1.0 Purpose

The purpose of this procedure is to define the method for the quantification and identification of Rebaudioside A and Rebaudioside M in raw materials and finished products by HPLC/UV.

2.0 Scope

This procedure applies to the quantification and identification of Rebaudioside A and Rebaudioside M in raw materials and finished products.

3.0 Responsibility

3.1 It is the responsibility of QC Chemists to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management 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 aligned with current practices.

4.0 Definitions

4.1 **HPLC/UV** – High Pressure Liquid Chromatography with Ultraviolet Detection

4.2 **QC** – Quality Control

4.3 **RBA** – Rebaudioside A

4.4 **RBM** – Rebaudioside M

4.5 **ACN** – Acetonitrile

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

4.7 **H_3PO_4** – 85% Phosphoric Acid

4.8 **H_2O** – Deionized Water

5.0 References

5.1 MV-LAB-19-047, Protocol, Validation of an Analytical Method for the Determination of Rebaudioside A and Rebaudioside M by HPLC/UV

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better

6.1.1 RBA Reference Standard

6.1.2 RBM Reference Standard

6.1.3 ACN

6.1.4 $NaH_2PO_4 \cdot H_2O$

6.1.5 H_3PO_4

6.1.6 Glassware

6.1.6.1 Volumetric glassware as required for standard and sample preparations

6.2 Disposables (as required for standard and sample preparations)

6.2.1 10mL Pipette Tips

6.2.2 1mL Pipette Tips

6.2.3 200µL Pipette Tips

6.2.4 Microcentrifuge tubes

6.2.5 16mL Test Tubes

6.2.6 Weigh paper

6.3 Equipment

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

6.3.2 Analytical Balance

6.3.3 Centrifuge

6.3.4 Adjustable Pipette

7.0 Procedure

7.1 Mobile Phase Preparation

7.1.1 Buffer Solution

7.1.1.1 Combine 1.56 g of $NaH_2PO_4 \cdot 2H_2O$ and 1000 mL of H_2O. Begin stirring and adjust the pH to 2.6 using H_3PO_4.

7.1.1.2 Other hydration states of NaH_2PO_4 may be used provided that correction is made for molecular weight.

7.1.2 Mobile Phase

7.1.2.1 Combine 730 mL of Buffer Solution with 270 mL of ACN.

7.1.3 Diluent

7.1.3.1 Combine 350 mL of H_2O with 150 mL of ACN.

7.2 Standard Preparation

7.2.1 Use the actual purity from the CofA for the reference standards in your calculations. The Standard Preparation reflects 100% of the label quantity. Standard solutions should be prepared fresh daily.

Example: RBA, 98.5% purity

Prepare 100 mL of a 0.5 mg/mL solution

0.5 mg/mL X 100 mL = 50 mg

50 mg / 0.985 = 50.76 mg

Dissolve 50.76 mg up to 100mL = 0.5 mg/mL RBA

7.2.2 All standards are prepared by weighing no less than 20 mg into an appropriately sized volumetric flask. Dissolve in diluent, and bring to final volume with diluent.

7.2.3 Dilutions should be prepared using 1 mL and 200 µL variable pipettes and/or volumetric glassware. Working standard concentration should approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label. Final dilutions may be prepared directly in HPLC vials.

7.2.4 Working standards and samples must be within the linear range of the method:

7.2.4.1 RBA: 0.10 – 0.74 mg/mL

7.2.4.2 RBM: 0.15 – 1.1 mg/mL

7.3 Sample Preparation

7.3.1 For solid dose finished products: at least 10 dosage units are pooled and ground by mortar and pestle. Based on the fill weight (for capsules) or tablet weight per dose, weigh no less than 40 mg of the pooled dosages into a suitably sized volumetric flask of no less than 100 mL to generate an analyte concentration that is within the validated linearity range. Dilute the sample to 2/3 of the flask

volume with diluent and swirl to dissolve. Shaking or sonication can also be used to assist dissolution. After the sample is dissolved, equilibrate the sample to room temperature (if sonicated), and dilute to volume using diluent.

7.3.2 For raw materials: weigh no less than 40 mg into a suitably sized volumetric flask of no less than 100 mL volume to generate an analyte concentration that is within the validated linearity range. Dissolve in and dilute to volume with diluent.

7.3.3 Perform further dilutions as required using diluent.

7.3.4 If particulates remain in the final sample preparation, a portion may be centrifuged at 10,000 rpm for 200 seconds prior to HPLC analysis.

7.3.5 For finished products or raw materials being analyzed for the first time using this method, an in process validation is required to demonstrate spectral purity, baseline separation of peaks, and extraction efficiency as a part of system suitability before data can be reported using this method.

7.4 HPLC Parameters

7.4.1 Column: Kinetex XB-C18, 5µm, 4.6 mm x 250 mm

7.4.2 Column Temperature: 40 °C

7.4.3 Flow rate: 1.0 mL/min

7.4.4 Wavelength: 210 nm with 4 nm bandwidth

7.4.5 Reference Wavelength: 250 nm with 50 nm bandwidth

7.4.6 Injection Volume: 10 µL

7.4.7 Run Time: 20 minutes

7.4.8 Spectral Range (for Identification)- 190nm to 400nm

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the diluent.

7.5.2 Make five (5) injections of Standard Solution.

7.5.3 Make a single injection of each Sample Preparation.

7.5.4 Make a single injection of the Standard Solution after every ten (10) sample injections or at the end of a run.

7.6 System Suitability Requirements

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

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

7.6.3 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.7 Retention Times

7.7.1 RBA: RT = 14.8 min, RRT = 1.000

7.7.2 RBM: RT = 6.2 min, RRT = 0.421

7.8 Example calculations for determining finished product % label or raw material % purity

7.8.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{Form_{wt}}{Spl_{wt}} \times \frac{V_{spl}}{LA} \times RRF \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg (correct for moisture if required)

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

P Purity of the reference standard in decimal format

$Form_{wt}$ The weight of a single dosage unit based on the formulation in mg (use 1 for raw materials)

Spl_{wt} Sample weight in mg

RRF Relative Response Factor for the analyte (see Section 7.8.2)

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

LA Label amount in mg (use 1 for raw materials)

7.8.2 Relative Response Factors (RRF)

7.8.2.1 $RRF_{RBM} = 1.46$

7.8.2.2 $RRF_{RBM} = 1.46$

7.8.3 Column Wash and Storage

7.8.4 Rinse the column with H_2O / ACN (80/20).

7.8.5 Store the column in H_2O / ACN (50/50).

8.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/26/19	New	N/A	S. Sassman
1	06/22/22	Updated logo and format.	CC-22-0288	K. Burris