A Specific, Sensitive and High-throughput Assay to Simultaneously Quantify Methylcobalamin, Cyanocobalamin and Cobamamide in Finished Goods using LC/MS/MS

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PURPOSE

Vitamin B12, including cyanocobalamin (cB12), methylcobalamin (mB12), adenosylcobalamin (cobamamide) and hydroxocobalamin, has a key role in the normal functioning of the brain and nervous system, and in the formation of red blood cells. Recently, mB12 is rapidly gaining popularity as the supplemental B12 form due to its higher bioavailability, longer retention time in tissue and it does not contain toxic cyanide. The Daily Value (%DV) of vitamin B12 has been revised from 6.0 µg downward to 2.4 µg as of May 2016, and its low content in supplements requires ultra-sensitive quantitation method. In this paper, we developed a specific, sensitive and high-throughput quantitative method for cB12, mB12 and cobamamide in finished goods using LC/MS/MS.

METHOD

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 and water
Mobile Phase B: Formic Acid and 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

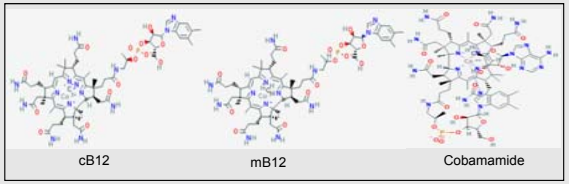

Figure 1: Chemical Structure of Cobalamins

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
mB12	673.6	665.6	2.16	75	75
cB12	678.6	358.9	1.80	75	71

Table 1: MS Condition for Cobalamins

RESULTS and DISCUSSION

During method development, standard solution stability was evaluated, and the data indicated that B12 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 (Fig. 2). Both protein and non-protein matrix samples were investigated, and it was found that the different diluents were necessary for different types of matrix. The method was successfully validated over the range of 2.00-200 ng/mL in both protein and non-protein matrix with the target concentration of sample preparation at 100 ng/mL. The specificity experiment showed that there was no significant contribution between analytes/IS and no visible interference peaks showed in blank diluent at the expected retention time (Fig. 2). The LLOQ has sufficient sensitivity (S/N> 10) (Fig. 3). System suitability consisted of six replicate injections of the middle standard solution and was injected before sample analysis, and RSD was ≤6% (Table 2). The response linearity study revealed that quadratic regression with 1/X weighing factor provides the best fit, and the correlation coefficient r^2 is ≥0.995 (Fig. 4 and Table 3). The accuracy experiment showed that the spiking recovery is within ±20% (Table 4). The %RSD of precision and repeatability was <3.6% (Table 4). The standard solution stability and extracted sample stability was established for up to 19 and 3 days at 1-8°C in light protection, respectively.

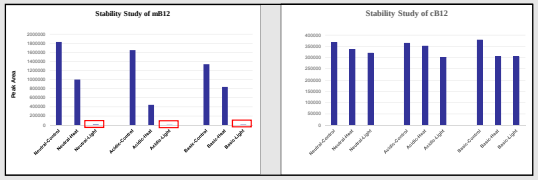

Figure 2: The Stability Study of Cobalamins

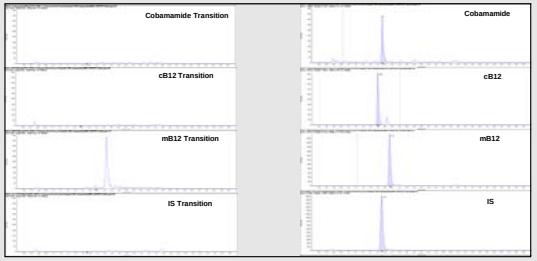

Figure 3: Blank Sample Chromatogram ***Figure 4***: LLOQ Standard Chromatogram

Compound	Cobabamide (Area)	cB12 (Area)	mB12 (Area)
Replicate 1	2643	3581	15510
Replicate 2	2491	3458	16740
Replicate 3	2487	3306	16860
Replicate 4	2445	3423	16210
Replicate 5	2709	3240	16370
Replicate 6	2475	3714	15410
Average	2542	3454	16183
Std Dev	107	175	609
RSD (%)	**4.2**	**5.1**	**3.8**

Table 2: System Suitability of Cobalamins

Analyte	Target (ng/mL)	Accuracy(%)	Regression
Cobamamide	1.84	88.9	Quadratic 1/x Weighting r^2 = 0.99986
	3.68	97.3	
	9.19	96.0	
	46.0	103	
	91.9	98.9	
	184	100	
cB12	2.00	98.1	Quadratic 1/x Weighting r^2 = 0.99930
	N/A	N/A	
	9.99	101	
	50.0	99.2	
	100	104	
	200	98.2	
mB12	1.82	102	Quadratic 1/x Weighting r^2 = 0.99922
	3.63	98.6	
	9.09	97.2	
	45.4	105	
	90.9	96.8	
	182	100	

Table 3: The Calibration Curve Analysis

Analyte		QC Levels		
		Low QC (10.0 ng/mL)	**Medium QC (50.0 ng/mL)**	**High QC (150 ng/mL)**
Cobamamide	Replicate 1	96.5	92.5	98.9
	Replicate 2	103	97.9	90.3
	Replicate 3	96.7	97.4	97.9
	Average	98.8	96.0	95.7
cB12	Replicate 1	93.2	91.0	96.5
	Replicate 2	89.2	98.3	97.4
	Replicate 3	93.1	103	104
	Average	91.8	97.4	99.4
mB12	Replicate 1	92.6	92.2	93.6
	Replicate 2	94.8	91.3	91.6
	Replicate 3	93.9	89.5	85.5
	Average	93.7	91	90.2

Table 3: The Post Spiking Accuracy (%)

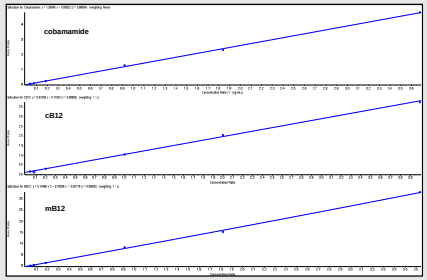

Figure 4: Typical Calibration Curve of Cobalamins

Analyte		Accuracy High QC (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**
cB12	Accuracy (%)	97.4
		104
		96.4
		97.1
		96.2
		101
	Average	98.8
	SD	3.3
	RSD%	**3.4**
mB12	Accuracy (%)	91.6
		85.5
		91.8
		90.9
		92.3
		87.0
	Average	89.8
	SD	2.8
	RSD%	**3.2**

Table 4: The Precision Data

CONCLUSIONS

This is the first known published specific, fast and high-throughput LC/MS/MS assay for quantification of cB12, mB12 and cobamamide in finished goods.