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High performance liquid chromatographic–mass spectrometric determination of ginsenoside Rg3 and its metabolites in rat plasma using solid-phase extraction for pharmacokinetic studies

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Abstract

To support pharmacokinetic studies of ginsenosides, a novel method to quantitatively analyze ginsenoside Rg3 (Rg3), its prosapogenin ginsenoside Rh2 (Rh2) and aglycone 20(S)-protopanaxadiol (ppd) in rat plasma was developed and validated. The method was based on gradient separation of ginsenosides present in rat plasma using high performance liquid chromatography (HPLC), followed by detection with electrospray ionization(ESI) mass spectrometry (MS) in negative ion mode with the mobile phase additive, ammonium chloride (500 μM). Differentiation of ginsenosides was achieved through simultaneous detection of the $[M^+Cl^-]$ adduct of ginsenoside Rg3 and $[M^+Cl^-]$ adducts of its deglycosylated metabolites Rh2 and ppd, and other ions after solid phase extraction (SPE). The /specific ions monitored were *m/z* 819.50 for Rg3, *m/z* 657.35 for Rh2, *m/z* 495.40 for ppd and *m/z* 799.55 for the internal standard (digitoxin). The mean recoveries for Rg3, Rh2 and ppd were 77.85, 82.65 and 98.33%, respectively using 0.1 ml plasma for extraction. The lower limits of quantification were 10.0, 2.0 and 8.0 ng/ml (equivalent to 0.1, 0.02 and 0.08 ng in each 10 μl injection onto the HPLC column) for Rg3, Rh2 and ppd, respectively. The method has been demonstrated to be highly sensitive and accurate for the determination of Rg3 and its metabolites in rat plasma.

Keywords: Ginsenoside Rg3; Rh2; 20(S)-Protopanaxadiol (ppd); Pharmacokinetics

1. Introduction

Panax ginseng is a medicinal herb commonly used worldwide for many years. Ginsenosides with a dammarane skeleton (Fig. 1) are classified into 20(S)-protopanaxadiol(ppd) or 20(S)-protopanaxatriol (ppt) compounds (except ginsenoside R_0), has long been regarded as the main active component accounting for the pharmacological properties of *P. ginseng* C.A. Meyer [1,2]. *G*insenoside Rg3 and Rh2 are ppd type compounds which are active components in Red *P. ginseng* [3]. Rg3 has been developed as a promising new anti-cancer substance and is under clinical evaluation in China [4–9]. Pharmacokinetic studies suggest that ginsenosides are very poorly absorbed following oral administration to rabbits [10], rats [11] and humans [9]. Studies using ^{3}H-labelled ginsenoside did suggest higher drug concentrations in the body than those when unlabelled active was used, and this is probably due to the nonspecific nature of the assay [12]. However, the many important findings concerning the pharmacodynamics of these actives [3], seem at odds with reports on their low bioavailability due to lack of suitable analysis methods. Bae et al. [13] reported that ginsenoside Rg3 is metabolized to

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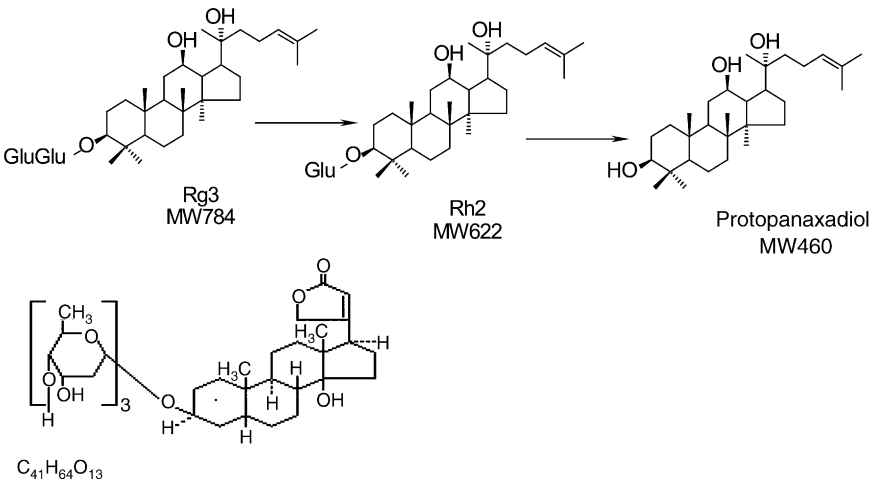

Fig. 1. Structures of ginsenosides used in this study and the internal standard digitoxin.

ginsenoside Rh2 and ppd when anaerobically incubated with human fecal microflora, and the deglycosylated metabolites have activities comparable to or higher than that of Rg3 [14]. This suggests that active metabolites might be responsible, at least in part, for the observed pharmacodynamics; hence research on the main metabolic pathways is being undertaken in our laboratory.

Analytical methods have been developed using radioimmunoassay (RIA) [15], thin layer chromatography (TLC) [11], high performance liquid chromatography with evaporative light scattering detection (HPLC/ELSD) [16] or HPLC with UV detection [17,18], and all are based on detection of the parent drug at higher doses. Moreover, the low wavelength (203 nm), required for detection of gisenosides makes UV detection problematic. HPLC/MS has been successfully applied for qualitatively analyzing ginsenosides extracted from raw *P. ginseng* plant material [19–22]; There are a few reports on HPLC/MS [23] and GC–MS [24] analysis of ginsenosides extracted from biological samples. Such analyses are essential for pharmacokinetic studies and for other fundamental or practical purposes, where it is necessary to analyze the main metabolic products of the target analyte in biological samples at low concentrations. The establishment and application of sensitive and reproducible method of determing Rg3 and it metabolites in rat plasma is presented in this paper.

2. Experimental

2.1. Chemicals, reagents and animals

The reference standards of ginsenoside Rg3, Rh2 and ppd (purity > 99.0%) were obtained from Bai Qiu-En Medical University, Jilin, Changchung, China and the internal standard digitoxin (No.: 51201) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout, Acetonitrile (Tedia Company, USA) and methanol (Merck, Germany) were of HPLC grade, ammonium chloride (NH_4Cl) is commercially available and was of analytical grade. Standard solutions were prepared at 5 mg/ml in DMSO and stored at 4 °C. Sprague–Dawley rats were obtained from the Jiangpu animal breeding center, Nanjing and the studies were approved by the Animal Ethics Committee of China Pharmaceutical University.

2.2. Apparatus and chromatographic conditions

All analytical procedures were performed on a Shimadzu 2010 LC–MS, with ESI probe (Q-array-Octapole-Quadrupole mass analyzer), a Shimadzu 10ADvp Pump, and a Shimadzu 10ATvp Autosampler. Nitrogen, the carrier gas was supplied by the Gas Supplier Center of Nanjing University, China. Separation was carried out on an ODS column (5 μm, 2.1 mm × 150 mm, Metachem, USA) with a C_{18} guard column (Security Guard, Phenomenex, USA). The mobile phase was acetonitrile: water containing 500 μM/l of ammonium chloride at the flow rate of 0.2 ml/min. The following optimized MS conditions were selected: gas flow: 4.5 l/min; CDL (curve dissolution line) voltage fixed as in tuning, CDL temperature: 250 °C; block temperature: 200 °C. Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and scan mode, accordingly. Mass vacuum was obtained by Turbo molecular pump. LC-Mass Solution Version 2.02 works on Windows 2000 operating system was used for data processing. The injection volume was 10 μl. Acetonitrile (solvent B): 500 μM NH_4Cl (solvent A) in water was run under a gradient program. The samples were eluted with 68% B for the initial 3.61 min followed 7 min linear gradient to 95% B and a 1 min isocratic period

2.3. Sample preparation

Stock solutions for ginsenoside Rg3, Rh2 and ppd were prepared with dimethylsulfoxide (DMSO) as solvent. Work-

ing solutions were diluted with 95% methanol before experiments. Plasma standards were prepared using drug-free rat plasma. Standard samples were prepared in 0.1 ml of blank rat plasma at concentrations of 10, 25, 50, 100, 250, 500 ng/ml for Rg3; 2, 5, 10, 20, 50, 100 ng/ml for Rh2 and 8, 20, 40, 80, 200, 400 ng/ml for ppd. Calibration curves were obtained by plotting peak area ratios of drug/internal standard versus drug concentrations in the standard samples.

2.4. Separation and extraction procedures

Plasma samples were thawed in a water-bath at 37 °C and 100 μl rat plasma was used for extraction. Plasma samples were slowly added onto the solid-phase cartridge (HLB 1cc,Oasis^{TM}, Waters, USA), which had been previously activated with 1 ml methanol and balanced with 1 ml water. After the sample had been absorbed by the cartridge, the cartridge was washed with 4 ml water and the ginsenosides were then eluted with 1ml 95% methanol in water, which was evaporated under an air stream at 40 °C in a water-bath. The residue was reconstituted in 100 μl 95% methanol with vortexing. After centrifugation at 20000 × *g*, 15 min, 70 μl supernatant was drawn and 10 μl injected into the HPLC/MS.

2.5. Drug administration and sample collection

12 Sprague–Dawley rats, weighing 260–320 g with both sex assigned randomly into two groups for intravenous (iv) and intra-gastic (ig) administration were used in this study. The rats were fasted for 12 h before dosing and 4 h afterwards, with free access to water. For oral route, dosing solutions were prepared by dissolving Rg3 powder in water containing 0.5% CMC-Na (croscarmellose sodium) and mixing well. For intravenous bolus, Rg3 powder was dissolved in isotonic saline containing 20% ethanol and dosing solution was delivered using a 1 ml syringe into a rat tail vein. The preparations were made immediately before drug administration.

A rat was fixed on a wooden plate under ether anesthesia, a cannula was inserted into the carotid artery. Blood samples (200 μl) were collected immediately before and at 2, 5, 15, 30 min, 1, 2, 4, 6, 8, 12 h for iv group and 15, 30, 45 min, 1, 2, 4, 6, 8, 12 h for ig group after Rg3 administration. After each blood sampling, the cannula was flushed with physiologic saline containing heparin. The blood sample was transferred into a heparinized Eppendoff tube and mixed gently, then centrifuged (1000 × *g*, 5 min) to obtain 100 μl plasma, which was kept at −20 °C until analysis.

2.6. Validation of the assay

Specificity was determined by analyzing blank rat plasma without adding the internal standard to determine possible interference with the analyte, metabolite and the internal standard.

Five calibration curves for each analyte were obtained on a single day using Rg3, Rh2 and ppd spiked rat plasma samples. Drug/internal standard peak-area ratios versus concentration were fit by a least-square linear regression method. The inter-day variabilities were calculated by analyzing each analyte at low, medium and high concentrations on five consecutive days. Extraction recoveries of ginsenoside Rg3,Rh2 and ppd from rat plasma were calculated by comparing the peak area ratios of authentic samples Rg3, Rh2 and ppd added to blank rat plasma and extracted by SPE to those of the same quantities added in the mobile phase. Validation was carried out at three concentration levels. The calibration samples and validation samples were processed exactly the same way as unknowns.

3. Results and discussion

3.1. Identification of Rg3 and its metabolites

The HPLC/MS total ion chromatogram scanned from *m*/*z* 200–1400 in the negative mode showed that chlorinated molecular ions [M^+Cl^-] were predominant. It was shown that ammonium chloride supplement in the mobile phase is essential to give robust signal as shown clearly in Fig. 2 A typical HPLC/MS chromatogram of an extract from rat plasma gave four major peaks which were not present in the chromatogram of blank samples. The four peaks, assigned as peak 1, 2, 3, 4 had the same *m*/*z* values as those obtained from authentic samples of internal standard, Rg3, Rh2 and ppd under the SIM mode (Fig. 3). Retention times were 3.2, 3.5, 6.6 and 10.0 min for digitoxin, Rg3, Rh2 and ppd, respectively. The LLOQ level for Rg3, Rh2 and ppd were shown in Fig. 4.

3.2. SPE method establishment

The aim for sample preparation was to remove interferences from plasma samples with a suitable recovery rate involving a minimum number of working steps. Several extraction procedures were tested including solid phase extraction using HLB cartridge and the liquid–liquid extraction method by ether. It was estimated that SPE could remove proteins and other interfering components in rat plasma with satisfactory drug recovery. Moreover, SPE could extract Rg3 and other metabolites simultaneously. Loading the sample should be slow, and different concentrations of acetonitrile or methanol were selected for the final eluting procedure. It was found that the SPE column eluted with 95% methanol not only provide sufficient sample clean-up and gave higher recovery, but also gave better chromatograms with few interferences.

3.3. Validation

The linearity was poor when stock samples of Rg3, Rh2 and ppd in DMSO were serially diluted with water; however,

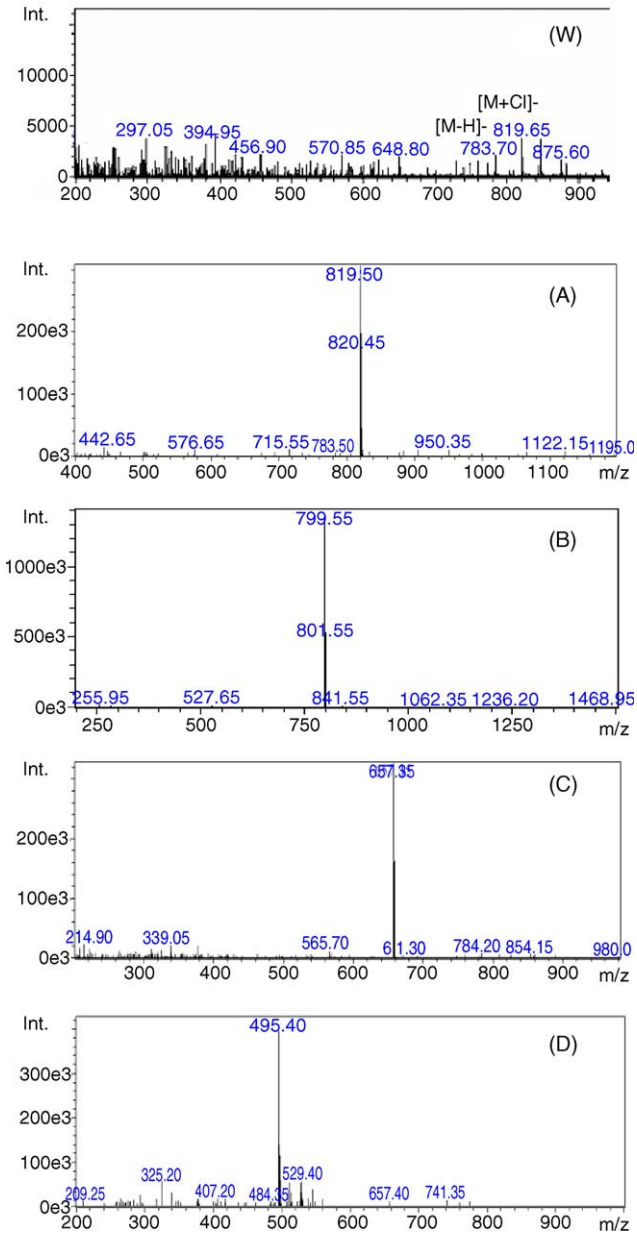

Fig. 2. Negative ion electrospray mass spectra obtained in a scan mode from authentic samples of Rg3, Rh2, ppd (each of 50 ng per injection) and internal standard (50 ng) with the abundance of [M^+Cl^-], (W) was without ammonium chloride supplement and with ammonium chloride supplement (A) Rg3, (B) digitoxin, (C) Rh2 and (D) ppd, respectively.

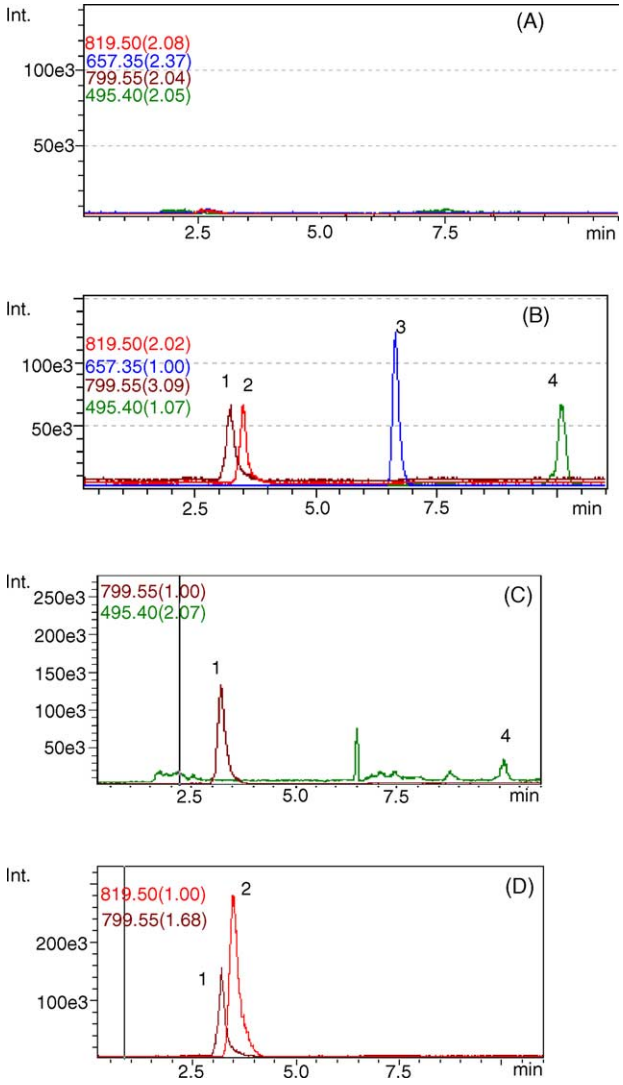

Fig. 3. Representative chromatograms of (A) blank rat plasma, (B) rat plasma spiked with Rg3, Rh2 and ppd standards, (C) sample from a rat 24 h after intra-gastric administration of 10 mg kg^{-1} Rg3 and (D) sample from a rat at 5 min after iv administration of 1 mg kg^{-1} Rg3. (1): IS, (2): Rg3, (3): Rh2 and (4): ppd.

an alternative solvent of 50–95% methanol showed satisfactory (>0.995) linearity. Within-day precision and between-day precision were evaluated by analyzing quintuplicate samples of rat plasma at three concentration levels as shown in Table 1. Within-day and between-day performance, expressed as the relative standard deviation (R.S.D.%), was less than 15% at all concentrations within the standard. The lower limit of of quantification (LLOQ) of 10, 2 and 8 ng/ml for Rg3, Rh2 and ppd, respectively was determined using 100 μl aliquots and five different blank rat plasma. Diluting plasma 2–20-fold with a blank matrix did not show any effects on the assay values, which will allow analysis after dilution for the samples which show values greater than the quantifiable limits at intravenous group. The extracted samples in vials were found to be stable for at least 48 h. Ion suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent. It was shown that SPE improving the sample clean-up to remove plasma components and thereby deceasing the amount of matrix injected onto the column, thus the ion suppression effect was minimized. The results showed that there was no significant difference in the signals of analytes extracted from rat plasma and

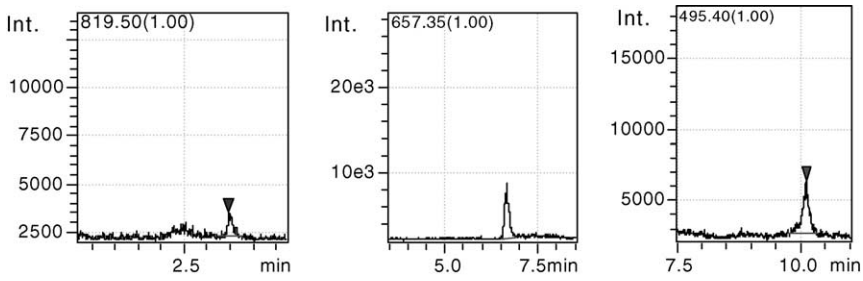

Fig. 4. Rat plasma spike with 10.0, 2.0 and 8.0 ng/ml (equivalent to 0.1, 0.02 and 0.08 ng in each 10 μl injection onto the HPLC column, LOQ level) for Rg3, Rh2 and ppd, respectively.

from the mobile phase, indicating that there were no matrix effects.

3.4. Linearity

The calibration curves were done at the concentration of 10, 25, 50, 100, 250, 500 ng/ml for Rg3, 2, 5, 10, 20, 50, 100 ng/ml for Rh2 and 8, 20, 40, 200, 400 ng/ml for ppd. The method was shown to be linear over the concentration range using least squares linear regression of samples versus internal standard gave correlation coefficients more than 0.99 for the three compounds defined. The regression equation were $Y = 0.0003X - 0.0006$ for Rg3, $Y = 0.0027X - 0.0178$ for Rh2 and $Y = 0.0043X - 0.0057$ for ppd, respectively, where Y represents the ratio of sample area and internal standard area, X represents concentration.

3.5. Freeze-thaw stability of Rg3, Rh2 and ppd in rat plasma

The stability of Rg3, Rh2 and ppd were assayed by analyzing triplicate samples at three concentration levels (the same level as recovery test) and submitting them to three freeze-thaw cycles.

The results obtained after the procedure of putting samples frozen at −20 °C for 14 days and thawed at room temperature 2 h demonstrated that more than 95% of the initial contents of the three analytes were recovered with R.S.D. less than 5%, indicating the analytes were stable under these conditions.

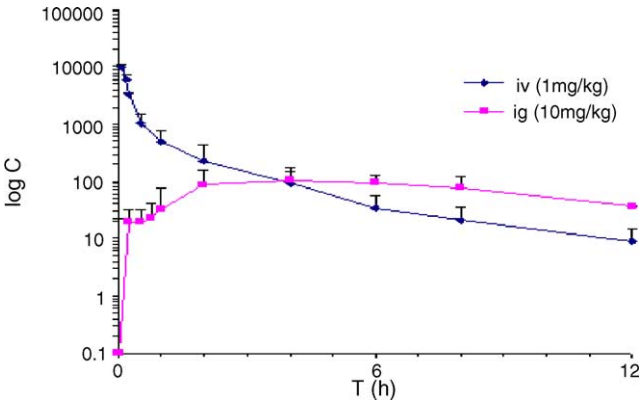

Fig. 5. Mean plasma Rg3 concentration-time curves ($\bar{x}$ + S.D.) in rats after ig administration at the dose of 10 mg kg^{-1} and iv administration at the dose of 1 mg kg^{-1}, respectively.

3.6. Pharmacokinetic studies

It was previously reported [13] that the uptake of ginsenosides occurs after complete or partial hydrolysis to prosapogenins or aglycons by acidic conditions in the stomach and intestinal micro flora. The method described in this paper was successfully used to monitor Rg3 and its metabolites in rat plasma. A typical concentration versus time profile for Rg3 after iv and ig administration were presented in Fig. 5. The plasma concentration time curves of Rg3 and its metabolites in rats following ig of 10 mg/kg Rg3 were de-

Table 1
Method recovery and precision of Rg3, Rh2 and ppd in blank rat plasma ($n = 5$)

Compounds	Spiked value (ng/ml)	Recovery (%)			Within-day (ng/ml)			Between-day (ng/ml)		
		$\bar{X}$	S.D.	R.S.D.(%)	$\bar{X}$	S.D.	R.S.D.(%)	$\bar{X}$	S.D.	R.S.D.(%)
Rg3	10.0	82.3	8.1	9.8	9.5	0.9	9.3	9.3	0.7	7.4
	50.0	78.7	6.1	7.7	52.1	3.6	6.8	51.3	2.7	5.2
	500.0	72.6	1.6	2.2	494.5	20.5	4.1	497.3	30.7	6.2
Rh2	2.0	80.3	5.9	7.4	2.2	0.2	9.4	2.2	0.3	11.5
	10.0	85.7	3.7	4.3	9.5	0.9	9.4	8.9	0.9	9.9
	100.0	82.0	2.5	3.0	104.9	10.3	9.8	103.3	11.2	10.8
ppd	8.0	98.3	8.3	8.4	7.5	0.7	9.8	7.7	0.7	8.4
	40.0	99.1	6.5	6.6	40.3	3.0	7.4	38.3	2.5	6.6
	400.0	97.6	9.4	9.6	396.1	19.9	5.0	397.1	13.6	3.4

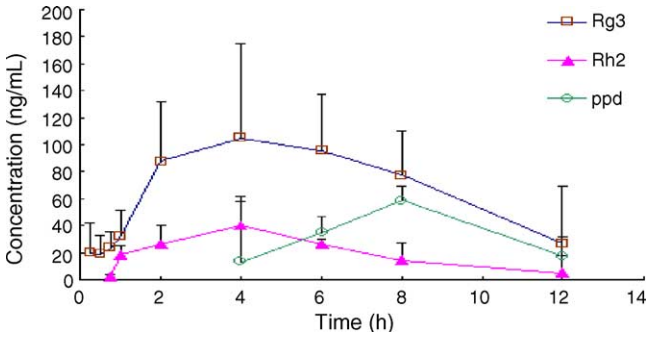

Fig. 6. Mean plasma concentration-time curves of Rg3 and its metabolites after ig administration of Rg3 (10 mg kg^{-1}).

picted in Fig. 6. Rg3 concentration time profile conformed to a two-compartment pharmacokinetic model after intravenous administration. First order elimination was assumed $T_{1/2\alpha}$ and $T_{1/2\beta}$ for Rg3 were 0.12 ± 0.03 (h) and 2.09 ± 0.50(h), respectively. For ig group, C_{max} and T_{max} was determined by visual inspection with $C_{max} = 104.07 \pm 59.95$ ng/ml, $t_{max} = 4.40 \pm 1.67$ h, The area under the concentration time curve (AUC_{0-12}) and the area under the first moment of the concentration-time curve ($AUMC_{0-12}$) were calculated using the linear trapezoidal rule, up to the last measured time point. Absolute baioavailability was determined from the ratio of dose normalized AUC values obtained for oral versus iv administered drug which was expressed as (AUC_{po}) ($Dose_{iv}$)/(AUC_{iv}) ($Dose_{po}$).The absolute bioavailability of ginsenoside Rg3 in rats is 2.63%.

4. Conclusion

The first pharmacokinetics study on Rg3 which had a low bioavailability in humans [9] was reported using HPLC–UV determination. Advances in analytical techniques have made further elucidation of the metabolic profile of this compound possible. In order to measure Rg3 and its metabolites selectively and precisely in rat plasma, the method for the simultaneous determination of three or more ginsenosides in rat plasma after SPE has been established and applied for pharmacokinetic studies. This is the first sensitive method for simultaneous determination of ginsenoside Rg3 and its metabolites in rat plasma by HPLC–MS. We have compared SPE with liquid–liquid extraction using ether etc and found that SPE is an appropriate extraction procedure. Choosing 1 ml of 95% methanol aqueous solution for eluting Rg3, Rh2, ppd and digitoxin from SPE cartridge showed satisfactory results. Concerning the mobile phase, 500 μM/l ammonium chloride in water was preferred to maintain abundant and stable signals. Plasma concentrations of Rg3, Rh2 and ppd could be quantified from 1 to 50 ng/100 μl, 0.2 to 10 ng/100 μl, and 0.8 to 40 ng/100 μl, respectively using 100 μl plasma sample, making it suitable for pharmacokinetic studies when blood can be taken from a single animal many times.

It was reported [13,14] that among ginsenoside Rg3 and its metabolites, ppd and Rh2 exhibited the most potent cytototoxicity against tumor cells. Investigation of the pharmacokinetic and metabolic profile of Rg3 in vivo is essential to clarify its mechanisms of action. It is also important to examine whether the pharmacological effects are derived from Rg3 alone or combined with its metabolites Rh2 and aglycone ppd. It was shown that followed by Rg3(10 mg/kg) administration, there was trace Rh2 and no ppd was detected from all the six rat plasma samples in the intravenous group while Rg3, Rh2 and ppd could be detected in the rat plasma of intragastric group and the standard deviation was quite big among individual rat (as shown in Fig. 5), Further studies are needed to elucidate the way of Rg3 metabolism and the absorption profile of Rh2 and ppd.

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