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99m-Technetium as a marker of liposomal deposition and clearance in the human lung

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Summary

The clearance of pulmonary delivered ^{99m}Tc-labelled dipalmitoyl phosphatidylcholine (DPPC) liposomes has been studied in 4 healthy volunteers using the technique of gamma-scintigraphy. The labelling process was dependent on the method of separation of free and liposomally bound activity and on the concentration of stannous chloride ($SnCl_2$) used. Gel chromatography resulted in lower labelling efficiencies on increased $SnCl_2$ concentration due to the progressive failure of liposomes to elute from the column. Higher labelling efficiencies were achieved following treatment with an anionic exchange resin as a result of efficient recovery of the liposomes. Transmission electron microscopy and X-ray microanalysis showed that liposomes (1 ml containing 40 mg phospholipid) labelled with 20–25 MBq ^{99m}Tc in the presence of 0.3 mg $SnCl_2$ appeared uncontaminated with technetium–tin colloid, and were used in the in vivo studies. Pulmonary deposition of multi-lamellar and small unilamellar vesicles, delivered from an air jet nebulizer, was dependent on droplet size of the aerosol product. Short term clearance of both liposome populations was typical of muco-ciliary transport resulting in statistically equivalent retentions at 6 h. Subsequent retention data suggested that faster process(es) than those described for insoluble particulates were contributing to the clearance of alveolar deposited liposomes.

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Introduction

The concept of delivering a drug directly to the target organ is widely utilized in the treatment of obstructive lung disease. The administration of minute amounts of drug by inhalation results in therapeutic levels concentrated at the site of action initiating a rapid pharmocological effect (Walker et al., 1972). However, this is often short-lived as drug is removed from the lung (Snider and Laguarda, 1972; Shenfield et al., 1976). Studies in rats on the pulmonary absorption of cytosine arabinoside have proved that liposomal encapsulation can confine the distribution of a drug to the respiratory tract (Juliano and McCullough, 1980). This may prove to be of considerable significance in the modulation of current inhalation therapy by confining locally active drugs to the lung that are released from liposomes at a rate sufficient to extend their duration of action. The performance of a liposomal system incorporated into an aerosol to achieve controlled delivery of a therapeutic agent will, in part, be dependent on clearance from the lung, and therefore the determination in vivo of pulmonary deposition and clearance of liposomes is a necessary pre-requisite to the evaluation of vesicle encapsulated drug formulations.

Gamma-scintigraphy has been widely employed to study the pattern of deposition and clearance of radiolabelled particles from the lung. Regional deposition can be divided into tracheobronchial (T-B, 'airways') and alveolar fractions on the basis that the amount of activity retained in the lung 24 h after inhalation is alveolar deposited (Camner and Philipson, 1978). It has been found that $> 80–85\%$ of T-B deposition is cleared within 6 h following inhalation (Pavia et al., 1983) thereby allowing the use of short-lived radionuclides, e.g. ^{99m}Tc, to monitor short-term clearance from which estimates of alveolar deposition can be made.

Preformed liposomes can be labelled with ^{99m}Tc as pertechnetate in the presence of a reducing agent such as $SnCl_2$ (Richardson et al., 1977, 1978). Initial reports of the labelling process, where free pertechnetate was removed by dialysis indicated that $> 97\%$ efficiency of attachment was achieved. More recent work from the same laboratories (Barratt et al., 1983) reported apparent variable efficiencies that fell as low as 50% with multilamellar vesicles (MLVs) and even lower with small unilamellar vesicles (SUVs) when free ^{99m}Tc was separated by gel chromatography. Another study has also demonstrated low values of efficiency for MLVs (50–70%) and SUVs (4–20%), where the differences between liposome types was cited as probably due to the tight molecular packing of SUVs hindering the efficient interaction of ^{99m}Tc–$SnCl_2$ with the phosphates of phospholipids (Caride, 1981). In the absence of lipid, a colloidal complex of technetium–tin is formed (Billinghurst, 1973). High concentrations of $SnCl_2$ used in the labelling procedure may encourage competition for ^{99m}Tc between colloidal tin and liposomes (De Ligny et al., 1976). The use, therefore, of closely defined amounts of $SnCl_2$ should preclude the formation of the labelled colloid within liposome suspensions so that the in vivo activity profiles following administration are representative of liposomal distributions only.

In this paper, the influence of $SnCl_2$ concentration and method of separation of liposomally bound ^{99m}Tc from free activity were investigated with a view to the formation of efficiently labelled liposomes free from technetium–tin colloid. The

subsequent assessment of pulmonary deposition and clearance of such liposomes administered to volunteers was monitored by gamma-scintigraphy so that comparison between multi-lamellar and small unilamellar vesicles could be made.

Materials and Methods

Lipids

L-α-Phosphatidylcholine, dimyristoyl (DMPC) and dipalmitoyl (DPPC), synthetic, approximately 99% were obtained from Sigma Chemicals, U.K.

L-α-Phosphatidylcholine, di-[1-^{14}C]palmitoyl ([^{14}C]DPPC) of specific activity 4.14 GBq · mmol^{-1} was obtained from Amersham International, U.K.

Other chemicals and reagents

0.9% w/v saline, sterile Polyfusor was donated by Boots, U.K.

Solutions of ^{99m}Tc as pertechnetate (100–120 MBq) were eluted from a generator (Amersham International, U.K.) with sterile 0.9% w/v saline.

Triton X-100 (BDH, U.K.) was sonicated with warm water to form a 1% v/v solution.

Stannous chloride (AnalaR), Chloroform (AnalaR) and Cocktail T (Scintran) were obtained from BDH, U.K.

1 × 10 cm columns of Sephadex G-25(M) were obtained prepacked from Pharmacia, Sweden (PD-10 columns).

Amberlite resin, IRA-400 (Cl) (BDH, U.K.) was weighed into 0.5 g aliquots, regenerated with 0.9% w/v saline and sterilized by autoclaving at 115°C for 30 min.

All water was glass distilled.

Preparation of liposomes

Amounts of DMPC or DPPC necessary to form suspensions containing 40 mg · ml^{-1} of lipid were weighed into 50 ml roundbottomed flasks and dissolved in small quantities of chloroform. The organic solvent was evaporated on a rotary evaporator under reduced pressure at 40°C to form a thin lipid film. The required amount of sterile 0.9% w/v saline was added and the film hydrated at a temperature approximately 15°C above the transition temperature of the lipid until MLVs had formed. The MLVs were further maintained at the hydration temperature for 30 min to anneal the liposomal structure (Lawaczek et al., 1976).

SUVs were formed from MLVs by probe sonication using a 150 mW sonicator (M.S.E., U.K.) with a titanium probe, 10 mm in diameter. Sonication was conducted at the hydration temperature under a constant stream of nitrogen gas (to minimize lipid oxidation) for eight bursts (16 μm amplitude, peak-to-peak) of 1 min interspersed with 1-min cooling periods.

Preparation of sterile aqueous solutions of $SnCl_2$

In order to avoid the rapid oxidation of $SnCl_2$ in solution to the stannic form, it was necessary to use deoxygenated water as the solvent. This was prepared by

vigorously boiling freshly distilled water for 10 min in a narrow-necked flask before cooling with nitrogen gas, bubbled through the water for 1 h. Aliquots (15 ml) of the deoxygenated water were transfered to multi-dose injection vials via a 0.22 μm pore size membrane filter. The vials were flushed with nitrogen and sealed before autoclaving at 115°C for 30 min.

5 ml volumes of a 2 mg · ml^{-1} $SnCl_2$ solution made to pH 1 with 0.1 M HCl were dispensed into separate vials and freeze-dried under vacuum (Edwards, U.K.). The lyophilized samples (equivalent to 10 mg) were sealed under aseptic conditions. $SnCl_2$ prepared in this way, when reconstituted with 10 ml of sterile deoxygenated water, gave stable solutions containing 1 mg · ml^{-1}.

Labelling procedure

1 ml of each of the liposome suspensions was shaken with 0.1–1.0 ml of the $SnCl_2$ solution followed by the addition of 0.5 ml of pertechnetate (20–25 MBq) in sterile 0.9% w/v saline. The mixture was shaken vigorously for 1 min and allowed to stand at room temperature for 30 min. Similarly, controls were prepared by replacing the liposome suspension or $SnCl_2$ solution with sterile 0.9% w/v saline. Separation of the free activity from liposomes was attempted by gel filtration or by an ion-exchange resin method. For gel filtration, the samples were eluted with sterile 0.9% w/v saline on PD-10 columns, collecting 1-ml fractions. For the ion-exchange resin method, liposome suspensions were shaken with 0.5 g IRA-400 (Cl) for 3 min to facilitate uptake of free ^{99m}Tc by the resin. Each suspension was then decanted from the resin and the resin washed with two 2.5-ml volumes of sterile 0.9% w/v saline to ensure removal of loosely adhering liposomes. The washing fractions were added to the suspension and the final volume recorded.

The gamma activity in each fraction following gel filtration and in the diluted liposome suspensions following treatment with IRA 400(Cl) resin was assessed in a calibrated well counter.

Electron microscopy of the labelled liposome suspensions

The presence of colloidal tin in ^{99m}Tc-labelled liposomes was determined by transmission electron microscopy as previously reported by Morgan et al. (1981). In addition, estimation of tin levels unassociated with the liposomes was assessed by X-ray microanalysis.

Stability of the label in vitro

Freshly prepared liposome suspensions were placed in firmly sealed dialysis sacks, prepared from regenerated Visking tubing, and stirred in 15 ml of 0.9% w/v saline for up to 18 h at room temperature. The dialysis membrane was porous to free pertechnetate allowing the extent of leaching of the label from liposomes to be detected by gamma scintigraphy of the external phase.

In vivo studies

The studies were approved by the Joint Ethics Committee, South Glamorgan Health Authority and all volunteers participated with informed consent. All manipu-

lations in the preparation and labelling of liposomes prior to administration were conducted under aseptic conditions. Labelled liposomes (containing 40 mg DPPC) were diluted to 8 ml with sterile 0.9% w/v saline before transferance to an air jet nebulizer fitted with a T piece and extension tube (Hudson; Henleys Medical Supplies, U.K.). Each of a group of 4 healthy male volunteers (aged 27–40 years, with normal lung function for age and height) inhaled the nebulized product, generated with compressed air at 172 kPa, via a mouthpiece while remaining undisturbed in a supine position above a wide angle gamma camera fitted with a medium energy parallel hole collimator. The volunteers were encouraged to maintain deep, slow inspirations with breath-holding before exhalation (breathing frequency 6–8 cycles $\cdot$ min^{-1}) in order to maximize deposition in the peripheral airways. Nebulization was continued for sufficient time (approximately 13 min) to accumulate initial lung burdens of 3.5–4 MBq. The volunteers remained supine for a further 5–10 min whilst a series of dynamic frames (30 s duration) were completed. Further images were taken at intervals up to 8 h post-inhalation with a terminal reading at 20–24 h employing acquisition times of 5 min at the beginning of the test to 20 min at the end of the experiment. All camera images were computed to produce a visual array in a 64×64 format of areas of activity in the lungs. Regions of interest were defined separately around the left and right sides of the lung and the activity within these regions quantified following correction for background radiation and decay which subsequently allowed the determination of clearance.

Size characterization of the liposomes and of the nebulized product

The determination of liposomal size before and after the labelling procedure and following nebulization was conducted using photon correlation spectroscopy for SUVs and Coulter counting for MLVs. The nebulized products were characterized by directing the aerosols generated from liposome suspensions composed of phospholipid spiked with 37 kBq [^{14}C]DPPC into a calibrated multi-stage liquid impinger. Mass median aerodynamic diameter (MMAD) and geometric standard deviation (σ_g) were derived from estimation of the deposition of lipid on each stage, obtained by scintillation counting (Rackbeta, LKB Wallac, U.K.) of 1 ml aliquots incorporated into 10 ml Cocktail T for 10 min. Quench correction was carried out using internal standardization resulting in counting efficiencies of typically 90%.

Results and Discussion

The labelling of liposomes with ^{99m}Tc

The use of gel chromatography in the separation of liposomally bound or entrapped moieties from the free fraction is well established, where the former appears in the void volume and the latter elutes at a slower rate. Fig. 1 shows typical elution profiles of activity for suspensions of DMPC MLVs prepared using 0.1–1 mg $SnCl_2$. It was apparent that serial increments in $SnCl_2$ concentration resulted in a concurrent reduction in the amount of liposomes and associated activity eluting in the void volume fractions (3–7), such that the use of 1 mg $SnCl_2$ in the labelling

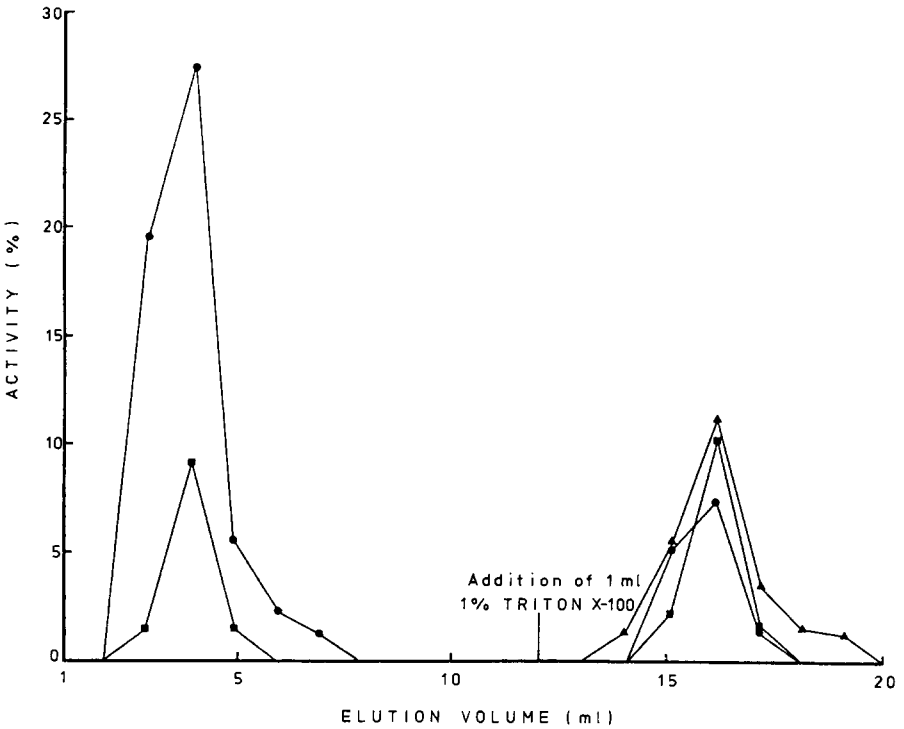

Fig. 1. Elution profiles of ^{99m}Tc-labelled liposome suspensions on G-25 columns. Elution profiles correspond to liposome suspensions labelled with pertechnetate by the addition of: 0.1 mg (●), 0.5 mg (■), and 1 mg (▲) $SnCl_2$.

procedure totally prevented elution from the G-25 column. The apparent, poor labelling efficiencies, therefore, at higher $SnCl_2$ concentrations were a measure of the recovery of the liposomes rather than a true efficiency of labelling. The adhesion of liposomes to the gel was highlighted by the treatment of poorly eluting columns with 1 ml of 1% v/v Triton X-100. Further elution resulted in peaks of activity for all suspensions studied as the adhering liposomes were disrupted. The theory of liposome adhesion as an explanation of poor labelling efficiency differs from that postulated by Barratt et al. (1983), who tentatively suggested that the affinity of Sephadex to bind reduced technetium (Billinghurst, 1973; Valk et al., 1973) may 'strip' activity from the eluting liposomes. It is conceivable, however, that a poor recovery of labelled liposomes would remain unnoticed during the gel filtration of optically clear suspensions of SUVs used by Barratt et al. (1983) unless a concurrent phospholipid assay was conducted on the eluting fractions.

Further studies with liposomes treated with $SnCl_2$ without pertechnetate demonstrated that the prevention of liposomal permeation through the gel was not due to a size effect; liposomes (40 mg DMPC in 1 ml) had a mean size of 2.20 ± 0.24 μm before and 2.09 ± 0.07 μm after the addition of 1 mg $SnCl_2$ ($n = 4$, $\pm$S.D.). Such observations infer that $SnCl_2$ is capable of either chemical modification or binding

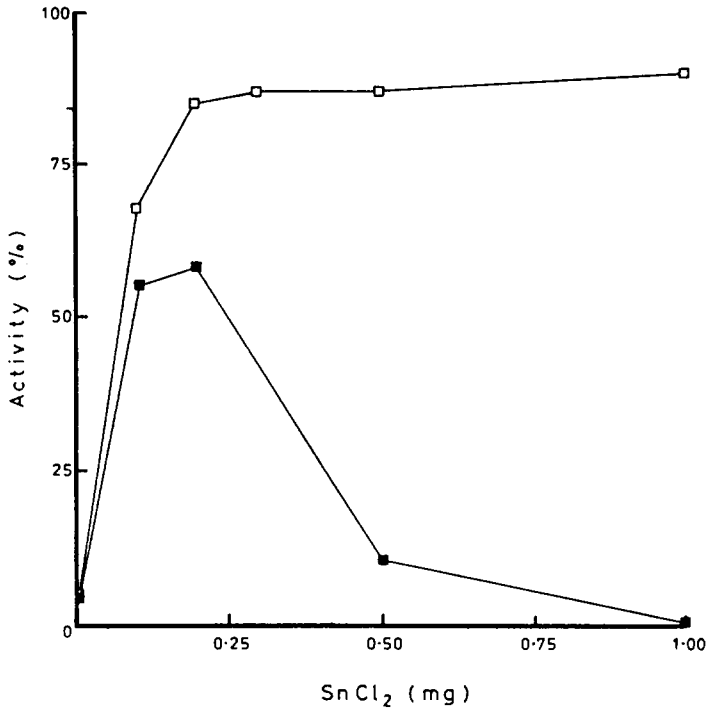

Fig. 2. The efficiency of labelling liposomes with ^{99m}Tc following elution through G-25 columns (■) or shaking with IRA 400 (Cl) resin (□). Each point is a mean of 3 determinations (± < 6%).

to the outer lipid bilayer of liposomes resulting in adsorption to G-25 gel. It is probable that these processes are important in the interaction of ^{99m}Tc with liposomes where, for example, ^{99m}Tc may bind to liposomes via a Stannous link. Previously, Jeyasingh (1982) suggested that complexation of ^{99m}Tc to liposomes occurs via the phosphate groups of lipid following chemical reduction of pertechnetate, although it has been stressed (Barratt et al., 1983) that little is known of the precise nature of the interaction between the lipid bilayer, $SnCl_2$ and the radionuclide.

IRA-400 (Cl) resin is a strongly basic anion exchange resin containing quaternary ammonium ion moieties. These have been found to rapidly and irreversibly bind pertechnetate (Theodorakis et al., 1982). This was supported in this study where shaking of 25 MBq of pertechnetate with 0.5 g IRA-400 (Cl) resin resulted in 100% association of activity with the resin in 3 min. A comparison of the results demonstrating the influence of $SnCl_2$ on the labelling efficiency of DMPC liposomes with ^{99m}Tc is displayed in Fig. 2 where free activity was removed by gel filtration or by the ion exchange resin method. For suspensions treated with IRA-400 (Cl) resin, a sharp increase in the labelling efficiency occurred as $SnCl_2$ level was increased to 0.25 mg. Subsequent increments in the amount of $SnCl_2$, however, produced only minimal changes in labelling efficiency. The resultant high labelling efficiencies at

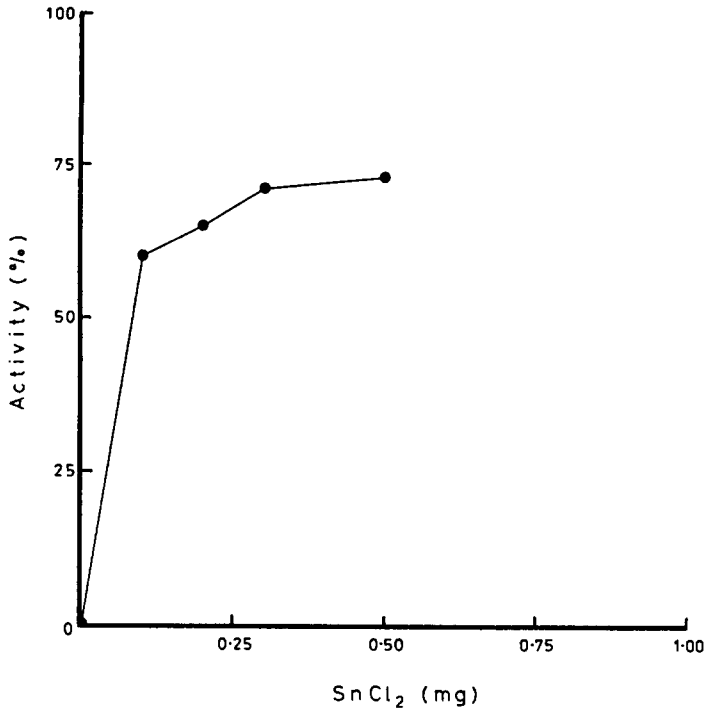

Fig. 3. The influence of $SnCl_2$ concentration on the amount of ^{99m}Tc remaining unassociated with IRA 400 (Cl) resin following 3 min shaking. The graph shows progressive formation of technetium–tin colloid on the addition of higher $SnCl_2$ concentrations.

higher levels of $SnCl_2$ were in direct comparison to those obtained following gel filtration and reflect the efficient recovery of the liposomes.

Both gel filtration and dialysis are unable to specifically identify liposomally attached technetium, but merely that in a high molecular weight complex. The failure of IRA-400 (Cl) resin to remove technetium–tin colloid from deliberately contaminated samples (Fig. 3) confirmed that the ion-exchange method was no exception. Comparative TEM demonstrated that liposome suspensions containing > 0.3 mg $SnCl_2$ were contaminated with the technetium–tin colloid. These observations were supported by X-ray microanalysis. Samples subjected to this technique were analyzed in terms of chemical elements and following computation (EDAX) of the results, gave semi-quantitative graphical displays of the component elements. Part of the scan for a sample containing 0.3 mg $SnCl_2$ in the absence of lipid is shown in Fig. 4. The semi-quantitative peak obtained for Sn was representative of the large contamination of the sample with the colloid. Further scans for labelled liposome suspensions containing 0.3 mg and 1 mg $SnCl_2$ are shown in Fig. 5. It was important to ensure that liposomes were absent in the areas subjected to this technique so that any resultant peaks of Sn were representative only of the fraction unassociated with the binding of ^{99m}Tc to liposomes. Diminution in the peak size until its disappearance at 0.3 mg supported the observations from TEM. At higher

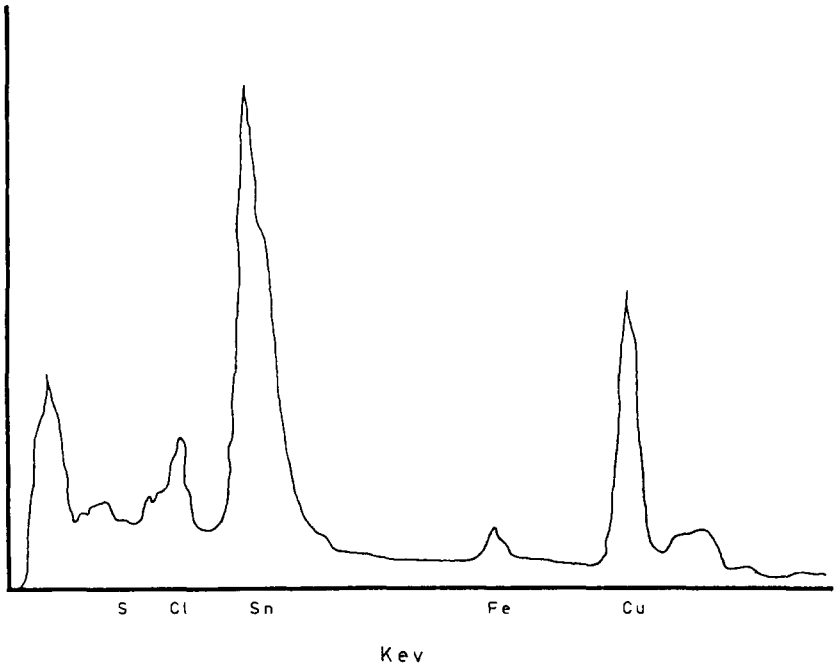

Fig. 4. X-Ray microanalysis of technetium–tin colloid after shaking with IRA 400 (Cl) resin.

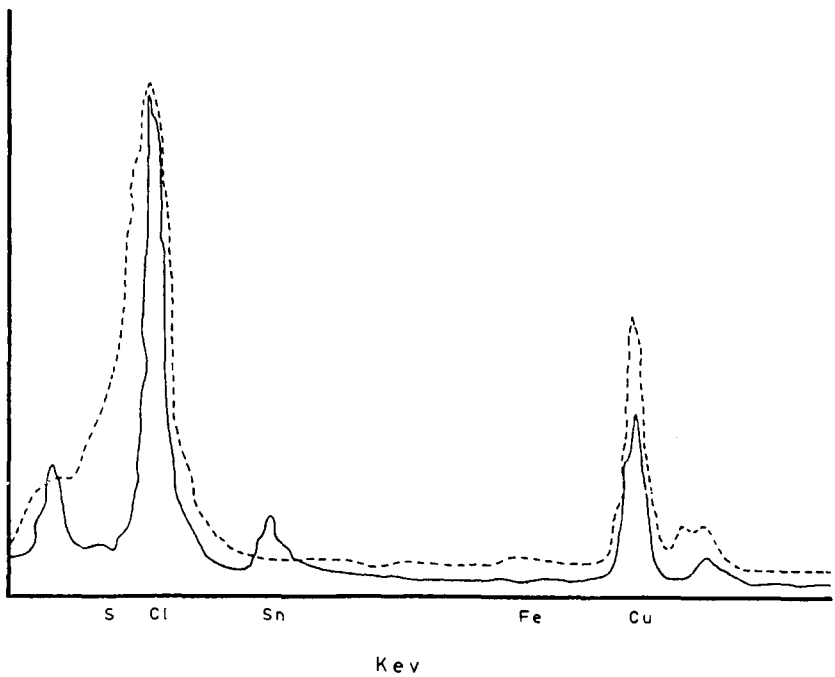

Fig. 5. X-Ray microanalysis of ^{99m}Tc-labelled liposome suspensions prepared using $SnCl_2$ after shaking with IRA 400 (Cl) resin. The solid line represents a liposome suspension containing 1 mg $SnCl_2$, the broken line 0.3 mg $SnCl_2$.

concentrations of $SnCl_2$, competition for ^{99m}Tc between liposomes and colloidal tin appears to occur, thereby agreeing with the work of Billinghurst (1973) and De Ligny et al. (1976).

This study elucidated that the addition of < 0.3 mg $SnCl_2$ to liposome suspensions containing 40 mg DMPC followed by the separation of free activity with IRA-400 (Cl) resin resulted in the formation of highly labelled liposomes free from the colloidal impurity. In addition, the label was stable for > 18 h in 0.9% w/v saline at room temperature. Whilst experiments on the labelling procedure were carried out using DMPC, problems were encountered in the formation of stable DMPC SUVs as storage of the liposomes for even short periods of time (10 h) resulted in a significant increase in liposomal mean size. Similar problems have been reported previously (Senior and Gregoriadis, 1982). Reversion to DPPC allowed the preparation of both MLVs and SUVs that were required for later in vivo studies. Moreover, it was apparent that the labelling process was independent of whether liposomes were composed of DMPC or DPPC, or prepared as MLVs or SUVs.

In vivo deposition and clearance of ^{99m}Tc-labelled liposomes

The apparent mean size of MLVs was reduced from 5.7 μm to 2.9 μm following nebulization probably due to breakdown of vesicle aggregates. SUVs were unaffected with a mean size of 0.07 μm. Aerosol size analysis gave values for MMAD and σ_g of 3.7 μm and 1.54 μm for MLVs and 3.2 μm and 1.53 μm for SUVs. Total lung clearance curves for each volunteer administered ^{99m}Tc-labelled MLVs and SUVs are depicted in Fig. 6. Short-term profiles were indicative of clearance via the muco-ciliary mechanism resulting in mean retentions at 6 h of $87.5 \pm 2.1\%$ and $76.8 \pm 5.1\%$ ($\pm$S.E.M.) for MLVs and SUVs, respectively. The fate of a nebulized pertechnetate solution followed in one volunteer (A) was vastly different with rapid absorption from the lung resulting in $< 25\%$ activity remaining after 1 h.

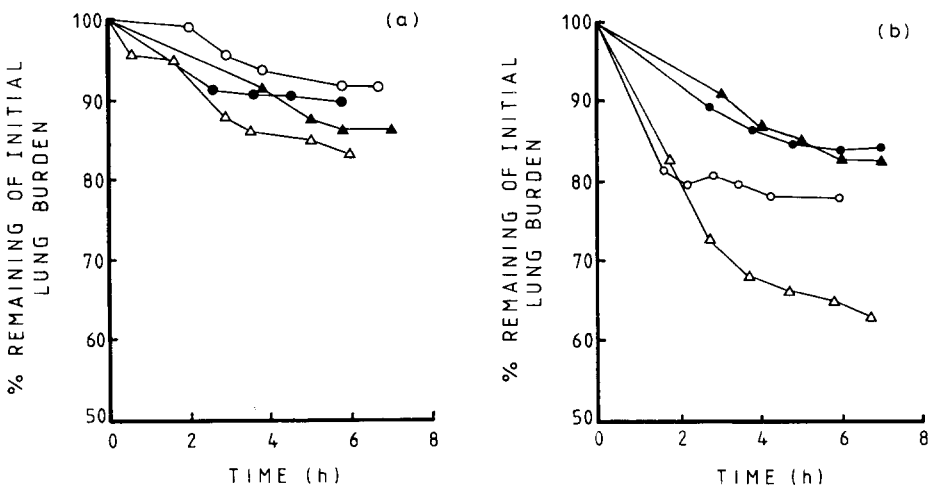

Fig. 6. Total lung clearance of activity following administration of ^{99m}Tc-labelled DPPC MLVs (a) and SUVs (b) to volunteers A (▲), B (●), C (○) and D (△).

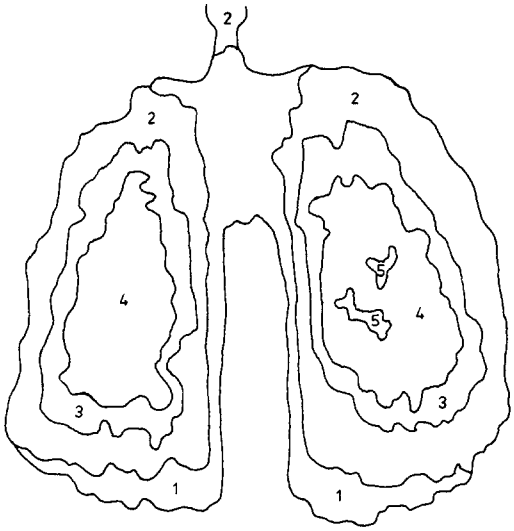

Fig. 7. Diagrammatic representation of the gamma activity contours obtained from a typical scintigram (posterior view) immediately after inhalation of ^{99m}Tc-labelled liposomes. Areas numbered 1 to 5 represent increasing intensity of activity. The left and right sides of the lung are apparent together with a small deposition of activity within the pharyngeal region.

Muco-ciliary transport becomes progressively faster from the peripheral to the central airways (Foster et al., 1980). Therefore, the rate of clearance is dependent on the depth to which aerosolized particles penetrate the lung which, in turn, is dependent on the aerosol characteristics and on breathing pattern. In all but one volunteer (D), the regional deposition of both MLVs and SUVs was similar which subsequently resulted in statistically equivalent values for retention at 6 h. Such data infer that deposition and clearance of liposomes were a function of droplet size of the nebulized product rather than liposome size. For normal subjects adopting a slow breathing cycle, it has been determined that droplets exhibiting a MMAD of 3–4 μm have a high probability of deposition in the alveolar region of the lung (Stahlhofen et al., 1981). The polydisperse nature of the emitted aerosol, however, would ensure a more diffuse pattern of deposition and is apparent from a typical scintigram (Fig. 7).

The importance of initial deposition on rate of clearance was highlighted in volunteer D following administration of SUVs. The initial lung image depicted significant activity in the perihilar zones of the left and right sides of the lung. These regions predominate in the central airways (Agnew et al., 1984) within which muco-ciliary transport is comparatively rapid. Serial images confirmed this by showing a rapid removal of activity from these regions to the stomach over the first 3 h of study. As muco-ciliary clearance is impaired in chronic respiratory disease (Lourenco, 1970; Agnew et al., 1981, 1984), it is probable that 6-h lung retentions displayed by the healthy volunteers would be significant underestimates of those

TABLE 1

A COMPARISON OF OBSERVED VALUES FOR ALVEOLAR DEPOSITION OF ^{99m}Tc-LABELLED LIPOSOMES WITH PREDICTIONS BASED UPON 6 h VALUES FOR RETENTION

Volunteer	Lung retention (%)			
	MLVs		SUVs	
	Obs.	Pred.	Obs.	Pred.
A	56	66–71	60	66–70
B	70	72–77	60	66–70
C	63	73–77	62	62–66
D	56	66–71	33	51–54

Obs. = observed; Pred. = predicted values.

obtained with diseased individuals. Prospectively, slower clearance of liposomes may prove to be beneficial in therapy by retaining entrapped drug within the conducting airways that is subsequently released at a controlled rate directly to the site of action.

Table 1 shows lung retentions of activity at 20 h. It is a reasonable assumption that 20-h retentions, in normal subjects, are equivalent to that which is alveolar deposited. Predicted data, derived from 6-h lung burdens, assuming 80–85% of T-B deposition was cleared at this time (Pavia et al., 1983), show some overestimation of the alveolar fraction. This suggests that alveolar-deposited liposomes are subjected to faster clearance mechanisms than those described for insoluble particulates (Green, 1973). Alternatively, dissociation of the label in vivo could account for differences between observed and predicted data although monitoring of the thyroid gland, where released ^{99m}Tc would concentrate, failed to detect any dissociated activity.

DPPC SUVs delivered to the respiratory tract of rabbits were found to be rapidly cleared from the airspaces by phospholipase-independent uptake into lung tissue (Oyarzun et al., 1980). Furthermore, similar studies in rats have determined specific accumulation within lamellar bodies before subsequent absorption, resulting in 51.4% of the phospholipid remaining in the lung 24 h after administration (Morimoto and Adachi, 1982). DPPC cleared from the lung has been shown to distribute to the liver and spleen (Geiger et al., 1975; Morimoto and Adachi, 1982), organs which rapidly accumulate intact liposomes following systemic administration. Such evidence infers that alveolar deposited liposomes may be absorbed intact from the lung. Conversely, others have failed to detect circulating phospholipid in any form following intratracheal administration to rats (Juliano and McCullough, 1980) although a significantly greater rate constant for removal from blood compared to absorption from the lung would lead to this phenomenon.

Whilst it was apparent from this study that liposomes depositing on the conducting airways were removed by the muco-ciliary clearance mechanism, the adoption of low lung burdens of the short-lived radiolabel, ^{99m}Tc, prevented the acquisition of data pertinent to the fate of alveolar deposited liposomes. Further studies employing liposomes composed of [^{123}I]phospholipid will allow imaging for a longer duration

after administration and therefore help to elucidate the process(es) contributing to the clearance of the alveolar deposited fraction. In addition, the adoption of a dual labelling technique incorporating an aqueous phase marker such as [^{99m}Tc]diethylenetriaminepentaacetic acid (DTPA) in conjunction with [^{123}I]phospholipid may provide information on the integrity of the liposome structure in vivo. A more rapid clearance of the former would suggest destabilization of liposomes, although correction for possible efflux of [^{99m}Tc]DTPA should be applied.

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