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Enhanced Oral Bioavailability of Puerarin Using Microemulsion Vehicle

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The main purpose of this study was to prepare puerarin microemulsion system to improve oral bioavailability of puerarin. The microemulsion formulations were prepared using soybean oil, soybean lecithin/ethyl lactate (1:1), and 1,2-propanediol/water. The presence of microemulsion regions were investigated by pseudo-ternary phase diagrams. The droplet size of microemulsion was characterized by photo-correlation spectroscopy. In vivo pharmacokinetic study was conducted in mice, and the results indicated that $AUC_{0\to\infty}$ was 15.82-fold higher than that of puerarin suspension upon oral administration. Particles of puerarin microemulsion were round and homogeneous. Puerarin microemulsion also showed good stability. These studies showed that microemulsion system of puerarin might be promising vehicles for the peroral delivery of puerarin.

Keywords puerarin; microemulsion; bioavailability; pharmacokinetics

INTRODUCTION

Puerarin, a traditional Chinese medicinal herb, is isolated from the root of *Pueraria lobata* (Willd.) Ohwi and P. thomsonni Benth. Puerarin is a mixture that mainly consists of four isoflavones, i.e., daidzein, daidzin, puerarin, and daidzein-4′,7-diglucoside, among which puerarin is the most active component. Puerarin was found clinically effective to treat a variety of diseases, such as hypertension, arteriosclerosis, arrhythmia, fever, liver fibrosis, and so forth. Studies on pharmacology and clinical practice have shown that puerarin dilates coronary arteries, decreases myocardial oxygen consumption, and improves microcirculation in both animal and human patients suffering from cardiovascular diseases (Gao, 2003; Shi & Zhang, 2003). In China, *P. lobata* is also used as a health dietary supplement for reducing risk factors of cardiovascular diseases.

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However, puerarin is a poorly water-soluble drug with low bioavailability in animals and humans upon oral administration. Orally administered puerarin is absorbed rapidly with a t_{max} of about 1–2 h and a $t_{1/2}$ of about 1.5–2 h. The clearance of puerarin is 73.8%. It is excreted via dejecta as its prototype, and only 0.78% is excreted in the urine (Zhang, You, Wei, He, & Li, 1997; Zhu, 1979).

It was also reported that the elimination half-life of puerarin in the volunteers and patients was so short that the plasma concentration was fairly low after 4-h single-dose i.v. bolus of 5 mg/kg (Jin, Cheng, & Zhu, 1991; Robinson, 1978). Recently, it was reported that puerarin injection caused adverse reactions, such as anaphylactic shock, allergic rashes, laryngeal edema, hemolytic anemia, and so forth.

It can be seen that low bioavailability mainly hampered the effectiveness of puerarin as disease remedy. Because oral administration avoids the pain and risk of adverse reactions with parenteral administration, it is necessary to prepare oral puerarin formulations to maintain a more even blood level and significantly improve bioavailability. There are several formulations published to improve the dissolution and bioavailability of puerarin, such as puerarin micro-powders (Tang, Guo, & Rong, 2005), solid dispersions (Zhou et al., 2003), and puerarin phytosomes (Li et al., 2006).

In recent years, much attention has focused on lipid-based formulations to improve the oral bioavailability of poorly water-soluble drug compounds. One of the promising technologies is microemulsion drug delivery system. Microemulsions, which were a thermodynamically stable system composed of at least water, oil, and surfactants, can be used to improve the bioavailability of poorly soluble drugs (Moulik & Paul, 1998; Schwuger & Stickdorn, 1995). Microemulsions are transparent dispersions of oil and water stabilized by an interfacial film of surfactant molecules, which have the droplet size less than 100 nm. Microemulsion provides ultralow interfacial tensions and large oil/water interfacial areas. Microemulsions have a higher solubilization capacity than simple micellar solutions, and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions. Microemulsions,

associated with the nano-sized droplets, would influence the transport properties of the drug due to enormous interfacial areas, which is an important factor in sustained and targeted drug delivery (Eccleston, 1994; Lawrence & Rees, 2000).

The absorption of drug is mainly intestinal. The specific components of microemulsion promote the intestinal lymphatic transport of drugs. Lymphatic transport and association with CM and other triglyceride-rich lipoproteins may lead to many pharmacokinetic and pharmacodynamic consequences (Brocks, Ala, & Aliabadi, 2006; Gershkovich, Shtainer, & Hoffman, 2007; Shayeganpour, Jun, & Brocks, 2005). Main mechanisms include increasing membrane fluidity to facilitate transcellular absorption, opening tight junction to allow paracellular transport, inhibiting P-gp and/or CYP450 to increase intracellular concentration and residence time by surfactants, and stimulating lipoprotein/chylomicron production by lipids (Charman & Stella, 1991; Humberstone & Charman, 1997; O'Driscoll, 2002; Swenson & Curatolo, 1992; Wang, Yang, Lu, & Zhu, 1997).

The oral bioavailability of puerarin microemulsion has not been reported in either animals or humans. In this study, we studied the phase behavior of puerarin microemulsion, through which optimal microemulsion formulations were determined. Also bioavailability of orally administered puerarin microemulsion was evaluated in mice.

MATERIALS AND METHODS

Materials

Puerarin was purchased from Nanjing Sorun Herbal Technology Company (Nanjing, China) with the purity of 98%. Puerarin standard with the purity of 99.5% was provided by the National Institute for the Control of Pharmaceutic and Biological Products (Beijing, China). Puerarin and glucose injections were provided by Yangtze River Pharmacy Group Company (Jiangsu, China). Pure soybean lecithin of medical grade was purchased from TaiWei Co. Ltd., Shanghai, China. Soybean oil was purchased from Jiangxi Jinhaitang Pharmaceuticals Company (Jiangxi, China). Ethyl lactate and 1,2-propanediol were purchased from Shanghai Chemical Reagent Company (Shanghai, China). High-performance liquid chromatography (HPLC)-grade methanol was supplied by Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical reagent grade.

Screening of Aqueous Phase and Surfactants for Microemulsion

In order to find out optimal solvent that had a good solubilizing capacity of puerarin and, thus, could be used as the aqueous phase in microemulsion, the solubility of puerarin in various solvents was measured. Solvent candidates employed were ethyl lactate, 1,2-propanediol, and glycerin, respectively. An excess amount of puerarin was added to 10 mL of each solvent and turbine reciprocally at 60°C, then equilibrated at 37°C for 72 h, and centrifuged with the speed of 11,560 × g for 10 min. After that, puerarin concentration in the filtrate was determined by HPLC at 250 nm.

Construction of Pseudo-Ternary Diagrams

On the basis of the solubility studies of the drug, 1,2-propanediol/water was selected as the aqueous phase. Soybean lecithin was used as surfactant and ethyl lactate used as cosurfactant. Pseudo-ternary phase diagrams were constructed using aqueous phase titration method at 37°C to obtain the components and their concentration ranges that could result in large existence area of microemulsion without the drug. Four phase diagrams were plotted, with the weigh ratios of soybean lecithin to ethyl lactate as 2:3, 1:1, and 3:2, respectively. With each ratio of surfactant to cosurfactant (S/CoS), the ratios of the oil to the mixture of surfactant and cosurfactant were varied as 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1, respectively. Aqueous phase was added drop-wise to each oily mixture, under gentle magnetic stirring. After equilibration, the mixtures were visually assessed as microemulsion, crude emulsion, or gel. Based on these diagrams, appropriate concentrations of different materials were selected and used in the preparation of puerarin microemulsion.

Preparation of Puerarin Microemulsion

Through pseudo-ternary phase diagram investigation, optimal formulation (Table 1) was selected. Because puerarin was practically insoluble in water, the aqueous solution was prepared by dissolving puerarin in 1,2-propanediol first and then diluted to a definite volume of deionized water. The puerarin solution was added drop-wise to bean oil and surfactant mixture, under magnetic stirring at 37°C.

Particle Size Analysis

The average droplet size and polydispersivity index of the microemulsion were determined at 25°C by photon correlation spectroscopy with a Zetasizer 3000HS (Malvern Zetamaster, Malvern Instruments, 3000 HSa, Malvern, UK).

Transmission Electron Microscopy

The morphology of puerarin microemulsion was also observed using transmission electron microscopy (TEM, JEM-100SX,

TABLE 1
Composition of the Optimal Puerarin Microemulsion Formulation

Formulation Components	% (by weight)
Puerarin	4.8
Soybean lecithin	22.2
Soybean oil	37.6
Ethyl lactate	22.2
1,2-Propanediol/water	13.2

Hitachi, Japan). One drop of sample was deposited on a polyvinyl acetate-coated copper specimen grid and allowed to stand for 10 min, after which any excess fluid was removed with filter paper. The grid was later stained with one drop of 3% phosphotungstic acid and allowed to dry for 5 min before examination.

Stability of Microemulsion

The centrifuge tests were carried out to assess the physical stability of microemulsion. Microemulsion vehicles were centrifuged for 30 min at 13,870 × *g* and for 6 h at 3,470 × *g*. Microemulsion vehicles were also stored at 4°C, 40°C, and ambient temperature for 6 months. Then the clarity, phase separation, particle size, and concentration of puerarin were investigated to judge the optimal storage temperature monthly.

Determination of Puerarin in Mice Plasma by RP-HPLC

The concentration of puerarin in plasma samples was determined by HPLC (Agilent1100, Milan, Italy) with the wavelength of 250 nm at a constant temperature of 40°C. Mobile phase consisted of methanol–citric acid–water mixture (30:0.07:70, by volume) with a flow rate of 1.0 mL/min. The injection volume was 30 μL.

Liquid phase extraction procedure was as follows: 15 μL methanol was added to 30 μL of resulting plasma and vortexed for 5 min to form the precipitate. Then 30 μL of perchloric acid solution (5%) was added to precipitated protein and vortexed mixture for another 2 min and centrifuged at 12,000 rpm for 15 min. The supernatant was evaporated to dryness in a 40°C water bath in the presence of nitrogen. After that, the residue was dissolved in 50 μL of mobile phase and 30 μL was injected for HPLC analysis.

The chromatograms of the blank mouse plasma and a plasma sample spiked with puerarin are shown in Figure 1.

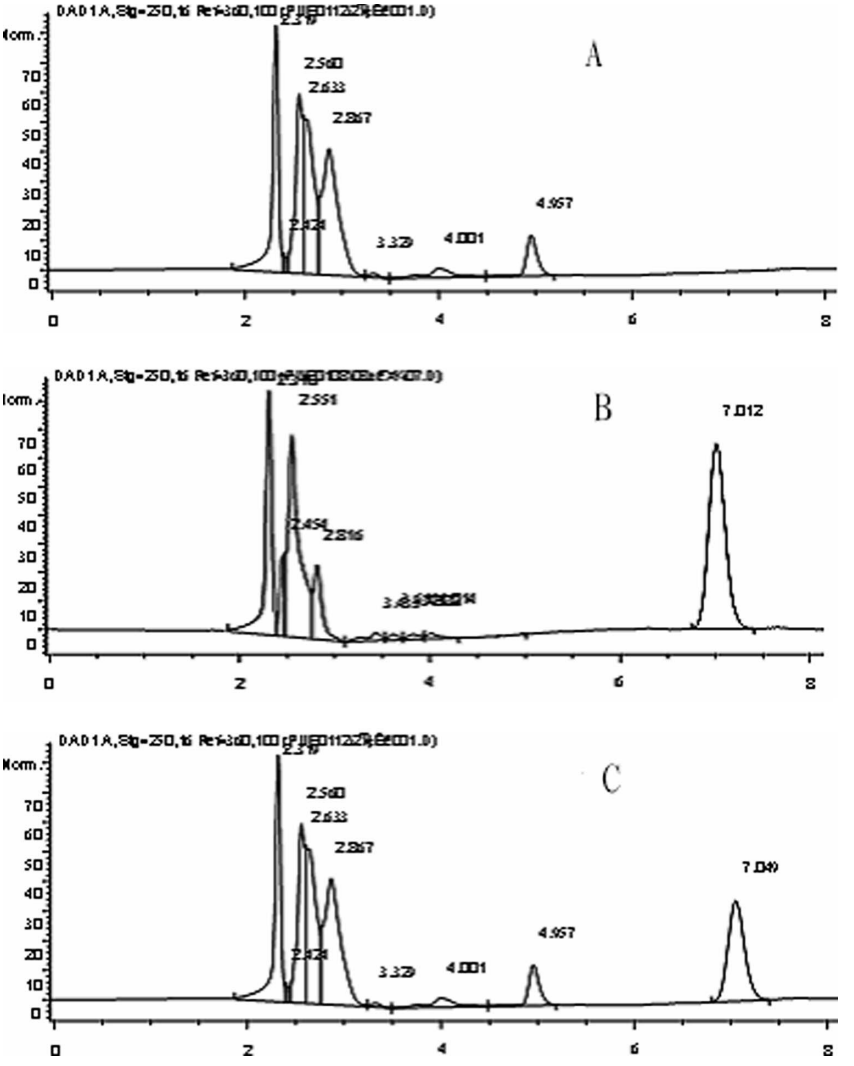


FIGURE 1. Typical chromatograms of puerarin. (A) Mouse blood plasma control (B) puerarin standard (C) mouse blood plasma spiked puerarin.

TABLE 2
Recovery of Spiked Puerarin from Mice Plasma

Groups	Spiked Puerarin (μg/mL)	Recovered Puerarin (μg/mL)	Recovery (%)
Group 1	0.0521	0.0534 ± 0.0019	102.63
Group 2	6.6667	6.6861 ± 0.0516	100.32
Group 3	53.3333	54.5211 ± 0.4	102.21

No interferences were detected during the elution of puerarin. The relationship between the peak area and appropriate concentrations was linear over the tested range (0.0521–66.6667 μg/mL). The correlation coefficient (*r*) of all calibration curves was >.99995.

The recovery of the three concentrations of puerarin samples (0.0521, 6.6667, 53.3333 μg/mL) are shown in Table 2. The standard deviations of reproducibility on intra- and inter-day analyses were also acceptable (<5%). The lower limit of quantification was determined to be 0.0261 μg/mL.

Long-term stability studies showed no significant degradation of puerarin in rat plasma samples stored >6 months at –20°C ($p < .05$). Overnight (~24 h) stability at room temperature was also determined for the post-preparation samples (extracts) (Table 3).

The concentration of the puerarin was determined by HPLC external standard method. Internal standards were not investigated in this study because the external standard method initially employed yielded acceptable accuracy, precision, and linearity, so correction by an internal standard was not necessary.

Bioavailability Studies

Kunming mice (25 ± 2 g, $n = 6$) were used for the study of both intravenously and orally administered puerarin. The animals were kept under standard laboratory conditions, that is, temperature at 25 ± 2°C and relative humidity at 55 ± 5%. The animals were housed in polypropylene cages, with free access to standard laboratory diet and water ad libitum. Before treatment, the animals were fasted overnight.

Bioavailability of puerarin microemulsion was compared with both puerarin suspension and marketed injection. Puerarin suspension was prepared by milling puerarin powder with a small amount of water and diluted to a definite volume using the same vehicle afterward.

Puerarin microemulsion was administrated to group 1 by gavage at a dose of 0.2 mg/g of puerarin. The blood samples were collected from the eye socket vein at specified time intervals of 10, 20, 30, 40, 50, 60, 90, 120, 180, 240, 360, 480, and 720 min after dosing.

Puerarin suspension was administrated to group 2 by gavage at a dose of 0.2 mg/g of puerarin. The blood samples were collected from the eye socket vein at specified time intervals of 10, 20, 25, 30, 35, 40, 50, 60, 75, 90, and 120 min after dosing.

Puerarin injection was intravenously administrated to group 3 at a dose of 0.01 mg/g of puerarin. The blood samples were collected from the eye socket vein at specified time intervals of 1, 5, 10, 15, 20, 30, 40, 60, 80, and 100 min after dosing.

Blood samples (~30 μL) were collected and placed into heparinized test tubes. Each sample was immediately centrifuged at 3,000 rpm for 10 min to obtain plasma. Then the plasma was transferred to a polypropylene plastic vial and stored in a deep freezer until used.

All data were subsequently processed using the computer program 3P97 (Chinese Pharmaceutical Association, 1997).

RESULTS AND DISCUSSION

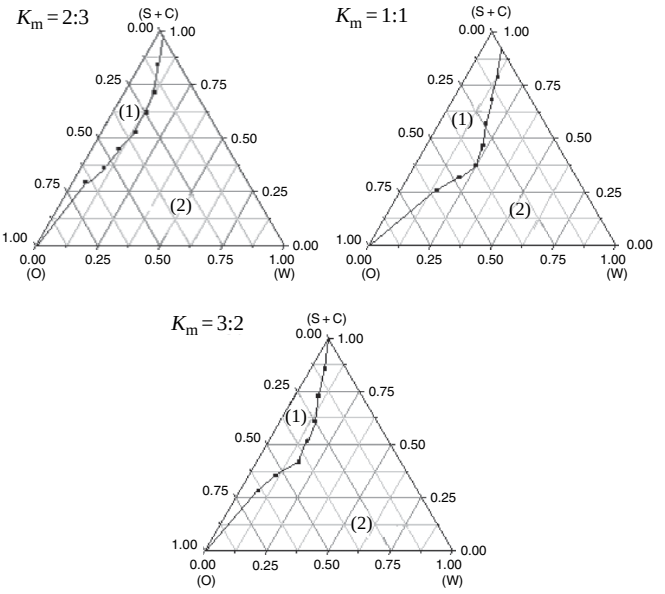
Pseudo-Ternary Phase Diagram Study

As shown in Figure 2, there were two areas in the diagram. With the addition of proper ratio of oil phase and surfactant

TABLE 3
Post-preparation (Extract) Stability of Puerarin at Room Temperature

	0.0521 (μg/mL)		54.5211 (μg/mL)	
	Original Injection (% rec)	Reinjection (% rec)	Original Injection (% rec)	Reinjection (% rec)
Sample 1	102.22	103.26	105.36	99.36
Sample 2	99.98	102.36	102.27	99.85
Sample 3	101.52	101.95	101.95	100.26
Sample 4	101.26	99.36	100.59	100.85
Sample 5	100.56	101.22	99.22	100.53
Average ± *SD*	101.11 ± 0.86	101.63 ± 1.47	101.63 ± 2.52	100.17 ± 0.58

% rec, percent recovery; *SD*, coefficient of variation.



(1) microemulsion region (2) multiphase region

FIGURE 2. Pseudo-ternary phase diagram of soybean oil–soybean lecithin–ethyl lactate–propylene glycol microemulsion for K_m = 0.67–1.5.

mixture, the system changed from microemulsion region to multiphase region followed by adding a drop of 1,2-propanediol/water. The multiphase region on the phase diagram included the turbid and conventional emulsion area based on visual observation, and the transparent and high-viscosity region included the gel area. At K_m ratio of 1/1, the area of existence in microemulsion reached the maximum.

Particle Size Analysis

The size distribution of puerarin microemulsion as measured by Zetasizer showed only one narrow sharp peak, which indicated that the nanoparticles size was unimodal distribution. The calculated mean size of puerarin microemulsion, based on three separate measurements, is 40.2 ± 5.9 nm.

Visualization by Transmission Electron Microscopy

Morphology of puerarin microemulsion characterized by TEM showed round particles (Figure 3).

Stability of Puerarin Microemulsion

Microemulsion formulations were stable at ambient temperature in either presence or absence of puerarin. No change of particle size, phase separation, and degradation of puerarin was observed during 6 months. The centrifuge tests showed that puerarin microemulsion had good physical stability. Puerarin microemulsion also showed good physical stability when vehicles were stored at 4 and 40°C for 6 months.

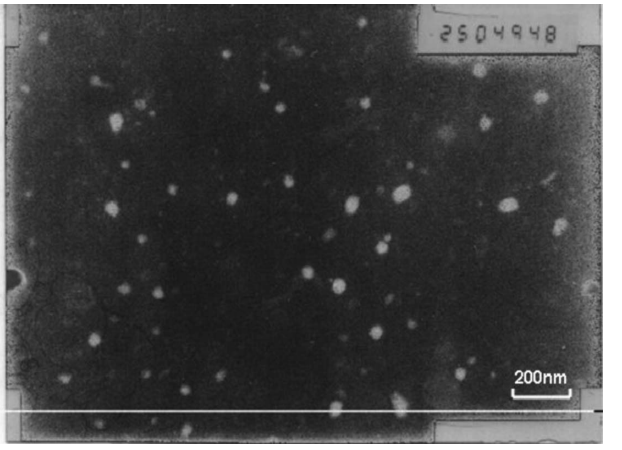


FIGURE 3. Microphotograph of puerarin microemulsion by transmission electron microscope (×25,000).

Bioavailability Studies

Pharmacokinetic parameters of puerarin microemulsion, suspension, and injection were calculated. Mean plasma puerarin concentration was plotted as a function of time and shown in Figure 4.

Compared with puerarin suspension, the higher plasma concentration of puerarin microemulsion after gavage administration might be due to the small size of nanoparticles. After intravenous administration of puerarin injection, plasma level of puerarin eliminated rapidly, most of which (after 100 min) was under limit of detection, with $AUC_{0\to\infty}$ of only 4.84 μg·h/mL.

Pharmacokinetic parameters of puerarin microemulsion and solution were shown in Table 4. Mean pharmacokinetic parameters for puerarin microemulsion and puerarin suspension were as follows: t_{max}, (0.81 ± 0.02) versus (0.46 ± 0.01) h; C_{max}, (5.21 ± 0.30) versus (1.62 ± 0.10) μg/mL; $AUC_{0\to\infty}$, (37.29 ± 2.83) versus (2.45 ± 0.47) μg·h/mL; $AUC_{0\to T_n}$, (31.32 ± 2.82) versus (1.98 ± 0.20) μg·h/mL, respectively. Based on $AUC_{0\to\infty}$

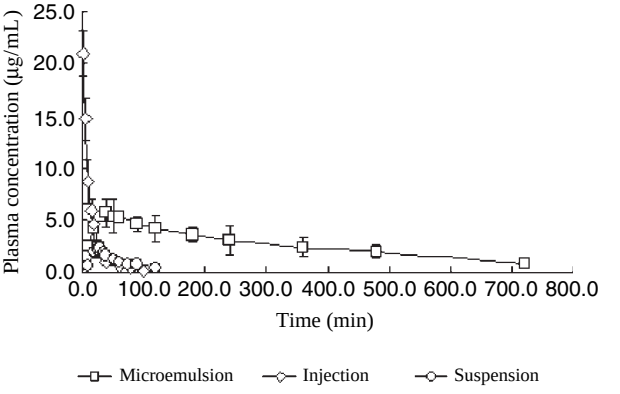


FIGURE 4. Time course of puerarin in mouse blood plasma after i.v. injection microemulsion and suspension.

TABLE 4
Pharmacokinetic Parameters of Puerarin Microemulsion and Suspension

Parameter	Puerarin Mean	Microemulsion *SD*	Puerarin Mean	Suspension *SD*
t_{max} (h)	0.81	0.02	0.46	0.01
C_{max} (μg/mL)	5.21	0.30	1.62	0.10
$AUC_{0\to\infty}$ (μg·h/mL)	37.29	2.83	2.45	0.47
$AUC_{0\to T_n}$ (μg·h/mL)	31.32	2.82	1.98	0.20
MRT (h)	4.39	0.17	0.83	0.01

calculations, the mean relative bioavailability of puerarin microemulsion was 15.82-fold higher than that of puerarin suspension. Compared with puerarin i.v. injection, the mean absolute bioavailability of puerarin microemulsion and that of suspension were 37.91 and 2.50%, respectively. The mean residence time (MRT) of puerarin microemulsion increased 5.289 times in plasma compared with the same dose of puerarin suspension, which may be due to the sustained release of puerarin from puerarin microemulsion.

In an attempt to improve the absorption of puerarin, water-in-oil microemulsion formulations were investigated as possible bioavailability enhancement. The medium-chain fatty acid lipid components of the microemulsion system were also putative absorption-enhancing agents and may therefore increase the permeability of the intestinal wall to the peptide (Lundin, Bojrup, Ljusberg-Wahren, Westrom, & Lundin, 1997; Lindmark, Schipper, Lazorova, de Boer, & Artursson, 1998; Yamamoto, Okagawa, & Kotani, 1997). High bioavailability of microemulsion may attribute to its promotion of lymphatic transport through transcellular pathway (Gershanik & Benita, 2000). It was reported that the long-chain oils promoted lipoprotein synthesis and subsequent lymphatic absorption (Charman & Stella, 1991).

CONCLUSIONS

Based on pseudo-ternary phase diagram, microemulsion formulation of puerarin contained soybean oil (38%, wt/wt), soybean lecithin (22%, wt/wt), and ethyl lactate (22%, wt/wt) as oil phase, surfactant, and cosurfactant, respectively. Particles of puerarin microemulsion were round and homogeneous. Puerarin microemulsion also showed good stability.

Compared with puerarin suspension and injection, puerarin microemulsion showed a higher bioavailability and a prolonged residence time as well. The pharmacokinetic studies in mice after oral administration of puerarin microemulsion and suspension showed that the AUC of puerarin microemulsion was superior to that of puerarin suspension.

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