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Qualitative and quantitative determination of oleanolic acid in a scalp tonic products by HPLC using response surface methodology for extraction optimization

Lin Xi Cai[1], Chong Woon Cho[1], Yan Zhao[3], Jong Seong Kang[1], Kyung Tae Kim[2,★], and Sang-Hun Jung[1,★]

[1]*College of Pharmacy, Chungnam National University, Daejeon 34134, Korea*
[2]*Department of Food Science & Technology, Dong-Eui University, Busan 47340, Korea*
[3]*School of Pharmacy, Yantai University, Yantai 264005, China*

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Abstract The simple and effective analytical method for the quality control of a novel scalp tonic formulation has been developed and optimized in terms of HPLC conditions and sample preparation method, meanwhile, the optimization of preparation condition was using response surface methodology (RSM) based on central composite design (CCD). Oleanolic acid was selected as marker compound because of its bioactivities for alopecia therapy. The developed analytical method and extraction condition were successfully qualified. Coefficient of determination (r^2) for the calibration was 0.9997 with a line passing through the origin point in the range of 0.1-100 mg/mL. The limit of detection (LOD) and the limit of quantitation (LOQ) were 17.5 ng/mL and 55.0 ng/mL, respectively. The intra-day and inter-day precision of the method were 0.5-1.4 % and 0.7-1.8 % in relative standard deviation, respectively, while those accuracy were 99.5-100.9 % and 100.0-102.2 %, respectively. The repeatability of oleanolic acid in samples ranged of 0.3-1.9 % based on peak area and 0.3-0.7 % for retention time. Recoveries from samples were 95.0-99.4 % with lower than 1.8 % in relative standard deviation. Overall, the developed analytical method will be used for quality control of this commercial scalp tonic products successfully.

Key words: Scalp tonic, oleanolic acid, response surface methodology (RSM), quality control, HPLC

1. Introduction

These days, good appearance is one of advantages in human relationship, however a lot of people are suffering hair losing called alopecia disease. The additional problem is the increasing number of alopecia patients in Taiwan,[1] United States of America,[2] France, Germany, Italy, the United Kingdom[3] and Korea[4] and so on. Various factors such as emotional distress and anxiety may incur the loss of hair.[5] In addition, synthetic chemical products sold in the market including shampoo to clean scalp can cause hair-losing problem due to their chemical irritation on scalp and skin.[6] Therefore, more natural and skin-

★ Corresponding author
Phone : +82-(0)51-890-1570, +82-(0)42-821-5939 Fax : +82-(0)505-182-6897, +82-(0)42-823-6566
E-mail : kimkkt@deu.ac.kr, jungshh@cnu.ac.kr

friendly products are interested and required by consumers. In accordance with the demand of customers, a new scalp tonic formulation was developed using natural herb extract.

A new scalp tonic formulation was developed with the extract of Pulsatillae Radix (PR) and other herbs. PR extract is main component of scalp tonic. PR is a traditional herbal drugs and defined as the root of *Pulsatilla koreana* Nakai or *P. chinensis* Regel (Ranunculaceae).[7] It has been widely used to treat bacterial infections, intestinal amebiasis[8,9] around in China, Korea and other eastern Asia countries. PR has been known as a medicinal herb to treat tumor and endotoxin.[10]

PR contains numerous compounds, including phytosterone[11] and triterpenoid saponins.[12] Oleanolic acid is one of main compounds in this herb,[13] which is a pentacyclic triterpenoid and owns various biological activities such as antioxidant, exhibiting potent antitumor and antibacterial activities and so on.[14-19] It involved into the hair regrowth in clinical experiment,[20] therefore, oleanolic acid is used as a main component in some patents for the treatment of baldness hair loss or hair regrowth.[21,22] Recently, therapeutic components are chosen as a chemical marker to apply for qualitative and quantitative evaluation.[23] Considering the bioactivity of alopecia treatment, oleanolic acid can be a key compound to determine the bioactivity and marker compound for the standardization and quality control of commercial scalp tonic. Therefore, oleanolic acid was selected as a marker compound in this study.

Up to now, several HPLC methods have been reported to quantify oleanolic acid contents in PR extract,[24-26] but there is no HPLC method to analyze oleanolic acid in PR and commercial products containing PR extract together. The quantitation of target compound in herb extract and commercial herbal products is completely different. Even though an analytical method can analyze marker compounds in herb, it is still hard to be used for quantify and qualify markers in commercial herbal products due to the different chemical compositions between herb and commercial herbal products. The problem of maker detection induces the standardization and quality control of herbal product and it interrupts to commercialize a natural product frequently.

The aim of this study is to develop a rapid and effective HPLC analytical method to apply both PR extract and scalp tonic product, qualitatively and quantitatively. Extraction conditions such as concentration of sulfuric acid and extraction time were optimized through a response surface methodology (RSM), which is an effective and powerful tool for optimizing experimental conditions due to its logical validity over a minimal number of runs. Optimum conditions were elucidated through central composite design (CCD), which is widely used because of its practical design. Oleanolic acid peak were identified from LC-ESI–MS by their spectral characteristics and by comparing with standard signatures.

2. Materials and Methods

2.1. Chemicals and reagents

Four scalp tonic products were made by a cosmetic company in Korea and coded EG01, EG02, EG03 and EG04 with different PR extract contents, EG01 is the only one without PR extract. Acetonitrile, methanol and ethyl acetate were purchased (HPLC grade) from Burdick & Jackson (Morris Plains, NJ, USA).Sulfuric acid and formic acid were obtained from Daejung Chemicals & Metals (Siheung, Korea). Oleanolic acid having purity of 98 % was purchased from ALB Technology (HongKong, China) for standard and its structure was shown in *Fig.* 1. Distilled water of over 18 MΩ was purified by Milli-Q

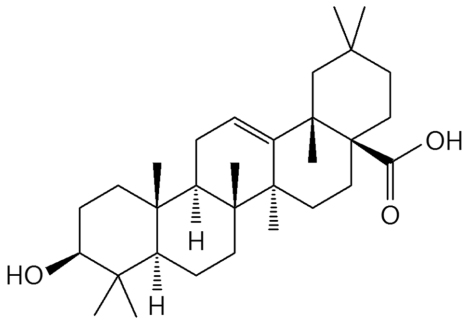

Fig. 1. The chemical structure of oleanolic acid.

purification system (Sinhan, Seoul, Korea). Syringe filter (25 mm, 0.22 μm, PVDF membrane) were made by Woongki Science (Seoul, Korea). All samples were stored at 4 °C before use.

2.2. Preparation of sample and standard solutions

PR (0.1 g) and scalp tonic solution (5 mL) were accurately weighted, and 10.0 mL of 2.8 M sulfuric acid was added to each sample for extraction. The solution was refluxed in water bath at 95 °C for 76 min. After cool down in the air, the solution was partitioned by adding 15.0 mL of ethyl acetate three times for liquid-liquid extraction. All ethyl acetate layers were combined together and evaporated to dry. 1.0 mL of methanol was added to dissolve the dried extract. This solution was filtered by 0.22 μm PVDF filter. 1.0 mg of oleanolic acid standard was dissolved in 1.0 mL methanol for stock standard solution and storage at 4 °C. Working standard solution was prepared by diluting the stock solution with methanol.

2.3. Development of HPLC method

HPLC analysis was carried out on a Shimadzu LC-20AD series system (Kyoto, Japan) using an Optimapak C_{18} column (4.6 × 250 mm, 5 μm, RStech, Korea) with mobile phase of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) at 35 °C. The elution condition (isocratic or gradient) and flow rate (0.5 mL/min to 1.0 mL/min) of mobile phase were optimized with several different HPLC runs. Overall consideration, the gradient program was with 95-99 % B at 0-8 min, 99 % B at 9-21 min and 99-95 % B at following 1 min, at a mobile phase flow rate of 0.5 mL/min and an injection volume of 5 μL.

2.4. Identification of oleanolic acid in sample by LC-ESI-MS.

To identify oleanolic acid in sample, mass spectrometric experiment was performed using Shimadzu LCMS-8040 system under ESI (electrospray ionization) interface mode. Mass condition was set drying gas (N_2) of 10 L/min at 200 °C, nebulizing gas of 3.0 L/min, the heat block temperature of 350 °C, the interface voltage of 3.5 kV in positive and negative mode. The other parameters were same as HPLC-UV conditions.

2.5. Optimal extraction conditions

For optimizing extraction efficiency of samples, response surface methodology (RSM) was performed based on the result of preliminary one-variable-at-a-time (OVAT). Acid types, different acid concentrations, hydrolysis time and material-solvent ratio were examined on OVAT experiments. According to the quantitation result of preliminary experiments, two remarkable factors were selected and designed as X1: concentration of sulfuric acid (1.0, 3.0 and 5.0 M), X2: extraction time (30, 60 and 90 min) for the further central composite design (CCD). Totally, 13 runs containing 5 replicates of central points, 2 variables at three levels (low, medium and high) and responses from these experiments (Y) were designed. The results were shown in *Table* 1. All data were analyzed by Design Expert Software Ver. 10 (30 trial version, Stat-Ease Inc., Minneapolis, Minnesota, USA). The model with the largest effect demonstrated the maximum values of each factor, and it was inferred from the three-dimensional response surface.

2.6. HPLC method qualification

Linearity, limit of detection (LOD), limit of

Table 1. Two-variables for central composite design

Run	Conc.	Time
1	3	75
2	3	75
3	5	75
4	1	120
5	1	30
6	3	75
7	3	120
8	5	30
9	3	30
10	3	75
11	3	75
12	1	75
13	5	120

quantitation (LOQ), precision, accuracy, repeatability and recovery were performed for method qualification. Linearity was verified by the coefficient of determination (r^2) of calibration curve between a series of standard solution and its corresponding peak area. LOD and LOQ were determined the concentration at the signal-to-noise ratio of 3:1 and 10:1 respectively. Accuracy and precision were evaluated at low, medium and high concentration of standard solution five times a day for intra-day and five consecutive days for inter-day. Precision was expressed relative standard deviation (RSD). Repeatability was examined by six continuous injections of sample and evaluated with value of RSD. Recovery was determined by spiking the known concentration in three levels (80 %, 100 % and 120 %) in sample solution. The fortified sample were dealt with same procedure as described in section 2.2. The result was calculated as recovery (%) = amount found/ (amount original + amount spiked) × 100 %. All results were expressed with RSD value.

3. Results and Discussion

3.1. Optimization of HPLC conditions

According to publications, acetonitrile is good for mobile phase to detect oleanolic acid in natural herb,[27] and in this research, wavelength was set at 200 nm, therefore water-acetonitrile was selected as mobile phase. Furthermore, adding formic acid, 0.1 % aqueous formic acid-acetonitrile showed better shape and symmetry of peak than water-acetonitrile. Comparing the resolution and other factors, gradient mode with 0.5 mL/min was adapted instead of 1 mL/min. Except 0.5 mL/min, other flow rate cannot be used to separate oleanolic acid with its front impurity peak. The effect of flow rate on retention time and resolution between oleanolic acid peak and impurity peaks was shown in *Fig.* 2. With the flow rate of 0.5 mL/min, the gradient elution program was set as follows: initial 0-8 min, mobile phase B changed from 95 % to 99 %; the following 12 min, B was kept at 99 %; B inclined back to 95 % in next 1 min. Another 10 min was required to equilibrate the column.

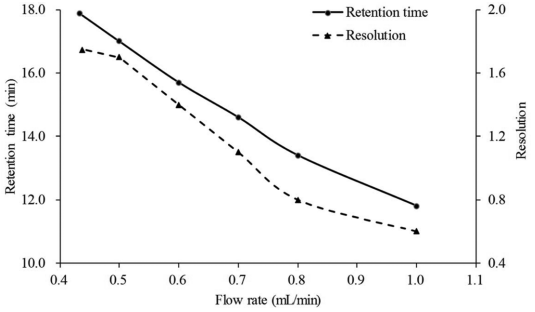

Fig. 2. Effect of flow rate on retention time of oleanolic acid and resolution of oleanolic acid and next available peak.

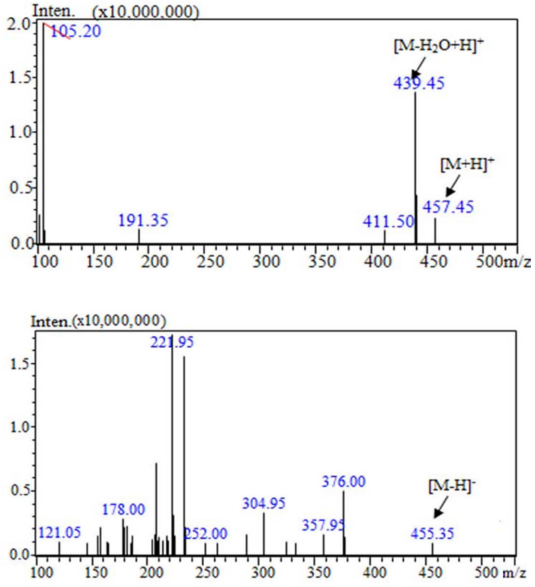

Fig. 3. LC-ESI-MS spectrum of oleanolic acid in sample at positive and negative mode.

Totally, 30 min was needed and the injection volume of each sample was 5 μL. Retention time of oleanolic acid standard and samples were all eluted at 17.0 min. Samples were analyzed by LC-ESI-MS to identify the oleanolic acid. The pseudo-molecular ions at 17.0 min in MS spectrum were *m/z* 439 $[M\text{-}H_2O + H]^+$ and *m/z* 457 $[M+H]^+$ in positive mode, and *m/z* 455 $[M\text{-}H]^-$ in negative mode (*Fig.* 3). It indicated that the detected compound was oleanolic acid. Additionally, same mass-to-charge ratio has been observed to do identification of oleanolic acid in published papers.[24]

3.2. Optimization of extraction of PR and product

The central points of two factors, sulfuric acid concentration and extraction time, were decided as mentioned in section 2.5, as 3.0 M and 60 min, respectively. From 13 experiments, built on 2-factor, 3-level CCD were used to design the three-dimensional model (*Fig*. 4). The maximum yield obtained from the optimal conditions values of two variables by RSM were 2.8 M sulfuric acid and 76 min of extraction time. The relationship between the two factors and peak area (Y) was positive at the beginning and finally got a form of parabola. However, their relation became negative and this was considered as the result of the dissolution of other constituents and high temperatures caused by extraction to marker compounds. Response surface methodology is one of the multivariable statistic techniques to figure out the optimal condition including the interactive effects among the variable parameters.

Table 2. Comparison between experimental and RSM values for analytical sample preparation (n=3)

	X1 (%)	X2 (min)	Y(AU × 10^3)
Predicted by RSM	2.8	76.2	3115
Experimental	2.8	76.0	3094±20
Matching (%)	-	-	99.3%

The experiment was performed under the optimized conditions obtained by RSM model and the results were in accordance with the predicted values, indicating that the applied RSM model in this study was suitable for sample preparation (*Table* 2).

3.3. HPLC method qualification

Linearity was evaluated at seven different concentrations of standard solution diluted with 100 % methanol. The coefficient of determination (r^2) was 0.9997 and it indicated that peak area and the concentration of marker compound showed good

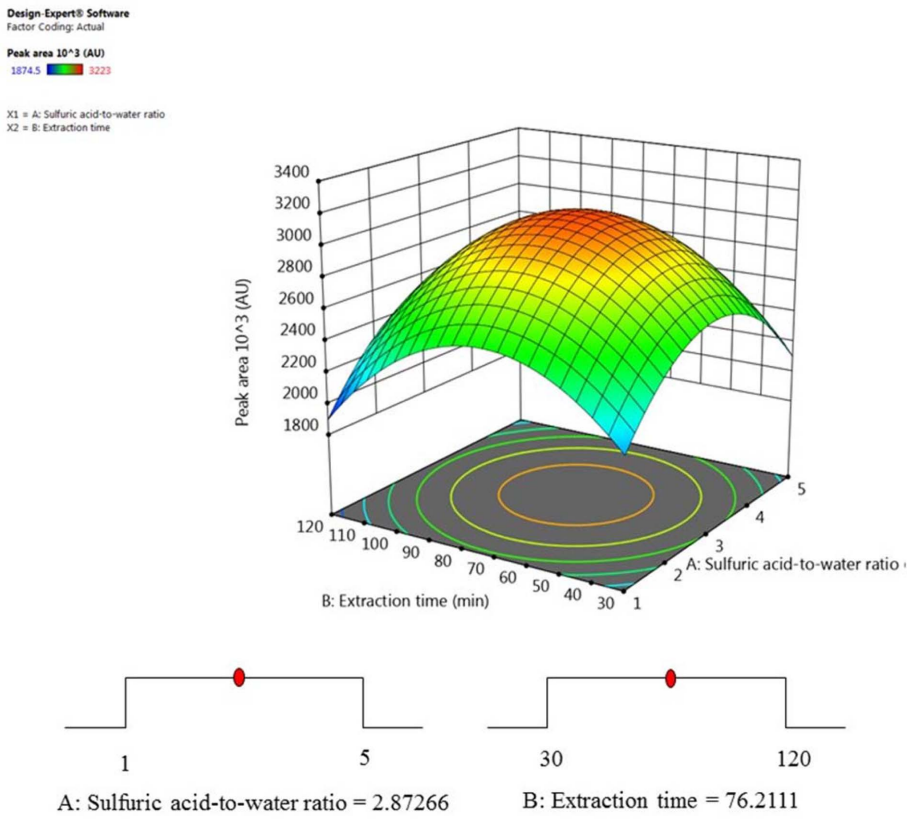

Fig. 4. Optimal extraction condition of scalp tonic by 3D response surface methodology.

Table 3. Intra-/inter-day accuracy and precisions of oleanolic acid (n=5)

Conc. (μg/mL)	Intra-day			Inter-day		
	Found (μg/mL)	Accuracy (%)	RSD (%)	Found (μg/mL)	Accuracy (%)	RSD (%)
0.61	0.63±0.01	100.9	1.43	0.64±0.01	102.2	1.83
12.50	12.45±0.19	99.6	1.53	12.50±0.14	100.3	1.12
100.0	99.50±0.54	99.5	0.54	100.1±0.71	100.0	0.71

Table 4. Recovery of the developed method (n=3)

Products	Concentration of oleanolic acid			Recovery (%)	RSD (%)
	Original (μg/mL)	Spiked (μg/mL)	Found (μg/mL)		
EG02	0.247	0.200	0.424±0.003	95.0	0.9
		0.250	0.490±0.002	98.7	0.3
		0.300	0.524±0.003	95.9	0.4
EG03	0.478	0.400	0.846±0.014	96.4	1.8
		0.500	0.941±0.004	96.2	0.4
		0.600	1.025±0.002	95.2	0.2
EG04	2.532	2.000	4.503±0.065	99.4	1.5
		2.500	4.997±0.033	99.3	0.7
		3.000	5.465±0.029	98.8	0.5

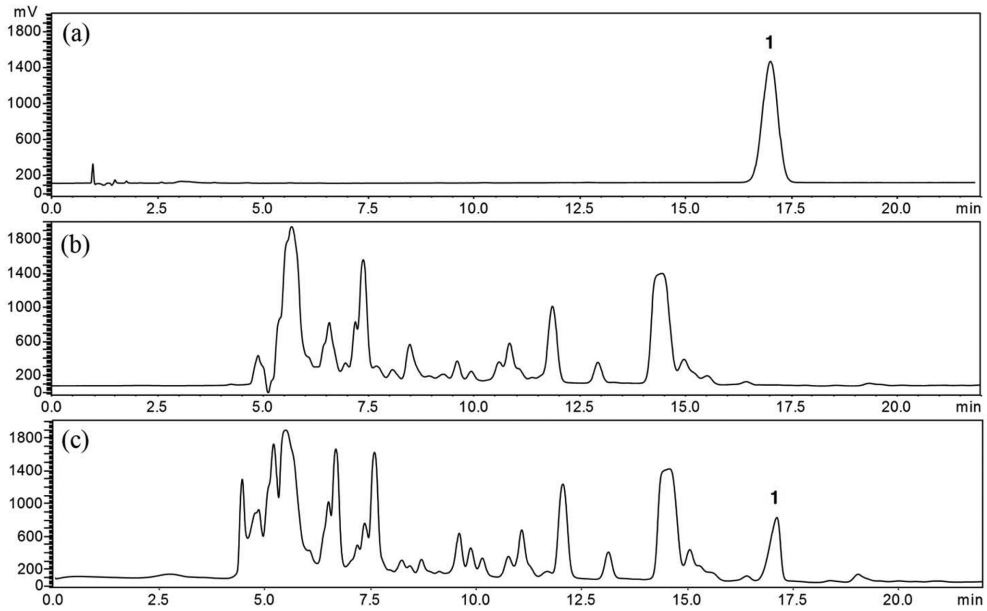

Fig. 5. HPLC chromatograms of (a) oleanolic acid standard, (b) EG01 and (c) EG04 solution. HPLC conditions: column; Optimapak C_{18} (4.6 × 250 mm), mobile phase; 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B), gradient program; 0 min 95 %B, 8 min 99 % B; 20 min 99 %B, flow rate; 0.5 mL/min, column temp.; 35 °C, Peak: 1. Oleanolic acid.

linearity in the concentration range of 0.1 to 120 mg/mL. LOD and LOQ were estimated as 17.5 ng/mL and 55.0 ng/mL, respectively. Intraday accuracy and precision were verified at three different concentrations (0.6, 12.5 and 100 mg/mL) of standard solution five times in a day. Inter-variabilities were performed in

five consecutive days. Accuracies of intra-day and inter-day were 99.5 % to 100.9 % and 100.0 % to 102.2 %, respectively. Precisions of them were 0.5 % to 1.4 % RSD and 0.7 % to 1.8 % RSD (*Table* 3). As shown in *Table* 4, recovery of each sample was at acceptable range. Sample preparation process was suitable because of low loss of marker compound and high extraction efficiency of marker compound during sample treatment. In overall, the developed method is well qualified in accordance with the guidelines of China and Korea.[28-29]

3.4. Application for the quality control of scalp tonic products

The qualified method was applied to the developed scalp tonic products, and as shown in *Fig.* 5, the marker compound, oleanolic acid, could be base line separated from other matrices in the products. EG01 showed no peak at 17.0 min, indicating that blank sample contains no oleanolic acid, while the marker compound could be determined from PR extract, EG02, EG03 and EG04 samples. The concentration of oleanolic acid in products could be converted into the concentration of PR extract using the concentration of oleanolic acid in the PR extract (156.9±0.7 mg/g). As shown in *Table* 5, the concentrations of PR extract in the products, EG02, EG03 and EG04 were 0.157 %. 0.305 % and 1.61 %, respectively.

4. Conclusions

This study was performed to develop a simple HPLC method for standardization and quality control of commercial scalp tonic product containing PR extract. The selected marker compound (oleanolic acid) was suitable for the quality control of PR and scalp tonic. The optimal conditions of analytical sample estimated by RSM coupled with CCD were 2.8 M sulfuric acid and 76.0 min of extraction time by sonication. The developed method was useful to apply for the quality control PR extract and scalp tonic together by qualitative and quantitative analysis. In addition, the developed method can be applied to set up efficiently for the quality control of other tonics and liquid products based on PR extract.

Table 5. Oleanolic acid content in EG products (n=3)

Products	Oleanolic acid (μg/mL)	PR Ex.in product (mg/mL)	PR Ex.in product (w/v%)
EG02	0.247±0.002	1.57	0.157
EG03	0.478±0.004	3.05	0.305
EG04	2.532±0.002	16.14	1.614

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Authors' Positions

Lin Xi Cai : Research Scientist
Chong Woon Cho : Research professor
Yan Zhao : Professor
Jong Seong Kang : Professor
Kyung Tae Kim : Professor
Sang-Hun Jung : Professor