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High-performance liquid chromatography of abietane-type compounds

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ABSTRACT

A sensitive, quantitative procedure was developed for the simultaneous determination of tanshinone IIA and I, cryptotanshinone and dihydrotanshinone I by high-performance liquid chromatography (HPLC) employing a normal-phase column. In addition, a method for the determination of the polar tanshinones tanshinone V and VI by reversed-phase HPLC, an HPLC method for ferruginol and a qualitative procedure for other tanshinones were also developed. These methods were applied to identify abietane-type compounds in various tissues of *Salvia miltiorrhiza* Bung, including cultured cells, fresh roots and regenerated plant roots.

INTRODUCTION

Dried roots (Tan-Shen in Chinese) of *Salvia miltiorrhiza* Bung (Labiatae), an ancient Chinese drug, have been used to treat haemorrhages, menstrual disorders and miscarriages [1]. Tan-Shen is known to contain abietane-type diterpenes (tanshinones), such as tanshinone I [2], cryptotanshinone [3] and tanshinone VI [4], which protect the myocardium against ischaemia [4], and also related orange-red pigments (shown in Fig. 1).

Usually, tanshinones have been resolved by thin-layer chromatography and cryptotanshinone and ferruginol determined by gas chromatography [5]. Recently, a reversed-phase high-performance liquid chromatographic (HPLC) method was applied to the determination of several tanshinones from *Salvia* species [6,7]. However, tanshinones are hydrophobic compounds and tanshinone IIA and cryptotanshinone, the major tanshinones, can be completely eluted from a silica gel column with chloroform. The objective of this study was to develop a simple method for the determination of tanshinones and ferruginol in various tissues using isocratic normal-phase HPLC. This procedure involves the extraction of tanshinones followed by fractionation and analysis by HPLC. Both qualitative and quantitative procedures for the polar tanshinones were accomplished by reversed-phase HPLC. With these

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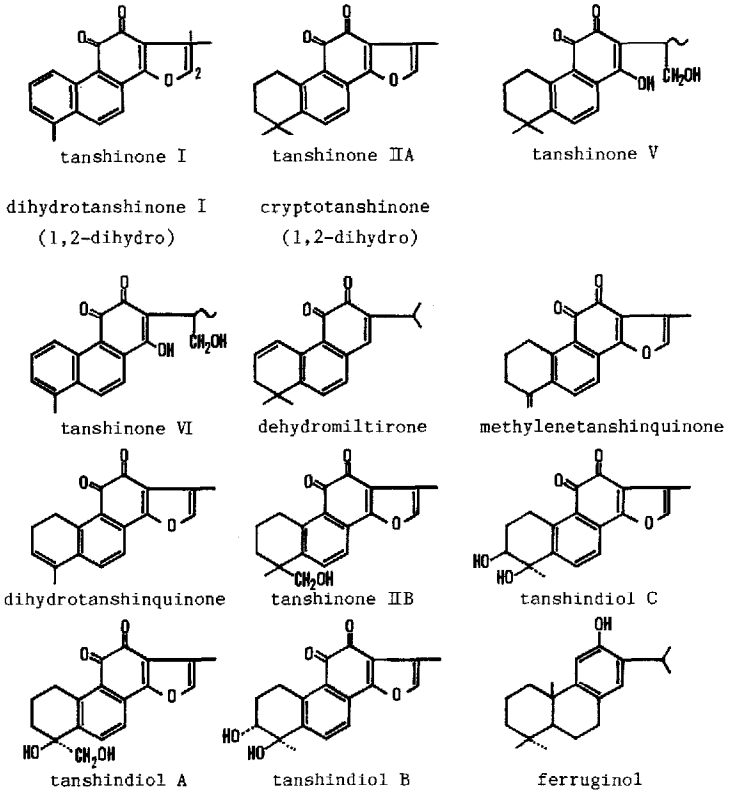

Fig. 1. Structures of abietane-type compounds.

methods it was possible to study the production of bioactive tanshinones in cultured cells and regenerated plants.

EXPERIMENTAL

Materials

Tan-Shen grown in China was purchased from Nakai-Koshindo (Kobe, Japan). *S. miltiorrhiza*, regenrated plants, water-cultured plants and callus derived from *S. miltiorrhiza* were supplied by Tsukuba Medicinal Plant Research Station (Ibaraki, Japan).

Adventitious root cultures were derived on Murashige-Skoog medium supplemented with 1-naphthaleneacetic acid (0.5 ml/l) and benzyladenine (0.1 ml/l) from segments of stems and petioles after 8 weeks in the dark at 25°C. Differentiated shoots were induced when the stem pieces were placed on Murashige-Skoog medium containing 3-indoleacetic acid (0.5 mg/l) and kinetin (1 mg/l) for 8 weeks under 16 h light (4000 lux) at 25°C. Young plantlets were derived from stem tips of differentiated shoots on hormone-free Murashige-Skoog medium for 8 weeks under 16 h light (4000 lux) at 25°C and transferred to a field in May. Regenerated plants were harvested in November and January. Water-cultured plants were grown in a greenhouse for

3 months. Ferruginol was kindly provided by Tomita (Niigata, Japan) and tanshinones were isolated from Tan-Shen [4]. The HPLC solvents were of HPLC grade and other solvents and chemicals were of analytical-reagent grade, all of which were obtained from Wako (Osaka, Japan). Iatrobeads (silica gel, 100-μm particle size) were from Iatron Laboratories.

Apparatus

The HPLC system consisted of two Tosoh CCPD pumps equipped with a Tosoh CCP controller connected to a dynamic mixer, a 5-μl sample loop and a Tosoh UV-8000 UV–VIS detector. The data were processed by means of a SIC Chromatocorder-11 integrator to evaluate the peak areas. The purity of the chromatographic peaks was estimated using a Waters M990J photodiode-array detector.

Extraction and fractionation of tanshinones and ferruginol

All procedures for the extraction, fractionation and analyses were performed in the dark as much as possible because some tanshinones readily undergo photo-oxidation [8]. The lyophilized sample was homogenized with ten volumes or more of chloroform–methanol (2:1, v/v). The homogenate was then centrifuged and the supernatant was transferred to a test-tube by decantation. The remaining insoluble material was sonicated in a sonic cleaning bath for 10 min with half the volume of the above homogenate. The pooled supernatant was washed according to the method of Folch *et al.* [9] and evaporated to dryness under nitrogen. An appropriate amount of extract, containing less than 14 mg, was taken up in a small volume of hexane–chloroform (1:1, v/v) and placed on a silica gel column containing 0.4 g of Iatrobeads. The column was eluted with 4 ml each of the above solvent and chloroform. The first fraction contained ferruginol and tanshinones were recovered in the second fraction. Both fractions were evaporated to dryness under nitrogen, dissolved in a known small volume of chloroform and then a portion was injected into the HPLC system. For the analysis of only tanshinones, the Iatrobeads chromatography was omitted.

Preparation for determination of tanshinone VI

The extract (10–100 mg) prepared by the method of Folch *et al.* [9] described above was dissolved in 2 ml of ethyl acetate and extracted with 2 ml of 5% aqueous sodium carbonate. The ethyl acetate layer was further extracted twice with 1 ml of the same solvent. Hydrochloric acid (1 *M*, 3.5 ml) was added to the pooled sodium carbonate layer and the mixture was extracted with 3 ml of ethyl acetate. The residual layer was further extracted once more in the same manner. The pooled ethyl acetate layer was washed three times with 1 ml of water and evaporated to dryness under nitrogen. The residue was dissolved in acetonitrile and an appropriate aliquot was used for reversed-phase HPLC analysis.

Normal-phase HPLC procedures

Normal-phase HPLC was performed on a stainless-steel column (250 × 4.6 mm I.D.) of Tosoh TSKgel silica 150 (5-μm particle size). The determinations of tanshinones and ferruginol was carried out under two sets of HPLC conditions. Elution was performed in an isocratic mode with two ratios of hexane to dioxane at a flow-rate of 1.0 ml/min throughout. HPLC system A for tanshinones employed

dioxane–hexane (8:92) and was monitored at 260 nm, whereas system B for ferruginol used dioxane–hexane (2.4:97.6) and was monitored at 285 nm. After use, the column was washed with dioxane–hexane (15:85) overnight.

Reversed-phase HPLC procedures

The reversed-phase column was made of stainless-steel (150 × 4.6 mm I.D.) and packed with a Tosoh TSKgel ODS-120T (5-μm particle size). The analytical procedures were performed with two HPLC systems, both of which utilized two solvents, (A) acetonitrile–0.01 *M* acetate buffer (pH 5.2) (20:80) and (B) acetonitrile. The flow-rate was maintained at 1.0 ml/min throughout. The qualitative and quantitative analysis of tanshinones was carried out with HPLC system C (see below), whereas the quantitative determination of tanshinone VI was accomplished by the use of system D following the pretreatment described above.

With system C, the elution programme was as follows: 0–3 min, 86% B; 3–20 min, linear change to 75% B; 20–30 min, 75% B (monitored at 275 nm). In order to prevent the peaks of tanshinone V and VI from broadening, the column was rinsed with 30 ml or more of 0.5% trifluoroacetic acid in acetonitrile prior to use.

With system D, the elution programme was as follows: 0–29 min, linear change from 14 to 33% B; 29–30 min, linear change to 90% B; 30–50 min, 90% B (monitored at 290 nm).

Partial synthesis of tanshinone VI

Dihydrotanshinone (169 mg) was dissolved in 1 ml of 1 *M* hydrochloric acid–50% methanol and heated for 1 h at 80°C. This reaction mixture was extracted with 4 ml of 5% aqueous sodium carbonate and washed three times with 1 ml of ethyl acetate. The residual layer was neutralized with 1 *M* hydrochloric acid and extracted three times with 1 ml of ethyl acetate. The pooled ethyl acetate extract was rinsed three times with 1 ml of water and evaporated to dryness under nitrogen. The residue was analysed using system C.

RESULTS AND DISCUSSION

Normal-phase HPLC

Table I gives the retention times (t_R) and capacity factors (k') of tanshinones and ferruginol standards in two systems with normal- and reversed-phase columns.

System A separated tanshinone IIA and I, cryptotanshinone and dihydrotanshinone I, but methylenetanshinquinone and 1,2-dihydrotanshinquinone eluted as a single peak (shown in Fig. 2). There was good linearity from 30 pmol to 10 nmol of tanshinone IIA and I, cryptotanshinone and dihydrotanshinone I with a correlation coefficient (r) of 0.999–1.000 with system A. In system A ferruginol emerged at 5.6 min, overlapping peaks corresponding to an impurity, and could not be detected. Pretreatment with Iatrobeads and the use of system B facilitated the detection of ferruginol (t_R = 12.0 min, k' = 2.00; shown in Fig. 3). A linear calibration graph for ferruginol with system B was obtained over the concentration range 0.1–100 nmol (r = 1.000). Tanshinones cannot be detected with system B.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS FOR TANSHINONES AND FERRUGINOL STANDARDS

Compound	System A		System C	
	t_R (min)	k'	t_R (min)	k'
Ferruginol	5.6	0.49		
Dehydromiltirone	6.4	0.71	22.2	12.21
Tanshinone IIA	7.3	0.95	23.4	12.93
Methylenetanshinquinone	8.2	1.19	22.2	12.21
1,2-Dihydrotanshinone	8.2	1.19	22.2	12.21
Tanshinone I	9.6	1.56	21.2	11.62
Cryptotanshinone	16.4	3.37	20.4	11.14
Dihydrotanshinone I	19.6	4.23	18.0	9.71
Tanshinone V			16.0	8.52
Tanshinone IIB			14.6	7.69
Tanshinone VI			13.0	6.74
Tanshindiol C			8.4	4.00
Tanshindiol B			6.8	3.05
Tanshindiol A			6.4	2.81

Reversed-phase HPLC

Tanshinone V and VI, tanshindiol A–C and tanshinone IIB, the polar tanshinones, cannot be detected using normal-phase HPLC. Therefore, we developed

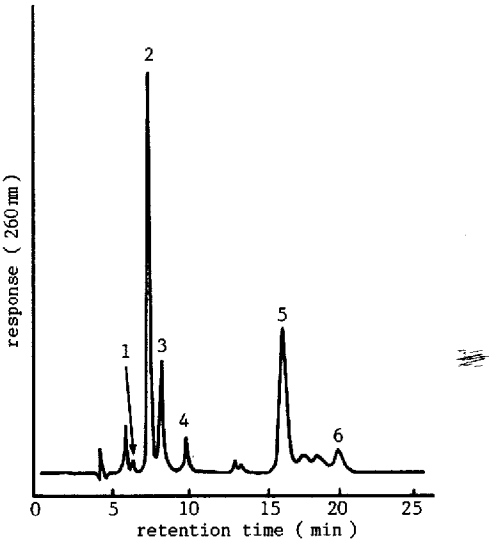

Fig. 2. Separation of tanshinones in the root of *S. miltiorrhiza* using system A. Peaks: 1 = dehydromiltirone; 2 = tanshinone IIA; 3 = methylenetanshinquinone and 1,2-dihydrotanshinquinone; 4 = tanshinone I; 5 = cryptotanshinone; 6 = dihydrotanshinone I.

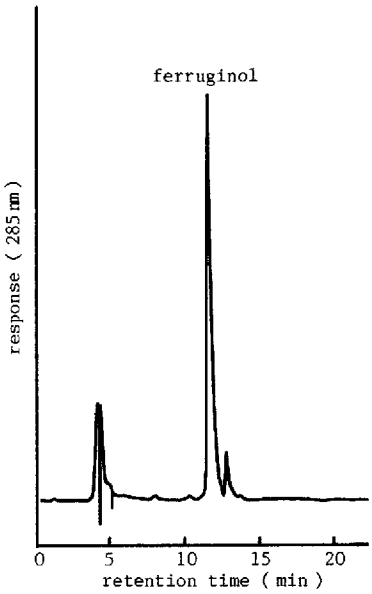

Fig. 3. Separation of ferruginol in Tan-Shen using system B.

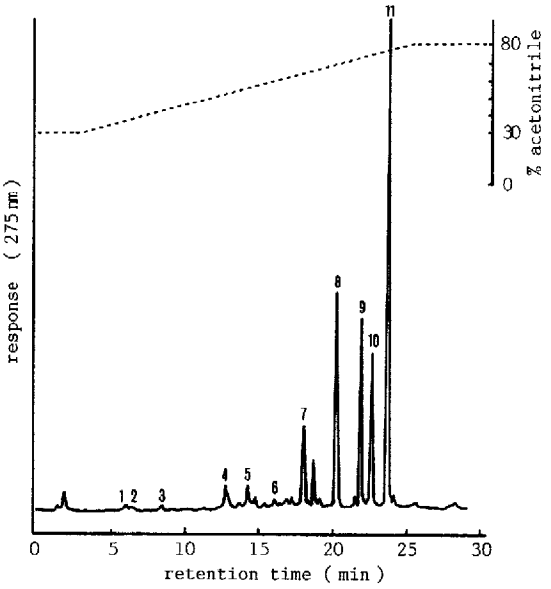

Fig. 4. Separation of tanshinones in Tan-Shen using system C. Peaks: 1 = tanshindiol A; 2 = tanshindiol B; 3 = tanshindiol C; 4 = tanshinone VI; 5 = tanshinone IIB; 6 = tanshinone V; 7 = dihydrotanshinone I; 8 = cryptotanshinone; 9 = tanshinone I; 10 = dehydromiltirone, methylenetanshinquinone and 1,2-dihydrotanshinquinone; 11 = tanshinone IIA.

a reversed-phase HPLC procedure by which the polar tanshinones can be studied both quantitatively and qualitatively.

System C resulted in the separation of most tanshinones, except dehydromiltirone, methylenetanshinquinone and 1,2-dihydrotanshinquinone, which completely overlapped each other (shown in Fig. 4). The calibration graphs for tanshinone V and VI and the less polar tanshinones (tanshinone IIA and I, cryptotanshinone, dihydrotanshinone I) in the concentration range 0.03–5 nmol followed a straight line (r = 0.999–1.000) with system C. Tanshindiol A–C and tanshinone IIB also yield distinct peaks, although the linear range was not examined.

Tanshinone VI is the most effective of the tanshinones in protecting the myocardium against ischaemia-induced derangements. It is difficult to measure tanshinone VI in tissues with system C, because it cannot be separated from impurities. It can be achieved, however, by using a sample pretreated with 5% aqueous sodium carbonate, even for low concentrations in tissues, with system D (t_R = 22.0 min, k' = 14.71), as shown in Fig. 5. Good linearity was obtained at levels of 0.1–2 nmol (r = 0.999).

We recommend system A for the less polar tanshinones and system C for the highly polar tanshinones. A sample containing tanshinones of high and low polarities can best be analysed by using a combination of both methods. Similar results were obtained with both systems A and C with the less polar tanshinones. The determination of tanshinone VI and ferruginol are achieved best with systems B and D, respectively.

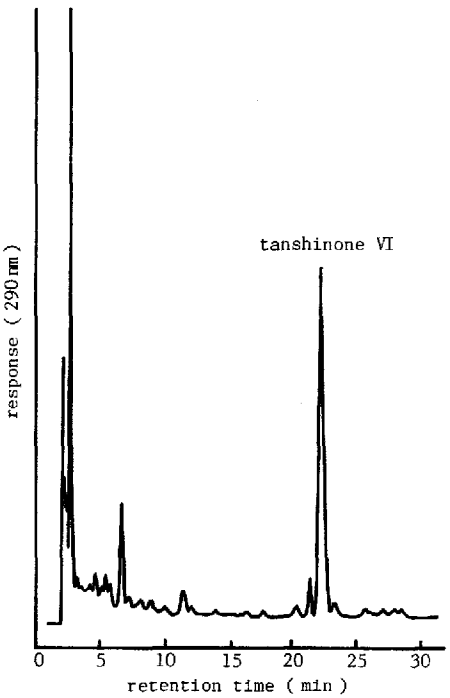

Fig. 5. Separation of tanshinone VI in the root of *S. miltiorrhiza* using system D.

TABLE II

RECOVERY AND PRECISION FOR TANSHINONES AND FERRUGINOL

Compound	System	Initial amount (μg)	Added (μg)	Recovery (%)[a]	Relative standard deviation (%)[b]
Ferruginol	B	108.1	66.0	97.1 ± 5.7	1.53
Tanshinone IIA	A	707.4	316.7	99.2 ± 0.9	0.53
Tanshinone I	A	116.6	55.6	96.5 ± 1.0	0.60
Cryptotanshinone	A	241.1	102.9	101.2 ± 2.7	0.54
Dihydrotanshinone I	A	46.3	18.5	96.3 ± 1.2	0.73
Tanshinone VI	D	22.5	9.6	89.8 ± 3.0	1.92

[a] Results are means ± standard deviation from three independent extractions.
[b] $n = 10$.

Recovery experiments

Recovery experiments were carried out by adding known amounts of tanshinones and ferruginol to the homogenate of regenerated plants. The mixture was extracted and assayed according to the above procedure. Table II summarizes the percentage recoveries and the statistical evaluation for tanshinones and ferruginol.

Determination of tanshinones and ferruginol

Ferruginol is found only in the roots of intact plants, as are tanshinones [10]. The contents of tanshinones and ferruginol in the roots of regenerated plant, the parent plant, water-cultured plant and Tan-Shen are given in Table III. One-year-old roots of regenerated plants contained 3–7 times more tanshinones than Tan-Shen, an amount similar to that in the parent plant (about 1 year old). This study on plant regeneration suggests useful and relatively rapid culture methods for the production of tanshinones; currently, commercial Tan-Shen is obtained from the roots of *S. miltiorrhiza* cultured for 4–5 years.

TABLE III

CONTENT OF TANSHINONES IN REGENERATED PLANT, PARENT PLANT, WATER-CULTURED PLANT AND TAN-SHEN DETERMINED USING SYSTEMS A AND B

Material	Concentration (% dry weight)				
	Tanshinone IIA	Tanshinone I	Cryptotanshinone	Dihydrotanshinone I	Ferruginol
Regenerated plant[a]	1.102	0.172	0.335	0.077	0.089
Parent plant[b]	1.524	0.141	1.141	0.104	0.117
Water-cultured plant[c]	0.011	0.007	0.018	0.015	0.012
Commercial Tan-Shen	0.150	0.050	0.050	0.025	0.074
Callus[d]	0.116	0.034	0.106	0.037	0.052

[a] Cultured in Tsukuba since May 1988 and harvested in June 1989.
[b] Cultured in Tsukuba and harvested in November 1987 (about 1 year old).
[c] Cultured for 3 months.
[d] Obtained by adventitious root culture.

TABLE IV

CONTENT OF TANSHINONES IN REGENERATED PLANT AND PARENT PLANT BY SYSTEMS A AND B

Material	Concentration (% fresh weight)				
	Tanshinone IIA	Tanshinone I	Crypto-tanshinone	Dihydro-tanshinone I	Ferruginol
Regenerated plant[a]:					
Nov. 1988[b]	0.31	0.04	0.07	0.01	0.06
Jan. 1989[b]	0.14	0.02	0.03	0.01	0.04
Parent plant[c], Tsukuba	0.48	0.04	0.36	0.03	0.12

[a] Cultured in Tsukuba since May 1988.
[b] Harvest time.
[c] Cultured in Tsukuba and harvested in November 1988 (about 1 year old).

Table IV shows a comparison of the contents of the tanshinones and ferruginol in the roots of regenerated plants harvested at different times. The content in tissue harvested in November was approximately twice that in tissue harvested in January. It appears that the production of tanshinones and ferruginol is greatly influenced by the season.

Determination and partial synthesis of tanshinone VI

Tanshinone VI is one of the most effective components of *S. miltiorrhiza*, as described previously. It is difficult to isolate sufficient for analysis because of the low levels found in various sources (Table V). Therefore, we studied the partial synthesis of tanshinone VI from dihydrotanshinone I with the use of system C. Dihydrotanshinone I was treated with 1 *M* hydrochloric acid in 50% methanol for 1 h at 80°C, giving a yield of tanshinone VI of 69.5%.

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TABLE V

CONTENT OF TANSHINONE VI IN COMMERCIAL TAN-SHEN, FRESH ROOT AND CALLUS DETERMINED USING SYSTEM D

Material	Concentration (% dry weight)
Fresh root[a]	0.0034
Callus[b]	0.0018
Commercial Tan-Shen	0.0072

[a] Cultivated in Fukuyama and harvested in November 1988.
[b] Obtained by adventitious root culture.

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