



Proliposome powder or tablets for generating inhalable liposomes using a medical nebulizer

Iftikhar Khan¹ · Sakib Yousof² · Mohammad Najlah³ · Waqar Ahmed⁴ · Abdelbary Elhissi⁵

Received: 29 May 2020 / Accepted: 28 July 2020
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Abstract

Purpose The aim of this study was to develop and compare proliposome powder and proliposome tablet formulations for drug delivery from a Pari-LC Sprint nebulizer.

Methods Proliposome powders were prepared by the slurry method and sorbitol or mannitol carbohydrate carrier were used in a 1:10 and 1:15 w/w lipid phase to carrier ratio. Beclometasone dipropionate (BDP; 2 mol%) was incorporated in the lipid phase. Proliposome powders were compressed into tablets, and liposomes were generated from proliposome powders or tablets within the nebulizer reservoir for subsequent aerosolization.

Results Comparatively, shorter sputtering times were reported for the tablet formulations ($\approx < 2.7 \pm 0.45$ min), indicating uniform aerosolization. Post-nebulization, liposomes size was larger in the nebulizer reservoir in the range of 7.79 ± 0.48 μm – 9.73 ± 1.53 μm for both powder and tablet formulations as compared to freshly prepared liposomes (5.38 ± 0.73 μm – 5.85 ± 0.86 μm), suggesting liposome aggregation/fusion in the nebulizer's reservoir. All formulations exhibited more than 80% mass output regardless of formulation type, but greater BDP proportions (circa 50%) were delivered to the Two-stage Impinger when tablet formulations were used. Moreover, the nebulized droplet median size and size distribution were lower for all tablet formulations in comparison to the powder formulations. Proliposome tablet and powdered formulations demonstrated the ability to generate vesicles that sustained the release of BDP.

Conclusion Overall, this study showed that proliposome tablets could be disintegrated within a Pari-LC Sprint nebulizer to generate inhalable aerosol, with high drug output and hence can be manufactured on large scale to overcome the storage problems associated with powder formulations.

Keywords Liposome · Proliposome · Jet-nebulizer · Two-stage impinger · Beclometasone dipropionate · Tablets

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s40005-020-00495-8>) contains supplementary material, which is available to authorized users.

✉ Iftikhar Khan
I.Khan@ljmu.ac.uk; iftikharkhans@yahoo.com

- ¹ School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool L3 3AF, UK
- ² School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston PR1 2HE, UK
- ³ Pharmaceutical Research Group, School of Allied Health, Faculty of Health, Education, Medicine and Social Care, Anglia Ruskin University, Chelmsford CM1 1 SQ, UK
- ⁴ School of Mathematics and Physics, College of Science, University of Lincoln, Lincoln LN6 7TS, UK
- ⁵ Office of The Vice President for Research and Graduate Studies, College of Pharmacy, Qatar University, Doha, Qatar

Introduction

Aerosol formulations have been employed for pulmonary drug delivery to achieve localized treatment for respiratory and other immune-mediated diseases (Waldrep. 1998; Godet et al. 2017). Moreover, the pulmonary system offers a large surface area (82–100 m²), facilitating fast drug absorption to the systemic circulation (Khan et al. 2013, 2016). However, deep lung deposition, associated with better treatment outcomes, is challenging. As a consequence, pulmonary formulations and devices are areas of continuous research (Khan et al. 2013). Medical nebulizers are devices used to generate inhalable aerosol, offering deep lung deposition, mostly for immediate drug release. Liposomes have been investigated for around five decades as drug carriers in pulmonary inhalation. This is due to their ability to entrap hydrophilic and hydrophobic drugs (Rojanarat et al. 2012; Kontturi

et al. 2019; Subramanian et al. 2016; Bnyan et al. 2020) and localize drug action in the lung for prolonged periods (Elhissi. 2017). Liposomes may minimize local irritation of the lung that may happen because of direct contact between the drug and pulmonary epithelium (Girod de Bentzmann et al. 1994). Furthermore, inhalation of drug in liposome formulations may provide uniform drug deposition in the lung, prolonging drug action via sustained release, and minimize local toxicities in the pulmonary epithelium (Darwis and Kellaway. 2001; Elhissi 2017)

Nebulization performance of liposomal formulations is dependent on an interplay of factors including liposome size, formulation composition, nebulizer type and design, and nebulization parameters (Bridges and Taylor. 1998; Taylor et al. 1990; Elhissi et al. 2015; Niven et al. 1991). Liposomes have demonstrated commercialization success in the field of inhalation therapy; for example Arikayce® (Insmad, NJ, USA) is a nebulizable formulation of amikacin-loaded liposomes approved recently by FDA for Mycobacterium avium complex lung disease (Shirley. 2019).

However, the poor stability of liposomes during storage is still a prominent drawback limiting their shelf life. The instabilities are attributed to oxidation and hydrolysis of phospholipids, causing aggregation and fusion of lipid vesicles in an aqueous environment, which may cause leakage of the originally entrapped material (Hunt and Tsang. 1981; Khan et al. 2018b). Proliposomes are stable formulations that can generate liposomes upon hydration with aqueous phase. Proliposomes are stable powders of carbohydrate coated or mixed with phospholipid, cholesterol and drug using small scale production techniques such as modified rotary evaporators (Payne et al. 1986) or large scale methods such as fluid-bed coating (Gala et al. 2015) or spray-drying (Omer et al. 2018). Alternatively, and as an interest of the present research investigation, the slurry-based method can be used on small or large scales as shown by our research group (Khan et al. 2015). Powders, in general, are susceptible to atmospheric factors (moisture, temperature, and humidity) and are difficult to store, due to their bulkiness and large surface area. Tablets are more convenient for patient administration and can offer advantages in terms of storage and handling when compared to powder formulations. In a recent investigation, we capitalized on the advantage of the slurry method by developing proliposome tablets having highly desirable characteristics (Khan et al. 2018a).

In the present study, we developed Beclometasone dipropionate (BDP) proliposome tablets, and investigated the ability of the tablets, upon crushing, to generate inhalable liposomal aerosol similar to that generated from their corresponding powder formulations. We studied the aerosol characteristics when sorbitol or mannitol were used as carbohydrate carriers in 1:10 or 1:15 w/w lipid to carrier. The characteristics of aerosols generated from the tablet

formulations were studied and compared to their corresponding powder formulations using laser diffraction size analysis. The output and output rate of the formulations were also reported to evaluate the delivery efficiency. Furthermore, the drug deposition profile was evaluated in vitro using inertial impaction by employing the Two-stage Impinger (TSI), and the drug release profiles of the formulations were also investigated. This is the first study that proposed anti-asthma proliposome tablets made from simple traditional phospholipid and carbohydrate compositions as a system for pulmonary delivery via nebulization.

Materials and methods

Materials

Beclometasone dipropionate (BDP), cholesterol, sorbitol (particle size; 250–700 µm) and D-mannitol (particle size; < 250 µm) were purchased from Sigma Aldrich, UK. Absolute ethanol was bought from Fischer Scientific, UK. Soya phosphatidylcholine (SPC) (Lipoid S-100) was kindly gifted by Lipoid, Switzerland. Ferric chloride and Ammonium thiocyanate were procured from VWR Scientific, UK. A Pari-LC Sprint Air-jet nebulizer fitted with a TurboBoy compressor were bought from PARI GmbH, Germany to perform all nebulization studies. All materials were used as received.

Methods

Preparation of proliposomes via the slurry method

Proliposome powder formulations were prepared via the slurry method (Khan et al. 2015, 2018b), using sorbitol or mannitol as carbohydrate carriers in 1:10 or 1:15 w/w lipid phase to carrier. Particle size of sorbitol ranged from 250 to 700 µm, whilst mannitol particle size was < 250 µm; both powders were used in the size range supplied. SPC and cholesterol were employed in combination as the lipid phase in 1:1 mol ratio, containing 166.66 mg and 83.34 mg, respectively. BDP was incorporated in 2 mol% (i.e. 4.48 mg) to the lipid phase (i.e. 250 mg). The lipid phase constituting SPC, cholesterol and BDP were dissolved in absolute ethanol (8.26 ml for the 1:10 formulation and 12.01 ml for the 1:15 formulation (i.e. 333 mg/ml)). Sorbitol or mannitol particles were placed in a round bottom flask (100 ml) and the ethanolic solution was poured onto the carbohydrate carrier to form a slurry, ensuring uniform distribution of the lipid phase and drug over the carrier particles (the amount of carbohydrate carrier was 2.5 g for 1:10 formulations and 3.75 g for 1:15 formulations). A rotary evaporator (Buchi Rotavapor R-114, Buchi, Switzerland) was utilised to evaporate

the ethanol from the slurry, using a vacuum pump (Buchi Vac V-501), and by setting up the water bath of the evaporator at 45 °C and the rotation speed at 280 rpm for 2 h. Proliposome granules constituting carbohydrate particles coated with lipid were then collected and stored at -18 °C for conducting subsequent studies.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Surface morphology of proliposome powders was determined using SEM. For SEM, proliposome powder formulations were transferred to aluminium stubs. Excess powder from the stub was removed via air spray, followed by gold coating using a JFC-1200 Fine Coater under vacuum for ~2 min. Proliposome samples were observed and images were captured under SEM (Quanta 200, Czech Republic) at 20 kV. For TEM, a drop of liposomes suspension (generated from proliposome via hydration) and a drop of negative stain (1% w/v phosphotungstic acid) were transferred to a carbon coated copper grid (400 mesh) (TAAB Laboratories Equipment Ltd., UK). After drying (1 h), liposomes vesicles were viewed and photographed using a Philip CM 120 Bio-Twin TEM (Philip Electron Optics BV, Netherlands).

Compression of proliposomes via the minipress single punch machine

Sorbitol or mannitol-based proliposome granules in either 1:10 or 1:15 w/w lipid to carrier ratio were compressed into round biconvex tablets (6 mm in diameter) using the Riva Minipress MII SA single punch machine (Cenova 4018 Ciudadela, Buenos Aires, Argentina). Tablet weights achieved for sorbitol-based proliposomes in 1:10 and 1:15 lipid to carrier were 90.17 ± 4.30 mg and 91.12 ± 2.66 mg, respectively; and for mannitol-based proliposomes were 91.44 ± 4.21 mg and 91.95 ± 3.95 mg, respectively.

Generation of liposomes from proliposomes

For the purpose of nebulization, 150 mg of proliposome powder or tablets formulation (10 tablets were gently crumbled in a mortar and pestle) and transferred into the reservoir of a Pari-LC Sprint nebulizer (fitted with a TurboBoy Compressor; PARI GmbH, Germany). Deionized water (DW; 5 ml) was poured in the nebulizer reservoir, followed by 3 min of manual shaking to permit tablet disintegration and liposome formation. The concentration (i.e. hydration) of proliposomes utilised was 30 mg/ml in the nebulizer reservoir.

Characterization of liposomes before and after nebulization

Liposomes generated from proliposome powders or tablet formulations were characterized for size (also referred to as volume median diameter; VMD) and size distribution (expressed as span) via laser diffraction, using the Malvern Mastersizer 2000 (Malvern Instruments LTD., UK). These analyses were conducted prior to nebulization (i.e. when liposomes were generated from proliposomes powders or tablets via hydration) and post-nebulization (i.e. samples were collected from the nebulizer reservoir, and the upper and lower stages of TSI). The liposome size and size distribution measurements following completed nebulization were compared to those of freshly generated vesicles prior to nebulization, in order to study the influence of shearing provided by the nebulizer on the integrity of the vesicles.

Stewart assay

The Stewart assay (Stewart 1980) was conducted to determine the lipid concentration in liposome formulations, so that how efficient was the process of coating the lipid onto the carrier particles can be evaluated. Individual samples of liposome suspension (1 ml) hydrated from sorbitol or mannitol-based proliposomes were placed in a glass centrifuge tube. Ethanol was added to dissolve the lipid, and samples were placed overnight in an oven (i.e. 90 °C). Chloroform (2 ml) and ammonium ferrocyanide solution (2 ml) (ammonium thiocyanate 15.20 g and ferric chloride 13.52 g were dissolved in 500 ml deionised water) were added in the centrifuge tube. Vortex mixing was conducted for 1 min, followed by centrifugation at 300 g for 5 min using a bench centrifuge (Jouan B4i, Thermo Electron Corporation, UK). The resultant lower chloroform layer containing lipid was separated from the upper maroon-coloured iron layer and quantified using UV-Visible spectrophotometer at 488 nm (Jenway, 7315, Bibby Scientific Ltd, UK).

Nebulizer performance

Nebulizer performance was assessed by examining a number of variables, including 'nebulization time' and 'sputtering time'. The time required for aerosol generation to start becoming erratic and intermittent was determined as the nebulization time. Whereas, further tapping of the nebulizer leads to continuation of the erratic aerosolization is referred to as 'sputtering'; this duration continued until complete cessation of aerosol generation (i.e. till dryness) and was reported as 'sputtering time'. Moreover, complete nebulization time was referred to the total time of both 'nebulization time' and 'sputtering time'. Nebulizer performance was conducted using sorbitol or mannitol-based proliposome

powders or tablets in both 1:10 and 1:15 w/w lipid phase to carrier ratio.

Mass output and output rate

After achieving nebulization to ‘dryness’, aerosol mass was determined by calculating the percentage difference in mass (i.e. weight) between the liposomal formulation in nebulizer (150 mg/5 ml), before and after completed nebulization. This was conducted for both sorbitol and mannitol-based proliposome (in 1:10 and 1:15 w/w lipid to carrier ratios) powders and tablets. The percentage mass output was determined using the following equation;

$$\text{Mass output(\%)} = \left(\frac{\text{Weight of nebulized formulation}}{\text{Weight of formulation present in the nebulizer prior to nebulization}} \right) \times 100$$

Output rate was also calculated gravimetrically using the following equation:

$$\text{Aerosol output rate (mg/min)} = \left(\frac{\text{Weight of nebulized formulation}}{\text{Complete nebulization time}} \right)$$

Nebulizer output efficiency of (BDP)

The output efficiency of the Pari-LC Sprint nebulizer was determined by calculating the concentration of BDP collected from the nebulizer reservoir, the upper stage and the lower stage of TSI after completed nebulization to ‘dryness’ (Section “[Nebulizer performance](#)”). BDP concentration was analysed and calculated via high performance liquid chromatography (HPLC) (Agilent 1200 HPLC instrument, UK). A mobile phase constituting methanol and HPLC-grade water was used in 75:25 (v/v) with a flow rate of 1.7 ml/min. The temperature was set at 40 °C, with an injection volume of 20 µl and detection wavelength of 239 nm. The HPLC instrument employed was set with C-18 column (4.6 mm × 150 mm, 5 µm; Agilent Technology, USA). A calibration curve for BDP in methanol was constructed in the concentration range of 0–50 µg/ml.

Assessment of drug deposition using the (TSI)

The Two-stage Impinger (TSI) was employed as an in vitro aerosol deposition model for the nebulized formulation (in the form of aerosol). Whilst the lower stage of TSI collects particles potentially eligible for ‘peripheral airway’ deposition (respiratory bronchioles and alveoli), the upper stage of the TSI collect particles that would be expected to deposit in

the upper respiratory tract. The aforementioned deposition eligibility assumption is based on typical application of 60 l/min air flow rate across the TSI (the cut-off aerodynamic size between the two stages at this flow rate is 6.4 µm) (Hallworth and Westmoreland, 1987). During assembly of the TSI, the upper and lower stages were loaded with 7 ml and 30 ml of DW, respectively. Each liposome formulation (i.e. sorbitol or mannitol-based proliposome powder and tablets for both 1:10 and 1:15 w/w ratios (150 mg/5 ml in DW)) was generated in the nebulizer reservoir, prior to nebulization (Section “[Generation of liposomes from proliposomes](#)”). The nebulizer position was positioned with its mouthpiece towards the TSI, negative pressure of 60 l/min was applied

by the vacuum pump and nebulization was started and completed to ‘dryness’.

Size analysis of nebulized droplets using laser diffraction

VMD and span of the aerosol droplets were characterized by laser diffraction, using a Malvern SprayTec (Malvern Instruments LTD, UK). Liposomes were generated from powder or tablet proliposome formulations (sorbitol or mannitol-based formulations in 1:10 and 1:15 w/w ratio) in the Pari-LC Sprint nebulizer (Section “[Generation of liposomes from proliposomes](#)”). In order to maintain uniform aerosol generation and measurement, the nebulizer was clamped securely at a fixed position of 2.5 cm distance from the laser beam. The aerosols generated from the nebulizer were traversed through the laser beam with the aid of a vacuum pump (Copley Scientific, UK). Aerosol droplet size and span values were recorded throughout nebulization until the ‘dryness’ status was reached (150 mg of proliposome powder or tablet were used in 5 ml of DW).

Release study of BDP from liposomes

The release study of BDP from liposomes was conducted using dialysis bags (a cut-off MW 3500 Da; this was selected based on the pore size, which should be between 5–10 times larger than the molecular weight of BDP i.e. 521.04 Da) followed by HPLC analysis. This method was adopted from the release study previously conducted for BDP (Khan et al. 2018b; Nasr et al. 2014). Hydrated liposomes (i.e. from proliposome powder or proliposome tablets) containing 500 µg BDP were sealed in a dialysis bag and placed in 15 ml phosphate buffer solution containing 20% of methanol at 25 °C, with a continuous stirring at 100 rpm. Methanol was used in

order to overcome solubility issues with the drug. Samples (200 µl) were withdrawn every hour, and replaced each time with the same amount of drug-free media. The BDP release study was conducted for 8 h using HPLC. BDP (500 µg), dissolved in methanol was also employed for comparison purposes and to find out its release profile as a control.

Statistical analysis

Analysis of variance (ANOVA) or Student's t-tests were performed as appropriate for statistical analysis using the SPSS software, to determine whether the difference between the compared groups was significant. A value of $p < 0.05$ was considered an indicator that the difference between the compared groups is statistically significant. All experiments were conducted in triplicate using three different batches.

Results and discussion

VMD and span analysis of liposomes prior and post-nebulization in TSI

To assess the effect of the nebulization on liposome size, measurements were taken before and after nebulization. Irrespective of carrier type and lipid concentration, liposomes generated from proliposome powders

or proliposome tablets had similar size ($p > 0.05$). However, post-nebulization into the TSI, liposome size varied depending upon the compartment of deposition (i.e. nebulizer reservoir, upper stage or lower stage). For example, compared to upper stage and lower stage, liposomes remaining in the nebulizer reservoir were larger in size for both tablets ($9.32 \pm 1.58 \mu\text{m}$ – $9.73 \pm 1.53 \mu\text{m}$) and powders (7.79 ± 0.48 – $9.67 \pm 0.35 \mu\text{m}$) for 1:10 and 1:15 ratios, respectively (Table 1). The increase in lipid concentration and decrease in temperature of nebulizer contents within the jet-nebulizer during nebulization increases the viscosity of formulations and the adherence of atomized primary aerosol to the inner walls of the nebulizer (McCallion et al. 1995; Finlay et al. 2000; Steckel and Eskandar. 2003). It has been previously reported that phospholipid may accumulate within the nebulizer reservoir during jet-nebulization, causing aggregation of liposomes and/or retention of large liposomes within the nebulizer (Ferron et al. 1976; Bridges and Taylor. 1998). This also agrees with our findings for liposomes generated from particulate-based proliposomes within air–jet (Elhissi and Taylor. 2005; Elhissi et al. 2012), ultrasonic and vibrating–mesh nebulizers (Elhissi and Taylor. 2005).

Moreover, the measured VMD of liposomes in the upper stage of the Impinger was independent of formulation type (tablet or powder), carrier type (sorbitol or mannitol) or lipid concentration (1:10 or 1:15 lipid to

Table 1 Volume median diameter (VMD) and span values for liposomes generated from sorbitol or mannitol-based proliposome powder and tablets in a 1:10 and 1:15 w/w lipid phase to carrier ratio by Pari-LC Sprint nebulizer before and after nebulization

Proliposome formulations		Before nebulization Liposome (freshly prepared)	After nebulization		
			Nebulizer reservoir	Upper stage	Lower stage
VMD	Powder				
	Sorbitol 1:10	5.49 ± 1.78	8.33±0.53	5.66±0.56	4.44±0.81
	Sorbitol 1:15	5.68 ± 1.55	7.79±0.48	5.19±0.35	3.56±0.21
	Mannitol 1:10	5.43 ± 0.74	9.67±0.35	4.62±0.34	3.71±0.25
	Mannitol 1:15	5.38 ± 0.73	9.06±1.81	4.59±0.14	3.17±0.62
	Tablets				
	Sorbitol 1:10	5.61±0.84	9.32±1.58	5.78±0.82	3.68±0.58
	Sorbitol 1:15	5.85±0.86	9.38±1.15	5.32±0.91	2.14±0.91
	Mannitol 1:10	5.47±0.65	9.73±1.53	5.34±0.62	3.21±0.67
	Mannitol 1:15	5.39±0.78	9.66±0.36	6.38±0.71	3.63±1.09
Span	Powder				
	Sorbitol 1:10	2.32 ± 0.71	2.58±0.27	3.07±1.26	3.70±0.60
	Sorbitol 1:15	2.62 ± 0.75	2.39±0.32	2.33±0.36	2.02±0.39
	Mannitol 1:10	3.80 ± 0.79	2.60±0.29	2.40±0.33	3.50±0.80
	Mannitol 1:15	3.46 ± 0.97	2.46±0.15	2.16±0.50	2.24±0.21
	Tablets				
	Sorbitol 1:10	2.51 ± 0.61	3.78±1.39	3.63±0.24	1.72±0.64
	Sorbitol 1:15	3.62 ± 0.72	2.52±0.84	2.62±1.23	1.18±0.96
	annitol 1:10	2.39 ± 0.53	2.58±1.16	1.72±0.87	2.51±1.14
	Mannitol 1:15	2.47 ± 0.62	3.12±1.67	3.41±0.71	2.86±1.11

Data are mean ± SD, n = 3; $p < 0.05$

carrier) ($p > 0.05$), indicating that nebulized droplets with similar size carry liposome vesicles of similar size (Table 1). VMD of liposomes in the upper stage of TSI were notably similar to the VMD of liposomes prior to nebulization, indicating that shearing during nebulization had minimal or no effect on size of vesicles depositing in the Impinger's upper stage. For all formulations, liposomes characterized in the lower stage of the TSI were found to have smaller size than those collected from the Impinger's upper stage and nebulizer reservoir (Table 1). This demonstrates that liposomes sheared into a smaller size within the nebulizer were incorporated into smaller droplets that deposited into the lower stage of the TSI; this 'fractionation behaviour' was observed in our previous investigations using liposomes generated from ethanol-based proliposomes (Elhissi et al. 2006).

Reduction in liposome size during nebulization was attributed to compressed air-driven shearing within the nebulizer and impaction of liposomes on the baffles and internal walls of the nebulizer, causing fragmentation of large liposomes into smaller vesicles (Taylor et al. 1990; Saari et al. 1999). Upon examination of span values, no significant difference ($p > 0.05$) was observed between proliposome powder and tablet formulations (regardless of carrier type, and lipid to carrier ratio) prior to or post-nebulization (including nebulizer reservoir, upper and lower stage of TSI) (Table 1).

Morphology of proliposome formulation via SEM and TEM

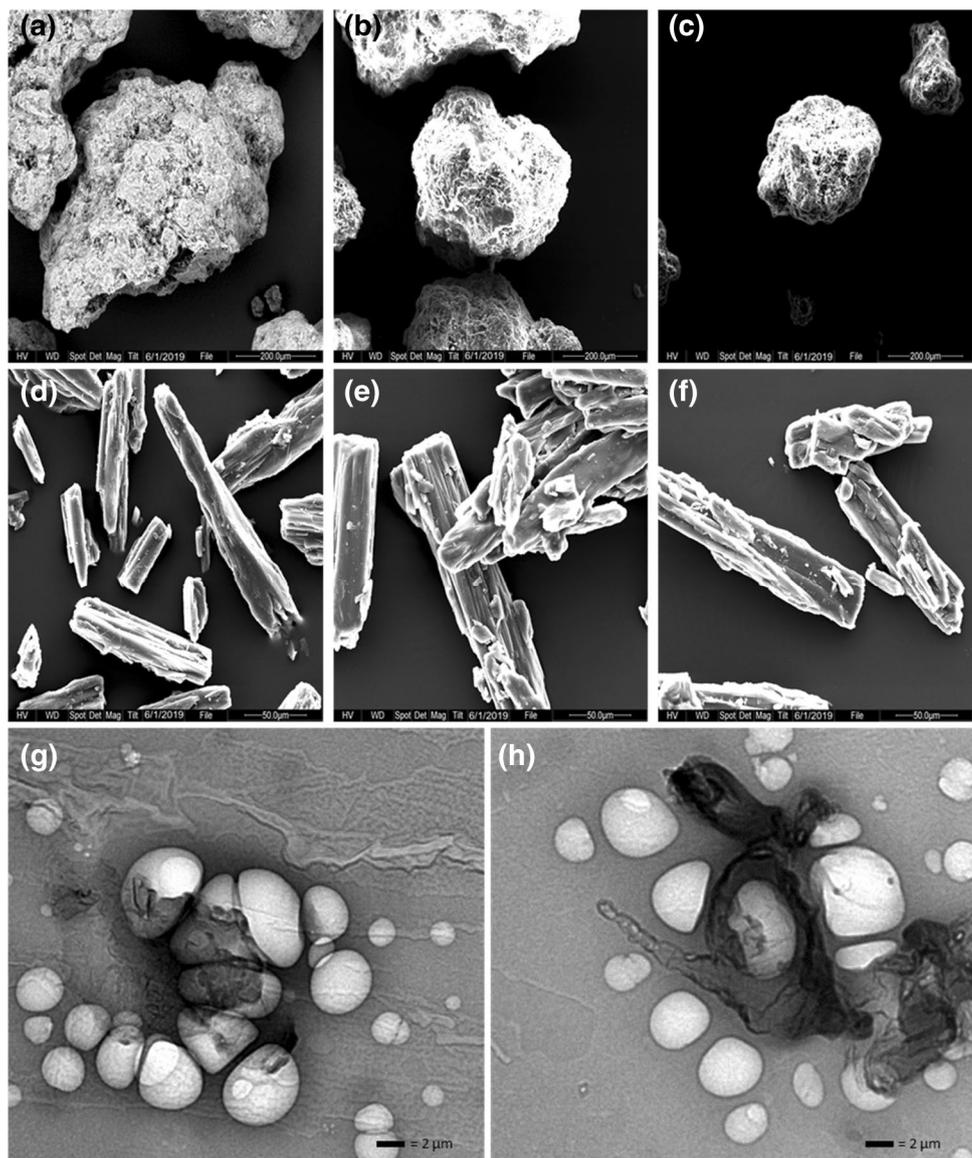
The surface morphology of coarse carbohydrate carriers i.e. sorbitol and mannitol as well as proliposome powders of sorbitol and mannitol-based formulations using both 1:10 and 1:15 w/w lipid phase to carrier ratios were examined using scanning electron microscopy (SEM) (Fig. 1a–f). Upon examination, it was found that the mannitol particles were non-porous in structure, whereas sorbitol particles were porous (Fig. 1a–f). Transmission electron microscopy (TEM) was used in order to determine the architecture of liposomes generated via hydration from proliposome powders and tablets (Fig. 1g, h). The generated liposomes from both sorbitol and mannitol-based proliposome powders and tablets were similar in terms of size and structure and therefore TEM images of liposomes hydrated from sorbitol-based proliposome powders and sorbitol-based proliposome tablets in a 1:10 w/w lipid phase to carrier ratios were used (Fig. 1g, h). All liposomes were observed to be spherical to oval in shape, confirming the successful generation of vesicles from proliposome powder and tablet formulations.

Nebulization and sputtering time analysis

Nebulization time to 'dryness' was determined for liposome suspension (5 ml) when generated from sorbitol or mannitol-based proliposome powder and tablets. Liposomes generated from sorbitol or mannitol-based proliposome powders in both 1:10 and 1:15 w/w ratios exhibited no significant difference ($p > 0.05$) in terms of nebulization time. Moreover, sorbitol-based proliposome tablets at 1:15 ratio exhibited a significantly shorter nebulization time ($p < 0.05$) (24.18 ± 0.16 min) compared to sorbitol and mannitol-based proliposome powders regardless of lipid concentration in the formulation (Fig. 2a). These findings indicate that sorbitol was more appropriate as proliposome carrier, if the aim was to accomplish nebulization quickly; this might be attributed to the greater solubility of sorbitol than mannitol (Bouchard et al. 2007), permitting faster formulation disintegration and completed nebulization.

Nebulization time for sorbitol-based tablets (1:15 w/w ratio) was a significantly shorter ($p < 0.05$) than that of mannitol-based tablets (for both 1:10 and 1:15 w/w ratios; 31.39 ± 2.66 and 31.36 ± 1.46 min, respectively) (Fig. 2a). The difference in nebulization performance might be attributed to different solubilities of the carriers or different dispersion viscosities resulting from using different carriers. Lipid dispersions with lower viscosities may facilitate faster delivery of liposomes from nebulizers (Elhissi et al. 2011). Thus, formulations having higher lipid contents may contribute to generating more viscous liposome formulations, resulting in prolonged nebulization times. This assumption was ascertained by using the Stewart lipid assay (Stewart. 1980) to determine lipid content in each type of carbohydrate carrier. Mannitol formulations were found to have higher lipid content than sorbitol proliposome formulations (Table 2), which may have increased the viscosity of the lipid dispersion (Narenji et al. 2017). This is associated with the smaller particle size of mannitol (less than $250 \mu\text{m}$) than sorbitol ($250\text{--}700 \mu\text{m}$), allowing more surface area for lipid deposition (Fig. 1a–f). Additionally, the decrease in temperature of the air-jet nebulizer contents may further elevate the viscosity of the nebulization fluid (Steckel and Eskandar. 2003). Higher viscosity may affect nebulization, as explained by Finlay et al. (2000), who posed that the higher the viscosity the greater the adherence of aerosol droplets to the inner walls of the nebulizer, followed by the reduced deflection of these droplets into the nebulizer reservoir, greatly prolonging the nebulization time (McCallion et al. 1995; Finlay et al. 2000). This was further confirmed by the larger measured liposome size for mannitol-based formulations in the nebulizer's reservoir (Table 1), indicating liposome aggregation and/or fusion due to increased lipid accumulation within the nebulizer towards the end of nebulization, causing prolonged nebulization (Elhissi et al. 2012).

Fig. 1 SEM images of coarse carbohydrate carrier and proliposome powder prepared with different w/w lipid phase to carrier ratios including; (a) coarse sorbitol, (b) sorbitol-based proliposome with 1:10 w/w, (c) sorbitol-based proliposome with 1:15 w/w, (d) coarse mannitol, (e) mannitol-based proliposome with 1:10 w/w and, (f) mannitol-based proliposome with 1:15 w/w. TEM images of liposome generated from, (g) sorbitol-based proliposome powder 1:10 w/w and, (h) sorbitol-based proliposome tablets 1:10 w/w ratio. These images are typical of three such different experiments



Sputtering time is identifiable by the sputtering behaviour of the nebulizer associated with intermittent nebulization (when there is a low volume of suspension remaining). Upon direct comparison, no significant difference ($p > 0.05$) was found between proliposome tablets (i.e. sorbitol and mannitol-based at both ratios). Similar results ($p > 0.05$) were also identified for powder formulations regardless of carrier type and ratio (Fig. 2b). Overall, all proliposome powders exhibited longer sputtering times when compared with proliposome tablet formulations (Fig. 2b). The exception to this trend was demonstrated by sorbitol-based proliposome tablets in a 1:15 w/w ratio, which exhibited a shorter sputtering time (2.42 ± 0.54 min). Trends in sputtering time exhibited by the aforementioned sorbitol formulation mirrored nebulization time findings (Fig. 2).

Thus, amongst all the formulations prepared via the slurry method (powder or tablet), sorbitol-based proliposome tablets (particularly a 1:15 w/w ratio) exhibited a notably shorter nebulization and sputtering time. This is indicative that proliposome formulations manufactured in tablet form, are comparatively superior to proliposome powder formulations (i.e. in terms of nebulization rapidity).

Nebulizer output and output rate

Nebulization was performed to 'dryness'. In general, complete nebulization of liposome dispersion is not achieved, and therefore an optimum or 100% mass output is not reached; this means that nebulizer at the end of atomization will have a fraction of residual components remaining in its reservoir (Manca et al. 2012). This is partially attributed

Fig. 2 Duration of; (a) nebulization time and, (b) sputtering time when liposomes were generated from sorbitol or mannitol-based proliposome powder and tablets in 1:10 and 1:15 w/w lipid phase to carrier ratio in deionised water. Data are mean \pm SD, $n=3$; $p < 0.05$

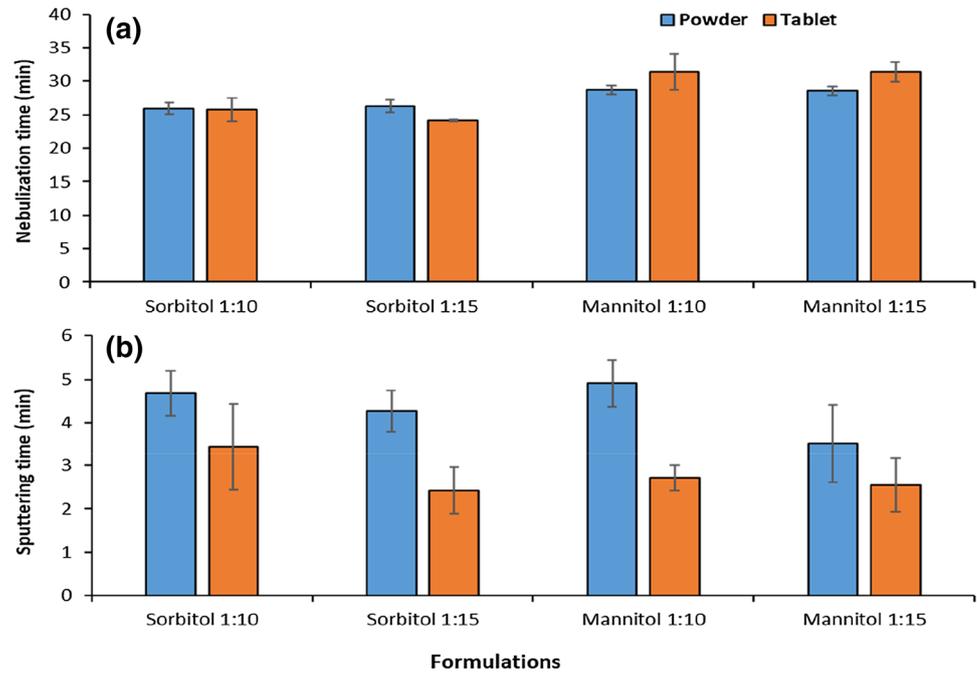


Table 2 Stewart assay establishing the amount of lipid phase coated on carbohydrate carriers, including: sorbitol or mannitol, with formulations ratios of 1:10 and 1:15 w/w lipid to carrier ratio.

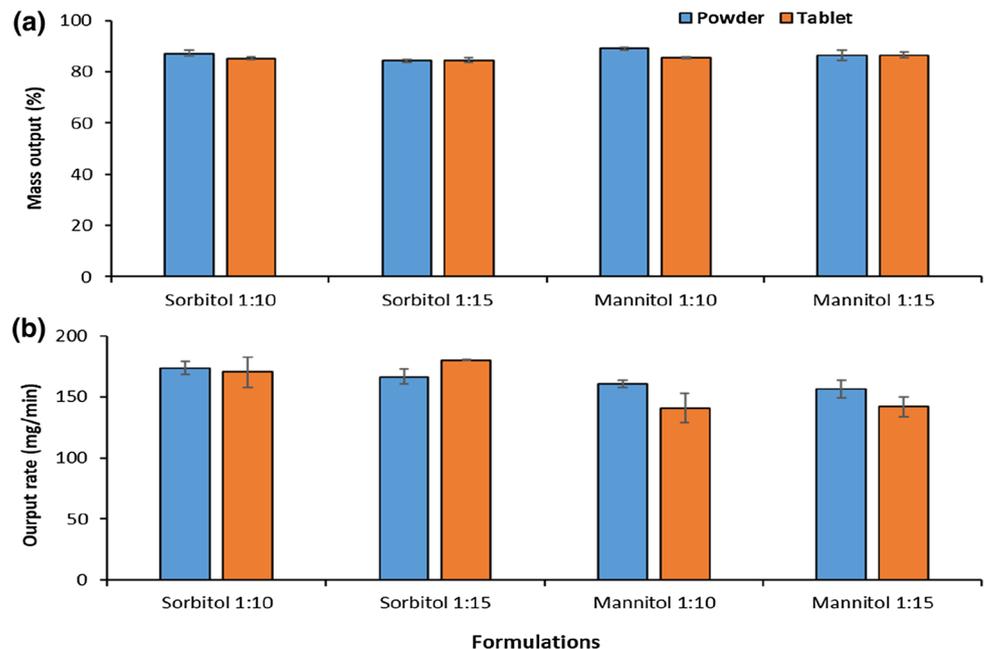
Carrier-based proliposome formulations	Percentage lipid concentration (%)	
	(1:10 w/w)	(1:15 w/w)
Sorbitol	1.93 \pm 0.14	1.42 \pm 0.11
Mannitol	2.21 \pm 0.64	1.69 \pm 0.29

Data mean \pm SD, $n=3$; $p < 0.05$

to the presence of large liposomes or liposome aggregates (Clay et al. 1983; Elhissi et al. 2006, 2012; Khan et al. 2016). The total mass of the formulation delivered from the nebulizer (i.e. output), and output rate were determined for tablet and powder proliposome formulations prepared using mannitol or sorbitol carriers.

Sorbitol and mannitol-based powder and tablets exhibited similar mass output, regardless of carrier type (Fig. 3a). All formulations had output values exceeding 80% (Fig. 3a),

Fig. 3 Liposome generated from sorbitol or mannitol-based proliposome in deionised water in 1:10 and 1:15 w/w lipid phase to carrier ratio were investigated post-nebulization utilising Pari-LC Sprint nebulizer for; (a) aerosol mass output (%) and, (b) aerosol output rate (mg/min). Data are mean \pm SD, $n=3$; $p < 0.05$



which are in concordance to nebulization studies conducted for proliposomes prepared using a different proliposome technique (Elhissi and Taylor. 2005; Khan et al. 2020a, 2020b). However, less than 80% mass output was found in earlier studies using the Pari-LC nebulizer to deliver traditional thin-film hydrated liposomes (Bridges and Taylor. 2000), indicating the advantage of the proliposome technology used in this study or the more efficient aerosolization offered by the more advanced Pari LC-Sprint nebulizer system used in the present investigation.

The output rate was similar for sorbitol and mannitol-based powder formulations. Amongst all formulations, the highest aerosol output rate (180.20 ± 0.31 mg/min) was observed for sorbitol-based proliposome tablets (in a 1:15 w/w lipid phase to carrier ratio). In contrast, the lowest output rate (140.92 ± 11.88 mg/min) was determined for mannitol-based proliposome tablets (i.e. 1:10 w/w ratio) (Fig. 3b). All proliposome powder formulations had similar output rates. In conclusion, sorbitol-based proliposome tablets demonstrated superiority with regard to output and output rate of the aerosols. Mannitol-based formulations exhibited higher lipid content (confirmed via Stewart assay; Table 2), when compared to sorbitol-based formulations. Furthermore, higher lipid content of formulations may increase viscosity and hence more formulation deposition in the internal walls of nebulizer reservoir during nebulization, offering poor deflection of formulation in the reservoir, also confirmed via longer nebulization time.

The output findings were validated in a subsequent section by investigating the drug output characteristics and deposition profile in the TSI (Section “Nebulizer output efficiency of BDP using TSI”). It is important here to report the fact that gravimetrically determined mass output and output rate, although useful for comparing between formulations,

are not very accurate, owing to solvent evaporation occurring during jet-nebulization, commonly resulting in overestimated nebulization output values. Thus, gravimetric output findings were paralleled with drug output determination using HPLC, as demonstrated in the subsequent section.

Nebulizer output efficiency of BDP using TSI

Following nebulization into the TSI, BDP concentration in the nebulizer reservoir and in the upper and lower stages of TSI were determined using HPLC. All formulations, regardless of carrier type and lipid concentration, demonstrated high drug output; thus, around 50% of BDP was emitted from the nebulizer and distributed between the upper and lower stages of the TSI. Importantly, proliposome tablets exhibited higher BDP output compared to proliposome powder formulations, supporting the advantage of compressing the proliposome powders into tablets for subsequent nebulization.

Upon looking into the drug distribution between the nebulizer reservoir and the Impinger stages, for the powder formulations BDP remaining in the nebulizer reached up to as high as 62.70% using mannitol carrier (Fig. 4). A similar trend was observed for tablets; but with no difference among the formulations ($p > 0.05$) (Fig. 4). Thus, when proliposome tablets were used, similar drug output was demonstrated and formulation had no effect on drug output. Drug remaining in the nebulizer reservoir might be attributed to liposome accumulation as a result of aggregation or fusion (Ferron et al. 1976; Niven et al. 1991; Bridges and Taylor. 2000).

In the upper and lower stages of TSI, sorbitol (1:15) proliposome powder and tablet formulation elicited a higher concentration of BDP depositing in the TSI when compared

Fig. 4 Beclometasone dipropionate (BDP) concentration was determined in the nebulizer reservoir, upper and lower stages of Two-stage Impinger individually for sorbitol or mannitol-loaded proliposome powder and tablet formulations in a 1:10 and 1:15 w/w lipid to carrier ratio, post-nebulization. Data are mean \pm SD, $n = 3$; $p < 0.05$

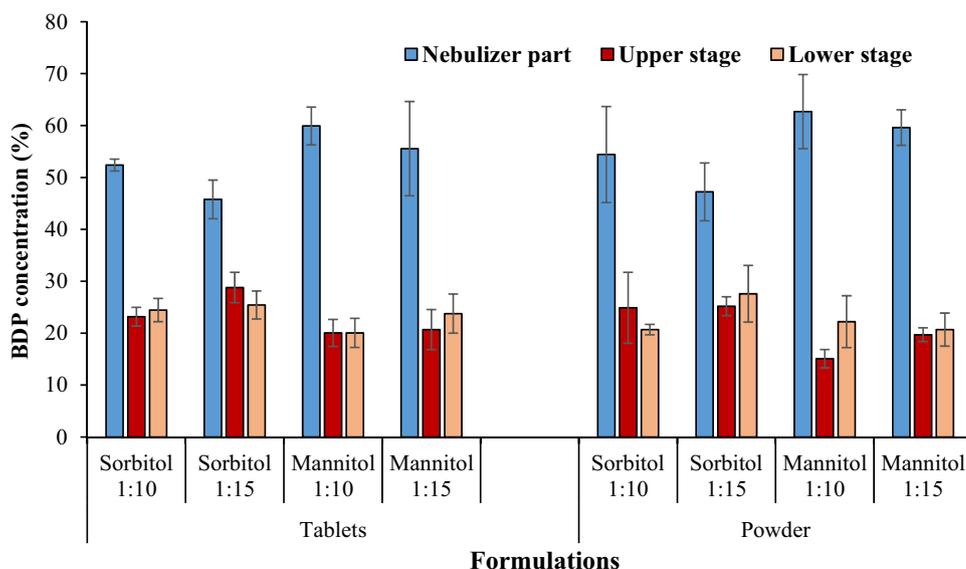


Table 3 Beclometasone dipropionate (BDP) output (cumulative concentration of BDP in the upper and lower stages of Two-stage Impinger together in percentage) for sorbitol or mannitol-loaded proliposome formulations in a 1:10 and 1:15 w/w lipid to carrier ratio, post-nebulization.

Proliposome formulations		BDP output (%)
Powder	Sorbitol (1:10)	45.58 ± 7.89
	Sorbitol (1:15)	52.78 ± 7.72
	Mannitol (1:10)	37.30 ± 7.68
	Mannitol (1:15)	40.38 ± 7.63
Tablets	Sorbitol (1:10)	47.62 ± 4.05
	Sorbitol (1:15)	54.22 ± 6.64
	Mannitol (1:10)	40.08 ± 5.41
	Mannitol (1:15)	44.45 ± 6.83

Data are mean ± SD, n = 3; $p < 0.05$

to the other powder and tablet formulations nebulized (Fig. 4 and Table 3).

The higher total mass output (Fig. 3a) compared to drug output (Fig. 4) is attributed to solvent evaporation caused by the forced gas during jet-nebulization and/or accumulation of BDP with the liposomes in the nebulizer's reservoir as a result of vesicle aggregation/fusion.

Proliposome formulation (i.e. powder or tablet) was deemed to have no significant effect upon nebulizer drug output efficiency, despite tablets exhibiting marginally higher values. Out of the carbohydrate carriers tested, sorbitol in a 1:15 w/w ratio was posed to generate liposomes that were able to withstand the nebulization process with no harm to vesicle integrity.

For tablet formulations, BDP output is higher when compared to powder formulations, and particularly in case of sorbitol formulations (Table 3). Again, high viscosity offered by mannitol formulation due to smaller particle size and more lipid deposition, remained in higher concentration in the nebulizer reservoir. Whereas, sorbitol with lower lipid concentration demonstrated higher BDP output concentration. BDP entrapment efficiency (please check supplementary data (Fig. a)) can be compared with the BDP output percentage (Table 3). All proliposome powder and tablet formulation exhibited high BDP entrapment than BDP output percentage, which may be related to the sticking of lipid content of powder formulations to the punch and die cavity during tableting and hence lower output was determined. Moreover, larger size of liposome in the nebulizer reservoir post nebulization (Table 1) may further demonstrated t lower output percentage of BDP.

VMD and span analysis of aerosol droplets via spraytec laser diffraction

In this section, we continued to investigate the characteristics of aerosol. The VMD of nebulized droplets generated upon dispersion of proliposome powders was not influenced by carrier type or lipid concentration ($p > 0.05$). Thus, VMD values (50% undersize) for aerosols nebulized from hydrated proliposome powders were between 4.16 μm and 4.71 μm , suggesting that aerosol droplet size was not affected by the formulation when the Pari-LC Sprint nebulizer was used (Fig. 5a). Furthermore, the size values of droplets generated from the hydrated tablets were similar to those of powder

Fig. 5 Representing; (a) volume median diameter (VMD) and, (b) span of the aerosol droplets generated from sorbitol or mannitol-based proliposome formulations for both 1:10 and 1:15 w/w lipid to carrier ratios using Pari-LC Sprint nebulizer via laser diffraction by Malvern SprayTec, mean ± SD, n = 3; $p < 0.05$

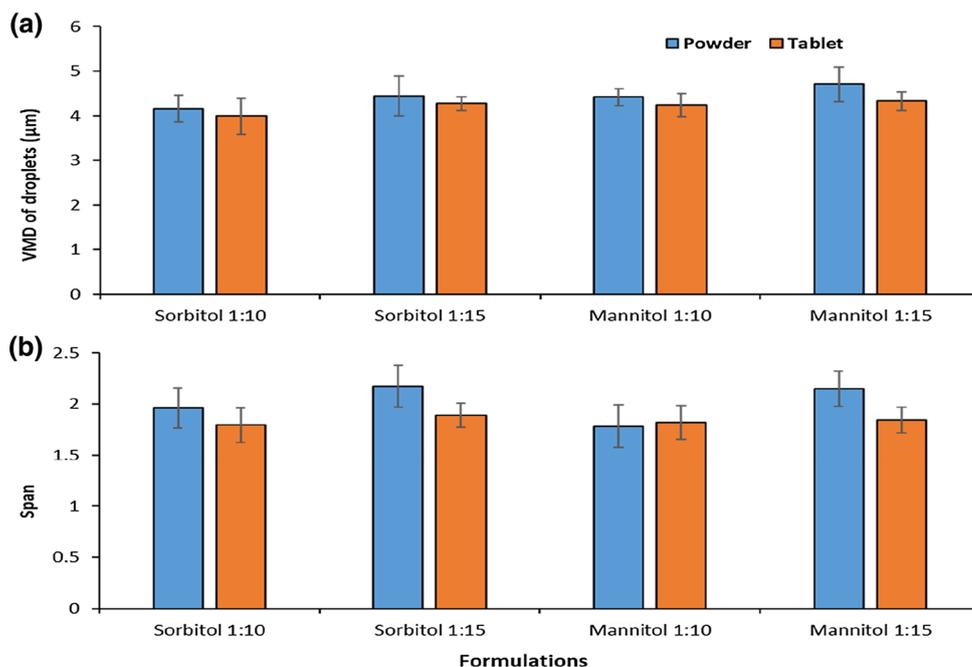
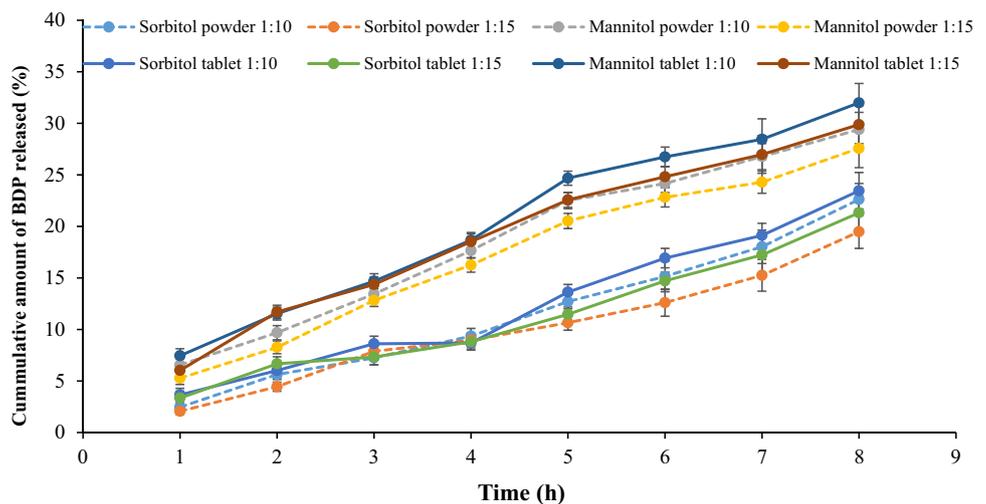


Fig. 6 In-vitro release of Beclometasone dipropionate (BDP) from liposomes generated from sorbitol or mannitol-based proliposome powder and tablet formulations for both 1:10 and 1:15 w/w lipid to carrier ratios. Data are mean \pm SD, $n=3$; $p < 0.05$



formulations (Fig. 5a). Laser diffraction results demonstrated aerosol droplet size to be less than 5 μm , indicating potential eligibility of aerosols generated from these formulations for deposition in “deep lung” (O’Callaghan and Barry, 1997).

Similar to VMD, hydrated tablet formulations exhibited a trend for generating aerosols with narrow size distribution (demonstrated by lower span values) when compared to powder formulation in both 1:10 and 1:15 w/w ratio (Fig. 5b). However, no significant difference in span values were found between powder and tablet formulations, regardless of carrier type and lipid to carrier ratio (Fig. 5b). Overall, the aerosolization of liposomes generated from the tablet formulations (in 1:10 and 1:15 w/w lipid phase to carrier ratio) were superior in terms of VMD and span of droplets (i.e. tended to have smaller median sizes and narrower size distribution), when compared to the corresponding powder formulations.

Release study of proliposome powder and tablets

BDP as a control, was released fully (i.e. 100% released) within the first 3 h (data not shown). However, over a period of 8 h, the maximum release of BDP was found in liposomes generated from mannitol-based proliposome powder and tablet in both 1:10 and 1:15 w/w formulations (i.e. $29.39 \pm 1.67\%$ and $27.57 \pm 1.87\%$; $31.98 \pm 1.88\%$ and $29.87 \pm 1.85\%$) (Fig. 6). Comparatively, slower release profiles ($p < 0.05$) of BDP incorporated in liposomes were exhibited by sorbitol-based proliposome powder and tablet ($22.61 \pm 1.55\%$ and $19.48 \pm 1.62\%$; $23.45 \pm 1.77\%$ and $21.32 \pm 1.63\%$) (Fig. 6). The crystalline structure of mannitol with its large surface area may be attributed to persuade lipid distribution over the carbohydrate carrier, whereas sorbitol carrier offer porous structure to accommodate phospholipid and therefore take longer time to release BDP (Khan et al. 2018b).

Conclusion

This research aimed to investigate and establish differences between proliposome powder and tablet formulations prepared in different ratios using BDP as a model drug. Upon investigation, proliposome powder or tablet formulations hydrated in nebulizer reservoir (Pari-LC Sprint nebulizer), exhibited similar nebulization time, however shorter sputtering time was found for all tablet formulations demonstrating more progressive and consistent aerosolization during nebulization. Post-nebulization, VMD of liposomes found in the nebulizer reservoir were significantly larger than prior to nebulization (for both powder and tablet formulations), indicating fusion/aggregation of liposome vesicles forming larger liposomes, whereas significantly smaller liposomes were found in the lower stage of TSI. Overall, all proliposome tablet formulations demonstrated circa 50% BDP delivery into the stages of TSI. Additionally, droplet size measurement exhibited a smaller VMD and span values for proliposome tablet formulations. In general, proliposome tablets also exhibited a sustained release of BDP. Thus, it was identified that tableting of proliposome powders may be conducted without detriment to any of the parameters investigated. Consequently, due to the advantages of tablet formulations i.e. ease in manufacture, dosing, transportation and storage and off course large scale manufacturing, it is posed that proliposome tablets offer a solution to the drawbacks associated with proliposome powder formulations.

Acknowledgment We are very thankful to Lipoid, Switzerland for their generous supply of phosphatidylcholine (SPC, S-100).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of Human and Animal Rights This article does not contain any studies with human and animal subjects performed by any of the authors.

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