1.0 Purpose

The purpose of this procedure is to define the method for the quantification and/or identification of Dehydroepiandrosterone and Piperine in raw materials and finished products by HPLC using UV-Vis Spectroscopy.

2.0 Scope

This procedure applies to the identification and quantification of Dehydroepiandrosterone & Piperine in raw materials and finished products. This method was validated under Protocol MV-LAB-19-137.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical 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 the procedure is being followed.

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

4.0 Definitions

4.1 **QC** – Quality Control

4.2 **H_3PO_4** – Phosphoric Acid

4.3 **MeOH** – Methanol

4.4 **DHEA** – Dehydroepiandrosterone

4.5 **H_2O** – Deionized Water

4.6 **PIP** – Piperine

4.7 **HPLC** – High Performance Liquid Chromatography

4.8 **UV/Vis** – Ultraviolet & Visible Electromagnetic Spectra

5.0 References

5.1 MV-LAB-19-137, Protocol, Dehydroepiandrosterone and Piperine Determination Using HPLC with UV/Vis Spectroscopy

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better

6.1.1 H_2O

6.1.2 MeOH

6.1.3 H_3PO_4

6.1.4 DHEA Reference Standard

6.1.5 PIP Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Volumetric glassware and/or adjustable pipettes and tips

6.2.2.1 **CAUTION:** Piperine solutions are not light stable. Minimize exposure to light during transfers and only use red or foil-wrapped glassware!

6.2.3 Weigh paper or funnels

6.2.4 10ml Syringes with 0.45u Nylon Syringe Filters

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 Sonicator bath

6.3.4 Wrist Action Shaker

7.0 Procedure

7.1 Mobile Phase, Diluent & Diluent Preparation

7.1.1 Mobile Phase A – 0.1% H3PO4

7.1.1.1 Combine 1 mL of H3PO4 with 1000 mL of H_2O. Mix well.

7.1.2 Mobile Phase B - MeOH

7.1.3 Diluent – MeOH

7.1.4 Preparations may be scaled as necessary

7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 25 mg each of DHEA and PIP reference standards into a 100-mL volumetric flask. Add 50mL of Diluent. Sonicate for 5min, equilibrate to room temperature, then QS to volume with Diluent.

7.2.2 Dilute 1:10 w/ Diluent for a Working Standard concentration of 25 µg/ml.

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 below.

7.3.2 The validated range for the analytical method is 5 – 40 mcg/mL for DHEA and 6 – 117 mcg/mL for PIP.

7.3.3 For raw materials: weigh no less than 25 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. Fill the flask to about 50% of the calculated volume with Diluent and shake mechanically for 15 minutes. Sonicate for 5 minutes, equilibrate to room temperature and bring up to volume with Diluent.

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 and liquids) or tablet weight per dose, weigh no less than 100 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. Fill the flask to about 50% of the calculated volume with Diluent and shake mechanically for 15 minutes. Sonicate for 5 minutes, equilibrate to room temperature and bring up to volume with Diluent.

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 no less than 400 mg 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 of no less than 50 mL to generate an analyte concentration that is within the validated linear range. Fill the flask to about 50% of the calculated volume with Diluent and shake mechanically for 15 minutes. Sonicate for 5 minutes, equilibrate to room temperature and bring up to volume with Diluent.

7.3.6 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

7.3.7 Centrifuge an aliquot of the final sample at 10,000 rpm for 5 min to remove particulates. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL before collecting a portion for analysis.

7.4 HPLC Parameters

7.4.1 Column: Thermo Scientific Accucore Polar Premium, 3.0 x 100mm, 2.6u

7.4.2 Column Temperature: 45°C

7.4.3 Flow rate: 0.5 mL/min

7.4.4 Wavelength: DHEA @ 210 nm, PIP @ 343 nm

7.4.5 Injection Volume: 5 µL

7.4.6 Run Time: 12 minutes.

7.4.7 Recommended 3-D Spectral Range (for Identification) - 200nm to 400nm

7.4.8 Mobile Phase Gradient – Isocratic: 50% A / 50% B

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the Diluent.

7.5.2 Make five (5) injections of Working Standard.

7.5.3 Make a single injection of each Sample Preparation.

7.5.4 Make a single injection of the Working Standard 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 2.0%

7.6.2 The %RSD of all standard injections is NMT 3.0%.

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

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

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

R_u Sample peak area

R_s Mean (n=5) standard peak area

Wt_{std} Weight of the reference standard in mg (corr. for water if applicable)

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 (use 1 for raw materials)

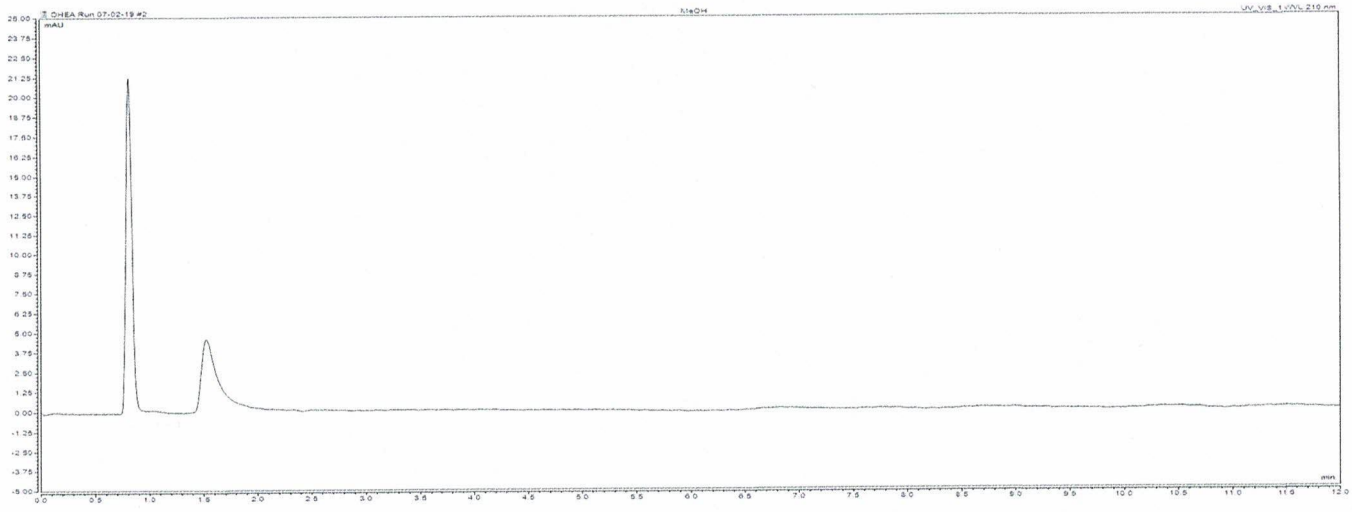
LA Label amount in mg of analyte (use 1 for raw materials)

7.8 Column Wash and Storage

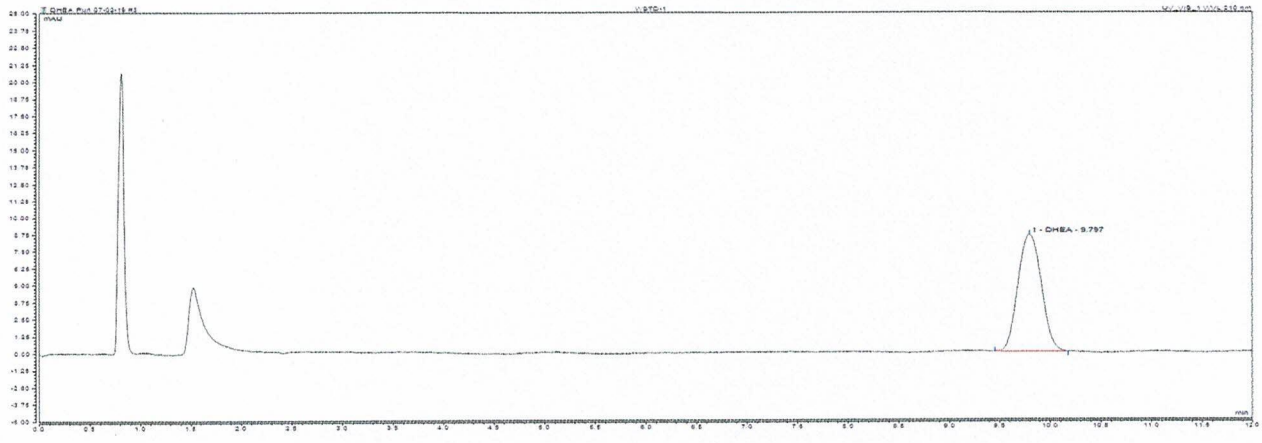
7.8.1 Wash and store the column in 75:25 ACN / Milli-Q Water.

8.0 Example Chromatograms

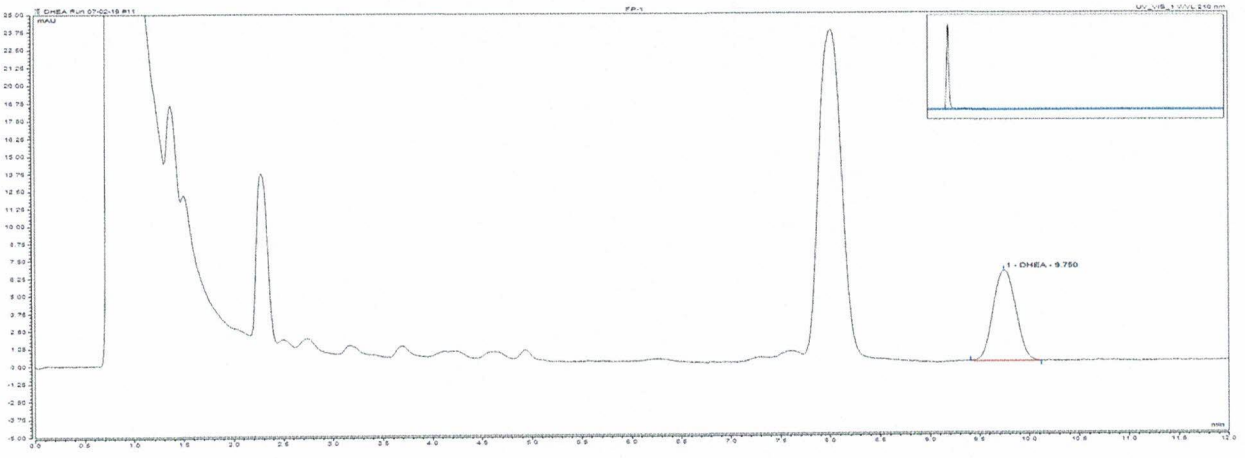
8.1 Typical DHEA Diluent Chromatogram



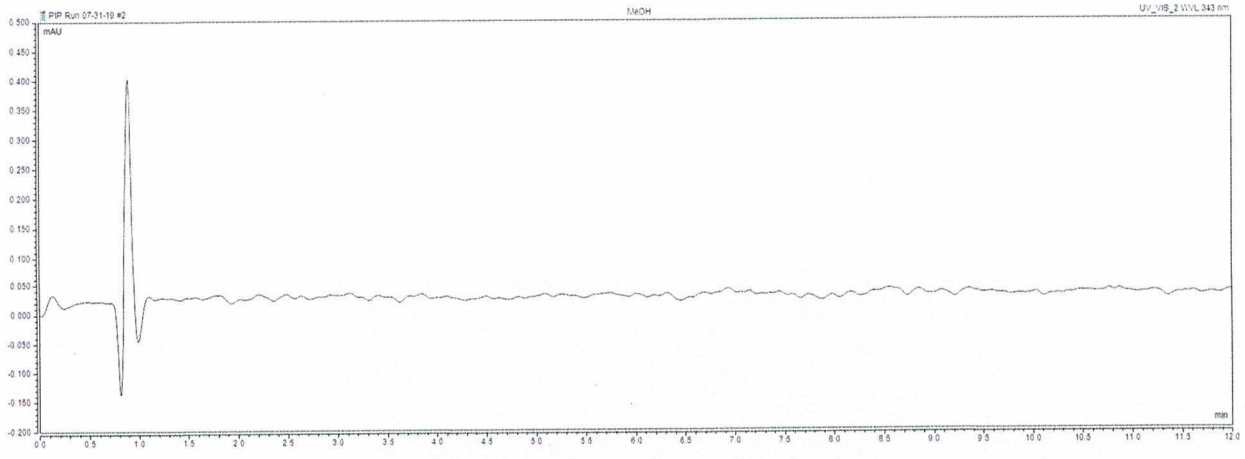
8.2 Typical DHEA Working Standard Chromatogram



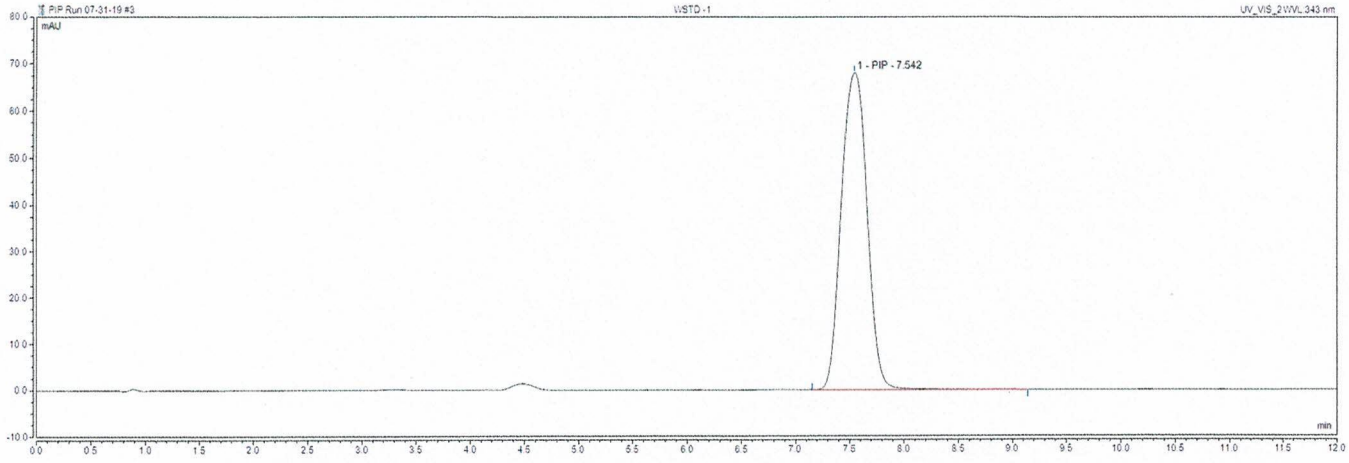
8.3 Typical DHEA Sample Chromatogram



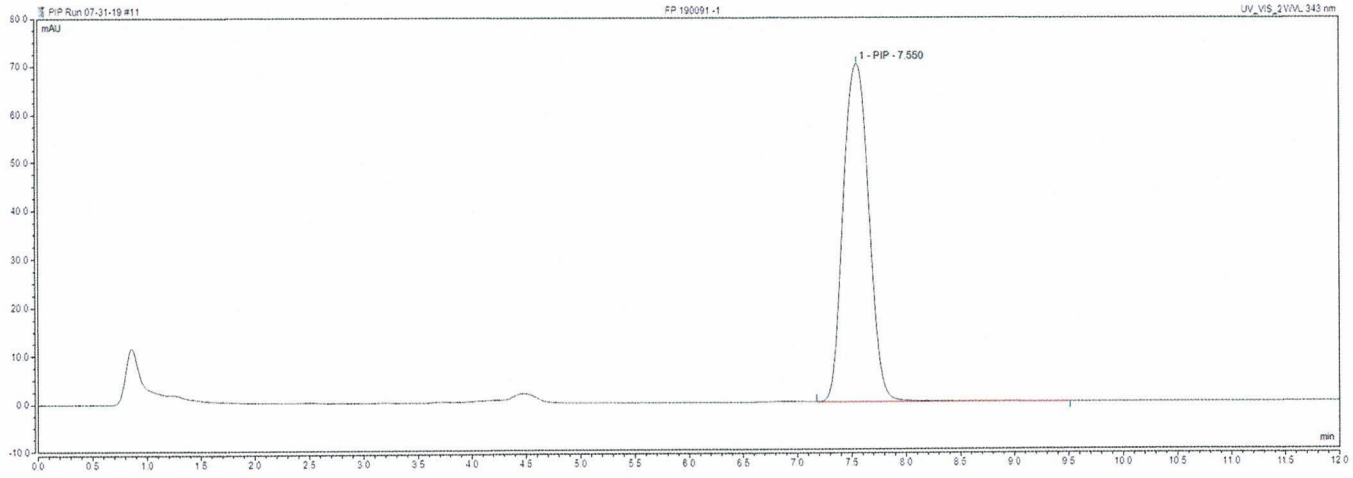
8.4 Typical PIP Diluent Chromatogram



8.5 Typical PIP Working Standard Chromatogram



8.6 Typical PIP Sample Chromatogram



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	09/11/19	New procedure.	N/A	C. Perry
1	07/21/22	Scheduled review: updated logo and format.	CC-22-0292	K. Burris
2	06/06/23	Minor edits for consistency with current methods, add instruction to follow test details for sample preparation, add specific instruction for different dosage forms. Update logo and format.	CC-23-0276	S. Sassman