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Extraction and HPLC Analysis of Tanshinone I, Tanshinone IIA and Cryptotanshinone from *Salvia miltiorrhiza Bunge*

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In the present work, simultaneous extraction and separation of tanshinone I, tanshinone IIA and cryptotanshinone has been developed and validated from *Salvia miltiorrhiza bunge*. The extracts of the tanshinones were separated on a C_{18} column with the mobile phase composed of methanol-water (78:22, v/v, containing 0.5 % v acetic acid) at a flow-rate of 0.5 mL/min. The calibration was linear in a range of 0.1-500.0 µg/mL for these three compounds. The extracted amounts are 0.0091 mg/g for the tanshinone I, 0.12 mg/g for the tanshinone IIA and 0.15 mg/g for the cryptotanshinone.

Key Words: Extraction, Tanshinone I, Tanshinone IIA, *Salvia miltiorrhiza bunge*, Cryptotanshinone.

INTRODUCTION

The root of *Salvia miltiorrhiza bunge* has been widely used in traditional Chinese medicine for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood, *et al.*[1,2]. *Salvia miltiorrhiza bunge* has received many interests due to its ability to accumulate large amounts of active compounds such as tanshinones[3,4]. Tanshinone I, tanshinone IIA and cryptotanshinone (molecular structures are shown in Fig. 1) are the main abietane-type diterpenes contained in *Salvia miltiorrhiza bunge*. It is reported that tanshinones can dilate coronary arteries, increase coronary flow, modulate mutagenic activity and protect the myocardium against ischaemia. They also have sedative and tranquilizing effects and some of them have been used to treat neurasthenic insomnia[5,6]. In addition, tanshinones have attracted particular attention because they exhibit significant anti-bacterial[7,8], anti-dermatophytic, antioxidant[9,10], antiinflammatory[11] and antiplatelet aggregation[12] activities.

The rapid extraction and separation of these tanshinone compounds is a central problem in medicinal chemistry and biotechnology. Extraction of tanshinones from *Salvia miltiorrhiza bunge* by organic solvents has been reported by several authors with subsequent analysis of HPLC[13,14] and spectrophotometry[15]. In order to simplify the operating procedure, new extraction and separation methods should be developed.

The complexity of tanshinone compounds makes it difficult to separate them in a single chromatographic run, so the primary aim of the present study is to develop a simple, sensitive and more reliable reversed-phase high performance liquid chromatography (RP-HPLC) method for the simultaneous extraction and separation of these tanshinone compounds from *Salvia miltiorrhiza bunge*.

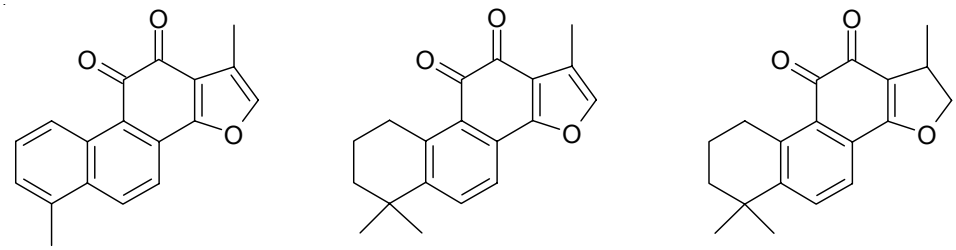

Fig. 1. Chemical structures of three tanshinones (A) tanshinone I, (B) tanshinone IIA, (C) cryptotanshinone

EXPERIMENTAL

Standards of tanshinone I, tanshinone IIA and cryptotanshinone were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Salvia miltiorrhiza bunge* was purchased from a local market. All the solvents were HPLC grade and were purchased from Duksan Pure Chemical. Co., Ltd. (Korea). Water was twice distilled and filtered (FH-0.45 μm, Advantec MFS, Inc., Japan) using a decompressing pump (Division of Millipore, Waters, USA).

The standards of tanshinone I, tanshinone IIA and cryptotanshinone were dissolved in methanol to yield a final concentration of 0.2 mg/mL and stored at 4 ºC in a refrigerator in darkness. The *Salvia miltiorrhiza bunge* roots were oven-dried under 40 ºC and then sliced into powder for the extraction experiments.

HPLC analysis: The chromatography system consisted of a Waters 600s Multi Solvent Delivery System and a Waters 616 liquid chromatography system (Waters Associates, Milford, MA, U.S.A.), a Rheodyne injector (Cotati, CA, USA) valve with a 20 mL sample loop and a variable wavelength 2487 UV dual channel detector. Millennium software (Ver. 3.2 Interface Eng., Korea) on a PC was used as a data acquisition system.

RESULTS AND DISCUSSION

Effect of different extraction solvents: The different extraction solvents used in the experiment for the extraction of tanshinone I, tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza bunge* were water, methanol, ethanol, ethyl

acetate and chloroform. 50 mL of each solvent was used to extract 0.5 g *Salvia miltiorrhiza bunge* powder for 12 h under room temperature, respectively. All the three compounds can be solved in polar and organic solvents[16], so as the Table-1 shown the highest extracted amount of the three compounds was obtained by methanol. Hence, methanol was used in the subsequent experiments.

TABLE-1
EXTRACTED AMOUNTS OF TANSHINONE I, TANSHINONE IIA AND CRYPTOTANSHINONE WITH DIFFERENT SOLVENTS (mean ± SD, n = 3)

Solvents	Compounds		
	Tanshinone I (μg g^{-1})	Tanshinone IIA (μg g^{-1})	Cryptotanshinone (μg g^{-1})
Methanol	0.3900 ± 0.01000	0.5200 ± 0.02000	0.1100 ± 0.00500
Ethanol	0.3200 ± 0.00900	0.4900 ± 0.01000	0.0800 ± 0.00200
Ethyl acetate	0.0760 ± 0.00020	0.1200 ± 0.00900	0.0200 ± 0.00100
Chloroform	0.0003 ± 0.00001	0.0006 ± 0.00002	0.0001 ± 0.00001
Water	Not detected	Not detected	Not detected

Effect of different extraction methods: The different extraction methods such as dipping extraction and ultrasonic extraction were investigated. In dipping extraction, 0.5 g *Salvia miltiorrhiza bunge* powder was mixed and stirred with 50 mL methanol for 1, 2, 4, 5, 7, 9 and 12 h. In Fig. 2, it is seen that the extracted amounts of the three compounds increased with the dipping time increasing from 1 to 9 h, there was no obvious increase after further prolonged extraction time.

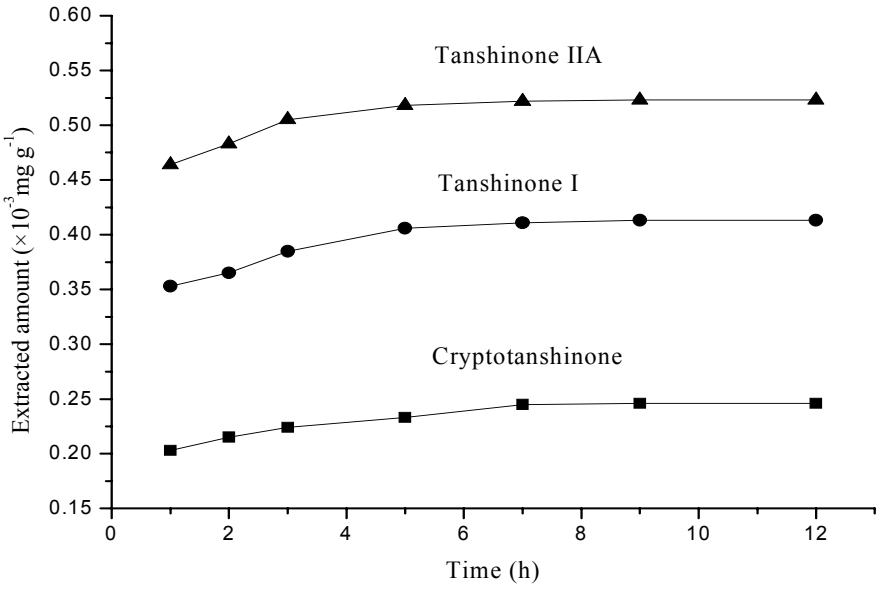

Fig. 2. Effect of different dipping times on extracted amounts of *Salvia miltiorrhiza bunge*

Equivalent samples were then prepared by ultrasonic method without dipping time. 5, 10, 15, 20, 30, 40 and 60 min were investigated and Fig. 3 shows that the extracted amounts of tanshinone I, tanshinone IIA and cryptotanshinone increased with the ultrasonic time increasing before 0.5 h. However, comparing the results of two methods, it was found that the extracted amounts *via* the ultrasonic method were higher in a short time (0.5 h). Thus, it was determined that the dipping method was not appropriate for this approach and ultrasonic method will be established for further experiments.

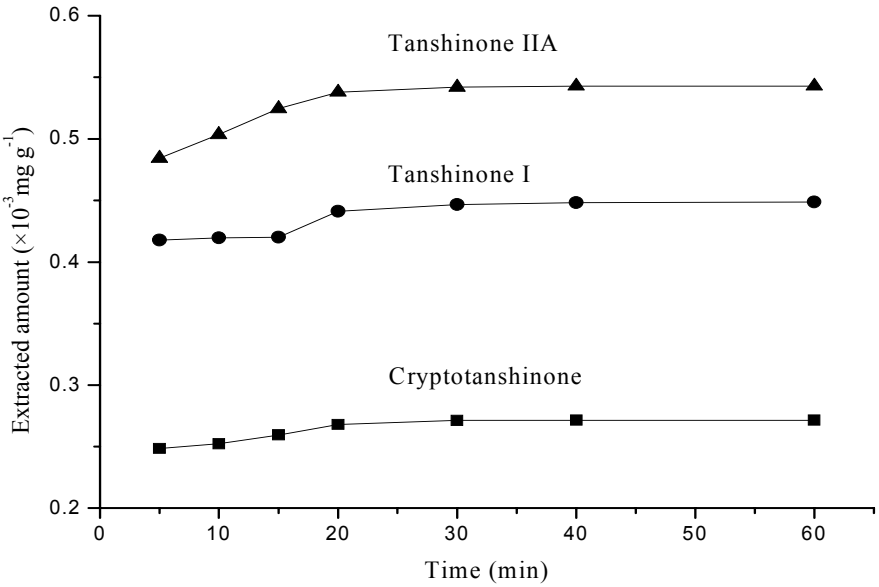

Fig. 3. Effect of different ultrasonic times on extracted amounts of *Salvia miltiorrhiza bunge*

Method validation: A series of samples containing of tanshinone I, tanshinone IIA and cryptotanshinone (0.1, 3.0, 10.0, 50.0, 100, 250.0 and 500.0 µg/mL) were prepared to detect the relationships between the peak areas and the concentrations of the tanshinones. The mobile phase was composed of methanol-water (78:22, v/v, containing 0.5 % v acetic acid) with 0.5 mL/min flow rate on a C_{18} column (4.6 × 150 mm, 5 µm, RS-Tech Corporation, Korea). The injection volume was 5 µL and the UV wavelength was set at 254 nm. Each concentration was injected 3 times and calibration curves of the three compounds showed good linearity ($r^2 > 0.9997$), the regression equations of tanshinone I, tanshinone IIA and cryptotanshinone were y = 53735x - 75327, y = 37353x - 88373 and y = 47839x - 6967, respectively.

In order to determine the accuracy and the stability of solutions, relative standard deviations (RSDs) were performed by injecting standard solutions of tanshinone I, tanshinone IIA and cryptotanshinone 5 times in a 7-day period. The results in Table-2 showed that there was no significant degradation within this period.

TABLE-2
CALIBRATION CURVE FOR THE QUANTIFICATION OF
TANSHINONE I, TANSHINONE IIA AND CRYPTOTANSHINONE

Analyte	Standard curve (r^2)	Test range (μg mL^{-1})
Tanshinone I	Y = 53735.0X - 75327 (0.9998)	0.1-500.0
Tanshinone IIA	Y = 37353.0X - 88373.0 (0.9997)	0.1-500.0
Cryptotanshinone	Y = 47839.0X - 6967.0 (0.9998)	0.1-500.0

Y = peak area; X = concentration of analyte (μg mL^{-1}).

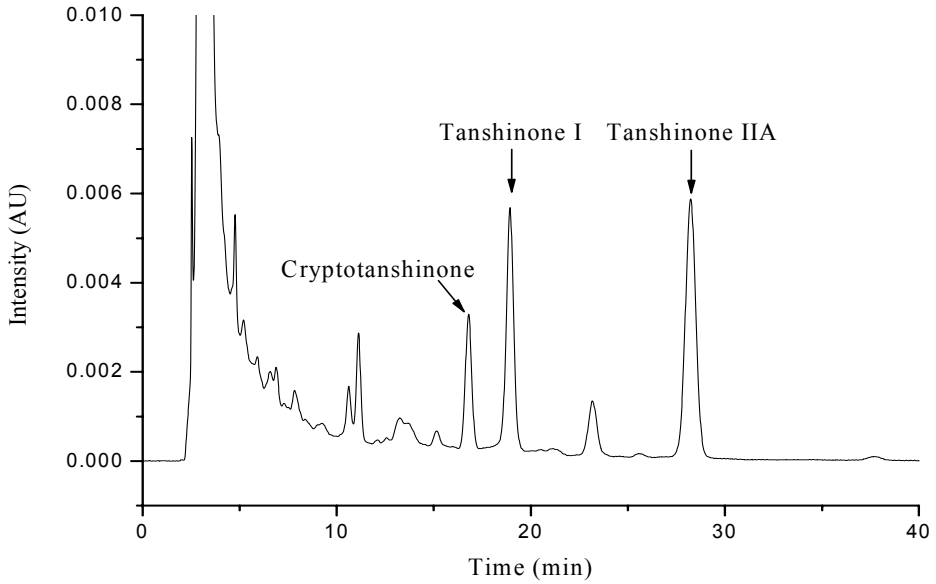

Fig. 4. Chromatogram of tanshinone I, tanshinone IIA and cryptotanshinone in methanol extract

The standard solvents of three compounds (1.0, 10.0, 40.0 μg/mL) were added to 3 mL of the extracts from licorice, respectively and to a final volume of 6 mL.

$$R = \frac{C_p - C_0}{C_m} \times 100\% \quad (1)$$

R: recovery rate, C_p: the total amount of the compound of final solvent, C_0: the amounts of the compound from licorice, C_m: the amount of the compound which was added. The measured concentration was compared with the theoretical concentration to calculate the recovery rate[17] by eqn. 1.

The standard solutions of the three compounds were diluted and injected until the limit of detection (LOD) was obtained at a signal/noise ratio of 3. The RSD of precision tests, the limit of detections (LOD) on standard solutions and the recovery rates are presented in Table-2. Comparison with the real sample analysis verified that the values noted above were of acceptable precision and accuracy.

TABLE-3
RECOVERY STUDIES OF TANSHINONE I, TANSHINONE IIA AND CRYPTOTANSHINONE IN *Salvia miltiorrhiza bunge* (n = 3)

Compounds	RSD (%)		Recovery rate			LOD (ng/mL)
	Intra-day	Inter-day	Amount added (μg/mL)	Average recovery (%)	RSD (%)	
Tanshinone I	4.6	4.5	1.0	89.5	4.3	85
	4.7	4.9	10.0	88.6	4.6	
	4.2	4.1	40.0	90.3	3.9	
Tanshinone IIA	3.6	3.8	1.0	90.5	3.8	93
	3.5	3.6	10.0	89.6	3.5	
	3.0	2.9	40.0	88.7	2.9	
Cryptotanshinone	4.1	4.0	1.0	86.2	3.9	76
	4.2	4.3	10.0	88.9	4.1	
	4.0	4.2	40.0	88.5	3.8	

Conclusion

In this study, a simple and convenient method for simultaneous extraction of tanshinone I, tanshinone IIA and cryptotanshinone from *Salvia miltiorrhiza bunge* was described. Methanol as extract solvent and 20 min ultrasonic time provide excellent extraction. Furthermore, methanol-water (78:22, v/v, containing 0.5 % acetic acid) as the mobile phase was the optimum condition for the separation. The extracted amounts are 0.0091, 0.12 and 0.15 mg/g for these three compounds, respectively.

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