APPLICATIONS

A Simple and Effective High pH LC/MS/MS Method for Determination of Underivatized Vitamin B1 and B6 in Human Whole Blood

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Introduction

Vitamins B1 and B6 are two water-soluble vitamins with clinical research interest. Thiamine Diphosphate (TDP) is the main biologically active form of Vitamin B1 and is required for various metabolic functions. Pyridoxal-5-phosphate (PLP) is the main biologically active form of Vitamin B6 and is a coenzyme for a number of transamination reactions.[1,2] Chromatographically, these are very challenging analytes for a reversed phase separation as they have negative values for the octanol/water partition coefficients. The structure of TDP and PLP are shown in **Figure 1**. The most common method for TDP and PLP analysis is a HPLC based assay with a pre-column derivatization with alkaline potassium ferricyanide and semicabazide, followed by fluorescence detection. This procedure is time and labor intensive and uses toxic reagent. Another approach uses ion pairing reagents, however these are not alternatives that are compatible with mass spectrometry. Presented here is a simple method that does not involve derivatization or ion pairing reagents. The method involves whole blood extraction at lower pH, cost effective internal standards, no derivatization and a reversed phase LC/MS/MS compatible method for the analysis of polar TDP and PLP. The assay is evaluated for precision, accuracy, linear range, and the results meet acceptance criteria.

Experimental Conditions

Optimized Sample Extraction Method

Human whole blood samples were frozen immediately at -20 °C after collection. It is important to freeze the sample for at least 24 hours prior to analysis in order to prevent the analyte from decomposition, especially TDP.

1. *Pipette 100 µL of thawed hemolyzed blood into a 1.8 mL centrifuge tube*
2. *Add 300 µL of working internal standard (20 ng/mL of Pyridoxine-D_2 and 50 ng/mL of Thiamine-$^{13}C_4$ in DI water) and mix for 30 seconds*
3. *Add 30 µL of 70 % $HClO_4$ and mix for 1 minute to precipitate proteins*
4. *Centrifuge sample at 14,000 rpms for 10 minutes to pellet the protein*
5. *Transfer 200 µL of supernatant into an autosampler vial for LC MS/MS analysis*

Note: Since the analytes are light sensitive, the extraction steps were performed in amber color centrifuge tube and were protected from light.

LC/MS/MS Method Parameters

Column:	Gemini® 5 µm C18
Dimensions:	50 x 4.6 mm
Part No.:	00B-4435-E0
SecurityGuard Cartridge:	AJ0-7597
Mobile Phase:	A: 10 mM NH_4HCO_3 in water, pH8.8 B: Methanol
Gradient:	**Time (min) B (%)** 0.01 0 1.5 0 5 60 6.5 60 6.51 0 9 0
Flow Rate:	600 µL/min
Injection Volume	10 µL
Instrument:	Agilent® 1260 LC
Detection:	MS/MS (ESI+) (SCIEX API 4500™)
Sample:	1. Pyridoxal 5-phosphate (PLP) 2. Thiamine Diphosphate (TDP) 3. Pyridoxine D_2 4. Thiamine-$^{13}C_4$

Figure 1. Structure of TDP and PLP

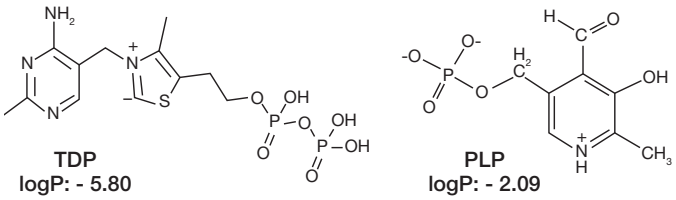

Table 1. MRM Transitions

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP	CE
PLP 1	248.1	149.8	125	75	15
PLP 2	248.1	94.1	125	75	25
TDP 1	425.1	122.1	25	60	15
TDP 2	425.1	81.0	25	60	52
TDP 3	425.1	303.9	25	70	20
Thiamine-13C4 1	270.1	122.9	75	40	15
Thiamine-13C4 2	270.1	148.1	75	40	15
Pyridoxine D2 1	172.1	155.0	75	40	15
Pyridoxine D2 2	172.1	136.0	75	40	15

Revision: 0
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Table 2. Accuracy and Precision

	Run 1		Run 2	
	TDP	PLP	TDP	PLP
Nominal Conc.	100 ng/mL			
Sample ID	Conc. Found (ng/mL)		Conc. Found (ng/mL)	
QC1_1	103	88.5	118	95.8
QC1_2	80.5	103	89.1	74.5
QC1_3	94.8	98.5	105	88.3
QC1_4	87.2	110	102	106
QC1_5	85.5	102	86.7	88
QC1_6	93.6)	73.6	110	85.8
Nominal Conc.	200 ng/mL			
QC2_1	222	170	215	176
QC2_2	226	201	217	190
QC2_3	218	196	259	260
QC2_4	211	215	192	209
QC2_5	216	202	228	205
QC2_6	255	228	227	160
Mean Conc. Found (ng/mL)	96.3	92.8	224	201
STDV	11.3	11.7	18.2	26.8
CV%	11.8	12.6	8.13	13.3
Accuracy (%)	96.3	92.8	112	101

Figure 2. Representative Chromatogram in Whole Blood at LLOQ (20 ng/mL)

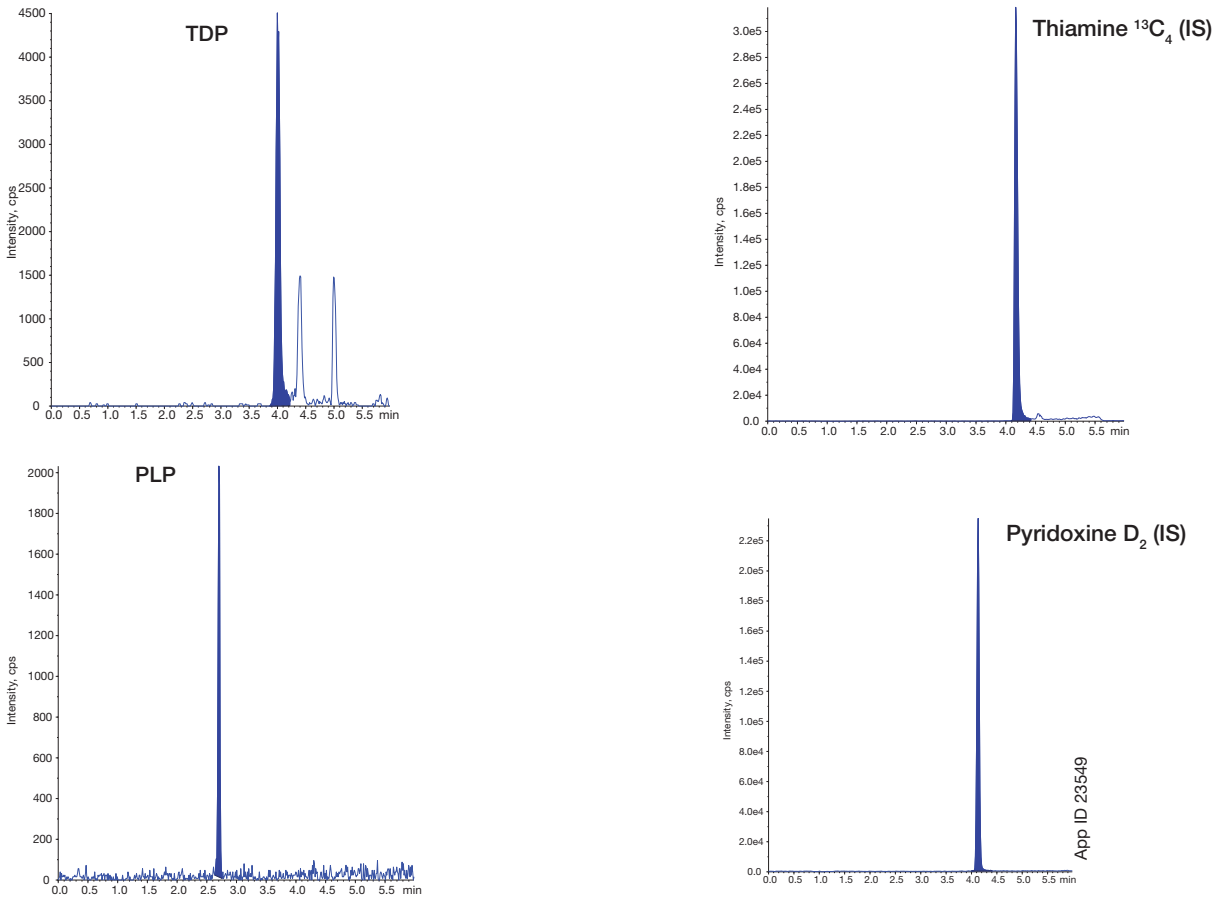


Figure 3. Representative Chromatogram in Whole Blood at ULOQ (250 ng/mL)

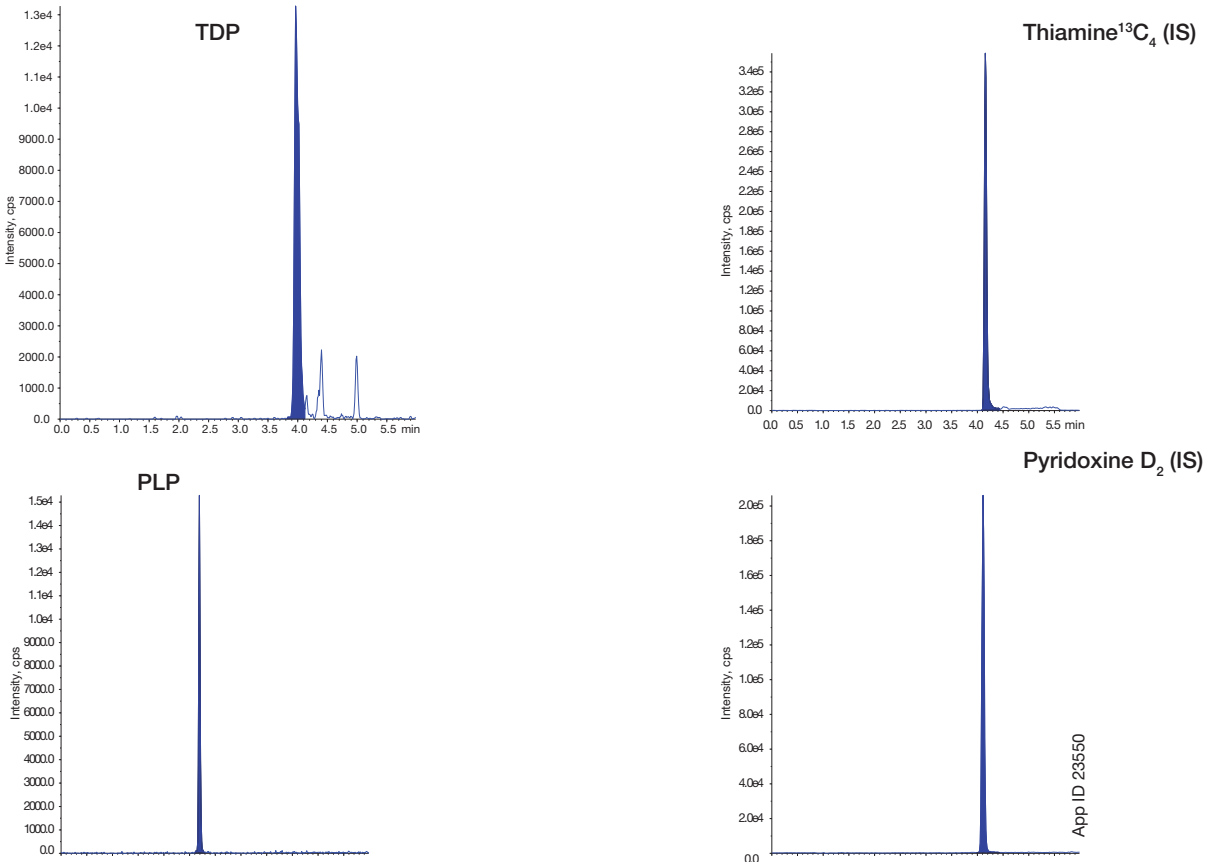


Figure 4. Interference Peak Analysis during TDP Detection

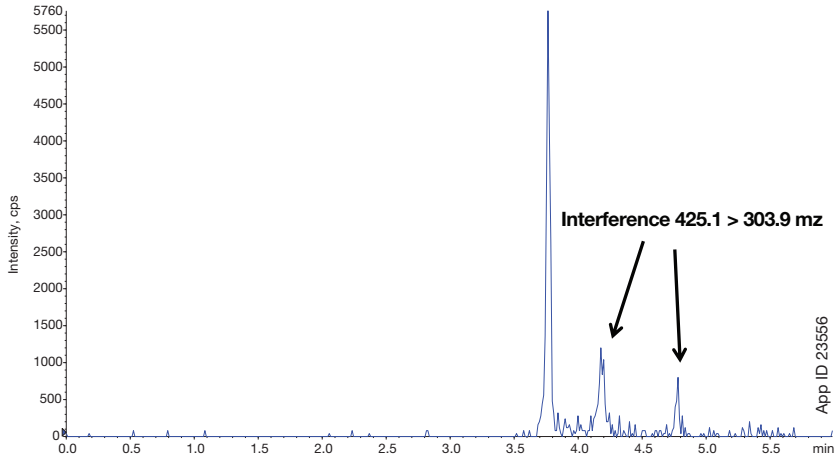


Figure 5. Plot Demonstrating Suitability of Internal Standard

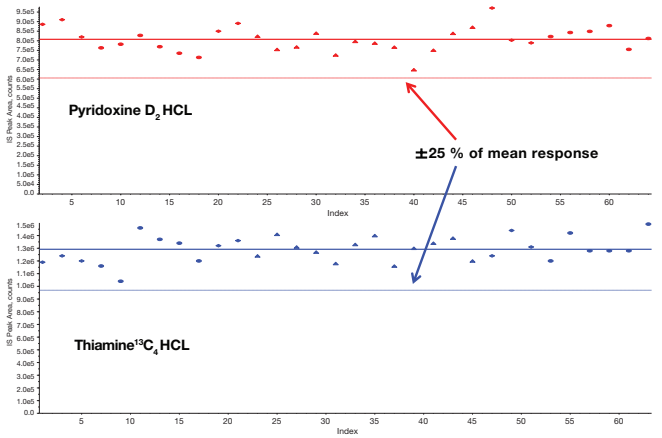


Figure 6. Representative Calibration Curve of TDP in Whole Blood

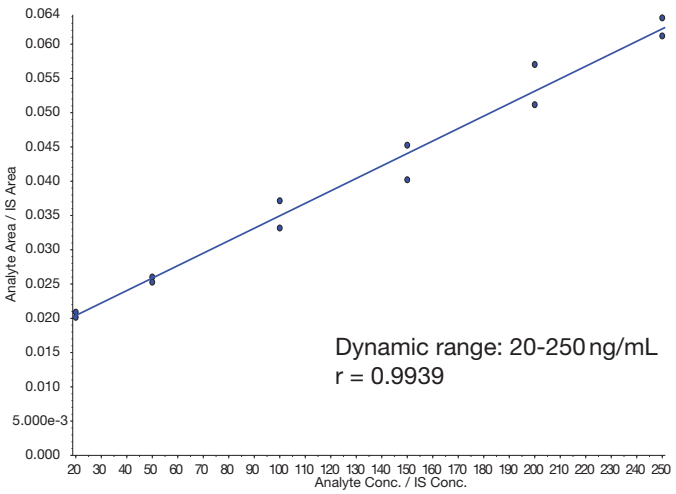


Figure 7. Representative Calibration Curve of PLP in Whole Blood

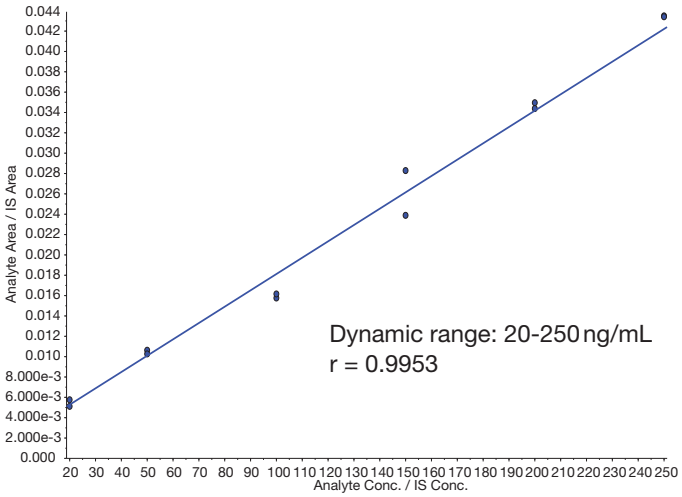
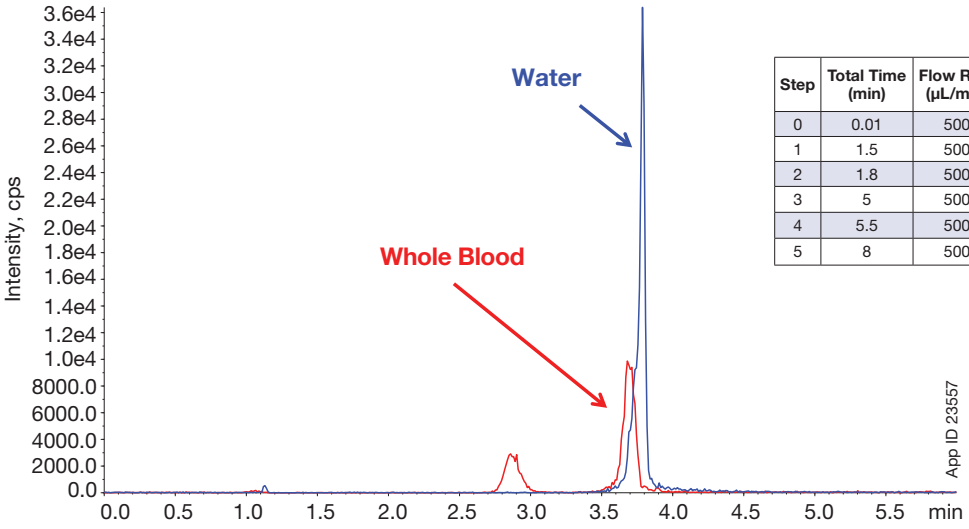


Figure 8. Matrix Effects on PLP Analysis: Water vs. Whole Blood



Step	Total Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0	0.01	500	100	0
1	1.5	500	100	0
2	1.8	500	40	60
3	5	500	40	60
4	5.5	500	100	0
5	8	500	100	0

Figure 9. Flow Rate Alteration to Separate Interference Peaks During PLP Detection

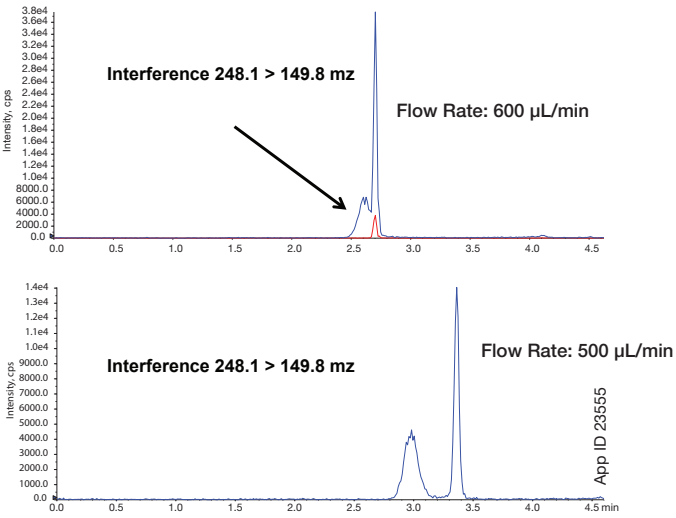


Figure 10. Matrix Effect on PLP Detection

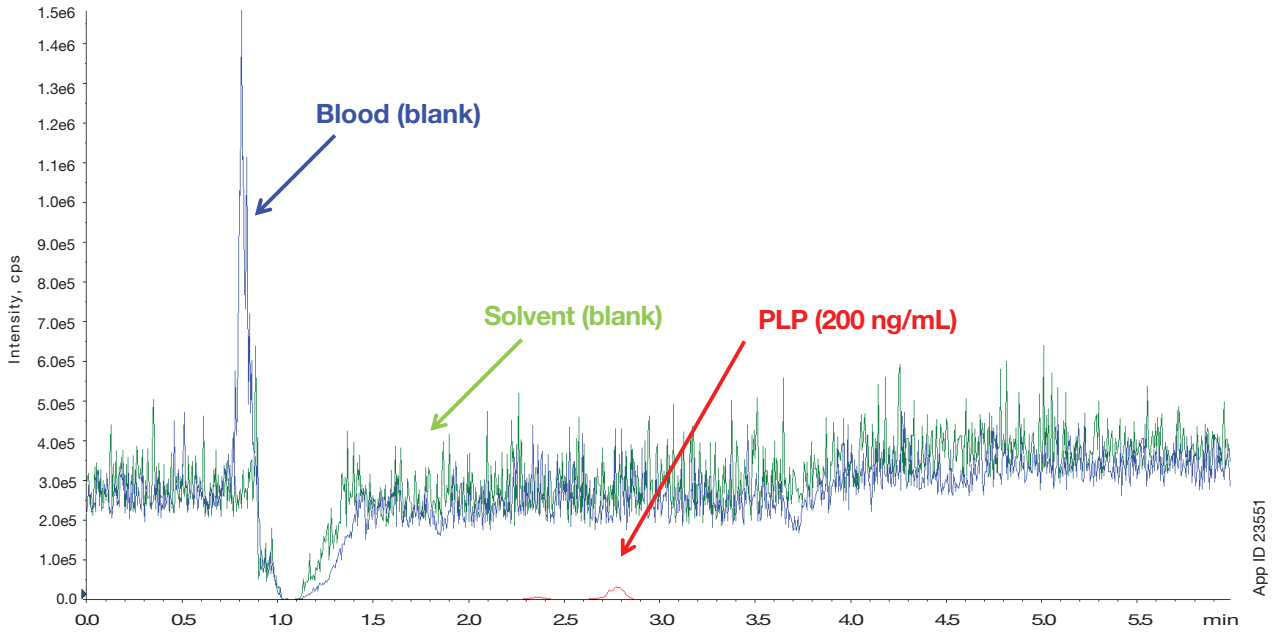


Figure 11. Matrix Effect on TDP Detection

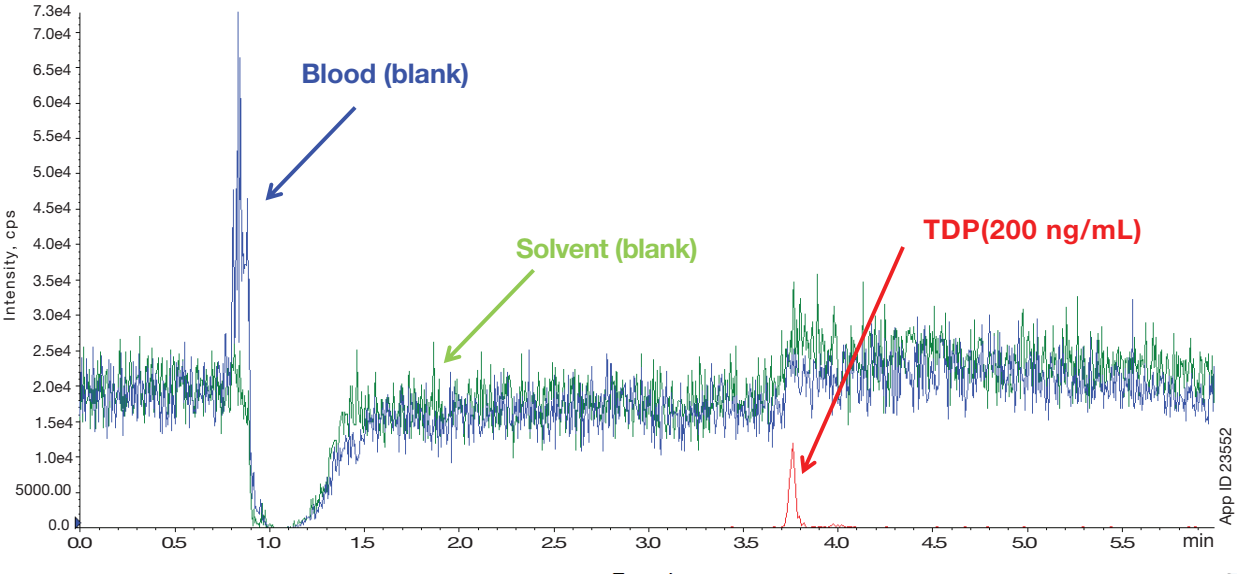


Figure 12. Endogenous Levels of PLP in Six Individual Lots of Blank Whole Blood

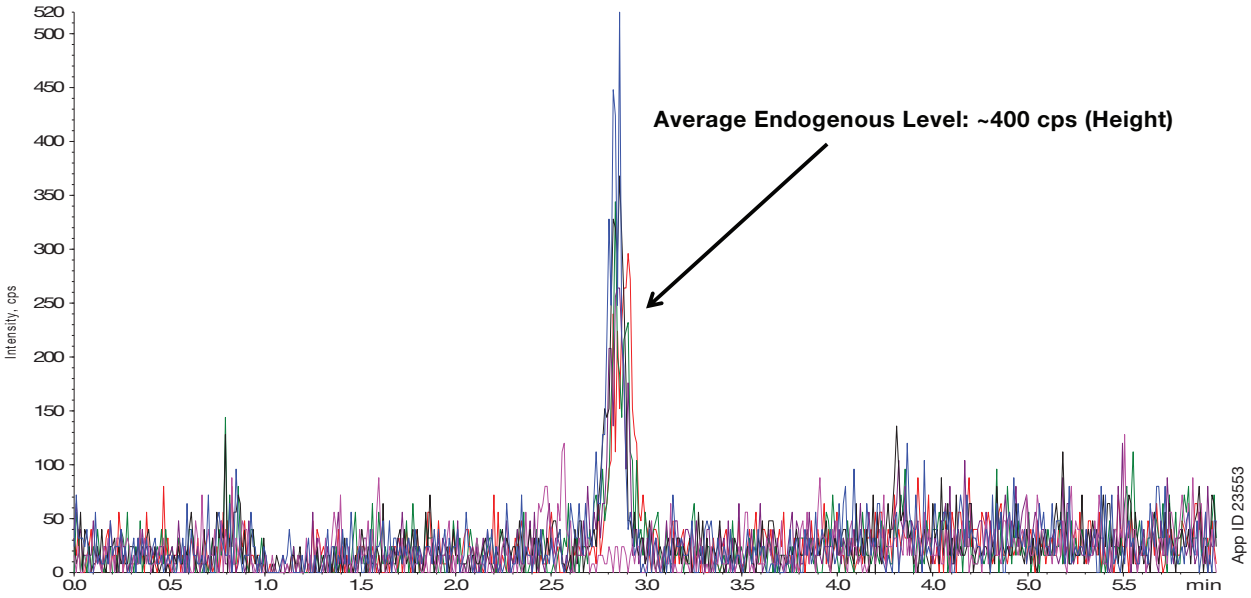
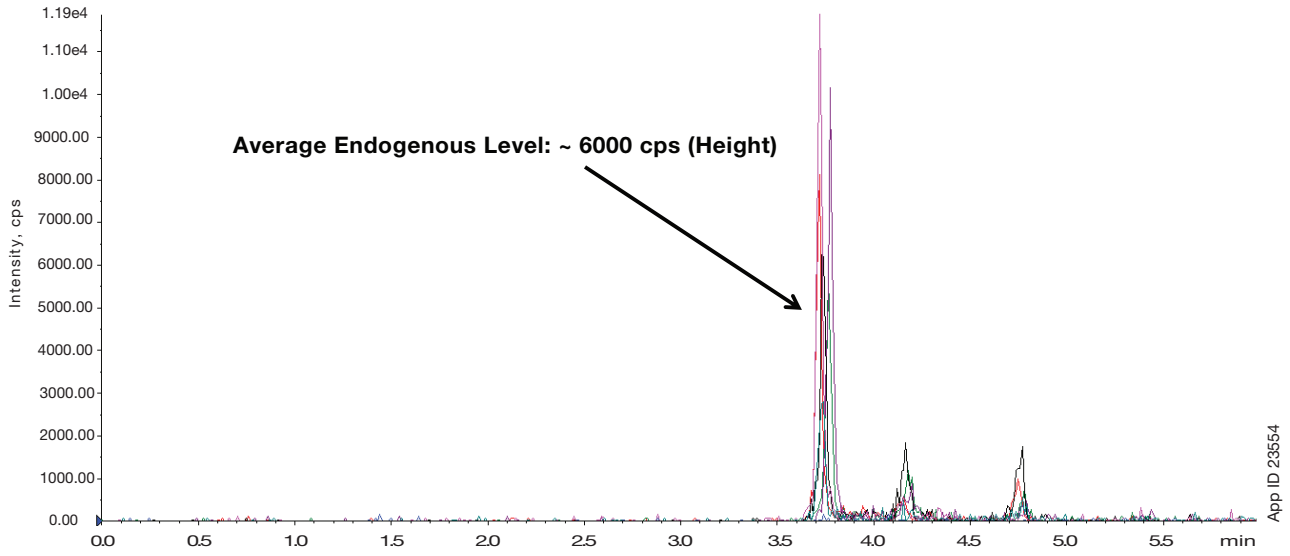


Figure 13. Endogenous Levels of TDP in Six Individual Lots of Blank Whole Blood



Results and Discussion

Presented here is a simplified extraction procedure for whole blood samples followed by a LC/MS/MS compatible reversed phase method for both TDP and PLP. Both analytes are highly polar (refer to structures in **Figure 1**.) and retaining both compounds under reversed phase conditions is challenging. Gemini® 5 µm C18 is a reversed phase HPLC column with an extreme pH resistance that offers flexibility to explore pH beyond conventional silica columns capabilities. For the present work, Gemini 5 µm C18, 50 x 4.6 mm was employed under high pH mobile phase conditions. The gradient started with 100 % aqueous mobile phase to promote retention of TDP and PLP and representative chromatogram at Lower Limit Of Quantitation (LLOQ) and Upper Limit Of Quantitation (ULOQ) are presented in **Figures 2** and **3**. These chromatograms provide evidence of successful retention of both the analytes on Gemini C18 column despite the use of any ion pairing reagent. In addition to improved reversed phase retention, interference peaks were also resolved from the analyte peaks as shown in **Figure 4**.

The extraction was performed under acidic conditions prior to LC/MS/MS which not only helped with the stability of TDP and PLP, but also allowed the extraction to take place at room temperature. Two sets of evaluation batches were run individually on 2 different days, on each day 2 concentration of QCs 100 ng/mL and 200 ng/ml were run 6 times to ensure precision and accuracy. Accuracy and precision data are presented in **Table 2**. The assay is accurate with a recovery of 92.8-112.0 % and precise with % CV of 8.13-12.6 % for human whole blood matrix. The method utilized cost effective alternative for internal standard which are commercially available. The response of the isotope internal standards is proven consistent within ±25 % of the mean response of the standards and QCs for all samples as shown in **Figure 5**.

The dynamic ranges of both TDP and PLP in whole blood are presented in **Figures 6** and **7** and the developed method proved to be linear from 20 to 250 ng/mL. Water as a matrix was also analyzed and was compared with chromatograms of whole blood extract as shown in **Figure 8**. It is clearly evident that, DI water matrix will have clean chromatography but when calibration curve is performed on DI water to quantitate TDP and PLP from whole blood, there will be matrix interference that will not be accounted as shown in **Figure 8**. For the same reason, we highly recommend using the whole blood calibration curve as it is the best practice for TDP and PLP analysis.

Challenging matrices, such as whole blood, usually require a rigorous sample cleanup due to the presence of matrix interferences. Especially due to the fact that whole blood as a matrix offers variability. In order to account for this variability and to demonstrate method flexibility, experiments were performed with change in mobile phase flow rate. The respective chromatograms are shown in **Figure 9**, which shows the separation of interference peaks with changes in the mobile phase flow rate. Further to demonstrate the success of the sample cleanup, matrix effects were monitored during the method development process by performing post column infusion tests as shown in **Figures 10** and **11**. Certainly, endogenous levels of TDP and PLP in the blank whole blood can affect the LLOQ of the assay and for the same reason, it is important to evaluate the level of endogenous interference. Six individual lots of commercially purchased human whole blood blanks were evaluated to determine the endogenous levels of TDP and PLP. The average endogenous level of six lots was ~6000 cps (peak height) for TDP and ~400 cps (peak height) for PLP when quantified by MS/MS (SCIEX API 4500™) as shown in **Figures 12** and **13**.

Overall, the method development involved changing the extraction pH, selecting the correct reversed phase column, and optimization of gradient profile. In addition, selection of cost effective internal standards produced a time and cost efficient alternative for the separation of TDP and PLP. Aside from being efficient, the method proved to be reproducible and gave consistent results for more than 500 injections on the LC/MS/MS.

Conclusion

A simple and rapid assay method is presented for the quantitation of Vitamin B1 and B6 in human whole blood by LC/MS/MS. The method is accurate with recoveries from 92.8-112.0 % and with %CV of 8.13-12.6 % it proves to be precise and the method is linear from 20-250 ng/mL of the analytes. Matrix effects and resolution of interferences from analyte peaks were also monitored for TDP and PLP, resulting in an acceptable detection of TDP and PLP. This assay is simple, time saving, cost effective and automation friendly.

References

1 Puts J, de Groot M, Haex M, Jakobs B (2015) Simultaneous Determination of Underivatized Vitamin B1 and B6 in Whole Blood by Reversed Phase Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry. PLoS ONE 10(7): e0132018. doi:10.1371/journal. pone.0132018

2 Trang, Hung Khiem, “Development of HPLC methods for the determination of water-soluble vitamins in pharmaceuticals and fortified food products” (2013). All Theses. Paper 1745.

APPLICATIONS

Ordering Information

Gemini® HPLC Columns

3 µm Microbore, Minibore and MidBore™ Columns (mm)										SecurityGuard™ Cartridges (mm)
Phases	50 x 1.0	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	4 x 2.0*
										10/pk
C18	00B-4439-A0	00M-4439-B0	00A-4439-B0	00B-4439-B0	00D-4439-B0	00F-4439-B0	00B-4439-Y0	00D-4439-Y0	00F-4439-Y0	AJ0-7596
										for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)						SecurityGuard™ Cartridges (mm)
Phases	30 x 4.6	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 3.0*
						10/pk
C18	00A-4439-E0	00B-4439-E0	00D-4439-E0	00F-4439-E0	00G-4439-E0	AJ0-7597
						for ID: 3.2-8.0 mm

5 µm Minibore and MidBore Columns (mm)									SecurityGuard™ Cartridges (mm)
Phases	30 x 2.0	50 x 2.0	150 x 2.0	250 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	250 x 3.0	4 x 2.0*
									10/pk
C18	00A-4435-B0	00B-4435-B0	00F-4435-B0	00G-4435-B0	00B-4435-Y0	00D-4435-Y0	00F-4435-Y0	00G-4435-Y0	AJ0-7596
									for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)						SecurityGuard™ Cartridges (mm)
Phases	30 x 4.6	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 3.0*
						10/pk
C18	00A-4435-E0	00B-4435-E0	00D-4435-E0	00F-4435-E0	00G-4435-E0	AJ0-7597
						for ID: 3.2-8.0 mm

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

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