Reduced Ion Suppression in an Automated Extraction of Vitamins B1 and B6 from Whole Blood for LC/ MS-MS Analysis.

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Introduction

Concentrations of Vitamin B1 (Thiamine Diphosphate, TDP) and B6 (Pyridoxal-5-Phosphate, PLP) in whole blood are measured clinically to measure various metabolic functions. Accurate concentrations of vitamins B1 and B6 are difficult to obtain due to ion suppression found in LC-MS/MS analysis. Phospholipids and proteins are a main cause of ion suppression. Currently, laboratories are struggling to create accurate, precise, and robust sample preparation methods focused on the removal of these interferences.

This study aims to provide a sample preparation method with accurate and precise concentrations of vitamin B1 and B6 through the removal of interfering phospholipids and proteins. This study further aids in accuracy and pre cision through the use of automation .

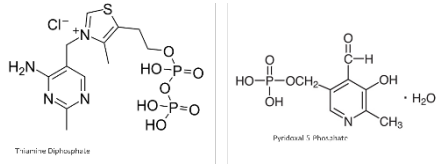

Figure 1: Structures of TDP and PLP

Methods

Chemicals and Reagents

HPLC-grade methanol and water were purchased from Honeywell. TDP and PLP standards were purchased from Cerilliant. Concentrated hydrochloric and perchloric acid were purchased from Sigma. Human whole blood was purchased from ZenBio. Mobile phase A (10 mM ammonium bicarbonate, pH 8.8) preparation: add 0.8024 g ammonium bicarbonate to 1 L of HPLC grade water. Adjust pH to 8.8 with ~2.3 mL of 5% ammonium hydroxide.

Whole Blood Extraction

The Biotage ISOLUTE® PLD+ Protein and Phospholipid Removal Plate is used in conjunction with the Biotage Extrahera® sample preparation workstation to automate the extraction of vitamins B1 & B6 from whole blood. 330 µL of precipitation solvent (0.1M HCl + 1.2 M perchloric acid) is added to each well -, followed by 200uL of whole blood. Each well is mixed thoroughly via repeat aspirate/ dispense steps. Analytes are extracted with positive pressure (10 psi) for approximately 5 minutes. For highly particulate-laden or viscous samples, increased pressure may be required.

Post Extraction

Dry the extract in a stream of air or nitrogen using a Biotage TurboVap® 96 Dual 50L/ min flow at 40°C (gas) and 60°C (plate) with a plate height of 55mm. If necessary, transfer samples via pipette to autosampler vials.

Phospholipid and Protein Removal

Phospholipid removal is measured by monitoring the 184-product ion in precipitated samples compared to the samples prepared with ISOLUTE® PLD+. Protein removal is measured through gel electrophoresis of precipitated samples compared to the samples prepared with ISOLUTE® PLD+. (Figures 4&5)

HPLC Conditions

Instrument : Shimadzu Nexera X2

Column: Phenomenex Gemini 5 µm C18 (100 x 3 mm id)

Mobile Phase:

A: 10 mM Ammonium bicarbonate pH 8.8 (aq)

B: Methanol

Column Temp: 40°C

Flow Rate: 0.6 mL/ min

Time	%A	%B
0.01	100	0
1.5	100	0
5	40	60
6.5	40	60
6.51	100	0
9	100	0

Table 1. HPLC gradient conditions.

MS/MS Conditions

Instrument : Sciex 5500 MSD

Ion Source Temperature : 425°C

ID	Q1	Q3	DP (volts)	CE (volts)	CXP (volts)
PLP1	248.0	150.1	40	25	6
PLP2	248.0	122.1	80	30	6
PLP3	248.0	94.1	60	40	7
TDP1	425.0	304.0	80	20	8
TDP2	425.0	81.0	60	25	7
TDP3	425.0	122.0	80	20	7

Table 2. Positive ions acquired in the multiple reaction monitoring (MRM) positive mode

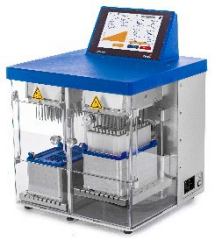

Figure 2: Biotage TurboVap® 96 Dual

Figure 3: Biotage Extrahera®

Results

The negative logP values for TDP (-5.9) and PLP (-2.2) allow for the aqueous acidic crash solvent to double as the extraction solvent. Recoveries based on endogenous levels of TDP/ PLP are presented in Table 3.

Chromatography

Good separation was observed between the TDP/PLP. Figure4 represents spiked whole blood extraction of TDP/PLP at 75 ng/mL .

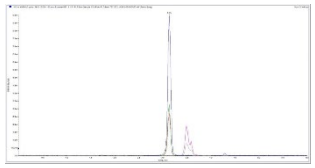

Figure 4: Spiked whole blood extraction of TDP/PLP at 75 ng/mL

Calibration Curves

Stripped whole blood was not available at the time of the study, so standard addition calibration was performed in a range of 10 -1000 ng/mL. Good linearity was observed (r2>0.99) over the calibration range. Figure 5 shows representative standard addition c alibration curves.

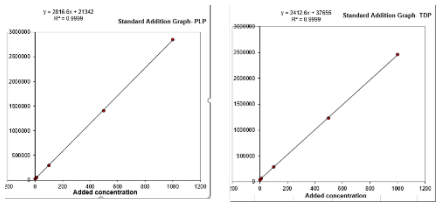

Figure 5: Standard addition calibration curves for PLP and TDP.

Analyte Recovery

Whole blood samples prepared by precipitation alone (no ISOLUTE® PLD+ sample prep) were compared to samples prepared with ISOLUTE PLD+ plates as described previously. The average of 5 injections was recorded and percent recovery and RSD values were obtaine d. 5 injections were performed, and percent recovery and RSD values were calculated. Percent recovery is based upon the calculated recovery of the TDP/PLP by precipitation alone, highlighting any analyte loss due to the use ISOLUTE PLD+ sample preparation plate. Table 3 summarizes the recovery and RSDdata.

Analyte	Precipitation alone (ng/mL)	PLD+ (ng/mL)	Recovery %	% RSD (n=5)
PLP	43.7	36.4	81.9	1.2
TDP	83.5	78.6	93.9	1.3

Table 3: PLP and TDP recoveries with ISOLUTE® PLD+ sample prep

Phospholipid and Protein Removal

The efficacy of the ISOLUTE PLD+ Phospholipid and Protein Removal Plate was investigated via LC-MS/MS analysis of residual phospholipids and gel electrophoresis analysis for proteins. Figure 4 shows no detectable protein i n electrophoresis data for samples treated with ISOLUTE PLD+. A significant reduction (>99%) of phospholipids is observed in the LC -MS/MS analysis of the common 184 product ion (Figure 5) when compared to whole blood precipitated alone.

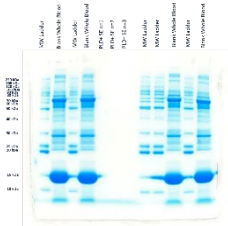

Figure 6. Gel Electrophoresis on whole blood precipitated with aqueous acidic crash solvent and extracted using ISOLUTE PLD+.

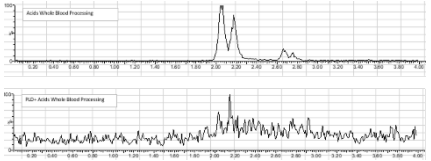

Figure 7. LC-MS/MS on whole blood precipitated with 100% aqueous acidic crash solvent and ISOLUTE PLD+. The 184-184 transition was monitored in precipitated whole blood (top), and whole blood precipitated on ISOLUTE PLD+ (Bottom).

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