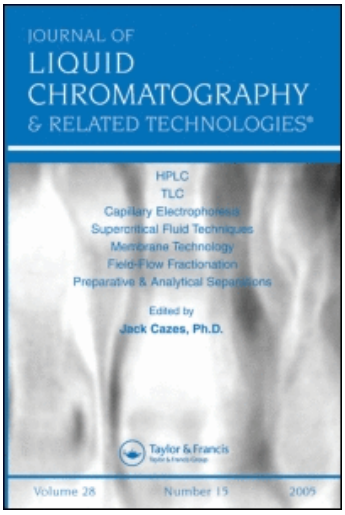

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A Rapid Simple Approach to Screen Components in Radix *Salviae Miltiorrhizae* Using Rapid Resolution Liquid Chromatography Coupled with Diode Array Detection and Electrospray Ionization Time-of-Flight Mass Spectrometry

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Abstract: An improved method based on rapid resolution liquid chromatography (RRLC) coupled with diode array detection (DAD) and electrospray ionization time-of-flight mass spectrometry (TOF/MS) has been developed for quantification of seven major components and identification of most components in extracts of Radix *Salvia Miltiorrhizae* (RSM). RRLC analysis was performed with a C_{18} column by gradient elution using acetonitrile and 0.4% formic acid (v/v) in water as the mobile phase; the detection wavelength was set at 280 nm. Seven major components in RSM were successfully separated. This quantitative method was fully validated with respect to the following performance criteria: linearity, precision, repeatability, stability, accuracy, limit of detection (LOD) and quantification (LOQ). The UV spectra, acquired by DAD, were used to distinguish the two groups of components roughly, water-soluble phenolic and nonpolar diterpenoid compounds. A formula database of known compounds in RSM was established, against which most of the reported components in the herbal extract were identified effectively, based on the extract masses acquired by

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TOF/MS. This qualitative and quantitative method was successfully used to analyze the components in 10 batches of RSM samples collected from different regions in China. This improved quality evaluation method consisted of RRLC assay of seven major components and unambiguous identification of forty components, is suitable for routine quantification and comprehensive quality control of RSM.

Keywords: Quality evaluation, Radix *Salvia Miltiorrhizae*, RRLC-DAD, RRLC-ESI-TOF/MS

INTRODUCTION

Radix *Salvia Miltiorrhizae* (RSM, the dried root of *Salvia Miltiorrhiza* BGE., Lamiaceae), is one of the most popular traditional Chinese herbal medicines and has been widely used for promoting circulation and improving blood stasis.[1,2] Many studies about its chemical components have been reported.[3–6] Depending on their chemical structures, the major bioactive constituents can be classified into two major groups: water-soluble phenolic compounds and nonpolar diterpenoid compounds. Of these constituents, the major phenolic components are danshensu, protocatechuic aldehyde, rosmarinci acid, and salvianolic acid B, and the major diterpenoid components are cryptotanshinone, tanshinone I, tanshinone IIA.[7–11] It has been reported that the contents of seven components are higher than other components in RSM and they have definite pharmacological action[12–14] and, therefore, to some extent, these components could reflect the quality of this crude herb. So, it is imperative to develop a quantitative method for analysis of the seven major bioactive components. Furthermore, various pharmacological activity studies *in vitro* and *in vivo* indicated that, not only the seven major components, but also other minor active components have important pharmaceutical actions, such as improving micro-circulation, dilating the coronary arteries, increasing the blood flow, and preventing myocardial ischemia.[15–18] So, it is valuable to identify the components in RSM unambiguously and to develop its unambiguous chemical fingerprints, which is a vital complement to multi-component quantification. This method can display the overall chemical characteristics of the crude herb, which is not represented by only the marker components.

During the last decades, several analytical methods have been reported for components in RSM, including HPLC,[19–23] capillary electrophoresis (CE),[24] and HPLC-MS/MS which have been used for the determination of salvianolic acids or diterpenoids.[25–27] But, these studies just determined several components in RSM either without identification, or only identification of components without determination.

Time-of-flight (TOF) mass spectrometry is a very useful tool and can yield accurate masses of most components; because of this, it always works in scan mode without loss of sensitivity, generating full data of the product ion spectra and avoiding "ion shopping."[28–30] Recently, there has been considerable interest in the application of RRLC-DAD-TOF/MS. RRLC-TOF/MS allows identification of non-target, unknown compounds based on accurate masses of these ions, which permits calculating their elemental compositions and searching chemical structures against formula databases.[28–30] In this paper, a rapid simple method, combined with quantitative analysis and unambiguous chromatographic fingerprints for quality evaluation of herbal medicines by novel RRLC-DAD-TOF/MS is reported. We expected that this work would be helpful for identification and the quality control of RSM.

EXPERIMENTAL

Materials and Reagents

Sodium danshensu, protocatechuic aldehyde, salvianolic acid B, cryptotanshinone and tanshinone IIA were purchased from the National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). Romarinic acid, and tanshinone I were isolated from the RSM by the authors. Their structures were fully characterized by nuclear magnetic resonance (NMR) spectroscopy and MS (Figure 1), and their purities were shown to be over 99.0%. The RSM crude herb was purchased from Shanghai Huayu Medicine Corporation.

Acetonitrile and methanol (Fisher, USA) were of RRLC grade and ultrapure water was prepared with a Milli-Q System (Millipore, Bedford, MA, USA). Formic acid used in the mobile phase was of analytical grade, purchased from the Beijing Chemical Corporation (Beijing, China).

Extraction Procedures of RSM

The dried roots were powdered to a homogeneous size by a mill, sieved through a No.40 mesh, and further dried at 60°C for 5 h. An accurately weighed 0.5 g powder sample was extracted for 30 min twice by sonication in 70% methanol. Extraction volume was 25 mL and made up the loss weight with 70% methanol after extraction. The sample solution was filtered through a 0.45 μm nylon filter into an RRLC amber sample vial for injection.

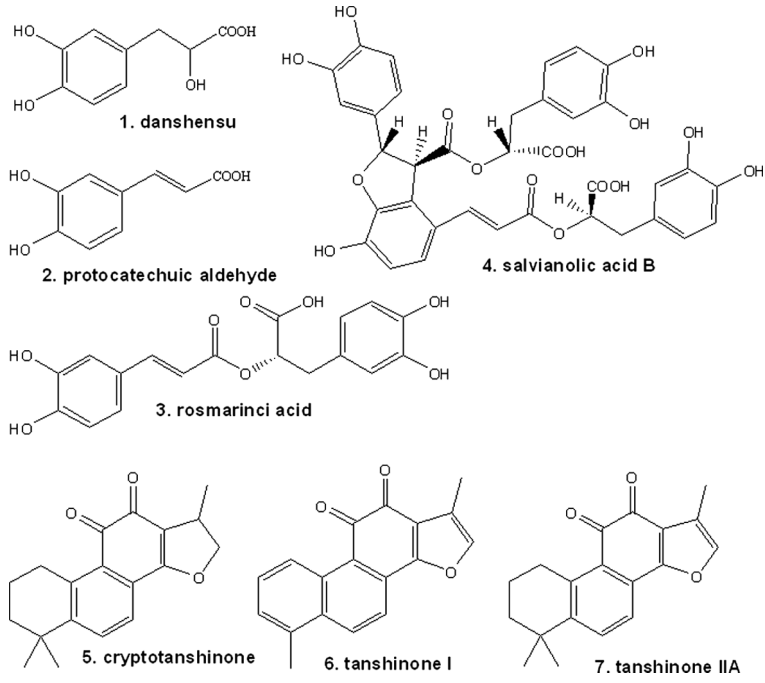

Figure 1. The chemical structures of chemical components in RSM.

RRLC-DAD Conditions

RRLC quantitative analyses were performed with an Agilent 1200 series RRLC system (Agilent, Waldbronn, Germany) consisting of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller, and a diode-array detector (DAD) interfaced with a 6210 TOF mass spectrometer (TOF/MS, Agilent Technologies, Santa Clara, CA, USA). The analytical column was Zorbax XDB-C_{18} column (3.0×50 mm, 1.8 μm). The column was maintained at 20°C. Detection wavelength was set at 280 nm. The mobile phase consisted of 0.4% (v/v) formic acid (A) and acetonitrile (B). A gradient program was used as follows: 0–15 min, 5–30% B; 15–20 min, 30–60% B; 20–30 min, 60–85% B, with a hold time of 10 min; the flowing rate was 0.4 mL · min^{-1}; injection volume: 4 μL.

RRLC-ESI-TOF/MS Conditions

The RRLC conditions were the same as those described above. The mass spectra were acquired by a 6210 time-of-flight mass spectrometer

equipped with an electrospray interface. The mass spectrometric parameters were optimized as follows: in the positive ion mode (ESI^+), capillary voltage 4000 V, nebulizer gas pressure 40 psig, drying gas flow rate 9 L/min, gas temperature 350°C, fragmentor 130 V and skimmer 60 V; in negative ion mode (ESI^-), capillary voltage 3500 V, nebulizer gas pressure 30 psig, drying gas flow rate 9 L/min, gas temperature 350°C, fragmentor 130 V and skimmer 60 V. The mass spectrometer was scanned from m/z 50–1500 in full scan mode. The RSM sample was detected in the negative mode during 0–18 min and in the positive mode during 18–30 min. Agilent Masshunter and Analyst QS software were used for data processing. The components in RSM were identified rapidly by RRLC-ESI-TOF/MS according to the accurate masses of these components represented by various ions, searching against the formula database of RSM.

Preparation of Standard Solutions

Standard stock solutions of 7 standards were prepared by dissolving accurately weighed standards in 70% methanol. The working standard solutions containing seven standards were prepared by mixing and diluting their original stock solutions with 70% methanol to reach the concentration of 135.3 $\mu g \cdot mL^{-1}$ for sodium danshensu, 45.28 $\mu g \cdot mL^{-1}$ for protocatechuic aldehyde, 118.8 $\mu g \cdot mL^{-1}$ for rosmarinci acid, 1012 $\mu g \cdot mL^{-1}$ salvianolic acid B, 133.0 $\mu g \cdot mL^{-1}$ for cryptotanshinone, 56.00 $\mu g \cdot mL^{-1}$ for tanshinone I, 127.0 $\mu g \cdot mL^{-1}$ for tanshinone IIA, respectively.

Calibration Curves, Limits of Detection and Quantification

The calibration curve of each individual standard was constructed by plotting the ratio of each analyte area versus analyte concentration with six appropriate concentrations, in triplicate. The studied concentration ranges were 1.353–135.3 $\mu g \cdot mL^{-1}$ for sodium danshensu, 0.4528–45.28 $\mu g \cdot mL^{-1}$ for protocatechuic aldehyde, 1.188–118.8 $\mu g \cdot mL^{-1}$ for rosmarinci acid, 10.12–1012 $\mu g \cdot mL^{-1}$ for salvianolic acid B, 1.330–133.0 $\mu g \cdot mL^{-1}$ for cryptotanshinone, 0.5600–56.00 $\mu g \cdot mL^{-1}$ for tanshinone I and 1.270–127.0 $\mu g \cdot mL^{-1}$ for tanshinone IIA. Good linearity ($r > 0.9997$) was observed for the calibration curves over the concentration ranges investigated. The results are demonstrated in Table 1.

The dilute solutions of the seven standards were further diluted to a series of concentrations with 70% methanol to determine the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise

Table 1. Regression equations, LODs and LOQs for seven reference standards

Compounds	Linear regression	Linear range ($\mu g \cdot mL^{-1}$)	R^2	LOD ($\mu g \cdot mL^{-1}$)	LOQ ($\mu g \cdot mL^{-1}$)
Danshensu	$y = 3.830x + 0.4840$	1.353–135.3	0.9991	0.02	0.08
Protocatechuic aldehyde	$y = 29.769x - 12.21$	0.4528–45.28	0.9996	0.04	0.10
Rosmarinci acid	$y = 10.328x + 1.337$	1.188–118.8	0.9997	0.03	0.08
Salvianolic acid B	$y = 5.578x + 8.040$	10.12–1012	0.9999	0.02	0.05
Cryptotanshinone	$y = 12.216x - 17.97$	1.330–133.0	0.9996	0.02	0.06
Tanshinone I	$y = 22.410x - 21.85$	0.5600–56.00	0.9993	0.01	0.05
Tanshinone IIA	$y = 22.934x + 8.885$	1.270–127.0	0.9997	0.02	0.05

Table 2. Precision of the intra-day and inter-day measurements of the analytical method

Compound	Concentration ($\mu g \cdot mL^{-1}$)	Intra-day (n = 5)			Inter-day (n = 3)		
		Mean detected conc. ($\mu g \cdot mL^{-1}$)	Deviation (%)	RSD (%)	Mean detected conc. ($\mu g \cdot mL^{-1}$)	Deviation (%)	RSD (%)
1	2.705	2.658	0.043	1.62	2.665	0.045	1.68
	13.53	13.65	0.03	0.19	13.54	0.12	0.87
	135.3	135.4	1.45	1.07	135.9	1.97	1.45
2	0.9056	0.9063	0.0159	1.75	0.9088	0.0102	1.12
	4.528	4.517	0.085	1.87	4.527	0.043	0.96
	22.64	22.53	0.23	1.04	22.66	0.13	0.55
3	2.375	2.356	0.058	2.45	2.347	0.039	1.68
	11.88	11.78	0.30	2.55	11.76	0.16	1.32
	59.38	59.24	0.52	0.88	59.30	0.52	0.87
4	20.24	20.15	0.27	1.36	20.21	0.23	1.15
	126.5	124.9	2.57	2.06	124.2	1.73	1.39
	506.0	507.8	3.30	0.65	506.9	7.76	1.53
5	2.660	2.633	0.085	3.22	2.648	0.062	2.34
	10.64	10.59	0.20	1.90	10.76	0.18	1.68
	66.50	66.72	1.34	2.01	66.67	0.51	0.77
6	1.120	1.132	0.01	0.97	1.136	0.02	1.96
	5.600	5.681	0.063	1.11	5.675	0.046	0.81
	28.00	28.41	0.59	2.09	28.91	0.52	1.78
7	2.540	2.556	0.053	2.08	2.534	0.033	1.32
	15.88	15.90	0.23	1.43	15.99	0.35	2.16
	63.50	63.63	0.76	1.19	63.52	0.92	1.45

1. Danshensu, 2. Protocatechuic aldehyde, 3. Rosmarinci acid, 4. Salvianolic acid B, 5. Cryptotanshinone, 6. Tanshinone I, 7. Tanshinone IIA.

(S/N) ratio of 3 and 10, respectively. LOD and LOQ for each compound are also shown in Table 1.

Precision and Accuracy

The intra- and inter-day precision was determined by analyzing quality control samples at low, medium, and high concentrations in a set of five in a single assay day and in duplicate in three consecutive days, respectively. The RSD was taken as a measure of precision, and the results are exhibited in Table 2.

Recovery

A recovery test was used to evaluate the accuracy of this method. Accurate amounts of seven standards were added to approximate 0.5 g of RSM and then extracted as described above. The average recoveries were

Table 3. Recovery experiment of the analytical method for seven components

Compound	Original (mg)	Spiked (mg)	Found (mg)	Recovery mean (%)	RSD% (n = 3)
1	1.995	1.015	3.016	100.2	1.55
		2.030	3.924	97.5	3.13
		3.044	4.944	98.1	0.95
2	0.390	0.226	0.606	98.3	2.19
		0.453	0.809	96.2	1.87
		0.679	1.071	100.2	1.73
3	2.190	1.069	3.367	103.3	0.58
		2.138	4.402	101.7	1.43
		3.208	5.522	102.3	1.92
4	11.26	5.060	16.548	101.4	2.39
		10.12	21.957	102.7	3.03
		15.18	26.070	98.6	1.33
5	0.925	0.466	1.389	99.9	0.77
		0.931	1.828	98.5	1.52
		1.397	2.354	101.4	0.67
6	0.825	0.420	1.244	99.9	0.86
		0.840	1.667	100.1	1.22
		1.260	2.079	99.7	1.83
7	1.250	0.635	1.883	99.9	1.95
		1.270	2.490	98.8	1.27
		1.905	3.127	99.1	0.91

1. Danshensu, 2. Protocatechuic aldehyde, 3. Rosmarinci acid, 4. Salvianolic acid B, 5. Cryptotanshinone, 6. Tanshinone I, 7. Tanshinone IIA.

calculated by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%. The results are given in Table 3.

Quantification of Seven Major Components in RSM

The developed RRLC quantitative method was applied to simultaneous determination of seven major components in RSM collected from different regions in China. Each sample was extracted and analyzed in triplicate. The representative chromatogram acquired by RRLC is shown in Figure 2. In this study, the highest total yield of phenolics was found in the samples from Shandong province. Among these phenolics, salvianolic acid B is the most abundant phenolic acid. The highest yield of total diterpenoids was found in the samples from Hebei province. Among these diterpenoids, tanshinone IIA is the most abundant diterpenoid compound. In addition, the contents of seven major compounds present in individual samples varied considerably and the the concentrations of individual components in different samples is also noticeably different. Variations could have occurred due to various factors such as

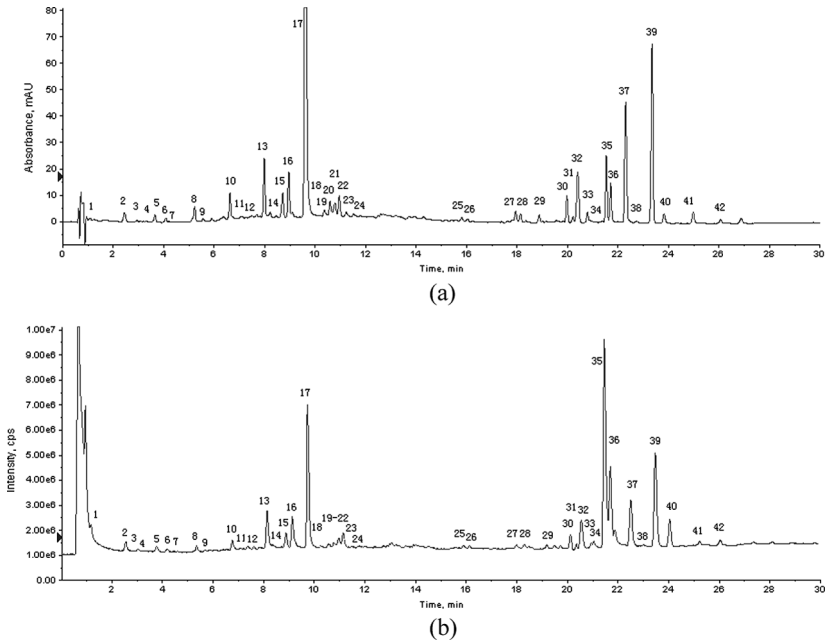

Figure 2. The HPLC-DAD chromatogram (a) and HPLC-TOF/MS TIC (b) of RSM sample solutions.

Table 4. Quantitative analytical results of seven components in RSMs collected from different regions in China

No.	Batch number	Province collected	Contents (mean ± SD) of seven components (mg/g) (n = 3)						
			Danshensu	Protocatechuic aldehyde	Rosmarinci acid	Salvianolic acid B	Cryptotanshinone	Tanshinone I	Tanshinone IIA
1	070205	Jiangsu	1.26 ± 0.01	0.16 ± 0.00	1.62 ± 0.01	11.29 ± 0.05	0.69 ± 0.02	0.75 ± 0.01	1.49 ± 0.01
2	070308	Shandong	2.25 ± 0.03	0.63 ± 0.00	3.07 ± 0.01	20.73 ± 0.43	1.38 ± 0.01	1.22 ± 0.01	1.74 ± 0.03
3	070224	Shandong	3.99 ± 0.02	0.78 ± 0.01	4.38 ± 0.04	22.51 ± 0.64	1.85 ± 0.05	1.65 ± 0.01	2.50 ± 0.01
4	070801	Guangxi	1.02 ± 0.01	0.09 ± 0.00	1.16 ± 0.04	8.76 ± 0.01	0.09 ± 0.00	0.17 ± 0.00	0.24 ± 0.02
5	070506	Guangdong	0.73 ± 0.00	0.22 ± 0.00	1.01 ± 0.01	3.81 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.18 ± 0.01
6	070315	Sichuan	2.84 ± 0.01	0.08 ± 0.00	3.05 ± 0.02	20.86 ± 0.28	0.81 ± 0.01	1.03 ± 0.01	1.16 ± 0.00
7	080205	Jiangxi	2.91 ± 0.01	0.17 ± 0.02	3.62 ± 0.01	21.29 ± 0.17	0.69 ± 0.02	0.76 ± 0.02	1.09 ± 0.00
8	080302	Henan	0.58 ± 0.01	0.33 ± 0.03	0.85 ± 0.00	2.34 ± 0.03	0.54 ± 0.01	0.43 ± 0.00	0.82 ± 0.01
9	080205	Anhui	2.03 ± 0.00	0.61 ± 0.00	2.76 ± 0.01	15.67 ± 0.21	1.67 ± 0.08	1.77 ± 0.01	2.17 ± 0.01
10	080503	Hebei	2.85 ± 0.01	0.68 ± 0.01	3.02 ± 0.01	19.37 ± 0.42	1.62 ± 0.02	1.89 ± 0.00	2.78 ± 0.01

geographical source, cultivation, harvest, storage, and processing, etc. The results of quantitative analysis are summarized in Table 4.

Qualification of Components in RSM by RRLC-DAD-ESI-TOF/MS

In order to identify the chemical components in the chromatogram, both HPLC-DAD and HPLC-ESI-TOF/MS were employed. The identification method in which standard compounds were used to compare was accurate and fast. There were 7 peaks identified by comparing their retention times and ultraviolet absorptions with standard compounds. The areas of these 7 peaks accounted for 50% of all peak areas. Among the 7 standards, there were 4 phenolic acids and 3 diterpenoid compounds. Seen from the chromatogram in Figure 2, most components were well separated under the present conditions.

The total-ion-current (TIC) chromatogram was acquired by RRLC-TOF/MS, which is shown in Figure 2. The accurate molecular weights of components represented by chromatographic peaks in the TIC of RSM were calculated using the Agilent software tool "formula_database_generator," and a formula database of components in RSM was established. Forty-two componentś were characterized by searching against the formula database established by the Agilent software.

RESULTS AND DISCUSSION

Extraction Method Development

To obtain optimal extraction conditions, several variables involved in the procedure, such as extraction means, extraction solvents, and extraction times were optimized. Pure methanol, aqueous methanol (30%, 50%, 70%) and pure water were investigated as the extraction solvent. The best solvent was found to be 70% methanol, which allowed complete extraction of all the components in high yield. Sonication was simpler and more effective for extraction compared with refluxing extraction. Hence, sonication was chosen as a preferred method. The extraction time was considered as a factor of the efficiency of extraction. Powdered samples were extracted with methanol for 15, 30, 45, and 60 min, respectively. The results suggested that the highest extraction efficiency was obtained by sonication twice for 30 min in 70% methanol, which is shown in Figure 3.

Optimization of RRLC-DAD-TOF/MS Conditions

The RRLC conditions were optimized using seven standards and a real RSM sample. Various columns (Agilent Zorbax SB-C_{18} and Agilent

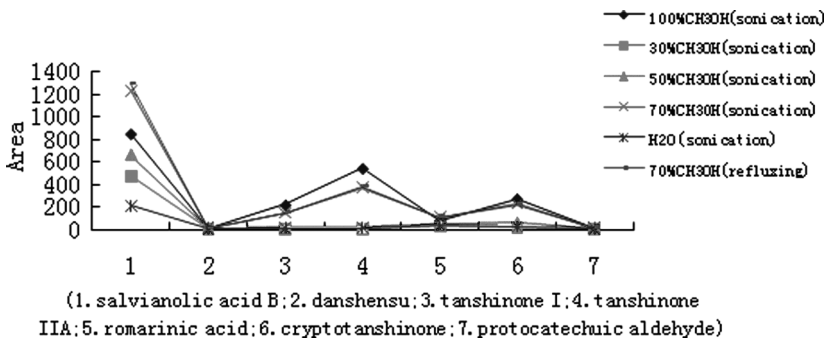

Figure 3. The typical UV spectra of phenolics and diterpenoids in RSM a. salvianolic acid B; b. tanshinone IIA.

Zorbax XDB-C_{18}) including particle (1.8, 5 μm), inner diameter (4.6, 3.0, 2.1 mm), column length (50, 250 mm) were examined. Firstly, a column (4.6 × 250 mm, 5 μm) was tested in gradient elution mode. The retention behavior of phenolics on columns was somewhat affected by the pH value of the mobile phase and acidic conditions apparently improved the resolution efficiency of phenolics. In order to develop a separation method compatible with ESI-TOF/MS, formic acid was added to the mobile phase. The addition of 0.4% formic acid in the mobile phase provided the best resolution and signal/noise ratio. It was noticed that the better separation was achieved when the column temperature was kept at 20°C. In order to optimize the RRLC-DAD detection conditions, seven standards were monitored in the scanning range between 190–400 nm. The optimal detection wavelength in the RRLC analysis was determined to be 280 nm. At this wavelength, more characteristic peaks in the chromatogram were observed, and the seven marker compounds were sensitively detected. The shorter C_{18} column, packed with smaller particle (1.8 μm), gave considerable separation at a rate of 0.4 mL/min in a short time, compared with columns packed with larger particles (5 μm), therefore, being suitable for TOF/MS detection. By comparison with the result which was obtained from the 5 μm column, the analytical time on the 1.8 μm column was reduced by over 2 times, from 80 min to 30 min, with similar resolution and without post column splitting. By comparison with the result which was obtained from the 2.1 mm inner diameter column, the resolution on the 3.0 mm inner diameter column increased and column pressure decreased, with the same analytical time. Thus, the column of 3.0 × 50 mm, 1.8 μm was selected for the analysis of RSM.

In this study, the electrospary mass spectra of seven components varied with drying gas flow rate, nebulizer gas pressure, fragmentor,

and skimmer. Through optimization experiments, optimal mass spectrometric conditions were applied to sample analysis.

Validation of Quantitative Method

Table 1 lists linear calibration with R^2, linear range, LOD, and LOQ of each compound. As a result, all calibration curves showed good linear regression ($R^2 > 0.999$) within test ranges; the LODs ($S/N = 3$) and the LOQs ($S/N = 10$) for the seven components are less than 40 ng and 100 ng at 280 nm. Table 2 lists the results of the tests of precision of seven

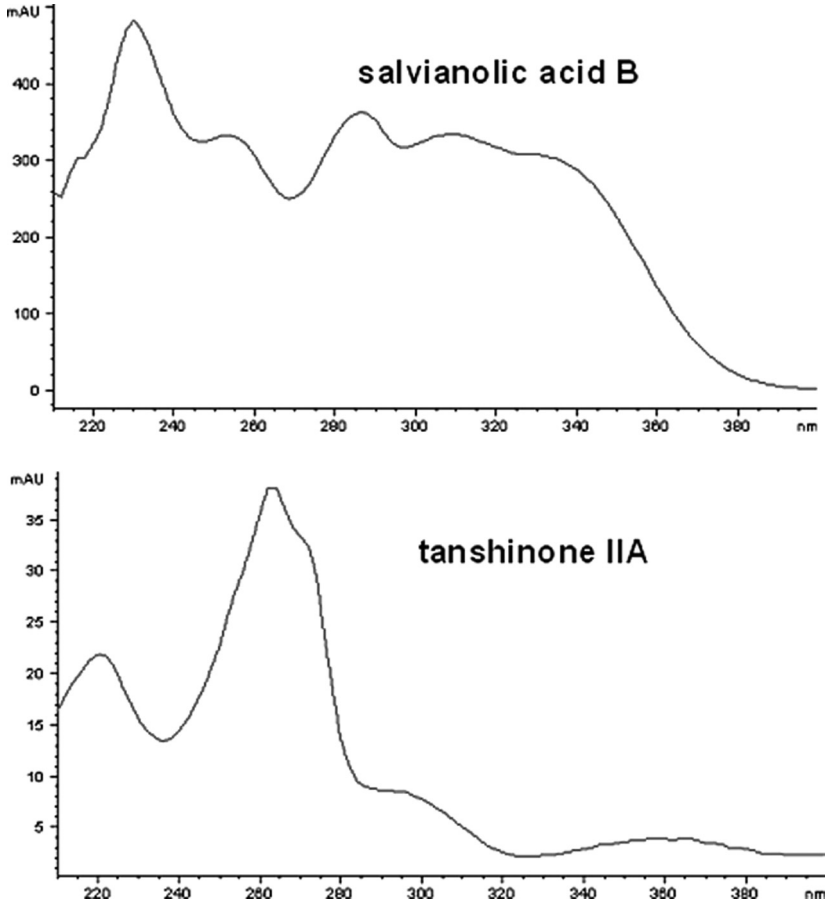

Figure 4. Effect of different extraction solutions and extraction methods on extraction efficiency of seven components in RSM.

Table 5. RRLC-ESI-TOF/MS accurate molecular weights and DAD maximum wavelengths of components in RSM

No.	Rt (min)	Assignment	Mw	P. M. or D. M	Expected m/z (Da)	Experimental TOF-MS (Da)	ppm error
1	1.021	Succinic acid	242	$[C_4H_5O_4]^-$	117.0188	117.0187	−0.8546
2	2.256	Danshensu	288	$[C_9H_9O_5]^-$	197.0450	197.0453	1.5225
3	2.963	Protocatechuic acid	294	$[C_7H_5O_4]^-$	153.0188	153.0185	−1.9605
4	3.175	Protocatechualdehyde	310	$[C_7H_5O_3]^-$	137.0239	137.0241	1.4596
5	3.779	Danshensu methyl ester	292	$[C_7H_5O_3]^-$	211.0606	211.0609	1.4214
6	4.081	Vanillic acid	292	$[C_8H_7O_4]^-$	167.0344	167.0345	0.5987
7	4.153	Caffeic acid	324	$[C_9H_7O_4]^-$	179.0344	179.0346	1.1171
8	5.167	Ferulic acid	322	$[C_{10}H_9O_4]^-$	193.0501	193.0498	−1.554
9	5.572	Prolithospermic acid	310	$[C_{18}H_{13}O_8]^-$	357.0610	357.0612	0.5601
10	6.852	Salvianolic acid	312	$[C_{17}H_{13}O_6]^-$	313.0712	313.0715	0.9582
11	7.087	Salvianolic acid D	328	$[C_{20}H_{17}O_{10}]^-$	417.0822	417.0826	0.9590
12	7.423	Caffeic acid methyl ester	325	$[C_{10}H_9O_4]^-$	193.0501	193.0503	1.0360
13	7.955	Salvianolic acid H	310	$[C_{27}H_{21}O_{12}]^-$	537.1033	537.1034	0.1862
14	8.179	Salvianolic acid G	313	$[C_{27}H_{21}O_{12}]^-$	339.0505	339.0508	0.8848
15	8.766	Rosmarinic acid	328	$[C_{18}H_{11}O_6]^-$	359.0767	359.0763	−1.1140
16	8.923	Lithospermic acid	310	$[C_{18}H_{15}O_8]^-$	537.1033	537.1037	0.7447
17	9.745	Salvianolic acid B	288	$[C_{36}H_{29}O_{16}]^-$	717.1456	717.1459	0.4183
18	9.901	Salvianolic acid E	330	$[C_{36}H_{29}O_{16}]^-$	717.1456	717.1453	−0.4183
19	10.223	Monomethyl lithospermate	310	$[C_{28}H_{23}O_{12}]^-$	551.1190	551.1191	0.1814
20	10.442	Salvianolic acid A	288	$[C_{26}H_{21}O_{10}]^-$	493.1135	493.1137	0.4056
21	10.615	Ethyl lithospermate	287	$[C_{29}H_{25}O_{12}]^-$	565.1346	565.1349	0.5308

22	10.917	Dimethyl lithospermate	323	$[C_{29}H_{25}O_{12}]^-$	565.1346	565.1347	0.1769
23	11.189	Dimethyl lithospermate B	310	$[C_{38}H_{33}O_{16}]^-$	745.1769	745.1775	0.8052
24	11.495	Salvianolic acid C	364	$[C_{26}H_{20}O_{10}]^-$	491.1213	491.1219	1.2217
25	15.892	Danshenxinkun A	264	$[C_{18}H_{16}O_4]^+$	297.1127	297.1130	1.0097
26	15.907	Methylenetanshinquinone	264	$[C_{18}H_{14}O_3]^+$	279.1021	279.1023	0.7166
27	17.903	Trijuganone A	245	$[C_{18}H_{14}O_4]^+$	295.0970	295.0975	1.6944
28	18.076	Tanshinone IIB	270	$[C_{19}H_{19}O_4]^+$	311.1283	311.1287	1.2856
29	18.939	Trijuganone C	260	$[C_{20}H_{21}O_5]^+$	341.1389	341.1393	1.1725
30	19.987	1,2-Didehydrotanshinone IIA	258	$[C_{19}H_{18}O_4]^+$	293.1178	293.1183	1.7058
31	20.176	15,16-dihydrotanshinone I	244	$[C_{19}H_{18}O_4]^+$	279.1021	279.1025	1.4332
32	20.223	Trijuganone B	276	$[C_{18}H_{15}O_3]^+$	281.1178	281.1181	1.0672
33	20.792	Methyl tanshinonate	246	$[C_{18}H_{15}O_3]^+$	339.1232	339.1237	1.4744
34	21.076	1,2-Didehydrocryptotanshinone	268	$[C_{18}H_{17}O_3]^+$	295.1334	295.1335	0.3388
35	21.514	Cryptotanshinone	264	$[C_{19}H_{21}O_2]^+$	297.1491	297.1492	0.3365
36	21.680	Tanshinone I	246	$[C_{20}H_{19}O_5]^+$	277.0865	277.0868	1.0827
37	22.218	1,2-Dihydrotanshinone I	244	$[C_{19}H_{19}O_3]^+$	279.1021	279.1025	1.4332
38	22.542	1,2-Didehydromiltirone	248	$[C_{19}H_{21}O_3]^+$	281.1542	281.1546	1.4227
39	23.265	Tanshinone IIA	268	$[C_{18}H_{13}O_3]^+$	295.1334	295.1337	1.0165
40	23.871	Tanshiniactone	264	$[C_{19}H_{19}O_3]^+$	269.1542	269.1545	1.1146
41	25.042	2,3-Didehydrotanshinone IIA	258	$[C_{18}H_{21}O_2]^+$	293.1178	293.1182	1.3646
42	26.025	Miltirone	258	$[C_{19}H_{23}O_2]^+$	283.1698	283.1697	−0.3531

Rt: retention time.
Mw: maximum wavelength.
P. M.: protonated molecular weight.
D. M.: deprotonated molecular weight.

analytes. It was indicated that the RSD of the overall intra- and inter-day variations were less than 4% for the seven analytes. Table 3 indicates that the developed analytical method has a good accuracy with recoveries from 96% to 104% for the analytes. Therefore, the RRLC quantitative method is precise, accurate, and sensitive enough for simultaneous quantitative evaluation of seven major active components in RSM.

Identification of Components in RSM by RRLC-DAD-ESI-TOF/MS

In photodiode array detection, the maximum absorption wavelengths of the target peaks were identified based on a comparison with the on line UV information with literature data or standards. The UV spectrum of peaks 1–24 showed two strong absorption bands, at 230 and 280–350 nm, which is characteristic of phenolics. For peaks 25–42, all the UV spectra showed typical tanshinone absorption bands, at 220 and 250–280 nm. The representative UV spectra of salvianolic acid B and tanshinone IIA are shown in Figure 4. According to the UV spectra and maximum absorption wavelength acquired by DAD, the two groups of components, water-soluble phenolics and nonpolar diterpenoid compounds, can be distinguished roughly.

Electrospray ionization-mass spectrometry (ESI-MS) is a soft ionization technique that produces predominately molecular ion peaks under appropriate fragmentor. The TIC from RRLC-ESI-TOF/MS exhibited good agreement with that obtained from RRLC-DAD (Figure 2). Since no mass spectral library was available for the chemical constituents in RSM, the accurate molecular weight of each chemical component was calculated by using the Agilent software tool "formula_database_generator." And, the formula database of the chemical constituents in RSM was established ourselves.

An examination of the UV spectrum and the MS data allowed the tentative identification of 42 components, which are listed in Table 5. The table lists the retention times, maximum absorption wavelengths, and TOF/MS ions, etc. Among these 42 peaks, peaks 1–24 corresponded to water-soluble phenolic compounds and peaks 25–42 represented nonpolar diterpenoid compounds. Seven of them were unambiguously identified by comparison with their retention times and their UV and mass spectra. The deprotonated or protonated molecular weights of all target compounds were calculated within 5 ppm error.

CONCLUSIONS

This study was intended to provide a comprehensive quality evaluation method of RSM through simultaneous determination of seven major

active components and unambiguous herbal fingerprints by rapid resolution liquid chromatography (RRLC) coupled with diode array (DAD) and time-of-flight mass spectrometry (TOF/MS). A simple RRLC method with a diode array ultraviolet detector was developed for simultaneous determination of three lipophilic components (cryptotanshinone, tanshinone I, and tanshinone IIA) and four major hydrophilic components (danshensu, protocatechuic aldehyde, rosmarinci acid, and salvianolic acid B) of RSM in gradient elution mode. A rapid RRLC fingerprinting method was established with the same chromatographic conditions as the above quantitative assay. Forty peaks in this RRLC fingerprint were rapidly identified by employing RRLC-DAD-ESI-TOF/MS techniques and matching their accurate molecular masses with the formulae of the compounds in the database. Significant variations were demonstrated in the yields of seven major components and their RRLC fingerprints among 10 batches of RSM samples collected from different regions of China. This simple, rapid, and reliable RRLC quantitative analysis and unambiguous fingerprints are suitable for routine analysis and quality control of traditional Chinese herbal medicines consisting of bioactive multi-components.

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