1.0 Purpose

The purpose of this procedure is to define the parameters for the analysis and/or identification of Water Soluble Vitamins and other chemically similar compounds in raw materials and dietary supplements using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the identification and/or quantification of pantothenate, riboflavin, pyridoxyl-5-phosphate, resveratrol and ascorbic acid in the QC laboratory at Ion Labs.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists who have verified their ability to execute this procedure to follow this procedure.

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

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development to keep this procedure is aligned with current practices.

4.0 Definitions

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

4.2 **HPLC** – High Performance Liquid Chromatography

4.3 **QC** – Quality Control

4.4 **H_3PO_4** – Phosphoric Acid

4.5 **Riboflavin** – Vitamin B_2

4.6 **Pantothenate** – Vitamin B_5

4.7 **Pyridoxal 5' Phosphate** – Vitamin B_6

4.8 **Ascorbic Acid** – Vitamin C

4.9 **K_2HPO_4** – Potassium Phosphate Dibasic

4.10 **$NaH_2PO_4 \cdot 2H_2O$** – Sodium Phosphate Monobasic Dihydrate

4.11 **NaOH** – Sodium Hydroxide

4.12 **NaCl** – Sodium Chloride

4.13 **$Na_2EDTA \cdot 2H_2O$** – Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate

4.14 **H_2O** – Water ($\geq$ 18.2 M$\Omega \cdot$cm)

5.0 References

5.1 HPLC Application ID# 2581 by Phenomenex, 2014

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

5.3 MV-LAB-14-031, Protocol, General Method for Water Soluble Vitamin and Other Analyte Determination using HPLC and UV/VIS Spectroscopy

5.4 MV-LAB-18-061, Protocol, Ascorbic Acid Determination by HPLC using UV/Vis Spectroscopy

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better.

6.1.1 H_2O

6.1.2 Methanol

6.1.3 Sodium 1-hexanesulfonate monohydrate

6.1.4 H_3PO_4

6.1.5 NaOH

6.1.6 NaCl

6.1.7 $Na_2EDTA \cdot 2H_2O$

6.1.8 Citric Acid

6.1.9 K_2HPO_4

6.1.10 $NaH_2PO_4 \cdot 2H_2O$

6.1.11 Reference Standards

6.1.11.1 Calcium Pantothenate

6.1.11.2 Riboflavin

6.1.11.3 Ascorbic acid

6.1.11.4 Pyridoxal 5' Phosphate

6.1.11.5 Resveratrol

6.2 Glassware

6.2.1 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 Volumetric glassware as required for standard and sample preparations

6.3 Disposables

6.3.1 Tips for adjustable pipets

6.3.2 Centrifuge tubes

6.3.3 Disposable plastic luer lock syringe and 0.45 µm syringe filters

6.3.4 Weigh paper

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 Eppendorf centrifuge

6.4.6 Adjustable pipets

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

7.1 Mobile Phase A

7.1.1 Transfer 2.5 g sodium 1-hexanesulfonate monohydrate to a 1000-mL bottle.

7.1.2 Add 1000 mL H_2O.

7.1.3 Add 2.0 ml H_3PO_4, and mix until dissolved.

7.1.4 Filter through 0.45 µm nylon membrane.

7.2 Mobile Phase B

7.2.1 Transfer 2.5 g sodium 1-hexanesulfonate monohydrate to a 1000-mL bottle.

7.2.2 Add 1000 mL of methanol.

7.2.3 Add 2.0 ml H_3PO_4, and mix until dissolved.

7.2.4 Filter through 0.45 µm nylon membrane.

7.3 Diluent/Extraction Solvent

7.3.1 Pantothenate

7.3.1.1 H_2O

7.3.2 Pyridoxal 5' phosphate

7.3.2.1 Transfer 1.25 g of NaCl to a suitable container.

7.3.2.2 Add 9.6 g of Citric Acid.

7.3.2.3 Add 93 mg of $Na_2EDTA{\cdot}2H_2O$.

7.3.2.4 Stir until dissolved.

7.3.2.5 Adjust to pH 5.0 using 50% NaOH.

7.3.3 Riboflavin

7.3.3.1 Transfer 1.74 g K_2HPO_4 to a l000-mL bottle.

7.3.3.2 Add 1000 mL H_2O, and mix to dissolve.

7.3.4 Resveratrol

7.3.4.1 Methanol

7.3.5 Ascorbic Acid

7.3.5.1 Transfer 2.34 g $NaH_2PO_4{\cdot}2H_2O$ to a suitable container.

7.3.5.2 Add 0.56 g of $Na_2EDTA{\cdot}2H_2O$.

7.3.5.3 Stir until dissolved.

7.3.5.4 Adjust to pH 3.0 with H_3PO_4.

7.4 Standard Preparation

7.4.1 The linear range of the method for each analyte is listed below. All standard and sample preparations must be within this range.

7.4.1.1 Pantothenate (210 nm) – 1.25 µg/mL – 1000 µg/mL

7.4.1.2 Riboflavin (270 nm) – 0.25 µg/mL – 100 µg/mL

7.4.1.3 Pyridoxal 5' Phosphate (295 nm) – 1.25 µg/mL – 800 µg/mL

7.4.1.4 Resveratrol (306 nm) – 0.25 µg/mL – 200 µg/mL

7.4.1.5 Ascorbic Acid (245 nm) – 10 µg/mL – 100 µg/mL

7.4.2 **Riboflavin, pyridoxal 5' phosphate, and resveratrol are light sensitive. Use low-actinic (red) glassware to minimize light exposure.**

7.4.3 The standard is prepared by weighing no less than the minimum weight of the analytical balance, transfer to an appropriately sized volumetric flask and bring up to two thirds the final volume using Diluent, shake until completely dissolved, and bring up to final volume.

7.4.4 Dilutions are prepared using Diluent. Dilutions can be made using volumetric flasks or using 10mL, 1mL, and 200µL variable 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 HPLC vials.

7.5 Sample Preparation

7.5.1 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.

7.5.2 The concentration of the final sample preparation must be within the linear range listed above.

7.5.3 **Riboflavin, pyridoxal 5' phosphate, and resveratrol are light sensitive. Use low-actinic (red) glassware to minimize light exposure.**

7.5.4 Samples can be dissolved in Diluent at any volume starting from 50 mL and any weight greater than the minimum weight of the analytical balance.

7.5.5 The sample is suspended in two third of the final volume and put in the wrist action shaker for 25 minutes. Then bring up to the final volume using Diluent.

7.5.6 Before injection, insoluble matter should be removed via filtration using a nylon syringe filter. Discard at least the first 0.5 mL of filtrate before collecting a portion

for analysis. Dilute filtrate as needed then add 1mL of the final dilution to an HPLC vial for analysis.

7.5.6.1 Alternatively, samples and standards can also be centrifuged at 10,000 RPM for 5 minutes in an Eppendorf centrifuge to pellet insoluble matter.

8.0 Test Conditions

8.1 Pantothenate (210 nm, retention time ≈ 5.6 min)

8.1.1 Gradient – Isocratic

Time	%A	%B
0.00	85	15
10.00	85	15

Gradient – Multistep

Time	%A	%B
0.00	85	15
10.00	85	15
10.10	60	40
14.00	60	40
14.01	85	15
20.00	85	15

8.2 Riboflavin (270 nm, retention time ≈ 8.2 min)

8.2.1 Gradient – Multistep

Time	%A	%B
0.00	70	30
5.00	70	30

7.00	65	35
10.00	65	35
10.01	70	30
14.00	70	30

8.3 Pyridoxal 5' Phosphate (295 nm, retention time ≈ 6.7 min)

8.3.1 Gradient – Isocratic

Time	%A	%B
0.00	93	7
12.00	93	7

8.4 Resveratrol (306 nm, retention time ≈ 22 min)

8.4.1 Gradient - Multistep

Time	%A	%B
0.00	90	10
5.00	80	20
15.00	70	30
15.10	55	45
21.00	50	50
21.10	90	10
25.00	90	10

8.5 Ascorbic Acid (245 nm, retention time ≈ 2.7 min)

8.5.1 Gradient – Isocratic

Time	%A	%B
0.00	93	7

12.00 93 7

8.6 Column – Kinetex XB C18, 5µm, 100Å, 250mm x 4.6mm

8.7 Flow Rate – 1.0mL/min

8.8 Injection Volume - 20µL

8.9 Column Temperature – 28°C

8.10 Recommended 3D Spectral Range – 200 nm – 500 nm

8.11 Recommended Sequence

8.11.1 Make at least two injections of the diluent.

8.11.2 Make five injections of Standard Solution.

8.11.3 Make a single injection of each Sample Preparation.

8.11.4 Make a single injection of the Standard Solution after every six samples and at the end of the run.

8.12 System Suitability Requirements

8.12.1 The %RSD of the first five standard injections is NMT 5.0%.

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

8.13 Column Wash and Storage

8.13.1 Wash the column with H_2O:Methanol (90:10) at 1 mL/min for at least 15 min.

8.13.2 Wash the column with H_2O:Methanol (10:90) at 1 mL/min for at least 15 min.

8.13.3 Store the column with H_2O:Methanol (10:90).

9.0 Example Calculations

9.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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 (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 Revision History

Revision	Date	Description of Changes	CCR #	By
0	11/12/14	New	14-0913	X. Shao
1	02/24/17	Biennial review: Fixed typographical error and updated stability requirement to 100%	16-1139	J. Maignan
2	07/03/18	Added Ascorbic acid to method.	18-0221	J. Maignan
3	04/15/22	Update to reflect current practices and for clarity, add reference to original validation, add recommended sequence, add system suitability, add column wash and storage, add retention times.	CC-22-0183	S. Sassman
4	02/22/23	Update for consistency with current methods, add instruction to check the product profile for test details, remove language requiring in-process validation for new products, remove spectral match and RT match from system suitability since these are requirement of the sample and not the system.	CC-23-0094	S. Sassman
5	11/28/23	Add analyte specific extraction solvent/diluent for P5P and ascorbic acid to improve analyte stability. Add note about light sensitivity of riboflavin, P5P, and resveratrol. Minor edits for consistency with current methods.	CC-23-0574	S. Sassman