Pharmacokinetics and Oral Bioavailability of Ganoderic Acid A by High Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT
Ganoderic acid A is one of the important active triterpenoid components of Ganoderma lucidum, however the study on pharmacokinetics and oral bioavailability of it is still lacking. The present study aims to investigate pharmacokinetic properties and the absolute oral bioavailability of Ganoderic acid A. A sensitive and selective LC-MS/MS method was developed for the determination of Ganoderic acid A. The validated method was successfully applied to the quantification of Ganoderic acid A in rat plasma after oral and intravenous administrations of triterpenoid extract from Ganoderma lucidum with different single dosages. Ganoderic acid A was rapidly absorbed with the time to maximum concentration (Cmax) <0.611 h after oral administrations for all oral dosage groups. The Cmax after oral administration were 358.733, 1378.20 and 3010.40 ng mL⁻¹ for 100, 200, 400 mg kg⁻¹ dosages, respectively. Area under the concentration-time curve from time zero to the last time point were 954.732, 3235.07 and 7197.23 h ng mL⁻¹ after oral administration for 100, 200, 400 mg kg⁻¹ dosages and 880.950, 1751.076 and 7129.951 h ng mL⁻¹ after intravenous administration for 10, 20, 40 mg kg⁻¹ dosages, respectively. The half-life ranged from 0.363-0.630 h and 2.183-2.485 h after intravenous and oral administration, respectively. Absolute bioavailability ranged from 10.38-17.97%.

Key words: Ganoderic acid A, Ganoderma lucidum, bioavailability, pharmacokinetics, LC-MS/MS

INTRODUCTION
Ganoderma lucidum, also known as “Ling Zhi” in China (Hsu et al., 2013) or "Reishi" in Japan (Yoshida et al., 2012), is a famous traditional Chinese medicinal mushroom that has been used for centuries in Asian countries for health improvement and life prolonging (Wang et al., 1997). Further researches show that G. lucidum could be effective with remedy of hepatitis (Zhang et al., 2002), neuroasthenia (Tang et al., 2005), fatigue (Zhao et al., 2012) and cancer (Lin and Zhang, 2004), etc. Polysaccharides and triterpenoids are two major bioactive components in G. lucidum (Lin et al., 2003). Triterpenoids have been shown to play an important role in the mechanism of G. lucidum therapeutical effect (Paterson, 2006). However, since the complexity of triterpenoid component, the pharmacokinetics (PK) and oral bioavailability of triterpenoids after oral administration are still unclear.

GA is a typical triterpenoid isolated from G. lucidum. It has been reported to have antitumor activity through its inhibition of oxidative stress-induced invasion of MCF-7 cells through suppression of IL-8 secretion (Thyagarajan et al., 2006). In addition, GA was able to suppress IL-6-induced signal transducer and activator of transcription 3 phosphorylation in HepG2 cells through JAK1 and JAK2 (Yao et al., 2012). Generally, G. lucidum are taken
as medicine or health care products after extraction and preparation process by pharmaceutical factories. Therefore, to better simulate the real condition and provide reference to further study of *G. lucidum* preparations, the current study investigated the pharmacokinetics of GA after administration of triterpenoids extraction of *G. lucidum* orally and intravenously.

**MATERIALS AND METHODS**

**Chemicals and reagents:** GA (with purity of 98.5%) was provided by Sichuan Industrial Institute of Antibiotics (Chengdu, China). Hydrocortisone (internal standard, IS) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Peking, China). Sodium carboxymethyl cellulose Na (CMC-Na) and Tween-80 was purchased from Kemiu Chemical Reagent Co., Ltd. (Tianjin, China). About 0.9% sodium chloride injection was purchased from Kelun Pharmaceutical Co., Ltd. (Hunan, China). Methanol, acetonitrile were purchased from Sigma (St. Louis, MO, USA), ammonium acetate from ROE Scientific Inc. (Newark, New Jersey, USA). The deionized water (DW) was distilled using a Millipore AFS-10 water purification system (Millipore, Billerica, MA, USA).

**Quantification of GA in triterpenoid extract (TE) from *G. lucidum*:** The Triterpenoid Extract (TE) from *G. lucidum* was provided by Sichuan Industrial Institute of Antibiotics. A stock solution of GA (204.6 μg mL⁻¹) was prepared to determine GA in TE. The stock solution was further diluted of by methanol to obtain the standard curve of GA concentrations of 20.46, 40.92, 136.4, 409.2, 818.4, 1637 and 2046 ng mL⁻¹ and then the working solutions were injected into the LC-MS/MS system. The GA content in TE was calculated through the standard curve.

**Drug administration and sample collection:** Thirty-six Sprague-Dawley rats weighing about 200 g were randomly divided into six groups, 6 rats each (Group I-VI). All the animals were adapted to a 12 h light/dark cycle under controlled room temperature and humidity conditions. Rats from group I-III were fasted overnight prior to administration, while food and water were given *ad libitum* for group IV-VI. The animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Sichuan University. The rats of Group I-III were administrated orally of 100, 200, 400 mg kg⁻¹ of TE (i.e., 10, 20, 40 mg kg⁻¹ of GA), respectively; the rats of Group IV-VI were administrated intravenously of 10, 20, 40 mg kg⁻¹ of TE (i.e. 1, 2, 4 mg kg⁻¹ of GA), respectively. The blood samples of Group I-III were collected via caudal vein at 0.17, 0.33, 0.5, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h and those of Group IV-VI were collected at 0.03, 0.08, 0.17, 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h after dosing. The blood samples were placed in heparinized tubes and centrifuged to obtain the plasma. The plasma was stored at -20°C until analysis.

**Instrumentation and chromatographic conditions:** LC-MS/MS analysis was performed on an SHIMADZU SCL-10A HPLC system (SHIMADZU, Japan) coupled to an API 3000 mass spectrometer (Applied Biosystems, Foster City, CA, USA). The chromatographic separation was achieved using a CAPCELL PAK C18 MG II column (5 μm, 2.0 mm ID × 50 mm, Cat. No. 92517 SHISEIDO, Japan) coupled with a Security Guard Cartridge (C18, 4×3.0 mm, Phenomenex). The column temperature was set at 25°C. An isotropic elution was carried out using a mobile phase containing 80% ACN and 20% DW (containing 0.1% formic acid). The flow rate was 0.3 mL min⁻¹.

The mass spectrometer was operated in the Multiple Reaction Monitoring (MRM) mode using negative ion electrospray. The Q1 scans and product ion scans of GA and IS are shown in Fig. 1-2; the chromatographic figures of GA and IS are shown in Fig. 3a-b. The target compounds were detected by monitoring m/z transition of 515.8-285.1 and 361.3-331.3 for GA and hydrocortisone, respectively. The API 3000 instrument parameters were set as follows: Nebulizer 12 psι, curtain gas 10 psι, collision gas 6 psι, ionspray voltage 4200 eV, ion source temperature 500°C. Data acquisition and analysis were performed using the Analyst software version 1.4.2. (Applied Biosystems, Foster City, CA, USA).

**Preparation of calibration curves and quality control samples:** A stock solution of GA and hydrocortisone with concentrations of 201.4 and 207.2 μg mL⁻¹, respectively, was prepared by dissolving the accurately weighed reference compounds in methanol. A series of standard working solutions were obtained by further dilution of the stock solution with DW. Calibration curves were prepared by spiking 25 μL of the appropriate standard solution into 50 μL blank rat plasma. The linear range of the calibration curve was over 5.829-5038 ng mL⁻¹. Quality Control (QC) samples were prepared by adding the stock solution of GA into blank plasma to obtain final concentrations of 17.49, 209.9 and 4030 ng mL⁻¹ for GA which represented low, medium and high concentration of QC samples, respectively. A 7.4 μg mL⁻¹ IS working solution was prepared by diluting a stock solution of hydrocortisone with DW. All of the solutions were stored at 4°C and brought to room temperature before use. The analytical standards and QC samples were stored at -20°C.

**Sample preparation:** Samples were thawed to room temperature prior to preparation. Twenty-five microliter of DW instead of the corresponding working solutions as mentioned above, 25 μL IS working solution and 50 μL of 1% formic acid were spiked. Samples were then mixed well by vortexing briefly. Liquid-liquid extraction was then performed by addition of 1 mL of an ethyl acetate/isopropanol (9:1, v/v) mixture, followed by vortex extraction for 3 min (Vortex Genie® 2 Vortex, Carlsbad, CA, US). After centrifuging at 12000 rpm for 5 min, the upper organic layer was transferred into another tube and completely evaporated to dryness at
Fig. 1(a-b): (a) Q1 and (b) Q3 scan of Ganodemic acid A

37°C under a stream of nitrogen (Turbovap Zymark, Hopkinton, MA, USA). The dry residue was reconstituted with 50 µL of mobile phase. After centrifuging at 13000 rpm for 5 min, 5 µL of supernatant was injected into the LC-MS/MS system.

Method validation: Specificity was evaluated in terms of the endogenous interference by analyzing blank plasma samples from six different rats spiked with only IS or GA. The curves were fitted by a linear weighted (1/c²) least squares regression method through measurement of the peak-area ratio of the analytes to the IS.

The precision and accuracy of the method were assessed by the determination of QC samples at three concentration levels in triplicate on the same day and three different days.
The accuracy was expressed as the percentage of the observed concentration to nominal concentration, while the precision as the Relative Standard Deviation (RSD) of the corresponding triplicate samples. The intra and inter-day precision was set at <20% for QC samples at low limits of quantification (LLOQ) concentration or 15% for other QC samples from nominal concentration. Extraction recovery of GA and IS was determined by comparing the peak areas of the extracted QC samples to those of the extracted plasma blanks spiked with the equivalent nominal concentration of GA or IS. The stability of GA and IS with regard to different conditions (time and temperature) was evaluated during the sample collection, storage and handling process using QC samples.
Fig. 3(a-b): Chromatographic figure of (a) Ganoderic acid A and (b) Hydrocortisone (Internal standard)

The absolute matrix effect for GA was evaluated by comparing the peak area of GA in extracted blank plasma samples obtained from six rats unexposed to any drug to that in neat solution. The variability in matrix factors, as measured by the coefficient of variation, should be less than 15%.

**Pharmacokinetic calculation:** The pharmacokinetic data was analyzed by non-compartmental method with the aid of Phoenix WinNonlin (version 6.3, Pharsight Corp, Mountain View, CA, USA). Main PK parameters after oral administration were calculated via a previous method (Wang et al., 2013). For intravenous administration, $C_0$ is the concentration just after intravenous administration and it is estimated by back-extrapolation from concentrations determined at any time after the dose.
RESULTS

Quantitative determination of GA in TE: A stock solution of TE (1076 µg mL⁻¹) was prepared and the stock solution was further diluted to 5380 ng mL⁻¹ to obtain a working solution of TE. The working solution was then injected into LC-MS/MS system five times and the content of GA in TE was calculated with reference to the standard curve. The GA accounted for 10.09% of the total extract.

Specificity: The specificity was evaluated by comparing blank, spiked and routinely prepared plasma samples. The GA and IS were eluted at about 1.04 and 1.07 min, respectively. No apparent interference was observed in the matrix.

Linearity of calibration curves and lower limit of quantification (LLOQ): All the calibration curves exhibited good linearity with correlation coefficients above 0.99. The LLOQs of this assay were 5.829 ng mL⁻¹. Under the present LLOQs, GA could be quantified in plasma samples until 24 h after administration. This result is sensitive enough to investigate the pharmacokinetic behaviors of GA.

Precision and accuracy: Table 1 summarizes the intra and inter-day precision and accuracy values for the QC samples. In this assay, the intra and inter-day precisions of GA were measured to be below 8.55 and 7.97%, respectively. These results indicated good precision, accuracy and reproducibility.

Recovery and stability: The recoveries of GA obtained from plasma (n = 6) were 78.52±5.94, 81.27±3.78 and 85.77±12.09% at concentrations of 17.49, 209.9 and 4030 ng mL⁻¹, respectively. The results of stability experiments showed that GA was stable for 15 days in DW, for 10 h after preparation at room temperature, for 6 h at room temperature and after three freeze/thaw cycles (-20°C to room temperature), as the relative error values were within ±5% for low, medium and high concentrations. Thus, the samples could be stored and prepared under routine laboratory conditions without special attention.

Matrix effect: Matrix effect was evaluated by comparing the signals produced by the same QC concentration in blank methanol and in blank plasma. The coefficient of variation values from six lots of plasma was less than 6.45%. No significant difference between the two matrices was observed. The results indicate the absence of obvious matrix effect for quantitative analysis.

Application of the method to pharmacokinetic studies in rats: The validated analytical method was applied to the assay of GA among the six groups (Group I-VI). Mean plasma concentrations versus time profiles are presented in Fig. 4 and 5. The main pharmacokinetic parameters following oral doses and intravenous doses are listed in Table 2.

DISCUSSION

A pre-clinical pharmacokinetic study of GA was conducted in SD rats which for the first time provided precise and comprehensive information of its pharmacokinetics and oral bioavailability.

This study first described the development of a sensitive and specific LC-MS/MS method for the determination of GA concentrations in rat plasma. In the full-scan Q1 mass spectrum, the parent negative ion peak of GA, [M-H]⁻, appeared at m/z = 515.8 amu and the abundance of this ion peak was sufficient for the quantification of GA. For hydrocortisone, the most abundant peak was the deprotonated molecular ion [M-H]⁻ found at m/z = 361.3 amu. Hence, deprotonated GA and hydrocortisone were targeted for fragmentation and the most stable and abundant ions in the product ion scan of GA and hydrocortisone were m/z = 285.1 and 331.3 amu, respectively.
Fig. 4: Mean plasma concentrations versus time profile of Ganoderic acid A in rats orally receiving different doses of triterpenoid extract (L, M, H doses were 10, 20 and 40 mg kg\(^{-1}\) of triterpenoid extract, respectively)

Fig. 5: Mean plasma concentrations versus time profile of Ganoderic acid A in rats intravenously receiving different doses of triterpenoid extract (L, M, H doses were 10, 20 and 40 mg kg\(^{-1}\) of triterpenoid extract, respectively)

During the optimization of chromatographic conditions, different ratios of organic and aqueous phase of 60:40, 70:30, 80:20 and 85:15 were tested. To avoid tailing peak shape and achieve short chromatographic runtime, the ratio of acetonitrile/water (80/20, v/v) was selected. As shown in Fig. 3a, GA peak was symmetric and the total runtime for a single sample was only 2 min.

Because TE had poor solubility in water, TE with a small amount of DMSO was resolved first and then added 0.9% sodium chloride injection (with 1% Tween-80, v/v) to obtain TE injections with different concentrations: 2, 4 and 8 mg mL\(^{-1}\). DMSO accounted for less than 1% of the total volume of the diluted injection. The 0.5 mL injection was administrated for each 100 g weight of the rats. On the other hand, the gavage suspension was made by suspending TE with 0.5% CMC-Na aqueous solution to obtain different concentrations: 10, 20 and 40 mg mL\(^{-1}\). One milliliter of the suspension was administrated for each 100 g weight of the rats.

Hydrocortisone displayed similar chromatographic retention behavior (with a retention time of 1.07 min) with GA and high extraction efficiency (79.80%). Additionally, there were no interferences of IS from GA and endogenous substances. Therefore hydrocortisone was chosen as IS.

Protein precipitation and liquid-liquid extraction were both assessed as sample preparation methods. Although protein precipitation would provide a simpler method, injection of precipitated samples by acetonitrile or methanol would cause an increase in column pressure, meanwhile, the LLOQ of the determination after protein precipitation was relatively high and was not sensitive enough for the quantification of biological samples. However, these problems
could be solved using a liquid-liquid extraction method. The efficiencies of dichloromethane, ethyl ether, ethyl acetate, Methyl-Tert-Butyl Ether (MTBE) and mixture of ethyl acetate-isopropanol as extract solvents were evaluated and the recoveries of GA were higher when ethyl acetate-isopropanol (v/v, 9/1) was used as compared with the others.

The main pharmacokinetic parameters of GA in the six groups were calculated using non-compartment modeling. GA was rapidly absorbed at all doses with the median tmax ranging from 0.417-0.611 h. However, its absolute bioavailability was quite low with a value being around 15%. This low bioavailability might attribute to significant first pass effect and poor permeability via the intestinal epithelial membrane after oral administration.

Only few literatures reported about the PK of some triterpenoid such as ganoderic acids C2, B, K and H (Wang et al., 2007) and ganoderic acid D (Cheng et al., 2013). Although Teekachanhanate et al. (2012) have studied the PK of Ganoderic Acid A (GA) and F. The present method has a quicker GA peak time and thusly shortened sample analysis time. Additionally, for the first time was studied PK of three different dosages of G. lucidum product and conducted an intravenous group in order to obtain the bioavailability of GA.

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REFERENCES


