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

Rory M Doyle, Agilent Technologies Inc., Wilmington, Delaware 19808

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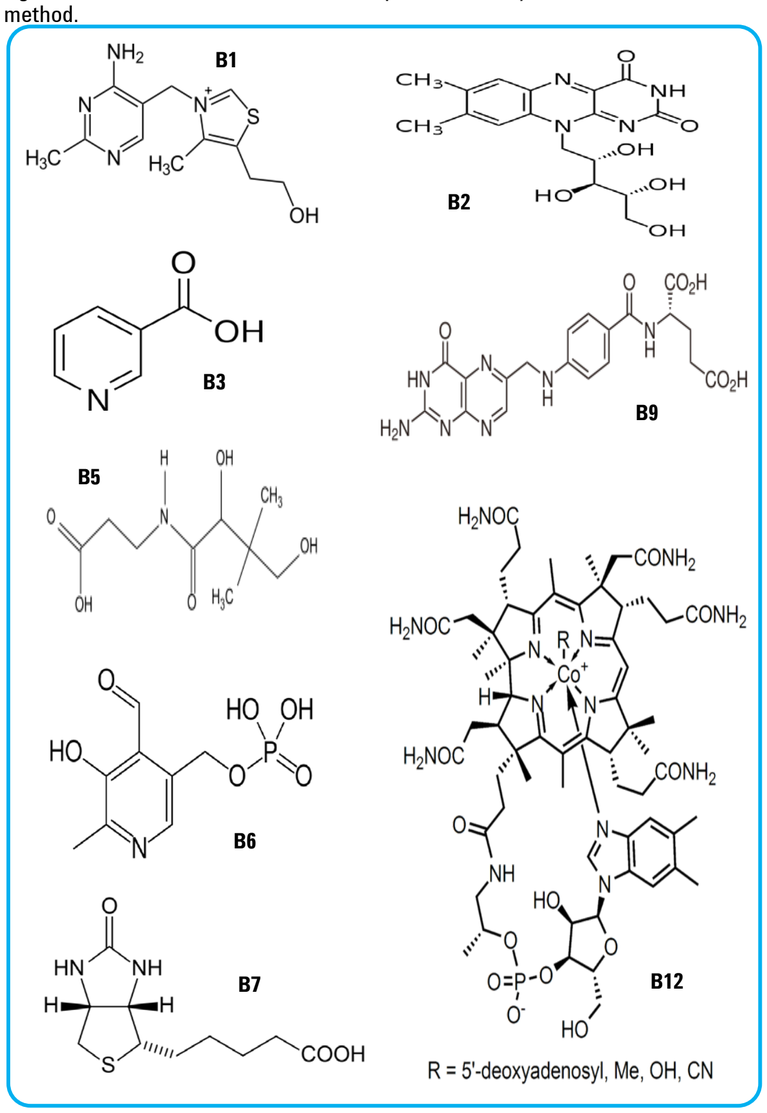
Agilent Technologies

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

Reagents, Standards, Calibrators and Controls

The following standards were obtained from Isociences

Standards	Internal Standards
Biotin	Biotin-$^{2}H_{8}$
Pyridoxal	Pyridoxal-$^{2}H_{3}$
Pyridoxine	Pyridoxine-2H3
Pyridoxamine	Pyridoxamine-$^{2}H_{3}$
Riboflavin	Riboflavin-$^{13}C_{4}$, $^{16}N_{2}$
Thiamine	Thiamine-$^{13}C_{4}$
Pantothenic Acid	Pantothenic Acid-$^{13}C_{3}$,$^{16}N_{1}$

The following standards were obtained from Cerilliant
Nicotinic Acid
Nicotinamide
Cyanocobalamin

Folic Acid — Sigma-Aldrich
Patient Samples: — 3 Serum samples
Methanol — Burdick and Jackson
Formic Acid: — Sigma Aldrich
Ammonium Formate — 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 tranferred 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
Run/Stop time:	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)	Collison Energy (V)
Thiamine	0.8	265.1 > 144/122*	87	36/8
Thiamine-$^{13}C_{4}$	0.8	269.1 > 122	82	8
Pyridoxamine	0.82	169.1 > 152*/134	87	8/20
Pyridoxamine-$^{2}H_{3}$	0.81	172.1 > 155	77	8
Pyridoxal	0.9	168.1 > 150*/94.1	82	8/24
Pyridoxal-$^{2}H_{3}$	0.93	171.1 > 97.1	72	24
Pyridoxine	0.93	170.1 > 152*/134	87	8/20
Pyridoxine-$^{2}H_{3}$	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-$^{13}C_{3}$,$^{16}N_{1}$	1.18	224.2 . 94.1	97	8
Biotin	3.04	245.1 > 227*/123	82	8/28
Biotin-$^{2}H_{8}$	3.02	253.1 > 235	102	12
Riboflavin	3.13	377.2 > 243*/172	132	24/40
Riboflavin-$^{13}C_{4}$, $^{16}N_{2}$	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 (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

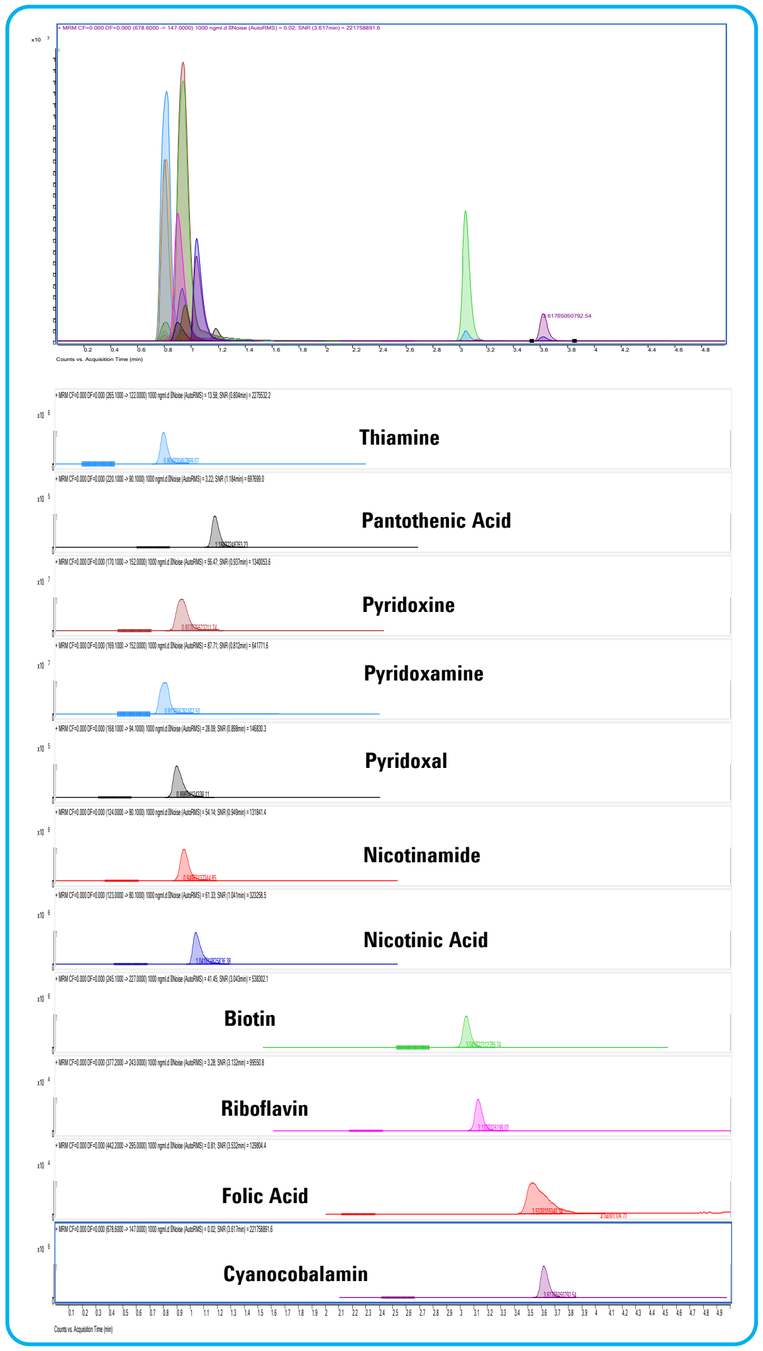
Results and Discussion

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

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