Detecting metabolites of different transition metal-lithospermate B complexes after intravenous injection in rats

Ying-Jie CHEN 1, Tse-Yu CHUNG 1, Wen-Ying CHEN 2, Chung-Yu CHEN 3, Maw-Rong LEE 3, Tzyy-Rong JINN 4, Jason TC TZEN 1, 4, 5, *

1Graduate Institute of Biotechnology, 2Department of Veterinary Medicine and 3Department of Chemistry, National Chung Hsing University, Taichung 40227, Taiwan, China; 4School of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, China; 5Agricultural Biotechnology Research Center, Academia Sinica, Taipei 11529, Taiwan, China

Aim: Lithospermate B (LSB) isolated from the traditional Chinese medicine danshen (Salvia miltiorrhiza) is an effective Na⁺/K⁺-ATPase inhibitor and used to treat congestive heart failure. The inhibition of LSB on Na⁺/K⁺-ATPase is potentiated by forming complexes with transition metal ions. Here we investigated the safety and metabolites of different transition metal-LSB complexes in rats.

Methods: LSB complexed with six different transition metal ions (Mg²⁺, Zn²⁺, Cr³⁺, Co²⁺, Ni²⁺ and Mn²⁺) were prepared. Adult male SD rats were injected with the different metal-LSB complexes (50 mg/kg, iv), and their bile and blood samples were collected. The metabolites of the metal-LSB complexes in the samples were analyzed using mass spectroscopy.

Results: In rats injected with LSB complexed with Mg²⁺, Zn²⁺, Cr³⁺, Ni²⁺ or Mn²⁺, LSB and its four putative metabolites were equivalently detected in their bile samples. Mn²⁺-LSB exhibited distinct metabolite profiles compared with the other four metal-LSB complexes. The four putative metabolites were identified as 3-monomethyl-LSB, 3,3′′-dimethyl-LSB, 3,3′′′-dimethyl-LSB and 3,3′′,3′′′-trimethyl-LSB. The tracking of successive bile samples of rats injected with Mg²⁺-LSB, Zn²⁺-LSB and Mn²⁺-LSB concurrently demonstrated that LSB was firstly methylated at position 3, then at position 3′, and, finally, the 3′′ hydroxyl group. All rats injected with Co²⁺-LSB died.

Conclusion: Zn²⁺-LSB, Cr³⁺-LSB, Ni²⁺-LSB or Mn²⁺-LSB produces identical four methylated metabolites of LSB in rats, and seemed to be as safe as LSB or Mg²⁺-LSB.

Keywords: lithospermate B; transition metal complex; drug metabolism; metabolite; methylation; Na⁺/K⁺-ATPase inhibitor; danshen; traditional Chinese medicine

Introduction

Na⁺/K⁺-ATPase, an active transporter of sodium and potassium ions, is responsible for maintaining membrane potentials, the cell volume, and the active transport of other solutes in animal cells[1]. The therapeutic effect of cardiac glycosides in the treatment of congestive heart failure depends on the reversible inhibition of the Na⁺/K⁺-ATPase located in the cell membrane of the human myocardium[2, 3]. Although the inhibition of the Na⁺/K⁺-ATPase produces beneficial effects in patients with congestive heart failure, severe side effects and the narrow therapeutic index of cardiac glycosides have evidently limited their clinical applications[4].

Many steroid-like compounds found in a variety of Chinese herbs used for promoting blood circulation were demonstrated to be inhibitors of Na⁺/K⁺-ATPase, and thus regarded as the active ingredients responsible for their cardiac therapeutic effects via the same molecular mechanism triggered by cardiac glycosides[5–9]. However, no appreciable level of steroid-like compounds were found in danshen (Salvia miltiorrhiza), a well-known Chinese herb traditionally used for promoting blood circulation[10]. Instead, lithospermate B (LSB) in complex with Mg²⁺ was found to be the major soluble ingredient in danshen and shown to be an effective inhibitor of Na⁺/K⁺-ATPase, which is presumably responsible for the cardiac therapeutic effect of this herb[11]. Being non-toxic antioxidants without apparent adverse effects, Mg²⁺-LSB and LSB may be used as substitutes for cardiac glycosides for the treatment of congestive heart failure[12].

To evaluate in vivo pharmacological activities, the metabolic fate of Mg²⁺-LSB was examined in rats[13]. Four major metabolites were excreted into bile after the intravenous injection of Mg²⁺-LSB, and identified via mass spectrometry.
as meta-O-methylated derivatives of LSB, namely 3-monomethyl-LSB, 3,3′′-dimethyl-LSB, 3,3′′′-dimethyl-LSB, and 3,3′′′,3′′′′-trimethyl-LSB. These methylated metabolites were found to be potent antioxidants, and thus assumed to be largely responsible for the pharmacological effects of Mg\(^{2+}\)-LSB.

In complex with Mg\(^{2+}\), LSB possesses a relatively rigid structure due to the formation of salt bridges between Mg\(^{2+}\) and the four oxygen atoms of the carboxyl groups on the four caffeic acid fragments[14]. Comparatively, the rigid structure around the salt bridges formed between Mg\(^{2+}\) and carboxyl groups partially mimics the core steroid structure of cardiac glycosides. Recently, we demonstrated that some transition metal ions were able to replace Mg\(^{2+}\) to form stable complexes with LSB[15]. The in vitro potencies (ie, the inhibition of Na\(^+\)/K\(^-\)-ATPase activity) of LSB complexed with Cr\(^{3+}\), Mn\(^{2+}\), Co\(^{2+}\), or Ni\(^{2+}\) increased by approximately 5 times compared with the naturally occurring LSB and Mg\(^{2+}\)-LSB. Thus, the transition metal-LSB complexes have the potential to be superior substitutes for cardiac glycosides in the treatment of congestive heart failure. To further explore this potential utilization, we aimed to examine, in this study, the safety and metabolites of transition metal-LSB complexes after intravenous injection in rats.

**Materials and methods**

**Chemicals and reagents**

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glacial acetic acid (>99.7%) was obtained from J T Baker Chemical Co (Phillipsburg, NJ, USA). Phosphoric acid (85%) and analytic grade formic acid were bought from Merck Millipore (Gibbstown, NJ, USA). Water was purified by a Millipore clear water purification system (Direct-Q, Millipore, Billerica, MA, USA). Puriﬁed LSB was a gift from KO DA Pharmaceutical Co (Taiwan, China). Mg(OH)\(_2\) was purchased from Showa Chemical Co (Tokyo, Japan), while NaOH, MnCl\(_2\), NiCl\(_2\), CrCl\(_3\), and CoCl\(_2\) were obtained from Sigma-Aldrich Co (St Louis, MO, USA).

**Preparation of metal-LSB complexes**

Metal-LSB complexes were prepared and characterized as described in our previous study[15]. Briefly, Mg\(^{2+}\)-LSB, and Zn\(^{2+}\)-LSB complexes were prepared in 20 mL of H\(_2\)O by mixing equimolar concentrations of LSB (a ﬁnal concentration of 50 mmol/L) with Mg(OH)\(_2\) and Zn(OH)\(_2\), respectively. To prepare Cr\(^{3+}\)-LSB, Mn\(^{2+}\)-LSB, Co\(^{2+}\)-LSB, and Ni\(^{2+}\)-LSB complexes, LSB (50 mmol/L) was ﬁrst precipitated with NaOH (100 mmol/L) in 20 mL of ethanol, and the precipitation was then dissolved by adding CrCl\(_3\), MnCl\(_2\), CoCl\(_2\), and NiCl\(_2\) (50 mmol/L) to form metal-LSB complexes, respectively. These metal-LSB complexes were lyophilized at -86 °C and stored at -20 °C prior to usage. The purity of metal-LSB complexes in powder was approximately 85% as estimated by HPLC analysis.

**Animal studies**

Male Sprague-Dawley rats weighing 250–270 g were purchased from BioLasco, Taiwan Co, Ltd (Taiwan, China). The animals were adapted in a standard controlled environment of 23±2°C, 60%±10% humidity and a 12-h light/dark cycle, and fed with hard rat chow pellets (Fwusow Ind Corp, Taiwan, China) and puriﬁed water ad libitum. The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC Approval No: 101–107(8)).

**Bile collection and preparation**

Thirty-three male Sprague-Dawley rats were fasted overnight but had access to water ad libitum. The animals were anesthetized with Zoletil 50® (40 mg/kg, ip; Virbac Laboratories, Carros, France) and remained anesthetized during the surgical operation. LSB or LSB complexes with Mg\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\), and Co\(^{2+}\) (50 mg/kg, iv) were dissolved in normal saline and Cr\(^{3+}\)-LSB complex was dissolved in 50% poly(ethylene glycol)–400 (v/v) (Fluka Chemie, Buchs, Switzerland); solutions were injected into the right femoral vein. Bile fистulae of the rats were cannulated with PE-20 polyethylene tubing for the collection of bile. The bile was collected into successive tubes on ice at 10 or 30 min intervals for 60 min after a single intravenous dosing. Bile samples of 200 μL were vortex-mixed with two volumes of methanol containing 0.1% H\(_3\)PO\(_4\) for 10 min, and centrifuged at 10000×g for 20 min at 4°C. The supernatant was ﬁltered through a 0.22 μm polyvinylidene diﬂuoride (PVDF) membrane ﬁlter (PALL Corp, Glen Cove, NY, USA), and used for the following analyses.

**Blood sampling and preparation**

The left femoral vein was cannulated with PE-50 polyethylene tubing and connected with a 23-gauge needle for blood sampling. After intravenous administration with 100 mg/kg of Zn\(^{2+}\)-LSB from the right femoral vein, blood samples of 300 μL were withdrawn in heparinized tubes on ice at 0, 5, 15, 30, and 60 min. Plasma was obtained by centrifugation at 3000×g for 15 min at 4°C. For analysis, plasma (100 μL) was mixed with methanol (200 μL) containing 0.1% H\(_3\)PO\(_4\) and vortexed for 10 min. After centrifugation at 10000×g for 20 min at 4°C, the supernatant was ﬁltered by a 0.22 μm PVDF membrane ﬁlter and subjected to HPLC and LC/MS/MS analyses.

**HPLC/UV and LC/MS/MS analyses**

Bile and plasma samples were analyzed by HPLC coupled to a Waters Corp 600 controller pump with a 2996 photodiode array detector and a 717 autosampler (Milford, MA, USA). The separation was achieved using a Syncronis column (250 mm×4.6 mm id, 5 μm) from Thermo Scientiﬁc (Waltham, MA, USA). The HPLC mobile phase comprised (A) water with 0.5% acetic acid (v/v) and (B) acetonitrile. The gradient for metabolism analysis started at 95% solvent A and 5% solvent B for 5 min, followed by a linear increase of solvent B to 70% for 20 min, and then a decrease of solvent B to 5% for 5 min. For a continual sample analysis, the column was equilibrated with 5% solvent B for 10 min before the next sample injection. The injection volumes of bile and blood samples were
20 and 150 μL, respectively. In all analyses, the column was kept at room temperature, and the flow rate was 1 mL/min. The metabolites of bile and blood were analyzed by a Thermo Finnigan™ LTQ™ linear ion trap mass spectrometer (Thermo LTQ XL, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface, and connected to a Thermo Scientific Surveyor LC plus system equipped with a Surveyor MS pump plus and a Surveyor autosampler (Thermo Scientific, San Jose, CA, USA). The mass spectra were obtained in a negative ESI mode. The spray voltage was 3.7 kV and the heated capillary temperature was 300°C. Sheath gas and auxiliary gas flow rates were 30 and 3 arbitrary units, respectively. The full mass spectra were obtained at a mass-to-charge ratio (m/z) scan rate from 150 to 1500. To obtain the product ion spectra, the relative collision energy of the collision-induced dissociation (CID) was set at 24% for m/z 717 and 23% for m/z 731, 745, and 759. Separations were performed using a Waters Corp Xbridge™ C18 column (100 mm×2.1 mm id, 3.5 μm; Milford, MA, USA) at room temperature, and the injection volume was 5 μL at a flow rate of 0.15 mL/min. The tray and column oven temperature were set at 4 and 30°C, respectively. The mobile phase comprised (C) water with 0.2% formic acid (v/v) and (D) acetonitrile with 0.2% formic acid (v/v). The program for gradient elution started at 95% solvent C and 5% solvent D for 5 min, followed by a linear increase of solvent D to 70% for 30 min, maintained at 70% solvent D for 8 min, and then a decrease of solvent D to 5% for 2 min. The detection wavelength was set at 288 nm.

Statistical analysis
The metabolite intensities (peak areas) were analyzed with Student’s t-test performed by SigmaStat (Version 3.5). P values less than 0.05 were considered statistically significant.

Results
Metabolites of metal-LSB complexes in rat bile
To examine the excretion of biliary metabolites, bile samples of three rats were collected after intravenous injection with 50 mg/kg of LSB complexed with Mg²⁺, Zn²⁺, Cr³⁺, Ni²⁺, Mn²⁺, or Co²⁺. Surprisingly, rats injected with Co²⁺-LSB perished. Three more rats were used to repeat Co²⁺-LSB intravenous injections; however, all three rats again perished in our experimental conditions. Regardless, similar profiles of metabolites in bile samples were observed when rats were injected with LSB and the rest of metal-LSB complexes except for Mn²⁺-LSB. For each metal-LSB complex, similar results were observed among the three injected rats; a representative pattern of each metal-LSB was shown to illustrate the metabolite profile (Figure 1).

Identification of biliary metabolites
Five peaks were consistently observed in the biliary metabolites of rats injected with any of the metal-LSB complexes, as observed in the metabolite profile of Zn²⁺-LSB in rat bile (Figure 2A). According to previous studies on the metabolites of Mg²⁺-LSB, the five peaks were putatively identified by LC/MS/MS as LSB and four meta-O-methylated metabolites of LSB, namely 3-monomethyl-LSB (M1), 3,3′′-dimethyl-LSB (M2), 3,3′′′-dimethyl-LSB (M3), and 3,3′,3′′′-trimethyl-LSB (M4; Figures 2B and 2C). The data displayed the extracted ion chromatograms [M-H]⁻ for LSB at m/z 717 and MS² ions at m/z 717 and 519 with a 23.3 min retention time, M1 at m/z 731 and MS² ions at m/z 731 and 533 with a 24.5 min retention time, M2 and M3 at m/z 745 and MS² ions at m/z 745 and 547 with 26.1 and 25.7 min retention times, respectively, and M4 at m/z 759 and MS² ions at m/z 759 and 547 with a 24.5 min retention time. ESI-MS showed that M1, M2, M3, and M4 had molecular ion peaks at m/z 731, 745, 745, and 759 [M+CH₃-H], respectively. The molecular weights of the four metabolites were 14, 28, 28, and 42 mass units higher than that of LSB, as expected of the four methylated metabolites. The same mass spectrometric outcomes were observed for the five equivalent peaks found in the biliary metabolites of rats injected with LSB, Mg²⁺-LSB, Cr³⁺-LSB, Ni²⁺-LSB, or Mn²⁺-LSB (data not shown).

In vivo metabolism of Mg²⁺-LSB, Zn²⁺-LSB, and Mn²⁺-LSB
To monitor the metabolism of metal-LSB complexes in detail, bile samples were collected at 10 min intervals for 60 min after rats were intravenously injected with 50 mg/kg of LSB complexed with Mg²⁺, Zn²⁺, and Mn²⁺. Detailed tracking of the three successive bile samples concurrently suggested that in the following metabolism, LSB was first methylated to form M1, which was further methylated to form M2 (relatively fast) and M3 (relatively slow); as a final note, both M2 and M3 were complementarily methylated to form M4 (Figures 3 and 4). It seemed that the methylation of LSB occurred sequentially at three sites, ie, first at position 3, then 3′, and, finally, the 3′′ hydroxyl group. Relatively speaking, the metabolism (ie, methylation at the three hydroxyl groups) of Mn²⁺-LSB was faster than those of Mg²⁺-LSB and Zn²⁺-LSB. However, the elimination rate of the M4 methylated from Mn²⁺-LSB was relatively slow and remained a major metabolite in bile compared with the results from Mg²⁺-LSB and Zn²⁺-LSB methylations.

Plasma metabolites of Zn²⁺-LSB
Because similar profiles of metabolites in bile samples were observed when rats were injected with LSB and the metal-LSB complexes, Zn²⁺-LSB was representatives selected to inspect plasma metabolites of metal-LSB complexes. Zn²⁺-LSB of 100 mg/kg was used for intravenous injections in rats, and blood samples were collected at 0, 5, 15, 30, and 60 min after injections. In the HPLC profile, M1 and M2 were barely detected in the plasma 5 min after injection (Figure 5A). Nevertheless, monomethyl-LSB (M1), dimethyl-LSB (M2) and trimethyl-LSB (M4) were all detectable in the LC/MS/MS analyses (Figures 5B–5F). In agreement with the HPLC profile, M1 and M2 were detected in the plasma 5 min after injection, while M4 was detected 15 min after injection. However, the relatively minor intermediate metabolite, M3, was undetectable in this analytic condition. Overall, the results suggest that the metabolites of metal-LSB complexes in plasma are fundamentally identical to those in bile.
Discussion

In the present study, four meta-O-methylated metabolites (M1, M2, M3, and M4) were detected in bile samples of rats after intravenous injections of LSB, Mg\textsuperscript{2+}-LSB, Zn\textsuperscript{2+}-LSB, Cr\textsuperscript{3+}-LSB, Ni\textsuperscript{2+}-LSB, and Mn\textsuperscript{2+}-LSB. These four methylated metabolites were identical to those detected in rat bile after intravenous administrations of LSB and Mg\textsuperscript{2+}-LSB in a previous study\cite{16}. Presumably, the four methylated LSB metabolites were sequentially formed by a hepatic enzyme, catechol O-methyltransferase (COMT), which catalyzed the transfer of the methyl group from S-adenosyl methionine to the meta-hydroxyl group of the catechol moiety prior to enterohepatic circulation in rats\cite{17}. The methylation of phenolic compounds tend to result in a lower polarity and a higher metabolic stability by preventing the conjugation of glucuronic acid and sulfate groups, and can thus, be regarded as a route to improve the NQO1-inducing activities of phenolic acids, such as LSB, in danshen\cite{18, 19}. Our results suggest that in the treatment of cardiovascular diseases, for at least a comparable dosage lower than 50 mg/kg, iv in rats, the artificial LSB complexes with transition metals Zn\textsuperscript{2+}, Cr\textsuperscript{3+}, Ni\textsuperscript{2+}, and Mn\textsuperscript{2+} have potential as safe, even superior therapeutic substitutes for LSB and Mg\textsuperscript{2+}-LSB naturally isolated from danshen.

According to our observations, the metabolic rates of metal-LSB complexes via methylation by COMT were comparable except for that of Mn\textsuperscript{2+}-LSB (Figure 1). M1 and M2 were found to be major metabolites of LSB and of Mg\textsuperscript{2+}-LSB, Zn\textsuperscript{2+}-LSB, Cr\textsuperscript{3+}-LSB, Ni\textsuperscript{2+}-LSB, and Mn\textsuperscript{2+}-LSB. These four methylated metabolites were identical to those detected in rat bile after intravenous administrations of LSB and Mg\textsuperscript{2+}-LSB in a previous study\cite{16}. Presumably, the four methylated LSB metabolites were sequentially formed by a hepatic enzyme, catechol O-methyltransferase (COMT), which catalyzed the transfer of the methyl group from S-adenosyl methionine to the meta-hydroxyl group of the catechol moiety prior to enterohepatic circulation in rats\cite{17}. The methylation of phenolic compounds tend to result in a lower polarity and a higher metabolic stability by preventing the conjugation of glucuronic acid and sulfate groups, and can thus, be regarded as a route to improve the NQO1-inducing activities of phenolic acids, such as LSB, in danshen\cite{18, 19}. Our results suggest that in the treatment of cardiovascular diseases, for at least a comparable dosage lower than 50 mg/kg, iv in rats, the artificial LSB complexes with transition metals Zn\textsuperscript{2+}, Cr\textsuperscript{3+}, Ni\textsuperscript{2+}, and Mn\textsuperscript{2+} have potential as safe, even superior therapeutic substitutes for LSB and Mg\textsuperscript{2+}-LSB naturally isolated from danshen.

![Figure 1. HPLC chromatogram and color of bile collected from rats at basal, 0–30 min and 31–60 min after intravenous administration of LSB (A), Mg\textsuperscript{2+}-LSB (B), Zn\textsuperscript{2+}-LSB (C), Cr\textsuperscript{3+}-LSB (D), Ni\textsuperscript{2+}-LSB (E), or Mn\textsuperscript{2+}-LSB (F). Rats were injected with each sample at 50 mg/kg.](image-url)
Cobalt ion is one of the necessary essential elements for humans as suggested by the World Health Organization (WHO), and its daily recommended intake is 5–40 μg/day\cite{20}. It stimulates the production of erythropoietin and red blood cells for the prevention of anemia. Additionally, cobalt ions raise the blood oxygen-carrying capacity to prevent ischemia and hypoxia. However, an overdose of cobalt ion may be harmful due to its toxic effects on the hematopoietic system\cite{21}, thyroid\cite{22}, and lungs\cite{23}; additionally, its neurotoxicity\cite{24}, cardiomyopathy\cite{25}, and carcinogenicity\cite{26} have been reported. The adverse effects were consistently observed when the concentration of cobalt ion in human blood exceeded 800 μg/L\cite{27}. The oral LD$_{50}$ values of Wistar and Sprague-Dawley rats for single administrations of cobalt ion were reported to be 42, 317, 631, and 3672 mg/kg for cobalt chloride, cobalt carbonate, cobalt sulfate, and tricobalt tetraoxide, respectively\cite{28-32}. The acute LD$_{50}$ values of cobalt chloride was 20 mg/kg (equivalent to 9.1 mg/kg cobalt ion) in rats after intravenous injection\cite{33}. In our experiment, the concentration of cobalt ion in rats injected with 50 mg/kg of Co$^{2+}$-LSB was equivalent to 3.8 mg/kg of cobalt ion, far below the reported harmful dosage. The reason why rats perished after intravenous injection with 50 mg/kg Co$^{2+}$-LSB should be clarified in follow-up studies. Meanwhile, whether a lower dosage of Co$^{2+}$-LSB can be used to develop a new substitute for cardiac glycosides in the treatment of congestive heart failure requires cautious evaluation.

**Acknowledgements**

The work was supported by a grant to Jason TC TZEN of National Chung-Hsing University (NCHU-102D604), Taiwan, China.

---

**Figure 2.** (A) UV and extracted ion chromatograms for the [M-H]$^-$ ions of Zn$^{2+}$-LSB at 717 m/z, the monomethyl-M1 metabolite at 731 m/z, the dimethyl-M2 and M3 metabolites at 745 m/z, and the trimethyl-M4 metabolite at 759 m/z. (B) MS/MS spectra of [M-H]$^-$ ions of Zn$^{2+}$-LSB, the monomethyl-M1, the dimethyl-M2 and M3, and the trimethyl-M4 metabolites. (C) Structures of metal-LSB complexes and four metabolites. Metal represents Mg$^{2+}$, Zn$^{2+}$, Cr$^{3+}$, Ni$^{2+}$, or Mn$^{2+}$. 

![UV and extracted ion chromatograms](image1)

![MS/MS spectra](image2)

![Structures of metal-LSB complexes and four metabolites](image3)
**Author contribution**

Jason TC TZEN and Tzyy-Rong JINN designed research; Ying-Jie CHEN performed the animal experiments and HPLC analysis; Tse-Yu CHUNG prepared the metal-LSB complexes; Wen-Ying CHEN guided the animal experiments; Chung-Yu CHEN and Maw-Rong LEE performed the LS/MS/MS analysis; Jason TC TZEN and Ying-Jie CHEN wrote the paper.

**References**

1. Skou JC, Esmann M. The Na,K-ATPase. J Bioenerg Biomembr 1992; 24: 249–61.
2. Li-Saw-Hee FL, Lip GY. Digoxin revisited. QJM 1998; 91: 259-64.
3. Melero CP, Medarde M, San Feliciano A. A short review on cardiotonic steroids and their aminoguanidine analogues. Molecules 2000; 5: 51–81.
4. Yang Z, Luo H, Wang H, Hou H. Preparative isolation of bufalin and cinobufagin from Chinese traditional medicine ChanSu. J Chromatogr Sci 2008; 46: 81–5.
5. Chen RYJ, Chung TY, Li FY, Lin NH, Tzen JTC. Effect of sugar positions in ginsenosides and their inhibitory potency on Na⁺/K⁺-ATPase activity. Acta Pharmacol Sin 2009; 30: 61–9.
6. Chen RYJ, Chung TY, Li FY, Yang WH, Jinn TR, Tzen JTC. Steroid-like compounds in Chinese medicines promote blood circulation via inhibition of Na⁺/K⁺-ATPase. Acta Pharmacol Sin 2010; 31: 696–702.
7. Chen YC, Liu YL, Li FY, Chang CI, Wang SY, Lee KY, et al. Antcin A, a steroid-like compound from *Antrodia camphorata*, exerts anti-inflammatory effect via mimicking glucocorticoids. Acta Pharmacol Sin 2011; 32: 904–11.
8. Chung TY, Li FY, Chang CI, Jinn TR, Tzen JTC. Inhibition of Na⁺/K⁺-ATPase by antcins, unique steroid-like compounds in *Antrodia camphorata*. Am J Chin Med 2012; 40: 953–65.
9. Tzen JTC, Chen RYJ, Chung TY, Chen YC, Lin NH. Active compounds in Chinese herbs and medicinal animal products which promote blood circulation via inhibition of Na⁺, K⁺-ATPase. Chang Gung Med J 2010; 33: 126–36.
10. Chen RYJ, Jinn TR, Chen YC, Chung TY, Yang WH, Tzen JTC. Active ingredients in Chinese medicines promoting blood circulation as Na⁺/K⁺-ATPase inhibitors. Acta Pharmacol Sin 2011; 32: 141–51.
11. Tzen JTC, Jinn TR, Chen YC, Li FY, Cheng FC, Shi LS, et al. Magnesium lithospermate B possesses inhibitory activity on Na⁺, K⁺-ATPase and neuroprotective effects against ischemic stroke. Acta Pharmacol Sin 2007; 28: 609–15.
12. Chen YC, Jinn TR, Chung TY, Li FY, Fan RJ, Tzen JTC. Magnesium lithospermate B extracted from *Salvia miltiorrhiza* elevates intracellular Ca²⁺ level in SH-SYSY cells. Acta Pharmacol Sin 2010; 31: 923–9.
13. Zhang Y, Akao T, Nakamura N, Hattori M, Yang XW, Duan CL, et al. Magnesium lithospermate B is excreted rapidly into rat bile mostly as methylated metabolites, which are potent antioxidants. Drug Metab Dispos 2004; 32: 752–7.
14. Lu Y, Foo LY. Polyphenolics of *Salvia*—a review. Phytochemistry 2002; 59: 117–40.
15. Lin NH, Chung TY, Li FY, Cheng FC, Shi LS, et al. Enhancing the potency of lithospermate B for inhibiting Na⁺/K⁺-ATPase activity by forming transition metal ion complexes. Acta Pharmacol Sin 2013; 34: 893–900.
16. Cui L, Chan W, Wu JL, Jiang ZH, Chan K, Cai Z. High performance liquid chromatography-mass spectrometry analysis for rat metabolism and pharmacokinetic studies of lithospermic acid B from danshen. Talanta 2008; 75: 1002–7.
17. Axelrod J, Tomchick R. Enzymatic O-methylation of epinephrine and other catechols. J Biol Chem 1958; 233: 702–5.
18. Walle T. Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. Int J Mol Sci 2009; 10: 5002–19.
19. Zhang X, Song Z, Xu J, Ma Z. Improving the NQ01-inducing activities of phenolic acids from radix *Salvia miltiorrhiza*: a methylation strategy. Chem Biol Drug Des 2011; 78: 558–66.
compounds WHO, Geneva; 2006.

21 Horiguchi H, Oguma E, Nomoto S, Arao Y, Ikeda K, Kayama F. Acute exposure to cobalt induces transient methemoglobinuria in rats. Toxicol Lett 2004; 151: 459–66.

22 Lantin AC, Mallants A, Vermeulen J, Speybroeck N, Hoet P, Lison D. Absence of adverse effect on thyroid function and red blood cells in a population of workers exposed to cobalt compounds. Toxicol Lett 2011; 201: 42–6.

23 Sauni R, Linna A, Oksa P, Nordman H, Tuppurainen M, Uitti J. Cobalt asthma—a case series from a cobalt plant. Occup Med (Lond) 2010; 60: 301–6.

24 Catalani S, Rizzetti MC, Padovani A, Apostoli P. Neurotoxicity of cobalt. Hum Exp Toxicol 2012; 31: 421–37.

25 Linna A, Oksa P, Groundstroem K, Halkosaari M, Palmroos P, Huikko S, et al. Exposure to cobalt in the production of cobalt and cobalt compounds and its effect on the heart. Occup Environ Med 2004; 61: 877–85.

26 Magaye R, Zhao J, Bowman L, Ding M. Genotoxicity and carcinogenicity of cobalt-, nickel- and copper-based nanoparticles. Exp Ther Med 2012; 4: 551–61.

27 Finley BL, Monnot AD, Gaffney SH, Paustenbach DJ. Dose-response relationships for blood cobalt concentrations and health effects: a review of the literature and application of a biokinetic model. J Toxicol Environ Health B Crit Rev 2012; 15: 493–523.

28 Singh P, Junnarkar A. Behavioural and toxic profile of some essential trace metal salts in mice and rats. Indian J Pharmacol 1991; 23: 153–9.

29 FDRL. Acute oral LD50 study of cobalt sulphate lot No S88336/A in Sprague-Dawley rats (FDRL Study No 8005D). Food and Drug Research Laboratories, Inc, Waverly, NY 1984.

30 FDRL. Study of cobalt (II) carbonate tech gr CoCO3, lot #030383 in Sprague-Dawley rats. Food and Drug Research Laboratories, Inc, Waverly, NY 1984.

31 FDRL. Acute oral toxicity study of cobalt oxide tricobalt tetroxide in Sprague-Dawley rats. Food and Drug Research Laboratories, Inc, Waverly, NY 1984.

32 Reagan EL. Acute oral LD50 study in rats with cobalt sulfate. Int J Toxicol 1992; 11: 688.

33 Domingo JL, Llobet JM, Corbella J. The effects of EDTA in acute cobalt intoxication in rats. Toxicol Eur Res 1983; 5: 251–5.
Figure 5. Identification of plasma metabolites after intravenous injection of Zn\(^{2+}\)-LSB with a dosage of 100 mg/kg. Zn\(^{2+}\)-LSB and its plasma metabolites at 0, 5, 15, 30, and 60 min after injection were analyzed by HPLC (A). Extracted ion chromatograms for the [M-H] \(^{-}\) ions of plasma metabolites were detected for the samples of 0 min (B), 5 min (C), 15 min (D), 30 min (E), and 60 min (F) at \(m/z\) 717, 731, 745, and 759.