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Determination of glucosamine sulfate in human plasma by precolumn derivatization using high performance liquid chromatography with fluorescence detection: Its application to a bioequivalence study

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Abstract

A simple, rapid, selective and specific high-performance liquid chromatography (HPLC) method with fluorescence detection was developed for determination of glucosamine sulfate in human plasma and application to a bioequivalence in healthy volunteers. Precipitation of plasma was accomplished with acetonitrile to separate interfering endogenous products from the compound of interest. After vortex mixing and centrifugation, the supernatant was transferred and derivatized with 9-fluorenylmethoxycarbonyl chloride–acetonitrile solution in borate buffer (pH = 8.0) at 30 °C for 30 min. The chromatographic separation was performed on a Diamonsil™ C_{18} column (150.0 mm × 4.6 mm, 5 μm) with a mobile phase gradient consisting of water and acetonitrile at a flow rate of 1 mL/min. The method was linear in the range of 0.1–10.0 μg/mL with a correlation coefficient (r) of 0.9996. The limit of detection was 15 ng/mL. Inter- and intra-day precisions were ≤6.28 and 7.41%, respectively, and the accuracy ranged from 95.20 to 104.92%. Extraction recoveries of glucosamine sulfate from plasma were more than 90.4%. Plasma samples containing glucosamine sulfate were stable for 40 days at −20 °C and for 24 h after derivatization at 4 °C. The method was successfully applied to the bioequivalence study of glucosamine sulfate in healthy volunteers.

Keywords: Glucosamine sulfate; High performance liquid chromatography; Derivatization; Fluorescence; Bioequivalence

1. Introduction

Glucosamine (2-amino-2-deoxy-D-glucose), an amino monosaccharide, is a natural component found in connective tissues and gastrointestinal mucosal membranes. The chemical structure of glucosamine is shown in Fig. 1. It is readily synthesized in the body from glucose. Because of its high concentration in joint tissues, the hypothesis that glucosamine supplements would provide symptomatic relief for osteoarthritis was developed as early as 30 years ago. Many clinical trials have tested this hypothesis [1] and glucosamine was shown to block NFκ B activation in IL-1-stimulated human chondrocytes. It also appeared to decrease matrix metalloproteinase expression in cultured chondrocytes and to increase the expression of a major cartilage proteoglycan [2]. Glucosamine supplements are widely used for the treatment of osteoarthritis. To meet the demand for human use, glucosamine is supplied as crystalline glucosamine sulfate.

Glucosamine is considered as a dietary supplement by the Food and Drug Administration. However, the bioavailability of glucosamine products in human is rarely determined. So, there is a need to accurately determine the bioavailability, especially because numerous products are marketed for the treatment of osteoarthritis. The drug is highly hydrophilic; hence its extraction from plasma with organic solvents is unfeasible. This renders the removal of many endogenous compounds with chemical structure similar to glucosamine. In addition, the drug has no chromophore; hence has no light absorption at the UV range. Recently, The AOAC International has established an official method for determination of glucosamine in

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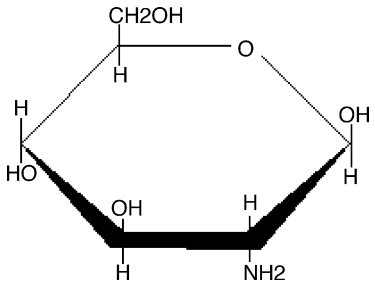

Fig. 1. The chemical structure of glucosamine sulfate.

raw materials and dietary supplements containing glucosamine sulfate and/or glucosamine hydrochloride by high-performance liquid chromatography (HPLC) with FMOC-Su derivatization [3,4]. Other literature methods have been reported in which glucosamine was analyzed in chitin sample [5] and pharmaceutical formulations [6–9]. To determine glucosamine bioavailability, previous analytical methods depended on radiolabeling a compound. However, using radioactivity to quantitate glucosamine may potentially confound results because the labeled compound and any degradation products will be quantified [9]. A precolumn derivatization HPLC method to quantitate glucosamine in beagle dog plasma sample was reported, but the lower limit of quantitation (LOQ) was 1.25 μg/mL [9], which was not sensitive enough to determine glucosamine in human plasma. Another HPLC method with precolumn derivatization and ion exchange purification was developed for the assay of glucosamine in rat plasma; however, the LOQ was also 1.25 μg/mL and glucosamine peak appeared at 29 min after injection [10], which was not suitable for routine assay for a large number of samples.

To study the bioavailability of glucosamine sulfate in human, a simple, rapid, selective and specific method should be developed. The present work describes a precolumn derivatization HPLC method with fluorescence detection for the determination of glucosamine sulfate in human plasma. The method was successfully applied to a pharmacokinetics and bioequivalence study of two capsule formulations after oral administration to 20 healthy Chinese male subjects.

2. Experimental

2.1. Materials

The test glucosamine sulfate capsule (250 mg/capsule, lot no. GSC Q026) was provided by Yongxin Pharm. Co., Ltd. (Jiangsu, China). D(+)Glucosamine sulfate (99.6% in purity) was offered by a local pharmaceutical company (Kangbao, Shanxi, China). The reference capsule was Viaereil-S capsule (lot no. C04090A) containing 250 mg of glucosamine sulfate from Rottapharm Pharm. Co. Ltd., Italy. Acetonitrile (HPLC grade) was purchased from Merck. 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl, purity 97%) was obtained from Aldrich (lot no. 05614BB). HPLC grade water was produced by the Millipore Direct-Q system.

2.2. Preparation of calibration standards and quality control samples

In order to prepare stock solution of glucosamine sulfate, 25.0 mg of glucosamine sulfate was dissolved in 25.0 mL of deionized water. The stock solution was stored at 4 °C. To 0.1 mL of the blank human plasma, the stock solution of glucosamine sulfate was added to yield final respective concentrations as 0.1, 0.3, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/mL of glucosamine sulfate in human plasma. Quality control (QC) samples (0.1, 1.0 and 5.0 μg/mL) were prepared in a similar manner.

2.3. Sample preparation [11]

Two hundred microliters of acetonitrile was added to 0.1 mL plasma to precipitate plasma proteins and to separate interfering endogenous products from the compound of interest. The mixture was vortex mixed for 1 min and centrifuged at 5000 × *g* for 5 min, and 100 μL of the supernatant layer was removed and used as a sample for derivatization. To 100 μL of each supernatant, 50 μL of 0.2 M borate buffer solution, pH 8.0, was added, followed by addition of 50 μL of 500 μg/mL FMOC-Cl-acetonitrile solution, and the resulting mixture was vortexed for 10 s. The derivatization was performed at 30 °C for 30 min in a water-bath. Then 20 μL of the resulting solution was injected into the equilibrated HPLC system.

2.4. Chromatographic conditions

The analysis of glucosamine sulfate was carried out using Agilent 1100 HPLC system consisting of G1322A Vacuum Degasser, G1312A Bin Pump, G1316A Thermostatted Column Compartment and G2710AA single instrument ChemStation for liquid chromatography (LC) systems. Chromatographic separation was achieved on Diamonsil^{TM} C_{18} (150.0 mm × 4.6 mm, 5 μm) analytical column guarded with Phenomenex Security-Guard Cartridges C_{18} (4 mm × 3.0 mm) column. The mobile phase solvent A was HPLC-grade water and the solvent B was acetonitrile. A gradient elution was used to elute glucosamine sulfate from the column (0–10 min, 30%A, 70%B; 10–16 min, 30%A, 70%B → 2%A, 98%B; 16–21 min, 2%A, 98%B → 30%A, 70%B). The flow rate was set at 1 mL/min and the temperature was maintained at 35 °C. Fluorescence detection was performed at an excitation wavelength of 263 nm and an emission wavelength of 315 nm.

2.5. Validation

2.5.1. Linearity and range

Linearity was evaluated using freshly prepared spiked plasma samples in the concentration range of 0.1–10.0 μg/mL. Five such linearity curves were analyzed. The linearity was evaluated by plotting the peak area of glucosamine versus the nominal concentration of glucosamine present in the plasma sample. The calibration curves were calculated by the equation: $y = mx + c$, using weighted least square regression. A correlation of more than 0.99 was desirable for all the calibration curves.

2.5.2. Specificity

Six randomly selected blank human plasma samples were processed by the similar derivatization procedure and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at retention time of analyte.

2.5.3. Precision and accuracy

To determine the inter-day precision, five replicates of each low, mid and high QC samples were analyzed on the same day. The intra-day precision was assessed at the same concentration on three different days. To determine the accuracy, replicates of each low, mid and high QC samples were analyzed by comparing the tested concentration with the theoretical concentration.

2.5.4. Extraction recovery

To assess the extraction recovery, two series of triplicate QC samples were prepared as described above; one with human plasma and the other without plasma. To the set without plasma, deionized water was added instead of plasma. All the samples were processed as described in Section 2.3. The extraction recovery was determined from the ratio of peak area of water standard to the peak area of corresponding plasma standard.

2.5.5. Stability

2.5.5.1. Long-term stability. To determine the long-term stability of glucosamine sulfate in human plasma the QC samples containing 5.0 μg/mL concentration were kept at −20 °C for 40 days. The samples were processed and analyzed on the 10th, 20th, 30th and 40th day. The concentrations obtained were compared with the nominal values and all values within ±15.0% qualified the test.

2.5.5.2. Short-term stability after derivatization. QC samples containing 5.0 μg/mL concentration were processed and derivatized as described in Section 2.3. The samples were kept at 4 °C for at least 24 h. The samples were analyzed at 0, 4, 8, 12 and 24 h after derivatization. Peak areas were compared to determine the stability of glucosamine derivative in plasma.

2.5.5.3. Freeze and thaw stability of frozen samples. Effect of several freeze and thaw cycles on stability of frozen plasma samples containing glucosamine sulfate was determined to establish the ruggedness of the method. Unextracted QC samples containing 5.0 μg/mL concentration were stored at −20 °C and subjected to several freeze-thaw cycles. After the completion of the last cycle, the samples were processed and analyzed, and results were compared with nominal values. The values were expected to fall within ±15.0% of the theoretical concentration.

2.6. Bioequivalence study

2.6.1. Study protocol

The above method was applied to compare the single dose oral relative bioavailability and to establish bioequivalence of two glucosamine sulfate capsule formulations.

The study was conducted as a single dose, randomized, two-period, two-treatment crossover study with a wash-out period of at least 7 days between the treatment in 20 healthy adult, male, human subjects under fasting conditions. Written informed consents were obtained from all the volunteers and the study protocol was approved by the ethical committee of Zhongshan hospital (Shanghai, China). All volunteers avoided using other drugs for at least 2 weeks prior to the study and until after its completion. Each volunteer received an oral dose of 1500 mg (six capsules) of glucosamine sulfate test or reference formulation along with 250 mL potable water. At 4 h after oral administration, all subjects were given standardized meals. Approximately 3 mL blood samples were collected via the cannula at the following times, predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 8 h after the administration. The heparinized normal saline injectable solution 2 mL was flushed after each blood sample. The blood sample was centrifuged at 1200 × *g* for 10 min and the plasma was separated and frozen at −20 °C until analyzed.

2.6.2. Pharmacokinetic analysis [12]

Individual subject glucosamine plasma concentration versus time profiles were analyzed by non-compartmental analysis. The maximum observed plasma concentration (C_{max}) and the time of maximum observed plasma concentration (T_{max}) were obtained directly from the concentration–time curve. The terminal slope (k) of the concentration–time curve was determined by log-linear regression of at least the last three data points. Elimination of half-life ($t_{1/2}\beta$) of the terminal log-linear phase was calculated utilizing the equation $0.693/k$. The $AUC_{0\to t}$ was calculated using the linear trapezoidal rule and was extrapolated to infinity according to the relationship $AUC_{0\to\infty} = (AUC_{0\to t} + C_t)/\beta$, where $AUC_{0\to\infty}$ is the area under the plasma concentration–time curve from zero to time infinity, C_t the last quantifiable concentration and β is elimination rate constant at terminal phase.

2.6.3. Statistical analysis

For the purpose of bioequivalence analysis $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} were considered as primary variables. Bioequivalence between the formulations was determined by calculating 90% confidence intervals (90%CI) for the ratio of $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} values for the test and reference formulations, using logarithmic transformed data. Analysis of variance (ANOVA) was used to assess product, group and period effects. The formulations were considered bioequivalent if the 90%CI of $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} fell within 80–125%.

3. Results and discussion

3.1. Method validation

3.1.1. Linearity and range

Calibration curves were found to be linear over the range of 0.1–10 μg/mL with the lower limit of quantitation (LOQ) of 100 ng/mL and the limit of detection (LOD) of 15 ng/mL at a signal-to-noise ratio of 3. The correlation coefficient was

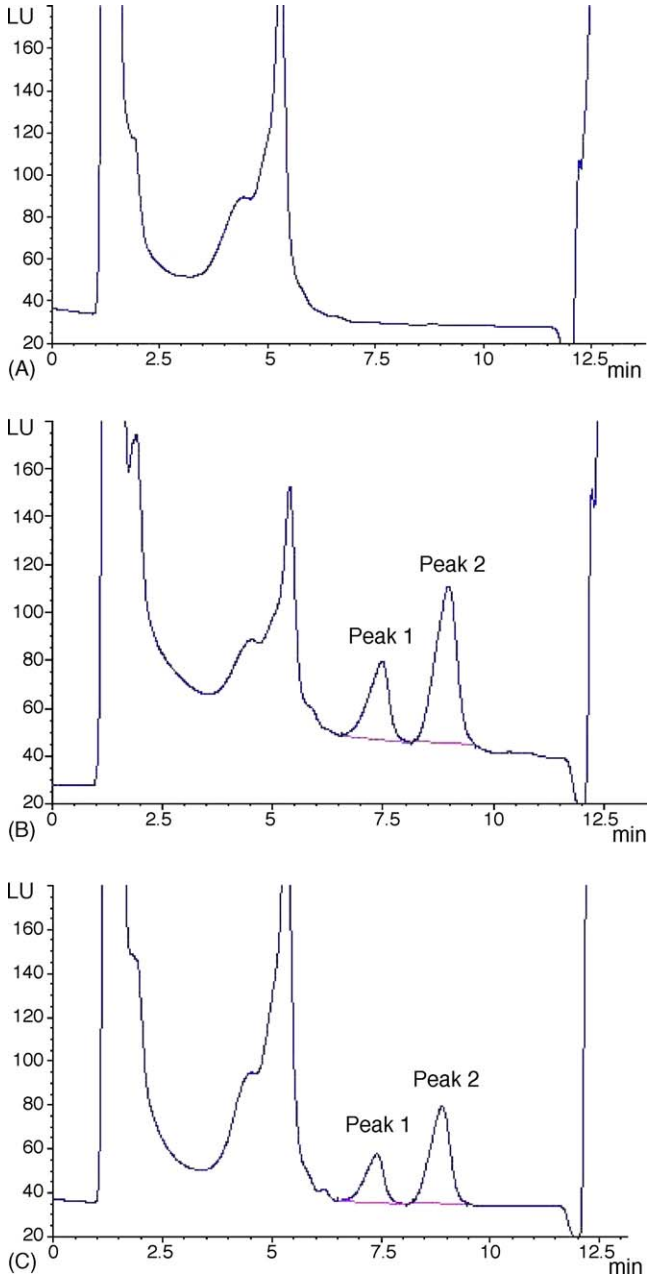

Fig. 2. HPLC chromatograms for glucosamine-FMOC-Cl resulting from analysis of: (A) blank (drug free) human plasma; (B) blank human plasma spiked with glucosamine sulfate (equivalent to 2 μg/mL); (C) plasma sample obtained at 0.5 h after a single oral dose of 6 × 250 mg glucosamine sulfate from a healthy volunteer.

found to be better than 0.99. A typical regression equation was $y = 320.51x - 9.0713$ ($r = 0.9996$, $n = 7$).

3.1.2. Specificity

Fig. 2 shows the representative chromatograms of the extracted blank human plasma, spiked plasma and a plasma sample of glucosamine sulfate. The analyte was well separated using the present chromatographic conditions. Because glucosamine has two natural stereoisomers (α and β), and the interconversion of these two in aqueous solution is not preventable, two peaks are shown in the chromatogram. The sum of the areas of these two peaks is used for the quantification of the glucosamine [4]. Glucosamine-FMOC-Cl derivative peak 1 and peak 2 were 7.5 min and 8.9 min, respectively. No interfering peaks from the endogenous plasma components were observed at the retention time of glucosamine sulfate.

Table 1
Accuracy of glucosamine sulfate in human plasma

Concentration added (μg/mL)	Mean concentration found (μg/mL)	Accuracy (%)	CV (%)	*n*
0.1	0.0952	95.20	7.35	15
1.0	1.0492	104.92	7.05	15
5.0	5.0437	100.87	5.60	15

3.1.3. Precision and accuracy

The intra-day R.S.D.s were 5.58, 7.41 and 6.12% and the inter-day R.S.D.s were 6.28, 5.68 and 4.86% for QC samples at concentrations of 0.1, 1.0 and 5.0 μg/mL, respectively. The accuracy ranged 95.20–104.92%, and the data are presented in Table 1.

3.1.4. Extraction recovery

The mean extraction recovery for glucosamine sulfate in human plasma ranged between 90.4 and 92.6% and data are presented in Table 2.

3.1.5. Stability

3.1.5.1. Long-term stability. The results demonstrated that there were no significant differences (≤±5%) among the observed concentrations at zero time and after 10, 20, 30 and 40 days stored at −20 °C, indicating glucosamine sulfate was stable in human plasma for at least 40 days when stored at −20 °C.

3.1.5.2. Short-term stability after derivatization. The results showed that no significant difference (≤±5%) was found among the peak areas at zero time and after 4, 8, 12 and 24 h at 4 °C after derivatization, which indicated the stability of FMOC-Cl derivative of glucosamine sulfate in plasma at 4 °C.

3.1.5.3. Freeze and thaw stability of frozen samples. The relative error (RE%) of the observed concentrations of before and after freeze-thaw cycles was 1.95% at the concentration of 5.0 μg/mL. The freeze-thaw cycle stability test revealed that plasma samples of glucosamine were stable after subjecting to several freeze-thaw cycles.

Table 2
Extraction recovery of glucosamine sulfate in human plasma

QC samples	Concentration added (μg/mL)	Mean recovery (%)	CV (%)	*n*
Low	0.1	91.8	5.15	5
Mid	1.0	90.4	4.26	5
High	5.0	92.6	4.18	5

Table 3
Pharmacokinetic parameters of glucosamine sulfate in 20 healthy human volunteers after oral administration

Pharmacokinetic parameter	Test formulation	Reference formulation	n
$AUC_{0\to t}$ (μg h/mL)	9.8546 ± 3.5875	9.8213 ± 4.0522	20
$AUC_{0\to\infty}$ (μg h/mL)	10.2121 ± 4.1542	10.3655 ± 4.0568	20
C_{max} (μg/mL)	3.2125 ± 2.1728	3.1065 ± 2.1410	20
T_{max} (h)	1.54 ± 0.73	1.76 ± 0.68	20
$T_{1/2}$ (h)	1.54 ± 0.35	1.57 ± 0.28	20

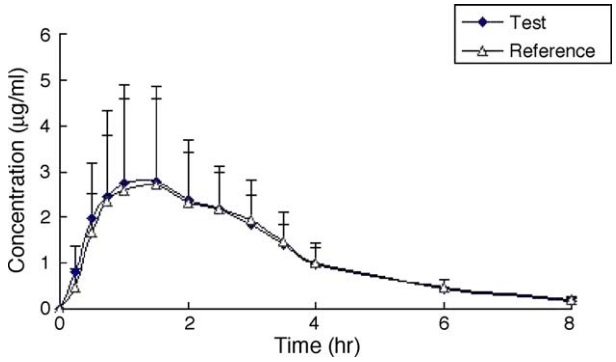

Fig. 3. Mean plasma concentration–time curves of glucosamine sulfate after administration of test and reference formulations to 20 healthy human volunteers.

Table 4
Analysis of variance (ANOVA) for the assessment of the product, period and group effects and 90% confidence intervals (90%CI) for the ratio of $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} values for the test and reference formulations, using logarithmic transformed data, after administration to 20 healthy volunteers ($\alpha = 0.05$)

Pharmacokinetic parameter	ANOVA (p-value) variation source			90%CI	n
	Product	Period	Group		
$AUC_{0\to t}$	0.2178	0.7689	0.8187	94.7 (−105.6%)	20
$AUC_{0\to\infty}$	0.6110	0.3226	0.8657	95.2 (−103.8%)	20
C_{max}	0.3624	0.1598	0.9202	96.0 (−114.1%)	20

3.2. Bioequivalence study

The HPLC method described herein was applied to a bioequivalence study of two glucosamine sulfate capsule formulations. The mean pharmacokinetic parameters and mean plasma concentration–time curves of glucosamine sulfate after administration of a single oral dose of 1500 mg for both formulations are shown in Table 3 and Fig. 3, respectively. These parameters showed close mean values, with only marginal differences between the test and reference formulations. Fig. 3 showed that the plasma concentration–time curves of the test and reference formulations were almost overlapping.

After logarithmic conversion, the analysis of variance for assessments of products, period and group effects and 90% confidence intervals for the ratio of $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} values for the test and reference formulations are shown in Table 4. The results did not show any statistically significant product, period or group effect with $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max}. Ninety percent confidence intervals with $AUC_{0\to t}$, $AUC_{0\to\infty}$ and C_{max} were comprised in the stipulated 80–125% range, indicating the bioequivalence of the test and reference formulations.

4. Conclusion

A rapid, simple, sensitive and selective HPLC method with fluorescence detector was developed for determination of glucosamine sulfate in human plasma, involving precolumn derivatization with FMOC-Cl for sample preparation. As glucosamine sulfate does not contain a chromophore absorbing in the wavelength range useful for liquid chromatography with ultraviolet detection, it is necessary to employ derivatization to facilitate HPLC analysis. As a derivatization reagent, FMOC-Cl reacts with primary and secondary amino sugars, and the derivative of glucosamine was stable for at least 24 h at 4 °C and can be eluted in a relatively short time, hence FMOC-Cl was chosen for derivatization. The method was validated and it satisfied the requirement of linearity, precision, accuracy, extraction recovery and stability. Recently, liquid chromatography with electrospray ionization mass spectrometry method for the assay of glucosamine sulfate in human plasma was reported [13]. However, compared with this HPLC method, the LC–MS method is not as widely accessible as HPLC method with fluorescence detector. Because of the low cost and short sample preparation time, this method is suitable for routine analysis. The method was successfully used to analyze glucosamine sulfate concentration in human plasma samples from a bioequivalence study. The results of the bioequivalence study indicated that the domestic capsule was bioequivalent to the imported capsule.

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