1.0 Purpose

This document describes the analytical procedure for the determination of chondroitin, N-acetylglucosamine and glucosamine using HPLC coupled with UVspectrophotometry.

2.0 Scope

This procedure applies to the identification and quantification of chondroitin, N-acetylglucosamine and glucosamine in raw materials and finished products.

2.1 Two separate methods are described.

2.1.1 See Sections 7.0 – 8.0 for analysis of chondroitin and N-acetylglucosamine.

2.1.2 See Sections 9.0 – 10.0 for analysis of glucosamine.

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 associated monographs and laboratory practices.

4.0 Definitions

4.1 **H_2O** – Water (≥18.2 MΩ·cm)

4.2 **ACN** – Acetonitrile

4.3 **ACS** – American Chemical Society

4.4 **H_3PO_4** – Phosphoric Acid

4.5 **K_2HPO_4** – Potassium Phosphate Dibasic

4.6 **NH_4OH** – Ammonium Hydroxide

4.7 **HCl** – Hydrochloride

4.8 **QC** – Quality Control

4.9 **CofA** – Certificate of Analysis

4.10 **HPLC** – High Performance Liquid Chromatography

4.11 **UV** – Ultraviolet (Detection)

5.0 References

5.1 MV-LAB-13-010, Protocol, Validation: Glucosamine Determination

5.2 MV-LAB-18-083, Protocol, Validation: Chondroitin Determination

5.3 MV-LAB-18-179, Protocol, Validation: N-acetylglucosamine Determination

5.4 PRTCL-22-0067, Protocol, Validation of an Analytical Method for the Determination of Glucosamine by HPLC-UV

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are ACS grade or better.

6.1.1 H_2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 K_2HPO_4

6.1.5 NH_4OH

6.1.6 Glucosamine HCl reference standard

6.1.7 N-acetylglucosamine reference standard

6.1.8 Chondroitin sulfate reference standard

6.2 Supplies and Glassware

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

6.2.2 1L mobile phase containers

6.2.3 Volumetric Glassware

6.2.4 50mL and 100mL beakers

6.2.5 200µL, 1mL, 10mL pipette tips

6.2.6 Plastic luer lock syringes

6.2.7 0.45µm, 25mm Nylon syringe filters

6.2.8 22mL screw cap vials

6.2.9 Micro centrifuge tubes

6.2.10 Weigh paper and weigh boats

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 Vortex Mixer

6.3.4 Stir Plate

6.3.5 Wrist action shaker

6.3.6 200uL, 1mL and 10mL pipettes

6.3.7 Micro-centrifuge

7.0 Preparation of Mobile Phase, Diluent, Standards, and Samples for Determination of Chondroitin and N-acetylglucosamine

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 1000mL of H_2O to a mobile phase bottle.

7.1.1.2 Add 1.0mL of H_3PO_4, and mix well.

7.1.2 Mobile Phase B (0.1% H_3PO_4 in ACN)

7.1.2.1 Transfer 1000mL of ACN to a mobile phase bottle.

7.1.2.2 Add 1.0mL of H_3PO_4, and mix well.

7.1.3 Diluent (50:50 H_2O:ACN)

7.1.3.1 Transfer 500mL of ACN to a mobile phase bottle.

7.1.3.2 Add 500mL of H_2O, and mix well.

7.1.3.3 **Allow to equilibrate to room temperature before using.**

7.2 Standard Preparation

7.2.1 The linear range of the method is listed below. All standard and sample preparations must be within the linear range of the method. If the analyte form is not the same as listed below, the linear range may need to be corrected for analyte form.

7.2.1.1 Chondroitin sulfate sodium – 0.1mg/mL to 1.0mg/mL

7.2.1.2 N-acetylglucosamine – 0.01mg/mL to 0.1mg/mL

7.2.2 Use the actual purity from the CofA in your calculations.

7.2.3 All Standards are prepared by weighing no less than the minimum weight of the analytical balance. Transfer to a suitably sized volumetric flask. Dissolve in and dilute to volume using Diluent.

7.2.4 Dilutions are prepared using Diluent. Dilutions can be made using volumetric flasks or using 10mL, 1mL and 200uL variable 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 can 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 of this method.

7.3.2 For finished products, 20 or more dosage units can be pooled and ground by mortar and pestle as necessary.

7.3.3 Based on the fill or tablet weight per dose for finished products or raw material potency, weigh a portion of the pooled dosages to generate an analyte concentration that is within the validated linear range.

7.3.4 Samples can be dissolved in Diluent at any volume starting from 50mL. To manage large volumes the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the analyte concentration into the linear range of measurement.

7.3.5 The final diluted sample must be filtered or centrifuged before analyzing by HPLC. Filter using a 0.45µm nylon syringe filter, discarding at least 0.5mL of filtrate before collecting the filtered sample in a vial for analysis.

7.3.6 For centrifugation using the final large scale diluted sample, fill an even number of 1.5 or 2.0mL microcentrifuge tubes and pellet insoluble matter for 5 minutes at 6000rpm.

8.0 Test Conditions for Determination of Chondroitin and N-acetylglucosamine

8.1 Gradient – Isocratic 60% A : 40% B

8.2 Column – Acclaim C18, 5µ, 120Å, 4.6 X 250mm

8.3 Flow Rate – 0.6mL/min

8.4 UV detection – 195nm

8.5 Injection volume – 20µL

8.6 Column Temperature – 35°C

8.7 Recommended 3-D Spectral Range – 190nm to 300nm

8.8 Recommended Sequence

8.8.1 Make at least 2 injections of a Blank (Diluent).

8.8.2 Make at least 5 injections of the Working Standard.

8.8.3 Make a single injection of each Sample Preparation

8.8.4 Make a single injection of the Working Standard after every 6 samples and at the end of the run.

8.9 System Suitability

8.9.1 The %RSD of 5 consecutive injections of Working Standard is NMT 5.0%

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

8.10 Column Wash and Storage

8.10.1 Rinse the column with H_2O / ACN (50/50) at 1 mL/min for at least 15 min.

8.10.2 Store the column with H_2O / ACN (50/50).

9.0 Preparation of Mobile Phase, Diluent, Standards and Samples for Determination of Glucosamine

9.1 Mobile Phase and Buffer Preparation

9.1.1 Mobile Phase A

9.1.1.1 Dissolve ~3.5g K_2HPO_4 in 1000mL of water, then add 0.25mL NH_4OH and pH to 7.5 with H_3PO_4.

9.1.2 Mobile Phase B

9.1.2.1 Use ACN.

9.1.3 Diluent (50:50 H_2O:ACN)

9.1.3.1 Transfer 500mL of ACN to a mobile phase bottle.

9.1.3.2 Add 500mL of H_2O, and mix well.

9.1.3.3 **Allow to equilibrate to room temperature before using.**

9.2 Standard Preparation

9.2.1 The linear range of the method is listed below. All standard and sample preparations must be within the linear range of the method. If the analyte form is not the same as listed below, the linear range may need to be corrected for analyte form.

9.2.1.1 Glucosamine HCl: 2.25 – 5.26mg/mL

9.2.2 Use the actual purity from the CofA in your calculations.

9.2.3 All Standards are prepared by weighing no less than the minimum weight of the analytical balance. Transfer to a suitably sized volumetric flask. Dissolve in and dilute to volume using Diluent.

9.2.4 Dilutions are prepared using Diluent. Dilutions can be made using volumetric flasks or using 10mL, 1mL and 200uL variable 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 can be prepared directly in HPLC vials.

9.3 Sample Preparation

9.3.1 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 of this method.

9.3.2 For finished products, 20 or more dosage units can be pooled and ground by mortar and pestle as necessary.

9.3.3 Based on the fill or tablet weight per dose for finished products or raw material potency, weigh a portion of the pooled dosages to generate an analyte concentration that is within the validated linear range.

9.3.4 Samples can be dissolved in Diluent at any volume starting from 50mL. To manage large volumes the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the analyte concentration into the linear range of measurement.

9.3.5 The final diluted sample must be filtered or centrifuged before analyzing by HPLC. Filter using a 0.45µm nylon syringe filter, discarding at least 0.5mL of filtrate before collecting the filtered sample in a vial for analysis.

9.3.6 For centrifugation using the final large scale diluted sample, fill an even number of 1.5 or 2.0mL microcentrifuge tubes and pellet insoluble matter for 5 minutes at 6000rpm.

10.0 Test Conditions for Determination of Glucosamine

10.1 Gradient – Isocratic 40% A : 60% B

10.2 Column – HyperSil GOLD Amino, 5µ, 175Å, 4.6 X 150mm

10.3 Flow Rate – 1.0mL/min

10.4 UV detection – 195nm

10.5 Injection volume – 5µL

10.6 Column Temperature – 35°C

10.7 Recommended 3-D Spectral Range – 190nm to 260nm

10.8 Recommended Sequence

10.8.1 Make at least 2 injections of a Blank (Diluent).

10.8.2 Make at least 5 injections of the Working Standard.

10.8.3 Make a single injection of each Sample Preparation

10.8.4 Make a single injection of the Working Standard after every 6 samples and at the end of the run.

10.9 System Suitability

10.9.1 The %RSD of 5 consecutive injections of Working Standard is NMT 5.0%

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

10.10 Column Wash and Storage

10.10.1 Rinse column with 60% ACN at 1 mL/min for at least 1 hour.

10.10.2 Store column on 100% ethanol.

11.0 Example Calculation

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 (solids) or mL (liquids)

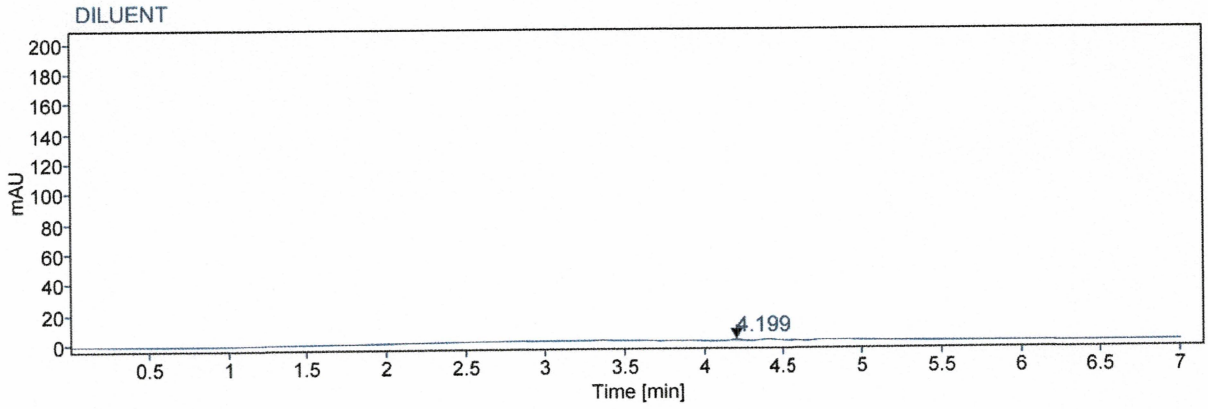
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

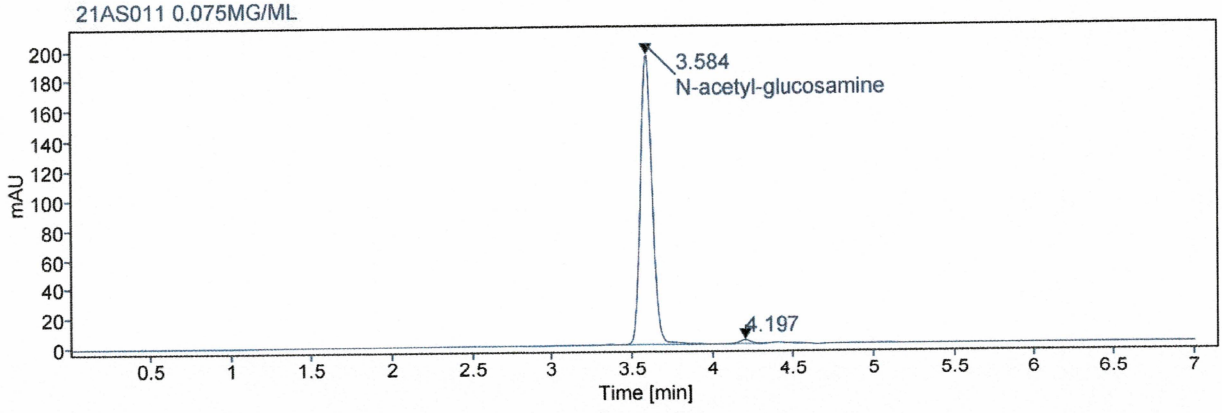
LA Label amount in mg per dose or 1 for raw materials

12.0 Example Chromatography

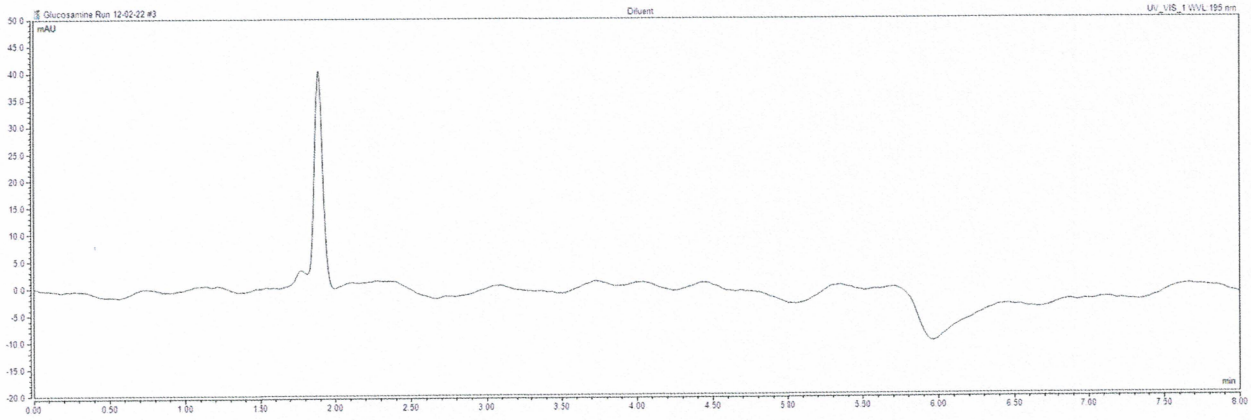
12.1 Chondroitin / N-acetylglucosamine Blank



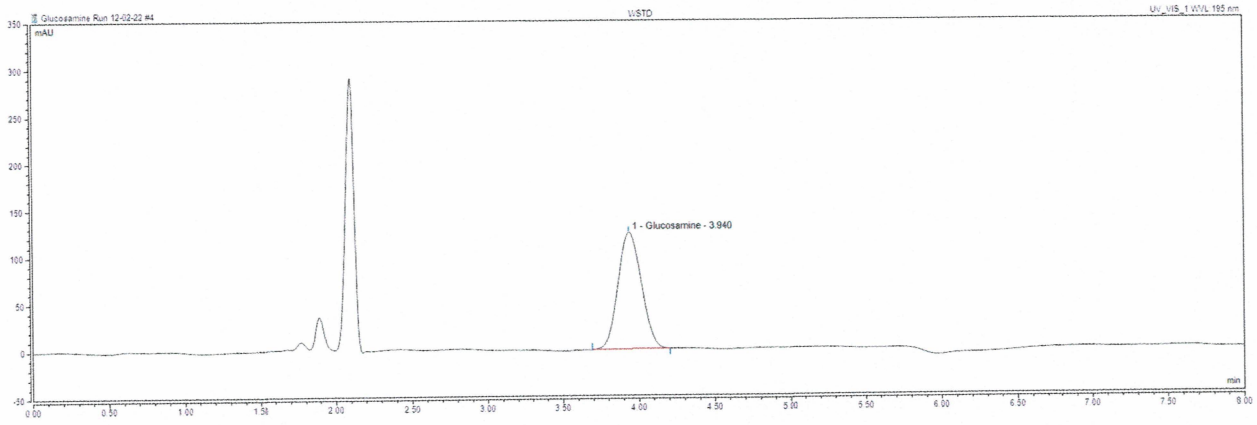
12.2 N-acetylglucosamine Working Standard



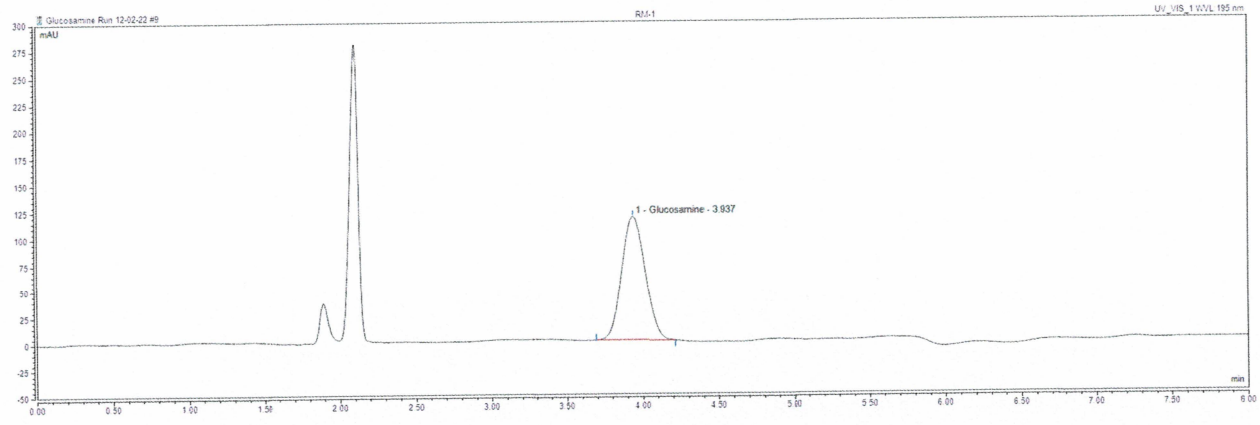
12.3 Glucosamine Diluent



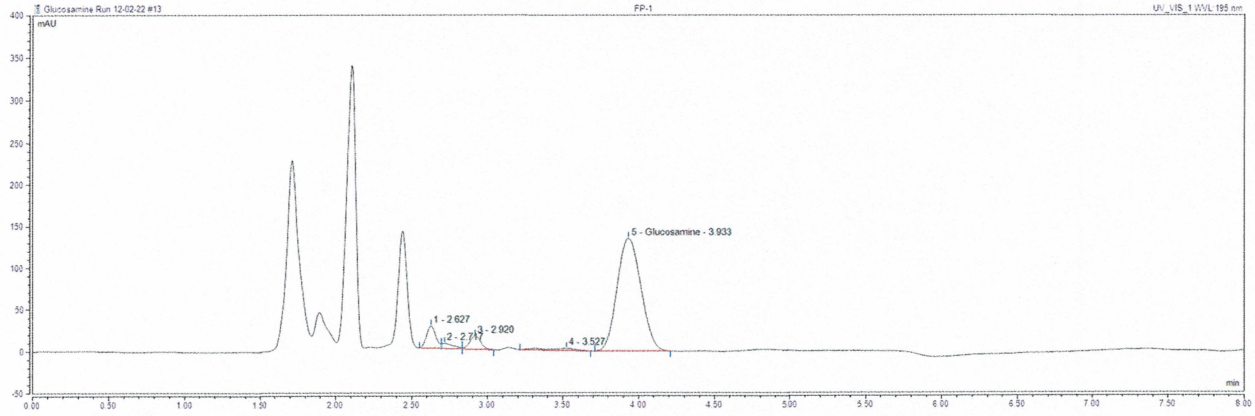
12.4 Glucosamine Standard



12.5 Glucosamine Raw Material



12.6 Glucosamine Finished Product



13.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	06/21/13	New	13-468	B. Johns
2	07/01/15	Biennial review: Updates SOP format. Aligned content of SOP with latest format.	15-0582	B. Johns
3	07/03/18	Biennial review: Update SOP to match stability requirement. Added Chondroitin to SOP based off protocol MV-LAB-18-083	18-0223	J. Maignan
4	01/02/19	Added N-acetylglucosamine to SOP based off validation protocol MV-LAB-18-179.	19-0001	J. Maignan
5	06/21/22	Update for consistency with current methods, add recommended sequence section, replace requirements with system suitability section, add column wash and storage, add example chromatography. Update logo and format.	CC-22-0279	S. Sassman
6	01/20/23	Added new glucosamine procedure. Added reference to product profile regarding specific testing details. Removed first time validation statement.	CC-23-0003	C. Perry