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A study of ^{99m}technetium-labelled beclomethasone dipropionate dilauroylphosphatidylcholine liposome aerosol in normal volunteers

M. Vidgren [a], J.C. Waldrep [b,*], J. Arppe [a], M. Black [b], J.A. Rodarte [c], W. Cole [d], V. Knight [b]

[a] *Department of Pharmaceutical Technology, University of Kuopio, P.O. Box 1627, FIN-70211, Kuopio, Finland*
[b] *Center for Biotechnology, Baylor College of Medicine, 4000 Research Forest Drive, The Woodlands, TX 77381, USA*
[c] *Pulmonary Section, Department of Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*
[d] *Nuclear Medicine Service, The Methodist Hospital, 6565 Fannin, Houston, TX 77030, USA*

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Abstract

^{99m}Technetium (^{99m}Tc) was bound to preformed beclomethasone dipropionate (Bec) dilauroylphosphatidylcholine (DLPC) liposomes (Bec-DLPC) in the presence of the reducing agent, stannous chloride. Labelling efficiency was 96–99% as determined by micropartition and chromatographic analysis. Andersen cascade impactor analysis showed close correlation of the distribution of ^{99m}Tc, DLPC, and Bec over the full range of particle sizes sampled. In mouse biodistribution studies, approximately one-half of ^{99m}Tc activity delivered to the lungs was retained at 24 h. ^{99m}Tc clearance was almost exclusively via the gastrointestinal tract. In contrast, free ^{99m}Tc (administered unbound to Bec-DLPC) liposomes was cleared from mouse lungs within a few minutes. Six normal volunteers inhaled 20 breaths of the labelled Bec-DLPC liposome aerosol from each of two nebulizers (Aerotech II, MMAD 1.5 μm, GSD 2.4) (Spira, MMAD 3.6 μm, GSD 2.5). Immediately following inhalation, the gamma camera analysis showed 17% pulmonary deposition of inhaled ^{99m}Tc-Bec-DLPC with the Aerotech II and 14% with the Spira nebulizer. At 3 h after Aerotech II exposure, 93% of deposited activity was retained in the lung and 82% was retained from the Spira nebulizer. These findings suggest that there is a stable association of ^{99m}Tc with the Bec-DLPC liposomes and that inhaled liposomes were cleared slowly from the lungs of normal volunteers. The smaller particles produced by the Aerotech II nebulizer are presumably responsible for the somewhat larger deposition and longer persistence of pulmonary activity. These findings encourage further study of this modality of treatment for asthma and related conditions.

Keywords: Liposome; Aerosol; Nebulizer; ^{99m}Technetium

* Corresponding author. Dept. of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA.

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1. Introduction

Metered dose inhalers (MDI) are commonly used to administer topical treatment of asthma with glucocorticoids (GC) (National Heart, Lung and Blood Institute, 1992; Chapman et al., 1993). These treatments, although beneficial, have limitations due to inefficient use by some patients, systemic GC effects due to swallowed drug, local *Candida* overgrowth, and dysphonia due to irritation of the vocal chords (Johansson et al., 1982; Reed, 1990; Packe et al., 1992; Brown et al., 1993). In an effort to increase therapeutic efficacy and to reduce untoward effects, we have prepared the topically active GC, beclomethasone dipropionate, in a liposome preparation with dilauroylphosphatidylcholine (Bec-DLPC) to be administered from aqueous suspension by jet nebulizer.

Liposomes are one of the carrier systems widely investigated for pulmonary drug delivery (Schreier et al., 1993). Liposome aerosols of appropriate particle size offer a means of increasing lung deposition, decreasing upper respiratory tract deposition and prolonging the residence time of deposited material in lower airways (Taylor and Farr, 1993).

In previous studies, we demonstrated that Bec-DLPC liposome aerosol can be administered in several different nebulizers which produce particle sizes (1–3 μm MMAD) suitable for lower respiratory tract deposition (Waldrep et al., 1993). In a previous study of this methodology, we gave 15-min treatments with DLPC alone and Bec-DLPC aerosol to 10 normal volunteers. Spirometric, biochemical and clinical observation disclosed no untoward effects (Knight et al., 1994). We now report the use in aerosol of Bec-DLPC liposomes labelled with ^{99m}Tc to visualize deposition and clearance of the radioactive preparation from the respiratory tract of six normal volunteers.

2. Materials and methods

2.1. Labelling of Bec-DLPC liposomes with ^{99m}Tc

Beclomethasone dipropionate (Bec) and dilauroylphosphatidylcholine (DLPC) liposomes were produced as described previously (Waldrep et al., 1994). Briefly, 1 mg Bec and 25 mg DLPC were dissolved in 10 ml *t*-butanol. After mixing, the Bec-DLPC solution was pipeted into glass vials, rapidly frozen in dry ice-acetone and lyophilized overnight to remove the organic solvent. The liposomes were produced by adding ultra-pure water to obtain a final drug concentration of 500 μg/ml. The mixture was stirred for 30 min at 37°C to allow hydration of liposomes.

The preformed Bec-DLPC liposomes were labelled with ^{99m}Tc in the presence of $SnCl_2$ (Merck, Darmstadt, Germany) as the reducing agent. In the labelling process the same lipid/$SnCl_2$/^{99m}Tc ratios as described previously by Barratt et al. (1983) were used. Thus, the formation of a colloidal complex of technetium and tin was avoided. In the preparation of $SnCl_2$ solution, it is important to exclude the possibility of oxidation of tin to the unreactive stannic form. Therefore, before dissolving stannous chloride (67 mg), 100 ml of sterile, pyrogen-free water was bubbled 30 min with helium and 30 min with nitrogen in order to expel all oxygen. 1 ml of preformed Bec-DLPC liposome suspension, containing 500 μg of Bec, was mixed with 0.5 ml of 3 mM $SnCl_2$ solution. Then, 1 ml of [^{99m}Tc]pertechnetate in sterile saline with radioactivity of 4 mCi was added, and the mixture (total volume 2.5 ml) was shaken vigorously for 1 min and left to react at room temperature for 30 min.

2.2. Assessment of ^{99m}Tc attachment to liposomes

The labelling efficiency was determined by using the Centrifree Micropartition system (Amicon, Beverly, MA) and paper chromatography (Whatman 31M chromatography paper). In the micropartition method 200 μl of the labelled Bec-DLPC suspension was placed in a micro-partition tube and centrifuged (5000 rpm) for 10 min. The radioactivity of the supernatant and the sediment was measured with a gamma counter (Ludlum Model 2200 Portable Scaler/Rate Meter) or gamma camera (Siemens ZLC 370). In the chromatographic method, an aliquot of the labelled Bec-DLPC liposome suspension was placed 1 cm from the bottom of a 0.5 cm × 6 cm strip of

chromatography paper. The paper was immersed in 1 ml of sterile saline in an open vial until the solvent had moved 5 cm up the paper. The paper was then cut in half cross-wise, and the two halves were counted separately in a gamma counter. The labelling yield was expressed as a percentage of the total amount of radioactivity applied into the testing systems.

To examine the quantities of Bec, DLPC and ^{99m}Tc in different particle size fractions, and the extent to which the amount of radioactivity corresponded to the amount of the Bec and the DLPC, cascade impaction studies were carried out by using the Andersen/ACFM non-viable ambient particle sizing sampler (Andersen Instruments Inc., Atlanta, GA). A jet nebulizer (Aerotech II, CIS-US, Bedford, MA or Spira, Respiratory Care Center, Hameenlinna, Finland) was connected to the Andersen sampler and the radioactive liposome suspension (2.5 ml) was aerosolized into the impactor using an input air flow of 10 l/min (Aerotech II) or 8 l/min (Spira) through the nebulizer. Aerosol samples were collected for 1 min as previously described (Waldrep et al., 1993). The radioactivity on different stages of the impactor was detected with a gamma counter, and the corresponding Bec and DLPC concentrations were determined by HPLC methods. The mass median aerodynamic diameters (MMAD) and geometric standard deviations (GSD) were determined from Bec, DLPC, or ^{99m}Tc on each stage of the sampler using a computer program (Kaleidograph, Version 2.0, Synergy Software, Reading, PA).

2.3. Glucocorticoid analysis

The HPLC assay is used to determine the Bec content of liposome formulations and the Bec content of aerosol samples obtained with the Andersen sampler and the AGI. Bec concentrations are determined by HPLC analysis using a Waters 710B WISP autosampler and a Waters Nova-Pak C18 (3.9 × 150 mm) column at room temperature (Waldrep et al., 1993). Peak detection for Bec was performed at 238 nm using a variable-wavelength detector with quantification on an integrator. The mobile phase utilized for these studies was 50:50 ethanol/water at a flow rate of 0.8 ml per min. Samples for analysis were dissolved directly into ethanol (to solubilize the liposomes).

2.4. DLPC analysis

A modification of the HPLC protocol of Grit et al. (1991) was used to measure DLPC. A Waters 717 WISP automatic sample injector and a Spherisorb S5 amino column (25 cm × 4.6 mm, 5 μm) is used with acetonitrile, methanol, and 10 mM ammonium/trifluoroacetic acid, pH 4.8 (64:28:8 v/v) mobile phase. Peaks were detected with a mass evaporative detector (Sedex 55, Sedere, France) and quantified with an integrator. Samples for analysis were dissolved directly in mobile phase (to solubilize the liposomes).

2.5. Animal studies

For the in vivo assessment of the labelling method, 40 μl of the radioactive Bec-DLPC suspension was administered intranasally into Balb/c mice under methoxyflurane anesthesia. The mice were killed at time 0, 1, 4, 8 and 24 h (three animals per time point) after the installation of the radioactive Bec-DLPC liposomes. The relative distribution (%) of radioactivity in different tissues (lung, GI tract, liver, kidney and oropharynx) was detected by using a gamma counter. Three parallel determinations in each time point were performed. To assess the absorption rate of free ^{99m}Tc, 40 μl of [^{99m}Tc]pertechnetate in saline was administered as a reference.

2.6. Aerosol administration to normal volunteers

An open, randomized, cross-over design was used to study pulmonary deposition and clearance of ^{99m}Tc-labelled Bec-DLPC liposomes in six normal volunteers, three males and three females ranging between 25 and 41 years of age. Routine spirometry was normal. The aerosol was delivered by Aerotech II and Spira jet nebulizers. Mouth-in and mouth-out breathing was chosen to reduce nasopharyngeal deposition and minimize possible local toxic effects. The study consisted of two gamma camera studies with a minimum washout period of 2 days between the aerosol administrations. Before the gamma camera study,

the particle size distributions of the generated aerosols were determined with the Andersen cascade impactor as well as by the laser diffraction method (Malvern laser diffractometer, type 2600C, Malvern Instruments Ltd, UK). The air flow through the Aerotech was adjusted to 10 l/min and with the Spira the air flow was 8 l/min. The MMADs (GSD) of the generated aerosols, measured with the cascade impactor, were 1.5 μm (2.4) and 3.6 μm (2.5) after delivery from the Aerotech and Spira jet nebulizers, respectively. In the laser diffraction analysis, the droplet size distribution of the generated aerosols was measured 8 cm from the nebulizer orifice. According to the laser diffraction analysis, the mass median diameters (MMD) were 1.5 μm for the Aerotech and 3.3 μm for the Spira. For the gamma scintigraphy study, for containment of radioactive aerosols, the ^{99m}Tc-Bec-DLPC liposome aerosols were generated during inhalation phase by a Pari Jet Interrupter valve system (Munich, Germany) between the air compressor and nebulizer. Particle size analysis of the radiolabelled Bec-DLPC aerosol (20 actuations, each 5 s) showed MMADs (GSD) of 1.2 μm (1.7) and 3.2 μm (2.7) for the Aerotech II and Spira nebulizers, respectively. Thus, the interrupted aerosol generation did not have significant effect on the particle size distributions. On average, there was a 37.6% higher output with the Spira nebulizer with 20 actuations than with the Aerotech II nebulizer. The individual devices used in the study were selected after a water aerosol output test in order to minimize nebulizer variation.

The radiolabelled Bec-DLPC liposome suspension (2.5 ml) with 4 mCi initial radioactivity was placed in the reservoir of the jet nebulizer equipped with a valve actuator. The valve enabled the volunteers to manually control the aerosol generation to coincide with inspiration. The volunteers placed the nebulizer tightly between their lips and inhaled deeply while simultaneously pressing the actuating valve. A deep inspiration was followed by a normal exhalation. Exhaled labelled Bec-DLPC liposomes were captured using a Hudson filter. This procedure was repeated 20 times according to the volunteer's own inspiratory cycle without any breath holding between the inhalations. After inhalation, counts were determined for the nebulizer and exhalation filter for comparison to the pre-nebulization counts for calculation of aerosol output, retention, and exhalation.

2.7. Measurement and calculation of pulmonary deposition and clearance

Prior to the liposome study the attenuation coefficient of each volunteer was determined using a flat ^{54}Co disc method (Fleming, 1979). Immediately after inhaling the test preparation, the volunteer was placed in a supine position. The radioactivity was measured in the whole lung area by a two-headed, large field-gamma camera (Dual Genesys, ADAC System) equipped with low-energy all-purpose collimators. The subject was instructed to keep the examined area immobile during the 5 min measuring period. To evaluate the mucociliary clearance of the inhaled liposomes from the lung region, a 5 min gamma camera detection was repeated 1, 2, 3 and 6 (for two volunteers) h after the aerosol delivery. The intrapulmonary radioactivity was quantified by using a ROI (region of interest) program (Pegasys, Version 2.1) and the geometric mean counts were calculated for the lung region after the correction of individual attenuation of radioactivity and the time decay of ^{99m}Tc. The ROI would contain the alveolated areas of the lung (Weibel generations 17–23) and the distal portions of the conducting airways (Weibel generations 0–16) (Weibel, 1963).

The radioactivity in the reservoir was quantitated before and after the inhalations by a gamma camera. In addition, the exhaled liposomes were collected by a back-up filter and the radioactivity was measured by a gamma camera. The fractional results (%) of the initial deposition were listed for the deposition in the whole lung area, in the oropharynx and in the GI tract as well as in the exhaled fraction.

3. Results

3.1. In vitro assessment of ^{99m}Tc-labelled liposomes

Micropartition and chromatographic analysis of labelled Bec-DLPC liposomes demonstrated a

high labelling efficiency (96–99%) with minimal free ^{99m}Tc. The ratio of [^{99m}Tc]pertechnetate, $SnCl_2$, and Bec-DLPC liposomes was critical for high labelling efficiencies. The $SnCl_2$-catalyzed reduction of $^{99m}Tc^{4+}$ ions reacts primarily with the phosphate portion of the DLPC forming a positive and a stable association between the radioactive tag and the liposomes. The established labelling method was shown to be reliable and reproducible. Furthermore, the simple labelling process took only 35 min to carry out under standard conditions.

Cascade impaction analysis showed a positive correlation between the Bec, DLPC, and ^{99m}Tc in the aerosol particles. The correlation coefficient of the Bec and ^{99m}Tc concentrations on different stages of the cascade impactor was 0.940. It was 0.978 between the DLPC and radioactivity and 0.943 for Bec and DLPC. The MMADs (GSD) of Bec-DLPC liposomes were 1.5 (2.3), 1.4 (2.2), and 1.7 (1.8) according to the drug, radioactivity and lipid analysis, respectively, after administration from the Aerotech II nebulizer.

After reconstitution and labelling, the Bec-DLPC liposomes were heterogeneous with sizes ranging from < 1 to about 50 μm in diameter. With a continuing passage through the nebulizer jet, the Bec-DLPC liposomes were reduced in size by shear forces associated with continuous re-cycling through the nebulizer (May, 1973). Although the liposomes were labelled before nebulization, the reduction of the initial particle size by the nebulizer did not cause any disintegration of the bond between the phospholipid and the radioactive tag. Thus, the ^{99m}Tc remained associated with the Bec-DLPC liposomes during nebulization and in the aerosol particles with homogeneous distribution of the radioactive tag throughout the lipid phase. Small differences were noted between the MMAD of the labelled and the unlabelled liposome preparations of 1.5 and 1.2, respectively, after administration from the Aerotech II nebulizer. Similarly, after aerosol generation with the Spira, the MMADs were 3.6 and 3.1 μm, respectively. These differences were within normal limits of the assay.

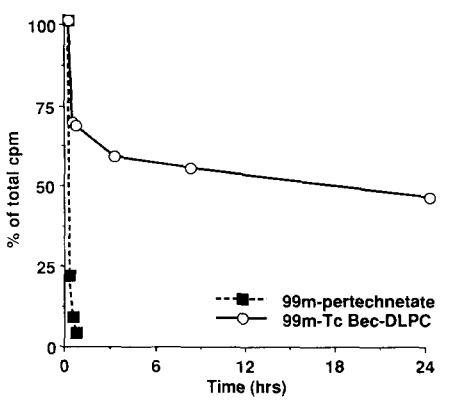

Fig. 1. Clearance of free ^{99m}Tc and ^{99m}Tc-labelled Bec-DLPC liposomes from the lungs of mice following intranasal administration under anesthesia.

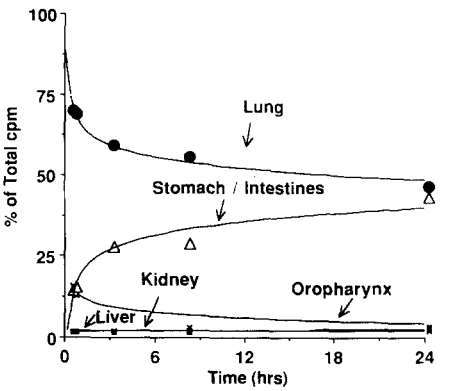

Fig. 2. Biodistribution of ^{99m}Tc-labelled Bec-DLPC liposomes in mice following pulmonary delivery via intranasal administration under anesthesia.

3.2. *In vivo clearance of ^{99m}Tc-Bec-DLPC liposomes in mice*

In mice, free technetium was cleared from the lung within minutes, whereas the radioactivity associated with the liposomes was retained in lungs for up to 24 h (Fig. 1). The ^{99m}Tc-Bec-DLPC liposomes were administered to the lungs via intranasal instillation under anesthesia. On average, 75% of the dose was distributed to the lungs, whereas about 12% was retained in the nasopharynx and 13% was swallowed. After 24 h, an average of 50% of the radioactivity was still detected in the lungs of the mice with smaller amounts in the gastrointestinal tract (Fig. 2).

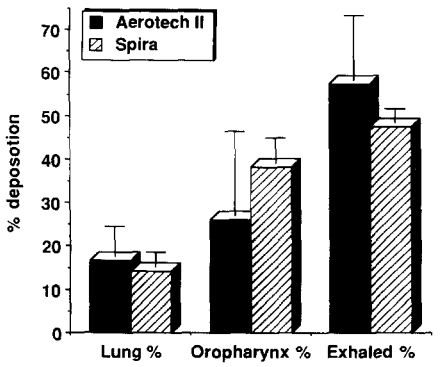

Fig. 3. Distribution of ^{99m}Tc following inhalation of Bec-DLPC aerosol.

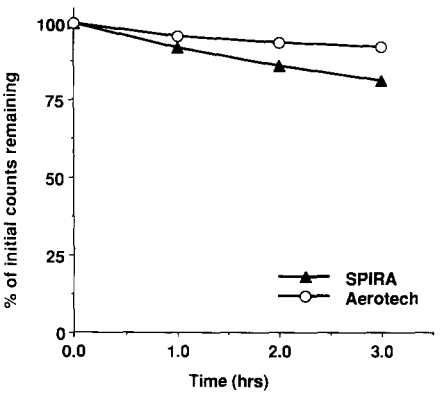

Fig. 4. Clearance of ^{99m}Tc from lungs of normal volunteers following 20 inhalations of ^{99m}Tc-labelled Bec-DLPC liposomes by mouth.

Thus, there is prolonged retention of ^{99m}Tc in the lungs.

3.3. Distribution and clearance of ^{99m}Tc in normal volunteers

Fig. 3 shows the distribution of ^{99m}Tc in six normal volunteers following 20 consecutive inspirations of labelled Bec-DLPC liposomes from each of the nebulizers studied. Inspired volumes were not recorded. Following exposure, gamma camera counts were acquired for 5 min. Shown in the figure are the pulmonary, oropharyngeal (including gastrointestinal tract and mediastinum) deposition percentages and the fraction exhaled. The values for pulmonary deposition were similar; 14 ± 3 for the Spira and 17 ± 7 for the Aerotech II. The major differences in deposition patterns was increased oropharyngeal/GI tract deposition of 38 ± 6 for the Spira and 26 ± 19 for the Aerotech II.

Fig. 4 shows a comparison of decay corrected ^{99m}Tc activity in the lungs of the volunteers at intervals following inhalation from each nebulizer. There was a progressive clearance of ^{99m}Tc-Bec-DLPC with both nebulizers in the 3 h period of observation. The clearance was greatest following inhalation of the larger particles produced by the Spira nebulizer. At 3 h, 93% of the original dose was detected in volunteers breathing from the Aerotech II nebulizer while it was only 82% following inhalation from the Spira nebulizer. The single volunteer who was examined 6 h following inhalation from each nebulizer showed further clearance of deposited ^{99m}Tc-Bec-DLPC liposomes.

The changes described above are visualized (Fig. 5) in anterior lung scintigraphs of a single volunteer after inhalation from each nebulizer. Results from the Aerotech II nebulizer (top row) showed intense activity in oropharynx, lungs and stomach shortly following inhalation. At 1 h, the oropharynx cleared with a resulting large burden

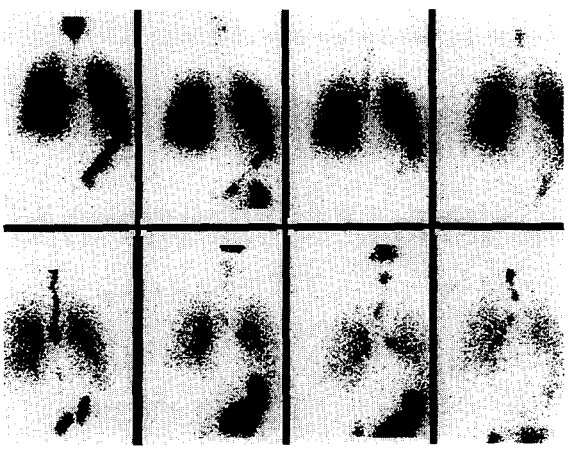

Fig. 5. Anterior chest scintigraphs of a normal volunteer at 0, 1, 2 and 3 h following 20 inhalations of ^{99m}Tc-labelled Bec-DLPC liposome aerosol by mouth. (Top four panels) Aerotech II nebulizer; (bottom four panels) Spira nebulizer. (Left to right) Time 0, 1, 2, and 3 h post-inhalation.

of radioactivity in the stomach. ^{99m}Tc activity remained intense in the lung at two and three h. Following inhalation from the Spira nebulizer, ^{99m}Tc activity was noted in the stomach and in the central portions of the lung. During observations at 1, 2 and 3 h, large amounts of ^{99m}Tc activity were present in the stomach and upper small bowel while there was appreciable clearing of deposition from the lung fields. Total deposition was not comparable because different amounts were initially delivered by the two nebulizers.

4. Discussion

This study has described the regional respiratory tract deposition and subsequent clearance of ^{99m}Tc bound to Bec-DLPC liposome aerosol following inhalation by six normal volunteers. In vitro data and mouse studies were consistent with continued linkage of ^{99m}Tc with the liposomes after deposition in the lung. The pulmonary area identified for deposition analysis in this study was determined by outlining the region of interest (ROI) using a computer program. The mediastinum was not included in the ROI. We propose that the ROI included all of Weibel's lung branching generations 17–23 and an appreciable proportion of the distal conducting airways (generations 0–16).

A way to judge the validity of this study is to compare it with deposition calculated for lung models using mouth breathing. Such data are available from Persons et al. (1987). Based on that model and using an MMAD of 1.5 μm with GSD 2.4, calculated pulmonary deposition (Weibel lung generations 17–23) would be 17%. This coincides approximately with pulmonary deposition (ROI) in the present study with the Aerotech II nebulizer of 17% (Fig. 3). The lung model predicted 20% deposition in the Weibel 17–23 generations of volunteers inhaling from the Spira nebulizer. Fig. 3 shows 14% deposition in the lung following inhalation from the Spira nebulizer. The small amount deposited in the 0–16 generations could not be quantified and is included in the respective ROI fractions. The measured exhaled ^{99m}Tc activity from the Spira nebulizer averaged 48% compared to the 51% predicted by the model. Exhaled ^{99m}Tc activity from the Aerotech II nebulizer was 57% compared to a predicted 74% exhaled in the model. Mouth deposition from the oropharynx and other sites was not comparable to 'mouth' deposition predicted by the model. The relatively large variation between pulmonary depositions in the present study with the two nebulizers probably resulted from variable breathing maneuvers. We feel that the present results are a reasonable representation of the regional distribution of this radiolabelled liposome aerosol in normal volunteers.

Once deposited, a large proportion of the liposomes remained in the lung during the 3 h period after inhalation. However, transfer of the ^{99m}Tc to other macromolecules (such as albumin) in the lung could not be ruled out (O'Doherty and Miller, 1993). Clearance was marginally greater with aerosols generated from the Spira. We believe that the larger aerosol particles of the Spira nebulizer were deposited higher in the respiratory tract and a greater proportion were cleared by mucociliary action. For the purpose of treatment of pulmonary disease, we would choose the Aerotech II or nebulizers producing similar aerosols (Waldrep et al, 1993). Fig. 4 illustrates visually (in one volunteer) the differences we describe above.

In a similar study, Farr et al. (1985) measured deposition and clearance of dipalmitoylphosphatidylcholine (DPPC) liposome aerosol after inhalation by normal volunteers. A similar method was employed with stannous chloride linkage of ^{99m}Tc to the liposomes. Volunteers were monitored for 6 h after inhalation when 56–70% of inhaled radioactivity was still present in the lungs. Their results were obtained using DPPC which has a phase transition temperature of 40°C. The phase transition temperature of DLPC is -2°C. In addition, Barker et al. (1994) have recently demonstrated that approx. 45% of deposited liposome aerosols are retained in the lung after 24 h. Thus, liposomal formulations are generally retained within the lung longer than most soluble drugs. Also, our findings seem consistent with a

continued association of ^{99m}Tc with liposomes for at least 3 h following deposition.

The results of this pilot study demonstrate the feasibility of using ^{99m}Tc for labelling and monitoring inhaled drug-liposome formulations. This approach should prove beneficial for monitoring the treatment of a variety of pulmonary disorders, such as asthma and bronchiolitis obliterans complicating lung transplantation. In addition, due to the retention of the Bec-DLPC liposomes, prolonged anti-inflammatory effects can be suggested.

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