Journal of Food and Drug Analysis

Volume 14 | Issue 3 Article 9

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Recommended Citation
Don, M.-J.; Ko, H.-C.; Yang, C.-W.; and Lin, Y.-L. (2006) "Detection of polyphenols and tanshinones in commercial Danshen by liquid chromatography with UV and mass spectrometry," *Journal of Food and Drug Analysis*: Vol. 14 : Iss. 3 , Article 9.
Available at: https://doi.org/10.38212/2224-6614.2473

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Journal of Food and Drug Analysis, Vol. 14, No. 3, 2006, Pages 254-259 藥物食品分析 第十四卷 第三期

Detection of Polyphenols and Tanshinones in Commercial Danshen by Liquid Chromatography with UV and Mass Spectrometry

MING-JAW DON[1,2], HAN-CHIEH KO[1], CHENG-WEI YANG[2] AND YUN-LIAN LIN[1,*]

[1] *National Research Institute of Chinese Medicine, Taipei City 112, Taiwan, R.O.C.*
[2] *Department of Chemistry, Chinese Culture University, Taipei City 111, Taiwan, R.O.C.*

(Received: January 25, 2006; Accepted: May 24, 2006)

ABSTRACT

Danshen, the dried rhizome of *Salvia miltiorrhiza* Bunge is a widely used herb in traditional Chinese medicinal preparations to treat cardiovascular diseases, renal and liver diseases. Tanshinones and salvianolic acids are used as markers to survey the commercial available products. In this study, a convenient high-performance liquid chromatographic method was developed to simultaneously separate and identify six main polyphenolic components, caffeic acid (**1**), danshensu (**2**), lithospermic acid (**3**), rosmarinic acid (**4**), salvianolic acid B (**5**) and salvianolic acid A (**6**), and four major abietane-type diterpenes, 15,16-dihydrotanshinone I (**7**), cryptotanshinone (**8**), tanshinone I (**9**), and tanshinone IIA (**10**), with UV and MS detectors by comparing their retention time, MS and MS^2 data with those data obtained from the authentic compounds. A good resolution of all the analytes was obtained by choosing methanol and 0.25% aqueous acetic acid (adjusted to pH 2.77 with 1N HCl) as the mobile phase. By using LC-UV (290 nm)-MS method, polyphenols (**1-6**) and tanshinones (**7-10**) were easily detected and unambiguously identified in commercial Danshen.

Key words: Danshen, *Salvia miltiorrhiza*, LC-UV-MS, polyphenols, tanshinones, chemical profile.

INTRODUCTION

Danshen, the dried rhizome of *Salvia miltiorrhiza* Bunge has been widely used in traditional Chinese medicinal combination for the treatment of various diseases[1-2]. Both lipid-soluble abietane-type diterpenoid tanshinones and water-soluble polyphenols in Danshen were considered its major and effective components for coronary arteries, inducing apoptosis on cancer cell line, liver protective effect, and preventing lipid peroxidation[3-15]. In earlier studies, high-performance liquid chromatography (HPLC) with UV[16] and various types of high-speed counter-current chromatography were developed for the separation and purification of tanshinones and salvianolic acid B[17-20], and non-aqueous capillary electrophoresis was used for tanshinone analysis[21]. A liquid chromatography-mass spectrometry (LC-MS) was used to characterize tanshinones[22] and polar components of *S. miltiorrhiza*[23]. Recently, HPLC and LC-MS-MS was developed to investigate multi-components of *S. miltiorrhiza*[24-25]. However the formic acid-containing mobile phase could not satisfactorily resolve the peaks of polyphenols. Furthermore, the materials of Danshen were incorrectly used due to the vast number and poor identification knowledge among *Salvia* species. Therefore, it is worthwhile to simultaneously identify both polyphenols and tanshinones in commercial available Danshen. The aim of this study is to develop a reproducible method for simultaneous analysis of lipophobic and lipophilic constituents in Danshen and to establish chemical profiles of commercial products.

* Author for correspondence. Tel: +886-2-28201999 ext. 6531; Fax: + 886-2-2825-0743; E-mail: yllin@nricm.edu.tw

MATERIALS AND METHODS

I. *Instruments and Materials*

HPLC analysis was carried out using a quaternary pump (SpectraSYSTEM P4000) equipped with an autosampler (AS 3000) and an UV detector (UV 2000, ThermoQuest-Finnigan Co., San Jose, CA, USA). Ten authentic compounds were separated on a C18 reversed-phase column (Inertsil ODS-3, 250 × 4.6 mm, 5 μm) at ambient temperature. Mass spectra were obtained using an ion trap mass spectrometer (LCQ, Finnigan MAT) equipped with an electrospray ion source. Caffeic acid was purchased from Acros Organics (Geel, Belgium). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Water was purified with Milli-Q system (Millipore, Bedford, MA, USA).

Slices of Danshen imported from mainland China were purchased from local Chinese herb retailer in Taipei. Sample was identified by comparison with the voucher specimen deposited earlier at the Herbarium of the National Research Institute of Chinese Medicine. For analysis, 1g of sample was sonicated with 15 mL of aqueous methanol (80% methanol/H_2O, v/v) for 30 min. After filtration and concentration, a total volume of 5.0 mL was made with methanol and then filtered with a 0.45 μM

PTFE syringe filter. An aliquot of the filtrate (20 μL) was directly injected into the analytical system.

II. *HPLC and ESI-MS Conditions*

The mobile phase consisted of (A) 0.25% aqueous acetic acid (adjusted to pH = 2.77 with 1N HCl) and (B) methanol using a gradient elution of 40–80% B at 0-25 min, 80% B at 25-30 min, 80-100% B at 30-50 min, 100% B at 50-60 min. The flow rate was set at 0.5 mL/min and the absorbance was measured at 290 and 254 nm. The injection volume was 20 μL. The mass spectrometric data were acquired in the negative ion mode for 0-30 min and positive ion mode for 30-60 min. The full scan mass spectrum was recorded over the range of *m/z* 100-1000. The conditions for ESI spectra were as follows: spray voltage, 4.5 kV; capillary voltage, 10 V; capillary temperature, 270ºC; sheath gas (N_2), 80 units; auxiliary gas (N_2), 20 units. Acquisition and processing of data from the mass spectrometer was performed using the Xcalibur software revision 1.0 (ThermoQuest-Finnigan Co., San Jose, CA, U.S.A.).

III. *Preparations of Reference Sample*

Danshensu **(2)**, lithospermic acid **(3)**, rosmarinic acid **(4)**, salvianolic acid B **(5)** and salvianolic acid A **(6)** were isolated from the aqueous methanol extract of *S. miltiorrhiza* by Diaion HP-20 and Sephadex LH-20 column chromatography. 15,16-Dihydrotanshinone I **(7)**, cryptotanshinone **(8)**, tanshinone I **(9)**, and tanshinone IIA **(10)** were isolated from the ethyl acetate extract of Danshen by silica gel column chromatography. Their structures were characterized by MS, 1D- and 2D-NMR spectra analyses and were compared with that from literature data[13,23,26-27]. A methanol stock solution of

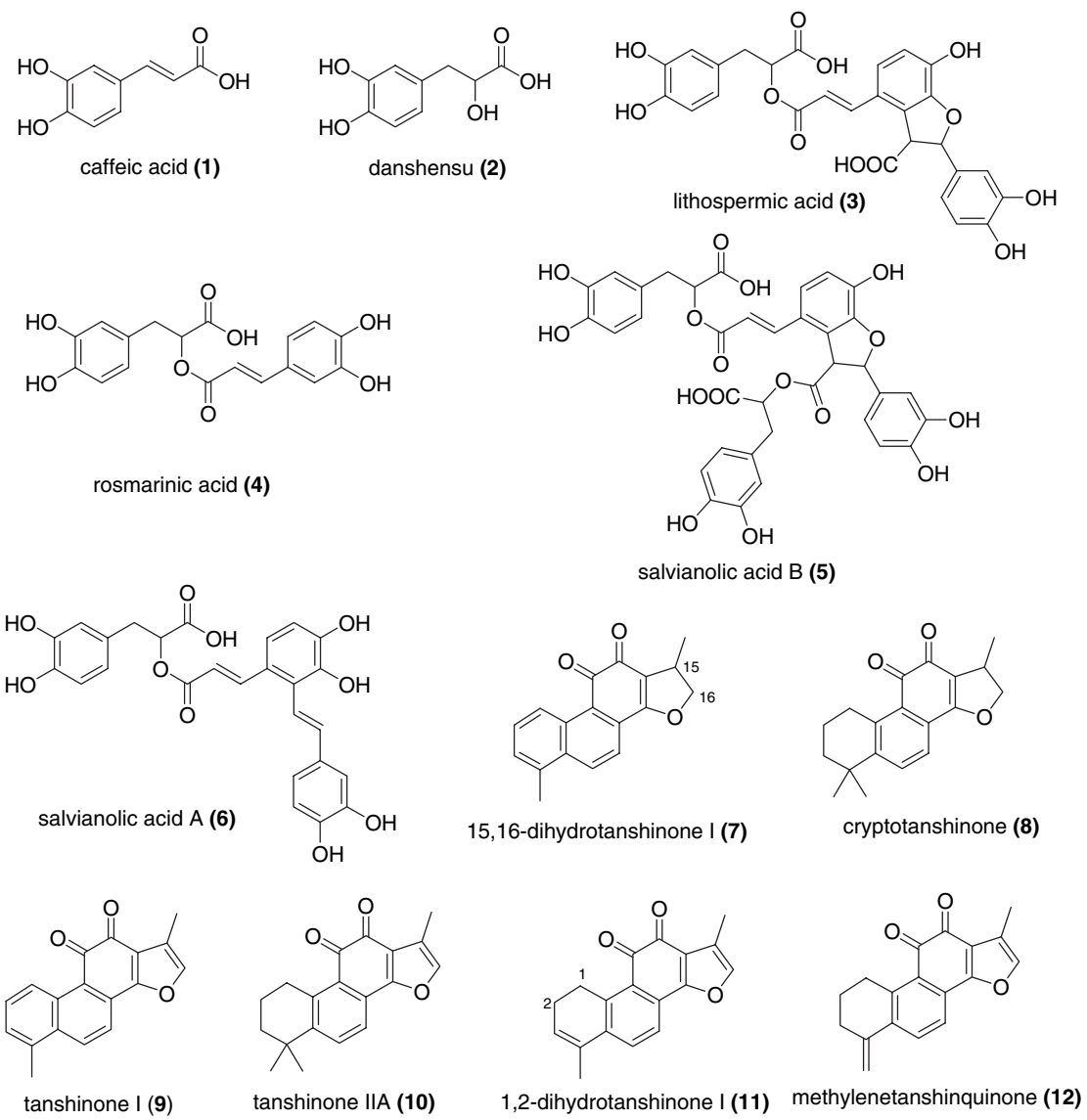

Figure 1. The structures of polyphenols and tanshinones.

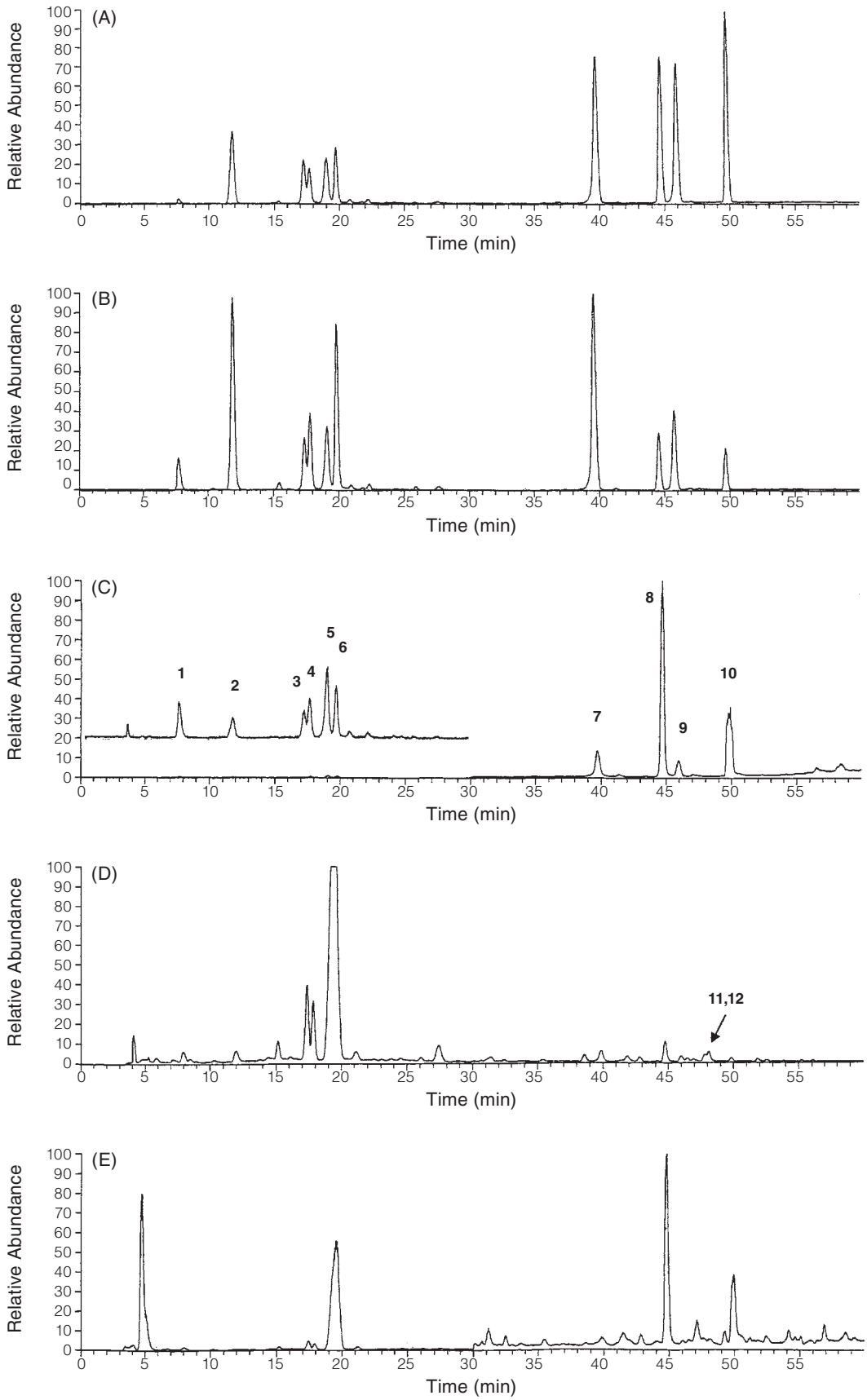

Figure 2. (A) UV chromatogram of ten authentic compounds at 254 nm, (B) UV chromatogram of ten authentic compounds at 290 nm, (C) Total ion current chromatogram (TIC) of ten authentic compounds, (D) UV chromatogram of 80% methanolic extracts of Danshen at 290 nm, (E) TIC of 80% methanolic extracts of Danshen.

Journal of Food and Drug Analysis, Vol. 14, No. 3, 2006

Table 1. Retention time (tR), MS data, MSn data for the main constituents in commercially available Danshen

Compound	t_R (min)	$[M-H]^-$ (*m/z*)	$[2M-H]^-$ (*m/z*)	$[M-H-CO_2]^-$ (*m/z*)	$[M+Cl]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	$[2M+Na]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	identity
1	7.97	197	395		233, 235			179	135	Danshensu
2	12.00	179			214, 216			135		Caffeic acid
3	17.40	537		493				493	295	Lithospermic acid
4	17.88	359	719		395, 397			161		Rosmarinic acid
5	19.23	717						519	321	Salvianolic acid B
6	19.83	493	987					295	277	Salvianolic acid A
7	39.87					279	579	261	233	15,16-Dihydrotanshinone I
8	44.76					297	615	279	251	Cryptotanshinone
9	45.99					277	575	249	221	Tanshinone I
10	49.83					295	611	277	249	Tanshinone IIA
11 or 12	47.76 48.10					279 279		261 261		Methylenetanshinquinone or 1,2-Dihydrotanshinone I

all reference compounds were prepared by dissolving 2 mg of each compound in 1 mL of methanol and 10 μL of each compound was subjected to LC-UV-MS analysis for comparison.

RESULTS AND DISCUSSION

Hu *et al.*[24] recently used HPLC-DAD-ESI MS to determine diterpenes, flavonolignans and phenolic compounds of Danshen. In addition to protocatechualdehyde and danshensu, other major polyphenols, such as salvianolic acids A, B, rosmarinic acid, were not mentioned in that paper. Zhao *et al.*[25] developed LC/MS/MS method to determine multi-components in Danshen as well. However, the peaks of polyphenols could not be well resolved in the above studies using formic acid-containing mobile phase. In order to obtain good resolution of hydrophilic and hydrophobic components in a run, 0.25% acetic acid and pH 2.77 was found satisfactory to establish the fingerprint of Danshen in this study.

Our preliminary experiments suggested that chromatographic separation of polyphenols depended on acid concentration. At a pH value higher than 3, peak tailing and bad resolution of polyphenols were observed. On the other hand, at pH value below 3, all the analytes eluted within 60 min gave satisfactory resolutions except lithospermic acid **(3)** and rosmarinic acid **(4)** which partially overlapped. Therefore, methanol and 0.25% aqueous acetic acid (adjusted to pH 2.77 with 1N HCl) were chosen as the mobile phase. A complete HPLC-UV profile obtained with the standard solution is shown in Figure 2A (detection at 254 nm) and Figure 2B (detection at 290 nm). Polyphenols and tanshinones could be detected at both 254 nm and 290 nm. However, compound **1** exhibited a very low absorption at 254 nm compared to others. A better absorption for all polyphenols of Danshen was found at 290 nm. Thus, 290 nm was used for the fingerprinting of Danshen products.

In previous studies, the mass detection with negative mode were suitable for polyphenols and deprotonated molecule ions $[M-H]^-$[20]. In contrast, the mass detection with positive mode were suitable for tanshinones and protonated molecule ions $[M+H]^+$[22]. On the basis of above information, a complete LC-ESI-MS profile obtained with the standard solution is shown in Figure 2C (total ion current chromatogram), indicating that authentic polyphenols appeared before 30 min and tanshinones appeared after 30 min using a 60 min single run. The mass abundance of polyphenols was lower than that of tanshinones, which is probably due to the acidic mobile phase favor for forming protonated molecule ion. Enlargement of Y-axis of the TIC from 0 to 30 min was shown in Figure 2C (upper layer) for better comparison. In the mass spectra of all authentic polyphenols, deprotonated molecular ions $[M-H]^-$ were observed as predominant peaks. Furthermore, deprotonated dimer molecular ions $[2M-H]^-$ were also evident for danshensu **(2)**, rosmarinic acid **(4)**, and salvianolic acid A **(5)**. In addition, adduct with chlorine ions $[M+Cl]^-$ appeared at *m/z* 233 and 235 for danshensu , at *m/z* 214 and 216 for caffeic acid, and at *m/z* 395 and 397 for rosmarinic acid. A fragment ion corresponding to the loss of CO_2 $[M-H-CO_2]^-$ was found at *m/z* 493 with relatively high intensity in the mass spectrum of lithospermic acid. In the mass spectra of all authentic tanshinones, protonated molecular ions $[M+H]^+$ were observed as predominant peaks. Adducts with sodium ion $[2M+Na]^+$ were also observed in a relatively low intensity.

The fragment ions (MS^n) of authentic compounds were also carried out through flow injection to ensure the structural identification of polyphenols and tanshinones. The corresponding retention time (t_R), MS, and MS^n data are summarized in Table 1. Fragment ions corresponding to the loss of CO_2 *m/z* 44 were found for caffeic acid **(1)**,

danshensu **(2)**, and lithospermic acid **(3)**, respectively. A fragment ion corresponding to the loss of danshensu **(2)** *m/z* 198 was found for lithospermic acid, rosmarinic acid, salvianolic acid B, and salvianolic acid A, respectively. Furthermore, two units of danshensu **(2)** (717 → 519 → 321) were found for salvianolic acid B on the basis of the MS^3 fragment experiment. The results of the MS^2 and MS^3 experiments for tanshinone IIA **(10)**, cryptotanshinone **(9)**, and 15,16-dihydrotanshinone I **(7)** indicated a loss of water and a carbonyl group, respectively. The information of MS^n of authentic polyphenols and tanshinones could provide useful information for unambiguous determination of those compounds in Danshen.

The HPLC-UV (290 nm)-MS total ion current chromatogram (TIC) of 80% methanolic extracts of *S. miltiorrhiz* are shown in Figure 2D and 2E, respectively. By comparing the retention times and mass values, polyphenols **(1-6)** and tanshinones **(7-10)** were detected and unambiguously identified. Among them, the content of salvianolic acid B was relatively high, and overlapped with salvianolic acid A. In addition, some minor peaks were present in both UV and TIC profiles. The mass spectra of two overlapped peaks at the retention time 47.76 and 48.10 min were identical and showed $[M+H]^+$ ion at *m/z* 279, which were assignable to a mixture of methylenetanshinquinone and 1,2-dihydrotanshiquinone I (**11** and **12**) according to literature[28]. Furthermore, the fragmentation pattern of methylenetanshinquinone and 1,2-dihydrotanshiquinone I was similar to that of 15,16-dihydrotanshinone I. On the basis of relative abundance of chromatogram (Figure 2D and 2E), polyphenolic compounds detected by using UV detector were more obvious than that using MS detector under this condition. Tanshinones detected by MS detector were better than that using UV detector. Further studies will be carried out to identify other minor peaks.

In conclusion, the reverse phase liquid chromatography coupled with on-line UV and ESI-MS provides a convenient method for the separation and identification of both polyphenols and tanshinones of Danshen in a single run. This analytical method can be considered a suitable analytical method for the quality control of Danshen and can be applied to the analysis of polyphenols and tanshinones in Danshen preparation.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Health of the Republic of China for the financial support (CCMP92-CT-04).

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