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A Simple HPLC Assay for Ginsenoside-Rh2 in Plasma and Its Application for Pharmacokinetic Study in Rats

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Abstract

Ginsenoside-Rh2 (G-Rh2) is an important constituent in ginseng and has significant anti-tumor property. A simple HPLC assay was developed to study pharmacokinetics of G-Rh2 in rat plasma. The HPLC consisted of a C_{18} reversed phase analytical column, a variable wavelength ultraviolet (UV) spectrophotometric detector, and a mobile phase made up of 0.005 M KH_2PO_4 (pH 7.2): acetonitrile: methanol (23:7:70). The system was operated at ambient temperature isocratically at a flow rate of 0.5 mL/min and wavelength at 203 nm. Extraction of G-Rh2 from plasma was achieved by solid phase extraction (SPE) using 100 mg/mL C_{18} SPE columns. The results showed that standard curves using 50 μL of plasma sample were linear from 0.25 to 100 μg/mL, with regression coefficient (r^2)>0.99. The intra- and inter-assay variations over a 3-month study period were <10% and <20%, respectively. After 10 mg/kg twice daily for 4 doses by subcutaneous (sc) injection, the mean maximum plasma concentration (C_{max}), and time to C_{max} (T_{max}) of G-Rh2 was 0.79 μg/mL and <0.5 hour, respectively. The described HPLC is readily performed in most laboratories and should have adequate sensitivity and specificity to study pharmacokinetics of G-Rh2 in rats following multiple doses.

Keywords: Ginsenoside-Rh2; Ginseng; HPLC; Pharmacokinetics; Rats

Introduction

Natural products such as traditional Chinese medicines (TCM) are increasingly used in our societies to enhance health and prevention of chronic diseases [1-9]. There are also evidences to suggest that these herbal products may enhance the therapeutic effects of conventional medicines and they should be incorporated in prescription drug therapies [8,10-12].

Ginseng is a well-known medicinal herb native to China and Korea, and has been used as a herbal remedy in eastern Asia for thousands of years [13]. In TCM, ginseng is used for a variety of illnesses such as anorexia, shortness of breath, palpitation, insomnia, impotence, hemorrhage and diabetes. In western medicines, however, it is recognized for its effect on physical and psychomotor performance, cognitive function, immunomodulation, diabetes mellitus, improving cardiovascular risk factors, quality of life, as well as for counteracting adverse effects [9]. It is known that ginseng contains close to 40 different ginsenosides some of which have potent and varying pharmacologic properties [14-16] including anti inflammatory and anti-tumor effects [17,18]. 20(S)-Ginsenoside-Rh2 (G-Rh2) is a trace constituent in ginseng which was first isolated from red ginseng by Kitagawa et al. [19]. It has an aglycone skeleton of 20(S)-protopanaxadiol dammarane structure (Figure 1) and exhibits cytotoxic effects in various cancer cells *in vitro* [20,21]. G-Rh2 has been shown to reverse resistance developed for daunomycin or vinblastine by P388/Adr cancer cells [22], synergistically enhance the activities of paclitaxel and mitoxantrone in prostate cancer cells [23], and increase the antitumor activity of cyclosposphamide while decreasing its genotoxic effects [24,25]. There is also evidence to suggest that G-Rh2 could increase the absorption of drugs which are substrates of P-glycoprotein in rats [26]. Thus G-Rh2 has considerable therapeutic potential for oncology therapy, and understanding the pharmacokinetics of G-Rh2 could help to better design dosage and route of administration for its optimal use to aid cancer chemotherapy.

There have been several HPLC methods reported for ginsenosides to-date [27-37]. Most of these assays, however, are either cumbersome requiring gradient separation or fluorescent derivatization, and that they also require expensive instrumentation such as mass spectrometer (MS) which is not readily available in most routine laboratories. Thus there is need for a rapid and less expensive method which could be adopted for pharmacokinetic and biopharmaceutical studies in a more general setting. The current paper describes development and validation of a simple HPLC assay which is readily accessible and has adequate sensitivity and specificity for determining plasma concentration of G-Rh2. The assay was applied successfully to study the pharmacokinetics of G-Rh2 in rats after receiving 4 doses of 10 mg/kg of G-Rh2 given twice daily by subcutaneously injection.

Materials and Methods

Chemicals

G-Rh2 was isolated and purified from Panax ginseng C.A. Meyer cv. Silvatica. purchased from the Institute of Frontier Medical Science of Jilin University (Changchun, China), and was >98% pure as determined by HPLC [38]. Imipramine (IMI) was purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA) (Figure 1). Other pharmaceutical products tested for interference with the assay were either received as gifts from their respective manufacturers, or purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). Solid phase extraction (SPE) columns were C_{18} materials (100 mg/mL) purchased commercially (Extra-Sep®, Chromatographic Specialties Inc., Brockville/ ON, Canada.). Solvents were HPLC grade and other chemicals were reagent grade (Fisher Scientific, Ont., Canada).

HPLC system

The HPLC system consisted of a Beckman 114 M solvent delivery

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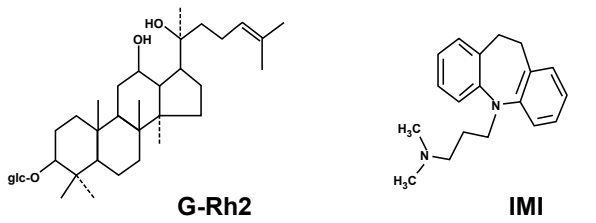

Figure 1: Chemical structures of G-Rh2 and imipramine (IMI).

module (Berkeley, CA, USA), a Rheodyne syringe loading injector (model 9725) with a 100 µL PEEK injection loop (Scientific Products & Equipment, Concord, ON, Canada), a Shimadzu ultraviolet (UV) spectrophotometric detector (UV VIS SPD-20A, Man-Tech Assoc. Inc., Guelph, ON, Canada), and a Hewlett-Packard HP3395 Integrator (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a 3 µm 110 A 150×3.0 mm i.d. C_{18} reversed phase analytical column (Gemini®-NX, Phenomenex, Torrance, CA 90501, USA) coupled with a 5 µm 4.0×3.0 mm i.d. C_{18} reversed phase guard column (Security® Guard Cartridges, Phenomenex, Torrance, CA 90501, USA) using a mobile Phase of a mixture of 0.005 M potassium phosphate buffer at pH 7.2 (KH_2PO4):acetonitrile:methanol (23:7:70).

The system was operated at room temperature isocratically with a flow rate of 0.5 mL/min at an operating pressure of 2.5 kpsi (ca. 183 kgf/cm^2). G-Rh2 and IMI were detected and quantified at 203 nm. Detector output was recorded by an integrator (Hewlett-Packard HP3395 Integrator/Palo Alto, CA, USA), and digitalized using the Peak Simple® software (Chromatographic Specialties Inc., Brockville/ ON, Canada).

Preparation of standard solutions

Stock solutions of G-Rh2 and IMI were prepared in methanol at 1 mg/mL and 0.1 mg/mL, respectively. Serial dilution of the stock solutions using HPLC water was performed to prepare standard spiking solutions for preparing plasma standards of 100, 40, 10, 4, 2, 1, and 0.25 µg/mL. The working internal standard (IS) solution of 2 µg/mL was prepared by diluting the stock IMI solution with the HPLC water. Intra-assay and inter-assay variations were assessed over a 3-month study period using quality control (QC) samples at 1 and 10 µg/mL. These spiking solutions should be prepared on the same day of the extraction.

Solid phase extraction (SPE)

50 µL of each of the spiking solution of G-Rh2 or HPLC water was added to 50 µL of rat blank plasma or study plasma sample in a 1.5 mL polyethylene micro-centrifuge tube, respectively, followed by 50 µL of 0.01 M KH_2PO_4 buffer solution (pH 6.5), and then 50 µL of the working IS solution containing 0.1 µg of IMI. Each tube was gently mixed, and add to it was 150 µL of 62% cold methanol followed by vortex mix for 5 min at 1200 rpm in room temperature (Eppendorf Model 5436 Thermomixer, Hamburg, Ger) to precipitate the plasma proteins. After centrifugation at 5000xg for 10 min at room temperature (Eppendorf Model 5415 Microcentrifuge, Hamburg, Ger), the supernatant fluid was collected and loaded on top of the C_{18} 100 mg/mL SPE column, and allowed to equilibrate for 5 min before passing it through the column slowly (ca. 1 mL/5 min) at a pressure of 10 inches of Hg (Vac-Elut™, Varian, Harbor City, CA, USA). Each SPE column was washed with 2×1 mL of 62% methanol in water and then air dried at a vacuum of 30 inches of Hg for 30 min. G-Rh2 and the IS were recovered from the column by eluting with 2×0.5 mL of 90% methanol in water. The filtrate was collected into a round bottom glass culture tube (Kimax®, Fisher Scientific Co., Ottawa, ON, Canada), and evaporated to dryness under a gentle stream of nitrogen at 55°C (Thermolyn Dri-Bath®, Fisher Scientific Co., Ottawa, ON, Canada). The residues were stored at -20°C until analysis. Each sample was reconstituted in 200 µL of mobile phase immediately prior to injection, and an aliquot (10-50 µL) was injected into the HPLC.

Pharmacokinetics study

The study protocol was approved by the Dalhousie University Committee on Laboratory Animals (UCLA) using the Canadian Council of Animal Care (CCAC) guidelines. Male SD rats (300-350 g) with a carotid artery catheter were purchased from Charles River Laboratories (Wilmington, DE, USA). They were each (n=6) given twice daily G-Rh2 (10 mg/kg) for 4 doses by subcutaneous (sc) injection. Blood samples (0.3 mL each) were obtained from the catheter serially before the last dose, and at 0.1, 0.25, 1, 1.2, 1.5, 2, 3, 4, 5 and 6 hours post dose. The plasma (>0.1 mL) was immediately separated by centrifugation (4°C, 1720×g, 5 min) and then stored at –80°C until analysis. An aliquot of the plasma samples (50 µL) was used for analysis of G-Rh2 by the described HPLC. All the samples were analyzed within 3 months after collection.

Data analysis

Recoveries of G-Rh2 and IMI from plasma samples were determined by measuring the amounts (expressed as peak heights) after the SPE extraction, and compared the values to the amounts added to the QC samples. Standard curves were plotted using known plasma concentrations of G-Rh2 (x-axis) and the peak height ratios (PHR) of G-Rh2 to the IS (y-axis) from 0.25 to 100 µg/mL, and the data analyzed by linear regression (Lotus 1-2-3, IBM Canada). The QC samples at each concentration were performed in 4 replicates. Intra- and inter-assay variations were assessed from the in study QC samples (1 and 10 µg/mL) for each batch analysis over a 3-month period. The intra-assay variation was the average coefficient of variation (%CV) assessed over the study period, whereas the inter-assay variation (%CV) was determined using the mean PHR of the QC samples obtained from each period. Sensitivity of the assay was assessed by determining the smallest amount of G-Rh2 injected on-column which resulted in a signal to noise ratio of greater than 3. The lower limit of quantization (LLQ) of the assay was determined by the lowest concentrations of G-Rh2 measurable with a CV of less than 15%. Accuracy was assessed by comparing the concentrations determined by the assay with the spiked concentrations of the QC samples. Pharmacokinetic variables such as area under the curve (AUC), maximum plasma concentration (C_{max}), time to maximum concentration (T_{max}) and half-life ($T_{½}$) were calculated using Rstrips® (MicroMath Scientific Software, St. Louis, MO, USA) assuming one compartment model after first order input (sc injection). The mean plasma concentration-time data from the rats (n=6) were used for data fitting and calculation of the pharmacokinetic parameter estimates.

Results

Under the described chromatographic conditions, the average retention times of IMI, and G-Rh2 were 7.6 and 24 minutes, respectively (Figure 2). The standard curves of the HPLC assay for G-Rh-2 constructed during method development were linear between 0.25 to 100 µg/mL with regression coefficients (r^2)>0.99. A typical standard curve is shown in (Figure 3). The recoveries using the described SPE were >85% for G-Rh2 and the IS (IMI). The sensitivity of the assay based on absolute on-column injection of G-Rh2 was 5 ng which produced a signal to noise ratio >3 (Figure 2). The LLQ was <0.25 µg/mL using 50

μL of plasma sample with CV of <15%. The intra assay variations at 1 and 10 μg/mL determined from 4 study batches (n=4 in each batch) over a 3-month period were <10%; and inter-assay variations were <20%. Accuracy of the assay was 100% and 107% for the 10 and 1 μg/mL QC samples, respectively, over the same study period (Table 1). We have tested a series of natural products and therapeutic agents which may be encountered in concurrent pharmacotherapy, and shown that there was no interference from any of the agents tested (Table 2).

Plasma concentration of G-Rh-2 was still detectable in some of the rats at the 0 h sample before the last injection. On average the plasma concentrations of G-Rh2 over the 6 h study period was 0.50 ± 0.24 μg/mL, and it was barely measurable in the rats 6 h after the injection, and thus the 6 h sample was not included in the pharmacokinetic analysis. The mean plasma concentration-time data could be adequately described by a one compartment open model following first order input with r^2=0.9976 and $T_{1/2}$=2.8 h. Using a 2-compartment model for data analysis produced similar terminal $T_{1/2}$ and r^2 value. Absorption of G-Rh2 was rapid after sc injection with T_{max} occurred in less than 0.5 h (Figure 4). There were considerable differences in the plasma concentrations of G-Rh2 between the rats particularly towards the end of the experiment (Figure 4) with coefficient of variation (CV) averaging close to 50% over the study period.

Discussion

Despite many HPLC assays which have been reported for ginsenosides to-date [27-29,31,32,34-37,39,40], there is still need for specific HPLC assay that is simple, easily accessible and sensitive enough for pharmacokinetics study of G-Rh2. Most of the HPLC assays reported for ginsenosides were based on tandem HPLC/MS techniques which are not readily amenable for routine analyses in general laboratory setting. On the other hand, the HPLC assay described by Shangguan and co-workers did not require mass-spectrometry, but it needed chemical derivatization followed by detection with fluorescent detector [28]. The only other non-mass spectrometric method as described by Wang and co-workers used similar UV detection, but it was not specific for G-Rh2 [27]. The HPLC method described in the current communication employed a 3 μm 150×3 mm ID column, which minimized mobile phase consumption and kept the cost down for the analysis. The method is specific for G-Rh2 as it is separated from other ginsenosides such as ginsenoside-Rg1 (G-Rg1) and ginsenoside-Rg3 (G-Rg3) (Table 2). Thus the method is simple, specific, economical to run, and should be easily adoptable in most laboratory settings.

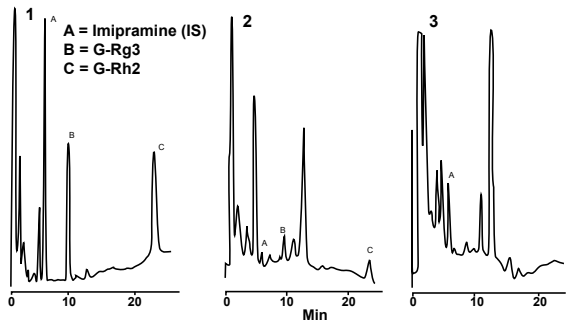

Figure 2: HPLC chromatograms of G-Rh2, G-Rg3 and the internal standard IMI. (1) Standard solution containing 0.1 μg of each of G-Rg3, G-Rh2 and IMI. (2) Plasma standard containing 10 μg/mL of G-Rh2. (3) Plasma blank.

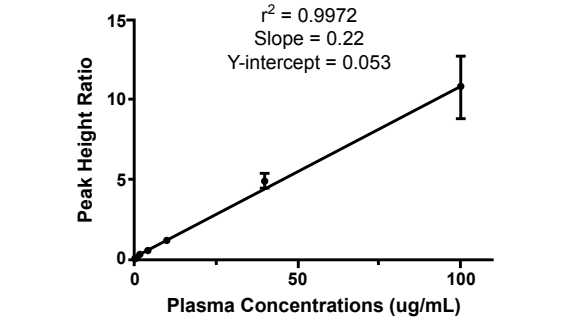

Figure 3: Typical standard curve of G-Rh2 in rat plasma. Each point represents mean ± SD of 4 replicates.

Period[a] / Concentrations	10 μg/mL		1 μg/mL	
	Intra-assay %CV	Accuracy %	Intra-assay %CV	Accuracy %
Batch No 1	8%	99%	4%	100%
Batch No 2	14%	100%	4%	110%
Batch No 3	9%	100%	9%	113%
Batch No 4	7%	100%	15%	105%
Mean	9.7%	99.8%	8.1%	107.0%
Inter-assay Variation (%CV)	17.1%		13.9%	
[a]Over 3 months				

Table 1: Intra- and inter-assay variations of the HPLC assay of G-Rh2 in plasma.

Drug test	Retention time (min)	Drug test	Retention time (min)
Ginsenoside-Rg1	24 min	Imipramine	7.6 min
Ginsenoside-Rg1	3.2 min	Digoxin	2.5 min
Ginsenoside-Rg3	10 min	Clomipramine	8.3 min
Hydroxysafflor Yellow A	5.4 min	Irinotecan	3.7 min
Neomycin	>60 min	Losartan	2.4 min
Bosentan	>30 min	Diltiazem	3.5 min
CoQ10	>60 min	Dipyridamole	3.6 min

Table 2: Retention times of drugs tested for interferences.

The method utilized SPE technique coupled with reversed phase HPLC separation and UV detection using 62% methanol in water as a cleanup step before desorbing the analytes with 90% methanol. The extraction method achieved over 85% recoveries for G-Rh2 and the IS. During method development we also explored G-Rg3 as internal standard as it is chemically similar to G-Rh2 and had a retention time of about 10 min which should make it more suitable for the HPLC assay. However, the recovery was poor (<20%) and highly variable using the described SPE, for that we chose to use IMI despite having a more distinct chemical structure and less retained in the column (retention time 7.6 min). We have found that separation of imipramine (IS) from endogenous plasma materials was sensitive to mobile phase composition. Using 60% methanol in water (*vs.* 62%) as a cleanup step resulted in much dirtier sample and more interference from plasma matrix. On the other hand, using higher concentration of methanol in the cleanup step led to significant reduction of recovery from the SPE. Both precision as well as accuracy of the assay were within acceptable limits (± 10%) for the high (10 μg/mL) and low (1 μg/mL) QC samples. The assay was also robust enough with an inter-assay variation of <20% over the 3-month period (Table 1). Further, none of the compounds we tested in (Table 2) interfered with the assay. These include commonly used therapeutic products that could be potentially taken together with G-Rh2 in other research study and/or clinical situations. These are desirable attributes for an analytical method particularly for pharmacokinetic and drug interaction studies.

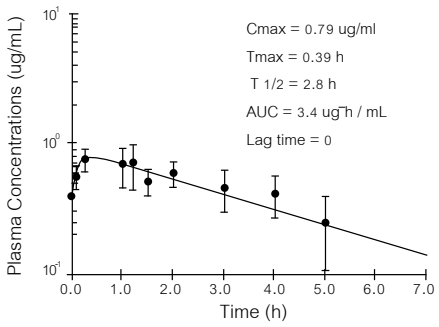

Figure 4: Mean plasma concentration – time profile of G-Rh2 in rats following 10 mg/kg given twice daily for 4 doses by sc injection. Each point represents mean ± SEM from 6 samples.

The current HPLC assay is not as sensitive as the LC/MS assays previously reported for G-Rh2 [32,36,40]. However despite the limited sensitivity, we were still able to measure plasma concentrations of G-Rh2 up to 5 h for pharmacokinetic measurement in rats following 4 doses of 10 mg/kg given by sc injection. Based on the pilot study, it appeared that G-Rh2 was rapidly absorbed following sc injection with T_{max} attained at <0.5 h after injection. After that plasma concentration of G-Rh2 followed a mono-exponential decline with $T_{1/2}$ about 3 h, which was adequately characterized by a one-compartment model with 1st order input. However, it should be noted that the 6 hr sampling time was inadequate for an accurate estimate of $T_{1/2}$. In addition, since G-Rh2 was present in the plasma samples collected before the last injection in some rats, it is probable that G-Rh2 was extensively distributed into extra-vascular tissues, and released back to circulation from tissue compartment beyond the 6 h sampling period. Thus the $T_{1/2}$ reported in this study was only an approximate which may underestimate the true terminal $T_{1/2}$. Very little is known of the pharmacokinetics of G-Rh2 in humans or animal models. One brief report which used a LC/MS method to measure plasma G-Rh2 in rat (n=3) up to 1 h following a single intravenous injection of 5 mg/kg [36]. The duration was too short in this study to provide meaningful estimates of pharmacokinetic parameters. Another study using LC/MS which measured G-Rh2 up to 24 h in rats following a single intragastric (ig) or intravenous (iv) administration. It reported for G-Rh2 a $T_{1/2}$ of 4-5 h which is similar to the 3 h found in the current study. The absolute oral bioavailability was <10% in rat, but was considerably higher in dog (20-30%) [41]. Thus despite the limitation, the current HPLC can provide a reasonable estimate of the pharmacokinetic of G-Rh2 in rats following multiple injections (Figure 4). However, it remains to be tested if the assay has adequate sensitivity for study requiring a lower dose or oral route of administration.

While no interference was found from a wide variety of therapeutic agents (Table 2), it is still questionable if the method has enough sensitivity for clinical therapeutic study which employs a lower dose. The current HPLC uses 50 µL of plasma sample and has a LLQ of about 0.25 µg/mL. It may be possible measuring lower than 20 ng/mL if a larger plasma sample size is used (e.g. 1 mL or larger). However, it will need to be evaluated further for therapeutic monitoring in a clinical setting.

In summary, the HPLC assay as described is simple, economical to run, readily accessible in general laboratory setting and have adequate sensitivity and specificity to determine G-Rh2 concentrations in plasma for pharmacokinetics studies in rats after multiple doses given by subcutaneous injection. The suitability for clinical study awaits further investigation.

Acknowledgements

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