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NANOSTUCTURED LIPID BASED DRUG DELIVERYSYSTEM OF LOPINAVIR- OPTIMIZATION AND EVALUATION

[1*]Mr. Sunil Rambhau Sawale, [2]Dr. R. V. Shete, Dr. Vishwas C. Bhagat. and [4]Dr. Madhuri T. Deshmukh

[1]B. Pharm., [2]M. Pharm. Ph. D., [3]M. Pharm. Ph. D., [4]M. Pharm. Ph. D.
Rajgad Dnyanpeeth's, College of Pharmacy, Bhor, Pune.

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***Corresponding Author**
Mr. Sunil Rambhau Sawale
B. Pharm. Rajgad Dnyanpeeth's, College of Pharmacy, Bhor, Pune.

ABSTRACT

By using the high-shear homogenization method Nanostructured lipid carriers (NLCs) loaded with lopinavir (LOP) were prepared. The LOP-NLCs formulations were freeze-dried using POLOXOMER 188 as a cryoprotectant. A burst release is shown by in vitro release studies in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8). The optimized freeze-dried formulation (LOP-NLC) had a particle size (PS), zeta potential (ZP) and % entrapment efficiency (%EE) of 159.5 _ 3.75 nm, 0.413 _ 0.017, -46 mV and 97.77 _ 4.46%, respectively. A spherical shape was observed in the optimized formulation by transmission and scanning electron microscopes. The absence of chemical interaction between the drug and lipids is observed in Differential scanning Calorimetry study. In vitro study is performed using the bulk-equilibrium reverse dialysis technique, to investigate LOP release from optimized LOP-NLC and pure drug suspension in different media. The optimized formulations stored in amber glass container were found to be physically and chemically stable for three months at room temperature. The bioavailability of LOP following oral administration of LOP-NLC in male Wistar rats was found 4.52-fold higher than the LOP-suspension. So the conclusion is:- for improving the oral bioavailability of lopinavir, the nanostructure lipid carriers are potential carriers.

KEYWORDS: lopinavir; lipid-based formulations; factorial design.

1. INTRODUCTION

A variety of complications caused by the human immunodeficiency virus (HIV) infection i.e., Acquired immune deficiency syndrome (AIDS). Mostly localized and replicated area in the

cell for HIV is (monocytes and CD4+ T lymphocytes), and anatomical (lymphatic system, central nervous system and genitals) levels. At present, many of the available anti-HIV drugs suppressing the viral replication within the peripheral blood circulation.[1] As a result, targeting the antiretroviral drug to a system it serves as a viral reservoir along with some advantages with a reasonable approach.

Belonging to protease inhibitor class, Lopinavir (LOP) is an antiretroviral drug. It plays important role in an effective treatment for HIV-1 infections in a combination therapy.[1] Due to poor aqueous solubility (0.01 mg/mL), high P-glycoprotein efflux and extensive first-pass metabolism by liver microsomal enzyme cytochrome P450 (CYP3A4) LOP exhibits limited oral bioavailability.[2] Subsequently, when administered alone, LOP fails to attain sufficient therapeutic concentration in the systemic circulation. In order to improve the oral bioavailability, LOP is currently marketed as a fixed dose co formulation with Ritonavir, under the names of Aluvia® and Kaletra®.[3] Ritonavir is an analogue of LOP that enhances the oral bioavailability of LOP due to its inhibitory effect on CYP3A4 and P-gp.[3,4] On the other hand, gastrointestinal intolerance, glucose intolerance, perioral paresthesia and lipid elevation etc. are the adverse reactions caused due to the use of Ritonavir in the combination therapy.[4] Hence, it is inevitable to develop LOP formulation without coadministration of ritonavir to improve the oral bioavailability of LOP.

In recent years, great potential in delivering the therapeutic agents to the intestinal lymphatic system and to avoid the first pass metabolism is shown by lipid-based nano formulations such as nanostructured lipid carriers (NLCs).[5,6] Due to controlled release properties and greater chemical and physical stability, NLCs are employed as an alternative to solid lipid nanoparticles (SLNs). Including lower drug loading and formulation instability due to polymorphic modification of similar lipid molecules, NLCs minimizes the limitations of SLNs.[7] LOP is a class II drug, based on Biopharmaceutics Classification System, which restricts its absorption via intestinal membrane. By enhancing the saturation solubility of loaded LOP and its dissolution, NLCs may overcome the solubility related absorption formulation. Also, in reducing the drug dose, NLCs possess more space for drug payload. High shear homogenizer and lyophilizer are the instruments and processing steps, used in the preparation of stable NLCs formulations can be easily employed in the pharmaceutical industry for the large-scale production.

In the present study, LOP-NLCs formulation for oral delivery was developed using

Poloxamer 188 as the solid lipid, and Labrafil M 1944 CS as the liquid lipid. NLCs can be prepared by various methods including hot and cold homogenization, solvent diffusion, solvent evaporation and microemulsion. The solvent diffusion method was used because it is simple and efficient on a lab scale. The formulations were characterized in term of particle size (PS), zeta potential (ZP), percent of entrapment efficiency (%EE) and percent yield. To improve the stability, the selected LOP-NLCs formulations were freeze-dried using cryoprotectant. In vitro release studies of the selected freeze dried LOP-NLC formulations were carried out in alkaline and acidic media. The in vitro cellular uptake of LOP from the optimized LOP-NLC formulation was performed using the bulk- equilibrium reverse dialysis technique. The in vivo study was carried out using male Wistar rats to evaluate the oral bioavailability of LOP.

2. MATERIALS AND METHODS

2.1. Chemicals

Lopinavir was obtained from Cipla Ind. ltd. Myristic acid from S.D. Fine chemicals, Mumbai, Maisine 35-1, Capryol 90 (Polyproylene glycol monocaprylate), Labrafac PG (propylene glycol caprylate/caprate) Labrafil M CS 1944 (oleoyl macrogolglycerides), Labrasol (Caprylo Caproyl macrogolglycerides), Gelucire 44/14 (Lauroyl polyoxyl- 32 glycerides), Transcutol P (purified diethylene glycol monoethyl ether) were procured from Gattefosse India ltd. Mumbai. Cremophore RH 40 (Polyoxyl 40 hydrogenated castor oil), Cremophore EL (polyethoxylated castor oil), Solutol HS 15 (Macrogol 15 hydroxy stearate), Vitamin E TPGS (Dalpha-Tocopheryl polyethylene glycol succinate, TPGS) from BASF, Mumbai, India Oleic acid, Sodium hydroxide, Hydrochloric acid, Methanol, Acetonitrite was obtained from S. D. Fine Chemicals Mumbai. Castor oil, Glacial acetic acid, Potassium dihydrogen phosphate, Potassium bromide, Chloroform from Research Lab. Mumbai.

2.2. Preparation of NLCs and LOP-NLCs

Using the hot high shear homogenization method NLCs were prepared.[9] The lipid phase consisted of Labrafil and Oleic acid in 1:1 ratio. The aqueous phase consisted of surfactants (Glycerol Monostearate i.e., GMS and Stearic acd) at a ratio of 4:4. Separately prepared the lipid and aqueous phases. The lipid phase was prepared in methanol with solid lipid (w/w) and liquid lipid (w/w). The aqueous phase consisted of distilled water (dH2O) and emulsifier (w/w). Lopinavir 0.2 (%) was dissolved in the lipid phase. Both the phases were heated

separately to 85˚C. The aqueous phase was added to the lipid phase and mixed using a high-shear homogenizer at 10,000 rpm for 10 min. The mixture was further subjected to ultrasonication for 10 min. to obtain a translucent nano emulsion of LOP-NLC. This was then placed in a vacuum desiccator for 24 h at room temperature to evaporate the residual organic solvent.

The pH of the above obtained NLC dispersion was adjusted to 1.20 by the addition of 0.1M hydrochloric acid to aggregate the nanoparticles. This dispersion was centrifuged (10,000 rpm for 30 min.) and the NLC precipitate was collected. The NLC precipitate was used to determine entrapment efficiency and drug loading [104-107].

2.3. Optimization of Various Variables

The three variables such as solid lipid, liquid lipid and surfactant were indicated in Preliminary experiments as the main factors that affected the NLC formulation characteristics. A variation in the concentration of any of these components causes a change in the droplet size, drug loading (DL), entrapment efficiency (EE) and other properties of the LOP-NLC. Thus, a central composite rotatable design–response surface methodology (CCRD–RSM) was used to systematically investigate the influence of these three critical formulation variables on particle size, drug loading and entrapment efficiency of the prepared NLC. Thus, concentration of solid lipid, liquid lipid and surfactant were chosen as the independent variables for the optimization. On the basis of the results of preliminary experiments, literature survey and the feasibility of preparing the NLC at the extreme values, the experimental range was selected. The value range of the variables was surfactant concentration (A) of 1-3 %, liquid lipid concentration (B) of 0.8-2.4 % and solid lipid concentration (C) of 2-6 %.

Independent variables

a) Glycerol monostearate (solid lipid)
b) Labrafil M 1944 CS (liquid lipid)
c) Poloxomer 188: Transcutol P (1:1) (surfactant and cosurfactant)

Table 2.31 Independent variables and their ranges for optimization of LOP-NLC Formulation.

Variable/Factor	Lower limit	Upper limit
Surfactant (A)	1 %	3 %
Liquid lipid (B)	0.8 %	2.4 %
Solid lipid (C)	2 %	6 %

Table 2.32: Response variables selected for optimization of LOP-NLC formulation.

Sr. No.	Response Variable	Unit	Type
1	Particle size (Y1)	nm	Numeric
2	Entrapment efficiency (Y2)	%	Numeric
3	Drug loading (Y3)	%	Numeric

2.4.Using the Desirability Function- Selections of NLCs

For the selection of the NLCs, the desirability function was used. Accordingly, the obtained results of responses such as PS, PdI and ZP were fitted into the desirability model of Zetasizer Ver. 6.34 (Malvern Instruments Ltd) and Nanophox Particle Size Analysis Windox 5. The desirability value 0 signifies as an unacceptable value, while the desirability value 1 signifies as an acceptable value (most desired value) for the responses.

2.5.Characterization of FormulationsNanoparticulate Properties

The average particle size, size distribution and polydispersity index of LOP–NLCs were determined using Zetasizer Ver. 6.34 (Malvern Instruments Ltd) and Nanophox Particle Size Analysis Windox 5. All samples were diluted with double distilled water to produce a suitable scattering intensity, prior to the measurements.

2.6.Drug Content Estimation

The encapsulation efficiency and drug loading were determined by dispersing 0.5 ml of the LOP-NLC in 1.5 ml of 1 % sodium dodecyl sulfate (SDS) solution and vortexed for 3 minutes to dissolve the free drug. The resulting dispersions were centrifuged for 10 min at 12,000 rpm. The drug content in the supernatant (free drug) after centrifugation was measured by using uv-visible spectrophotometer (Shimadzu vision pro software). SDS solution was used as reference solution. The % EE and %DL in LOP-NLCs were calculated from calibration equation.

$\% EE = Wa - Ws / Wa \times 100$

$\% DL = Wa - Ws / WL \times 100$

Where, Wa, Ws and WL were the weight of drug added to the system, free drug in

supernatant and weight of lipid added in system, respectively.

Table 2.6 Particle size, % EE and % DL of pre-optimization batches.

Batch	Absorbance	% EE	% DL	Particle size (nm)
F1	0.067 ± 0.0023	97.38	3.47	174
F2	0.027 ± 0.0089	98.24	5.45	170.04
F3	0.551 ± 0.0012	87.01	3.62	173.45
F4	0.067 ± 0.0023	97.38	3.47	174
F5	0.024 ± 0.0072	98.3	2.8	190
F6	0.170 ± 0.0030	95.18	3.07	195.54
F7	0.153 ± 0.0022	95.54	2.98	180.27
F8	0.067 ± 0.0023	97.38	3.47	174
F9	0.468 ± 0.0042	88.79	4.22	165.51
F10	0.067 ± 0.0023	97.38	3.47	174
F11	0.093 ± 0.0077	96.82	2.54	235.73
F12	0.117 ± 0.0018	96.31	3.43	175.84
F13	0. 067 ± 0.0023	97.38	3.47	174
F14	0. 185 ± 0.0042	94.85	3.79	169
F15	0. 276 ± 0.0097	93.03	3.32	186.29

2.7.Freeze-Drying Study

2.7.1. Screening of lipids, surfactants and co-surfactants

The lipids, surfactants and co-surfactants are selected on the basis of their solubilization ability, properties and stability of preliminary formulations.

For development of a suitable nanostructured lipid carrier, selection of appropriate lipids on the basis of their solubilization potential for loponavir, biocompatibility and acceptability of lipid is a prime requirement. For this, the saturation solubility of lopinavir in various lipids and lipid combinations was determined as per solubility studies. Solid lipids (stearic acid and glycerol monostearate (GMS)) and liquid lipids (oleic acid and labrafil M 1944 CS) were selected on the basis of above criteria.

Surfactant and co-surfactant were selected on the basis of solubilization potential for loponavir, biocompatibility and acceptability of excipients. For this, the saturation solubility of lopinavir in various surfactants and co-surfactants was determined as described solubility study. Surfactants selected on the basis of solubilizing capacity for lopinavir were poloxamer 188, transcutol P and tween 80.

In order to obtain optimal excipient concentrations of lopinavir loaded NLC formulation,

central composite rotatable design (CCRD)[108-113] was applied. For this Design-Expert software (8.0.7.1 version) of Stat-Ease, Inc. Minneapolis, USA was used. From the preliminary studies appropriate batch was selected for further optimization. The lyophilized samples were reconstituted in distilled water and analyzed in term of mean PS and PdI. The excipients, that produced the smallest PS and PdI was selected for further study.

2.8.In Vitro Release Study

The *in vitro* release studies of optimized LOP-NLC were carried out by the bulk-equilibrium reverse dialysis technique [104-107]. The release medium was 0.1 N HCl (pH 1.2) and phosphate buffer (pH 6.8) at 37^{o}C. 50 rpm stirring speed was maintained. The dialysis bags (molecular weight cut off 12-14 kDa) containing of 2 ml of sink solution were equilibrated in release medium for 12 h prior to experiment. Aliquots of 1 ml of LOP-NLC were directly placed into 900 ml of release medium. At intervals of 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours one dialysis bag was withdrawn from the release solution and the same volume of fresh medium was added in dialysis bag to maintain a constant volume. Then the release media in the dialysis bags was analyzed by uv-visible spectrophotometry.

2.9.Differential Scanning Calorimetry (DSC)

Mettler Toledo DSC 821e model was used to perform DSC study, with the help of data recording software STAR® SW 9.20 and calibrated with indium as per the standard procedure. The equipment was provided with an auto-cooling accessory for programmed cooling. The sample was weighed accurately into a standard aluminium pan, hermitically sealed and heated from 25 to 300°C at a constant rate of 10°C /min under constant purging of nitrogen at 20 ml/min. For a reference an empty sealed pan was used. DSC graph of ptimized LOP-NLC formulation was recorded and the melting point, peak maxima, the presence and absence of endotherm peaks were observed in the DSC graphs.

2.10. Investigations using an Electron Microscope

The surface morphology of optimized LOP-NLC was visualized by scanning electron microscopy (SSX-550, Shimadzu, Japan).

2.11. Oral Bioavailability Study

In vivo studies were carried out in order to determine bio-availability of LOP-NLC formulation. The protocols for the animal studies were approved by Institutional Animal Ethics Committee (IAEC) and all experiments were conducted as per the norms of the

committee for the purpose of control and supervision of experimental animals (CPCSE), India. Male wistar rats weighing 200–250 g were housed at temperature of 22± 2^{o}C and 50-60 % relative humidity and were supplied with food and water *ad libitum*. All the rats were divided randomly into two groups (test and standard) comprising six animals in each. The rats were fasted for 12 hours before experiment but allowed tofree access to water.

The rats were given LOP-NLC and drug suspension (40 mg/kg or equivalent dose) orally to test and standard group respectively in both studies. Formulation was administered to rats by oral gavage with the help of an animal feeding needle. The rats were anesthetized using ether, and blood samples (1.5-2 ml) were withdrawn from the retro-orbital plexus at time intervals of 0.20, 0.40, 1, 1.5, 2, 4, 6 and 24 hrs after administration. All the blood samples were collected in eppendorffs and immediately centrifuged at 8,000 rpm for 10 min. at temperature of 4^{o}C to separate the plasma. The plasma obtained was stored at −20 ^{O}C until analysis [104-107, 114].

The concentrations of LOP in rat plasma were determined by a validated bio- analytical HPLC method. AUC of drug suspension and LOP-NLC formulation were determined at various time intervals and compared.

2.12. Stability Studies

The optimized LOP-NLC was stored at room and accelerated temperature for three months in stability chamber as shown in table. Particle size, entrapment efficiency, drug loading and organoleptic properties were assessed for the stored samples using the same procedures adopted for the fresh samples.[104, 106]

Sr. No.	Sample	Temperature (^{0}C)	Humidity (%)
1.	LOP-NLC	25 (R.T.)	40
2.	LOP-NLC	40 (Accelerated)	65

3. RESULTS AND DISCUSSION

3.1.ANOVA for response surface quadratic model

a) Particle size

The Model F-value of 8.12 implies that the model is significant. Values of "Prob > F" less than 0.0500 indicates that the model terms are significant. Here C, C^2 are significant model terms.

Table 3.1: Analysis of variance table [Partial sum of squares - Type III] for particle size.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F
Model	3882.535	431.3928	8.11751	0.0164
A-Surfactant	54.60125	54.60125	1.02743	0.3573
B-Liquid lipid	23.2562	23.2562	0.43761	0.5375
C-Solid lipid	2157.588	2157.588	40.5993	0.0014
AB	219.1339	219.1339	4.12344	0.098
AC	17.09644	17.09644	0.32170	0.5951
BC	70.85577	70.85577	1.33329	0.3004
A^2	0.028115	0.028115	0.00052	0.9825
B^2	32.17083	32.17083	0.60535	0.4717
C^2	928.4068	928.4068	17.4698	0.0087

Final Equation

PS = Particle size= 175.9841 - 3.69463 * Surfactant + 2.411234 * Liquid lipid + 23.22492* Solid lipid + 10.46742 * Surfactant * Liquid lipid + 2.923734 * Surfactant * Solid lipid - 5.95213 * Liquid lipid * Solid lipid + 0.06037 * Surfactant^2 - 2.04213 * Liquid lipid^2 + 10.97037 * Solid lipid^2.

b) Entrapment efficiency

The Model F-value of 38.69 implies the model is significant. There is only a 0.04% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Here A, B, AB, A^2 and B^2 are significant model terms.

Table 3.12: Analysis of variance table [Partial sum of squares - Type III] for entrapment efficiency.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F
Model	156.7432	17.41591	38.69422	0.0004
A-Surfactant	5.3792	5.3792	11.95137	0.0181
B-Liquid lipid	36.38045	36.38045	80.82915	0.0003
C-Solid lipid	1.0082	1.0082	2.239993	0.1947
AB	17.54743	17.54743	38.98643	0.0015
AC	1.039135	1.039135	2.308724	0.1891
BC	0.360664	0.360664	0.801314	0.4117
A^2	8.27113	8.27113	18.37658	0.0078
B^2	57.61867	57.61867	128.0157	< 0.0001
C^2	1.200802	1.200802	2.66791	0.1633

Final Equation

EE= Entrapment efficiency = 97.19741 + 1.159655 * Surfactant + 3.01581 * Liquid lipid - 0.50205 * Solid lipid - 2.96205 * Surfactant * Liquid lipid + 0.72081 * Surfactant * Solid lipid + 0.424655 * Liquid lipid * Solid lipid - 1.03546 * Surfactant^2 - 2.73296 * Liquid lipid^2 + 0.394537 * Solid lipid^2.

c) Drug loading

The Model F-value of 139.85 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, AB, A^2, B^2, C^2 are significant model terms.

Table 3.13: Analysis of variance table [Partial sum of squares - Type III] for drug loading.

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F
Model	6.302736	0.700304	139.8536	< 0.0001
A-Surfactant	0.00605	0.00605	1.20821	0.321
B-Liquid lipid	0.2048	0.2048	40.8994	0.001
C-Solid lipid	4.23405	4.23405	845.5573	< 0.0001
AB	0.487757	0.487757	97.4070	0.0002
AC	0.005258	0.005258	1.05006	0.352
BC	0.012448	0.012448	2.48582	0.175
A^2	0.050868	0.050868	10.1586	0.024
B^2	0.108699	0.108699	21.7075	0.005
C^2	0.403825	0.403825	80.6456	0.000

Final Equation

DL = Drug loading = 3.489259 + 0.038891 * Surfactant - 0.22627 * Liquid lipid - 1.02884 * Solid lipid - 0.49384 * Surfactant * Liquid lipid - 0.05127 * Surfactant * Solid lipid + 0.078891 * Liquid lipid * Solid lipid - 0.0812 * Surfactant^2 - 0.1187 * Liquid lipid^2 + 0.228796 * Solid lipid^2.

3.1.1. Effects of Variables on the Mean Particle Size

The 3D surface plot is a three-dimensional representation of the response across the select factors. Two factors at a time can be displayed with the full range. The scale on the left of the 3D response surface plot depicts the different regions, based on particle size in various colours from blue to red. The blue region indicates lower particle size (i.e., the desirable

region), while the red region corresponds to larger particle size. The entrapment efficiency and drug loading is also depicted in the red region (desirable region) and blue region. The following results were obtained from the 3D response surface plot.

a. There is significant increase in particle size of NLC formulation by increasing concentrations of solid lipid and surfactant. But in case of liquid lipid there is slight increase in particle size with increasing concentration of liquid lipid as shown in figure: 5.27.(a) and (b).

b. A special trend was found in case of entrapment efficiency i.e. with increasing concentrations of liquid lipid and surfactant there is also increase in EE. There is slight increase in EE with increase in concentration of solid lipid as shown in figure: 5.28. (a) and (b). These results indicate that the liquid lipid increases solubility of drug and due to the surfactant the stability is increased.

c. There is increase in drug loading with increasing concentrations of liquid lipid and surfactant. But there is decrease in DL with increase in concentration of solid lipid as shown in figure: 5.29. (a) and (b).

It is clear from the above plots that on using the optimum concentration of each component, the desired formulation with specified characteristics can be obtained.

a) Particle size

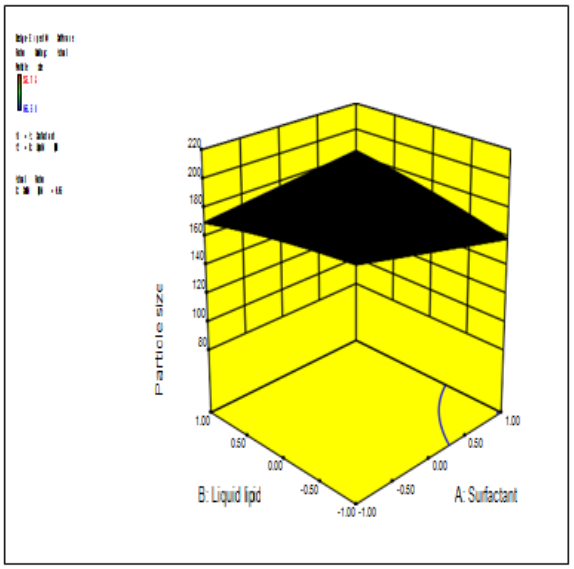


Figure 5.27 (a) 3D response surface plot of particle size particle size vs. variable factors.

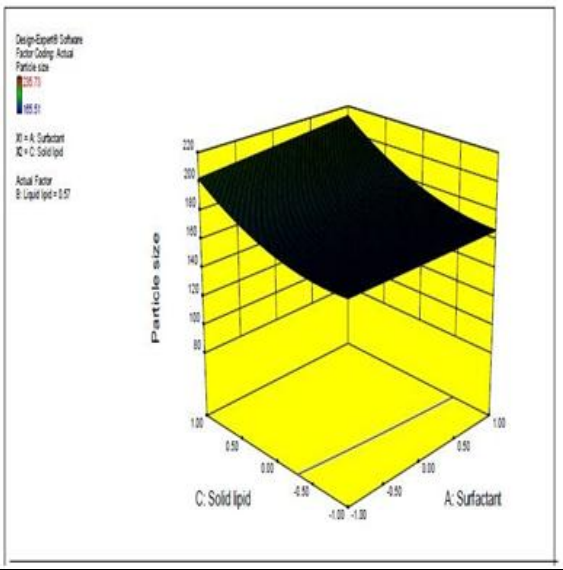


Figure 5.27 (b) 3D response surface plot of vs. Variable size.

a) Entrapment efficiency

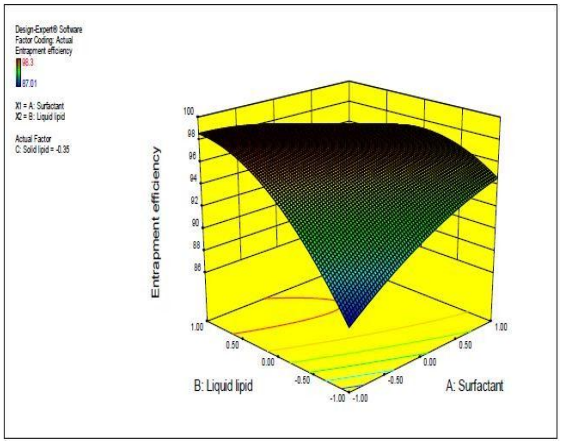


Figure 5.28 (a) 3D response surface plot of entrapment efficiency vs. variable factors

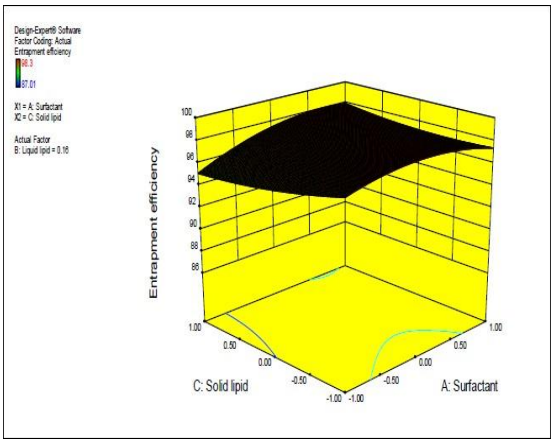


Figure 5.28 (b) 3D response surface plot of entrapment efficiency vs. variable factors

b) Drug loading

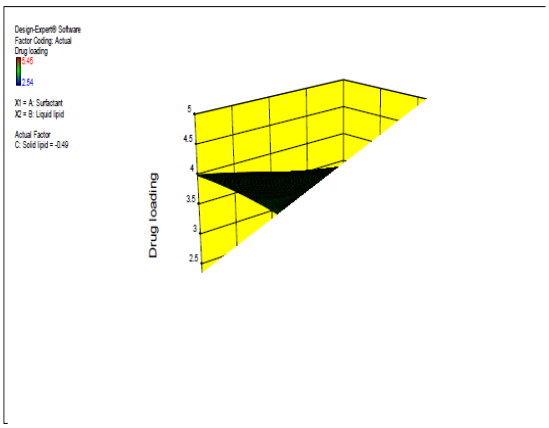


Figure 5.29 (a) 3D response surface plot of ofdrug loading vs. variable factors

Figure 5.29 (b) 3D response surface plot drug loading vs. variable factors

4. EVALUATION OF OPTIMIZED LOP-NLC FORMULATION

In this study, glycerol monostearate was selected as for the solid lipid matrix and labrafil M 1944 CS was used as the liquid lipid of the matrix which made the NLC differes from the formulation of solid lipid nanoparticles (SLN). The non-toxic, non-ionicsurfactant poloxamer 188 was used in combination with transcutol P as co- surfactant. NLCs can be prepared by various methods including hot and cold homogenization, solvent diffusion, solvent evaporation and microemulsion. The solvent diffusion methodwas used because it is simple and efficient on a lab scale.

4.1. Particle size and zeta potential measurement

The mean size of optimized LOP-NLC formulation was found to be 159.5 nm and the polydispersity index value was 0.35. This reveals that the size distribution of the particles

was quite narrow and they had a uniform size as shown in figure: 4.11.

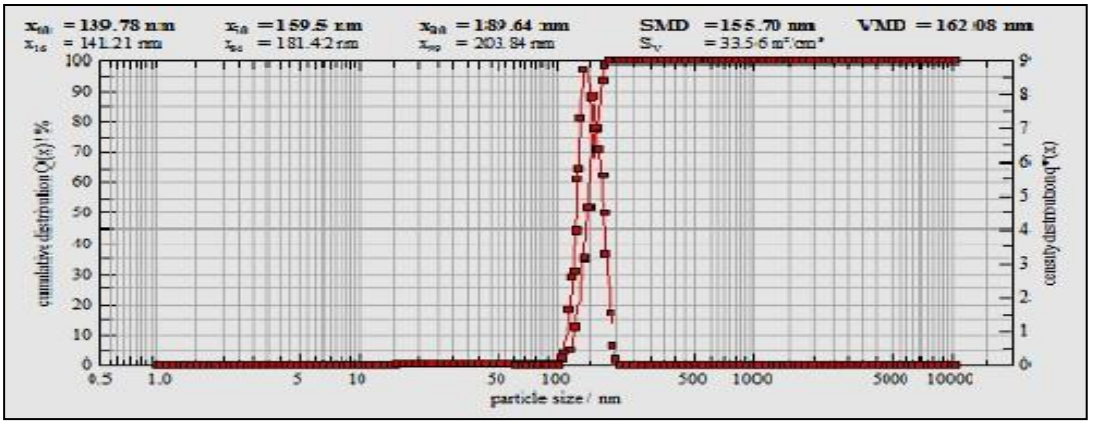


Figure 4.11: Particle size of optimized LOP-NLC formulation.

The measurement of zeta potential, the electric potential at the plane of shear is a useful method to predict the physical stability of nanoparticles during storage. The nanoparticles had higher absolute value of zeta potential, indicating a better stability of this colloid system. It has been established that without steric stabilizer a colloid system was not stable. In this study hence, poloxamer 188 and transcutol P were chosen as the stabilizers. Poloxamer 188 had the typical characteristics of surfactants and self- associated into large micelles due to the zeta potential values decreased with combined surfactants. The less polar propylene oxide i.e. poly chain segments were dissolved and segregate into a hydrophobic micelle core surrounded by a soft brush of highly hydrated, flexible poly(ethylene oxide) chain. Formation of complex micelles is due to surfactant molecules were arranged around the interface of the nanodroplet. The zeta potential of optimized LOP-NLC was found to be -46 mV as shown in figure: 4.12. The decrease in zeta potential is thus due to addition of poloxamer 188.

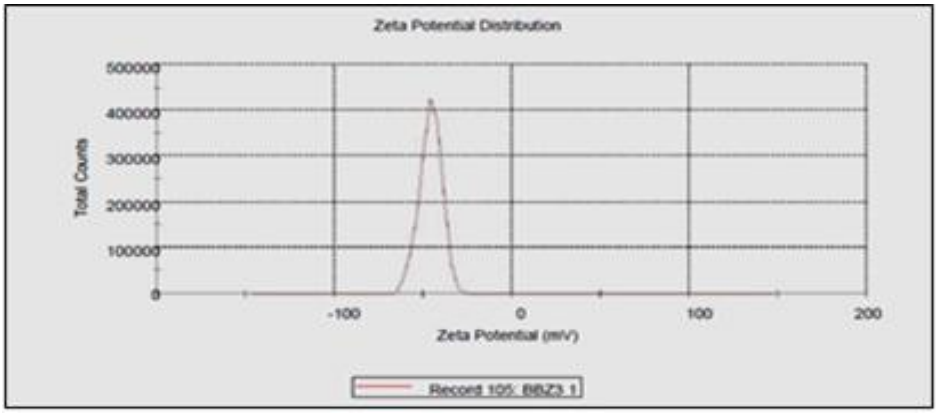


Figure 4.12 Zeta potential of optimized LOP-NLC formulation.

4.2.Drug Entrapment Efficiency (% EE) and Drug Loading (% DL)

An important issue with respect to the use of lipid nanoparticles, as drug carriers is their higher capacity for drug entrapment and loading. The effects of labrafil M 1944 CS on drug entrapment efficiency and loading capacity of glycerol monostearate NLC were investigated. It is clear that the drug entrapment efficiency and drug loading capacity of nanoparticles were increased with increasing the concentration of labrafil M 1944 CS. Optimized LOP-NLC formulation was showed 97.77 % EE and 4.46 % DL, respectively. The incorporation of liquid lipids in solid lipids could lead to massive crystal order disturbance and the resulting matrix of lipid particles indicates great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, thus leading to improved drug loading capacity and drug entrapment efficiency.

4.3.Determination of viscosity

Viscosity studies are important to characterize the NLC system physically and to control its stability. The viscosity of the optimized formulations was determined and the values are shown in table: 4.3.

Table 4.3: Observation for viscosity of optimized formulations.

Sr. No.	Viscosity (cp)	Speed (rpm)	Temperature (^{0}C)
1.	0.1356	1000	25

4.4.Thermal analysis

Pure lopinavir showed one endothermic peak at about 126^{0}C. The excipients GMS and LOP-NLC formulation did not show the lopinavir endothermic peak over the entire range of the tested temperatures. No obvious peak for lopinavir was found for the LOP-NLC, indicating that the drug must be present in an amorphous or molecularly dissolved state in the formulation.

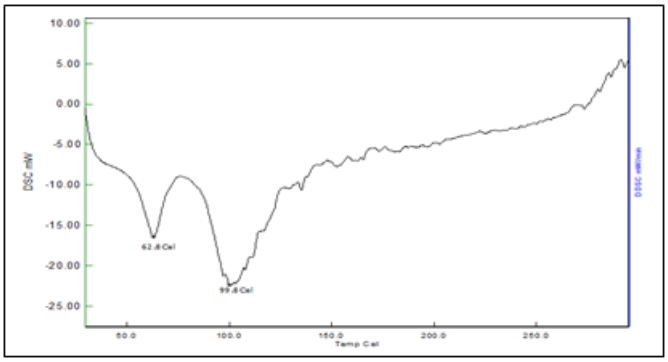
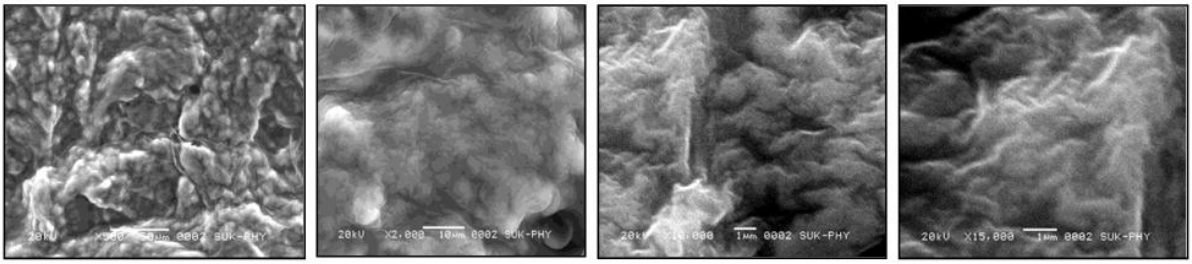


Figure 4.4 DSC graph of optimized LOP-NLC.

4.5.Morphology of particles

In order to give information on the morphology and size of the optimized LOP-NLC, SEM was used to take images of the optimized LOP-NLC. Figure: 4.5 show the images of surface morphology and internal structure of LOP-NLC after freeze-drying. The results indicated that the particles were round and homogeneous with a smooth surface and fixed in the bulk and grid structure formed by cryoprotectants. No aggregation or drug crystal of particles was visible.



4.5 SEM morphology of LOP-NLC showing the surface structure of the lyophilized powder of LOP-NLC at500, 2000, 10000, 15000 resolution.

4.6.*In vitro* drug release studies

The bulk-equilibrium reverse dialysis technique was selected to investigate LOP release from optimized LOP-NLC and pure drug suspension in different media. This method is quite different from conventional dialysis bag diffusion which is widely used for measuring the drug release from colloidal solutions. In dialysis diffusion method, the sample is added in the dialysis bag and is not diluted with enough medium, thus diffusion process of drug is slow because of the low concentration gradient and a barrier of dialysis. In the bulk-equilibrium reverse dialysis method, the LOP-NLC colloidal solution is directly placed in the release solution where it has the opportunity to release the drug under maximum dilution (perfect sink conditions). The nanoparticles were directly exposed to a large volume of sink release medium and better diluted. Therefore the diffusionrate of the drug from nanoparticles to sink solution was high.

The LOP release profiles in 0.1N HCl at pH 1.2 and phosphate buffer at pH 6.8 were shown in figure: 4.61 and 4.62 respectively. Comparing of the release curves of LOP-NLC in pH 1.2 and pH 6.8, we concluded that LOP-NLCs showed no burst release at initial stage in both medium. This was real evidence that, there was no unencapsulated drug fusing to the surface of the particles. A sustained release profile was demonstrated in pH 6.8. The results shows

that a maximum of about 47.36 % LOP was released in acidic medium. A maximum release was seen in phosphate buffer pH 6.8 (92.70 %) suggesting that, the LOP entrapped in NLC was protected from the strong acidic environment of the stomach and then subsequently released small intestine. The results suggested that the major content of LOP in NLC seem to be taken up by the intestinal cells and enter the systemic circulation thus sustaining drug release.

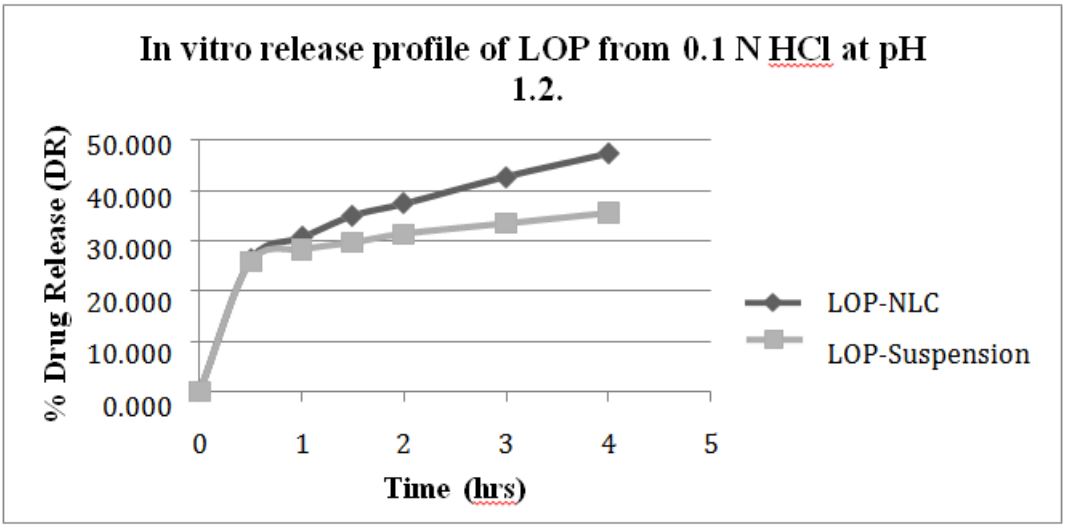


Figure 4.61 In vitro drug release profile of lopinavir from 0.1 N HCl vehicle at pH 1.2.

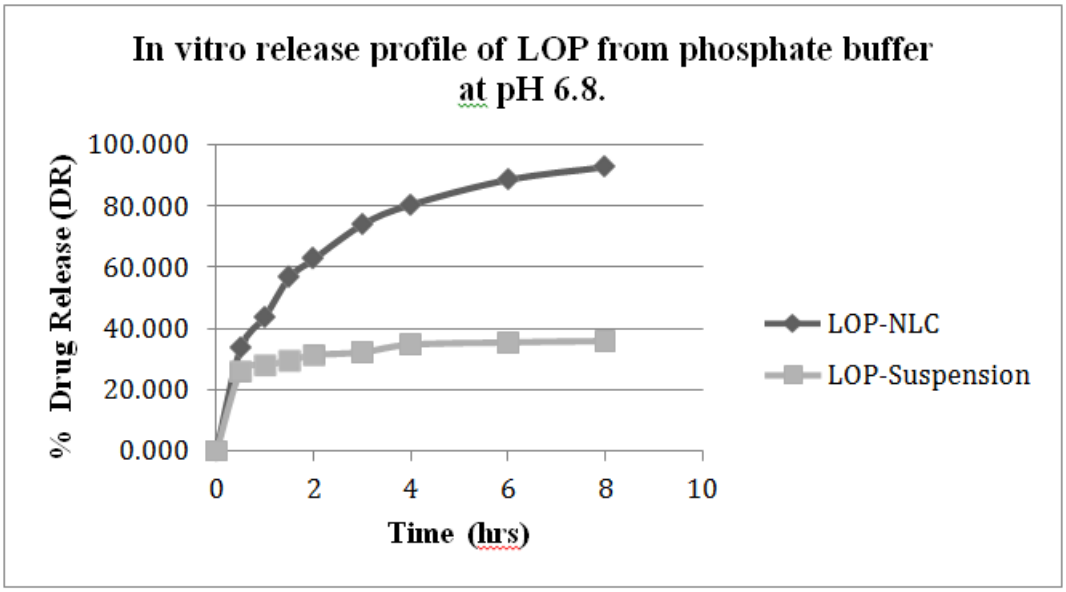


Figure 4.62 In vitro drug release profile of lopinavir from phosphate buffer vehicle at pH 6.8.

4.7.Oral Bioavailability Study

A validated bio-analytical HPLC method was used to determine lopinavir concentration in rat plasma. LOP-NLC and LOP drug suspension were orally administrated to male wistar rats. The plasma concentration time plots in rats after oral administration were shown in figure: 4.7. The Tmax was 90 min. and the Cmax value was 956.75 ± 40.25 ng/ml after oral administration of LOP suspension. However the Tmax value of LOP-NLC was 60 min. which is a half hour earlier than that of LOP suspension. The visible difference between Tmax values of LOP-NLC and LOP suspension manifested that the rates of absorption of the two formulations were not the same. LOP in suspension had probably not dissolved completely in the GI tract. Therefore, it reached peak concentration slowly at 90 min. However LOP in NLC could be released into the gastrointestinal tract quickly at 60 min. as supported by in vitro release studies. Therefore, the intact LOP-NLCs were directly absorbed into the blood circulation and released the drug gradually. The Cmax value of LOP-NLC was 1657.28 ± 65.87 ng/ml which was significant higher than that obtained with the LOP suspension (956.75 ± 40.25 ng/ml). Meanwhile at all time points the LOP plasma concentrations in rats administered LOP-NLC were remarkably higher than those administered LOP suspension. Twenty-four hours after oral administrationof LOP-NLC, the LOP plasma concentrations was still 467.58 ng/ml; whereas the drug was undetectable after 6 hours of rat treated with same dose of LOP suspension. The corresponding pharmacokinetic parameters were listed in table: 4.71. The AUC0-24 hrs after oral administration of LOP-NLC and LOP suspension was found to be 21,717.72 ±273.78 ng h/ml and 4802.28 ± 152.95 ng h/ml respectively. Which was approximately 4.52 fold higher than that of LOP suspension. The results indicated that systemic absorption of LOP was significantly enhanced by incorporating into NLC compared with LOP suspension. The NLCs thus showed a promising potential for enhancing oral bioavailability of poorly water-soluble drugs. Paired and unpaired t-test was calculated by GraphPad Software. The two tailed P value for paired and unpaired t-test was found to be 0.0027 and 0.0091 respectively. This difference is considered to be very statistically significant by conventional criteria.

Table 4.71 Pharmacokinetic parameters after oral administration of LOP drug suspension and optimizedLOP-NLC.

Sr. No	Formulation	Cmax (ng/ml)	Tmax (min.)	AUC024 (ng h/ml.)	t-Test
1.	LOP drug suspension	956.75 ± 40.25	90	4802.28	Very significant
2.	LOP-NLC	1657.28 ± 65.87	60	21717.72	

The possible reasons for low bioavailability of LOP are its poor water solubility, extensive first pass metabolism, efflux majorly mediated by cytochrome P450 and P-glycoprotein which limit intestinal uptake. We have summarized the possible mechanism of improved oral bioavailability of LOP by employing a NLC formulation. NLC firstly was composed of solid and liquid lipids which were showing structural similarity to fat rich in food. The lipids could induce bile secretion in the small intestinal and the LOP-NLC were associated with bile salt to form mixed micelles which helped the intact NLC avoid the liver first pass metabolism by get into the lymphatic vessels. The uptake and lymphatic transport of intact NLC play a dominant role in promoting absorption. Secondly the particle size range of NLC formulation was less than 200 nm, and a reduction in particle size resulted in a great increase in surface area of the particles. The NLC with high dispersibility was positive to a steady and sufficient absorption in the intestinal tract. On the other hand, LOP which was directly absorbed into the blood circulation would undergo extensive first pass metabolism. In the case of LOP-NLC first-pass effect could be largely avoided because that the drug was encapsulated in nanoparticles and they were available for the lymphatic absorption pathway. Another reason of the improved absorption might be attributed to the use of liquidlipid labrafil M 1944 CS in the NLC formulations. Liquid lipid might be able to inhibit p-glycoprotein efflux pump and increase LOP transport across theintestinal mucosa and also increase lymphatic absorption of drug.

4.8.Stability Studies

The optimized formulations stored in amber glass container were found to be physically and chemically stable for three months at room temperature. From table: 4.8 it is observed that there is no significant change in the particle size, EE and DL of LOP-NLC formulation at 25^0C. This indicates that the drug remains solubilised even at room temperature for three months and lopinavir remained chemically stable in the NLC formulation. The particle size of NLC stored at 40°C was found to be increase due to increased kinetic energy of the system at high temperature (40 °C) which could increase the impact of particles and as a result

improved the possibility of aggregation for nanoparticles. At high temperature EE and DL of NLC also decreased significantly.

Table 4.8: Stability study of LOP-NLC formulation.

Temperature (^{0}C)	Time	Particle size(nm)	EE (%)	DL (%)
25	Initial	159.5	97.7	4.46
	3 months	175.8	95.4	4.22
40	Initial	159.5	97.7	4.46
	3 months	254.61	88.6	3.05

5. CONCLUSION

LOP–NLC for oral administration was successfully prepared by a high shear homogenization method. Glycerol monostearate, labrafil M 1944 CS and poloxamer 188 with transcutol P were selected as the solid lipid (V3), liquid lipid (V2) and surfactant with co-surfactant combination (V1) respectively. Numerical optimization was then used to predict the levels of the factors V1, V2 and V3 required for obtaining an optimum formulation with minimum solid lipid, liquid lipid and surfactant concentration with maximum drug entrapment efficiency, drug loading and minimum particle size. The optimal formulation of LOP–NLC was composed of glycerol monostearate 3 % (V3), labrafil M 1944 CS 1.38 % (V2) and emulsifier 2.28 % in the ratio of 1:1 of transcutol P and poloxomer 188 (V3). LOP–NLC under the optimized conditions showed small homogeneous particle size (159.5 nm) with high encapsulation efficiency (97.77 %) and drug loading (4.46 %). Experimental responses were very close with the predicted values of the optimized formulation, thereby representing the possibility of the optimization method in developing NLC. Investigations on the drug lipid interaction using FTIR and DSC confirmed the compatibility of LOP with the lipid carrier. SEM images indicate that the particles were round and homogeneous with a smooth surface.

In vitro release by reverse dialysis membrane demonstrated that LOP-NLC showed biphasic drug release pattern with burst release at the initial stage and prolonged release afterwards. The rate of release of lopinavir from NLC formulation was found to be significantly higher than the pure drug suspension. Thus major content of LOP in NLC seems to be taken up by the intestinal cells and enter body circulation showing sustained release *in vivo* up to 8 hours.

An oral pharmacokinetic study was conducted in rats and the results showed that NLC produced a significant improvement in the bioavailability as compared to the lopinavir drug

suspension. The AUC0-24 hrs after oral administration of LOP-NLC and LOP suspension was found to be 21,717.72 ± 273.78 ng h/ml and 4802.28 ± 152.95 ng h/ml respectively. Which was approximately 4.52 fold higher than that of LOP suspension. The oral bioavailability of LOP is expected to improve due to the enhanced solubility of drug, higher intestinal lymphatic uptake and inhibition of first pass metabolism of LOP-NLC. The optimized NLC formulation was found to be physically stable for three months at 25^0C but physically unstable at 40^0C. Thus NLC prepared offer a potential approach to enhance the oral bioavailability of poorly water soluble drugs. Based on these findings, we will further study the precise and specific mechanisms of improvement of oral absorption of poorly water soluble drugs in NLC formulations.

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