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Research Article
Efavirenz nanoemulsion: Formulation Optimization by Box-Behnken Design, *in vivo* Pharmacokinetic Evaluation and Stability Assessment

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Abstract

Background and Objective: The emergence of HIV/AIDS and its therapy has revolutionized antiretroviral therapy. Nevertheless, antiretroviral therapy in pediatric HIV infection still faces challenges. Pediatric antiretroviral therapy faces inaccurate dosing and thus reduced efficacy and/or safety. **Materials and Methods:** Therefore, in the present research, a nanoemulsion formulation of efavirenz was developed to overcome such issues. The nanoemulsion was prepared using a high-pressure homogenization technique using Box-Behnken experimental design with three factors namely surfactant level, homogenization cycle and pressure at three levels. **Results:** Electron microscopy results confirmed the globule size in the range of 30-40 nm. Non-everted gut sac method showed a 2.24 ($p<0.05$) fold increase in the drug permeability when compared to suspension in 2 hrs. Pharmacokinetic studies confirmed an assuring *in vivo* absorption pattern as compared to the suspension of efavirenz ($p<0.05$). In plasma, a 2.2-fold increment in the value of $AUC_{0\rightarrow\infty}$ was found for nanoemulsion when compared with aqueous suspension. **Conclusion:** The results confirmed that this formulation of efavirenz can serve as an effective dose adjustable formulation with improved bioavailability for HIV therapy especially in pediatric population.

Key words: Nanoemulsion, surfactants, efavirenz, AIDS box-behnken, bioavailability, pharmacokinetics

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

According to the report from UNAIDS, globally about 38 million people are living with human immunodeficiency virus (HIV) infection in 2019. Among this nearly 2.5 million patients are children under age 15. HIV infection causes AIDS a distressing disease. Now, HIV is one of the lethal epidemics globally, having considerable economic, social as well as political challenge. Till the end of June 2020 nearly 26 million people were accessing antiretroviral therapy[1]. Among children the deaths by HIV are almost null in high-income countries. Regarding AIDS the most challenging people in pharmacotherapy is pediatric population. There is substantial accomplishments in the last 25 years concerning better status regarding disease prevention, diagnosing techniques and up-to-date therapeutic policy development[2].

Though, a large dissimilarity in number of death and loss to follow-up can be observed in different regions, with sites in West African province showing the most terrible outcome. Here nearly 9% of children die in 18 months after starting the treatment and additionally a 24% failed to follow-up, based upon concerns regarding adherence. When drug development and financing is considered, regrettably, children are often a low priority. Investors must grasp pharmaceutical manufacturers and government authorities are answerable for the development and approval of antiretroviral drugs and formulations in time which are suitable for children's needs[3].

Almost 90% of the HIV-positive children does not have a proper reach to anti-retroviral drugs due to the lack of proper pediatric dosage forms. Poor adherence, inadequate potency and pharmacological issues like sub optimal plasma drug concentrations as well as sanctuary sites are the major reasons for therapeutic failure. New targets for managing of AIDS became very much relevant particularly in the case of excessive drug resistance, high pill burden usually observed with the conventional anti-retrovirals[4].

Efavirenz (Sustiva®) a first line antiretroviral drug (ARV) in both adult and pediatric pharmacotherapy. It is acts by non-nucleoside reverse transcription inhibition (NNRTI) of HIV-1. It is extremely lipophilic and belongs to BCS class II. Efavirenz directly bind to the enzyme reverse transcriptase and causes inhibition of RNA as well as DNA dependent DNA polymerase activity. It acts by producing a conformational change, which leads to destruction of enzyme's catalytic site. Efavirenz is not soluble in water and have a very feeble absorption in gastric fluid. This low aqueous solubility (less than 4 μg mL^{-1}) is one of the major reasons for its low bioavailability[5,6].

The correct dose adjustment and an easy way of administration of efavirenz is not possible for children since liquid formulation of efavirenz is not available worldwide. Even though suspension is available in market but with very low bioavailability than tablets. High dose of efavirenz administration is required to ensure the therapeutic plasma concentration.

Since the conventional antiretroviral agents are suffering from side effects and extensive drug resistance and high pill burden, there is a critical requirement to achieve novel treatment approach for the effective management of HIV infection. Lower doses will result in a lower treatment cost, possibly better adherence as well as a considerable enhancement in the accessibility of more patients to efavirenz[4]. With the use of nanotechnology a range of novel strategies like nanosuspensions[5-7], nanoparticles[8-10], liposomes[11], dendrimers[12] are presently being developed for the successful delivery of ARV drugs.

Nanoemulsion composed of a stable isotropic system of two immiscible phases formulated with suitable surfactants with a droplet diameter less than 100 nm. The solubilization ability of nanoemulsion is comparatively higher than simple micellar solutions. The stability aspects are also exceptional as compared to conventional suspensions and emulsions. Nanoemulsions possess a relatively long shelf life. Since the nanodroplets present huge interfacial area, the transport properties of the drug would be enhanced. O/w nanoemulsions have the ability to include hydrophobic drugs of the oil phase to improve the solubility and thereby bioavailability[13-15], thus they are of high attraction.

BCS class II drugs, almost all of them are having an adequate bond globally between solubility enhancement and bioavailability augmentation[16,17]. For enhancing the availability and distribution of efavirenz to anatomical reservoir areas and cellular level, formulations based on nanotechnology could provide an exceptional advantage[18]. Bioavailability enhancement of efavirenz using nanotechnology viz nano particles[19-22], nanostructured lipid carrier[23], self-nanoemulsifying drug delivery system[24] freeze-dried nanoemulsion[25] flax seed-oil nanoemulsion[26] has been reported by some research groups. Our research group investigated and published a low energy method of efavirenz nanoemulsion formulation[27].

In this study nanoemulsion of efavirenz was developed by homogenization under high pressure with the help of Box-Behnken design. *In vivo* studies were conducted in addition to e*x vivo* permeability studies in optimized formulations using albino Wistar rats and pharmacokinetics

parameters were calculated and compared with efavirenz suspension. Also shelf life of the formulation was determined using stress stability studies.

MATERIALS AND METHODS

Study area: The study was carried out in 2015 in research lab, Department of Pharmaceutics, School of Pharmaceutical Education and Research, Jamia Hamdard, India.

Drug Efavirenz was received from Lupin Ltd. India as a gift sample. Transcutol® HP (Diethylene glycol monoethyl ether) and Capryol 90 (propylene glycol mono caprylic) were received from Gattefosse (Saint Priest, Cedex, France) as a gift sample. Poly-oxyethylene sorbitan monolaurate (Tween 20) was supplied by Merck (Germany). All of the other reagents, chemicals and solvents used in this experiment were of analytical grade and procured from Merck and S.D. Fine Chem.

Coarse emulsion formulation: Capryol 90 was used oil phase for emulsion formulation as it showed a higher solubility. Gelucire 44/14 and transcutol HP was selected as surfactant and cosurfactant respectively. Drug loaded oil phase was mixed thoroughly with the S_{mix} (surfactant mixture: Gelucire 44/14 and transcutol) using magnetic stirrer. After that distilled water added as the aqueous medium to the oil-surfactant mixture with stirring[27].

Nanoemulsification by high pressure homogenization using Box-Behnken Design: In this study we used Box-Behnken design selected because it needs a smaller number of runs when compared to central composite design, for 3 or 4 variables. The experimental design contains a number of points located at the middle of every edge and also the simulated center point of the multidimensional cube that represents the region of interest. A regression model is built by approximation which is closest to the original regression model is achieved by Response Surface Methodology (RSM). Since the experiment investigates the effects of different blend of more than one factor and its levels on different response variables, a multiple regression analysis is required. Factorial experiments, analyzes the main effects as well as the interaction effects[28,29]. Table 1 showed the dependent and independent variables with their levels.

Optimized formula: Polynomial equations were developed for the responses including percentage transmittance and PDI as independent variables and the formulations were optimized that would result a maximum value of percentage transmittance and a minimum value of PDI. Effect on Globule size and drug release were explained in our previous research[27].

Table 1: Independent and dependent variables and their levels

Independent variables	Levels		
	Low (-1)	Medium (0)	High (1)
A = Surfactant (%)	8	13	18
B = Pressure (mPa)	22	55	88
C = No. of cycles	2	5	8
Dependent variables			Constraints
R1 = Transmittance			Maximize
R2 = PDI			Minimize

High pressure homogenization: The coarse emulsion was passed through microfluidizer (Stansted Fluid Power, UK) working from 22 Mega pascal to 88 Mega pascals[24]. The equipment is fitted with a pneumatic pump, an interaction chamber and a filter. The operations could be continuous or recycled. Homogenization was carried out at different pressure and number of cycles. For each pressure level, 10 mL coarse sample was passed through the microfluidizer for 1 cycle at the set pressure. Then 500 µL of sample was taken out from the microfluidizer for size determination. The remaining amount was again passed for next cycle and so on. All experiments were performed twice[27].

Preparation of suspension of efavirenz: For comparing the bioavailability and distribution of efavirenz to cellular and anatomical reservoir sites, sodium carboxy methyl cellulose as suspending agent (0.5%) was used to prepare aqueous suspension of efavirenz.

Characterization of optimized formulation

Particle size and size distribution examination: DLS technique by Malvern Zetasizer 1000 HS was used to measure the hydrodynamic oil droplet diameter (Malvern Instruments, UK). 100 folds diluted sample was used for the analysis[30,31].

Percentage transmittance: By using UV Visible spectrophotometer (Shimadzu, Japan), Percentage transmittance of the formulations was analyzed spectrophotometrically. 100 times diluted samples were analyzed at 232.6 nm.

Determination of zeta potential: Zeta potential was determined using ZetasizerZS90 (Malvern Instruments Ltd, Worcestershire, United Kingdom) after proper dilution.

Determination of viscosity: The viscosity of the optimized nanoemulsion formulation was determined with Brookfield R/S CPS Plus Rheometer (Middleboro, MA, USA) at 25±0.5°C. RHEO3000software was used. 1 mL sample was used for analysis. The spindle speed was 70 rpm at a temperature of 25±0.5°C. 413 per min of shear rate were applied and spindle diameter was 50 mm.

Transmission electron microscopy (TEM): To examine the morphology of globules in the optimized nanoemulsion formulation and also to observe any precipitation of drug with the addition of water TEM study was performed 50 µL nanoemulsion placed to 200-mesh form ware-coated copper sample holders and stained with negatively stained with 1.5% phosphotungstic acid at room temperature. The nanoemulsion was analyzed by transmission electron microscope (Morgagni-268D, USA) functioning at 60-80 kV with a1550X magnification.

Efavirenz analysis by chromatographic method: A reverse phase HPLC method utilized for pharmacokinetic evaluation as well as *in vitro* release of efavirenz. The data was analyzed using HPLC (Merck, Darmstadt, Germany) with C18 reverse-phase column (particle size 5 µm, 250 mm×4.6 mm) using photo diode array detector (Waters 2998) at a wavelength of 247 nm (software Class VP, version 5.032). 0.1M formic acid and acetonitrile in the ratio 45:55 v/v was the mobile phase at a flowrate of 1 mL per minute. Run time was 15 min with a 9±0.4 min retention time. Diluted and filtered samples were directly injected in case of *in vitro* release study.

About 100 µL of plasma samples were precipitated with 200 µL of IS solution in a 1.5 mL centrifuge tubes. 1 mL of ethyl acetate the mixture was added to this and mixed by vertexing for about 1 minute and then centrifuged for 10 min at 10000×g. Then the organic layer was collected to a sample vial and vaporized and reconstituted mobile phase (0.5 mL) and analyzed[34].

Intestinal permeability studies: Approval for animal study was sanctioned from Institutional Animal Ethics Committee, (Registration No. 841/CPCSEA) instructions were followed for the entire study. Small intestine removed after sacrificing the rats by spinal dislocation method. Full length of small intestine was cut and taken out from upper end of duodenum to lower end of ileum. The mesentery was stripped manually. Then it was carefully flushed out using 0.9% oxygenated saline solution using a syringe. The cleaned intestinal tract was made into sacs having with 3.0±0.4 mm diameter and 8±0.4 cm length.

About 1 mL of the optimized nanoemulsion and efavirenz suspension were filled in the sacs using a blunted needle. Thread was used to tie the two sides of the intestine firmly. Each sac was kept in 100 mL of Tyrode's solution in distilled water in conical flask. 1% SLS was added to maintain sink condition. The entire system was placed in water bath (37°C) and aerated. Aliquot amount of the samples was taken from outside the sac on every half an hour interval. Fresh medium was used to replace the withdrawn amount. The study was carried out for 2 hrs. Analysis of the samples were done using HPLC. Studies were performed in triplicate[32,33].

Cytotoxicity analysis: Cell viability of optimized nanoemulsion was determined by MTT assay using culture medium DMEM media supplemented with 10% of fetal bovine serum containing 100 units/mL of penicillin-G and 100 µg mL^{-1} of streptomycin. About 100 µL of cell suspension containing 10^5 cells/mL was placed into 96 well plate (Nest Biotech, China). The plates were incubated at a temperature of 37°C for 24 hrs with CO_2 (5%) in an incubator (New Brunswick, USA). 200 µL of serially diluted nanoemulsion formulation, pure drug and placebo were added into the plate in triplicates and kept for incubation for 24 hrs at the same conditions. About 20 µL of MTT (5 mg mL^{-1} in DPBSA) was placed to the plate to ensure the cell viability and incubated at the same conditions for 4 hrs. To terminate the assay, the solution was poured out and replaced by DMSO (200 µL) and mixed for 60 sec. The absorbance was measured (570 nm) by ELISA microplate reader (Biorad, CA). Positive control was untreated cells and media only was taken as blank[34].

Pharmacokinetics studies

Animal protocol for oral administration: Pharmacokinetic studies were carried out using adult Wistar albino rats obtained from institutional animal house, weighing about150-200 g). Approval for *in vivo* study (Registration No. 841/CPCSEA) was acquired from Institutional Animal Ethics Committee. All the protocols and guidelines were strictly followed during entire study. Rats were fasted for 24 hrs before the absorption study. Rats were randomly divided into two groups for receiving nanoemulsion and suspension. A single dose of 20 mg kg^{-1} of optimized nanoemulsion as well as suspension were administered to the animals[35,36]. Drug formulations were orally administered to each conscious animal by gavage, by the help of a stomach tube. Blood samples of the animals were collected through retro-orbital vein at determined time points intervals of 0 (before dosing), 1, 2, 4, 6, 8 and 24 hrs in to EDTA tubes, then plasma was separated out by 20 min centrifugation at 2000 rpm at 37°C.

Efavirenz from plasma was extracted and analyzed using a previously established and validated reverse phase HPLC method.

Pharmacokinetic and dynamic behavior of drugs may be changed by continuous blood sampling by fluid loss. therefore, in this study maximum blood volume of 3 mL as total was only extracted during 24 hrs period. Since this volume is within the limit of maximal recommended amount (3.5 mL) it was assured that loss of blood while experimental procedures were not affected the pharmacokinetic characteristics of the drug.

Non-compartmental pharmacokinetic analysis: Pharmacokinetic parameters like maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), half-life ($t_{1/2}$), mean residence time (MRT), area under the curve from zero to 24 hrs ($AUC_{0\to24}$), are under the curve from zero to infinity ($AUC_{0\to\infty}$), are aunder momentum curve from zero to 24 hrs ($AUMC_{0\to24}$) and the are aunder momentum curve from zero to infinity ($AUMC_{0\to\infty}$) were estimated for oral administration of nanoemulsion as well as suspension. The relative plasma bioavailability of optimized efavirenz nanoemulsion was calculated by comparing with the standard aqueous suspension using the given equation. (The dose administered was same):

$$\text{Relative bioavailability} = \frac{AUC_{(nanoemulsion)}}{AUC_{(suspension)}}$$

Data analysis: All readings were reported as Mean±SD of minimum four experimental trials. Student's t-test used to determine the statistical differences among the groups. ANOVA was used to compare results with more than two groups. The pharmacokinetic data between nanoemulsion and suspension was compared by the one-way ANOVA. Tukey-Kramer multiple comparisons test was used for checking statistical significance with the software GraphPadInstat (GraphPad Software Inc., CA, USA).

Stress stability study and shelf-life determination: Three batches of the optimized nanoemulsion were kept at a temperature of 40±2°C and75±5% RH for three months' time period. Samples were taken out at different time points (0, 30, 60 and 90 days) for inspection of any physical change. Globule size, zeta potential and % drug remaining were determined with HPLC after each time period. A graph was plotted between log of % drug remaining and time in days.

For determining of shelf life Arrhenius method was used. The optimised nanoemulsion was stored for 3 months at 30±2°C; 40±2°C and 50±2°C at 55±5% RH. Samples were taken out at predetermined time intervals which were 0, 30, 60 and 90 days and percentage drug content measured by HPLC. Zero-time samples were considered as controls.

Graph was plotted between log of % drug remaining and time in days. At each elevated temperature the degradation rate constant 'k' was determined from slope by the given equation (Eq. 1):

$$\text{Slope} = \frac{-k}{2.303} \quad (1)$$

Arrhenius plot was drawn between logarithm of k values at several raised up temperatures and reciprocal of absolute temperature. At 25°C value of K was determined by from the plot. Shelf life was determined using the below given equation (Eq. 2):

$$t_{0.9} = \frac{0.1052}{k_{25}} \quad (2)$$

$t_{0.9}$ stands for shelf-life and which is defined as the time required for 10% degradation of the drug.

RESULTS AND DISCUSSION

Formulation optimization using Box behnken design: Nanoemulsion was formulated as mentioned in the method section. As suggested by the experimental design software 17 batches were prepared. All the formulations were examined for size, % transmittance, polydispersity index (PDI) and drug release. Effect of independent factors on globule size and drug release were explained in detail in our previous research[27]. The effect on % transmittance and polydispersity index (PDI) were shown in Table 2.

Fitting the response surface models: RSM was employed to predict the difference in the percentage transmittance and PDI. Statistical analysis was performed on the data to decide the best fitted model.

A positive value denotes the effect which favors optimization owing to a synergistic effect in the regression equation whereas a negative sign denotes an opposite relationship between the factor and the response[28,29,37].

Table 2: Data obtained for the experimental design

Run	Surfactant (%)	Pressure (mPa)	No. of cycles	Transmittance (%)±SD	PDI±SD
1	13	22	8	88.93±2.34	0.361±0.015
2	8	22	5	84.83±2.04	0.456±0.014
3	13	88	8	96.01±0.13	0.644±0.034
4	8	55	8	85.92±2.32	0.379±0.021
5	13	22	2	93.10±3.21	0.223±0.032
6	13	55	5	94.62±2.72	0.431±0.033
7	18	22	5	98.41±2.98	0.748±0.029
8	13	88	2	93.73±3.62	0.232±0.016
9	13	55	5	92.12±3.05	0.417±0.028
10	13	55	5	96.95±0.19	0.812±0.018
11	13	55	5	91.07±2.18	0.421±0.027
12	13	55	5	93.02±3.76	0.443±0.019
13	18	55	8	97.91±0.20	0.808±0.025
14	8	88	5	82.15±3.71	0.403±0.024
15	18	55	2	98.57±0.17	0.391±0.027
16	18	88	5	98.31±2.88	0.637±0.032
17	8	55	2	80.38±2.92	0.252±0.018

Effect of independent factors on transmittance: F-value of 21.05 shows the significance of the model. Values of "Prob> F" less than 0.0500 designate model terms are significant. 1.97 "Lack of Fit F-value" means that the Lack of Fit is not significant (p value = 0.2613). Here A, C, A2 are significant model terms. The "Pred R-Squared" of 0.8322 is in accordance with the "Adj R-Squared" of 0.9585. "Adeq Precision" was 14.278. 'Adeq Precision' shows the ratio between signal and noise. A value greater than 4 was desirable. Here the ratio of 20.295 shows a sufficient signal. Therefore, this model was useful to read the design space.

Polynomial equation suggested by the model for transmittance is shown:

$$R1 = +92.76+7.53A-0.20B+1.20C+0.65AB-1.00AC +0.000BC-2.58A2+0.72B2+0.52C2$$

where, R1 is the % transmittance, A is the concentration of surfactant used (%), homogenization pressure is represented by B and the no. of cycles of homogenization by C.

Factor A (amount of surfactant used) and C (number of cycles) affected the transmittance in positive direction. The factor B that is the homogenization pressure showed a little effect on the value of % transmittance. With higher concentrations of surfactant and more number of cycles, a higher value of percentage transmittance was observed. This might be due to the fact that increased level of surfactant and no. of cycle reduced the size of globule and a nanosized formulation might be formed. Contour plots (Fig. 1a) show the effects of surfactant concentrations on percentage transmittance. Factor A and C shown to have extra intense effect on percentage transmittance other than C. Figure 1b shows the predicted versus actual plot for the response % transmittance. Visible transmittance is a small fraction of spectrum of light (400-700 nm) is extensively used in colloidal solution for checking clarity[16]. Higher amount of surfactant and more number of cycles might have reduced the globule size. Percentage transmittance value close to 100 implies the clarity of the formulations. Since nanoemulsions are liquid formulations, clarity as well as and transparency are major aspects in giving acceptance to the formulation by the patient. In addition to this it also suggests that the size of the droplets were in nanometer range. This in turn reveals that the drug release and absorption in biological media will be fast and promising.

Effect of independent factors on PDI (R2): 30.14 F-value of represents a significant model. Here C and C2 are significant. "Lack of Fit F-value" of 1.61 denotes the lack of fit is not significant comparative to the pure error. A value of "Pred R Squared" of 0.7618 is almost similar to "Adj R Squared" of 0.9425. The signal to noise ratio shows the "Adeq Precision". A ratio above 4 is desirable. The Equation suggested by the design is as follows:

$$PDI = +0.42+4.500E-003A-5.750E-003B+0.069C+8.250E-003AB +2.750E-003AC+2.250E-003BC+0.017A2375E-003B2-0.12C2$$

It is clear that two independent variable (A and B) display a little effect on PDI (R2). According to the design only C and C2 are significant model terms. Figure 2a shows the contour plots showing the effects of PDI on percentage transmittance. Figure 2b shows the predicted versus actual plot for the response PDI.

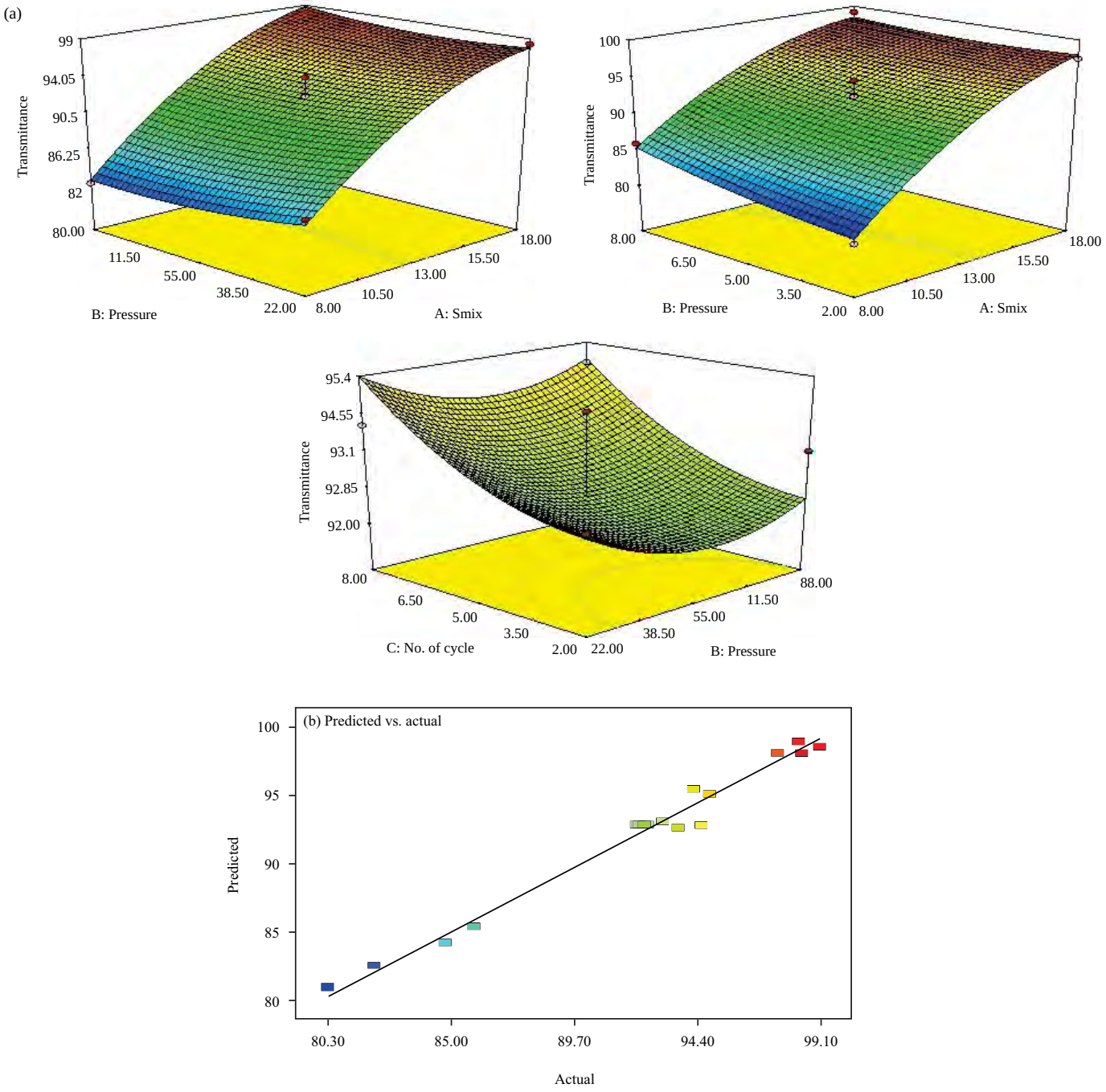

Fig. 1(a-b): (a) Surface plot of factor A, B and C for transmittance and (b) Plot of actual versus predicted values for percentage transmittance

From the figure it was clear that as the number of cycles of homogenization is increasing, the value of poly dispersity index is also increasing. This may be due the fact that during passage of emulsion through the piston there are number of droplets of different size are producing based on the distance of globules with respect to center of the piston. The variation in PDI may be due to the in homogeneities at certain points while the passage of emulsion through the piston. A small value of PDI indicates the uniformity in the size of the dispersed oil droplets. From the Fig. 2 the level of S_{mix} and pressure used for homogenization did not show any effect on PDI.

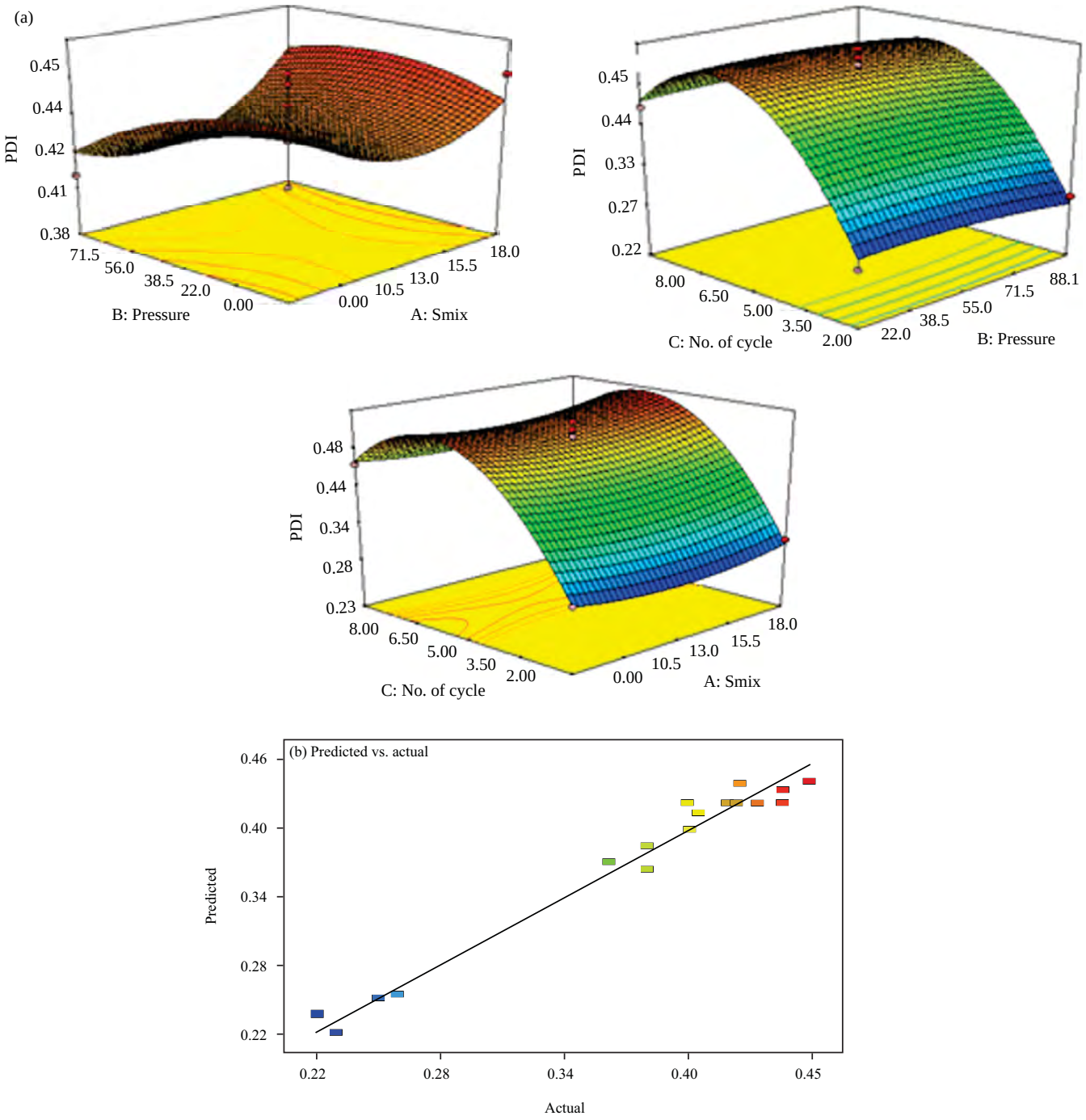

Fig. 2(a-b): (a) Surface plot of factor A, B and C for PDI and (b) Plot of actual versus predicted values for PDI

Optimization of responses: Numerical optimization was used for formulation optimization. After considering the effect of independent parameters on response, the final formulas were determined. Subsequent to selection of each variable and setting their goal along with limits, the optimized formula was generated. The selected optimization constraints for the selected variable and also the optimized formula obtained with software displaying the predicted as well as experimental values achieved was shown in Table 3. After setting the limits for individual variable, the software created the optimized formula automatically. Nanoemulsion was formulated using optimized formula suggested by software and the experimental values were obtained. Percentage prediction of error was found to be very less (less than 8%) for all the factors.

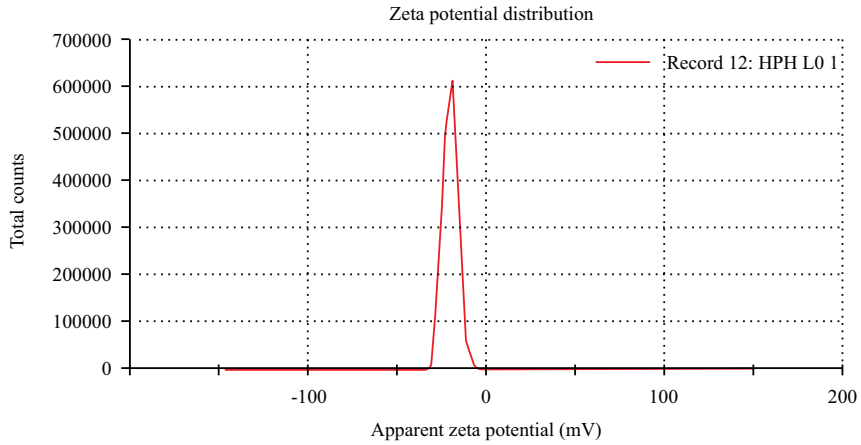

Fig. 3: Zeta potential of optimized formulation

Table 3: Predicted values and experimental values on the basis of optimized formula

Factors			Responses	
Surfactant (%)	Pressure (MPa)	No. of cycles	Transmittance (%)	PDI
Predicted values				
17.8	54.49	2	99.02	0.3856
Experimental values				
18	55	2	98.6±8.95	0.391±0.02
Percentage prediction error =			-0.4242	0.6873

This showed correctness of prediction as well as the usefulness of the experimental design for formulating the nanoemulsion with preferred parameters.

Characterization of nanoemulsion

Particle size and size distribution: Mean globule size of the optimized formulation was found to be 24.08±5.06. Particle size distribution of the optimized formulation was examined. The PDI of optimized formulation was found to be 0.391±0.02. Lower value of PDI shows the uniformity of the globule size in the formulation[38-40].

Percentage transmittance: The percentage transmittance of the optimized formulation was found to be 98.6±8.95. In this study, water was used as a dispersion medium, which strongly absorbs most of the wavelengths in the electromagnetic spectrum, but it has a narrow window of transparency that includes 400-700 nm. It doesn't absorb in the wavelength range of visible light, because there is no physical mechanism which produces transitions in this region. A value near to 100 implies that the optimized formulation was clear and transparent[16].

Zeta potential determination: The zeta potential of optimized formulation was shown in Fig. 3. The optimized formulation showed a value of -23.2±2.1. The stability of colloidal dispersions can be predicted in terms of zeta potential. Zeta potential indicates the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed globules. Zeta potential could serve as a partial indicator for the physical stability of the emulsion being formed. In a dispersion zeta potential indicates degree of repulsion between adjacent similarly charged particles. For small molecules a high zeta potential will resist aggregation and increases stability. Zeta potential can control charge interaction. Usually, a high zeta potential may be high in positive or negative value like -30 or +30 mV[41]. Negative signs designate that the formulations were negatively charged and high values indicate the stability of the system. Since zeta potential indicates the degree of inter-particulate repulsion having similar charge, when the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. Therefore, dispersions with high zeta potential whether it is negative or positive value are electrically stabilized while colloids with low zeta potentials tend to coalescence or flocculate. Thus the probability of coagulation in the biological fluid is less and also during its shelf life[42,43].

Viscosity: Viscosity measurements are essential for characterizing the physical stability of system. Viscosity of the final nanoemulsion was low (17.85cp±1.96), because the optimized formulation has more than 80% of water.

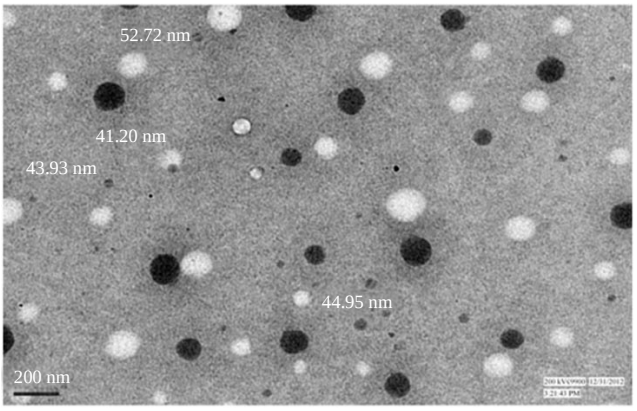

Fig. 4: TEM image of optimized formulation

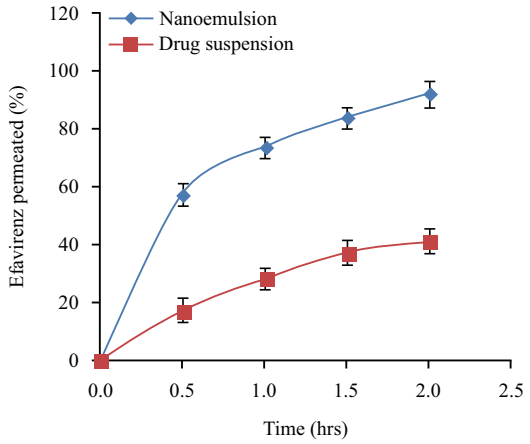

Fig. 5: Intestinal permeation profiles for the optimized nanoemulsion and drug suspension

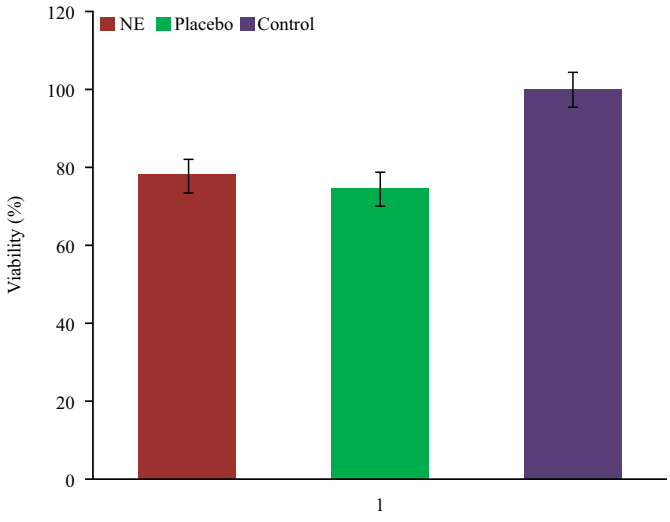

Fig. 6: Comparative percentage cell viability for nanoemulsion (NE), placebo and cells only as control

Transmission electron microscopy: Negatively stained samples were observed under TEM for the examination of physical properties of the oil droplets[44]. Bright field imaging with increasing magnification revealed oil globules in the nanoemulsion as dark and the background bright (Fig. 4). Since point-to-point resolution is possible droplet sizes can be measured accurately. Oil droplets were clearly visible and the sizes were almost as same sizes as observed with zetasizer dynamic light scattering measurement. Moreover, droplet showed a spherical morphology with no evidence of drug precipitation in oil and aqueous phase.

Intestinal permeability studies: Rat intestinal sac everted and non-everted type are commonly used to study the drug absorption. According to Genty and associates[33]. the permeability will be high for when the sacs was in case of actively transported molecules. The permeability of drugs which absorb by passive diffusion mechanism will be same regardless the sac was everted or non-everted. Since the mode of absorption of efavirenz is passive diffusion the non-everted gut sac model will be sufficient to explain the process of absorption[32]. Also, the non-everted sac model have reported advantages like simplicity, easiness of consecutive collection of samples with minimum changes to intestinal morphology, need for minimum amount test sample etc. over the everted sac model. The intestinal permeation profiles of nanoemulsion and drug suspension was shown in Fig. 5.

It was found that significantly very less amount of drug was permeated from the suspension to the outside medium as compared to nanoemulsion. Non-everted gut sac method showed a 2.24 ($p<0.05$) fold increase in the drug permeability when compared to suspension in 2 hrs. This considerable improvement in permeation may be due to the molecular structure of phospholipid with two fat-soluble tails and a water-soluble head of the micellar system. Moreover, the nano size of the system also contributed to the faster and increased the permeability dramatically. The small droplet dimension of nanoemulsion provided an increased area for partitioning of the drug through intestinal membrane.

Cytotoxicity analysis: In living cells MTT test accesses the mitochondrial dehydrogenase activity. Cytotoxic effects of the formulation were checked by the measurement of mitochondrial activity after re-incubating for a 24 hrs. Damaged cells will show decreased or no recovery while incubation. The optimized nanoemulsion exhibited a similar mitochondrial activity as that of control, representing a complete recovery (Fig. 6).

Table 4: Pharmacokinetics parameters after oral administration of efavirenz nanoemulsion and aqueous suspension

Pharmacokinetic parameters	Efavirenz nanoemulsion	Efavirenz suspension
t_{max} (h)	4	4
*C_{max} (μg mL^{-1})	2.9±0.18	1.80±0.13
AUC_{0-24h} (μg.h/mL)	35.61±10.94	20.22±9.16
*$AUC_{0-\infty}$ (μg.h/mL)	82.24±18.13	37.76±10.37
$AUMC_{0-24h}$ (μg.h^2/mL)	350.38±38.22	195.20±29.12
$AUMC_{0-\infty}$ (μg.h^2/mL)	3782.61±128.32	1198.07±93.65
$T_{1/2}$ (h)	34.36±3.19	34.0±5.73
K_e (h^{-1})	0.02016±0.0032	0.03019±0.0062
$MRT_{0-24hrs}$ (hrs)	9.83±0.78	9.65±0.87

*Highly significant (p<0.001)

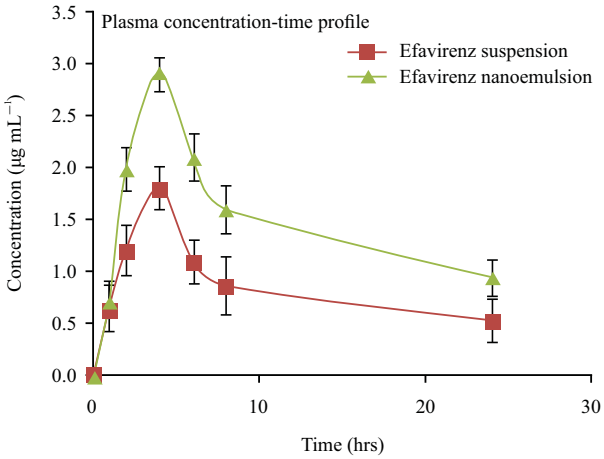

Fig. 7: Plasma concentration versus time profile of efavirenz following oral administration of optimized nanoemulsion and aqueous suspension

Pharmacokinetics studies

Plasma concentrations following oral administration: Even though significant advances have accomplished in HIV therapy in last few years many of the strategies are unable to provide adequate level of drug in plasma. Failure of effective drug absorption and distribution will lead to failure in maintaining drug level in therapeutic window, has significant clinical consequence. The major problem is development of multidrug resistance. So, there is a serious requirement of developing new delivery technique for anti-HIV drugs which can improve solubility and permeability and thereby oral bioavailability as well as distribution to major reservoir sites.

In this study, enhanced bioavailability following oral administration, nanoemulsion formulations and aqueous suspension as control were administered to conscious albino Wistar rats were evaluated. Plasma drug concentration after oral administration was plotted against time for optimized nanoemulsion as well as aqueous suspension of efavirenz.

The results of the study evidently showed that significantly higher plasma efavirenz concentrations can be achieved when efavirenz given by oral route in the form of nanoemulsion when compared with the aqueous suspension. So, the optimized formulation was found to be more efficient in increasing the oral absorption of efavirenz as that of suspension. The maximum plasma concentration of 2.9±0.18 μg mL^{-1} was observed after 4 hrs of administration of nanoemulsion, but only 1.8± 0.13 μg mL^{-1} for suspension (Fig. 7).

Pharmacokinetic analysis after oral administration: To determine the plasma parameters after oral dosage, pharmacokinetic analysis (non- compartmental model) was carried out. The results of analysis for efavirenz nanoemulsion and aqueous suspension were shown in Table 4. A significantly increased maximum plasma concentration was observed for the nanoemulsion formulations compared to control formulation. Mean T_{max} value was 4 hrs for both nanoemulsion and suspension.

Relative bioavailability in plasma: The relative bioavailability of efavirenz after oral administration as nanoemulsion and aqueous suspension were normalized based on $AUC_{0-\infty}$ values. Nanoemulsion exhibited a significant ($p<0.05$) improvement in the bioavailability of efavirenz in blood after oral administration. Here we can find that 2.2 times rise in the relative bioavailability of nanoemulsion than that of aqueous suspension. This may be due to the use of medium-chain fatty acid esters for solubilizing the drug and also, the nanometric size of the formulation. These factors might have enhanced the absorption of efavirenz. Current result was in compliance with the work done by Tiwari and Amiji[45]. The comparison study in blood showed significant higher C_{max} and AUC when efavirenz was given as nanoemulsion than the aqueous suspension and this confirms the successfulness developed formulation[18]. Previously efavirenz nanoemulsion formulated

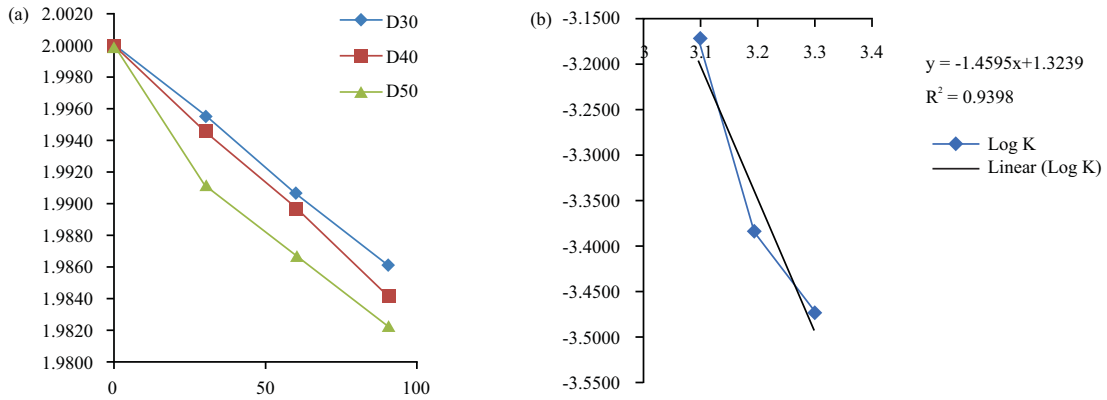

Fig. 8(a-b): (a) Log percent drug remaining versus time plot for optimized nanoemulsion and (b) Arrhenius plot for optimized nanoemulsion

Table 5: Globule size, zeta potential and drug (%) remaining of optimized nanoemulsion stored at 40±2°C and75±5% RH

Time (months)	*Mean globule size (μ)±Sd (N = 3)	*Mean zeta potential (Mv)±Sd (N = 3)	Drug remaining±Sd (%) (N = 3)
0	24.08±5.06	-22.2±2.21	100
1	25.30±7.52	-24.5±4.12	99.0651
2	25.80±10.1	-23.6±3.21	98.6744
3	26.10±8.43	-22.7±4.3	97.6529
6	26.90±7.31	-23.2±2.9	96.0132

*Not significant (p>0.05)

by phase inversion composition method also proved a promising result in the enhancement of bioavailability. Previous studies by Vyas *et al.*[18], found that nanoemulsion of saquinavir (SQV) using flax-seed oil showed 3-fold higher concentrations in the systemic circulation as compared to the control suspension.

Accelerated stability study and shelf-life determination: At time intervals of 0, 1, 2, 3 and 6 months, the samples were tested to examine the changes in globule size, potential as well as % drug remaining. The results of the study were shown in Table 5. From the table, it can be observed that values were slightly increased as the time passes but the changes were actually not significant (p>0.05). Accelerated stability tests at 40±2°C and a relative humidity of 75±5% predicted degradation of about 3.96% of drug in the optimized nanoemulsion at 90 days.

Shelf life was determined by Arrhenius method. It was found that the percentage of efavirenz remaining unchanged was 96.8631, 96.4321 and 96.015 at 30±0.5, 40±0.5 and 50±0.5°C respectively. The degradation reaction followed a first-order kinetics (Fig. 8a).

'K' (degradation reaction rate constant) was calculated from the slope of the line at each temperature. Arrhenius plot was made by plotting the logarithm of K at different temperature versus inverse of absolute temperature and shown in Fig. 8b. From this graph, K value at 25°C was used to calculate the shelf life of the optimized formulation. The shelf-life of the optimized nanoemulsion was found to be1.09 years at room temperature.

CONCLUSION

In this work, improvement of oral bioavailability of efavirenz an anti-HIV agent of BCS class II, is achieved. Oral nanoemulsion formulation was made using medium-chain fatty acid esters of propylene glycol as oil phase and blend of gelucire and transcutol as surfactants. The optimized formulation showed a significant improved oral bioavailability as compared to the aqueous suspension formulation. Cytotoxicity studies proved that the formulation is not toxic. In general, the results of this research have proved the potential of nanoemulsions for enhancing oral bioavailability of anti-HIV agents. The formulated nanoemulsion was palatable due to the tasteless nature of efavirenz and moreover 75% of the formulation contains water. Since this is a liquid formulation it is very useful for pediatric population hence it is dose adjustable.

SIGNIFICANCE STATEMENT

Overall, the research proved that optimized nanoemulsion of efavirenz performed satisfactorily in improving the bioavailability without any cytotoxicity. This

strategy could be successfully exploited for delivering ant-HIV agents effectively to pediatric populations. This study will help the researcher in drug delivery field to make sure about the potential of nanoemulsions for enhancing oral bioavailability of anti-HIV agents.

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