ABSTRACT

Objective: Carbamazepine (CBZ) is used as a first line in the treatment of grand mal and partial seizures, but it suffers from many side effects on different systems of the body. The objective of the present study was optimization of CBZ vesicular structures using ²⁴ multivariate design for the most efficient targeting of CBZ to the brain via the intranasal route.

Methods: The concentration of CBZ (10 and 20%), type of vesicles (niosomes and spanlastics) and speed of rotation (200 and 300 rpm) were considered as the independent variables X₁, X₂, and X₃ respectively, while the dependent variables were particle size PS (Y₁), polydispersity index PDI (Y₂), zeta potential ZP (Y₃) and entrapment efficiency EE (Y₄). The study of the effect of different formulation variables was carried out using Design-Expert ® software. CBZ-loaded spanlastics and niosomes were prepared by the ethanol injection method and thin film hydration method, respectively. The optimized formulation was subjected to viscosity measurement, in vitro drug release and physical stability studies. In vivo evaluations in rats for the optimized formulation in comparison to oral CBZ suspension was carried out using behavioral assessment by elevated plus maze test, determination of endothelial nitric oxide synthase (e-NOS), reduced glutathione (GSH) and ELISA estimation of TNFs.

Results: The selected optimized formulation (F₀) containing 20% CBZ and spanlastic vesicular structure showed PS, PDI, ZP, and the EE % of 350.09 nm, 0.830, 16.124 mV and 82.777%, respectively. In vitro release study of F₀ demonstrated the ability of the F₀ to increase drug release in the range time from 10-60 min (p<0.05) when compared with CBZ suspension. The viscosity of F₀ was nearly uniform (65 cps). The photomicrograph taken by the transmission electron microscopy (TEM) reveals the spherical shape of F₀. Good physical stability for six months of storage at 25 °C was found for F₀. The optimized spanlastic formulation F₀ showed a decrease in latency time in behavior assessment test using elevated plus Maze test, a decrease in serum eNOS and TNF-α and increase in GSH when compared with the oral CBZ suspension, in addition to the histopathological study that revealed the more CBZ uptake by the brain.

Conclusion: The optimized spanlastic formulation F₀ achieved better results when compared with the oral CBZ suspension for targeting the CBZ spanlastic vesicular structure to the brain via the nasal route.

Keywords: Optimization, Carbamazepine, Spanlastics, Niosomes, Brain targeting, Elevated Plus Maze Test, Histopathology and Intranasal route

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by the occurrence of two or more unprovoked seizures which are signs of excessive and synchronous neuronal discharge [1].

CBZ is an iminostilbene derivative; it has a similar structure to a tricyclic antidepressant. CBZ is used as a first line for the treatment of generalized tonic-clonic or partial seizure, and also it is used in psychiatric disorders, trigeminal neuralgia and schizophrenia [2]. CBZ has a specific mechanism to treat epilepsy by blocking sodium channel which leads to the delay of the recovery and decreases the firing’s rate [3]. CBZ commonly exhibits the slow onset of action and systemic toxicity when administered via oral or intravenous (IV) routes due to the complex tight junctions between the endothelial cells of brain capillaries [4]. The intranasal route is the best choice to deliver CBZ directly to the brain, as this route exhibits several advantages as the good penetration and absorption of the small molecules, rapid delivery of therapeutic effect to blood and it can avoid metabolism by the first pass effect of the liver. The intranasal route delivers the drug to the brain through different pathways as the olfactory pathway, trigeminal nerve pathway or vascular pathway. Different dosage forms as Nasal drops, sprays, gel, solution, and suspension can be used via the intranasal route [4].

Nanotechnology played an important critical role in different biomedical applications not only in drug delivery. Nowadays it is utilized in targeted areas such as cancer targeting, brain targeting, and gene delivery. Nanotechnology is classified into nano-devices and nanomaterials which are classified into nanocrystalline and nanostructured which may be organic and inorganic particles [5]. The organic nanoparticles may contain polymeric nanosphere, nanocapsule, micelle, niosomes or liposomes. The inorganic nanoparticles are like dendrimers nanoparticle or carbon nanotube. Niosomes are the novel systems for drug targeting, they consist of cholesterol which is responsible for the rigidity and stability of the carrier system, and nonionic surfactant which can carry either hydrophilic or hydrophobic drug [6–8]. They have many advantages as enhancement oral bioavailability, enhancement skin permeability and have better patient compliance. Niosomes or non-ionic surfactant vesicles (NSVs) show a high level of encapsulated drugs in the animals’ brain which was reported in several types of research [9]. Increased brain uptake of doxorubicin reported when prepared as niosomes and when compared to the commercial doxorubicin [10].

Spanlastic vesicles (SV) are non-conventional types of niosomes composed of Span or Tween as edge activator and nanocapsule, micelle, niosomes or liposomes. The inorganic nanoparticles are like dendrimers nanoparticle or carbon nanotube. The organic nanoparticles may contain polymeric nanosphere, nanocapsule, micelle, niosomes or liposomes. The inorganic nanoparticles are like dendrimers nanoparticle or carbon nanotube. Niosomes are the novel systems for drug targeting, they consist of cholesterol which is responsible for the rigidity and stability of the carrier system, and nonionic surfactant which can carry either hydrophilic or hydrophobic drug [6–8]. They have many advantages as enhancement oral bioavailability, enhancement skin permeability and have better patient compliance. Niosomes or non-ionic surfactant vesicles (NSVs) show a high level of encapsulated drugs in the animals’ brain which was reported in several types of research [9]. Increased brain uptake of doxorubicin reported when prepared as niosomes and when compared to the commercial doxorubicin [10].

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optimize the best vesicular structure for brain targeting via intranasal route.

The aim of the present study was to optimize the CBZ nano-vesicular structures for rapid drug release, minimize the potential side effects and fasten the brain targeting via intranasal route and to study the cognitive enhancing mechanism of the CBZ by behavioral analysis using elevated plus maze test, histopathological examination and biochemical evaluation of the brain oxidative stress and inflammation.

MATERIALS AND METHODS

Materials

Carbamazepine (CBZ) was a gift from SEDICO pharmaceutical, Giza (Egypt). Cholesterol was purchased from Bio Basic (Toronto, Canada). Span 60, was purchased from Oxford Lab Chem, Mumbai (India). Polyvinyl alcohol (PVA) was purchased from Sigma-Aldrich, St. Louis, USA. Pentylentetrazol (PTZ) was purchased from Merck industrial company. All other chemicals and solvents were of pharmaceutical grade, from ADWIC Company (Qalyubia Egypt).

Methods

Preparation of spanlastics formulations

CBZ-loaded spanlastics (F1, F4, F 6 and F7) were prepared by ethanol injection method as showed in table 1. CBZ and span were dissolved in 10 ml absolute ethanol and injected slowly in the preheated aqueous phase (60 °C) containing PVA and was stirred for 1 h on magnetic stirrer then, the formed dispersion was sonicated for 2 min. Eventually, the prepared formulations were left in the refrigerator until further investigation [13]. Experimental runs, independent variables and the measured dependent variables for the prepared formulations were given in table 1.

Preparation of niosomes formulations

Noisome formulations (F2, F3, F 5 and F8) as shown in table 1 were prepared by thin film hydration method. CBZ and span cholesterol mixture (mole ratio 1:1) were dissolved in 10 ml of methanol: chloroform mixture (volume ratio 1:2). The dried film was obtained by evaporation of the solvent using a rotary evaporator at 60 °C and under low pressure for 45 min. This dried layer was then rehydrated using 5 ml of phosphate buffer (pH 6.8) and rotated for 1 h. The vesicle suspension was sonicated for 2 min at 60 °C [14].

Table 1: Experimental runs, independent variables and the measured dependent variables for the prepared formulations

| Formula code | Percentage of CBZ (%) (X₁) | Type of vesicles (X₂) | Speed of rotation (rpm) (X₃) | Particle size (nm) (Y₁)* | Polydispersity index (Y₂)* | Zeta potential (mV) (Y₃) | Entrapment efficiency (%) (Y₄) |
|--------------|---------------------------|----------------------|-----------------------------|-------------------------|--------------------------|--------------------------|-----------------------------|
| F1           | -1                        | Spanlastics          | -1                          | 448±13.5                | 0.95±0.04                | -9.9±0.4                 | 69.4±5.1                    |
| F2           | -1                        | Niosomes             | -1                          | 2309.4±105.3            | 0.65±0.07                | -14.6±0.5                | 96.9±4.3                    |
| F3           | 1                         | Niosomes             | -1                          | 2990±117.2              | 0.59±0.03                | -57.9±3.6                | 66.2±5.2                    |
| F4           | 1                         | Spanlastics          | 1                           | 413±6±21.5              | 0.95±0.05                | -5.3±0.4                 | 91±4.6                      |
| F5           | -1                        | Niosomes             | 1                           | 767.3±11.3              | 0.7±4.04                 | -60.6±5.2                | 95.1±3.8                    |
| F6           | -1                        | Spanlastics          | 1                           | 263.8±4.76              | 0.85±0.05                | -10±6.6                  | 78±3.4                      |
| F7           | -1                        | Spanlastics          | 1                           | 295±5.9                 | 0.99±0.05                | -4.6±5.1                 | 44±2.5                      |
| F8           | 1                         | Niosomes             | -1                          | 1914.8±50.3             | 0.88±0.04                | -58.9±3.4                | 97.6±4.2                    |

X₁: Percentage of CBZ (%): Low level (-1) = 10%, High level (1) =20%, X₂: Type of vesicles: Spanlastics or niosomes, X₃: Speed of rotation (rpm): Low level (-1):-200 rpm, High level (1):300 rpm, 'All results are the mean±SD, n=3

Statistical optimization of the formulations

A 2³×2×2 multifactorial factorial design was utilized to develop and optimize eight formulations and to investigate the relationship between independent variables and their responses. Design-Expert 10.0.1.1® software (Stat-Ease Inc., Minneapolis, United States of America) was used to by changing the utilized concentrations of CBZ, type of vesicles and speed of rotation which were considered as the independent variables X₁, X₂ and X₃ respectively, as shown in table 1. On the other hand, dependent variables were PS (Y₁), PDI (Y₂), ZP (Y₃) and EE (Y₄). Desirability values were measured to identify the optimum formula based on analyzing the response surface of the obtained data [13].

Characterization of the prepared vesicles structures

Determination of particle size (PS), zeta potential (ZP) and polydispersity index (PDI)

PS, size distribution (expressed in term of PDI) and ZP of the prepared vesicular systems were evaluated by dynamic light scattering (DLS) technique employing Zetasizer (Nano ZS-90, Malvern Inst., Worcestershire, UK). This technique analyzes the fluctuations in light scattering as a result of the Brownian motion of particles to estimate Z-average. Representative samples had been taken from each formula and diluted with water in ratio 1:10 v/v as dispersant at 25 °C [15]. The results are the mean of three readings±standard deviation.

Entrapment efficiency (EE)

Samples from each formula were centrifuged at 15000 rpm for 90 min at 4 °C using an ultra-cooling centrifuge for separation of supernatant containing a non-entrapped drug. After that, the supernatant was collected by micropipette and analyzed using a UV-visible spectrophotometer (UV-1800 spectrophotometer, Shimadzu, Kyoto, Japan) against a blank at the predetermined λ_max (284 nm) to quantify the contribution of the components to UV absorption [16]. The amount of drug entrapped was expressed as a percentage (EE%) and calculated from the following equation (equation 1):

\[
EE\% = \frac{T - F}{T} \times 100
\]

Where T is a total drug amount that theoretically present in the taken sample, F is the free drug amount that actually presents in the supernatant. The experiment was done in triplicate and represented as a mean±standard deviation.

Selection of the optimized formulation

The desirability values were used as a differentiating parameter to compare between the different prepared formulations. The optimized formulation (F0) was planned to be with the least PDI and PDI in addition to the highest ZP and EE. The optimization of all responses was performed by calculating the overall desirability. Then, the optimized formulation was prepared and subjected to more characterization [17].

Transmission electron microscopy (TEM)

Morphological features of the optimized formulation were conducted to evaluate structural attributes including the lamellarity, uniformity of size and shape. In brief, one drop of appropriately-diluted dispersion was spotted on a carbon-coated copper grid. The excess was removed using a filter paper, dried at room temperature and was stained with 2% w/v phosphotungstic acid for 10 min, then washed with distilled water for three times and left to dry before the examination. Photomicrograph was taken by TEM (Philips 208 S, USA.) [18]
Viscosity measurement

The viscosity of the optimized formulation (F₀) was measured without dilution using cone and plate viscometer (Brookfield DV-III, USA) at 25±0.5°C, attached with Spindle No CP42. The experiment was done in triplicate and represented as a mean±standard deviation.

In vitro drug release study

In vitro release study was carried out using vertical type Franz diffusion cells (Hanson research, Los Angeles, California, USA) with a 1 cm² diffusion area. The optimized formulation (F₀) and the CBZ suspension equivalent to 5 mg was placed in the donor compartment to compare the release of CBZ from the optimized vesicular structures and the CBZ suspension. The receptor compartment was filled using 7 ml phosphate buffer at pH 6.8, the temperature was maintained at 37±0.5°C and the rotation was adjusted at 500 rpm. The formulation was separated from the receptor compartment using the cellophane membrane (Spectrum Medical Inc., Los Angeles, CA, USA cut-off 12,000–14,000).

Samples were withdrawn (400 µl) at the following time intervals; 10, 20, 30, 45, 60, 70, 90, 120, 150 and 180 min and were replaced with fresh buffer solution kept under the same conditions. The drug concentrations were analyzed using a UV spectrophotometer at predetermined λ max (284) nm. The readings are the mean of three experiments±standard deviation.

The percent of the drug release was calculated and plotted against time (min) for each of the optimized formulation and the pure CBZ suspension. The data obtained from the release study was subjected to kinetic analysis and the order of drug release was determined regarding zero and first-order kinetics as well as the Higuchi diffusion model.

Physical stability study

The optimized CBZ formulation (F₀) was kept for 6 mo in a tightly closed glass container at 25±2°C. EE %, PS and drug release were re-evaluated at the end of the six months. The results were analyzed statistically using Design Expert software (version 10.0.1.0, Stat-Ease, Inc, USA). The difference was considered significant at P ≤ 0.05. Similarity factor (f₂) was used and the release of both, the fresh and the stored formulations was compared. Similarity factor was calculated according to Moore and Flanner model (1996) using the following equation (equation 2):

\[ f_2 = 50 \log \left[ \left( \frac{1}{R} \right) \sum_{i=1}^{n} \left( \frac{R_t - T_t}{R_t} \right)^2 \times 100 \right] \]

R and T are the percentages of CBZ released at time t for fresh and test formulations, respectively, while n is the number of the sampling point. The release of both the fresh and the test formulations were considered similar when f₂ value falls within the range of 50–100.

In vivo studies

Based on the pharmaceutical in vitro evaluation, the optimized formulation (F₀) was selected for the in vivo evaluations in rats in comparison to oral CBZ suspension. All animal experiments were approved by the Pharmaceutical Ethical Committee of MSA University (PT 2/EC2/2017 PD).

Male Wistar rats weighing 220-250g, were purchased from the Egyptian Company for Production of Vaccines, Sera and Drugs (EGYVAC; Cairo, Egypt) and allowed free access to water and standard pellet chow. They were kept under constant conditions (temperature 25±3°C and humidity 50%) with 12/12 h light/dark cycles. The study was carried out according to the guidelines of the Ethics Committee, Faculty of Pharmacy, October University for Modern Sciences and Arts.

Experimental design

A preliminary study was carried out on 10 rats to determine the animals’ behavior and survival rate after the administration of PTZ (50 mg/kg, i. p) [19]. This was achieved by observing the animals for 60 min following the injection of PTZ. The kindling score was recorded immediately after PTZ administration according to a prevalidated 5-point scoring scale (0:no convulsion; 1:occasional body twitches; 2:partial clonic seizures in the body; 3:generalized clonic seizures; 4:tonic seizures).

In the main study, 32 rats were randomly divided into four groups, 8 rats in each group:

Group I: received normal saline to serve as a negative control group.

Group II: received a single dose of PTZ (50 mg/Kg; i. p) [19] dissolved in normal saline for the induction of kindling.

Group III: received oral CBZ (20 mg/kg) 30 min before PTZ administration[20].

Group IV: received the optimized formulation (F₀) (1 mg/kg intranasal) in equal amount in both nostrils 20 min after PTZ administration.

After performing the behavioral tests, 60 min after PTZ administration, blood was collected from each rat aseptically by sterile disposable 3 ml syringe through cardiac puncture for serum separation and estimation of the eNOS level. Rats were sacrificed using thiopental (50 mg/kg; i. p) [21] and brains were removed, cleaned with cold saline (0.9%) and either fixed in 10% formalin for histopathological examination or homogenized in phosphate buffer saline to measure TNF-α and GSH content.

Fig. 1: Elevated plus maze device; fig. 1a: The maze is a wooden device consists of central platform 10 x 10 cm with 10 x 50 cm two open arms and two 10 x 50 x 40 cm closed arms with an open roof. Fig. 1b: Rats were individually placed at the distal end of the open arm (@) at T=0 min, T=15 min and T=60 min. Fig. 1c: time taken for the rat entering to either of the closed arm (*) was recorded.
Behavioral assessment by elevated plus maze test

Kindling-induced cognitive impairment was assessed using elevated plus maze and passive avoidance test. The maze is a wooden device consisting of a central platform (10 x 10 cm) with two open arms (10 x 50 cm) and two closed arms with an open roof (10 x 50 x 40 cm) as shown in fig. 1a. The height of the maze was 50 cm from the floor. Each rat underwent 3 trials. In the first trial, the rat was individually placed at the distal end of the open arm as shown in fig. 1b. 20 min after PTZ administration and the time taken for the rat to enter any of the closed arms were recorded as the initial transfer latency (T transfers). The second and third trials were performed similarly 30 and 40 min after PTZ administration and the retention transfer latency (T transfer) and (T transfers) were recorded respectively [22]. After each trial, the maze was cleaned with alcohol to remove confounding olfactory cues. A 90 seconds cut-off was set. To become familiar with the maze, rats were allowed to move freely in the maze after reaching the closed arm for another 10 seconds [23].

Characterization of the prepared vesicles

According to equation (3), the positive value of drug's percent XA reveals that the increase of drug's percent will lead to increase the PS of niosomes as the more increase in CBZ's percent, the more encapsulation of CBZ into the vesicle. This is because of the probability of the interaction between the CBZ, the surfactant head groups and the interaction with cholesterol which leads to the increase of the PS. While with spanlastics the more increase the drug’s percent, the more decrease the PS due to the absence of cholesterol as shown by the negative coefficient of Xb in equation (5) [27]. The negative coefficient with cholesterol of Xc reveals that, increasing the speed of rotation lead to the decrease the PS of both noisome and spanlastics. This is due to the formation of a thin uniform lipid layer result in the spherical vesicles upon the hydration of the vesicles [28].

Equation (3) shows that PS is affected adversely by the presence of the vesicles of niosomes and spanlastics as can be deducted from the negative coefficient of Xc. Spanlastics have smaller PS than niosomes and this due to; spanlastics is prepared by using span60 which has low hydrophilic-lipophilic balance (HLB) leading to decrease the surface energy and formation a smaller PS. Also, it contains polyvinyl alcohol which contributing to this small size by decreasing the membrane [29]. On the other hand, Niosomes prepared by using cholesterol which makes a rigid bilayer through reducing the vesicle's phase transition temperature peak and increasing the liquid bilayer's chain [30, 31]. As showed in equation (3) and table 2, the PS of both the niosomes and spanlastics is significantly affected by all factors except Xc, which has an insignificant effect on PS as shown in equation (4) and (5).

The effect of independent formulation variables on the polydispersity index (PDI)

PDI refers to the vesicle size's uniformity within the formulation. PDI is used as a measure for the formulation homogeneity. It is calculated through the ratio of the standard deviation to the mean vesicle size. The higher of the PDI, the lower of the vesicle size's uniformity [32]. The PDI for the prepared formulation ranged from 0.594-0.99 as shown in table 1. The response Y2, which is the PDI is represented by the following polynomial equation (equation 6):

\[ Y2 = 0.080 - 0.015 XA + 0.10 XB + 0.061 XC - 0.015 XAB + 0.018 XAC - 0.028 XBC \]

• For niosomes represented in equation 7:
  \[ PDI = 0.70 + 3.75E - 004 XA + 0.08 XC + 0.018 XAC \]

• For spanlastics represented in equation 8:
  \[ PDI = 0.90 - 0.029 XA + 0.033 XC - 0.018 XAC \]

As shown in table 2, PDI is significantly affected by all factors except Xa, Xb and Xc which have an insignificant effect on PDI.

According to equations (7) and (8), the positive coefficient of Xc reveals that increasing the speed of rotation increases the PDI of both noisome and spanlastics. This is due to the formation of a thin uniform lipid layer that results in the formation of spherical vesicles upon the hydration of both vesicles which leads to increase the PDI [28].

As shown in equation (6), the positive coefficient (Xc) reveals that PDI is affected by the presence of the vesicular structure of both niosomes and spanlastics. Niosomes have larger PS so they show lower PDI. On the other hand, spanlastics which have smaller PS show larger PDI that may be due to the difference in composition between both types of vesicular structures [33].

The effect of independent formulation variables on the zeta potential (ZP)

ZP measures the repulsion's degree between the same charged particles in the dispersion system and this will determine the vesicle's stability [34]. The ZP ranged from -4.63 mV-60.6 mV as represented in table 1. ZP is significantly affected by all factors except Xa which has an insignificant effect on ZP as shown in table 2.

According to equation (4), the positive value of drug's percent Xa reveals that the increase of drug's percent will lead to increase the
Equation (9) showed the negative coefficient of X to an increase the ZP for both niosomes and spanlastics. Decreasing the ratio of both drug’s percent and rotation’s speed leads in the ZP in the spanlastics. According to equations (10) and (11), on the other hand, increasing the speed of rotation leads to a decrease aggregation and achieves higher repulsion between the particles [28]. Due to the formation of a thin film that prevents the increase in the ZP and the more stability will be achieved this could be the more increase in the speed of rotation in niosomes, the more.

The ZP is affected by the type of the vesicles whether niosomes or spanlastics. Equation (9) showed the negative coefficient of X which show that ZP is affected by the type of the vesicles whether niosomes or spanlastics which can be attributed to the presence of edge activator on the surface of the vesicular layers leading to the decrease in the ZP in case of spanlastics [35].

The effect of independent formulation variables on entrapment efficiency (%EE)

EE is an important parameter in the vesicular formulation’s design. The more the EE, the more drug’s bioavailability and also the higher the drug’s concentration will be targeted to the specific site leading to decrease in the required dose [32]. The EE ranges for all prepared formulations was ranged from 4%-97.6%.

Final equation in terms of coded factors for EE is represented by the following polynomial equation (equation 12)

\[ Y_4 = +83.80 + 7.23 X_A - 12.87 X_B - 1.83 X_C + 6.59 X_A B + 4.95 X_A C - 1.60 X_B C \]

According to equations (13) and (14), the positive coefficient of Xs reveals that the EE increased as the drug amount which is used to load the niosomes or the spanlastics increased. This is may be due to the increase in the medium’s saturation with the drug which will then be available to be encapsulated into the vesicles [32].

As shown in table 2, the EE is insignificant for XAs as the p-value is=0.05).

The EE of niosomes is higher than that of spanlastics, this may be attributed to the EE of niosome will be affected by both components; cholesterol and span 60. Cholesterol increases the membrane’s rigidity by increasing the membrane bilayer hydrophobicity making it more rigid and this will prevent the drug’s leakage [36]. While the spanlastics showed lower EE than niosomes this may refer to the presence of span 60 and absence of cholesterol. Span 60 has a low HLB value (5.3), long carbon chain (C16) and it has a high phase transition temperature which decreases the leakage and the fluidity of the bilayer that leads to lower EE [33, 37].

The Pred. R² for PS, PDI, ZP and EE was found to be 0.96, 0.94, 0.86 and 0.76, respectively and in reasonable agreement with the Adj. R² of 0.96, 0.94, 0.89 and 0.89 for PS, PDI, ZP and EE, respectively.

Fig. 2: 3D surface plot of (a) PS, (b) PDI, (c) ZP and (d) EE% of niosomes and spanlastics
Table 2: Sum of squares, degree of freedom (df), mean squares, F-value for the model coefficients estimated from the factorial design for (a)PS, (b) PDI, (c) ZP and (d) EE% of niosomes and spanlastics

| Source               | Sum of squares | df | Mean square | F value | p-value | Prob>F* |
|----------------------|----------------|----|-------------|---------|---------|---------|
| PS                   |                | 1  | 1.753E+005  | 12.36   | 0.0079  |         |
| A-% of Drug          | 1.753E+005     | 1  | 1.753E+005  | 12.36   | 0.0079  |         |
| B-Types of vesicular structure | 7.970E+006 | 1  | 7.970E+006  | 561.61  | <0.0001 |         |
| C-speed of rotation  | 2.965E+006     | 1  | 2.965E+006  | 208.95  | <0.0001 |         |
| AB                   | 2.367E+005     | 1  | 2.367E+005  | 16.68   | 0.0035  |         |
| AC                   | 1203.05        | 1  | 1203.05     | 0.085   | 0.7738  |         |
| BC                   | 2.943E+006     | 1  | 2.943E+006  | 207.35  | <0.0001 |         |
| PDI                  |                | 1  | 3.393E-003  | 0.059   | 0.7738  |         |
| A-% of Drug          | 3.393E-003     | 1  | 3.393E-003  | 0.17    | 0.6351  |         |
| B-Types of vesicular structure | 5.293E-003 | 1  | 5.293E-003  | 0.17    | 0.9085  |         |
| C-speed of rotation  | 5.059          | 1  | 5.059       | 0.17    | 0.7738  |         |
| AB                   | 3.570E-003     | 1  | 3.570E-003  | 3.13    | 0.1150  |         |
| AC                   | 5.293E-003     | 1  | 5.293E-003  | 4.64    | 0.0635  |         |
| BC                   | 0.012          | 1  | 0.012       | 10.94   | 0.0107  |         |
| A-% of Drug          | 5.499.00       | 1  | 5.499.00    | 7.78    | 0.0236  |         |
| ZP                   |                | 1  | 6268.68     | 88.67   | <0.0001 |         |
| B-Types of vesicular structure | 404.61    | 1  | 404.61      | 5.72    | 0.0437  |         |
| C-speed of rotation  | 359.10         | 1  | 359.10      | 5.08    | 0.0542  |         |
| AB                   | 413.72         | 1  | 413.72      | 5.85    | 0.0419  |         |
| AC                   | 728.19         | 1  | 728.19      | 10.30   | 0.0124  |         |
| BC                   | 836.94         | 1  | 836.94      | 23.18   | 0.0013  |         |
| % EE                 |                | 1  | 2651.22     | 73.44   | <0.0001 |         |
| B-Types of vesicular structure | 53.36     | 1  | 53.36       | 1.48    | 0.2587  |         |
| C-speed of rotation  | 695.38         | 1  | 695.38      | 19.26   | 0.0023  |         |
| AB                   | 391.45         | 1  | 391.45      | 10.84   | 0.0110  |         |
| AC                   | 40.90          | 1  | 40.90       | 1.13    | 0.3182  |         |
| BC                   | 82.777%        | 1  | 82.777%     |         |         |         |

*Values of "Prob>F" less than 0.05 indicate model terms are significant.

Optimization

The Design-Expert 10.0.1.0® software suggested one spanlastics optimized formulation. The composition of the optimized formulation was based on selecting the lowest PS, lowest PDI, highest ZP and highest EE. The compositions of the prepared CBZ optimized formulation (F0) was represented in table 3, in addition to the observed and predicted responses. The predicted value for PS, PDI, ZP, and the EE% were 350.09 nm, 0.830, 16.124mV and 82.777%, respectively. The experimentally observed results were in reasonable agreement with the model prediction; thus, the validity of the model was established. The optimized spanlastics formulation (F0) was prepared and formulated using the same method and under the same conditions of the originally prepared experimental formulations (F1-F8), then further investigations were carried on the optimized spanlastics formulation (F0). The 3-D plot and the desirability were illustrated as a normal plot in fig. 4a and fig. 4b, respectively.
Table 3: The predicted, observed and % biased of the optimized formulation (Fo)

| Composition of the optimized formulation (Fo) | Dependent variables | Predicted | Observed | % Biased |
|----------------------------------------------|---------------------|-----------|----------|----------|
| $X_1 = 0.95$                                 | PS (nm)             | 350.09    | 360      | -2.75    |
| $X_2 = \text{Spanplastics}$                  | PDI                 | 0.8310    | 0.89     | -6.74    |
| $X_3 = -1.00$                                | ZP (mv)             | -16.124   | -14.7    | 9.68     |
|                                              | EE (%)              | 82.777    | 85.6     | -3.29    |

*% Biased = (Predicted-Observed)/Observed

**Transmission electron microscopy (TEM)**

The photomicrograph of the optimized spanplastics formulation was taken by the transmission electron microscopy (TEM) as shown in fig. 5. The photomicrograph reveals the spherical shape of the optimized spanplastics formulation. The structure of well-identified sealed spherical vesicles was noticed which are nearly homogenous in shape [38].

**Viscosity measurement**

The viscosity of the CBZ optimized spanplastics formulation was studied as an indication for the in vitro prolong retention after administration via the intranasal route. The viscosity was nearly uniform and constant through the whole shear rate values (nearly 65 cps). This nearly high viscosity due to the presence of two highly viscous surfactants in the composition of spanplastics formulation so that the prepared formulation could be used without the need of incorporating a gelling agent [13]. The almost constant viscosity indicated that the flow of the optimized formulation is Newtonian flow.

**In vitro drug release study**

In vitro release study was carried out using vertical type Franz diffusion cells (Hanson research, Los Angeles, California, USA) with a 1 cm²-diffusion area. The CBZ optimized spanplastics formulation and the CBZ suspension release were compared in order to study the effect of formulation on drug release, results are represented in fig. 6. The amount of CBZ (95.85%) was detected in the tested medium after 180 min. The results demonstrated the ability of the optimized spanplastics formulation to increase drug release in the range time from 10-60 min (p<0.05) when compared with the release of CBZ suspension. While after 90 min the release showed almost the same profile and no significant difference was noticed at the end of the release profile (p=0.05), while for the CBZ suspension the release profile showed the release of 61.11% after 180 min. This might be due to the vesicular structure of spanplastics and the incorporation of span 60 and polyvinyl alcohol which can improve the release of the CBZ from the vesicles [12, 32]. Both formulations were found to fit the Higuchi diffusion equation with "r" values of 0.976 and 0.992 for the optimized spanplastics formulation and CBZ suspension, respectively.

**Physical stability study**

The appearance of the CBZ optimized spanplastics formulation (Fo) showed no visual change during the six-month period of storage at 25°C. Table 4 represents the measured parameters of the EE%, the PS and the release after one h Q1, the results of the stored optimized formulation showed no significant difference (p>0.05) when compared with the fresh formulation. Fig. 7 showed that the stored and the fresh formulation were almost similar. The similarity factor (f2) was found to be 68 which indicates that the storage condition did not affect the drug [38].
**In vivo studies**

The results of the preliminary study revealed that approximately 80% of the animals survived in the first 60 min of the experiment. In the first 10 min, the animals did not display any convulsions and were assigned a kindling score 0; however, during the following 10 min (from 10-20 min), the animals started to exhibit some convulsions and were assigned kindling score 1. Meanwhile, from 20 to 40 min the severity of the convulsions increased and was given a kindling score 3. Only 20% of the animals experienced generalized clonic seizures (score 4) and died in the last 10 min of the experiment. Based on these observations, an elevated plus maze test was used to observe the animal’s latency to reach any of the closed arms at T\textsubscript{20 min}, T\textsubscript{30 min} and T\textsubscript{40 min} after pentylenetetrazol (PTZ) administration.

**Behavioral assessment by elevated plus maze test**

Animals groups received either saline, intraperitoneal PTZ, oral CBZ or intranasal CBZ. Latency time was recorded after 20 min, 30 min, and 40 min after PTZ administration. The rats in the normal control group (group I) reached the closed arm at T\textsubscript{20 min}, T\textsubscript{30 min} and T\textsubscript{40 min} within a latency of 8.125, 7.916, and 8.25 seconds respectively. Rats injected with PTZ (group II) displayed a delay in latency time by 319% at T\textsubscript{20 min} this delay was due to the small tremors (score 1) that started 10 min after PTZ administration. However, after 30 min (score 2) and 40 min (score 3), PTZ injected rats demonstrated some clonic convulsions which resulted in a further delay in latency time by 9.35 and 12 folds, respectively compared to the normal control group. The administration of either oral CBZ (group III) or intranasal optimized formulation F\textsubscript{0} (group IV) improved latency time compared to untreated rats. However, the effect of the intranasal dosage form was more pronounced resulting in a 31% decrease in latency time at T\textsubscript{30 min} and 43% at T\textsubscript{40 min} compared to the oral dosage form. Values are expressed as mean±S. E (8 animals). Statistical analysis was performed using the one-way ANOVA followed by Tukey’s post hoc test (P<0.05) where (a) significant difference from normal control group, (b) PTZ control and (c) CBZ oral. T\textsubscript{20 min} latency time after 20 min of PTZ administration; T\textsubscript{30 min} latency time after 30 min of PTZ administration and T\textsubscript{40 min} latency time after 40 min of PTZ administration as shown in fig. 8. This is revealed to the rapid CBZ release from the optimized spanlastic formulation and the rapid absorption of the CBZ from the nasal route to reach rapidly the brain via the olfactory region.
Determination of serum endothelial nitric oxide synthase (e-NOS), tissue reduced glutathione (GSH) and ELISA estimation of tissue TNFα

PTZ injected rats exhibited an increase in brain inflammation as manifested by the significant increase in TNF-α as compared to the normal control group as represented in table 5. This increase has also been reported in previous studies [39, 40]. In the present study, the administration of CBZ by either route significantly decreased TNF-α content in the brain tissue as compared to the PTZ control group. Oral administration of CBZ is known to present anti-inflammatory effects, these effects might be accredited to the ability of CBZ to block Na channels [41]. Nevertheless, rats which received intranasal CBZ displayed a better anti-inflammatory effect as depicted by the decrease in brain content of TNF-α as compared to oral CBZ. In addition, rats on intranasal dosage form displayed enhanced antioxidant activity as evident by the increase in the brain content of GSH. Similarly, serum eNOS was significantly increased in the PTZ control group as compared to normal control rats. This increase can be attributed to the acute inflammatory state created by the epileptic seizures that were detected in the current as well as previous studies where eNOS expression has been shown to be increased in endothelial and Schwann cells of sciatic nerves during experimental autoimmune neuritis [42]. Epileptic seizures by itself can induce an increase in NO production, and this increase may be involved in seizure-induced neurodegeneration [43]. In the present study, the administration of CBZ attenuated the PTZ-induced increase in eNOS. Although the effect on nasal CBS was more pronounced than oral CBZ, yet the difference was not statistically significant.

Table 5: The effect of oral CBZ and intranasal CBZ on serum level of endothelial nitric oxide synthase (eNOS) and brain content of tumor necrosis factor alpha (TNF-α) and glutathione (GSH) in PTZ-induced epilepsy in rats

| Parameters groups | Serum Enos (ng/ml) | Brain TNF-α (Pgf/mg) | Brain GSH (mg/g) |
|-------------------|--------------------|----------------------|-----------------|
| Normal Control    | 2.000±0.134        | 86.070±2.630         | 275.315±6.701   |
| PTZ Control       | 5.95±0.380         | 127.555±0.704        | 161.510±5.237   |
| CBZ Oral          | 3.650±0.201ab      | 107.600±2.111ab      | 211.795±5.118bc|
| CBZ Nasal         | 2.950±0.190b       | 96.270±3.568bc       | 257.120±5.720bc|

Values are expressed as mean±SE (8 animals). Statistical analysis was performed using one-way ANOVA followed by Tukey’s post hoc test (P<0.05), where (a) significant difference from the normal control group, (b) PTZ control and (c) CBZ oral.

Fig. 8: Elevated plus maze test for normal control, PTZ positive control, CBZ oral group and CBZ (F0) nasal spanlastic group, a: significantly different from normal the control group at P≤0.05. b: significantly different from the PTZ control group at P≤0.05. c: significantly different from CBZ oral group at P≤0.05. Data are presented as the mean±SE Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

Fig. 9: Histopathological images of brain tissue sections: (a) examination of normal brain negative group showed intact neurons in the different layers (→). (b) Positive control group showed single scattered degeneration (●) and glial cells infiltration (●●). (c) Oral CBZ showed mild glial cells infiltration (●●). (d) Optimized intranasal (F0) showed almost intact neurons (●●)
Histological examination
The histopathological image for the brains of the rats in the normal control group I showed intact neurons in the different layers and zones of the hippocampus (hilus and fascia dentata) ( fig. 9a). PTZ administration group II caused a single scattered degeneration (●) in some neurons in the layer of CA zone with pyramidal cells and congested blood vessels with moderate-high glial cells infiltration (●, ●) (fig. 9b). Rats treated with oral CBZ group III displayed relatively intact neurons, which appeared with milder glial cells infiltration (●, ●) in CA zones and milder edema in dentate gyrus of the hippocampus compared to PTZ injected rats (fig. 9c). In the meantime, brains from the rats treated with intranasal optimized CBZ F0 group IV presented intact neurons (●, ●) in CA region, however they showed edema with higher glial cells infiltration in the gyrus region as compared to oral CBZ (fig. 9d). Interestingly in the intranasal CBZ group, there was higher glial cells infiltration. Glial cells play an important role in maintaining and supporting neural cells. Much like fibrocytes in other tissues, glial cells undertake an important part of wound healing following an insult to the brain. Thus, more glial cell infiltration and gliosis suggests that more extensive damage has occurred in this group compared to the oral CBZ. This might indicate more drug uptake by the brain from the intranasal route [38].

CONCLUSION
In the present work, optimization of carbamazepine (CBZ) nano vesicular structures was achieved using 24 factorial design. The novel CBZ spantastic (Fx) was successfully formulated showing high viscosity that allowed its use in the nasal route without the need of a gelling agent, also improvement of the in vitro release was noticed in addition to its stability for six months of storage at 25 °C with similarity decrease in latency time in behavior assessment test using elevated plus maze test. F0 also showed a decrease in serum eNOS and TNF-α and an increase in GSH when compared with the oral CBZ suspension. The histopathological study revealed the more CBZ uptake by the brain. CBZ novel spantastic vesicles were successfully formulated and examined to be administered via nasal route for brain targeting.

AUTHORS CONTRIBUTIONS
All the author have contributed equally

CONFLICT OF INTERESTS
No potential conflicts of interest are reported by the authors.

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