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

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# **Agilent Technologies**

### Introduction

The major water soluble vitamins such as Vitamin B1 (Thiamine), Vitamin B2 (Riboflavin), Vitamin B3 (Nicotinic Acid and Nicotinamide), Vitamin B5 (Panothenic Acid), Vitamin B6 (Pyridoxamine, Pyridoxal, and Pyridoxine), Vitamin B7 (Biotin), Vitamin B9 (Folic Acid) and Vitamin B 12 (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**

# Method

#### **HPLC Conditions**

Agilent 1260 Infinity HPLC series binary pump, well plate, thermo-statted column compartment Agilent Technologies Poroshell 120, EC-CN, 2.1 x 100 mm Column: Column Temperature: 25 °C Injection Volume: 5 μl 4 °C Autosampler Temperature:

Needle Wash: Flush port (50%Methanol:50%Water) 5 seconds Mobile Phase A: Mobile Phase B: Flow Rate: Gradient:

0.1% Formic Acid+5mM Ammonium Formate Water 0.1% Formic Acid in Methanol 0.3 ml/min100%A:0%B 0 min-5%A:95%B 5 min-5 minutes/3 minutes

### **MS** Conditions

Run/Stop time:

Agilent 6460 Triple Quadruple Mass Spectrometer- Dynamic MRM					
lon 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)	Collison 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**

#### 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...



### **Experimental**

Reagents, Standards, Calibrators	s and Controls
The following standards were ob	tained from Isociences
Standards	Internal Standards
Biotin	Biotin- <sup>2</sup> H <sub>8</sub>
Pyridoxal	Pyridoxal- <sup>2</sup> H <sub>3</sub>
Pyridoxine	Pyridoxine-2H3
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

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  $\mu$ l of serum sample, calibrators, controls was taken and 10  $\mu$ l ISTD at 1000 ng/ml were added to each

•400  $\mu$ l of HPLC grade Water was added to each tube and vortexed briefly prior to centrifugation for 10 minutes at 13000 rpm

•The supernatant was tranferred to MS vials for analysis

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

### **Results and Discussion**

#### Linearity

The assay was linear over the calibration curve shown in the table below with a mean of coefficient of determinations (R2) > 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

# 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 Technolgies, Inc, Application Note, 2008

•Multi-analyte Quantification of Vitamin  $B_6$  and  $B_2$  Species in the Nanomolar Range in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry Clinical Chemistry, 2005, 52, 1206-1216

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