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Research Article

Formulation and Evaluation of Lipid Based Nanoparticles of Etravirine

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Abstract

Engineered nanoparticles have the potential to revolutionize the diagnosis and treatment of many diseases like HIV/AIDS. Etravirine is one of the key components of highly active antiretroviral therapy used for the treatment of HIV-1 infections. The aim of the present study was to formulate and evaluate nanostructured lipid carriers of etravirine, intended for targeted delivery to macrophages, using solvent emulsification - evaporation technique. Estimates of drug solubility were employed for selection of solid lipids, liquid lipids and stabilizers for the preparation of NLCs. Design of experiments was used to optimize the formulation with respect to drug-lipid ratio and concentration of stabilizer in the external phase using 3^2 full factorial design. Particle size of the carriers and drug release characteristics were the responses which were set to suitable levels for optimization. The optimized formulation was prepared and characterized for size, poly dispersity index, zeta potential, entrapment efficiency and appearance. The nanostructured lipid carriers of etravirine were prepared using stearylamine and glyceryl monostearate as solid lipids, Capryol 90 as liquid lipid and polyvinyl pyrrolidone as stabilizer. All experimental batches showed high drug loading efficiencies nearing 99%, indicating that etravirine remained closely associated with the lipids. The nanostructured lipid carriers displayed a zeta potential of -10.1 mv and a particle size of 261.6 nm with a polydispersity index of 0.374. *In vitro* release of etravirine from the optimized formulation at 2 h was 9% indicative of a low burst; and 56% of the entrapped drug was released after 24 h, suggesting prolonged release characteristics. Thus, etravirine loaded lipidic nanoparticles with potential for targeting cellular reservoirs of the AIDS virus such as macrophages were successfully developed.

Keywords: Etravirine, Nanostructured lipid carriers, full factorial design

INTRODUCTION

Drug release from NLC Human immune deficiency (HIV) infection constitutes one of the most serious infectious disease challenges to public health globally[1]. The causative agent is a lenti virus from the retroviridae family, responsible for the acquired immune deficiency syndrome (AIDS). HIV and other lenti viruses are unique due to their ability to infect and replicate in dividing cells including those of the monocyte/macrophage (M/M) lineage.[2,3,4]

HIV is known for its long period of latency. This is achieved to a certain extent, by the formation of cellular or anatomical reservoirs or sanctuaries by the virus. Three types of cells are representative of cellular reservoirs; quiescent $CD4^+$ lymphocytes (non-productive HIV-1 infected lymphocytes), macrophage and dendritic cells, and follicular dendritic cells. Macrophages constitute one of the most important viral reservoirs because of the ability of virus to reside in multiple tissue compartments and their relative longevity once infected [5,6]. Nearly 99% of all viral replications occur in activated and productively infected $CD4^+$ T cells of the blood and lymphoid tissues such as the peripheral secondary lymphoid organs, spleen, lymph nodes and gut associated lymphoid tissue. Monocyte/Macrophage (M/M) and cells derived from that lineage are other major cellular targets of HIV-1 infection. M/M become more important as a viral reservoir only when the majority of $CD4^+$ T cells have been lost later in HIV-1 disease. Therefore, a critical step for eradicating HIV infection requires the targeted treatment to infected M/M cells.

Based on the extensive knowledge about the HIV replication cycle, drug targets have been identified over the years and effective treatment options are currently available. The mainstay of clinical therapy, known as Highly Active Anti-Retroviral Treatment or HAART is considered as one of the most significant advances in contribution towards reducing the mortality in patients. Chronic intake of antiretrovirals is mandatory to control HIV infection without which viral replication resumes several weeks after withdrawal. Many antiretroviral drugs undergo significant first pass metabolism and gastro intestinal degradation leading to low and erratic bioavailability. Their half life is short, which leads to the requirement of frequent administration of doses leading to decreased patient compliance[7]. The current HIV treatment is also associated with short residence time and also low concentrations of antiretrovirals at certain inaccessible and latent viral reservoir sites which includes infected M/M cells, leading to relapse of the disease[8,9,10].

The use of a novel drug delivery system can present an opportunity to overcome the challenges associated with anti-retro viral therapy. Novel systems refer to strategies,

technology, formulation-based approaches and customized systems developed for safe and effective drug delivery.

Nanocarriers constitute one such versatile drug delivery system, which may be employed for the delivery of antiretroviral drugs, due to their ability to overcome physiologic barriers and guide the drug to specific cells or intracellular compartments due to their small size, typically in 10-1000 nm range [11,12]. They could be engineered to reach specific sites in the body either by passive or ligand mediated targeting mechanisms.

Among the various nano systems, lipid-based particles are recognized as a subset with several advantages including good biocompatibility, high *in vivo* stability, ability to protect the drug, sustain its release and alter its biodistribution. Further, they possess a broad application spectrum for administration via several routes[13]. Nano structured lipid carriers (NLCs), frequently referred to as second generation lipidic nanoparticles are composed of a lipid matrix formulated using a mixture of lipids, both solid and liquid, with embedded active compounds. The presence of liquid lipid renders a largely amorphous matrix since the presence of liquid prevents crystallization of the solid lipids, which in turn results in a suitable environment for holding the drug and preventing its premature expulsion.

Etravirine (ETR) is the first drug in the second generation of Non-Nucleoside Reverse Transcriptase inhibitors (NNRTIs) and has been marketed for the treatment of HIV infection. ETR is more effective than other first generation NNRTIs due to its activity against NNRTI-resistant HIV-1[15]. It is a diaryl pyrimidine compound allowing flexible binding to the reverse transcriptase in multiple conformations, therefore resulting in less susceptibility to drug resistant mutations. The drug is highly bound to plasma proteins and is primarily metabolized by cytochrome P450 CYP 3A4, 2C9, and 2C19 enzymes. This highly lipophilic agent has an octanol: water partition coefficient (log P) greater than 5 along with an ionization constant (pka) of 3.75. The recommended dose of ETR is 200 mg taken twice daily following a meal [16] . However, there are some significant drawbacks to its oral use, which include very low aqueous solubility, poor permeability (since it is a BCS class IV agent) and limited bioavailability because of its highly hydrophobic nature. ETR loaded nanoparticles when administered parenterally can not only overcome the limitations of oral bioavailability, but also serve to target the drug to the cellular hosts used by the HIV for multiplication, thereby increasing its therapeutic efficacy. The aim of the present study was to formulate and characterize etravirine nanoparticles with suitable properties for parenteral targeting of cellular viral reservoirs such as macrophages using design of experiments.

MATERIALS AND METHODS

Etravirine was kindly provided by Hetero Pharma, Hyderabad, India. Capryol 90, Labrafac and Labrasol were received as gift samples from Gattefosse, Mumbai, India. Tween 20 Tween 80, Poloxamer188, and Poloxamer 407 were purchased from Sigma Aldrich. Dichloro methane was procured from SD Fine chemicals Limited Mumbai, India**.** Poly vinyl alcohol (Degree of polymerization 1700-1800) was purchased from Qualikems fine chemicals Pvt ltd, Vadodara, India, stearylamine from SRL chemicals, Maharashtra, India and glyceryl monostearate from RX chemicals, Maharashtra, India.

Solubility of ETR in solid lipids

The solubility of ETR in several solid lipids was determined semi-quantitatively. Solid lipids were shortlisted for the study based on an elaborate survey of literature. One gram of each of the lipids was accurately weighed and transferred into a boiling tube which was then heated in a water bath at a temperature 5 °C above the melting point of the lipid. ETR was added to the molten lipid in 5 mg increments and stirred with a glass rod. Solubilisation of drug was checked by visual inspection[17]. If the drug completely dissolved, the next increment of 5 mg was added and the procedure continued. The maximum quantity of ETR which could be dissolved, after which the subsequent increment failed to dissolve was considered as solubility of ETR in the respective oils.

Solubility of ETR in oils

To determine ETR solubility in oils, 2 ml of different liquid lipids was taken into vials and excess amount of drug was added to each vial. These vials were kept on rotary shaker for 48 h under ambient conditions. The undissolved drug was filtered, required volume of the liquid lipid was suitably diluted in methanol and analysed using UV spectrophotometry. The experiment was carried out in triplicate.

Selection of combination of solid and liquid lipids

Based on the outcome of the solubility estimates, selected oils and lipids were examined further for choosing their relative proportions. Here, lipids taken together in different ratios were molten, mixed together and cooled till 37°C. The combination which allowed maximization of the liquid lipid yet yielded a solid mass at body temperature was selected for preparation of NLC.

Selection of surfactant

Various surfactants were screened for ability to solubilize the drug in aqueous medium and also for NLC stabilization. For the former study, 10 ml of a 1% w/v solution of different surfactants was prepared in distilled water and transferred to clean glass vials. Excess amount of drug was introduced in to each vial. These vials were kept on rotary shaker for 48 h under ambient conditions[18]. The solutions were filtered, suitably diluted and analysed. The surfactants showing limited ability to solubilize ETR were further screened in preliminary trials for preparation of NLC and the most suitable stabilizer was selected by its ability to form a stable NLC dispersion.

Formulation of NLC

Nanostructured lipidic carriers of ETR were prepared using solvent emulsification and evaporation technique. Measured amounts of solid and liquid lipids along with 20 mg ETR were dissolved in 5 ml of dichloromethane. The external phase (40 ml) was kept stirred at 2000 rpm using an overhead stirrer (Remi motors). During this time, the organic phase was slowly injected into it with the help of a syringe and needle over a period of 2 minutes with continuous stirring resulting in emulsification. The crude dispersion was immediately subjected to probe sonication (Q Sonica Q55) for 30 min at 80% amplitude. Next, the nano-dispersion produced was once again transferred to the overhead stirrer to allow for complete solvent evaporation. The entire procedure was conducted at 25° C. The NLC dispersion was promptly subjected to evaluation studies as described in later sections.

Formulation optimization by design expert (DOE)

An Ishikawa fish bone diagram was used to identify the processing conditions as well as formulation variables likely to impact the Critical Quality Attributes (CQAs) of the ETR loaded NLC. Particle size, entrapment efficiency of ETR and drug release were considered to be the CQAs. Preliminary experiments were used to further identify the critical variables to be optimized via the DOE for achieving target CQAs.

A full factorial design with two independent variables, each at three levels was used to arrive at an optimal formulation of NLCs. Design Expert Software (Version 13 Stat-Ease 360[R]

software trial) was used for the exercise. The DOE design suggested a total of 9 experimental runs in random sequence. These were prepared and analysed for particle size, entrapment efficiency and drug release. The data was analysed statistically and ANOVA was applied to assess the significant factors. Contour plots and equations were generated to understand and quantify the effect of the independent variables on the selected properties of the NLC.

Characterization of the NLCs

Particle size and zeta potential

The particle size and zeta potential of the formulations were determined using a zeta sizer (Malvern, USA). The sample to be analysed was diluted ten times with Milli Q water (Refractive index- 1.40; Viscosity- 0.887 cps). The analysis was done at a scattering angle of 90° and at a temperature of 25°C.

Each sample was analysed for average particle size and poly dispersity index. For zeta potential determination the sample was diluted similarly and introduced in the electrophoretic cells. All analyses were done in triplicate.

Entrapment efficiency

The entrapment efficiency of the developed ETR-NLC was determined by centrifugation method. ETR-NLC dispersion (5 ml) was centrifuged using research centrifuge -Remi R-24 at 17,000 rpm for 1hr. The dispersion medium was filtered through 0.22µm filter and further diluted using methanol for analysis. The ETR content was analysed using UV spectrophotometry at 311 nm and the entrapment efficiency was assessed using the equation[20].

Drug Entrapment Efficiency (%w/w) = W_{total}– W_{drug} X 100/ W_{total}

Where W_{total} = Total amount of ETR used in the formulation

W_{drug} = Amount of ETR in supernatant

***In vitro* drug release study**

The release study of ETR-NLCs was performed by dialysis bag method using a dialysis membrane with a molecular weight cut off between 12 kDa and 14 kDa (Hi-Media, India). ETR-NLC equivalent to 2.5 mg of ETR and 2 ml of the dissolution medium was added into the dialysis bag. The ends of the dialysis bag were sealed with clips and each dialysis bag was introduced into a beaker containing 50 ml of phosphate-buffered saline (PBS) pH 7.4 with 1 % w/v tween 80 at 37°C as the dissolution medium. The release medium was kept stirred using a magnetic bar. Aliquots (2 ml) were withdrawn at predetermined intervals and drug release was determined using UV spectrophotometry. The volume of the dissolution medium was maintained with the addition of fresh equivalent medium after each withdrawal[21]. Similar process was adopted for estimating the passage of unentrapped ETR across the dialysis membrane by taking 2.5 mg of drug dispersed in 5 ml of pH 7.4 phosphate buffer with 1 % w/v tween 80 in to the dialysis bag. Samples were taken at regular intervals and drug release was determined.

Table 1: Independent variables with their levels used for the 3^2 full factorial design and dependent variables and target used for optimization

Independent variables	**Actual levels and their codes**
	Low (-1) Medium (0) High (1)
Total amount of lipid per 20 mg ETR	100mg 200mg 300mg
% of PVA w/w	1.5% 2.25% 3%
Dependent variables	Particle size ---- Target 250nm Drug release at 2hr --- minimal Drug release at 24hr ---maximum

Table 2: NLC Formulations according to design expert

SNO	**Formulations**	**Factor A: Concentration of PVA in external phase**	**Factor B: Amount of lipid in internal phase**
1	F1	-1	-1
2	F2	0	-1
3	F3	1	-1
4	F4	-1	0
5	F5	0	0
6	F6	1	0
7	F7	-1	1
8	F8	0	1
9	F9	1	1

Preparation and evaluation of the optimized batch of NLC

The optimized batch as suggested by the software was prepared and further analysed for particle size, entrapment efficiency, drug release as described earlier. The particles were also visualized using scanning electron microscopy. SEM includes placing of dried nanoparticles on brass stub, which will be gold coated in an ion sputter to render them electrically conductive. Photomicrographs of nanoparticles were taken at different magnifications.

RESULTS AND DISCUSSION

Solubility of ETR in oils and solid lipids

To enable high loading of the drug into the lipidic carrier, ability of the lipid excipients used for preparation of the NLC to dissolve the drug was considered as a critical attribute for selection of individual lipidic components. Different solid lipids like stearic acid, glyceryl monostearate, Compritol®, Precirol® and stearylamine were screened during the study and overall, all the solid lipids examined were found to have limited capacity to dissolve the drug. Besides being practically insoluble in aqueous media, ETR is also reported to exhibit poor solubility in lipidic systems[22]. ETR was found to have the highest solubility in stearylamine and glyceryl monostearate (between 10-15 mg/ gm of lipid) and hence, these were selected as solid lipids for formulation of NLC.

Likewise, several oils like Labrafac®, Labrasol® (Lsol) oleic acid, Capryol 90®, isopropyl myristate (IPM) and Transcutol® were screened for solubilization of ETR. Amongst them Capryol 90® (10.288±0.67 mg/ml) and oleic acid (11.496±0.871 mg/ml) showed highest solubility (**figure 1)** and therefore both were chosen for further screening as liquid lipids for the formulation of NLCs.

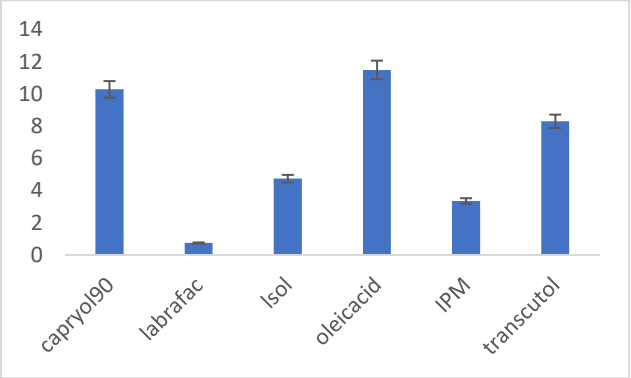

Figure 1: Solubility of Etravirine in different liquid lipids

Combination of solid and liquid lipids

The rationale for the use of NLCs for delivery of ETR was based on the fact that NLCs contain heterogenous lipids which in turn produced less ordered lipidic core. This imperfection of internal arrangement aids more drug accommodation and reduces the risk of drug expulsion due to lipid crystallization[23]. Typically, for preparation of NLC, solid lipids are reported to be mixed with liquid lipids in a ratio of 70:30 up to a ratio of 99.9:0.1[24]. In case of the ETR loaded NLCs, an approach of selecting a highly heterogenous lipidic composition with maximal amount of liquid lipid was attempted. This would be expected to lead to the preparation of NLCs classifiable as a Type II model with advantages of minimal drug leakage and sustained release along with high entrapment efficiencies[25]. The relative proportions of lipids were chosen based upon the ability of the mixture to remain solid near body temperature despite a high load of liquid lipids (**Table 3).**

Table 3: Results of melt-congealing experiments on combination of solid lipid and liquid lipid

Combination of solid and liquid lipid	Texture	Melting Point
Oleic acid + stearylamine (1:1)	Yellow soft shiny solid	35-37°C
Oleic acid + glyceryl monostearate (1:1)	Light yellow soft solid	35-37°C
Capryol 90® + glyceryl monostearate (1:1)	Waxy hard solid	65-67°C
Capryol 90® + stearylamine (1:1)	Waxy soft solid	65°C
Capryol 90® +stearylamine+glyceryl monostearate (3:1:2)	Waxy soft solid	65-66°C

Although oleic acid had good solubility for ETR, it was found that its mixtures with the solid lipids were considerably low melting. From the melting point and texture, a combination of Capryol 90®, Stearylamine and Glyceryl monostearate was selected for preparation of the lipid matrix of the NLCs.

Solubility of ETR in stabilizers

The NLCs were prepared by an emulsion-solvent evaporation method wherein the lipid-drug solution in a volatile solvent is emulsified into an aqueous phase containing a suitable stabilizer. Different stabilizers were screened as 1% aqueous solutions for the solubility of ETR with the intent of selecting a stabilizer which has the least ability to solubilize ETR allowing for maximal entrapment in the lipid phase. Thus, poloxamer-188 (P-188), poloxamer-407 (P-407), Tween 80 (T-80), Tween 20 (T-20), Poly vinyl alcohol (PVA) and Sodium deoxycholate (SDC) were evaluated. ETR showed least solubility in SDC, P-188, P-407 and PVA solutions (**figure 2)**. Further, during preliminary trials, it was found that SDC, P-188 and P-407 failed to allow for suitable emulsification. Emulsions with suitable stability and fine globule size could be prepared using PVA as the stabilizer in the external phase. PVA is also widely reported as a stabilizer for the emulsion solvent evaporation-based preparation of various drug carriers including those based on lipids and polymers[26]

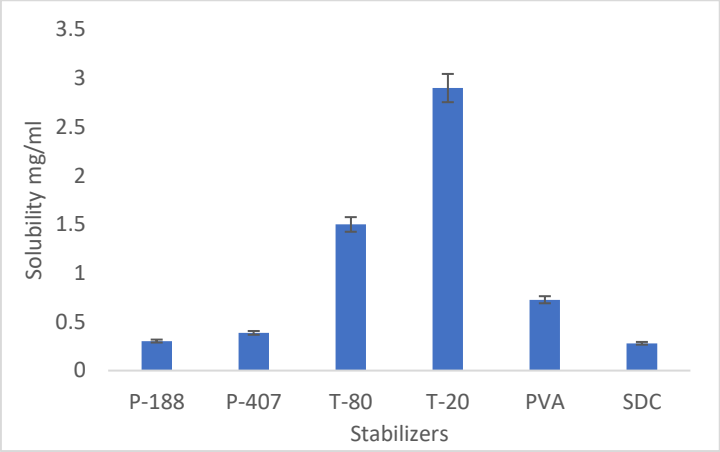

Figure 2: Solubility of Etravirine in different stabilizers

Formulation of Etravirine NLC by DOE

The Ishikawa fish bone diagram prepared for identifying the critical process and formulation variables are given in (**figure 3)**

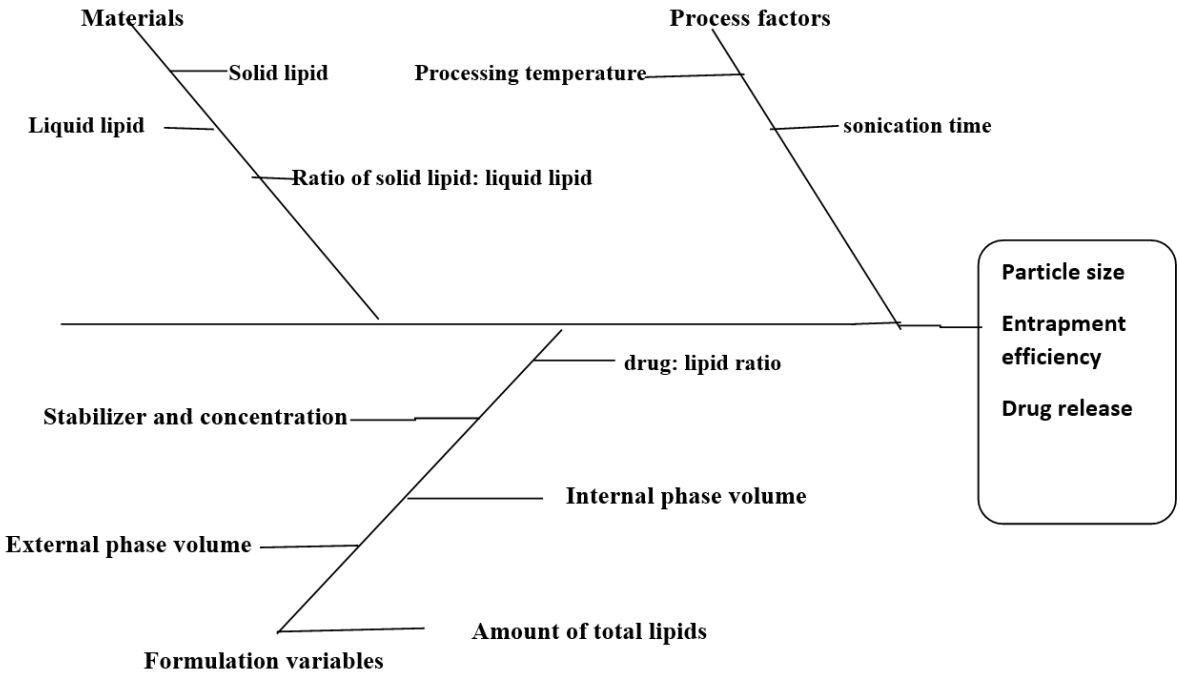

Figure 3: Fish bone diagram for selection of variables

In case of the material related variables, the type of lipids, their relative proportions and the stabilizer to be used were established through preliminary experiments described above. The amount of dichloromethane used as internal phase was dictated by solubility of ETR and 5 ml of the class 2 solvent was found to be necessary to dissolve the drug and lipid mixtures. Likewise, 40 ml of the external phase was also found to be a suitable process volume. In case of the process variables, sonication amplitude and time were considered as important parameters for optimization. However, it was found through preliminary experiments that operation at the upper end of the equipment settings (80% amplitude) for a substantially long duration (30 min) was necessary to obtain the desired size. Hence these parameters were also fixed at the above-mentioned levels during the optimization exercise. Early experiments to examine the temperature during preparation led to the conclusion that preparation at lower temperature (15°C) led to the lipid phase being highly viscous and therefore difficult to emulsify, whereas use of high temperature (35°C) led to very rapid evaporation of the internal phase solvent, again resulting in a rapid thickening of the internal phase and preventing fine globule sizing of the emulsion. Hence the temperature of the medium during preparation was maintained at 25°C.

For optimization of the product, the total amount of lipids used (which also impacted the lipid: drug ratio) and the

concentration of stabilizer in the external phase were the independent variables evaluated during the DOE exercise.

Similarly, during preliminary experiments it was noticed that irrespective of change in temperature, sonication speed and other parameters, at all times more than 99% of the etravirine added was entrapped within the NLCs. The observation was attributed to the solubility of ETR in the lipid phase and poor partitioning ability into the external PVA containing aqueous medium. This led to the conclusion that the CQAs which entailed risk with change in the formulation parameters were particle size and drug release from the NLC. Further, in case of the drug release behaviour, release at the end of 2 h and 24 h were chosen as response variables.

Table 4: Formulations prepared as per DOE and the results of the dependent variable estimations

Sr. No.	Formulation code	Factor A: Concentration of PVA %	Factor B: Amount of lipid mg	Response 1 Particle size nm	Response 2 Release at 2 h %	Response 3 Release after 24 h %	Entrapment efficiency % w/w
1	F1	-1	-1	236	13.7	76.5	99.00
2	F2	0	-1	214	18.2	81.2	98.56
3	F3	1	-1	201	20.9	88.3	97.45
4	F4	-1	0	305	8.4	55.3	98.73
5	F5	0	0	273	15.4	69.7	98.98
6	F6	1	0	259	16.9	71.9	99.37
7	F7	-1	1	508	4.6	19.8	99.40
8	F8	0	1	461	6.5	28.6	98.70
9	F9	1	1	398	10.3	32.6	99.00

Prior to the optimization exercise, targets were set for the dependent variables.

It is widely reported that nanoparticles with a size greater than 200-300 nm is effective in passively targeting the payload to various viral reservoir sites, chiefly the macrophages[27,28]. Hence for the present optimization exercise, the target size for the NLCs was set at 250 nm. To avoid premature release of the drug, the burst release measured as the percentage of drug released at the end of 2 h was minimized whereas the drug release at 24 h was maximized. The 2 h window is intended to ensure uptake by macrophages[29] both at tissue and blood level and the ensuing sustained drug release to allow for virucidal action in infected macrophages.

The response analysis model was based on the lack of fit test, sequential and model summary statistics. Simultaneously analysis of variance (ANOVA) was applied to determine the significance of the variables and their effects. The results of the characterization studies for the DOE batches are presented in **table 4.**

Effect of independent variables on particle size: The mean particle size of the different batches ranged between 200-500 nm. The developed model relating the particle size with the independent variables were found to be significant with p value 0.0007.

The final equation describing the impact of the levels of the independent variables on particle size is

Particle size = + 277.8 - 31.8 Concentration of PVA +119.3 Amount of lipid - 18.75 Concentration of PVA* Amount of lipid + 1.83 Concentration of PVA^2 +57.33 Amount of $lipid^2$**.**

As seen from the equation, the size of the lipid nanoparticles was strongly influenced by both the concentration of PVA in the external phase and the amount of lipid used during preparation. The signs of the coefficients in the equation indicate that as the amount of lipid was increased, the particle size increased whereas the concentration of PVA had an inverse impact, as it increased, the particle size was found to decrease. This is as expected since more lipid would mean a denser internal phase which is more difficult to break up, whereas more stabilizer in the external phase would aid globule size reduction. The level of PVA in the external phase has been reported to be an influential factor which dictates the particle size of lipidic nanoparticles when produced by the emulsion solvent evaporation method[30]. The contour plot generated also clearly reflects this (**figure 4).**

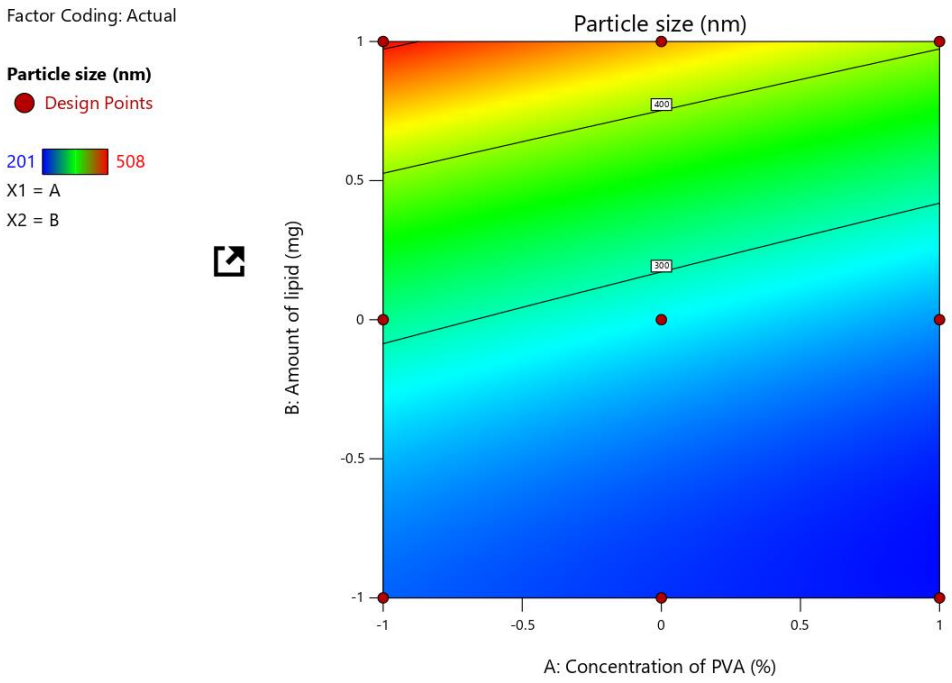

Figure 4: Contour plot showing the effect of PVA% and amount of lipid on particle size

Effect of independent variables on drug release:

The drug release profiles obtained from the various DOE batches are depicted in **(figure 5)**.

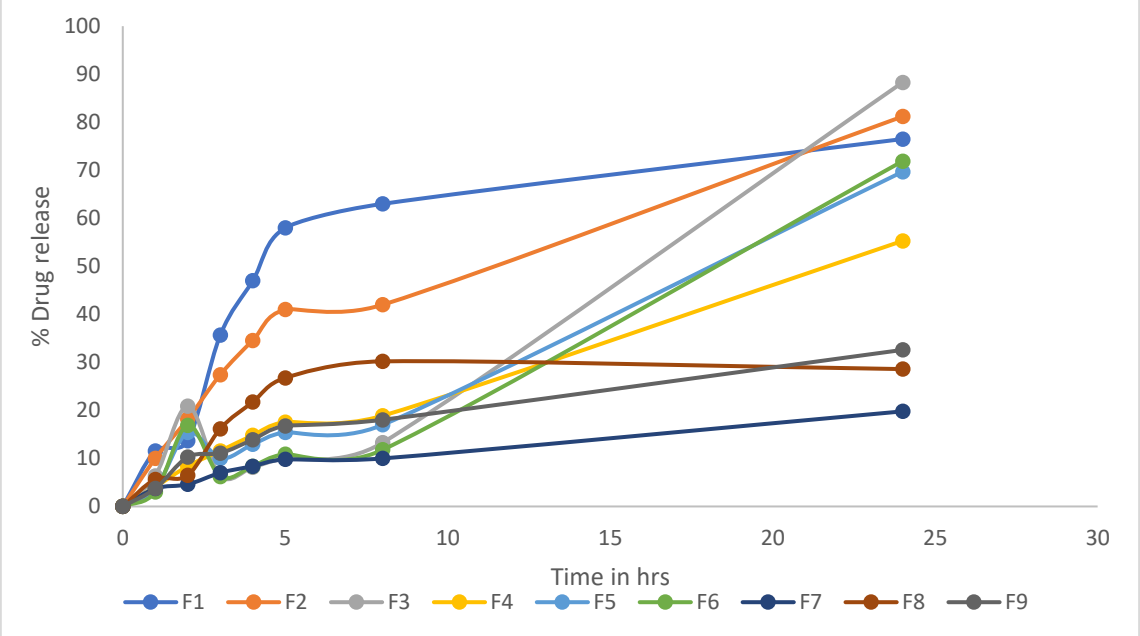

Figure 5: % Drug release from the experimental batches of ETR NLC

Release at 2hr

The drug released at the end of two hours was used as an estimate of burst release. The different DOE batches showed burst release between 4.5 to 21% of the entrapped drug. This release was considerably less than that often reported from NLCs[31,32,33] possibly due to the preference of the drug for the lipidic environment within the particles than the aqueous release medium. The release at two hours showed a linear relationship with the independent variables, increasing with the amount of PVA in solution and decreasing with the amount of lipid as shown in (fig 5). This effect can be simplistically analysed based on the impact of the two variables on the particle size. Since increase in PVA levels and decrease in the amount of lipid used for entrapment lead to smaller particles with a larger surface area, the burst release was found to be more. Besides, the use of more lipid content for encapsulation of the same amount of ETR also leads to superior drug entrapment and lower burst release.

The model was found to be significant (with a p value of 0.0001) and the equation relating the variables was obtained as

Release at 2 hour = + 12.8 + 3.6 Concentration of PVA -5.23 Amount of lipid.

The contour plot is presented in (**figure 6)**

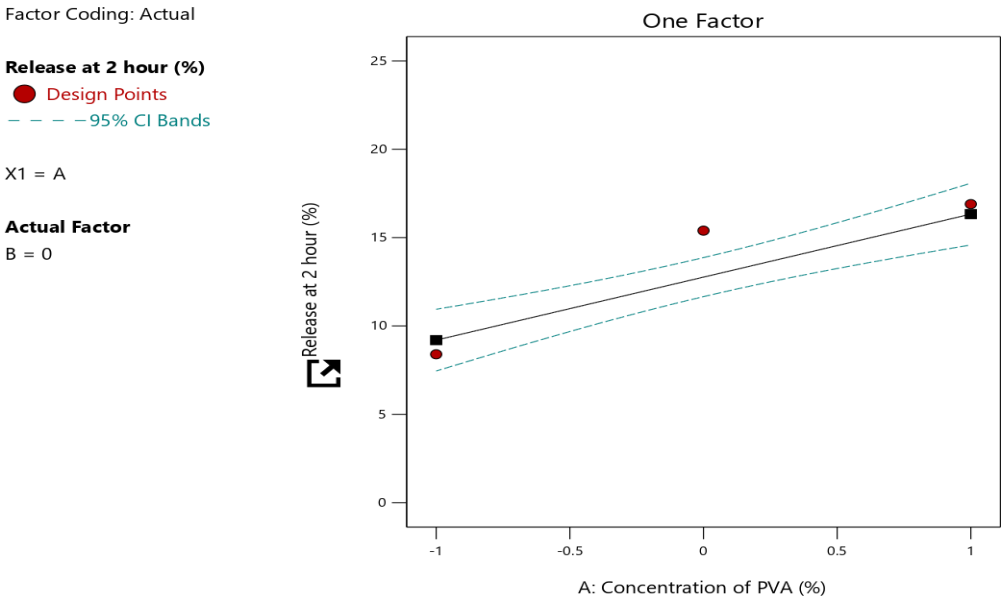

Figure 6: Contour plot showing release at 2hr

Release after 24 hr

The drug release from NLCs is reported to be a function of several factors including the partition coefficient of the drug molecule, type and concentration of the emulsifier used, type and amounts of lipids used, and the method and conditions used for preparation including the temperature [34,35]. For the present studies, the actual percentage of ETR released from the experimental batches ranged from about 19% to nearly 90%. To bring about the complete release of the drug after the nanoparticles were engulfed within the macrophages, the target set was that the release at the end of 24 h was intended to be maximized.

The ANOVA test reported a significant quadratic model with p value of 0.0011. As seen from the equation generated and the contour plot **(figure 7)**, the release corelated directly with the amount of PVA used as stabilizer and inversely with the total amount of lipid used for preparation.

Release after 24 hr = + 67.25 + 6.9 Concentration of PVA -27.5 Amount of lipid + 0.25 Concentration of PVA* Amount of lipid - 2.43 Concentration of PVA^2-11.13 Amount of lipid^2

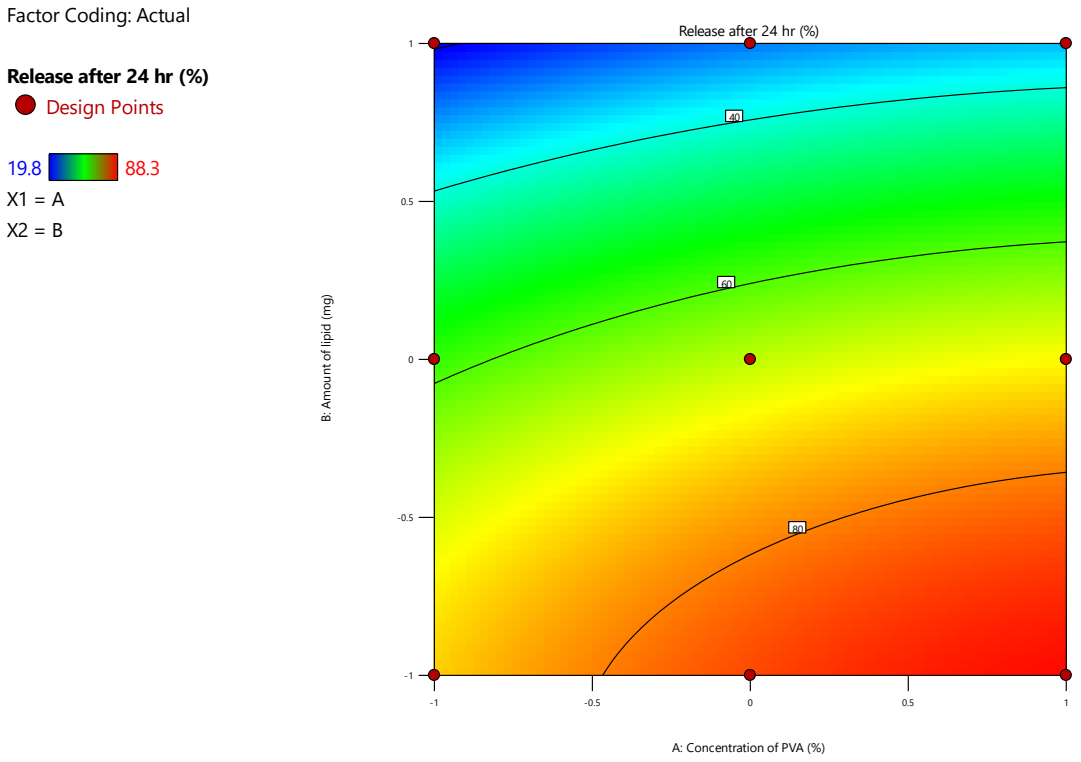

Figure 7: Contour plot for release after 24hr

Preparation and evaluation of the optimized batch of NLC:

Once the relationships between the particle size and release with the independent variables were understood, the next step was to identify an optimal formulation which would allow generation of nanoparticles meeting the target profile.

A total of 11 formulae with various levels of desirability were suggested by the software. The formulation with the highest desirability as shown in (**figure 8)** was identified, prepared and characterized for its critical quality attributes.

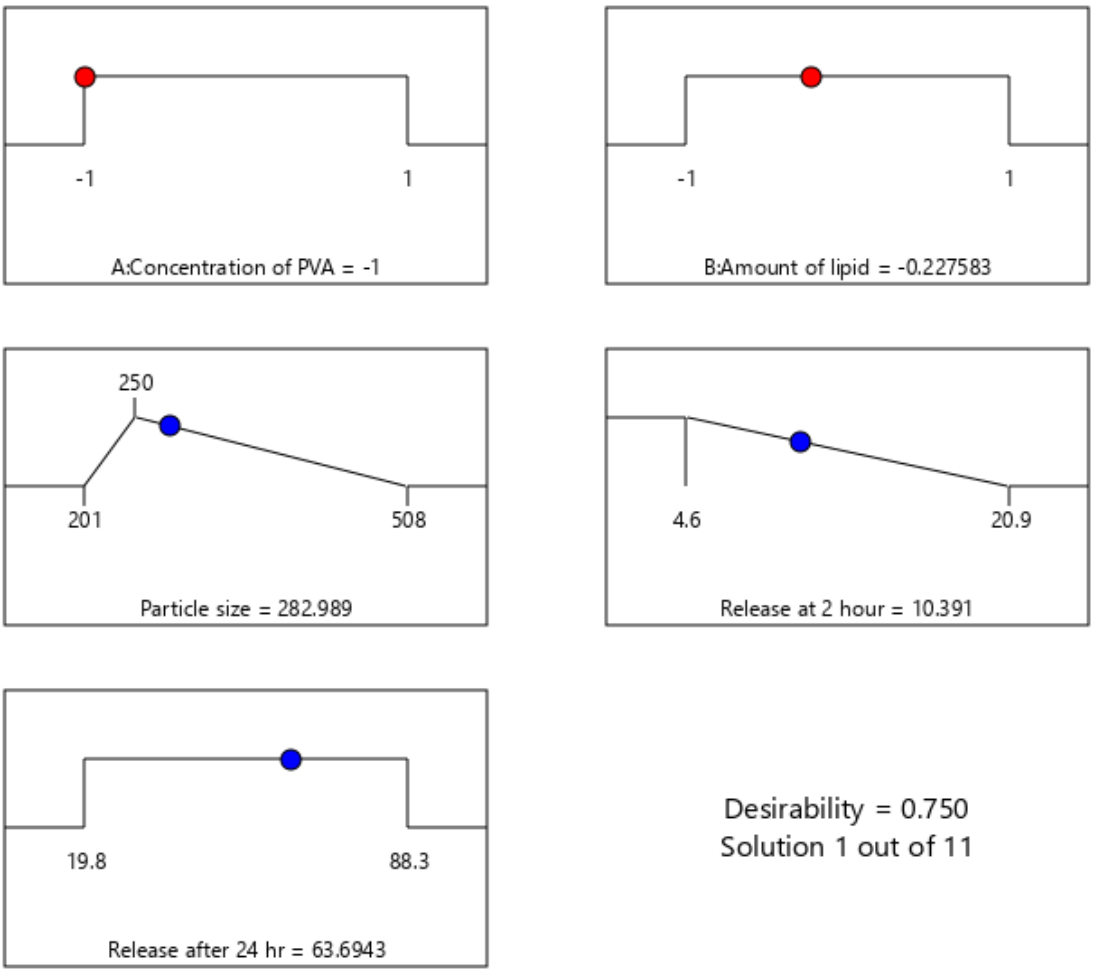

Figure 8: Desirability of optimal formula as suggested by DOE software

Table 5: Optimized batch of NLC and its predicted and experimental properties

Independent variable	**value**	
Amount of lipid in internal phase	180 mg: Stearylamine – 27mg Glyceryl monostearate –54mg Capryol 90® -- 99mg	
Stabilizer solution used as external phase	1.5% w/w PVA solution	
Response	Predicted	Experimental
Particle size (nm)	250	261.6
Drug release after 2h Drug release after 24 h	10.39 % 63.69%	9.05% 59.80%

From **table 5** it is observed that the experimental values of the prepared NLCs were close to the predicted values as given by the design software.

			Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	261.6	Peak 1:	283.5	94.6	133.5
PdI:	0.374	Peak 2:	5182	5.4	485.4
Intercept:	0.966	Peak 3:	0.000	0.0	0.000

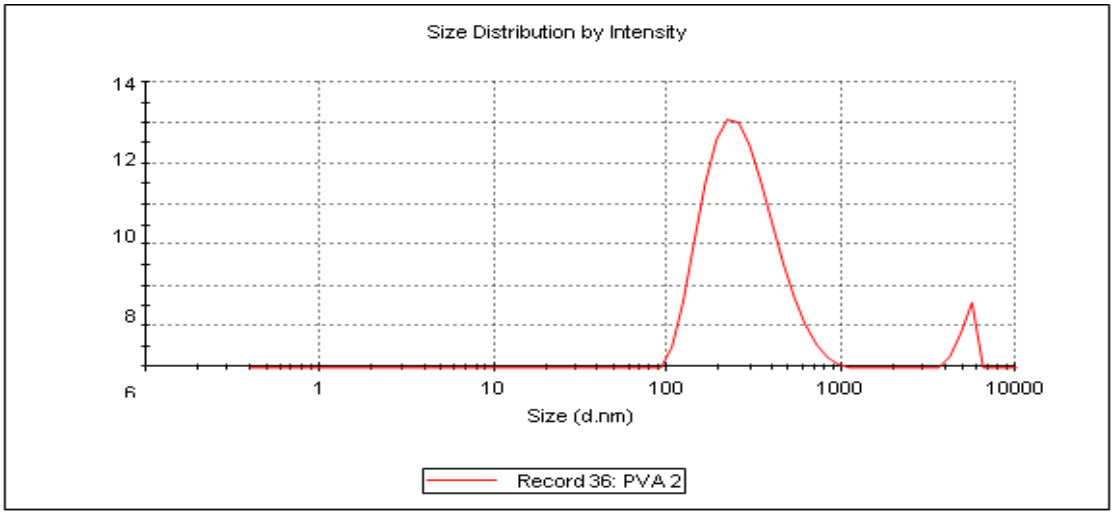

Figure 9: Average particle size of NLC formulation

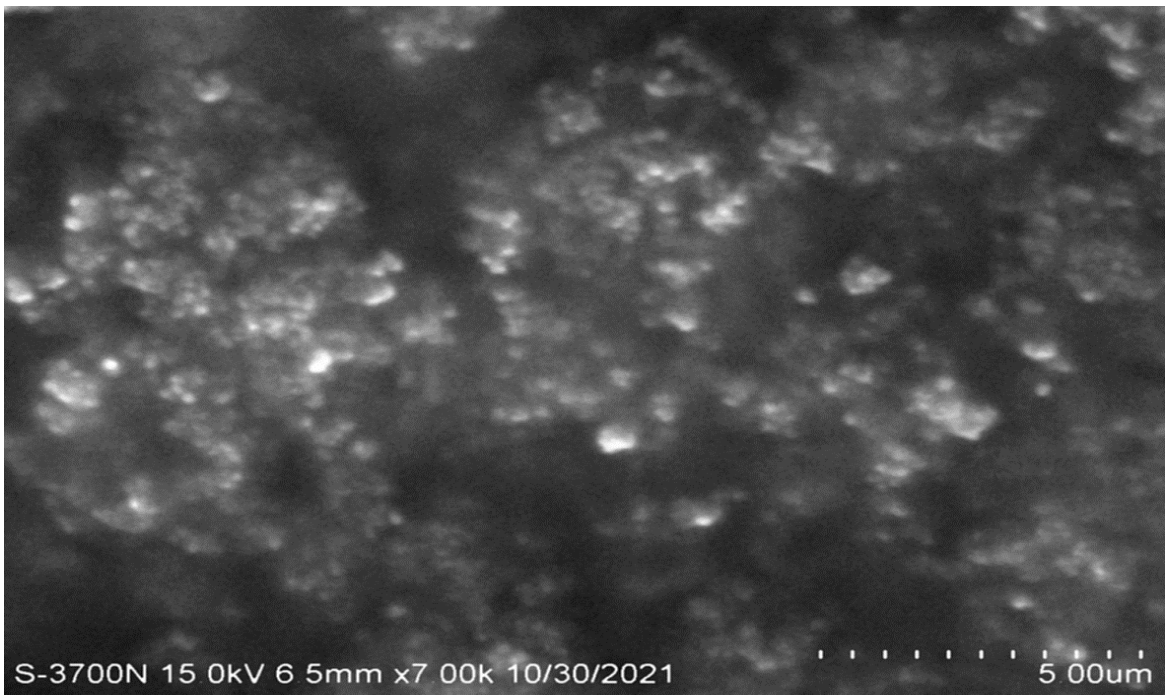

Figure 10: SEM image of optimized NLC formulation

Average particle size of the prepared NLC was 261.6 nm (**figure 9**) which is reported to be ideal size for targeting to tissues like liver, spleen and other macrophage associated tissues according to literature[36]. PDI of 0.374 indicates that size range of nanoparticles obtained were narrowly distributed. The SEM image of the NLCs seen in (**figure 10**) also reflects this finding.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -10.1	Peak 1:	-10.1	100.0	7.11
Zeta Deviation (mV): 7.11	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.407	Peak 3:	0.00	0.0	0.00
Result quality Good				

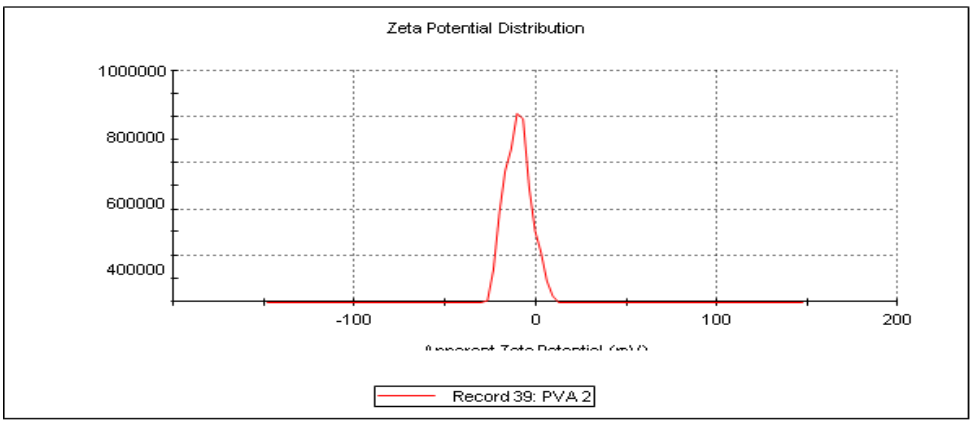

Figure 11: Zetapotential of optimized etravirine loaded NLC formulation

Zeta potential of the optimized formulation was found to be -10.1 mv (**figure 11)** It is known that zeta potential of more than ± 30 mv are considered a good indication for the stability of the nanoparticles. In order to maintain the stability of nanoparticles the suspension can be lyophilized and reconstituted at the time of administration.

Release studies of optimized formulation

From **figure 12** it is observed that 95.48% percent of the unentrapped ETR permeated through the dialysis membrane after 5 h from whereas the optimized NLC formulation released 59.8% of drug over a period of 24 h which indicates sustained release from NLCs.

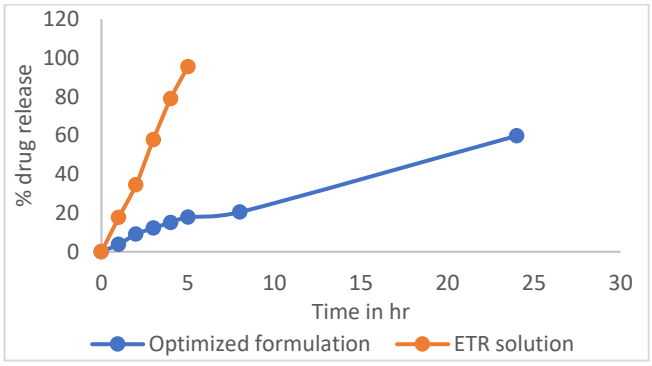

Figure 12: Comparison of drug release for optimized formulation and ETR solution

CONCLUSION

In the present study, ETR loaded NLCs were formulated successfully by solvent emulsification and evaporation technique employing a 3^2 full factorial design. Lipidic particles with high drug loading efficiency (greater than 99%) could be obtained. During the optimization exercise, it was noticed that drug release and particle size were significantly affected by concentration of PVA as stabilizer in the external phase and drug lipid ratio. Perfectly spherical NLCs with average particle size of 261.6 nm and a narrow size distribution were obtained. Optimized product was found to show low burst release and allow sustained release of ETR for over 24h. Further, *in vivo* studies are required to confirm their targeting to the reservoir sites and in the management of HIV infection.

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