Validation on the Analytical Method of Ginsenosides in Red Ginseng

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Abstract

A cross-examination between KT&G Central Research Institute and Guangzhou Institute for Drug Control was carried out in order to select optimum conditions for extraction, separation and determination of ginsenosides in red ginseng and to propose a better method for the quantitative analysis of ginsenosides. The optimum extraction conditions of ginsenosides from red ginseng were as follows: the extraction solvent, 70% methanol; the extraction temperature, 100℃; the extraction time, 1 hour for once; and the repetition of extraction, twice. The optimum separation conditions of ginsenosides on the SepPak C_{18} cartridge were as follows: the loaded amount, 0.4 g of methanol extract; the washing solvents, distilled water of 25 ml at first and then 30% methanol of 25 ml; the elution solvent, 90% methanol of 5 ml. The optimum HPLC conditions for the determination of ginsenosides were as follows: column, Lichrosorb NH_2 (25 x 0.4 cm, 5 μm, Merck Co.); mobile phase, a mixture of acetonitrile/water/isopropanol (80/5/15) and acetonitrile/water/isopropanol (80/20/15) with gradient system; and the detector, ELSD. On the basis of the optimum conditions a method for the quantitative analysis of ginsenosides were proposed and another cross-examination was carried out for the validation of the selected analytical method conditions. The coefficient of variances (CVs) on the contents of ginsenoside-Rg, -Re and -Rb_1 were lower than 3% and the recovery rates of ginsenosides were 89.4~95.7%, which suggests that the above extraction and separation conditions may be reproducible and reasonable. For the selected HPLC/ELSD conditions, the CVs on the detector responses of ginsenoside-Rg, -Re and -Rb_1 were also lower than 3%, the regression coefficients for the calibration curves of ginsenosides were higher than 0.99 and two adjacent ginsenoside peaks were well separated, which suggests that the above HPLC/ELSD conditions may be good enough for the determination of ginsenosides.

Key words: cross-examination, validation, ginsenosides, SepPak C_{18} cartridge, HPLC/ELSD.

Introduction

Ginseng saponins have been regarded as the principal components responsible for the pharmacological activities of ginseng. In addition, the ginseng saponin content is being used as the most important specification for evaluating qualities of ginseng and ginseng products in many countries including China, Japan and Germany as well as in Korea.

A number of methods for the identification of ginseng saponins and their quantitative analysis are available in the literature. The analytical methods of ginseng saponins may be classified into four categories of gravimetry [1-4], spectrophotometry [5-7], thin-layer chromatography (TLC) [1-6] and high performance liquid chromatography (HPLC) [1, 5-6, 8-12]. With gravimetry, the yield of butanol-soluble materials can be measured but the content of total ginsenosides or individual ginsenoside cannot be measured. The gravimetry is prescribed as a standard method for the inspection of ginseng quality in the Regulations of Ginseng Industry [2], the Korea Pharmacopoeia [3] or the Official Methods for the Analysis of Food [4]. By spectrophotometry the contents of total ginsenosides can be measured indirectly, but the individual ginsenoside content also cannot be measured. The spectrophotometry is prescribed as a standard method for the inspection of ginseng saponins in the Grade Quality Standards of Products of Processed Ginseng [5], the China Pharmacopoeia [6] and the German Pharmacopoeia [7]. The TLC method has been widely used for the identification of ginsenosides, which is prescribed as a standard method in China as well as in Korea. The HPLC method has been widely used for the quantitative analysis of each ginsenoside, which is prescribed as a standard method only in the Grade Quality Standards of Products of Processed Ginseng and the China Pharmacopoeia.

Refluxing [1-12] is mostly used for the initial extraction of ginsenosides from ginseng, but ultrasonic extraction [5-6] is also used instead of refluxing, where aqueous alcohol is generally used as a solvent. Phase-separation [1-6, 8-11] has been mostly used for the separation of ginsenosides from ginseng extract, but much time is required for the phase-separation. Lee et al. [12] introduced a new technique using a SepPak C_{18} cartridge, by which the disadvantage of phase-separation may be overcome. Refractive index (RI) detector, ultra-violet (UV) detector and evaporative light scattering detector (ELSD) have been used as HPLC detectors for the quantitative analysis of ginsenosides. RI detector has been widely used for the analysis of ginsenosides, but it is not adequate for the analysis of minor ginsenosides because of poor sensitivity and resolution. Soldati et al. [8] introduced a HPLC method using UV detector instead of RI detector in 1980's

and some other scientists [9-10] also used UV detector, but it has been not so widely used for the analysis of ginsenosides due to poor chromopore of ginsenosides. In 1996 Park et al. [11] introduced an evaporative light scattering detector (ELSD) as an excellent HPLC detector for the analysis of ginsenosides. ELSD is a kind of universal detector like RI detector but its sensitivity is as good as UV detector, so it is expected to be very useful detector for the simultaneous analysis of multi-ginsenosides.

Korea Ginseng Corporation and some other companies have been trying to register Korean red ginseng as a drug to State Drug Administration (SDA) of China since 1999. National Institute for the Control of Pharmaceutical & Biological Products (NICPBP) which is a major subsidiary organization of SDA have been investigating the analysis method of ginsenosides in order to establish better quality standards applicable for the inspection of imported red ginseng. Recently, our research team carried out a cross-examination on the analysis method of ginsenosides by using a SepPak C_{18} cartridge and a HPLC/ELSD technique with a Chinese research team in Guangzhou Institute for Drug Control by the recommendation of NICPBP. Through the cross-examination, validities on the analytical conditions such as extraction of ginsenosides from red ginseng and their separation and determination were examined.

Materials and Methods

Ginseng sample

Korean red ginseng in root-size of 30 Ji (=38 roots per 600g) was used as a ginseng sample. Rhizome of the ginseng sample was removed, and then its main body and lateral roots (=big tails) were ground to 60 mesh-size just before the analysis.

Reagents

All reagents used in this study were GR or HPLC grades.

Instruments

A high performance liquid chromatograph (Waters Associates model 510, Waters Co.; U.S.A.) equipped with an evaporative light scattering detector (ELSD 2000, Alltech Co.; U.S.A.) was used for the quantitative analysis of ginsenosides.

HPLC/ELSD conditions for the determination of ginsenosides

KGTRI's method. The HPLC/ELSD analytical conditions suggested by Korea Ginseng and Tobacco Research Institute were as follows: (i) Column, Lichrosorb NH_2 (25 x 0.4 cm, 5 μm, Merck Co.). (ii) Mobile phase, mixtures of solvent A (acetonitrile/water/isopropanol = 80:5:15) and solvent B (acetonitrile/water/isopropanol = 80:20:15). (iii) Gradient profile of solvent A to solvent B, from 70:30 to 0:100 for 0-20 min; 0:100 for 20-55 min; from 0:100 to 70:30 for 55-65 min; flow rate, 1.0 ml/min. (iv) Detector, ELSD (ELSD 2000, Alltech Co.; U.S.A.); detection temperature, 92°C; nebulizing gas, nitrogen, 2.0 l/min.

GIDC's method. The HPLC/ELSD analytical conditions suggested by Guangzhou Institute for Drug Control were as follows: (i) Column, Lichrosorb NH_2 (25 x 0.4 cm, 5 μm, Merck Co.). (ii) Mobile phase, mixtures of acetonitrile (A), water (B) and isopropanol (C). (iii) Gradient profile of A/B/C, 94.9:5:0.1 for 0-4 min; from 94.9:5:0.1 to 91.9:8:0.1 for 4-15 min; from 91.9:8:0.1 to 89.5:10:0.5 for 15-30 min; from 89.5:10:0.5 to 87:10:3 for 30-30.01 min; from 87:10:3 to 77:15:12 for 30.01-60 min; flow rate, 1.0 ml/min. (iv) Detector, ELSD (SEDEX 55, Sedere Co.; France); detection temperature, 40°C; nebulizing gas, nitrogen, 2.0 l/min.

Extraction of ginsenosides from red ginseng and their separation

KGTRI's method. One gram of red ginseng sample powder (60 mesh) was extracted with 80% ethanol at 80°C for 1 hours and filtered. The residue was extracted with 80% methanol at 80°C for 1 hours and filtered, which procedure was carried out once again. All the filtrates were collected together and concentrated at 40°C under vacuum. The concentrate was suspended in 5 ml of distilled water and 2 ml of it were applied to a SepPak C_{18} cartridge (Sep-Pak Plus, Waters Co.; U.S.A.). The cartridge was washed with 8 ml of water at first, and then washed with 8 ml of 25% methanol before ginsenosides in the cartridge were eluted with 5 ml of 90% methanol.

GIDC's method. One gram of red ginseng sample powder (60 mesh) was extracted with 70% ethanol at 100°C for 1 hour and filtered, and then the residue was extracted with 70% methanol at 100°C for 30 minutes and filtered once again. The two filtrates were collected together and concentrated at 40°C under vacuum. The concentrate was suspended in 5 ml of distilled water, all of which were applied to a SepPak C_{18} cartridge (Sep-Pak Plus, Waters Co.; U.S.A.). The cartridge was washed with 5 ml of water at first, and then washed with 5 ml of 30% methanol before ginsenosides in the cartridge were eluted with 5 ml of 100% methanol.

Results and Discussion

HPLC/ELSD conditions for the determination of ginsenosides

In order to establish a quantitative analytical method of ginsenosides in red ginseng, several HPLC/ELSD conditions suggested by KGTRI and GIDC for the determination of ginsenosides were examined. A standard mixture containing ginsenoside-Rh_1, -Rh_2, -Rg_2, -Rg_1, -Rg_3, -Rf, -Re, -Rd, -Rc, -Rb_2 and -Rb_1 was analyzed by HPLC/ELSD. Fig. 1 shows HPLC patterns of ginsenosides analyzed by KGTRIs method and GIDCs method. Such HPLC/ELSD analytical conditions as mobile phases, detector model and detection temperature were different between KGTRI's method and GIDC's method, but all the ginsenoside peaks except for ginsenoside-Rh_1 and -Rh_2 were well separated not only by KGTRI's method but by GIDC's method. This result indicates that the peak resolutions of most ginsenosides may be very good as long as ginsenosides are analyzed under the HPLC conditions using a Lichrosorb NH_2 column and an ELSD. Therefore, the HPLC/ELSD method of ginsenosides were established as described in HPLC/ELSD conditions for the determination of ginsenosides of Materials and Methods.

Validation on the established HPLC/ELSD method

Ginsenosides were analyzed by the established HPLC/ELSD method and their peak resolution, repeatability, linearity and detection limit were examined.

The resolution values (R) of two adjacent peaks of ginsenoside-Rg_2 and -Rg_1, -Rg_1 and -Re, -Re and -Rd, -Rd and -Rc, -Rc and -Rb_2, and -Rb_2 and -Rb_1 in their HPLC chromatogram (Fig. 1)

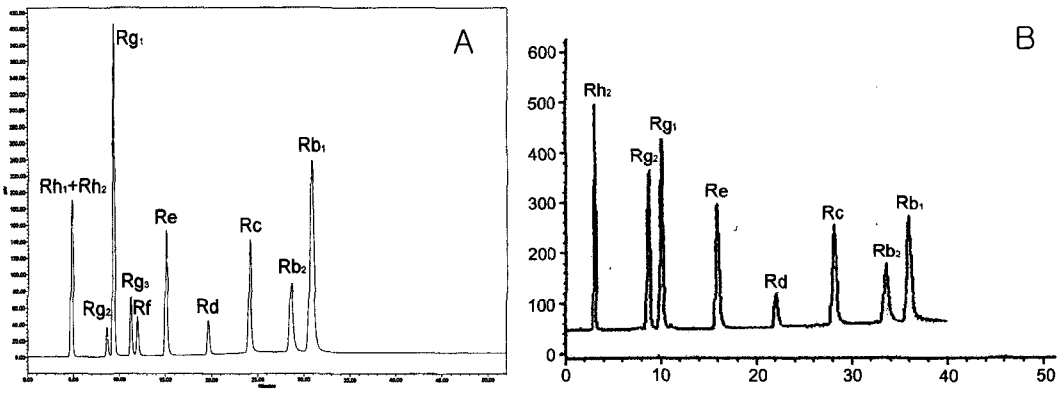

Fig. 1. HPLC/ELSD patterns of standard mixture of ginsenosides.
(A: KT & G, B: GDIC)

Table 1. The resolution of two adjacent ginsenoside peaks on the HPLC chromatogram

Ginsenosides	R value
Rg2 - Rg1	0.529
Rg1 - Rg3	1.519
Rg3 - Rf	0.638
Rf - Re	2.078
Re - Rd	2.950
Rd - Rc	2.956
Rc - Rb2	2.303
Rb2 - Rb1	0.847

R value were calculated as follow. R=D/(W1+W2)
D: distance between adjacent peak, W1, W2 : each width of adjacent peaks

Table 2. The repeatability result of ginsenoside- Rg1, -Re and -Rb1 presented by KT&G

Ginsenoside	Average (mg/g)	Standard deviation	Coeffcient of Variation (%)
Ginsenoside-Rg1	2.99	0.06	2.1
Ginsenoside-Re	1.34	0.04	2.7
Ginsenoside-Rb1	4.20	0.12	2.9
Sum	8.52	0.19	2.3

The data are means of 12 injections

were shown in Table 1. This result indicates that most ginsenosides except ginsenoside-Rg_2 and Rf can be well separated from each other in the HPLC pattern.

The repeatability for HPLC/ELSD analysis was shown Table 2. For the check the error of mechanical deviation (injection), 12 injections of same sample solution were done by autosampler. The Coefficient of variation of repeatability results of ginsenoside- Rg1, -Re and -Rb1 were below 3%. Therefore, the analysis system which was HPLC, autosampler and detector was valid.

When the standard mixtures of ginsenoside-Rg_1, ginsenoside-Re and ginsenoside-Rb_1 were analyzed by using an ELSD of ELSD 2000 (KGTRI's method), the calibration graph of either ginsenoside-Rg_1, -Re or -Rb_1 was quadratic as shown in Fig. 2. The regression coefficient (r) for each graph of ginsenoside was 0.9999 or higher. In addition, there were good linear relationships between the detector responses of ginsenosides and their amount injected to the HPLC (r = 0.9990-0.9999) when the standard mixtures of ginsenoside-Rg_1 of 3.8-18.9 μg, ginsenoside-Re of 3.4-17.2 μg and ginsenoside-Rb_1 of 5.0-25.3 μg were analyzed by another ELSD of SEDEX 55 (GIDC's method). These results indicate that the linearities of both the HPLC/ELSD methods

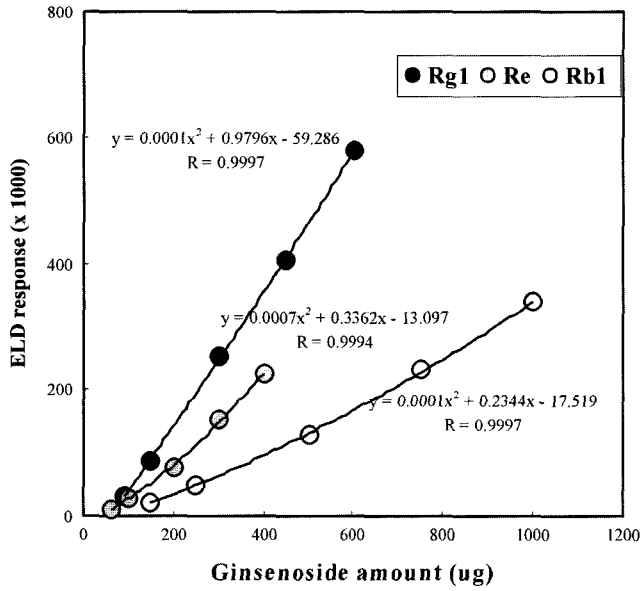

Fig. 2. The calibration curves of ginsenoside-Rg_1, -Re and -Rb_1.

would be good. The detection limits of ginsenoside-Rg_1, -Re and -Rb_1 were 9 ng, 28 ng and 108 ng when analyzed by using an ELSD of ELSD 2000 model but the detection limits of the three ginsenosides were 80 ng all when analyzed by using another ELSD of SEDEX 55, as shown in Table 3. These results suggest that linearity of ELSD for the analysis of ginsenosides and its sensitivity may be different from the HPLC conditions such as the mobile phase and the operating temperature of ELSD. Park et al. [11] reported that the there were linear relationships between the ginsenoside amounts of 0.5-10 μg and their ELSD responses and the detection limits of ginsenosides were 35-155 ng.

Conditions for the extraction and separation of ginsenosides

Table 3. The detection limits of ginsenoside-Rg_1, -Re and -Rb_1

Ginsenoside	Detection limits (ng)	
	KT&G	GDIC
Ginsenoside-Rg1	9	80
Ginsenoside-Re	28	80
Ginsenoside-Rb1	108	80

Detection limits : the signal to noise level (S/N) was below 3.0

The extraction conditions of ginsenosides and their separation conditions using a SepPak C_{18} cartridge suggested by KGTRI and GIDC were compare to establish a better method.

When the red ginseng powder was extracted with 80% ethanol at 80°C for 1 hr three times and ginsenosides in the ethanol extract were separated on a SepPak C_{18} cartridge by KGTRI's method, the contents of ginsenoside-Rg_1, -Re and -Rb_1 were 2.99 mg/g, 1.34 mg/g and 4.06 mg/g, respectively, as shown in Table 4. On the other hand, the contents of ginsenoside-Rg_1, -Re and -Rb_1 were 3.02 mg/g, 1.30 mg/g and 3.69 mg/g when the red ginseng powder was extracted with 70% ethanol at 100°C for 1 hr at first and then for 30 minutes and ginsenosides in the ethanol extract were separated on a SepPak C_{18} cartridge by GIDC's method. This result suggests that either the extraction efficiencies or the separation efficiencies of ginsenosides may be a quite similar between KGTRI's method and GIDC's method.

For the extraction of ginsenosides from red ginseng, 70% ethanol may be used as a solvent instead of 80% ethanol due to good penetration to ginseng powder particles. Most of ginsenosides were extracted in the first extraction and the second extraction, so third extraction may not be necessary. Therefore, the initial extraction conditions of ginsenosides from red ginseng for the cross-examination of validation study were established as follows: the extraction solvent, 70% ethanol; the extraction temperature, 100°C; and the extraction time, twice (1 hour for once).

When ginsenosides in the ginseng extracts are separated on the SepPak C_{18} cartridge, to load a smaller quantity of ginseng is recommendable in considering capacity of the cartridge. It is reported that ginseng extract equivalent to 0.4 g of ginseng powder was desirable for being loaded to the cartridge [12]. The cartridge should be washed with water at first for removing sugars, and then washed with 25~30% methanol for removing pigments after ginseng extract being loaded before eluted with 85~100% methanol for the separation of ginsenosides. Lee et al. [12] reported that 30% methanol can be used as the second washing solvent, and GIDC also suggested

Table 4. The ginsenoside contents analyzed by KGTRI's method and GIDC's method

Ginsenoside	KT&G	GIDC
	Average (mg/g)	Average (mg/g)
Ginsenoside-Rg1	2.99	3.02
Ginsenoside-Re	1.34	1.30
Ginsenoside-Rb1	4.06	3.69
Sum	8.39	8.01

The data are means of 12 replications. Analysis was done by KT&G.

that 30% methanol can be used as the second solvent. However, the ginsenoside-Rb_1 contents analyzed by GIDC's method were lower than those by KGTRI's method, which indicates that small quantity of ginsenoside-Rb_1 can be removed when the cartridge being washed with 30% methanol. Both the two washing solvents of up to 25 ml can be used for removing sugars and pigments completely. In addition, more polar ginsenosides such as ginsenoside-Rb_1 cannot be eluted completely from the cartridge if methanol concentration in the eluting solvent is lower than 85% and other impurities can be also eluted from the cartridge if methanol concentration in the eluting solvent is up to 100%. Therefore, the separation conditions of ginsenosides on the SepPak C_{18} cartridge for the cross-examination of validation study were established as follows: the loaded quantity of ginseng extract, equivalent to 0.4 g of red ginseng powder; the first washing solvent, water of 25 ml; the second washing solvent, 25% methanol of 25 ml; and the eluting solvent, 90% methanol of 5 ml.

Validation on the established extraction and separation method

A cross-examination of validation on the extraction and separation method of ginsenosides was carried out between KGTRI and GIDC. The reproducibility for the contents of ginsenoside-Rg_1, -Re and -Rb_1 and the recovery rates of the three ginsenosides were examined. The results are

Table 5. The coefficient of variance (CV) of ginsenoside-Rg_1, -Re and -Rb_1 contents analyzed by the established method in KGTRI and GIDC

Ginsenoside	coefficient of variation (%)	
	KT&G	GDIC
Ginsenoside-Rg1	2.7	2.0
Ginsenoside-Re	3.0	2.5
Ginsenoside-Rb1	3.5	2.0
Sum	3.0	Not presented

The data are means of 12 replications in KT &G and 5 replications in GDIC.

Table 6. The recovery rates of ginsenoside-Rg_1, -Re and -Rb_1

Ginsenoside	Average (%)	Standard deviation
Ginsenoside-Rg1	95.7	4.1
Ginsenoside-Re	93.4	6.6
Ginsenoside-Rb1	89.4	6.3

The recovery test was done by addition of known amounts of authentic ginsenosides to the red ginseng powders and extracted and fractionated as method written above.

shown in Table 5 and Table 6.

The coefficient of variance (CV) of the three ginsenoside contents were 2.7%, 3.0% and 3.5%, respectively and the CV of ginsenoside-Rg_1, -Re and -Rb_1 contents analyzed five times in GIDC were less than 3%, too. Therefore, entire procedure from weighing to HPLC analysis was valid. In addition, the recovery rates of ginsenoside-Rg_1, -Re and -Rb_1 for the extraction and separation method were 95.7 ± 4.1%, 93.4 ± 6.6% and 89.4 ± 6.3%, respectively, which are similar to Lee et al.'s data [12]. Recovery of ginsenosides was satisfactory, but ginsenoside Rb1 was slightly poor. But In this case, the recovery of ginsenoside- Rg1, -Re and -Rb1 were good enough components of quality control.

Conclusion

The optimized methods of extraction and fractionation methods were as follow. The extraction solvent is 70% ethanol and temperature is 100C with 1.5hr (1hr and 0.5hr twice extractions) and the loading amounts are 0.4gr equivalent. For the elimination of pigments, 30% methanol was chosen for washing solvents and 100% methanol was adopted as elution solvents. The order of each ginsenoside elution was same such as ginsenoside-Rh1, -Rh2, -Rg2, -Rg1, -Rg3, -Rf, -Re, -Rd, -Rc, -Rb2, and -Rb1. The calibration graphs of each ginsenosides are quadratic. The detection limits of ginsenoside- Rg1, Re and Rb1 were 9ng, 28ng, 108ng, respectively. The full procedure including from extraction to analysis was also valid through test of reproducibility which C.V. was below 3%. The recovery of the authentic ginsenoside compounds were between 89.4~95.7%.

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