Quantitative Analysis of Eight Ginsenosides in Red Ginseng Using Ginsenoside Rg1 as Single Reference Standard

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Abstract

Objective: To develop a reversed-phase high-performance liquid chromatography method for the quantification of major ginsenosides in red ginseng (RG, the steamed and dried root of the cultivar of Panax ginseng C. A. Mey). Methods: A feasible method was developed in strict accordance with chromatographic properties of eight ginsenosides. Their contents could be unveiled with conventional external standard method, or as an alternative, using ginsenoside Rg1 as the single reference standard by means of seven conversion factors. Those parameters had been validated on different chromatographic columns and instruments. Results: Twenty-one batches of RG samples were determined. In addition, the chromatograms of RG and confusing species, including Panax ginseng, Panax quinquefolium, and Panax notoginseng, were apparently different. Conclusions: The method was proved to be efficient for the quality control of RG.

Keywords: Ginsenosides, high-performance liquid chromatography, quantification, red ginseng

INTRODUCTION

Red ginseng (RG, the steamed and dried roots of Panax ginseng) is a valuable traditional Chinese medicine with a long clinical history and still popular nowadays. It shares the same medicinal plant origin as Asian ginseng named Panax ginseng (PG) C.A. Meyer in Latin (family, Araliaceae) but undergoes steaming and drying process, while Asian ginseng has only been cleaned and dried after collection from the field.

In Chinese Pharmacopoeia (Ch.P, 2020 version),[1] RG is mainly used for indications including tending to collapse caused by body deficiency, coldness of limbs and faint pulse, qi failing to control the blood, flooding, and spotting. Modern research has demonstrated RG’s various pharmacological activities, such as improving blood circulation,[2] free-radical scavenging effect,[3] immunostimulating effect,[4] anticancer potential,[5] protecting effect against vestibular/hearing disfunction,[6] beneficial effects on sleeping behaviors,[7] antidiabetic effect,[8] and protecting against renal failure.[9] A plenty of research has unearthed that ginsenosides in RG play a pivotal role in the pharmacological activities of RG. For example, ginsenoside Rg1 inhibits platelet activation, arterial thrombosis,[10] and vascular intimal hyperplasia.[11] Ginsenoside Rb1 inhibits the carotid neointimal hyperplasia.[12] Ginsenoside Rd promotes the proliferation of neural stem cells.[13]

In the RG quality monograph in Ch.P, Rg1, Re, and Rb1 were chosen as quantitative chemical markers in the assay, while in Korean Pharmacopoeia[14] and Japanese Pharmacopoeia,[15] only Rg1 and Rb1 are quantitatively determined. In United States Pharmacopoeia (USP), Rg1 and Rb1 were selected as quantitative markers for Asian ginseng (PG),[16] but Rg1, Re, Rb1, Re, Rb2, and Rb3 for American ginseng (Panax quinquefolium [PQ]).[17] In the present research, eight
ginsenosides were quantified by using single standard for the determination of multiple component (SSDMC) method. They are ginsenosides Rg1, Re, Rf, Rb1, Ro, Rc, Rb2, and Rd, respectively, in which Rg1 was selected as the single reference standard for the SSDMC method. Their structures are shown in Figure 1.[18]

**Methods**

**Instruments and reagents**

**Instruments**

High-performance liquid chromatography (HPLC) separation was performed on two Agilent 1260 Infinity series and one Agilent 1100 series. Each instrument consists of a quaternary pump, an autosampler, a thermostatted column compartment, and a diode Array Detector.

**Reagents**

Acetonitrile UV (UV, Honeywell, Muskegon, USA, LOT DH499), methanol (AR, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and phosphoric acid (85%, TEDIA, Fairfield, USA, LOT 1203176) were purchased. Rg1 (LOT 2099/9863), Re (LOT 2070/9866), Rf (LOT 2337/10024), Rb1 (LOT 2326/10487), Ro (LOT 1397/10488), Rc (LOT 2303/10026), Rb2 (LOT 2358/10489), and Rd (LOT 2302/10025) were obtained from Shanghai Standard Biotech Co., Ltd.

**Samples**

Twenty-one batches of RG were gathered from Jilin and Liaoning provinces in China. PG, PQ, and Panax notoginseng (PN) were also collected as confusing materials. All samples were identified by Prof. De-An Guo (Shanghai Research Center for Modernization of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, Chinese Academy of Sciences). The powdered extract of RG was made in our laboratory.

**Preparation method of sample solutions**

Samples of 0.5000 g RG was transferred to a 100-mL glass-stoppered conical flask. Totally, 25 mL of a mixture of methanol and water (7:3, v/v) was added into the flask, sonicated for 30 min (565 W, 37 kHz), and filtered. Then, above extraction was repeated once. The flask and test specimen was rinsed with 15 mL of a mixture of methanol and water (7:3, v/v). The washing solution was added to the filtrate. The filtrate was evaporated under reduced pressure to dryness and dissolved in the mixture of methanol and water (7:3, v/v). The solution was transferred to a 10-mL volumetric flask and diluted with the mixture of methanol and water (7:3, v/v) to volume. The solution was mixed to volume and passed through a nylon filter of 0.22-μm pore size, with the first portion of the filtrate discarded. Above parameters were optimized by solvents, methods, frequency and the time-length of extraction, and the volume of extraction solvent.

**Chromatographic procedure**

HPLC separation was performed on an Agilent Zorbax SB-C18 column (4.6 mm × 150 mm, 2.7 μm). The mobile phase consisted of acetonitrile (A) and 0.01% aqueous solution of phosphoric acid (B). Elution program: 0/25/35/75 min, 18%/21%/28%/32% B. Injection volume: 20 μL. Flow rate: 1.2 mL/min. Detection wavelength: 203 nm. Column temperature: 40°C. Above parameters were optimized by mobile phase system, mobile phase acidity, elution program of mobile phase, flow rate, column temperature, and injection volume. Different from the RG monograph in the current pharmacopeias, phosphoric acid was used in mobile phase in the present method because Ro was found to be separated only in acidic conditions.

**Determination and validation of relative retention times**

Stable relative retention times (RRTs) and conversion factors (CFs) are key parameters for an SSDMC method. RRTs were obtained on three different days and calculated using Formula 1.[19] RRTi, RTi, and RTRg1 are the RRTs of the relevant marker, the retention time of the relevant marker, and the retention time of Rg1, respectively. Validation of RRT was carried out through different experiments: (a) three columns and (b) three instruments.

Formula 1: \[ \text{RRT}_i = \frac{\text{RT}_i}{\text{RT}_{Rg1}} \]

**Determination and validation of conversion factors**

CFs were obtained with standard solutions and calculated using Formula 2.[19] CFi, Slopei, and SlopeRg1 were the CF of the relevant
marker, the slope of the relevant standard curve, and the slope of the standard curve of Rg1, respectively. Validation of RRT was carried out through different experiments: (c) three standard curves, (d) three columns, (e) three instruments, and (f) different calculating methods. The first method was the external standard method (ES method) using the mixture containing eight reference standards (Method I). The second method was also an ES method, but with standard solution prepared with powdered extract of RG with known concentration of eight components (Method II). The third one is the SSDMC method (Method III). \[ \text{Formula 2: } CF_i = \frac{\text{Slope}_\text{RG1}}{\text{Slope}_i} \]

**Method validation**
Method validation was carried out according to the conventional requirements of USP, including recovery, precision (repeatability and intermediate precision), linearity, specificity, stability, and robustness.

**RESULTS**

**Relative retention time**
RRT and the result of validation are presented in Table 1. Results of experiments (a) ~ (b): The relative standard deviations (RSDs) of RRT were <2%, indicating that no significant difference was shown.

| Table 1: Determination and validation of relative retention times |
|----------------------|------------------|------------------|------------------|------------------|
| RRT                  | Average | RSD (%) | Experiment (a) | Average | RSD (%) | Experiment (b) | Average | RSD (%) |
| Rg1                  | 1.00    | 0.00    | 1.00            | 0.00    | 1.00    | 0.00            | 1.00    | 0.00    |
| Re                   | 1.04    | 0.00    | 1.04            | 0.00    | 1.04    | 0.00            | 1.04    | 0.00    |
| Rf                   | 1.76    | 0.57    | 1.77            | 0.56    | 1.77    | 0.56            | 1.77    | 0.56    |
| Rb1                  | 2.38    | 0.24    | 2.38            | 0.64    | 2.40    | 0.87            | 2.40    | 0.87    |
| Ro                   | 2.44    | 0.24    | 2.45            | 0.47    | 2.46    | 1.02            | 2.46    | 1.02    |
| Rc                   | 2.56    | 0.23    | 2.57            | 0.81    | 2.58    | 1.03            | 2.58    | 1.03    |
| Rb2                  | 2.78    | 0.21    | 2.78            | 0.90    | 2.80    | 1.15            | 2.80    | 1.15    |
| Rd                   | 3.23    | 0.18    | 3.23            | 0.93    | 3.25    | 0.99            | 3.25    | 0.99    |

**Conversion factor**
The regression coefficient of the standard curves for eight components ranged from 0.9998 to 0.9999, respectively. CF and the result of validation are presented in Table 2. Results of experiments (c) ~ (f): The RSDs of CF and content were <2%, and thus no significant difference was observed.

| Table 2: Determination and validation of conversion factors |
|------------------|------------------|------------------|------------------|------------------|
| Experiment (c)   | Average | RSD (%) | Experiment (d) | Average | RSD (%) | Experiment (e) | Average | RSD (%) | Experiment (f) | Average | RSD (%) |
| Rg1               | 1.00    | 0.00    | 1.00            | 0.00    | 1.00    | 0.00            | 1.00    | 0.00    | 0.302            | 0.19    |
| Re                | 1.00    | 0.59    | 1.00            | 0.50    | 1.00    | 0.00            | 1.00    | 0.00    | 0.105            | 0.00    |
| Rf                | 0.83    | 0.84    | 0.83            | 0.60    | 0.83    | 0.69            | 0.83    | 0.69    | 0.066            | 0.87    |
| Rb1               | 1.27    | 0.87    | 1.27            | 0.39    | 1.27    | 0.00            | 1.27    | 0.00    | 0.450            | 0.56    |
| Ro                | 1.03    | 1.79    | 1.02            | 0.49    | 1.02    | 1.70            | 1.02    | 1.70    | 0.320            | 0.83    |
| Rc                | 1.22    | 1.32    | 1.22            | 0.41    | 1.22    | 0.00            | 1.22    | 0.00    | 0.174            | 0.66    |
| Rb2               | 1.22    | 1.04    | 1.22            | 0.48    | 1.21    | 0.48            | 1.21    | 0.48    | 0.175            | 0.57    |
| Rd                | 1.02    | 0.14    | 1.02            | 0.80    | 1.02    | 0.57            | 1.02    | 0.57    | 0.060            | 1.67    |

**Method validation**
In the accuracy test, the average recoveries ranged 99.39% to 101.8% with RSDs ranging from 0.32% to 0.84%. In the repeatability test, the RSDs of content at different concentrations were <2.00%. In the intermediate precision test, the RSDs of content obtained with different days, analyzers, and equipment were <2.00%. The linearity of eight ginsenosides was satisfactory. In the specificity test, eight peaks were pure in the purity test of Agilent Chemstation, and blank solution had no interference on the chromatogram. In the robustness test, the results showed that the ratio of acetonitrile in mobile phase, the time length of elution program, column length, flow rate, injection volume, and column temperature could be slightly changed, but the wavelength of UV-visible detector must be strictly controlled.

**Content**
The average total content of 21 batches of RG samples ranged from 0.886% to 2.531%, with an average content of 1.802% [Figure 2]. The content ratio of Ro to the total ginsenosides content ranged from 12.98% to 30.77%.

**Chromatograms of confusing materials**
Chromatograms of confusing materials are shown in Figure 3. There was a peak of Rf shown in the chromatograms of RG and PG, but not in PQ and PN. For PG, two small peaks were shown as neighboring peaks before Rc (Peak 1) and Rb2 (Peak 2), which could be used as distinguishing characteristics from RG. For PQ, the peak of Re was higher than that of Rg1; however, for RG, PG, and PN, the situation was just opposite. Four species were successfully differentiated with the developed method.

**Discussion**
With the application of mobile phase containing phosphoric acid, the slope of the relevant standard curve, and the slope of the standard curve of Rg1, respectively. Validation of RRT was carried out through different experiments: (c) three standard curves, (d) three columns, (e) three instruments, and (f) different calculating methods. The first method was the external standard method (ES method) using the mixture containing eight reference standards (Method I). The second method was also an ES method, but with standard solution prepared with powdered extract of RG with known concentration of eight components (Method II). The third one is the SSDMC method (Method III).
acid, and help of core-shell columns, eight markers in RG could be baseline separated. In particular, superior to the current RG monograph in ChP, Ro was successfully determined. Chromatographic parameters and sample preparation method were carefully optimized. Only one reference standard was needed in the SSDMC determination so that expense to use eight individual reference standards instead was dramatically reduced. Key parameters of RRT and CF were strictly validated. Method validation was performed and different samples were determined successfully.

CONCLUSION

The method has been proven to be precise and feasible for the quality control of RG. It was also worth noting that confusing materials like PG, PN and PQ could be apparently differentiated from RG.

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Conflicts of interest

There are no conflicts of interest.

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