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Freeze-drying of nanostructure lipid carriers by different carbohydrate polymers used as cryoprotectants

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ABSTRACT

Freeze-drying technique preserves the stability of nanoparticles. The objective of this study was optimization of freeze-drying condition of nano lipid carriers (NLCs). NLCs were prepared by emulsion-solvent evaporation followed by ultra-sonication method. Different carbohydrate and polymeric cryoprotectants including Microcelac® (mixture of lactose and Avicel), Avicel PH102 (microcrystalline cellulose), mannitol, sucrose, Avicel RC591 (mixture of microcrystalline cellulose and sodium carboxymethyl cellulose), maltodextrine, Aerosil and PEG4000 were tested initially. The NLCs showing lower particle size growth and greater absolute zeta potential after freeze drying were chosen for further investigation using Taguchi optimization method. Studied factors included cryoprotectant type and concentration, freezing temperatures applied at different time periods and sublimation time. Sucrose, Avicel RC591 and Aerosil were selected as cryoprotectants from initial screening tests. Increasing their concentration increased the particle size. 1% of Avicel RC591, 24 h of freezing at −70 °C and 48 h sublimation time showed lower growth in particle size.

1. Introduction

Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) have been the main colloidal lipid systems studied due to their low toxicity, their ability to carry hydrophilic or lipophilic drugs, their ability to control and localize the release of the active drug, easy to scale up and their small size. They have been proposed for several administration routes, such as oral, parenteral, nasal and topical routes. It has been reported that optimized SLN formulations could maintain their physicochemical stability up to 3 years (Muhlen, Schwarz, & Mehnert, 1998). However, the physicochemical stability of the lipid carriers showed variations due to their numerous compositions and structures. Nanoemulsions are in the liquid state and undergo instabilities such as flocculation, membrane permeability, drug release, creaming, gelling, and particle aggregation. Instabilities are the effect of hydrolysis oxidation, crystallization, polymorphism, phase inversion and zeta potential (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003). Alternative ways to avoid most of these instabilities is the elimination of the water in the sample using freeze drying, fluid bed drying and spray drying techniques (Freitas & Muler, 1998; Heurtault et al., 2003). Solid dosage forms are more stable and more preferred than liquids in nanoparticle formulations (Konan, Gurny, & Allémann, 2002; Lee, 2003). Freeze-drying or lyophilization is an industrial process, which removes water from a frozen sample using sublimation and desorption under vacuum, wherein a solid is converted to the vapor state without first passing through the liquid phase. This process stabilizes biomaterials so that they can be stored. Lyophilization is utilized as a critical technique to convert the lipid dispersion to a solid state to extend the stability and to avoid particle aggregation (Mehnert & Mader, 2001). Vaccines, pharmaceuticals, and other delicates, heat sensitive materials are mostly dried by this technique. Freeze-drying process improves the long-term physico-chemical stability of SLNs and prevents degradation reactions such as hydrolysis and growth of initial particle size (Vighi, Ruozi, Montanari, Battini, & Leo, 2007).

This technique is more favorable for lipid nanoparticles (Lim & Kim, 2002). A successful nanoparticles freeze-drying requires a deep investigation of the formulation and the process conditions to retain the properties of nanoparticles in spite of various stresses during the process. In order to prevent aggregation of nanoparticles and ensure re-dispersability after freeze–thawing, cryoprotectants have been added (Kesisoglou, Panmai, & Wu, 2007). These additives help to decrease particle aggregation and improve re-dispersion of the dry product (Vighi et al., 2007). Commonly used cryoprotective agents are polyvinylpyrrolidone (PVP), and water soluble sugars such as sorbitol, mannose, maltose, trehalose and glucose (Heurtault et al., 2003; Kesisoglou et al., 2007; Mehnert & Mader, 2001). In literatures, different changes in particle size distribution

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Table 1
Freeze drying conditions studied by the 3-level, 4-factor L_9 orthogonal array Taguchi design.

Batch no.	X_1 (freezing temperature applied at different time periods)	X_2 (cryoprotectant concentration) (%w/w)	X_3 (sublimation time)	X_4 (cryoprotectant type)
1	−89°/5 min	1%	72 h	Aerosil
2	−89°/5 min	2%	48 h	Avicel RC591
3	−89°/5 min	3.5%	56 h	Sucrose
4	−20°/24 h	1%	56 h	Avicel RC591
5	−20°/24 h	2%	72 h	Sucrose
6	−70°/24 h	3.5%	72 h	Avicel RC591
7	−70°/24 h	1%	48 h	Sucrose
8	−20°/24 h	3.5%	48 h	Aerosil
9	−70°/24 h	2%	56 h	Aerosil

during lyophilization are observed but it could be minimized using optimization of the process. The present study focuses on the application of some new carbohydrate excipients as cryoprotectants in freeze drying of NLCs. Although the solid state is chemically and physically more stable than liquid dispersions, the effect of lyophilization process and the process of re-dispersion should be considered on the overall stability of colloidal dispersions once they are reconstituted. The negative effects of these processes can be lessened by employing the optimum freezing velocity by selecting the best re-dispersion method, i.e. either sonication or manual shaking (Schwarz & Mehnert, 1997). For these reasons the present study was carried out to optimize the cryoprotectant type and concentration, freezing temperatures applied at different time periods and sublimation time during lyophillization of nano lipid carriers (NLCs). To do this an optimization procedure based on Taguchi L_9 method was used to optimize and evaluate the effect of different variables on freeze drying of NLCs using carbohydrates.

2. Materials and methods

2.1. Materials

Valproic acid (VPA) was provided by Fabric Beck Chemi (Germany), cetyl palmitate (CP) (mp 46–53 °C) (Croda, England), Poloxamer 188 (Sigma Aldrich Chemie, Germany), soy lecithin S100 (Lipoid, Germany), octyldodecanol (Sasol, Germany), sucrose (Merck Chemical Company, Frankfort, Germany), Avicel RC591 (FMC, Brussels, Belgium), Aerosil (Evonik, Frankfort, Germany), Avicel 102 (FMC, Brussels, Belgium), mannitol (Merck Chemical Company, Frankfort, Germany), maltodextrine (Luzhou Bio-Chem, Shandong, China), Microcelac (Meggle, Wasserburg, Germany), polyethylene glycol 4000 (Merck Chemical Company, Frankfort, Germany), polyvinyl alcohol (Merck Chemical Company, Frankfort, Germany), acetone (Merck Chemical Company, Germany), ethanol (Merck Chemical Company, Germany), acetonitrile HPLC grade (Merck Chemical Company, Germany), caproic acid (Merck Chemical Company, Germany) and chloroform (Merck Chemical Company, Germany).

2.2. Preparation of VPA loaded NLC

NLCs of VPA as a candidate for nasal delivery of this drug were prepared by an emulsion-solvent diffusion and evaporation method followed by ultrasonication as reported before (Varshosaz, Eskandari, & Tabbakhian, 2009; Varshosaz, Eskandari, Kennedy, Tabbakhian, Minaiyan, in press). Briefly, the hot lipid phase containing 400 mg of Cp, 100 mg soy lecithin S100, 0.1 mL octyldodecanol and 400 mg VPA were dissolved in 10 mL of a mixture of acetone and absolute ethanol on water bath at 60 °C. In the second step, the oily phase was dispersed in an aqueous phase containing Poloxamer 188 and premixed by magnetic stirrer for 1 min at 2000 rpm. The resulting pre-emulsion was then ultrasonicated for 2 min using a probe sonicator (Bandelin, Germany) by probe TT13 in amplitude 40% to produce an O/W nanoemulsion. In the last step the obtained nanoemulsion (O/W) was cooled down at room temperature while stirring on magnetic stirrer 600 rpm for about 1 h.

2.3. Freeze drying the NLCs

In preliminary tests the particles were lyophilized by using different kinds of cryoprotectants; PEG4000, Microcelac®, Avicel PH102, mannitol, sucrose, Avicel RC591, Aerosil and maltodextrine. Freezing temperature was −20 °C for about 24 h and 3% (w/v) concentration of cryoprotectants were used. Lyophilization time lasted for 72 h. The freeze-dryer (Christ, Alpha 2-4 LD plus, Germany) operating conditions were temperature of −40 °C and pressure of 0.001 bar. After choosing the best cryoprotectants which caused the lowest size growth of the particles, the Taguchi design was used for optimization of the conditions to have the least change in particle size, zeta potential, pH, and osmolality of NLCs after freeze drying. In this study 3 kinds of cryoprotectants each in 3 concentrations were chosen, different freezing temperatures applied at different time periods and sublimation times were included (Table 1). Re-dispersion of freeze dried nanoparticles was done using PVA 0.01% in distilled water using a bath sonicator for about 10 min.

2.4. Experimental design and analysis

Table 1 displays the independent variables and their levels studied in a L_9 orthogonal array using Taguchi design. A standard orthogonal array L_9 (Taguchi & Konishi, 1987) was used to examine four-factors each in three levels. L and subscript 9 denote the Latin square and the number of the experimental runs, respectively. A run involved the corresponding combination of levels to which the factors in the experiment were set. All experiments were performed in triplicate. Four studied responses included size (nm), zeta potential (mV), pH and osmolality. The experimental results were then analyzed by the Design Expert software to extract independently the main effects of these factors, followed by the analysis of variance (ANOVA) to determine which factors were statistically significant. Identifying controlling factors and qualifying the magnitude of effects, as well as identifying the statistically significant effects, was emphasized.

2.5. Determination of particle size, zeta potential and osmolality of nanoparticles

Size and zeta potential of all drug loaded NLC samples were measured by photon correlation spectroscopy (PCS, Zetasizer 3000, Malvern, UK). All the samples were diluted one to ten ratio with distilled water to get optimum 50–200 kilo counts per second (kcps) for measurements. *Z*-Average particle size, polydispersity index and zeta potential were measured in triplicate. Osmolality was

measured using cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany) after calibration using Gonotech calibration standard 300.

2.6. Analytical method of VPA

Determination of VPA in release medium and its entrapment efficiency in the nanoparticles were conducted using Kishore method (Kishore, Rajani Kumar, Satyanarayana, & Krishna, 2003) with some modifications. Briefly, a reversed-phase HPLC (Waters, USA) method with UV detector at 210 nm using a Nova pack, reversed-phase C18 column, 250 mm × 4.5 mm employing a mobile phase of 40% acetonitrile and 60% phosphate buffer solution 0.02 M (pH 3) with delivery at a flow-rate of 1 mL/min was used. The retention time of the drug was found to be 10.0 ± 0.1 min. Data analysis and processing was done by Millennium software.

2.7. Determination of entrapment efficiency and drug loading in the NLCs

Drug loading percent was determined by measuring the concentration of unentrapped free drug in aqueous medium (Venkateswarlu & Manjunath, 2004). The aqueous medium was separated by centrifugation (Sigma 3K30, Germany). About 1.5 mL of the NLC dispersion was placed in the eppendorf tubes and acidified with HCl 1 N to pH 1.5 and centrifuged at 25,000 rpm for 30 min. The amount of VPA in the aqueous phase was estimated by HPLC method described aforementioned. The loading percentage was calculated using Eq. (1):

$$\text{Loading}\,\% = \left(\frac{\text{analyzed weight of drug in NLCs}}{\text{analyzed weight of NLCs}}\right) \times 100 \qquad (1)$$

2.8. In vitro release of VPA from NLCs

To determine the release rate of VPA from nanoparticles 3 mL of aqueous dispersion of formulation (containing 8 mg/mL VPA) was added to the dialysis bags with molecular weight cutoff of 12,400 Da and the sealed bags were placed in the glass test-tube in 200 mL of the phosphate buffer solution (PBS) 0.1 M (pH 6) containing 0.1% polysorbate 80 to provide sink conditions with agitation of 200 rpm. Samples were withdrawn at predetermined time intervals of 2, 24, 72, 96, 168 (7 days) and replaced with fresh PBS maintained at the same temperature. The content of VPA in the samples was determined by the described HPLC method.

2.9. Atomic force microscopy (AFM)

To study the morphological changes and also the particle size of NLCs before and after lyophillization AFM micrographs were taken. AFM observation was performed by a Nanosurf mobile S, Atomic Force Microscope (Nanosurfe AG, Liestal, Switzerland). AFM images were obtained by measurement of the interaction forces between the tip and the sample surface (Heydenreich, Westmeier, Pedersen, Poulsen, & Kristensen, 2003). The experiments were done in air at room temperature (25 °C) operating in non-contact mode (NC-AFM). Droplets of the final suspension (20 μL) were deposited onto a small mica disk. After the drop was dried, the non-contact mode was used at room temperature. The measurements were performed in different sample locations. The mean size of NLCs was obtained by processing the AFM images with the Nanosurf mobile S software. The amplitude AFM images were taken before and after freeze-drying the NLCs in the optimized condition of freeze-drying, i.e., the freezing temperature of −70 °C applied at a time periods of 24 h, 1% of Avicel RC591 (as cryoprotectant) and sublimation time of 48 h.

Table 2
Results of a preliminary screening study of different cryoprotectants and freezing temperature of −20 °C for 24 h, 3% (w/v) concentration and sublimation time of 72 h (n = 3).

Zeta potential (mV)	Size (nm)	Cryoprotectant
2.92 ± 0.2	2863 ± 320.0	PEG4000
9.47 ± 0.4	520.8 ± 147.0	Microcelac®
−0.71 ± 0.2	599.2 ± 150.4	Avicel PH102
−0.70 ± 0.1	558.2 ± 128.2	Mannitol
−12.10 ± 0.1	446.5 ± 95.1	Sucrose
−27.50 ± 0.9	448.7 ± 88.4	Avicel RC591
−22.50 ± 0.4	401.2 ± 101.2	Aerosil
−20.00 ± 0.3	708.6 ± 234.0	Maltodextrine

2.10. Transmission electron microscope (TEM) observation

A drop of nanoparticles suspension was placed on a carbon film coated on a copper grid for TEM. Observation was done at 80 kV in a Leo 906 (Germany).

2.11. DSC analysis

DSC thermograms were obtained using (DSC 131 Setaram, Germany). A certain amount of dried nanoparticle powder was crimped in a standard aluminum pan and heated from 25 to 125 °C at a heating rate of 5 °C/min under constant purging of nitrogen.

3. Results and discussion

3.1. Physicochemical properties of NLCs after freeze drying

The particle size, zeta potential, drug loading percent and drug release of the optimized NLCs of VPA (containing 0.8% cetyl palmitate, 1% poloxamer 188, 0.2% lipoid, 0.2% octyldodecanol and 0.8% VPA) were 138.5 nm, −12.2 mV, 44% and 69%, respectively. The polydispersity of these nanoparticles was 0.2. Freeze-drying is a way for ensuring stability, ease in storage, handling and formulation into solid-dosage forms. Because the presence of water accelerates degradation of various types of polymers and lipids used in nanoparticles. The results of preliminary screening study of different cryoprotectants of Table 2 shows that in most formulations the size and zeta potential of NLCs after re-dispersion is high which is related to the effect of each cryoprotectant. PEG4000 caused an increase in size to about 3 m and zeta potential to about 3 mV, which indicates significant aggregation of nanoparticles with poor re-dispersability property. Previous results showed that when the concentration of PEG was low, concentrations up to 15 wt.% acts as an aggregation promoter but in concentration of 25 or 40 wt.% an increase in particle size was not observed (Lee, Kim, Kim, & Lee, 2009). Mannitol, maltodextrine, Avicel PH102 and Microcelac (mixture of lactose and Avicel) showed poor re-dispersability with particle size of more than 0.5 μm that may be related to precipitation of cryoprotectant. Sucrose, mannitol and maltodextrine are sugars which are used as cryoprotectant. The most popular cryoprotectants encountered in the literature for freeze-drying nanoparticles are sugars.

In the study of Zimmermann, Müller, & Mader (2000) trehalose was found to be the most effective, while PVP was found to be least effective in lyophilization of SLNs. The level of stabilization afforded by sugars generally depends on their concentrations (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006). The other results showed that effect of mannitol, lactose and maltodextrine on particle size might be related to their concentration and freezing temperatures applied at different time periods (Lee et al., 2009). Although using some cryoprotectants are usual but are not always helpful. Table 2 shows that sucrose, Avicel RC591 and Aerosil cause less increase in

Table 3
Observed responses[a] after re-dispersion of freeze-dried formulations of L_9 orthogonal array Taguchi design in water.

Batch No	Y_1 size (nm)	Y_2 Zeta (mV)	Y_3 pH	Y_4 Osmolality (mOsmol/kg)
1	436.8	−27.9	5.8	249
2	444.8	−34.3	5.9	351
3	670.0	−14.3	5.0	500
4	252.2	−35	6.2	290
5	530.7	−12.4	5.2	397
6	278.6	−22.5	6.5	359
7	407.4	−17.1	5.0	296
8	390.7	−28.2	6.1	334
9	287.5	−25.9	6.0	264

[a] Standard deviation of the responses did not exceed 10% of the measured value

particle size (446.5, 448.7 and 401.2 nm, respectively) and zeta potential (−12.1, −27.5 and −22.5 mV, respectively) after re-dispersion of dried nanoparticles in distilled water. However, the effect of concentration, freezing temperatures applied at different time periods and sublimation time should be considered for these cryoprotectants. The freezing procedure also affects the crystal structure and properties of the lyophilizate (Vighi et al., 2007). Cooling may be done either rapidly or slowly. Rapid cooling can be performed by dipping the vial containing the preparation into liquid nitrogen or by adding the dispersion drop wise to liquid nitrogen (Mehnert & Mader, 2001). This results in the formation of small, heterogeneous crystals, whereas slow cooling causes large crystals to form as is done by placing the vials in a freeze drier having a shelf temperature of −25 °C for 24 h (Mehnert & Mader, 2001). Each type is associated with certain advantages and disadvantages. While rapid cooling decreases freezing out effects, it causes slower sublimation. Therefore, freeze-drying must be done in a sample-specific manner by optimizing the lyophilization process (Mehnert & Mader, 2001). Analysis of data in Table 3 shows that fast freezing in sucrose, Avicel RC591 and Aerosil causes increase in particle size while lower particle size growth obtains regardless of the concentration and time of sublimation when deep freezing in −70 °C is used (Fig. 1). The results show increasing in particle size of NLCs at high freezing temperature (absolute value) applied at short time for all formulations. This may be related to the competition between diffusion rate of cryoprotectant in cryo-concentrated liquid phase and freezing temperature applied at different time periods, which causes more aggregation. However, previous studies showed that fast freezing was beneficial for re-dispersibility, where nanoparticles would not have enough time to move around and aggregate (Lee et al., 2009). It may be concluded that the presence of liquid oils in the structure of NLCs may

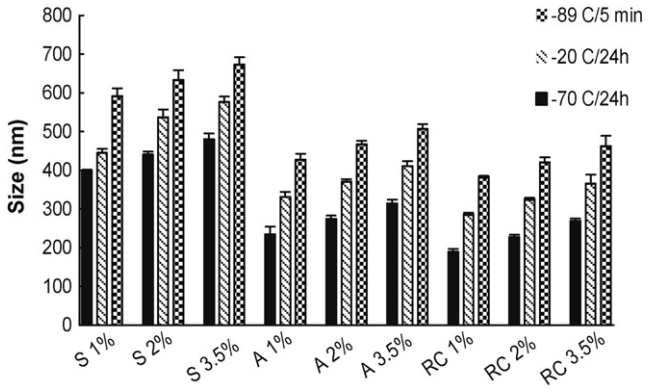

Fig. 1. Effect of different cryoprotectants, concentration and freezing rate on particle size. Sublimation time has not significant effect on particle size. S = sucrose, A = Aerosil, and RC = avicel RC 591.

cause such a difference between the behaviors of these nanostructures with solid nanoparticles. Fig. 1 shows that increasing the concentration of sucrose, Aerosil and Avicel RC591 cause particle size growth regardless of the time of sublimation. However, growth in particle size by sucrose is the highest compared to other cryoprotectants (sucrose > Aerosil > Avicel RC591). Previous results showed that low concentration of sucrose increased aggregation and re-dispersability was better in higher concentrations to about 20% (Saez, Guzman, Molpeceres, & Aberturas, 2000). Fig. 1 also shows that increasing the concentration of Avicel RC591 causes the increase in particle size of NLCs. Avicel RC591 is a type of microcrystalline cellulose containing sodium carboxymethyl cellulose, which is dispersible and partially soluble in water. The information from the supplier revealed that approximately 60% of the crystallites in dispersion were below 200 nm (Rowe, Sheskey, & Owen, 2005). This was confirmed by measuring the particle size of a suspension of Avicel RC591 prior to its application as the cryoprotectant. The results showed a *Z*-average of 180 nm. It helps promote more uniform, stable and elegance formulation. Avicel RC 591 uses as an emulsion stabilizer by reducing oil droplet movement and possible coalescence. Its crystalline structure showed that the two hydroxyl dominant sides of crystal have an affinity for water while the remaining two sides of crystals are wetted by lipid nanoparticles. Being so, it forms a permanent barrier for nanoparticle agglomeration during freeze drying. Concentrations more than 1% are prone to form gel which inhibits effective evaporation of water in freeze drying process whereas concentrations equal to 1% form colloid dispersion. Results of particle size analysis reveal that increasing concentration of Aerosil causes increase in particle size which might be due to gel forming effect of Aerosil in high concentrations. The most frequently used glidant in tabletting process is colloidal silicon dioxide (Aerosil), which has particle size of below 20 nm (Rowe et al., 2005). It has the advantage of acting as a moisture scavenger. Residual water in the formula binds to the silica, thereby providing a drier environment. Schaffazick, Pohlmann, Dalla-Costa, & Guterres (2003) showed that Aerosil could prevent aggregation of freeze-dried diclofenac polymeric nanocapsules without leakage of drug or disturbing the structural integrity of the capsule wall. The results of data analysis on particle size show that sublimation time is not an effective factor on particle size of nanoparticles. Results of data analysis in Table 3 show that zeta potential is fit in the linear model and the range of zeta potential for all formulations is low with the range of −12.4 to −35.0 mV. Re-dispersion of the freeze dried formulations in PVA 0.01% minimizes the aggregation of nanoparticles and enhances dispersion. Table 3 shows that Avicel RC591 causes more decrease in zeta potential whereas sucrose causes less decrease in zeta potential. Possibly because the suspensions of Avicel and Aerosil have themselves negative zeta potential (near −14 to −16 mV) which increases the absolute values of zeta potential of the nanoparticles while, sucrose is a neutral compound that dissolves in water. Therefore, in the presence of sucrose the nanoparticles show their own surface charge. Entrapment of nanoparticles in some polymers normally changes the zeta potential because the coating layers shield the surface charge and move the shear plane out wards from the particle surface (Hawley, Illum, & Davis, 1997; Tobio, Gref, Sanchez, Langer, & Alonso, 1998). Redhead, Davis, & Illum (2001) have reported a similar reduction in the zeta potential of PLGA nanoparticles after coating with amphiphilic polymers like poloxamer 407 and poloxamine 908. The lower shielding effect of sucrose also explains the lower absolute values for zeta potential of NLCs prepared with sucrose as cryoprotectant (Table 3).

Another explanation for increased absolute values of zeta potential of nanoparticles with Avicel and Aerosil is the fact that both are water insoluble products. Being so, they form a permanent barrier for nanoparticles agglomeration while this barrier is not

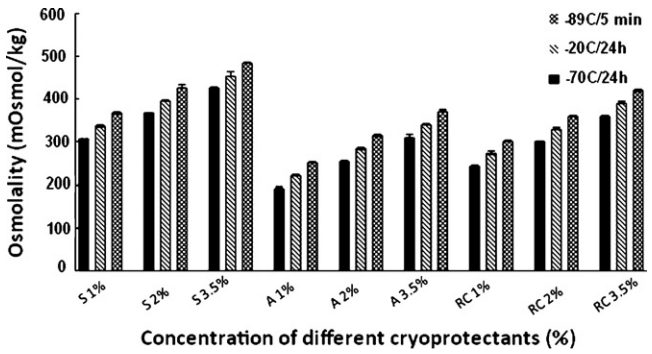

Fig. 2. Effect of different cryoprotectants, freezing temperature applied at different time periods and concentration on osmolality. Sublimation time have not significant effect on osmolalityl. S = sucrose, A = Aerosil, and RC = avicel RC 591.

permanent in the case of sucrose. During freeze-drying, the latter system evolves from a sucrose solution to a concentrated solution that is then transformed into a hygroscopic, glassy state that finally crystallizes out and after re-dispersion of particles it will be dissolved so can not affect on the zeta potential of nanoparticles significantly unlike the other two materials (Bernard et al., 2008). In most studies on cryoprotectants, the higher the concentration of cryoprotectant, the better the stability of the nanoparticles. However, the trend was reverse in some cases as our study (Saez et al., 2000; Sameti et al., 2003). Possible rational for this observation is related to specific interactions among cryoprotectants, solvents, particles and crystallization behavior of the cryoprotectants. Analysis of zeta potential data revealed that increasing the freezing temperature applied at different time periods and sublimation time does not have any significant effect on zeta potential respect to the other conditions. The results of Table 3 show that after re-dispersion of NLCs in PVA 0.01%, the pH of all formulations is in the suitable range of 5.0–6.5 as for nasal formulations. To avoid nasal irritation, efficient drug permeation and prevention the growth of bacteria, the pH of the nasal formulation should be adjusted to 4.5–6.5 (Arora, Sharma, & Garg, 2002). The results show that sucrose, Aerosil and Avicel RC591 cause pH 4.7–5.3, 5.6–6 and 5.8–6.5, respectively, which all are in suitable range. The results of osmolality in Fig. 2 show that cryoprotectant type and its concentration are the most effective factors on osmolality. However, the most formulations except those containing sucrose 2% and 3.5% are near an isotonic solution (260–330 mOsmol) after re-dispersion. Osmolality is another influencing factor in drug absorption from nasal cavity. Usually an isotonic solution (260–330 mOsmol) is preferred for nasal administration (Aulton, 2007). Ohwaki, Ando, & Kakimoto (1987) studied the effect of osmolality on the absorption of secretin in rats and found that absorption and permeation reached a maximum at an isotonic solution because of the shrinkage of the nasal epithelial mucosa (Jadhav, Gambhire, Shaikh, Kadam, & Pisa, 2007; Ohwaki et al., 1987).

3.2. Optimization of freeze drying conditions of NLCs

Computer optimization process and a desirability function determined the effect of the levels of independent variables on the responses. All responses were fitted to the linear model. The constraints of particle size was $252.2 \leq Y_1 \leq 670$ nm with targeting the particle size on minimum, for zeta potential was

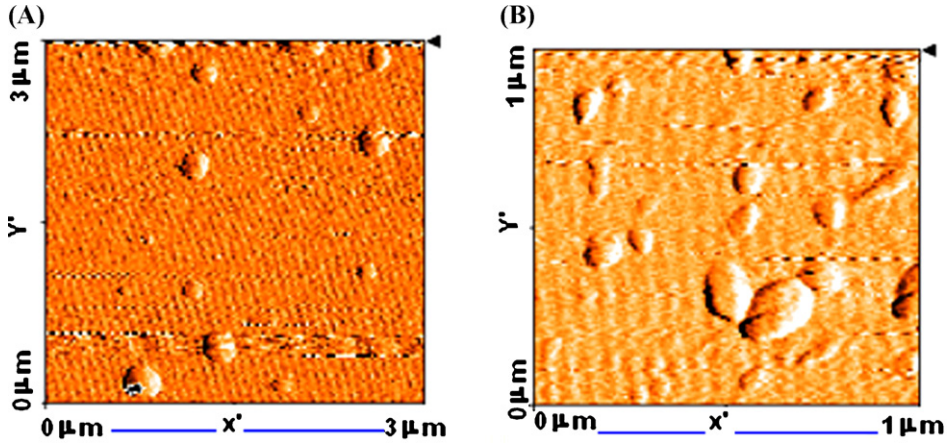

Fig. 3. Amplitude mode atomic force microscopy images of NLCs of valproic acid (A) before and (B) after freeze drying the NLCs in the optimized condition of freeze-drying, i.e., the freezing temperature of −70 °C applied at 24 h, 1% of Avicel RC591 and sublimation time of 48 h.

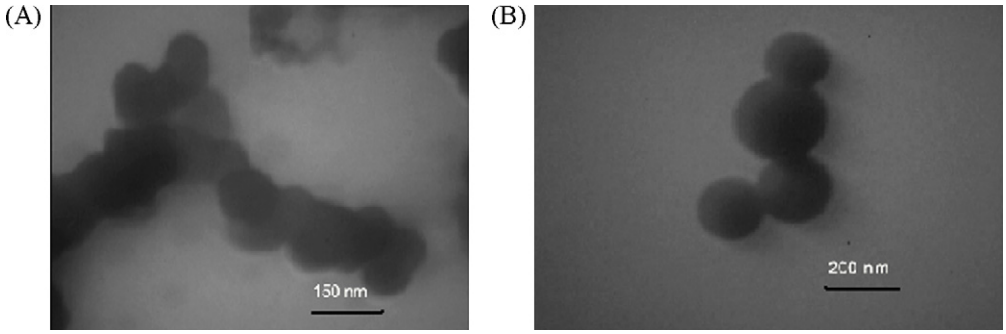

Fig. 4. Transmission electron microscopy photographs of NLCs of valproic acid (A) before and (B) after freeze drying the NLCs in the optimized condition of freeze-drying, i.e., the freezing temperature of −70 °C applied at 24 h, 1% of Avicel RC591 and sublimation time of 48 h.

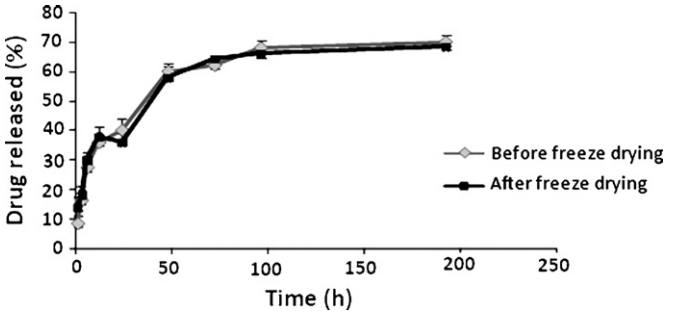

Fig. 5. Release profiles of valproic acid from NLCs before and after freeze drying of the NLCs in the optimized condition of freeze-drying, i.e., the freezing temperature of −70 °C applied at 24 h, 1% of Avicel RC591 and sublimation time of 48 h.

$-35.0 \leq Y_2 \leq -12.4$ mV while the target was maximum absolute value of zeta potential, for pH the constraint was $5.0 \leq Y_3 \leq 6.5$ with the goal of the pH in range of 5–6.5 and the osmolality constraint was $249 \leq Y_4 \leq 500$ mOsmol/kg with the target between 260 and 330 mOsmol/kg. The predicted values of Y_1, Y_2, Y_3 and Y_4 were 251.6 nm, −35.03 mV, pH of 6 and 260.6 mOsmol, respectively, when X_1, X_2, X_3 and X_4 levels are: freezing temperature of −70 °C applied at 24 h, 1% of Avicel RC591 and sublimation time of 48 h with 100% desirability. To confirm the predicted model, the optimized freeze drying condition was applied and the observed responses were measured after re-dispersion of dried NLCs in solution of 0.01% PVA. The measured Y_1, Y_2, Y_3 and Y_4 are as follows: 165 nm, −26 mV, pH of 5.6 and osmolality of 310 mOsmol/kg that are in close agreement with the predicted values demonstrating the reliability of this method in predicting a desirable freeze drying condition which is in compatible with standards of a nasal delivery formulation. In order to confirm the particle size data, AFM analysis of the NLCs sample was performed before and after optimized freeze drying condition (Fig. 3). Processing the AFM images of NLCs before freeze drying (Fig. 3A) show length of 152.8 nm, width 82.03 nm and height 128.9 nm which is in accordance with the results of PCS technique. The results of AFM analysis after freeze drying (Fig. 3B) show length of 147.4 nm, width of 78.12 nm and height of 125 nm. Some of the bigger particles are shown in the Fig. 3, which may be related to the cantilever probe of the AFM that pushes and warms the particles and may cause a deformation in the original morphology (Ruozi, Tosi, Forni, Fresta, & Vandelli, 2005). TEM photographs (Fig. 4) of nanoparticles prior and following freeze-drying also showed the similarity of the measured particle size by Malvern device. As can be seen before freeze drying (Fig. 4A) their diameters are less than 150 nm while after freeze drying (Fig. 4B) diameters of the most particles are below 200 nm. These results obtained from PCS, AFM and TEM images are in near accordance. Based on these results, optimized freeze-drying condition maintains the particle size of the NLCs of VPA after re-dispersion. The results of loading percent and 1 week drug release test on the optimized formulation after re-dispersion showed 46% drug loading and 68.5% drug release form nanoparticles (Fig. 5). The results of drug release after one week showed no significant change in the burst effect and drug release percent from NLCs before and after freeze drying (Fig. 5).

DSC thermograms of cetyl palmitate as the lipid base of nanoparticles and also optimized nanoparticles prior and following freeze-drying process (Fig. 6) showed shifting the endothermic peak in the nanoparticles prior to freeze-drying from 96.8 °C to 51.7 °C following freeze-drying which may represent a change in crystalline form of nanoparticles. Considering the enthalpy changes of this endothermic peak in comparison to the lipid base

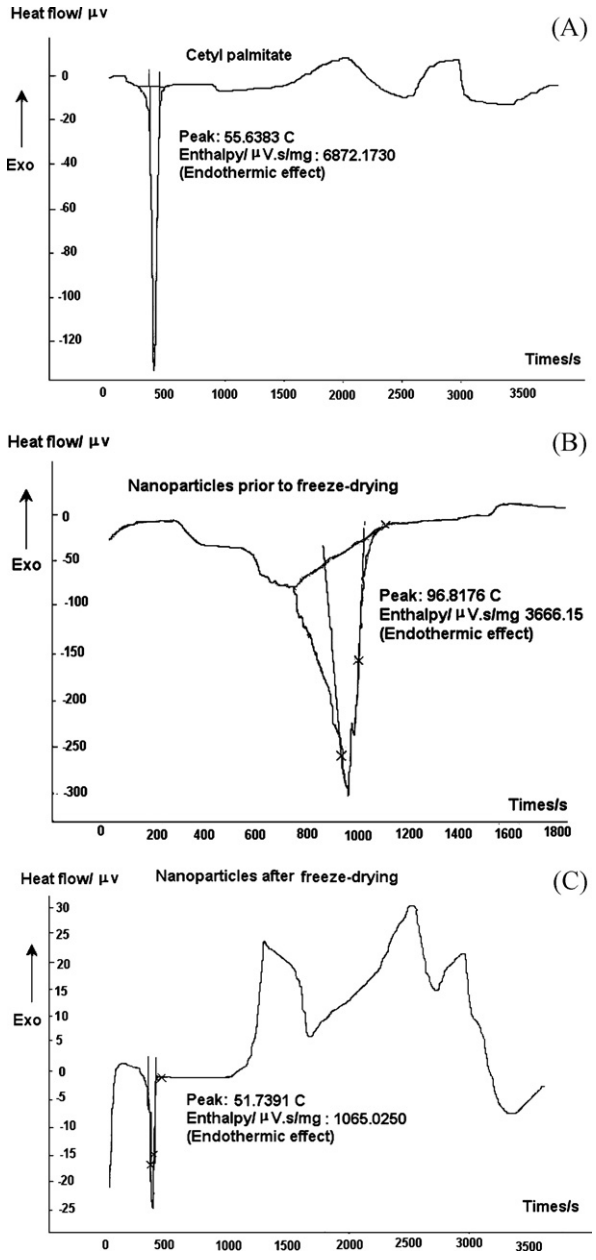

Fig. 6. DSC thermograms of (A) cetyl palmitate (lipid base of NLCs), nanoparticles (B) prior and (C) after freeze-drying in the optimized condition of freeze-drying, i.e., the freezing temperature of −70 °C applied at 24 h, 1% of Avicel RC591 and sublimation time of 48 h.

of nanoparticles may also indicate some changes in crystallinity percent (Han, Li, Yin, Liu, & Xu, 2008):

$$C\% = \frac{\Delta H_{\text{freeze-dried NLC}}}{\Delta H_{\text{bulk}}} \times 100 \quad (2)$$

In this case if the crystallinity of bulk lipid (cetyl palmitate) is considered 100%, then taking the enthalpy of cetyl palmitate as 6872 µV s/mg, nanoparticles prior to freeze-drying as 3666 15 µV s/mg and nanoparticles after freeze drying as 1065 µV s/mg (Fig. 6), the crystallinity percent will change from 53% before freeze-drying to 15.5% after that. In other words incorporation of liquid lipids has reduced the regular lattice structure of cetyl palmitate and even after freeze-drying this order will be decreased to 15.5%.

4. Conclusions

In this study NLCs of VPA are prepared using solvent diffusion method following probe sonication and then freeze dried at conditions which is assessed with different factors. Cryoprotectant type, its concentration, freezing temperature applied at different time periods and sublimation time are evaluated in three levels using Taguchi method with an L_9 orthogonal array. The optimized condition obtained using Avicel RC591 with 1% (w/v) concentration, freezing temperature of −70 °C applied at 24 h and sublimation time of 48 h with desirability of 100%. The results obtained for particle size of 165 nm, zeta potential of −26 mV, pH of 5.6 and osmolality of 310 mOsmol, drug release of 68.5% after 1 week and drug loading of 46% after re-dispersion in PVA 0.01% (w/v) solution reveal that freeze drying of the NLCs has not so difference with the characteristics of NLCs of VPA before freeze drying. Also physicochemical properties of formulation after re-dispersion are compatible for nasal delivery of NLCs. This provides maximum stability and could be beneficial for preserving the shelf-life of the product. As a general conclusion the mixture of polymeric carbohydrates of microcrystalline cellulose and sodium carboxymethyl cellulose (Avicel RC591) is promising in freeze drying the NLCs.

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References

Abdelwahed, W., Degobert, G., Stainmesse, S., & Fessi, H. (2006). Freeze-drying of nanoparticles: Formulation, process and storage considerations. *Advanced Drug Delivery Reviews*, *58*, 1688–1713.

Arora, P., Sharma, S., & Garg, S. (2002). Permeability issues in nasal drug delivery. *Drug Discovery Today*, *7*, 967–975.

Aulton, M. (2007). *Aulton pharmaceutics: the design and manufacture of medicines*. Leicester, UK: Churchill Livingstone.

Bernard, V. E., Sofiem, V., Johan, M., Jan, V., Ludo, F., Jan, V. H., Guy, V. M., & Patrick, A. (2008). Microcrystalline cellulose, a useful alternative for sucrose as a matrix former during freeze-drying of drug nanosuspensions – A case study with itraconazole. *European Journal of Pharmaceutics and Biopharmaceutics*, *70*, 590–596.

Freitas, C., & Muler, R. H. (1998). Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN) dispersions. *International Journal of Pharmaceutics*, *168*, 221–229.

Han, F., Li, S., Yin, R., Liu, H., & Xu, L. (2008). Effect of surfactants on the formation and characterization of a new type of colloidal drug delivery system: Nanostructured lipid carriers. *Colloids and Surfaces A: Physicochemical Engineering Aspects*, *315*(1–3), 210–216.

Hawley, A. E., Illum, L., & Davis, S. S. (1997). Preparation of biodegradable, surface engineered PLGA nanospheres with enhanced lymphatic drainage and lymph node uptake. *Pharmaceutical Research*, *14*, 657–661.

Heurtault, B., Saulnier, P., Pech, B., Proust, J. E., & Benoit, J. P. (2003). Physico-chemical stability of colloidal lipid particles. *Biomaterials*, *24*, 4283–4300.

Heydenreich, A. V., Westmeier, R., Pedersen, N., Poulsen, H. S., & Kristensen, H. G. (2003). Preparation and purification of cationic solid lipid nanospheres-effect on particle size, physical stability and cell toxicity. *International Journal of Pharmaceutics*, *254*, 83–87.

Jadhav, K. R., Gambhire, M. N., Shaikh, I. M., Kadam, V. J., & Pisa, S. S. (2007). Nasal drug delivery system – Factors affecting and applications. *Current Drug Therapy*, *2*, 27–38.

Kesisoglou, F., Panmai, S., & Wu, Y. (2007). Nanosizing – Oral formulation development and biopharmaceutical evaluation. *Advanced Drug Delivery Reviews*, *59*, 631–644.

Kishore, P., Rajani Kumar, V., Satyanarayana, V., & Krishna, D. R. (2003). HPLC determination of valproic acid in human serum. *Pharmazie*, *58*, 378–380.

Konan, Y. N., Gurny, R., & Allémann, E. (2002). Preparation and characterization of sterile and freeze-dried sub-200 nm nanoparticles. *International Journal of Pharmaceutics*, *233*, 239–252.

Lee, J. (2003). Drug nano- and microparticles processed into solid dosage forms: Physical properties. *Journal of Pharmaceutical Sciences*, *92*, 2057–2068.

Lee, M. K., Kim, M. Y., Kim, S., & Lee, J. (2009). Cryoprotectants for freeze drying of drug nano-suspensions: Effect of freezing rate. *Journal of Pharmaceutical Sciences*, *98*, 4808–4817.

Lim, S. J., & Kim, C. K. (2002). Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. *International Journal of Pharmaceutics*, *243*, 135–146.

Mehnert, W., & Mader, K. (2001). Solid lipid nanoparticles production, characterization and applications. *Advanced Drug Delivery Reviews*, *47*, 165–196.

Muhlen, A. Z., Schwarz, C., & Mehnert, W. (1998). Solid lipid nanoparticles (SLN) for controlled drug delivery – Drug release and release mechanism. *European Journal of Pharmaceutics and Biopharmaceutics*, *45*, 149–155.

Ohwaki, T., Ando, H., & Kakimoto, F. (1987). Effects of dose, pH, and osmolarity on nasal absorption of secretin in rats. II: Histological aspects of the nasal mucosa in relation to the absorption variation due to the effects of pH and osmolarity. *Journal of Pharmaceutical Sciences*, *76*, 695–698.

Redhead, H. M., Davis, S. S., & Illum, L. (2001). Drug delivery in poly (lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: In vitro characterization and in vivo evaluation. *Journal of Controlled Release*, *70*, 353–363.

Rowe, R. C., Sheskey, P. J., & Owen, S. C. (2005). *Handbook of pharmaceutical excipients* (5th ed.). London, UK: Pharmaceutical Press.

Ruozi, B., Tosi, G., Forni, F., Fresta, M., & Vandelli, M. A. (2005). Atomic force microscopy and photon correlation spectroscopy: Two techniques for rapid characterization of liposomes. *European Journal of Pharmaceutics*, *25*, 81–89.

Saez, A., Guzman, M., Molpeceres, J., & Aberturas, M. R. (2000). Freeze drying of polycaprolactone and poly-(D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs. *European Journal of Pharmaceutics and Biopharmaceutics*, *50*, 379–387.

Sameti, M., Bohr, G., Ravi Kumar, M. N. V., Kneuer, C., Bakowsky, U., Nacken, M., Schmidt, H., & Lehr, C. M. (2003). Stabilization by freeze-drying of cationically modified silica nanoparticles for gene delivery. *International Journal of Pharmaceutics*, *266*, 51–60.

Schaffazick, S. R., Pohlmann, A. R., Dalla-Costa, T., & Guterres, S. S. (2003). Freeze-drying polymeric colloidal suspensions: Nanocapsules, nanospheres, and nanodispersion. A comparative study. *European Journal of Pharmaceutics and Biopharmaceutics*, *56*, 501–505.

Schwarz, C., & Mehnert, W. (1997). Freeze-drying of drug-free and drugloaded solid lipid nanoparticles (SLN). *International Journal of Pharmaceutics*, *157*, 171–179.

Taguchi, G., & Konishi, S. (1987). *Orthogonal arrays and linear graphs*. Dearborn, MI: American Supplier Institute, Inc.

Tobio, M., Gref, R., Sanchez, A., Langer, R., & Alonso, M. J. (1998). Stealth PLA–PEG nanoparticles as protein carriers for nasal administration. *Pharmaceutical Research*, *15*, 270–275.

Varshosaz, J., Eskandari, S., & Tabbakhian, M. (2009). Production and optimization of valproic acid in nanostructured lipid carriers by the Taguchi design. *Pharmaceutical Technology and Development*, *14*, 2–29.

Varshosaz, J., Eskandari, S., Kennedy R., Tabbakhian, M., Minaiyan, M. Effective factors in production of nanostructure lipid carriers of valproic acid. *Journal of Biomedical Nanotechnology*, in press.

Venkateswarlu, V., & Manjunath, K. (2004). Preparation characterization and *in vitro* release kinetics of clozapine solid lipid nanoparticles. *Journal of Controlled Release*, *95*, 627–638.

Vighi, E., Ruozi, B., Montanari, M., Battini, R., & Leo, E. (2007). Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. *European Journal of Pharmaceutics and Biopharmaceutics*, *67*, 320–328.

Zimmermann, E., Müller, R. H., & Mader, K. (2000). Influence of different parameters on reconstitution of lyophilized SLN. *International Journal of Pharmaceutics*, *196*, 211–213.