Conversion of salvianolic acid B into salvianolic acid A in tissues of Radix Salviae Miltiorrhizae using high temperature, high pressure and high humidity

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Abstract

Salvianolic acid A (Sal A)，an important component of *Radix Salviae Miltiorrhizae* (RSM), is effective for the treatment of myocardial infarction (MI) and coronary heart disease due to its potential in the improvement of acute myocardial ischemia. However, its content is very low in RSM. So it is obvious to find a rich source of Sal A or to improve its content by conversion of other related components into Sal A modifying reaction conditions. In this research we focused on the conversion of Sal B into Sal A in aqueous solutions of RSM by using different reaction conditions including pH, temperature, pressure and humidity. During the reactions, the contents of Sal A, Sal B and danshensu in the RSM were analyzed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LCMS). The results indicated that the conversion of Sal B into Sal A in RSM tissues under the conditions of a high temperature, high pressure and high humidity was efficient and thereby, was readily utilized to prepare rich Sal A materials in practice.

Keywords: Radix Salviae Miltiorrhizae (RSM); conversion; salvianolic acid A; salvianolic acid B; danshensu

Introduction

Radix Salviae Miltiorrhizae (RSM), commonly known as Danshen, is the dried root of *Salvia miltiorrhiza Bunge* which is widely used in traditional Chinese medicines (TCM) for the treatment of cardiovascular and cerebrovascular diseases such as angina pectoris, hyperlipidemia, and acute ischemic stroke (Zhou *et al.*, 2005). Salvianolic acid A (Sal A) and salvianolic acid B (Sal B) are the two most important phenolic acids of the effective constituents in RSM. Sal B, a main effective component of *Salvia miltiorrhiza Phenolic Acid Salt Injection* that was approved by China Food Drug Administration (CFDA) in 2005, is widely used in clinical practice for the treatments of myocardial infarction (MI) and coronary heart disease in China. Sal A, the most effective salvianolic acids of RSM, is demonstrated to have cardiovascular protection (Ho and Hong, 2011), cardioprotection (Pan *et al.*, 2011), and anti-hepatic fibrosis (Tsai *et al.*, 2010). In particularly, the treatments for acute myocardial ischemia in rat models with doses of Sal A 10, 5 mg/kg, respectively, are more effective than that with Sal B 10 mg/kg, and the effect of Sal A 2.5 mg/kg on improving acute myocardial ischemia in rats is equivalent to that of Sal B 10 mg/kg (Song *et al.*, 2007). However, the chromatographic fingerprint analysis of RSM displayed that the content of Sal A was very low in RSM (Liu *et al.*, 2007). Due to these facts, scientists have focused on Sal A to improve its contents in RSM. The degradation of Sal B in 0.9% (w/v) sodium chloride aqueous solution could produce nine products including a very small amount of Sal A (Zhou *et al.*, 2011). The decoction of Sal B in an aqueous solution of pH 4.9 at 100 °C for 24 h produced 13 compounds including tiny amount of Sal A (Lee *et al.*, 2012). When Sal B and lithospermic acid were separately dissolved in aqueous solution and were kept in a reactor under high temperature and high pressure (120 °C, 0.2 MPa) for 4 h, the major product was Sal A (Wang *et al.*, 2011a), and this conversion could be conducted in acidic solution with pH scale from 1.0 to 5.0 for reaction time period from 1 h to 5 h, but the yields was very low, particularly of 2.48%, 2.34% and 0.69% Sal A from the extracts with 10%, 28% and 100% Sal B, respectively (Wang *et al.*, 2011b). This might be due to the fact that Sal A in hot aqueous solution was very unstable (Xu *et al.*,

2011).

The research group of Shandong University have been involved in exploring the bioactive constituents of the root of *S. miltiorrhiza* Bunge var. *alba* C. Y. Wu et H. W Li (Cao *et al.*, 2008; Cao *et al.*, 2009a, b; Zhu *et al.*, 2012a, b), which is used as RSM in clinical practice in China. The present research aimed to obtain a rich Sal A resource by converting Sal B into Sal A in the tissues of RSM using high temperature, high pressure and high humidity. The conditions, such as pH values of aqueous solutions to wet RSM materials, reaction temperatures and reaction time for converting Sal B into Sal A, were investigated. Optimal conditions and a more applicable method for preparing rich Sal A contents have been described in this report.

Experimental

Chemicals and materials

High-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1260 series HPLC system with an auto sampler and diode array detector. Liquid chromatography-mass spectrometry (LC-MS) analysis was conducted on a Shimadzu LC-MS 2020 system, which consisted of a Shimadzu HPLC analysis system and Shimadzu mass spectrometer with 10-2000 ESI and API interfaces (Shimadzu, Japan). Ultrasonic wave extraction was conducted in a KO-250B ultrasound apparatus (Kunshan, China). The pH values of solutions were measured by a Mettler Toledo FE20-Kit FiveEasy pH Meter with pH Electrode LE438 (Mettler Toledo, Switzerland). Raw RSM materials were grounded in a pulverizer GF-150 (Zhongnan, China), and were treated in an inner-loop-pressure steam sterilization pot LDZX-50KB (Shenan, China). The treated powder was dried in Christ Alpha 1-4 LSC freeze dryer (Marin Christ, Germany).

The roots of *S. miltiorrhiza* Bunge (RSM) were collected from Tai'an (Shandong, China) in November 2010 and were authenticated by Professor Lan Xiang. The voucher samples (No. 2012003) were kept at the laboratory for future reference.

Deionized water was used to wet the crude materials, and Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for HPLC or LC-MS

analysis. HPLC grade acetonitrile (SayFo, USA) and phosphoric acid (Merck, Darmstadt, Germany) were used for HPLC analysis. HPLC grade methanol (SayFo, USA) was used for extraction and sample preparation from RSM. Other chemicals and solvents were of analytical grade.

Preparation of solutions with different pH values

The solutions with different pH values from pH 1.0 to pH 13.0 were prepared by diluting an appropriate amount of 1N aqueous hydrochloric acid (HCl) solution or 1% (w/v) sodium hydroxide (NaOH) solution with deionized water, and their pH values were measured by a pH meter calibrated with standard buffer solutions (B4, 0.05 mol/kg potassium hydrogen phthalate solution, pH 4.003; B6, 0.025 mol/kg mixed phosphate solution, pH 6.864; and B9, 0.01 mol/kg borax solution, pH 9.182) at 25 °C, respectively.

Thermal degradation of Sal B in RSM root tissue

The RSM materials were grounded into coarse powder (less than 2 mm in diameter). 2.0 grams of the powder was put in a watch glass and wetted by 4.0 ml of a required solution with a pH value (pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, or 13.0). The watch glass was then placed in an air-removed, high-temperature steam sterilization pot, and was kept under various experimental conditions such as a high temperature (105 °C, 110 °C, 115 °C, 120 °C, or 125 °C), high pressure (0.16 - 0.20 MPa) and high humidity (saturated vapor, 90% - 100% relative humidity) for reaction time (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, or 8 h). Later, the treated powder was taken out of the pot and was kept into an ultra-low temperature freezer. Then it was dried in a freeze dryer for 24 h.

Preparation of sample solutions

The freeze-dried powder (0.5 g) was extracted with 2.0 ml methanol at room temperature for 30 min by ultrasonic wave extraction. The supernatant methanol solution was filtered through a 0.22 μm membrane for HPLC or LC-MS analysis.

HPLC analysis

The HPLC analysis was carried out on an Agilent TC-C18 column (4.6 × 250 mm, 5 μm). The operational conditions of HPLC included the followings: flow rate at 1.0 ml/min, injection volume at 10 μl, column temperature at 30°C, wavelength of diode array detector ranged from 200 to 400 nm, and detection at 286 nm. The mobile phase consisted of 0.05% (v/v) phosphate solution (A) and acetonitrile (B). A gradient program was used as follows: 0-15 min, 10-20% B; 15-35 min, 20-25% B; 35-45 min, 25-30% B; 45-55 min, 30-90% B; and 55-70 min, 90% B.

The methanol extraction of original or treated RSM powder was analyzed by HPLC under the analytical conditions described above. The content of Sal B, Sal A, and danshensu, one of the effective constituents in RSM for cardiovascular disease treatment, were described using their peak areas measured by HPLC, since their areas in the HPLC chromatogram were directly proportional to their actual content in the same extraction (Jiang *et al*., 2011; Shi *et al*., 2005).

LC-MS analysis

The HPLC mobile phase consisted of 0.03% (v/v) formic acid aqueous solution (A) and acetonitrile (B), and its gradient program was the same as those HPLC analysis described above. The mass spectrometer was operated in negative ion mode with an ion spray voltage of 4.5 kV. Nebulizer gas flow rate was 1.5 L/min, fragmentor was 130 V, and skimmer was 60 V. The mass spectrometer was scanned ranging *m/z* 100-1000 in full scan mode. The sample was detected in the negative mode from 0 to 70 min. Data were processed by LCMS solution Version 5.11 Software.

The methanol extraction of original or treated RSM powder was analyzed by LC-MS under the conditions of LC-MS analysis described above.

Analytical method validation

Analytical method was validated for the stability, precision and repeatability, following the International Conference on Harmonization (ICH) guidelines (ICH Topic Q2B 1996) and several reports on quantitative determination (Liu *et al*., 2012;

Jiang *et al*., 2011)

Results and discussion

Validation of the analytical method

Stability and precision

Stability of sample solution was determined at the time of 0, 8, 16, 24, 32, 40 and 48 h under room temperature by HPLC. The compounds were found to be relatively stable in methanol solution within 48 h (RSD < 0.68, Table 1).

Intra- and inter-day precisions were tested by analyzing a sample solution for five replicates in one day and by duplicating the experiments once a day for five consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. The results were shown in Table 1, and the RSD values of the tested compounds were all less than 1.23%.

Repeatability

Six independently prepared sample solutions using the same RSM powder, wetted by an aqueous solution with pH 4.0 and treated under 120 °C for 1 h, were analyzed to test the repeatability of the method. As shown in Table 1, this method had good repeatability with RSD less than 1.98%.

HPLC analysis of phenolic acids in original and treated RSM

The chromatographic fingerprint analysis of phenolic acids in original RSM and treated RSM were shown in Figure 1, in which the peaks were assigned based on the results of LC-MS analysis (Figure 2 and 3, Table 2 and 3). In original RSM，compared with treated RSM, the content of Sal B was the highest, but the content of Sal A was very low. In treated RSM, the content of Sal B decreased along with the reaction time period prolonged, while the content of Sal A increased at earlier stage, but decreased later (Figure 1).

LC-MS analysis of phenolic acids in the original RSM

The qualitative LC-MS analysis of phenolic acids in original RSM was shown in

Figure 2 and Table 2. The total-ion chromatogram (TIC) was in good agreement with the HPLC-ultraviolet (UV) chromatogram. The chemical constituents of the main negative ion peaks in the TIC were identified with the previous reports (Hu *et al.*, 2005; Lee *et al.*, 2012; Zheng and Qu, 2012), which were listed in Table 2. The most abundant phenolic acid was conformed as Sal B.

LC-MS analysis of phenolic acids in treated RSM

The qualitative LC-MS analysis of phenolic acids in treated RSM was shown in Figure 3 and Table 3. The TIC chromatogram was in good agreement with the HPLC-UV chromatogram. The chemical constituents of the primary negative ion peaks in TIC chromatogram was identified with the previous reports (Hu *et al.*, 2005; Lee *et al.*, 2012; Zheng and Qu, 2012), which were listed in Table 3. The main phenolic acid was conformed as Sal A.

By comparing the phenolic acids of the treated RSM with that of the original RSM, the contents of Sal B and lithospermic acid obviously decreased; while the contents of Sal A, danshensu, protocatechualdehyde, and salvianolic acid D (Sal D) significantly increased in the treated RSM. By analyzing the structural features of these compounds, the thermal degradation pathway of Sal B was deduced as shown in Figure 4. It was inferred that Sal B was decomposed into lithospermic acid by the 'pathway a'，and then lithospermic acid was transformed into Sal A by decarbonylation through the 'pathway b'，which was different from the degradation of Sal B in aqueous solution (Zhou *et al.*, 2011), since Sal B could be converted into Sal A at a pH value from 7.0 to 12.0 in tissues of root. On the other hand, lithospermic acid and Sal A could be decomposed into Sal D, protocatechualdehyde or danshensu，as shown in Figure 4.

Effect of solutions with various pH values on conversion of Sal B into Sal A

The effect of an aqueous solution with a pH value from 1.0 to 13.0 on the conversion of Sal B into Sal A in RSM root tissue was investigated under conditions of different reaction temperatures and reaction time (at 120 °C for 2 h, at 120 °C for 4 h,

at 125 °C for 2 h, and at 125 °C for 4 h). The contents of Sal A, Sal B, and danshensu were measured in the RSM treated under above different conditions, and the results were shown in Figure 5A, 5B, and 5C, respectively.

The contents of Sal A and Sal B in RSM wetted by a solution with pH 1.0 both were found to be higher than those in RSM wetted by solutions with other pH values, but the content of danshensu was just the reverse. Hence, it could be confirmed that Sal B and Sal A were relatively stable, and the decomposition of Sal B into danshensu was less at pH 1.0, producing more Sal A. The contents of Sal A, Sal B, and danshensu in RSM treated with solutions at different pH values from pH 2.0 to pH 11.0 were fluctuated in a relatively small range. This may be attributed to their similar stability and any operational errors involving temperature, pressure, drying or extraction. In RSM treated with other solutions with a pH value range 2.0 - 12.0, by comparing with the materials wetted by a solution with pH 1.0, the content of Sal A and Sal B were obviously reduced, but the content of danshensu distinctly increased. The peak areas of Sal A and Sal B in RSM wetted by a solution with pH 1.0 and treated at 120 °C for 4 h were 27038.3 and 10573.0, respectively. However, they were 14056.4 and 4499.2, respectively, in RSM wetted by a solution with pH 2.0 and treated at 120°C for 4 h. At the same time, the peak area of danshensu was 6043.8 in RSM wetted by the solution with pH 1.0, but up to 11997.7 in RSM wetted by the solution with pH 2.0.

The amount of Sal A and Sal B decreased more in RSM wetted by a solution with pH 12.0, along with much amount of danshensu was generated, but they were all zero in the materials wetted by the solution with pH 13.0. These conclusions illustrated that Sal A and Sal B were unstable with the rise of pH value of solution, and were completely decomposed, together with danshensu. Therefore, the optimal pH value of aqueous solution for wetting materials was pH 1.0, and the amount of Sal A in treated RSM was the largest under this condition.

Effect of reaction time on conversion of Sal B into Sal A

When the RSM powder was wetted by a solution with a pH value (1.0, 4.0, 8.0, or 11.0), and was treated in a steam sterilization pot with the following conditions:

120 °C, 0.124 MPa and saturated vapor for different reaction time (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, or 8 h), the changes of Sal B, Sal A and danshensu contents were investigated, as shown in Figure 1, 6A, 6B, and 6C.

During the reaction time from 1 h to 4 h, the content of Sal A was increasing and reached the maximum at 4 h, but it decreased from 5 h to 8 h (Figure 1 and 6A). The results illustrated that the amount of Sal A was produced more than that decomposed from 1 h to 4 h, but it was produced less than that decomposed from 5 h to 8 h. In the meantime, the content of Sal B in treated RSM always decreased along with the treated time prolonged, especially from 1 h to 4 h，it increased quickly, but it kept a constant from 5 h to 8 h, while the content of danshensu was in inverse proportion to that of Sal B from 1 h to 4 h and similar to Sal B from 5 h to 8 h (Figure 6B and 6C). So the optimal reaction time period was 4 h for the conversion of Sal B into Sal A.

Effect of reaction temperature on conversion of Sal B into Sal A

The RSM powder was treated with a solution with a pH value (1.0, 4.0, 8.0 or 11.0) in a steam sterilization pot under 105, 110, 115, 120, or 125 °C for 4 h, respectively, and the contents of Sal A, Sal B and danshensu in it were measured (Figure 7A, 7B and 7C).

The content of Sal A was lower at 105 °C, indicating that Sal A was produced less at this temperature (Figure 7A), and it increased slowly with the reaction temperature going up from 105 to 115 °C, but sharply increased at the temperatures from 115 to 120 °C. Ultimately, it reached the maximum at 120 °C. With the reaction temperature going up from 120 °C to 125 °C, the content of Sal A declined, but very little. Based on the results, the optimal reaction temperature was 120 °C.

At the same time, the changes of Sal B and danshensu content were also presented certain regularity in treated RSM (Figure 7B and 7C). With the rising of temperature, the content of Sal B was negatively correlated to the reaction temperature, while the content of danshensu was in positive correlation with it.

In summary, the conversion of Sal B into Sal A was conducted under the

conditions of a wide pH range from 1.0 to 12.0, a wide temperature range from 105 °C to 125 °C and a wide reaction time period from 1 h to 8 h. The produced Sal A was stable in root tissue, compared with the yield of that in acidic aqueous solution (Wang *et al.*, 2011a). This conversion method was superior to that in acidic aqueous solution.

Conclusion

Sal B in RSM root tissue could be converted into Sal A by thermal degradation, and the possible degradation pathway was deduced. When the RSM materials were grounded into coarse powder (less than 2 mm in diameter), wetted by a solution with pH 1.0, and treated at 120 °C for 4 h, the amount of produced Sal A was the largest than that in RSM treated with other conditions. The results indicated that the method to convert Sal B into Sal A in RSM tissues under the conditions of high temperature, high pressure and high humidity was reliable, and was readily utilized to prepare rich Sal A materials in practice.

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Tables:

Table 1 Stability, precisions and repeatability of three compounds

Compound	Stability(n=6)		Precision(n=5)		Repeatability(n=6)	
	Average (peak area)	RSD (%)	Intra-day RSD(%)	Inter-day RSD(%)	Average (peak area)	RSD (%)
Danshensu	5988.5	0.27	0.036	0.27	5990.48	0.12
Salvianolic aicd B	8948.2	0.51	0.081	1.23	8938.22	1.98
Salvianolic acid A	7009.5	0.68	0.055	0.78	7003.57	1.22

Table 2. HPLC-ESIMS negative ions of phenolic acids in original RSM

Peak	t_R(min)	Assignment	MW	MS (*m/z*)
1	34.21	Rosmarinic acid	360	359.0 $[M-1]^-$, 719.0 $[2M-1]^-$
2	36.40	Lithospermic acid	538	537.1 $[M-1]^-$, 492.9 $[M-COOH]^-$
3	41.52	Salvianolic acid B	717	717.0 $[M-1]^-$
4	45.54	Salvianolic acid b/e/isomer	717	717.0 $[M-1]^-$

Table 3. HPLC-ESIMS negative ions of phenolic acids in treated RSM

Peak	t_R(min)	Assignment	MW	MS (*m/z*)
1	7.29	Danshensu	198	196.8 $[M-H]^-$, 395.0 $[2M-1]^-$
2	12.54	Protocatechualdehyde	138	136.8 $[M-H]^-$, 274.9 $[2M-1]^-$
3	29.45	Salvianolic acid D	418	417.0 $[M-H]^-$
4	34.71	Rosmarinic acid	360	359.0 $[M-H]^-$, 719.0 $[2M-1]^-$
5	42.23	Salvianolic acid B	718	717.0 $[M-H]^-$
6	47.06	Salvianolic acid A	494	493.1 $[M-H]^-$, 987.1 $[2M-1]^-$

Figures:

Figure 1. HPLC chromatogram of original RSM and the RSM wetted with a pH 1.0 hydrochloric acid solution and treated at 120 °C in a sterilization pot for reaction time (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, or 8 h). Peaks: 1 = danshensu, 2 = protocatechualdehyde, 3 = salvianolic acid D, 4 = rosmarinic acid, 5 = salvianolic acid B, 6 = salvianolic acid A.

Figure 2. HPLC-UV-MS chromatogram of phenolic acids in original RSM.
A = HPLC-UV chromatogram; B = TIC

Figure 3. HPLC-UV-MS chromatogram of phenolic acids in the RSM wetted by two times weight of an aqueous solution with pH 1.0 and treated at 120 °C for 6 h in a saturated steam sterilization pot. A = HPLC-UV chromatogram; B = TIC.

Figure 4. Possible thermal degradation pathway of Sal B in RSM tissues under a high temperature, high pressure and high humidity

Figure 5. Effects of solutions with different pH values on the content of Sal A (A), Sal B (B) and danshensu (C) in the RSM treated solutions at different pH values scale from 1.0 to 13.0 at 120 °C for 2 h and 4 h or at 125 °C for 2 h and 4 h, respectively. A = the content of Sal A; B = the content of Sal B; C = the content of danshensu.

Figure 6. Effect of different reaction time on the content of Sal A (A), Sal B (B) and danshensu (C) in the RSM treated with a solution of a pH value (1.0, 4.0, 8.0, or 11.0) at 120 °C in a sterilization pot for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, or 8 h, respectively. A = the content of Sal A; B = the content of Sal B; C = the content of danshensu.

Figure 7. Effect of different reaction temperatures on the content of Sal A (A), Sal B (B) and danshensu (C) in RSM treated with a solution of pH 1.0, 4.0, 8.0, or 11.0 at 105 °C, 110 °C, 115 °C, or 120 °C in a sterilization pot for 4 h, respectively. A = the content of Sal A; B = the content of Sal B; C = the content of danshensu.