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The effect of deep eutectic solvent on the pharmacokinetics of salvianolic acid B in rats and its acute toxicity test

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ABSTRACT

Deep eutectic solvent (DES), the benign green solvent with uniquely physical properties, has been widely applied in various fields. Our previous study indicated that DES could improve the stability and extraction efficiency of salvianolic acid B (SAB). In this work, with SAB as a model drug, the feasibility of DES as a drug carrier for oral preparation was investigated by evaluating the influence of DES on the pharmacokinetics of SAB and the toxicity of DES. Acute oral toxicity test illustrated that choline chloride-glycerol (ChCl-GL, molar ratio 1:2) was non-toxic with the median lethal dose of 7733 mg/kg. To comparison the difference of pharmacokinetics between SAB dissolved in ChCl-GL (1:2) and in water, a rapid and sensitive ultra-performance liquid chromatography coupled with mass spectrum was established to determine SAB and its metabolites in rat plasma. The method validation was also tested for the specificity, linearity (r^2 > 0.9980 over two orders of magnitude), precision (*intra*-day relative standard deviation (RSD) < 2.73% and *inter*-day RSD < 7.72%), extraction recovery (70.96–80.78%) and stability under three different situations. Compared to water, the pharmacokinetic parameters clarified that ChCl-GL (1:2) could promote the absorption of SAB, the peak concentration (C_{max}) of 0.308 ± 0.020 mg/L was slightly higher than 0.277 ± 0.024 mg/L (SAB dissolved in water), and the peak time (T_{max}) was significantly decreased from 30 min (SAB dissolved in water) to 20 min. There was no significant difference on the metabolites between SAB dissolved in ChCl-GL (1:2) and in water. This is the first report on the pharmacokinetic study of DES as a candidate of drug carrier, and the results provide a meaningful basis for the application of DES in pharmaceutical preparation.

1. Introduction

Deep eutectic solvent (DES) is a thermodynamically stable eutectic system, consisting of two or more compounds [1,2]. Owing to the charge delocalization between the anion and the hydrogen-donor with hydrogen bonding, the melting point of DES is much lower than any of the individual compound [3]. The physical properties of DES, such as viscosity and polarity, can be flexibly adjusted by choosing appropriate constituents and water content [4,5]. The advantages of non-toxicity, low cost, biocompatibility and biodegradability make DES attract more and more attention instead of ionic liquids and organic solvents [6–8]. There have been considerable references based on the applications of DES as green medium for catalysis [9], electrochemistry [10], biological compounds [11,12] and herbal medicines extraction [13,14]. In addition, several publications reported that DES plays an important role of solubilization and storage of insoluble and unstable compounds in plant cell [15,16]. Based on this, DES was used as drug vehicles for poorly soluble compounds [17] and the solubilization solvent for phenolic compounds [13,18]. Moreover, DES with biodegradable properties has been applied to synthetic of antimicrobial wound dressing [19], and therapeutic-DES (menthol: ibuprofen 3:1) has been studied for the development of controlled release drug delivery systems [20]. Hence, DES has a great potential as drug carrier for oral preparation.

In this study, salvianolic acid B (SAB) was selected as a model drug for investigating whether DES is suitable as a drug carrier for oral preparation. SAB is one of the most abundant water-soluble compounds of Radix *Salviae miltiorrhizae* (named danshen in Chinese), which has been reported to exhibit strong anti-oxidation [21], anti-inflammatory activities [22,23] and multi-organ protective effects [24]. SAB has been successfully used for the treatment of coronary vascular diseases in clinical case [25]. The pharmacokinetics study of SAB indicated that SAB has the poor stability and low permeability [26–29]. Our previous research shows that DES can obviously improve the stability of SAB, especially choline chloride-glycerol with the common molar ratio (ChCl-GL, 1:2) [30]. Thus, SAB may be an appropriate object for

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exploring the feasibility of ChCl-GL (1:2) as a drug carrier for oral formulations.

Toxicological studies are urgently needed for new "green" solvent, especially for drug carrier. So far as we known, choline is an important component of lecithin and sphingomyelin, which plays important physiological functions. In view of the benign hypothesis that the DES is non-toxic when individual components in DES are generally non-toxic, choline-based DES is considered to have negligible toxicity or cytotoxicity profiles [17]. However, recent studied showed that choline chloride-based DES had some antibacterial activity and cytotoxicity towards brine shrimp [31,32]. The lack of toxicological data limits the applications of DES for drug carrier. Therefore, as a novel solvent candidate, it is necessary to evaluate the toxicity of ChCl-GL (1:2) in animal for further application.

Hence, one of the objectives of this work was to evaluate the safety of ChCl-GL (1:2) through acute oral toxicity test. In addition, the pharmacokinetic of SAB dissolved in water and in ChCl-GL (1:2) were investigated in this experiment. Moreover, the UPLC–MS method was developed and validated to analyze SAB and its metabolites in rat plasma.

2. Experimental

2.1. Chemicals and reagents

SAB (purity > 98%) and chloramphenicol (internal standard, IS) were obtained from Chengdu Must Bio-Technology Co. Ltd. (Chengdu, China). HPLC-grade acetonitrile and formic acid were purchased from Merck (Merck, Darmstadt, Germany). Ethyl acetate was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water for the LC mobile phase was deionized water quality. ChCl-GL (1:2) was prepared by the method used in a previous report [30]. All other reagents were of analytical grade.

2.2. Animals

Totally 70 mice (male equal female) for acute toxicity test, 4–5 week-old and weighing 20–22 g, were obtained from the Laboratory Animal Center of Shanxi Medical University (Shanxi, China). Pharmacokinetics of SAB dissolved in different solvents were analyzed by 12 Sprague-Dawley (SD) male rats (220–250 g), which were provided by the Animal Center, Beijing HFK Bioscience Co., Ltd (Beijing, certificate No. SCXK 2014-0004). All animals were kept at a controlled temperature of 22 ± 2 °C and humidity of 40% under a 12 h light-dark cycle. A standard pelleted feed and water ad libitum were provided. After acclimatization for one week, the animals were fasted for 12 h before experiment but allowed free access to water. All animals were handled according to the guidelines of Shanxi University Animal Research Committee.

2.3. Instrument and chromatographic conditions

The UPLC–MS analyses were performed using a Waters Acquity H-class UPLC® apparatus (Waters, Milford, MA, USA) equipped with a quaternary solvent manager, an auto-sampler, a column heater, UPLC®-photodiode array (PDA) detector and QDa detector. The QDa detector was operated in positive and negative ion mode with the ESI capillary voltage set at +0.8 kV and the cone voltage at 15 V. The probe temperature was 600 °C. A full mass spectrum between *m/z* 100 and 1000 was acquired at a sampling rate of 2.0 points/s. The chromatographic column used an a ACQUITY UPLC®BEH C18 (50 mm × 2.1 mm, 1.7 μm, Waters, USA) with a corresponding guard column at a column temperature of 20 °C. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile (B). The optimal gradient elution was as follows: 10–50% B at 0–8 min, 50–80% B at 8–12 min. The flow rate was 0.3 mL/min and the injection volume was 10 μL. Acquisition and analyses of data were performed by Empower 2 software (Waters, Milford, MA, USA).

2.4. Acute oral toxicity test of ChCl-GL (1:2)

An initial dose of 5000 mg/kg ChCl-GL (1:2) to mice did not cause any observable signs or symptoms of toxicity. With the dose increased to 12,000 mg/kg, all tested mice were dead. Therefore, totally 70 mice in half genders were randomly divided into 7 groups with ten mice in each group. Group 1 animals received distilled water only, while groups 2, 3, 4, 5, 6 and 7 received graded dose of ChCl-GL (1:2) at doses of 5236, 6160, 7247, 8526, 10,030 and 11,800 mg/kg body weight gain, respectively. ChCl-GL (1:2) was administered orally using gavage following 4 h fast but with access to water. Then the animals were maintained on standard animal diet and water. Toxic signs of mice were observed including symptoms of toxicity, changes in body weight and mortality for the first 4 h, then for 7 and 14 days. And the median lethal dose (LD_{50}) was estimated though the Karber method:

$$LD_{50} = \lg^{-1}[x_m - i\,(\Sigma P - 0.5)] \quad (1)$$

$$95\%\ \text{confidence interval} = \lg^{-1}\left[LD_{50} \pm 1.96 \times i\sqrt{\sum \frac{P(1-P)}{n}}\right] \quad (2)$$

Where x_m represents logarithm of the maximum dose, i represents the difference of the logarithm of the two adjacent groups, P represents the mortality in each dose group, n represents number of mice in each group.

2.5. Pharmacokinetics analysis of SAB dissolved in ChCl-GL (1:2)

The 12 fasted SD rats were randomly divided into two groups. One group received a single oral dose of SAB (dissolved in water), the other group was oral administration of SAB (dissolved in ChCl-GL). The concentration of salvianolic acid B dissolved in different solvents was 100 mg/mL, and the volume of intragastric administration was 5 mL/kg according to the body weight of mice. Blood samples (approximately 0.4 mL) were collected in heparinized tubes at 0, 2, 5, 10, 20, 30, 60, 120, 180, 240 and 300 min. Blood samples were immediately centrifuged at 3500 rpm for 10 min and the plasma samples were stored at −20 °C until analysis.

For the analysis, plasma samples (200 μL), 10% hydrochloric acid (10 μL) and chloramphenicol solution (IS, 50 μL) were added to a 2 mL tube and vortexed for 1 min, the mixture was extracted with ethyl acetate (1.5 mL) and also vortexed for 1 min. Then the tube was centrifuged at 4000 rpm for 10 min at 4 °C, the supernatant was transferred into a new tube and dried with nitrogen. The residue was re-dissolved with 100 μL acetonitrile and centrifuged at 12,000 rpm for 10 min, then 10 μL of the supernatant was detected by UPLC–MS. Drug and Statistics (DAS) version 3.2.8 software (Modern Research Center for Traditional Chinese Medicine of Shanxi University, Shanxi, China) was employed to determine the pharmacokinetic parameters of SAB dissolved in different solvents. All data were expressed as mean ± SD.

2.6. Preparation of stock and working solutions, calibration standards and quality control samples

Standard stock solution of SAB was prepared in water at 1.0 mg/mL and serially diluted to working solutions with water. The working solution of IS was diluted to a final concentration of 1.0 μg/mL. Calibration standards and quality control samples were prepared by spiking 50 μL SAB and 50 μL IS working solution into 200 μL of blank rat plasma. Then the plasma samples were prepared as Section 2.5. The concentrations of SAB in calibration standards were 0.05, 0.15, 0.75, 3.75, 7.00 and 10.00 μg/mL. Quality control samples were obtained with SAB concentrations of 0.05, 0.75, and 10.00 μg/mL. All stock and working solutions were stored at −20 °C.

Table 1
Effects of DESs on body weights and survival numbers of mice with a single dose in acute toxicity test.

No.	Treatment (mg/kg)	Sex	Amount	Mortality (*n*)	death rate (*P*)	body weight			Weight gained (%)
						Initial	7 day	14 day	
1	Control	male	5	0	0	20.92 ± 0.95	26.61 ± 0.82	31.77 ± 0.83	51.88
		female	5	0		20.91 ± 1.72	23.77 ± 0.41	28.75 ± 0.72	37.50
2	5236	male	5	0	0	21.32 ± 0.86	26.97 ± 1.47	32.62 ± 1.20	52.98
		female	5	0		19.65 ± 0.24	23.92 ± 0.77	28.87 ± 0.48	46.90
3	6160	male	5	1	0.1	20.67 ± 0.45	24.86 ± 1.32	32.16 ± 0.92	55.61
		female	5	0		19.46 ± 0.26	23.57 ± 0.52	28.83 ± 0.53	48.15
4	7247	male	5	2	0.4	20.73 ± 0.57	26.73 ± 0.19	32.60 ± 0.32	57.27
		female	5	2		19.06 ± 0.69	23.66 ± 0.76	29.77 ± 0.44	56.21
5	8526	male	5	3	0.8	21.40 ± 0.97	27.39 ± 0.93	32.18 ± 0.71	50.40
		female	5	5		19.75 ± 0.77	0	0	
6	10030	male	5	3	0.8	21.87 ± 1.16	27.39 ± 0.77	31.96 ± 0.84	46.09
		female	5	5		19.87 ± 0.99	0	0	
7	11800	male	5	4	0.9	22.04 ± 0.58	26.94	33.63	52.59
		female	5	5		20.19 ± 0.84	0	0	

3. Results and discussion

3.1. Acute toxicity test

The acute toxicity test was intended to assess the safety of ChCl-GL (1:2) before further clinical trials. With the dose increased to 6160 mg/kg and above, the majorities of mice appeared to excited, jump up and down, and then reduce activity, shortness of breath, convulsions, tremor and other symptoms within 20–30 s, then 30 mice died within 4 h, and the surviving mice returned to normal within 2 h.

As shown in Table 1, the mortality was increasing with the increase in the oral dose of ChCl-GL (1:2). And there was no significant influence on body weight gain between the control and the treated groups during the observation period. According to the Karber method, the estimated LD_{50} was 7733 mg/kg with a 95% confidence interval of 7130–8387 mg/kg for oral administration. This indicates that ChCl-GL

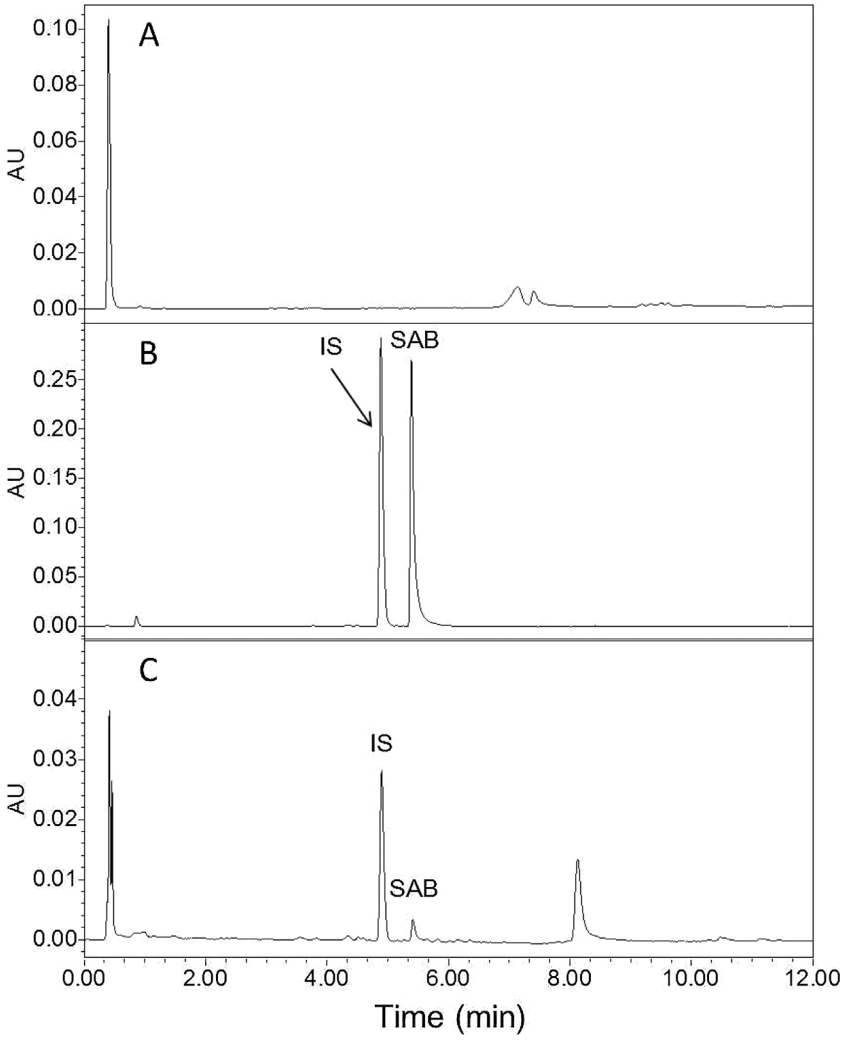

Fig. 1. (A) Blank plasma; (B) Blank plasma spiked with SAB and internal standard; (C) Plasma sample at 30 min after oral administration spiked with internal standard.

Table 2
Linearity and range of SAB.

No. (day)	Calibration curve	r^2	LOQ (ng/mL)	LOD (ng/mL)
1	$y = 6.6696x + 0.4752$	0.9980	47.62	15.33
2	$y = 6.7103\,x - 0.7756$	0.9992		
3	$y = 6.6222\,x - 0.5199$	0.9991		

(1:2) is non-toxic, and is relatively safe on clinical application for oral dosage forms.

3.2. Method validation

To validate the plasma sample preparation and UPLC–MS method, the specificity, linearity, precision, recovery and stability were determined.

3.2.1. Specificity

The specificity of the method was demonstrated by comparing the UPLC–MS chromatograms for blank rat plasma sample (A), blank rat plasma sample spiking with SAB and IS working solutions (B), and plasma sample collected at 30 min after the oral administration spiking with IS working solutions (C). As shown in Fig. 1, the retention times were 4.91 and 5.38 min for IS and SAB, respectively. All the major constituents were well separated and no interference peaks were observed in the drug-free rat plasma.

3.2.2. Linearity

Blank plasma samples spiked with six different concentrations of SAB were processed as described in Section 2.6. The calibration curves were measured daily for three days by calculating the peak area ratios (y) of SAB to IS against SAB standard concentrations (x). Table 2 showed that calibration curves of SAB had good linear regressions ($r^2 > 0.998$) in the tested range. The limit of quantification (LOQ, based on signal/noise = 10) and the limit of determination (LOD, based on signal/noise = 3) were 47.62 ng/mL and 15.33 ng/mL in rat plasma for SAB, respectively.

3.2.3. Accuracy and precision

The accuracy and precision of the method were estimated by duplicate determination of quality control samples with low, middle and high concentrations on the same day for three consecutive days. The results were presented in Table 3. The relative standard deviation (RSD) of *intra*-day ($n = 6$) and *inter*-day ($n = 3$) precisions for the concentration of SAB were in the range of 2.01%–2.73% and 1.29%–7.72%, respectively. The corresponding accuracy was range from 93.52 to 100.60%.

3.2.4. Extraction recovery and stability

The extraction recovery was calculated in plasma samples ($n = 6$) spiked with SAB standard solution on concentrations of 0.05, 0.75 and 10 μg/mL, respectively. The measured concentration of SAB in spiked plasma samples was compared with same original concentration. As shown in Table 4, the extraction recovery of SAB was within the range 70.96-80.78%. The recovery of the IS was more than 92.11%.

Several stability experiments were performed and the results were summarized in Table 5. SAB concentrations were no significant changes after storage for 12 h at room temperature, storage at 4 °C for 24 h and three freeze-thaw cycles at −20 °C. These results indicated that the analytes were stable during sample storage and preparation.

3.3. Pharmacokinetic of SAB dissolved in ChCl-GL (1:2)

In order to determine the influence of ChCl-GL (1:2) on the pharmacokinetic of SAB, 100 mg/kg SAB dissolved in ChCl-GL (1:2) was

Table 3
Precisions and accuracies of SAB ($n = 3$).

SAB	Nominal concentration (μg/mL)	Found concentration (μg/mL)	RSD (%)	Accuracy (%)
Intra-day (n = 6)	0.05	0.0503 ± 0.0014	2.73	100.60
	0.75	0.7423 ± 0.0150	2.01	98.97
	10.00	9.3519 ± 0.1994	2.13	93.52
Inter-day (n = 3)	0.05	0.0483 ± 0.0037	7.72	96.60
	0.75	0.7407 ± 0.0447	6.04	98.76
	10.00	9.4930 ± 0.1225	1.29	94.93

Table 4
Recovery of SAB and IS ($n = 3$).

No.	Spiked plasma concentration (μg/mL)	Extraction recovery (%, mean ± SD)
SAB	0.05	77.50 ± 2.93
	0.75	70.96 ± 2.75
	10.00	80.78 ± 3.71
IS	1.00	92.11 ± 1.49

Table 5
Stability of SAB ($n = 3$).

SAB	Nominal concentration (μg/mL)	Found concentration (μg/mL)	RSD (%)	Recovery (%)
At 20 °C for 12 h	0.05	0.0498 ± 0.0005	1.10	99.60
	0.75	0.7067 ± 0.0166	2.35	94.23
	10.00	8.9759 ± 0.1586	1.77	89.76
At 4 °C for 24 h	0.05	0.0490 ± 0.0011	2.34	98.00
	0.75	0.7312 ± 0.0117	1.60	97.49
	10.00	9.3082 ± 0.1746	1.88	93.08
After three freeze-thaw cycles at −20 °C	0.05	0.0489 ± 0.0005	1.10	97.80
	0.75	0.6515 ± 0.0094	1.44	86.87
	10.00	8.9496 ± 0.0563	0.63	89.50

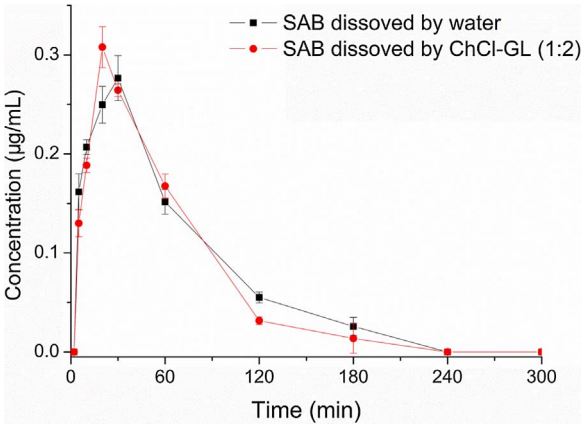

Fig. 2. Plasma concentration-time profiles of SAB after oral administration at a dose of 100 mg/kg (x ± SD, $n = 6$).

oral administration to rats as a test group, and the same dosage of SAB dissolved in water was as a control group. The concentrations of SAB in plasma samples were determined by the developed and validated method, and the mean plasma concentration versus time profile of SAB ($n = 6$) was illustrated in Fig. 2. The plasma concentration of SAB in two groups both declined rapidly after 30 min, this phenomenon

Table 6
Main pharmacokinetic parameters of SAB (n = 6).

Parameters		SAB-water	SAB-DES
T_{max}	min	30	20
C_{max}	mg/L	0.277 ± 0.024	0.308 ± 0.020
K_e	1/min	0.023 ± 0.001	0.020 ± 0.001
$t_{1/2}$	min	30.338 ± 1.091	34.880 ± 0.757
CL	L/min/kg	4.572 ± 0.307	4.790 ± 0.184
$AUC_{(0-t)}$	mg min/L	21.903 ± 1.416	20.847 ± 0.791*
$MRT_{(0-t)}$	min	60.192 ± 3.428	53.559 ± 1.390

SAB-water means SAB dissolved in water, SAB-DES means SAB dissolved in ChCl-GL (1:2).
* p > 0.05 when compared with SAB-water.

suggested that SAB was distributed and metabolized rapidly in tissues and organs. SAB could only be detected within 240 min after oral administration owing to the low content and rapid elimination in rat plasma.

The corresponding pharmacokinetic parameters presented in Table 6 were analyzed by non-compartmental methods with DAS software, the P-value less than 0.05 was considered to be significant difference using T-test. Compared with water, ChCl-GL (1:2) could promote the speed of the absorption of SAB in the gastrointestinal tract after administration. The peak plasma concentration (C_{max}) of SAB dissolved in ChCl-GL (1:2) was slightly increased from 0.277 ± 0.024 mg/L (SAB dissolved in water) to 0.308 ± 0.020 mg/L. Time to reach C_{max} (T_{max}) of SAB was significantly decreased from 30 min (SAB dissolved in water) to 20 min, and the half-life of elimination ($t_{1/2}$) was prolonged from 30.338 ± 1.091 min (SAB dissolved in water) to 34.880 ± 0.001 min. However, compared to others pharmacokinetic parameters of SAB dissolved in water, SAB dissolved in ChCl-GL (1:2) showed more rapid distribution and elimination with the mean residence time (MRT_{0-t}) of 53.559 ± 1.390 min and the clearance (CL) of 4.790 ± 0.184 L/min/kg. There is no significant difference of the area under concentration-time curve (AUC_{0-t}) between SAB dissolved in ChCl-GL (1:2) and in water. The results above indicated that ChCl-GL (1:2) could accelerate the absorption and elimination of SAB, and the reason might be that eutectic system could enhance the membrane penetration ability of drug [33]. Based on the pharmacokinetic characteristics such as rapid absorption, long half-life,

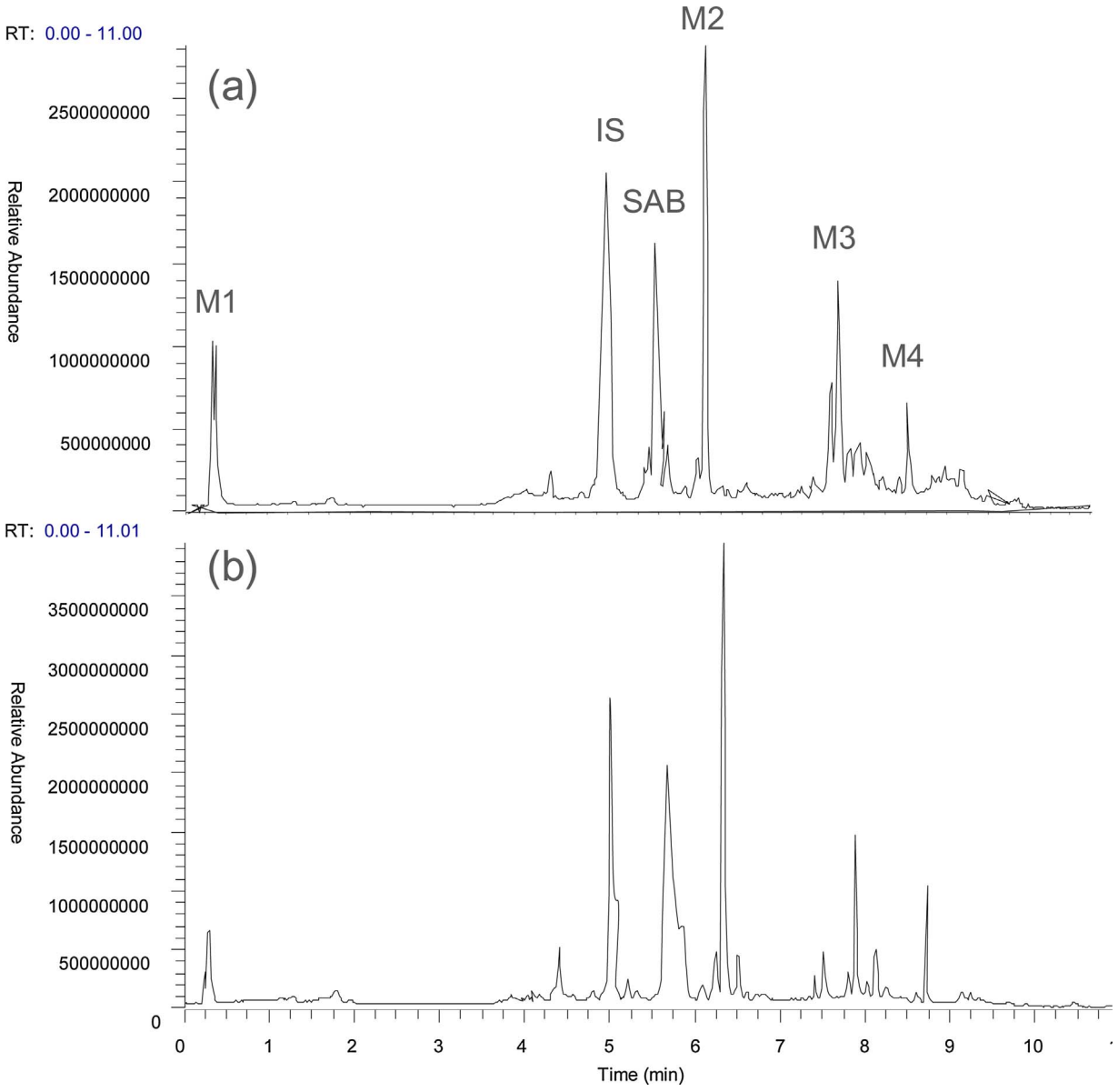

Fig. 3. Total ion chromatograms of metabolites and IS in rat plasma after oral administration of SAB for 30 min: (a) SAB dissolved in water, (b) SAB dissolved in ChCl-GL (1:2).

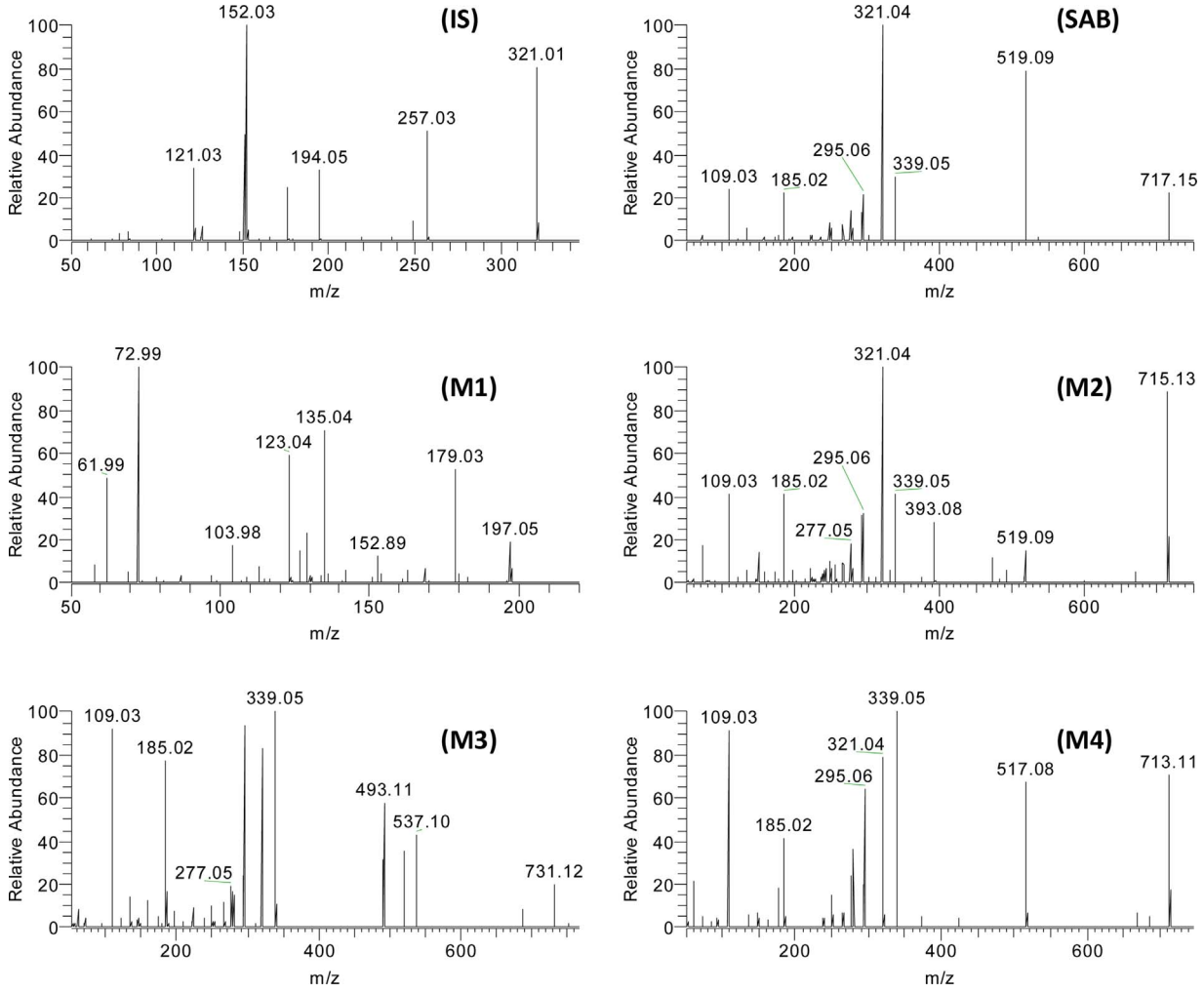

Fig. 4. Ion mass spectra of $[M-H]^-$ of metabolites and IS in rats plasma after oral administration of SAB.

and fast elimination after 60 min, ChCl-GL (1:2) provides good prospect in drug carrier of oral administration for rapid absorption.

3.4. Metabolites identification

Background subtracted, targeted and non-target extracted ion chromatogram of MS/MS analysis was used for identification of metabolites. Similar metabolites have been determined of SAB dissolved in water and in ChCl-GL (1:2) after oral administration. Total ion chromatograms and ion mass spectra of metabolites were provided in Figs. 3 and 4. IS produced an $[M-H]^-$ ion at *m/z* 321.01. SAB showed an $[M-H]^-$ ion at *m/z* 717.15. The fragments of SAB derived from the loss of one or two molecules of danshensu (198 u), resulting in fragment ions at 519.09 and 321.04. M1 showed an $[M-H]^-$ ion at *m/z* 197.05 and specific fragment ion of $[M-H-O]^-$ ion at *m/z* 179.03, indicating that M1 might be danshensu. M2 produced ions of $[M-H]^-$ at *m/z* 715.13 and [M−H-caffeic acid]$^-$ at *m/z* 321.04. The ions of $[M-H]^-$ at *m/z* 731.12 and characteristic product ions of [M−H-danshensu-caffeic acid]$^-$ at *m/z* 339.05 suggested that M3 might be monomethyl-SAB. SAB removed substituents to form ions of [M−H-danshensu]$^-$ at *m/z* 519, and then combined with glucuronic acid in plasma to formation the M4 with *m/z* 713.12. According to the metabolites identification, there was no difference on the metabolites between SAB dissolved in water and in ChCl-GL (1:2) after oral administration.

4. Conclusion

In this paper, the acute toxicity of ChCl-GL (1:2) was preliminary studied for the first time, and the result demonstrated that ChCl-GL (1:2) was non-toxic with LD_{50} 7733 mg/kg. A sensitive and stable UPLC–MS method was established and validated for the determination of SAB and its metabolites after oral administration. The drug-time curve of SAB dissolved in different solvents had the similar change tendency basically. Considering the characteristics of higher C_{max} and shorter T_{max} of SAB dissolved in ChCl-GL (1:2), we could draw the conclusion that ChCl-GL (1:2) was conducive to promote the absorption of SAB by enhancing the membrane penetration ability. The main metabolites were basically consistent between SAB dissolved in water and in ChCl-GL (1:2). This is a first report in this field and will be helpful for further clinical development of DES, and DES shows great potential as a candidate of oral carrier for rapid absorption of drugs.

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