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Short communication

Capillary electrophoresis determination of glucosamine in nutraceutical formulations after labeling with anthranilic acid and UV detection

Nicola Volpi*

Department of Biologia Animale, University of Modena and Reggio Emilia, Via Campi 213/d, 41100 Modena, Italy

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ABSTRACT

A new robust CE method for the determination of the glucosamine (GlcN) content in nutraceutical formulations is described after its derivatization with anthranilic acid (2-aminobenzoic acid, AA). The CE separation of derivatized GlcN with AA was performed on an uncoated fused-silica capillary tube (50 μm I.D.) using an operating pH 7.0 buffer of 150 mM boric acid/50 mM NaH_2PO_4 and UV detection at 214 nm. The method was validated for specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantitation (LOQ). The detector response for GlcN was linear over the selected concentration range from 240 to 2400 pg (40–400 μg/mL) with a correlation coefficient greater than 0.980. The intra- and inter-day variations (CV%) were between 0.5 and 0.9 for migration time, and between 2.8 and 4.3 for peak area, respectively. The LOD and the LOQ of the method were approximately 200 and 500 pg, respectively. The intra- and inter-day accuracy was estimated to range from 2.8% to 5.1%, while the percent recoveries of GlcN in formulations were calculated to be about 100% after simple centrifugation for 10 min, lyophilization and derivatization with AA. The CE method was applied to the determination of GlcN content, in the form of GlcN–hydrochloride or GlcN–sulfate, of several nutraceutical preparations in the presence of other ingredients, i.e. chondroitin sulfate, vitamin C and/or methylsulfonylmethane (MSM) as well as salts and other agents. The quantitative results obtained were in total conformity with the label claims.

1. Introduction

Glucosamine (GlcN) (2-amino-2-deoxy-D-glucose, CAS number 3416-24-8) is an amino-derivative of glucose in C2, biosynthesized endogenously in animals and in man normally in almost all human tissues. GlcN is structurally incorporated and used in the synthesis of several macromolecular compounds, such as glycosaminoglycans, in particular hyaluronic acid and glucosaminoglycans, i.e. heparin and heparan sulfate, glycoproteins and proteoglycans [1,2]. Although no definitive clinical efficacy has been reported [3], GlcN is believed to be effective in treating osteoarthritis pain, rehabilitating cartilage, renewing synovial fluid and repairing joints that have been damaged from osteoarthritis [1,2]. As a consequence, several nutraceutical supplements as well as pharmaceutical preparations containing GlcN as a single component or combined with other principles and various additives and ingredients are marketed worldwide and frequently used in the treatment of osteoarthritis [4–6]. As a therapeutic agent for human use, GlcN is supplied as crystalline GlcN–HCl (glucosamine hydrochloride) or GlcN–sulfate (glucosamine sulfate), also in the presence of KCl [4–6].

There is no official method in the European Pharmacopoeia [7] regarding the assay of GlcN, while the United States Pharmacopoeia [8] reports an HPLC method for GlcN determination in bulk drug as well as in tablets by using octylsilane as the stationary phase and phosphate buffer pH 3.0/acetonitrile as the mobile phase and UV detection. As a consequence, a simple, sensitive, convenient and specific analytical procedure needs to be established for quality assurance in quality control laboratories.

HPLC is often utilized to determine GlcN in nutraceuticals [4,5,9] or in human plasma after its administration by using mass spectrometry [10–12], pulsed amperometric detection [13] and fluorimetric detection [14] after its derivatization. Other suitable techniques for the determination of GlcN involve thin-layer chromatography/densitometry [15], UV spectrophotometry after derivatization with ninhydrin [16], and FACE analysis [17].

Besides the HPLC method, capillary electrophoresis (CE) is often utilized to determine GlcN. However, due to the lack of chromophores in the molecules of common carbohydrates as well as in the GlcN molecule, it is difficult to detect these analytes in

Abbreviations: AA, anthranilic acid, 2-aminobenzoic acid; CCD, contactless conductivity detection; CE, capillary electrophoresis; CV%, standard deviation percentage; CZE, capillary zone electrophoresis; FACE, fluorophore-assisted carbohydrate electrophoresis; GlcN, glucosamine, 2-amino-2-deoxy-D-glucose; LIF, laser-induced fluorescence; LOD, detection limit; LOQ, quantitation limit; MSM, methylsulfonylmethane; MT, migration time; PA, peak area; REC%, recovery ratio percentage.

* Tel.: +39 059 2055543; fax: +39 059 2055548.
E-mail address: volpi@unimo.it.

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CE with direct UV detection. However, the use of strongly alkaline borate buffers results in the formation of complexes with enhanced absorption at 190 nm [18] or GlcN derivatization allows direct [19,20] or indirect [21] UV detection, and laser-induced fluorescence (LIF) evaluation [22,23]. Finally, capillary zone electrophoresis (CZE) with contactless conductivity detection (CCD) has been recently introduced for the determination of GlcN in pharmaceuticals [6]. However these CE applications require expensive detectors, LIF [22,23] or CCD [6], or complex procedures during the derivatization process [19] or CE separation [18], or a relatively lengthy time of analysis [21] which is a somewhat limiting feature for routine analysis. In this paper, a new CE method for the determination of GlcN content in nutraceutical formulations is reported by means of CE at 214 nm after a simple derivatization step with anthranilic acid (AA). The CE method has been applied to the determination of GlcN content of several nutraceutical preparations that also contain other ingredients, i.e. chondroitin sulfate, vitamin C and/or methylsulfonylmethane (MSM), as well as salts and other agents.

2. Experimental

2.1. Materials

D-(+)-GlcN hydrochloride, anthranilic acid (2-aminobenzoic acid, AA) and sodium cyanoborohydride were from Sigma–Aldrich. All the other reagents were analytical grade.

Various dietary supplement samples in the form of tablets, caplets or capsules containing GlcN in different formulations and amounts and in the presence of several other ingredients and excipients (Table 1) were obtained from several USA suppliers and pharmaceutical companies.

2.2. Sample preparation

Stock solutions of GlcN standard were prepared by dissolving an accurately weighed amount of 50 mg in 5 mL (10 mg/mL) of bidistilled water. A series of standard solutions were obtained by dilution of the stock solution in a standard volume of water (200 μL) and lyophilized.

For the analysis of GlcN in formulations, tablets and caplets were carefully crushed and 50 mg were dissolved in 50 mL of bidistilled water. After centrifugation at 10,000 rpm for 10 min, 200 μL (200 μg of sample) were lyophilized and derivatized with AA as reported below.

Table 1
The various dietary supplement samples analyzed in this study with their formulation and GlcN content as claimed on the product label.

Sample	Formulation	Declared GlcN Content	Measured GlcN%
A	Chondroitin sulfate MSM	GlcN–HCl, 500 mg/caplet	101.9
B	Chondroitin sulfate Vitamin C Dietary fiber	GlcN–HCl, 500 mg/caplet	103.6
C	Chondroitin sulfate Red Beetroot Juice Powder	GlcN–HCl, 750 mg/tablet	99.3
D	Chondroitin sulfate Vitamin C Dietary fiber	GlcN–HCl, 500 mg/caplet	98.7
E	Chondroitin sulfate MSM	GlcN–HCl, 750 mg/capsule	104.7
F	Cellulose Polyvinyl pyrrolidone Polyethylenglycol	GlcN–sulphate, 750 mg/caplet	96.7

2.3. Derivatization of GlcN with AA

Lyophilized GlcN standard solutions or dietary supplement samples were dissolved in 100 μL 1% fresh sodium acetate and 100 μL of AA (30 mg) and sodium cyanoborohydride (20 mg) dissolved in 1 mL of methanol–acetate–borate solution (120 mg sodium acetate and 100 mg boric acid in 5 mL methanol) were added [24]. Tubes were heated at 80 °C for 60 min. After cooling to room temperature, the samples were made up to 500 μL with bidistilled water and analyzed by CE.

2.4. Capillary electrophoresis

Capillary electrophoresis was performed on a Beckman HPCE instrument (P/ACE system 5000) equipped with a UV detector set at 214 nm. Separation and analysis were performed on an uncoated fused-silica capillary tube (50 μm I.D., 85 cm total length and 65 cm from the injection point to the detector) at 25 °C. The operating buffer was composed of 150 mM boric acid and 50 mM NaH_2PO_4 buffered at pH 7.0 with NaOH solution. The buffer was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath. Before each run, the capillary tube was washed with 0.1 M NaOH for 1 min, double distilled water for 5 min, and then conditioned with the operating buffer for 5 min. The samples to be analyzed were injected automatically, using the pressure injection mode, in which the sample is pressurized for 10 s. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer, giving an estimated volume of 6 nL per second of injection time. Electrophoresis was performed at 15 kV (about 35 μA) using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

2.5. Validation of the analytical method

The quantitative CE/UV method validations were established according to the Guidance for Industry, Bioanalytical Method Validation from the U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) published in May 2001 [25], including specificity, linearity, detection (LOD) and quantitation (LOQ) limit, precision, accuracy, recovery, and robustness tests. The detection limits were estimated as the quantity of GlcN producing a signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The specificity of the CE/UV technique was determined with migration time (MT) and peak area (PA) of the GlcN peak through the precision analysis assay. The calibration curve was constructed from PA versus concentrations of GlcN standard. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (r^2) of the calibration curve. The precision of the method was assessed by determination of GlcN standard with five replicates ($n = 5$) of five different concentrations (from 240 to 2400 pg) of standard solutions. Intra- and inter-day precision, and accuracy of the method were estimated by relative standard deviation percentage (CV%) from the analysis of freshly prepared solutions on 3 separate days. For recovery of GlcN from solid formulations, GlcN standard was added to the supplements A, B, C and F (see Table 1) dissolved in 50 mL bidistilled water, as previously described. The solutions were spiked with GlcN standard at three concentration levels (120%, 150%, and 200% of the normal GlcN concentration in the preparations) and then analyzed. The solutions were replicated three times each, and the GlcN amounts determined were compared to the theoretical amounts. The recovery ratio percentage (REC%) and their CV% were calculated. Robustness was assessed by analysis of the GlcN standard in different analytical conditions, in particular temperatures, voltage,

and buffer composition no more and no less than 10% of the adopted values.

3. Results and discussion

According to previous studies [24,26,27], GlcN (and other monosaccharides) is derivatized with AA in methanol–acetate–borate reaction medium to produce a derivative capable of strong absorption at UV 214 nm. After a short time, 1 h, required for the derivatization process, samples are separated by CE in approximately 10 min (Fig. 1) at a high sensitivity having a limit detection of approximately 200 pg (Fig. 2).

The intra- and inter-day variations (CV%), under the experimental conditions adopted, were between 0.5 and 0.9 for MT, and between 2.8 and 4.3 for PA, respectively. The calibration curve showed good linearity for the examined concentration range, 240–2400 pg (40–400 μg/mL), with an average correlation coefficient greater than 0.980 (Fig. 2). The LOD and the LOQ of the method were approximately 200 and 500 pg, respectively. The intra- and inter-day accuracy was estimated to range from 2.8% to 5.1%, while the percent recoveries of GlcN in formulations were calculated to be 97% for 120% of the normal GlcN concentration, 103% for 150% and 95% for 200% of the GlcN concentration in the preparations. As a consequence, the GlcN recovery from solid formulations was found to be about 100% after simple centrifugation for 10 min, lyophilization and derivatization with AA as previously illustrated. Variations in temperatures, voltage, and buffer composition in comparison with adopted conditions within a 10% limit do not modify the electrophoresis results. Finally, GlcN showed to be stable after 1 month at −20 °C.

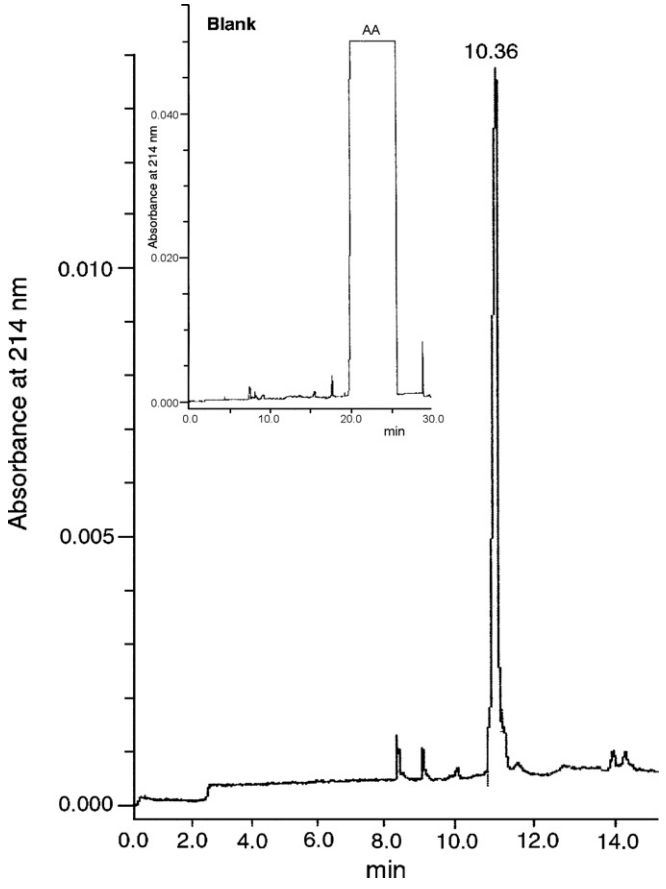

Fig. 1. CE electrophoregram of 500 pg GlcN derivatized with AA and detected at 214 nm. The inlet panel illustrates the CE electrophoregram of blank with AA indicating the derivatization agent peak.

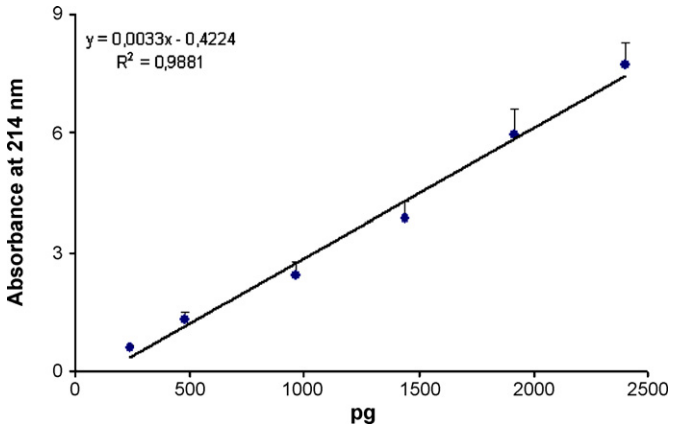

Fig. 2. Calibration curve of increasing amounts (pg = picograms) of GlcN derivatized with AA and detected at 214 nm. The equation and the correlation coefficient are reported.

CE was applied for the determination of GlcN in several solid nutraceutical formulations also in the presence of other ingredients, in particular chondroitin sulfate, vitamin C and/or MSM as well as salts and other agents (Fig. 3). The GlcN quantitative results obtained using this analytical approach are shown in Table 1, in total conformity with the label claims.

AA (2-aminobenzoic acid) is well known for its utility in the determination of the monosaccharide composition, included the different aminosugars, of glycoproteins [24,27] and glycosaminoglycans [28,29] with high sensitivity by using HPLC or CE. It is also well known that reductive amination of hexosamines is inhibited to a maximum of 30% at 50% water content [24]. As a consequence, a lyophilization step was performed before derivatization to ensure the removal of all the possible water present in the formulations.

The LOD and the LOQ of the method were found to be approximately 200 (33.3 μg/mL) and 500 pg (100 μg/mL), respectively, very similar to the values calculated for HPLC without any derivatization process (LOD = 37 μg/mL) [5], but greater than those observed for HPLC with precolumn derivatization with phenylisothiocyanate (1.25 μg/mL) [4] and dansyl chloride (2.0 μg/mL) [20], and for CE with CCD (LOD = 9.3 μg/mL) [6]. However, due to the fluorescence properties of AA [30], a strong increase in sensitivity may be obtained by using a laser-induced fluoresence (LIF) detector.

CE separation of GlcN after derivatization with AA and direct UV detection was found to be a useful approach for quantifying GlcN in nutraceutical formulations. This is a key point due to the very complex nature of these products owing to the presence of other ingredients such as chondroitin sulfate, vitamin C, MSM, salts and other agents. However, the interference from the formulations matrix to the analysis was adequately avoided by the CE conditions, by the buffer composition and UV wavelength, and in particular by the derivatization process of GlcN. Furthermore, samples for CE analysis were prepared in a short time after their lyophilization, 1 h, suitable for the processing of several samples in a day also considering the rapid analysis enabled by CE, approximately 10 min (plus 5 min for the rinse procedure). Finally, the GlcN in the nutraceuticals was quantitatively evaluated by CE with no specific pretreatment apart from centrifugation to remove insoluble material. As a consequence, this CE procedure should be generally applicable to other types of formulation for quantitative purposes. Furthermore, due to the fluorescence properties of AA, a very sensitive CE separation with LIF detection is expected for GlcN determination in biological fluids, tissues and cells.

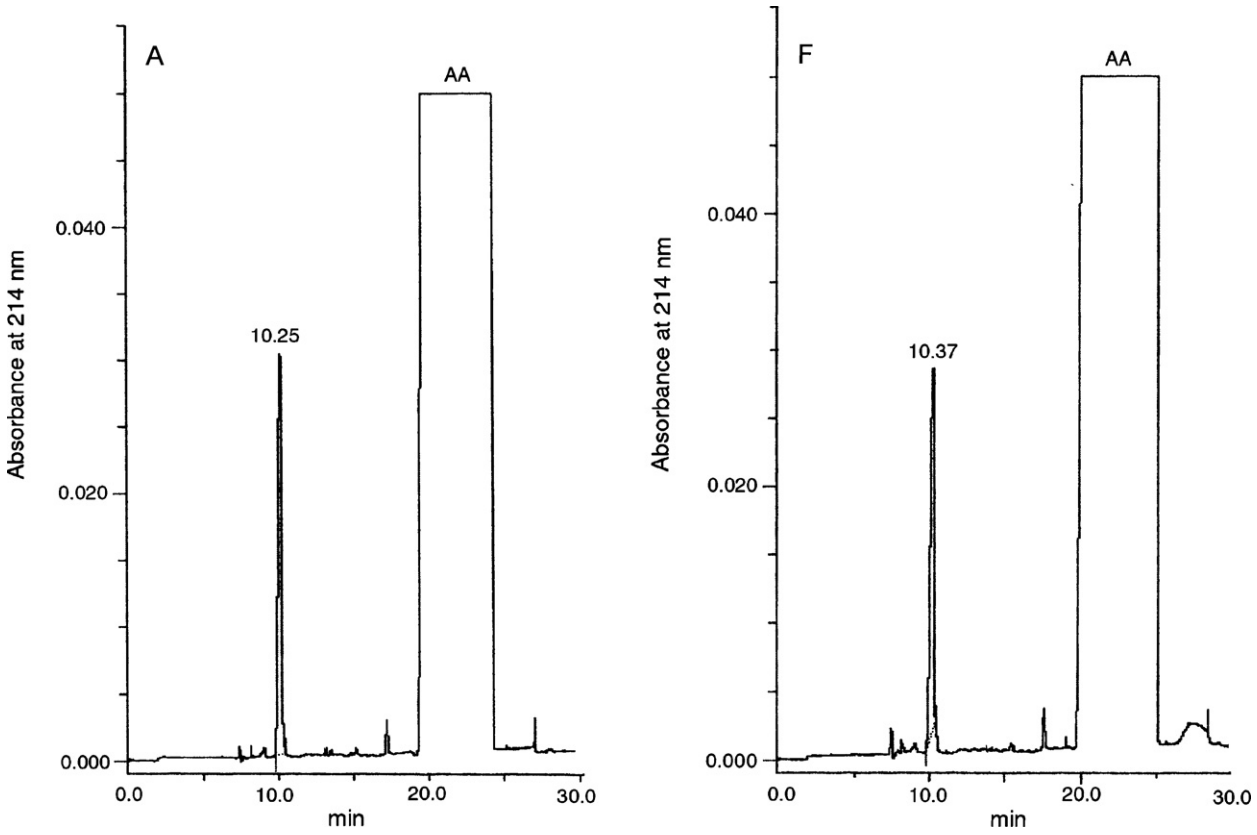

Fig. 3. CE electrophoregrams of GlcN derivatized with AA in two different (A and F as an example) nutraceutical formulations detected at 214 nm. AA indicates the derivatization agent peak.

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