Analysis of active components in *Salvia miltiorrhiza* injection based on vascular endothelial cell protection

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Correlation analysis based on chromatograms and pharmacological activities is essential for understanding the effective components in complex herbal medicines. In this report, HPLC and measurement of antioxidant properties were used to describe the active ingredients of *Salvia miltiorrhiza* injection (SMI). HPLC results showed that tanshinol, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, protocatechuic acid and their metabolites in rat serum may contribute to the efficacy of SMI. Assessment of antioxidant properties indicated that differences in the composition of serum powder of SMI caused differences in vascular endothelial cell protection. When bivariate correlation was carried out it was found that salvianolic acid B, tanshinol and protocatechuic aldehyde were active components of SMI because they were correlated to antioxidant properties.

Keywords: *Salvia miltiorrhiza* injection, HPLC, antioxidant activity, correlation analysis, salvianolic acid

Herbal medicines and their derivatives are widely used as therapeutics in many countries (1). *Radix Salviae miltiorrhizae*, called "Danshen" in China, is one of the most important traditional Chinese herbs and is accepted as a health product in western countries owing to its remarkable and reliable biological activities, especially in the treatment of cardiovascular disorders (2). *Salvia miltiorrhiza* products commonly include tablets, pills, and injections (3). Formulation of *Salvia miltiorrhiza* dripping pills has been certified by the US FDA Phase II clinical trials. *Salvia miltiorrhiza* injection (SMI) is believed to be another potential herbal product and has been the subject of recent studies.

SMI made from the aqueous extract of *Radix Salviae miltiorrhizae* is widely prescribed to patients with coronary disease, unstable angina, brain hemorrhage, cerebral thrombosis and cerebrovascular diseases in Chinese clinics (3–5). In addition, it is also applied to treat other diseases, such as liver dysfunction, renal deficiency and diabetic vascular complications (4). Studies of SMI showed that phenolic acids are beneficial compounds, responsible for therapeutic efficacy (5–8). Moreover, it is worth noting that phenolic acids of *Radix Salviae miltiorrhizae* such as salvianolic acid A, salvianolic acid B and rosmarinic acid have been reported to exhibit antioxidant properties and were protective against peroxidative

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damage (9, 10). Other studies showed that the water soluble extract of *Radix Salviae miltior-rhizae* exhibited high reactive oxygen species scavenging activity (11, 12). Many methods have been developed for chemical and pharmacological investigations of SMI, including chemical analysis with HPLC-UV, HPLC-MS, UPLC-MS, HPLC-CL and *in vivo / in vitro* pharmacological studies (13–18). However, there are few reports on ingredient analysis of SMI correlated to its biological activity.

Though we have already reported a simultaneous detection of seven phenolic acids in SMI (13), investigations in which phenolic acids have higher correlation to cardiovascular protection are more meaningful but have not been published yet. Therefore, in this paper, we present a study correlating phenolic acids in serum powder of SMI to its vascular en-dothelial cell protection by the use of bivariate correlation analysis (*e.g.*, ref. 19).

EXPERIMENTAL

Chemicals

Standards including tanshinol (99.8 %), protocatechuic aldehyde (99.8 %), protocate-chuic acid (99.8 %), rosmarinic acid (99.8 %) and salvianolic acid B (99.5 %) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Salvianolic acid A was isolated from the aqueous extract of *Radix Salviae miltior-rhizae* and was identified on the basis of mass spectrum data; its purity was over 97 % (by HPLC analysis). SMI was kindly supplied by Qinchunbao Pharmaceutical Co., Ltd. (China). Methanol, acetonitrile and formic acid (Merck, Germany) were of HPLC grade. Cho-lestan-3β,5α,6β-triol (triol), superoxide dismutase (SOD) kits and 1,1-diphenyl-2-picrylhy-drazyl (DPPH) were purchased from Sangon Biotech Co., Ltd. (China). Materials used for cell experiments including human umbilical vein endothelial cells (HUVECs), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), recombinant human basic fibro-blast growth factor (bFGF), bovine serum albumin (BSA) and 4′,6-diamidino-2-phenylin-dole (DAPI) were also purchased from Sangon Biotech Co., Ltd. Deionized water used throughout the experiments was produced using a Mill-Q water purification system (Mil-lipore, USA).

Animals and blood samples

Female Sprague Dawley rats (clean grade), weighting 220–250 g, were obtained from the Zhejiang Academy of Medical Sciences (Hangzhou, China). The studies were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (Hangzhou, China). Rats were fasted overnight, with free access to water, before the experiment. They were then randomly assigned into six groups (groups 1–6, n = 6 each). Groups 1–6 were given SMI, 630 mg kg^{-1} by intravenous injection.

Blood samples (0.2 mL) were collected from the ophthalmic venous plexus in heparin-ized polythene tubes just before drug injection and 5 (group 1), 10 (group 2), 15 (group 3), 20 (group 4), 25 (group 5) and 30 (group 6) min after injection. Serum was separated quick-ly using centrifugation at 1600×g for 10 min. One mL acetone was added to serum and centrifuged at 3000×g for 10 min to remove proteins. The supernatant was concentrated to

the serum powder of SMI with a vaccum concentrator (Savant SPD121P, Savant SpeedVac Concentrator, Thermo Scientific, USA) set at 4 °C. Finally the serum powder of SMI was stored at –80 °C until analysis. Serum powder of SMI was dissolved in methanol and the solution was injected into HPLC system to analysis. Serum powder of SMI when dissolved in deionized water was used for the cells.

Cells

HUVECs were seeded at 5×10^4 cm^{-2} and cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 20 % (*V/V*) FCS and 10 ng mL^{-1} bFGF for 24 hours. The medium was changed to DMEM supplemented with 0.1 % BSA, 10 ng mL^{-1} bFGF and various concentrations of cholestan-3β,5α,6β-triol. The number of cells in the confluent culture did not change significantly in the serum-free medium over the subsequent 48 hours unless triol was added. Cells between passages 2 to 7 were used for experiments.

HUVECs were stained with 500 ng mL^{-1} of DAPI for 10 min at room temperature. The number of apoptotic cells (approximately 1000–2000 cells per well) was counted for nine high power fields under a fluorescent microscope. Apoptotic cells were defined as cells with fragmented and/or more apoptotic bodies and their percentage was calculated.

HPLC

The HPLC equipment used was an Agilent 1100 series system (Agilent Technologies, USA) consisting of a binary pump, online vacuum degasser, autosampler, thermostated column compartment and multiple wavelength detection. A Tigerkin C$_{18}$ column (200×4.6 mm i.d., 5.0 µm) from Dalian Sipore Co. (China) was used for all chromatographic separations. The HPLC analytical conditions, which we developed before, were used in the assay. Linear gradient elution of eluents A [0.5 % (*V/V*) aqueous formic acid] and B [0.5 % (*V/V*) formic acid in acetonitrile] was used to run the separation. The first linear gradient was 5–20 % eluent B in the range of 0–10 min, the second was 20–25 % eluent B in the range of 10–17 min, and the last one was 25–55 % eluent B in the range of 17–35 min. The system was restored to initial conditions after 5 min. The solvent flow rate was 1.0 mL min^{-1}, injection volume was 20 µL, and column temperature was maintained at 30 °C. Chromatograms were recorded at 280 nm in the range of 0–13 min, 326 nm in the range of 13–23.5 min, and at 286 nm in the range of 23.5–35 min. Chemstation software (Agilent Technology) was used for peak detection and peak area calculation.

Free radical scavenging activity and antioxidant activity of serum powder of SMI

Free radical scavenging activity was determined as described by Blois (20). Briefly, acetate buffer (100 mmol L^{-1}, pH 5.5), ethanol (100 %) and freshly prepared 500 nmol L^{-1} DPPH solution in ethanol were mixed in a volume ratio 1:1:0.5. After the test compound was added, the mixture was incubated at 25 °C for 90 min. The change in absorption at 517 nm was then determined with a spectrophotometer (TU-1901 double-beam UV-Vis spectrophotometer, China).

Antioxidant properties were evaluated by measuring SOD activity. The experiment was performed using the SOD kit according to the manufacturer's instructions.

Drug preparation

SMI was prepared by the Qinchunbao Pharmaceutical Co., Ltd (China). It was made from the extract of *Radix Salviae miltiorrhizae* and prepared according to the standard injection production process. It was stored at 4 °C. SMI was diluted with a 50 % glucose injection in a volume ratio 1:5; the dilution should be freshly prepared prior to injecting it to the rats.

Statistical analysis

The data of free radical scavenging activity and antioxidant properties were presented as mean ± standard error of the mean (SEM). Bivariate correlation was tested using the SPSS Version 17.0 software package (SPSS, USA) to analyze the correlation between the peak areas and antioxidant properties. Pearson correlation coefficients were obtained by bivariate correlation.

RESULTS AND DISCUSSION

HPLC chromatograms of SMI serum powder

Six groups of SMI serum powder were analyzed by HPLC. Tanshinol, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, protocatechuic acid and some metabolites were found in the serum powder of SMI (Fig. 1). As shown in Fig. 1 the retention time of peak 4a was close to the retention time of rosmarinic acid; however, this peak did not appear in the chromatogram of SMI. Thus it may be the metabolite of rosmarinic acid. Similar cases were peak 5a (the metabolite of salvianolic acid B) and peak 6a (the metabolite of salvianolic acid A). These metabolites and protocatechuic acid were found only in the serum powder of SMI. In addition, tanshinol, protocatechuic aldehyde, rosmarinic acid and salvianolic acid B which existed in SMI were also found in the serum powder of SMI (Table I). The ingredients that exist in the serum may be the active components. Therefore, tanshinol, protocatechuic aldehyde, rosmarinic acid, salvianolic acid B, protocatechuic acid and the metabolites may contribute to the efficacy of SMI.

Serum powder of SMI inhibits endothelial cell apoptosis

Serum powder of SMI prevented the triol-induced apoptosis of human umbilical vein endothelial cells *in vitro* (Fig. 2). In the assay, 5 μg mL^{-1} of triol induced apoptosis in HUVECs in a time-dependent manner *in vitro*. Triol induced significant apoptosis in HUVECs as early as 24 h from the start of incubation, and the number of apoptotic cells increased up to 48 h. When 100 μg mL^{-1} of SMI serum powder was added into the HUVECs with 5 μg mL^{-1} of triol, the number of apoptotic cells decreased significantly (inhibition 47.6 %, $p < 0.05$) *vs.* reference drug probucol (inhibition 72.1 %, $p < 0.01$), in contrast to the blank control. In short, serum powder of SMI could inhibit the triol-induced apoptosis of HUVECs and protect normal HUVECs *in vitro*.
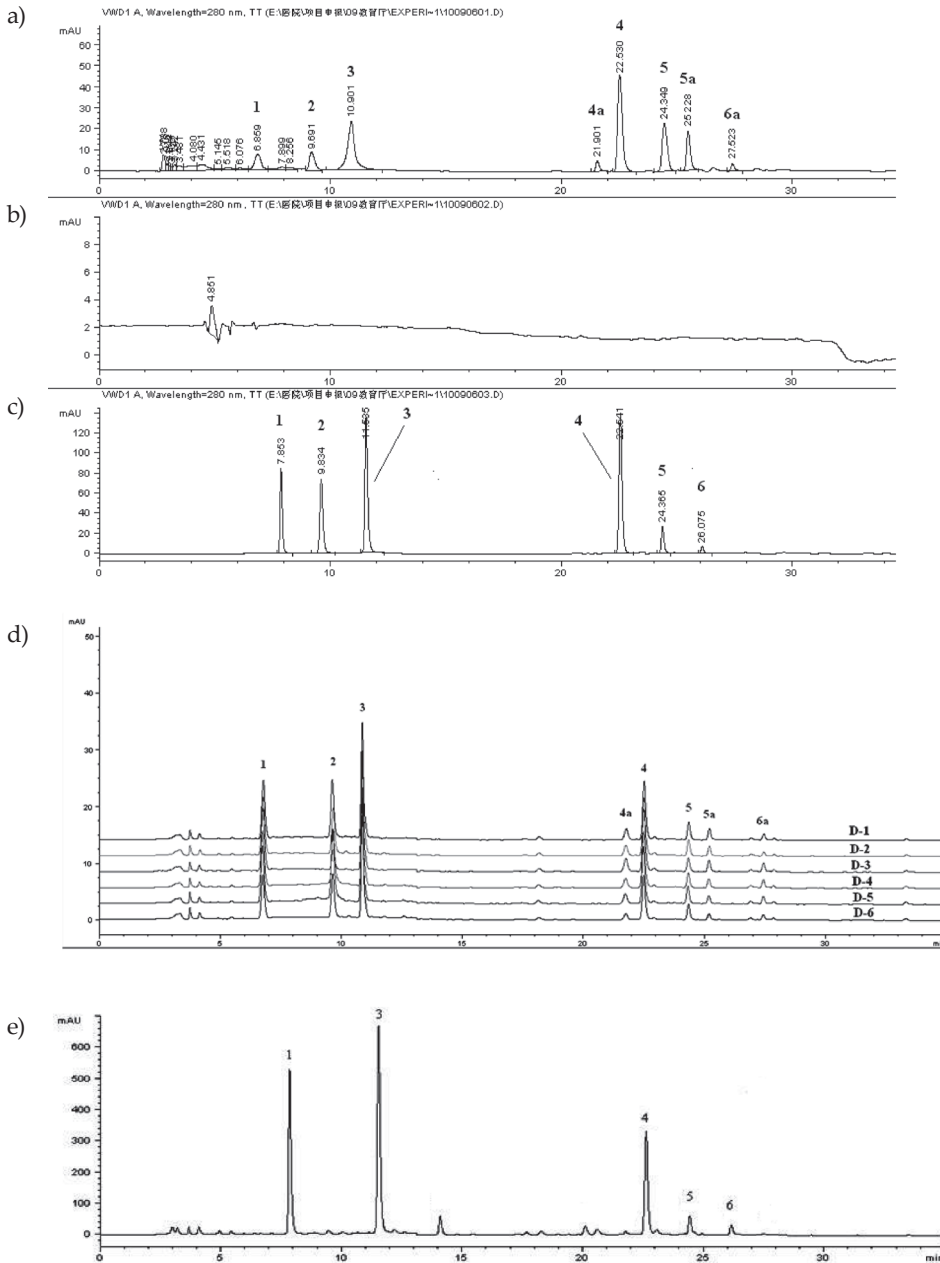
Fig. 1. HPLC chromatograms of: a) serum powder of SMI, b) blank control, c) standard, d) six groups of SMI serum powder (D-1 to D-6) and e) SMI. Components: 1 – tanshinol, 2 – protocatechuic acid, 3 – protocatechuic aldehyde, 4 – rosmarinic acid, 5 – salvianolic acid B, 6 – salvianolic acid A, 4a – metabolite of rosmarinic acid, 5a – metabolite of salvianolic acid B, 6a – metabolite of salvianolic acid A.

Table I. Components in SMI and SMI serum powder

Component	Content in SMI (mol L^{-1})[a]	Content in serum powder of SMI (mol L^{-1})[a]
Tanshinol	2.34 ± 0.04	(9.10 ± 1.74) × 10^{-2}
Protocatechuic aldehyde	1.96 ± 0.02	(1.70 ± 0.05) × 10^{-2}
Salvianolic acid B	0.42 ± 0.01	(1.56 ± 0.04) × 10^{-2}

[a] Mean ± SD (n = 6).

Table II. Radical scavenging activity and SOD activity of SMI serum powder in HUVECs induced by triol[b]
for 48 h by DPPH assay

Group	IC_{50} (μg mL^{-1})	SOD (U mL^{-1})
1	57.6 ± 2.4[c]	0.53 ± 0.05
2	52.4 ± 1.9[c]	0.58 ± 0.05[c]
3	49.3 ± 1.7[c]	0.63 ± 0.03[c]
4	54.7 ± 2.2[c]	0.54 ± 0.02
5	56.9 ± 2.2[c]	0.51 ± 0.05
6	58.3 ± 2.9[c]	0.46 ± 0.04
Negative control (triol)	174.1 ± 6.5	0.21 ± 0.04
Positive control (probucol)	35.6 ± 1.3[c]	0.79 ± 0.03[c]

Values were mean ± SEM (n = 6).
[a] Concentration of SMI: 100 μg mL^{-1}.
[b] Concentration of triol: 5 μg mL^{-1}.
[c] $p < 0.05$ compared with triol

Six groups of serum powder of SMI and probucol were added into the triol-induced apoptotic HUVECs. Apoptosis of endothelial cells was inhibited significantly ($p < 0.05$) in groups of SMI serum powder (Fig. 3).

Free radical scavenging activity and antioxidant activity of SMI serum powder

Serum powder of SMI exhibited free radical scavenging activity in the 1,1-diphenyl-2--picrylhydrazyl (DPPH) assay. The results are listed in Table II. As shown in Table II, all groups of SMI serum powder showed good radical scavenging activity, with the IC_{50} values from 49.3 ± 1.7 to 58.3 ± 2.9 μg mL^{-1} in contrast to triol (174.1 ± 6.5 μg mL^{-1}). Groups 3 and 2 were the best. The radical scavenging activity of serum powder of SMI was 1.5 times lower than that of the reference drug probucol (35.6 ± 1.3 μg mL^{-1}).
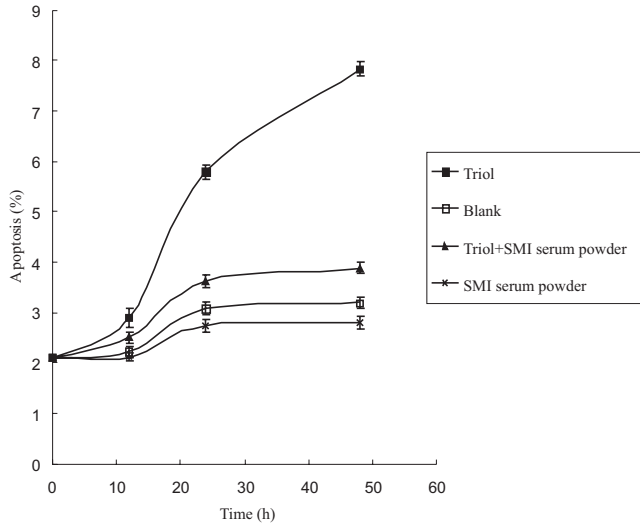
Fig. 2. The time course for HUVECs cultured in SMI serum powder (X-type), SMI serum powder with 5 μg mL^{-1} of triol (closed triangle), blank control (open square) or 5 μg mL^{-1} of triol (closed square) for 48 h. Each point represents the mean ± SEM ($n = 6$).
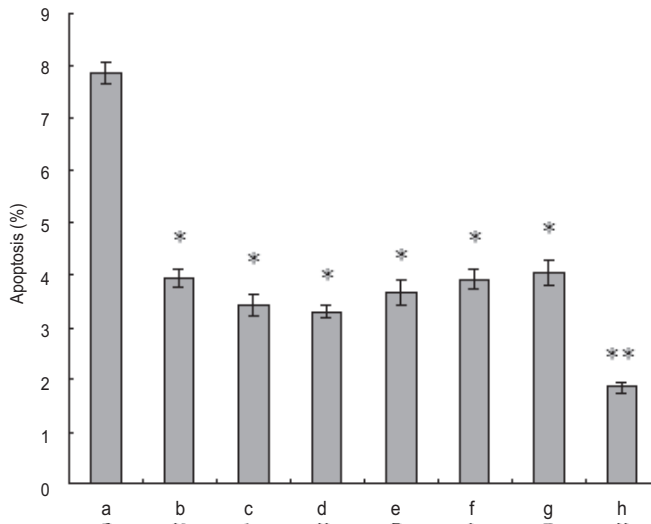

Fig. 3. The effect of SMI serum powder on the triol-induced apoptosis of HUVECs. a – triol, b – group D-1 (5 min), c – group D-2 (10 min), d – group D-3 (15 min), e – group D-4 (20 min), f – group D-5 (25 min), g – group D-6 (30 min), h – probucol. Each point represents the mean ± SE ($n = 6$). Significantly different from triol: * $p < 0.05$, ** $p < 0.01$.

Table III. Pearson correlation coefficient between peak area and antioxidant property of ingredients in SMI serum powder

Compound	Retention time (min)	Peak area *vs.* IC_{50}	Peak areas *vs.* SOD
Tanshinol	7.899	−0.880[a]	0.861[a]
Protocatechuic acid	9.691	0.398	−0.440
Protocatechuic aldehyde	10.901	−0.722[a]	0.807[a]
Metabolite of rosmarinic acid	21.901	0.005	−0.139
Rosmarinic acid	22.530	−0.773	0.654
Metabolite of salvianolic acid B	23.388	−0.227	0.129
Salvianolic acid B	24.349	−0.954[b]	0.911[a]
Metabolite of salvianolic acid A	27.523	−0.449	0.279

[a] Significance level of correlation ($p < 0.05$).
[b] Significance level of correlation ($p < 0.01$).

The activity of superoxide dismutase was used as an index of oxidative damage in the assay. All groups of serum powder of SMI increased the low activity of SOD caused by triol (SOD value of 0.21 ± 0.04 U mL^{-1}). As shown in Table II, group 3 was better (0.63 ± 0.03 U mL^{-1}) than the other groups.

Correlation analysis based on the chromatograms and antioxidant properties of SMI serum powder

According to the results shown in Table III, the area of four peaks in HPLC chromatograms showed higher correlation to radical scavenging activity than the others, with the absolute correlation coefficient value higher than 0.7. These four compounds were salvianolic acid B, tanshinol, protocatechuic aldehyde and rosmarinic acid. Among them, salvianolic acid B reached the significance level with $p < 0.01$ and tanshinol and protocatechuic aldehyde with $p < 0.05$. Since correlation between IC_{50} and peak area appeared in protocatechuic acid and rosmarinic acid metabolite, we suggest that the two components had no free radical scavenging activity. Neither did metabolites of salvianolic acid B and A, due to their low *R*. Peak areas of salvianolic acid B, tanshinol, protocatechuic aldehyde and rosmarinic acid showed higher correlation to SOD activity than the other components (Pearson correlation coefficient $R > 0.6$). Among them, salvianolic acid B, tanshinol and protocatechuic aldehyde reached the significance level with $p < 0.05$. Our findings suggest that protocatechuic acid and the rosmarinic acid metabolite had almost no antioxidant activity. Furthermore, low *R* between peak area and SOD activity suggested that metabolites of salvianolic acid B and A were unrelated to antioxidant activity.

This method enables rapid identification of active components in herbal medicine and provides a meaningful tool for their quality control.

CONCLUSIONS

Based on these results, it was concluded that salvianolic acid B, tanshinol and protocatechuic aldehyde correlated well with the antioxidant properties of serum powder of SMI. They contributed to the protection of vascular endothelial cells by inhibiting the triol-induced apoptosis of HUVECs and protecting normal HUVECs *in vitro*. They were shown for the first time to be antioxidants by correlation analysis. Salvianolic acid B was demonstrated to be the most potential antioxidant of all the SMI ingredients, since it was closely related to the free radical scavenging activity and SOD activity with the Pearson correlation coefficient higher than 0.9. In future experiments, we will identify the metabolites of SMI and a systematic study of pharmacokinetics of SMI will be carried out.

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