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Improved and simplified liquid chromatography/electrospray ionization mass spectrometry method for the analysis of underivatized glucosamine in human plasma

Sheng Zhong [a,b], Dafang Zhong [a], Xiaoyan Chen [a,*]

[a] *Center for Drug Metabolism and Pharmacokinetics Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 646 Songtao Road, Shanghai 201203, PR China*
[b] *Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China*

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Abstract

Glucosamine is an amino monosaccharide reagent. It is difficult to assay using typical reversed-phase column due to the early elution, by optimizing the chromatographic conditions, especially the analytical column and the mobile phase composition, an improved analytical method was developed and validated, which offers rapid, sensitive and specific determination of glucosamine in human plasma. Following protein precipitation, the analyte and internal standard (valibose) were separated using an isocratic mobile phase on an Inertsil CN-3 column and detected by mass spectrometry in the multiple reaction monitoring mode using the respective precursor to product ion combinations of *m/z* 180/72 for glucosamine and *m/z* 252/198 for valibose. The chromatographic time was just 4.2 min for each sample, which made it possible to analyze more than 120 human plasma samples per day. The method exhibited a linear dynamic range of 4.00–4000 ng/mL for glucosamine in human plasma. The lower limit of quantification (LLOQ) was 4.00 ng/mL with a relative standard deviation of less than 10.9%. Acceptable precision and accuracy were obtained for the plasma concentrations over the standard curve range. By monitoring the two different MRM transitions, it was proved that no endogenous glucosamine was found in human plasma. The validated method has been successfully used to analyze human plasma samples for application in a bioequivalence study.

Keywords: Glucosamine; Chromatography optimization; LC/MS/MS; Plasma concentration

1. Introduction

Glucosamine (2-amino-2-deoxy-D-glucose, Fig. 1), an amino monosaccharide, is the first clinical proven disease modifying drug for osteoarthritis. Contrary to non-steroidal anti-inflammatory drugs, glucosamine interferes with the disease both in terms of symptoms and of the joint structure changes. After administration, the drug is preferentially incorporated by chondrocytes into the components of the glycosaminoglycan chains in the intact cartilage [1]. Moreover, it can stimulate the synthesis of proteoglycans and inhibit its degradation, so as to restore the articular function [2]. Meanwhile, it can also inhibit the proinflammatory mediators in human osteoarthritis chondrocytes stimulated with interleukin-1β [3].

The bioanalytical component of a pharmacokinetics study requires an analytical method with simplicity, selectivity, sensitivity and rapid turn-around time. A few methods for the quantification of glucosamine in biological fluids have been reported [4–7]. Some were based on precolumn derivatization using HPLC [4,5] or liquid chromatography/mass spectrometry (LC/MS) [6]. Precolumn derivatization techniques enabled the drug to be separated on typical reversed-phase C_{18} column by increasing its hydrophobicity, but it had drawbacks of time-consuming sample preparation which could not meet the requirement of high throughput analysis.

Quantification of drugs in biological matrix by liquid chromatography/tandem mass spectrometry (LC/MS/MS) is becoming more common due to the improved sensitivity and selectivity of this technique. Recently, Roda et al. [7] described an LC/MS/MS method to determine glucosamine by a gradient elution program to separate glucosamine from human plasma

* Corresponding author. Tel.: +86 21 50800738; fax: +86 21 50800738.
E-mail address: xychen@mail.shcnc.ac.cn (X. Chen).

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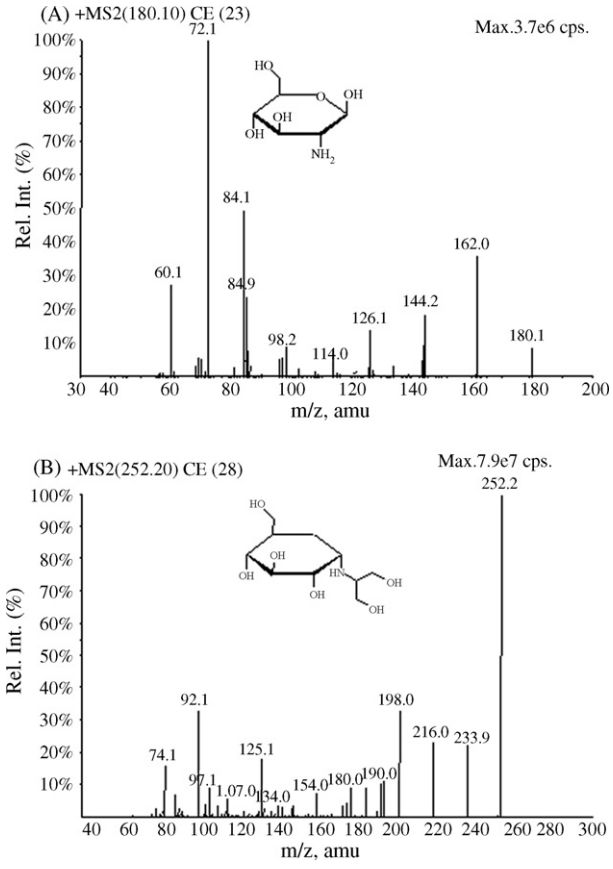

Fig. 1. Full-scan production spectra of $[M+H]^+$ of glucosamine (A) and valibose (IS) (B).

on an amino column. The chromatographic run time per sample was about 30 min. The lower limit of quantification (LLOQ) was 10 ng/mL using 490 μL plasma.

In the study presented here, by optimizing the chromatographic conditions, especially the analytical column and the mobile phase composition, a novel rapid and sensitive LC/MS/MS method suitable for the determination of glucosamine using a low volume (100 μL) of human plasma was developed. After full validation, it was expected that this method would be efficient in analyzing large batches of plasma samples obtained for pharmacokinetics, bioavailability or bioequivalence studies after therapeutic doses of administration of glucosamine sulfate.

2. Experimental

2.1. Chemicals

D-Glucosamine sulfate·2NaCl (101.5% purity) and valibose (internal standard, 99.4% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol and acetonitrile were purchased from Sigma (Steinheim, Germany). HPLC-grade ammonium acetate and acetic acid were purchased from Tedia (Fairfield, OH, USA). Blank (drug free) heparinized human plasma was provided by Shanghai Shuguang Hospital (Shanghai, China). Distilled water, prepared from demineralized water, was used throughout the study. Viartril-S™ (Glucosamine sulfate capsule, 750 mg), purchased from Rottapharm Ltd. (Mulhuddart, Dublin 15, Ireland), was used as reference formulation.

Table 1
Major working parameters of API 4000 tanderm mass spectrometer

Parameters	Values
Source temperature (°C)	500
Dwell time per transition (ms)	200
Ion source gas 1 (psi)	60
Ion source gas 2 (psi)	60
Curtain gas (psi)	10
CAD gas (psi)	3
Ion spray voltage (V)	4200
Declustering potential (V)	50
Collision energy (eV)	23 (analyte) and 28 (IS)
Mode of analysis	Positive
Ion transition for glucosamine (*m/z*)	180/72
Ion transition for IS (*m/z*)	252/198

2.2. LC/MS/MS instruments and conditions

An Agilent 1100 series LC system (Agilent, Waldbronn, Germany) equipped with a G1311A quaternary pump, a G1379A degasser, a G1313A autosampler and a G1316A thermostatted column was applied. The Chromatographic separation was achieved on an Inertsil CN-3 column (5 μm, 250 × 4.6 mm, GL Science Inc., Shinjuku-ku, Tokyo, Japan) at temperature of 25 °C. The isocratic mobile phase composition was a mixture of 2 mM ammonium acetate/acetonitrile (60:40, v/v), which was pumped at a flow rate of 0.6 mL/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (Applied Biosystems, Concord, Ontario, Canada) in multiple reaction monitoring (MRM) mode. A Turbo Ionspray electrospray ionization (ESI) interface in positive ionization mode was used. The major working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed with Analyst 1.4.1 software (Applied Biosystems, Concord, Ontario, Canada).

2.3. Preparation of calibration standards and quality control samples

Standard stock solutions of glucosamine and the IS were separately prepared at 400 μg/mL in methanol. The stock solution of glucosamine was then serially diluted with methanol to obtain the desired concentrations. Calibration curves were prepared by spiking 50 μL of the appropriate standard solution to 100 μL of drug-free heparinized human plasma. Effective concentrations in plasma samples were 4.00, 10.0, 25.0, 80.0, 200, 500, 1000, 2000 and 4000 ng/mL. The IS working solution (1.0 μg/mL) was also prepared by diluting its stock solution with methanol.

The quality control samples (QCs) were prepared at concentrations of 10.0, 500 and 3600 ng/mL with blank human plasma, by an independent weighing of the reference standard.

The standard samples and QCs were prepared on each analytical batch along with the unknown samples. All stock solutions and working solutions were kept at 4 °C.

2.4. Sample preparation

Frozen plasma samples were thawed to room temperature prior to preparation. After vortexing, a 50 μL aliquot of internal standard (valibose, 1.0 μg/mL in methanol), 50 μL of methanol and 200 μL of acetonitrile were added to 100 μL of human plasma samples, respectively. The mixture was vortexed for 1 min and centrifuged at 11,000 rpm for 5 min to remove the protein precipitate. The supernatant (200 μL) was transferred into a glass test tube and was evaporated to dryness under nitrogen in a TurboVap LV evaporator (Caliper, Hopkinton, MA, USA) at 40 °C. The residue was dissolved in 200 μL of the mixed solution of acetonitrile/water/acetic acid (50:50:2.5, v/v/v). A 20 μL aliquot of the solution was injected onto the LC/MS/MS system.

2.5. Bioanalytical method validation

To ensure the accuracy, selectivity, reproducibility and specificity, the method was fully validated on the items described as follows:

For the evaluation of linearity, calibration curves with nine levels covering the total range 4.00–4000 ng/mL of glucosamine were prepared in duplicates and analyzed in three separate analytical runs. Calibration curves were calculated based on the measurement of the ratio of peak area of glucosamine to that of the internal standard. Least-squares linear regression using $1/x^2$ as weighting factor was used to fit the measured signal versus the theoretical concentration. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within ±15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at ±20%.

The matrix effect (ME), the possible suppression or enhancement of ionization induced by the endogenous substances, was evaluated by comparing the chromatographic peak areas of glucosamine from the spike-after-extraction samples at two levels in triplicates with the neat standards at the same concentrations.

Quality control samples at three concentration levels (10.0, 500 and 3600 ng/mL) were analyzed to evaluate the precision and accuracy of the present method. The three concentrations were chosen to encompass the range of the calibration curve corresponding to the glucosamine levels expected to occur in plasma samples. The QCs were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. Precision was expressed by the relative standard deviation (RSD) within a single run and between different assays, and the accuracy by the percentage of deviation between nominal and measured concentrations. The acceptance criteria for intra- and inter-day precision and accuracy were ≤15%.

The lower limit of quantification (LLOQ) of glucosamine was experimentally chosen as the minimal concentration at which both precision and accuracy were less than or equal to 20% and it was evaluated by analyzing samples which were prepared in six replicates.

The recovery of the protein precipitation procedure was assessed by comparing the mean peak areas of the regularly prepared samples at three concentrations (10.0, 500 and 3600 ng/mL) with the mean peak area of spike-after-extraction plasma samples, which represented the 100% recovery. To prepare the spike-after-extraction samples, blank human plasma was processed according to the sample preparation procedure as described above. All the supernatant was mixed with the appropriate standard solutions of glucosamine at concentrations corresponding to the final concentration of the pretreated plasma samples. After vortexing, 200 μL of mixture was evaporated to dryness and the residue was reconstituted with the mixed solution of acetonitrile/water/acetic acid (50:50:2.5, v/v/v). Similarly, recovery of IS was also evaluated by comparing the mean peak areas of six regularly prepared samples to mean peak areas of six standard solutions spiked in pretreated drug free plasma samples.

The stability of glucosamine and IS in human plasma under different temperature and timing conditions was evaluated by assaying triplicate plasma samples of glucosamine at low and high concentration levels (25.0 and 3600 ng/mL). The plasma samples were analyzed after storage at ambient temperature for 2 h, in auto-sampler for 24 h at room temperature after protein precipitation, at −20 °C for 30 days and after three freeze–thaw cycles from −20 °C to room temperature. Peak area obtained from the analysis of the stored samples was compared to those obtained from the analysis of freshly prepared plasma samples. The analyte was considered stable in the biological matrix when the ratio was within the range of 85–115%. The stability of standard solutions was also tested at 4 °C for 15 days.

2.6. Application to bioequivalence study

The method was applied to determine the plasma concentrations of glucosamine from a clinical trial study in which 22 healthy male volunteers received a single dose of 750 mg glucosamine from the test and reference formulations, with a 7 days washout periods. Blood samples were collected into heparinized tubes according to the following time schedule: 0.0, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12 and 24 h post-dosing. Blood samples were centrifuged immediately at 3500 rpm for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry conditions

As glucosamine is a highly polar and basic compound, (+) ESI was selected as the ionization mode for the analysis in this work. In the Turbo Ionspray interface, glucosamine formed predominately protonated molecules $[M+H]^+$. No solvent adduct ion was observed. The fragmentation behavior of the $[M+H]^+$ ion at m/z 180 is strongly dependent on the collision energy. A

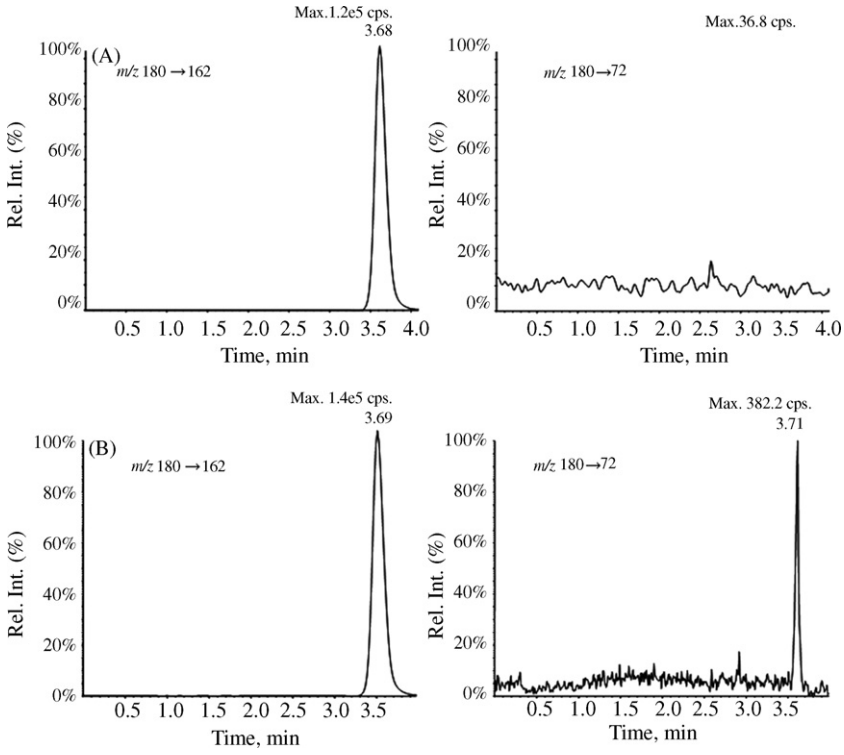

Fig. 2. Typical MRM chromatograms with the transitions of *m/z* 180 → 162 and *m/z* 180 → 72 employed for glucosamine in human plasma. It was demonstrated that the peak at 3.68 min is an endogenous compound. (A) Blank plasma sample and (B) plasma sample spiked with 4.00 ng/mL of glucosamine.

major fragment ion at *m/z* 162 was formed by the neutral loss of a H_2O molecule using 12 eV collision energy (CE). When higher collision energy (23 eV) was used, the parent ion was further fragmented, forming product ion of *m/z* 72 (Fig. 1A). The absolute intensity of the product ion at *m/z* 72 was considerably lower than that of the ion at *m/z* 162 obtained using 12 eV collision energy; however, high background noise and interference from plasma endogenous impurities were observed using the transition of *m/z* 180 → 162 (Fig. 2). Thus, the fragment ion at *m/z* 72 was used in MRM acquisition mode to obtain high specificity and low noise. Valibose, structurally relevant to glucosamine, was used as internal standard, which showed the $[M+H]^+$ ion at *m/z* 252 as the base peak in the full-scan Q1 mass spectrum and the major fragment ion at *m/z* 198 in the corresponding product ion spectrum. Hence, the transition *m/z* 252 → 198 was chosen for the MRM acquisition of the IS. The MRM state file parameters were optimized to maximize the response for the analyte and IS. The parameters in Table 1 were the result of this optimization.

3.2. Chromatographic conditions

Glucosamine is a highly polar compound, which makes it difficult to extract from plasma with organic solvents. The protein precipitation is the only method for plasma preparation, which might lead to ion suppression when LC/ESI-MS/MS was applied. Therefore, the appropriate chromatographic column and suitable mobile phase are needed for accurate quantification of glucosamine in human plasma.

A number of reversed-phase C8 or C18 columns, such as Zorbax SB C8 and C18, Atlantis dC18, Gemini C18 and Diamonsil C18, were tested during method development. Strong ion suppression was observed on all tested reversed-phase columns, which was attributed to the early elution of polar compound. Eddington and coworkers [4], Sha and coworkers [5] and Duan and coworkers [6] adopted precolumn derivatization to improve glucosamine retention time on C18 columns and assay sensitivity. A normal-phase chromatography using an amino column was also successfully utilized to assay the polar compound [7]. But a long chromatographic run time (30 min) was needed to equilibrate the column and to avoid ion suppression. In order to retain the analyte better to achieve the desired separation and to increase the sample throughput, an Intersil CN-3 normal-phase column was considered in the experiment. Through several trials (as shown in Fig. 3A), glucosamine and the IS were well retained on the column, but the retention time, peak shape, and MS response were found strongly affected by the composition of the mobile phase.

When using 2 mM ammonium acetate/acetonitrile (60:40, v/v) as the mobile phase, the retention time of glucosamine was about 6 min with a broad peak. As shown in Fig. 3A, the retention time of the analyte was prolonged as the organic concentrations increased and the chromatographic peak became broader. When a small amount of acidic modifiers, e.g. 0.1% formic acid or acetic acid, were used in the mobile phase, the retention time of glucosamine was about 2.5 min, which was equal to the dead time, and poor MS response was also observed due to the ion sup-

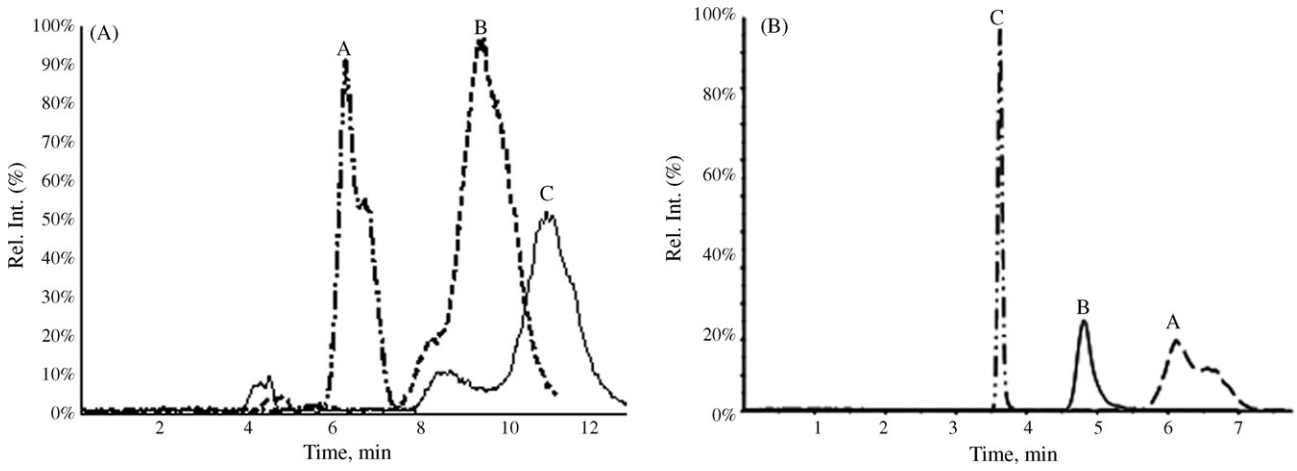

Fig. 3. (A) Representative chromatograms of glucosamine in human plasma obtained at different mobile phase compositions on an Inertsil CN-3 column to investigate the effect of the mobile phase. Peak A: 2 mM ammonium acetate/acetonitrile (60:40, v/v), t_R = 6.11 min, MS response: 2194.6 cps. Peak B: 2 mM ammonium acetate/acetonitrile (50:50, v/v), t_R = 8.65 min, MS response: 2364.7 cps. Peak C: 2 mM ammonium acetate/acetonitrile (40:60, v/v), t_R = 10.8 min, MS response: 1240.2 cps. (B) Representative chromatograms of glucosamine in human plasma obtained on an Inertsil CN-3 column when 2 mM ammonium acetate/acetonitrile (60:40, v/v) was used as the mobile phase to investigate the effect of the reconstitution solvent on the chromatographic peak shape and MS response. Peak A: 2 mM ammonium acetate/acetonitrile (60:40, v/v), t_R = 6.11 min, MS response: 2194.6 cps. Peak B: Water/acetonitrile/acetic acid (50:50:0.l, v/v/v), t_R = 4.76 min, MS response: 2839.5 cps. Peak C: Water/acetonitrile/acetic acid (50:50:2.5, v/v/v), t_R = 371 min, MS response: l.2 × 10^4 cps.

pression from plasma sample. The optimization of the pH values of the mobile phase was tried to obtain a reasonable retention time and to circumvent ion suppression. But even if 0.05% of acidic modifier was used, the significant ion suppression was still observed.

As the mobile phase containing acidic modifiers could significantly shorten the retention time of glucosamine and improve peak shape, adding various percentages of acetic acid to reconstitution solvents was attempted. As shown in Fig. 3B, a sharp chromatographic peak with suitable retention time (3.7 min) was obtained when the mixed solution of acetonitrile/water/acetic acid (50:50:2.5, v/v/v) was used as reconstitution solvent. Chromatographic run time of each sample was completed within 4.2 min. After each sample was analyzed, there was about 1 min interval for the inject preparation of the autosampler, which could be also considered as column wash time. During the determination of human plasma samples, a batch of 104 plasma samples per run were analyzed continuously for the bioequivalence study of glucosamine sulfate capsule following a column wash using acetonitrile/water (90:10, v/v), which was pumped at a flow rate of 0.3 mL/min for about 10 h, and no deterioration of the chromatogram or decrease in MS response was observed.

3.3. Internal standard selection

Choosing an appropriate IS was an important aspect to achieving acceptable method performance, especially with LC/MS/MS. Use of stable isotope-labeled analogues as internal standard is highly recommended since matrix effect should not affect the relative efficiency of the ionization of the analyte and IS, but the problem such as "cross-talk" between MS/MS channels used and isotopic purity should also be carefully observed. Moreover, there was no good isotope-labeled IS commercially available for glucosamine, as they were either radioactive or bear a single C_{13} atom making them unsuitable for use in bioanalysis.

In this experiment, valibose, structurally similar to glucosamine, was adopted as internal standard, which has an almost identical retention time to that of the analyte. According to this, potential matrix effect for the target analyte and the IS caused by co-eluted endogenous matrix components could be compensated, for their similar chromatographic and mass spectrometric properties.

3.4. Method validation

3.4.1. Selectivity and matrix effect

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 4 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with glucosamine at the LLOQ and IS, and a plasma sample from a healthy volunteer 1.5 h after the administration. No significant interference from endogenous substances with analyte or IS was detected. Typical retention times for glucosamine and IS were around 3.70 min.

Matrix effect recovery of blank plasma samples in the six batches spiked after the sample preparation with 10.0 and 3600 ng/mL of glucosamine was found to be within the acceptable limits (91.7–103.8%). The same evaluation was performed on the IS and no significant peak area differences were observed. Thus, we concluded that ion suppression or enhancement from plasma matrix was negligible for this method.

3.4.2. Linearity of calibration curves and lower limit of quantification

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 4.00–4000 ng/mL in human plasma. A typical equation of

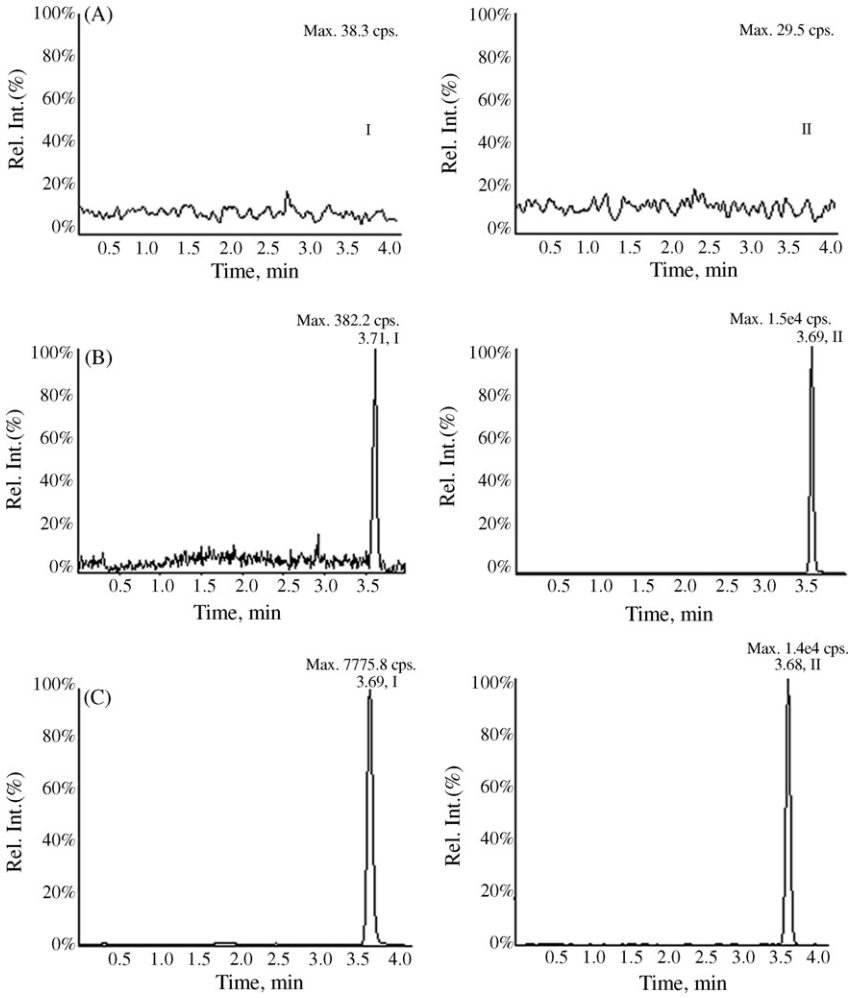

Fig. 4. Typical MRM chromatograms of glucosamine (I) and IS (valibose, II) in human plasma sample. (A) Blank plasma sample, (B) plasma sample spiked with glucosamine (4.00 ng/mL) and IS (500 ng/mL) and (C) a volunteer plasma sample 1.5 h after oral administration of 750 mg glucosamine sulfate capsule.

the calibration curves is as follows: $y = 0.0128 + 0.0024x$ ($r = 0.9983$), where y is the peak area ratio of analyte to IS, and x is the plasma concentration of glucosamine. Good linearity ($r > 0.9963$) was seen in this concentration range over all analytical runs.

The lower limit of quantification (LLOQ) was 4.00 ng/mL for determination of glucosamine in plasma and the data are listed in Table 2. At the LLOQ level, the intra-day precision was 10.9% and the accuracy was 102%. Under the present LLOQ, the glucosamine concentration could be determined in plasma samples until 24 h after oral administration of 750 mg of glucosamine sulfate capsules, which is sensitive enough to investigate the pharmacokinetic behaviors of the drug.

3.4.3. Precision and accuracy

Table 2 summarizes the intra- and inter-day precision and accuracy for glucosamine evaluated by assaying the QC samples. In this assay, the intra- and inter-day precisions ranged from 6.5% to 8.2% and from 0.6% to 8.6% for each QC level, respectively. The accuracy was better than 96.2%. The results, calculated using one-way ANOVA, indicated that the values were within the acceptable range and the method was accurate and precise.

3.4.4. Recovery and stability

Mean recoveries of glucosamine at 10.0, 500 and 3600 ng/mL were $104 \pm 4\%$, $98.3 \pm 5.6\%$ and $99.2 \pm 2.7\%$, respectively

Table 2
Precision and accuracy for the analysis of glucosamine in human plasma in method validation (in validation, $n = 6$ for LLOQ and $n = 18$ for QC samples)

Added concentration (ng/mL)	Found concentration (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (%)
4.00	4.08	10.9	–	102.0
10.0	10.2	6.5	8.6	102.5
500	481	8.2	2.1	96.2
3600	3613	6.6	6.6	100.4

Table 3
Stability of glucosamine in human plasma

Nominal concentration (ng/mL, $n=3$)	Found concentration (ng/mL)	RSD (%)	Accuracy (%)
Short-term stability (at ambient temperature for 2 h			
25.0	27.8	2.2	111.1
3600	3589	4.8	99.7
Long-term stability (−20 °C for 30 days)			
25.0	26.4	4.8	105.5
3600	3275	1.8	91.0
Autosampler stability (room temperature for 24 h after treatment)			
25.0	25.3	4.0	101.3
3600	3307	12.6	91.9
Three freeze–thaw cycles			
25.0	27.0	7.7	107.9
3600	3792	5.3	105.3

($n=6$). Mean recovery of the internal standard (500 ng/mL) was 96.1 ± 6.3% ($n=6$).

The stabilities of glucosamine on bench top, in auto-sampler, after three freeze–thaw cycles and after long period of storage at −20 °C were investigated. The results are listed in Table 3, which indicate that the analyte was stable under the storage conditions described above, with the 91.0–111.1% accuracy. The good stability of glucosamine simplified the precautions needed for laboratory manipulations during the analytical procedures. In addition, standard stock solutions of glucosamine were shown to be stable for at least 15 days at 4 °C.

3.5. Application

It is well known that glucosamine is an endogenous compound in human [4–10]. In the present experiment, no endogenous glucosamine was found in human plasma, which might be due to ethnic and/or individual differences between the subjects enrolled here and in the other studies.

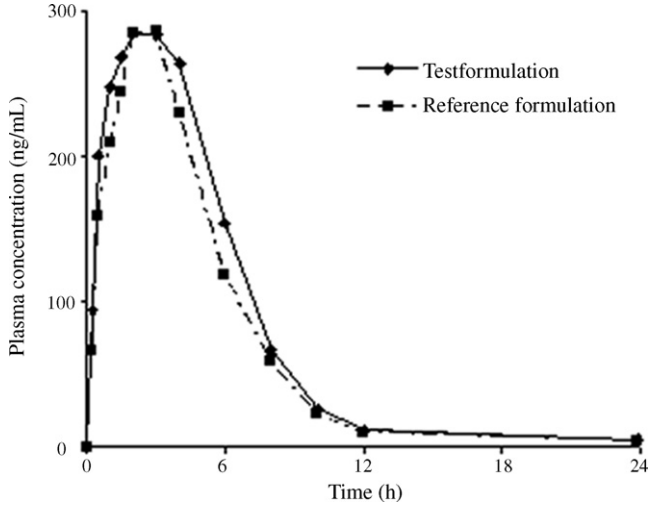

Fig. 5. Mean plasma concentration–time curve of glucosamine after an oral administration of 750 mg of glucosamine sulfate formulations to 22 healthy volunteers.

Table 4
The main pharmacokinetics parameters of glucosamine after an oral administration of 750 mg of glucosamine sulfate formulations to 22 healthy volunteers ($\bar{x} \pm s$, $n = 22$)

Parameter	Test formulation	Reference formulation
$t_{1/2}$ (h)	4.80 ± 1.73	4.73 ± 1.32
T_{max} (h)	2.86 ± 0.93	2.64 ± 0.79
C_{max} (ng/mL)	343 ± 93	312 ± 92
AUC_{0-t} (ng h/mL)	1843 ± 623	1637 ± 447
$AUC_{0-\infty}$ (ng h/mL)	1878 ± 639	1666 ± 460

This validated analytical method has been successfully applied to determine the plasma concentration of glucosamine following a single 750 mg oral administration to 22 healthy subjects in a bioequivalence study. Mean plasma concentration–time profiles of glucosamine obtained from 22 healthy volunteers are presented in Fig. 5. And the main pharmacokinetics parameters are summarized in Table 4.

In order to establish if the formulations tested are bioequivalent, the 90% confidence intervals of C_{max} and AUC_{0-t} were calculated, they were 104.0–119.3% and 101.1–120.8%, respectively. The results indicate that the valued data were within the bioequivalence acceptance range. Based on these, the two formulations are found to be bioequivalent.

4. Conclusion

By developing the chromatographic conditions, a rapid, sensitive and specific LC/(+) ESI-MS/MS method was developed and validated to directly determine glucosamine in human plasma. Compared with the analytical methods reported previously, the current method showed more rapid chromatographic time (4.2 min per sample) and more sensitivity with an LLOQ of 4.00 ng/mL. After a total of 572 plasma samples were analyzed for the bioequivalence study of glucosamine sulfate capsule, deterioration of the column or decrease in MS response was not observed. It allows the determination of glucosamine up to 24 h after an oral administration only using 100 μL of plasma sample.

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