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Original Research Article

ENHANCEMENT OF AQUEOUS SOLUBILITY OF EZOGABINE: PREPARATION AND CHARACTERIZATION OF EZOGABINE NANOSUSPENSION ANTICIPATED FOR NOSE TO BRAIN TARGETING BY 3² FACTORIAL DESIGN

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Abstract

Context: Adverse effects after oral administration, as well as low solubility, present a substantial contest for the suitability of formulations for intranasal drug delivery.

Objectives: The objective of the present work was to develop ezogabine nanosuspension for enhancement of solubility and direct olfactory administration by 3² full factorial design and determine its pharmacokinetic and pharmacodynamic profile in rats.

Methods: The ezogabine nanosuspension was developed by nanoprecipitation-ultrasonication method. The formulation was planned to develop by using a 2-factor, 3-level factorial design. The formulations were subjected to various in-vitro characterizations like particle size analysis, saturation solubility, in-vitro drug diffusion, zeta potential, SEM and TEM. The selected formulation was intranasally instilled into the nostrils of rats with the help of cannula for determining pharmacokinetic and pharmacodynamic profile in-vivo.

Results: The formulation showed better results in terms of in-vitro and in-vivo study. The statistical analysis of data revealed that polymer concentration had significant effect on particle size and no significant effect on saturation solubility and in-vitro drug diffusion. Whereas, ultrasonication time had significant effect on particle size, saturation solubility and in-vitro drug diffusion (solubility). The C_{max} revealed concentration of ezogabine in brain and plasma as 0.1812 µg/ml and 0.183 µg/ml, resp., for plain suspension and concentration of ezogabine in brain and plasma as 0.7885 µg/ml and 0.7483 µg/ml, resp, for nanosuspension at same dose of 1 mg/ml when administered intranasally.

Conclusion: The present investigation confirmed that the potential of ezogabine nanosuspension for enhancement of aqueous solubility and providing direct nose-to-brain delivery.

Keywords: Ezogabine, Nanosuspension, Nanoprecipitation, Factorial design, Aqueous solubility.

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Introduction

Epilepsy is among the most prevalent of the serious neurological disorders, affecting, from 0.5 to 1% of the world population. Interestingly, the prevalence of epilepsy in developing countries is generally higher than in developed countries⁽¹⁾. This disease has a multifactor etiology and is the result of abnormal synchronical discharges in a group of neurons, which are produced by a deviant dynamism of neuronal networks⁽²⁾. Epileptic seizures are paroxysmal clinic events arising from neuronal hyper-excitability and hyper-synchrony of the cerebral cortex, either locally (partial seizures) or diffusely in both hemispheres (generalized seizures). The agitated neuronal activity that occurs during a seizure is caused by a sudden imbalance between the inhibitory and excitatory signals in the brain with gamma amino butyric acid and noradrenaline respectively being the most important

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neurotransmitters involved⁽³⁾. Ezogabine is an anticonvulsant used for the treatment of partial epilepsies. Ezogabine acts as a neuronal KCNQ/Kv7 potassium channel opener. This mechanism of action is unique among antiepileptic drugs, and may hold promise for the treatment of other neurological conditions, including migraine and neuropathic pain⁽⁴⁾.

Intranasal delivery of a formulation is possible and easily accessible. The segments of nasal cavity include the olfactory epithelium which is a direct pathway for brain targeting. Nanosuspension has a size range from 1-1000nm. So the nanosuspension can be administered intranasal and it crosses the tight cell junctions of the Blood Brain Barrier easily. Thus the nanosuspension is targeted to brain and this therapy is painless and effortless⁽⁵⁾. Advantages of intranasal administration include a large surface area for delivery, rapid achievement of target drug levels, circumvent blood brain barrier (BBB) and avoidance of first pass metabolism; furthermore, this delivery route is non-invasive, maximizing patient comfort and compliance⁽⁶⁾. The nanoprecipitation technique for nanoparticle manufacture was first developed and patented by Fessi and coworkers⁽⁷⁾. This technique presents numerous advantages, in that it is a straight forward technique, rapid and easy to perform⁽⁸⁾. Different hydrophobic drugs have already been formulated successfully in this way, for instance donepezil⁽⁶⁾, olmesartan medoxomil⁽⁹⁾, felodipine⁽¹⁰⁾, acetazolamide⁽¹¹⁾, esomeprazole⁽¹²⁾, nitrendipine⁽¹³⁾.

Currently marketed potassium channel opener is found entirely in oral dosage form. However, alternative routes, in particular intranasal administration, may provide benefits relative to oral dosing. Since, oral dose of potassium channel opener available currently in the market is as thrice a daily tablet (50, 200, 300 or 400 mg). These potassium channel opener also showed the gastrointestinal disorders (nausea, constipation, dyspepsia and dry mouth), metabolism and urinary disorders (dysuria, urinary hesitation, haematuria, chromaturia, urinary retention and nephrolithiasis)⁽¹⁴⁾ etc. As a result, it is very important to develop non-gastrointestinal delivery system of these potassium channel opener for treatment of epilepsy. The postulate for this study was that, the formulation gives nose to brain targeted drug delivery via olfactory pathway. In the present investigation we used different polymer conc. (X_1) and ultrasonication time (X_2) to study the effect on particle size (Y_1), saturation solubility (Y_2) and in-vitro drug diffusion (Y_3) by 3^2 full factorial designs. The graphs and mathematical models were computed using Design Expert 10 (Stat- Ease, USA) software.

MATERIALS

Chemicals and reagents

Ezogabine [Ethyl-N-(2-amino-4-[(4-fluorophenyl) methyl] amino} phenyl) carbamate] as a drug candidate was received as a gift sample from Lupin Pharma, Aurangabad, Maharashtra, India. The tween 80, poloxamer 188, polyvinyl pyrrolidone K 25, polyethylene glycol 400 and ortho phosphoric acid were purchased from Loba Chemie Pvt. Ltd, Mumbai. Methanol was purchased from Molychem lab, Mumbai. HPLC grade methanol, acetonitrile and water were purchased from Rajesh chemicals, Mumbai. All other chemicals were of diagnostic grade or highest grade commercially accessible.

Animals

Healthy, young, male Albino-Wistar rats weighing 200–220 g were selected for the study and adapted to the laboratory environments for minimum five days earlier to the trial and casually allotted to weight-matched investigational sets. Animals were acquired from National Institute of Biosciences, Pune, Maharashtra, India. The investigational procedure was sanctioned by the Institutional Animal Ethics Committee (IAEC) of protocol number (1211/PO/ac/08/CPCSEA). The animals were kept in polypropylene cages in sets of six in each cage and were kept in a room maintained at $25 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle. They were delivered standard laboratory animal feedstuff (Prashant Enterprises, Pune), water ad libitum.

METHODS

Preparation of ezogabine nanosuspension

Ezogabine nanosuspension was prepared by the nanoprecipitation–ultrasonication method with different conc. of polymer [Poloxamer 188 (10, 20, 30 mg)] and ultrasonication time. Tween 80 and Poloxamer 188 were dissolved in distilled water (10 ml), mixed and labeled as mixture 1. This mixture was kept on magnetic stirrer for 15 min. to get homogeneous mixture. Ezogabine (10 mg) was dissolved using defined volume of methanol (2ml). This solution was slowly added dropwise to mixture 1 with constant stirring on magnetic stirrer for 1 hr. After 15 minutes, the solutions were kept in ultrasonicator (Citizen Digital Ultrasonic Cleaner, CD-4820) for specified time (10, 20 and 30 min). Hence, the nanosuspension was formed ^(15, 16). Formula for the preparation of ezogabine nanosuspension using 3² factorial design is given in table 1.

Table 1: Formulation table for the preparation of ezogabine nanosuspension using 3² factorial design

Sr. No.	Formulation code	EZG (mg)	Tween 80 (ml)	Poloxamer 188 (mg)	Ultrasonication time (min)	Methanol (ml)	Distilled Water (ml)
1	NS ₁	10	0.2	10	10	2	10
2	NS ₂	10	0.2	20	10	2	10
3	NS ₃	10	0.2	30	10	2	10
4	NS ₄	10	0.2	10	20	2	10
5	NS ₅	10	0.2	20	20	2	10
6	NS ₆	10	0.2	30	20	2	10
7	NS ₇	10	0.2	10	30	2	10
8	NS ₈	10	0.2	20	30	2	10
9	NS ₉	10	0.2	30	30	2	10

Preparation of plain suspension using tragacanth

The tragacanth mucilage was prepared and mixed with equal volume of distilled water. PVP K25 and PEG 400 were dissolved in distilled water and mixed with tragacanth mucilage. The ezogabine was dissolved in methanol. This solution was slowly added to tragacanth mucilage using mortar and pestle. Then this solution was filtered through muslin piece and final volume was adjusted with distilled water. The suspension was formed and preserved for further use⁽¹⁷⁾.

CHARACTERIZATION OF NANOSUSPENSION

Particle Size Analysis

The particle size was measured using a Zetasizer Nano ZS⁽¹³⁾ (Malvern Instruments, Malvern, UK).

Saturation solubility studies

Nanosuspension equivalent to 10 mg of ezogabine were taken and individually hosted into 25 ml stoppered conical flask having 10 ml distilled water. The flasks were closed and retained in rotary shaker for 24 hrs. at 37°C and equilibrated for 2 days. The samples were collected afterwards the definite time pause and it was filtered and diluted with distilled water suitably. The diluted samples were investigated by means of UV spectrophotometer⁽¹⁸⁾ (Shimadzu- 1800, Japan) at 218.2 nm.

***In-vitro* drug diffusion study**

The freshly removed goat nasal mucosa excluding the septum portion was collected from the slaughter house in phosphate buffer (PB), pH 6.4. The superior nasal conche was identified and separated from the nasal membrane; the excised superior nasal membrane was mounted on Franz diffusion cell (Electro lab, India). The tissue was stabilized using PB (pH 6.4) in both the compartments and allowed to stir for 15 min. on a magnetic stirrer (FHMS-3491, Remi lab, Mumbai). The temperature of the receiver chamber containing 30 ml of diffusion media (PB, pH 6.4) was controlled at $37^{\circ}\text{C} \pm 1$ under continuous stirring with teflon-coated magnetic bar at a constant rate, in a way that the nasal membrane surface just flushes the diffusion fluid. A volume of 1 ml of each selected formulation was placed in the donor compartment of Franz diffusion cell. Samples from the receptor compartment were withdrawn at predetermined time intervals and analyzed. Each sample removed was replaced by an equal volume of diffusion media. Each study was carried for a period of 2.5 hrs. during which the drug in receiver chamber (mg/ml) across the goat nasal membrane was calculated at each sampling point⁽¹⁹⁾.

Similarity factor determination

The similarity factor f_2 was used to check the similarities between release profile of reference standard and drug from the test formulation. It was adopted by FDA Centre for Drug Evaluation and Research (CDER) as an assessment criterion of similarity between different *in-vitro* profiles. Its value is from 50 to 100, the values larger than 50 shows similarities⁽²⁰⁾.

Drug release kinetics

To analyze the mechanism of drug release kinetics from the dosage form, the data obtained were fitted into zero order, first order, Higuchi's model, Korsmeyer-Peppas and Hixson-Crowell's model using Microsoft Office Excel 2010⁽²¹⁾.

Drug Content

The drug content of nanosuspension was analyzed by dispersing a 1ml of nanosuspension in mobile phase (Water:Acetonitrile:Methanol, 50:20:30 v/v) followed by sonication and filtering through 0.22 μm filter. The amount of drug was determined by HPLC (Isocratic LC-NET II/ADC, JASCO Corporation, Japan). The drug content was determined by two methods, external standard and calibration curve method⁽²²⁾.

Zeta Potential

The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Measurements were performed in distilled water adjusted with 0.9% (w/v) sodium chloride solution to a conductivity of 50 S cm^{-1} and a pH of 5.5–6.0 to obtain information on the surface charge⁽²³⁾.

Scanning Electron Microscopy

The SEM was used to attest the consistency of particle shape and size. The sample was smeared on a small piece of adhesive carbon tape which was fixed on a brass stub. The sample, then subjected to gold coating using sputtering unit (Model: JFC1600) for 10 sec at 10 mA of current. The gold coated sample placed in chamber of SEM (JEOL/JSM6390LA, Japan) and secondary electron/back scattered electron image was recorded⁽²⁴⁾.

Transmission Electron Microscopy

An extremely small amount of nanosuspension was suspended in water (just enough to obtain a slightly turbid solution). The solution was ultrasonicated to disperse the particles. A drop of the solution was then pipetted out and cast the drop on carbon-coated grids of 200 mesh. This grid was then mounted in the instrument and photograph was taken using TEM instrument ⁽²⁵⁾(JEOL/JEM2100, Japan).

Stability study

Stability studies for nanosuspension were conducted at two different storage conditions, viz., room temperature and refrigerated conditions (2–8°C) for 3 months. The three batches of selected nanosuspension were used for each storage condition. At periodic time intervals, the samples were withdrawn and analyzed for particle size and drug content⁽²⁶⁾.

Pharmacodynamic study

The anticonvulsant activity of ezogabine nanosuspension was studied against maximal electro-shock-induced convulsion in rats. The 9 rats were weighed and numbered. They were divided in to three groups each consisting of 3 rats. The first group was treated as control, second group as standard and third group as test. The first group remains untreated, second group was treated intranasally with phenytoin (25 mg/kg) and third group was treated intranasally with ezogabine nanosuspension. Electroconvulsions were produced by applying current (150 mA, 0.2 s) through ear clip electrodes using electroconvulsimeter (Besto, India). After 30 min of administration the different phases of seizures were measured. Concisely after application of current an instant severe tonic phase was observed which was characterized by maximal extension of the anterior and posterior legs. At the end of tonic phase clonic phase starts which was characterized by paddling movement of the hind limb and shaking of body. During stupor phase which was observed after tonic and clonic phase rat remained silent without any movement. Recovery time was recorded as total time from starting of tonic phase till animal regains its normal movement⁽²⁷⁾.

Estimation of drug content in the brain by HPLC method

Male Albino-Wistar rats (n= 6) weighing between 200-220 g were selected for the present study. The dose of plain suspension and selected nanosuspension of ezogabine (0.1 ml) was administered at same dose of 1 mg/kg through the intranasal route with the help of cannula fitted in the 1 ml syringe and the rats were kept in upright position at an angle of 90°, so that maximum drug concentration can reach to the brain. After specific time interval of intranasal dosing the animals were sacrificed under ether anesthesia and the whole brain was excised, isolated and weighed. The brain tissue was then homogenized in 1% acetic acid using tissue homogenizer (Remi, Mumbai, India), and particulate matter removed by centrifugation (Tanco Pvt. Ltd, India) and filtration. The clarified supernatant was analyzed for drug content in brain via HPLC (Isocratic LC-NET II/ADC, JASCO, Japan) method. The mobile phase consisted of Water:Acetonitrile:Methanol (50:20:30 v/v). The mobile phase was set at a flow rate of 1 ml/min. The run time of the sample was kept 10 min. The ultra-violet detection of ezogabine was performed at 220 nm. Pharmacokinetic parameters were evaluated using PK solver software (non-compartmental modeling). Pharmacokinetic parameters such as C_{max} , t_{max} , $AUC_{0 \rightarrow 24}$, $AUC_{0 \rightarrow \infty}$, and MRT were calculated for both the plain suspension and nanosuspension group⁽⁶⁾.

Results and Discussion

Factorial design

The responses observed for all the formulations such as particle size (Y_1), saturation solubility (Y_2) and *in-vitro* drug diffusion (Y_3) with variables polymer conc. (X_1) and ultrasonication time (X_2) are given in table 2.

Table 2: Formulations with their responses

Formulation Code	Variables		Responses		
	X_1	X_2	Y_1	Y_2	Y_3
NS ₁	10	10	416.8	0.356	87.19
NS ₂	20	10	399.6	0.378	88.18
NS ₃	30	10	449.6	0.356	88.48
NS ₄	10	20	261	0.5681	94.24
NS ₅	20	20	238	0.553	92.62
NS ₆	30	20	287	0.5795	93.57
NS ₇	10	30	212.2	0.7477	96.43
NS ₈	20	30	155.2	0.7718	95.13
NS ₉	30	30	230.6	0.7265	98.91

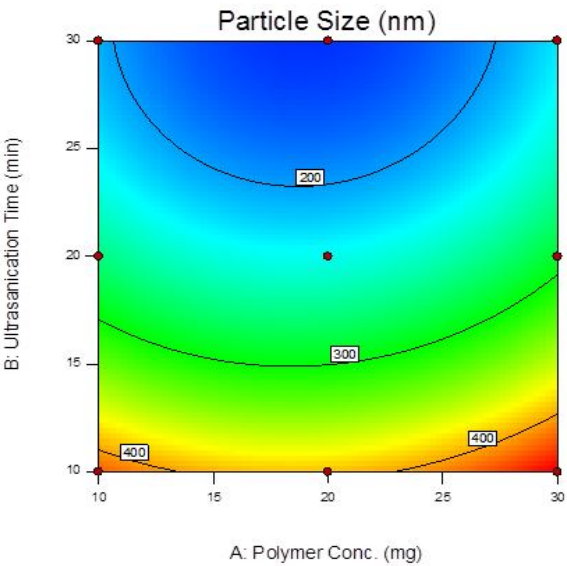


Figure 1: (a) Contour surface plot of particle size (b) Three-dimensional response surface plots of particle size.

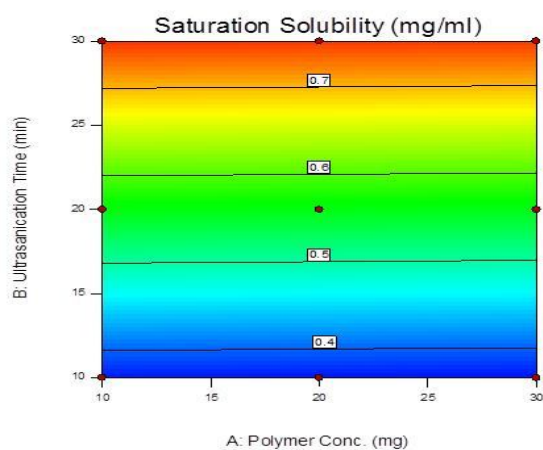
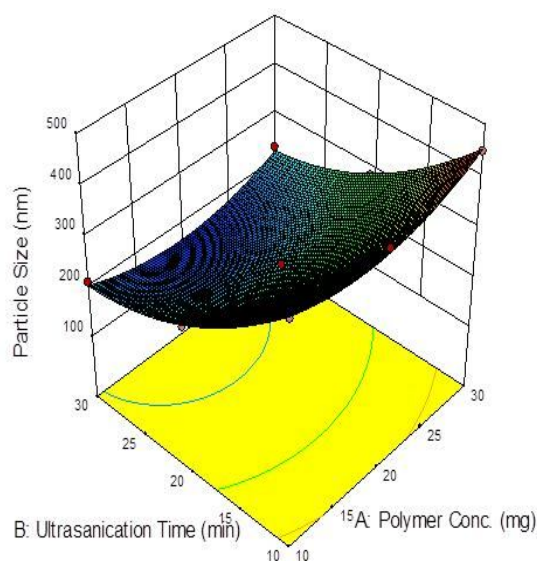
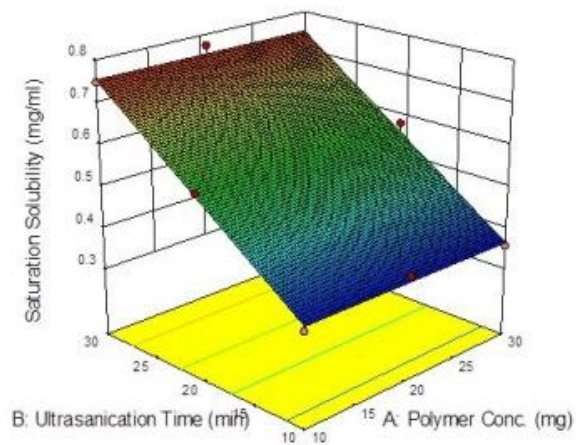


Figure 2: (a) Contour surface plot of saturation solubility (b) Three-dimensional response surface plots of saturation solubility.



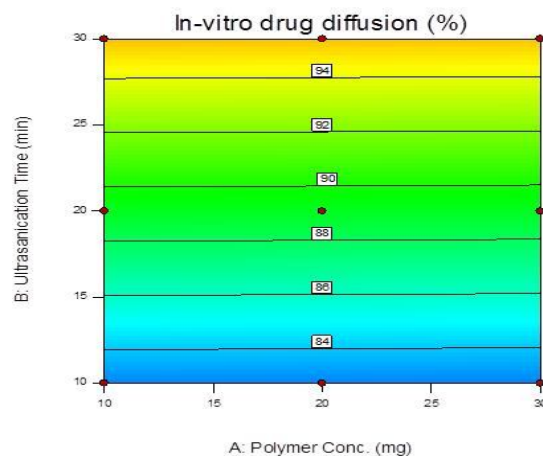
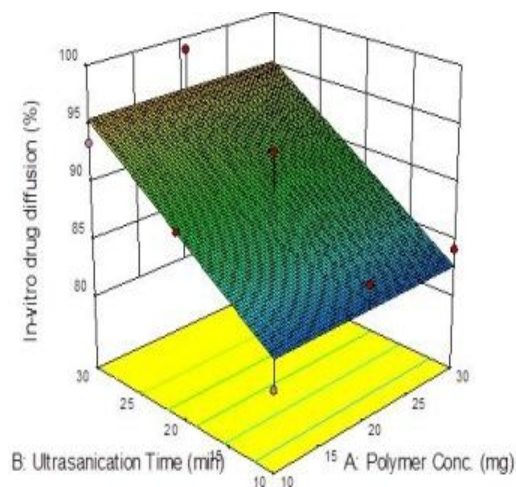


Figure 3: (a) Contour surface plot of *in-vitro* drug diffusion (b) Three-dimensional response surface plots of *in-vitro* drug diffusion.



For particle size the model F-value was 114.84 which suggest that the model was significant. In this case X_2 , X_1^2 and X_2^2 was significant model terms. For saturation solubility the model F-value was 347.36 which suggest that the model was significant. In this case X_2 was a significant model term. For *in-vitro* drug diffusion the model F-value was 10.09 which suggest the model was significant. In this case X_2 was a significant model term. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Summary of regression analysis of particle size, saturation solubility and *in-vitro* drug diffusion and ANOVA is given in table 3 and table 4 respectively.

Table 3: Summary of results of regression analysis of particle size, saturation solubility and *in-vitro* drug diffusion.

Source	Std. Dev.	R Squared	Adjusted R Squared	Predicted R Squared	PRESS	Suggested Model
Particle Size (Y1)						
Linear	39.43	0.8899	0.8532	0.7433	21742.43	
2FI	43.07	0.8905	0.8248	0.3539	54715.08	
Quadratic	12.11	0.9948	0.9861	0.9415	4958.24	Suggested
Cubic	9.27	0.9990	0.9919	0.8152	15650.01	Aliased
Particle Size (Y2)						
Linear	0.018	0.9914	0.9886	0.9786	4.81E-003	Suggested
2FI	0.019	0.9919	0.9871	0.9551	0.010	
Quadratic	0.021	0.9943	0.9847	0.9528	0.011	
Cubic	0.033	0.9952	0.9618	0.1290	0.20	Aliased
In-vitro Drug Diffusion (Y3)						
Linear	3.46	0.7708	0.6944	0.5311	147.12	Suggested
2FI	3.75	0.7760	0.6415	0.2727	228.17	
Quadratic	3.03	0.9122	0.7659	-0.0256	321.75	
Cubic	1.71	0.9906	0.9250	-0.7086	536.04	Aliased

Table 4: ANOVA Table

Source	Sum of Squares	df	Mean Square	F Value	p- value Prob.>F	
Y₁ Particle Size						
Model [Quadratic]	84250.84	5	16850.17	114.84	0.0013	Significant
X ₁	993.31	1	993.31	677	0.0802	
X ₂	74370.67	1	74370.67	506.87	0.0002	
X ₁ X ₂	51.84	1	51.84	0.35	0.5941	
X ₁ ²	4098.14	1	4098.14	27.93	0.0132	
X ₂ ²	4736.89	1	4736.09	32.98	0.0108	
Y₂ Saturation Solubility						
Model [Linear]	0.22	2	0.11	347.36	<0.0001	Significant
X ₁	1.601E-005	1	1.601E-005	0.050	0.8306	
X ₂	0.22	1	0.22	694.68	<0.0001	
Y₃In-vitro Drug Diffusion						
Model [Linear]	241.82	2	120.91	10.09	0.0120	Significant
X ₁	8.067E-003	1	8.067E-003	6.730E-004	0.9801	
X ₂	241.81	1	241.81	20.17	0.0041	

Particle size

The average particle size of all the formulations was of nano size, ranging from 155.2 to 449.6nm. The quadratic model was suggested for particle size analysis. The statistical analysis of data revealed that polymer conc. (X₁) and ultrasonication time (X₂) has significant effect on particle size. Associated contour plots & 3 D surface plot are shown in Figure 1 (a, b).

Saturation solubility

The saturation solubility of all formulations was found to be in the range of 0.356 to 0.7718 mg/ml. The linear model was suggested for saturation solubility. The statistical analysis of data revealed that polymer conc. (X_1) has no significant effect, whereas ultrasonication time (X_2) has significant effect on saturation solubility. Related contour plots & 3 D surface plot are shown in Figure 2 (a, b). The water solubility of ezogabine is 0.0307 mg/ml. The solubility of drug in selected nanosuspension NS₈ was found to be 0.7718 mg/ml and there was 25.14 fold increased in solubility of ezogabine.

In-vitro drug diffusion

The *in-vitro* drug diffusion for all the formulation was found to be in the range of 80.19 to 98.91% at 2.5 hrs. The linear model was suggested for *in-vitro* drug diffusion. The statistical analysis of data revealed that surfactant conc. (X_1) has no significant effect, whereas ultrasonication time (X_2) has significant effect on *in-vitro* drug diffusion. Related contour plots & 3 D surface plot are shown in Figure 3 (a, b). The percent drug release of nanosuspension and plain suspension is given in table 5 and Comparison of *in-vitro* drug diffusion of selected nanosuspension NS₈ (series1) and plain suspension (series2) is given in figure 4.

Table 5: Percent drug release of nanosuspension and plain suspension

Formulation code	Percent Drug Release, Mean $\pm$ SD, n=3						
	15	30	45	60	90	120	150
NS ₁	29.55 $\pm$ 0.22	36.66 $\pm$ 0.14	47.47 $\pm$ 0.35	59.46 $\pm$ 0.24	73.64 $\pm$ 0.19	82.11 $\pm$ 0.25	87.19 $\pm$ 0.26
NS ₂	30.32 $\pm$ 0.22	38.46 $\pm$ 0.35	49.44 $\pm$ 0.37	57.26 $\pm$ 0.29	67.72 $\pm$ 0.24	84.02 $\pm$ 0.32	88.18 $\pm$ 0.21
NS ₃	31.39 $\pm$ 0.42	37.60 $\pm$ 0.24	48.52 $\pm$ 0.33	55.44 $\pm$ 0.25	74.69 $\pm$ 0.23	85.39 $\pm$ 0.30	88.48 $\pm$ 0.21
NS ₄	32.42 $\pm$ 0.23	40.44 $\pm$ 0.33	49.69 $\pm$ 0.25	61.82 $\pm$ 0.32	79.46 $\pm$ 0.42	89.45 $\pm$ 0.38	94.24 $\pm$ 0.14
NS ₅	31.44 $\pm$ 0.40	38.46 $\pm$ 0.82	46.11 $\pm$ 0.34	60.06 $\pm$ 0.33	79.63 $\pm$ 0.35	88.29 $\pm$ 0.36	92.62 $\pm$ 0.46
NS ₆	30.42 $\pm$ 0.40	39.51 $\pm$ 0.31	46.30 $\pm$ 0.30	58.41 $\pm$ 0.26	76.66 $\pm$ 0.46	89.47 $\pm$ 0.36	93.57 $\pm$ 0.30
NS ₇	33.26 $\pm$ 0.15	39.04 $\pm$ 0.31	48.66 $\pm$ 0.23	62.35 $\pm$ 0.24	79.53 $\pm$ 0.28	92.46 $\pm$ 0.26	96.43 $\pm$ 0.23
NS ₈	34.37 $\pm$ 0.24	44.33 $\pm$ 0.21	55.74 $\pm$ 0.20	66.94 $\pm$ 0.13	82.24 $\pm$ 0.24	96.48 $\pm$ 0.39	98.91 $\pm$ 0.31
NS ₉	32.73 $\pm$ 0.40	38.44 $\pm$ 0.40	52.51 $\pm$ 0.18	63.06 $\pm$ 0.18	76.53 $\pm$ 0.33	91.44 $\pm$ 0.26	95.13 $\pm$ 0.41
Plain suspension	23.95 $\pm$ 0.88	33.96 $\pm$ 0.54	49.89 $\pm$ 0.77	58.56 $\pm$ 0.91	74.65 $\pm$ 0.85	89.13 $\pm$ 0.56	91.95 $\pm$ 0.86

Figure 4: Comparison of *in-vitro* drug diffusion of selected nanosuspension NS₈ (series1) and plain suspension (series2)

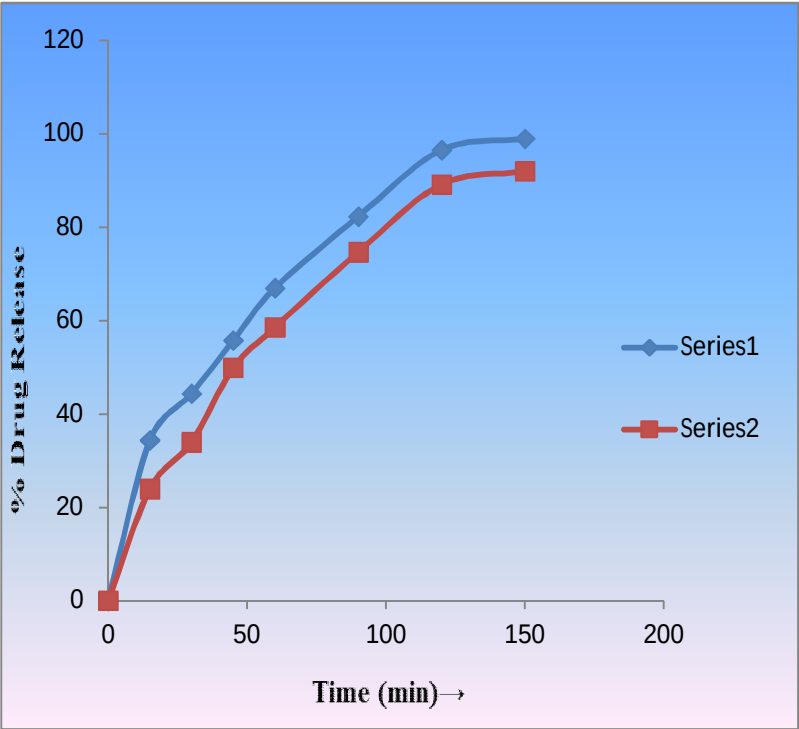


Table 6: Similarity factor data of ezogabine nanosuspension and plain suspension

Time	R _t	T _t	(R _t -T _t)	(R _t -T _t) ²
15	23.95	34.37	10.42	108.57
30	33.96	44.33	10.37	107.53
45	49.89	55.74	5.85	34.22
60	58.56	66.94	8.38	70.22
90	74.65	82.24	7.59	57.60
120	89.13	96.48	7.35	54.02
150	91.95	98.91	5.96	35.52
SUM (R _t -T _t)			55.92	
SUM (R _t -T _t) ²			467.71	
SUM R _t			422.09	
Similarity factor f ₂			54	
Difference factor f ₁			13	

R_t = Cumulative percentage dissolved of ezogabine plain suspension at time t.

T_t = Cumulative percentage dissolved of ezogabine nanosuspension at time t.

Similarity factor

The calculated value of f₂ for the plain suspension and nanosuspension (NS₈) was 54, which showed the similarities in release profile of both formulations. The similarity factor data of ezogabine nanosuspension and plain suspension is given in table 6.

Drug release kinetics

Table 7: Drug release kinetics of ezogabine nanosuspension and plain suspension

Sr. No	Formulation	R ² Value for determination of model					Suggested Model
		Zero Order	First Order	Higuchi	Korsmeyer-Peppas	Hixson-Crowell	
1.	NS ₈	0.9496	0.9575	0.9843	0.9888	0.9897	Hixson- Crowell
2.	Plain Suspension	0.948	0.863	0.9861	0.9849	0.9884	Hixson- Crowell

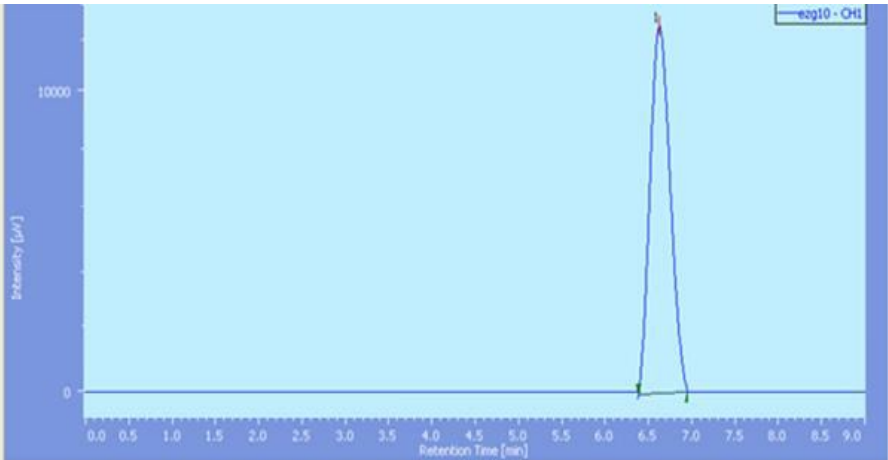
The mechanism of release was determined by finding the R²value for each kinetic model like, zero-order, first-order, Higuchi, Korsmeyer Peppas, Hixson-Crowell cube root law etc. corresponding to the release data of NS₈ and plain suspension. The R²value of Hixson-Crowell model was very close to one than the R²values of other kinetic models. Thus, it could be thought that the drug release monitors Hixson-Crowell's release kinetics. In the case of nanosuspension diffusion occurred in planes that were parallel to the drug surface (Particle size).Drug release kinetics of ezogabine nanosuspension and plain suspension is given in table 7.

Drug Content

The drug content of selected ezogabine nanosuspension (NS₈) was found to be 93.60% and 93.00% by external standard method (ESM) and calibration curve method (CCM), respectively (Table 8). The HPLC chromatogram for A. Ezogabine std. and B. Ezogabine nanosuspension is given in figure 5.

Figure 5: HPLC chromatogram of A. Ezogabine std. and B. Ezogabine nanosuspension

A



B

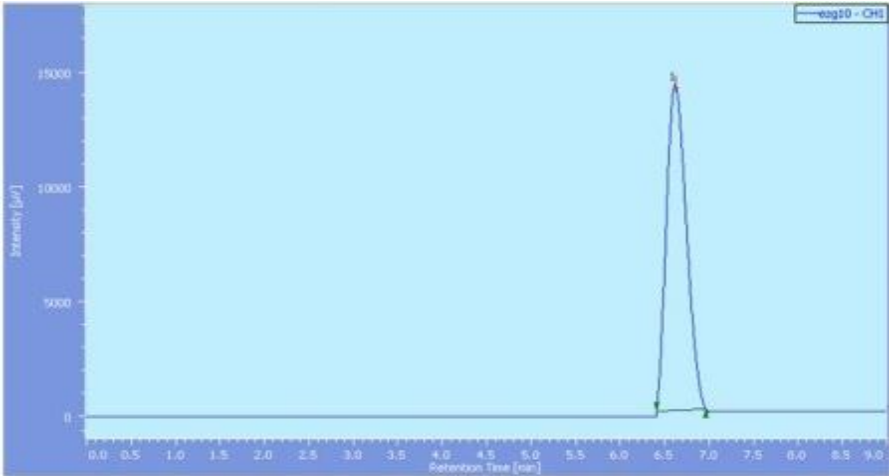
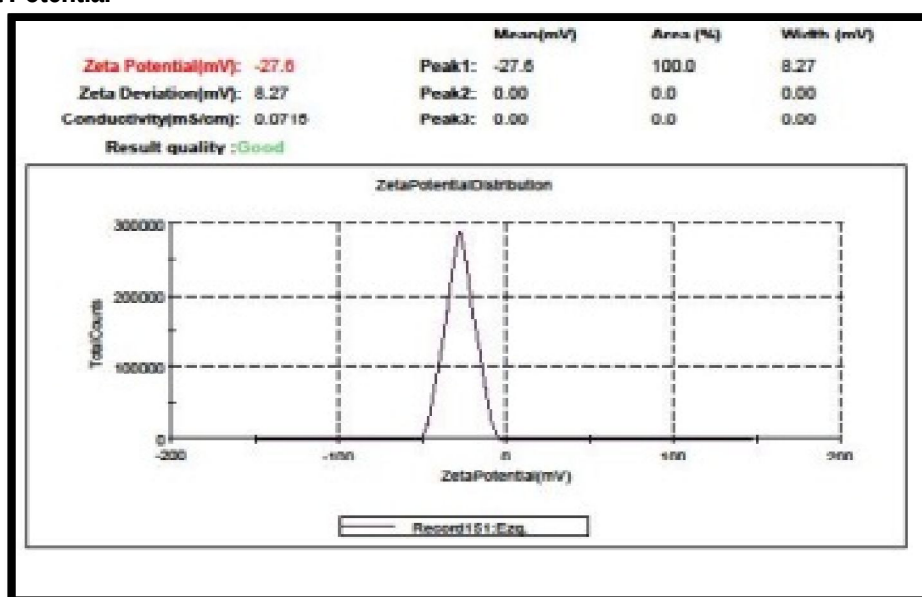


Table 8: Drug content of ezogabine nanosuspension by ESM and CCM.

Sr. No	Peak Name	Retention Time (Min.)	Peak Area	Peak Height	Symmetry Factor	Drug Content (%)
1	Ezogabine Std.	6.617	236236	14651	1.153	100
2	Ezogabine Nanosuspension	6.610	221114	14201	1.253	93.60 (ESM) 93.00 (CCM)

Zeta Potential

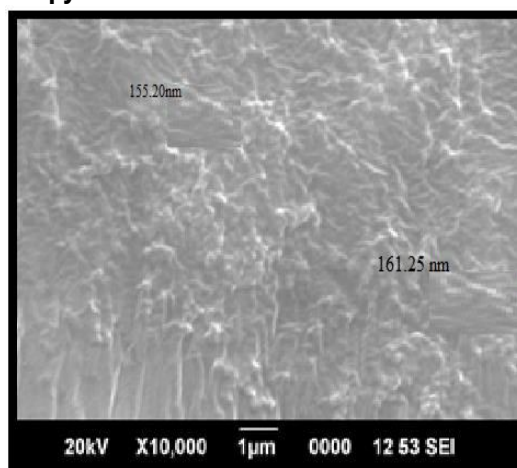
Figure 6: Zeta potential of the selected nanosuspension (NS₈)



The zeta potential of formulations was found to be -27.6 mV. The negative charge is due to the amine and/or ester group. These result showed that the nanosuspension was stable. The Zeta potential of the selected nanosuspension (NS₈) is given in figure 6.

Scanning Electron Microscopy

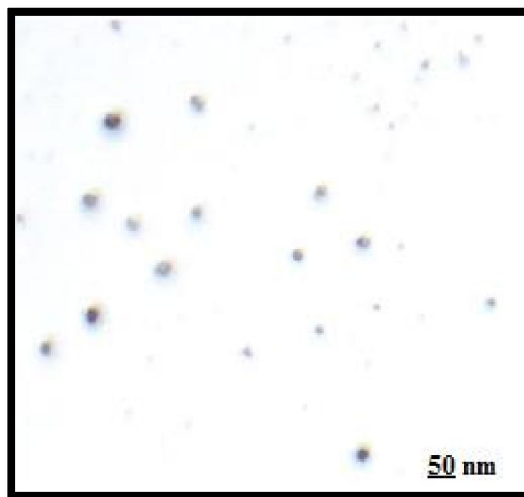
Figure 7: SEM photomicrograph of selected nanosuspension(NS₈)



Morphology of precipitated drug particles in the suspension afterwards air drying followed by oven-drying. The drug particles were orbicular and the size ranges from 155.20 to 161.25 nm. The particles were distinct and uniform in size and there was no indication of accumulations. The SEM photomicrograph of selected nanosuspension(NS₈) is given in figure 7.

Transmission Electron Microscopy

Figure 8: TEM photomicrographs of selected nanosuspension (NS₈)



The study shown that utmost of the nanoparticles were legitimately elliptical in shape. The surface of the particles indicated a characteristic smoothness which helps to improve solubility. The TEM photomicrographs of selected nanosuspension (NS₈) is given in figure 8.

Stability Study

Table 9: Result of the stability studies of selected nanosuspension

Sr. No	Time (Day)	Stability Data of nanosuspension			
		Particle Size (nm)		Drug Content (%)	
		Room Temp.	Refrigerator	Room Temp.	Refrigerator
1	Initial	155.2 ±0.80	155.2 ±0.80	99.12 ±0.02	99.12 ±0.02
2	10	162.1 ±1.12	158.6 ±0.99	99.13 ±0.16	99.05 ±0.27
3	20	172.3 ±0.86	165.5 ±1.15	99.18 ±0.45	99.14 ±0.81
4	30	190.5 ±0.68	168.2 ±0.86	98.93 ±0.75	99.01 ±0.19
5	45	198.9 ±1.45	171.4 ±1.87	99.01 ±0.28	98.89 ±0.87
6	60	215.2 ±1.16	175.8 ±1.19	99.09 ±0.27	99.08 ±0.56
7	90	224.2 ±0.99	177.1 ±0.85	99.14 ±0.45	99.15 ±0.14

The outcomes of the stability studies are shown in table 9. In the situation of nanosuspension kept at room temperature, the particle size increased from 155.2-224.2nm in 90 days. However, under refrigerated storage situations, there was insignificant rise from 155.2-177.1nm showing superior stability under these situations. The results indicated that temperature has an impact on accumulation of nanoparticles and at room temperature; accumulation was greater compared to refrigerator situation for liquid nanosuspension. The inference is that greater temperature marks a rise in particle size. Alternative cause may be the Ostwald ripening causing from variations in room temperature. It

can be detected that there is no substantial variation in the drug content of the preparation, in any of the two storage circumstances viz. room temperature or refrigerated conditions. Thus the nanosuspensions are chemically stable at both the storage situations.

Pharmacodynamic study

Table 10: Pharmacodynamic study of ezogabine nanosuspension in rats

Sr. No	Treatment (Dose)	Time (sec) in various phases of convulsion				
		Flexion	Extensor	Clonus	Stupor	Recovery/ Death
1	Control	8 ±1	12±2	132.5±2.52	52.3±2.2	Recovery
2	Standard	11.3±1.53	4.3±0.58	147.5±2.52	59.3±4.04	Recovery
3	Test	9±1	4±1	154.3±4.04	42.3±2.52	Recovery

From the table 10 it was observed that there is reduction in tonic extensor phase of rats treated with phenytoin (standard) and ezogabine nanosuspension (test). Both phenytoin and ezogabine showed reduction in tonic phase as compared to control; therefore we could say that the drug had anticonvulsant activity which was found out by maximal electro-shock-induced convulsions in rats.

Estimation of drug content in the brain by HPLC method

The drug content was determined in brain tissue which was analyzed by HPLC showed the retention time of 6.3 min and compared with the standard graph with a retention time of 6.6 min, thus the two peaks were correlated each other and signifies the presence of drug in the brain homogenate. Pharmacokinetic parameter data of selected nanosuspension and plain suspension is given in table 11. When the AUCs of both the groups were compared, the intranasal administration of nanosuspension group had higher AUC [212.16 µg/mL*min in plasma and 240.00 µg/mL*min in brain] as compared to plain suspension [34.35 µg/mL*min in plasma and 39.82 µg/mL*min in brain] which showed presence of greater drug conc. in brain. When the C_{max} and t_{max} were compared, nasally administered plain suspension reached its peak within 75 min (C_{max} = 0.183 µg/ml) in plasma and 45 min. (C_{max} = 0.1812 µg/ml) in brain and nanosuspension reached its peak within 45 min. (C_{max} = 0.7483 µg/ml) in plasma and 30 min. (C_{max} = 0.7885 µg/ml) in brain. When ezogabine concentrations in the brain were studied after intranasal administration of nanosuspension it attained 4 times higher concentration in the brain as compared to plain suspension.

The intranasal route of administration was an achievement to deliver nanoparticulate suspension and thus can be used for treating syndromes of brain. This research effort has two components. Firstly we developed a novel formulation of potassium channel opener in the form of a nanosuspension by factorial design, which has the potential utility for treatment of brain syndromes, by reducing the dose and reducing the side effects which have been reported for the marketed formulations. Secondly we used this formulation to address the question of the significance of the olfactory transport pathway to brain for potassium channel opener after intranasal administration.

Table 11: Pharmacokinetic parameter of ezogabine nanosuspension and plain suspension upon intranasal administration in rats

Sr. No	Pharmacokinetic Parameter	Nanosuspension		Plain Suspension	
		Plasma	Brain	Plasma	Brain
1	C _{max} (µg/ml)	0.7483	0.7885	0.183	0.1812
2	T _{max} (min)	75	45	75	45
3	AUC _{0-t} (µg/ml*min)	212.16	240.00	34.35	39.82
4	AUC _{0-∞} (µg/ml*min)	293.15	240.00	37.46	60.02
5	MRT (min)	358.35	680.54	178.11	414.79

The nasal cavity has about a total volume of 15–20 ml with a total surface area of 150 cm²; therefore, volume that can be delivered into the nasal cavity is restricted to 25–200 µl. The total volume of developed potassium channel opener nanosuspension administered to the nasal cavity of the animals was 0.1 ml (100µl), which showed a safe delivery system since; no toxicity in nasal mucosa and brain was observed, with the treated animals. Thus nanosuspension is a good approach to deliver drugs to brain via nasal route of administration.

Conclusion

In the current study, we described the development of ezogabine nanosuspension by means of nanoprecipitation-ultrasonication method. The main objective of this study was to enhance the aqueous solubility and to develop brain targeted drug delivery of ezogabine by 3² factorial design. The plain suspension was prepared for comparison with nanosuspension. The factorial design showed promising approach to study the effect of polymer conc. (X₁) and ultrasonication time (X₂) on particle size (Y₁), saturation solubility (Y₂) and *in-vitro* drug diffusion (Y₃). The formulation showed better results in terms of drug content, zeta potential, SEM, TEM and stability study. The formulation showed better antiepileptic activity when checked against maximal electro-shock-induced convulsions in rats. This preparation was capable to show higher drug concentration in brain and no mortality when formulation was administered intranasally. Thus it was concluded that ezogabine nanosuspension was capable of providing direct nose-to-brain delivery, thereby enhancing drug concentration in the brain.

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Conflicts of interest

Declared None

REFERENCES

- White H S. Preclinical Development of Antiepileptic Drugs: Past, Present, and Future Directions.*Epilepsia*. (2003) 44(7): 2–8.
- Rubio C, Lopez M, Osornio M and Marquez S. *In Vivo* Experimental Models of Epilepsy.*Bentham Science Publishers Ltd*. (2010) 10:298-309.
- Sakthivel M, Kannan K, Manavalan R and Senthamarai. Formulation and *in vivo* Evaluation of Niosomes containing Oxcarbazepine.*J. Pharm. Sci. & Res. Vol*. (2013) 5(1): 8–11.
- Rajurkar V G, Aucharmal A L and Dethe S. Development of Ezogabine Co Crystal Formation: An Efficient Approach to Enhance Aqueous Solubility.*British Biomedical Bulletin*.(2015) 3(3): 350-363.

- Kinjal B, Patel A, Talele A and Jadhav A G. An overview of Intranasal Nanosuspension for Brain Targeted Drug Delivery, *Indo American Journal of Pharmaceutical Research*.(2015) 5(2):730-740.
- Bhavna, Shadab M, Mushir A, Rashid A, Aseem B, Sanjula B and Javed A. Donepezil Nanosuspension Intended for Nose to Brain Targeting: *In Vitro* and *In Vivo* Safety Evaluation. *International Journal of Biological Macromolecules*.(2014) 67: 418-425.
- Fessi H. Process for the Preparation of Dispersible Colloidal Systems of a Substance in the Form of Nanoparticles. 1988, *US Patent* US5118528, 2.
- Quintanar-Guerrero D. Preparation Techniques and Mechanisms of Formation of Biodegradable Nanoparticles from Preformed Polymers. *Drug Dev. Ind. Pharm.*(1998) 24: 1113–1128.
- [Thakkar](#) H P, [Patel](#) B V and [Thakkar](#) S P. Development and characterization of nanosuspensions of olmesartan medoxomil for bioavailability enhancement. *J Pharm Bioallied Sci.*(2011) 3(3): 426–434.
- Sahu B P, Das M K. Optimization of felodipine nanosuspensions using Full Factorial Design, *International Journal of PharmTech Research*. (2013) 5(2): 553-561.
- Maurya P, Mittal A, Sharma K and Alam S. Fabrication of Acetazolamide Loaded Nasal Nanosuspension: An *In Vitro* and *Ex Vivo* Characterization. *Analele Universitatii Dunarea De Jos Galati*.(2013) 93-105.
- Agarwal V and Bajpal M. Preparation and Optimization of Esomeprazole Nanosuspension using Evaporative Precipitation–Ultrasonication. *Tropical Journal of Pharmaceutical Research*.(2014) 13 (4): 497-503.
- Dengning X, Peng Q, Hongze P, Hongyu P, Shaoping S, Yongmei Y and Fude C. Preparation of stable Nitrendipine nanosuspensions using the precipitation–ultrasonication method for enhancement of dissolution and oral bioavailability. *European Journal of Pharmaceutical Sciences*.(2010) 40: 325–334.
- Summary of Product Characteristics, <http://www.ema.europa.eu/>.
- Sandhya J, Pavani A and Reddy R. Formulation and evaluation of nanosuspension of Nisoldipine. *International Journal of Pharmaceutical Sciences Review and Research*.(2014) 24(1): 177-181.
- Pandya V M, Patel J K and Patel D J. Formulation, Optimization and characterization of Simvastatin Nanosuspension prepared by nanoprecipitation technique. *Scholars Research Library Der Pharmacia Lettre*.(2011) 3(2): 129-140.
- R. M. Mehta, *Pharmaceutics II*, Vallabh Prakashan 3rd edition, 132-133.
- Nagajyothi N, Dhanalakshmi M, Thenmozhi S, Natarajan R and Rajendran N. Formulation and evaluation of Pitavastatin nanosuspension. *International Journal of Pharmacy & Life Sciences*.(2014) 5(2): 3318-3324.
- Patel RB, Patel M R, Bhatt K K and Patel B G. Formulation consideration and characterization of microemulsion drug delivery system for transnasal administration of carbamazepine. *Bulletin of Faculty of Pharmacy, Cairo University*.(2013) 51: 243–253.
- Ullah S S. Formulation and evaluation of directly compressed Ofloxacin Ethocel controlled release Tablets: A Kinetic Approach. *International Journal of Research in Ayurveda and Pharmacy*.(2015) 2(3): 801-809.
- Snehamayee M. Application of Central Composite Design For Optimization of Effervescent Floating Tablets using Hydrophilic Polymers. *Asian Journal of Pharmaceutical and Clinical Research*.(2015) 8(1): 293-298.
- Attari Z, Bhandari A and Jagdish P C. Enhanced ex-vivo intestinal absorption of olmesartan medoxomil nanosuspension: Preparation by combinative technology. *Saudi Pharmaceutical Journal*.(2015) 03: 1-7.
- Pardeikea J, Strohmeir D M, Schrodri N, Voura C and Zimmer A. Nanosuspensions as advanced printing ink for accurate dosing of poorly soluble drugs in personalized medicines, *International Journal of Pharmaceutics*.(2011).
- Sahu B P and Das M K. Nanosuspension for enhancement of oral bioavailability of felodipine. *App/ Nanosci*. (2014) 4: 189–197.

Venkatesh D N, Baskaran M, Karri V, Mannemala S, Radhakrishna K and Goti S. Fabrication and in vivo evaluation of Nelfinavir loaded PLGA nanoparticles for enhancing oral bioavailability and therapeutic effect. *Saudi Pharmaceutical Journal*.(2015), 23: 667–67.

[Thakkar](#) H P, Patel B V and Thakkar S P. Development and characterization of nanosuspensions of olmesartan medoxomil for bioavailability enhancement. *J Pharm Bioallied Sci*.(2011) 3(3): 426–434.

Sheetal P. Preparation and evaluation of transnasal microemulsion of carbamazepine. *Asian Journal of Pharmaceutical Sciences*.(2013) 8: 64-70.



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