AAPS PharmSciTech, Vol. 19, No. 1, January 2018 (© 2017)
DOI: 10.1208/s12249-017-0793-2

Research Article

Proliposome Powders for the Generation of Liposomes: the Influence of Carbohydrate Carrier and Separation Conditions on Crystallinity and Entrapment of a Model Antiasthma Steroid

Iftikhar Khan,[1,2,3] Sakib Yousaf,[2,3] Sneha Subramanian,[2,3] Mohamed Albed Alhnan,[2,3] Waqar Ahmed,[4] and Abdelbary Elhissi[5,6]

Received 31 December 2016; accepted 24 April 2017; published online 11 July 2017

Abstract. Formulation effects on the entrapment of beclometasone dipropionate (BDP) in liposomes generated by hydration of proliposomes were studied, using the high-density dispersion medium deuterium oxide in comparison to deionized water (DW). Proliposomes incorporating BDP (2 mol% of the lipid phase consisting of soya phosphatidylcholine (SPC) and cholesterol; 1:1) were manufactured, using lactose monohydrate (LMH), sorbitol or D-mannitol as carbohydrate carriers (1:5 *w/w* lipid to carrier). Following hydration of proliposomes, separation of BDP-entrapped liposomes from the unentrapped (free) BDP at an optimized centrifugation duration of 90 min and a centrifugation force of 15,500*g* were identified. The dispersion medium was found to have a major influence on separation of BDP-entrapped liposomes from the unentrapped drug. Entrapment efficiency values were higher than 95% as estimated when DW was used. By contrast, the entrapment efficiency was 19.69 ± 5.88, 28.78 ± 4.69 and 34.84 ± 3.62% upon using D_2O as a dispersion medium (for LMH-, sorbitol- and D-mannitol-based proliposomes, respectively). The similarity in size of liposomes and BDP crystals was found to be responsible for co-sedimentation of liposomes and free BDP crystals upon centrifugation in DW, giving rise to the falsely high entrapment values estimated. This was remedied by the use of D_2O as confirmed by light microscopy, nuclear magnetic resonance (^{1}HNMR), X-ray diffraction (XRD) and entrapment studies. This study showed that carrier type has a significant influence on the entrapment of BDP in liposomes generated from proliposomes, and using D_2O is essential for accurate determination of steroid entrapment in the vesicles.

KEY WORDS: characterization; inhalation; liposome; phospholipid; proliposome.

INTRODUCTION

Pharmaceutical and biomedical science milestones have been established, with an ever-increasing library of drug delivery systems, including niosomes (1), solid lipid nanoparticles (2), dendrimers (3), quantum dots (4), carbon nanomaterials (3–6), liposomes (3,7–9) and many others. Beclometasone dipropionate (BDP) is a synthetic steroid that is commercially available in the market in the form of inhaled therapy for prophylaxis against asthma, under a number of brands (e.g. Clenil and QVAR, both available as pressurized metered dose inhaler products, and Asmabec, as a dry powder inhaler). As a model drug, BDP has been formulated in a plethora of micro- and nanocarrier formulations, including microspheres (10,11), niosomes (12,13), solid lipid nanoparticle (14), dendrimers (15), micelles (16), extra fine BDP particles (17,18) and liposomes (9,19–21) for potential application in pulmonary delivery, aiming to solubilize the drug and localize its action in the lung for prolonged periods of time.

Niosomes and liposomes have been introduced as promising drug delivery systems [9; 13]. Whilst liposomes offer modified release, instability manifestations are a major hurdle, giving rise to physical aggregation and fusion of the vesicles, and chemical degradation of phospholipid *via* oxidation and hydrolysis. To reduce instability, powdered proliposomes, made by coating phospholipid onto

[1] School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, L3 3AF, Liverpool, UK.
[2] Institute of Nanotechnology and Bioengineering, University of Central Lancashire, Preston, PR1 2HE, UK.
[3] School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, PR1 2HE, UK.
[4] School of Mathematics and Physics, College of Science, University of Lincoln, Lincoln, LN6 7TS, UK.
[5] Pharmaceutical Sciences Section, College of Pharmacy, Qatar University, P.O. Box 2713, Doha, Qatar.
[6] To whom correspondence should be addressed. (e-mail: aelhissi@qu.edu.qa; aelhissi@gmail.com)

1530-9932/18/0100-0262/0 © 2017 American Association of Pharmaceutical Scientists

carbohydrate carriers, have been developed for subsequent hydration to generate liposomes (22,23). Proliposomes have demonstrated potential for pulmonary delivery *via* nebulization (24,25) or by using dry powder inhalers (26,27). Furthermore, compared to proniosomes (13), proliposomes have higher biocompatibility and biodegradability since they are made from materials that are very similar to those constituting the mammalian membranes.

The entrapment of BDP in liposomes has been reported in a range of studies (19,20,28,29). As a hydrophobic drug, BDP exhibits an affinity for the fatty acid chain of liposomal phospholipid molecules, resulting in entrapment of the steroid within the liposomal bilayers. Whilst successful entrapment has been established and substantiated *via in vivo* studies (19), reliable determination of the entrapment efficiency is challenging, owing to the difficulty of separating the free BDP from the liposome-entrapped fraction of the drug.

Several methods of separation are commonly utilized in the characterization of liposomes containing entrapped drug. These include the Column Switch HPLC System, where free and entrapped drug (doxorubicin) in liposome preparations are separated through the aid of three different columns and two mobile phases. Initially, both entrapped and unentrapped fractions are separated *via* two separate columns with two separate mobile phases, and finally, with the assistance of a gradient method, both fractions are passed through a third analytical column for quantification (30). The efficacy of this method was subsequently demonstrated through separation and quantification of insulin loaded into liposomes (31).

Liquid chromatography-mass spectroscopy is an alternative method which can be employed with the aid of solid-phase extraction and protein precipitation for the separation of the free drug from the entrapped drug (32). Some therapeutic agents have narrow therapeutic indexes such as amphotericin B, resulting in serious toxicity manifestations (33). It is known that the injection of amphotericin B in liposomal suspensions (e.g. Ambisome®) can ameliorate these toxicities (33). For this reason, an effective separation protocol of the unentrapped drug is needed to determine the proportion of the free drug which may cause toxicity.

Ultracentrifugation is another alternative separation method, where the unentrapped (i.e. free) drug is separated from the drug-loaded liposomes. This method relies upon gravitational force or high centrifugation speed, in order to achieve the desired separation. A key example where this has been successfully implemented is in the analysis of resveratrol entrapped in liposomes. Entrapment, using this method, was reported to be in the range of 92–97% (34).

Filters (Ultrafiltration or Millipore) have also been used in combination with bench centrifugation in order to separate free drug from drug-entrapped liposomes. In this method, liposomal suspensions are passed through ultra-filters. Liposomes are retained (with drug-entrapped fraction), allowing free drug to pass through the filter pores. Upon using this method, around 97% drug entrapment efficiency was observed for quercetin and resveratrol (35). It is a challenging task to detect nanoparticles present in the filtrate or supernatant, *via* the naked eye or even using optical microscopy. Importantly, it has been shown that hydrophobic drugs such as steroids might be entrapped in liposomes only partially, since marked proportions of the drug may form crystals on the surfaces of liposome bilayers (29). Hence, traditional centrifugation may sediment these crystals with the liposome-entrapped drug, resulting in marked overestimation of the determined entrapment efficiencies (29). Thus, designing effective and reliable separation procedures is extremely important.

In this study, proliposome formulations incorporating BDP were developed using a range of carbohydrate carriers to generate liposomes upon hydration. A simple centrifugation method was developed by using deuterium oxide (D_2O; also known as heavy water) as dispersion medium and *via* manipulating the centrifugation speed and duration. The method of separation implemented in this research utilizing D_2O as dispersion medium was validated using HPLC, light microscopy, ^{1}HNMR and X-ray diffraction. The present study aims to further question whether the entrapment efficiency of hydrophobic drugs in many liposome formulations reported in the literature merits reinvestigation using the separation principle adopted in this report. Importantly, parallel to establishing an accurate separation protocol, the influence of carbohydrate carrier type on BDP entrapment was also evaluated.

MATERIALS AND METHODS

Materials

Lactose monohydrate (LMH; <250 μm), sorbitol (250–700 μm), D-mannitol (<250 μm), cholesterol, beclometasone dipropionate (BDP), loratadine, paclitaxel, erythromycin, amphotericin B and deuterium oxide (D_2O; density = 1.105 g/ml) were purchased from Sigma-Aldrich, UK. Absolute chloroform, deuterated chloroform, ethanol, HPLC-grade methanol and water were all procured from Fischer Scientific Ltd., UK. Ammonium thiocyanate and ferric chloride were supplied by VWR, UK. Soya Phosphatidylcholine (SPC; Lipoid S-100) was gifted by Lipoid, Switzerland. Prednisolone was purchased from Severn Biotech Ltd., UK.

Preparation of Proliposome Formulation Using the Slurry-Based Method

A slurry-based method for preparing proliposomes that can generate liposomes upon hydration has recently been introduced by our research group (36). SPC and cholesterol were used as the lipid phase (250 mg; in 1:1 mole ratio) and dissolved in absolute ethanol within a glass vial. This was followed by the addition of BDP at a concentration of 2 mol% (4.48 mg) of the total lipid phase. Carbohydrates used were LMH, D-mannitol and sorbitol. Carbohydrate carrier particles (1.25 g) were placed in a round-bottom flask (100 ml). The lipid phase was poured over the carbohydrate carrier in the flask to form a slurry, using a total of 4.51 ml absolute ethanol (333 mg/ml), in order to form proliposomes in a 1:5 *w/w* lipid to carrier ratio. The flask was attached to a rotary evaporator (Buchi Rotavapor R-114, Buchi, Switzerland) and partially immersed in a water bath (45°C). The organic solvent was evaporated *via* a vacuum pump (Buchi Vac V-501), adjusted to 280 rpm for 2 h. After complete

evaporation, the flask was detached and the prepared proliposomes were collected and stored at −18°C. A prolonged evaporation time was maintained aiming for complete evaporation of the ethanol from formulations and the avoidance of the possible occurrence of interdigitation and subsequent drug leakage from the vesicles following hydration (37).

Proliposome Morphology Study Using Scanning Electron Microscopy (SEM)

The surface morphology of proliposomes was examined using SEM and compared with the morphology of carbohydrate particles before coating with the lipid. Thus, proliposomes were placed onto carbon-coated aluminium stubs. Samples were gold coated for 2 min using a sputter coater (JEOL, Tokyo, Japan), followed by examination using a scanning electron microscope (Quanta-200, FEI, Holland). The surface morphology was evaluated after imaging using the instrument's software.

Hydration of Proliposomes Using DW or D_2O

Proliposomes were hydrated to form liposomes. This was achieved by adding 30 mg of proliposome powder to 1 ml of DW or D_2O. Each proliposome formulation prepared from each individual carbohydrate carrier was treated identically to generate liposomes. Proliposome powder (30 mg) was initially vortex-mixed (Fisions WhirliMixer, Fisions Scientific Equipments, UK) for 2 min in 50 μl of hydration medium (DW or D_2O), followed by 1 min vortex-mixing in the remaining 950 μl of the solvent. This process resulted in dissolution of the carbohydrate carrier and hydration of the lipid to generate liposomes dispersed in the carbohydrate solution.

Size Analysis of Liposomes *Via* Laser Diffraction

Size, expressed as volume median diameter (VMD; 50% undersize), and polydispersity, expressed as span value, were measured *via* laser diffraction using the Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). The span, a term introduced by Malvern Instruments Ltd. to express size distribution, was calculated mathematically *via* the software of the instrument using the following formula:

$$\text{span} = (90\%\text{undersize} - 10\%\text{undersize}) / \text{VMD}$$

Separation of BDP-Entrapped Liposomes from Free BDP *Via* Centrifugation

After hydration in the respective media (DW or D_2O) ('Hydration of Proliposomes Using DW or D_2O'), 1 ml of liposome suspension was transferred to a centrifuge tube, and separation was conducted on the basis of centrifugal force, utilizing a bench centrifuge (Spectrafuge 24D, Jencons, UK). This process was conducted in order to separate BDP-entrapped liposomes from the unentrapped or free BDP crystals. A number of different centrifugal forces were utilized in this experiment, including 11,000, 12,000 or 13,000 rpm (11,100, 13,200 or 15,500 gravity), in combination with two separation durations (60 or 90 min).

Light Microscopy for Visualization of Separation

Following the separation process, light microscopy (Novex, Holland) was employed to study the supernatant and pellet fractions to investigate whether free BDP crystals are present in the samples. All three liposome suspensions were individually analysed at the various stages of separation (i.e. time intervals and centrifugation speed).

Determination of BDP-Entrapment Using HPLC

Post-centrifugation, BDP-entrapped liposomes were separated from the unentrapped drug (i.e. either free BDP crystals or dissolved BDP). Each of the individual fractions (the upper creamy layer, middle layer and the sedimented pellet) was analysed to quantify the BDP. Individual fractions were completely solubilized in methanol and analysed with HPLC using the Agilent 1200 Series instrument equipped with a UV detector (Agilent, UK). HPLC grades of methanol and water (75:25 *v*/*v*) were employed as the mobile phase with a flow rate of 1.7 ml/min. An Agilent column of 150 mm × 4.6 mm and C-18 (Agilent Technology, UK) was used with a UV detection wavelength of 239 nm. A sample injection volume of 20 μl was set with a column temperature of 40°C.

Crystallinity Studies Using ^{1}HNMR

^{1}HNMR was employed to analyse and identify the crystalline structure of the compound, separated as a pellet post-centrifugation in D_2O. The pellet was dried at room temperature to eliminate the remaining moisture. Deuterated chloroform was used to dissolve the pellet. Upon transfer to the NMR tube, the solution was capped to prevent solvent evaporation. Post sample preparation, proton (^{1}H) NMR (Bruker, Magnetic system 300 MHz/54 mm, UltraShield, UK) was conducted to scan the sample. The same procedure was repeated for pure BDP, as a reference sample to allow comparison through various proton peaks in the spectra.

Crystallinity Studies *Via* XRD

The crystalline properties of the starting materials and the formulations were studied by XRD (X-ray diffractometer, D2 PHASER with LYNXEYE, Bruker, Germany). Samples were loaded onto inert polymeric discs and operated with a power of 45 kV voltage and 40 mA current. All samples were scanned at 2θ ranging from 5 to 50°, utilizing a scintillation counter and a one-dimensional LYNXEYE detector. The angular positions of the intense peak from the individual excipients, formulations and physical mixtures were examined and compared to the crystallinity of the pure BDP sample.

Lipid Quantification *Via* Stewart Assay

Stewart assay was performed to quantify the lipid concentration in the formulation (38). Quantification of lipids in liposomes was adapted from a protocol designed

previously (24). Lipid (10 mg) was dissolved in chloroform (2 ml) and subsequently evaporated in a 100-ml round-bottom flask using a rotary evaporator. A thin film which formed in the flask was initially hydrated with 1 ml of aqueous media (DW or D_2O), followed by addition of 1 ml of ethanol to form an ethanolic solution. This solution was stored in an oven overnight, set at 90°C to ensure complete evaporation of the solvent. The dried sample was then dissolved in 100 ml of chloroform in order to construct a calibration curve ranging from 40 to 80 μg/ml through serial dilutions.

Statistical Analysis

Statistical analysis was performed on the data obtained using one-way analysis of variance (ANOVA) or Student's *t* test by employing the SPSS software, to determine whether the difference was statistically significant between more than two groups or two sets of data, respectively. All experiments were conducted in triplicate using three different batches, and the difference was judged to be statistically significant when the calculated *p* value was lower than 0.05.

RESULTS AND DISCUSSION

Proliposome Morphology and Lipid Content

LMH was observed to be irregular in shape (Fig. 1a). LMH was non-porous; however, when formulated as proliposomes in a 1:5 *w*/*w* lipid to carrier ratio, they appeared to be densely coated, which was attributed to the sticky nature of the phospholipid (23) (Fig. 1b). Stewart assay (i.e. practical yield, which is the actual total amount of lipid in 1 ml of formulation) showed that LMH-based proliposomes had higher lipid concentrations (94.15 ± 5.49%) (Table I). Sorbitol particles are relatively large (250–700 μm), whilst LMH and mannitol particles are smaller than 250 μm. Thus, the smaller particles of LMH and mannitol offer a large surface area to volume ratio as compared to sorbitol carriers. Therefore, the total lipid yield using sorbitol particles was less than the yields obtained using LMH or mannitol carriers. Sorbitol particles were found to be irregular to spherical in shape, with micro-needles protruding from the surfaces (Fig. 1c). These structures were believed to be responsible for accommodating the lipid phase. Hence, the void spaces of the particles may inhibit agglomeration (23,39,40). Meyer and co-workers have demonstrated that sorbitol particles may retain their flowability even when coated with egg phosphatidylcholine in a 1:10 *w*/*w* phospholipid to carrier ratio (41). However, using a higher lipid content such as 1:5 *w*/*w* lipid to carrier ratio, the porosity of sorbitol was reduced in comparison to coarse sorbitol powder; these findings are also in concordance with the data presented by our research group using another proliposome preparation approach (Fig. 1c, d) (40). D-Mannitol particles were observed to be oblong/ellipse in shape with finely engraved lines on the surfaces (Fig. 1e) (42). D-Mannitol-based proliposomes were coated in the lipid phase, when formulated in a 1:5 *w*/*w* lipid to carrier ratio. However, smaller particle sizes may induce distribution of the lipid phase over the carrier, exhibiting poor agglomeration as quantified with a practical yield of 89.50 ± 3.34% (Fig. 1f; Table I).

Size Analysis of Liposomes

The measured size of liposomes was studied upon hydration of the carrier-based proliposomes (i.e. LMH, sorbitol or D-mannitol). Volume median diameter (VMD) values of liposomes prepared from LMH- and D-mannitol-based proliposomes (4.09 ± 0.64 and 4.30 ± 0.36 μm, respectively) were significantly ($p < 0.05$) smaller than liposomes hydrated from sorbitol-based proliposomes (i.e. 6.11 ± 0.83 μm) (Table II). The relatively slow dissolution of LMH and mannitol may create a hypertonic environment outside the liposomes, causing the vesicles to shrink and have a smaller size than those generated using sorbitol carrier particles which have a higher dissolution rate (40). Span (size distribution) values of liposomes generated from D-mannitol-based proliposomes, were significantly ($p < 0.05$) larger than liposomes prepared from LMH-based proliposomes, indicating greater vesicle aggregation caused by the D-mannitol carrier when the proliposome powder was hydrated (Table II). The selection of carrier type, porosity and size is important in controlling the size and size distribution of the subsequently generated liposomes (22). Size and size distribution findings in this study are in agreement with size analysis observation using the traditional liposomes made by thin-film hydration (37), indicating that the technique used in the present investigation and any residual traces of ethanol remaining after solvent evaporation had no effect on vesicle size distribution following hydration of the proliposomes.

Optimization of Separation Speed and Time

Centrifugation speed and time both play a pivotal role in the separation of BDP-entrapped liposomes from the unentrapped drug (either dissolved or free BDP crystals). The purpose of the centrifugation studies was to optimize the separation conditions, as conventional centrifugation usually results in a debatably high BDP entrapment. In order to examine the validity of this conventional method of separation, DW and D_2O were used as separate dispersion media for hydration of proliposomes into liposomes ('Hydration of proliposomes using DW or D2O'). For optimization of the centrifugation speed, the Stewart Assay was employed to quantify the percentage of lipid present in the sample. Additionally, HPLC was implemented to determine drug entrapment efficiency.

Upon hydration of proliposomes in aqueous media (DW or D_2O), liposome suspensions were formed (Fig. 2a). Liposomes in DW were centrifuged at 11,000, 12,000 or 13,000 rpm (i.e. 11,100, 13,200 or 15,500 g). Post-centrifugation, using DW for dispersion, two distinct fractions were formed in the centrifuge tube (Fig. 2b); the upper supernatant fraction was found to contain the unentrapped drug, whilst a pellet formed at the bottom of the centrifuge tube was found to contain BDP-entrapped liposomes (Fig. 2b). Literature findings indicate that liposomes and BDP crystals may fall within a similar size or density range (29). On the basis of this information, both BDP-entrapped liposomes and free BDP crystals would possibly co-sediment at the bottom of the tube

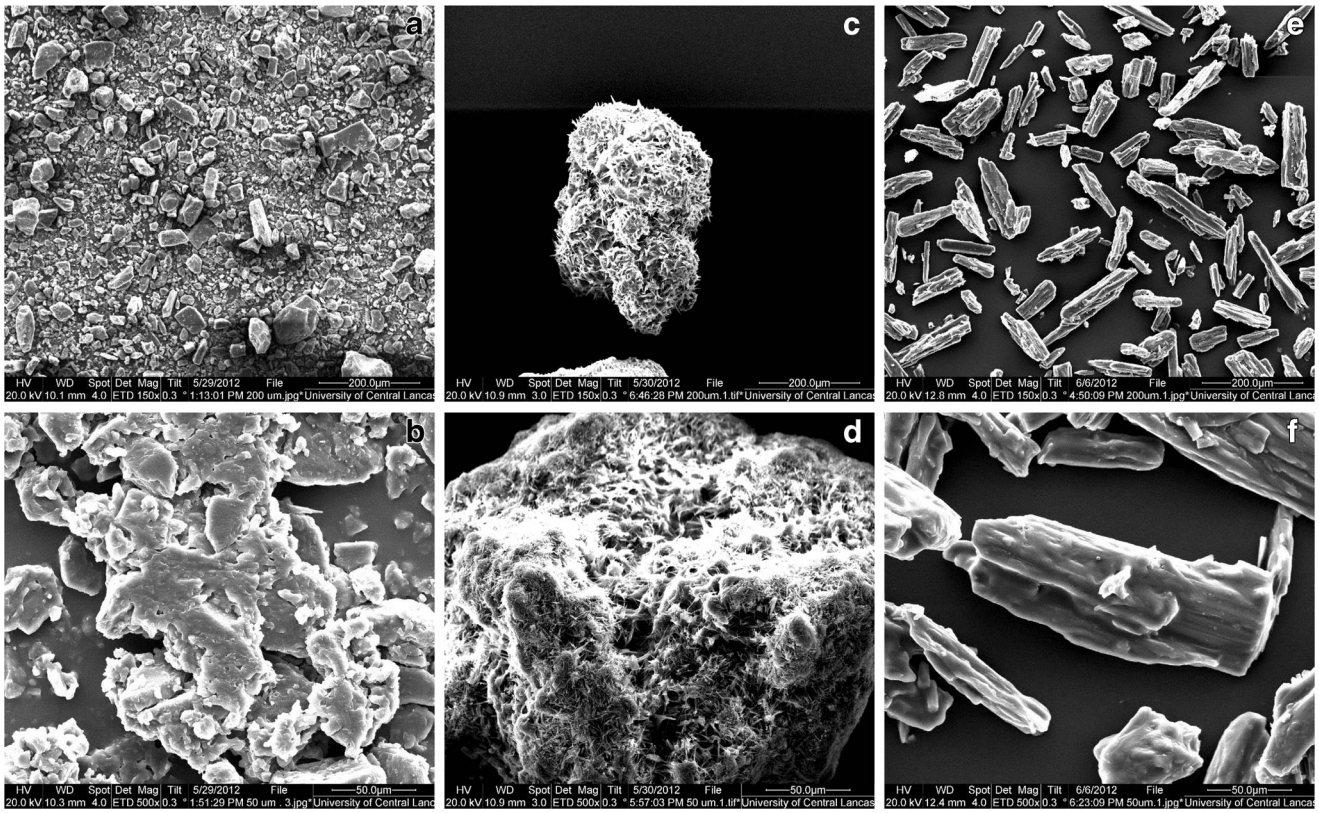

Fig. 1. SEM pics of proliposome formulations utilizing various carbohydrate carriers, **a** coarse LMH carrier, **b** LMH carrier loaded with lipid phase in a 1:5 *w/w* lipid to carrier ratio, **c** coarse sorbitol carrier, **d** sorbitol carrier loaded with lipid phase in a 1:5 *w/w* lipid to carrier ratio, **e** coarse D-mannitol carrier, **f** D-mannitol carrier loaded with lipid phase in a 1:5 *w/w* lipid to carrier ratio. These images are typical of three such different experiments

upon centrifugation, giving rise to overestimated entrapment values when DW is used for dispersing the liposomes.

In order to optimize the separation of liposome-entrapped drug from the free BDP, an alternative solvent was employed as dispersion medium, namely D_2O (deuterium oxide). This solvent was selected as a dispersion medium to examine and analyse the effect of medium density on the separation and subsequently drug entrapment efficiency. D_2O possesses a higher density (1.053 g/ml at 20°C) than water (0.9982 g/ml at 20°C), and according to literature, a higher density medium would potentially allow separation of the liposome-entrapped hydrophobic drug from the unentrapped drug (i.e. free drug) (29,43). Importantly, D_2O was solely implemented on the premise of entrapment confirmation. This solvent is not to be used clinically owing to its toxic nature and restricted limits in terms of human consumption

Table I. Stewart Assay Analysis Exhibiting the Percentage Lipid Content (in the Middle Fraction Post-Centrifugation in D_2O for 60 or 90 min Employing Various Speeds) Coated on Carbohydrate Carriers, Including LMH, Sorbitol or D-Mannitol, with a Formulation Ratio of 1:5 *w/w* Lipid to Carrier Ratio

Time	Centrifugal force	LMH	Sorbitol	D-Mannitol
60 min	11,100*g*	5.35 ± 0.53	15.61 ± 2.79	12.35 ± 1.85
	13,200*g*	4.98 ± 0.62	12.85 ± 2.27	10.24 ± 1.92
	15,500*g*	3.73 ± 0.64	10.66 ± 2.31	7.82 ± 1.67
90 min	11,100*g*	1.60 ± 0.31	4.77 ± 1.23	4.19 ± 0.49
	13,200*g*	1.56 ± 0.04	4.03 ± 1.50	3.08 ± 1.27
	15,500*g*	1.13 ± 0.24	3.35 ± 1.33	2.50 ± 0.46
Practical yield		94.15 ± 5.49	66.97 ± 5.06	89.50 ± 3.34

Data are mean ± SD, $n = 3$
LMH lactose monohydrate

Table II. Size (μm) and Span of Liposomes Generated from Proliposomes in Aqueous Media Using Carbohydrate Carriers of LMH, Sorbitol or D-Mannitol with 1:5 *w/w* Lipid to Carrier Ratio

Parameters	LMH	Sorbitol	D-Mannitol
Size (μm)	4.09 ± 0.64	6.11 ± 0.83	4.30 ± 0.36
Span	2.30 ± 1.01	3.07 ± 0.98	4.54 ± 0.89

Data are mean ± STD, $n = 3$
LMH lactose monohydrate

(i.e. no greater than 20 g daily) (44). Thus, the purpose of using D_2O was only to determine the entrapment efficiency accurately, not to suggest its use in further formulation development.

Upon using D_2O as a dispersion medium in combination with various centrifugation speeds, post-centrifugation, three distinct fractions were formed (Fig. 2c). The uppermost supernatant fraction appeared as a creamy-white layer, the middle fraction was identified as the unentrapped component (comprising of a low concentration of dissolved or free BDP crystals along with some dispersed lipid molecules) and the lowermost fraction was a pellet (formed at the bottom of the centrifuge tube) which was comprised of free BDP crystals. BDP present in the various fractions (either in DW or D_2O) was quantified *via* HPLC.

Both Stewart assay and light microscopy were conducted in order to establish the optimum separation speed and duration. Stewart assay was employed to quantify the amount of lipid in the middle fraction following centrifugation in D_2O; the lower the lipid concentration found within the middle layer, the better the separation. Light microscopy was employed to confirm and identify the crystalline structure of BDP in the various fractions.

Initially, 1 ml of liposome suspension was quantified to determine the total lipid concentration. A drop of normal liposome suspension was also examined, and light microscopy images were taken (Fig. 3). Centrifugation speeds of 11,100, 13,200 or 15,500*g* were utilized in order to separate the distinct layers in D_2O for 60 min (Table I). Post-centrifugation, the supernatant and pellet fractions were examined through light microscopy. It was found that all liposomes generated from the various carrier-based proliposomes had BDP crystals in the upper creamy-white fraction in combination with lipid content (Fig. 4a–e), suggesting insufficient separation time when centrifugation duration was as short as 60 min. Contrastingly, the pellet was comprised solely of BDP crystals (Fig. 4b–f). Stewart assay analysis also suggested that as the centrifugation speed was gradually increased, the lipid content in the middle fraction decreased (Table I). The two time intervals (60 and 90 min) and three centrifugation forces (11,100, 13,200 or 15,500*g*) were selected following extensive optimization studies to show how appropriate centrifugation conditions are important to provide adequate separation of unentrapped drug from the liposome-entrapped BDP.

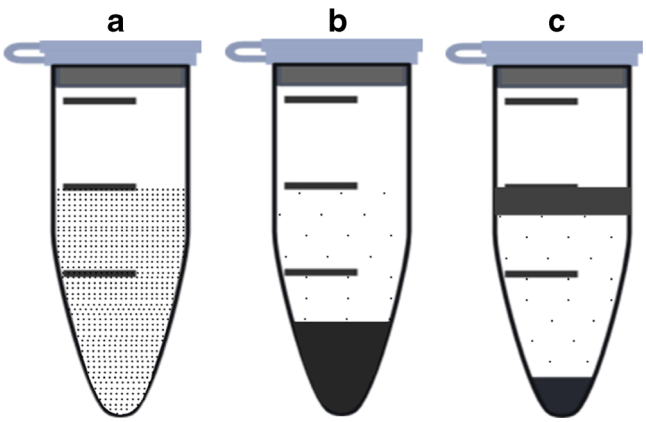

Fig. 2. Separation of BDP-entrapped liposomes from free BDP crystals upon employing different dispersion media; **a** normal distribution of liposomes in D_2O or DW before centrifugation, **b** dispersion of liposomes in DW after centrifugation, **c** dispersion of liposomes in D_2O after centrifugation

Based on findings using 60 min centrifugation time, the longer duration of 90 min was experimented. Subsequent Stewart Assay (Table I) showed a reduction in the lipid content observed in the middle layer. Moreover, the upper creamy-white layer was observed to be free from BDP crystals. Thus, the changes in composition of both layers examined indicated a greater degree of separation of the free BDP from BDP-entrapped liposomes. Finally, pellet formations at the bottom of the tube were isolated and analysed (Fig. 5) by HPLC, ^{1}HNMR and XRD, in order to confirm and quantify the free drug crystals settled at the bottom of the centrifuge tube.

To substantiate the separation method, preliminary experiments using a number of other drugs such as prednisolone, loratadine, paclitaxel, erythromycin and amphotericin B were conducted following preparation of proliposomes based on sorbitol carrier particles in 1:5 *w/w* lipid to carrier ratio. All formulations showed drug crystals sedimenting at the bottom of the centrifuge tube as pellets, and separation was achieved (Fig. 6). Thus, it was concluded that the separation method (utilizing D_2O as dispersion medium), centrifugation speed, and time play key roles in achieving improved and effective separation as compared to alternative traditional methods using normal DW along with ultracentrifugation.

Analysis of Pellet *Via* HPLC

HPLC peaks in this section were analysed and compared to establish the difference between the isolated pellet fraction post-centrifugation and pure BDP. The peak retention time of the pellet was determined at 3.765 ± 0.4 min, while the standard BDP peak was observed at 3.711 ± 0.4 min (Fig. 7). This indicated that both samples exhibited peaks/retention time for BDP, and therefore, these results support the assumption that the pellet is actually composed of free BDP crystals that sedimented at the bottom of the tube upon centrifugation. This section confirms the successful separation of free BDP from the entrapped drug in liposomal suspensions, when D_2O was employed as dispersion medium.

Analysis of Pellet *Via* ^{1}HNMR

^{1}HNMR was further employed to analyse and substantiate pellet composition. Upon pellet isolation,

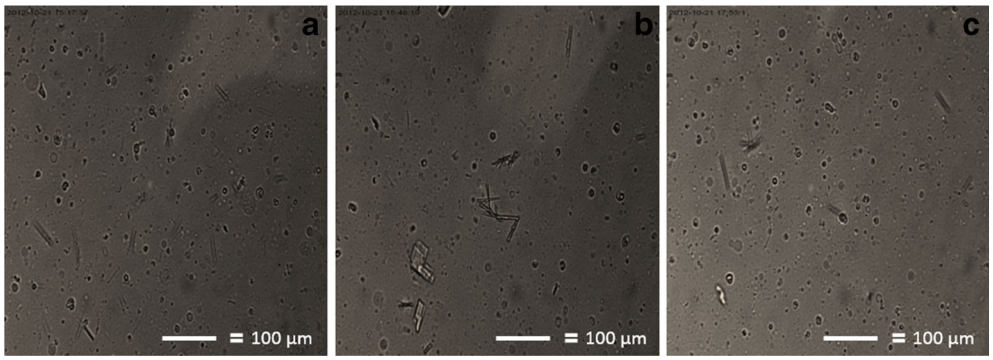

Fig. 3. Images of light microscopy; magnification ×100 presenting liposomes generated from proliposomes using various carbohydrate carriers after annealing in DW or D_2O; **a** LMH in a 1:5 *w/w* lipid to carrier ratio, **b** sorbitol in a 1:5 *w/w* lipid to carrier ratio, **c** D-mannitol in a 1:5 *w/w* lipid to carrier ratio. These images are typical of three such different experiments

^{1}HNMR was conducted and compared with a BDP standard. Both spectra exhibited common peaks; however, weak peaks (lower intensity) were exhibited by the pellet, due to the lower concentration of BDP isolated from the formulation upon centrifugation (Fig. 8). This technique was used to further identify the crystals found in the isolated pellet to be BDP. Further confirmation of free drug separation from BDP-entrapped liposomes by ^{1}HNMR supports that D_2O is a more appropriate dispersion medium for quantification of BDP entrapment efficiency. Moreover, it highlighted that the separation method achieved in this work is an effective and tangible technique for the separation of free (i.e. unentrapped) drug from the liposome-entrapped drug.

Analysis of Pellet *Via* XRD

XRD is typically used for studying the crystallinity of pharmaceutical powders (45). BDP was firstly tested to identify its nature of state, which was crystalline in appearance. All coarse carbohydrate carriers (i.e. LMH, sorbitol or D-mannitol) exhibited intense peaks, suggesting their structure is crystalline (Fig. 9). As ethanol was used to prepare proliposomes, each carrier was individually treated with ethanol alone in a round-bottom flask (in accordance to the section 'Preparation of Proliposome Formulation Using the Slurry-Based Method'), to determine the effect of alcohol on the carbohydrate carrier. These samples were also found to

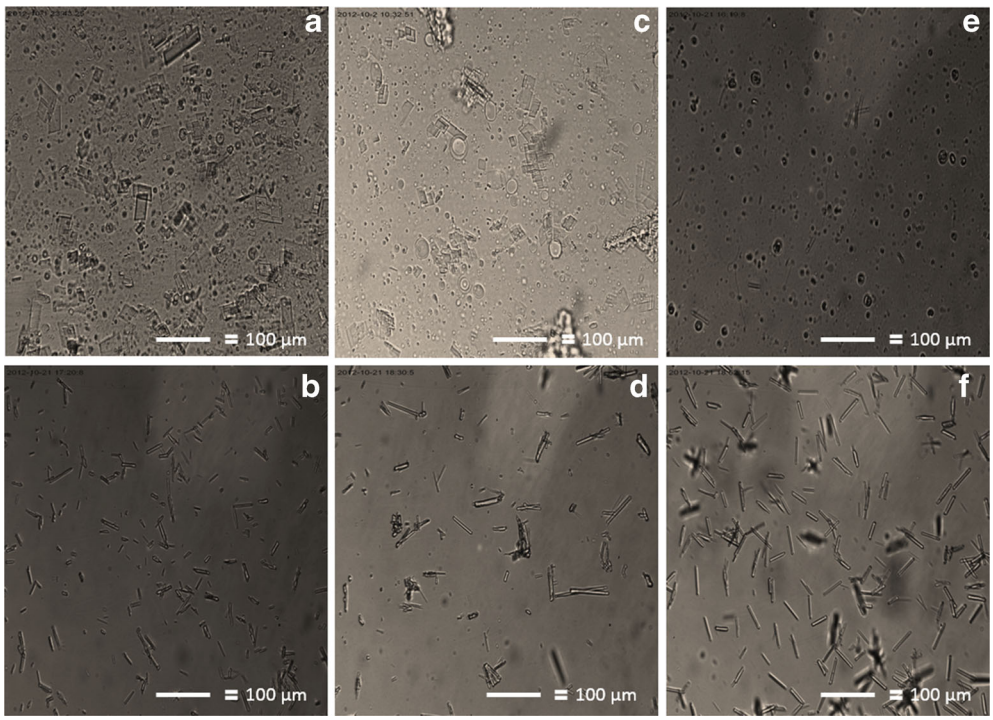

Fig. 4. Images of light microscopy; magnification ×100 exhibiting supernatant containing more liposomes and relatively less BDP crystals after centrifugation (i.e. at 13,000 RPM for 60 min) when liposomes were generated from proliposomes prepared with 1:5 *w/w* lipid to carrier ratio using various carbohydrate carriers including; **a** LMH, **c** sorbitol, **e** D-mannitol. Sedimented pellet (at the bottom of the centrifuge tube) containing a smaller amount of liposomes and more BDP crystals **b** LMH, **d** sorbitol, **f** D-mannitol. These images are typical of three such different experiments

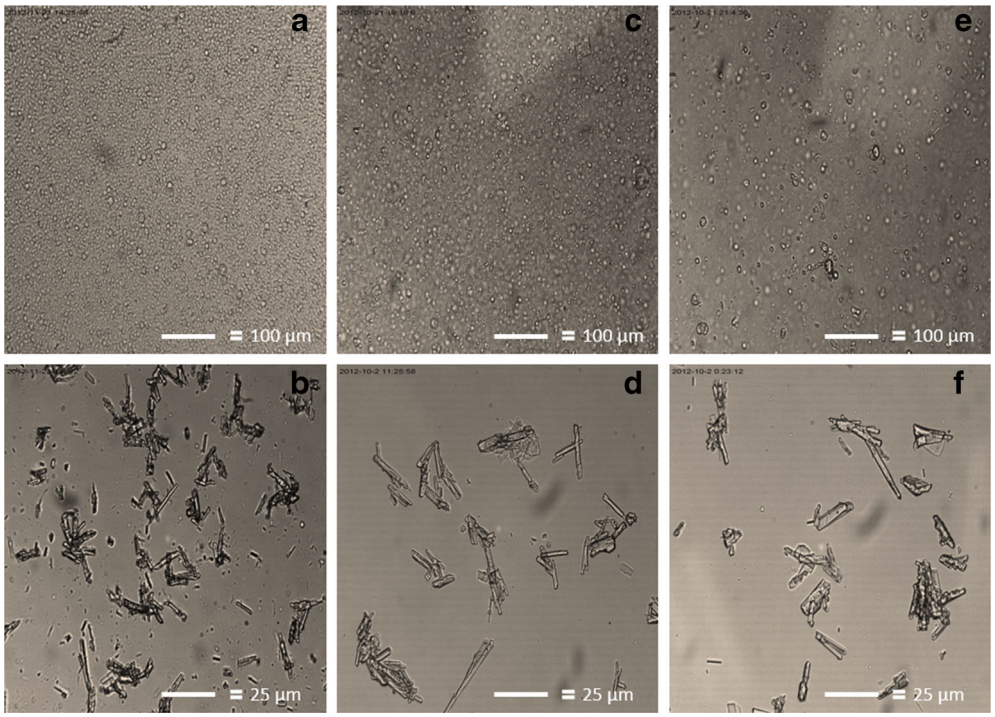

Fig. 5. Light microscopy images with magnification of ×100 representing liposomes in the upper creamy-white fraction (supernatant) of the centrifuge tube post-centrifugation for 90 min at 13,000 RPM when liposomes were generated from proliposomes prepared in a 1:5 *w*/*w* lipid to carrier ratio using a carbohydrate carriers of **a** LMH, **c** sorbitol, **e** D-mannitol. Free BDP crystals sedimented (as pellet) at the bottom of the centrifuge tube were photographed with magnification of ×400 *via* light microscopy using carbohydrate carriers of, **b** LMH, **d** sorbitol, **f** D-mannitol

be crystalline, indicating that ethanol did not affect the crystallinity of the carbohydrate carriers (Fig. 9).

Proliposome powder formulations using 2 mol% of BDP were examined to establish the crystallinity of BDP (within the sample). Due to the high intensity peaks of proliposome excipients, BDP peaks were undetectable, and it was further assumed that BDP was present in an amorphous form as opposed to crystalline status (Fig. 9). This was confirmed by preparing a physical mixture of proliposome formulations using 2 mol% of BDP. Even in the physical mixture, no BDP

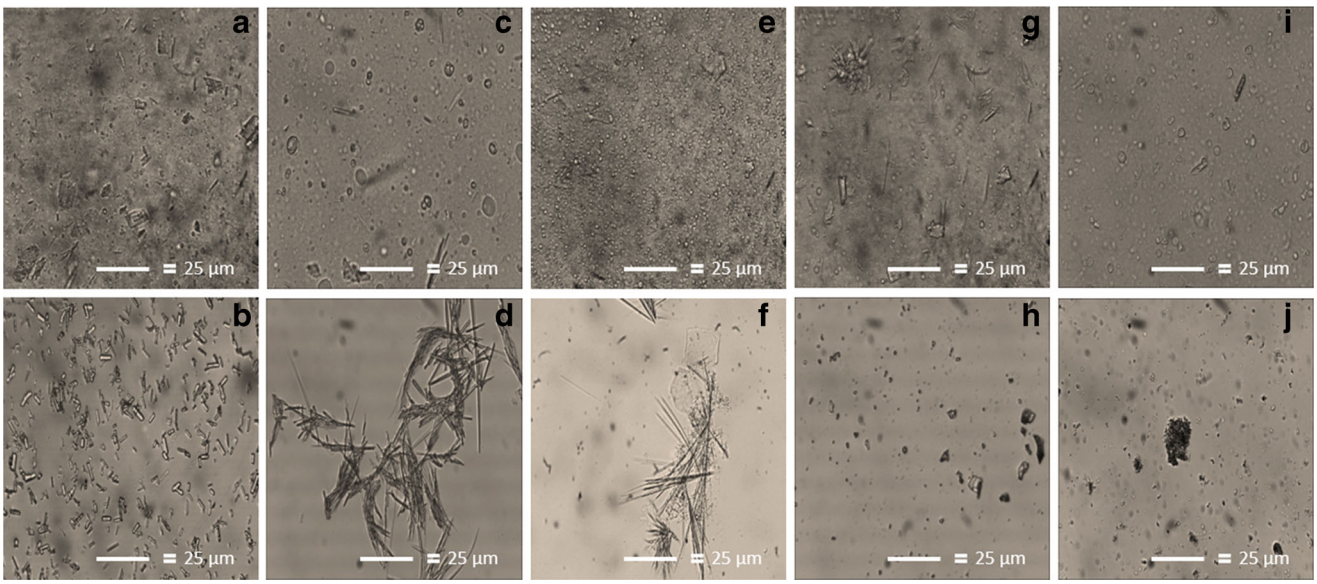

Fig. 6. Images of light microscopy; magnification ×400 exhibiting normal liposome suspension containing lipid and drug crystals prior to centrifugation (i.e. at 13,000 RPM for 90 min) when liposomes were generated from proliposomes prepared with 1:5 *w*/*w* lipid to carrier ratio using various drugs including; **a** prednisolone, **c** loratadine, **e** paclitaxel, **g** erythromycin, **i** amphotericin B. Free drug crystals sedimented (as pellet) at the bottom of the centrifuge tube were photographed with magnification of ×400 *via* light microscopy using various drugs including **b** prednisolone, **d** loratadine, **f** paclitaxel, **h** erythromycin, **j** amphotericin B

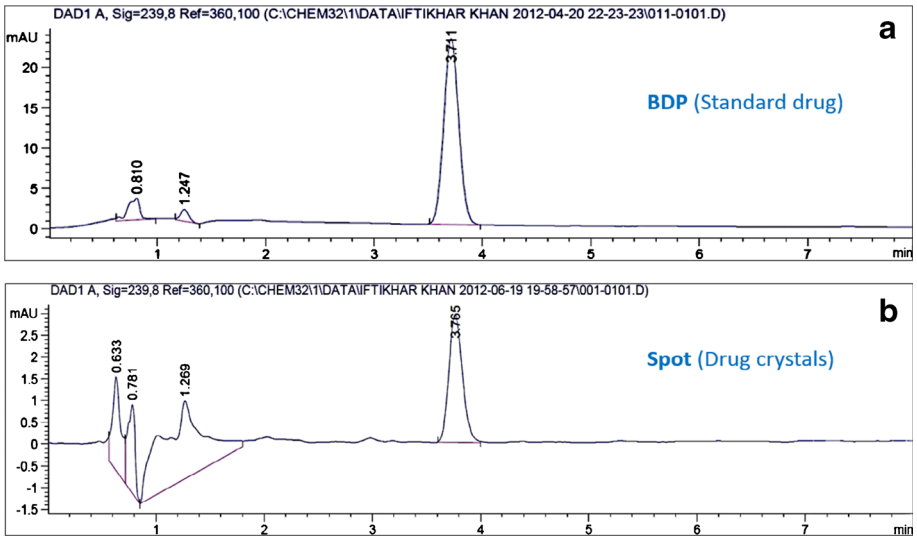

Fig. 7. HPLC chromatograms, demonstrating the retention time peak of the sedimented pellet post-centrifugation for 90 min at 13,000 RPM in D_2O. This was in comparison to pure BDP in methanol; **a** retention time peak of pure BDP during calibration, **b** retention time peak of pellet (free BDP crystal). Data are mean of three such different experiments

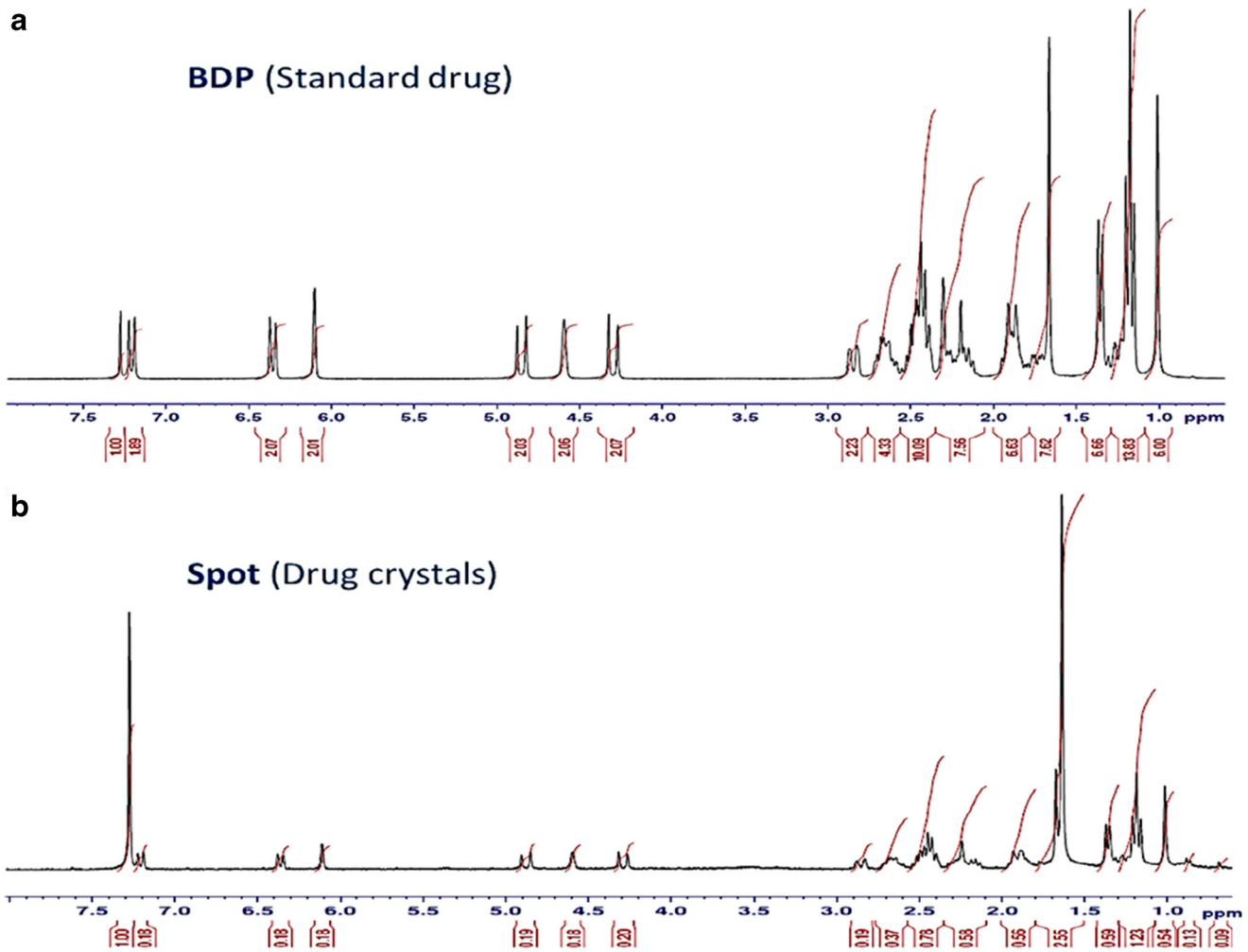

Fig. 8. ^{1}HNMR spectra demonstrating the **a** molecular structure of pure BDP in comparison to **b** molecular structure of pellet (free BDP crystal), isolated after centrifugation for 90 min at 13,000 RPM in D_2O. Data is typical of three such different experiments

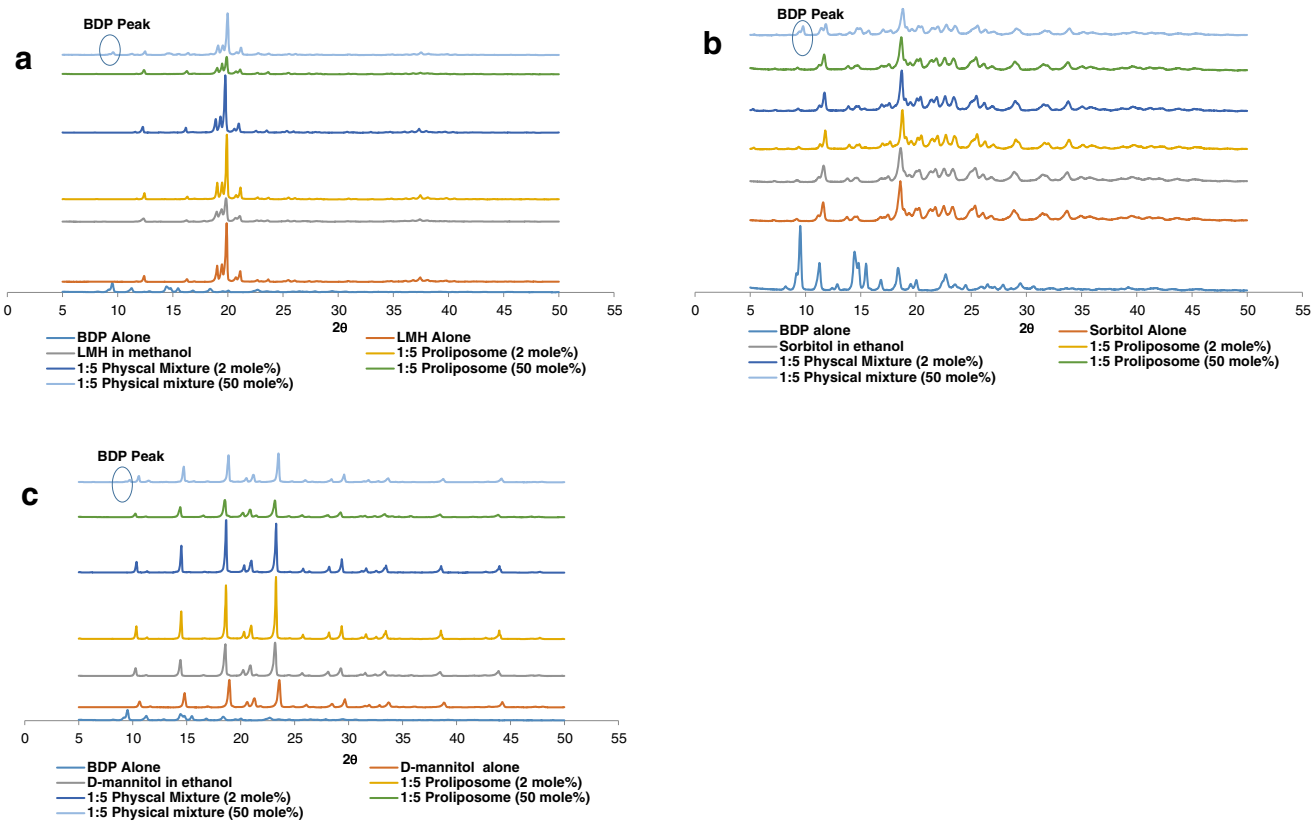

Fig. 9. X-ray diffraction spectra for **a** BDP, LMH, LMH treated with ethanol, and LMH-based proliposome formulations (1:5, *w*/*w* lipid to carrier ratios) with BDP in a concentration of 2 mol% to the lipid phase. Additionally shown are the spectra for the 50 mol% concentration of BDP to the lipid phase with the 1:5 *w*/*w* lipid to carrier ratio. LMH-based proliposomes (without BDP) in a 1:5 *w*/*w* lipid to carrier ratio were physically mixed individually with 2 mol% of BDP and, alternatively, 50 mol% of BDP to the lipid phase. The same procedure was repeated for **b** sorbitol and **c** D-mannitol. This data is typical of three such different experiments

peaks were identified. This may be explained by the fact that the incorporated BDP concentration of 2 mol% (i.e. 4.48 mg) was substantially low for detection by XRD, when compared to the other proliposome excipients, making it challenging to detect the small drug concentration. For this reason, a proliposome formulation was prepared (in accordance to the section 'Preparation of Proliposome Formulation Using the Slurry-Based Method') employing 50 mol% of BDP to the lipid phase. This formulation was analysed *via* XRD; again, BDP peaks were absent in the spectra, indicating formation of amorphous structures of the steroid. Once again, a physical mixture of BDP and proliposome excipients was studied, this time with a 50 mol% BDP concentration. Upon examination, a peak was present, indicating BDP was in crystalline form (Fig. 9).

These findings suggest that in pure form, BDP is present in crystalline form; however, upon its incorporation into proliposome formulation, the crystalline structure transformed into amorphous. Thus, irrespective of BDP concentration (i.e. 2 or 50 mol%), upon formulation in proliposomes, drug crystallinity has converted into amorphous. Interestingly, upon hydration of these formulations, the BDP structure reverted to crystalline form and was collected at the bottom of the centrifuge tube following centrifugation.

Entrapment Efficiency in DW or D_2O

In section 'Optimization of Separation Speed and Time', the formation of two distinct fractions post-centrifugation (at 15,500*g* for 90 min) of hydrated proliposome formulations in DW was identified. Both fractions were taken and analysed using HPLC, exhibiting that the drug entrapment efficiency exceeded 95%.

The entrapment efficiency was quantified for individual formulations when proliposomes were prepared from LMH, sorbitol or D-mannitol (i.e. 97.80 ± 0.57, 98.33 ± 0.24% or 99.37 ± 0.14%, respectively) (Fig. 10a). Upon direct comparison, no significant difference ($p > 0.05$) in terms of entrapment was observed between the different carrier-based proliposome formulations, when DW was used as dispersion medium. The entrapment findings generated in DW were in concordance with the results described in literature (46,47). On the basis of previous literature and data generated in this research, it is suggested that separation of BDP using DW as dispersion medium and traditional high-speed centrifugation were inefficient in completely separating the free steroid from the BDP entrapped in liposomes; this is supported by the presence of free BDP crystals with the sedimented BDP-entrapped liposomes following centrifugation, as discussed earlier.

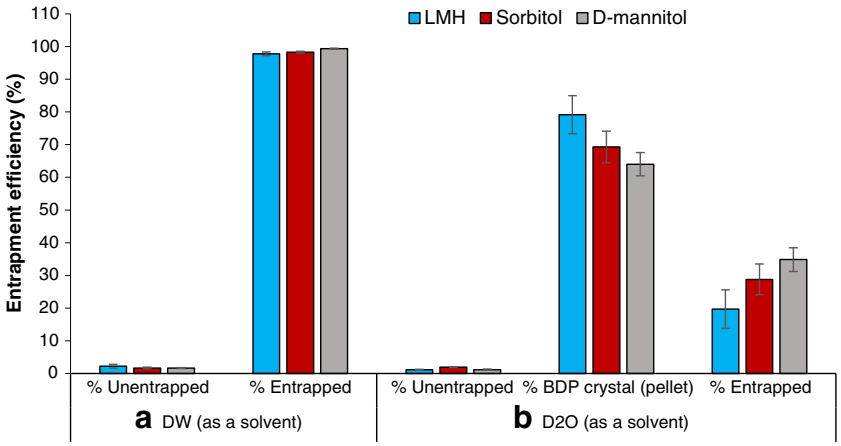

Fig. 10. Entrapment efficiency of BDP in liposomes was generated from proliposome powders manufactured from carbohydrate carriers including LMH, sorbitol or D-mannitol, in formulation ratios of 1:5 *w/w* lipid to carrier ratio in **a** DW, **b** D_2O. Data are mean ± STD, $n = 3$

In contrast to DW, three distinct fractions were identified when D_2O was used as dispersion medium post-centrifugation. Each of the distinct fractions was analysed *via* HPLC. Upon evaluation of the isolated pellet, it was found that it possessed the greatest concentration of BDP (as free drug crystals). This trend was examined for all proliposome formulations, regardless of carrier type (79.18 ± 5.8, 69.30 ± 4.82 and 64.01 ± 3.55%, respectively, for formulations prepared using LMH, sorbitol and D-mannitol) (Fig. 10b). An inverse relationship between BDP entrapment and free BDP concentration was identified, i.e. decreasing free BDP concentration with increasing BDP entrapment (Fig. 10b). The highest entrapment was found in liposomes hydrated from D-mannitol-based proliposomes, e.g. 34.84 ± 3.62%, followed by 28.78 ± 4.69 and 19.69 ± 5.88%, respectively, for liposomes generated from sorbitol- and LMH-based proliposome formulations (Fig. 10b). The only significant difference ($p < 0.05$) in terms of entrapment was noted between liposomes prepared from LMH- and D-mannitol-based proliposomes, since D-mannitol showed the highest steroid entrapment efficiency.

Differences in entrapment were only examined *via* the use of different dispersion media. In D_2O, the entrapment efficiency was lower than 50% of the original entrapment calculated when using conventional centrifugation methods, utilizing DW as separation medium. Thus, it is proposed that the separation method utilizing D_2O as dispersion medium, in addition to separation speed and time, played a key role in achieving improved and convenient separation as compared to alternative methods. Importantly, using the right dispersion medium to facilitate separation has shown the significant difference of steroid entrapment in accordance with using different carbohydrate carriers (D-mannitol > sorbitol > LMH). These findings trigger a debate on many literature reports of drug entrapment in which DW was used as centrifugation medium. Furthermore, the findings here highlighted the influence of carbohydrate carrier type on the entrapment efficiency of the model antiasthma steroid used (i.e. BDP). Noteworthy, when the *in vitro* release profile over 6 h was compared between the formulations, the cumulative drug released from liposomes generated from mannitol-based proliposomes was higher than the release from liposomes made using sorbitol or lactose carriers, being 26.67, 16.12 and 13.91%, respectively. This indicates that carrier affects both BDP entrapment and release profile (data not shown).

In general, steroids exhibit weak accommodation within liposome bilayers (29,48) and the unentrapped drug fraction is present in a crystalline form at the bilayer/water interface (29,36,37,49,50). This behaviour is influenced by the composition of lipid phase (20), and the length of ester chain (49), hydrophobicity and molar mass of the steroid (50). BDP is poorly entrapped in liposomes (20,29,37). Darwis and Kellaway (20) have shown that BDP included in multilamellar vesicles (MLVs) at the maximum entrapment concentration (determined by HPLC) resulted in lowering the onset temperature of the main transition of the phospholipid. However, in an earlier study, Carlson and co-workers (51) have reported that progesterone had a minimal effect on the phase transitions of liposomes even when 33 mol% steroid concentration was incorporated into formulation, indicating that different steroids have different modes of interaction with the liposome bilayers and hence different crystallization behaviours at the phospholipid/water interface. It is hence suggested that the steric fitness of steroids within the liposome bilayers and crystallization behaviour at the bilayer/water interface depend on the molecular structure of the steroid.

Importantly, using highly sensitive differential scanning calorimetry (HSDSC), our research group has found that also other excipients such as ethanol and carbohydrate may influence the nature of BDP interaction with the bilayers. Whilst carbohydrates such as sucrose tended to slightly depress the pretransition of the bilayers and broaden the main transition, ethanol in very small concentrations abolished the pretransition phase and caused bilayer interdigitation, resulting in very poor interaction between BDP and liposome bilayers (37). In the present work, complete ethanol evaporation upon solvent evaporation from the slurry-based proliposomes

was not ascertained analytically. It is known that ethanol exceeding certain concentrations (depending on lipid phase composition) may cause liposome instability, bilayer interdigitation (52), very rapid drug leakage (53) and very poor drug entrapment (approaching 0% for BDP) (37). The relatively high drug entrapment values obtained (Fig. 10) and the similarity of liposome size using the slurry technique (Table II) with the previously reported size of thin-film hydrated vesicles (20,37) indicate that no bilayer interdigitation or instability manifestations have occurred. Furthermore, the entrapment of this steroid in liposomes (Fig. 10) was compared to the entrapment of the drug in niosomes generated from proniosomes (13), possibly suggesting similar steroid crystallization behaviour due to the steric properties of BDP in the bilayers of non-ionic surfactants. Using niosomes generated from particulate-based proniosomes, it was also shown that sucrose carrier may enhance the entrapment of BDP compared to sucrose-free niosomes vesicles (13). This indicates that sucrose may play a positive role at retarding the crystallization of BDP at the bilayer/water interface and promote the accommodation of the steroid in the bilayers.

To further evaluate the possible influence of ethanol (in case trace residues were not evaporated by the rotary evaporator) and the effect of carbohydrate carrier on liposome stability, a drug release study over 6 h was conducted (data not shown). The cumulative BDP released from liposomes was in the range of 13.91–26.67%, depending on carrier type. These findings confirmed that residual ethanol traces, if any, did not affect the short-term stability of the liposomes.

CONCLUSIONS

Liposomes prepared from proliposomes using different carriers (i.e. LMH, sorbitol or D-mannitol) elicited no significant difference in the determined BDP entrapment when DW was used as the dispersion medium, owing to the poor separation of the unentrapped BDP from the liposome-entrapped drug. When the dispersion medium was D_2O, the separation speed of 15,500*g* and separation time of 90 min have influenced the separation of BDP entrapped in liposomes from the unentrapped BDP. In proliposome formulations, BDP achieved an amorphous structure, which reverted to crystalline upon hydration using aqueous media (DW or D_2O). The similar size or density of liposomes and BDP crystals resulted in co-sedimentation at the bottom of the centrifugation tube when DW was employed for dispersion. Separation was identified to play a pivotal role in determining accurate drug entrapment values, with a strong emphasis placed upon the separation medium used; D_2O demonstrated a clear superiority. The usage of XRD and ^{1}HNMR in further analysing the entrapment efficiency highlighted the structural changes of BDP upon formulation and hydration, allowing accurate quantification. The study of hydrophobic drugs other than BDP further confirmed the validity of using D_2O for accurate determination of drug entrapment efficiency, as demonstrated by light microscopy images. Importantly, by using the right separation procedure, carrier type has shown an effect on the estimated proportion of drug entrapped in liposomes generated from proliposomes.

ACKNOWLEDGEMENTS

We thank Lipoid, Switzerland, for supplying us with soya phosphatidylcholine (SPC, Lipoid S-100).

COMPLIANCE WITH ETHICAL STANDARDS

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

REFERENCES

1. Moghassemi S, Hadjizadeh A, Omidfar K. Formulation and characterization of bovine serum albumin-loaded niosome. AAPS PharmSciTech. 2016 (in press).
2. Zheng J, Wan Y, Elhissi A, Zhang Z, Sun X. Targeted paclitaxel delivery to tumors using cleavable PEG-conjugated solid lipid nanoparticles. Pharm Res. 2014;31:2220–33.
3. Mody N, Tekade RK, Mehra NK, Chopdey P, Jain NK. Dendrimer, liposomes, carbon nanotubes and PLGA nanoparticles: one platform assessment of drug delivery potential. AAPS PharmSciTech. 2014;15:388–99.
4. Mehra NK, Jain K, Jain NK. Design of multifunctional nanocarriers for delivery of anti-cancer therapy. Curr Pharm Des. 2015;21:6157–64.
5. Elhissi AM, Ahmed W, Hassan IU, Dhanak VR, D'Emanuele A. Carbon nanotubes in cancer therapy and drug delivery. J Drug Deliv. 2012 837327.
6. Yao HJ, Sun L, Liu Y, Jiang S, Pu Y, Li J, Zhang Y. Monodistearoylphosphatidylethanolamine-hyaluronic acid functionalization of single-walled carbon nanotubes for targeting intracellular drug delivery to overcome multidrug resistance of cancer cells. Carbon. 2016;96:362–76.
7. Khan I, Elhissi A, Shah M, Alhnan MA, Waqar A. Liposome-based carrier systems and devices used for pulmonary drug delivery. In: Davim JP, editor. Biomaterial and medical tribology research and development. Cambridge, UK: Woodhead Publishing Limited; 2013. p. 395–442.
8. Chen R, Li R, Liu Q, Bai C, Qin B, Ma Y, *et al*. Ultradeformable liposomes: a novel vesicular carrier for enhanced transdermal delivery of procyanidins: effect of surfactants on the formation, stability, and transdermal delivery. AAPS PharmSciTech. 2016 (in press).
9. Elhissi A. Liposomes for pulmonary drug delivery: the role of formulation and inhalation device design. Curr Pharm Des. 2016 (in press).
10. Sakagami M, Kinoshita W, Sakon K, J-i S, Makino Y. Mucoadhesive beclomethasone microspheres for powder inhalation: their pharmacokinetics and pharmacodynamics evaluation. J Cont Rel. 2002;80:207–18.
11. Smola M, Vandamme T, Sokolowski A. Nanocarriers as pulmonary drug delivery systems to treat and to diagnose respiratory and non respiratory diseases. Int J Nanomedicine. 2008;3:1–19.
12. Terzano C, Allegra L, Alhaique F, Marianecci C, Carafa M. Non-phospholipid vesicles for pulmonary glucocorticoid delivery. Eur J Pharm Biopharm. 2005;59:57–62.
13. Elhissi A, Hidayat K, Phoenix DA, Mwesigwa E, Crean S, Ahmed W, Faheem A, Taylor KM. Air-jet and vibrating-mesh nebulization of niosomes generated using a particulate-based proniosome technology. Int J Pharm. 2013;444:193–9.

14. Jaafar-Maalej C, Andrieu V, Elaissari A, Fessi H. Beclomethasone-loaded lipidic nanocarriers for pulmonary drug delivery: preparation, characterization and in vitro drug release. J Nanosci Nanotechnol. 2011;11:1841–51.
15. Nasr M, Najlah M, D'Emanuele A, Elhissi A. PAMAM dendrimers as aerosol drug nanocarriers for pulmonary delivery via nebulization. Int J Pharm. 2014;461:242–50.
16. Gaber NN, Darwis Y, Peh KK, Tan YT. Characterization of polymeric micelles for pulmonary delivery of beclomethasone dipropionate. J Nanosci Nanotechnol. 2006;6:3095–101.
17. Xu L-M, Zhang Q-X, Zhou Y, Zhao H, Wang J-X, Chen J-F. Engineering drug ultrafine particles of beclomethasone dipropionate for dry powder inhalation. Int J Pharm. 2012;436:1–9.
18. Barnes N, van Noord JA, Brindicci C, Lindemann L, Varoli G, Perpiña M, Guastalla D, Casula D, Patel S, Chanez P, FACTO (Foster® As Complete Treatment Option) Study Group. Stepping-across controlled asthmatic patients to extrafine beclometasone/formoterol combination. Pulm Pharmacol Ther. 2013;26:555–61.
19. Saari M, Vidgren MT, Koskinen MO, Turjanmaa VMH, Nieminen MM. Pulmonary distribution and clearance of two beclomethasone liposome formulations in healthy volunteers. Int J Pharm. 1999;181:1–9.
20. Darwis Y, Kellaway IW. Nebulisation of rehydrated freeze-dried beclomethasone dipropionate liposomes. Int J Pharm. 2001;215:113–21.
21. Subramanian S, Khan I, Korale O, Alhnan MA, Ahmed W, Najlah M, Taylor KM, Elhissi A. A simple approach to predict the stability of phospholipid vesicles to nebulization without performing aerosolization studies. Int J Pharm. 2016;502:18–27.
22. Payne NI, Timmins P, Ambrose CV, Ward MD, Ridgway F. Proliposomes: a novel solution to an old problem. J Pharm Sci. 1986;75:325–9.
23. Payne NI, Browning I, Hynes CA. Characterization of proliposomes. J Pharm Sci. 1986;75:330–3.
24. Elhissi A, Taylor KMG. Delivery of liposomes generated from pro liposomes using air-jet, ultrasonic and vibrating-mesh nebulisers. J Drug Del Sci Technol. 2005;15:261–5.
25. Elhissi A, Gill H, Ahmed W, Taylor K. Vibrating-mesh nebulization of liposomes generated using an ethanol-based proliposome technology. J Liposome Res. 2011;21:173–80.
26. Desai TR, Wong JP, Hancock RE, Finlay WH. A novel approach to the pulmonary delivery of liposomes in dry powder form to eliminate the deleterious effects of milling. J Pharm Sci. 2002;91:482–91.
27. Rojanarat W, Changsan N, Tawithong E, Pinsuwan S, Chan HK, Srichana T. Isoniazid proliposome powders for inhalation-preparation, characterization and cell culture studies. Int J Mol Sci. 2011;12:4414–34.
28. Waldrep JC, Keyhani K, Black M, Knight V. Operating characteristics of 18 different continuous-flow jet nebulizers with beclomethasone dipropionate liposome aerosol. Chest. 1994;105:106–10.
29. Batavia R, Taylor KMG, Craig DQM, Thomas M. The measurement of beclomethasone dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method. Int J Pharm. 2001;212:109–19.
30. Ohnishi N, Tomida H, Ito Y, Tahara K, Takeuchi H. Characterization of a doxorubicin liposome formulation by a novel in vitro release test methodology using column-switching high-performance liquid chromatography. Chem Pharm Bull (Tokyo). 2014;62:538–44.
31. Ohnishi N, Tanaka S, Tahara K, Takeuchi H. Characterization of insulin-loaded liposome using column-switching HPLC. Int J Pharm. 2015;479:302–5.
32. Deshpande NM, Gangrade MG, Kekare MB, Vaidya VV. Determination of free and liposomal amphotericin B in human plasma by liquid chromatography-mass spectroscopy with solid phase extraction and protein precipitation techniques. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878:315–26.
33. Stone NR, Bicanic T, Salim R, Hope W. Liposomal amphotericin B (AmBisome(®)): a review of the pharmacokinetics, pharmacodynamics, clinical experience and future directions. Drugs. 2016;76:485–500.
34. Isailović BD, Kostić IT, Zvonar A, Đorđević VB, Gašperlin M, Nedović VA, Bugarskia BM. Resveratrol loaded liposomes produced by different techniques. Innovative Food Sci Emerg Technol. 2013;19:181–9.
35. Cadena PG, Pereira MA, Cordeiro RBS, Cavalcanti IMF, Netoc BB, Pimentel MCCB, Filhoa JLL, Silvab VL, Santos-Magalhãesa NS. Nanoencapsulation of quercetin and resveratrol into elastic liposomes. Biochim et Biophys Acta (BBA) - Biomembranes. 2013;1828:309–16.
36. Khan I, Yousaf S, Subramanian S, Korale O, Alhnan MA, Ahmed W, Taylor KM, Elhissi A. Proliposome powders prepared using a slurry method for the generation of beclometasone dipropionate liposomes. Int J Pharm. 2015;496:342–50.
37. Elhissi AM, O'Neill MAA, Roberts SA, Taylor KMG. A calorimetric study of dimyristoylphosphatidylcholine phase transitions and steroid–liposome interactions for liposomes prepared by thin film and proliposome methods. Int J Pharm. 2006;320:124–30.
38. Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem. 1980;104:10–4.
39. Newman AW, Vitez IM, Mueller RL, Kiesnowski CC, Findlay WP, Rodriguez C, *et al*. Sorbitol. In: Harry GB, editor. Analytical profiles of drug substances and excipients, vol 26. Academic Press; 1999, p. 459–502.
40. Elhissi AMA, Ahmed W, McCarthy D, Taylor KMG. A study of size, microscopic morphology, and dispersion mechanism of structures generated on hydration of proliposomes. J Dispers Sci Technol. 2011;33:1121–6.
41. Meyer VR. Practical high-performance liquid chromatography. 5th ed. John Wiley & Sons, Inc: Switzerland; 2010. 412 p.
42. Row RC, Sheskey PJ, Owen SC. Handbook of pharmaceutical excipients. 5th ed. London, Uk: Pharmaceutical Press; 2006. p. 385–748.
43. Vecellio L, Abdelrahim ME, Montharu J, Galle J, Diot P, Dubus J-C. Disposable versus reusable jet nebulizers for cystic fibrosis treatment with tobramycin. J Cyst Fibros. 2011;10:86–92.
44. Kushner DJ, Baker A, Dunstall TG. Pharmacological uses and perspectives of heavy water and deuterated compounds. Can J Physiol Pharmacol. 1999;77:79–88.
45. Sharma R, Bisen D, CShukla U, Sharma B. X-ray diffraction: a powerful method of characterizing nanomaterials. Recent Res Sci Technol. 2012;4:77–9.
46. Radhakrishnan R. Novel liposome composition for sustained release of steroidal drugs. 1991. US Patent 5043165 A.
47. Jaafar-Maalej C, Charcosset C, Fessi H. A new method for liposome preparation using a membrane contactor. J Liposome Res. 2011;21:213–20.
48. Kulkarni SB, Vargha-Butler EI. Study of liposomal drug delivery systems 2. Encapsulation efficiencies of some steroids in MLV liposomes. Colloids Surf B. 1995;4:77–85.
49. Fildes FJT, Oliver JE. Interaction of cortisol-21-palmitate with liposomes examined by differential scanning calorimetry. J Pharm Pharmacol. 1978;30:337–42.
50. Arrowsmith M, Hadgraft J, Kellaway IW. The interaction of cortisone esters with liposomes as studied by differential scanning calorimetry. Int J Pharm. 1983;16:305–18.
51. Carlson JC, Gruber MY, Thompson JE. A study of the interaction between progesterone and membrane lipids. Endocrinology. 1983;113:190–4.
52. Veiro JA, Nambi P, Herold LL, Rowe ES. Effect of n-alcohols and glycerol on the pretransition of dipalmitoylphosphatidylcholine. Biochim Biophys Acta. 1987;900:230–8.
53. Elhissi AM, Giebultowicz J, Stec AA, Wroczynski P, Ahmed W, Alhnan MA, Phoenix D, Taylor KM. Nebulization of ultradeformable liposomes: the influence of aerosolization mechanism and formulation excipients. Int J Pharm. 2012;436:519–26.