Research Article

Determination of Glucosamine in Human Plasma by High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Source-Tandem Mass Spectrometry

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Received 22 March 2011; Revised 13 April 2011; Accepted 13 April 2011

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A sensitive, specific, and rapid high-performance liquid chromatography-atmospheric pressure chemical ionization source-tandem mass spectrometry (HPLC-APCI-MS/MS) method for the determination of glucosamine in human plasma was developed and validated. Plasma samples were processed by protein precipitation with dehydrated ethanol, and the chromatographic separation was performed on an Agilent XDB-C18 column with a mobile phase of methanol—0.2% formic acid solution (70:30, v/v). Mass spectrometric quantification was carried out in the multiple reaction monitoring (MRM) mode, monitoring ion transitions of m/z 180.1 to m/z 162.1 with collision energy (CE) of 2 eV for glucosamine and m/z 181.1 to m/z 163.1 with CE of 2 eV for the internal standard (IS) in positive ion mode. The linear calibration curves covered a concentration range of 53.27–3409 ng/mL with a lower limit of quantification (LLOQ) of 53.27 ng/mL. The extraction recovery of glucosamine was greater than 101.7%. The intra- and interday precisions for glucosamine were less than 10%, and the accuracies were between 93.7% and 102.6%, determined from quality control (QC) samples of three representative concentrations. The method has been successfully applied to determining the plasma concentration of glucosamine in a clinical pharmacokinetic study involving 20 healthy Chinese male volunteers.

1. Introduction

Glucosamine is an amino monosaccharide that is an essential component of mucopolysaccharides and chitin. The molecular formula of glucosamines is \( C_6H_{13}O_5N \), also known as 2-amino-2-deoxy-D-glucose [1]. The chemical structure of glucosamine is shown in Figure 1. Glucosamine has diverse pharmacological actions. As the normal composition of cartilage matrix and synovial fluid, glucosamine not only stimulates cartilage cells and synovial cell to synthesize proteoglycans and hyaluronic acid glucosamine but also inhibits the enzyme cartilage damage [2–4]. The anti-inflammatory effects of glucosamine are also reported [5, 6]. Glucosamine has no impacts on glucose metabolism under the common use, so it is widely used to relieve symptoms from osteoarthritis for its safety [7–12]. Glucosamine can soothe pain and improve the function in patients with the cartilage degeneration diseases. Glucosamine is also beneficial for the vascular properties and cartilage metabolism.

Glucosamine is absorbed rapidly after oral administration, and nearly 90% is absorbed through the gastrointestinal path [13]. But because of the liver first pass effects, the absolute bioavailability is only 26% [14]. The plasma protein-binding rate of glucosamine is less than 10%, and the peak plasma concentrations (\( C_{\text{max}} \)) occur on an average of 4 h after drug administration. There are no obvious adverse effects in animals after an oral administration of 8000 mg/kg glucosamine [15]. Both safety trials and toxicology studies show that glucosamine has a large safe dose range [7, 16–19].
There are few articles about the determination methods for glucosamine [20–23]. In this study, a sensitive, specific and rapid high-performance liquid chromatography-atmospheric pressure chemical ionization source-tandem mass spectrometric (HPLC-APCI-MS/MS) method is developed and validated, which has never been used for the determination of glucosamine in human plasma. This method has been successfully applied to determining the plasma concentration of glucosamine and a clinical pharmacokinetic study involving 20 healthy Chinese male volunteers.

2. Experimental

2.1. Chemicals and Reagents. Glucosamine hydrochloride (purity > 99.8%) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), while stable isotope-labelled D-[1-13C] glucosamine hydrochloride (13C-GLcN, isotope purity > 99%) was purchased from Sigma (St. Louis, USA). Methanol of HPLC grade was obtained from Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Distilled water was purified in a Millipore purification system (Molsheim, France). All other chemicals were of the analytical grade from commercial sources.

2.2. Instrumentation and Analytical Conditions. The chromatography was carried out using an Agilent 6410B triple quadrupole LC/MS system (Agilent, Waldbronn, Germany) equipped with an atmospheric pressure chemical ionization (APCI) source.

The chromatographic separation was performed on an XDB-C18 column (5 μm, 250 mm × 4.6 mm i.d., Agilent, Waldbronn, Germany), operated at 25°C. A Gemini C18 column (5 μm, 3.0 mm × 4.0 mm i.d., Phenomenex, Torrance, USA) was employed as the guard column. The mobile phase, formed by methanol-0.2% formic acid solution (70 : 30, v/v), was delivered with a flow rate of 0.25 mL/min, and the injection volume was 5 μL.

Mass spectrometric quantification was carried out in the multiple reaction monitoring (MRM) mode, monitoring ion transitions of m/z 180.1 to m/z 162.1 with collision energy (CE) of 2 eV for glucosamine and m/z 181.1 to m/z 163.1 with CE of 2 eV for the IS in positive ion mode. Possible fragmentation mechanisms of zidovudine and tinidazole are shown in Figure 1. The APCI source operating parameters were set as follows: drying gas (N2) flow of 4 L/min, nebulizer pressure of 45 psi, drying gas temperature of 325°C, capillary voltage of 3.5 kV, and the fragmental voltage of 70 V.

2.3. Preparation of Standard Solutions and Quality Control (QC) Samples. The stock standard solution of glucosamine was prepared in 5% methanol at the concentrations of 852.3 μg/mL. The glucosamine stock solution was further serially diluted in 5% methanol to 6818, 3409, 1705, 852.3, 426.1, 213.1, and 106.5 ng/mL. The series of solutions were then diluted with blank human plasma to give calibration samples at seven concentration levels of 3409, 1705, 852.3, 426.1, 213.1, 106.5, and 53.27 ng/mL. The QC samples were set at the concentration levels of 3409, 852.3, and 106.5 ng/mL. The stock standard solution of internal standard, 13C-GLcN, was prepared in 5% methanol at the concentrations of 1150 μg/mL, and the solution was then diluted in 5% methanol to prepare internal standard working solution at 2.0 μg/mL. All the solutions were stored at 4°C.

2.4. Sample Preparation. A 150 μL of plasma sample was mixed with 50 μL IS solution. The mixture was processed by protein precipitation with 400 μL of dehydrated ethanol. After the mixture was vortex-mixed for 3 min and then centrifuged for 5 min at 12000 g, 400 μL of the supernatant was evaporated to dryness under a gentle stream of nitrogen gas at 45°C. The residue was reconstituted in 200 μL of dehydrated ethanol, and a 5 μL aliquot was injected into the LC-MS system.

2.5. Method Validation

2.5.1. Selectivity. The selectivity of the assay was assessed by processing samples of six different batches of blank human plasma and then comparing the chromatograms of blank plasma samples with the corresponding plasma samples spiked with glucosamine (852.3 and 53.27 ng/mL) and IS. Chromatograms were visually inspected for endogenous interferences.
2.5.2. Linearity of Calibration Curves and Lower Limits of Quantification. Linearity was tested at seven levels of concentrations covering a range of 53.27–3409 ng/mL. Calibration curve ($Y = aC + b$) was confirmed by plotting the peak area ratios ($Y$) of glucosamine to IS versus glucosamine concentration ($C$) in the calibration samples. The regression parameters were calculated by linear least square regression ($1/C^2$ weighting). The LLOQ was defined as the lowest...
2.5.3. Precision and Accuracy. To evaluate the precision and accuracy of the method, QC samples at concentrations of 106.5, 852.3, and 3409 ng/mL were analyzed on five replicates in three validation days. The concentration of each sample was determined by using calibration curve prepared on the same day. Precision was expressed using the relative standard deviation (RSD%). The accuracy is defined as the relative deviation in the determined concentration of a standard from that of its nominal concentration expressed as a percentage.

2.5.4. Recovery and Matrix Effects Evaluation. The recoveries were determined at three concentrations (106.5, 852.3, and 3409 ng/mL) by comparing the peak areas that were extracted from plasma samples with standard solutions without extraction procedure at the same nominal concentrations. Matrix effect was determined quantitatively by comparing the peak area counts from plasma samples spiked with analyte and internal standard postextraction, to samples from neat reference standard solutions at the same concentrations for analyte and internal standard. Three different concentration levels of glucosamine (106.5, 852.3, and 3409 ng/mL) were evaluated by analyzing five samples at each concentration.

2.5.5. Stability. QC samples at concentrations of 106.5, 852.3, and 3409 ng/mL were employed to investigate the stability of glucosamine in plasma under a variety of storage and handling conditions. To evaluate short-term stability, samples were kept at room temperature before analysis. Long-term storage stability was determined after freezing at −20°C for 30 days. For freeze-thaw stability, QC samples were tested after three freeze (−20°C) and thaw (room temperature) cycles. To evaluate post-preparative stability, extracted QC samples were kept under the autosampler conditions (25°C) for 20 h.

2.6. Pharmacokinetic Study. The method described above was applied to the analysis of plasma samples, as a part of a pharmacokinetic study. Twenty healthy Chinese male volunteers aged 21–30 years and weighing from 52 to 76 kg were selected for this study after a clinical screening procedure. Each volunteer was fasted and administered randomly a single dose of 1.44 g glucosamine hydrochloride test dispersible tablets produced by Anbeite Pharm.Co.Ltd. (Zhejiang, China) or reference tablets produced by Xinsidun Pharm.Co.Ltd. (Sichuan, China) in cycle. Venous blood samples (5 mL) were collected in heparin tubes at the times of 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 24.0 h after oral administration of glucosamine tablets. The whole blood samples were immediately centrifuged (2800 rpm × 10 min), and the plasma obtained was frozen at −20°C until analysis.

The pharmacokinetic parameters of glucosamine including maximum plasma concentration (Cmax), time point of maximum plasma concentration (Tmax), area under the plasma concentration versus time curve from 0 h to the last measurable concentration (AUC0−t), area under the plasma concentration versus time curve from 0 h to infinity (AUC0−∞), and elimination half-life (t1/2) were acquired.

3. Results and Discussion

3.1. Assay Selectivity. The typical MRM chromatograms of blank human plasma, spiked plasma samples with glucosamine (53.27, 852.3 ng/mL) and the IS, and a plasma sample from a healthy volunteer after oral administration
Glucosamine was 53.27 ng/mL with the signal-to-noise ratio (S/N) of 0.994, indicating a good linearity. The LLOQ for glucosamine was 106.5 ng/mL was 93.3 ± 2.1, 2.25 ± 0.644, and 107.4 ± 6.9, respectively. There were no obvious interferences at the retention times of the analyte and the IS. The results showed that the method exhibited good specificity and selectivity.

3.2. Calibration Curve and Sensitivity. The regression equation for calibration curves in the range of 53.27 ~ 3409 ng/mL was Y = 1.9487X + 0.0249. The correlation coefficient was 0.999, indicating a good linearity. The LLOQ for glucosamine was 53.27 ng/mL with the signal-to-noise ratio (S/N) ≥ 10. The precision at this concentration was 6.50% (n = 5), and accuracy was 88.7%, indicating a good sensitivity of the established method (Table 1).

### Table 3: Absolute recoveries and matrix effect evaluation of glucosamine in human plasma (n = 5).

<table>
<thead>
<tr>
<th>Nominal concentration (ng/mL)</th>
<th>Mean recovery ± S.D. (%)</th>
<th>R.S.D. (%)</th>
<th>Mean matrix effect ± S.D. (%)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>106.5</td>
<td>93.3 ± 2.1</td>
<td>2.25</td>
<td>107.4 ± 6.9</td>
<td>6.44</td>
</tr>
<tr>
<td>852.3</td>
<td>96.3 ± 3.1</td>
<td>3.19</td>
<td>101.7 ± 3.8</td>
<td>3.69</td>
</tr>
<tr>
<td>3409</td>
<td>102.2 ± 3.1</td>
<td>3.08</td>
<td>104.9 ± 3.2</td>
<td>3.09</td>
</tr>
</tbody>
</table>

### Table 4: Stability of glucosamine in human plasma at three QC concentrations (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>106.5 ng/mL</th>
<th>852.3 ng/mL</th>
<th>3409 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Determined mean concentration (ng/mL) ± S.D.</td>
<td>R.S.D. (%)</td>
<td>Mean accuracy (%)</td>
</tr>
<tr>
<td>Short-term stability</td>
<td>95.2 ± 3.7</td>
<td>3.86</td>
<td>89.4</td>
</tr>
<tr>
<td>Postpreparatory stability</td>
<td>97.9 ± 1.2</td>
<td>1.26</td>
<td>92.0</td>
</tr>
<tr>
<td>Freeze-thaw stability</td>
<td>111.1 ± 10.7</td>
<td>9.62</td>
<td>104.3</td>
</tr>
<tr>
<td>Long-term stability</td>
<td>100.5 ± 4.0</td>
<td>3.40</td>
<td>94.4</td>
</tr>
</tbody>
</table>

were shown in Figure 2. As shown, the retention times of glucosamine and IS were approximate 3.3 min and 3.3 min, respectively. There were no obvious interferences at the retention times of the analyte and the IS. The results showed that the method exhibited good specificity and selectivity.

3.3. Precision and Accuracy. Intra- and interday precision and accuracy values are summarized in Table 2. As shown, all the accuracy values were within 85 ~ 115%, and the intra- and interday precisions were all less than 15%. The data demonstrate that the precision and accuracy of this assay are within the acceptable range and the method is accurate and precise.

3.4. Recovery and Matrix Effects Evaluation. The absolute recoveries of glucosamine at 106.5, 852.3, and 3409 ng/mL were 107.4%, 101.7%, and 104.9% (n = 5), respectively (Table 3), showing a good extraction recovery. The matrix effects for glucosamine at 106.5, 852.3, and 3409 ng/mL were calculated by dividing the area of plasma-extracted sample spiked with analyte and IS, by the area of the respective neat solution and were equal to 93.3%, 96.3%, and 102.2% (n = 5), respectively (Table 3).

3.5. Stability. The data of the short-term, freeze-thaw, and long-term stability, as well as for the postpreparative test of glucosamine are summarized in Table 4. It is shown that glucosamine in human plasma was stable at room temperature for 6 h or at −20°C for 30 days. The results of the freeze-thaw stability test indicated that the samples underwent three freeze-thaw cycles were stable. The postpreparative stability tests showed that no significant degradation occurred in the sample extracts under the autosampler conditions (25°C) for 20 h.

3.6. Pharmacokinetic Study. The method was successfully applied to a pharmacokinetic study including 20 healthy male Chinese volunteers after an oral dose of 1.44 g glucosamine hydrochloride preparation. The mean plasma concentration time curve is shown in Figure 3. The main pharmacokinetic parameters of glucosamine were calculated and...
summarized in Table 5. In this study, the relative bioavailability of the test formulation was 103.0%, based on the test-reference ratio of AUC. There were no remarkable differences in bioavailability between the test glucosamine dispersible tablet and the reference formulation.

4. Conclusion

A sensitive and efficient HPLC-APCI-MS/MS method was developed and validated for the determination of glucosamine in human plasma. The described method showed acceptable specificity, precision, accuracy, and linearity, allowing the determination of glucosamine in the clinical pharmacokinetic study.

References


Table 5: The main pharmacokinetics parameters of glucosamine in 20 healthy volunteers after an oral administration of 1.44 g of glucosamine hydrochloride.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test tablet</th>
<th>Reference tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2/h</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Tmax/h</td>
<td>2.0 ± 0.6</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>Cmax/μg·L⁻¹</td>
<td>1890 ± 568</td>
<td>1826 ± 618</td>
</tr>
<tr>
<td>AUCC₀→₀/μg·h·L⁻¹</td>
<td>6033 ± 1560</td>
<td>5909 ± 1543</td>
</tr>
<tr>
<td>AUCC₀→∞/μg·h·L⁻¹</td>
<td>6365 ± 1622</td>
<td>6284 ± 1478</td>
</tr>
</tbody>
</table>
