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ORIGINAL RESEARCH

RETRACTED ARTICLE: Optimized Nanostructured Lipid Carriers Integrated into In Situ Nasal Gel for Enhancing Brain Delivery of Flibanserin

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Background and Aim: Flibanserin (FLB) is a multifunctional serotonergic agent used for treating hypoactive sexual desire disorder in premenopausal women via oral administration. FLB has a reported limited oral bioavailability of 33% that could be attributed to the drug's first-pass metabolism. In addition, FLB has a pH-dependent solubility that could be a challenging factor for drug dissolution in the body neutral fluid, and consequently, absorption via mucosal barriers. Thus, this work aims at investigating the potential of utilizing nanostructured lipid carriers (NLCs) to overcome the aforementioned drawbacks and to enhance nose-to-brain drug delivery.

Methods: Box-Behnken design was applied to explore the impact of solid lipid % (SL%, X_1), liquid lipid % (LL%, X_2) and sonication time (ST, X_3) on particle size. The optimized NLC formulation was characterized and incorporated into gellan gum in situ gel. The prepared gel was subjected to in vitro drug release, in vivo pharmacokinetic performance, and histopathological assessment in rats.

Results: Statistical analysis revealed a significant negative effect for both SL% and ST on NLCs size and contrast, a significant positive effect was observed for the LL%. The optimized formulation showed spherical shape with vesicular size of 114.63 nm. The optimized FLB-NLC in situ gel exhibited adequate stability and enhanced in vitro release compared to raw FLB control gel. The plasma and brain concentrations of the drug after nasal administration in rats increased by more than 3–6-fold, respectively, compared to raw FLB in situ gel. In addition, the histopathological studies revealed the absence of any pathological signs.

Conclusion: The aforementioned results highlight the safety of FLB-NLC in situ nasal gel and its potential to improve the drug bioavailability and brain delivery.

Keywords: flibanserin, nanostructured lipid carrier, Compritol® 888 ATO, almond oil, gellan gum, pharmacokinetics

Introduction

Flibanserin (FLB) was initially developed as an antidepressant.[1] The observed increased libido as a side effect in female patients led to its adoption for the treatment of female sexual interest/arousal disorder (FSIAD).[1,2] Following its widespread adoption, FLB was approved in 2015 by the Food and Drug Administration (FDA) as the first drug for treatment of hypoactive sexual desire disorder (HSDD) in premenopausal women.[3] The postulated mechanism of drug action is based on being a multifunctional serotonergic agent that has an agonist action on postsynaptic 5-HT_{1A} receptors and an antagonistic action on postsynaptic

5-HT_{2A} receptors. Thus, this action on the serotonin receptors in the prefrontal cortex (PFC) is hypothesized to enhance sexual desire through increasing downstream release of dopamine and norepinephrine, while decreasing serotonin release.[4,6] However, FLB suffers from limited oral bioavailability of about 33%, which could be attributed mainly to the drug's considerable first-pass metabolism.[1] In addition, FLB is a weakly basic drug with a pH-dependent solubility; it is soluble in 0.1N HCL and insoluble in phosphate buffer at pH 6.8.[6] This solubility feature may be a challenging factor for drug dissolution in the body neutral fluid, and subsequently, absorption via mucosal barriers.

Nasal delivery has recently gained a great consideration as a highly vascular, convenient, and non-invasive route for drug delivery that is able to surpass the presystemic metabolism of many drugs.[7] In addition, the unique anatomy of the nasal cavity, which permits absorption via the olfactory trigeminal regions, could provide a route that surmounts the blood–brain barrier (BBB), and, thus, offers direct delivery of the drugs to the brain.[8] Previous researchers have evaluated and demonstrated the effectiveness of the nasal route for brain delivery of drugs.[9,10]

Various lipid-based nanocarriers have installation [illegible] improving the solubility and, consequently, the bioavailability of hydrophobic drugs.[11,13] Moreover, they have demonstrated high potential for brain targeting by virtue of their nano-scale size and their lipid content that [illegible] the passage of small particles through the nasal route and directly across the BBB via passive diffusion. They also have the advantage of reduced toxicity and better biocompatibility compared to polymeric micelles.[14,15]

Liposomes, the primary lipid nanocarriers, were applied for drug targeting to the brain despite their limitations that include low drug loading efficiency, rapid clearance through the reticuloendothelial system, and poor stability. Solid lipid nanoparticles (SLN) have been introduced as an alternative to liposomes and polymeric nanocarriers. Additionally, they have a better safety profile than the polymeric nanoparticles due to avoidance of organic solvents during preparation. In addition, they offer prolongation of drug release and higher stability in comparison to liposomes.[14,16] However, the major disadvantage of SLN is the possibility of drug expulsion and uneven drug incorporation owing to their crystalline structure. Accordingly, nanostructured lipid carriers (NLCs) have been proposed as a second-generation SLN to overcome the aforementioned drawbacks.[17,18] NLCs comprise a blend of solid and liquid lipids with different spatial arrangements that provide a larger distance between the glycerides' fatty acid chains and result in defects in the crystalline structure. The unique unstructured matrix of NLCs could potentially improve drug loading and impede expulsion of active molecules or leakage during storage.[17,19,23] Recently, NLCs have been widely employed as a promising delivery system for brain targeting of versatile drugs via the nasal route.[8,24,25]

In this work, the potential of employing nanostructured lipid carriers loaded in situ gel for enhancing nose-to-brain delivery of FLB has been explored. Box–Behnken design was applied for optimization of FLB-NLCs. The optimized FLB-NLC formulation was assessed for morphology and stability, and then incorporated into gellan gum in situ gel. The prepared FLB-NLC in situ gel was then evaluated for in vitro release behavior and in vivo pharmacokinetic performance in rats.

Materials and Methods

Materials

Flibanserin was purchased from Qingdao Sigma Chemical Co., Ltd. (Qingdao, China). Compritol® 888 ATO (Glyceryl behenate) and Gelucire® 44/14 were provided by Gattefossé (Saint-Priest, France); Sweet almond oil was purchased from Sigma-Aldrich Co. (St Louis, MO, USA), and L-phosphatidylcholine (soya 95%) was purchased from Avanti Polar Lipids (Birmingham, UK). All other reagents and chemicals were of analytical grade.

Preparation of FLB-NLCs

FLB-NLCs were prepared by hot emulsification–ultrasonication method.[26] In all formulations, the total lipid phase was kept constant at 10% w/v. Briefly, specified amounts of glyceryl behenate (solid lipid, SL), sweet almond oil (liquid lipid, LL), L-phosphatidylcholine (amphiphilic emulsifier, 2%w/v), and FLB (50 mg) were blended and heated to 70°C under stirring to form a uniform lipid phase. Gelucire® 44/14 (hydrophilic emulsifier, 1.5% w/v) was then dissolved in distilled water and heated to 70°C. The hot aqueous phase was then added dropwise to the melted lipids, and the formed dispersion was homogenized by an IKA Ultra-Turrax T8 homogenizer (IKA, Wilmington, NC, USA) at 20,000 rpm at the same temperature for 3 minutes. The obtained pre-emulsion was ultrasonicated by Sonics Vibra Cell VCX750 (Sonics & Materials Inc., CT, USA) at 35% amplitude, 750 W, 20 kHz at the specified sonication time (ST). The dispersion was cooled for 12 hours at 4°C. The prepared formulations were then kept in a refrigerator for further investigations.

Experimental Design for Optimization of FLB-NLCs

A three-variable Box–Behnken design was employed to optimize the formulation of FLB-NLCs using Statgraphics Plus, Version 4 (Manugistics Inc., Rockville, MD, USA). Solid lipid percent (X_1), liquid lipid percent (X_2), and sonication time (X_3) were studied as variables in the ranges of 0.6–0.9% w/v, 0.1–0.4% w/v, and 1–5 minutes, respectively, while the particle size (Y_1, nm) was investigated as a response parameter. The ranges used in the study and the percentage of both amphiphilic and hydrophilic emulsifiers were selected based on preliminary investigations (data not shown). The coded levels of each factor and their corresponding actual values are depicted in Table 1. Fifteen experimental runs were generated as per the experimental design. The actual values of the independent variables and the observed values for the response parameter are compiled in Table 2. Analysis of Variance (ANOVA) was applied to statistically analyze the measured response data. The polynomial equations corresponding to the best fitting model, Pareto chart, and contour plots were generated to assess the effect of the investigated variables and interaction between them at 95% level of significance. Furthermore, the composition of the optimized FLB-NLC with minimized particle size was predicted utilizing numerical optimization. The proposed optimum formulation was practically prepared and characterized for particle size. The measured particle size was compared to the predicted value for residual calculation to ensure the validity of the optimization process.

Particle Size Measurement

Dynamic light scattering technique was utilized to determine the particle size of FLB-NLCs (z-average) using a Nano-ZS particle size analyzer (Malvern Instruments, Worcestershire, UK). Samples were sufficiently diluted with the formulation's aqueous phase prior to measurement to achieve an optimum count of 50–200 kilo-counts per second (kcps). The mean particle size was computed as the average of five measurements.

Table 1 Independent Variables and Responses Used in the Box–Behnken Design for the Formulation and Optimization of FLB-NLCs

Independent Variables	Levels		
	(−1) (0)		(+1)
X_1: Solid lipid % X_2: Liquid lipid % X_3: Sonication time (min)	0.6 0.1 1	0.75 0.25 3	0.9 0.4 5
Responses	**Desirability constraints**		
Y_1: particle size (nm)	Minimize		

Abbreviation: FLB-NLCs, flibanserin nanostructured lipid carriers.

Table 2 Experimental Runs and the Observed of Particle Size of FLB-NLCs Prepared According to Box–Behnken Design

Experimental Run #	Independent Variables			Particle Size (nm)±SD
	SL (%)	LL (%)	ST (min)	
NLC-1	0.75	0.25	3	173±2.56
NLC-2	0.9	0.1	3	86±1.21
NLC-3	0.75	0.25	3	174±2.14
NLC-4	0.6	0.4	3	284±5.28
NLC-5	0.9	0.1	1	205±3.56
NLC-6	0.6	0.4		2±1.47
NLC-7	0.75	0.25	3	1 ±1.89
NLC-8	0.6	0.4		198 2.19
NLC-9	0.6	0.4	5	1 ±1.59
NLC-10	0	0.1	3	41±3.36
NLC-11		0	5	154±1.98
NLC-12	0.75	0.25	1	115±2.11
NLC-13	0.75	25		65±0.98
NLC-14	0.75	0.2	1	287±4.45
NLC-15	75	0.25	5	241±3.65

Abbreviation: FLB-NLCs, flibanserin nanostructured lipid carriers.

Characterization of Optimized FLB-NLCs

For investigation of vesicle size, polydispersity index (PDI), and zeta potential of the optimized FLB-NLC, the same method mentioned in section (2.4) using a Malvern size analyzer was followed. Also, transmission electron microscopy (Philips XL30, Eindhoven, Netherlands) was used to investigate the shape of the optimized FLB-NLC. One drop of the diluted NLC dispersion was applied on a carbon coated grid and left for 3 minutes to allow its adsorption on the carbon film. The adsorbed NLCs were then stained with phosphotungstic acid (1% w/v, pH 6.8). The grid was air-dried thoroughly after removal of excess stain. The sample was examined with 30,000X magnification. To study the stability of the optimized FLB-NLC, three freeze–thaw cycles (between −20°C and +25°C) were performed. The particle size was then measured and compared to those of freshly prepared NLCs.

Preparation of FLB-NLC in situ Nasal Gel

Briefly, gellan gum (0.6%, w/v) was spread over boric/borax buffer (pH 7.4) at 80°C and the mixture was subjected to continuous stirring till obtaining a clear polymeric dispersion.[27,28] The resulting dispersion was left overnight to cool. The formed dispersion was then integrated into the prepared gellan gum dispersion to yield a final FLB concentration of 10 mg/g. The prepared gel was kept for 24 hours in the refrigerator before evaluation. A control in situ gel formulation containing raw FLB was prepared at the same concentration for comparison.

The prepared FLB-NLC in situ *nasal gel* formulation was evaluated for viscosity and gelation before and after addition of simulated nasal fluid (SNF).[29,30]

In vitro Release Study

The dialysis bag technique was carried out to study in vitro release of FLB from the optimized FLB-NLC in situ nasal gel in comparison to control raw FLB in situ gel. The specified weight of the gel was placed in the dialysis bags with a 14 kDa molecular weight cutoff (MWCO) (Sigma-Aldrich Co.) and immersed in 500 mL simulated nasal fluid, PH 6.5 at 37°C in a using USP Dissolution Tester, apparatus II (Erweka, Germany) rotating at a speed of 50 rpm. Aliquots (5 mL) were taken from the dissolution medium after 0.5, 1, 2, 4, 6, 8, 12, and 24 hours; FLB content was analyzed using high-performance liquid chromatography with an ultraviolet (UV) diode array detection (HPLC-DAD) method developed and validated in our laboratory in terms of linearity, accuracy, and precision. High-performance liquid chromatography (HPLC) Agilent 1200 system with diode array detector was used. The system was equipped with an auto-sampler, a quaternary pump, and column compartment (Palo Alto, CA, USA). The system was equipped with ChemStation software (Rev. B.01.03 SR2 (204)). Isocratic elution was employed at a flow rate of 0.6 mL/min. The utilized mobile system comprised acetonitrile:water containing 0.1% formic acid (9:1). A volume of 5 µL was injected on a Zorbax Extend C18 column (4.6*150 mm, 5 µm) for LC-DAD analysis. The detection wavelength was 260 nm. The concentration of FLB in the injected samples was calculated with reference to the constructed calibration curve at 260 nm.[31]

In vivo Study

Study Protocol

The pharmacokinetic performance of the optimized FLB-NLC in situ nasal gel was studied in male Wister rats (n=84), weighing between 200–250 g compared to raw FLB in situ nasal gel. The study procedure has been approved at the King Abdulaziz University, Kingdom of Saudi Arabia Research Ethics Committee with approval no. (PH-124-41); The panel guarantees that animals use in compliance with the European Union Directive 2010/63/EU on animal welfare and the Guiding Principles on animal welfare. (DHEW publication NIH 80–23). The animals were maintained in a restricted-access room with controlled temperature (23°C) and light–dark cycle (12 hours–12 hours) and were housed in rack-mounted cages with a maximum of four rats per cage. Rats were allowed to drink and feed *ad libitum*. The experimental animals were divided into two groups (I and II). Each animal received a FLB dose of 10 mg/kg intranasally as follows: Group I, the positive control group that received raw FLB in situ nasal gel and group II, the treatment group that received optimized FLB-NLCs in situ nasal gel. Blood samples (0.25 mL) were collected from the tail vein at 0, 0.5, 1, 2, 3, 6, and 8 hours after the administration of FLB. The blood samples were centrifuged at 5000 rpm for 5 minutes to obtain plasma. At each time interval, six animals were euthanized by cervical dislocation and brains were collected, and homogenized with phosphate buffer saline (pH 7.4) at 7000 rpm for 2 min. The separated plasma and the homogenized brain samples were stored at −80°C until analysis.

For histopathological evaluation, 12 rats were divided into four groups, gp1, of untreated rats; gp 2, for plain in situ gel without drug, gp 3 for raw FLB in-situ gel, and gp 4 for optimized FLB-NLCs in situ gel. The same dosing procedure previously described in the pharmacokinetics study was utilized. After 8 hours, histopathological examination was conducted according to the method of Young.[32] In brief, the head was removed, and the brain and jaw were removed from the head along with any other listed tissues. The nasal cavity was initially fixed in a solution of 10% formalin and then decalcified in a solution of 10% EDTA. The tissue was then placed in 70% ethanol before being embedded in paraffin, sectioned, and stained with hematoxylin and eosin stain prior to microscopic visualization.

FLB Assay in Plasma

A specified volume of plasma or brain homogenate was transferred to a screw-capped test tube, mixed with 50 µL internal standard solution (valsartan, 625 ng/µL) and 1 mL acetonitrile. The prepared mixture was vortexed for 1 minute, and then centrifuged at 5300 rpm for 8 minutes. An aliquot of about 500 µL of the clear supernatant was transferred to a total recovery autosampler vial, and a volume of 7 µL was

injected for LC-MS/MS-DAD analysis. The MS system was connected to an HPLC- Agilent 1200 system equipped with an autosampler, a quaternary pump, and a column compartment (Palo Alto, CA, USA). The system was equipped with ChemStation software (Rev. B.01.03 SR2 (204)). The IT–MS was controlled using 6300 series trap control version 6.2 Build No. 62.24 (Bruker Daltonik GmbH), and the general MS adjustments were: capillary voltage, 4200 V; nebulizer, 37 psi; drying gas,12 L/min; desolvation temperature, 330°C; ion charge control (ICC) smart target, 200,000; and max accumulation time, 200 milliseconds (ms). The MS scan range was 50–550 m/z. For quantitative monitoring, single positive molar ion mode was applied at a programed time segment, 0–4.0 min, m/z 391.2 $[M^+H]^+$ FLB; 4.0–10 min, m/z 436.3 $[M^+H]^+$ internal standard. Isocratic elution was conducted at a flow rate of 0.5 mL/min with a mobile system composed of 52% acetonitrile and 48% water containing 0.1% formic acid FLB content in the assayed samples was quantified with reference to a calibration curve that was constructed in the range of 1–1000 ng/mL.

Data Analysis

Kinetica™ software (Version 4; Thermo Fisher Scientific, Waltham, MA, USA) was used to compute the maximum plasma concentration (C_{max}), and area under the plasma concentration-time curve ($AUC_{0-\infty}$), and time to maximum plasma concentration (T_{max}). Both C_{max} and $AUC_{0-\infty}$ were expressed as mean±standard deviation, while T_{max} was presented as median.

Statistical analysis of the measured plasma concentrations and the computed parameters was performed using Prism® (version 8.4.0, GraphPad Software Inc., La Jolla, CA, USA) at 95% level of significance. Two-way ANOVA followed by Sidak's multiple comparisons test was applied to analyze the plasma concentrations. The determined C_{max} and $AUC_{0-\infty}$ were statistically analyzed using unpaired *t*-test with Welch's correction, while T_{max} was analyzed using the nonparametric Mann–Whitney *U*-test (Wilcoxon rank sum test) at $P<0.05$.

Results and Discussion

Preparation of FLB-NLCs

Hot emulsification–ultrasonication method was used for the preparation of FLB-NLCs. It is worthy to note that a prior study was conducted to test for FLB stability at the lipid melting point for a period long enough to form the proposed NLCs. The study confirmed no drug degradation under the testing conditions (data not shown). Compritol® 888ATO and almond oil were selected based on their reported successfulness for the preparation of NLCs. The used lipid materials are known for their good biocompatibility and biodegradability; in addition, almond oil has a good safety profile being a natural oil. Many researchers have reported the use of these lipids for the formulation of NLCs in previous studies.[33,36] L-phosphatidyl choline was utilized as an amphiphilic surfactant to enhance the stability of the NLCs, in addition, the combination of hydrophilic and lipophilic surfactants is reported to cause reduced particle sizes in comparison to using either alone.[37]

Experimental Design

A three-level Box–Behnken design was employed for formulation and optimization of FLB-NLCs with minimized particle size. Box–Behnken is an independent, rotatable, or nearly rotatable three-level response surface design that is widely utilized for optimization in pharmaceutical research.[38] The experimental runs composition is determined according to the combinations at the center midpoints of edges of design space. The adequate model for expressing the response is based on maximizing determination coefficient R^2, in addition, the values of R^2 and adjusted R^2 should be close to each to ensure the validity of the model to explore the design space. Pareto charts were generated to illustrate the effect of the experimental variables on the measured response (particle size).

Effect on Particle Size

The particle size is a crucial parameter for nanocarriers, as it has a significant impact on its biopharmaceutical characteristics including release pattern, absorption, and distribution in the biological system.[39] The measured particle size ranged from 65±0.98 to 287±4.45 nm indicating that the prepared NLCs were within the nano-size range (Table 2). In addition, the small standard deviation obtained indicates the homogeneity of the dispersions. The observed small size has an important role in enhancing the permeation via the nasal mucosa directly to the brain, in addition to facilitating crossing the BBB. Regression analysis of the particle size revealed that the data is best fitted to the quadratic model based on its highest R^2 and adjusted R^2 (Table 3). The model was statistically significant at a 95% confidence level. The polynomial equation representing the quadratic model was generated as indicated in equation (1).

Table 3 Statistical Analysis Output of the Measured Particle Size of FLB-NLCs, The Composition of the Optimized Formulation, and Its Predicted and Observed Response

Factor	Optimum Level		Low Level		High Level		
X_1 X_2 X_3	0.899 0.1 4.97		0.6 0.1 1.0		0.9 0.4 5.0		
Response	**Predicted**		**Actual**		**Residual**		
NLCs particle size (nm)	110.49		114.63		4.1 (3.72%)		
Statistical analysis output of NLCs particle size	**R^2** 0.9474		**Adjusted R^2** 0.8526		**SEE** 1.528	**MAE** 10.422	
	***P*-value**	**X_1**	**X_2**	**X_3**	**X_1X_2**	**X_2^2**	**X_2X_3**
		0.0001	0.0005	0.0004	0.003	0.0154	0.0126

Abbreviations: SEE, standard error of estimate; MAE, mean absolute error; FLB-NLCs, flibanserin nanostructured lipid carriers.

FLB NLCs SIZE = 575.553–605.0*SL + 695.741*LL - 14.25*ST + 118.519*SL^2 −444.444*SL*LL + 10.8333*SL*ST - 281.481*LL^2 - 22.5*LL*ST - 0.395833*ST^2 …. (1)

Analysis of variance (ANOVA) revealed a significant negative effect for both SL% (X_1) and the ST (X_3) on NLCs size with *P*-values of 0.0001 and 0.0004, respectively, as shown in Table 3 and in the Pareto chart (Figure 1). In contrast, a significant positive effect was observed for the LL% (X_2) with *P*-value 0.000. In addition, the quadratic term X_2^2 corresponding to LL%, and the interaction terms corresponding to the interaction between LL% and either SL% (X_1X_2) or ST (X_2X_3) were found to be significant at the tested significance level.

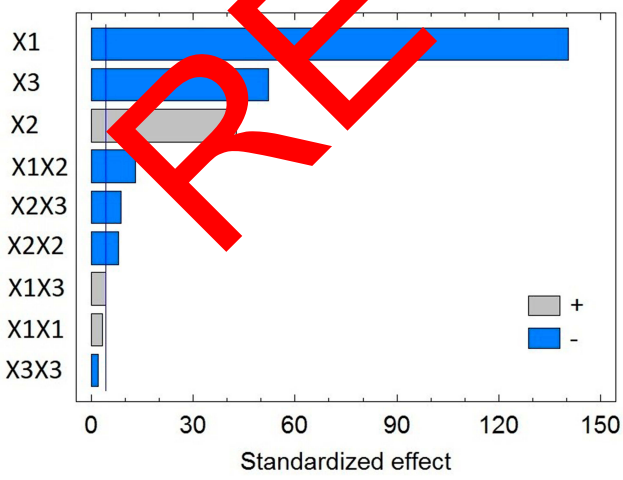

Figure 1 Standardized Pareto Chart for the particle size of flibanserin nanostructured lipid carriers (FLB-NLCs).

The contour plot for the effect of the investigated variables on the NLCs size illustrated in Figure 2 shows that the particle size of the prepared FLB-NLCs significantly decreases with increasing SL and decreasing LL content of the nanocarriers, ie, the particle size decreases with increasing SL:LL ratio at a constant total lipid phase content. This result is in agreement with previously reported studies. Upon investigating the effect of solid-to-liquid lipid ratio on properties of curcumin nanostructured lipid carriers prepared using Compritol® and Labrafac®, Snagsen et al[40] reported a particle size increase with increasing liquid oil amount. Furthermore, Mishra et al[35] reported the lowest NLCs size at the highest solid-to-liquid lipid ratio for carvedilol nano lipid carriers prepared using stearic and oleic acid as solid and liquid lipids, respectively. Higher LL concentration could facilitate lipid coalescence and increase the size of the produced vesicles. The LL induce size growth through disruption of the NLCs wall.[41] In addition, swelling of the NLCs wall due to increased LL content may result in increased NLCs size.[42]

It was evident that increasing sonication time (ST) from led to a significant reduction in the particle size, as depicted in Figure 2. A similar result was found by Ghaderi et al,[43] who reported a significant reduction in the particle size of gammaoryzanol nanoparticles upon increasing ultra-sonication time up to 5 minutes. In addition, Lasoń et al[44] reported a significant decrease in the size of NLCs with increasing ultra-sonication time. This effect could be credited to the cavitation (compression) forces generated by the passage of the ultrasonic waves through the dispersion. These forces could lead to

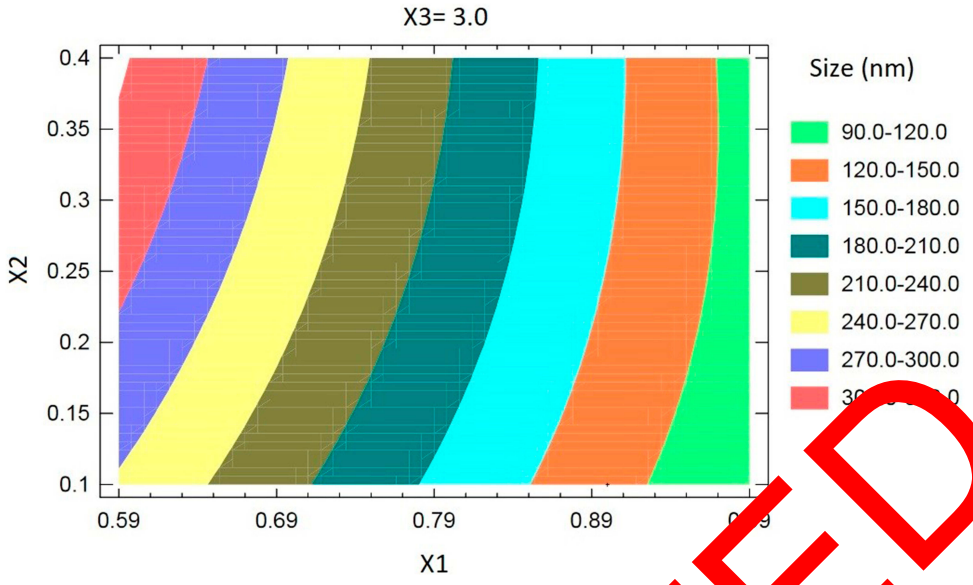

Figure 2 Contour plot for the effect of solid lipid % (X_1), liquid lipid % (X_2), and sonication time (X_3) on the particle size of flibanserin nanostructured lipid carriers (FLB-NLCs).

fractionation of the particles and reduction of their size.[45] In addition, The effect of surfactant on lipid carrier particle size has been previously reported.[46,48] The previous investigations revealed that the surfactant hinders lipid particles aggregation through reduction of the surface tension and stabilization of the newly formed surfaces. The surfactant's surface-active potential enhances lipid particles wettability and enables reduction of the particle size.

Characterization of Optimized FLB-NLCs

In order to explore the shape of the optimized FLB-NLC, transmission electron microscopic analysis was performed. As indicated in Figure 3, the NLC showed almost spherical mono-disperse appearance with no lumping or adherence. The image revealed uniform size distribution of NLC, and the observed diameter was consistent with the results obtained by dynamic light scattering size measurements. In addition, the PDI of the optimized formulation was found to be 0.241±0.052. Further, the zeta potential was found to be 8.4±1.22 mV.

The optimized formulation exhibited adequate dispersion with no significant difference in the particle size after subjection to freeze–thaw cycles during stability testing.

Characterization and in vitro Release of FLB-NLC in situ Gel

At the used gellan concentration, the prepared gel formulation showed adequate viscosity that allows easy installation into the nose as a liquid. After addition of SNF, this viscous solution was transformed into gel as evidenced by the increased gelling factor. The observed gelation that might be credited to cross-linking effect of the cations present in the SNF on the gellan gum helices could provide an improved drug residence time in the nasal cavity.[49]

In vitro release profile of FLB-NLC in situ nasal gel compared to raw FLB in situ gel in simulated nasal

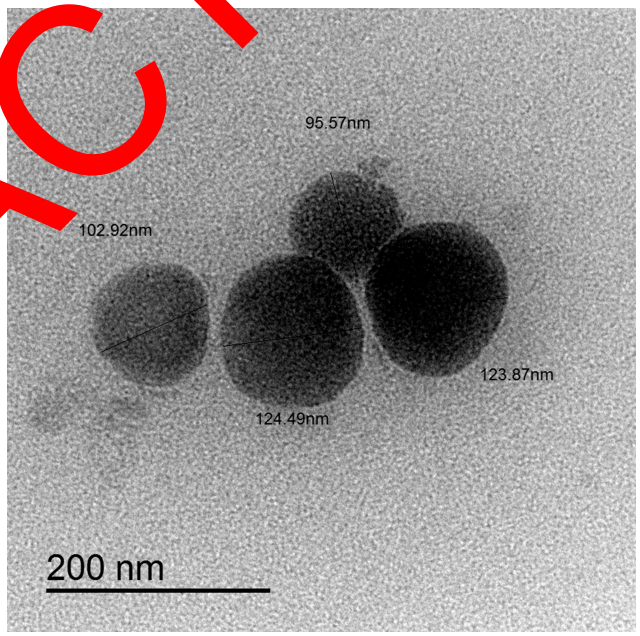

Figure 3 Transmission electron microscope (TEM) of optimized flibanserin nanostructured lipid carriers (FLB-NLCs) with 30,000X magnification.

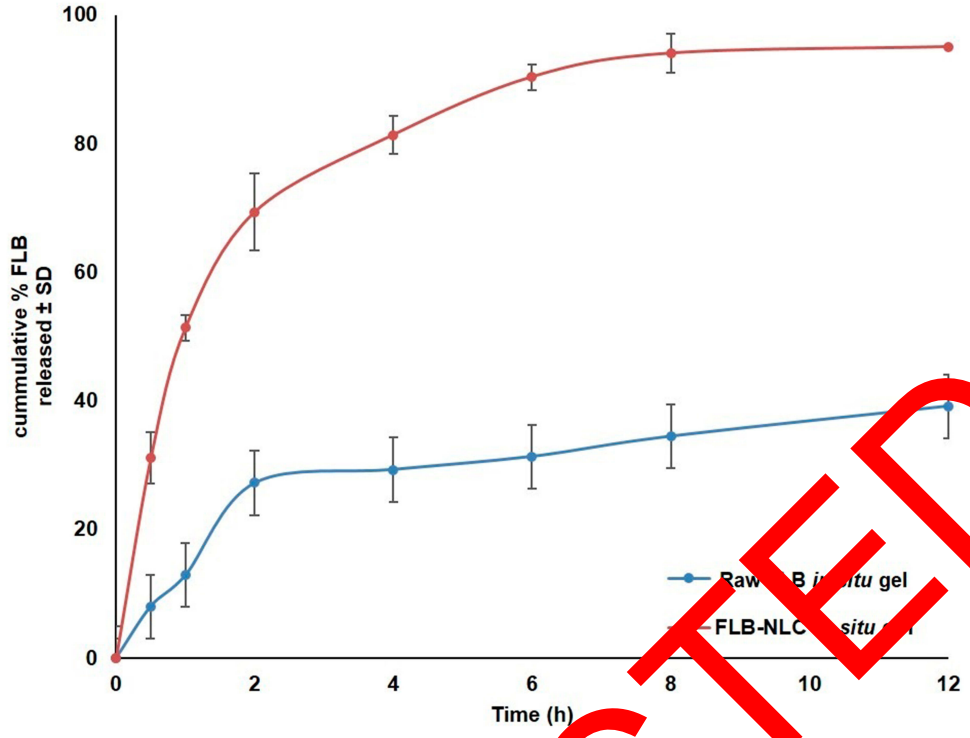

Figure 4 In vitro release profile of optimized flibanserin nanostructured lipid carriers (FLB-NLCs) in situ nasal gel compared to raw FLB in situ gel in simulated nasal fluid, pH 6.5 at 35°C (results presented as mean±SD, n=3).

fluid, PH 6.5 is graphically illustrated in Figure 4. It was evident that FLB-NLC in situ gel exhibited enhanced drug release compared to raw FLB control gel with almost complete drug release (about 98%) after 8 hours. Release efficiency after 8 hours (RE_{8h}) computed for FLB-NLC gel (74.41%±3.42) was significantly higher than that computed for the control gel (36.67% ±1.49) at $P<0.05$, confirming the ability of the proposed formulation to provide improved drug release. It is worthy to note that FLB is a water insoluble drug; accordingly, the in-situ gel matrix could have two possible mechanisms for hindering the drug release into aqueous medium. The first mechanism is the increased viscosity of the in situ gel matrix that hinders or reduces the diffusion of FLB. The second mechanism is the formation of an insoluble gel matrix that could favor the residence of raw FLB crystals within the matrix.

According to the highest coefficient of determination (R^2), kinetic analysis of FLB-NLC gel release profile showed that drug release data is best fitted to the Weibull model. The computed shape parameter (β) was 0.71, indicating that the main mechanism of drug release is Fickian diffusion combined with case II transport.[50,51]

In vivo Assessment

FLB concentrations spiked in plasma and brain homogenate were linearly correlated with the peak area ratios, with coefficients (R) of 0.9992 and 0.9984, respectively. The used assay depicted an acceptable precision with relative standard deviation (RSD) being in the range of 4.3–7.1% and 7.1–8.9% for the intra-day assay and the inter-day assay, respectively. The FLB-spiked plasma and brain samples showed that the mean extraction recovery was 94.8±5.4% to 92.6±7.6%, respectively. Mean concentrations of FLB in rats' plasma and brain following intranasal administration of optimized FLB-NLC and raw FLB in situ gels are graphically illustrated in Figure 5A and B. Compared to raw FLB gel, the optimized FLB in situ gel demonstrated significantly higher C_{max} and AUC in both plasma and brain ($P<0.05$), while no significant difference was observed for T_{max}, Table 4.

The observed higher absorption extent from optimized FLB in situ gel compared to the raw FLB gel could be ascribed to the enhanced solubility and permeability of the drug by the lipidic nature of the carrier. FLB taken intranasally is delivered along the olfactory nerves, either extracellular or intracellular, in the olfactory region (upper region) of the nasal cavity passages to the central

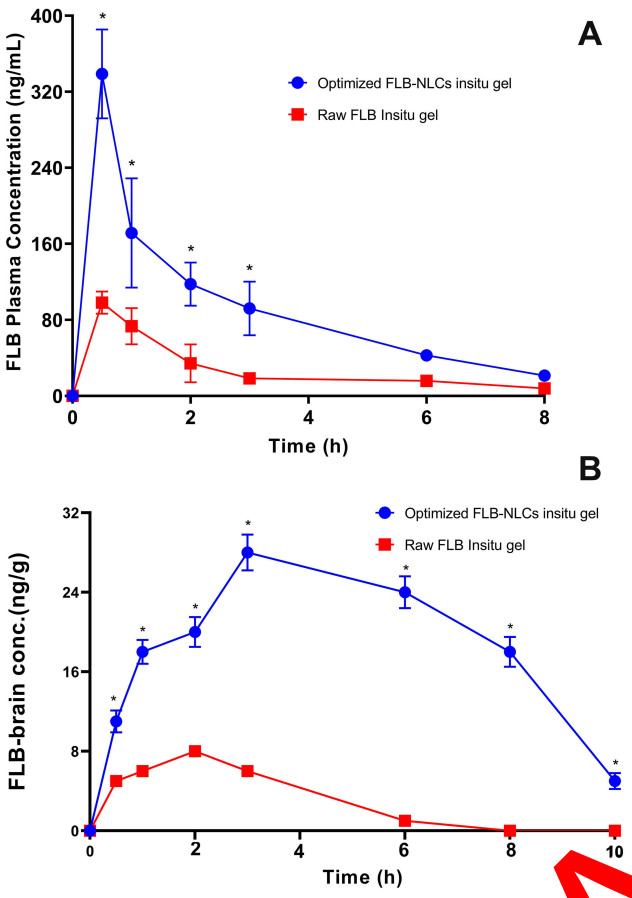

Figure 5 Mean (**A**) plasma concentrations and (**B**) brain concentrations versus time of flibanserin (FLB) in rats after nasal administration of optimized nanostructured lipid carriers (FLB-NLCs) in situ gel compared to control raw FLB in situ gel at a dose of 10 mg/kg. Results presented as mean±SD, n=6. *Significant at $P<0.05$ Sidak's multiple comparisons test.

Table 4 In vivo Pharmacokinetic Parameters Following Intranasal Administration of Optimized FLB-NLC in situ Gel Compared to Raw FLB in situ Control Gel

Pharmacokinetic Parameter	Plasma Data		Brain Data	
	Raw FLB in situ gel	FLB-NLC in situ gel	Raw FLB in situ gel	FLB-NLC in situ gel
C_{max} [&] (ng/mL, plasma) (ng/g, brain)	98.20 ±11.6	338.80 ±46.7[#]	8.11 ±1.23	28.11 ±3.28[#]
$AUC_{0-\infty}$ [&] (ng.h/mL, plasma) (ng.h/ng, brain)	255.33 ±33.2	805.72 ± [illegible]	30.38 [illegible]34	192.75 ±18.65[#]
T_{max} (h) [$]	0.5	[illegible]	3.0	4.0
Relative bioavailability (%)	–	315 [illegible]	–	634.46

Notes: [&]Data represent the mean value±standard deviation (SD), n=6. [$]Data represent the median. [#]Significant at $P<0.05$ unpaired *t*-test (two-tailed) with Welch's correction compared to raw FLB group.
Abbreviations: FLB-NLCs, flibanserin nanostructured lipid carrier.

nervous system (CNS).[52,53] The extracellular mechanism includes the enhanced flow of FLB molecules among nasal epithelium cells. This mechanism enhanced the delivery of FLB to the olfactory bulb and the CNS (within few minutes) after the intranasal administration of optimized FLB formula. Furthermore, the intracellular mechanism includes the endocytosis mechanisms or passive diffusion of FLB molecules within the olfactory receptor neuron that is followed by the slow (within several hours) FLB axonal transport to the olfactory bulbs and the other brain areas.[54,57] Also, it is reported that part of the trigeminal nerve ends in the olfactory bulbs.[58] Accordingly, there is a possibility that FLB intranasally administered from the optimized formula could reach the olfactory bulb and other rostral brain areas through trigeminal pathways. Furthermore, the improved FLB brain delivery could be attributed to enhanced absorption and permeation as a result of reduction in the nasal mucociliary clearance, P-gp efflux transporters modulation, and paracellular transport.[59,60] Finally, the elevated concentration of the drug in the brain highlights the potential of the NLC to improve the brain delivery of the drug by virtue of their nanosize and their lipid content that enhances the passage of the drug molecules directly across the BBB through nasal olfactory region.

Histopathological images of the nasal mucosal tissue showed a normal nasal wall with normal intact epithelial lining (black arrow), average submucosa with average blood vessels, average submucosal cellularity (yellow arrow), and average nasal cartilage (white arrow), which indicate no increasing in submucosal cellularity or tissue up normality in all groups, as represented in Figure 6. These observations revealed the absence of any pathological signs of epithelial damage or hyperplasia, edema, or inflammatory infiltration, indicating the safety and biocompatibility of the optimized formulation. It is worthy to note that the selection of NLCs was based, in addition to its advantages as a drug carrier, on the avoidance of organic solvent use during NLCs preparation. Avoidance of organic solvents use ensures the safety of the formula as the components are previously reported for their bio-compatibility.[61,62]

Conclusion

Compritol®/almond oil-based FLB-NLCs were successfully prepared and optimized using Box–Behnken

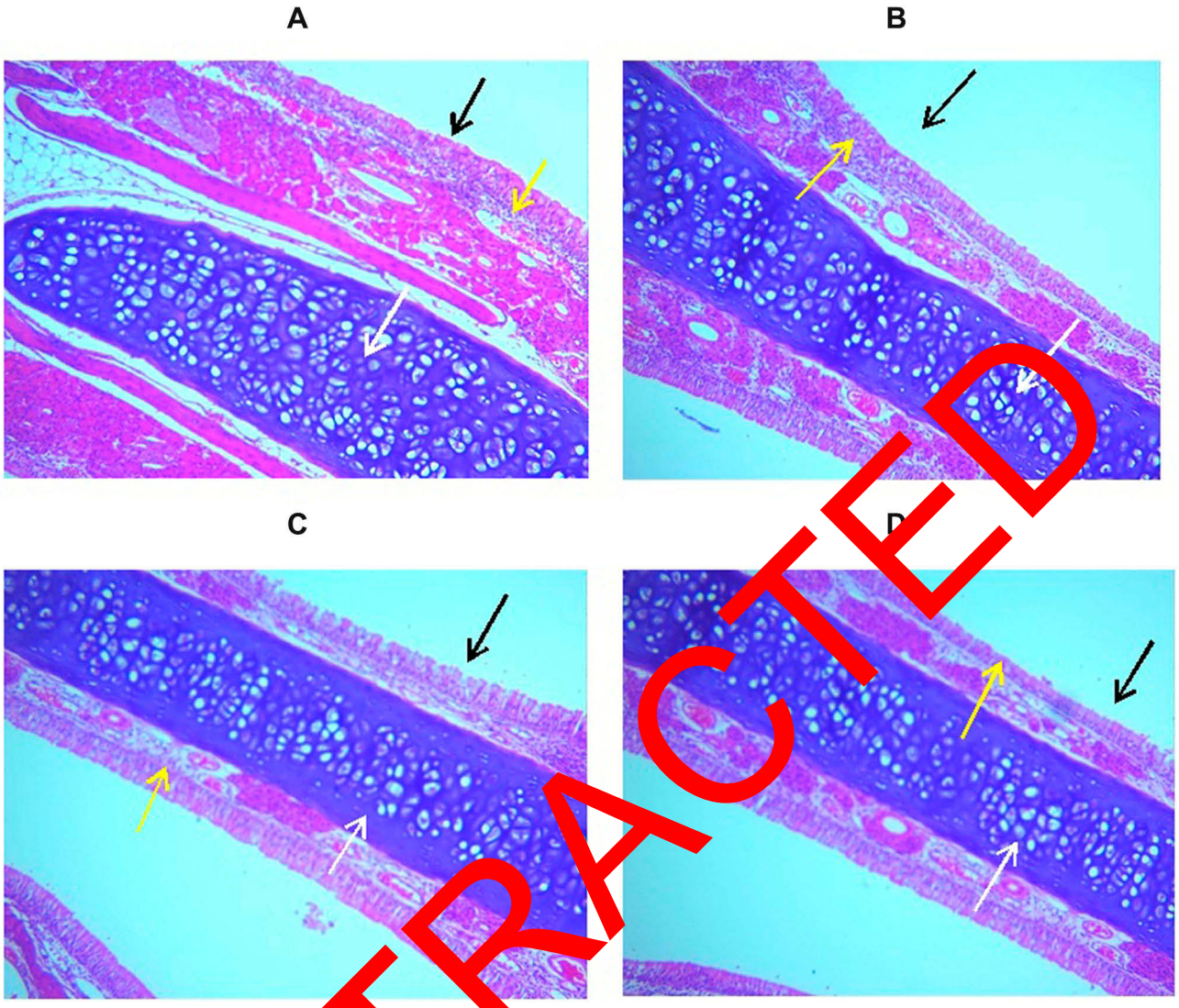

Figure 6 Histopathological images of the nasal mucosal tissue stained with H&E (x200). (**A**) Control untreated group, (**B**) plain in situ gel group, (**C**) raw FLB loaded in situ gel group, and (**D**) optimized FLB-NLC in situ gel.

design. The particle size of the NLCs was significantly affected by solid lipid%, liquid lipid%, and sonication time. The optimized formulation selected based on minimized particle size showed spherical mono-dispersed morphological characteristics with adequate stability. The optimized formulation integrated into gellan gum in situ gel showed enhanced drug release compared to raw FLB control gel. In vivo pharmacokinetic assessment in rats demonstrated higher plasma and brain concentrations of FLB from in situ gel integrating optimized NLC compared to raw FLB in situ gel. According to these results, the proposed optimized FLB-NLC in situ gel could be utilized as a potential delivery system to enhance the nose to brain delivery of the drug and circumvent its poor oral bioavailability.

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Disclosure

Solomon Okbazghi is an employee of Alexion Pharmaceuticals. The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper.

References

1. English C, Muhleisen A, Rey JA; English C, Muhleisen A, Rey JA. Flibanserin (Addyi): the first FDA-approved treatment for female sexual interest/arousal disorder in premenopausal women. *P T*. 2017;42(4):237–241.
2. Lodise NM. Female sexual dysfunction: a focus on flibanserin. *Int J Women's Health*. 2017;9:757–767. doi:10.2147/IJWH.S83747
3. Baid R, Agarwal R. Flibanserin: A controversial drug for female hypoactive sexual desire disorder. *Ind Psychiatry J*. 2018;27(1):154. doi:10.4103/ipj.ipj_20_16
4. Stahl SM, Sommer B, Allers KA. Multifunctional pharmacology of flibanserin: possible mechanism of therapeutic action in hypoactive sexual desire disorder. *J Sex Med*. 2011;8(1):15–27. doi:10.1111/j.1743-6109.2010.02032.x
5. Stahl SM. Mechanism of action of flibanserin, a multifunctional serotonin agonist and antagonist (MSAA), in hypoactive sexual desire disorder. *CNS Spectr*. 2015;20(1):1–6. doi:10.1017/S1092852914000832
6. Rolf-Stefan Brickl W, Wagner JBM, Karl Gerhard B. US20110045090A1 - Formulations of flibanserin - US Patents.
7. Ahmed OAA, Badr-Eldin SM. In situ misemgel as a multifunctional dual-absorption platform for nasal delivery of raloxifene hydrochloride: formulation, characterization, and in vivo performance. *Int J Nanomedicine*. 2018;13:6325–6335. doi:10.2147/IJN.S181587
8. Jain K, Sood S, Gowthamarajan K. Optimization of artemether-loaded NLC for intranasal delivery using central composite design. *Drug Deliv*. 2015;22(7):940–954. doi:10.3109/10717544.2014.885999
9. Parashar P, Diwaker N, Kanoujia J, et al. In situ gel of lamotrigine for augmented brain delivery: development characterization and pharmacokinetic evaluation. *J Pharm Investig*. 2020;50(1):95–105. doi:10.1007/s40005-019-00436-0
10. Erdő F, Bors LA, Farkas D, Bajza Á, Gizurarson S. Evaluation of the intranasal delivery route of drug administration for brain targeting. *Brain Res Bull*. 2018;143:155–170. doi:10.1016/j.brainresbull.2018.10.009
11. Williams, H.D., Trevaskis, N.L., Charman, S.A. Strategies to address low drug solubility in discovery and development. *Pharmacol Rev*. 2013;65(1):315–499.
12. Kawabata Y, Wada K, Nakatani M, Yamada S, Onoue S. Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications. *Int J Pharm*. 2011;420(1):1–10. doi:10.1016/j.ijpharm.2011.08.032
13. Kumar S, Dhanushkodi N, Jayaran R, Ranjana G. Nanotechnology as emerging tool for enhancing solubility of poorly water-soluble drugs. *Bionanoscience*. 2012;2(4):227–250. doi:10.1007/s12668-012-0070-7
14. Agrawal M, Saraf S, Saraf S, et al. Recent strategies and advances in the fabrication of nano lipid carriers and their application towards brain targeting. *J Control Release*. 2020;321:372–415. doi:10.1016/j.jconrel.2020.02.020
15. Puri A, Loomis K, Smith B, et al. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. *Crit Rev Ther Drug Carrier Syst*. 2009;26(6):523–580. doi:10.1615/CritRevTherDrugCarrierSyst.v26.i6.10
16. Talegaonkar S, Bhattacharyya A. Potential of lipid nanoparticles (SLNs and NLCs) in enhancing oral bioavailability of drugs with poor intestinal permeability. *AAPS PharmSciTech*. 2019;20(3):3. doi:10.1208/s12249-019-1337-8
17. Naseri N, Valizadeh H, Zakeri-Milani P. Solid lipid nanoparticles and nanostructured lipid carriers: structure preparation and application. *Adv Pharm Bull*. 2015;5(3):305–313. doi:10.15171/apb.2015.043
18. Das S, Chaudhury A. Recent advances in lipid nanoparticle formulations with solid matrix for oral drug delivery. *AAPS PharmSciTech*. 2011;12(1):62–76. doi:10.1208/s12249-010-9563-0
19. Joshi M, Patravale V. Nanostructured lipid carrier (NLC) based gel of celecoxib. *Int J Pharm*. 2008;346(1–2):124–132. doi:10.1016/j.ijpharm.2007.05.060
20. Doktorovova S, Souto EB, Silva AM. Nanotoxicology applied to solid lipid nanoparticles and nanostructured lipid carriers – A systematic review of in vitro data. *Eur J Pharm Biopharm*. 2014;87(1):1–18. doi:10.1016/J.EJPB.2014.02.005
21. Gaba B, Fazil M, Ali A, Baboota S, Sahni JK, Ali J. Nanostructured lipid (NLCs) carriers as a bioavailability enhancement tool for oral administration. *Drug Deliv*. 2015;22(6):691–700. doi:10.3109/10717544.2014.898110
22. Beloqui A, Del Pozo-Rodríguez A, Isla A, Rodríguez-Gascón A, Solinís MÁ. Nanostructured lipid carriers as oral delivery systems for poorly soluble drugs. *J Drug Deliv Sci Technol*. 2017;42:144–154. doi:10.1016/j.jddst.2017.06.0
23. Das S, Ng WK, Tan RBH. Are nanostructured lipid carriers (NLCs) better than solid lipid nanoparticles (SLNs): development, characterizations and comparative evaluations of clotrimazole-loaded SLNs and NLCs? *Eur J Pharm Sci*. 2012;47(1):139–151. doi:10.1016/j.ejps.2012.05.0
24. Pokharkar V, Patil-Gadhe A, Palla S. Efavirenz loaded nanostructured lipid carrier engineered for brain targeting through intranasal route: in-vivo pharmacokinetic and toxicity study. *Biomed Pharmacother*. 2017;94:150–164. doi:10.1016/j.biopha.2017.07.067
25. Jazuli I, Annu, Nabi B, Nabi B, et al. Optimization of nanostructured lipid carriers of lurasidone hydrochloride using box-behnken design for brain targeting: in vitro and in vivo studies. *J Pharm Sci*. 2019;108(9):3082–3090. doi:10.1016/j.xphs.2019.05.001
26. Kar N, Chakraborty S, De AK, Ghosh S, Bera T. Development and evaluation of a cedrol-loaded nanostructured lipid carrier system for in vitro and in vivo susceptibilities of wild and drug resistant Leishmania donovani amastigotes. *Eur J Pharm Sci*. 2017;104:196–211. doi:10.1016/j.ejps.2017.03.046
27. Li P, Zhu L, Ao J. A novel in situ gel base of deacetylase gellan gum for sustained ophthalmic drug delivery of ketotifen: in vitro and in vivo evaluation. *Drug Des Devel Ther*. 2015;9:3943. doi:10.2147/DDDT.S87368
28. Cai Z, Song X, Sun F, Yang Z, Hou S, Liu Z. Formulation and evaluation of in situ gelling systems for intranasal administration of gastrodin. *AAPS PharmSciTech*. 2011;12(4):1102–1109. doi:10.1208/s12249-011-9678-y
29. Sayed EG, Hussein AK, Khaled KA, Ahmed OAA. Improved corneal bioavailability of ofloxacin: biodegradable microsphere-loaded ion-activated in situ gel delivery system. *Drug Des Devel Ther*. 2015;9:1427–1435. doi:10.2147/DDDT.S80697
30. Farid RM, Etman MA, Nada AH, Ebian AEAR. Formulation and in vitro evaluation of salbutamol sulphate in situ gelling nasal inserts. *AAPS PharmSciTech*. 2013;14(2):712–718. doi:10.1208/s12249-013-9956-y
31. Tian H, Pharmacist HW-C. Determination of Content and Related Substances of Flibanserin by HPLC-DAD. *Pharmacist*. 2017;20(10):1777–1780.
32. Young J. Histopathologic examination of the rat nasal cavity. *Fundam Appl Toxicol*. 1981;1(4):309–312. doi:10.1016/s0272-0590(81)80037-1
33. Pinto F, de Barros DPC, Fonseca LP. Design of multifunctional nanostructured lipid carriers enriched with α-tocopherol using vegetable oils. *Ind Crops Prod*. 2018;118:149–159. doi:10.1016/j.indcrop.2018.03.042
34. Ribeiro LNM, Breitkreitz MC, Guilherme VA, et al. Natural lipids-based NLC containing lidocaine: from pre-formulation to in vivo studies. *Eur J Pharm Sci*. 2017;106:102–112. doi:10.1016/j.ejps.2017.05.060

35. Mishra A, Imam SS, Aqil M, et al. Carvedilol nano lipid carriers: formulation, characterization and in-vivo evaluation. *Drug Deliv*. 2016;23(4):1486–1494. doi:10.3109/10717544.2016.1165314
36. Bashiri S, Ghanbarzadeh B, Ayaseh A, Dehghannya J, Ehsani A, Adun P. Essential oil-loaded nanostructured lipid carriers: the effects of liquid lipid type on the physicochemical properties in beverage models. *Food Biosci*. 2020;35:100526. doi:10.1016/j.fbio.2020.100526
37. Akhoond Zardini A, Mohebbi M, Farhoosh R, Bolurian S. Production and characterization of nanostructured lipid carriers and solid lipid nanoparticles containing lycopene for food fortification. *J Food Sci Technol*. 2018;55(1):287–298. doi:10.1007/s13197-017-2937-5
38. Khuri AI, Mukhopadhyay S. Response surface methodology. *WIREs Comput Stat*. 2010;2(2):128–149. doi:10.1002/wics.73
39. Jain AK, Thareja S. *In vitro* and *in vivo* characterization of pharmaceutical nanocarriers used for drug delivery. *Artif Cell Nanomed Biotech*. 2019;47(1):524–539. doi:10.1080/21691401.2018.1561457
40. Sangsen Y, Laochai P, Chotsathidchai P, Wiwattanapatapee R. Effect of solid lipid and liquid oil ratios on properties of nanostructured lipid carriers for oral curcumin delivery. *Adv Mater Res*. 2014;1060:62–65. doi:10.4028/www.scientific.net/AMR.1060.62
41. Hu FQ, Jiang SP, Du YZ, Yuan H, Ye YQ, Zeng S. Preparation and characterization of stearic acid nanostructured lipid carriers by solvent diffusion method in an aqueous system. *Colloids Surf B Biointerfaces*. 2005;45(3–4):167–173. doi:10.1016/j.colsurfb.2005.08.005
42. Tamjidi F, Shahedi M, Varshosaz J, Nasirpour A. Design and characterization of astaxanthin-loaded nanostructured lipid carriers. *Innov Food Sci Emerg Technol*. 2014;26:366–374. doi:10.1016/j.ifset.2014.06.012
43. Ghaderi S, Ghanbarzadeh S, Mohammadhassani Z, Hamishehkar H. Formulation of gammaoryzanol-loaded nanoparticles for potential application in fortifying food products. *Adv Pharm Bull*. 2014;4 (Suppl 2):549–554. doi:10.5681/apb.2014.081
44. Lasoń E, Sikora E, Ogonowski J. Influence of process parameters on properties of nanostructured lipid carriers (NLC) formulation. *A Biochim Pol*. 2013;60(4):773–777. doi:10.18388/abp.2013_2056
45. El-Helw A-RMA-RM, Fahmy UA. Improvement of fluvastatin bioavailability by loading on nanostructured lipid carri *Int J Nanomedicine*. 2015;10:5797–5804. doi:10.2147/IJN.S1550
46. Hosny KM, Bahmdan RH, Alhakamy NA, Alfalel HA, Ahm OA, Elkomy MH. Physically optimized nano-lipid ca rs augm ifene and vitamin d oral bioavailability in l dthy for management of osteoporosis. *J Pharm S* 2020;109(2145–2155. doi:10.1016/j.xphs.2020.03.009
47. Helgason T, Awad TS, Kristbergs n K, M lements DJ, eiss J. Effect of surfactant surface c erage on for ion of solid lipid nanoparticles (SLN). *J Co d Interface Sci*. 2 ;334(1):75–81. doi:10.1016/j.jcis.2009.0 12
48. Shah M, Pathak K. De opment d statistical optimization of solid lipid nanoparticles of simva y using 23 -factorial design. *AAPS PharmSciTech*. 2 2):489 96. doi: 208/s12249-010-9414-z
49. Paulsson M, Hägerström H, Edsman K. Rheological studies of the gelation of deacetylated gellan gum (Gelrite®) in physiological conditions. *Eur J Pharm Sci*. 1999;9(1):99–105. doi:10.1016/S0928-0987(99)00051-2
50. Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. *Eur J Pharm Sci*. 2001;13(2):123–133. doi:10.1016/S0928-0987(01)00095-1
51. Papadopoulou V, Kosmidis K, Vlachou M, Macheras P. On the use of the Weibull function for the discernment of drug release mechanisms. *Int J Pharm*. 2006;309(1–2):44–50. doi:10.1016/j.ijpharm.2005.10.044
52. Jansson B, Björk E. Visualization of in vivo olfactory uptake and transfer using fluorescein dextran. *J Drug Target*. 2002;10(5):379–386. doi:10.1080/1061186021000001823
53. Ruigrok MJR, de Lange ECM. Emerging for translational pharmacokinetic and pharmacokineti harmacod mic studies: towards prediction of nose-to-brain nsport in hum s. *AAPS J*. 2015;17(3):493–505. doi:10.1208/s122 015-9724-x
54. Dhuria SV, Hanson LR, Frey H. Intranasal delivery to the central nervous system: mechani s and experim al siderations. *J Pharm Sci*. 2010;99(4): 4–1673 :10.1002/j .21924
55. Haque S, Md S, Sahni A , Baboota S. Development and evaluation of br targeted ntranasal ginate nanoparticles for treatment of pression. *J ych Res*. 2014;48(1):1–12. doi:10.101 psyc s.2013.10.01
56. Misra A, Jogani V, J rkar K, Vyas T. Recent patents review on intra administration r CNS drug delivery. *Recent Pat Drug D v Formul*. 2008;2(1):25 40. doi:10.2174/187221108783331429
57. G mez D, Martin z JA, Hanson LR, Frey WH, Toth CC. Intranasal t atment of neur generative diseases and stroke. *Front Biosci Sch*. 2 :4S(1):74–8 doi:10.2741/s252
58. Tale kar S ishra PR. Intranasal delivery: an approach to bypass the blood brain barrier. *Indian J Pharmacol*. 2004;36(3):140–147.
59. BJ, Broadwell RD, Salcman M, El-Kalliny M. Avenues for entry of peripherally administered protein to the central nervous system in mouse, rat, and squirrel monkey. *J Comp Neurol*. 1986;251(2):260–280. doi:10.1002/cne.902510209
60. Pardeshi CV, Belgamwar VS. Direct nose to brain drug delivery via integrated nerve pathways bypassing the blood-brain barrier: an excellent platform for brain targeting. *Expert Opin Drug Deliv*. 2013;10(7):957–972. doi:10.1517/17425247.2013.790887
61. Prasad PN. *Introduction to Nanomedicine and Nanobioengineering*. John Wiley & Sons; 2012.
62. Desai P, Patlolla RR, Singh M. Interaction of nanoparticles and cell-penetrating peptides with skin for transdermal drug delivery. *Mol Membr Biol*. 2010;27(7):247–259. doi:10.3109/09687688.2010.522203