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Research Article

ISOLATION OF TANSHINONE IIA AND CRYPTOTANSHINONE IN SALVIA MILTIORRHIZA USING TWO CONVENTIONAL EXTRACTION TECHNIQUES AND QUANTIFICATION BY VALIDATED HPLC METHOD

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ABSTRACT

This study describes a simple, easy and inexpensive method for extracting tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza* by sonication or decoction. A comparison of sonication and decoction showed a similar yield for cryptotanshinone, while the tanshinone IIA yield was slightly higher using sonication. An analytical method for detecting tanshinone IIA and cryptotanshinone in extracts was developed and validated. Optimal conditions for separation and detection were achieved using a Grom Sil 120 ODS-5 ST column (250 X 4 mm, 5 µm) with an isocratic mixture of methanol:water (80:20) containing 0.5% acetic acid, at flow rate 0.5 ml/min, detected at 254 nm. The analysis run time was 25 min. The recovery of the method was >87% for cryptotanshinone and >92% for tanshinone IIA and both compounds showed good linearity (r^2 > *0.9911).*

Keywords: Tanshinone IIA, Cryptotanshinone, Salvia miltiorrhiza, Danshen, HPLC

INTRODUCTION

Salvia miltiorrhiza is frequently used in traditional Chinese medicine, and is also known as danshen or Chinese red sage. In other countries, such as the United States, European countries, Japan and Korea, danshen is used as an herbal medicine[1]. It is commonly used to treat angina pectoris, myocardial infraction[2], cerebrovascular disease, liver disease, diabetes mellitus, menstrual disorders and miscarriage. It has many significant pharmacological effects including antineoplastic[3], anticancer[4], antiinflammation[5], antibacterial, antidermatophytic, antioxidant[6], antithrombic and antiplatelet aggregation.

Danshen contains both hydrophilic and lipophilic compounds. The lipophilic compounds are cryptotanshinone, tanshinone I, IIA, IIB[7-9] and the hydrophilic compounds are phenolic acids[10] and other related compounds such as protocatechuic aldehyde, salvianic acid A and protocatechuic acid. The biological activities of tanshinone II A and cryptotanshinone have been extensively studied. Tanshinone IIA and crytotanshinone are the main abietance-type diterpenes in danshen (Figure 1).

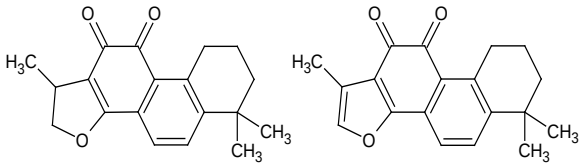


Fig. 1: Chemical structure of a) cryptotanshinone and b) tanshinone II A.

Table 1: Extraction methods and techniques for isolation and detection of *Salvia miltiorrhizia*

Compound	Techniques	Extraction method	Reference
Tanshinone IIA, Tanshinone I Cryptotanshinone, Dihydrotanshinone I	HPLC	Micelle-mediated extraction	14
Tanshinone I, Tanshinone IIA Cryptotanshinone, Dihydrotanshinone I	HPLC-MS/MS	-	15
Tanshinone I, Tanshinone IIA Cryptotanshinone	HPLC-UV	Microwave-assisted techniques	18
Tanshinone IIA	HPLC-UV	Supercritical fluid extraction, Phytosol extraction	19
Tanshinone IIA, Cryptotanshinone	HPLC-UV	Microwave-assisted Soxhlet, Heat reflux	20
Tanshinone I, Cryptotanshinone Tanshinone II A	Capillary electrophoresis	-	21
Tanshinone IIA, Tanshinone I Cryptotanshinone, Dihydrotanshinone Danshenxinkun B	HPLC-UV	Simple extraction	22
Tanshinone IIA, Tanshinone I, Salvonic acid, Cryptotanshinone	HPLC-MS/MS	Ultrasonication	23
Tanshinone II A, Tanshinone I	HPLC-MS/MS	Pressurized liquid extraction, Pressurized hot water extraction	24

Several techniques have been developed for separating and detecting danshen, including high performance liquid chromatography with ultraviolet detection (HPLC-UV) or diode array detector (DAD)[11-14], liquid chromatography-mass spectrometry(LC-MS)[15], high-speed counter-current chromatography (HSCCC)[16] and nonaqueous capillary electrophoresis (NACE)[17].

Several efficient extraction techniques have been developed such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), phytosol extraction, micelle-mediated extraction, ultrasonication, pressurized liquid extraction (PLE), and pressurized hot water extraction (PHWE). These are commonly used for isolation of tanshinone IIA and cryptotanshinone from *S. miltiorrhiza* roots. (Table 1).

In this paper, we used two different extraction techniques, sonication and decoction, and compared their results. An HPLC-UV method was validated for simultaneous detection of tanshinone IIA and cryptotanshinone in *S. miltiorrhiza* root extracts. The results indicate that this method is fast, sensitive and suitable for quantitative assessment of danshen samples.

MATERIAL AND METHOD

Chemicals and reagents

HPLC-grade methanol was from Fisher Scientific (Toronto, Canada). Triple deionized water was purified using a Millipore system (Millipore, Milford, MA, USA). Acetic acid was analytical grade (Merck, Germany). Ethanol was from Duksan Pure Chemicals (South Korea). Tanshinone IIA and cryptotanshinone standards were from Sigma (USA). *S. miltiorrhiza* root was from Erae Pharmaceutical Company (Paju, Kyungki, Korea).

Extraction procedure

The plant extracts were prepared as described by Reddy et. al.[25] with some modifications. Extraction of *S. miltiorrhiza* was carried out in two ways, decoction using a heating mantle (Method I) and sonication using a sonicator (Method II). Weighed, dried roots (600 gm) and 1.5 liters of ethanol were added to a conical flask or round bottom flask and 3 hrs of extraction performed with continuous sonication (Branson, Danbury, CT) or decoction. The process was repeated three times, replacing the 1.5 liters of ethanol extraction solvent each time, for complete extract recovery of the root samples. Collected solutions were filtered through 0.26-mm filter paper (Advantech, No. 2). Sample extracted by both methods were treated equally. Aliquots from the filtered samples were evaporated at 40 ºC using a one-liter rotary evaporator (EYELA, Japan), as described by Zahin et. al.[26] with some modifications. Ethanol-free concentrated extracts were placed in freeze dryers (Ilshin, Hong Kong) to generate powder, which was packed in airtight containers and kept at 4ºC until HPLC analysis.

Chromatography

HPLC equipment was an auto sampler (Model S5200), solvent delivery system (Model S2100) and UV-Vis detector (Model S3210S), all from Sykam (Munich, Germany). Tanshinone IIA and cryptotanshinone were separated on a Grom Sil 120 ODS-5 ST column, 250 X 4 mm i.d., 5-µm particle size. The mobile phase was methanol:water (80:20 v/v) containing 0.5% acetic acid, which gave better results that other mixtures of methanol/water or water/acetonitrile that were tested. The mobile phase was filtered through a 0.45-µm membrane filter (Osmonics), which had been deaerated ultrasonically. The flow rate was 0.5 mL/min, the UV wavelength was 254 nm and the column temperature was 50°C. The chromatographic peaks of the analytes were confirmed by comparing their retention time and UV spectra with reference standards. Quantification was carried out by peak integration using an external standard method.

Preparation of standard solution and sample

Stock solutions of tanshinone IIA and cryptotanshinone were prepared by dissolving 10 mg in 10 mL methanol to a final concentration of 1 mg/mL and storing at 4°C. Stock solutions were diluted with methanol to obtain solutions of different concentrations. Powdered danshen extract was ground into a fine powder using a mortar and pestle. About 100 mg was weighed into 10 ml of methanol in a volumetric flask. The sample was extracted at room temperature using a sonicator for 15 minutes. Before injection, the mixture was filtered through a 0.45-µm nylon syringe filter and 10 µL injected into the HPLC system. The final concentration of the sample was 10 mg/mL.

RESULTS AND DISCUSSION

Selection of mobile phase

Different gradient and isocratic system was studied for various concentrations of acetonitrile: water and methanol: water. Four mobile systems were tried to find the optimum elution conditions: methanol: water (50:50 %), acetonitrile: water (60:40 %), methanol: water (70:30), methanol: water (80:20%, 0.5% acetic acid). Additions of 0.5% acetic acid improved the sensitivity. Thus, methanol: water (80:20%, 0.5% acetic acid) was selected for the analysis of danshen extract.

Validation of the method

Linearity

Calibration graphs were obtained by measuring peak area versus concentration. Calibration curves of the two compounds were constructed by measuring different concentrations. A good linearity was obtained for both compounds (Figure 2). Regression equations were calculated as y = mx + c where y indicates the area of the analytes and x indicates the sample concentration (Table 2).

Table 2: Regression equation, correlation coefficient, linear range

Compound	Regression equation	Correlation coeff. (r^2)	Linear Range (µg/mL)
Tanshinone IIA	59467x+296829	0.9911	30-230
Cryptotanshinone	62354x-109248	0.9921	60-220

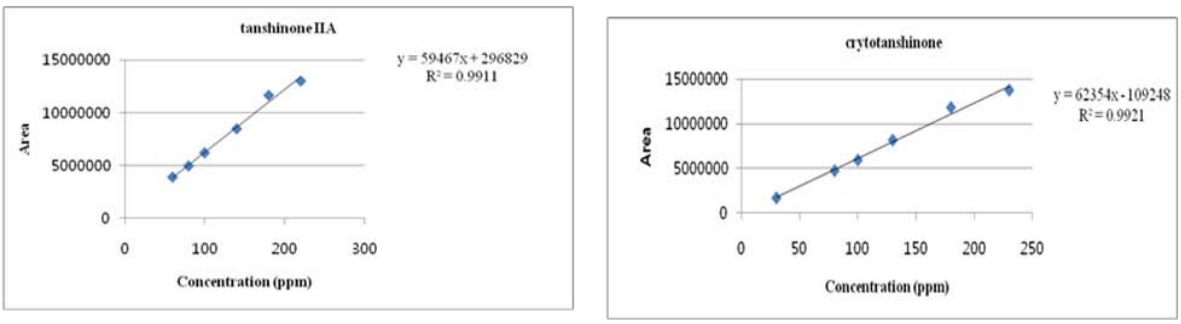


Fig. 2: Calibration curve of (a) tanshinone II A (b) cryptotanshinone

Recovery

Danshen extract was spiked with reference standard at various concentrations. After HPLC analysis, recoveries of the reference standards were calculated (Table 3) by the following equation:

Recovery (%) = (amount found – original amount)/ amount spiked × 100%

Relative Standard Deviation (RSD) (%)= (SD/mean) × 100%

Accuracy and precision

The intra- and interday precision was determined by analyzing three different concentrations on a single day and on three different days. The intraday variation was determined by analyzing five replicates on the same day, and interday variation determined on three consecutive days (n = 3). The RSD was taken as a measure of precision (Table 4).

Quantification of tanshinone IIA and cryptotanshinone from different extracts of *salvia miltiorrhzia*

Sample solutions of *S. miltiorrhiza* extracts were dissolved in methanol and injected and separated under the conditions described above. Typical HPLC-UV profiles are in Figure 3. Good separation was achieved within 25 min. An isocratic system was developed for the separation of tanshinone II A and cryptotanshinone. By increasing the column temperature, tanshinone IIA and cryptotanshinone eluted faster than in earlier studies [13, 25] and reduced retention time is important for large samples. The retention time was 14.8 minutes for cryptotanshinone and 21.6 minutes for tanshinone IIA. The content of tanshinone IIA and cryptotanshinone in *S. miltiorrhiza* was determined by the regression equation. The contents (n = 5) of two batches are in Table 5. The tanshinone II A content was found to be slightly higher using the sonication than the decoction method, while for cryptotanshinone, similar yields were obtained with both methods.

Table 3: Recovery of tanshinone IIA and cryptotanshinone in extracts (n = 3)

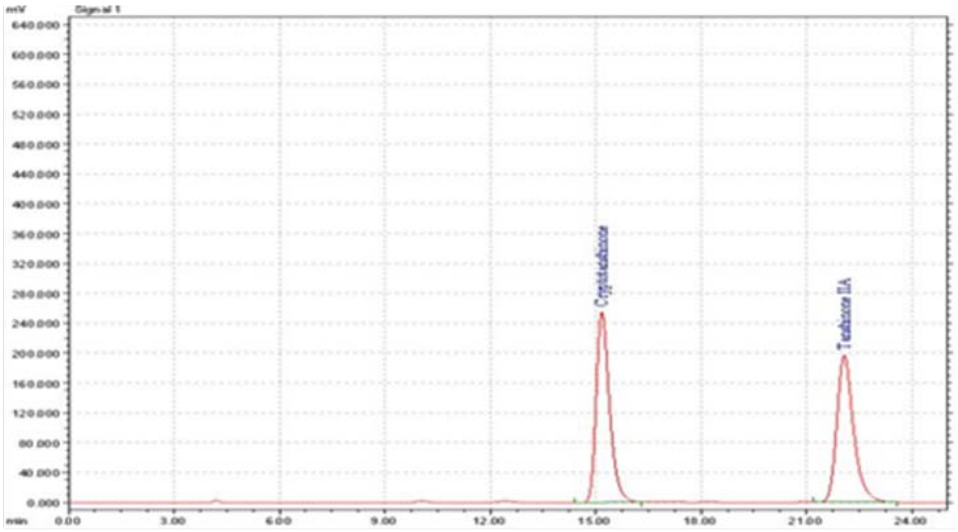
Compound	Amount added (µg/ml)	Recovery (%)
TanshinoneIIA	25	92.394
	50	101.394
Cryptotanshinone	25	87.996
	50	91.399

Table 4: Precision and accuracy

Compound	Actual concentration (µg)	Observed concentration ± SD	Precision (CV %)
Intraday (n = 5)			
Tanshinone IIA	25	22.849	11.66
	50	43.966	12.21
Crytotanshinone	25	19.376	7.966
	50	52.320	2.4
Interday (n = 3)			
Tanshinone IIA	25	22.849	11.66
	50	43.998	12.466
Cryptotanshinone	25	23.098	4.454
	50	50.921	4.76

Table 5: Measurement of tanshinone II A and cryptotanshinone (n=5)

Extraction method	Compound	Content (µg/mg) ± SD
Sonication	Tanshinone IIA	18.58 ± 1.86
	Cryptotanshinone	13.16 ± 1.224
Decoction	Tanshinone II A	10.96 ± 4.75
	Cryptotanshinone	10.65 ± 6.231



a)

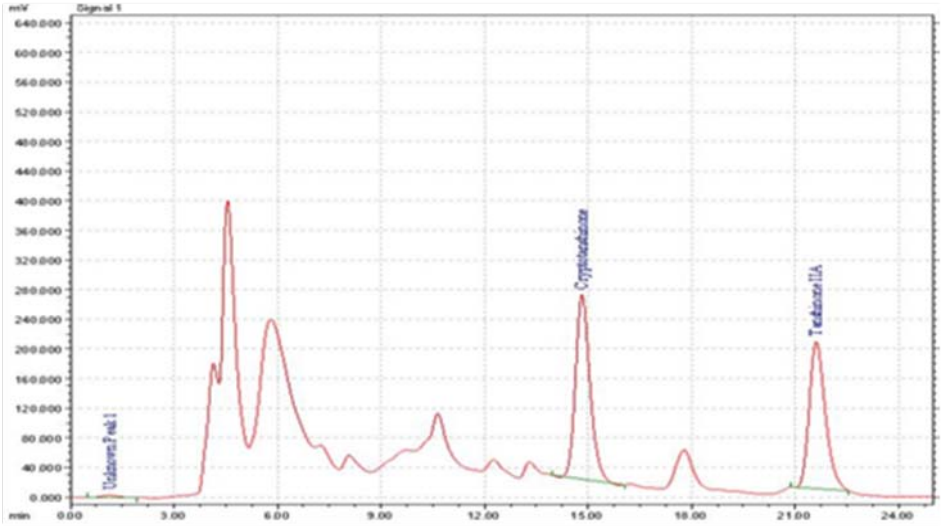


Fig. 3: Chromatogram of (a) standard cryptotanshinone and tanshinone IIA (b) danshen extract (10 mg/mL)

CONCLUSION

Modern extractions methods, which include PLE, MAE, and SFE, are usually expensive. In this study, we used two conventional methods of sonication and decoction, which are simple, easy and inexpesive. The yields from both methods were similar for cryptotanshinone, while the yield for tanshinone IIA was slightly higher content using sonication. Our separation was validated method for good separation and simultaneous detection of tanshinone IIA and cryptotanshinone in extracts of *S. miltiorrhiza* root, with a short retention time, good sensitivity, simplicity and precision.

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