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Short communication

Determination of ginsenoside Rg3 in human plasma and urine by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Here we report a method capable of quantifying ginsenoside Rg3 in human plasma and urine. The method was validated over linear range of 2.5–1000.0 ng mL^{-1} for plasma and 2.0–20.0 ng mL^{-1} for urine using ginsenoside Rg1 as I.S. Compounds were extracted with ethyl acetate and analyzed by HPLC/MS/MS (API-4000 system equipped with ESI$^-$ interface and a C_{18} column). The inter- and intra-day precision and accuracy of QC samples were ≤8.5% relative error and were ≤14.4% relative standard deviation for plasma; were ≤5.6% and ≤13.3% for urine. The Rg3 was stable after 24 h at room temperature, 3 freeze/thaw cycles and 131 days at −30 °C. This method has been applied to pharmacokinetic study of ginsenoside Rg3 in human.

1. Introduction

Ginsenoside Rg3 (Fig. 1), extracted from Red Panax ginseng, is a tetracyclic triterpenoids saponins monomer. Pharmacological experiments showed that ginsenoside Rg3 significantly inhibited tumor growth and metastasis in mice and *in vitro* tumor cell invasion, as well as enhanced body immunity [1–8]. The anti-tumor drug-Rg3 Shenyi Capsule, which mainly consists of ginsenoside Rg3, has been approved by State Food and Drug Administration as a first-class new drug in China in 2003, and some commercial products containing ginsenoside Rg3 are applying for clinical trail.

Numerous methods have been introduced during recent years for the determination of many kinds of ginsenosides. They include gas chromatography coupled with mass spectrometry (GC/MS), high performance liquid chromatography (HPLC), HPLC coupled with mass spectrometry (HPLC/MS) and tandem mass spectrometry (HPLC/MS/MS) [9–25]. However, these methods appear to have either a complicated extraction procedure, low sensitivity, or a long chromatographic run-time, or perderivatization. Using the GC/MS method, 20(S)-protopanaxadiol and 20(S)-protopanaxatriol were measured as the genuine aglycones of ginsenosides in human urine [25]. HPLC methods with UV detection suffer from limited selectivity due to the presence of more than 20 structurally similar ginseng saponins and endogenous interferences from biological fluids.

Several methods have been established for evaluate pharmacokinetics profiles of ginseng and ginseng preparations in animals or human [16–18,23,24]. Li et al. [16] used an HPLC/UV coupled with solid-phase extraction method for determination of four active saponins (ginsenoside Rg1, Rb1, Rd and notoginsenoside R1) from Panax notoginseng in rat urine. Xu et al. [18] used another HPLC/UV method to evaluate the pharmacokinetics and bioavailability of ginsenoside Rb1 and Rg1 from Panax notoginseng in rats. Cai and Qian [17,24] established an HPLC/Q/TOF analytical method for the determination of ginsenoside Rg3 in rat plasma and its major metabolites in samples from *in vitro* and *in vivo* metabolism studies. Xie et al. [23] established an HPLC/MS method for the determination of ginsenoside Rg3 and its metabolites in rat plasma using solid-phase extraction for pharmacokinetic studies.

However, there are few reports on the determination of ginsenoside Rg3 in human for pharmacokinetic study [9,13]. Wang et al. reported some pharmacokinetic parameters of Rg3 after oral administration of the ginsenoside in human at 3.2 mg kg^{-1}. The human plasma was prepared with solid-phase extraction and the extract was analyzed by using HPLC/UV for Rg3 concentrations. Human pharmacokinetic parameters, including C_{max} of 15.67 ± 6.14 ng mL^{-1} and t_{max} of 0.66 ± 0.01 h were obtained. The lower limit of quantitation of this method was 2.5 ng mL^{-1}, and the run-time for each sample was 20 min [13].

Summing up the above, there is no specific and rapid HPLC/MS/MS method reported for determining ginsenoside Rg3 for evaluating the pharmacokinetics of it after administration in human until now. In order to provide a useful clinical tool for proper medication, we develop an HPLC/MS/MS method for quantitation of ginsenoside Rg3 in human plasma and urine, which is accurate, sensitive, specific, and with a high throughput. This method is applied to approximately 1000 samples from healthy male and female volunteers.

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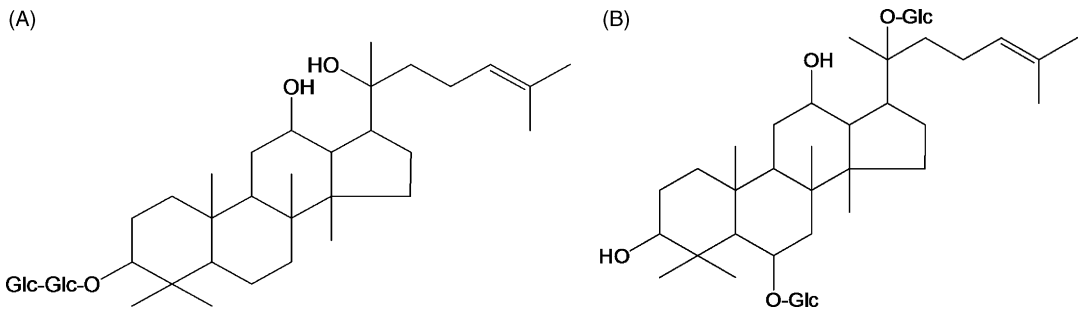

Fig. 1. Chemical structures of ginsenoside Rg3 and Rg1. (A) Ginsenoside Rg3 and (B) ginsenoside Rg1.

2. Experimental

2.1. Chemicals and reagents

Ginsenoside Rh3 and Rg1 (internal standard, Fig. 1) were obtained from Beijing Xinlinheng Company (China). Ammonium acetate was purchased from Beijing Chemical Reagents Company (China). HPLC grade methanol was purchased from Fisher (USA). All liquids used for experiments were filtered through 0.22 μm membrane filters from Millipore (USA). Heparinized blank human plasma and blank urine were obtained from volunteers at Peking Union Medical College Hospital, Beijing, China.

2.2. Standard curve and quality control sample preparation

Stock solutions of ginsenoside Rg3 were prepared from separate weightings for standards and quality control samples (QCs). The stock solutions and subsequent working solutions were prepared in methanol and stored at −30 °C during the validation.

Calibration standards were prepared by spiking an appropriate amount of concentrated stock solutions into blank control plasma and urine. The calibration ranges were 2.5–1000.0 ng mL^{-1} for plasma and 2.0–20.0 ng mL^{-1} for urine. Three levels of QC samples were prepared at 7.5 ng mL^{-1}, 80.0 ng mL^{-1} and 800.0 ng mL^{-1} for plasma and 3.0 ng mL^{-1}, 7.5 ng mL^{-1} and 15 ng mL^{-1} for urine. The QC concentrations were chosen near the low, medium and high calibration standard concentrations being prepared. QCs were stored at −30 °C with clinical sample to be analyzed.

2.3. Instrumentation

The HPLC system consisted of a Shimadzu DGU-20A pump (Japan), a Shimadzu DGU-20A autosampler (Japan) and a Waters SymmetryShield® RP18 column (4.6 mm × 100 mm, 3.5 μm). The HPLC system was operated isocratically at a flow rate of 1 mL min^{-1}. The mobile phase consisted of methanol:10 mM ammonium acetate (95:5, v/v).

The mass spectrometer was an AB API-4000 triple quadrupole mass spectrometer (Thornhill, Canada) equipped with a turboionspray (ESI) interface. The heated nebulizer was set at 500 °C and the needle voltage (IS) was set at −4500.00 V; the nebulizer gas (GS1), the auxiliary nitrogen gas (GS2), the curtain gas (CUR) and the collision gas (CAD) were set at 11 units, 12 units, 10 units and 10 units, respectively. Ion monitored in the multiple reaction monitoring (MRM) mode were m/z 783.5 (parent ion) to m/z 160.9 (daughter ion) for ginsenoside Rg3 and m/z 799.5 (parent ion) to m/z 637.4 (daughter ion) for ginsenoside Rg1 (I.S.). Nitrogen was used as the collision gas. The electron multiplier was set at 2000 V.

2.4. Extraction procedure

To 200 μL heparinized plasma (300 μL urine) samples, 50 μL I.S. in methanol (2.5 μg mL^{-1} for plasma samples and 250 ng mL^{-1} for urine samples) was added. After vortex-mixing briefly, 900 μL ethyl acetate was added. Then vortex-mixed for 1 min. The organic phase was then separated from the aqueous phase by centrifugation at 3500 × g for 3 min. The organic phase was transferred to a clean tube. After evaporation to dryness under nitrogen gas in 40 °C water bath, the residue was reconstituted in 300 μL of methanol:water (95:5 v/v), and 10 μL was injected onto the HPLC/MS/MS system.

2.5. Assessment of validation

The specificity, precision/accuracy of the assay and the stabilities of ginsenoside Rg3 in human plasma and urine were determined using three levels of quality control samples.

2.5.1. Specificity and selectivity

Blank human plasma and urine from six different lots were prepared and tested for endogenous interference. One blank matrix sample spiked with I.S. only and one blank matrix sample spiked with ginsenoside Rg3 alone at 1000 ng mL^{-1} without I.S. were analyzed in order to assess potential interferences that may affect either the analyte or the I.S.

Ginsenoside Rh2 (loss of one glucose from ginsenoside Rg3), the potential metabolite of ginsenoside Rg3 in human which was reported in rats study was analyzed to assessing the interferences for ginsenoside Rg3 and I.S.

2.5.2. Precision and accuracy

Five replicates of each quality control samples were analyzed on three batches and the intra- and inter-day means, relative standard deviation (RSD%) and relative error (RE%) were calculated.

2.5.3. Stability testing

The stability of ginsenoside Rg3 stock solution was calculated by comparing the peak area of stock solution after stored for a period of time with that of stock solution freshly prepared at the same concentration (freshly weighing) under the same determination condition. To mimic the possible freezing and thawing conditions of samples, quality control samples were subjected to multiple cycles of freezing and thawing and then analyzed. The values of quality control samples after three freeze/thaw cycles were comparable to the theoretical concentration. The autosampler stability was evaluated by comparing the concentration of quality control samples after being stored in autosampler for a period with the theoretical concentration of quality control samples. Short-term stability test was done by comparing the data from quality control samples thawed and stored on the bench to nominal value. Long-term storage stability test was done by comparing the data from quality control samples stored in biological matrix in freezer conditions

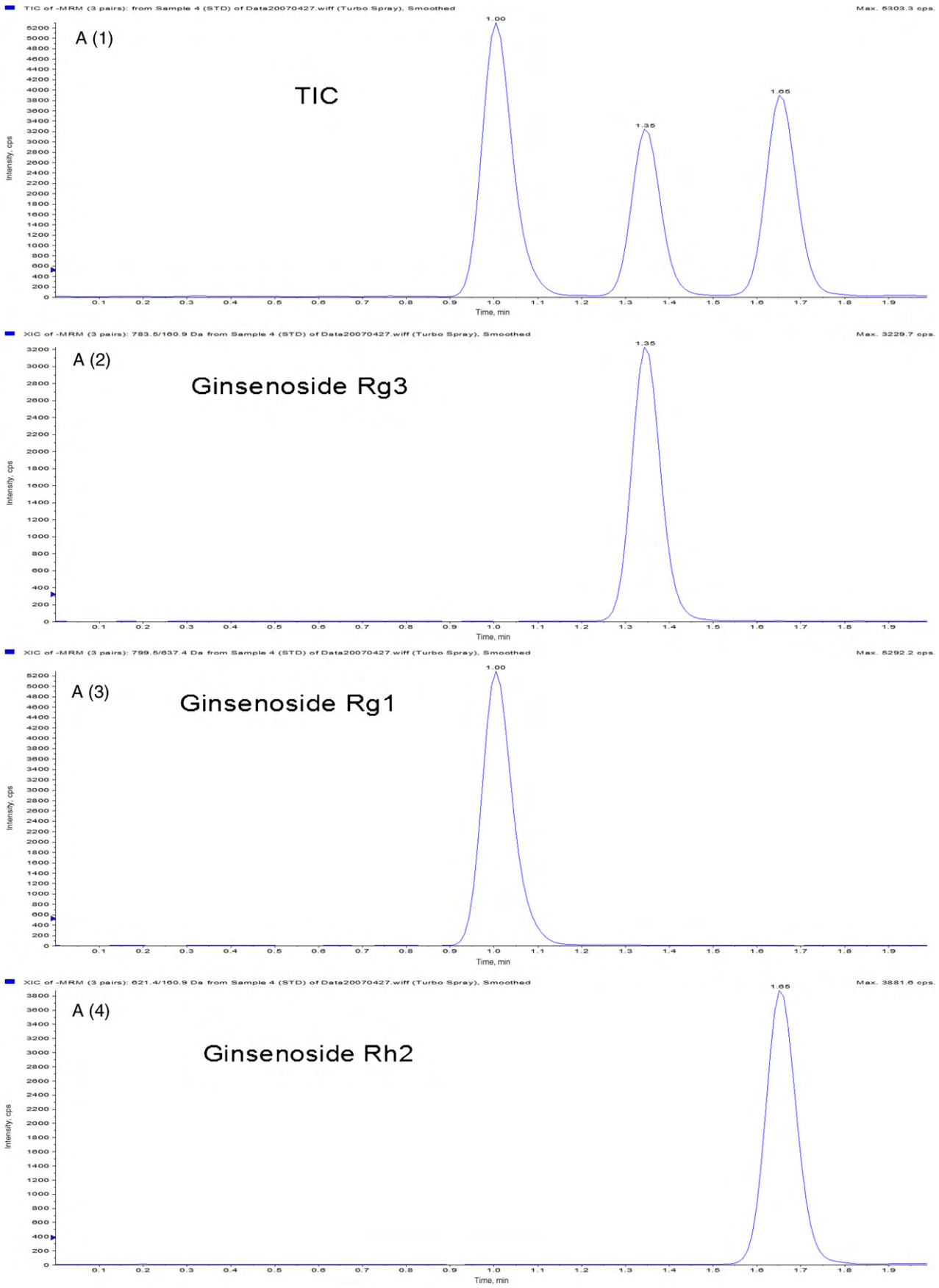

Fig. 2. Representative chromatograms of ginsenoside Rg3 in human plasma and urine. (A) Plasma containing 300 ng mL^{-1} ginsenoside Rg3, 600 ng mL^{-1} ginsenoside Rh2 and 300 ng mL^{-1} ginsenoside Rg1 standard; (B) blank plasma (blank urine is quite similar); (C) LLOQ sample of ginsenoside Rg3 (2.5 ng mL^{-1} for plasma, 2.0 ng mL^{-1} for urine) and (D) representative unknown study sample (plasma, 133 ng mL^{-1}).

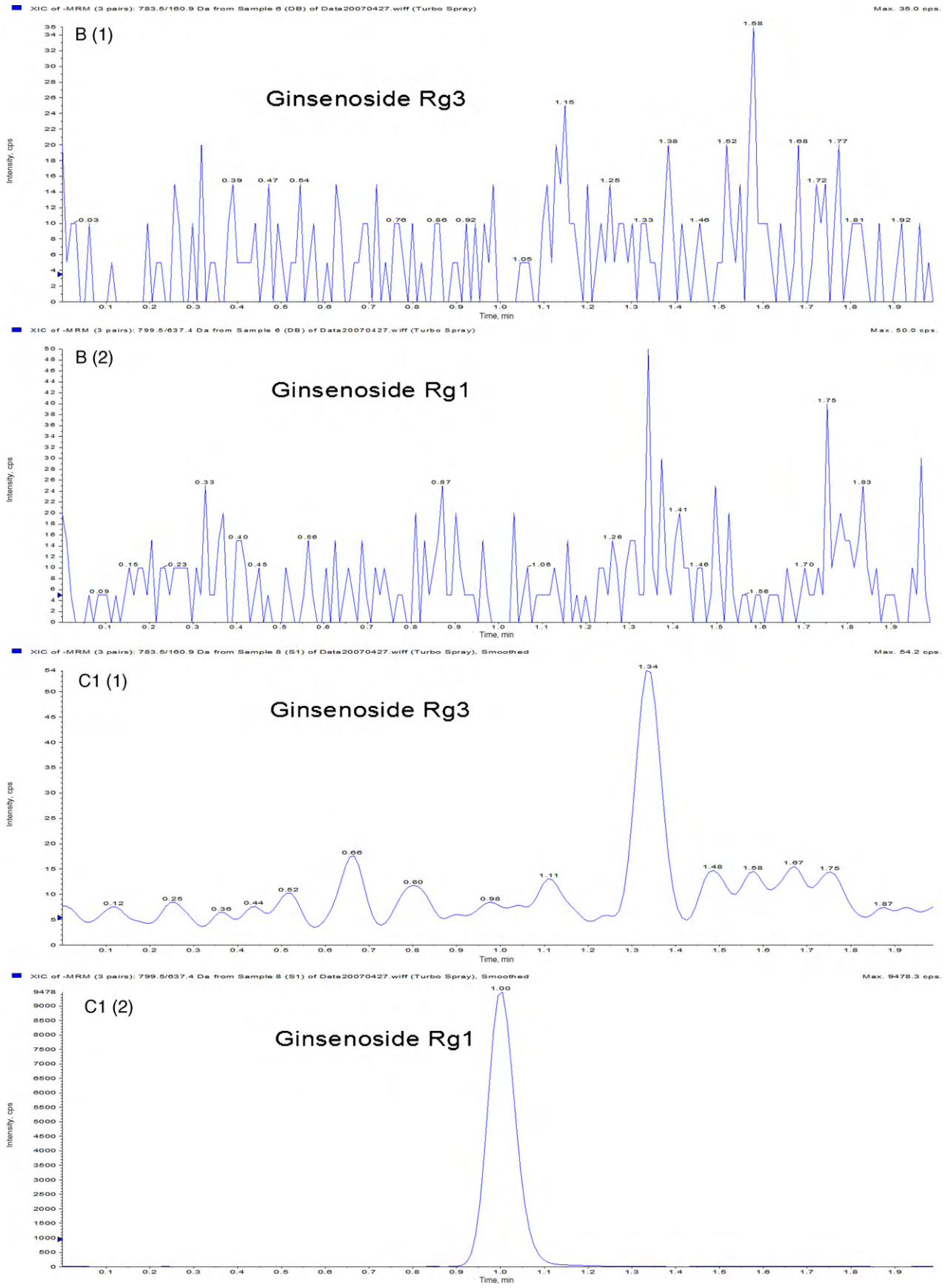

Fig. 2. (*Continued*).

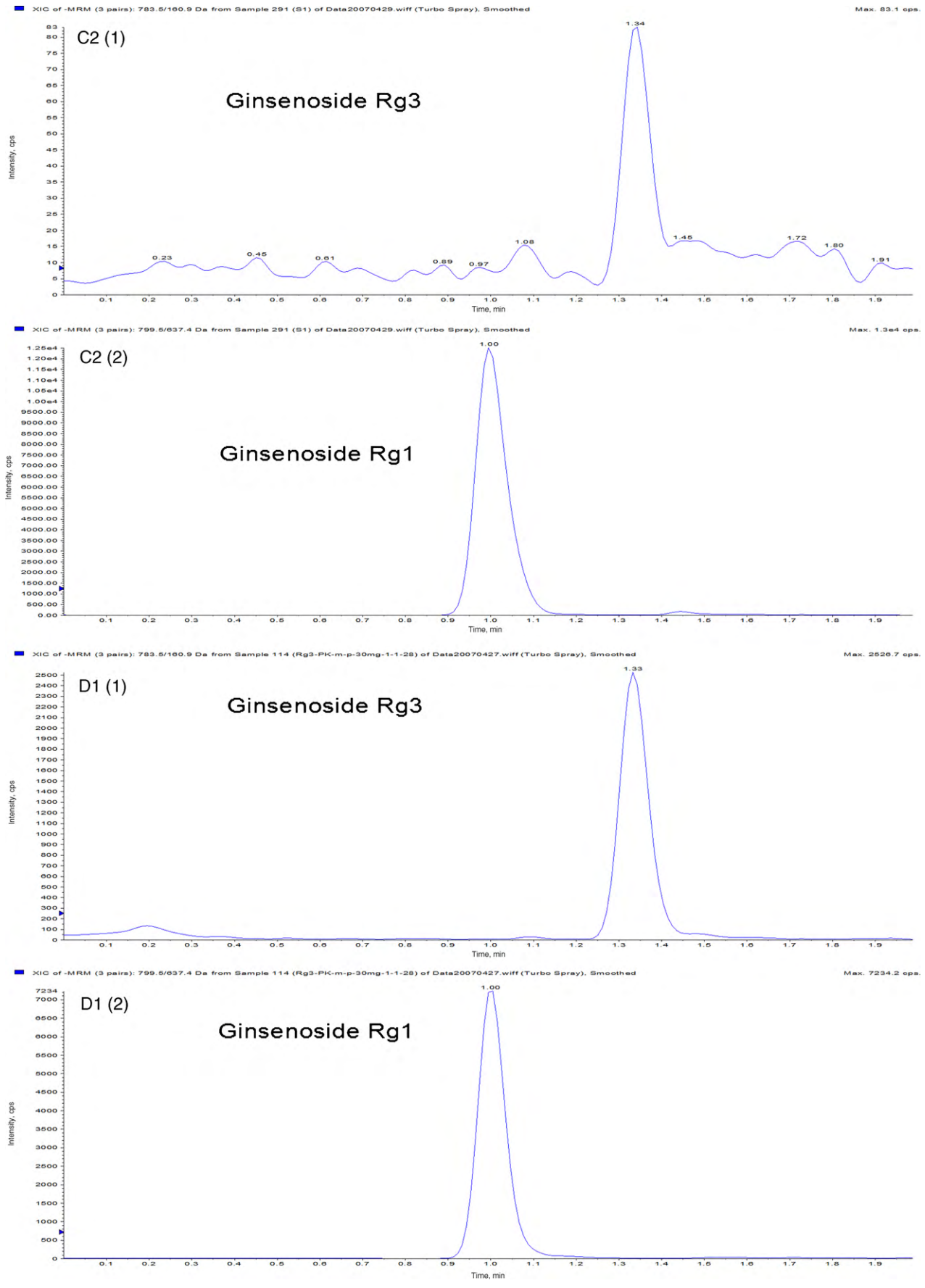

Fig. 2. (*Continued*).

Table 1
Intra- and inter-day accuracy and precision of quality control samples.

Theoretical conc. (ng mL^{-1})	Intra-day (n = 5)			Inter-day (n = 15)		
	Found conc. (mean ± SD, ng mL^{-1})	Precision (RSD%)	Accuracy (RE%)	Found conc. (mean ± SD, ng mL^{-1})	Precision (RSD%)	Accuracy (RE%)
Plasma						
7.5	7.4 ± 0.4	4.8	−1.4	7.6 ± 0.7	9.9	0.9
80.0	77.7 ± 5.5	7.0	−2.9	78.4 ± 9.3	11.8	−2.0
800.0	867.6 ± 115.7	13.3	8.5	762.7 ± 109.5	14.4	−4.7
Urine						
3.0	3.1 ± 0.3	8.9	+3.6	2.9 ± 0.3	11.7	−3.0
7.5	7.5 ± 1.0	13.3	−0.3	7.5 ± 0.8	10.4	+0.2
15.0	15.3 ± 1.2	8.0	+2.3	15.8 ± 1.6	9.9	+5.6

anticipated for study samples to data from the theoretical concentration after preparation.

2.5.4. Recovery

The recovery of ginsenoside Rg3 was calculated by direct comparison of the peak area of extracted three levels of QCs to the analyte spiked post extraction to the blank biological matrix at the same concentration.

2.5.5. Matrix effect

The matrix effect of ginsenoside Rg3 was evaluated with the ratio of the average peak areas obtained from analysis of extracted double blank plasma and urine which were spiked with ginsenoside Rg3 after extraction relative to the average peak areas of working solution. Plasma and urine from 6 health volunteers were prepared for every concentration levels of the QC samples.

The matrix effect of internal standard was evaluated with the ratio of the average peak areas obtained from analysis of extracted double blank plasma and urine with I.S. after extraction relative to the average peak areas obtained from working solution. Plasma and urine from 6 different donors were prepared for I.S. samples.

2.6. Data treatment

Concentrations were analyzed by Analyst (version 1.4.1) software. Descriptive statistics (mean, SD, RSD%, RE%) were calculated using Microsoft Excel 2003.

A weighted $1/x^2$ quadratic regression was used to determine slopes, intercepts and correlation coefficient, where y was the ratio of the compound peak area to the I.S. peak area and x was the concentration of the compound. The resulting ratios were used to calculate ginsenoside Rg3 from the following equation: $y = ax^2 + bx + c$.

3. Results and discussion

3.1. Specificity and selectivity

Fig. 2(A) shows a typical chromatogram for ginsenoside Rg3, ginsenoside Rh2 (potential metabolite of Rg3) and ginsenoside Rg1 standard. The blank plasma and urine from 6 lots not spiked with any analytes showed no peaks which would interfere with either ginsenoside Rg3 or ginsenoside Rg1 (I.S.), see Fig. 2(B). Fig. 2(D) shows a typical chromatogram for study sample in which all the analytes of interest were all baseline separated from each other,

Table 2
Stability of quality control samples (n = 5).

Stability tests	Time period	Theoretical conc. (ng mL^{-1})	Found conc. (mean ± SD, ng mL^{-1})	Precision (RSD%)	Accuracy (RE%)
Stock solution stability (−30 °C)	224 days	51,280 ± 2178[a]	49,120 ± 3859	7.9	−4.2
Plasma					
Freeze/thaw stability	3 times	7.5	7.5 ± 0.8	10.9	+0.2
(from −30 °C to 25 °C)		80.0	87.0 ± 8.3	9.5	+8.8
		800.0	801.4 ± 76.8	9.6	+0.2
Autosampler stability	24 h	7.5	7.8 ± 0.8	10.4	+4.0
(25 °C)		80.0	83.0 ± 4.4	5.3	+3.7
		800.0	710.2 ± 59.0	8.3	−11.2
Short-term stability	24 h	7.5	7.6 ± 0.7	9.8	+1.0
(25 °C)		80.0	77.9 ± 3.9	5.0	−2.7
		800.0	815.2 ± 25.7	3.2	+1.9
Long-term stability	4 months	7.5	7.2 ± 0.8	11.5	−3.6
(−30 °C)		80.0	76.6 ± 8.3	10.9	−4.3
		800.0	767.8 ± 50.7	6.6	−4.0
Urine					
Freeze/thaw stability	3 times	3.0	2.7 ± 0.2	6.2	−10.6
(from −30 °C to 25 °C)		7.5	7.5 ± 0.4	5.8	+0.4
		15.0	13.6 ± 0.8	5.7	−9.6
Autosampler stability	24 h	3.0	2.8 ± 0.3	10.5	−6.7
(25 °C)		7.5	8.2 ± 0.7	8.9	+9.5
		15.0	13.4 ± 1.2	8.8	−10.7
Short-term stability	24 h	3.0	3.0 ± 0.3	10.0	+0.8
(25 °C)		7.5	8.0 ± 0.8	10.6	+6.4
		15.0	13.8 ± 1.4	10.5	−8.1
Long-term stability	4 months	3.0	2.9 ± 0.2	5.7	−4.7
(−30 °C)		7.5	7.6 ± 0.2	6.8	+0.8
		15.0	14.6 ± 0.5	3.6	−2.5

[a] Fresh stock solution (mean ± SD).

the chromatograms are clean and with good resolution. The retention times were about 1.00 min and 1.35 min for ginsenoside Rg3 and I.S., respectively. The mobile phase with a high percentage of organics made the run-time as short as 2.0 min.

The presence of "cross-talk" between channels used for monitoring the analytes was evaluated by the analysis of plasma/urine samples containing Rg1 at the working concentration in the absence of Rg3 and the analysis of plasma/urine samples containing Rg3 at the upper limit of quantitation (ULOQ, 1000 ng mL^{-1}) in the absence of Rg1. No "cross-talk" was observed in either experiment.

To ensure the selectivity of the chromatogram, ginsenoside Rh2 (loss of one glucose from ginsenoside Rg3), the potential metabolite of ginsenoside Rg3 were tested, they were baseline separated from each other, avoiding the effect on the determination of ginsenoside Rg3 induced by in source collision, see Fig. 2(A).

3.2. Relative sensitivity

The lower limit of quantitation (LLOQ) was defined as the lowest standard level, which meets the acceptance criteria in accuracy and precision of 9.7% RSD% and 6.7% RE% for plasma and 3.1% RSD% and 1.4% RE% for urine. The LLOQs were set at 2.5 ng mL^{-1} for plasma (S/N = 8) and 2.0 ng mL^{-1} for urine (S/N = 12). Fig. 2(C) presents a typical chromatogram of a spiked plasma and urine sample containing 2.5 ng mL^{-1} and 2.0 ng mL^{-1} ginsenoside Rg3, respectively.

Different ionization model (positive and negative ion ionization), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) were selected to detect the ginsenoside Rg3 and Rg1. The result showed that there had no significant difference on sensitivity between two kinds of ion sources, and the negative ion ionization model had the higher efficiency for ionization because of the glucosyl group of Rg3 and Rg1. Finally, the ESI$^-$ was used as the interface in this method. It had consistent response to the analytes under the MRM mode and could offer better sensitivity.

3.3. Linearity

Linearity was established in the range 2.5–1000 ng mL^{-1} for plasma and 2.0–20.0 ng mL^{-1} for urine. All curves had correlation coefficients greater than or equal to 0.996. Table 2 shows inter-day precision and accuracy for each standard concentration.

The linearity range and LLOQ for plasma were decided to be ideal to cover the clinical samples from pharmacokinetic studies. The highest Rg3 concentration from healthy volunteers after intramuscular injecting a single dose of 10 mg, 30 mg or 60 mg ginsenoside Rg3 was 754.0 ng mL^{-1}. The concentration level of LLOQ was lower than 1/10–1/20 of C_{max} and the determined concentration of biological samples after 3–5 times $t_{1/2}$ of intended study drug.

The concentrations of Rg3 in most urine samples were from 3 ng mL^{-1} to 17 ng mL^{-1}, and the highest concentration from volunteers after dosing was 21.9 ng mL^{-1}. The lower limit of standard curve in urine was not low enough to analyze all samples from pharmacokinetic study, but considering the low ratio of extraction from urine of Rg3 (<1%), it was not necessary to set-up a more sensitive method for urine samples.

3.4. Precision and accuracy

Table 1 displays the inter- and intra-day precision and accuracy of three quality control levels for plasma and urine. The data shows that this HPLC/MS/MS method is very consistent and reliable with overall good RSD% and low RE%.

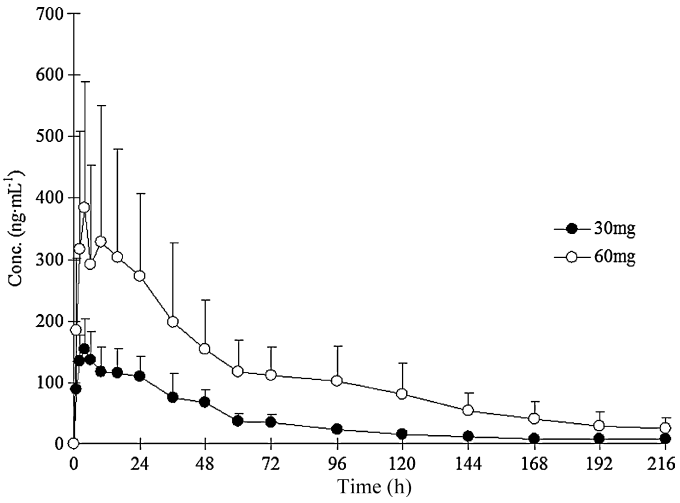

Fig. 3. . Pharmacokinetic profile of 30 mg and 60 mg ginsenoside Rg3 by intramuscular injected (n = 16).

3.5. Stability

Stabilities of stock solution storage and sample in biological matrix (freeze/thaw cycles, autosampler storage, short-term and long-term storage) were established during the method validation. The stability of ginsenoside Rg3 stock solution was tested for 224 days at −30 °C. Ginsenoside Rg3 was stable when stored under the following conditions: for 131 days at −30 °C, for 24 h at room temperature (25 °C), after 3 freeze/thaw cycles (from −30 °C to 25 °C), and for 24 h in the autosampler at 25 °C after sample preparation for plasma samples; for 152 days at −30 °C, for 24 h at room temperature (25 °C), after 3 freeze and thaw cycles (from −30 °C to 25 °C), and for 24 h in the autosampler at 25 °C after sample preparation for urine samples. The data is presented in Table 2.

3.6. Recovery

A simple one-step extraction was introduced to extract analytes from human plasma and urine. Ginsenoside Rg3 recovery was 41.0 ± 1.1%. All recoveries had RSD% less than 2.62% throughout the low, mid and high concentration plasma standards, showing great consistency.

3.7. Matrix effect

No apparent matrix effect was found to affect assay precision for ginsenoside Rg3 and internal standard. The mean matrix effect of ginsenoside Rg3 and Rg1 was 105.2% (ranged from 96.5% to 114.3% for different QC levels, RSD% was 8.5%) and 103.5% for plasma sample; and 103.4% (ranged from 92.5% to 111.9% for different QC levels, RSD% was 9.6%) and 98.1% for urine samples, respectively.

4. Clinical application

This HPLC/MS/MS method was used to evaluate the pharmacokinetic profiles of ginsenoside Rg3 in human plasma and urine successfully. Twenty-four healthy male Chinese volunteers were enrolled. All subjects were intramuscular injected a single dose of 10 mg, 30 mg or 60 mg ginsenoside Rg3, and subsequently sampled at specific time points. Drawn blood was heparinized, centrifuged, and the plasma was collected. A plot of ginsenoside Rg3 concentrations (mean ± SD) in plasma versus time after treatment is shown in Fig. 3. No significant problems such as pressure ascend, shift in retention times and interferences were observed with columns

being used continuously for at least 720 plasma samples and 170 urine samples during the bioanalysis.

5. Conclusion

An HPLC/MS/MS method with ESI interface was developed and validated for the quantitative determination of ginsenoside Rg3 in human plasma and urine. The sensitivity of ginsenoside Rg3 in plasma and in urine was suitable for pharmacokinetic studies of ginsenoside Rg3 formulations.

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