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Development and validation of a sensitive HPLC–ESI-MS/MS method for the direct determination of glucosamine in human plasma

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Abstract

A sensitive and specific HPLC–ESI-MS/MS method for the direct determination of glucosamine in human plasma has been developed and validated. Plasma samples were analyzed after a simple, one-step protein precipitation clean-up with trichloroacetic acid using a polymer-based amino high-performance liquid chromatography (HPLC) column and a water/acetonitrile mobile phase elution gradient, with D-[1-^{13}C]glucosamine as the internal standard. Detection was performed by mass spectrometry, using an electrospray source and employing multiple reaction monitoring to separately monitor glucosamine and the internal standard. The limit of quantification of the method was 10 ng/ml of glucosamine and the calibration curve showed a good linearity up to 1000 ng/ml. The precision (R.S.D.) and the accuracy (bias) of the method at the limit of quantification were 13.8 and 4.0%, respectively, and the mean recovery of glucosamine at three concentration levels was 101.6 ± 5.7%. The method was applied for the determination of glucosamine concentrations in human plasma samples collected from untreated healthy volunteers and, in a separate bioavailability study, to evaluate plasma glucosamine pharmacokinetics profiles after oral administration of crystalline glucosamine sulfate.

Keywords: Bioavailability; Glucosamine; HPLC; Mass spectrometry; Osteoarthritis

1. Introduction

The amino monosaccharide glucosamine (GLcN) is a natural component of the glycoproteins present in connective tissue and gastrointestinal mucosal membrane and acts as a building block of glycosaminoglycans [1–5]. *In vitro* studies have shown that GLcN stimulates the production of proteoglycans via chondrocytes activation and increases sulfate uptake by articular cartilage, thus helping to rebuild cartilage. Hypothetical mechanisms by which GLcN exerts its effects relates to glucosamine-induced reversal of the pro-inflammatory and joint-degenerating effects of interleukin-1 (IL-1), via an inhibitory effect on the IL-1 intracellular signalling cascade and especially by the reduction in the activation and nuclear translocation of the transcription factor NF-kB. Other putative mechanisms involve GLcN as an inhibitor of catabolic enzymes, including matrix metalloproteinases [6]. Glucosamine sulfate was found to be a symptom- and structure-modifying drug in osteoarthritis (OA) when administered at the oral dose of 1500 mg/day [7].

The analysis of GLcN in biological fluids is important to understand its physiological role and metabolism, as well as to study GLcN pharmacokinetics, fate and mechanism of action when crystalline GLcN sulfate is used as a drug. However, the quantitative determination of GLcN in biological matrices poses a challenge to bioanalysis due to the low concentration of this molecule and its peculiar physicochemical properties. Most biological fluids, such as plasma and sinovial fluid, contain large amounts of structurally related compounds (such as glucose, galactose, and other sugars and aminosugars) that could interfere in the analysis of GLcN. Chromatographic separation of GLcN from these potentially interfering compounds is a complex task

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because sugars and aminosugars are highly polar molecules, which are not retained on the common hydrophobic column packing materials. In addition, sensitive detection of GLcN is difficult because GLcN presents a poor absorbivity, which precludes the use of conventional UV/vis spectroscopic detection techniques [8].

In order to improve the detectability of GLcN by UV/vis spectroscopy and obtain adequate retention on hydrophobic stationary phases, high-performance liquid chromatography (HPLC) methods with pre-analytical derivatization procedures involving the reaction of the GLcN amino group with hydrophobic chromophores have been developed [9–11]. However, the analytical performance of these methods were not satisfactory because, due to the aspecificity of the derivatization reaction, other sample components could be also derivatized, resulting in an increased noise in the separation and detection steps. Phenylisothiocyanate [12,13] and 1-naphthyl isothiocyanate [14] were the most used derivatization reagents, allowing GLcN analysis with detection limits ranging from 1.25 to 0.3 ng/ml. A HPLC method employing preanalytical GLcN derivatization with 1-naphthyl isothiocyanate has been applied to the evaluation of pharmacokinetics and bioavailability of GLcN in the rat [15], although its high limit of quantitation (0.63 μg/ml) did not allow good definition of pharmacokinetic profiles.

Electrochemical detection has been used to improve GLcN detectability but, although this detection technique is more specific and sensitive than UV/vis spectroscopy, the requirement of extensive sample preparation and clean-up procedures makes difficult to achieve low detection limits in complex matrices. As a result, the limits of detection were sometimes improved with respect to the methods based on UV/vis detection, while basal GLcN plasma levels were still undetectable [16,17]. High-performance liquid chromatography methods with mass spectrometry detection have been also developed for the analysis of GLcN. Upon reaction with phenylisothiocyanate, quantitative measurement of GLcN levels in plasma could be performed with detection limits ranging between 1.25 μg/ml [18,19] and 35 ng/ml [20]. Even though the latter limit of detection could be satisfactory for pharmacokinetics studies and, at least partially, for the measurement of endogenous GLcN plasma levels, these methods still involve pre-analytical GLcN derivatization procedures, which are time-consuming and could negatively affect the performance of the assay.

In the present investigation, a simple and sensitive HPLC–ESI-MS/MS method for the direct quantitative determination of GLcN in human plasma has been developed. Glucosamine was separated from other endogenous sugars and aminosugars on a polymer-based amino column, while mass spectrometry detection was performed by using a triple quadrupole mass spectrometer connected to the chromatographic system through an electrospray source operating in the positive ionization mode. The efficiency of the chromatographic separation combined with the high sensitivity and selectivity of the detection allowed us to employ a simple, one-step sample preparation procedure (protein precipitation using trichloroacetic acid). The method was validated for GLcN analysis in plasma, achieving a limit of detection low enough for the measurement of endogenous GLcN plasma levels. Therefore, in addition to the evaluation of pharmacokinetics and metabolism of exogenous GLcN after administration of GLcN during bioavailability/pharmacokinetic studies [21], the availability of this method will also allow the conduction of biochemical studies to assess the physiological role of endogenous GLcN.

2. Experimental

2.1. Materials and reagents

D-glucosamine hydrochloride (purity > 99.8%) was obtained from Rotta Reseach Laboratorium (Monza, Italy), while stable isotope-labelled D-[1-^{13}C]glucosamine hydrochloride (^{13}C-GLcN, isotope purity 99%) was purchased from Omicron (South Bend, IN, USA). Acetonitrile (HPLC grade, LiChrosolv®) was from Carlo Erba Reagents (Rodano, Italy) and deionized water was obtained using a Milli-Q Synthesis A10 system (Millipore, Molsheim, France). All the other reagents were of analytical grade and were used without further purification.

2.2. Calibration standards and quality control samples

Stock solutions of GLcN and ^{13}C-GLcN (100 μg/ml) were prepared in 10 mM ammonium acetate buffer, pH 7.5, and stored at −20 °C until use. The working solutions of GLcN, as well as the ^{13}C-GLcN internal standard working solution at fixed concentration of 12.5 μg/ml, were prepared by further dilution of aliquots of stock solutions with ammonium acetate buffer. For the preparation of calibration standards and quality control (QC) samples a large supply of plasma was obtained by pooling selected human plasma samples with a GLcN content below the limit of detection (LOD) of the method. Plasma calibration standards of GLcN in the concentration range between 2 and 1000 ng/ml, a high-concentration sample at 9000 ng/ml for assay parallelism assessment, and QC samples at 10, 100, and 800 ng/ml were prepared by spiking the appropriate GLcN working standard solution into the human plasma pool.

2.3. Sample preparation

Four-hundred ninety microliter of plasma sample were mixed with 10 μl of internal standard working solution to achieve a final ^{13}C-GLcN concentration of 250 ng/ml, then 250 μl of a 200 mg/ml solution of trichloroacetic acid in water were added. After brief stirring with a vortex mixer, the sample was centrifuged at 2000 × *g* for 20 min and the supernatant was transferred in a vial for HPLC–ESI-MS/MS analysis.

2.4. HPLC–ESI-MS/MS analysis

HPLC separation was performed on an Alliance 2695 chromatograph (Waters, Milford, MA, USA) equipped with a built-in autosampler. The analytical HPLC column was a Shodex® Asahipak NH_2P-50 2D column (5 μm, 2.0 mm i.d. × 150 mm), protected by a Shodex® Asahipak NH_2P-50G 2A guard column (5 μm, 2.0 mm i.d. × 30 mm); both columns were from Showa

Denko K.K. (Kanagawa, Japan). The sample volume injected was 3 μl, and the separation was achieved using a gradient composed of solvent A (Milli-Q water) and solvent B (acetonitrile) at a flow rate of 0.3 ml/min. The gradient elution program was as follows: 0–7 min, 20% A; 7–8 min, linear increase from 20 to 50% A; 8–16 min, 50% A; 16–17 min, linear decrease from 50 to 20% A; 17–27 min, 20% A. The eluent was introduced directly into the electrospray source of a triple quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd., UK) that was set in the positive ionization mode. Optimization of instrument response was performed by direct infusion in the mass spectrometer of a 1 μg/ml GLcN solution in 1:4 (v/v) water/acetonitrile. The electrospray source was operated using nitrogen at flow rates of 85 l/h (nebulizer gas) and 550 l/h (desolvation gas). The ion source block and desolvation temperatures were set at 120 and 250 °C, respectively. The capillary voltage was 3.6 kV and the cone voltage was 18 V. The molecular ions of GLcN and ^{13}C-GLcN were fragmented at a collision energy of 19 eV using argon as collision gas. Specific precursor/product ion transitions were employed. Multiple reaction monitoring (MRM) mode was used for the quantification of the analytes by monitoring the ion transitions *m/z* 180 → 72 and 181 → 73 for GLcN and ^{13}C-GLcN, respectively. To achieve the highest detectability the acquisition was performed at a relatively low resolution (∼0.8 *u* FWHM), which however still allowed the separate detection of the analyte and the internal standard. In order to increase the ion current intensity, we also modified the instrument by slightly enlarging the sample cone pinhole, thus allowing a higher amount of analyte(s) to enter in the mass analyzer. MassLynx version 4.0 software (Micromass UK Ltd.) was employed for instrument control and data acquisition and analysis.

2.5. GLcN quantitative analysis

Calibration curves in plasma were generated by analyzing the human plasma calibration standards according to the above described clean-up and analysis procedure. Linear calibration curve parameters were obtained from the plot of the GLcN/^{13}C-GLcN peak area ratio versus the nominal GLcN concentration by using a least squares fitting procedure. The concentrations of unknown samples were then calculated by interpolating their GLcN/^{13}C-GLcN peak area ratios on the calibration curve.

2.6. Applications

The HPLC–ESI-MS/MS method was employed to measure plasma concentrations of GLcN in 12 healthy volunteers (five females and seven males, mean age 25.9 ± 4.3 years) participating in a GLcN sulfate bioavailability study [21], which is briefly described in the following. Subjects were considered to be appropriate for study after screening medical examination and clinical laboratory analysis to assess general health. The volunteers understood and signed the Informed Consent Form, and the study protocol was approved by the local Ethics Committee (G. D'Annunzio University, Chieti, Italy). The study was conducted in accordance with the current revision of the Declaration of Helsinki concerning medical research in human and with current Good Clinical and Laboratory Practice Guidelines. Drug treatment consisted of one (single dose) or three consecutive once-daily (repeated dose) administrations of 1880 mg crystalline GLcN sulfate, corresponding to 1500 mg of GLcN sulfate, formulated as an oral soluble powder presented as a sachet. Plasma samples from each subject were collected before and up to 48 h after drug administration to obtain GLcN concentration versus time profiles. The possible contribution of endogenous GLcN to the measured GLcN concentration was taking into account by subtracting the basal GLcN plasma level measured in each subject before drug administration from the concentrations determined in all subsequent samples collected from the same individual. In order to define the physiological levels of endogenous GLcN we also measured plasma GLcN concentrations in other healthy subjects, for an overall of 82 individuals (mean age 28.5 ± 5.7 years).

3. Results

3.1. Mass spectra of GLcN and ^{13}C-GLcN and HPLC–ESI-MS/MS analysis

Full-scan and product ion mass spectra of GLcN and ^{13}C-GLcN are reported in Fig. 1a and b, respectively. The positive electrospray ionization of GLcN and ^{13}C-GLcN produced the protonated molecular ions $[M+H]^+$ at *m/z* 180 and 181, respectively [22], and other less intense peaks at *m/z* 162 and 163, which can be ascribed to the loss of one water molecule. Protonated molecular ions were selected as the precursor ions and subsequently fragmented in MS/MS mode to identify fragment ions suitable for performing MRM quantitative analysis. The most intense peaks in the product ion mass spectra corresponded to the ion fragments at *m/z* 72 (for GLcN) and 73 (for ^{13}C-GLcN) with molecular formula $[C_3H_6NO]^+$, which could derive from the fragmentation scheme depicted in Fig. 2. Due to the relative abundance of the *m/z* 72 and 73 ions in the product ion spectra and to their specificity for GLcN and ^{13}C-GLcN, the ion transitions *m/z* 180 → 72 and 181 → 73 were chosen for the quantification of the analyte and the internal standard by MRM mass spectrometry.

Typical chromatograms obtained in MRM mode for GLcN in the human plasma pool used for the preparation of plasma calibration standards and QC samples and in plasma calibration standards containing 10 and 250 ng/ml GLcN are reported in Fig. 3a–c, respectively. Fig. 3c also shows the chromatographic trace of the internal standard ^{13}C-GLcN. As expected, both GLcN and the internal standard ^{13}C-GLcN exhibited the same retention time of 4.4 min. No other significant signals are present in the chromatograms, suggesting that the HPLC method with MRM mass spectrometry detection is suitable for the selective detection of GLcN in human plasma without interference from endogenous compounds.

3.2. Calibration curves

Calibration curve parameters derived by the statistical analysis of six independently obtained calibration curves in plasma

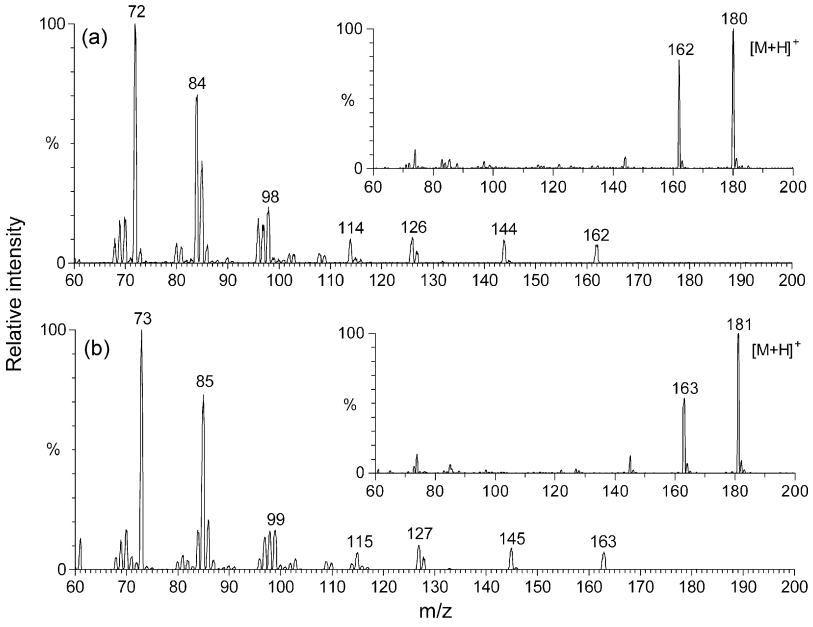

Fig. 1. Product ion mass spectra of (a) GLcN and (b) ^{13}C-GLcN recorded in the conditions reported in Section 2 by using the GLcN and ^{13}C-GLcN molecular ions as parent ions; the inserts show the full-scan mass spectra of each compound.

are reported in Table 1. The calibration curve showed a good linearity in the concentration range between the LOQ (10 ng/ml, see below) and 1000 ng/ml GLcN, and the precision of the assay was poorly affected by the concentration, the R.S.D. being below 5.0% for all calibration standards except the 10 ng/ml one.

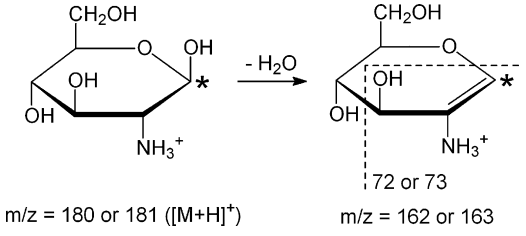

Fig. 2. Possible fragmentation sequence originating the ions at *m/z* 72 (for GLcN) and 73 (for ^{13}C-GLcN); the asterisk indicates the isotopically-labeled carbon atom in ^{13}C-GLcN.

Table 1
Mean linear calibration curve parameters obtained by least squares fitting of six independent six-points calibration curves in the 10–1000 ng/ml GLcN concentration range[a]

Curve	Slope	Intercept	Correlation coefficient
1	0.00326	0.008	0.9943
2	0.00330	0.011	0.9978
3	0.00322	0.008	0.9889
4	0.00329	0.011	0.9962
5	0.00315	0.013	0.9990
6	0.00325	0.009	0.9923
Mean	0.00324	0.010	
S.D.	0.00005	0.002	
(%) R.S.D.	1.6	19.7	

[a] Plasma calibration standards were obtained by spiking GLcN standard solutions into a human plasma pool with a GLcN concentration below the LOD of the method.

3.3. Precision, accuracy, and limit of quantification

The within-assay precision (repeatability) of the method was determined by performing six consecutive assays in the same day on QC samples spiked at three different GLcN concentration levels, i.e. 10 (low level), 100 (medium level) and 800 (high level) ng/ml, which are within the range of the calibration curve. The QC samples were also analyzed in six different days to assess the between-assay precision (reproducibility) of the method.

The accuracy of the method was evaluated at the same GLcN concentration levels by comparing the measured GLcN concentrations of the QC samples with their nominal values. These data are summarized in Table 2.

The LOQ of the method was defined according to the guidance for industry on the validation of bioanalytical methods [23].

Table 2
Within-assay and between-assay precision (R.S.D.) and accuracy (bias) of the analytical method obtained from the analysis of QC samples[a]

Theoretical GLcN concentration (ng/ml)	Mean back calculated GLcN concentration (ng/ml)	% R.S.D.	% Bias
Within-assay			
10.0[b]	10.7	9.6	7.0
100.0	97.0	4.1	−3.0
800.0	798.4	4.6	−0.2
Between-assay			
10.0[b]	10.4	13.8	4.0
100.0	100.1	5.3	0.1
800.0	789.6	4.3	−1.3
Over-range sample diluted 1:10 (v/v) before analysis, within-assay			
9000	8839	3.3	−1.8

Data are the mean values of six experiments.
[a] QC samples were obtained by spiking GLcN standard solutions into a human plasma pool with a GLcN concentration below the LOD of the method.
[b] Concentration corresponding to the LOQ of the method.

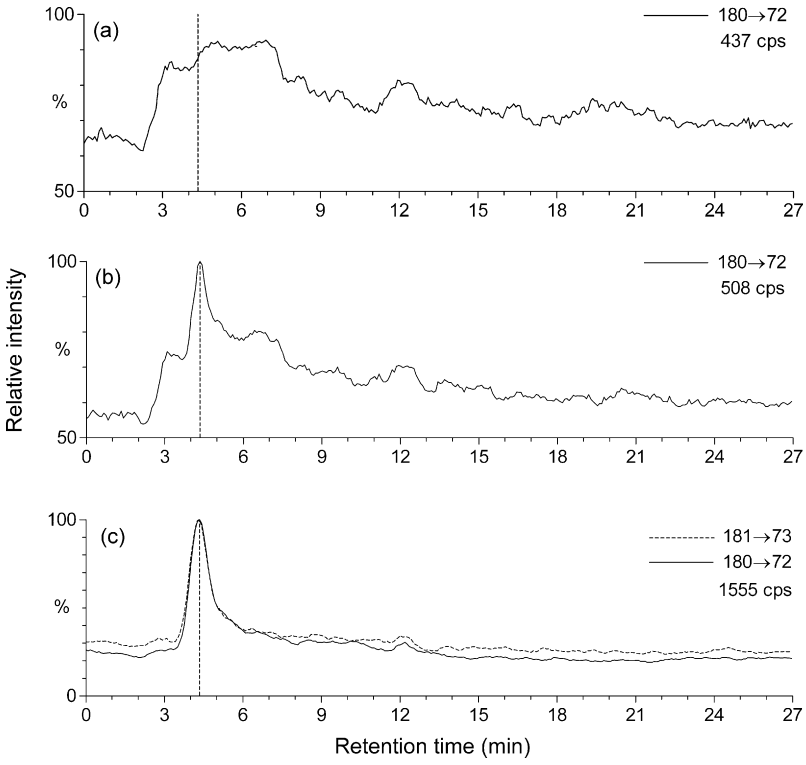

Fig. 3. Chromatograms obtained for GLcN in the MRM acquisition mode in (a) the human plasma pool used for the preparation of plasma calibration standards and QC samples, (b) a 10 ng/mL GLcN plasma calibration standard (the concentration corresponding to the LOQ of the method), and (c) a 250 ng/ml GLcN plasma calibration standard (in this chromatogram the signal of the internal standard ^{13}C-GLcN is also shown); chromatographic traces correspond to the ion transitions *m/z* 180 → 72 (GLcN, —) and 181→73 (^{13}C-GLcN, - - -).

In particular, the LOQ was defined as the concentration of the lowest standard on the calibration curve for which (a) the analyte peak is identifiable and discrete, (b) the analyte response is at least five times the response of the blank sample, and (c) the analyte response is reproducible with a precision higher than 20% and accuracy better of 80–120%. According to these criteria, the concentration value of 10 ng/ml GLcN, for which the between-assay precision (R.S.D.) and accuracy (bias) of the method were 13.8 and 4.0%, respectively, was set as the LOQ of the method. On the basis of the signal-to-noise ratio of the chromatograms, the LOD of the method could be also set, which corresponded to about 5 ng/ml GLcN.

3.4. Assay parallelism assessment

A parallelism check was performed by analyzing a high-concentration GLcN plasma sample diluted 1:10 (v/v) with the pooled plasma used for preparation of standards and QC samples. The results obtained (Table 2) indicate that plasma GLcN levels above the top calibration standard and up to 9000 ng/ml can be measured upon dilution of the sample with precision and accuracy comparable to those achieved for GLcN concentrations within the calibration range.

3.5. Process efficiency

The method process efficiency was measured by comparing the peak area ratios obtained for QC samples with those obtained for GLcN standard solutions in buffer at the same concentrations. The results, summarized in Table 3, indicates a good total process efficiency with recoveries ranging from 96.1 to 107.6% (mean recovery 101.6 ± 5.7%). In addition, we evaluated the absolute recovery of ^{13}C-GLcN by comparing the ^{13}C-GLcN peak areas for plasma extracts and ^{13}C-GLcN standard solutions

Table 3
Method process efficiency calculated from the analysis of QC samples[a]

GLcN concentration (ng/ml)	Mean area ratio in plasma extract (% R.S.D.)	Mean area ratio in buffer solution (% R.S.D.)	% Recovery
10.0[b]	0.0449 (8.4)	0.0418 (9.1)	107.6
100.0	0.3212 (4.4)	0.3342 (5.1)	96.1
800.0	2.638 (4.2)	2.609 (3.1)	101.1

Data are the mean values of six experiments.
[a] QC samples were obtained by spiking GLcN standard solutions into a human plasma pool with a GLcN concentration below the LOD of the method.
[b] Concentration corresponding to the LOQ of the method.

in buffer at the concentration used to spike the plasma samples (250 ng/ml). The absolute recovery of GLcN at this concentration level was 97.4 ± 4.6% ($n = 6$), thus demonstrating again the high process efficiency and, moreover, suggesting the absence of any significant matrix effect in the ionisation process.

3.6. Stability of plasma and processed samples

The stability of plasma and processed samples during analysis and in the usual storage conditions was investigated. No decrease in the measured GLcN concentration or change of the chromatographic behavior due to degradation of the analyte were observed in GLcN stock solutions and GLcN-spiked plasma samples or extracts maintained at room temperature for periods of up to 24 h. Spiked human plasma samples stored at −20 °C were stable for at least 4 months, and at least after three freeze-thaw cycles.

3.7. Determination of endogenous GLcN levels and application to bioavailability studies

The developed HPLC–ESI-MS/MS method was employed to measure endogenous GLcN plasma levels in healthy volunteers. The endogenous GLcN plasma concentrations showed high inter-subject variability (the values ranged from below the LOQ up to 204.0 ng/ml) with a mean value of 64.3 ± 47.2 ng/ml ($n = 82$), and no significant sex difference was observed. In fact, the mean endogenous GLcN plasma levels of male ($n = 38$) and female ($n = 44$) subjects were 62.2 ± 44.5 ng/ml and 66.4 ± 47.2 ng/ml, respectively, which were not significantly different ($P < 0.05$). On the contrary, the intra-subject variability was relatively low, as reported in [21]: indeed, it was found that the endogenous GLcN levels were rather constant, and in the majority of the subjects the variability (% R.S.D.) of basal GLcN levels between the three different study periods was <40%. Thus, the contribution of endogenous GLcN can be reliably removed from the pharmacokinetics profiles by subtracting the initial baseline GLcN values. Representative mean exogenous GLcN plasma concentration versus time profiles obtained in the bioavailability study are shown in Fig. 4. After the administration of crystalline GLcN sulfate, the plasma concentrations of GLcN rapidly increased reaching a peak of 980 ± 265 ng/ml (single dose) and 1510 ± 490 ng/ml (repeated dose) 3 h after administration ($n = 12$).

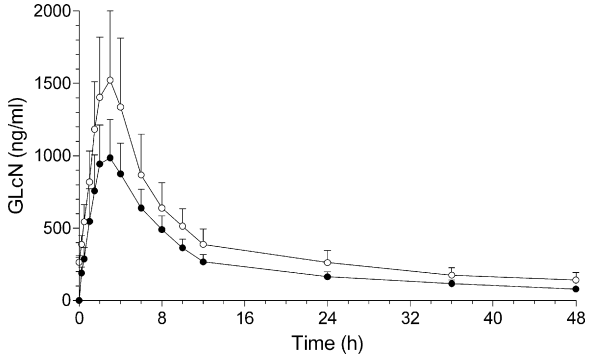

Fig. 4. Mean pharmacokinetic profiles of plasma GLcN concentration measured in healthy volunteers ($n = 12$) after single (●) and repeated (◯) oral administration of 1500 mg GLcN sulfate.

4. Discussion

The aim of this study was the development and validation of a method for the quantitative determination of GLcN in human plasma characterized by sensitivity, precision and accuracy suitable for biochemical, pharmacological and clinical applications. As mentioned in Section 1, such assay must include both a sensitive and selective GLcN detection method and an efficient separative technique in order to avoid interference due to other endogenous sugars and aminosugars contained in biological samples. In addition, the assay should not require pre-analytical derivatization steps, which lengthen the analytical procedure and may negatively affect the performance of the assay.

Among the various techniques, mass spectrometry detection proved suitable for the direct sensitive and selective measurement of GLcN. The electrospray ionization in the positive mode produced intense positive ion currents, thus enabling the detection of small concentrations of GLcN without any derivatization procedure, and quantification of GLcN performed in the MRM mode allowed to achieve high selectivity, thus reducing interferences from other sample components. In addition, the accuracy and precision of the assay was increased by employing the stable isotope-labeled D-[1-^{13}C]glucosamine as the internal standard. Even though GLcN and ^{13}C-GLcN have identical physicochemical properties, and therefore exhibit the same retention time, mass spectrometry in the MRM mode allowed their separate detection by monitoring the ion transitions at m/z 180 → 72 and 181 → 73, respectively. Such peculiar aspect of this method is, at least in part, responsible for its better performance in comparison to other methods for the determination of GLcN, which mostly used galactosamine as an internal standard [13,14,18–20].

Even if the analyte and the internal standard can be separately detected, the choice of the analytical column is still important. Although the high selectivity of the MRM mass spectrometry detection mode allowed avoiding specific interference from plasma components, other substances present at relatively high concentrations in plasma could affect the performance of the technique by causing unpredictable interferences in the ionization process (e.g., by determining ion suppression). In particular, plasma contains many sugars and aminosugars (such as glucose, galactose and galactosamine) that may be present at high concentrations: chromatographic separation of GLcN from such endogenous sugars is required in order to achieve optimal and reproducible analytical performance. This is particularly important as concerned, for example, the possible interference from endogenous galactosamine, because this aminosugar has the same molecular weight as GLcN and a very similar structure, thus shows the same mass spectroscopic behaviour.

Hydrophilic and highly polar compounds like sugars and aminosugars cannot be fractionated on the widely employed reverse-phase columns unless they are derivatized to increase their hydrophobicity. In order to perform the direct chromatographic separation of such compounds we selected a polymer-

based amino column, in which the stationary phase is a polyvinyl alcohol gel incorporating amino groups. Such column is able to separate monosaccharides, oligosaccharides and sugar alcohols by partition/adsorption mode in normal phase using water, acetonitrile or ethanol, either singly or in mixtures, as eluents. Indeed, in the optimized analytical conditions GLcN and ^{13}C-GLcN are retained with a relatively short (4.4 min) retention time (Fig. 3c). However, the overall analysis time was much longer (about 30 min) in order to elute strongly retained sample components and to re-equilibrate the column with the initial mobile phase composition after the elution gradient. The nature of the mobile phase also facilitates the coupling of the HPLC system with the mass spectrometer, since the large amount of acetonitrile leads to more efficient evaporation and desolvation processes in the electrospray interface.

The combination of an efficient HPLC separation and a selective mass spectrometry detection allowed to employ a very simple and fast sample treatment procedure. Protein precipitation with trichloroacetic acid removed all the potentially interfering endogenous compounds, as demonstrated by the absence of any significant signal in the chromatographic trace shown in Fig. 3a. Also, we did not detect potentially interfering endogenous galactosamine, which in our experimental conditions had a retention time of 4.0 min. The GLcN recovery values ranged from 96.1 to 107.6%, indicating that sample treatment did not cause a significant loss of the drug. This was confirmed by the high absolute recovery of ^{13}C-GLcN (97.4 ± 4.6%), which also demonstrated the absence of any matrix effect in the ionization process.

The LOQ of the method was 10 ng/ml (the corresponding chromatogram is reported in Fig. 3b), which is significantly lower than those of previously reported GLcN assays (mostly based on pre-analytical GLcN derivatization) and the calibration curve in plasma showed a good linearity in the 10–1000 ng/ml GLcN concentration range. It should be noted that the analyte and the ^{13}C-GLcN internal standard only differ by $m/z = 1$, therefore the natural ^{13}C isotopic contribution of GLcN is expected to affect the signal of the internal standard, especially at high GLcN concentrations. Indeed, for a 1000 ng/ml GLcN solution the peak in the 181 → 73 chromatographic trace is about 2–3% of that in the 180 → 72 trace, which agrees with the natural ^{13}C abundance and the detection of a C_3 fragment. This cross-contribution causes an increase in the internal standard peak area and, more importantly, determines a nonlinear, second-order calibration curve [24]. However, the experimental calibration curve in the 10–1000 ng/ml GLcN concentration range was still nearly linear, as demonstrated either by simulation of the calibration curve and by the high correlation coefficients obtained in the linear least squares fitting procedure (Table 1). Thus, a linear calibration curve could be used in this GLcN concentration range without introducing important errors in the calculated concentrations. Nevertheless, the pronounced nonlinearity of the calibration curve at GLcN concentrations greater than 1000 ng/ml prevented the direct analysis of samples with high GLcN levels using the linear calibration approach. Such samples were thus analyzed upon dilution with GLcN-free plasma to achieve analyte concentrations within the linear range. The R.S.D. values measured during the repeatability study did not exceed 15% and were in most cases lower than 5%, while the bias of the assay ranged between −3.0 and 7.0% (precision and bias at the LOQ were 13.8 and 4.0%, respectively). These excellent analytical performances have been exploited both in pharmacokinetics and bioavailability studies and for the determination of endogenous GLcN levels in healthy volunteers. The low LOQ of the method allowed the measurement of physiological GLcN plasma levels, which varied from below the LOQ up to approximately 200 ng/ml, while GLcN plasma concentration profiles were evaluated after single and repeated oral administration of GLcN sulfate at the therapeutic dose of 1500 mg/day. In this context, the long-term stability (at least 4 months) of GLcN in plasma and processed plasma samples stored at −20 °C and the absence of GLcN degradation in samples maintained at ambient temperature for up to 24 h were demonstrated, thus enabling parallel sample treatment and automated analysis in HPLC systems equipped with autosamplers.

5. Conclusion

The new HPLC–ESI-MS/MS method for the measurement of GLcN fulfills all the required analytical characteristics for the analysis of GLcN in human plasma. The LOQ of the method (10 ng/ml) is significantly lower than those of previously reported methods for analysis of GLcN, thus allowing both the determination of GLcN in subjects receiving this drug and the study of endogenous GLcN plasma levels. In addition, unlike the previously reported methods (including those based on mass spectrometry detection), no pre-analytical GLcN derivatization is required and the analysis can be performed directly on the plasma sample after a simple and fast protein precipitation step, thus reducing analytical variability. This assay can thus be applied for the analysis of GLcN in human plasma samples collected during clinical and pharmacokinetic studies.

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