Title Page

Metabolism of Salvianolic Acid A and Antioxidant Activities of Its Methylated Metabolites

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Abbreviations:

SA, salvianolic acid; SAA, salvianolic acid A; COMT, catechol O-methyltransferase; SAM, S-adenosyl-L-methionine; MDA, malondialdehyde; TCA, trichloroacetic acid; TBA, thiobarbituric acid; BHT, butylatedhydroxytoluene; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; SPE, solid-phase-extraction; ESI, electrospray ionization; TMS, tetramethylsilane; SAB, salvianolic acid B; XIC, extracted ion current; TIC, total ion current; RHC, rat hepatic cytosol; AA, ascorbic acid;
Metabolism of salvianolic acid A (SAA) both in vivo and in vitro was investigated in the present study. LC-MS analysis on drug-containing rat bile and the bile sample hydrolyzed by glucuronidase revealed a series of methylated conjugates of SAA and its glucuronides, as well as the predominance of the methylation pathway of SAA in rats. For the first time, four major methylated metabolites present in vivo were prepared for structure characterization and bioactivity evaluation using in vitro co-incubation systems with rat hepatic cytosol protein as enzyme donor. By NMR and other spectroscopic methods, these metabolites were unambiguously elucidated as 3-\(O\)-methyl- (M1), 3′-\(O\)-methyl- (M2), 3, 3″-\(O\)-dimethyl- (M3), and 3′, 3″-\(O\)-dimethyl- salvianolic acid A (M4), respectively. Along with the results from enzyme inhibition study, selective formation of these meta-\(O\)-methylated derivatives indicated that catechol \(O\)-methyltransferase (COMT) is responsible for methylated transformation of SAA. All these metabolites displayed fairly high antioxidant potency against in vitro rat liver lipid peroxidation with half-maximal inhibitory concentrations much lower than those of the positive controls and even SAA. Overall, the findings from the present study demonstrated that SAA is a metabolically unstable compound that would undergo rapid methylation metabolism catalyzed by COMT, and these generated \(O\)-methylated metabolites may be largely responsible for its in vivo pharmacological effects.
Introduction

Danshen is a kind of well-known traditional Chinese herbal medicine prepared from the dried roots and rhizomes of *Salvia Miltiorrhiza* Bge. (Labiatae). With the ability to enter the channel of heart, pericardium and liver, Danshen has the effects to promote blood circulation and remove stasis, and is extensively used in clinic to treat and prevent many diseases, including hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea, neurasthenic insomnia, and ischemic injuries [Cheng, 2006; Wasser et al., 1998; Ji et al., 2000; Zhou et al., 2005]. During the past two decades, research interest toward the water-soluble components of Danshen has been initially motivated by its usage as water decoction in traditional Chinese prescriptions and as injection for the treatment of cerebral and coronary vascular diseases [Wang et al., 2007]. Caffeic acid derivatives occur as the major water-soluble components of Danshen. Up to now, more than twenty salvianolic acids (SAs), the caffeic acid oligomers, have been isolated from Danshen [Lu and Foo, 2002; Ho and Hong, 2011].

Salvianolic acid A (SAA) is a kind of caffeic acid trimer biosynthesized from condensation of caffeic acid and Danshensu at a molar ratio of 2:1 (Fig. 1) [Li et al., 1984]. In recent years, SAA has drawn great research attention for its diverse potent bioactivities, including antioxidative, antiplatelet and antithrombotic effects [Huang and Zhang, 1992; Wang et al., 2005; Fan et al., 2010]. Among various SAs, SAA has been found to display the most potent protective action against peroxidative damage to biomembranes [Liu et al., 1992]. Considerable evidences have also demonstrated the potent protective effects of SAA against ischemia-induced injury both *in vitro* and
As is well known, phenolic acids (including SAs) are likely to undergo phase II conjugation metabolism, and biliary route of elimination is considered to be of the utmost importance to the in vivo disposition of these compounds [Baba et al., 2004; Zhang et al., 2004; Lv et al., 2010]. Pharmacokinetic studies indicate that SAA has extremely low bioavailability and very short plasma elimination half-time [Hou et al., 2007; Pei et al., 2008]. Our previous study on rat after intravenous administration of SAA (unpublished data) also revealed that there were various metabolites found in bile, and SAA excreted as unchanged form was very limited (0.57% and 2.74% into urine and bile, respectively). All these findings suggest that metabolism is an important elimination pathway of SAA, and pharmacological activities in vivo may be related to its metabolic fate. Five conjugated metabolites of SAA, including methylated, glucuronidated and the both, were identified from rat plasma, indicating two main in vivo metabolic pathways of SAA in rats, methylation and glucuronidation [Shen et al., 2009]. The kinetics of SAA glucuronidation by pooled human microsomes and recombinant UDP-glucuronosyl transferases (UGTs) isozymes have recently been studied, and UGT1A1 and 1A9 were identified as the major human UGT isoforms that catalyze the glucuronidation of SAA [Han et al., 2012]. However, the methylation characteristics of SAA have not been systematically evaluated, which is the objective of the present study package.

On the basis of analyzing in vivo metabolites profile in rat and in vitro enzyme-catalyzed methylation, the enzyme responsible for methylation of SAA were
confirmed, and four major methylated metabolites of SAA present in vivo were prepared using in vitro incubation system. For the first time, the chemical structures of these methylated metabolites were unambiguously identified by spectroscopic methods, and their antioxidant activities were evaluated. The findings from the present study have laid a solid foundation from the viewpoint of metabolism to expound in vivo pharmacodynamic substance species of SAA.

**Materials and Methods**

**Chemicals and Other Reagents.** SAA with 99.5% purity was provided by Shandong Target Drug Co. Ltd (Yantai, China). S-adenosyl-L-methionine (SAM), entacapone, trichloroacetic acid (TCA), thiobarbituric acid (TBA), and β-glucuronidase from *E. coli* were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,6-di(tert-butyl)-4-hydroxytoluene (BHT) and α-tocopherol (95%) were obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China) and FlukaChemieAG (Buchs, Switzerland), respectively. All the remaining chemicals and solvents used were of standard analytical or HPLC grade. Ultrapure water prepared by a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the study.

**Animals.** Male Sprague-Dawley rats weighing 180-220g were obtained from the Laboratory Animal Center of Shandong Luye Pharmaceutical Co. Ltd (Yantai, China). They were provided with standard laboratory food and water and maintained on a 12-h light/dark cycle in an air-conditioned animal quarter at constant temperature (22-24°C) and humidity (50±10%). The animals were fasted overnight with free access to water before any experiment. The animal experiments were conducted in
accordance with the local institutional guidelines for care and use of laboratory animals at Yantai University.

**In Vivo Metabolism in Rat.** Eight rats were evenly divided into two groups at random. The animals were fixed on wooden plate and anesthetized with diethyl ether. An abdominal incision was made and the common bile duct was cannulated with PE-10 tubing (i.d. 0.8mm, Becton Dickinson, USA) for bile collection. When recovered from anesthesia, one group received a single 20 mg/kg intravenous dose of SAA in saline and the drug-containing bile samples were collected into successive vials on ice at different time points over 12-h period. The blank samples were collected from the other group after intravenously dosing of the same volume of saline. Hydrolysis of drug-containing bile sample with $\beta$-glucuronidase was performed according to the method of Han *et al.* [2012]. For subsequent analysis, all the bile samples were first subjected to liquid-liquid extraction as follows. An aliquot of 2 mL of bile sample was extracted with 3 volumes of ethyl acetate after addition of 300 $\mu$l hydrochloric acid (1 M). The supernatant was separated after vortex-mixing for 5 min and centrifugation at 8,000 g for another 5 min, then evaporated to dryness under a stream of nitrogen at 35°C. The residue was reconstituted into 300 $\mu$l mobile phase by vortex-mixing for 1 min and then centrifuged at 15,000 g for 10 min to obtain supernatant for analysis.

**In Vitro Methylation.** Immediately after sacrifice, the rat livers were removed and homogenized with 9 volumes of 1.15% KCl, then centrifuged at 12,000 g for 15 min at 4°C. The supernatant (S9 fraction) was further centrifuged at 100,000 g for 90 min
to obtain microsomal and cytosol fractions. The incubation systems contained 6.8 mg rat hepatic cytosolic protein, 5 mM MgCl₂, and 1 mM SAM as exogenous methyl donor in 50 mM phosphate buffer solution (pH 7.4). When confirming the enzyme responsible for methylation, entacapone, a selective catechol O-methyl transferase (COMT) inhibitor, was added at concentration of 3 μM. After pre-incubation at 37°C for 5 min, SAA was added into the mixture at a final concentration of 0.25 mM and subjected to further incubation for 15 min at 37°C. Then the reaction was terminated by adding 5 M HCl, and the mixture was extracted with 3 volumes of ethyl acetate. After vortex-mixing for 5 min and centrifugation at 8,000 g for 10 min, the supernatant was separated and evaporated to dryness for subsequent HPLC assay and preparative separation.

Separation of Methylated Metabolites by Column Chromatography. The terminated incubation samples from several batches were combined, diluted with water, and applied to a Strata-X solid-phase-extraction (SPE) column (Phenomenex, Torrance, CA, USA). After washing with water and MeOH - H₂O (20 : 80) both for eight column volumes, the column was then eluted with ten column volumes of MeOH - H₂O (55 : 45) to obtain eluent for further repeated column chromatography using C18 (i.d. 10 mm × 200 mm, YMC-pack ODS-A, 5 μm), which was gradient eluted with the mixture of MeOH and 0.05% formic acid. According to the elution order, four compounds named M1 (15 mg), M2 (12 mg), M3 (8 mg), and M4 (5 mg) were obtained.

Chemical Structure Characterization. UV and IR spectra were measured on a
Shimadzu UV-2550 (Shimadzu, Kyoto, Japan) and Nicolet 6700 FTIR spectrometer (Thermo Nicolet Corp., Madison, WI, USA), respectively. Electrospray ionization mass spectroscopy (ESI-MS) data were determined in negative ion mode using a triple quadrupole mass spectrometer from Thermo-Fisher Scientific Inc. (Waltham, MA, USA). 1D and 2D NMR spectra were recorded on a Bruker AV400 NMR spectrometer (Bruker Co., Faellanden, Switzerland) operating at 400 MHz for protons and 100 MHz for carbon in methanol-\(d_4\) at room temperature. Chemical shifts were expressed as values (parts per million) relative to the internal standard, tetramethylsilane (TMS).

**Analytical HPLC Conditions.** The chromatographic system used for detecting metabolites was Agilent 1100 HPLC (Agilent, USA) equipped with binary pump, autosampler, column manager, and PDA detector. HPLC analysis was performed on an Xterra RP18 column (150 mm × 4.6 mm i.d., 3.5 μm, Waters, USA) and the column temperature was maintained at 30°C. The mobile phase composed of mixture of acetonitrile (A) and 0.05% formic acid (B) was delivered at a flow rate of 1 mL/min. For \textit{in vivo} metabolism studies, a linear gradient elution was performed to run the separation, and the elution programme was conducted as follows: 0-29 min, holding at 25% A; 29-31 min, a linear increase from 25% A to 30% A; 31-60 min, holding at 30% A, 60-62 min, a linear decrease from 30% A to 25% A; 62-75 min, holding at 25% A. An isocratic elution mode with 30% A and 70% B was used for \textit{in vitro} incubation systems. Injection volume was set at 20 μl, and HPLC detection was conducted at 285 nm according to the maximum absorption of SAA.
**LC-MS Qualitative Analysis.** A TSQ Quantum Access triple-quadrupole tandem mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was connected to HPLC system via electro-spray ionization (ESI) interface. Ultra-high purity helium was used as the damping and collision gas, and nitrogen as the sheath and auxiliary gas. The ESI source was operated in negative ion mode from \( m/z \) 150 to 900. Other MS conditions for maximum detection of metabolites were set as follows: spray voltage 3.5 kV, capillary temperature 350°C, capillary voltage -30 V, sheath gas 30 psi and 0.75 L/min, auxiliary gas 5 psi and 0.15 L/min, collision gas 1.5 milli-Torr and 0.75 L/min. The HPLC conditions were set as described above. Both system control and data processing were performed by Xcalibur workstation (version 1.4.1).

**Assay of Lipid Peroxide in Rat Liver Homogenate.** This assay is based on the reaction of malondialdehyde (MDA) with TBA forming an MDA-TBA2 adduct and performed as described by Chai *et al.* [2012] with slight modification. Male SD rats with body weight of 220 ± 20 g were sacrificed and livers were isolated after disposing of blood. Then the livers were cut into slices and homogenated with saline to obtain 2% tissue homogenate for use. An aliquot of 0.25 mL liver homogenate was mixed with 1.0 mL test sample solution (dissolved with PBS at different concentrations), 50 \( \mu l \) ferrous ammonium sulphate solution (1 mmol/L), and 50 \( \mu l \) vitamin C solution (1 mmol/L) to initiate reaction. The mixtures were incubated at 37°C for 1 h, and then 1.0 mL of TCA solution (20%) was added to stop the oxidation reaction. After mixing and standing for 10 min, the reaction solution was centrifuged at 3,000 g for 10 min. The supernatant was added with 0.25 mL TBA solution (3.2%)
and delivered to a boiling water bath for 20 min. After cooling down, the absorption of the solution was determined at 532 nm. The inhibitory rate on lipid peroxidation was calculated by referring to blank control. All the experiments were performed in triplicate, and data are expressed as mean ± S.D. of three independent experiments. Statistical analysis of the data was performed using one-way ANOVA. Differences with \( p < 0.05 \) were considered as statistically significant.

Results

In Vivo Metabolism of SAA in Rat. Rat urine was collected and quickly analyzed. However, excretion of SAA conjugative metabolites was minimal in this matrix, and thus, the analysis focused on bile. Taking into account the composition complexity of the drug-containing bile sample, an LC-MS assay based on gradient chromatographic elution mode was developed to fully display the biliary metabolites profile as far as possible. A linear gradient elution of eluents A (acetonitrile) and B (0.05% formic acid) was well optimized and performed to run the separation within an overall runtime of 70 min. For more exact qualitative characterization, the extracted ion current (XIC) chromatograms created via a special data-mining process were recovered from the entire data set for a chromatographic run, the total ion current (TIC) chromatogram. In view of the two main in vivo metabolic pathways of SAA in rats, methylation and glucuronidation [Shen et al., 2009], the extracted \( m/z \) values of \([M - H]^-\) ions representing SAA and all its conjugative metabolites of interest included 493 (SAA), 507, 521, 535 (mono-, di-, and tri-methyl SAA), 669, 683, 697, and 711 (glucuronides of SAA, and mono-, di-, tri-methyl SAA). The HPLC-UV, TIC and XIC
chromatograms of the drug-containing bile and blank bile sample were shown in Fig. 2 and the detailed LC-MS data were listed in Table 1.

Based on LC-MS profiling of bile, more than twenty metabolites with peak intensity larger than $3.5 \times 10^4$ were detected, including SAA, ten methylation products of SAA (three mono-, five di-, and two tri-methyl), one glucuronide of SAA, as well as thirteen underlying methylated metabolites showing both methylation and glucuronidation (as shown in Table 1). The XIC filtered chromatograms (Fig. 2F) clearly demonstrated that both the two largest metabolite peaks in bile marked as L1 and L2 in Fig. 2D were di-methyl glucuronide of SAA with an $m/z$ value of 697. By comparison with the reference standards obtained from in vitro preparation as discussed below, the other four major methylation products with retention times of 29.69 min, 34.58 min, 41.57 min, and 44.87 min were chromatographically identified as $3'-O$-methyl-salvianolic acid A (M1), $3'-O$-methyl-salvianolic acid A (M2), $3',3''-O$-dimethyl-salvianolic acid A (M3), and $3',3''-O$-dimethyl-salvianolic acid A (M4), respectively. So many methylated and underlying methylated conjugates of SAA abundant in drug-containing bile provide convincing evidence for the predominance of its in vivo methylation pathway in rat. These findings also revealed that SAA in rats is prone to undergoing rapid hepatic metabolism and high biliary excretion in the form of methylated conjugates, which is similar to the metabolic characteristics observed in some other natural phenolic acids, including salvianolic acid B (SAB), caffèic and rosmarinic acids [Baba et al., 2004; Xu et al., 2007; Lv et al., 2010].
In order to examine the relative distribution of all these methylation conjugates of SAA, hydrolysis study was further conducted on the drug-containing rat bile sample using β-glucuronidase to cleave all the glucuronides of SAA back to their aglycones. LC-MS analysis was performed for the hydrolyzed bile under the same conditions as the untreated sample, which clearly revealed the full set of methylation products in the absence of all the glucuronides, as well as the difference in methylation products profiling between these two bile samples. As shown in Fig. 3 and Table 1, glucuronidase hydrolysis resulted in significant changes in the relative distribution of the mono-methyl SAA at 30.68 min to the whole mono-methylation products, and the di-methyl SAA at 44.87 min to the whole di-methylation products, from very a low level (~3%) to 16%, and 23% to 30%, respectively. According to the retention time and meta-O-methylation of SAA catalyzed by COMT, these two metabolites could be deduced as 3′′-O-monomethyl-salvianolic acid A, and 3′, 3′′-O-dimethyl-salvianolic acid A (M4), respectively. All these findings suggest that 3′′-O-methyl-salvianolic acid A is much more reactive than M1 or M2, and is very easily subjected to conjugative reaction (including methylation and glucuronidation). Eventually this methylation product is rapidly transformed into more stable subsequent conjugative metabolites of SAA, such as M3, M4, and 3′′-O-methyl-salvianolic acid A glucuronide. This may be why there is very limited amount of this 3′ mono-methyl metabolite but fairly high levels of M1 to M4 found in rat bile.

**In Vitro Methylation in RHC Incubation System.** Based on *in vivo* findings above that SAA could be rapidly metabolized into a series of methylated conjugates
by rat hepatic enzymes, *in vitro* enzyme-catalyzed incubation using rat hepatic subcellular fraction as enzyme donor was performed to elucidate the mechanism of *in vivo* methylation and prepare major methylated metabolites of SAA for further studies on structure and activity. The COMT activities of rat liver subcellular fractions were determined as 0.753±0.106, 0.742±0.100, and 0.146±0.032 pmol·mg⁻¹·min⁻¹ (*n* = 6) for S9, cytosol, and microsomes, respectively. The hepatic cytosol displayed much higher COMT activity than microsome (*P* < 0.05) and contained much less endogenous interfering substances than S9 fraction. Therefore the *in vitro* enzyme-catalyzed biotransformation of SAA was carried out in the incubation system using RHC as enzyme donor. The optimal incubation conditions were established according to our previous study on the enzyme kinetics of this incubation system.

Several different incubation systems were constructed as controls, including the blank control without SAA, the negative control containing SAA, SAM and heat-inactivated RHC, and the control containing entacapone, a selective COMT inhibitor. Firstly, a feasible HPLC method differing from that for *in vivo* metabolism study was developed for fast and effective separation of methylation products using an isocratic elution of acetonitrile and 0.05% formic acid (30 : 70, v/v) within an overall runtime of 30 min. In order to verify the consistency between *in vitro* and *in vivo* metabolism, the drug-containing rat bile sample was used a reference and subjected to further HPLC detection under the conditions for these *in vitro* incubation systems. The discrepancy between Fig. 4 G and Fig. 2 D revealed the difference in chromatographic elution mode, as well as the reliability of this LC method for *in vitro*
study. Four major metabolites with the same retention times as those present in
drug-containing rat bile (Fig. 4 G) could be detected in RHC system just after 2 min
incubation (Fig. 4 D). With very limited amount of SAA left, no significant increase in
amount of these metabolites was observed after 20 min incubation (Fig. 4 E),
indicating metabolism of SAA in this RHC system was completed within 20 min. As
shown in Fig. 4 F, addition of entacapone would significantly inhibit
biotransformation of SAA and accordingly the generation of these metabolites. All
these evidences therefore confirmed the hepatic contribution to disposition of SAA in
rat, and also the fact that COMT is the enzyme responsible for methylation of SAA.

**Structure Elucidation of Methylated Metabolites of SAA.** Using Phenomenex
Strata-X and repeated ODS column chromatography, four metabolites named M1
through M4 were separated from the *in vitro* RHC incubation system and their purities
were all determined as more than 90% by HPLC analyses (Fig. 5). By comprehensive
spectroscopic analysis and comparing the spectral data with those of SAA [Sun et al.,
2009], the chemical structures of these metabolites were unambiguously elucidated.
The spectral data of metabolites M1 through M4 were shown as follows.

* M1: A slight yellow powder, [α]D20 +41.5° (c 0.1, EtOH). UV (MeOH) λmax (log ε): 288 (4.44), 305 nm (4.39). IR (KBr) νmax: 3385, 1689, 1601, 1519, 1283, 1167, 969,
807 cm⁻¹. ESI-MS m/z: 507 [M−H]⁻, 1037 [2M+Na−2H]⁻. ¹H-NMR (in methanol-d4, 400 MHz) δ: 7.16 (1H, d, J=1.0 Hz, H-2), 6.79 (1H, d, J=8.2 Hz, H-5), 7.00 (1H, d, J=8.2 Hz, H-6), 7.19 (1H, d, J=16.4 Hz, H-7), 6.65 (1H, d, J=16.4 Hz, H-8), 6.75 (1H, d, J=8.4 Hz, H-5'), 7.12 (1H, d, J=8.4 Hz, H-6'), 8.06 (1H, d, J=15.8 Hz, H-7'), 6.30
(1H, d, $J$=15.8 Hz, H-8'), 6.72 (1H, d, $J$=2.0 Hz, H-2''), 6.61 (1H, d, $J$=8.0 Hz, H-5''), 6.55 (1H, d, $J$=8.0 Hz, H-6''), 3.06 (1H, d, $J$=11.4 Hz, H-7'a), 2.93 (1H, d, $J$=13.8 Hz, H-7''b), 5.12 (1H, d, $J$=6.9 Hz, H-8''), 3.89 (3H, s, 3-OCH$_3$). 13C-NMR (in methanol-d$_4$, 100 MHz) $\delta$: 128.2 (C-1), 120.4 (C-2), 149.2 (C-3), 146.9 (C-4), 110.5 (C-5), 120.6 (C-6), 137.8 (C-7), 116.3 (C-8), 126.3 (C-1'), 131.3 (C-2'), 146.1 (C-3'), 148.2 (C-4'), 116.1 (C-5''), 121.9 (C-6''), 147.8 (C-7''), 116.2 (C-8''), 168.8 (C-9''), 130.0 (C-1''), 117.3 (C-2''), 145.1 (C-3''), 144.4 (C-4''), 114.8 (C-5''), 121.4 (C-6''), 38.3 (C-7''), 74.6 (C-8''), 173.4 (C-9''), 56.4 (C3-'OCH$_3$);

$M_2$: A slight yellow powder, $[\alpha]_{D}^{20}$+40.2° (c 0.1, EtOH). UV (MeOH) $\lambda_{\text{max}}$(log $\varepsilon$): 287.5 (4.45), 306 nm (4.30). IR (KBr) $\nu_{\text{max}}$: 3389, 1686, 1608, 1516, 1276, 1173, 977, 811 cm$^{-1}$. ESI-MS $m/z$: 507 [M--H] $^·$, 1037 [2M+Na--2H] $^·$. $^1$H-NMR (in methanol-d$_4$, 400 MHz) $\delta$: 7.06 (1H, s, H-2), 6.74 (1H, d, $J$=8.4 Hz, H-5), 6.90 (1H, d, $J$=8.2 Hz, H-6), 7.17 (1H, d, $J$=16.4 Hz, H-7), 6.65 (1H, d, $J$=16.4 Hz, H-8), 6.97 (1H, d, $J$=8.4 Hz, H-5''), 7.12 (1H, d, $J$=8.4 Hz, H-6''), 8.01 (1H, d, $J$=15.8 Hz, H-7''), 6.30 (1H, d, $J$=15.8 Hz, H-8''), 6.71 (1H, s, H-2''), 6.63 (1H, d, $J$=8.0 Hz, H-5'''), 6.56 (1H, d, $J$=8.0 Hz, H-6'''), 3.08 (1H, d, $J$=12.7 Hz, H-7'a), 2.92 (1H, m, H-7''b), 5.11 (1H, br.s, H-8''), 3.86 (3H, s, 3'-OCH$_3$). 13C-NMR (in methanol-d$_4$, 100 MHz) $\delta$: 127.9 (C-1), 120.3 (C-2), 146.1 (C-3), 146.4 (C-4), 113.7 (C-5), 120.6 (C-6), 137.3 (C-7), 116.6 (C-8), 126.3 (C-1'), 132.5 (C-2'), 149.2 (C-3'), 148.1 (C-4'), 114.8 (C-5'), 121.9(C-6'), 147.8 (C-7'), 116.2 (C-8'), 169.0 (C-9'), 130.6 (C-1''), 117.3 (C-2''), 146.0 (C-3''), 144.4 (C-4''), 113.7 (C-5''), 121.0 (C-6''), 38.5 (C-7''), 74.7 (C-8''), 173.4 (C-9''), 56.4 (C3'-OCH$_3$);
M3: A slight yellow powder, $[\alpha]_D^{20} +43.8^\circ$ (c 0.05, EtOH). UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$):

287.5 (4.38), 305 nm (4.30). IR (KBr) $\nu_{\text{max}}$: 3385, 1688, 1605, 1520, 1282, 1170, 969, 808 cm$^{-1}$. ESI-MS $m/z$: 521 [M−H]$^-$, 1043 [2M−H]$^-$.

$^1$H-NMR (in methanol-$d_4$, 400 MHz) $\delta$: 7.16 (1H, s, H-2), 6.79 (1H, d, $J=8.2$ Hz, H-5), 7.00 (1H, d, $J=8.0$ Hz, H-6), 7.20 (1H, d, $J=16.4$ Hz, H-7), 6.65 (1H, d, $J=16.4$ Hz, H-8), 6.77 (1H, d, $J=7.0$ Hz, H-5$'$), 7.11 (1H, d, $J=8.4$ Hz, H-6$'$), 8.08 (1H, d, $J=15.8$ Hz, H-7$'$), 6.29 (1H, d, $J=15.8$ Hz, H-8$'$), 6.81 (1H, s, H-2$''$), 6.64 (1H, d, $J=8.2$ Hz, H-5$''$), 6.74 (1H, d, $J=8.0$ Hz, H-6$''$), 3.13 (1H, d, $J=11.8$ Hz, H-7$''a$), 2.99 (1H, m, H-7$''b$), 5.15 (1H, d, $J=7.6$ Hz, H-8$''$), 3.89 (3H, s, 3-OCH$_3$), 3.76 (3H, s, 3$''$-OCH$_3$).

$^{13}$C-NMR (in methanol-$d_4$, 100 MHz) $\delta$:

128.2 (C-1), 120.4 (C-2), 149.2 (C-3), 146.8 (C-4), 110.6 (C-5), 120.6 (C-6), .137.8 (C-7), 116.4 (C-8), 126.2 (C-1$'$), 131.4 (C-2$'$), 146.3 (C-3$'$), 148.2 (C-4$'$), 116.0 (C-5$'$), 121.4 (C-6$'$), 147.8 (C-7$'$), 116.2 (C-8$'$), 168.7 (C-9$'$), 130.9 (C-1$''$), 114.8 (C-2$''$), 148.7 (C-3$''$), 144.4 (C-4$''$), 114.0 (C-5$''$), 123.0 (C-6$''$), 38.6 (C-7$''$), 74.6 (C-8$''$), 173.3 (C-9$''$), 56.5(C3-OC$_3$H$_3$), 56.3 (C3$''$-OC$_3$H$_3$);

M4: A slight yellow powder, $[\alpha]_D^{20} +42.6^\circ$ (c 0.02, EtOH). UV (MeOH) $\lambda_{\text{max}}$ (log $\varepsilon$):

288 (4.42), 307 nm (4.29). IR (KBr) $\nu_{\text{max}}$: 3386, 1690, 1600, 1521, 1277, 1173, 975, 810 cm$^{-1}$. ESI-MS $m/z$: 521 [M−H]$^-$, 1043 [2M−H]$^-$.

$^1$H-NMR (in methanol-$d_4$, 400 MHz) $\delta$: 7.08 (1H, s, H-2), 6.74 (1H, d, $J=8.4$ Hz, H-5), 6.91 (1H, d, $J=8.2$ Hz, H-6), 7.18 (1H, d, $J=16.4$ Hz, H-7), 6.70 (1H, d, $J=16.4$ Hz, H-8), 6.97 (1H, d, $J=8.6$ Hz, H-5$'$), 7.10 (1H, d, $J=8.4$ Hz, H-6$'$), 8.04 (1H, d, $J=15.8$ Hz, H-7$'$), 6.29 (1H, d, $J=15.8$ Hz, H-8$'$), 6.82 (1H, s, H-2$''$), 6.64 (1H, d, $J=8.0$ Hz, H-5$''$), 6.75 (1H, d, $J=8.0$ Hz, H-6$''$), 3.14 (1H, d, $J=12.9$ Hz, H-7$''a$), 2.99 (1H, m, H-7$''b$), 5.14 (1H, d, $J=5.8$ Hz,
H-8"), 3.86 (3H, s, 3′-OCH3), 3.76 (3H, s, 3″-OCH3). 13C-NMR (in methanol-d4, 100 MHz) δ: 127.9 (C-1), 120.2 (C-2), 146.2 (C-3), 146.5 (C-4), 113.7 (C-5), 120.6 (C-6), 137.3 (C-7), 116.6 (C-8), 126.3 (C-1′), 132.6 (C-2′), 149.2 (C-3′), 148.2 (C-4′), 114.9 (C-5′), 121.0 (C-6′), 147.8 (C-7′), 116.0 (C-8′), 168.8 (C-9′), 130.4 (C-1″), 112.8 (C-2″), 148.7 (C-3″), 144.5 (C-4″), 113.9 (C-5″), 123.0 (C-6″), 38.7 (C-7″), 74.7 (C-8″), 173.4 (C-9″), 56.4 (C3′-OCH3), 56.3 (C3″-OCH3).

The UV and IR spectra of the four metabolites were similar to those of SAA, suggesting that they possessed the same skeleton. The ESI-MS data showed that M1 and M2 had the same molecular ion peaks ([M−H]−) at m/z 507, and M3 and M4 at m/z 521, which were 14 and 28 mass units higher than SAA, and suggested they were the isomer pair of monomethyl ether, and dimethyl ether of SAA, respectively. The 1H NMR spectra confirmingly showed signals of one and two methoxyl groups in these two pairs. Since these compounds had the same structure apart from the differences in methoxyl groups, the methylation position could be further determined by detailed analysis including 2D-NMR spectra and comparing the chemical shifts with those of SAA.

When compared with the parent compound SAA, M1 displayed an additional methoxy carbon signal at δ 56.4 and methoxy protons signal at δ 3.89 (s, 3H). Meanwhile, the chemical shifts of H-2 (δ 7.16, 1H) and H-6 (δ 7.00, 1H) were shifted to lower field by 0.1, C-3 (δ 149.2) to lower field, and C-4 (δ 110.5) to higher field by 3.0, respectively, which reflected the substitution effect of methoxy group on electron density of the neighbouring groups. Moreover, long-range correlations between the
methoxy protons (δ 3.89, 3H) and phenolic carbon at δ 149.2 (C-3) were observed on HMBC spectrum. The chemical structure of M1 was therefore unambiguously determined as 3-0-methyl-salvianolic acid A. By the same way, the structures of M2, M3, and M4 were identified as 3′-O-methyl-, 3, 3″-O-dimethyl-, and 3′, 3″-O-dimethyl-salvianolic acid A, respectively. Such structure characteristics of these metabolites demonstrated that O-methylation exclusively resulted in the selective formation of meta-O-methylated derivatives of SAA. In combination with the findings from in vitro enzyme inhibition experiment, these results further confirmed that COMT catalyzes the transfer of methyl group from SAM to the meta-hydroxyl group of SAA, which is the same for other phenolic compounds with a catechol structure [Axelrod and Tomchick, 1958].

**Antioxidant Activities of Methylated Metabolites.** It has been proven that antioxidant properties are the basis for various biological activities of SAA [Lu and Foo, 2002]. Contrastive investigation on antioxidant potency thus was conducted to evaluate biological activities of these obtained methylated metabolites of SAA. In vitro rat liver homogenate lipid peroxidation assay was applied, and BHT (synthetic antioxidant) and α-tocopherol (representative natural antioxidant) were used as positive controls.

As shown in Fig. 6, all the test compounds displayed significant inhibitory effect on lipid peroxidation induced by ferrous ascorbate system and the action was dose-dependent within the test concentration ranges. The half-maximal inhibitory concentrations (IC₅₀) on lipid peroxidation reaction were further calculated for
quantitative comparison based on dose-response curves. As to the positive controls, the IC₅₀ values were determined as 146.5 μM and 64.7 μM for α-tocopherol and BHT, respectively. These data were almost the same as those reported in references [Fagali and Catalá, 2012; Wang et al., 2000] and therefore indicated the reliability of the test results from the present study. SAA and its four methylated metabolites displayed the IC₅₀ values of 7.48, 0.58, 3.74, 2.77, and 1.65 μM for SAA, and M1 to M4, respectively, indicating much stronger antioxidant activities than α-tocopherol and BHT (p < 0.05). Moreover, the inhibitory effects on lipid peroxidation significantly increased with methylation of meta-hydroxyl groups on the skeleton, and both methyl-conjugated positon and the number of methylation had a certain influence on antioxidant activity of methylated metabolites of SAA.

**Discussion**

Metabolism involving phase II conjugative reactions occurs generally in SAs and other natural polyphenol compounds [Lv et al., 2010]. It has been shown that SAA in rat is metabolized by way of methylation and glucuronidation into a series of methyl conjugates and their glucuronides, which have previously been found in plasma [Shen et al., 2009]. Such unique metabolic fate produces a rather complicated metabolism profile of SAA, and making isolation of these metabolites from excreta is quite challenging. Thus it becomes a new challenge for SAA to thoroughly understand its metabolism mechanism and the active species in vivo on the basis of structure elucidation and bioactivities evaluation of metabolites.

In summary, methylation metabolism of SAA both in vivo and in vitro was
systematically investigated in the present study. Hydrolysis study using glucuronidase to cleave all the glucuronides were performed for the drug-containing rat bile. To display the full set of methylation products, LC-MS analysis was conducted for both the hydrolyzed bile sample and the untreated bile, in which XIC filtered chromatograms were applied to resolve suspected co-eluting substances, highlight potential metabolite isomers, and then provide clean chromatograms of the metabolite types of interest. The results demonstrated the presence of various methylated metabolites of SAA and its glucuronide in drug-containing bile of rats intravenously administered SAA, which indicated rapid hepatic metabolism and biliary elimination of SAA predominantly in the form of its methylated conjugates. For the first time, four major methylated metabolites present in vivo were prepared by in vitro enzyme-catalyzed incubation with SAM as methyl donor and RHC protein as COMT donor. Their chemical structures were unambiguously identified by NMR and other spectroscopic methods, and in vitro antioxidant activities against rat liver lipid peroxidation were evaluated. The findings confirmed that all these metabolites are meta-O-methylated and COMT is responsible for methylation of SAA. These methylated metabolites displayed fairly high antioxidant potency that was kept at the level of SAA or even significantly increased, suggesting in vivo pharmacological effects of SAA may be largely related to these metabolites. In order to acquire a more complete understanding of the metabolism of SAA and the potential pharmacological relevance of its metabolites, further research will be carried out on those underlying methylated metabolites derived from both methylation and glucuronidation. The
problems to be addressed may include conjugation position, bioactivity evaluation, SAR (structure-activity relationship) analysis, and quantitative mass balance of these metabolites.
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**Authorship Contributions**

Participated in research design: Xu, and Liu.

Conducted experiments: Xu, Li, Che, Tian, and Fan.

Preformed data analysis: Xu, and Li.

Contributed to the writing of the manuscript: Xu, and Li.
References:


Baba S, Osakabe N, Natsume M, and Terao J (2004) Orally administered rosmarinic acid is present as the conjugated and/or methylated forms in plasma, and is degraded and metabolized to conjugated forms of caffeic acid, ferulic acid and m-coumaric acid. *Life Sci* **75**: 165–178.


Fagali N and Catalá A (2012). The antioxidant behaviour of melatonin and structural analogues during lipid peroxidation depends not only on their functional groups but also on the assay system. *Biochem Biophys Res Commun* **423**: 873–877.


Footnotes:

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Legends For Figure:

Figure 1. Chemical structure of SAA

Figure 2. HPLC-UV, TIC and XIC chromatograms of the drug containing rat bile and the blank control. A, B and C indicated the HPLC-UV (A), TIC (B) and XIC (C) chromatograms of the blank bile, and D, E and F indicated the HPLC-UV (D), TIC (E) and XIC (F) chromatograms of the drug containing rat bile sample, respectively. SAA, M1, M2, M3, M4, L1, and L2 labeled in D indicated the parent compound, the four isolated methylation products and the two largest peaks present in rat bile, respectively. The extracted m/z values in the XIC chromatograms (C and F) were 493, 507, 521, 535, 669, 683, 697, and 711 from top to bottom.

Figure 3. HPLC-UV (A), TIC (B) and XIC (C) chromatograms of the drug containing rat bile hydrolyzed by β-glucuronidase. The extracted m/z values in the XIC chromatograms were 493, 507, 521, and 535 from top to bottom.

Figure 4. HPLC chromatograms for incubation study. A indicated the reference control of SAA. B and C indicated the blank control containing RHC and SAM, and the negative control containing heat-inactivated RHC, SAM and SAA, respectively, which were both subjected to 20 min incubation. D and E indicated the systems containing RHC, SAM and SAA for 2 min and 20 min incubation, respectively. F indicated the system containing RHC, SAM, entacapone and SAA for 20 min incubation. G indicated the chromatogram of drug-containing rat bile sample obtained under the same HPLC conditions as those of A to E. Peaks M1~M4 indicated the four major metabolites with the same retention time as those present in drug-containing rat bile.

Figure 5. HPLC chromatograms for purity check of the four methylated metabolites separated from in vitro RHC incubation system. A, B, C, D indicated M1, M2, M3, and M4, respectively.
Figure 6. Inhibitory effects on the formation of TBA reactive substance induced by ferrous ascorbate system in rat liver homogenate \textit{in vitro}. 
Table 1. LC-MS data of drug-containing rat bile (Ⅰ) and the bile sample hydrolyzed by β-glucuronidase (Ⅱ)

<table>
<thead>
<tr>
<th>Retention time /min</th>
<th>I</th>
<th>II</th>
<th>m/z of [M-H]- in XIC</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.56 ~ 16.61</td>
<td>4.76×10^5 (100)</td>
<td>1.03×10^6 (100)</td>
<td>493</td>
<td>SAA</td>
</tr>
<tr>
<td>34.54 ~ 34.58</td>
<td>7.23×10^5 (100)</td>
<td>7.39×10^5 (100)</td>
<td>507</td>
<td>monomethyl-SAA</td>
</tr>
<tr>
<td>29.69 ~ 29.76</td>
<td>4.34×10^5 (60)</td>
<td>1.99×10^5 (27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.74 ~ 30.76</td>
<td>3.61×10^4 (5)</td>
<td>1.85×10^5 (25)</td>
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<td></td>
</tr>
<tr>
<td>41.57 ~ 41.55</td>
<td>4.66×10^5 (100)</td>
<td>1.74×10^6 (100)</td>
<td>521</td>
<td>dimethyl-SAA</td>
</tr>
<tr>
<td>44.33 ~ 44.31</td>
<td>3.73×10^5 (80)</td>
<td>5.22×10^5 (30)</td>
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<td></td>
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<tr>
<td>44.87 ~ 44.91</td>
<td>3.35×10^5 (72)</td>
<td>1.09×10^6 (63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38.51 ~ 38.60</td>
<td>1.72×10^5 (37)</td>
<td>3.31×10^5 (19)</td>
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</tr>
<tr>
<td>47.89 ~ 47.95</td>
<td>1.11×10^5 (24)</td>
<td>6.96×10^4 (4)</td>
<td></td>
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<tr>
<td>62.80 ~ 62.84</td>
<td>2.98×10^5 (100)</td>
<td>3.65×10^5 (100)</td>
<td>535</td>
<td>trimethyl-SAA</td>
</tr>
<tr>
<td>52.19 ~ 52.22</td>
<td>4.47×10^4 (15)</td>
<td>4.02×10^4 (11)</td>
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<td></td>
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<tr>
<td>10.50 ~ 10.52</td>
<td>1.37×10^6 (100)</td>
<td></td>
<td>669</td>
<td>SAA glucuronide</td>
</tr>
<tr>
<td>18.25 ~ 18.28</td>
<td>1.14×10^6 (100)</td>
<td></td>
<td>683</td>
<td>monomethyl-SAA glucuronide</td>
</tr>
<tr>
<td>25.16 ~ 25.18</td>
<td>2.84×10^6 (100)</td>
<td></td>
<td>697</td>
<td>dimethyl-SAA glucuronide</td>
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<tr>
<td>31.23 ~ 31.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.40 ~ 16.42</td>
<td></td>
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<td></td>
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</tr>
</tbody>
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| 10.86 ~ 10.88 | 1.51×10⁶ (53) |
| 19.34 ~ 19.36 | 4.97×10⁵ (27) |
|               | 3.86×10⁵ (21) |
|               | 3.68×10⁵ (20) |

| 34.64 ~ 34.66 | 3.95×10⁵ (100) |
| 29.36 ~ 29.39 |                |
| 21.92 ~ 21.94 | 2.37×10⁵ (60)  |
|               | 1.58×10⁵ (40)  |

* Only those peaks with intensity larger than 3.5×10⁴, were listed.
Figure 1

Salvianolic acid A (SAA)
(C_{26}H_{22}O_{10} MW 494.45)

Danshensu (DSS)
(C_{9}H_{10}O_{5} MW 198.17)

Caffeic acid (CA)
(C_{9}H_{8}O_{4} MW 180.16)
Figure 3

A

B

C

Relative Abundance

Time (min)

100

0