Natural Product Sciences
17(3) : 225-229 (2011)

Similarity Analysis of Chemical Profiles of *Scrophularia buergeriana* Based on HPLC-DAD†

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Abstract – A new approach for similarity analysis of chemical profiles of *Scrophularia buergeriana* was developed. The roots of *S. buergeriana* collected in Andong, Korea were analyzed using HPLC-DAD and the peak areas of the chromatograms were used to construct the chemical profiles of *S. buergeriana*. The analytical conditions were validated for specificity, precision and repeatability. The standard chemical profiles were established from the chromatograms of nine standard analytical extracts from three batches. The correlation coefficients of peak areas of characteristic compounds between the chromatograms of the standard and test samples were calculated for quantitative expression of the chemical profiles. The results showed that the HPLC chemical profiles of the samples of same geographic origin were relatively consistent. This new approach could be applied to the quality assessment of herbal medicines with complex chemical compositions.
Keywords – chemical profile, similarity analysis, correlation coefficient, *Scrophularia buergeriana*

Introduction

Nowadays, there is an increasing trend towards the use of herbal medicine worldwide. Herbal medicines comprise a lot of complex phytochemicals and their curative effects are known to be principally based on the synergistic effect of their multi-targeting, multi-ingredient preparation (Xie *et al*., 2008). Since chronic, and degenerative diseases, and diseases with multiple causes are increasing these days, herbal medicines are suggested as new type of drugs replacing chemical drugs often focusing on a single target. However, those above mentioned characteristics of herbal medicines make it more difficult to identify all the chemical constituents and the active components among them, which means that standardization and quality maintenance of herbal medicines are also very difficult. Despite their chemical complexity, the current practice for assessing quality of herbal medicines is by measuring the contents of one or very few markers or active components, which is referred to as the marker approach (Mok and Chau, 2006). This strategy has proved to be insufficient for the quality control of herbal medicines (Li *et al*., 2010). To solve this problem, recently, significant efforts have been made to devise methods for the quality control of herbal medicines by utilizing the chemical profile data acquired by various analytical instruments including HPLC, GC, NMR and so on. A detailed chemical profile of an herbal medicine is certainly necessary to ensure the quality and pharmacological effects. According to the concept of phytoequivalence, other herbal extracts fitting the profile of the clinically proven profile is assumed to have the same pharmacological activities (Mok and Chau, 2006). Chromatograms have been suggested as a good source of chemical profiles of herbal medicines by presenting separated chemical patterns. Many methods have been proposed to evaluate the similarity or difference of herbal medicines based on the calculation of the similarity between the chromatograms (Gan and Ye, 2006). Although there has been great achievement in the area of chemometrics, there are still some problems in extracting chemical profiles from digitized chromatograms, which is mainly due to the variations in peak heights and retention times for a given sample running under the same analysis conditions. Thus sometimes it may be better to construct the chemical profile from peak areas in the chromatogram, since peak areas corresponding to the concentrations of the compositions of herbal medicine are relatively stable (Gan and Ye, 2006).

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In our previous study, the five active constituents of *Scrophularia buergeriana* were simultaneously determined by HPLC-DAD and the resulted data were applied to multivariate analysis including principal component analysis (PCA) to discriminate the samples according to their geographic origins (Kim *et al*., 2010). Choosing the right raw material is very important for herbal medicines. Once the raw material of a specific geographic origin has been selected, then it should be also considered how to ensure its quality consistency. Thus, in the present study, we tried to develop new method for similarity evaluation of *S. buergeriana* using its chemical profiles derived from HPLC chromatograms.

Materials and methods

Plant materials and chemicals – Three samples of *S. buergeriana* originated from Andong were purchased from Kyungdong traditional herbal market (Seoul, Korea). All the samples were authenticated by Dr. Jong Hee Park, professor of Pusan National University. Voucher specimens (EC0010-1 ~ EC0010-3) have been deposited at the Institute for Life Science, Elcomscience Co. HPLC grade solvents (acetonitrile, water and methanol) and reagents were obtained from BDH chemicals (Poole, UK). Acetic acid (analytical grade) was purchased from Merck (Darmstadt, Germany). Triply deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

HPLC-DAD conditions – The Waters ACQUITY UPLC® System with the PDA detectors were used for the experiment. The output signal of the detector was recorded using a Empower™ 2 Software. Chromatographic separation was achieved on a Capcell Pak C18 UG120 (150 mm × 4.5 mm i.d., 5 μm) using a linear gradient elution of A (acetonitrile) and B (0.1% formic acid in water) at a flow rate of 1.0 mL/min (0 min 20% A; 20 min 32% A; 22 min 40% A). The wavelength for quantification was set at 280 nm (Lee *et al*., 2007).

Sample preparations for HPLC – Dried *S. buergeriana* roots were ground and sieved. Particles with the size between 1.5 cm and 2.0 cm i.d. were collected for the study. An accurately weighed sample powder of 5.0 g was introduced into a 250 mL round bottom flask and 100 mL of 70% ethanol was added. The flask was attached to a reflux condenser and extracted for 2 h at 90 °C using a heating mantle. This extract was filtered and evaporated in vacuum, and then suspended to 10 mL with 50% methanol in a volumetric flask. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC. Three batches of *S. buergeriana* extract were prepared according to the above mentioned procedure. Three analytical samples were collected from each batch.

Similarity evaluation – The chemical profile of each analytical sample was constructed from the areas of the selected peaks in the chromatogram. Similarities between the test chromatograms and the standard chromatogram were evaluated using correlation analysis.

Results and Discussion

Optimization of chromatographic conditions – The quality of each chromatogram directly affect the quality of its chemical profile. Thus the HPLC chromatographic conditions should meet the requirements of providing adequate chemical information, good separation, and repeatability of the assay (Xie *et al*., 2007). In the previous report, various chromatographic conditions were tried to develop the optimal analytical condition for *S. buergeriana* using HPLC-DAD and LC-ESI-MS (Lee *et al*., 2007). During the process, it was found that most of compounds in *S. buergeriana* showed strong UV absorbance at 280 nm. Various analytical columns, Capcell Pak C18 UG120 (250 mm × 4.5 mm i.d., 5 μm), Capcell Pak UG120 (150 mm × 4.5 mm i.d., 5 μm), Capcell Pak C18 MG (150 mm × 4.5 mm i.d., 5 μm), Xterra RP18 (150 mm × 4.5 mm i.d., 5 μm), Luna C18 (150 mm × 4.5 mm i.d., 5 μm), and Motor C18 (150 mm × 4.5 mm i.d., 5 μm), were tested and the chromatograms were compared. In contrast to the previous report, the preferred chromatographic condition was found to be using Capcell Pak UG120 (150 mm × 4.5 mm i.d., 5 μm) with a mixture of 0.1% formic acid in water and acetonitrile. A gradient elution was chosen to obtain the efficient separation of peaks from the extracts.

Method validation of HPLC-DAD method – Using the optimized HPLC method, separation of the five major peaks showed satisfactory resolution (Fig. 1A). Except these five peaks, the other peaks could not provide enough signal intensity and repeatability. Hence these peaks were chosen for the establishment of chemical profiles. Their specificity was determined by the calculation of peak purity facilitated by DAD. The absorption spectrum of a single peak remained invariable at each time point in one peak, which supported the singularity of each peak (Fig. 1B). The intermediate precision of the method was assessed by three independent injections of the sample on three sequential days (1, 3, 5 days). The analytical samples were independently prepared from one batch. The relative peak area (RPA) and relative retention time

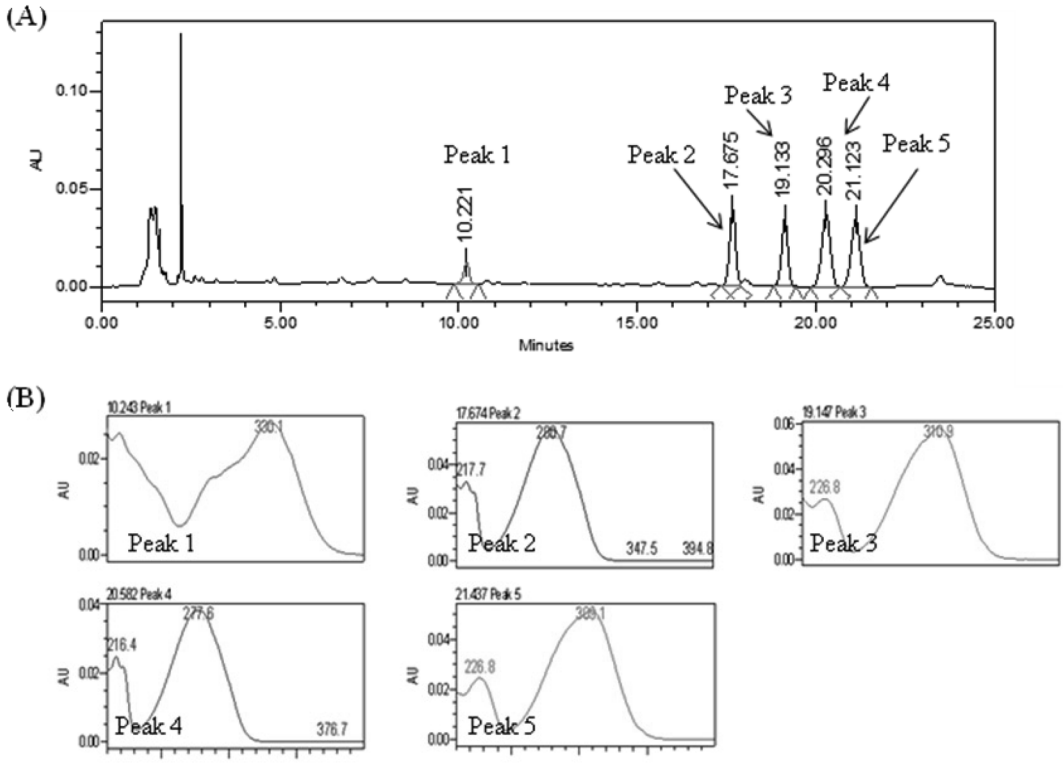

Fig. 1. The typical HPLC chromatogram of *S. buergerian* extract (A) and UV spectra of the five major characteristic peaks (B).

Table 1. Intermediate precision of RPA and RRT

RPA[a]	Day 1	Day 3	Day 5	Average	RSD (%)[c]
Peak 1	0.197	0.195	0.205	0.199	2.598
Peak 2	0.755	0.749	0.751	0.751	0.398
Peak 3	0.643	0.663	0.667	0.657	1.963
Peak 4	1.000	1.000	1.000	1.000	0.000
Peak 5	0.953	0.945	0.944	0.947	0.506
RRT[b]	Day 1	Day 3	Day 5	Average	RSD (%)[c]
Peak 1	0.502	0.503	0.507	0.504	0.572
Peak 2	0.854	0.864	0.867	0.862	0.766
Peak 3	0.924	0.936	0.937	0.932	0.758
Peak 4	1.000	1.000	1.000	1.000	0.000
Peak 5	1.041	1.041	1.041	1.041	0.033

[a]Original values for average peak areas were 126049.0, 475764.3, 416354.7, 633231.7 and 599665.0, respectively.
[b]Original values for average retention times were 10.42, 17.81, 19.27, 20.67 and 21.52, respectively.
[c]RSD (%) = (standard deviation of measured value / mean of measured value) × 100 (n = 3)

(RRT) of each characteristic peak related to the reference peak were calculated for semi-quantitative expression of the chemical properties in the HPLC chromatograms of *S. buergeriana*. The peak 4 was used as the reference peak as it has a large and stable peak area. The RSD values of RPA and RRT of the five peaks were found in the range of 0.51 – 2.60% and 0.03 – 0.77%, respectively (Table 1).

The method repeatability was determined by analyzing six independently prepared analytical samples. The RSD values of RPA and RRT were lower than 0.45 and 3.03%, respectively (Table 2). These validation results indicated that the conditions used in this analytical method were acceptable.

Table 2. Repeatability of RPA and RRT

RPA[a]	1st	2nd	3rd	4th	5th	6th	Average	RSD (%)[c]
Peak 1	0.200	0.196	0.197	0.196	0.197	0.197	0.197	0.627
Peak 2	0.748	0.749	0.750	0.751	0.753	0.755	0.751	0.329
Peak 3	0.667	0.669	0.624	0.625	0.624	0.643	0.645	3.026
Peak 4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
Peak 5	0.939	0.940	0.939	0.938	0.951	0.953	0.943	0.708
RRT[b]	1st	2nd	3rd	4th	5th	6th	Average	RSD (%)[c]
Peak 1	0.506	0.505	0.501	0.503	0.503	0.502	0.503	0.342
Peak 2	0.864	0.862	0.859	0.859	0.855	0.854	0.859	0.451
Peak 3	0.935	0.933	0.929	0.930	0.926	0.924	0.929	0.431
Peak 4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000
Peak 5	1.041	1.041	1.041	1.041	1.042	1.041	1.041	0.032

[a]Original values for average peak areas were 124805.7, 475324.0, 408212.7, 632959.3 and 597095.2, respectively.
[b]Original values for average retention times were 10.41, 17.77, 19.23, 20.69 and 21.54, respectively.
[c]RSD (%) = (standard deviation of measured value / mean of measured value) × 100 (n = 3)

Table 3. Peak areas and retention times of the characteristic peaks of three batches of *S. buergeriana* extract

Peak area	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Batch 1	124578	476622	406132	632814	602027
	123510	473155	403051	627067	597304
	130351	477573	424241	636056	600338
Batch 2	145809	660992	404654	654525	557207
	147768	665055	406655	659639	569163
	147361	669708	407780	663810	573448
Batch 3	141192	672642	378345	1010245	681004
	144464	688015	385432	1027078	694985
	141611	678925	381233	10179943	685788
Mean	138516.0	606965.2	399730.3	769575.2	617918.2
Retention time	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Batch 1	10.45	17.77	19.24	20.78	21.65
	10.48	17.84	19.30	20.88	21.74
	10.45	17.85	19.31	20.60	21.45
Batch 2	10.49	17.90	19.37	20.81	21.67
	10.50	17.93	19.40	20.65	21.49
	10.47	17.94	19.40	20.59	21.43
Batch 3	10.52	17.95	19.42	20.55	21.39
	10.51	17.98	19.44	20.54	21.39
	10.51	17.96	19.43	20.56	21.39
Mean	10.49	17.90	19.37	20.66	21.51

Standard chemical profiles of *S. buergeriana* – The quality consistency among the differenct batches of *S. buergeriana* extracts can be described by their chemical similarity to a certain reference extract. To establish a representative chemical profile, a standard chemical profile, three authentic batches of *S. buergeriana* extract were analyzed using the established HPLC method. Three measurements were performed for each batch. Among the acquired chromatograms, representative five peaks existing in all batches were designated to the common characteristic peaks and were used to construct the chemical profile for *S. buergeriana*. The standard variation of each peak area and retention time was calculated from the data. The RSD value of retention time of each peak

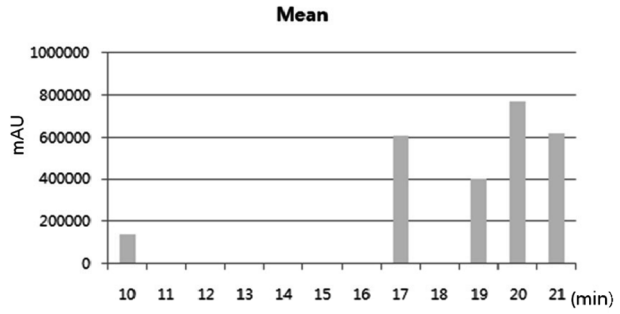

Fig. 2. Standard chemical profile of *S. buergeriana* extract.
Each bar depicts mean value of peak area at mean retention time calculated from the nine chromatograms of standard analytical extracts.

was less than 0.64%, implying good precision and repeatability of the analytical method. However, there were relatively large variations in the area of peak 2 and peak 4, although all three types of raw *S. buergeriana* for three batches of extract, respectively, had the same geographic origin, Andong, Korea. The standard chemical profile should be able to reflect those possible variations. Accordingly, the standard chemical profile of *S. buergeriana* from Andong was generated using the mean values of peak area and retention time of the five major peaks obtained from the nine chromatograms of three batches (Fig. 2, Table 3) .

Similarity evaluation of the chemical profiles – Correlation coefficients were the most commonly used statistical methods for the evaluation of similarity of the multivariate systems (Liang *et al*., 2004). The correlation coefficients of the samples constructing the standard chemical profile to the standard chemical profile were calculated. The results were all higher than 0.9638. Then another *S. buergeriana* samples collected from Andong were extracted using the same reflux method to test the feasibility of similarity evaluation based on chemical profiles. The correlation coefficient of the test sample to the standard chemical profile was relatively high, 0.9906. These results indicated that *S. buergeriana* extract was highly consistent in quality from batch to batch when raw materials with the same geographic origin were extracted in a controlled method.

Acknowledgments

This research was supported by a grant of the Korea Food & Drug Administration.

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Received June 8, 2011
Revised August 29, 2011
Accepted August 31, 2011