A Sensitive and Specific and Comprehensive Assay to Quantify Methylcobalamin, Cyanocobalamin and Adenosylcobalamin in Dietary Supplements using LC/MS/MS

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INTRODUCTION

Vitamin B_{12}, also called as cobalamin, has different forms like cyanocobalamin (cB_{12}), methylcobalamin (mB_{12}) and adenosylcobalamin (cobamamide) and plays a key role in the normal functioning of the brain and nervous system. These vitamin B_{12} present analytical challenges due to it's unique chemical properties and low concentration in dietary supplements. To our knowledge, there is no published ultra-sensitive LC/MS/MS method to quantitate B_{12} in finished goods. In 2017, AOAC has an open call for a quantitative vitamin B_{12} assay in dietary supplement. Here we report a comprehensive, sensitive and specific LC-MS/MS assay for cB_{12}, mB_{12} and cobamamide (***Figure 1***) in dietary supplements.

Figure 1: Chemical Structure of Cobalamins

METHODOLOGY

Sample Preparation and Extraction:
About 2 g of sample was extracted with water and methanol. The extracted sample was cleaned by 0.20 µM filtration before analysis.

UPLC-MS Conditions
UPLC system: Nexera UPLC system including SIL-30AC auto-sampler, controller, column heater and binary pump (Shimadzu)
Column: 100x2.1 mm, 1.6 µm CORTECS C_{18} (Waters)
Mobile Phase A: Formic Acid in water
Mobile Phase B: Formic Acid in acetonitrile
Flow rate: 0.40 mL/min
Pump Gradient Cycle time: 6.0 minutes
MS detector: Triple Quadrupole 4000 MS (AB Sciex)
MS Parameters: see Table 1

MS CONDITIONS					
Scan Mode	**Ion Mode**	**Source Temperature (°C)**		**Dwell Time (ms)**	
MRM	Positive	500		100	
Compounds Parameters					
Analyte	**Q1**	**Q3**	**RT (min)**	**Typical DP**	**Typical CE**
Cobamamide	791.4	665.7	1.92	52	32
mB_{12}	673.6	665.6	2.16	75	75
cB_{12}	678.6	358.9	1.80	75	71

Table 1: MS Conditions for Cobalamins

RESULTS and DISCUSSIONS

Stability

During method development, standard solution stability was evaluated, and the data indicated that B_{12} is very sensitive to light and temperature. In order to minimize the effect of the light and temperature, the sample is processed without light exposure at reduced temperature conditions (***Figure 2***). With light protection and storage at 1–8 °C, the standard solutions and extracted sample are stable for up to 9 days.

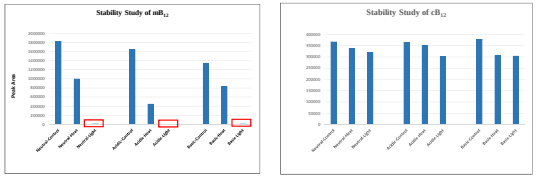

Figure 2: Stability Study of Cobalamins

Specificity

The specificity results indicated that there is no interference between analyte and IS and the method is specific and the blank sample has no interference at analyte and IS expected retention time. The representative chromatograms are shown in ***Figures 3-4***.

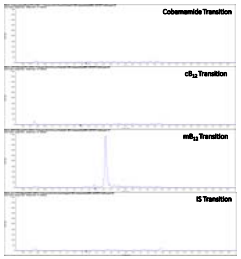

Figure 3: The chromatogram of blank sample.

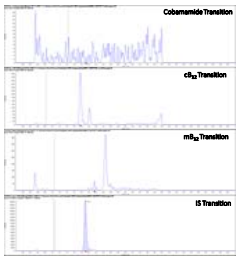

Figure 4: The chromatogram IS only sample.

Linearity

The curve range of 2.00-200 ng/mL was successfully validated. The regression is linear with 1/x as the weighing factor (***Figure 5***). The Correlation Coefficient R^2 is > 0.995. The % variance (deviation from nominal concentration) of LLOQ was 11.1% and all other points on the curve do not exceed 5.00% (***Table 2***). The representative chromatograms for LLOQ and ULOQ were in ***Figure 6***.

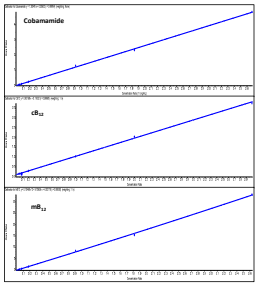

Figure 5: Typical Calibration Curve of Cobalamins

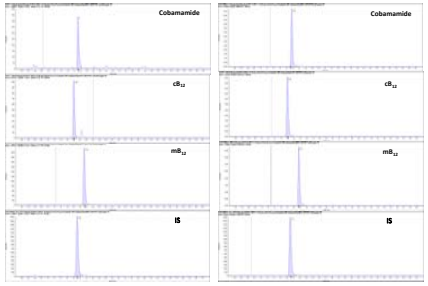

Figure 6: Representative chromatograms of LLOQ (left) and ULOQ (right).

Analyte	**Nominal Conc. (ng/mL)**	**Measured Conc. (ng/mL)**	**Accuracy(%)**	**Variance (%)**
Cobamamide	1.84	1.64	88.9	-11.1
	3.68	3.58	97.3	-2.70
	9.19	8.82	96.0	-4.00
	46.0	47.4	103	3.00
	91.9	90.9	98.9	-1.10
	184	184	100	0.00
cB_{12}	2.00	1.96	98.1	-1.90
	N/A	N/A	N/A	N/A
	9.99	10.1	101	1.00
	50.0	49.6	99.2	-0.800
	100	104	104	4.00
	200	196	98.2	-1.80
mB_{12}	1.82	1.86	102	2.00
	3.63	3.58	98.6	-1.40
	9.09	8.84	97.2	-2.80
	45.4	47.7	105	5.00
	90.9	87.99	96.8	-3.20
	182	182.00	100	0.00

Table 2: Calibration Curve Analysis

Accuracy and Precision

The accuracy and precision were investigated with post-spiking cobalamin in placebo matrix at lower, medium and high regions of the established range of the calibration curve (***Tables 3-4***).

Analyte		**QC Levels**		
		Low QC (10.0 ng/mL)	**Medium QC (50.0 ng/mL)**	**High QC (150 ng/mL)**
Cobamamide	Rep. 1	96.5	92.5	98.9
	Rep. 2	103	97.9	90.3
	Rep. 3	96.7	97.4	97.9
	Average	98.8	96.0	95.7
cB_{12}	Rep. 1	93.2	91.0	96.5
	Rep. 2	89.2	98.3	97.4
	Rep. 3	93.1	103	104
	Average	91.8	97.4	99.4
mB_{12}	Rep. 1	92.6	92.2	93.6
	Rep. 2	94.8	91.3	91.6
	Rep. 3	93.9	89.5	85.5
	Average	93.7	91	90.2

Table 3: Post-Spiking Accuracy (%)

Analyte		**%Accuracy at 150 ng/mL**
Cobamamide	Accuracy (%)	98.9
		90.3
		97.9
		99.0
		98.5
		94.6
	Average	96.5
	SD	3.5
	RSD%	**3.6**
cB_{12}	Accuracy (%)	97.4
		104
		96.4
		97.1
		96.2
		101
	Average	98.8
	SD	3.3
	RSD%	**3.4**
mB_{12}	Accuracy (%)	91.6
		85.5
		91.8
		90.9
		92.3
		87.0
	Average	89.8
	SD	2.8
	RSD%	**3.2**

Table 4: Precision

CONCLUSIONS

This is the first known published LC-MS/MS assay for quantification of cB_{12}, mB_{12} and cobamamide in dietary supplements.