Intranasal Zotepine Nanosuspension: intended for improved brain distribution in rats

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Abstract

Background Zotepine (ZTP), an antipsychotic drug is well tolerated and particularly effective for treating negative symptoms of psychosis. But is limited by low oral bioavailability caused by substantial first pass metabolism and thereby less amount of drug reaches the brain due to blood brain barrier (BBB).

Objectives Since ZTP displays dose dependent side effects, purpose of the contemporary study is to develop zotepine loaded nanosuspension (ZTP-NS) for increased brain targeting in rats at lower doses.

Methods ZTP-NS is prepared by two techniques viz., sonoprecipitation (SP) and combination technique (high pressure homogenization preceded by precipitation) by employing various stabilizers. Optimized ZTP-NS was characterized for particle size, solid state, morphology and solubility. In vitro drug release of ZTP and formulations was conducted using Franz diffusion cell. Stability study was performed at different temperature conditions. Pharmacokinetic study was performed in Wistar rats to determine the bioavailability and brain distribution of ZTP after intra-nasal (IN) and intravenous (IV) administration. Histopathology of brain was done after repeated administration of IN ZTP dispersion and NS up to 14 days.

Results The optimized ZTP-NS formulated with Pluronic F-127 (0.3%w/v), Hydroxypropyl methyl cellulose E15 (0.3%w/v) and soya lecithin (0.4%w/v) showed particle size of 519.26 ± 10.44 nm & 330.2 ± 12.90 nm and zeta potential of -21.7 ± 1.39 mV and -18.26 ± 1.64 mV with sonoprecipitation and combination technique respectively. In vitro drug release was high ($81.79 \pm 3.23\%$) for ZTP-NS prepared by combination technique. Intranasal NS resulted in high brain concentrations of 8.6 fold (sonoprecipitation) and 10.79-fold hike in AUC_{0-24h} in contrast to intravenous ZTP solution. Histopathology results reveal no significant changes in brain microscopic images.

Conclusion ZTP-NS was successfully developed, characterized and found that nanosuspension is a favorable approach for intranasal delivery of zotepine.

Keywords Antipsychotics · Blood brain barrier · High pressure homogenization · Histopathology · Pharmacokinetics · Sonoprecipitation

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Introduction

Psychosis is a clinical syndrome with symptoms like delusions, hallucinations, catatonia and thought disorder [1]. Anti-psychotics are classified into two categories: Typical and atypical, the latter are mostly preferred, owing to their less extra pyramidal effects (EPS) compared to typical [2]. ZTP, an atypical antipsychotic belonging to dibenzothiepine derivative (Fig. 1) is marketed as Sirilept (Sun Pharma Pvt. Ltd) for oral use in India since 2010 [3, 4]. It was first invented and introduced in Japan in 1982. Though it was not approved by FDA, it has been marketed in Germany and countries of Europe and

Fig. 1 Structure of zotepine

Asia (brand names- Lodopin, Zoleptil, Nipolept) [5]. However, very limited clinical research was done on zotepine. As per the available literature, ZTP is more efficient compared to Haloperidol, Quetiapine, Aripiprazole, Ziprasidone, Chlorpromazine, Asenapine, Lurasidone, Iloperidone [6].

ZTP acts against dopamine (D2) receptor and serotonin 5-HT_{2A} receptor [5]. ZTP belongs to BCS class II that is practically insoluble in water (0.046 µg/L) and highly lipophilic (log P 5.6) [7]. The low solubility, dissolution rate and extensive first pass metabolism of zotepine diminish its bioavailability, which has been reported to be 7–13% [8]. In oral route, 30% of the drug metabolizes into Nor-zotepine (active metabolite) and remaining 70% of drug transforms to inactive metabolites such as 3-hydroxyzotepine, 2-hydroxyzotepine and zotepine-S-oxide [9]. Also, the drug cannot reach brain due to blood brain barrier (BBB) as it possesses tight junctions of endothelial cells forming a seal and restricts the entry of any foreign substance to the brain. Consequently, BBB also restricts paracellular diffusion of drug molecules to the brain and thus leads to lower permeability of drug. Exclusively small and oleophilic molecules could traverse the BBB via transcellular diffusion [10]. However, P-glycoproteins also hamper the drug entry into the brain. Although some of the molecules are expected to diffuse across BBB depending on molecular weight and lipophilicity [11]. Currently, ZTP is taken in the form of tablet with dosing range of 75–300 mg/day due to which it has dose related side effects like QTc prolongation, tachycardia and hypotension [12, 13] that could be reduced/subsided by decreasing the dose. Else, ZTP is effective in treating negative symptoms and recurrence of psychosis with less extrapyramidal side effects. In this regard, research was done on SMEDDS and cyclodextrin complexes of ZTP to overcome certain drawbacks. However, authors reported benefits only in terms of solubility and have not considered the oral bioavailability [7, 14]. As witnessed, the complex anatomy and physiology of central nervous system (CNS) poses a big challenge in transporting drugs to brain through conventional route of drug delivery. Though oral delivery of drugs is superior regarding patient compliance, shortcomings like slow onset of action, first pass effect, low bioavailability, rapid clearance and presence of absorption barriers

in the brain makes it less efficient for brain targeting. In this regard, nose to brain delivery has come up as a surrogate route of drug delivery to attain high concentrations in brain with low dose. Nerve endings of CNS in nasal cavity can take-up the drug directly from nose by means of olfactory pathway and trigeminal nerves by circumventing BBB [15–17]. Different formulation approaches like nanosuspension based gels [18], polymeric nanoparticles [19], solid lipid nanoparticles [20], nanostructured lipid nanoparticles [21], microemulsions [22], cyclodextrin complexes [23] etc., are being experimented to carry drug directly to brain. However, the surface area of nasal cavity is 150 cm² with total volume of 15–20 mL nasal fluid and thus the volume of intranasal drug delivery is restricted to 25–200 µL [19, 24, 25]. In this facet, among the different approaches nanosuspension was selected owing to its advantages like high drug loading capacity, increasing solubility and uncomplicated manufacturing process.

NS, also known as nanocrystal, is described as ‘a system consisting of drug stabilized with particle ranging in between 10–1000 nm’. In general, NSs are produced by techniques namely bottom-up and top-down processes based on the difference in manufacturing process [26]. In the former process, drug is dissolved in an organic phase and precipitated by anti-solvent addition in the presence of stabilizers [27]. Bottom up techniques are very simple and cost effective. The latter involves the dispersion of drug in water along with stabilizers and high energy input is necessary in which particle size gets reduced by mechanical comminution processes (microfluidization, wet milling and high pressure homogenization) [28, 29]. However, combination technology involves solvent precipitation as pretreatment followed by high energy input for the formulation of NS resulting in more stable systems [30].

Hence, the purpose of the current research is to formulate NS by sonoprecipitation and combination technique, compare the particle size, drug release, stability and also to investigate the brain targeting efficiency of ZTP-NS via intranasal delivery.

Materials and methods

Materials

Zotepine was acquired as a gift from Symed Laboratories, Hyderabad, India. Pluronic F-68, Pluronic F-127 and Vitamin E-TPGS were procured from Sigma® Aldrich, India. Hydroxypropyl methyl cellulose (HPMC)-E15 and Soya lecithin (SL) were purchased from Hi-media, India. Tween-80, calcium chloride (CaCl₂), potassium chloride (KCl) and sodium chloride (NaCl) were procured from TCI chemicals, India. HPLC grade solvents were purchased from Merck®, Mumbai, India. Methanol, ethanol and acetone were of analytical grade.

Methods

Preparation of ZTP-NS by sonoprecipitation method (SP)

Sonoprecipitation method was employed to formulate ZTP-NS as reported earlier in literature [31]. In a nutshell, accurately weighed quantity of ZTP (1%w/v) was dissolved in organic solvent and stabilizers (Tween-80, TPGS, Pluronic F-68, SL, HPMC E15, Pluronic F-127) were dissolved in triple distilled water to prepare solvent and anti-solvent phases respectively. Organic phase was forthwith added to aqueous phase with 22-gauge syringe to precipitate the drug under continuous stirring at 800 rpm on magnetic stirrer (Ika, India). The precipitated suspension was immediately kept for probe sonication (Sonics & materials Inc. Vibra cell VCX 750) under ice cold conditions for further control of particle growth. During ultrasonication, ultra sound burst was set to 6 s on/3 s off with 40% amplitude. After sonication, the NS was placed on magnetic stirrer at 800 rpm for 3 h to ensure the total evaporation of solvent. The prepared ZTP-NS was freeze dried (Freeze dryer, FD5508, Skadi-Europe) at -54°C under vacuum and stored in an air tight container for long term storage.

Optimization of formulation and process parameters For the optimization of ZTP-NS, the effect of different formulation parameters like stabilizer type (Tween-80, TPGS, Pluronic F-68, Pluronic F-127, SL and HPMC E-15),

Table 1 Various stabilizers and their combinations used for screening

Formulation code	Type of stabiliser	Stabilizer (% w/v)
F1	T-80	0.3
F2	F-127	0.3
F3	TPGS	0.3
F4	F-68	0.3
F5	HPMC	0.3
F6	Soya lecithin	0.3
F7	T-80	0.3
	HPMC	0.3
F8	TPGS	0.3
	HPMC	0.3
F9	F-127	0.3
	HPMC	0.3
F10	Soya lecithin	0.3
	HPMC	0.3
F11	F-127	0.3
	HPMC	0.3
	Soya lecithin	0.3

Table 2 Concentrations of stabilizers studied for optimization of ZTP-NS

Formulation code	F-127:SL:HPMC ratios (%w/v)
F12	0.1: 0.2: 0.3
F13	0.1: 0.4: 0.1
F14	0.1: 0.4: 0.5
F15	0.1: 0.6: 0.3
F16	0.3: 0.2: 0.1
F17	0.3: 0.2: 0.5
F18	0.3: 0.4: 0.3
F19	0.3: 0.6: 0.1
F20	0.3: 0.6: 0.5
F21	0.5: 0.2: 0.3
F22	0.5: 0.4: 0.1
F23	0.5: 0.4: 0.5
F24	0.5: 0.6: 0.3

combination of stabilizers (Table 1), concentration of stabilizer (0.1–0.6%w/v) (Table 2) and sonication time (5–15 min) were considered for sonoprecipitation method. The effect of all the above parameters on particle size, PDI and zeta potential was measured.

Combination technique (CT)

ZTP-NS was prepared by combination technique i.e. precipitation succeeded by high pressure homogenization (HPH). In brief, ZTP (1%w/v) was dissolved in acetone to prepare organic phase and stabilizers were dissolved in aqueous phase. The organic phase was injected into aqueous phase under continuous homogenization using high shear homogenizer (Ultra-Turrax, IKA T18) at 12000 rpm/10 min. The organic phase was vaporized using rotaevaporator (Heidolph, Germany) at 40°C for 30 min. The coarse suspension ($<5\ \mu$ size) was then subjected to HPH for 3–24 cycles at 1500 bar pressure under ice cold condition using Microfluidizer (LM 20, Micro fluidics, Trident) [32, 33]. The NS was freeze-dried at -54°C under vacuum using freeze drier for further studies.

Characterization and evaluation of ZTP-NS

Particle size, polydispersity index (PDI) and zeta potential (ZP)

Mean particle size, PDI and ZP of the NS were analyzed by dynamic light scattering using Zeta sizer (Malvern Zeta sizer). Samples were diluted with triple distilled water and measured at room temperature.

Attenuated Total reflectance Fourier transform infra-red spectroscopy (ATR-FTIR)

IR spectra of ZTP, HPMC E15, SL, Pluronic F-127, physical mixture and freeze-dried NS prepared by both techniques were recorded on IR spectrophotometer (Perkin-Elmer 1600, V-650 Jasco) in the range of $4000\text{--}400\text{ cm}^{-1}$ wave number to study drug excipient interaction.

Differential scanning calorimetry (DSC)

Thermal behavior of ZTP, SL, HPMC E15, Pluronic F-127, physical mixture and freeze-dried NS prepared by both techniques were examined using DSC (DSC Shimadzu, DSC-60, Kyoto, Japan). The samples were filled in aluminium pans with a perforated lid and analyzed at a temperature range of $40\text{--}170\text{ }^{\circ}\text{C}$ with an increment of $10\text{ }^{\circ}\text{C}/\text{min}$ under inert atmosphere.

Powder X-ray diffraction (PXRD)

X-ray diffractograms of ZTP and freeze-dried NS were recorded using powder x-ray diffractometer (D8Advance, Bruker, Germany) with Cu as a source of radiation. Assessment was conducted at a voltage of 40 kV and 25 mA. The samples were scanned at an angle of $20 \leq 2\theta \leq 80^{\circ}$ with a scanning rate of $2^{\circ}/\text{min}$.

Scanning electron microscopy (FESEM)

Surface morphology of plain ZTP, ZTP recrystallized in acetone, freeze dried NS prepared by SP and CT were scanned using a SEM (FEI, Quanta 250 model). The samples were staged on aluminium stub coated with Au through a sputter coater under vacuum and examined under microscope with ET detector run at an accelerating voltage of 10 kV at $10000\text{--}80000\times$ magnification.

Saturation solubility

Excess amount of ZTP and freeze-dried ZTP-NS were added to 5 mL screw cap vials in triple distilled water and simulated nasal fluid (SNF) (pH adjusted to 5.5 with HCl) respectively. The vials were kept in shaker (Lab Companion, SI300) at $37\text{ }^{\circ}\text{C}$ for 48 h; centrifuged at 8000 rpm/10 min. The clear liquid was separated and clarified with $0.22\text{ }\mu$ filter (Millipore) and examined for ZTP content using UV-Visible spectrophotometer at 264 nm [34, 35].

In vitro drug release

The drug release study of ZTP and ZTP-NS was conducted using Franz diffusion cell apparatus (Orchid, EMFDC08,

India). The diffusion cell was stocked with 28 mL of SNF (pH 5.5) in receptor compartment as release media and in donor compartment plain ZTP and ZTP-NS (Equivalent to 5 mg ZTP) dispersed in 5 mL of media was added and both the compartments were separated by a dialysis membrane (Hi-media, Molecular weight cutoff - 12 KD). A fraction of 2 mL sample was withdrawn at predetermined time intervals (15, 30, 45, 60, 75, 90, 105 and 120 min) and restored with same volume of fresh media. The samples were clarified with $0.22\text{ }\mu$ filter and after appropriate dilutions estimated the ZTP content using UV-Visible spectrophotometer at λ_{max} of 264 nm. The cumulative percentage drug release was compared from plain drug and NS [36].

Stability study

The optimized freeze dried ZTP-NS were stored at different temperatures ($25 \pm 2\text{ }^{\circ}\text{C}$ and $4 \pm 2\text{ }^{\circ}\text{C}$) for 6 months. The samples were analyzed at regular intervals (15 days, 1 month, 3 months and 6 months) for particle size and ZP using Malvern zeta-sizer.

In vivo evaluation

Animal protocol

The protocol (NIP/8/2016/PE/203) was reviewed and approved by Institutional Animal Ethical Committee (IAEC) for Care and Use of Laboratory animals. Male Wistar rats (230–250 g) were nourished with routine diet and water ad libitum. Animals were acclimatized to in-house animal facility for 1 week with 12 h light/dark cycle and temperature of $22 \pm 2\text{ }^{\circ}\text{C}$. Animals were segregated into four groups: Group A as positive control injected with intravenous (IV) ZTP solution (20% ethanol and 80% propylene glycol); Group B as positive control administered with intranasal (IN) ZTP solution; Group C was administered with oral ZTP dispersion (0.5% sodium carboxy methyl cellulose); Group D and E were administered with intranasal NS (SP and CT techniques). Further, each group was subdivided into eight groups for eight time points (0.5, 1, 2, 4, 6, 8, 12 and 24 h) with three rats in each [37, 38]. For intravenous injection, ZTP solution was diluted to 0.5 mL with saline and injected through tail vein. For intranasal dosing, animals were mildly anaesthetized with isoflurane inhalation before dosing and 50 μL sample (dose eq. to 4.4 mg/kg of ZTP) was instilled in each nostril using micropipette. While dosing, rats were held at slant position on their back for proper instillation of dose [39]. At fixed time intervals, blood specimens were collected in EDTA-coated tubes by retro-orbital puncture under mild anesthesia and immediately brain was excised from the sacrificed (CO_2 exposure) animal. The collected blood was centrifuged at 8000 rpm/10 min/ $4\text{ }^{\circ}\text{C}$, plasma was separated and preserved at $-20\text{ }^{\circ}\text{C}$ until further

analysis with HPLC. Similarly, brain samples were rinsed with saline to get rid of blood and dried up with filter paper and homogenized in 1.5 mL of phosphate buffer saline and acetonitrile (50:50 ratio) using Teflon homogenizer (Remi Motor, India) followed by centrifuging at 12000 rpm/15 min/10 °C [22, 40, 41]. The clear liquid was separated and preserved at −20 °C for further analysis with HPLC.

HPLC method for bio analysis

HPLC method was developed for analysis of bio samples using Waters HPLC system with PDA detector. Fortis column (C18, 250 mm × 4.5 μm, 5 μ) was used for analysis with ammonium acetate (20 mM): acetonitrile: methanol (10:45:45%v/v) as mobile phase at 1 mL/min flow rate and estimated at 264 nm. The stock solution (1 mg/mL) was made in methanol and working standards of ZTP were prepared by suitable dilutions with mobile phase. Linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) were validated for the developed method. Recovery was calculated by spiking ZTP working standards to blank plasma or brain homogenate to give ZTP concentrations of 0.1, 0.5, 1, 2, 4, 6, 8 and 10 μg/mL. The samples were analyzed and the concentration of extracted standard was compared with unextracted standard concentration.

Drug extraction

Plasma samples were precipitated by adding 300 μL acetonitrile (ACN) to 100 μL of plasma and vortexed. Deproteinized plasma was centrifuged at 12000 rpm/10 min/10 °C and the supernatant was injected (100 μL) into HPLC system. Deproteinization of supernatant obtained from brain homogenization was carried by adding 500 μL ACN to 300 μL of brain sample and vortexed. Each sample was centrifuged (12,000 rpm/10 min/10 °C), supernatant was collected and dried using nitrogen evaporator (Biotage, TurboVap®LV). Upon drying, the samples were reconstituted with 250 μL ACN, centrifuged and supernatant was injected (100 μL) to HPLC system for determining ZTP concentration.

Pharmacokinetic study

Pharmacokinetic parameters such as area under the curve (AUC), maximum concentration (C_{max}), time at maximum concentration (T_{max}), half-life ($T_{1/2}$) and mean residence time (MRT) were calculated using Phoenix WinNonlin software (version 8.1). Two indices such as: (i) Drug targeting efficiency and (ii) Drug transport percentage were calculated to evaluate the brain targeting efficiency of ZTP-NS [20, 42].

$$\begin{aligned} \text{Drug targeting efficiency (\%DTE)} &= \frac{B_{i,n}/P_{i,n}}{B_{i,v}/P_{i,v}} \\ &\times 100 \text{ Drug targeting percentage (DTP)} \\ &= \frac{B_{i,n} - B_x}{B_{i,n}} \times 100 \end{aligned}$$

Where, $B_x = B_{i,v}/P_{i,v} \times 100$.

$B_{i,v}$ is the AUC_{0-24h} (brain) after IV dosing.

$P_{i,v}$ is the AUC_{0-24h} (blood) after IV dosing.

$B_{i,n}$ is the AUC_{0-24h} (brain) after IN dosing.

$P_{i,n}$ is the AUC_{0-24h} (blood) after IN dosing.

Acute toxicity

In order to perform sub-acute toxicity study at low dose and high dose, Wistar rats ($n = 3$) were randomly separated into six groups. Group I is normal control; Group II blank NS; Group III and IV received NS (F18); group V and VI received NS(CT). Group I animals were administered with saline, group III and V were given a dose of 2.2 mg/kg equivalent to ZTP and group IV and VI were given a dose of 4.4 mg/kg. All the treatment groups received intranasal dosing once daily up to 14 days. Blood samples were collected and estimated for various hematological parameters. Animals were then euthanized with CO₂ inhalation and brain was extracted. The collected brain samples were washed with phosphate buffer saline (pH 7.4) and fixed in 10% formalin buffer for 24 h. The dehydrated organs were fixed in paraffin blocks and sectioned at 4 μm using slide microtome. The tissue sections were deparaffinized followed by staining (eosin and hematoxylin) and observed under optical microscope for any histopathological changes among different groups [22, 43].

Statistical analysis of data

All the data is given as mean ± SD. Results were interpreted using Prism software (version 6.01; Graph Pad, San Diego, CA) by applying un-paired student's t test and one-way ANOVA followed by Tukey's-Kramer multiple comparison tests. The results were considered notable at $p < 0.05$.

Results and discussion

Sonoprecipitation method

Screening of excipients

In this study, ZTP-NS was formulated by sonoprecipitation and combination technique. In sonoprecipitation method, selection of solvent phase plays a critical role in the particle size.

In our preliminary study, methanol and acetone were utilized as organic phase, as they solubilized maximum amount of drug. Use of methanol resulted in larger particles (2775 ± 126.90 nm) and aggregation with non-uniform distribution ($PDI = 0.808 \pm 0.05$). Whereas, NS prepared with acetone resulted in non-aggregated (1407 ± 84.46 nm and 0.328 ± 0.012), physically stable (2 days) system and thus acetone was used for further optimization. The impact of sonication time on particle size and PDI was evaluated at 5, 10, 15 and 20 min (Supplementary Fig. 1) and it was found that there is no reduction in particle size/PDI after 10 min (PS). Thus, sonication time was fixed as 10 min (6 s on/3 s off) for all the formulations. Further, stabilizer type and concentration were optimized as it helps in reducing the particle size by increasing the activation energy and reducing agglomeration [44, 45]. To achieve a stable nanosuspension of ZTP, different polymeric stabilizers (HPMC E15) and surfactants (Pluronic F-68, Pluronic F-127, SL, TPGS and T-80) were screened. All the stabilizers were screened at fixed concentrations (0.3% w/v) and its effect on particle size and PDI was determined. From the results, (Supplementary Fig. 2A) it was noticed that the particle size lowered in the order of $SL > F-68 > F-127 > T-80 > TPGS > HPMC E15$. The particle size of the ZTP-NS was higher with surfactants of low and high HLB values (SL-8; F 68–29; P-127-18-23) than with surfactants of medium HLB values (T80–15, TPGS-13.2). Furthermore, the particle size of ZTP-NS was less with polymer (F1-HPMC E15) when compared to formulations composed of surfactants (F2, F3, F4, F5 and F6). The zeta potential of the formulations was in the decreasing order of $SL > F-68 > T-80 > F-127 > HPMC E15 > TPGS$ (Supplementary Fig. 2B). From these results it is evident that particle size and ZP depends on stabilizer type. However, the NS prepared with single stabilizer was not physically stable (except pluronic F-127) for more than 3 days as noticed with increased particle size and PDI which may be ascribed to Ostwald ripening effect [46, 47]. Hence, combination of stabilizers was tried to achieve a stable formulation with less particle size and PDI. Further, all the surfactants were tried in combination with HPMC E15, a polymeric surfactant as it produced NS with comparatively less particle size (1026 ± 68.3 nm). HPMC E15 reduced the particle size and stabilized the formulation by inhibiting the crystal growth via formation of intermolecular interactions (hydrogen bonding) between ZTP and HPMC E15 and sterically hinders the crystallization process [48]. ZTP has four hydrogen bond acceptor sites and HPMC E15 has the ability to donate eight hydrogen bonds to ZTP, thereby showcasing the chances of hydrogen bonding. Also, HPMC E15 increases the viscosity of aqueous vehicle, thereby decreasing the rate of diffusion of drug from bulk solution to the crystal surface impeding the crystallization process [49]. The combination of HPMC E15 and Pluronic F-127 (F9) demonstrated lesser particle size (781.4 ± 49.3 nm) and PDI (0.462 ± 0.04) than other combinations

(F8, F9 and F10). This may be ascribed to additive effect of the chosen stabilizers. Hence, taking particle size and PDI into consideration the combination of HPMC E15 and F-127 was continued for further screening. However, the NS prepared with HPMCE15 and F-127 was showing less ZP (-10.1 ± 1.39 mV). From the preliminary screening it was observed that SL when used alone has shown better ZP (-17.3 ± 1.29). SL, being an ionic stabilizer gets adsorbed on particle surface and thus the surface charge and electrostatic repulsion between the particles prevent the aggregation, leading to a stable system. Also, it is reported to be a good penetration enhancer with no toxicity [46]. Hence to prepare NS, SL was considered for optimization. NS (F11) prepared with three stabilizers (SL, HPMC E15 and F-127) each at 0.3% w/v level resulted in particle size of 576.5 ± 27.8 nm, PDI 0.302 ± 0.05 and ZP of -21.8 ± 2.9 mV.

Optimization of ZTP-NS by sonoprecipitation method

From the preliminary screening studies, HPMC E15, Pluronic F-127 and SL were selected for the formulation of ZTP-NS. Based on the FDA inactive ingredients data base, limit (F-127: 0.1–0.5% w/v; SL: 0.2–0.6% w/v; HPMC E15: 0.1–0.5% w/v) was fixed for each stabilizer for IN route. Within these limits, NSs were formulated at different concentrations to achieve a stable system and the data of their composition along with the particle size, PDI and ZP is shown in Fig. 2.

In the prepared NS, particle size was in the range of 519.26 ± 10.44 to 1169 ± 42.41 nm and PDI from 0.272 ± 0.023 to 0.547 ± 0.023 with ZP ranging from 12.2 ± 1.94 to 28.8 ± 14.7 mV. The formulations prepared with combination of surfactants were studied for short term stability by storing at room temperature for 15 days and observed for particle size, PDI and physical stability. The formulation (F18) containing F-127 (0.3% w/v), SL (0.4% w/v) and HPMC E15 (0.3% w/v) resulted in particle size of 519.26 ± 10.44 nm, PDI 0.272 ± 0.023 and ZP of -21.7 ± 1.39 mV. Among all the combinations, F18 showed better stability i.e. there is no major difference in particle size, PDI and physical appearance after 15 days. Hence, F18 was selected for further studies.

Combination technique (CT)

In general, combining precipitation with HPH was recommended as it results in less particle size by inhibiting crystal growth further with increased stability of NS [44]. In this method, ZTP-NS was prepared with F-127 (0.3% w/v), SL (0.4% w/v) and HPMC E15 (0.3% w/v) by precipitation, succeeded by HPH. The consequence of pressure and number of cycles passed on particle size and PDI were determined in order to optimize the process parameters and the same is shown in Fig. 3a. Particle size and PDI of NS was measured from 300 to 1500 bar. At homogenization pressure (HP) of

Fig. 2 The effect of stabilizer concentrations on particle size, PDI and zeta potential

300 bars for 3 cycles the particle size and PDI were 1708 ± 70.9 nm and 0.84 ± 0.09 respectively. When the pressure was gradually elevated from 300 to 1500 bar, particle size and PDI decreased from 1708 ± 70.9 nm to 742 ± 32.4 nm and PDI from 0.84 ± 0.09 to 0.329 ± 0.056 respectively [34, 50]. Hence, 1500 bar pressure was fixed and effect of number of

cycles was studied. The ZTP-NS was homogenized at 1500 bar up to 24 cycles and the results are shown in Fig. 3b. On increasing the number of cycles, the particle size of NS reduced at 12 cycles with particle size, PDI and ZP of 330 ± 12.90 nm, 0.205 ± 0.029 and 18.26 ± 1.64 mV respectively. Further rise in the number of cycles resulted in increased size

Fig. 3 a The effect of homogenization pressure on particle size and PDI; and (b) the effect of number of homogenization cycles on particle size, PDI and zeta potential

Fig. 4 Particle size, PDI and zeta potential of (a) NS optimized by sonoprecipitation method (F18-NS); and (b) NS optimized by combination technique (CT-NS)

at 15 cycles (374 ± 17.39 nm). High pressure homogenizer decreases the particle size via collision and mechanical attrition of particles [51]. Considering these observations, it was of no use to exceed 12 cycles and thus the processing condition of 12 cycles at 1500 bar was fixed.

Characterization and evaluation

Particle size, PDI and ZP

The particle size, PDI and zeta potential were analyzed using Malvern zeta sizer by diluting the sample with triple distilled water. The optimized formulation prepared by sonoprecipitation (F18) gave particle size of 519.26 ± 10.44 nm, PDI of 0.272 ± 0.023 zeta potential of -21.7 ± 1.39 mV (Fig. 4a). The formulation produced by combination technique (CT) showed particle size, PDI and ZP of 330 ± 12.90 nm, 0.208 ± 0.029 and 18.26 ± 1.64 mV respectively (Fig. 4b).

ATR-FTIR

ATR-FTIR has been used to evaluate the compatibility among drug and stabilizers used in preparation of NS. The IR spectra

of ZTP, and excipients (SL, Pluronic F-127, HPMC E15), physical mixture and freeze-dried NS (F18 and CT) are manifested in Fig. 5. The plain ZTP has shown absorption peaks at 3064 , 1097 , 1052 and 754 cm^{-1} corresponding to C-NH₂ stretch, C-S stretch, C-O stretch and C-Cl stretch respectively. The distinctive peaks at 3064 , 1097 , 1052 and 754 cm^{-1} were maintained in NS formulations indicating the original composition of drug was retained.

Differential scanning calorimetry (DSC)

The DSC thermograms of plain ZTP, physical mixture (SL, HPMC, pluronic F-127 and ZTP) and freeze-dried ZTP-NS (F18 and CT) are shown in Fig. 6. The plain ZTP demonstrated a sharp endothermic peak at 94 $^{\circ}\text{C}$ corresponding to its melting point. Whereas in physical mixture endothermic peak of ZTP was seen at 92.2 $^{\circ}\text{C}$ and pluronic F-127 showed its characteristic melting point at 57 $^{\circ}\text{C}$. However, it was observed that a characteristic endothermic peak of ZTP at 94 $^{\circ}\text{C}$ have shifted to 85.2 $^{\circ}\text{C}$ and 88.19 $^{\circ}\text{C}$ in F18 and HPH formulations respectively indicating that the crystallinity of ZTP has changed or a probable transformation to amorphous state. To further confirm the nature of the drug PXRD was done.

Fig. 5 ATR-FTIR spectra of (a) SL; (b) Pluronic F-127; (c) HPMC E15; (d) ZTP; (e) physical mixture; (f) F18-NS and (g) CT-NS

Powder X-ray diffraction

The PXRD patterns of plain ZTP, lyophilized ZTP-NS (F18 and CT) were recorded and the same are shown in Fig. 7. The plain ZTP displayed strong peaks at 2θ of 19.8° , 21° , 22° , 24° and 28.3° revealing the crystalline nature of drug. In contrary, NS demonstrated peaks with low intensity, thereby indicating the decreased crystallinity or conversion to amorphous form. PXRD also confirmed the results of DSC.

Scanning Electron microscopy

The surface morphology of plain ZTP, precipitated in acetone and ZTP-NS are shown in Fig. 8. The plain ZTP and precipitated ZTP shows rectangular block shaped crystals ($>5 \mu\text{m}$ in size). While the NS prepared by sonoprecipitation converted the drug to spherical particles with consistent size range from

201.3 to 273.3 nm. The NS with CT also produced spherical particles ranging from 139.3 to 178.6 nm. The study proved the outcome of nanosizing upon precipitation and drug particles in NS have not shown any aggregation which could be attributed to the potential of stabilizers in preventing aggregation.

Saturation solubility

The saturation solubility of plain ZTP, ZTP-NS (F18 and CT) in water was $3.787 \pm 1.08 \mu\text{g/mL}$, $77.14 \pm 2.71 \mu\text{g/mL}$ and $84.791 \pm 3.65 \mu\text{g/mL}$ respectively. Similarly, the solubility of ZTP, F18 and CT-NS in SNF was $29.57 \pm 3.18 \mu\text{g/mL}$, $103.03 \pm 4.11 \mu\text{g/mL}$ and $112.53 \pm 5.263 \mu\text{g/mL}$ respectively (Supplementary Fig. 3). The increase in solubility with significant difference ($p < 0.001$) was observed for F18 and CT formulations in both media when compared to plain ZTP.

Fig. 6 DSC thermogram of (a) physical mixture, ZTP and F18-NS and (b) CT-NS

This may be due to genesis of nano-sized particles with high surface area, decrease in crystallinity of ZTP and improved wettability due to surfactants in NS. Formulations prepared by combination technique (CT) resulted high solubility as compared to sonoprecipitation method (F18) which could be ascribed to difference in particle size.

In vitro drug release

The drug release study was conducted to differentiate the release of ZTP from plain ZTP and optimized ZTP-NS (F18 and CT) and the same is shown in Supplementary Fig. 4. ZTP-NS (F18 and CT) has shown $77.3 \pm 3.57\%$ and $81.79 \pm 3.23\%$ drug release respectively within 2 h as compared to PD with

a release of $19.34 \pm 2.01\%$ ($p < 0.001$). The NS exhibited improved release due to reduced particle size, eventually higher surface area that enabled the rapid release of drug and due to amorphous nature of NS [31].

Stability study

Stability study of ZTP-NS (F18 and CT) was examined at two conditions for 6 months at particular time intervals (15 days, 1, 3 and 6 months) and the effect of stability conditions on particle size, PDI and ZP was deliberated and the data is given in Supplementary Table 1A and 1B respectively. NS prepared by sonoprecipitation and combination technique were stable without any significant difference in particle size, PDI and

Fig. 7 PXRD spectra of (a) NS prepared by combination technique (CT-NS); (b) Plain ZTP and (c) NS prepared by sonoprecipitation (F18-NS)

zeta potential up to 1 month at various storage conditions. An increase in particle size of F18 and CT from 607.3 ± 25.48 nm and 362.5 ± 11.57 nm to 1021 ± 37.28 nm and 493.21 ± 29.81 nm respectively was noticed at refrigerated condition at the end of sixth month. Similarly, an increase in particle size

of F18 and CT from 591.71 ± 29.18 nm and 371.5 ± 14.37 nm to 1273.9 ± 52.7 nm and 552.45 ± 38.72 nm respectively was observed which might be due to loss of polymer stabilization integrity with time resulting in physical instability/particle aggregation due to Ostwald ripening.

Fig. 8 SEM images of (a) plain drug, (b) ZTP recrystallized in acetone, (c & d) NS prepared by sonoprecipitation (F18-NS) and (e & f) NS prepared by combination technique (CT-NS)

Fig. 9 Concentration of ZTP after intranasal and intravenous administration of ZTP-NS in (a) plasma and (b) brain

HPLC method for bioanalysis

A mobile phase composition of ammonium acetate (20 mM): Methanol: ACN (10:45:45) was developed and further validated. The analytes were differentiated with a peak symmetry factor of <1.2 and retention time of 8.5 ± 0.33 min. Linearity was detected in the concentration range of 0.1–10 $\mu\text{g/mL}$. The LOD and LOQ of ZTP in plasma and brain homogenate were 0.05 $\mu\text{g/mL}$ and 0.075 $\mu\text{g/mL}$ respectively. Recovery of ZTP from plasma and brain homogenate was $>90\%$ and the results were also reproducible.

Pharmacokinetics of plasma and brain

The concentration of ZTP in plasma and brain samples were measured after intravenous (IV) and intranasal (IN) administration of PD, ZTP-NS to male Wistar rats and the same is shown in Fig. 9a and b respectively. Table 3 presents the pharmacokinetic

data of all groups administered with intravenous ZTP solution and intranasal NS. Plasma and brain concentrations of oral ZTP were not detectable by HPLC. As per the literature, the plasma concentrations of ZTP after single oral dose (50 mg) in healthy volunteers is approximately 13 to 31 $\mu\text{g/L}$ after 3 h. The reason for low plasma concentrations can be attributed to first pass metabolism due to which most of the drug metabolizes in liver [52]. Nada K et al. have reported that the concentration of unchanged zotepine in serum is higher in humans than rats [53]. Plasma concentration of ZTP was high in intravenous PD followed by intranasal PD and significantly lesser in case of intranasal NS. IN NS attained higher C_{max} (13.36 ± 1.82 $\mu\text{g/g}$ and 14.86 ± 1.11 $\mu\text{g/g}$ for F18 and CT respectively) in brain compared to that of IN PD and IV PD (1.90 ± 0.37 $\mu\text{g/g}$ and 2.37 ± 0.65 $\mu\text{g/g}$ respectively). The results showed significant difference ($p < 0.00001$) between IN NS and IV PD but there is no significant difference ($p < 0.547$) between

Table 3 Pharmacokinetic parameters

		ZTP solution (IV)	ZTP solution (IN)	F18-NS (IN)	CT-NS (IN)
AUC_{0–24}					
(µg/ml*h)	Plasma	14.270 ± 2.493	6.760 ± 0.249	0.575 ± 0.059	0.775 ± 0.061
	Brain	2.4061 ± 0.361	5.878 ± 0.691	32.702 ± 2.66	40.692 ± 5.002
C_{max}					
(µg/ml)	Plasma	8.186 ± 0.977	3.009 ± 0.447	0.417 ± 0.062	0.467 ± 0.072
	Brain	2.378 ± 0.651	2.120 ± 0.374	13.366 ± 1.82	14.867 ± 1.111
T_{max}(h)	Plasma	0.5	0.5	0.5	0.5
	Brain	0.5	1	1	1
T_{1/2} (h)	Plasma	2.41 ± 0.36	2.49 ± 0.21	5.14 ± 0.48	4.26 ± 0.44
	Brain	1.33 ± 0.21	4.0 ± 1.33	3.56 ± 0.60	3.75 ± 1.07
MRT (h)	Plasma	2.06 ± 0.44	3.95 ± 0.28	5.82 ± 0.49	4.97 ± 0.58
	Brain	0.97 ± 0.15	3.04 ± 0.24	4.29 ± 0.63	4.03 ± 0.89

IN PD and IV PD. Area under the curve (AUC_{0-24}) values in brain also established the supremacy of IN nano-suspension (32.70 ± 2.6 h* μ g/g, 40.69 ± 5.0 h* μ g/g for F18 and HPH respectively) over IN PD (3.77 ± 0.4 h* μ g/g) and IV PD (2.40 ± 0.36 h* μ g/g) with significant difference ($p < 0.00001$). The literature reveals that, uptake of drug from nasal cavity to the brain happens typically by two pathways: (i) Systemic pathway-in this pathway some amount of the drug directly goes into systemic circulation and hits the brain by traversing BBB; (ii) Olfactory pathway - In this path the drug moves straightly into CSF/brain from nasal cavity. Hence, it can be assumed that the proportion of drug reaching brain followed by intranasal administration may be contributed by the above two pathways. The excipients used in NS may also lead to higher absorption levels of drug. Soya lecithin increases nasal absorption by enhancing the permeability of drug [54]. Similarly, pluronic F-127 increases the transcellular transport of drug via nasal cavity by decreasing the particle size, viscosity and elasticity of mucus [55]. Whereas, HPMC E15 helped in improving the drug residence time in nasal cavity due to its mucoadhesive property [56]. The DTE % and DTP correspond to the percentage of drug transported straightway to the brain by the olfactory pathway. F18 formulation exhibited highest DTE% and DTP ($33,711.61 \pm 2232.6$ & 97.28) among the tested formulations followed by CT ($35,147 \pm 1945.7$ & 96.51%) when compared to PD (520 ± 108.87 & 80.6%) (Supplementary Table 2). The higher DTE% and DTP values implies that NS has superior brain targeting efficiency and the results are in agreement with the previously reported works [57].

Rapid transport of drug to the brain followed by intranasal administration of NS is attributed to its lower particle size and allowing the drug to be transported by transcellular route [58].

Acute toxicity

In vivo safety of ZTP-NS was evaluated in-terms of hematology and histopathological changes of brain after intranasal administration in rats.

Hematology

Hematology was performed for rats administered with ZTP NS (2.2 and 4.4 mg/kg) for 14 days and compared with results of the zeroth day. Results of all the hematological parameters on 14th day are comparable with zeroth day indicating that the NS did not alter any of the hematology parameters and can be considered as safe for administration and the data of the same is shown in Table 4.

Histopathology of brain

The microscopic images of brain tissue are shown in Fig. 10. Histopathology of brain exposed healthy appearance without any notable changes after 14 days treatment. There was no significant change in microscopic structure of brain treated with ZTP-NS as compared to control group. Hence, intranasal ZTP-NS could be an effective and safe approach for treating the disease condition.

Table 4 Hematological parameters after intranasal administration of blank NS, F18-NS and CT-NS up to 14 days

	Day	Group I	Group II	Group III	Group IV	Group V	Group VI
RBC (10^6 cells/ μ L)	0	8.68 ± 0.11	8.87 ± 0.07	8.61 ± 0.05	8.72 ± 0.09	8.83 ± 0.03	8.53 ± 0.061
	14	8.71 ± 0.09	9.47 ± 0.13	8.55 ± 0.13	8.32 ± 0.15	9.17 ± 0.23	8.72 ± 0.034
WBC (10^3 cells/ μ L)	0	15.18 ± 1.46	15.79 ± 1.15	9.85 ± 2.75	13.18 ± 1.36	15.47 ± 0.82	15.63 ± 0.67
	14	16.31 ± 1.21	15.41 ± 0.93	8.48 ± 2.24	12.62 ± 1.72	13.84 ± 0.73	15.54 ± 0.83
Hemoglobin (g/dL)	0	11.5 ± 1.46	15.6 ± 0.14	16.3 ± 0.135	16.2 ± 0.223	15.47 ± 0.53	11.35 ± 0.42
	14	11.1 ± 2.139	16 ± 0.329	17.2 ± 0.42	15 ± 0.957	16.36 ± 0.262	10.24 ± 0.39
Platelets (10^3 cells/ μ L)	0	412 ± 78.3	894 ± 54.39	564 ± 73.38	916 ± 32.7	950 ± 10.38	764.29 ± 53.2
	14	475 ± 35.38	724 ± 43.6	710 ± 21.15	869 ± 47.33	921 ± 17.3	721.37 ± 26.4
Hematocrit (%)	0	34.4 ± 2.42	44 ± 2.47	42.5 ± 1.22	42.4 ± 1.32	43.1 ± 1.32	42.37 ± 0.83
	14	32.3 ± 1.41	46.3 ± 1.8	42.1 ± 0.92	41 ± 1.36	42.78 ± 1.26	41.92 ± 0.98
MCV (fL)	0	49.6 ± 2.37	48.1 ± 1.44	51.2 ± 2.37	48 ± 1.31	49.6 ± 2.19	39.12 ± 1.30
	14	47.5 ± 1.83	48.9 ± 2.64	49.3 ± 1.12	48.9 ± 1.23	47.37 ± 1.38	40.25 ± 1.41
MCH (pg)	0	18.1 ± 0.01	18.4 ± 0.03	18.2 ± 0.02	18.1 ± 0.01	18.1 ± 0.02	18.37 ± 0.02
	14	18 ± 0.01	18.7 ± 0.04	18.1 ± 0.02	17.7 ± 0.03	17.9 ± 0.012	18.21 ± 0.03

Fig. 10 Histopathology images of sections of brain: (a) Control, (b) intranasal blank NS, (c) intranasal NS prepared by sonoprecipitation (2.2 mg/kg), (d) intranasal NS prepared by CT (4.4 mg/kg)

Conclusion

In the present study, ZTP-NS was formulated by both sonoprecipitation and combination method. The NS prepared by combination technique was physically more stable than sonoprecipitation method indicating the effect of production method on stability. With intranasal dosing, ZTP concentration in brain ($C_{\max}$ and AUC_{0-24h}) was significantly improved thereby suggesting that desired response can be achieved at lower doses. No considerable changes were noted in hematology and brain histopathology in intranasally treated animals signifying ZTP to be safe. Hereby, it can be concluded that ZTP-NS is an effective and safe approach in targeting drug to brain.

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Author's contribution SR designed the study, conducted experiments and drafted the manuscript. ST: analytical and bioanalytical method development. NR: reviewed and revised the manuscript. RR: helped in preparation of nanosuspension. VS: supported in oral, intranasal and intravenous dosing and blood collection & brain extraction of all the animals. ND: helped in acute toxicity study. SS: supervision of all the experiments.

Compliance with ethical standards

Conflict of interest On behalf of all the authors, we report no conflict of interest.

Human participants and/or animals Humans are not involved in the study. Wistar rats were used in pharmacokinetics and toxicity study. The animal protocol (NIP/8/2016/PE/203) was approved by Institutional Animal Ethical Committee (IAEC) for Care and Use of Laboratory animals and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

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