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Quantitative Analyses for the Quality Evaluation of Salviae Miltiorrhizae Radix by HPLC

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Abstract – In this study, quantitative analysis for the quality evaluation of Salviae Miltiorrhizae Radix using HPLC/UV was developed. For quantitative analysis, six major bioactive compounds were determined. The separation conditions employed for HPLC/UV were optimized using ODS C_{18} column (250 × 4.6 mm, 5 μm) with gradient condition of A (1% formic acid in H_2O) and B (acetonitrile : methanol : formic acid = 100 : 75 : 1) as the mobile phase at a flow rate of 1.0 mL/min and a detection wavelength of 280 nm. These methods were fully validated with respect to the linearity, accuracy, precision and recovery. The HPLC/UV method was applied successfully to the quantification of six major compounds in the Salviae Miltiorrhizae Radix. The results indicate that the established HPLC/UV method is suitable for the quantitative analysis.
Keywords – Salviae Miltiorrhizae Radix, HPLC, Quality control

Introduction

Salviae Miltiorrhizae Radix is the radix of *Salvia miltiorrhiza* Bunge in the Korean Pharmacopoeia (K.P.) and Chinese Pharmacopoeia (C.P.). Salviae Miltiorrhizae Radix is controlled to contain not less than 0.2% of tanshinone IIA and 3.0% of salvianoic acid B in C.P. Pharmacological studies have revealed in to have antioxidant (Choi *et al*., 2004), antibacterial (Lee *et al*., 1999), antiinflammatory (Kang *et al*., 2000), antiallergenic (Choi *et al*., 2004), neuroprotective (Lam *et al*., 2003), cytotoxicity (Wang *et al*., 2005), antitumor (Li *et al*., 2002), anti-fibrosis (Liu *et al*., 2000) and antithrombotic activities (Li *et al*., 2004).

Some HPLC/UV analytical methods have been developed for the analysis of Salviae Miltiorrhizae Radix and its related products (Son *et al*., 1999; Lay *et al*., 2003; Li *et al*., 2002). In the present study, a simple, sensitive and precise reverse-phase HPLC/UV method has been developed for the quantitative determination of six marker components, rosmarinic aicd (**1**), salvianolic acid B (**2**) 15,16-dihydrotanshinone I (**3**), cryptotanshinone (**4**), tanshinone I (**5**) and tanshinone IIA (**6**) for the quality control of Salviae Miltiorrhizae Radix extract. The sixteen Salviae Miltiorrhizae Radix samples purchased from China and Korea were analyzed by HPLC after extraction with 75% methanol.

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Experimental Section

Plant material – Sixteen Salviae Miltiorrhizae Radix samples purchased in 2005 for this study include the following accessions: four and twelve commercial Salviae Miltiorrhizae Radix samples purchased from Korea and China, respectively (Table 1).

Reagents – All of the standard compounds were provided by Prof. K. H. Son, Andong National University, Andong, Korea. Their structures were unambiguously identified by NMR and MS data, with the published data, such as rosmarinic acid (Kovatcheva *et al*., 1996), salvianolic acid B (Tang *et al*., 2002), 15,16-dihydrotanshinone I (Ryu *et al*., 1999), cryptotanshinone, tanshinone IIA (Danheiser *et al*., 1995) and tanshinone I (Lee *et al*., 2006). Their purities were above 95% as determined by HPLC and LC-MS/MS analysis, and the standard compound structures were shown in Fig. 1. Internal standard, 4-hydroxycinnamic acid (**7**), was purchased from Sigma Chemicals (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were purchased from Merck K GaA (Darmstadt, Germany). All other

Table 1. HPLC conditions for the assay of analytes in Salviae Miltiorrhizae Radix

	Time (min)	A (H_2O : HCOOH = 100 : 1) %	B (MeOH : CH_3CN : HCOOH = 100 : 75 : 1) %
Mobile phase	0	75	25
	1	60	40
	14	60	40
	22	40	60
	23	11	89
	40	11	89
Detector		UV (280 nm)	
Column		YMC ODS-H80 (4.6 × 250 mm)	
Flow rate		1 mL/min	
Column temp.		20 °C	
Injection volume		10 μL	

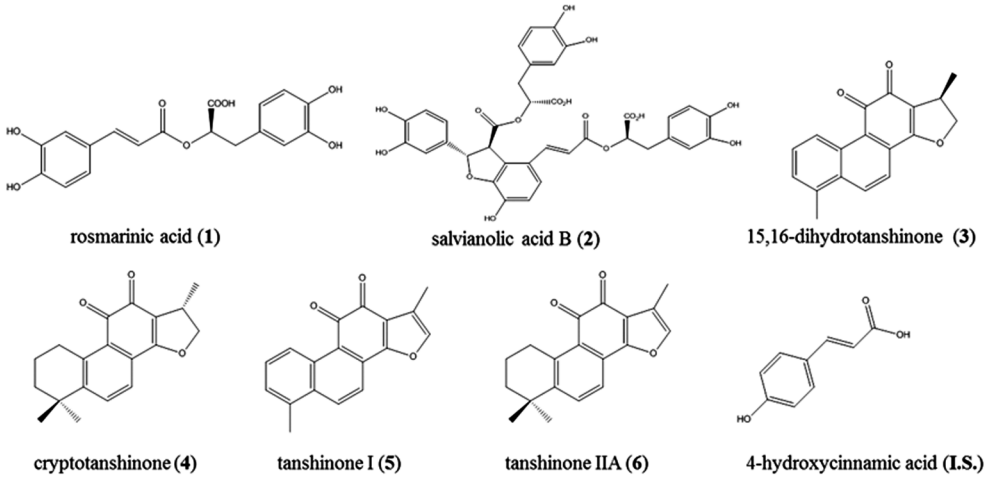

Fig. 1. Structures of the compounds from Salviae Miltiorrhizae Radix and an internal standard.

chemicals used were of analytical grade unless otherwise noted. Distilled water was prepared using Milli-Q purification system (Millipore, Bed-ford, MA, USA).

Sample preparation – To determine the content of three phenolic compounds of Salviae Miltiorrhizae Radix samples, the dried root powder was used for each extraction. Salviae Miltiorrhizae Radix sample was powdered and sieved through 100 mesh, and about 0.3 g of the powder were accurately weighed and added 50 mL of 75% methanol, accurately measured weight and sonicated for 30 min. at room temperature. The solution was cooled, weighed again, and made up the loss in weight with 75% methanol. The solution was filtered through a 0.45 μm membrane filter and the filtrate was used as the test solution. 10 μL of sample solution was subjected to injection into the HPLC system.

HPLC/UV condition – The HPLC equipment was a Gilson HPLC system (Unipoint 2.0) with Gilson 321 pumps, a UV/VIS-151 Detector and 321 XL sampling injector. YMC ODS-H80 (250 × 4.6 mm, 4 μm), Shiseido capcell pak (250 × 4.6 mm, 5 μm) and Shodex ODS pak (250 × 4.6 mm, 5 μm) columns were tested with the guard columns filled with the same stationary phase. A (H_2O : HCOOH = 100 : 1) and B (MeOH : CH_3CN : HCOOH = 100 : 75 : 1) were used as the mobile phase, using a gradient condition to analyze samples (Table 2). The mobile phase was filtered under vacuum through a 0.45 μm membrane filter and degassed prior to use. The analysis was carried out at a flow rate of 1.0 mL/min with the detection wavelength set to 280 nm, and the total run time was 40 min. All compounds could be resolved with baseline separation at 280 nm with the maximum absorption. Hence, characteristic chromatographic patterns were obtained at 280 nm. The chromatograms were processed using software Empower pro system.

Analytical method validation – The calibration curves

were made by diluting the stock solutions with 100% methanol. The reference solutions of the salvianolic acid B at concentrations of 0.2~800 μg/mL and the other five compounds at concentrations of 0.2~40 μg/mL were analyzed by HPLC/UV. The regression equations were calculated in the form of $y = ax + b$, where y and x correspond to peak area and compound concentration, respectively (Table 3). For the preparation of the crude extract, the powders of the dried radix of Salviae Miltiorrhizae Radix were sieved through a 100 mesh. The recovery, precision and accuracy tests were executed by mixing a powdered sample (0.3 g) with three control levels (salvianolic acid B as 20%, 50%, 100% and other compounds as 50%, 100%, 150%) of the reference compounds. The mixture was then sonicated with 50 mL of 75% methanol at room temperature for 30 min. The extract solution was filtered through a 0.45 μm membrane. The HPLC/UV analysis experiments were performed in triplicate for each control level. The data was compared with those from the standard solution and extracted sample.

Table 2. Salviae Miltiorrhizae Radix samples

Samples	Purchased
K-1	Cheorwon, Gangwondo, Korea
K-2	Miryung market, Korea
K-3	Jegidong, Seoul, Korea
K-4	Yangyeongsi, Daegu, Korea
C-1	Gansu, China
C-2	Hebei, China
C-3	Shandong, China
C-4	Sichuan, China
C-5	Changsha, China
C-6	Chengdu, China
C-7	Kunming, China
C-8	Lanzhou, China
C-9	Lanzhou, China (counterfeiting)
C-10	Kunming, China
C-11	Changsha, China
C-12	Xishuangbanna, China

Results and Discussion

Optimization of chromatographic condition – The HPLC conditions were selected by the requirement for obtaining the chromatograms with a better resolution of the adjacent peaks within a short retention time. All compounds could be resolved with baseline separation at 280 nm with the maximum absorption. Hence, characteristic chromatographic patterns were obtained at 280 nm. The typical chromatograms of samples and standard mixture are shown in Fig. 2 from which one can observe that all target compounds and an internal standard are completely separated within 40 minutes. 4-Hydroxycinnamic acid (**7**) was selected as an internal standard. The chromatographic peaks of the analytes in sample solution were identified by comparing their retention times with those of the reference standards and further confirmed by spiking samples with the reference compounds.

Optimization of sample preparation condition – Three extracting solvents, 100% methanol, 75% methanol and 50% methanol were compared with regard to sample assays using sonication extraction for 30 min at room temperature. When sample was extracted with 75% methanol, the sample assay was higher than the other solvent samples. Therefore, we employed 75% methanol as an extracting solvent throughout this work (Fig. 3). Two extraction methods, ultra-sonication and reflux using 75% methanol extraction solvent, were compared with regard to sample assays. When used for sonication extraction method, the sample assay was higher than reflux one (Fig. 4). To determine the time needed to obtain complete extractions, extraction of a sample was performed for three different lengths of time (15, 30, and 60 min). The rest of the variables employed were: 75% methanol solvent and sonication extraction method at room temperature. When extraction time was 30 min, the

Table 3. Calibration graphs, linear ranges, LOD and LOQ

Analytes	Linear range (μg/mL)	Slope (a)	Intercept (b)	Correlation coefficient (r)	LOD (ng/mL)	LOQ (ng/mL)
Rosmarinic acid (**1**)	0.2~40	0.0663	−0.0149	0.9999	36.2	120.6
Salvianolic acid B (**2**)	0.2~800	0.0393	0.0060	0.9999	51.5	155.0
15,16-dihydrotanshinone I (**3**)	0.2~40	0.1522	−0.0431	0.9999	30.1	100.4
Cryptotanshinone (**4**)	0.2~40	0.0881	−0.0031	1.0000	34.1	113.6
Tanshinone I (**5**)	0.2~40	0.1529	−0.0424	0.9999	36.7	122.2
Tanshinone IIA (**6**)	0.2~40	0.1510	−0.0154	0.9999	32.4	108.1

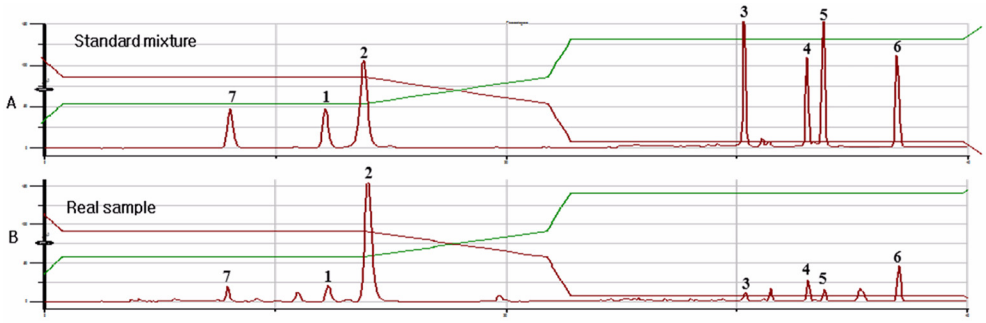

Fig. 2. HPLC chromatograms of standard mixture (A) and sample (B) of Salviae Miltiorrhizae Radix.
1 rosmarinic acid, **2** salvianolic acid B, **3** 15,16-dihydrotanshinone I, **4** cryptotanshinone, **5** tanshinone I, **6** tanshinone IIA, **7** 4-hydroxycinnamic acid (I.S.).

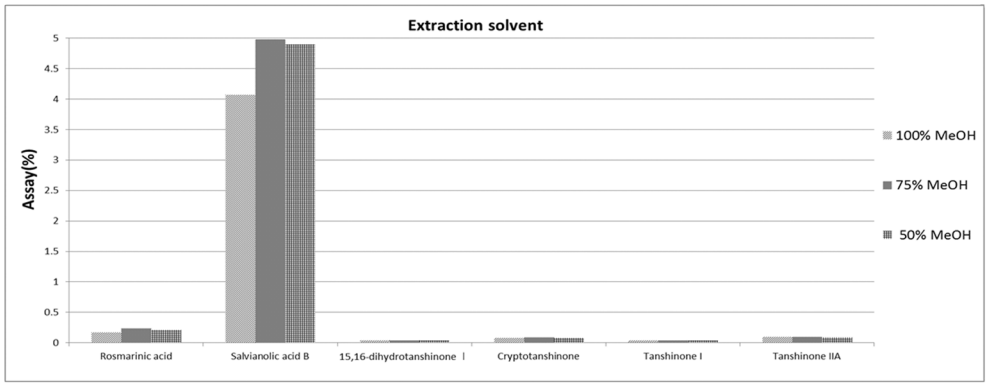

Fig. 3. Comparison of the extraction solvents for extraction efficiencies of marker compounds.

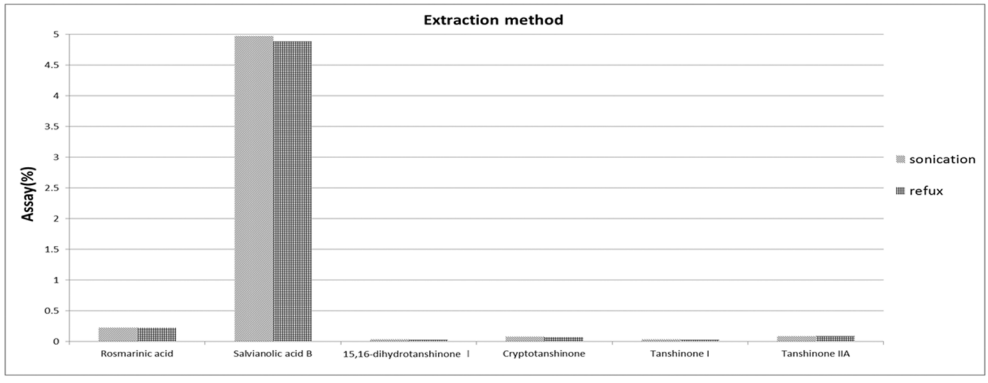

Fig. 4. Comparison of the extraction methods (reflux and sonication) for extraction efficiencies of marker compounds.

sample assay was same as 60 min, and higher than 15 min. Therefore, when extraction time was 30 min, all of the compounds were sufficiently extracted (Fig. 5).

Validation – The linearity of the peak area ratio with respect to the concentration was examined under optimal HPLC/UV conditions and is described as a regression equation. Each coefficient of correlation (r^2) was > 0.999, as determined by least square analysis, suggesting good linearity between the peak areas and the compound concentrations over a wide concentration range (Table 3). The limits of detection (LOD) and the limits of quantification (LOQ) were evaluated based on the lowest detectable peak in the chromatogram having a signal-to-noise (S/N) ratio of 3 and 10, respectively. Under our experimental conditions, we listed LOD and LOQ in Table 3. The obtained values for both LOD and LOQ for these six standards were shown to be low enough to detect traces of these compounds in either crude extract or its

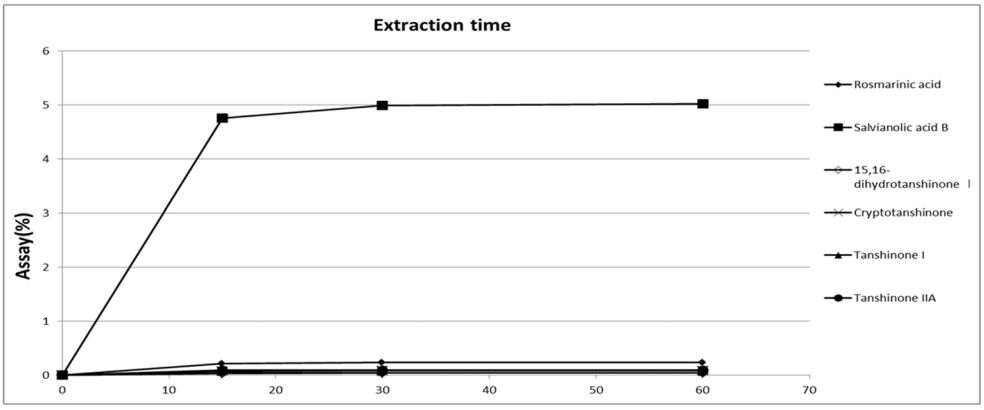

Fig. 5. Comparison of the extraction time for extraction efficiencies of marker compounds.

Table 4. Recovery of marker compounds through standard addition (n = 5)

Analyte	Fortified conc. (μg/mL)	Observed conc. (μg/mL)	Recovery mean (%)	Recovery cv (%)
Rosmarinic acid (**1**)	0.0	13.66 ± 0.18	–	–
	5.0	18.79 ± 0.16	102.6	3.0
	10.0	23.78 ± 0.19	101.2	3.9
	15.0	28.71 ± 0.25	100.4	5.1
Salvianolic acid B (**2**)	0.0	332.84 ± 1.81	–	–
	60.0	382.41 ± 3.06	82.6	5.2
	150.0	465.82 ± 4.37	88.6	4.7
	300.0	591.85 ± 5.62	86.3	6.2
15,16-dihydrotanshinone I (**3**)	0.0	1.96 ± 0.02	–	–
	1.0	3.07 ± 0.04	110.9	2.4
	2.0	4.07 ± 0.04	109.5	3.3
	3.0	5.12 ± 0.09	105.4	9.6
Cryptotanshinone (**4**)	0.0	5.05 ± 0.07	–	–
	2.0	6.98 ± 0.03	96.6	1.6
	4.0	8.99 ± 0.09	98.7	4.4
	6.0	11.00 ± 0.17	99.2	8.6
Tanshinone I (**5**)	0.0	2.39 ± 0.04	–	–
	1.0	3.24 ± 0.03	84.9	3.4
	2.0	4.22 ± 0.03	91.4	3.1
	3.0	5.24 ± 0.05	95.8	5.0
Tanshinone IIA (**6**)	0.0	5.64 ± 0.04	–	–
	1.0	7.56 ± 0.05	96.1	2.5
	2.0	9.56 ± 0.09	98.0	4.7
	3.0	11.57 ± 0.14	98.8	6.9

preparation.

The extraction recovery test was performed by extracting a known amount of the six compounds from the Salviae Miltiorrhizae Radix powder samples. The % recovery of each standard ranged from 82.6 to 110.9%, and the RSD was less than 9.6% (Table 4). The average recovery was represented by the formula: R (%) = [(amount from the sample spiked standard – amount from the sample)/amount from the spiked standard] × 100. Precision and accuracy were determined by multiple analysis (n = 3) of quality control samples prepared at lower, medium and higher concentrations spanning the

Table 5. Precision and accuracy of analytical results

Analyte	Nominal conc. (μg/mL)	Intraday (n = 3)				Interday (n = 3)			
		Observed	SD	Accuracy	Precision	Observed	SD	Accuracy	Precision
Rosmarinic acid (**1**)	5.0	5.02	0.07	101.6	1.40	5.08	0.10	100.4	2.04
	10.0	10.04	0.92	100.7	0.92	10.07	0.15	100.4	1.50
	15.0	15.01	0.05	99.9	0.32	14.99	0.28	100.1	1.87
Salvianolic acid B (**2**)	60.0	64.31	3.24	107.2	4.03	56.71	3.34	94.5	5.89
	150.0	144.38	3.12	96.3	4.01	144.28	3.76	96.2	4.21
	300.0	294.19	3.01	98.1	3.01	303.52	4.12	101.2	4.46
15,16-dihydrotanshinone I (**3**)	1.0	0.96	0.03	97.7	3.45	0.98	0.02	96.1	2.41
	2.0	2.04	0.04	104.2	2.01	2.08	0.04	102.0	1.89
	3.0	3.04	0.05	99.8	1.75	3.00	0.08	101.5	2.59
Cryptotanshinone (**4**)	2.0	1.97	0.03	102.3	1.67	2.05	0.04	98.7	1.80
	4.0	3.96	0.11	99.8	2.65	3.99	0.04	99.1	1.04
	6.0	6.02	0.10	98.9	1.72	5.94	0.08	100.4	1.35
Tanshinone I (**5**)	1.0	0.96	0.01	99.7	0.73	1.00	0.02	96.3	2.39
	2.0	1.96	0.03	97.4	1.49	1.95	0.03	98.2	1.65
	3.0	3.00	0.06	99.7	2.01	2.99	0.06	99.9	1.93
Tanshinone II A (**6**)	2.0	2.01	0.05	98.9	2.74	1.98	0.02	100.5	0.82
	4.0	3.95	0.05	100.2	1.36	4.01	0.12	98.7	2.94
	6.0	6.00	0.17	99.5	2.76	5.97	0.08	100.1	1.41

Table 6. Analytical results (w/w %) of the marker compounds in Salviae Miltiorrhizae Radix (n = 3)

Sample	Analyte content (w/w %)											
	Rosmarinic acid (**1**)		Salvianolic acid B (**2**)		15,16-dihydro-tanshinone I (**3**)		Cryptotanshinone (**4**)		Tanshinone I (**5**)		Tanshinone IIA (**6**)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
K-1	0.2201	0.0012	7.2352	0.0712	0.0542	0.0075	0.2438	0.0046	0.2532	0.0022	0.4769	0.0039
K-2	0.1127	0.0008	2.2792	0.0023	0.0346	0.0030	0.1073	0.0010	0.1175	0.0002	0.1503	0.0012
K-3	0.1010	0.0089	3.7430	0.0379	0.0086	0.0010	0.0638	0.0080	0.0417	0.0060	0.2961	0.0039
K-4	0.2170	0.0026	5.6834	0.0127	0.0398	0.0005	0.1099	0.0006	0.0897	0.0030	0.1340	0.0008
C-1	0.3337	0.0023	6.7917	0.1036	0.0134	0.0010	0.0344	0.0128	0.0553	0.0045	0.1079	0.0078
C-2	0.3586	0.0041	7.2463	0.0192	0.0487	0.0001	0.0695	0.0003	0.0685	0.0007	0.1011	0.0013
C-3	0.6717	0.0032	9.7992	0.1055	0.0772	0.0023	0.2696	0.0088	0.1976	0.0007	0.3729	0.0079
C-4	0.2688	0.0075	3.5783	0.1325	0.0071	0.0006	0.0230	0.0036	0.0211	0.0040	0.1188	0.0012
C-5	0.5912	0.0097	9.1417	0.0269	0.065	0.0002	0.2309	0.0042	0.0827	0.0024	0.1126	0.0024
C-6	0.1685	0.0062	2.4274	0.0316	0.0157	0.0039	0.0151	0.0067	0.0403	0.0037	0.0451	0.0007
C-7	0.2328	0.0009	4.8186	0.0034	0.0058	0.0001	0.0338	0.0014	0.0275	0.0001	0.1827	0.0004
C-8	0.4190	0.0033	6.4239	0.0576	0.0488	0.0005	0.0403	0.0032	0.0718	0.0017	0.0526	0.0005
C-9	0.1490	0.0089	0.3192	0.0093	0.0864	0.0013	0.2120	0.0007	0.3053	0.0046	0.4872	0.0040
C-10	0.1108	0.0099	4.7821	0.0768	0.0105	0.0012	0.0272	0.0009	0.0302	0.0002	0.0576	0.0003
C-11	0.2992	0.0003	5.9090	0.0019	0.0272	0.0013	0.0523	0.0006	0.1040	0.0006	0.1331	0.0003
C-12	0.3151	0.0026	7.1792	0.0233	0.0227	0.0002	0.0650	0.0002	0.0784	0.0004	0.1224	0.0004

calibration range. The remaining quality control samples had the intra-assay precision below 4.03% and accuracy between 96.3% and 107.2%. The remaining quality control samples had the inter-assay precision lower than 5.89% and accuracy between 94.5% and 102.0%. The above data reflects that the developed method is highly reproducible and precision and accuracy data are presented in Table 5.

Sample analysis – The developed HPLC/UV method was then applied to the simultaneous determination of the six compounds, rosmarinic acid (**1**), salvianolic acid B (**2**), 15,16-dihydrotanshinone I (**3**), cryptotanshinone (**4**), tanshinone I (**5**) and tanshinone IIA (**6**) in the Salviae Miltiorrhizae Radix. Commercially available sixteen Salviae Miltiorrhizae Radix samples were obtained from Korea and China. The developed analytical method was subsequently applied to the simultaneous determination of the six components in Salviae Miltiorrhizae Radix extract. The quantity of each compound present in samples was determined and the results are summarized in Table 6. Each sample was analyzed in triplicate to ensure the reproducibility of the quantitative result. The results indicated that, the assay of salvianolic acid B showed 2.28, 2.43 and 0.32% in K-2, C-6 and C-9, and the other samples showed 3.58~9.80%, not less than 3% (C.P. provision). The assay of tanshinone IIA showed 0.48, 0.30, 0.37 and 0.49% in K-1, K-3, C-3 and C-9 (counterfeiting), not less than 0.2% (C.P. provision), and the other samples showed 0.05~0.18%, not consistent with C.P. provision. In the purchased Korean and Chinese samples, the assay of rosmarinic acid, 15,16-dihydrotanshinone I, cryptotanshinone and tanshinone I showed 0.10~0.67%, 0.01~0.09%, 0.02~0.27% and 0.02~0.31%, respectively.

Conclusions

A rapid and optimized chromatographic method with UV detection was designed for the quality control of Salviae Miltiorrhizae Radix, well-known Korean traditional medicine. The developed HPLC/UV method for quantitative analysis of six major bioactive compounds can provide the promising prospect to comprehensive quality control of Salviae Miltiorrhizae Radix and its related herbal medicine.

In the assay of salvianolic acid B, Salviae Miltiorrhizae Radix counterfeiting sample C-9 showed 0.32%, and K-2 and C-6 showed 2.28 and 2.43%, not consistent with C.P. provision, and the other samples showed 3.58~9.80%, not less than C.P. provision. In the tanshinone IIA assay, almost of the commercial samples showed less than 0.2%, not consistent with C.P. provision except K-1, K-3, C-3 and C-9. Therefore, almost the commercial Salviae Miltiorrhizae Radix samples were not consistent with C.P. provision.

Acknowledgments

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