Preparation Process of Hydroxypropyl Tetrahydropyrantriol Liposomes

Chun BAI△, Xin ZHANG△, Jiayi DING, Jing XIONG, Tingting LIU, Hua JIN, Fang YANG, Peijue CHEN, Lili HE*

College of Pharmacy, Southwest Minzu University, Chengdu 610041, China

Abstract [**Objectives**] To explore the optimal process for preparing hydroxypropyl tetrahydropyrantriol liposomes. [**Methods**] A refractive index method was used to determine the content of hydroxypropyl tetrahydropyrantriol. Using particle size distribution and encapsulation rate as evaluation indicators, the effects of hydration time, ratio of organic phase to aqueous phase, granulation method, as well as thin film dispersion and reverse evaporation methods on liposomes preparation were investigated, and the optimal preparation method was selected. Single factor experiments were used to screen the drug phospholipid ratio, ultrasound time, and phospholipid cholesterol ratio, and the preparation process was optimized through orthogonal experiments. [**Results**] The optimal process of preparing hydroxypropyl tetrahydropyrantriol liposomes was as below: 1∶10 of drug phospholipid ratio, 6 min of ultrasound time, 4∶1 of phospholipid cholesterol ratio, (60.94% ±7.24%) of entrapment efficiency, (86.44 ±6.08) nm of particle size, (0.195 ±0.077) of PDI. [**Conclusions**] The optimal preparation process of hydroxypropyl tetrahydropyrantriol liposomes selected by orthogonal experiment could effectively improve the encapsulation efficiency of hydroxypropyl tetrahydropyranotriol and reduce particle size. Moreover, the method was stable and reliable.

Key words Hydroxypropyl tetrahydropyrantriol, Reverse evaporation, Liposome, Orthogonal design, Preparation technology

1 Introduction

Hydroxypropyl tetrahydropyrantriol is a xylose derivative with anti-aging activity, and its chemical structure is shown as Fig. 1. Previous studies have shown that hydroxypropyl tetrahydropyrantriol can stimulate the synthesis of mucopolysaccharides (GAGs) and promote the production of collagen and hyaluronic acid[1-2]. It can also promote the close connection between the dermis and epidermis, help maintain the elasticity of the dermis, and prevent skin aging. Moreover, it will not accumulate in organisms, is easy to biodegradate, and is non-toxic[3]. Hydroxypropyl tetrahydropyrantriol is a water-soluble component with a large molecular weight, only a small amount can enter the dermis, and it is easily decomposed by related enzyme proteins in the skin[4]. Therefore, a method is needed to improve the bioavailability of hydroxypropyl tetrahydropyrantriol.

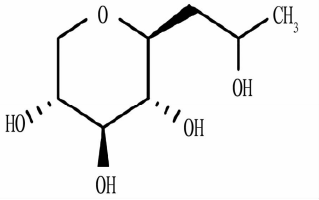

Fig. 1 Chemical structure of hydroxypropyl tetrahydropyrantriol

Liposome is a microcapsule formed by encapsulating drugs within a lipid like bilayer. Liposomes can encapsulate lipophilic and water-soluble drugs, similar in structure to biofilms. Therefore, liposomes have strong affinity with cell membranes, which can increase the ability of encapsulated drugs to penetrate cell membranes and enhance therapeutic effects. At the same time, it can also reduce the rate of drug elimination, extend the action time, and improve drug stability. Therefore, preparing hydroxypropyl tetrahydropyrantriol into liposomes can not only increase its stability, but also improve its bioavailability and expand its application range.

2 Materials

2.1 Reagents Hydroxypropyl tetrahydropyrantriol (content: 98.49%, CAS: 439685-79-7, Chengdu Yunxi Chemical Co., Ltd.); soy lecithin (CAS: 8002-43-5, Chengdu Kelong Chemical Co., Ltd.); cholesterol (CAS: 57-88-5, Chengdu Kelong Chemical Co., Ltd.); chloroform (CAS: 67-66-3, Chengdu Kelong Chemical Co., Ltd.); PBS buffer (dry powder, lot No.: P1010).

2.2 Instruments Laser particle size analyzer (Nano ZS, Malvern Instruments Co., Ltd., England); rotary evaporator (RE-5203, Zhengzhou Bilang Instrument Co., Ltd.); ultrasonic cell pulverizer (JY96-IIN, Ningbo Xinzhi Biotechnology Co., Ltd.); constant temperature cultivation vibrator (ZWY-240, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.); 1/10 000 electronic analytical balance (LT224D, Jiangsu Changshu Tianliang Instrument Co., Ltd.); centrifuge (H2050R Changsha Xiangyi Centrifuge Instrument Co., Ltd.); Abbe refractometer (WZS-1, Shanghai Optical Instrument Factory).

Received: May 4, 2023 Accepted: July 10, 2023

Supported by Innovation and Entrepreneurship Training Program Project of Southwest Minzu University (S202210656134); Project of Sichuan Department of Science and Technology (2022NSFSC1437); Project of Sichuan Administration of Traditional Chinese Medicine (2021MS121).

△These authors contributed equally to this paper.

* Corresponding author. E-mail: lilihes@163.com

3 Methods

3.1 Establishment of content analysis method of hydroxypropyl tetrahydropyrantriol In the experiment, the linear relationship between the refractive index of hydroxypropyl tetrahydropyrantriol solution and its concentration was used. By measuring the refractive index of hydroxypropyl tetrahydropyrantriol at different concentrations, standard curve and regression equation were formed to determine hydroxypropyl tetrahydropyrantriol at unknown concentrations[5], thereby determining the encapsulation efficiency of hydroxypropyl tetrahydropyrantriol liposomes.

3.1.1 Establishment of standard curve. 25 mg of hydroxypropyl tetrahydropyrantriol standard sample was weighed accurately, and PBS solution was used to fix the volume to 5 mL, thereby obtaining 5 mg/mL of hydroxypropyl tetrahydropyrantriol solution. 100, 300, 500, 700, 900, and 1 100 μL of hydroxypropyl tetrahydropyrantriol solution was taken accurately, and PBS solution was used to fix the volume to 5 mL, thereby obtaining a series of sample solutions (0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 mg/mL). Abbe refractometer was used to measure the refractive index of the above concentration solution at 20 ℃, and each sample was read three times, and the mean was taken.

3.1.2 Repeatability test. According to the preparation method of Section **3.1.1**, three lots of hydroxypropyl tetrahydropyrantriol solutions (0.3, 0.5, 0.7 mg/mL) were prepared. Three parallel measurements were performed, and the refractive index of each sample solution was recorded, and the relative standard deviation was calculated.

3.1.3 Precision test. 300, 500, and 700 μL of hydroxypropyl tetrahydropyrantriol solution (5 mg/mL) was taken, and PBS solution was used to fix the volume to 5 mL, and 6 sets of parallel samples were made separately. By the detection method of Section **3.1.1**, the refractive index of each sample solution was measured and recorded separately, and the relative standard deviation was calculated.

3.1.4 Stability test. According to the preparation method of Section **3.1.1**, 0.5 mg/mL of hydroxypropyl tetrahydropyrantriol sample solution was prepared. It was set for 0, 2, 4, 6, 8, and 10 h at room temperature, and the refractive index of each sample solution was measured and recorded separately, and the relative standard deviation was calculated.

3.1.5 Sample recovery rate test. 6 portions of test solutions with known content were accurately taken and added in the reference solution to the volumetric flask, and PBS solution was added to fix the volume. According to the detection method of Section **3.1.1**, the refractive index of each sample solution was measured and recorded separately, and the average recovery rate and relative standard deviation of the reference substance were calculated.

3.1.6 Determination of encapsulation efficiency. Encapsulation efficiency is an important evaluation index for liposome delivery systems. At present, the common methods for determining the encapsulation efficiency of water-soluble drugs include ultracentrifugation, dialysis, ultrafiltration centrifugation, *etc*[6]. This experiment used ultrafiltration centrifugation to determine the encapsulation efficiency of hydroxypropyl tetrahydropyrantriol liposomes.

Pretreatment of ultrafiltration centrifuge tube (0.5 mL 100 kD): the new ultrafiltration centrifuge tube needed to be soaked in distilled water before use to wet the filter membrane. Then, 40 mg/mL of hydroxypropyl tetrahydropyrantriol solution was filtered, and the adsorption of membrane was saturated. It was washed with distilled water (do not allow the membrane to dry again), and set aside for use[7].

Ultrafiltration centrifugation method: 0.4 mL of hydroxypropyl tetrahydropyranotriol liposome was transferred into a pre treated ultrafiltration centrifuge tube, and the lid of the centrifuge tube was tightened. The ultrafiltration tubes were placed symmetrically in a centrifuge to centrifuge[8] at a speed of 10 000 rpm for 30 min, with a controlled temperature of 4 ℃. After centrifugation, the lower filtrate was collected.

The concentration of hydroxypropyl tetrahydropyranetriol in the filtrate of different samples after ultrafiltration was measured on the Abbe refractometer. Each sample was measured three times, and the mean was taken. Based on the plotted standard curve, the concentration of free drugs was determined.

Encapsulation efficiency (EE)% = [Total amount of drug − Free drug dosage)/Total amount of drug] × 100

3.2 Screening of preparation conditions of hydroxypropyl tetrahydropyranetriol liposomes

3.2.1 Hydration time. When phospholipids combine with aqueous phase, the rearrangement and orientation of molecules occur, and the formation of lipid bilayer[9] is a key step in the preparation of liposomes. In order to achieve complete hydration of phospholipids and hydroxypropyl tetrahydropyranetriol, the particle size was controlled to make them uniform. The hydration time was controlled for 20, 40, and 60 min, respectively. The effect of hydration time on the particle size distribution of liposomes was explored, and the optimal hydration time was selected.

3.2.2 Ratio of organic phase to aqueous phase. That was volume ratio of chloroform to PBS aqueous solution. The ratio of organic phase to aqueous phase was controlled as 2∶1, 3∶1, 4∶1, to explore the effect of the ratio of organic phase to aqueous phase on particle size distribution, and select the optimal ratio of organic phase to aqueous phase.

3.2.3 Granulation method. Particle size is an important part of liposome characterization. By comparing the effects of ultrasound granulation and microporous membrane filtration granulation on the particle size distribution of liposomes, a more optimal granulation method was selected. Ultrasound granulation of the prepared liposome suspension was conducted for 10 min (5 sec on, 5 sec off) to investigate the feasibility of ultrasound granulation steps. The prepared liposome suspension was filtered by 0.22 μm of microporous filter membrane to explore the feasibility of the filtration steps using microporous membrane filtration.

3.3 Methodology study of preparation of hydroxypropyl tetrahydropyranetriol liposomes Liposomes were prepared using

thin film dispersion method and reverse evaporation method. Moreover, the particle size, PDI, Zeta potential, and encapsulation efficiency of blank and drug loaded liposomes by both methods were investigated to select the optimal preparation method[10].

3.3.1 Preparation of blank liposomes. Blank liposomes were prepared using thin film dispersion method. 40 mg of lecithin and 10 mg of cholesterol were taken to dissolve in 3 mL of chloroform, then it was poured into a 100 mL of round-bottom flask. Use a rotary evaporator, the organic solvent was removed at 40 ℃ and 80 rpm, and a yellowish film was formed on the round bottom flask. After that, 1 mL of PBS (pH = 7.2) aqueous solution was added to continue to rotate and hydrate for 10 min. It was transferred to a constant temperature culture vibrator, and then hydrated for 30 min at 40 ℃ and 120 rpm, until the film was fully hydrated, forming a light yellow suspension. Finally, the granulation was conducted by ice bath ultrasound for 10 min (5 sec on, 5 sec off) at a power of 30%.

Blank liposomes were prepared using reverse evaporation method. 40 mg of lecithin and 10 mg of cholesterol were taken to dissolve in 3 mL of chloroform to form an organic phase, and 1 mL of PBS solution without drugs was added. After ultrasound in an ice bath at 30% power on a cell grinder for 8 min (5 sec on, 5 sec off), a rotary evaporator was used to remove the organic solvent by reduction vaporization at 40 ℃, 80 rpm until no bubbles were generated. Then, 2 mL of PBS solution without drugs (pH = 7.2) was added, to continue to rotate and evaporate for 10 min. After that, it was transferred to a constant temperature culture shaker and hydrated at 40 ℃ and 120 rpm for 30 min to form a uniform aqueous solution. Finally, the granulation was conducted by ice bath ultrasound for 10 min (5 sec on, 5 sec off) at a power of 30%.

3.3.2 Preparation of drug loaded liposomes. Drug loaded liposomes were prepared using thin film dispersion method. 40 mg of lecithin and 10 mg of cholesterol were taken to dissolve in 3 mL of chloroform, and it was poured into a 100 mL round-bottom flask. Using a rotary evaporator, the organic solvent was removed at 40 ℃ and 80 rpm, and a yellowish film was formed on the round-bottom flask. After adding 1 mL of hydroxypropyl tetrahydropyranetriol solution (8 mg/mL), it continued to rotate and hydrate for 10 min. Then, it was transferred to a constant temperature culture vibrator, and hydrated for 30 min at 40 ℃ and 120 rpm, so that the two can fully react. The film was fully hydrated to form a light yellow suspension. Then, granulation was conducted by ice bath ultrasound for 10 min (5 sec on, 5 sec off) under the power of 30%, and the ultrasound treated liposomes were brought to a constant volume of 5 mL.

Drug loaded liposomes were preparaed using reverse evaporation method. 40 mg of lecithin and 10 mg of cholesterol were taken to dissolve in 3 mL of chloroform to form an organic phase, and 1 mL of hydroxypropyl tetrahydropyranetriol solution (8 mg/mL) was added. After ultrasound in an ice bath at 30% power on a cell grinder for 8 min (5 sec on, 5 sec off), a rotary evaporator was used to remove the organic solvent by reduction vaporization at 40 ℃ and 80 rpm until no bubbles were generated. 2 mL of PBS solution without drugs was added, and it continued to rotate and evaporate for 10 min. Then, it was transferred to a constant temperature culture shaker and hydrated at 40 ℃ and 120 rpm for 30 min to form a uniform aqueous solution. Finally, the granulation was conducted by ice bath ultrasound for 10 min (5 sec on, 5 sec off) at a power of 30%, and the ultrasound treated liposomes were brought to a constant volume of 5 mL.

3.4 Optimization of preparation process through single factor and orthogonal experiments

3.4.1 Preparation method of liposomes. The liposome was prepared by reverse evaporation method. A certain amount of lecithin and cholesterol were weighed and dissolved in 3 mL of chloroform to form an organic phase. 1 mL of PBS solution with a certain drug concentration was added. The ice bath ultrasound was performed on a cell grinder at 30% power for a certain time (5 sec on, 5 sec off). Then, the rotary evaporator was used to remove the organic solvent by decompression evaporation at 40 ℃ and 80 rpm until no bubbles were generated. After adding 2 mL of drug-free PBS solution, it continued to rotate and evaporate for 10 min. Then, it was transferred to a constant temperature culture shaker, and hydrated at 40 ℃ and 120 rpm for 30 min to form a uniform aqueous solution. Finally, granulation was conducted by ice bath ultrasound for 10 min (5 sec on, 5 sec off) under 30% power, and the ultrasound treated liposomes were brought to a constant volume of 5 mL.

3.4.2 Drug phospholipid ratio. The amount of lecithin was fixed at 40 mg, and the ultrasound time was controlled to 8 min, and the phospholipid cholesterol ratio was adjusted to 4 ∶ 1. According to the method of Section **3.4.1**, the liposomes were prepared. The effect of drug phospholipid ratio of 1 ∶ 10, 2 ∶ 10, 3 ∶ 10 on the particle size distribution and encapsulation efficiency of liposomes was investigated.

3.4.3 Ultrasound time. The drug phospholipid ratio was fixed as 1 ∶ 10, and phospholipid cholesterol ratio was controlled as 4 ∶ 1. According to the method of Section **3.4.1**, the liposomes were prepared. The effect of ultrasound time of 2, 4, 6, 8, and 10 min on the particle size distribution and encapsulation efficiency of liposomes was investigated.

3.4.4 Phospholipid cholesterol ratio. Lecithin and cholesterol are the main raw materials for liposomes. Lecithin and cholesterol are separated from each other and arranged in a directional manner to form a lipid membrane. Cholesterol can alter the arrangement order and fluidity of phospholipids in the lipid bilayer.

The amount of lecithin was fixed at 40 mg, and the drug phospholipid ratio was controlled to 1 ∶ 10, and the ultrasound time was set to 8 min. According to the method of Section **3.4.1**, the liposomes were prepared. The effect of phospholipid cholesterol ratio at 1 ∶ 1, 2 ∶ 1, 3 ∶ 1, 4 ∶ 1, 5 ∶ 1 on the particle size distribution and encapsulation efficiency of liposomes was investigated.

3.4.5 Orthogonal experiment. According to experiment results,

two levels were selected from three factors (A drug phospholipid ratio, B ultrasound time, C phospholipid cholesterol ratio), and $L_6(2^3)$ was used to conduct orthogonal experiment. Based on particle size distribution and encapsulation efficiency, the optimal process for preparing hydroxypropyl tetrahydropyranotriol liposomes was selected, and factor and level were shown as Table 1.

Table 1 Orthogonal design for preparation of hydroxypropyl tetrahydropyranotriol liposomes

Level	Drug phospholipid ratio A	Ultrasound time B	Phospholipid cholesterol ratio C
1	1∶10	6	4∶1
2	2∶10	8	3∶1

4 Results and analysis

4.1 Establishment of an analytical method for the content of hydroxypropyl tetrahydropyranotriol According to Section **3.1.1**, the standard regression equation was obtained: $y = 0.001\,252x + 1.337\,2$ ($R^2 = 0.998\,6$), with good linear relationship. According to Section **3.1.2**, repeatability test was conducted. The *RSD* values of samples from different batches were all less than 2%, indicating good repeatability in the preparation of samples from different batches. According to Section **3.1.3**, precision test was conducted. The *RSD* values of the refractive index obtained by continuous injection were all less than 2%, indicating that the detection method had good precision. According to Section of **3.1.4**, stability test was conducted. The *RSD* values of the refractive index obtained from the same sample solution at 0, 2, 4, 6, 8, and 10 h were less than 2%, indicating good stability of the test solution within 10 h. According to Section **3.1.5**, sample recovery rate test was conducted. The recovery rate of hydroxypropyl tetrahydropyranotriol was between 97.60% and 101.82%, and the *RSD* value was less than 2%, indicating that the recovery rate of this method was relatively good.

4.2 Screening of liposomes preparation conditions

4.2.1 Hydration time. Seen from Table 2, the particle size and PDI decreased as the hydration time increased, and there was no significant difference in the particle size and PDI under 40 and 60 min of hydration operations. From an economic and resource perspective, hydration for 40 min was chosen.

Table 2 Effects of hydration time on particle size and PDI

Hydration time//min	Particle size//nm	PDI
20	138.6 ± 6.33	0.231 ± 0.063
40	135.9 ± 7.12	0.200 ± 0.051
60	130.1 ± 5.43	0.199 ± 0.042

4.2.2 Ratio of organic phase to aqueous phase. Seen from Table 3, the change in the ratio of organic phase to aqueous phase can affect particle size distribution. As the ratio of organic phase to aqueous phase increased, the particle size and PDI first decreased and then increased, and reached the minimum at 3∶1. Therefore, the ratio of organic phase to aqueous phase was 3∶1.

Table 3 Effects of VO/VW on particle size and PDI

Ratio of organic phase to aqueous phase	Average particle size//nm	PDI
2∶1	191.33 ± 5.23	0.340 ± 0.054
3∶1	177.10 ± 6.41	0.296 ± 0.044
4∶1	221.65 ± 4.46	0.395 ± 0.061

4.2.3 Granulation method. Seen from Table 4, after 10 min of ice bath ultrasound at 30% and 45 W power, the particle size of liposomes decreased, and their distribution became more uniform. The microporous filter membrane filtration had almost no effect on the particle size and PDI of liposomes. Therefore, ultrasonic granulation was required in the preparation process.

Table 4 Effects of different granulation methods on particle size and PDI

Granulation method	Particle size//nm	PDI
Blank control group	137.5 ± 6.22	0.218 ± 0.049
Ultrasonic granulation	80.75 ± 5.36	0.206 ± 0.055
Microporous filter membrane granulation	133.8 ± 7.31	0.196 ± 0.044

The final preparation conditions for hydroxypropyl tetrahydropyranotriol liposomes were as follows: hydration time of 40 min, organic phase to aqueous phase ratio of 3∶1, ultrasound granulation for 10 min (5 sec on, 5 sec off).

4.3 Methodology screening for liposomes preparation Seen from Table 5, the particle size of liposomes by the reverse evaporation method was smaller than that of the thin film dispersion method, making it more suitable for transmembrane absorption. Smaller PDI indicated more stable physical properties. Moreover, the reverse evaporation method carried a large amount of net charge and had a greater electrostatic repulsion force, making it more stable, less prone to settling, and less prone to accumulation. Meanwhile, it had high encapsulation efficiency, more drugs loaded, and better quality[10]. Therefore, considering both particle size distribution and encapsulation efficiency, the reverse evaporation method was chosen as the preparation method for hydroxypropyl tetrahydropyranotriol liposomes.

Table 5 Methodology screening results of liposomes preparation

Sample	Average particle size//nm	Average PDI	Average potential//mV	Entrapment efficiency//%
Blank liposomes by thin film evaporation method	96.22 ± 5.76	0.253 ± 0.058	−20.1 ± 1.27	
Drug loaded liposomes by thin film evaporation method	94.90 ± 4.05	0.227 ± 0.045	−21.0 ± 1.19	31.25 ± 5.22
Blank liposomes by reverse evaporation method	87.63 ± 5.09	0.223 ± 0.060	−21.2 ± 2.01	
Drug loaded liposomes by reverse evaporation method	83.67 ± 5.12	0.221 ± 0.055	−22.3 ± 1.78	54.69 ± 6.42

4.4 Optimization of preparation process through single factor and orthogonal experiments

4.4.1 Drug phospholipid ratio. Seen from Fig. 2, the liposome particle size increased as the drug phospholipid ratio increased, and PDI first decreased and then increased, and the potential increased. When the drug phospholipid ratio was 1 ∶ 10 and 2 ∶ 10, the difference in particle size was not significant, but PDI of 2 ∶ 10 was smaller than that of 1 ∶ 10. When the drug phospholipid ratio was 3 ∶ 10, the reason for the increase in particle size and PDI may be that as the dosage of drugs increased, the lipid membrane cannot completely encapsulate the drugs, and the drugs separated from the lipid membrane, resulting in an increase in particle size.

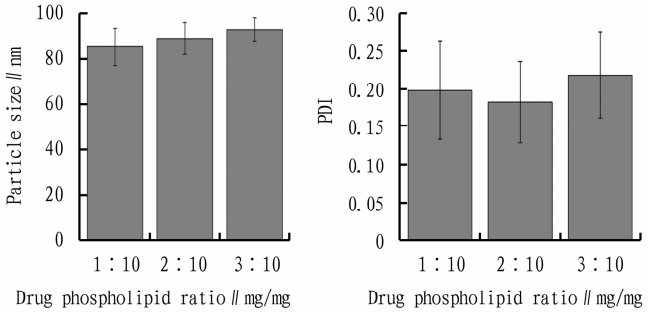

Fig. 2 Effects of drug phospholipid ratio on particle size distribution and PDI

Seen from Fig. 3, the encapsulation efficiency decreased as the drug phospholipid ratio increased. When the drug phospholipid ratio was 1 ∶ 10, the encapsulation efficiency was the highest, which was (65.26% ±6.14%). It can be seen that the internal loading space formed by phospholipid was limited. As the concentration of hydroxypropyl tetrahydropyranotriol increased, the lipid membrane was insufficient to encapsulate hydroxypropyl tetrahydropyranotriol, resulting in an increase in free drugs and a decrease in encapsulation efficiency[11].

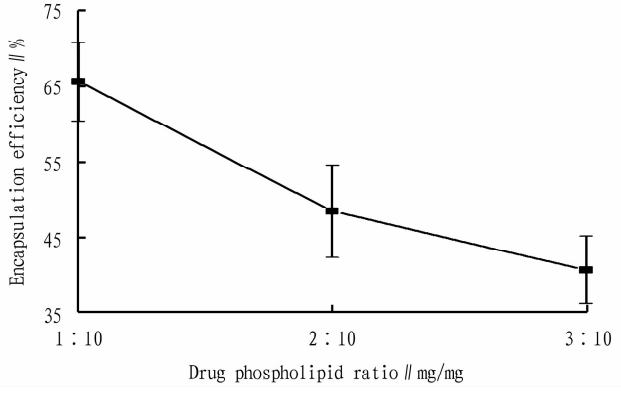

Fig. 3 Effects of drug phospholipid ratio on encapsulation efficiency

4.4.2 Ultrasound time. Seen from Fig. 4, the particle size and PDI of hydroxypropyl tetrahydropyranotriol liposomes first decreased and then increased as the ultrasound time increased from 2 to 10 min, and reached the minimum at 6 min. The reason was that as the ultrasound time increased, the proportion of aqueous phase entering the oil phase increased. At 6 min, the organic phase and aqueous phase combined to form a uniform W/O type of emulsion. With the increase of ultrasound time, some hydroxypropyl tetrahydropyranotriol separated from the oil phase, resulting in an increase in particle size. The potential difference at each ultrasound time was not significant.

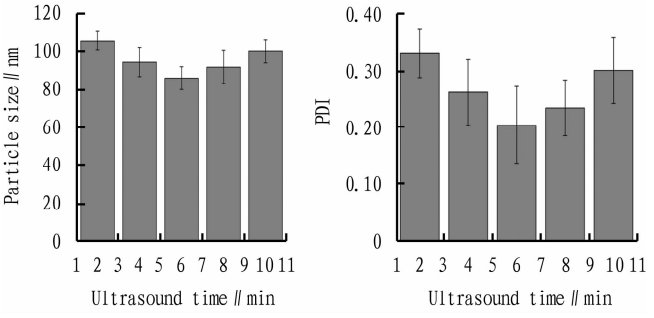

Fig. 4 Effects of ultrasound time on particle size distribution and PDI

Seen from Fig. 5, ultrasound time affected the encapsulation efficiency of hydroxypropyl tetrahydropyranotriol liposomes. When the ultrasound time increased from 2 to 6 min, the maximum encapsulation efficiency was obtained (68.75% ±6.91%). Continuing to increase the ultrasound time, the encapsulation efficiency of liposomes was reduced. When the ultrasound time was less than 6 min, the ultrasound time was too short to form a uniform W/O type of solvent.

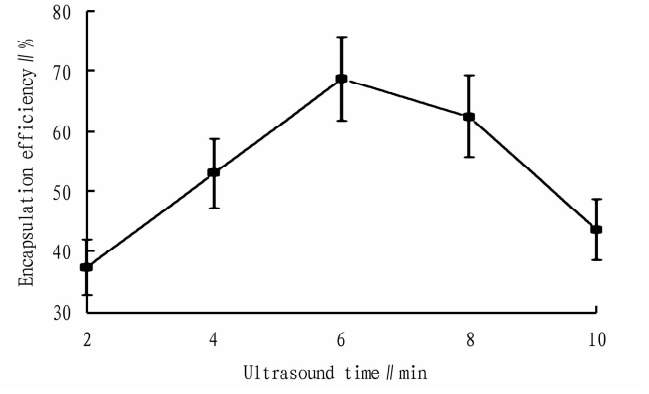

Fig. 5 Effects of ultrasound time on encapsulation efficiency

4.4.3 Phospholipid cholesterol ratio. Seen from Fig. 6, the particle size decreased sharply when phospholipid cholesterol ratio was from 1 ∶ 1 to 2 ∶ 1, and the decrease was slow when phospholipid cholesterol ratio was from 2 ∶ 1 to 5 ∶ 1. When phospholipid cholesterol ratio was 4 ∶ 1 and 5 ∶ 1, there was almost no difference in particle size. PDI first sharply decreased and then gradually increased, and reached the minimum (0.182 ±0.057) when phospholipid cholesterol ratio was 3 ∶ 1. The higher the phospholipid cholesterol ratio during the experiment, the easier it was to hydrate. The possible reason was that the amount of phospholipids used made hydration difficult and easy to agglomerate. As the ratio of phospholipid cholesterol increased, hydration became easier. When the phospholipid drug ratio was 3 ∶ 1, phospholipids and cholesterol formed a stable bilayer[12]. When the phospholipid cho-

lesterol ratio was less than 3 ∶ 1, the lipid bilayer was not closely arranged, forming fewer liposomes, and resulting in larger particle size and PDI[13].

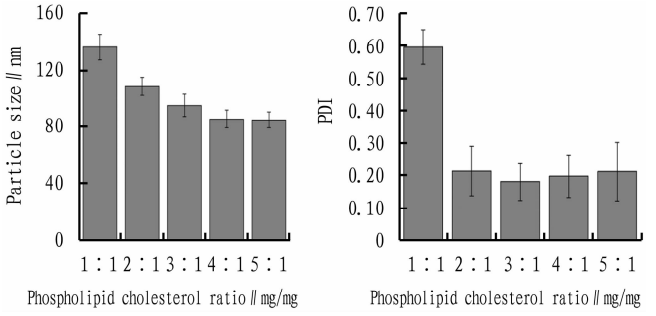

Fig. 6 Effects of phospholipid cholesterol ratio on particle size distribution and PDI

Seen from Fig. 7, the encapsulation efficiency first increased and then decreased as the amount of cholesterol decreased. When pcholesterol cholesterol ratio was 4 ∶ 1, the encapsulation rate was the maximum (65.62% ±6.91%). The reason for the increase in encapsulation efficiency from 1 ∶ 1 to 4 ∶ 1 was that the increase in phospholipids improved the viscosity of the lipid membrane, making the bilayer more stable. But when the ratio increased to 5 ∶ 1, the bilayer was destroyed, and the asymmetry, permeability, and rigidity of the lipid bilayer membrane increased, leading to drug penetration and a decrease in encapsulation efficiency[14].

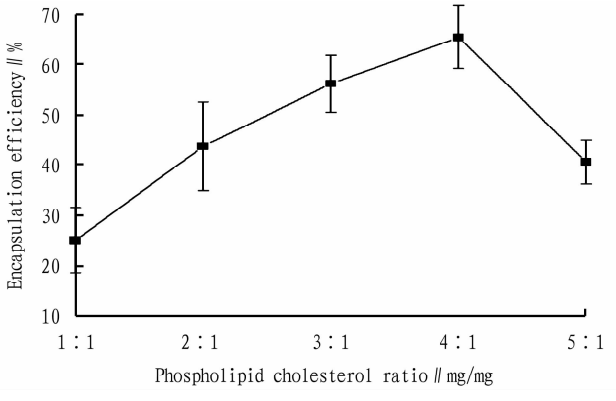

Fig. 7 Effects of phospholipid cholesterol ratio on encapsulation efficiency

4.4.4 Orthogonal experiment. Orthogonal experiment was conducted according to factor and level of Section **3.4.5**, and the results were shown in Table 6 – 7.

Table 6 Results of particle size and PDI in orthogonal experiment

No.	A	B	C	Average particle size // nm	Average PDI
1	1	1	1	86.44 ±6.08	0.195 ±0.077
2	1	2	2	95.12 ±8.34	0.182 ±0.089
3	2	1	2	88.42 ±7.35	0.230 ±0.062
4	2	2	1	85.96 ±7.71	0.183 ±0.107
K_1	181.56	174.85	172.39		
K_2	174.37	181.08	183.54		
R	5.08	4.40	7.88		

Through range analysis of orthogonal experiments, it can be concluded that the influence sequence of various factors on particle size was C > A > B, namely phospholipid cholesterol ratio > drug phospholipid ratio > ultrasound time.

Table 7 Results of encapsulation efficiency in orthogonal experiment

No.	A	B	C	Encapsulation efficiency // %
1	1	1	1	60.94 ±7.24
2	1	2	2	62.50 ±7.51
3	2	1	2	48.44 ±6.32
4	2	2	1	54.69 ±8.19
K_1	115.62	123.44	109.37	
K_2	110.94	103.12	117.19	
R	0.033	0.144	0.055	

Through range analysis of orthogonal experiments, it can be concluded that the influence sequence of various factors on the encapsulation efficiency was B > C > A, namely ultrasound time > phospholipid cholesterol ratio > drug phospholipid ratio.

Considering both particle size and encapsulation efficiency, it was concluded that the optimal process for preparing hydroxypropyl tetrahydropyranotriol liposomes by reverse evaporation method was $A_1B_1C_1$. The optimal preparation process was as follows: drug phospholipid ratio of 1 ∶ 10, ultrasound for 6 min, phospholipid cholesterol ratio of 4 ∶ 1.

5 Discussion

The commonly used preparation methods for liposomes include ethanol injection method, reverse evaporation method, pH gradient method, thin film dispersion method, *etc.* The most commonly used thin film dispersion method is simple to operate, but cannot encapsulate water-soluble drugs in large quantities[15]. The reverse evaporation method is applicable to encapsulating water-soluble drugs. Liposomes with smaller particle sizes and more uniform distribution can be obtained through ultrasound, but the preparation process is more complex than the thin film dispersion method. This experiment used thin film evaporation method to prepare hydroxypropyl tetrahydropyranotriol liposomes, which had low experimental cost and high repeatability. In consideration of the later application of the liposome in cosmetics, its absorption effect and skin irritation are the key points of the main process, so the membrane material is cholesterol and soybean lecithin. When the environmental conditions change (such as temperature, osmotic pressure, pH, *etc.*), cholesterol can play a role in enhancing the structural stability of the liposome[16]. In the single factor experiment, as the ratio of cholesterol to soybean lecithin increased, the encapsulation efficiency of liposomes showed a trend of first increasing and then decreasing. The possible reason is that the excessive content of hydroxypropyl tetrahydropyranotriol in liposomes reduces the surface charge and electrostatic repulsion between the bilateral vesicles of liposomes, leading to a decrease in encapsulation efficiency. The drug phospholipid ratio is also an important

factor affecting the encapsulation efficiency of liposomes. If the ratio of drugs to phospholipids is too small, it will lead to the inability of drugs to be fully encapsulated, which cannot achieve the ideal effect of improving bioavailability. If the ratio is too small, it will cause drug waste. In the preparation of liposomes, ultrasound time mainly affects the stratification of the liposome solution. Under the stable and non layered conditions, the liposome solution is conducive to the formation of W/O type emulsions, which in turn affects the quality of the liposome. As the ultrasound time increased, the encapsulation first increased and then decreased. This may be due to the uneven film formation of liposomes caused by a short ultrasound time, which is prone to rupture. Hydroxypropyl tetrahydropyranotriol also flows out, resulting in a decrease in the encapsulation efficiency of liposomes. If the ultrasonic time is too long, the temperature will rise, causing the degradation of hydroxypropyl tetrahydropyranetriol. Moreover, the high temperature will lead to the increase of energy input, which will destroy the Van Der Waals force between phospholipid molecules[17], thus improving the fluidity of lipid bilayer. Therefore, an appropriate ultrasound time can increase the stability of liposome suspensions. After the ultrasonic process is completed, the mixture also needs to be hydrated, which can make the particle size distribution of liposomes more uniform and prevent the phenomenon of liposome clumping and aggregation.

This experiment initially studied the effects of hydration time, ratio of organic phase to aqueous phase, and granulation method on the particle size distribution of liposomes. Finally, hydration for 40 min, organic phase to aqueous phase of 3 ∶ 1, and ultrasound granulation were selected as the conditions for preparing liposomes. Then, the effects of film dispersion method and reverse evaporation method on the particle size distribution and encapsulation efficiency of liposomes were compared. From the particle size distribution, the film dispersion method was superior to the reverse evaporation method. From the perspective of encapsulation effect, the reverse evaporation method was superior to the thin film dispersion method. So the reverse evaporation method was chosen from these two aspects comprehensively. Next, the reverse evaporation preparation process was optimized. Single factor and orthogonal experiments were conducted to investigate the effects of drug phospholipid ratio, ultrasound time, and phospholipid cholesterol ratio on particle size distribution and encapsulation efficiency. The optimal preparation process was obtained, with a drug phospholipid ratio of 1 ∶ 10, ultrasound time of 6 min, and phospholipid cholesterol ratio of 4 ∶ 1. The prepared liposome appeared as a light yellow transparent and clear liquid, with entrapment efficiency of (60.94% ± 7.24%), particle size of (86.44 ± 6.08 nm), and PDI of (0.195 ± 0.077).

References

[1] JITSUKAWA S, HARA K. A new xylose-derived active cosmetic ingredient for anti-aging: Toward sustainable development[J]. Fragrance Journal, 2006, 34(10): 35.

[2] PINEAU N, BERNERD F, CAVEZZA A, *et al.* A new C-xylopyranoside derivative induces skin expression of glycosaminoglycans and heparan sulphate proteoglycans[J]. European Journal of Dermatology, 2008, 18(1): 36 – 40.

[3] DALKO M, CAVEZZA A, PICHAUD P, *et al.* Pro-xylane, a new xylose-derivative as an activator of glycosaminoglycan biosynthesis[R]. European Academy of Dermatology and Venereology, EADV, Proceedings of the Congress, 15th, Rhodes, Greece, Oct.4-8, 2006(2006): 621 – 625.

[4] Jiangsu Ruibeili Biotechnology Co., Ltd. A method for preparing nanoemulsions to improve the bioavailability of bosin: China (202010629024.9)[P]. 2020-09-18. (in Chinese).

[5] Shanghai Keqin Technology Co., Ltd. A method for determining the concentration of Pro-Xylane using refractive index (CN201911396367.9)[P]. 2020-05-22. (in Chinese).

[6] SUN JL, HOU XL, ZHU LB. Research progress in the entrapment efficiency of Chinese traditional medicine liposomes[J]. Heilongjiang Animal Science and Veterinary, 2017(1): 95 – 97.

[7] LI MY, ZHANG H, MEI XG, *et al.* Preperation and quality evaluation of vincristine sulfate thermosensitive liposomes[J]. Chinese Pharmaceutical Journal, 2014, 49(18): 1615 – 1619. (in Chinese).

[8] WU Q. Preparation and transdermal properties of collagen liposomes[D]. Jilin: Jilin University, 2018. (in Chinese).

[9] YANG CR. Liposome technology and applications[M]. Harbin: Heilongjiang Education Press, 2013. (in Chinese).

[10] XU X, ZHOU QM, FENG WY, *et al.* Comparative study on the preparation of matrine alkaloid transporters using thin film dispersion method and reverse evaporation method[J]. Modernizing Agriculture, 2016(12): 46 – 47. (in Chinese).

[11] WANG RY, JIN Q, LIN H, *et al.* The technical study of grape-seed polyphenol liposomes with reverse phase evaporation[J]. Cereals & Oils, 2019, 32(10): 93 – 96. (in Chinese).

[12] ZHANG L, YUAN Y, LIU CS, *et al.* Preparation of brusine liposomes[J]. Chinese Traditional Patent Medicine, 2005, 27(6): 632 – 637. (in Chinese).

[13] SONG GS, LIU JY, GONG JY, *et al.* Study on the preparation, surface modification and in vitro digestion stability of gardenia yellow liposomes[J]. Journal of Nuclear Agricultural Sciences, 2021, 35(12): 2799 – 2809. (in Chinese).

[14] DENG YJ. Liposome technology[M]. 2nd edition. Beijing: People's Health Publishing House, 2007. (in Chinese).

[15] SHERRYM, CHARCOSSETC, FESSI H, *et al.* Essential oils encapsulated in liposomes: A review[D]. Journal of Liposome Research, 2013, 23(4): 268 – 275.

[16] GONG P, FANG WJ, ZHAO WJ, *et al.* Preparation technology of Ganoderma lucidum polysaccharide liposome[J]. Acta Edulis Fungi, 2022, 29(5): 101 – 108. (in Chinese).

[17] FAN YP, WANG DY, HU YL, *et al.* Preparation condition optimization of astragalus polysaccharide liposome by orthogonal test[J]. Chinese Traditional and Herbal Drugs, 2011, 42(3): 470 – 473. (in Chinese).