

EXHIBIT 5

Glucosamine Rapid Assay

METHOD:

1. Place sample (containing 0.5 - 10 μg GlcN) in a Pyrex screw capped tube.
2. Add HCl to a final concentration of 2N and a final volume of 0.6 ml. Flush with nitrogen.
3. Stopper tightly and hydrolyze 16h at 100°C.
4. Prepare standards containing 0 - 20 μg GlcN from a stock solution of 1.5 mg/ml. (Use 0, 3, 5, 8, 10, 12 ml of stock solution and add water to make up to 300 μl . Then add 300 μl 4N HCl to a final volume of 0.6 ml and final concentration of 2N). Standards need not be heated.
5. Neutralize samples and standards with 0.4 ml 2M Na_2CO_3 (10.6 g in 50 ml). (pH - 7 with the slight excess of Na_2CO_3)
6. Shake gently and add 0.5 ml (freshly prepared) 2% acetyl acetone in 1.5M Na_2CO_3 . (15.9 g Sodium Carbonate + 2 ml acetyl acetone made up to 100 ml).
7. Stopper tightly and heat in boiling water bath for 20 min.
8. Cool. Add 1 ml EtOH.
9. Add 0.5 ml Ehrlich's reagent. (1 g p dimethylaminobenzaldehyde in 15 ml EtOH and 15 ml c/HCl)
10. Shake tubes vigorously to expel excess CO_2 .
11. Maximum colour development is reached in 5 min. Chromophore stable for 1 to 2 hours.
12. Read absorbance at 530 nm.

NOTE: NaCl affects colour. Therefore standards and sample should contain the same amount of salt to avoid colour depression and erroneous results. Dialysis helps to remove NaCl.

Methods



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Determination of Glucosamine by HPLC Method 121.000

Assay Title: Determination of glucosamine raw material by HPLC with pre-column PITC derivatization

Scope: This assay can be used to determine glucosamine purity in glucosamine sulfate and glucosamine HCl raw materials. Results are reported as free base.

NOTE: There are several available forms of glucosamine sulfate. This method is only applicable to salts of glucosamine and not to covalently-bonded glucosamine sulfate.

Background: Phenylisothiocyanate (PITC) reacts with both primary and secondary amines and the resultant derivative is stable and a UV-detectable chromophore. Because glucosamine has diastereomeric forms (alpha & beta), two peaks are observed in the chromatography and their relative ratios may vary according to equilibrium times. An alternative to PITC is 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which has been commercialized by Waters, Inc. as AccQ-Tag.

Safety: Consult the Material Safety Data Sheet (MSDS) for any chemical used that is unfamiliar. All chemicals should be considered hazardous - avoid direct physical contact. For more safety information go to <http://hazard.com/msds/>.

Caution: When handling PITC, wear gloves and eye/face protection. Do not inhale vapor.

Standards: D-(+)-glucosamine HCl, (2-amino-2-deoxy-D-glucosamine) ChromaDex, PN 07256-1, $\geq 98\%$, $C_6H_{14}ClN_5O_5$, MW 215.63

Apparatus:

- High-Performance Liquid Chromatography System as described in USP chapter <621>. Verify and document that apparatus, software, and subsystems meet performance requirements for Installation Qualification/Operation Qualification (IQ/OQ).
- Analytical balance, calibrated
- 1000- and 250-mL graduated cylinders
- Ultrasonic bath
- Transfer pipets, Class A, assorted sizes
- Reaction vials with caps, 5-mL or centrifuge tubes with caps

Reagents:

- Water, HPLC grade or Nanopure
- Acetonitrile, HPLC grade
- Methanol, HPLC grade
- Sodium acetate, anhydrous, ACS reagent grade
- Phosphoric acid, 85%, HPLC grade
- Phenyl isothiocyanate, 99+%, Sigma Catalog No. 13974-2, or equivalent

Sodium Acetate Solution Preparation:

In an appropriate container, add about 4.1 g of sodium acetate to 500-mL of water. Stir until dissolved. The approximate concentration of the resultant solution is 0.1M.

Standard Preparation:

Accurately weigh approximately 50.0 mg of glucosamine · HCl reference standard and transfer into a 50-mL volumetric flask. Add approximately 40 mL of 0.1M NaOAc solution and sonicate until dissolved. Dilute to volume with 0.1M NaOAc and mix for stock standard. The final concentration of this stock standard is approximately 1.0 mg/mL.

Note: Preparation of the standard in duplicate is recommended. **Correct standard concentration to reflect free base concentration.**

Pipet 5 mL of stock standard into a 50-mL volumetric flask. Add 400 μ L of PITC. Add 15 mL of methanol and mix by hand until all the PITC dissolves. Dilute to volume with MeOH/H₂O (60/40) and mix.

Transfer approximately 5 mL of the solution to a reaction vial or centrifuge tube and tightly seal. Heat in a water bath or dry block heater at 80°C for 15 minutes. Allow the solution to cool to room temperature. Add approximately 5 mL of heptane to the reaction vessel and shake mechanically or by hand for 1 minute to extract unreacted PITC. Remove an aliquot from the methanolic layer and place into an HPLC vial for analysis. The approximate concentration of the working standard is 0.1 mg/mL.

Sample Preparation:

Accurately weigh 40 mg of raw material and transfer into a 50-mL volumetric flask. Add approximately 40 mL of 0.1M NaOAc solution and sonicate until dissolved. Dilute to volume with 0.1M NaOAc solution and mix.

Pipet 5 mL of the sample solution into a 50-mL volumetric flask and add 400 μ L of PITC. Add 15 mL of methanol and mix by hand until all of the PITC dissolves. Dilute to volume with MeOH/H₂O (60/40) and mix.

Transfer approximately 5 mL of the solution to a reaction vial or centrifuge tube and tightly seal. Heat in a water bath or dry block heater at 80°C for 15 minutes. Allow the solution to cool to room temperature. Add approximately 5 mL of heptane to the reaction vessel and shake mechanically or by hand for 1 minute to

extract unreacted PITC. Remove an aliquot from the methanolic layer and place into an HPLC vial for analysis.

Chromatographic Conditions:

Column: Phenomenex Luna C18, 4.6 x 150 mm

Mobile Phase: Isocratic ACN/H₂O/H₃PO₄ (10/90/0.1)

Flow Rate: 1.5 mL/min

Detector: UV at 240 nm

Injection Vol.: 10 µL

Run Time: 10 minutes

Relative Retention Times: (on Phenomenex Luna C18(2), 4.6 x 150mm)

Glucosamine 1 1.00

Glucosamine 2 1.20

NOTE: After 10-12 injections, flush column with 100% acetonitrile to remove unreacted PITC build up on column. Flush for 10 minutes.

Procedure:

Make 5 replicate injections of the 0.1 mg/mL glucosamine · HCl working standard.

Make single injections of the sample preparations.

Quality Control:

The relative standard deviation (RSD) of the glucosamine peak area response for at least five replicate, consecutive injections is not more than 2.0%.

The resolution between the two glucosamine peaks must be greater than 1.5.

Calculations:

Calculate the % purity of glucosamine, as free base, by:

$$\% = \frac{R_x}{R_y} \times C \times \frac{50mL}{W} \times \frac{50mL}{5mL} \times 100\%$$

Where:

R_u is the sum of peak area ratios for peak 1 and peak 2 in the sample preparation.

R_s is the average sum of peak area ratios for peak 1 and peak 2 in the standard preparation.

C is the concentration of glucosamine free base in the working standard preparation. For example, if using glucosamine-HCl, multiple the standard concentration (mg/mL) by $(179.2 \div 215.63)$ or 0.8330.

W is the sample weight (mg).

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GLUCOSAMINE BY HPLC FOLLOWING AUTOMATED DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE

1 Objective

- 1.1 To extract, isolate from matrix interferant, and quantify glucosamine from nutritional and/or OTC pharmaceutical products. This method employs an *o*-phthaldialdehyde precolumn derivatization automated via a programmable autosampler, followed by reversed-phase HPLC for separation and quantification. *This revision incorporates a glutamate internal standard that automatically controls for volumetric variability thus returning significant enhancements in precision and accuracy.*

2 Instrument/Apparatus

- 2.1 High-performance liquid chromatograph (HPLC) with variable-wavelength detector and programmable autosampler
- 2.2 Genesis C18, 3.0 x 50 mm, 4 μ m particle
- 2.3 Sonication bath with temperature control
- 2.4 Stomacher with appropriate stomacher bags
- 2.5 Volumetric flasks (25 and 100 ml)
- 2.6 Variable volumetric pipets
- 2.7 Microfuge and tubes
- 2.8 Autosampler vials with PTFE/silicone septa screw caps
- 2.9 Two-liter vacuum filtration glassware with 0.2 μ m nylon membrane filters
- 2.10 Magnetic stirrer and stir bars

3 Reagents

- 3.1 Methanol, HPLC grade
- 3.2 Water, reagent grade (≥ 10 M Ω , carbon- and submicron-filtered)
- 3.3 Sodium acetate, trihydrate, reagent grade
- 3.4 Hydrochloric acid, reagent grade
- 3.5 Glacial acetic acid, reagent grade
- 3.6 Glucosamine hydrochloride, Aldrich, G220-6 (COA: 99.9% by HPLC)
- 3.7 FluoraldehydeTM OPA reagent solution (Pierce Chemical)
- 3.8 L-(+)-Glutamic acid hydrochloride 99%, (Aldrich)

4 Comments

- 4.1 Fluoraldehyde™ is a stabilized OPA reagent that greatly improves assay precision over traditional OPA preparations.
- 4.2 Methanol is flammable and toxic, OPA is an irritant and likely a sensitizer, hydrochloric acid and glacial acetic acid are corrosive, and buffers are variably irritants. Contact, vapor exposure, and ignition sources should be avoided accordingly.

5 Procedure

5.1 Reagent/standard preparation

- 5.1.1 Diluent with internal standard (5 mM acetic acid in 5% acetonitrile):
Transfer 290 µl acetic acid and 50 ml acetonitrile and 400 mg glutamic acid (99%) as internal standard to ca 200 ml water in a 1000 ml volumetric flask; QS to volume with water. Stir until dissolved.
- 5.1.2 Glucosamine standards
 - 5.1.2.1 Standard Stock Solution: Accurately weigh approximately 50 mg of standard glucosamine HCl and transfer to a 25-ml volumetric flask. QS to volume with diluent and stir for 15 minutes at room temperature. **Ensure total solubility.** If incompletely dissolved, sonicate 15 minutes in 65° C bath then magnetically stir another 5 minutes.
 - 5.1.2.2 From the above standard stock solution, prepare working standard solutions in diluent equivalent to 10-fold and 2-fold dilute concentrations of the standard stock solution. Standard thus prepared must be allowed to age for 1 h at room temperature to achieve anomeric equilibrium before use (cf. below). Prepare a three-point calibration curve including the stock and the two standard dilutions.

5.2 Mobile phase preparations

- 5.2.1 *Buffer A* [50 mM sodium acetate (pH 5.9):methanol, (85:15)]
 - 5.2.1.1 In a 1-liter volumetric flask, dissolve 6.80 g of sodium acetate trihydrate in ca. 700 mL water. Adjust to pH 5.9 with dilute acetic acid, and QS to volume with water. Filter/degas under aspirator vacuum through a 0.2 µm nylon membrane (Gelman, or equivalent).
 - 5.2.1.2 Combine 150ml methanol with 850 ml acetate buffer. Mix thoroughly. [N.B. This solution may be prepared in any volume desired, as long as weights and volumes remain in the same proportions.]
- 5.2.2 *Buffer B* [50 mM sodium acetate (pH 5.9):methanol, (20:80)]
 - 5.2.2.1 In a 1-liter volumetric flask, dissolve 6.80 g of sodium acetate trihydrate in ca. 700 mL water. Adjust to pH 5.9 with dilute acetic

acid, and QS to volume with water. Filter/degas under aspirator vacuum through a 0.2 μm nylon membrane (Gelman, or equivalent).

- 5.2.2.2 Combine 800 ml methanol with 200 ml acetate buffer. Mix thoroughly. [N.B. This solution may be prepared in any volume desired, as long as weights and volumes remain in the same proportions.]

5.3 Sample preparation

5.3.1 Tablets, capsules, raw materials

- 5.3.1.1 Weigh and finely pulverize at least 20 tablets. Transfer an accurately weighed portion of the powdered sample equivalent to approximately 25 mg of glucosamine into a 25-ml volumetric flask. QS with diluent and vortex to suspend the powder. Sonicate in a 65° C water bath for 20 minutes.
- 5.3.1.2 Pellet approximately 1.5 mL of the suspension for 1 minute at 16,000xg. Transfer approximately 1 mL of the supernate to an autosampler vial, then cap the vial.
- 5.3.1.3 Place the vials containing standards and sample extracts in the “sample rack” of the autosampler.

5.3.2 Bars

- 5.3.2.1 Prepare a uniform composite of 12 bars (grinder or blender, do not soften in the microwave). Use 1/4 of each bar for the composite.
- 5.3.2.2 Weigh approximately 8 grams of sample into a stomacher bag.
- 5.3.2.3 Add 200 mL of diluent.
- 5.3.2.4 Stomach on normal for 60 seconds, then stomach on high for 120 seconds.
- 5.3.2.5 Centrifuge prior to injection.

5.3.3 Move Free Softlets

- 5.3.3.1 Weigh and finely pulverize at least 20 softlets. Sieve the powdered sample using Sieve #25 or higher to separate the coating.
- 5.3.3.2 Transfer an accurately weighed portion of the powdered sample equivalent to approximately 25 mg of glucosamine into a 25-ml volumetric flask. QS with diluent and vortex to suspend the powder. Sonicate in a 65° C water bath for 20 minutes.
- 5.3.3.3 Pellet approximately 1.5 mL of the suspension for 1 minute at 16,000xg. Transfer approximately 1 mL of the supernate to an autosampler vial, then cap the vial
- 5.3.3.4 Place the vials containing standards and sample extracts in the “sample rack” of the autosampler.

5.4 Pre-column derivatization

- 5.4.1 An appropriate autosampler program must be specified in the instrument method.

- 5.4.2 An appropriate “sample rack” and “inject rack” must be specified in the instrument method.
- 5.4.3 Pre-column derivatization is carried out by an autosampler program that transfers 100 μL of standard or sample extract to a vial containing 600 μL of Fluoraldehyde reagent, mixes, allows the derivatization to proceed for 1 minute, then injects the derivatized solution.
- 5.4.4 Prepare one vial containing 600 μL of Fluoraldehyde reagent for each injection that will be performed. Cap the vials and place them in the “inject rack” in vial positions that correspond to the vial positions occupied by the sample vials in the “sample rack”. **Note: These vials must be replaced if a re-injection of a sample or standard is desired.**
- 5.4.5 Derivatization is carried out by the autosampler as part of the injection process as the sample series is analyzed.

5.5 Chromatography

- 5.5.1 Injection volume: 25 μL .
- 5.5.2 Monitor absorbance at 340 nm. [Alternatively, monitoring fluorescence emission at 455 nm (excitation at 340 nm) yields low-femtomolar sensitivity.]
- 5.5.3 Flow rate: 1.0 ml/minute
- 5.5.4 Run Time: 15 minutes
- 5.5.5 Gradient:

Time (minutes)	Mobile Phase A (%)	Mobile phase B (%)
0	100	0
6.5	100	0
7.0	0	100
9.0	0	100
9.1	100	0

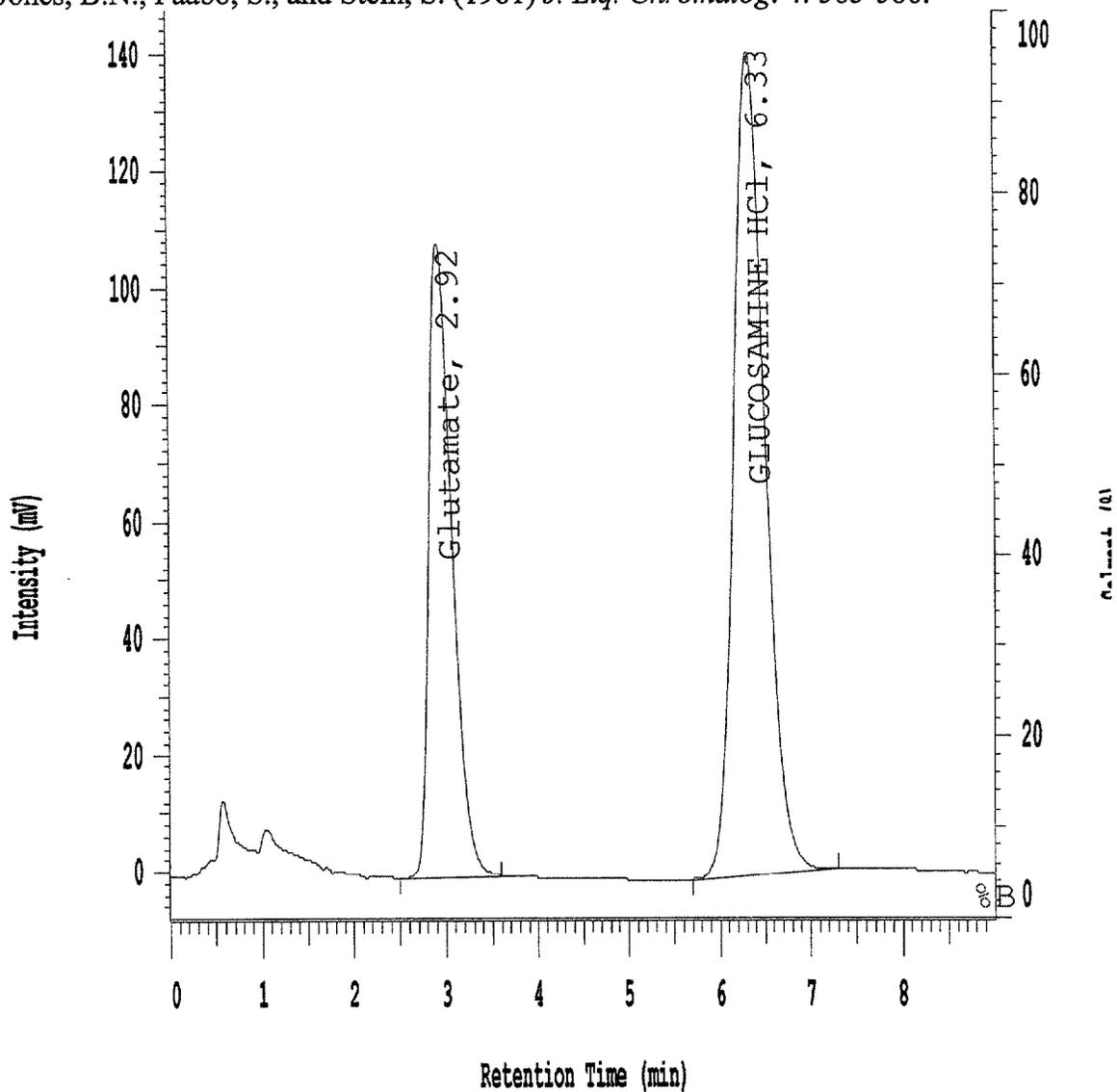
- 5.5.6 The automated pre-column derivatization process is several minutes in duration. On some HPLC instrumentation it may be possible to initiate the pre-column derivatization process for an injection while gradient elution and column equilibration is in process for the previous injection. The timing of the two processes should be programmed to assure that derivatization time remains accurate and constant for all injections.
- 5.5.7 Perform single injections of all standards and samples.
- 5.5.8 The relative retention time of glutamate (internal standard) compared to glucosamine is approximately 0.5.
- 5.5.9 The principle OPA-glucosamine anomer (β -pyranose) has a retention time of ~ 6 minutes and is the sole peak that is calibrated and quantified. [Note: The α -pyranose peak would elute at ~ 10 minutes. Anomer distribution reaches equilibrium in ≤ 20 minutes at room temperature. Since sample workup includes heating at 65°C for that period, equilibrium will be reached so only the more prominent former peak is quantified.]

[Quantification of both anomeric forms is possible; however, no significant improvement in RSD is observed.]

5.5.10 Glucosamine is quantitated using the glutamate internal standard method.

6 Reference

Jones, B.N., Paabo, S., and Stein, S. (1981) *J. Liq. Chromatog.* 4: 565-586.





PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Bc

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1: J Rheumatol 2002 Nov;29(11):2407-9

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Active ingredient consistency of commercially available glucosamine sulfate products.

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OBJECTIVE: To assess the content of active ingredient in over-the-counter (OTC) glucosamine sulfate (GLS) preparations. **METHODS:** We analyzed in a coded, blind manner 14 commercially available capsules or tablets of GLS, plus one herbal mixture as a control. We used a high performance liquid chromatography system as described. **RESULTS:** The amount of free base varied from 41 to 108% of the mg content stated on the label; the amount of glucosamine varied from 59 to 138% even when expressed as sulfate. **CONCLUSION:** If GLS is used as a therapeutic agent, it is important that the products conform to a standard in their description. The content is probably best expressed in terms of free base.

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