

# Development and Evaluation of Aloperine-Loaded Nanostructured Lipid Carriers for the Treatment of Pulmonary Arterial Hypertension

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**Objective:** This study focuses on the development and evaluation of nanostructured lipid carriers (NLCs) loaded with aloperine as a potential therapeutic approach for the treatment of pulmonary arterial hypertension.

**Methods:** The NLCs were designed to enhance the solubility, stability, and bioavailability of aloperine, a compound with vasodilatory and anti-inflammatory properties. Through a series of experiments including single-factor experimentation, transmission electron microscopy, high-performance liquid chromatography, in vivo pharmacokinetics, and tissue distribution studies, we assessed the physicochemical properties, drug release profiles, and in vitro and in vivo performance of this novel nanocarrier.

**Results:** The prepared aloperine-loaded NLCs exhibited a milky white and translucent suspension appearance, presenting a quasi-spherical shape under a transmission electron microscope, with an average particle size of (509.48±30.04) nm and an entrapment efficiency of (64.18±1.14)%. The drug release profile demonstrated good sustained-release characteristics in vitro, and the formulation remained stable for up to 15 days when stored at 4°C. Compared to the aloperine solution group, the  $t_{1/2}$ ,  $AUC_{(0 \rightarrow t)}$ ,  $AUC_{(0 \rightarrow \infty)}$ ,  $MRT_{(0 \rightarrow t)}$ , and clearance rate of the aloperine-loaded NLCs were 2.3, 2.96, 3.06, 3.03, and 0.22 times higher, respectively. This indicates that formulating aloperine into NLCs can prolong its circulation time in the body. Furthermore, the concentrations of aloperine in the lungs of the NLCs group were 1.79, 3.78, and 2.30 times higher than those in the solution group at three time points (0.25 h, 1.5 h, 4 h), suggesting that NLCs can increase the accumulation of aloperine in the lungs.

**Conclusion:** Our findings suggest that NLCs loaded with aloperine could offer a promising strategy for the treatment of pulmonary arterial hypertension.

**Keywords:** aloperine, nanostructured lipid carrier, aerosol inhalation, pharmacokinetics

## Introduction

Pulmonary arterial hypertension (PAH) is a malignant pulmonary vascular disease characterized by progressive occlusion of pulmonary arteries, leading to a gradual increase in pulmonary vascular resistance and subsequent right ventricular failure. It has high prevalence and incidence rates, with a current 5-year survival rate of only 57%.<sup>1-4</sup> The pathogenesis of PAH is complex, involving structural or functional changes in pulmonary blood vessels caused by multiple heterogeneous diseases (etiologies).<sup>5</sup> Clinical treatments for PAH primarily include calcium channel blockers, endothelin receptor antagonists, guanylate cyclase agonists, and type 5 phosphodiesterase inhibitors.<sup>6</sup> However, the efficacy of existing therapies is limited, as they cannot fundamentally cure the disease, and they are constrained by high costs.<sup>7</sup> Therefore, the development of inexpensive natural drugs for the treatment of pulmonary hypertension is of great research importance.

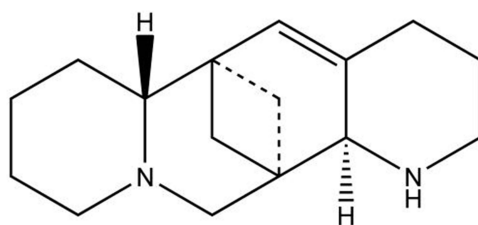
*Sophora alopecuroides* L. is a perennial dry herb in the family of Leguminosae and genus *Sophora*.<sup>8</sup> It is widely distributed in the northwestern part of China, such as Ningxia, Inner Mongolia, and Gansu, and has the efficacy of clearing heat and removing toxins.<sup>9,10</sup> Recent studies have shown that bitter bean seeds have anti-tumour, immunomodulatory, anti-inflammatory, analgesic, antimicrobial, cardiovascular system protection and hepatoprotective properties.<sup>11,12</sup> Aloperine is a natural alkaloid obtained from bitter bean seeds and its chemical structure formula is shown in Figure 1. Clinical use in the treatment of acute dysentery has made considerable progress.<sup>13</sup> The group's previous studies have confirmed that aloperine can treat rats with wild larkspur alkaloid-induced pulmonary arterial hypertension by regulating the RhoA/ROCK signalling pathway, inhibiting the inflammatory response of rat lung tissues, improving pulmonary vascular remodelling, and ultimately lowering the pulmonary arterial pressure,<sup>14,15</sup> suggesting that aloperine has a good therapeutic effect on pulmonary arterial hypertension.

However, aloperine has side effects such as central neurotoxicity and hepatotoxicity, and some studies have confirmed that intraperitoneal injection of 16 mg/kg aloperine in mice can cause liver and kidney damage.<sup>16,17</sup> Another study showed that the elimination half-life of aloperine was only 2 h after intravenous injection into rabbits; and it was widely distributed in the body, in the heart, liver, lungs, and fat, suggesting that the drug was less selective; and after intravenous injection into rats, it was hardly excreted through bile, suggesting that the drug might be metabolised by the liver.<sup>18,19</sup> In addition, the drug is thermally unstable, and all of the above suggest some limitations in its clinical application.<sup>20</sup> Some investigators have made enteric capsules of aloperine to prolong the duration of action of the drug in vivo, which achieved enteric targeting and long duration of action, but still failed to improve the selectivity of lung tissue.<sup>21</sup>

Nanostructured lipid carriers (NLC) represent a cutting-edge nanocolloidal delivery system developed based on solid lipid nanoparticles. They consist of biocompatible, low-toxic, and biodegradable solid lipids, liquid lipids, and emulsifiers.<sup>22,23</sup> The incorporation of liquid lipids disrupts the originally ordered carrier spatial structure, creating special lattice defects. This enhancement allows for improved encapsulation efficiency and drug loading capacity of the entrapped drug, along with greater stability of the encapsulated drug.<sup>24</sup> Additionally, NLC can protect active substances from adverse environmental factors such as temperature, further enhancing the stability of the entrapped drug. They also offer advantages such as improved drug solubility and bioavailability.<sup>25</sup> Therefore, nanostructured lipid carriers are also good carriers for the treatment of pulmonary hypertension.<sup>26,27</sup>

Nebulization inhalation is a drug delivery method that uses a special nebulizer to disperse a drug solution into small droplets, which are then inhaled in the form of an aerosol through the respiratory tract. Due to the special physiological structure of the lungs, nebulization inhalation, compared to other routes of administration, allows drugs to directly target organs, offering advantages such as minimal side effects and excellent efficacy. Additionally, the lungs have a large absorption area for drug administration, resulting in fast onset of action. Furthermore, the low activity of lung biometabolic enzymes can avoid the liver's first-pass effect.<sup>28,29</sup> However, nebulised inhalation also has drawbacks, such as the rapid elimination of the drug by the body after inhalation.<sup>30</sup> As a result, patients often require frequent medication and are less compliant. Nanoformulations are able to encapsulate drugs in lipids, which has a slow release effect and improves patient compliance.<sup>31</sup> Nanoformulations are able to avoid recognition by lung macrophages and clearance by respiratory mucosal cilia due to their smaller particle size and the addition of special modifying components, thus prolonging the residence time of the drug in the lungs.<sup>32</sup>

Therefore, the preparation of aloperine into NLC combined with nebulization inhalation can deliver the drug directly to the lungs, increase drug accumulation in the lungs, reduce toxicity to organs such as the liver, and decrease systemic



**Figure 1** Chemical structure formula of Picrasidine.

adverse reactions. Additionally, the unique structure of NLC can enhance the stability of aloperine, prolong the residence time of the drug in the body, and improve patient compliance. The development of aloperine nanostructured lipid carriers (ALO-NLC) may provide a new approach for the treatment of pulmonary arterial hypertension.

## Materials and Methods

### Drugs and Reagents

Aloperine (purity>98%) was purchased from Ningxia Dushun Biotechnology Co. Stearic acid was purchased from Shanghai Yuanye Biological Co. Medium chain triglycerides were purchased from Beijing Fengli Jingquan Co. Soya lecithin was purchased from Dalian Meilun Biotechnology Co. Sodium deoxycholate and polyoxyethylene castor oil were purchased from Shanghai Yi En Chemical Technology Co. Matrine (purity >98%) was purchased from Chengdu Aifa Biotechnology Co. Methanol (chromatographic pure) and acetonitrile (chromatographic pure) were purchased from Thermo Fisher Scientific (China) Co.

### Animals

Male SD rats (230–250 g) and male ICR mice (22–25 g) were provided by the Experimental Animal Center of Ningxia Medical University, with production license numbers SYXK (Ning) 2020–0001 and SCXK (Ning) 2020–0001, respectively. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Experimental Animal Center of Ningxia Medical University (IACUC-NYLAC-2020-015). The study also adhered to the “Guidelines for the Ethical Review of Experimental Animals” of the People’s Republic of China. All rodents were housed under a 12-hour light/dark cycle in a temperature-controlled environment and were acclimated for 3–7 days. The animal center is located within the Ningxia Collaborative Innovation Laboratory of Traditional Chinese Medicine with Ningxia Characteristics at Ningxia Medical University. All procedures involving feeding, housing, modeling, administration, anesthesia, and other surgical procedures for rats and mice in this experiment were conducted in the spf-grade environment of the Ningxia Collaborative Innovation Laboratory of Traditional Chinese Medicine with Ningxia Characteristics at Ningxia Medical University.

### Main Solution Preparation

0.2% Phosphoric Acid Solution: Take 500 mL of bi-pure water, add 1 mL of phosphoric acid solution, filter by pumping and sonicate for 20 min to exhaust, and use immediately.

Heparin saline solution: take 1 mL of heparin sodium injection (12500 IU) and 50 mL of saline, dissolve and mix well, then store at 4°C.

## Preparation and Optimisation of ALO-NLC

### Method for the Determination of the Content of Aloperine

The content was determined on an Agilent C18 column (250 mm×4.6 mm, 5 µm) under the following chromatographic conditions: the mobile phase was methanol and 0.2% phosphoric acid (40:60, v/v), the injection volume was 20 µL, the flow rate was 0.8 mL/min, the temperature of the column was kept at 40 °C, and the detection wavelength was set at 205 nm.

### Selection of ALO-NLC Preparation Methods

ALO-NLC was prepared by double emulsion solvent evaporation, microemulsion and emulsion sonication, respectively, to screen the most suitable preparation method.

### Single-Factor Experiments for ALO-NLC

#### (I) Selection of Different Liquid Lipid Types

We weighed 5 mg of aloperine into a 1.5 mL centrifuge tube and added a small amount of oleic acid and medium chain triglyceride. After vortexing, the solubility of aloperine in these two oil phases was observed.

## (2) Effects of Different Emulsifier Types

With other factors remaining constant, various emulsifiers were used as variables, including Polyoxyethylene castor oil, Poloxamer 188 (P188), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-MPEG 2000). The particle size and stability of the NLC were evaluated as indicators, and the optimal emulsifier type was selected based on the results. The experimental formulations are presented in [Appendix Table 1](#).

## (3) Impact of Different Emulsifier Composition Ratios

With other factors remaining constant, the ratios of SPC to SDC (3:7, 5:5, 6:4, 7:3, 8:2) were varied, and the particle size and stability of NLC were used as evaluation criteria to optimize the emulsifier dosage based on the results.

## (4) Effect of Different Stirring Time

With other factors remaining constant, different Stirring time (2 h, 4 h, 6 h, 8 h, 24 h) were used as variables, and the particle size and stability of NLC were used as evaluation criteria to select the optimal Stirring time based on the results.

## (5) Influence of Different Quantity of Main Drug

With other factors remaining constant, various quantity of main drug (5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg) were tested as variables. The particle size and stability of NLC were evaluated to determine the optimal quantity of main drug based on the outcomes.

## (6) The Effect of Varying Amounts of Organic Solvent

With all other factors remaining constant, different volumes of organic solvent (0.5 mL, 0.8 mL, 1.0 mL, 2.0 mL) were used as variables, and the particle size and stability of NLCs were evaluated as indicators to optimize the amount of organic solvent based on the results.

## (7) The Effect of Varying Lipid Concentrations

Keeping all other factors constant, different lipid concentrations (0.5%, 1.0%, 1.5%) were tested as variables. The particle size and stability of the NLCs were assessed as evaluation criteria to determine the optimal lipid concentration based on the outcomes.

## (8) The Impact of Different Stirring Speeds

With all other parameters held constant, various stirring speeds (800 rpm, 1200 rpm, 1500 rpm, 1600 rpm) were examined. The NLC particle size and stability were evaluated to select the best stirring speed according to the results.

## (9) The Influence of Different Ultrasonication Times

When all other conditions were fixed, different ultrasonication durations (1 min, 2 min, 5 min) were explored. The NLC particle size and stability were used as metrics to choose the optimal ultrasonication time based on the experimental findings.

## (10) The Influence of Different Ultrasonic Powers

With other factors remaining constant, ultrasonic power (100 W, 200 W, 400 W) was varied, and the particle size and stability of NLC were used as evaluation criteria to optimize the ultrasonic power based on the results.

## (11) The Influence of Different Organic Solvent Types

With other factors remaining constant, the type of organic solvent (methanol, ethanol, methanol and ethanol, methanol and trichloromethane, ethanol and trichloromethane; see [Appendix Table 11](#) for formulations) was varied, and the particle size and stability of NLC were used as evaluation criteria to optimize the organic solvent type based on the results.

### (12) The Influence of Different Temperatures

With other factors remaining constant, temperature (room temperature, 37°C, 40°C) was varied, and the particle size and stability of NLC were used as evaluation criteria to optimize the temperature based on the results.

### (13) The Influence of Different Amounts of Solid Lipid

With other factors remaining constant, the amount of solid lipid (1 mg, 2 mg, 3 mg) was varied, and the particle size and stability of NLC were used as evaluation criteria to optimize the amount of solid lipid based on the results.

## Evaluation of Physical and Chemical Properties

### Morphology, Particle Size and Zeta Potential Determination of ALO-NLC

A small amount of ALO-NLC suspension was diluted to the appropriate concentration with doubly purified water, dropped on the surface of the carbon sprayed copper mesh and then stained, dried naturally and then observed the morphology under a transmission electron microscope and photographed.

Take 1 mL of ALO-NLC suspension and add it to the cuvette at room temperature, the sample does not need to be diluted and bubbles should be avoided when adding. The particle size and potential were determined using a Nano-ZS 90 Malvern potentiometric particle sizer, with three measurements in parallel for each sample.

### ALO-NLC Encapsulation Percentage and Drug Loading Measurement

Take 0.1 mL of ALO-NLC suspension, add 1.9 mL of methanol, break the emulsion, vortex and mix, sonicate for 30 min, repeat the sonication three times, and feed the sample to measure the total amount of the drug according to the method of 2.4.1.

Take 0.1 mL of ALO-NLC suspension, add 1.9 mL of purified water, mix well and take 0.5 mL in an ultrafiltration centrifuge tube. The free drug was separated by centrifugation at 4°C and 4000 rpm for 30 min. The filtrate was collected from the outer tube of the ultrafiltration tube each time of centrifugation and the volume was recorded, and the inner tube of the ultrafiltration tube was replenished with an equal volume of purified water, and the filtrate was collected for a total of six times to obtain the sample solution of the free drug. The filtrate collected on each occasion was separately injected for free drug measurement according to 2.4.1.

Encapsulation percentage =  $1 - (\text{free drug} / \text{total drug}) \times 100\%$  loading efficiency =  $\text{packaged drug volume} / (\text{excipient drug volume} + \text{packaged drug volume}) \times 100\%$

### ALO-NLC in vitro Drug Release Study

Three portions of ALO-NLC suspension of the same concentration, 3 mL each, with the total mass of the drug contained therein recorded as  $m_0$ , were loaded into a dialysis bag (14,000 Da) and added to a pyre containing 150 mL of PBS buffer (pH 7.4). The in vitro release experiments were carried out in a thermostatic water-bath oscillator (37°C, 100 rpm), and the appropriate amount of solution outside the dialysis bag was taken at 0.00, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 24 and 48 h, and the release medium was replenished immediately at the same temperature and volume. The same method was used to take an equivalent amount of aloperine raw material drug as ALO-NLC, and the release method was the same as above. The collected renewed filtrate was used to determine the aloperine content according to the method of 2.4.1, calculate the total drug release at each time point (recorded as  $m_t$ ) and plot the cumulative percentage release of aloperine over time.

Cumulative release (%) =  $m_t / m_0 \times 100\%$ .

### Preliminary Stability Study of ALO-NLC

Three portions of ALO-NLC suspension were prepared in parallel and sealed in vial, placed in a refrigerator at 4°C for a certain period of time, and sampled at fixed time points (0, 7, 15, and 30 days). The changes in appearance, particle size, and encapsulation rate are examined according to the methods under 2.5.1 and 2.5.2 to evaluate their initial stability.

**Table 1** Mass Spectrometry Conditions

Mass Spectrometry Conditions	Result
detector	Triplex four-stage pole
Quantitative analysis of ion pairs (m/z)	Aloperine:233.2/97.8; Internal standard (IS):249.3/148.0
Ion source	ESI+
Ion monitoring mode	Multi-ion reaction monitoring (MRM)
De-cluster Voltage	Aloperine:85V;IS:110V
Collision energy	Aloperine:45V;IS:50V
Source temperature	450°C
Impingement gas	4psi
Air curtain	10psi
Output voltage	12V
Input voltage	10V
Spray Voltage	5500V

## Pharmacokinetic Studies

### Dosing Regimen and Plasma Sample Collection

Twelve male SD rats (weighing 230–250 g) were randomly divided into two groups, with six rats in each group. The rats were fasted for 12 hours before administration but had free access to water. Based on previous research findings from our team, the optimal dose for SD rats is 100 mg/kg.<sup>14,15</sup> Therefore, a compressed nebulizer was used to administer both aloperine solution and ALO-NLC, with an inhaled dose of 100 mg/kg (calculated based on the administered drug amount). Approximately 0.5 mL of blood was collected from the rats' inner canthus before and after administration at 0.033 h, 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, and 48 h. The blood samples were placed in heparinized centrifuge tubes, centrifuged at 4000 rpm for 10 minutes at 4°C, and the plasma was separated and stored at –80°C for future testing.

### Plasma Sample Pre-Treatment

Plasma sample preprocessing methods: Take 100 µL of blank plasma sample, add 10 µL of internal standard solution (210.20 ng/mL Matrine), 10 µL of 50% methanol water, 500 µL of acetonitrile, vortex for 5 min, centrifugation for 20 min (4 °C, 14,000 rpm), and then the supernatant was injected into the sample, and then the plasma aloperine concentration was measured at each time point, and the average blood concentration-time curve was plotted.

### HPLC-MS/MS Analytical Methods

Matrine was chosen as the internal standard, and the mass spectrometry conditions are shown in Table 1 and the liquid phase conditions are shown in Table 2.

**Table 2** Liquid Phase Conditions

Liquid phase conditions	Result
Chromatographic column	Shim-pack XR-ODS C18 (2.0 mm × 100 mm, 2.2 µm)
Mobile phase	A: acetonitrile
(VA/VB=5:95)	B:0.2% formic acid aqueous solution
Elution method	Isocratic elution
Column temperature	40°C
Velocity of flow	0.3 mL/min
Run time	3 min
Injection volume	10 µL



## Data Processing

Statistical analysis was performed using SPSS 28.0. Data were processed by Phoenix 8.3.0 pharmacokinetic software according to the non-atrial model, pharmacokinetic parameters were calculated, and results were expressed as Mean $\pm$ SD.

## Organizational Distribution Studies

### Dosing Regimen and Tissue Sample Collection

Forty-eight male ICR mice (weighing 22–25 g) were randomly divided into two groups, with 24 mice in each group. The mice were fasted for 12 hours before administration but had free access to water. Based on previous research findings from our team, the optimal dosage for SD rats is 100 mg/kg.<sup>14,15</sup> By converting the dosage, we determined that the appropriate dosage for ICR mice is 140 mg/kg. A compressed nebulizer was used to administer either aloperine solution or ALO-NLC, with an inhalation dose of 140 mg/kg (calculated based on the administered drug amount). Organs including heart, liver, spleen, lungs, kidneys, and brain were collected before nebulization and at 0.25, 1.5, and 4 hours after nebulization (with 12 mice sacrificed at each time point, six from each group). The organs were rinsed clean with 0.9% sodium chloride solution, excess water was removed with filter paper, and approximately 0.2 to 0.3 g of tissue samples from the heart, liver, spleen, lungs, kidneys, and brain were precisely weighed (if the tissue weighed less than 0.2 g, the entire tissue was taken). The samples were then homogenized with 800  $\mu$ L of normal saline and stored at  $-80^{\circ}\text{C}$  for future testing.

### Tissue Sample Pre-Treatment

Methods for pre-treatment of tissue homogenization samples: Take 100  $\mu$ L of blank tissue homogenate, add 10  $\mu$ L of internal standard solution (210.20 ng/mL Matrine), 10  $\mu$ L of 50% methanol in water, 500  $\mu$ L of acetonitrile, vortex for 5 min, and centrifuge for 20 min ( $4^{\circ}\text{C}$ , 14,000 rpm), then take the supernatant and inject it into the sample according to mass spectrometry conditions and liquid-phase conditions of 2.6.3, and then measure the concentration of aloperine in each tissue and plot the concentration-time distribution characteristic plots for each tissue. The concentration of aloperine in each tissue was measured, and the concentration-time distribution of each tissue was plotted.

## Data Processing

SPSS 28.0 statistical software was used to analyze the data, and all the above data were expressed as Mean $\pm$ SD.

## Results

### Selection of Preparation Method for ALO-NLC

Experimental results indicate that the colloidal systems of NLC prepared using the double emulsion solvent evaporation method and the microemulsion method are unstable, exhibiting stratification and flocculation phenomena soon after preparation. However, the NLC prepared using the emulsification-ultrasonication method appears as a uniform, milky white suspension with good stability, showing no aggregation after storage. Therefore, the emulsification-ultrasonication method was ultimately selected for preparation.

### Single-Factor Experiments for ALO-NLC

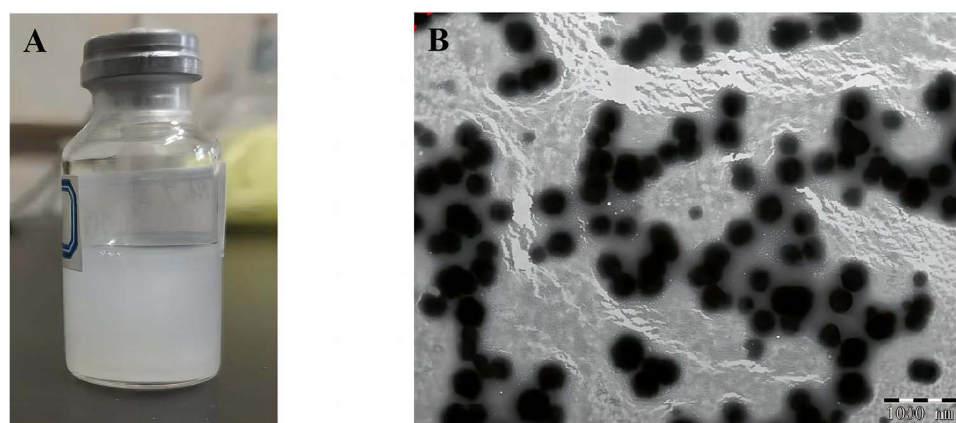
Experimental results with different types of liquid lipids reveal that the solubility of aloperine in medium chain triglyceride is higher than in oleic acid. Hence, medium chain triglyceride were chosen as the liquid lipid. Additional single-factor investigation results are presented in [Appendix Tables 1 to 13](#). Based on these findings, the preparation parameters for ALO-NLC were finalized as follows: Precisely weigh 0.0072 g of SPC and place it in a 100 mL pear-shaped flask, add 10 mL of purified water, vortex for 5 minutes to ensure uniform dispersion. Then, accurately weigh 0.2251 g of polyoxyethylene castor oil and 0.0048 g of SDC as the aqueous phase. Separately, precisely weigh 0.0015 g of aloperine, 0.0002 g of stearic acid, and 0.0041 g of medium chain triglyceride, placing them in a 100 mL pear-shaped flask, and add 1 mL of ethanol as the oil phase. Both the aqueous and oil phases are magnetically stirred at room temperature for 0.5 hours. Subsequently, the aqueous phase is slowly dripped into the oil phase and mixed with magnetic

stirring at 37°C for 24 hours. The mixture is then treated with an ultrasonic cell disruptor for 5 minutes (200 W, ultrasonic pulse of 3 seconds on and 3 seconds off) to obtain ALO-NLC.

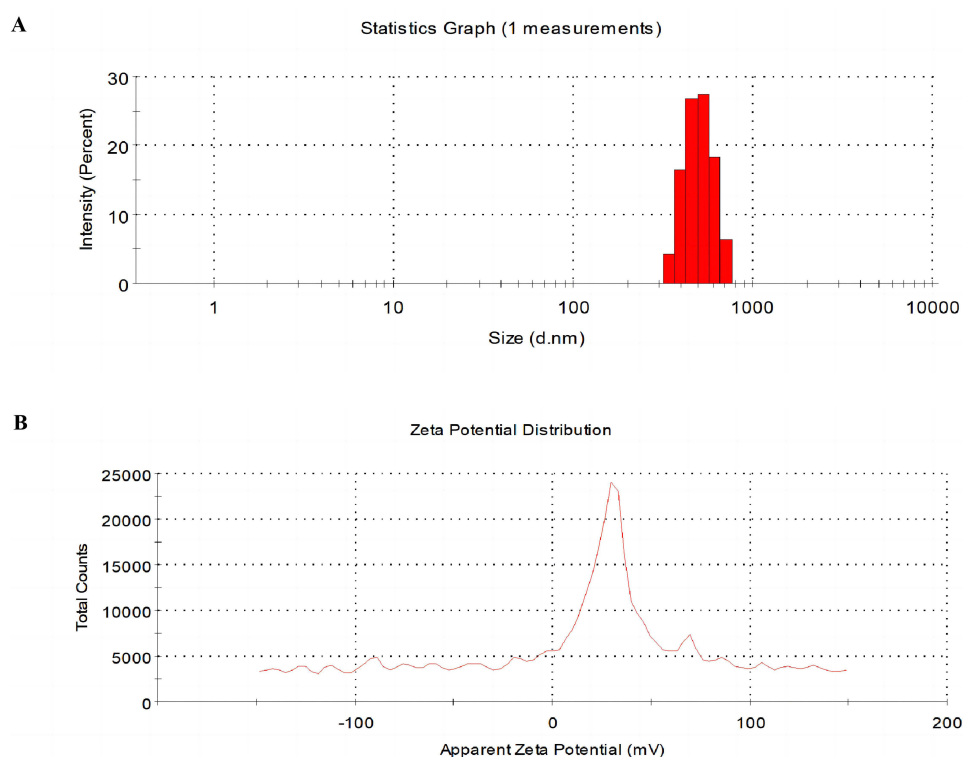
## Evaluation of Physicochemical Properties of ALO-NLC

### Morphology, Particle Size, Zeta Potential, Encapsulation Percentage and Drug Loading Measurement

The ALO-NLC suspension (n=3) obtained from the preparation of the preferred prescription was taken for observation as shown in Figure 2, which is a milky white homogeneous suspension, which is spherical-like under transmission electron microscopy. As shown in Figure 3, the PDI, particle size, and zeta potential of ALO-NLC were measured to be ( $0.33 \pm 0.07$ ), ( $509.48 \pm 30.04$ ) nm, and ( $12.11 \pm 3.94$ ) mV, respectively. Operating according to 2.5.2, the encapsulation percentage and drug loading were calculated to be ( $64.18 \pm 1.14$ )% and ( $2.43 \pm 0.04$ )%, respectively.

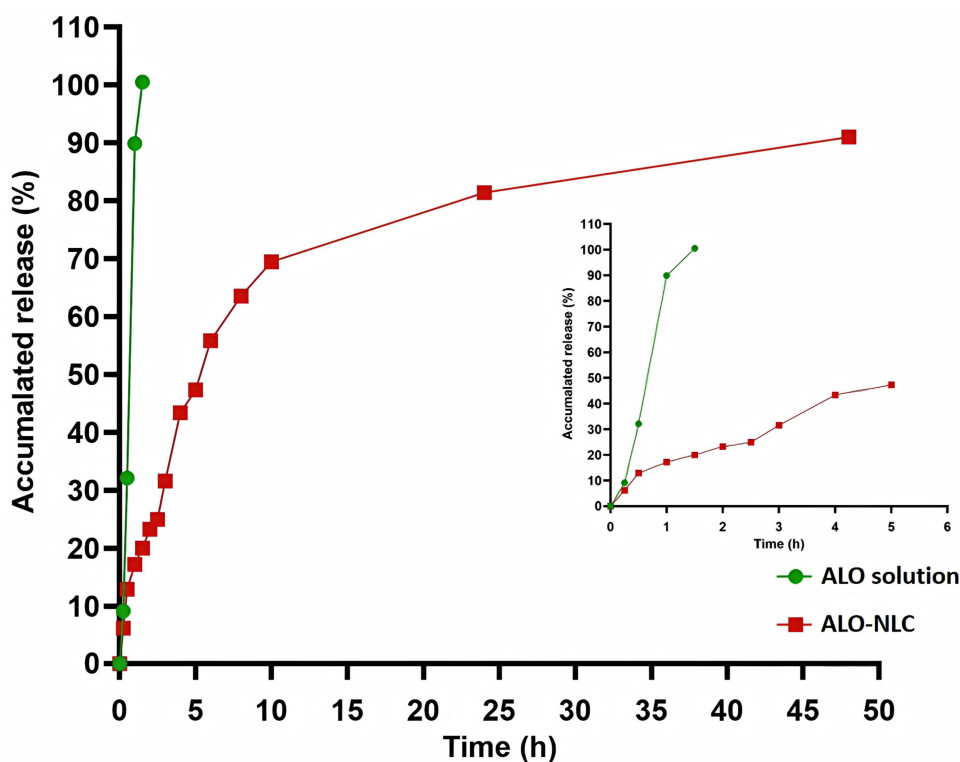


**Figure 2** ALO-NLC (A) appearance, (B) transmission electron microscope (magnification  $\times 3000$ ).



**Figure 3** ALO-NLC (A) particle size distribution diagram, (B) potential distribution diagram).





**Figure 4** In vitro release profile.

### In vitro Drug Release Study

As shown in [Figure 4](#), the aloperine solution group was rapidly released in PBS medium at pH 7.4, and the cumulative release of the solution reached 89.95% at 1.0 h and 100.46% at 1.5 h, indicating that there was no adsorption of aloperine by the dialysis bag. Compared with the aloperine solution, the release of ALO-NLC was relatively smooth, with the amount of release less than 40% at 0.5 h, indicating that there was no sudden release phenomenon, and the initial release was faster, with the cumulative amount of release reaching 69.42% at 10 h, and 90.98% at 48 h, which was basically a complete release, indicating that the ALO-NLC had a good drug release characteristics.

### Initial Stability Experiments

As shown in [Appendix Figure 1](#) and [Table 3](#), the ALO-NLC prepared with the optimal formula was placed under 4°C conditions, and samples were taken at 0, 7, 15, and 30 days. The appearance color showed no significant change and remained as a milky white liquid. With the extension of time, the particle size gradually increased, and the encapsulation efficiency decreased accordingly. The encapsulation efficiency decreased most significantly at 30 days, while there was little change within 15 days. Therefore, it is considered that ALO-NLC is relatively stable within 15 days.

**Table 3** Preliminary Stability Test Results of ALO-NLC (Mean±SD, n=3)

Time (days)	Particle size (nm)	Encapsulation percentage (%)
0	582.36±67.57	71.48±1.83
7	635.49±81.92	68.44±1.32
15	654.43±45.61	63.66±1.61
30	759.17±108.18	54.92±5.18

## Pharmacokinetic Study of ALO-NLC

The mean blood concentration-time curves of the nebulized inhalation of equal amounts of aloperine solution and ALO-NLC suspension in rats are shown in Figure 5, which shows rapid administration by nebulized inhalation and direct distribution without absorption phase. The  $T_{max}$  of the two groups, aloperine solution and ALO-NLC, did not differ much, and the  $C_{max}$  of the aloperine solution was slightly larger than that of ALO-NLC. The drug was eliminated faster in the aloperine solution group, with lower concentrations measurable after 4 h and almost undetectable at 12 h. However, the elimination rate was slower in the ALO-NLC group, with lower concentrations measurable after 10 h and still detectable after 24 h. The drug was eliminated more rapidly in the ALO-NLC group, with lower concentrations measurable after 10 h, and still detectable after 24 h. The drug was eliminated more rapidly in the ALO-NLC group. The blood concentration results of aloperine solution group and ALO-NLC group were used to calculate the pharmacokinetic parameters according to the non-atrial model using Phenix 8.3.0 operating software, and the results are shown in Table 4. As can be seen from the results, the  $AUC_{(0 \rightarrow t)}$  of the ALO-NLC group was 2.96 times higher than that of the aloperine solution group, which suggests that NLC can improve the bioavailability of aloperine;  $t_{1/2}$  was 2.3 times higher in the aloperine solution group; the  $MRT_{(0 \rightarrow t)}$  was 3.03 times higher than that of the solution group and was different between the two groups ( $P < 0.05$ ); the clearance rate in the ALO-NLC group was only 0.22 of that in the solution group, and the results were different ( $P < 0.05$ ), indicating that  $t_{1/2}$  and  $MRT_{(0 \rightarrow t)}$  were significantly prolonged and the clearance rate was reduced after making aloperine into NLC. These results indicate that NLC significantly prolongs the circulation time of aloperine in vivo with a slow-release effect compared to aloperine solution.

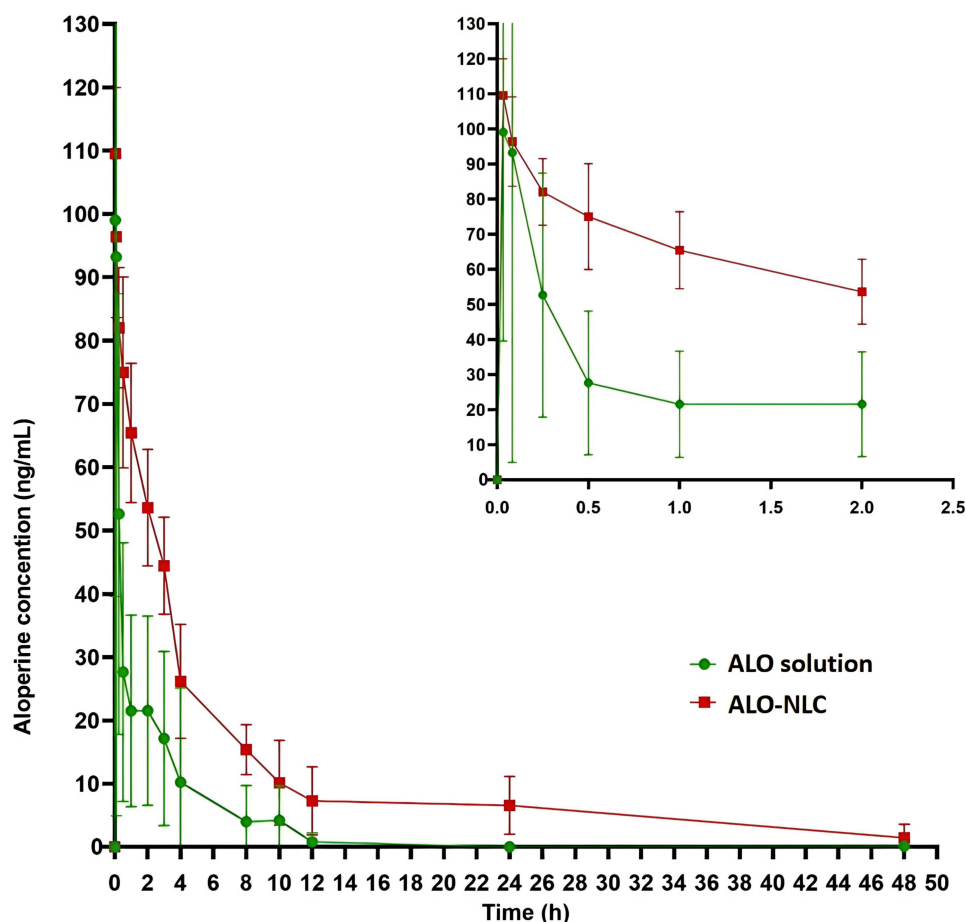


Figure 5 Mean plasma concentration-time curves (Mean $\pm$ SD, n = 6).

**Table 4** The Pharmacokinetic Parameters of Rats (Mean  $\pm$ SD, n=6)

Parameters	Aloperine Solution	ALO-NLC
$t_{1/2}$ (h)	3.64 $\pm$ 2.99	8.38 $\pm$ 5.30
AUC <sub>(0<math>\rightarrow</math>t)</sub> (h*mg/L)	161.34 $\pm$ 137.26	476.78 $\pm$ 101.35*
AUC <sub>(0<math>\rightarrow\infty</math>)</sub> (h*mg/L)	168.28 $\pm$ 134.32	515.40 $\pm$ 99.58*
C <sub>max</sub> (mg/L)	117.38 $\pm$ 73.80	110.92 $\pm$ 11.54
T <sub>max</sub> (h*mg/L)	0.10 $\pm$ 0.09	0.04 $\pm$ 0.02
Vd (L/kg)	3.43 $\pm$ 1.09	2.26 $\pm$ 1.48
CL (L/h/kg)	0.93 $\pm$ 0.58	0.20 $\pm$ 0.04*
MRT <sub>(0<math>\rightarrow</math>t)</sub> (h)	2.93 $\pm$ 1.47	8.88 $\pm$ 4.44*

Note: \*,  $P < 0.05$  compared to aloperine solution.

## In vivo Tissue Distribution Studies in Mice

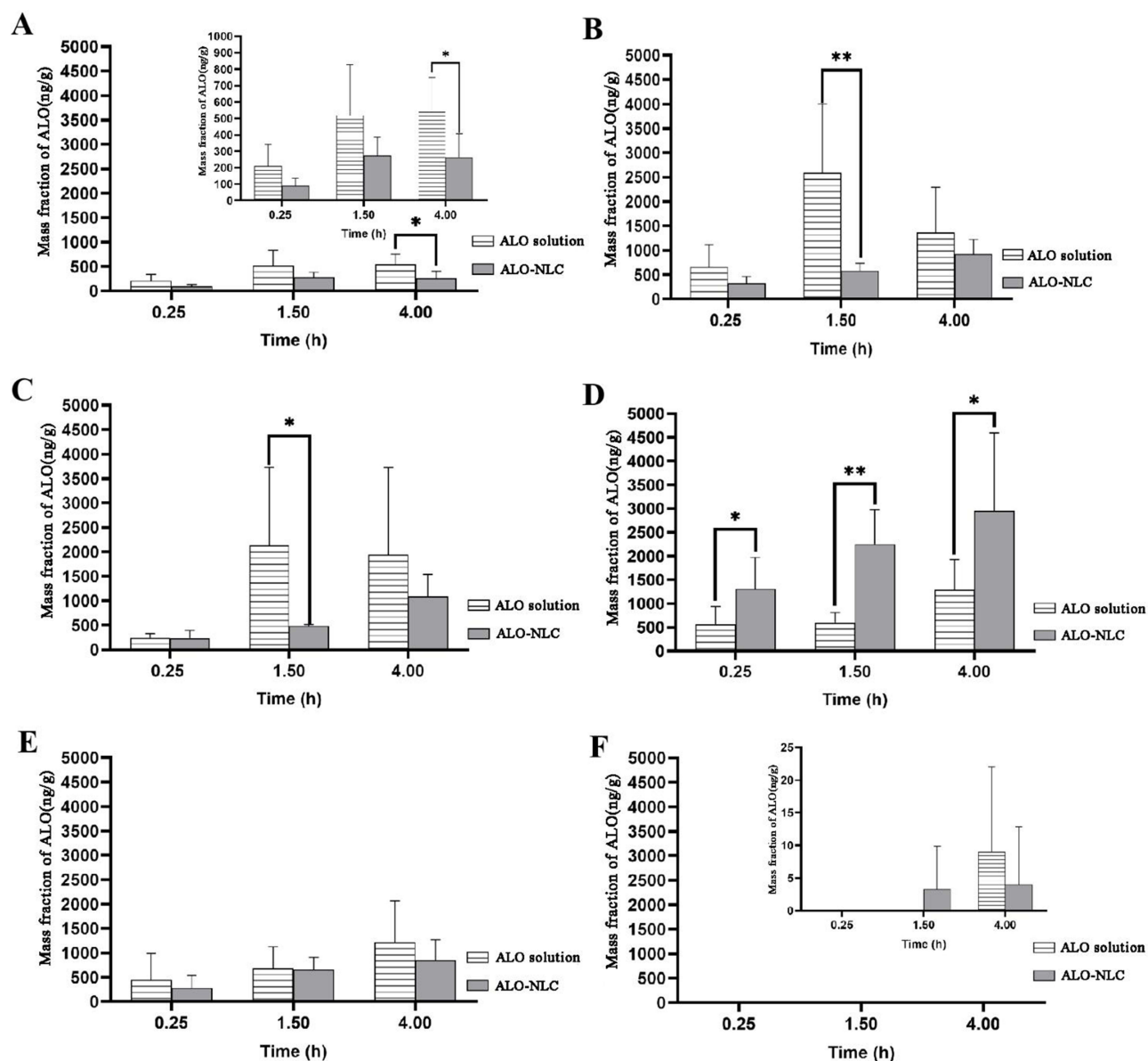
As shown in Figure 6, after mice were nebulized and inhaled aloperine solution and ALO-NLC, respectively, the tissue distribution of the ALO-NLC group exhibited significantly different characteristics from that of the aloperine solution group at different time points (0.25 h, 1.5 h, and 4 h), and there was a wider distribution of nebulized and inhaled aloperine solution in mice, which was mainly concentrated in the liver and spleen tissues. In contrast, the preparation of aloperine into ALO-NLC significantly improved the distribution of the drug in vivo, resulting in a significant decrease in the distribution of the drug in the liver and spleen tissues and a much higher concentration in the lungs, with the concentration of aloperine in the lungs of the ALO-NLC group at the three time points (0.25 h, 1.5 h, and 4 h) being 1.79, 3.78, and 2.30 times higher than that of the solution group, respectively. This indicated that making NLC increased the accumulation of aloperine in the lungs.

## Discussion

NLC preparation methods are similar to liposome preparation methods, and common preparation methods include solvent dispersion, microemulsion, high-pressure homogenization, double emulsion solvent evaporation, and emulsification sonication.<sup>33</sup> Among them, double emulsification solvent evaporation method is suitable for the preparation of water-soluble drugs and microemulsion method is suitable for the preparation of temperature-sensitive drugs under mild preparation conditions,<sup>34,35</sup> but the NLC obtained from the above methods was not stable in this experiment. The emulsification sonication method is a commonly used preparation method in the laboratory, which has the advantages of simple operation, short time-consumption, no need to use special equipment and a large number of organic solvents, and high stability of the particles produced by this method. Therefore, the emulsification sonication method was chosen for the preparation of ALO-NLC.

The commonly used methods for determining the encapsulation rate of nanostructured lipid carriers include dextran gel column method, microcolumn centrifugation method, dialysis method, and ultrafiltration centrifugation method. Among them, the ultrafiltration centrifugation method has a wide range of applicability and accurate results, which can be used to determine the encapsulation rate of water-soluble drugs, and it is one of the most commonly used methods for determining the encapsulation rate, which has the advantages of simple operation and so on.<sup>36</sup> According to the recovery of ultrafiltration centrifugation method to investigate the method has no adsorption effect on aloperine, can meet the determination of aloperine encapsulation rate, so the ultrafiltration centrifugation method was chosen for the determination of encapsulation rate and drug loading. The measured encapsulation percentage and drug loading were (64.18  $\pm$  1.14)% and (2.43  $\pm$  0.04)%, respectively.

Stability is a critical parameter for evaluating ALO-NLC, determining the storage conditions and duration of the formulation. When ALO-NLC was stored at 4°C, the particle size increased over time, accompanied by a decrease in encapsulation efficiency. This phenomenon could be attributed to the continuous reduction of surface energy on the NLC, which accelerated particle aggregation. Experimental results demonstrated that there were minimal changes in particle size and encapsulation efficiency of ALO-NLC within 15 days of storage at 4°C, indicating relative stability during this



**Figure 6** The quality score of ALO at different time points in tissues after aerosol inhalation (Mean $\pm$ SD, n=6, (A) heart, (B) liver, (C) spleen, (D) lung, (E) kidney, (F) brain). **Note:** \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  compared to ALO solution.

period. However, given the relatively poor stability of the formulation, further modifications to its composition are necessary to enhance its stability.

The pharmacokinetics of drug metabolism in the two groups of rats after administration were studied using nebulized inhalation of aloperine solution and ALO-NLC in rats, respectively. The selection of internal standards for HPLC-MS/MS analytical methods requires that their chromatographic behaviors are similar to those of the analytes. Under the selected chromatographic conditions, we tried to use Matrine, Oxymatrine, Sophorine, Oxysophocarpine, and N-methylnordicine, which are similar in physicochemical properties to aloperine, as internal standards, and the response of Matrine was the best, and its retention time was close to that of aloperine, which were 0.97 min and 1.00 min, finally, Matrine was selected as the internal standard. The protein precipitation method is easy to operate and has a wide range of applications, and the commonly used precipitants are methanol and acetonitrile.<sup>37</sup> In this study, it was found that when using acetonitrile as the protein precipitating agent for precipitation, the precipitation effect was better and the sensitivity could meet the requirements of analytical tests. The final choice was to use acetonitrile protein precipitation method to detect the amount of aloperine in rat plasma and tissues.

As shown by the results of pharmacokinetic metabolism parameters, the  $AUC_{(0 \rightarrow t)}$  of the ALO-NLC group was 2.96 times higher than that of the aloperine solution group, and the  $t_{1/2}$  was 2.3 times higher than that of the aloperine solution group; the  $MRT_{(0 \rightarrow t)}$  was 3.03 times higher than that of the solution group, and the clearance of the ALO-NLC group was only 0.22 of that of the solution group, which indicated that the made NLC was able to reduce the clearance of the drug from the plasma and prolong the circulation time of the drug in the body, and improved the bioavailability of the drug in the body. Similar results can be observed in the mean blood concentration-time profiles, where the aloperine solution is rapidly cleared after administration and the plasma drug concentration is at a very low level and essentially undetectable at 12 h, whereas ALO-NLC maintains a certain distribution up to 24 h after administration, which is most likely due to the rapid clearance of NLC from the systemic circulation through conditioning and its uptake by the reticuloendothelial organs.<sup>38</sup> Compared to the solution group, the  $AUC_{(0 \rightarrow t)}$  was significantly larger in the ALO-NLC group, which may stem from the lower uptake of NLC from positive ions in endoplasmic reticulum organs (eg, liver and spleen) and attract more negative NLC.<sup>39</sup> The  $C_{max}$  values of the preparation group were slightly lower than those of the solution group, but the results of the two groups were not significantly different. The two  $T_{max}$  values are relatively close and not significantly different. This is due to the fact that nebulized inhalation is similar to intravenous administration and is characterized by rapid absorption.

Tissue distribution studies in mice were further evaluated on the basis of plasma pharmacokinetic studies in ALO-NLC rats. Since the concentration and drug distribution in various tissues of the body is a dynamic process affected by many factors, the concentration of the drug at a single time point cannot truly reflect the distribution of the drug in the body, therefore, the present experiment examined the tissue distribution characteristics of mice at three time points (0.25 h, 1.5 h, 4 h) after the administration of aloperine solution and ALO-NLC nebulization, and the distribution of mice in heart, liver, spleen, lung, kidney, and brain tissues was determined at different time the distribution of heart, liver, spleen, lung, kidney and brain tissues in mice.

The tissue distribution of the ALO-NLC group showed significantly different characteristics from that of the aloperine solution group, with the nebulized inhaled aloperine solution having a wider distribution in mice, mainly concentrated in liver and spleen tissues. On the one hand, this is because drugs are usually regarded as foreign substances upon entry into the body, and thus preferentially recognized or phagocytosed by the reticuloendothelial organs (liver and spleen tissues), which are rich in macrophages and monocytes.<sup>40</sup> On the other hand, because the liver is the main metabolic organ in the body, most drugs are metabolized by the liver. In contrast, making NLC significantly improved the distribution of the drug in the body, resulting in a significant decrease in the distribution of the drug in the liver and spleen tissues. In addition, the concentration in the lungs was much higher in the ALO-NLC group, suggesting that NLC production reduces the clearance of aloperine from the lung tissue. This is most likely due to the fact that the manufactured ALO-NLC has a particle size of about 500 nm, which allows it to enter the alveoli smoothly and avoids being exhaled with the respiratory tract due to the small particle size, thus effectively depositing it in the lungs.<sup>41,42</sup> On the other hand it may be due to the fact that NLC increases adhesion to lung surface-active substances, thus avoiding clearance by mucus.<sup>43</sup> Compared to the aloperine solution group, the concentrations in the heart and spleen were decreased in the ALO-NLC group. It may stem from the fact that the smaller particle size is more inclined to reach the heart and spleen tissues through blood or lymphatic circulation, thus accumulating in the heart and spleen tissues.<sup>44</sup> After the preparation of aloperine into ALO-NLC, the distribution of the drug in the tissues showed specificity, and the distribution in the lungs was significantly higher, improving the selectivity to the lungs. Whereas the concentration of aloperine solution group and ALO-NLC group in renal tissues did not differ much, the ALO-NLC group was slightly lower than the aloperine solution group.

In summary, the study demonstrates that the prepared ALO-NLC combined with nebulization administration can directly deliver aloperine to the lungs, increasing its distribution in lung tissue. Additionally, the unique structure of NLC enhances the stability of aloperine, prolongs the drug's residence time in the body, improves patient compliance, and holds practical therapeutic significance. However, this study has some limitations. Firstly, the preparation process of the formulation is cumbersome, and the stability of the resulting ALO-NLC is relatively poor, which is not conducive to preservation and unsuitable for expanded production. Secondly, long-term toxicity studies have not been conducted. Therefore, further modifications to the ALO-NLC formula are needed to improve its stability, and a comprehensive safety evaluation is required.

## Conclusion

In this experiment, a aloperine nanostructured lipid carrier was prepared and applied for the first time in the treatment of pulmonary hypertension. Initial physicochemical evaluations revealed that the ALO-NLC exhibited a milky white, translucent suspension appearance, with a quasi-spherical shape under transmission electron microscopy. It demonstrated good sustained-release characteristics in vitro and remained stable for 15 days when stored at 4°C. Pharmacokinetic results indicated that compared to aloperine solution, the  $t_{1/2}$ ,  $AUC_{(0 \rightarrow t)}$ ,  $AUC_{(0 \rightarrow \infty)}$ , and  $MRT_{(0 \rightarrow t)}$  of ALO-NLC were 2.3, 2.96, 3.06, and 3.03 times higher, respectively, while there was no significant difference in  $C_{max}$  and  $T_{max}$  between the two formulations. The clearance rate in the ALO-NLC group was only 0.22 times that of the solution group, suggesting that NLC can significantly prolong the circulation time of aloperine in the body and exhibit a sustained-release effect. Tissue distribution results showed that the aloperine concentration in the lungs of the ALO-NLC group was 1.79, 3.78, and 2.30 times higher than that of the solution group at 0.25 h, 1.5 h, and 4 h, respectively. This indicates that NLC can increase the accumulation of aloperine in the lungs, which has practical therapeutic significance. Therefore, the nebulized administration of ALO-NLC can be considered an effective method for the treatment of PAH. This provides a theoretical basis for the preparation of aloperine formulations and the development of new drugs for PAH, offering potential therapeutic strategies for the treatment of PAH.

## Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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## Disclosure

The authors report no conflicts of interest in this work.

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