



**NANOSTRUCTURED LIPID CARRIER – A NOVEL DOSAGE FORM
TO IMPROVE THE ORAL BIOAVAILABILITY OF LOPINAVIR**

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ABSTRACT

Lopinavir (LPV) is used in combined chemotherapy which is also known as Highly Active Anti-Retroviral Therapy (HAART). It is a potent and one of the frequently administered protease inhibitors used in the therapeutic treatment of the HIV. LPV has very poor oral bioavailability which is due to P-glycoprotein efflux transport and high first pass metabolism primarily mediated by cytochrome P450. LPV is given in combination with Ritonavir (RTV) in most of the marketed formulation due to its high first pass metabolism because RTV inhibits or suppress the cytochrome P450 enzyme. On the other hand, LPV and RTV combination therapy leads to major adverse effects such as

Perioral Parasthesias, Elevations of lipid level, Glucose as well as Gastrointestinal intolerance. Hence, there is a need of a RTV free formulation strategy to improve the oral bioavailability of LPV. Nanostructured Lipid Carriers (NLCs) of LPV were prepared using High Speed Homogenization. LPV were successfully encapsulated in the lipid mixture of Glyceryl Behenate & Miglyol 812 by using Poloxamer 188 as a surfactant. Quality target product profile was achieved by using optimized formulation as well as process parameter. Optimized formulation have mean particle size <200 nm with Polydispersity index around 0.1 which indicates very narrow particle size distribution. Entrapment efficiency was around 90 %. Slow drug release profile indicates the homogeneous dispersion of LPV in lipid matrix. TEM image of the LPV loaded NLCs indicates that NLCs have spherical feature with better uniformity. Also they are not aggregated. Absence of characteristic & intense peaks of LPV

in NLCs indicates presence of LPV in more amorphous form and efficient miscibility of LPV in lipids. This further proves presence of molecular level dispersion of drug in lipid matrix after NLCs formation which results in the crystalline reduction of drug and lipid. Also there is no significant change upon the Stability. For LOP-NLCs, the AUC was found to be around 280 % than LOP Solution. This bioavailability increment indicates higher GI uptake of LOP-NLC in comparison to LOP solution. In a nutshell, Nanoformulation such as Nanostructured Lipid Carrier is one of the promising approach to enhance the Oral Bioavailability of LPV.

KEYWORDS: Lopinavir (LPV), Nanostructured Lipid Carrier (NLCs), Encapsulated, Homogeneous Dispersion, Oral Bioavailability.

INTRODUCTION

Lopinavir (LPV) is used in combined chemotherapy which is also known as Highly Active Anti-Retroviral Therapy (HAART).^[1] It is a potent and one of the frequently administered protease inhibitors used in the therapeutic treatment of the HIV.^[2,3] LPV has very poor oral bioavailability which is due to P-glycoprotein efflux transport and high first pass metabolism primarily mediated by cytochrome P450.^[4,5] LPV is given in combination with Ritonavir (RTV) in most of the marketed formulation due to its high first pass metabolism because RTV inhibits or suppress the cytochrome P450 enzyme.^[6,7] On the other hand, LPV and RTV combination therapy leads to major adverse effects such as Perioral Parasthesias, Elevations of lipid level, Glucose as well as Gastrointestinal intolerance.^[8,9] Hence, there is a need of a RTV free formulation strategy to improve the oral bioavailability of LPV.^[10]

Nanostructured Lipid Carriers (NLCs) are the second generation lipid nano carriers containing solid and liquid lipids.^[11] NLCs have the advantages such as high oral bioavailability, low toxicity, biodegradability, drug protection, controlled release and avoidance of organic solvents.^[12,13] NLCs have the capability to strongly immobilize drugs and reduce the particles from coalescing.^[14,15] Mobility of the incorporated drug molecules is drastically reduced in the solid phase. Also the liquid oil droplets in the solid matrix increase the drug loading capacity as compared to Solid Lipid Nanoparticles.^[16] The NLCs were developed with a perspective to meet industrial standards line scale up, technology transfer, qualification and validation, etc.

Several methods had been utilized for the formulation of NLCs like High pressure homogenization, Micro-emulsion, Sonication, Solvent diffusion, Solvent evaporation.^[17]

Being highly lipophilic, LPV seems to be a suitable candidate for entrapment in the lipid matrix.^[18-20] Poloxamer 188 has been used to coat the NLCs prepared with the lipid matrix of Miglyol 812 as a solid lipid and Glyceryl Behenate (Compritol 888 ATO) as a liquid lipid to entrap LPV. In the present research, High speed homogenization was utilized followed by ultrasonication for the preparation of NLCs. Prepared NLCs were characterized mean particle size, zeta potential, entrapment efficiency, assay, *in-vitro* and *in-vivo* study of the formulation. Also, TEM and XRPD evaluation were performed on the Initial and Stability samples of the optimized formulation batch.^[21]

MATERIALS

LPV (melting point 124–127°C) was kindly supplied by Hetero Labs Limited, Andhra Pradesh, India as a gift sample. Miglyol 812 and Glyceryl Behenate (Compritol 888 ATO) were obtained as a gift sample from Croda and Gattefosse respectively. Poloxamer 188 were supplied by Sigma–Aldrich, Bangalore, India and Pearlitol-PF was supplied by Signet Chemicals, Mumbai, India as a gift sample. All remaining reagents and chemicals were of analytical grade. Purified water used for all experiments was MilliQ Plus, Millipore.

METHODS

1.1 Analytical Method Development using HPLC-UV Technique

The HPLC system used consists of LC-10AD/20AD pumps coupled with a Ultra-violet (UV) detector. The conditions on which these instruments run are as below.

Mobile Phase: Methanol, Acetonitrile, Deionise water.

HPLC Column: Luna 5 μ Phenyl-Hexyl column.

Wavelength: 210 nm.

Injection Volume: 10 μ l.

The LPV stock solutions were prepared by dissolving appropriate quantities of LPV in methanol, sonicated for 30 minutes for complete dissolution and make up to the mark in the volumetric flask to yield a final concentration of 1mg/ml (25mg or 0.025gms of LPV was accurately weighed and dissolved in 25ml of ethanol).^[21]

1.2 Pre-formulation Studies

1.2.1 Solubility Studies in Different Solvent

The solubility of LPV was determined by mixing an excess quantity of drug with approximately 2 ml of the solvent which was taken in a screw-capped bottle. The bottles were rotated on a Glass-Col (Terre Haute, IN) laboratory rotator at room temperature. Preliminary studies indicated that this time period was adequate to obtain equilibrium solubility. After the particles had settled, the supernatant was carefully withdrawn and filtered through a 0.22- μ m filter and analysed by UV.^[21]

1.2.2 Physical Compatibility Study

Compatibility studies were carried out for appropriate selection of excipients. Studies were carried out by mixing the drug with various excipients in required proportion in glass vials. Vials were closed with rubber stopper and kept at three conditions, namely 40°C/75 % RH; 25°C/60 % RH; and Photo stability for 1 month. Physical observations of the blend were done during the study at regular intervals.^[21]

1.3 Formulation Development of LPV Loaded NLCs

1.3.1 Preparation of NLCs

NLCs were prepared using homogenization process followed by ultrasonication. LPV 100 mg was weighed accurately and added to a suitable amount of Miglyol 812 and Glyceryl Behenate (which was previously melted at 80°C). Poloxamer 188 was dissolved in double distilled water and heated up to 80°C in a beaker. When a clear homogenous lipid phase was obtained the hot aqueous surfactant solution was added to hot lipid phase and homogenization was carried out using a high-speed homogenizer (Ultra Turrax T25, IKA, GmbH). The temperature was maintained at 80°C or above during homogenization. The obtained pre-emulsion was ultrasonicated using a probe sonicator t 100 W for 5 min. The obtained nanoemulsion (o/w) was cooled down in an ice bath to form NLCs and finally diluted up to 200 ml with deionised water. The NLC dispersions were stored at 2°C-8°C until further analysis.

1.3.2 Optimization of the Composition & Process

In present study concentration of surfactant (Poloxamer 188) was increased from 30% to 70% and proportionally concentration of lipid (Glyceryl Behenate) was decreased from 60% to 20%. Process parameter for the High Speed Homogenization will keep constant which is

15000 RPM for 15 min. The effects of lipid and surfactant concentration on the LPV NLC were evaluated as mentioned in Table 1.

Table 1: Composition for the Preparation of the Nlcs

Batch No.		NLC1	NLC2	NLC3	NLC4	NLC5
Sr. No.	Ingredients	% w/w (Solid Content)				
1	LPV	1.2	1.2	1.2	1.2	1.2
2	Miglyol 812	8.8	8.8	8.8	8.8	8.8
3	Glyceryl Behenate	60.0	50.0	40.0	30.0	20.0
4	Poloxamer	30.0	40.0	50.0	60.0	70.0
5	Purified Water	Quantity Sufficient				
Total		100%	100%	100%	100%	100%

High speed homogenizations are used widely as efficient and promising technique for NLCs preparation. Optimized and robust process of High Speed Homogenization may help to achieve the desired LPV loaded NLCs. Process parameter for High Speed Homogenization are optimized as mentioned in Table 2.

Table 2: Process Parameter for High Speed Homogenization

Batch No.	NLC1	NLC2	NLC3	NLC4	NLC5
Parameter	2500 RPM for 2 min	5000 RPM for 2 min	10000 RPM for 2 min	10000 RPM for 5 min	10000 RPM for 10 min

1.4 Evaluation of the NLCs

1.4.1 Particle Size Analysis

The average particle size and Polydispersity index as a measure of the particle size distribution were assessed by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments, UK). NLC formulations were diluted with double distilled water to weaken opalescence before particle size analysis.

1.4.2 Zeta Potential Measurement

The surface charge was determined by measuring the zeta potential of NLC using Zetasizer Nano ZS (Malvern Instruments, UK). Zeta potential measurements were run at 25°C with electric field strength of 23 V/cm.

1.4.3 Entrapment Efficiency

The entrapment efficiency was calculated by determining the amounts of non-encapsulated LOP in the aqueous surfactant solution, against the total amount of drug added to the formulation. LPV loaded NLCs were centrifuged in a cooling centrifuge and the filtrate was

diluted appropriately and analysed by HPLC. Entrapment Efficiency was determined by using following formula.

$$\text{Entrapment Efficiency} = [(\text{Amount of LOP in NLCs}) / (\text{Total weight of LOP})] \times 100$$

1.4.4 Assay

Assay was determined by estimating concentration of LOP in 100 mg freeze dried NLCs against the lipid content of NLCs using following formula.

$$\text{Assay} = [(\text{Amount of LOP in NLCs}) / (\text{Weight of Lipid in NLCs})] \times 100$$

1.5 Freeze Drying

Diluted NLCs dispersions were filtered using a stirred cell ultra filtration unit 8050 (Millipore, Milan, Italy) equipped with a polyethersulfone membrane. Filtration was carried out by applying suitable positive pressure with Nitrogen. The lipid suspension was freeze-dried in table top freeze drier using Pearlitol-PF as a cryoprotectant. The pre-freezing and freeze drying times, temperature and vacuum conditions were appropriately adjusted to recover completely dried nanoparticles. The obtained freeze-dried NLCs were stored at 2°-8°C.

1.6 Characterization of the NLCs using TEM and XRPD

LPV loaded NLCs was visualized by a high-resolution Transmission Electron Microscope using suitable magnification. Powder X-ray diffractometry of LPV and lyophilized sample were obtained at room temperature using Xpert MPD - XRD instrument by Philips, Holland. The sample was spread on a graticule and pressed in such a way that sample did not fall on keeping the graticule in vertical position. The graticule was placed in sample holder and exposed for radiation.

1.7 *In-vitro* Release Study

In-vitro drug release study was performed for optimized NLC formulation. Dispersion containing 10.0 mg LPV in 750 ml of 0.1 N HCl at 50 RPM for 2 Hour followed by 1000 ml of pH 6.8 Phosphate Buffer at 50 RPM for 12 Hour.

1.8 *In-vivo* Study

Single oral dose bioavailability studies of the optimized NLC formulation was carried out.

Species: Wistar Albino Rats.

Weight : 250 ± 20 gm

Gender: Male.

The Particular species are being selected for the *in-vivo* study to determine pharmacokinetic studies of the new drug formulations. Rat is considered as the most feasible animal for the determination of the plasma level profile of the new drug formulations are to be investigated. Experiments were approved by the Institutional Animal Ethics Committee of the Ganpat University. The first group was given the suspension of lopinavir in distilled water containing methyl cellulose as suspending agent and the second group received NLCs dispersion containing the drug. Then blood samples will be collected at predetermined intervals (30, 60, 120, 240 and 300 min) of post-dose into heparinized tubes from the orbital sinus. The plasma will be separated immediately by cold centrifugation (Remi Equipments Ltd., Mumbai, India) at 5000 rpm for 15 min and the plasma will be stored at -20°C until analysis. Drug present in rat plasma will be measured using HPLC. The plasma concentrations at different time intervals were evaluated using Win Lin software.

1.9 Stability Study

Stability studies were carried out with the optimized LPV loaded NLC. Sealed vials of freshly prepared freeze-dried NLC were placed in stability chamber maintained at $2-8^{\circ}\text{C}$ for 6 month and analysed.

RESULTS AND DISCUSSION

A suitable stability-indicating analytical method development is very critical. The standard curve was generated for the entire range from 0 to 100 $\mu\text{g/ml}$. The results of standard curve preparation are shown in Figure 1.

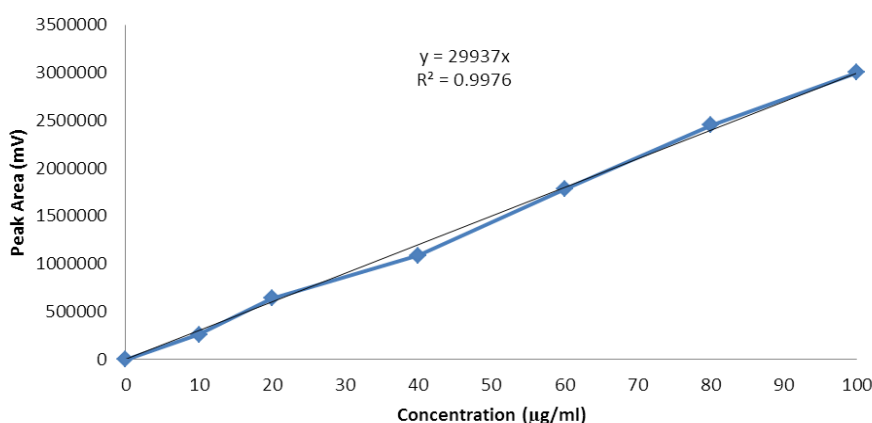


Figure 1: Linearity Curve of LPV

The solubility of LPV in various solvents was estimated. LPV is insoluble in water and aqueous buffer of pH 1 to 8. It is Soluble in organic solvents like methanol, ethanol, dichloromethane and DMF.

From the extensive literature search and considering regulatory points, excipients were selected for the compatibility study with LPV. Studies were carried out using USP type I glass vials. Vials were closed with seal and kept at 40°C/75 % RH; 25°C/60 % RH; and Photo stability for 1 Month. Drug Excipients compatibility study was performed with the ratio mentioned in above Table 3. There is no significant change in the appearance of the binary mixture as well as in physical mixture 1 & 2 after 1 month accelerated condition.

Table 3: Observation of Drug-Excipients Compatibility Studies

Sr. No.	Raw Materials	Ratio	Observation	Initial and after 1 Month		
				Light	25°C / 60% RH	40°C / 75% RH
1.	LPV	—	White powder	NC	NC	NC
2.	LPV + Miglyol 812	10:90	White powder	NC	NC	NC
3.	LPV + Glyceryl Behenate	10:90	White powder	NC	NC	NC
4.	LPV + Poloxamer 188	10:90	White powder	NC	NC	NC
5.	LPV + Pearlitol-PF	10:90	White powder	NC	NC	NC
6.	LPV + Miglyol 812 + Glyceryl Behenate + Poloxamer 188 (Physical Mixture 1)	10:50:20:20	White powder	NC	NC	NC
7.	LPV + Miglyol 812 + Glyceryl Behenate + Poloxamer 188 + Pearlitol-PF (Physical Mixture 2)	05:50:20:20:05	White powder	NC	NC	NC

Ideal NLC have small particle size with narrow particle size distribution. PDI (Polydispersity Index) <1 % and higher % Entrapment efficiency depends on the formulation composition and process parameter. In present study concentration of surfactant (Poloxamer 188) was increased from 30% to 70% and proportionally concentration of lipid (Glyceryl Behenate) was decreased from 60% to 20%. Process parameter for the High Speed Homogenization will keep constant which is 15000 RPM for 15 min. The effect of lipid concentration on the LPV NLCs was evaluated as mentioned in Table 4.

Table 4: Evaluation of the LPV NLCs

Sr. No.	Evaluation	NLC1	NLC2	NLC3	NLC4	NLC5
1	Mean Particle Size (nm)	335 ± 19	218 ± 13	193 ± 10	234 ± 35	222 ± 30
2	Polydispersity Index	0.12 ± 0.04	0.11 ± 0.02	0.14 ± 0.03	0.72 ± 0.14	0.95 ± 0.08
3	Zeta Potential (mv)	-27.3 ± 0.36	-34.1 ± 0.17	-29.4 ± 0.25	-15.3 ± 0.16	-17.7 ± 0.07
4	% Entrapment Efficiency	88.1 ± 0.9	87.4 ± 1.5	89.2 ± 1.2	59.8 ± 5.6	41.2 ± 5.1
5	Assay	89.4 ± 1.5	94.1 ± 1.1	92.1 ± 0.9	84.6 ± 2.8	93.1 ± 0.8

The effect of lipid concentration on the % entrapment efficiency & particle size were shown in Figure 2.

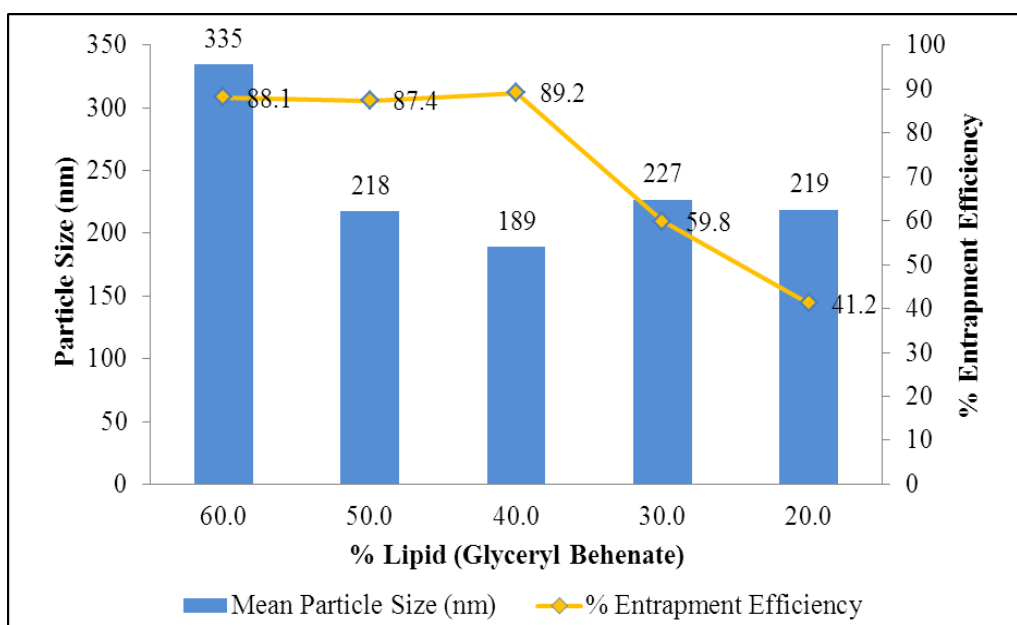


Figure 2: Effect of Lipid Concentration on % Entrapment Efficiency & Mean Particle Size

As the lipid concentration is increased with the simultaneous surfactant reduction, the entrapment efficiency was also increased. % Entrapment efficiency was increased from $41.2 \pm 5.1\%$ to $88.1 \pm 0.9\%$ by increasing lipid concentration from 20% to 60%. Formulation NCL1, NCL2 and NCL3 has around 85 – 90 % Entrapment Efficiency. Entrapment efficiency of formulation NCL4 and NCL5 are 59.8 ± 5.6 and 41.2 ± 5.1 % respectively. % lipid content is responsible for the size distribution of the NLC. Wide variation in particle size of NLC leads to content uniformity issues. Also it impacts the release of the drug from the formulation. Increasing % lipid content resulted in more uniform distribution of particle size (Decrease in the PDI). Formulation NCL1, NCL2 and NCL3 has the PDI of around 0.11 to 0.14 which is indicate very narrow particle size distribution. The viscosity of the system was also increased with the increased in lipid Concentration which may resulted in the larger particle Size of the NLC. Formulation NCL1 has the particle size of 335 ± 19 nm and NCL2 has 218 ± 13 nm Composition of Formulation NCL3 is taken forward for time and RPM optimization of the high speed homogenization process.

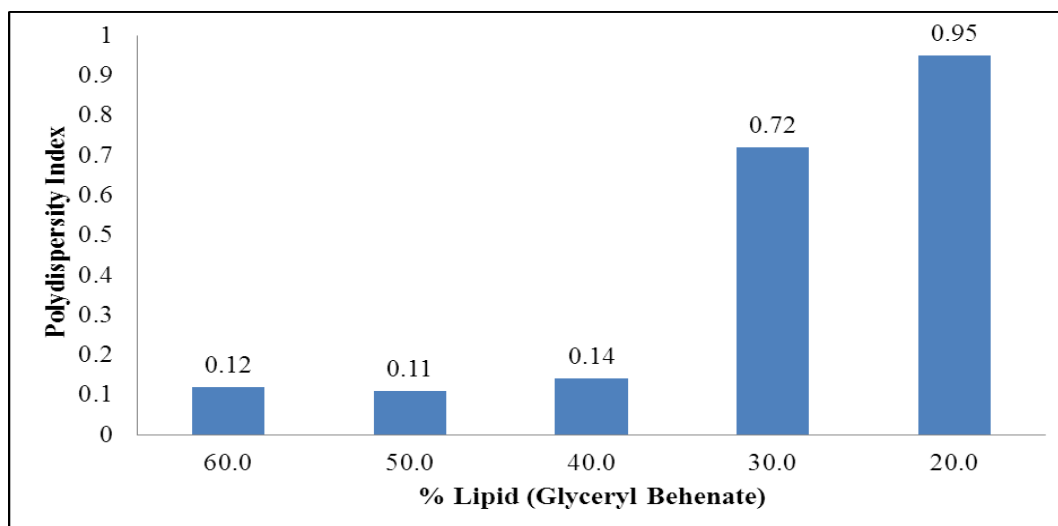


Figure 3: Effect of Lipid Concentration on Polydispersity Index

Table 5: Process Parameter for High Speed Homogenization

Batch No.	NLC6	NLC7	NLC8	NLC9	NLC10
Parameter	2500 RPM for 2 min	5000 RPM for 2 min	10000 RPM for 2 min	10000 RPM for 5 min	10000 RPM for 10 min

Above prepared batches are evaluated as mentioned in Table 6.

Table 6: Evaluation of the NLCs

Batch Code	NCL6	NCL7	NCL8	NCL9	NCL10
Mean Particle Size (nm)	Phase Separation Observed.		173 ± 19	179 ± 11	192 ± 7
PDI			1.21 ± 0.04	0.49 ± 0.12	0.09 ± 0.01
Zeta Potential (mV)			-29.2 ± 0.21	-26.7 ± 0.27	-37.3 ± 0.14
% Entrapment Efficiency			51.9 ± 2.1	71.3 ± 1.5	89.8 ± 1.1
Assay			90.8 ± 0.8	91.2 ± 0.6	92.0 ± 0.5

Phase separation was observed in formulation NCL6 and NCL7 which may be due to low RPM and time of the High Speed Homogenization, resulted in improper mixing of the aqueous and lipid phase. In batch no. NCL8, RPM was increasing by keeping time constant as 2 min. Particle size of the NLC in formulation NCL8 was 173 ± 19 nm as increasing homogenization speed resulted in decrease in particle size due to increase of applied shear forces which helps to breaking of the droplets with significant reduction of the particle size. But, the % Entrapment Efficiency was around 50 % in formulation may be due to improper mixing time which may be responsible for the leakage. In formulation NCL9 and NCL10 time was increase by keeping RPM 10000. The mean particle size of optimized NLC formulation NCL10 was found to be 192 ± 7 nm with a PDI of 0.09 ± 0.01 . Mean zeta potential value was about -37.3 ± 0.14 mV.

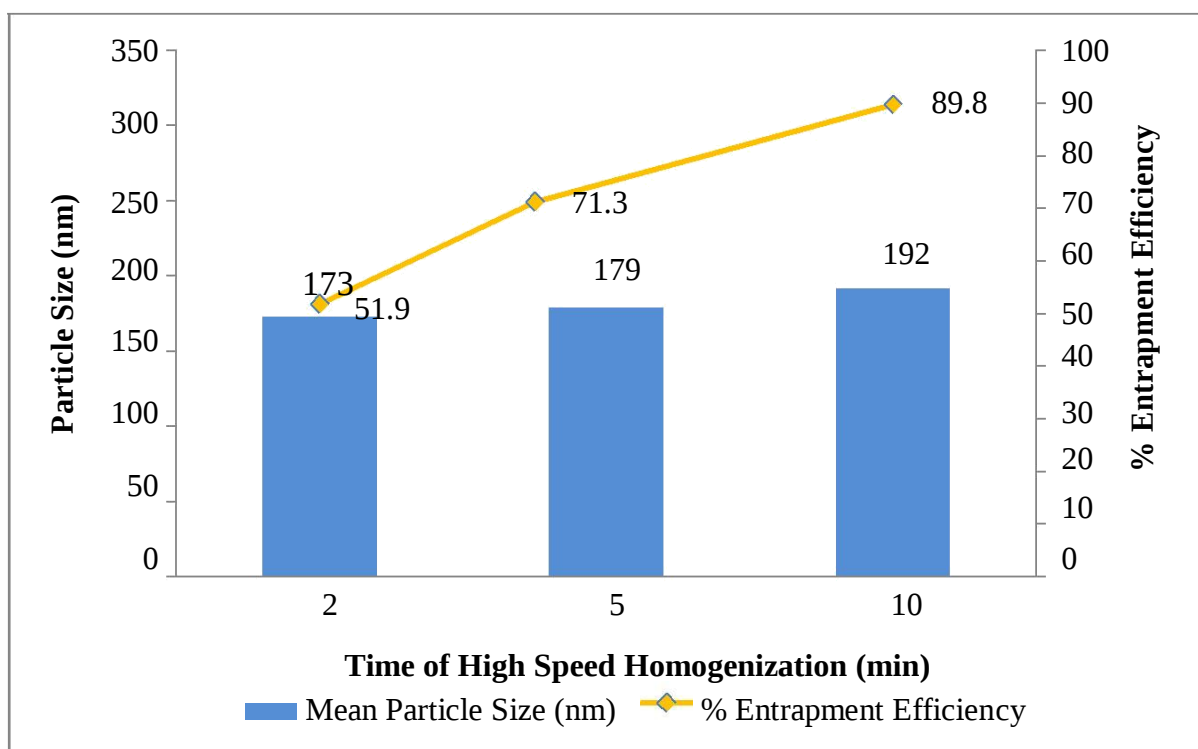


Figure 4: Effect of High Speed Homogenization Time on Particle Size and Entrapment Efficiency.

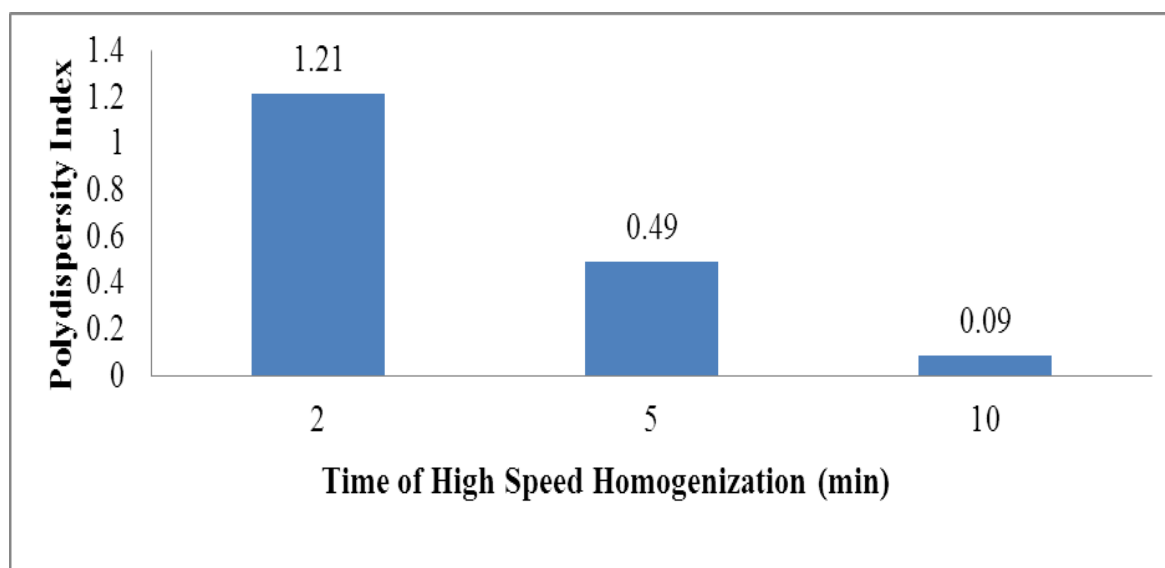


Figure 5: Effect of High Speed Homogenization Time on Polydispersity Index

In-vitro drug release study was performed for formulation NCL10 in 750 ml of 0.1 N HCl at 50 RPM for 2 Hour followed by 1000 ml of pH 6.8 Phosphate Buffer at 50 RPM for 12 Hour as mentioned. Drug release in 0.1 N HCl after 2 Hour was NMT 5 % and around 90% at the end in phosphate buffer as mentioned in Figure 6. Slow drug release is due to homogeneous dispersion of LPV in lipid matrix. For stability study freeze dried NLCs were kept at 2-8°C

up to 6 months. There is no significant change in mean particle size as well as drug release profile after 6 months stability data as mentioned in Table 7. This indicates that freeze dried NCLs were remain stable up to 6 months at 2-8°C.

Table 7: Stability Data of Batch No. NCL10

Evaluation	2° - 8°C Condition				
	Initial	1 Month	2 Month	3 Month	6 Month
Mean Particle Size (nm)	192 ± 7	195 ± 5	199 ± 8	201 ± 8	201 ± 8
Polydispersity Index	0.09 ± 0.01	0.12 ± 0.02	0.11 ± 0.01	0.13 ± 0.03	0.14 ± 0.05
Zeta Potential (mv)	-37.3 ± 0.14	-35.3 ± 0.08	-38.2 ± 0.11	-34.9 ± 0.12	-36.1 ± 0.13
% Entrapment Efficiency	89.8 ± 1.1	91.7 ± 0.7	90.8 ± 1.2	88.2 ± 1.5	90.0 ± 1.3
Assay	92.0 ± 0.5	90.3 ± 0.5	91.7 ± 0.8	89.2 ± 0.7	91.1 ± 1.1

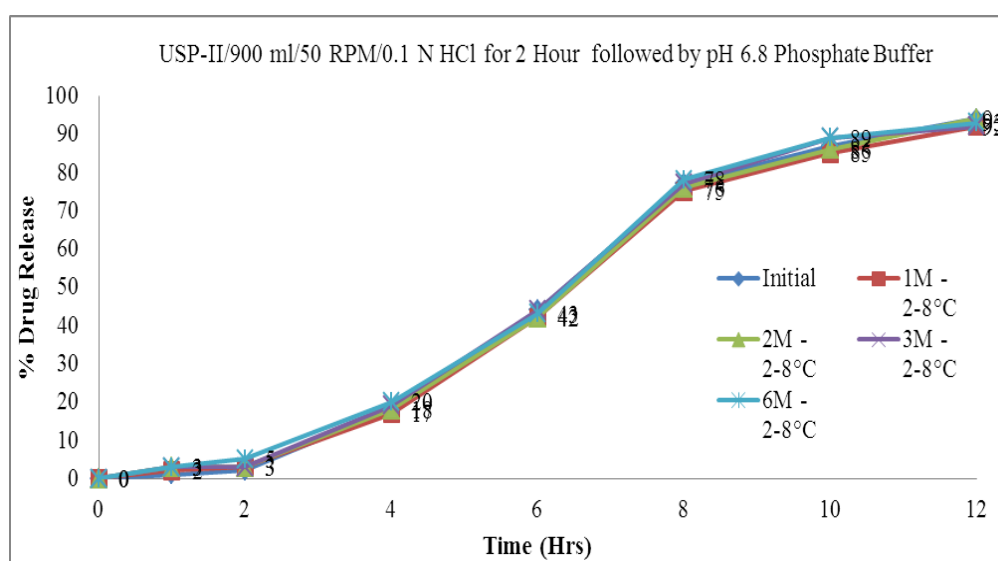


Figure 6: IN-VITRO Dissolution of The LPV NLCs

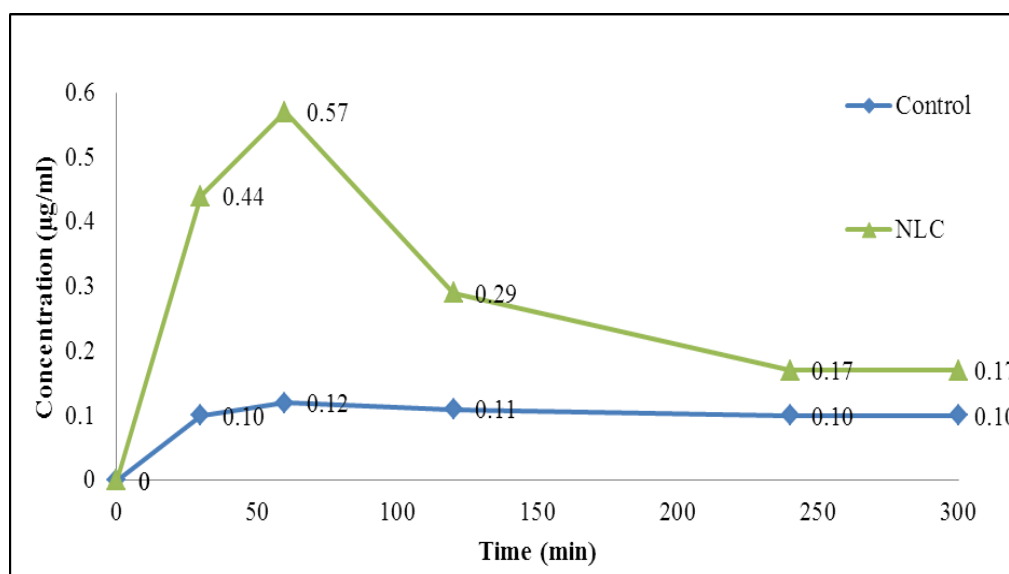


Figure 7: Comparison of % Relative Bioavailability

The plasma profiles of LOP-MC and LOP-NLCs were compared as mentioned in Figure 7. For LOP- NLCs the AUC was found to be around 280 % than LOP-MC ($p < 0.05$) as mentioned in Table 8. This bioavailability increment indicates higher GI uptake of LOP-NLC in comparison to LOP solution.

Table 8: Pharmacokinetic Parameter of LPV NLCs

Sr. No.	Formulation	C _{max} (µg/ml)	AUC (µg min/ml)	% Relative Bioavailability
1	LPV - Control	0.12 ± 0.02	30.3 ± 1.17	--
2	LPV - NLC	0.57 ± 0.12	85.35 ± 3.44	281.68

Figure 8 shows the typical TEM image of the LPV loaded NLCs which indicates that NLCs have spherical feature. In addition to this, there is no significant difference in the NLCs sphericity, surface properties and size upon the stability study.

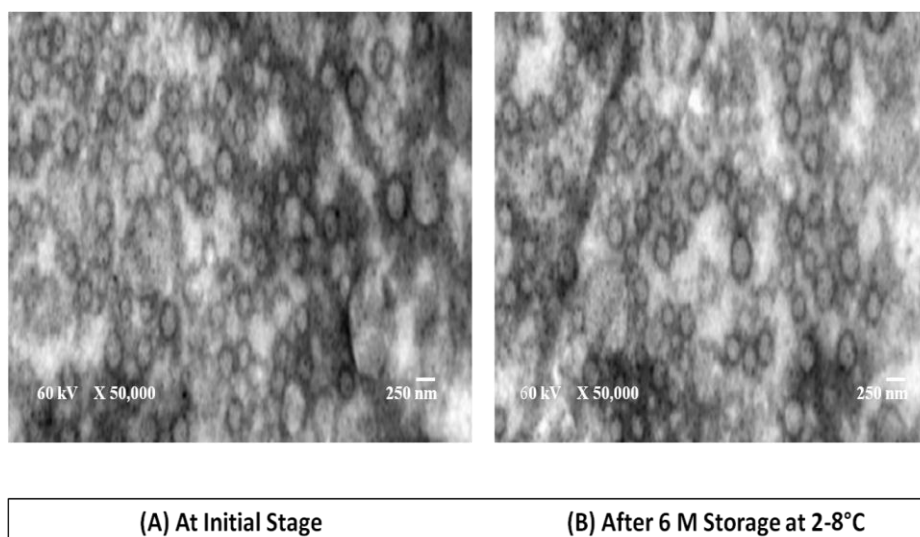
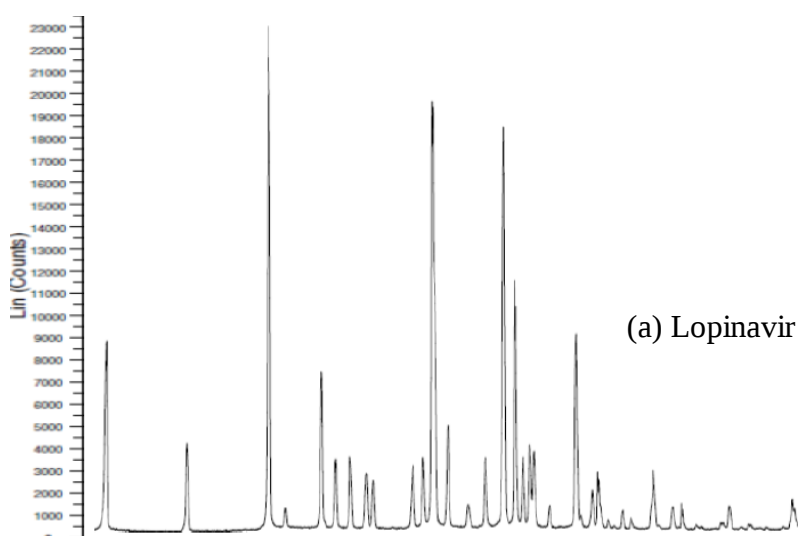


Figure 8: TEM Images of the LPV NLC (A) Initial And (B) After 6 Month 2-8°C



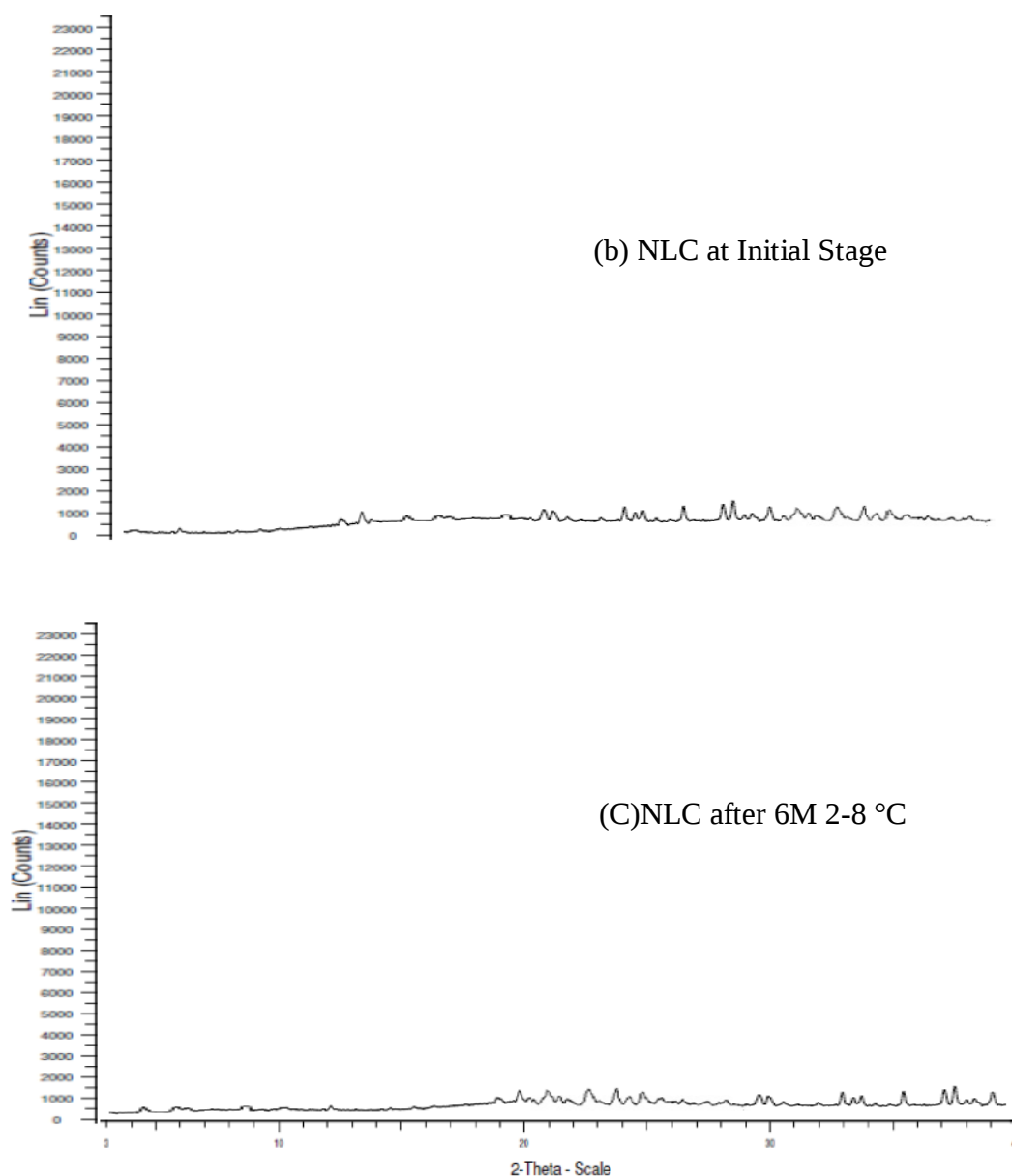


Figure 9: XRPD Spectra of (a) Lopinavir (b) NLC at Initial (c) NLC after 6M 2°-8°C Storage

In the XRPD evaluation as mentioned in Figure 9, the drug sample showed intense peaks as LPV is crystalline in nature. XRPD pattern of NLCs at initial and stability sample's looks similar. Absence of intense peaks of LPV in NLCs indicates presence of LPV in more amorphous form and efficient miscibility of LPV in lipids. There is no significant change in the intensity of the peaks at same or nearer 2θ values on the stability samples which indicates that formulation is stable as there is no form conversion upon the stability.

CONCLUSION

In the present investigation, LPV loaded NLCs were successfully manufactured using High speed homogenization technique. The effect of the formulation composition and process parameter on the LPV loaded NLCs were evaluated and optimized. Optimized formulation shows desired particle size with narrow particle size distribution, high % entrapment efficiency, longer *in-vitro* drug release profile with increase in oral bioavailability. By suitable selection of the lipids and surfactant, we can achieve desired characteristics of NLCs. Stability data shows that there is no significant difference in the optimized formulation of LPV loaded NLCs after 6 Month 2-8°C conditions. The study opens the chances of manufacturing of the LPV loaded NLCs by competitive cost at commercial level.

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