International Journal of Pharmaceutics, 58 (1990) 57–61
Elsevier

IJP 01965

The stability of liposomes to nebulisation

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(Received 24 May 1989)
(Accepted 26 July 1989)

Key words: Liposome; Liposomal entrapment; Nebulisation; Nebuliser; Sodium cromoglycate; Stability

Summary

The stability of liposomes to aerosolization was determined by analyzing the aerosol produced from liposomal formulations using a mulistage liquid impinger. These investigations indicated a large loss of entrapped drug on passage of multilamellar vesicles and reverse-phase evaporation vesicles through an air-jet nebuliser, due to fragmentation of vesicles. Reducing the size of vesicles by extrusion of preparations through polycarbonate membrane filters decreased the extent of drug loss during nebulisation. Regional deposition of the aerosolized product within the multistage liquid impinger was governed by the size of the aerosol droplets rather than that of the liposomes.

Introduction

Liposomal entrapment of the antitumour drug cytarabine, prior to pulmonary administration to rats, prolonged drug retention within the lung altering its pharmacokinetics, and resulting in localised drug action within the respiratory tract. (Juliano and McCullough, 1980). Sodium cromoglycate (SCG) is widely used in the prophylactic treatment of bronchial asthma. SCG is highly polar and is not administered orally due to poor absorption from the gastro-intestinal tract. It is, however, rapidly absorbed from the lungs and rapidly excreted, necessitating frequent dosing (Moss et al., 1970). Pulmonary delivered liposomally encapsulated SCG might prolong the local action of SCG in the asthmatic lung.

Nebulisation would appear the simplest method available for delivery of drugs to the human lung. Farr et al. (1985) in a gamma scintigraphy study, used a nebuliser to deliver ^{99m}Tc-labelled liposomes to volunteers, in an investigation of the fate of pulmonary deposited vesicles. This present study used a calibrated multistage liquid impinger (MLI) to investigate the stability of various liposome formulations to nebulisation; to assess the extent of drug loss during nebulisation and to determine changes in liposomal vesicle size during the process.

The MLI, first described by May (1966), is a 'wet-stage' cascade impactor and is used routinely for aerosol analysis (Hallworth and Andrews, 1976). The MLI can be calibrated with respect to the effective cut off diameter (ECD) for each stage, at a particular airflow rate. 60 $l \cdot min^{-1}$ is usually employed, as this is the estimated mini-

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mum attainable inspiratory flow rate for an asthmatic subject (Hallworth and Andrews, 1976). The MLI is particularly useful for in vitro aerosol characterisation, as the presence of water at the impaction site resembles the humid conditions of the human lung.

Materials and Methods

Materials

Unless otherwise stated materials in this study were AnalaR grade and obtained from BDH (U.K.). Water was glass distilled. Sodium cromoglycate (micronised) was a gift from Fisons (U.K.). Egg phosphatidylcholine (EPC; about 90%, Sigma, U.K.) was subsequently purified as described by Bangham et al. (1974). Dipalmitoyl-L-α-phosphatidylcholine (DPPC) and cholesterol (Chol; 99 + %) were obtained from Sigma (U.K.).

Methods

Preparation of liposomes All liposome preparations had bilayers comprising equimolar quantities of phospholipid and cholesterol, since these formulations had a high entrapment efficiency of SCG (Taylor et al., 1989).

The required amounts of phospholipid and cholesterol were weighted into a quickfit round bottom flask and dissolved in a small volume of chloroform. Organic solvent was slowly removed at reduced pressure, on a rotary evaporator, at 40 °C, such that a thin film of dry lipid was deposited on the inner wall of the flask. Aqueous phase (SCG in 0.9% saline) was added at 40 °C for EPC and at 56 °C for DPPC. The flask was maintained at that temperature for 1 h, then shaken on a mechanical agitator for 2 min to produce multilamellar liposomes (MLVs).

Reverse-phase evaporation liposomes (REVs) were prepared by the method of Szoka and Papahadjopoulos (1978). Lipid components were weighed into a long-necked 200-ml quickfit round bottom flask and dissolved in chloroform/diethylether (1 : 1). SCG in 0.9% saline was added such that the organic to aqueous phase ratio was 6 : 1. The flask was sealed under nitrogen and the mixture sonicated for 4–6 min at 40 °C (EPC) or 50 °C (DPPC) in an ultrasonic bath. An emulsion was produced from which organic solvent was slowly removed at 45 °C with a rotary evaporator to produce liposomes. The flask remained on the evaporator until organic solvent could not be detected by olfactory means and was then flushed with nitrogen.

The particle size of some REV preparations was reduced by extrusion through polycarbonate membrane filters (Nucleopore Inc., USA, pore size 1.0 μm) held in 25-mm holders.

Following production, all liposomes were maintained for 1 h at a temperature exceeding the phospholipid T_c (MLVs: EPC = 37 °C, DPPC = 56 °C and REVs: EPC = 40 °C, DPPC = 45 °C) to anneal the liposome structure.

Determination of SCG entrapment by liposomes

Duplicate 5-ml samples were centrifuged at 200 000 × *g* and 37 °C for 30 min in a temperature pre-equilibrated head. The concentration of free SCG was calculated from the UV absorbance of the supernatant at 326 nm. A knowledge of the total drug in the preparation allowed the amount of drug associated with the liposomes to be calculated by difference. Entrapment was expressed as mg SCG entrapped per 100 mg lipid.

Determination of liposome size

Vesicle size was determined using a Coulter Counter, TAII, with a 50 μm aperture tube and using 0.9% saline, filtered through a 0.05 μm membrane filter, as a conductive medium. Photon correlation spectroscopy (Malvern RR144) was used when Coulter analysis indicated a mean particle size less than 3 μm. Measurements were carried out at a scattering angle of 90 ° at 37 °C in filtered 0.9% saline.

Characterization of aerosols produced by an air-jet nebuliser

Aerosols were generated from 8 ml of liposome formulation, from which free drug had previously been removed by dialysis for 120 h at 4 °C against 100 volumes of 0.9% saline, continuously stirred and changed 4 times in 24 h. The nebulised product was produced by an air-jet nebuliser (Hudson, Henleys Medical Supplies) fitted with a T-piece and extension tube, and directed into a calibrated

MLI. The nebuliser was driven by nitrogen from a compressed gas cylinder at 172 kPa for 10 min, and air drawn through the impinger at 60 l · min^{-1} by means of a vacuum pump via a terminal filter. Calculation of free and total SCG as described above allowed the amount of SCG entrapped within liposomes deposited on each stage of the MLI to be determined.

Results and Discussion

Preliminary investigations indicated that formulations having high concentrations of lipid and SCG would be required such that a 'therapeutic' dose of drug would be delivered by the nebuliser in an acceptable time. The maximum concentration of SCG in the aqueous phase was considered to be 30 mg · ml^{-1} since higher concentrations of drug could not exceed its solubility during liposome production. Liposomes containing greater than 50 mg · ml^{-1} lipid produced clogging of the smallest jets of the MLI, consequently 40 mg · ml^{-1} lipid was the chosen level for the investigations.

Dialysed liposome formulations contained less than 4% of their total SCG content as free drug.

Analysis of the solution on the stages of the MLI indicated that 7.5 mg SCG was deposited. 50.8 ± 0.5% (mean ± SE) of the entrapped drug was lost during nebulisation. The distribution of free and entrapped SCG over the stages of the MLI, throat adaptor and terminal filter is shown in Fig. 1a, with the majority of free and entrapped drug deposited on stages 3 and 4.

Nebulisation was associated with a significant decrease in mean vesicle diameter from 5.4 μm to 2.7 μm. This suggested that liposomes and liposome aggregates were broken up on passage through the nebuliser, resulting in a net loss of entrapped material. Vesicle fragmentation is most likely to occur as liquid is drawn up the nebuliser liquid inlet tube and mixed with the high speed air-jet, and as aerosolized droplets strike the filtering baffle. Spontaneous reformation of liposomes is likely to occur; in the bulk nebuliser liquid as the large droplets removed from the airstream by the baffle are recycled, in the aerosolised droplets, or in the stages of the MLI, resulting in re-entrapment of some released drug. Thus the deposited liposome-associated drug is probably entrapped in the smaller vesicles of the original preparation and in vesicles formed from phospholipid fragments of the ruptured vesicles.

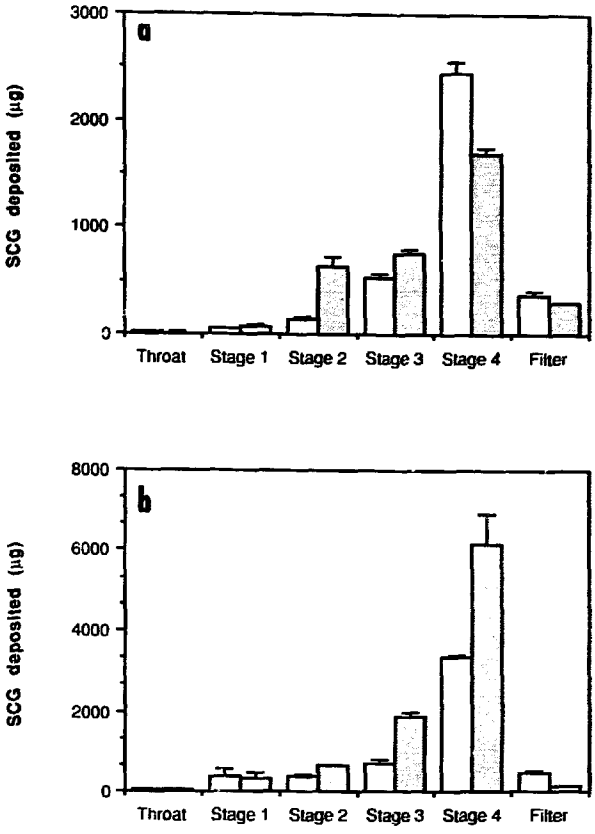

Fig. 1. Deposition of free SCG (□) and liposome entrapped SCG (■) in the MLI following nebulisation of (a) EPC/Chol MLVs and (b) EPC/Chol REVs. Mean ± S.E., $n = 3$.

A significant decrease in mean vesicle size from 3.4 μm to 2.5 μm occurred when EPC/Chol REVs were nebulised into the MLI. The distribution of free and entrapped drug in the MLI was similar to that for MLVs with predominant distribution on stages 3 and 4 (Fig. 1b). A total of 14.5 mg SCG was deposited in the MLI with 31.9% of the previously entrapped drug being released on passage through the nebuliser. This is probably the result of fragmentation of the large oligolamellar vesicles in the preparation and possibly due to break-up of some of the fragile large unilamellar vesicles also present in this formulation (Taylor et al., 1989).

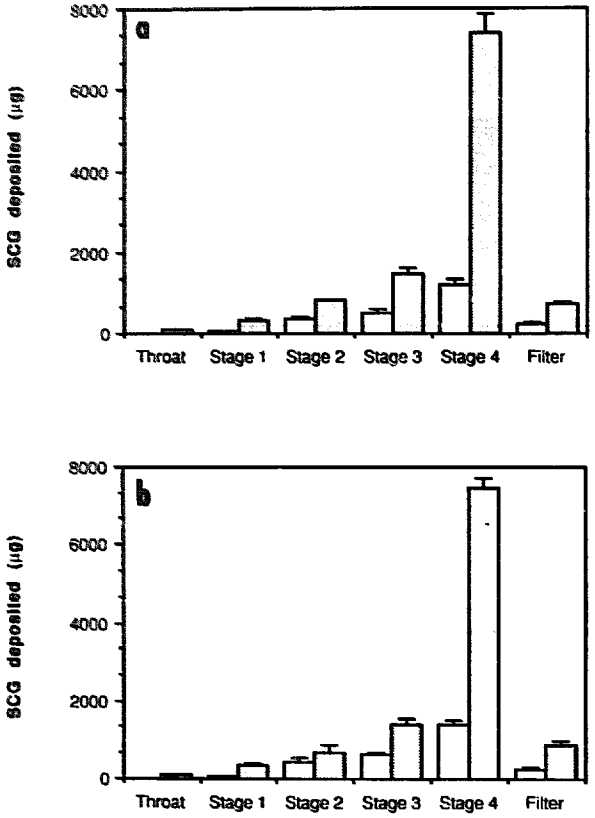

Fig. 2. Deposition of free SCG (□) and liposome entrapped SCG (■) in the MLI following nebulisation of: (a) filtered EPC/Chol REVs and (b) filtered DPPC/Chol REVs. Mean ± S.E., $n = 3$.

During the time taken to collect material deposited in the MLI and determine free and entrapped SCG, the liposomes were in very dilute conditions, consequently some of the free drug will also have originated through leakage from deposited intact vesicles; although the inclusion of cholesterol in the preparations reduced this release to a minimum (Taylor et al., 1989).

In an attempt to reduce drug loss on nebulisation REVs were extruded through polycarbonate membrane filters, and unentrapped SCG removed by dialysis as before. Fig. 2a and b shows the distribution of free and entrapped SCG in the MLI following nebulisation of filtered EPC/Chol REVs and filtered DPPC/Chol REVs, respectively. The pattern of SCG distribution was very similar for these formulations, with predominant distribution again on stages 3 and 4 of the MLI. Thus the deposition of free and liposome entrapped drug was for all formulations dependent upon the nebuliser droplet size rather than on vesicle size. Loss of liposome-associated drug was reduced in filtered preparations: 16.6% of entrapped SCG was lost from EPC/Chol REVs and 14.2% was lost from DPPC/Chol REVs during nebulisation. Total SCG deposited in the MLI was 13.6 mg and 13.3 mg for EPC/Chol and DPPC/Chol REVs, respectively.

The loss of drug from filtered REVs on nebulisation was accompanied by an insignificant change in mean vesicle size vesicles of both formulations being 1.2 µm prior to nebulisation and 1.1 µm after nebulisation.

Particle size-cumulative % undersize plots were made for deposited free, entrapped and total SCG against the effective cut-off diameter (ECD) of each stage. The ECD of a 90° bend at an airflow of 60 $l \cdot min^{-1}$ has been calculated at 20 µm (Hallworth and Andrews, 1976). The linearity of these plots on log-probability axes indicated that the sizes of deposited droplets were log-normally distributed. The plot for free and entrapped SCG was similar for each liposome formulation, indicating that the deposition site was dependent on the size characteristics of the aerosol rather than on vesicle size or type. These plots have allowed estimation of the mass medium aerodynamic diameter (MMAD) for each formulation, from the 50% cumulative level. The geometric standard deviation (σg) was determined from the ratio of the size at the 84.2% cumulative level to that at 50% (Table 1).

Analysis of variance indicated that the MMAD of the aerosol produced by the nebuliser was

TABLE 1

Mean size and size distribution of aerosols deposited in the multistage liquid impinger following nebulisation of liposomal formulations of SCG.

Formulation	MMAD (µm)	(σg)
EPC/Chol MLV	2.72	1.95
EPC/Chol REV	2.81	1.88
EPC/Chol REV (filtered)	2.59	1.95
DPPC/Chol REV (filtered)	2.63	1.93

significantly greater in the presence of phospholipid vesicles than in their absence, whilst all aerosols were polydispersed. Only small changes were seen in the size distribution of the final aerosol because of the size-selective characteristics of the nebuliser for retaining primary droplets (Mercer, 1973).

The small increase in MMAD of the final aerosol is unlikely to affect the regional distribution of the nebulised product in the human respiratory tract. The ECD for stage 2 of the MLI (5.51 μm) dictates that the aerosol deposited on stages 3 and 4 and on the filter have aerodynamic diameters less than 5–6 μm. These droplets can then be considered the 'respirable fraction' of the aerosolized dose, being small enough to penetrate to the alveoli (Stahlhofen et al., 1980). 94% of the total aerosol produced from the SCG solution was in this respirable range as was 86.7% for EPC/Chol MLVs, 83.7% for EPC/Chol REVs, 88.1% for filtered EPC/Chol REVs and 87.2% for filtered DPPC/Chol REVs.

The high respirable fraction for all the aerosols was a measure of the ability of the nebuliser to filter out large primary aerosol droplets. Filtered REVs would appear to be the most suitable formulation for delivery of SCG to the human lung since a high proportion of drug delivered to the lower stages of the MLI was liposomally entrapped. Maintenance of the integrity of the liposome structure during nebulisation may be less important if a bilayer-associated hydrophobic drug is to be delivered to the lung, whilst small unilamellar vesicles may prove more stable to nebulisation than filtered REVs and hence be better suited for delivery of hydrophilic drugs with a low therapeutic dose.

Acknowledgements

The authors are grateful to SERC and Fisons plc for supporting this study.

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