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A Simple and Effective Method for HPLC Quantification of Thiamin Diphosphate (Vitamin B1) from Whole Blood using a Luna C18(2) HPLC Column and Fluorescence Detection

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The purpose of this experiment was to develop a simple and robust HPLC method for the extraction of Vitamin B1 (TDP) from whole blood.

Introduction

Thiamin (Thiamine), or Vitamin B1, is an essential nutrient. The biologically active diphosphate form of thiamin, thiamin diphosphate (TDP), is required for various functions, including branched-chain amino acid and carbohydrate metabolism, as well as neurotransmitter and myelin production[1]. A prolonged deficiency can cause beriberi, a debilitating disease which can affect either the peripheral nervous system or circulatory system[2]. Standard methods for thiamin analysis include indirect measurement of erythrocyte transketolase activity. However, the majority of thiamin in serum is bound to proteins, mainly albumin. Approximately 90% of total thiamin in blood is in erythrocytes. Direct measurement of TDP in whole blood or erythrocytes by HPLC with fluorometric detection is the preferred route[3].

In this study, we report a method for concentration determination of TDP from whole blood. Samples were precipitated with TCA, followed by a derivatization using alkaline potassium ferricyanide. The limit of quantification for TDP was established at 50 nmol/L, which is below clinically relevant deficiency levels of <70 nmol/L[4].

Materials and Methods

Reagents and Chemicals

Thiamin, Thiamin Diphosphate (TDP) and Thiamin Monophosphate (TMP) standards were obtained from Sigma-Aldrich Corporation, (St. Louis, MO).

Reagent Preparation Procedure

1. 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.
2. 0.04 % Potassium ferricyanide in 15 % NaOH was prepared fresh daily by adding 6 mg of potassium ferricyanide to 15 mL of 15 % NaOH.
3. 10 % Trichloroacetic acid (TCA) was prepared by adding 10 g of TCA to 100 mL of DI water.
4. 10 % H_3PO_4 was prepared by adding 2.35 mL of 85 % H_3PO_4 to 17.65 mL of DI water.
5. Water saturated methyl tert-butyl ether (MTBE) was prepared by adding 100 mL of DI water to 100 mL MTBE.

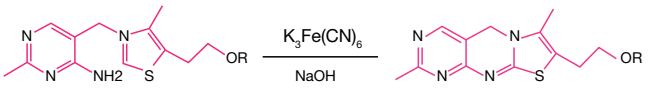
Experimental Conditions

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 > 24hr at -20°C) after the 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. 250 µL of thawed blood samples, water blank, calibrators and quality control standards (50, 320, and 400 nmol/L standards prepared in water), were aliquoted into 1.8 – 2 mL microcentrifuge tubes.

Protein precipitation was performed by adding 250 µL of 10 % TCA to all samples and vortexing for 15 seconds. Each sample was left on ice or at 2-8 °C for 15 minutes, protected from the light. A liquid-liquid extraction was performed by centrifuging tubes at 14,000 rpm for 10 minutes at 5 °C, then the supernatant was transferred into tubes (100 x 16 mm) and washed twice with 750 µL of water saturated methyl tert-butyl ether (MTBE) to remove TCA. Tubes were centrifuged at 3,500 rpm for 5 minutes and the top layer was discarded. The 80 µL of extract was transferred to an autosampler vial. 20 µL MeOH was added to the extract followed by 50 µL of 0.04 % potassium ferricyanide in 15 % sodium hydroxide. The sample was mixed and was then placed on ice or in the refrigerator (4-8 °C) for 20-30 min to complete the derivatization. To prevent the conversion of TDP to Thiamin, extracts were neutralized with 50 µL 10 % phosphoric acid prior to HPLC analysis. The addition of 10 % phosphoric acid also neutralized the pH of the solution which helped to extend the HPLC column lifetime.

Scheme of the Derivatization Reaction:



R=H	Thiamin	Thiochrome
R=PO_3^{2-}	ThMP	ThcMP
R=$P_2O_6^{3-}$	ThDP	ThcDP
R=$P_3O_9^{4-}$	ThTP	ThcTP
R=$P_3O_8^{3-}$ -adenosine	AThTP	AThcTP

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Experimental Conditions cont.

HPLC Conditions

HPLC analysis 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 in HPLC Conditions.

HPLC Conditions:

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 in water, pH 7.0
B: Methanol

Gradient:

Time (min)	B (%)
0.01	5
1.8	15
3.5	50
4.5	50
4.51	5
6.5	5

Flow Rate: 600 µL/min
Temperature: 28 °C
Pressure 60 bar
Injection Volume: 10 - 30 µL
Detection: Fluorescence detector
Excitation: 375 nm and Emission: 435 nm

Figure 1.
Endogenous levels of TDP in human whole blood

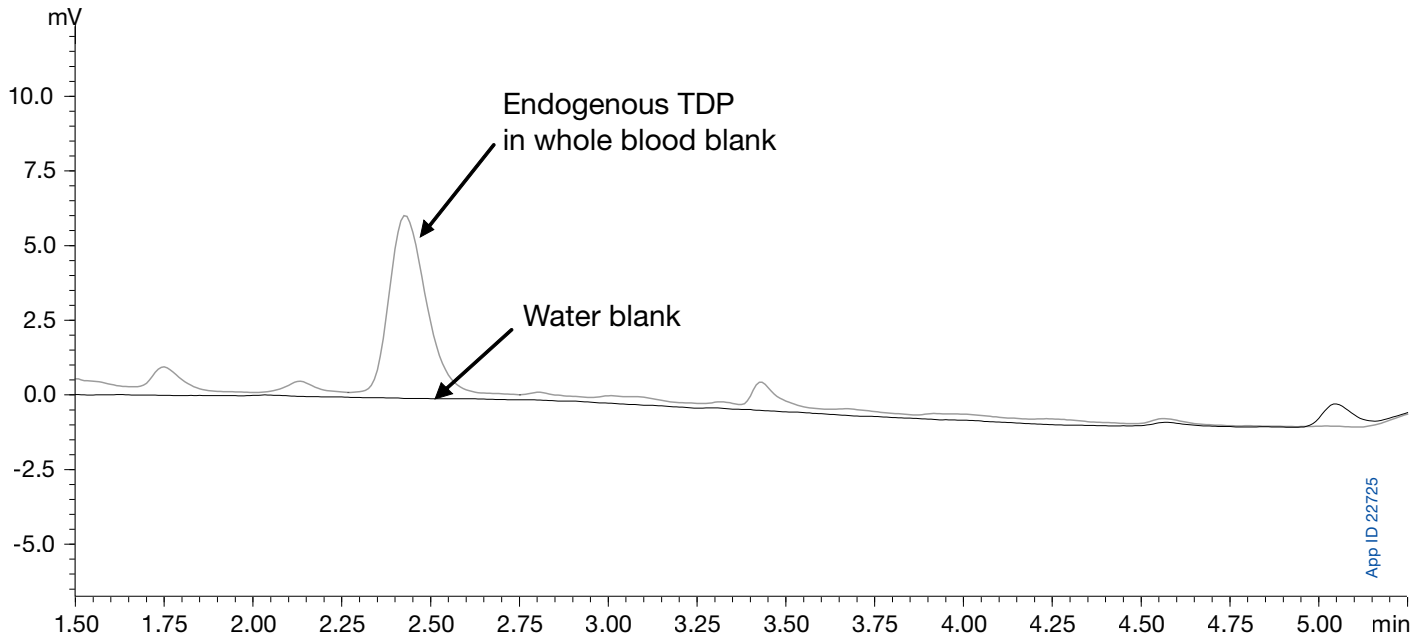

Figure 2.
Chromatogram of TDP, Thiamin, and TMP

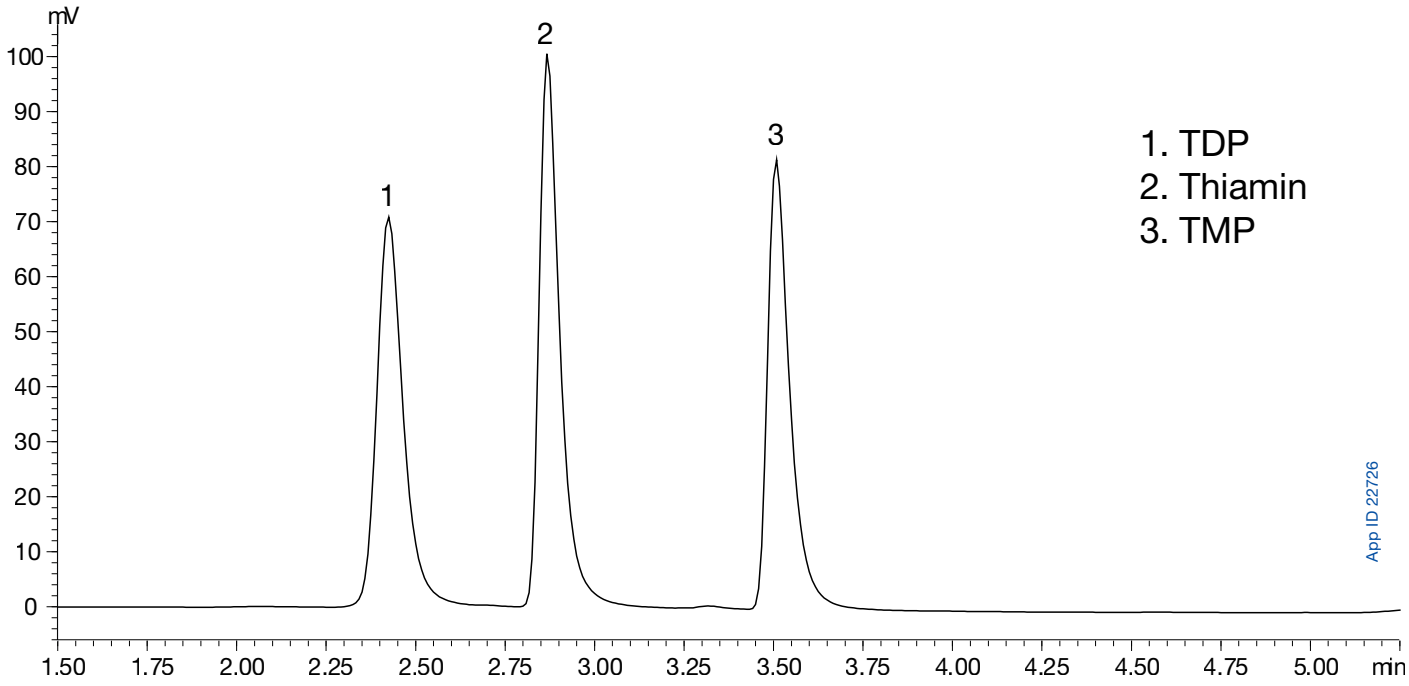

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Figure 3.
Chromatograms of TDP in Water and Blood Sample to Determine LLOQ (50 nmol/L)

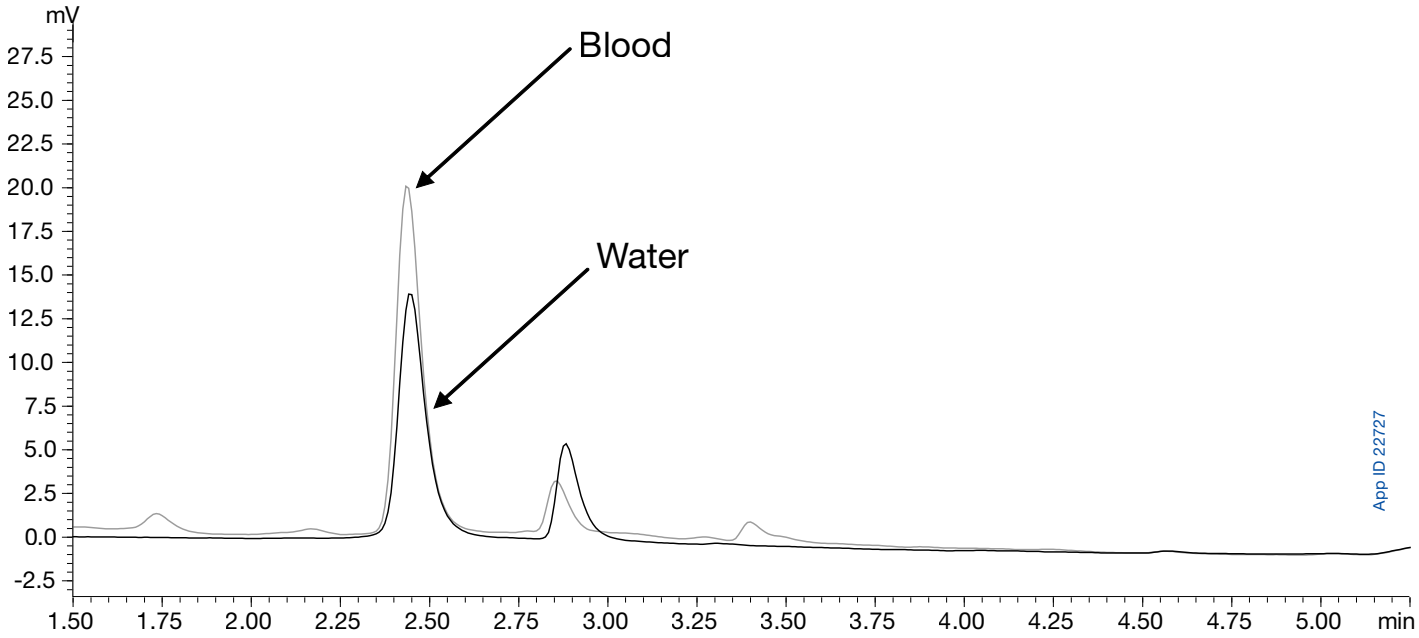
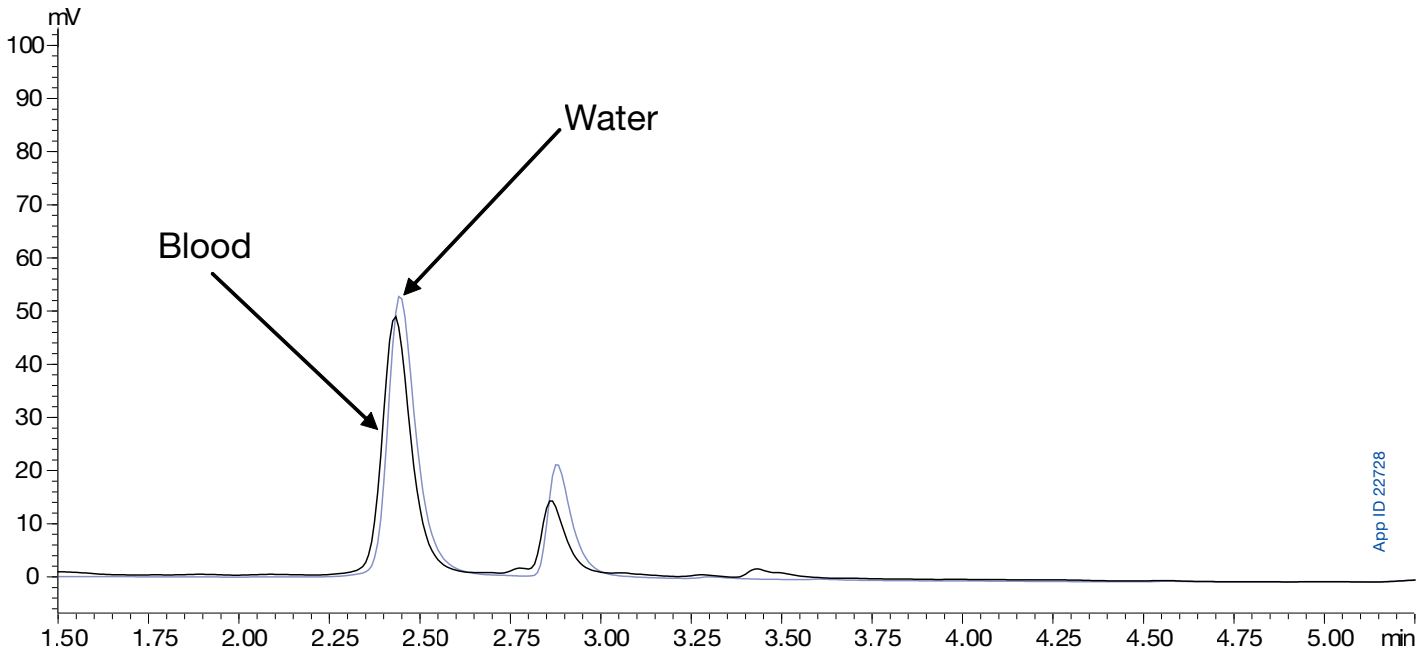


Figure 4.
Chromatograms of TDP in Water and Blood Sample to determine ULOQ (400 nmol/L)



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Results and Discussion

Figure 1 shows endogenous levels of TDP extracted from blank human whole blood when compared to a water blank.

Figure 2 shows good separation of the three major forms of Vitamin B1 (TDP, Thiamin, and TMP) using our proposed HPLC method. Although this study was for TDP quantitation in whole blood samples only, other matrices like plasma, urine, or breast milk, can be run using this HPLC method to analyze all major forms of Vitamin B1 however sample preparation procedures may differ.

Figures 3 and **4** show TDP extracted from human whole blood compared to a spiked water standard at 50 nmol/L and 400 nmol/L (respectively).

Conclusions

The method presented in this study could be used for the determination of clinically relevant levels of TDP in whole blood. Because it is specific for all three forms of Vitamin B1 (TMP, TDP, and Thiamin), the method could also be used for other biological matrices as well.

The study showed that our method resulted in reproducible chromatography after more than 500 injections (without an analytical guard column), however, using an analytical guard column such as SecurityGuard™ will further extend the lifetime of the Luna® C18(2) HPLC column.

References

[1] Serra, A. Wernicke's Encephalopathy: New Clinical Settings And Recent Advances In Diagnosis And Management. *The Lancet Neurology*, 442-455.

[2] Stanley, N. N.. "Cardiac Beriberi: Two Modes of Presentation." BMJ: 567-569.

[3] Delacoux, E.. "Comparison of erythrocyte transketolase activity with thiamine and thiamine phosphate ester levels in chronic alcoholic patients." Clinica Chimica Acta: 91-100

[4] Stuetz W, Carrara VI, McGready R, Lee SJ, Biesalski HK, et al. **(2012)** Thiamine Diphosphate in Whole Blood, Thiamine and Thiamine Monophosphate in Breast-Milk in a Refugee Population. PLoS ONE 7(6): e36280. doi:10.1371/journal.pone.0036280

Luna® HPLC Column Ordering Information

3 µm Microbore and Minibore Columns (mm)							SecurityGuard ™ Cartridges (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	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 Narrow Bore and Analytical Columns (mm)									SecurityGuard Cartridges (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	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	AJ0-4287
									for ID: 2.0-3.0 mm	3.2-8.0 mm

5 µm Microbore and Minibore Columns (mm)								SecurityGuard Cartridges (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	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 Narrow Bore and Analytical Columns (mm)								SecurityGuard Cartridges (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	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	AJ0-4287
								for ID: 2.0-3.0 mm	3.2-8.0 mm

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

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