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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

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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

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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

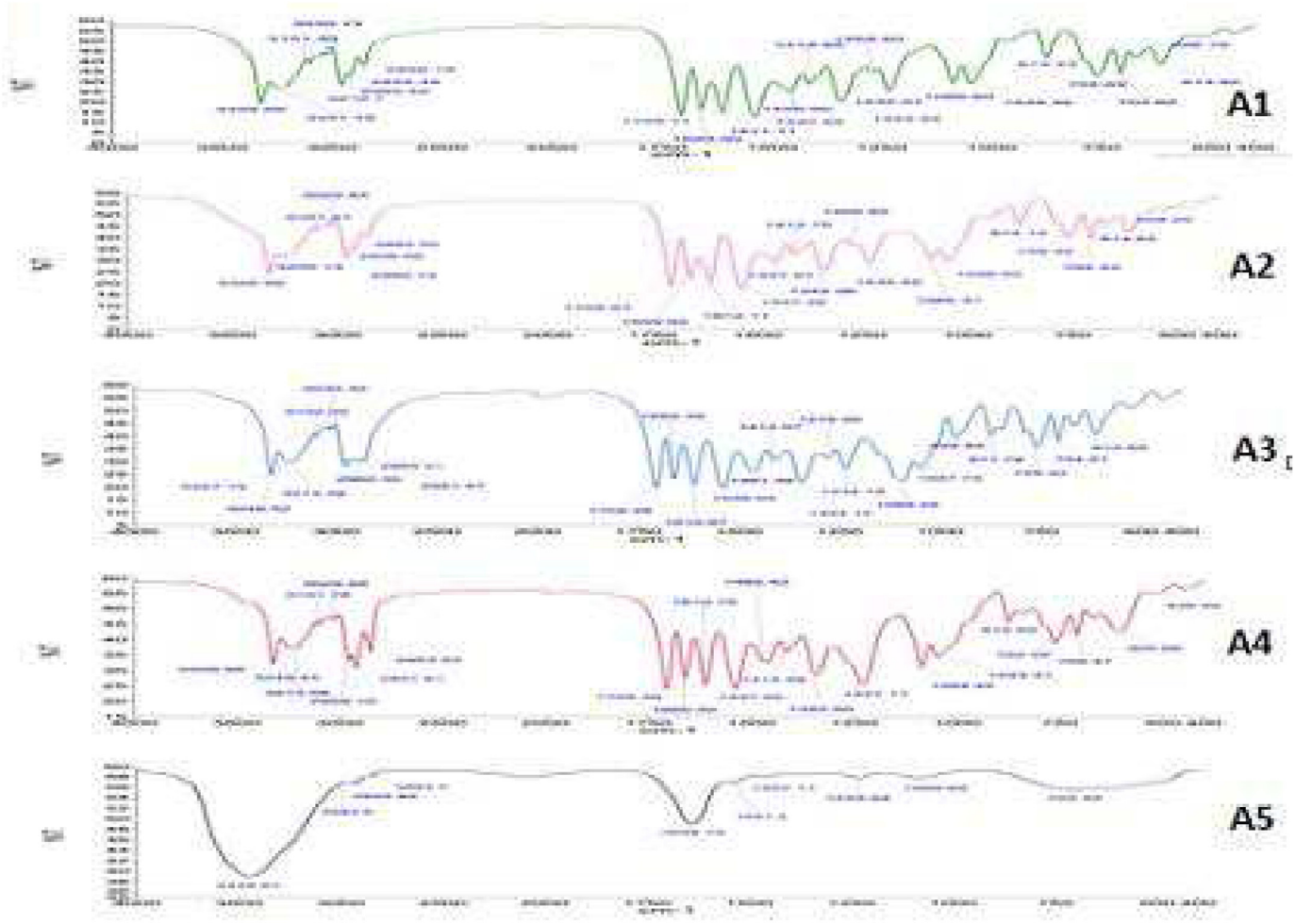

Figure 1. FTIR stacked spectra (Ritonavir-A1: Ritonavir, A2: Ritonavir + HPMC, A3: Ritonavir + Poloxamer, A4: Ritonavir + SLS, A5: Formulation.).

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

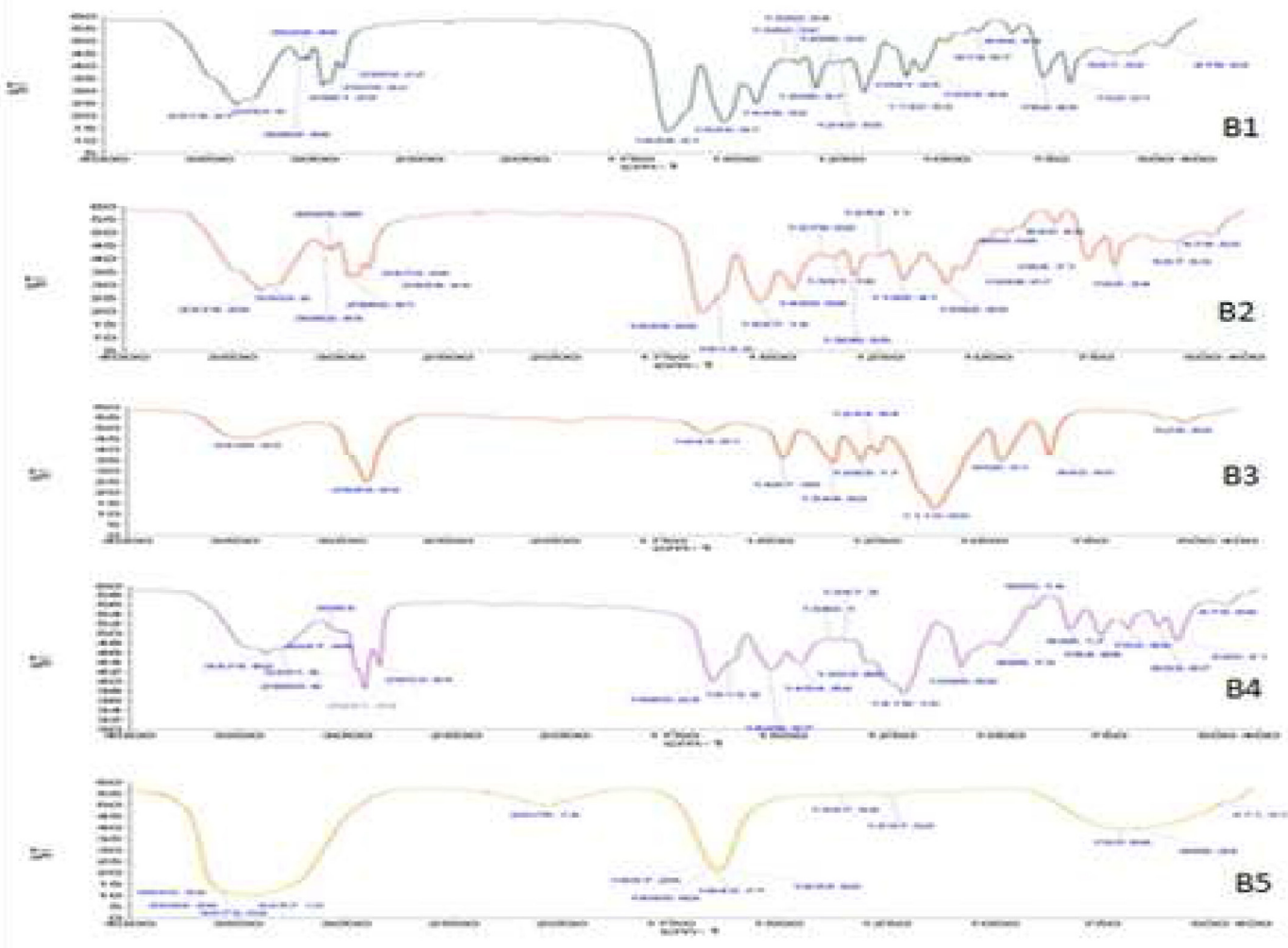

Figure 2. FTIR stacked spectra (Lopinavir. B1: Lopinavir, B2, Lopinavir + HPMC, B3: Lopinavir + Poloxamer, B4: Lopinavir + SLS, B5: Formulation).

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

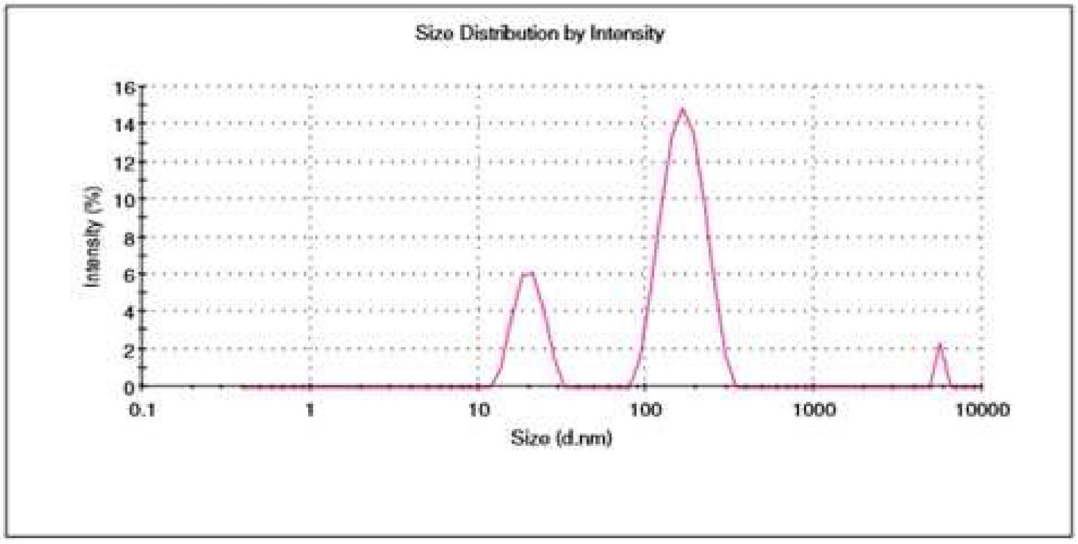

Figure 3. Particle size of Ritonavir A3 batch.

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

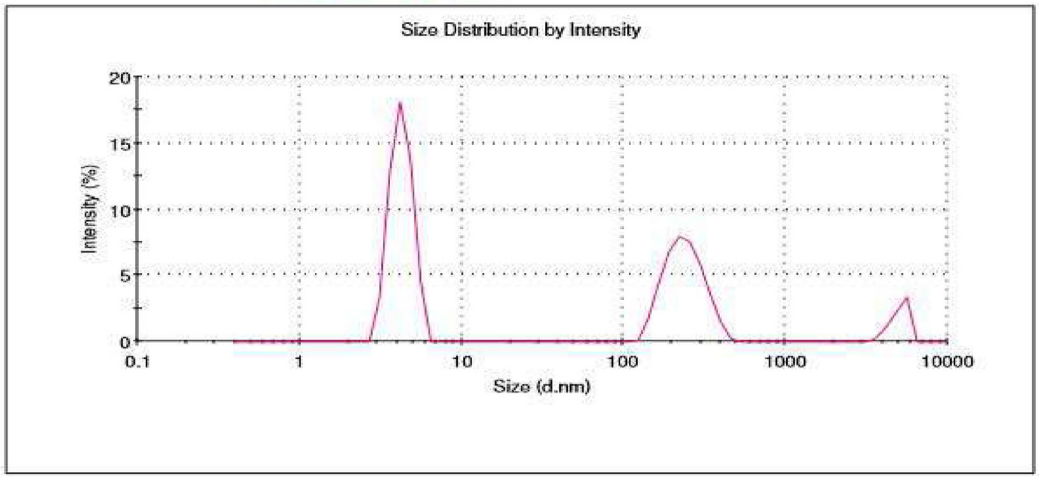

Figure 4. Particle size of Lopinavir B2 batch.

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

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -22.7	Peak 1:	-22.7	100.0	4.17
Zeta Deviation (mV): 4.17	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0790	Peak 3:	0.00	0.0	0.00

Result quality : Good

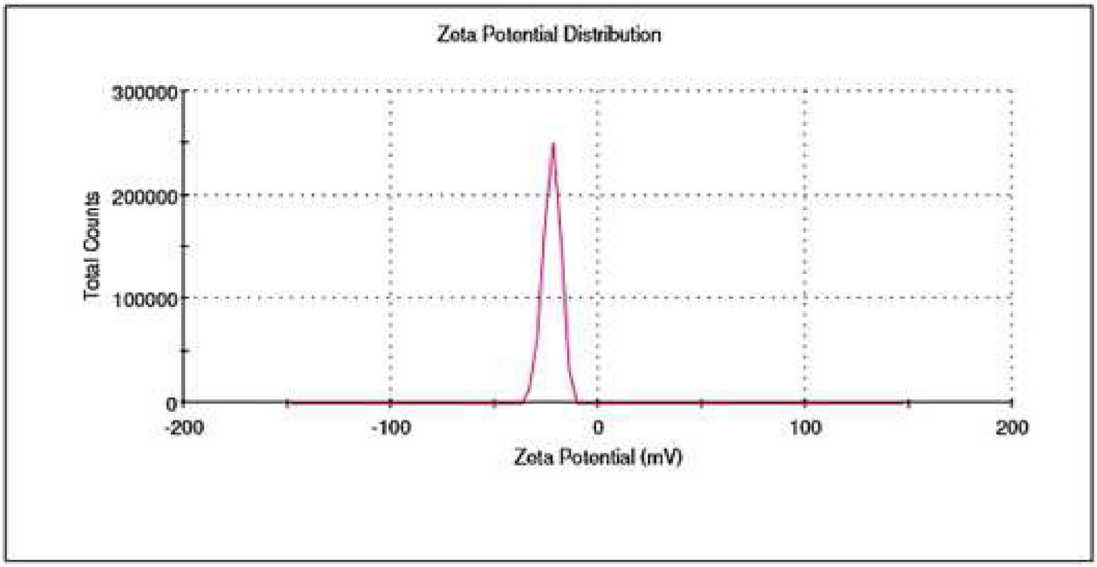

Figure 5. Zeta potential of Ritonavir A3 batch.

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -19.1	Peak 1:	-19.1	100.0	4.92
Zeta Deviation (mV): 4.92	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0847	Peak 3:	0.00	0.0	0.00

Result quality : Good

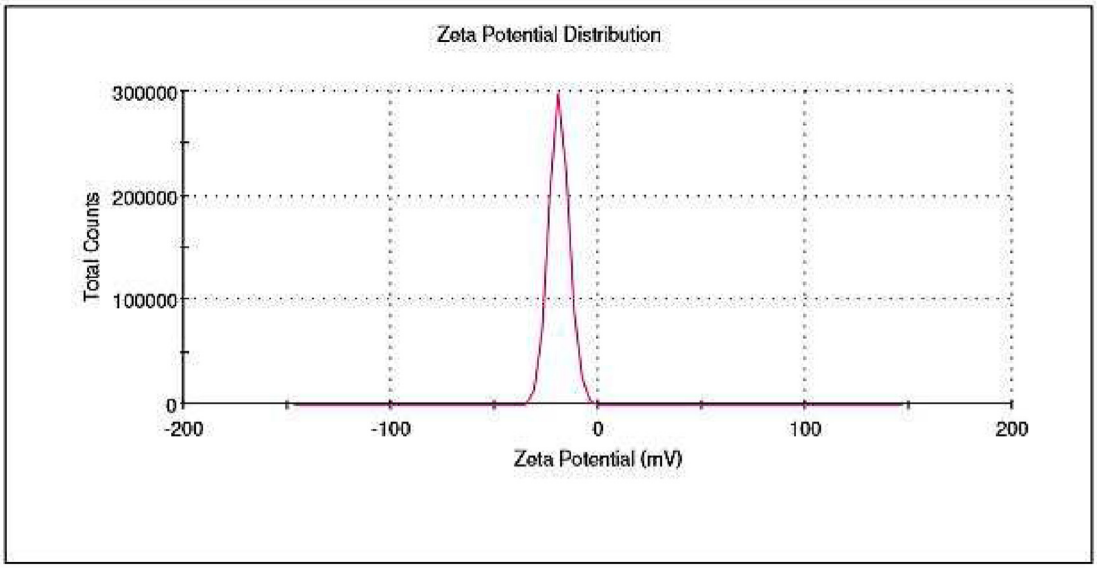

Figure 6. Zeta potential of Lopinavir B2 batch.

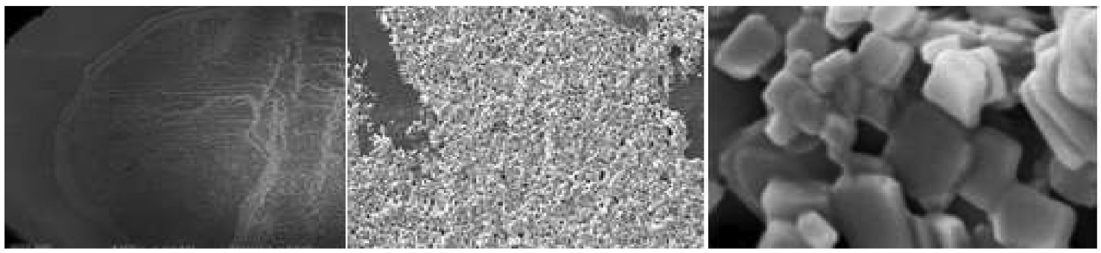

Figure 7. SEM photograph (Ritonavir nanosuspension with magnification 90 X, 100 KX and 50 KX).

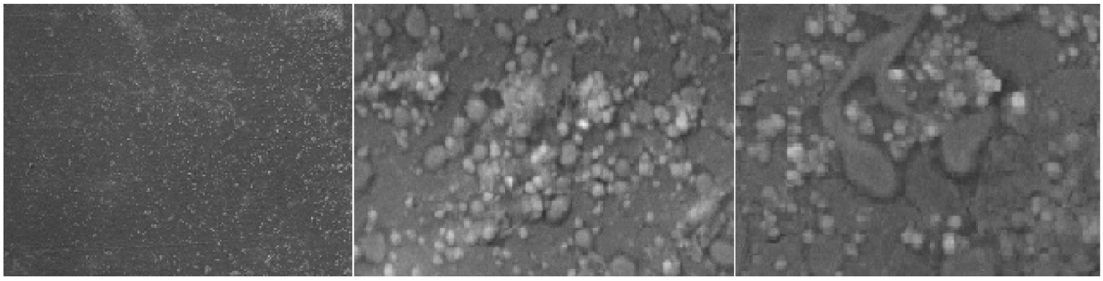

Figure 8. SEM photograph (Lopinavir Nanosuspension with magnification 90 X, 100 KX and 50 KX).

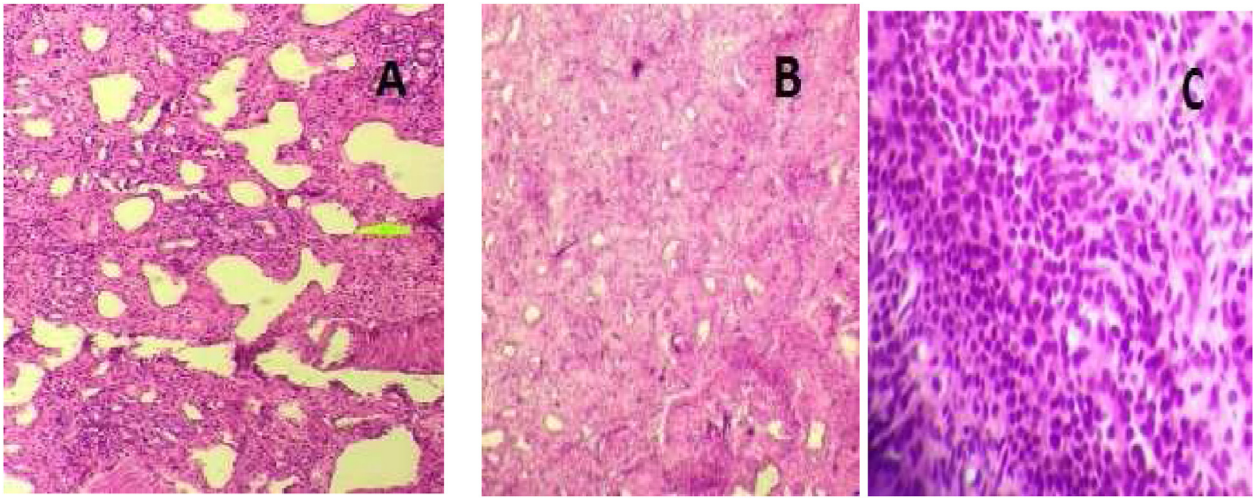

Figure 9. Histopathology study. (A- Positive control, B – Negative control, C –Nanosuspension).

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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