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Determination of 4 Fat-soluble Components in Salvia Miltiorrhiza by UPLC-MS/MS

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To establish a UPLC-MS/MS method for simultaneous of 4 fat-soluble components in Danshen, such as tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone. Method: The separation and analysis was performed on a Hypersil Gold C18 (2.1 mm×100 mm, 1.9 μm). The mobile phase consisted of 0.1% acetic acid and methanol with gradient elution. The column temperature was set at 30°C with flow rate of 0.300 mL·min-1. Electrospray ionization (ESI) source was applied and operated in positive mode. Selective reaction monitoring (SRM) mode was used to quantify the 4 compounds. Results: The assay linearity of the 4 compounds was confirmed in the range of 0.20-1000 ng·mL-1.The average recoveries were 83.4%~112%, the detection limits were 0.10~0.50 ng·mL-1 and the relative standard deviation was less than 4.3%. Conclusion: This method is rapid, accurate, reproducible and sensitive, and has been successfully applied in the simultaneous quantification of 4 components in danshen. It can be used for study and quality control.

1. Introduction

Salvia miltiorrhiza known as red ginseng, purple salvia miltiorrhiza, red root, which is the dry root and rhizome of labiatae plants danshen is an important traditional Chinese medicine. Salvia has been used in research of traditional Chinese medicine for thousands of years in history. In recent years, foreign investment and private capital start the mass involved in the field of traditional Chinese medicine, main customer group of Salvia miltiorrhiza health products are the old man and the young people of working pressure with irregular life. Heart cerebrovascular disease are at higher risk in recent years, and the younger age trend obviously. Consumer health products demand has risen sharply for the prevention of disease of heart head blood-vessel. Salvia miltiorrhiza supplements to prevent cardiovascular and cerebrovascular diseases can have stable and reliable function: 1. expansion of coronary artery, myocardial nutrition; 2. antioxidation, protecting endothelial function; 3. promoting blood circulation to remove blood stasis, resistance to atherosclerosis; 4. improve microcirculation, improve the ability of hypoxia; 5. inhibit platelet aggregation and thrombosis; 6. strengthen the ability of red blood cells carry oxygen, promote tissue repair and regeneration; 7. enhance the capacity of deformation of red blood cells; 8. yangxin nerves of the central nervous have a calming effect; 9. other function. Apart from the above function, salvia miltiorrhiza and anti-tumor, enhance immunity, protect liver, blessed are except vexed, etc. So, it mainly used in the treatment of diseases of the cardiovascular system in clinically.The active ingredients of salvia miltiorrhiza mainly include two categories: the first category is fat-soluble 2 terpenoid compound, such as tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone, the second category is water-soluble multi-poly phenolic acids, such as protocatechualdehyde, salvianolic acid B, rosmarinic acid, etc.

At present, the detection form of salvia miltiorrhiza is mainly capsule (Tang et al., 2015), oral liquid (Zhang and Luo, 2015), dropping pill (Li et al. 2014) and tablet (Suo et al., 2005), and some of them are even for the detection of salvia miltiorrhiza (Liu, 2015). The method for determination of active ingredients of salvia miltiorrhiza was high performance liquid chromatography (HPLC) (Zhang et al., 2015) and high performance liquid chromatography coupling diode array detection (HPLC - DAD) method (Zhang et al., 2010). But the analysis time is long. In recent years, UPLC-MS/MS has developed rapidly as a new detection method. Yu dicusses in detail the application of UPLC-MS in detection of pesticide residues in his doctoral thesis (YU, 2009). Wang dicusses the application research of online HPLC-MS technich for tood safety analysis in her

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doctoral thesis (Wang, 2008). In this paper, the ultra high performance liquid chromatography - mass spectrometry(UPLC-MS/MS) was used to detect 4 kinds of ingredients of salvia miltiorrhiza in fat-soluble, and it can be finished within 7 min, itprovided a fast and accurate analysis method for the comprehensive evaluation of the immanent quality of salvia miltiorrhiza drugs.

2. Instrument and reagent

UltiMate 3000 Ultra high performance liquid chromatograph; TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo, USA); Allegra 64R Centrifuge high-speed refrigerated centrifuge (BECKMAN COULTER); solid-phase extraction apparatus. (Waters).
Standard substance: tanshinone I (batch number: 110867-200406) provided by the Chinese pharmaceutical and biological products offices; cryptotanshinone (batch number: 110852-110852), tanshinone IIA (batch number: 110766-200619) provided by national institutes for food and drug control; dihydrotanshinone (batch number: MUST - 14062317) provided by Chengdu Manstite Bio-Technology CO.,LTD. methanol, formic acid as chromatography (Merck);Water for the high pure water (Millipore system);
Fufang Danshen tablets (Inner Mongolia Renze pharmaceutical co., LTD., national medicine permission number Z15020566), Fufang Danshen tablets (guangdong yili pharmaceutical co., LTD., national medicine permission number Z44022225), compound danshen dropping pill (Tasly pharmaceutical group co., LTD., national medicine permission number Z1095011).

3. Method sand results

3.1 Format Conditions of chromatography - mass spectrometry

3.1.1 Chromatographic conditions

Chromatographic column: Hypersil Gold C18 (2.1 mm*100 mm, 1.9μm); Mobile phase: A 0.1% formic acid solution, B for methanol, gradient elution conditions are shown in table 1; Flow rate 0.300 mL·min-1; Column temperature of 30°C; Sample size 5 μL.

Table 1: Gradient elution faction

Time/min	Mobile phase A /%	Mobile phase B/%
0	90.0	10.0
1	90.0	10.0
4	10.0	90.0
8	10.0	90.0
8.1	90.0	10.0

3.1.2 Mass spectrometry conditions

Ion source: electrospray ion source (ESI); Test mode: positive ion detection;Spray: voltage 3 kv. The vapor pressure temperature: 400°C; Sheath gas pressure: 40 psi.Assist gas pressure: 10 psi. Capillary tube (ion transport) temperature: 320°C. Test method: select reaction monitor (SRM). Each component monitoring of ion pair and related parameters setting are shown in table 2.

Table 2: MS parameters

components	retention time/min	precursor ion/m/z	daughter ion/m/z	collision energy/eV
tanshinone I	6.43	277	249*	20
			178	36
tanshinone IIA	6.82	295	277*	17
			249	22
cryptotanshinone	6.45	297	251*	22
			254	25
dihydrotanshinone	6.15	279	261*	16
			233	22

* indicates quantitative ions

3.2 The preparation of the solution

3.2.1 Standard solution

4 kinds of reference substance were weighed accurately according to 2.5 mg, and they were constanted to 25 mL with methanol solution and stored under 4°C. The reference substance solution was measured 1.0 mL respectively and constanted to 10 mL with methanol solution, they were also stored under 4°C.

3.2.2 The test sample

The test sample were weighed accurately according to 1.0 g (solid samples were needed to wipe off icing and grind to powder), and be dissolved in methanol respectively. ultrasonic 10 min. they were constanted to 100 mL with methanol solution when they were cold, and filtered with membrane of 0.22μm.

3.3 The LOD and LOQ

The right amount reference substance solution of "3.2" were diluted with 50% methanol and determined according to the "3.1" under chromatography - mass spectrometry conditions. According to the 3 times and 10 times the signal-to-noise ratio, the limit of detection (LOD) and the limit of quantitation (LOQ) were calculated. The result is as follows: tanshinone I (LOD: 0.1 ng·mL-1; LOQ: 0.2 ng·mL-1); tanshinone IIA (LOD: 0.1 ng·mL-1; LOQ: 0.2 ng·mL-1); cryptotanshinone (LOD: 0.1 ng·mL-1; LOQ: 0.2 ng·mL-1); dihydrotanshinone (LOD: 0.5 ng·mL-1; LOQ: 1 ng·mL-1).

3.4 Linear relationship

The right amount reference substance solution of "3.2" were measured accurately and diluted into 0.1, 0.2, 0.5, 1, 5, 10, 50, 100, 500, 1000ng·mL-1 series with 50% methanol solution. They were analyzed with UPLC/MS/MS after 0.22μm filter membrane filtration, The result of chromatograph is shown in figure 1.

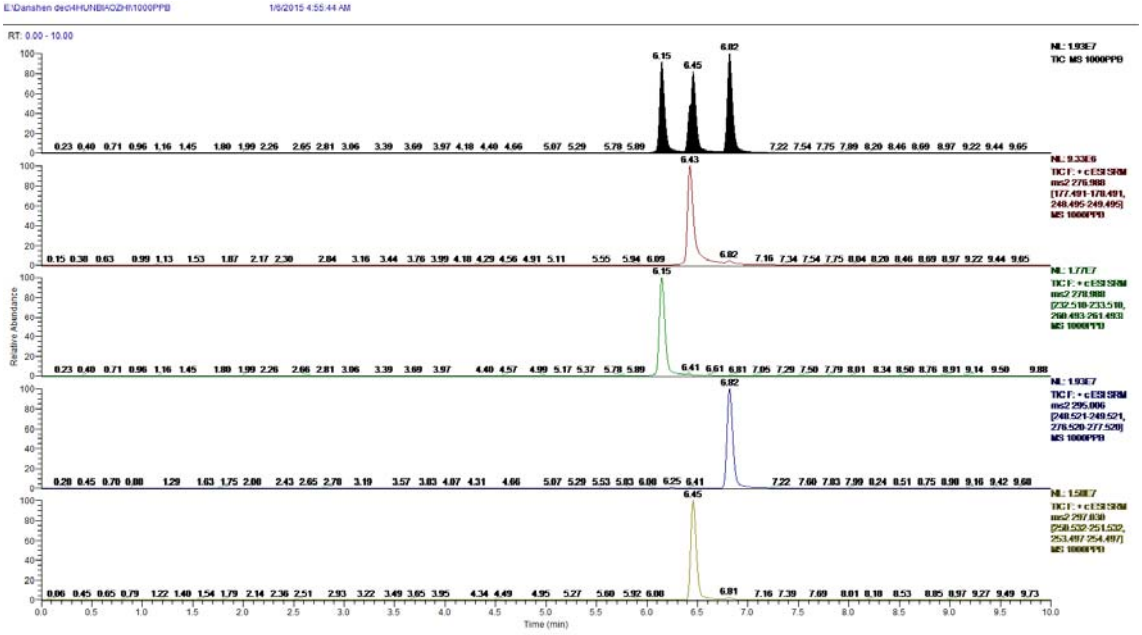

Figure 1: UPLC-MS/MS chromatograms of 4 standard substances

Mix the above series of reference substance solution respectively according to the "3.1" chromatography - mass spectrometry conditions under sample analysis, the linear regression was taken with the concentration of the 4 kinds of compounds ($ng \cdot mL^{-1}$) as the abscissa and the peak area for the vertical linear regression. The experimental results are shown in table 3.

Table 3: The regression equations and correlation coefficients of the 4 chemical drugs

components	linear regression equation/ ng·mL^{-1}	Regression equation	r
tanshinone I	0.20-1000	Y = 33773.6+35015.4*X	0.9917
tanshinone IIA	0.20-1000	Y = 63732.9+46096.4*X	0.9982
cryptotanshinone	0.20-1000	Y = 25587.0+35225.4*X	0.9970
dihydrotanshinone	1.0-1000	Y = 63430.3+38818.3*X	0.9967

3.5 The real sample determination

The above analysis method is used for all kinds of danshen medicinal material content determination of 4 kinds of ingredients, the chromatogram of UPLC-MS/MS are shown in Figure 2.

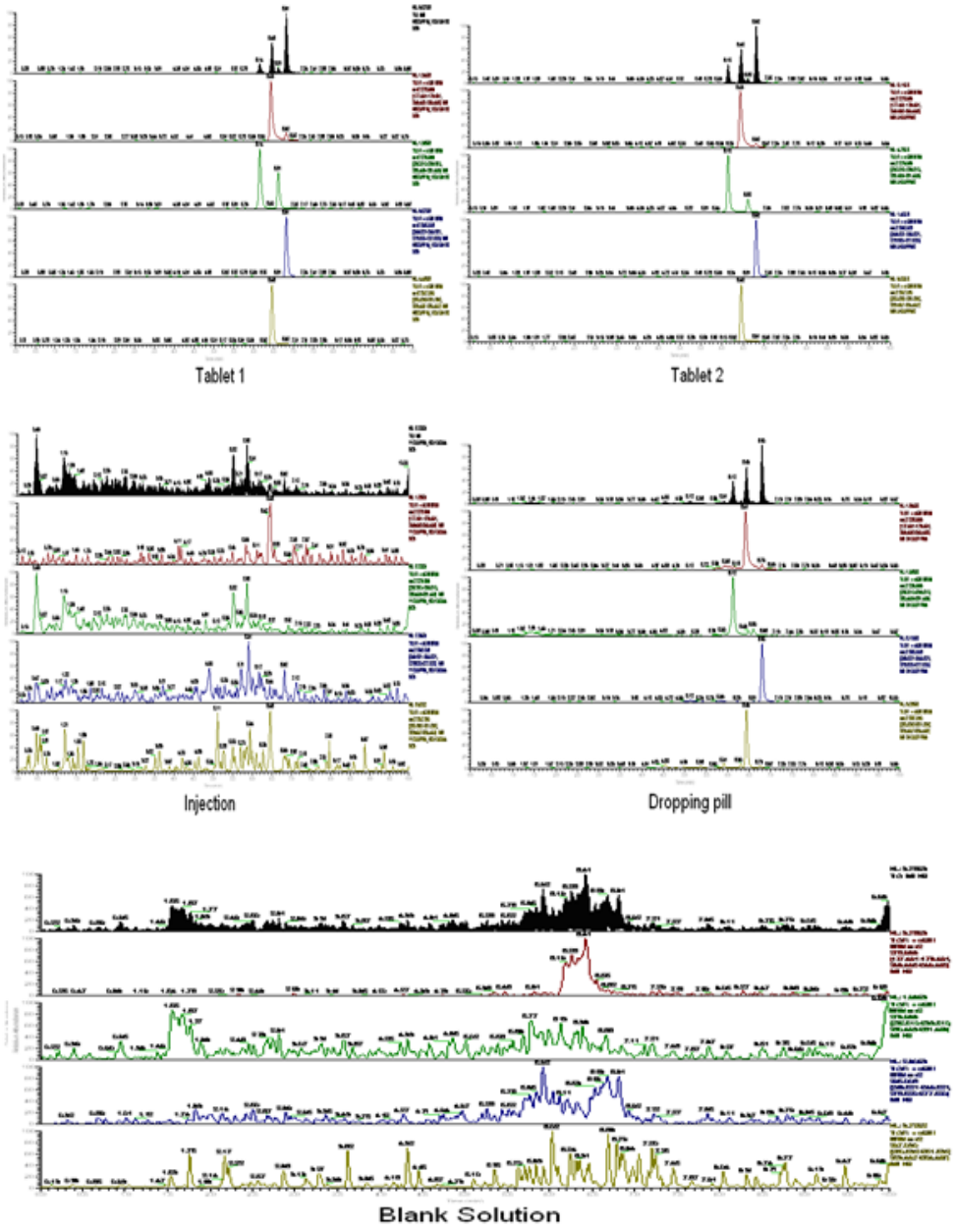

Figure 2: UPLC-MS/MS chromatograms of different types of Danshen and blank solution

The results of quantitative measurement are shown in table 4. Different kinds of preparation of danshen medicinal materials content of active ingredients is different obviously.

Table 4: Determination results of samples

Components	Content(μg·g-1)			
	Tablet 1	Tablet 2	Injection	Dropping pill
tanshinone I	236.42	418.20	NF	10.67
tanshinone IIA	682	650.12	NF	21.73
cryptotanshinone	445.506	511.64	NF	17.19
dihydrotanshinone	127.548	253.54	NF	9.403

3.6 Sample recovery

Adding concentration equivalent to 10 ng·mL-1, 100 ng·mL-1, 1000 ng·mL-1of high, medium and low concentration of three kinds of hybrid reference substance solution in 100μg·mL-1 sample, Determination of each concentration solution repeated six times, The result of measured recovery are shown in table 5. The average recovery of low concentration is 105% ~ 107%, and RSD is less than 4.3%.The average recovery of medium concentration is 104% ~ 112%, RSD is less than 2.9%; The average recovery of high concentrations is 83.4% ~ 87.6%, RSD is less than 2.2%. Test results show that the chemical recovery rate and precision is good.

Table 5: Recoveries of 4 chemical drugs (n=6)

components	recovery at low concentration		recovery at medium concentration		recovery at high concentration	
	mean/%	RSD/%	mean/%	RSD/%	mean/%	RSD/%
tanshinone I	107	2.3	106	1.7	84.6	0.98
tanshinone IIA	106	3.5	111	0.9	86.8	1.6
cryptotanshinone	106	2.9	104	2.9	87.6	2.2
dihydrotanshinone	105	4.3	112	1.4	83.4	1.3

3.7 Repeatability

Take the same copy of the test solution, respectively at 0, 6, 12, 18, 24 h sample determination, and results show that the RSD of peak area of each component are 0.45% (tanshinone I); 0.82% (tanshinone IIA); 1.1% (cryptotanshinone); and 0.91% (dihydrotanshinone), it showed that the test sample solution was stable within 24 h.

4. Discussion

4.1 The optimization of the extraction conditions

This paper examines the methanol, methanol and water (50:50), such as acetonitrile extraction solvent. The results show that using methanol and acetonitrile extraction effect is good. Considering extracting efficiency and cost, and ultimately choose methanol as extraction solvent.

4.2 Chromatographic separation conditions

This paper examines the methanol - water, methanol 0.1% formic acid aqueous solution, methanol 0.1% formic acid aqueous solution (including 5mmol·L-1 ammonium acetate) and acetonitrile - water as mobile phase, the results showed that the methanol - 0.1% formic acid aqueous solution is best as mobile phase chromatographic separation system.

4.3 Summary

Experimental results show that the established super high performance liquid chromatography - tandem mass spectrometry can multicomponent testing drugs salvia miltiorrhiza effective component content at the same time.This method is a strong specificity, high sensitivity, good accuracy and fast inspection speed.

Acknowledgments

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