Pharmacokinetic Study of Ginsenosides Rb1 and Rg1 in Rat by ELISA Using Anti-ginsenosides Rb1 and Rg1 Monoclonal Antibodies

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Abstract: Enzyme-linked immunosorbent assay (ELISA) systems using anti-ginsenoside Rb1 (G-Rb1) and Rg1 (G-Rg1) monoclonal antibodies (MAbs) were established for pharmacokinetic investigations of G-Rb1 and G-Rg1 in rat serum. The systems not only allowed sensitive detection of G-Rb1 at the level as low as 20 ng/ml and of G-Rg1 at 300 ng/ml, but showed strong capacity for detecting the two agents in a broad concentration range (20 to 400 ng/ml for G-Rb1 and 0.3 to 10 µg/ml for G-Rg1, respectively). In this respect, these assay systems are superior to other methods using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). In addition, another advantage of these immunoassays is the comparably low quantities of specimen required; as little as 5 µl of serum suffices the need for determination of ginsenosides. We report in this article the application of this immunoassay in pharmacokinetic study of G-Rb1.

Keywords: Ginsenosides; Monoclonal Antibody; Enzyme-linked Immunosorbent Assay; Eastern Blotting; Pharmacokinetics.

Introduction

Ginseng is one of the most important and highly valued traditional Chinese medicines (TCM) with a documented history of application for therapeutic purposes for over 2000 years in China, and is now used worldwide as a tonic in treatment of stress, cancer, central nervous system disturbances, hypothermia and radio-protection (Tanaka, 1973; Shibata,
1982). It contains many dammarane and oleanane saponins (Besso et al., 1982; Koizumi et al., 1982), polyacetylene derivatives (Hansen and Boll, 1986) and polysaccharides (Tomoda et al., 1993), whose biological activities have been studied widely.

Ginsenoside Rb1 (G-Rb1), one of the main 20 (S)-protopanaxadiol type saponins, possesses vasodilating and anti-inflammatory capacities, and is capable of depressing the central nervous system (CNS). Ginsenoside Rg1 (G-Rg1), a 20 (S)-protopanaxatriol type saponin with hemolysis and anti-fatigue activities, produces, however, a CNS-exciting effect (Zheng and Cai, 2003) (Fig. 1). Odani et al. (1983) investigated the pharmacokinetics of G-Rb1 using TLC, but the amount of G-Rb1 absorbed from the digestive tract of rats remained rather low. Recently, Xu et al. (2003) reported the pharmacokinetics of G-Rb1 and -Rg1 in rats on the basis of HPLC and suggested the necessity of serum sample pretreatment and the minimal requirement of 1 ml of blood sample for the determination.

In our ongoing study of the formation of MAbs against naturally occurring bioactive compounds, we have prepared MAbs against G-Rb1 (Tanaka et al., 1999) and G-Rg1 (Fukuda et al., 2000), respectively. Currently, there are no other reports of MAbs related to ginsenosides that have been published, besides our own, although an immunological approach for quantitative assay of ginsenosides using polyclonal antibodies had been investigated by Sankawa et al. (1982). In this article, we reported the establishment of a highly sensitive assay, namely competitive enzyme-linked immunosorbent assay (ELISA) with highly specific MAbs against ginsenosides for determination of G-Rb1 and G-Rg1, with which we performed a pharmacokinetic study of ginsenosides in rats.

Figure 1. Chemical structures of ginsenosides.

Materials and Methods

Chemicals and Immunochemicals

G-Rb1 and -Rg1 were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). BSA and HSA were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided by ICN Biochemicals, Inc. (Aurora, OH, USA). All other chemicals were standard commercial products of analytical grade.

Synthesis of Antigen Conjugates

G-Rb1 and -Rg1 carrier protein conjugates were synthesized by a modification of the procedure already used for solamargine (Ishiyama et al., 1996), which is based on the method of Erlanger and Beiser (1964). G-Rb1 or G-Rg1 (0.7 ml, 10 mg individually) was added dropwise into an H2O solution (0.5 ml) containing NaIO4 (4 mg), 80% MeOH solution and stirred at room temperature for 1 hour. A carbonate buffer solution (pH 9.6, 1 µg/ml) containing BSA (10 mg) was then added into the mixture and stirred at room temperature for 5 hours. The reaction mixture was dialyzed 5 times against H2O (deionized), and then lyophilized to prepare G-Rb1 or G-Rg1 conjugate.

Purification of MAb

A MAb was purified using a Protein GFF column (0.46 × 11 cm, Amersham Biosciences Corp., Piscataway, NJ, USA). The culture medium (500 ml) containing the IgG was adjusted to pH 7.0 with 1 M Tris-HCl buffer (pH 9.6) and subjected to the column, and the column was then washed with 10 mM phosphate buffer (pH 7). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 3). Eluted IgG was neutralized with 1 M Tris-HCl buffer, then dialyzed 3 times against phosphate buffered saline (PBS) (pH 7.4), and finally lyophilized.

Direct ELISA Using G-Rb1-HSA

The reactivity of MAb to G-Rb1-HSA was determined by an ELISA. G-Rb1-HSA conjugate (100 µl, 1 µg/ml) was adsorbed to the wells of a 96 well-immunoplate (Nalge Nunc Int., Corp., Rochester, NY, USA) then treated with 300 µl of PBS containing 5% of skim milk (SPBS) for 1 hour to reduce non-specific adsorption. The plate was washed three times with PBS containing 0.05% of Tween 20 (TPBS) and reacted with 100 µl of testing MAb solution for 1 hour. The plate was washed three times with TPBS, and then MAb combined with 100 µl at a 1:1000 dilution of peroxidase-labeled anti-mouse IgG solution (ICN Biochemicals, Inc.) for 1 hour. After washing the plate three times with TPBS, 100 µl of substrate solution, [0.1 M citrate buffer (pH 4.0) containing 0.003% H2O2, and 0.3 mg/ml ABTS (Wako Pure Chemical Ind., Ltd., Osaka, Japan)] was added to each well and incubated for 20 min. Absorbance was measured by a microplate reader (ImmunoMini NJ-2300, Nalge Nunc Int., Corp., Rochester, NY, USA) at 405 nm. All reactions were carried out at 37°C.
Competitive ELISA

G-Rb1-HSA (5 molecules of G-Rb1 per molecule of HSA) (100 µl, 1 µg/ml) was adsorbed to the wells of a 96 well-immunoplate and then treated with 300 µl SPBS for 1 hour to reduce non-specific adsorption. Fifty microliters of various concentrations of G-Rb1 dissolved in 20% of MeOH solution was incubated with 50 µl (IgG: 1.0 µg/ml) of anti-G-Rb1 MAb solution for 1 hour. The plate was washed three times with TPBS; peroxidase-labeled anti-mouse IgG (100 µl, at 1:1000) was added to each well and incubated for 1 hour. After washing the plate three times with TPBS, 100 µl of ABTS solution was added to each well and incubated for 15 min. The absorbance was measured by the microplate reader at 405 nm. Competitive ELISA for G-Rg1 was carried out in the same manner as for G-Rb1.

HPLC Instruments and Conditions

The chromatograph used in this study consisted of a LC-10AD equipped with a SPD-10A vp UV detector (Shimadzu, Kyoto, Japan) and a Chromatopac C-R8A data analyzer system (Shimadzu, Kyoto, Japan). The analytical column was a Cosmosil 5 C18-AR column (4.6 × 150 mm i.d., Nacalai Tesque, Kyoto, Japan) maintained at room temperature. The mobile phase for G-Rb1 was acetonitrile-water (30:70, by volume) containing 50 mM KH$_2$PO$_4$, and that for G-Rg1 was acetonitrile-water (20:80, by volume) containing 0.5% H$_3$PO$_4$. The flow rate was 1.0 ml/min. UV absorbance detection at 202 nm was used for monitoring the effluent. In these conditions, the retention times of G-Rb1 and G-Rg1 were 13 min and 22 min, respectively.

Animals

The experimental animals used were male Sprague-Dawley rats, weighing 180–220 g, from Kyudo Co., Ltd. (Kumamoto, Japan).

Sample Preparation

The blood sample was centrifuged at 10,000 rpm for 10 min to obtain the serum, which was then mixed with 9 volumes of methanol, followed by thorough vortex shaking. After centrifugation at 10,000 rpm for 10 min, the supernatant was first diluted by 4 volumes of H$_2$O, and then with 20% methanol solution to prepare the sample solution for ELISA.

Recovery of G-Rb1 and G-Rg1 Added to Serum Samples

After the extraction procedures, recoveries of G-Rb1 and G-Rg1 from the serum spiked with different amounts of G-Rb1 and G-Rg1 were calculated by comparing the data of
spiked samples with those of non-spiked samples. Standard deviations were determined from individual triplicate experiments using the method mentioned above.

**Determination of G-Rb1 and G-Rg1 in Blood Samples**

G-Rb1 or G-Rg1 solution in NS (normal saline) of 2 mg/ml was injected in rats through the tail vein at the dose of 5 mg/kg. Blood samples of rats with G-Rb1 administration were collected at 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 60, 72 hours after injection, and those of rats given G-Rg1 were collected at 0.08, 0.17, 0.33, 0.5, 1, 2, 4, 8, 12 hours after injection. Blood samples were centrifuged at 10,000 rpm for 10 min to obtain the sera, which were stocked at −20ºC until analysis. The concentrations of G-Rb1 and G-Rg1 in the sera were determined by competitive ELISA described above.

**Pharmacokinetic Analysis**

The 3p87 program (a software for pharmacokinetic analysis specially developed by the China Pharmacology Society) was used for pharmacokinetic analyses.

**Eastern Blotting for G-Rb1 on Polyethersulfone (PES) Membrane**

G-Rb1 of different amount were loaded onto PES membrane, then developed with acetonitrile-water (25:75, by volume) containing acetic acid (1%). After drying, the blotted PES membrane was dipped into NaIO₄ solution (10 mg/ml) and stirred at room temperature for 1 hour. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and stirred at room temperature for 3 hours. After washing the PES membrane with TPBS, the membrane was treated with PBS containing 5% skim milk for 2 hours to reduce nonspecific adsorption. The PES membrane was immersed in anti-G-Rb1 MAb solution and stirred at room temperature for 3 hours. After washing the PES membrane twice with TPBS and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in phosphate buffer containing 0.2% gelatin (GPBS) was added, and the mixture stirred at room temperature for 1 hour. The PES membrane was washed twice with TPBS and water and then exposed to freshly prepared 1 mg/ml 4-chloro-1-naphthol-0.03% H₂O₂ in phosphate buffer for 15 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane allowed to dry.

**Image Analysis System and Image Acquisition**

A graphic analysis system consisting of a personal computer (Macintosh Power Book G4, Apple Computer Inc., Irvine, CA, USA), a public domain program NIH Image 1.62 (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/), a desktop scanner (GT-9700F, Seiko Epson Corporation, Nagano, Japan) and scanning software, Photoshop CS (Adobe Systems Inc., San Jose, CA,
USA) was used. Images were captured as 256 levels grayscale. The file size was 300 k bytes (200 dots per inch resolution). Digital output was transferred from the scanner to the computer and stored as a PICT file.

**Image Analysis Using NIH Image Software**

The immunostained PES membrane grayscale image was loaded and thresholded to make a binary image for the separation of objects and background. Then, we selected the analyze menu options and the area, ellipse major and ellipse minor axis commands to calculate the objects area as previously described (Masters *et al.*, 1992; Root and Wang, 1993).

**Results**

**Assay System**

First, the blood samples were centrifuged at 10,000 rpm for 10 min to obtain the sera. The deproteinization by the addition of 9 volumes of methanol to the sera was carried out as an indispensable process in the preparation of samples for competitive ELISA. After deproteinization, the supernatant was diluted with 20% methanol solution that can be used directly for ELISA. As far as the calibration curve was concerned, 20% methanol solutions of ginsenosides were found to be reasonable. No specially treated working buffer was needed because we found that the calibration curves for spiking standard chemicals in the blank sera were almost the same as those in 20% methanol as shown in Fig. 2.

**Cross-Reactivity**

Cross-reactivity is the most important factor in determining the value of an antibody. Since ELISA for G-Rb1 was established for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross reactivities of the MAb with related various compounds. It became evident that the MAb reacted only very weakly with a small number of structurally related ginsenosides, G-Rc (0.024%) and G-Rd (0.020%); it did not react with other steroidal compounds resulting in that the MAb against G-Rb1 exhibited high specificity. Further, anti-G-Rg1 MAb had a weak cross-reactivity against G-Re (3.3%), and was very weak for G-Rd, G-Rc and G-Rb1 (0.93% or lower) indicating high specificity. From these results it became clear that these two MAbs against G-Rb1 and G-Rg1 could be used for pharmacokinetic studies as well as for phytochemical investigations, as previously reported (Tanaka *et al.*, 1999; Fukuda *et al.*, 2000a).

**Sensitivity**

The sensitivity of the newly established ELISAs was higher than that of the TLC (Tani *et al.*, 1981) or HPLC methods (Sticher and Solidati, 1979; Solidati and Sticher, 1980).
The free MAb following competition is bound to polystyrene microtiter plates precoated with G-Rb1-HSA. Under these conditions, the full measuring range of the assay extends from 20 to 400 ng/ml (Tanaka et al., 1999). On the other hand, the concentration of G-Rg1 can be analyzed between 0.3 to 10 µg/ml (Fukuda et al., 2000a).

**Correlation Between ELISA and HPLC**

The correlation coefficient was calculated from fitting a straight line analyzed by ELISA and the HPLC method. There were good correlations for G-Rb1 ($\gamma = 0.997$) and G-Rg1 ($\gamma = 0.998$) between assay values from both methods. This methodology can be utilized for the assay of G-Rb1 and G-Rg1. Therefore, it is possible to study a large number of animal samples and a small sample size *in vitro* with these systems.

**Recoveries of G-Rb1 and G-Rg1 by ELISAs**

Spiked serum samples of G-Rb1 were prepared and analyzed using the above procedure for ELISA. For recovery experiments, 100 µl of serum was used. At each level, three samples were analyzed. Recovery was calculated from spiked G-Rb1 (1, 2 and 5 µg, respectively) in the same concentration ranges, indicating 102 ± 3, 96.5 ± 0.6 and 106 ± 10 %, respectively. In the case of G-Rg1, 1, 10 and 100 µg of G-Rg1 were used for recovery experiments,
resulting in 109 ± 8, 101 ± 2 and 104 ± 6%, respectively. From these results, it is evident that the ELISA using the MAbs can be routinely applied to animal fluids and/or TCM in pharmacokinetic studies without any complicated pretreatment as had previously been necessary with HPLC (Xu et al., 2003), because as documented above the MAbs show high sensitivity and specificity.

**Concentration Changes of G-Rb1 in Serum Corresponding to Time**

Five rats were used to carry out the pharmacokinetic study of G-Rb1. G-Rb1 concentration in serum changes corresponding to the time as shown in Table 1 and Fig. 3. As for G-Rg1, although the assay system was established, however, our attempts to determine its concentration in rat serum after injection was unsuccessful.

<table>
<thead>
<tr>
<th>Time after injection (hr)</th>
<th>Concentration of G-Rb1 in Serum (µg/ml)</th>
<th>Mean (µg/ml)</th>
<th>SD (µg/ml)</th>
<th>RSD</th>
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<td>Rat No. 3</td>
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<td>6.01</td>
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Figure 3. Mean serum G-Rb1 concentration-time profile after i.v. administration at 5 mg/kg to SD rats.
Pharmacokinetic Parameters

Analysis with the aid of the 3p87 program showed that the metabolism of G-Rb1 fit a 2-compartment model, similar to previous studies (Xu et al., 2003). The principal pharmacokinetic parameters were: $V_c$ (0.054 ± 0.020) l·kg$^{-1}$, $CL$ (0.0037 ± 0.0008) mg·h$^{-1}$·kg$^{-1}$, $K_{12}$ (1.02 ± 0.39) h$^{-1}$, $K_{21}$ (0.46 ± 0.38) h$^{-1}$, $K_e$ (0.074 ± 0.020) h$^{-1}$, $t_{1/2a}$ (0.79 ± 0.18) h, $t_{1/2b}$ (26.7 ± 1.84) h, $AUC$ (1420 ± 358) mg·h·l$^{-1}$, respectively.

Eastern Blotting for Ginsenoside Rb1 in Serum

On the PES membrane, only one clear cyanous spot representing G-Rb1 could be seen. Primary analysis (data not shown) indicated that the area and density of the spots were in direct proportion to the amount of G-Rb1 loaded (Fig. 4).

Discussion

Currently, several options for pharmacokinetic study of ginsenosides have been available, including TLC (Odani et al., 1983; Chen et al., 1980; Takino et al., 1982), HPLC (Xu et al., 2003; Li et al., 2004) and liquid chromatography-mass spectrometry (LC-MS) (Sun...
et al., 2005; Xie et al., 2005; Li et al., 2005; Cai et al., 2005). Xu et al. (2003) have reported their successful investigation of the pharmacokinetics and bioavailability of G-Rb1 and G-Rg1 in rats. With these analytical methods, the limits of detection of G-Rb1 and G-Rg1 have been effectively lowered to around 40 and 12 ng, respectively. Nevertheless, all these above methods necessitate a serum amount of as much as about 1 ml, and require laborious pretreatments of the specimen. In our ongoing studies of MAb against naturally occurring bioactive compounds, we found that MAb-based immunoassay may provide a much more sensitive means for determining these compounds, and therefore attempted to develop a new immunoassay system for measuring serum G-Rb1 and G-Rg1 using MAbs against G-Rb1 and G-Rg1 for pharmacokinetic study of G-Rb1 and G-Rg1.

We previously experienced failure to use the serum directly in the determination experiment, probably because of considerable endogenous interference in the serum, but after deproteination with methanol, we obtained satisfactory results. In this study, we investigated the time course of ginsenoside pharmacokinetics after its intravenous administration and ended up with findings similar to those reported by other researchers. Within 1 hour after G-Rb1 administration in rats, the serum G-Rb1 concentration fell sharply from about 100 µg/ml to a level of 55 µg/ml, followed by further declination at much lower rate even 72 hours after the injection, G-Rb1 at a concentration of 6.88 µg/ml could still be detected. Xu et al. (2003) reported that the final concentration of G-Rb1 72 hours after injection was approximately 10 µg/ml, which was 1.5 times higher than what we observed. But considering the much smaller injection dose (5 mg/kg) of G-Rb1 we had chosen as compared with the dose of 15.5 mg/kg they injected (Xu et al., 2003), our result seemed only reasonable.

On the other hand, G-Rg1 injected in the same manner as G-Rb1 exhibited quite a different pharmacokinetic pattern. The serum concentration of G-Rg1 rapidly decreased to an undetectable level after administration within a time period of several minutes, which resembled the findings reported by Xu et al. (2003). The discrepancy in the metabolic rate between G-Rb1 and -Rg1 in rat blood may be attributed to the different molecular structure of the two ginsenosides as substrates of β-glucosidase. Given the recognized high activity of β-glucosidase in the blood, we can readily infer that G-Rg1 is hydrolyzed to produce an aglycon, protopanaxatriol. However, G-Rb1 might be unable to be cleaved at the sugar moiety by β-glucosidase in the blood although it can be another story with intestinal enzymes (Bae et al., 2000; Hasegawa, 2004). This phenomenon might provide an important clue for investigations into the availability of ginsenosides.

On the whole the ELISA systems established in this study possess advantages over previously reported methods in respect to the amount of serum required for analysis and the necessity of tedious pretreatment.

For detection and quantification of ginsenoside Rb1 in the serum, we also attempted a new Eastern blotting system. At the time we first reported the technique Eastern blotting (Tanaka et al., 1997; Fukuda et al., 2000b; Fukuda et al., 2001; Shan et al., 2001), we used PVDF membrane and had to transfer the target substances from TLC to PVDF membrane. In the newly developed immunostaining system, another kind of membrane, PES, was used, which allows omission of the step of transferring the blots from TLC to
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the PES membrane as ginsenosides are developed and directly separated by the solvent systems like acetonitrile-water-acetic acid (25:75:1, by volume) (Morinaga et al., 2005). After separating the components of the samples on the PES membrane and proper staining, we successfully detected the spots of G-Rb1 in the serum samples on PES membrane, as shown in Fig. 4. Primary analysis prompted us to consider the possibility of its use in quantitative analysis of ginsenosides in serum, and our endeavors to test the feasibility of this method are in progress.

Acknowledgments

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References


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