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Pulmonary distribution and clearance of two beclomethasone liposome formulations in healthy volunteers

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Abstract

The pulmonary distribution and clearance of ^{99m}Tc-labelled beclomethasone dipropionate (Bec) dilauroylphosphatidylcholine (DLPC) and dipalmitoylphosphatidylcholine (DPPC) liposomes were compared in 11 healthy volunteers using gamma scintigraphy. As delivered by using the Aerotech jet nebulizer both liposome aerosols had a suitable droplet size (mass median aerodynamic diameter 1.3 μm) allowing deep pulmonary deposition. However, in the total drug output during the inhalation there was a relatively large difference between DLPC and DPPC of 11.4 and 3.1 μg, respectively. In a gamma camera study no significant differences existed in the central/peripheral lung deposition between the DLPC and DPPC formulations. Progressive clearance of both Tc-labelled Bec liposomes was seen: 24 h after inhalation, 79% of the originally deposited radioactivity of DLPC liposomes and 83% of that of DPPC liposomes remained in the lungs. Thus there was slightly slower clearance of inhaled liposomes using DPPC instead of DLPC. We conclude that both liposome formulations are suitable for nebulization, although aerosol clouds were more efficiently made from the DLPC liposome suspension. Our results support the view that liposome encapsulation of a drug can offer sustained release and drug action in the lower airways.

Keywords: Aerosol; Dilauroylphophatidylcholine (DLPC); Dipalmitoylphophatidylcholine (DPPC); Liposome; Nebulizer; ^{99m}Technetium

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1. Introduction

The administration of liposome-encapsulated drugs by aerosols seems to be a feasible way of targeting drugs into the lungs. Previous studies have indicated that liposome encapsulation of a drug offers a prolonged release and drug action in the lower airways (Mihalko et al., 1988), limited redistribution to other tissues (Meisner et al., 1989) and decreased incidence of side-effects (McCalden and Abra, 1989). The tolerability and safety of liposome aerosols have been previously tested in animals as well as in human volunteers (Thomas et al., 1991; Myers et al., 1993; Knight et al., 1994; Waldrep et al., 1997). No untoward effects have been reported. The beclamethasone (Bec)–dilauroyldiphosphatidylcholine (DLPC) liposomes used in our studies have proved to be technically suitable for nebulization (Waldrep et al., 1994a) and have offered good pulmonary deposition with slow clearance. In addition, Bec–DLPC liposomes were well tolerated by patients (Waldrep et al., 1997).

In our previous study, we evaluated the deposition and clearance of inhaled Bec–DLPC liposomes in severe and mild asthmatics (Saari et al., 1998). The clearance of inhaled ^{99m}Tc-labelled Bec–DLPC was found to be strikingly slow in both groups of asthmatics. However, a clear difference in distribution patterns in mild and severe asthmatics could be detected.

To extend the previous evaluation, the aim of this study was to compare the intrapulmonary distribution and clearance of two different liposome formulations in healthy volunteers. There were several reasons for including beclomethasone–dipalmitoylphosphatidylcholine (Bec–DPPC) in this study. First, DPPC is a major component of the pulmonary surfactant found in the respiratory tract and is thought to be a response to the stability of the surface tension reducing film (Wright and Clemets, 1987). Secondly, DPPC is the main phospholipid derivative in exogenous surfactant therapy in the treatment of neonatal respiratory distress syndrome (RDS) (Hennes et al., 1991; Morley 1991). Besides these physiological arguments, we were interested in the aerosol properties of nebulized DPPC liposomes. Thus, before entering clinical trial, the particle size distribution and aerosol properties of nebulized liposome preparations were analysed.

2. Materials and methods

2.1. Liposome preparation and aerosol characteristics

Multilamellar (MLV) beclomethasone dipropionate (Bec) liposomes were prepared by a freeze-drying technique from the phosphatidylcholine (PC) derivatives dilauroylphophatidylcholine (DLPC) and dipalmitoylphophatidylcholine (DPPC) as previously described by Waldrep et al. (1994b). Briefly, 1 mg of drug and 25 mg of the phospholipid were dissolved in 10 ml of *t*-butanol. After mixing, the Bec–phospholipid solution was pipetted into glass vials, rapidly frozen in dry ice–acetone and lyophilized overnight to remove the organic solvent. The MLV liposomes were reconstructed by incubating the freeze-dried samples in sterile water above the phospholipid phase transition temperature (Bec–DLPC 37°C, Bec–DPPC 50°C). The final drug concentration was 500 μg/ml.

In order to analyze the effect of nebulization (Aerotech II CIS-US, Bedford, USA) on liposome size, the liposome aerosol was collected for 4 min in a specially constructed collection chamber (All-glass Impinger, AGI, Ace Glass Co., Vineland, NJ, USA) using an air flow of 12.5 l/min. Liposome size distribution was determined before and after nebulization by using the quasi-elastic light scattering method (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, USA). The particle size distribution were determined as mean diameter on the basis of vesicle volume. The AGI device was used also to measure the total Bec aerosol output. The standard sampling period was 2 min after 1 min of operation of the nebulizer.

Furthermore, the mass median aerodynamic diameter (MMAD) and geometric standard deviation (G.S.D.), as well as a small particle size fraction of the aerosol cloud (< 5.8 μm), were determined by using the Andersen cascade impactor (Andersen Instruments Inc., Atlanta, GA,

USA). The Bec liposome aerosols generated from the jet nebulizer were collected through a metal throat (USP) to the sampler at a sampling time of 4 min. The air flow through the impactor was adjusted to 28.3 l/min.

The Bec content of the samples was determined by high-performance liquid chromatography (HPLC) analysis using a Promis II autosampler (Spark Holland BV, Emmen, The Netherlands) and a Supelcosil® LC-18-DB column (14 cm × 4.6 mm, 5 μm particle size; Supelco Inc., Bellefonte, PA, USA) at room temperature. Peak detection for Bec was performed at 254 nm using a variable-wavelength detector (LKB 2151, Bromma, Sweden) with an integrator (Hitachi D-2500, Hitachi Ltd., Tokyo, Japan). The mobile phase utilized for these studies was methanol–water (80:20) at a flow rate of 1.2 ml/min. Samples for analysis were dissolved in methanol or ethanol.

2.2. Labelling of Bec–DLPC and Bec–DPPC liposomes with ^{99m}Tc

The reconstructed liposomes were labelled with ^{99m}Tc using the stannous reduction method described by Barratt et al. (1983). In the labelling process, 1 ml of the liposome suspension was mixed with 0.5 ml of 3 mM $SnCl_2$ solution. In the preparation of the stannous chloride solution, it is important to exclude the possibility of the oxidation of tin to the unreactive stannic form. Therefore, before dissolving stannous chloride (67 mg per 100 ml), sterile, pyrogen-free water was bubbled for 30 min with nitrogen in order to expel most of the oxygen. Then 1 ml of technetium pertechnetate in sterile saline was added; the mixture (total volume 2.5 ml) was shaken vigorously for 1 min and left to react at room temperature for 30 min. The radioactivity in 1 ml of technetium pertechnetate was approx. 27 mCi.

Labelling efficiency was determined by paper chromatography (ITCL-SG, product 61885; Gelman Sciences, Ann Arbor, MI). Free pertechnetate migrates to the top of the paper, while liposomally entrapped material remains at the application point. The labelling yield was expressed as a percentage of the total amount of radioactivity applied in the testing system.

2.3. Subjects studied

Eleven healthy non-smoking volunteers (three males and eight females) whose mean age was 37 years (range 23–50 years) took part in the study (Table 1). Medical histories were taken and physical examinations carried out by the attending pulmonary physician at the beginning of the study. None of the subjects had had an upper viral infection within the previous 4 weeks. Spirometric measurements (Vitalograf, Buckingham, UK) were performed before inhalation of corticosteroid liposomes. At least three technically correct manoeuvers for forced maximal expiratory flow volume (FEV) curves were performed, and the curve with the greatest sum of FEV_1 and forced expiratory vital capacity (FVC) was utilized to obtain data.

This trial was an open, randomized, two-period cross-over study. It was conducted according to the Declaration of Helsinki. Written, informed consent was obtained from all subjects, and the study protocol was approved by the Ethical Committee of Tampere University Hospital.

2.4. Corticosteroid liposome delivery

Bec liposome suspensions were inhaled by each subject. The time between the two test days was a minimum of 3 days and a maximum of 2 weeks. ^{99m}Tc-labelled liposome suspension was delivered from the commercially available jet nebulizer connected to an automatic, inhalation-synchronized dosimeter (Spira Elektro 2, Respiratory Care Cen-

Table 1
Demographic data of healthy volunteers

Characteristic[a]	Mean	S.D.	Range
Sex (M/F)	3/8		
Age (years)	37	8.9	23–50
Body mass index	22.7	1.9	20.4–25.5
FVC	4.8	0.9	3.5–6.4
FVC % of predicted	107	12.0	82–121
FEV_1	4	0.6	2.9–5.1
FEV_1 % of predicted	104	10.6	83–117

[a] FVC, forced expiratory vital capacity; FEV_1, forced expiratory volume.

ter, Hämeenlinna, Finland). This dosimeter is triggered by a very low inspiratory flow rate with a threshold of < 2 l/min digitally, and the inhalation flow rate is controlled by a flow indicator (Nieminen et al., 1987, 1988, 1991). A breath-actuated, variable-time circuit regulates air through a solenoid valve to a nebulizer set to a flow rate of 10 l/min. The dosimeter was set to commence nebulization at the beginning of inhalation but after the patient had inhaled a volume of 10 ml, with a 0.5-s nebulization period.

A total dose of 500 μg of beclomethasone dipropionate within the labelled liposomes (2.5 ml), having an initial radioactivity of approx. 990 MBq (27 mCi), was placed in the jet nebulizer. Subjects were instructed to place the nebulizer tightly between their lips and inhale deeply. With a noseclip and mouthpiece in place, the subject controlled breathing with a flow indicator (an LED screen) so that the inspiratory flow rate of each breath reached but did not exceed 30 l/min. Inhalation was followed by normal exhalation. Exhaled Bec liposomes were captured using a Hudson filter. This inspiration procedure was repeated 20 times according to the subject's own inspiratory cycle, without breath holding between inhalations. Prior to the experiment, each subject practised inhalation from the dosimeter nebulizer with saline.

2.5. Gamma camera measurements

Immediately after inhalation, anterior and posterior views of the lungs and an anterior view of the oropharynx were measured in a supine position by a large field gamma camera (GE, CamStar XR/T, Wisconsin, USA) equipped with a low-energy high resolution parallel collimator. In order to evaluate the mucociliary clearance of the inhaled liposomes, scans were repeated 1, 2, 4 and 24 h after the aerosol delivery. In addition, a posterior ventilation scan was obtained after the liposome study by inhaling the noble gas ^{133}Xe with a radioactive dose of 460 MBq (12.5 mCi). All images were stored on a computer (Hermes, Nuclear Diagnostics, Hägersten, Sweden) for subsequent data analysis.

^{133}Xe posterior images were used when regions of interest (ROI) were manually drawn around central (C) and peripheral (P) lung zones. ROIs were subsequently superimposed upon each liposome aerosol view, enabling the quantity of aerosol dose in each of the zones to be determined. Each image was manually aligned, i.e. each lung view was shifted to adapt to the superimposed ROIs. The lungs were divided into inner and outer regions, with the central zone encompassing 33% ($\pm$ 2%) of the total lung area and the outer region the remaining area (Smaldone et al., 1988, 1989). Lung distribution of the liposome aerosol was described as the ratio between central and peripheral lung areas (C/P ratio) and the total lung clearance curve as a plot of the percentage of initial lung burden versus time after inhalation.

The numbers of counts and pixels in each region of interest were measured and saved on a file in the Hermes computer. Subsequently, the data were transferred via a local area network to a personal computer and analysed with a program specially made for this study. Counts from the anterior and posterior views of the lungs were combined by taking geometric mean values. The camera-to-patient distance was standardized by placing the collimator close to the chest for the anterior view and in contact with the imaging bed for the posterior view. Geometric mean counts were corrected for the room's background—measured separately from each image—and for radioactive decay.

An approximate tissue absorption correction was carried out using the method described by Macey and Marshall (1982). Briefly, individual transmission images of each subject's lung region were taken prior to the liposome study using a flat radiation source and keeping the imaging geometry similar both in transmission and ventilation scans. This transmission method was used to correct the individual emission counts recorded with the gamma camera.

2.6. Statistical analysis

The absolute values at the baseline (0 h), the percentage of ^{99m}Tc detected in the lungs after 24 h and C/P ratio were used for the final analysis.

Table 2
Characteristics of the Bec–DLPC and Bec–DPPC liposomes

Bec liposome formulation	Mean liposome size[a] (μm)		MMAD (μm)	G.S.D.	Bec output (μg/min)
	Before aerosolization	After aerosolization			
DLPC	3.49 (1.16)[b]	0.83 (0.08)	1.3	2.7	68.6
DPPC	5.07 (2.06)	0.91 (0.26)	1.3	2.2	18.3

[a] Mean liposome size is the mean diameter on the basis of vesicle volume.
[b] S.D. in parentheses.

The analysis of variance for repeated measurements for cross-over designs was used to test the differences between pulmonary distribution and clearance of Bec–DLPC and Bec–DPPC at 24 h.

A conventional model including treatment, period and carryover effect of the treatment as independent factors was used. The 95% confidence interval (CI) of the difference between treatments was also calculated.

3. Results

Using the quasi-elastic light scattering method, the mean liposome size (S.D.) was determined as 3.49 (1.16) and 5.07 (2.06) μm before nebulization and 0.83 (0.08) and 0.91 (0.26) μm after nebulization for the DLPC and DPPC liposomes, respectively. The cascade impactor analysis showed a high fine particle size fraction of the delivered drug dose, being 93% for the DLPC and 96% for the DPPC liposomes. The MMAD (G.S.D.) was 1.3 (2.7) μm for the DLPC and 1.3 (2.2) μm for the DPPC liposomes. There was a relative large difference in drug rate of output between the liposome formulations. The output of Bec–DLPC and Bec–DPPC liposomes nebulized with the Aerotech II nebulizer was 68.6 and 18.3 μg/min, respectively. The total rate of output of the DLPC aerosol was 11.4 μg and that of the DPPC liposome 3.1 μg. A smaller output of the Bec–DPPC aerosol was also seen as a lower level of the radioactivity measured in respiratory tract after inhalation, being 427 400 counts for the DLPC and 171 200 counts for the DPPC. The characteristics of the two liposomes are summarized in Table 2.

Instant thin-layer chromatography (ITLC) was carried out after every labelling process of the liposomes. ITLC analysis showed a high labelling efficacy (96–99% for the DLPC and 97–99% for the DPPC).

Fig. 1 shows the regional pulmonary distribution of radiolabelled Bec liposomes in healthy subjects. Immediately after liposome inhalation, no significant differences existed in central and peripheral depositions (C/P ratio) between the DLPC and DPPC formulations: 0.66 (S.D. 0.05) and 0.64 (S.D. 0.07), respectively ($p = 0.21$). The similar homogeneous distribution pattern of both formulations was retained during the entire fol-

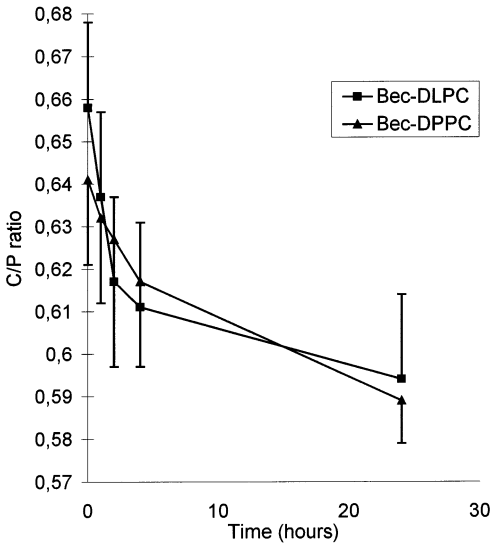

Fig. 1. C/P ratio as a function of time in healthy subjects following inhalation of ^{99m}Tc-labelled Bec–DLPC and Bec–DPPC liposome aerosols. Values are presented as mean ± S.E.

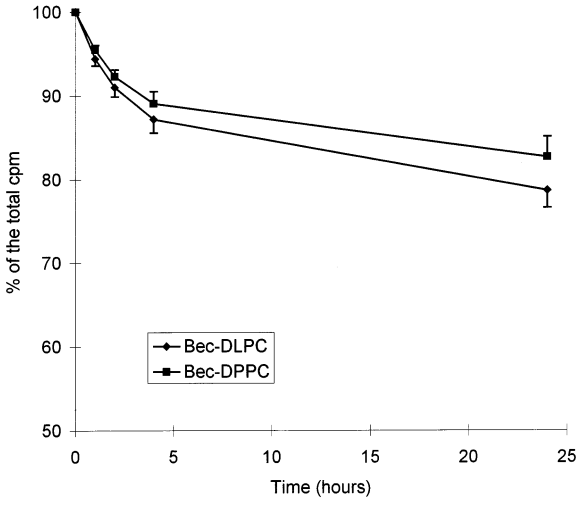

Fig. 2. The clearance of ^{99m}Tc from the lungs of healthy volunteers following inhalation of radiolabelled Bec–DLPC and Bec–DPPC liposome aerosols. Values are presented as mean ± S.E.

low-up period. The C/P ratio of the DLPC liposomes 24 h after inhalation was 0.60 (S.D. 0.06) and of the DPPC formulation 0.59 (S.D. 0.05).

The progressive clearance of both ^{99m}Tc-labelled Bec liposomes in healthy volunteers is shown in Fig. 2 and Table 3. However, DLPC liposomes were cleared somewhat faster than DPPC liposomes. At the 4-h measurement point, a mean of 87% (S.D. 5.4) of the total pulmonary dose was detected in the lungs after Bec–DLPC inhalation, whereas after Bec–DPPC inhalation the figure was 89% (S.D. 4.8). On average, 79% (S.D. 6.8) of the DLPC liposomes was detected in the whole lung area after 24 h, whereas on average 83% (SD 8.1) of the DPPC liposomes remained in the lungs. The difference between the clearance of the two liposomes after 24 h was 4% (95% CI: 0.9–7.2%, $p = 0.001$).

The cross-over analysis revealed a slight period effect ($p < 0.05$), indicating that the total measured counts from the lung region was higher during the second liposome inhalation experiment. This might be due to the slightly higher concentration of radioactivity in the test preparation used on the second study day. Furthermore, the learning process of the inhalation manoeuver was apparent, in spite of the fact that nebulization was practised by each subject before the experiment. More importantly, no carryover effect was detected between the two experiments.

3.1. Adverse events

One male subject felt shortness of breath and cough after both inhalations of Bec liposomes. The spirometry during symptoms was normal with no significant changes to baseline values prior to inhalation. Dyspnea, described as moderate, was relieved after salbutamol inhalation.

4. Discussion

The nebulization of liposome suspensions has proved to be a simple and suitable way of delivering liposomes to the lower airways. During nebulization, liposome vesicles are reduced in size by shear forces associated with continuous re-cycling through the nebulizer. The pulmonary deposition of multilamellar vesicles delivered from an air jet nebulizer is dependent on the droplet size of the aerosol product rather than liposome vesicle diameter before nebulization (Farr et al., 1985; Gilbert et al., 1988; Barker et al., 1994). Aerosols with a so-called 'fine particle fraction' (i.e. capable of efficient penetration to the bronchioles and alveoli) require a particle size of 5–6 μm or less (Stahlhofen et al,. 1980). In our study, the Aerotech II jet nebulizer was chosen for liposome delivery for having a small MMAD for aerosol droplets, as well as for our good experiences in aerosol delivery to the lower airways in previous studies with liposomes (Saari et al,. 1998; Vidgren et al., 1995). Using the quasi-elastic light scattering method, a marked decrease in mean vesicle size was found in both Bec–DLPC liposomes and Bec–DPPC liposomes as extruded through the Aerotech II nebulizer. There were no significant differences in vesicle sizes between these corticosteroid liposomes. The small particle size after nebulization as well as a high fine particle fraction showed that, in theory, both liposome formulations could be effectively delivered to the lungs. After inhalation of radioactive liposomes, the distribution patterns of the formulations could be

Table 3
Total lung counts in the lung region immediately after inhalation of ^{99m}Tc-labelled Bec–DLPC and Bec–DPPC liposomes and the clearance of ^{99m}Tc in a 24-h follow-up

	Time (h)	Bec–DLPC		Bec–DPPC		p-value
		Mean	S.D.	Mean	S.D.	
Total lung counts	0	427394	106654	171218	67720	<0.0001
^{99m}Tc in lungs (as % of initial amount)	0	100		100		
	1	94.4	2.6	95.5	1.9	
	2	91	3.6	92.3	2.7	
	4	87.2	5.4	89.1	4.8	
	24	78.7	6.8	82.7	8.1	0.001[a]

[a] In cross-over analysis a significant ($p<0.05$) period effect. The level of the measurements was higher during the second period.

detected and quantified in healthy subjects. No significant differences existed in the C/P index values between the DLPC and DPPC formulations. Both liposome formulations were thus similarly distributed in the central and peripheral parts of the lungs. This phenomenon could be detected throughout the study.

However, there was a significant difference in total Bec output between the two Bec liposomes. The output of DPPC was markedly lower than that of DLPC. That seems to be due to the high T_c of DPPC ($+41$°C) compared with DLPC (T_c -2°C) producing more solid and rigid liposomes which are inefficiently extruded through the nebulizer jet orifice. Thus it is important to evaluate the aerosol properties of each formulation before the final liposome formulation is chosen for inhalation.

In previous studies, the clearance of originally liposome-associated ^{99m}Tc proved to be strikingly slow (Farr et al., 1985; Barker et al., 1994; Vidgren et al., 1995). Vidgren et al. (1995) monitored the clearance of ^{99m}Tc-labelled Bec–DLPC liposomes in healthy volunteers in whom 93% of the original dose was still detected in the lungs after 3 h. In a similar study, Farr et al. (1985) measured the deposition and clearance of DPPC liposome aerosol after inhalation by normal volunteers. Subjects were monitored for 6 h after inhalation; 88% of the inhaled radioactivity was still present in the lungs. Barker et al. (1994) have recently studied liposome (DPPC) entrapped ^{99m}Tc-DTPA and demonstrated that approx. 45% of originally deposited radioactivity remained in the lungs after 24 h. This represented the fraction of radiolabel remaining intact in alveolar-deposited vesicles, since free ^{99m}Tc-DTPA was removed from the airways with a half-life of 75 min. Our data correspond well with previously published data showing 79% of the original dose of DLPC and 83% of DPPC in the lungs 24 h after inhalation.

In our study, the clearance of liposome-bound ^{99m}Tc was strikingly slow and the clearance kinetics were similar in both groups of liposome formulations. However, the DLPC liposomes were cleared somewhat faster than the DPPC liposomes. This difference might be due to the different binding to the pulmonary surfactant and biological membranes as well as to the different cellular uptake of the liposomal phospholipid. The difference in phase transition temperatures (DLPC -2°C, DPPC $+41$°C) could also have some effect upon the clearance of liposomes from the lungs. The slightly higher C/P ratio immediately after inhalation of the DLPC formulation also indicates a greater degree of centrally deposited liposomes, which could quite evidently result from the G.S.D.s for the DLPC aerosols being greater than for the DPPC aerosols. Thus, they are likely more widely distributed over the respiratory tract, including greater deposition in the ciliated airways.

The male subject who felt shortness of breathing and cough after both inhalations of corticosteroid liposomes is a former national speed skater. Constant outdoor exposure to cold air may, in the

long run, lead to hyperreactivity of the lower airways without asthma-related symptoms or changes in spirometry (Bjermer and Larsson, 1996; Sue-Chu et al., 1996). The clinical expression was that the side-effects that occurred were due to mild bronchial hyperreactivity rather than adverse effects of the two liposome formulations tested. Unfortunately, we were not able to carry out the methacholine provocation test on the subject after the study in order to verify our assumption about bronchial hyperreactivity.

There are great expectations for the future regarding inhaled liposome corticosteroids in asthma therapy. They might permit once a day or even more infrequent steroid inhalation, while yielding fewer local and systemic side-effects. In the present study, we radiolabelled only the phospholipid part of the complex. Therefore, one must regard the present data with caution concerning corticosteroid treatment.

We conclude that the clearance of ^{99m}Tc, bound originally to either DLPC or DPPC liposomes, was markedly slow in both liposome groups, although the DLPC liposomes were cleared slightly faster than the DPPC liposomes. Both liposome formulations were suitable for nebulization, although aerosol clouds were more efficiently made from the DLPC liposome suspension. Our results confirm the view that liposomes delivered via the pulmonary route may act as a local sustained drug release reservoir, even though no direct conclusions concerning the retention of steroids in the liposome matrix can be drawn from this study design.

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