

Original Article



Fingolimod Slns: Preparation, In Vitro Evaluation And Optimization Of Lyophilization Using D-Optimal Experimental Design

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Abstract

Multiple Sclerosis (MS) is one of the most common neurological disorders diagnosed in young adults. There are no current cure for the disease or its underlying causes. Some drugs have been developed that can decrease or delay disease progression. Fingolimod is an immunomodulating drug, mostly used for treating multiple sclerosis (MS). It approximately halves the rate of relapse in relapsing-remitting multiple sclerosis over a two-year period; however, Fingolimod causes a number of off-target effects including cardiovascular complaints. A different delivery method may alleviate some of these effects. Lipid-based nanoparticles containing Fingolimod were prepared using cholesterol as a biocompatible lipid through a high-pressure homogenization technique. The physical and chemical properties of the resulting particles, including particle size, zeta potential, morphology, drug-loading capacity and release profile were investigated. A preferred formula was lyophilized using mannitol as the cryoprotectant. A D-optimal model was used to determine optimum freeze-drying conditions that minimized size enlargement and maximized the zeta potential of the processed particles. The optimized Fingolimod SLNs are 150 nm in diameter with a Zeta potential of 19 mV. AFM imaging confirms that the particles remain spherical. Fingolimod loading efficiency was found to be 89%, and 85% of the loaded drug was released over 16 days. Results demonstrated that nanotechnology could help to prolong the drug efficacy duration as well could increase compliance of the patient to continue MS control, whole life duration. Sustained release Fingolimod SLNs using cholesterol as the matrix is effective and may provide an alternative to oral drug administration to prolong drug administering intervals and increase the chance of treatment following by patients.

Keywords: Fingolimod; Freeze-drying; MS; Solid lipid nanoparticles; Sustained release.

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Introduction

Multiple Sclerosis (MS) is one of the most common neurological disorders diagnosed in young adults (Ziemssen, 2016). MS damages and destroys the myelin protective insulation which surrounds the nerves. This leads to nerve damage which causes the loss of body function symptomatic of the disease. Although there are no current cures for the disease or its underlying causes, some drugs have been developed that can decrease or delay disease progression (Martin, 2016, Murray, 2006). One of these next-generation orally active immunomodulatory drugs is Fingolimod (Novartis) which was approved by the Food and Drug Administration (FDA) in 2010. Fingolimod (FTY720) interacts with the lymphocyte membrane sphingosine 1-phosphate receptor, causing it to internalize into the cell, and thereby inhibits lymphocyte release from the lymph nodes. (Brinkmann, 2010). Fingolimod is administered orally daily and continuously and patient compliance with this routine might decrease over time. Ideally, a slow release formulation would be beneficial as would decrease the number of doses and the inconvenience of the drug. Furthermore, an alternative formulation could also decrease the severity of the observed side effects while increasing the bioavailability of the drug. The design of an effective oral sustained drug delivery system (DDS) requires a predictable, reproducible, controlled the drug release profile to ensure a sufficient constant drug concentration in the target tissue. (Kumar, 2012). Nanostructures have repeatedly been shown to act as useful scaffolds for sustained drug release (Ochekpe, 2009). The functionality and performance of drug delivery systems are continuously being enhanced and extended to both maximize therapeutic activity and minimize undesirable side-effects (Kamaly, 2016; Bozzuto, 2015; Safari, 2014). Nanoparticles can offer significant advantages over traditional delivery mechanisms in terms of higher stability, higher specificity, higher drug carrying capacity, the ability for

controlled release, possibility to use in different dosage forms and the capability to transport both hydrophilic and hydrophobic molecules simultaneously (Jain, 2010).

Over recent few decades, drug delivery systems based on Solid Lipid Nanoparticles have made tremendous progress (Mandawgade, 2008), and have become a favored iteration among the colloidal drug delivery system options (Garud, 2012). These solid-lipid nanoparticles, biodegradable nanocarrier-based drug delivery system, are normally aqueous colloidal dispersions of nanoparticles (diameter 40–1000 nm) comprising a solid-lipid core stabilized by a surfactant corona. The surfactant is generally introduced during the lipid-matrix solidification process. These SLNs have several advantages over conventional dosage forms in terms of tunable controlled-release kinetics, capability for target or site-specific delivery and enhanced stability (Kumar and Randhawa, 2013). A number of different lipids, meeting the requirement that they are solid and stable at physiological temperatures, have been used as SLN cores, including glyceride mixtures, triglycerides, and waxes (highly purified). They are particularly useful for the controlled release of water-sensitive drugs, as the amorphous form of the SLNs creates irregular hollow cavities within the particles. Co-solidification with the hydrophobic drug of choice encapsulates them in these highly hydrophobic environments.

Incorporation of SLNs into an oral sustained release drug delivery system is an innovative system with several advantages over traditional ones but avoid some of their major drawbacks (Muller, 2000; Hu, 2004). A sustained release orally introduced SLN-Fingolimod is a particularly useful potential formulation. As mentioned above, compliance is a challenge with this particular drug, and SLNs will reduce the number of doses required by increasing the dose intervals. Finally, physical encapsulation of drugs has been shown to remarkably improve the pharmacokinetics and therapeutic index of the drugs compared to introducing the drug as a standard pill (Varshosaz, 2012).

To address these challenges, Fingolimod was loaded into SLNs, and the constructs were evaluated for their drug-release kinetics. We have found that these parameters are highly sensitive to the freeze-drying conditions used to prepare the nanoparticles, and we have sought to optimize the freeze-drying conditions using a D-optimal study design. We wish to report our progress towards this objective.

Materials and method

Materials

Cholesterol, Tween 80, mannitol, ethanol, and acetone were purchased from Merck, Germany and used without further purification. Fingolimod was kindly donated by Tofigh Pharmaceutical Co, Iran.

Methods

Preparation of SLNs: 300 mg of cholesterol was added to 4 mL of acetone and 12 mL of ethanol, and the mixture was heated on a hot-plate/stirrer (IKA, T 25, Germany) at 70°C until the cholesterol fully melted and dissolved into a homogenous mixture. Fingolimod (150 mg) was then dispersed 60 ml of purified water containing 780 mg of tween 80 at ambient temperature. The hot organic phase was then added to the water phase under and homogenized (IKA, C-MAG HS, Germany) at 21000 rpm for 15 minutes. SLNs formed as the temperature decreased to room temperature. This mixture was then sonicated at 25°C for 2 minutes using a standard sonicator bath system (James, Sonic 6D, China).

Characterization of Fingolimod-SLNs

Particle size evaluation

Particle sizing and zeta potential measurements were carried out using a Dynamic Light Scattering (ZEN3600; Malvern Instruments, Malvern, Britain).

Drug loading Efficiency

The SLN dispersion was centrifuged at 30,000 rpm for 45 minutes at -4°C using a Sigma, 3K30 laboratories centrifuge (Germany). The drug concentration in the supernatant was determined using ultraviolet spectroscopy at 220 nm (Shimadzu, Japan) and the loading efficiency percent (LE%) was calculated using the reverse method equation 1, (Ghaffari, 2012).

Equation 1:

$$\text{Drug-loading efficiency (LE\%)} = \frac{\text{Drug}_{\text{total}} - \text{Drug}_{\text{supernatant}}}{\text{Drug}_{\text{total}}} * 100$$

Release study

The dialysis sink method was used to characterize the drug release profile. First, 3 ml of prepared SLN dispersion was placed in a dialyzing membrane by DO405 dialysis membrane (Sigma, Germany). The drug release profile was studied in two release media: dialysis membranes were placed in either 25 ml of water or 25 ml buffer (PH 7.4). At the desired sampling time points, 2 ml aliquots were obtained from release medium to investigate by spectrophotometer.

Scanning Electron Microscopy

The nanoparticles were mounted on aluminum stubs, sputter-coated with a thin layer of Au/Pd, and examined by using an SEM (Philips XL30, Almelo, Netherlands) instrument.

Freeze-drying of SLNs

The SLNs were freeze-dried using a machine. The amount of mannitol (cryoprotectant), freeze-drying time, and freeze-drying temperature were modified to determine the optimal conditions for retaining the desired properties as measured by comparing the release profile and differential scanning calorimetry thermographs of both the freeze-dried and untreated SLNs (Varshosaz,2010; Schwarz,1997).

A D-optimal methodology was used to determine the optimal values for the parameters using the Design-Expert (version 7.0.0, Stat-Ease,

Inc., Minneapolis, MN, USA): cryoprotectant concentration (X1), Freezing temperature (X2), Freezing time (X3), particle size (Y1) and dispersity (Y2) and zeta potential (Y3). Table 1 and 2 illustrate the level of the studied factors. Table 3 shows the total of 19 runs whose order was fully randomized in order to choose the best model for optimization of freeze-drying

conditions.

The statistical software package, Design-Expert, was used to design matrix to study the effect of cryoprotectant concentration (X1), Freezing temperature (X2), Freezing time (X3) on the particle size (Y1) and Poly disparity index (Y2) and Zeta potential (Y3).

Table 1: Independent Variables used in D-optimal experimental design in freeze-drying of SLNs of Fingolimod

Independent variables	Units	Symbol	Levels		
Percentage of cryoprotectant	mass %	X1	5	12.5	20
Freezing Temperature	C°	X2	-80	-20	-
Freezing Time	Hours	X3	18	28	48

Table 2: Dependent Variables used in D-optimal experimental design in the freeze-drying of SLNs of Fingolimod

Dependent variables	Units	Symbol	
Particle size	Nm	Y1	Minimize
Poly disparity index	-	Y2	Minimize
Zeta potential	mV	Y3	Maximize

Results and Discussion

Particle Size and Zeta Potential

The size and zeta potential of the particles were 175 and 150 nm for size and +24.7 and +19mV for zeta potential before and after lyophilization, respectively.

Drug Loading Efficiency

Our standard formulation provides an 89.79% Fingolimod loading efficiency as calculated using equation 1. UV spectroscopy method with Shimadzu spectrophotometer (Cary 100 Conc) was used to detect Fingolimod at 220 nm wavelength.

Drug Release

Figure 2 shows the Fingolimod release profile of the nanoparticles. As can be seen, Fingolimod release from SLNs was observed and no significant burst effect was seen, although before 154 hours the release rate of the drugs from freeze-dried SLNs was rapid. No significant changes were detected in release profile of both nanoparticles before and after freeze-drying, on the whole, freeze-dried SLNs show slower release rate in comparison with other SLNs. Fingolimod's started to release within the first

Table 3: D-optimal experimental design input parameters of various runs and the corresponding response in freeze-drying of SLNs of Fingolimod

Independent Variables				Dependent Variables		
No.	Time(h)	Mannitol (%)	Temp.(°C)	DLS	Pdi	Zeta
	X3	X1	X2	Y1	Y2	Y3
1	48	20	-20	250	-0.19	17.9
2	48	5	-20	25.4	0.064	8.2
3	48	20	-20	21.2	0.064	8.2
4	48	5	-20	28.4	0.168	7.04
5	48	12.5	-80	17.4	0.065	3.3
6	48	5	-80	24	0.065	-0.8
7	48	20	-80	18.4	0.065	1.5
8	48	5	-80	50.1	-0.035	-31.01
9	28	12.5	-20	50.9	0.013	-0.4
10	28	5	-20	335	-0.233	33.5
11	28	20	-80	26.7	0.041	38
12	18	12.5	-70	210	0.041	21.8
13	8	5	-20	82.4	-0.139	-5.7
14	8	20	-20	20.1	-0.042	-14.7
15	8	12.5	-20	64.9	-0.164	16
16	8	20	-20	15.5	0.241	45.1
17	8	5	-70	15	-0.239	-5.1
18	8	20	-70	61.2	0.127	24.9
19	8	5	-70	72.9	0.085	34

30 minutes. The significantly sustained release was seen between the hours 3 to 72; therefore, it can be concluded that freeze drying method can be satisfactory for storage of these nanoparticles during their shelf lives.

Freeze drying

SLNs have seen limited use in the clinic due to

their physical and chemical instability. Lyophilization was used to prolong the stability of Fingolimod loaded SLNs. In this method, water is to be withdrawn to achieve a better physical and chemical stability. Cryoprotectants like mannitol were used to limit the risk of aggregation of particles and inhibit the size enlargement.

The evaluation of optimum condition for ly-

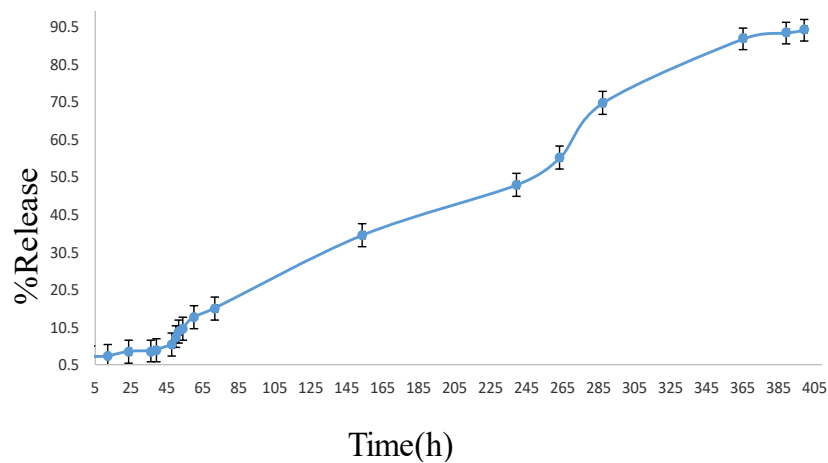


Figure 1: Release profile of Fingolimod from SLNs

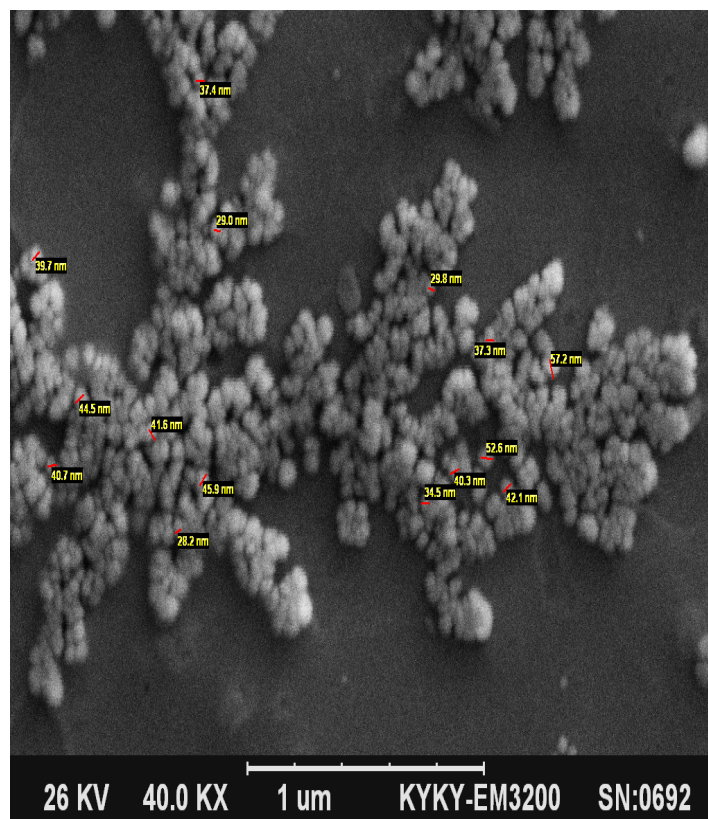


Figure2: SEM photograph of lyophilized SLNs of Fingolimod.(Scanning Electron Microscopy)

ophilization for the type and percentage of cryoprotectant and freezing temperature was done and the best results achieved with 5% w/w of Mannitol as cryoprotectant at -80°C during 42 hours. In this condition, as the results are shown, the increase in particle size

was minimum, the release profile changes were minimum, and AFM pictures showed spherical particles similar prior to freeze-drying and this figure confirms that freeze-drying doesn't have any significant impact on the shape and size of SLNs.

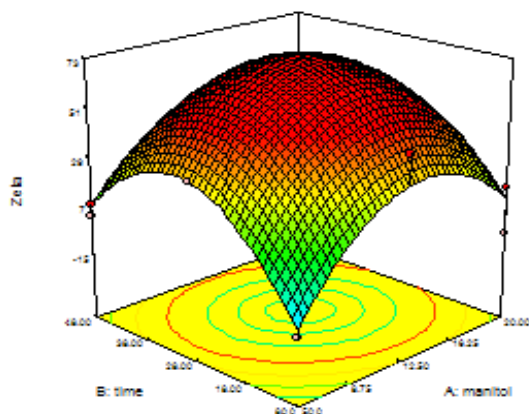


Figure 3: Response surface plot indicating the effect of Mannitol (%) and time on Zeta potential of Fingolimod-loaded nanoparticles.

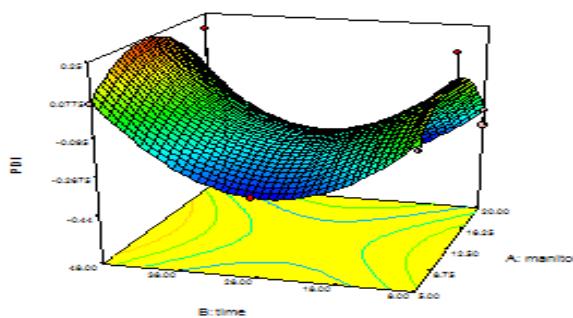


Figure 4: Response surface plot indicating the effect of Mannitol (%) and time on disparity (Pdi) of Fingolimod-loaded nanoparticles.

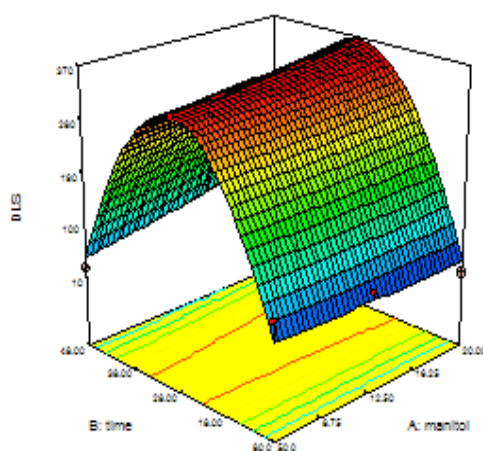


Figure 5: Response surface plot indicating the effect of Mannitol (%) and time on the size of Fingolimod-loaded nanoparticles.

DSC Analysis

DSC graphs of cholesterol, Fingolimod alone, Mannitol and prepared Fingolimod loaded SLNs are shown in Figure 4. The shift of SLNs Fingolimod melting point from 125°C to 160°C before and after freeze-drying respectively in the DSC thermogram, indicate the drug molecular dispersion within cholesterol crystals and the probability of hydrogen bond between cholesterol and Fingolimod, which are the reasons for sustained release in these nanoparticles.

Conclusion

The aim of this study was to prepare and evaluate of the Fingolimod SLNs with cholesterol carrier before and after freeze-drying, to use the freeze-drying method in order to reach more stability in nanoparticles and to both achieve the optimized formulation of freeze-dried particles and evaluate their physical and chemical characteristics.

The most important parameters, among different

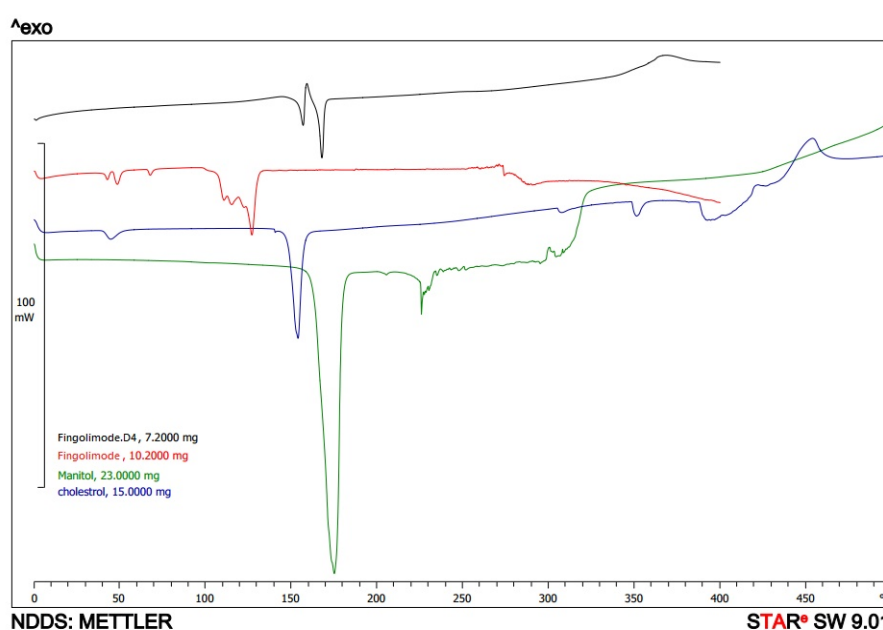


Figure 6. DSC thermogram of raw materials and freeze-dried SLNs of Fingolimod AFM

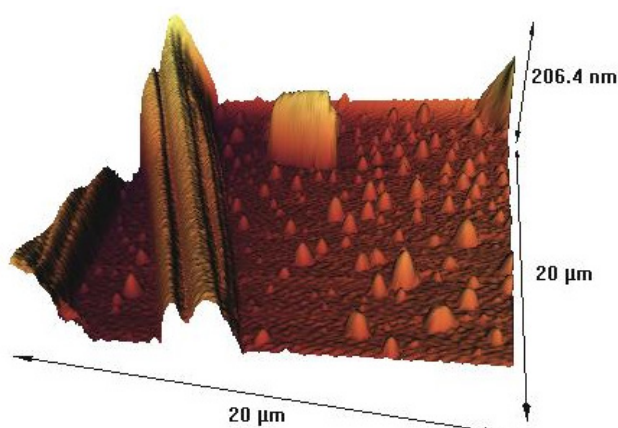


Figure 7. AFM photograph of lyophilized SLNs of Fingolimod

studied variables, on the particle size of SLNs of Fingolimod, were the amount of cryoprotectant, freezing temperature and time. The optimum level of these effective factors was achieved by the use of D-optimal design. The optimum values of independent variables to conclude the lowest particle growth after lyophilization were found to be the freezing temperature of -80°C , 5% Mannitol as a suitable amount of cryoprotectant and 42 hours. Particle size, release profile, DSC analysis and AFM of the small lyophilized SLNs were studied after lyophilization of SLNs of Fingolimod in optimum condition and the results showed decreasing on particle size, long release profile during 16 days. Probable interaction between Fingolimod and cholesterol used as cryoprotectant were shown by DSC analysis. The AFM photographs showed spherical shape of the optimized freeze-dried particles. As the final conclusion, it seems nanotechnology could be a choice in the development of sustained release Fingolimod dosage forms to increase patient compliance with increasing drug taking intervals and minimizing drug side effect due to slower release.

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