Pharmacokinetics of magnesium lithospermate B after intravenous administration in beagle dogs[1]

Xiao-chuan LI, Chen YU [2], Wei-kang SUN, Gang-yi LIU[2], Jing-ying JIA[2], Yi-ping WANG[3]

Department of Pharmacology, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203; [2]Central Laboratory, Shanghai Xuhui Central Hospital, Shanghai 200002, China

KEY WORDS *Salvia miltiorrhiza*; magnesium lithospermate B; pharmacokinetics; liquid chromatography-tandem mass spectrometry

ABSTRACT

AIM: To develop a specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the pharmacokinetic study of magnesium lithospermate B (MLB), and study the pharmacokinetics of MLB after iv administration in beagle dogs. **METHODS:** Each beagle dog was iv administered MLB 3, 6, and 12 mg/kg random. The serum drug concentration was determined by specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays. The pharmacokinetic parameters were calculated by Drug and Statistics version 1.0 program. **RESULTS:** The calibration curve for MLB was linear over a range of 16-4096 μg/L with coefficients of correlation >0.999. The intra- and inter-day precisions (CV) of analysis were <10 %, and accuracy ranged from 90 % to 113 %. After iv administration of MLB at the doses of 3, 6, and 12 mg/kg, the C_0 values for MLB were estimated to be of 24, 47, and 107 mg/L, respectively. The AUC increased with the increasing doses for iv administration, and the mean $AUC_{0\text{-}t}$ values were 109.3, 247.9, and 582.4 mg·min·L^{-1}, respectively. MLB was distributed and eliminated quickly from central compartment, the mean $T_{1/2\alpha}$ values for MLB at doses of 3, 6, 12 mg/kg were 2.2, 2.7, and 2.9 min, and the mean $T_{1/2\beta}$ values were 43, 42, and 42 min, respectively. **CONCLUSION:** This LC-MS/MS method is rapid, sensitive, and specific for the pharmacokinetic study of MLB. The kinetic process of MLB in beagle dogs *in vivo* was best fitted to a two-compartment model. For iv administration, the pharmacokinetic parameters of C_0 and AUC have good linearity among the doses, and MLB was distributed and eliminated quickly in beagle dogs.

INTRODUCTION

The Chinese medicine, danshen, is the dried root and rhizome of *Salvia miltiorrhiza* Bge (Labiatae)[1]. Traditionally, danshen is believed to be effective in eliminating blood stasis, relieving pain, promoting blood flow, stimulating menstrual discharge as well as easing the mind[1]. Therefore, it is widely used in many Chinese medicine preparations and formulae. Recent pharmacological studies have indicated that magnesium lithospermate B (MLB, also named lithospermic B magnesium salt, or magnesium tanshinoate B) (Fig 1A) isolated from the aqueous extract of danshen is a biologically active component[2]. MLB has been found to have strong antioxidative and free radical scavenging effect [3-8]. In addition, MLB has been shown to protect against the pathological processes of the organ system, such as renal dysfunction, liver damage, and lung fibrosis[9-16].

[1] Project supported by the National High Technology Research and Development Program (863 Program, 2003AA2Z3269).
[3] Correspondence to Prof Yi-ping WANG.
Phn 86-21-5080-6733. Fax 86-21-5080-7088.
E-mail ypwang@mail.shcnc.ac.cn
Received 2004-04-15 Accepted 2004-07-15

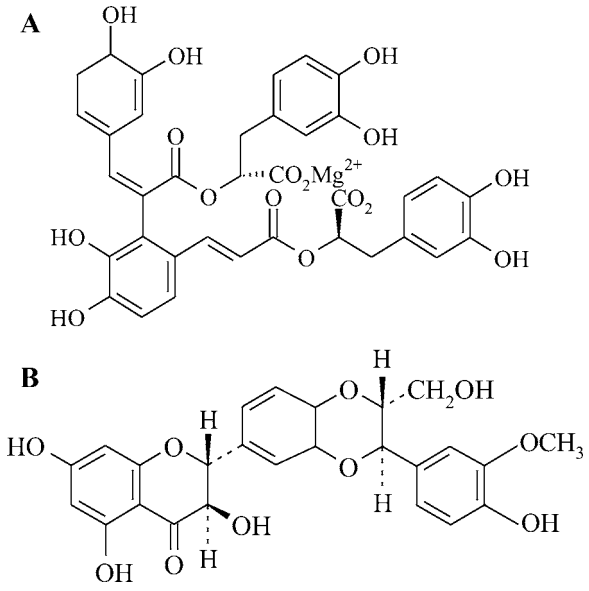

Fig 1. Chemical structure of MLB (A) and silibinin (B) (internal standard).

As a result, there is great interest in the therapeutic potentials of MLB.

MLB is easily oxidative and extremely unstable in water, and has high affinity to proteins in blood; recently Zhang *et al* found that MLB had extremely low systemic bioavailability in rats by using HPLC-ECD method to detect the plasma[17]. However, to our knowledge, no publication describing quantitative analysis of MLB using mass spectrometry has been published until now. And the pharmacokinetic parameters of iv administration are useful and necessary for the clinical study of MLB. In this paper we present a rapid and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to detect serum concentrations of MLB, and describe the pharmacokinetics of MLB after intravenous administration in beagle dogs.

MATERIALS AND METHODS

Chemicals and reagents MLB and silibinin (internal standard, IS) were provided by Department of Phytochemistry, Shanghai Institute of Materia Medica. The purity of these compounds was above 99.8 %, which was verified by the supplier using HPLC methods. Acetonitrile (HPLC grade) were purchased from Fisher (Fair Lawn, NJ, USA). Ethyl acetate and acetone (AR grade) were purchased from Shanghai Chemical Reagent Co Ltd (Shanghai, China). All other chemicals were purchased from commercial sources and used as received.

Animal treat Beagle dogs (male and female, 11.3±0.9 kg, Certificate No SCXKC2002.0004) were purchased from the Animal Center of School of Agriculture and Biology, Shanghai Jiaotong University (Shanghai, China). Six beagle dogs were used in this experiment for determining serum concentrations of MLB after iv administration. Each beagle dog was given a single dose of MLB 3, 6, or 12 mg/kg at random every 3 d, respectively, and the assay was done in three period. The dose of MLB 6 mg/kg administered to beagle dogs was decided according to the dose conversion factors between beagle dog and human. Beagle dogs were used without fasting before iv administration. The solutions of MLB were formulated shortly before administration such that the desired dosages were delivered in a volume of 10 mL. The solution of MLB was diluted with sodium chloride injection (0.9 %).

Blood samples (0.5 mL) were collected into tubes from the vein of each beagle dog. After iv injection (1 min) in beagle dogs, the sampling was performed at 1, 3, 6, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, and 150 min. After stored at 4 ºC for 2 h, blood was processed for serum by centrifugation at 3000×*g* for 10 min. The serum samples with high concentrations of MLB were diluted with blank serum. All serum samples were frozen and maintained at -20 ºC until analysis.

Extraction procedures The serum sample (100 μL) was mixed with 10 μL of the IS solution (silibinin 4 mg/L) and 15 μL of formic acid-water (1:3, v:v). Then 0.2 mL of acetone-water (70:30, v:v) solution was added using an Eppendorf repeater pipette, followed by the addition of 1 mL ethyl acetate to each tube. The tubes were then vortex-mixed for 1.5 min on a vortexer and centrifuged at 16 000×*g* for 2 min. The organic layer was transferred to a clean test tube and evaporated to dryness under gentle stream of nitrogen at 30 ºC. The residue was dissolved with 200 μL of water (containing 25 % acetone), vortexed, and then centrifuged at 16 000×*g* for 1 min, and a volume of 10 μL of the supernatant was introduced into the LC-MS/MS system.

LC-MS/MS analysis The LC-MS/MS analysis was performed using a 5- μm CAPCELL PAK C18 column (50 mm×2 mm ID, SHISEIDO, JAPAN), and a Phenomenex C_{18} guard column was used before the analytical column with a mobile phase consisting of 56 % water (containing a mass fraction of 0.5 % formic acid) and 44 % acetonitrile at a flow-rate 0.25 mL/min for an isocratic elution at room temperature.

The LC-MS/MS system comprised an HPLC system (including a vacuum degasser, a quaternary pump and an autosampler; SHIMADZU, JAPAN) coupled to Perkin-Elmer SCIEX API-3000 triple-quadruple mass spectrometer (Foster city, CA, USA) equipped with ESI source. The LC-MS/MS system was controlled by Analyst 1.3.1 software. The operating parameters of the ion source, including the compound-dependent and the source-dependent ones, were optimized to obtain the best performance from the mass spectrometry for the analysis of MLB and silibinin. The sensitivity of detection in negative ion mode was found to be much higher than that in positive ion mode. Therefore, instrumental parameters were selected that maximized generation of the molecule ($[M-H]^-$) of the test compound, and that also efficiently produced characteristic fragment ions. The source-dependent parameters of MLB and silibinin consisted of the flow rates of the nebulizer gas, the curtain gas, the collision gas, the ionspray voltage, and the temperature of heated gas, with optimum values of 12, 10, 12 L/min, -3300V, and 300 ºC, respectively. The compound-dependent parameters were also tuned for the test compound to achieve the highest instrument response. The mass spectrometer was operated at low mass resolution for both Q1 and Q3 in multiple reactions monitoring (MRM) mode. The precursor-to product ion transitions were *m/z* 717.2→519.2 for MLB, and *m/z* 481.0→300.9 for silibinin.

Preparation of calibration curves The primary stock solutions of MLB and silibinin (1.000 g/L) were prepared in methanol and serially diluted to working solution with water. All the stock and working solutions were stored at -20 ºC. The working solutions of MLB were prepared from the stock solutions by dilution with water to the concentrations of 40.96, 20.48, 10.24, 5.12, 2.56, 1.28, 0.64, 0.32, and 0.16 mg/L. Blank serum (90 μL) was mixed with 10 μL of the working solutions, 10 μL of the IS working solutions (silibinin 4 mg/L), and 15 μL of the 25 % formic acid. The resultant serum concentrations were 4 096, 2 048, 1 024, 512, 256, 128, 64, 32, and 16 μg/L. The serum samples for calibration were extracted with ethyl acetate and prepared under the same conditions as the test samples. Calibration curves were constructed using a linear regression of the test compound/IS peak area ratio (Y) to nominal serum concentrations of the test compound (X, μg/L) with weighting of reciprocal concentration ($1/X$).

Validation procedures The intra-day and inter-day accuracy and precision of the method presented here was investigated by analyzing serum samples of known amounts at four different concentrations 16, 20, 1600, and 3200 mg/L for MLB. The stability of the analytes in serum at autosampler temperature (4 ºC) was investigated by assessing QC samples after 0, 2.5, 4, and 8 h. Freeze-thaw cycle's stability was done after three times of cycles. The assay of dilution was also done to test the recoveries and RSD. The recoveries of all these samples were 90 %-105 % and the RSD were less than 10 %.

Pharmacokinetic analysis The pharmacokinetic parameters were calculated by Drug and Statistics version 1.0 (Anhui Provincial Center for Drug Clinical Evaluation, China) program. We used a two-compartment model and a weighing function of $1/C^2$ for data fitting and parameter estimation. All data were expressed as mean±SD. Statistical analysis was performed using statistical program SPSS. To evaluate dose proportionality, the AUC- and C_0-relationships were analyzed by linear regression. Statistical differences of the pharmacokinetic parameters were determined by means of analysis of variance (ANOVA).

RESULTS

Method validation Under the selected LC-MS condition, typical retention time for MLB and silibinin were 1.15 min and 1.45 min with no interferences from endogenous serum components at the retention time corresponding to the analytes. The limit of detection (LOD), defined as the lowest concentration that could give a signal-to-noise ratio of 3:1, was 1.5 μg/L. The typical chromatograms of MLB in beagle dog serum were illustrated in Fig 2.

Calibration curves exhibited excellent linearity over a range of 16-4096 μg/L for MLB, the typical regression equation for MLB was: Y=0.00367X-0.0206 (r=0.9994, X: 16-4 096 μg/L), and the precision and accuracy values of calibration curves were shown in Tab 1. The intra- and inter-assay precision and accuracy values of the method, assessed using spiked serum samples at concentration of 16, 20, 1600, and 3200 μg/L for MLB were shown in Tab 2. The intra-day and inter-day RSD were similar throughout the range of the calibration with determined concentration within 10 % of the theoretical values. The stability of the analytes in serum at autosampler temperature was great, and the CVs were determined to be <6.9 % and the recoveries were 90 %-102 %.

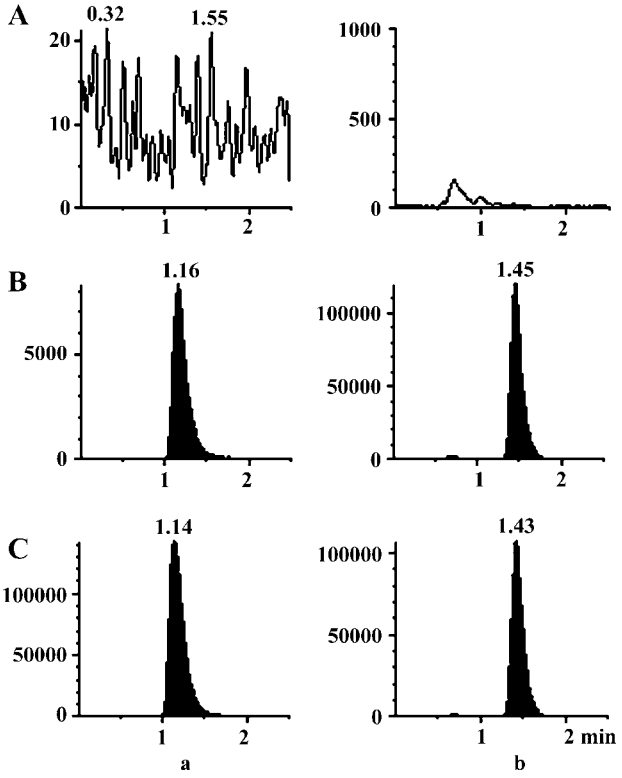

Fig 2. Chromatograms of MLB and silibinin (internal standard, IS) in beagle dog serum. A) Blank serum sample; B) Serum sample spiked with MLB 256 μg/L and IS; C) Serum sample 1 h after iv administration of MLB 6 mg/kg to a beagle dog. Peak a, b refer to MLB and IS, respectively.

Tab 1. Accuracy and precision of the calibration curve in validation method. *n*=6.

Nominal conc /μg·L^{-1}	Mean back-calculation conc/μg·L^{-1}	CV/%	Accuracy/%
16	17	0.4	108.4
32	30	2.2	95.2
64	69	1.3	108.4
128	124	2.8	97.0
256	246	1.5	96.1
512	496	5.7	96.8
1024	974	3.5	95.2
2048	2095	0.6	102.3
4096	4124	3.2	100.7
Slope	0.0367		
Intercept	-0.0206		
R	0.9994		

Tab 2. Accuracy and precision of the LC-MS/MS method to determine MLB in beagle dog serum.

Concentration/ μg·L^{-1}	Intra-day (*n*=6) CV/%	Accuracy/%	Inter-day (*n*=5) CV/%	Accuracy/%
16	1.9	113.0	8.5	100.6
20	2.9	97.6	5.0	95.5
1600	2.1	92.4	5.3	98.0
3200	1.1	90.3	9.1	96.7

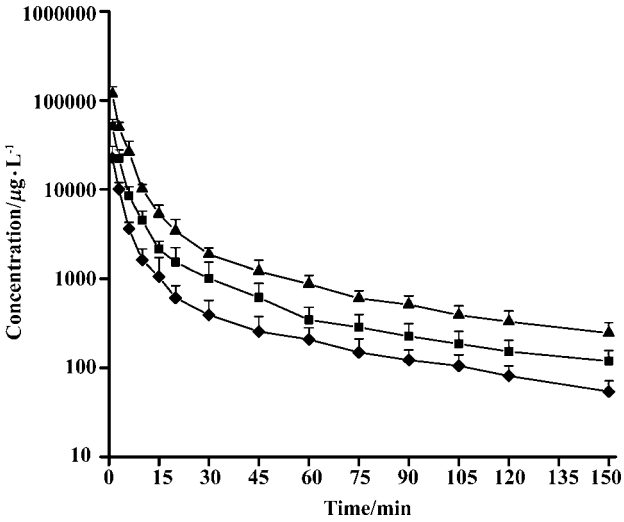

Fig 3. Profiles of mean serum concentration of MLB versus time after iv administration of MLB to beagle dogs. (◆ 3 mg/kg ; ■ 6 mg/kg; ▲ 12 mg/kg.)

Tab 3. Pharmacokinetic parameters of MLB after iv administration to beagle dogs. *n*=6. Mean±SD.

Parameter	Dose/mg·kg^{-1} 3	6	12
C_0/mg·L^{-1}	24±4	47±12	107±15
$AUC_{0\text{-}tn}$/mg·min·L^{-1}	109±24	248±55	582±84
$AUC_{0\text{-}\infty}$/mg·min·L^{-1}	109±24	248±55	583±84
$MRT_{0\text{-}\infty}$/min	16.3±3.8	14.8±2.9	14.0±0.8
*k*10/h^{-1}	13.2±2.1	11.6±1.5	11.2±1.3
*k*12/h^{-1}	5.2±1.6	3.9±2.2	3.1±0.5
*k*21/h^{-1}	1.5±0.5	1.4±0.4	1.4±0.3
V/L·kg^{-1}	1.7±0.5	1.6±0.6	1.3±0.3
$T_{1/2\alpha}$/min	2.2±0.2	2.7±0.5	2.9±0.3
$T_{1/2\beta}$/min	43±9	42±7	42±10
CL/mL·min^{-1}·kg^{-1}	28±5	26±7	21±3

Pharmacokinetics in beagle dogs The mean serum concentration-time profiles of MLB after iv administration were shown in Fig 3. Serum concentration versus time data was analyzed and the pharmacokinetic parameters of three representative doses were shown in Tab 3. Model discrimination was assessed by the analysis of the data, and actually most concentration-time profiles were best fitted to a two-compartment model.

After iv administration of MLB at doses of 3, 6, and 12 mg/kg, the C_0 values for MLB were estimated to be of 24, 47, and 107 mg/L, respectively. The AUC increased with increasing doses for iv administration, and the mean AUC_{0-t} values were 109, 248, and 582 $mg \cdot min \cdot L^{-1}$, respectively.

$T_{1/2\alpha}$ values for MLB at doses of 3, 6, and 12 mg/kg were 2.2±0.2 min, 2.7±0.5 min, 2.9±0.3 min, and k_{12} for all doses were larger than k_{21}. So MLB was distributed quickly at these doses. And MLB at doses of 3, 6, and 12 mg/kg was eliminated quickly from central compartment, and the mean $T_{1/2\beta}$ values were 43±9 min, 42±7 min, and 42±10 min, respectively. The *V* values were 1.7±0.5, 1.6±0.6, 1.27±0.27 L/kg, and CL was relatively constant from 28±5 to 21±3 $mL \cdot min^{-1} \cdot kg^{-1}$.

The analysis of variance of CL, $T_{1/2\beta}$, *V*, and MRT showed no difference among the three doses of treatments after iv administration ($P>0.05$). The linearity of the kinetics of MLB after iv administration was found in the regression analysis of the AUC-dose plot, and these plots indicated good linearity ($r>0.94$, $P<0.01$), and the C_0-dose relationship was also linear ($r>0.96$, $P<0.01$).

DISCUSSION

In this study, a sensitive and specific liquid chromatography-tandem mass spectrometry method was developed for the quantitative determination of MLB in beagle dog serum. The method has been well validated and applied to measure serum concentrations of biological samples of MLB in beagle dogs. We know that MLB has its optical isomer from its structure (Fig 1A), which existed less than 0.5 % in the sample, and both of them were measured as the concentration of MLB in the experiment. MLB is easily oxidative and extremely unstable; it is difficult to keep the stability in the extraction procedure and detection process. In the extraction procedure of serum samples, formic acid and acetone were used to increase the extraction recoveries and stability of MLB. And the sample dissolved with acetone-water is stable even keeping in the autosampler (4 ºC) for more than 24 h. The RSD and recoveries of all samples were good. In a word, this LC-MS/MS method was stable, sensitive, and specific.

It can be seen that the large values of CL (28-21 $mL \cdot min \cdot kg^{-1}$) and *V* (1.73-1.27 L/kg) after MLB was intravenously administered to beagle dogs at the dose of 3, 6, and 12 mg/kg, and Zhang *et al* reported that excretion of the unchanged MLB in urine and bile was extremely low (6 %)[17]. These results suggested MLB had rapid distribution and metabolism in tissues. The serum concentrations of MLB at the dose of 3 mg/kg were extremely low after 2.5 h of iv administration, the serum concentrations of MLB at 1 min after iv administration were almost 500 times of that at 150 min. The low values of $T_{1/2\alpha}$ and $T_{1/2\beta}$ also indicated that MLB had a wide distribution in beagle dogs, which might be due to their high affinity to tissues and organs.

Moreover, though the linearity between AUC and doses was good ($r=0.947$) for iv administration, the AUC value at the high dose increased more than the proportional increase from that at the low dose, and the CL and *V* values obtained at the high dose were lower than those at the low dose. These data suggested a saturated distribution and metabolism might occur at the high dose. In the next assay, we will explore the distribution of MLB in tissues and organs.

In conclusion, a method of liquid chromatography-tandem mass spectrometry to detect the serum concentrations of MLB has been developed and validated in dog serum. The assay was rapid, sensitive, specific, and reproducible. These studies have shown the linear pharmacokinetics of C_0 and AUC of MLB after iv administration in beagle dogs.

ACKNOWLEDGEMENTS We thank Prof Yan-lin ZENG (Department of Pharmacology, Shanghai Institute of Materia Medica) for his helpful suggestion on the calculation of pharmacokinetic parameters.

REFERENCES

1 The State Pharmacopoeia Commission of China. Pharmacopoeia of the People's Republic of China; v 1. Beijing: Chemical Industry Press; 2000.

2 Tanaka T, Morimoto S, Gen-Ichiro N, Nishioka I, Yokozawa T, Hae YC, *et al*. Magnesium and ammonium-potassium lithospermate B, the active principles having an uremia-preventive effect from *Salvia miltiorrhiza*. Chem Pharm Bull 1989; 37: 340-4.

3 Shigematsu T, Tajima S, Nishikawa T, Murad S, Pinnell SR,

Nishioka I. Inhibition of collagen hydroxylation by lithospermic acid magnesium salt, a novel compound isolated from *Salviae miltiorrhizae* Radix. Biochim Biophys Acta 1994; 1200: 79-83.

4 Fung KP, Wu J, Zeng LH, Wong HN, Lee CM, Hon PM, *et al*. Lithospermic acid B as an antioxidant-based protector of cultured ventricular myocytes and aortic endothelial cells of rabbits. Life Sci 1993; 53: 189-93.

5 Yokozawa T, Chung HY, Dong E, Oura H. Confirmation that magnesium lithospermate B has a hydroxyl radical-scavenging action. Exp Toxicol Pathol 1995; 47: 341-4.

6 Kasimu R, Tanaka K, Tezuka Y, Gong ZN, Li JX, Basnet P, *et al*. Comparative study of seventeen *Salvia* plants: aldose reductase inhibitory activity of water and MeOH extracts and liquid chromatography-mass spectrometry (LC-MS) analysis of water extracts. Chem Pharm Bull (Tokyo) 1998; 46: 500-4.

7 Wu XJ, Wang YP, Wang W, Sun WK, Xu YM, Xuan LJ. Free radical scavenging and inhibition of lipid peroxidation by magnesium lithospermate B. Acta Pharmacol Sin 2000; 21: 855-8.

8 O K, Lynn EG, Vazhappilly R, Au-Yeung KK, Zhu DY, Siow YL. Magnesium tanshinoate B (MTB) inhibits low density lipoprotein oxidation. Life Sci 2001; 68: 903-12.

9 Yokozawa T, Lee TW, Chung HY, Oura H, Nonaka G, Nishioka I. Renal responses to magnesium lithospermate B. J Pharm Pharmacol 1990; 42: 712-5.

10 Yokozawa T, Lee TW, Oura H, Nonaka G, Nishioka I. Effect of magnesium lithospermate B in rats with sodium-induced hypertension and renal failure. Nephron 1992; 60: 460-5.

11 Kamata K, Noguchi M, Nagai M. Hypotensive effects of lithospermic acid B isolated from the extract of *Salviae miltiorrhizae* Radix in the rat. Gen Pharmacol 1994; 25: 69-73.

12 Hase K, Kasimu R, Basnet P, Kadota S, Namba T. Preventive effect of lithospermate B from *Salvia miltiorrhiza* on experimental hepatitis induced by carbon tetrachloride or *D*-galactosamine/lipopolysaccharide. Planta Med 1997; 63: 22-6.

13 Yokozawa T, Dong E, Oura H, Kashiwagi H, Nonaka G, Nishioka I. Magnesium lithospermate B suppresses the increase of active oxygen in rats after subtotal nephrectomy. Nephron 1997; 75: 88-93.

14 Wang W, Wang YP, Sun WK, Xu YM, Xuan LJ. Effects of magnesium lithospermate B on aggregation and 5-HT release in rabbit washed platelets. Acta Pharmacol Sin 2000; 21: 859-63.

15 Luo WB, Wang YP. Magnesium lithospermate B inhibits hypoxia-induced calcium influx and nitric oxide release in endothelial cells. Acta Pharmacol Sin 2001; 22: 1135-42.

16 Au-Yeung KK, Zhu DY, O K, Siow YL. Inhibition of stress-activated protein kinase in the ischemic/reperfused heart: role of magnesium tanshinoate B in preventing apoptosis. Biochem Pharmacol 2001; 62: 483-93.

17 Zhang Y, Akao T, Nakamura N, Duan CL, Hattori M, YangXW, *et al*. Extremely low bioavailability of magnesium lithospermate B, an active component from *Salvia miltiorrhiza*, in rat. Planta Med 2004; 70: 138-42.