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Simultaneous rapid quantification of ginsenoside Rg_1 and its secondary glycoside Rh_1 and aglycone protopanaxatriol in rat plasma by liquid chromatography–mass spectrometry after solid-phase extraction

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Abstract

Ginseng saponins isolated from ginseng, have been regarded as the principal constituents responsible for the biological activities. The aim of this study was to set up a liquid chromatography–mass spectrometry (LC–MS) method for simultaneously determine the concentration of Ginsenoside Rg_1 and its secondary glycoside Rh_1 and aglycone protopanaxatriol (PPT) in rat plasma so as to study the pharmacokinetics of Rg_1 after intraveneous (i.v.) and intragastric gavage (i.g.) administration. One hundred microliters or 1.0 ml of rat plasma samples from i.v. or i.g. treated rats were used respectively for analysis. After solid-phase extraction (SPE) and high performance liquid chromatography (HPLC) separation, the chloride adduct anions $[M + Cl]^-$ of Rg_1, Rh_1 and PPT were analyzed by LC–MS in selected ions monitoring (SIM) mode. Rg_1 could be determined by this LC–MS method over the ranges of 1.56–250 ng/ml and 250–20,000 ng/ml with the correlation coefficients of 0.999 and 0.9998, respectively. The detection limits (LOD) of this method was 20 pg (S/N>3) for Rg_1, 100 pg for Rh_1 and 10 pg for PPT. Chromatographic separation was achieved in less than 8 mins. The method has been used for the pharmacokinetic study of Rg_1 in rats.

Keywords: Saponin; Ginsenosides; Rg_1; Rh_1; PPT; LC–MS; SPE

1. Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), has been used in China for more than 4000 years and now is widely used all around the world. Ginseng saponins isolated from ginseng, have been regarded as the principal constituents responsible for the pharmacological activities [1,2]. More than 30 different ginsenosides have been identified and can be divided into two groups according to their sapogenins with a dammarane skeleton, protopanaxatriol (PPT) and protopanaxadiol (PPD) groups except for ginsenoside Rg_0 (the only oleanolic acid-type saponin identified in the roots of *P. ginseng*)[3]. Ginsenoside Rg_1 (one of the active components of *P. ginseng*, see Fig. 1), is known to have several physiological effect including the promotion of activity DNA, protein and lipid synthesis in animal bone marrow cells, the stimulation of central nervous system, the increasing of blood pressure, the increase of initial learning performance and anti-fatigue activity, and is a "functional ligand" for glucocorticoid receptors. [3,4].

Several studies report the analysis of gisenosides. The analytical methods for determining ginsenosides featuring thin layer chromatography (TLC) [5–7], enzyme immunoassay (EIA) [8–10], high performance liquid chromatography with ultraviolet detection (HPLC–UV), high performance liquid chromatography with fluorescence detection (HPLC–FLD) [11], liquid chromatography–evaporative light-scattering detection (LC–ELSD) [12,13], and LC–MS[14] and liquid chromatography-tadem mass spectrometry (LC–MS/MS) [15,16] were published. The TLC method involves a tedious sample cleanup procedure, complex developing solvents sys-

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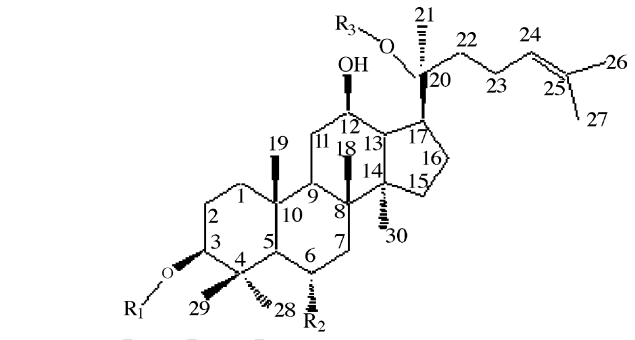

	R_1	R_2	R_3
Rg1:	-H	-O-Glc[1)]	- Glc
Rh1:	-H	-O-Glc	-H
PPT:	-H	-OH	-H

1) β-D-glucopyranosyl,

Fig. 1. Structure of ginsenoside Rg_1, Rh_1 and PPT.

tem and low sensitivity. The HPLC–UV method has a poor sensitivity of 0.1 μg/ml of Rg_1 in water solution at wavelength of 203 nm, according to the experimental data in our laboratory (data not shown). The HPLC–FLD method involves a tedious procedure of sample pretreatment and derivatization. Some researchers [16] have dealt with the qualitative detection of Rg_1 by LC–MS using positive ion mode which is not suitable for the quantification of Rg_1 in biological fluids due to the insufficient sensitivity. Other study [15] reports the negative ion mode for qualitative study but so far, little information appears to be available on the quantification of Rg_1 in bio-fluids by LC–MS. Due to the increasing requirements of the pharmacokinetic research of traditional Chinese medicine (TCM) in vivo, we intend to develop a LC–MS method sensitive enough for the pharmacokinetic study of Rg_1 and its secondary glycoside and aglycone in biological fluid samples. The method was validated in rat plasma by spiking the rat plasma with pure standards. This method was also successfully extended to analyze other ginsenosides, as well as some glycosides from herbs, such as astragaloside IV, icariin, digitoxin, etc. in plasma, with high sensitivity.

2. Experimental

2.1. Chemicals and reagents

The reference standard of ginsenoside Rg_1, and internal standard digoxin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and secondary glycoside Rh_1 and aglycone PPT were from Bai Qiu-En Medical University (Changchung, Jilin, China). HPLC-grade methanol and acetonitrile were from Fisher Scientific (Fair Lawn, New Jersey, USA). Solid-phase cartridge was from Waters Chromatography (HLB 1cc, Oasis™, Waters, USA). Dimethyl sulfoxide (DMSO) was from Nanjing Chemistry Company (Nanjing, China). Milli-Q water was generated by passing distilled water through Quantum EX ultrapure organex cartridge (Cat No. QTUM 000EX).

Table 1
Composition of mobile phase with gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)	Gradient curve
0.0	75	25	–
1.5	25	75	Linear
3	25	75	–
3.5	10	90	Linear
5	10	90	–
5.01	75	25	Linear
6.5	75	25	–

A: 0.1 mmol/L ammonium chloride; B: acetonitrile.

2.2. Liquid chromatograph–mass spectrometry (LC–MS)

The HPLC system consisted of a DGU-14 AM degasser, two Shimadzu 10ADvp Pump, a high pressure mixer, a CTO-10Avp column oven and a Shimadzu 10ATvp Autoinjector (Shimadzu, Kyoto, Japan). A Shimadzu 2010 liquid chromatograph–mass spectrometer (Shimadzu, Kyoto, Japan) equipped with ESI (Electrospray Ionization) probe, QOQ system (Q-array-Octapole-Quadrupole mass analyzer) was used in the study. The analysis was carried on a Chromasil C18 analytical column (5 μm particle size, 150 mm × 2.1 mm i.d., Metachem, USA), equipped with C18 guard column (4 mm × 2.0 mm i.d., Security Guard, Phenomenex, USA), at a flow-rate of 0.2 ml/min. The isocratic mobile phase of acetonitrile-Milli-Q water (75:25), containing 0.1 mmol/L of ammonium chloride was used for method optimization. A step gradient of (A) 0.1 mmol/L ammonium chloride, and (B) acetonitrile was used for the validation and the final analysis of plasma samples (Table 1).

Mass spectrometric conditions were optimized to obtain maximum sensitivity. The final ESI conditions used was as follows: curve dissolution line (CDL) voltage was fixed as that in tuning and probe high voltage was set at 4.5 kV, Q-array voltage −35 V and RF 150 V. Mass spectra were obtained at a dwell time of 0.2 s in SIM mode and 1 s in scan mode. Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing Univesity, China) was used as the nebulizing gas at 4.5L/min. LCMass Solution Version 2.02 worked on Windows 2000. Vacuum in the Mass detector was obtained by Turbo molecular pump (Edwards 28, England).

2.3. Preparation of stock solutions, calibration samples and validation samples

Stock solutions for ginsenoside Rg_1, Rh_1 and PPT were prepared in DMSO separately. Working solutions were prepared by Milli-Q water before experiment, by mixing known amount of Rg_1, Rh_1 and PPT together. Calibration samples were prepared using drug-free rat plasma spiked with known amount of Rg_1, Rh_1 and PPT all-in-one (Rats were sacrificed by bleeding from the femoral artery after anesthetized

by ether. The blood was gathered into heparinized tube and plasma was separated by centrifugation). Absolute extraction recoveries of Rg_1, Rh_1 and PPT from rat plasma spiked with different amount of Rg_1, Rh_1 and PPT were calculated by comparing the peak area of samples in plasma after solid phase extraction (SPE) procedure with those of samples in water without SPE. The calibration samples and validation samples were processed exactly the same way as unknowns.

2.4. Sample pretreatment

Plasma samples were thawed in thermostatic water bath at 37 °C and thoroughly vortex shaken for 30 s. One hundred micro-liters or 1.0 ml of plasma samples from i.v. or i.g. treated rats were used, respectively. Twenty micro-liters of internal standard (20 μg/ml) were added to the plasma samples and vortex shaken for 20 s. The samples from i.v. group were diluted to 1 ml by Milli-Q water and then loaded onto the preconditioned SPE cartridge (The cartridge was preconditioned with 1 ml methanol followed by 2 ml Milli-Q water). The samples from i.g. group were diluted two-fold by Milli-Q water and loaded onto the cartridge. The cartridge was then rinsed by 4 ml Milli-Q water, simply dried by passing 5 ml air, and then eluted with 1 ml of 95% methanol. The eluates were then centrifuged at 21,000 × *g* for 10 min (Micromax RF, Thermo IEC, USA). Ten microliters of the supernatant was used for analysis.

2.5. Validation of the assay

The method was validated in rat plasma after solid phase extraction. Samples were spiked with different amount of Rg_1, Rh_1 and PPT. The linearity was studied over the ranges of 1.56–250.0 ng/ml, 6.25–500.0 ng/ml and 0.78–250.0 ng/ml for Rg_1, Rh_1 and PPT, respectively, when 1.0 ml of plasma was used. The linearity ranges of 250.0–10000.0 ng/ml for Rg_1, Rh_1 and PPT were studied when 100 μl of plasma was used for the analysis. Precision of the assay was calculated as the relative standard deviation (RSD). The intra-day precision and accuracy were evaluated for Rg_1, Rh_1 and PPT at three different plasma concentrations by analysis of five spiked control plasma samples on the same day. Inter-day variation was evaluated by analyzing of spiked control plasma samples stored at −20 °C and analyzed with each run over 5 days. The extraction recovery was studied at four concentration levels by comparing the area of interested peak of the sample clean-up procedures relative to aqueous solutions. The freeze and thaw stability study of Rg_1, Rh_1 and PPT was carried out in triplicate at three concentration levels (62.5, 500, 5000 ng/ml).

2.6. Application

The assay has been applied in the pharmacokinetic studies of Rg_1 in rats after i.v. or i.g. administration of Rg_1 in 0.9% saline. Rats were fasted for 12 h before dosing, with free access to water. For i.v. experiments, a cannula was inserted into carotid artery of the rat under slight anesthesia by ether before dosing. A 0.4 mg/ml solution of Rg_1 in saline at a dose of 1 mg/kg was given to the rat via the femoral vein. Blood samples (ca 250 μl) were collected prior to and at 2, 5, 10, 15, 30 min, 1, 2, 4, 6, 8 h after i.v. dosing into heparinized Eppendoff tubes and mixed gently, and then centrifuged at 1200 × *g* at room temperature for 5 min using a centrifuge (Micromax RF, Thermo IEC, USA). Plasma (100 μl) was separated and frozen at −20 °C until analysis. For i.g. experiments, rats were sacrificed by bleeding at 5, 15, 30 min, 1, 1.5, 2, 3, 8 h after i.g. administration of Rg_1 at dosage of 20 mg/kg. Blood was collected into heparinized disposable plastic tubes and mixed gently, then centrifuged at 1200 × *g* at room temperature for 5 min using a centrifuge (Model 0412-1, Shanghai Surgical Instruments Factory, Shanghai) to obtain 1.0ml plasma, which was kept at −20 °C until analysis.

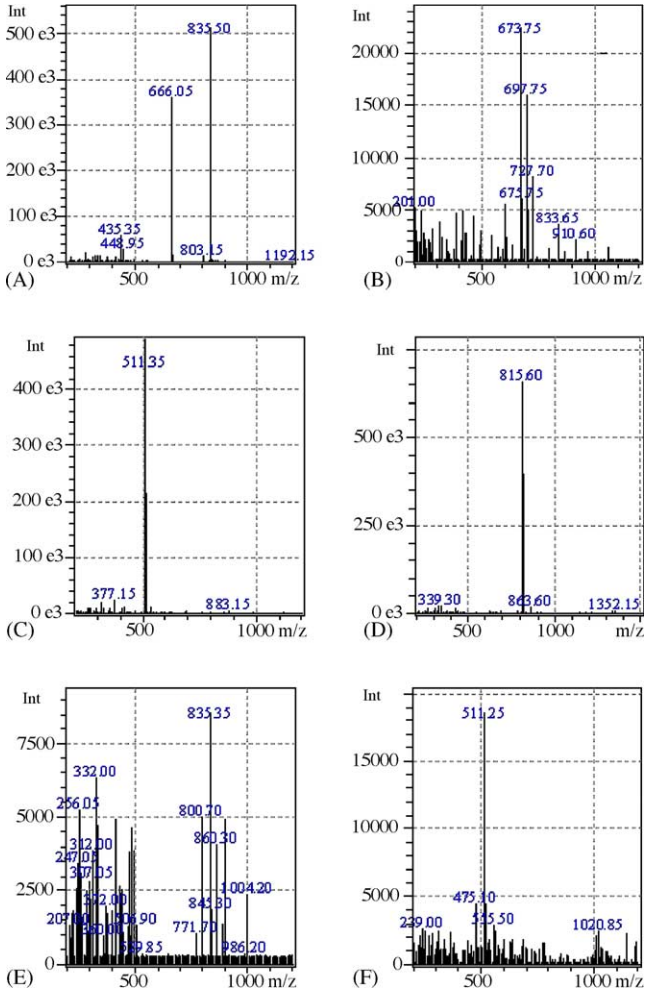

Fig. 2. Mass spectra of Rg_1 (A), Rh_1 (B), PPT (C), and internal standard digoxin (D), obtained when using isocratic mobile phase of 0.1 mmol/L ammonium chloride–acetonitrile (25:75). And mass spectra of Rg_1 (E), PPT (F), obtained when using isocratic mobile phase of Milli-Q water–acetonitrile (25:75). See Section 2.2 for mass scan conditions.

3. Results and discussions

3.1. Specificity, linearity and sensitivity

Resolution, sensitivity and mass number calibration were optimized by auto tuning program using polyethylene glycol mixture, before analysis. Fig. 2 shows mass spectra of ginsenoside Rg_1 and its secondary glycoside Rh_1 and aglycone PPT. In the mass spectra, negative adduct ions $[M + Cl]^-$ were observed at *m/z* 835.50, 673.75 and 511.35, for Rg_1, Rh_1 and PPT, respectively. Sensitivity of the method was further improved by addition of ammonium chloride to the mobile phase. Since the LOQ of Rg_1 detected was 100 pg in deprotonated ion mode$[M - H]^-$ and 12.5 pg in adduct ion mode $[M + Cl]^-$ (Fig. 3), we used the latter mode for validation in the following research.

This method was applied to determination of several kinds of saponins including Rg_1 and Rh_1, PPT. The specificity of the method was evaluated by screening blank plasma of different rats in selected ions monitoring (SIM) mode. The chromatography achieved by the current method showed a low baseline noise and complete separation between the interested compounds and the interferences (Fig. 4). The peak area ratios of interests/internal standard were linearly related to amounts over the ranges of 1.56–250.0 ng/ml, 6.25–500 ng/ml and 0.78–250 ng/ml for Rg_1, Rh_1, and PPT, respectively. Good linearity for Rg_1, Rh_1 and PPT was confirmed over the assay range of 250–10,000 ng/ml, when 1 ml plasma was used for analysis (Table 2). The LOQ was 20 pg for Rg_1, 40 pg for PPT, and 100 pg for Rh_1.

For the plasma samples, sensitivity could be further improved by concentrating the eluate of SPE. After evaporated

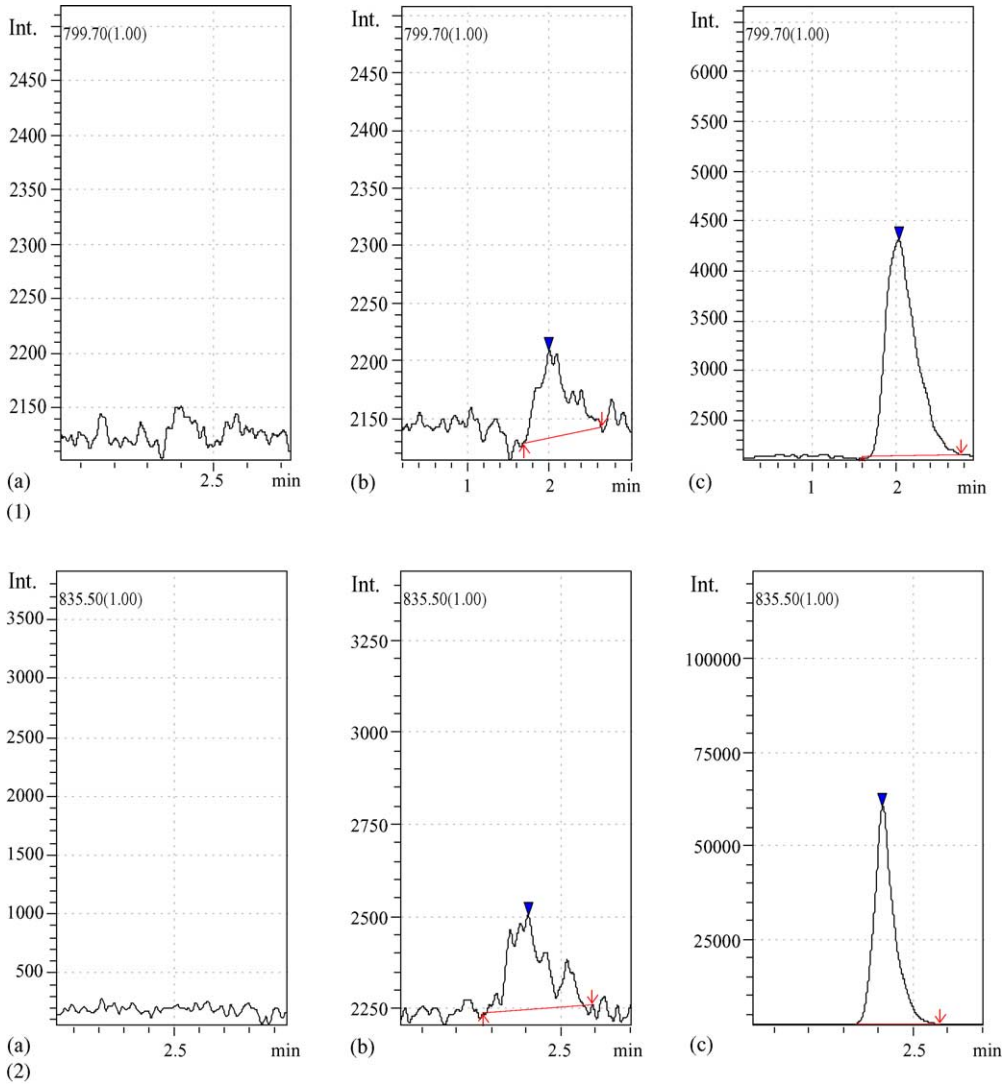

Fig. 3. (1) SIM Chromatograms of Rg_1 in deprotonated ion mode $[M - H]^-$: control (a), standard of Rg_1 in water (10 ng/ml) (b), standard of Rg_1 in water(500 ng/ml) (c). Separation was carried out with an isocratic mobile phase of Milli-Q water–acetonitrile (25:75); (2) MS chromatograms of Rg_1 in adduct ion mode $[M + Cl]^-$: control (a), standard of Rg_1 in water (1.25ng/ml), (b) standard of Rg_1 in water (200 ng/ml), (c). Separation was carried out with an isocratic mobile phase of Milli-Q water–acetonitrile (25:75).

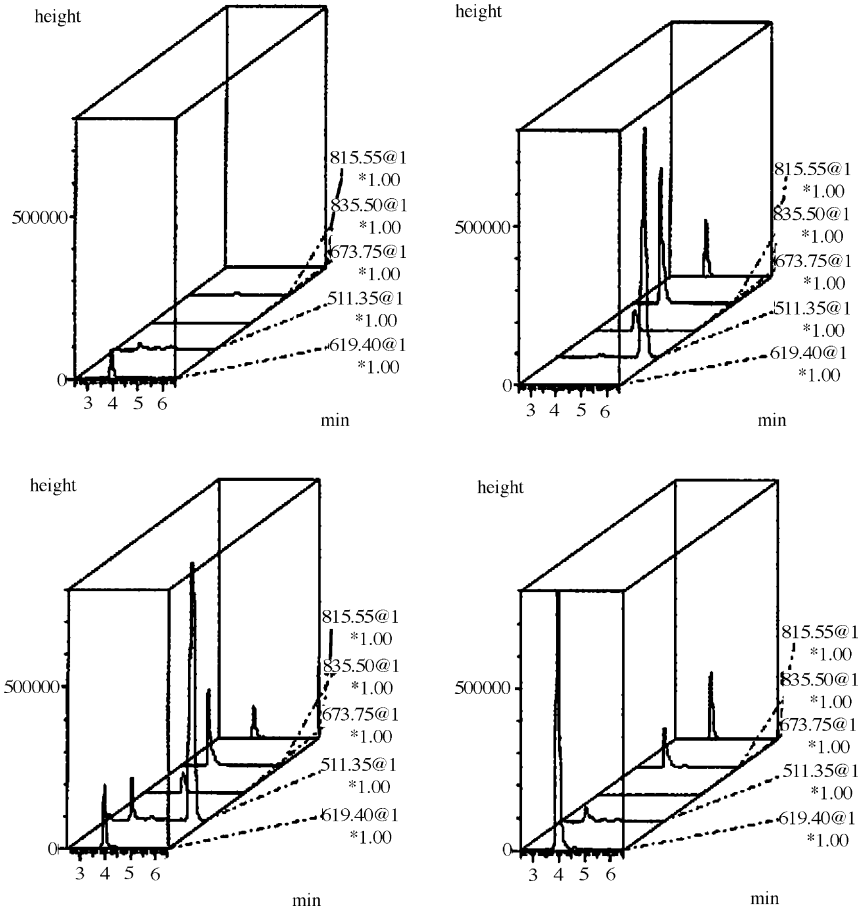

Fig. 4. SIM Chromatograms of blank plasma (a), standard of Rg_1, Rh_1 and PPT (b), plasma spiked with 500 ng/ml of ginsenoside Rg_1, internal standard, 500 ng/ml of Rh_1, and 1000 ng/ml of PPT (c), plasma sample from rat at 1.5 h after drug administration (d). *m/z* 815.65, 835.50, 673.75, 511.35, 619.40 for Rg1, internal standard, Rh1, PPT, and unidentified, respectively. See Section 2.2 for mass condition and Table 1 for HPLC condition.

to dryness and reconstituted by mobile phase, the concentration of the sample can be condensed 10-fold around and thus the sensitivity of the method improved.

3.2. Precision and accuracy

Results show that good precision and accuracy were achieved using the current method. Table 3 shows the inter-day, intra-day assay precision and accuracy results for Rg_1, Rh_1 and PPT. The intra- and inter-assay precision were less than 12.3, 13.5 and 10.8% for Rg_1, Rh_1 and PPT, respectively.

3.3. Recovery

The absolute extraction recoveries of ginsenoside Rg_1 and Rh_1 and PPT from rat plasma were estimated at four concentration levels (Table 3). Since the sample loading speed was the crucial step in SPE, we diluted the plasma samples prior to sample loading in order to facilitate the interaction of the interests with the active bonds on surface of the solid phase, resulted in extension of the loading time. Rg_1 can be eluted by methanol and acetonitrile in the same efficient way, though, Rh_1 and PPT was eluted more efficiently by 95% methanol. A 95% methanol solution was thus chosen as the

Table 2
Regression statistics for the calibration curves ($n = 5$)

Analytes	Amount added (ng/ml)	Slope $m \pm S_m$	Intercept $m \pm S_m$	r
Rg1	1.56–250.0	$7.65 \times 10^{-4} \pm 2.9 \times 10^{-5}$	$1.3 \times 10^{-3} \pm 5.8 \times 10^{-4}$	0.998
	250.0–10000.0	$9.9 \times 10^{-4} \pm 2.4 \times 10^{-5}$	$1.7 \times 10^{-2} \pm 3.1 \times 10^{-2}$	0.999
Rh1	6.25–500.0	$4.2 \times 10^{-4} \pm 2.5 \times 10^{-4}$	$2.7 \times 10^{-3} \pm 1.6 \times 10^{-3}$	0.997
	250.0–10000.0	$2.89 \times 10^{-3} \pm 5.8 \times 10^{-6}$	$1.2 \times 10^{-2} \pm 6.2 \times 10^{-3}$	0.999
PPT	0.78–250.0	$3.4 \times 10^{-3} \pm 1.3 \times 10^{-4}$	$-7.4 \times 10^{-3} \pm 2.7 \times 10^{-3}$	0.998
	250.0–10000.0	$5.3 \times 10^{-3} \pm 1.6 \times 10^{-4}$	$-4.5 \times 10^{-1} \pm 2.0 \times 10^{-1}$	0.999

Table 3
Recovery, accuracy and precision of Rg1, Rh1 and PPT assay in rat plasma ($n = 5$)

Analytes	Amount added (ng/ml)	Amount found (ng/ml)	Recovery (%)	Within-day R.S.D. (%)	Between-day R.S.D. (%)
Rg1	3.12	3.42	85.1 ± 10.1	11.4	12.3
	31.2	32.1	81.3 ± 5.2	6.4	9.8
	500.0	498.2	78.6 ± 3.1	3.9	6.1
	5000.0	4993.2	80.8 ± 2.9	2.8	5.2
Rh1	12.5	11.9	88.5 ± 11.2	12.7	13.5
	125.0	124.2	85.4 ± 6.8	8.0	8.4
	500.0	502.5	86.8 ± 3.9	4.5	6.3
	5000.0	4988.2	83.7 ± 3.8	4.0	5.8
PPT	3.12	2.98	95.1 ± 9.2	9.7	10.8
	31.2	30.7	93.4 ± 8.7	8.9	9.2
	500.0	504.2	98.2 ± 4.5	4.6	6.9
	5000.0	5021.0	96.8 ± 2.8	2.9	7.1

eluent for validation study. The recovery was about 78.6–85.1 %, 85.4–88.5%, 93.4–98.2% for Rg_1, Rh_1 and PPT, respectively.

3.4. Freeze and thaw stability of Rg_1 in rat plasma

The stability in rat plasma after freeze and thaw procedure was studied by storing the plasma spiked with known amount of Rg_1, Rh_1 and PPT separately in −20 °C and then thawed at 37 °C after 1 week. The samples were then processed the same way as the procedure of rat plasma and analyzed by LC–MS. The results showed that Rg_1, Rh_1 and PPT were stable under the present storage and preparation conditions (Table 4).

3.5. Effect of mobile phase on the sensitivity of LC–MS in detecting saponins

Saponins were mostly detected as the protonated or cationized ions in positive mode [16–18] and only few references [18,19] were found to deal with the negative mode. The study of Xu on negative ions of ginsenoside was limited to the qualitative study rather than the quantification [18]. In their study, ammonium acetate was used as mobile phase additive to get $[M + CH_3CO_2]^-$ adducts, which was proven to be unstable and influenced greatly by minor changes of mobile phase. The LC–MS method we developed focused on the quantification study. The interferences in negative mode were much less than that in positive mode in LC–MS detection of ginsenoside according to our research. Since the response of deprotonated ion $[M - H]^-$ was low (Fig. 2.), and the addition of ammonium chloride could enhance the formation of chloride adduct of the ginsenoside molecule $[M + Cl]^-$, the present mobile phase (0.1 mmol/L of ammonium chloride in the water phase) was used and thus a higher sensitivity was obtained. The LOQ of Rg_1 in the present study is 20 pg.

Table 4
Freeze and thaw stability study ($n = 3$)

Amount added (ng/ml)	Amount found (ng/ml)		
	Rg1	Rh1	PPT
62.5	59.6 ± 2.9	62.4 ± 2.5	59.5 ± 2.4
500.0	498.0 ± 8.0	502.6 ± 6.1	503.3 ± 6.2
5000.0	4989.4 ± 30.1	4996.2 ± 30.8	5023.6 ± 84.1

3.6. Effect of drug adsorption to surface of glass vial on the signal intensity

During our research, we found that the signal of the sample in water solution dropped significantly during the analysis. We owed it to the adsorption of Rg_1, Rh_1, PPT and the internal standard by the active surface of glassware. This disadvantage could be effectively eliminated by changing the solvent with 90% acetonitrile.

3.7. Pharmacokinetic study

Previously, the pharmacokinetic study was reported on ginsenoside Rg_1 (100mg/kg) as a representative of

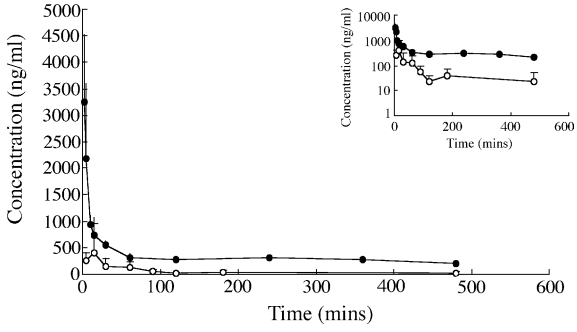

Fig. 5. Mean plasma concentration–time profiles of ginsenoside Rg_1 in rats after i.v. (solid circle) or i.g. (hollow circle) administration of Rg_1 at dosage of 1 mg/kg ($n = 3$), 20 mg/kg ($n = 5$), respectively. For i.v. experiment, blood was collected according to time from the carotid artery in one rat under anesthetic. For i.g. experiment, rats were sacrificed by bleed under ether anesthetic at different time and blood was collected. The small figure represents the semi-logarithm scale.

protopanaxatriol-type ginsenosides [20]. Ginsenoside Rg_1 was absorbed rapidly from the upper parts of the digestive tract in rats. According to our research, the plasma concentration of Rg_1 decreased quickly after intravenous injection. Fig. 5 shows the concentration–time profiles of Rg_1 in rats after i.v. (1 mg/kg) and i.g. (10 mg/kg) administration. The profile of i.v. fit a three-compartment pharmacokinetic model. Rg_1 can be absorbed in rat after i.g. administration. The absolute bioavailbility of Rg_1 after i.g. is about 1.33%. Neither Rh_1 nor PPT was found in the rat blood after i.v. or i.g. treated with Rg_1.

4. Conclusions

A specific, sensitive, rapid, precise and accurate LC–MS method for the simultaneous determination of Rg_1, Rh_1 and PPT after solid phase extraction was developed and validated, suitable for quantification in pharmacokinetics research. The analysis was finished within 8 min. The assay has been used for the analysis of plasma samples obtained from rats after i.v. and i.g. administration of Rg1.

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