for HCY. See *Materials and Methods* for details.

Table 2. Within- and between-run imprecision of the PLP enzymatic assay.^a

Plasma	Within-assay			Between-assay		
	PLP, nmol/L	SD, nmol/L	CV, %	Mean, nmol/L	SD, nmol/L	CV, %
A (n = 20)	22.4	2.53	11	21.6	2.74	13
B (n = 20)	77.2	7.45	9.6	74.6	9.0	12
C (n = 20)	125.1	9.96	7.9	122.4	12.5	10
Mean			9.6			12

^a The three plasma samples were run in 20 duplicates. Between-assay imprecision is based on results from 20 succeeding analytic set-ups performed on 10 different days. Five microliters of plasma was used for the PLP enzymatic assay with HCY. See *Materials and Methods* for details.

were 103% at 10 nmol/L, 96% at 50 nmol/L, and 94% at 100 nmol/L, with a mean recovery of 98%.

Precision. The within-assay variation was estimated after three samples, containing 22.4, 77.2, and 125.1 nmol/L PLP, were analyzed in 20 parallel determinations. Each sample was assayed in duplicate. The between-assay variation was determined from 20 successive analytic set-ups that analyzed aliquots from a single sample on 10 different days over a 1-month period. The within- and between-assay mean CVs were 9.6% and 12%, respectively (Table 2).

Correlation. The concentration of PLP in 45 different human serum samples was measured by the present method and the vitamin B₆ ³H REA method. As shown in Fig. 2, excellent agreement was observed between the

methods by linear regression analysis (Fig. 2A), which yielded the equation $y = 0.9367x + 10.569$ ($R^2 = 0.9201$), and by a Bland-Altman plot (21) of the difference between the results obtained by the two methods as a function of their mean value ± 2 SD (Fig. 2B).

INTERFERENCE

Bilirubin at 125 and 250 μ mol/L had no appreciable effect on the assay (Table 3). Hemoglobin at 2500 mg/L had no appreciable effect, but at 5000 mg/L produced a -13% change in the result (Table 3). Triglycerides at 3.32 and 6.65 mmol/L had a negligible effect on the assay (Table 3).

Discussion

We report here the first homogeneous, nonradioactive assay for determining vitamin B₆ (PLP) in plasma. Only a single enzyme was necessary in this assay. We used a three-step procedure to prepare apo-rHCYase by use of hydroxylamine phosphate, a carbonyl-specific reagent known to reversibly dissociate PLP from PLP-dependent enzymes (22-25). To prevent the apoenzyme from re-binding free PLP, the enzyme was precipitated rapidly with ammonium sulfate to remove free PLP and hydroxylamine phosphate.

We observed that 10 mmol/L citric phosphate buffer (pH 5.3) was more effective for dissociation of PLP and the subsequent apoenzyme reconstitution. The apo-rHCY-

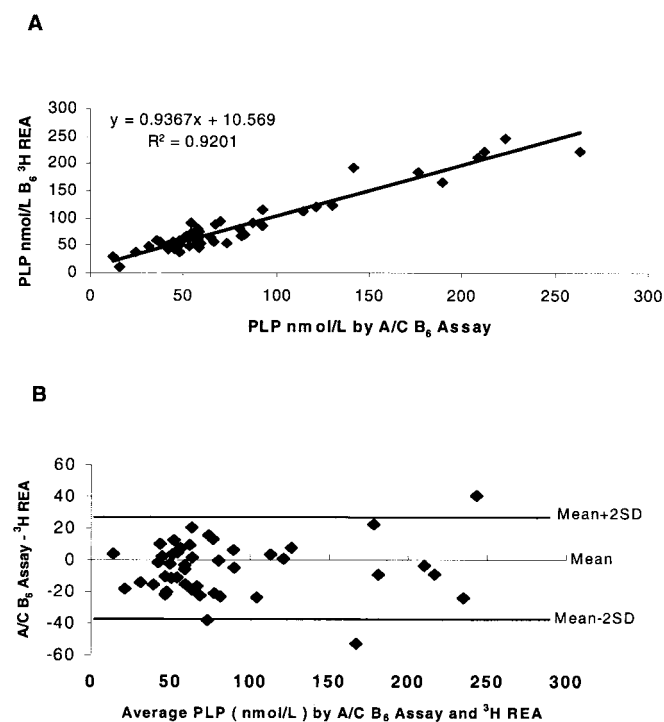


Fig. 2. Correlation between the PLP enzymatic assay (y) and vitamin B₆ ³H REA (x).

(A), linear regression analysis: $y = 0.9367x + 10.569$ ($R^2 = 0.9201$; $n = 45$). (B), Bland-Altman plot of the difference in PLP values between the two methods as a function of their mean value ± 2 SD (21).

Table 3. Interference from bilirubin, hemoglobin, and triglycerides added to plasma samples.

Compound	PLP, nmol/L	Interference, ^a %
Bilirubin added, μ mol/L		
0	55.2	
125	52.2	-5.4
250	57.2	3.6
Hemoglobin added, mg/L		
0	77.2	
2500	73.2	-5.2
5000	67.2	-13
Triglycerides added, mmol/L		
0	133.2	
3.32	139.2	4.5
6.65	143.2	7.5

^a (Result for sample with compound added - Result for native sample)/sample $\times 100 =$ % interference.

ase typically retained 0.5–1.0% of the original enzyme activity, which indicates that a small, residual amount of PLP remained bound to the PLP-free enzyme (26). The apo-rHCYase, however, had lower residual enzyme activity and a high recovery of enzyme activity with PLP.

We previously described a simple, single-enzyme assay method for measurement of HCY by modified methylene blue determination of H₂S (27). The PLP method uses the amplification principle of the HCY enzymatic assay, which allows nanomolar concentrations of PLP to be measured, with the signal conversion of millimolar concentrations of HCY to H₂S. Amplification of the PLP signal is on the order of 10⁶, which enables PLP concentrations as low as 5 nmol/L to be measured.

We observed an excellent correlation between our new method and the vitamin B₆ ³H REA method. The new analytic method may be useful in the diagnosis of vitamin B₆ deficiency in large, population-based studies because PLP appears to be an independent risk factor for cardiovascular and other diseases. We are now developing a fully automated method based on this assay system. A main goal will be to shorten the reconstitution time to further reduce the total assay time.

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