Journal of Pharmaceutical Analysis 2012;2(1):19–28

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

www.elsevier.com/locate/jpa
www.sciencedirect.com

ORIGINAL ARTICLE

Precolumn derivatization LC–MS/MS method for the determination and pharmacokinetic study of glucosamine in human plasma and urine

Min Song[a], Tai-Jun Hang[a,*], Cheng Wang[a], Lin Yang[b], Ai-Dong Wen[b]

[a]*Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China*
[b]*Department of Pharmacy, Xijing Hospital, Xi'an 710032, China*

Received 11 July 2011; accepted 30 August 2011
Available online 24 October 2011

KEYWORDS

Glucosamine;
Pharmacokinetics;
Precolumn derivatization;
LC–MS/MS

Abstract A selective precolumn derivatization liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the determination of glucosamine in human plasma and urine has been developed and validated. Glucosamine was derivatized by o-phthalaldehyde/3-mercaptopropionic acid. Chromatographic separation was performed on a Phenomenex ODS column (150 mm × 4.6 mm, 5 μm) using linear gradient elution by a mobile phase consisting of methanol (A), and an aqueous solution containing 0.2% ammonium acetate and 0.1% formic acid (B) at a flow rate of 1 mL/min. Tolterodine tartrate was used as the internal standard (IS). With protein precipitation by acetonitrile and then the simple one-step derivatization, a sensitive bio-assay was achieved with the lower limit of quantitation (LLOQ) as low as 12 ng/mL for plasma. The standard addition calibration curves suitable for clinical sample analysis showed good linearity over the range of 0.012–8.27 μg/mL in plasma and 1.80–84.1 μg/mL in urine. The fully validated method has been successfully applied to a pharmacokinetic study of compound glucosamine sulfate dispersible tablets in health Chinese volunteers receiving single oral doses at 500, 1000 and 1500 mg of glucosamine sulfate, as well as multiple oral doses of 500 mg t.i.d. for 7 consecutive days.

*Corresponding author. Tel./fax: +86 25 83271090.
E-mail address: hangtj@cpu.edu.cn (T.-J. Hang).

Peer review under responsibility of Xi'an Jiaotong University.
doi:10.1016/j.jpha.2011.08.003

Production and hosting by Elsevier

1. Introduction

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino sugar and a prominent precursor involved in the biosynthesis of glycosylated proteins and lipids, acting as a preferred substrate for the biosynthesis of glycosaminoglycan chains, and subsequently, for the production of aggrecan and other proteoglycans of cartilage [1]. Glucosamine is believed to be effective in easing osteoarthritis pain, rehabilitating cartilage, renewing synovial fluid and repairing joints that have been damaged from osteoarthritis [2]. Hence, glucosamine is often prescribed

for the treatment of osteoarthritis [3]. Numerous nutraceutical supplements as well as pharmaceutical preparations containing glucosamine as a single active component or combined with other principles (such as chondroitin) are marketed worldwide [4].

Because of the perceived benefits and important clinical application, the determination of glucosamine in human body fluids will give a further understanding of its action and make the medication more effective and safe.

Many reports have been made about the bio-assay of glucosamine directly or after derivatization. The direct detection methods were performed with normal chromatographic separation in most cases [5–8]. However, there were a large number of endogenous substances similar with glucosamine in structure or polarity, such as glucose, galactosamine, amino acids and peptides etc, in the bio-fluids, having similar retention behavior as glucosamine. Therefore, a long chromatographic run time as long as 27 min [5,6] was necessary to separate the strongly retained endogenous substances to circumvent the matrix effects or interferences even with the most selective MS/MS detection and to re-equilibrate the column for repeated runs, which was inadequate for high throughput analysis. Moreover, the use of a ^{13}C labeled glucosamine as the internal standard [5–8] was not sound since there was only one atomic mass unit difference between the analyte and the internal standard [9], the isotopic form of the analyte would also contribute significantly to the response of the IS. Relatively quick separation was also achieved on Cyano or Carbopack columns for underivatized glucosamine in human plasma [10–12]. However, for the two anomeric forms of glucosamine in equilibrium only one single peak was recorded on the chromatograms. Therefore, the separation might also suffer from endogenous interferences.

Several reversed-phase chromatographic methods were also reported for the determination of glucosamine in bio-fluids. Meulyzer et al. [13] determined the glucosamine directly with LC–MS on a C18 column (100 mm × 2 mm, 5 μm). The retention time of glucosamine and IS was only 2.4 min and 1.4 min, respectively. Although the short retentions had the merits of high throughput, the unavoidable matrix effects would affect the accuracy of determination [7].

In order to separate the glucosamine from endogenous polar substances as much as possible, to improve the resolution and to enhance the selectivity of glucosamine on reverse-phase chromatography, precolumn derivatization methods have been developed for optimal and reproducible assay performances. Phenylisothiocyanate (PITC) [14–17], 1-naphthyl isothiocyanate [18], 9-fluorenylmethyl chloroformate (FMOC-Cl) [19–21] and 6-aminoquinolyl-N-hydroxylsuccinimidyl carbamate (AQC) [22,23] have been reported as derivative reagents for glucosamine. Since the samples are often of large number in bio-study, the derivatization method should be as simple and rapid as possible. However, the PITC [14–16] includes steps of removing the excess of derivative reagent and drying the samples under vacuum. The AQC [22] has steps of separating the aqueous layer from the organic for the derivatization. For FMOC-Cl and 1-naphthyl isothiocyanate, the derivative products are unstable, and the buffer concentration will give marked influences on the reaction [21,23]. In addition, the sensitivity of the derivative methods [14–23] was not appropriate for human bio-fluids in most cases for the best LOD achieved among them was only 15 ng/mL [19].

In this study, a precolumn derivatization LC–MS/MS method was developed and fully validated for the quantitation of glucosamine in human plasma and urine. O-phthalaldehyde/3-mercaptopropionic acid (OPA/3-MPA) was used as the derivative reagent which is remarkable for its simple derivative procedures [24–27]. The significantly improved chromatographic retention and separation behavior post the derivatization, and the highly selective and sensitive detection afforded by the MS/MS selective reaction monitoring have made the LLOQ of the method as low as 12 ng/mL in human plasma. The method was successfully applied to a pharmacokinetic study of compound glucosamine sulfate dispersible tablet in healthy Chinese volunteers receiving single oral doses at 500, 1000 and 1500 mg of glucosamine sulfate, as well as multiple oral doses of 500 mg t.i.d. for 7 consecutive days.

2. Experimental

2.1. Chemicals and reagents

Compound glucosamine sulfate dispersible tablets (each contains 250 mg glucosamine sulfate and 200 mg chondroitin sulfate, Lot No.: 071201) was supplied by the Guangzhou Yipinhong Pharmaceutical group Co. Ltd. (Ghangzhou, China). The chemical reference standards of glucosamine hydrochloride (>99.8% purity) and tolterodine tartrate (IS) (>99.8% purity) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC-grade methanol was the product of Tedia Company Inc. (Fairfield, OH, USA). Formic acid, ammonium acetate, sodium tetraborate, o-phthalaldehyde (OPA) and 3-mercaptopropionic acid (3-MPA) were all of analytical grade obtained from Nanjing Chemical Reagent Company Inc. (Nanjing, PR China). Water was prepared with double distillation.

2.2. Instrumentation

The LC–MS/MS system consisted of a Thermo-Finnigan TSQ Quantum Ultra AM tandem mass spectrometer equipped with an ESI source (San Jose, CA, USA), a Surveyor LC pump and a Surveyor auto-sampler. The system control and data acquisition were performed with Xcalibur 1.2 software (Thermo-Finnigan, San Jose, CA, USA). Peak integration and calibration were carried out using LC Quan software (Thermo-Finnigan).

2.3. Preparation of derivative reagent

The derivative reagent was prepared by dissolving 50 mg of anhydrous OPA with 1 mL of methanol, and then mixing thoroughly with 10 mL of 0.05 mol/L borate buffer (pH 9.3) and 0.1 mL 3-MPA. The solution was freshly prepared and kept at 4 °C on daily basis and no signs of deterioration were observed within this period.

2.4. Calibration standards and quality control samples in human plasma and urine

Endogenous level of glucosamine in human plasma was found to be 10–200 ng/mL [8]. Therefore, the pooled human blank plasma was used for the preparation of calibration standards

and quality control samples with the glucosamine content determined before use by standard addition calibration with linear regression of a series of spiked standards. Then the actual concentration of calibration standard was defined as the sum of the endogenous and the spiked concentration. The actual concentration as free base was used hereafter unless indicated otherwise. The blank urine was collected from a volunteer after two day bland diet.

Glucosamine hydrochloride stock solution of 1 mg/mL was prepared in distilled water. The working solutions with concentrations in the range of 0.1–80 μg/mL for plasma and 4–800 μg/mL for urine were prepared by serial dilution of the stock solution with water. The IS solutions of 0.4 and 2 μg/mL were prepared similarly for the determination of glucosamine in plasma and urine, respectively. All the stock and working solutions were prepared in brown volumetric flasks, stored shaded from light at 4 °C and used within one week.

The standard addition calibration plasma samples were prepared by spiking aliquots of 0.4 mL of the pooled blank human plasma (the glucosamine concentration was estimated to be 3.8 ng/mL) with 40 μL of the corresponding working solutions, respectively to yield concentrations of 0.012, 0.020, 0.037, 0.087, 0.169, 0.417, 0.831, 1.66, 4.14 and 8.27 μg/mL. The calibration urine samples of 1.80, 2.21, 3.04, 5.52, 9.65, 17.9, 42.7 and 84.1 μg/mL were prepared similarly with blank human urine (the glucosamine concentration was estimated to be 1.4 μg/mL). The QC samples were 0.020, 0.169 and 1.66 μg/mL in plasma and 2.21, 9.65 and 42.7 μg/mL in urine. All the samples were stored at −20 °C until analysis.

2.5. Sample preparation and derivatization

For plasma sample, an aliquot of 0.4 mL plasma in a 2 mL Eppendorf tube was mixed with 40 μL water (except for the calibration and QC standards), 40 μL IS solution (0.4 μg/mL tolterodine tartrate in water) and 0.8 mL acetonitrile by vortex-mixing for 1 min to free the analyte and precipitate protein. 150 μL of the supernatant obtained after centrifuging at 16,000*g* for 10 min at 5 °C was transferred into an auto-sampler vial, then mixed with 0.35 mL of 0.05 mol/L borate buffer (pH 9.3) and 0.15 mL of derivative reagent to carry out the derivatization at 25 °C water bath for 15 min under dim light. Then the resultant solution was kept refrigerated at −20 °C before 25 μL was injected into the LC–MS/MS system for analysis within 8 h. The derivatization reaction of glucosamine with OPA/3-MPA is shown in Fig. 1.

The urine sample was processed in the same manner as plasma described above except that the 2 μg/mL tolterodine tartrate was used as the IS solution.

2.6. Chromatography and mass spectrometry condition

Liquid chromatography was performed on a Phenomenex ODS column (150 mm × 4.6 mm, 5 μm) preceded by an ODS guard cartridge (2 mm × 4 mm). The mobile phase consisted of methanol (A) and an aqueous solution containing 0.2% ammonium acetate and 0.1% formic acid (B) pumped at a flow rate of 1 mL/min with the following steep linear gradient elution steps: 0.0 min (45%A–55%B)→1.0 min (45%A–55%B)→2.5 min (95%A–5%B)→4.9 min (95%A–5%B)→5.0 min (45%A–55%B)→6.5 min (45%A–55%B). The auto-sampler tray temperature was set at 4 °C for the optimal sample stability.

To avoid excessive entry of complex endogenous components into the MS system, only 30% of the eluent was split into the inlet of the ESI probe of the mass spectrometer and the flow was directed into the instrument only during the window time for the target compounds analysis, after 2.8 min of each chromatographic run.

The MS/MS detections were performed with positive ion ESI and selected reaction monitoring. The spray voltage, heated capillary temperature, nitrogen sheath and auxiliary gases were optimized and set at 5 kV, 350 °C, 275 kPa and 70 kPa, respectively. The argon gas collision induced dissociations were used with a pressure of 0.20 Pa and collision energy of 35 eV for both glucosamine derivatives and IS. Their product ion-scan spectra are shown in Fig. 2. The most stable and strongest ion transitions were selected for the quantitation of glucosamine with m/z 384→118 for the glucosamine-OPA/3-MPA derivatives and m/z 326→147 for IS. The run time for each LC–MS/MS analysis was 6.5 min.

2.7. Method validation

The method validation was carried out according to the guidelines for bio-assay of the U.S. Food and Drug Administration (FDA) [28], including specificity, matrix effect, precision, accuracy, linearity, sensitivity, extraction recovery and stability.

The specificity of this assay was investigated by analyzing six individual human blank plasma and urine samples.

The matrix effect was evaluated by comparing the chromatographic peak areas of the analyte from the spike-after protein precipitation samples with the neat standards in duplicates at the QC concentrations.

Bio-samples were quantitated using the peak area ratios of the analyte to the IS. The calibration curves were established through a linear least squares regression with a weighting factor of $1/C^2$, where C is the concentration of the calibration standards. The criteria for the calibration include a correlation coefficient (r) of 0.996 or better, and the deviation of the found from the nominal being within ±15% except for LLOQ of ±20%.

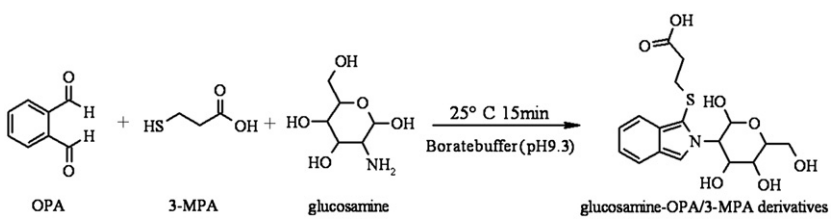

Figure 1 The derivatization reaction of glucosamine with OPA/3-MPA.

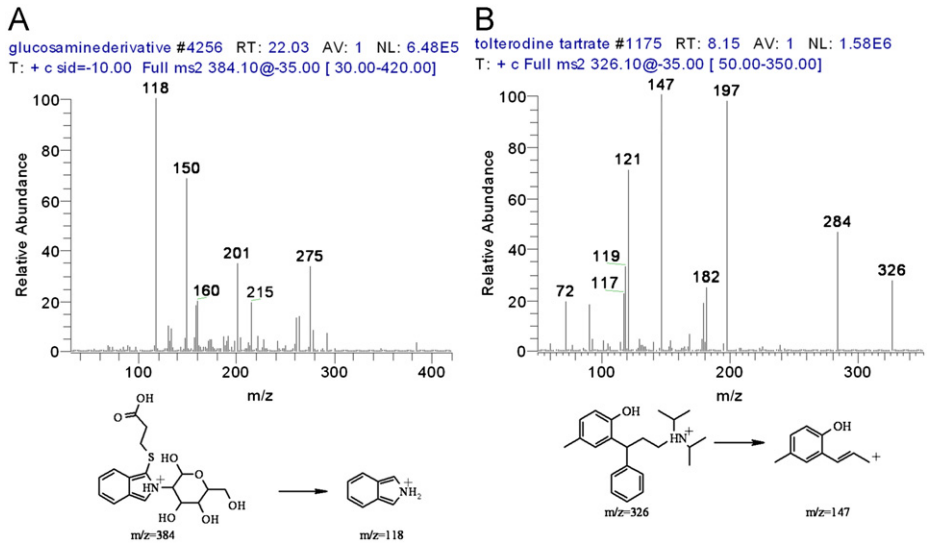

Figure 2 The product ion spectra and proposed fragmentation processes of glucosamine-OPA/3-MPA derivatives (A) and IS (B).

The extraction recoveries was determined by comparing the responses of the analyte extracted from quintuplicate QC samples with the responses of the analyte spiked after protein precipitation samples at an equivalent concentration.

The precision and accuracy of the method were evaluated by analyzing quintuplicate samples at three QC concentration levels and assessed by within- and between-batch validation. The acceptance criteria for within- and between-batch precision (expressed as relative standard deviation, RSD%) were $\pm 20\%$ for LLOQ and $\pm 15\%$ for the other concentrations. For accuracy, the acceptance criteria were $100 \pm 20\%$ for LLOQ and $100 \pm 15\%$ for the other concentrations.

Post-preparative stability of the processed samples, i.e. the stability of derivative products, was investigated carefully, including the residence time in auto-sampler tray at 4 °C and refrigerator at −20 °C for 24 h.

Stability of glucosamine in plasma and urine samples under different storage and processing conditions were evaluated after the stability of the glucosamine-OPA/3-MPA derivatives were thoroughly established. QC samples of low and high concentration, 0.020 and 1.66 μg/mL for plasma, 2.21 and 42.7 μg/mL for urine, were used for the stability evaluation.

Freeze-thaw stability samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 h under the same condition and the freeze-thaw cycles were repeated up to three times. Freeze-thaw samples were then analyzed with comparison to the freshly prepared references.

Short-term stability was determined by analyzing QC samples kept at room temperature for 8 h. To investigate long-term stability, samples were kept at −20 °C for up to 60 days.

2.8. *Application to pharmacokinetic study*

The established method was applied to a pharmacokinetic study of compound glucosamine sulfate dispersible tablets with an open-label, randomized, parallel-group design. Thirty healthy Chinese volunteers (15 males and 15 females) with mean age 34.8 ± 3.3 years, mean weight 58.7 ± 4.3 kg and mean height 164.1 ± 6.8 cm, were randomized into three parallel-groups (5 men and 5 women in each group), who received a single oral dose of 500, 1000 and 1500 mg (the dose was expressed as glucosamine sulfate) with 250 mL of water after an overnight fast. Venous blood samples about 3.5 mL were drawn before administration (0 h) and after 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10, 12, 14 and 24 h in heparinized tubes. The plasma samples were obtained by centrifuged at 1000*g* force for 10 min. Urine samples of the 1000 mg dose group were collected before (0 h) and after administration for time segments of 0–3, 3–6, 6–10, 10–14 and 14–24 h. The 500 mg group was continued to be given multiple oral doses of 500 mg t.i.d. for 7 consecutive days, and the steady-state was evaluated by blood sampling before morning doses on day 4 through 7 and at the same time points as those after a single oral dose on day 7. The separated plasma and urine samples were all stored at −20 °C until analysis. The study was conducted at Xijing hospital. The study protocol was approved by the local Ethical Review Committee in accordance with the principles of the Declaration of Helsinki, and the recommendations of the State Food and Drug Administration of China. Written informed consent was obtained from all subjects. All the subjects were instructed to abstain from taking any medication for 2 weeks before and during the whole study period unless otherwise deemed as necessary by the doctor.

The maximum observed plasma concentration (C_{max}) and the time of its occurrence (T_{max}) were obtained directly from the concentration–time curve. Other pharmacokinetic parameters were calculated by non-compartmental method and statistical analysis was performed using the DAS program (Drugs and Statistics version 2.0, Chinese Pharmacological Society, China). Analysis of variance (ANOVA) was used to assess the gender or treatment group differences. $P < 0.05$ was taken as statistically significant in all tests.

3. Results and discussion

3.1. *LC–MS/MS condition*

The reversed phase HPLC and volatile mobile phase were chosen based on the physicochemical properties of the analyte

and characteristics of MS detection. Various types and brands of analytical column (C18, C8 and PFP) and mobile phase compositions were evaluated. It was found that the retention behaviors of the glucosamine derivatives and the IS were influenced significantly by the pH and composition of the mobile phase (methanol, ammonium acetate, formic acid or acetic acid in water). In the pH range of 2.0–7.5 tested, the lower pH tends to give a better retention and sharper peaks for the glucosamine-OPA/3-MPA derivatives. However, the peak of the IS became broad and asymmetric when the pH of the mobile phase was below 3.0, such as the mixture of 50 volumes of 0.2% formic acid in water and 50 volumes of methanol. Finally sharp chromatographic peaks with suitable retention time for both analyte and IS were obtained using a mixture of methanol (A) and an aqueous solution containing 0.2% ammonium acetate and 0.1% formic acid (B) as mobile phase with the following steep linear gradient elution steps: 0.0 min (45%A–55%B)→1.0 min (45%A–55%B)→2.5 min (95%A–5%B)→4.9 min (95%A–5%B)→5.0 min (45%A–55%B)→6.5 min (45%A–55%B).

Moreover, glucosamine is present with two anomeric forms in equilibrium [23]. Therefore two peaks are found on the chromatogram (Figs. 3 and 4) with retention time of 3.2 and 4.1 min for the corresponding glucosamine-OPA/3-MPA derivatives. The area sum of these two peaks was handled together for the quantitation.

Triple quadrupole tandem mass spectrometry has great advantage in reducing interferences and enhancing sensitivity over other MS detection methods [29]. The mass detection was carried out with positive electrospray ionization and SRM. Parameters such as the spray voltage, capillary temperature, desolvation nitrogen gas flows were tuned to maximize the specificity and sensitivity for the detection. What is more, the argon gas collision induced dissociation behaviors of both compounds concerned were strongly dependent on the gas pressure and collision energy. The product scan spectra are shown in Fig. 2. The most stable SRM transition was selected with m/z 384→118 for glucosamine derivatives and m/z 326→147 for IS accordingly with the argon gas pressure and collision energy set at 0.2 Pa and 35 eV, respectively.

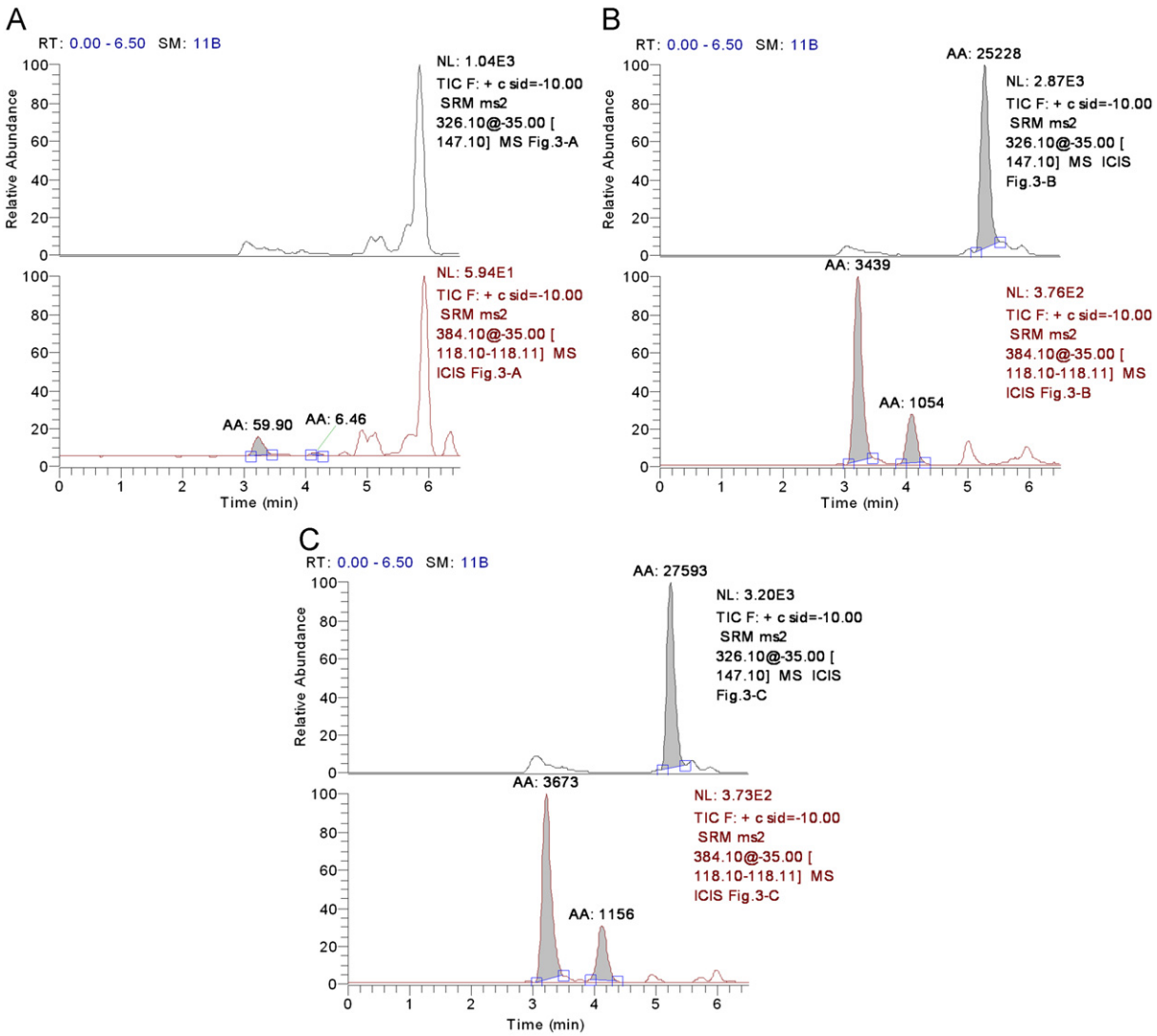

Figure 3 Representative chromatograms of (A) the blank plasma sample, (B) a calibration standard of plasma sample 0.169 μg/mL glucosamine and (C) a plasma sample from a healthy volunteer 2 h after oral administration of 500 mg compound glucosamine sulfate dispersible tablets. The retention time for the two glucosamine derivatives and IS is 3.2, 4.1 and 5.2 min, respectively.

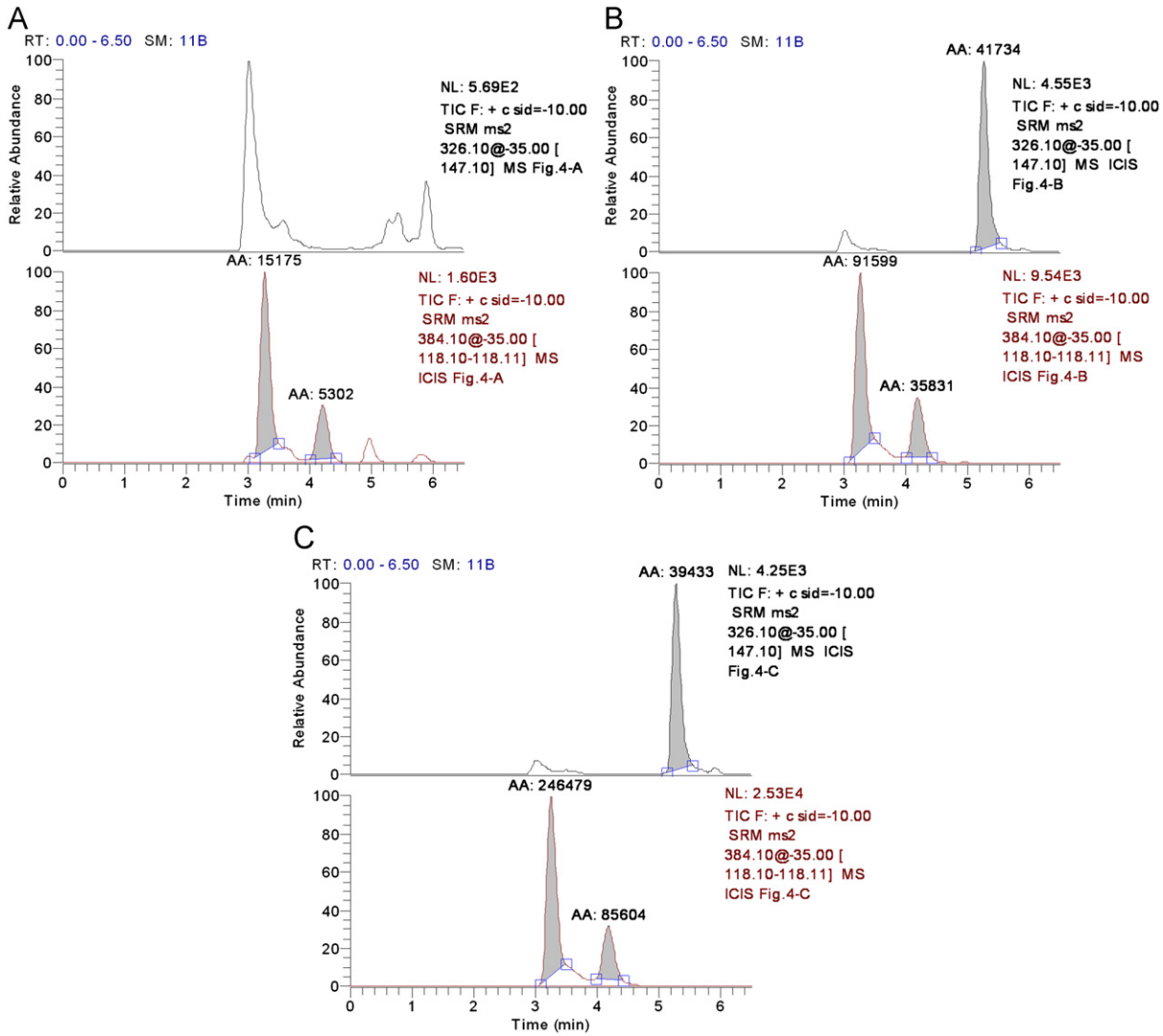

Figure 4 Representative chromatograms of (A) the blank urine, (B) a calibration standard of urine sample 9.65 μg/mL of glucosamine and (C) a urine sample from a healthy volunteer 3–6 h after oral administration of 1000 mg compound glucosamine sulfate dispersible tablets. The retention time for the two glucosamine derivatives and IS is 3.2, 4.1 and 5.2 min, respectively.

3.2. Sample preparation and derivatization

A simple protein precipitation was used for sample pretreatment based on the high polarity, intrinsic stability and freely water solubility of glucosamine. Acetonitrile was found to give best results with regard to the recovery and derivatization for both plasma and urine samples.

The key point of this study was to establish an optimal derivatization method to improve the retention, which could overcome the matrix effects and enhance the sensitivity and selectivity of glucosamine. The method should be also as rugged and simple as possible to meet the demand of high throughput clinical bio-assay. Among the common derivate reagents for amino compounds, such as PITC, FMOC-CL, naphthyl or phenylisothiocyanate, OPA, AQC, Dabsyl-Cl and Dansyl-C1, OPA is remarkable for its water compatibility and simplicity [24–27].

The OPA/3-MPA combination was used for the derivatization of glucosamine. And three main factors were investigated to improve derivative yield. They were the amount of the derivative reagent, reaction temperature and reaction time as well. The optimization was conducted for plasma and urine samples. The optimum results revealed that the derivative conditions were almost the same for these two types of samples with a reagent volume of 0.15 mL, reaction temperature at 25 °C for 15 min.

However, the OPA derivatives of some amino compounds tend to have relatively poor stability [24,25]. Therefore, the stability of glucosamine-OPA derivatives was investigated in great detail at the very beginning of this study. The stability test results demonstrated that both plasma and urine sample derivatives did not show obvious degradation under cool places, i.e. the deviations were less than 10% in autosampler tray at 4 °C and in the refrigerator at −20 °C for 12 and 24 h. Therefore, all the derivatized samples were kept frozen at −20 °C immediately after the derivative reaction and the LC–MS/MS analysis was carried out within 8 h in order to guarantee the suitability and accuracy of the determination.

3.3. Selection of internal standard

The selection of internal reference standard is an important part for accurate and precise LC–MS/MS analysis of bio-samples. Primary amino group containing compounds, which could also react with the OPA/3-MPA, such as tranexamic acid, fudosteine, ganciclovir and penciclovir were investigated at first. For they all have similar physicochemical properties as glucosamine except for amino acids, which would suffer from endogenous interferences. However the intrinsic poor stability of the derivatives of these compounds and the obvious differences between them made these internal standard candidates no contribution.

The use of stable isotope labeled analogs as internal standard is always highly recommended for MS detection since the influences of matrix effects and differences of ionization efficacies would all be eliminated. Though, problems such as cross-talk between MS channels and isotopic purity should also be carefully addressed. But there was no commercially available multiply stable isotope labeled glucosamine for bio-analysis [10].

Finally, tolterodine tartrate, a tertiary amine group containing compound, was chosen as the internal standard. Because it was stable and easily recovered at the protein precipitation and derivatization processes, and it worked as a good indicator for the recovery and stability of glucosamine. Moreover, the similar chromatographic and mass spectrometric features with the glucosamine-OPA/3-MPA derivatives made tolterodine tartrate an eligible internal standard here.

3.4. Method validation

3.4.1. Specificity and matrix effects

Due to the endogenous glucosamine and the highly sensitive method, there is no glucosamine free blank plasma and urine. Therefore, the blank plasma and urine samples were prepared and assayed in advance for the preparation of calibration and QC standards. The mean endogenous concentrations of glucosamine in the blank plasma and urine used were found to be about 3.8 ng/mL and 1 μg/mL, respectively. At the same time, the blank plasma and urine did not show any interferences of IS. The retention time of the two glucosamine derivatives and IS was 3.2, 4.1 and 5.2 min, respectively. Typical chromatograms of plasma and urine samples are shown in Figs. 3 and 4, respectively.

Matrix effect values in the plasma and urine were varied from 87.6% to 103.2% and 94.1% to 118.7%, respectively, which were all within the acceptable limits. The same evaluation was performed on the IS. There was no significant ion suppression or enhancement for the targeted ions, which ensured the accuracy and precision of the assay.

3.4.2. Linearity and LLOQ

The method showed good linear response over the glucosamine concentration range of 0.012–8.27 μg/mL for plasma and 1.80–84.1 μg/mL for urine. Typical calibration curves were $Y=0.9798C-0.0014$ with $r=0.998$ in plasma, and $Y=0.3365C+0.3660$ with $r=0.999$ in urine, where Y is the peak area ratio of the analyte to IS and C is the concentration of the glucosamine. Because of the high endogenous glucosamine level in the blank plasma and urine, the lower limit of quantitation (LLOQ) was set at 12 ng/mL for plasma and 1.80 μg/mL for urine. The LOD of the method might have been driven much lower.

3.4.3. Accuracy, precision and extraction recovery

The within- and between-batch accuracy and precision data are summarized in Table 1. They were within the limits of acceptance criteria set by the guidelines for bioanalytical methods validation [28].

Extraction recoveries as shown in Table 2 were found to be all over 85% at all the QC levels for both plasma and urine samples with acceptable deviation.

3.4.4. Stability

The glucosamine-OPA derivatives were found to be stable and no significant degradation within 24 h when kept at 4 °C or −20 °C (RSD < 10%). However, due to intrinsic instability of the OPA derivatives of amino carbohydrates, all the processed samples were immediately put into a −20 °C refrigerator and kept at that status for no more than 24 h before the LC–MS/MS determination. The residence time of the sample in the auto-sampler tray prior to the analysis was managed to be less than 8 h.

The stability test results of glucosamine in plasma and urine under different storage conditions investigated showed that they were stable after three freeze-thaw cycles, a short-term of 8 h bench-top duration and a long-term storage at −20 °C up to 2 months, since no significant degradations were observed in all the cases for the deviations in the concentrations tested were all within 10% of their nominal values with RSD < 15%. The stability data pertaining to glucosamine in human plasma and urine are summarized in Table 3.

Table 1 Within- and between-batch precision and accuracy for glucosamine in plasma and urine (mean ± SD).

Sample	Conc. (μg/mL)	Within-batch ($n=5$)			Between-batch ($n=15$)		
		Mean conc. found (μg/mL)	Accuracy (%)	RSD (%)	Mean conc. found (μg/mL)	Accuracy (%)	RSD (%)
Plasma	0.020	0.024 ± 0.001	120.0	4.17	0.022 ± 0.002	110.0	9.09
	0.169	0.160 ± 0.005	94.7	3.13	0.172 ± 0.012	101.8	6.98
	1.66	1.580 ± 0.058	95.2	3.67	1.660 ± 0.009	100.0	5.42
Urine	2.21	2.13 ± 0.10	96.4	4.69	2.14 ± 0.18	96.8	8.41
	9.65	10.10 ± 0.47	104.7	4.65	9.82 ± 0.40	101.7	4.07
	42.7	47.30 ± 0.96	110.8	2.03	45.90 ± 2.01	107.5	4.38

Table 2 Extraction recovery of glucosamine in plasma and urine (mean ± SD, $n=5$).

Sample	Conc. (μg/mL)	Mean conc. found (μg/mL)	Mean recovery (%)
Plasma	0.020	0.018 ± 0.001	90.0
	0.169	0.180 ± 0.009	106.5
	1.660	1.658 ± 0.078	99.9
Urine	2.21	1.96 ± 0.09	93.3
	9.65	9.69 ± 0.50	100.4
	42.7	46.12 ± 3.07	108.5

Table 3 Stability data pertaining to glucosamine in human plasma and urine (mean ± SD, $n=2$).

Storage conditions	Plasma			Urine		
	Conc. (μg/mL)	Mean conc. found (μg/mL)	Accuracy (%)	Conc. (μg/mL)	Mean conc. found (μg/mL)	Accuracy (%)
Derivatives at 4 °C for 24 h	0.020	0.023 ± 0.001	115.0	2.21	1.81 ± 0.04	81.9
	1.660	1.776 ± 0.020	117.7	42.7	39.80 ± 1.96	93.2
Derivatives at −20 °C for 24 h	0.020	0.022 ± 0.002	110.0	2.21	2.16 ± 0.04	97.4
	1.660	1.685 ± 0.073	101.5	42.7	42.40 ± 1.01	99.3
Freeze-thaw ($n=3$)	0.020	0.020 ± 0.002	100.0	2.21	2.36 ± 0.09	106.6
	1.660	1.747 ± 0.037	105.2	42.7	45.00 ± 4.29	105.3
Short-term after 8 h (25 °C)	0.020	0.023 ± 0.002	115.0	2.21	2.31 ± 0.08	104.2
	1.660	1.633 ± 0.285	98.4	42.7	47.50 ± 1.60	111.2
Long-term after 60 d (−20 °C)	0.020	0.018 ± 0.001	90.0	2.21	2.20 ± 0.20	99.2
	1.660	1.770 ± 0.099	106.6	42.7	44.30 ± 4.10	103.7

3.4.5. *Application to clinical pharmacokinetic study*

The plasma glucosamine baseline levels found in the recruited Chinese volunteers were in the range of 0–120 ng/mL with a mean of 16.7 ± 26.9 ng/mL ($n=30$), and urine glucosamine levels were in the range of 2.79–8.07 μg/mL with a mean of 4.96 ± 1.74 μg/mL ($n=10$). The plasma glucosamine baseline levels found in the Chinese volunteers were much lower than those in the Caucasian volunteers reported by Persiani [8], which ranged from 10.4 to 204.0 ng/mL with a mean value of 45.3 ng/mL. These might be due to the racial and food differences between the subjects.

The validated LC–MS/MS method was successfully applied to a pharmacokinetic study of compound glucosamine sulfate dispersible tablets in healthy Chinese volunteers after single oral doses of 500, 1000 or 1500 mg, as well as multiple oral doses of 500 mg t.i.d. for 7 consecutive days. The mean plasma glucosamine concentration–time profiles are shown in Fig. 5. The main pharmacokinetic parameters are presented in Table 4. It was shown that glucosamine was rapidly absorbed and the peak concentration was reached at about 2 h after oral administration. The plasma glucosamine was eliminated to the baseline level at about 10 h post-dosing. Therefore, faster excretion was found in Chinese than Caucasian volunteers [8].

The main pharmacokinetic parameters (T_{max}, MRT and $t_{1/2}$) did not show any statistical difference ($P>0.05$) at the three dose levels as well as gender differences ($P>0.05$). While the dose-normalized values of the area under plasma concentration–time curves (AUC), the mean peak concentrations (C_{max}) for the 1500 mg dose group were both significantly lower than the corresponding values calculated at the 500 and 1000 mg dose groups ($P<0.05$). Therefore, a non-linear absorption at higher doses was obvious with oral administration. This may be due to

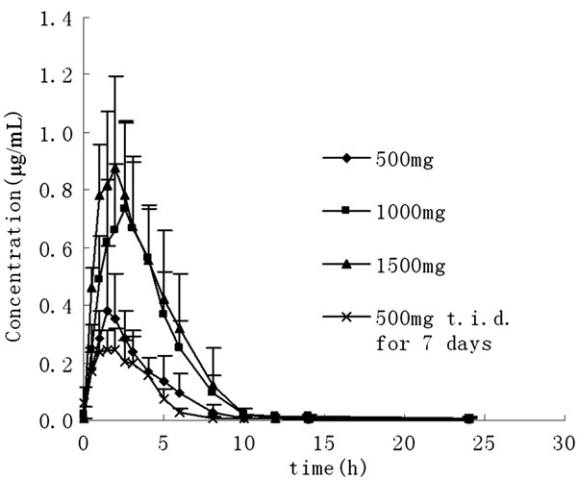

Figure 5 Mean (±SD) plasma concentration–time profiles of glucosamine in healthy Chinese volunteers ($n=10$) after a single oral administration of 500, 1000 and 1500 mg and after multiple doses of 500 mg t.i.d. on the 7th day to healthy volunteers.

Table 4 The pharmacokinetic parameters for glucosamine after single oral doses of 500, 1000, 1500 mg and after multiple doses of 500 mg t.i.d. on the 7th day to healthy volunteers (mean ± SD, $n = 10$).

Parameters	500 mg	1000 mg	1500 mg	Multiple-dose
C_{max} (μg/mL)	0.417 ± 0.190	0.795 ± 0.280	0.925 ± 0.280	0.294 ± 0.076
T_{max} (h)	2.60 ± 1.39	2.70 ± 0.86	1.90 ± 0.84	1.90 ± 1.10
AUC_{0-10} (μg h/mL)	1.42 ± 0.20	3.37 ± 1.02	4.03 ± 1.28	1.02 ± 0.24
$AUC_{0-\infty}$ (μg h/mL)	1.43 ± 0.21	3.42 ± 1.01	4.18 ± 1.40	1.02 ± 0.24
MRT_{0-10} (h)	3.18 ± 0.71	3.57 ± 0.24	3.30 ± 0.61	2.70 ± 0.34
$t_{1/2}$ (h)	1.17 ± 0.40	1.32 ± 0.35	1.36 ± 0.94	1.41 ± 0.76
CL/F (mL/h)	0.280 ± 0.042	0.245 ± 0.061	0.321 ± 0.140	0.407 ± 0.120
C_{ss_min} (μg/mL)	–	–	–	0.059 ± 0.056
C_{ss_av} (μg/mL)	–	–	–	0.125 ± 0.031
AUC_{ss} (μg/mL h)	–	–	–	1.00 ± 0.24
DF (%)	–	–	–	1.90 ± 0.93

Table 5 Urine excretion (% of dose) of glucosamine after single oral dose of 1000 mg to healthy volunteers (mean ± SD, $n = 10$).

Time after dosing (h)	Urine excretion (%)
0–3	0.434 ± 0.300
0–6	1.39 ± 1.45
0–10	1.83 ± 1.84
0–14	1.90 ± 1.86
0–24	1.99 ± 1.86

a saturation of the absorption process, which was also evinced by the higher CL/F values observed at the 1500 mg dose group. The same non-linear absorption phenomenon was also found in the Caucasian subjects [8].

The pharmacokinetic parameters of glucosamine after multiple oral doses of 500 mg compound glucosamine sulfate dispersible tablets t.i.d. for 7 consecutive days were similar with those after a single-dose. Therefore, no significant accumulation effects were seen in Chinese subjects. This was due to the short elimination half-life time observed.

The urinary excretion (% of dose) of glucosamine after single oral dose of 1000 mg compound glucosamine sulfate dispersible tablets is shown in Table 5. The excretion was almost completed in the first 10 h after the oral administration with no statistically significant gender differences ($P > 0.05$).

4. Conclusion

The established precolumn derivatization LC–MS/MS method improved the chromatographic retention and reduced the matrix effects significantly, which made the LLOQ down to 12 ng/mL for human plasma. The robust acetonitrile protein precipitation in combination with a rapid OPA/3-MPA derivatization also simplified the sample pretreatment processes significantly, which was very important for clinical investigation. Meanwhile, the good stability of the derivatives of glucosamine in the auto-sampler tray at 4 °C and in the refrigerator at −20 °C within 24 h made the determination feasible. These characteristics of the precolumn derivatization LC–MS/MS method guaranteed the sensitive, specific and accurate determination of glucosamine in bio-fluids. The fully validated method has been successfully applied to the pharmacokinetics in health Chinese volunteers.

References

[1] I. Setnikar, R. Cereda, M.A. Pacini, et al., Antireactive properties of glucosamine sulfate, Arzneimittelforschung 41 (1991) 157–161.
[2] S. Laverty, J.D. Sandy, C. Celeste, et al., Synovial fluid levels and serum pharmacokinetics in a large animal model following treatment with oral glucosamine at clinically relevant doses, Arthritis Rheum. 52 (2005) 181–191.
[3] R. Thakral, U.K. Debnath, C. Dent, Role of glucosamine in osteoarthritis, Curr. Orthop. 21 (2007) 386–389.
[4] P. Ják, P. Los, Z. Spácil, et al., Fast assay of glucosamine in pharmaceuticals and nutraceuticals by capillary zone electrophoresis with contactless conductivity detection, Electrophoresis 29 (2008) 3511–3518.
[5] A. Roda, L. Sabatini, A. Barbieri, et al., Development and validation of a sensitive HPLC-ESI-MS/MS method for the direct determination of glucosamine in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 844 (2006) 119–126.
[6] S. Persiani, R. Rotini, G. Trisolino, et al., Synovial and plasma glucosamine concentrations in osteoarthritic patients following oral crystalline glucosamine sulphate at therapeutic dose, Osteoarthritis Cartilage 15 (2007) 764–772.
[7] E. Pastorini, R. Rotini, M. Guardigli, et al., Development and validation of a HPLC-ES-MS/MS method for the determination of glucosamine in human synovial fluid, J. Pharm. Biomed. Anal. 50 (2009) 1009–1014.
[8] S. Persiani, E. Roda, L.C. Rovati, et al., Glucosamine oral bioavailability and plasma pharmacokinetics after increasing doses of crystalline glucosamine sulfate in man, Osteoarthritis Cartilage 13 (2005) 1041–1049.
[9] F. Beaudry, P. Vachon, Determination of glucosamine in horse plasma by liquid chromatography tandem mass spectrometry, Biomed. Chromatogr. 22 (2008) 1–4.
[10] S. Zhong, D. Zhong, X. Chen, Improved and simplified liquid chromatography/electrospray ionization mass spectrometry method for the analysis of underivatized glucosamine in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 854 (2007) 291–298.
[11] Y. Zhu, J. Zou, D. Xiao, et al., Bioequivalence of two formulations of glucosamine sulfate 500-mg capsules in healthy male Chinese volunteers: an open-label, randomized-sequence, single-dose, fasting, two-way crossover study, Clin. Ther. 31 (2009) 1551–1558.
[12] E. Pashkova, A. Pirogov, A. Bendryshev, et al., Determination of underivatized glucosamine in human plasma by high-

performance liquid chromatography with electrochemical detection: application to pharmacokinetic study, J. Pharm. Biomed. Anal. 50 (2009) 671–674.
[13] M. Meulyzer, P. Vachon, F. Beaudry, et al., Comparison of pharmacokinetics of glucosamine and synovial fluid levels following administration of glucosamine sulphate or glucosamine hydrochloride, Osteoarthritis Cartilage 16 (2008) 973–979.
[14] J. Du, N. White, N.D. Eddington, The bioavailability and pharmacokinetics of glucosamine hydrochloride and chondroitin sulfate after oral and intravenous single dose administration in the horse, Biopharm. Drug Dispos. 25 (2004) 109–116.
[15] Z. Liang, J. Leslie, A. Adebowale, et al., Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC, J. Pharm. Biomed. Anal. 20 (1999) 807–814.
[16] I.A. Tekko, M.C. Bonner, A.C. Williams, An optimized reverse-phase high performance liquid chromatographic method for evaluating percutaneous absorption of glucosamine hydrochloride, J. Pharm. Biomed. Anal. 41 (2006) 385–392.
[17] T.M. Huang, L. Cai, B. Yang, et al., Liquid chromatography with electrospray ionization mass spectrometry method for the assay of glucosamine sulfate in human plasma: validation and application to a pharmacokinetic study, Biomed. Chromatogr. 20 (2006) 251–256.
[18] A. Aghazadeh-Habashi, S. Sattari, F. Pasutto, et al., Single dose pharmacokinetics and bioavailability of glucosamine in the rat, J. Pharm. Pharm. Sci. 5 (2002) 181–184.
[19] L.J. Zhang, T.M. Huang, X.L. Fang, et al., Determination of glucosamine sulfate in human plasma by precolumn derivatization using high performance liquid chromatography with fluorescence detection: its application to a bioequivalence study, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 842 (2006) 8–12.
[20] T.M. Huang, C.H. Deng, N.Z. Chen, et al., High performance liquid chromatography for the determination of glucosamine sulfate in human plasma after derivatization with 9-fluorenylmethyl chloroformate, J. Sep. Sci. 29 (2006) 2296–2302.
[21] X. Zhu, J. Cai, J. Yang, et al., Determination of glucosamine in impure chitin samples by high-performance liquid chromatography, Carbohydrate Res. 340 (2005) 1732–1738.
[22] X. Wang, X. Chen, L. Chen, et al., Optimizing high-performance liquid chromatography method for quantification of glucosamine using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatization in rat plasma: application to a pharmacokinetic study, Biomed. Chromatogr. 22 (2008) 1265–1271.
[23] J. Diaz, J.L. Lliberia, L. Comellas, et al., Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection, J. Chromatogr. A 719 (1996) 171–179.
[24] S.K. AlDeeb, I.I. Hamdan, S.M. AlNajjar, Spectroscopic and HPLC methods for the determination of alendronate in tablets and urine, Talanta 64 (2004) 695–702.
[25] M. Eikenes, M. Fongen, L. Roed, et al., Determination of chitosan in wood and water samples by acidic hydrolysis and liquid chromatography with online fluorescence derivatization, Carbohydrate Polym. 61 (2005) 29–38.
[26] M. Yoshitake, H. Nohta, S. Ogata, et al., Liquid chromatography method for detecting native fluorescent bioamines in urine using post-column derivatization and intramolecular FRET detection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 858 (2007) 307–312.
[27] N.I. Larionova, D.K. Zubaerova, D.T. Guranda, et al., Colorimetric assay of chitosan in presence of proteins and polyelectrolytes by using o-phthalaldehyde, Carbohydrate Polym. 75 (2009) 724–727.
[28] US Department of Health and Human Services Food and Drug Administration, 2001. Website: 〈http://www.fda.gov/cder/guidance/index.htm〉.
[29] M. Song, L. Wang, H. Zhao, et al., Rapid and sensitive liquid chromatography-tandem mass spectrometry: assay development, validation and application to a human pharmacokinetic study, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 875 (2008) 515–521.