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Short communication

Quantitation of ursolic acid in human plasma by ultra performance liquid chromatography tandem mass spectrometry and its pharmacokinetic study

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ABSTRACT

Ursolic acid is a hydroxy pentacyclic triterpene, which proved to have sedation, anti-inflammatory, antibacterial, antiulcer and anti-cancer activities. An ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) method with high selectivity, sensitivity and throughput has been established and validated for quantitation of total ursolic acid in human plasma. Plasma samples were pretreated by liquid–liquid extraction with ethyl acetate and were chromatographed by an ACQUITY UPLC BEH C_8 column (100 mm × 2.1 mm, I.D., 1.7 μm) using mobile phase consisting of acetonitrile and 10 mM ammonium formate (90:10, v/v) at 0.2 mL/min. The duration of chromatography analysis was 3 min. The multiple reaction monitoring (MRM) was performed at m/z 455.1 → 455.0 for ursolic acid and m/z 469.3 → 425.2 for glycyrrhetinic acid (internal standard, IS) in the negative ion mode with electrospray ionization (ESI) source. The assay showed good linearity over the range of 10–5000 ng/mL for ursolic acid in human plasma with a lower limit of quantitation of 10 ng/mL. The mean extraction recovery was 73.2 ± 4.5% and the matrix ion suppression ranged from −11.4% to −5.6%. The intra- and inter-day precisions were less than 7.0% and 7.2%, respectively, and the accuracy was within ±2.0%. Ursolic acid was stable during the analysis and the storage period. The validated method has been successfully applied to a pharmacokinetic study after intravenous infusion of Ursolic Acid Nano-liposomes to healthy volunteers.

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1. Introduction

Ursolic acid is a pentacyclic triterpene acid, which exists widely in the natural plants in the form of free acid or aglycones for triterpenoid saponins [1], including *Calendula officinalis* L., *Eugenia jaumbolana* Lam., *Glechoma hederacea* L. and so on. It has been long-recognized to have anti-inflammatory and anti-hyperlipidemic properties. Recently, ursolic acid has been noted for its antitumor-promotion effect, as for the mechanism of which, it is reported capable of eliminating various types of cancer cells by inhibiting the STAT3 activation pathway [2,3].

The ability to quantify ursolic acid in biological matrixes is important for evaluating the pharmacokinetic/pharmacodynamic (PK/PD) relationship in support of their preclinical and clinical development. Hence, a reliable bioanalytical method with high selectivity, sensitivity, accuracy, precision, and throughput is desirable for further study of ursolic acid.

As for analysis, liquid chromatography tandem mass spectrometry (LC/MS/MS) assays have provided high throughput and sensitivity for quantitation of small molecules. Ultra performance liquid chromatography (UPLC), which has been developed recently, offers better separation, faster analysis, and higher resolution than LC analysis [4,5].

Two LC/MS methods for ursolic acid quantitation in plants with liquid–liquid extraction (LLE) [6], and supercritical fluid extraction (SFE) [7] have been reported. However, both methods required complicated pretreatment of samples and high consumption of chemical agents with low sensitivity. Another published LC/MS method [8] was developed for determination of ursolic acid in rat plasma after oral administration of Lu-Ying extract. The quantification was achieved by selected ion monitoring (SIM) mode for ursolic acid and IS. Besides, the method needed large amount of plasma (500 μL), but supplied a narrower linear range (10–1000 ng/mL). Therefore, to investigate the PK/PD properties of ursolic acid in human following intravenous infusion, a more simple and sensitive method with wide dynamic range is required.

In this study, a simple, rapid and sensitive UPLC/MS/MS assay for the quantitation of total ursolic acid in human plasma was developed. The method involved a small sample volume (100 μL) with an LLE using 1 mL ethyl acetate for sample preparation, a run-time of 3 min, and a linear range over 10–5000 ng/mL, which has been successfully applied in a clinical pharmacokinetic study of

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ursolic acid in human after intravenous infusion of Ursolic Acid Nano-liposomes.

2. Experimental

2.1. Chemicals and reagents

Ursolic acid (>99.5% purity) and glycyrrhetinic acid (IS, >99.5% purity) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ursolic Acid Nano-liposomes (>99% purity) were obtained from Hubei Gedian Human well Pharmaceutical (Hubei, China). 5% glucose injection was purchased from China Otsuka Pharmaceutical (Tianjin, China). HPLC grade of acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other reagents were of analytical grade. Deionized water, prepared from demineralized water using a SYZ550 quartz pure water distiller (Tianjin Xinzhou Tech Co. Ltd., Tianjin, China), was used for all aqueous solutions during the experiment.

2.2. UPLC–MS analysis

Chromatographic separation was performed on an ACQUITY UPLC BEH C_8 column (100 mm × 2.1 mm, I.D., 1.7 μm, Waters Corp., Milford, MA, USA) using an ACQUITY™ UPLC system equipped with a binary pump, a vacuum degasser and an autosampler (Water Corp., Milford, MA, USA). The column oven was maintained at 40 °C. The mobile phase consisting of acetonitrile and 10 mM ammonium formate (90:10, v/v) was performed at a flow rate of 0.2 mL/min. The samples were kept at 10 °C in the autosampler.

The mass spectrometry detection was performed on a Waters ACQUITY TQD™ triple quadrupole system with a Z-spray ionization source (Waters Corp., Milford, MA, USA). MassLynx v.4.1 software was used for data acquisition and analysis (Waters Corp., Milford, MA, USA). Ionization was operated using an electrospray ionization (ESI) source in the negative ion mode. The ionization source parameters were as follow: capillary voltage 3.8 kV, cone voltage 80 V, source temperature 110 °C, desolvation temperature 350 °C, desolvation gas flow 600 L/h, cone gas flow 50 L/h. The collision gas (Ar) for MS/MS was maintained at 3.0×10^{-3} mbar. Quantification was performed in multiple reaction monitoring (MRM) mode at m/z 455.1 → 455.0 for ursolic acid, and m/z 469.3 → 425.2 for IS, respectively. The optimized collision energy (CE) was 20 eV for ursolic acid, and 34 eV for IS, respectively.

2.3. Preparation of standards, quality control (QC) samples and test samples

Ursolic acid and IS stock solutions were prepared in acetonitrile to give a final concentration of 1.0 mg/mL. Calibration standard solutions with concentrations of 10, 20, 100, 500, 2000, 4000 and 5000 ng/mL were prepared by serial dilution of the stock with acetonitrile. Working solutions for quality control samples with concentrations of 20, 500 and 4000 ng/mL were prepared in the same manner. The IS working solution (10,000 ng/mL) was prepared in acetonitrile from the 1.0 mg/mL IS stock solution. All the solutions were kept at 4 °C and were brought to room temperature before use. The 100 μL of calibration standard solution was evaporated to dryness at 40 °C under a stream of nitrogen, added with 100 μL of blank human plasma, and then vortex-mixed for 30 s. This yielded calibration standard concentrations of 10, 20, 100, 500, 2000, 4000, and 5000 ng/mL in each sample. QC samples were prepared in the same manner at low, medium and high levels (20, 500, 4000 ng/mL). To prepare test solutions, Ursolic Acid Nano-liposomes was dissolved in 5% glucose injection and then diluted at concentrations of 500 ng/mL, 10 μg/mL and 100 μg/mL in 5% glucose injection. The test solutions were then spiked into human plasma to prepare test plasma samples at the concentrations of 20, 500 and 4000 ng/mL. Subsequently, all the spiked plasma samples were treated according to sample preparation procedure mentioned in section 2.4. Extraction effect of two kinds of plasma samples (in triplicate) were determined by comparing the peak areas of test plasma samples to QC samples made from working solutions of ursolic acid at three QC levels using the UPLC–MS/MS method specified above.

2.4. Sample preparation

Human plasma samples were treated by liquid–liquid extraction with ethyl acetate. An aliquot of 100 μL plasma sample was added with 100 μL IS working solution (10,000 ng/mL), and 1 mL ethyl acetate in the glass tubes. Subsequently, the tubes were vigorously vortex-mixed for 2 min, and centrifuged at 2000 × g for 10 min. 200 μL of the upper organic layer was transferred and evaporated to dryness at 40 °C under nitrogen. The residue was reconstituted in 200 μL mobile phase, vortex-mixed for 30 s and centrifuged at 12,000 × g for 5 min. The supernatant was transferred to an injection vial and then an aliquot of 5 μL was injected for UPLC/MS/MS analysis.

2.5. Method validation

The UPLC/MS/MS method presented in this work was rigorously validated according to the FDA guideline [9] for bioanalytical method validation.

Selectivity was assessed by extracting six different lots of human blank plasma to investigate potential interferences of endogenous compounds. The chromatogram of blank plasma sample was compared with those of plasma sample spiked with analyte and IS and incurred plasma sample collected after intravenous infusion.

Calibration curves, consisting of seven concentration levels (10, 20, 100, 500, 2000, 4000 and 5000 ng/mL of ursolic acid in human plasma), were prepared and analyzed in triplicate on three consecutive days. Calibration curves were constructed based on the peak area ratio (analyte/IS) versus the spiked concentrations by least square linear regression analysis with a weighting factor of $1/x^2$. Deviations of these back-calculated concentrations from calibration standard samples were set within ±15% of nominal concentrations (±20% for the lower limit of quantitation).

Precision and accuracy were assessed by determining six replicates of the low, medium and high QC samples (20, 500, 4000 ng/mL) on three consecutive days. The precision was expressed as the relative standard deviation (RSD) and the accuracy was described as the relative error (RE). The criterion of intra- and inter-day precisions is that RSD is within ±15%; for accuracy RE of all QC samples within ±15%.

The lower limit of quantitation (LLOQ) of the assay was assessed as the lowest concentration of the calibration curve that can be quantitatively determined with acceptable precision less than 20% and accuracy within ±20%. The LLOQ was established based on six replicates on three consecutive days.

The matrix effect (ME) was defined as the ion suppression/enhancement on the ionization of analyte. The matrix effect of ursolic acid and IS was investigated by comparing the corresponding peak areas of the post-extraction spiked samples from six different lots of blank plasma to those of the QC and IS working solutions reconstituted in mobile phase. The variability of the ratios, as measured by the coefficient of variation (CV %), should be less than 15% [10]. Experiments were performed at the three QC levels, in six replicates.

The extraction recoveries of ursolic acid and IS were calculated by comparing the peak areas of blank plasma samples spiked with

analyte pre-extraction with those of the post-extraction spiked samples at the same concentrations. Experiments were performed at three QC concentration levels, in six replicates.

The stability of ursolic acid in human plasma was investigated by comparing the measured concentrations of low, medium and high QC samples (in triplicate) with the spiked concentrations under the following four storage conditions: (1) short-term temperature stability at room temperature for 6 h; (2) freeze-thaw stability over three cycles; (3) post-preparative stability at 10 °C for 24 h; (4) long-term temperature stability at −20 °C for 90 days. The analyte was considered stable when the percent deviation was within ±15%.

2.6. Pharmacokinetic study

The method was put in practice to quantitate ursolic acid in the plasma of 8 healthy volunteers in a clinical pharmacokinetic trial, who were treated with intravenous infusion of Ursolic Acid Nano-liposomes (Hubei Gedian Humanwell Pharmaceutical Co., Ltd., Hubei, China) at the dose of 98 mg/m^2. The pharmacokinetic study was approved by the Ethical Committee of Tianjin Medical University Cancer Institute and Hospital, Tianjin Medical University. About 4 mL blood samples were collected from elbow vein into heparinized tubes before dosing (0 h), and 0.5, 1, 2, 4 h during the 4 h-continuous infusion and 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 h after the end of the infusion. The blood samples were immediately centrifuged at 4000 × *g* for 5 min, and then the supernatant (plasma) was transferred into polypropylene test tubes and stored at −20 °C until analysis. Major pharmacokinetic parameters, including C_{max}, T_{max}, $t_{1/2}$, CL, AUC_{0-t}, and $AUC_{0-\infty}$ were calculated for each person by DAS 2.0 (Drug and Statistics) software (edited by Chinese Mathematical Pharmacology Society).

3. Results and discussion

3.1. Chromatographic separation

Both C_{18} and C_8 columns (100 mm × 2.1 mm, I.D., 1.7 μm, Waters Corp., Milford, MA, USA) were tested to achieve ideal separation. Compared with the C_{18} column, to which the binding of analyte was stronger, the separation by C_8 column showed shorter retention time and better peak shape, even at low concentration. The use of ACQUITY UPLC BEH C_8 column allowed separation of analyte and IS from endogenous compounds within 2 min with an isocratic mobile phase. As the mobile-phase modifier, 10 mM ammonium formate promoted the deprotonation in ESI negative mode than 0.1% formic acid. The addition of 10 mM ammonium formate helped enhance the ionization and improve peak shape. The mobile phase consisting of acetonitrile and 10 mM ammonium formate (90:10, v/v) was optimal for ursolic acid quantitation.

3.2. Mass spectrometric detection

Due to the carboxyl in the chemical structures of ursolic acid and IS, the full-scan mass spectrum showed that the signal intensity in the negative ion mode was much higher than that in the positive ion mode for both ursolic acid and IS. After fragmentation in the collision cell, the signal from the *m/z* 469.3 → 425.2 transition was observed to be the most abundant and stable transition for IS. Other product ions including 409.3 and 355.2 were observed (Fig. 1B) as well. However, further research was carried out and results showed that compared to 469.3 → 425.2, the sensitivity of 469.3 → 409.2 and 469.3 → 355.3 was lower and both transitions resulted in an increased signal-to-noise ratio.

Ursolic acid could not be collided into fragments when collision energy was lower than 40 eV, or no dominant product ions

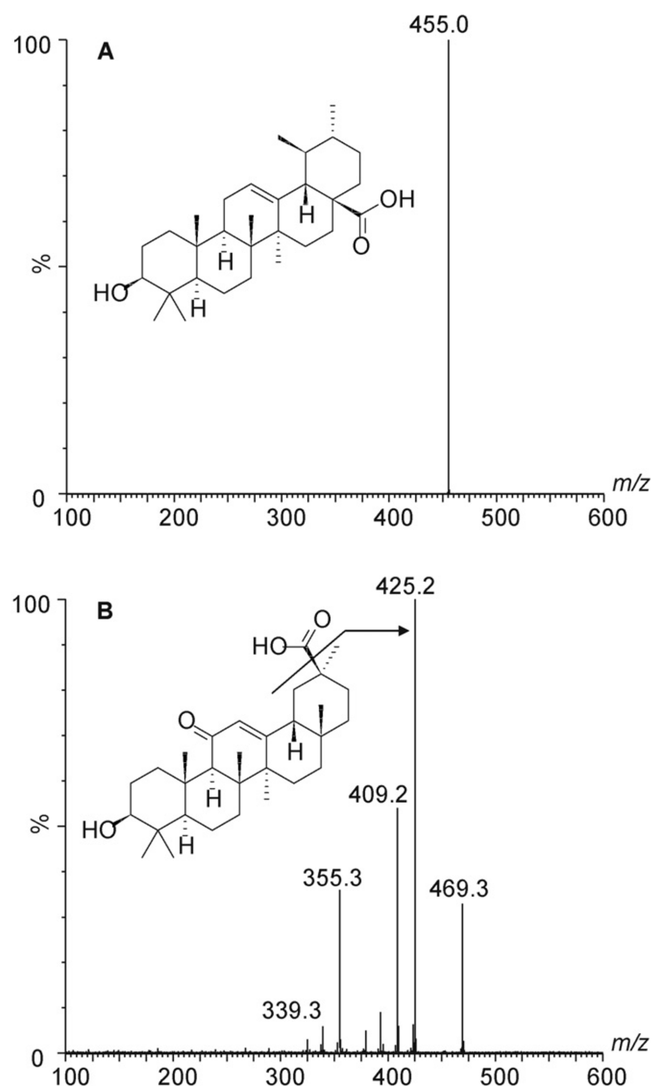

Fig. 1. Chemical structures and product ion mass spectra of $[M-H]^-$ of (A) ursolic acid, and (B) glycyrrhetinic acid (IS).

were detected if collision energy was higher than 50 eV, which indicated that routine MRM with different parent and product ion was not suitable for ursolic acid quantitation. The resolution of Waters TQD^{TM} is unit mass resolution. In the MRM mode of our experiment, the CE in Q2 was set to a low value to minimize fragmentation. Therefore, the parent ion isolated in Q1 (455.1) passed through Q2 (20 eV) without fragmentation. In Q3 (455), the same ion was monitored. We have investigated that compared to the SIM mode using Q1 or Q3, the signal of background noise in the MRM mode was much lower and this MS method derived from MRM showed higher sensitivity and selectivity. Fig. 1 shows the full-scan product ion MS/MS spectra of ursolic acid and IS. Other parameters of the sensitivity were optimized to obtain the best mass spectrometric conditions.

3.3. Sample preparation

Several sample preparation procedures were tested to achieve acceptable recovery, and reduce matrix effect. Direct protein precipitation with acetonitrile or methanol generally resulted in poor recovery (15–20%) and strong ion suppression (∼80% suppression). 1 mL ethyl acetate was selected as extraction solvent, which provided acceptable analyte recovery (∼70%) and weak ion sup-

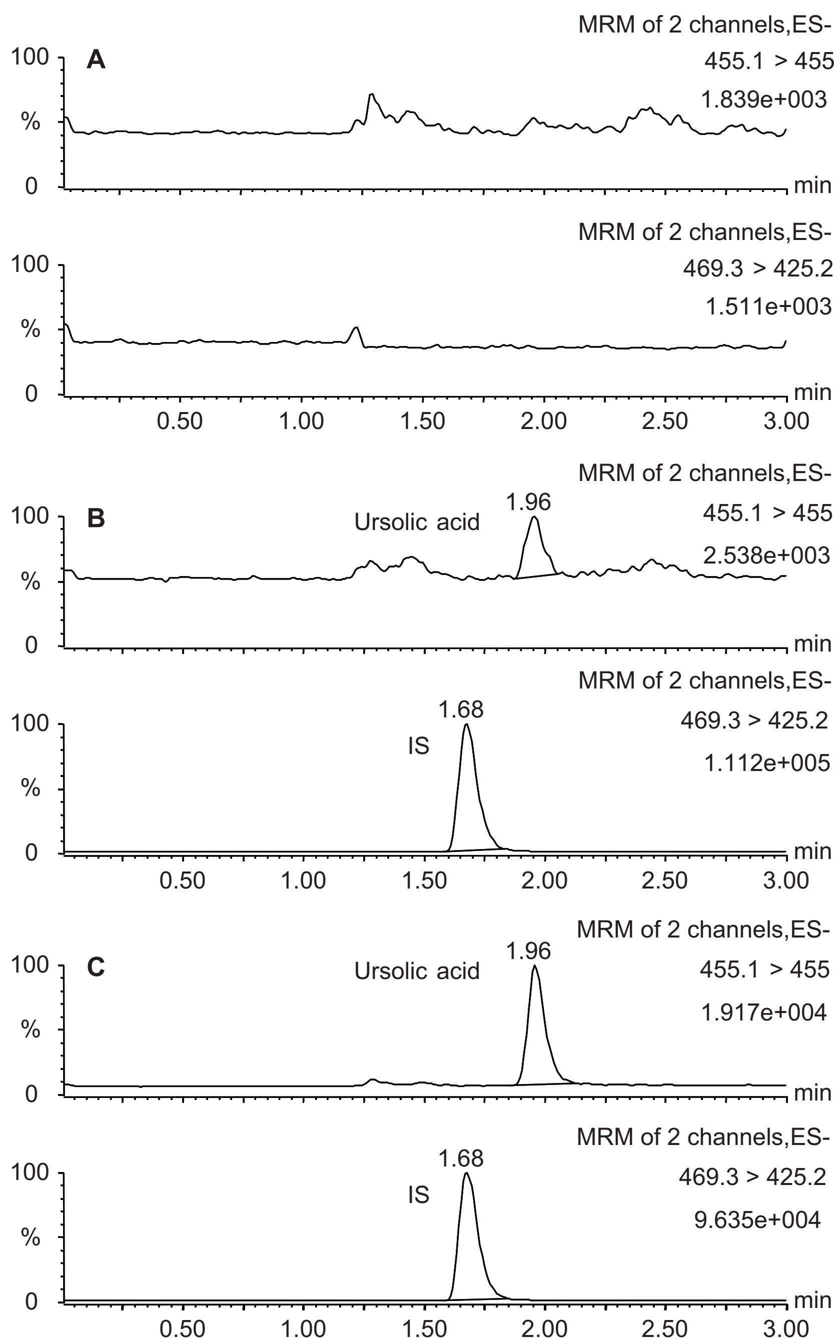

Fig. 2. Representative MRM chromatograms of ursolic acid and IS in human plasma. (A) Blank plasma sample; (B) plasma sample spiked with ursolic acid at 10 ng/mL and IS at 10, 000 ng/mL; (C) a plasma sample collected at 3.0 h post-infusion of 98 mg/m^2 ursolic acid.

pression (~10% suppression). In order to modulate pH, addition of glacial acetic acid was tested for higher extraction recovery and sensitivity since ursolic acid was a pentacyclic triterpene acid, however the results indicated it did not work as expected. During the sample preparation, 1 mL ethyl acetate was added and 200 μL of upper organic layer was transferred and evaporated to dryness, and then the residue was reconstituted in 200 μL mobile phase, which meant that both analyte and IS were diluted 10 times. We have investigated this approach can not only maintain the sensitivity, but also suppress matrix effect significantly. Different volumes of upper organic layer were transferred to obtain less matrix effect and results showed that the more organic layer transferred the stronger matrix suppression appeared. The reason for it might be that the suppression of ionization of analytes by the presence of matrix components in the biological samples was related to the amount of endogenous compound in the sample matrix that came into the surface of the ion source.

The results of comparison of ursolic acid peak area for QC samples ($n = 3$) were 209.6 ± 27.4, 5059.6 ± 367.3 and 38,327.7 ± 938.1 at concentrations of 20, 500 and 4000 ng/mL; for test samples ($n = 3$) those were 202.2 ± 11.5, 5088.8 ± 97.7 and 39,116.8 ± 1535.7 at concentrations of 20, 500 and 4000 ng/mL. The peak areas of QC samples at three levels were basically the same as those of test samples, which indicated that nano-liposomes could be efficiently disrupted for releasing ursolic acid by our sample preparation procedure.

Table 1
Precision and accuracy of ursolic acid in human plasma ($n = 6$).

Compound	Concentration (ng/mL)		Precision (RSD (%))		Accuracy (RE (%))
	Spiked	Measured	Intra-day	Inter-day	
Ursolic acid	10	10.0 ± 0.8	5.4	10.9	−2.2
	20	20.4 ± 1.4	7.0	4.6	2.0
	500	505.8 ± 25.2	4.6	7.2	1.2
	4000	3922.5 ± 176.1	4.4	4.9	−2.0

Table 2
Stability of ursolic acid in human plasma ($n = 3$).

Storage conditions	Concentration (ng/mL)		RE (%)
	Spiked	Measured (mean ± SD)	
At room temperature for 6 h	20	20.6 ± 2.9	2.9
	500	498.2 ± 24.3	−0.4
	4000	3693.1 ± 15.4	−7.8
Three freeze-thaw cycles	20	19.7 ± 0.8	−1.5
	500	511.5 ± 13.1	2.3
	4000	3825.7 ± 151.1	−4.3
At 10 °C in the autosampler for 24 h	20	21.3 ± 1.4	6.5
	500	522.1 ± 9.0	4.4
	4000	4130.7 ± 139.3	3.3
Long-term stability (at −20 °C for 90 days)	20	19.2 ± 2.0	−5.0
	500	502.9 ± 31.4	0.6
	4000	3953.5 ± 147.1	−1.0

3.4. Method validation

3.4.1. Selectivity and specificity

Representative chromatograms of blank human plasma, spiked human plasma, and incurred human plasma are presented in Fig. 2. The retention time is 1.96 min for ursolic acid and 1.68 min for IS. The chromatograms of blank plasma samples from six different lots showed no significant peak in either the analyte or the IS MRM channel, indicating that the method was selective.

3.4.2. Linearity and lower limit of quantitation

The calibration curve was ranged from 10 to 5000 ng/mL for ursolic acid, which was determined according to the concentrations anticipated in the incurred samples. Correlation coefficients of triplicate calibration curves were >0.99 on each validation day. The typical regression equation obtained by least squared regression was $y = 0.00106x + 0.000115$, $r^2 = 0.998$. At the LLOQ concentration of 10 ng/mL, the intra- and inter-day precisions were less than 5.4% and 10.9%, and the accuracy was less than −2.2% (Table 1). A signal-to-noise ratio of ∼50 was also observed at this concentration, which was sufficient for the pharmacokinetic study.

3.4.3. Precision and accuracy

QC samples at three concentrations were analyzed in six replicates in order to determine the assay accuracy and precision. As shown in Table 1, the intra- and inter-day precisions were less than 7.0% and 7.2%, and the accuracy was within ±2.0% for ursolic acid.

3.4.4. Matrix effect

Matrix effect may result in ion suppression or enhancement by co-eluting endogenous substances in biological matrix and therefore affects the sensitivity or reproducibility. The results of matrix effect for ursolic acid were 94.4 ± 5.1%, 89.6 ± 4.9%, and 90.1 ± 1.6% with the CV% of 5.4%, 5.4%, and 1.7% at concentrations of 20, 500, and 4000 ng/mL, respectively. The result for IS was 72.9 ± 3.7% with the CV% of 5.0%.

3.4.5. Recovery

The extraction recoveries of ursolic acid were 72.3 ± 6.5%, 75.1 ± 2.8%, and 72.2 ± 3.6% at concentrations of 20, 500, and 4000 ng/mL, respectively. The recovery of IS was 64.2 ± 2.2% at the concentration of 10,000 ng/mL.

3.4.6. Stability

Results of the stability tests were summarized in Table 2. Ursolic acid was stable in human plasma after being placed at ambient temperature for 6 h, in the autosampler at 10 °C for 24 h, after being stored at −20 °C for 90 days and through three freeze-thaw cycles.

3.5. Pharmacokinetic study

The UPLC/MS/MS method developed in this study yielded satisfactory results for the quantitation of ursolic acid in human plasma and has been successfully applied to the pharmacokinetic study of Ursolic Acid Nano-liposomes. The mean plasma concentration versus time curve is presented in Fig. 3. The major pharmacokinetic parameters of ursolic acid were calculated by non-compartment model based on statistical moment and presented in Table 3.

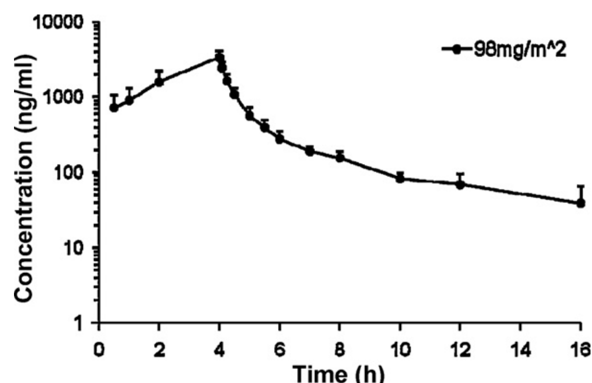

Fig. 3. Mean plasma concentration versus time curve of ursolic acid after intravenous infusion at dose of 98 mg/m^2 to volunteers ($n = 8$, mean ± standard deviation).

Table 3
Main pharmacokinetic parameters of ursolic acid after intravenous infusion of Ursolic Acid Nano-liposomes (98 mg/m^2) to volunteers (n = 8, mean ± SD).

Parameters	98 mg/m^2 Mean ± SD
C_{max} (ng/mL)	3404.6 ± 748.8
T_{max} (h)	4.0 ± 0.0
AUC_{0-t} (ng h/mL)	9644.1 ± 1193.2
$AUC_{0-\infty}$ (ng h/mL)	9918.4 ± 1215.2
$t_{1/2}$ (h)	3.9 ± 2.1
CL (L/h/m^2)	10.0 ± 1.2

4. Conclusions

A rapid, sensitive and specific UPLC/MS/MS method was developed and validated for determination of total ursolic acid in human plasma for the first time. The method was successfully applied to characterize the pharmacokinetics of ursolic acid in human after intravenous infusion of Ursolic Acid Nano-liposomes. A liquid–liquid extraction procedure was taken to extract ursolic acid from human plasma, followed by chromatography with tandem mass spectrometry detection. The method showed good sensitivity, reproducibility, precision and accuracy, as well as recovery for ursolic acid. Compared with other methods for quantifying ursolic acid reported in the literature, the present UPLC/MS/MS method is much simpler and faster, with higher specificity and wider linear range.

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