1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis and spectral identification of water soluble vitamins in finished products and raw materials using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure has been validated for use in identifying and quantifying seven common water soluble vitamins. The quantitation and/or identification can be completed individually or multiple vitamins can be quantified within a single chromatogram. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of individual vitamins which may require the use of an alternate wavelength that does not exhibit interference.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to understand and work within the guidelines of 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/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **Ascorbic Acid** – Vitamin C

4.2 **Niacin** – Vitamin B_3

4.3 **Thiamine** – Vitamin B_1

4.4 **Riboflavin** – Vitamin B_2

4.5 **Folic Acid** – Vitamin M or Vitamin B_9

4.6 **Cyanocobalamin** – Vitamin B_{12}

4.7 **Pyridoxine** – Vitamin B_6

4.8 **H_2O** – Water ($\geq$ 18.2 MΩ·cm)

4.9 **ACN** – Acetonitrile

4.10 **H_3PO_4** – Phosphoric Acid (85%)

4.11 **K_2HPO_4** – Potassium Phosphate Dibasic

4.12 **$Na_2EDTA{\cdot}2H_2O$** – Ethylenediaminetetraacetic acid disodium salt dihydrate

4.13 **$Na_2B_4O_7{\cdot}10H_2O$** – Sodium borate decahydrate

4.14 **HCl** – Concentrated hydrochloric acid (~37%)

4.15 **WSMV** – Water Soluble Multi-Vitamin

4.16 **CofA** – Certificate of Analysis

4.17 **USP** – United States Pharmacopeia

4.18 **HPLC** – High Performance Liquid Chromatography

4.19 **UV/Vis** – Ultraviolet and Visible Spectroscopy

5.0 References

5.1 MV-LAB-12-011, Protocol, Water Soluble Vitamins in Multicomponent Formulations Determination by HPLC

5.2 RPT-23-0063, Report, Extend Pyridoxine Linearity for D-728

5.3 NCR-23-0010, Product Specific Method Optimization, Riboflavin in SEC00448 and SCT00447

6.0 Supplies

6.1 Chemicals: all reagents are HPLC grade or better. Vitamins when used as standards should be USP traceable when available.

6.1.1 H2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 K_2HPO_4 (MW 174.18)

6.1.5 $Na_2EDTA \cdot 2H_2O$

6.1.6 $Na_2B_4O_7 \cdot 10H_2O$

6.1.7 HCl

6.1.8 Reference Standards

6.1.8.1 Ascorbic Acid

6.1.8.2 Niacin

6.1.8.3 Thiamine

6.1.8.4 Riboflavin

6.1.8.5 Folic Acid

6.1.8.6 Cyanocobalamin

6.1.8.7 Pyridoxine

6.1.8.8 Pantothenate

6.1.8.9 Biotin

6.2 Glassware

Note: Low actinic glassware recommended for riboflavin. Glassware may also be shielded from light using aluminum foil.

6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa.

6.2.2 Scintillation vials

6.2.3 1L mobile phase container

6.2.4 50mL, 100mL, and 500mL volumetric flasks

6.3 Disposables

6.3.1 200uL, 1mL, and 10mL pipette tips

6.3.2 Disposable plastic luer lock syringe- 3mL, 6mL or 10mL

6.3.3 Nylon syringe filters, 25mm or larger diameter.

6.3.4 Weigh boats

6.3.5 1.5mL and 2.0mL Eppendorf centrifuge tubes

6.4 Equipment

6.4.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.4.2 Analytical Balance

6.4.3 Vortex

6.4.4 Stir Plate

6.4.5 Wrist Action Shaker

6.4.6 Eppendorf centrifuge

6.4.7 200uL, 1mL and 10mL pipettes

7.0 Preparation of Mobile Phase, Dissolution Buffer, Samples and Standards

7.1 Mobile Phase A (0.1% H_3PO_4 in H_2O) - add 1.0 mL H_3PO_4 to 1000 mL H_2O.

7.2 Mobile Phase B (0.1% H_3PO_4 in ACN) - add 1.0 mL H_3PO_4 to 1000 mL ACN.

7.3 WSMV Dissolution Buffer (0.01M K_2HPO_4, used for all analytes except riboflavin) - add 1.7418g of K_2HPO_4 to 1000 mL H_2O, and mix well to dissolve.

7.4 Riboflavin Dissolution Buffer (90 mM EDTA + 125 mM Borate, pH=8.0) – Dissolve 33.5 g of $Na_2EDTA{\cdot}2H_2O$ and 47.7 g of $Na_2B_4O_7{\cdot}10H_2O$ in 1 L of H_2O by stirring under gentle heat, adjust to pH 8.0 using HCl.

7.5 Standard Preparation

7.5.1 Linear range – all standard and sample preparations must be within the linear range of the method. The values listed below are valid for a 20 µL injection volume. If another injection volume is used, the range should be corrected for injection volume (e.g. if the range is 0.01 – 0.1 mg/mL at 20 µL injection volume, then the range would be 0.02 – 0.2 mg/mL at 10 µL injection volume).

7.5.1.1 Ascorbic Acid (270 nm) – 0.1 mg/mL – 1.0 mg/mL

7.5.1.2 Cyanocobalamin (225 nm) – 0.00025 mg/mL – 0.1 mg/mL

7.5.1.3 Folic Acid (225 nm) – 0.003 mg/mL – 0.04 mg/mL

7.5.1.4 Niacin (230 nm) – 0.0025 mg/mL – 1.0 mg/mL

7.5.1.5 Pyridoxine (225 nm) – 0.0003 mg/mL – 1.0 mg/mL

7.5.1.6 Riboflavin (225 nm) – 0.0025 mg/mL – 0.06 mg/mL

7.5.1.7 Thiamine (225 nm) – 0.0025 mg/mL – 1.0 mg/mL

7.5.2 For low concentration standards and samples, it may be necessary to first prepare a higher concentration stock and subsequently dilute to the final concentration. The suggested maximum concentration of stock solutions with consideration to solubility are listed below.

7.5.2.1 Ascorbic Acid - 1.0mg/mL

7.5.2.2 Cyanocobalamin - 0.1mg/mL

7.5.2.3 Folic Acid - 0.1mg/mL

7.5.2.4 Niacin - 1.0mg/mL

7.5.2.5 Pyridoxine - 1.0mg/mL

7.5.2.6 Riboflavin - 0.1mg/mL

7.5.2.7 Thiamine - 1.0mg/mL

7.5.3 Use the actual purity from the CofA or the standard certification for the vitamin reference material for calculations. The Stock Standard Preparation reflects 100% content for the vitamin assayed.

7.5.4 Standards are prepared by weighing no less than the minimum weight of the analytical balance then bring up to two thirds of the final volume in an appropriate volumetric flask using Dissolution Buffer. Dissolution can be achieved with the use of a wrist action shaker or sonicator. Avoid long sonication times for heat labile compounds such as thiamine, ascorbic acid, and cyanocobalamin. The standard may require dilution to final volume to achieve complete dissolution.

7.5.5 Dilutions are prepared using Dissolution Buffer. Dilutions can be made using volumetric flasks or using 10mL, 1mL and 200uL variable pipettes. Specific standard working concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from label. Dilutions can be prepared directly in HPLC vials.

7.6 Sample Preparation

7.6.1 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 of this method.

7.6.2 For solid form finished products, recommended sample preparation- 20 or more dosage units pooled and ground by mortar and pestle as necessary.

7.6.3 Based on the average fill weight per dose weigh a portion of the parent sample to achieve the target analyte concentration for the selected sample volume giving consideration to the solubility and linearity requirements.

7.6.4 Samples can be dissolved or diluted in Dissolution Buffer at any volume no less than 25mL. To manage large volumes the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion diluted to bring the analyte concentration into the range of measurement. The final diluted sample must be filtered or centrifuged before analyzing by HPLC.

7.6.4.1 If analysis is required at a concentration below the validated linear range, a minimum of a three point standard curve is required and the coefficient of determination (R^2) must be no less than 0.99. Additionally, the sample concentration must be within the demonstrated range of linearity for result to be valid. At very low concentrations spectral identification may not be suitable and 3-D spectra of the chromatogram may be used to visually determine that no interference is present at the wavelength used in the quantitative analysis.

7.6.5 Optimizing the dissolution process for a given matrix can be characterized during system suitability. The recommended starting point is dissolution in 2/3 final volume for 30 minutes on a wrist action shaker. Sonication and other methods of dissolution may be used with proper verification of recovery and stability. Avoid long sonication times for heat labile compounds such as thiamine, ascorbic acid, and cyanocobalamin.

7.6.6 Particulate must be removed from working sample prior to injection. Particulate can be removed by filtration using a plastic syringe coupled with a 0.2µM to 0.45µM nylon syringe filter. Discard at least the first 0.5mL of filtrate before collecting a portion for analysis. Particulate can also be removed by centrifuging for 3 minutes at 6000rpm.

8.0 Test Conditions

8.1 Gradient (acceptable for all target analytes)

Time	%A	%B	Gradient type

0.00	98	2	0
2.80	98	2	0
2.81	90	10	step
7.30	50	50	linear
7.40	98	2	linear
10.00	98	2	0

8.2 Isocratic (only for ascorbic acid, niacin, pyridoxine, and thiamine)*

Time	%A	%B	Gradient type
0.00	98	2	0
4.00	98	2	0

* wash column with H2O/ACN (10/90) as outlined below after use when using isocratic

8.3 Column- Luna C5, 5µm, 150mm X 4.6mm

8.4 Flow Rate- 1.0mL/min

8.5 Recommended spectral range for identification- 205nm to 500nm

8.6 Injection volume- 20uL (other volumes may be used to optimize separation provided that the standard and sample are within the range of linearity).

8.7 Column Temperature- 45°C

8.8 Approximate Retention Times

8.8.1 Ascorbic Acid – 2.4 min (isocratic)

8.8.2 Cyanocobalamin – 7.0 min (gradient)

8.8.3 Folic Acid – 7.0 min (gradient)

8.8.4 Niacin (nicotinic acid) – 2.2 min (isocratic)

8.8.5 Niacin (niacinamide) – 1.8 min (isocratic)

8.8.6 Pyridoxine – 1.7 min (isocratic)

8.8.7 Riboflavin – 7.3 min (gradient)

8.8.8 Thiamine – 1.2 min (isocratic)

9.0 Recommended Sequence

9.1.1 Make at least 2 injections of the diluent.

9.1.2 Make five (5) injections of Standard Solution.

9.1.3 Make a single injection of each Sample Preparation.

9.1.4 Make a single injection of the Standard Solution after every six (6) samples and at the end of the run.

9.2 System Suitability Requirements

9.2.1 The %RSD of the first five (5) standard injections is NMT 5.0%.

9.2.2 The %RSD of all standard injections is NMT 5%.

9.3 Column Wash and Storage

9.3.1 Wash the column with H_2O:ACN (50:50) at 1 mL/min for at least 30 min.

9.3.2 Store the column with H_2O:ACN (50:50).

10.0 Example Calculation

10.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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

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

SS Serving size: Weight of a single dosage unit in mg, or 1 for raw materials.

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

11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	01/14/13	New	-	-
2	05/20/13	Updated format. Added calculations for direct % label measurement. Added more detailed instructions on sample preparation.	13-237	B. Johns
3	03/12/14	Increased readability with new format. Standardized wording. Added pantothenate to the list of vitamins.	14-0191	B. Johns
4	09/08/16	Updated to new SOP format. Added expanded information on current practices.	16-0823	N. Zhang
5	07/03/18	Updated SOP to match current Ion practices. Added Biotin to list of approved vitamins.	18-0219	J. Maignan
6	04/15/22	Update to reflect current practices, remove biotin and pantothenate as analytes, add recommended sequence, add column wash and storage, add retention times, add option to reduce wavelength range used for spectral match.	CC-22-0182	S. Sassman
7	12/20/22	Added test details section. Minor edits.	CC-22-0475	J. Sassman
8	10/19/23	Expand linear range for pyridoxine, allow long gradient for all analytes.	CC-23-0521	S. Sassman
9	10/31/23	Add new Dissolution Buffer for riboflavin.	CC-23-0537	S. Sassman