European Journal of Pharmaceutical Sciences 44 (2011) 127–135

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Preparation of an anhydrous reverse micelle delivery system to enhance oral bioavailability and anti-diabetic efficacy of berberine

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ARTICLE INFO

Article history:
Received 24 February 2011
Received in revised form 1 June 2011
Accepted 25 June 2011
Available online 1 July 2011

Keywords:
Lyophilization
Emulsion
Phosphatidylcholine
Oily formulation
Hypoglycemic effect
Pharmacokinetics

ABSTRACT

To enhance oral bioavailability and anti-diabetic efficacy of berberine (BER), an anhydrous reverse micelle (ARM) delivery system was prepared through lyophilization of water-in-oil (W/O) emulsions. Using soy phosphatidylcholine as emulsifiers, BER-containing W/O emulsions were prepared and then lyophilized to form dry products which, upon addition of oil, formed clear ARMs containing amorphous BER nanoparticles. BER-loaded ARMs or free BER solutions were administered to streptozocin-induced diabetic mice. In vivo measurements demonstrated that the blood glucose levels (BGLs) of diabetic mice reduced on average to 22% of the initial values 4 h after intravenous injection of BER solution at the dose of 2.5 mg/kg body weight, while the average BGL reduction was 57% in the group gavaged with ARMs at the dose of 100 mg/kg body weight. No significant BGL reduction was noticed in mice orally received BER solutions. Compared to BER solutions, the oral bioavailability of BER-loaded ARMs was enhanced 2.4-fold, and the maximum blood concentration of BER was enhanced 2.1-fold with a 2-h time lag leading to a prolonged efficacy. Thus, this novel ARM delivery system provides a valid method to improve oral bioavailability and anti-diabetic efficacy of BER, offering a promising product alternative to other hypoglycemic drugs for diabetes therapy.

1. Introduction

Berberine BER, Fig. 1) is a quaternary ammonium salt from the group of isoquinoline alkaloids. It is found in such plants as *Coptis chinensis*, goldenseal (*Hydrastis canadensis*), and Berberis, usually in the roots, rhizomes, and barks. Herbs containing affluently BER have a long traditional history that is used in Chinese, Ayurvedic, and Middle-Eastern folk medicine mostly for its antimicrobial and antiprotozoal properties (Imanshahidi and Hosseinzadeh, 2008). Also, these BER-rich herbs have been used in traditional Chinese medicine for the treatment of many other chronic ailments or diseases including tumor, depression, hypertension, hypercholesterolemia, mania, and diabetes mellitus, as recorded in a variety of ancient Chinese classical medicinal works (Chen and Lin, 2006). For example, the Tang dynasty (1300 years ago) physician Simiao Sun prescribed in his famous work *Thousand Golden Prescriptions* 9 formulations containing Coptis chinensis as the main component for the treatment of XiaoKeZheng (wasting-thirst syndrome), namely diabetes mellitus (Sun, 2008), which is a group of metabolic diseases characterized by high blood sugar levels, either because the body does not produce enough insulin (type 1 diabetes), or because cells do not respond to insulin (type 2 diabetes). And diabetes has long since afflicted human beings and is now still a refractory disease (Li et al., 2004). Nowadays, proof-of-concept research on BER has revealed various pharmacological properties and medicinal uses similar to BER-rich herbs (Li et al., 2004; Vuddanda et al., 2010), suggesting BER one of the main active ingredients rendering the efficacies.

Recently, many research groups have showed a strong impact of BER on glucose homeostasis and marked anti-diabetic effects on both human beings and rodent models, especially, with type 2 diabetes (Wang et al., 2011; Yin et al., 2008a; Zhang et al., 2008). The action mechanism of anti-diabetic effect of BER has been widely investigated (Yin et al., 2008b). The up-to-date reports argued that the anti-diabetic activity of BER is related to activation of AMP-activated protein kinase (AMPK), acute initiation of glucose transport activity of glucose transporter GLUT1, and improvement of insulin signal transduction in insulin-resistant myotubes in cultured cells (Cok et al., in press; Liu et al., 2010; Yin et al., 2008a). Also, recent research proved BER able to differentially modulate the activities of ERK, p38 MAPK, and JNK to suppress Th17 and Th1 T cell differentiation to recover the T cell-mediated destruction of β cells in type 1 diabetic mice (Cui et al., 2009). Now, the anti-diabetic property of BER attracts more and more research interests

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doi:10.1016/j.ejps.2011.06.015

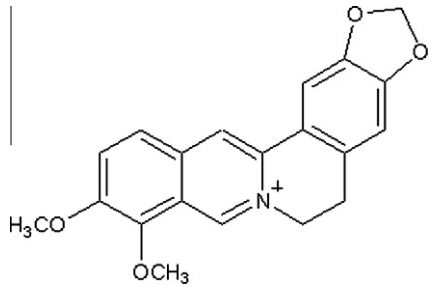

Fig. 1. Molecular structure of berberine.

because the safe and convenient-to-use natural products are rather desired than some currently available drugs with problems of adverse effects and drug resistance (Li et al., 2004). However, the anti-diabetic effects of BER have been reported in only long term studies which support its insulinotropic and insulin-sensitizing capabilities (Zhang et al., 2008). Moreover, up to now only the conventional dosage forms, such as solutions and tablets, have been used to perform the experiments and trials to test the hypoglycemic effects of BER on hyperglycemic subjects (Cui et al., 2009; Derosa et al., 2009; Zhang et al., 2008). BER is usually commercially available as berberine chloride dihydrate, which is slightly soluble in water. So far as the use of BER for anti-diabetic therapy is concerned, the conventional dosage forms are not efficient ones for oral administration, because the intestinal absorption of BER has proved very poor due to its limited membrane permeability and the bioavailability is usually less than 5% (Cicero and Ertek, 2009; Zuo et al., 2006). This may explain why previous studies on BER focused mostly on long term treatment, which usually takes several weeks or even months (Zhang et al., 2008); and this may also explain why the drug anti-diabetic effects observed were, sometimes, not as potent as expected (Li et al., 2004; Zhang et al., 2008). For these reasons, controversy on the anti-diabetic property of BER arose and still now exists (Zuo et al., 2006). Obviously, an efficient drug delivery system that can effectively improve oral bioavailability of BER will be beneficial in confirming its anti-diabetic effects and the related therapy.

In previous reports, novel procedures based on freeze-drying of emulsions were used to prepare drug-loaded liposomes and an oily formulation based on phospholipid and glyceride (Wang et al., 2009a,b, 2006, 2010). The oily formulation contained special reverse micelles and had been used as a carry for hydrophilic peptide, insulin, for oral delivery (Wang et al., 2010). In contrast to conventional reverse micelles having an independent inner water phase (Chen et al., 2008; Walde et al., 1990), the novel oily formulation trapped only dry hydrophilic agents in phospholipid-surrounding nano-cores and, therefore, was described as an anhydrous reverse micelle (ARM) drug delivery system (Wang et al., 2010). It confirmed that the ARMs could effectively protect hydrophilic peptide from damage in harsh gastrointestinal conditions and could enhance enormously anti-diabetic efficacy of insulin (Wang et al., 2010). The ARMs are an attractive oral drug delivery system for a number of reasons. They are normal dietary components and have barrier properties that can protect against damage from bulk water and other gut components such as proteolytic enzymes and protons, and they have also penetration-enhancing properties (Fricker et al., 2010; New and Kirby, 1997; Pouton and Charman, 1997). Moreover, oil phases can form a continuum with other lipidic barriers in the body, for example skin lipids and cell membranes, and in this way might allow passage of dissolved components which would otherwise be excluded (Kirby, 2000; Torchilin, 2007).

In this report, the simple FWE (freeze-drying of water-in-oil emulsions) procedure was deployed to prepare phospholipid-based BER-entrapped ARMs with purposes to enhance drug oral bioavailability and to verify and improve its anti-diabetic efficacy. The ARM preparation methodology includes preparation of submicron water-in-oil (W/O) emulsions containing phospholipids as emulsifiers in oil phase and hydrophilic BER in water, lyophilization to remove solvents, and addition of glyceride oil to form ARMs. The BER-loaded ARMs were orally administered to streptozocin (STZ)-induced type 1 diabetic mice to investigate the in vivo hypoglycemic effects, oral bioavailability, pharmacokinetics and anti-diabetic efficacy of the drug.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC, Lipoid S 100, purity > 94%) was purchased from Lipoid (Ludwigshafen, Germany). Medium-chain triglyceride (MCT, C_8 56.5%, C_{10} 43%) was provided by Greenwell Oleochemicals Co., Ltd. (Shah Alam, Malaysia). Berberine hydrochloride (BER, purity > 98%) was obtained from Xieli Pharmaceutical Co., Ltd. (Pengzhou, Sichuan Province, China). Streptozocin (STZ) was a product of Sigma–Aldrich (Shanghai, China). The solvents used for the chromatographic mobile phases were of HPLC grade. Bidistilled water was self-prepared. All other chemicals were of analytical reagent grade.

2.2. Preparation of ARMs by freeze-drying W/O emulsions (FWE)

The BER-containing ARMs were prepared according to a previous method with little modification (Wang et al., 2010). A mixture of cyclohexane/dichloromethane/diethyl ether (4:1:1, v/v/v) containing SPC was used as outer oil phase (O). BER was dissolved in water to give an initial drug concentration of 2 mg/ml and used as the inner aqueous phase (W). Different mass ratios of SPC-to-BER (from 5:1 to 50:1) were investigated to find out the minimum amount of SPC needed to solubilize the drug, namely to form an ARM system. To form submicron W/O emulsions, a mixture of W and O (1:4, v/v) was added to an ampoule (for large scale an appropriate container can be used). And then, using an ice/water bath to keep the temperature under 25 °C, the mixture was sonicated with a probe-type sonicator (JY92-II, Scientz Biotechnology Co., Ltd., Ningbo, China) in pulse mode (pulse on, 3 s; pulse off, 5 s; 80 W). Then the uniformly opalescent submicron W/O emulsions were immediately transferred into 5-ml freeze-drying vials with a fill volume of 2 ml, and rapidly frozen at −80 °C in an ultra-low temperature freezer (MDF-U73V, Sanyo Electric Co., Ltd., Japan). After being frozen at −80 °C for 4 h, the emulsion samples were lyophilized in a freeze-dryer (Eyela FDU-1100/DRC-1000, Tokyo Rikakikai, Japan) using the following procedure: primary drying at −37, −30, −20 °C for 4, 2 and 2 h periods, respectively; and secondary drying at 20 °C for 4 h. The chamber pressure was maintained below 10 Pa during the drying process. When the freeze-drying process was complete, the vials were immediately filled with nitrogen gas, sealed, and stored protected from light at 4 °C. When an appropriate amount (no less than half the initial volume of W/O emulsions, i.e. 1 ml in this case) of MCT oil was added into the vial of lyophilates, BER-loaded ARMs were obtained.

2.3. ARM formation condition analysis

Water content of the FWE products was analyzed using a trace moisture analyzer (KLS-411, Leici Precision & Scientific Instrument Co., Ltd., Shanghai, China).

The ARM formation conditions were analyzed by measuring the optical absorbance (OA) at 660 nm of the oily formulations

(Hammad and Muller, 1998), using a double-beam UV–vis spectrophotometer (TU-1901, Purkinje General Instrument Co., Ltd., Beijing, China), and MCT oil served as a blank control. Then the data are plotted with OA versus SPC-to-drug mass ratio. The OA of zero indicates complete solubilization of the hydrophilic drugs in oil, namely formation of an ARM system. The inflection point corresponding to the sudden increase of OA value was considered as the minimum value of SPC-to-drug mass ratio required to form ARMs (Hammad and Muller, 1998).

To determine the amount and recovery of BBR in oily formulations, 0.1 ml of an oily formulation and 10% Triton-X100 and 9.8 ml of water were mixed homogeneously, and then the mixture was analyzed by HLPC (LC-6AD liquid chromatograph, SPD-20A UV detector, SIL-10AF autosampler, Shimadzu, Japan), using an ODS column (4.6 mm × 250 mm, 5 μm particle diameter, Hanbon S&T Co., Ltd., Huaian, Jiangsu, China). The mobile phase was water (0.2% triethylamine, pH 3.0 by phosphoric acid)–methanol (75:25, v/v), filtered through a 0.45 μm Millipore filter and degassed by sonication prior to use. The flow-rate was 1.0 ml/min. The injection volume was 20 μL. Detection was performed at a wavelength of 347 nm at room temperature (Kheir et al., 2010).

2.4. Dynamic light scattering (DLS)

The size of the inner water droplet in the initial W/O emulsions and the size of particles in ARMs were analyzed by DLS using a Malvern Zetasizer NS90 (Malvern, Worcestershire, UK). Quartz cuvettes were used for DLS tests and the scattering light is collected at an angle of 90°. The DLS test was carried out at 20 °C. For emulsion size determination, the blank solvent was a mixture of cyclohexane/dichloromethane/diethyl ether (4:1:1, v/v/v) with a viscosity of 0.83 cP and a refractive index of 1.37 at 20 °C; and for ARMs the blank solvent was MCT oil with a viscosity of 29.7 cP and a refractive index of 1.45 at 20 °C. The blank solvent scattering was subtracted from the total sample scattering to determine the excess Rayleigh ratio, from which particle/droplet size was determined. For each sample the drug concentration was fixed at 0.1 mg/ml, and the DLS measurements are repeated three times. The gained values for ARMs are the "hydrodynamic" diameters of the micelles.

2.5. Characterization of the FWE lyophilates

2.5.1. Scanning electron microscopy (SEM)

Samples of the FWE lyophilates were fixed on an SEM-stub using conductive double-sided tape, coated in a vacuum with a thin layer of gold/palladium, and examined using a field emission scanning electron microscope (Superscan SSX-550, Shimadzu, Japan) equipped with a complete-digital computer control system. An accelerating voltage of 15 kV was used.

2.5.2. Thermal analysis

Differential scanning calorimetry (DSC) analysis was performed using DSC-60 Series Differential Scanning Calorimeter (Shimadzu DSC 60, Japan). Approximately 5 mg of BER or 15 mg of the lyophilized powders were accurately weighed in an aluminum pan and sealed for analysis. A heating rate of 10 °C/min was employed in the temperature range of 30–250 °C. An empty pan was used as a reference.

2.6. In vitro drug release assay

An ultrafiltration method was used for drug release assay (Wang et al., 2010). Briefly, 0.5 ml of oil-based formulations was mixed with 9.5 ml of an acidic buffer (0.01 M HCl, 0.021 M NaCl, pH 2.5) or a neutral buffer (0.06 M NaH_2PO_4, pH 6.8). Then the mixture was transferred into a 10-ml stirred ultrafiltration cell (Amicon 8010, Millipore Corporation, Bedford, USA) with a membrane of 10,000 Da cutoff, stirring at certain speed (120 or 60 rpm) at 37 °C. Then at pre-determined time points an aliquot of aqueous solution (0.5 ml) was separated from the ARM-buffer mixture through ultrafiltration driven by nitrogen gas pressure, and an equal volume of fresh medium was immediately added after each ultrafiltration. Then the amount of the drug released from oil into the medium at different time points could be calculated through assaying of the ultra-filtrates using the above-mentioned HLPC method.

2.7. In vivo experiments

The animals employed for testing different BER-containing formulations were male Kunming mice (20 ± 2 g) provided by the Experimental Animal House of Jining Medical College (JMC). All animal experiment protocols were approved by the Animal Care Committee of JMC Pharmaceutics Section and carried out in compliance with *the Declaration of Helsinki for Care and Use of Laboratory Animals* and *Guide for the Care and Use of Laboratory Animals* (Clark et al., 1996).

2.7.1. Streptozocin (STZ)-induced type 1 diabetic models in mice

Mice were rendered diabetic by once daily intraperitoneal injection of STZ (dissolved in 10 mM citrate buffer at pH 4.5) at a dose of 40 mg/kg body weight for 5 days (Wu and Huan, 2008). Mice were considered diabetic when their fasting BGL was higher than 8 mM, 2 weeks after the STZ treatment.

2.7.2. Hypoglycemic effects of once administration of BER formulations

Four groups of type 1 diabetic mice were employed and each group contained 18 mice. All the mice were fasted overnight prior to and during the experiment but had free access to water. The first group of mice was gavaged with BER-loaded ARMs at a dose of 100 mg/kg body weight. The second group was injected through tail vein a BER water solution that had been sterilized by filtering through 0.22-μm microporous membranes, at a dose of 2.5 mg/kg body weight. The third group was intragastricly given a BER water solution at a dose of 100 mg/kg body weight; and, likewise, the fourth with equal amount of MCT oil containing SPC, and used as controls. For blood glucose level (BGL) test, blood samples were collected from the tail veins of mice prior to drug administration and at different time intervals after dosing. The BGLs were immediately determined using a SureStep Plus blood glucose meter and OneTouch test strips (Johnson & Johnson LifeScan Inc., Milpitas, California, USA), with the testing range of 1.1–33.3 mM and detection limit of 0.1 mM. Following each BGL test after dosing, the mice were immediately anesthetized through ether inhalation and then their blood was sampled for drug bioavailability and pharmacokinetics study.

2.7.3. Drug bioavailability and pharmacokinetics study

To evaluate bioavailability and pharmacokinetics of BER formulations, the above four groups of mice were used, but the iv group and each gavage group included 6 and 3 more mice, respectively, and their blood samples were collected from heart cavities after anesthesia. Due to limited blood volume, at each time point three mice were used and then sacrificed immediately after sampling. Thus, the iv group contained 24 mice and each oral group contained 21 mice.

Each collected blood sample was immediately transferred to a 1.5-ml heparinized centrifuge tube and centrifuged at 23,000 *g* for 15 min. An aliquot of 0.2 ml of the supernatant was then transferred to a 1.5-ml centrifuge tube, and mixed with 0.8 ml of

methanol by vortex for 1 min. The resulting denatured protein precipitate was separated by centrifugation at 23,000 *g* for 15 min. An aliquot of 0.5 ml of the supernatant was then put in a 1.5-ml centrifuge tube. They were left to be evaporated at 37 °C, and then the residue was dissolved in 0.5 ml of methanol. The supernatant was separated by another centrifugation at 23,000 *g* for 15 min and then transferred to a 1-ml sample glass bottle and sealed. A 20 μl volume of each sample solution was injected with the autosampler into the HPLC system for BER analysis using the above-described HPLC method which has been proven valid in quantifying BER in vivo in mice (Kheir et al., 2010). Also, the linearity, accuracy, recovery of BER in mouse plasma, and limits of quantification and detection with the HPLC method were evaluated according to reference (Kheir et al., 2010). Data from these samples were used to evaluate the bioavailability of BER formulations and to construct pharmacokinetic profiles by plotting drug concentration versus time. The plasma BER concentration–time curves after administration were evaluated using SPSS Version 13 software. The maximum blood concentration (C_{max}) and the time of maximum blood concentration (t_{max}) were obtained directly by observation from the plasma drug concentration–time curves.

Data from the iv group were used to evaluate the pharmacokinetic compartment model. A log scale plot of the blood level decay curve of a 1-compartment model yields a straight line, while a log scale plot of the blood level decay curve of a 2-compartment model yields a biphasic line (Shargel et al., 2004).

The $AUC_{(0-16)}$ was the area under the plasma concentration–time curve from time 0 h to final tested concentration time point 16 h after oral administration and was calculated using the linear trapezoidal rule. The $AUC_{(0-\infty)}$ was the area under the plasma concentration–time curve from time 0 h to infinity after oral administration and was calculated using parameters obtained from the constructed pharmacokinetic profiles. The calculation for parameters including others such as $t_{1/2ka}$ (half-life of drug absorption), $t_{1/2\alpha}$ (half-life of drug distribution, for 2-compartment model), $t_{1/2\beta}$ (half-life of drug clearance, for 2-compartment model), and k_{cl} (the actual drug clearance rate), was performed according to the reference (Shargel et al., 2004).

The bioavailability (BA) and relative bioavailability (RBA) of test BER formulations after oral administration were calculated using the following formulas.

$$\mathrm{BA}\ (\%) = \frac{\mathrm{AUC_{oral}} \times \mathrm{Dose_{iv}}}{\mathrm{AUC_{iv}} \times \mathrm{Dose_{oral}}} \times 100 \quad (1)$$

$$\mathrm{RBA}\ (\%) = \frac{\mathrm{AUC_{oral\ ARM}} \times \mathrm{Dose_{oral\ solution}}}{\mathrm{AUC_{oral\ solution}} \times \mathrm{Dose_{oral\ ARM}}} \times 100 \quad (2)$$

2.7.4. Anti-diabetic efficacy of long term administration of BER-loaded ARMs

Two groups of type 1 diabetic mice were employed and each contained five mice. Prior to drug administration, the BGLs of all mice were tested after they were fasted overnight. Then, the first group of mice was once daily gavaged with BER-loaded ARMs at a dose of 100 mg/kg body weight for 7 days. The second group was orally given equal amount of MCT oil containing SPC, and used as controls. For each drug administration except for the last time, the mice were fasted continuously for 8 h, i.e. 4 h prior to and 4 h after dosing. On the seventh day, after being fasted overnight, the mice was gavaged with ARMs. Also, the BGLs of mice was tested prior to drug administration and at different time intervals after dosing using the above-mentioned method. Water was supplied to mice all the time.

2.8. Statistical analysis

Data are presented as mean ± SD (standard deviation). For group comparison, a one-way analysis of variance (ANOVA) was applied. Significant differences in mean values were evaluated by a Student's *t*-test. The *P* values less than 0.05 were considered statistically significant. SPSS Version 13 software was used for data analysis.

3. Results

3.1. Droplet size of the W/O emulsions

DLS examination indicated that the inner water droplet size of the W/O emulsions was below 500 nm with a mean diameter (MD) of 330 ± 280 nm (MD ± SD, $n = 3$).

3.2. ARM formation condition

The water content in the FWE lyophilates or oily formulations was less than 0.5% (w/w). Therefore water molecules could not aggregate as an independent aqueous microphase to form conventional reverse micelles, which can only be formed with molecular ratio of water-to-SPC higher than 8 (Walde et al., 1990).

The minimum value of SPC-to-BER mass ratio needed to form an ARM system was 25:1, as shown in Fig. 2.

The HPLC analysis proved that the recoveries of model drug BER in triplicate from the ARMs of SPC/BER (10:1) were 97.7%, 95.5% and 95.9%.

3.3. Particle size of the ARMs

DLS examination of the oily formulations with zero OA revealed that the particle size of the ARMs was below 60 nm. The mean diameter (MD) of the BER-containing ARMs was 36 ± 21 nm (MD ± SD, $n = 3$).

3.4. SEM

A representative SEM image of the BER-containing FWE lyophilates is shown in Fig. 3. It is clear that in the FWE lyophilates there were numerous particles with a MD of 28 nm.

3.5. DSC

The DSC thermograms of BER and the BER-containing FWE lyophilates are shown in Fig. 4. It can be seen that, in contrast to BER raw materials, the FWE lyophilates demonstrated no sharp

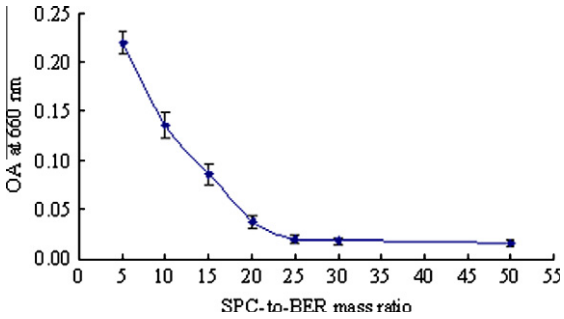

Fig. 2. The optical absorbance of the BER-containing oily formulations with different SPC-to-drug mass ratios ($n = 3$). The inflection point corresponding to the sudden increase of OA value was considered as the minimum value of SPC-to-drug mass ratio required to form ARMs.

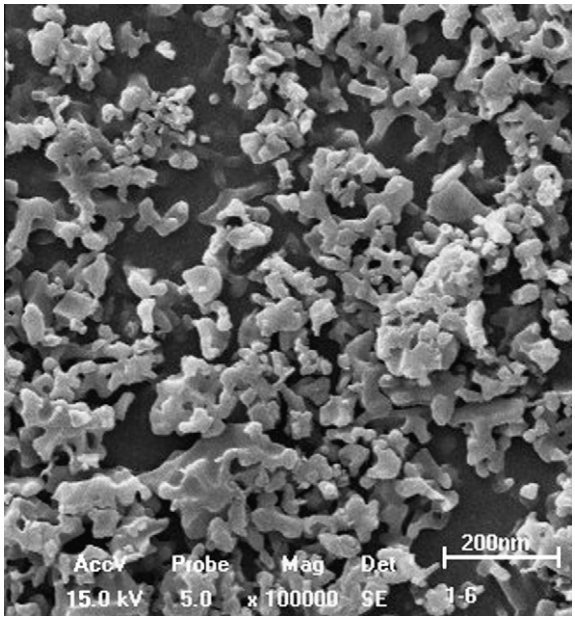

Fig. 3. A representative SEM of the FWE lyophilates with composition of SPC/BER (25:1, w/w). The particles with size of about 28 nm are BER crystal covered with SPC.

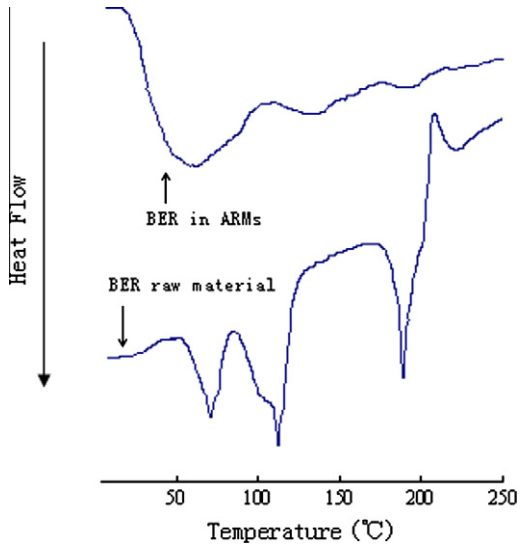

Fig. 4. The DSC thermogram of the BER-entrapped FWE lyophilates with composition of SPC/BER (25:1, w/w). There are no sharp endothermic transition peaks at 116 and 193 °C of crystal BER thermal characteristics, indicating BER in amorphous form.

endothermic transition peaks at 116 and 193 °C of crystalline BER. This indicates BER in the FWE lyophilates was mostly in an amorphous state.

3.6. In vitro drug release

The results of the release of BER from the ARM formulation under acidic and neutral conditions are shown in Fig. 5. It is clear that pH of the medium had little effect on BER release, but fast stirring increased the drug release rate markedly. In both acidic and neutral mediums, stirred at 60 rpm about 50% of total BER was released from the ARM formulation within 4 h. But when the stirring was 120 rpm 70% of BER was released within 4 h.

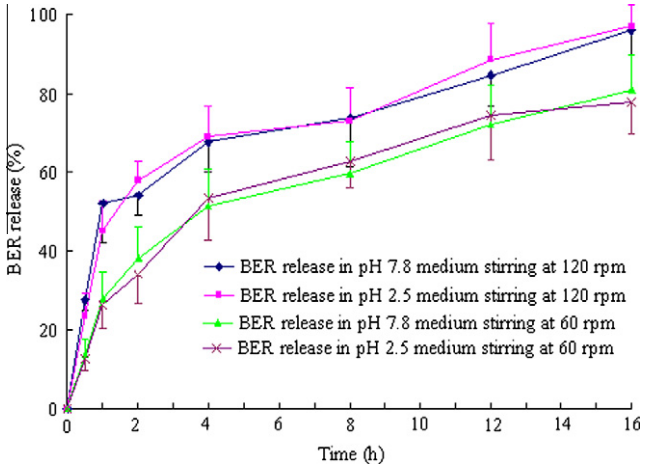

Fig. 5. The results of the in vitro release of BER from the oily formulations into the aqueous mediums. The release was influenced markedly by stirring rate ($n = 3$).

3.7. Anti-diabetic efficacy of BER formulations

The potential anti-diabetic effects of different BER formulations were evaluated in type 1 diabetic mice. Fig. 6 illustrates changes in plasma glucose levels of the diabetic mice after one treatment with BER products. Significant differences in plasma glucose reduction (percentage relative to the initial value) among groups of the control, BER water solution and BER-containing ARMs were observed after drug administration ($p < 0.05$). Intravenous injection of BER solution at a dose of 2.5 mg/kg body weight and oral administration of BER-entrapped ARMs at 100 mg/kg reduced within the first 4 h the BGLs of the fasting diabetic mice to 22%, 57% of the initial values, respectively. Especially in BER-loaded ARM group the glucose reduction continued for 12 h and returned to 87% after 16 h. In contrast, there was no significant BGL reduction in the group orally received BER water solution, but in the MCT/SPC group, the BGLs were not reduced but increased slightly to 107% within the first 4 h and then returned to 105% and continued till 16 h.

In the long term (6-day period) drug administration group, remarkably, the BGLs of the type 1 diabetic mice returned to normal range (3–7 mmol/L) after gavage once daily with BER-entrapped ARMs for 6 days continuously. Interestingly, further drug administration on the seventh day did not induce further BGL reduction in mouse models, as shown in Fig. 7. This might suggest that the STZ-induced type 1 diabetic mice may recover from illness after oral administration of BER-entrapped ARMs and BER cannot reduce BGLs of normal mice.

3.8. Drug bioavailability and pharmacokinetics

The HPLC method was valid for quantification of BER in mouse blood. The equation of the calibration curve constructed for mice blood plasma was $y = 531.6x + 2019.7$ (in the range from 0.02 to 5.0 μg/ml), where x is the concentration of BER in the blood and y is the area under the curve. The correlation coefficient (R^2) was 0.996. The recovery values of 0.02, 0.5 and 5 μg/ml from blood plasma were 78.6%, 81.3% and 80.4%, respectively. The coefficients of variation for intra- and inter-day precision were less than 10%, and the limit of quantification was 0.02 μg/ml, and the limit of detection was 0.0054 μg/ml.

The corresponding plasma BER concentration–time profiles and a log scale plot of the blood level decay curve of iv group are shown in Figs. 8 and 9, respectively. Fig. 9 shows clearly that a log scale plot of the blood level decay curve of iv group yields a biphasic line (linear α phase and linear β phase) featuring a 2-compartment

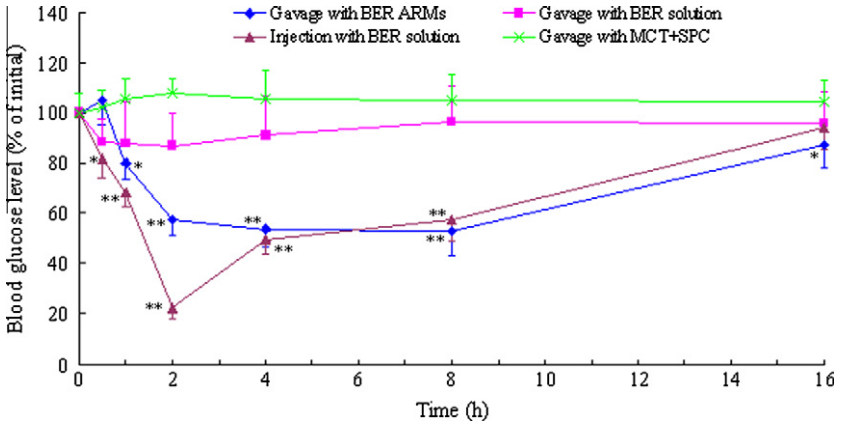

Fig. 6. Changes in BGL versus time profiles following administration of BER formulations in diabetic male Kunming mice ($n = 3$) fasted for 24 h. The dose of BER for injection was 2.5 mg/kg body weight, and the dose of BER for gavage was 100 mg/kg body weight. Each value represents mean ± S.D., and statistically significant difference from control: $^{*}p < 0.05$; $^{**}p < 0.01$.

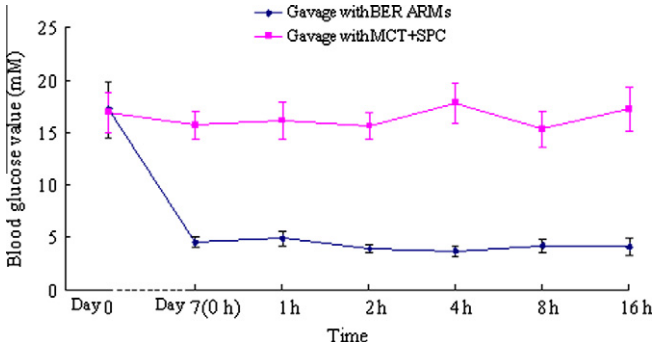

Fig. 7. Changes in BGL versus time profiles following repeat oral administration of BER-loaded ARMs in diabetic male Kunming mice ($n = 5$). The dose of BER was 100 mg/kg body weight. Each value represents mean ± SD, and statistically significant difference from control: $^{*}p < 0.05$; $^{**}p < 0.01$.

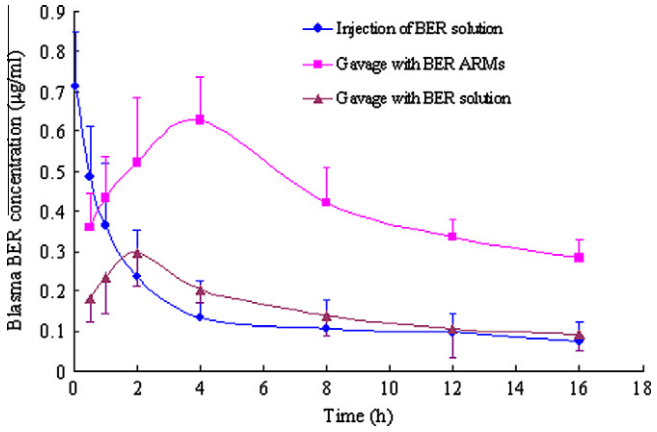

Fig. 8. The time-course of BER blood concentrations after administration of different BER formulations through different routes. The dose of BER for injection was 2.5 mg/kg body weight, and the dose of BER for gavage was 100 mg/kg body weight. Each point and bar represents the mean ± SD ($n = 3$).

model. Also, as shown in Fig. 8, in all formulations BER does not equilibrate rapidly throughout the body, as is assumed for a one-compartment model, but follows the pharmacokinetics of a two-compartment model: the drug distributes into two compartments, the central compartment and the tissue (peripheral compartment). The related pharmacokinetic parameters are shown in Table 1. The

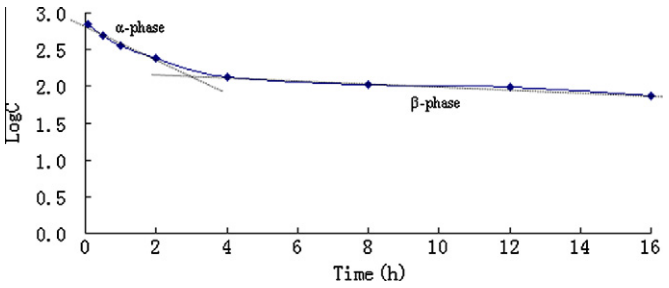

Fig. 9. A log scale plot of the blood level decay curve of iv group. A biphasic line (linear α phase and linear β phase) featuring a 2-compartment model (drug concentration, C, unit: μg/L). Each point represents the mean value ($n = 3$).

group gavaged with the free-form BER solution resulted in a maximum plasma concentration at 2 h post-administration, whereas the oral administration of BER-loaded ARMs showed a maximum plasma concentration at 4 h. The AUC (0–16 h) for the group orally treated with BER-loaded ARMs was 6 μg h/L, corresponding to a 2.4-fold enhancement of BA. These results demonstrated that the intestinal absorption of BER was significantly enhanced by the ARMs, thus markedly increasing its bioavailability. Considering

Table 1
Pharmacokinetic parameters of BER in diabetic mice after intravenous injection of the BER solution or oral administration of BER-loaded ARMs or BER solution ($n = 3$).

Parameter[a]	BER solution (iv)	BER ARMs (oral)	BER solution (oral)
Dose (mg/kg)	2.5	100.0	100.0
C_{max} (μg/mL)	0.712	0.628	0.298
t_{max} (h)	0.083	4.0	2.0
$t_{1/2\alpha}$ (h)	0.75	1.47	2.15
$t_{1/2\beta}$ (h)	35	16.18	23.15
k_{el} (h^{-1})	0.174	0.061	0.032
$t_{1/2ka}$ (h)	–	1.11	2.04
$AUC_{(0-16)}$ (μg h/L)	2.37	6.01	2.49
$AUC_{(0-\infty)}$ (μg h/L)	4.10	12.71	4.64
BA (%)	–	12.69	5.27
RBA (%)	–	241.0	100

[a] C_{max}: maximum plasma concentration; t_{max}: time at which C_{max} is attained; $t_{1/2\alpha}$: half-life of drug distribution; $t_{1/2\beta}$: half-life of drug clearance; k_{el}: the actual drug clearance rate; $t_{1/2ka}$: half-life of drug absorption; $AUC_{(0-16)}$: area under the plasma concentration–time curve from time 0 h to final tested timepoint 16 h after oral administration; $AUC_{(0-\infty)}$: area under the plasma concentration–time curve from time 0 h to infinity after oral administration; BA: bioavailability; RBA: relative bioavailability.

significant BGL reduction was not observed in the mice gavaged with drug water solution, it can be concluded that BER has oral anti-diabetic activity only on the ground that the drug be administered in an efficient delivery system, such as ARMs, to be absorbed enough.

4. Discussion

The above data have clearly demonstrated that BER has a potent anti-diabetic efficacy and can be solubilized in oily formulations to form an ARM system which has the ability to enhance oral bioavailability of the drug, and hence its anti-diabetic efficacy in type 1 diabetes. Though numerous clinical researches focused BER therapy on type 2 diabetic patients which are the most common cases, our research results indicate that BER delivered in ARMs can decrease BGL in type 1 diabetic mice. It is reported that BER increases glucose uptake through a mechanism distinct from insulin by stimulating the GLUT1-mediated glucose uptake by activating GLUT1 via AMPK pathway (Cok et al., in press; Kim et al., 2007; Zhou et al., 2007). This suggests that BER will exert its anti-diabetic efficacy both in presence of insulin (type 2 diabetes) and in absence of insulin (type 1 diabetes). In addition, the anti-diabetic effects of BER in type 1 diabetic mice had also been observed by other researchers, and the possible mechanisms were explored (Cui et al., 2009).

The ARM formation mechanism has been proposed in a previous report where insulin was used as a model drug (Wang et al., 2010). In this investigation, using the FWE procedure BER was loaded in ARMs where it was surrounded by SPC and existed mostly in amorphous form, as confirmed by DSC pattern. The decision factors for the drug state in ARMs are thought to be related to the formulation, freezing protocol and drug properties. For example, in the previous report insulin raw material contained trivial amount of zinc ions which facilitated insulin crystallization during the FWE process (Wang et al., 2010). Super-cooling and rapid freezing have been reported to inhibit crystallization (Craig et al., 1999) and should be responsible for BER amorphous state, which was also indirectly supported by the fact that BER release from ARMs into aqueous medium was much faster than crystalline insulin. It is known that amorphous state favors drug dissolution (Yu, 2001). On the other hand, the amorphous BER in ARMs resulted in large and loosen instable nanoparticles which also facilitate drug release. However, the slow partitioning of BER between the hydrophilic core and the external oil phase, for which it has limited affinity, makes drug escape from ARMs difficult. And the main drug release from the oily formulations occurred only when BER is in contact with the aqueous medium which is in fact separated from the drug by two barriers: SPC membranes and bulk oil. Thus, the drug release is enormously inhibited. Notably, the release rate of BER in both acidic and neutral medium was remarkably influenced by mechanic stirring. This is not the case in insulin ARMs (Wang et al., 2010). Perhaps the vigorous stirring breaks down the small oil droplets and SPC membranes that surrounded loosen BER nanoparticles leading to faster drug release. A schematic diagram of the BER release from the ARMs is shown in Fig. 10. It should be pointed out that, in small intestine, the drug release rate of ARMs may be significantly increased, because the oil phase will be emulsified by bile salts and the oily vehicle may be degraded by digestive enzymes (Jones et al., 2008). In addition, an increase in interfacial specific area may act as another driving force for encapsulated drug release (Jones et al., 2008). Despite these influential factors, the BER-containing ARMs provide a prolonged therapeutic effect, as confirmed by the in vivo tests.

Although BER is considered as one of the main ingredients in several Chinese herbal medicines that had long been used for

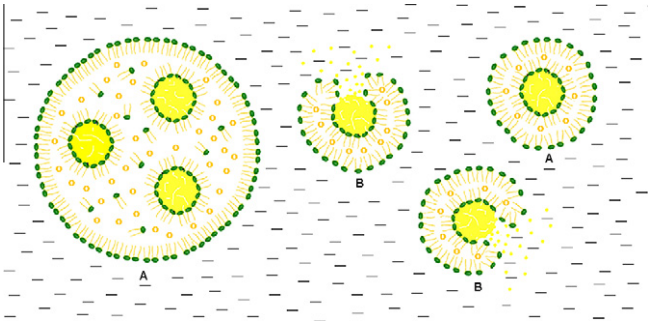

Fig. 10. A schematic diagram of the BER release from the FWE ARMs. (A) Oil droplets containing BER-loaded ARMs which are formed when the oily formulation is dispersed in an aqueous solution. (B) Broken BER-loaded ARMs from which BER is released. Small golden hollow dots represent oil phase, short black bars represent water phase.

anti-diabetic therapy in numerous ancient prescriptions (Chen and Lin, 2006; Sun, 2008), and modern clinical trials often presented positive effects on diabetic patients after long term treatment with BER (Imanshahidi and Hosseinzadeh, 2008; Yin et al., 2008a; Zhang et al., 2008), there is still a controversy on its anti-diabetic efficacy due to lack of direct evidence (Zuo et al., 2006). In this investigation, the in vivo experiments confirmed that BER has indeed a strong hypoglycemic effect on STZ-induced diabetic mice after treatment with intravenous injection of BER solution or oral administration of BER-loaded ARMs. However, when free BER solution was given only once intragastricly to model mice, no significant hypoglycemic effects were observed, due to, perhaps, poor intestinal absorption and too low blood concentration that was below the minimum effective level. This may be the reason why previous reports on BER hypoglycemic efficacy focused mainly on long term treatment, but few on short term effects. This also suggests that instead of conventional dosage forms an effective drug delivery system, such as ARMs, that can efficiently enhance intestinal absorption and bioavailability is required for oral administration of BER to engender an immediate anti-diabetic efficacy. Notably, as shown in Fig. 8, after a 6-day period of repeat treatment with BER-loaded ARMs, the BGLs of all STZ-induced diabetic mice returned to normal range (2–7 mM). This suggests the ARM-based BER delivery system a promising medication for curing diabetes mellitus which afflicts human beings for a long time.

The in vivo experiments show that the maximum concentration (C_{max}) of oral BER loaded ARMs was enhanced 2.1-fold compared to that of oral solution in model mice, while the bioavailability (BA) of ARMs was enhanced 2.4-fold. Although the BA of oral BER loaded ARMs was still no higher than 20%, the enhancement enables BER to exert significant hypoglycemic effects in diabetic mice. The time of maximum concentration (t_{max}) in the mice treated with BER loaded ARMs appeared 2 h posterior to that received orally free BER solution. The explanation for this drug lag may be that BER-loaded ARMs contained much MCT oil and the latter prolonged remarkably mammals' gastric emptying time. Correspondingly, this prolonged gastric emptying time resulted in sustained drug release in gastrointestinal tract and, thereby, longer efficacy. This sustained drug release can find practical applications and has advantages in the oral administration of drugs for extended therapy for the chronic disease such as diabetes mellitus. The half time of BER absorption ($t_{1/2ka}$) was shortened to half by ARMs indicating the ability of the carrier to accelerate drug absorption. Unexpectedly, the constants of actual drug clearance rate ($k_{1/2cl}$) of different dosing groups were completely different values and there was a wide gap between each other. A possible explanation for this is that certain enzymes that influenced BER metabolism are induced by and in dependence on drug blood concentrations, leading

to distinct clearance rates for the drug in different dosage forms that have exhibited different absorption features. Another explanation might be that when absorbed from gastrointestinal tracts and circulated in circulation systems BER was still in the ARM-carried form, therefore the ARM delivery system is responsible for drug pharmacokinetic profiles. Notably, the BER clearance half life ($t_{1/2\beta}$) in mice for different formulations ranks in the order of $t_{1/2\beta}$ of BER ARM (oral) < $t_{1/2\beta}$ of BER solution (oral) < $t_{1/2\beta}$ of BER solution (iv), while the amount of absorbed BER (AUC) ranks in just order of AUC of ARM (oral) > AUC of BER solution (oral) > AUC of BER solution (iv), as can be seen from Table 1. It seems that the higher BER blood concentration the more rapidly it is cleared. Often, the rate of drug metabolism and subsequent clearance can be accelerated by drugs themselves which are categorized as inducers of drug-metabolizing enzymes. BER might be a potent inducer of drug-metabolizing enzymes (but this remains to be further proved). Thus, more BER in mouse circulatory system could have induced more in vivo metabolic enzymes, resulting in accelerated drug clearance.

It should be pointed out that the correlation between the anti-diabetic efficacy and the blood drug concentration was not set up among different administration patterns, and this effort received confusion results (data are not shown). Nor did the AUC relate to drug efficacy. It is difficult to make an appropriate explanation for these since the processes and mechanisms of anti-diabetes of BER remain mostly unclear up to now despite of several related reports (Al-masri et al., 2009; Cui et al., 2009). However, we argue that, when affected or activated by BER, the mouse tissue cells might take certain time to initiate up-taking of glucose, but both the initiating time and up-taking rate may be dependent on and influenced by many factors, including, perhaps, drug dosage form and administration route. These deserve further investigation so that the optimal formulation based on a novel drug delivery system can be obtained. Also, making clear of these aspects is of remarkable significance for the safe administration of BER which manifests numerous pharmacologic activities and potent cytotoxicity (Imanshahidi and Hosseinzadeh, 2008), including anticancer efficacy and side effects on normal cells. In the in vivo experiments, when sterile BER solution was intravenously injected into mice at a dose of 10 mg/body weight, BER showed powerful inhibitory effects on central nervous system, and all the mice fell into lethargic sleep or even exanimation, and some even died immediately (data are not shown). But these serious adverse effects were not noticed at all in the mice that received BER-loaded ARMs. Although BER tablets are also a safe dosage form, the hypoglycemic effects were usually observed only in patients who continuously received the medicine for a long period of several months (Yin et al., 2008a; Zhang et al., 2008). Thus, the ARM drug delivery system has advantages over conventional dosage forms of BER in safety, convenience and efficacy.

In conclusion, in this investigation BER is proved a potent anti-diabetic agent but this efficacy can hardly be achieved using conventional oral dosage forms. A novel drug delivery system of ARMs was successfully prepared and used as an oral carrier for BER. BER-loaded ARMs are safe and can remarkably enhance drug oral bioavailability and thereby anti-diabetic efficacy. This novel ARM-based BER delivery system may have practical applications for oral anti-diabetic therapy.

Acknowledgments

This work was partly supported by both Shandong Provincial Natural Science Foundation (Grant No.: ZR2010HL067) and JMC PhD Faculty S&R Project Startup Fund. The authors would like to thank Dr. Lingxin Meng from People's Hospital of Sunshine City for the assistance in BGL tests. The authors are grateful to Lecturer Ruxia Qi from the Department of Pharmacology of JMC for her kind help in the work related to animals.

References

Al-masri, I.M., Mohammad, M.K., Tahaa, M.O., 2009. Inhibition of dipeptidyl peptidase IV (DPP IV) is one of the mechanisms explaining the hypoglycemic effect of berberine. J. Enzyme Inhib. Med. Chem. 24, 1061–1066.

Chen, S., Lin, Y., 2006. Quintessence of Material Medica, first ed. Chinese Military Medical Science Publishing House, Beijing.

Chen, C.L., Chang, S.F., Lee, D., Yang, L.Y., Lee, Y.H., Hsu, C.Y., Lin, S.J., Liaw, J., 2008. Bioavailability effect of methylprednisolone by polymeric micelles. Pharm. Res. 25, 39–47.

Cicero, A.F., Ertek, S., 2009. Metabolic and cardiovascular effects of berberine: from preclinical evidences to clinical trial results. Clin. Lipidol. 4, 553–563.

Clark, J.D., Baldwin, R.L., Bayne, K.A., Brown, M.J., Gebhart, G.F., Gonder, J.C., Gwathmey, J.K., Keeling, M.E., Kohn, D.F., Robb, J.W., Smith, O.A., Steggerda, J.A.D., Vandenbergh, J.G., White, W.J., Williams-Blangero, S., VandeBerg, J.L., 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, DC.

Cok, A., Plaisier, C., Salie, M.J., Oram, D.S., Chenge, J., Louters, L.L., in press. Berberine acutely activates the glucose transport activity of GLUT1. Biochimie. doi:10.1016/j.biochi.2011.04.013.

Craig, D.Q., Royall, P.G., Kett, V.L., Hopton, M.L., 1999. The relevance of the amorphous state to pharmaceutical dosage forms: glassy drugs and freeze dried systems. Int. J. Pharm. 179, 179–207.

Cui, G., Qin, X., Zhang, Y., Gong, Z., Ge, B., Zang, Y.Q., 2009. Berberine differentially modulates the activities of ERK, p38 MAPK, and JNK to suppress Th17 and Th1 T cell differentiation in type 1 diabetic mice. J. Biol. Chem. 284, 28420–28429.

Derosa, G., Salvadeo, S.A., D'Angelo, A., Ferrari, I., Mereu, R., Palumbo, I., Maffioli, P., Randazzo, S., Cicero, A.F., 2009. Metabolic effect of repaglinide or acarbose when added to a double oral anti-diabetic treatment with sulphonylureas and metformin: a double-blind, cross-over, clinical trial. Curr. Med. Res. Opin. 25, 607–615.

Fricker, G., Kromp, T., Wendel, A., Blume, A., Zirkel, J., Rebmann, H., Setzer, C., Quinkert, R.O., Martin, F., Muller-Goymann, C., 2010. Phospholipids and lipid-based formulations in oral drug delivery. Pharm. Res. 27, 1469–1486.

Hammad, M.A., Muller, B.W., 1998. Increasing drug solubility by means of bile salt–phosphatidylcholine-based mixed micelles. Eur. J. Pharm. Biopharm. 46, 361–367.

Imanshahidi, M., Hosseinzadeh, H., 2008. Pharmacological and therapeutic effects of *Berberis vulgaris* and its active constituent, berberine. Phytother. Res. 22, 999–1012.

Jones, M.C., Gao, H., Leroux, J.C., 2008. Reverse polymeric micelles for pharmaceutical applications. J. Controlled Release 132, 208–215.

Kheir, M.M., Wang, Y., Hua, L., Hu, J., Li, L., Lei, F., Du, L., 2010. Acute toxicity of berberine and its correlation with the blood concentration in mice. Food Chem. Toxicol. 48, 1105–1110.

Kim, S.H., Shin, E.J., Kim, E.D., Bayaraa, T., Frost, S.C., Hyun, C.K., 2007. Berberine activates GLUT1-mediated glucose uptake in 3T3-L1 adipocytes. Biol. Pharm. Bull. 30, 2120–2125.

Kirby, C.J., 2000. Oil-based formulations for oral delivery of therapeutic peptides. J. Liposome Res. 10, 391–407.

Li, W.L., Zheng, H.C., Bukuru, J., De Kimpe, N., 2004. Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. J. Ethnopharmacol. 92, 1–21.

Liu, L.Z., Cheung, S.C., Lan, L.L., Ho, S.K., Xu, H.X., Chan, J.C., Tong, P.C., 2010. Berberine modulates insulin signaling transduction in insulin-resistant cells. Mol. Cell. Endocrinol. 317, 148–153.

New, R.R.C., Kirby, C.J., 1997. Solubilisation of hydrophilic drugs in oily formulations. Adv. Drug Deliv. Rev. 25, 59–69.

Pouton, C.W., Charman, W.N., 1997. The potential of oily formulations for drug delivery to the gastro-intestinal tract. Adv. Drug Deliv. Rev. 25, 1–2.

Shargel, L., Wu-Pong, S., Yu, A.B.C., 2004. Pharmacokinetics of oral absorption. In: Shargel, L., Wu-Pong, S., Yu, A.B.C. (Eds.), Applied Biopharmaceutics and Pharmacokinetics, fifth ed. McGraw-Hill 714 Medical, New York, pp. 161–184 (Chapter 7).

Sun, S., 2008. Thousand Golden Prescriptions, first ed. People's Publishing House of Inner Mongolia, Hohhot.

Torchilin, V.P., 2007. Micellar nanocarriers: pharmaceutical perspectives. Pharm. Res. 24, 1–16.

Vuddanda, P.R., Chakraborty, S., Singh, S., 2010. Berberine: a potential phytochemical with multispectrum therapeutic activities. Expert Opin. Invest. Drugs 19, 1297–1307.

Walde, P., Giuliani, A.M., Boicelli, C.A., Luisi, P.L., 1990. Phospholipid-based reverse micelles. Chem. Phys. Lipids 53, 265–288.

Wang, T., Deng, Y., Geng, Y., Gao, Z., Zou, J., Wang, Z., 2006. Preparation of submicron unilamellar liposomes by freeze-drying double emulsions. Biochim. Biophys. Acta 1758, 222–231.

Wang, N., Wang, T., Li, T., Deng, Y., 2009a. Modulation of the physicochemical state of interior agents to prepare controlled release liposomes. Colloids Surf. B Biointerfaces 69, 232–238.

Wang, T., Wang, N., Jin, X., Zhang, K., Li, T., 2009b. A novel procedure for preparation of submicron liposomes-lyophilization of oil-in-water emulsions. J. Liposome Res. 19, 231–240.

Wang, T., Wang, N., Hao, A., He, X., Li, T., Deng, Y., 2010. Lyophilization of water-in-oil emulsions to prepare phospholipid-based anhydrous reverse micelles for oral peptide delivery. Eur. J. Pharm. Sci. 39, 373–379.

Wang, Y., Campbell, T., Perry, B., Beaurepaire, C., Qin, L., 2011. Hypoglycemic and insulin-sensitizing effects of berberine in high-fat diet- and streptozotocin-induced diabetic rats. Metabolism 60, 298–305.

Wu, K.K., Huan, Y., 2008. Streptozotocin-induced Diabetic Models in Mice and Rats Current Protocols in Pharmacology, vol. 5. Wiley Interscience, San Francisco, CA, pp. 1–14.

Yin, J., Xing, H., Ye, J., 2008a. Efficacy of berberine in patients with type 2 diabetes mellitus. Metabolism 57, 712–717.

Yin, J., Zhang, H., Ye, J., 2008b. Traditional Chinese medicine in treatment of metabolic syndrome. Endocr. Metab. Immune Disord. Drug Targets 8, 99–111.

Yu, L., 2001. Amorphous pharmaceutical solids: preparation, characterization and stabilization. Adv. Drug Deliv. Rev. 48, 27–42.

Zhang, Y., Li, X., Zou, D., Liu, W., Yang, J., Zhu, N., Huo, L., Wang, M., Hong, J., Wu, P., Ren, G., Ning, G., 2008. Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid berberine. J. Clin. Endocrinol. Metab. 93, 2559–2565.

Zhou, L., Yang, Y., Wang, X., Liu, S., Shang, W., Yuan, G., Li, F., Tang, J., Chen, M., Chen, J., 2007. Berberine stimulates glucose transport through a mechanism distinct from insulin. Metabolism 56, 405–412.

Zuo, F., Nakamura, N., Akao, T., Hattori, M., 2006. Pharmacokinetics of berberine and its main metabolites in conventional and pseudo germ-free rats determined by liquid chromatography/ion trap mass spectrometry. Drug. Metab. Dispos. 34, 2064–2072.