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Research Article

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Simultaneous quantitative determination of salvianolic acid B and tanshinon IIA in the *Salviae miltiorrhizae Radix et Rhizoma* semisolid extracts by HPLC

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Abstract An HPLC method was developed for the simultaneous quantification of salvianolic acid B (Sal-B) and tanshinone IIA (Tan-IIA) in the *Salviae miltiorrhizae Radix et Rhizoma* semisolid extracts (SME), which had been prepared from *Salviae miltiorrhizae Radix et Rhizoma* by hot aqueous-alcoholic extraction. The chromatography was established using a C_{18} (250 x 4.6 mm; 5 µm) column (set at 20 ^{o}C) and a UV detector set at 270 nm; The mobile phase consisted of acetonitrile - 0.1% phosphoric acid solution (gradient program, flow rate of 1 mL/min); Injection volume was 10 µL. The method was validated according to the AOAC requirement of specificity, linearity (ranging of Sal-B and Tan-IIA from 29.96 to 958.63 µg/mL and 1.02-32.53 µg/mL, respectively), precision and accuracy. Using this method to analyse some SME samples showed the content of Sal-B and Tan-IIA ranging from 7.16% to 7.72% and 0.011% to 0.785%, respectively.

Keywords *Salvia miltiorrhiza, Salviae miltiorrhizae Radix et Rhizoma* semisolid extracts, salvianolic acid B, tanshinon IIA, HPLC

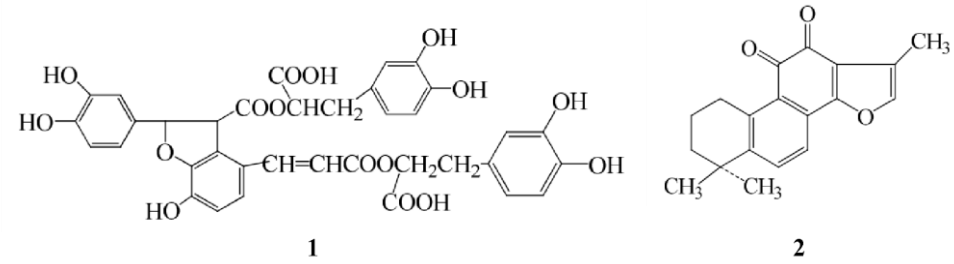
Introduction

Salviae miltiorrhizae Radix et Rhizoma (SMR) is the dried root and rhizome (collected in spring or autumn [1]) of *Salvia miltiorrhiza* Bunge (Lamiaceae family). SMR has been used for the treatment of cardiovascular and cerebrovascular diseases in Asia, the United States, and other European countries [2]. The SMR extracts (extracted with various solvents) have been reported to have therapeutic effects on a variety of diseases including cardiovascular diseases [3], liver diseases [4], nervous system diseases [5].

The active components of the SMR are divided into two groups by chemical and pharmacological investigations: One group is water-soluble phenolics containing salvianolic acid A (Sal-A), salvianolic acid B (Sal-B), lithospermic acid, rosmarinic acid, and danshensu [6]; The other group is lipophilic tanshinones containing tanshinone I, tanshinone IIA (Tan-IIA), tanshinone IIB, cryptotanshinone, and dihydrotanshinone I [7]. Salvianolic acids (including Sal-B), the most abundant compounds from *Salvia miltiorrhiza*, are known to exhibit diverse biological activities such as antioxidant [8], antithrombotic [9], inhibit lipid uptake, anti-atherosclerotic [10], and cardioprotective activities [11], while tanshinones show cardioprotective [12], neuroprotective, antitumor activities [13]. Sal-B and Tan-IIA are both considered by Chinese Pharmacopoeia 2015 [1] and Vietnamese Pharmacopoeia 2017 [14] to be marker compounds for the quality control of the SMR. The *Salviae miltiorrhizae Radix et Rhizoma* semisolid extracts (SME), prepared from the SMR by hot aqueous-alcoholic extraction method (to use in preparation of pharmaceutical products), could then have the same active compounds as in SMR.

Up until now, the quantitative determination of the active constituents in the SMR had been focused only on hydrophilic or lipophilic compounds [1], [14-16]. There were only some reports on the simultaneous

quantitative of Sal-B and Tan-IIA in SMR [17] but not any in SME. However, a new, simple, yet reliable HLPC method to simultaneously assay both Sal-B and Tan-IIA (figure 1) had been developed in this article.



1 **2**

Figure 1: Chemical structure of Sal-B (1) and Tan-IIA (2)

Materials and Methods

Materials

SMR conformed to the Chinese Pharmacopoeia 2015 [1] was supported by VCP Pharmaceutical joint stock Company, Hanoi, Vietnam. SME was extracted from SMR (sliced to 2-4mm thick) at 80 ^{o}C using ethanol-water as solvent, followed by solvent evaporation at low pressure then at 80 ^{o}C until semisolid form (humidity < 20%).

The Sal-B ($C_{36}H_{30}O_{16}$, 99.34%, Lot No. 200105) and Tan-IIA ($C_{19}H_{18}O_3$, 98.09%, Lot No. 191206) as reference substances were from Xinyang Zhongjian Metrology Biological Technology Co., Ltd. (China). Other chemical substances and HPLC solvents (methanol, acetonitril, phosphoric acid) were from Merck (Germany).

The HPLC analysis was carried out on a Shimadzu Corporation (Japan) equipped with a low-pressure gradient pump (LC-30AD), DAD detector (SPD-M20A), Autosample (SIL-20A), column oven (CTO-10AS). All data acquired were proceeded by LabSolutions software (Shimadzu Japan).

Methods

Sample preparation

The *soluble solvent* was the most suitable for extraction (possible to extract the highest content) of Sal-B and Tan-IIA from a SME sample, determined by comparing the analyzed results obtained from the test solutions prepared separately with 30%, 50%, 70%, and 100% of methanol as soluble solvent.

Standard solutions were the solutions of Sal-B and Tan-IIA (reference substances) in soluble solvent with the concentration ranging from 29.96 to 958.63 ⌈g/mL and 1.02 to 32.53 ⌈g/mL, respectively.

Test solution was prepared as follows: 25.0mL of soluble solvent was added into a stoppered conical flask contained about 0.1g (exact mass) of SME. The total mass was weighted and replenished (by soluble solvent) after an ultrasonication of 30 minutes. The obtained solution was mixed, filtered through a 0.45⌈m filter to give the test solution.

Chromatographic condition

Chromatographic separation was performed using a Shim-pack GIST C_{18} (250 x 4 mm, 5 µm) column. The mobile phase consisted of acetonitril (A) and 0.1% solution of phosphoric acid (B). The gradient program was as follows: 0-20 min, 26% A; 20-21 min, 26-86% A; 21-35 min, 86% A; 35-36 min, 86-26% A; 36-40 min, 26% A. The mobile phase was delivered to the column at the flow rate of 1.0 mL/min. 10 µL of samples were injected into the HPLC system for analysis.

The wavelength detection was determined by analysing the UV-Vis spectra of the peaks corresponding to the Sal-B and Tan-IIA in the chromatographies of standard solution. The column temperature and gradient program were chosen to obtain a good separation.

Method validation

The quantification procedure was validated according to the AOAC [18] and ICH [19] guidelines. The *selectivity* and *specificity* were carried out by comparison the retention time and UV-vis spectra of principal

peaks on the chromatographies of placebo, sample, Sal-B and Tan-IIA standard solutions. The *system suitability* was evaluated based on the %RSD < 2 of retention time and peak area obtained from 6 injections of a standard solution contained 239.66 µg/mL of Sal-B and 8.13 µg/mL of Tan-IIA. The *linearity* of the method was evaluated by analyzing a series of 6 standard solutions of Sal-B and Tan-IIA ranging from 29.96-958.63 µg/mL and 1.02-23.53 µg/mL respectively. The correlation coefficient (r) must be not less than 0.999. The *repeatability* (intra-day) and *intermediate precision* (inter-day) were obtained by recovery study of the test sample (n = 6) on the same day and on 2 different days, respectively. The relative standard deviation (RSD) must be not over 2.7%. The *accuracy* was determined by recovery of known amounts (equivalent to 75%, 100% and 150% of sample concentration) of Sal-B and Tan-IIA standard added to the working sample solution. The recovery had to be 97.0-103.0% for the content of Sal-B and 95.0-105.0% for the content of Tan-IIA.

Application

The validated method was used to evaluate the quality of some SME samples.

Results and Discussion

Choosing of soluble solvent

Table 1 presented the Sal-B and Tan-IIA content obtained from different solvent.

Table 1: Sal-B and Tan-IIA content extracted from different solvent.

TT	Solvent (% methanol in water)	Content of Sal-B (%)	Content of Tan-IIA (%)
1	30%	7.62	0.014
2	50%	7.39	0.132
3	70%	7.49	0.297
4	100%	7.49	0.302

The results showed that with the increase of methanol concentration from 30% to 70%, the Sal-B content changed trivial, while the Tan-IIA content was increased significantly. Compared to 100% methanol, with 70% methanol as soluble solvent, the Sal-B content kept the same, and the Tan-IIA content changed less than 2%. Therefore, 70% methanol was selected as the soluble solvent.

Chromatographic condition

The spectrum in the range of 220-350 nm of Tan-IIA standard solution (Figure 2-b) showed an absorption maximum at 270 nm, while Sal-B spectrum (Figure 2-a) showed an absorption minimum at the same wavelength. Through the survey, the Tan-IIA content is much lower than the Sal-B content in SME samples, so the 270 nm was chosen to determine both compounds.

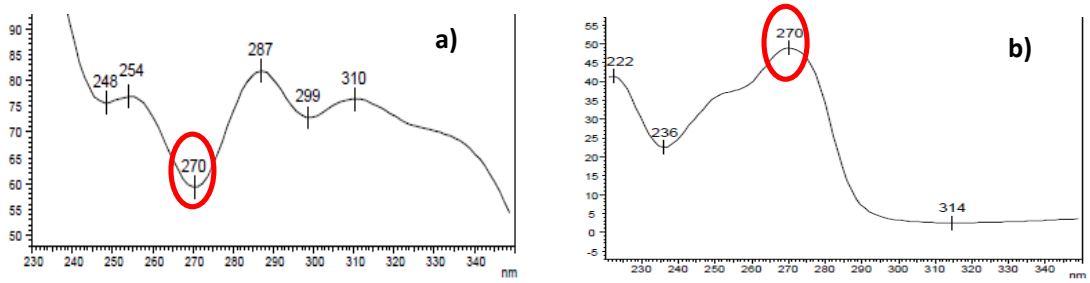


Figure 2: UV-vis spectra of Sal-B (a) and Tan-IIA (b)

After several test, 20 ^{o}C was selected as column temperature, and the gradient program was fixed as follows: 0-20 min, 26% A; 20-21 min, 26-86% A; 21-35 min, 86% A; 35-36 min, 86-26% A; 36-40 min, 26% A.

Method validation

Selectivity and specificity

Figure 3 showed the chromatographies of 4 solutions: soluble solvent, standard, test sample and test sample added standard solution.

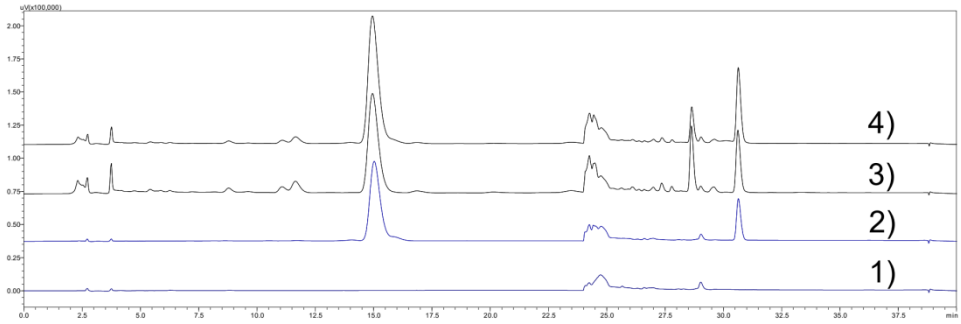


Figure 3: Chromatographs of solutions

1) soluble solvent, 2) Sal-B and Tan-IIA standard, 3) test sample, 4) test sample + standard

In figure 3, 2 well-separated peaks (at 15 min and 31 min) on the chromatographies (2 and 4) of test sample and test sample added standard solution appeared at the same retention times (Rt) of the peaks on the chromatography of standard solution (1) showed these peaks correspond to Sal-B and Tan-IIA. The soluble solvent chromatography without any peak at Rt of Sal-B and Tan-IIA proved that soluble solvent was not influent to the analysis.

On the other hand, the UV-Vis spectra of the sample and standard peaks (figure 4) showed a superposition coefficient of 1.0000 and 0.9970 (> 0.99) for Sal-B and Tan-IIA, respectively confirmed the identification of these peaks. Therefore, the selectivity and specificity of this method were ensured.

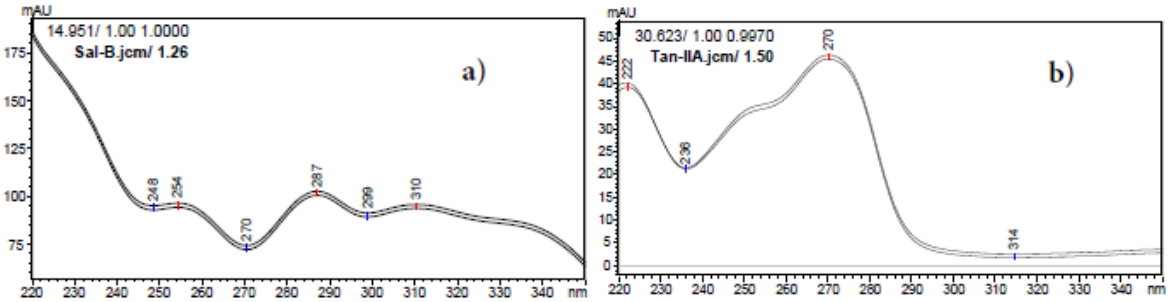


Figure 4: Spectra of the sample and standard samples Sal-B (a) and Tan-IIA (b)

System suitability

Table 2 showed the results of 6 injections from one standard solution.

Table 2: System suitability (n=6)

Compounds	**Sal-B**		**Tan-IIA**	
	Rt (min)	Peak area (mAU.s)	Rt (min)	Peak area (mAU.s)
Mean	15.030	1930732	30.647	402251
RSD (%)	0.16	0.24	0.02	0.18

The relative standard deviations (RSD) of the peak area and retention time of both Sal-B and Tan-IIA were less than 2%, conformed to the requirement of AOAC.

Linearity

The results presented in Table 3 and Figure 5 showed that there was a linear dependence between peak area and concentration with correlation coefficient r = 0.9999 for Sal-B and r = 0.9998 for Tan-IIA in the investigated concentration range. Thus, the standard curves were built with high linearity ensuring to perform the quantitative analysis of Sal-B and Tan-IIA.

Table 3: Quantitative linear range of Sal-B and Tan-IIA

Compounds	Sal-B	Tan-IIA
Calibration equation	y = 8116.3x - 33842	y = 54300x - 14671
r	0.9999	0,9998
Linear ranges (µg/mL)	29.96-958.63	1.02-32.53

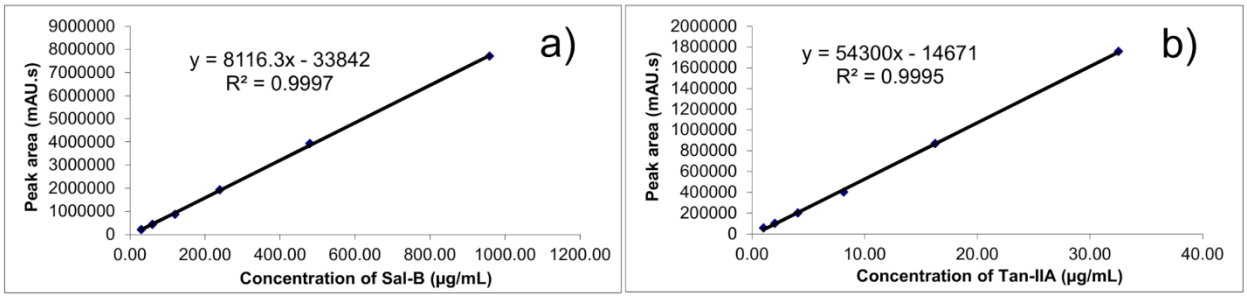


Figure 5: The calibration curves of Sal-B (a) and Tan-IIA (b)

Precision

The results presented in Table 4 showed that the method has high precision, the RSD values of intra-day and inter-day of quantification were < 2.7%, conformed to the requirement of AOAC.

Table 4: Results of intra-day and inter-day precisions

Compounds	Sal-B		Tan-IIA	
	Intra-day (n=6)	Inter-day (n=12)	Intra-day (n=6)	Inter-day (n=12)
Mean (%)	7.41	7.40	0.29	0.29
RSD (%)	0.68	0.50	0.67	0.62

Accuracy

The results presented in Table 5 showed that Sal-B recovery rates at each concentration range were in the range of 97-103% and Tan-IIA recovery rates at each concentration were in the of 95-105%, demonstrated the selected HPLC method to ensure the correctness for the quantification of Sal-B and Tan-IIA.

Table 5: Recovery of Sal-B and Tan-IIA (n=3)

Compounds	Sal-B			Tan-IIA		
Spiked (µg/mL)	62.57	125.13	250.26	2.82	5.63	11.26
Found (µg/mL)	62.38	124.45	251.14	2.73	5.42	10.91
Recovery (%)	99.71	99.69	100.35	97.14	96.26	96.90
RSD (%)	0.19	0.30	0.86	0.20	0.30	1.03

In general, the validation results had proved that this method was conformed to the requirements of AOAC for Standard method performance [18], and could be applied into quality control procedure of semisolid extract of SMR. It was an avantage in the fact of without previous research mentioned to this problem (simultaneously assay both Sal-B and Tan-IIA in SME). In SMR, Li-lan Lu *et al.* [17] used an almost similar Rp-HPLC method with a gradient programe of acetonitrile and phosphoric acid to analyze 5 compounds at the same injection. However, this method was realise at a different temperature (30 ^{o}C) and using 0.5% phosphoric acid with a complicated (9 steps) gradient program - more difficult than our method (20 ^{o}C, 0.1% phosphoric acid and 5 steps gradient program). Anyways, both methods were accurate, precise, and reproducible.

Method application

The validated method was applied to determine the contents of Sal-B and Tan-IIA in 6 semisolid extracts of *Salviae miltiorhizae Radix et Rhizoma*. The results showed a range of 7.08% to 7.63% and 0.011% to 0.785% for Sal-B and Tan-IIA, respectively (Table 6).

Table 6: Sal-B and Tan-IIA contents in SME

Sample	Sal-B content (%)	Tan-IIA content (%)
SME1	7.16	0.011
SME2	7.72	0.033
SME3	7.33	0.121
SME4	7.41	0.296
SME5	7.60	0.331
SME6	7.52	0.785

Table 6 showed an abundant amount of Sal-B in comparison to Tan-IIA. This result was the same as reported in the Chinese Pharmacopoeia [1].

All these samples were prepared from the same lot of raw material by six different procedures. The results showed the difference of content of Tan-IIA in these samples and proved that the best procedure was the 6th which collected the biggest amount of Sal-B (7.52%) and Tan-IIA (0.785%).

Conclusion

A simple, reliable, and accurate HPLC assay method for simultaneous determination of the Sal-B and Tan-IIA in the *Salviae miltiorhizae Radix et Rhizoma* semisolid extracts was successfully established. The contents of Sal-B and Tan-IIA in the *Salviae miltiorhizae Radix et Rhizoma* semisolid extracts ranged from 7.08% to 7.63% and 0.011% to 0.785%, respectively.

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