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Liposomal Budesonide for Dry Powder Inhaler: Preparation and Stabilization

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ABSTRACT The purpose of the study was to prepare stable liposomally entrapped budesonide (BUD) for a dry powder inhaler (DPI) formulation. BUD liposomes composed of egg phosphatidyl choline and cholesterol were prepared by lipid film hydration technique and sonicated to have the desired size (< 5 µm). A rapid method was used for separation of free drug by centrifugation at a lower centrifugal force (G value). Liposomal dispersion was subjected to lyophilization after blending BUD with cryoprotectant in varying bulk and mass ratios, and percent drug remaining entrapped after lyophilization was optimized. Comparative drug retention studies on storage of DPI formulations were carried out in accordance with International Conference on Harmonization guidelines. Critical relative humidity of the formulations was determined and reported as one of the manufacturing controls. Sucrose was found to be the most effective cryoprotectant when present on both sides of the lamellae of liposomes in a bulk strength of 500 mM and mass ratio of lipid:sugar; 1:10. Blending of sorbolac before lyophilization showed better retention of encapsulated drug (95.59%). The respirable fraction of the product (20.69 ± 1.50%) was comparable with that of the control (26.49 ± 1.52%), suggesting that the liposomal BUD can be successfully delivered throughout the broncho-pulmonary tree. The findings demonstrate that liposome of BUD can be prepared with a high entrapment value, stabilized by lyophilization, and delivered as an aerosolized DPI. The stability studies of lyophilized product suggests a shelf-life of one year when stored under refrigeration (2°C-8°C).

Keywords: Liposomes, dry powder inhaler, budesonide, lyophilization, drug retention.

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INTRODUCTION

Improving drug delivery to the pulmonary system has been an area of increasing interest among several disciplines. Particulate carriers such as liposomes have many attractive features as pulmonary drug delivery systems, particularly with respect to controlled delivery. One of the difficulties in practical application of these products has been the long-term stability of the liposomes. The stability problems of liposomes have been addressed [1] . The presence of certain sugars has been shown to enhance stability. The best evidence available suggests that there is a direct interaction between the sugar and the polar head group of the phospholipid, the result of which is a depression of the transition temperature of the lipid and its maintenance in a fluid state even in the absence of water [2,3] . Similarly, under appropriate lyoprotective conditions, liposomes retain their vesicle size, and hydrophobic drugs will remain associated with the bilayer upon lyophilization and rehydration. Lyophilization is presently used to achieve long-term stability of liposomes as a drug carrier system.

Budesonide (BUD) is a corticosteroid used in the prophylactic management of asthma. Local and systemic adverse effects remain a major concern for such inhaled corticosteroids. Liposomal encapsulation of BUD controls the release of drug and maintains therapeutic concentration of the drug in the lungs for longer periods; hence, it is expected to maintain lower drug concentration in blood, reduce systemic toxicities, and improve therapeutic efficacy. Prolong stay of the drug in the lungs (site of action) will reduce frequency of dosing. We have studied the feasibility of developing liposomal BUD dry powder inhaler (DPI) and enhancing the stability of the liposomal formulation using lyophilization. Efforts were made to find the ideal cryoprotectant and its optimum strength for better percent drug remaining entrapped (PDRE). Then the long-term drug retention within the liposomes and the in vitro dispersion behavior of developed DPI

formulations were evaluated. The drug retention studies of DPI formulations were carried out in different storage conditions according to the International Conference on Harmonization (ICH) guidelines, and a twin-stage impinger was used for preliminary screening for aerosol dispersion characteristics.

MATERIALS AND METHODS

Materials

Budesonide was received as a gift sample from Astra-IDL, Banglore, India. Egg phosphatidyl choline (EPC) (Sigma Chemicals, Mumbai, India); cholesterol (CHOL) (SD Fine Chemicals, Mumbai, India); a-tocopherol (E. Merck India Ltd, Mumbai, India); dextrose monohydrate (Roferose ST, ROQUETTE, Crow Chemicals, Indianapolis); maltose (Advantose 100, SPI Polyols, New castle, Delaware); lactose (Sorbolac-400, Meggle, Germany), and sucrose (Pokkie Odcaynnlki Chemicals, Poland) were purchased. All other reagents and chemicals used were of analytical grade or pharmacopoeial grade.

Preparation of liposomes

Multilamellar vesicles (MLVs) of BUD were prepared by the modified Bangham method, reported by Juliano and Daoud [4] . Drug (5 mg), EPC, CHOL, and a-tocopherol (1% of EPC) were co-precipitated to a thin film in the ratios shown in **Table 1**, using a solvent system ($CHCl_3$:CH_3 OH [2:1], 10 mL) for a solvent stripping technique in a rotary flask evaporator under vacuum in a nitrogen atmosphere. The dried film was then hydrated at 25 ± 2°C for 1 hour with 5 mL of phosphate buffer saline, pH 7.4 (0.17 µ ionic strength), containing 1 mM EDTA and 500 mM sucrose. Liposomal dispersion thus formed was subjected to sonication under nitrogen atmosphere in an ice bath for 30 minutes. Sonicated vesicles were further stabilized by hydration for 8 hours at ambient temperature and separated from free drug by centrifugation. The liposomal dispersion of BUD so obtained was filled in an amber-colored vial under nitrogen atmosphere and sealed and stored in a refrigerator.

Separation of free drug

For separation of free drug, the liposomal dispersion was centrifuged at 4.38 x 10^3 x g for 90 seconds. **Figure 1** shows the photomicrograph (Olympus, BX 40F4, Tokyo, Japan) of coarse liposomal dispersion, purified liposomal dispersion, and the drug crystals in pellet form following centrifugation. BUD was estimated in supernatant after it was dissolved in 10% Triton-X 100 in methanol by reverse-phase high-performance liquid chromatography (HPLC) [5] . Separation of free drug from liposomally entrapped drug was attempted by four reported procedures [6,7] : gel filtration, centrifugation, dialysis, and protamine aggregation. The results are recorded in **Table 2** .

Preparation of DPI

The purified dispersion was further dispersed with the required quantity of hydrating medium to obtain a lipid-sugar ratio of 1:10. An equivalent proportion of sorbolac calculated based on percent drug entrapped (PDE) was added to create a final strength of 100 µg of

Table 1. Composition of Optimized Liposomal Batches

Batch	COMPOSITION PER CAPSULE						PC:CHOL (molar ratio)	PDE*	PDRE* after lyophiliz-ation
	Drug (µg)	EPC (mg)	α-Tocopherol (µg)	CHOL (mg)	Sucrose (mg)	Lactose (mg)			
BUD1	100	3.86	38.62	-	38.62	107.38	1:0	95.80 (0.99)	95.59 (1.72)
BUD2	100	2.99	29.93	0.73	37.20	108.92	2:1	82.42 (1.90)	95.83 (2.18)
BUD3	100	2.13	21.31	1.04	31.70	115.00	1:1	86.91 (1.29)	95.84 (2.29)

*Mean (± SEM), n = 6.
EPC indicates egg phosphatidyl choline; CHOL, cholesterol; PC, phosphatidyl choline: PDE = percent drug entrapment; PDRE, percent drug remaining entrapped.

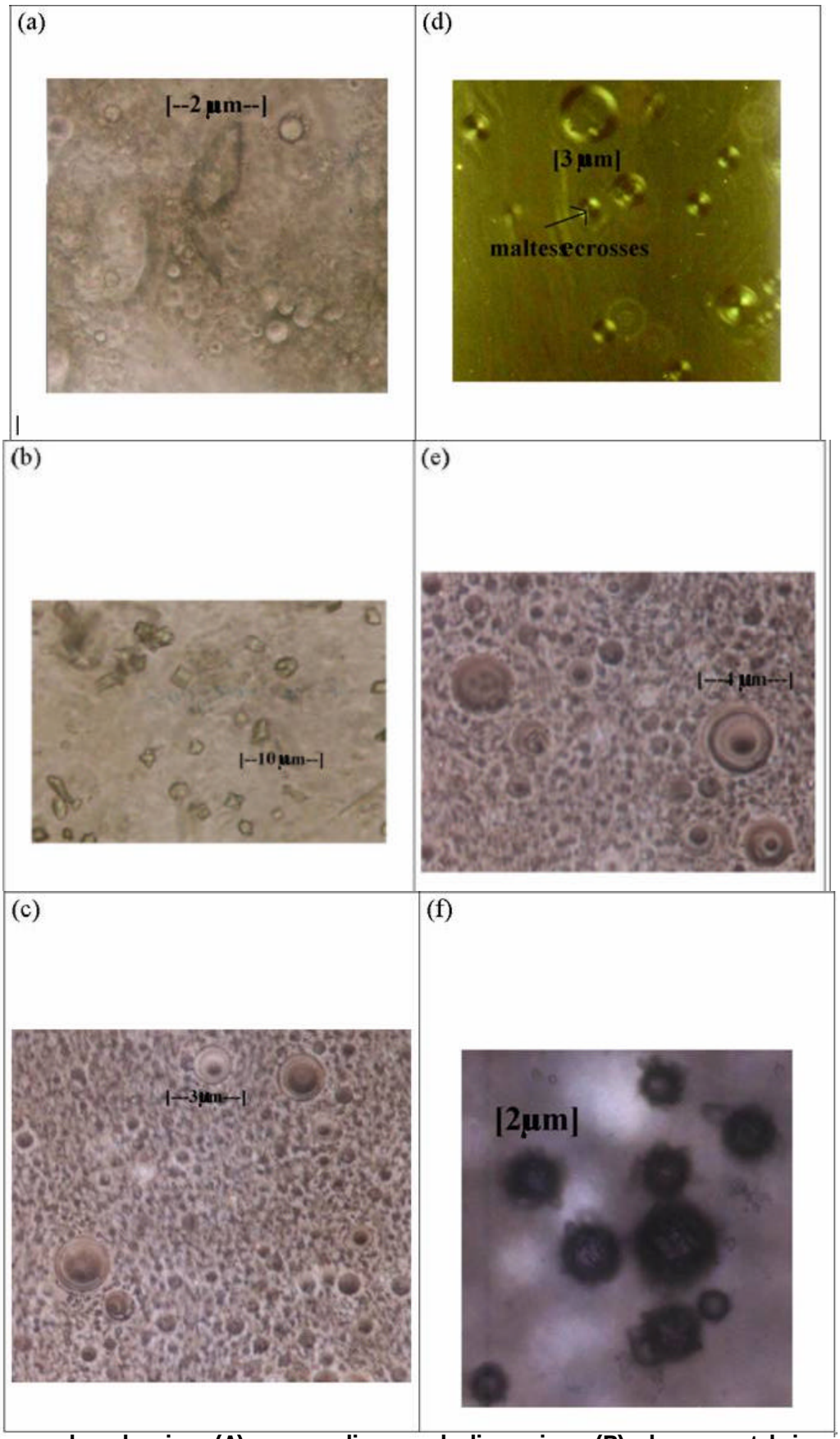

Figure 1. Photomicrographs showing (A) coarse liposomal dispersion; (B) drug crystal in pellet after centrifugation; (C) purified dispersion before lyophilization; (D) purified dispersion under polarizing filter; (E) purified dispersion after lyophilization; and (F) dry powder inhaler upon immediate suspension in water showing the initiation of rehydration.

Table 2. Separation of Free Budesonide (BUD) from Liposome

Sl. No.	Purification Technique	Batch No.	PDE*	Inference
1	Centrifugation ($G = 5.33 \times 10^6 \times g$)	BUD1(a)	97.13 (0.89)	Drug crystals get pelleted along with the liposomes.
2	Gel filtration (Sephadex G 50)	BUD1(b)	42.01 (6.92)	Process nonreproducible, tenuous, and erroneous. Requires modification for each liposome preparation.
3	Dialysis	BUD1(c)	99.25 (0.96)	Insoluble drug cannot pass through the membrane.
4	Protamine aggregation	BUD1(d)	87.01 (0.926)	Efficient yet destructive method; cannot be used for large-scale purification.
5	Centrifugation ($G = 4.38 \times 10^3$ x g)	BUD1(e)	89.72 (0.95)	Comparable and efficient to protamine aggregation as well as nondestructive. Fastest and simplest compared with all the existent methods.

*Mean (± SEM), n = 6.
PDE indicates percent drug entrapment.

entrapped drug per 150 mg of the formulation. The dispersion was frozen at -70°C and dried under negative displacement pressure (Heto Drywinner model DW1 0-60E, Holten, Denmark) for 24 hours. The porous cake thus formed was sized successively through #120 and #240 sieves and rotated in a ball mill (200 rpm) without balls for 10 minutes for further deaggregation of the particles. Capsules (size 2) were filled with powder (150 mg) containing 100 µg of BUD and packed under a nitrogen atmosphere in high density polyethylene bottles containing silica bags as a dehumectant. The bottle was sealed with polyvinyl chloride-coated aluminum foil and stored in a desiccator under refrigeration (2°C-8°C) until further use. A fraction of the powder was rehydrated with triple-distilled water with gentle and occasional agitation. The rehydrated liposomal dispersion was separated from the leaked drug by centrifugation and analyzed for PDRE.

Characterization of liposomes

Assay

BUD uptake, described as PDE, PDRE, and percent drug retention on storage (PDRS) was determined by reverse-phase HPLC [5] . EPC was quantified by ion-pair complexing as described by Stewart [8] ; CHOL was quantified by complexing it with acetic acid and ferric chloride, as described by Zlatkis [9] .

Photomicrography

All the batches of the liposomes prepared were viewed under an Olympus (BX 40F4, Tokyo, Japan) with a polarizing attachment to study their shape and lamellarity. Photomicrographs (x1000 magnification) before and after lyophilization; are shown in **Figure 1** .

Laser light scattering measurement

The mean vesicle size of pre-sonicated liposomes, sonicated liposomes, and rehydrated liposomes after lyophilization (**Table 4**) was determined by the laser diffraction technique using Mastersizer (Malvern Instruments Ltd, Worceftershire, UK) operating at a beam length of 2.40 mm and range of lens at 300 mm.

Table 3. Optimization of Sugar Concentration as Cryoprotectants in Lyophilization

Variable Studied	PDRE* after Lyophilization
Selection of Cryoprotectant	
Lactose	13.42 (2.10)
Sucrose	20.35 (2.34)
Dextrose	08.52 (1.93)
Maltose	14.26 (1.91)
Phase of Cryoprotectant Addition	
External	20.35 (2.35)
Internal	03.72 (1.26)
Both	46.33 (2.01)
Bulk Strength of Sugar (mM)	
100	46.33 (2.01)
250	57.55 (2.44)
400	55.74 (2.34)
500	60.77 (3.02)
600	49.9 (1.79)
Mass Ratio of Sugar (Lipid: Sugar)	
1:04	60.77 (3.02)
1:08	80.06 (1.51)
1:10	92.42 (2.48)
1:12	89.10 (2.41)
1:16	86.78 (1.44)
Phase of Diluent Addition	
Prelyophilization	95.59 (1.72)

*Mean (± SEM), n = 6.
PDRE indicates percent drug remaining entrapped.

Table 4. Comparative Characterization of Potential Batches of Liposomal Budesonide (BUD)*

Variable Studied	Potential Liposomal Batches		
	BUD1	BUD2	BUD3
Mean size ± SEM (size range) (µm)			
Presonication	9.20 ± 0.12 [1.04-51.15]	8.37 ± 0.09 [0.89-125.6]	5.20 ± 0.07 [1.04-69.00]
Postsonication	1.80 ± 0.08 [0.77-28.11]	1.98 ± 0.06 [0.56-32.65]	2.38 ± 0.04 [0.68-37.92]
Postrehydration	1.89 ± 0.14 [0.67-32.65]	2.02 ± 0.15 [0.59-37.92]	2.43 ± 0.11 [0.77-32.65]
Trapped volume† (µL/µmole)	0.520 (0.01)	0.610 (0.01)	0.760 (0.02)
Water content (%)†	0.93 (0.15)	0.77 (0.08)	0.69 (0.10)
Respirable fraction†	14.10 (1.75)	19.50 (1.02)	20.69 (1.50)
Effective index†	37.25 (1.98)	42.71 (1.24)	42.78 (1.78)
Critical relative humidity (%)†	72.32 (1.22)	73.54 (1.37)	74.93 (1.40)

*Control: Budecort (Astra IDL, India): FPF = 26.49 (1.52), Effective Index (EI) = 46.92 (1.86).
*†Mean (± SEM), n = 6.

Determination of trapped volume

Liposomes were spun at high centrifugal force (3.6 x 10^6 x g for 30 minutes) to get a tight pellet, and the supernatant was decanted to remove every drop of excess fluid (including some liposomes if necessary). The pellet was solubilized in 0.1% Triton-X 100 in methanol (10 mL). A small aliquot (0.1 mL) was removed for the quantification of EPC, and the remainder was used to obtain water content by the Karl Fischer method [10] . The trapped volume values (as µL/µmole EPC) are summarized in **Table 4** .

Solid State Characterization

Water Content Determination

Water content of the DPI formulation (1 g) was determined in triplicate on 2 consecutive days by Karl Fischer titration (**Table 4**).

Determination of Critical Relative Humidity

The critical relative humidity (RHo) was determined by measuring the steady-state moisture uptake rate at relative humidity above RHo and then extrapolating the relative humidity at which the moisture uptake is zero [11] . The saturated solutions of different percentages of various salts [12] were placed in desiccators and used at 20°C for creating each respective relative humidity condition. The samples were subjected to respective relative humidity and intermittently weighed for determination of the moisture uptake rate. The results are recorded in **Table 4** .

Fine Particle Fraction

The in vitro aerosol behavior of the formulation was investigated in terms of respirable fine particle fraction (FPF). The FPF values obtained using a twin-stage impinger (apparatus A), official in British Pharmacopoeia [13] , and summarized in **Table 4** . Because the apparatus was prepared in-house per the specifications of British Pharmacopoeia, the FPF value of a marketed preparation was also determined as a control. The flow rate and the actuation time of the twin-stage impinger were 60 L/min and 5 seconds, respectively. The airflow was controlled by a flow control valve, and the flow rate was measured with a Rotameter (Gilmont, model GF-2500, New York, USA). The volume of capturing solvent (methanol) in

the upper (stage 1) and lower (stage 2) chamber were 7 and 30 mL, respectively. FPF was calculated from the amount of drug collected in the lower impingement chamber as a percentage of the dose taken for inhalation. The powder was dispersed using a Rotahaler (Cipla, Mumbai, India); the device was rinsed with methanol to determine the fraction remaining in the device for calculating the effective index.

Drug Retention Studies

Comparative drug retention studies were carried out on the potential DPI formulations at freezer (-10°C±2°C), refrigerated (2°C±2°C), controlled room temperature (25°C±2°C and 60 ± 5% RHo), and accelerated (40°C±2°C and 75±5 % RHo) conditions. The product storage conditions were selected in accordance with ICH guidelines [14] for countries in Zone III (hot, dry) and Zone IV (very hot, humid). The product in its final packing was stored separately in all storage conditions. The samples of each batch stored at various storage conditions were withdrawn at definite time intervals, rehydrated with triple-distilled water for 30 minutes, and analyzed for PDRS within the liposomes. The results calculated in terms of percent BUD retained in liposomes are shown in **Figure 2** . The samples were also examined for the evidence of caking and discoloration.

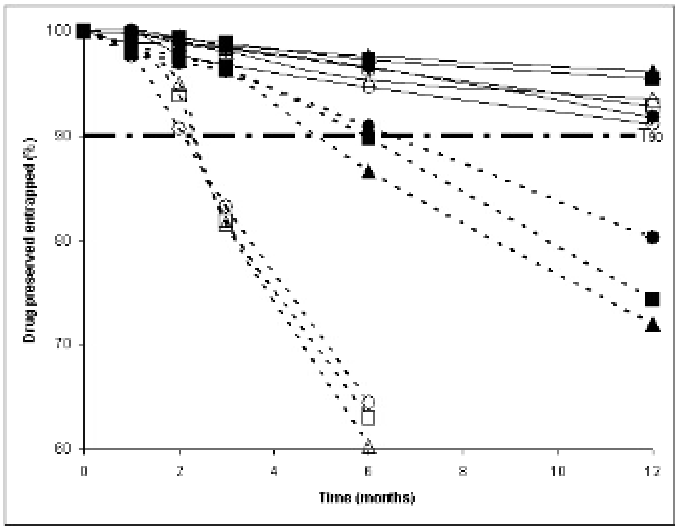

Figure 2. Drug retention studies of budesonide liposome, storage under different conditions, and its effect on T_{90}. Freezer: BUD3 (—s—), BUD2 (—n—), BUD1 (—l—). Refrigerator: BUD3 (—△—), BUD2 (—Ž—), BUD1 (—m—). Controlled room temperature: BUD3 (----s----), BUD2 (-----n-----), BUD1 (-----l-----). Accelerated: BUD3 (----△----), BUD2 (-----Ž-----), BUD1 (-----m-----).

Statistical analysis

Each batch was prepared 6 times and data from all experiments are expressed as mean ± standard error of the mean. Process variables were studied by comparing PDE of 2 batches having all other variables the same.

T_{90} refers to 90% drug retained within the liposomes. Effective index is the geometric mean of the emission fraction and FPF [15] , represented by the following equation:

$$EI = \sqrt{(100 - DF) \times FPF}$$

where DF is the device fraction.

Data were compared using analysis of variation and Student *t* -test, and differences greater than $p > 0.05$ were considered significant.

RESULTS

The liposomes of BUD were prepared using reported [4] lipid film hydration technique and were sized smaller than 5µm by sonication to a reproducible mean liposomal size [16] . The mean liposomal size before sonication decreased with the increased proportion of CHOL (**Table 4**), whereas it increased with the increase in CHOL after sonication. This may be due to the enhanced rigidity of the membrane by inclusion of CHOL.

Separation of free drug

Gel filtration, centrifugation, dialysis, and protamine aggregation techniques were used for separation of free drug. Centrifugation at lower g value was found to be most appropriate for separation of free BUD. Comparison of various techniques used for separation of free drug (crystals) has been recorded in **Table 2**. Separated liposomes were found to possess 89.72±0.95 PDE (BUD1; **Table 2**). PDE in liposomes was confirmed by the protamine aggregation technique. No significant difference in the PDE values was observed when estimated by either of the techniques. Complete separation of free drug was further confirmed by microscopy, as shown in **Figure 1** .

The prepared liposomes of BUD were lyophilized to prepare the DPI, and prepared DPI formulations were characterized and subjected to drug retention studies.

Lyophilization and optimization of cryoprotectants in the preparation of DPI

Initially, the formed lipid film was hydrated with phosphate buffer pH 7.4 containing 1 mM EDTA and free drug was separated by centrifugation. The liposomal dispersion so obtained was re-centrifuged at higher centrifugal force to pelletize liposomes. Liposomal pellets were suspended in phosphate buffer pH 7.4 containing 1 mM EDTA and 100mM lactose, maltose, sucrose, or dextrose, and the PDRE within the liposomal vesicles after lyophilization and rehydration was determined. The results are recorded in **Table 3** . The data reveal that sucrose gives the highest PDRE within liposomes (20.35±2.34) compared with maltose (14.26±1.91), dextrose (8.52±1.93), and lactose (13.42±2.10). This is in agreement with the findings of Madden and coworkers [17] , who examined the effectiveness of a number of sugars in maintaining structural and functional properties of microsomal membranes at low mean liposomal size and found trehalose to be the most effective cryoprotectant. At higher concentrations, however, sucrose was found to be equally effective for liposomes of large mean size and gave a porous cake with no sign of shrinkage. When sucrose as a cryoprotectant was provided only on the outside or the inside of the vesicles, a marked increase in permeability of liposome membrane was observed because of a difference in osmotic pressure across the liposomal membrane. For providing sucrose only inside the vesicles, the hydrated liposomes were spun at high centrifugal force; the pellet obtained was then resuspended into the hydration medium with the same composition without sucrose. Similarly, for providing sucrose outside only, the formed film was hydrated with the same hydration medium without sucrose and centrifuged; the pellet so obtained was then resuspended in the hydration medium containing sucrose before lyophilization. When sucrose was present only outside or only inside, less than 20.35±2.35 and 3.72±1.26 PDRE resulted, respectively. When sucrose was present on both the sides of the bilayer (**Table 3**), 46.33 ± 2.01 PDRE was obtained. Crowe [17] and others [18] have reported similar findings.

The batches of liposomal BUD were lyophilized in the presence of different concentrations of sucrose. The PDRE was found to be dependent on cryoprotectant concentration (**Table 3**). With the increase in the concentration of sucrose from 100 mM to 600 mM, PDRE within the liposomes was found to be highest (60.77±3.02) at the 500 mM concentration. When the concentration of sucrose was further increased from 500 mM to 600 mM, there was a reduction in PDRE within the liposomes after lyophilization. This may be due to the fact that in the process of dehydration, the liposomes were constricted and coated on the surface of internally deposited sugar. This stabilization by coating is in synergism with the hydration of polar head groups with the hydroxyl group of sucrose, which replaces the lyophilizing water molecule. If a less than optimum sucrose concentration was used, the internally deposited sugar did not provide adequate surface for the adherence of constricted bilayers. Similarly, if the sucrose concentration was high, the deposited sugar may pierce out of the bilayer membrane this is based on the assumption that the vesicles, once dehydrated in the presence of sucrose, retain their contents and do not re-encapsulate. It may be concluded that the bulk concentration of sugar required as cryoprotectant depends upon the type of sugar selected and upon the saturation of the polar head groups of the bilayer by drug or other formulation components. The lamellarity and size of liposomes are expected to change these requirements.

In another set of experiments, the PDRE was also studied by changing the mass ratio between sugar and lipid in the dry state. The samples were sequentially diluted to produce mixtures with varying lipid:sucrose mass ratio, but with the same concentration of sucrose (500mM). The results recorded in **Table 3** reveal that the mass ratio between the lipid and the sugar is equally important for maintaining the permeability barrier of liposomal vesicles. A mass ratio of 1:10 for lipid:sucrose was found to be the most effective and found to possess 92.42±2.48 PDRE after lyophilization. A further increase in sucrose (1:12) was found to decrease PDRE after lyophilization (89.10±2.41). The addition of sorbolac before lyophilization further increased PDRE after lyophilization to 95.59±1.72. The presence of lactose (sorbolac) resulted in the formation of a relatively more porous cake, thereby reducing the physical damage to the bilayer membrane during lyophilization. Thus, lactose being in an insoluble dispersed state helped in reducing the damage by glassy matrix [2] . However, when sorbolac was incorporated both internally and externally along with sucrose at the same ratio, no further improvement in PDE was observed (78.93±2.58), and the PDRE after lyophilization was 95.59±1.72.

Finally, these 3 formulations-BUD1, BUD2, and BUD3-were made having EPC:CHOL ratios 1:0, 2:1, and 1: 1 (**Table 1**) with PDE of 95.80±0.99, 82.42±1.90, and 86.91±1.29 and PDRE values of 95.59±1.72, 95.83±2.18%, and 95.84±2.28%, respectively. These formulations were characterized and evaluated for PDRS.

Characterization

Estimations of different components of liposomes were carried out to confirm the mass balance and recoveries of drug; EPC and CHOL were found to be between 98% and 100%.

Polarizing microscopy confirmed the formation of multilamellar and spherical liposomes (**Figure 1**). Multilamellar vesicles were also identified by the presence of Maltese crosses. Microscopy after immediate dispersion of DPI in water (**Figure 1F**) reveals the particle size of DPI formulation to be smaller than 5 µm, thereby confirming its dispersibility in air. Laser light scattering microscopy revealed mean liposomal sizes of rehydrated liposomes after lyophilization to be highest for BUD3 (2.43±0.11 µm), followed by BUD2 (2.02±0.15 µm) and BUD1 (1.89±0.14 µm). With the increase in the proportion of cholesterol and mean liposomal size from batch BUD1 to BUD3, the trapped volume also increased (**Table 4**), which confirmed its direct relationship to mean liposomal size. From the trapped volume values recorded in Table 4 and the studies reported in the literature [19,20] , it may be concluded that the majority of vesicles produced in this study are multilamellar even after downsizing by sonication to small sizes as 1.80±0.08 µm (BUD1). By extrapolation of the graph for unilamellar vesicles of size 1.8 µm [19] , the value of the trapped volume is 50 µL. It is many-fold higher than the actual value of 0.52 µL; the volume that cannot be accounted for is occupied by the internal bilayers [20] of an MLV. The formulation had a low moisture content (< 0.93±0.15, **Table 4**), which confirms its low aggregation tendency.

During early development, it is imperative to characterize the moisture sorption and desorption attributes of the formulation. This results in a number of advantages, including improved flow properties and dispersion, as well as enhanced physical stability in the bulk and final dosage form resulting from minimal moisture. Hence, RHo for finding the moisture uptake rate at different relative humidities was determined using the method reported by Zografi et al [11] ; results are recorded in **Table 4**. Unlike the individual components, the formulation did not deliquesce when stored at a relative humidity (RH) greater than the RHo (BUD1, 72.32±1.22; BUD2, 73.54±1.37%; and BUD3, 74.93±1.40%); instead, the formulation started forming adhered flocs followed by caking and finally creaming (pasty mass). It may be concluded from the above behavior that storage and processing of the formulation above RHo can lead to stability problems because EPC undergoes rapid hydrolysis [21] . Similarly, if the processing is carried out under extremely dry conditions, the level of adsorbed moisture may not be sufficient to dissipate attractive electrostatic forces, resulting in particle adhesion. The presence of moisture in the air provides a conducting medium through which the electrostatic charge can easily dissipate [22] . Hence, determination of RHo and stringent processing at a RH below RHo, which is still not too dry, is a necessary manufacturing condition [23, 24] .

From the 2 devices described in the British Pharmacopoeia [13] for particle characterization of FPF, a twin-stage impinger (apparatus A) was used. The results obtained from the studies suggest the fraction of the DPI likely to deposit in the lower airways. Ideal DPI formulation should provide small device fraction (effective emission from the device) and large FPF when inhaled. The FPF value (**Table 4**) for the optimized formulation (BUD3, 20.69 ± 1.50) was comparable with that of the marketed control (26.49 ± 1.52), suggesting substantial deposition of the developed liposomal BUD DPI formulation in the lung. The FPF ratios of control to that of the developed liposomal DPI formulations are 1.87, 1.36, and 1.28; effective index ratios are 1.25, 1.10, and 1.10 for BUD1, BUD2, and BUD3, respectively. The reduced ratio of Effective Index (EI) as compared to the ratio of FPF is indicative of efficient dispersion of BUD1 from the device, but, unlike the control, a higher proportion of the dispersed powder has been deposited in the upper respiratory tract [15] .

DISCUSSION

Drug retention studies

At accelerated storage, T_{90} (90% drug retention) was found to be between 2 months to 2.5 months, for controlled room temperature storage it was 5 months to

6 months, for refrigerated and freezer storage it was above 1 year (**Figure 2**). With the increase in CHOL content of the formulation, the PDRS at freezer condition after 1 year increased 91.81±0.92 for BUD1, 95.46±1.09 for BUD2, and 96.13±1.16 for BUD3 (**Figure 2**). Incorporation of CHOL is known to cause strong reduction in the permeability of the liposome system and thus reduce leakage of drug from the liposomes [25] . However, under the present anhydrous state of storage, the incorporation of CHOL was shown to proportionately increase the PDRS (**Figure 2**). This gives an understanding that reduction in the permeability of the membrane may not be the only mechanism acting, because in the anhydrous state there is not any possibility of drug diffusion; therefore, drug retention cannot be increased by reducing the permeability alone. It was therefore concluded that CHOL might have some association with EPC at the molecular level through weak bonding, thereby reducing the level of stress vector introduced during lyophilization as proposed by Van Winden et al [2] . Contradictory to the above result, batches stored at 25°C, 60% RH for 1 year showed decrease in the PDRS with an increase in CHOL in BUD1 (80.27±1.29), BUD2 (74.23±2.38), and BUD3 (71.98±1.62). This improved retention with higher proportion of amphiphilic EPC is due to the re-encapsulation of the drug by the vesicles and a higher proportion of EPC (BUD1), which will provide for a higher re-encapsulation capacity. The drug, being very hydrophobic, undergoes re-encapsulation rapidly and more preferentially. Lyophilization provided a shelf life of 1 year with storage under refrigerated conditions.

Caking and discoloration (cream color) was noted under accelerated storage conditions after 3 months. These phenomena were less evident at long-term storage and were not observed at refrigerated and freezer storage. Even the flow and dispersion properties of the formulation stored at long-term refrigerated and freezer conditions were same as the initial formulation.

CONCLUSION

Recently, considerable interest has been generated in the use of the liposomal system in the delivery to the lungs of drugs and other biologically active molecules[26, 27] . The ability of liposomes to encapsulate a drug within multilamellar vesicles and store the vesicles conveniently in a dehydrated form using a cryoprotectant offers a number of advantages over conventional pulmonary drug delivery procedures. Before such an aim can be commercially realized, the liposomally entrapped drug must be obtained in a form that can conveniently and selectively deliver it to the lung. In this study, the small multilamellar liposomal vesicles loaded with BUD were successfully prepared and stabilized by lyophilization in DPI formulations with a reasonable shelf-life. The findings of this investigation also demonstrate the possibility of delivery of liposomally entrapped BUD to terminal bronchioles in comparable doses. Hence, developed liposomal BUD formulated as DPI offers exciting possibilities of liposomal delivery in the anhydrous state.

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