The Study to Reduce the Hemolysis Side Effect of Puerarin by a Submicron Emulsion Delivery System

Peng-Fei YUE,a,b Hai-Long YUAN,*,a Wei-Feng ZHU,b Long-Bo CONG,b Huan XIE,a,c Zhi-Guo LIU,a Lu-Jun WANG,a and Xiao-He XIAOa

a 302 Hospital of PLA&PLA Institute of Chinese Materia Medica; Beijing 100039, China: b Key Lab of Modern Preparation of TCM, Ministry of Education; Nanchang 330004, China: and c Chengdu University of TCM; Chengdu 610075, China. Received May 16, 2007; accepted October 21, 2007; published online November 5, 2007

A safe and effective delivery system with a submicron emulsion for puerarin was studied. Puerarin submicron emulsion was prepared by a novel complex-phase inversion-high press homogenization technology. The mechanism to reduce the hemolysis side effect of puerarin was studied by blood cell counts in rabbits. The average diameter, zeta potential and entrapment efficiency of the emulsion prepared was 198.14±8.61 nm, −29.45±1.47 mV; 87.32±0.34%, respectively. Compared with control group, the red blood cell values, packed cell volume, plasma hemoglobin level, haptoglobin level and osmotic fragility of puerarin i.v. group was significantly different (p<0.05) at 42, 43 d, respectively. The blood cell parameter values of puerarin submicron emulsion group were not significantly different (p>0.05) in contrast to control group. Such observations indicated that the intravascular hemolysis occurred at 42, 43 d in puerarin i.v. group rabbits, the hemolysis did not occur for puerarin emulsion group rabbits. As an explanation for these results, it was proposed that the puerarin was either incorporated into the lipophilic core or intercalated between the phospholipid molecules at the interface. It could be concluded that puerarin submicron emulsions prepared markedly reduced the hemolysis effect of puerarin.

Key words puerarin; submicron emulsion; hemolysis; blood cell count

Puerarin,1) a naturally occurring isoflavone C-glycoside, was isolated from Pueraria lobota. It was traditionally used to reduce febrile symptoms, dilat aeteria coronaria and cerebral disease.5—7) However, the clinical efficacy of puerarin i.v. is limited by severe and acute side effects which develop after several weeks of therapy. Especially, the intravenous hemolysis is a key factor to limit the clinical utilization of puerarin i.v.8,9) So it is very necessary to seek a drug delivery system for puerarin in order to reduce its side effects in clinic.

Submicron emulsion is potentially interesting drug delivery system because of its ability to incorporate drugs into the dispersal phase.10) By the submicron emulsion delivery system, drug can be avoided direct contact with the body fluid and tissues in order to reduce the possible side effects. Submicron emulsions had increasing importance for the administration of these drugs which are poorly soluble in water, poorly soluble in oils and simultaneously toxiciferous for intravenous injection, and meanwhile it can enhance their activity and their bioavailability.11,12) Numerous reports have appeared in the literature that the systems can be able to enhance the solubility and efficacy.13,14) Some cases such as amphotericin B,15) prostaglandin E1,16) diazepam17) were reported.

Puerarin is slightly soluble in water and poorly soluble in oils of parenteral emulsions. The objective of this study is: puerarin emulsions were prepared by a novel complex-phase inversion-high press homogenization (CPHH) technology. Comparing the blood cell parameters of puerarin emulsion group after administration with those of puerarin i.v. group, we evaluated the protective effect to reduce the hemolytic effect of puerarin by submicron emulsions systems.

MATERIALS AND METHODS

Materials Puerarin was obtained from Xian-guochui Ltd., purity 99.05% (Xi-AN, China). Egg lecithin was purchased from Hua-qing-mei-hen Ltd. (Beijing, China). Synperonic F68 was obtained from Hua-qing-mei-hen Ltd. (Beijing, China). Purified soybean oil for parenteral use was purchased from Tieling BeiYa Pharmaceutical Co. (Tieling, China). Puerarin i.v. (marketed in China and included in Chinese pharmacopeia) was purchased from the local market. All other reagents used in this experiment were of analytical grade.

Preparation of Puerarin Intravenous Emulsion Puerarin intravenous emulsion contained 1% puerarin, 10% soybean oil, 1.2% egg lecithin, 0.2% synperonic F68, 0.3% α-tocopherol. Firstly, the complex was prepared with puerarin and phospholipid at a weight ratio of 1:1.2. Weighed amount of puerarin and phospholipid were taken in a 100 ml round bottom flask and 60 ml of absolute alcohol was added. The mixture was refluxed at a temperature not exceeding 60°C for 3 h. The resultant clear solution was evaporated at 40°C under vacuum to remove traces of solvents.10) Then the puerarin–phospholipid complexes were obtained. Secondly, the lipid phase was prepared by dissolving puerarin–phospholipid complexes in soybean oil by grinding at 55°C. The water phase was prepared by mixing water, synperonic F68, α-tocopherol and glycerol at 55°C. Glycerol was used to make the emulsion isotonic. The lipid phase were stirred at 55°C at 2000 rpm by high-speed stirrer for 15 min, and the water phase was slowly influxed into the lipid phase in order to achieve phase inversion. A fine emulsion was prepared by...
passing the coarse emulsion through high-pressure homogenizer (GYB40-10S, donghua homogenization apparatus, China) at 60 MPa for 6 cycles. After the pH was adjusted to 6—7, the emulsion was packed in 15 ml sterile glass vials under nitrogen. The vials were sealed and the emulsions were sterilized by autoclaving at 121°C for 15 min.

**Particle Size** Particle size and mean diameter of the emulsion were performed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer (Malvern Instruments, Malvern/U.K.). For size analysis approximately 50 μl emulsion was added to 10 ml distilled water in order to obtain the optimum scattering intensity.

**Zeta Potential Measurement** Zeta potential analyzer (Zetasizer Nano, Malvern Instruments, U.K.) were used to study zeta potential of emulsion. Puerarin emulsions were prepared according to the method described previously. The sample was diluted with distilled water until the appropriate concentration of particles was achieved, and the sample was measured to calculate zeta potential.

**Transmission Electron Microscopy (TEM)** Transmission electron microscope was performed using a HD-600 transmission electron microscope (Hitachi, Japan). A drop of the resultant emulsions was placed onto a carbon-coated copper grid, leaving a thin liquid film. The films on the grid were negatively stained by immediately adding a drop of 2.5% (w/w) sodium phosphotungstate (pH 6.8), removing the excess staining solution with a filter paper, and followed by through air-dry. The stained films were then viewed on a transmission electron microscope and photographed.

**Entrapment Efficiency (EE%) of Puerarin Emulsion** Chromatographic column Spherisorb ODS C18 (250 mm × 4.6 mm, 5 μm) was used for chromatographic separation. Mobile phase consisted of a mixture of methanol–H₂O (30:70, v/v) delivered at a flow rate of 1.0 ml/min. The injection volume was 20 μl. Detection was performed at 250 nm at room temperature.

The controlled solution (1.25—25 μg·ml⁻¹) was prepared by dissolving puerarin (precisely weighed) in mobile phase. The amount of puerarin penetrated into the receptor compartment was determined with the high-performance liquid chromatography (HPLC) described previously. The integral calculation of the chromatographic peak area (A) was recorded as Y-axis, and the concentration of puerarin (C) as X-axis. Drug recovery was calculated from the following equation. Drug recovery=(analyzed weight of drug in emulsion/theoretical weight of drug loaded in system)×100%.

The emulsions with drugs were centrifuged at 23000×g for 30 min (4°C) in a Beckman Optima MAX ultracentrifuge (BeckmanCoulter, Fullerton, CA, U.S.A.) in order to separate the incorporated drug and the non-incorporated drug. The drug was analyzed by HPLC for the non-incorporated drug concentration to determine the entrapment percentage.

The concentrations of puerarin in the emulsion (n₁) and free drug in the aqueous (the non-incorporated drug) (n₂) were assayed by HPLC after dilution with methanol. EE% could be achieved by the following equation: EE%=(n₁−n₂)/n₁×100%.

**In Vivo Hemolysis Study. Experiment Design** Male rabbits weighed 2—2.5 kg were provided by National Resource Center for Rodent Laboratory Animal. The rabbits (Specific Pathogen Free grade, SPF) were maintained with the condition of sterilized, 45—55% relative humidity and room temperature. Four groups of rabbits were designed to follow the evaluation of hemolysis. The animals were divided into control group A and test groups B, C, D, and each rabbit was injected by ear vein in one of the following dosage forms per body weight everyday: A (control group) 154 mM NaCl, B 25 mg (low dose) puerarin of submicron emulsion, C 50 mg (high dose) puerarin of submicron emulsion, D 25 mg puerarin of puerarin i.v. All the rabbits were administered through ear vein at predetermined time. Blood sample was obtained by puncture of ear vein after administration every day. The rabbits were fed with water and food. Ten rabbits were utilized in each group for each time interval.

**Blood Sample Collection and Assay** Cell counts were obtained from blood samples collected in 2.7 mM Na₂-EDTA, as described by Sano-Martins et al. Red blood cell (RBC) counts were determined by a Serono-Baker hematologic counter. Reticulocyte counts were determined by blood cell films with new methylene blue (0.5%) staining, immediately after blood collection. Plasma hemoglobin and haptoglobin were determined by colorimetric assays in EDTA-anticoagulated plasma samples. The whole blood samples were assayed within 6 h after collection to avoid the cell destruction.

**Osmotic Fragility** Osmotic fragility was performed according to Dacie and Lewis. Rabbit RBC was incubated with increasing salt concentrations, ranging from 17 to 154 mM NaCl, for 30 min at 24—28°C. NaCl concentration was plotted against percent hemolysis and curves were obtained for each animal. NaCl concentrations that induced 50% hemolysis (mean osmotic fragility, H₅₀%) were used as a parameter to compare groups at different time intervals.

**Statistical Analysis** Statistical analysis were performed using SAS (version8.01). Statistical analysis of differences between different treatments was performed using unpaired Student’s t-test. A 0.05 level of probability was taken as the level of significance. An analysis of variance (ANOVA) test was also used. In regard to osmotic fragility, data were transformed to logit before analysis, in order to achieve normality and homecedasticity; osmotic fragility curves were compared by regression analysis using SPSS 10.0 software. Data are expressed as mean±standard error of mean.

**RESULTS AND DISCUSSION**

**Preparation of Puerarin Submicron Emulsion** Preparation of puerarin—phospholipid complex was a key step. We prepared the phospholipid complexes as the previous method described. According to the solubility property that the complexes and phospholipids are easily soluble in chloroform, but puerarin not. It was proved by chloroform solution as described that the proportion of puerarin complexed was 99.86±0.53% in the puerarin—phospholipid complexes. Puerarin emulsion was prepared by the CPHH technology. Incorporation of drugs into the lecithin layer by this method (Fig. 1) and avoiding the stimulus effect for vasculum can lead to a reduction of side effects such as intravascular hemolysis of drugs.

**Particle Size and Zeta Potential** An adequate characterization of the submicron emulsion is a necessity for the control of the quality of the product. The average diameter and zeta potential are very important parameters to control
The quality and stability of submicron emulsion. The average diameter of puerarin submicron emulsion measured was 198.14 ± 8.61 nm in Fig. 2. The submicron emulsion loading puerarin showed a considerable small particle size.

The measurement of the zeta potential predicts the storage stability of emulsion. In general, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electric repulsion. The mean zeta potential of puerarin emulsion was -29.45 ± 1.47 mV, but the mean zeta potential of free emulsion without puerarin was -33.12 ± 1.64 mV. Therefore, the zeta potential of puerarin emulsion and free emulsion were not significantly different. The zeta potential of puerarin emulsion was not significantly decreased because of loading the drug. A relative high stability and good dispersion quality were obtained by this method.

Transmission Electron Microscopy (TEM) TEM graphs of the emulsions were taken by placing them on a copper grid, letting the water evaporate and staining them with phosphotungstic acid. In comparison to the free emulsions without drug (Fig. 3a), the puerarin emulsions showed a different appearance at the droplet surface (Fig. 3b). The change in the interfacial appearance might be localized to the certain regions. It was reported that a three-phase model, which assumed that the poor soluble drug of emulsion could be present in the oil phase or at the oil–water interface. It supported the hypothesis that puerarin was not only encapsulated in oil droplets but also distributed into the lecithin layer by special bind with lecithin, and our study data demonstrated that the puerarin mostly distributed into the lecithin interfacial layer of submicron emulsion. We think that such a certain ordered, localized structure might be explainable by a specific interaction between certain numbers of lecithin and drug molecules, comparable to a complex formation, but the structures are being presently under further investigation.

**Entrapment Efficiency of Puerarin Emulsion** The regression equation of puerarin was \( A = 11526C - 8342.2 \) \((r=0.9997)\). The assay was linear in the concentration range 1.25—25 μg·mL\(^{-1}\). The percentage recoveries ranged from 98.72 to 101.80%, and the mean was 99.46%.

To decrease the hemolysis side effect of puerarin, a low concentration of free puerarin in water of emulsion was required. Entrapment efficiency of puerarin emulsion was 87.32 ± 0.34%. It showed that a high incorporation efficacy could be obtained by the CPHH technology because of the formation of the drug–phospholipid bind to decrease the aqueous solubility and increase the liposolubility of puerarin.

**In Vivo Hemolysis Study** Rabbits (Group D) which were injected i.d. with 25 mg puerarin of parenteral solution showed a decrease in RBC counts, packed cell volume (PCV) values and hemoglobin levels at 42 d and 43 d (Figs. 4—6). RBC counts (Fig. 4) dropped significantly at 42 d \((p<0.001)\), 43 d \((p<0.001)\). Statistically significant differences for hemoglobin levels were noticed at 42 d \((p<0.001)\), 43 d \((p<0.001)\) (Fig. 6), and for PCV values at 42 d \((p<0.05)\) and 43 d \((p<0.001)\) (Fig. 5). Mean cell hemoglobin concentration were significantly increased in the experimental group \((p<0.001)\) at 43 d after injecting puerarin i.v. And rabbits presented a decrease in plasma haptoglobin levels (Fig. 7) and an accompanying increase in plasma hemoglobin.
levels (Fig. 8), which initiated at 42 d after injection of puerarin i.v. And reticulocyte counts (i.e., young RBC) (Fig. 9) were significantly increased in blood stream of rabbits injected puerarin i.v., peaking at 43 d ($p < 0.001$). Such parameters reached normal levels again at 45 d after stopping injection, despite no other drug has been administered. However, above-mentioned parameters were not significant different between group B, C and control group ($p > 0.05$).

Regression analysis showed that osmotic fragility curves of administration of puerarin i.v. and control rabbits were coincident at 1 and 42 d ($p < 0.05$), but not at 43 d ($p < 0.01$). Mean osmotic fragility ($H_{50\%}$) values of control and group D animals were not statistically significant ($p > 0.05$) at 1 and 42 d, ranging from 80 to 86 mM NaCl. However, Group D rabbits showed a statistically significant increase ($p < 0.05$) in $H_{50\%}$ values at 43 d (92.46±1.47 mM NaCl) in comparison with controls rabbits (83.21±1.32 mM NaCl) at the same time interval (Fig. 10). After stopping administration of puerarin of parenteral solution, osmotic fragility curves of group D and control group were again coincident at 44 and 45 d.
In a word, it was notable that the presence of oil droplets or in the core of micelles) or in the interface. That the drug would be incorporated in the lipophilic core (of other molecules as a complex. The third theory suggested that phospholipids protect membranes by being adsorbed as a monolayer onto the surface, thereby creating a hydrophobic barrier which results in a reduction of hemolysis because of long term injection of puerarin i.v. It was reported that the reasons of side effect of intravenous hemolysis was supported mainly by the following views: puerarin itself might have the stimulus effect for patient blood vessel. This stimulus effect might be very slightly, it can not induce the hemolysis effect at once after administration. But with long term and frequent administration of puerarin, it can induce the hemolysis effect. The accumulation damage effect of puerarin might be ideal spiritual state of the hemolysis. These are consistent with the side effect reports for submicron emulsion in clinical. But the submicron emulsion is potentially interesting drug delivery system because of its ability to incorporate drugs with poor solubility within the dispersal phase and lecithin interface, and to avoid utilization of solubilizing agent.

Submicron emulsions as a novel system to reduce the hemolytic effect, its mechanism of the protective effect was reported as follows: The first theory by Lichtenberger et al. correlated this phase and lecithin interface, and to avoid utilization of solubilizing agent.

Our results showed that the intravascular hemolysis occurred at 42 d, 43 d in the rabbits injected i.d. 25 mg puerarin of puerarin i.v. per body, as demonstrated by the increased plasma hemoglobin levels (Fig. 8), the increased reticulocyte count (Fig. 9) and decreased plasma haptoglobin levels (Fig. 7). Hemoglobin released in blood stream, due to any hemolytic process, interacted with plasma haptoglobin, forming a hemoglobin–haptoglobin complex that was rapidly removed from circulation by hepatocytes. When large amounts of hemoglobin were released directly into circulation, plasma haptoglobin was totally consumed and free hemoglobin was excreted into urine. Decreased RBC counts (Fig. 4) and PCV values (Fig. 5), mainly at 42 and 43 d after injection, also support the idea that intravascular hemolysis occurred in rabbits injected i.d. with puerarin of parenteral solution per body weight probably due to both indirect hemolytic activity present in puerarin i.v. And the increased plasma hemoglobin levels, decreased plasma haptoglobin levels and decreased RBC counts and PCV values of group of puerarin i.v. recovered to normal values without administration of anti-hemolysis drug at 44 and 45 d. These can also confirm that intravascular hemolysis occurred in rabbits injected i.d. puerarin i.v. However, the intravascular hemolysis did not occur in rabbits group B and C, as demonstrated by the nearly normal plasma hemoglobin levels, plasma haptoglobin levels, RBC counts and PCV values. It can be concluded that the adverse effect of intravenous hemolysis may occur in the rabbits which were successively injected long term with puerarin i.v., but the adverse effect of intravenous hemolysis can not occur in the rabbits which were successively injected long term with high dose of puerarin submicron emulsion under the same condition.

On the other hand, there were some clinical cases reported that some patients occurred the adverse effect of intravenous hemolysis because of long term injection of puerarin i.v. It was reported that the reasons of side effect of intravenous hemolysis was supported mainly by the following views: puerarin itself might have the stimulus effect for patient blood vessel. This stimulus effect might be very slightly, it can not induce the hemolysis effect at once after administration. But with long term and frequent administration of puerarin, it can induce the hemolysis effect. The accumulation damage effect of puerarin might be ideal spiritual state of the hemolysis. These are consistent with the side effect reports for puerarin i.v. in clinical. But the submicron emulsion is potentially interesting drug delivery system because of its ability to incorporate drugs with poor solubility within the dispersal phase and lecithin interface, and to avoid utilization of solubilizing agent.

Submicron emulsions as a novel system to reduce the hemolytic effect, its mechanism of the protective effect was reported as follows: The first theory by Lichtenberger et al. suggested that phospholipids protect membranes by being adsorbed as a monolayer onto the surface, thereby creating a hydrophobic barrier which results in a reduction of hemolysis. The second theory by Martin et al. correlated this behavior to the protective function of phospholipids themselves, as they were able to form mixed micelles with the other molecules as a complex. The third theory suggested that the drug would be incorporated in the lipophilic core (of the oil droplets or in the core of micelles) or in the interface. In a word, it was notable that the presence of oil droplets or

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**Figure 9.** Reticulocyte Count in Blood of Control Rabbits (A) (n=10) and Rabbits Experimentally Injected by the Following Formulations: B, Low Dose of Puerarin Emulsion (25 mg, i.d.) (n=10); C, High Dose of Puerarin Emulsion (50 mg, i.d.) (n=10); D, Puerarin i.v. (25 mg, i.d.) (n=0)

Data are expressed as mean±standard error of mean. p value was marked.

**Figure 10.** Osmotic Fragility of Red Blood Cells of Group A, B, C and D Rabbits at 43 d (n=10)

Data are expressed as mean±standard error of mean. Curves were non coincident by regression analysis (p<0.01) and mean values of H50% of control group A and experiment group D rabbits were statistically different (p<0.05). Curves and mean values of H50% of control group A and experiment group B, C rabbits were not significantly different (p>0.05).
the phospholipids membranes could play a very important role.

CONCLUSIONS

In this study, we prepared puerarin submicron emulsion by the CPHH technology. The entrapment efficiency of puerarin emulsion was 87%. The free puerarin dissolved in water phase was very little. The puerarin was incorporated into the lipophilic core (of the oil droplets or in the core of micelles) and in the interface, which resulted in a reduction of the hemolytic side effect. And soybean oil and lecithin having very good biocompatibility or bioavailability was applied, which avoided multiplicity utilization of some solubilizing agents such as propylene glycol. The data of hemolysis experiment in vivo showed that the adverse effect of intravenous hemolysis did not occur in the rabbits of low and high dose group injected puerarin emulsions, but the intravenous hemolysis occurred significantly in the rabbits which injected puerarin i.v. Puerarin submicron emulsion can reduce markedly the adverse effect of puerarin. But the mechanism to reduce the hemolysis of puerarin submicron emulsion is being further studied.

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