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Development of a sensitive LC-ESI-MS assay for 20(*R*)-ginsenoside Rh_2 and its pharmacokinetic application in dogs: A case for the influence of micronization on traditional Chinese medicine

Yi Gu, Guangji Wang *, Jianguo Sun, Haitang Xie, Yuanwei Jia

Key Laboratory of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, 1 Shennong Road, Nanjing City 210038, China

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Abstract

20(*R*)-ginsenoside Rh_2 is being developed as a new antitumor drug. To contribute to its biopharmaceutical and pharmacokinetic study, a novel LC-ESI-MS method was described in this paper and was proved to be accurate, precise and rugged with a linearity range of 0.5–200 ng/ml ($r^2 = 0.9998$) in dog plasma. The method procedure consisted of an economical and simple liquid–liquid extraction with satisfactory recovery (>70%), and a subsequent rapid analysis (within 9.5 min) which was performed on a Shimadzu LCMS2010A system (electrospray ionization, Q-array-octapole-quadrupole mass analyzer), with an ODS column (150 mm × 2.0 mm i.d., 5 μm) plus a C18 guard column for separation and ammonium chloride (500 μmol) as mobile phase additive. Chlorinated adducts of molecular ions $[M + Cl]^-$ of 20(*R*)-Rh_2 at *m/z* 657.35 and internal standard digitoxin at *m/z* 799.55 were monitored in select ion monitoring (SIM) mode of negative ions. The method showed an excellent sensitivity that the limit of detection (LOD) and the lower limit of quantitation (LLOQ) of 20(*R*)-Rh_2 were 0.1 and 0.5 ng/ml, respectively. This method was applied to a pharmacokinetic study of 20(*R*)-Rh_2 in six dogs and the evaluation of the influence of micronization on pharmacokinetics. The results indicated micronization could remarkably improve the absolute bioavailability of 20(*R*)-Rh_2.

Keywords: 20(*R*)-ginsenoside Rh_2; LC-ESI-MS; Pharmacokinetic; Bioavailability; Micronization; Traditional Chinese medicine

1. Introduction

Ginseng, the roots of Panax ginseng C. A. Meyer (Araliaceae) has been used as traditional Chinese medicine (TCM) and in traditional medicine practice in many oriental countries for treatment of various disorders for over 2000 years. Ginseng has gained popularity in the west during the last decades for its historical experience-based safety and "supernatural" effectiveness. Numerous biochemical and pharmacological studies reveal that the major active ingredients of ginseng are ginsenosides [1]. There are about 30 individual ginsenosides in the saponins of the ginseng root [2]. In regard to the molecule structures, the ginsenosides, glycosides containing an aglycone with a dammarane skeleton, can be classified into protopanaxadiols and protopanaxatriols. There is a chiral center at the position C-20 in the molecular structure, which connected with a 6-carbon-atom hydrocarbon chain.

Ginsenoside Rh_2 (Fig. 1), which belongs to protopanaxadiol group, was first isolated from red ginseng (Radix Ginseng Destillata) by steaming and drying the 6-year-old Panax ginseng C. A. Meyer [3]. Ginsenoside Rh_2 mainly exhibits anti-tumor activities through induction of apoptosis. The relative complex mechanisms of action are based on antiproliferation, cell cycle suppression, over-differentiation, activation of caspase pathway, and generation of reactive oxygen species [4–11]. Up to now only limited data are available in the literatures on pharmacokinetics of Rh_2. Xie et al. [12] have reported the preliminary pharmacokinetic results following administration of racemic Rh_2 to dogs. And Qian et al. [13] have reported the studies

Abbreviations: LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; SIM, selective ion monitoring; LOD, limit of detection; LLOQ, lower limit of quantitation; TCM, traditional Chinese medicine; IS, internal standard; QC, quality control; CDL, curved desolvation line; DC, direct current; RF, radiation frequency; RE, relative error; CV, coefficient of variation; CMC-Na, croscarmellose sodium; i.g., introgastric; i.v., intravenous; SPE, solid phase extraction; t_0, dead time; AIC, Akaike's Information Criterion; AUC, area under the concentration–time curve; MRT, mean residence time

* Corresponding author. Tel.: +86 25 85391035; fax: +86 25 85306750.
E-mail address: guangjiwang@yahoo.com.cn (G. Wang).

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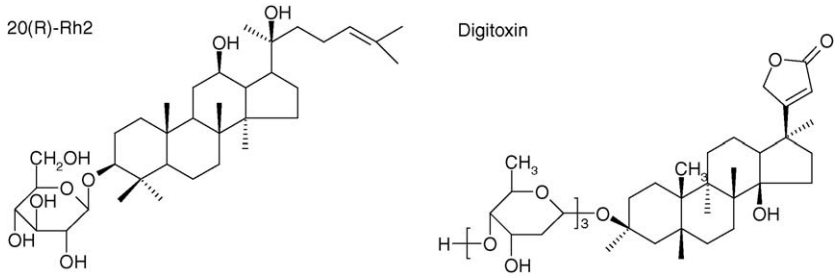

Fig. 1. Chemical structures of ginsenoside 20(*R*)-Rh_2 and internal standard digitoxin.

of 20(*R*)-Rh_2 in rats. However, in the latter, the number of animals was not sufficient and no Rh_2 was detected in the plasma at dosage of 100 mg/kg through oral route. The lower plasma concentration of Rh_2 requires a sensitive and selective method for the determination in biological fluids. LC-MS method has already been applied to the detection of ginsenosides in biosamples [12–17]. In contrast to the literatures about analysis of Rh_2 [12,13], we describe a validated LC-ESI-MS method with relative highly improved sensitivity, economy and reliability (the LC-MS quantitation in literature [13] was based on the external standard method) for the determination of 20(*R*)-ginsenoside Rh_2 in dog plasma in this paper.

Compared with intravenous administration, the bioavailability for the oral administration of Rh_2 is low. To improve its bioavailability, we have explored the feasibility of employing micronization technology, which has been proved to be a satisfactory small-particle engineering [18], for the improvement of the dissolution rate of Rh_2 and thus to enhance its absorption rate from oral dosage form. In this paper, we also report the absorption characteristics of both native drug and micronized preparation of 20(*R*)-Rh_2 following oral administration to beagle dogs, focusing on their similarities and differences.

2. Experimental

2.1. Chemicals, reagents and animals

Both the native drug (purity > 97%) and micronized preparation (purity > 98.3%) of 20(*R*)-ginsenoside Rh_2, as well as the standard of 20(*R*)-ginsenoside Rh_2 (purity > 99.9%), were kindly provided by Research Institute of Natural Medicine, Yunnan Baiyao Group Co., China. The internal standard (IS) digitoxin (Fig. 1) 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 (Fisher, USA) and methanol (Merck, Germany) were of HPLC grade, ammonium chloride and diethyl ether were commercially available and of analytical grade. Beagle dogs were obtained from the Jiangpu Animal Breeding Center, Nanjing and the studies were approved by the Animal Ethics Committee of China Pharmaceutical University. The animals were cared for according as the regulations of the Animal Committee under a constant temperature at 22 ± 1 °C, 12 h light/12 h dark cycle and 10–15 air changes per hour.

2.2. Standards and controls

A stock standard solution containing 20(*R*)-Rh_2 was prepared in methanol at a concentration of 1 mg/ml. Appropriate serial dilutions of the stock solution were made in methanol for spiking blank dog plasma. Aliquots (10 μl) of the appropriately diluted stock solution of 20(*R*)-Rh_2 were added to 1 ml amounts of blank dog plasma to yield working standards of the desired plasma concentrations. A stock standard solution of the IS digitoxin was also prepared in methanol at a concentration of 1 mg/ml, and diluted to 5 μg/ml with methanol to yield the IS working solution. Calibration curves were obtained with 20(*R*)-Rh_2-spiked dog plasma samples in the range 0.5–200 ng/ml. Additionally, spiked plasma samples were processed within each run as a means of assay quality control (QC).

2.3. Apparatus and chromatographic conditions

A Shimadzu 2010A LC-MS system with ESI interface, and Shimadzu LCMSsolution Workstation software (Ver 2.02) for the data processing, were utilized to perform the analytical procedures. The system consisted of two Shimadzu LC-10ADvp pumps, a Shimadzu SIL-HTc autosampler, a Shimadzu CTO-10Avp column oven, a Shimadzu DGU-14AM online degasser and a Shimadzu FCV-12AH flow channel selection valve. A Q-array-octapole-quadrupole mass analyzer was used as the detector. Separation was carried out on a Cosmosil-packed-ODS column (5C18-MS-II, 150 mm × 2.0 mm i.d., 5 μm, Code No. 38025–91, Nacalai Tesque, Japan) with a C18 guard column (Security Guard, Phenomenex, USA). The mobile phase, at the flow rate of 0.2 ml/min, was made up of acetonitrile and water containing 500 μmol/l of ammonium chloride. Acetonitrile (solvent B): 500 μmol/l NH_4Cl in water (solvent A) was drained from separated pumps under an elution program (Table 1). A flow-switching technique was used by commanding the flow channel selection valve. The liquid was only loaded to MS around the times when the target peaks emerged, in order to prevent redundant co-elutes and interferences from polluting the analyzer and thus to increase the targets' MS signals. The optimized MS parameters were selected as followed: CDL (curved desolvation line) temperature, 250 °C; the block temperature, 200 °C; the probe temperature, 250 °C; detector gain, 1.6 kV; probe voltage, −4.5 kV; CDL voltage, −5 V; Q-array DC (direct current) voltage, 0 V; RF (radiation frequency) voltage, 150 V. Nitrogen, supplied by the Gas Supplier Center of Nanjing

Table 1
LC program running for chromatographic separation

Time (min)	0.03	0.03	0.03	3.00	5.00	5.03	7.00	7.50	7.70	9.50
Units	Pump	Pump	Other	Other	Pump	Pump	Pump	Pump	Other	Pump
Command	B.Conc	B.Curve	RV.A(2-Pos.)	RV.A(2-Pos.)	B.Conc	B.Conc	B.Conc	B.Conc	RV.A(2-Pos.)	STOP
Value	60%	−3	0	1	80%	99%	99%	40%	0	0

LC program was set in the Shimadzu LCMSsolution workstation. Pump B served for acetonitrile. "B.Conc" represented the proportion of acetonitrile. "B.Curve" represented setting acetonitrile in the gradient mode. The curve at $0.03 < t \leq T$ could be calculated by the following equation: $C(t) = C1 + (C2 - C1)((\exp(|a|\,(1 - t/T)) - 1)/(\exp(|a|) - 1))$. Where $C(t)$ was the proportion of the solvent B at the relative time (t) in each interval; T was the whole curve time (5 min); a was the setting value (−3); $C1$, initial value of "B.Conc" (60%); $C2$, final value of "B.Conc" (80%). RV.A(2-Pos.) was the command of flow channel selection valve. When its value was 0, liquid switched to waste; when it was 1, liquid was loaded to MS analyzer.

University, China, served as nebulizer gas (flow rate: 1.5 l/min) and curtain gas (pressure: 1 MPa). Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and scan mode, respectively. The MS acquisition was performed in SIM mode of negative ions. The chlorinated adducts of molecular ions $[M + Cl]^-$ of 20(*R*)-Rh_2 at *m/z* 657.35 and IS digitoxin at *m/z* 799.55 were monitored.

2.4. Sample preparation

Vials containing frozen plasma samples were placed in 37 °C water to thaw. One milliliter of plasma and 10 μl of IS working solution were added to a 15-ml plastic stoppered conical extraction tube. After vortex-mixing for 30 s, 5 ml of diethyl ether were added; the tube was stoppered well and shaken vigorously for 3 min. Following centrifugation at 1200 × *g* for 10 min, 4 ml of the upper organic layer was transferred into another glass tube and evaporated to dryness at 50 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl acetonitrile, and centrifuged at 21000 × *g* at 4 °C for 10 min. The supernatant (65 μl) was pippetted to an autosampler vial, and 5 μl was injected onto column for analysis.

2.5. Method validation

Six pre-dose plasma samples from different dogs were used to evaluate the specificity. Blank samples were extracted and analyzed by LC-MS assay for potential interfering peaks within the range of the retention time of 20(*R*)-Rh_2 and IS.

Five sets of calibration curves of 20(*R*)-Rh_2 were constructed by plotting the peak-area ratios of target/IS versus plasma additive concentrations on a single day. The linearity was detected by calculating the correlation coefficient (r^2) of the curves by means of non-weighted least-squared linear regression method. The limit of detection (LOD) and the lower limit of quantitation (LLOQ) for 20(*R*)-Rh_2 were determined as the plasma concentration of 20(*R*)-Rh_2 giving a signal-to-noise ratio of 3:1 and 10:1, respectively.

To calculate intra-day and inter-day precision, QC samples with actual concentrations of 1, 10 and 100 ng/ml were reanalyzed for five times on a single day and once on five consecutive days, respectively. The determined concentrations, which were obtained from a calibration curve prepared on the same day, were used to evaluate the method accuracy and precision. The accuracy was determined by the relative error (RE), which was calculated by the equation: (mean of determined concentration − actual concentration)/actual concentration × 100%, and the precision was evaluated by the coefficient of variation (CV).

The absolute recovery of 20(*R*)-Rh_2 from dog plasma was calculated at three levels of concentration by comparing the peak areas obtained from extracts of spiked plasma samples and the peak areas obtained from direct injection of known amounts of standard solution of 20(*R*)-Rh_2.

Within-run stability was tested by reanalyzing QC samples at three different concentration levels kept under the autosampler conditions (4 °C) ever and again till the end in every routine analysis. The amount of QC samples was at least 5% of the total samples. The long-term stability and the effect of three freeze-thaw cycles were also studied by analyzing QC samples which were stored at −20 °C for a whole month and then followed by three freeze–thaw cycles, each of which contained a storage at −20 °C for 24 h and thaw at 37 °C.

2.6. Pharmacokinetic application: study design and procedure

Six beagle dogs, half male and half female with the average weight of 13.5 kg, were used in a randomized, self-control, and crossover study, which involved three test periods with three types of treatments for each dog (Table 2). One week was arranged as a washout phase between test periods in order to eliminate the influence left by last administration. During each

Table 2
Treatment schedule

Study period	Dog A	Dog B	Dog C	Dog D	Dog E	Dog F
1	Treatment 1	Treatment 1	Treatment 2	Treatment 2	Treatment 3	Treatment 3
2	Treatment 2	Treatment 3	Treatment 3	Treatment 1	Treatment 2	Treatment 1
3	Treatment 3	Treatment 2	Treatment 1	Treatment 3	Treatment 1	Treatment 2

Treatments 1, 2, 3 represents intravenous route, oral administration of native drug and oral administration of micronized preparation, respectively.

Table 3
Tested extraction solvents with pH conditions

Types	1	2	3	4	5	6	7	8	9	10	11	12
Tried solvents	Diethyl ether	Ethyl acetate	Cyclohexane	*n*-Hexane	Dichloromethane	Mixture A	Mixture B	Mixture C	Diethyl ether	Ethyl acetate	Diethyl ether	Ethyl acetate
pH Conditions	Acidification by 10 μl of 0.1 mol/l HCl								Basification by 10 μl of 0.1 mol/l NaOH		Neutral	

Mixture A: *n*-Hexane:diethyl ether, 2:1; mixture B: ethyl acetate:isopropyl alcohol, 4:1; mixture C: diethyl ether:acetone, 4:1.

period, the dogs were fasted for 12 h before dosing and 3 h afterward, with free access to water. The dosages of 20(*R*)-Rh_2 were 0.1 mg/kg with the volume of 0.5 ml/kg for intravenous route and 1 mg/kg with the volume of 1 ml/kg for oral administration. The dosing solution for intravenous route, prepared by dissolving the 20(*R*)-Rh_2 powder of native drug in isotonic saline containing 20% ethanol and 0.1% polysorbate (Tweens-80), was delivered using a 10 ml syringe into a dog forelimb vein. The dosage preparation for oral approach was made by dispersing the 20(*R*)-Rh_2 powder, of either native drug or micronized preparation, in water containing 0.7% CMC-Na (croscarmellose sodium), and mixing well. All preparations were accomplished immediately before drug administration.

A 3.5 ml volume of whole blood samples was collected by phlebotomizing from the forelimb vein prior to and at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 5, 7, 9, 12, 24, 36 h for introgastric (i.g.) groups and 0.033, 0.083, 0.25, 0.5, 0.75, 1.0, 2, 3, 5, 7, 9, 12, 24 h for intravenous (i.v.) group after drug administration into heparinized centrifuge tubes. Plasma was transferred out and stored at −20 °C till analysis after the centrifugation.

3. Results and discussion

3.1. Choice of the extraction method

With increasing the numbers of sugar moieties binding to aglycone, polarity and lipo-solubility of ginsenosides tend to increase and decrease, respectively. Therefore, solid phase extraction (SPE) and direct deproteinization were the main sample preparation for ginsenosides in plasma or serum [12–16,19–21]. However, liquid–liquid extraction by ethyl acetate has been used for extraction of Rg_3, a two-glucose-conjugated ginsenoside [17]. And *n*-butanol has also been used to extract Rh_2, the deglycosidation product of Rg_3, from feces and urine samples [13]. So we explored the feasibility of utilizing liquid–liquid extraction for the extraction of Rh_2 from plasma samples. Because using protein precipitation as sample preparation always reduces the MS sensitivity with an increased number of subsequent injections. Moreover, if deproteinization is not thorough, the impurity in sample liquid might block the LC-MS pipelines. In addition, liquid–liquid extraction avoids the high cost of SPE.

Twelve types of solvents with different pH conditions were tested to extract the analyte and IS (Table 3). 20(*R*)-Rh_2 could be extracted with diethyl ether, ethyl acetate, dichloromethane, mixture of ethyl acetate and isopropyl alcohol, and mixture of diethyl ether and acetone. Acidification of the plasma sample using 0.1 mol/l hydrochloric acid followed by extraction with diethyl ether gave the best recovery, while the result of neutralized condition with the same extraction solvent was also satisfactory. Extraction efficiency with ethyl acetate was acceptable likewise, similar to diethyl ether. Considering the possible unstability of the glycosidic bond in Rh_2's structure at acidic pH and the convenience of following evaporation, acidification was abandoned and diethyl ether was used. Moreover, IS (digitoxin), which has a similar structure with ginsenosides,

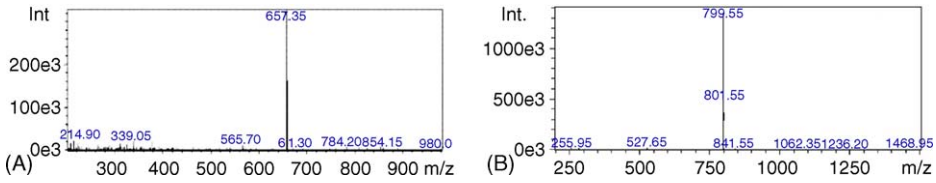

Fig. 2. Negative ion electrospray mass spectra obtained in scan mode from authentic samples of 20(*R*)-Rh_2 (A) and IS digitoxin (B) with abundance of $[M+Cl]^-$.

even results in better recovery than 20(*R*)-Rh_2 under current conditions.

3.2. Identification of analytes and optimization of analysis procedure

Previously most of the work on quantification of ginsenosides was operated in SIM mode of negative-ions of $[M-H]^-$. However, our laboratory recently reported that chlorinated adduct $[M+Cl]^-$ of ginsenosides, supplied by NH_4Cl water solution as a part of the mobile phase, was found to exhibit more sensitive, well-reproducible results [12,14,16]. Besides, the responses of 20(*R*)-Rh_2 to ESI were evaluated by recording the mass spectra scanned from *m/z* 200–1000 in both positive and negative ionization modes with this mobile phase condition. The negative mode yielded strong signals of $[M+Cl]^-$ of 20(*R*)-Rh_2; on the contrary, no obvious signals were obtained in positive mode under the same signal intensity. As shown in Fig. 2, $[M+Cl]^-$ of IS was also predominant under such conditions. So chlorinated adducts of molecular ions were chosen for detection in the SIM mode at *m/z* 657.35 for 20(*R*)-Rh_2 and *m/z* 799.55 for IS.

In order to improve the 20(*R*)-Rh_2's signal, we increased detector gain to 1.6 kV, at which the best signal-to-noise ratio could be obtained. Besides, setting the gain too high might cause an excess current to flow through the detector making it likely that the detector would get damaged. The other MS parameters were adjusted to maximize the 20(*R*)-Rh_2's signal by evaluating the total peak areas which were obtained in scan mode of negative ions by injecting standard solution of 20(*R*)-Rh_2 into MS analyzer without column separation. As shown in Table 4, Q-array DC voltage and CDL voltage played relative important role to achieve good fragmentation.

To optimize chromatographic conditions, columns packed with different types of C18 material (Nucleosil, Cosmosil, Kromosil) were tested; 20(*R*)-Rh_2 and the IS were extensively retained on these columns. To achieve symmetrical peak shapes and short chromatographic cycle times, the Cosmosil C18 column was used. The elution program was optimized for both 20(*R*)-Rh_2 and IS. Elution of 20(*R*)-Rh_2 needed high percentage (at least 80%) of organic solvent in mobile phase. But using high proportion of organic solvent (95% or so) directly resulted in unshapely peaks of 20(*R*)-Rh_2. This could be resolved by increasing the percentage of acetonitrile stepwise, which therefore might cause an enrichment effect of 20(*R*)-Rh_2 on the column. However, digitoxin eluted more rapidly as organic solvent proportion increased, so low initial and start proportions of

Table 4
Optimization of MS parameters

	Probe high voltage (kV)	CDL voltage (V)	Q-array voltage (V)		Results (total peak areas)
			DC	RF	
1	fix	fix	−70	150	246000
2	−4.5	fix	−70	150	259000
3	−3.5	fix	−70	150	216500
4	−4	fix	−70	150	176100
5	−4.5	fix	−70	150	206000
6	−4.5	−200	−70	150	135000
7	−4.5	−150	−70	150	184000
8	−4.5	−100	−70	150	133200
9	−4.5	−50	−70	150	158575
10	−4.5	−30	−70	150	212250
11	−4.5	−10	−70	150	281620
12	−4.5	−5	−70	150	313000
13	−4.5	0	−70	150	246000
14	−4.5	−2	−70	150	266500
15	−4.5	−7	−70	150	245000
16	−4.5	−5	−70	150	274870
17	−4.5	−5	−30	150	331000
18	−4.5	−5	−10	150	449000
19	−4.5	−5	−5	150	483000
20	−4.5	−5	0	150	629550

The total peak areas were obtained from 2 μg/ml standard solution of 20(*R*)-Rh_2 injected to MS directly without column separation. If "fix" was selected, the settings defined in the machine tuning file to be used for the analysis were used, which were listed as follow: probe high voltage, −3.5 kV; CDL voltage, −25 V. Parameters in group 20 were selected.

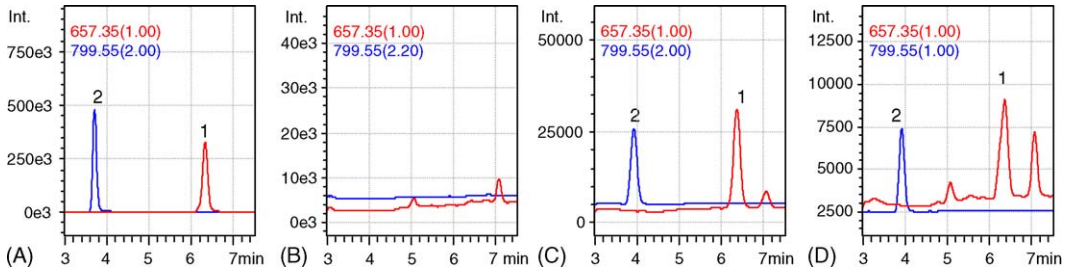

Fig. 3. Typical SIM chromatograms of (A) standard solution of 20(*R*)-Rh_2 and digitoxin injected directly, (B) blank beagle dog plasma, (C) blank dog plasma spiked with 20(*R*)-Rh_2 and digitoxin standards and (D) sample from a dog 0.75 h after oral administration of 1 mg/kg 20(*R*)-Rh_2 (micronized preparation). (1) 20(*R*)-Rh_2; (2) IS.

Table 5
Recovery, accuracy and precision of the method for analysis of 20(*R*)-Rh_2 in dog plasma ($n = 5$)

Spiked concentration (ng/ml)	Recovery		Intra-day			Inter-day		
	Mean ± S.D. (%)	CV (%)	Measured concentration (mean ± S.D.) (ng/ml)	RE (%)	CV (%)	Measured concentration (mean ± S.D.) (ng/ml)	RE (%)	CV (%)
1.0	74.7 ± 7.1	9.6	1.1 ± 0.1	8.9	11.1	0.9 ± 0.1	−8.0	14.1
10.0	76.3 ± 3.5	4.6	10.3 ± 1.1	2.9	10.7	10.5 ± 1.1	4.8	11.3
100.0	72.4 ± 6.2	8.7	95.3 ± 6.72	−4.7	7.0	89.5 ± 6.30	−10.5	7.0

acetonitrile were set and the increase was tuned gently. The last 2 min were set to achieve equilibrium of the chromatographic conditions.

3.3. Assay specificity

In the SIM mode of negative ions, blank dog plasma yielded relative clean chromatograms without significant interfering peaks both to 20(*R*)-Rh_2 and IS. A typical chromatogram of an extract from a dosed dog's plasma containing 20(*R*)-Rh_2 and IS gave peaks not presented in the chromatogram of blank sample (Fig. 3.). The representative peaks had the same *m*/*z* values as those from standard samples. The retention times of 20(*R*)-Rh_2 and IS were about 6.4 and 3.7 min, respectively.

3.4. Method validation

Five sets of the calibration curves showed good linearity from 0.5 to 200 ng/ml for 20(*R*)-Rh_2 in dog plasma. The following mean regression equation was derived from these calibration curves: $y = 0.0244x + 0.0088$ ($r^2 = 0.9998$). The LOD and LLOQ, based on a signal-to-noise ratio of 3 and 10, respectively, were determined respectively by comparing five appropriate spiked samples with five different blank dog plasma samples. The LOD and LLOQ of 20(*R*)-Rh_2 were 0.10 ± 0.02 and 0.5 ± 0.03 ng/ml, with the CV of 19.6 and 17.7%, respectively. The sensitivity of this method for determining plasma concentrations of 20(*R*)-Rh_2 is higher than any other ever reported [12–14,17]. Analytical accuracy and precision data are shown in Table 5. Intra-day and Inter-day precision values, expressed as CV, were less than 15% at all concentrations within the standard. Recovery results were all more than 70%, indicating the liquid–liquid extraction efficiency was well-acceptable. Stability results shown in Table 6 illuminated 20(*R*)-Rh_2 was stable for at least 48 h at the autosampler's conditions (4 °C) after extraction. Meanwhile, storing at −20 °C for a whole month followed by three freeze–thaw cycles did not affect its stability markedly.

Degradation of 20(*R*)-Rh_2 in dog plasma stored at 25 °C was investigated by spiking blank dog plasma to three concentration levels of 5, 50, 100 ng/ml. One milliliter of plasma was sampled immediately and 0.5, 1, 4, 8, 24 h after spiking for extraction and

Table 6
Stability of 20(*R*)-Rh_2 in dog plasma under different conditions ($n = 5$)

Spiked concentration (ng/ml)	Within-run (kept in autosampler for 48 h)			Long-term and freeze–thaw (stored at −20 °C for 1 month and followed by three freeze–thaw cycles)		
	Measured concentration (mean ± S.D.) (ng/ml)	Mean accuracy (%)	CV (%)	Measured concentration (mean ± S.D.) (ng/ml)	Mean accuracy (%)	CV (%)
1.0	1.02 ± 0.11	101.9	10.8	0.93 ± 0.11	93.2	11.5
10.0	9.08 ± 0.53	90.8	5.8	9.28 ± 0.36	92.8	3.9
100.0	94.56 ± 2.66	94.6	2.8	95.29 ± 3.06	95.3	3.2

Accuracy (%): measured concentration/spiked concentration × 100%.

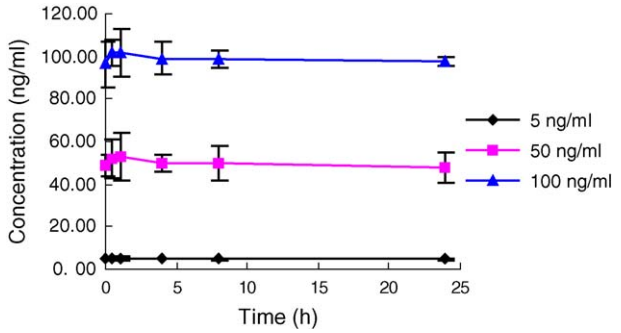

Fig. 4. Degradation curves of 20(*R*)-Rh_2 in dog plasma stored at 25 °C for 24 h.

analysis. Result curves (Fig. 4) showed degradation of 20(*R*)-Rh_2 in dog plasma stored for at least 24 h at 25 °C could be almost neglected.

3.5. Matrix effects

Matrix effects from co-eluting endogenous substances provide possible source of problems regarding assay interference and ion signal suppression, although matrix-matched calibration standards were used. In chromatographic separation, the retention times of 20(*R*)-Rh_2 and IS were prolonged relative far behind the dead time (t_0) of this system (about 1.7 min) to avoid main endogenous interferences, which were mostly eluted before or around t_0. Moreover, ion suppression effect was evaluated by comparing the peak areas of 20(*R*)-Rh_2 (50 ng/ml) and the IS (50 ng/ml) in six QC samples with those of standard solutions that had been prepared in the same way as the QC samples except that water was substituted for drug-free plasma. For 20(*R*)-Rh_2 and IS, the mean peak areas from the six QC samples had relative errors of 4.1% and 2.4%, respectively, when compared with that for these standard solutions. These observations indicated that no endogenous substances significantly influenced the ionization of these analytes.

Besides, diluting plasma 1 to 10 fold with blank matrix did not show any effects on the assay values, which allowed analysis after dilution for the samples which showed values greater than the quantifiable limits.

3.6. Pharmacokinetic and biopharmaceutical study in beagle dogs

Plasma concentrations of 20(*R*)-Rh_2 in dog after oral administration of native drug and micronized preparation and intravenous administration of native drug were determined respectively based on the validated LC-ESI-MS method. A three-session crossover study was involved to evaluate pharmacokinetic parameters. Fig. 5 shows the mean plasma concentration–time profile in six beagle dogs following the three types of treatments. Based on Akaike's Information Criterion (AIC), the plasma concentration–time data of i.v. 20(*R*)-Rh_2 in dogs was fitted to a three-compartment model, which might indicate cumulation at a certain extent in body and was partly proved in our subsequent research, and the data of oral native 20(*R*)-Rh_2 and micronized preparation were fitted to a one-compartment model. The pivotal pharmacokinetic parameters were calculated using DAS software (Ver 1.0, Medical College of Wannan, China) and results for three treatments are delineated in Table 7. Absolute bioavailability was determined by dividing the dose-normalized area under the concentration–time curve (AUC) resulting from oral administration by that resulting from intravenous administration, which was expressed as $(AUC_{i.g.}) \times (Dose_{i.v.})/(AUC_{i.v.}) \times (Dose_{i.g.}) \times 100\%$. The absolute bioavailabilities of native drug and micronized preparation were 16.1 ± 11.3 and $31.2 \pm 18.8\%$, respectively.

T_{max} and C_{max} of native 20(*R*)-Rh_2 administered orally to dogs were 2.6 ± 1.3 h and 371.0 ± 199.6 ng/ml. T_{max} was consistent with the data observed for racemic Rh_2 recently published [12], in an experimental set-up very similar to the one used in this study. While C_{max} of native 20(*R*)-Rh_2 in our study was a little lower, maybe due to a statistical difference. Elimination half life for 20(*R*)-Rh_2 was prolonged against racemic Rh_2. No significant differences were observed among the three treatments for mean residence time (MRT), indicating absorption was not a rate-limiting step, distribution and excretion might play a more important role during in vivo processing. It is well known that ginsenosides are absorbed poorly and many metabolism studies of ginsenosides are ever reported. 20(*R*)-Rh_2, if was dispersed well enough, might have a relative good penetrability through intestinal epithelial cells. However, relative low bioavailability

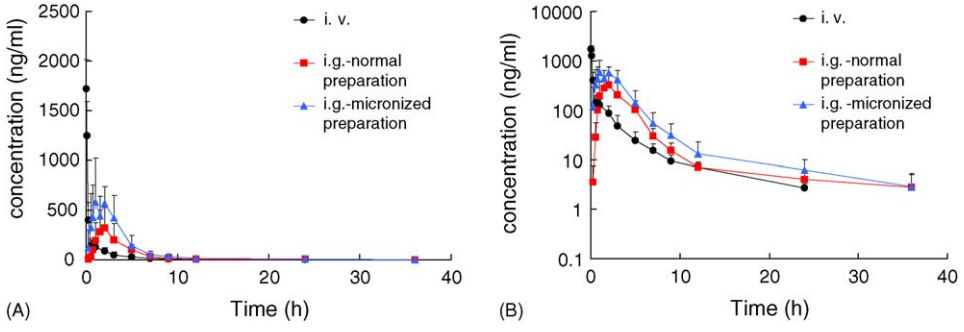

Fig. 5. Mean 20(*R*)-Rh_2 plasma concentration-time profile in six beagle dogs following three types of treatments. (B) Semi-logarithmic plot.

Table 7
Mean pharmacokinetic parameters for 20(*R*)-Rh_2 in dogs after three types of treatments

	Parameters							
	T_{max} (h)	C_{max} (ng/ml)	$T_{1/2}$ (h)	MRT (0–∞) (h)	CL/F (L/kg/h)	V/F (L/kg)	AUC_{0-36} (ng·h/ml)	$AUC_{0-\infty}$ (ng·h/ml)
i.g.-Native drug	2.6 ± 1.3	371.0 ± 199.6	5.8 ± 2.6	5.5 ± 2.0	1.1 ± 0.8	9.1 ± 5.9	1207.4 ± 601.3	1215.7 ± 598.6
i.g.-Micronized preparation	1.8 ± 0.7	730.0 ± 325.5	4.6 ± 2.9	4.2 ± 1.1	0.5 ± 0.2	3.4 ± 2.7	2371.9 ± 882.3	2375.9 ± 882.5
	Parameters							
	$C_{2\,min}$ (ng/ml)	$T_{1/2\gamma}$ (h)	MRT (h)	CL (L/kg/h)	V (L/kg)		AUC_{0-24} (ng·h/ml)	$AUC_{0-\infty}$ (ng·h/ml)
i.v.	1735.8 ± 266.4	8.0 ± 2.8	4.1 ± 0.6	0.1 ± 0.03	1.4 ± 0.5		824.3 ± 201.6	857.0 ± 209.6

Mean ± S.D. (n = 6).

might be caused by metabolism and degradation in gastrointestinal environment.

Result differences between native 20(*R*)-Rh_2 and micronized preparation were rather amazing. C_{max} and AUC of micronized preparation were nearly two times than those of native drug, which led to an approximate double absolute bioavailability. Elimination half life of the two preparations showed no significant difference ($P > 0.05$), however, T_{max} of micronized preparation was shortened. 20(*R*)-Rh_2 has very poor solubility in water, thus disperse extent in intestinal may be most important to its absorption. As a result, micronization improved bioavailability of 20(*R*)-Rh_2 evidently. 20(*R*)-Rh_2 in the micro-particle formulation seems to be better dissolved in the intestinal juice and well transported across the intestinal epithelial cells.

TCM, treasury of natural drugs, has been used for thousands of years. But it often suffers poor absorption and low bioavailability. Using modern science and technologies to research and develop TCM is a basic and urgent national policy in China. The results of oral micro-particle 20(*R*)-Rh_2 suggest that micronization of pharmaceutics could increase the absorption rate and extent of bioavailability of TCM.

4. Conclusion

In this paper, a simple, rapid and economical LC-ESI-MS method was validated to be accurate, precise and rugged for determination of 20(*R*)-Rh_2 in dog plasma. With respect to Rh_2 plasma concentration, the sensitivity of this method is higher than any other ever reported [12–14,17]. The assay was applied to a randomized, self-control and three-session crossover study to estimate the pharmacokinetics and absolute bioavailability of oral administration of 1 mg/kg native or micronized preparation of 20(*R*)-Rh_2 in dogs. The results showed that the bioavailability of oral micronized 20(*R*)-Rh_2 was almost 2-fold higher than that of native 20(*R*)-Rh_2 in dogs, suggesting the feasibility of micronization for optimizing the absorption of TCM.

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