e-ISSN: 2320-4230, p-ISSN: 2961-6085

Journal of Drug Discovery and Therapeutics

Available Online at www.jddt.in

CODEN: - JDDTBP (Source: - American Chemical Society)

Volume 12, Issue 06; 2024, 50-60

Invitro Study of Phospholipid Assisted Nano-Suspensions of Efavirenz

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Received: 22-09-2024 / Revised: 25-10-2024 / Accepted: 28-11-2024

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Conflict of interest: No conflict of interest.

Abstract:

According to WHO, AIDS caused by HIV has killed over 770,000 people worldwide in 2018 withover 37.9 million people living with HIV. With advancement in science, the current regimen termedas Highly active antiretroviral therapy (HAART) hasincreased the life expectancy of patients. Since HAART generally includes a combination of three drugs, patient compliance as well as side-effects due to high doses of the drugs are common drawbacks of the regimen. Nano systems have been known to possess various advantages such as sustained and targeted delivery of drugs which often causes improvement-in bioavailability and reduction in side effects Efavirenz, a non nucleoside reversetranscriptase inhibitor (NNRTI) is a first line drug given in combination with oth er drugs as part of the HAART. Due to its high dose and erratic absorption patterns, EFV has been reported to show poor bioavailability. Moreover, absorption of EFV has been shown to increase in presence of food.The aim of the work presented in the thesis was to design phospholipid assisted nanosuspension for delivery of EFV, to characterize them by *in vitro.*

Keywords: Efavirenz, Nanosuspension, Antiretroviral, Lipolysis

INTRODUCTIO

There are two strains of HIV namely H IV1 and HIV2, of which HIV1 is patho genic an prevalent. HIV targets the $CD4^+$ T cells and spreads to the lympho id organs. The virus becomedetectable i n the blood after about 10 days,the infe ction spreads exponentially over time. H IV causes progressive reduction in $CD4^+$ T cell count with count <200cells per µl and presence of the virus being term ed as AIDS.[4]

Antiretroviral therapy (ART) has aided i n combating AIDS to a large extent by reducingthe viral load, improving the lif e expectancy of patients, and reducing t he transmission. Currentregimen for AID S is called HAART which is a combina tion of three drugs Which target different enzymes in the HIV life cycle.

Drawbacks of Current Regimen

Even though HAART has been useful in controlling viral replication and decreas ing mortality, there are certain disadvant ages associated with the therapy. Some of them include:

- Antiretroviral drugs are often poorly soluble in nature which leads to decr

ease in their oral bioavailability.

- Shorter residence times of the drugs mean lesser concentration in the reservoir siteslike thelymphoid tissues, central nervous system,and lungs. Hence, prolonged duration with higher doses of drugs are needed to achieve optimal concentrations of the drug in the body which also leads to viral resistance.[7]
- Patient compliance has also been low due to side effects and toxicity of the drugs associatewith taking them for a longer time.
- The high cost of HAART is another problem which increases the burden on the developing countries where the prevalence of the infection is the highest.[8]

Nanosystems are versatile drug delivery systems, with an ability to overcome physiologic barriers and to guide the drug to specific cells or intracellular compartments due to their small size, typically in the 10 to 1000 nm range. Nanosystems offer several advantages,such as the protection of drugs against degradation, targeting of drugs to specific sites, and tailoring the release kinetics to provide prolonged release of the drugs.[8] Polymeric nanoparticles, solid lipidnanoparticles, liposomes, nanosuspensions and nanoemulsions have been reported to enhancethe effective delivery of the drugs.

Efavirenz, a BCS Class II drug, is practically insoluble in water with solubility <10µg/ml and low intrinsic dissolution rate of 0.037 mg/cm^2/min.[12] Due to its poor solubility and erratic oral absorption, the bioavailability of EFV is reported to be 40%.[13] Efavirenz has shown to have increased absorption in the presence of food with 28% increase in mean AUC and a 79% increase in mean C_{max} of EFV relative to the fasted condition.[14]

Hence, lipid assisted drug delivery systems likenanosuspensions with phospholipid as stabilizer was hypothesized to improve solubility and aid lymphatic uptake[15] of the poorly soluble Efavirenz.

Preparation and Characterization of EFV nano- Suspensions

Phospholipid was dissolved in acetone while the other stabilizers were dissolved in water.The organic phase was added to the aqueous phase under constant vortexing until a uniform dispersion was formed. The dispersion was stirred on a magnetic stirring till complete evaporation of acetone.
Phospholipon® 90 G and Tween® 80 formed a nanosuspension which did not show any instability
immediately on preparation as well as for a week after preparation. Hence, EFV nanosuspension using a mixture of phospholipid and Tween® 80 (PL TNS) were prepared using the procedure
mentioned above, where in EFV was dissolved with acetone along with the phospholipid.

Optimization of EFV nanosuspension (PL-T NS) using factorial design

A 2^3 factorial design was used to optimize the particle size and polydispersity index of PL TNS. The preparation of nanosuspension by antisolvent precipitation method is influenced by various parameters and process variables viz. stabilizer concentration, concentration of drug, amount of organic solvent, amount of antisolvent.

In Vitro Studies

In Vitro Lipolysis Studies:

In vitro lipolysis study considers the role of excipients which can undergo hydrolysis in the presence of the enzyme lipase which is present in the gastrointestinal tract for assisting the dissolution of the drug.

Materials:
Sodium chloride, calcium chloride dihyd

rate, tris maleate were purchased from S D Fine chemicals ltd. Bile salts and pan creatic lipase were purchased from Him edia. Lipoid, GmbH gifted the sample of Phospholipon® 90 G and Col orcon, India provided the gift sample of Methocel K4M(hydroxypropyl methyl ce llulose).

Preparation of Lipolysis Medium

The medium for lipolysis studies was pre pared according to the formula mentione d in Table 1.

Table.1-. Formula for lipolysis medium for 1 formulation.

Ingredients	Quantity taken (g)
Sodium chloride	0.351
Calcium chloride dihydrate	0.0294
Tris maleate	0.0947
Bile salts	0.086
Lecithin (Phospholipon 90G)	0.038
Water	36 ml

Preparation of EFV dispersion

EFV, 50 mg, was triturated with 25mg of Methocel K4M by adding small quan tities ofMilliQ water till dispersion beca me pourable. The volume of the dispers ion was made up to 10 ml with MilliQ water. Concentration of EFV in the dispersion was 5mg/ml.

Procedure for in vitro lipolysis study:

36ml of the lipolysis medium was taken in beaker to which 2ml of formulation was added. The pH of the medium wast hen adjusted to 6.5 with 0.1 N NaOH.4 0 mg pancreatic lipase enzymedispersed in 3 ml water was added to the mediu m which was stirredfor 1 hour on magn etic stirrer and pH of medium wasmonit ored and adjusted to 6.5 whenever requi red. Aliquots were withdrawn at the end of 30 minutes and1 hour. Aliquots wer e then centrifuged at 40,000 rpm for 45 minutes using ultracentrifuge (Beckm an Coulter Allegra TM 64 R Centrifuge, USA).

Supernatants were separated and analyze d using reverse phase HPLC with UV d etector set at 247nm.

IN VITRO RELEASE STUDIES:

Dissolution testing is a widely used tool to study the release profile of formulati ons which in turn helps in predicting th e behavior of the formulations in vivo. I n vitro release studies for the nano-suspensions were performed using USP Type II and USP Type IV apparatus. T he release profiles of the nanosuspension s were compared to that of the EFV dis persion and EFV solution using phosphate buffer pH 7.4 with 2% Twee n 80 as the release medium.

Materials:

Sodium hydroxide, potassium dihydrogen phosphate, Tween®80 (SD fine chemica ls ltd),Transcutol®HP (Gattefosse India Pvt. Ltd) and Dialysis membrane (Himedia).

Preparation of EFV dispersion-

EFV dispersion using Methocel K4M w as prepared.

Preparation of EFV solution-

50mg EFV was accurately weighed an dissolved in 10ml of Transcutol® HP to form a solution with 5mg/ml of EFV.

Preparation of release medium-

0.780g sodium hydroxide and 3.40g pot assium dihydrogenphosphate weredissolv ed in water and volume was made to 5 00ml. 2% Tween 80 was added to this solution and shaken thoroughly

for complete dissolution of Tween 80 in the buffer.

Protocol for release study using USP Type II Apparatus:

1. Apparatus: Modified USP Dissolution apparatus Type II (paddle type) (Electrolab, Mini jar apparatus) and dialysis sac as barrier (Molecular weight cut off 12,000to14,000Daltons, pore size 2.4nm)
2. Formulations under study
EFV dispersion, EFV solution, PL-T NS, PL-G NS, PL-P NS
3. Volume of sample used: 0.5 ml
4. Dialysis medium: Phosphate buffer pH 7.4 + 2% Tween 80
5. Volume of release medium: 100 mL
7. Speed of rotation: 100 rpm
8. Aliquot: 1 mL at each time points were withdrawn and replaced with 1 l of release medium
9. Time Points: 0.5,1, 2, 4, 6, 8, 10, 12 hours
10. Analytical Method: Reverse phase HPLC using UV detector set at 247nm.

Procedure using USP Type II apparatus:

Dialysis bags were cut to required length (around 8 cm) and were soaked in pH 7.5 phosphate
buffer for a period of 12 hrs prior to the study. 0.5 mL formulation was filled in the dialysis bags and bags were sealed from both ends using cotton thread. Dialysis bags were then tied to paddle
using thread and paddles were lowered into release media maintained at37±0.5°C Aliquots were withdrawn at predetermined time intervals and media was replaced with same volume of buffer solution. Aliquots were centrifuged (Minispin) at 5,000 rpm for 5min and suitably diluted before analysis using reverse phase HPLC with UV detector set at 247nm.
Graph of cumulative release versus time was plotted to represent the results.

Protocol for release study using USP Type IV Apparatus:

1. Apparatus: USP Dissolution apparatus Type IV (flow through) (Electrolab,) and dialysis sac as barrier (Spectra/Por ® Dialysis membrane, Molecular weight cut off: 20 KDa)
2. Formulations under studyEFV solution, PL-T NS, PL-G NS
Volume of sample used: 0.5 ml
3. Dialysis medium: Phosphate buffer pH 7.4 + 2% Tween 80
4. Volume of dialysis medium: 100 mL
7. Flow: 8ml/min
8. Aliquot: 1 mL at each time points were withdrawn and replaced with 1ml of release medium
9. Time Points: 0.5,1, 2, 4, 6, 8, 10, 12 hours
10. Analytical Method: Reverse phase HPLC using UV detector set at 247nm.

Procedure using USP Type IV apparatus:

USP dissolution apparatus 4, flow through cell (Electrolab) was used for the study. The flow
through cell units were prepared for the experiment. Each cell unit consisted of a flow through cell, 22.6mm in diameter, and a filter assembly which snugly fit on top of it and a ruby bead placed atth e bottom with glass beads filled up toth e lower mark. The filter assembly comprised of three stainless steel mesh placed alternating with Teflon ring supports and GF/D filter placed on the topmost mesh. The formulations (0.5mL) were filled in dialysis sacs, secured to dialysis adapters
and placed on top of glass beads in each cell. The cells were then closed with filter assemblies prepared as mentioned previously. These cell units were then placed in flow through cell and aclosed loop system was employed for release testing of formulations. The pump stoke rate washeld constant at 120ppm throughout the experiment. Aliquots of 0.5mL

were withdrawn from the reservoir medi a and fresh medium replaced at predeter mined time points. The content of EFV was determined using reverse phase HP LC.

ANTIHIV Testing using cell based as say:

AntiHIV testing was carried out Nationa l Aids Research Institute, Pune. Efaviren z as the puredrug and the nanosuspensio ns PL-T NS, PL G NS, PLP NS were studied for their e ffect on TZM-bl cells infected with two viral strains, namely HIV1 UG070 & VB28. The stu dy was carried outin the following steps :

1. Maintenance of cell lines: The TZ Mbl cells were maintained in DMEM su pplemented with 10%FCS and antibiotics; penicillin (50U/ ml) and 50ug/ml Streptomycin at 37°C with 5% CO_2.

2. Preparation of Virus stocks: Fre shly collected blood from healthy, HIV seronegative individual was obtained. Th e peripheral blood mononuclear cells (P BMCs) were separated on Ficoll-hypaque and stored at -196°C in liquid nitrogen and sti mulated with PHA(Phytohaemaggluti ninA) as and when needed. Two HIV1 strains namely UG070(X4 tropic) and V B28 (R5 tropic) strains were obtained fr om NARI virus repository. These strains were then allowed to infect the two separate sets of PHA activated PBMCs. Each virus c ulture was maintained in RPMI 1640 m edium supplemented with 10% FCS, 10 U IL2 and antibiotics, Penicillin+Streptomycin (50U/ml + 50u g/ml) at 37°C with 5% CO_2. Culture su pernatant was collected and tested for P24 antigen ELI SA. Culture supernatant (Virus stock) w as stored at 70°C Subsequently, the 50 % tissue culture infectivity dose (TCID50) of each isolate was determined in the TZM-bl cell line.

3. Seeding of 96 well plates: The TZM-bl cells were trypsinized, enumerated and then added to the 96 well flat bottom plates. The plates then incubated at 37°C with 5% CO_2 for 10 to 12 hrs. The cytotoxicity and antiHIV assays were performed in these pre-seeded TZM-bl plates.

4. Preparation of Test compound: The test compound was dissolved in suggested solvent, filtered, sterilized, and used for the assays. EFV was dissolved in DMSO and the nanosuspensionwere dissolved in phosphate buffer saline.

5. Cytotoxicity Assay: The cytotoxicity assay was carried out by preparing double dilutions of Test compoundin pre-seeded TZM bl plate. The cell viability was determined using MTT assay and percent viability and CC50 value (the concentration of a compound at which 50% cells are viable) was calculated by comparing with the Cell Control (only cells without TEST compound), sub toxic concentration were selected for anti-HIV assays.[22]

5. AntiHIV testing: Thisactivity is measured as a function of reduction in Luciferase reporter gene expression after virus infection in TZMbl cells. This testing was carried out using cell associatassay (to test the replication inhibition of HIV1) against two Primary isolates of HIV1 (UG070, X4 tropic and VB028, R5 tropic).

Cell associated Anti-HIV assay: The TZM-bl cells were infected with the pre-titrated HIV-1 UG070 & VB28 viruses. were overlaid on to the infected cells and incubated at 37^0C with 5% CO_2 for 48 hrs. After 48 hrs,

the supernatant was tested for Lucifera se activity using Britelite Assay. This a ssay was to respective Drug control was tested in each assay to compare the IC50 value of Test compound demonstrate the ability of Test compound to inhibit HIV1 replication. The activity of Test compound is compared with drug control. The results were expressed in terms of Relative Luminescence Units (RLU) and then the IC50 value was calculated for each virus.(Concentrati onof compound at which 50% virus isi nhibited).Also, the inhibition of virus g rowth was monitored and compared withvirus growth in absence of drug[23].

Result & Discussion

The lipolysis model used for the study of EFV nanosuspensions has been devel oped by our research group and has been previously studied for lipid nanoparticles of quercet in[15] and nelfinavir mesylate.[16] The lipol ysis profile shows maximum improveme nt in solubilization of EFV in PLT NS and PLP NS as compared to the EFV d ispersion which showed only 6% solubil ization at the end of one hour. The pres ence of phospholipids in the nanosuspen sions enhance the solubilization of EFV. However, nanosuspension with Gelucire as one of the stabilizers fared poorly in the lipolysis. study. Subramanian et al have reported inhibition of pancreatic lipase by certain lipid excipients.[13]. Inhibition of lipase by Gelucire did not allow digestion of the lipids reducing th esolubilization of EFV causing the poor performance of PLG NS. Nonionic surfa ctants aregenerally digestible by lipases which prevents precipitation of the drug s after digestion of the formulation. However, higher concentrati ons of surfactants in combination with l ower amountsof glycerides have been re ported to reduce solubilization of drug.[13] Hence, proportion of lipids to surfactan ts in the formulation is critical in estima tion of solubilization capacity by the in vitro lipolysis study.

Table.2 % EFV solubilization (mean ± S.D.)

Formulation	% EFV Solubilization	
	30 min	60 min
EFV dispersion	3.7±0.14	5.58±0.39
PL-T NS	5.49±0.25	24.5±0.42
PL-G NS	3.58±0.14	1.83±0.07
PL-P NS	6.81±0.11	25±0.29

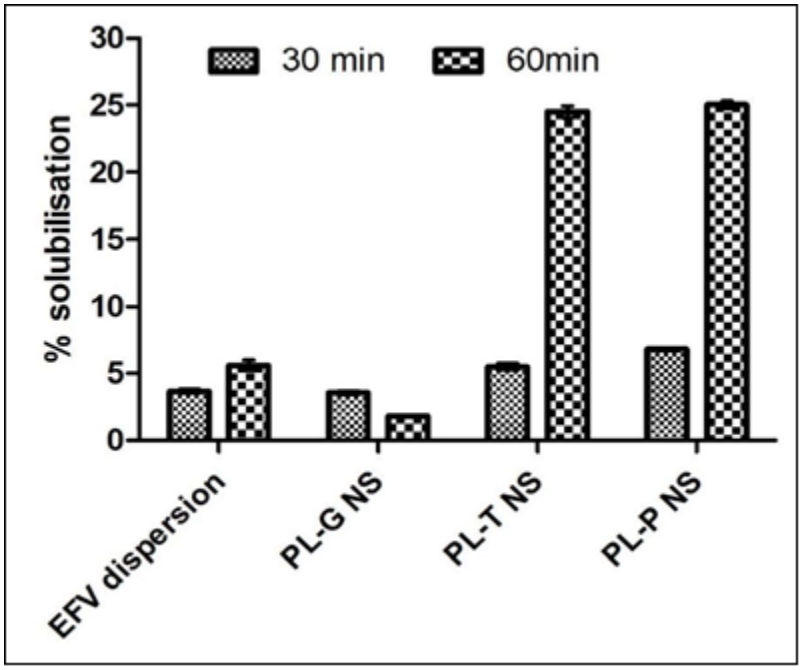

Figure 1 In vitro lipolysis profile of nanosuspensions

In vitro release profiling of nanosuspens ions was performed by two methods. Ba sed onsolubility of EFV phosphate buffe r pH 7.4+ 2% Tween 80 was chosen as the release medium.[10] Fig.2 shows the release profile generated by u sing modified USP Type II dissolution apparatus. EFV solution in Transcutol H P showed close to 100% release in 12 hours. Dialysis membrane is thought to act as a barrier causing the complete r elease of EFV from the solution only after 12 hours. The use of phospho lipid in various nanosystems to retard the release of drugs has been widely reported. [15] More than 90% of EFV release in 2 hours, attributed to the larg e surface area of the nanosuspensions co ntaining soya lecithin and poloxamer 40 7 has been reported by Taneja et al.[13] R educed particle size due to formulation of nanosuspensions increased dissolution rate of EFV with 99% of drug dissolve d in one hour was reported by Patel et a l.[12] EFV nanosuspensions wereprepared with phospholipid as one of the stabiliz ers and have shown sustained release of EFVover 12 hours. PLT NS showed m aximum release as compared to EFV di spersion due to the presence of Tween 80 since EFV exhibits superior solubilit y in Tween 80.[15]

The release profile generated by using USP Type IV dissolution tester is show n in Fig.3.The closed loop system was used for the stu dy where the release medium is circulat ed within the cells without complete rep lacement with fresh medium. Based on results from the experiment performed using USP Type II appara tus, PL-T NS and PL-G NS were thenanosuspensions chosen for further release testing. From the gr aph, it is observed that about 60% relea se was observed from EFV solution at t he end of 12 hours with poor release of 25% observed in case of PLT NS and P LG NS. A comparison of the results fro m both the apparatus show that USP Type II apparatus gave a better release profile of EFV as compared to USP Type IV.

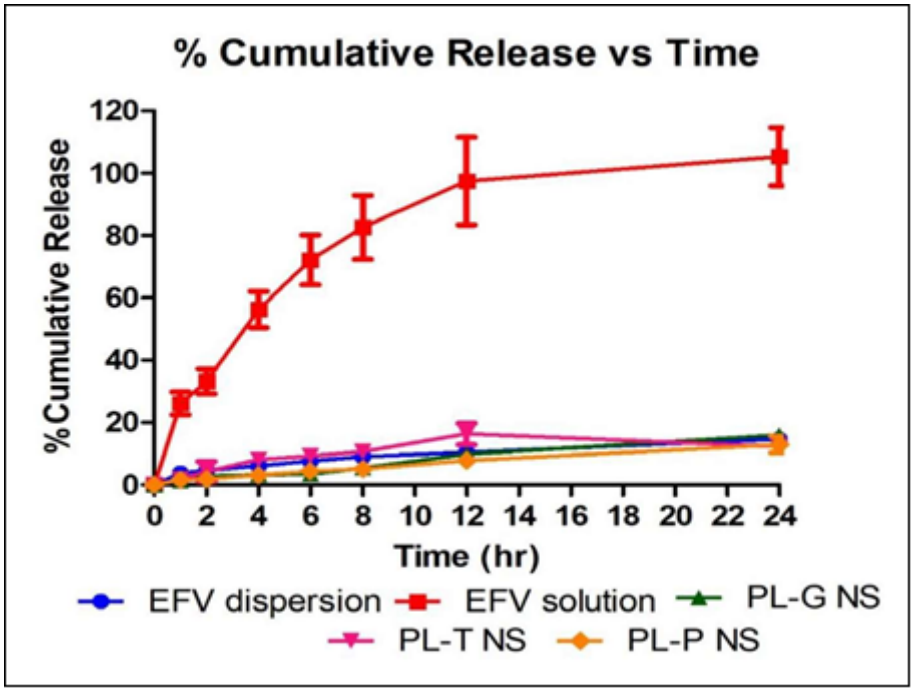

Figure 2 *In vitro* release profile of nanosuspensions (Type II apparatus)

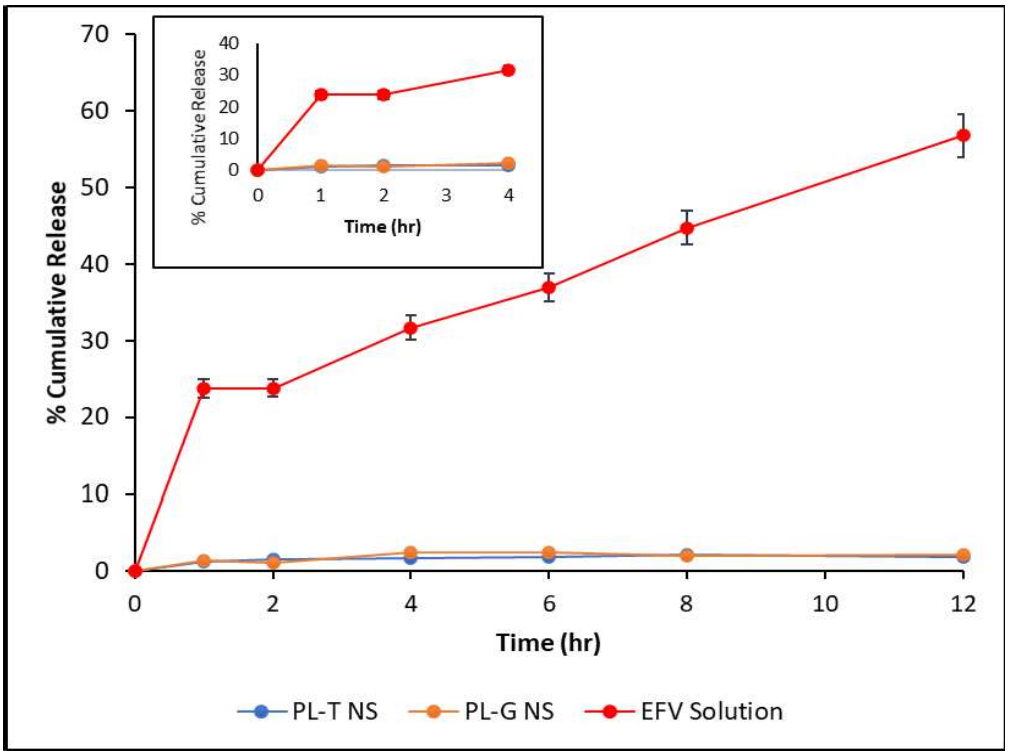

Figure3. *In vitro* release profile of nanosuspensions (Type IV apparatus)

Anti-HIV testing using cell based assay

Cytotoxicity of drugs or formulations is commonly tested using MTT (3-[4,5-dimethylthiazol- 2-yl]2,5 diphenyl tetrazoliumbromide) assay. The assay m easures viable cells in ahigh throughput setting like a 96 well plate without elaborate cell counting. It is based on th e principle that decrease in mitochondrial activity is the indication in decrease in n umber of viable cells. Mitochondrial activity is reflected by the conversion of pale.yellow tetrazolium salt MTT into red formazan

crystals which can be measured by the optical density. MTT assay can be perfo rmed on primarycell lines and the OD v alues of cells incubated with drugs is g enerally compared to the cells which ar e not exposed to drugs.[14]

In the present study, CC_{50} values were measured which is the concentration of t he drug at which 50% cells are viable. Table.3 show the CCanvalues for EFV, nanosuspensions. Hari et al have reported that efavirenz loaded eudragit n anoparticles were less toxic as compared to the pure drug in an MTT assay performed using C8166 cell lines.[145] In the present study, the CC_{50} value of EFV was found to be lower than that of the nanosuspension s which attributes to higher toxicity of the drug and confirms the advantage of using nanosuspensions for delivery of E FV.

AntiHIV testing has been carried out by different types of neutralizing antibody a ssays using various cell lines[16]. TZMbl cell line is a derivative of HeLa cell w hich expresses CD4 and CCR5 and is engineered to contain Tatresponsive r eporter gene for Luciferase (Luc). These features of TZMbl cell line make it hig hly susceptible to HIV1 infection in neu tralizing antibody assays.[17] In the current study, cytotoxicity study was car ried out on pure EFV as well as nanosu spensions to determine toxic concentrations.Sub toxic concentrations were used to perform cellassociated antiHIV assay by measuring Luciferase activity using Britelite plus substrate. In case of EFV and the nanosuspensions, I C_{50} values were below detection range 0.001 µg/ml which is indicative of the potency of the drug and formulations in inhibition of viral growth.

Table.3 Results for cytotoxicity measurement using MTT assay and anti-HIV assay

Product	**CC50 (µg/ml)**	**Cell associated assay**	
		IC50 (µg/ml)	
		HIV-1 X4 tropic	**HIV-1 R5 tropic**
EF V	12.02	<0.001	<0.001
PL-T NS	28.14	<0.001	<0.001
PL-G NS	17.65	<0.001	<0.001
PL-P NS	20.35	<0.001	<0.001

Conclusion

Phospholipid assisted nanosuspensions of EFV were prepared by antisolvent prec ipitation method with acetone as the solv ent and water as the antisolvent. Phosph olipid (Phospholipon® 90 G) along with Tween® 80 (PLT NS), Poloxamer 188 (PLP NS) and Gelucire® 50/13 (PLG N S) were used as stabilizers to form the nan osuspensions. 2^3 factorial design was used for optimization of PLT NS with p hospholipid, surfactant and drug load as the factors and particle size, polydispersity index as th e responses.

In vitro lipolysis was a tool used to stu dy the solubilization of EFV in presenc e of theenzyme lipase and other constitu ents of the lipolysis medium such as bil e salts, lecithin, and salts. At the end of onehour PLT NS and PLP NS s

howed 24.5% and 25% increase respectively, in solubilization of EFV as compared to EFV dispersion which showed 5% solubilization. Inhibition of pancreatic lipase by Gelucire probably caused reduced solubilization of EFV in PLG NS. *In vitro* release profile of the nanosuspensions was constructed using phosphate buffer 7.4 containing 2% Tween® 80 as the release medium using both USP Type II and USP Type IV dissolution apparatus. The nanosuspensions showed sustained release over a period of 12 hours as compared to theEFVsolution with USP Type II apparatus giving a better discriminatory profileas compared to theflow through dissolution apparatus. EFV and nanosuspensions were tested for cytotoxicity using the MTT assay on the TZMbl cell lines which showed that the nanosuspensions were less toxic than the pure drug. IC50 values were below detection limits of concentrations indicating excellent antiviral activity of EFV and that it is retained in nanosuspensions.

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