ACTA CHROMATOGRAPHICA, NO. 15, 2005

DEVELOPMENT AND VALIDATION OF AN HPTLC–DENSITOMETRY METHOD FOR ASSAY OF GLUCOSAMINE OF DIFFERENT FORMS IN DIETARY SUPPLEMENT TABLETS AND CAPSULES

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SUMMARY

A quantitative method using silica gel HPTLC plates, automated bandwise sample application, detection with ninhydrin chromogenic reagent solution, and automated visible mode densitometry has been developed for determination of glucosamine in nutritional supplements containing a variety of other active and inactive ingredients. Accuracy was validated by analysis of spiked blank and standard addition samples and precision by performing replicate analysis on a single day and different days. Recoveries of glucosamine hydrochloride from the spiked blank and standard addition samples were 100.0% and 101.5%, respectively. Repeatability for one sample, which was analyzed six times on a single plate, was 1.72% relative standard deviation (RSD). The intermediate precision was 1.20% RSD for a sample analyzed in duplicate once per plate on five different days over a seven-day period. A survey was made of free glucosamine content compared to the label values for nine commercial supplement products using the new method, which is shown to be suitable for routine use in nutritional supplement analysis for manufacturing quality control or governmental regulatory purposes.

INTRODUCTION

Glucosamine is an amino sugar produced from the shells of chitin (shellfish) and a key component of cartilage. Glucosamine works to stimulate joint function and repair. It has been proven effective in numerous scientific trials for easing osteoarthritis pain, aiding in the rehabilitation of cartilage, renewing synovial fluid, and repairing joints that have been da-

maged from osteoarthritis [1]. As stated by the companies of the product tested for analysis, glucosamine provides the building blocks for constructing cartilage and enhances lubrication.

Having ample glucosamine in your body is essential to producing the nutrients needed to stimulate the production of synovial fluid, which lubricates cartilage and keeps joints healthy. Without enough glucosamine, the cartilage in the hips, knees, and hands deteriorates. Each person naturally produces a certain amount of glucosamine within his or her body, but the amount might not be sufficient for healthy joint maintenance, especially as age increases. This has led to the worldwide consumption of large amounts of a great variety of over-the-counter glucosamine preparations, and reliable analytical methods are needed to test the contents of these commercial products.

A computer-assisted search of the literature through Chemical Abstracts, the ISI Web of Science, and Camag Bibliography Service (CBS) found a reversed phase ion-pairing column liquid chromatography (HPLC) method for determining glucosamine in nutritional supplements using a C_{18} column; methanol–0.05 M octanesulfonate, pH 2.1 (1:9) mobile phase; and refractive index detector [2]. Another study was published in which HPLC was used to assess the active ingredient consistency of glucosamine products [3]. Thin layer chromatography (TLC) methods have been published for analysis of glucosamine in plants [4] and biological materials [5] and for the separation and detection of free [6] and derivatized [7] glucosamine standard in mixtures with other amino compound standards. No TLC methods have been published for the determination of glucosamine in nutritional supplements.

Glucosamine is not a stable compound in its free-base form, and there are numerous more-stable forms of glucosamine available to consumers. In this research, a high-performance TLC (HPTLC) method was developed for analysis of glucosamine supplements of varying complexity and form using a silica gel layer, detection with ninhydrin spray reagent, and automated bandwise sample application and densitometric scanning. The new method was validated for sensitivity, linearity, accuracy, precision (repeatability), and ruggedness (intermediate precision), and it was applied in consumer-relevant content surveys of nine different supplement products with differing, and often unclear, label specifications.

EXPERIMENTAL

Preparation of Standard and Sample Solutions

A stock standard solution of D-(+)-glucosamine hydrochloride (#G-4875; Sigma, St Louis, MO, USA; CAS registry no. 66-84-2; 99.0% purity) was prepared at a concentration of 1.00 mg mL^{-1} in methanol–distilled water (3:1). Magnetic stirring for 30 min was required to complete dissolution. The TLC standard was prepared at 0.500 mg mL^{-1} in the same solvent by appropriate dilution of the stock solution. For the spiked blank validation, a 75.0 mg mL^{-1} glucosamine hydrochloride stock standard in pure deionized water was prepared; magnetic stirring for 30 min was required for complete dissolution. A third stock standard was prepared for the standard addition validation at a concentration of 100.0 mg mL^{-1} in deionized water, again with magnetic stirring for 30 min.

Nine different glucosamine dietary supplement products were purchased from local health and diet food stores. Each of the products had glucosamine in different labeled forms. Table I shows the exact labeling and amounts (mg $tablet^{-1}$ or mg $capsule^{-1}$) of each product analyzed. A sample stock solution of each was prepared by grinding one tablet or the con-

Table I

Exact labeling and amounts (mg $tablet^{-1}$ or mg $capsule^{-1}$) of the nine glucosamine products analyzed

Product	Label information on active ingredients (mg)
Product 1 (tablet)	Glucosamine HCl (750)
Product 2 (tablet)	Glucosamine advanced w/ arginine and boron: glucosamine HCl (375)
Product 3 (capsule)	Glucosamine sulfate: from 1000 mg glucosamine sulfate potassium chloride (750)
Product 4 (tablet)	Glucosamine, chondroitin, and MSM: glucosamine sulfate KCl (500)
Product 5 (capsule)	Glucosamine chondroitin formula: as glucosamine sulfate K, glucosamine HCl, and *N*-acetyl D-glucosamine (500)
Product 6 (tablet)	Glucosamine sulfate: from 625 mg on a 100% basis (500)
Product 7 (tablet)	Glucosamine and MSM: glucosamine sulfate from 667 mg glucosamine sulfate potassium chloride (500)
Product 8 (capsule)	Glucosamine sulfate: stabilized (500)
Product 9 (tablet)	Glucosamine with MSM: glucosamine sulfate potassium chloride; providing 750 mg glucosamine sulfate (525)

tents of one emptied capsule to a fine powder using a mortar and pestle and transferring the powder through a funnel into a 100-mL volumetric flask by washing with ca. 70 mL deionized water. The contents of the flask were stirred magnetically for 90 min, after which the stirbar was removed by use of a magnetic rod. The solution was diluted to the line with distilled water and sonicated for 30 min to completely dissolve the glucosamine. Undissolved inert ingredients were removed from Products 1, 4, 7, and 9 by filtering ca. 5 mL of solution through a Pall Gelman (Arbor, MI, USA) Acrodisc LC 13 mm syringe filter with 0.45 μm PVDF membrane into a capped vial. The glucosamine sample TLC test solutions were each prepared by mixing 100 μL glucosamine sample stock solution with 650 μL of methanol–deionized water (85:15). Digital Drummond (Broomall, PA, USA) microdispensers (10, 100, and 1000 μL) were used to measure volumes for preparation of various solutions throughout this research. The theoretical concentrations of the test solutions of Products 1 and 2 were 2.00 mg mL^{-1} of glucosamine hydrochloride; the theoretical concentrations of the other products could not be calculated based on the hydrochloride standard because of inexact content labeling.

Thin Layer Chromatographic Analysis

Analyses were preformed on 20 cm × 10 cm high-performance silica gel $60F_{254}$ GLP plates (no. 5613/6, EMD Chemicals, Gibbstown, NJ, USA; an affiliate of Merck, Darmstadt, Germany). The plates were precleaned by development to the top with dichloromethane–methanol (1:1) and dried in a fumehood before use. Standard and sample solutions were applied to the plate by means of a Camag (Wilmington, NC, USA) Linomat IV automated spray-on band applicator equipped with a 100 μL syringe and operated with the following settings: band length 6 mm, application rate 4 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from the plate edge 7 mm, and distance from the bottom of the plate 1.5 cm. The volumes applied for each analysis were 2.00 μL, duplicate 4.00 μL, and 8.00 μL of the TLC standard (1.00–4.00 μg). For each of the nine products, a different sample volume was applied in duplicate so that the sample zone scan areas matched the scan areas of the middle (2.00 μg) glucosamine hydrochloride standards. Table II shows the volume of each product that had to be applied to obtain these matching sample and standard scans.

Table II

Amount (mg) and percent free glucosamine compared with the label value (%G) in the nine products analyzed

Product (µL spotted)	Sample	Average (mg)	Avg. (% G)	n[a]	RSD (%)[b]
Product 1 (spot 2 µL)	Tablet 1	652.9	87.1	2	
	Tablet 2	564.1	75.2	2	
	Tablet 3[c]	623.3	83.1	6	1.72
Product 2 (spot 2 µL)	Tablet 1	348.3	92.9	2	
	Tablet 2	304.6	81.2	2	
	Tablet 3	329.6	87.9	2	
Product 3 (spot 2 µL)	Capsule 1	573.4	76.4	2	
	Capsule 2	613.9	81.8	2	
	Capsule 3	1025.3	136.7	2	
	Capsule 4	512.6	68.3	2	
	Capsule 5	528.2	70.4	2	
Product 4 (spot 4 µL)	Tablet 1	235.2	47.0	2	
	Tablet 2	266.4	53.8	2	
	Tablet 3	292.9	58.6	2	
	Tablet 4	294.5	58.9	2	
Product 5 (spot 5 µL)	Capsule 1	240.6	48.1	2	
	Capsule 2	197.6	39.5	2	
	Capsule 3	179.5	35.9	2	
Product 6 (spot 3 µL)	Tablet 1	320.9	64.2	2	
	Tablet 2	325.1	65.0	2	
	Tablet 3	335.5	67.1	2	
	Tablet 4[d]	337.5	54.0	5	1.20
Product 7 (spot 3 µL)	Tablet 1	299.2	59.8	2	
	Tablet 2	311.6	62.3	2	
	Tablet 3	359.4	71.8	2	
Product 8 (spot 3 µL)	Capsule 1	349.0	69.8	2	
	Capsule 2	326.2	65.2	2	
	Capsule 3	339.7	67.9	2	
Product 9 (spot 4 µL)	Tablet 1	282.8	53.9	2	
	Tablet 2	264.9	50.5	2	
	Tablet 3	290.6	55.4	2	

[a]n = number of sample zones applied to the layer
[b]RSD = relative standard deviation
[c]Repeatability result (precision)
[d]Time study result (intermediate precision)

Plates were developed 6 cm beyond the origin with 1-butanol–glacial acetic acid–deionized water (3:1:1) mobile phase in a vapor-equilibrated Camag HPTLC twin-trough chamber containing a saturation pad (Analtech, Newark, DE, USA). The development time was ca. 1.5 h.

After development, the mobile phase was evaporated from the plate by drying in a fumehood for 10 min. The plate was then sprayed heavily and evenly with ninhydrin reagent (0.3 g of ninhydrin dissolved in 100 mL of 1-butanol plus 3 mL of glacial acetic acid) and dried in the fumehood for ca. 10 min. The plate was then heated on a Camag plate heater at 115°C for several min to produce red zones of glucosamine on a white background. The sample and standard zone areas were measured by use of a Camag TLC Scanner II in a single beam reflectance mode with the tungsten source set at 580 nm, slit length 4, slit width 4, and scanning rate 4.0 mm s^{-1}. The CATS-3 software controlling the densitometer produced a polynomial regression calibration graph relating the standard zone weights to their scan areas and interpolated the sample zone weights from the graph. Because a glucosamine hydrochloride standard was used, all results were obtained on the basis of this compound. Multiplication by the ratio 179.17/215.6 (molecular weight of glucosamine/molecular weight of glucosamine hydrochloride) converted the results to free glucosamine for all nine products. This value was then multiplied by the ratio 750 μL (the total test solution volume) divided by the number of μL spotted to find the weight of free glucosamine (mg) in each product analyzed. For each analysis, percent glucosamine was then calculated by dividing the amount of free glucosamine by the label value and multiplying by 100. This gave a consumer-relevant comparison of the true content of glucosamine versus the number printed on the label for the different forms.

Validation

The accuracy of the new method was validated by spiked blank and standard addition analyses. A 1500 mg MSM (methylsulfonylmethane) joint maintenance tablet from the same manufacturers as glucosamine Product 1 was chosen as the blank because it contained all of the inert ingredients (excipients) present in all of the products, except Product 2. These inert ingredients included crospovidone, cellulose, magnesium stearate, silica, and stearic acid. Also, the MSM active ingredient separated from glucosamine on the layer. To prepare the blank solution, an MSM tablet test solution was prepared as described above, and 10.0 mL of the 75.0 mg mL^{-1} glucosamine stock standard was added by pipet to the 100

mL volumetric flask before dilution to the line to simulate a 750 mg glucosamine hydrochloride tablet solution containing exactly the label amount. An unspiked solution of the blank tablet was prepared identically. One hundred-µL amounts of the spiked and unspiked solutions were mixed with 650 µL of methanol–deionized water (85:15) and analyzed as described above. Recovery was calculated by comparing the mean analytical result for the spiked blank solution with the theoretical value of the weight of glucosamine hydrochloride added.

A standard addition validation analysis for Product 2 was performed as follows. A tablet solution was prepared in a 100 mL volumetric flask as described above, and a 1000-µL aliquot was placed in a 6 mL vial and mixed with 37.50 µL of the 100 mg mL^{-1} glucosamine hydrochloride stock solution and 3212.5 µL of methanol–deionized water (85:15). The unspiked solution was prepared by the usual 100 µL + 650 µL dilution with methanol–deionized water (85:15). The solutions were analyzed on the same plate by applying the usual four standard aliquots, duplicate 4.00 µL aliquots of the unspiked solution, and duplicate 2.00 µL aliquots of the spiked solution. The mean result of the spiked sample was compared to the theoretical result (the mean result of the unspiked sample plus the added weight) to calculate the recovery.

The precision (repeatability) of the method was evaluated by analyzing a Product 1 test solution six times on the same plate and calculating the relative standard deviation (RSD) values of the recovery results. Intermediate precision was determined by analyzing a Product 6 test solution on different plates on five days over a seven-day period.

RESULTS AND DISCUSSION

In preliminary studies, it was found that methanol–deionized water (3:1) was an excellent solvent for glucosamine standard compound. Because of low solubility in methanol, deionized water was found best to completely dissolve glucosamine from crushed nutritional supplements with 90 min of magnetic stirring and 30 min of ultrasonic mixing. After initial dissolving of products in a 100 mL volumetric flask and, if needed, removal of any undissolved excipients by filtration, a 100 µL to 750 µL dilution was required to bring the sample zone concentrations within the calibration range of the standards. This dilution was made with methanol–deionized water (85:15) to correct for the alcohol content to match the standard.

Glucosamine formed a narrow, flat, red, band-shaped zone with an R_F value of 0.36 on the silica gel HPTLC layer developed with 1-butanol–acetic acid–water (3:1:1) mobile phase (Fig. 1). The scanning wavelength of 580 nm was the absorption maximum of the in situ spectrum of a glucosamine standard zone measured with the spectral mode of the Camag scanner.

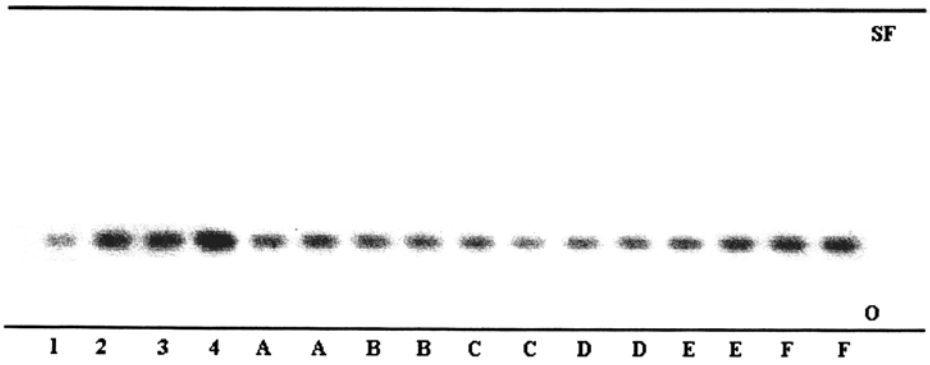

Fig. 1

Chromatograms of tablets 1, 2, and 3 (Product 5) and tablets 1, 2, and 3 (Product 6) analyzed on a high-performance silica gel $60F_{254}$ GLP plate by the described method. The plate was photographed under white light using a Camag Videostore Image Documentation System. SF, mobile phase front; O, origin; lanes 1–4, glucosamine standards; lanes A–C, duplicate ($n = 2$) samples of Product 5 tablets and lanes D–F duplicate samples of Product 6 tablets

Preparation of the calibration graphs using polynomial regression led to superior analytical results during the validation experiments compared to linear regression. As recommended by International Conference on Harmonization (ICH) guidelines [8], a calibration graph was established for glucosamine hydrochloride using five concentrations (2.00, 3.00, 4.00, 6.00, and 8.00 μL zone^{-1} of the TLC standard), representing 1.00–4.00 μg. The *r*-value (correlation coefficient) of this graph (scan areas versus weights) was 0.999. For routine analytical procedures, a three-concentration calibration graph within this range was used, produced by applying 2.00, duplicate 4.00, and 8.00 μL of the glucosamine hydrochloride TLC standard on each plate. The *r*-value for polynomial regression of these calibration graphs was always 0.999.

The limit of detection for glucosamine with ninhydrin reagent was determined by viewing developed plates containing 0.500 to 2.00 μg standard zones. The zone from 2.00 μL of the 0.500 mg mL^{-1} solution (1.00 μg) was barely visible, and the zone from 2.00 μL of a 0.250-mg mL^{-1} solution (0.500 μg) was not visible. From these results, the limit of detection and quantification were taken as 1.00 μg for glucosamine, and this amount was the lowest weight used for the respective calibration graphs as described above. The only other zone detected in the sample chromatograms was arginine (R_F = 0.18) in Product 2.

Percent recoveries for Product 1 and 2 were determined by analysis with the described procedure with n = 2. These were the only two products in the form of glucosamine hydrochloride, so percent recoveries could be calculated. The recoveries compared to the label value are shown in Table III. Values for both products were within 90–110% of the label value, which is the range usually specified for pharmaceutical dosage forms in the USP [9]. Published ranges of legally acceptable ingredient content comparable to USP are not available for nutritional supplements.

Table III

Recovery of glucosamine hydrochloride from Products 1 and 2 relative to the label values

Product	Sample	Recovery (%)
Product 1	Tablet 1	104.8
	Tablet 2	90.5
	Tablet 3	96.1
Product 2	Tablet 1	108.7
	Tablet 2	97.8
	Tablet 3	105.8

Preparing and analyzing a spiked blank sample as described above validated accuracy. Recovery of added glucosamine from the spiked MSM tablet solution was 100.0%, which represented a 0.0% error. Analysis of the unspiked MSM tablet solution showed that no interference occurred at the R_F value of glucosamine, and therefore, no correction of the scan areas of the spiked blank solution was required.

Accuracy was also validated by standard addition analysis of the fourth tablet of Product 2 for which no suitable blank was available because of the number of inactive ingredients, including ascorbic acid, natural ve-

getable fiber, hydroxypropylcellulose, dextrin, titanium dioxide, hydroxypropylmethylcellulose, corn starch, niacinamide, PEG, soy lecithin, blue 2 lake, dextrose, and cyanocobalamin. Spiked and unspiked solutions of this sample were analyzed on the same plate as described above. The tablet solution initially assayed at 97.5% recovery compared to the label value, and the recovery was within 101.5% of the amount of glucosamine hydrochloride added to this solution (1.5% error).

Repeatability was determined by spotting six 2.00 μL aliquots (n = 6) of Product 1 sample solution instead of the usual duplicate sample aliquots (n = 2). As shown in Table II, the RSD was 1.72%. As a second measure of precision, differences between scan areas of duplicate sample aliquots ranged from 0.253–2.03%, with an average of 1.27%. Intermediate precision was evaluated by five repeated analyses of a fourth tablet of Product 6 on different plates, i.e., on the day of sample preparation, the next day, and the fifth, sixth, and seventh day after preparation. The average amount of glucosamine of this sample was 337.5 mg, 54.0% of the label value, and the RSD of the analytical results was 1.20%. All of these precision values were excellent considering that application of a detection reagent by spraying was a step in the method.

Products 1 and 2 contained glucosamine hydrochloride as the active ingredient, and recovery values obtained by analysis using the glucosamine hydrochloride standard could be compared directly to the label values (Table III). The other seven products contained different forms of glucosamine, for which standards were not available, so these were analyzed using the hydrochloride standard and the hydrochloride-based results were converted to corresponding amounts of free glucosamine by calculation. Table II shows the normalized free glucosamine results for all nine products. When consumers purchase a glucosamine supplement product, they often do not realize that the number on the label is the content of a particular form of glucosamine and is not comparable to a number for a different form in another product. Data such as that in Table II allow comparison of products in terms of actual free glucosamine content. The lowest analytical result relative to the label number was found for Product 5, which contained glucosamine as sulfate K, glucosamine HCl, and *N*-acetyl D-glucosamine. This is probably because the portion of glucosamine in the *N*-acetyl form would not react with ninhydrin unless the sample was hydrolyzed with an acid before TLC. The greatest amounts overall were in Product 1 and 2, which contained glucosamine hydrochloride. Table II shows that content uniformity of Product 3 was not as great as the other

products and what we usually find when multiple tablets or capsules of regulated drug products are analyzed.

It has been demonstrated above that validation data for the new glucosamine quantitative HPTLC method meet the acceptance criteria for accuracy, precision, linearity, and detection and quantification limits set by the International Conference on Harmonization (ICH) for assay of pharmaceutical products [8]. Further, the validation data is at least as good as values reported regularly in the literature for HPTLC and HPLC analysis of pharmaceutical products [10–13] and nutritional supplements [14].

The described method is suitable for routine use by manufacturers for product quality control. It is simpler than HPLC and faster because up to sixteen samples (applied singly with the minimum three standard concentrations) can be analyzed on each plate. Cost of solvent purchase and disposal is very low because no more than 10 mL of mobile phase is required in the chamber trough containing the plate to develop these 19 chromatograms, and an additional 10 mL for vapor saturation in the other trough. The processing of samples and standards together at the same time (in-system calibration) leads to improved reproducibility and accuracy. The new method is more selective than visible-region spectrophotometric analysis with ninhydrin reagent, which could not be used for products containing glucosamine plus other ninhydrin-positive inactive or active ingredients (e.g. arginine in Product 2).

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