Simultaneous Determination of Rosmarinic Acid, Lithospermic Acid B, and Related Phenolics in *Salvia miltiorrhiza* by HPLC

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A gradient reversed-phase HPLC method was developed for the simultaneous separation and determination of rosmarinic acid, lithospermic acid B, caffeic acid, and other phenolic compounds. This method showed a satisfactory result. The limits of detection for these phenolic compounds were ≤0.05 mg/L. The developed method was applied to the determination of phenolic compounds in the dried roots of *Salvia miltiorrhiza* (Danshen) and the transformed cells of *S. miltiorrhiza*. In the transformed cells, rosmarinic acid was the predominant phenolic compound. Small amounts of lithospermic acid B and caffeic acid were also detected. In the dried roots, in contrast, lithospermic acid B was the main phenolic compound and only a very small amount of rosmarinic acid was found. In addition, danshensu and some unidentified phenolic compounds were also detected from the dried roots.

Keywords: *Phenolics; rosmarinic acid; lithospermic acid B; caffeic acid; Salvia miltiorrhiza; HPLC*

INTRODUCTION

Salvia miltiorrhiza, which has been used widely in traditional Chinese medicine for the treatment of the various kinds of diseases (Liu et al., 1992), has received much interest due to its ability to accumulate large amounts of active compounds such as tanshinones (Chen et al., 1997) and phenolic compounds (Tanaka et al., 1989; Kamata et al., 1993, 1994; Morimoto et al., 1994; Hase et al., 1997). The antioxidant activity of the plant phenolics may have potential benefits in human health (Pearson et al., 1997). The antioxidative activity of phenolics is generally ascribed to their hydroxyl groups (Chen and Ho, 1997). Seven phenolic compounds isolated from the aqueous extract of *S. miltiorrhiza* demonstrate a strong protective action against peroxidative damage to liver microsomes, hepatocytes, and erythrocytes (Liu et al., 1992). Lithospermic acid B shows endothelium-dependent vasodilation in the aorta (Kamata et al., 1993) and may be useful in the treatment of hypertension (Kamata et al., 1994). The salt of lithospermic acid B has a potent hepatoprotective activity in not only in vitro but also in vivo experiments on liver injuries (Hase et al., 1997) and exhibits an improved effect on uremic symptoms (Tanaka et al., 1989). In addition, rosmarinic acid (Mazumder et al., 1997; Arda et al., 1997), lithospermate, and rosmarinate (Lim et al., 1997) may be used as potent active substances against human immunodeficiency virus type one (HIV-1).

The rapid qualitative and quantitative analyses of these phenolic compounds have been a central problem in medicinal chemistry and biotechnology. Several reversed-phase HPLC and TLC methods have been developed for the determination of some of these compounds. An isocratic reversed-phase HPLC method was described to measure lithospermic acid B and rosmarinic acid in the dried tissue of callus, regenerated plantlets, or cultivated plants of *S. miltiorrhiza* (Morimoto et al., 1994). Gradient reversed-phase HPLC methods have also been used for the analysis of lithospermates and rosmarinates in an aqueous extract of *Cordia spinescens* (Lim et al., 1997) and of rosmarinic acid, lithospermic acid, and lithospermic acid B in hairy root cultures of *Ocimum basilicum* (Tada et al., 1996). A TLC-densitometric method has been reported for the parallel determination of rosmarinic acid and caffeic acid in five *Salvia* species (Janicsak and Mathe, 1997). In addition, a combined analytical method has been developed for the identification of lithospermic acid B in the aqueous extract of *S. miltiorrhiza* by using TLC and FAB-MS techniques (Kamata et al., 1993) and of rosmarinic acid and caffeic acid in *Sanicula europaea* L via spectral data and TLC comparisons with authentic samples (Arda et al., 1997).

However, no studies concerning the simultaneous determination of rosmarinic acid, lithospermic acid B, caffeic acid, and other related phenolic compounds have been reported. The complexity of phenolic compounds makes it difficult to separate them in a single chromatographic run. The primary aim of the present study is to develop a gradient reversed-phase HPLC method for the simultaneous separation and determination of these phenolic compounds in the dried roots (Danshen) and transformed cells of *S. miltiorrhiza*.

EXPERIMENTAL PROCEDURES

Transformed Cells and Dried Roots of *Salvia miltiorrhiza*. A transformed cell culture of *S. miltiorrhiza*, obtained after infecting the sterile plantlets of *S. miltiorrhiza* with *Agrobacterium tumefaciens* strain C58, was cultivated in 250-mL shake flasks containing 100 mL of hormone-free liquid 6,7-V medium supplemented with 20 g/L sucrose (Chen et al., 1997). The pH of the medium was adjusted to 5.7 prior to autoclaving at 121 °C for 15 min. Cultivation was performed on an orbital shaker at 160 rpm in darkness at 25 °C. Danshen (the dried roots of *S. miltiorrhiza*) was purchased from the local market.

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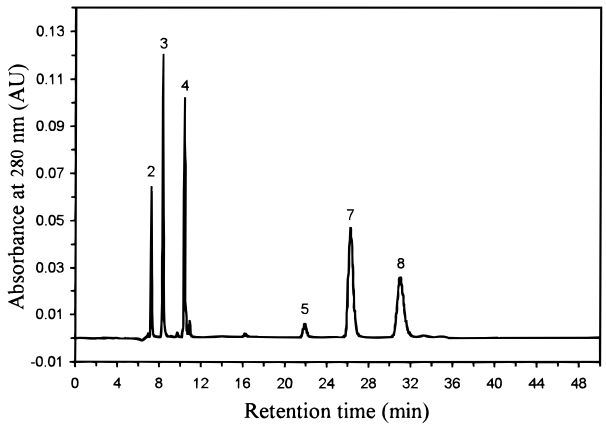

Figure 1. HPLC chromatogram of phenolic compound standards. Peaks: 2, protocatechuic acid; 3, protocatechualdehyde; 4, caffeic acid; 5, phenolic compound A; 7, rosmarinic acid; 8, lithospermic acid B.

Extraction. Cells were collected by separating the medium from the cell aggregates on Whatman no. 1 filter paper under vacuum and washing the cell aggregates three times with distilled water. The cells were then freeze-dried using a Heto FD3 freeze-dryer (Heto-holten, Denmark). The dry powdered cells (30 mg) and roots (15 mg) of *S. miltiorrhiza* were extracted with 5 mL of methanol, respectively. The mixture was then separated by centrifugation at 10 000*g* for 5 min, and the supernatant was collected. The extraction procedure was repeated at least three times. The total extracts were filtered through a 0.45 μm filter and then directly injected into the HPLC for the determination of phenolic compounds.

Chemicals and Reagents. Formic acid and HPLC-grade methanol were obtained from BDH Laboratory Supplies (Poole, England). Caffeic acid, protocatechualdehyde, and protocatechuic acid were purchased from Sigma (St. Louis, MO). Rosmarinic acid was purchased from ICN (Costa Mesa, CA). Lithospermic acid B was obtained as gifts from Dr. K. Kamata (Hoshi University, Hoshi, Japan) and Dr. Y. M. Xu (Shanghai Institute of Materia Medica, Academia Sinica, China), respectively. Danshensu was kindly provided by Shanghai Medical University.

HPLC Method. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The extract solution was separated and analyzed (20 μL aliquots) by using a 250 × 4.6 mm Beckman Ultrasphere C_{18} (5 μm) column at 30 °C. The mobile phase consisted of solvent A (methanol/water/formic acid, 14.0:85.2:0.8, v/v) and solvent B (methanol/water, 65:35, v/v). For the simultaneous separation of rosmarinic acid, lithospermic acid B, caffeic acid, and other related phenolic compounds, the following gradient elution procedure was used: 0% of B for 2 min; a linear gradient from 30% to 45% of B for 8 min. The flow rate was set at 1.0 mL/min. The tridimensional chromatogram was recorded from 200 to 500 nm. Peaks were measured at a wavelength of 280 nm to facilitate the detection of rosmarinic acid and related phenolic compounds. Chromatographic peaks were identified by comparing retention times and spectra against known standards.

RESULTS

A gradient elution procedure for separating rosmarinic acid, lithospermic acid B, caffeic acid, and related phenolic compounds was developed and used. The typical chromatogram and chromatographic data obtained from the standard mixture solution and the tested samples are shown in Figure 1 and Table 1, respectively. As shown in Figure 1, protocatechuic acid, protocatechualdehyde, caffeic acid, rosmarinic acid, and lithospermic acid B were all well separated.

The calibration curves of the peak-area (A) ratio against the concentration (C), for rosmarinic acid ($A = 45\,043C - 14\,816$, $r = 0.9991$), lithospermic acid B ($A = 20\,922C + 5135.3$, $r = 0.9992$), caffeic acid ($A = 69\,276C + 27\,549$, $r = 0.9984$), protocatechuic acid ($A = 25\,686C + 22\,913$, $r = 0.9998$), and protocatechualdehyde ($A = 88\,728C + 99\,913$, $r = 0.9995$), gave a linear response over a wide range of concentrations (Figure 2). The relative standard deviations of replicate injections ($n = 5$) for these compounds were 2.27% for rosmarinic acid, 2.67% for lithospermic acid B, 3.95% for caffeic acid, 5.06% for protocatechualdehyde, and 2.86% for protocatechuic acid, respectively. The recovery was determined by the standard addition method in order to demonstrate the accuracy of analysis. Known amounts of the standards were added to the samples. Three determinations with two spiking levels were carried out. The contents of the five phenolic compounds in the sample and the recoveries of these compounds are shown in Table 2.

As an application, the phenolic compounds in the dried roots and the transformed cells of *Salvia miltiorrhiza* were separated and determined. Figure 3 shows the typical chromatograms of the extracts of the dried roots and the transformed cells of *S. miltiorrhiza*. The identification of these phenolic compounds was achieved by comparing retention times and spectra against known standards. The absorption spectra of the five phenolic compound standards and five unknown compounds in the dried roots and transformed cells of *S. miltiorrhiza* were measured by the HPLC photodiode array detector, and the spectral data are shown in Table 1. The external standard method was used for the determination of phenolic compounds. The contents of the five unidentified compounds (compounds A−E) were measured by comparing the peak areas with rosmarinic acid. The results of determination are shown in Table 3.

DISCUSSION

In the present study, we used the mixed solvent of methanol, formic acid, and water as the mobile phase, to separate rosmarinic acid and related phenolic compounds from the extracts of *Salvia miltiorrhiza*. The results indicated that the presence of formic acid could minimize the peak tailing and improve the separation of these phenolic compounds. The retention behavior and separation effect of these phenolic compounds were affected significantly by the content of methanol in the mobile phase, especially for lithospermic acid B and rosmarinic acid. A higher content of methanol could reduce the retention times of rosmarinic acid and lithospermic acid B and improve their peak shapes, but increasing the content of methanol enabled worse separation of rosmarinic acid and lithospermic acid B to occur. The elution sequence of rosmarinic acid and lithospermic acid B might change while further increasing the content of methanol. In comparison with rosmarinic acid and lithospermic acid B, protocatechuic acid, caffeic acid, and protocatechualdehyde were poorly retained on the reversed-phase columns. It is difficult to simultaneously separate rosmarinic acid, caffeic acid, lithospermic acid B, protocatechualdehyde, and protocatechuic acid by using the isocratic elution method due to their larger differences in polarity. The HPLC methods reported were unsuitable for the simultaneous determination of rosmarinic acid and caffeic acid (Jan-

Table 1. Identification of Phenolic Compounds in the Dried Roots (Danshen) and Transformed Cells of *S. miltiorrhiza*

peak no.	retention time (min)	absorption maxima (nm)				compds identified	detection limit (mg/L)
1	6.6	233.5			280.6	danshensu	
2	7.2	230.0	258.2		295.8	protocatechuic acid	0.01
3	8.3	234.6	281.9		310.2	protocatechualdehyde	0.005
4	10.4	239.8			324.4	caffeic acid	0.004
5	21.8	230.0	286.8		324.2	phenolic compound A	
6	24.7	228.8	252.3	290.0	309.0	phenolic compound B	
7	26.3	229.9			329.2	rosmarinic acid	0.02
8	31.0	230.0	253.5	286.6	310.2	lithospermic acid B	0.05
9	33.7	233.5			285.3	phenolic compound C	
10	38.3	228.8	251.9	290.1	304.3	phenolic compound D	
11	45.5	228.8	252.3	290.1	309.0	phenolic compound E	

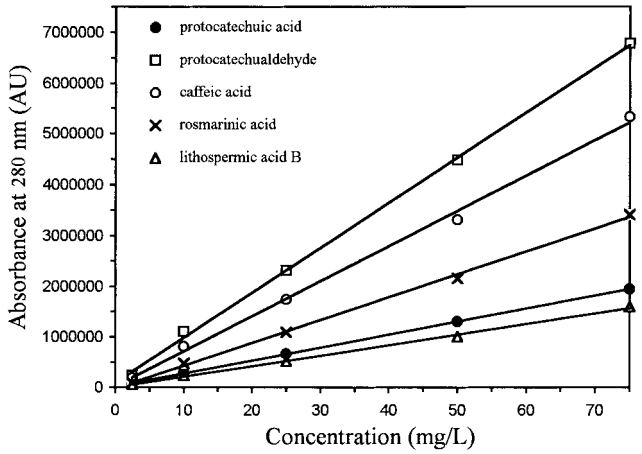

Figure 2. Calibration curves for the determination of the phenolic compounds.

Table 2. Recoveries of Standard Addition in the Transformed Cells of *S. miltiorrhiza*

compd	content (mg/g)	amt added (mg/g)	found (mg/g)	recovery (%) ± RSD
rosmarinic acid	9. 90	6.0	15.63	95.5 ± 1.4
		15.0	24.40	96.7 ± 2.1
lithospermic acid B	0.76	8.0	8.31	94.4 ± 1.8
		15.0	15.16	96.0 ± 1.6
caffeic acid	0.15	8.0	7.85	96.3 ± 1.3
		15.0	14.88	98.2 ± 1.7
protocatechualdehyde	0.02	8.0	7.82	97.5 ± 2.3
		15.0	15.22	101.3 ± 2.7
protocatechuic acid	not detected	8.0	7.74	96.8 ± 2.5
		15.0	15.11	100.7 ± 1.9

icsak and Mathe, 1997). Therefore, in the present work, we used the gradient elution procedure to separate rosmarinic acid and related phenolic compounds.

The results of determination indicated that, as shown in Figure 3 and Table 3, the composition of phenolic compounds in the transformed cells was significantly different from that in the natural dried roots of *S. miltiorrhiza*. Rosmarinic acid, which has been found to have an anti-HIV activity (Mazumder et al., 1997; Arda et al., 1997), is the major phenolic compound in the transformed cells of *S. miltiorrhiza*. Very small amounts of lithospermic acid B and caffeic acid were detected. In the dried roots of *S. miltiorrhiza*, lithospermic acid B, danshensu, and two unidentified compounds are the main phenolic compounds. Only a small amount of rosmarinic acid was found in the dried roots.

It has been reported that seven phenolic compounds, including rosmarinic acid, salvianolic acid A, salvianolic acid B (lithospermic acid B), caffeic acid, protocatechualdehyde, protocatechuic acid, and danshensu, have been isolated from the water-soluble extract of *S. miltiorrhiza*

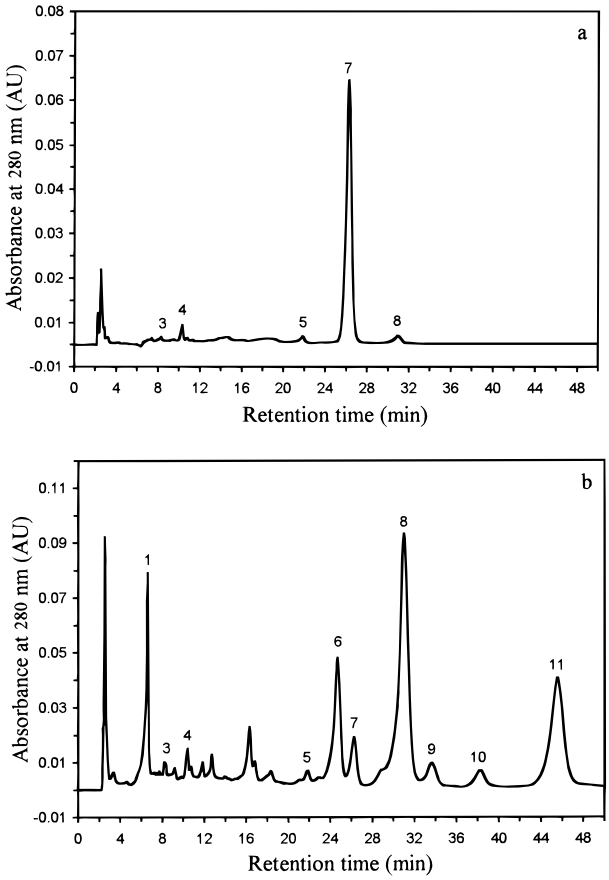

Figure 3. HPLC chromatograms of the extract from (a) the transformed cells and (b) the dried roots (Danshen) of *Salvia miltiorrhiza*. Peaks: 1, danshensu; 3, protocatechualdehyde; 4, caffeic acid; 5, phenolic compound A; 6, phenolic compound B; 7, rosmarinic acid; 8, lithospermic acid B; 9, phenolic compound C; 10, phenolic compound D; 11, phenolic compound E.

(Liu et al., 1992). In this work, no protocatechuic acid was detected from both the transformed cells and the dried roots of *S. miltiorrhiza*. For salvianolic acid A, since no standard could be obtained, it could not be identified from the chromatogram of the dried roots (Figure 3b). The absorption spectra of phenolic compound B and compound E are almost the same (see Table 1), indicating that phenolic compounds B and E have similar structures. Phenolic compound A (peak 5) in Figure 1, which was an impurity from the standards of phenolic compounds and has been detected in the rosmarinic acid standard, was also detected from the transformed cells and the dried roots (Figure 3a,b, respectively) of *S. miltiorrhiza*.

The developed method for the simultaneous separation and determination of these phenolic compounds on

Table 3. Results of Determination of Phenolic Compounds in the Dried Roots (Danshen) and Transformed Cells of *S. miltiorrhiza* (mg/g)

	transformed cells		dried roots (Danshen)	
phenolic compd	content (mg/g)	% tot. phenolics	content (mg/g)	% tot. phenolics
rosmarinic acid	9.90 ± 0.21	90.1	1.66 ± 0.04	3.0
lithospermic acid B	0.76 ± 0.02	6.9	28.72 ± 0.78	52.7
caffeic acid	0.15 ± 0.01	1.4	0.27 ± 0.01	0.5
protocatechualdehyde	0.02 ± 0.01	0.2	0.03 ± 0.01	0.1
protocatechuic acid				
danshensu			8.71 ± 0.31	16.0
phenolic compound A	0.16 ± 0.01	1.4	0.20 ± 0.01	0.4
phenolic compound B			4.88 ± 0.10	8.9
phenolic compound C			1.27± 0.03	2.3
phenolic compound D			0.96 ± 0.02	1.8
phenolic compound E			7.80 ± 0.17	14.3

one chromatographic run is very effective and has shown a satisfactory result. It can be applied to the simultaneous determination of rosmarinic acid, lithospermic acid B, caffeic acid, and other phenolic compounds in plants and cell cultures.

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