1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis and identification of 5-hydroxytryptophan (5-HTP) in finished products and raw materials using HPLC coupled with UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to the quantification and identification of 5-HTP. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of 5-HTP.

3.0 Responsibility

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

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **H_3PO_4** – Phosphoric Acid

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

4.4 **5-HTP** – 5-hydroxytryptophan

4.5 **QC** – Quality Control

4.6 **CofA** – Certificate of Analysis

4.7 **HPLC** – High Performance Liquid Chromatography

4.8 **UV/VIS** – Ultraviolet and Visible Spectroscopy

5.0 References

5.1 MV-LAB-13-033, Protocol, Validation: 5-Hydroxytryptophan Determination by HPLC

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

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are HPLC grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 5-HTP reference standard

6.2 Supplies and Glassware

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

6.2.2 Mobile phase containers

6.2.3 Volumetric glassware as required by sample and standard preparations

6.2.4 50mL and 100mL beakers

6.2.5 200µL, 1mL, and 10mL pipette tips

6.2.6 10mL plastic luer lock syringe

6.2.7 0.2 or 0.45µm Nylon syringe filters

6.2.8 22mL screw cap vials

6.2.9 HPLC vials, 12mm X32mm with screw cap enclosures w/ septa

6.2.10 Micro centrifuge tubes

6.2.11 Weigh Paper and weigh boats

6.3 Equipment

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

6.3.2 Analytical Balance

6.3.3 Vortex

6.3.4 Stir Plate

6.3.5 Wrist action shaker

6.3.6 200µL, 1mL, and 10mL pipettes

6.3.7 Microcentrifuge

7.0 Procedure

7.1 Mobile Phase and Buffer Preparation

7.1.1 Mobile Phase A - 0.1% H_3PO_4 in H_2O

7.1.1.1 Transfer 1000 mL H_2O to a suitable container.

7.1.1.2 Add 1.0 mL H_3PO_4, and mix well.

7.1.1.3 Scale as necessary.

7.1.2 Mobile Phase B - 0.1% H_3PO_4 in ACN

7.1.2.1 Transfer 1000 mL ACN to a suitable container.

7.1.2.2 Add 1.0 mL H_3PO_4, and mix well.

7.1.2.3 Scale as necessary.

7.1.3 Diluent- Mobile Phase A

7.2 Standard Preparation

7.2.1 The linear range of the analytical method is 0.01 mg/mL – 0.4 mg/mL. The standard and sample preparations must be within this range.

7.2.2 The standard is prepared by weighing no less than the minimum weight of the analytical balance, then bring up to two thirds their final volume in an appropriate volumetric flask using Diluent. Mix on a wrist action shaker for 30 minutes then inspect to ensure complete dissolution. Once the standard is fully dissolved, bring standard to final volume before using.

7.2.3 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. Working 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. Final dilutions may be prepared directly in HPLC vials.

7.3 Sample Preparation

7.3.1 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 listed above.

7.3.2 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. Dilute to 2/3 flask volume using Diluent, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.3 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and 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. Dilute to 2/3 flask volume using Diluent, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.4 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. Dilute to 2/3 flask volume using Diluent, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.5 To manage large volumes, the sample 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.

7.3.6 Centrifuge a portion of the final sample at 10,000 rpm for 5 min to remove particulates prior to HPLC analysis. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 2 – 3 mL.

8.0 Test Conditions

8.1 Gradient-multistep

8.1.1

Time	%A	%B
0.00	98	2
5.00	98	2
7.00	60	40
7.10	98	2
13.0	98	2

8.2 Column- Luna 5µm C5, 100Å, LC column, 150mm X 4.6mm or equivalent

8.3 Flow Rate- 1.0mL/min

8.4 UV detection- 225nm

8.5 Injection volume- 20uL

8.6 Column Temperature- 30°C

8.7 Recommended 3-D Spectral Range- 210nm to 330nm

8.8 Retention Time – about 4.5 min

8.9 Recommended Sequence

8.9.1 Make at least 2 injections of the diluent.

8.9.2 Make five (5) injections of Standard Solution.

8.9.3 Make a single injection of each Sample Preparation.

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

8.10 System Suitability Requirements

8.10.1 The %RSD of five (5) injections of the Working Standard is NMT 5.0%.

8.10.2 The %RSD of all injections of the Working Standard is NMT 5%.

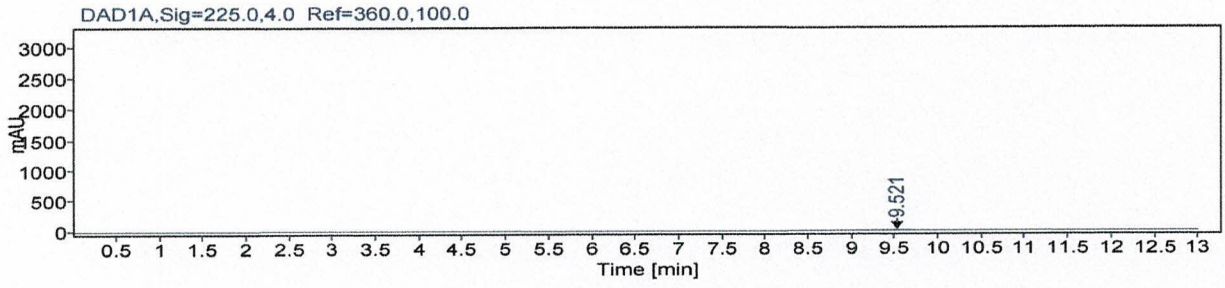
8.11 Example calculations for determining finished product % label or raw material % purity

8.11.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

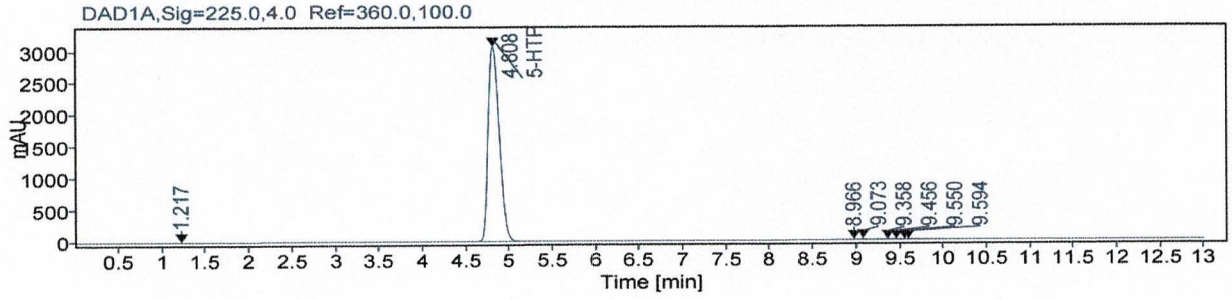
R_u	Sample peak area
R_s	Mean standard peak area
Wt_{std}	Weight of reference standard in mg
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SA	Sample amount in mg
V_{spl}	Volume of the sample preparation accounting for dilutions 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

8.12 Example Chromatography

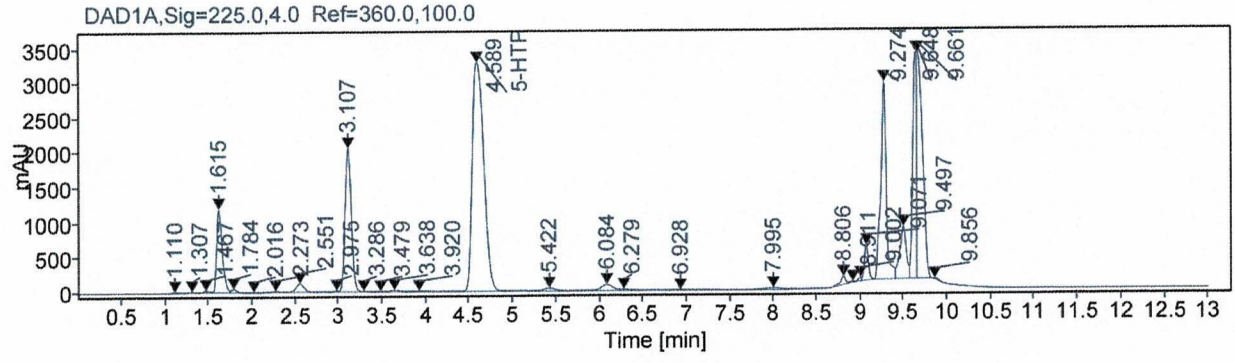
8.12.1 Blank



8.12.2 Standard



8.12.3 Sample



10.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	06/17/13	New	13-0416	B. Johns
2	07/01/15	Scheduled review: updated SOP format. Updated HPLC method format.	15-0585	B. Johns
3	01/02/19	Scheduled review: updated SOP format, stability requirement, weight requirement, and number of pooled tablets.	19-0008	J. Maignan
4	04/11/22	Updated for consistency with current methods and lab practices. Added recommended sequence. Added system suitability section. Added example chromatography. Removed extraneous information. Narrowed the range for spectral match.	CC-22-0171	S. Sassman
5	05/22/23	Removed unnecessary information and aligned with current SOP format. Added instruction to follow test details containing product specific sample preparation. Added specific sample prep instructions for different dosage forms. Updated logo.	CC-23-0252	S. Sassman