Quality Evaluation of Moutan Cortex Radicis Using Multiple Component Analysis by High Performance Liquid Chromatography

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A simple high performance liquid chromatographic method was developed to evaluate the quality of Moutan Cortex Radicis based on chromatographic fingerprints that characterize eight pharmacological compounds, namely, gallic acid, paeoniflorin, galloyl paeoniflorin, benzoic acid, quercetin, benzoylpaeoniflorin, paeoniflorigenone, and paeonol. These compounds were identified by their characteristic UV profiles and the mass spectroscopy data, and their contents were determined by HPLC. The chromatographic separation was performed on a C_{18} column by gradient elution with 0.05% formic acid in water and acetonitrile. The methodological validation gave acceptable linearities ($r = 0.9996$) and recoveries (ranging from 99.4 ~ 103.1%). The limits of detection (LOD) of these compounds ranged from 10 to 30 μg/mL. The representative chromatographic fingerprints of Moutan Cortex Radicis were obtained by analyzing 20 batches of samples collected from markets in Korea and China. For the efficient evaluation of quality for the commercial Moutan Cortex Radicis it is recommended that the total content of the six characteristic compounds should contain more than a minimum of 2% and that the content of total paeoniflorin and paeonol should exceed a minimum of 1.5% of dry weight of Moutan Cortex Radicis.

Key Words: Moutan Cortex Radicis, HPLC, Qualitative evaluation, Chromatographic fingerprint

Introduction

Herbal drugs have been playing an important role in the medical therapy system in the orient for thousands of years. In order to ensure the stability and efficiency of herbal drugs in clinical use, the quality control system of herbal medicines should reflect the phytoequivalence and pharmacological effects.[1] Thus, it is necessary to develop a method of evaluating the quality of herbal medicines, which is capable of comprehensive elucidation of constituents based on chemical fingerprints and quantitative analysis of key pharmacological compounds simultaneously. Moutan Cortex Radicis, the root bark of *Paeonia suffruticosa* ANDR., is one of the famous herbal medicines widely used for treatment of rheumatoid arthritis, cardiovascular disease, and anaphylaxis for hundreds of years.[2,3] Recently, new biological activities have been discovered for this herbal medicine.[4-8]

Korea Food and Drug Administration (KFDA) specified paeonol and paeoniflorin exhibits specific biological activities on human as the marker compounds for the quality control of Moutan Cortex.[9] Paeonol, which is the major active component of Moutan Cortex, is known to have anti-aggregatory, anti-oxidant and anti-inflammatory effect, and it can be used as the pharmaceutical material for treating myalgia, rheumatic pain and neuralgia.[10,11] Some other biological activities have been discovered using modern methods[12-14] indicating that paeonol could be the most important compound in this herbal medicine. Paeoniflorin, a natural antihyperlipidemic agent, is another important component of Moutan Cortex. It also has broad pharmacological effects, such as analgesic, antidiuretic, anti-inflammatory, anticonvulsant, vasodilatic and antithrombotic effects.[15,16] In addition to paeonol and paeoniflorin, many other biological active constituents were isolated from Moutan Cortex, and phytochemical and pharmacological studies have proved their biological activities such as analgesic, antibacterial, anti-inflammatory, anti-carcinogenic, antioxidative, and cardiacprotection activities.[17-21] Hence, not only paeonol and paeoniflorin but also other biological active compounds have to be included for efficient quality control of the Moutan Cortex. Although in studies that analyzed only a few samples, marker compounds have been used to characterize Moutan Cortex by HPLC-UV,[22-24] HPLC-MS[25-27] or by CE,[28,29] there has been no attempt on the determination of multiple compounds of the samples collected from the wide areas of Korea and China to present new criteria for the quality of commercially available Moutan Cortex.

In this study, a simple and rapid HPLC analytical method was developed to evaluate the quality of Moutan Cortex Radicis through simultaneous identification and determination of the multiple active compounds. Based on the established analytical method, the contents of eight key pharmacological components were determined in twenty different samples collected from Korea and China, and recommendations are made for policy decision in the quality control of Moutan Cortex.

Experimental Section

Instrument and apparatus. The HPLC system consists of a Gilson 321 pump, UV/Vis-151 detector, GX-271 liquid handler auto-sampler injector with solvent mixer 402 syringe pump, and a trilution LC 1.4 software for data processor (Gilson, USA). HPLC grade reagents, acetonitrile, methanol and water were purchased from SK Chemicals, Korea.

Standard chemicals and plant materials. Eight selected compounds – gallic acid, paeoniflorin, benzoic acid, galloyl paeoniflorin, quercetin, benzoylpaeoniflorin, paeoniflorigenone, and paeonol – were supplied by Prof. K. Bae of Chungnam National University at Daejeon, and their purities were identified by

HPLC (purity ≥ 97%). An internal standard of bisphenol A was obtained from Prof. B. S. Min, College of Pharmacy, Catholic University of Daegu, Korea. Analytical samples of the herbal drug Moutan Cortex Radicis was purchased from several markets in Korea and China. The voucher specimens were deposited at the herbarium at the College of Pharmacy, CNU.

Standard solution and calibration. Stock solution (1 mg/mL) of gallic acid, paeoniflorin, benzoic acid, galloyl paeoniflorin, quercetin, benzoylpaeoniflorin, paeoniflorigenone, paeonol, was prepared in methanol and kept below 4 °C. Standard solutions were prepared by serial dilution of the stock solution to working ranges of these substances with mobile phase. The solution of internal standard (bisphenol A) was prepared with methanol to the concentration of 625 μg/mL.

Sample preparation. Each dried Moutan Cortex Radicis was pulverized and weighed 200 mg into 50 mL volumetric flask. One milliliter of internal standard solution and 70% ethanol were added to mark the volume. After ultrasonical extraction for 30 min at 40 °C, the sample mixture was filtered through 0.2 μm membrane filters, then 10 μL aliquots from the filtrate were subjected to HPLC analysis.

Chromatographic conditions. HPLC analysis was achieved on a C_{18} column (4.6 × 250 mm, 5 μm, Phenomenex, USA) using the guard column of C_{18} cartridge (3.0 × 4.0 mm). A gradient elution was used for separation with solvent A, 0.05% formic acid in water and solvent B, acetonitrile. At a flow rate of 1.0 mL/min, the following gradient was used: 0 min, 10% B; 20 min, 20% B; 30 min, 40% B and held at 40% for 20 min. The chromatogram was monitored at a wavelength of 230 nm throughout the experiments. The injection volume was 10 μL.

Optimization of extraction efficiency of main compounds. The extraction solvent (70% and 100% ethanol, and 70% and 100% methanol), extraction time (10, 20, 30, and 60 min), extraction method (shaking, refluxing, and ultrasonication) were investigated by measuring the extracted amount of three main compounds of paeonol, paeoniflorin and benzoylpaeoniflorin.

Linearity and limit of detection. Three of eight reference standards were dissolved in methanol to the final concentration of 400 μg/mL for paeonol, 600 μg/mL for paeoniflorin, and 200 μg/mL for benzoylpaeoniflorin. The solution was diluted to proper concentration to establish the calibration curve by setting up the ratio for the peak areas (peak area of target compound/peak area of internal standard) versus concentration.

Precision and accuracy. Intra-day precision was examined by analyzing the standard solution 3 times within 1 day, and interday precision was determined for 3 independent days. Both assays were determined by performing three different concentration levels (high, medium and low) of the reference standards. The recovery test was performed to evaluate the accuracy of the method by spiked known quantities of the mixed standards to the samples with known contents of each standard. Then the resultants were extracted and analyzed immediately. The added standards were prepared at two different concentration levels, and each concentration was analyzed in triplicates. The content of each standard was determined by the corresponding calibration curve. The recovery of the method was calculated by using the ratio of detected amounts to added amounts.

Results and Discussion

Optimization of extraction methods. The extraction efficiencies of three major compounds by ultrasonication were similar to those by refluxing; however, the ultrasonic extraction was applied in the experiments since it was more convenient. Alcohols (methanol and ethanol) were selected as extraction solvents because they could extract the components in the wide range of polarity and be relatively less harmful. The extraction power of alcohols for paeonol was increased about 35% when 30% of water was added to the solvent. However, the difference between methanol and ethanol in extraction was not significant. The times required for complete extraction of paeonol, paeoniflorin and benzoylpaeoniflorin were about 30, 20 and 10 min, respectively. Hence, ultrasonication in 70% ethanol for 30 min was adapted as standard extraction condition for Moutan Cortex in this study.

Linearity and limit of detection. The calibration was carried out for the eight characteristic compounds. The calibration curves showed good linearity ($r = 0.9996$) over the range of 25 to 600 μg/mL. The limits of detection (LOD) ranged between 10 ~ 30 μg/mL depending on the absorbance of the compounds.

Precision, accuracy and stability. These parameters were evaluated for three major components of paeonol, paeoniflorin and benzoylpaeoniflorin because of the higher consumption of standard materials. As shown in Table 1, the intra- and inter-day

Table 1. Intra- and inter-day precisions in the assay of the three constituents.

Comp.[a]	Theoretical (μg/mL)	Intra-day (n = 3)			Interday (n = 3)		
		Observed (μg/mL)	RSD (%)	Accuracy (%)	Observed (μg/mL)	RSD (%)	Accuracy (%)
1	22.5	22.1 ± 0.2	0.86	98.2	22.9 ± 0.2	0.67	101.7
	45	45.5 ± 0.1	0.24	101.3	44.8 ± 0.4	0.78	99.6
	90	90.7 ± 0.3	0.33	100.8	91.5 ± 0.3	0.37	101.6
2	67.5	68.8 ± 0.2	0.32	101.9	67.5 ± 0.5	0.68	99.9
	135	134.7 ± 0.5	0.40	99.8	136.1 ± 1.1	0.78	100.8
	270	270.0 ± 1.2	0.46	100.0	271.4 ± 0.5	0.17	100.5
3	22.5	22.8 ± 0.1	0.67	101.3	22.4 ± 0.1	0.36	99.3
	45	44.6 ± 0.0	0.60	99.1	45.1 ± 0.1	0.18	100.1
	90	92.3 ± 0.3	0.95	102.6	91.4 ± 0.1	0.12	101.6

[a]Components: 1. paeonol, 2. paeoniflorin, 3. benzoylpaeoniflorin.

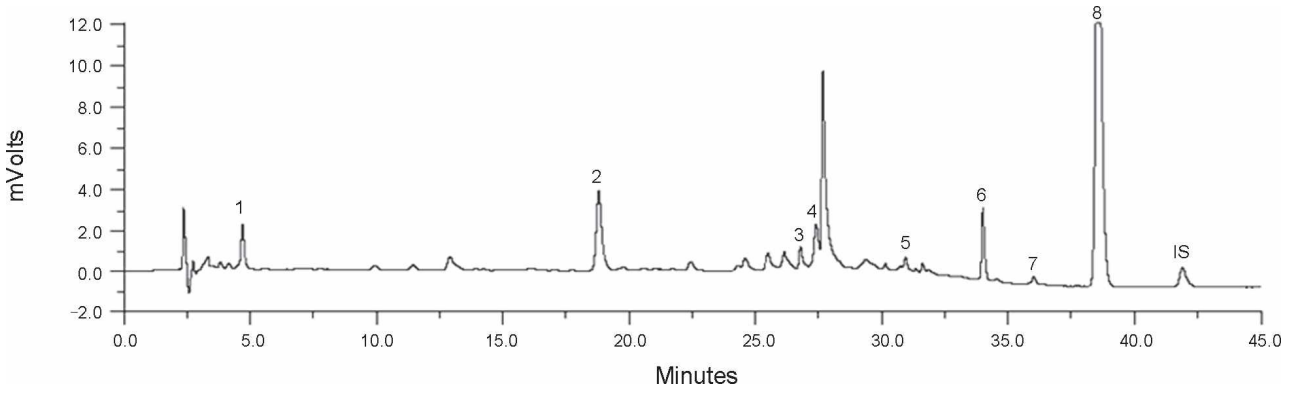

Figure 1. HPLC chromatogram of 70% ethanol extract. 1: gallic acid; 2: paeoniflorin; 3: benzoic acid; 4: galloyl paeoniflorin; 5: quercetin; 6: benzoylpaeoniflorin; 7: paeoniflorigenone; 8: paeonol; IS: bisphenol A.

Table 2. Recoveries of the three constituents in Moutan Cortex Radicis (n = 3).

Comp.[a]	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)
1	0.0	244.6	-	-
	50.0	296.5	101.0 ± 2.4	2.34
	100.0	346.6	101.0 ± 3.2	3.18
2	0.0	222.0	-	-
	150.0	383.5	103.1 + 2.7	2.58
	300.0	529.9	101.5 + 1.1	1.03
3	0.0	71.4	-	-
	50.0	121.8	100.4 + 1.3	1.29
	100.0	170.7	99.4 ± 2.1	2.07

[a]Components: 1. paeonol, 2. paeoniflorin, 3. benzoylpaeoniflorin.

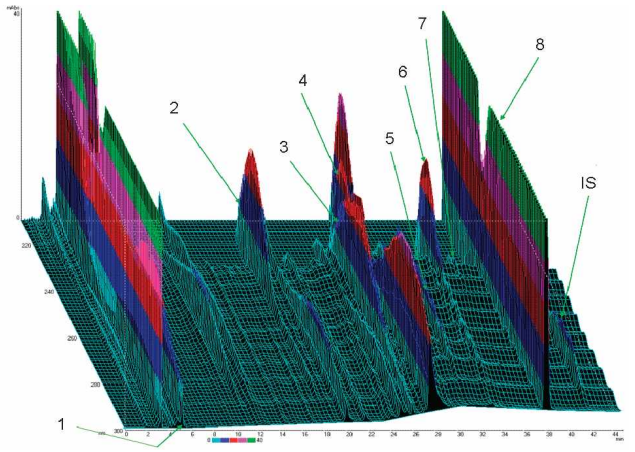

Figure 2. Three-dimensional chromatogram for identification of components in Moutan Cortex by LC-DAD. Peak identification was same as in Figure 1.

precision for three components ranged between 0.24 ~ 0.95% and 0.12 ~ 0.78%, respectively. The accuracy in standard solution ranged between 98.2 to 102.6%. The recoveries of three major compounds (Table 2), calculated by adding the known amount of standard to Moutan Cortex samples, ranged from 99.4 to 103.1%. There were no significant changes of concentration for three major components when the extracts were stored at 0 °C and 25 °C for two weeks. The observed linearity, recovery, accuracy and precision indicated that this method is suitable and applicable for qualitative and quantitative evaluation of the characteristic compounds in Moutan Cortex samples.

Analysis and evaluation of Moutan Cortex samples. Eight marker compounds were positively identified by comparing the retention time, UV spectra and MS fragmentation data of the analyzed samples with the corresponding data obtained from the analysis of reference compounds. The eight characteristic compounds and internal standard identified are shown in Figure 1. The three-dimensional chromatogram (Figure 2) was helpful in identifying the characteristic compounds from individual samples. Paeonol was the main compound in Moutan Cortex samples. Paeoniflorin, galloyl paeoniflorin, benzoylpaeoniflorin, and paeoniflorigenone, which have a similar structure, showed the same UV profile. Gallic acid and benzoic acid showed similar UV profiles. Twenty Moutan Cortex Radicis samples collected from different locations (Korea and China) were analyzed, and the content of each reference compound was calculated. The mean contents of gallic acid, paeoniflorin, quercetin, benzoylpaeoniflorin, paeoniflorigenone and paeonol were 1.36, 8.30, 0.05, 0.22, 0.10, 2.50, 0.45 and 15.97 mg/g, respectively. Paeonol showed the highest concentration, and then paeoniflorin and benzoylpaeoniflorin followed. Three samples containing significantly high content of gallic acid, paeoniflorin or benzoylpaeoniflorin were observed. The analysis results showed that there are clear variations in the contents of paeonol, paeoniflorin, gallic acid and paeoniflorigenone in different samples determined. Their contents ranged between 10.59 ~ 20.51 mg/g, 1.36 ~ 17.46 mg/g, 0.26 ~ 2.94 mg/g, and 0.00 ~ 1.26 mg/g, respectively. However, no significant variations were observed in the case of benzoylpaeoniflorin and quercetin, whose contents ranged between 0.96 ~ 4.53 mg/g and 0.01 ~ 0.22 mg/g, respectively. Moreover, the result showed that benzoic acid and galloyl paeoniflorin whose content ranged between 0 ~ 0.15 mg/g and 0 ~ 1.16 mg/g, respectively, were minor constituents, and in some samples, they did not even exist. For the purpose of quality control the total amounts of components would be more useful than the individual amounts because of the high variation of the content. The KFDA regulation has

Table 3. Contents (%) of 6 components in 20 Moutan Cortex samples.

No.	Components[a]						1 + 2[b]	1 ~ 6[c]	KFDA[d]	Recom[e]
	1	2	3	4	5	6				
MS01	1.38	0.84	0.25	0.11	0.06	0.018	2.22	2.66	Pass	Pass
MS02	1.43	0.62	0.10	0.04	0.01	0.006	2.05	2.21	Pass	Pass
MS03	1.65	0.17	0.16	0.20	0.06	0.001	1.82	2.24	Fail	Pass
MS04	2.05	0.96	0.26	0.10	0.03	0.012	3.01	3.41	Pass	Pass
MS05	1.80	0.42	0.22	0.11	0.06	0.007	2.22	2.61	Fail	Pass
MS06	1.31	0.83	0.16	0.13	0.02	0.007	2.14	2.46	Pass	Pass
MS07	1.58	0.14	0.18	0.08	0.08	0.001	1.71	2.06	Fail	Pass
MS08	1.44	0.56	0.24	0.15	0.05	0.004	2.01	2.45	Pass	Pass
MS09	1.44	0.89	0.29	0.21	0.03	0.007	2.34	2.88	Pass	Pass
MS10	1.10	1.29	0.20	0.03	0.03	0.010	2.39	2.65	Pass	Pass
MS11	1.71	1.75	0.26	0.18	0.05	0.013	3.45	3.95	Pass	Pass
MS12	1.58	0.89	0.33	0.15	0.06	0.018	2.47	3.02	Pass	Pass
MS13	2.02	0.74	0.25	0.11	0.05	0.005	2.75	3.16	Pass	Pass
MS14	1.78	0.81	0.29	0.15	0.10	0.012	2.59	3.14	Pass	Pass
MS15	1.94	0.94	0.29	0.13	0.07	0.007	2.89	3.39	Pass	Pass
MS16	1.79	0.58	0.19	0.21	0.02	0.006	2.37	2.79	Pass	Pass
MS17	1.92	0.93	0.31	0.13	0.02	0.014	2.85	3.32	Pass	Pass
MS18	1.43	1.05	0.25	0.07	0.00	0.022	2.48	2.82	Pass	Pass
MS19	1.06	0.97	0.31	0.29	0.00	0.014	2.03	2.65	Pass	Pass
MS20	1.52	1.22	0.45	0.14	0.13	0.017	2.74	3.48	Pass	Pass

[a]Components: 1. paeonol. 2. paeoniflorin. 3. benzoylpaeoniflorin. 4. gallic acid. 5. paeoniflorigenone. 6. quercetin. [b]Total content of paeonol and paeoniflorin. [c]Total content of six components. [d]Controlled by the KFDA criteria. [e]Controlled by the recommended criteria.

assigned paeonol and paeoniflorin as marker compounds for the quality control of Moutan Cortex. The minimum levels for qualification are 10 mg/g for paeonol and 5 mg/g for paeoniflorin. Among the 20 samples analyzed whose quality has been approved as good by a herbal medicine specialist, 3 samples contained less than 5 mg/g of paeoniflorin and they failed to meet the KDFA regulation (Table 3). However, these 3 samples showed a higher concentration of paeonol and other compounds, indicating that the quality control by one or two marker components only is not good enough. Therefore, it is necessary to include as many characteristic compounds as possible in the quality control of herbal medicines in order to show the complex matrices. For Moutan Cortex, the quality could be controlled by the total amount of six characteristic compounds, namely, gallic acid, paeoniflorin, quercetin, benzoylpaeoniflorin, paeoniflorigenone and paeonol. Benzoic acid and galloyl paeoniflorin are excluded because they are so easy to get and can be intentionally included in the unqualified compounds. Based on the result of statistical evaluation of the 20 samples analyzed, we suggest that the following recommendation for the quality control of Moutan Cortex. The minimum content of the total paeonol and paeoniflorin should be 1.5% of dry weight of Moutan Cortex, because the required levels of these components by KFDA are 1% and 0.5%, respectively. The total content of six characteristic compounds should exceed a minimum of 2%, because the average total content of benzoylpaeoniflorin, gallic acid, paeoniflorigenone and quercetin was about 0.5%. All the samples used satisfied the new criteria, and these criteria will be served as an efficient quality control of Moutan Cortex Radicis.

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