INTRODUCTION

Psychotic disorders included severe mental diseases with disordered thinking, loss of connections with reality due to delusions and/or hallucinations. Schizophrenia is one of the well-established and common psychotic disorders [1]. Majority of the schizophrenia cases require psychosocial treatment along with pharmacotherapy [2]. The anti-psychotic drugs generally have poor bioavailability apart from their adverse drug reactions [3]. The lack of drug availability at the site of action is mainly attributed to the blood-brain barrier (BBB), which restricts the penetration of drugs into the central nervous system (CNS) [4]. Hence, many efforts are being made to develop novel drug delivery systems such as solid-lipid nanoparticles, nanomulsions, liposomes and polymeric nanoparticles to transport the drug to the brain [4, 5].

Liposomes have desirable biological properties, including biocompatibility and biodegradability [6]. Liposomes, due to their subcellular size allow relatively higher intracellular uptake than other particulate systems. Apart from other routes of drug administration, nano-sized drug-loaded systems can be delivered through the nasal route. Upon nasal instillation of the formulation, the drug is directly transported to the brain by circumventing BBB and provide a rapid onset of action due to the presence of high vascularisation [7]. The drugs can reach the central nervous system through extracellular or intracellular transport along olfactory nerves and also by the trigeminal pathway [8]. Liposomes through intranasal administration, provide benefits in terms of improved penetration into the brain [9]. Liposomes are increasingly being explored for the intranasal delivery of drugs and vaccines for enhanced systemic availability and targeting. Some examples of the drugs tried.

Olanzapine (OZ) is a thieno benzodiazepine class second-generation or atypical antipsychotic that selectively binds to central dopamine D2 and serotonin (5-HT2c) receptors used for the treatment of schizophrenia and bipolar disorder. It has poor bioavailability due to hepatic first-pass metabolism and low permeability into the brain due to efflux by P-glycoproteins. It was approved by the FDA for the management of schizophrenia and the treatment of moderate to severe mania associated with bipolar disorders [10]. Following oral administration, the drug suffers from low brain permeability due to efflux by P-glycoproteins in the blood-brain barrier (BBB) as well as extensive hepatic first-pass metabolism where 40% of the dose is approximately metabolized before reaching the systemic circulation [11]. Increasing the dosage and/or dosing frequencies is commonly associated with extrapyramidal effects, orthostatic hypotension, weight gain, dry mouth, tremors, akathisia and somnolence.

Therefore, in the present study, liposome-loaded OZ is prepared and optimized using Box Behnken Design (BBD) and target the brain via the intranasal route, to improve the bioavailability and minimize its peripheral adverse effects owing to its direct delivery to the brain.

MATERIALS AND METHODS

Materials

Olanzapine (OZ) was a gift sample from MSN Laboratories Pvt Ltd, Hyderabad. phosphatidylcholine (PC) was obtained as a gift sample from LIPOID, Germany. Cholesterol was procured from Sigma Aldrich, India. All other reagents and chemicals used were of laboratory/analytical grade.

Preparation of OZ loaded liposomes

OZ loaded conventional liposomes consisting of phosphatidylcholine (PC) and cholesterol were prepared by lipid film hydration method as per the literature with slight modification [14]. Accurately weighed amount of drug, PC and cholesterol were added to a 250 ml round
bottom flask (RBF) and then dissolved in 10 ml of chloroform. The RBF was attached to the rotavapour (Superfit, India) and maintained at 40 °C. The rotation speed of RBF was adjusted to 40 rpm and a vacuum was applied for 20 min to aid the evaporation of chloroform and to form a thin layer of lipids on the inner wall of the RBF. The vacuum was released and RBF was kept in a vacuum desiccator for 24 h to completely remove the residual traces of chloroform. Phosphate buffer pH 6.4 (10 ml) was added as the hydration media and lipid film was removed by hand-shaken method in a water bath at 40 °C. A milky white uniform multilamellar liposomal suspension was obtained, which were further reduced in size by probe sonication (in an ice bath) at amplitude 60%, time 5 min and 2 pulse/s. Uniform suspension of liposomes obtained was transferred to sterilized vials and stored at 4 °C until use [15].

Optimization and validation of the experimental design

The liposome formulations contained different variables such as lipid ratio and lipid to drug ratio. The liposomal formulation was optimized by using Design-Expert® Software Version 11-Stat-Ease, Inc. Box-Behnken can be used to derive three factors (X1, X2 and X3) and three levels (−1, 0, +1) design can be developed by the inclusion of a central point. The present study consists of 3 variables/factors (X1: Phospholipid and X2: Cholesterol and X3: Sonication time) and the response variables were R1: Entrapment Efficiency, R2: Size, R3: Zeta Potential. The experimental trials being performed at all 15 possible combinations, as the batch containing central point was prepared three times to compute the results (table 1).

Table 1: Selected level of independent and dependent variables used for BBD for the preparation of liposomes

| Factors/independent variables | Name of the variables | Minimum | Maximum |
|-------------------------------|----------------------|---------|---------|
| X1                            | Lipid (mg)           | 300.00  | 500.00  |
| X2                            | Cholesterol (mg)     | 20.00   | 80.00   |
| X3                            | Sonication time (min)| 5.00    | 15.00   |
| Responses/Dependent variables |                      |         |         |
| R1: Entrapment Efficiency (%) | Maximum              |         |         |
| R2: Size                      | Minimum              |         |         |
| R3: Zeta potential (mV)       | In range             |         |         |

Entrapment efficiency

The entrapment efficiency of OZ loaded liposomes was determined by separating the free drug from the liposomes. The unentrapped drug was separated from the entrapped drug by ultracentrifugation of the risperidone loaded liposomal formulation at 60,000 rpm for one hour. Pellet of the entrapped drug was collected and supernatant (free drug) was separated by decantation. Further, the pellets were dispersed in phosphate buffer pH 6.4 (3 ml) and vortexed for 10 min followed by bath sonication for 5 min for complete dispersion of liposomes. A 50 μl of dispersed liposomes were taken and then 50 μl of 10% Triton-X was added to break the liposomes and release the entrapped drug. The volume was made up to 1 ml with phosphate buffer pH 6.4. The drug content was analysed in both pellets and the supernatant by the UV spectrometry method at 273 nm [16]. The entrapment efficiency was calculated by the formula [17]

Entrapment efficiency (%) = \( \frac{C_f - C_t}{C_t} \times 100 \)

Where \( C_t \) and \( C_f \) are the concentrations of the total and free drug, respectively.

Vesicle size and zeta potential

The mean vesicle size of OZ loaded liposomes was determined by the dynamic light scattering method using Malvern NanoZS (Malvern Instruments Ltd., UK). The system was equipped with a 4 mW Helium/Neon laser at 633 nm wavelength and measured the liposome sample with non-invasive backscatter technology at a detection angle of 173°. Zeta potential measurement was carried out using the same instrument by the Laser-Doppler electrophoresis technique. Liposomal samples were diluted with Milli-Q water and all measurements were carried out in at 25 ± 1 °C.

Morphological studies by transmission electron microscopy (TEM)

To study the surface characteristics of the liposomes and individual vesicle size TEM analysis was carried out by negative staining method. A sample containing 50 μl of liposomes dropped in the paraffin and copper grid was kept above the samples and waited for 1 min, then the copper grid was placed in the 2% phosphotungstic acid and waited for 30 s. Afterward, copper grid was taken out and dried with the help of tissue paper and then placed under the TEM microscope. The picture was taken at various magnifications (100 k) and accelerating voltage 80 kV and data was analysed.

In vitro release studies

In vitro release studies of OZ loaded liposomal formulation was carried by dialysis sac method using pH 7.4 phosphate buffer (PBS) as a medium. The dialysis sac membrane was previously soaked for 12 h and fixed at one side of the diffusion cell, having openings on both sides. A 25 ml of diffusion medium was taken in a 50 ml beaker and kept for stirring at 300 rpm on a magnetic stirrer at room temperature. OZ loaded liposomal suspension (1 ml) was placed on the dialysis sac and the diffusion cell was immersed in a diffusion medium [15]. At intervals of 1, 2, 4, 6, 8, 12, 16, 20 and 24 h, 1 ml of the dialysed sample was withdrawn and an equal volume of PBS pH 7.4 was replaced to maintain sink condition. The samples were analysed using UV spectrometry at 273 nm. The experiment was performed and the percentage of drugs released was calculated. For evaluating the kinetics and mechanism of drug release from the liposomes, Korsmeyer-Peppas model, Higuchi model, first-order and zero-order mathematical models were used, and best-fitted model was selected based on high correlation coefficient (R) value for the release data.

RESULTS AND DISCUSSION

Formulation design of OZ liposomes

OZ liposomes were prepared by the lipid film hydration method. To optimize the formulation parameters and to obtain a formulation with maximum entrapment with better in vitro release, the Box Behnken Design (BBD) was used. A total of 15 experimental runs for the prepared formulations with 3 centre points has been formulated. Responses obtained from these runs were shown in table 2. Batches were prepared based on the experimental plan from Design-Expert® Software. The formulations were evaluated for Entrapment efficiency, zeta potential and zeta potential [18]. The results obtained from all the formulations were fed into the Design-Expert® Software to generate a study design and the response surface plots. The quadratic model obtained for the formulations was suitably fitted. The values of R2, SD, and % coefficient of variation of each of the 3 responses are shown in table 3. The effect of independent variables on phospholipid, Cholesterol, and sonication time is presented on a three-dimensional graph (fig. 1). Moreover, a quantitative comparison that is resulted from experimental values of the responses with that of the predicted values can also be analyzed from fig. 2.

Response 1 (Y1): effect of independent variables on % entrapment efficiency

The entrapment efficiency of the formulation was a range between 72.9 and 85.1 % [19]. The Model F-value of 215.35 implies the model
is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values<0.05 indicate model terms are significant. In this case, X1, X2, X3, X1X2, X1X3, X2X3, X2², X3² are significant model terms. The regression equation for entrapment efficiency is

\[
\% \text{EE} = 80.93 + 2.91X_1 + 3.01X_2 + 0.975X_3 - 1.45X_1X_2 + 0.975X_1X_3 + 0.075X_2X_3 - 1.84X_1^2 + 1.36X_2^2 - 2.12X_3^2
\]

The positive effect on entrapment efficiency was found in correlation with lipid and cholesterol concentration. A significant effect was also found with interaction effects of lipid and cholesterol [20-22].

**Response 2 (Y2): effect of independent variables on size**

The average particle size of all the 15 experimental runs was found to be 299.20 nm; hence these value lies between the minimum and maximum value of the size 258.33 to 325.32 nm, respectively [23]. The Model F-value of 63.47 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values <0.0500 indicate model terms are significant. In this case, X1, X2, X3, X1X2, X1X3, X2X3, X2², X3² are significant model terms. The Lack of Fit F-value of 6.31 implies the Lack of Fit is not significant relative to the pure error. There is a 13.99% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R² of 0.8726 is in reasonable agreement with the Adjusted R² of 0.9757. All the factors have an effect size of the liposomes as the increase in sonication time the size reduction has been found in several formulations. The regression equation for size is

\[
\text{Size} = 294.87 - 6.83X_1 - 20.15X_2 - 4.66X_3 + 9.42X_1X_2 - 5.78X_1X_3 - 5.22X_2X_3 + 0.5987X_1^2 - 11.20X_2^2 + 19.92X_3^2
\]

**Response 3 (Y3): effect of independent variables on zeta potential**

The zeta potential of the 15 formulations ranges from -27.53 to -11.46 mV [24-26]. The Model F-value of 103.24 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values<0.05 indicate model terms are significant. In this case, X2, X3, X3² are significant model terms. Values>0.1 indicate the model terms are not significant. The Lack of Fit F-value of 10.27 implies there is a 9.00% chance that a Lack of Fit F-value this large could occur due to noise.

\[
\text{Zeta potential} = -17.43 + 0.5612X_1 + 1.11X_2 - 6.99X_3 - 0.58X_1 X_2 - 0.3125X_1 X_3 - 0.28X_2 X_3 + 0.1163X_1^2 + 0.3587X_2^2 - 2.70X_3^2
\]

**Optimization and validation**

The response surface plots elucidate the effect of variables on the responses and interactions between the variables. Minimum and maximum levels were provided for each response parameter, the goals are combined into an overall desirability function (0.971). The optimized formulation was fixed by the point prediction method. The list of solutions was sorted with the highest desirability. The contour plot for the optimized formulation and its desirability was shown in fig. 3. Analysis of variance (ANOVA) was used to identify the significant effect of factors on response regression coefficients [15]. To evaluate the chosen experimental design, the optimized formulations were prepared and subjected to evaluation parameters. The resulting experimental values were compared with predicted values. The constraints criteria were maximum entrapment of drug in formulation, optimum zeta potential, the minimum size of liposomes.

![Fig. 1: 3D-response surface plot showing the effect of independent variables on (a) Entrapment efficiency, (b) Size, and (c) Zeta potential](image-url)
Table 2: Observed responses in BBD design for optimization of liposomes

| Run | Factor 1  | Factor 2  | Factor 3  | Response 1   | Response 2   | Response 3   |
|-----|-----------|-----------|-----------|--------------|--------------|--------------|
|     | A: lipid (mg) | B: Cholesterol (mg) | C: Sonication time (min) | Entrapment efficiency (%) | Size (nm) | Zeta potential (mV) |
| 1   | 500       | 80        | 10        | 85.1±0.78    | 268.25±11.62 | -15.53±2.53  |
| 2   | 500       | 50        | 5         | 77.8±0.50    | 314.63±7.46  | -12.42±0.50  |
| 3   | 300       | 50        | 5         | 74.2±0.70    | 321.45±14.30 | -13.24±1.12  |
| 4   | 300       | 80        | 10        | 81.9±1.07    | 258.33±11.36 | -16.42±1.89  |
| 5   | 400       | 20        | 15        | 78.1±1.54    | 293.65±18.16 | -17.64±1.09  |
| 6   | 400       | 50        | 10        | 81.4±1.04    | 295.37±17.48 | -27.42±0.62  |
| 7   | 500       | 50        | 15        | 81.7±1.68    | 316.72±18.61 | -19.54±2.06  |
| 8   | 300       | 20        | 10        | 72.9±1.23    | 316.72±18.61 | -19.54±2.06  |
| 9   | 400       | 80        | 15        | 84.2±1.25    | 272.39±10.54 | -25.59±2.13  |
| 10  | 400       | 20        | 5         | 76.3±0.40    | 324.35±14.25 | -14.52±0.44  |
| 11  | 500       | 20        | 10        | 81.9±1.06    | 280.97±52.31 | -16.33±1.10  |
| 12  | 300       | 50        | 15        | 74.2±1.82    | 325.32±10.82 | -26.99±1.30  |
| 13  | 400       | 50        | 10        | 80.8±2.86    | 294.22±16.21 | -17.13±0.17  |
| 14  | 400       | 50        | 10        | 80.6±1.36    | 296.74±19.23 | -17.52±1.12  |
| 15  | 400       | 80        | 5         | 82.1±1.25    | 293.76±6.49  | -11.46±1.95  |

±SD (n=3) of three determinations

Table 3: Summary results of regression analysis, SD, and %CV with responses Y1, Y2, and Y3 for the quadratic model equation

| Quadratic model            | R²       | Adjusted R² | Predicted R² | SD    | % CV |
|----------------------------|----------|-------------|--------------|-------|------|
| Entrapment efficiency (%)  | 0.9974   | 0.9927      | 0.9829       | 0.31  | 0.40 |
| Size (nm)                  | 0.9913   | 0.9757      | 0.8725       | 3.36  | 1.12 |
| Zeta potential (mV)        | 0.9946   | 0.9850      | 0.9188       | 0.68  | 3.66 |

SD–Standard deviation; CV–Coefficient of variation

Fig. 2: Linear correlation plots (a, b, c) between actual and predicted values and the corresponding residual plots (d, e, f) for entrapment efficiency, size and zeta potential, respectively

Transmission electron microscopy (TEM)
The optimized liposomes were examined by TEM and appeared predominantly spherical in shape and had uniform size distribution. The surface of the liposome was found to be bilayer, smooth and regular. The aggregation of vesicles was not observed. The shape and surface morphology analysis indicated the stability of the prepared liposomes [27].

In vitro release studies
The optimized liposomes showed better release; initially, the drug releases rapidly from the lipid surface, following the slow release of the drug owing to degradation of the lipid core. The maximum release was found to be 98.43±1.2 % up to 24 h. The in vitro drug release plot was shown in fig. 4. The R² zero-order kinetics and Korsmeyer-Peppas model were found to be 0.9919 and 0.9664, respectively [28]. The
zero-order shows the best fit model with the highest $R^2$ value exhibiting better correlation and the 'n' value was also found to be 0.85; this lies between 0.43 and 0.85, an indication of both diffusion-controlled and swelling-controlled drugs release that is anomalous transport [29, 30].

CONCLUSION
OZ in the form of liposomes could be successfully used for delivering to the brain through nasal administration. Design-Expert® Software assisted in designing the experimental protocol in optimizing the formulations. The liposomes were discrete spherical vesicles with the smooth bilayered surface with optimum size and surface charges. The drug releases further support and proves to have a better-controlled release for 24 h. Hence, the formulation supports in all aspects and meet the in vitro characterization in the process of targeting the brain through nasal delivery.

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All authors have contributed equally.

CONFLICTS OF INTERESTS
The authors declared that they have no conflicts of interest.

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