APPLICATIONS

Fast, Accurate and Reproducible Method for Simultaneous Quantitation of Pyridoxal 5'-phosphate and Pyridoxic Acid in Human Plasma by HPLC with Fluorescent Detection

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Background

Pyridoxal 5'-phosphate (PLP) is the primary biologically active form of vitamin B6. Low levels of vitamin B6, based on plasma concentrations of PLP, have been identified in inflammatory diseases including cardiovascular disease, rheumatoid arthritis, inflammatory bowel disease, and diabetes. Plasma PLP levels were also inversely related to the risk of breast carcinoma, colon cancer, etc.

Fluorescence detection has been used nearly exclusively by a majority of reference laboratories in HPLC analysis of vitamin B6 due to its selectivity and high sensitivity. The natural florescence of the vitamin B6 compounds is low so chemical derivatization methods are necessary to improve the fluorescence signals. However, the published derivatization procedures suffer from tedious sample preparation and short HPLC column lifetimes. Our goals were to simplify the derivatization and sample preparation steps as well as to select the most efficient and durable HPLC column when analyzing PLP and pyridoxic acid (PA) by HPLC-FLD.

Materials and Methods

Sample Preparation

1. Thaw patient plasma samples and plasma/serum spiked calibrators or pre-manufactured calibration standards and controls at ambient temperature. Protect from light.
2. Pipette 200 µL of the plasma blank, calibration standards, controls and patient plasma specimens into appropriately labeled 0.6 mL amber microcentrifuge tubes.
   a. Briefly vortex the calibrators and controls immediately prior to sampling.
   b. Mix the plasma samples by gentle inversion immediately prior to sampling.
   c. Protect the tubes from light.
3. Add 30 µL of 100 mg/mL semicarbazide/glycine solution into all the tubes containing samples; cap the tubes, vortex for 15 seconds.
4. Incubate in the dark at room temperature (RT) for 30 minutes.
5. Uncap the tubes; add 25 µL of 20 % meta-phosphoric acid to the controls and patient samples.
6. Recap the tubes and vortex for 30 seconds.
7. Centrifuge for 5 minutes at 14,000 RPM at RT.

   Note: The relative centrifugal force (RCF) = 16,000 g.
8. Transfer 150 µL of supernatant to an amber autosampler glass Verex™ vial.
9. Cover the vial with a screw cap and place it in the autosampler at RT.
10. Inject 30 µL.

HPLC Conditions

An optimized Agilent® 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used with a Shimadzu RF-20A Prominence Fluorescence Detector (Shimadzu, Japan) for LC/FLD analysis.

Column: Gemini® 3 µm NX-C18
Dimensions: 100 x 4.6 mm
Part No. : 00D-4453-E0
Guard Column: SecurityGuard™ Cartridge C18, 4 x 3.0 mm
Part No. : AJ0-8368
Mobile Phase: A: 20 mM Sodium Phosphate and 1.0 mL Acetic Acid in DI water; pH ~ 6.0; no pH adjustment
B: Acetonitrile/Methanol (70:30)

Gradient:

Time (min)	% B
0.00	5
5.00	60
5.10	95
6.00	95
6.10	5
7.00	5

Standard Preparation

A 1 mM/L stock solution of PLP (26.5 mg/100 mL water), PA (18.4 mg/100 mL water), and PL (20.4 mg/100 mL water) were stored separately in amber Eppendorf tubes with 1 mL in each tube (-20 °C). The working calibration solution was prepared daily and further diluted with stripped and delipidated human plasma to obtain calibrators with 200, 100, 50, 25, 12.5, and 6.25 nmol/L for each calibrator. Calibrators were prepared in parallel with the plasma/serum samples prior to injection into the HPLC system.

Figure 1.
Sample chromatogram of semicarbazide derivatized PLP, PA and PL using a Gemini 3 µm NX-C18 HPLC column.

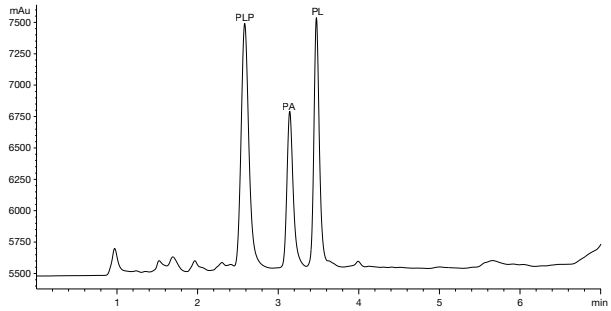

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Figure 2a.
Fluorescence wavelength selectivity studies of PLP, PA and PL by fixing the excitation wavelength at 370 nm and changing emission wavelength.

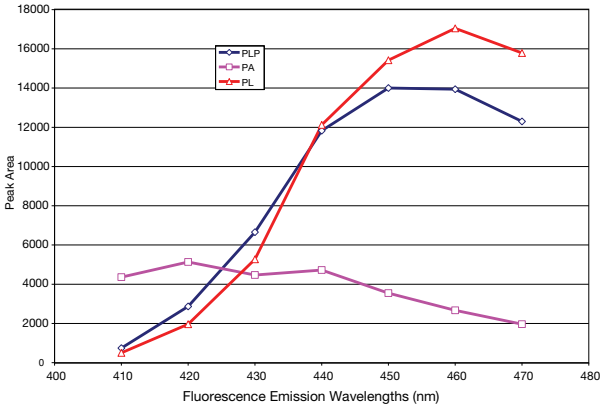

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Figure 2b.
Fluorescence wavelength selectivity studies of PLP, PA and PL by fixing the emission wavelength at 450 nm and changing excitation wavelengths

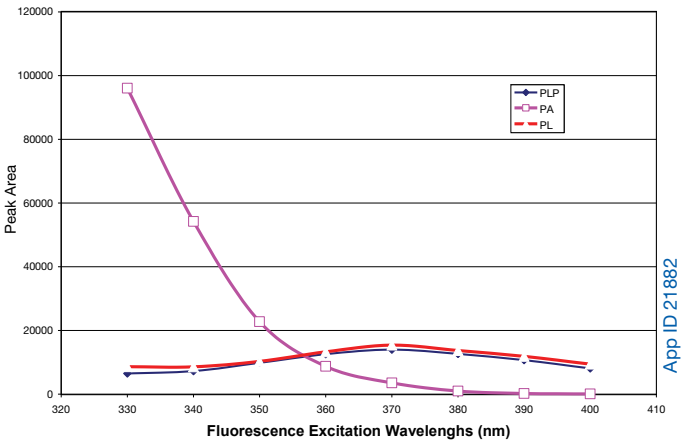


Figure 3a.
Fluorescence wavelength selectivity studies of PLP, PA and PL under different combinations of excitation and emission wavelengths.

Ex 390 nm
Em 450 nm

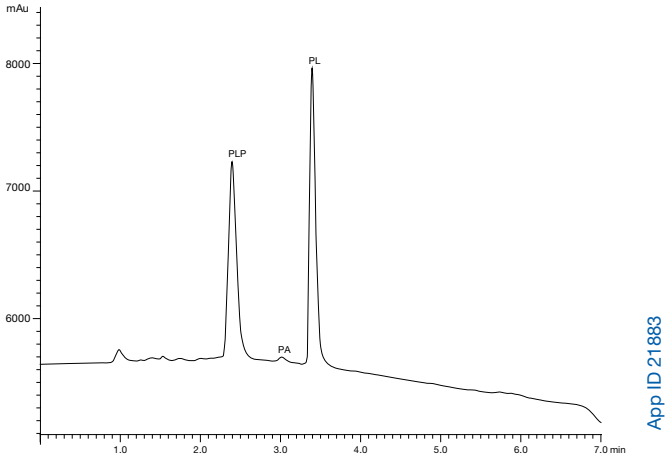


Figure 3b.
Ex 360 nm
Em 450 nm

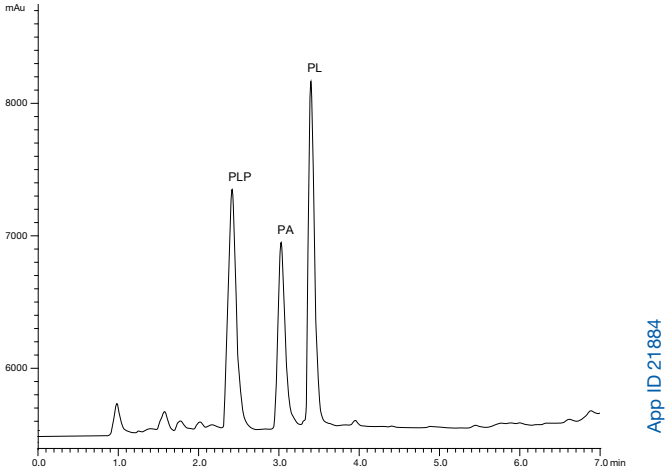


Figure 3c.
Ex 340 nm
Em 450 nm

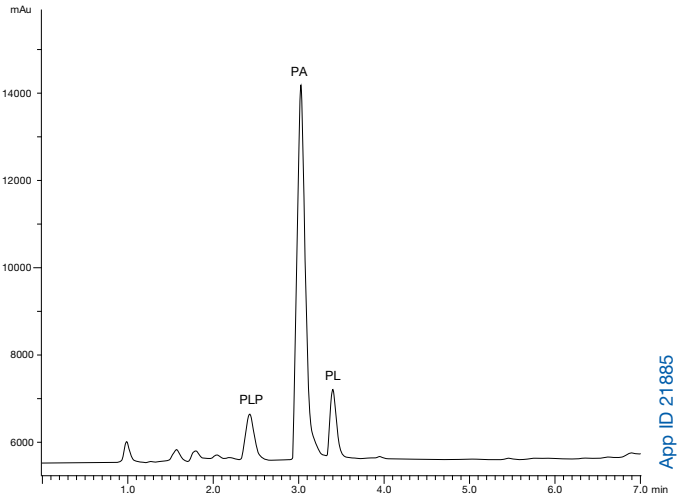


Figure 4.
Example standard curve from 6.25 nmol/L to 200 nmol/L for PLP in human serum (external standard method).

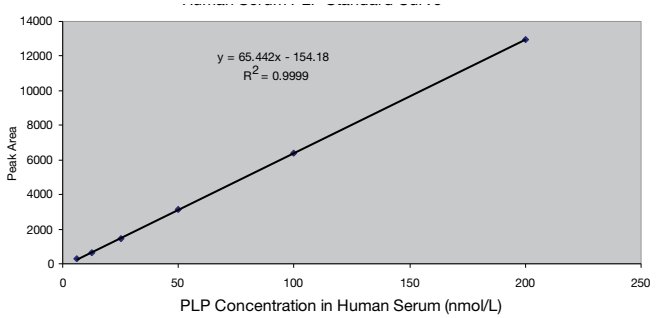
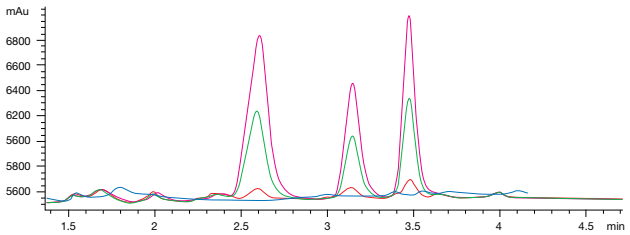


Figure 5.
Examples of HPLC chromatograms for plasma QC samples.



Red = Low level plasma QC sample (8 nmol/L of PLP, PA and PL in plasma)
Green = Medium level plasma QC sample (80 nmol/L of PLP, PA and PL in plasma)
Pink = High level plasma QC sample (160 nmol/L of PLP, PA and PL in plasma)
Blue = blank plasma control

Figure 6.
Sample precision study from 3 injections of a PLP, PA and PL spiked plasma QC Control level 3 (160 nmol/L).

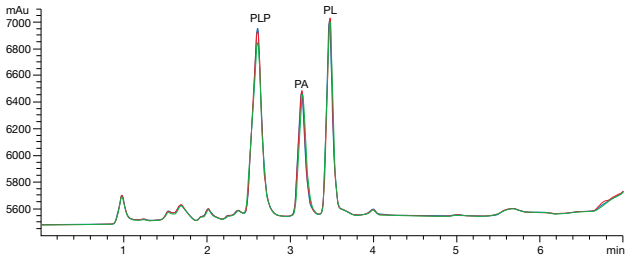


Table 1.
Statistical data of PLP, PA and PL in plasma by HPLC-FLD.

Analyte	LOD	LOQ	Intra Assay Precision (N=3)					
	nmol/L	nmol/L	8 nmol/L		80 nmol/L		160 nmol/L	
			Mean Recovery	RSD %	Mean Recovery	RSD %	Mean Recovery	RSD %
PLP	2	4	86.95	2.50	97.82	0.91	95.60	0.88
PA	2	4	94.96	4.33	95.14	3.30	95.09	2.15
PL	2	4	91.59	4.50	97.79	2.76	94.77	4.24

Results and Discussion

PLP, PA and PL Performance on Gemini® 3 µm NX-C18 HPLC Column, 100x4.6 mm.

Figure 1 is an example chromatogram of derivatized PLP, PA and PL using the Gemini 3 µm NX-C18 HPLC column. The results illustrate the good baseline-to-baseline separations for PLP, PA and PL as well as good resolution of each. Note, the PL was used as a marker for monitoring potential peak shifting. All results were generated using a fluorescence detector. The detected PLP, PA and PL were products of a semicarbazide derivatization. The selection of the optimized fluorescence wavelength was determined by the following detailed fluorescence wavelength selectivity studies which are outlined below.

Fluorescence Wavelength Selectivity Study

Although the fluorescence characteristics of different forms of vitamin B6 were thoroughly studied, the selectivity could be affected by slight changes in laboratory conditions. Following the recommendations from publications, we noticed low PA signals in some experiments. Because fluorescence signals are very sensitive and compound dependent, the accurate tuning of fluorescence wavelengths became very critical. The experiments in **Figure 2a** were designed by using a fixed excitation wavelength at 370 nm while monitoring the fluorescent signal strength by changing the fluorescence emission wavelength. The plasma sample concentrations analyzed were 200 nmol/L for PLP, PA and PL in this study. The results from **Figure 2a** clearly illustrate that the optimized fluorescence signals for PLP and PL were at Em 450 nm under our HPLC conditions. In contrast, the PA fluorescence signal remained flat when the emission wavelengths changed from Em 410 nm to Em 440 nm under the same conditions. The fluorescence signal decreased significantly for PA under the combination of Ex 370 nm and Em 450 nm.

In the experiments (**Figure 2b**) a fixed emission wavelength at Em 450 nm was used and fluorescent signal strength was monitored by changing the fluorescence excitation wavelength. The results from **Figure 2b** clearly illustrate that the optimized fluorescence signals for PLP and PL are Ex 370 nm under our HPLC conditions. In contrast, the PA fluorescence signal was relatively weak under Ex 370 nm and Em 450 nm. The fluorescence signal for PA started to increase between Ex 370 nm and Ex 360 nm and significantly increased between excitation wavelengths of Ex 350 nm and Ex 330 nm. However, the combination of Ex 370 nm and Em 450 nm displayed the highest signals for both PLP and PL.

Figure 3a-c shows selected chromatograms from the above mentioned fluorescence selectivity studies. **Figure 3a** shows that the fluorescence signal of PA could be "turned-off" at Ex 390 nm and Em 450 nm. **Figure 3b** shows that PLP and PL display high fluorescent signals however PA does not emit as strong of a signal. Because the goal of our work was to study PLP and PA, a combination of Ex 340 and Em 450 (**Figure 3c**) was chosen as our ideal conditions because we were able to achieve a much higher fluorescence signal for PA without losing a significant amount of signal for PLP as compared to Ex 360 and Em 450 (seen in **Figure 3b**).

The experiment demonstrated that carefully studying and applying fluorescence wavelength knowledge was critical to maximizing the PLP signal as well as to be able to detect the critical clinically relevant analyte, PA.

Analytical Performance

The linear regression analysis data for a six point calibration plot shows a good linear relationship over the concentration range of 6.25-200 nmoL/L for both PLP (R^2=0.9999; **Figure 4**) and PA (R^2=0.9993; not shown). Three concentration levels of plasma quality control (QC) samples were prepared at 8, 80 and 160 nmol/L (**Figure 5**). The percentage of coefficients of variation (CV%) for the intra-assay precision were 0.9 % to 2.5 % for PLP, and 2.15 % to 4.33 % for PA (**Table 1**). The mean recoveries were 86.9 % to 97.8 % for PLP, and 95.0 % to 95.1 % for PA (**Table 1**). The limit of detection (LOD) and limit of quantitation (LOQ) was 2 nmoL/L and 4 nmoL/L for both PLP and PA, respectively (**Table 1**). PL showed similar results; however, it was used as a marker for monitoring potential peak shifting. The performance of the Gemini 3 µm NX-C18 HPLC column was reproducible (**Figure 6**). The column was durable and column life was extended significantly by changing guard cartridges after every 200 injections.

Conclusion

A simple HPLC method for simultaneous determination of plasma PLP and PA was developed and validated using direct fluorescent detection. Detailed fluorescent wavelength selectivity studies showed that it is necessary to optimize the excitation and emission wavelengths to accommodate all analytes. The optimized fluorescence wavelengths were the combination of Ex 340 nm and Em 450 nm for accurate quantitation of PLP and PA in human plasma. The use of the Gemini 3 µm NX-C18 column allowed for clear baseline-to-baseline separation of PLP, PA and PL against other peaks from plasma. The chromatographic retention is based primarily on the PLP and PL interaction with the silica particles. Conventional analytical columns with a 4.6 mm ID and 100 mm length produced the best performance. The use of guard cartridges will extend the column lifetime and performance. The new improved sample preparation procedure with the meta-phosphoric acid reduces the number of steps in the sample preparation procedure and does not require a pH adjustment. The Gemini column has wide pH range tolerance which is ideally suited for the new sample preparation procedures. The sample volume used was 200 µL for routine clinical practice. The analyses can be performed with 100 µL plasma or serum which is very critical to pediatric patients. The fast HPLC method, with a total run time of 7 minutes, allows for high-throughput sample preparation and automation.

The method is selective, sensitive, precise and accurate. This new sample preparation procedure combined with a separation using the Gemini 3 µm NX-C18 column has been tested by a large clinical reference laboratory with large number of different patient samples which has demonstrated its effectiveness.

APPLICATIONS

Ordering Information

Gemini® HPLC Columns

3 µm Microbore, Minibore and Narrow Bore Columns (mm)

Phases	20 x 2.0	30 x 2.0	50 x 2.0	100 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard™ Cartridges (mm) 4 x 2.0*
									/10pk
NX-C18	00M-4453-B0	00A-4453-B0	00B-4453-B0	00D-4453-B0	00F-4453-B0	00B-4453-Y0	00D-4453-Y0	00F-4453-Y0	AJ0-8367

for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4453-E0	00D-4453-E0	00F-4453-E0	00G-4453-E0	AJ0-8368

for ID: 3.2-8.0 mm

5 µm Minibore and Narrow Bore Columns (mm)

Phases	30 x 2.0	50 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	250 x 3.0	SecurityGuard Cartridges (mm) 4 x 2.0*
								/10pk
NX-C18	00A-4454-B0	00B-4454-B0	00F-4454-B0	00B-4454-Y0	00D-4454-Y0	00F-4454-Y0	00G-4454-Y0	AJ0-8367

for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4454-E0	00D-4454-E0	00F-4454-E0	00G-4454-E0	AJ0-8368

for ID: 3.2-8.0 mm

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