Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 181–186

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Application of a liquid chromatographic/tandem mass spectrometric method to a kinetic study of derivative glucosamine in healthy human urine

Yue Guan[a], Yun Tian[a], Yan Li[a], Zhifu Yang[a], Yanyan Jia[a], Taijun Hang[b], Aidong Wen[a,*]

[a] Department of Pharmacy, Xijing Hospital, The Fourth Military Medical University, Changle West Street 15, Xi'an, Shaanxi 710032, China
[b] Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China

ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
14 December 2010
Accepted 14 January 2011
Available online 22 January 2011

Keywords:
Glucosamine
LC/MS/MS
Urine
Pharmacokinetics

ABSTRACT

A sensitive, selective and efficient liquid chromatographic/tandem mass spectrometric (LC/MS/MS) method was developed and validated for the determination of glucosamine in healthy human urine. Urine samples were extracted by acetonitrile and derivatized with o-phthalaldehyde/3-mercaptopropionic acid. Analysis was then carried out using ESI source and methanol/0.2% ammonium acetate-0.1% formic acid mobile phase gradient elution, with tolterodine tartrate as the internal standard. The linear calibration curve ranged from 0.41 μg/ml to 82.7 μg/ml. The intra-day and inter-day precisions were less than 3.93% and 10.0%, respectively. The extraction recoveries determined at three concentration levels were higher than 88.6%. The method was successfully applied for determining the urine concentration of glucosamine up to 24 h after oral administration of 1 g glucosamine sulfate dispersible table (containing 785.08 mg glucosamine) from a clinical pharmacokinetic study in healthy volunteers.

Published by Elsevier B.V.

1. Introduction

Glucosamine sulfate was the active ingredient of a dispersible tablet formulation, which has been used clinically for the treatment of degenerative osteoarthris for many years. Acute toxic studies in animals indicated that it is well tolerated without obvious toxicity after oral administration of very large doses (5–15 g/kg body weight) [1]. Several studies have compared glucosamine with non-steroidal anti-inflammatory drugs (NSAID) for arthritis. The prevalence of side effects in patients using glucosamine was less common than with ibuprofen [2].

Several papers have focused on the determination of glucosamine in human plasma and synovial fluid with pre-column derivatization using 9-fluorenylmethoxycarbonyl chloride (FMOC) [3,4], 8-aminopyrenesulfonic acid (APTS) [5] or phenylisothiocyanate [6] as the derivatization reagents. Gradient elution HPLC method was generally performed with water and acetonitrile [3], 0.1% acetic acid in water and acetonitrile [4] or water and 0.2 M sodium hydroxide [7]. The run time was above 10 min in the reported methods.

Glucosamine sulfate was reported to be cleared by the liver and kidney and excreted in urine in Beagle dogs [8]. However, so far as we know, no analytical method has been developed for the determination of glucosamine in human urine after oral administration. The aim of the present study was to develop a reliable and sensitive LC/MS/MS assay [1,9,10] after derivatization with o-phthalaldehyde and 3-mercaptopropionic acid for the determination of glucosamine sulfate in healthy volunteers' urine based on optimizing the sample extraction and the mass spectrometric conditions and shortening the run time.

2. Experimental

2.1. Chemicals and reagents

Glucosamine sulfate dispersible tablets and glucosamine hydrochloride standard (purity >99.0%) were provided by Yipinhong Pharmacy (Guangdong, China). Tolterodine tartrate, the internal standard (IS), was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were HPLC grade purchased from Tedia (Ohio, USA). All the other reagents, such as formic acid, ammonium acetate, 3-mercaptopropionic acid and borax, were of analytical grade. Water was triply distilled obtained using a Milli-Q Synthesis A10 system (Millipore, France). Blank urines were supplied by Xijing Hospital, Fourth Military Medical University, China.

2.2. Derivatization conditions

0.05 M aqueous borax solution was used as buffer solution at a pH of 9.3. 2.5 g o-phthalaldehyde dissolved in 50 ml methanol was stored under protection from light at −20 °C. The mixture

* Corresponding author. Tel.: +86 29 8477 3636; fax: +86 29 8477 3636.
E-mail address: adwen-2004@hotmail.com (A. Wen).

0731-7085/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jpba.2011.01.012

of 0.5 ml of this solution, 5 ml buffer solution and 50 μl of 3-mercaptopropionic acid was used as the derivatization reagent, freshly prepared everyday.

2.3. Preparation of calibration curve and QC samples

Stock solutions of glucosamine hydrochloride were prepared by dissolving the standard compound in distilled water to give concentrations of 5, 10, 20, 50, 100, 200 and 500 μg/ml, equivalent to 4.134, 8.268, 16.54, 41.34, 82.68, 165.4 and 413.4 μg/ml of glucosamine base. Tolterodine tartrate (IS) solution was diluted to final concentration of 2 μg/ml. Calibration curve samples were prepared by spiking 0.4 ml portions of blank urine to 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100 μg/ml glucosamine hydrochloride concentrations, equivalent to 0.413, 0.827, 1.654, 4.134, 8.268, 16.54, 41.34 and 82.68 μg/ml of glucosamine base. Quality control (QC) samples were similarly prepared at glucosamine concentrations of 2.213, 9.654 and 42.73 μg/ml in urine. All samples were stored at −20 °C until analysis.

2.4. Sample preparation

0.4 ml urine samples were transferred into plastic centrifuging tubes and spiked with 40 μl IS solution. Then 800 μl acetonitrile was added and the solution vortexed for 1 min. After centrifugation at 1.58×10^4 r/min for 10 min, 150 μl of the supernatant liquid was transferred to a liquid chromatography vial, 350 μl buffer solution and 150 μl derivatization reagent were added and mixed immediately. After the derivatization reaction at 25 °C in a water bath, for 15 min protected from light, samples were stored at −20 °C until analysis within 12 h.

2.5. LC/MS/MS conditions

A Finnigan Surveyor system (Thermo Finnigan, San Jose, CA) containing a Surveyor LC pump, a Surveyor auto-sample, a TSQ Quantum Ultra AM triple-quadrupole tandem mass spectrometer with an ion max source was used. Separation was performed on an ODS column (150 mm × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 35 °C. The mobile phase consisted of methanol (A) and 0.2% ammonium acetate–0.1% formic acid buffer solution (B), using a gradient elution of 45:55 (0–1 min) to 95:5 (1–5 min) to 45:55 (5.0–6.5 min) A–B. The flow rate was 1.0 ml/min and the injection volume was 20 μl. The eluate was introduced directly into the ESI source, operating in positive ionization mode for SRM detection. The LC/MS/MS method was carried out using nitrogen to assist nebulization with nebulizer pressure of 40 psi, drying gas temperature of 350 °C, capillary voltage of 5.0 kV. Quantitative determination was performed in SRM scan mode using the following transitions: m/z 384.1 → 118.1 and 326.1 → 147.1 for glucosamine derivative product and IS, respectively (see Fig. 1).

2.6. Method validation

2.6.1. Selectivity

Analyses were performed on six blank urine samples collected from different healthy volunteers without addition of internal standard and then with addition of the internal standard or glucosamine hydrochloride. Following the proposed sample preparation procedure and LC/MS/MS conditions no interference was found from the urine matrix (see Fig. 2).

2.6.2. Linearity

The linearity was calculated by means of calibration curves obtained from spiked urine samples in the range of 0.41–82.7 μg/ml in five replicates. Glucosamine is present in two isoforms. The sum of the two chromatographic peak areas corresponding to these was considered to be the glucosamine derivative peak area. The calibration curves were generated by a weighted linear least-squares regression of the peak area ratios (y) of the glucosamine to IS versus the concentrations (c) of the calibration standards. The lower limit of quantification (LLOQ) of glucosamine was defined as the lowest concentration of the non-zero calibration sample based on five samples from the same urine.

2.6.3. Precision and accuracy

To assess the accuracy, intra-day and inter-day precisions of the method, QC sample at three different concentrations (2.213, 9.654 and 42.73 μg/ml) of glucosamine were analyzed in five replicates on three consecutive days. The precision and accuracy for replicated quality controls at various concentrations must be situated within ±15%.

2.6.4. Stability

The stability of glucosamine was tested under a variety of storage and handling conditions. Urine samples kept at the ambient temperature were analyzed after 0, 4, 6 and 8 h, respectively. Freeze-thaw stability (−20 °C) was checked undergoing three freeze-thaw cycles. To investigate long-term stability, spiked urine samples were stored below −20 °C for 60 days, and then tested. The stability of handled urine sampled kept at 4 °C and −20 °C for 0, 8, 12, 24 h, respectively, was also evaluated. All the tests of each condition were carried out in five replicated QC samples at two concentrations (2.213 and 42.73 μg/ml) of glucosamine.

2.6.5. Extraction recovery

The extraction recovery was determined on five samples at three concentrations of QC samples of glucosamine by comparing the acetonitrile extracted from urine samples with standard solution without extraction. Extraction recovery was calculated as the peak area ratio of urine samples prepared normally against unextracted standard solution.

2.7. Pharmacokinetic study design

Ten healthy volunteers (evenly divided between men and women) aged 25–39 years were recruited to determine the urine concentration of glucosamine in a phase I clinical trial, which study protocol had been approved by the Ethics Committee of Xijing Hospital of the Forth Military Medical University. Consent forms were obtained from all subjects after explaining the aims and risks of the study. The volunteers were free from cardiac, hepatic, renal, pulmonary, neurologic, gastrointestinal and hematologic disease, as determined by medical history, physical examination and routine laboratory tests (hematology, blood biochemistry and urine analysis). The study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of good clinical practice (GCP). Each volunteer was fasted and administered a single dose of 1 g glucosamine sulfate dispersible table (containing 785.08 mg glucosamine). Urine samples were collected pre-dose and in the intervals 0–3, 3–6, 6–10, 10–14 and 14–24 h post-dose. The volume of urine collected was recorded and 5 ml of each sample was frozen and stored at −20 °C before analysis.

3. Results

3.1. Chromatograms and specificity

Representative LC/MS/MS chromatograms of blank urine samples, QC samples containing 8.268 μg/ml glucosamine and

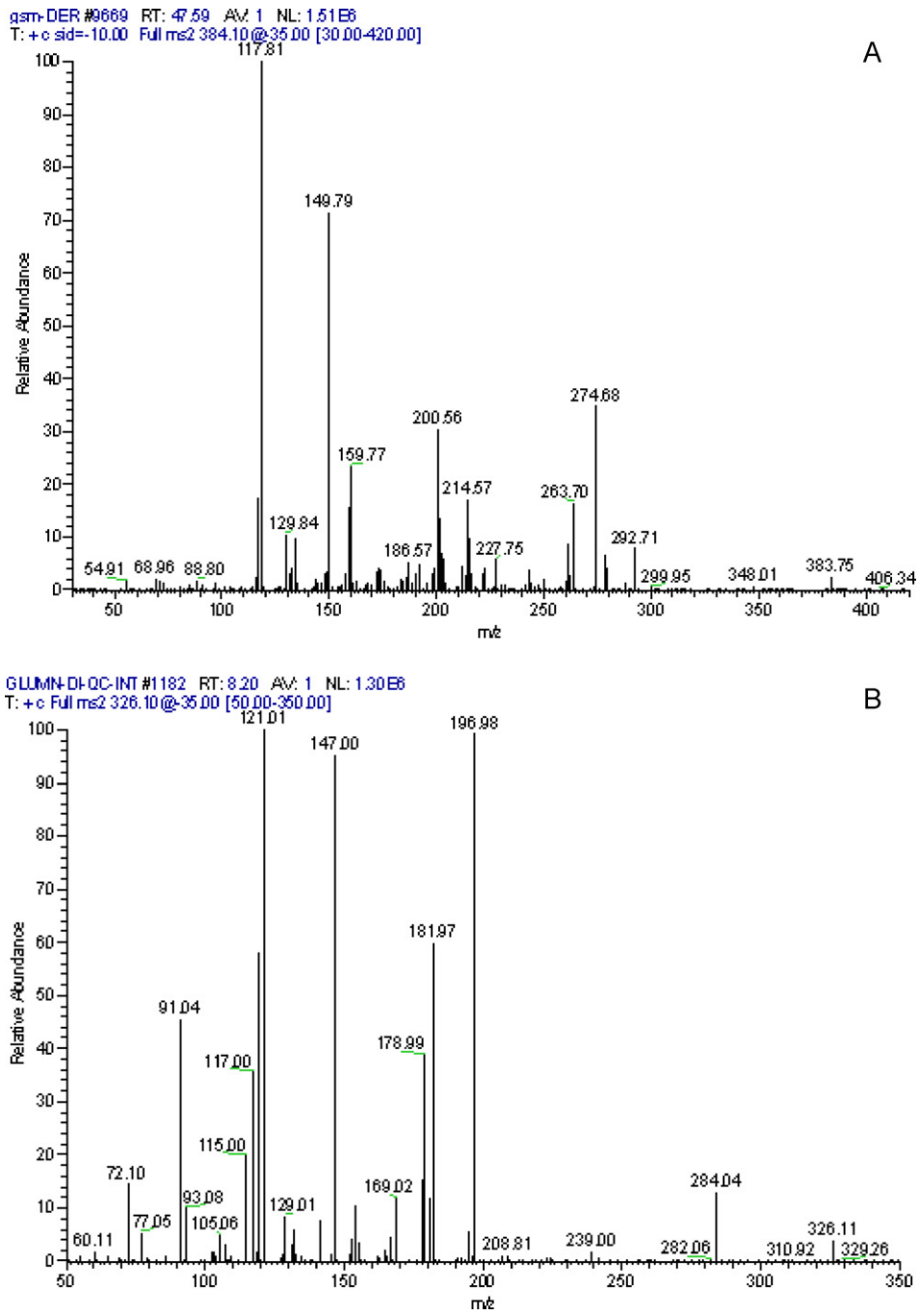

Fig. 1. Product ion mass spectra of (A) derivatized glucosamine and (B) IS.

unknown samples from a subject after oral administration of 785.08 mg glucosamine are shown in Fig. 2. The chromatograms show that due to the efficient sample preparation and the optimized HPLC gradient elution system (see Section 2.5) no endogenous interference was found and the system is suitable for the determination of glucosamine in urine. The retention times of the two glucosamine derivatized products and IS were 3.2, 4.1 and 5.2 min, respectively. The results manifested that the method exhibited good specificity and selectivity and was applicable to clinical use.

3.2. Linearity

The linear range of the method was optimized for the urine samples after single oral administration of glucosamine sulfate dispersible table. The regression equation for calibration curves was $y = 0.3035\ c + 0.0226$. The correlation coefficient was 0.9999, indicating a good linearity. The LLOQ was established at 2 ng/ml, which was sensitive enough for the pharmacokinetic study.

3.3. Assay precision and accuracy

Table 1 summarizes intra- and interday precision and accuracy results from the analysis of QC urine samples. The data in the table showed that intra- and inter-day variability values were less than 3.93% and 10.0%, respectively, and the relative error was within ±7.45%, all within the acceptable range, suggesting that the method is accurate and precise.

Table 1
Intra and interday precision and accuracy for the analysis of glucosamine in urine samples ($n = 5$).

Glucosamine concentration (μg/ml)		R.S.D. (%)		R.E. (%)
Added	Measured	Intra-day	Inter-day	
2.213	2.143	3.52	9.98	−3.16
9.654	9.818	3.93	4.95	1.70
42.73	45.91	3.30	8.30	7.45

3.4. Extraction recovery

The extraction recoveries of glucosamine in urine determined for five samples at three concentration levels of 2.213, 9.654 and 42.73 μg/ml were 88.6 ± 4.04%, 100.4 ± 5.13% and 107.9 ± 7.18%, and the RSD% were 4.56%, 5.10% and 6.65%, respectively. The results shown in Table 2 indicate that the recovery of glucosamine was over 85% in urine in the concentration range evaluated.

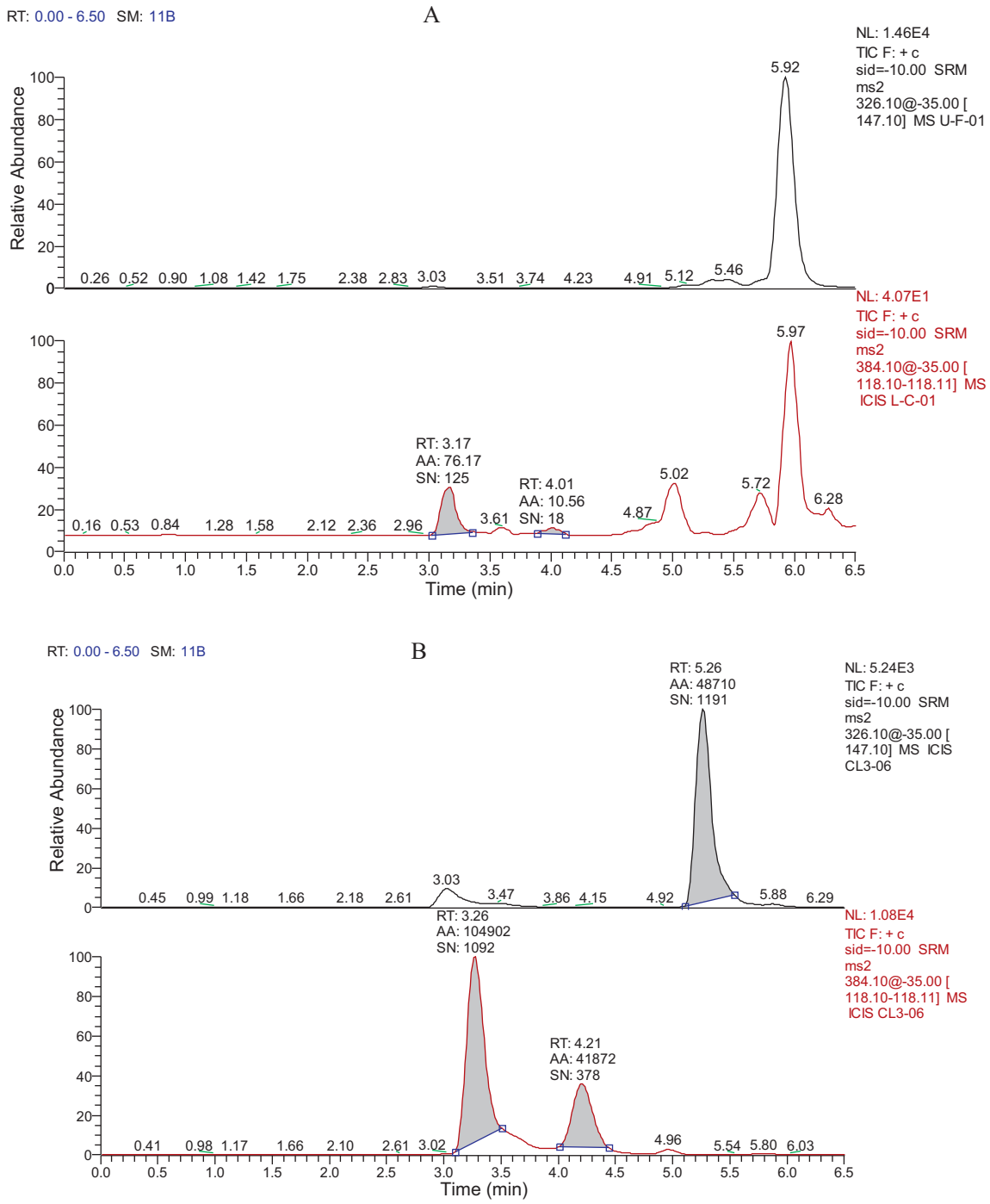

Fig. 2. Representative LC/MS/MS chromatograms for two glucosamine derivatized products and IS in healthy human urine samples. (A) Blank urine sample; (B) a blank urine sample spiked with glucosamine (t_R = 3.2 and 4.1 min, C = 8.268 μg/ml) and IS (t_R = 5.2 min, C = 0.2 μg/ml); (C) a urine sample from a volunteer in the interval 6–10 h after oral administration of glucosamine sulfate dispersible table.

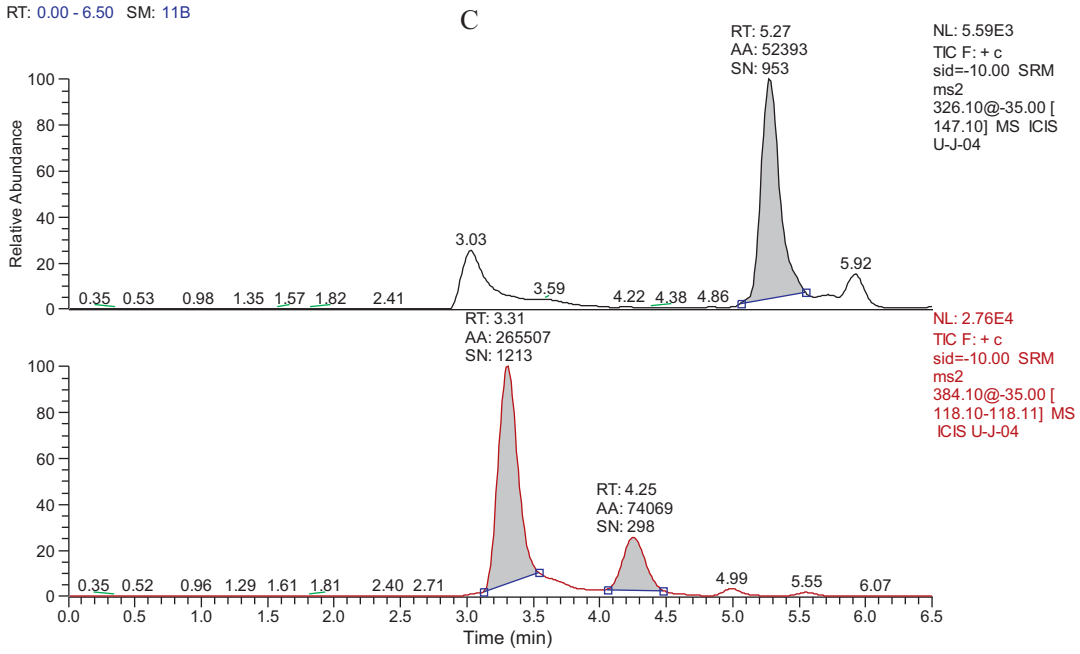

Fig. 2. (*Continued*).

Table 2
Extraction recovery of glucosamine in urine samples.

Added concentration (μg/ml)	Mean recovery (%) ± S.D.
2.213	88.6 ± 4.04
9.654	100.4 ± 5.13
42.73	107.9 ± 7.18

3.5. Stability

The results of stability analysis showed that there were no significant differences (<= ± 5%) among the urine samples of two concentrations (2.213 and 42.73 μg/ml) for at least three freeze-thaw cycles (R.S.D below 9.57%). The urine samples were found to be stable for 8 h at room temperature (R.S.D below 11.2%). After handled, QC samples also showed no significant degradation occurred when stored at 4 °C and −20 °C for 24 h (R.S.D within ±8.1% and ±8.6%), respectively. In addition, the QC samples of glucosamine were stable at −20 °C for at least 60 d (R.S.D. below 10.1%).

3.6. Pharmacokinetic application

The method described above was successfully applied to a pharmacokinetic study in healthy volunteers who with administration of 1 g glucosamine sulfate dispersible table (containing 785.08 mg glucosamine) in a single dose. The urinary pharmacokinetic profile for glucosamine excretion is presented in Fig. 3. The mean cumulative urinary excretion amount of 10 volunteers up to 24 h was 1.810 ± 0.985%. Then major PK parameters of urine were calculated by DAS Ver 2.0 software (see Table 3).

Table 3
Main pharmacokinetic parameters of glucosamine in urine after an oral dose of 785.08 mg glucosamine ($n = 10$).

Parameters	Mean ± S.D.
$t_{1/2}$ (h)	3.957 ± 1.209
Ke (1/h)	0.189 ± 0.051
Total urinary excretion amount (mg)	14.213 ± 7.730
Total urinary excretion rate (%)	1.810 ± 0.985

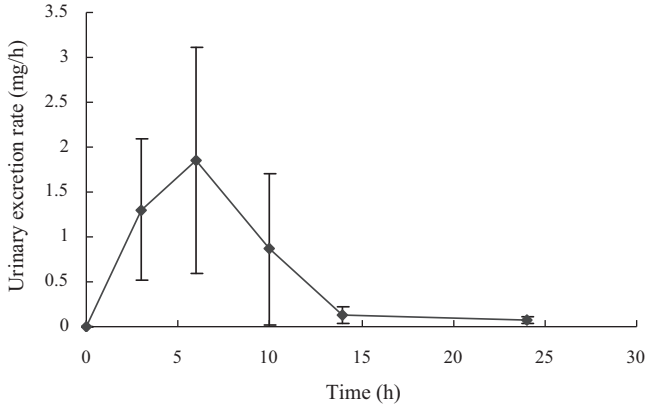

Fig. 3. Urinary excretion rate-time curve of glucosamine in 10 healthy volunteers after oral administration of one glucosamine sulfate dispersible table.

4. Discussion

To our best knowledge no pharmacokinetic study of glucosamine in urine samples has been reported yet. Ibrahim and Jamali did not completely validate the determination of glucosamine in urine [4], but they discussed their results which suggest 1.2% cumulative urinary excretion over 6 h following 200 mg/kg orally dosed to rats. Our results reached cumulative urinary excretion amount about 1.810% up to 24 h with oral 1 g glucosamine sulfate dispersible table (containing 785.08 mg glucosamine). The mean body weight of healthy adults was estimated as 60 kg, i.e. 13.08 mg/kg doses were administrated. Relative to Ibrahim and Jamali's data, our results are much higher. These differences could be explained by inter-species effects but also due to different study design and methods.

Glucosamine presented two isoforms in body, the C-4 epmeric form, known as galactosamine which could be found in urine either as a macromolecular degradation product or as an epimeric form of glucosamine. Some studies used galactosamine as an internal standard when determined glucosamine [6,10], but in our method, better performance of tolterodine made it finally

selected as our IS. For more accurately calculating the concentration of glucosamine, the summation of two chromatogram peaks (glucosamine and galactosamine) areas were calculated here as derivative glucosamine peak area in our study.

Comparing with the reported methods such as detection of underivatized glucosamine samples in human plasma and synovial fluid [9], and derivatized glucosamine with fluorescence detection in human and rat plasma [4], we developed a simple and effective method for shorter run time of glucosamine and IS. Because the structure of glucosamine contains no conjugated group, HPLC with UV or fluorescence detection could not satisfied the biological specimen analysis in our experiments. Simultaneously, glucosamine displayed strong polarity thus chromatographic retention weakness in HPLC/MS determination. Then, o-phthalaldehyde and 3-mercaptopropionic acid pre-column derivatization was introduced for the LC/MS/MS assay resulting in better peak form and shorter run time of glucosamine than other derivatization methods.

The acquisition of positive ions in SRM scanner mode provided lower background noise and higher response: the interference was so small that could be ignored when calculating the drug′s concentration.

During the study, none of the subject had any serious or less serious clinical or laboratory adverse effects.

The potential limitation of this study was single-dose administration rather than multiple-dose of glucosamine sulfate dispersible tablets. Single-dose study might not adequately characterize the pharmacokinetic properties in patients receiving ongoing therapy. Whether continuous administration for several days affect drug metabolism in urine would be of interest in future studies.

In conclusion, a sensitive and efficient LC/MS/MS method with high selectivity was developed and successfully applied to characterize the pharmacokinetics of glucosamine in volunteers after a single oral administration of 1 g glucosamine sulfate dispersible tablets.

References

[1] A. Roda, L. Sabatini, A. Barbieri, M. Guardigli, M. Locatelli, F.S. Violante, L.C. Rovati, S. Persiani, Development and validation of a sensitive HPLC-ESI-MS/MS method for the direct determination of glucosamine in human plasma, J. Chromatogr. B 844 (2006) 119–126.
[2] J.W. Anderson, R.J. Nicolosi, J.F. Borzelleca, Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy, Food Chem. Toxicol. 43 (2005) 187–201.
[3] L.J. Zhang, T.M. Huang, X.L. Fang, X.N. Li, Q.S. Wang, Z.W. Zhang, X.Y. Sha, 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 842 (2006) 8–12.
[4] A. Ibrahim, F. Jamali, Improved sensitive high performance liquid chromatography assay for glucosamine in human and rat biological samples with fluorescence detection, J. Pharm. Pharmaceut. Sci. 13 (2010) 128–135.
[5] K. Racaityte, S. Kiessig, F. Kalman, Application of capillary zone electrophoresis and reversed-phase high-performance liquid chromatography in the biopharmaceutical industry for the quantitative analysis of the monosaccharides released from a highly glycosylated therapeutic protein, J. Chromatogr. A 1079 (2005) 354–365.
[6] 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.
[7] E. Pashkova, A. Pirogov, A. Bendryshev, E. Ivanaynen, O. Shpigun, 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.
[8] I. Setnikar, C. Giacchetti, G. Zanolo, Pharmokinetics of glucosamine in the dog and in man, Arzneim-Forsch. Drug Res. 36 (1986) 729–735.
[9] E. Pastorini, R. Rotini, M. Guardigli, S. Vecchiotti, S. Persiani, G. Trisolino, D. Antonioli, L.C. Rovati, A. Roda, 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.
[10] T.M. Huang, L. Cai, B. Yang, M.X. Zhou, Y.F. Shen, G.L. Duan, 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.