Quantification of vitamins B1, B2, and B6 in human blood by liquid chromatography-tandem mass spectrometry for clinical research

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Goal

Development and implementation of a robust and reliable analytical method for quantification of vitamins B1, B2, and B6 in human blood using a Thermo Scientific™ TSQ Fortis™ triple quadrupole mass spectrometer and a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Introduction

Active variants of B vitamins are found in varying concentrations in biofluids and tissues in both free and phosphorylated forms. A number of analytical approaches have been developed for the determination of B vitamins including microbiological assays, immunoassays, and HPLC coupled to electrochemical, ultraviolet, or fluorescence detection. However, most studies focus on analysis of individual or a small subset of vitamers. B vitamins are often extracted by acid and enzymatic hydrolysis and determined by the total content of vitamins, while the chemically distinct bioactive forms are not individually measured. Hence, there is a need to develop a comprehensive method to measure each of the native forms of the B vitamins in blood.

In this study, a robust and reliable analytical method for clinical research for quantification of vitamins B1, B2, and B6 in human blood is reported. Whole blood samples were extracted by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system for LC separation. Detection was performed using either a TSQ Fortis triple quadrupole mass spectrometer or a TSQ Quantis triple quadrupole mass spectrometer with a heated electrospray ionization (HESI) source operated in positive mode by selected reaction monitoring (SRM). Method performance was evaluated using the ClinMass® LC-MS/MS Complete Kit for Vitamins B1, B2, B6 in Whole Blood from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for all analytes.

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Experimental

Target analytes

The concentration ranges covered by the calibrators (MS13013 batch #1477) are reported in Table 1.

Table 1. Concentration ranges covered by calibrators

Analyte	Internal standard	Concentration range (µg/L)
Thiamine pyrophosphate (TPP)	d_3-Thiamine pyrophosphate	14.2–152
Flavin adenine dinucleotide (FAD)	$^{13}C_5$-Flavin adenine dinucleotide	62.4–516
Pyridoxal (PL)	d_3-Pyridoxal	10.1–41.4
Pyridoxal phosphate (PLP)	d_3-Pyridoxal phosphate	5.5–91.5
Vitamin B1 (Thiamine pyrophosphate - TPP)	d_3-Thiamine pyrophosphate	14.2–152
Vitamin B2 (Flavin adenine dinucleotide - FAD)	$^{13}C_5$-Flavin adenine dinucleotide	62.4–516
Vitamin B6 (Pyridoxal - PL)	d_3-Pyridoxal	10.1–41.4
Vitamin B6 phosphate (Pyridoxal phosphate - PLP)	d_3-Pyridoxal phosphate	5.5–91.5

Table 2. LC method description

Gradient profile		
Time (min)	**Flow rate (mL/min)**	**%B**
0.00	0.30	0
0.15	0.30	0
0.16	0.30	12
0.20	0.45	12
0.70	0.45	12
2.00	0.45	40
2.10	0.45	100
2.20	0.45	100
2.30	0.45	0
4.30	0.45	0
4.31	0.30	0
5.50	0.30	0
Other parameters		
Column temperature		25 °C
Injection volume TSQ Quantis MS		15 µL
Injection volume TSQ Fortis MS		25 µL

Sample preparation

Reagents included four calibrators and two controls from RECIPE, as well as four isotopically labeled internal standards for quantification. Samples of 50 µL of whole blood were protein-precipitated using 100 µL of precipitating solution following addition of 20 µL of the internal standards solution. Precipitated samples were vortex-mixed and centrifuged. The supernatant was transferred to a clean vial.

Liquid chromatography

LC separation was achieved using mobile phases and an analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Injection volumes were 15 µL on the TSQ Quantis instrument and 25 µL on the TSQ Fortis instrument. The total run time was 5.5 minutes.

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Fortis triple quadrupole mass spectrometer and a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. A summary of the MS conditions is reported in Table 3. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation. SRM transitions with optimized collision energy, RF lens, and tube lens values are summarized in Table 4.

Table 3. Settings for TSQ Quantis MS and TSQ Fortis MS

Parameter	Value
Source type	Heated electrospray ionization (H-ESI)
Vaporizer temperature	350 °C
Capillary temperature TSQ Quantis TSQ Fortis	 325 °C 300 °C
Spray voltage (positive mode)	3500 V
Sheath gas	50 AU
Sweep gas	0 AU
Auxiliary gas	10 AU
Data acquisition mode	Selected reaction monitoring (SRM)
Source fragmentation	5 V
Collision gas pressure	1.5 mTorr
Cycle time	0.400 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for all analytes. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at

Table 4. SRM transitions, collision energies, RF lens (TSQ Quantis MS), and tube lens (TSQ Fortis MS) values

Analyte / internal standard	Quantification			Confirmation			RF lens (V)	Tube lens (V)
	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)		
Thiamine pyrophosphate	425.0	122.1	22	425.0	304.0	16	123	125
d_3-Thiamine pyrophosphate	428.0	125.1	22				123	125
Flavin adenine dinucleotide	786.2	348.1	20	786.2	439.1	25	209	150
$^{13}C_5$-Flavin adenine dinucleotide	791.2	353.1	20				209	150
Pyridoxal	168.1	150.1	11	168.1	94.1	23	66	64
d_3-Pyridoxal	171.1	153.1	11				66	64
Pyridoxal phosphate	248.0	150.1	15	248.0	94.1	27	120	120
d_3-Pyridoxal phosphate	251.0	153.1	15				120	120

two different levels provided by RECIPE (MS13082 batch #1477), prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear interpolation with 1/× weighting was used for vitamins B2 and B6 (pyridoxal and pyridoxal phosphate) and a quadratic interpolation with 1/× for vitamin B1. The percentage bias between nominal and back-calculated concentration was always within ±7% for all calibrators in all runs in both systems evaluated. Representative chromatograms for the lowest calibrator for vitamin B2 and B6 and their internal standards are reported in Figures 1 and 2 for the TSQ Quantis mass spectrometer and TSQ Fortis mass spectrometer, respectively. Representative calibration curves for the same analytes are reported in Figures 3 and 4 for the TSQ Quantis instrument and TSQ Fortis instrument, respectively.

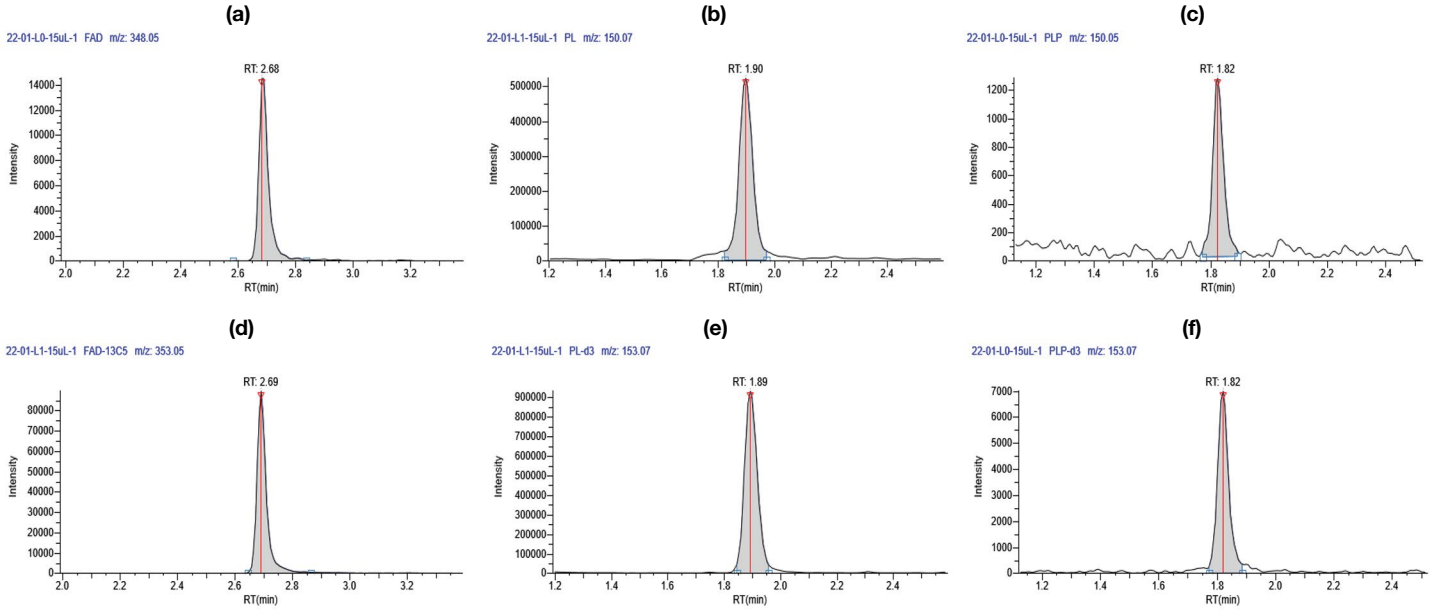

Figure 1. Representative chromatograms of the lowest calibrator for (a) Flavin adenine dinucleotide, (b) Pyridoxal, (c) Pyridoxal phosphate, (d) $^{13}C_5$-Flavin adenine dinucleotide, (e) d_3-Pyridoxal, and (f) d_3-Pyridoxal phosphate. TSQ Quantis MS.

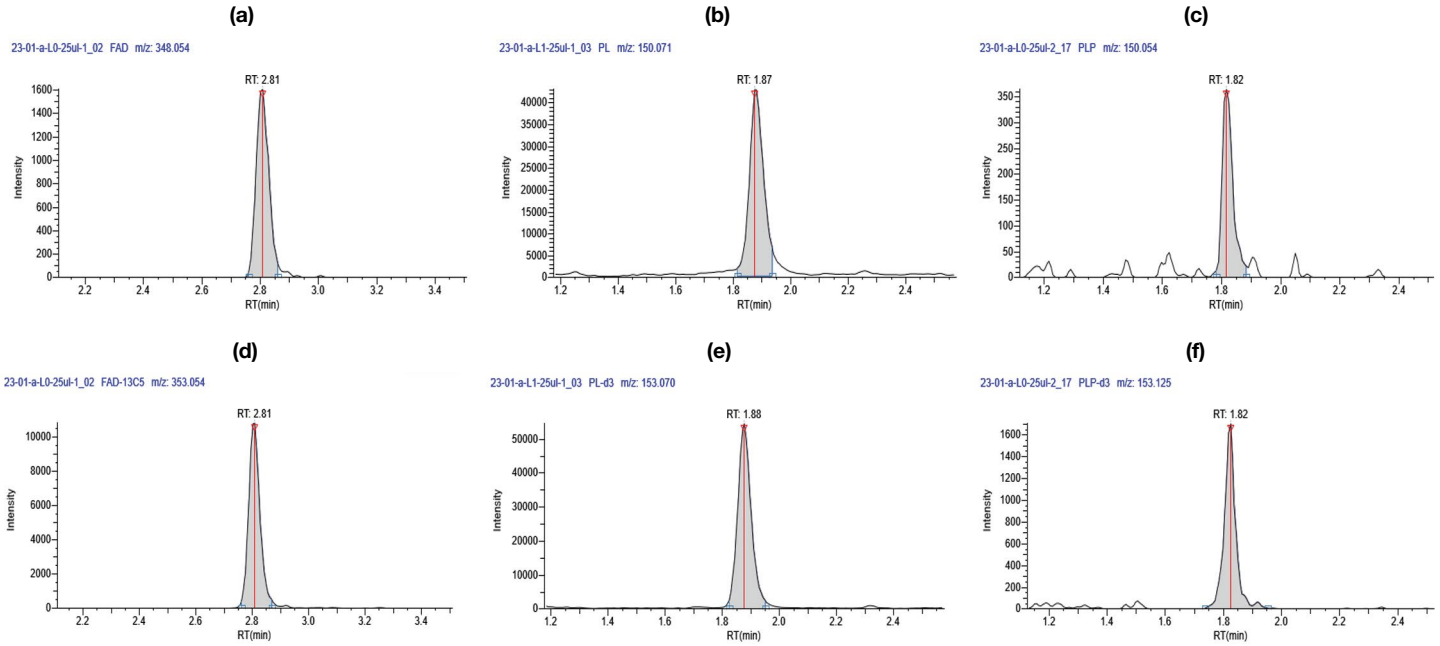

Figure 2. Representative chromatograms of the lowest calibrator for (a) Flavin adenine dinucleotide, (b) Pyridoxal, (c) Pyridoxal phosphate, (d) $^{13}C_5$-Flavin adenine dinucleotide, (e) d_3-Pyridoxal, and (f) d_3-Pyridoxal phosphate. TSQ Fortis MS.

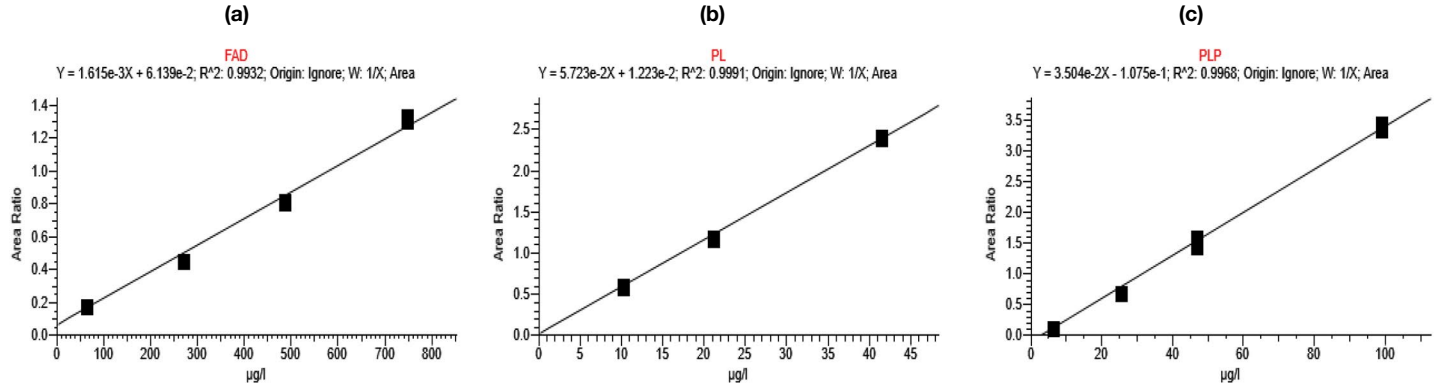

Figure 3. Representative calibration curves for (a) Flavin adenine dinucleotide, (b) Pyridoxal, and (c) Pyridoxal phosphate. TSQ Quantis MS.

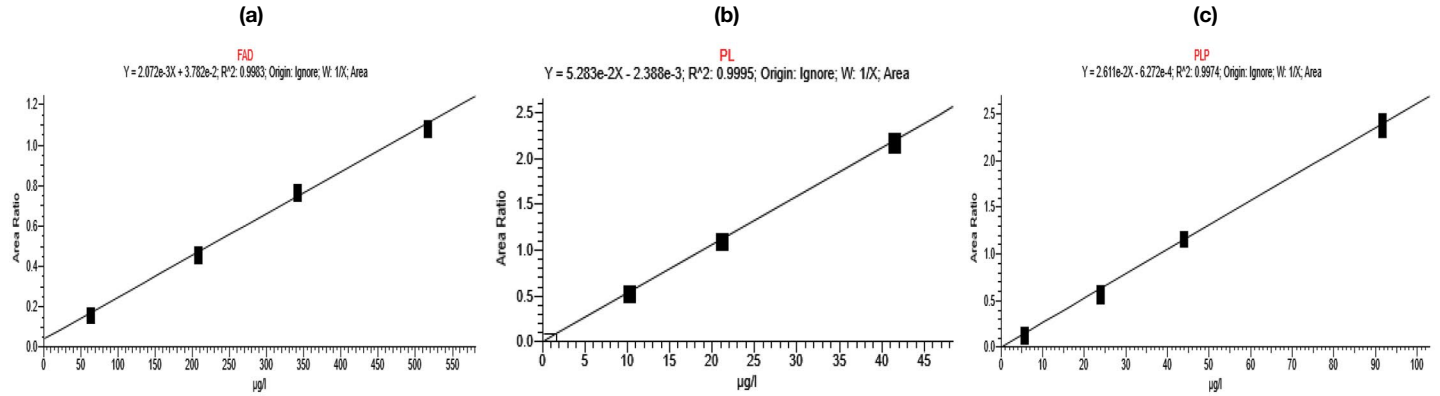

Figure 4. Representative calibration curves for (a) Flavin adenine dinucleotide, (b) Pyridoxal, and (c) Pyridoxal phosphate. TSQ Fortis MS.

The data demonstrate an accurate method, with a percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -8.1% and 1.6% in the TSQ Fortis mass spectrometer and between -6.0% and 3.0% on the TSQ Quantis mass spectrometer (Table 5). Internal standard reproducibility was below 12% on the TSQ Fortis instrument, and below 9% on the TSQ Quantis instrument.

The %CV for intra-assay precision was always below 10.3% for all the analytes on the TSQ Fortis instrument, and below 5% on the TSQ Quantis instrument. The maximum %CV for inter-assay precision including all the analytes was 11.6% for the TSQ Fortis instrument, and 9% for the TSQ Quantis instrument. Results for intra- and inter-assay precision are reported in Tables 6 and 7.

Table 5. Analytical intra-assay precision results for control MS13082 batch #1477

Analyte	Control	TSQ Quantis MS						TSQ Fortis MS					
		Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
Thiamine pyrophosphate	Level I (LOT #1477)	39.9	4.8	45.2	2.7	37.4	3.9	36.7	8.9	41.8	6.0	39.1	6.5
	Level II (LOT #1477)	104	3.7	97.4	4.9	106	3.6	103	10.2	93.3	10.3	107	7.4
Flavin adenine dinucleotide	Level I (LOT #1477)	158	3.9	150	4.7	152	4.1	141	8.2	161	8.0	151	5.7
	Level II (LOT #1477)	172	3.9	157	4.2	152	3.4	156	7.7	177	6.3	159	7.0
Pyridoxal	Level I (LOT #1477)	15.7	4.1	14.0	1.8	14.7	2.3	14.8	9.2	14.0	5.5	14.8	7.6
	Level II (LOT #1477)	22.3	3.1	21.0	3.4	22.0	2.0	21.2	6.2	22.4	6.2	21.1	5.3
Pyridoxal phosphate	Level I (LOT #1477)	15.0	2.1	13.3	4.3	13.7	4.8	15.3	10.1	13.2	8.1	12.8	8.0
	Level II (LOT #1477)	26.5	1.1	26.4	2.4	27.2	2.1	26.9	10.2	26.5	9.1	24.7	7.0

Table 6. Analytical inter-assay precision results for control MS13082 batch #1477

Analyte	Control	TSQ Quantis MS		TSQ Fortis MS	
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
Thiamine pyrophosphate	Level I (LOT #1477)	40.8	9.0	39.4	8.6
	Level II (LOT #1477)	102.4	2.6	101	10.2
Flavin adenine dinucleotide	Level I (LOT #1477)	153.3	4.5	151	8.9
	Level II (LOT #1477)	160.4	6.6	165	8.2
Pyridoxal	Level I (LOT #1477)	14.8	5.6	14.5	7.4
	Level II (LOT #1477)	21.8	3.7	21.6	6.3
Pyridoxal phosphate	Level I (LOT #1477)	14.0	6.5	13.7	11.6
	Level II (LOT #1477)	26.7	2.3	26.1	9.2

Table 7. Analytical accuracy results for control MS13082 batch #1477

Analyte	Control	Nominal concentration (µg/L)	TSQ Quantis MS		TSQ Fortis MS	
			Average calculated concentration (µg/L)	Bias (%)	Average calculated concentration (µg/L)	Bias (%)
Thiamine pyrophosphate	Level I (LOT #1477)	39.6	40.8	3.0	39.4	-0.5
	Level II (LOT #1477)	99.8	102.4	2.6	101	0.9
Flavin adenine dinucleotide	Level I (LOT #1477)	149	153.3	2.9	151	1.6
	Level II (LOT #1477)	164	160.4	-2.2	165	0.5
Pyridoxal	Level I (LOT #1477)	14.8	14.8	-0.1	14.5	-2.0
	Level II (LOT #1477)	21.6	21.8	0.9	21.6	0.2
Pyridoxal phosphate	Level I (LOT #1477)	14.2	14.0	-1.4	13.7	-3.4
	Level II (LOT #1477)	28.4	26.7	-6.0	26.1	-8.1

Conclusions

A robust, reproducible, and sensitive liquid chromatography-tandem mass spectrometry method for clinical research for quantification of vitamins B1, B2, and B6 in human blood was implemented. The ClinMass LC-MS/MS Complete Kit for Vitamins B1, B2, B6 in Whole Blood from RECIPE was used.

The method was analytically validated on a Vanquish Flex Binary UHPLC system connected to a TSQ Fortis or a TSQ Quantis triple quadrupole mass spectrometer, and both triple quadrupole mass spectrometers provided valid quantification results.

The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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