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Research Article

Preparation and characterization of Nelfinavir Mesylate nanocrystals by ball milling

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ABSTRACT

The objective of the present study was to formulate Nelfinavir mesylate nanocrystals by ball milling to improve its pharmacokinetic behavior in turn bioavailability. Nanocrystals were prepared by dispersing the drug in Poloxamer 407 solution and subjected for size reduction by ball milling. The nanocrystals were lyophilized using mannitol ascryoprotectant. The free flowing powder forms of nanocrystals powder was evaluated for its particle size, zeta potential, solubility, in vitro dissolution. Solid-state characteristics were studied by Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC) and Fourier Transform Infra-Red Spectroscopy. The particle size and zeta potential of optimized formulation was found to be 740.12 ± 79.21 nm and 23.31 ± 1.10 mV respectively with PDI of 0.20 ± 0.07. DSC analysis and SEM revealed that there was slight decrease in crystallinity in the nanocrystals compared to raw crystals. Increased dissolution velocity was observed along with increased saturation solubility. Thus, it can be concluded that increase in solubility and dissolution velocity of Nelfinavirmesylate was observed by formulating it as nanocrystals.

Keywords: Nelfinavirmesylate; Nanocrystals; Ball milling; Poloxamer 407; Dissolution Velocity

INTRODUCTION

A number of drug molecules entered to the market are discovered by modern drug discovery processes such as high throughput screening. However majority of drugs launched in recent years, are having problems associated with solubility and oral bioavailability. Anti-HIV drugs are one among them, are required in large doses to produce desired effect. This failure may be prevented by improving solubility of active molecule by using different types of solubility enhancement techniques. Traditionally uses of co-solvents, solid dispersion, complexation and micronization methods were used. However these methods were not successful for all the drugs (Du et al, 2013). Micronization method was found to be suitable as in this only drug molecule was involved, but surface area was not increased sufficiently as desired. This led to the development of nanonization, an effective solubility enhancement technique. Thus, by increasing the solubility the bioavailability can be increased and also the dose to be administered can be reduced (Johansson and Paterson, 2008; Gadaet al, 2012). Decreasing the size of the particles leads to increased curvature and dissolution pressure, as size decreases below 1 µm, saturation solubility of drug will also increase. Nanocrystals are exploited to improve the bioavailability of many drugs such as curcumin (Rachmavathi, 2013)

The protease inhibitors are a major class of drugs used to treat HIV infected patients. These drugs bind with high affinity to the aspartic HIV protease. Nelfinavir is used along with other anti-retroviral drugs to control progression of HIV infection. Nelfinavir helps to prevent further damage in HIV infected person by slowing down the production of new viruses (Shibata et al, 2000). Compared to all other protease inhibitors Nelfinavir has higher dose i.e., 1250 mg twice daily for adults. It comes under BCS class II and has dissolution rate limited absorption problems because of low solubility and high permeability, it possesses very low intrinsic dissolution rate. Therefore, many efforts have been made to develop formulation with improved bioavailability so as to reduce dose and improve the patient compliance in HIV/AIDS therapy.

Nelfinavir Mesylate is well absorbed in gastric region being soluble in acidic pH, however it may undergo precipitation in alkaline environment and therapeutic activity may decrease due to low absorption. It was reported that the presence of food in GI tract, increases the extent of absorption of Nelfinavir (Physician Desk Reference, 2000). Hence by formulating it as nanocrystals its solubility in intestine can be increased to some extent and also it is possible to increase its bioavailability through dissolution velocity improvement.

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Thus the objective of the present study was to prepare Nelfinavir Mesylate nanosuspension and translate it to nanocrystals by lyophilization.

MATERIALS AND METHODS

Materials

Nelfinavirmesilyte was obtained as gift sample from Aurobindo Pharma, Hyderabad. Poloxamer 407 was purchased from Sigma Aldrich Pvt. Ltd., Germany. Mannitol was purchased from Loba Chemie Pvt. Ltd., Mumbai. HPLC grade Acetonitrle and Methanol, were purchased from Merck Specialties Ltd., Mumbai. Milli-Q water was produced in the lab using the Milli-Q water generator (Millipore (India) Pvt. Ltd., Bangalore). All other chemicals and reagents were used are of analytical grade.

Solubility Studies

The solubility of Nelfinavirmesylatein various buffers with pH 1.2, 4.6, 6.8, 7.4 and Milli-Q water was determined. An excess amount of drug was placed in 2.0 ml of buffer solution in a 2.0 ml Ependroff tubes. The Ependroff tubes were incubated in a roto spin at room temperature for 24 hrs at 50 rpm. After 24 hrs, solutions were centrifuged at 15000 rpm for 10 min. and the supernatants were analyzed for the drug content. The pH of each supernatant was measured after addition of drug. These were analyzed by HPLC after appropriate dilution. All studies were performed in duplicate. The chromatographic conditions are given in Table 1.

Table 1: Chromatographic conditions for analysis of Nelfinavirmesylate

Chromatographic mode	Isocratic Reverse phase
Stationary phase	Grace Smart C_{18} (250.0mm × 4.6mm, 5µ)
Mobile phase	Acetonitrile: 25.0mM KH_2PO_4 in water pH 3.2 (55:45 % v/v)
Run time	8.0min
Detection wavelength	254.0 nm (UV detector)
Flow rate	1.0 ml/min
Injection volume	20.0 µL
Column oven Temperature	25°C
Autosampler temperature	4°C
Retention time (min)	
NelfinavirMesylate	6.60 ±0.02 min

Table 2: Composition and process parameters of formulation batches

B. No	N-1	N-2	N-3	N-4	N-5	N-6
Drug (mg)	500	500	500	500	250	250
Poloxamer 407 (% w/w)	10	20	10	20	20	20
Balls size (mm)	10	10	5	5	5	5
Ball Weight (g)	80	80	105	105	105	105
RPM	250	250	250	250	250	500
Volume	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml

Table 3: Results of solubility study of Nelfinavir at various pH buffers

S. No.	pH	Mean PD ± SD (mg/ml)
1	1.2	0.027 ± 0.01
2	4.6	0.005 ± 0.00
3	6.8	0.004 ± 0.00
4	7.4	0.0001 ± 0.00
5	Milli-Q	7.78 ± 1.10

Table 4: Major IR peaks (wave numbers) of Nelfinavirmesylate

Groups	Wave number (cm^{-1})
=C-H (aromatic stretching)	3078.35
$-CH_3$	2929.97
C_6H_5-CO-	1676.20
-CO-NH-	1639.55
-C-S	691.50
-COCN	1168.90

Table 5: Characterization data for formulation batches of nanocrystals

Formulation Code	Time (min)	P. size (nm)	PDI	Zeta Potential
N-1	0	1744.83 ± 45.12	0.84 ± 0.02	-
	15	1586.75 ± 20.23	0.74 ± 0.04	-
	30	945.26 ± 98.60	0.68 ± 0.055	15.61 ± 0.17
	60	2553.42 ± 730.94	1.00 ± 0.07	-
N-2	0	1666.61 ± 54.96	1.00 ± 0.08	-
	15	1473.63 ± 188.17	0.76 ± 0.04	-
	30	871.95 ± 119.60	0.51 ± 0.11	20.13 ± 0.14
	60	2087± 120.32	0.52 ± 0.02	-
N-3	0	1699.33 ± 30.15	0.84 ± 0.02	-
	15	1238.96 ± 111.48	1.00 ± 0.08	-
	30	782.35 ± 119.16	0.61 ± 0.02	18.41 ± 1.12
	60	967.37 ± 133.13	0.83 ± 0.04	18.82 ± 0.76
N-4	0	1758.41 ± 244.78	1.00 ± 0.2	-
	15	1172.14 ± 256.36	0.51 ± 0.14	-
	30	753.04 ± 132.65	0.28 ± 0.03	21.13 ± 0.41
	60	954.22 ± 106.27	0.44 ± 0.09	20.87 ± 1.02
N-5	0	1805.46 ± 150.24	1.00 ± 0.37	-
	15	1029.38 ± 193.14	0.25 ± 0.06	-
	30	740.12 ± 79.21	0.20 ± 0.07	23.31 ± 1.10
	60	754.22 ± 89.23	0.20 ± 0.06	22.98 ± 0.94
N-6	0	1836.71 ± 214.25	1.00 ± 0.64	-
	15	1005.85 ± 157.23	0.26 ± 0.04	-
	30	726.16 ± 82.38	0.21 ± 0.32	20.14 ± 0.85
	60	760.31 ± 91.62	0.20 ± 0.10	21.32 ± 1.34

Compatibility Studies

Fourier Transform Infrared Spectroscopy

Infrared Spectra were recorded using a Shimadzu FTIR 8300 Spectrophotometer. The sample containing drug Nelfinavir Mesylate or Nelfinavir Mesylate - Poloxamer 407 mixture was dispersed in potassium bromide and compressed into discs in a hydraulic press and analyzed in the region of 4000 to 400 cm^{-1}.

Differential Scanning Calorimetry (DSC)

DSC scan was performed using DSC-60, Shimadzu, Japan. The samples (Nelfinavir Mesylate or Nelfinavir Mesylate - Poloxamer 407) were placed in an aluminum pans and sealed, heated under nitrogen flow (30 ml/min) at a scanning rate of 5°C/min from 25°C to 260°C.

Preparation of nanocrystals using ball milling technique

Nelfinavir Mesylate nanocrystals were prepared by using a ball mill (PM100,Retsch Inc., Newtown, PA, USA), equipped with stainless steel milling jar with volume of 125 ml and sample capacity of 15-50 ml. The milling process was optimized by studying the effects of various process parameters on the size of nanocrystals. In this method, the drug (10 %w/w with respect to water) was dispersed in Poloxamer 407 solution (10% w/w with respect to drug). The drug suspension was wet milled with two different grinding media (5 mm and 10 mm diameter beads) (Ghoshet al, 2012). High shear force generated during the collision of the balls with the drug crystals allows fracture of the particles into nanosized crystals. During the operation of ball mill, process was stopped for 5 min and samples were withdrawn for particle size and zeta potential at different time points. The process was continued for 60 min. Various parameters such as drug amount, stabilizer concentration, milling speed, weight and size of the milling balls and milling time were varied and their effect on particle size was analyzed. The composition and process parameters of various batches are given in the Table 2. The optimized nanocrystals were lyophilized using mannitol as cryoprotectant.

Characterization and In vitro Evaluation of Nanocrystals

Particle Size and Size Distribution

Samples were examined for particle size, polydispersity index (PDI) and zeta potential by using Zetasizer Nano ZS (Malvern instruments, U.K.). The samples were analyzed at 25°C.

Drug Content Analysis

The freeze dried product was analyzed by dissolving 10.0 mg of it into 10.0 ml of methanol. The sample was sonicated for 15 min and filtered using 0.22 µ membrane filter and after sufficient dilution, the amount of drug was determined by HPLC.

$$\% \text{ Drug content} = \frac{\text{Amount of drug substance in 10.0 mg formulation} \times 100}{10}$$

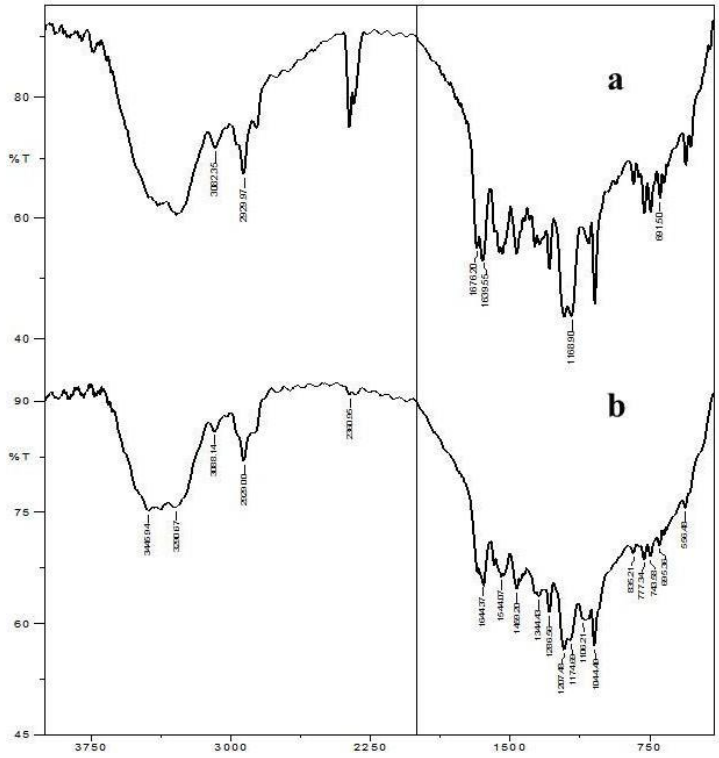

Figure 1: IR Spectra of a) Nelfinavirmesylateb) Nelfinavirmesylate + Poloxamer 407

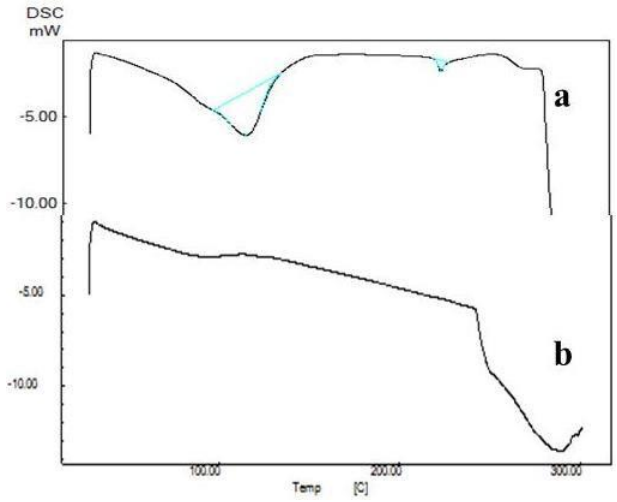

Figure 2: DSC Thermogram of a) Nelfinavirmesylate, b) Nelfinavirmesylate+ Poloxamer 407

Saturation Solubility

An excess amount of the freeze dried product of optimized formulation was added separately to 1.5 ml each of Milli-Q water, pH 1.2 HCl buffer, pH 4.6 acetate buffer, pH 6.8 phosphate buffer and pH 7.4 phosphate buffer in Ependroff tubes. Then the tubes were mounted on rotospin apparatus for 24 hrs at room temperature. Then the samples were centrifuged at 15,000 rpm for 10 minutes. After that supernatant was separated and filtered through 0.22 µ membrane filter. Finally the filtrate was analyzed by HPLC.

Solid-state Characterization

To detect the change in crystalline state of the formulation, the optimized formulation was subjected to FTIR and DSC analysis and compared with that of pure drug as described earlier. The optimized formulation was analyzed for surface topography by Scanning Electron Microscopy (Zeiss, EVO 18, Carl Zeiss SMT Ltd, UK). The sample was deposited on a double-sided carbon tape and examined.

In vitro dissolution studies

The in vitro dissolution study of optimized freeze dried formulations containing 100 mg of Nelfinavirmesylate (N-5) and 100 mg pure drug was carried out using USP type I dissolution apparatus containing 900 ml of 0.1 N HCl. The basket rotation speed was set at 100 rpm. The temperature of the dissolution medium was maintained at 37±0.5ºC by thermostatically controlled water bath. Samples were withdrawn at predetermined intervals of 5, 10, 20, 30, 60, 90, 120 and 180 min. At

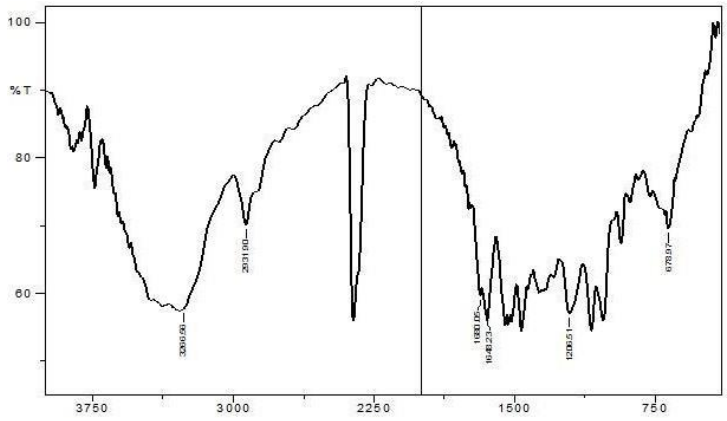

Figure 3: IR Spectra of Nelfinavirmesylate nanocrystals (N-5)

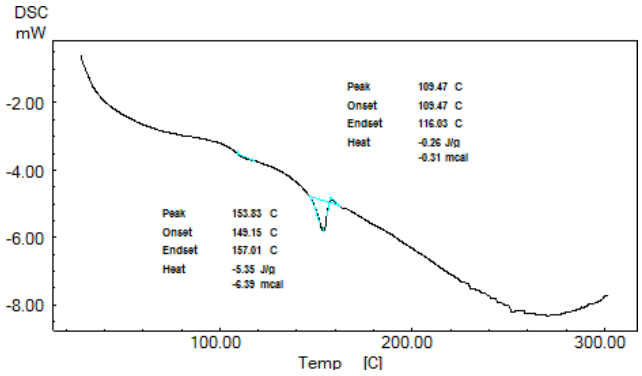

Figure 4: DSC Thermogram of Nelfinavirmesylate nanocrystals (N-5)

each time point 5 ml sample was withdrawn and replaced with fresh 0.1 N HCl. The withdrawn sample was filtered and analyzed by HPLC.

RESULTS AND DISCUSSION

Solubility Studies

Saturation solubility of Nelfinavir Mesylate was carried out in various buffers and the results are shown in Table 3. In basic buffers drug was precipitating out and UV-method was unable to detect the samples because of very less solubility of the drug. Hence HPLC method was used to analyse the samples. Drug showed highest solubility in Milli-Q water followed by 0.1 N HCl. Nelfinavir Mesylate is a basic drug (pKa 14) and hence it is in the ionized form in acidic media when compared to basic media. Hence the solubility in pH 1.2 was more compared to other buffers.

Compatibility Studies

The success of any formulation development depends on careful selection of excipients/ stabilizers that added in the formulation. The drug must be stable with the added excipients. The possible interaction between the drug and excipients was studied by FTIR and DSC studies.

FTIR-Spectroscopy

FTIR spectral analysis for drug alone and in combination with excipients was carried out to detect the presence of incompatibility. The essential peaks of the drug substances are listed in the Table 4. The spectrum recorded is showing all the essential characteristic peaks of pure drug to confirm that it is Nelfinavir mesylate. IR spectroscopic analysis combination of drug with poloxamer was carried out and the spectra were recorded and shown in Figure 1.

In the event that if there is no change in the essential peaks of drug in mixture when compared to pure drug, it indicates that there is no interaction between drug and excipients. By observing the above IR spectra of drug and drug excipient mixture it can be concluded that there is no considerable changes in the IR peaks when the drug mixed with excipients. So, based on this evidence it can be confirmed that there were no interactions between drug and excipients used in the formulation.

Differential Scanning Calorimetry

DSC analysis is a useful tool in the investigation of solid-state interactions. Thermograms were generated for pure drug alone and physical mixtures of drug with excipients. In the absence of any interaction, the thermograms of mixtures showed endothermic peak with similar intensity corresponding to that of pure drug. If any interaction occurs, this is indicated in the thermogram of a mixture by the shift in the value of melting point of the pure drug (Nayaket al, 2009). Pure Nelfinavir mesylate showed a sharp endotherm peak at 130°C corresponding to its melting point/ transition temperature. Based on the thermograms observed, it

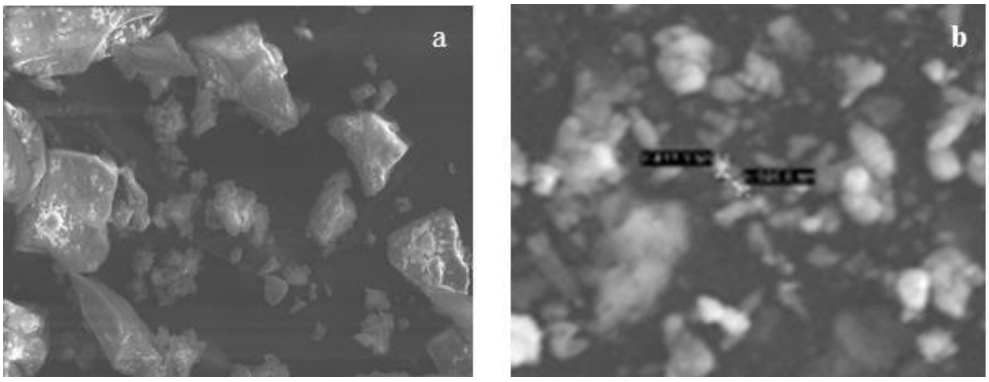

Figure 5: SEM images of a) Nelfinavirmesylateand b) Nelfinavirmesylatenanocrystals (N-5)

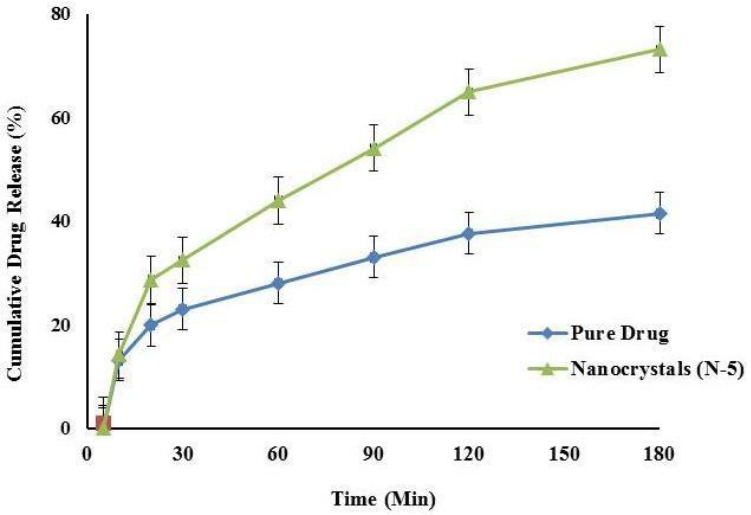

Figure 6: Dissolution profile of pure drug and nanocrystals

was found that intensity of drug peak was reduced significantly and there was a shift in melting point of the Nelfinavir (Fig. 2). It can be concluded that this may be due to slight interaction between drug and excipient mixture. However, there was no change in the IR peaks when drug was mixed with poloxamer as well as with PVA as discussed previously. The DSC and IR study was performed at drug-polymer ratio of 1:1, the amount of stabilizer used in the formulation development was very minute compared to the drug. Considering these aspects, same stabilizer was used in the study.

Characterization of Nanocrystals

In the current study, nanocrystals of Nelfinavir Mesylate were prepared by top down technique using ball milling technology. Various parameters such as drugamount, stabilizer concentration, milling speed, weight and size of the milling balls andmilling time were varied and their effect on particle size was studied. The results of particle size and poly dispersity index (PDI) are mentioned in the Table 5.

Initially with batch N-1 reduction in particle size was observed after 15 min processing in ball milling. However increase in the time of milling led to increased particle size, which may due to the heat generated by attrition of balls, which might have led to aggregation of the particles. Poly dispersity indices were also found to be very high for all the formulations indicating instability. Hence in N-2 the concentration of stabilizer was increased to 20 % with respect to drug. Increase in stabilizer concentration decreased the particle mobility and at 60 min particle size measurement indicated decrease in coagulation (Peltonen and Hirvonen, 2010). Then the smaller size balls were incorporated and batches were taken at two different stabilizer concentration. N-3 and N-4 showed good results with 782.35 ± 119.16 and 753 ± 132.65 nm respectively at the end of 30 min. However at 60th min there was marginal increase in size was observed. Further to see the effect of amount of drug, the drug quantity was reduced to 250 mg by keeping all other parameters same as that of N-4 containing high concentration of stabilizer.There was no significant difference in the particle size, however PDI was very low with N-5. At the end of 60 min, there was marginal variation in the particle size. N-6 batch was taken at higher milling speed, further slight decrease in particle size was observed. Nanocrystals of which particle size was less than 1000 nm were measured for their zeta potential. It was observed that formulations were stable at higher stabilizer concentration. Thus batch N-5 was considered as optimized formulation and selected for further study. It was observed that 30 min milling was sufficient to reduce the particle size at optimized conditions. Increase in milling

time increased rate of agglomeration as evidenced by increase in particle size.

Solid-state Characterization

FTIR study revealed presence of all functional groups in the optimized nanocrystals formulation as shown in Fig.2. DSC study indicated decrease in the melting point to 109ºC and also peak intensity was decreased drastically, hence it confirmed the reduction in crystallinity(Fig. 3). The optimized freeze dried nanocrystal formulation of each stabilizer and pure Nelfinavir were observed for shape and surface morphology by SEM. Nanocrystals were discrete in nature and images confirm reduction in size in the case of nanocrystals. Morphology study indicated decrease in crystallinity for nanocrystals (Fig. 4).

In vitro dissolution studies

In vitro dissolution study of pure drug and optimized Nelfinavir nanocrystals (N-5) was carried. Dissolution data was summarized in terms of cumulative percentage drug dissolved with respect to time as shown in the in Fig. 5. From the data it was observed that nanocrystals shown significantly higher dissolution velocity than pure drug. This was supported by high saturation solubility (11.56 mg/ml in Milli-Q water). Increased dissolution velocity is attributed due to the nano size of the particles as well as reduction in crystallinity.

CONCLUSION

Nelfinavir Mesylate nanocrystals were prepared by ball milling technology. Increased dissolution velocity was observed along with increased saturation solubility with optimized nano crystals. Thus it can be concluded that nanonization and reduction in crystallinity was observed by formulating Nelfinavir mesylateas nanocrystals that will help in increasing dissolution velocity and thereby bioavailability. Further in vivo pharmacokinetic studies may give better result with regard to bioavailability enhancement.

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