Fast determination of glucosamine in pharmaceutical formulations by high performance liquid chromatography without pre-column derivatization

Determinación rápida de glucosamina en formulaciones farmacéuticas mediante cromatografía líquida de alta resolución sin derivatización pre-columna

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ABSTRACT

The goal of this work was to achieve fast determination of glucosamine in pharmaceutical formulations by high performance liquid chromatography. Commercial tablets were dissolved in water and after filtration, 6 µL were injected to Luna C18 column (250 mm × 4.6 mm, 5µm). Diode Array Detector (DAD) was set at 193 nm. Using sodium perchlorate (50 mM, pH 6.5): acetonitrile (99:1, v/v) at 0.8 mL min^{-1} as a mobile phase, glucosamine eluted with the retention time 2.834 min ± 0.003 min and the peak purity in real sample was 998.9 ± 3.3. The detection limits evaluated for peak height and peak area measurements were 0.05 µg mL^{-1} and 0.02 µg mL^{-1}, respectively. The between-day precision relative standard deviation (RSD) evaluated at two concentration levels was always below 1%.

RESUMEN

El objetivo de este trabajo fue lograr la determinación rápida de glucosamina en formulaciones farmacéuticas mediante cromatografía líquida de alta resolución. Las tabletas comerciales se disolvieron en agua y después de filtración la solución (6 µL) fue introducida en la columna Luna C18 (250 mm × 4.6 mm, 5µm). La detección UV se llevó a cabo en 193 nm. La fase móvil fue perclorato de sodio (50 mM, pH 6.5): acetonitrilo (99:1, v/v) con el flujo total de 0.8 mL min^{-1}. En estas condiciones, el tiempo de retención fue 2.834 min ± 0.003 min con la pureza de pico en la muestra real de 998.9 ± 3.3. Los límites de detección evaluados para altura y área del pico fueron 0.05 µg mL^{-1} y 0.02 µg mL^{-1}, respectivamente. La precisión evaluada para dos niveles de concentración con una desviación estándar relativa (RSD, por sus siglas en inglés) fue siempre inferior al 1%.

INTRODUCTION

An amino monosaccharide glucosamine (2-amino-2-deoxyglucose) is widely used in the treatment of degenerative joint diseases (Chiusaroli *et al.*, 2011; Distler & Anguelouch, 2006; Ragle & Sawitzke, 2012). It has also been accepted as a dietary supplement by the US Food and Drug Administration; however its effectivity is now under discussion (Vlad, LaValley, McAlindon & Felson, 2007). The natural source of glucosamine is chitin, typically obtained form sea shrimp and crab shells (López-Cervantes, Sánchez-Machado & Delgado-Rosas, 2007; Zhu, Cai, Yang & Su, 2005). The producion of pharmaceuticals and dietary supplements involves the liberation of glucosamine from chitin by means of acid hydrolysis; poor control of glucosamine content in commercial products has been highlighted as an important cause of inconsistent outcomes in clinical trials (Aghazadeh-Habashi & Jamali, 2011). In this regard, analytical control of glucosamine in the commercial products becomes an issue. Several procedures are available for this purpose, including

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simple spectrophotometric assays (Gaonkar, Khanvilkar, Shettigar & Gadgoli, 2006), thin layer chromatography (Solomon, Kumar, Anand, Sivakumar & Venkatnarayanan, 2010), liquid chromatography (LC) or capillary electrophoresis (CE) separations with spectrophotometric, contactless conductivity, amperometric or fluorimetric detection (Ander, Karlsson & Ohrlund, 2001; Campo, Campo, Ferlazzo, Vinci & Calatroni, 2001; Chaisuwan *et al.*, 2011; Cheng & Kaplan, 2003; Qi, Zhang, Zuo & Chen, 2006; Soga & Heiger, 1998). Among various studies reported previously, the most common approach relies of suitable derivatization followed by LC separation with spectrophotometric or spectrofluorimetric detection (Anumula, 1994; Cohen & De Antonis, 1994; Domínguez & Dunn, 1987; Eikenes, Fongen, Roed & Stenstrømc, 2005; Liang, Leslie, Adebowale, Ashraf & Eddington, 1999; Palace & Phoebe, 1997; Yu, Cai, Zuo & Duan, 2005; Zhou, Waszkuc & Mohammed, 2004; 2005; Zhu *et al.*, 2005). The derivatization agents used so far have been: o-phtaldialdehyde (OPA) (Eikenes *et al.*, 2005; Nemati, Valizadeh, Ansarin & Ghaderi, 2007) 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) (Zhu *et al.*, 2005), 9-fluorenylmethyl-chloroformate (López-Cervantes *et al.*, 2007), N-(9-fluorenyl-methoxycarbonyloxy) succinimide (FMOC-Su) (Zhou, Waszkuc & Mohammed, 2004; 2005), dansyl chloride (Qi *et al.*, 2006) 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Cohen & De Antonis, 1994; Palace & Phoebe, 1997), 2-aminobenzoic acid (Anumula, 1994) and phenylisothiocyanate (Liang *et al.*, 1999). Even though precolumn derivatization enables for enhanced selectivity and sensitivity of determination, it requiers additional procedural steps and makes the whole procedure less attractive for routine analytical applications. To meet the desired simplicity and speed, the direct chromatographic method has been proposed by Shao, Alluri, Mummert, Koetter & Lech (2004). In that work however, the separation of glucosamine was achieved on the specific amine column and the separation time was 15 min.

In the present study, the direct determination of glucosamine has been undertaken, aiming the development of a new, simple and fast reversed phase chromatographic procedure, suitable for routine analytical control of glucosamine-based pharmaceutical formulations.

MATERIAL AND METHODS

Instrumentation

An Agilent Series 1200 liquid chromatograph equipped with a quartenary pump, well plate autosampler, a diode array detector and Chemstation (Agilent Technologies, Palo Alto, CA, USA). The chromatographic column was Luna C18 (250 mm × 4.6 mm, 5µm) from Phenomenex.

Reagents and samples

All chemicals were of analytical reagent grade. Deionized water (18.2 MΩ cm, Labconco, USA), HPLC grade acetonitrile and methanol (Fisher Scientific, Pittsburgh, USA) were used throughout. D-(+)-Glucosamine hydrochloride (99.9% purity) from Sigma was used as a standard. The following Sigma reagents were used: hydrochloric acid, perchloric acid, boric acid, sodium hydroxide, sodium acetate and *o*-phthalaldehyde (OPA). Three commercial pharmaceutical formulations were purchased in the local market.

Procedures

Standard/sample preparation

A stock solution of glucosamine was prepared by weighing precisely 900 mg of the Sigma reagent and dissolving in 100 mL of deionized water (ultrasonication for 15 min). Working solution corresponding to the highest calibration standard (0.9 mg mL^{-1}) was prepared daily by appropriate dilution. For each pharmaceutical product, 25 tablets were weighed and ground in porcelane mortar. Five aliquots corresponding to the average mass of one tablet were precisely weighed and transferred into a volumetric flasks. For better solubilization, each suspension was ultrasonicated (10 min) and the volume was brought to 100 mL with deionized water. The samples were centrifuged (10000 g, 10 min), diluted 1:10 (v/v) with the mobile phase and introduced into the HPLC autosampler vials.

Reversed phase separation without pre-column derivatization

Isocratic elution was carried out with the mobile phase composed of sodium perchlorate (50 mM perchloric acid adjusted to pH 6.5 with 1 M sodium hydroxide) – acetonitrile (99:1, v/v) at a flow rate 0.8 mL min^{-1}. The UV detection was at 193 nm (bandwidth 6 nm) and the injection volume 6 µL. The column was termostated (20 °C), the chromatographic run was set at 4 min and the next injection was performed with no need for column cleanning and equilibration. The calibration was performed in automatic mode, setting the concentrations at 0 mg mL^{-1}; 0.15 mg mL^{-1}; 0.30 mg mL^{-1}; 0.60 mg mL^{-1}; 0.90 mg mL^{-1}. For recovery experiments, the samples were spiked also in automatic mode. In

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brief, the sample volume was set at 6 µL, then 0 µL, 4 µL and 8 µL of standard solution (0.5 mg mL^{-1}) were added, the volume was brought to 20 µL with the mobile phase and injected.

Reversed phase separation after OPA derivatization

The derivatization of glucosamine in the calibration solutions and in the real samples was performed in the automatic mode.The following steps were programmed: (1) draw 5 µL of borate buffer (0.4 M, pH 10); (2) draw 5 µL of methanolic OPA solution (40 mM); (3) wash needle, 1 s; (4) draw 6 µL of sample (or calibration solution); (5) draw mobile phase to complete 20 µL; (6) mix in wash-port, 6 times at maximum speed; (7) needle wash in the flush port, 1 s; (8) wait 2 min and (9) inject. The mobile phases A – acetonitrile, B - methanol, C – sodium aceta-te (30 mM, pH 7.0) were used in the following gradient: 0 min 15% A, 5% B, 40% C; 7.0 min 50% A, 5% B, 15% C; 7.5 min 70% A, 5% B, 15% C; 8.5 min 40% A, 5% B, 20% C; 9.5 min 15% A, 5% B, 40% C. Total flow rate was 0.8 mL min^{-1}, column was thermostated at 30 °C and the UV detection was at 340 nm. The calibration range and the sample preparation was the same as in the procedure without derivatization.

Statistical analysis

Descriptive statistics was performed to obtain means and standard deviations. To detect possible differences between the results obtained by two chromatographic procededures, ANOVA (Analysis of variance) was carried out using Statistica for Windows software (StatSoft Inc, Tulsa, OK). Significance level was established at $p < 0.05$.

RESULTS AND DISCUSSION

Chromatographic determination of non-derivatized glucosamine by reversed phase chromatography with spectrophotometric detection is hampered by its high polarity and the lack of absorption band in visible wavelength range; however this particular approach would be quite attractive for routine analytical applications. In this work, several buffering and/or ion-pairing reagents have been examined as components of the mobile phase in order to achieve acceptable retention of glucosamine on typical analytical column (Luna C18). Specifically, phosphate, borate and acetate sodium or ammonium salts, heptafluorobutyric acid and sodium perchlorate in the range of pH from 3.0 to 7.5 were examined. The effect of the mobile phase ionic strength has also been explored, using the concentration ran-

ge of the above salts from 10 mM up to 50 mM. The proportion between aqueous solution and organic modifier (acetonitrile) has been tested from 99.5:0.5 to 95:5 (v/v). Finally, the flow rate has been varied in the range 0.5 mL min^{-1}-1.5 mL min^{-1}. In each case, the selection criterions were: short retention time of glucosamine with acceptable capacity factor and high peak purity. The conditions finally selected are presented in Materials and methods.

Considering the absorption spectrum of glucosamine in UV region, the detection wavelenghts 193 nm, 196 nm and 200 nm were tested, varying the band-width in the range 6 nm to 20 nm. With the selection criterion of possible high signal-to-noise ratio, the analytical wavelength 193 nm and bandwidth 6 nm were taken for further work. The injection volume was 6 µL and the column was termostated (20 °C).

The analytical parameters evaluated for this procedure are summarized in table 1 and the chromatograms obtained in the analysis of real sample and this same sample after standard addition are presented in figure 1a. As can be observed, the retention time of glucosamine was 2.834 min (dead volume at 1.65 min) with acceptable reproducibility as demonstrated by low relative standard deviation (RSD) obtained for 10 non-succesive injections performed during one week (RSD < 0.1%). Owing to the simple chemical composition of glucosamine-based pharmaceutical formulations, the capacity factor 0.72 was considered acceptable. The peak purity measured between 2.78 min and 2.88 min was 999.1 ± 3.0 and 998.9 ± 3.3, for the standard solution and for the real sample respectively.

Calibration solutions were prepared to cover concentration range expected in ten times diluted solutions of pharmaceutical formulations; linear regression function was obtained with r^2 = 0.9998, indicating good linearity (table 1). Within-day and between-day precisions presented in table 1 were evaluated as relative standard deviation for 10 succesive measurements and as RSD for 10 measurements performed during one week, respectively. The replicate measuremets were carried out using standard solutions 0.1 mg mL^{-1} and 0.9 mg mL^{-1}, each of them prepared daily from this same stock glucosamine standard (9 mg mL^{-1}). Slightly better precision was obtained for peak height measurements, so this signal mode could be used for routine applications. It should be noted that the analytical performance achieved in this simple chromatographic procedure, especially in terms of detection limit, precision and short time of analytical run, are comparable to those recently repoted for capillary

electrophoresis with capacitively coupled contactless conductivity detector (DL = 30 µg mL^{-1}, RSD < 1.9% and 3 min run time) (Campo, Campo, Ferlazzo, Vinci & Calatroni, 2001).

To examine stability of sample solution, similar repetitive measurements were carried out during one week, while keeping the sample at 4 °C. For the peak height signal mode, the RSD value obtained for sample solution containing 0.1 mg mL^{-1} was 1.14% as compared to 0.96%

evaluated for glucosamine standard solution at this same concentration. These results confirm good stability of both, sample and calibration solutions over at least one week period of time. For recovery checking, glucosamine standard was added to the sample solution, as described in Materials and methods. Doing so, the concentrations of standard added in the final solution were 0.1 mg mL^{-1} and 0.2 mg mL^{-1}; as presented in tables 1 and 2, practically complete recovery was obtained in each case both, for peak height and peak area siglal modes.

Table 1.
Analytical parameters evaluated for the proposed procedure (without derivatization) and for OPA method.

Parameter	Without derivatization		OPA method
	Peak height	Peak area	Peak area
Retention time ± SD [1], min	2.834 ± 0.003		6.275 ± 0.013
Linear regression [2]	$A = 317.89\ c + 1.76$	$A = 978.75\ c - 5.87$	$A = 4320.6\ c - 4.94$
Standard error of the slope	4.62	10.09	108.20
Standard error of the intercept	2.55	6.55	14.61
R^2	0.9998	0.9998	0.9991
DL [3], µg mL^{-1}	0.05	0.02	0.07
QL [4], µg mL^{-1}	0.16	0.06	0.23
Within-day precision [5]:			
RSD (0.1 mg mL^{-1}, n=10), %	0.42	0.60	0.86
RSD (0.9 mg mL^{-1}, n=10), %	0.15	0.13	0.51
Between-day precision [6]:			
RSD (0.1 mg mL^{-1}, n=10), %	0.96	1.02	0.86
RSD (0.9 mg ml^{-1}, n=10), %	0.57	0.79	0.51
Recovery (0.1 mg mL^{-1}) [7], %	100.8	100.4	100.8
Recovery (0.2 mg mL^{-1}) [7], %	100.5	100.6	100.6

[1] – evaluated for 10 non-succeeding injection (realized during one week);
[2] – A = absorbance value, mAU, c = glucosamine concentration, mg mL^{-1};
[3] – detection limit based on S/N 3:1;
[4] – quantification limit based on S/N 10:1;
[5] – 10 succeeding injection of this same solution;
[6] – 10 not-succeeding injections of this same solution (realized during one week);
[7] – evaluated for 3 replicates of standard addition 0.1 mg mL^{-1} and 0.2 mg mL^{-1} (see Materials and methods).
Source: Authors own elaboration.

Table 2.
Results of recovery experiments obtained for the proposed procedure, mean concentration values determined in sample solution are given with respective standard deviation values ($n = 3$).

Standard added, µg mL^{-1}	Peak height signal mode		Peak area signal mode	
	Mean ± SD (µg mL^{-1})	Recovery (%)	Mean ± SD (µg mL^{-1})	Recovery (%)
0	313.1 ± 0.9	-	304.8 ± 2.6	-
100	413.9 ± 1.1	100.8	405.2 ± 1.9	100.4
200	514.1 ± 1.3	100.5	506.0 ± 2.1	100.6

Source: Authors own elaboration.

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It is generally accepted that the conformity between results obtained in the analysis of this same sample by means of different analytical procedures indicate that the "true" concentration value for the target species had been approached. *O*-phthalaldialdehyde (OPA) has been previously used for the derivatization of glucosamine (on –NH$_2$) prior to reversed phase separation (Domínguez & Dunn, 1987; Eikenes *et al.*, 2005; Nemati *et al.*, 2007) and this procedure has been selected in the present work for comparativepurposes. The reaction was carried out in automatic mode, as described in Materials and methods. The separation and detection conditions are also listed in Materials and methods. Since the analysis of pharmaceutical formulations does not require high sensitivity, spectrophotometric detection (340 nm) was used. The calibration range was the same as for the procedure without derivatization, however all solutions were diluted with the mobile phase (1:50) prior to injection. In figure 1b, the chromatograms obtained for the solution of pharmaceutical formulation with and without standard addition are shown. It should be stressed that the chromatographic run time was 10 min, more than two times longer with respect to the procedure without derivatization (4 min). Furthermore, the elution of two anomers of the OPA-glucosamine product can be observed in figure 1b, in agreement with the previous reports (Eikenes *et al.*, 2005). For quantification, the sum of the two peak areas was taken. The analytical parameters evaluated for OPA method are given in table 1. As can be observed, the recovery values were 100.8% and 100.6% for two levels of standard addition (0.1 mg mL^{-1} and 0.2 mg mL^{-1}), confirming the accuracy of this procedure.

Three commercial products were analyzed using the two chromatographic procedures. En each case, five sub-samples were run and the results are shown in table 3. No statistically significant differences were found between results obtained by the procedure without derivatization and those obtained by OPA method (ANOVA), which confirms good accuracy of the determination. On the other hand, for the two pharmaceuticals, the results

obtained were consistent with the values reported by the manufacturer; however, the glucosamine concentration found in product 1 corresponded to about 70 % of the value given on the label. This finding confirms previous study, in wich similar discrepancies were observed while analyzing 14 different commerial products available in Canada (Russell, Aghazadeh-Habashi & Jamali, 2002) and also indicates the need for routine analytical control of these products, in consistency with the recent comprehensive review (Aghazadeh-Habashi & Jamali, 2011).
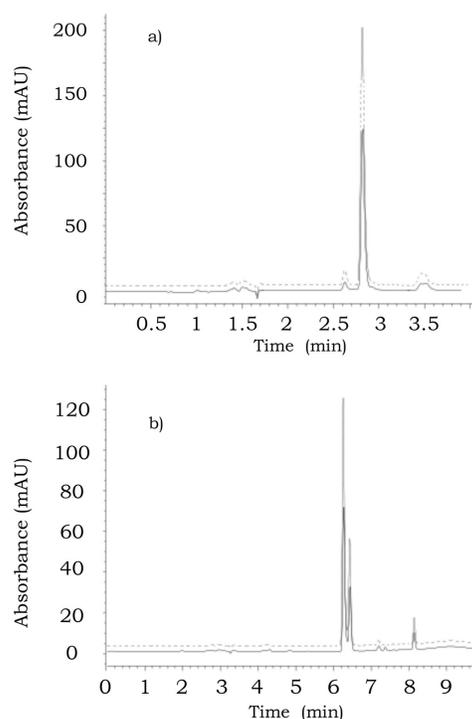

Figure 1. Reversed phase chromatograms obtained for product 2 by the two procedures: *a*) without derivatization and *b*) with OPA derivatization. (—) the diluted sample and (----) after standard addition (8 μL 0.5 mg mL^{-1} glucosamine, final concentration in the injected solution 0.2 mg mL^{-1}). For better comparison of the graphs, they have been offset on y axis.
Source: Authors own elaboration.

Table 3.
Analytical results obtained for glucosamine in the commercial pharmaceuticals in the application of the proposed procedure (without derivatization) and OPA method.

Pharmaceutical formulation	Mean result ± SD, mg/tablet (*n* = 5)		Peak area signal mode
	Without derivatization		OPA
	Peak height	Peak area	Peak area
Product 1 (500 mg/tablet)	361.2 ± 0.8	346.1 ± 0.3	363.6 ± 3.7
Product 2 (300 mg/tablet)	313.1 ± 0.9	304.8 ± 2.6	306.5 ± 1.4
Product 3 (500 mg/tablet)	495.0 ± 1.2	477.0 ± 5.6	501.5 ± 1.0

Source: Authors own elaboration.

CONCLUSIONS

In this work, the feasibility of reversed phase chromatographic determination of glucosamine in pharmaceutical products without pre-column derivatization was demonstrated in terms of sensitivity, within-day and between-day precision and also in the recovery experiments. For accuracy checking, three commercial products were analyzed by the procedure proposed and after derivatization of glucosamine with OPA; no statistically significant differences were detected between the results obtained in each procedure, confirming accuracy. The main features that make this procedure attractive for high throughput routine analyses, are the following: (1) no pre-column derivatization; (2) the use of typical reversed phase analytical column; (3) isocratic elution; (4) high peak purity with short retention time; (5) chromatpographic run completed in 4 min without necessity of column cleanning and equilibration; (6) good precision and accuracy with extremely simple procedure and low cost.

ACKNOWLEDGMENTS

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