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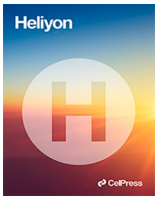

Research article

Pharmacokinetics study of ginsenoside Rg1 liposome by pulmonary administration

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ABSTRACT

Ginsenoside Rg1 (Rg1), a monomer saponin component, is one of the components with the highest content in total saponins of Panaxnotoginseng. It had various pharmacological effects. The bioavailability of oral tablets is only 1–20 %, and it is eliminated quickly in the blood. The development of new dosage forms and new routes of administration of ginsenoside Rg1 with sustained release and high bioavailability has become a significant problem to be solved. The Rg1 liposomes study used a thin film dispersion ultrasound method for its preparation. This study focused the pharmacokinetic parameters of ginsenoside Rg1 liposomes in rats through the lung perfusion method. Ginsenoside Rg1 liposomes were round and uniform in shape, the particle size was 2–3 μm, and the encapsulation efficiency of ginsenoside Rg1 liposome was 51.2 %. Results showed that, after pulmonary administration of ginsenoside Rg1, the time of ginsenoside Rg1 detected by Rg1 liposomes was longer than that of Rg1 solution, the relative bioavailability of ginsenoside Rg1 liposome lung administration $AUC_{liposome}/AUC_{solution} = 122.67$ %. These results provided the scientific theoretical and experimental basis for further development of new dosage forms and new routes of administration of ginsenoside Rg1.

1. Introduction

Panaxnotoginseng is the dried root of panaxnotoginseng (Burk.) F.H.Chenis, a traditional Chinese plant, that belongs to the Araliaceae family. This traditional plant is being used in herbal medicines because of its pharmacological properties and the main component of Yunnan Baiyao. This mainly contains saponins, polysaccharides, amino acids, and other chemical components [1]. According to reports, the total saponins Rg1, Rb1, Rd, and R1 of Panaxnotoginseng were mainly contain active saponins (their total content in PNS is $\geq$ 60 %). Until now, most pharmacokinetic studies had only focused on these four panaxnotoginseng saponins [1]. Ginsenoside Rg1 (Rg1, Fig. 1) is one of the most abundant components in Panaxnotoginseng saponins [2], and it is also a pharmacologically active component for clinical application. Ginsenoside Rg1 is a monomer saponin extracted from Panaxnotoginseng, which is dammarane-type saponin [3].

The 20 (S) - proto ginseng atrial group represented by Rg1 has the characteristics of exciting the central nervous system, anti-fatigue, and hemolysis. In recent years, some studies had confirmed that ginsenoside Rg1 also has anti-diabetes, liver protection, anti-inflammatory, antidepressant, and neuroprotective effects, especially neuroprotective and disease-improving effects on Parkinson's disease (P.D.). In addition, studies had confirmed the immunoprotective effect of ginsenoside Rg1 on granulocytes. Ginsenoside

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Rg1 can serve as an adjuvant in cancer treatment to alleviate the immunosuppressive effect on granulocytes caused by chronic stress [4–8]. Ginsenoside Rg1 had been successfully prepared into oral enteric-coated tablets, which had obtained the national approval number, and were widely used in clinical treatment. However, since Rg1 was easily degraded by enzymes produced by intestinal bacteria, the bioavailability of oral tablets was only 1–20 %, and it was quickly eliminated in the blood. Oral administration was only one of the most suitable routes of administration [9]. Therefore, developing new dosage forms and new routes of administration of ginsenoside Rg1 with long-term sustained release and high bioavailability has become a crucial problem to be solved as earliest possible.

A liposome is the ultra-micro spherical drug carrier prepared by encapsulating the drug in a lipid-like bimolecular layer. Because of its structure, which is similar to biofilm, it could encapsulate water-soluble and fat-soluble drugs. It had the advantages of reducing drug dose, toxicity, allergic reaction, and immune reactions, delaying release, reducing elimination rate *in vivo*, changing the distribution of drugs *in vivo*, and targeting drug release. It had received extensive attention and in-depth research. Therefore, it was of great value and significance to study the new dosage form of ginsenoside Rg1 liposome by using liposome as the drug carrier of ginsenoside Rg1 to improve its bioavailability and clinical efficacy.

Pulmonary drug delivery was a non-invasive route of administration, which had a rapid onset, reduced the dosage and side effects, and avoided the first-pass effect of the liver [10]. It was a promising development field. In recent years, encouraging results had been achieved in studying drug-loaded liposomes for pulmonary administration. A liposome-based pulmonary drug delivery system was a new study direction of pulmonary drug delivery. Liposome-based phospholipid bilayer could achieve slow, controlled release of drugs, stabilize blood drug concentration, and protect drugs from enzymatic decomposition during absorption and transport. It could significantly improve its bioavailability, effectively avoid and reduce the irritation and toxicity of drugs to the lungs, there by improving drug efficacy [11,12]. Drug-loaded liposome lung administration exhibited many advantages in treating lung and systemic diseases and had broad application prospects. Therefore, it was of great theoretical and practical significance to combine the new technology of modern pharmaceutical preparations with the pulmonary administration route and to make the liposome through the pulmonary administration route for targeted treatment of lung and systemic diseases. However, the study of Rg1 liposomes for pulmonary administration had not yet been reported.

In recent years, the pulmonary drug delivery market had rapid growth and high-value potential. According to statistics, the global market for pulmonary drug delivery technology reached USD 15 billion in 2005 and increased yearly at a rate of 10 % [13]. The research and development of liposome pulmonary drug delivery preparation was expected to create enormous economic and social benefits.

In this study, Rg1 liposome was prepared and administered through pulmonary pathway to the lungs. The pharmacokinetic parameters were observed, the bioavailability of Rg1 liposome was evaluated, and the feasibility and superiority of Rg1 liposome for lung administration were discussed. This study would lay scientific, theoretical, and experimental basis for ginsenoside Rg1 in the future. Therefore, it would contribute significantly to study the new dosage form and the new route of administration of ginsenoside Rg1 by using new preparations of modern drugs and new drug delivery technologies to improve its bioavailability and clinical efficacy.

2. Materials and methods

2.1. Chemicals and reagents

Ginsenoside Rg1, with a purity greater than 99.98 %, was purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Natural soybean lecithin and cholesterol were obtained from Tianjin Institute of Photorefining Fine Chemicals. Methanol and

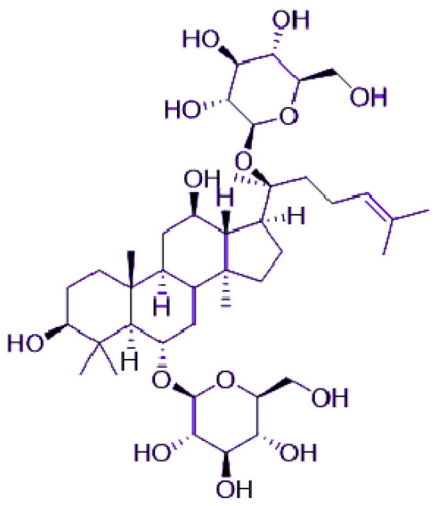

Fig. 1. The chemical structure of Ginsenoside Rg1.

acetonitrile (HPLC grade) were purchased from Merck Company (Germany). Anhydrous ethanol (analytical grade) was bought from Tianjin Chemical Corporation (China). 75 % ethanol, phosphate buffer solution, normal saline, and chloral hydrate were purchased from Solarbio Science Co., Ltd. (Beijing, China). Triply distilled water was from Kunming Medical College.

2.2. Chromatographic conditions

HPLC (High-performance liquid chromatography) 1100 purchased from Agilent Technologies, Inc. (U.S.A.) was equipped with HP1100 separation system (quaternary pump, column temperature chamber, and automatic sampler), HP1100 ultraviolet detector, Agilent chemstation workstation. HPLC was used to determine free ginsenoside Rg1. In addition, the chromatographic column of Diamonsil ODS C18 (250 mm × 4.6 mm, 5 μm) was provided by DIMA HOLDINGSCo., Ltd.(U.S.A.). The mobile phase was acetonitrile and water (30:70) with a flow rate of 1.0 mL/min and a run time of 8 min. The injection volume was 10 μL, the detection wavelength was 203 nm, and the column temperature was 35 °C.

2.3. Preparation process method

2.3.1. Preparation of Rg1 liposome

Ginsenoside Rg1 liposomes were prepared using the film dispersion-ultrasonic method [14]. The prescription amount of soybean lecithin and cholesterol for injection were accurately weighed, and an appropriate amount of organic solvent was dissolved and transferred to a 250 mL round-bottomed flask and placed in a constant temperature water bath at 35–40 °C to remove the organic solvent by vacuum evaporation, so that the lipid formed a uniform film on the bottle wall. After 5 mL of normal saline containing the right amount of Rg1 was added, the membrane was rotated and washed in a 35 °C constant temperature water bath for 30 min, and then ultrasonicated for 1 min. A milky white suspension was obtained, that was, ginsenoside Rg1 liposome (Fig. 2) (Oxygen should be avoided during ultrasound treatment).

2.3.2. Process factors

According to the preliminary test and related data [14], the following aspects were investigated by single factor: thin-film dispersion method, including rotary hydration (10 min), magnetic stirring (30 min), and ultrasound treatment (1 min); organic solvent, including anhydrous ethanol, methanol, and acetonitrile; ratio of phospholipid to cholesterol, including 1:1, 2:1, 4:3, 4:1, 3:1; types of hydration medium, including normal saline (pH = 6.5), pure water (pH = 7.0), and phosphate buffer (pH = 6.86); mass concentration, including 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL.

2.3.3. Optimization by orthogonal design

Based on a single-factor analysis, factors affecting the encapsulation efficiency and drug loading in the preparation of ginsenoside Rg1 liposomes were screened: phospholipid ratio, mass concentration, and organic solvent. Each factor had three levels, as shown in Table 1.

2.3.4. Statistical analysis

This experimental study was conducted under the Windows operating environment, and SPSS 12.0 was used for statistical analysis.

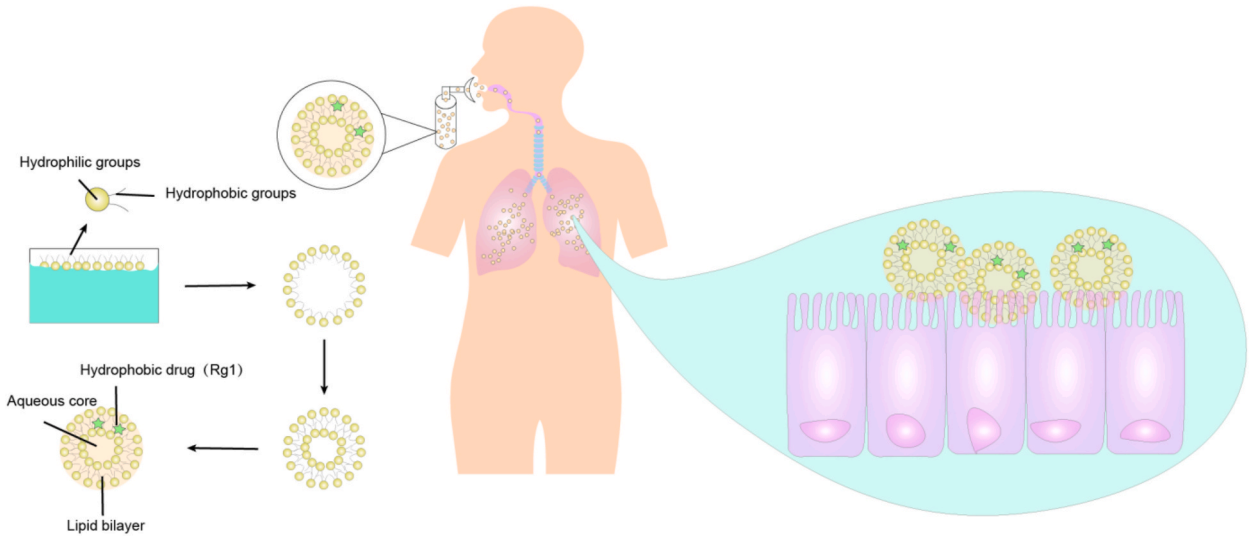

Fig. 2. The schematic diagram of liposome formation.

Orthogonal design analysis of variance was performed. Results of this study were expressed as mean ± standard deviation ($\bar{x} \pm s$), and a $P < 0.05$ was considered statistically significant.

2.4. Preparation of Rg1 sample solution and standard solution

Preparation of Rg1 sample solution: Rg1 liposome suspension was added in a 1.5 mL centrifuge tube, placed in a low-temperature freezing centrifuge, centrifuged at 13000 × *g* at 4 °C for 40 min, then supernatant was taken.

Preparation of Rg1 standard solution: 10 mg of ginsenoside Rg1 was weighed accurately, placed in a 10 mL volumetric flask, dissolved in methanol, diluted to volume, and shaken well.

2.5. Analysis method

2.5.1. Specificity

Rg1 liposome may affect its peak time due to the influence of excipient encapsulation, so a specificity test was needed. The reference solution, blank liposome demulsification solution, and Rg1 liposome demulsification solution were accurately measured. Each 10 μL was for injection analysis, and the chromatogram was obtained, as shown in Fig. 3 (A-C). It could be observed that under this chromatographic condition, the excipients and reagents had no interference with the drug determination, and the retention time of the Rg1 peak was about 6.0 min.

2.5.2. Linear range

The standard solutions of 1, 5, 10, 15, and 20 μL were accurately measured for injection. Linear regression was performed on the sample amount (X, μg) with the peak area of the reference substance (Y). The regression equation was $Y = 362163.1412X+37031.9593$ ($r = 0.99987$). Rg1 showed a good linear relationship in the range of 1–20 μg.

2.5.3. Instrument precision

10 μL of the above standard solution was injected under the determination conditions and repeated 5 times. The peak area of Rg1 was determined, and the RDS was 0.56 %, indicating the good precision.

2.5.4. Solution stability

The above sample solutions were taken at 0, 2, 4, 6 and 8 h, respectively, and 10 μL was injected. The peak area was determined. The RSD of ginsenoside Rg1 was 2.33 %, and the sample solution was stable within 8 h.

2.5.5. Reproducibility

The samples were prepared according to the above sample solution, and 6 samples were treated in parallel. The peak area was measured, and the relative standard deviation was calculated. The RSD was 1.89 %.

2.5.6. Recovery rate

10 mg Rg1 was precisely weighed, placed in a 100 mL flask, dissolved in methanol, diluted to volume, and shaken well. 0.5, 1.5, and 2.5 mL of the solution were taken into a 10 mL volumetric flask. Blank liposomes was added at the prescription amount and diluted with methanol to volume. Solutions with low, medium, and high concentrations of 5 μg/mL, 15 μg/mL, and 25 μg/mL were prepared. The 10 μL sample was injected separately, and the peak area was measured. The actual concentration was calculated by substituting the standard curve, and the recovery rate (Recovery rate = measured concentration/concentration to be measured × 100 %) was calculated. The results are shown in Table 2.

2.6. Encapsulation efficiency

The Rg1 liposome suspension was placed in a 1.5 mL centrifuge tube, centrifuged at 4 °C, 13000 × *g* for 40 min in a low-temperature refrigerated centrifuge, and the supernatant was taken. The volume of the supernatant was precisely measured, that was, the volume of the free drug.

The concentration of the supernatant was measured by HPLC and calculated according to the following formula:

$$\text{Encapsulation efficiency} = (W_{total} - C_{free} \times V_{free}) / W_{total} \times 100\ \%$$

Table 1
Influencing factors and levels.

Levels	Factors		
	Ratio of lecithin to cholesterol(mg/mg)	Mass concentration (mg/mL)	Organic solvent
1	2:1	1.0	methanol
2	3:1	1.5	ethyl alcohol
3	4:1	2.0	acetonitrile

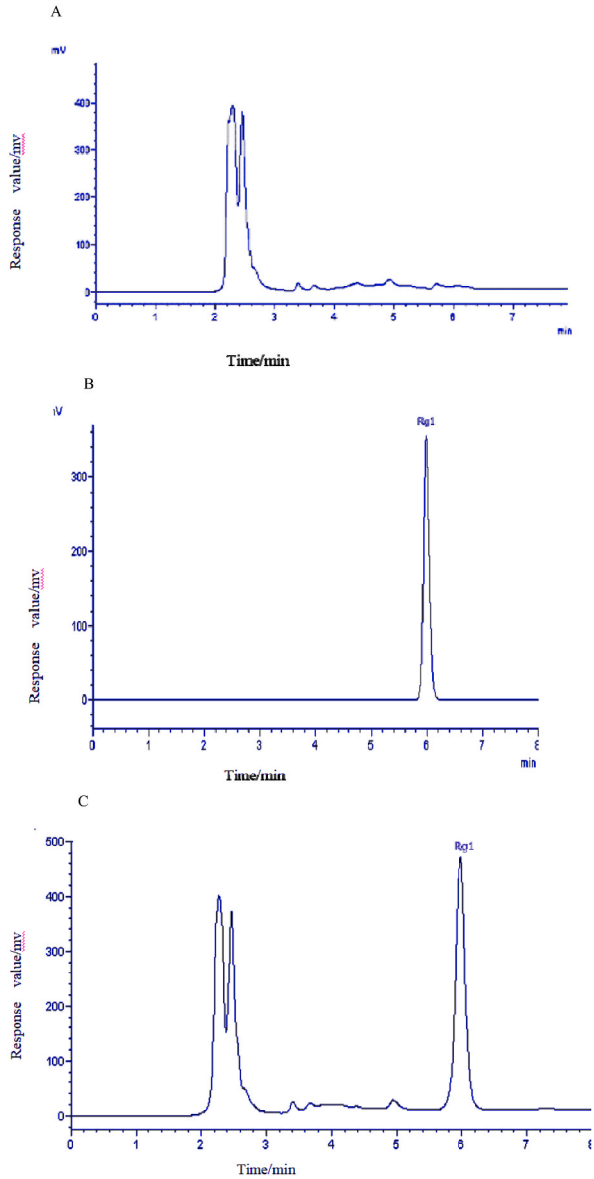

Fig. 3. The chromatogram of Rg1 specificity experiment (A-blank liposomes, B-Standard Rg1, C-Rg1 liposome).

Table 2
Recovery test.

No	Concentration to be measured (μg/mL)	Measured concentration (μg/mL)	Recovery rate (%)
1	5	5.04	100.8
2		4.98	99.74
3		5.13	102.6
4	15	15.13	100.9
5		15.09	100.6
6		14.64	97.65
7	25	24.94	99.87
8		26.15	104.6
9		24.67	98.73

W_{total}: the total mass of drug.
C_{free}: free drug mass concentration in the supernatant.
V_{Free}: Free drug volume in the supernatant.
The free drug mass concentration in the supernatant was calculated by the external standard method

$$A_{free}/A_{standard} = C_{free}/C_{standard}$$

2.7. Particle size and morphology of liposomes

The Rg1 liposomes were observed by transmission electron microscopy (TEM). The sample was prepared using the harmful staining method of phosphotungstic acid. A drop of Rg1 liposome suspension was taken and dropped into the groove of the drop reaction porcelain plate. The sprayed carbon copper mesh was placed on the test solution (film face down). After 1–2 min, the copper mesh was taken out, and the excess liquid was sucked from the edge of the copper mesh with filter paper. According to the same method as above, the copper mesh was placed on 4 drops of phosphotungstic acid solution (pH = 6.5) for about 30 s, and the excess dye solution was dried from the edge of the copper mesh with filter paper and evaporated naturally. The morphology, distribution, and particle size of liposomes were observed and photographed by TEM [15].

2.8. Stability

2.8.1. Effect of different temperatures on the stability of Rg1 liposomes

20 mL of Rg1 liposome suspension was divided into brown glass bottles and stored in a refrigerator at 4 °C or room temperature. The appearance, pH value, and encapsulation efficiency were observed at 4, 10, 20, and 28 d, and three small bottles were taken each time to study the stability of liposomes.

2.8.2. Effect of different media on the stability of Rg1 liposomes

Two batches of Rg1 liposome suspension were incubated with 10-fold 0.9 % sodium chloride (pH = 6.5) and rat plasma (pH = 7.4) in a water bath at 37 °C for 30 min, respectively. The free drug released from the liposome was centrifuged at 10 °C, 14000 × *g* for 60 min, separated, and determined by HPLC.

2.9. Pharmacokinetics study of ginsenoside Rg1 liposome by pulmonary administration

2.9.1. Animals and drug administration

SPF (Specific-pathogen-free) healthy adult S.D.(Sprague Dawley) rats (Certificate No. SCXK 2005-0008), half male and half female, weight at 300 ± 20 g were provided by the Experimental Animal Center of Kunming Medical College. Twelve healthy S.D. rats were fasted for 12–14 h before the experiment andwere randomly divided into 2 groups (Rg1 solution group and Rg1 liposome group, n = 6). The Rg1 solution group was administered by tracheal instillation of 0.3 mL Rg1 solution. The Rg1 liposome group was given by tracheal instillation of 0.3 mL Rg1 liposome solution. Rg1 liposome and Rg1 solution were intratracheally instilled at 1.0 mg/kg. Because chloral hydrate had the advantages of rapid induction of anesthesia, maintenance of anesthesia, and recovery time that could be adjusted by dose [16]. 10 % chloral hydrate (3 mL/kg) was injected intraperitoneally before administration. At the same time, the induction time of anesthesia with 3 mL/kg chloral hydrate injection was (6.1 ± 0.4) min [16], and chloral hydrate injection was given 6 min before Rg1 administration. Then, the rats were hung on a wooden board with an inclination of about 80°. The rats could breathe spontaneously. The Y-tube was connected to the ventilator for tracheal intubation. The ventilator was used to prolong the inspiratory time of the rats. The microinjector was used for tracheal instillation through intubation. After administration, the rat plate was kept tilted at 80° for 60 s and then placed horizontally.

2.9.2. Collection and processing of blood samples

Blood samples were collected from the caudal vein at 0.5, 1, 1.5, 2, 2.5, 3, 24, 48 and 72 h, respectively. 0.5 mL of blood was placed in a 1.5 mL small plastic tube for about 60 min and centrifuged (4000 $r \cdot min^{-1}$ × 10 min). 0.2 mL of serum was taken, and 0.5 mL of acetonitrile was added for protein precipitation [17]. The obtained solution was vortexed for 30 s and then centrifuged in a low-temperature high-speed centrifuge at 10000 $r \cdot min^{-1}$ for 10 min. 10 μL of the supernatant was taken and analyzed by HPLC.

2.9.3. Preparation of the standard curve

5.0 mg of Rg1 reference substance was accurately weighed and placed in a 10 mL volumetric flask, dissolved in purified water, and diluted to volume as a stock solution. 0.5 mL of rat blank serum was taken, and 1–20 μg/mL of Rg1 reference substance stock solution was accurately measured. The serum was added appropriately to prepare a series of standard serum samples with mass concentrationsof 1, 5, 10, 15, and 20 μg/mL. The linear regression equation was obtained with the mass concentration C of the substance to be measured as the abscissa and the peak area Y of the substance to be measured as the ordinate: Y = 362163.1412X+37031.9593 (r = 0.99987), Rg1 in 1–20 μg/mL had a good linear relationship. The minimum detection concentration was 1 μg/mL.

2.9.4. *Method specificity investigation*

Under the above chromatographic analysis conditions, the chromatograms of blank serum, blank serum added with Rg1, and serum samples after pulmonary administration were measured, as shown in Fig. 4 (A-C). Under the experimental conditions, the retention time of Rg1 was at 6 min, and plasma endogenous substances did not interfere with the determination of Rg1 [18].

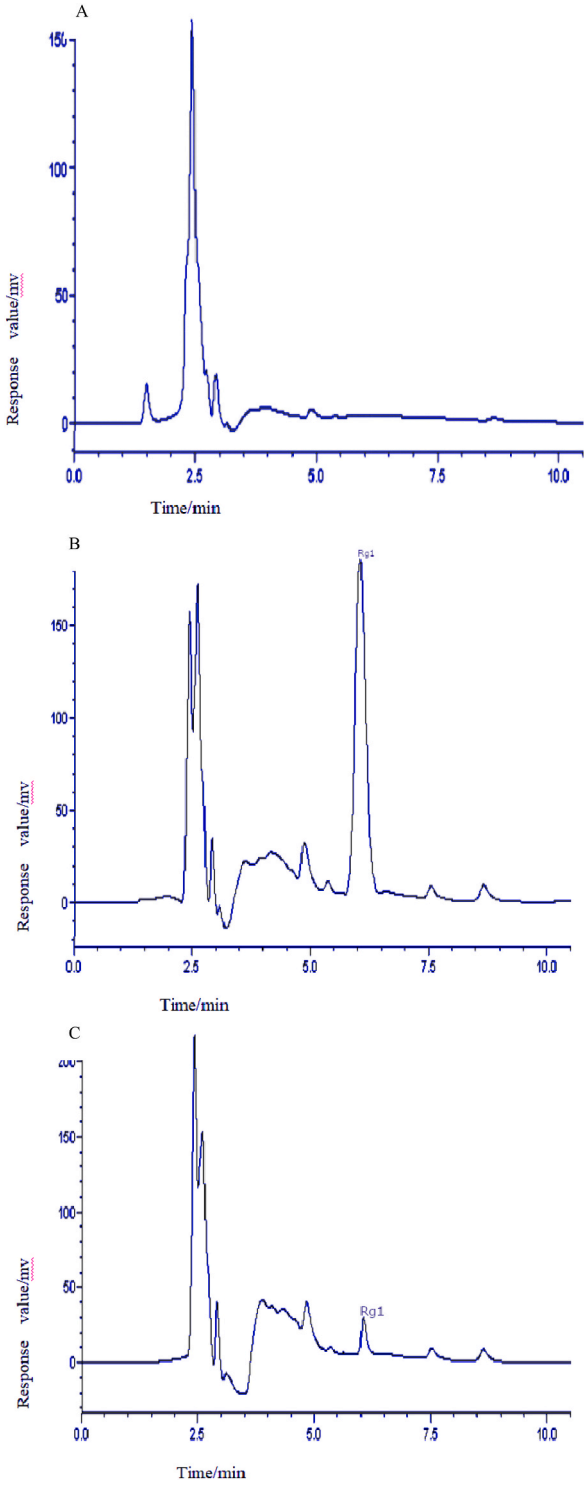

Fig. 4. The chromatogram of Rg1 in rat serum (A-blank serum, B-blank serum added with Rg1, C-Serum samples after pulmonary administration).

2.9.5. Intra-day and inter-day precision

Rg1 standard serum samples at low, medium and high concentrations (5, 25, 50 μg/mL) were prepared. According to the 2.9.3, 10 μL of the supernatant was injected into the HPLC chromatograph to determine the peak area of ginsenoside Rg1. The intra-day and inter-day precisions were determinedby measuring 5 times on the same day and for 5 consecutive days. The results are shown in Table 3.

2.9.6. Extraction recovery

Sample1: Rg1 standard serum samples with low, medium and high mass concentrations (5, 25, 50 μg/L) were prepared, and 5 portions were prepared separately. According to the operation under 2.9.3, 10 μL of supernatant was injected into the HPLC chromatograph, and the peak area Y1 was recorded.

Sample 2: Rg1 standard solutions with low, medium, and high mass concentrations (5, 25, 50 μg/L) were prepared, and 5 portions were prepared separately. The average peak area Y2 was calculated according to the formula: Extraction recovery rate = Y1/Y2 × 100 %, and the extraction recovery rate of ginsenoside Rg1 was calculated, shown in Table 3.

2.9.7. Blood concentration data processing

The plasma concentrations of the Rg1 liposome group and Rg1 solution group at each time point (n = 6) were measured by HPLC. The plasma concentration data were processed by the DAS 2.0 pharmacokinetic degree compiled by the Mathematical Pharmacology Committee of the Chinese Pharmacological Society.

3. Results

3.1. Factors affecting liposome preparation process

The effects of thin film dispersion-rotation hydration (10 min), magnetic stirring (30 min), and ultrasound treatment (1 min) on the entrapment efficiency were determined, while keeping other conditions the same. After the liposome was prepared using the thin film dispersion method, the liposome solution was subjected to rotational hydration (10 min), magnetic stirring (30 min), and ultrasound treatment (1 min). The entrapment efficiency was similar, but the liposome prepared by ultrasonic method had a small particle size and was suitable for pulmonary administration. Therefore, the film dispersion-ultrasonic method was selected.

The effect of organic solvents on the encapsulation efficiency of liposomes was determined, while keeping other conditionsthe same. Anhydrous ethanol, methanol, and acetonitrile were selected to dissolve cholesterol and lecithin. The encapsulation efficiency of acetonitrile was the highest, but the toxicity was high, and the film formation was uneven. The encapsulation efficiency of anhydrous ethanol was higher than that of methanol, the toxicity was low, the cost was low, and the film formation was uniform. Therefore, ethanol was more suitable.

The effect of the ratio of phospholipid to cholesterol on the encapsulation efficiency was determined, while keeping other conditions same. The amount of cholesterol tophospholipid was 1:1, 2:1, 3:1, 4:1, 4:3. The encapsulation efficiency was the highest when the amount of phospholipid tocholesterol was 2:1, more bubbles were generated during the preparation process, the ultrasound was complete, and the quality of the prepared liposomes was also good. Therefore, the ratio of phospholipid to cholesterol was fixed at 2:1.

The effect of the type of hydration medium on the encapsulation efficiency was determined, while keeping other conditions same. Physiological saline (pH = 6.5), purified water (pH = 7.0), and phosphate buffer (pH = 6.86) were used to elute the liposome membrane attached to the bottle wall. The encapsulation efficiency of normal saline (pH = 6.5) was the highest, the cost was low, so it was suitable.

The effect of mass concentration on encapsulation efficiency was determined, while keeping other conditions same. The mass concentrations of Rg1 were 1.0, 1.5, and 2.0 mg/mL. When the concentration was 2.0 mg/mL, the encapsulation efficiency was highest.

3.2. Determination of the optimal formulation of Rg1 liposomes

Rg1 liposomes were prepared in random order under various experimental conditions, and the encapsulation efficiency and drug loading were determined according to the above method. According to the principle of the orthogonal test [19], the experimental design was carried out according to Table L9 (3^4), and the test scheme in Table 4 was listed.

Ginsenoside Rg1 liposomes under various experimental conditions were prepared in random order, and the encapsulation efficiency and drug loading were determined according to the above conditions and methods. The results were shown in Table 4. The encapsulation efficiency and drug loading were used as indicators for variance, as shown in Tables 5 and 6. The encapsulation

Table 3
Method precision and extraction recovery ($n = 5$).

Mass concentration (μg/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Extraction recovery (%)
5	7.28	6.02	86.91
25	4.98	5.15	83.23
50	4.21	7.65	90.52

efficiency and drug loading were used as comprehensive indicators, and the data of encapsulation efficiency and drug loading were divided by the sum of all the data in the group to obtain the proportion. The sum of the proportion of the two data of encapsulation efficiency and drug loading in each test was used as the comprehensive evaluation result, and the variance analysis was carried out. The results were shown in Table 7.

According to the results of the orthogonal test, the variance analysis was carried out with the encapsulation efficiency as the indicator, and there was no significant difference between the three factors ($P > 0.05$). From the perspective of drug loading as the indicator and the comprehensive indicator of encapsulation efficiency and drug loading, only the mass concentration of the three factors was statistically significant ($P < 0.05$), and the optimal concentration was 2.0 mg/mL. The other two factors could be selected from the table with a significant average level. Therefore, the optimized prescription was preliminarily screened as follows: the ratio of phospholipid to cholesterol was 2:1, the organic solvent was anhydrous ethanol (relatively low toxicity), and the mass concentration was 2.0 mg/mL.

According to the experimental results, the ginsenoside Rg1 liposomes were prepared according to the optimized preparation conditions. The ginsenoside Rg1 liposomes were prepared using the thin film dispersion-ultrasonic method. 70 mg soybean lecithin for injection and 35 mg cholesterol were accurately weighed, dissolved withan appropriate amount of anhydrous ethanol and transferred to a 250 mL round-bottom flask. Anhydrous ethanol was removed by vacuum evaporation in a constant temperature water bath at 35–40 °C, so that the lipid formed a uniform film on the wall of the bottle. 5 mL of normal saline containing 2.0 mg/ml Rg1 was added, the membrane was washed for 30 min at 35 °C in a constant-temperature water bath, and the ultrasound treatment was performed for 1 min. The milky white suspension was obtained. That was, the encapsulation rate was 51.2 %, measured by HPLC, and the liposome particle size was about 2–3 μm.

3.3. Determination of particle size and morphology of liposomes

The liposomes were observed by TEM. It was found that the measured liposomes were mainly small single-chamber liposomes, round or oval, with a smooth shape and uniform morphological distribution, shown in Fig. 5 (A-D). The average particle size was (2.5 ± 0.5) μm. And the encapsulation efficiency was (51.20 ± 1.5) %.

3.4. Stability evaluation of Rg1 liposome

3.4.1. Effect of different temperatures on the stability of liposomes

Appearance: The appearance of Rg1 liposomes stored at 4 °C for 28 days was uniform without significant change. Rg1 liposomes stored at 25 °C had a little white flocculent deposition, but the deposition was dispersed after shaking, and the white suspension returned to its original state.

PH value and encapsulation efficiency: The pH value and encapsulation efficiency of Rg1 liposomes stored for 28 days were shown in Table 8. The results showed that the pH values of Rg1 liposomes stored at 4 °C and 25 °C decreased, and the decreasing trend of Rg1 liposomes stored at 4 °C was slower than that at 25 °C. The encapsulation efficiency of Rg1 liposomes stored at 4 °C was relatively stable, and the encapsulation efficiency of Rg1 liposomes stored at 25 °C was decreased rapidly.

3.4.2. Effect of different media on the stability of liposomes

After incubation in a 37 °C water bath, the encapsulation efficiency of liposomes in rat plasma was 50.23 %, which was close to that in 0.9 % sodium chloride, with no statistical significance ($P < 0.05$). It may be due to the effect of temperature (37 °C) leading to partial leakage of liposomes rather than the effect of protein in plasma.

3.5. Rg1 pulmonary pharmacokinetics of Rg1 liposome

3.5.1. The concentration-time curve of Rg1 liposome in rats after pulmonary administration

The plasma concentration-time data were fitted with DAS 2.0. The results showed that after pulmonary administration (1.0 mg/mL) of Rg1 liposome and Rg1 solution in rats, the Akaike's Information Criterion (AIC) and F-test were used for comprehensive

Table 4
Factors and results of orthogonal test.

No	Factors			Results	
	Lecithin: cholesterol (mg: mg)	Mass concentration(mg/mL)	Organic solvent	Encapsulation efficiency(%)	drug loading(10^{-2}mg)
1	2:1	2.0	methanol	63.68	636.80
2	2:1	1.5	ethyl alcohol	42.26	316.95
3	2:1	1.0	acetonitrile	28.22	141.10
4	3:1	1.5	acetonitrile	34.28	257.10
5	3:1	1.0	methanol	18.70	93.50
6	3:1	2.0	ethyl alcohol	47.14	471.40
7	4:1	1.0	ethyl alcohol	37.64	188.20
8	4:1	2.0	acetonitrile	43.50	435.00
9	4:1	1.5	methanol	47.34	355.05

Table 5
Variance analysis with encapsulation efficiency as an indicator.

Resource of gap	Deviation from mean *SS*	Degree of freedom *V*	Mean square *M.S.*	Statistic *F*	The valve of *P*
Lecithin: cholesterol	107.97	2	53.99	0.91	> 0.05
Mass concentration	553.23	2	276.61	4.66	> 0.05
Organic solvent	157.09	2	78.55	1.33	> 0.05
Error	118.61	2	59.30		
Total variation	1312.85	8			

Note: $F_{0.05(2,2)} = 19.0$.

Table 6
Variance analysis with drug loading as an indicator.

Resource of gap	Deviation from mean *SS*	Degree of freedom *V*	Mean square *M.S.*	Statistic *F*	The valve of *P*
Lecithin: cholesterol	5220.03	2	2610.01	1.09	> 0.05
Mass concentration	156996.49	2	78498.25	32.78	< 0.05
Organic solvent	15209.88	2	7604.94	3.18	> 0.05
Error	4790.08	2	2395.04		
Total variation	242356.78	8			

Note: $F_{0.05(2,2)} = 19.0$.

Table 7
Variance analysis with encapsulation efficiency and drug loading as comprehensive indicators.

Resource of gap	Deviation from mean *SS*	Degree of freedom *V*	Mean square *M.S.*	Statistic *F*	The valve of *P*
Lecithin: cholesterol	27.08	2	13.54	0.94	> 0.05
Mass concentration	405.41	2	202.71	28.05	< 0.05
Organic solvent	59.15	2	29.58	2.06	> 0.05
Error	28.71	2	14.36		
Total variation	709.54	8			

Note: $F_{0.05(2,2)} = 19.0$.

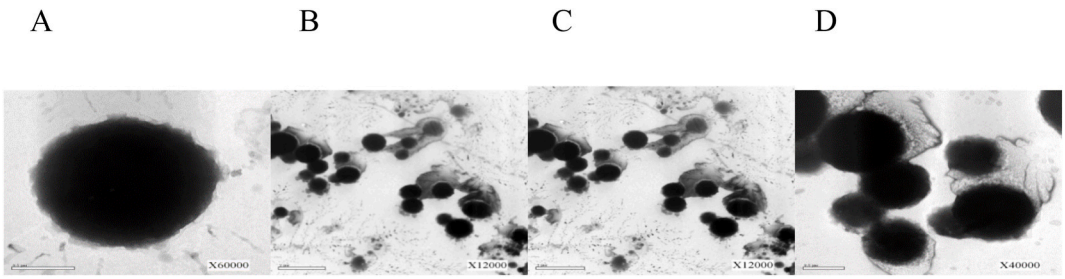

Fig. 5. TEM photo of Rg1 liposome (A-Structure of Rg1 liposome, B-Rg1 liposome distribution, C-Rg1 liposome distribution, D-Morphology of Rg1 liposome).

Table 8
The changes in pH value and encapsulation efficiency at different storage temperatures ($\bar{x} \pm s$, $n = 3$).

Time/d	pH value		Encapsulation efficiency	
	4 °C	25 °C	4 °C	25 °C
4 d	6.50 ± 0.01	6.50 ± 0.01	53.23 %	53.34 %
10 d	6.36 ± 0.01	6.00 ± 0.01	50.12 %	33.44 %
20 d	6.22 ± 0.01	6.00 ± 0.01	48.11 %	21.89 %
28 d	6.10 ± 0.01	5.93 ± 0.01	47.23 %	18.84 %

determination, and the two preparations in rats conformed to the two-compartment model, as shown in Fig. 6 (A-B).

3.5.2. Pharmacokinetic parameters

The plasma concentration data was processed by DAS 2.0 software. The results showed that the apparent absorption rate and elimination rate of liposomes were slower than those of the solution. The peak time of liposomes was at 3 h, with apeak concentration of 62.38 mL, and the peak time of solution was at 1.5 h, with a peak concentration of 68.72 mL. Ginsenoside Rg1 could not be detected in the serum of the Rg1 solution group on the third day after administration. While ginsenoside Rg1 could still be detected in the serum of the Rg1 liposome group on the third day after administration (Fig. 6).

The AUC_{last} and AUC_{tot} of liposomes were 1999.31 and 6480.35, respectively. The AUC_{last} and AUC_{tot} of solution were 848.024 and 925.563, respectively. The relative bioavailability of Rg1 liposomes: $AUC_{liposomes}/AUC_{solution} = 122.67\ \%$. According to the method of the pharmacokinetic compartment model, it was determined that both liposomes and solutions were two-compartment models in rats, which was consistent with the results of Rg1 injection in our laboratory's preliminary experiment [20]. The absolute bioavailability of Rg1 liposomes was $AUC_{liposomes}/AUC_{injection} = 69.27\ \%$.

The results of the hybrid parameters (A, B), the fast configuration rate constant (α), the slow configuration rate constant (β), the interventricular transport rate constant (K_{21}), the absorption phase half-life ($t_{1/2\alpha}$), and the end phase half-life ($t_{1/2\beta}$) of the two formulations were shown in Table 9.

4. Discussion

Liposomes are ultra-micro spherical drug carrier prepared by encapsulating the drug in a lipid-like bimolecular layer. In 1971, liposomes were first used as drug carriers by Gregoriadis and Rymen [21].

Liposomes were very attractive for the following reasons. First, the materials of phospholipids and cholesterol are the main components of biological cell membranes, are endogenous substances in the body, and have good biocompatibility and degradability, and no toxicity and no immunogenicity. Second, the composition and structure of liposomes are similar to those of biological cells, which are easily absorbed, fused, exchanged, and endocytosed by cells. Third, it has certain elasticity and deformability and is easier to enter the lesion tissue than other types of nanoparticles with the same particle size [22–24].

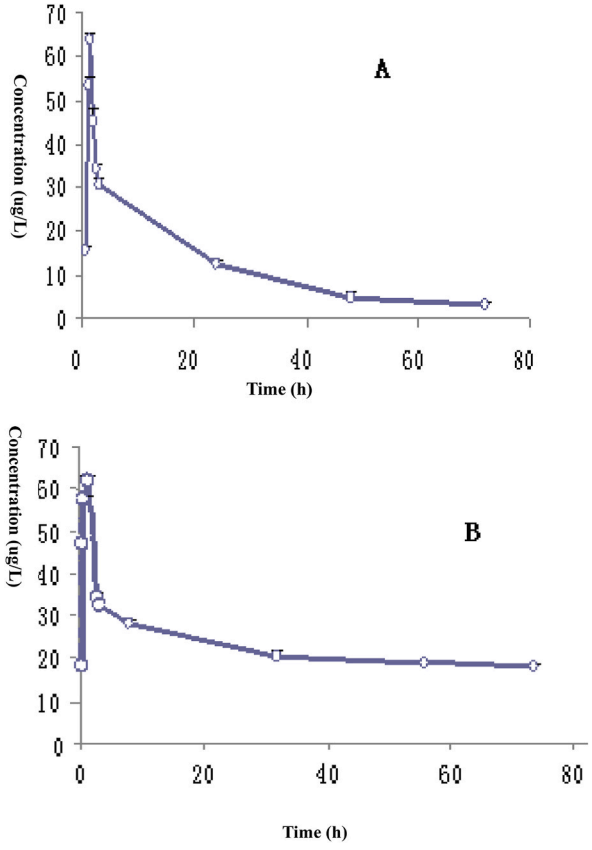

Fig. 6. Drug-time curve of ginsenoside Rg1 after pulmonary administration in rats (n = 6) (A-Rg1 solution group drug time curve, B-Rg1 liposome group drug time curve).

Table 9
Pharmacokinetic parameters of Rg1 liposome and solution in rats after pulmonary instillation (n = 6).

Parameter	Unit	Liposomes	Solution
A	μg/mL	29.45 ± 25.73	164.51 ± 30.77
B	μg/mL	46.91 ± 20.54	67.25 ± 30.12
α	/h	0.075 ± 0.11	1.59 ± 0.27
β	/h	0.01 ± 0.002	0.026 ± 0.001
K_{21}	/h	0.065 ± 0.017	0.34 ± 0.15
$t_{1/2\alpha}$	h	4.39	1.65
$t_{1/2\beta}$	h	49.32	42.84

In recent years, practical parts or monomers of traditional Chinese medicinal plants have been extracted and separated to prepare liposomes. However, the preparation method was not mature enough, and the encapsulation efficiency was not very satisfactory. In particular, there is no complete report on the preparation optimization and stability of Rg1 liposomes.

At present, there are many methods to prepare liposomes [19,25–29], including film dispersion method, ultrasonic dispersion method, injection method, freeze-drying method, freeze-thaw method and reverse phase evaporation method. The liposomes prepared by these methods were not uniform in size and poor in stability. Therefore, in this study, the thin film dispersion-ultrasound combined method was used to prepare Rg1 liposomes. The results showed that the encapsulation efficiency reached 51.2 %, and the particle size of the prepared Rg1 liposomes was 2–3 μm, which met the requirements of pulmonary administration for particle size in the range of 1–5 μm. It was suitable for pulmonary administration and could be used as a new dosage form, which provided an experimental and theoretical basis for further pharmacokinetic studies of pulmonary administration.

According to the results of the orthogonal test, the variance analysis was carried out by taking the drug loading as the indicator and the comprehensive indicator of encapsulation efficiency and drug loading. It was found that the encapsulation efficiency and drug loading were higher when the mass concentration was 2.0 mg/mL, indicating that the drug concentration might have the most significant influence on the drug loading when the encapsulation efficiency was constant. The higher the concentration is, the higher the drug loading is.

We achieved preparation of liposomes by the thin film dispersion-ultrasonic method. When the solvent was evaporated to form a film, it should be rotated faster to form a thin and uniform film. After adding normal saline, the slower speed of rotation was beneficial for the formation of liposomes. Liposomes should be placed for a certain period of time in the final stage of preparation to facilitate the encapsulation of liposomes. Ginsenoside Rg1 is sparingly soluble in water, soluble in methanol and ethanol, and the solubility in methanol is more excellent than that in ethanol. Methanol has good resolution and less interference, but ethanol has the characteristics of low toxicity, uniform film formation, low cost, and simple preparation process, which is more suitable for solvents for mass production and has a better potential of development. Therefore, absolute ethanol was selected as the solvent in this study. To determine the entrapment efficiency, the centrifugal speed should be as high as possible, and the temperature should be controlled in the room temperature range. It was difficult to separate the small liposome from the lower layer when the speed was too low. The increase in temperature made it easy to break the liposome and affected the determination of entrapment efficiency. Ultrasound treatment could change the particle size of liposomes and change multi-compartment liposomes into micro single-chamber liposomes, but too long ultrasound treatment time may destroy the stability of liposomes, so the time was selected at 1 min.

The stability of liposomes has become a significant problem in its application. The stability problem [30,31] mainly referred to the fact that, during storage, due to the influence of temperature, phospholipids were prone to oxidative hydrolysis, bimolecular membranes were prone to phase change, small-size liposomes were easy to aggregate and fuse, and the drugs encapsulatedin them were easy to leak [32].

In this study, the stability of liposomes was investigated, and it was observed that: (1) In the determination of the particle size of the prepared liposomes, there were very few liposomes with a particle size exceeding 3 μm, which may be due to the fusion of liposomes. (2) With the increase in temperature, not only the membrane fluidity formed by liposome phospholipid and cholesterol was enhanced, the membrane material was easy to perforate, but the liposome phospholipid membrane was prone to hydrolysis and oxidation reaction, which was consistent with the literature [31]. Hydrolysis reaction increased the acid value of liposomes, and oxidation reaction produced lipid peroxide, which increased the peroxide value of liposomes. Therefore, the stability of liposomes could be affected by the enhancement of membrane fluidity and the oxidative hydrolysis of the phospholipid membrane, resulting in drug leakage and decrease of encapsulation efficiency. If the temperature was too low, it could form ice crystals in the internal and external water phases of liposomes, increasing the permeability of the bilayer membrane and the defects in the membrane structure. The activity of liposomes was unchanged when they were refrigerated at 4 °C. For example, the preparation of lyophilized liposomes could effectively improve the stability of liposomes [33].

The current focus of ginsenoside Rg1 liposomes should be on further medication of the surface of liposomes to make it an active targeting preparation, transformation of it into a suitable precursor drug, improvement of the distribution behavior of drugs, and further elucidation of the mechanism and clinical efficacy of liposomes. In this study, ventilator-assisted tracheal instillation was used. The pharmacokinetic parameters of Rg1 liposomes and Rg1 aqueous solution in rats were observed. The half-life of the absorption phase was 4 times that of the solution, and the half-life of the B elimination phase was 1.2 times that of the solution. The drug transport rate K_{21} from the peripheral to the central chamber was prolonged from 0.343 h^{-1} to 0.065 h^{-1}, suggesting that after pulmonary administration of ginsenoside Rg1, the time of ginsenoside Rg1 being detected by Rg1 liposome was longer than that of Rg1 solution, which had a certain long circulation effect and might have a long-term sustained release effect. Compared with Rg1 solution, the

relative bioavailability of Rg1 liposome was 122.67 %, and the absolute bioavailability of Rg1 liposome was 69.27 %, indicating that the pulmonary administration of Rg1 liposome improved its bioavailability in rats [34,35].

This study found that pulmonary administration of Rg1 liposomes had a long-term sustained release effect and could improve its bioavailability, which was related to liposomes and lung tissue characteristics. As a drug delivery site, the lung had the following characteristics compared with other parts: (1) Lung absorption area was large, the number of adult alveolar was up to 3.4 billion, and the total area of that was about 25100 m^2, which was more than 25 times the body surface area. (2) There were two layers of cell membrane between the alveoli and the circulating blood, and the thickness of each layer was only 0.51 m, which was conducive to the rapid absorption of drugs. (3) Lung blood volume was sufficient, almost all the blood from the right ventricle went through the lung, and most of the alveoli were closely connected with the surrounding capillaries. (4) The activity of biological hydrolytic metabolic enzymes inside and outside the lung cells was low, and there was no first-pass effect in the liver [36–38]. (5) Liposome lung administration had the characteristics of good histocompatibility, high bioavailability, reduced toxicity and stimulation, and long-term sustained release [39].

This study showed that the effect of ginsenoside Rg1 liposome was better than that of ginsenoside Rg1 aqueous solution, which might be due to the following reasons: (1) The dosage form factor of the liposome. Ginsenoside Rg1 had a specific solubility in water. After ginsenoside Rg1 was made into a liposome, ginsenoside Rg1 was encapsulated in a lipid bilayer, so the drug had strong lipophilicity. Liposomes could significantly improve the absorption of ginsenoside Rg1, the drug's bioavailability, and its bioavailability. (2) The role of phosphatidylcholine. As the raw material for the preparation of liposomes, lecithin contains phosphatidylcholine (P.C.), a precursor of choline, and could increase the bioavailability of choline. (3) Lecithin could effectively regulate blood lipid metabolism, improve lipid metabolism, result in smaller emulsificationofserum cholesterol and neutral fat particles, remain suspended to avoid deposition in the blood vessel wall, reduce blood viscosity, maintain vascular patency, and improve blood circulation, which were conducive to absorption and utilization [40,41].

Liposomal drugs entered the body in at least three forms: released-free, drug-containing liposomes (encapsulated drug), and drug-free liposomal empty carriers. Usually, the concentration of free drugs released in blood or normal tissues was positively correlated with the degree of adverse reactions. In contrast, the concentration of free drugs released in target organs, such as tumor tissues, was positively correlated with efficacy. Therefore, the decreased liposome encapsulation rate might lead to decrease efficacy and increase toxicity totarget organs.

Although the application of Rg1 liposome as a drug carrier had many advantages, there were still some limitations. It was well-known that the most suitable drugs for liposomes were two types of drugs with good fat solubility or good water solubility. Generally, the LogP of drugs in the n-octanol-water two-phase was used to represent this property. Only fat-soluble drugs with LogP > 4.5 or water-soluble drugs with LogP < 0.3 could be encapsulated into liposomes with high encapsulation efficiency and stability. After encapsulating liposomes with intermediate values, the drugs would also leak quickly. The encapsulation efficiency of ginsenoside Rg1 liposomes was low. Therefore, their liposomes were further modified to improve their water solubility, fat solubility, and targeting, thus improving their encapsulation efficiency and bioavailability.

In summary, the research on pulmonary delivery of liposomes had begun to transition from animal experiments to human experiments in recent years, and encouraging studyresults had been obtained. Lung administration of liposomes showed unique advantages in treating local lung diseases and had broad application prospects. In addition, it had great application potential in the systemic administration of protein and polypeptide macromolecular drugs [42–48].

5. Conclusion

In the study, the shape of the ginsenoside Rg1 liposome was round and uniform, and the particle size was 2–3 μm, which met the requirements of lung administration for a particle size of 1–5 μm. The encapsulation efficiency of Rg1 liposome was 51.2 %, which was in line with the guiding principle of liposome preparation in the Pharmacopoeia of the People's Republic of China. The appearance, pH value and encapsulation efficiency of Rg1 liposomes did not change significantly after storage at 4 °C for one month. The stability was good. In addition, after pulmonary administration of ginsenoside Rg1, the time of ginsenoside Rg1 being detected by Rg1 liposome was longer than that of Rg1 aqueous solution, and there was a specific long circulation effect, indicating that it might have a long-term sustained release effect. The relative bioavailability of ginsenoside Rg1 after liposome lung administration was $AUC_{liposome}/AUC_{solution} = 122.67$ %, indicating that Rg1 liposome through lung administration improved its bioavailability in rats to a certain extent. Studying the new dosage forms and new administration routes of ginsenoside Rg1 was of great significance, which would improve its bioavailability and clinical efficacy.

Ethics statement

All experiments were conducted by the guides of the Animal Care and Use Committee at the Fourth Hospital of Hebei Medical University.

Funding statement

None.

Data availability statement

Data are available upon reasonable request.

CRediT authorship contribution statement

Ping Liang: Conceptualization. **Jie Zhang:** Visualization, Validation, Supervision, Conceptualization. **Juan Hou:** Investigation, Formal analysis, Data curation. **Rui Feng:** Software, Resources, Project administration, Methodology. **Jintuo Yin:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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