Scutellaria baicalensis Extract-phospholipid Complex: Preparation and Initial Pharmacodynamics Research in Rats

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Abstract: Background: Baicalin, a flavonoid glycoside compound present in Scutellaria baicalensis, has shown a wide spectrum of biological activities, but its liposolubility, water-solubility and mucosal permeability are all very poor, which leads to the low concentration in brain and poor bioavailability by oral or intravenous injective administration.

Objectives: The primary objective of this study was to formulate the Scutellaria baicalensis extract (SBE) with phospholipid to yield Scutellaria baicalensis extract-phospholipid complex (SBEPC) , and to evaluate its pharmacodynamics in the middle cerebral artery occlusion (MCAO).

Methods: The optimal preparation technology of SBEPC was obtained through single-factor test and central composite design-response surface methodology (CCD-RSM), and was characterized with various analytical techniques including SEM, FT-IR and NMR. The storage conditions of SBEPC were established through stability study and the MCAO rat model was investigated through conducting pharmacodynamic studies to screen the appropriate administration and dose of SBEPC as well as to verify the neuroprotective effect of SBEPC on cerebral ischemia-reperfusion injury.

Results: The optimized preparation conditions of SBEPC were summarized as follows: the ratio of phospholipid to drug was 2:1, the drug concentration was 3.5 mg/ml, the reaction temperature was 50 °C, and the entrapment efficiency was over 93.00%. Stability studies have demonstrated that SBEPC should be stored under 40 °C in a dry and ventilated place away from light and below 37% humidity. Furthermore, pharmacodynamic studies have found that, compared with SBE, SBEPC could introduce drugs into the brain and better exert the neuroprotective effect on MCAO rats, and the optimal administration and dose concentration of SBEPC were nasal administration and 40 mg/ml, respectively.

Conclusion: These findings demonstrate that SBEPC is successfully prepared by CCD-RSM. SBEPC can enhance drugs’ ability to enter the brain and improve the bioavailability of drugs in brain, and can effectively exert the neuroprotective effect on cerebral ischemia-reperfusion injury as compared with SBE.

Keywords: Scutellaria baicalensis extract-phospholipid complex, central composite design-response surface methodology, stability study, pharmacodynamics, cerebral ischemia.

1. INTRODUCTION

Scutellaria baicalensis Georgi. (named as Huang-Qin in China), belonging to the genus Scutellaria (Lamiaceae), is commonly used as traditional Chinese medicine for thousands of years. Baicalin (Fig. 1A), one of the indispensable active ingredients in S. baicalensis, has shown a wide spectrum of pharmacological activities such as anti-inflammatory [1], antiviral [2], antitumor [3, 4], antioxidant [5], and antibacterial effects [6, 7], and could be used to treat respiratory tract infections, pneumonia, hepatitis, colitis, and allergic diseases. Qingkailing injection, which contains baicalin from S. baicalensis, is a patent traditional Chinese medicine formulation approved by the China Food and Drug Administration (CFDA) for the treatment of ischemic stroke and it has been...
clinically used for more than 30 years [8]. Recently, several studies have shown that baikalin possesses salutary effects against cerebral ischemia [9-14]. However, its liposolubility, water-solubility and mucosal permeability are all very poor, which leads to the low concentration in brain and poor bioavailability by oral or intravenous injective administration. As a result, its therapeutic effect on cerebral ischemia cannot be fully exerted, which limits the clinical application of the effective components of S. baicalensis. Therefore, it is crucial to improve the liposolubility of baikalin by some method, which would significantly expand its clinical values.

In recent years, several approaches have been explored to improve the bioavailability of drugs, among which the complexation technology of plant drugs with phosphatidylcholine (Fig. 1B) has become one of the most successful methods to improve the bioavailability and therapeutic effect of some malabsorbed herbal constituents and natural extracts [15-17]. Several studies have shown that the solubility, in vivo absorption, and bioavailability of baikalin can be improved through preparation of its phospholipid complex [18, 19].

Herein, the objective of this work is to complex the Scutellaria baicalensis extract (SBE) with phosphatidylcholine (PC) to generate Scutellaria baikalensis extract-phospholipid complex (SBEPC) via a solvent evaporation method, and to optimize the optimal preparation through both quality by design (QbD) approach and central composite design-response surface methodology (CCD-RSM) [20-22]. We further discussed the pharmacodynamics of SBEPC on the middle cerebral artery occlusion (MCAO) rats in this study.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Scutellaria baikalensis Georgi. extract containing 85% baikalin was prepared by the same procedures reported in our previous work (NO. 20100403). Soybean lecithin and phosphatidylcholine were purchased from Shanghai Ai Kang Fine Chemical Co., Ltd. (China) and Sigma-Aldrich (China), respectively. All other reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co., Ltd. (China) and used directly without further purification.

2.2. Preparation of SBEPC

The SBEPC was prepared with SBE and PC at different ratios, i.e., 2:1, 3:2, 1:1, 1:2 and 1:3, via a solvent evaporation method. Firstly, SBE and PC were dissolved in ethanol and chloroform, respectively. Then, the mixture was poured into a 200 ml round bottom flask and refluxed for different periods (1-4 h) and at different temperatures (30-60 °C). After removal of any solvent trace by evaporation and vacuum drying, the resulting transparent residues were collected and re-dried under the vacuum to obtain SBEPC. The complexation efficiency of the phospholipids was calculated as W/W₀ × 100%, where W₀ is the initial amount of SBE, and W is the amount of SBE in the SBEPC.

2.3. Single Factor Experiment and Central Composite Design-response Surface Methodology

2.3.1. Optimal Formulation and Process for Preparation of SBEPC by Single-factor Experiment

The effects of seven factors on the complexation efficiency of SBEPC were investigated, including reaction solvent, soybean lecithin with different PC content, phospholipids/drug ratio, drug concentration, reaction temperature, reaction time, and magnetic stirring speed.

2.3.2. Central Composite Design-response Surface Methodology

Based on the single factor experiment, CCD-RSM was applied to optimize the critical independent variables affecting the entrapment efficiency of SBEPC, i.e., phospholipids/drug ratio (X₁, m/m), reaction temperature (X₂, °C) and drug concentration (X₃, mg/ml). Three independent variables (X₁, X₂ and X₃) were selected at three different levels to obtain in total twenty possible combinations. The entrapment efficiency of SBEPC was taken as the dependent variable. The experimental trials were conducted with all twenty possible combinations of variables. The mathematical model including coefficient effects, interactions, and polynomial terms was set up to evaluate the reaction using the following equation: Y = b₀ + b₁X₁ + b₂X₂ + b₃X₃ + b₁₁X₁² + b₂₂X₂² + b₃₃X₃² + b₁₂X₁X₂ + b₁₃X₁X₃ + b₂₃X₂X₃, where Y is the dependent variable, b₀ is the intercept representing arithmetic mean reaction of the 20 runs, bᵢ is the estimated coefficient for the factor (Xᵢ, i = 1, 2, 3), (X₁, X₂, and X₃) are the coded levels of independent variables. The interaction terms (X₁X₂, X₁X₃, and X₂X₃) showed how the response changed when independent variables were changed at the same time. The polynomial terms (X₁², X₂², and X₃²) were used to describe nonlinearity [22]. The coded levels and the real values of the independent variables are shown in Table 1. The experimental compositions as well as complexation efficiency are shown in Table 2.
Table 1.  Coded levels and real values for each factor under study.

| Variables | Levels | -1.732 | -1 | 0 | 1 | 1.732 |
|-----------|--------|--------|----|---|---|-------|
| X1        | 1.00   | 1.42   | 2.00 | 2.58 | 3.00 |
| X2        | 30.00  | 36.34  | 45.00 | 53.66 | 60.00 |
| X3        | 2.50   | 3.56   | 5.0  | 6.44 | 7.50 |

Table 2.  CCD-RSM design experiments and results.

| Std | Actual Value Variables | Response Value |
|-----|------------------------|----------------|
|     | X1/(m/m) | X2°C | X3/(mg/ml) | Y/%   |
| 1   | 1.42 | 36.34 | 3.56 | 83.12 |
| 2   | 2.58 | 36.34 | 3.56 | 90.54 |
| 3   | 1.42 | 53.66 | 3.56 | 79.86 |
| 4   | 2.58 | 53.66 | 3.56 | 90.42 |
| 5   | 1.42 | 36.34 | 6.44 | 67.13 |
| 6   | 2.58 | 36.34 | 6.44 | 88.17 |
| 7   | 1.42 | 53.66 | 6.44 | 68.58 |
| 8   | 2.58 | 53.66 | 6.44 | 89.05 |
| 9   | 1.00 | 45.00 | 5.00 | 63.42 |
| 10  | 3.00 | 45.00 | 5.00 | 92.34 |
| 11  | 2.00 | 30.00 | 5.00 | 80.97 |
| 12  | 2.00 | 60.00 | 5.00 | 82.06 |
| 13  | 2.00 | 45.00 | 2.50 | 87.11 |
| 14  | 2.00 | 45.00 | 7.50 | 75.18 |
| 15  | 2.00 | 45.00 | 5.00 | 88.47 |
| 16  | 2.00 | 45.00 | 5.00 | 88.66 |
| 17  | 2.00 | 45.00 | 5.00 | 88.85 |
| 18  | 2.00 | 45.00 | 5.00 | 89.05 |
| 19  | 2.00 | 45.00 | 5.00 | 89.14 |

The results of CCD-RSM were summarized in Table 2. The optimal preparation conditions were X1 (2:1), X2 (50 °C) and X3 (3.5 mg/ml), which were further validated through six parallel experiments under the optimized preparation conditions (Table 4).

2.4. Physicochemical Properties of SBEPC

2.4.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy has been used to determine surface morphology. Dried SBEPC sample was placed on an electron microscope brass stub and coated with gold in an ion sputter using JEOL JSM-6510LV Scanning microscope (Japan). Digital images of SBEPC were taken by random scanning of the stub at 500 × and 2500 × magnifications.

2.4.2. Particle Size Distribution

The particle size analysis of the SBEPC sample was analyzed with dynamic light scattering laser particle size analyzer and zeta potential analyzer ZS90 (Malvern). The isopropanol
dispersion of SBEPC was taken and placed into the laser particle size analyzer to determine the particle size distribution and average particle size.

2.4.3. Fourier Transform Infrared Spectroscopy (FT-IR)

The analysis of the SBEPC sample was carried out using KBr compact. The influence of the residual moisture was theoretically removed by subjecting the sample to vacuum drying before obtaining spectrum. FT-IR spectrum of the SBEPC was taken (PerkinElmer Spectrum 400, America) in the transmission mode with the wave number region 4000-450 cm\(^{-1}\).

2.4.4. Nuclear Magnetic Resonance (NMR)

The SBEPC sample was dissolved in deuterated chloroform (CDCl\(_3\)) and transferred to an NMR tube for analysis. \(^1\)H-NMR spectrum was recorded on a Avance NEO 600 MHz (Bruker Biospin AG, Switzerland) and chemical shifts were reported relative to tetramethylsilane (TMS).

2.5. Stability Study

Factors such as temperature, illumination, and humidity on the effect of the freshly prepared SBEPC were investigated, and appearance, content of SBEPC and PC were determined at 0, 1, 3, 5, 10, and 15 days to evaluate the stability under the different storage conditions.

2.6. Pharmacodynamic Study in Rats

2.6.1. Raising Animals

Healthy adult male Sprague Dawley rats (SPF grade, weighing 250-270 g) were purchased from Chengdu Dashuo experimental animal research institute and raised in the experimental animal center of Chengdu University of Traditional Chinese Medicine. The rats were kept at room temperature 25 ± 1 °C, and humidity 60 ± 5%, maintained with good ventilation and provided with free food and water. All the experimental procedures were performed in accordance with the Code of Ethics of the World Medical Association. This study protocol was approved by the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (No. 2018BL-002).

2.6.2. Establishment of a CI Model and a Method for Administering Drugs

After 7 days of acclimation, 100 rats were randomly divided into 5 groups as follows: Sham operation group, CI model group, SBEPC nasal administration group (NA group),
intraperitoneal administration group (IPA group), and intragastric administration group (IGA group). Considering the difficulty and success rate of establishing an appropriate model, there were 20 rats in each group. After 5 days of administration (twice a day), we established a MCAO rat model by suture method on the 6th day. Then, the NA, IPA and IGA groups were administered with the appropriate formulations at a dose of 8 mg/kg. The sham operation group and the model group were administered with 0.1 ml of saline intranasally.

2.6.3. Neurological Deficit Score of Rats (Longa score)

The Zea-Longa MCAO scoring method was used to score neurological deficit [23].

2.6.4. Determination of Cerebral Infarction by Triphenyltetrazolium Chloride (TTC) Staining

Brain tissues were taken out and frozen at −20 °C for 30 min, and then quickly prepared into 6 slices and placed in a glass dish containing 3% TTC solution [23]. Next, the slices were incubated at 37 °C for 30 min and turned over periodically to ensure uniform staining. After 30 min, the infarction area was stained white, and the rest was stained red (Fig. 2). After color development, the slices were fixed with 4% paraformaldehyde solution. The images of each group were obtained under the same settings, and the infarction area was calculated 5 times with Image J software. Cerebral infarction ratio = white area / whole area × 100%.

![Fig. (2). TTC staining in rat brain slices (A higher resolution/colour version of this figure is available in the electronic copy of the article).](image)

2.6.5. Screening of Effective Dose of Nasal Administration

After 7 days of acclimation, 100 rats were randomly divided into 5 groups as follows: Sham operation group, CI model group, low dose group of SBEPC (LD group, 10 mg/ml), medium dose group of SBEPC (MD group, 20 mg/ml), and high dose group of SBEPC (HD group, 40 mg/ml). The rats were pre-administrated with drugs or saline for 5 days (b.i.d). On the sixth day, the focal cerebral ischemia model was set by suture method. The rats were administrated three times before 2 h of modeling and after 2 h and 23 h of modeling. All rats were decapitated after 24 h of modeling and their brains were kept at −20 °C.

2.7. Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Statistical comparisons were performed with GraphPad Prism version 8.0. Student t-test was used to analyze the statistical significance of differences between two groups. Statistical significant difference was set as p < 0.05, and an extremely significant difference was defined as p < 0.01.

3. RESULTS

3.1. Factors Affecting the Formation of SBEPC

3.1.1. Reaction Solvent

Based on the complexation principle, the nitrogen atom in phospholipids is prone to lose electrons, while the oxygen atom of the phenolic hydroxyl group in baicalin tends to obtain electrons, which makes the formation of complexes possible [24]. Hence, non-protonated transfer solvents (e.g., tetrahydrofuran, ethanol, ethyl acetate, acetone, and chloroform) are necessary to provide a reaction environment that does not disturb the exchange of electrons (protons) between drugs and phospholipids. The complexation efficiencies of each complex prepared with a series of solvents were investigated as follows: 100% in dioxane-methanol (7:3), 76.7% in tetrahydrofuran, 48.3% in ethyl acetate, 36% in ethanol, and 24.8% in methanol [25]. It is noted that dioxane, tetrahydrofuran, ethyl acetate and methanol are toxic and expensive, and that dioxane with a high boiling point of 101 °C is difficult to be completely removed from the complex. Furthermore, using the same PC content of soybean lecithin (80%), starting molar ratio (1:2), concentration of the drug in the reaction system (5 mg/ml), reaction temperature (55 °C), magnetic stirring speed (120 r/min), and reaction time (2 h), the complexation efficiencies of complexes prepared with four solvents vary a lot among tetrahydrofuran (97.71%), petroleum ether-ethanol (7:3, 10.46%), ethanol (91.09%), and anhydrous ethanol (95.67%). In comparison, the complexes prepared with anhydrous ethanol and tetrahydrofuran had a higher complexation efficiency than those with other solvents. With the additional concerns of the feasibility of large-scale production and safety problems caused by residual solvents, anhydrous ethanol was preferred as the optimal reaction solvent.

3.1.2. Soybean Lecithin with Different PC Content

Complexation efficiencies of complexes prepared at four PC contents were found as follows: 75.10% (PC: 60%), 84.51% (PC: 70%), 95.48% (PC: 80%), and 95.87% (PC: 90%). It could be concluded that the different PC contents in soybean phospholipid cast a great influence on the complex reaction, and that the complexation efficiency increased with an increase of PC content. Interestingly, complexation efficiencies were quite similar when the content of PC reached 80% and 90%, indicating completion of complex reaction. Therefore, considering the atom economy, soybean phospholipid with 80% PC content was selected as the experimental raw material.

3.1.3. Starting Molar Ratio

Complexation efficiencies of complexes prepared at different molar ratios (drug/phospholipids) were found as follows: 59.43% (3:1), 66.01% (2:1), 78.59% (3:2), 82.45% (1:1), 95.40% (1:2), 95.44% (1:3) and 95.50% (1:4). It found that starting molar ratio had a significant effect on the complexation reaction, and that it complexed more efficiently...
when the ratio was below 1. Taking into account the high cost of soybean phospholipid and the maximum drug loading of the complex, the molar ratio from 1:1 to 1:3 was considered as the optimal range.

3.1.4. Concentration of the Drug in the Reaction System

Complexation efficiencies of complexes prepared at four concentrations of the drug in the reaction system were found as follows: 95.47% (2.5 mg/ml), 95.10% (5.0 mg/ml), 78.72% (7.5 mg/ml), and 64.98% (10 mg/ml). Obviously, the concentration played an important role in the complex reaction, where the complexation efficiency decreased with an increase of drug concentration in the reaction. The concentrations of 2.5 and 5.0 mg/ml led to much higher complexation efficiency, while the concentration of 10 mg/ml yielded the lowest. On the other hand, increasing the reaction concentration could reduce the amount of solvent, thereby simplifying the selection of production equipment and saving costs. Taking both factors into consideration, the optimal concentration range was selected as 2.5-7.5 mg/ml.

3.1.5. Reaction Temperature

Complexation efficiencies of complexes prepared at six reaction temperatures were found as follows: 80.15% (20 °C), 89.55% (30 °C), 92.41% (40 °C), 95.45% (50 °C), 95.47% (60 °C), and 95.85% (70 °C). The complexation efficiency increased with an increase of reaction temperature, but the increasing range was quite limited and reached a plateau when the temperature was above 50 °C. In addition, the properties of phospholipids turned to be poor at 70 °C. This analysis is in line with the literature report that the oxidation of soybean lecithin was accelerated rapidly when the temperature exceeded 60 °C [26]. Overall, to promote the complexation reaction and minimize the oxidation of soybean lecithin, the optimal temperature range was determined to be 30-60 °C.

3.1.6. Reaction Time

Complexation efficiencies of complexes prepared at four kinds of reaction time were found as follows: 92.71% (1.0 h), 95.45% (2.0 h), 95.47% (3.0 h), and 95.46% (4.0 h). It was noted that the complexation efficiency increased slightly with an increase of reaction time, and that it was stabilized after 2 h, indicating a completion of the reaction. Overall, reaction time had little effect on the complexation efficiency, the difference of which is less than 2%, and 2 h are sufficient for the reaction to complete.

3.1.7. Magnetic Stirring Speed

Complexation efficiencies of complexes prepared at magnetic stirring speeds were found as follows: 93.58% (60 r/min), 95.39% (120 r/min), 95.40% (180 r/min), and 95.41% (240 r/min). It was found that the complexation efficiency increased gently with an increase of magnetic stirring speed, and it was stabilized from a speed of 120 r/min. Since further higher speeds would require more mechanical work and energy consumption, 120 r/min was chosen as the optimal magnetic stirring speed.

3.2. Optimal Formulation and Process for Preparation of SBEPC by Central Composite Design-response Surface Methodology

In present research, it could be seen that three independent variables (X1, X2, and X3) had a remarkable effect on the entrapment efficiency of SBEPC. The measured values of entrapment efficiency in Table 2 revealed a wide range (63.42-92.34%), and the 15-20th trials were six repeated central point experiments to investigate the model error. In the design expert 7.0 software, a response surface regression analysis was adopted to establish mathematical relationship between factors and parameters. The three-dimensional response surface plots for the most statistically significant variables on the evaluated responses were presented in Fig. (3). The equations represented the quantitative effect of process variables (X1, X2, and X3), and their interactions on response Y were as follows:

\[ Y = 6.88 + 32.31X_1 + 2.07X_2 - 1.70X_3 - 9.82X_1^2 - 0.03X_2^2 - 1.05X_3^2 + 0.064X_1X_2 + 3.53X_1X_3 + 0.06X_2X_3. \]

It could be learnt from Table 3 that the polynomial model for Y was significant (F = 80.57, p < 0.01). The value of correlation coefficient \( R^2 \) was 0.9172, indicating a good fit to the quadratic model.

The changes in the Y (%) as a function of A, B, and C were acquired by the response surface plots based on the central composite design. The interpolated values were generated based on all the data of 20 batches of the central composite design by software. The response surface plots indicated that the tested factors (A, B, and C) had a significant influence on entrapment efficiency. It was found that increasing levels of A, B, and C were favorable conditions to obtain higher entrapment efficiency. Based on these observations, along with the multiple regression models, the optimal values of the tested factors, namely, the phospholipids/drug ratio, reaction temperature, and drug concentration were 2:1, 50 °C, and 3.5 mg/ml, respectively.

In order to evaluate the optimization ability of the model generated from the results of CCD-RSM, an additional batch of SBEPC was prepared using the optimized values of the variables, that is, X1, X2, and X3 2:1, 50 °C, and 3.5 mg/ml, respectively. To verify this, six parallel tests were carried out under the optimized preparation conditions (Table 4). The predicted (theoretical) value (%) obtained from the developed model was compared with the observed value (%) acquired from the prepared formulation. The model-predicted value for the entrapment efficiency of SBEPC was 93.87%, while the average observed value (%) from the prepared batches was (92.73 ± 0.22)%, indicating both applicability and validity of the developed model. The bias (%) calculated with the equation was less than 2%, which indicated the relative robustness of the model.

Bias% = \( \frac{\text{predicted value} - \text{observed value}}{\text{observed value}} \times 100\% / \text{predicted value} \)

3.3. Physicochemical Properties of SBEPC

3.3.1. Scanning Electron Microscopy (SEM)

Fig. (4A and 4B) indicate that the surface morphology of the SBEPC at 500 × and 2500 × magnifications are irregular particles on account of the complex formation.

3.3.2. Particle Size Distribution

The mean particle size of SBEPC as shown in Fig. (4C) was measured with dynamic light scattering technique.
3.3.3. FT-IR Analysis

The FT-IR spectrum of SBEPC is shown in Fig. (4D). Based on comparison of the IR spectral data of baicalin, the main compound of SBE and phospholipid with the initial literature [27], the IR spectrum of SBEPC had the characteristics of baicalin and phospholipid, indicating that the formation of phospholipid complex did not produce new chemical bonds between molecules. It could be seen that the IR spectrum of SBEPC had changed greatly, the stretching vibration peak of hydroxyl group in the region of 3300-3600 cm\(^{-1}\) disappeared, and only the characteristics of phospholipid in this region could be seen. The peak of 1739.97 cm\(^{-1}\) appeared in the range of 2800-1700 cm\(^{-1}\), which was similar to the stretching vibration peak of ester in phospholipid (C=O, 1739.37 cm\(^{-1}\)), while the carbonyl peak (C=O, 1600.96 cm\(^{-1}\)) of baicalin C ring moved to 1667.62 cm\(^{-1}\), and the skeleton vibration peak (C=C, 1609.13, 1573.41, 1551.97, 1496.32 cm\(^{-1}\)) of baicalin benzene ring changed obviously, among which 1609.13 cm\(^{-1}\) moved to the high wave number of 1616.79 cm\(^{-1}\), while the medium strong peak of 1573.41 cm\(^{-1}\) and the weak peak of 1551.97 cm\(^{-1}\) disappeared, and a new weak peak of 1587.58 cm\(^{-1}\) appeared. However, the main peaks of phospholipid at non-polar terminal such as the hydrocarbon stretching vibration peak (C-H, 2925.61 cm\(^{-1}\), 2854.13 cm\(^{-1}\)) and the bending vibration peak of methylene (CH\(_2\), 1466.36 cm\(^{-1}\)) in the saturated long fatty chain of phospholipid almost did not change.

3.3.4. NMR Analysis

The \(^1\)H-NMR spectrum of SBEPC was shown in Fig. (4E). \(^1\)H-NMR signals of SBEPC showed obvious changes based on the comparison of its spectroscopic data with those of baicalin, the main compound of SBE and phospholipid in the literature [27]. In the \(^1\)H-NMR spectrum of the SBEPC, signals of the protons in baicalin belonging to aromatic hydrogen and phenolic hydroxyl in \(\delta_H\) 6-8 had weakened remarkably to the extent that they could not be observed, and only some signals belonging to baicalin were widened, indicating these protons were involved in the formation of the complex. The signals of non-polar terminal fatty chain (\(\delta_H\) 1.2541) of phospholipid were clearly visible, whereas the signals of glycerol part (\(\delta_H\)
3.9961-4.3716) and choline part ($\delta_H$ 3.2369) of polar terminal in phospholipid were slightly widened.

3.4. Stability Study of SBEPC

3.4.1. Effect of Temperature on the Stability of SBEPC

Temperature stability studies of optimized SBEPC under four storage conditions were carried out, and the changes in content of SBEPC and PC were evaluated as parameters. Fig. (5A) demonstrated that the content of SBEPC was found to be 86.29% and to be 86.06, 86.23, 86.05 and 86.01% after 15 days storage at 4, 25, 40, and 60 °C, respectively. Fig. (5B) illustrated that the content of PC was found to be 52.21% and to be 52.66, 52.12, 52.53 and 37.56% after 15 days storage at 4, 25, 40, and 60 °C, respectively. There was no obvious change in content of SBEPC, indicating that temperature had...

Fig. (4). Physicochemical properties of SBEPC. Surface morphology of SBEPC (SEM) at (A) 500 × and (B) 2500 × magnifications. (C) Particle size analysis of SBEPC. (D) IR spectrum of SBEPC. (E) $^1$H-NMR spectrum of SBEPC (CDCl$_3$, 600 MHz) (A higher resolution / colour version of this figure is available in the electronic copy of the article).
little effect on SBEPC content. When the temperature was higher than 40 °C, the color of SBEPC became dark, and gradually changed from light yellow to yellow, brown yellow and even brown. The content of PC decreased significantly during storage at 60 °C, therefore, the storage temperature of phospholipids should be lower than 40 °C.

3.4.2. Effect of Illumination on the Stability of SBEPC

Illumination stability studies of optimized SBEPC under two storage conditions were carried out, and the changes in content of SBEPC and PC were evaluated as parameters. Fig. (5C) showed that the content of SBEPC was found to be
86.29% and to be 85.86, and 86.31% after 15 days storage at 0 and 4500 Lux, respectively. Fig. (5D) illustrated that the content of PC was found to be 52.21% and to be 52.21 and 42.13% after 15 days storage at 0 and 4500 Lux, respectively. The results showed that illumination had a great influence on the appearance and color of SBEPC, but had little effect on the content of SBEPC, and it had a great influence on the content of PC with the increase of storage time. Therefore, SBEPC should be stored away from light.

3.4.3. Effect of Humidity on the Stability of SBEPC

Humidity stability studies of optimized SBEPC under four storage conditions were carried out, and the changes in content of SBEPC and PC were evaluated as parameters. SBEPC sample was precisely weighed and placed in a stopped surface plate, the saturated solutions of four kinds of salts (K₂CO₃, NaBr, NaCl, and KCl) were prepared and placed in a glass dryer respectively at room temperature for 48 h. The internal humidity was balanced by placing the surface plate in it to form the relative humidity (42.7, 57.7, 75.3, and 84.3%), and then placed at 25 °C in dark area. Samples were taken at 0, 1, 3, 5, 10 and 15 days for determination of various indicators. The experimental results were shown in Fig. (5E and 5F).

The experimental results showed that humidity had a great influence on the properties of SBEPC. When the humidity reached 57.7%, due to the strong hygroscopicity of SBEPC, there were different degrees of caking, while the increase of humidity had little effect on other indexes of SBEPC, indicating that the complex had strong hygroscopicity. Therefore, the storage humidity of SBEPC should be kept below 42.7%.

3.4.4. Calculation of Critical Relative Humidity of SBEPC

The SBEPC sample was precisely weighed and placed in a stopped surface plate, the saturated solutions of six kinds of salts (CH₃COOK, MgCl₂, K₂CO₃, NaBr, NaCl, and KCl) were prepared and placed in a glass dryer respectively at room temperature for 48 h. The internal humidity was balanced by placing the surface plate in it to form the relative humidity (22.5, 33.0, 42.7, 57.7, 75.3, and 84.3%), and then placed at 25 °C in dark area. Samples were taken at 10 days for determination of various indicators, and the moisture absorption rate was calculated. Take the relative humidity as X and the moisture absorption rate as Y, draw the moisture absorption curve, as shown in Fig. (5G). The abscissa corresponding to the intersection of two tangent lines of the curve was the critical relative humidity. The experimental results were shown in Table 5.

According to the moisture absorption curve, the critical relative humidity was about 37%. The hygroscopicity of SBEPC was slight when the humidity was under 37%, while it was obviously increased with the humidity over 37%. Combined with the Table 5, the SBEPC should be kept below 37% humidity.

3.5. Pharmacodynamic Study in Rats

3.5.1. Neurological Deficit Scoring

The neurological function scores of rats in different experimental groups (Fig. 6A) showed that the score of model group was significantly higher than that of the sham operation group ($p < 0.01$), which indicated that the modeling was successful. The scores of IPA and NA groups were significantly lower than that of model group ($p < 0.01$), indicating that SBEPC could alleviate brain injury, thus showing the neurological function deficit was significantly improved after reperfusion. There was no significant difference in neurological function score between IGA group and the model group (1.67 ± 0.52 vs 2.33 ± 0.52). But there was a certain mortality in the IPA group. Considering the convenience and safety of administration, the NA group showed a better curative effect than the IGA group (0.83 ± 0.75 vs 1.67 ± 0.52), suggesting the drug through the olfactory delivery could achieve a higher delivery efficiency.

3.5.2. Determination of Cerebral Infarction by TTC Staining

The results of cerebral infarct volume measurement were shown in Fig. (6B). In the MCAO model group, the average infarct volume ratio was higher than that of NA and IPA groups, indicating that the surgery caused the damage of the brain. There was no significant difference in average infarct volume rate between IGA group and the model group (21.69 ± 5.79 vs 20.06 ± 8.57). The average infarct volume rate of NA group was lower than that of IPA group (15.29 ± 5.36 vs 15.74 ± 6.64), indicating that nasal administration might have a better therapeutic effect on MCAO model. It further demonstrated the feasibility of nasal administration in the treatment of the ischemic stroke.

Table 5. Investigation results of relative humidity.

| No. | Relative Humidity (%) | Saturated Salt Solution | Moisture Absorption Rate (%) |
|-----|-----------------------|-------------------------|-----------------------------|
| 1   | 22.5                  | CH₃COOK                 | 6.3                         |
| 2   | 33.0                  | MgCl₂                   | 7.2                         |
| 3   | 42.7                  | K₂CO₃                   | 11.8                        |
| 4   | 57.7                  | NaBr                    | 28.2                        |
| 5   | 75.3                  | NaCl                    | 50.2                        |
| 6   | 84.3                  | KCl                      | 60.5                        |
3.5.3. Screening of Effective Dose of Nasal Administration

The results of the neurological deficit function score evaluation were summarized in Fig. (6C). After MCAO operation, the scores of the rats in this group were much higher than other groups ($p < 0.01$), indicating that the MCAO model was successfully prepared. Also, the HD administration showed a better curative effect than the MD and LD treatments ($0.67 \pm 0.52$ vs $0.83 \pm 0.41$ and $1.00 \pm 0.89$). Additionally, the results of cerebral infarction rate measurement were shown in Fig. (6D). The average infarct volume rate of HD group was lower than those of MD and LD groups ($15.66 \pm 4.93$ vs $16.93 \pm 6.02$ and $18.10 \pm 5.39$), indicating that high dose (40 mg/ml) of nasal administration might have a better protective effect on the cerebral infarction after cerebral ischemia-reperfusion.

4. DISCUSSION

Cerebral ischemia is the second-leading cause of death in the world after heart disease, and it is also the main cause of permanent adult disabilities worldwide [28]. Qingkailing injection is a patent traditional Chinese medicine formulation approved by the CFDA for the treatment of ischemic stroke, and it has been clinically used over 30 years [8]. Baicalin, the main ingredient of Qingkailing injection, has been widely used to treat ischemic stroke, and its preventive and therapeutic effects on focal cerebral ischemia model are remarkable and definite [29, 30]. Many pharmacological studies suggest that its single-use effect is no less than Qingkailing prescription [29-31]. However, due to low water-solubility and liposolubility caused by glucosyl group in the molecular structure...
of baicalin, the bioavailability after oral administration is only 2.2%, which limits its clinical application [32]. This study attempts to improve bioavailability of SBE via its complexation with phospholipids and initially discuss the pharmacodynamics of SBEPC on MCAO rats.

The preparation of phospholipid complex needs to be carried out in aprotic solvent, tetrahydrofuran and ethyl acetate are the main reaction solvents in the literature [24-25], and anhydrous ethanol is rarely reported. Through the investigation of the four reaction solvents, it is found that when anhydrous ethanol is used as the solvent, the same complexation efficiency of tetrahydrofuran can be obtained in the improved device. Considering the feasibility of large-scale production, anhydrous ethanol was selected as the reaction solvent, and a series of factors affecting the complexation efficiency of SBEPC were investigated by single factor to determine the main factors affecting the complexation reaction. The formulation and process variables were optimized by CCD-RSM. The optimized preparation conditions of SBEPC were as follows: with a certain quantity of SBE and PC content of 80% soybean lecithin, at phospholipids/drug ratio of 2:1, by addition of proper amount of anhydrous ethanol, drug concentration was 3.5 mg/ml, in the constant temperature water bath at 50 °C for 2 h in a magnetic stirring which keeping the speed of 120 r/min, until the medium cleared. SBEPC was obtained after removal of the solvent and dissolved in chloroform, achieving the complexation efficiency of over 93.00%. Firstly, the SEM, particle size distribution, FT-IR and NMR studies indicated the successful formation of SBEPC. Next, the factors affecting the stability of SBEPC: temperature, illumination, and humidity were investigated, and stability studies have demonstrated that SBEPC should be stored under 40 °C in a dry and ventilated place away from light and below 37% humidity. Finally, conducted pharmacodynamic studies on the MCAO rat model to screen the appropriate administration and dose of SBEPC and verify the neuroprotective effect of SBEPC on cerebral ischemia-reperfusion injury. However, the presence of the obstacles such as the blood- brain- barrier (BBB) still makes it difficult to enter brain effectively. Meanwhile, several studies have shown that baicalin-phospholipid complex can be transported into brain through intranasal administration without the BBB, so as to enhance its brain pharmacological effect [33-35]. Thus in this research, taking neurological function score and cerebral infarction rate as evaluation indexes, through the investigation of three administration ways of SBEPC: nasal administration, intraperitoneal administration, intragastric administration, and nasal administration of SBE, it was finally determined that nasal administration of SBEPC was the best administration mode. The results suggested that SBEPC could overcome the disadvantages of poor solubility and low bioavailability of SBE. Meanwhile, the results indicated that high dose (40 mg/ml) of nasal administration might have a better protective effect on the cerebral infarction after cerebral ischemia-reperfusion.

Yet this study still has some limitations. Firstly, in the process of stability experiment, it was found that the color change of phospholipids was related to the change of PC content. It was concluded that the discoloration was caused by the transformation of PC into another component. Secondly, the safety and reasons of the transformed components should be investigated in the next step. Finally, in the initial pharmacodynamic experiment, only neurological function score and cerebral infarction rate were used as evaluation indexes, and the selection of indexes might be single and subjective. Therefore, more systematic indexes should be considered in future evaluations.

CONCLUSION

In conclusion, we have optimized the optimal preparation process of SBEPC by central composite design-response surface methodology. The initial pharmacodynamic studies have demonstrated that compared with SBE, SBEPC could enhance drugs' ability to enter the brain and improve the bioavailability of drugs in brain, and it is more effective for neuroprotection in focal cerebral ischemia. The successful preparation and good therapeutic effect on cerebral ischemia-reperfusion of SBEPC can provide new ideas for the further development and utilization of traditional Chinese medicine preparations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study protocol approved by the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine, China (approval no. 2010BL-007).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All procedures performed in studies involving animal participants were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine. All the experimental procedures were performed in accordance with the Code of Ethics of the World Medical Association (No. 2018BL-002).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are included within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Liao, H.; Ye, J.; Gao, L.; Liu, Y. The main bioactive compounds of Scutellaria baicalensis Georgi. for alleviation of inflammatory cytokines: A comprehensive review. Biomed. Pharmacother., 2021, 133, 110917.

[2] Yang, F.; Feng, C.; Yao, Y.; Qin, A.; Shao, H.; Qian. Antiviral effect of baicalein on Marek’s disease virus inCEF cells. BMC Vet. Res., 2020, 16(1), 371.

[3] Orzechowska, B.U.; Wróbel, G.; Tulej, E.; Jatczak, B.; Sochocka, M.; Chaber, R. Antimutator effect of baicalein from the Scutellaria baicalensis radix extract in B-acute lymphoblastic leukemia with different chromosomal rearrangements. Int. Immunopharmacol., 2020, 79, 106114.

[4] Yang, B.; Bai, H.; Sa, Y.; Zhu, P.; Liu, P. Inhibiting EMT, stemness and cell cycle involved in baicain-induced growth inhibition and apoptosis in colorectal cancer cells. J. Cancer, 2020, 11(8), 2303-2317.

[5] Lei, K.; Shen, Y.; He, Y.; Ye, Z.; Zhang, L.; Zhang, J.; Tong, W.; Xu, Y.; Jin, L. Baicalein represses C/EBPβ via its antioxidative effect in Parkinson’s disease. Oxid. Med. Cell. Longev., 2020, 8959107.

[6] Gao, X.; Guo, M.; Zhang, Z.; Shen, P.; Yang, Z.; Zhang, N. Baicalein promotes the bacteriostatic activity of lysozyme on S. aureus in mammary glands and neutrophil granulocytes in mice. Oncotarget, 2017, 8(12), 18994-19001.

[7] Jiang, M.; Li, Z.; Zhu, G. Protective role of flavonoid baicalin from Scutellaria baicalensis in periodontal disease pathogenesis: A literature review. Complement. Ther. Med., 2018, 38, 11-18.

[8] Ma, C.; Wang, X.; Xu, T.; Zhang, S.; Liu, S.; Zhai, C.; Wang, Q. Qingkailing injection ameliorates cerebral ischemia-reperfusion injury and modulates the AMPK/NLRP3 inflammasome signalling pathway. Brain Res., 2020, 1726, 116-124.

[9] Dai, J.; Qiu, Y.M.; Ma, Z.W.; Yan, G.F.; Zhou, J.; Li, S.Q.; Wu, H.; Jin, Y.C.; Zhang, X.H. Neuroprotective effect of baicalin on focal cerebral ischemia in rats. J. Neurosci. Res., 2018, 15193, 28184027.

[10] Li, N.; Zhang, R. Pharmacokinetics, pharmacodynamics and tox- tokines: A comprehensive review. Oxid. Med. Cell. Longev., 2020, 8959107.

[11] Su, J.; Wang, T. The intranasal administration of Carthamus tinctorius L. extract/phospholipid complex in the treatment of cerebral infarction via the TNFα-MAPK pathway. Biomed. Pharmacother., 2020, 110563.

[12] Wei, J.; Wang, B.; Li, M.; Shi, Y.H.; Wang, C.; Kang, Y.G. Network pharmacology identification of mechanisms of cerebral ischemia injury amelioration by baicalin and geniposide. Eur. J. Pharmacol., 2019, 859, 172484.

[13] Yang, H.; Zhang, Q.; Zhao, H.; Gu, X. Effect of baicalin-loaded liposomes on cerebral ischemia-reperfusion injury rats via intranasal administration. Brain Res., 2020, 1726, 1465-1465.

[14] Zhang, Y.; Liu, S.; Wan, J.; Yang, Q.; Xiang, Y.; Ni, L.; Long, Y.; Cui, M.; Ci, Z.; Tang, D.; Li, N. Preparation, characterization, and in vivo study of borneol-baicalin-liposomes for treatment of cerebral ischemia-reperfusion injury. Int. J. Nanomedicine, 2020, 15, 5977-5989.
PEGylated cationic solid lipid nanoparticles modified by OX26 antibody on regulating the levels of baicalin and amino acids during cerebral ischemia-reperfusion in rats. *Int. J. Pharm.*, **2015**, 489(1-2), 131-138.
http://dx.doi.org/10.1016/j.ijpharm.2015.04.049 PMID: 25895718

Zhou, Z.Q.; Li, Y.L.; Ao, Z.B.; Wen, Z.L.; Chen, Q.W.; Huang, Z.G.; Xiao, B.; Yan, X.H. Baicalin protects neonatal rat brains against hypoxic-ischemic injury by upregulating glutamate transporter 1 via the phosphoinositide 3-kinase/protein kinase B signaling pathway. *Neural Regen. Res.*, **2017**, 12(10), 1625-1631.
http://dx.doi.org/10.4103/1673-5374.217335 PMID: 29171427

Xing, J.; Chen, X.; Zhong, D. Absorption and enterohepatic circulation of baicalin in rats. *Life Sci.*, **2005**, 78(2), 140-146.
http://dx.doi.org/10.1016/j.lfs.2005.04.072 PMID: 16107266

[33] Li, N.; Je, Y.J.; Yang, M.; Jiang, X.H.; Ma, J.H. Pharmacokinetics of baicalin-phospholipid complex in rat plasma and brain tissues after intranasal and intravenous administration. *Pharmazie*, **2011**, 66(5), 374-377.
https://dx.doi.org/10.1691/ph.2011.0783 PMID: 21699072

Shi, Y.J.; Yang, M.; Shi, J.H.; Liu, J.Y.; Tang, M. [Study on formulation of *Scutellaria baicalensis* extract phospholipid complex nasal preparation]. *Zhong Yao Cai*, **2013**, 36(10), 1697-1701.
PMID: 24761683

[35] Li, N.; Ma, J.H.; Yang, M.; Ye, Y.J.; Xu, R.C. Intranasal administration of baicalin-phospholipid complex improves brain targeting and ischemic-reperfusion injuries. *J. Pharm. Sci. Technol.*, **2012**, 4(7), 958-971.