Quantitative Determination of Vitamin B12 in rodent diet by High Performance Liquid Chromatography-UV detection

Joe Warnick (jwarnick@eplbas.com) and Brett Prater (bprater@eplbas.com)

Abstract

Vitamin B_{12}, also called cobalamin, is a water-soluble vitamin with a key role in the formation of blood and the normal functioning of the brain and nervous system. It is involved in the metabolism of every cell of the human body, especially affecting DNA synthesis and regulation, fatty acid synthesis, and energy production. It is one of the eight B vitamins and it contains the biochemically rare element cobalt.

A new procedure for analysis of formulated rodent diet utilizing High Performance Liquid Chromatography with UV detection (HPLC-UV) was developed and validated. The use of a Vitamin B_{12} immunoaffinity solid phase extraction (SPE) column provided excellent selectivity and allowed for concentration of the sample on the column, further increasing the sensitivity of the method. The HPLC column selected for the assay contained a C18 bonded phase with integral polar functionality which is resistant to phase collapse even with 100% aqueous mobile phase. The method precision was 13% (relative standard deviation) for seven replicates and the overall mean spiking recovery was 82.5% for three replicates.

Objective

The objective of the research was to develop and validate an analytical method for the determination of vitamin B_{12} in rodent diet. The developed analytical procedure was based on an AOAC method for analysis of infant formula *(1), (2)*.

Materials and Methods

Chemicals and Reagents

See Figure 1 for the chemical structure of Vitamin B_{12} (Cobalamin).

- Individual stock standard solutions of vitamin B_{12} were prepared in deionized water at a concentration of 100 µg/mL.
- Subsequent dilutions of the stock solutions were prepared in 90:10 0.025% trifluoroacetic acid in DI water: 0.025% trifluoroacetic acid in ACN to achieve concentrations needed for laboratory fortification and instrument calibration.
- All standard solutions were stored in amber glass vials in a refrigerator (ca. 4°C) when not needed in the laboratory.
- Vitamin B_{12} (Cobalamin), Analytical Standard, Sigma-Aldrich, St. Louis, MO. 98%
- All standard solutions were stored in amber glass vials in a refrigerator (ca. 4°C) when not needed in the laboratory.
- Acetonitrile (ACN), HPLC grade, EMD, Billerica, MA
- Trifluoroacetic acid (TFAA), HPLC grade, EMD, Billerica, MA
- Sodium acetate trihydrate, ACS, Fisher Scientific, St. Louis, MO
- Methanol, HPLC grade, EMD, Billerica, MA
- α-amylase, Sigma-Aldrich, St. Louis, MO
- pepsin, Sigma-Aldrich, St. Louis, MO
- sodium cyanide, Sigma-Aldrich, St. Louis, MO
- NANOpure water system.

Equipment

- Balances, 0.1 and 1 mg accuracy
- Class A volumetric glassware, including actinic
- Waters Total recovery autosampler vials
- 50 mL disposable amber plastic centrifuge tubes
- Pasteur pipettes
- Bottle Top Dispenser
- Pipeters, fixed and adjustable volume
- Centrifuge
- Amber storage bottles
- 20 mL disposable syringes
- Vortex mixer
- Culture tubes, glass, 15 mL
- Platform shaker with orbital rotation
- Vortex mixer, VWR, Radnor, PA
- Nitrogen evaporation unit, N-Evap, Organomation Berlin, MA
- HPLC autosampler vials, 1.5 mL, glass, snap caps, Waters Corporation, Milford, MA
- HPLC Column: ACE C18 III AQ 3 micron 150 x 3.0 mm, Scotland
- HPLC, 2695 Separations Module, Waters Corporation, Milford, MA
- HPLC UV detector, 2487 UV/Vis, Waters Corporation, Milford, MA
- Vitamin B12 Immunoaffinity columns, R-Biopharm AG, Scotland
- SPE vacuum manifold, 24 position, Supelco (Sigma-Aldrich), St. Louis, MO
- 25mm GxF Glass syringe filter, VWR, Radnor, PA
- Syringes, 20 cc, plastic, VWR, Radnor, PA
- Waterbath, VWR, Radnor, PA

Test Matrixes

The method was validated using Purina Lab Diet 5002. Diet samples were stored in a freezer (ca. -20°C) when not needed in the laboratory.

Extraction and Cleanup Procedures:

- Weigh 5 g (4.500-5.499 g) of diet into an amber 50 mL centrifuge tube.
- Add approximately 0.05 g α-amylase and 0.5 g pepsin into the centrifuge tube.
- Laboratory fortified diet sub-samples were spiked with 0.250 mL of a 1 µg/mL vitamin B_{12} working standard solution.
- 40 mL of 0.2M sodium acetate solution was added using a bottle top dispenser or equivalent and 2 mL of the sodium cyanide solution was added to each sample vial.
- Capped and vortexed each sample for approximately 15 seconds. Samples placed into a 40 °C water bath for at least 30 minutes, shaking the samples approximately every 10 minutes.
- Samples transferred to a 100 °C water bath for at least 30 minutes, shaking the samples approximately every 10 minutes.
- Immunoaffinity columns prepared according to the manufacturer's guidelines
- Samples removed from water bath and cooled to room temperature, then centrifuged at 3000 rpm for 10 minutes.

Extraction and Cleanup Procedures:

- Filtered and transferred 9.0 mL of the supernatant into a prepared immunoaffinity column.
- Capped and shook the immunoaffinity column by hand to distribute the gel contained in the column.
- Columns shaken horizontally on an orbital shaker for approximately 15 minutes and allowed to settle for 10 minutes when removed from shaker.
- Columns placed on SPE manifold, drained, and washed with 10 mL Deionized water.
- Columns dried under slight vacuum to remove all water.
- Vitamin B_{12} was eluted with 4 mL of methanol while under vacuum.
- Evaporated the methanol solution to dryness under a stream of nitrogen using a heating block temperature setting of 50°C.
- The dried extract was reconstituted with 0.5 mL of 90:10 0.025% Trifluoroacetic acid in DI water: 0.025% Trifluoroacetic acid in ACN.
- Sample vortexed and transferred to a HPLC autosampler vial and analyzed by HPLC-UV.

HPLC-UV Parameters

- Flow Rate: 0.25 mL/min.
- Injection Volume: 100 µL
- Colum Temp.: 30°C
- UV wavelength: 361nm
- UV AUFS: 0.1
- HPLC Gradient: Linear
- Expected Retention Times (Figures 2, 3 and 4): Vitamin B_{12}: 19.93-19.95 min.

Mobile Phase Gradient Program

Time (min.)	%A	%B
0.0	99	1
3.5	99	1
11.0	85	15
19.0	75	25
20.0	90	10
25.0	99	1
30.0	99	1

A- 0.025% TFAA in DI water. B- 0.025% TFAA in ACN

A linear regression calibration curve was constructed using peak area (y-axis) and the standard solution concentration (x-axis). Sample solution concentrations were calculated using the straight line equation from the calibration curve.

Results/Discussion

Method Validation

Vitamin B_{12} content was expressed on a fresh weight basis (FW). Analyst precision (Table 1) was the relative standard deviation (RSD) obtained by a single analyst during the validation of the analytical method. Analyst accuracy (Table 2) is represented by the mean recovery of laboratory fortified samples by a single analyst during the validation of the analytical method. Measure values agreed with the certified values of 0.0273 (mg/kg FW) for Purina Lab Diet 5002.

Table 1. Analyst Precision

Matrix	Mean (n=7) (mg/kg FW)	Std. Dev.	RSD (%)	Range (mg/kg FW)
rodent diet	0.0283	0.00365	12.9	0.0248-0.0351

Results/Discussion

Table 2. Analyst Accuracy

Matrix	Fortification Level (mg/kg Fw)	Mean (n=3) Recovery (%)	(%)
rodent diet	0.05	82.5	80.7-85.4

Limit of Quantitation (LOQ):

The method LOQ for vitamin B_{12} was established at 0.25 mg/kg. LOQ's were based on peak signal to noise ratios (S/N) for rodent diet samples. S/N at the LOQ was approximately 10.

Linearity:

The linear concentration range for vitamin B_{12} was approximately 10-250 ng/mL (Figure 5).

Conclusions

A method for the extraction and HPLC-UV analysis of vitamin B_{12} in rodent diet was developed and validated. The method demonstrated acceptable accuracy, precision, sensitivity, selectivity and linear range.

Figure 1. Vitamin B_{12} structure

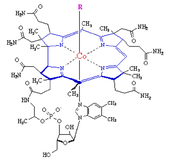


Figure 2. HPLC-UV Chromatogram- Vitamin B_{12} Calibration Standard

51 ng/mL vitamin B_{12}

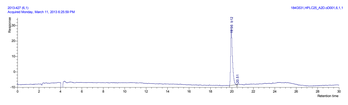


Figure 3. HPLC-UV Chromatogram- Vitamin B_{12} Calibration Standard

5 ng/mL vitamin B_{12}

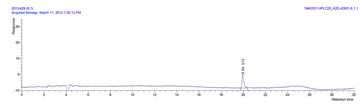


Figure 4. HPLC-UV Chromatogram- Rodent Diet Sample

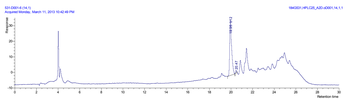
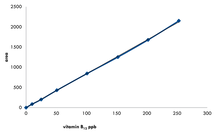


Figure 5. Vitamin B_{12} Linear Range

10-253 ng/mL

Calibration Equation =	84349815.7654x - 7.5781
Coefficient of Determination	0.9995
Correlation coefficient	0.9998
Standard Error of Calibration	18.5339



References

1) Campos-Giménez, E.; Fontannaz, P.; Trisconi, M.-J. Kilinic, T.; Gimenez, C., Andrieux, P. Determination of Vitamin B_{12} in Infant Formula and Adult Nutritionals by Liquid Chromatography/UV Detection with Immunoaffinity Extraction: First Action 2011.08. *J. AOAC Int.* **2012**, 95, No. 2.

2) Kirchner, U.; Degenhardt, K.; Raffler, G. Determination of Vitamin B12 in Infant Formula and Adult Nutritionals Using HPLC After Purification on an Immunoaffinity Column: First Action 2011.09. J. AOAC Int. 2012, 95, No. 4.