1.0 Purpose

The purpose of this procedure is to define the method for the determination and/or identification of Methylcobalamin in finished products and raw materials using HPLC and UV/VIS spectroscopy.

2.0 Scope

This procedure applies to methylcobalamin quantification and identification. Methylcobalamin is quantified at 351 nm. Alternate wavelengths may be used with justification to avoid interferences.

3.0 Responsibility

3.1 It is the responsibility of QC and analytical development personnel to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and Analytical Development personnel to keep this procedure current with latest Ion Labs practices.

4.0 Definitions

4.1 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.2 **H_3PO_4** – Phosphoric Acid

4.3 **KH_2PO_4** – Potassium phosphate monobasic

4.4 **ACN** – Acetonitrile

4.5 **KOH** – Potassium Hydroxide

4.6 **H_2O** – Water

4.7 **QC** – Quality Control

5.0 References

5.1 HPLC Application ID# 2581 by Phenomenex, 2014

5.2 MV-LAB-15-004, Protocol, Methylcobalamin Determination using HPLC and UV/VIS Spectroscopy.

5.3 Novel RP-HPLC method for the Simultaneous Estimation of Thiamine Mononitrate, Calcium Methylcobalamin, L-Cystine and Para Amino Benzoic Acid in Multi Vitamin Dosage Forms. Tamma Narendra Kumar *et al.*, IJSID **2011**, 1 (20), 226-242.

5.4 D-793, SOP, Cryogenic Grinding of Chewable Gels

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 KH_2PO_4

6.1.4 H_3PO_4

6.1.5 KOH

6.1.6 Methylcobalamin, Reference Standard, MW 1344.40, CAS No: 13422-55-4

6.2 Glassware

6.2.1 Low Actinic HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.2 Scintillation Vials

6.2.3 Mobile Phase Containers

6.2.4 25mL, 50mL, 100ml Low Actinic Volumetric Flasks

6.3 Disposables

6.3.1 Pipette Tips

6.3.2 Microfuge tubes

6.3.3 Disposable Plastic Luer Lock Syringe – 3mL, 6mL, or 10mL

6.3.4 Nylon Syringe Filters, 0.45µm

6.3.5 Weigh paper

6.4 Equipment

6.4.1 Suitable HPLC system consisting of a pump, autosampler, column compartment, and diode array detector. Analytical Balance

6.4.2 Vortex

6.4.3 Stir Plate

6.4.4 Wrist-action mechanical shaker

6.4.5 Eppendorf Centrifuge

6.4.6 Adjustable Pipettes

7.0 Preparation of Mobile Phase, Diluent, Samples, and Standards

7.1 Mobile Phase and Buffer Preparation

7.1.1 Mobile Phase A – Prepared by dissolving 680.5mg KH_2PO_4 , in 950ml of Millipore water adjusted pH to 5.6 with phosphoric acid or potassium hydroxide, then bring up to 1 liter

7.1.2 Mobile Phase B – 100% ACN

7.1.3 Diluent– 95:5 (Mobile A : Mobile B)

7.2 Standard Preparation

Note: Methylcobalamin is especially susceptible to degradation in solution. Ensure the autosampler chiller is set to 4°C, and place the working sample and/or standard

into a vial in the autosampler quickly to avoid degradation. Ensure that it is not exposed to light/heat sources and samples should be analyzed soon after preparation.

7.2.1 The standard is prepared by weighing no less than the scale's limit at the time of use, then bringing up to two thirds the final volume in an appropriate low actinic volumetric flask using Diluent then shaking for up to 20 minutes (until no solid material remains) and bringing up to final volume with Diluent.

7.2.2 Dilutions can be made using low actinic volumetric flasks, volumetric glass pipettes, and/or adjustable pipettes. Specific standard concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label. Dilutions can be prepared in low actinic HPLC vials.

7.3 Sample Preparation

Note: Avoid exposure to light during sample preparation. Samples should be prepared in low actinic (red) glassware, placed in the chilled autosampler, and used within 2 hours.

7.3.1 The linear range of the method is 0.01 – 0.5 mg/mL. All standards and samples to be injected must be within this range.

7.3.2 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, then follow preparation procedure as described below, maintaining concentration within the linear range listed above.

7.3.3 For raw materials: weigh no less than 20 mg into a suitably sized volumetric flask of no less than 25 mL volume to generate an analyte concentration that is within the validated linearity range. Add Diluent to 2/3 of the flask volume, shake mechanically for 10 min, dilute to volume with Diluent, and mix well.

7.3.4 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (capsules), serving

size (powders) or tablet weight per dose, weigh no less than 50 mg of the pooled dosages into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linear range. Add Diluent to 2/3 of the flask volume, shake mechanically for 10 min, dilute to volume with Diluent, and mix well.

7.3.5 For chewable gels (gummies), homogenize at least 10 dosage units according to the procedure outlined in D-793 Cryogenic Grinding of Chewable Gels. Quickly weigh a portion of the pooled and homogenized dosages into a beaker. Use several small portions of Diluent to completely transfer the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Add Diluent to 2/3 of the flask volume, shake mechanically for 10 min, dilute to volume with Diluent, and mix well.

7.3.6 When the sample matrix is found to cause greater instability to methylcobalamin, vigorous shaking for 1 minute can be used in place of mechanical shaking to reduce degradation.

7.3.7 To manage large volumes, the standard can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials

7.3.8 Filter an aliquot of the sample through a 0.45 µm membrane discarding the first 3 – 4 mL before collecting a portion for analysis.

8.0 Test Conditions

8.1 Gradient

Time	%A	%B	Gradient Type
0.00	85	15	Linear
5.00	85	15	Linear

10.00	75	25	Linear
11.50	55	45	Linear
11.60	50	50	Linear
14.00	90	10	Linear

8.2 Column – Phenomenex Luna C8 (2), 5µm, 100Å, LC column, 150mm x 4.6mm

8.3 Flow Rate – 1.0 mL/min

8.4 UV Detection – 351nm

8.5 Injection Volume - 20µL

8.6 Column Temperature – 30°C

8.7 Peltier Sample Chiller - 4°C, equilibrated before adding test samples

8.8 Retention Time – about 9 min

8.9 Recommended Sequence

8.9.1 Perform 2 Blank (Diluent) injections.

8.9.2 Perform 5 injections of the Working Standard.

8.9.3 Perform a single injection of each sample preparation.

8.9.4 Perform 1 injection of the Working Standard after every 6 samples and at the end of the run.

8.10 System Suitability

8.10.1 %RSD of 5 injections of working standard is NMT 3%.

8.10.2 %RSD of all injections of working standard is NMT 5%.

9.0 Example Calculation

$$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount in mg (solids) or mL (liquids)

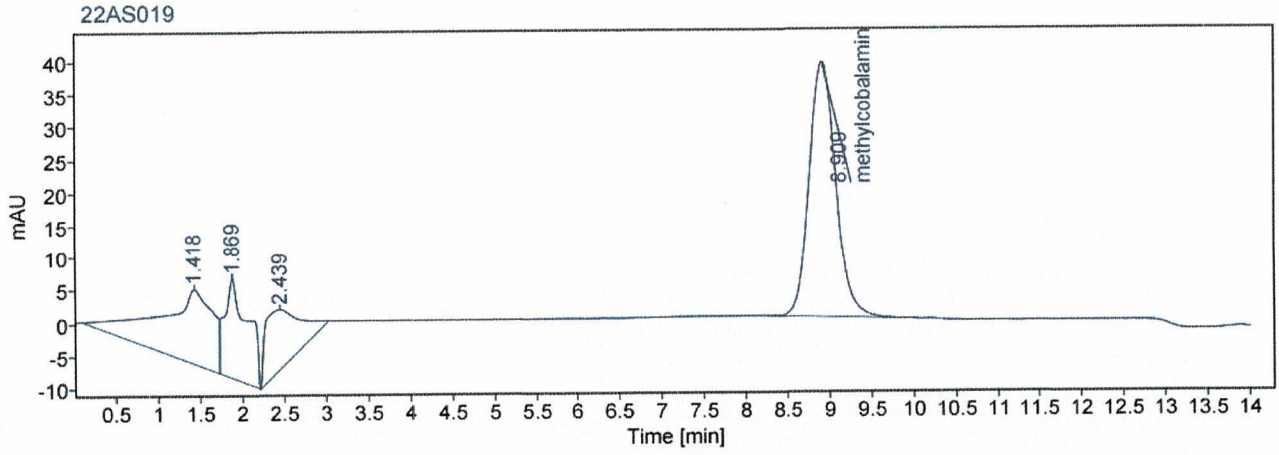
V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

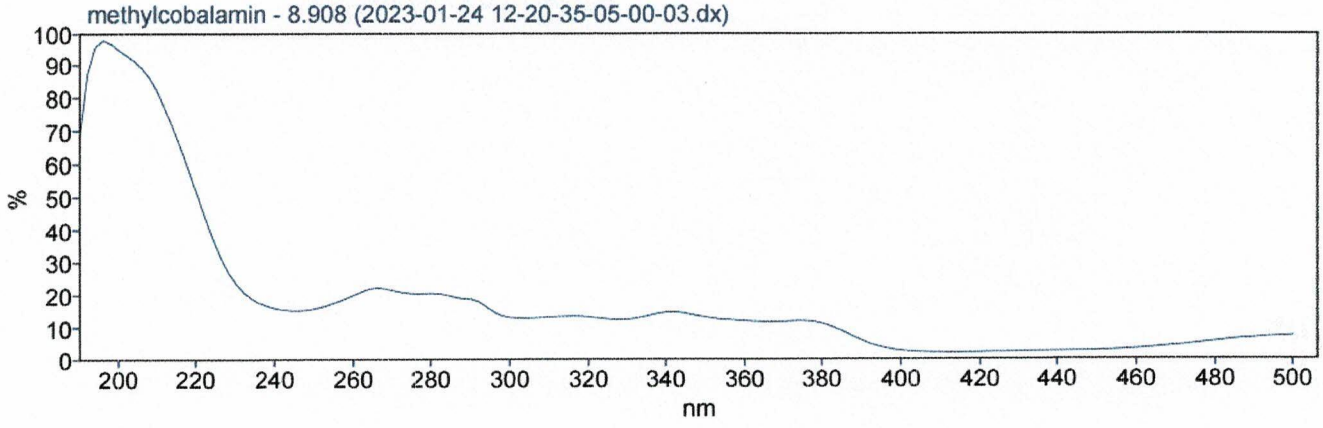
LA Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography and Spectrum

10.1 Standard



10.2 UV Spectrum



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	08/21/15	New	15-0800	X. Shao
1	04/19/17	Updated testing procedure to meet current practices.	17-0435	B. Johns
2	04/15/20	Scheduled review: Added information about the lack of stability of Methylcobalamin. Removed release requirements	CC-20-0293	J. Maignan
3	03/23/22	Updated to match current practices. Updated format. Removed references to specific HPLC systems.	CC-22-0113	S. Sassman
4	05/17/23	Removed unnecessary information and align with current SOP format, Added instruction to follow product specific test details. Added specific sample prep for different dosage forms. Added example chromatography and spectrum. Changed logo.	CC-23-0224	S. Sassman