Optimized Preparation, Characterization and Biodistribution in Heart of Breviscapine Lipid Emulsion

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Breviscapine is a Traditional Chinese Medicine treating cardiovascular diseases by promoting blood circulation and removing blood stasis. The major active component of breviscapine has low aqueous solubility, poor chemical stability, short biological half-life and rapid elimination rate from the plasma. The use of a lipid emulsion formulation containing breviscapine might improve chemical stability, increase drug loading, exhibit sustained release profile. In the present study, we developed an optimized formulation and technological method for the preparation of sterile and stable breviscapine lipid emulsion (Bre-LE) for intravenous infusion. The average particle size, polydispersity index, zeta potential, stability constant \((K_s)\) value and content of final product were \((225.3 \pm 8.8)\,\text{nm}\), \(0.221 \pm 0.020, (–29.6 \pm 1.5)\,\text{mV}, (24.3 \pm 2.9)\%\) and \((94.5 \pm 0.6)\%\) respectively \((n=3)\). The results of \textit{in vitro} release experiment suggest that lipid emulsion as breviscapine carrier showed a desirable sustained release profile. Dilution stability and long-term stability were also researched in the present paper. The results show the carrier could protect drug from degradation after dilution by phosphate buffered saline and fetal calf serum. And Bre-LE was stable for up to 6 months at room temperature storage condition. The biodistribution of drug in heart of mice increased dramatically after encapsulation into lipid emulsion which was beneficial to heart disease therapy.

**Key words** breviscapine; lipid emulsion; scutellarin; optimized preparation; biodistribution

Cardiovascular diseases are the world’s largest killers. Traditional Chinese Medicine has special advantages in treating cardiovascular diseases, such as low toxicity and well-known therapeutic effects. Breviscapine is a flavone glucuronide extracted from a Chinese herb \textit{Erigeron breviscapinus} (VANT.) HAND.-MAZZ.\(^{1}\) It is widely used in the treatment of angina pectoris, coronary heart disease, cerebral infarction and its sequelae. It contains mainly scutellarin (4’,5,6-tetrahydroxylavone-7-O-glucuronide, primary active ingredient) for chemical structure see Fig. 1) and little apigenin-7-O-glucuronide. Scutellarin, the major active component of breviscapine, has low aqueous solubility, poor chemical stability, short biological half-life and rapid elimination rate from the plasma.\(^{2,3}\) Breviscapine and its preparation (\textit{Injectio Breviscapine}) were listed in the Pharmacon Criteria (Chinese Traditional Patent Medicine). \textit{Injectio Breviscapine} is aqueous solution of water-soluble salt of breviscapine.

![Chemical Structure of Scutellarin](image)

Fig. 1. Chemical Structure of Scutellarin

Micelles. Furthermore lipid emulsion can be produced on large industrial scale and sterilized by autoclaving but avoiding drug leakage from carriers like liposomes.\(^{11,12}\) The use of a lipid emulsion formulation containing breviscapine might improve the chemical stability, increase drug loading, decrease irritation on the surrounding tissue as well as control and modify its pharmacokinetics and tissue distribution. In the present study, we developed an optimized formulation and technological method for the preparation of sterile and stable breviscapine lipid emulsion (Bre-LE) for intravenous infusion. The characterization and biodistribution in heart of Bre-LE was also researched.

**Experimental**

**Materials** Breviscapine was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co., Ltd. (Jiangsu, China). Scutellarin standard (purity >98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lipoid E80 (egg yolk lecithin with 80—85% of phosphatidylcholine) was purchased from Lipoid GmbH (D-Ludwigshafen, Germany). Poloxamer 188 was purchased from BASF (China) Co., Ltd. (Shanghai, China). Triton X-100 was purchased from Sigma. \textit{Injectio Breviscapine}, which is an injection solution of scutellarin (20 mg/5 ml) was produced by Gejiu Bio-Medicine Industry Ltd. (Yunnan, China). Other chemicals used were of analytical grade.

**Preparation of Bre-LE** Bre-LE was prepared as follows. The oil phase was prepared by dissolving scutellarin (0.04%) in the mixture of required amounts of Lipoid E80, oleic acid, vitamin E (abbreviated V_{E} (0.6%) and soybean oil using a sonicator (B5200S-DT Sonicator, Branson Ultrasonics Co., Ltd., Shanghai, China). To make the aqueous phase, required amounts of Poloxamer 188 dissolved in a mixture of glycerol (2.25%) and double distilled water. A preemulsion was prepared by mixing the oil phase and the aqueous phase with a constant speed stirrer (XHF-1 Stirrer, Shanghai Xinda BioChem Instrument Co., Ltd., Shanghai, China). Final emulsification was completed by passing the preemulsion through a homogenizer (EmulsiFlex-05 High Pressure Homogenizer, Avestin Inc., Canada) and autoclaved. The formulation and technological parameters of breviscapine lipid emulsion were optimized on the basis of univariate analysis and orthogonal experiment design as shown in Table 1. Composite grade method was used to evaluate the preparation. The composite grade index \((S)\) was calculated accord-
Table 1. Factors and Levels of Orthogonal Experiments Design

<table>
<thead>
<tr>
<th>Factors</th>
<th>Levels</th>
<th>A (Lipoid E80, %)</th>
<th>B (Poloxamer 188, %)</th>
<th>C (Oleic acid, %)</th>
<th>D (Pressure, psi)</th>
</tr>
</thead>
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<td>0.6</td>
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<td>17500</td>
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<td>1.4</td>
<td>2.4</td>
<td>1.2</td>
<td>20000</td>
<td></td>
</tr>
</tbody>
</table>

where $S_i = \left[ \frac{(D_{max} - D)(D_{max} - D_{min})}{100} \right]^{\%}$

Long-term stability of Bre-LE was evaluated after storage at room temperature for up to 6 months. The particle size, polydispersity index, zeta potential, stability constant $K_s$ and content of scutellarin in Bre-LE were determined as a function of the storage time. The content of scutellarin was determined by HPLC. The particle size, polydispersity index, zeta potential and stability constant $K_s$ were measured as described previously.

Biodistribution of Bre-LE in Heart

Kunming mice with an average weight of 20 g were used in this study. The mice were divided into two groups of three to five animals. Breviscapine lipid emulsion and Injectio Breviscapine were injected intravenously into the tail vein of the mice (25 mg/kg). Animals were sacrificed under ether anesthesia at indicated intervals after administration. Heart was removed, weighed and homogenized (10%, w/v) in a solution of 1% sodium bisulfate in physiological saline. All samples were immediately frozen at −20 °C until analysis. A 200 μl aliquot of methanol was added to 100 μl aliquot of homogenate.

Results and Discussion

Univariate Analysis for Formulation and Technological Method

To study effects of technological method on properties of preemulsion and final emulsion during the preparation, the lipid emulsion were prepared with the fixed composition: breviscapine (0.04%), Lipoid E80 (1.2%), V E (0.06%), oleic acid (1.0%), soya bean oil (10%), poloxamer 188 (2.0%) and glycerol (2.25%). The results of mixing temperature, stirring time and stirring rate on preemulsion preparation are shown in Figs. 2–4. Smaller particle size, polydispersity index and $K_s$ value were achieved at higher temperature of mixing. The content of scutellarin decreased to below 98% beyond 80 °C. Thus, the optimized temperature of mixing was chosen as 80 °C. It was also seen that particle size and polydispersity index decrease first, then increase with stirring time and rate increasing. It was due to the dispersed oily droplets coagulated under high shearing force provided by constant speed stirrer during oil phase dispersion simultaneously. Another reason was the bubbles formed in the process of high speed stir could weaken shearing force and then increase the collision of oily droplets. Thus, the optimized stirring time and rate of the preemulsion mixture were chosen as 60 s and 8000 rpm.

Homogenization could lower the interfacial tension between oil and water phase resulting in a more stable emulsion. The results of cycle number and operating pressure of carriers of to protect drug from degradation after dilution by PBS (pH 7.4) and PBS containing 10% fetal calf serum, Bre-LE without V E (Bre-LE-1) was used. The same procedures were applied for preparing Bre-LE-1 as it for Bre-LE except for no V E added to the oil phase. Breviscapine solution (Bre-Sol) was also prepared as control just before used. In details, 40 mg of breviscapine dissolved in 5 ml double distilled water with 17 mg NaHCO₃ as pH adjuster. After drug dissolved, this solution was diluted with normal saline to 100 ml. Dilution stability experiment as follows: Bre-LE-1 and Bre-Sol were diluted 10-fold with PBS or 10% fetal calf serum at 37 °C. The samples were withdrawn at appropriate intervals and prepared for analysis. The scutellarin remained in the diluted samples was determined by HPLC.
homogenization on final emulsion are shown in Figs. 5 and 6. It was seen that smaller particle size and polydispersity index were observed at higher number of cycles and operating pressure of homogenization. But there was no significant difference between 20 and 25 passes. Thus, the optimized cycle number was chosen as 20 passes.

Figure 7 summarizes the effects of sterilization method on particle size, polydispersity index, zeta potential, $K_s$ value and scutellarin assay. As the results show, larger particle size was found at higher temperature of sterilization due to the combination of oily droplets. The physical stability of breviscapine lipid emulsion were improved and the particle was uniform which could be concluded by smaller polydispersity index and $K_s$ value, higher absolute value of zeta potential achieved at higher temperature of sterilization. But significant loss (more than 5%) in scutellarin content was observed when the heat temperature increased beyond 100 °C. Thus, the optimized sterilization method was chosen as 100 °C for 30 min. Although the heat-treatment may be insufficient, we could take measures to remove microorganism for compensation, such as: some excipients in the formulation adopted were intravascular administration grade, the preparation was passed through a 0.22 μm sterilizing-grade membrane filter performed under aseptic conditions. And the preparation has no pyrogen examined by limulus test.

Oleic acid concentration was an important factor on the physical stability of breviscapine lipid emulsion. Results show that the properties of breviscapine lipid emulsion changed with different oleic acid concentration before and after sterilization on final emulsion. It was seen that smaller particle size and polydispersity index were observed at higher number of cycles and operating pressure of homogenization. But there was no significant difference between 20 and 25 passes. Thus, the optimized cycle number was chosen as 20 passes.

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Figure 5. Particle Size and Polydispersity Index as a Function of Number of Cycles through the Homogenizer (20000 psi, Mean±S.D., n=3)

Fig. 6. Particle Size and Polydispersity Index as a Function of Operating Pressure (20 Cycles, Mean±S.D., n=3)
There was no data shown with formulation composition of 0% and 20% oleic acid after sterilization, because drug precipitation (0% oleic acid) and emulsion broken (20% oleic acid) were observed during heating process, respectively. Breviscapine solubility in oil phase was improved with oleic acid added. Otherwise, overdose of oleic acid could not be emulsified by Lipoid E80 and Poloxamer 188. Oleic acid could not only increase breviscapine solubility, but also contribute to increase the absolute value of zeta potential and then improve physical stability properties conferred to the emulsion due to strengthening of the molecular interactions occurring between phospholipid and Poloxamer emulsifiers in the presence of an ionized form of oleic acid at the o/w interface of the emulsified oily droplets.15)

Orthogonal Experiment Design for Formulation and Technological Method Based on univariate analysis above and experience found in preparation, orthogonal experiment design was performed to further optimize the formulation and technological parameter of homogenization. The emulsion was prepared with some of fixed parameters: temperature of mixing (80 °C), stirring time and rate (60 s and 8000 rpm) for preemulsion preparation, number of cycles for final emulsion homogenization (20 passes) and autoclave method parameters for sterilization (100 °C for 30 min). The composition scale: breviscapine (0.04%), Lipoid E80 (0.8—1.4%), VE (0.06%), oleic acid (0.6—1.2%), soybean oil (8.8—9.4%), poloxamer 188 (1.8—2.4%), glycerol (2.25%). The results of orthogonal experiment design were given in Table 2. The sequence of effect on composite grade to evaluate the preparation was: D>A>C>B. The optimum preparation parameters were A3B2C4D4, that is Lipoid E80 1.2%, poloxamer 188 2.0%, oleic acid 1.2%, pressure 20000 psi.

Optimum Formulation and Technological Method The oil phase was prepared by dissolving breviscapine (0.04%) in the mixture of Lipoid E80 (0.8—1.4%), VE (0.06%), oleic acid (0.6—1.2%), soybean oil (8.8—9.4%), poloxamer 188 (1.8—2.4%), glycerol (2.25%). The results of orthogonal experiment design were given in Table 2. The sequence of effect on composite grade to evaluate the preparation was: D>A>C>B. The optimum preparation parameters were A3B2C4D4, that is Lipoid E80 1.2%, poloxamer 188 2.0%, oleic acid 1.2%, pressure 20000 psi.

Characterization of Bre-LE Follow the optimum
preparation found above, the average particle size, polydispersity index, zeta potential, $K_s$ value and content of final product were (225.3 ± 8.8) nm, 0.221 ± 0.020, (−29.6 ± 1.5) mV, (24.3 ± 2.9)% and (94.5 ± 0.6)% respectively ($n = 3$). Bre-LE is safe for intravenous administration because the size distribution was narrow and the particle size was smaller than 1 μm. The results of zeta potential and $K_s$ value suggest Bre-LE is stable.

**In Vitro Release** The release of scutellarin was studied in both PBS and fresh rat plasma. As shown in Fig. 9, free scutellarin in control samples (*Injectio Breviscapine*) diffused freely across the membrane of dialysis bag, and in fewer than 6 h of dialysis, nearly 100% of the free drug had crossed the membrane to the release medium of both PBS and fresh rat plasma. However, less than 80% of drug was released from Bre-LE after 12 h in fresh rat plasma. These results suggest that lipid emulsion as breviscapine carrier showed a desirable sustained release profile. The drug leaked from oil phase to aqueous phase in the preparation could be negligible because the saturated solubility of scutellarin was very low and the amount of drug leaked was lower than or equal to the saturated solubility regarding no precipitation observed in the preparation. Therefore the leakage could not influence the results of release test.

**Dilution Stability and Long-Term Stability** Drug was diluted when it was injected into body. The dilution stability experiment was designed to simulate the chemical stability *in vivo*. The degradation mechanism of scutellarin was mainly oxidation. The lipid emulsion could protect drug in oil phase from meeting oxygen in the air because the aqueous phase was a barrier between the air and oil phase. We need to investigate the protective ability of lipid emulsion carriers itself, not the ability of stabilizer to protect drug from degradation. Therefore Bre-LE without VE was used for removing the disturbance of stabilizer on the results. The results of dilution stability in PBS and 10% fetal calf serum are shown in Figs. 10 and 11. The results show there was a nearly linear degradation of scutellarin in both PBS and 10% fetal calf serum. The first-order rate constant of Bre-Sol and Bre-LE-1 were 0.0060 and 0.0050 in PBS, 0.2339 and 0.1484 in fetal calf serum, respectively. The results show the carrier could protect drug from degradation after dilution by PBS and fetal calf serum. The dilution stability is very important to drug with poor chemical stability. Because drug degradation happened after injection is one of the key factors inducing shorter half-life in plasma.

The long-term stability data for Bre-LE under storage at room temperature is summarized in Table 3. The particle size, polydispersity index and $K_s$ value increased slowly. And absolute value of zeta potential and content of scutellarin decreased gently. Based on the data, Bre-LE was physically

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**Table 2. The Results of Orthogonal Design for Optimization of Bre-LE Formulation and Technological Method**

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$K_s/4 = 58.2$, $K_s/4 = 58.1$, $K_s/4 = 57.7$, $K_s/4 = 48.6$

$K_s/4 = 61.1$, $K_s/4 = 62.2$, $K_s/4 = 61.3$, $K_s/4 = 54.6$

$K_s/4 = 65.4$, $K_s/4 = 60.9$, $K_s/4 = 58.6$, $K_s/4 = 67.7$

$K_s/4 = 57.9$, $K_s/4 = 61.4$, $K_s/4 = 65.1$, $K_s/4 = 71.9$

$R = 7.5$, $R = 4.0$, $R = 7.4$, $R = 23.2$
and chemically stable for 6 months at room temperature.

**Biodistribution of Bre-LE in Heart**

The mean concentration vs. time profile of scutellarin following intravenous administration of *Injectio Brevicscape* and breviscapine lipid emulsion is showed in Fig. 12. The area under concentration (AUC) was calculated by logarithmic trapezoidal method. The maximum tissue concentration (C_max) was obtained directly from the individual concentration–time profiles. Mean retention time between time 0 and the infinite (MRT_0→∞) was determined using a log-linear trapezoidal method 3P97 (Mathematic Pharmacological Committee, Chinese Pharmacological Society, China). The tissue distribution advantages in heart of lipid emulsion carrier were very obvious: the AUC values of Bre-LE (623.26 µg · min/ml) were much higher than that of *Injectio Brevicscape* (5.39 µg · min/ml). And the C_max of Bre-LE (9.84 µg/g at 30 min after administration) increased more than 18-fold versus *Injectio Brevicscape* (0.54 µg/g at 5 min after administration). The MRT_0→∞ of Bre-LE and *Injectio Brevicscape* were 62.62 and 8.17 min, respectively. The MRT_0→∞ of breviscapine increased more than 7-fold after encapsulation into lipid emulsion. It was possibly because breviscapine was a chemically unstable drug and easily degrade in vivo. Lipid emulsion, as a drug carrier, could protect breviscapine from degradation and therefore produced a significant change in the heart. Increasing amount and prolonging retention time of drug in heart were all beneficial to heart disease therapy.

**Conclusion**

We prepared a lipid emulsion of Traditional Chinese Medicine—breviscapine with its process easily produced on large industrial scale and sterilized by autoclaving. Bre-LE optimixedly prepared in this paper could protect scutellarin from degradation, exhibited sustained release, and was stable for up to 6 months at room temperature storage condition. Based on the results of biodistribution in heart, higher cardioprotective activity of Bre-LE than that of *Injectio Brevicscape* in further studies on pharmacodynamics may be expected.

**Acknowledgments**

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**References**