LC/MS/MS Quantitative Analysis of Water Soluble Vitamins in Blood

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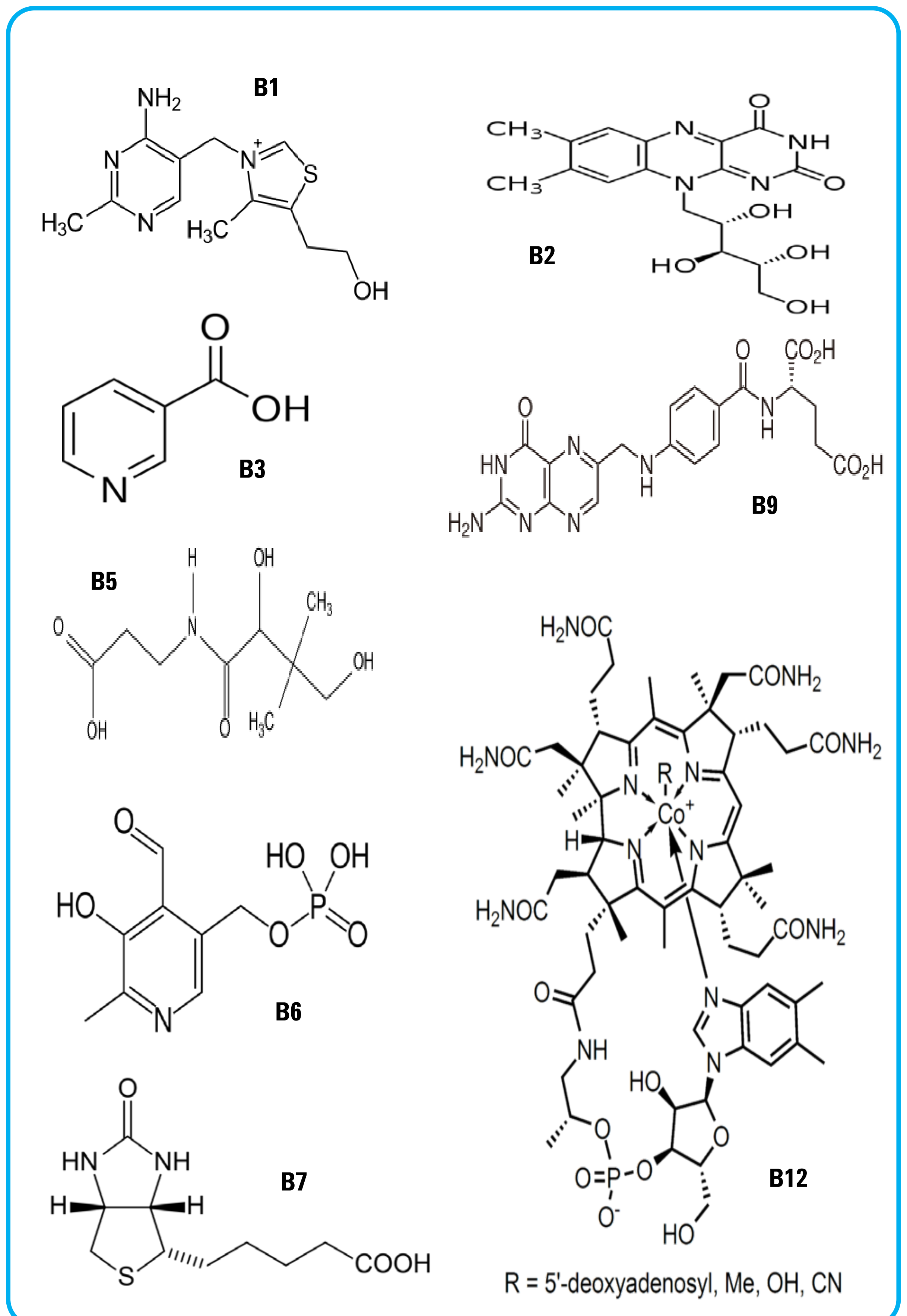
Introduction

The major water soluble vitamins include Vitamin B1 (Thiamine, Thiamine Pyrophosphate), Vitamin B2 (Riboflavin), Vitamin B3 (Nicotinic Acid and Nicotinamide), Vitamin B5 (Panothenic Acid, Pantothenol and Pantethine), Vitamin B6 (Pyridoxamine, Pyridoxal, Pyridoxine and Pyridoxal-5-Phosphate), Vitamin B7 (Biotin), Vitamin B9 (Folic Acid, Folinic Acid and 5-Methyltetrahydrofolate) and Vitamin B 12 (Cyanocobalamin, Adenosylcobalamin, Methylcobalamin and Hydroxocobalamin).

Therefore, we developed LC/MS/MS analytical methods and evaluated various columns and solvent combinations in order to demonstrate the chromatographic separation, detection and quantification of the water soluble vitamins in blood. The sample preparation was a simple protein precipitation and the methodologies were developed on an Agilent 1260 HPLC and 6460 Mass Spectrometer with a 8 minute analytical gradient method in positive ionization mode.



Experimental

Reagents, Standards, Calibrators and Controls

Standards/Calibrators

Biotin:	Isosciences	Biotin-$^{2}H_8$:	Isosciences
Pyridoxal:	Isosciences	Pyridoxal-$^{2}H_3$:	Isosciences
Pyridoxine:	Isosciences	Pyridoxine-$^{2}H_3$:	Isosciences
Pyridoxamine:	Isosciences	Pyridoxamine-$^{2}H_3$:	Isosciences
Riboflavin:	Isosciences	Riboflavin-$^{13}C_4$,$^{15}N_2$:	Isosciences
Thiamine:	Isosciences	Thiamine-$^{13}C_4$:	Isosciences
Pantothenic Acid:	Isosciences	Pantothenic Acid-$^{13}C_3$$^{15}N_1$:	Isosciences
Nicotinamide:	Cerilliant	Nicotinamide-$^{13}C_3$,$^{15}N_1$:	Isosciences
Nicotinic Acid:	Cerilliant	Nicotinic Acid-$^{13}C_3$,$^{15}N_1$:	Isosciences
Folic Acid:	Sigma	Folic Acid-$^{13}C_5$,$^{15}N_1$:	Isosciences
Folinic Acid:	Sigma-Aldrich		
5-Methyltetrahydrofolate:	Cayman Chemicals		
Cyanocobalamin	Cerilliant		
Adenosylcobalamin:	Sigma-Aldrich		
Methylcobalamin:	Sigma-Aldrich		
Hydroxocobalamin:	Sigma Aldrich		
Pantothenol:	Sigma-Aldrich		
Pantethine:	Sigma-Aldrich		
Thiamine Pyrophosphate:	Sigma-Aldrich		
Pyridoxal-5-Phospahte:	Sigma-Aldrich		

Reagents

Methanol:	Honeywell	Ammonium Formate:	Sigma-Aldrich
Formic Acid:	Sigma-Aldrich	Trichloroacetic Acid:	Sigma-Aldrich
Sodium Hydroxide:	Sigma-Aldrich		

Experimental

Sample Preparation

- 200 µl of serum sample, calibrators, controls was taken and 20 µl ISTD at 1000 ng/ml were added to each
- 100 µl of 5% Trichloroacetic Acid was added to each tube and vortexed for 5 minutes prior to centrifugation for 10 minutes at 13000 rpm
- 25 µl of 1N Sodium Hydroxide was added to quench the acid and prevent damage to the column
- The supernatant was transferred to MS vials for analysis

All in-house calibrators were prepared in DC Mass Spec Gold Serum (Golden West Biological, Inc.)

Method

HPLC Conditions

Agilent 1260 Infinity HPLC series binary pump, well plate, thermostatted column compartment

Column:	Agilent Technologies Poroshell 120, SB-AQ, (or Extend) 3 x 100 m, 2.7 µm Agilent Technologies Poroshell 120 Fast Guard 2.1 x 5 mm
Column Temperature:	25 ºC
Injection Volume:	5 µl
Autosampler Temperature:	4 ºC
Needle Wash:	Flush port (50%Methanol:50%Water) 5 seconds
Mobile Phase A:	0.1% Formic Acid+5mM Ammonium Formate Water
Mobile Phase B:	0.1% Formic Acid in Methanol
Flow Rate:	0.5 ml/min
Gradient:	0 min- 100%A:5%B 6 min- 5%A:95%B
Run/Stop time:	8 minutes/3 minutes

MS Conditions

Agilent 6460 Triple Quadruple Mass Spectrometer- Dynamic MRM

Ion mode:	Agilent Jet Stream Positive Mode
Gas Temperature:	300ºC
Gas Flow:	8 L/min
Nebulizer:	38 psi
Sheath Gas Temperature:	400ººC
Sheath Gas Flow:	112 l/min
Capillary Voltage:	2100V
Nozzle Voltage:	0V
Q1/Q2 Resolution:	0.7/0.7 unit
Delta EMV/CAV:	+400V/2

Table 1: MRM Acquisition Table- * Quantifier Ion

Compounds	R (Min)	MRM	Fragmentation Voltage	Collision Energy
Thiamine	1.44	265.1 – 144/122*	97	8/12
Thiamine Pyrophosphate	1.19	425.1 – 122.1*	122	44
Thiamine-13C4	1.42	269.2 – 122.1	82	12
Riboflavin	4.48	277.2 – 243.1*/198.1	167	20/40
Riboflavin-13C4,15N2	4.45	383.2 – 202.1	167	40
Nicotinic Acid	1.72	124 – 80.1*/53.1	107	20/32
Nicotinic Acid-13C3,15N	1.69	128.1 – 54.1	102	36
Nicotinamide	2.31	123.1 – 80.*1/53.1	112	20/32
Nicotinamide- 13C3,15N	2.29	127.1 – 83.1	112	20
Pantothenic Acid	2.39	220.1 – 90.1*/72.1	107	12/20
Pantothenic Acid-13C3,15N	2.36	224.1 – 94.1	97	12
Panthothenol	2.36	206.1 – 76.1/58.1	92	8/20
Pantethine	4.73	555.1 – 425.1/295.1	147	8/20
Pyridoxamine	1.29	169.1 – 152.1*/134.1	82	8/20
Pyridoxamine-2H3	1.26	172.1 – 155.1	82	8
Pyridoxal	1.58	168.1 – 150.1*/94.1	82	24/28
Pyridoxal-2H3	1.55	171.1 – 153.1	87	8
Pyridoxine	1.65	170.1 – 152.1*/134.1	92	8/20
Pyridoxine-2H3	1.62	173.1 – 155.1	97	12
Pyridoxal-5-Phosphate	1.49	247.9 – 150/94.1	142	12/28
Biotin	4.13	245.1 – 227.*1/123	102	12/28
Biotin-2H8	4.11	253.2 – 235.1	122	8
Folic Acid	4.54	442.2 – 295.1*/176	102	12/40
Folic Acid-13C5,15N	4.52	448.2 – 295.1	117	12
Folinic Acid	4.43	474.2 – 327.1/120	122	16/40
5-Methyltetrahydrofolate	3.72	460.2 – 313.1/180	122	16/48
Cyanocobalamin	4.42	678.2 – 359.1/147.1	152	20/40
Adenosylcobalamin	4.94	790.3 – 147.1/227.1	152	32/88
Methylcobalamin	5.06	673 – 359.1/147.1	132	20/48
Hydroxocobalamin	3.99	665.1 – 359.1/147.1	157	20/40

Linearity

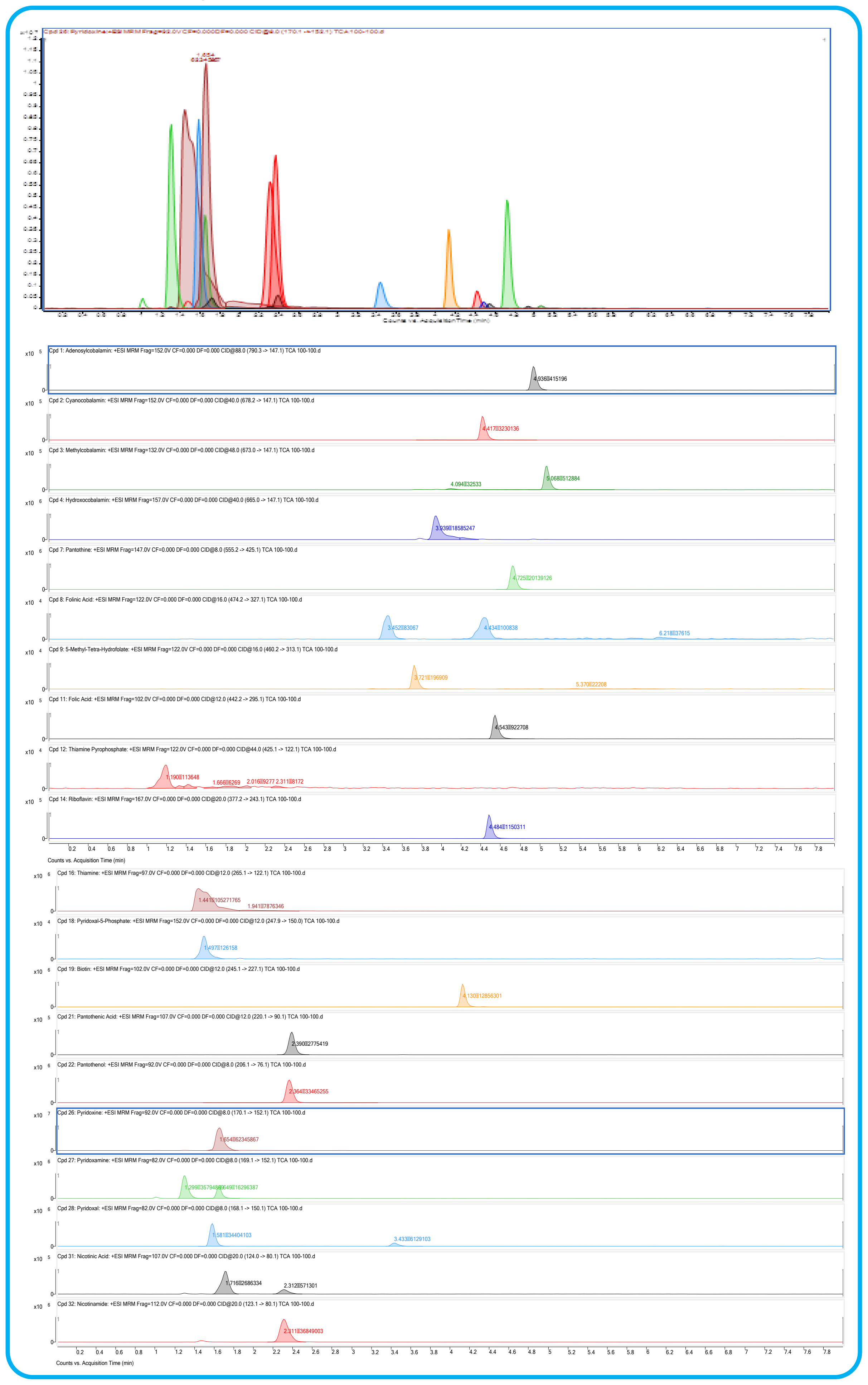
The assay was linear over the calibration curve from 0.1 to 5000 ng/mL with a mean of coefficient of determinations (R2) > 0.998 and %CV <10%. Standards need to be made fresh in water and away from light at all times to avoid oxidation.

Results and Discussion

Table 2- Sensitivity

Compound (ng/mL)	LOD	LOQ	Compound (ng/mL)	LOD	LOQ
Thiamine	0.1	0.25	Pyridoxine	0.1	0.25
Thiamine pyrophosphate	10	25	Pyridoxal-5-Phosphate	10	25
Riboflavin	5	10	Biotin	0.25	0.5
Nicotinic Acid	0.1	0.25	Folic Acid	5	10
Nicotinamide	0.1	0.25	Folinic Acid	25	50
Pantothenic Acid	2.5	5	5-Methyltetrrahydrofolate	10	25
Pantothenol	0.25	0.5	Cyanocobalamin	2.5	5
Pantethine	1	2.5	Adenosylcobalamin	5	10
Pyridoxal	0.25	0.5	Methylcobalamin	5	10
Pyridoxamine	0.1	0.5	Hydroxocobalamin	0.1	0.25

Table 3-Chromatograms



Conclusions

- Baseline separation of the water soluble vitamins was achieved within a 8 minute run on a Poroshell 120 SB-AQ column.
- Excellent linearity (>998) of calibration curves with great accuracy, precision and reproducibility was achieved for all vitamins
- Further evaluate different sample preparation techniques and water soluble vitamin free serum matrixes available to determine which gives the best results while maintaining low cost and ease of use.
- Further evaluate the different Water Soluble Vitamins per a group method rather than a global method for better analytical needs, chromatography and results.