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Short communication

Clinical adaptation of a high-performance liquid chromatographic method for the assay of pyridoxal 5′-phosphate in human plasma

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Abstract

Vitamin B6, measured as pyridoxal 5′-phosphate (PLP), is a co-enzyme in the transsulfuration pathway of homocysteine metabolism. Since depletion of PLP has been suggested as an independent risk factor for coronary artery disease, PLP is frequently measured to guide patient care. By a change and utilization of an Aquasil C18 column and the addition of an acetonitrile clean-up gradient to the potassium phosphate, with sodium perchlorate and bisulfite buffer between samples we report the modification of a previously described method for analysis of PLP. The result is a more practical, efficient, reliable and robust method for daily clinical use. We also determined and report that it is critical to protect freshly prepared standard PLP samples from light exposure during assay preparation. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Pyridoxal 5′-phosphate; Vitamin B6

1. Introduction

It is well known that vitamin B6, clinically measured as pyridoxal 5′ phosphate (PLP) has an integral role as a coenzyme in the transsulfuration pathway of homocysteine metabolism. Depletion of pyridoxal 5′ phosphate has also been suggested as an independent risk factor for development of coronary artery disease [1,2]. Additionally, PLP analysis has proven clinical relevance in assessing dietary and vitamin status in patients with acute myocardial infarction or renal failure as well as diagnosing suspected malnutrition in the hospitalized, hyperhomocysteinemic, or the elderly patient [3–10]. Various methods have been developed for PLP analysis; however, most are technically too difficult and labor intensive for the routine clinical laboratory. These methods for PLP analysis have been previously described and employ microbiological, radioenzymatic, and high-performance liquid chromatography (HPLC) principles [11–18]. Kimura et al. [19] described a highly sensitive and simple PLP method utilizing HPLC. In our experience, however, this basic method was limited by excessive sample carry-over making it unacceptable for routine use in our clinical laboratory. With a modification in column type and the addition of a ‘‘clean-up’’ gradient

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between samples we report that this method has become both reproducible and reliable for analysis of PLP in the clinical setting.

2. Experimental

Five ml samples of venous blood were collected into EDTA treated tubes, immediately protected from light, and stored on ice. The blood was centrifuged for 10 min at 3000 *g* and the plasma removed and stored at −80°C for batch analysis. Thawed plasma samples (500 ul) were prepared for analysis according to the method of Kimura et al. [19]. Sample preparation consisted of plasma protein precipitation by the addition of 0.5 ml 0.8 *M* perchloric acid (Fisher Scientific, St. Louis, MO, USA) to 0.5 ml of plasma. This mixture was then vigorously vortex-mixed for 1 min. Following centrifugation at 1700 *g* for 10 min, the supernatant was removed and analyzed for PLP. For quantification, the peak area from each sample was used as compared with a standard calibration curve. B6 vitamers analyzed included pyridoxamine (PM), pyridoxal 5′ phosphate (PLP), pyridoxal (PL), and pyridoxine (PN) (Sigma, St. Louis, MO, and USA). Deionized double distilled water was used to prepare all reagents and vitamers. For mobile phase A, 0.1 mol/l potassium dihydrogen phosphate (Fisher Scientific) buffer containing 0.1 mol/l sodium perchlorate (Sigma) and 0.5 g/l sodium bisulfite (Fisher Scientific), was adjusted to pH 3.0 with *o*-phosphoric acid (Fisher Scientific). Sodium bisulfite was utilized for on-line derivitization. A clean-up mobile phase (B) consisted of 30% acetonitrile/water (v/v). After experimentation, this concentration of acetonitrile allowed maximum column clean-up, required the shortest re-equilibration time, and resulted in optimal peak separation. It should be noted that lessor concentrations of acetonitrile did not completely eliminate carry over from prior injections. All reagents were HPLC grade, when available, and reagent grade otherwise. Organic solvents were of spectrophotometric grade. All mobile phases were filtered with a 0.45 μm filter, degassed with helium, and sonicated under a vacuum. The HPLC instrumentation consisted of an Alliance System (Waters Assoc., Milford, MA, USA), utilizing Millennium software for chromatogram analysis. A model 474 fluorescence detector (Waters Assoc.) equipped with a 12 μl flow cell was utilized. Wavelengths of 300 nm excitation and 400 nm emission were found to be optimal. A C18 Aquasil guard cartridge to extend column life was used, followed by an Aquasil C18, 5 μm, 150×4.6 I.D. column (Keystone Scientific, Inc., Bellefonte, PA, USA). An injection size of 50 μl provided the optimal results. The column was operated at ambient temperature, with a flow-rate of 1 ml/min. Mobile A was run for 5 min, followed by a partial clean-up with Mobile B for 10 min, and finally a equilibration phase of Mobile A for 5 min. The clean-up phase eliminated the elution of aberrant peaks in subsequent injections and the equilibration phase allowed for stabilization of the column, as noted by a return to baseline by the fluorescence detector. At the completion of a days run, Mobile B was run overnight for complete column clean-up.

3. Results

A standard PLP, and a PLP spiked plasma calibration curve (concentrations: 404, 202, 101, 50.5, 25.25, and 12.63 nmol/l) were analyzed to establish linearity and reportable ranges (r=0.9999 in both calibration curves). Regression statistics were calculated using the following equation:

$$y = 11899.69x + 12700.57$$

Standard error of the slope was 24004. All plasma concentrations were calculated utilizing data derived from an aqueous standard curve. An aqueous standard curve was utilized due to the unavailability of plasma completely void of PLP, the lacking of a clinically accepted standardized plasma PLP calibrator, as well as ease of use. Controls for PLP analysis were pooled patient plasma from our laboratory's homocysteine testing. Separate pools of plasma with homocysteine levels of >25 nmol/l (high) and <8 nmol/l (low) were collected, aliquoted, and stored at −80°C. The high homocysteine control served as a low PLP control and the low homocysteine control served as a high PLP control. Mean PLP high and low control values (66 and 40 nmol/l respectively, as determined by the described method) were statistically different with a $P<0.0001$. To

validate reproducibility, intra- and inter-assay precision testing was performed. Intra-assay precision testing consisted of ten replicate injections of standard PLP (202 nmol/l) and produced a coefficient of variation (C.V.) of 1.12%. Additional intra-assay precision testing was performed on ten replicate injections of PLP spiked plasma which produced a C.V. of 0.52%. The high and low pooled plasma controls were assayed in the same manner, producing a C.V. of 2.36% and 1.10%, respectively. The above 30 assays were run in one batch to validate the clean-up/re-equilibration time and sample to sample carry over. Inter-assay precision was performed on standard PLP (202 nmol/l) and both pooled plasma controls over a period of ten days with C.V.'s of 2.89, 5.28 and 3.95% respectively. Again, no intra-run (sample-to-sample) or inter-run (day-to-day) carry over was observed. Analytical recovery studies were performed on PLP by adding water or a known standard concentration to pooled plasma prior to protein precipitation. In this study, recoveries of 100, 50, 25, and 13 nmol/l in human plasma were 103, 102, 91 and 92%, respectively. The detection limit was observed to be 0.1 nmol/l. Adequate separation of PLP, PM and PN was observed and all vitamers were eluted by 13 min. PLP, the most clinically important (biologically active) form, was eluted first at 2.4 min and PM at 3.6 min (see Fig. 1). This retention time remained constant over a 10 h analysis. A retention time of 2.136±0.007 min was maintained for over a month's time with constant column utilization. Mobile B was initiated at 5 min since PLP was the peak of clinical interest.

Sample integrity continues to be a concern with all clinical assays, particularly regarding PLP and light exposure. In view of this, studies were performed exposing both PLP standard and human plasma to normal laboratory lighting. Standard PLP and human plasma were prepared at the time of draw and at the following subsequent times post draw, 0.5, 1, 2, 4, and 6 h. Samples were exposed to normal laboratory lighting during these time periods. Peak area was utilized to measure concentration and values were calculated based on the mean area of all freshly prepared standard PLP samples, prepared at the above defined times. Freshly prepared standard PLP values were utilized to control for preparation variation. Statistical analysis compared PLP concentrations at time zero (post draw) to 0.5, 1, 2, 4, and 6 h. Standard PLP significantly ($P<0.05$) degraded by 0.5 h, while human plasma left exposed to light did not significantly change by 6 h. The half-life of the PLP standard in our laboratory ($n=4$) occurred approximately at 4 h. Based on these observations, it is critical to protect PLP standard from light exposure during assay preparation (see Table 1).

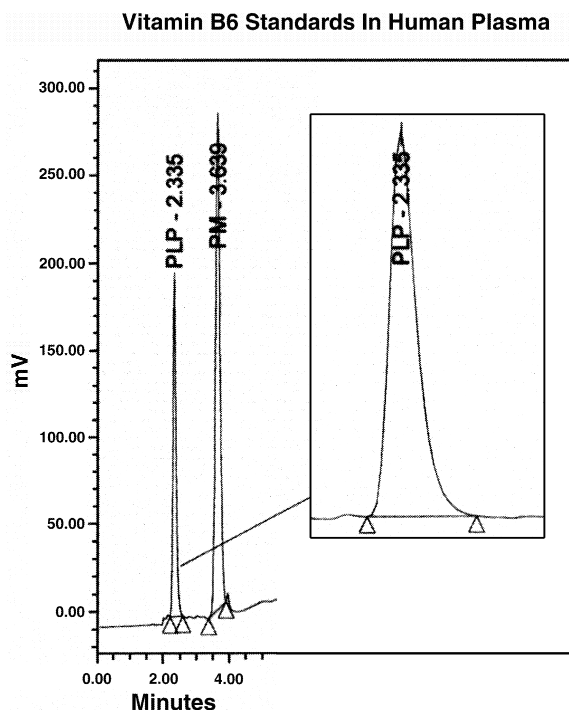

Fig. 1. Chromatogram of vitamin B6 standards in human plasma. Pyridoxal 5′ Phosphate (PLP) 200 nmol/l eluted at 2.335 min; pyridoxamine (PM) 40 nmol/l eluted at 3.639 min.

An additional extension of our work and to demonstrate its clinical application was to statistically compare PLP levels in 12 coronary artery diseased patients with normal/low (<8 nmol/l) and elevated (>25 nmol/l) homocysteine levels. Analysis indicated a tendency towards an inverse relationship between elevated homocysteine and PLP, however, statistical significance was not achieved most likely due to the low number of patients analyzed. An additional group of 12 was selected from a pool of individuals with no known coronary artery disease. The non-diseased group was sex and age matched (within 2 years) to the diseased group. Homocysteine

Table 1
PLP standard and sample concentrations response to light exposure

Standard PLP exposure time	Standard PLP concentration (nmol/l)[a]	Plasma concentration based on standard PLP time zero (nmol/l)[a]
Time Zero (freshly prepared.)	200	53
0.5 h	177*	40
1 h	161*	44
2 h	143*	45
4 h	115*	45
6 h	93*	45

[a] All concentrations were calculated using time zero prepared standard PLP area.
*Significance: ($P<0.05$).

and PLP results were obtained from the non-diseased group as a basis of comparison. When samples were controlled for age and sex, there was no statistical difference in PLP levels among diseased versus non-diseased patients with low/normal homocysteine levels. However, PLP levels were significantly different ($P<0.02$) among diseased versus non-diseased with elevated homocysteine levels (see Table 2). These data lend further evidence to the contribution of PLP in the transsulfuration pathway with regards to hyperhomocystemia and further support the thought that depletion of PLP acts as an independent risk factor for the development of coronary artery disease.

4. Discussion

Much data have recently been reported which support the significance of hyperhomocysteinemia as a risk factor for myocardial infarction, stroke, peripheral vascular disease and venous thrombosis. Vitamin B6 (PLP), in combination with folic acid and vitamin B12, appears to be an effective therapy for reducing elevated levels of homocysteine. Vitamin B6 deficient ranges have been defined as <14 nmol/l and 15–20 nmol/l defined as marginal low. Risk assessment levels of Vitamin B6 in regards to hyperhomocystemia have not been defined to date. It is possible that using past determined deficient ranges for assessing subclinical vitamin deficiencies might not accurately define PLP levels which predispose patients at risk for developing premature coronary heart disease. An efficient and practical method for PLP has been previously lacking to help guide this treatment. While this methodological adaptation requires the use of two pumps, the actual analysis of the vitamin is isocratic. In addition, we demonstrate that it is important to protect PLP from light exposure during calibrator preparation. This

Table 2
PLP levels in human plasma[a]

Sample	Sample Size (n)	Mean (nmol/l)	SD	
Non-diseased with low homocysteine	12	111	121.81	$P=0.79$
CAD with low homocysteine	12	99	86.06	
Non-diseased with high homocysteine	12	113	104.99	$P=0.02$*
CAD with high homocysteine	12	32	33.33	

[a] Non-diseased versus coronary artery diseased (CAD) with low/normal homocysteine levels, and non-diseased versus CAD with high homocysteine levels.
*Significance: ($P<0.05$).

modification of the analysis of vitamin B6, pyridoxal 5′ phosphate, described above is a simple, time-efficient, and reproducible method and is clearly sensitive enough for meaningful PLP analysis in human plasma. It should be mentioned that the time efficiency of this adaptation does omit the analysis of pyridoxamine and pyridoxine, however, this was not significant for our clinical purposes, especially since it has been recognized that PLP is the major transport form of vitamin B6 [16]. Additional chromatogram variation from Kimura's [19] work includes transposition of the pyridoxamine (PM), and pyridoxal 5′ phosphate (PLP) peaks. Internal standardization is recognized as an important validation tool, however, due to the quality of the results we felt that an internal standard was not necessary for this application. The quality and the precision of the chromatograms facilitated this decision. In addition, this methodological adaptation is robust enough to hold up to the rigors of a clinical laboratory.

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