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PAEDIATRIC- FRIENDLY MULTI-DRUG POLYMERIC NANOPARTICLES FOR MANAGEMENT OF AIDS AND TUBERCULOSIS

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ABSTRACT
AIDS is one of the most widely prevalent diseases affecting the paediatric population and the treatment becomes difficult due to the age related barriers. Further anti-retroviral therapy has been less effective due to lack of efficacy and other drug related issues. Nanoparticulate approach is preferred for improving the physicochemical attributes of these classes of drugs. Nevirapine, Lamivudine, Stavudine and isoniazid nanoparticles were prepared as a part of the effective ART plan of WHO by nanoprecipitation and double emulsification method respectively. The prepared nanoparticles were optimised by factorial design using design expert with the nanoparticles having particle size <300 nm with a spherical and smooth surface as per SEM analysis. IR analysis proved that the drugs were compatible with the excipients used for the nanoparticulate formulation. The entrapment was found to be >60 % with the nanoparticles showing an *ex-vivo* permeation of 24 hours across sheep mucosa. Cellular uptake studies showed a greater internalisation of NP's in the Hela cells hence supporting the greater permeation of NP's as compared to pure drugs. This will further help develop a platform technology for incorporation of the NP's into a suitable drug delivery system compatible with pediatric population.

KEYWORDS: Anti-retrovirals, Nanoparticles, Scanning Electron Microscope (SEM), Cellular Uptake, *ex-vivo* permeation.

INTRODUCTION
Acquired immune deficiency syndrome (AIDS) is a spectrum of conditions inflected by the human immunodeficiency virus (HIV).[1] Worldwide, an estimated 34 million people are suffering from this dreaded HIV; 3.4 million (approximately 10%) are younger than 15 years. Nearly all (95%) children younger than 15 years acquired HIV infection perinatally; with a bulk of the number of the 2 million adolescents (ages 10-19 years) with HIV infection worldwide thought to be long-term survivors of perinatal HIV infection.[2,3] Non-availability of Anti-retroviral (ARV) preventive interventions, in non breast feeding populations has been observed. 25% to 30% of infants born to HIV-infected women become infected with an increased risk to as high as 50% for infants with prolonged breastfeeding. Further, due to AIDS, much of the paediatric population also becomes susceptible to acquire tuberculosis as a secondary complication.[3, 4]

The use of single dose Nevirapine (sd-NVP) at the onset of labour significantly reduces peri-partum HIV transmission.[4,5] However, this approach is less effective than other ARV prophylactic regimen and may be associated with acquisition of viral resistance to non-nucleoside reverse transcriptase inhibitor (NNRTI) drugs. Additionally, the use of sd-NVP does not reduce HIV transmission risk during the breastfeeding period.[5] However, World Health Organization (WHO) has implemented the anti-retro viral (ART) therapy which includes drugs like lamivudine, stavudine and Neverapine in combination for effective therapy. Important considerations for ART regimens for infants and children include: the availability of a suitable formulation that can be taken in appropriate doses; simplicity of the dosage schedule; the taste and palatability, and thus the potential for compliance in young children.[6] Fixed-dose combinations (FDCs) are increasingly available for younger children, and are preferred to syrups and single drugs because they promote and support treatment adherence and reduce the cost of treatment. Adult tablets that require cutting up can result in under dosing or overdosing when given to children, and this may lead to an increased risk of resistance or toxicity.[7] In view of the availability of pediatric formulations, use of adult dose solid formulations is usually not resorted to. Dosing of antiretroviral drugs in children is usually based on either body surface area, or weight, or more conveniently by weight band (as in the National programme). As these change with growth, drug doses must be adjusted for weight in order to avoid under-dosing. Hence in this

regard a suitable carrier for these drugs in combination is favourable.[6,7]

Nanotechnology is a vigorous research area and one of its important applications is in targeted drug delivery. Nanostructured particles and scaffolds have been widely studied for increasing treatment efficacy and specificity of present treatment approaches. The advantages of using NPs may include: (1) carrying the drug to its destination while keeping the drug concentrated so that once endocytosed by cells, the drug can maximize its effect; (2) protecting the drug from being dispersed or degraded by body fluids and increasing the circulation time or retention time in the body; (3) carrying more drug molecules and increasing the solubility of some hydrophobic drugs due to the large surface area of NPs; and (4) achieving better permeability profile for low permeable drugs.[8, 9]

Hence in the present research an attempt has been made to formulate and evaluate nanoparticles of the above mentioned drugs with an aim of improving their permeability and cellular uptake, which will facilitate a better efficacy for the therapy.

MATERIALS AND METHODS

Materials

Drugs namely Nevirapine, Stavudine, Lamivudine and Isoniazid were obtained as gift samples from Mylan Laboratories Limited, Medak, Andhra Pradesh. Eudragit S-100 was obtained from Evonik India Pvt. Ltd, Mumbai. Chitosan, 65-75% deacetylated, was a product of Himedia Pvt. Ltd., India. Surfactants like Poloxamer 407 and didodecyldimethylammonium bromide were obtained from Sigma-Aldrich Pvt. Ltd, India, while Polyvinyl Alcohol and span 60 were obtained from SD fine chem. Ltd, Mumbai. Solvents like methanol and Acetone were obtained from SD Fine-Chem Limited, Mumbai. Sodium tripolyphosphate was obtained from Sigma-Aldrich Pvt. Ltd, India. Dialysis membrane 12-14 kDa molecular weight cut-off was procured from Himedia, Laboratories Pvt. Ltd., Mumbai.

METHODS

Preparation of Nevirapine loaded Eudragit S-100 nanoparticles by nanoprecipitation method

Nanoparticles were prepared by a nanoprecipitation method. Eudragit S100 and the drugs were differentially dissolved 10 mL of solvent to prepare individual batches of nanoparticles for each of the respective drugs. The internal organic phase solutions were slowly injected under ultra-homogenization into 20 mL of the external aqueous solution containing different % of surfactant, and the mixtures were then ultra-homogenised at 16000 rpm for 30 minutes. Organic solvent was completely removed by placing the product on a magnetic stirrer. The solvent used for internal organic phase should completely dissolve both drug and eudragit in it. The Drug: polymer ratio was kept constant i.e., 50:75. The results were interpreted based on particle size and PDI.[10] Further optimization was carried out.

Optimization of Nevirapine loaded Eudragit nanoparticles formulation by 3^2factorial design

Following the preliminary work which resulted in identifying crucial process and formulation variables the formulations were optimized according to a 3^2full factorial design, allowing the simultaneous evaluation of three independent variables and their interaction. Two factors were evaluated each at two levels. The drug to polymer ratio (*X1*), and Concentration of Surfactant (*X*2) were selected as independent variables. The dependent variables selected for study was particle size (*Y1*) and % PDI (*Y2*). Further Nevirapine loaded Eudragit nanoparticles were prepared at room temperature (25±2°C) in batches, OA1 to OA10.[11]

Preparation of drug (Stavudine, Lamivudine and Isoniazid) loaded Eudragit S-100 nanoparticles by double emulsion method

Polymeric nanoparticles loaded with drugs (stavudine, Lamivudine and Isoniazid) were prepared by dissolving the polymer in acetone (1% w/w) to which the different drugs were added individually, producing different ratio of drug and polymer. Further, drug/polymer solution was then emulsified using different concentrations of different surfactants. The stirring was continued for 4 h for the particles to solidify. After that, the particles were collected by centrifugation, and the supernatant decanted off. These collected nanoparticles were resuspended in different concentrations of different co-surfactants to produce slurry of wet nanoparticles, which were oven-dried at 40 °C to produce dry nanoparticles.[12]

Optimization of drug (Stavudine, Lamivudine and Isoniazid) loaded Eudragit nanoparticles formulation by 3^3 factorial design

Following the preliminary work which resulted in identifying crucial process and formulation variables the formulations were optimized according to 3^3 factorial design, allowing the simultaneous evaluation of three independent variables and their interaction. Three factors were evaluated each at two levels. The drug to polymer ratio (*X1*), Concentration of Surfactant (*X*2) and Concentration of co-surfactant (*X*3) were selected as independent variables. The dependent variables selected for studies were particle size (*Y1*), % PDI (*Y2*) and %EE.[13]

Evaluation of Nanoparticles

Particle size and polydispersity index analysis: The mean particle diameter and size distribution of the PNPs were characterized by Dynamic Light Scattering (DLS) method using Particle size analyser (Zetasizer Nano S-90, Malvern UK). Each sample was measured in triplicate and results were expressed as mean ± SD.[14]

% encapsulation efficiency (EE): The encapsulated drug was estimated by taking the residue of formulation

remained in the eppendorf tube after estimation of free drug, as described above. The quantity remained in eppendorf tube was added (10mL) to dissolve Eudragit S100, methanol was used as solvent in case of stavudine and isoniazid, sonicate to obtain a homogenous distribution. The sample was analysed using UV-Visible spectrophotometer after suitable dilutions against a similarly treated blank ES100 formulation. Each sample was measured in triplicate and results were expressed as mean ± SD. In case of lamivudine the residue of the drug in the eppendorf tube is first dissolved with acetone and centrifuged to remove the eudragit part and later the residue of drug is dissolved in water.[15]

% Encapsulation efficiency was calculated using the formula,

$$\% \text{ Encapsulation efficiency} = \frac{\text{Initial amount of drug} - \text{Amount of drug in the residue}}{\text{Initial amount of drug}} \times 100 \quad (1)$$

Ex-vivo permeation of nanoparticles: *Ex-vivo* permeability studies of the various nanoparticles were conducted to understand the amount of drug which gets permeated through the animal buccal mucosa. The study was carried out using a Franz diffusion cell. Briefly the individual NP's were taken and dissolved in 1ml of distilled water and taken in the receptor compartment. The donor compartment consisted of 85 ml of pH 7.4 PBS. The study was carried out *ex-vivo* using excised sheep buccal mucosa mounted between the donor and receptor compartment. The entire assembly was arranged on a magnetic stirrer. The temperature maintained was 37ºC at 50 R.P.M. Similarly, the drug were dispersed in water and added to the receptor compartment to conduct the study.[16]

FTIR (Fourier transformed infrared) studies: Integrity of pure drugs and nanoparticles was checked by taking an IR spectrum. The spectra were obtained using Shimadzu FTIR- 8700 spectrophotometer. In this study potassium bromide (KBr) disc method was employed. Potassium bromide was completely dried at 100°C for one hour and then cooled following which it was thoroughly mixed with the sample in the ratio of 1 part of sample and 100 parts of KBr. The mixture was compressed to form a disc using dies under very high pressure and compression force. This disc was placed in the sample chamber and a spectrum was obtained through the software program which was further subjected to interpretation.[17]

Scanning Electron Microscopy (SEM) studies: **S**urface morphology (roundness and formation of aggregates) of optimized nanoparticle formulation of each drug was measured by SEM (Jeol JSM 5600 LV, Jeol, Tokyo, Japan) equipped with 15 kV. Samples of dried NPs were dispersed in water, and then drops of the dispersion were placed on aluminium slide coated with gold, to a thickness of about 300 Å under an argon atmosphere.[18]

Cellular Uptake of Nanoparticles: HeLa cells were seeded in a 96 well plate Lab-Tek® Chamber Slides (NalgeNuncInternational, Naperville, IL, USA) at a density if $5x10^4$ cells/well in a 100µL. The cells were cultured in a CO_2 incubator at 37°C. The cells were then treated with 1% of the test compound treatment namely Nevirapine nanoparticles suspension tagged with rhodamine in DMEM, Isoniazid nanoparticles suspension tagged with rhodamine in DMEM, Lamivudine nanoparticles suspension tagged with rhodamine in DMEM and Stavudine nanoparticles suspension tagged with rhodamine in DMEM. Samples which served as control were also seeded in the cultured wells. These treated well plates were further incubated for 24 h at 37ºC and the cell monolayers were fixed with 0.5 ml of 4% paraformaldehyde. Final concentration of drug was maintained at 1% throughout the plate. After incubation, the cells were fixed using 4% PFA for imaging. The fixed cells were imaged using CELLOMICS® ARRAYSCAN® VTI (Thermo Scientific) modular florescence microscope as objective lenses at 40×magnifications.[19]

RESULTS

Optimization of nevirapine loaded Eudragit nanoparticles formulation by 3^2factorial design:

3^2 factorial design was performed to determine the relationship between formulation parameters *viz*., drug to polymer ratio and percentage of surfactant on response variables for the optimization of NPs formulations of NVP. The observed responses were particle size and PDI. (Table 1).

Optimization of Stavudine loaded Eudragit nanoparticles formulation by 3^3 factorial design (STV NPs): The optimization of Stavudine loaded Eudragit nanoparticles formulation by 3^3 factorial design is exhibited in table 2. 3^3 factorial design was performed to determine the relationship between formulation parameters *viz*., the drug to polymer ratio (*X1*), Concentration of Surfactant (*X*2) and Concentration of co-surfactant (*X*3) were selected as independent variables. The dependent variables selected for study was particle size (*Y1*), % PDI (*Y2*) and %EE.

Optimization of Lamivudine loaded Eudragit nanoparticles formulation by 3^3 factorial designs (LAM NPs): The Optimization of Lamivudine loaded Eudragit nanoparticles formulation by 3^3 factorial design is given in table 3. 3^3 factorial design was performed to determine the relationship between formulation parameters *viz*., the drug to polymer ratio (*X1*), Concentration of Surfactant (*X*2) and Concentration of co-surfactant (*X*3) were selected as independent variables. The dependent

variables selected for study was particle size (*Y1*), % PDI (*Y2*) and %EE.

Optimization of isoniazid loaded Eudragit nanoparticles formulation by 3^3 factorial designs (ISO NPs): The optimization of isoniazid loaded Eudragit nanoparticles formulation by 3^3 factorial design is depicted in the table 4. 3^3 factorial design was performed to determine the relationship between formulation parameters *viz*., The drug to polymer ratio (*X1*), Concentration of Surfactant (*X*2) and Concentration of co-surfactant (*X*3) were selected as independent variables. The dependent variables selected for study was particle size (*Y1*), % PDI (*Y2*) and %EE.

Response surface graphs as shown in figures 2, 3, 4 indicate direct relationship of concentration of surfactant and concentration of co-surfactant on particle size and polydispersity index. As the drug: polymer ratio increases, the particle size and PDI increase and vice-versa. These graphs also indicate a direct relationship of drug: polymer ratio on entrapment efficiency. As the drug: polymer ratio increases, the entrapment efficiency increases and vice-versa.

Evaluation of polymeric nanoparticles
Evaluation of optimized NVP nanoparticles (OA3)
Determination of Particle size
The particle size and PDI of NVP OA3 formulation was found to be 178.9 nm and 0.287 respectively.

Ex-vivo drug release of Nevirapine Nanoparticles.
Comparative drug release graphs for ex-vivo permeation studies of Nevirapine loaded nanoparticles and Nevirapine pure drug in given in figure 6.

FT-IR of Nevirapine loaded nanoparticles: FT-IR of Nevirapine loaded nanoparticles and pure drug is shown in Figure 7.

Evaluation of optimized STV nanoparticles (F-06)
Determination of Particle size
The particle size and PDI of STV F-06 formulation was found to be 118.9 nm and 0.184 respectively.

Ex-vivo release of Stavudine loaded nanoparticles and pure drug: Comparative drug release graphs for ex-vivo permeation studies of Stavudine loaded nanoparticles and Stavudine pure drug in given in figure 9.

FT-IR of Stavudine loaded nanoparticles: FT-IR of Stavudine loaded nanoparticles and pure drug is shown in Figure 10.

Evaluation of optimized LAM nanoparticles (F-05)
Determination of Particle size
The particle size and PDI of LAM F-05 formulation was found to be 86.33 nm and 0.228 respectively.

Ex-vivo release of Lamivudine loaded nanoparticles and pure drug: Comparative drug release graphs for ex-vivo permeation studies of Lamivudine loaded nanoparticles and Lamivudine pure drug in given in figure12.

FT-IR of Lamivudine loaded nanoparticles: FT-IR of Lamivudine loaded nanoparticles and pure drug is shown in Figure 13.

Evaluation of optimized ISO nanoparticles (F-08)
Determination of Particle size
The particle size and PDI of ISO F-08 formulations was found to be 104.0 nm and 0.159 respectively.

Ex-vivo drug permeation studies of Isoniazid loaded nanoaprticles and pure drug: Comparative drug release graphs for ex-vivo permeation studies of Isoniazid loaded nanoparticles and isoniazid pure drug in given in figure15.

FT-IR of Isoniazid loaded nanoparticles and pure drug: FT-IR of Isoniazid loaded nanoparticles and pure drug is shown in Figure 16.

Scanning Electron Microscopy of polymeric nanoparticles of Nevirapine, Stavudine, Lamivudine and Isoniazid: Scanning Electron Microscopy of polymeric nanoparticles of Nevirapine, Stavudine, Lamivudine and Isoniazid is depicted in figure 17.

Cellular uptake studies of nanoparticles of Nevirapine, Stavudine, Lamivudine and Isoniazid: The cellular uptake studies of nanoparticles of Nevirapine, Stavudine, Lamivudine and Isoniazid was conducted using Florescence Microscopy and the images of the same are shown in Figure 18.

Table 1: Optimization of nevirapine loaded Eudragit nanoparticles formulation by 32factorial design.

Formulation code	Runs	Independent variables		Response variables	
		X_1 (w/w)	X_2 (%)	Y_1 D_{nm}	Y_2 PDI
OA1	1	+1	0	226.4	0.362
OA2	2	0	+1	287.3	0.413
OA3	3	-1	-1	178.9	0.287
OA4	4	-1	0	209.5	0.341
OA5	5	-1	+1	279.2	0.424
OA6	6	0	-1	182.2	0.302
OA7	7	0	0	215.7	0.351
OA8	8	+1	-1	187.6	0.316
OA9	9	+1	+1	291.2	0.424
OA10	10	0	0	214.2	0.358

Table 2: Experimental plan and observed response values of STV NPs.

Batch Code	Variable level in coded form			Response Variables		
	X_1*	X_2#	X_3**	*Y*1 (Dnm)	*Y*2 (PDI)	Y3 (%EE)
F1	-1.00	0.00	0.00	126.22	0.198	52.2
F2	-1.00	-1.00	-1.00	194.2	0.28	53.2
F3	-1.00	1.00	-1.00	181.25	0.42	52.8
F4	0.00	0.00	1.00	127.11	0.194	57.9
F5	1.00	0.00	0.00	124.15	0.204	62.0
F6	1.00	0.00	-1.00	118.9	0.184	58.3
F7	0.00	0.00	-1.00	126.59	0.195	59.1
F8	1.00	-1.00	1.00	173.29	0.26	65.48
F9	1.00	-1.00	-1.00	177.14	0.246	63.0
F10	1.00	1.00	1.00	189.15	0.4	61.0
F11	0.00	0.00	0.00	128.48	0.199	57.5
F12	0.00	0.00	0.00	124.29	0.202	59.2
F13	0.00	-1.00	0.00	174.19	0.295	56.1
F14	0.00	1.00	0.00	185.25	0.421	58.0
F15	-1.00	1.00	1.00	179.2	0.42	53.7
F16	-1.00	-1.00	1.00	187.38	0.192	54.0

Table 3: Experimental plan and observed response values of LAM NPs.

Batch Code	Variable level in coded form			Response Variables		
	X_1*	X_2#	X_3**	*Y*1 (Dnm)	*Y*2 (PDI)	Y3 (%EE)
F1	-1.00	0.00	0.00	99.5	0.198	52.2
F2	-1.00	-1.00	-1.00	158.4	0.31	53
F3	-1.00	1.00	-1.00	181.25	0.41	52.8
F4	0.00	0.00	1.00	100.2	0.194	57.9
F5	1.00	0.00	-1.00	86.33	0.228	69.7
F6	1.00	0.00	0.00	141.7	0.295	58.3
F7	0.00	0.00	-1.00	100.8	0.275	59.1
F8	1.00	-1.00	1.00	147.2	0.26	56.4
F9	1.00	-1.00	-1.00	135.4	0.32	66
F10	1.00	1.00	1.00	189.4	0.4	64.5
F11	0.00	0.00	0.00	109.6	0.199	57.8
F12	0.00	0.00	0.00	97.7	0.202	59.2
F13	0.00	-1.00	0.00	98.5	0.204	58.3
F14	0.00	1.00	0.00	185.25	0.421	58.1
F15	-1.00	1.00	1.00	179.2	0.42	53.7
F16	-1.00	-1.00	1.00	139.5	0.309	54

Table 4: Experimental plan and observed response values of ISO NPs.

Batch Code	Variable level in Coded Form			Response Variables		
	X_1*	X_2#	X_3**	Y_1 (D_{nm})	Y_2 (PDI)	Y_3 (%EE)
F1	-1.00	0.00	0.00	111.2	0.198	52.2
F2	-1.00	-1.00	-1.00	158.4	0.31	53.2
F3	-1.00	1.00	-1.00	181.25	0.41	52.8
F4	0.00	0.00	1.00	112	0.211	57.9
F5	1.00	0.00	1.00	115.2	0.204	62
F6	1.00	0.00	0.00	141.7	0.295	58.3
F7	0.00	0.00	-1.00	100.8	0.275	59.1
F8	1.00	0.00	-1.00	104	0.159	65.48
F9	1.00	-1.00	-1.00	135.4	0.308	63
F10	1.00	1.00	1.00	189.4	0.41	61
F11	0.00	0.00	0.00	114.3	0.199	57.5
F12	0.00	0.00	0.00	114	0.202	59.2
F13	0.00	-1.00	0.00	147.2	0.26	56.1
F14	0.00	1.00	0.00	185.25	0.405	58
F15	-1.00	1.00	1.00	179.2	0.42	53.7
F16	-1.00	-1.00	1.00	139.5	0.309	54

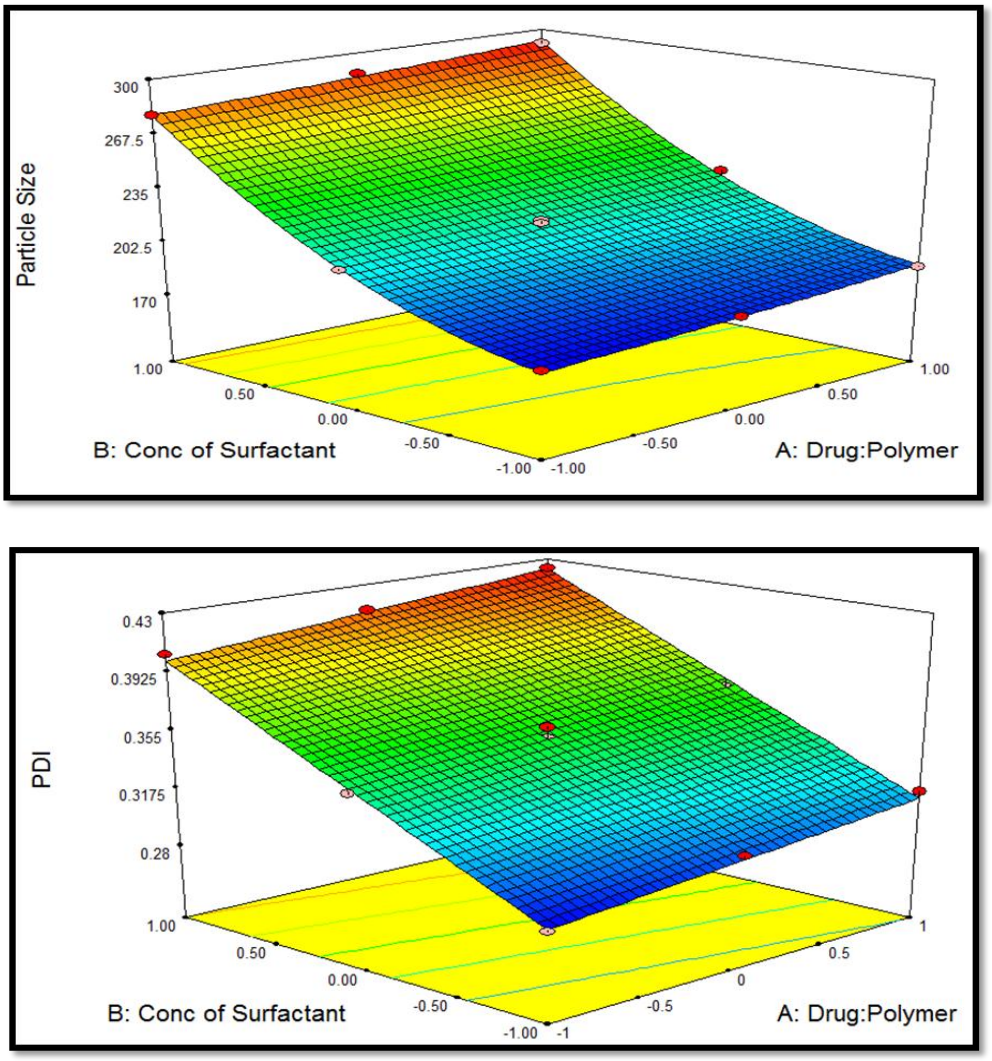

Figure 1: Response surface plot showing the influence of surfactant and polymer on particle size and PDI for NVP nanoparticles.

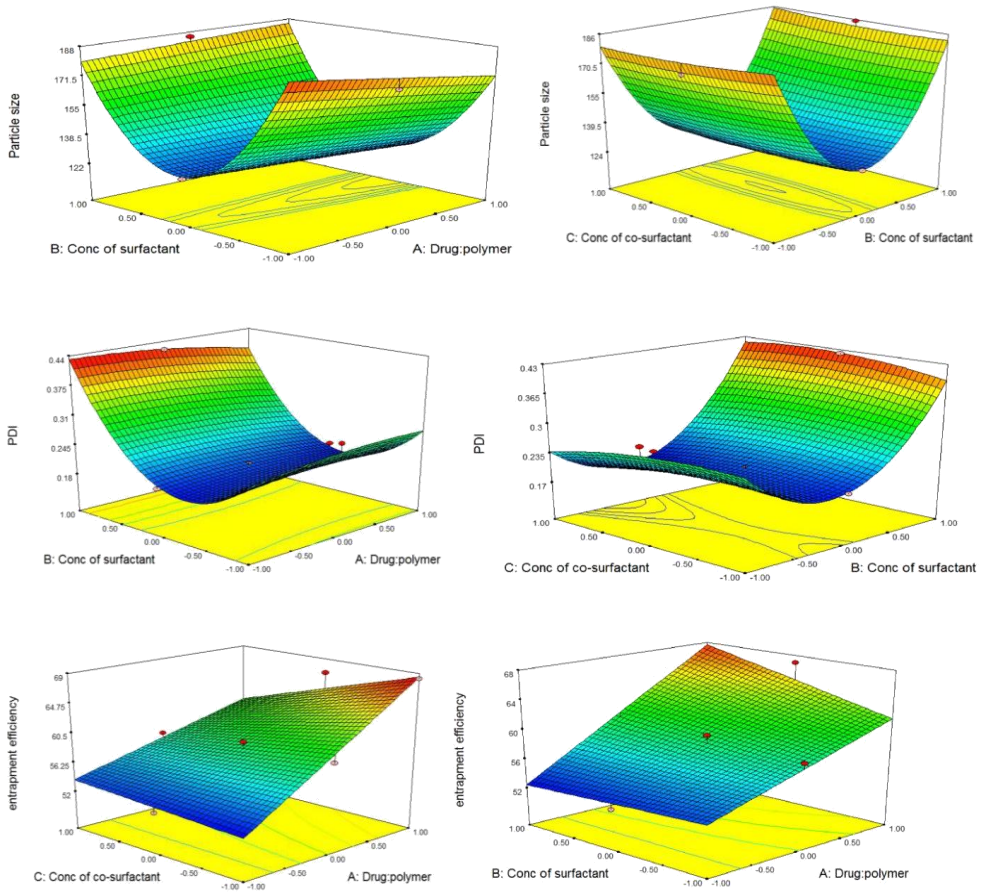

Figure 2: Response surface plots of Particle Size, PDI and Entrapment efficiency and their relationship with drug: polymer ratio and concentration of surfactant and co-surfactant (STV-NPs).

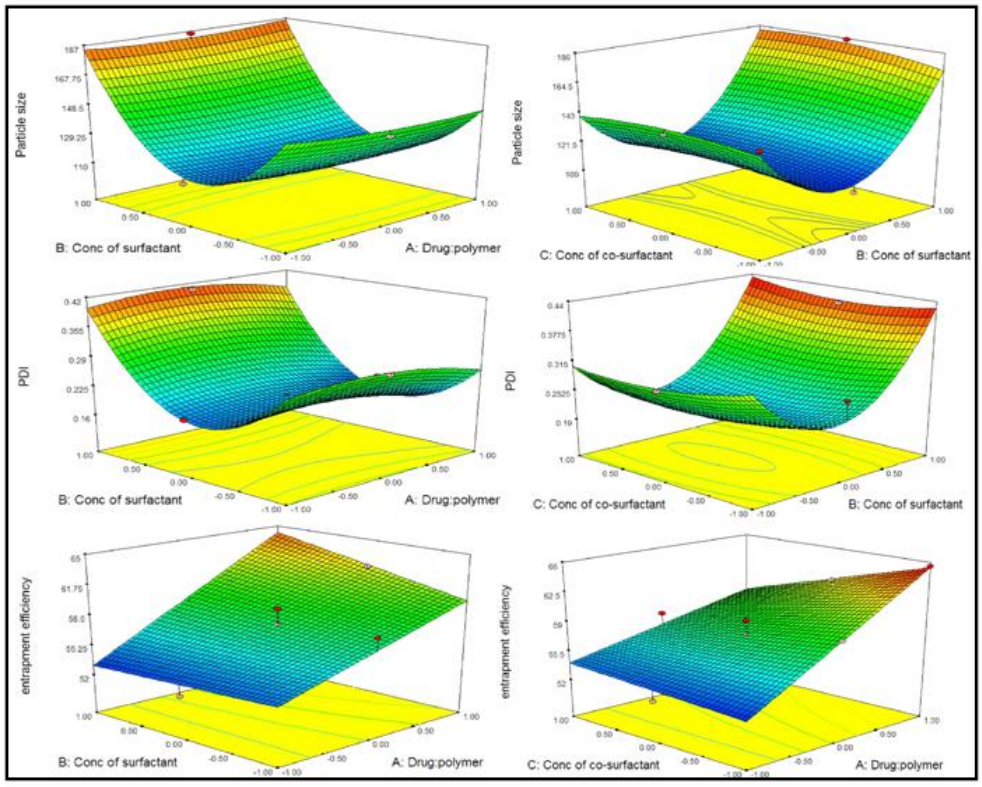

Figure 3: Response surface plots of Particle Size, PDI and Entrapment efficiency and their relationship with drug: polymer ratio and concentration of surfactant and co-surfactant (LAM-NPs).

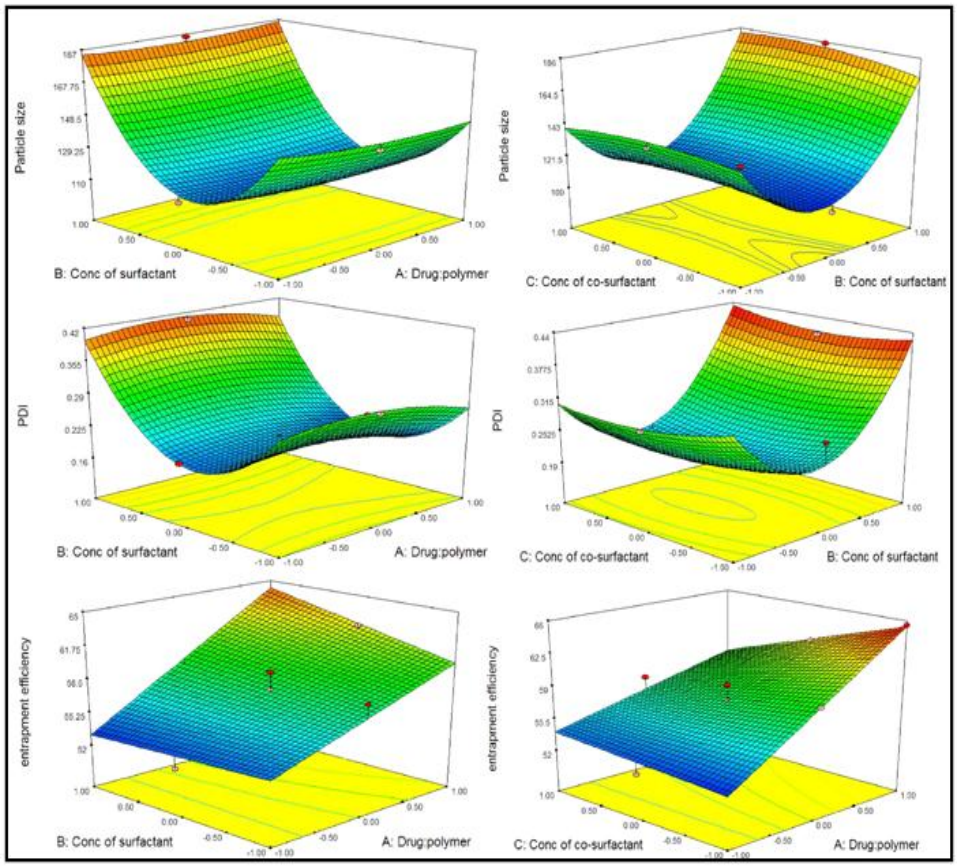

Figure 4: Response surface plots of Particle Size, PDI and Entrapment efficiency and their relationship with drug: polymer ratio and concentration of surfactant and co-surfactant (ISO- NPs).

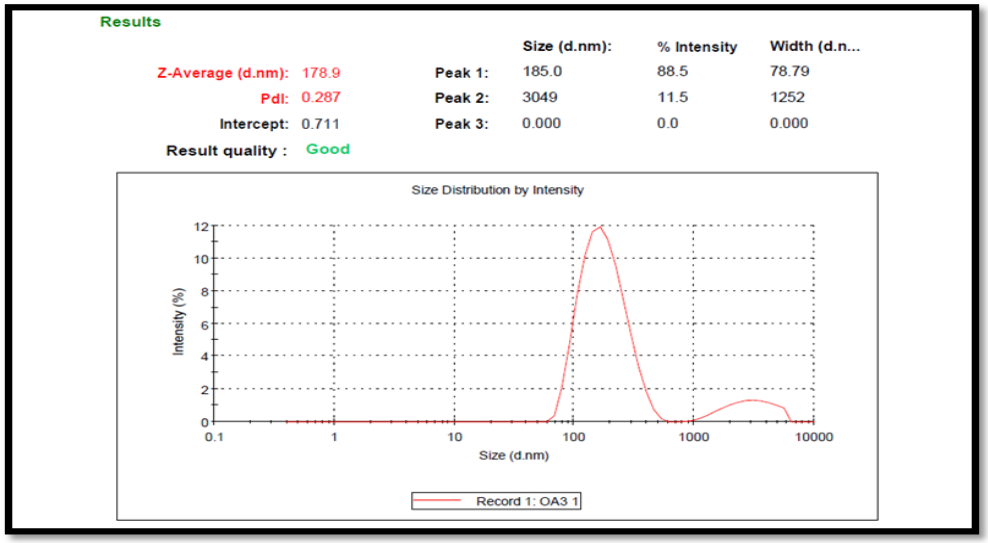

Figure 5: Particle size and PDI of (OA3) formulation loaded with Nevirapine.

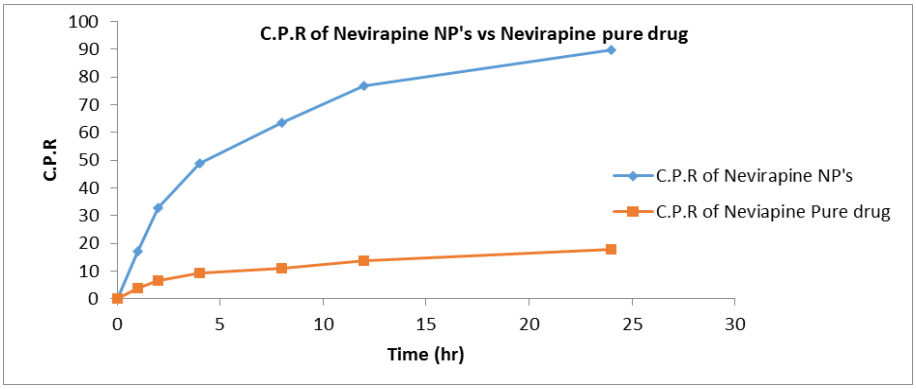

Figure 6: Comparison of *ex-vivo* drug release of Nevirapine NP's and pure drug.

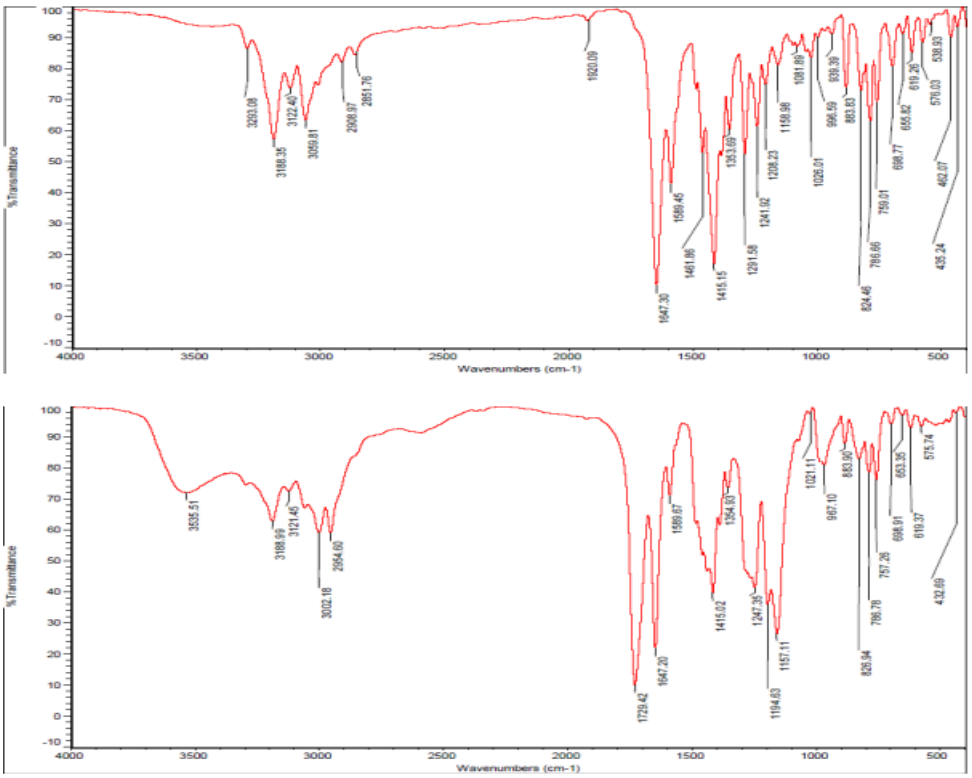

Figure 7: Comparision of IR spectra of NVP pure drug and NVP NP's.

		Size (d.nm):	% Intensity	Width (d.n...
Z-Average (d.nm): 118.9	Peak 1:	138.8	100.0	58.10
PdI: 0.184	Peak 2:	0.000	0.0	0.000
Intercept: 0.949	Peak 3:	0.000	0.0	0.000
Result quality : Good				

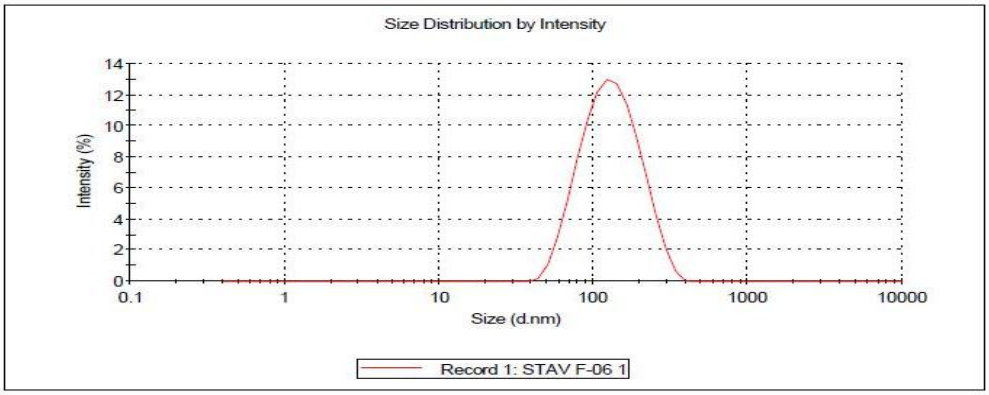

Figure 8: Particle size and PDI of (F-06) formulation.

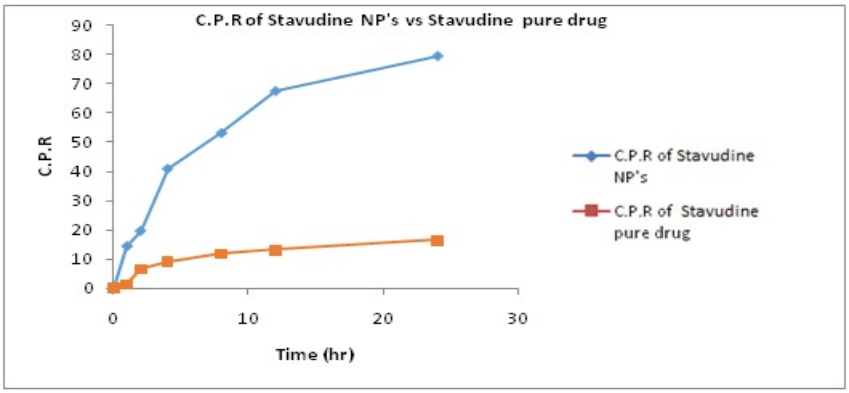

Figure 9: Comparison of *ex-vivo* drug release of Stavudine NP's and pure drug.

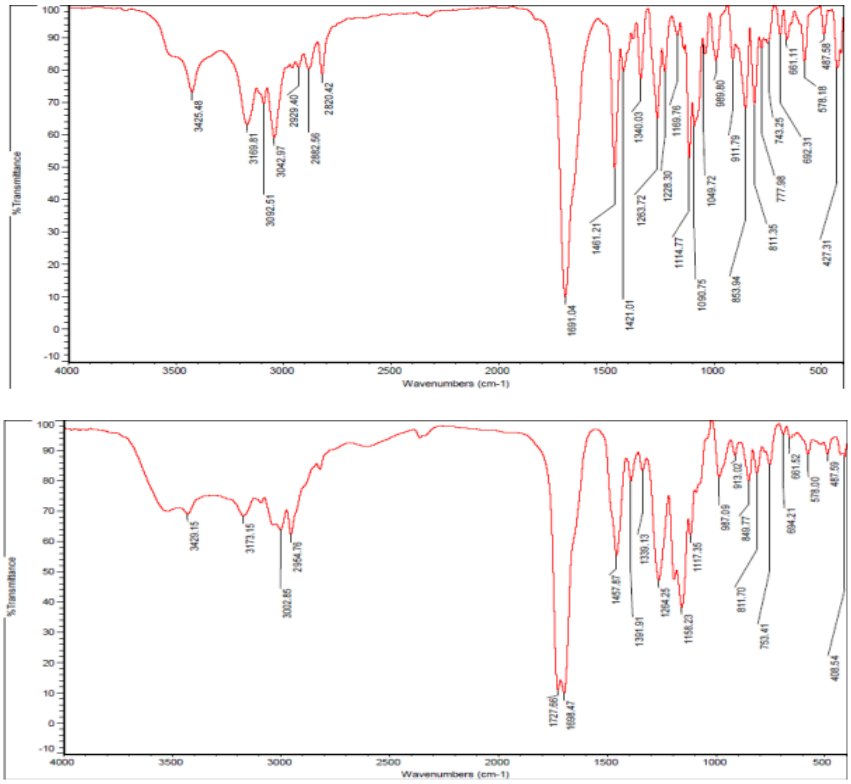

Figure 10: Comparision of IR spectra of STV pure drug and STV NP's

		Size (d.nm):	% Intensity	Width (d.n...
Z-Average (d.nm): 86.33	Peak 1:	105.7	100.0	47.99
PdI: 0.228	Peak 2:	0.000	0.0	0.000
Intercept: 0.931	Peak 3:	0.000	0.0	0.000
Result quality : Good				

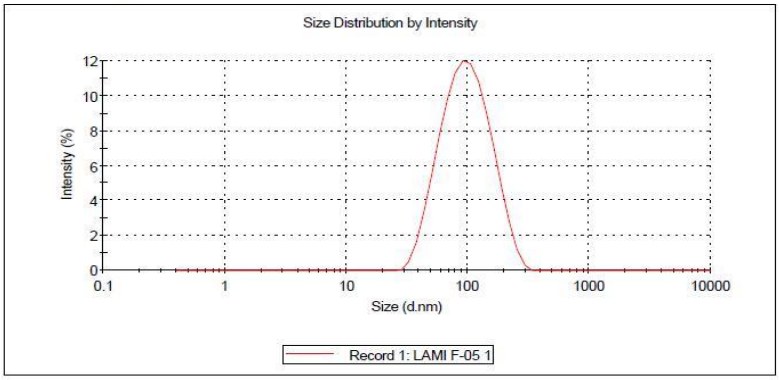

Figure 11: Particle size and PDI of (F-05) formulation.

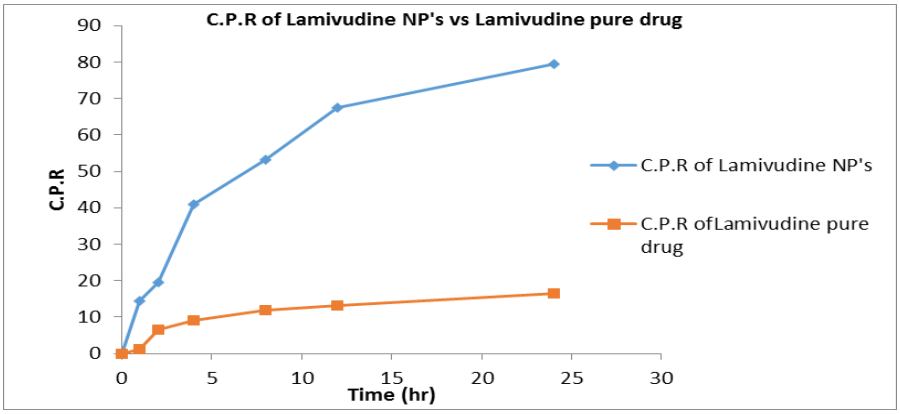

Figure 12: Comparison of *ex-vivo* drug release of Lamivudine NP's and pure drug.

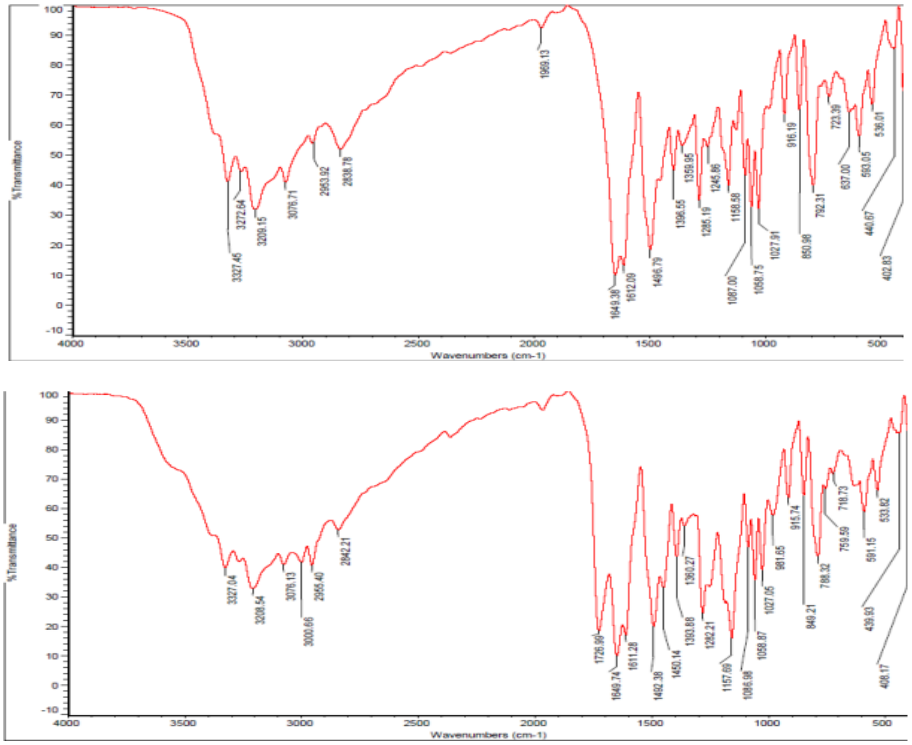

Figure 13: Comparision of IR spectra of LMV pure drug and LMV NP's.

		Size (d.nm):	% Intensity	Width (d.n...
Z-Average (d.nm): 104.0	Peak 1:	123.2	100.0	53.25
PdI: 0.159	Peak 2:	0.000	0.0	0.000
Intercept: 0.947	Peak 3:	0.000	0.0	0.000
Result quality : Good				

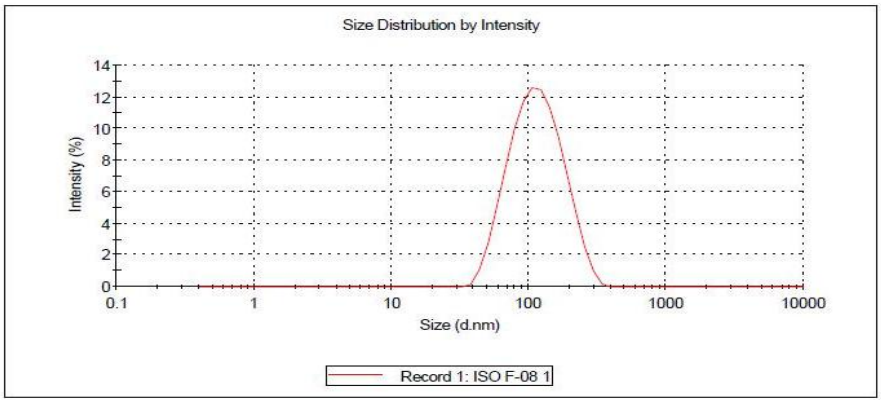

Figure 14: Particle size and PDI of (F-08) formulation.

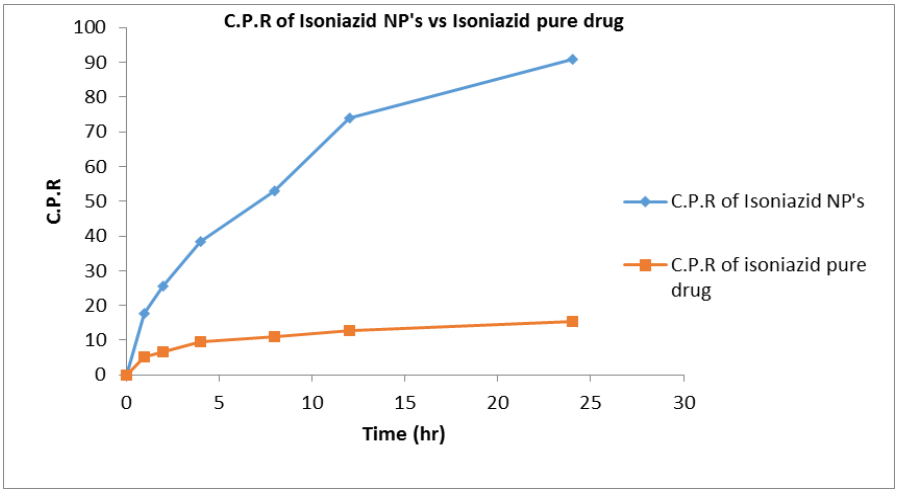

Figure 15: Comparison of *ex-vivo* drug release of Isoniazid NP's and pure drug.

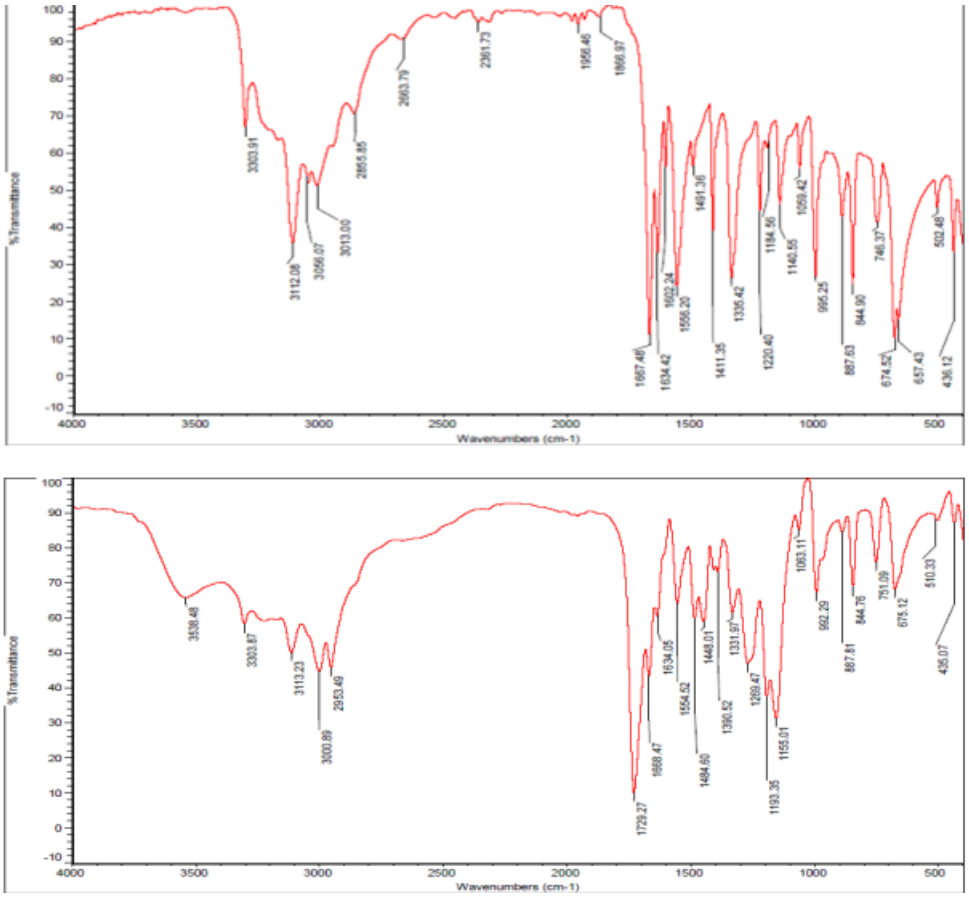

Figure 16: Comparision of IR spectra of INH pure drug and INH NP's.

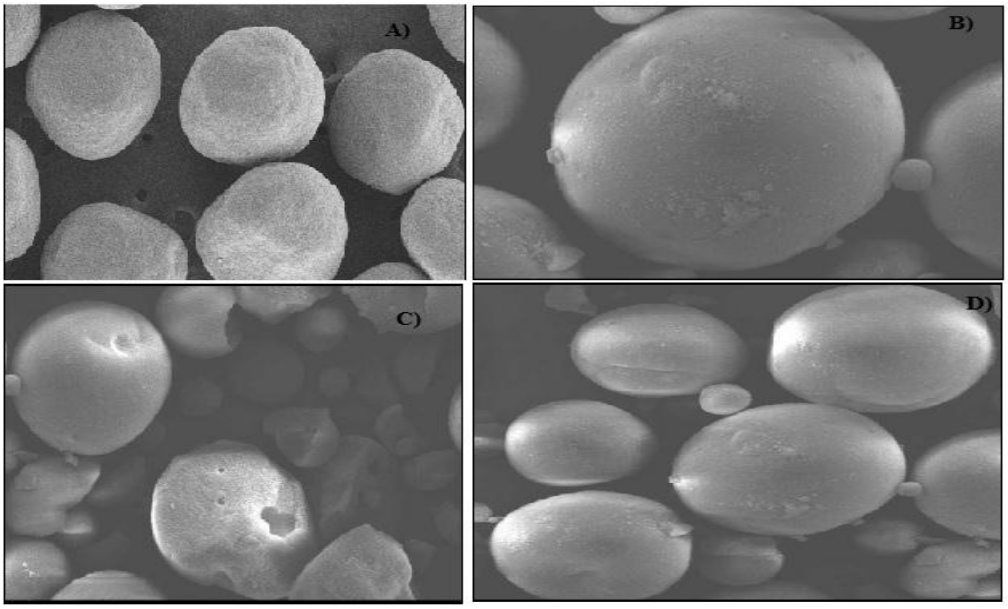

Figure 17: SEM of (A) NEV NPs (B) STA NPs (C) LAM NPs and (D) ISO NPs.

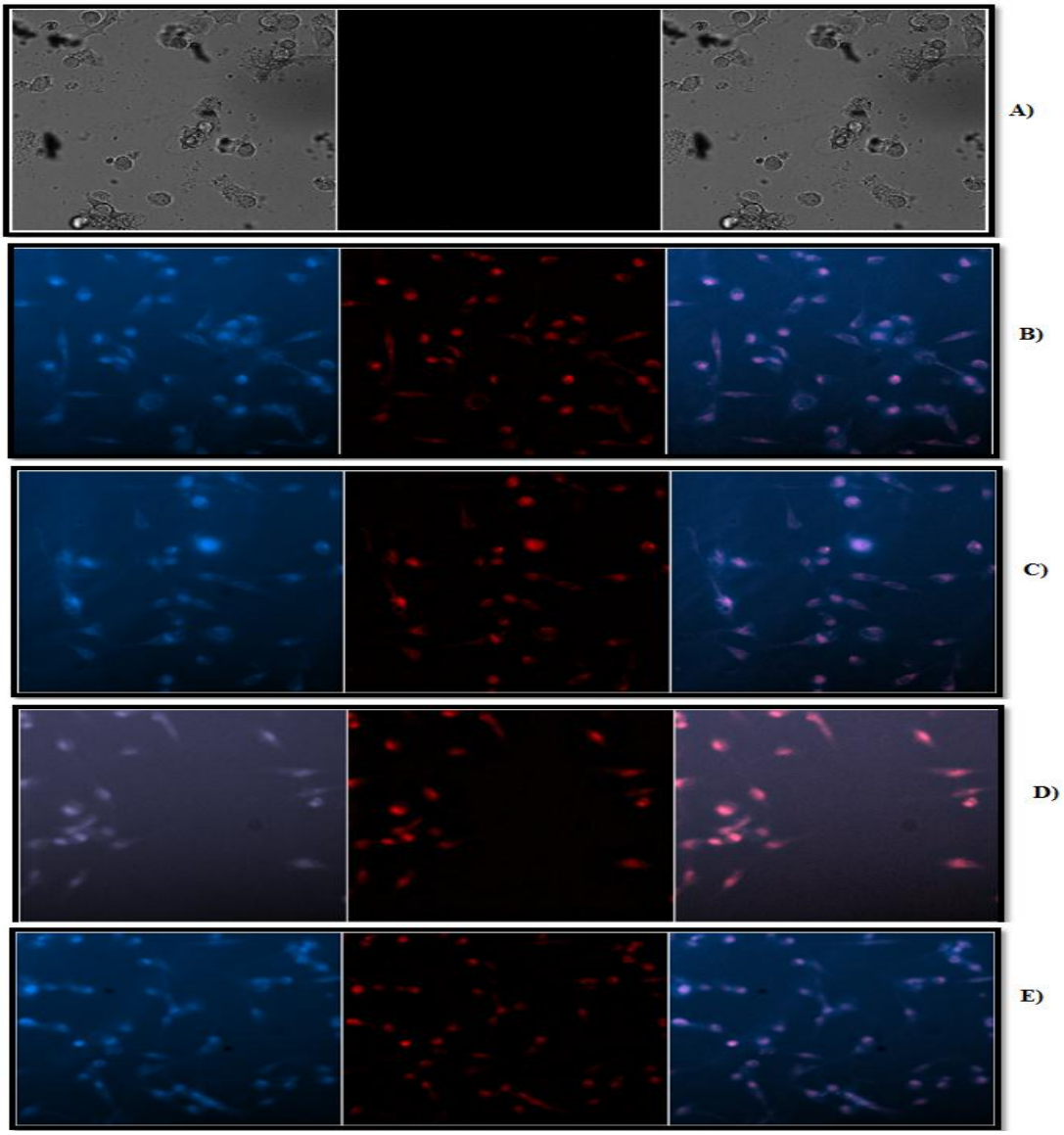

Figure 18: Cellular uptake of (A) Blank nanoparticles (B) NEV NPs (C) STA NPs (D) LAM NPs and (E) ISO NPs.

DISCUSSION

Nanoparticles were prepared by a nanoprecipitation method. Eudragit S100. Drug loaded Eudragit S-100 nanoparticles were prepared by a nanoprecipitation method and evaluation was done. Results showed that methanol if used as the solvent there precipitation was seen. Hence, further trials were carried out with acetone as the solvent and formulation A3 was selected for further optimization studies.

Following the preliminary work which resulted in identifying crucial process and formulation variables the formulations were optimized according to a 3^2 full factorial design, allowing the simultaneous evaluation of three independent variables and their interaction. Two factors were evaluated each at two levels. The drug to polymer ratio (*X1*), and Concentration of Surfactant (*X*2) were selected as independent variables. The dependent variables selected for study were particle size (*Y1*) and % PDI (*Y2*). Further Nevirapine loaded Eudragit nanoparticles were prepared at room temperature (25±2°C) in batches, OA1 to OA10. The observed responses were particle size and PDI. The results showed that formulation OA3 with least amount of surfactant and least drug: polymer ratio showed the best result.

Response surface graphs were plotted in three-dimensional model for optimization of NPs. The coefficients of the polynomial equation, generated using MLRA (Multi Linear Regressions Analysis) for particle size and PDI, which formed excellent fits to the data, with the values of r^2 ranging between 0.9986 and 0.9944. Figures portray the respective three-dimensional response surfaces for various response variables.

Graphical presentation of the data can help to show the relationship between response and independent variables. Graphs gave information similar to that of the mathematical equations obtained from statistical analysis.

The response surface plot illustrated that as drug:polymer ratio increases, the value of dependent variables, i.e. particle size and PDI increases. As the percentage of surfactant increases the value of dependent variables, i.e. particle size and PDI increases. The mean particle diameter and size distribution of the PNPs were characterised by Dynamic Light Scattering (DLS) method using Particle size analyser (Zetasizer Nano S-90, Malvern UK). The particle size and PDI of NVP OA3 formulation was found to be 178.9 nm and 0.287 respectively. The optimized NVP formulation was lyophilized and % yield was calculated. % yield of the NVP OA3 formulation was 92±1.6033. % free drug of NVP OA3 formulation was found to be 33.95±2.0408. % Encapsulation efficiency (EE) of NVP OA3 formulation was found to be 64.68±1.5510. Nanoparticles of NVP of desired particle size for buccal delivery have been achieved.

For the nanoparticles of water soluble drugs namely Stavudine, Lamivudine and Isoniazid, screening and formulation procedures were carried out separately. Eudragit was selected for further trails. Eudragit S 100 nanoparticles were prepared by a nanoprecipitation method. Evaluation of Eudragit S-100 blank nanoparticles was carried out. Nanoparticles were prepared by a nanoprecipitation method for all three drugs lead to formation of precipitation and hence double emulsification method for preparation was adopted. Nanoparticles were successfully prepared by using this method and screening of different surfactants and co-surfactants was carried out, Tween-80 as surfactant and PVA as co-surfactant gave the best results. Further these parameters were selected and process parameters were standardized. Effect of time and speed of homogenization is important and effect of probe sonication is negligible. Selected parameters were chosen for further optimization. Following the preliminary work which resulted in identifying crucial process and formulation variables the formulations were optimized according to a 3^3 full factorial design, allowing the simultaneous evaluation of three independent variables and their interaction. Two factors were evaluated each at two levels. The drug to polymer ratio (X1), Concentration of Surfactant (X2) and Concentration of co-surfactant (X3) were selected as independent variables. The dependent variables selected for study was particle size (Y1), % PDI (Y2) and %EE. Optimization was carried out for all 3 drugs.

Response surface graphs were plotted in three-dimensional model for optimization of NPs. The coefficients of the polynomial equation generated using MLRA (Multi Linear Regressions Analysis) for particle size and PDI, which formed excellent fits to the data, with the values of r^2 values within the limits as mentioned in results part. Figures portray the respective three-dimensional response surfaces for various response variables.

Graphical presentation of the data can help to show the relationship between response and independent variables. Graphs gave information similar to that of the mathematical equations obtained from statistical analysis.

The mean particle diameter and size distribution of the PNPs were characterised by Dynamic Light Scattering (DLS) method using Particle size analyser (Zetasizer Nano S-90, Malvern UK). The particle size and PDI of STV F-06 formulation was found to be 118.9 nm and 0.184 respectively. The optimized STV F-06 formulation was lyophilized and % yield was calculated. % yield of the STV F-06 formulation was 92±1.023. % free drug of STV F-06 formulation was found to be 28.66±2.521. % Encapsulation efficiency (EE) of STV F- 06 formulation was found to be 66.38±2.251. The mean particle diameter and size distribution of the PNPs were characterised by Dynamic Light Scattering (DLS)

method using Particle size analyser (Zetasizer Nano S-90, Malvern UK). The particle size and PDI of LAM F-05 formulation was found to be 86.33 nm and 0.224 respectively. The optimized LAM F-05 formulation was lyophilized and % yield was calculated. % yield of the LAM F-05 formulation was 91.14±1.423. % free drug of LAM F-05 formulation was found to be 25.18±1.025. % Encapsulation efficiency (EE) of LAM F-05 formulation was found to be 68.37±2.036. The particle size and PDI of ISO F-08 formulations was found to be 104nm and 0.159 respectively. The optimized formulation was further lyophilized and the % yield was calculated which was found to be 90.5±1.023. % free drug of ISO F-08 formulation was found to be 26.23±2.425. % Encapsulation efficiency (EE) of ISO F-08 formulation was found to be 64.36±1.736.

The aim of formulating drug loaded nanoparticles was to improve the permeability characteristics of the drug. As the drugs are poorly permeable, incorporating the drugs directly in a buccal delivery system would not favorably help in the drug permeation across the mucosal barrier. Herein we describe the *ex-vivo* permeability of drug loaded NP's through sheep mucosa. The results indicated that the drug permeated through the membrane when formulated in a NP system. The drugs had a higher permeation as compared to the pure drugs; owing to the smaller particle size of the NP's which can passively diffuse through the buccal area. SEM images showed that all the nanoparticles exhibited a smooth and a spherical surface showing the feasibility of the method of preparation of nanoparticles.

Cell uptake studies were performed with epithelial HeLa cell lines, which is a good indicator of permeation across the mucosal barrier. The nanoparticulate formulations were tagged with Rhodamine dye which is a fluorescent label used to tag compounds for imaging in a fluorescent microscope. From the images it is clear that all the formulations have achieved sufficient degree of cellular penetration owing to the smaller size of the nanoparticles. As evident from the cellular uptake images, the NP's could penetrate the epithelial barrier and permeate into the cells as evident from the florescence microscopy images. It could be concluded that NP's have an added advantage of increase cellular uptake which supports the results of the *ex-vivo* permeation data. Phagocytic uptake and destruction is one of the major causes for the lack of activity of nanoparticles, however this is valid for particles in the size range of 1000 nm, hence by formulating particles of around 200nm, we have ensured that the NP's reaches its target site as shown by the results of cellular uptake.

CONCLUSION

HIV stigma and associated disorders are major barriers for a healthy society. Adherence to HIV care for children and its treatment becomes more difficult as the regimen for the therapy involves multiple drugs which are needed to be administered regularly to children, a population opposing any medication. Hence, by developing an effective nanoparticulate therapy in a combination dosage form, the disease mitigation can be effected in a more patient friendly manner. The research also opens further horizons for incorporating the NP's in a paediatric friendly platform.

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