APPLICATIONS

A Simple and Effective Method for HPLC Quantification of Simultaneous Vitamin B1 (Thiamine Diphosphate) and Vitamin B6 (Pyridoxal 5-Phosphate) from Whole Blood

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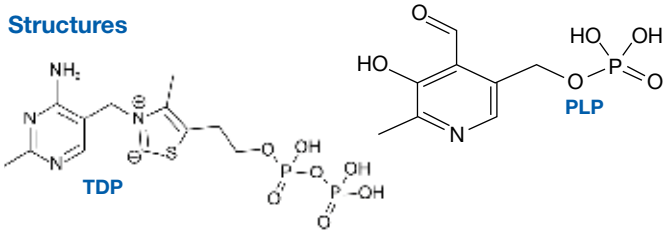
Abstract
The purpose of this experiment was to develop a simple and robust method for the simultaneous analysis of biologically active Vitamin B1 (TDP) and Vitamin B6 (PLP) from whole blood.

Introduction
Water-soluble B Vitamins are important cofactors in cell metabolism. Two water-soluble vitamins with clinical relevance are Vitamins B1 and B6. Thiamine Diphosphate (TDP) is the biologically active form of Vitamin B1 and is required for various metabolic functions. Prolonged deficiency can cause beriberi, a debilitating neurological disease[1]. Pyridoxal 5-phosphate (PLP) is the biologically active form of Vitamin B6 and is a coenzyme for a number of transamination reactions. It plays critical roles in chronic disease and pro-inflammatory response[2]. Additionally, both Vitamin B6 and B12 have also been linked to increased survival rates in the elderly[3].

Both TDP and PLP present unique challenges for reversed phase HPLC analysis. TDP quantitation from whole blood typically involves precipitation, followed by pre-column derivatization prior to HPLC analysis[4]. In plasma, PLP is the predominant form of Vitamin B6. The plasma PLP concentration is considered as an indicator of Vitamin B6 status and is reported to be well correlated with tissue PLP concentrations. However, vitamin concentrations in blood cells tend to be a better marker of cellular stores.

In this study, we present a method for direct quantitation of PLP and TDP from whole blood. This method is novel since it analyzes both TDP and PLP, and performs the derivatization steps prior to protein precipitation with perchloric acid. This streamlined protocol is an improvement from previously reported methods, which involve longer extraction times and more solvent.

Structures



Materials and Methods
Reagents and Chemicals

TDP and PLP standards were obtained from Sigma-Aldrich Corporation. (St. Louis, MO).

Reagent Preparation Procedure
1. 50 mg/mL alkaline potassium ferricyanide was prepared fresh daily by adding 100 mg of $K_3Fe(CN)_6$ to 2 mL of 15 % NaOH.
2. 25 % NaOH was prepared by adding 10 mL of 50 % NaOH to 10 mL DI Water.
3. 25 mM Na_2HPO_4 (pH 7) was prepared by adding 6.7 g of $Na_2HPO_4.7H_2O$ to 1 L of DI water. pH was adjusted with 85 % H_3PO_4.
4. 250/250 mg/mL semicarbazide/glycine was prepared by adding 500 mg of semicarbazide and 500 mg of glycine to 2 mL of DI water.

Sample Preparation
All extraction steps were performed while protecting the sample from light and under cold conditions, such as on ice. Human whole blood samples were frozen immediately (-70 °C or > 24 hr at -20 °C) after collection. It is important that the samples be frozen prior to analysis in order to lyse the cells, thus releasing the TDP to be further analyzed. Standards and quality control samples were analyzed to determine the accuracy, precision, and linearity of the method.

250 µL of thawed, hemolysated blood samples were added to 250 µL of deionized water and vortexed for 3-5 seconds. Then, Vitamin B1 derivatization was performed by adding 30 µL of 50 mg/mL alkaline potassium ferricyanide to each sample and vortexing for one minute. 40 µL of 250/250 mg/mL semicarbazide/glycine was added to the derivatized Vitamin B6 samples, again vortexing for one minute. Samples were incubated at 2-8 °C for 30 minutes.

Protein precipitation was performed by adding 40 µL of 70 % perchloric acid to each sample, then mixing vigorously. Samples were centrifuged at 12,000-14,000 rpm for 10 minutes. To neutralize samples, 10 µL of 25 % NaOH was added to 200 µL of supernatant. 20-25 µL of sample was injected onto the LC system.

HPLC Conditions
HPLC was performed using a Luna® C18(2) 5 µm 50 x 3.0 mm HPLC column (p/n 00B-4252-Y0) on a Shimadzu Prominence® LC-20 AD system (Shimadzu Corporation, Kyoto, Japan) with an upper pressure limit of 400 bar, equipped with a Shimadzu RF-20A fluorescence detector. Running conditions and detector settings are indicated below.

Column:	Luna 5 µm C18(2)
Dimensions:	50 x 3.0 mm
Part No.:	00B-4252-Y0
Mobile Phase:	A: 25 mM Na_2HPO_4, pH 7 B: Methanol
Gradient:	**Time (min) B (%)** 0.01 5 1.80 15 3.50 50 4.50 50 4.51 5 6.50 5
Flow Rate:	600 µL/min
Injection Volume:	20-25 µL
Temperature:	28 °C
Detector:	Fluorescence detector Excitation: 380 nm, Emission: 450 nm, after 2.2 min switch to Excitation: 375 nm, Emission: 435 nm

Results and Discussion

All standards and QCs were prepared in whole blood. All QC samples had an accuracy and precision within 91.25-105 % and 0.663-5.39 %, respectively. The assay was linear from 20-250 ng/mL for both TDP and PLP. The Lower Limit of Quantitations (LLOQ's) were also determined to be 20 ng/mL for PLP and 50 nmol/L for TDP.

Table 1. Accuracy and precision for QC samples of PLP and TDP

	PLP	TDP
	QC 2 (200 ng/mL)	
CV (%)	2.34	5.39
Accuracy (%)	93.6	91.25
	QC 1 (120 ng/mL)	
CV (%)	2.27	0.663
Accuracy (%)	105	99.7

Figure 1. Representative calibration curve of PLP from whole blood

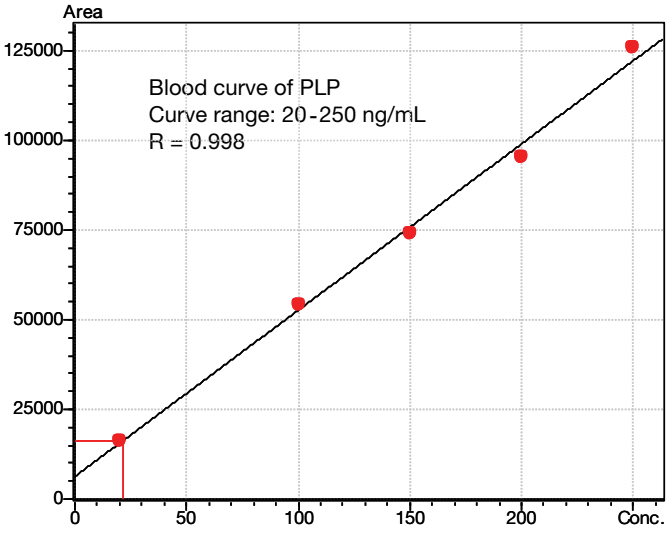


Figure 2. PLP and TDP standards at 250 ng/mL in water vs. human whole blood

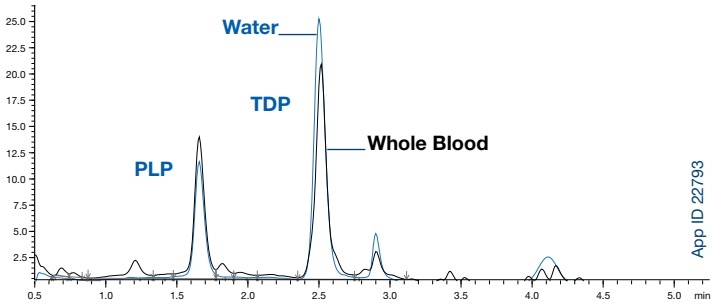


Figure 3. Lower limit of quantitation of TDP and PLP (20 ng/mL) in human whole blood

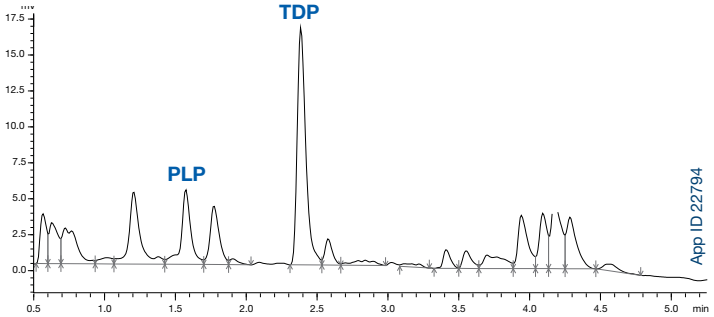


Figure 4. Upper limit of quantitation of TDP and PLP (250 ng/mL) in human whole blood

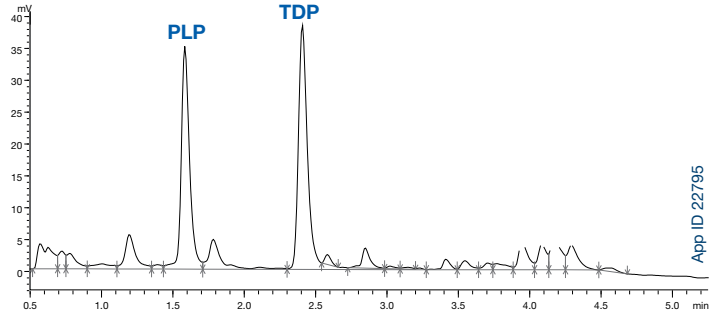


Figure 5. Endogenous levels of TDP and PLP in human whole blood

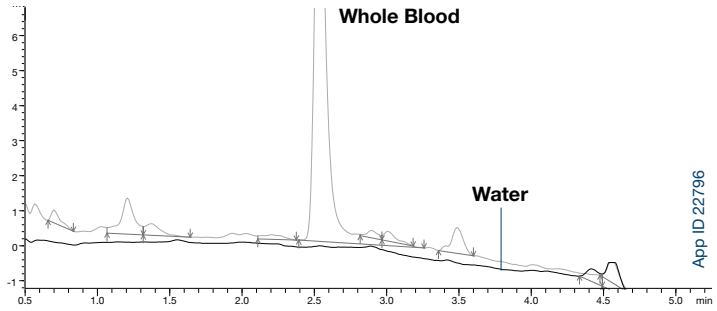


Figure 6. System ruggedness test

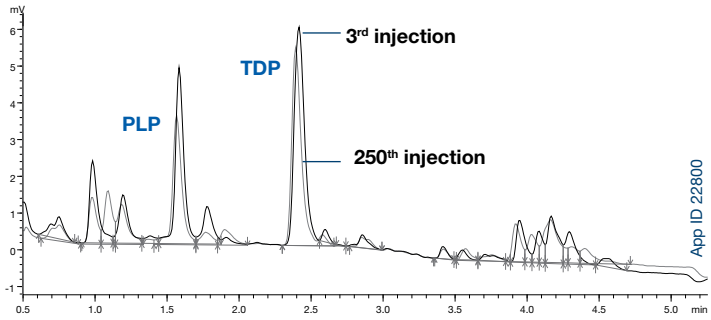


Figure 1 shows a representative calibration curve of PLP in human whole blood with R=0.998. **Figure 2** shows good chromatographic separation of TDP and PLP derivatives. Although not shown, the method could be used for other forms of thiamine (e.g. TMP) and pyroxidine (e.g. PM, PMP, PL). **Figures 3** and **4** show chromatograms for PLP and TDP at LLOQ and ULOQ levels, respectively. **Figure 5** shows endogenous levels of TDP and PLP in human whole blood.

Figure 6 shows a ruggedness test indicating that after 250 injections, no peak shifting occurs indicating that the assay is not only simple but is also robust.

Conclusion

Because Vitamin B1 and B6 are unstable under normal conditions, it is preferred for labs to perform extractions from whole blood for direct quantitation. However, a combined extraction of both vitamins from whole blood is very challenging. In this method, we have optimized a derivatization and extraction protocol that addresses the challenges of working with water-soluble vitamins from whole blood.

The final optimized assay is simple, fast, and easy to perform. Both levels of TDP and PLP are within clinically relevant levels, and the linearity, accuracy, and precision data presented provide guidance for the method validation. Furthermore, the assay could be fully automated for high-throughput applications.

References

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2. Huang, et al. “Vitamin B6 Supplementation Improves Pro-inflammatory Responses in Patients with Rheumatoid Arthritis.” European Journal of Clinical Nutrition (2010): 1007-013
3. Huang, et al. “Prediction of All-cause Mortality by B Group Vitamin Status in the Elderly.” Clinical Nutrition (2011): 191-98.
4. Delacoux, E.. “Comparison of erythrocyte transketolase activity with thiamine and thiamine phosphate ester levels in chronic alcoholic patients.” Clinica Chimica Acta: 91-100
5. Edwards, P.: A Simple Liquid-Chromatographic Method for Measuring Vitamin B6 Compounds in Plasma, Clinical Chemistry 35/2, 1989: 241-245
6. Talwar, D. “Pyridoxal Phosphate Decreases in Plasma but Not Erythrocytes during Systemic Inflammatory Response.” Clinical Chemistry (2003): 515-18w

APPLICATIONS

Luna® HPLC Column Ordering Information

3 µm Microbore and Minibore Columns (mm)

Phases	50 x 1.0	150 x 1.0	30 x 2.0	50 x 2.0	100 x 2.0	150 x 2.0	SecurityGuard™ Cartridges (mm) 4 x 2.0*
C18(2)	00B-4251-A0	00F-4251-A0	00A-4251-B0	00B-4251-B0	00D-4251-B0	00F-4251-B0	AJ0-4286 for ID: 2.0-3.0 mm

3 µm MidBore™ and Analytical Columns (mm)

Phases	30 x 3.0	50 x 3.0	150 x 3.0	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard Cartridges (mm) 4 x 2.0*	4 x 3.0*
C18(2)	00A-4251-Y0	00B-4251-Y0	00F-4251-Y0	00A-4251-E0	00B-4251-E0	00C-4251-E0	00D-4251-E0	00F-4251-E0	AJ0-4286 for ID: 2.0-3.0 mm	AJ0-4287 3.2-8.0 mm

5 µm Microbore and Minibore Columns (mm)

Phases	50 x 1.0	150 x 1.0	250 x 1.0	30 x 2.0	50 x 2.0	150 x 2.0	250 x 2.0	SecurityGuard Cartridges (mm) 4 x 2.0*
C18(2)	00B-4252-A0	00F-4252-A0	00G-4252-A0	00A-4252-B0	00B-4252-B0	00F-4252-B0	00G-4252-B0	AJ0-4286 for ID: 2.0-3.0 mm

5 µm MidBore™ and Analytical Columns (mm)

Phases	30 x 3.0	50 x 3.0	150 x 3.0	250 x 3.0	30 x 4.6	50 x 4.6	75 x 4.6	SecurityGuard Cartridges (mm) 4 x 2.0*	4 x 3.0*
C18(2)	00A-4252-Y0	00B-4252-Y0	00F-4252-Y0	00G-4252-Y0	00A-4252-E0	00B-4252-E0	00C-4252-E0	AJ0-4286 for ID: 2.0-3.0 mm	AJ0-4287 3.2-8.0 mm

* SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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