

Research Article

Preparation of a Sustained-Release Nebulized Aerosol of *R*-terbutaline Hydrochloride Liposome and Evaluation of Its Anti-asthmatic Effects *via* Pulmonary Delivery in Guinea Pigs

Qingrui Li,¹ Shuyao Zhan,¹ Qing Liu,¹ Hao Su,¹ Xi Dai,¹ Hai Wang,¹ Huimin Beng,¹ and Wen Tan^{2,3}

Received 18 April 2017; accepted 23 May 2017

Abstract. An aerosolized liposome formulation for the pulmonary delivery of an anti-asthmatic medication was developed. Asthma treatment usually requires frequent administration of medication for a sustained bronchodilator response. Liposomes are known for their sustained drug release capability and thus would be a suitable delivery system for prolonging the therapeutic effect of anti-asthmatic medication. Liposomes prepared by thin film hydration were loaded with a model drug, *R*-terbutaline hydrochloride (*R*-TBH), using an ammonium sulfate-induced transmembrane electrochemical gradient. This technique provided an encapsulation efficiency of up to 71.35% and yielded *R*-TBH liposomes with a particle size of approximately 145 ± 20 nm. According to stability studies, these *R*-TBH liposomes should be stored at 4°C before usage. Compared to *R*-TBH solution, which showed 90.84% release within 8 h, liposomal *R*-TBH had a cumulative release of 73.53% at 37°C over 192 h. A next generation impactor (NGI) was used to analyze the particle size distribution in the lungs of *R*-TBH liposome aerosol *in vitro* at 5°C. The therapeutic efficacy of the nebulized aerosol of the *R*-TBH liposomes was assessed *via* pulmonary delivery in guinea pigs. The results showed that, compared to the *R*-TBH solution group, the *R*-TBH liposome group had a prolonged anti-asthma effect.

KEY WORDS: *R*-terbutaline nebulized aerosol; pulmonary delivery; liposome; sustained-release; guinea pigs.

INTRODUCTION

Asthma is a chronic pulmonary disease currently affecting more than 300 million people, and its prevalence is rising. The β_2 adrenergic receptor agonist terbutaline (TBT) is a cellulosic sympathomimetic amine widely used for treating bronchial asthma, chronic bronchitis, and emphysema (1). Similar to many other sympathomimetic drugs, TBT has two enantiomers: an *R*-isomer and an *S*-isomer. The bronchodilating property of β -adrenoceptor agonists has been shown to arise from the *R*-enantiomers, which, for salbutamol and TBT, have been extensively studied regarding their pharmacokinetics and pharmacodynamics (2–6). Studies have shown that the *R*-isomer of TBT has a much more potent

anti-asthmatic effect than the *S*-isomer (3,4,7); moreover, the latter may be responsible for some of the adverse effects of racemic TBT, such as hyperresponsiveness. Commercial pharmaceutical formulations of TBT include tablets, granules, aerosols, and atomization solution. However, the oral administration of high doses of TBT is often associated with adverse effects (8). Moreover, when taken orally, TBT is subject to first-pass elimination in the liver, and its oral bioavailability is only 10% when administered together with food (9). Among the alternative administration routes, pulmonary delivery stands out, because it directly targets drugs to the lungs, reducing unwanted systemic activity or toxicity.

Because conventional TBT aerosol formulations have a fast pharmacokinetic profile, the dosage must be repeated many times to sustain a bronchodilator response. However, such a dose *schedule* may be inconvenient for patients. Therefore, a sustained-release TBT formulation is desirable to improve patient compliance (10–14). To deliver an efficacious dose of the drug to the lungs, a suitable drug carrier is required. Considerable research on pulmonary drug delivery applications has been focused on liposomes because of their potential advantages, including biocompatibility, biodegradability, sustained drug release, and local irritation reduction. Furthermore, liposomes are the only sustained-

Electronic supplementary material The online version of this article (doi:10.1208/s12249-017-0816-z) contains supplementary material, which is available to authorized users.

¹ School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China.

² Institute of Biomedical and Pharmaceutical Sciences, Guangdong University of Technology, Guangzhou, China.

³ To whom correspondence should be addressed. (e-mail: went@gdut.edu.cn)

release strategy for pulmonary delivery that has reached clinical development (15).

Liposomes are mainly produced from neutral, positively charged or negatively charged natural or synthetic phospholipids. The most commonly used phospholipids are lecithins, phosphatidylethanolamines, sphingomyelins, phosphatidylglycerol, and phosphatidylserines (16). Liposomes comprise an aqueous volume surrounded by a single layer or bilayer of lipids and are capable of encapsulating both hydrophobic and hydrophilic drugs (17). Studies have revealed that liposomal-based aerosol formulations increase drug stability and bioavailability, protect drugs from enzymatic degradation, prolong the retention half-life, enable intracellular delivery, and overcome pulmonary clearance (18).

Using liposomes for topical administration may cause TBT to be retained and localized within a reservoir at the site of administration. Thus, liposome formulations with high TBT carrying capacities and improved retention times are desired. In this study, the *R*-isomer of TBT was chosen as the model drug because of its better therapeutic effect on asthma than the *S*-isomer (3,4,7). The encapsulation efficiency (EE) and drug loading (DL) of *R*-terbutaline hydrochloride (*R*-TBH) liposomes were optimized, and the *in vitro* lung distribution and stability of liposomal *R*-TBH were studied. In addition, its anti-asthmatic effects were evaluated in guinea pigs *via* pulmonary delivery.

MATERIALS AND METHODS

Materials

R-TBH ($\geq 99\%$) was provided by Key-Pharma Biomedical Inc., Dongguan, China. Soya lecithin (for injection) was purchased from Taiwei Pharmaceutical Co., Ltd. (Shanghai, China). Cholesterol ($\geq 99\%$) and histamine were supplied by Sigma-Aldrich. Methanol and acetonitrile (both HPLC grade) were purchased from Fisher Scientific. Sodium 1-octanesulfonate monohydrate was obtained from Adamas Reagent Co., Ltd. Chloroform (synthesis grade), phosphate buffered saline (PBS 1 $\times$), and ammonium sulfate (chemical purity) were obtained from various vendors in China. Water was purified by a Milli-Q Ultrapure Water Purifier (Darmstadt, Germany).

Animals

Guinea pigs of 18 weighing 290 ± 40 g were obtained from the Laboratory Animal Center of Southern Medical University (Guangzhou, China) and housed in acrylic cages with food and water *ad libitum* under a 12-h light/dark cycle. All procedures involving animals were carried out according to the guidelines of the Ethics Committee of Animal Welfare in the School of Bioscience and Bioengineering, South China University of Technology (Guangzhou, China).

Preparation of Liposomes

Briefly, lipids (111.80 mg of soybean lecithin and 12.65 mg of cholesterol) were dissolved in a 7:3 mixture of chloroform and methanol and dried for 15 min at 65°C at a

speed of 100 rpm to form a thin film in a pear-shaped flask using a rotary evaporator (IKA®RV 10, Germany) under reduced pressure. The drug was incorporated by active loading. For active loading, a dried lipid film was first rehydrated with 5 mL of 250 mM ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$ solution (*pH* 4.0) for 1.5 h at 40°C . The resulting liposomes were extruded through polycarbonate membrane filters (100 nm) using a high-pressure extruder (Morgue Machinery Co. Ltd., Shanghai), and small liposomes were collected following 20 cycles of extrusion from the opposite side of the extruder. A transmembrane gradient was generated using dialysis by exchanging external ammonium sulfate with ammonium ion-free PBS (1 $\times$, *pH* 7.4) for 2 h. The drug was encapsulated by incubating it with ammonium sulfate entrapped in liposomes for 1 h at 40°C . The unencapsulated drug was removed by dialysis in PBS (1 $\times$, *pH* 7.4) for 4 h, which was refreshed at certain times (0.5, 1, and 2 h). Liposomes thus prepared were stored at 4°C for future studies.

Characterization of the *R*-TBH Liposomes

The average particle size, polydispersity index (PDI), and zeta potential of the liposomes were determined on a Malvern Zetasizer (Malvern® Instruments Limited, Worcestershire, UK). Samples were placed in plastic disposable cuvettes and equilibrated at 25°C . Each measurement was made at least three times at 25°C .

The morphological observation of *R*-TBH liposomes was performed by transmission electron microscopy (Philips Tecnai-10, Netherlands). The *R*-TBH liposome suspension was dropped on a 200-mesh copper grid followed by negative staining with 2% phosphotungstic acid for 1 min. The excess solution was removed by blotting.

To determine EE and DL, *R*-TBH liposomes (50 μL) were lysed in 3 mL of 1:1 (*v/v*) methanol-water and centrifuged to separate the drug from the lipids. The amount of *R*-TBH in the supernatant was measured at 280 nm using HPLC. The mobile phase was a buffer comprising sodium 1-octanesulfonate monohydrate in water (6.5 mM, *pH* 3.40) mixed with acetonitrile (77:23, *v/v*). HPLC was conducted on an Agilent C18 column (150 mm $\times$ 4.6 mm ID, 5 μm ; Agilent Company, USA). The mobile phase flow rate was adjusted to 1 mL/min at 40°C , and the volume of automatically injected sample was set to 20 μL . The standard curve of *R*-TBH between 0.90 and 57.37 $\mu\text{g/mL}$ was determined to be $y = 8631x - 250.0$ ($r^2 = 1$, where x is the concentration of *R*-TBH, and y is the chromatographic peak area of *R*-TBH). The EE and DL were calculated using Eqs. 1 and 2, respectively.

$$\text{EE}(\%) = \frac{W_{\text{encapsulated}}}{W_{\text{total}}} \times 100\% \quad (1)$$

$$\text{DL}(\%) = \frac{W_{\text{encapsulated}}}{W_{\text{lipid}} + W_{\text{encapsulated}}} \times 100\% \quad (2)$$

where W_{total} , $W_{\text{encapsulated}}$, and W_{lipid} are the weight of the drug added to the liposomes, the detected weight of the drug

A Sustained-Release Nebulized Aerosol of *R*-terbutaline Hydrochloride Liposome

encapsulated in the liposomes, and the weight of the lipid added to the system, respectively.

In vitro Drug Release

In vitro drug release was measured using the dialysis method. Liposome suspension (2 mL) was added to a dialysis cassette (molecular weight cut-off of 12,000–14,000 Da, BIOSHARP, USA) using a pipettor. Plain *R*-TBH was used as a control to evaluate whether the dialysis cassettes themselves have any influence on drug release. The cassettes were immersed in 50 mL of PBS in a capped bottle and incubated at 37°C with stirring at a speed of 50 rpm. Aliquots were drawn at predetermined time points (0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, 35, 48, 75, 96, 120, 144, 168, and 192 h), and the medium was immediately replenished with fresh PBS. The amount of *R*-TBH released into the medium was estimated using the HPLC method described above. The experiments were carried out in triplicate.

Study of Liposomal Stability

The storage stability of *R*-TBH-loaded liposomes was evaluated for 30 days. In this study, *R*-TBH liposomes (2 mL) were stored at 4 or 25°C, and samples were withdrawn after 0, 10, 20, and 30 days and analyzed for the particle size, PDI, and zeta potential, as described above. Then, a short-term release study was performed to evaluate the storage stability of the *R*-TBH liposomes further. EE, DL, and release dose were calculated after the 6-h release. Furthermore, post-nebulization liposomal *R*-TBH stability was evaluated. Briefly, an aliquot of liposomal *R*-TBH suspension was aerosolized with the PARI Turbo boy nebulizer (PARI Respiratory Equipment Inc.), and fine liposomal droplets collected in the next generation impactor (NGI) were evaluated for particle size, PDI, and zeta potential, as described above. Subsequently, an 8-h release study was performed to evaluate the liposomal *R*-TBH stability after nebulization. Finally, EE, DL, and release dose were calculated.

In vitro Aerosol Characterization

The aerodynamic particle size distribution was measured using the NGI (MSP Company, USA). The NGI was operated at a flow rate of 15 L/min using a high capacity pump after cooling for 90 min at 5°C according to the specifications of Chapter 2.9.44 in the EP (European Pharmacopoeia) (19). All tests used an LC Plus nebulizer cup filled with 4 mL of *R*-TBH liposome suspension or *R*-TBH solution and were operated as specified in the instruction manual to deliver aerosol for 15 min into the impactor circuit. The pump was kept running for an additional 10 s after each 15 min test to wash out the remaining liquid from the nozzle. After nebulization, the *R*-TBH liposomes or *R*-TBH solution were deposited on the throat, seven impactor stages and micro-orifice collector (MOC). The impactor was then dismantled, and the samples were washed with the solvent. Then, *R*-TBH liposomes were transferred into volumetric flasks filled with the prepared solvent, i.e., sodium 1-octanesulfonate monohydrate in water (6.5 mM, pH 3.40) mixed with methanol(1:1, v/v); *R*-TBH solution were removed into volumetric flasks which were filled with the water. Then, the

amount of *R*-TBH distributed at different stages was determined by HPLC.

Anti-asthmatic Effect on Guinea Pigs

Animal Model

A sensitive guinea pig model was established according to the methods described in previous reports (19,20). Briefly, each guinea pig weighing 240–330 g was placed in a plastic box (4 L), and then 0.6% histamine saline solution was sprayed into the box at a speed of 0.15 mL/min for 15 s. The asthma latent period was defined as the time it took for the animal to twitch and fall down after spraying of histamine. Guinea pigs with an asthma latent period exceeding 120 s were not included in this study. These tests were carried out prior to the following experiments.

Selected guinea pigs were randomly divided into three groups of six. Each group of guinea pigs inhaled either an aerosol of blank liposomes, of *R*-TBH liposomes, or of free *R*-TBH solution. Each guinea pig was anesthetized with urethane (1.25 g/kg, intraperitoneal injection) and then intubated with a tracheal cannula and chest tube. The jugular vein was cannulated to administer histamine. The guinea pig was placed in a body plethysmograph (a 45 cm × 11 cm × 15 cm plastic box) in supine position. A 10-min stabilization period was conducted to record baseline values of the respiratory flow rate, tidal volume, and intrathoracic pressure. An asthmatic response was evoked with histamine (15 µg/mL, 0.5 mL, intravenous injection) at 1 mL/min (19). The changes in R_{aw} (airway resistance) and C_{dyn} (dynamic compliance of lung) after the histamine challenge were expressed as percentages of the baseline before the histamine challenge. After being provided, additional time for stabilization, the same animal was given a second histamine challenge (15 µg/mL, 0.5 mL, intravenous injection) at 4 min after a 2-min inhalation of the aerosol of blank liposome, *R*-TBH liposome (the concentration of *R*-TBH was 1 mg/mL) or *R*-TBH solution (1 mg/mL). Then, the last histamine challenge (15 µg/mL, 0.5 mL, intravenous injection) was given at 4 h post-administration. The peak changes in R_{aw} and C_{dyn} after the histamine challenges were compared to the baseline values before the histamine challenge and expressed as percentages of the baseline. The prolonged anti-asthmatic effects were determined by the changes in R_{aw} and C_{dyn} after the treatments with the blank liposomes, *R*-TBH liposomes, or *R*-TBH solution.

Analytical Methods and Statistics

Pulmonary function was characterized with two indexes, R_{aw} and C_{dyn} , which were calculated from the recorded parameters using the following equations (21,22):

$$R_{aw} = \Delta P_R / \Delta V \quad (3)$$

$$C_{dyn} = V_T / \Delta P_R \quad (4)$$

where ΔP_R is the intrathoracic pressure (cmH₂O), ΔV is the flow rate (derivation of tidal volume, cmH₂O/s), and V_T is the tidal volume (cmH₂O).

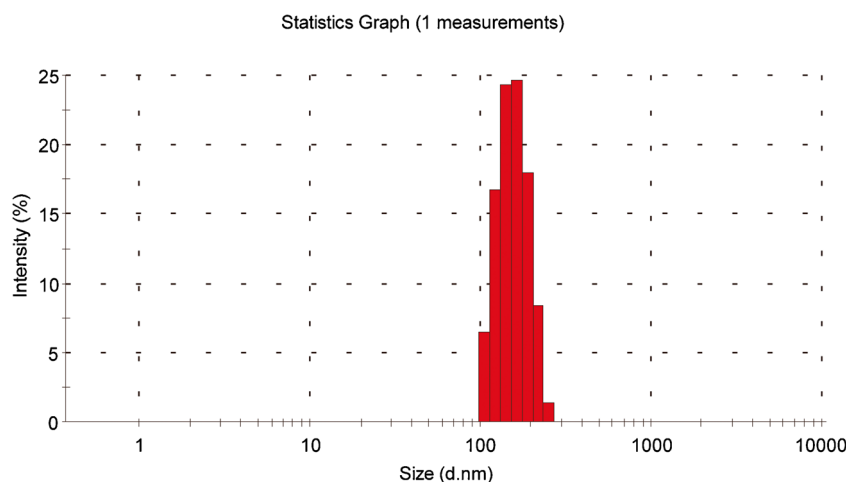


Fig. 1. Size distribution by intensity of the *R*-TBH liposomes

The percent change from the baseline for R_{aw} and C_{dyn} was calculated using the following equations:

$$R_{aw} \% = \{(R_1 - R_0)/R_0\} \times 100\% \quad (5)$$

$$C_{dyn} \% = \{(C_1 - C_0)/C_0\} \times 100\% \quad (6)$$

where R_1 is the maximum value of R_{aw} after the challenge, R_0 is the mean of R_{aw} before the challenge, C_1 is the minimum value of C_{dyn} after the challenge, and C_0 is the mean of C_{dyn} before challenge.

The percent change from the baseline for R_{aw} and C_{dyn} at each time point after inhalation was compared with that before the inhalation (control) in each group using a *t* test. * $P < 0.05$ was significant.

RESULTS

Characterization of the *R*-TBH Liposomes

Upon encapsulation of *R*-TBH into liposomes, the particle size, PDI, and zeta potential of the *R*-TBH liposomes were evaluated. The particle size for the *R*-TBH liposomes was found to be 145.02 ± 5.74 nm ($n = 3$), and the PDI was 0.024 ± 0.005 ($n = 3$). As shown in Fig. 1, the size distribution of the *R*-TBH liposomes was narrow. The zeta potential of *R*-TBH liposomes was -7.63 ± 0.49 mV ($n = 3$), which contributed to the stability of the liposomes. To obtain the information about the morphology of *R*-TBH liposomes, transmission electron microscope analysis was performed. Figure 2 shows that *R*-TBH liposomes had spherical-like shapes. The content of *R*-TBH in liposomes was detected by reversed-phase HPLC method, and the HPLC chromatogram of *R*-TBH was shown in Fig. 3. Using the optimal prescription enabled *R*-TBH to be entrapped in liposomes with an EE of up to 71.35% and a DL of approximately 9.11%.

In vitro Release Studies

In vitro drug release studies were performed to test the hypothesis that encapsulating *R*-TBH into liposomes provides sustained drug release in physiological fluid. The *in vitro* release profile of the liposomal formulations obtained in PBS

buffer at 37°C showed continuous release of the drug over 192 h. The release studies indicated that the *R*-TBH liposome formulation released approximately 16% of *R*-TBH after 6 h and 42.68% after 48 h. At 192 h (Fig. 4), the cumulative release was 73.53%. By contrast, the *R*-TBH solution formulation released 90.84% of the drug into the PBS medium within 8 h, indicating that the dialysis bag was not a limiting factor in the sustained release of *R*-TBH from the *R*-TBH liposomes (Fig. 4). Overall, the *R*-TBH liposome formulation was able to release the *R*-TBH in a sustained manner, and most of the drug (>73%) was released after 192 h *in vitro* (Fig. 4).

Stability of the *R*-TBH Liposomes

Storage Stability

To examine the stability of the *R*-TBH liposomes, we assessed the changes in particle size, PDI, zeta potential, and

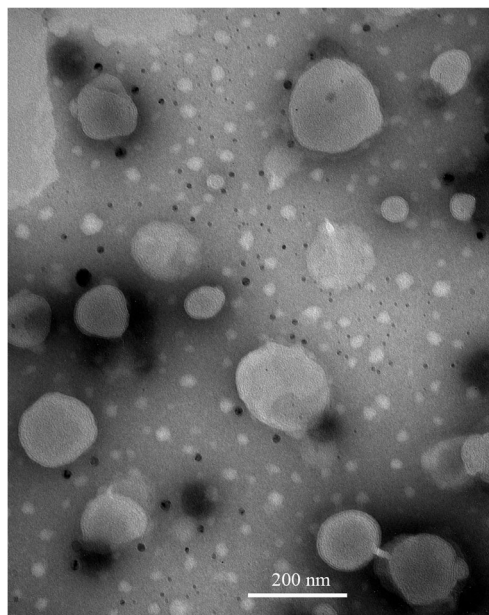


Fig. 2. Transmission electron microscopy micrograph of *R*-TBH liposomes

A Sustained-Release Nebulized Aerosol of *R*-terbutaline Hydrochloride Liposome

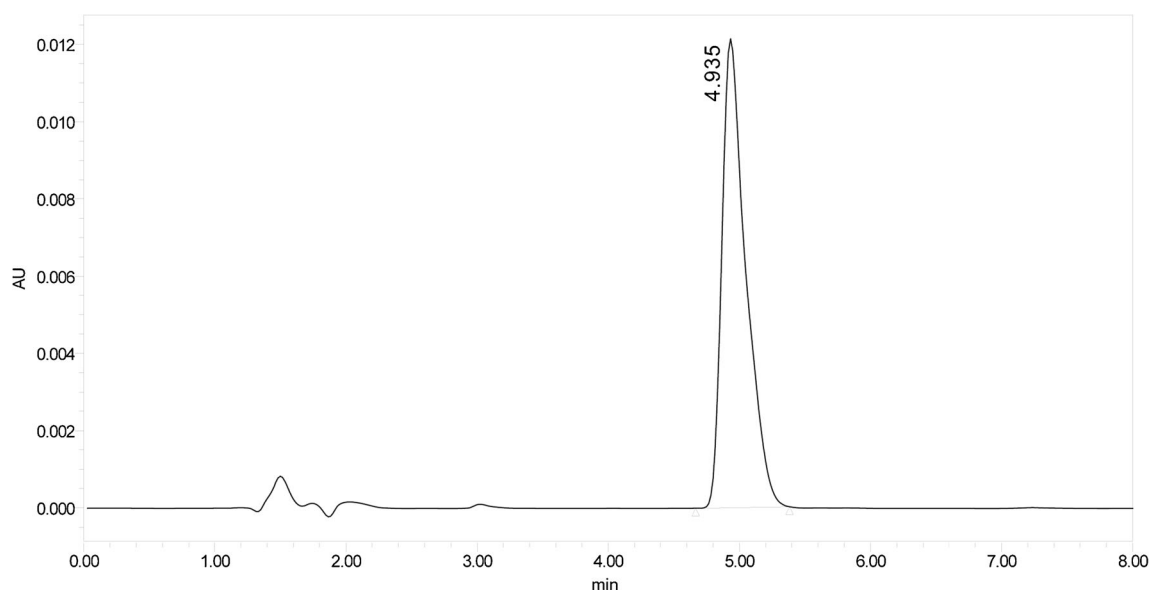


Fig. 3. The HPLC chromatogram of *R*-TBH

EE of *R*-TBH liposomes stored at 4°C for 30 days or at 25°C for 20 days. The properties of the *R*-TBH liposomes were monitored at certain intervals (0, 10, 20, and 30 days). The data presented in Table I show that the storage temperature of 4°C and length of storage within 30 days have little influence on the particle size, PDI, or zeta potential (values are within a reasonable range), suggesting that while stored at 4°C, the liposomes remained well separated from each other and formed no aggregates. The data presented in Table I also show that the storage temperature and length of storage have little influence on the particle size or PDI at 25°C within 20 days, although the zeta potential increased over time. As shown in Fig. 5 and Table I, compared to the control (0 day), the liposomes stored at 4°C for 10 days exhibited no significant change in drug entrapment, whereas those stored at 4°C for 20 days exhibited an 8–16% reduction in drug entrapment. After 30 days, the reduction in drug entrapment was 11–22%. However, storage at room temperature (25°C) for 20 days resulted in approximately 57–64% drug loss,

which is 4–5 times greater than the drug loss that occurred upon storage at 4°C. Subsequently, a short-term release study was performed to evaluate the storage stability of the *R*-TBH liposomes (Fig. 6). According to the release profiles, *R*-TBH was released from the liposomes slowly when stored at 4°C for 30 days. However, more than 80% *R*-TBH was released from the liposomes stored at 25°C for 20 days. These results indicate that these liposomes should be stored at 4°C instead of at room temperature for no more than 10 days before usage, because these conditions are conducive to maintaining a stable and high EE.

Nebulization Stability

In addition to studying the liposomal stability at different storage temperatures and lengths, we also evaluated their stability upon nebulization. Nebulization breaks larger droplets into smaller ones and delivers the formulation in the form of fine mists. However, the force applied during nebulization may rupture the liposomes and release the encapsulated drug prematurely, resulting in drug leakage from certain liposomes. Therefore, we sprayed the formulation three times into the next generation impactor to mimic intratracheal administration and collected the liposomes after nebulization. Next, various physicochemical properties of the *R*-TBH liposomes were evaluated. The data presented in Table II show that the PDI and EE, but not in the particle size and zeta potential, of the *R*-TBH liposomes before and after nebulization were significantly different, suggesting that liposomal integrity was affected by the force applied by the PARI Turbo boy nebulizer to administrate the formulations.

A proportion (8–11%) of the drug was released prematurely from the liposomes upon nebulization (Table II). A short-term release study performed to evaluate the nebulization stability of the *R*-TBH liposomes further (Fig. 7) suggested that the drug was released from the liposomes faster after nebulization. However, the cumulative release rates after 8 h of the drug from the liposomes was approximately 17–25% after nebulization and 16–21% before

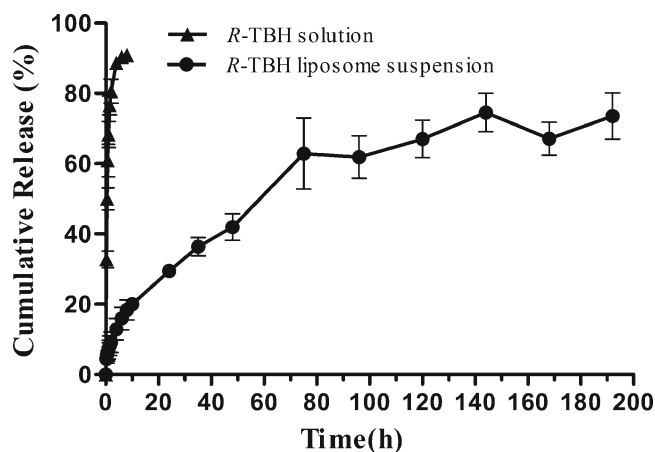


Fig. 4. *In vitro* release profiles of the *R*-TBH solution and *R*-TBH-loaded liposomal formulations in PBS (pH 7.40). Data are expressed as the mean $\pm$ standard deviation ($n = 3$)

Table I. Influence of Storage Temperature and Length of Storage on the Parameters of *R*-TBH Liposomes

Storage temperature and length (days)	Release dose (%) ^a	Drug loading (%) ^a	Entrapment efficiency (%) ^a	Particle size (nm)	Polydispersity index	Zeta potential (mV)
0	15.94 ± 3.21	8.11 ± 0.31	62.90 ± 2.67	145.43 ± 9.78	0.033 ± 0.011	-7.53 ± 0.22
4°C, 10	15.87 ± 1.62	8.19 ± 0.84	60.13 ± 2.69	139.94 ± 1.44	0.047 ± 0.031	-12.31 ± 1.93
4°C, 20	30.50 ± 0.86	6.66 ± 0.13	50.79 ± 1.07	159.01 ± 1.62	0.038 ± 0.018	-10.51 ± 0.79
4°C, 30	32.88 ± 1.17	6.12 ± 0.27	46.39 ± 2.18	146.41 ± 1.72	0.04 ± 0.010	-13.49 ± 0.54
25°C, 10	71.23 ± 16.71	3.18 ± 2.42	23.69 ± 18.48	160.51 ± 7.54	0.071 ± 0.022	-5.74 ± 1.17
25°C, 20	87.92 ± 1.13	0.40 ± 0.06	2.85 ± 0.38	141.93 ± 1.54	0.037 ± 0.011	-4.69 ± 0.55

Data represent mean ± standard deviation ($n = 3$)

^aSample parameters after the release for 6 h

nebulization, indicating that after nebulization, a slow release is still achieved over 8 h.

In vitro Aerosol Characterization

Generally, drug particles from a nebulizer will be deposited in different parts in lungs. Those drug particles will gradually accumulate the in primary bronchi, bronchi, terminal bronchi, and alveoli in accordance with the particle size from large to small (23). Studies have found that inflammation at distal portion of lungs play a significant role in development and persistence of asthma symptoms, and the aerodynamic diameter and geometric standard deviation (GSD) may impact the delivery as well as therapeutic response to inhaled medications (24).

To determine drug particles' aerodynamic behavior in the lungs *in vitro*, freshly prepared *R*-TBH liposomes or *R*-TBH solution were nebulized into the NGI. The particle size distribution profiles of *R*-TBH liposome aerosol and *R*-TBH solution aerosol from stage 1 to the MOC are shown in Fig. 8. Two major indexes can be generated with CITDA software according to the amount of drug deposited at different stages: fine particle fraction (FPF%, <5 μ m) and mass median aerodynamic diameter (MMAD). The FPF shows that more than 60% of the *R*-TBH liposome aerosol were below 5 μ m (Table III). MMAD is a measure of central tendency,

whereas GSD indicates the magnitude of the dispersity from the MMAD value (25). Particles MMAD values between 1 and 5 μ m are subject to sedimentation by the gravitational force in smaller airways and respiratory bronchioles (26). Using these three indices to evaluate the particle size distribution in the lungs, *in vitro* of the *R*-TBH liposome aerosol and *R*-TBH solution aerosol (Table III) showed that these two formulations are significantly different in MMAD, PPF, and GSD.

Evaluation of Anti-asthmatic Effects of *R*-TBH Liposome Aerosol via Pulmonary Delivery in Guinea Pigs

The anti-asthmatic effect of the *R*-TBH liposome aerosol was studied in guinea pigs with histamine-induced asthma. The asthmatic response evoked by histamine (15 μ g/mL, 0.5 mL) was indicated by an increase in airway resistance and a decrease in dynamic compliance. Aerosols of *R*-TBH liposomes, free *R*-TBH solution, or blank liposomes were administered to three groups of guinea pigs ($n = 6$ per group) via pulmonary delivery to monitor their anti-asthmatic effect. The results showed that there were no significant differences ($P > 0.05$) in control treatment between groups.

In the control treatment of *R*-TBH liposome group, the peak R_{aw} and C_{dyn} expressed as percentages of the baseline values during the asthma response before the inhalation of *R*-TBH liposome aerosol were $1477.38 \pm 619.08\%$ and $-91.30 \pm 2.08\%$, respectively. However, when an animal was pretreated with inhaled *R*-TBH liposome aerosol for 2 min

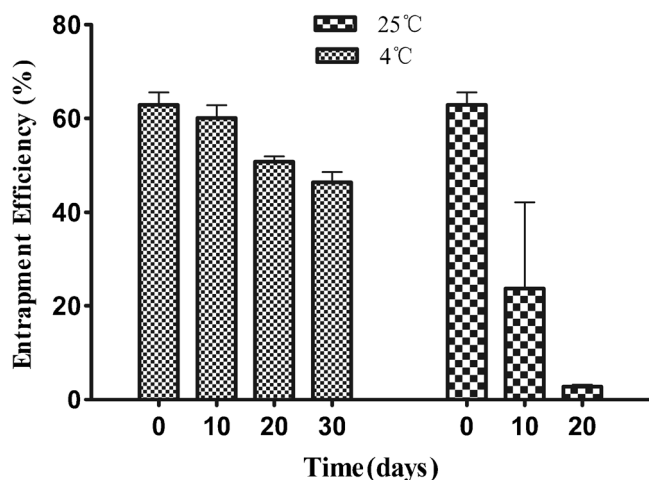


Fig. 5. Influence of temperature and length of storage on drug EE. Formulations were stored at 4°C for 30 days or 25°C for 20 days. Data are expressed as the mean ± standard deviation ($n = 3$)

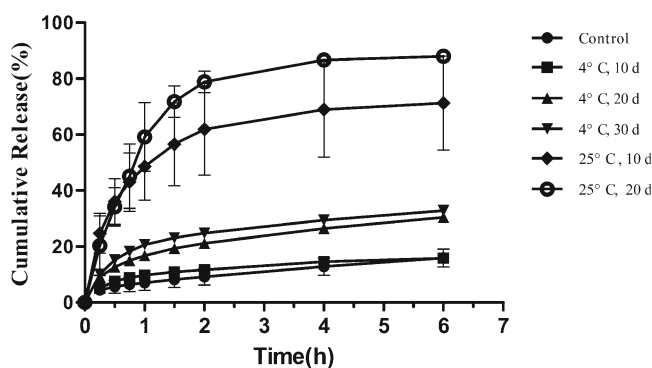


Fig. 6. Short-term release profile of *R*-TBH from liposomes in PBS at 37°C. These *R*-TBH liposomes were stored at different temperatures and for different intervals. Data are expressed as the mean ± standard deviation ($n = 3$)

Table II. Characterization of the *R*-TBH Liposomes before and after Nebulization

Nebulization	Size (nm)	Polydispersity index	Zeta potential (mV)	Entrapment efficiency (%)
No	128.222 ± 0.385	0.07 ± 0.004	-7.7 ± 0.035	66.08 ± 0.533
Yes	130.411 ± 6.139	0.08 ± 0.003**	-7.5 ± 0.460	56.33 ± 0.604**

Data represent mean ± standard deviation ($n = 3$, ** $P < 0.01$)

(the concentration of *R*-TBH is 1 mg/mL), the increase in the peak R_{aw} during histamine-induced asthma after 4 min of administration was $44.36 \pm 23.26\%$, which was much lower than the increase observed for the control; furthermore, the peak C_{dyn} was $-58.56 \pm 11.53\%$, which was a significant increase ($P < 0.01$) compared to the control. At 4 h post-administration, the peak R_{aw} and C_{dyn} during the asthma response were $623.23 \pm 358.95\%$ and $-86.99 \pm 3.37\%$, respectively. Compared to the control, the former was decreased significantly ($P < 0.01$), and the latter was increased significantly ($P < 0.05$), indicating that the inhalation of the *R*-TBH liposomes can significantly relieve asthma, even at 4 h post-administration (Fig. 9a, b).

A similar test was conducted by administering the same dose of the *R*-TBH solution aerosol before the histamine challenge. The asthmatic response was recorded at certain intervals (at 4 min and 4 h post-administration). In the control treatment, the peak increase in R_{aw} and peak decrease in C_{dyn} before the inhalation of *R*-TBH solution aerosol were $1206.98 \pm 383.09\%$ and $-90.82 \pm 4.55\%$, respectively. However, compared with the control, the peak R_{aw} reduced greatly and peak C_{dyn} increased greatly ($P < 0.01$) at 4 min post-inhalation of the *R*-TBH solution aerosol, whereas no significant changes in R_{aw} and C_{dyn} were detected at 4 h post-administration. The above results indicated that the anti-asthmatic effect of the *R*-TBH solution aerosol on guinea pigs was not sustained up to 4 h after inhalation (Fig. 9a, b).

The two groups of guinea pigs treated with the *R*-TBH liposome suspension or free *R*-TBH solution displayed an anti-asthmatic effect, whereas no anti-asthmatic effects were observed for the blank liposome group. As shown by the data (Fig. 9a, b), the R_{aw} or C_{dyn} in the blank liposome group were

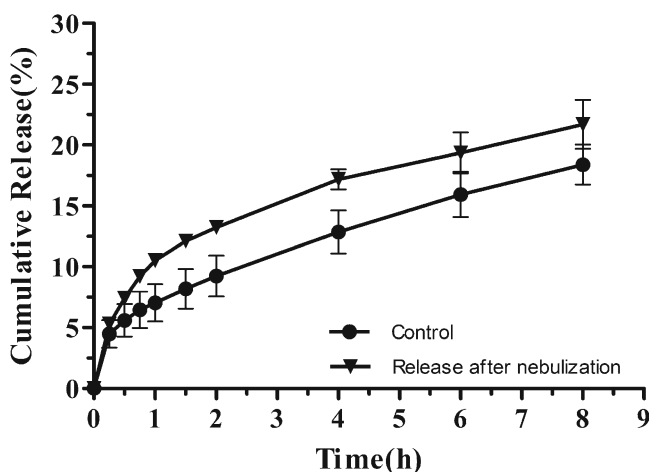


Fig. 7. Short-term release profile of *R*-TBH from liposomes before and after nebulization. Data are expressed as the mean ± standard deviation ($n = 3$)

not significantly different before and after inhalation of the aerosol in terms of the percent change from the baseline.

The results from our pharmacodynamic experiments proved that *R*-TBH liposome aerosol exhibited a more prolonged anti-asthmatic effect in guinea pigs than free *R*-TBH solution aerosol.

DISCUSSION

TBT is a short-acting β_2 -adrenergic receptor agonist and has a short elimination half-life (27,28). Several studies in the literature have focused on developing a long-acting TBT formulation to improve patient compliance (29–33). *R*-TBH, a more potent enantiomer of terbutaline, was chosen as the model drug for developing a sustained-release *R*-TBH formulation in this study. For the treatment of the asthma, inhalation therapy is accepted as the prioritized therapy (34). Liposomes are particularly suitable drug delivery vehicles for pulmonary administration, because liposomes are not only safe and well tolerated by the lungs but also capable of serving as sustained release carriers for the drug. Several studies of TBT liposomes have shown that encapsulation in liposomes prolongs the residence time of TBT in the lungs, thus providing the possibility of prolonged therapeutic efficacy (35–37). In the studies on TBT liposomes, intratracheal instillation was used for pulmonary delivery; however, the distribution and absorption of inhaled aerosols in the lungs and airways are different from those of instilled liquids (36–39), and therefore, that the therapeutic efficacy of aerosol formulations may differ considerably from

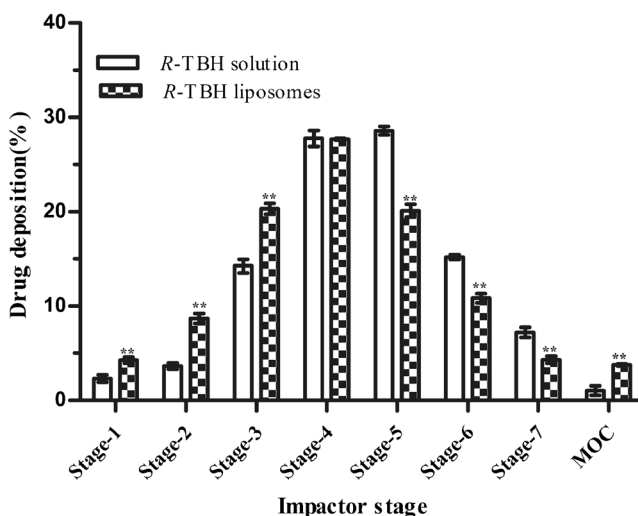


Fig. 8. Drug deposition rate for the *R*-TBH solution aerosol and *R*-TBH liposome aerosol at different stages. Data are expressed as the mean ± standard deviation, ($n = 3$, ** $P < 0.01$)

Table III. Particle Size Distribution under Cooling for Two Aerosol Formulations

Sample	MMAD (μm)	FPF%	GSD
<i>R</i> -TBH	3.28 ± 0.20	74.14 ± 3.26	1.87 ± 0.04
<i>R</i> -TBH liposomes	$4.00 \pm 0.10^{**}$	$62.16 \pm 1.48^{**}$	$1.97 \pm 0.003^{**}$

Cooling condition: 5°C

Data are expressed as mean $\pm$ SD, $n = 3$, $^{**}P < 0.01$

MMAD mass median aerodynamic diameter, FPF fine particle fraction, GSD geometric standard deviation

therapeutic efficacy of instilled formulations. Thus, in this study, we developed a sustained-release nebulized aerosol of

R-TBH liposomes that may contribute to obtaining a prolonged anti-asthmatic effect.

First, negative charged liposomes were prepared by the thin film dispersion method, and small-sized particles were obtained from an extruder. Particle size is a critical factor in the nebulization process. The smaller the particle size, the greater the number of nanoparticles accommodated in the micron-sized aerosol droplets, thereby improving the delivery of the drug to the deep lung because of the increased diffusional mobility (40). Smaller particle size also increases the rate of drug absorption by promoting a more uniform drug distribution (26). Since TBT is a weakly basic hydrophilic molecule with a low affinity for phospholipids, achieving a high EE in lipid-based formulations is difficult (41,42). For this reason, we optimized the EE of TBT in liposomes using active loading methods by varying the pH of the $(\text{NH}_4)_2\text{SO}_4$ medium, the incubation time of the drug with blank liposomes, and the concentration of the drug added into the liposomes. The results showed that with a liposomal internal pH of 4, a 1-h incubation time and a drug concentration of 3.5 mg/mL (data are shown in the supplemental material), optimal drug entrapment is achieved. According to the optimal prescription of *Rac*-TBT sulfate liposomes (data are shown in the supplemental material), *R*-TBH was efficiently encapsulated into liposomes with an EE of up to 71.35% because of the similar physicochemical properties of the two drugs. Storage stability studies showed that greater than 80% *R*-TBH was released from the liposomes stored at 25°C for 20 days (Table I). Therefore, extending the stability test at room temperature to 30 days was not necessary. Generally, liposomes are not stored at room temperature. Because liposomes comprise phospholipids and cholesterol, temperature affects the hydrolysis of liposomes, and 4°C is the optimum temperature for storing liposomes. Moreover, the cholesterol and phospholipids used to prepare liposomes are unlikely to undergo degradation during storage at 4°C (43). Furthermore, liposomal formulations have been shown to release drugs over intervals ranging from 2 weeks to several months (44), indicating that the integrity of liposomes is unlikely to be adversely affected in physiological fluid.

The anti-asthmatic study of liposomal *R*-TBH conducted in guinea pigs showed that the *R*-TBH liposome aerosol exerted an anti-asthmatic effect at 4 h post-administration, whereas no anti-asthmatic effect was observed for the *R*-TBH solution aerosol at 4 h post-administration. Encapsulating *R*-TBH in liposomes likely protects the drug from being metabolized quickly, and the efficacious dose of *R*-TBH released from the liposomes was available in the lungs of guinea pigs at 4 h post-administration, which plays a significant role in the observed anti-asthmatic activity. By contrast, 4 h after inhalation of the aerosol of *R*-TBH not encapsulated in liposomes, the drug may have been metabolized from the lungs, and asthma was not relieved. The rapid decline in R_{aw} and increase in C_{dyn} was shown 4 min after inhaling the *R*-TBH liposome aerosol and indicated that some

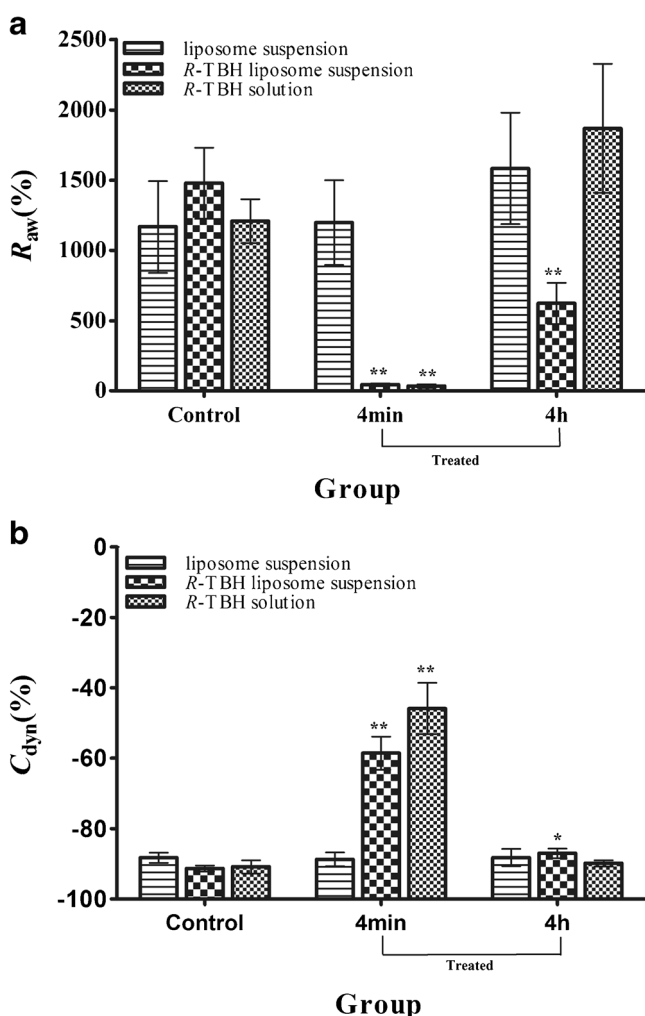


Fig. 9. Changes in R_{aw} (a) and C_{dyn} (b) at 4 min and 4 h after inhaling blank liposomes, *R*-TBH liposomes, or *R*-TBH solution. The animals were divided into three groups (each group has six guinea pigs, three males and three females): I, the control treatment followed by inhaling blank liposomes (2 min); II, the control treatment followed by inhaling *R*-TBH liposomes (1 mg/mL, 2 min); and III, the control treatment followed by inhaling *R*-TBH solution (1 mg/mL, 2 min). Data are expressed as the mean $\pm$ SE, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control treatment

free *R*-TBH was available in the *R*-TBH liposome suspension after nebulization. According to the results of the stability test of *R*-TBH liposomes during nebulization, a small amount of drug was released from the liposomes prematurely because of spray damage, which may play a significant role in the anti-asthma effect observed at 4 min after administration. However, the remaining drug in the liposomes continued to be released over 4 h and significantly relieved asthma (Fig. 9a, b). Because the duration of anesthesia restricts the tests of normal physiological indicators to only approximately 4–5 h, the anti-asthmatic effect of the *R*-TBH liposomes was monitored for only 4 h in our study. However, the anti-asthma effect of the *R*-TBH liposomes was significant at 4 h post-administration, indicating that a sustained release of *R*-TBH from the liposomes for at least for 4 h *in vivo* was achieved. Thus, the results of anti-asthmatic study of liposomal *R*-TBH conducted in guinea pigs led to the conclusion that the *R*-TBH liposome aerosol exerted a more prolonged anti-asthmatic effect than the *R*-TBH solution aerosol because of the sustained release of *R*-TBH from the liposomes.

CONCLUSION

In this study, water-soluble *R*-TBH was efficiently encapsulated into liposomes. The high EE and *in vitro* sustained release of *R*-TBH from the liposomes were achieved. The MMAD and FPF% of the *R*-TBH liposomal aerosols are beneficial for its anti-asthmatic efficacy. The pharmacodynamic study in a guinea pig model suggests that liposomes prolonged the anti-asthmatic effect of *R*-TBT administered *via* pulmonary delivery in comparison to the immediate bronchodilating effect of unencapsulated *R*-TBH. Future studies may need to further improve the stability of this liposomal aerosol formulation for pharmaceutical therapy.

ACKNOWLEDGEMENTS

The authors thank Key-Pharma Biomedical Inc. for providing *R*-terbutaline hydrochloride. This work was supported by the Ministry of Science and Technology of China under Grant 201305017 and the Ministry of Science and Technology of Guangdong Province, China under Grant 2015B010109004.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCES

1. Emami J, Varshosaz J, Jafari E. Comparison of hydrophilic natural gums and cellulosic polymers in formulation of

- sustained-release matrix tablets of terbutaline sulfate. *Res Pharm Sci.* 2012;1(1):30–9.
2. Hartley D, Middlemiss D. Absolute configuration of the optical isomers of salbutamol. *J med Chem.* 1971;14(9):895–6.
3. Wetterlin K. Resolution of terbutaline, a new. beta.-sympathomimetic amine. *J med Chem.* 1972;15(11):1182–3.
4. Borgstrom L, Nyberg L, Jonsson S, *et al.* Pharmacokinetic evaluation in man of terbutaline given as separate enantiomers and as the racemate. *Br J Clin Pharmacol.* 1989;27(1):49–56.
5. Guan S, Hu C, He M, *et al.* Comparative pharmacokinetics and bile transformation of R-enantiomer and racemic bambuterol after single-dose intravenous, oral administration in rats and beagle dogs. *Eur J Drug Metab Pharmacokinet.* 2015;40(4):453–60.
6. Zhou T, Zhao T, Cheng Q, *et al.* A sensitive LC-MS/MS method for simultaneous determination of R-bambuterol and its active metabolite R-terbutaline in human plasma and urine with application to a clinical pharmacokinetic study. *Biomed Chromatogr.* 2014;28(7):994–1002.
7. Jeppsson AB, Johansson U, Waldeck B. Steric aspects of agonism and antagonism at β -adrenoceptors: experiments with the enantiomers of terbutaline and pindolol. *Basic Clin Pharmacol Toxicol.* 1984;54(4):285–91.
8. Aagaard L, Hansen EH. Paediatric adverse drug reactions following use of asthma medications in Europe from 2007 to 2011. *Int J Clin Pharm.* 2014;36(6):1222–9.
9. Borgström L, Nilsson M. A method for determination of the absolute pulmonary bioavailability of inhaled drugs: terbutaline. *Pharm res.* 1990;7(10):1068–70.
10. Stewart IC, Rhind GB, Power JT, *et al.* Effect of sustained release terbutaline on symptoms and sleep quality in patients with nocturnal asthma. *Thorax.* 1987;42(10):797–800.
11. Dahl R, Pedersen B, Hägglöf B. Nocturnal asthma: effect of treatment with oral sustained-release terbutaline, inhaled budesonide, and the two in combination. *J Allergy Clin Immunol Pract.* 1989;83(4):811–5.
12. Westermann CJJ, Van Weelden BM, Laros CD. Sustained-release terbutaline in nocturnal asthma. *Allergy.* 1986;41(4):308–10.
13. Dahl R, Harving H, Säwedal L, *et al.* Terbutaline sustained-release tablets in nocturnal asthma—a placebo-controlled comparison between a high and a low evening dose. *Br J Dis Chest.* 1988;82:237–41.
14. Holimon TD, Chafin CC, Self TH. Nocturnal asthma uncontrolled by inhaled corticosteroids. *Drugs.* 2001;61(3):391–418.
15. Loira-Pastoriza C, Todoroff J, Vanbever R. Delivery strategies for sustained drug release in the lungs. *J Appl Physiol.* 2014;75:81–91.
16. Lasic DD. Recent developments in medical applications of liposomes: sterically stabilized liposomes in cancer therapy and gene delivery *in vivo*. *J Control Release.* 1997;48(2):203–22.
17. El-Sherbiny IM, El-Baz NM, Yacoub MH. Inhaled nano- and microparticles for drug delivery. *Glob Cardiol Sci Pract.* 2015;2.
18. Schreier H, Gonzalez-Rothi RJ, Stecenko AA. Pulmonary delivery of liposomes. *J Control Release.* 1993;24(1):209–23.
19. Zhang X, Liu Q, Hu J, *et al.* An aerosol formulation of R-salbutamol sulfate for pulmonary inhalation. *Acta Pharm sin B.* 2014;4(1):79–85.
20. Yang Z, Lu A, Chi Kwan Wong B, *et al.* Effect of liposomes on the absorption of water-soluble active pharmaceutical ingredients via oral administration. *Curr Pharm des.* 2013;19(37):6647–54.
21. Vargas MH, Sommer B, Bazán-Perkins B, *et al.* Airway responsiveness measured by barometric plethysmography in guinea pigs. *Vet res Commun.* 2010;34(7):589–96.
22. Begin R, Renzetti AD, Bigler AH, *et al.* Flow and age dependence of airway closure and dynamic compliance. *J Appl Physiol.* 1975;38(2):199–207.
23. Saini D, Biris AS, Srirama PK, *et al.* Particle size and charge distribution analysis of pharmaceutical aerosols generated by inhalers. *Pharm dev Technol.* 2007;12(1):35–41.
24. Martin RJ. Therapeutic significance of distal airway inflammation in asthma. *J Allergy Clin Immunol.* 2002;109(2):S447–60.
25. Carvalho TC, Peters JI, Williams RO. Influence of particle size on regional lung deposition—what evidence is there? *Int J Pharm.* 2011;406(1):1–10.

26. Yang W, Peters JI, Williams RO. Inhaled nanoparticles—a current review. *Int J Pharm.* 2008;356(1):239–47.
27. Murthy SN, Hiremath SRR. Physical and chemical permeation enhancers in transdermal delivery of terbutaline sulphate. *AAPS PharmSciTech.* 2001;2(1):1–5.
28. Gillies HC, Rogers HJ, Spector RG, Trousseau JR. A textbook of clinical pharmacology. 2nd ed. London: Edward Arnold; 1986. p. 473.
29. Sahin S, Selek H, Ponchel G, *et al.* Preparation, characterization and *in vivo* distribution of terbutaline sulfate loaded albumin microspheres. *J Control Release.* 2002;82(2):345–58.
30. Hashem FM, Nasr M, Fathy G, *et al.* Formulation and *in vitro* and *in vivo* evaluation of lipid-based terbutaline sulphate bi-layer tablets for once-daily administration. *AAPS PharmSciTech.* 2016;17(3):727–34.
31. Khattab I, Bandarkar F, Lila A. Formulation and optimization of sustained release terbutalinesulfate microspheres using response surface methodology. *Drug Discov Ther.* 2009;3:123–35.
32. Al-Akayleh F, Al Remawi M, Rashid I, *et al.* Formulation and *in vitro* assessment of sustained release terbutalinesulfate tablet made from binary hydrophilic polymer mixtures. *Pharm dev Technol.* 2013;18(5):1204–12.
33. Hadi MA, Rao AS, Abhinetri V, *et al.* Design and in-vitro evaluation of multiparticulate drug delivery system of terbutaline sulphate for the treatment of nocturnal asthma. *Pharm Lett.* 2012;4(6):1777–85.
34. Dyreborg A, Krogh N, Backer V, *et al.* Pharmacokinetics of oral and inhaled terbutaline after exercise in trained men. *Front Pharmacol.* 2016;7:150.
35. Palakurthi S, Govardhanachary M, Vyas SP, *et al.* Biodistribution of liposomes of terbutaline sulfate in guinea pigs. *Drug dev Ind Pharm.* 2000;26(10):1099–105.
36. Joshi MR, Misra AN. Liposomes of terbutaline sulphate: *in vitro* and *in vivo* studies. *Indian J Exp Biol.* 1999;37(9):881–7.
37. Fielding RM, Abra RM. Factors affecting the release rate of terbutaline from liposome formulations after intratracheal instillation in the guinea pig. *Pharm res.* 1992;9(2):220–3.
38. Brown RA, Schanker LS. Absorption of aerosolized drugs from the rat lung. *Drug Metab Dispos.* 1983;11(4):355–60.
39. Brain JD, Knudson DE, Sorokin SP, *et al.* Pulmonary distribution of particles given by intratracheal instillation or by aerosol inhalation. *Environ res.* 1976;11(1):13–33.
40. Patlolla RR, Chougule M, Patel AR, *et al.* Formulation, characterization and pulmonary deposition of nebulized celecoxib encapsulated nanostructured lipid carriers. *J Control Release.* 2010;144(2):233–41.
41. Schilt Y, Berman T, Wei X, *et al.* Using solution X-ray scattering to determine the high-resolution structure and morphology of PEGylated liposomal doxorubicin nanodrugs. *Biochim Biophys Acta.* 2016;1860(1):108–19.
42. Gupta V, Gupta N, Shaik IH, *et al.* Liposomal fasudil, a rho-kinase inhibitor, for prolonged pulmonary preferential vasodilation in pulmonary arterial hypertension. *J Control Release.* 2013;167(2):189–99.
43. Haran G, Cohen R, Bar LK, *et al.* Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. *Biochim Biophys Acta.* 1993;1151(2):201–15.
44. Lindner LH, Hossann M. Factors affecting drug release from liposomes. *Curr Opin Drug Discov Devel.* 2010;13(1):111–23.

1 **Supplemental materials**

2 Table I. EE(%)and DL(%)at different pH values. The concentration of the drug is 15
3 mg/mL, and the incubation time is 1 h.

4	pH value inside of the	Encapsulation	Drug Loading(%)
	liposome	efficiency(%)	
5			
6	pH=3	18.09	9.85
7	pH=4	20.67	11.04
8	pH=5	20.17	10.81

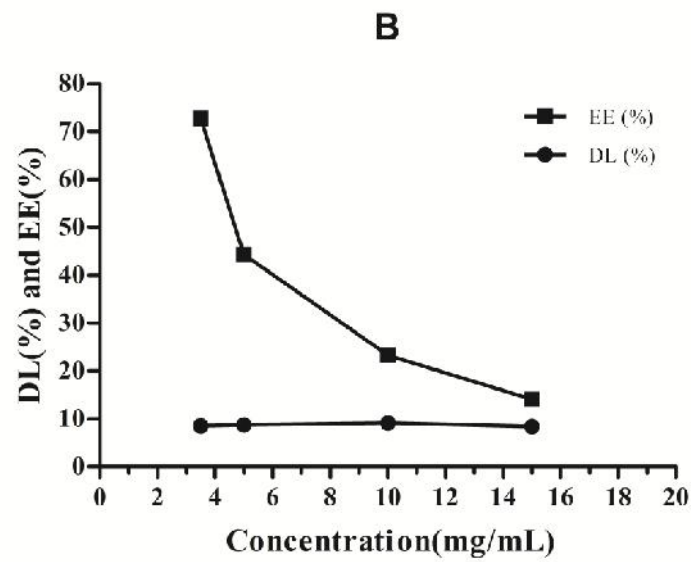
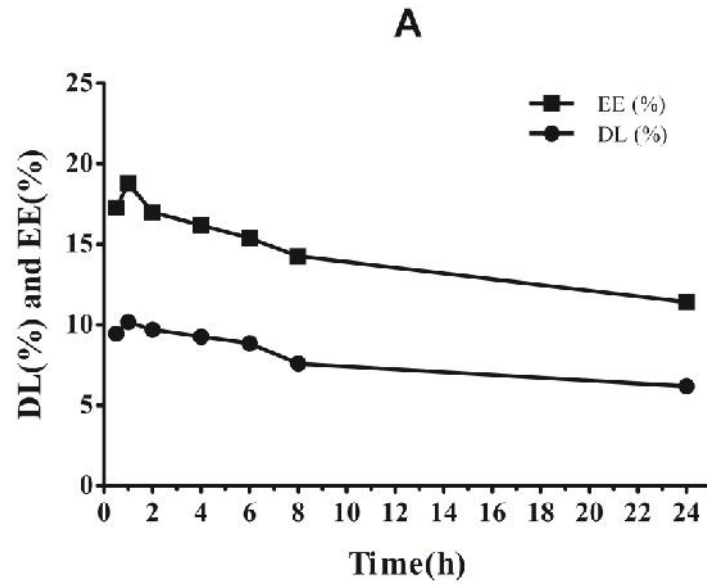


Figure 1. Effect of the incubation time and drug concentration on the EE (%) and DL (%) of *Rac*-TBT liposomes. A, The concentration of the drug is 15 mg/mL, and the liposomal internal pH is 4. B, The incubation time is 1 h, and the liposomal internal pH is 4.