Acta Pharmacologica Sinica (2014) 35: 937–944

www.nature.com/aps

Original Article

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

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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 (Mg^{2+}, Zn^{2+}, Cr^{3+}, Co^{2+}, Ni^{2+} and Mn^{2+}) 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 Mg^{2+}, Zn^{2+}, Cr^{3+}, Ni^{2+} or Mn^{2+}, LSB and its four putative metabolites were equivalently detected in their bile samples. Mn^{2+}-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 Mg^{2+}-LSB, Zn^{2+}-LSB and Mn^{2+}-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 Co^{2+}-LSB died.
Conclusion: Zn^{2+}-LSB, Cr^{3+}-LSB, Ni^{2+}-LSB or Mn^{2+}-LSB produces identical four methylated metabolites of LSB in rats, and seemed to be as safe as LSB or Mg^{2+}-LSB.

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

Acta Pharmacologica Sinica (2014) 35: 937–944; doi: 10.1038/aps.2014.37

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 Mg^{2+} 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, Mg^{2+}-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 Mg^{2+}-LSB was examined in rats[13]. Four major metabolites were excreted into bile after the intravenous injection of Mg^{2+}-LSB, and identified via mass spectrometry

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Received 2014-01-24 Accepted 2014-03-31

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_2O 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(R)).

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 fistulas 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_3PO_4 for 10 min, and centrifuged at 10 000×*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_3PO_4 and vortexed for 10 min. After centrifugation at 10 000×*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™ C18 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 Mg^{2+}, Zn^{2+}, Cr^{3+}, Ni^{2+}, Mn^{2+}, or Co^{2+}. Surprisingly, rats injected with Co^{2+}-LSB perished. Three more rats were used to repeat Co^{2+}-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 Mn^{2+}-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 Zn^{2+}-LSB in rat bile (Figure 2A). According to previous studies on the metabolites of Mg^{2+}-LSB[13, 16], 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 MS^2 ions at *m/z* 717 and 519 with a 23.3 min retention time, M1 at *m/z* 731 and MS^2 ions at *m/z* 731 and 533 with a 24.5 min retention time, M2 and M3 at *m/z* 745 and MS^2 ions at *m/z* 745 and 547 with 26.1 and 25.7 min retention times, respectively, and M4 at *m/z* 759 and MS^2 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 $[M+CH_3-H]^-$, 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, Mg^{2+}-LSB, Cr^{3+}-LSB, Ni^{2+}-LSB, or Mn^{2+}-LSB (data not shown).

In vivo metabolism of Mg^{2+}-LSB, Zn^{2+}-LSB, and Mn^{2+}-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 Mg^{2+}, Zn^{2+}, and Mn^{2+}. 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 Mn^{2+}-LSB was faster than those of Mg^{2+}-LSB and Zn^{2+}-LSB. However, the elimination rate of the M4 methylated from Mn^{2+}-LSB was relatively slow and remained a major metabolite in bile compared with the results from Mg^{2+}-LSB and Zn^{2+}-LSB methylations.

Plasma metabolites of Zn^{2+}-LSB

Because similar profiles of metabolites in bile samples were observed when rats were injected with LSB and the metal-LSB complexes, Zn^{2+}-LSB was representatively selected to inspect plasma metabolites of metal-LSB complexes. Zn^{2+}-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.

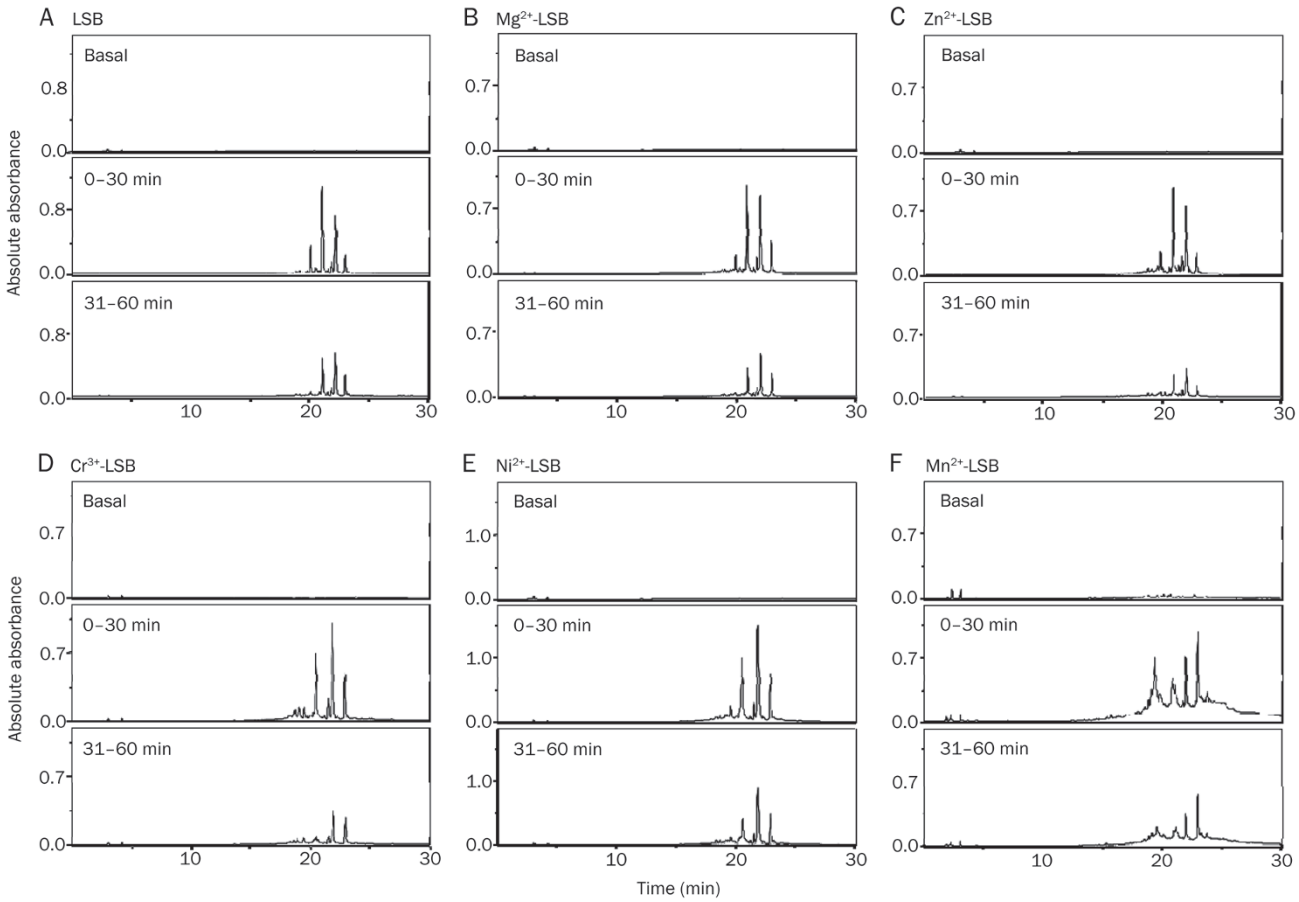

Figure 1. HPLC chromatogram and color of bile collected from rats at basal, 0–30 min and 31–60 min after intravenous administration of LSB (A), Mg^{2+}-LSB (B), Zn^{2+}-LSB (C), Cr^{3+}-LSB (D), Ni^{2+}-LSB (E), or Mn^{2+}-LSB (F). Rats were injected with each sample at 50 mg/kg.

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^{2+}-LSB, Zn^{2+}-LSB, Cr^{3+}-LSB, Ni^{2+}-LSB, and Mn^{2+}-LSB. These four methylated metabolites were identical to those detected in rat bile after intravenous administrations of LSB and Mg^{2+}-LSB in a previous study[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[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[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^{2+}, Cr^{3+}, Ni^{2+}, and Mn^{2+} have potential as safe, even superior therapeutic substitutes for LSB and Mg^{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^{2+}-LSB (Figure 1). M1 and M2 were found to be major metabolites of LSB and of Mg^{2+}-LSB, Zn^{2+}-LSB, Cr^{3+}-LSB, and Ni^{2+}-LSB, while M2 and M4 were the major metabolites of Mn^{2+}-LSB. In contrast with the metabolism of other metal-LSB complexes, Mn^{2+}-LSB-converted M1 was immediately methylated to form M2; however, the elimination rate of its further methylated metabolite, M4, was relatively slow, which resulted in its accumulation as a major metabolite of Mn^{2+}-LSB. It is likely that Mn^{2+}-LSB and its monomethylated metabolite (M1) are better substrates of COMT than other metal-LSB complexes and their monomethylated metabolites, while the trimethylated metabolite (M4) of Mn^{2+}-LSB is relatively resistant to additional metabolism in rats. Whether the different metabolic fate of Mn^{2+}-LSB, in comparison with those of other metal-LSB complexes, leads to a distinctive pharmacological effect on the therapeutic activity of LSB remains to be evaluated.

Unexpectedly, all the rats died within 10 min after intravenous administration with 50 mg/kg of the Co^{2+}-LSB complex. In a preliminary test, rats survived when the intravenous administration of Co^{2+}-LSB was reduced to less than one-fifth

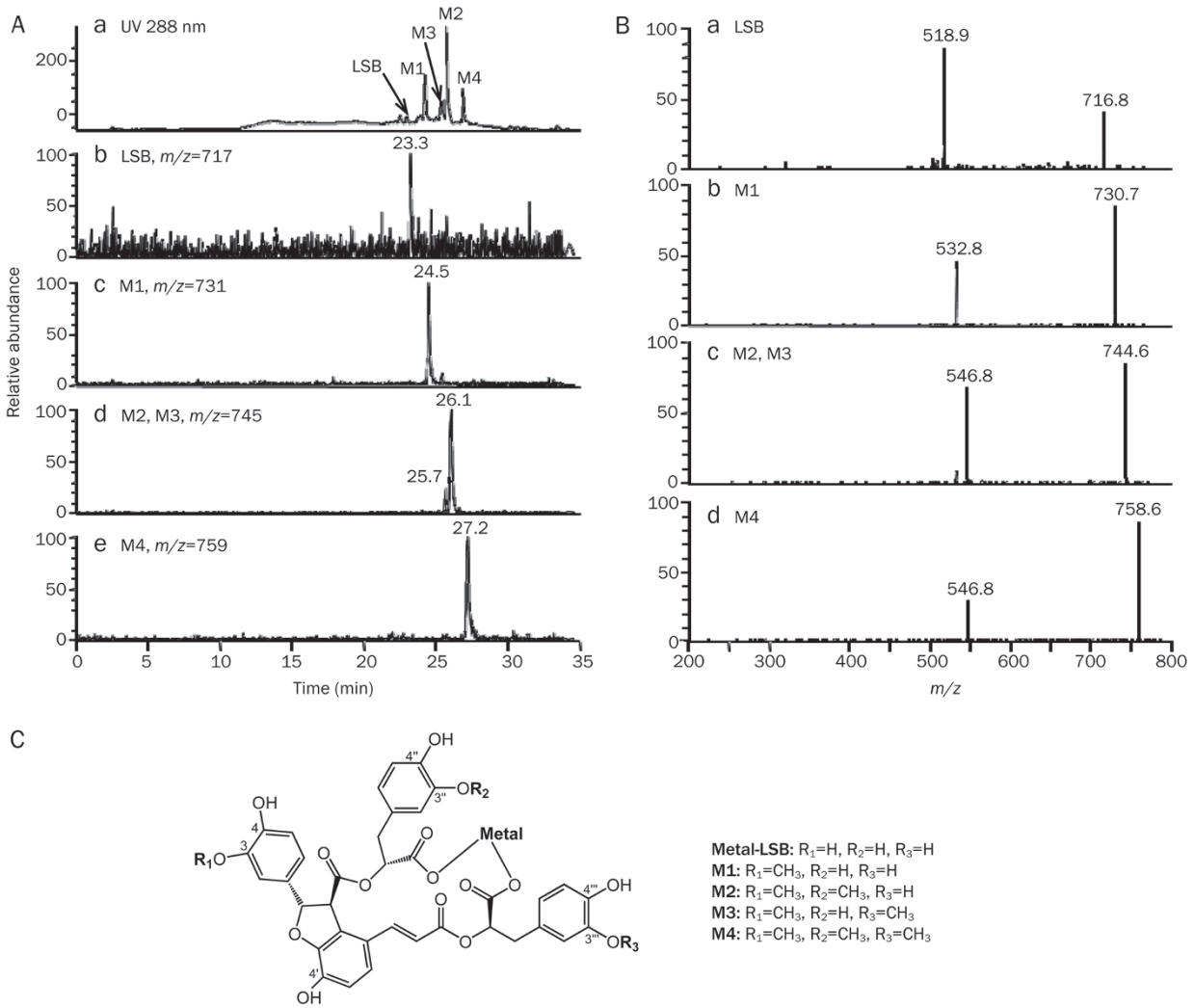

Figure 2. (A) UV and extracted ion chromatograms for the $[M-H]^-$ ions of Zn^{2+}-LSB at 717 *m/z*, the monomethyl-M1 metabolite at 731 *m/z*, the dimethyl-M2 and M3 metabolites at 745 *m/z*, and the trimethyl-M4 metabolite at 759 *m/z*. (B) MS/MS spectra of $[M-H]^-$ ions of Zn^{2+}-LSB, the monomethyl-M1, 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+}.

(10 mg/kg; data not shown). 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[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[21], thyroid[22], and lungs[23]; additionally, its neurotoxicity[24], cardiomyopathy[25], and carcinogenicity[26] have been reported. The adverse effects were consistently observed when the concentration of cobalt ion in human blood exceeded 800 μg/L[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[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[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.

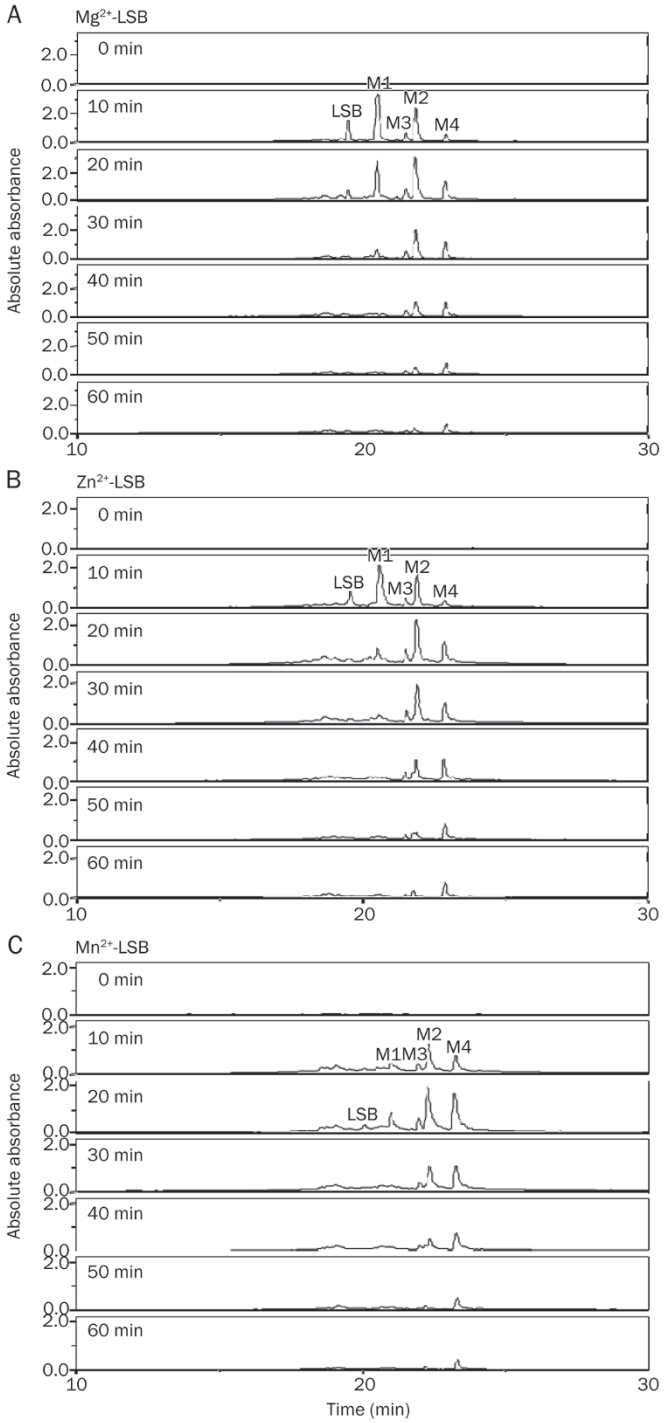

Figure 3. HPLC profiles of bile metabolites collected from rats injected with Mg^{2+}-LSB (A), Zn^{2+}-LSB (B), and Mn^{2+}-LSB (C) complexes. Rats were injected with each metal-LSB at 50 mg/kg, and bile samples were collected at 0, 10, 20, 30, 40, 50, and 60 min after injection.

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.

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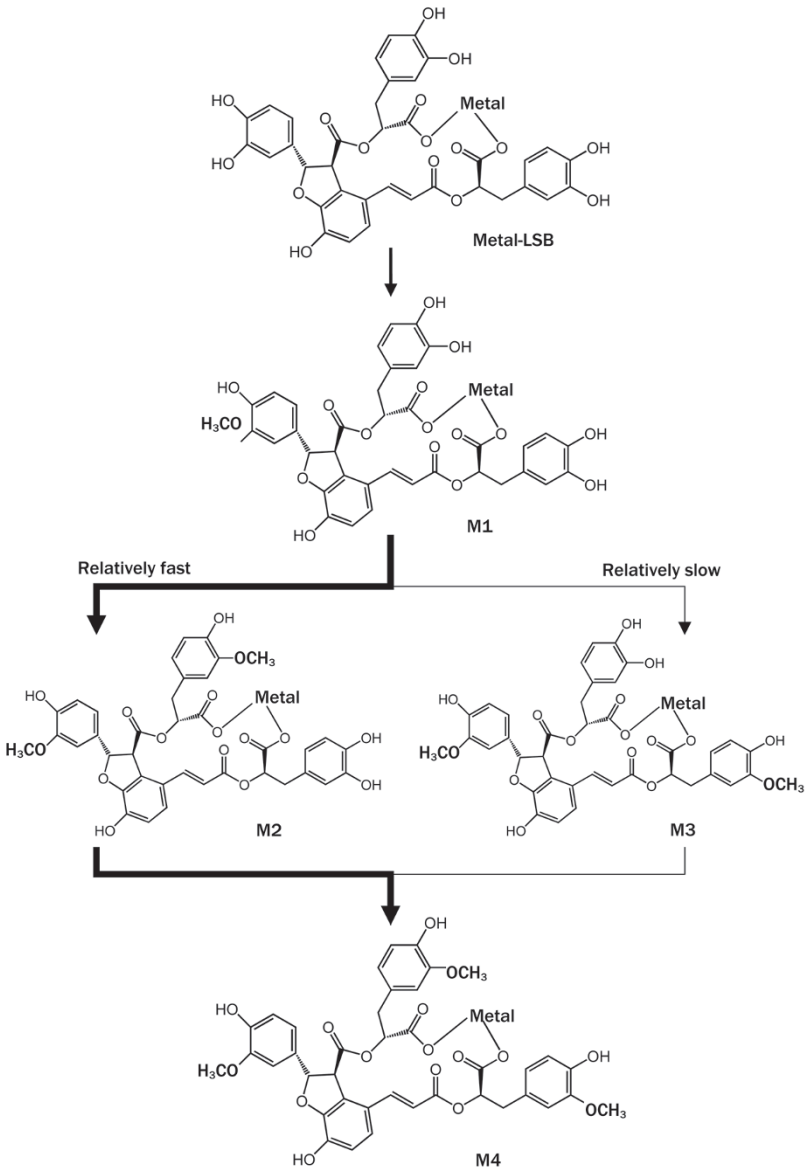

Figure 4. Proposed metabolic pathway of metal-LSB complex. M1, M2, M3, and M4 stand for 3-monomethyl-LSB, 3,3′′-dimethyl-LSB, 3,3′′′-dimethyl-LSB, and 3,3′′,3′′′-trimethyl-LSB, respectively. Metal represents Mg^{2+}, Zn^{2+}, Cr^{3+}, Ni^{2+}, or Mn^{2+}.

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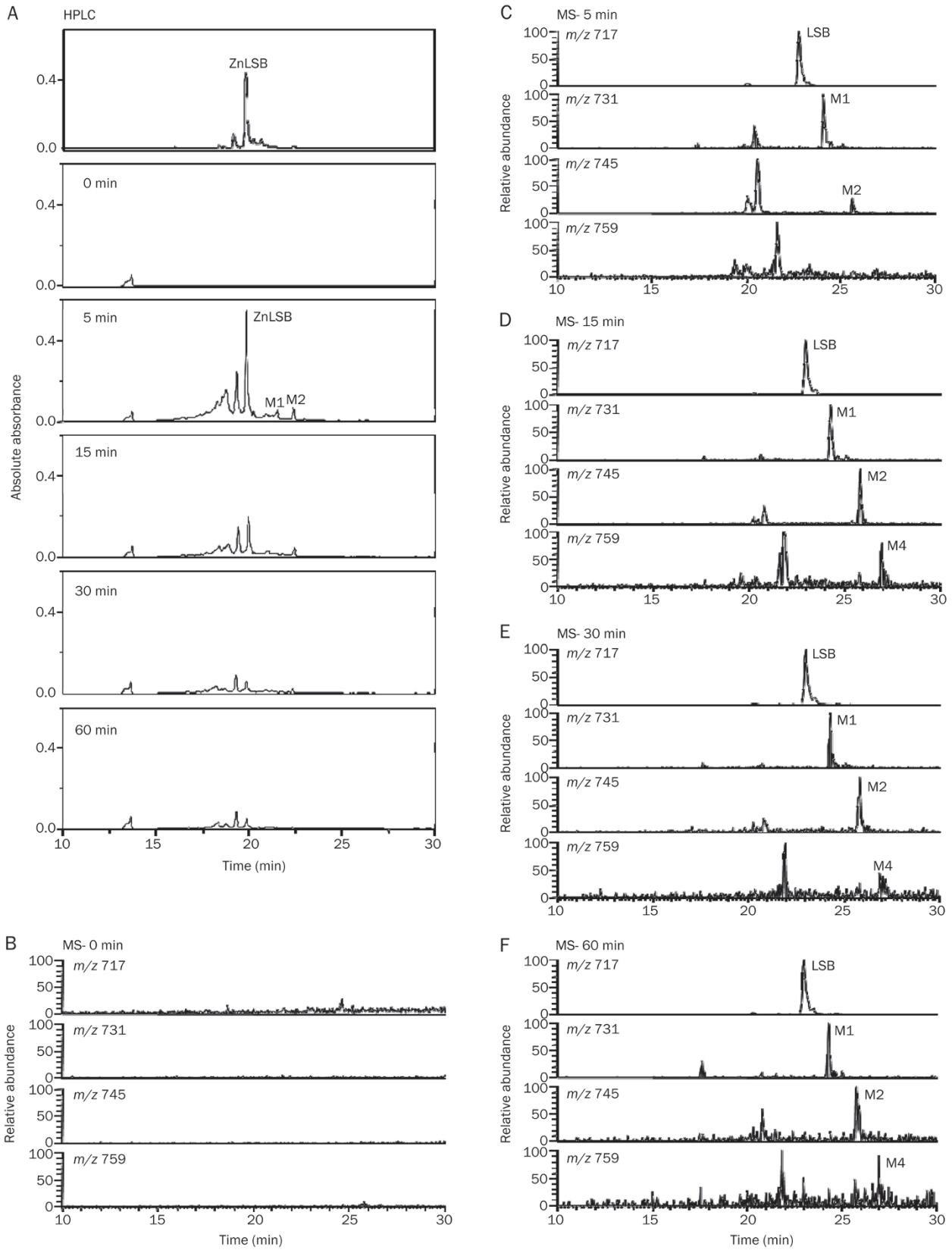

Figure 5. Identification of plasma metabolites after intravenous injection of Zn^{2+}-LSB with a dosage of 100 mg/kg. Zn^{2+}-LSB and its plasma metabolites at 0, 5, 15, 30, and 60 min after injection were analyzed by HPLC (A). Extracted ion chromatograms for the $[M-H]^-$ ions of plasma metabolites 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.