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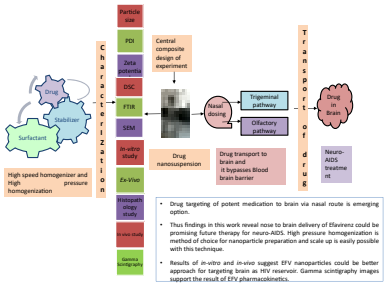
Research article

Nose to brain delivery of Efavirenz nanosuspension for effective neuro AIDS therapy: *in-vitro*, *in-vivo* and pharmacokinetic assessment

Smita Kakad *, Sanjay Kshirsagar

Department of Pharmaceutics, MET's Institute of Pharmacy, Nashik, Affiliated to Savitribai Phule Pune University, Pune, 422003, India

GRAPHICAL ABSTRACT



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ABSTRACT

Efavirenz is inhibitor of non-nucleoside reverse transcriptase enzyme; BCS class II drug. The objective of the present research was to prepare and evaluate nanosuspension of Efavirenz for the treatment of neuro-AIDS. Efavirenz is the substrate for drug resistant proteins at BBB prone to efflux and could not reach brain with effective levels. Current need of the therapy is to develop drug delivery systems targeting viral reservoirs at effective concentration in the brain. With this need we developed Efavirenz nanosuspension for nose to brain drug transport to bypass blood brain barrier. Nanosuspension prepared with high-pressure homogenization had a mean particle size of 223 nm, PDI of 0.2 and -21.2 mV zeta potential. Histopathology study on goat nasal mucosa showed no adverse effects of formulation on nasal tissues. Gamma scintigraphy study and *in-vivo* study on Wistar rat model reveals drug transport to the CNS after nasal administration. Pharmacokinetic parameters and drug targeting potential of 99.46 % suggest direct nose to brain transport of Efavirenz nanoparticle. Results reveal that nose to brain delivery of Efavirenz is the best possible alternative for neuro –AIDS treatment.

1. Introduction

UNAIDS (The Joint United Nations Programme on HIV and AIDS) report says that "Prevention and end of AIDS as a common health threat can be interpreted quantitatively as a 90% reduction in newly developed HIV infections and deaths caused by AIDS (Acquired Immunodeficiency Syndrome) elated illness by year 2030 compared to 2010 baselines." 25.4 million People were accessing antiretroviral therapy and 38 million people worldwide living with HIV indicate its huge prevalance [1, 2].

Invasion of human immunodeficiency virus (HIV) to central nervous system (CNS) is associated with neurologic condition called neuro-AIDS. Neuro-AIDS prevails among AIDS patients [3].

HIV gains entry into brain via blood-derived macrophages or trans-migration across blood brain barrier [4]. After entering into brain HIV

* Corresponding author.
E-mail address: smitadarade87@gmail.com (S. Kakad).

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follows both pathophysiologic infectious pathway and degenerative pathway [5]. HIV associated neurocognitive disorders (HAND) are of three different categories as (a) Asymptomatic neurocognitive impairment, (b) Mild neurocognitive disorder and (c) HIV-associated dementia [6].

Alessandro et al (2018); reported that ARVs (anti-retroviral) show difficulties in penetrating BBB due to active efflux transporters (AETs). To avoid efflux of ARV via efflux transporters drug can deliver bypassing blood brain barrier [7]. EFV could not cross BBB efficiently after IV administration as active efflux transporter protein hinders in the transport of EFV at BBB. EFV is a substrates for breast-cancer-resistance protein (BCRP) active efflux transporters at BBB. EFV is a ligand of both human receptors nuclear pregnane X (PXR) and human constitutive androstane (hCAR). Nuclear receptor activity of EFV restricts its ability to enter the brain by increasing P-gp expression at BBB [7].

Nose to brain drug delivery via trigeminal and olfactory pathway is a success for migraine therapy and vaccine delivery, which may prove a better alternative for ARV to reach the brain with an effective drug level. The design of promising drug delivery approaches for life-threatening HIV infection may save the brain from Neuro-AIDS and help to enhance the socio-economic life of HIV patients [8]. Intranasal nanoformulations enhances drug bioavailability to the CNS as this route protects the drug from chemical and biological degradation, it prevents P-gp efflux proteins associated extracellular transport of drug, nano size of drug facilitates transcellular transport via neuronal pathway [9]. Thus nose to brain delivery of the antiretroviral may prove effective solution for treating neuro-AIDS pathogenesis.

EFV is non-nucleoside reverse transcriptase inhibitor (NNRTI). It is a first line medication in the treatment of HIV infection. EFV is BCS class II drug with poor solubility in water about 4 μg/mL, lipophilicity in terms of log p is 5.4, and limited oral bioavailability of 40–50%, with high intra and inter-individual variability of 19–24% and 55–58%, respectively [10, 11]. EFV is low molecular weight (315.67 g/mol) drug. Lipophilic molecules with molecular weights less than 1 kDa are rapidly and efficiently absorbed transcellularly across the nasal membrane [12, 13]. HIV-patients on oral EFV therapies possibly have lesser drug concentration in the CNS contributes to persistence of HAND [14].

2. Materials and methods

EFV was generously suplied by Mylan Laboratories (Nashik, India) as a gift sample. Hydroxypropyl methylcellulose Premium LV (HPMC E3) generously gifted by Colorcon Asia Pvt., Ltd. (Goa, India). Poloxamer 407 (P407) was purchased from Signet Chemicals Corporation Pvt., Ltd. (Mumbai, India). Sodium lauryl sulphate (SLS) was purchased from Sisco Research Laboratories Pvt., Ltd. (Mumbai, India). All other chemicals and solvents were of analytical grade. The experimental procedures on animal were conducted after the approval of institutional animal ethics committee (IAEC) as per the guidelines of the CPCSEA for care of laboratory animals. Approval number is MET-IOP-IAEC/2019–20/02.

2.1. Preparation of nanosuspensions

Nanosuspensions were prepared by high speed (IKA T25, Ultra Turrex) 45 min at 15000 rpm) and high pressure homogenization technique (Gea Niro Soavi, 750 barr pressure with 20 cycles). To establish the process of nanosuspension trial batches were prepared. Combination of HPMC and poloxamer 407 (stearic stabilizer) and SLS (surfactant) were used for the preparation of nanosuspension. Response surface methodology (RSM) with central composite design (quadratic model) was used for the optimization of nanosuspension (Design-Expert® software, version 12, Stat-Ease, Inc., Minneapolis, MN, USA) with 2 independent factors as mention in Table 1. According to the design, 13 runs including factorial points (4), central points (5) and axial points (4) were analysed. Dependant variables were screened for all the batches, these are particle size, PDI and zeta potential. The polynomial fitting quality was employed

Table 1. Independent variable factors of design with coded value.

Factor	Name	Minimum (-α)	Maximum (+α)	Coded Low(-1)	Coded High(+1)
A	Poloxamer 407	31.72	88.28	-1 ↔ 40.00	+1 ↔ 80.00
B	SLS	1.89	23.11	-1 ↔ 5.00	+1 ↔ 20.00

to quantify the effect of independent formulation variable A and B on the response variables Y with applied constraints. Quadratic model generated following Eq. (1):

$$Y = A + B + AB + A^2 + B^2 + A^2B + AB^2 + AB^2 + A^3 + B^3 \quad (1)$$

Where Y is the measured response and A, B are the independent formulation variables.

2.2. Lyophilization of nanosuspension

The optimized batch of formulation was processed for lyophilization, to further increase the stability of product. Mannitol (1%) was used as a cryoprotectant. Filled 20mL of nanosuspension in flint coloured vials having rubber stoppers and frozen in deep freezer at −75 °C for 24h. These frozen mass was freeze-dried in lyophilizer (Virtis Benchtop, Bombay, India) for 48 h to produce free flowing dry powder [15, 16].

2.3. Characterization of EFV nanosuspension

2.3.1. Determination of particle size, size distribution and zeta potential

Nanosuspensions were characterized for zeta potential, mean particle size, and polydispersity index (PDI) by dynamic light scattering (DLS) method using the Malvern Zetasizer (Malvern Instruments Ltd. version 6.20, UK). Samples were suitably diluted with the double distilled water before analysis.

2.3.2. Morphology of nanosuspension

The scanning electron microscope (Supra 55- Carl Zeiss, Germany) with focused electron beam was utilized for the visualization of shape and morphology of the prepared nanosuspension [17].

2.3.3. DSC study

Thermal analysis of pure EFV and freeze-dried product using DSC (DSC 60, Shimadzu, Japan). The sample scanning rate of 20 °C/min over a temperature of 35–300 °C.

2.3.4. X-ray powder diffraction characterization

Crystalline structure of API and potential changes in the freeze dried nanosuspension were analysed by X-ray powder diffractometer (XRD-7000, Shimadzu, Japan) [16, 18].

2.3.5. Determination of saturation solubility

To determine saturation solubility added an excess amount of EFV and freeze dried nanosuspension separately in distilled water, to obtain a saturated solution. Samples were agitated in orbital shaker (Remi Electrotechnik) for 48 h at 25 °C. After centrifugation and filtration through a 0.1 mm membrane filter (Millipore Corporation), the filtrate was diluted and analysed at 247nm with UV spectrophotometer [19].

2.3.6. Nasal mucociliary transport time

As per approved animal study protocol rats were anesthetized by intramuscular injection of Ketamine 50 mg/ml IP. Animals were divided in two group (Test and control). Instilled 20 μL of test formulation added with the solution of methylene blue to a rat nose (5 mm depth into the right nostril) using a micropipette (Labline, ECO). The appearance of the dye at the pharynx and nasopalatine region of the oral cavity was recorded as the appearance time by taking swab with moistened cotton-

tipped applicators. For control group instilled normal saline added with methylene blue dye (5 mg/mL) and the appearance time of the blue dye was recorded [20, 21].

2.3.7. Nasal histopathology study

Histopathological studies were performed on goat nasal mucosa to check safety of the formulation when administered intranasal. Goat nasal mucosa was collected from a local slaughter house and placed in phosphate buffer solution (pH 6.5). It was cleaned with phosphate buffer and cut into nine symmetrical pieces with uniform thickness. The first 3 pieces were treated with formulation and the next 3 pieces were treated with the positive control 70% isopropyl alcohol, while the last three pieces were treated with negative control (PBS 6.5). After 1 h of treatment, all samples were washed thoroughly with phosphate buffer solution and stored directly in 10% solution of formalin for 24 h. It was important to ensure that the volume of formalin used was ten times more than the volume of the fixed sample. After 24 h, the formalin solution was replaced with 70 % ethanol and the samples stored at 4 °C for dehydration. The dehydrated sections were then embedded in agar and paraffin block and cut to 5 μm thickness by using a microtome. Finally, the samples were stained with a combination of haematoxylin and eosin (H&E) dye and observed under optical microscope to detect any damage [22].

2.3.8. In-vitro drug release study

The nanosuspension equivalent to 10 mg EFV was placed in a cellulose dialysis bag, (MWCO 12,000 g/mol; Himedia Laboratories Pvt. Ltd.), this was then sealed at both ends and immersed into a dialysis reservoir containing 150 mL phosphate-buffered saline (PBS, pH 6.5) preconditioned and maintained at 34 ± 0.5 °C on a water bath. Withdrawn 1 mL sample at specified time intervals (5, 15, 30, 45 and 60 min) and added same amount of fresh PBS to monitor sink conditions. Cumulative drug release was determined [21].

2.3.9. In-vivo drug release study

All the procedures related to animal handling were followed as per the approved animal study protocol. Animals were acclimatized to animal house facility for 2 week with 12 h light/dark cycle and temperature of 22 ± 2 °C. Animals were divided into two groups. Group A (Positive control) was injected via tail vein (IV) and group B (Test) was administered intranasal (IN). Further, animals from each group were studied for six time points (1, 4, 6, 12, 24, and 72h) with three rats in each group. Mild anaesthesia was given with Ketamine 50 mg/mL prior to intranasal dosing.

20μL dose (equivalent to 4 mg/kg of drug) was instilled in each nostril using micropipette. At fixed time intervals, blood specimens were collected in EDTA-coated tubes by retro-orbital puncture under mild anaesthesia and immediately brain were excised from the sacrificed animal. The collected blood was centrifuged at 8000 rpm/10 min/4 °C, plasma was separated and preserved at −20 °C. Homogenized brain samples in phosphate buffer saline and acetonitrile in the ratio of 50:50; followed by the centrifugation at 12000 rpm/15 min/10 °C. The clear liquid separated and preserved at −20 °C [23, 24, 25, 26, 27, 28, 29]. Further, samples were analysed with previously developed and validated RP-HPLC method [30].

Pharmacokinetic parameters c_{max}, t_{max}, t_{half} and AUC were determined. Drug targeting index and % drug transport were determined to check brain targeting potential. Eqs. (2), (3), (4), and (5) were used for calculation.

$$\text{Drug targeting index} = \frac{(\text{AUC brain}/\text{AUC plasma})\ \text{in}}{(\text{AUC brain}/\text{AUC plasma})\text{iv}} \quad (2)$$

$$\frac{\text{Biv}}{\text{Piv}} = \frac{\text{Bx}}{\text{Pin}} \quad (3)$$

$$\text{Bx} = \left(\frac{\text{Biv}}{\text{Piv}}\right) \times P_{in} \quad (4)$$

$$\%\text{DTP} = \frac{(\text{Bin} - \text{Bx})}{\text{Bin}} \times 100 \quad (5)$$

AUC_0-t of EFV after intravenous and intranasal administration in the brain and the plasma are Biv, Piv, Bin, Pin respectively. Bx is the AUC fraction of EFV in brain obtained after intranasal dosing it might come up with systemic circulation via blood brain barrier [31].

2.3.10. Gamma scintigraphy study

In-vivo biodistribution of drug was studied by gamma scintigraphy. The experimental procedures were conducted after the approval of institutional animal ethics committee (IAEC) as per the guidelines of the CPCSEA for care of laboratory animals. Nine healthy male Wistar rats (180–250g, aged 2–3months) were selected for this study. Animals were grouped as mentioned in Table 2; rats from first group were administered with intravenous EFV solution, animals from second group were administered with nasal dosing of EFV solution and third group was administered with nasal formulation. Drug solution (prepared in 0.9% saline solution and 30% propylene glycol as a co-solvent) added with reducing agent stannous chloride 20 min prior to radio labelling with 99mTc (1mci) by direct labelling method. Similarly drug nanosuspension (equivalent to 25 mg/mL drug) was radiolabelled. Incubated both mixtures for 30 min [7, 31]. Forty microliter of radiolabelled 99mTC- drug solution (1mg drug) was intravenously administered by tail vein of Wistar rat using 1-mL Helminton syringe, while radiolabelled 99mTC- drug solution (40μL) and 99mTc- drug nanoparticles (40μL) were administered intranasal in both nostrils using micropipette adaptor. Animals were held at supine positon with 90° angle for the deposition of maximum dose at olfactory region. Rats were anesthetized using ketamine 50 mg/mL and placed on the imaging platform. Radio images were taken at predetermined time interval (1hr, 2hr and 6hr) using gamma camera (Tandem_Discovery_630) at HCG Manvata Cancer Centre, Nashik, India. In between gamma scanning intervals, the animals were freed and allowed for normal activities [7, 31]. All the procedures involved in Gamma scintigraphy studies, were performed at nuclear medicine section of HCG Manvata Cancer Centre, Nashik, India.

2.3.11. Stability study

EFV-LNS (Efavirenz lyophilized nanosuspension) were analysed under accelerated stability conditions as per ICH guidelines. Sample was kept at temperature of 40 °C ± 2°C/and humidity of 75% RH ± 5% RH. Physical stability was assessed by checking zeta potential and particle size. Chemical stability was confirmed by analysing drug content of EFV-LNS [32].

3. Results

Process variables number of homogenization cycles and pressure were kept constant after taking trial batches and effect of formulation variables were analysed. Trial batches suggest the combination of HPMC 3cps, poloxamer 407 and SLS for better stabilization to EFV nanosuspension measured in terms of zeta potential. Nanosuspension batches prepared were as mention in Table 3.

Table 2. Animals grouped for gamma scintigraphy study.

Group Code	Compound No.	Dose (mg/kg)	No. of animals	Route
I	Drug solution	4	3	Intravenous
II	Drug solution	4	3	Intranasal
III	Drug formulation	4	3	Intranasal

Table 3. Batches prepared by high pressure homogenization.

Batch	Drug mg	HPMC 3cps mg	Poloxamer 407 mg	SLS mg	Particle size (Z- Avg nm)	PDI	Zeta potential (mV)
1	100	100	60	12.5	223.3	0.262	-21.2
2	100	100	80	20	146	0.414	-12.3
3	100	100	40	5	248.3	0.596	-7.09
4	100	100	12.5	31.71	223.2	0.431	-9.25
5	100	100	60	12.5	223.3	0.262	-21.2
6	100	100	88.28	12.5	423.4	0.630	-0.835
7	100	100	60	12.5	223.3	0.262	-21.2
8	100	100	60	12.5	223.3	0.262	-21.2
9	100	100	60	23.10	191.4	0.554	-11.8
10	100	100	60	1.89	364.3	0.716	-10.6
11	100	100	40	20	119.5	0.59	-10.5
12	100	100	60	12.5	223.3	0.262	-21.2
13	100	100	80	5	149.2	0.615	-7.78

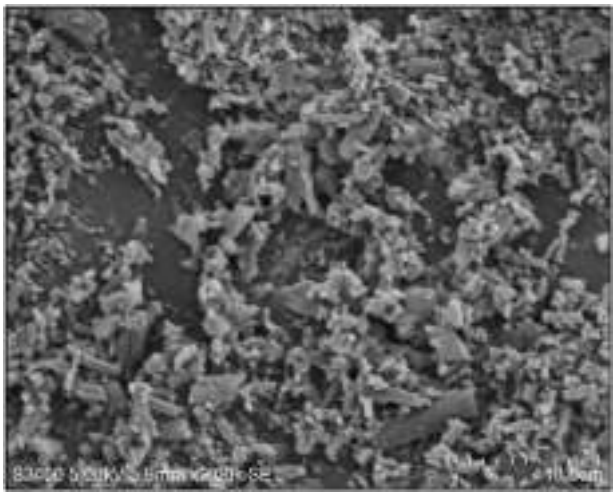

Figure 1. SEM of EFV-LNS

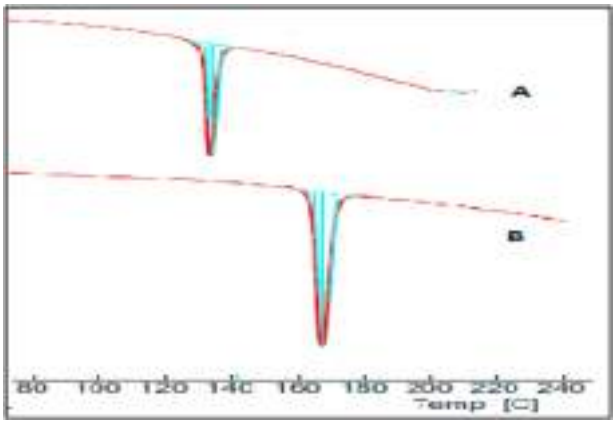

Figure 2. DSC thermogram of EFV (A) and EFV-LNS (B).

3.1. Influence of formulation variables on particle size, polydispersity index (PDI) and zeta potential

Polynomial Eqs. (6), (7), and (8) showing effect of variables on response.

$$\text{Particle size} = +223.30 + 26.32A - 47.06B + 31.40AB + 16.29A^2 - 6.43B^2 \quad (6)$$

$$\text{PDI} = +0.2620 + 0.0156A - 0.0545B - 0.0487AB + 0.127A^2 + 0.1793\,B^2 \quad (7)$$

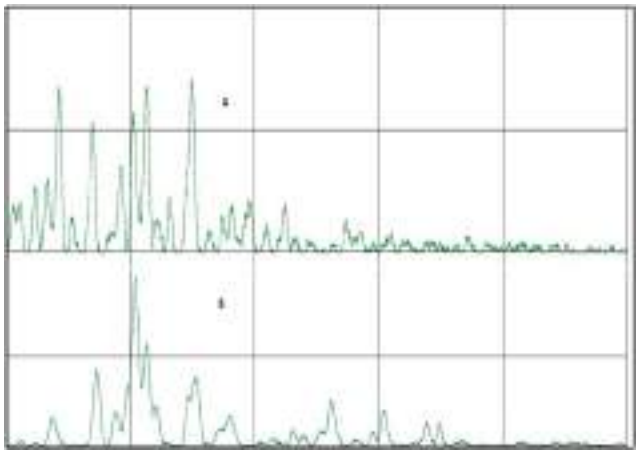

Figure 3. XRD spectra of EFV (A) and EFV-LNS (B).

$$\text{Zeta potential} = -21.20 + 1.18A - 1.20B - 0.2775AB + 7.75\,A^2 + 4.68\,B^2 \quad (8)$$

Poloxamer concentration (A) and SLS concentration (B) both formulation variables shown significant impact on zeta potential and particle size as compared to PDI. Refer Table 3 for the observation. PDI for nanoparticles was found in the range of 0.262–0.716. F1 formulation with particle size 223.3 nm, PDI of 0.262 and zeta potential -21.2 mV with desirability one was selected as the optimized batch. Optimized formulation F-1 with desirability one was found in good agreement with the predicted values generated by model with applied constraint.

3.2. Characterization of lyophilized EFV nanosuspension

3.2.1. Drug content, pH and saturation solubility

EFV content of the lyophilized product was found to be 96.56 ± 0.85. The apparent pH of the formulation was in the range of 6–6.5. Saturation solubility of lyophilized product was found to be 110.43 ± 0.8 μg/ml whereas the plain drug solubility was 6.97 ± 0.01 μg/ml in distilled water.

3.2.2. Morphology of nanosuspension

Scanning electron microscopy (SEM) image of optimized batch is revealed in Figure 1. It showed clusters of amorphous product after lyophilization rather than crystalline form of pure drug.

3.2.3. Differential scanning calorimery (DSC)

Figure 2 shows DSC thermogram of EFV lyophilized nanosuspension and EFV. Sharp melting endothermic peak of EFV was observed at 141.63

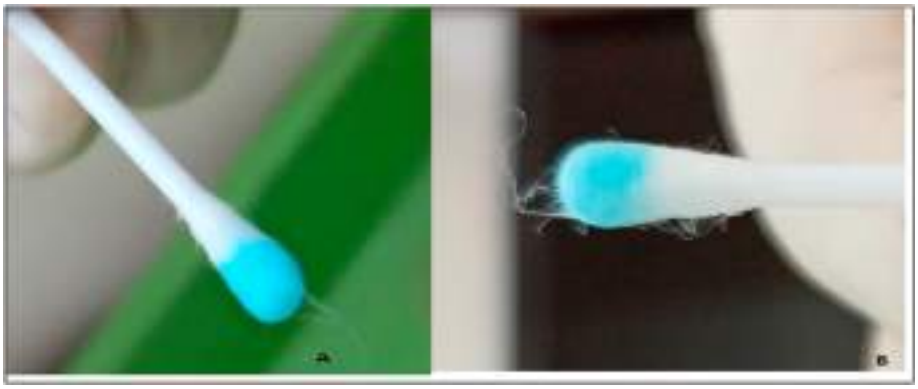

Figure 4. (A) Control solution swab collected after 2min and (B) formulation swab collected after 30 min.

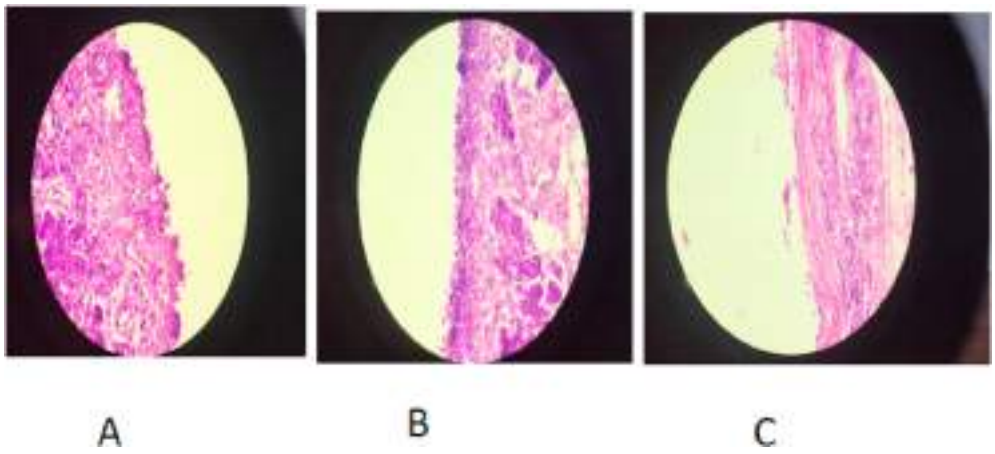

Figure 5. Histopathology study of EFV-LNS. (A: Formulation, B: Negative Control and C: Positive Control).

°C. Characteristic endothermic peak of EFV was absent in the DSC thermogram of EFV-LNS as drug was converted to amorphous form. EFV LNS thermogram showed endothermic peak at 171 °C corresponding to the melting point of mannitol used for lyophilization [34].

3.2.4. X-ray powder diffraction characterization (XRD)

Figure 3 shows XRD spectra of EFV and EFV-LNS. Characteristics multiple sharp peaks in the XRD spectrum of EFV at $2\Theta = 14$, $2\Theta = 21$ and $2\Theta = 24$ showed crystalline nature of EFV. XRD spectra of EFV LNS showed broad peaks at $2\Theta = 17$, $2\Theta = 20$ and $2\Theta = 21$. Absence of characteristics sharp peak of EFV indicates loss of crystalline nature of drug during formulation as it was converted to amorphous form [34].

3.2.5. Nasal mucociliary transport time

Nasal mucociliary transport time was observed between 27 to 32 min for the test formulation. It showed better residence of formulation in the nasal cavity [35]. Figure 4 showed images for swab after nasal administration of sample.

Table 4. Pharmacokinetic analysis.

Parameter	Plasma (IV)	Plasma (IN)	Brain (IV)	Brain (IN)
	Solution	NPS	Solution	NPS
C_{max}(μg/ml)	125.97 ± 2.61	6.65 ± 0.16	0.725 ± 0.002	4.49 ± 0.001
$T_{max\ (min)}$	15 ± 0.00	240 ± 0.00	360 ± 0.00	360 ± 0.00
$AUC_{0\text{-}72}$(μg/ml)*hr	856.33 ± 19.66	68.8 ± 1.26	8.427 ± 0.12	127.63 ± 0.23
$t_{half\ (hr)}$	18.4358 ± 0.12	13.977 ± 0.1058	14.1315 ± 3.1054	15.5931 ± 0.2622
$MRT_{(hr)}$	26.6802 ± 0.264	20.1621 ± 0.1537	19.6518 ± 5.001	22.4202 ± 0.4283

3.3. Nasal histopathology study

As shown in Figure 5C the positive control (70% isopropyl alcohol) shows changes in the epithelium cell i.e. they were detach from the membrane and also the regular sequence of the epithelium cells were distorted as compared to the negative control (Figure 5B). There was no significant destructive effect seen in the epithelial cell membrane after treated by EFV-LNS. So there was no damage to the mucosal cells seen in Figure 5C. Observations confirmed that the formulation found safe and non-irritant on intranasal administration [22].

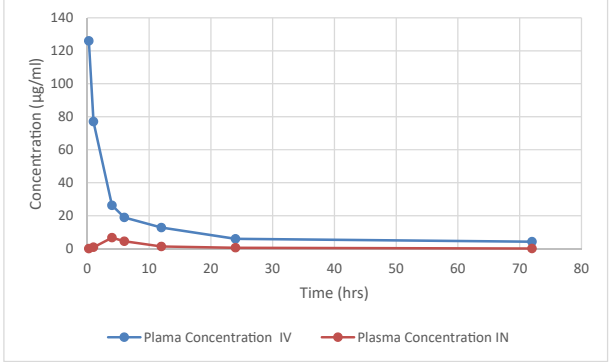

Figure 6. Plasma EFV concentration profile after IV and IN dose administration.

3.3.1. In-vitro drug release study

The *in-vitro* drug release study was performed using dialysis bag showed more than 81% cumulative drug released after 30 min. Mathematical models were applied to analyze the data for the drug release kinetics. Higuchi model was best fitted with the r^2value of 0.9546. Higuchi model reveals diffusion control drug release. The suspended nanoparticles showed better release profile with the polymer HPMC.

3.3.2. In-vivo drug release study

Pharmacokinetics of EFV after intravenous and intranasal administration were assessed by non-compartmental analysis and results are mentioned in Table 4. Figures 6 and 7 reveals comparison between plasma and brain EFV concentration.

C_{max} for brain after intranasal administration was significantly higher than obtained after IV dosing. $AUC_{0\text{-}72}$ of brain after intranasal administration was six fold higher than intravenous administration. Plasma $AUC_{0\text{-}72}$ of 68.8 ± 1.26 μg/mL*h showed partial drug transport to the systemic circulation absorbed via respiratory circulation. After IV administration $AUC_{0\text{-}72}$ of brain was small compared to plasma $AUC_{0\text{-}72}$ might be due to protein binding of EFV (>99.5%) and efflux by BCRP (breast cancer-resistant protein) present at brain endothelium [35]. The brain AUC fraction (Bx) contributed by systemic circulation after intranasal dosing was 0.68. Drug targeting index of 186.58 and drug targeting potential of 99.46 % suggest direct transport of EFV nanoparticle [31].

3.3.3. Gamma scintigraphy study

Gamma scintigraphy results reflect biodistribution of radiolabelled EFV. Figure 8 clearly depicts high radioactivity in the brain region after IN administration of nanoparticles as compared to IV drug solution. After IV dosing radioactivity was seen in abdomen at about 2 h of administration and later it diminishes up to 6 h.

3.3.4. Stability studies

Physical stability of nanosuspension was confirmed with insignificant changes observed in zeta potential and particle size. EFV-LNS found chemically stable as drug content of the formulation was observed unchanged during accelerated stability study. Table 5 gives results obtained after stability study.

4. Discussion

Optimum blend of stearic and electrostatic surfactant is required to produce the stable nanosuspension. HPMC and poloxamer 407 were good combination for stearic stability of nanosuspension also it has mucoadhesive properties required for good retention time [21, 35]. SLS was electrostatic stabilizer in the formulation [36]. Low PDI values of NPs indicate that the NPs with the narrow size range can be obtained with the size range of 119.5nm–423.4 nm are obtained in experiments performed.

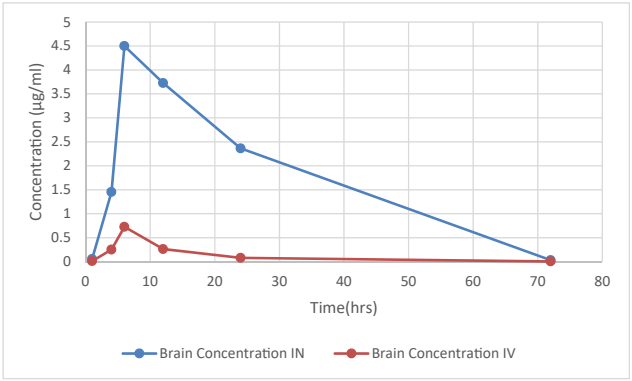

Figure 7. Brain EFV concentration profile after IV and IN dose administration.

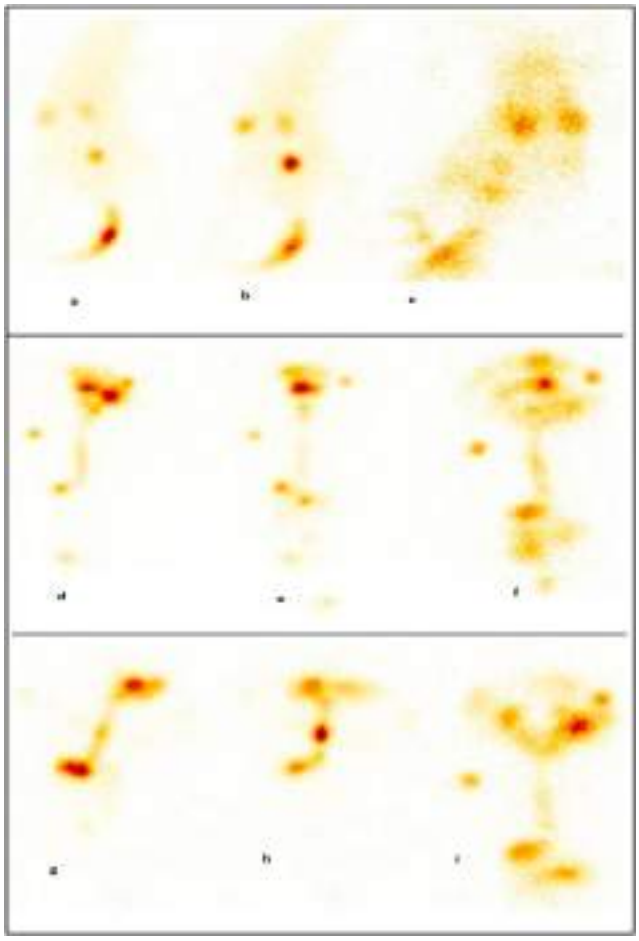

Figure 8. Gamma scintigraphy images of rat after intravenous administration of radiolabeled EFV solution (a = 1hr, b = 2hr, c = 6hr). Images of rat after intranasal administration of radiolabeled EFV nanosuspension (d = 1hr, e = 2hr, f = 6hr). Images of rat after intranasal administration of radiolabeled EFV solution (d = 1hr, e = 2hr, f = 6hr).

EFV is lipophilic and low molecular weight drug thus nanoparticle facilitates faster mucosal transport and transcellular transport via trigeminal pathway. Presence of both positive and negative charges at the surface of cell gives high binding affinity to NPs due to electrostatic interactions. Negatively charged nanoparticles bind at positively charged domains of cell membrane facilitates endocytosis and cell uptake. Nanoparticles could rapidly cross the mucosal barrier due to small size and charges developed on the surface [37]. Zeta potential values were dramatically affected with surfactant and stabilizer concentration as reported in Table 1. Zeta potential > ±20mV is required for stable nanosuspension [33]. Larger value for zeta potential (either negative or positive) imparts repulsion between suspended particles and prevents aggregate formation.

Lyophilized nanosuspension found with 15 fold increase in solubility as compared to pure EFV. This increase in the solubility was associated with conversion of drug to amorphous form and nanosizing of drug [19].

DSC, SEM and XRD study reveals that the lyophilized EFV nanoparticles are amorphous in nature [34]. Nasal mucocilliary clearance

Table 5. Stability study.

Sr. no.	Parameter	0 month	6 months
1	Drug content	90.55 ± 2.12	88.60 ± 2.02
2	Particle size nm	223.3	224.1
3	Zeta potential mV	-21.5	-19.1

study reveals that mucoadhesive nature and viscosity of the formulation achieved with polymer HPMC prolongs its retention in nasal cavity [35]. Drug transport may occur extracellularly from perineural spaces present around olfactory axons. Drug transport to CFS occurs via tight junctions at epithelium of olfactory nerves and connected in submucosa and subarachnoid space [38]. Drug transport is combination of olfactory and trigeminal pathway. Drug transported by passive diffusion from axons to olfactory bulb by endocytosis and pinocytosis.

Intranasal (IN) administration showed better CNS uptake of EFV NPS contributed by direct transport to CNS via trigeminal and olfactory region which bypasses BBB. Brain concentrations found higher than IC90-95 value of EFV [31, 39]. Drug targeting index and findings of *in-vivo* study suggest nose to brain delivery is promising strategy for neuro-AIDS treatment. Gamma scintigraphy supports the in vivo study and shown transport of drug to brain via nasal route.

5. Conclusion

For drug targeting of potent medication to brain nasal route is emerging option. Hurdles in nanosuspension development could be solved with selection of smart excipient. High pressure homogenization is method of choice for nanoparticle preparation and scale up is easily possible with this technique. Results of *in-vitro* and *in-vivo* suggest EFV nanoparticles could be better approach for targeting brain as HIV reservoir. Gamma scintigraphy images support the result of EFV pharmacokinetics. Thus findings in this work reveal nose to brain delivery of antiviral medication could be promising future therapy for neuro-AIDS.

Declarations

Author contribution statement

Smita Kakad: Conceived and designed the experiments; Performed the experiments; analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sanjay Kshirsagar: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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RESEARCH Open Access

Development of reverse phase high-performance liquid chromatographic method for the estimation of HIV non-nucleoside reverse transcriptase inhibitor drug efavirenz in the rat brain

Smita Prakash Kakad* and Sanjay Jayprakash Kshirsagar

Abstract

Background: The brain is the potential viral reservoir, and estimating the antiviral drug concentration in the brain is a hurdle to the researchers as very few animal models are available for this study. The objective of the study was to develop and validate the RP-HPLC method for the estimation of antiviral drug efavirenz (EFV) in the brain of healthy Wistar rats. EFV was the first-line antiretroviral medication. The optimized HPLC condition used for the analysis had the mobile phase methanol to water (9:1) ratio. The flow rate was set at 0.8 mL/min, while the detection wavelength was 248 nm.

Results: The retention time was found to be 5.7 min, and the % RSD was found within the limit. Recovery was found to be nearly 78%. The validation results were found to be within the limit range; hence, the obtained method was accurate, specific, rapid, and repeatable for estimation of EFV in the brain.

Conclusion: This method for estimation of EFV in the rat brain can be applicable for pharmacokinetic and toxicology study of EFV in the brain after administration of EFV to rats.

Keywords: Efavirenz, HPLC, Brain pharmacokinetic

Background

Efavirenz (EFV) is an antiretroviral drug classified as non-nucleoside reverse transcriptase inhibitors (NRTI). It is one of the first-line agents in HIV-1 therapy [1]. Chemically, it is (*S*)-6-chloro-(cyclopropylethynyl)1,4-dihydro-4-(trifluoromethyl)-2*H*-3,1-benz-oxazin-2-one. The empirical formula for the same is $C_{14}H_9ClF_3NO_2$, and it is practically water-insoluble [2]. The structure of EFV is shown in Fig. 1.

During HIV infection, the sensory neuropathy is one of the major causes of morbidity; toxic effects of antiretroviral drugs in the peripheral nervous system may increase this incidence. After entering into the brain, HIV follows both pathophysiologic infectious pathway and degenerative pathway [3]. As the immune system starts to deteriorate, neurological complications are seen and will finally result in dementia [4, 5]. Novel therapies to treat inflammatory mechanisms can be effective [6]. Safe and effective drug delivery to the brain is essential to protect its structure and function [5, 7]. To develop validated bioanalytical methods and absolute animal models for brain pharmacokinetic study are thrust area of research for the brain targeted drug delivery systems.

There are several applications of HPLC in pharmaceutical analysis at different stages starting from the

* Correspondence: smitadarade87@gmail.com
M.Pharm Pharmaceutics, Department of Pharmaceutics, MET Institute of Pharmacy, Nashik, affiliated to Savitribai Phule Pune University, Pune, Maharashtra 422003, India

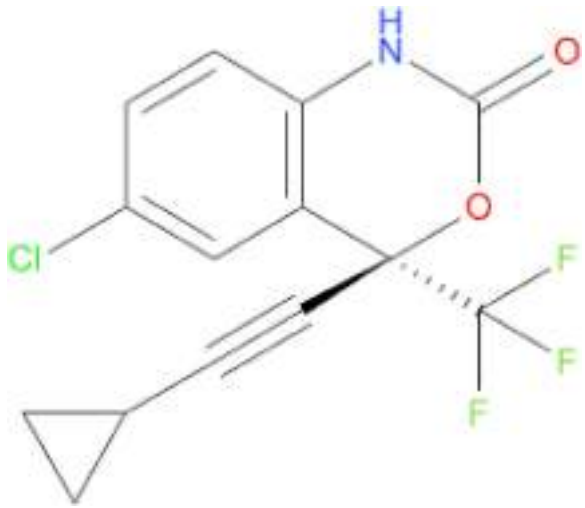

Fig. 1 Efavirenz structure

discovery of a new drug until it is ready to use for patients. HPLC technique is remarkable in the identification of drug targets and specific ligands by performing bioassay. In the RP-HPLC, less polar analytes could be retained by non-polar stationary phase for a longer time than polar ones; this principle was used in this work [8].

HPLC methods have been previously developed for the estimation of EFV in the plasma of patients on highly active antiretroviral therapy (HAART) [9]. The RP-HPLC method for EFV was developed and validated on human plasma [9, 10]. Drug safety and effectiveness can be evaluated with validated methods to provide us supportive critical data [11]. Methods for analysis of EFV in human plasma could work out for plasma sample analysis, but brain pharmacokinetics can be established well with specific methods developed with brain models. In this research work, we made attempt for the same.

The following are the applications of the developed method:

1. Study of the pharmacokinetics of EFV in the brain after oral, nasal, or parenteral route of administration
2. Study drug targeting index of EFV for the brain targeted drug therapy; example: Neuro-AIDS treatment where the brain is the viral sanctuary site for HIV
3. Study of the toxicological potential of EFV in the different drug delivery approaches like nanoparticulate drug delivery, etc.

The RP-HPLC system has been used for the analysis of the antiviral agent with an ultraviolet (UV) detector. A novel perspective for the determination of the EFV in the brain is described in the paper. The entire analysis was performed as per the Bioanalytical Method Validation Guidance for Industry given by the US Department of Health and Human Services Food and Drug Administration [11].

Methods

Chemicals and reagents

Pure EFV sample was obtained as a gift sample from Mylan Laboratories, Sinnar, Nashik. HPLC grade solvents methanol and water were used as mobile phase in the analysis obtained from Thermo Fisher Pvt. Ltd. Solvents were degassed by sonication and then filtered through ultrafiltration unit prior to use.

Instrumentation

RP-HPLC (HPLC 3000 series) of Analytical Technology Pvt. Ltd. with a binary gradient system with a UV detector was used for analysis. C18 column (Cosmosil) was used for the HPLC analysis. Data were obtained and processed in the HPLC Workstation software. A sonicator (WUC-4L, WENSAR) was used for the degassing of the mobile phase. Cellulose membrane filter 0.45 μ size (Borosil) was used. Tissue homogenizer and cooling centrifuge (Electrotechnik) were required for the processing of brain tissue. High-precision weighing balance-PGB 100 (Wensar) was used.

Chromatographic conditions

The optimized condition used was methanol to water in a ratio of 9:1 with a 0.8-mL flow rate per minute. The wavelength for the detection was set at 248 nm. The separation was carried out in a C18 Cosmosil column with 250 mm × 4.6ID, particle size 5 μ dimension. The sample volume injected was 20 μL. Table 1 shows the optimum chromatographic conditions. The effectiveness of the theoretical plates was within 5000 to 10,000 while the tailing factor was less than 1.75. It ensures the performance of the analytical system.

Sample preparation

Stock solutions of EFV were prepared with the solvent in the same ratio of the mobile phase used for the

Table 1 Chromatographic conditions

Equipment	HPLC 3000 series (Analytical Technology)
Mobile phase	Methanol to water (9:1)
Column	C18 (Cosmosil) 250 mm × 4.6ID, particle size 5 μ dimension
Column temperature	Ambient
Injection volume	20 μL
Flow rate	0.8 mL/min
Wavelength	248 nm
Sample cooler	5 °C

validation at the concentration of 1000 μg/mL which was further diluted to 100 μg/mL. Further dilutions were formed using the 100-μg/mL stock solution for the calibration curve in the range of 0.4 to 8 μg/mL.

Linearity

The calibration curve for the linearity parameter of EFV was obtained by injecting solutions of the concentration ranging from 0.4 to 8 μg/mL. The dilutions were prepared from the stock and the working solution of standard EFV of 1000 μg/mL and 100 μg/mL, respectively. Quality control standards LQC of 1.2 μg/mL, MQC of 4.8 μg/mL, and HQC of 8 μg/mL were used. The calibration graph was plotted as the concentration of the drug solutions versus the peak area of each. The value of the slope, regression coefficient, and intercept were determined.

Recovery

Recovery was determined by calculating the amount of drug detected in the brain after the addition of the dose and was compared with that of the standard injected in the column.

Accuracy

Accuracy of the method was calculated by injecting the five replicates of the quality control standards of lower (LQC) 1.2 μg/mL, medium (MQC) 4.8 μg/mL, and higher (HQC) 8 μg/mL. The abovementioned concentration or dose of LQC, MQC, and HQC was injected into the rat brain sample to perform the accuracy data. The rat brain was isolated, and the dose of the three concentrations was added separately into the brain samples which were processed and further injected in the HPLC column. After the analysis, the peak area was observed to calculate the standard deviation and percentage relative standard deviation.

Sensitivity

The sensitivity of the method was demonstrated as the limit of detection (LOD) and limit of quantification (LOQ) for EFV. LOD and LOQ can be calculated by using the standard deviation and the slope of the calibration curve. From the following formula, LOD and LOQ can be calculated:

$$\mathrm{LOD} = 3.3\sigma/s \qquad \mathrm{LOQ} = 10\sigma/s$$

where:
σ = standard deviation of the response
s = slope of the calibration curve

Specificity

The specificity of the developed method was confirmed by analyzing the chromatogram of the standard EFV drug and the deproteinized brain extract. Both chromatograms were observed for any interference in the brain sample at the standard retention time of the EFV. This study was done to ensure the presence of impurities or proteins or plasma bio-matrix at the retention time of the EFV.

Precision

A random error that occurs in the method was considered as the precision parameter. The errors can be eliminated by analyzing the replicates of the observed results. The relative standard deviation (RSD) or the coefficient of variation of the analytical results were obtained from separately prepared quality control standards to evaluate the precision of the method.

Extraction of drug from the brain

For extracting the EFV from the brain, the following method was carried out.

The experimental procedures were conducted after the approval of the Institutional Animal Ethics Committee (IAEC) as per the guidelines of the CPCSEA for the care of laboratory animals. Male Wistar rats (250 g) were nourished with routine diet and water, ad libitum. Animals were acclimatized to an in-house animal facility for 1 week with a 12-h light/dark cycle and a temperature of 22 ± 2 °C. Ketamine 50 mg/mL IP (Ketamax 50) was used to induce anesthesia in the rat before the removal of the brain. The rat was sacrificed to isolate the brain. Brain tissue samples were homogenized in a homogenizer (T-25 IKA, Ultraturrex) in saline at 20,000 rpm for 5 min. To the homogenized brain sample, EFV was added linearly in different concentrations for the calibration plot with the six points of 0.4 ppm, 0.8 ppm, 2.4 ppm, 4.8 ppm, 6.4 ppm, and 8 ppm in six different isolated homogenized brain samples. This homogenized brain sample with the addition of the EFV was sonicated for 10 min [12–17]. Deproteinization of the sample was carried out by adding 5 mL of ethyl acetate which can be done by the addition of any non-polar solvent. Which further vortexed for 5 min, after 5 min, it was centrifuged in the cooling centrifuged at 1000 rpm/10 min/4 °C to separate the organic layer which was transferred to the sample vial and allowed to evaporate to dryness. After complete evaporation, 10 mL of the mobile phase was added to the vial, sonicated properly, and injected in the HPLC column (C-18). The graph was plotted for linearity response.

Stability studies

Stability study of EFV in the brain was carried out for the short and long terms. Three quality control

concentrations LQC, MQC, and HQC were analyzed in triplicates for the stability study. The four sets of the quality samples were prepared and placed in the sample vials and analyzed for the short-term and long-term stability study. One sample was analyzed freshly at the same time while the three were analyzed after 6 h, 12 h, and 24 h of the preparation for the short-term study. The percent deviation was calculated. For the long-term study, one sample was analyzed freshly on the same day of preparation. The other three were analyzed on the 10th day, 20th day, and 30th day.

Result

Linearity

The calibration plot was obtained by injecting extracted drug from the brain into the HPLC column. The calibration curve is given in Fig. 2. The equation of the calibration curve is $y = 21{,}023x + 14{,}253$. The regression coefficient was found to be 0.9867 which ensures the high precision of the developed method. Table 2 shows the data for the linearity. The spectrum for a blank sample of brain homogenate is given in Fig. 3. The spectrum for standards EFV is mentioned in Fig. 4. And Fig. 5 gives the presence of EFV in the brain homogenate.

Accuracy

The accuracy parameters were calculated by taking the quality control samples with low, medium, and high concentrations of the drug EFV (LQC, MQC, and HQC, respectively) with five replicates of each three concentration, i.e., 1.2 μg/mL, 4.8 μg/mL, and 8 μg/mL, respectively. The extraction was carried out in the same manner as mentioned above. Table 3 shows the values for accuracy.

Recovery

Recovery data was calculated by comparing the standard drug 4 μg/mL concentration with that of the brain sample. Added 4 μg/mL in the brain (extracted similarly as mentioned above) and calculated in the three replicates. Recovery was found to be nearly 78%.

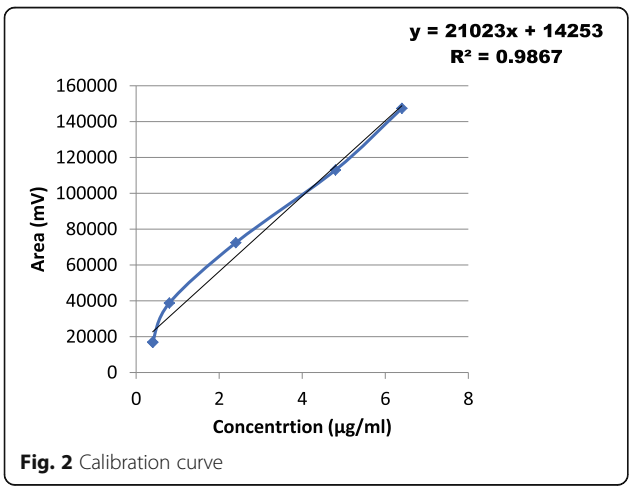

Fig. 2 Calibration curve

Table 2 Linearity ranges

Sample name	Sample Conc. (μg/mL)	Area (mV)
Standard A	0.4	16,734 ± 485.1979
Standard B	0.4	16,927 ± 330.5768
Standard C	0.8	38,729 ± 549.3806
Standard D	2.4	72,371 ± 1424.877
Standard E	4.8	112,938 ± 4922.987
Standard F	6.4	147,364 ± 1450.975
Standard G	8.0	196,834 ± 6006.519

Sensitivity

The values of the LOD and the LOQ were found to be 0.1126 and 0.3414, respectively.

Stability studies

Short-term stability

In the short-term study, the brain sample was kept at RT for three different time intervals which were 6, 12, and 24 h. It is found that degradation was increasing over time. During 6 h, 32% degradation was observed, and at 12 h, it was found nearly 53% while during 24 h, it was 70%.

Long-term stability

Long-term stability was done for the number of days (10 days, 20 days, and 30 days) to check the amount of degradation. It was observed that 98% degradation was seen during the course of 10 days.

Discussion

Rat is a widely used animal model in the preliminary in vivo studies of pharmaceutical formulation. For in vivo characterization of brain-targeted drug delivery systems, it is required to perform biodistribution study of the drug in the brain as well as CSF. For such experiments, highly sensitive analytical methods are required. After oral administration, a very small fraction of the drug reaches the brain due to physiological barriers, and hence, sensitive methods are required for the estimation of the quantities present from micro- to nanogram per milliliter. A low-dose EFV gives virologic failure, and high doses can cause CNS toxicity [14]. Thus, the developed method will help researchers in the evaluation of novel drug delivery systems to estimate the CNS EFV level. Penetration of antiretroviral in the brain is the current focus for the effectiveness of HAART and C-ART. Very scattered data were availed on ARV drug penetration into brain tissue [15]. EFV has limited

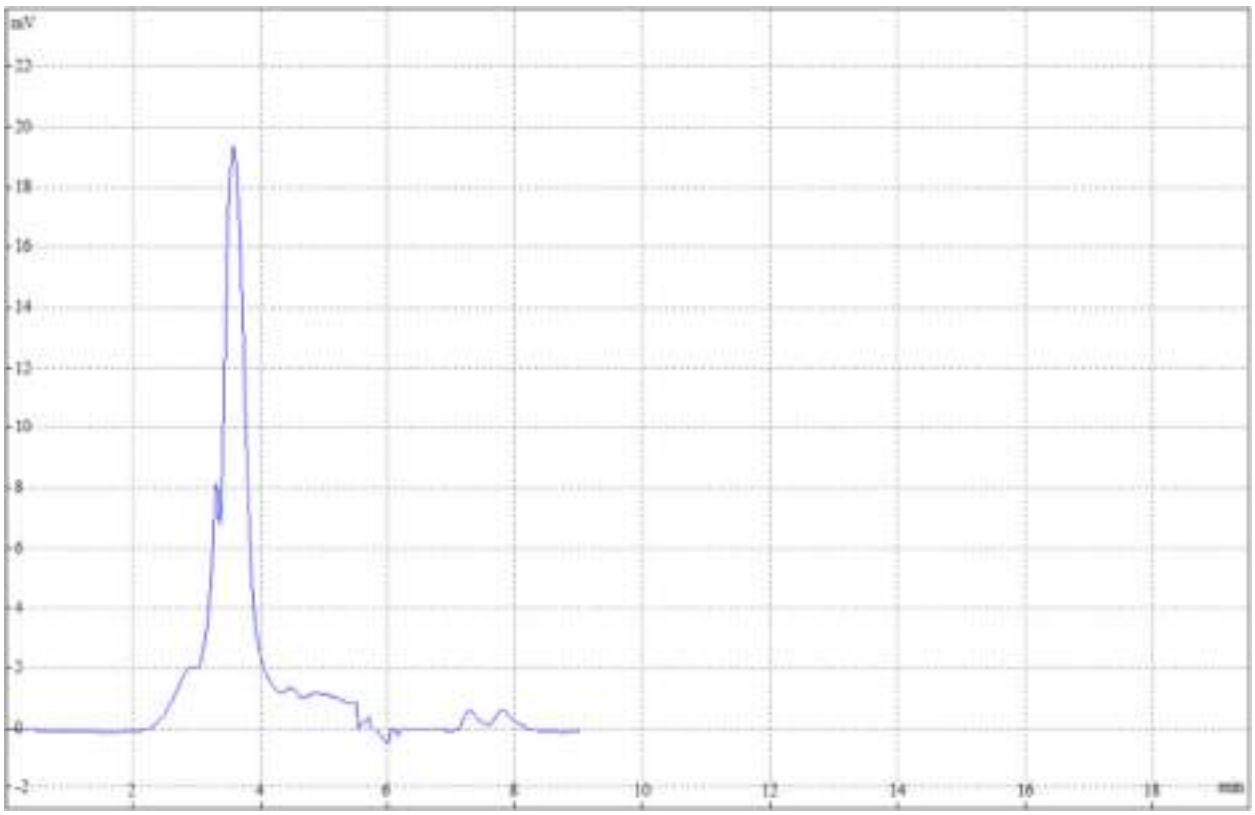

Fig. 3 Spectrum obtained for a blank sample of brain homogenate

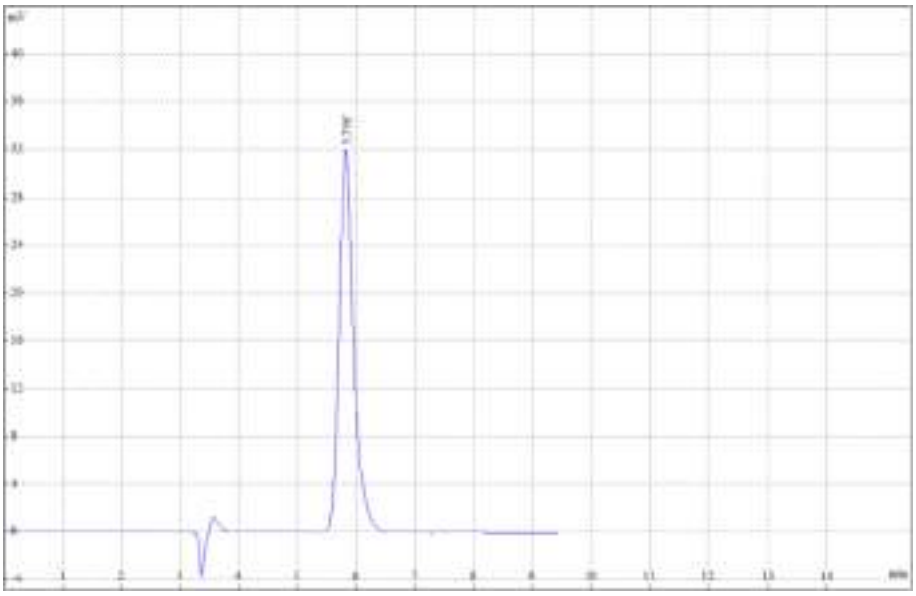

Fig. 4 Standard efavirenz spectrum

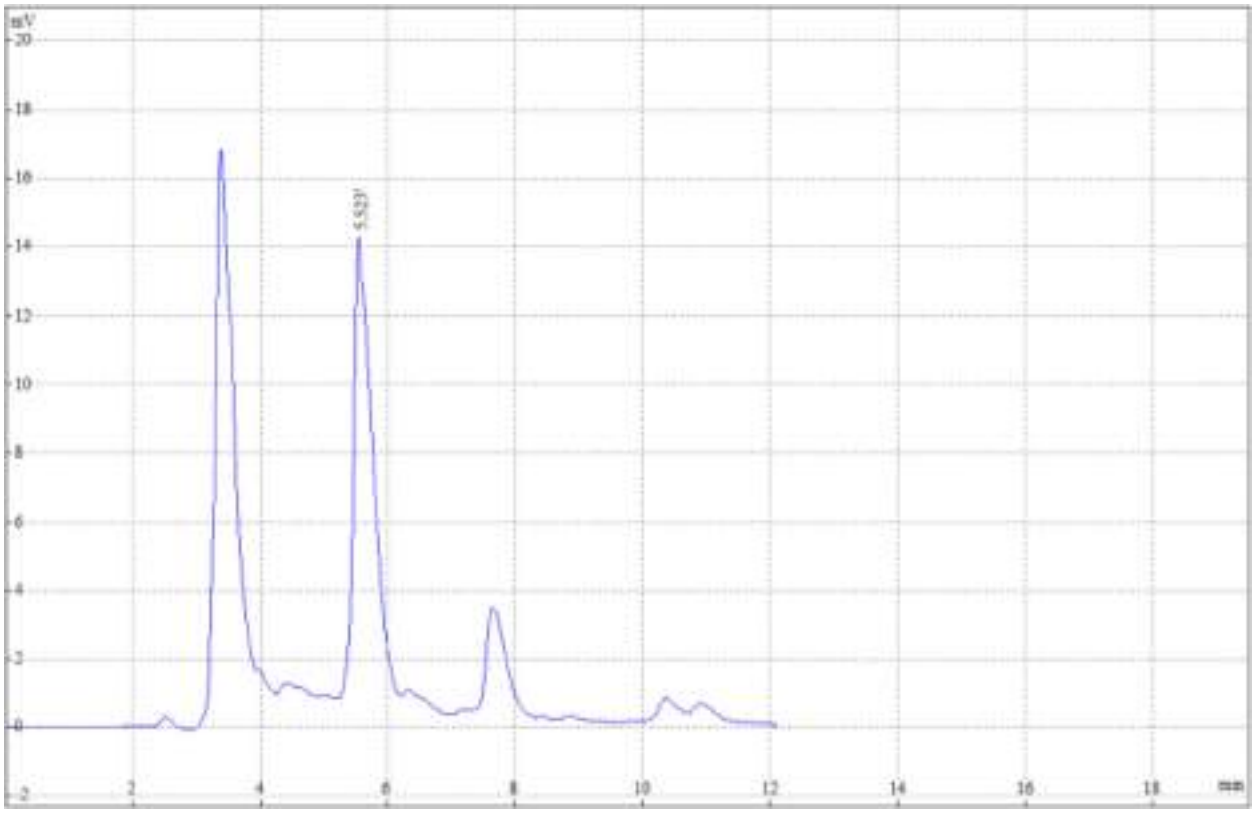

Fig. 5 Efavirenz in brain homogenate

central nervous system (CNS) penetration [16]. Thus, the developed method is suitable for the estimation of EFV in the rat brain.

Conclusion

Previously reported methods were for the estimation of EFV in the plasma. This method is for the estimation of brain EFV concentration in a rat model. It will be helpful for studying the brain targeting the index of EFV in the rat model and further pharmacokinetic estimation of EFV in the brain. We have developed a reliable method for the estimation of antiretroviral drug EFV in the rat brain, and it was found to be sensitive, specific, simple, and accurate. The system suitability parameters, % RSD, recovery, and regression coefficient were found to be within the limit range. The developed method could be carried out in routine analysis as it is precise.

Table 3 Data for accuracy

Sample name	Sample Conc. (μg/mL)	Area (mV)
LQC	1.2	56,117 ± 717.9001
MQC	4.8	116,927 ± 1541.274
HQC	8.0	197,239 ± 3576.957

Abbreviations

EFV: Efavirenz; RP-HPLC: Reverse phase high-performance chromatography; NRTI: Non-nucleoside reverse transcriptase inhibitors; HAART: Highly active antiretroviral therapy; C-ART: Current antiretroviral therapy; RSD: Relative standard deviation; QCs: Quality control samples; IAEC: Institutional Animal Ethics Committee; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals

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Authors' contributions

SPK (main author and corresponding author of the research paper): concept, performed laboratory experiments, statistical analysis with graph, and prepared the final manuscript. SJK (co-author): supervised and monitored all the experiments and activities. All authors read and approved the final manuscript.

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The study was done through self-finance.

Availability of data and materials

All data and materials are available upon request.

Ethics approval and consent to participate

The study was approved by the Institutional Animal Ethical Committee of METs Institute of Pharmacy, Nashik (Approval number MET-IOP-IAEC/2019-20/02), India.

Consent for publication

Not applicable

Competing interests
The authors declare that they have no competing interests.

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REVIEW ARTICLE

Neuro-AIDS: Current Status and Challenges to Antiretroviral Drug Therapy (ART) for Its Treatment

Smita P. Kakad[1,*] and Sanjay J. Kshirsagar[1]

[1]*Department of Pharmaceutics, MET's Institute of Pharmacy, Adgaon, Nashik, Savitribai Phule Pune University, Maharashtra, 422003, India*

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Abstract: ***Introduction*:** The infiltration of HIV into the brain alters the functions of the nervous system known as Neuro-AIDS. It leads to neuronal defects clinically manifested by motor and cognitive dysfunctions.

***Materials/Methods*:** Current antiretroviral therapy can prevent viral replication but cannot cure the disease completely. HAART-Highly active antiretroviral therapy is used for the treatment of HIV infection. Challenges in neuro-AIDS therapy are as shown in the graphical abstract. One of the challenges is latent viral reservoirs like the brain; which act as a sanctuary site for viruses. Nearly ~50% of HIV patients show neuropathological signs. Nervous system related disorders, including AIDS dementia, sensory neuropathy, and myelopathy have a 25% of prevalence in patients having access to a highly active combination of antiretroviral therapy.

***Results/Conclusions*:** Brain is one of the viral sanctuary sites for HIV. The current need of neuro-AIDS therapy is to target the brain as a viral reservoir. Drugs should cross or bypass the blood-brain barrier to reach the brain with effective concentrations. Current research on novel drug delivery approaches may prove helpful in treating neuro-AIDS and related disorders effectively.

Keywords: HIV, Neuro-AIDS, brain, current antiretroviral therapy, dementia, neuro-AIDS challenges.

1. INTRODUCTION

The neuroinvasion of HIV leads to Neuro-AIDS among HIV infected patients. Nearly ~50%HIV patients demonstrate neuro-pathological signs [1-3]. Neurological disorders are primarily caused by HIV access to the CNS. Examples of neuro-AIDS related disorders include sensory neuropathy, AIDS dementia and myelopathy [4]. Advanced HIV disease showed HIV- associated dementia (HIV-D) and HIV-related sensory neuropathies (HIV-SN) with a combined prevalence of about 30-50%, suggesting that highly active antiretroviral therapy (HAART) does not provide complete protection against neurological damage. HIV-D remains a common cause of dementia worldwide, and HIV-SN represents the commonest neurological disorders associated with AIDS [1]. Nearly, 80% of autopsies show a range of neuropathology in AIDS patients [5].

UNAIDS report says that "Prevention and end of AIDS as a common health threat can be interpreted quantitatively as a 90% reduction in newly developed HIV infections and deaths from AIDS-related illness by the year 2030 compared to 2010 baselines." HIV has a huge prevalence, according to the UNAIDS report; 'Global HIV and AIDS statistics'- 2018 facts sheet [6].

In 2017-

- 36.9 million Patients- Suffer from HIV.
- 21.7 million Patients -Received antiretroviral therapy.
- 1.8 million Patients – Acquired a new HIV infection.
- 35.4 million Patients -Died of the AIDS-related illness from the beginning of the epidemic.

About 5000 New HIV infections found to develop a day.

HAART-Highly active antiretroviral therapy is a combination of ARV (Antiretroviral) therapy (cART) like nucleoside analogs (NA), protease inhibitors (PI) and a non-nucleoside inhibitor of the reverse transcriptase (NNRTI), with a neuro prophylactic value to the manifestation of HIV-I associated CNS disease. Current antiretroviral therapy has a major limitation of its inability to penetrate the CNS [7].

Drug delivery strategies under research are size reduction/nanosizing of drugs enabling to cross BBB. Kuo YC *et al*., in 2007 developed solid lipid nanoparticles of stavudine, delavirdine, and saquinavir for transport across the blood-brain barrier using poly butyl cyanoacrylate and methyl-methacrylate derivatives [8]. Burke *et al*., (2019) worked on

*Address correspondence to this author at the Department of Pharmaceutics, MET's Institute of Pharmacy, Adgaon, Nashik, Savitribai Phule Pune University, Maharashtra, 422003, India; E-mail: smitadarade87@gmail.com

dolutegravir HIV integrase inhibitor and reported that the oxidative stress-related metabolites were found to be completely attenuated when dolutegravir was administered as a nanoformulation [9]. Patel *et al*., (2018) mentioned several advantages of nanomedicine for intranasal delivery to enhance brain uptake like improved bioavailability, reduced dose and fast onset of action and better patient compliance [10].

Nanoformulations based strategies have the potential to increase the bioavailability of drugs at the potential reservoir site. Change of conventional oral route of administration to non-invasive intranasal route could bypass the blood-brain barrier (BBB). Shweta Gupta (2019) in her research proved the success of solid lipid nanoparticles to enter CNS *via* nasal route [11].

With the advent of HAART, Neuro-AIDS therapy is facing challenges as drugs are unable to cross the BBB, hence, there is a need for personalized therapy to deal with resistance to ARVs and drug interactions. The current demand is a promising drug therapy that resolves existing lacunas, which indirectly alter the socioeconomic status of the patient, as improvement in neuro-AIDS treatment will maintain the mental wellbeing of HIV patients.

As per the WHO, Global Health Sector Strategy on HIV, 2016–2021," Global target is to end the AIDS epidemic by 2030." Preventive means are available, still prevalence levels are high and noticeable due to lack of awareness and knowledge about ART in developing countries. Neuro-AIDS challenges are mentioned in the graphical abstract [2].

2. NEURO-AIDS PATHOGENESIS

HIV enters the brain *via* several pathways as shown in Fig. **1**. It gains entry *via* blood-derived macrophages or trans-migration across BBB [12]. After entering into the brain, HIV follows both infectious pathophysiologic pathway and degenerative pathway [13]. In the brain, HIV starts

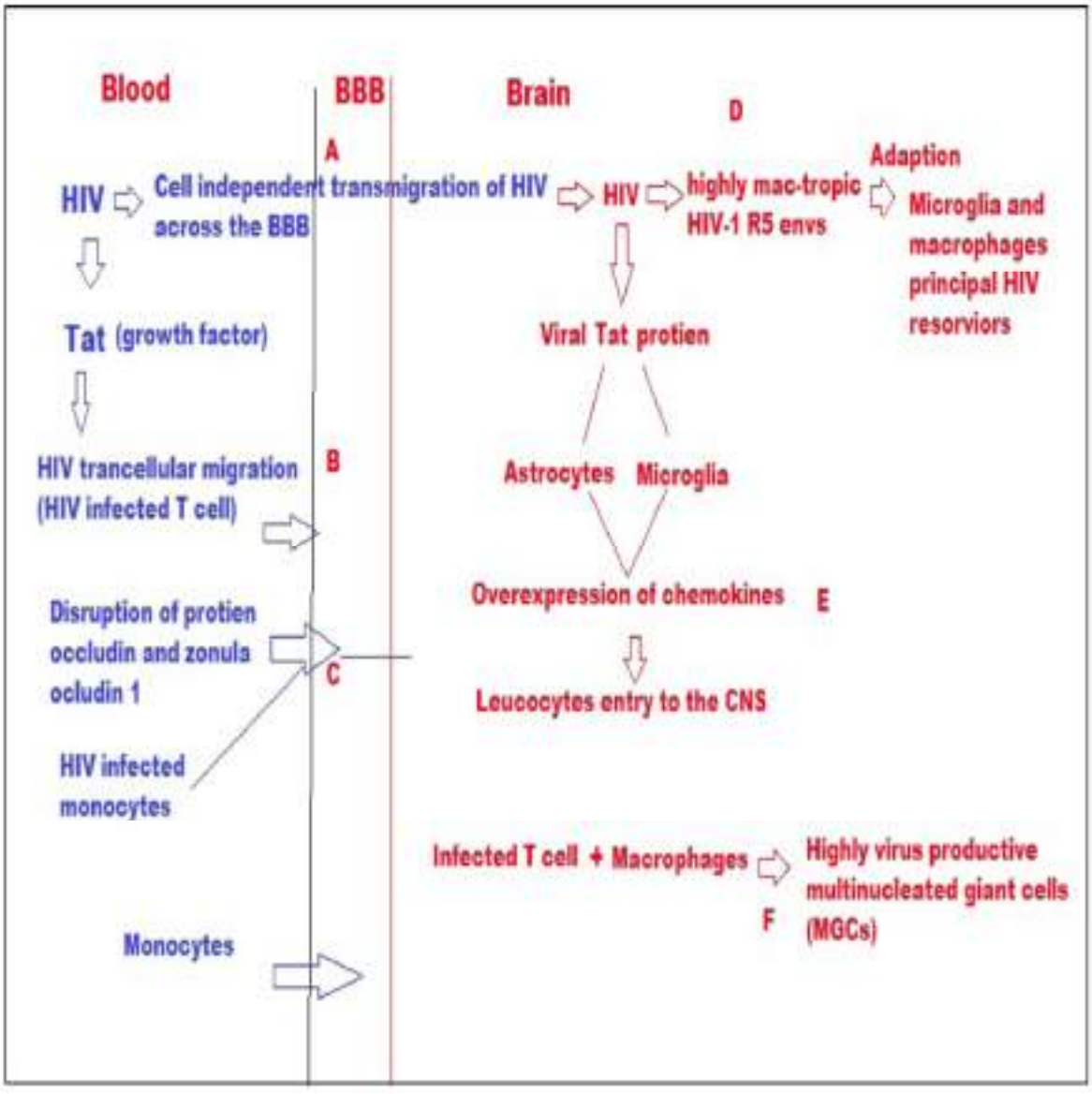

Fig. (1). Mechanism for Neuro-AIDS pathogenesis. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

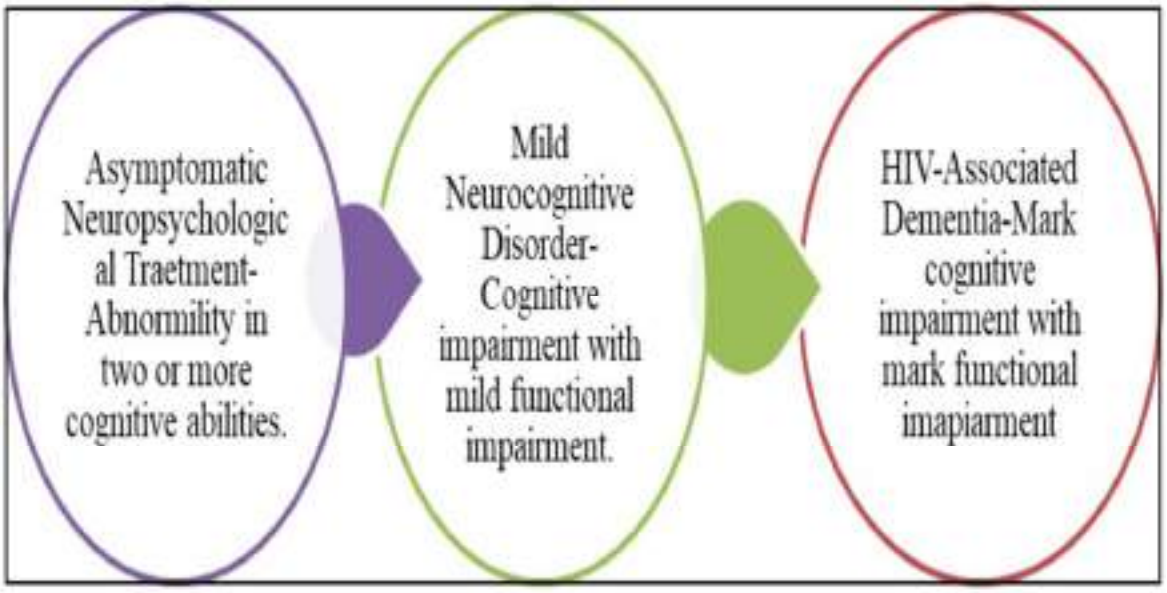

Fig. (2). HAND-HIV associated neurocognitive disorders [20, 21]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

replication in macrophages, it does not replicate in neurons, astrocytes, oligodendrocytes or microvascular endothelial cells in the brain [14-16]. As the immune system starts to deteriorate, neurological complications are seen and will finally result in dementia [12]. Some neurons put up with dendritic pruning, and simplification occurs at synaptic contacts with loss of free cells [17, 18]. As shown in Fig. **1**, the following are the possible ways for HIV to cross the BBB and get entry into the brain [19].

A. Transmigration of HIV across the blood-brain barrier. (Independent of the cell).

B. Transcellular migration of HIV infected cells into the brain. HIV 1 Tat protein acts as a growth factor in transport.

C. Transmigration of monocytes and macrophages into the brain by the disruption of proteins occludins present at endothelial tight junction.

D. Highly mactropic HIV1 R5 envelope glycoprotein is responsible for the adoption of replication in microglia and macrophages, which forms HIV reservoirs in brain tissue.

E. Viral Tat proteins are responsible for the entry of leukocytes into CNS.

F. Infected T cells interact with macrophages and lead to the generation multinucleated giant cells called MGCs; these are highly virus productive.

3. MAJOR CNS-RELATED IMPAIRMENT WITH CHRONIC HIV INFECTION

HIV associated neurocognitive disorders (HAND) are of three different categories as (a) asymptomatic neurocognitive impairment, (b) mild neurocognitive disorder and (c) HIV-associated dementia [15]. Impaired cognitive and motor dysfunctions may affect many people with AIDS [4, 20, 21]. Fig. **2** shows the fate of HAND.

Impaired memory and concentration are early presentations of AIDS-Dementia [22]. Individuals infected with HIV have higher levels of anxiety, depression, apathy, and irritability and experience decrements in dual-task performance [23]. Neurocognitive deficits are present with progressive brain atrophy in HIV/AIDS. Neurocognitive deficits affect concentration, psychomotor skills and information processing. This could affect the social life of the patient [24]. Neurocognitive dysfunction is memory impairment, poor concentration, slower psychomotor activity. Emotional disturbance can slowly lead to social withdrawal and it can be considered as depression. Irritability, mental inflexibility, and decreased sex drive are other signs. Motor abnormalities are weakness, ataxia, clumsy gait, slow motor skills, tremor, diffuse increase in tone, hyperreflexia, spasticity, abnormal eye movements and Parkinson's [25].

Sharma *et al.*, (2017) conducted a retrospective observational study on 91 patients with the neurological manifestation of HIV (Age group 18-67; male: female-1:1.05). Out of this group, 29% had a primary neurological illness, whereas 62% had a secondary illness, as mentioned in Table **1**. Tuberculosis meningitis is a secondary infection with 40% of incidence in the study population, followed by Cryptococcus meningitis with 13% of incidence. Suspicious neurological involvement of HIV patient helps in earlier diagnosis and initiation of treatment, which will eventually decrease the morbidity and mortality [26].

As per memorial Sloan Kettering cancer care scale [27], six clinical stages of HIV associated dementia are: normal (0), subclinical (0.5), mild (1), moderate (2), severe (3) and end-stage (4). Patient's ability changes in different stages as mentioned in Table **2**.

4. CHALLENGES IN DIAGNOSIS

Early HIV-1 infection is typically asymptomatic; therefore, for its detection, there is a need to conduct cerebrospinal fluid analysis for detection of markers. Neuroimaging

Table 1. Neurological illnesses found in HIV infected patients. ("n = no of patients") [26].

Types	Neurological Illness	Number (%) n=91
Primary	Distal symmetric polyneuropathy (DSPN)	15 (16.4%)
	AIDS Dementia complex (ADC)	6 (6.59%)
	Acute inflammatory demyelinating polyneuropathy (AIDP)	3 (3.29%)
	Stroke syndromes	5(5.49%)
Secondary	Tuberculosis bacterial meningitis	40(43.9%)
	Cryptococcus meningitis	13(14.2%)
	Toxoplasmosis	2(2.19%)
	Progressive multifocal leukoencephalopathy (PML)	1(1.09%)
	Neuro Syphilis	6(6.59%)

Table 2. Sloan Kettering cancer care scale for clinical stages of HIV associated dementia [27].

Stage-0	**Normal:** Patient observed with the normal mental and motor function.
Stage-0.5	**Subclinical:** Patient observed with the capacity to perform daily living activity. Absent or minimal symptoms. No impairment observed in work. Mild signs observed like snout reflex, slowed ocular movements or extremity movements might be present.
Stage-1	**Mild-** The patient can execute all activities of daily living, but with unequivocal evidence. Patients can be observed with functional, intellectual and /or motor impairment. Patient can walk without help or support.
Stage-2	**Moderate-** Patient can do basic activities like self-care, but cannot work or extend to the more demanding aspects of daily life.
Stage-3	**Severe-** Patients cannot follow the news or personal events, cannot sustain a complex conversation, considerable slowing of all outputs. Patients with major intellectual incapability and motor disability need help to walk.
Stage-4	**End-stage-** The patient is almost in a vegetative status with intellectual and social comprehension and output at a rudimentary level. Almost mute with paraparetic or paraplegic with double incontinence.

with magnetic resonance spectroscopy (MRS) is a non-invasive technique to measure brain's biochemistry [13]. Multi-marker assay predicts the progress of HIV-1. Functional MRI (fMRI) is another noninvasive technique used to measure blood flow changes related to neural activity in the brain. Quantitative changes in neuroanatomical structures can be studied with morphometry models [13, 24, 28, 29].

Brain imaging MRI with gadolinium is necessary to exclude other causes of dementia-like cryptococcus infection, toxoplasmosis, cytomegalovirus encephalitis, CNS lymphoma and progressive multifocal leukoencephalopathy which may show the HAD (HIV associated dementia) clinical picture. Brain MRI reveals the ill-defined or defuse areas of the hyperintense signal with cerebral atrophy in patients of HAD.

Proton magnetic resonance spectroscopy (MRS) detects the myoinositol and choline levels in the grey and white matter and N -acetyl aspartate in grey matter [30].

- Increased myoinositol- Indicating gliosis
- Increased choline- Indicating demyelination
- A decrease in levels of N -acetyl aspartate- Indicating neuronal/axonal loss.

Diagnostic methods now in use have some limitations such as biopsy of neural tissue and collection of cerebrospinal fluid (CSF) are quite difficult techniques and associated with slight risks [31, 32]. The biomarker proves helpful in predicting disease onset and progression to find the effectiveness of novel therapies. Other advantages like objective assessment, the precision of measurement, reliability, established validity and disease mechanisms are often studied [31].

New emerging science, "Metabolomics," is used for the measurement of metabolite fluxes and concentrations in cells. It is applicable in the toxicological profiling of drugs and biomarker studies. Identification of reliable biomarkers

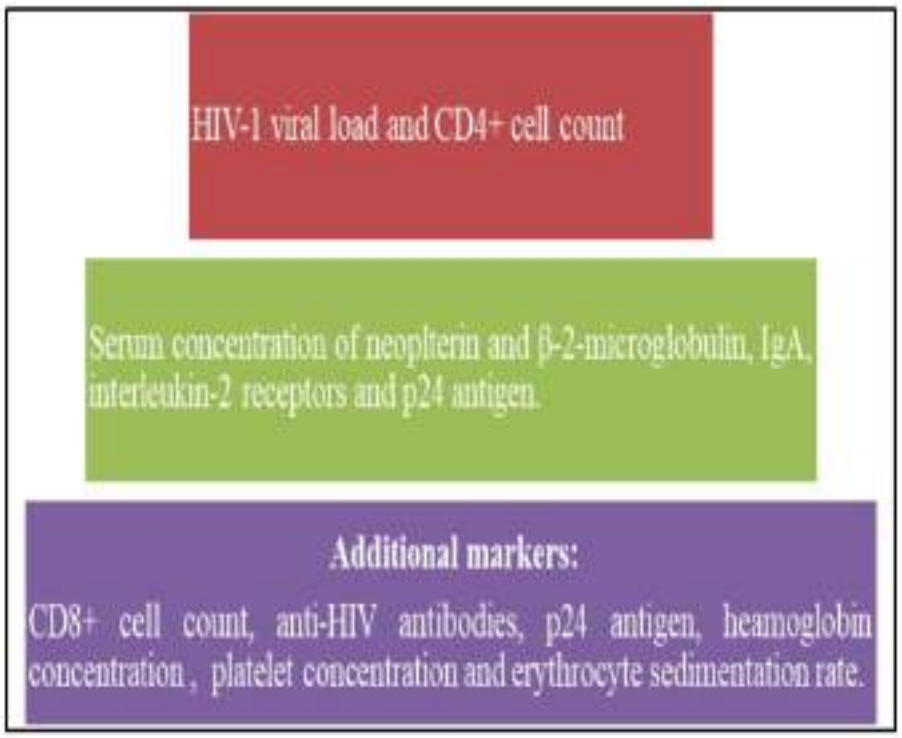

Fig. (3). Biomarkers used to detect and monitor HIV-1 progression. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

for Neuro-AIDS will lead to detect the presence and growth of HIV in the CNS to take maximal benefit from ART and to study the mechanism of neuro-pathogenesis. The most commonly used biomarkers are mentioned in Fig. **3**. Following are current diagnosis methods [31].

- Neuropsychological assessment: HIV-Dementia Score (HDS) method.
- Detection of viral markers *e.g*. HIV RNA and DNA in CSF and plasma, HIV p24 antigen with amplification by PCR(Polymerase Chain Reaction)
- Post-contrast magnetic resonance enhancement.
- Neopterin in the CSF.
- Proteomics for the CSF analysis.
- INF (Interferon) γ in brain
- TNF (Tumour necrosis factor) α and IL (Interleukin) 1 in plasma.
- TNF α, MCP (Monocyte chemotactic protein), and M-CSF (Macrophages colony-stimulating factor) in CSF and plasma.

5. CHALLENGES IN CURRENT ART

ARVs are antiretroviral drugs and ART is an antiretroviral therapy to treat HIV. Antiretroviral drugs are prescribed as combination therapy and, in very few cases, they are taken as single drug therapy, for example, during the first trimester in pregnant mothers. Treatment could slow down the virus and progress of HIV disease, but complete eradication of viruses from a viral reservoir is the need for patients.

The NA penetrates the cerebrospinal fluid and positively influences HIV-1-associated brain disease. NNRTIs are also CNS effective. HAART potentially reduces mortality and morbidity rates among HIV-infected patients, improves their quality of life and provides an effective treatment option for treatment-naive and treatment-experienced patients [14, 15, 17].

Table 3. Goals of ART as per NACO guidelines [33].

Clinical goals	Prolongation of life and improvement in life quality.
Virological goals	Greatest possible reduction in viral load for as long as possible
Immunological goals	Immune reconstitution that is both quantitative and qualitative
Therapeutic goals	Rational sequencing of drugs in a fashion that achieves clinical, virological and immunological goals while maintaining treatment options, limiting drug toxicity and facilitating adherence
Reduction of HIV transmission in individuals	Reduction in HIV transmission by suppression of viral load.

Current ART has a major limitation of their inability to penetrate the CNS [26]. AS per the NACO guidelines, following are goals set for ART therapy to HIV patient as mentioned in Table **3** [33].

Hurdles in the antiretroviral therapy (ART) are drug toxicity, resistance to existing therapy, different stages of infection, age group of patients, mother to fetus transmission, availability of treatment option and awareness in a patient about the severity of conditions [34, 35]. CNS escape is a phenomenon where ART controls HIV in the periphery but not in the CNS [34]. Few challenges revoke difficulty in treating Neuro-AIDS. Selection and choice of ARV regimen depend on the following factors [35].

- Cost of therapy- As therapy is an intimation of lifelong treatment; affordability of drugs is an important factor
- Availability
- Convenience
- Likelihood of adherence to the treatment.
- Regimen potency, tolerability, and adverse effect profile
- Potential drug interactions
- Availability of alternate treatment options where the existing drug regimen fails.
- Age of patient
- Clinical stage of infection

Currently available ARV medications do not cure HIV-1 or AIDS. ARV medications can reduce the risk but do not eliminate the risk of transmission of HIV-1 to others. Not all medications are right for all people, and treatment may be different for each person [29]. Antiretroviral agents used are from different classes, and their mechanisms of action are given in Table **4**.

The adverse effects of ARV are another challenge to the treatment. Nucleoside analog causes lactic acidosis and hepatic steatosis [39]. The patient developed hepatic toxicity with nevirapine (NNRI's), so treatment discontinued with this drug. Efavirenz (NNRI's) contraindicated during the first trimester of pregnancy. Protease inhibitors cause bleeding in hemophilia, increased liver function test, bone disorders, metabolic abnormalities like resistance to insulin, increased triglycerides, cholesterol and lipodystrophy [40].

Table 4. Antiretroviral drugs with their mechanism of action.

Class of ARV Drug	Drugs	Mechanism
Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	Tenofovir Alafenamide (TAF), Tenofovir Disoproxilfumerate (TDF) Abacavir (ABC) Zidovudine (AZT, ZDV) Stavudine (d4T) Lamivudine (3TC) Emtricitabine (FTC)	NRTIs are active inhibitors of the reverse transcriptase enzyme. HIV-1 needs this enzyme to make copies of itself. [36-39]
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz (EFV) Nevirapine (NPV) Etravirine (ETV) Rilpivirine (RPV)	NNRTIs bind to and alter reverse transcriptase, an enzyme HIV-1 needs to make copies of itself [36, 37, 39].
Fusion Inhibitors	Enfuvirtide (ENF)	Fusion inhibitors act extracellularly; prevent fusion of HIV 1 and cells. Thus blocks HIV-1 from entering the CD4 cells or other target cells of the immune system [36, 37, 39].
Chemokine receptor Antagonist (CCRs)	Maraviroc (MVC)	Maraviroc (CCR5 antagonists), specifically and reversibly bind CCR5, block CCR5, a protein on the CD4 cells that a certain type of HIV-1 needs to enter the cell [36, 37, 39, 40].
Protease inhibitors (PIs)	Atazanavir (ATV) Nelfinavir (NFV) Darunavir (DRV) Fosamprenavir (FPV) Tipranavir (TPV) Ritonavir (RTV) Lopinavir (LPV) Saquinavir (SQV) Indinavir (IDV)	Protease inhibitors competitively inhibit the HIV protease enzyme. HIV-1 protease, an enzyme HIV-1 needs to make copies of it. [29, 34, 36]
Integrase inhibitors	Raltegravir (RAL) Elvitegravir (EVG) Dolutegravir (DTG)	Integrase strand transfer inhibitors block HIV-1 integrase, an enzyme HIV-1 needs to make copies of it. HIV integrase enzyme is required for the transport and attachment of pro-viral DNA to the chromosomes of the host cell, thus it allows transcription of viral proteins and leads to assembly and synthesis of the virus particles [32, 37, 39].

Resistance, adverse effects, pregnancy, and co-infection with the hepatitis B virus, or hepatitis C virus present important challenges to clinicians when selecting and maintaining therapy. Resistance testing is a new cornerstone of HIV therapy; recommended for newly diagnosed persons and in patients in whom ART has failed [41]. The genetic diversity of HIV-1 and their constant mutation during infection lead to develop ARV resistance. To solve the resistance issue, personalized therapy is needed, wherein the resistance testing is performed to decide the right kind of therapy for each patient. Transmission of drug-resistant virus spreads infection with 10% of prevalence in resource-rich countries [42]. One of the important causes of resistance to NRTI's is the mutation in the pro-viral nucleic acid chain. Mutations associated resistance will alter the enzyme-binding capacity of some ARVs like NNRTI's. For protease inhibitor, drug resistance develops as primary mutations induce conformational changes in the protease-binding site, followed by secondary compensatory mutations that improve enzymatic activity. In the fusion inhibitor, drug resistance develops as amino acid substitutions occur in the 36-45 regions of gp41. Chemokine receptor inhibitors showed amino acid substitutions in the V3 loop of gp120 that will develop resistance. An integrase inhibitor leads to mutations, which aid in resistance [38].

Interaction between ARV drugs is the next challenge to treatment. Interaction between antiretroviral agents, if given in combination, alters the predicted effect of combination therapy. To overcome this, either the co-administration of interacting drugs needs to be stopped or the dose of interacting drugs needs to be changed. For example, atazanavir and tenofovir, if administered together, there will be a decline in the atazanavir concentration. So, atazanavir can be administered with ritonavir. Ritonavir is a pharmacokinetic booster for other protease inhibitors [43, 44]. Interactions between antiretroviral drugs described in Table **5**.

As per the WHO data and statistics, in mid-2017, globally, 20.9 million people were living with HIV receiving ART and 37.9 million people were living with HIV at the end of 2018. In 2018, the global ART coverage for pregnant

Table 5. Interaction between antiretroviral agents and their management [43].

Antiretroviral Drug	Interacting Antiretroviral Drug	Predicted Effect	Management
Atazanavir (ATV)	Tenofovir	↓ATV	Administer ATV 300 mg with RTV 100 mg
	Etravirine	↓ATV, ↑ETV	Do not co-administer.
	Nevirapine	↓ATV, ↑NVP	
	Efavirenz	↓ATV	Administer ATV 400 mg with RTV 100 mg in treatment-naive; do not co-administer in treatment-experienced.
Abacavir (ABC)	Tipranavir	↓ABC	Do not co-administer.
Etravirine (ETV)	Tipranavir	↓ETV	Do not co-administer.
Fosamprenavir (FPV)	Lopinavir/ ritonavir, tipranavir	↓FPV	Do not co-administer.
	Etravirine	↑FPV	Do not co-administer.
Didanosine (ddI)	Tenofovir	↑ddI	Decrease ddI dose (250 mg qd)
Indinavir (IDV)	Tipranavir	↓IDV	Do not co-administer
Lopinavir/ritonavir (LPV/r)	Efavirenz, Nevirapine	↓LPV	LPV/r 500/125 mg bid (tablet) or 533/133 mg bid (liquid)
	Tipranavir	↓LPV	Do not co-administer.
Darunavir (DRV)	Lopinavir/ ritonavir, saquinavir	↓DRV	Do not co-administer.
Maraviroc (MVC)	Efavirenz, etravirine	↓MVC	Increase MVC dose (600 mg bid)
	Protease Inhibitors (except tipranavir)	↑MVC	Decrease MVC dose (150 mg bid)
	Delavirdine	↑MVC	Decrease MVC dose (150 mg bid)
Nelfinavir (NFV)	Tipranavir	↓NFV	Do not co-administer.
Saquinavir (SQV)		↓SQV	
Zidovudine (ZDV)		↓ZDV	

"↑" indicate an increase in concentration; "↓" indicates a decrease in concentration.

and breastfeeding women living with HIV was high being 82%. One of the challenges is to decline transmission from mother to child (MTCT) to reduce new emerging infections. Antiretroviral therapy with a single or dual drug regimen is not recommended except for the prevention of MTCT and post-exposure prophylaxis of HIV [38]. Availability of ART does not suppress HIV replication in all patients, and the emergence of drug-resistant virus hinders subsequent treatment. Chronic therapy is proving difficult as it can also result in toxicity and side effects. Thus, challenges remain and there is a need for research on novel drugs and delivery strategies to gain complete control over chronic infections [45]. Latent reservoirs (lymphoid tissue, testes, liver, kidney, gut and CNS) are the main perpetrator of the HIV cure [46-48].

For the CNS drug delivery, the key obstruction is a tight junction at the blood-brain barrier [48]. The CNS acts as a potential reservoir for HIV as brain cells, like astrocytes, perivascular macrophages, and microglial cells of CNS, are capable of inducing latency [49]. The virus enters the CNS at the early infection stage; it compartmentalizes in the different CNS compartments and accumulation of the virus starts in the CNS [50, 51].

The similarity of the brain and lymph node virus suggested that the meninges may be a part of the lymphatic system, which could offer the virus an alternative entry to the brain than through the blood-brain barrier [50]. Rahimy *et Al.*, (2017) reported that during the early stages of HIV infection, neuropathogenesis of BBB occurs.

The following are biological barriers for drug delivery to CNS [48].

- **Drug stability-** Enzymatic degradation, poor stability.
- **Epithelial and endothelial barrier-** Blood-brain barrier.
- **Immunological barrier-** Opsonization, RES uptake.
- **Extracellular barrier- Mucin** penetration, extracellular matrix penetration.
- **Intracellular barrier-** Endosomal entrapment, efflux pump.

Many cases of neuro-AIDS are asymptomatic at the early stages of infection [51, 52]. With the development of genetic diversity of virus pre-cART, [53] challenges emerged in treating perinatally HIV (PHIV)-infected adolescents with particular emphasis on non-adherence, resistance and management strategies. Significant numbers of perinatally HIV infected children newly diagnosed, later in childhood, only start cART as they approach adolescence. Many children diagnosed and treated early will survive to adolescence and adulthood [54].

Table **6** shows the adverse effects of antiretroviral agents; E.g. Zalcitabine (Hivid, ddC) deoxycytidine nucleoside ana-

Table 6. Adverse effects of antiretroviral agents.

Antiretroviral Agents/Brand Name	Adverse Effects
Abacavir (Ziagen)	Hypersensitivity reaction, diarrhea, musculoskeletal pain, hyper-triglyceridemia, depression, fever/chills, viral respiratory infections, thrombocytopenia [40, 60].
Didanosine (Videx, Videx EC)	Peripheral neuropathy, pancreatitis, nausea, lactic acidosis, noncirrhotic portal hypertension [40, 60]
Emtricitabine (Emtriva)	Minimal toxicity, hyperpigmentation, neuritis, paresthesia, diarrhea, insomnia, asthenia, nausea, rhinitis [40, 60]
Lamivudine (Epivir)	Nervous system neuropathy, exacerbation of hepatitis, anorexia, arthralgia, dyspepsia, pancreatitis [40, 60].
Stavudine (Zerit)	Lactic acidosis, hepatic steaosis [40].
Tenofovir (Viread)	Anorexia, depression, myalgia nausea, asthenia, renal insufficiency, neutropenia [40, 60].
Zalcitabine (Hivid)	Peripheral neuropathy, pancreatitis, lactic acidosis, stomatitis (Product discontinued) [55, 56].
Zidovudine (Retrovir)	Granulocytopenia headache, severe leukopenia, somnolence, hyperpigmentation of nails (bluish-brown), dyspepsia, changes in platelet count, paresthesia [40].
Delavirdine (Rescriptor)	Rash, headache (Product discontinued)[57,58]
Efavirenz (Sustiva)	Rash, somnolence, vivid dreams, confusion, visual hallucinations, hyperlipidemia [40].
Rilpivirine (Edurant)	Depressive disorders, insomnia, headache, rash
Atazanavir (Reyataz)	Hyperbilirubinemia, prolonged PR interval, hyperglycemia, skin rash, hyperlipidemia [40, 60].
Darunavir (Prezista)	Anorexia, rash, nausea, diarrhea, hyperlipidemia, hyperglycemia, angioedema, pruritus, Stevens-Johnson syndrome, urticarial, rhabdomyolysis [40, 60].
Fosamprenavir (Lexiva)	Hyperlipidemia, hyperglycemia, pruritus, Stevens-Johnson syndrome, angioedema, myocardial infarction, nephrolithiasis, hypercholesterolemia [40, 60].
Elvitegravir (Vitekta)	Immune reconstitution syndrome [40, 60].
Enfuvirtide (Fuzeon)	Nodules, erythema, pruritus, pain, and ecchymosis, bacterial pneumonia [40, 60].

log, discontinued in 2006, has severe side effects like peripheral neuropathy [55, 56]. Delavirdine (Rescriptor), discontinued in 2018, quickly develops cross-resistance to other NNRTIs [57, 58]. Patients taking stavudine suffer from hepatic steatosis; physicians should consider it as a possible cause of higher hepatic aminotransferase levels [38]. There is a need for therapy with least or no developing resistance and adverse reactions. ART-associated anemia increases the chances of late virological failure. Management of anemia and monitoring level of hemoglobin for all patients on ART will be an effective strategy to improve virological success rates [59].

6. CURRENT RESEARCH STRATEGIES FOR DRUG TARGETING IN NEURO-AIDS

Current research on neuro-AIDS drug therapy focuses on overcoming the drawbacks of current therapy; Table **5** mentions some novel drug delivery approaches to increase bioavailability and distribution of drugs at the target sites. Marketed dosage forms of ARV drugs are available as tablets, capsules, powder form, and liquid dosage form for oral use [36, 38]. Fusion inhibitor drug enfuvirtide is available as a subcutaneous injection [60]. Groothuis *et al*., (2009) reported that after systemic administration of zidovudine, brain concentrations of zidovudine are likely lower than that necessary to inhibit HIV-1 replication [61].

6.1. Major Challenges Associated with Drug Delivery Systems are [61]

1. Poor concentration of ARV drugs at viral reservoirs sites like brain, macrophages and lymphatic system
2. Poor oral and systemic bioavailability
3. Poor efficacy of existing anti-HIV drugs

6.2. Strategies to Overcome Neuro-AIDS Challenges: [62-66]

1. Early diagnosis for neurological disturbance
2. Effective HAART for every patient/personalize therapy.
3. Novel formulations to cross the BBB.
4. Drug targeting to brain.
5. Nose to brain drug delivery of ART (Bypass the BBB)

Delivery of antiviral drugs to the brain remains a major treatment challenge. Since under the treatment of brain infection, the brain works as a viral reservoir that maintains the body in a normal state [61].

Ramya Devi Durai in 2015 reported several drug delivery approaches using acyclovir as a nanoemulsion by emulsion polymerization, nanosuspension by nanoprecipitation, quasi-emulsion by solvent evaporation method, complexation, floating drug delivery, nanospheres, polymeric micelles, nanocapsules, liposomes, ocular implants, and hydrogels [63].

Nano drug delivery of ARV may improve bioavailability with non-invasive routes like buccal, nasal and transdermal [64]. Nanotechnology-based drug delivery strategies may enhance the CNS penetration of drugs; these may prove efficacious over existing therapy to get benefits in treating neu-

Table 7. Current research on some ARV's for CNS therapy.

ARV Drug	Formulation Approaches	Refs.
Lopinavir	Solid lipid nanoparticle	[70]
Tenofovir	Magnetic nanoparticles	[71]
Enfuvirtide	Amphiphilic polymer myst coating on drug –iron oxide nanoparticles	[72]
Saquinavir	Nanoemulsion-based intranasal drug delivery system	[73]
Azidothymidine 5′-triphosphate	Magnetoelectric nanoparticle (MENPS)	[74]
Lopinavir	Mucoadhesive thermoreversible gels	[75]
Indinavir	Tween 80 containing lipid nanoemulsions	[76]
Zidovudine	Nanostructured lipid carriers	[77]
Nevirapine	Nanosuspensions	[78]
Atanzavir, ritonavir	NanoArt with human monocyte-derived macrophages	[79]
Saquinavir	Transferrin-conjugated quantum rods (QRs) nanoparticle	[80]
Azidothymidine 5′-triphosphate	Magnetic AZTTP liposomes prepared using a mixture of phosphatidylcholine and cholesterol	[81]
Saquinavir	Cationic solid lipid nanoparticles	[82]
Atanzavir	Solid lipid nanoparticles	[83]
Stavudine, delavirdine, and saquinavir	Polybutylcyanoacrylate (PBCA) nanoparticles, methyl methacrylate- sulfopropyl methacrylate (MMA-SPM) nanoparticles, and solid lipid nanoparticles.	[8]
Dolutegravir	Nanoparticles suspended in Poloxamer 407	[9]

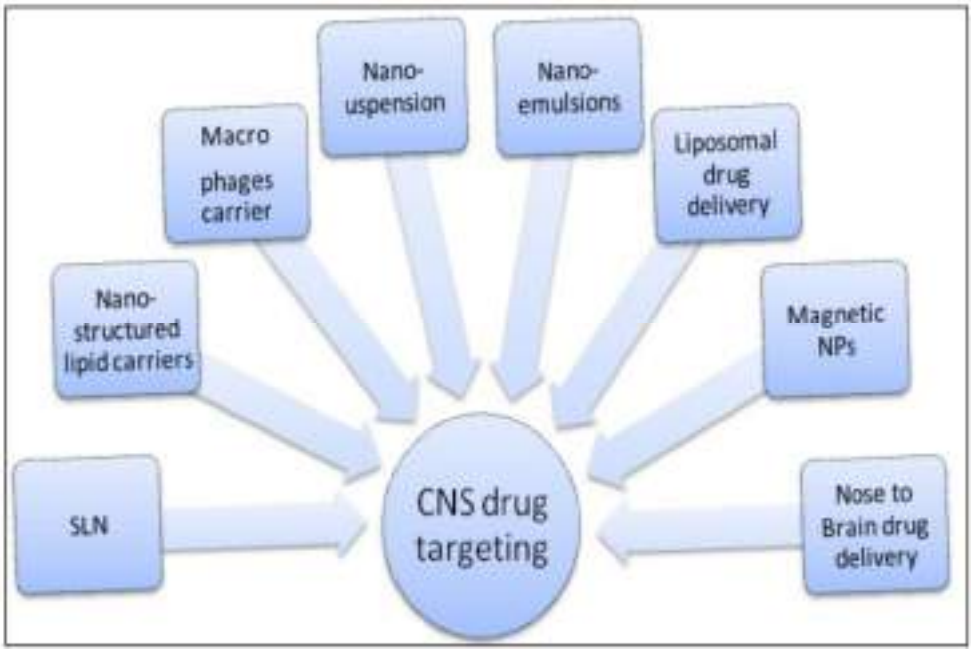

Fig. (4). Potential research strategies for CNS drug targeting [85-102]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

rocognitive disorders [65]. To prevent chronic viral infection or transmission from mother to fetus, the development of neonatal vaccines and other immune-based approaches may prove helpful [66].

Saravanan *et al.*, (2018) reported novel approaches for neuro-HIV eradication and neuroprotection with nanomedicine *in-vitro* and on the small animal models [67]. For the future therapy of antiretroviral nanomedicine, preclinical studies are needed to be performed on some small and large animal models [68]. Moreover, electro-magnetically delivered nanoparticle bound delivery of the BECN1 gene is an effective mechanism to attenuate HIV-1 replication and viral-induced inflammation in the context of the BBB [69].

Lipidic nanocarriers like solid lipid nanoparticles, liposomes, microemulsion and nanoemulsion showed better potential for targeting the anti-HIV drug to the viral reservoir at the brain macrophages and lymphatic drug delivery system could improve the efficacy of ARV drug [70-84].

Current research is focused on novel approaches to treat neuro-AIDS effectively. Table **7** shows some works on this line. Fig. **4** depicts some of the potential research strategies for CNS drug targeting [85-102].

CONCLUSION

The current article summarizes the challenges to Neuro-AIDS and reviews current research on antiretroviral therapy to reduce HIV load in the brain. Novel drug delivery approaches may prove effective in the management of Neuro-AIDS. Patients could better accept non-invasive administration methods and cost-effective formulations as drug therapy persists for a long time. Development of novel safe treatmet options like nanoparticulate drug delivery, liposomal drug delivery, microemulsion-based drug delivery to target viral reservoirs may prove effective with an increase in the bioavailability. Nose to brain drug delivery *via* trigeminal and olfactory pathway is successful for migraine therapy and vaccine delivery, which may prove a better alternative for ARV to reach the brain and CSF at an effective drug level. The design of promising drug delivery approaches for such life-threatening infections may save the brain from Neuro-AIDS and help to improve the socio-economic life of HIV patients.

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Review on Current Drug Therapy and Use of Natural Products in the Management of COVID 19

[1]Kakad Smita P*,Kshirsagar Sanajy J., Asole Rahul, K., Pathade Nikhil V., Vyavahare Maheshwari D.
MET's Institute of Pharmacy, Adgaon, Nashik, 422003, Savitribai Phule Pune University, Maharashtra, India.

E-mail ID of corresponding author: smitadarade87@gmail.com

Abstract:

The novel corona virus infection firstly found in China in December 2019.The virus is almost spreading in all the continents and has become a global economic crises.The virus is mutating fastly and thus it becomes challenging task for all research scientist in the development of a vaccine. In this paper introduction to the virus and the current drugused in the first line treatment of the novel corona virus infection are mentioned in detailed.The key drugs used in therapy are azithromycin, ciclesonide, colchicine, famotidine, apn01, anticoagulant, favipiravir, interferon beta, lopinavir, ritonavir, remdesivir, intravenous Vit C etc.The review also summarizes about the home remedies given by Ministry of AYUSH, Government of Indiawhich has proven effective in some cases and some natural products which serve as immune boosting in the global pandemic of novel corona virus.

Keywords:

COVID-19, Azithromycin, Favipiravir, Lopinavir,Ritonavir, Remdesivir.

1. INTRODUCTION:

For the success of drug therapy to treat novel corona virus infection the detailed structure and metabolic pathways of virus must be knownalso pathophysiology of the infection is important to identify possible drug targets.Structure of corona virus is given in figure 1.

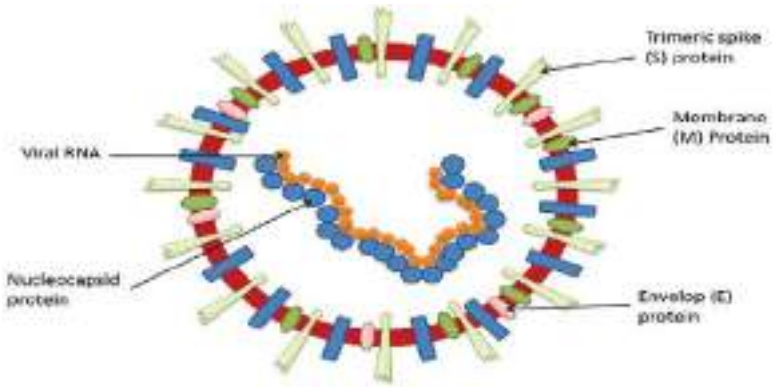

Figure 1. Structure of Corona Virus
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The life cycle of corona virus in the host cell:

In the attachment step the spike proteins of the coronavirus binds to cellular receptor angiotensin-converting enzyme 2 (ACE2) facilitate entry of the viral RNA genome into the host cell and translation of viral proteins. Open reading frames are translated to produce polyproteins, which are cleaved by the proteases to yield 16 non-structural proteins. Next step is assembly and budding into the lumen of the Endoplasmic Reticulum Golgi Intermediate Compartment (ERGIC). Virions released from the infected cell through exocytosis. [1,2]

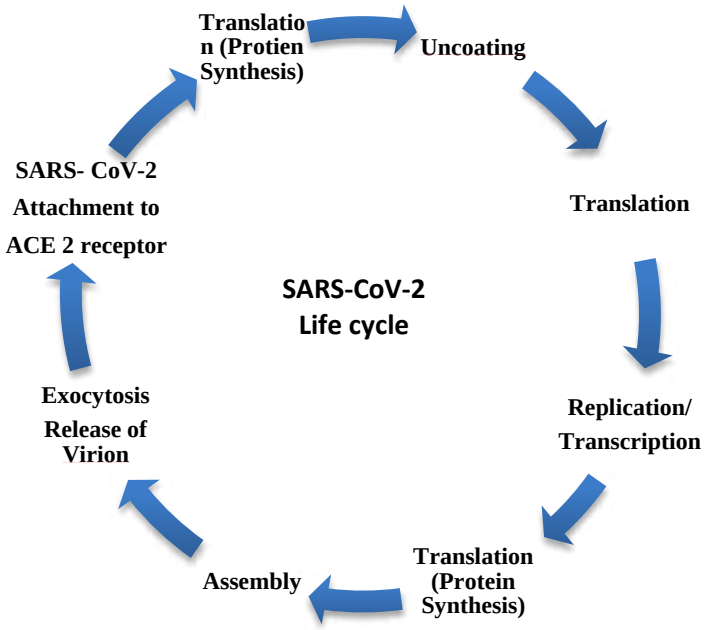

Figure 2. The life cycle of coronavirus in host cells. [1,2]

Possible therapeutic options:

Currently, no antiviral medication is recommended to completely cure COVID-19, and no cure is available for COVID-19. Antibiotics aren't effective against viral infections such as COVID-19. Current research focused on trials on drugs that could be effective for treating severe COVID-19.
Treatment is directed at relieving symptoms and may include:

- Pain relievers (ibuprofen or acetaminophen)
- Cough syrup or medication
- Rest
- Fluid intake

There is no evidence that ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDS) need to be avoided.[3]Researchers in Hong Kong have tried combination of antiviral drugs lopinavir and ritonavir with the hepatitis drug ribavirin and the multiple sclerosis treatment interferon-beta with a control group given just lopinavir-ritonavir. Researchers found that patients suffering milder illness caused by the novel coronavirus recover more quickly if they are treated soon after symptoms appear. This was small trial with 127 patients and detailed study with larger patient group is required. The human immunodeficiency virus (HIV) that causes AIDS is best treated with combinations of different drugs and this could also be the case with COVID-19.[4]Baricitinib, fedratinib, and ruxolitinib are powerful anti-inflammatories are likely to be effective against the elevated levels of cytokines (interferon-γ) typically observed in people with COVID-19·[5]

2. Immunity Boosting Measures for Self-care[6]

A) Measures for Enhancing Immunity:

1. Drink warm water throughout the day.
2. Daily practice of Yogasana, Pranayama and meditation as advised by Ministry of AYUSH (India).

3. Indian spices are recommended in cooking. Example- Haldi (Turmeric), Jeera (Cumin), Dhaniya (Coriander) and Lahsun(Garlic)

B) Ayurvedic Immunity Enhancing Measures:

1. Take Chyavanprash in the morning. Diabetics should take sugar free Chyavanprash.
2. Drink decoctionmade from Tulsi (Basil), Shunthi (Dry Ginger), Dalchini (Cinnamon), Kalimirch (Black pepper), and Munakka (Raisin) or herbal tea.
3. Golden Milk- Drink hot milk with Haldi (turmeric) powder.

C) Simple Ayurvedic Procedures:
1. Nasal applicationof sesame oil or coconut oil or ghee in the nostrils as per method given in Ayurvedic therapy.
2. Oil pulling therapy- Take 1 table spoon sesame or coconut oil in mouthas per method given in Ayurvedic therapy.
D) Actions During dry cough and sore throat:
1. Steam inhalation with Ajwain (Caraway seeds) or fresh Pudina (Mint) leaves.

2. Lavang (Clove) powder mixed with natural sugar or honey can be taken 2-3 times a day in case of cough or throat irritation. However, it is best to consult doctors if these symptoms persist.

3. Drugs Used in COVID 19:-

A.Azithromycin

Introduction: Azithromycin antibiotic used for the treatment of a many bacterial infections. Most common are pneumonia, infection of throat, middle ear infections, traveller'sdiarrhoea, and certain other intestinal infections.

Mechanism of action: Azithromycin interfering with their protein synthesis and inhibit bacterial growth.
Side effects: Common side effects include nausea, vomiting, diarrhea and upset stomach. An allergic reaction, such as anaphylaxis, QT prolongation, or a type of diarrhea caused by Clostridium difficile is possible. No harm has been found with its use during pregnancy. Its safety during breastfeeding is not confirmed, but it is likely safe.Azithromycin is an azalide, a type of macrolide antibiotic.It works by decreasing the production of protein, thereby stopping bacterial growth.[7-9]

Clinical Trials: Azithromycin has been shown to be active in vitro against Zika and Ebola viruses and to prevent severe respiratory tract infections when administrated to patients suffering viral infection.Azithromycin with hydroxychloroquine was significantly more efficient for virus elimination in some trials.Azithromycin is being studied together with other medications in COVID-

19.There is no strong evidence to support combining azithromycin with hydroxychloroquine to treat COVID-19, though such use is being studied. [10-12]

B.Ciclesonide

Introduction: Ciclesonide is a glucocorticoid used to treat asthma and allergic rhinitis. It is marketed under the brand names Alvesco for asthma and Omnaris, Omniair, Zetonna, and Alvesco for hay fever in the US and Canada.[13]

Mechanism of action: Ciclesonide is a glucocorticoid receptor agonist. Ciclesonide is glucocorticoid it can inhibitleukocyte infiltration at the inflammation site, interfere with mediators of inflammatory response, and suppress humoral immune responses alsoreduces inflammatory reaction by limiting the capillary dilatation and permeability of the vascular structures.
Side effects: Side effects of the medication include headache, nosebleeds, and inflammation of the nose and throat linings.[14] The drug was approved for adults and children 12 and over by the US Food and Drug Administration in October 2006.[15]

Clinical trials: According to *In-vitro* studies, ciclesonide showed good antiviral activity against severe acute respiratory syndrome SARS-CoV-2. Clinical trials are going for evaluating the antiviral effect in COVID-19.Luxembourg and Zug(Switzerland) May 2020 announced initiation of a Phase 3 clinical study."There is promising scientific evidence that Alvesco, an inhaled glucocorticoid, may both reduce COVID-19 symptoms and suppress viral replication," said Michael Blaiss, M.D., Clinical Professor of Pediatrics, Medical College of Georgia at Augusta University in Augusta, Georgia. [13, 16]
The Phase 3 study of Alvesco, a metered-dose inhaler onthe 400 patientstartedin the United States. Early results of this study are expected to be released in September 2020.There are currently no antiviral drugs approved by the FDA for COVID-19 with the exception of Gilead's Antiviral Remdesivir which has received FDA Emergency Use Authorization for the treatment of COVID-19. According to the website of the Centres for Disease Control and Prevention (CDC), clinical management of COVID-19 includes prompt implementation of recommended infection prevention and control measures in healthcare settings and supportive management of complications. The World Health Organization (WHO) advises that people of all ages can be infected by the new coronavirus (2019-nCoV). WHO recommends people of all ages to take steps to protect themselves from the virus, for example by following good hand and respiratory hygiene.[17]

C.Colchicine

Introduction: Colchicine is a medication used to treat gout and Behcet's disease.[18,19]In gout, it is less preferred to NSAIDs or steroids.Other uses include the prevention of pericarditis and familial Mediterranean fever.[18, 20]

Mechanism of action:Colchicine inhibits multiple proinflammatory mechanism and increases level of anti-inflammatory mediators.[22] **C**olchicine inhibits mitosis and neutrophil motilityto give anti-inflammatory effect.[21]

Side effects: Deaths – both accidental and intentional – have resulted from overdose of colchicine. Typical side effects of moderate doses may include gastrointestinal upset, diarrhea, and neutropenia.

Over dosage damage bone marrow, anemia, hair loss. Side effects can result from inhibition of mitosis, which may include neuromuscular toxicity and rhabdomyolysis.[21]

Clinical trials: A clinical trial of 6000 people with COVID-19 infection, funded by the Government of Quebec, began in March 2020 to test the potential efficacy of using colchicine over a 30 day period to reduce disease symptoms.[22]The clinical study, named COLCORONA, coordinated by the Montreal Health Innovations will evaluate the phenomenon of major inflammatory storm present in adults suffering from severe complications related to COVID-19. The researchers hypothesized that the treatment could reduce the complications associated with COVID-19. [23, 24]

D.Famotidine

Introduction: Famotidine, sold under the brand name Pepcid among others, is a medication that decreases stomach acid production. It is used to treat peptic ulcer disease, gastroesophageal reflux disease, and Zollinger-Ellison syndrome.It is taken by mouth or by injection into a vein.It begins working within an hour.[25, 28]

Mechanism of action: It is a histamine H2 receptor antagonist.[25]Activation of H2 receptors located on parietal cells stimulates the proton pump to secrete acid. Famotidine (H2 antagonist) blocks the action of histamine in the parietal cells, ultimately blocking acid secretion in the stomach.[28]

Side Effects: Common side effects include headache, intestinal upset, and dizziness.[25] Serious side effects may include pneumonia and seizures.[25,26] Use in pregnancy appears safe but has not been well studied while use during breastfeeding is not recommended.[27]

Clinical trials: A research study by Columbia University, Northwell Health and Massachusetts General Hospital shows that Famotidine, a common heartburn drug improved the clinical outcomes of hospitalized COVID-19 patients.[29]
The study involving 1620 patients from more than 10 hospitals in the United States showed that Famotidine associated with reduced risk of intubation or death in hospitalized COVID-19 patients. However further randomized controlled trials are warranted and also detailed studies are needed to understand the mode of its efficacy.
The drug was chosen as earlier drug repurposing computer modelling studies showed that the drug molecular structure had very good potential to block ‘docking sites’ on the spike protein structures of the SARS-CoV-2 coronavirus, rendering the virus to become inactive and not capable of entering host cells and replicating.

E.APNO1

Clinical trials: Austrian immuno-oncology firm Apeiron Biologics today announced that it has received regulatory approvals in Austria, Germany and Denmark to initiate a Phase II clinical trial of APN01 to treat COVID-19.APN01 is the recombinant form of the human angiotensin-converting enzyme 2 (rhACE2), and has the potential to block the infection of cells by the novel SARS-CoV-2 virus (COVID19), and reduce lung injury. The Phase II trial aims to treat 200 severely infected COVID-19 patients, and the first patients are expected to be dosed shortly.
APN01 has a unique dual mode of action. The virus binds to soluble ACE2/APN01, instead of ACE2 on the cell surface, which means that the virus can no longer infect the cells. At the same time,

APN01 reduces the harmful inflammatory reactions in the lungs and protects against acute lung injury (ALI/acute respiratory distress syndrome (ARDS).[30]

F. Favipiravir

Introduction:Favipiravir, sold under the brand name Avigan, is an antiviral medication used to treat influenza in Japan.[31] It is also being studied to treat a number of other viral infections. It became a generic drug in 2019.[32]Favipiravir has been approved to treat influenza in Japan. It is, however, only indicated for novel influenza (strains that cause more severe disease) rather than seasonal influenza.[33]

Mechanism of Action: The mechanism of its actions is thought to be related to the selective inhibition of viral RNA-dependent RNA polymerase. Human hypoxanthine guanine phosphoribosyl transferase (HGPRT) is key enzyme in the activation of prodrug favipiravir. Active form of favipiraviris favipiravir-ribofuranosyl-5'-triphosphate (favipiravir-RTP), available in both oral and intravenous formulations. Favipiravir is not toxic to mammalian cells as it does not inhibit RNA or DNA synthesis in mammalian cells.In Japan favipiravir was approved against influenza pandemics in 2014. However, favipiravir has not been shown to be effective in primary human airway cells, casting doubt on its efficacy in influenza treatment.[34]

Sideeffects: There is evidence that use during pregnancy may result in harm to the baby.[35]

Clinical Trials: In February 2020, favipiravir was being studied in China for experimental treatment of the emergent COVID-19. Trials are also being planned in Japan.[36]The drug has been approved for use in clinical trials of coronavirus disease 2019 in China.[37]In February 2020, favipiravir was being studied in China for experimental treatment of the emergent COVID-19. Italy approved Favipiravir for the experimental use in COVID-19 during March 2020, and has started conducting trials. The Italian Pharmaceutical Agency, however, reported that existing evidence in support of this drug is scant and preliminary.The drug was approved for the treatment of COVID-19 in the hospital settings in Russia on May 29, 2020, after an ongoing open-label randomized clinical trial had recruited 60 subjects on favipiravir. On May 30, 2020, the Russian Health Ministry approved a generic version offavipiravir named Avifavir. RDIF backed the development of Avifavir and found it highly effective in the first phase of clinical trials.[38]Research in 2014 suggested that favipiravir may have efficacy against Ebola based on studies in mouse models; efficacy against in humans was unaddressed.[39]

During the 2014 West Africa Ebola virus outbreak, a French nurse who contracted Ebola while volunteering for MSF in Liberia reportedly recovered after receiving a course of favipiravir. A clinical trial investigating the use of favipiravir against Ebola virus disease began in Guéckédou, Guinea, in December 2014. Preliminary results presented in 2016 at the Conference on Retroviruses and Opportunistic Infections (CROI), later published, showed a decrease in mortality in patients with low-to-moderate levels of virus in blood, but no effect on patients with high levels (the group at a higher risk of death). The trial design was concomitantly criticised for using only historical controls.[40]

G. Interferon Beta

Introduction: Interferon beta-1a (also interferon beta 1-alpha) is a cytokine in the interferon family used to treat multiple sclerosis (MS).It is produced by mammalian cells, while interferon beta-1b is

produced in modified E. coli. Some claims have been made that Interferons produce about an 18–38% reduction in the rate of MS relapses.[41-43]

Mechanism Of Action:, Interferon beta therapy leads to a reduction of neuron inflammation. Also increases the production of nerve growth factor and consequently improve neuronal survival. In vitro, interferon beta reduces production of Th17 cells which are a subset of T lymphocytes believed to have a role in the pathophysiology of MS.[44]

Side Effects: Interferon beta-1a is available only in injectable forms, and can cause skin reactions at the injection site that may include cutaneous necrosis. They usually appear within the first month of treatment albeit their frequency and importance diminish after six months of treatment.[45]Clinical studies on the efficacy of type I interferons, including interferon alfa and interferon beta, in the treatment of SARS-CoV had variable results.[46]

Clinical Trials: Since there is evidence that insufficient production of interferon beta-1a in lung cells in older people can lead to their increased susceptibility to respiratory viral infections such as SARS-CoV-2 and MERS-CoV. Company Synairgen began clinical tests of SNG001, a special inhalation formulation of interferon beta-1a in patients with COVID-19.[47]

H. Lopinavir/ Ritonavir

Introduction: Lopinavir/ritonavir (LPV/r), sold under the brand name Kaletra among others, is a fixed dose combination medication for the treatment and prevention of HIV/AIDS. It combines lopinavir with a low dose of ritonavir.It is taken by mouth as a tablet, capsule, or solution.[48]It is on the World Health Organization's List of Essential Medicines, the safest and most effective medicines needed in a health system.[49]

Mechanism of action: Mechanism of Action: Lopinavir/ritonavir inhibits the HIV protease enzyme by forming an inhibitor-enzyme complex thereby preventing cleavage of the gag-pol polyproteins. Immature, noninfectious viral particles are subsequently produced.
Administered alone, lopinavir has insufficient bioavailability; however, like several HIV protease inhibitors, its blood levels are greatly increased by low doses of ritonavir, a potent inhibitor of intestinal and hepatic cytochrome P450 3A4, which would otherwise reduce drug levels through catabolism.Abbott, therefore, pursued a strategy of co-administering lopinavir with doses of ritonavir sub-therapeutic with respect to HIV inhibition; hence, lopinavir was only formulated and marketed as a fixed dose combination with ritonavir.[50]

Medicinal uses: As of 2006, lopinavir/ritonavir forms part of the preferred combination for HIV first-line therapy recommended by the US United States Department of Health and Human Services in 2006[51]

Side effects: Common side effects include diarrhea, vomiting, feeling tired, headaches, and muscle pains. Severe side effects may include pancreatitis, liver problems, and high blood sugar. It is commonly used in pregnancy and it appears to be safe.Both medications are HIV protease inhibitors. Ritonavir functions by slowing down the breakdown of lopinavir.[48]

Clinical trials: While data for SARS-CoV-1 looked promising, the benefit in COVID-19 is unclear as of 23 March 2020. In 2020, a non-blinded, randomized trial found lopinavir/ritonavir was not useful

to treat severe COVID-19. In this trial the medication was started typically around 13 days after the start of symptoms.[52]

I. Remdesivir

Introduction: Remdesivir is a broad-spectrum antiviral medication developed by the biopharmaceutical company Gilead Sciences.[53] It is administered via injection into a vein. As of 2020, remdesivir is being tested as a specific treatment for COVID-19, and has been authorized for emergency use in the US, India[54] and approved for use in Japan for people with severe symptoms. It also received approval in the UK in May 2020, however will be rationed due to limited supply. It may shorten the time it takes to recover from the infection.[55]
Side effects may include liver inflammation and an infusion-related reaction with
SARS-CoV-2 nusea, low blood pressure, and sweating[56]
It is a pro-drug that is converted in the body into GS-441524, a ribonucleotide analog.
Earlier studies found antiviral activity against several RNA viruses including SARS coronavirus and MERS coronavirus, but it is not approved for any indication. Remdesivir was originally developed to treat hepatitis C and was then tested against Ebola virus disease and Marburg virus disease, but was ineffective for all of these viral infections.[53]

Mechanism of action: As an adenosine nucleoside triphosphate analog, the active metabolite of remdesivir interferes with the action of viral RNA-dependent RNA polymerase and evades proofreading by viral exoribonuclease (ExoN), causing a decrease in viral RNA production. In some viruses such as the respiratory syncytial virus it causes the RNA-dependent RNA polymerases to pause, but its predominant effect (as in Ebola) is to induce an irreversible chain termination. Unlike with many other chain terminators, this is not mediated by preventing addition of the immediately subsequent nucleotide, but is instead delayed, occurring after five additional bases have been added to the growing RNA chain. For the RNA-Dependent RNA Polymerase of MERS-CoV, SARS-CoV-1, and SARS-CoV-2 arrest of RNA synthesis occurs after incorporation of three additional nucleotides. Hence, remdesivir is classified as a direct-acting antiviral agent that works as a delayed chain terminator.[57,58]

Sideeffects: The most common adverse effects in studies of remdesivir for COVID 19 include respiratory failure and organ impairment, including low albumin, low potassium, low count of red blood cells, low count of platelets that help with clotting, and yellow discoloration of the skin. Other reported side effects include gastrointestinal distress, elevated transaminase levels in the blood (liver enzymes), and infusion site reactions.[59]

Other possible side effects of remdesivir include:

- Infusion-related reactions. Signs and symptoms of infusion-related reactions may include: low blood pressure, nausea, vomiting, sweating, and shivering.

- Increases in levels of liver enzymes, seen in abnormal liver blood tests. Increases in levels of liver enzymes have been seen in people who have received remdesivir, which may be a sign of inflammation or damage to cells in the liver.[60]

Clinical trials: Remdesivir for 5 or 10 Days in Patients with Severe Covid-19. A randomized, open-label, phase 3 trial in patients with severe Covid-19 not requiring mechanical ventilation, this trial did

not show a significant difference between a 5-day course and a 10-day course of remdesivir. With no placebo control, however, the magnitude of benefit cannot be determined. [61,62]

J. Intravenous vitamin C

Introduction: Intravenous Ascorbic Acid (also known as vitamin C or L-ascorbic acid), is a type of therapy that delivers soluble ascorbic acid directly into the bloodstream, either administered via injection or infusion. Intravenous ascorbic acid is a dietary supplement for nutritional deficiencies.Some recent research suggests its ability to decrease inflammation in the patient and to improve symptoms related to disease processes, and side effects of standard cancer treatments.[63-65]
Mechanism of action: Ascorbic acid operates as an anti-oxidant and essential enzyme cofactor in the human body. Although many in vitro studies have studied hydrogen peroxide generation by ascorbic acid, the pharmacological mechanism of intravenous ascorbic acid in vivo is still unclear.[66,67]
Side effects: Vitamin C is a water-soluble vitamin, with dietary excesses not absorbed, and excesses in the blood rapidly excreted in the urine, so it exhibits remarkably low acute toxicity. More than two to three grams may cause indigestion, particularly when taken on an empty stomach. Other symptoms reported for large doses include nausea, abdominal cramps and diarrhea. These effects are attributed to the osmotic effect of unabsorbed vitamin C passing through the intestine. [68] There is a longstanding belief among the mainstream medical community that vitamin C increases risk of kidney stones.[69,70]

Clinical trials:
As of April 2020, there are ten ongoing clinical trials of intravenous vitamin C for people who are hospitalized and severely ill with COVID-19; two placebos controlled (China, Canada) and one with no control (Italy).[71]

K. Oralvitamin D

Introduction: Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and multiple other biological effects. In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D2 (ergocalciferol).[72]
The major natural source of the vitamin is synthesis of cholecalciferol in the lower layers of skin epidermis through a chemical reaction that is dependent on sun exposure (specifically UVB radiation).Cholecalciferol and ergocalciferol can be ingested from the diet and from supplements. Flesh of fatty fish, naturally contain significant amounts of vitamin D. Cow milk and plant-derived milk substitutes are fortified with vitamin D, as are many breakfast cereals. [73]

Side effects: Dehydration,omitting,Diarrhea,Decreased appetite,Irritability,Constipation,Fatigue, Muscle weakness,Metastatic calcification of the soft tissues

Infectious diseases and covid-19: In general, vitamin D functions to activate the innate and dampen the adaptive immune systems. Deficiency has been linked to increased risk or severity of viral infections, including HIV. Supplementation slightly decreases the risk and severity of acute respiratory tract infections, and also the exacerbation of asthma. There is no evidence for vitamin D affecting respiratory infections in children under five years of age.

The COVID-19 pandemic raised concerns that vitamin D deficiency may be a risk factor for respiratory infection, but there is only preliminary evidence of a direct association between vitamin D deficiency and COVID-19 infection.[74]
One UK study found no association between previously measured vitamin D levels and the incidence of COVID-19 infection when adjustments were made for potential confounding factors, such as ethnicity. Vitamin D deficiency is prevalent in many countries with the highest numbers of COVID-19 cases and deaths, such as the United States, Spain, the UK, Italy, and Iran.[75,76]
Table 1 listed some natural remedies for preventive measures as well as used to get relief from symptomatic treatment.

Table 1.Role of Natural Products In The Management of Covid-19

Natural Product	**Health Benefits**	**Marketed Preparation**	**References**
Ocimumtenuiflorum (Tulsi)	Relief from Sore throat and Cough, common cold	Herbal Tea	[77,78]
Curcuma longa (Turmeric)	Immunity Booster	Golden Tea	[79]
Chyavanprash	Immunity Booster	Dabur Chyavanpraash, ZandukesariJivanchyavan praash.	[80,81]
Allium sativum (Garlic)	Treat Common cold, Vit C.	Health juices	[82,83]
Glycyrrhiza glabra(Licorice)	Respiratory System well maintained. Immunity booster.	Himalaya Yashtimadhu.	[84]
Honey	Sore throat and cough, Common cold	Dabur Honey, Patanjali Honey.	[85,86]
Trigonella foenum-graecum(Fenugreek)	Strengthens Immunity and Anti-oxidant	Fenugreek extract	[87]
Zingiber officinale(Ginger)	In Cold and Cough symptoms	Ginger Tea	[87]

Conclusion:

The paper focus on the virus structure along with virus's life cycle in host cell and different drugs which are being used currently in the management of the COVID-19. The different immunity boosting majors for self-care are discussed. The various dugs from different classes of disease are being used alone or in the combination of other drugs which are showing positive impact in curing the

disease. The clinical trials are conducted on many patients in various universities all over the world. Many traditional and natural remedies like Tulsi, Garlic, Honey, Turmeric, Ginger etc. are used for immunity boosting purposes and also for symptomatic reliefin COVID-19.

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FORMULATION AND OPTIMIZATION OF RITONAVIR NASAL NANOSUSPENSION FOR BRAIN TARGETING

Tapasya R. Mulam[a]*, Sanjay J. Kshirsagar[a] and Smita P. Kakad[a]

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ABSTRACT

Nowadays, HIV associated neurological disorder especially HIV-1 virus infection is enhanced. Current available HIV therapies only reduce the plasma viral level and do not kill the virus completely. Administered dosage form does not reach the central nervous system (CNS) completely by the conventional approach. The oral route of drug administration,causes gastrointestinal irritation, hepatic metabolism and slow onset of action and some methods are invasive, resulting in the patient's non compliance. To overcome these problems, an effective novel formulation that will directly reach the CNS or brain needs to be developed.This study aims to formulate intranasal nanosuspension of ritonavir. Ritonavir is widely used as an antiretroviral agent and it is a protease enzyme inhibitor which is poorly soluble in water. High pressure homogenization technique was used for preparation prepare and optimization of nanosuspension by using 2 factors 3 level full factorial design, which is further characterized for particle size, polydispersity index, zeta potential, pH, drug content, *in vitro* drug diffusion and *ex vivo* permeation study. For stability of nanosuspension, lyophilization of optimized formulation was done. A comparison study between plain drug, nanosuspension and the lyophilized formulation was carried out, and it showed a significant increase in drug release from the membrane.

Keywords: Intranasal, antiretroviral, blood brain barrier, ritonavir, nanosuspension, optimization, lyophilization

INTRODUCTION

Around 36 % worldwide and 6 % population in India are reportedly suffering from neurological disorders. The main cause of acquire immunodeficiency syndrome (AIDs) is the human immunodeficiency virus (HIV). The prevalence of HIV-1 virus is found more in CNS/ brain where the endurance of virus found in macrophages, microglia and neurons cells[1]. HIV principally infects CD4 cells, monocytes, macrophages and T-lymphocytes. Infection of the brain cells results in neurological impairments and can cause HIV associated dementia. During HIV infection, CD4 cell count decreases by 250 cell/mm^3 and prevalence of other opportunistic infections increases. Monocytes and these CD4 cells serve as entry points for these cells in the brain, where they multiply and infect new cells. Release of leukotrienes, chemokines and tumor necrosis factor- alpha (TNF-a) occurs when there is transmigration of leukocytes through the blood brain barrier (BBB), which disturbs BBB integrity[2]. Intranasal administration is a non-invasive route for active low dose drugs and local, systemic, central nervous effects a achieved[3]. Problems related to BCS class II drugs result in dissolution-rate-limited step which affects the bioavailability[4]. 40 % of recent pharmaceutical drugs are poorly water soluble and lipophilic in nature. There are conventional methods available for enhancing solubility such as salt formation, pH adjustment, emulsion, micellar dispersion and complexation with cyclodextrin. Alternative method is nanonization of drug particles, which reduces particle size to nanometre range and leads to improved bioavailability[5]. Nanosuspension successfully deals with solubility issue. Micronization increases surface area as well as dissolution velocity of drug, but does not enhance the saturation solubility. In case of very low saturation solubility, there is increase in dissolution velocity but not enhancement in bioavailability[6]. Nanosuspensions are very finely dispersed drug particles, suspended in continuous phase. They are colloidal dispersions stabilized by surfactants, polymers or both. Average particle size ranges from 200-600nm. Oral route fails to deliver the dosage form to the brain due to the enzymes present in gut and strong network of blood brain barrier; intranasal delivery is the superior route to transport drugs to the brain[7]. Now-a-days, intranasal delivery is the promising route for many drugs due to its properties like manageable

[a]Department of Pharmaceutics, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik - 422 003, Maharashtra, India
*For Correspondence: E-mail: mulam20tapasya@gmail.com

surface area, low enzymatic action, high permeability, high vascularity and avoidance of hepatic metabolism[8]. Area of nasal cavity is 180cm^2 and olfactory area occupies around 3 % of nasal cavity. Volume of cerebrospinal fluid in adult human is 160 mL and it is replace of every 5 h^2.Didanosine chitosan-loaded nanoparticles, used to treat HIV infection, is one of the examples of brain targeted intranasal drug delivery[7].

MATERIALS AND METHODS

Ritonavir, Poloxamer 407 and HPMC 3Cps were gifts from Glenmark Pharmaceuticals Ltd R & D Centre, Sinnar. Mannitol, sodium lauryl sulphate and potassium dihydrogen orthophosphate were purchased from Thomas Baker (Chemical) Pvt. Ltd., Mumbai. HPLC grade methanol and sodium hydroxide pellets were purchased from S. D. Fine Chem Lab. Limited, Mumbai, India.

Preparation of nanosuspension

Nanosuspensions were prepared by high pressure homogenization technique followed by high speed homogenization. A polymer solution was made in water and then Poloxamer 407 and sodium lauryl sulphate were added. Addition of above excipients was done on a magnetic stirrer. After proper mixing, drug was added in polymeric solution. The suspension was subjected to high speed homogenization for 1 h at 15000 rpm. Further, this coarse suspension was exposed to high pressure homogenization at 750 bar with 8 cycles.

Saturation solubility study

Saturation solubility was performed by using orbital shaker. Excess amount of drug was added in 25mL of different vehicles in conical flask separately and kept in orbital shaker for 100rpm for 48 h at room temperature. After 48 h, samples were centrifuged at 3000 rpm for 15 mins and the supernatant was taken and filtered by using Whatman filter paper. The supernatants were measured by using UV- spectrophotometer and appropriate dilutions were made with water and phosphate buffer (pH 6.5) at 233 and 238nm, respectively. Then, drug solubility (mg/mL) was calculated.

Characterization by FT-IR analysis

Fourier transform infrared (FTIR) spectroscopy analysis provides structural and functional information by spectra. The FTIR spectrum of drug, polymer and surfactants were recorded by FTIR spectrophotometer (Carry 630 FTIR Agilent Tech.). Analysis was done by using KBr (use as carrier and it is optically transparent) in the ratio of 1:9 of drug: KBr and then mixing well. Small amount of mixture was placed in the cuvette and then sample was analysed on FTIR spectrometer.

Compatibility study with drugs, polymer and surfactant

The physical mixture of drug, polymers and surfactants was kept in vials. Vials were sealed and stored at room temperature for a period of 21 days and then these samples were analysed for any variation in IR spectrum[9]. Mixtures of drug+ polymer and drug+ surfactant were made, if there is change in colour or visual properties and even by spectral changes we can say that they are incompatible and may cause adverse reaction, antagonistic in action and not safe for formulation. Inactivation of drug can occur through either decomposition or loss of drug by its conversion to a less favourable physical or chemical form.

Trial batches for nanosuspension

Trial batches were taken into consideration to get desired particle size (<250 nm) and polydispersity index ($\approx$0.6) by varying in formulation parameters (polymer, surfactant concentration) and the process parameters (cycle and pressure) of high pressure homogenizer. Parameters of high speed homogenizer were kept such as duration of 60 mins at 15000 rpm for all batches as shown in Table I.

Table I: Trial batches of high pressure homogenization

Batch No.	Drug (mg)	HPMC 3cps (mg)	Poloxamer 407 (mg)	SLS (mg)	Cycle (min)	Pressure (bar)
T1	100	100	45	25	6	650
T2	100	100	45	-	8	750
T3	100	100	65	25	6	650
T4	100	100	65	25	7	750

Optimization by 3 levels and 2 factor

Design of experiment was used to determine the optimum nanosuspension formulation. After the formulation of nanosuspension, it was characterized for particle size, particle size distribution and zeta potential. Poloxamer 407 used as a stabilizer in the formulations and cycles were selected as independent variables. Particle size and drug release were considered as dependent variables. For statistical design, Design Expert® Version 11 was used. A 3 level factorial design was used in this study. Experimental trials were performed with all 9 possible combinations. Factors were selected as cycles and drug release on the levels of -1, 0, +1 (low, medium and high), as shown in Table II. The number of cycles (min) on high pressure homogenization process (6, 7, and 8) and Poloxamer 407 concentration (45, 55, and 65 mg in formulation) were selected. Interactions between formulation variables were analysed using ANOVA at $p< 0.05$ as minimum level of significance.

Determination of pH of nanosuspensions

The pH of all batches (F1-F9) was determined by using Digital pH meter (Systronics, India). To determine pH of formulation, around 10mL of nanosuspension was put into beaker and then pH probe was put into formulation and pH of formulation was displayed on digital pH meter.

Determination of particle size and polydispersity index (PDI)

Particle size (average particle size) and polydispersity index of nanosuspension was determined by photon correlation spectroscopy (Zeta-sizer Ver. 7.01, Malvern Instruments). It is based on the principle of dynamic light scattering. The sample is illuminated with a laser beam and the intensity of the resulting scattered light produced by the particles fluctuates at a rate that is dependent upon the size of the particles. A drop of nanosuspension by syringe was taken into test tube and it was diluted by distilled water, mixed thoroughly with vigorous shaking and placed in small volume disposable polystyrene cuvette and light scattering was monitored at 25 °C.

Determination of zeta potential

For determination of zeta potential, same instrument was used as that of particle size i.e. photon correlation spectroscopy (Zeta-sizer Ver. 7.01, Malvern Instruments). There is a different cuvette used for zeta potential analysis. Surface charges were determined in zeta potential.

Characterization by differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a method to evaluate the crystallinity of drug as well as final formulation and heat absorb or released by sample. It also evaluates possible polymorphic transformations of ritonavir in nanosuspension. DSC can provide thermodynamic information of drug and identify the change in melting point temperature and enthalpy[10]. DSC measurements for pure drug and lyophilized powder were performed (Mettler Toledo India Pvt. Ltd, Switzerland). The samples were dried and then 2-5 mg accurately weighed, then placed in an aluminum pan and then hermetically sealed with an aluminum lid. The system was purged with nitrogen gas at a flow rate of 50mL/min and heated at a rate of 10 °C/min.

Table II:Optimization of nanosuspension by 3 level factorial design

Composition formulation code	Drug ritonavir (mg)	HPMC 3cps (mg)	SLS (mg)	Stabilizer Poloxamer 407 (mg)	Cycle (min)	Pressure (bar)
F1	100	100	25	55	**7**	750
F2	100	100	25	45	7	750
F3	100	100	25	65	8	750
F4	100	100	25	65	7	750
F5	100	100	25	45	8	750
F6	100	100	25	65	6	750
F7	100	100	25	55	6	750
F8	100	100	25	45	6	750
F9	100	100	25	55	8	750

Drug content for nanosuspension

From nanosuspension, 1mL of aliquot was removed and diluted with methanol. Then it was filtered by Whatman filter paper (0.45 µm). The samples were analysed by UV-spectrophotometer at λ max 239 nm. Total drug content was calculated by following formula[11]:

$$TDC = \frac{\text{Vol. Total}}{\text{Vol. aliquot}} \times \text{Drug amount in aliquot X 100 … (A)}$$

Lyophilization of optimized batch (F3)

Nanosuspension was prepared in aqueous medium using high pressure homogenizer. Nanosuspension has property to aggregation due to the charges present on surface of particles, so lyophilization is best way to stabilize the formulation. Lyophilization not only increases the stability but also enhances the shelf life of the finished product by stable dry state which will be reconstituted with water[12] . Proper selection of cryprotecting agent is important as it governs the efficiency of freeze drying process. Nanosuspension was kept with mannitol (1 %w/V) as cryoprotectant at -80 °C for 12h and then freeze dried in lyophilizer (LABOGENE).

Drug content for lyophilized powder

Lyophilized powder equivalent to 5mg was weighed and placed in 50mL of volumetric flask, 5mL of methanol taken and volume made upto 50 mL by phosphate buffer (pH 6.5). Then, the sample was analysed by using UV-spectrophotometer at λ max 238 nm and blank was made by corresponding solution of excipients[12]. By the above formula, drug content of freeze dried powder was calculated by equation (A).

In vitro diffusion study

The *in vitro* drug release study was performed by using Franz diffusion cell of 16 mL capacity. The nanosuspension drug amount equivalent to 5 mg was placed on a cellulose dialysis membrane, (MWCO 12,000 g/mole; Himedia Laboratories Pvt. Ltd.)**.** The dialysis membrane was placed in-between receptor and donor compartment, which was stirred continuously at 100 rpm maintained at 37 °C. The receiver compartment was filled with phosphate buffer (pH 6.5) and samples were withdrawn at regular time intervals and the same volume was replaced with fresh medium. Drug release was analysed by UV- spectrophotometer at λ max 238 nm[14].

Ex vivo permeation study

Ex vivo permeation study was carried out by using goat nasal mucosa for the optimized batch (F3). Nasal mucosa was procured from local slaughter house and it was washed with phosphate buffer (pH 6.5). Mucosal membrane acts as a permeation membrane for release of drug, mucosal surface was facing the donor compartment and kept in between donor and receiver compartment. Phosphate buffer pH 6.5 was used as diffusion medium, stirred continuously at 100 rpm and maintained at 37 °C. Samples were withdrawn at regular time intervals and the same volume was replaced with fresh medium. The samples were measured by UV spectrophotometer at 238 nm[14].

X-ray diffraction

XRD (Bruker AXS D8 advance) is the method used to evaluate the crystallinity, amorphous and polymorphism characterization of drug and formulation. XRD study was performed in X-ray diffractometer using Cu X-ray source (wavelength 1.5406 A^o) with voltage of 40kV and current of 25mA to determine the effect on pure as well as lyophilized formulation. The x-ray diffraction profiles were obtained using continuous scan mode with 2-theta ranging from 2^o to 50^o at the rate of 1^o/min at 40 kv[15].

Scanning electron microscopy

Scanning electron microscopy (JEOL model JSM-6390LV) is a method for high resolution surface imaging and particle surface morphology is observed. Small drop of the suspension was air dried followed by oven drying and was fixed on an SEM stub using double-sided adhesive tape. A scanning electron microscope with a secondary electron detector was used to obtain digital images of the samples at an accelerating voltage of 15 KV[16].

Histopathological study

Histopathological study was carried out on goat's nasal mucosa to confirm the irritation or cell damage to the nasal membrane by intranasal administration of optimized formulation. Fresh goat nasal mucosa was collected from a local slaughter house and transported in phosphate buffer solution (pH 6.4). It was cleaned properly with phosphate buffer and cut into 3 symmetric pieces. First one was treated as positive control by using 70 % isopropyl alcohol, second piece was treated as negative control by use of phosphate buffer pH 6.4 and the last piece was treated with formulation. After 1 h of treatment, all samples were washed thoroughly with phosphate buffer solution and stored directly in 10 % formalin solution for

24 h. After 24 h, the formalin solution was replaced with 70 % ethanol and the samples stored at 4 °C for dehydration. The dehydrated sections were then embedded in agar and paraffin block and cut to 5 µm thickness by using a microtome. Finally, the samples were stained with a combination of haematoxylin and eosin (H&E) dye and observed under optical microscope (TUCSEN- USB2.0H) to detect any damage to the membrane[16].

Stability study

For stability study ICH guidelines were followed. Study was carried out for stability of final formulation. Lyophilized powder was dispersed in phosphate buffer (pH 6.5) and kept in borosilicate glass vials and sealed, then these vials were store at 40 °C ± 2 °C / 75 % RH ± 5 % for 1 month[16].

RESULTS AND DISSCUSION

Solubility study

Ritonavir is practically insoluble in water and the reported solubility is 0.00126 mg/mL at 25 °C. It is slightly soluble in phosphate buffer (pH 6.5). From the practical work, solubility of ritonavir was found to be 0.0029 mg/mL and 0.0366 mg/ mL, respectively, as given in Table III. By observing the above results, we come to know that there is increase in solubility of formulation due to nanosizing. This happen because of reduction in particle size with the use of different polymers and surfactants by the method called as high pressure homogenizer. Surfactants help to reduce the hydrophobicity of the drug[17]. Since reduction in particle size causes increase in surface area it makes the particles surface more exposed to the medium.

Table III: Saturation Solubility of pure drug

Sr. No	Solvent	Solubility (mg/mL) Pure drug	Solubility (mg/mL) Nano suspension
1.	Water	0.0029 ±0.26	0.056 ±0.25
2.	Phosphate buffer (pH 6.5)	0.0366±0.24	0.120 ±0.27

FTIR analysis of drug and excipients

As observing Fig.1, the principle peaks of 3449.67 (cm^{-1}), 2923.93(cm^{-1}), 2864.4(cm^{-1}), 1638(cm^{-1}), 1457.11(cm^{-1}), 1229.64(cm^{-1}), 1089.69 (cm^{-1}) correspond to aliphatic secondary amine, - NH stretch, aromatic ethers, aryl-O stretch, C-H stretch alkane, amide, methyl C-H asym bend aromatic ethers, aryl-O stretch, primary amine and -CN stretch, respectively. The principle peaks are retained for drug and excipients, from this we can conclude that there is no interaction and the excipients are compatible[22].

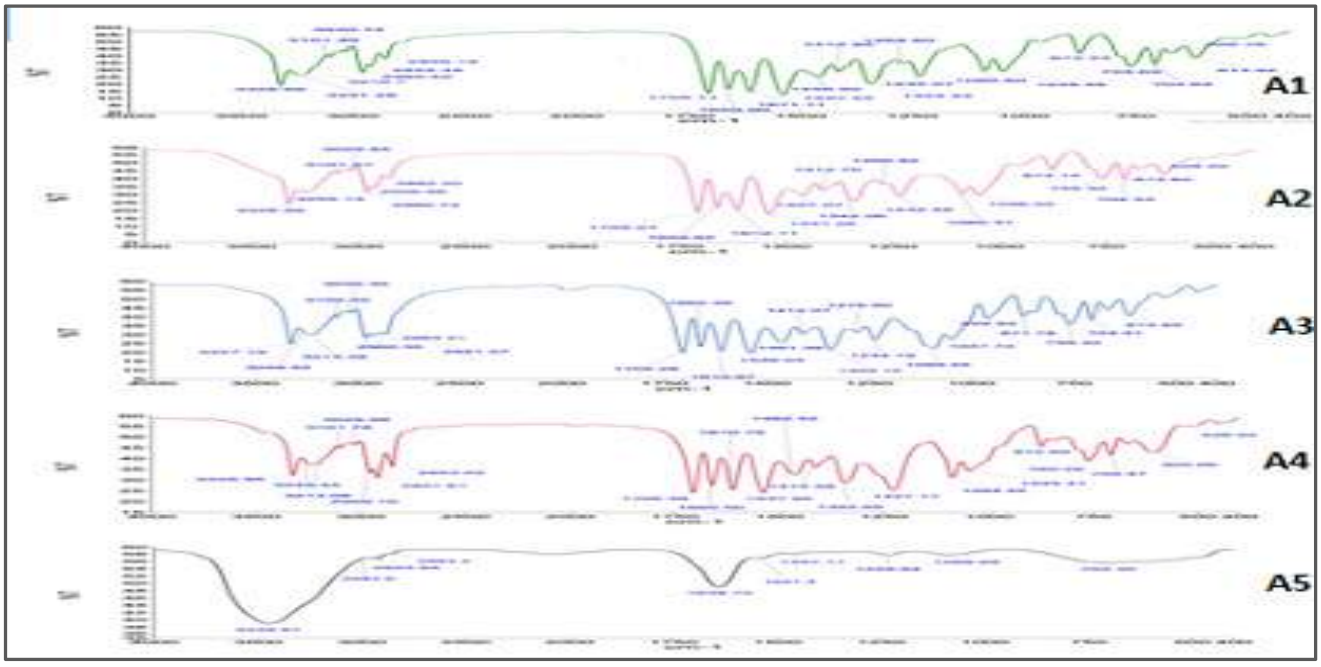

Fig. 1: FITR spectra of A1: Ritonavir, A2, Ritonavir+ HPMC, A3: Ritonavir + Poloxamer, A4: Ritonavir+ SLS, A5: Formulation

Differential scanning calorimeter (DSC)

DSC for pure drug

Melting point of ritonavir was found to be 127.58 °C Spectrum shows a sudden drop in heat flux which was a sharp endothermic peak implying melting of drug having happened. The actual melting point of ritonavir is 121-123 °C. From the spectrum it is observed that the onset of melting starts from 123.13 °C, so we conclude that rito-navir is in pure form (Fig. 2).

DSC for lyophilized formulation

The spectrum indicates (Fig. 3) well defined transition at 156.02 °C. The actual melting point of ritonavir is 121-123 °C. Shift in the peak to exothermic peak of ritonavir is due to change in physical state by use of high pressure homogenizer technique which helps in reduction in particle size. We can conclude that during appliction of thermal energy, the drug has changed its amorphous nature to crystalline nature. A sharp endothermic peak at 55.31 °C is of the polymer.

Optimization by 3 level factorial design

A 3 level factorial design was used in this study. Experimental trials were performed at all 9 possible combinations. The amount of Stabilizer Concentration (X1) and Cycles (X2) were selected as Factors which are shown in Table IV. A statistical model incorporating interactive and polynomial terms was used to evaluate the responses such as particle size and % drug release. The statistic optimization procedure was performed with the help of optimization software such as Design Expert version 11.

Table IV: Design summary for factors and levels

Factor	Name	Units	Type	Low Actual	Medium Actual	High Actual	Low Coded	Medium Coded	High Coded
A	Conc. of stabilizer	mg	Numeric	45	55	65	-1	0	+1
B	Cycles	mins	Numeric	6	7	8	-1	0	+1

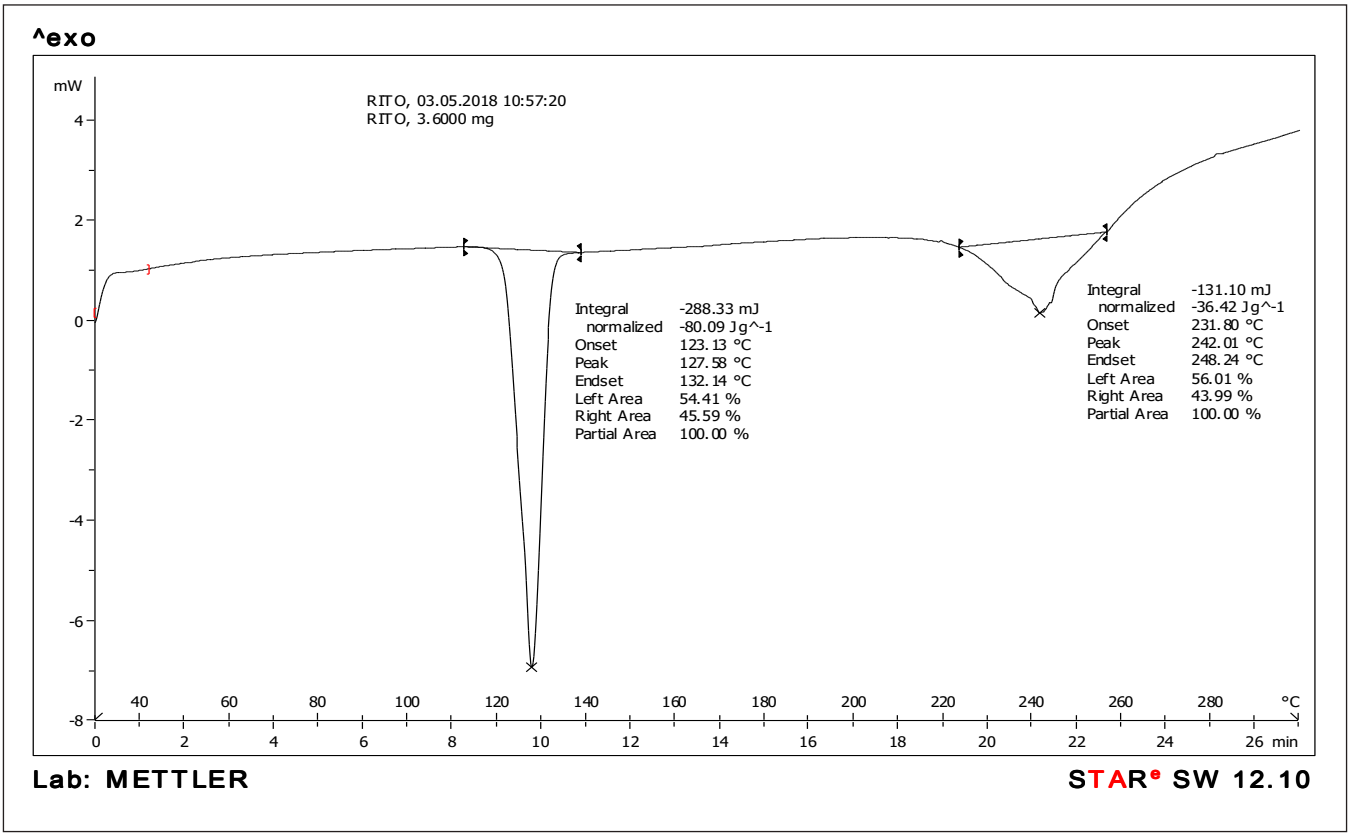

Fig. 2: DSC for pure drug ritonavir

a) Final equation in terms of coded factor for particle size

Particle size = +211.29- 43.25*A-39.37*B

The “Predicted R-Square” of 0.5127 is in reasonable agreement with the Adjusted R-Square” of 0.7418”. "Adequate Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 9.985 indicates an adequate signal. This model can be used to navigate the design space. There is only a 0.73 % chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate that the model terms are significant.

From the above equation, it was concluded that Stabilizer (factor A) and Cycle (factor B) are having an individual effect on the particle size of nanosuspension. ANOVA result of the experimental model for particle size. The Model F-value of 12.49 implies that the model is significant. There is only a 0.73 % chance that an F-value this large could occur due to noise. F test used to check the statistical significance of equation shows that the fitted model is significant at 95 % confidence interval (P-value < 0.05). Values of “Prob> F” less than 0.05 indicate model terms are significant. In this case, A and B are significant model terms. Values greater than 0.1 indicate the model terms are not significant.

The same table also shows the other adequacy measure R^2, adjusted R^2 and predicted R^2 . All the adequacy measures are in reasonable agreement as they indicate significant relationships, as shown in Fig.4.

By observing the contour plot form (Fig. 5) we can say that as the concentration of stabilizer increases, particle size decreases and hence they have an inverse relation.

b) Final equation in terms of coded factor for % drug release

% Drug release = +76.66+7.18*A+4.04*B

From the above equation, it was concluded that Stabilizer (factor A) and Cycle (factor B) are having an

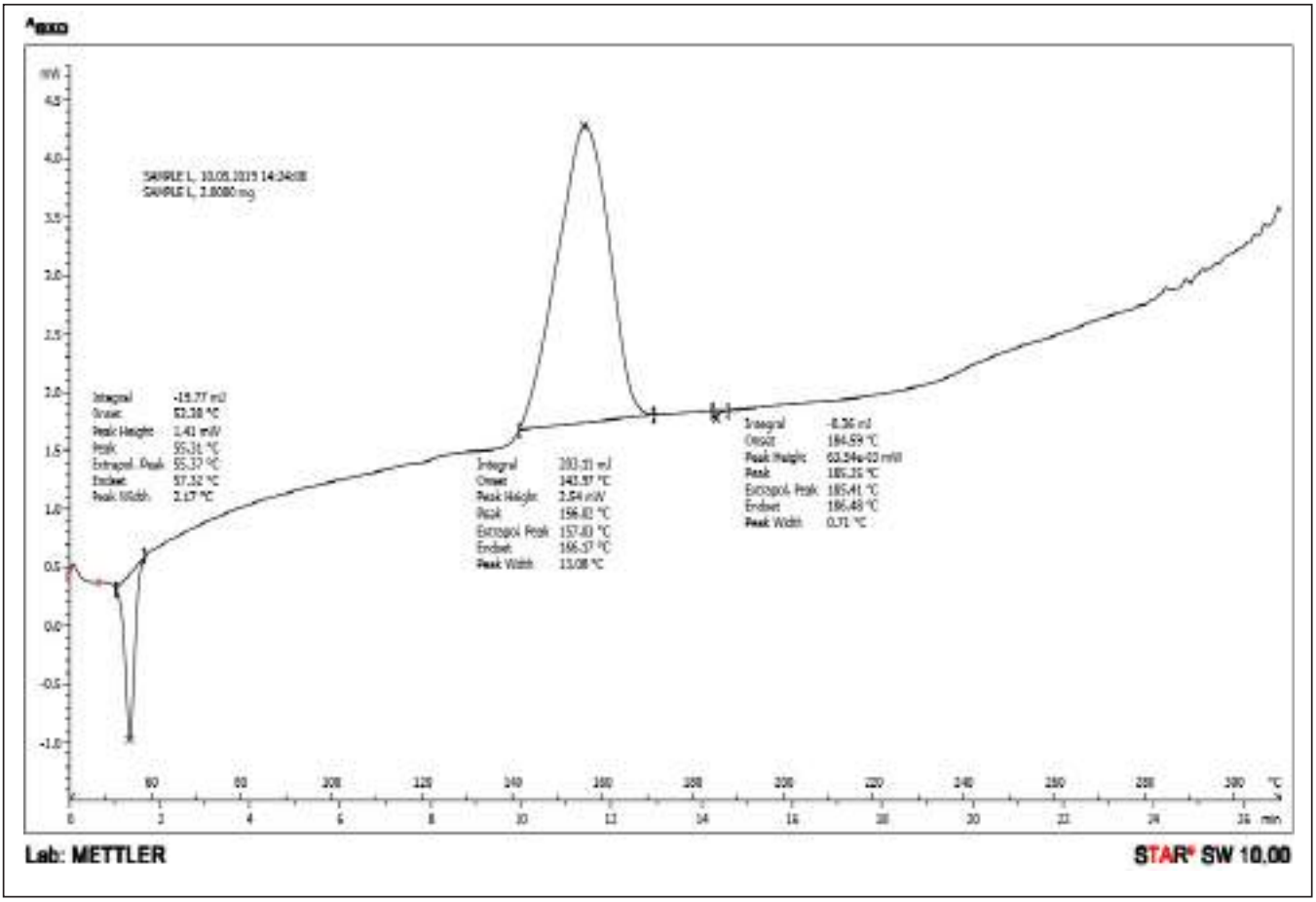

Fig. 3: DSC for lyophilized formulation

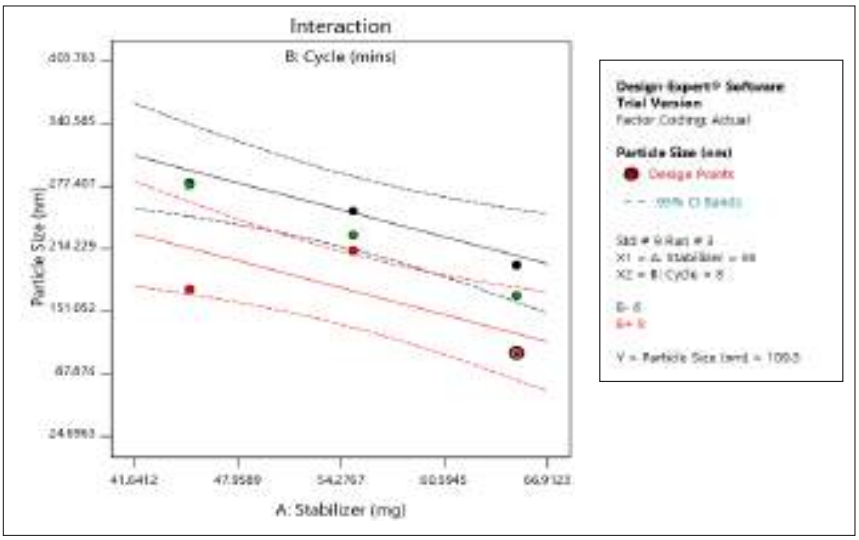

Fig. 4: Effect of experimental variables on the response

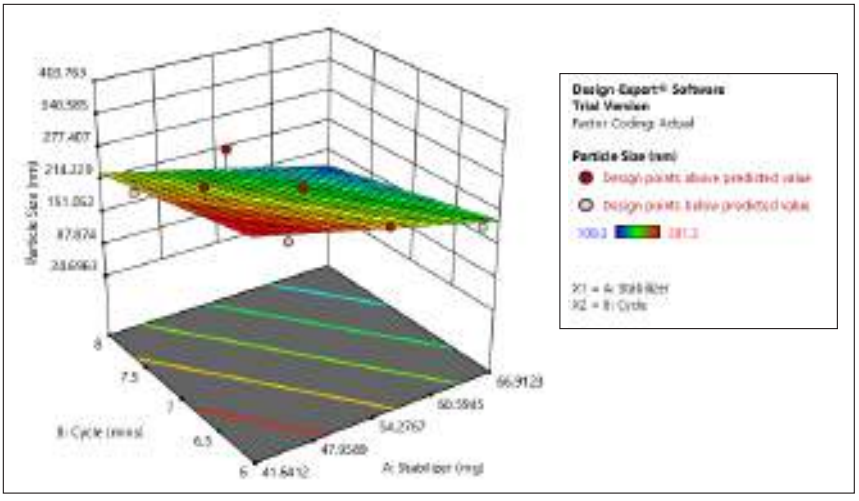

Fig. 5: Three dimentional view of particle size with respect to stabilizer and cycles

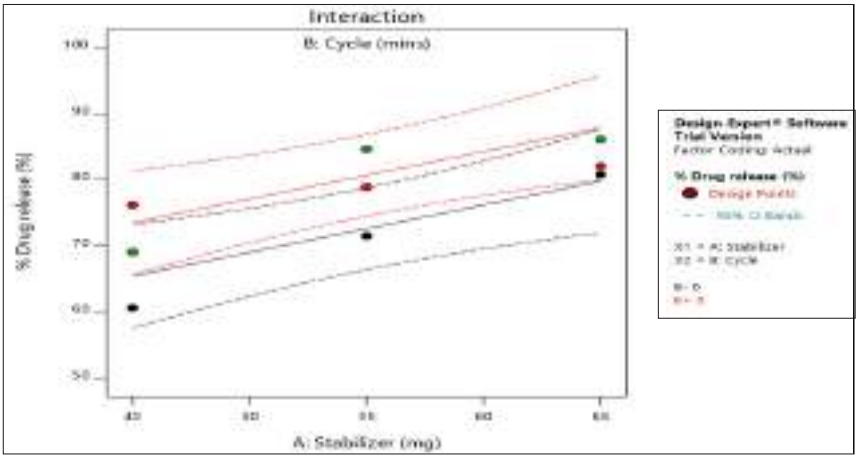

Fig. 6: Interaction plot for stabilizer and cycles on % drug release

individual effect on the % drug release of nanosuspension.

ANOVA result of the experimental model % drug release as a response. The Model F-value of 8.81 implies the model is significant. There is only a 1.64% chance that an F-value this large could occur due to noise. F test used to check the statistical significance of equation shows that the fitted model is significant at 95% confidence interval (P-value < 0.05). Value of "Prob> F" less than 0.05 indicate model terms are significant. In this case A and B are significant model terms. Value greater than 0.1 indicate the model terms are not significant.

The same table also shows the other adequacy measure R^2, adjusted R^2 and predicted R^2 all the adequacy measures are in reasonable agreement as they indicate the significant relationships, as shown in Fig.6.

By observing Fig. 7, we can say that the increase in concentration and runs (cycle) have a direct effect on % drug release.

Particle size and Polydispersity index (PDI) of trial batches

Trial batches were taken for optimization of formulation by high pressure homogenizer. From above Table V, we can say that at the low concentration of surfactant and minimum cycles as well as pressure there was maximum particle size and PDI (T1 batch). But when we increased surfactant concentration and pressure, there was reduction seen in particle size and PDI (T2 batch) towards desire range. Accordingly the batches were taken for further optimization studies.

Particle size and Polydispersity index (PDI)

High pressure homogenizer method shows reduction in particle

Table V: Particle size and PDI of trial batches

Batch No.	Particle size (nm)	Polydispersity index (PDI)
T1	522.1	0.827
T2	363.3	0.595
T3	326	0.545
T4	123	0.399

Table VI: pH of nanosuspension formulation

Sr. No.	Batch no.	pH of nanosuspension
1.	F1	5.4± 0.912
2.	F2	5.2± 0.453
3.	F3	6.0± 0.233
4.	F4	6.6± 0.861
5.	F5	5.4± 0.794
6.	F6	5.5± 0.995
7.	F7	5.8± 0.363
8.	F8	4.8± 0.989
9.	F9	5.0± 0.457

Table VII:*In vitro* diffusion study of nanosuspension

Sr. No.	Batch no.	% Drug release
1.	F1	84.65
2.	F2	69.07
3.	F3	82.07
4.	F4	86.12
5.	F5	76.20
6.	F6	80.79
7.	F7	71.5
8.	F8	60.64
9.	F9	78.91

size. Reduction in particle size occurs on changing in formulation and process parameters. The desired range for brain targeted drug delivery is < 300 nm[18]. Batch F3 shows minimum particle size of 109.3 nm with PDI of 0.391 at the cycle of 8 and pressure of 750 bar. The lower the polydispersity, higher the uniformity of the particle size in the formulation. 0.391 PDI is within desired range and indicates narrow particle size distribution and mono dispersed system. Particle size distribution of nanosuspension is an important parameter than can be used to assess both stability and biopharmaceutical aspects. The small particle sizes of nanosuspension provide large interfacial surface area for absorption or permeation of the drug across biological membrane and thus the relative bioavailability of the incorporated drug is improved as shown in Fig. 8.

Zeta potential analysis of optimized batch of nanosuspension

Standard zeta potential range is ±30 mV for dispersed system but particularly for nanosuspension the range should be ±20 mV[15]. Zeta potential value for optimized batch (F3) was found to be -10mV, which falls within the range, as shown in Fig. 9. There is effect of zeta potential on long term stability of nanosuspension formulations. Combinations of stabilizer, surfactants and polymers were used to get stable nanosuspension. Zeta potential was stabilized by using steric and electrostatic stabilizers in the formulation. Use of combinations of stabilizers improved the stability and there was reduction in particle aggregations.

Determination of pH

Intranasal pH ranges lies in between 4.5-6.5, so there is a need to formulate a dosage form which has pH range similar to intranasal pH, this will not create any irritation and discomfort during delivery of formulation. This pH also increases the absorption of formulation at that site[19]. From the above Table VI, pH of optimized batch was found to be 6.0, which was within range.

Drug content of nanosuspension

Drug content was found to be in range of 87-93 % for nanosuspension formulation. For optimized batch (F3) drug content was found to be 92.15 %. There, somehow drug content is less. This is because of processing in high pressure homogenizer and also due to human error.

In vitro diffusion study

From the diffusion study, it has been observed that the formulation batch F3 with lower particle size shows

Table VIII: *Ex vivo* permeation studyof optimized (F3) nanosuspension formulation

Sr. No	Time (min)	% Drug release
1.	5	48.16327
2.	10	50.03673
3.	15	51.10204
4.	30	62.78367
5.	45	75.33878
6.	60	81.42449

Table IX: Drug release profile (Pure drug, Nanosuspension and Lyophilized powder)

Drug release (%)			
Time (min)	Pure drug	Nano suspension	Lyophilized powder
5	23.67	50.61	51.83
10	24.68	63.11	63.93
15	25.62	68.32	67.1
30	28.12	74.78	76.42
45	34.38	82.9	84.14
60	45.89	85.76	89.86

higher diffusion. Nanosuspension with particle size 109.3 nm shows 82.07 % of drug release in 60 min as shown in Table VII. By high pressure homogenization, the crystalline state of drug is transformed to amorphous, hence there is increase in solubility by reduction in particle size with increase in surface area thus further helps to increases drug diffusion from the membrane and enhancement in bioavailability. The drug release kinetics were fitted into four different models (zero order, first order, Higuchi, Kormeyer-Peppas model). The highest R^2 value was obtained for the Higuchi model[20].

Ex vivo permeation study

Ex vivo study was carried out on goat nasal mucosa by using optimized batch (F3). From the Table VIII. 81.42 % of drug released or permeated through nasal membrane. The drug diffuses across nasal mucosa at faster rate and total percentage diffusion is less than that of drug diffusing through dialysis membrane. Permeation from goat nasal membrane is less and may be due to tightly bound epithelial cells of the nasal mucosa. High permeability across the mucosa is advantageous *in vivo*, considering that mucocilliary clearance rapidly removes the drug away from the nasal mucosa. Smaller the particle size, more will be the permeation through nasal membrane. Hence this will potentially allow nanosuspension to be transported transcellularly through olfactory and trigeminal nerve to the brain via various endocytic pathways of sustentacular or neuronal cells[16].

Histopathological study

Nasal histopathological study was done to ensure that the optimized nanosuspension formulation has no significant damage to nasal mucosa and there is sequential

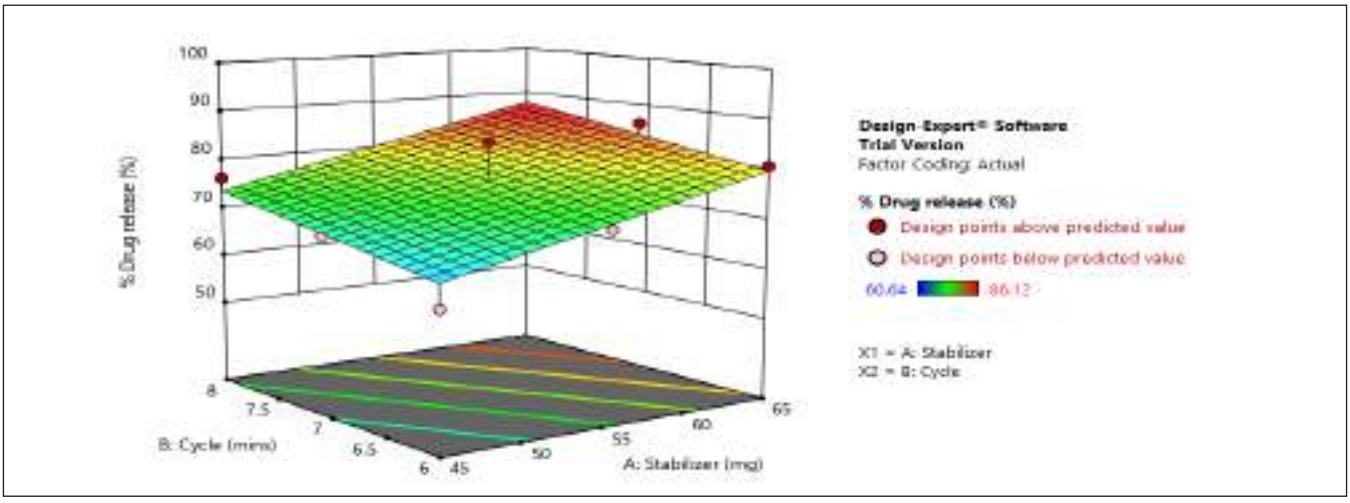

Fig. 7: Three dimentional view of particle size with respect to stabilizer and cycles

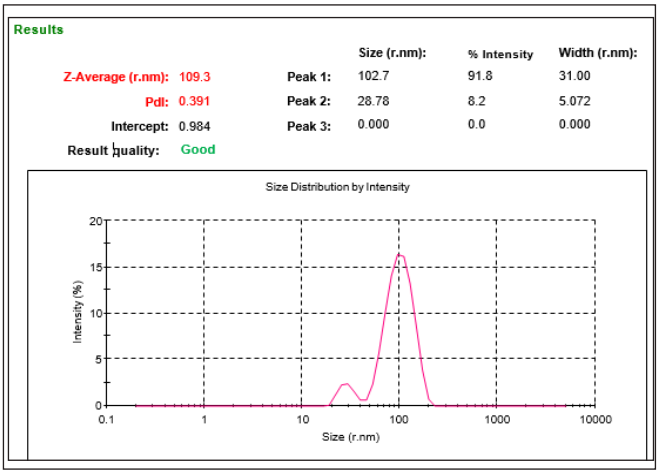

Fig. 8: Particle size and PDI of optimized batch (F3) of nanosuspension

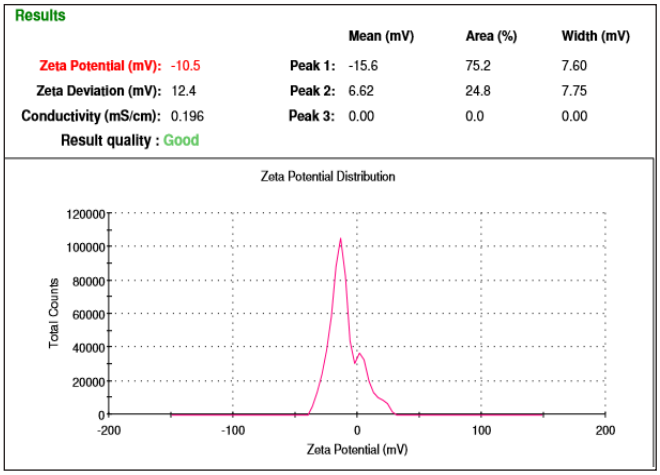

Fig. 9: Zeta potential of optimized batch (F3) of nanosuspension

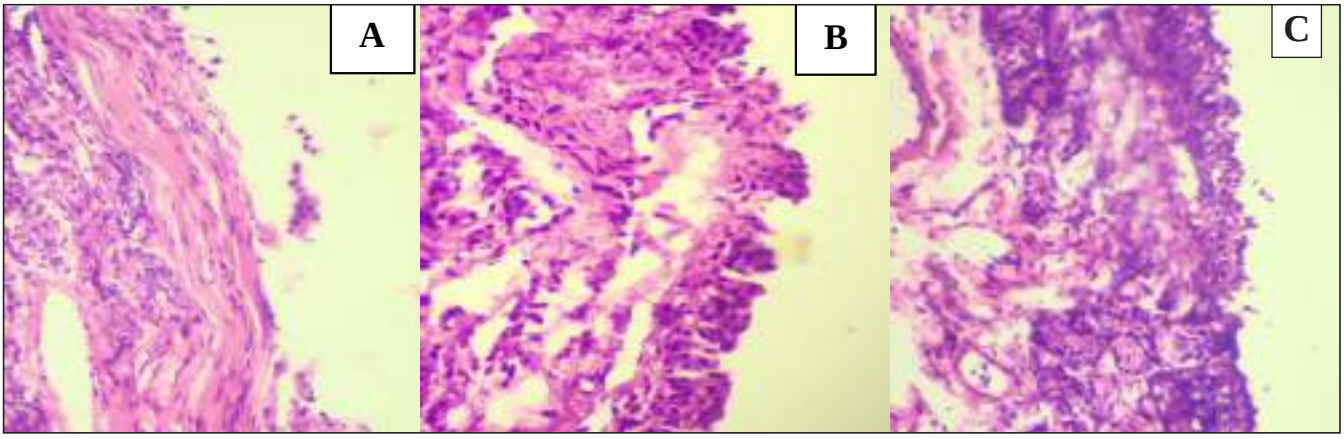

Fig. 10: Histopathological study of nanosuspension
A: Positive control (70% iso-propyl alcohol); B: Negative control (pH 6.4 phosphate buffer) and C: Formulation

arrangement of epithelial cells. As shown in Fig. 10 A: the positive control (70 % isopropyl alcohol) shows damage to the epithelium cell i.e. they were detached from the membrane and also the regular sequence of the epithelium cells were distorted as compared to negative control sample (Fig.10B). There was no significant destructive effect seen in epithelial cells after being treated by nanosuspension. So there was no damage to the mucosal cells (epithelial cells) as seen in Fig.10C sample; this confirmed that the formulation is safe and non-irritant on intranasal administration[16].

Lyophilization of optimized batch (F3)

Lyophilization was done for improving the stability issue of nanosuspension and to improve long term storage of formulation. Mannitol was selected as cryoprotectant as it decreases the osmotic activity of water and crystallization and favors the glassy state of the frozen sample, thus preventing aggregation[21]. Mannitol is freely soluble in nanosuspension. Cryoprotectant is used to protect nanosuspension from particle aggregation. As the HPMC provides steric stability and SLS provides electrostatic stability, so this provides better zeta potential value in combination.

The particle size and PDI of reconstituted lyophilized nanosuspension in distilled water was analysed by Malvern zeta sizer. Particle size of lyophilized product was found to be 123.4 nm and PDI 0.461. Compared to nano- suspension, there is an increase in particle size, this is due to mannitol or may be due to storage of nanosuspension before going for lyophilization. But from the above particle size, it seems that the size lies within limits. Therefore, no significant interruption is seen in drug release.

Table X: Stability study for nanosuspension

Temperature and humidity	Parameter	Months	
		0	1
40 °C ± 2 °C; 75 % ± 5 % RH	Drug content (%) F3	93.57 ± 1.020	92.05 ± 1.960
	Drug release (%) F3	86.91 ± 0.186	87.43 ± 0.679
Room temperature	Drug content (%) F3	93.57 ± 1.020	91.07 ± 1.222
	Drug release (%) F3	86.91 ± 0.186	85.84 ± 0.285

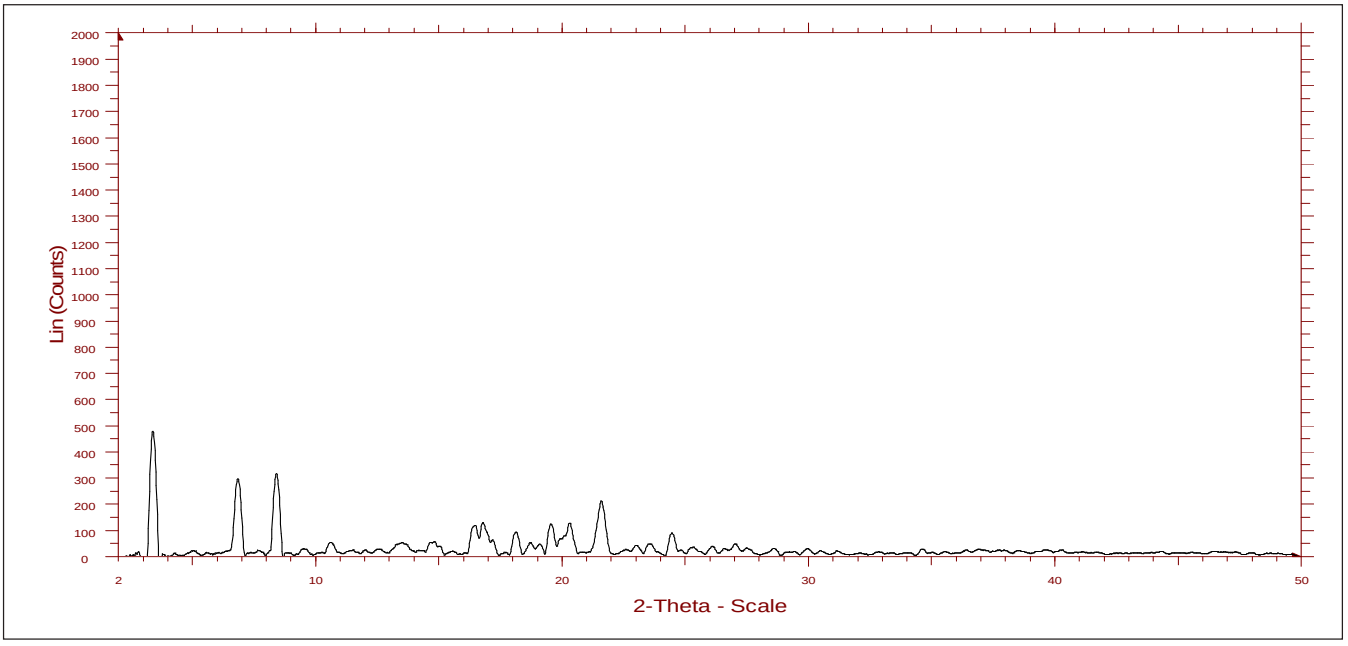

Fig. 11: XRD of untreated drug (ritonavir)

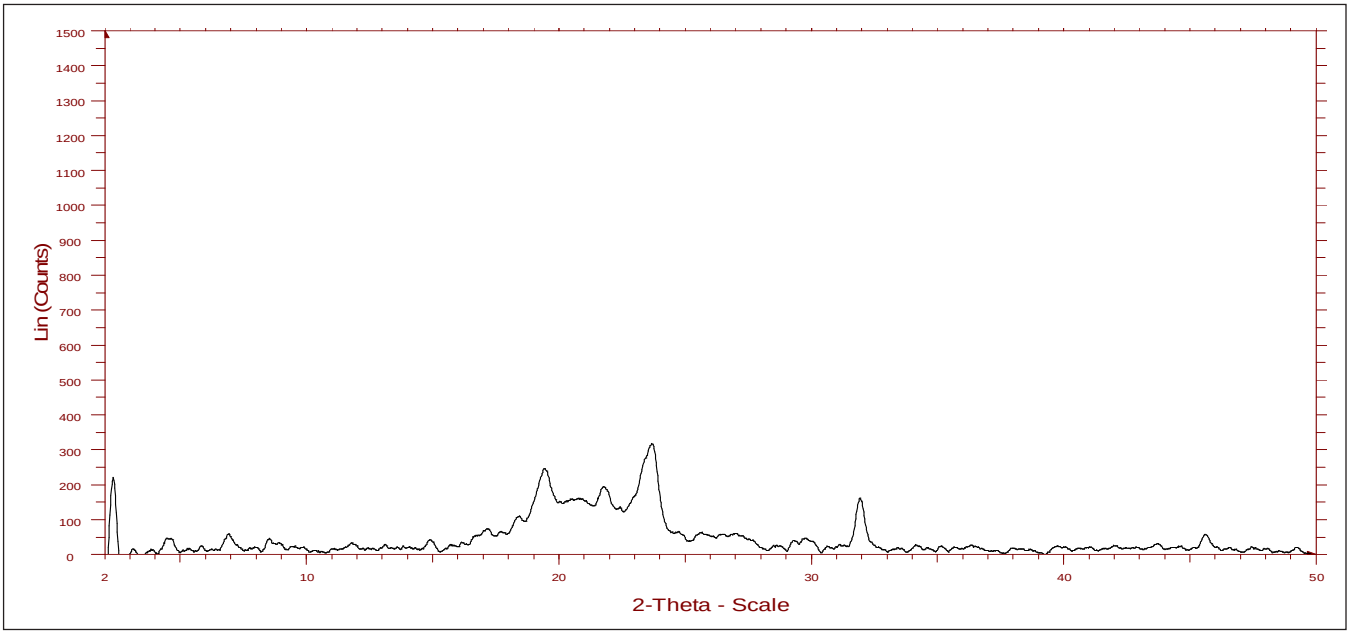

Fig. 12: XRD of lyophilized nanosuspension

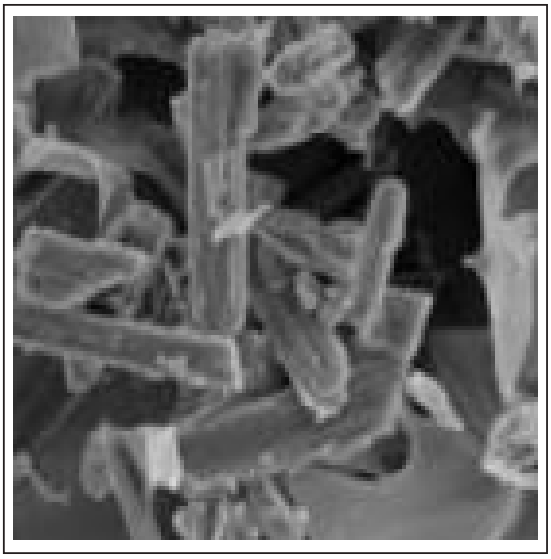

Fig. 13: SEM of ritonavir

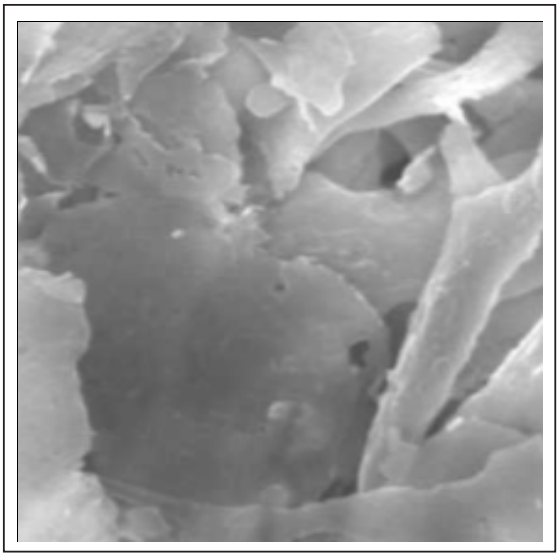

Fig. 14: SEM of lyophilized nanosuspension

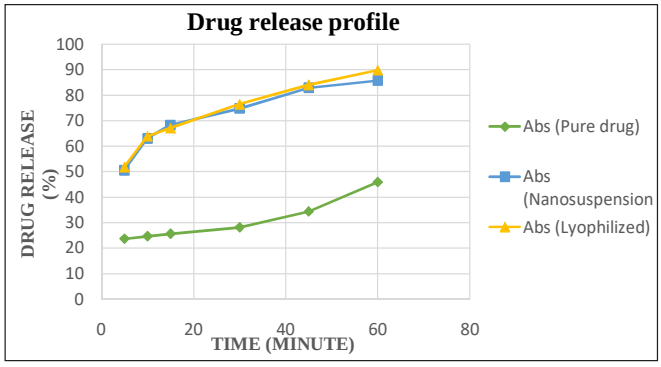

Fig. 15: Drug release profile (pure drug, nanosuspension and lyophilized powder)

X-ray diffraction (XRD)

XRD was carried out to know the crystalline structure of ritonavir in the preparation of nanosuspension[13]. Diffractogram of ritonavir (API) and lyophilized nanosuspension was recorded as shown in Fig.11 and 12. The untreated ritonavir (pure drug) exhibited a crystalline state with sharp peaks. Lyophilization and high pressure homogenization step effected the crystalline structure of drug. Lyophilized nanosuspension pattern shows partially amorphous nature of ritonavir in our study.

Scanning electron microscopy (SEM)

Morphology of ritonavir nanosuspension was evaluated by SEM (Figs. 13 and 14). Initial shape of ritonavir (pure drug) was rod-like and rough[15]. After HPMC, Poloxamer and SLS were added to the formulation, the rough surface seemed smooth. Changes in morphology of the particle were due to high pressure homogenization technique.

Drug release profile

The comparison study between pure drug, nanosuspension and lyophilized product was done for drug release. This comparison study was done because there is no nasal formulation containing ritonavir drug available in the market for comparative study. From Table IX, we can say that drug release form pure drug is 45.89 % which was less for therapeutic effect. When compared to nanosuspension and lyophilized powder, we found that drug release has been increased in lyophilized formulation i.e. 85.76 % and 89.86 %, respectively, as shown in Fig. 15. This is may be due to concentrated formulation formed as well as stable formulation when lyophilization process is done. Lyophilized product helps in proper dose calculation for further redispersion.

Stability study

For stability, ICH guidelines were followed. Effect of temperature and humidity on formulation was carried out on optimized formulation (F3). Parameters for stability were kept as 40 °C ± 2 °C and 75 % ± 5 % relative humidity in stability chamber for one month. Formulation were analyzed for drug content (%) and drug release (%). Before keeping the formulation in stability chamber, Initial analysis were done for drug content (%) and drug release (%). Then the same formulations were kept in stability chamber for suggested temperature and humidity conditions for one month. After completion of one month, the formulations were analyzed for drug content (%) and drug release (%) as given in Table X. From the study we conclude that there were no major changes in the formulation after storage conditions on physical parameters of optimized formulations.

CONCLUSION

From the above results, we can conclude that high pressure homogenizer is an effective instrument to reduce the particle size followed by high speed homogenizer. Particle size and zeta potential are the important parameters to be considered for nanosuspension development, so the selection of surfactants, drugs and media is very important and crucial. Appropriate selection of excipients, drug and media can give desired particle size and zeta potential, which ultimately affects the stability and bioavailability of the formulation. The lower the particle size, the more is the bioavailability brain targeting then becomes possible as the drug will pass efficiently through the transcellular route. Further, this drug can be used as model drug in combination therapy.

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Research article

Nose to brain delivery of nanosuspensions with first line antiviral agents is alternative treatment option to Neuro-AIDS treatment

Smita P. Kakad *, Trupti D. Gangurde, Sanjay J. Kshirsagar, Vaishali G. Mundhe

Department of Pharmaceutics, MET Institute of Pharmacy, Nashik, Maharashtra, India

HIGHLIGHTS

- Neuro-AIDS is current treatment challenge in chronic HIV patients.
- Nanosuspension formulated using first line antiretroviral drug Ritonavir and Lopinavir.
- High pressure homogenizer is best method for nanosuspension preparation.
- Prepared nanosuspension were optimized for nasal drug delivery.

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ABSTRACT

Intranasal drug delivery is one of the uprising areas of the research in targeting drug to the brain. Nose to brain drug delivery follows the olfactory pathway and purportedly known to be more efficient to deliver neuro-therapeutics to the brain by circumventing the BBB and thereby increasing bioavailability of drugs in the brain. The advantage of this method is non-invasiveness, rapid onset of action and helps to achieve site specific delivery. In this research work nanosuspension were prepared using combination of antiretroviral agents for Neuro-AIDS treatment. Nanosuspensions were prepared by high-speed homogenization, wet milling and high-pressure homogenization techniques. Formulations were analysed by SEM, FTIR, and DSC. Morphology and stability analysis was done by analysing zeta potential, particle size, and PDI. *Ex-vivo* diffusion study and histo-pathological analysis was performed using goat nasal mucosa. High pressure homogenization was found to be best technique for formulation of nanosuspension. Antiviral drugs could be delivered successfully by optimizing nasal dosage form.

1. Introduction

Human immunodeficiency infection (HIV) affects over 36.7 million people worldwide, according to the UNAIDS report 2017, and 1.8 million people were newly infected with HIV in 2016. Since the emergence of the epidemic AIDS-related illnesses has killed over 35 million people globally [1]. It is well fact that HIV has a wrecking impact on the human immune system which results in AIDS and furthers various opportunistic diseases that cause the death of the patient. The initial symptoms of HIV infection include immune suppression, but it also has peculiar neurological manifestations targeting the Central Nervous System (CNS). When HIV enters the brain, it remains there for an extended period of time, presumably until the individual dies. HIV-associated dementia (HAD) is characterized by neurological, motor, and cognitive abnormalities when the virus enters the brain directly [2]. Survival of HIV-positive persons has been improved due to the application of increasingly powerful and highly active antiretroviral agents (HAART). Combination therapies (cART) have endeavored for fast and efficacious treatment. Various drug delivery approaches are invented to overcome the drawback associated with antiretroviral therapy [3, 4]. Even though the use of increasingly intense and dynamic antiretroviral agents such as HIV protease inhibitors (PIs), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitor (NNRTIs), and viral entry inhibitors has improved the survival of HIV-infected individuals, symptoms of neuro-aids persist in 30–50 percent of the HIV population [5]. Antiretroviral therapy (ART) drugs that can cross the blood-brain barrier (BBB) can help reduce viral load, limit the virus's development in the brain parenchyma, and improve neurocognitive impairment. Infected

* Corresponding author.
E-mail address: smitadarade87@gmail.com (S.P. Kakad).

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cells, on the other hand, are unaffected by HIV-1 transcription and translation from viral genomes. All of the patients who have been treated and have complete viral suppression have low-level viremia. To reduce HIV-1 transcription and residual viruses, new treatment drugs are required. Many nanoparticulate preparations are developed nowadays to directly reach the CNS [5]. Nano emulsion, nanosuspension, micellar carriers, nanoparticles, liposomes, and other formulation boosts brain permeability, hence lowering virus load. BCS Class IV medicines Ritonavir (RTV) and Lopinavir (LPV) can be made into nanosuspension for the nose to brain delivery to improve solubility and permeability with the application of surfactants. A colloidal dispersion of drug particles in an aqueous phase with particle sizes smaller than 1 micron is known as nanosuspension. The drug's solubility is improved in both the aqueous and lipid phases [6]. The primary goal of this study is to create nanosuspensions of the antiretroviral agents Ritonavir and Lopinavir using appropriate methods such as wet milling, high-speed homogenization, and high-pressure homogenization. RTV and LPV are used as double-boosted PIs in this study. In combination therapy with two PIs, a tiny dose of RTV is administered as a booster [7]. A combination of RTV and LPV formulation was made by combining the two suspensions.

2. Materials and methods

Ritonavir, Lopinavir, HPMC 3CPS (Methocel E3), Poloxamer 407 (Pluronic F127) were received as gift samples from Glenmark Pharmaceuticals Ltd R & D Centre (Sinnar, Nashik). Sodium Lauryl sulfate purchased from Thomas Baker Chemicals Pvt. Limited.

2.1. High speed homogenization

A high-speed homogenizer (IKAT25 Ultra Turrax®) was used to make the nanosuspension [8]. HPMC, Poloxamer 407, and Sodium lauryl sulfate were accurately weighed and dissolved in water on a magnetic stirrer [9]. This solution was then subjected to high-speed homogenization. The drugs (RTV and LPV) were disseminated gently into the solution and homogenized for 90 min at 20,000 rpm.

2.2. Wet milling technique

Nanosuspension was produced by using Dyna mill® (Glenmark Pharmaceuticals Ltd R & D Centre, Nashik). Coarse pre-suspension of drug and excipients (HPMC 3 CPS, Pluronic F127, Sodium lauryl sulfate) was prepared using high-speed homogenization. Further, this suspension of a drug was fed into the Dyna mill containing small beads of zirconium oxide with a size of 2.3 mm. The grinding chamber and the beads rotate at high shearing speed [8]. The drug particles bombard over the sides of the grinding chamber. The force of friction and impaction of particles in the chamber results in particle size reduction [6, 10].

2.3. High pressure homogenization

To begin, the micronized drug particles were pre-suspended in a surfactant and polymer solution using a high-speed homogenization technique. Then the coarse micronized pre-suspension had been subjected to high-pressure homogenization [11] at different pressure/cycles (each cycle 90 s/min). For particle. size reduction, many cycles were used [12, 13].

2.4. Particle size and zeta potential determination

The particle size of the developed formulations was measured to asses if any difference in particle size depending on the method used to make the nanosuspension. The formulation was diluted in water at 1:100 ratios and particle size were measured. Using a Zeta sizer ZS 90 (Malvern Instrument Ltd., UK) at a 90° angle, fluctuations in light scattering (due to Brownian motion) are identified in photon correlation spectroscopy [14]. The batches with lower particle sizes were selected for further characterization like zeta potential determination. The Nanosuspension was deposited in a folded capillary cell and then into the analyzing chamber of a Zetasizer ZS 90 (Malvern Instrument Ltd., UK), which uses the Electrophoretic Light Scattering (ELS) technique to detect zeta potential [15].

2.5. pH determination

At 25 °C, the pH of the formulation was determined using a pH meter (Systronic 362 mpH system, India). For pH testing, 5 mL of sample was placed in a beaker, and measurements were taken in triplicate [16].

2.6. Drug content determination

An aliquot (1 ml) was pipette out and dissolved completely in Methanol. This solution was further filtered with 0.45 μm filter paper. The samples were examined with a UV spectrophotometer at lambda max 238 nm for RTV and 259 nm for LPV [15]. Eqs. (1) and (2)) were used to calculate the total drug content (TDC) and percent TDC· [16, 17].

$$\mathrm{TDC} = \frac{(\text{Total Volume} \times \text{Drug amount in aliquot})}{\text{Volume of aliquot}} \times 100 \quad (1)$$

$$\%\ \mathrm{TDC} = \frac{\mathrm{TDC}}{TAD} \times 100 \quad (2)$$

Where, TAD is Total amount of drug.

2.7. FTIR analysis

The FTIR spectrum of drugs, polymer, and surfactants were recorded by FTIR spectrophotometer (Carry 630 FTIR, Agilent Tech.) In an FTIR spectrophotometer, all samples were scanned between wave numbers 500 and 4000 cm^{-1} [18]. FTIR was performed to analyze the inactivation or loss of the drug due to its conversion to a less therapeutically efficacious physical or chemical form. The compatibility of the drug-drug and drug- excipient and excipient- excipient is checked. Vials containing samples were sealed and maintained for 21 days at room temperature before being analyzed for any differences in the IR spectra [19].

2.8. Saturation solubility

The excess amount of the pure drug or equivalent drug in the formulation was added to 25 mL of each vehicle 0.1N HCl, distilled water, and phosphate buffer solution (PBS 6.5) in a conical flask and agitated for 48 h at room temperature in an incubator orbital shaker (Remi Ekectrotechnik Limited). The equilibrated samples were then taken out of the shaker and centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the membrane filter (0.45 m) was used to filter the filtrate. After suitable dilution with 0.1N HCl, Water, and PBS (6.5), the concentration of the medication in the supernatant was determined using a UV spectrophotometer (UV 1700, Shimadzu, Japan) at 238 nm and 259 nm for RTV and LPV, respectively. The solubility of the drug in milligrammes per millilitre (mg/mL) was calculated. For formulation, the solubility was calculated in PBS (6.5) [20].

2.9. In- vitro diffusion study

Nanosuspension having drug amounts equivalent to 5 mg and 16 mg of RTV and LPV respectively was taken and poured into a Dialysis bag (MWCO 12,000 g/mol; Himedia Laboratories Pvt. Ltd.) the ends of the bag were sealed to avoid leakage of the formulation. The dialysis bag was then plunged into the diffusion medium, which was constantly swirled at 100 rpm at 37 °C. At regular intervals, sample aliquots of the same volume were extracted and the volume was replaced with diffusion medium.

The samples were evaluated by UV spectrophotometer (UV 1700, Shimadzu, Japan)to determine drug (RTV and LPV)diffused at specific time intervals [21, 22].

2.10. Ex-vivo permeation study

An ex-vivo investigation was conducted using goat nasal mucosa that had been freshly brought from a local slaughterhouse. The nasal tissue sample was fixed between the receiver and donor chamber of Franz diffusion cells of 12.5 ml capacity. The receiver chamber was pre-incubated for 20 min after being filled with PBS6.5 and held at 37 °C. The donor chamber was filled with the best possible formula (A3-RTV and B2- LPV). After aliquots were removed from the receiver chamber, PBS was replaced. A UV spectrophotometer was used to assess the amount of medication in PBS (UV 1700, Shimadzu, Japan) [23, 24, 25].

2.11. Histopathological studies

Histopathological research was conducted to see if the RTV and LPV formulations caused any harm to the nasal mucosa. Freshly excised goat nasal mucosa was collected from the local slaughter house; it was well cleaned and cut into nine symmetrical pieces, and transferred to PBS6.5. All the nine pieces were treated with RTV and LPV formulation, positive control (70% Isopropyl Alcohol), and negative control (PBS 6.5) [26]. After an hour, all tissues were properly cleaned in PBS and placed in 10% formalin solution for 24 h. The formalin solution samples were treated with 70% ethanol and stored at 4 °C for dehydration after 24 h. The dehydrated sections were then embedded in agar and paraffin block and a thin section was cut using a microtome. The slides were prepared for observation in the light of an optical microscope. Structural changes in the mucosa were observed and noted [27].

2.12. Differential scanning calorimetry (DSC)

DSC of drugs and formulations were performed on Mettler Toledo India Pvt. Ltd. (Switzerland) to study physical state characterization [28]. The liquid formulation DSC measurements were done on a TA Instruments Q20. The samples were dried, and a 5 mg sample was placed in an aluminum pan, which was hermetically sealed with an aluminum lid. The system was purged with nitrogen gas at a flow rate of 50 mL/min, and heated at a rate of 10° C/min [29].

2.13. Scanning electron microscopy

Particle morphology was observed using scanning electron microscopy (SEM) Carl Zeiss (Germany). A drop of the sample was dried in an oven (Dolphin, Mumbai)and firmed on an SEM stub with the assistance of double-sided adhesive tape. At a 15 Kv accelerating voltage, a scanning electron microscope with a secondary electron detector was employed to obtain digital photographs of the materials [14, 30].

2.14. Stability study

Temperature and humidity have an impact on formulation it was tested using the approach outlined in the ICH stability testing standards. The samples were sealed in borosilicate glass vials and stored in a stability chamber at 40 °C 2 °C/75 percent RH 5% in stability chamber for one month (Remi, Electrolab, India). Drug content (percentage), drug release (percentage), and FTIR analyses were performed [14,31–33].

3. Results and discussion

3.1. Solubility study

From the experimental work, the solubility was noticed to be 0.0080 ± 0.36 mg/mL for RTV and 0.0166 ± 0.21 mg/mL for LPV which are close to the reference values. Nano sizing has resulted in a 10-fold increase in solubility of APIs [34]. The results show that particle size reduction increases the drug's solubility and can also boost its bioavailability [11]. Reduced particle size increases the surface area of the drug particle, which improves solubility. (Li X et al. 2009) [35]. The surfactants employed in the formulation help to lower the drug's hydrophobicity, which aids in its solubilization [24].

3.2. FTIR analysis

FTIR stacked spectra (Figures 1 and 2) showed that principle peaks of RTV at 3449.67 (cm^{-1}), 2923.93(cm^{-1}), 2864.4(cm^{-1}), 1638(cm^{-1}), 1457.11(cm^{-1}), 1229.64(cm^{-1}), 1089.69 (cm^{-1}) for –N\H stretching amide, H-bonded acid within the molecule, C–H Stretch Alkane, Methyl C–H asymmetrical bend Aromatic Ethers, Methyl C–H asymmetrical bend, Aryl-O-Stretch, C–N stretching [30] respectively and principle peaks of LPV at 3437.02 cm^{-1}, 1657.26 cm^{-1}, 1237.02 cm^{-1}, 1347 cm^{-1}, 2076.14 cm^{-1} functional group Amines N–H Stretching Vibration, amide C=O stretching, Imines = N–H, NO_2 stretching nitro compound, alcohol are present [24]. The principle peaks for drug and excipients are retained, indicating that no chemical changes have occurred in the drug. As a result, drug excipients are discovered to be appropriate for one another.

3.3. Differential scanning calorimetry

RTV DSC thermogram, showed a sudden drop in heat flux which depicts a sharp endothermic process. A well-defined transition is observed 123–132 °C (Centered on a peak of 127 °C) and RTV formulation DSC thermogram indicates well defined exothermic transition at 109–123 °C (Peak centered on 123.59 °C) [36]. A minor endothermic peak ranging from 64.89 °C to 84.54 °C (centered on a peak of 78.78 °C) and a sharp endothermic peak ranging from 90.76 °C to 102.25 °C (centered on a peak of 97.07 °C) were seen on the LPV DSC thermogram. DSC analysis of the LPV formulation revealed a well-defined exothermic transition between 65.04 and 120.33 °C (peak at 114.2 °C) [37]. The above results show that endothermic peaks are in the melting temperatures ranges, indicating that both pharmaceuticals are crystalline. These endothermic peaks have been converted to exothermic, which possibly as a result of high-pressure homogenization of both medicines [38]. During the application of thermal energy, the drug in the formulation has changed its amorphous nature to crystalline. The change in physical state because of the high-pressure homogenization process, which aids in particle size reduction and may lead to drug amorphism.

3.4. Particle size and PDI

Variation in particle size were observed as we change method of preparation as shown in Table 1. Table 1 shows different particle size with different method of preparation of nanosuspension. The required particle size distribution development nose to brain delivery was up to 100–200 nm. Trials batches were taken with high-speed homogenization, wet milling, and high-pressure homogenization method. The particle size was determined using wet milling technique was greater and with high pressure, homogenization was lowest [11]. The smaller the particle size the larger is the surface area and the more the absorption of the drug. As the area for absorption or permeation increases relative bioavailability is enhanced [39]. The lower the polydispersity index (PDI), the higher the uniformity of the particle size in the formulation. Particle size and PDI results are mentioned in Table 2. Results indicate that A3 and B2 Nanosuspension formulation approaches mono dispersed stable systems with smaller particle sizes. The higher stability and optical clarity of nanosuspension can be the result of low particle size [37]. The nanosuspension particle size is also affected by the type and concentration of surfactants used [12]. An optimum increase in the concentration

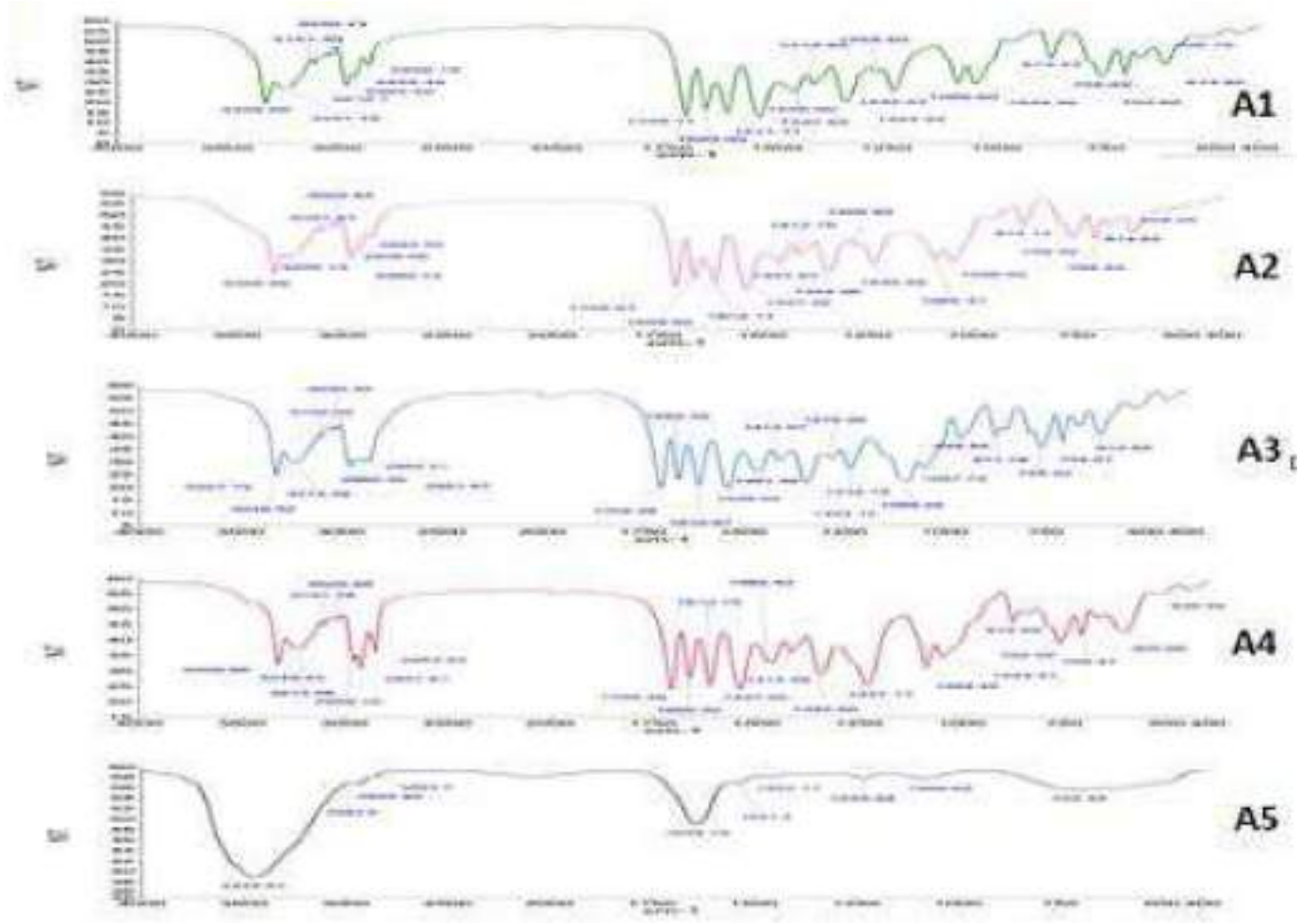

Figure 1. FTIR stacked spectra (Ritonavir-A1: Ritonavir, A2: Ritonavir + HPMC, A3: Ritonavir + Poloxamer, A4: Ritonavir + SLS, A5: Formulation.).

of surfactants helps to stabilize the obtained particle size in nanosuspension. Particle size results of optimized batches are mentioned in Figure 3(A3 Ritonavir) and Figure 4(B2 Lopinavir.).

3.5. Zeta potential measurement

The stabilizers HPMC (3CPS), Poloxamer 407, and Sodium lauryl sulfate helps in stabilizing the Nanosuspension. HPMC is a polymer used to prevent the settling of particles (Duro R et al. 1998) by increasing viscosity of the formulation and particle size decrease (Verma Set al. 2009), Poloxamer 407 is a non-ionic surfactant used for steric stabilization [40], and Sodium lauryl sulfate is an anionic surfactant used for electrostatic stabilization [41]. When compared to the negative or neutral charged particles, the positively charged particles are rapidly attracted to the mucosal membrane. Although this type of electrostatic interaction could improve the drug's bioavailability it may produce irritation and/or other toxicity in the membranes. The particle charge has an impact on the pharmacokinetics of the drug in the body. A stable dispersion of the particles could be obtained when zeta potential values are above ±30 mV [42]. Zeta potential values for RTV and LPV were discovered to be −22.7 and −19.1 respectively as mentioned in Table 2. This is as a result of the existence of repulsive forces acting between particles. Lower zeta potential values may lead to inter particulate interaction and unstable formulations could be formed [19]. However, according to research, combining stabilizers can produce a stable nanosuspension. Zeta potential results are mentioned in Figures 5 and 6 respectively for Ritonavir and Lopinavir.

3.6. pH determination

Intranasal solutions should ideally have a pH of 4.5–6.5 to avoid discomfort or irritation the mucosa of the nasal mucosa caused by acidic pH and bacteria development caused by basic pH. If the pH of the formulation is 4.5–6.5 the drug absorption from the formulation is more [43]. The changes in pH due to the changes in surfactant concentration are reported in Table 2.

3.7. Drug content

The medication concentration of all manufactured intranasal nanosuspension formulations was discovered to range between 82 and 92%. Uniformity of content was found in all formulations. The loss of drug possibly as a result of processing of formulation on high-pressure homogenization and the remaining losses can be during recovery of batches. Drug content of batches is mentioned in Table 2.

3.8. In vitro drug release

A dialysis membrane (MWCO 12,000 g/mol; Himedia Laboratories Pvt. Ltd.) was employed in the drug release investigation. According to the findings, the formulations A3 and B2 with smaller particle sizes have higher diffusion. RTV with 125.5 nm particle size was observed with 70.185 ± 0.196% drug release and LPV with 82.79 nm particle size was observed with 84.457 ± 1.020% drug release for 2 h (Table 2). The

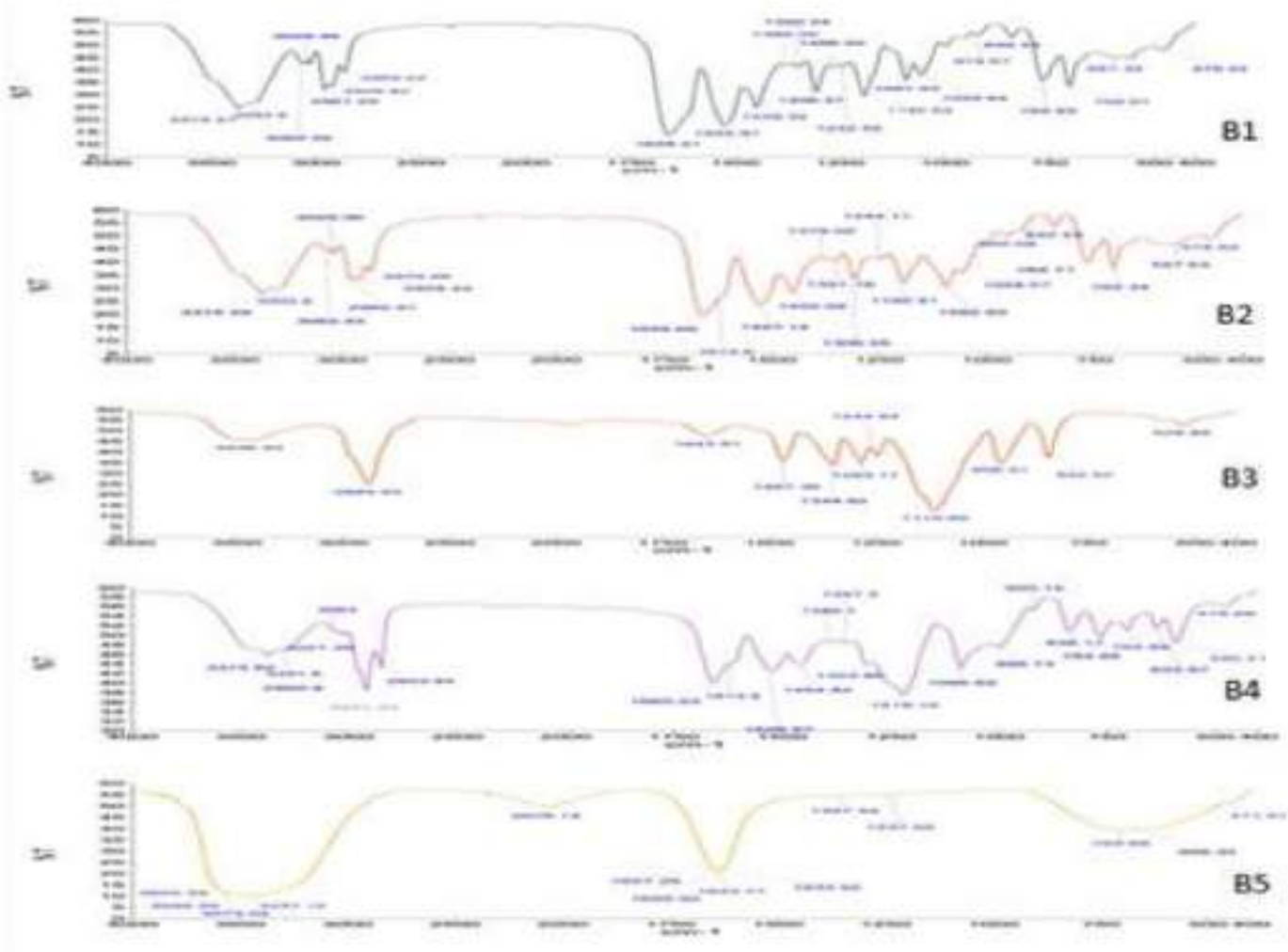

Figure 2. FTIR stacked spectra (Lopinavir. B1: Lopinavir, B2, Lopinavir + HPMC, B3: Lopinavir + Poloxamer, B4: Lopinavir + SLS, B5: Formulation).

Table 1. Comparison of particle size obtained with different techniques.

Sr. no.	Wet milling	High speed homogenization	High pressure homogenization
Ritonavir	4828 ± 4.51 nm	1069 ± 2.11 nm	125.5 ± 0.53 nm
Lopinavir	3133 ± 3.62 nm	859.5 ± 2.54 nm	82.79 ± 0.25 nm

crystalline structure of the medication is converted to amorphous as a consequence high-pressure homogenization, increasing solubility, and increasing surface area. These modifications to the medical-aid increase diffusion as well as bioavailability [44]. Four alternative models were employed to make it fit drug release kinetics (Zero order, First order, Higuchi and Korsmeyer-Peppas model). The highest regression coefficient R^2 value was obtained for the Higuchi model [29]. Drug release results are mentioned in Table 2.

3.9. Ex-vivo permeation study

Ex-vivo permeation through goat nasal mucosa was performed on the batches A3 and B2. The study reveals that 65.606 ± 0.122% and 78.255 ± 0.554% drug was released from RTV and LPV formulation respectively. The drug diffused from nanosuspension across nasal mucosa is at a faster rate [27] and the total percentage diffusion is less than the drug spread throughout the room dialysis membrane. This can be because of the tightly bound epithelial cells of the nasal mucosa. The smaller particle

Table 2. Characterization of Nanosuspension (Prepared with high pressure homogenization method).

Batch no.	Particle size Nm	PDI	pH	Drug content	Drug release
A3	125.5 ± 0.53	0.361 ± 0.01	4.6 ± 0.2	84.769 ± 3.0483	70.185 ± 0. 196
A4	158 ± 0.34	0.326 ± 0.14	5.5 ± 0.5	84.594 ± 2.8711	67.924 ± 0.351
A6	143.7 ± 0.21	0.302 ± 0.02	6 ± 0.1	86.383 ± 1.990	64.796 ± 0.237
B2	82.79 ± 0.25 nm	0.265 ± 0.03	5.8 ± 0.3	92.578 ± 2.1499	84.457 ± 1.020
B3	178.8 ± 0.63	0.420 ± 0.02	4.7 ± 0.1	87.155 ± 1.2576	56.317 ± 0.746
B4	313.7 ± 0.45	0.572 ± 0.06	5.7 ± 0.2	82.088 ± 0.8491	42.796 ± 6.750

Results

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 125.5	Peak 1:	170.8	75.1	46.14
PdI: 0.361	Peak 2:	20.20	22.5	3.788
Intercept: 0.614	Peak 3:	5560	2.4	0.000

Result quality : Refer to quality report

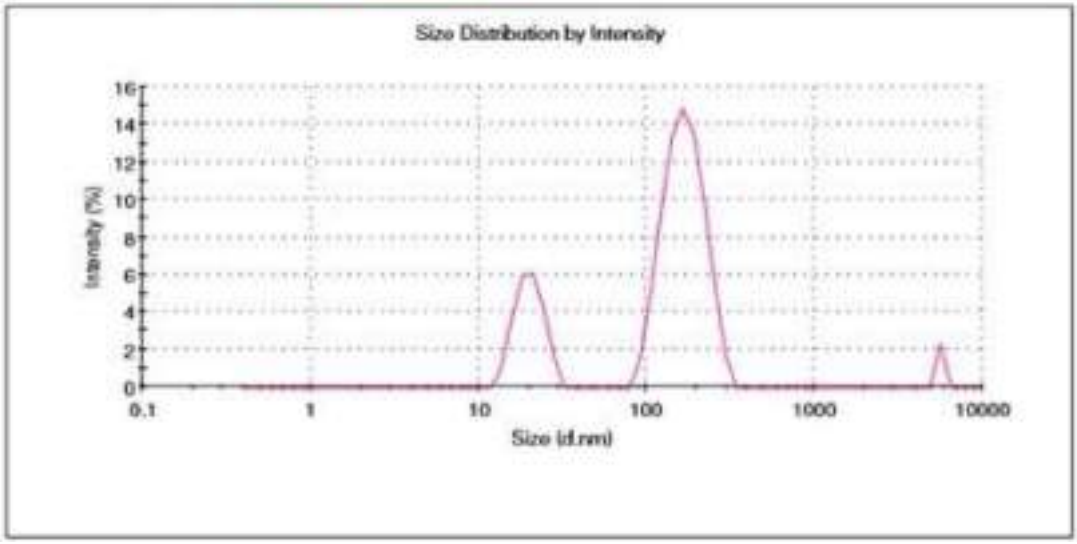

Figure 3. Particle size of Ritonavir A3 batch.

Results

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 82.79	Peak 1:	4.277	52.7	0.6600
PdI: 0.265	Peak 2:	242.2	40.2	65.92
Intercept: 0.549	Peak 3:	4994	7.2	611.6

Result quality : Refer to quality report

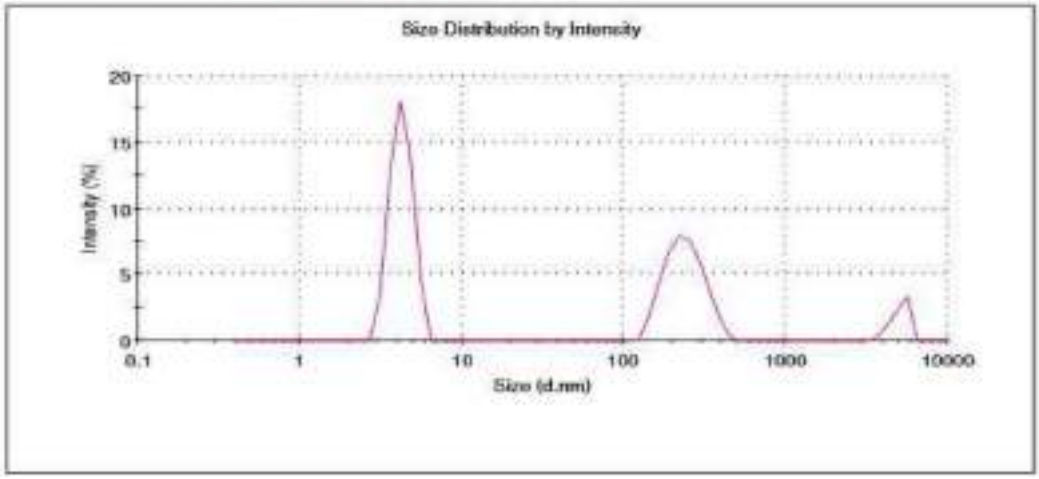

Figure 4. Particle size of Lopinavir B2 batch.

size of the medicine in nanosuspension allows for faster drug absorption. It will assist the actual transcellular transportation of nanosized particles via olfactory neurons to the brain [14]. HPMC was added in formulation as mucoadhesive polymer to enhance retention in nasal cavity [14]. HPMC also acts as stabilizer and suspending agent in nano suspension formulation.

3.10. Scanning electron microscopy

Morphology of nano formulated drug particles (shown in Figures 7 and 8) was analyzed by air drying followed by oven-drying the nano-suspension. There were no agglomerates observed and the particles appeared distinct and homogenous in size. The RTV drug particle

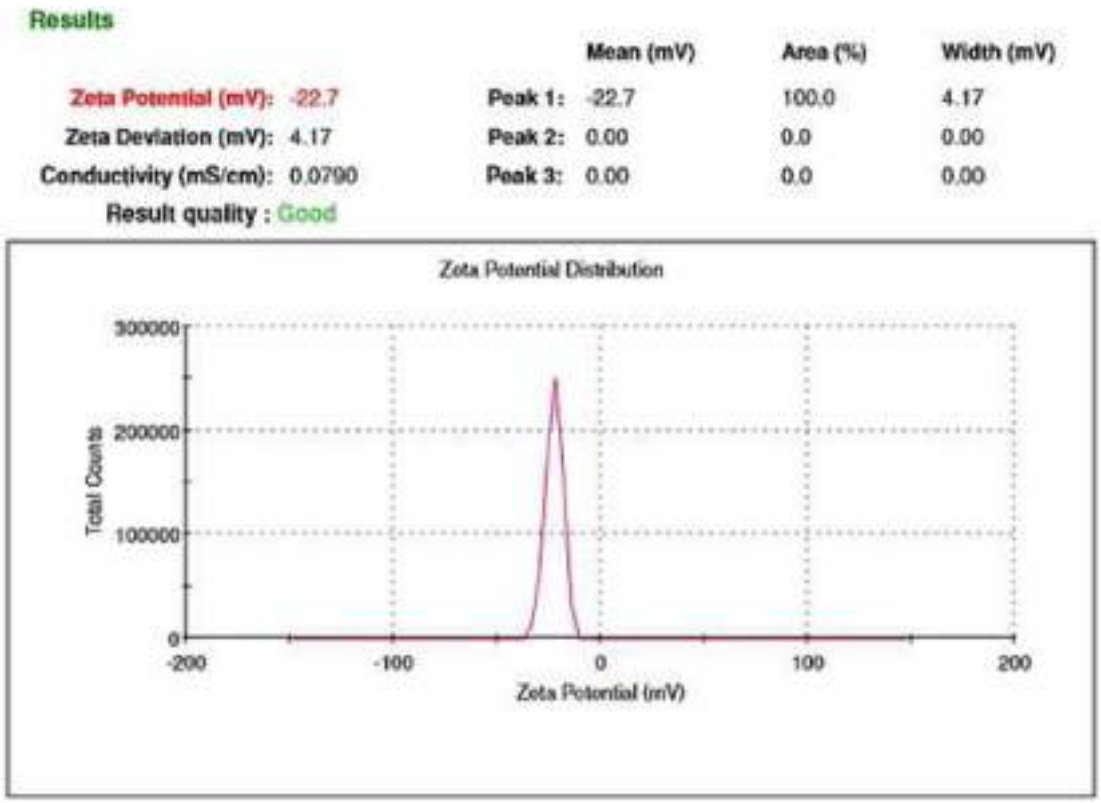

Figure 5. Zeta potential of Ritonavir A3 batch.

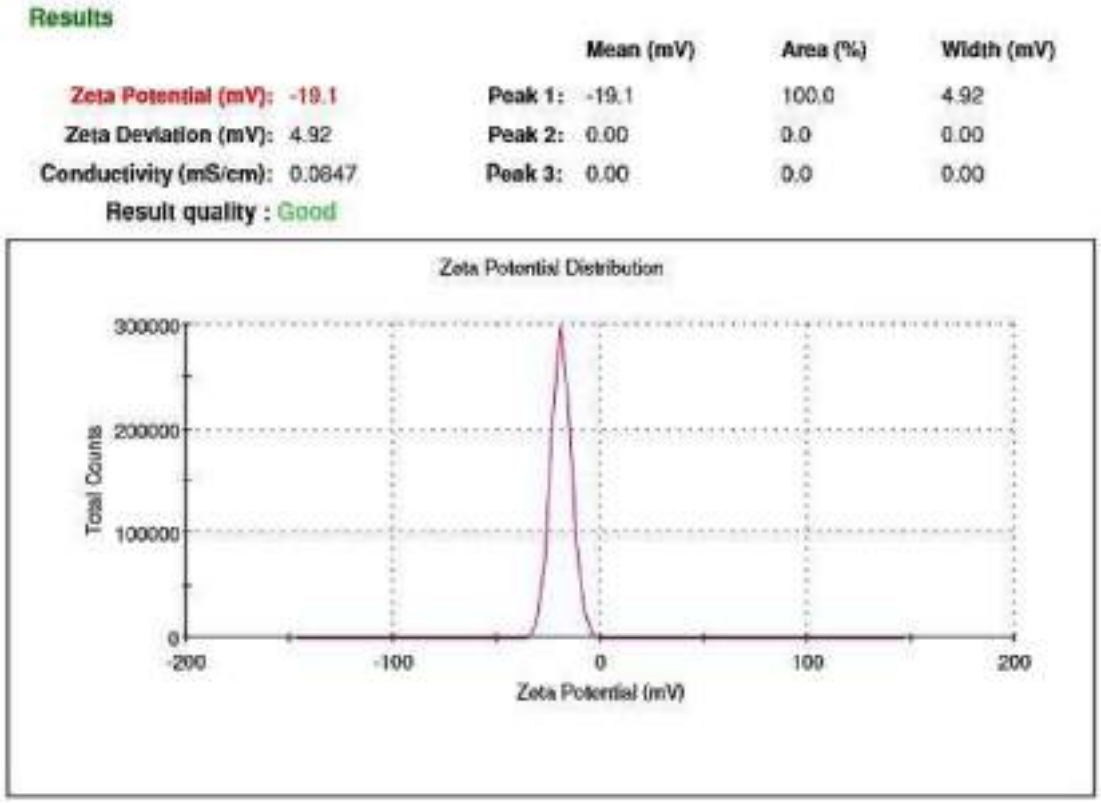

Figure 6. Zeta potential of Lopinavir B2 batch.

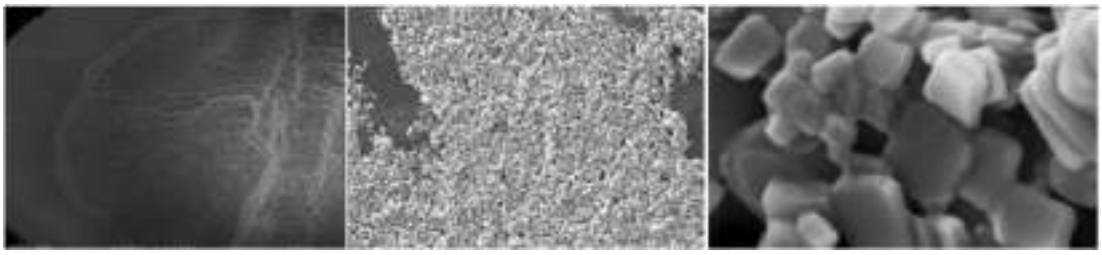

Figure 7. SEM photograph (Ritonavir nanosuspension with magnification 90 X, 100 KX and 50 KX).

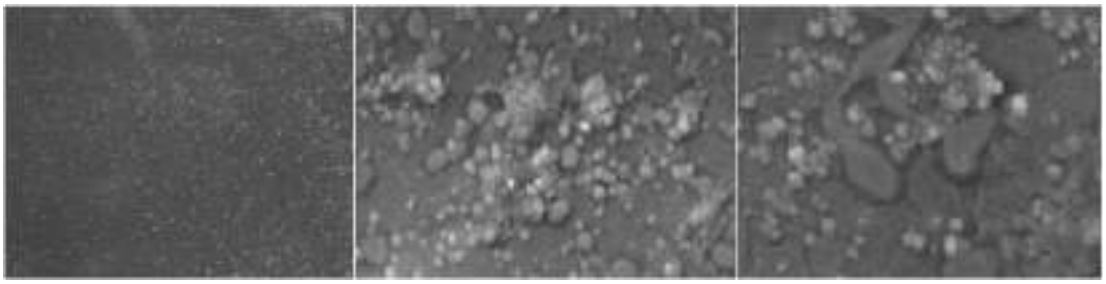

Figure 8. SEM photograph (Lopinavir Nanosuspension with magnification 90 X, 100 KX and 50 KX).

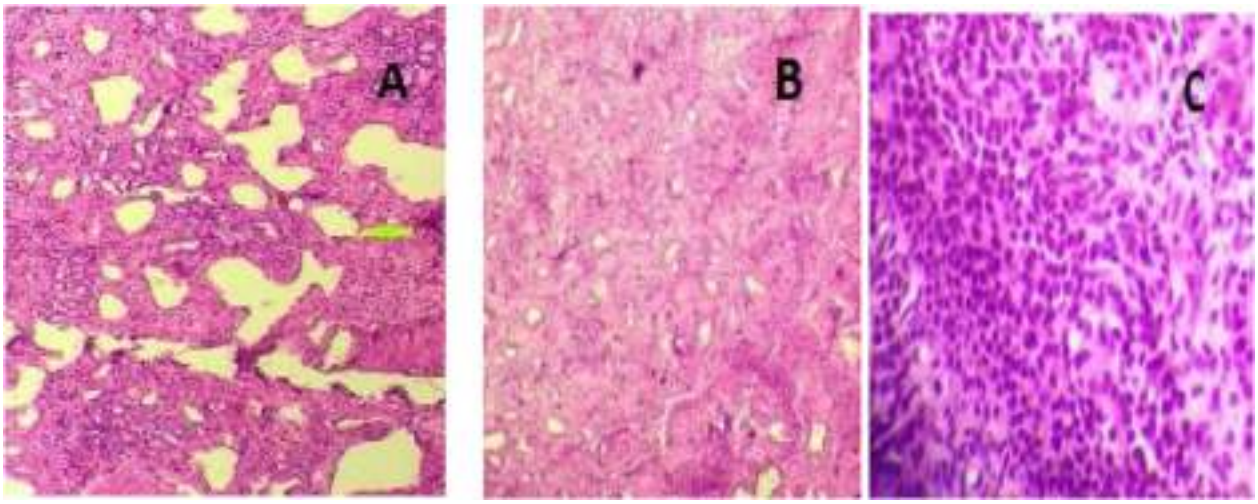

Figure 9. Histopathology study. (A- Positive control, B – Negative control, C –Nanosuspension).

formulation is relatively cuboidal, while the LPV drug particle formulation is found to be spherical and free of agglomerations. From SEM, we can conclude that the prepared nanosuspensions were formed uniform and stable [14, 45].

3.11. Nasal histopathology study

A nasal histopathology analysis shown that the improved nanosuspension formulations did not cause any significant damage at nasal mucosal lines. As compared to the normal conditions in the nasal mucosa treated with PBS 6.5, the samples treated with the positive control (70 percent Isopropyl Alcohol) showed cilia loss and tissue damage in the interior structure (Figure 9). However, in the mucosal tissues of the nose treated with nanosuspension, no significant change observed over cilia and nasal mucosa linings, confirming the formulation's safety for nasal administration [14, 46].

3.12. Stability study

Stability Study was carried out according to the procedure described by ICH guidelines [Q1A (R2)] on stability testing. Effect of temperature and humidity on formulation was carried out by analyzing the optimized formulation keeping at 40 °C ± 2 °C/75% ± 5% relative humidity in stability chamber for one month. Table 3 shows that during stability investigations, in-vitro drug release and diffusion for the optimized formulation batches were verified; readings for in-vitro drug release and diffusion were compared before and after the optimized batch's stability study. As a result, we can infer that storage conditions did not significantly affect physical parameters, drug release, or diffusion of optimal formulations generated using high-pressure homogenization [37, 47]. Results of drug content and drug release of both drugs are mentioned in Table 3.

Table 3. Stability study of nanosuspension.

Temperature and humidity	Parameter	Months	
		0	1
40 °C ± 2 °C; 75% ± 5%RH	Drug content (%) A3	84.769 ± 3.0483	85.284 ± 0.375
	Drug content (%) B2	92.578 ± 2.1499	91.278 ± 1.222
	Drug release (%) A3	70.185 ± 0. 196	67.984 ± 0.228
	Drug release (%) B2	84.457 ± 1.020	80.954 ± 0.239
Room temperature	Drug content (%) A3	84.769 ± 3.0483	80.777 ± 0.154
	Drug content (%) B2	92.578 ± 2.1499	85.234 ± 0.595
	Drug release (%) A3	70.185 ± 0. 196	66.931 ± 0.420
	Drug content (%) B2	84.457 ± 1.020	80.877 ± 0.679
Refrigeration 4 °C – 8 °C	Drug content (%) A3	84.769 ± 3.0483	85.284 ± 0.375
	Drug content (%) B2	92.578 ± 2.1499	91.278 ± 1.222
	Drug release (%) A3	70.185 ± 0. 196	67.984 ± 0.228
	Drug release (%) B2	84.457 ± 1.020	83.55 ± 0.547

4. Conclusion

Nanosuspension of combination of antiviral agents Ritonavir and Lopinavir was prepared using stabilizers. The increase in pressure leads to decrease in particle size and finally the drug release is increased. The study demonstrates that the developed Nanosuspension A3 (Ritonavir) and B2 (Lopinavir) formulation have particle size 125.5 nm and 82.79 nm and zeta potential – 22.7 mV and – 19.1 mV respectively. The cumulative release of Ritonavir and Lopinavir was found to be 70.185 ± 0.196% and 84.457 ± 1.020% respectively. From the above results we conclude that High pressure homogenizer is an effective instrument to reduce the particle size than the Wet milling technique and High speed homogenizer. The low the particle size the more is the bioavailability the brain targeting then becomes possible as the drug will pass efficiently through the transcellular route.

Declarations

Author contribution statement

Dr. Smita Kakad: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Trupti Gangurde: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Dr. Sanjay Kshirsagar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Vaishali Mundhe: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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RESEARCH

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Development of nanoemulsion of antiviral drug for brain targeting in the treatment of neuro-AIDS

S. M. Nemade[1], S. P. Kakad[2*], S. J. Kshirsagar[3] and T. R. Padole[3]

Abstract

Background: Delivery of drugs via the nasal route directly to the brain utilizing the olfactory pathway is purportedly known to be a more efficient method to deliver neuro-therapeutics to the brain by circumventing the BBB, thereby increasing the bioavailability of these drugs in the brain. The main objective of the project work is to improve the bioavailability of the antiretroviral drug and to minimize the side effects of this therapy which are observed at the higher side in the chronic HIV treatment. The advantage of nasal drug delivery is its noninvasiveness and self-administration. Nanoformulation provides fast onset of action and helps to achieve site-specific delivery. In the current work, nanoemulsion formulation was developed with a ternary phase system. In vitro characterization of nanoemulsion was performed.

Result: Optimized batch B2 had a zeta potential of − 18.7 mV showing a stable emulsion system and a particle size of 156.2 nmin desirable size range. Batch B2 has the least variation in globule size with PDI 0.463. Results from ex vivo studies revealed that developed nanoemulsion (B2) possessed a higher rate of drug release compared to other formulations.

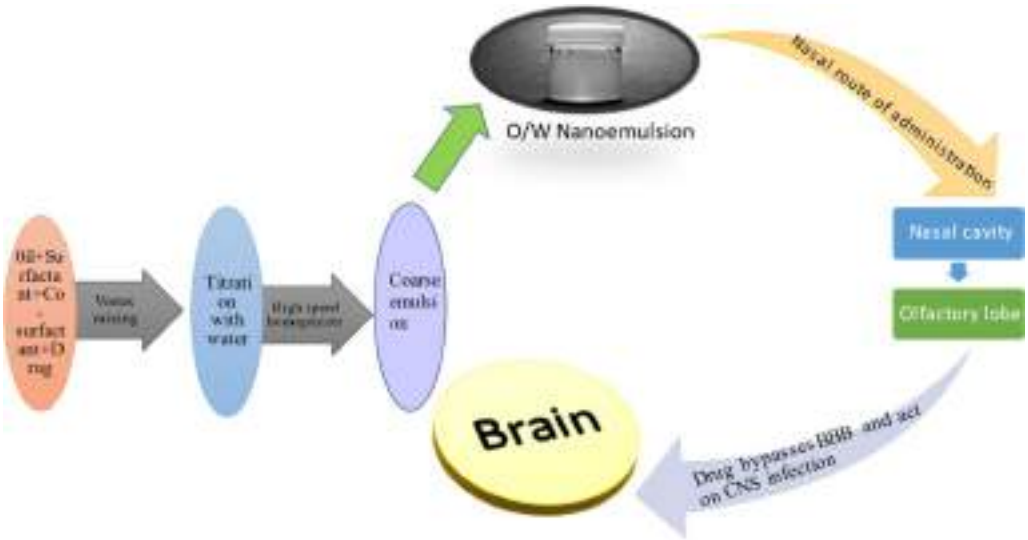
Conclusion: Phase diagrams indicated more width of the nanoemulsion region with an increase in surfactant ratio. Stable nanoemulsion was prepared with a combination of surfactant and co-surfactants. Nanoemulsions could prove one of the best alternatives for brain delivery of potent medications.

Keywords: Antiretroviral therapy, Nanoemulsion, Nose-to-brain delivery, Ex vivo diffusion study, Cytotoxicity study

*Correspondence: smitadarade87@gmail.com

[2] Department of Pharmaceutics, MET's Institute of Pharmacy, BKC, Adgaon, Nashik, Maharashtra 422002, India
Full list of author information is available at the end of the article

Graphical Abstract



1 Background

The delivery of drugs to the brain has been fraught with the low bioavailability of drugs in the brain. This is caused by the "Blood–Brain Barrier" (BBB) and the "Blood–Cerebrospinal Fluid Barrier" (BCSFB) which block therapeutics from gaining access to the (CNS) [1, 2]. Drugs that are administered via oral and intravenous are faced with this challenge of BBB, thereby making the treatment of neurodegenerative diseases difficult to manage [3]. Delivery of drugs via the nasal route directly to the brain utilizing the olfactory pathway is more significant, delivering successively to the brain by passing the BBB, thereby increasing the bioavailability of drugs in the brain [4, 5].

Nervous system alterations occur due to direct or indirect effects of HIV infection, collectively known as neuro-AIDS [6, 7]. The estimated overall prevalence of nervous system disorders among patients receiving highly active antiretroviral therapy but also requiring neurological care is over 25% [8]. According to WHO (Global HIV & AIDS statistics—2020), there are ~ 34 million people in the world infected with HIV [8]. Out of 95 percent of these cases as well as deaths from AIDS that occur in the developing world [9], dementia (HIV-associated dementia) is becoming common in HIV-infected adults having a prevalence of up to 40% in western countries [10, 11].

The evaluation of the nanoemulsions includes (1) appearance testing by visual as well as under radiation observation; (2) stability testing by centrifugation; (3) stability under differential atmospheric conditions includes temperature, humidity, and change in forms such as cracking (flocculation) and creaming; (4) viscosity with respect to (i) change in time and (ii) change in RPM; and (5) pH of the formulation of such parameters indicates whether the formulation remains stable under certain circumstances. Characterization of the formulation includes (i) droplet size analysis, (ii) zeta potential, (iii) percent transmittance, (iv) morphology studies by transmission electron microscopy, (v) pH of the formulation, (vi) refractive index studies, and (vii) drug content and some other tests like polydispersity test, dye test, fluorescence test, dilution test, conductance test, and filter paper test. Furthermore, ex vivo diffusion study with the help of sheep nasal mucosa and cytotoxicity studies using cultured cell beds so that any damaging effects to tissues can be determined [12, 13].

Tenofovir disoproxil fumarate, the oral pro-drug of tenofovir, is a nucleotide reverse transcriptase inhibitor [14]. It inhibits viral polymerases by directly competing with the natural deoxyribonucleotide substrate and, after incorporation into deoxyribonucleic acid (DNA), by DNA chain termination [15, 16]. Tenofovir disoproxil fumarate is used to treat HIV and chronic hepatitis. Tenofovir has poor water solubility and low bioavailability (25%), thus a suitable candidate to formulate nanoemulsion for nasal drug delivery [12, 16].

2 Materials and methods

Tenofovir disoproxil fumarate was gifted by Mylan Pharmaceutical Private Limited, Nashik, India. Oleic acid (oil) was gifted by S.D. Fine-Chem Limited, Mumbai, India. Tween 60 and tween 80 were purchased from Merck Specialties Pvt. Ltd. Mumbai, India. Ethanol and methanol were purchased from Changshu Yangyuan Chemicals, China. The water used was semi-quartz distilled. All other chemicals and reagents used were of analytical grade, procured commercially, and used as received.

2.1 Determination of partition coefficient

The partition coefficient of the drug in oil and water was determined with the shake flask method. The drug was partitioned into the oil and water phase. After shaking for 1.5 h, the mixture was kept aside after appropriate dilution. The concentration in oil was determined on the UV spectrophotometer (UV 1800 Shimadzu) at 260 nm [17].

2.2 Screening of oil for nanoemulsion

The solubility of tenofovir disoproxil fumarate (TDF) in various oils like oleic acid, olive oil, and castor oil was determined by dissolving the excess amount of TDF in 5 ml of each selected oil in stoppered vials separately for the determination of solubility. The mixture vials were then kept in a shaker for 48 h to get equilibrium. The equilibrated samples were then centrifuged at 9000 RPM for 10 min. The supernatant was collected and filtered through a 0.45-µm membrane filter. The concentration of drug was determined in each oil by a UV spectrophotometer (UV 1800 Shimadzu) with suitable dilution with 0.1 N HCl at a wavelength of 260 nm [6].

2.3 Screening of surfactant and co-surfactant

Surfactants and co-surfactants were selected based on their capability to form stable nanoemulsion with relevant surfactants at minimum concentration. Of the several surfactants, tween 80 provided better outputs. Based on trials, ethanol was chosen as a co-surfactant [6].

2.4 Construction of phase diagram

On the basis of drug solubility in various nanoemulsion components, different combinations of oil, water, and surfactant/co-surfactant were selected. The pseudo-ternary phase diagrams of oil, surfactant/co-surfactant, and water were developed using the surfactant titration method [18]. The mixtures of oil and water at different weight ratios varying from 1:9 to 9:1 were titrated with surfactant/co-surfactant mix in a dropwise manner. Pseudo-ternary phase diagram was achieved by titrating with four different ratios of surfactant and co-surfactant (1:1, 1:2, 2:1, and 4:1) until it turns from hazy to transparent. After the identification of nanoemulsion region in the phase diagrams, formulation component ratios were selected in order to form the nanoemulsion [19, 20].

On the basis of the solubility study, oleic acid was selected as the oil phase. Tween 80 and ethanol were selected as surfactant and co-surfactant, respectively. Distilled water is used as an aqueous phase. The drug was dissolved in the required quantity of oil, surfactant, and co-surfactant with varying ratios. Distilled water was added to the above mixture as a fixed ratio. Surfactant and co-surfactant were added gradually with continuous stirring, which resulted in the formulation of a transparent and homogenous nanoemulsion [20, 21].

2.5 Characterization of nanoemulsion

2.5.1 Thermodynamic stability studies

The formulation consists of a couple of immiscible phases, so as to overcome the problems such as instability these thermodynamic stability studies were performed. Prepared formulations were centrifuged at 3000 rpm for 30 min and then examined for phase separation. Those formulations that did not show any phase separation were taken for the heating and cooling cycles at a temperature of 4 °C and 45 °C for 48 h. The formulations were then observed for phase separation. The formulations which were stable at these temperatures passed the thermodynamic stability test and were selected for further evaluation studies [21, 22].

2.5.2 Evaluation of the nanoemulsion

The nanoemulsion prepared with optimized composition was evaluated for the parameters like zeta potential, viscosity, pH, conductivity, and refractive index [23]. The results are given in Tables 3 and 4.

2.5.3 Transmittance test

To assess the transparency and clarity of the nanoemulsion, this test was performed. The transparency of nanoemulsion was checked by measuring transmittance at 650 nm with 0.1 N HCl as blank by using a UV spectrophotometer (UV 1800 Shimadzu). It was determined with the formula in Eq. 1. The results are given in Table 5.

$$\text{Absorbance} = -\log(\%T/100). \tag{1}$$

2.5.4 Drug content estimation

Nanoemulsion containing 100 mg drug was dissolved in 100 ml 0.1 N HCl taken in a volumetric flask. Then, the solvent was filtered; 1 ml was taken in 50 ml volumetric solution and diluted up to the mark with 0.1 N HCl and analyzed spectrometrically at 260 nm (UV 1800 Shimadzu). The concentration of tenofovir in mg/ml was obtained by using a standard calibration curve of the drug. Drug content studies were carried out in triplicate for each formulation batch [2, 24].

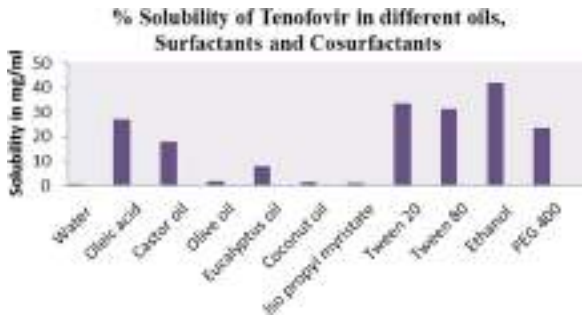


Fig. 1 Solubility of TNF in different oils, surfactants, and co-surfactants

2.5.5 Particle size and zeta potential measurement

The particle size and zeta potential of the optimized nanoemulsion were determined by dynamic light scattering with Zetasizer ver.7.12 (Malvern Instruments Ltd.) [22, 24]. The results are shown in Tables 3 and 4.

2.5.6 Ex vivo diffusion studies

Egg membrane and sheep nasal mucosa were used for preliminary preclinical evaluation of nasal dosage forms. Sheep nasal mucosa is used for an experiment as it mimics human nasal vasculature. A diffusion study was carried out using an isolated egg membrane for the trial batches (B1–B4) in phosphate buffer pH 6.4 (PBS 6.4) for a period of 3 h using a Franz diffusion cell [25, 26]. Diffusion of drug from egg membrane was observed. Later, best batch B2 was forwarded to diffusion study from sheep nasal mucosa. The sheep nose piece was obtained from a local slaughterhouse; the nasal mucosa layer was excised and used for diffusion study. Nasal mucosa was placed in Franz diffusion cells having a diffusion area of 0.785 cm^2. PBS pH 6.4 was added to the receiver chamber maintained at 37 °C temperature. Franz cell was pre-incubated for 20 min, and formulation equivalent to10 ml of B2 sample was placed in the donor chamber. Withdrawn 1 mL samples from the receiver chamber at predetermined time intervals, added 1 mL of PBS 6.4 after each sampling to maintain sink condition. All the samples were filtered and analyzed using a UV spectrophotometer at 224 nm, and cumulative drug release was determined [27, 28].

Observations after diffusion from goat nasal mucosa are mentioned in Table 6.

3 Results

3.1 Formulation and optimization of nanoemulsion

The solubility of tenofovir in various oils was investigated and found to be highest in oleic acid, i.e., 26.69 ± 0.3 mg/ml (Fig. 1). Among surfactants, in tween 80 the drug showed the highest solubility of 30.31 ± 1.5 mg/ml. In ethanol, the drug showed the highest solubility among the co-surfactants of 41.57 ± 0.6 mg/ml, followed by PEG 400. The nanoemulsion existence region was determined by constructing phase diagrams. From the pseudo-ternary phase diagrams, it was concluded that the highest nanoemulsion zone was obtained for the nanoemulsion having tween 80 and ethanol in the ratio of 4:1 as shown in Fig. 2 and (Table 1) [29, 30].

3.2 Dispersion stability studies

The objective of thermodynamic stability is to evaluate the phase separation and effect of temperature on formulation stability. In the thermodynamic stability studies, the formulation selected was subjected to stress tests like heating–cooling cycle and centrifugation. It was observed that all formulations were stable, clear liquid, and no phase separation occur under stress condition. This confirms the liquid formulations were stable for the storage [31, 32]. Nanoemulsions are thermodynamically stable formulation composed of a fixed proportion of oil, surfactant, co-surfactant, and water which does not tend to show any phase separation after multiple changes in the temperature and centrifugation. After centrifugation at 3000 RPM for 20 min, all the formulations were still stable, clear liquid, and no phase separation occurred under stress conditions. The conditions are mentioned in Table 2. It proves that the formulations are thermodynamically stable [33, 34].

3.3 Evaluation of the nanoemulsion composition

The nanoemulsion prepared by the selected composition was evaluated for the parameters like droplet size, PDI, zeta potential, viscosity, pH, conductivity, and refractive index. The result is given in Tables 3 and 4.

It was observed that there was no any significant difference between placebo formulation and formulation with drug. It indicates the formulation has isotropic nature. This confirms the drug was in a dissolved state and uniformly distributed in liquid formulation.

3.4 Transmittance test

From the results of the transmittance test in Table 5, it was observed that the transparency of the formulation goes on decreasing as the concentration of S-mixture. So it shows high transparency of the first formulation, and later it gets decreased [18].

The viscosity of four formulations was observed in the range of 400–500 cps, and after the variations in the RPM it is concluded that the system of all the nanoemulsion formulations was observed as a shear thinning system.

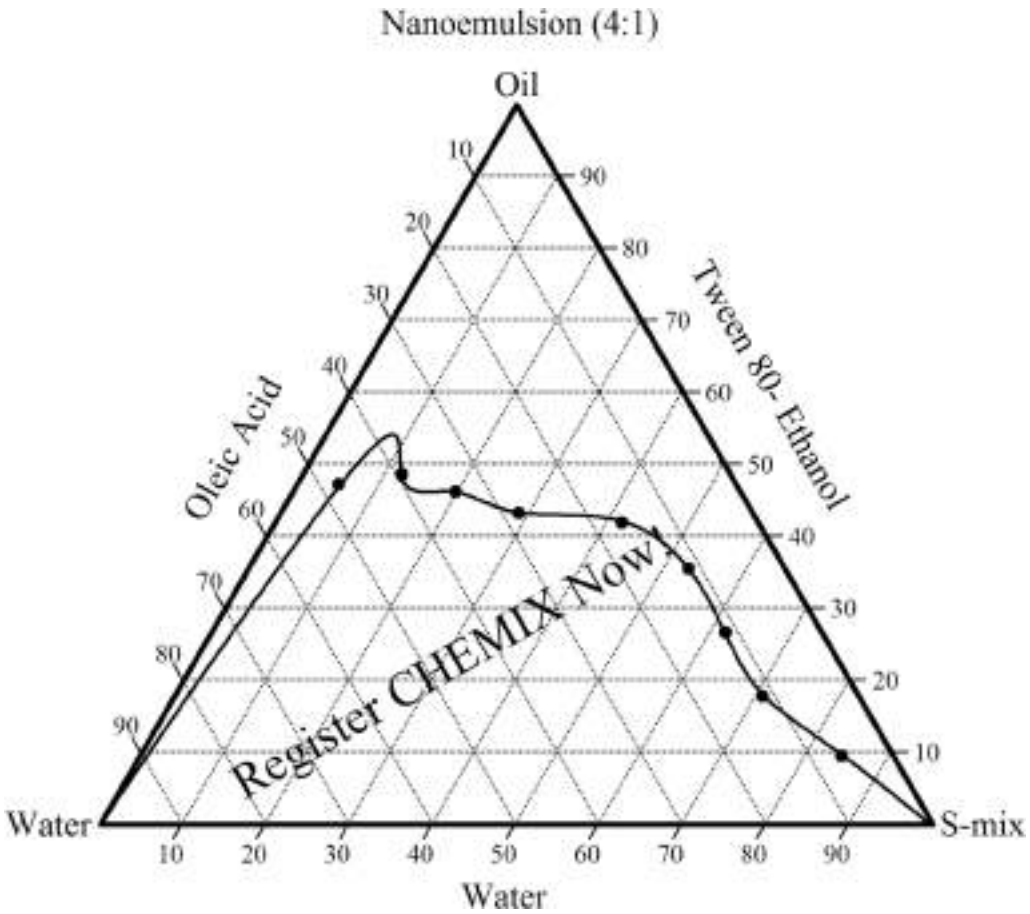


Fig. 2 Ternary phase diagram studies for ratio 4:1

That is viscosity gets decreased by a sudden increase in the resistance. But with respect to time the viscosity of the formulations remained stable and no major fluctuations were observed in it.

Nasal pH has a range which is between 5.6 and 6.5, and the observed values of the pH of all the formulations were in the range so it proves that the developed nanoemulsion formulations are applicable to the nasal drug delivery system.

3.5 Drug content estimation

The concentration of tenofovir in mg/ml was obtained by using a standard calibration curve of the drug. Drug content studies were carried out in triplicate for each formulation batch. The results are given in Table 5. Drug content determination helps to find out the amount of drug entrapped in the formulation. From results, more drug content was observed in batch B2 than in other batches [23, 35].

Table 1 Trial batches preparation

Batch no.	Oil %	S-mixture % (4:1)	Water %	Appearance	Stability
B1	10	84	6	Transparent	Stable
B2	18	70	12	Transparent	Stable
B3	26	61	13	Transparent	Stable
B4	36	53	11	Transparent	Stable
B5	42	42	16	Turbid	Unstable
B6	44	28	28	Cloudy	Unstable
B7	46	20	34	Cloudy	Unstable
B8	48	12	40	Cloudy	Unstable
B9	47	5	48	Cloudy	Unstable

3.6 Particle size, polydispersity index, and zeta potential measurement

The particle size and zeta potential of the optimized nanoemulsion were determined by dynamic light

Table 2 Thermal and centrifugation stability of tenofovir nanoemulsion

Nanoemulsion formulation	Thermal stability			Centrifugation stability at 3000 rpm
	Storage at 4 °C	Storage at R.T. (27 °C)	Storage at 45 °C	
B1	√	√	√	√
B2	√	√	√	√
B3	√	√	√	√
B4	√	√	√	√

scattering with Zetasizer ver.7.12 (Malvern Instruments Ltd.). The results are shown in Figs. 3 and 4. The zeta potential is −18.7 and the size (z average) is 156.2 nm with PDI 0.463. The ideal size of globules in nanoemulsion is in between the range of 100 and 500 nm, the polydispersity index should be narrow, and the zeta potential should be within the range of −15 to +20 mV for prediction of stability of nanodroplets in emulsion [35, 36]. The obtained results are appropriate with these values. Batch B2 has a large globule size, but it has the minimum polydispersity index that means the variation in the globule size throughout the formulation is very less in the batch B2 [37, 38].

3.7 Ex vivo diffusion studies

Ex vivo study was performed using egg membrane in nanoemulsion batches B1, B2, B3, and B4. Here, batch B2 shows greater drug diffusion that is 74.98 ± 1.06% after 3 h. For the sheep nasal mucosal membrane, B2 batch showed 75.9841 ± 0.14% of diffusion in the system, which is determined by taking triplicate readings with standard deviation ($n = 3$) [35]. From the above studies, it is concluded that B2 is the optimized batch and has a greater diffusion ratio compared to all other batches. The diffusion of the drug from the formulations is comparable through egg membrane and sheep nasal mucosa (Fig. 5; Table 6) [39, 40].

Table 5 Drug content and percent transmittance

Sr. no.	Batch	% Transmittance	Drug content
1	B1	88.93 ± 0.4%	92.77 ± 0.3%
2	B2	87.82 ± 0.8%	94.19 ± 0.6%
3	B3	87.79 ± 0.6%	93.25 ± 0.6%
4	B4	87.51 ± 0.5%	93.79 ± 0.8%

4 Discussion

HIV treatment is a combination of antiretroviral drugs. Most people who treat their HIV will take two or more drugs each day for the rest of their lives. Sticking to the treatment plan isn't always easy. The main purpose of this study was to improve the bioavailability of the antiretroviral drug and to minimize the dose of the antiretroviral drug and ultimately reduce the side effects of this therapy which is observed at the higher side in the other formulations/administration process [41]. Nasal route delivery to the brain utilizing the olfactory pathway is purportedly known to be more efficient and deliver

Table 3 Evaluation of nanoemulsion

Sr. no.	Batch label	Conc of oil (%)	Conc. of S. mix (%)	Drug amount (mg)	Droplet size (nm)	PDI	Zeta potential
1	B1	4.69	42.26	25	155.8	0.508	−12.1
2	B2	4	45	25	156.2	0.463	−18.7
3	B3	8.85	35.41	25	160.3	0.289	−5.29
4	B4	10	33	25	241.7	0.497	−2.26

Table 4 Physicochemical parameters of developed tenofovir nanoemulsion

Sr. no.	Batch label	Drug amount (mg)	Viscosity (cps)	Refractive index	pH
1	B1	25	428 ± 0.2	1.47 ± 0.5	5.2 ± 0.1
2	B2	25	420 ± 0.7	1.472 ± 0.3	5.5 ± 0.3
3	B3	25	412 ± 0.1	1.45 ± 0.9	5.7 ± 0.2
4	B4	25	491 ± 0.5	1.47 ± 0.4	6.1 ± 0.3

			Size (d.nm)	% Intensity	StDev
Z-Average(d.nm)	156.2	Peak1	221.9	92.2	98.45
PdI:	0.463	Peak2	43.83	4.6	9.197
Intercept:	0.954	Peak3	5008	3.2	604.0

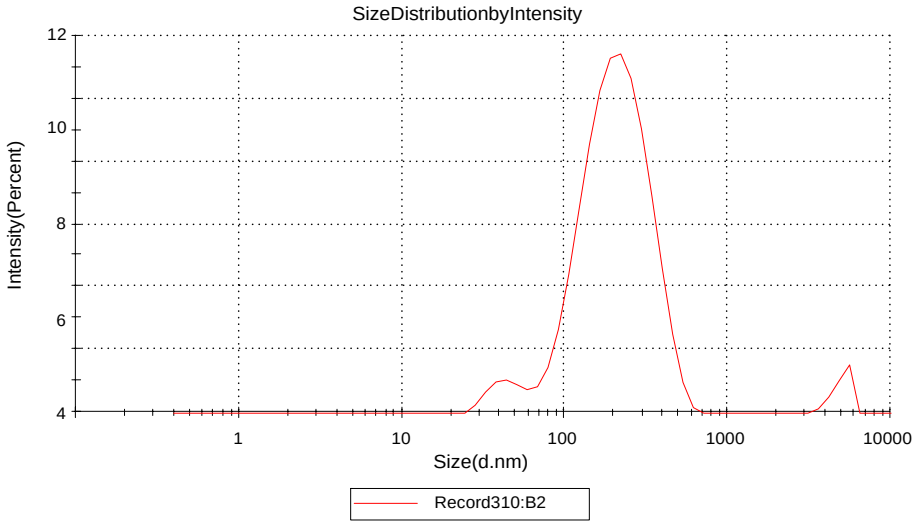


Fig. 3 Particle size and polydispersity index graph of optimized nanoemulsion batch

			Mean (mV)	Area(%)	StDev(mV)
Zeta Potential (mV):	-18.7	Peak1	-18.7	100.0	4.52
Zeta Deviation(mV):	4.52	Peak2	0.00	0.0	0.00
Conductivity (mS/cm):	0.0463	Peak3	0.00	0.0	0.00

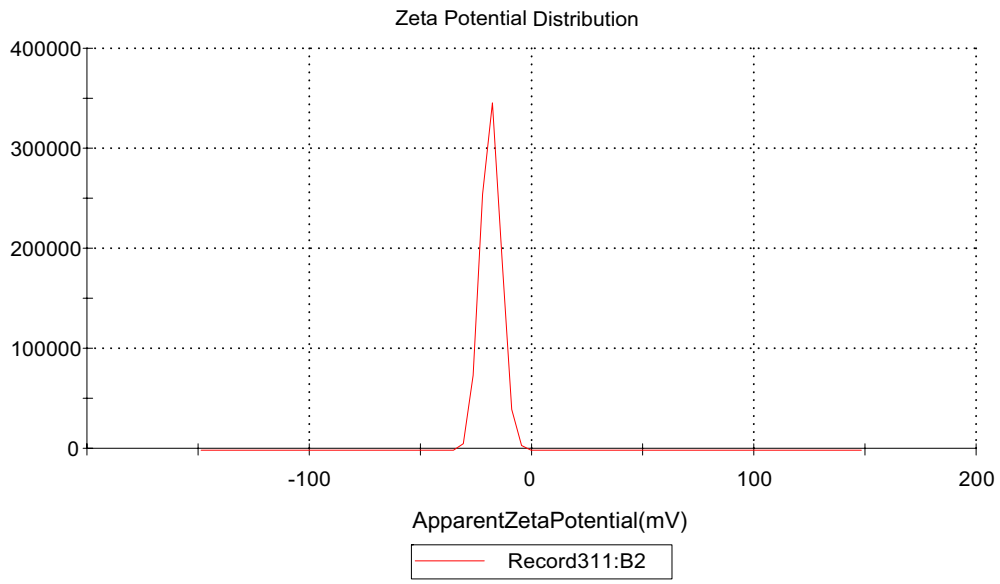


Fig. 4 Zeta potential graph of optimized nanoemulsion batch

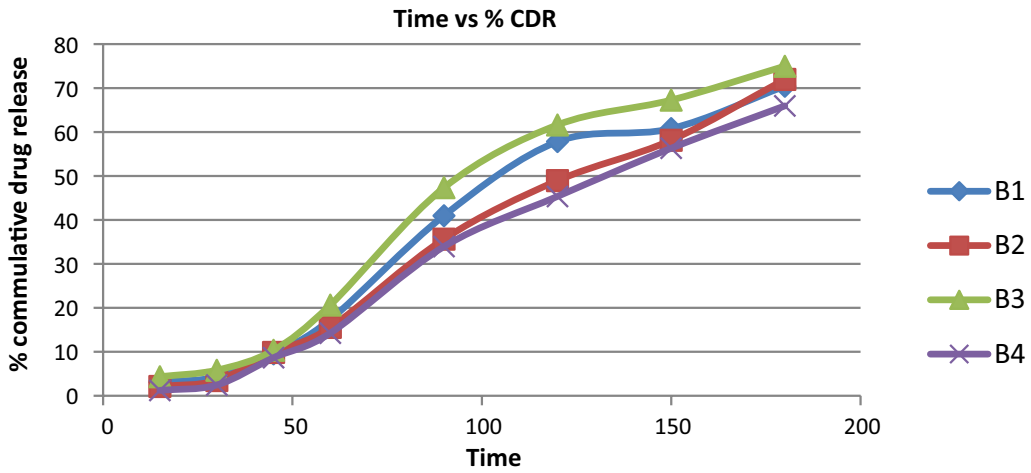


Fig. 5 Comparison of diffusion study profiles of batches B1–B4

neuro-therapeutics to the brain by passing the BBB, thereby increasing the bioavailability of drugs in the brain. The advantage of this method is that nasal drug delivery administration is noninvasiveness in nature, essentially painless, and particularly suited for children [42].

Tenofovir disoproxil fumarate is selected for the study. On the basis of drug solubility in various nanoemulsions components, different combinations of oil, water, and surfactant/co-surfactant were selected. The pseudo-ternary phase diagrams of oil, surfactant/co-surfactant, and water were developed using the surfactant titration method. Phase diagrams indicated more width of the nanoemulsion region with an increase in surfactant ratio. Diffusion study was carried out using egg membrane for the trial batches (B1–B4) and sheep nasal mucosa for the optimized batch (B2) in phosphate buffer (PB) pH 6.4 for a period of 3 h using diffusion cell apparatus. Firstly, in the study using egg membrane in batches B1, B2, B3, and B4 of nanoemulsions, batch B2 shows greater diffusion that is 74.98 ± 1.06, and for the sheep nasal mucosal membrane B2 batch showed 75.9841 ± 0.14 of drug release.

The drug amount was kept fixed in all batches (25 mg). Oil (%) and surfactant mix (%) are variable in all batches. B2 batch has the highest surfactant mix (%), showing comparatively better diffusion. With respect to the dependent variable zeta potential, batch B2 was showing the highest magnitude (− 18.7 mV), showing good stability of emulsion. The polydispersity index of 0.463 is within the acceptable range. Other batches (B1, B3, and B4) have a very small magnitude of zeta potential which may lead to droplet coalescence and aggregation during storage. Surfactant mix concentration of 45% was found

Table 6 % CDR values after diffusion profile studies

Time (min)	B1	B2	B3	B4	B2
	Diffusion study through egg membrane				Diffusion study through sheep nasal mucosa
15	3.11 ± 0.214	2.11 ± 1.13	4.34 ± 0.913	1.22 ± 0.753	4.34532 ± 0.14
30	4.27 ± 1.35	3.47 ± 0.87	5.82 ± 1.03	2.45 ± 0.434	5.78417 ± 0.25
45	9.55 ± 0.587	9.88 ± 0.741	10.34 ± 0.642	8.66 ± 1.23	10.2446 ± 0.15
60	17.34 ± 0.623	15.55 ± 0.61	20.66 ± 1.20	14.26 ± 0.946	20.4604 ± 0.11
90	40.98 ± 0.975	35.65 ± 1.22	47.34 ± 0.946	33.94 ± 0.846	46.9353 ± 0.16
120	57.82 ± 0.813	48.96 ± 1.34	61.62 ± 1.09	45.36 ± 1.115	60.7482 ± 0.11
150	60.77 ± 1.29	58.14 ± 0.974	67.26 ± 0.976	56.31 ± 0.643	65.7842 ± 0.09
180	70.42 ± 0.750	74.98 ± 1.06	71.96 ± 0.853	65.94 ± 1.054	75.9841 ± 0.14

to give good stability to internal phase globules. Considering the results of four batches, B2 is found to be the optimum combination of drug, oil, and surfactant mix.

5 Conclusion

Nanoemulsion was found to be one of the potential drug delivery strategies for nose-to-brain delivery. For poorly soluble and poorly permeable drugs, nanoemulsion approach increases the surface area and gives lipophilic nature to disperse the drug. The development of nanoemulsion formulation was done with ternary phase studies. Surfactant selection is the heart of preparing stable dispersions. TNF could be given via the nasal route after performing clinical studies on such preparations. For CNS HIV infection, nose-to-brain delivery options are emerging strategies. The advantage of this method is that nasal drug delivery administration is noninvasiveness in nature, essentially painless, and particularly acceptable for all age-groups. Nanoformulation provides fast onset of action and helps to achieve site-specific delivery.

Abbreviations

TDF: Tenofovir disoproxil fumarate; BBB: Blood–brain barrier; RPM: Revolutions per minute; BCSFB: Blood–cerebrospinal fluid barrier; HIV: Human immunodeficiency virus; PB: Phosphate buffer; AIDS: Acquired immune deficiency syndrome; CNS: Central nervous system.

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Competing interests

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Author details

[1]Department of Quality Assurance Techniques, MET's Institute of Pharmacy, BKC, Adgaon, Nashik, Maharashtra 422003, India. [2]Department of Pharmaceutics, MET's Institute of Pharmacy, BKC, Adgaon, Nashik, Maharashtra 422002, India. [3]Department of Pharmaceutics, MET's Institute of Pharmacy, BKC, Adgaon, Nashik, Maharashtra 422003, India.

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