Research Article

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The Optimization and Production of Ginkgolide B Lipid Microemulsion

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Abstract: In this study, we introduce a method to formulate Ginkgolide B lipid microemulsion (GB-LM). We have assessed its general characteristics and pharmacokinetics in animals. Soybean oil (10.0%) and oleic acid (2.4%) were chosen as the oil phase, refined egg yolk lecithin (PC98T) (1.8%) as the surfactant, and glycerol (2.25%) as the co-surfactant. The optimized formulation process resulted in particles with average diameter of 185.9 ± 52.5 nm and the zeta potential of -19.8 ± 1.3 mV. The GB-LM remained steady for three months at room temperature. Giving each dose of 4 mg/kg to rats through the vein, a clearance rate of CL (L/h): 2.594 and C_{max} (ng/mL) was achieved: 353.8 through pharmacokinetic analysis and statistical analysis. The AUC of GB-LM was about 1.57-fold higher than that of the products available in the market. In addition, brain tissue distribution studies show that after 0.5 h administration of GB, the concentration of GB in brain tissue can reach its maximum, and then significantly decrease after 2 hours. Therefore, the improved formulation of GB-LM shows encouraging results compared with present products in several features.

Keywords: Ginkgolide B; Lipid microemulsion; Optimization.

1 Introduction

Parkinson's disease is a nervous-system disease which frequently occurs among the elderly. Its manifestations include slowness of movement, muscular rigidity, tremor at rest, and difficulty with walking. Non-motor symptoms such as sense of smell constipation, depression, and anxiety are also common [1]. As a typical elderly disease, its rate doubles when age grows; 1%~2% of people over 65 suffer from Parkinson's disease [PD], and the rate increases to 3%~5% for people over 85 [2, 3]. According to the prediction, because of PD's low-rate of death and high-rate of disability, it will bring challenge to the societal and medical institutions in China, which is now experiencing aging [4].

Ginkgolides (GG) is the main effective component in Ginkgo biloba. It has a wide range of medical use, mainly for nervous-system and cardiovascular diseases [5, 6]. GG is fat soluble; it includes Ginkgolide A, B, C, and also Bilobalide B (BB), all of which have a small solubility: (259.21±3.39), (69.26±2.63), (512.51±3.83), (1247.50±4.09) mg/L in Soybean Oil at 50-60°C [7].

As the main chemical component in the extract of Ginkgo biloba, GB can protect nerve cell and prevent it from decaying. Its effectiveness in treating nerve-system diseases has already been recognized [8]. GB is a natural free radical scavenger, it can protect the body from the damage of free radicals, improve brain circulation and neuronal dysfunction. The mechanism of action of GB may be that M1 receptors are upregulated in brain tissue of aged animals and Alzheimer's disease [9-11], in addition, GB also promotes learning and memory, promotes the development of the nervous system and differentiation of neural stem cells into neurons, and prevents and treats neurodegenerative diseases such as Alzheimer's disease [12]. GB's CAS number is [15291-77-7], $C_{20}H_{24}O_{10}$ with molecular weight 424.40. It has a high melting point (about 300°C); it is soluble in many organic solvents, such as acetone and ethanol. It hardly dissolves in ether or water (2.5×10^{-4} mol/L) and is completely soluble in cyclohexane, benzene, chloroform and carbon tetrachloride. GB is

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relatively stable in mineral acid. The oil-water distribution coefficient is 1.72; the ionic product constants are $pK_{a1} = 7.14$, $pK_{a2} = 8.60$, $pK_{a3} = 11.889$ (13, 14). GB has a wide range of clinical applications. However, due to its low solubility, short half-life and low bioavailability, its application is limited. Therefore, it is necessary to develop a new pharmaceutical preparation of GB with improved pharmaceutical parameters.

Lipid microemulsion (LM) is a vegetable oil based nanosphere covered with phospholipid. It is a stable Oil/Water disperse system consisting of oil phase, emulsifier, water phase, the diameter of particle size is between 100~200 nm, which is also called submicroemulsion. Medicament with high dissolvability in oil can be sealed in oil phase and phospholipid interface membrane. The medicament can rapidly turn into active material after the system carries it to a target site and it seldom leaks in advance [15]. LM has become a popular preparation because of its safety, stability, and biocompatibility [16, 17]. By adjusting the composition of oil phase, the polarity of the oil phase, emulsifier, ionic strength and preparation method to increase drug solubility, this system would be able to transfer medicament more effectively and safer than traditional preparations. The onset time is shortened [18, 19] while the side-effects are also prevented since there is few medicament outside the target site [20]. In this research, we designed a drug delivery system based on LM to transport medicament, and developed an optimized formulation of Ginkgolide B-Lipid microemulsion (GB-LM) using single factor and orthogonal experiment design, the physicochemical properties and pharmacokinetic parameters of GB-LM in rats were also established.

2 Experimental Procedure

2.1 Materials and equipment

Ginkgolide B (GB>98.0%) and bilobalide reference substance were purchased from Sichuan Pharmaceutical Co., Ltd (Sichuan, China). Glycerol was purchased from Beijing Chemical Works. PC98T were purchased from Q.P. Corporation. Soybean oil and oleic acid were purchased from Tieling Beiya Medicinal Oil Co. Ltd. Refined egg yolk lecithin and commercial GB injection (The dosage of GB injection 20 mg/mL) was provided by the general hospital of the people's liberation army. Methanol (MREDA) and tetrahydrofuran (SIGMA-ALORICH) were chromatographic grade in particular. Distilled water was

from Guangzhou Watsons, and pure water was drinking water from Rahaha.

Shimadzu LC-2010A HT high performance liquid chromatograph, Artorius CRA225D2 electronic balance of one in one hundred thousand, SEDERE LT-ELSD SEDEX80 evaporative light scattering detector, Kunshan KQ3200DB ultrasonic cleaner.

2.2 Preparation of Ginkgolide B Lipid Microspheres

According to the prescription, soybean oil was weighed and heated to 50-60°C. Ginkgolide, oleic acid and refined egg yolk lecithin phospholipid (PC98T) were added to soybean oil, stirred about 2 minutes at 6000rpm until phospholipid was completely dissolved, this process was protected by nitrogen during heating, it was used as oil phase, heat preservation; According to the prescription, water phase was heated to 50-60°C, oil phase was added to water phase slowly, stirred about 10 minutes at 3000-10000 rpm high speed to form colostrum. It was homogenized 8 times (1000bar, homogenization temperature 40°C), adjusted pH to 6.8 with hydrochloric acid, then filled and sealed with nitrogen, sterilized for 8 minutes at 121°C. The process of GB-LM preparation is illustrated in Figure 1.

Formulation and process parameters for GB-LM were selected based on the single-variable analysis and orthogonal experimental showed in Table 1. Through the equations below, an index S for composite grade is computed to evaluate the production.

$$S = (S_1 + S_2 + S_3 + S_4 + S_5)$$

$$S_1 = D - 200, S_2 = D_{90} - 200, S_3 = D_{50} - 100,$$

$$S_4 = \text{PDI} \times 100, S_5 = D_{400} \% \times 1000$$

Where D is the particle size, PDI is polydispersity index, D_{90} is the maximum particle size of 90% particles, D_{50} is the maximum particle diameter of 50% particles, D_{400} is the (%) particle size ≥ 400 nm.

2.3 Measurement of particle size and zeta potential

The mean particle size, polydispersity index and zeta potential were determined by Zetasizer Nano ZS90 (Malvern Instruments Ltd) dynamic light scattering particle size analyzer at the dispersion medium coefficient

Table 1: Orthogonal experimental design.

Levels	Factors			
	A (Soybean oil, %)	B (Lecithin, %)	C (Oleic acid, %)	D (Glycerin, %)
1	9.0	1.4	0.30	0.20
2	10.0	1.6	0.32	0.25
3	11.0	1.8	0.34	0.30

The % stands for the mass percentage of this substance in the prescription

Figure 1: Manufacturing process of GB lipid microemulsion.

of 1.33 at 25°C where the refractive indices of soybean oil, oleic acid, refined egg yolk lecithin (PC98T), and glycerol are 1.474, 1.377, 1.405 and 1.474, respectively. All samples were diluted 100 times with glycerine (2.25%) at 25°C. All experiments mentioned above were performed at least three times in order to get creditable values.

2.4 Method for determination of GB in lipids

HPLC-ELSD system was used to determine the concentration of GB in lipid microspheres. First, 200 µL of GB was dissolved in 10 mL methanol for breaking

emulsion. They were then filtered by a 0.45 µm membrane filter; 10 µL aliquot of the supernatant fluid of broken GB was injected into HPLC-ELSD system, a Diamonsil C18 column (250 mm×4.6 mm, 5µm) was employed to separate samples, the ratio of mobile phase was methanol: tetrahydrofuran : water = 25 : 10 : 65, with the flow rate 1.0 mL/min. All HPLC analyses were performed at (30±1)°C, the drift tube temperature was 40°C, the carrier gas pressure was 2.5 bar, Gain 7. The retention time was 12. 618 min.

GB (15 mg) was accurately weighed and dissolved in 50 mL methanol as a stock solution (the concentration of GB about 0.3 mg/mL). The mother liquid was diluted 8 mL, 7 mL, 5 mL and 3 mL to 10 mL by methanol. The concentration of GB was 0.24 mg/mL, 0.21 mg/mL, 0.15 mg/mL and 0.09 mg/mL. Based on GB standard solutions, the working calibration curve showed visible linearity within the range of 0.9-2.5 mg. The regression line was approximately $Y=1.6677X+1.9786$ ($R^2 = 0.9998$, $n = 5$), where X was the logarithm of concentration and Y was the logarithm of the peak area.

2.5 Pharmacodynamics studies

Sprague–Dawley rats weighing 200±20 g were supplied by Peking Union Medical College Hospital Laboratory Animal Center (Beijing, China). 12 rats, both male and female, were randomly separated into GB-LM group and GB commercial product group. The rats were fasted for 12 h and had free access to water before dosing. The GB-LM and GB commercial product were injected intravenously at a dose of 4 mg/kg. All animal care was directed according to the Guiding Principles in the Use of Animals in Toxicology by the Society of Toxicology. Serial blood samples (0.5mL) were collected from the eye socket vein at seven specified time intervals after dosing, and all were placed into heparinized tubes. Another sample of the rat plasma were centrifuged at 3,000 rpm for 10 min. The plasma supernatant (0.5 mL) was mixed with 50 µL of hydrochloric

acid (1 mol/L), and plasma samples were frozen at -20°C until analysis. Plasma samples (100 μL) were mixed with 10 μL per ml of fluconazole solution as the standard, 1.0 mL of ethyl acetate was added, vortexed for 2 min and centrifuged at 5000 rpm for 5 min. Supernatants (800 μL) were collected, passed through a 0.45 μm membrane filter and evaporated to dryness at 40°C under nitrogen gas. The residues were reconstituted in 200 μL acetonitrile and the concentrations of GB in plasma samples were analyzed by LC-MS.

LC-MS was implemented on a Shimadzu 8050 system attached to a Shimadzu 30AD LC system and an 8050 triple quadrupole mass spectrometer (Shimadzu Corporation UFLC XR, Kyoto, Japan). A Shimadzu LC-30AD series chromatographic system consisted of two LC-30A binary pumps, a SIL-30AC autosampler, a DGU-20A3 degasser, SPD-M30A Diode Array Detector and a CTO-20A column oven.

Chromatographic separation of GB in plasma samples was achieved on a Phenomenex Kinetex® C18 column (100 $\times$ 2.1 mm, 2.6 μm). The column temperature was set to 30°C . Mobile phase consists of acetonitrile and 0.2% formic acid at a ratio of 35: 65.

The triple quadrupole mass spectrometer operated under multiple reaction monitoring mode for quantitative and qualitative analysis. The optimized electrospray ionization condition was: gas flow 10 L/min, gas temperature 400°C , sheath gas flow 10 L/min, sheath gas temperature 300°C , capillary voltage 3000 V and nozzle voltage 1800 V.

2.6 Brain tissue distribution studies

Six rats were fasted for 12 hours but had water before the treatment. The GB-LM were injected intravenously at 4 mg/kg via the femoral vein. Rats were euthanized at 0.5 and 2 h after dosing. The brain tissues were separated and washed with saline and then were weighed and homogenized in saline solution (100 mg/mL). The homogenates (100 μL) were mixed with 10 μL of fluconazole (10 $\mu\text{g}/\text{mL}$) solution as an internal standard, 50 μL of methanol was added, vortexed for 1 min and centrifuged at 10000 rpm for 5 min. Supernatants (100 μL) were collected the tissue homogenates were stored at -20°C .

2.7 Pharmacokinetic data analysis

The analysis used program WinNonlin version 4.1 (Pharsight Co.), which used the WinNonlin method to

calculate AUC_{0-240} (integral of the time concentration curve between 0 min and 240 min), Mean Residence Time (MRT), half-life ($t_{1/2}$), steady state volume of distribution (Vss), total clearance (Cl), distribution half-life ($t_{1/2\alpha}$), elimination half-life ($t_{1/2\beta}$), elimination rate constant from central compartment (K_{10}), rate constant for drug transfer from central to peripheral compartment (K_{12}), rate constant for drug transfer from peripheral to central compartment (K_{21}). The above data were presented as the mean $\pm$ standard derivation. Analysis of variance was employed to estimate the pharmacokinetic difference between GB-LM and the commercial product.

3 Results

3.1 The effects of homogenization pressure and temperature on the preparation of GB-LM

In order to explore the effects of the preparation method on the physicochemical properties of GB-LM, the GB-LM were prepared with the following composition: soybean oil (10.0%), GB (0.2%), PC98T (1.8%), oleic acid (2.4%) and glycerol (2.25%). The effect of homogeneous pressure on the GB-LM preparation is shown in Table 2 the effect of homogeneous temperature on the GB-LM preparation is shown in Table 3. The results showed that a higher pressure and temperature of homogenization led to a decrease in both particle size and PDI, respectively.

With the increasing pressure in homogenization, the pressure compresses the structure of the emulsion membrane, making the particle size smaller, and dispersing the grease microemulsions in the solvent, the interfacial tension between oil phase and water phase declines, so do PDI and the diameter of the particles. Considering the feasibility of production and the duration of equipment, homogeneous pressure was set at 1000 bar. The homogeneous temperature study indicates that high temperature can decrease the particle diameter of lipid microemulsion but destroy the stability of GB. Because Ginkgolide B is a diterpene lactone compound, the molecule contains multiple reducing hydroxyl functional groups that are thermally unstable; higher temperature can make the structure of emulsified membrane become looser and looser, resulting in rupture, therefore, the temperature should be between $38-40^{\circ}\text{C}$.

Table 2: Effect of homogeneous pressure on the preparation of GB-LM.

Pressure (bar)	Size (nm)	PDI
700	225.9	0.147
750	220.2	0.132
800	210.7	0.117
900	198.4	0.102
1000	185	0.091

Table 3: Effect of mixing temperature on preparation of GB-LM.

Temperature (°C)	Size (nm)	PDI
25	197.3	0.131
30	188.2	0.084
40	183.7	0.079

Table 4: Effect of cycle number of homogenization on the GB-LM preparation.

Homogenization times	Size(nm)	PDI
1	672.3	0.437
2	321.2	0.214
3	200.9	0.142
4	195.3	0.129
5	190.3	0.111
6	191.2	0.101
7	186.3	0.087
8	185.0	0.091

3.2 The effect of cycle number of homogenization on the GB-LM preparation

GB-LM was prepared as previously described, we studied the effect of homogenization times on the production of GB-LM (Table 4). As homogenization times increases, PDI and the diameter decreases at 1st-3rd times. The decreasing speed slows down at 3rd-8th times, and the data satisfies the requirement at the 7th time. However, we choose the 8th time, as later experiment shows that it meets the stability requirement.

Table 5: The results of orthogonal experimental design for GB-LM.

Level	Factors				S
	A	B	C	D	
1	1	1	1	1	472.8
2	1	2	2	2	283.5
3	1	3	3	3	399.4
4	2	1	2	3	245.6
5	2	2	3	1	385.1
6	2	3	1	2	190.2
7	3	1	3	2	416.2
8	3	2	1	3	435.8
9	3	3	2	1	303.6
K	K1	385.233	378.200	366.267	387.167
	K2	273.633	368.133	277.567	296.633
	K3	385.200	297.733	400.233	360.267
Range		111.600	80.467	122.666	90.534

Table 6: Optimum formulation of GB-LM.

Material name	prescription
Soybean oil	100g
GB	2g
PC98T	18g
Oleic acid	3.2g
Glycerol	2.5g
HCL	appropriate amount
Aquae pro injectione	1000ml

3.3 Formulation optimization by orthogonal experiment design

Orthogonal experimental design is used for studying the effect of multiple factors at different levels. It takes representative samples; these samples have evenly distributed discrete factors. Orthogonal experimental design is an effective, fast, economical test design [21,22].

On the basis of univariate analysis, orthogonal experiment design to optimize the formulation and the results were obtained as presented in Table 5. K is calculated, which is the average of one certain level with a certain factor; Range is the extremum, which is the largest K minus the smallest K. The sequence of effects on composite grade to evaluate the preparation was

Table 7: Stability of fat emulsion stored at room temperature for three months.

storage time (months)	Size (nm)	PDI	Zeta potential (mV)	GB content (%)
0	185.9±52.5	0.091±0.023	-19.8 ± 1.3	93.1±0.5
3	191.6±42.1	0.108±0.006	-26.5 ± 4.2	90.2±1.9

Table 8: Pharmacokinetic parameters of GB after intravenous administration of GB-LM and the commercial product at a dose of 4 mg/kg as GB in rats.

Pharmacokinetic parameter	GB-LM	The commercial product
AUC _{0-240min} (min·μg·ml ⁻¹)	0.365±0.12*	0.232±0.18
MRT(mean residence time) (h)	1.384±0.39*	0.665±0.43
C _{max} (ng·ml ⁻¹)	353.8	489.7
T _{max} (h)	0.08	0.08
T _{1/2α} (h)	0.127±0.036	0.075±0.05
T _{1/2β} (h)	1.506±0.43*	0.780±0.51
K ₁₀ (h ⁻¹)	1.312±0.81*	4.542±2.41
K ₁₂ (h ⁻¹)	3.229±4.58	6.142±3.59
K ₂₁ (h ⁻¹)	3.972±5.67	3.292±1.09
Vss (L)	1.977±1.2*	2.826±1.46
CL (L·h ⁻¹)	2.594±0.25*	3.885±0.47

Each value represents the mean ± S.D. (n=8). * p<0.05 compared with the commercial product

A>B>C>D. The optimal composition was A₂B₃C₂D₂, which means 10.0% soybean oil, 1.8% lecithin, 0.32% oleic acid and 0.25% glycerol (Table 6).

Based on the results obtained, the optimal preparation and critical process parameters can be summarized as follows: the oil phase was set by adding GB (2.0%), PC98T (1.8%) and oleic acid (0.32%) into soybean oil 10.0%, stirring until dissolved at 50-60°C, nitrogen protection; The aqueous phase was prepared by dissolving glycerol (0.25%) in distilled water. Then the oil phase was poured into aqueous phase at 3000-10000 rpm high-speed mixing for 10 min to form the coarse emulsion. The resultant coarse emulsion passed through a homogenizer for one cycle at 200bar, eight cycles at 1000bar to obtain the final emulsion. The pH was adjusted to 6.8, passed through a 0.45 μm membrane filter and sterilized at 125°C for 8 min.

As a result, the average particle size, polydispersity index and zeta potential of the final formulation were 185.9±52.5nm, 0.091±0.023 and -19.8±1.3mV, respectively

Figure 2: Zeta potential and particle size of optimized GB lipid emulsion. Each value represents the average ±S.D. (n=3).

(Table 7, Figure 2). The optimal preparation parameters mentioned above did not differ significantly even after storage at room temperature for three months, the content of GB was only slightly decreased by 2.9%. These results indicate that GB-LM was stable.

3.4 Study on pharmacokinetics of rats in vivo

The pharmacokinetic parameters of GB were determined by intravenous administration of GB-LM and the in-market products at a dose of 4 mg/kg as GB to rats. Figure 3 and Table 8 showed the mean plasma concentration time profiles and the pharmacokinetic parameters of GB. The total plasma concentrations with the lipid micro emulsion formulations were considerably higher than with the commercial product. In particular, the AUC of GB from the lipid micro emulsion was about 1.57-fold higher than that from the commercial product, which indicates that GB had greater bioavailability compared to the other one. In addition, the T_{1/2α} and T_{1/2β} in the GB group were 0.127±0.036 min and 1.506±0.43 min respectively, which were 69.3% and 93.1% longer than those of the

Figure 3: Plasma concentration-time profiles of GB. GB-LM (○) and the commercial product (●). Each value represents the mean $\pm$ S.D. (n = 8).

Figure 4: The concentration of GB in the brain tissues distribution.

commercial product group, respectively. It was clear that incorporating GB into lipid microemulsion resulted in a significant alteration of the pharmacokinetic parameters such as a marked increase in $AUC_{0-240min}$, MRT, plasma half-life and elimination rate (2.594 L/h vs 3.885 L/h). Thus, the enhanced bioavailability of drug from such lipid microemulsion could be beneficial to its therapeutic effect.

The result shows that the AUC, MRT, $T_{1/2\beta}$, K_{10} , V_{ss} , and CL for GB-LM and commercial products differs statistically. In particular, t-test indicates that the in-equivalence between two samples. AUC has a 90% confidence interval between 143% and 182%. The relative bioavailability of GB-LM is $158\% \pm 28\%$.

3.5 Brain tissue distribution

The GB-LM were injected intravenously at a dose of 4 mg/kg via the femoral vein. The brain tissues were dissected respectively at 0.5 and 2 h after dosing, the concentration of GB in the brain tissues were shown in Figure 4. The concentration of GB reached a peak at 0.5h, which indicated GB-LM could pass through the blood brain barrier; the concentration of GB in the brain tissues decreased significantly after 2 h, the MRT was 1.384, these indicate that the drug will not accumulate in the body. With the extension of time, the concentration of the drug in the tissues and organs rapidly decays, and the drug will be quickly removed from the body.

4 Conclusion

In conclusion, the optimized formulation of the lipid microemulsion containing GB was developed using orthogonal experimental design. The average particle size, polydispersity index and zeta potential of the optimized formulation were 185.9 ± 52.5 nm, 0.091 ± 0.023 , and -19.8 ± 1.3 mv, respectively. The formulation was stable for at least three months at room temperature. In addition, GB-LM can pass through the blood brain barrier and reach brain cell. The pharmacokinetic study indicates that it provided a higher AUC and longer terminal half-life value than did the commercial product, indicating that it improved the bioavailability of GB in rats compared with the commercial product. Taken together, the optimized formulation of this lipid microemulsion was a more effective and stable delivery system for GB compared to the existing commercial products.

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