Simultaneous quantification of ginsenoside Rg1 and its metabolites by HPLC–MS/MS: Rg1 excretion in rat bile, urine and feces

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Abstract Ginsenoside Rg1 (Rg1), the major effective component of ginseng, has been shown to have multiple bioactivities, but low oral bioavailability. The aim of this study was to develop a simple, sensitive and rapid high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method, which could be used to validate and quantify the concentrations of Rg1 and its metabolites in Sprague-Dawley rat bile, urine, and feces after oral administration (25 mg/kg). Calibration curves offered satisfactory linearity (r > 0.995) within the determined ranges. Both intra-day and inter-day variances were less than 15%, and the accuracy was within 80–120%. The excretion recoveries of Rg1, ginsenoside Rh1 (Rh1), and protopanaxatriol (Ppt) in bile, urine, and feces combined were all greater than 70%. The fecal excretion recoveries of Rg1, Rh1, and Ppt were 40.11%, 22.19%, and 22.88%, respectively, whereas 6.88% of Rg1 and 0.09% of Rh1 were excreted in bile. Urinary excretion accounted for only 0.04% of Rg1. In conclusion, the observed excretion profiles for Rg1 and its metabolites after oral administration are helpful for understanding the poor oral bioavailability of Rg1 and will aid further investigations of Rg1 as a pharmacologically active component.

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**1. Introduction**

Ginsenoside Rg1 (Rg1) is one of the major active saponins originating from *Panax ginseng* of the Araliaceae family and is classified as a panaxatriol saponin. The chemical structure of Rg1 is shown in Fig. 1A. It has long been regarded as the main active component responsible for the pharmacological activities of *Panax* herbs. Multiple bioactivities of Rg1 have been studied, including stimulation of the central nervous system, improvement of memory, attenuation of fatigue, and formation of acetylcholine, proteins and lipids. It has also been demonstrated that Rg1 can improve the hematopoietic function of bone marrow in a model of cyclophosphamide-induced myelosuppression. Furthermore, Rg1 was reported to significantly improve survival of septic mice.

A few reports on the metabolism and pharmacokinetics of Rg1 have been published. Other reports revealed that Rg1 could be biotransformed via deglycosylation in intestine. Ginsenoside Rh1 (Rh1) and protopanaxatriol (Ppt) are major metabolites in human intestine, whereas another ginsenoside F1 (F1), an isomer of Rh1, is also found in rat intestine. In addition, metabolites of Rg1 have even greater biological effects than Rg1. However, pharmacokinetic studies indicate that Rg1 has very poor oral bioavailability, and Odani et al. and Xu et al. reported that the amount of ginsenoside Rg1 absorbed via oral administration was the range of 1.9%–20.0% of the dose. Poor membrane permeability and active biliary excretion could be primary factors limiting the oral bioavailability of Rg1 and its metabolites. Therefore, comprehensive studies of the excretion of Rg1 are required and a validated bioanalytical method is necessary to support any pharmacokinetic studies. Some methods for quantifying concentrations of Rg1 have been published, such as thin-layer chromatography (HPTLC), which has low sensitivity and long analysis time, but such methods are not suitable for pharmacokinetic studies. Although high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometer (LC–MS) are the most commonly employed analytical methods, such methods have not been applied to the comprehensive study of Rg1 and its metabolites in bile, urine and feces. Thus, the development of a much more sensitive, accurate and simple LC–MS/MS method was necessary to determine Rg1 in complex matrices. The successful method should also be capable of separating the analytes from interfering substances (including analyte degradation products) from biological samples.

The objective of this study was to develop and validate a reliable and rapid high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneously quantifying the concentrations of Rg1 and its metabolites (Rh1 and Ppt) in bile, urine, and feces of Sprague–Dawley (SD) rats. Combining the advantages of HPLC and MS/MS, we examined the overall excretion of Rg1 and its metabolites in bile, urine, and feces, and provide an *in vivo* excretion profile for Rg1 after oral administration.

**2. Material and methods**

**2.1. Chemicals and reagents**

Rg1 (No. 110703-201027, purity > 98%) and androlin (No. 100008-200505, purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rh1 (No. 200612, purity > 95%) and Ppt (No. 200612, purity > 95%) were supplied by Jilin University (Jilin, China). HPLC grade acetonitrile was acquired from Fisher (Fairlawn, NJ, USA), and water was purified by a Milli-Q Plus water purification system (Millipore, Ltd., Billerica, MA, USA). Formic acid was of LC–MS grade (99% purity, Wako, Osaka, Japan). The other chemical reagents were of analytical grade and were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

**2.2. HPLC–MS/MS conditions**

HPLC–MS/MS was carried out using the HPLC–MS/MS8040 (Shimadzu Corp., Kyoto, Japan). A Shim-Pack XR-ODS II (75 mm × 2 mm, 2.3 μm) column was used for separation. The mobile phase consisted of 0.05% formic acid (A) and 0.05% formic acid in acetonitrile (B) with a gradient elution. The linear

![Figure 1](image-url) The chemical structures of (A) Rg1, Rh1 and Ppt, and (B) IS.
gradient increased solvent B from 22% to 80% within 7 min. After changing back to 22% solvent B at 7.01 min, the mobile phase gradient was maintained for 3 min for column equilibration. The flow rate was 0.5 mL/min, and the column temperature was kept at 40 °C.

A triple-quadrupole mass spectrometer (Shimadzu Corp.) equipped with an electrospray ionization (ESI) interface was used. Mass spectrometer conditions were optimized to obtain maximal sensitivity. For positive ESI analysis, the parameters were as follows: nebulizer gas, 3 L/min; drying gas, 15.0 L/min; interface, −4.5 kV; collision-induced dissociation gas, 230 kPa; DL temperature, 250 °C; and heat block temperature, 400 °C. Quantification was carried out using the selected reaction monitoring (SRM) mode.

2.3. Preparation of working stock solutions

Mixed stock solutions of Rg1, Rh1, and Ppt were prepared in methanol at the concentration of 400 μg/mL. Working solutions were prepared at 0.02–40 μg/mL by dilution of the stock solution with methanol. The internal standard (IS, androlin) was prepared at 1 μg/mL in methanol. The chemical structure of IS is shown in Fig. 1B.

2.4. Calibration curves and quality control samples

The samples for standard calibration curves were prepared by spiking blank rat bile (50 μL), urine (100 μL), or feces homogenate (50 μL) with 10 μL of a working stock solution to obtain concentrations of 10, 25, 50, 125, 250, 500, 1000, 5000, 10,000, 20,000, 40,000 ng/mL. Quality control (QC) samples were prepared at appropriate concentrations in blank bile, urine, and feces.

2.5. Sample preparation

Fecal samples were porphyrized by sonication for 15 min after addition of methanol (1:20, v/v). The supernatant was collected as feces homogenate. Then, the test sample of bile (50 μL), urine (100 μL), or feces (50 μL) was extracted with 1 mL of a mixture of ether, dichloromethane, and n-butanol in a ratio of 2:1:1 (v/v/v) after addition of IS (10 μL). Samples were mixed in a shaker for 30 min and centrifuged at 5000 r/min for 5 min. Supernatant fractions were then dried under N2 flow at 40 °C, and residues were reconstituted in 100 μL solution of acetonitrile, water and formic acid in a ratio of 20:80:0.05 (v/v/v). All samples were vortex-mixed for 60 s and centrifuged at 10,000 r/min for 10 min. Aliquots of 5 μL were injected into the HPLC–MS/MS system for analysis.

2.6. Rg1 excretion

Excretion experiments were performed using Sprague-Dawley (SD) rats (male, 250–290 g), which were bred and maintained by the Experimental Animal Science Department of Peking University Health Science Center (certificate number: SCXK 2009-0017). Five animals were fasted for 12 h before the experiments with free access to water. After oral administration of Rg1 (25 mg/kg of an Rg1 solution in water), rats were anesthetized with ether, and the bile duct was cannulated with tubing (Portex Nylon tubing, 0.75 mm internal diameter, 0.94 mm external diameter). Bile samples were collected via the bile cannula before drug administration as control and at 0–3, 3–6, 6–12, 12–24, 24–36, and 36–48 h after administration. The test samples of bile were weighed, and 50 μL of each sample was prepared with IS (10 μL) as described for sample analysis. Urine and feces samples were collected from another five rats kept in metabolic cages before drug administration as control and
at 0–4, 4–8, 8–12, 12–24, 24–36, 36–48, 48–54, 54–60 and 60–72 h after Rg1 administration. Rats were given free access to food and water after administration for 3 h. The volume of urine collected was measured. After air drying, feces samples were weighed and stored at −20°C until use. After thawing, samples were prepared as described in the “Sample preparation”. All animal experiments were conducted in accordance with the institutional guidelines and ethics and approved by the Laboratory Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

3. Results and discussion

3.1. Optimization of LC–MS/MS for quantitative analysis

Optimization of the mobile phase was performed with different compositions of acetic acid, formic acid, and ammonium acetate alone or in combination at different concentrations. A mixture of 0.1% formic acid and 0.1% formic acid in acetonitrile with gradient elution was selected for satisfactory peak shape, resolution and appropriate ionization in the SRM acquisition. Rg1, Rh1, Ppt, and IS were eluted at retention times of 1.5, 2.8, 5.0 and 6.8 min, respectively. The typical mass spectrum of the Rg1 and its metabolites in rat bile, urine and feces is shown in Fig. 2. Using this method, we were able to quantify the concentrations of Rg1 and all metabolites with high sensitivity within 10 min following sample injection.

Results in both positive and negative ionization modes were compared. The molecular ions for Rg1, Rh1, and Ppt were the most abundant ions in the positive ion scan mode. Auto product ion scan and SRM optimization were helpful for determining the following selective detection ion pairs: m/z 621.40→405.45 for Rg1, m/z 441.40→441.40 for Rh1, m/z 441.20→123.20 for Ppt, and m/z 345.10→97.10 for IS.

3.2. Selection of IS

The use of an IS in the present methodology facilitates the accurate determination of analyte concentration. The selected IS should have a chemical structure and retention time similar to those of the relevant analytes. Androlin, a stable compound, was selected as IS in our study, since its chemical structure is similar to Rg1 and it did not interact with the sample components. Although androlin is an endogenous compound that might cause matrix interference, its concentration in blank samples was below LLOQs and did not affect the detection of Rg1 or its metabolites in bile, urine and feces samples in the specificity experiment of Fig. 2. Thus, in the absence of an ideal stable isotope-labeled analyte, androlin served as an appropriate IS for our study.

3.3. Specificity

Assay selectivity was evaluated by analyzing blank plasma samples obtained from six rats. No interfering peaks were observed during the detection of Rg1 or its metabolites in these samples.

3.4. Calibration curve and lower limit of quantification

The linear range and typical regression equations for the quantification of Rg1, Rh1, and Ppt in bile, urine, and feces are presented in Table 1. The correlation coefficients for these calibration curves were all greater than 0.995. The lower limits of quantification (LLOQs) for Rg1, Rh1, and Ppt in bile were 48.75, 20.80 and 20.40 ng/mL, respectively, and those in urine were 48.75, 26.00 and 25.50 ng/mL, respectively. The LLOQs for Rg1, Rh1, and Ppt in feces were the same as those in urine. Each LLOQ was determined as the lowest concentration point on the standard curve that could be quantitated with an accuracy within ±15% bias of nominal concentration and precision not exceeding 15% coefficient of variation.

3.5. Accuracy and precision

The relative standard deviation (RSD) of the intra- and inter-day analyses was used to assess the precision of the method. Nominal concentration (Cnom) and the mean value of the observed concentration (Cobs) were used to calculate accuracy (%) in the following equation: accuracy (%) = [(Cobs − Cnom)/Cnom] × 100. Precision and accuracy were determined by analyzing high, medium and low standard concentrations of QC samples on the same day (intra-day, n = 5) and continuously for 3 days (inter-day, n = 15). Intra- and inter-day precision and accuracy are summarized in Table 2. The results demonstrated good precision and accuracy of this method.

| Table 1 | Standard curves of Rg1, Rh1, and Ppt in bile, urine and feces. |
|---------|---------------------------------------------------------------|
| Sample  | Compound | Regression equation | R      | Range (ng/mL) |
| Bile    | Rg1      | y = 0.0003x – 0.007 | 0.9955 | 48.75 – 39,000.00 |
|         | Rh1      | y = 0.01x – 0.29    | 0.9988 | 20.80 – 2080.00  |
|         | Ppt      | y = 0.008x – 0.001  | 0.9964 | 20.40 – 2040.00  |
| Urine   | Rg1      | y = 0.0006x + 0.89  | 0.9995 | 48.75 – 9750.00  |
|         | Rh1      | y = 0.003x + 0.10   | 0.9975 | 26.00 – 1040.00  |
|         | Ppt      | y = 0.02x + 0.41    | 0.9982 | 25.50 – 1020.00  |
| Feces   | Rg1      | y = 0.003x + 0.12   | 0.9999 | 48.75 – 9750.00  |
|         | Rh1      | y = 0.02x + 1.03    | 0.9999 | 26.00 – 10,400.00 |
|         | Ppt      | y = 0.009x + 0.47   | 0.9997 | 25.50 – 10,200.00 |
3.6. Extraction recovery

To minimize interference for mass analysis, the liquid–liquid extraction procedure was established for sample preparation. Extraction solvents of ether, dichloromethane, n-butanol and ethyl acetate alone or in combination in different proportions were compared. The extraction recoveries of Rg1, Rh1 and Ppt from rat bile, urine or feces were obtained by comparing the peak area for each extracted analyte (ER1) with that for blank extracts spiked with standard solution (ER2), which were expressed as (ER1/ER2) × 100%. QC samples at three concentrations were evaluated. The best extraction was achieved using ether-dichloromethane-n-butanol (2:1:1, v/v/v). The extraction recoveries of Rg1, Rh1 and Ppt in bile, urine and feces exceeded 70% and were consistent and reproducible.

3.7. Stability

Sample stability was evaluated by calculating the peak areas for the analytes after storing QC samples in the autosampler at 4 °C and at ambient temperature for 24 h. The concentrations of Rg1, Rh1 and Ppt in bile, urine and feces samples deviated less than ±15% from those in fresh QC samples, indicating no significant substance loss during storage.

3.8. Excretion of Rg1 and its metabolites

After oral administration of Rg1 to SD rats at a dose of 25 mg/kg, the concentrations of Rg1 and its metabolites Rh1 and Ppt were simultaneously determined by HPLC-MS/MS to assess the excretion profile of Rg1 in bile, urine and feces samples. Within 12 h, Rg1 excretion via urine was completed, and within 48 h, Rg1 excretion via bile and feces was completed. Furthermore, the maximum concentrations of Rg1 were observed from 0–4 h in urine, 0–3 h in bile, and 10–24 h in feces. The Rh1 concentration in urine and the Ppt concentration in urine and in bile could not be determined. The maximum concentration of Rh1 was observed from 0–3 h in bile and 10–24 h in feces, and the maximum concentration of Ppt was observed from 24–36 h in feces. The mean recoveries of Rg1 in bile, urine, and feces were 6.88 ± 2.56%, 0.038 ± 0.021%, and 40.11 ± 12.74%, respectively. Those of Rh1 in urine and feces were 0.085 ± 0.018% and 22.19 ± 5.80%, respectively, and that of Ppt in feces was 22.88 ± 7.00%. The results are summarized in Tables 3–5, and accumulated excretion of Rg1 and its metabolites in bile, urine and feces were shown in Fig. 3.

As we know, ginsenosides were reported to show high polarity, and thus exhibit very low oral bioavailability. However, the well-established efficacies of these drugs (e.g., modulating blood pressure, metabolism, and immune functions) have been validated...
Table 3  The excretion of Rg1 and its metabolites in rat bile.

| Time (h) | Rg1 | Rh1 |
|---------|-----|-----|
|         | Excretion amounts in bile (μg) | Excretion rate in bile (%) | Excretion amounts in bile (μg) | Excretion rate in bile (%) |
| 0–3     | 138.14 ± 88.07 | 2.08 ± 1.28 | 1.29 ± 0.60 | 0.02 ± 0.01 |
| 3–6     | 56.72 ± 35.53  | 0.85 ± 0.53  | 0.77 ± 0.29  | 0.01 ± 0.006 |
| 6–12    | 57.01 ± 21.83  | 0.86 ± 0.33  | 0.50 ± 0.32  | 0.009 ± 0.006 |
| 12–24   | 125.67 ± 42.70 | 1.90 ± 0.67  | 0.97 ± 0.83  | 0.02 ± 0.01 |
| 24–36   | 71.29 ± 24.28  | 1.08 ± 0.38  | 0.69 ± 0.41  | 0.01 ± 0.008 |
| 36–48   | 7.39 ± 3.34    | 0.11 ± 0.06  | 0.34 ± 0.17  | 0.006 ± 0.003 |
| 48–72   | ND             | ND            | ND           | ND          |
| Total   | 456.23 ± 173.54| 6.88 ± 2.56  | 4.56 ± 1.15  | 0.08 ± 0.02 |

ND: Not detected. Data are mean ± SD, n=5.

Table 4  The excretion of Rg1 and its metabolites in rat urine.

| Time (h) | Excretion amounts of Rg1 in urine (μg) | Excretion rate in urine (%) | Excretion amounts of Rh1 and Ppt in urine (μg) |
|---------|---------------------------------------|-----------------------------|-----------------------------------------------|
| 0–4     | 1.30 ± 0.56                           | 0.02 ± 0.01                 | ND                                            |
| 4–8     | 0.42 ± 0.36                           | 0.008 ± 0.007               | ND                                            |
| 8–12    | 0.41 ± 0.44                           | 0.007 ± 0.008               | ND                                            |
| 12–18   | ND                                    | ND                          | ND                                            |
| 18–24   | ND                                    | ND                          | ND                                            |
| Total   | 2.13 ± 1.09                           | 0.04 ± 0.02                 | ND                                            |

ND: Not detected. Data are mean ± SD, n=5.

Table 5  The excretion of Rg1 and its metabolites in rat feces.

| Time (h) | Rg1 | Rh1 | Ppt |
|---------|-----|-----|-----|
|         | Excretion amounts in feces (μg) | Excretion rate in feces (%) | Excretion amounts in feces (μg) | Excretion rate in feces (%) |
| 0–10    | ND  | ND  | ND  |
| 10–24   | 1519.15 ± 696.71 | 27.43 ± 12.94 | 491.77 ± 246.63 | 8.87 ± 4.02 |
| 24–36   | 754.54 ± 258.70 | 15.35 ± 1.91  | 629.36 ± 280.42 | 11.23 ± 4.34 |
| 36–48   | 22.36 ± 45.92  | 0.68 ± 1.06  | 137.93 ± 167.63 | 2.51 ± 2.66 |
| 48–54   | ND             | ND            | 8.14 ± 10.38    | 0.15 ± 0.16 |
| 54–60   | ND             | ND            | 5.00 ± 3.55     | 0.26 ± 0.24 |
| 60–72   | ND             | ND            | 3.01 ± 2.39     | 0.31 ± 0.43 |
| Total   | 2231.03 ± 684.40 | 40.11 ± 12.74 | 1236.36 ± 349.55 | 22.19 ± 5.80 |

ND: Not detected. Data are mean ± SD, n=5.

Figure 3  Accumulated excretion of Rg1 and its metabolites in bile (A), urine (B) and feces (C) (n=5).
after oral administration\textsuperscript{21,22}, seemingly in contrast with the low bioavailability. The present excretion results may help to resolve the paradox, since Rg1 is poorly absorbed into blood and instead quickly excreted through feces. Moreover, previous reports indicated that intestinal bacteria, which are rich in β-glucosidase, could transfer the drug, which contains a glycosidic bond, via a hydrolysis reaction\textsuperscript{23}. The cumulative excretion results for Rh1 and Ppt, the deglycosylated metabolites of Rg1, in feces indicated that the gastrointestinal tract is likely the major metabolic site of Rg1.

4. Conclusions

In the present study, we have developed a rapid, highly selective, and reliable HPLC–MS/MS method for simultaneous quantification of Rg1, Rh1, and Ppt concentrations in biological samples after oral administration. This method was successfully applied to a comprehensive quantification study of Rg1 and its metabolites in bile, urine and feces. In addition, the validated bianalytical method exhibited sufficient specificity to separate the analytes from interfering substances in complex matrices, which is necessary to support the pharmacokinetic studies. Furthermore, orally-administered Rg1, known to produce powerful systemic actions, exhibited low systemic pharmacokinetic studies. Furthermore, orally-administered Rg1, known to produce powerful systemic actions, exhibited low systemic absorption and pharmacokinetic behavior of ginsenoside Rg1 from Panax notoginseng in rats. J Ethnopharmacol 2003;84:187–92.

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