

•Research article•

Development of liquiritigenin-phospholipid complex with the enhanced oral bioavailability

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[ABSTRACT] In the present study, liquiritigenin-phospholipid complex (LPC) was developed and evaluated to increase the oral bioavailability of liquiritigenin. A single-factor test methodology was applied to optimize the formulation and process for preparing LPC. The effects of solvent, drug concentration, reaction time, temperature and drug-to-phospholipid ratio on encapsulation efficiency were investigated. LPCs were characterized by UV-visible spectroscopy, differential scanning calorimetry (DSC), fourier transform infrared spectroscopy (FTIR), and powder X-ray diffractometry (PXRD). The apparent solubility and n-octanol/water partition coefficient were tested. The pharmacokinetic characteristics and bioavailability of the LPC were investigated after oral administration in rats in comparison with liquiritigenin alone. An LPC was successfully prepared. The optimum level of various parameters for liquiritigenin-phospholipid complex was obtained at the drug concentration of 8 mg·mL⁻¹, reaction time for 15 min, reaction temperature of 30 °C, a ratio of 1 : 4.5 (W/W) drug-to-phospholipid and anhydrous ethanol as reaction solvent. Compared to liquiritigenin, the *AUC*₀₋₄ of the LPC was increased by 239%. The liquiritigenin-phospholipid complex significantly increase the lipid solubility and bioavailability of liquiritigenin, suggesting that it is an effective formulation for further development and clinical applications.

[KEY WORDS] Liquiritigenin; Liquiritigenin-phospholipid complex; Solubility; Characterization; Bioavailability

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Introduction

Liquiritigenin (Fig.1) is a flavonoid derived from the polyphenol structures in the roots of licorice plants. It has anti-tumor, anti-inflammatory, anti-hyperlipidemic, relieving a cough liver protective, and proapoptotic effects, etc. [1-7]. In the previous studies, liquiritigenin was reported to inhibit the growth of human colon cancer Lovo cells and induce apoptosis in human hepatoma SMMC-7721 cells, indicating its good anti-tumor effect [8, 9]. However, liquiritigenin has low aqueous solubility and lipid solubility resulting the low bioavailability *in vivo* [10].

Phospholipids are known to be capable of encapsulating drugs *via* intermolecular charge interactions. The lipid solubility and bioavailability of the drug can be improved by forming a phospholipid complex. The chemical structures of flavonoids usually contain hydrogen donor groups, which can form the stable complex with the positive charged phosphol-

ipids under certain conditions. Increasing attention has been paid to the development of phospholipid complex with active ingredients of Chinese traditional medicine to achieve better pharmacokinetic performance. [11-16]. In this study, a liquiritigenin-phospholipid complex (LPC) was prepared and characterized in order to improve the bioavailability of poorly water soluble liquiritigenin.

Materials and Methods

Liquiritigenin and apigenin were purchased from Chunqiu Bioengineering Co., Ltd. (Nanjing, China). Soybean phospholipid was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Methanol, ethanol, ethyl acetate, tetrahydrofuran, and hydrochloric acid were obtained from Nanjing Chemical Reagent Co., Ltd.. Methanol and acetonitrile were HPLC grade, and all other chemical reagents employed in the experiments were analytical grade.

Determination of the content of liquiritigenin by HPLC

Ten milligrams of LPC were dissolved in 100 mL of methanol and filtered through a 0.45 μm filter [17]. The content of liquiritigenin was determined by a Tigerkin C8T column (4.6 mm × 250 mm, 5 μm) at 30 °C with 10 μL of in-

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These authors have no conflict of interest to declare.

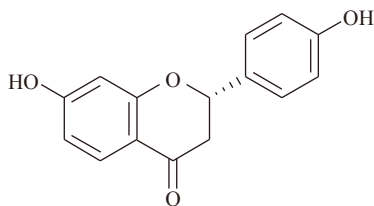


Fig. 1 Chemical structure of liquiritigenin

jection volume. A mobile phase of acetonitrile-water (40 : 60, V/V) was set at a flow rate of 1.0 mL·min⁻¹. The detection wavelength was monitored at 274 nm.

Preparation of LPC

A certain amount of liquiritigenin and soybean phospholipid were weighed and dissolved in an organic solvent. The mixture was stirred for 2 minutes, and then the solvent was removed by rotary evaporation under reduced pressure. The residual solid was collected and freeze-dried for 12 h, thus the dried material was stored by sealing into the vials under nitrogen. The encapsulation efficiency of the phospholipids was calculated as follows [18], and the encapsulation efficiency = $(W_0 - W)/W_0 \times 100\%$. W_0 is the initial amount of liquiritigenin, and W is the amount of liquiritigenin in the phospholipid complex;

Optimal formulation and process for preparation of LPC by single-factor experiment

Phospholipids, including soya lecithin and lecithin, can be used in phospholipid complexes. Liquiritigenin was encapsulated by CS-70 soybean phospholipid and PL-100M egg phospholipid at a ratio of 6 : 1 (W/W). The encapsulation efficiency of CS-70 soybean phospholipid and PL-100M egg phospholipid was 99.7% and 89.8%, respectively. Soybean phospholipids have high encapsulation efficiency, so soybean phospholipids were selected for inclusion. The effects of factors such as solvent, drug concentration, reaction time, temperature, drug-to-phospholipid ratio and the encapsulation efficiency of phospholipids were investigated.

Characterization of LPCs

Determination of apparent solubility

Solubility determination of liquiritigenin, LPC and Physical mixture was carried out by adding excess samples to phosphate buffer (pH 1.2, 4.5, 6.0, 6.8), medium-chain triglyceride (MCT), and soybean oil (LCT) in sealed glass container at room temperature. Each experiment was performed in triplicate. The samples were shaken for 48 hour at 37 °C and then centrifuged at 12 000 r·min⁻¹ for 10 min. The supernatant was subsequently filtered by a 0.22 μm membrane. The filtrates were suitably diluted with methanol and a 10 μL aliquot was measured by HPLC.

N-Octanol/water partition coefficient of the LPC

LgP of liquiritigenin and LPC was carried out by adding excess of liquiritigenin and LPC into 10.00 mL phosphate buffer (pH 1.2, 4.5, 6.0, 6.8) in sealed glass containers at 25 °C, respectively. The samples were dissolved in solvent as fully as possible under ultrasonic condition. Each experiment was performed in triplicate. And then all solutions were cent-

rifuged at 10 000 r·min⁻¹ for 15 min to get the supernatants. 4 mL supernatant and 4 mL n-octanol (water saturated) were mixed and then shaken for 24 h in a shaker at 37 °C. The solutions of the upper layer and the sub-layer suitability was diluted with methanol, respectively. Liquiritigenin and LPC amount was measured by HPLC. The oil-water partition coefficient can be calculated by the equation as follows: $\log P = \log(C_o/C_w)$ [19].

Ultraviolet (UV)-visible spectroscopy

Liquiritigenin was dissolved in methanol to prepare a reference solution (10 μg·mL⁻¹). 50 mg Phospholipids, 100 mg LPC and 100 mg physical mixture of liquiritigenin and phospholipids (50/50) were mixed with 50 mL petroleum ether. The samples were shaken for 3 minutes and then filtered by 0.45 μm filters. The 1 mL filtrates were evaporated and the residues were redissolved in methanol for UV-vis analysis. UV spectra were recorded for liquiritigenin, phospholipid, the physical mixture and the LPC. the absorption spectrum was scanned in the wavelength range of 200–400 nm to obtain the UV spectra.

Differential scanning calorimetry (DSC)

Small amounts of liquiritigenin, phospholipid, the physical mixture and the LPC were weighed in aluminum pan. An empty aluminum pan was used as a reference. The sample was heated at a rate of 10 °C·min⁻¹ from 40 to 300 °C.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) spectra of liquiritigenin, phospholipid, the physical mixture and the LPC were obtained by IR spectrophotometer. The samples were mixed with dry KBr and scanned over the range 4000–400 cm⁻¹.

Powder X-ray diffractometry (PXRD)

Samples of certain weight were added into the slide for packing prior to X-ray scanning. Spectra of graphs were plotted from 3.00° to 40.00° of 2θ with a step width of 0.02° and step time of 0.2 s at room temperature.

In vivo oral pharmacokinetic study

Animals and dosing

Female Sprague Dawley (SD) rats of 200 ± 20 g were obtained from Qinglong Mountain Animal Breeding Farm. Animal experiments were performed according to the requirements in the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Animals Ethics Committee of the University. The rats were maintained at 25 ± 2 °C and 50%–60% relative humidity (RH) under natural light/dark conditions for one week before the experiment. They were randomly distributed into two groups, each containing 6 rats. Before the experiment, both groups were fasted overnight for 12 h with free access to water. One group was orally administered the LPC suspended in water at a dose equivalent to 20 mg·kg⁻¹ body weight of liquiritigenin, while the other group was administered by liquiritigenin aqueous suspension. Blood samples were collected from the orbital venous plexus of the rats at 0, 3, 5, 10, 15, 30, 60, 120, 240, 360, 480, and 600 min after administration and were anticoagulated with heparin sodium. The samples were centrifuged

at 1500 r·min⁻¹ for 5 min, and the upper layer of plasma was taken and stored in a refrigerator at -20 °C.

Quantification of LPC in plasma samples

Rat plasma (100 µL) and internal standard solution (10 µL) were placed in a 1.5 mL centrifuge tube and vortexed for 30 s. The plasma sample was added to 500 µL of acetonitrile, and the protein was precipitated by vortexing for 3 min and centrifuging at 12 000 r·min⁻¹ for 5 min. The supernatant was transferred to another centrifuge tube and centrifuged (12 000 r·min⁻¹) for 5 min. 2 µL of the supernatant was injected into the LC-MS system for analysis.

The mobile phase was composed of water and acetonitrile. A is water and B is acetonitrile. Gradient elution: 0–1.0 min, 5%–50% B, 1.0–3.0 min, 50%–90% B, 3.0–4.0 min, 90% B, 4.0–5.0 min, 90%–5% B, 5.0–5.5 min, 5% B. The column flow rate was 0.3 mL·min⁻¹ with 2 µL of injection volume. The column temperature was 35 °C.

The ion source was an ESI source; CUR: 35; TEM: 500; IS: 5500; GAS 1: 35; GAS 2: 35; DP: -60.73; CE: -33.03; EP: -12.87; and CXP: -12.96. The ion pairs used for the analysis were *m/z* 255.0→119.0 (liquiritigenin) and *m/z* 269.0→117.0 (apigenin).

Statistical analysis

The pharmacokinetic parameters were computed using the software program DAS 2.0. All values were expressed as the mean ± the standard deviation (SD). Statistical analysis was carried out using a *t*-test. Differences were considered statistically significant at *P* < 0.05.

Results and Discussion

Optimal Formulation of LPC

Solvent is the important factor for preparing LPC. Since methanol and ethanol have a larger dielectric constant than tetrahydrofuran, the resulting reverse potential is not favorable for phospholipid inclusion. In this experiment, we compared different solvents and the anhydrous ethanol was found to achieve a higher encapsulation efficiency than other solvents (Table 1). Compared with other solvents, anhydrous ethanol is also less toxic, which would be beneficial for drug development and production in terms of residual solvent. The encapsulation efficiency of liquiritigenin and phospholipid in a 1 : 3 ratio was only 8%. By contrast, the encapsulation efficiency reached more than 98% when the drug-to-phospholipid ratio was 1 : 4.5. The encapsulation efficiency did not change with the extension of the reaction time, as the encapsulation was complete at 15 min. The drug concentration has little effect on the encapsulation efficiency. The complexes have the same encapsulation efficiency for different concentrations of drugs. The test results led to the selection of the drug concentration at 8 mg·mL⁻¹. Finally, The results showed that there was no difference in encapsulation efficiency at different temperatures, so the temperature was selected at 30 °C.

The following process parameters were determined by a single-factor test: anhydrous ethanol as reaction solvent, a ratio of liquiritigenin to soybean phospholipid of 1 : 4.5, a mass concentration of 8 mg·mL⁻¹, a reaction time of 15 min, and a reaction temperature of 30 °C. Three verification

Table 1 Effect of different factors on the encapsulation efficiency of LPCs

Formulation	solvent	drug-to-phospholipid ratio	<i>t</i> /min	<i>c</i> /mg·mL ⁻¹	<i>T</i> /°C	encapsulation efficiency (%)
1	methanol	1 : 6	120	8	60	78.30
2	ethanol	1 : 6	120	8	60	89.20
3	ethyl acetate	1 : 6	120	8	60	86.70
4	tetrahydrofuran	1 : 6	120	8	60	81.70
5	ethanol	1 : 3	120	8	60	8.30
6	ethanol	1 : 4.5	120	8	60	98.10
7	ethanol	1 : 6	120	8	60	98.40
8	ethanol	1 : 4.5	15	8	60	94.30
9	ethanol	1 : 4.5	30	8	60	93.70
10	ethanol	1 : 4.5	60	8	60	90.20
11	ethanol	1 : 4.5	90	8	60	91.80
12	ethanol	1 : 4.5	15	4	60	94.50
13	ethanol	1 : 4.5	15	8	60	92.60
14	ethanol	1 : 4.5	15	12	60	92.50
15	ethanol	1 : 4.5	15	8	30	95.60
16	ethanol	1 : 4.5	15	8	40	93.10
17	ethanol	1 : 4.5	15	8	60	94.60
18	ethanol	1 : 4.5	15	8	30	96.80
19	ethanol	1 : 4.5	15	8	30	95.10
20	ethanol	1 : 4.5	15	8	30	97.30

batches prepared according to the determined process parameters showed that the liquiritigenin encapsulation efficiency was high and the process was feasible.

Apparent solubility

Compared with the concentration of liquiritigenin, the concentration of the LPC in water, hydrochloric acid solution (pH 1.2) and phosphate buffer (pH 4.5, 6.0 and 6.8) did not increase significantly (Table 2). The solubility of physical mixture was significantly higher than that of LPC in the pH

1.2 hydrochloric acid solution. It was observed that the solids of LPC were quite sticky in the acidic condition. Compared to the physical mixture, the solids of LPC were not dispersed well in the medium during the solubility test. The formation of a gel-like chunk of LPC in the pH 1.2 medium may yield the lower solubility.

The solubility of LPC in soybean oil was not very different from that of liquiritigenin, but the solubility in MCT was increased by approximately 20 times (Table 3).

Table 2 Apparent solubility of liquiritigenin and the LPC in different media at 37 °C ($\mu\text{g}\cdot\text{mL}^{-1}$) (mean \pm SD, $n = 3$)

	water	pH 1.2	pH 4.5	pH 6.0	pH 6.8
Liquiritigenin	63.89 \pm 2.34	120.39 \pm 3.14	50.24 \pm 0.85	79.06 \pm 0.87	129.01 \pm 2.18
LPC	88.21 \pm 1.23	106.6 \pm 2.14	90.63 \pm 0.94	89.58 \pm 1.14	122.32 \pm 1.33
Physical mixture	88.90 \pm 1.41	142.02 \pm 3.38	93.14 \pm 1.37	85.41 \pm 1.38	123.03 \pm 1.36

N-Octanol/Water Partition Coefficient

N-Octanol/Water Partition Coefficient is an important physio-chemical property of drugs. It plays a crucial role in absorption, distribution, metabolism and excretion (ADME) [20]. As shown in Table 4, compared with the concentrations of liquiritigenin, the concentrations of LPC in water, phosphate buffer (pH 1.2, 4.5, 6.0, 6.8) and n-octanol solution increased significantly. The *P* value is proportional, so the Log*P* value did not increase significantly. However, The lipophilicity of LCP was significantly increased by formation of phospholipid complex.

Ultraviolet (UV)-visible spectrophotometry

The UV spectra in Fig. 2 show that liquiritigenin and

Table 3 Apparent solubility of liquiritigenin and the LPC in different oils at 37 °C ($\mu\text{g}\cdot\text{mL}^{-1}$) (mean \pm SD, $n = 3$)

	LCT	MCT
Liquiritigenin	440.01 \pm 5.63	1490.21 \pm 12.14
LPC	339.63 \pm 4.54	28060.75 \pm 36.72

Table 4 N-octanol/water partition coefficient (log *P*) of liquiritigenin and the LPC in water and buffered saline (mean \pm SD, $n = 3$)

	Solvent	$c_{\text{water}}/\mu\text{g}\cdot\text{mL}^{-1}$	$c_{\text{Octanol}}/\mu\text{g}\cdot\text{mL}^{-1}$	Log <i>P</i>
Liquiritigenin	water	3.68 \pm 0.27	34.30 \pm 1.35	0.97
	pH 1.2	4.08 \pm 0.58	65.07 \pm 2.34	1.20
	pH 4.5	3.32 \pm 0.34	50.51 \pm 2.18	1.15
	pH 6.0	3.64 \pm 0.64	47.19 \pm 1.22	1.15
	pH 6.8	2.52 \pm 0.14	58.98 \pm 1.10	1.37
LPC	water	5.04 \pm 0.64	230.39 \pm 3.82	1.66
	pH 1.2	12.45 \pm 0.87	269.09 \pm 4.94	1.33
	pH 4.5	6.23 \pm 0.93	139.53 \pm 2.36	1.35
	pH 6.0	11.78 \pm 1.24	162.48 \pm 4.14	1.14
	pH 6.8	5.53 \pm 0.94	164.52 \pm 2.35	1.47

LPC have maximum absorption peaks at 274 nm. Liquiritigenin was dissolved in methanol to prepare a reference solution. The Liquiritigenin in the phospholipid complex can be extracted by petroleum ether, while liquiritigenin in the physical mixture could not be dissolved in petroleum ether during the extraction step, as suggested by the absence of absorption peak at 274 nm. The difference in the UV spectra of LPC and physical mixture upon the extraction by petroleum ether indicated that the lipophilicity of liquiritigenin was significantly increased after phospholipid inclusion.

Differential scanning calorimetry (DSC)

DSC traces of liquiritigenin, phospholipid, the physical mixture and the LPC were displayed in Fig. 3. The liquiritigenin shows an endothermic peak at around 210 °C and that there is no obvious endothermic peak in the DSC of the soybean phospholipids. The physical mixture did not show an endothermic peak at approximately 210 °C. It was possible that the liquiritigenin dissolved in the soybean phospholipid during the slow heating process, showing the melting point depression. The DSC trace of the LPC exhibit no endothermic peak, indicating that no liquiritigenin crystals remained. The liquiritigenin was dispersed in the phospholipid as the amorphous form.

Fourier transform infrared spectroscopy (FTIR)

Fig. 4 showed the FTIR spectra of liquiritigenin, phospholipids, Physical mixture and LPC. Liquiritigenin exhib-

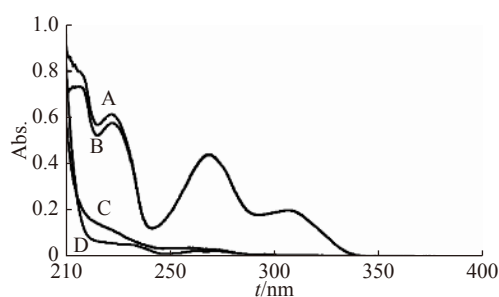


Fig. 2 UV spectra of liquiritigenin(A), the LPC (B), physical mixture (C) and phospholipid (D)

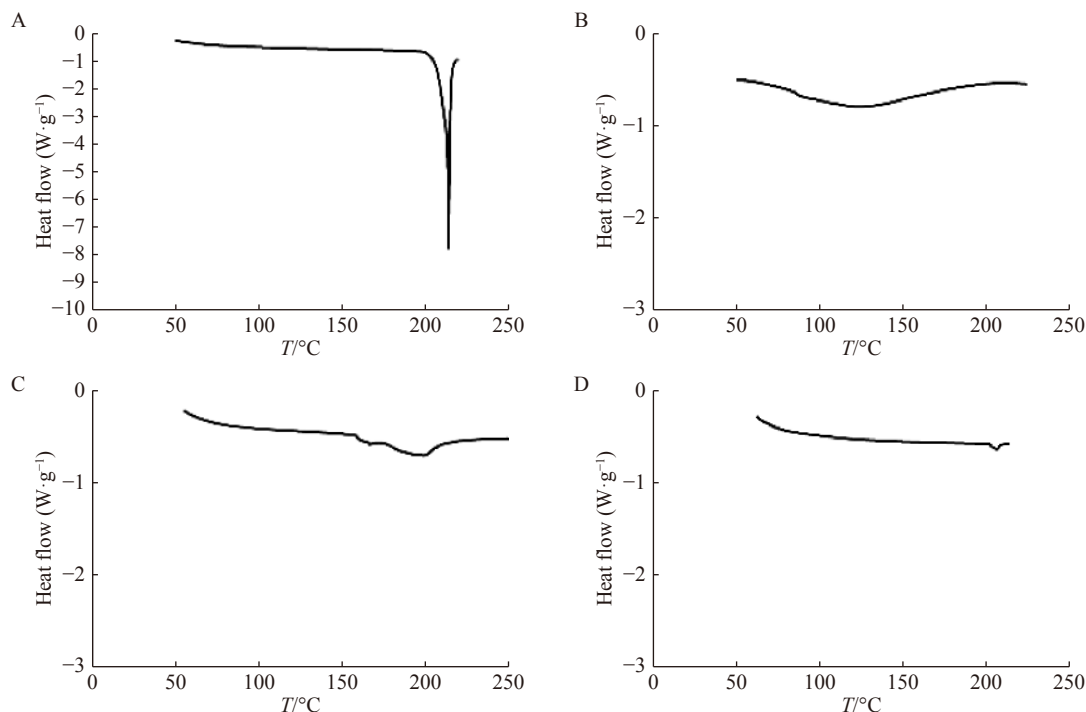


Fig. 3 DSC traces of liquiritigenin(A), phospholipid (B), physical mixture (C), and the LPC (D)

ited the characteristic bands at 3237 cm⁻¹ (phenolic O-H stretching) and 1653 cm⁻¹ (C=O stretching). The FTIR spectrum of pure phospholipid showed the characteristic signals at 2918 cm⁻¹ and 2850 cm⁻¹ (C-H stretching of long fatty acid chain); 1738 cm⁻¹ (C=O stretching in the fatty acid ester); 1236 cm⁻¹ (P=O stretching); 1091 cm⁻¹ (P-O-C stretching); and 970 cm⁻¹ (N⁺(CH₃)₃ stretching) [21]. The spectrum of the physical mixture exhibited a summation of the vibrational frequencies of the individual components. For the spectrum of LPC, the peaks of phenolic O-H stretching were broadened and the peak of C=O stretching of liquiritigenin was significantly shifted from 1653 cm⁻¹ (C=O stretching) to 1680 cm⁻¹, suggesting the formation of intermolecular interactions between liquiritigenin and phospholipids.

Powder X-ray diffractometry (PXRD)

Figs. 5A and 5C showed that there were diffraction peaks in the pure liquiritigenin sample and the physical mixture because that liquiritigenin existed in the crystalline state. In the PXRD pattern of LPC, the liquiritigenin crystal diffrac-

tion peaks were absent, indicating that the crystallization of liquiritigenin were inhibited and that liquiritigenin were highly dispersed in the phospholipid in the amorphous form. Liquiritigenin was complexed with phospholipids physically without forming a new chemical specie.

Pharmacokinetics

The plasma concentration-time profiles of liquiritigenin and LPC in rats were shown in Fig. 6. the corresponding pharmacokinetic parameters are summarized in Table 5. Fig. 6 shows that after oral administration of liquiritigenin and LPC, the C_{max} values was 326.55 and 1051.30 ng·mL⁻¹, respectively, a 3.22 times increase. Compared to that of liquiritigenin, the AUC_{0-t} of the LPC was increased by 239%. The pharmacokinetic study showed that LPC significantly improved the oral bioavailability of liquiritigenin. The development of phospholipid-drug complex is an effective solution to deliver poorly soluble natural products for potential applications in clinical practice.

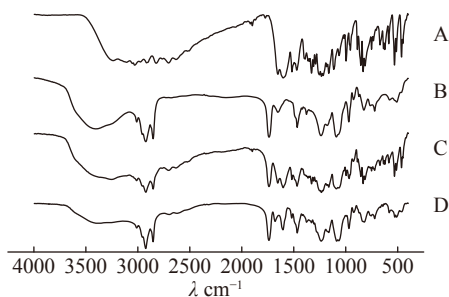


Fig. 4 FTIR spectra of liquiritigenin(A), phospholipid (B), the physical mixture (C), and the LPC (D)

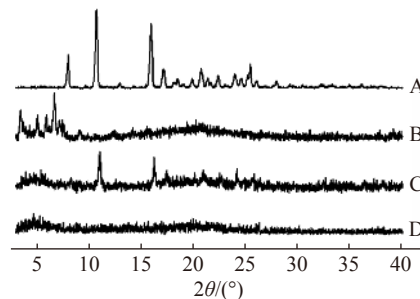


Fig. 5 PXRD patterns of liquiritigenin(A), phospholipid (B), the physical mixture (C), and the LPC (D)

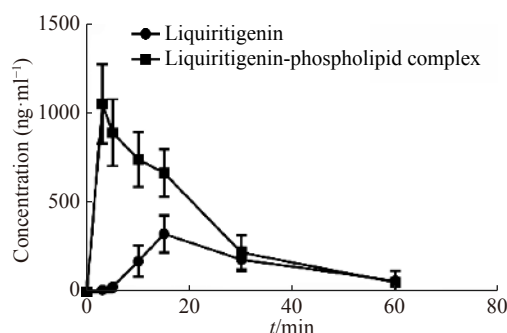


Fig. 6 Mean plasma drug concentration-time curve of liquiritigenin in rats after oral administration of liquiritigenin and LPC equivalent to 20 mg·kg⁻¹ of liquiritigenin. Values are the mean ± SD (*n* = 6/group/time point).

Table 5 Pharmacokinetic parameters of liquiritigenin and the LPC after a single oral administration of 20 mg·kg⁻¹ of body weight dose to rats (mean ± SD, *n* = 6)

Parameter	Liquiritigenin	LPC
<i>t</i> _{1/2} (min)	19.19 ± 3.98	13.35 ± 3.44
<i>T</i> _{max} (min)	15.15 ± 2.85	3.06 ± 1.46*
<i>C</i> _{max} (ng·mL ⁻¹)	326.55 ± 63.41	1051.30 ± 187.4*
<i>AUC</i> _{0-t} (ng·mL ⁻¹ ·min)	9356.02 ± 876.32	22 374.81 ± 2140.21 *

**P* < 0.05 is statistically significant for LPC versus liquiritigenin.

Conclusion

In this study, we successfully prepared LPC by the solvent-evaporation method. DSC, FTIR and PXRD data of phospholipid complex showed that the liquiritigenin crystals were converted into the amorphous form after forming the liquiritigenin-phospholipid complex (LPC). The n-octanol/water partition coefficient (*P*) of LPC studies showed that LPC remarkably increased the lipophilicity of LPC. Compared to liquiritigenin crystals, the LPC can significantly improve the bioavailability of liquiritigenin *in vivo*. The phospholipid complexation is a useful approach to improve the bioavailability of natural product with poor solubility.

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