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Original Article

LYOPHILIZATION OF SOLID LIPID NANOPARTICLES FOR BRAIN TARGETING

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ABSTRACT

Objective: The aim of this work is to investigate formulation and process parameters that could impact freeze drying of loperamide-loaded Solid Lipid Nanoparticles (Loperamide loaded SLN).

Methods: Loperamide loaded SLN was prepared by high shear homogenization method and freeze dried in vials without and with different types and concentrations of cryoprotectants (lactose, glucose, mannitol and povidon) using pilot freeze dryer.

Results: The best result has been obtained by using 5 % of monosaccharide glucose solution, although Nanoparticles size has been increased 6 folds. However, the total concentration of loperamide hydrochloride (Loperamide HCl) remained unchanged after freeze drying. It has been found that the type and ratio of surfactants adsorbed at the surface of SLN have impact on the success of the drying process.

Conclusion: Results revealed that polymeric nanoparticles could withstand freeze drying stress more than SLN because polymers have higher mechanical resistance in comparison with waxes and lipids.

Keywords: Solid lipid Nanoparticles, Lyophilization, Stability, Brain targeting.

INTRODUCTION

In recent years, it has become evident that the development of new active ingredients is not sufficient to ensure progress in drug therapy. Promising experimental data obtained *in vitro* are very often followed by unsuccessful results *in vivo*. Main reasons for this failure include:

- Insufficient drug concentration due to poor absorption, rapid metabolism and elimination.
- Drug distribution to other tissues combined with high drug toxicity.
- Poor drug solubility, which excludes intravenous injection of aqueous drug solution.
- High fluctuation of plasma levels due to unpredictable bioavailability after per oral administration [1].

During last decades, new drug delivery systems have been developed to overcome the previous problems. These delivery systems offer a possibility to provide targeted delivery of drugs, improved bioavailability, or controlled drug release in target tissue

In the middle of the 1990s, new type of nanoparticles made from solid lipids (SLN or lipospheres) appeared. The SLNs combine the advantages of other innovative carry systems such as; physical stability, protection of incorporated labile drugs from degradation, controlled release, excellent tolerability, avoidance of organic solvents and the possibility of large scale production and sterilization [2].

Solid lipid nanoparticles can be defined as submicron colloidal solid systems made from lipids such as waxes. The main disadvantages of SLN are the lack of physical (agglomeration and fusion of particles) and chemical (Oxidation of lipids) stability during long-term storage. To improve the physical and chemical stability of SLN, water should be removed from the colloidal suspension. Freeze drying is the most common technique applied for making SLN system to allow extended periods of shelf-lives. Lyophilization or freeze drying can transform colloidal suspensions into stable solid cakes for long term storage.

Freeze drying is an industrial process which involves water removal from frozen samples by sublimation under vacuum. However, it is a complex process that generates stress sources itself, which can destabilize the nanoparticles formulation in the process such as freezing and drying stresses. Cryoprotectants have been used to decrease SLN aggregations due to the stress during the process of freeze-drying. For this reasons, both formula and process should be carefully studied to select the suitable excipients and optimal freeze drying conditions [3].

Successful nanoparticles lyophilizate should have:

- Elegant aspect.
- Short reconstitution time in water.
- Physical and chemical stability (ex: particle size, encapsulation efficiency)
- Low residual water [4].

Loperamide hydrochloride is a common-used anti diarrhea drug, an opioid agonist that is unable to cross the blood-brain barrier (BBB). In this work, Loperamide was loaded on nanoparticles to improve its crossing to BBB and to have a strong central analgesic effect [5].

So far, there have been few studies into achieving freeze-dried SLNs using different cryoprotectants. The aim of this research was to study the freeze drying of solid lipid nanoparticles loaded with loperamide and to find the suitable excipients and conditions for successful lyophilization process. To the best of our knowledge, this is the first study of freeze drying of loperamide loaded SLN.

MATERIALS AND METHODS

Materials

Loperamide hydrochloride was supplied by sigma-Aldrich (Italy). Beeswax, carnauba wax, and egg lecithin was all obtained from Carl Roth (Germany). The surfactant Tween 80 was purchased from Sigma-Aldrich (France). Triton x was supplied by Rasayan Laboratories and finally, HPLC grade acetonitrile was provided from Scharlau (spain).

Preparation of solid lipid nanoparticles

Loperamide loaded SLN was prepared by High shear homogenization method. Briefly, a mixture of beeswax (0.48 g), carnauba wax (0.12 g), egg lecithin (0.12 g) and Loperamide hydrochloride (50 mg) was melted in a water bath at 65ºC. Then 0.18 mg of tween 80 was mixed

with 20 mL of de ionized water at 80 ℃ under magnetic stirring (1000 rpm) for the few minutes and added to the molten lipid-drug mixture. The resulting emulsion was homogenized at 24000 rpm during 5 min using a rotor-stator (ultra-Turrax, IKA T18 B, Germany), and then dispersed in cooled water under stirring.

Particle size and zeta potential analysis

The mean size of nanoparticles and poly dispersity index (PdI) was determined by photon correlation spectroscopy (PCS), using a Malvern Zetasizer Nanoseries (Zetasizer Nano ZS, Malvern instruments, England). Zeta (ζ) potential measurements were made with the same instrument by Smoluchowski's equation from electrophoretic mobility of nanoparticles. All measurements were performed at 25 °C.

Determination of encapsulation efficiency

Loperamide association efficiency (AE) was indirectly determined to quantify the amount of active ingredient effectively entrapped into the produced SLN. Total loperamide concentration (TL) was determined after dissolution of 1 mL of the nano suspension in 3 mL of triton x (5%) then sonicated for 25 min. after that strong magnetic stirring was applied for about 25 min. The final volume was adjusted to 10 mL with the mobile phase used for high performance liquid chromatography (HPLC) analysis method.

Free loperamide concentration (FL) was determined after separation of loaded-NPs from the aqueous medium by ultracentrifugation (CP 80 WX Himac preparative ultracentrifuge, Hitachi, Japan). 1 mL of samples was centrifuged at 40000 rpm for 30 min at 25 °C The free loperamide concentration was then determined in the supernatant by a HPLC-UV method which was applied exactly as reported in USP 34 NF29 2011 pharmacopeia.

HPLC separation was performed with Agilent Liquid Chromatographer (1260 Infinity, Agilent, Germany). Quantitative measurement of loperamide content was done at 214 nm. Loperamide was separated on a silica gel column (EC 150/4.6 Nucleodur 100-3 C8, Macherey-Nagel, Germany) with a mobile phase of buffer solution: acetonitrile (63:37) (v/v) at 1.5 ml/min. The samples were run in triplicate and all measurements were performed at 25 °C. The encapsulation efficiency was calculated as follows:

$$\text{Encapsulation efficiency (\%)} = (TL–FL/TL) \times 100$$

Thermal analysis

To measure the glass transition temperature of maximally cryo concentrated suspension (Tg'), a thermal analysis was performed by a differential scanning calorimeter DSC TA 125 (TA instrument, USA). A certain amount of samples heated from (-100 to 30 °C), and a heating rate of 10 °C/min was applied throughout the analysis. The instrument was calibrated with indium for melting point and heat of fusion.

Freeze–Thaw study of nanoparticles

In order to evaluate the resistance of nanoparticles during freezing, this is the first step of lyophilization. 0.5 mL of nanoparticles suspension was filled into a 7 mL freeze drying vials after mixing with 0.5 mL of cryoprotectant solution. Samples freezing was performed on a shelf of pilot-scale freeze dryer (EPSILON 2-6D Martin Christ, Germany). Freezing was hold for two hours at–45 °C using cooling rate of 1 °C/min. The frozen preparations were kept at room temperature for thawing. The particle size was determined before freezing and after thawing, and the final to initial size ratio (Sf/Si) was also calculated.

Freeze drying of nanoparticles

SLN were freeze-dried using lactose, glucose, mannitol and povidon as cryoprotectants. Thus, 0.5 mL of cryoprotectant solutions was added to 0.5 mL of nanoparticles suspension and homogenized. Samples were poured into 7 mL freeze drying vials.

The lyophilization of nanoparticles was realized on a pilot freeze dryer (EPSILON 2-6D Martin Christ, Germany). The applied conditions during our study were as following: freezing for 2 h at–45 °C with a temperature ramp of 1 °C/min, sublimation at-30° C and 10 pascal for 12 h and finally, secondary drying was carried out at 20 °C and 5 pascal for 6 h.

RESULTS AND DISCUSSION

In this work, solid lipid nanoparticles were prepared by High shear homogenization method. The optimized nanoparticles formulation was produced with a mean diameter in the colloidal range (about 80 nm) less than 200 nm, which is suitable for brain targeting.

From table 1, it can be observed that loperamide loaded SLN size and poly dispersity index do not change significantly in Comparison with unloaded nanoparticles. However, the value of zeta potential values was highly changed from about-32 mV to about+17 mV. This result could be explained by the adsorption of Loperamide on the particles surface, which indicates the high compatibility between the drug substance and the lipids forming the lipospheres.

Table 1: Characterization of loperamide loaded and unloaded solid lipid nanoparticles

Nanoparticles	Size (nm)±SD	PDI±SD	Zeta potential(mV)±SD	Encapsulation efficiency (%)±SD
Without loperamide	90±7	0.43±0.07	-32±0.8	-
loperamide loaded	79.8±0.2	0.43±0.01	+17.7±0.8	54±0.6

N = 3, SD: standard deviation between the three assays

Loperamide hydrochloride is a salt of strong acid and the acid group ionizes in water and produces protons and chloride negative ions which results in the protonation of the nitrogen atom in the Pepperdine cycle. This positive proton is responsible of the appearance of the positive charge on the nanoparticles surface. Consequently, zeta potential values have changed to the positive value (fig. 1).

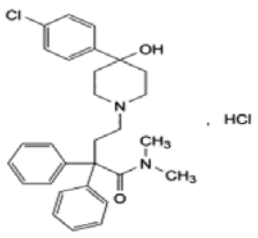

Fig. 1: Chemical structure of loperamide hydrochloride

Freeze drying of nanoparticles needs experience and knowledge of both formulation and freeze drying conditions in order to keep nanoaprticles properties and protect them from degradation.

Freezing is considered the most aggressive and critical step during lyophilization. This step can cause aggregation or destruction of nanoparticles. Normally, freeze drying study starts with freeze–thaw experiments to evaluate the effect of freezing conditions and protecting excipients on nanoparticles properties. In this study, four different types of protecting excipients have been investigated which are: Mannitol (polyol), lactose (disaccharide), glucose (monosaccharide) and povidone (polymer) [6]. Freezing of SLN was conducted in the presence of 5% solution of cryoprotectant at–45 °C and cooling rate 1° C/min. Table 2 shows the results of this study.

The used cryoprotectant can be considered more effective in the protection of nanoparticles during freezing when the properties of particles before and after freezing are unchanged (Particles size, poly dispersity index, zeta potential). Furthermore, the calculation of

Sf/Si ratio can help in the evaluation of excipients as a value close to 1 indicates good preservation of nanoparticles.

Table 2: Effect of Loperamide loaded SLN freezing by using of 5% of cryoprotectant solution

	Before freezing (average±SD)			After freezing (average±SD)			
Cryoprotectant	**Mean size (nm)**	**PdI**	**Z. P(mV)**	**Mean size (nm)**	**PdI**	**Z. P.(mV)**	**Sf/Si**
Without excepients	79.8±0.2	0.43±0.01	+17.7±0.8	visible particles formed			-
Manitol	Nanoparticles properties have not changed after mixing with			1490.5±298	0.64±0.06	+12.05±0.45	18.6
Lactose	cryoprotectant solution			3097±442	o.18±0.11	+13.5±0.8	38.8
Glucose				1514.5±145	0.99±0.01	+12.95±0.15	18.9
Povidone				1693±156	0.68±0.03	+8.2±0.18	21.2

N = 3, SD: standard deviation between the three assays

After freezing, zeta potential value was slightly reduced which indicates a good association of loperamide with the surface of nanoparticles and shows that freezing dissociates only small part of loaded loperamide (table 2).

However, particle size and poly dispersity index were highly increased when using all excipients which indicates the aggregation of nanoparticles under the stress of ice crystals. For this reason, the concentration of cryo protectants was increased to 10 %.

Table 3 shows that the size and poly dispersity index of SLN clearly increased after freezing in the presence of 10 % of cryoprotectant. By comparing these results with these in table 2, it can be noticed that the results were better in the presence of 5 % of cryoprotectant solution. Furthermore, zeta potential reduced after freezing more significantly than its reduction when using 5 % of excipient solution. This result confirms a dissociation of larger part of loperamide from the particle's surface after freezing. In general, glucose and mannitol gave the best results.

Table 3: Effect of Loperamide loaded SLN freezing by using of 10% of cryoprotectant solution

	Before freezing (average±SD)			After freezing (average±SD)			
Cryoprotectant	**Mean size (nm)**	**PdI**	**Z. P(mV)**	**Mean size (nm)**	**PdI**	**Z. P.(mV)**	**Sf/Si**
Without excepients	93.38±5	0.514±0.1	+9.83±0.1	visible particles formed			-
Manitol				4571±203	0.638±0.03	+0.89±0.03	48.9
Lactose	Nanoparticles properties have not changed after mixing with			2439.5±204	0.673±0.05	+3.5±0.1	26.1
Glucose	cryoprotectant solution			7524.5±135	0.921±0.27	+9.98±0.45	80.5
Povidone				5999±722	0.638±0.22	-6.19±0.3	64.2

N = 3, SD: standard deviation between the three assays

Freeze drying may generate many stresses that could destabilize colloidal suspension of nanoparticles, especially, the stress of freezing and dehydration. It is well known that during freezing of a sample there is a phase separation into ice and cryo-concentrated solution. In the case of suspension of nanoparticles this cryo-concentrated phase is composed of nanoparticles and the other components of the formulation [3].

This high concentration of particulate system may induce the aggregation and in some cases irreversible fusion of nanoparticles. Furthermore, the crystallization of ice may exercise a mechanical stress on nanoparticles leading to their destabilization. For these reasons, special excipients must be added into nanoparticles suspension before freezing to protect these fragile systems.

Sugars are the most cryoprotectants used in freeze drying of nanoparticles especially, trehalose, sucrose and glucose. These sugars are known to vitrify at a specific temperature denoted Tg' (glass transition temperature of maximally cryo-concentrated solutions) [7]. The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals.

Generally, freezing must be carried out below Tg' of a frozen amorphous sample or below Teu (crystallization temperature of soluble component as a mixture with ice) if it is in the crystalline state to ensure the total solidification of the sample [7].

The level of stabilization afforded by sugars generally depends on their concentrations. It has been proved that trehalose is more effective for stabilizing both comprotol solid lipid nanoparticles and glycerol trilaurate SLN during freeze drying at concentration 15 %, whereas 2 % of trehalose was not sufficient to protect the nanoparticles [8]

On the other hand, in some cases, increasing the cryoprotectant concentration to a certain level may eventually reach a limit of stabilization or even destabilize nanoparticles. For example, particle aggregation increased with higher glucose concentration during freeze drying of cationically modified silica nanoparticles [9].

This finding may explain the better result obtained in this work with the concentration 5 % in comparison with the concentration 10 % of cryoprotectant during freezing.

In our previous work, using monosaccharides at concentration 5 % was sufficient to protect polymeric (poly (D, L-lactide-co-glycolide) and poly caprolactone) nanoparticles during freezing. This result indicates that polymeric nanoparticles are more resistant to freezing stresses than solid lipid nanoparticles as polymers have higher mechanical resistance and are more solid than waxes and lipids [6].

SLN were freeze dried according to the conditions presented in table 4 by using 5 % of lyoprotectant.

It could be observed that during freezing, the sample temperature was slightly higher than the shelf temperature because of the low thermal conductivity of vial glass, whereas during primary drying the sample temperature was lower than the shelf temperature because of the cooling resulted from ice sublimation.

Table 4: The conditions of nanoparticles freeze drying

	Shelf temperature (C °)	Sample temperature(C °)	Cooling rate (C °/min)	Condenser temperature(C °)	Time (hour)	Pressure (pascal)
Freezing step	-45	-42	1	-	2	-
Primary drying	-30	-32	-	-80	12	10
Secondary drying	+20	+18	-	-80	6	5

Fig. 2 presents the resulted lyophilizates of solid lipid nanoparticles. Collapse of lyophilizate could be clearly observed when using glucose as lyoprotectant, whereas partial collapse was obtained by using lactose. Samples protected with mannitol and PVP have a good aspect and occupy a volume equals to the original volume of solution, which affirm the absence of collapse.

Fig. 2: Image shows formulated lyophilizates appearance using (from right to left): glucose, lactose and mannitol

Collapse of lyophilizate has negative effects on the freeze dried samples: collapsed samples have long reconstitution time and high percentage of residual moisture, in addition to the unacceptable aspect [10].

It can be seen from table 4 that glucose and lactose lyophilizates have relatively long reconstitution time comparing with mannitol lyophilizate which has shorter reconstitution time.

Table 4: Reconstitution time of nanoparticles lyophilizate

Nanoparticles lyophilizate	Reconstitution time (sec)±SD
SLNs with lactose	90±7
SLNs with glucose	144±15
SLNs with mannitol	66±4

N = 3, SD: standard deviation between the three assays

The collapse of glucose samples could be explained by the heating of the sample during primary drying into a temperature, that is higher than the collapse temperature of glucose lyophilizates, which is about–42 °C (table 7), while the sample temperature was about-32 C. Lactose sample temperature was below the collapse temperature of lactose; however, the approach of sample temperature from the collapse temperature may enhance the undesired partial collapse.

The preservation of mannitol and PVP lyophilizates was due to the crystallization of mannitol and the relative high collapse temperature of PVP (about-21 C).

Table 5: Collapse temperature of some excipients used for nanoparticles protection during of freeze drying

Excipient	Collapse temperature (glass transition temperature)
Manitol	Crystallized
Lactose	-30.5
Glucose	-42
Povidone	-21

Freeze drying results indicate a clear increase in the nanoparticles size after drying which affirms particles aggregation. These results comply with the freezing results because glucose gave the best results (Sf/Si ratio was 6.6). In the case of using lactose as a lyoprotectant the ratio Sf/Si was about 27 (high particles aggregation). In addition, zeta potential slightly reduced in the case of glucose and mannitol and this reduction was more evident in the case of lactose.

These results indicate the efficacy of monosaccharide lyoprotectants for the preservation of polymeric and lipid nanoparticles during freeze drying as glucose presented excellent results in the case of freeze drying of polymeric nanoparticles prepared from polycaprolactone and poly lactide co glycolide [6].

Table 6: Study of loperamide loaded SLNs lyophilization with different excipients

	Before lyophilization			After lyophilization			
Cryoprotectant	**Mean size (nm)**	**PdI**	**Z. P(mV)**	**Mean size (nm)**	**PdI**	**Z. P.(mV)**	**Sf/Si**
Without excepients	110.5	0.485	+14.1	visible particles formed			-
Manitol	Nanoparticles properties have not changed after mixing with			1653±120	0.639±0.18	+5.57±0.37	14.9
Lactose	cryoprotectant solution			3006±108	0.906±0.16	-1.67±0.76	27.2
Glucose				739.6±60	0.774±0.06	+6.41±0.29	6.6

N = 3, SD: standard deviation between the three assays

Table 7: Total concentration of Loperamide hydrochloride loaded SLNs suspension before and after lyophilization

	Total concentration of Loperamide hydrochloride before lyophilization (µl/ml)±SD	Total concentration of Loperamide hydrochloride after lyophilization (µl/ml)±SD	Cf/Ci %
Nanoparticles with lactose	2154.3±112	1842.1±23	0.85
Nanoparticles with mannitol		2085.8±45	0.96
Nanoparticles with glucose		2054.6±78	0.95

N = 3, SD: standard deviation between the three assays

It can be observed that the polymeric nano particles freeze drying results are better than these of solid lipid nanoparticles. Perhaps this result may be attributed to the more solid structure of polymeric nanoparticles.

Furthermore, many researches [11] confirmed that the composition of surfactants on the solid lipid nanoparticles surface has the crucial effect on the success of the lyophilization process. Best results have been obtained using weight percent 54:46 of eggs lecithin to Tween whereas the percent 40:60 was used in our work.

The same research affirmed that the SLN size clearly increased after freeze drying when using more than 46 % of Tween 80 and this result complies with our results.

About 54% encapsulation efficiency was achieved with loperamide hydrochloride loaded SLN, which indicates a good affinity of the active ingredient with waxes and lipid forming the particles.

Table 7 shows the results of loperamide HCl assay before and after freeze drying. A slight reducing in the total concentration of the active ingredient after freeze drying has been obtained (about 15 %) in the case of using lactose, whereas the results were perfect with the other excipients without any reduction of active concentration. These results comply with the zeta potential measurement results which indicate the desorption of lopera mide from the nanoparticles surface stabilized by lactose after freezing. This desorption may be the cause of its partial degradation. Also, this result complies with the result of polymeric nanoparticles freeze drying prepared from poly lactide co glycolide [6]. According to Abdel wahed the total concentration of active ingredient was reduced by 15 % when using lactose as lyoprotectant.

From these results, it can be concluded that the disaccharides are less effective as lyoprotecatnt in comparison with monosaccharides or polyols for both polymeric and lipid nanoaprticles.

CONCLUSION

The type and concentration of protective excipient has important role in the success of freeze drying process and in the conservation of solid lipid nanoparticles. Best results were obtained by using 5 % of glucose. Using glucose at higher concentration resulted in the degradation and instability of particles. However, the freeze-drying conditions should be precisely controlled to prevent the collapse of lyophilizate as glucose has low collapse temperature (about-40 C). Furthermore, freeze-drying of solid lipid nanoparticles was less successful in comparison with polymeric nanoparticles because of their lower mechanical resistance. Freeze drying of SLN led to an increase of particle size about six folds when glucose was used as lyoprotectant. In addition, the adsorbed stabilizers on the particles surface play an important role in the success of freeze-drying that they must be added at certain percent. Finally, the lyoprotecatnt may keep the active ingredient attached to the surface of particles and protect it from degradation. Mono saccharides afforded better results than disaccharides.

CONFLICT OF INTERESTS

Declared None

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