

# Quantitative analysis of the major Water Soluble Vitamins in human serum by Liquid Chromatography Triple Quadruple mass Spectrometry on the Agilent Triple Quad 6460 Mass Spectrometer

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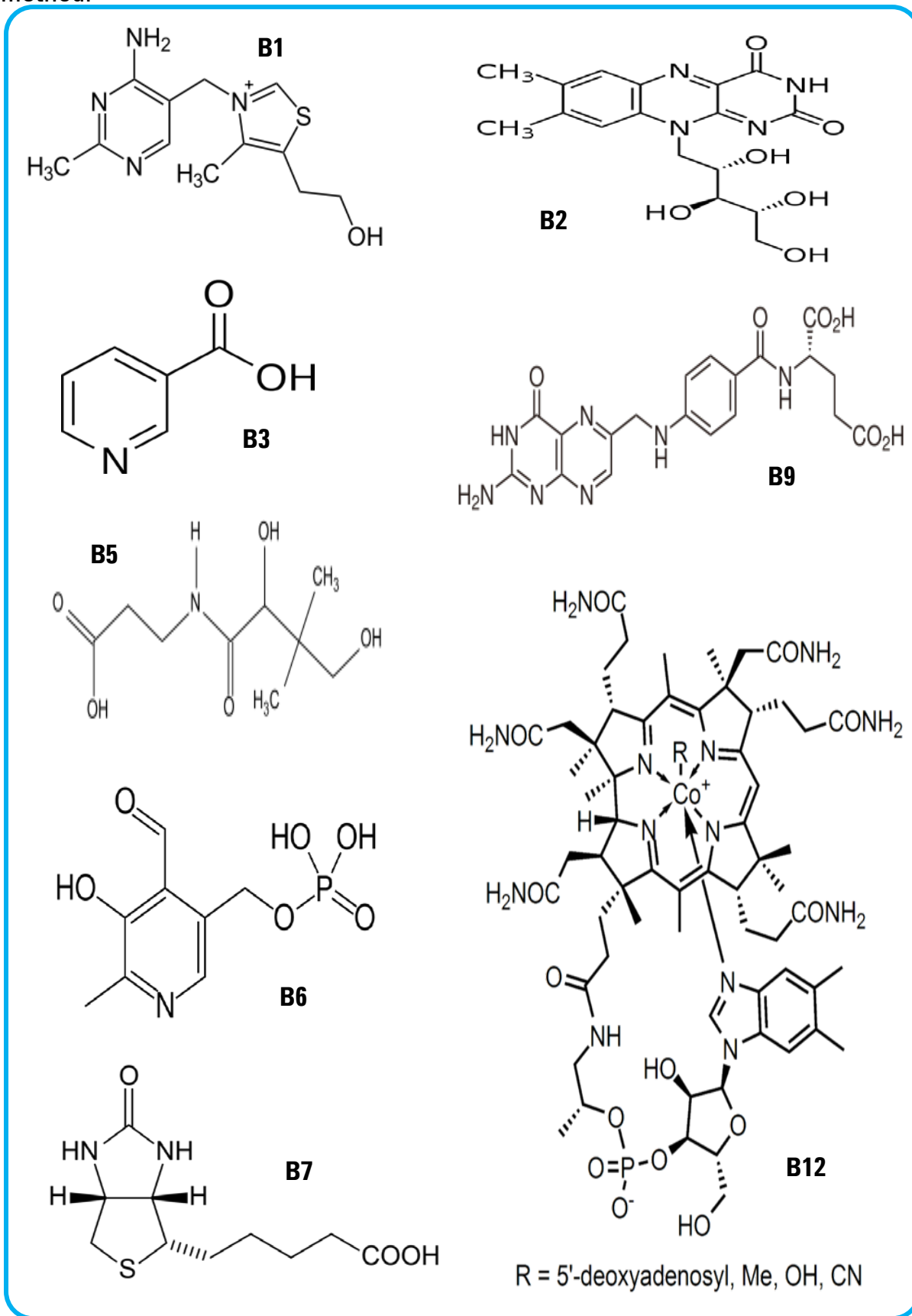
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## Introduction

The major water soluble vitamins such as Vitamin B1 (Thiamine), Vitamin B2 (Riboflavin), Vitamin B3 (Nicotinic Acid and Nicotinamide), Vitamin B5 (Pantothenic Acid), Vitamin B6 (Pyridoxamine, Pyridoxal, and Pyridoxine), Vitamin B7 (Biotin), Vitamin B9 (Folic Acid) and Vitamin B12 (Cyanocobalamin) are essential nutrients required for normal body functioning that can either cannot be synthesized by the body at all or in insignificant amounts. These compounds are acquired from the diet and can be toxic in large doses and can cause significant medical issues when deficient.

A simple, sensitive, specific and accurate quantitative analytical method was developed for the chromatographic baseline separation and measurement of the water soluble vitamins in human serum. A Poroshell 120 EC-CN column on an Agilent 1260 HPLC and 6460 Mass Spectrometer system was used for this method.



## Experimental

### Reagents, Standards, Calibrators and Controls

The following standards were obtained from Isosciences  
Standards Internal Standards  
Biotin Biotin-<sup>2</sup>H<sub>8</sub>  
Pyridoxal Pyridoxal-<sup>2</sup>H<sub>3</sub>  
Pyridoxine Pyridoxine-<sup>2</sup>H<sub>3</sub>  
Pyridoxamine Pyridoxamine-<sup>2</sup>H<sub>3</sub>  
Riboflavin Riboflavin-<sup>13</sup>C<sub>4</sub>, <sup>16</sup>N<sub>2</sub>  
Thiamine Thiamine-<sup>13</sup>C<sub>4</sub>  
Pantothenic Acid Pantothenic Acid-<sup>13</sup>C<sub>3</sub>, <sup>16</sup>N<sub>1</sub>

The following standards were obtained from Cerilliant

Nicotinic Acid  
Nicotinamide  
Cyanocobalamin

Folic Acid  
Patient Samples:  
Methanol  
Formic Acid:  
Ammonium Formate

Sigma-Aldrich  
3 Serum samples  
Burdick and Jackson  
Sigma Aldrich  
Sigma Aldrich

### Sample Preparation

- 200 µl of serum sample, calibrators, controls was taken and 10 µl ISTD at 1000 ng/ml were added to each
- 400 µl of HPLC grade Water was added to each tube and vortexed briefly prior to centrifugation for 10 minutes at 13000 rpm
- 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)

## Experimental

### Method

#### HPLC Conditions

Agilent 1260 Infinity HPLC series binary pump, well plate, thermo-statted column compartment

Column: Agilent Technologies Poroshell 120, EC-CN, 2.1 x 100 mm  
Column Temperature: 25 °C  
Injection Volume: 5 µl  
Autosampler Temperature: 4 °C  
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: 0.3 ml/min  
Gradient: 0 min- 100%A:0%B  
5 min- 5%A:95%B  
5 minutes/3 minutes

#### MS Conditions

Agilent 6460 Triple Quadruple Mass Spectrometer- Dynamic MRM  
Ion mode: Agilent Jet Stream Positive Mode  
Gas Temperature: 300°C  
Gas Flow: 8 L/min  
Nebulizer: 38 psi  
Sheath Gas Temperature: 400°C  
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

Compound	R (Min)	MRM	Fragmentor (V)	Collision Energy (V)
Thiamine	0.8	265.1 > 144/122*	87	36/8
Thiamine- <sup>13</sup> C <sub>4</sub>	0.8	269.1 > 122	82	8
Pyridoxamine	0.82	169.1 > 152*/134	87	8/20
Pyridoxamine- <sup>2</sup> H <sub>3</sub>	0.81	172.1 > 155	77	8
Pyridoxal	0.9	168.1 > 150*/94.1	82	8/24
Pyridoxal- <sup>2</sup> H <sub>3</sub>	0.93	171.1 > 97.1	72	24
Pyridoxine	0.93	170.1 > 152*/134	87	8/20
Pyridoxine- <sup>2</sup> H <sub>3</sub>	0.93	173.1 > 155	97	12
Nicotinic Acid	0.95	123 > 80.1*/53.2	117	20/32
Nicotinamide	1.04	124 > 80.1/53.2	112	20/32
Pantothenic Acid	1.18	220.1 > 202/90.1*	77	8/8
Pantothenic Acid- <sup>13</sup> C <sub>3</sub> , <sup>16</sup> N <sub>1</sub>	1.18	224.2 > 94.1	97	8
Biotin	3.04	245.1 > 227*/123	82	8/28
Biotin- <sup>2</sup> H <sub>8</sub>	3.02	253.1 > 235	102	12
Riboflavin	3.13	377.2 > 243*/172	132	24/40
Riboflavin- <sup>13</sup> C <sub>4</sub> , <sup>16</sup> N <sub>2</sub>	3.12	383.2 > 249	112	20
Folic Acid	3.51	442.2 > 295*/176	92	8/44
Cyanocobalamin	3.61	678.6 > 359/147*	172	28/24

## Results and Discussion

### Linearity

The assay was linear over the calibration curve shown in the table below with a mean of coefficient of determinations (R<sup>2</sup>) > 0.998

Compound	Curve Range (ng/ml)	LOD/LOQ (ng/ml)	S/N	%CV C1 2.5 ng/ml	%CV C2 25 ng/ml	%CV C3 250 ng/ml
Thiamine	0.1 - 1000	0.1	812.3	6.42	3.59	2.92
Pyridoxamine	0.1 - 1000	0.1	270.3	7.23	3.65	2.45
Pyridoxal	0.25 - 500	0.25	220.9	11.2	6.58	4.56
Pyridoxine	0.1 - 1000	0.1	775.1	7.62	4.57	1.66
Nicotinic Acid	0.1 - 100	0.1	76.9	9.89	4.63	N/A
Nicotinamide	0.1 - 1000	0.1	67.2	7.2	3.6	2.89
Pantothenic Acid	0.25 - 1000	0.25	9.817	8.9	4.57	3.27
Biotin	0.25 - 1000	0.25	119	8.36	4.22	4.15
Riboflavin	5 - 1000	5	7	N/A	3.03	4.13
Folic Acid	N/A	N/A	N/A	N/A	N/A	N/A
Cyanocobalamin	0.1 - 1000	0.1	5777.7	11.3	8.2	4.61

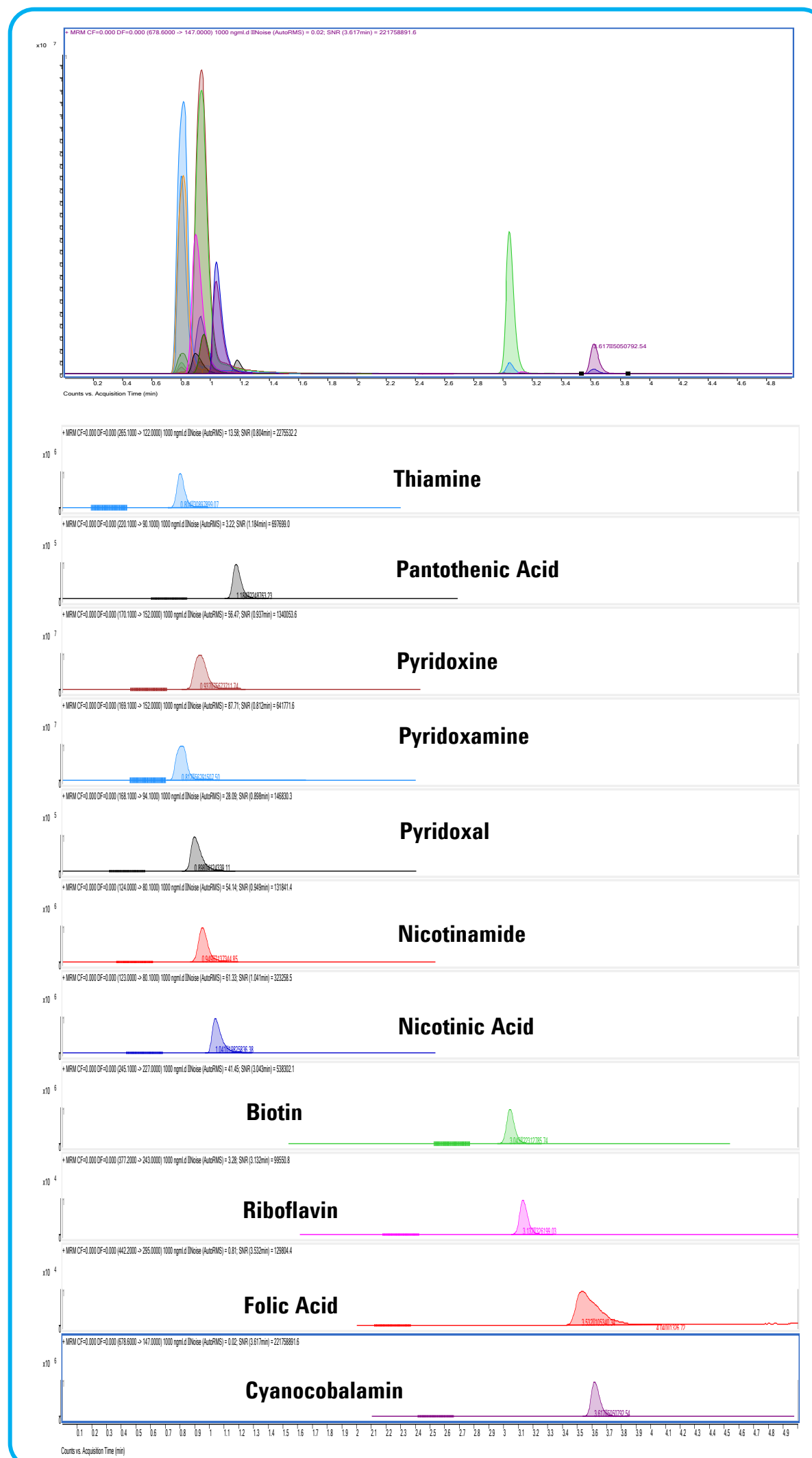
## Results and Discussion

### Precision

The inter-assay precision for the Water soluble vitamins were determined by extracting and quantifying five replicates of in-house tri-level QC material as shown. Folic Acid proved difficult to obtain consistent results due to break down in solution.

### Sample Analysis

Three healthy adult male samples were analyzed for the presence of Water soluble vitamins and it was discovered that Biotin, Folic Acid and Cyanocobalamin were not present in the serum samples analyzed..



## Conclusions

- Baseline separation of the water soluble vitamins was achieved within a 5 minute run on a Poroshell 120 EC-CN column. Other columns were evaluated but did not offer the same degree of fast separation
- Excellent linearity (>998) of calibration curves with great accuracy, precision and reproducibility was also achieved down to low clinical levels for the majority of the analytes except for Folic Acid, Riboflavin and Nicotinic Acid
- Further investigation into the best sample preparation will be carried out in order to achieve lower LOQ and to achieve consistent results for all the clinically relevant water soluble vitamins
- References
- Quantitative Analysis of Water-Soluble B-Vitamins in Cereal Using Rapid Resolution LC/MS/MS  
Agilent Technologies, Inc, Application Note, 2008
- Multi-analyte Quantification of Vitamin B<sub>6</sub> and B<sub>2</sub> Species in the Nanomolar Range in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry  
Clinical Chemistry, 2005, 52, 1206-1216