DESIGN AND OPTIMIZATION OF CLOZAPINE PRONIOSOMES USING DESIGN OF EXPERIMENTS

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Abstract:
Clozapine is atypical antipsychotic agent used in treatment of patients with schizophrenia, it is available in oral route, Clozapine is class II drug having a poor bioavability (27%), hence the aim of present study to develop proniosomes of Clozapine for brain targeted delivery through olfactory pathway. Proniosomes drug delivery system is a promising drug delivery system derived from Niosomes, Proniosomes proves to be potential carriers for efficient drug delivery for Lipophilic and amphphillc drug using oral route, nasal route, transdermal route and other route. 3-factor and 3 levels box Behnken design used to explain multiple regression analysis and counter plot response. The independent variable selected were Cholesterol, Span 60 and Sonication time; dependent variables % entrapment efficiency, particle size analysis and PDI value. Based on Box-Behnken design 17 runs trials studied and optimized. The formulation evaluate for Scanning electron Microscopy (SEM), FT-IR Study, DSC Study, Particle size analysis. From DOE the derived polynomial equation and counter plots give support to predicating the values of selected independent variable for the preparation of the optimum formulation with required properties

Keywords: Clozapine, Proniosomes, Box –Behnken, Brain targeted delivery, Cholesterol

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Introduction

The Central nervous system (CNS) offers a unique challenge for drug delivery system. The Blood-brain Barrier significantly prevents treatment of CNS Disorder by preventing drug entry into the brain, 98 % of drug do not cross the blood brain barrier due to their molecular mass or physicochemical properties.[1] The different type of disease of Central nervous system (CNS) for instance Schizophrenia, Parkinson's disease and Alzheimers disease required deliver the drug to brain for treatment. Hence this type of transports remains difficult particularly for hydrophilic drug and large molecular mass due to the impervious nature of the endothelial membrane separating the systemic circulation and central interstitial fluid.[2] For drug delivery to the CNS and Systemic circulation, the nasal route offer several advantage over conventional oral or parenteral route, Drugs administered in to the nasal cavity can be rapidly absorbed into systemic circulation without enzymatic degradation and hepatic first pass metabolism associated with oral administration, resulting in enhanced bioavability and on set of action[3]

Nose to brain delivery is possible by olfactory region situated at the roof of nasal cavity and its neuroepithelium is the only exposed portion of the CNS to the external environment.[4] drug delivery through intranasal route transported through olfactory sensory neurons to yield significant concentration in the CSF and olfactory bulb. The olfactory region of nasal mucosa that provide direct connection between nose and brain is used in condition like Alzheimer,s disease, depression, Migrranine, schizophrenia etc [5]

Novel drug delivery system have emerge various route of administration, to attain targeted and controlled drug delivery. Drug encapsulation in the vesicles is one such system which helps to prolong drug duration in systemic circulation and decrease the toxicity by selective uptake. Based on this technique a number of vesicular drug delivery system such as liposomes, niosomes and provesicular system like proliposomes and proniosomes have been developed. Liposomes are colloidal, vesicular structure that are prepared by several concentric phospholipidic bilayer and variety of substances and drug. liposomes have certain limitation like Physicochemical stability, oxidation, etc. To overcome this problem Proniosome approach provide major advantage over the liposomes. It provide more stability during sterilization and storage. Proniosomes are dry formulation of water-soluble carrier particles that are coated with surfactant and can be measured out as needed and dehydrated to form niosomal dispersion immediately before use on brief agitation in hot aqueous media within minutes.[6]

Clozapine is an atypical antipsychotic agent with a dibenzodiazepne structure and indicated for the management of severely ill schizophrenic patients who are refractory or intolerant to standard trratments.[7] Clozapine is classified in the Biopharmaceutics classification system (BCS) as a Class II drug with low aqueous solubility and low permeability, it having low bioavailability of 27 % due to first fast metabolism.[8] Clozapine is a 'gold standard' against treatment resistant schizophrenia. Schizophrenia is a server mental disorder with a lifetime risk of about Characterized by hallucinations, delusions and cognitive deficits with heritability estimated at upto 80%.treatment strategies of schizophrenia include the use of typical and atypical antipsychotics.[9]

The aim of present study to improve therapeutic efficacy of antipsychotic drug through proniosomal encapsulation. Clozapine is synthetic drug commonly used in treatment of schizophrenia it having poor bioavailability due to first pass metabolism, it is difficult to cross Blood brain barrier due to having poor aqueous solubility, develop Proniosomes for Clozapine with nasal route to omit Blood brain barrier. Proniosomes prepared by slurry method using Maltodextrin ,Span 60 and cholesterol as Carrier, Surfactant and membrane stabilizer respectively to enhance Aqueous solubility of Clozapine.

Material and Method

Material

Clozapine was procured form CTX Lifescience, Maltodextrin and Cholesterol obtained from Loba chemie, Span 60, Methanol obtained from SD fie chem. Mumbai India.

Preparation of Proniosomes

Clozapine Proniosomes were prepared by slurry method using maltodextrin as a carrier. Weigh required amount of lipid mixture contains Cholesterol and span 60 were dissolved in 20 ml methanol: Chloroform (1:1), further add Clozapine above mixture. The solution was transferred in round bottom flask and maltodextrin added to form slurry. The flask was rotated at 60-70 rpm by using roatatory evaporator to evaporate organic solvent at a temperature 45 °C± 2°C and reduce pressure to 600 mm Hg. A resultant powder further dried in a vacuum oven overnight at room temperature to obtain free flowing powder. Stored Proniosomal powder at 4°C in tightly closed container.

Preparation of Niosmes from Proniosomes:
To prepared Niosomes from Proniosomal powder Hydrating with 25 ml 7.4 phosphate buffer. The suspension was sonicated for 45 min in probe sonication to achieve optimum particle size and drug entrapment efficiency.

Box –Behnken Experiments Design
Box behnken design with 3 factors and 3 levels was built up to estimate to significant effect of different variables on the response namely % drug Entrapment efficiency, Vesicles size measurement and Polydispersity index and then predicts the optimized Proniosomes formulation. Box Behnken design is appropriate to explore quadratic response surface and assemble second order polynomial equations. The design includes sets of points in the middle of each side and 5 centre point of the multidimensional cube. The polynomial equation was derived using Design Expert software. The independent and dependent variable were tabulated in table.

$Y_i = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$

Where Y_i is the dependent variable, b0 is the intercepts b_1 to b_3 are regression coefficient and x_1, x_2 and x_3 are the independent variables.

Table.No.1 Independent Variables

Independent Variable	Levels		
	Low	**Medium**	**High**
X1 =Cholesterol	28.70 mg	36.95 mg	45.20 mg
X2 = Span 60	25.00 mg	35.00 mg	45.00 mg
X3 = Sonication Time	25 min	35 min	45 min

Table.No.2 Dependent Variable

Dependent variable	
Y1	Vesicles size (nm)
Y2	% Drug entrapment
Y2	Polydispersity Index

Table No.3 Different levels in Box behnken design

Run	X1	X2	X3	Y1	Y2	Y3
1.	1	1	0	76.52	590.4	0.412
2.	0	1	1	72.23	624.8	0.362
3.	0	0	0	78.25	586.4	0.451
4.	1	-1	0	66.25	590.4	0.628
5.	0	-1	1	76.25	658.8	0.358
6.	0	0	0	85.46	553.5	0.452
7.	1	0	-1	77.45	753.8	0.362
8.	-1	1	0	78.52	563.4	0.625
9.	0	1	-1	82.12	886.4	0.725
10.	-1	-1	0	71.36	536.4	0.785
11.	0	-1	-1	62.54	732.5	0.624
12.	0	0	0	85.24	538.4	0.524
13.	-1	0	1	72.52	624.5	0.625
14.	0	0	0	80.25	558.4	0.485
15.	1	0	1	82.25	423.8	0.351
16.	0	0	0	82.74	546.5	0.625
17.	-1	0	-1	67.25	634.8	0.783

Characterization of Prepared Proniosomes
DSC Thermogram of Clozapine
Differential scanning colorimetry (DSC) of pure drug Clozapine was analysis using DSC instrument. Clozapine was sealed in standard DSC aluminium pans, crimped it and then scanned over a temperature from 50°C to 300 °c at heating rate 10 °C/min.

FTIR Spectroscopy
FTIR was performed for estimating the possible physical interaction between Clozapine and other excipients, the compatibility study

Vesicle size measurement
The Vesicle size of niosomes were determined by using Particle size analyser by Malvern

(MALVERN INSTRUMENTS, STARTECH LABS PVT. LTD.) The apparatus consisted of He-Ne lease beam 632.8 nm focussed with maximum power of 4 mW using Fourier lens and small volume sample holding cell. The sample was carried out before determining the particle size.

Transmission Electron Microscope[12]

The morphological appearance of niosomes was observed by transmission electron microscopy (TEM). In order to prepare the samples for the microscopy, niosome dispersion was dropped onto carbon-coated 200-mesh copper grids and held horizontally to allow the penetration. The excess sample was removed by filter paper, and one drop of 2% uranyl acetate was added to the grid for staining. The negatively stained samples were then imaged on a FEG - transmission electron microscope (HR-TEM), Thermo Fisher Scientific, Talos F200i S/TEM

Determination of drug entrapment efficiency (% DEE)

The entrapment efficacy of Clozapine in the primed Proniosomes was determined by assay of the Clozapine content in the Nano-vesicles. Proniosomes was hydrated with 25 ml Phosphate buffer pH 7.4 manually shaking for 5 minutes, to form proniosomal dispersion. For separation of unentrapped drug the proniosomal dispersion was centrifuged at 15000 rpm for 30 minutes at 20°C and analysed in UV spectroscopy at 292 nm. The entrapment efficacy was calculated using the formula

$$\% \text{ entrapment efficiency} = \frac{\text{Total Drug} - \text{diffused drug}}{\text{Total drug}} \times 100$$

Optimum formula and design Checkpoint analysis:

From the developed polynomial equation, the software optimization process predicts the value of independent variable (X1, X2 and X3) that achieve optimum formulation with the desire response. The optimization process predicted two optimized promiosomal formulae, formula P6 and P14 achieve maximum drug entrapment efficiency and lowest particle size with good PDI Value.

To conform the role of polynomial equation in expecting the responses, checkpoint analysis was performed. The factors value of the two optimized formulae were compared with theoretical values that derived from polynomial equation.

Results and discussion

FTIR Spectroscopy

FTIR Spectra of the pure drug and Clozapine, cholesterol, Maltodextrin and span-60 mixture are illustrated Fig1 and Fig.2. The IR spectra showed the characteristic peak of Clozapine at 2800.73 (C-H Stretching), 3294.53 (NH stretching) and 1739.95 (C=0 Stretching).This peaks appeared in Mixture with no significance change, The Results reveled the compatibility between Clozapine and other excipients.

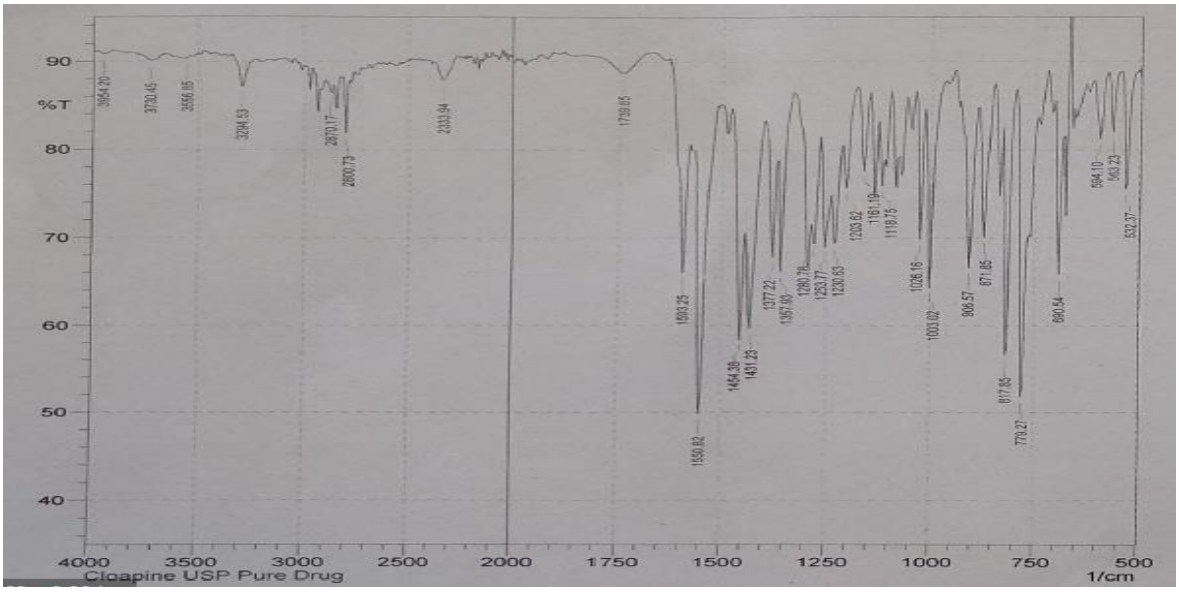


Fig.1: FTIR Spectra f Pure drug (Clozapine)

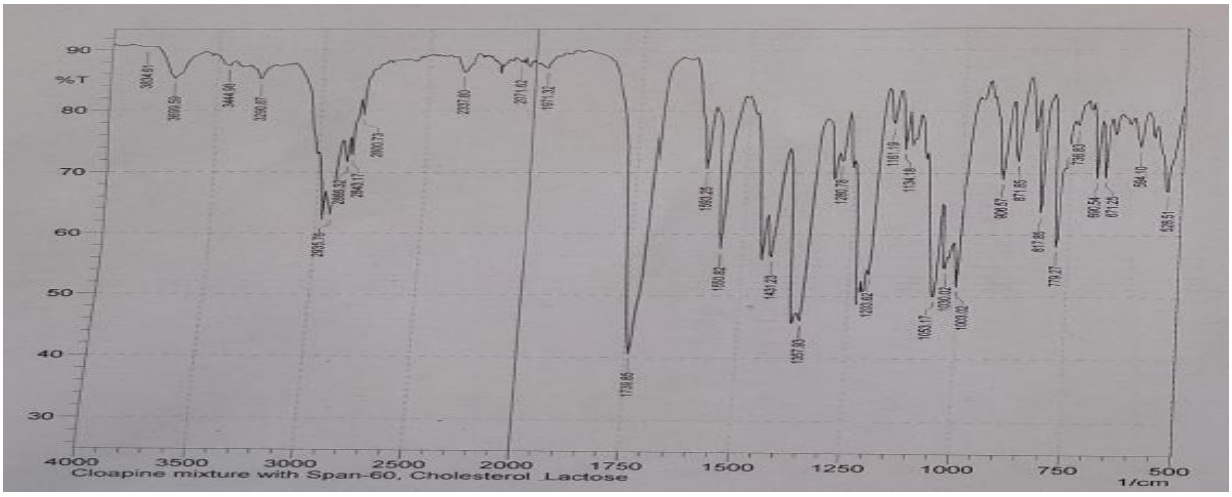


Fig 2: FTIR Spectra of Drug and excipients mixture

DSC Thermogram of Clozapine

The DSC thermogram of Clozapine showed a sharp endothermic peak at 197.58 °C which correspond to the melting point of Clozapine which indicate crystalline and purity of drug.

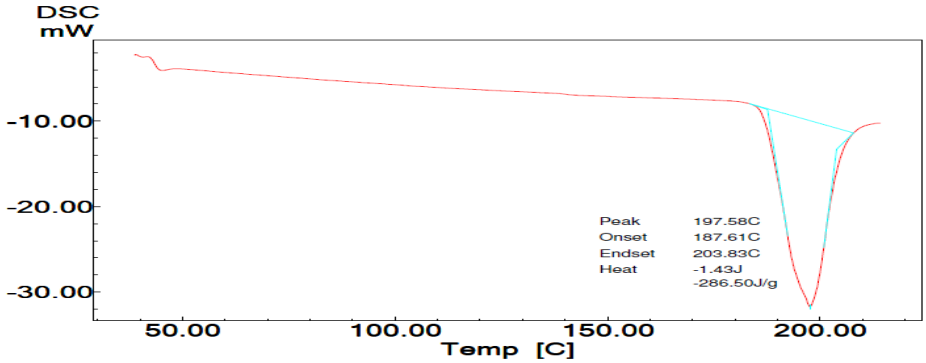


Fig.3: DSC Thermogram of pure drug

Box Behnken Design

Box –Behnken experimental design with 3 factors (Cholesterol, Span 60 and sonication time) at 3 levels were assemble to study the effect of these factors on the following response (Vesicle size, Drug entrapment efficiency and Polydispersity Index) of the niosomal suspension got upon hydration of prepared Proniosomes The quadratic model showed the highest adjusted R^2 and Predicted R^2 was selected to analyse these response individually using linear regression. The experimental design proved numbers of runs (17 runs) of experiments. All dependent variables were exposed to multiple regressions to get a second order polynomial equation (Full model). Averaging of the changes of the factors from its low levels to its high levels was defined as the main effect of X_1, X_2 and X_3. The interaction terms of (X_1X_2, X_1X_3, X_2X_3, X_1^2,X_2^2 and X_3^2) disclose changes in response when two factors are simultaneously changed.

Table no.3 shows the 17 trials with composition and their responses. Proniosomes formulations were prepared using slurry method. Dried film was hydrated with 7.4 phosphate buffer to form niosomal suspension. Malodextrin having high water solubility, easy for hydration and poor solubility in the loaded mixture solution. Therefore maltodextrin used as a carrier to increase surface area of hydration and so improve drug loading efficacy and cholesterol used to increase the membrane stability and drug entrapment efficiency of the vesicles.

Drug Entrapment efficacy:

The amount of Clozapine entrapped was determined by UV method. The calibration curved was applied over the concentration range 10 to 90 µg/ml with regression coefficient of 0.943.

The drug entrapment efficiency value of the prepared the Proniosomes were range for 62.54 % to 85.40%.Multiple regression were done to these value at the various levels of three factors (X_1,X_2 and X_3) to give up a second order regression equation.

$Y1 = 82.39+1.60A+4.12B+1.74C-0.12AC-5.90BC-3.82A^2-5.4B^2-3.70C^2$

The determination Coefficient R^2 value was 0.8097 which indicated good fitting model. The result clarifies the amount of cholesterol P<0.05 had a strong effect on drug entrapment efficacy. The Positive coefficient (1.62) i.e. at high concentration of cholesterol, drug entrapment efficacy increased. The positive values of the interaction coefficient of the 2 factors (AC,BC) showed gracious effect on drug entrapment efficacy. The contour plot figure 7 revealed that the relation between amount of cholesterol and sonication time at 0 levels to X1 illustrated contour plot that showed the interaction effect of amount of cholesterol and sonication time on Drug entrapment efficacy. Its shows the relation between both the variable was liner at 80.86% drug entrapment efficacy.

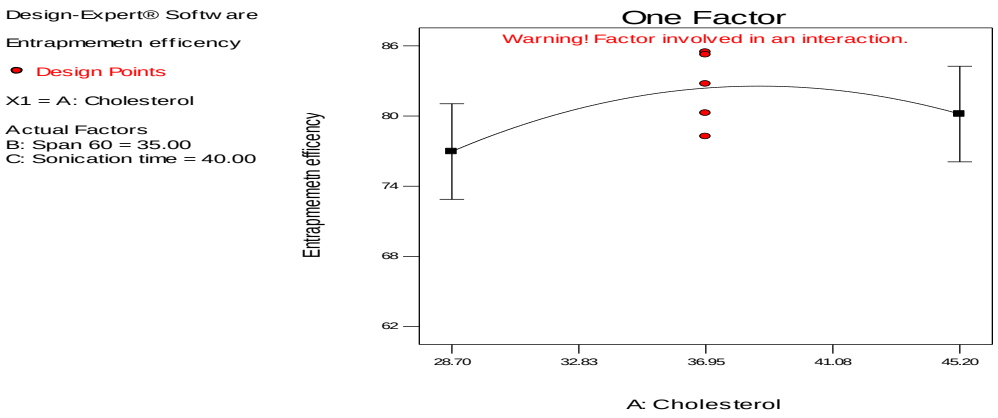
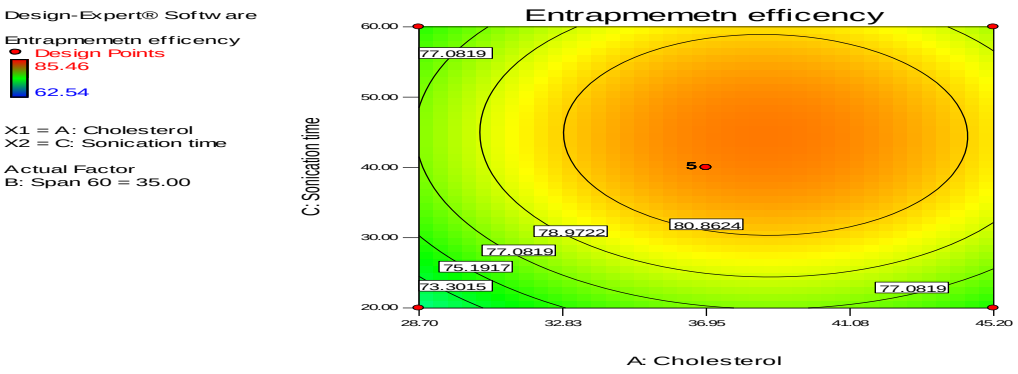


Fig 4: one factor showing the effect of Cholesterol on Drug entrapment efficiency



.Fig 5: The contour plot showing effect of interaction between Cholesterol and sonication time on the Drug entrapment efficiency.

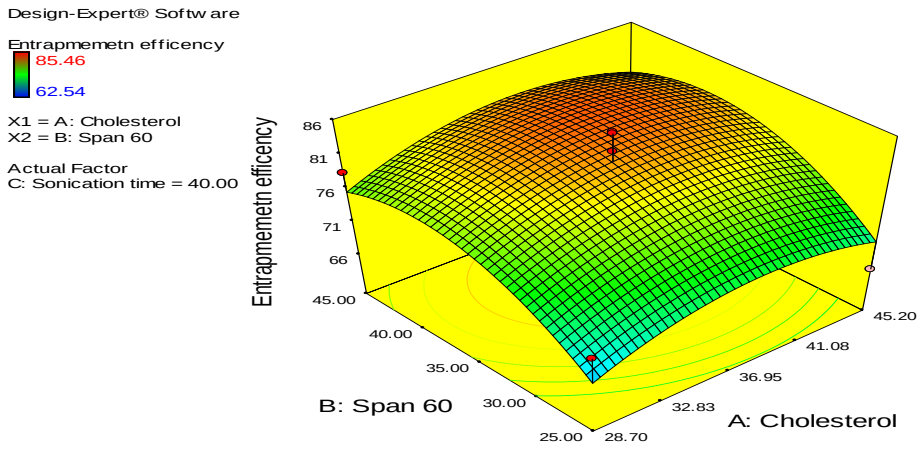


Fig 6: The Response surface plot showing effect of interaction between Cholesterol and sonication time on the Drug entrapment efficiency.

Vesicle size measurement:

The Vesicle size of Proniosomes formulae range 423 to 753 nm as shown in table no 3. These results were in concord with Yoshioka et al. [10] described that the mean size of niosomes increased with increasing the hydrophilic balance of the surfactant as the free energy of surfactant decrease with decreasing the hydrophobicity. They found that preparing niosomes with span 60 (HLB 4.7) as surfactant direct to a vescicles with a mean particle size.

Analysis of variance (ANOVA) was implemented to recognize the significant term of the quadratic model on Vesicle size. Multiple regression were done to vesicle size values at various levels of the three factors (X1,X2 and X3) to give second order regression equation with a determination coefficient of 0.96

Y2 = 556.64 - 0.087A + 18.36B - 84.45C - 6.75AB - 79.83AC - 46.98BC - 51.45A2 + 64.95B2 +103.03C2

It was found that only X3 (Sonication time) significantly influenced on Vesicle size ($P<0.05$). Also coefficients of X3 represent positive value that indicated a desirable effect. The Three replicated center point in design clarified the pure error of experiments and checked lack of fit model P value was found to be 0.129 which indicated that there was no lack fit. The main effect of amount of cholesterol on the vesicle size is illustrated as a line plot in figure 7, figure 8 illustrated contour plot that showed the interaction effect of amount of cholesterol and sonication time on vesicle size. Its shows the relation between both the variable was liner at vesicle size 595 nm.

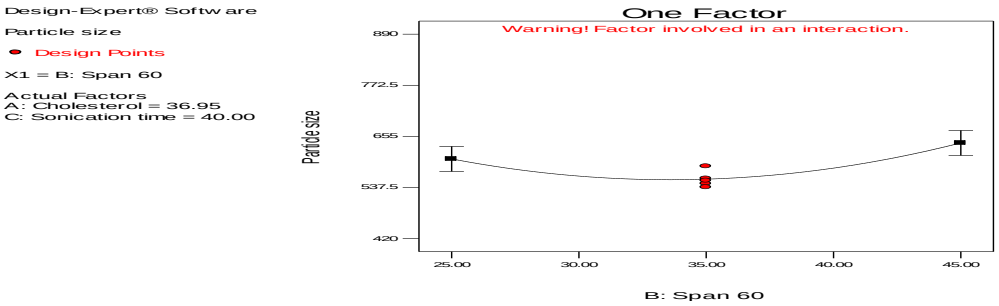


Fig 7: one factor showing the effect of span 60 on vesicle size

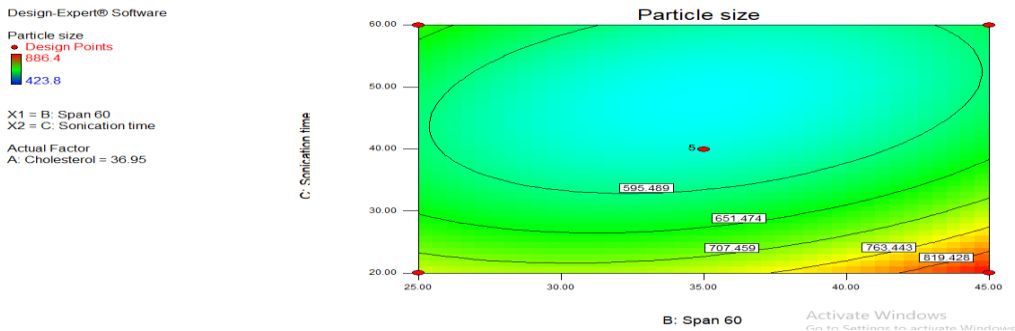


Fig 8: The contour plot showing effect of interaction between Span 60 and sonication time on the particle size.

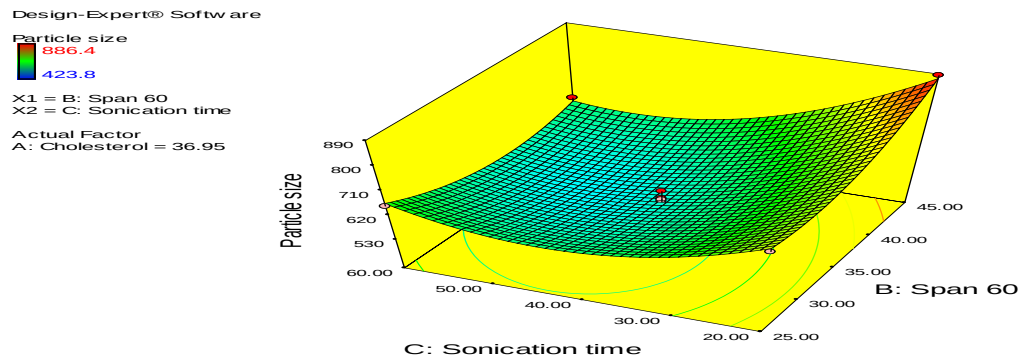


Fig 9: Response Surface plot showing interaction between Span 60 and Sonication time

Polydispersity Index:

The Polysispersity index of Proniosomes formulation in a range 0.350 to 0.790 was shown in table 3 The contour plot figure 10 revealed that the relation between amount of cholesterol and S[an 60 at 0 levels to X3 illustrated contour plot that showed the interaction effect of amount of cholesterol and Span 60 on PDI. Its shows the relation between both the variable was liner at 0.539. The effect of independent variable shown in equation.

Y3= +0.54-0.13A-0.034B-0.100C

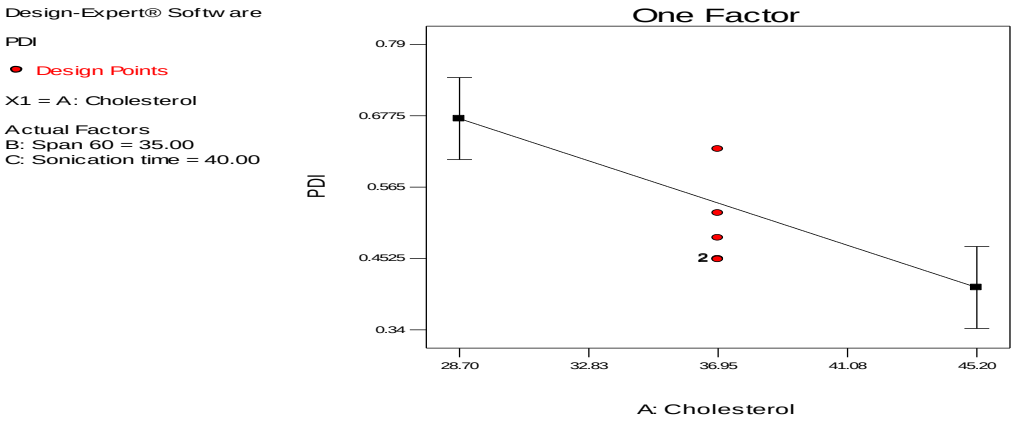


Fig 10 : one factor showing the effect of Cholesterol on PDI

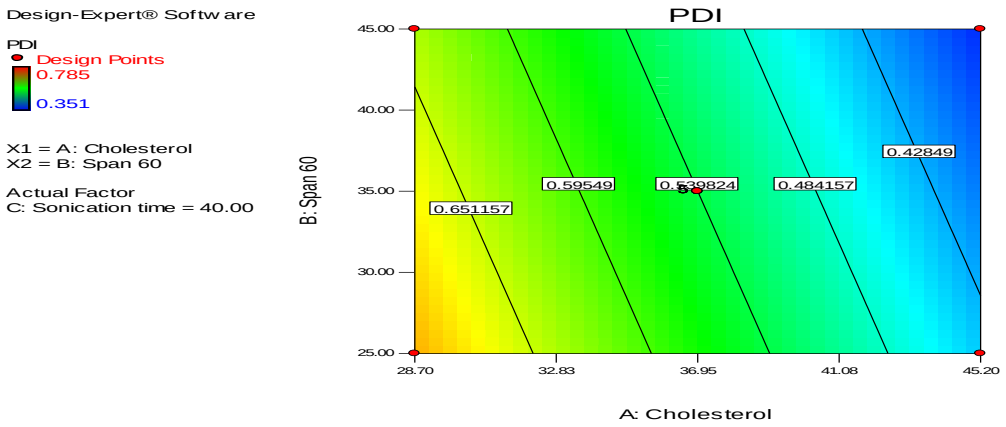


Fig 11: The contour plot showing effect of interaction between Span 60 and Cholesterol on the PDI.

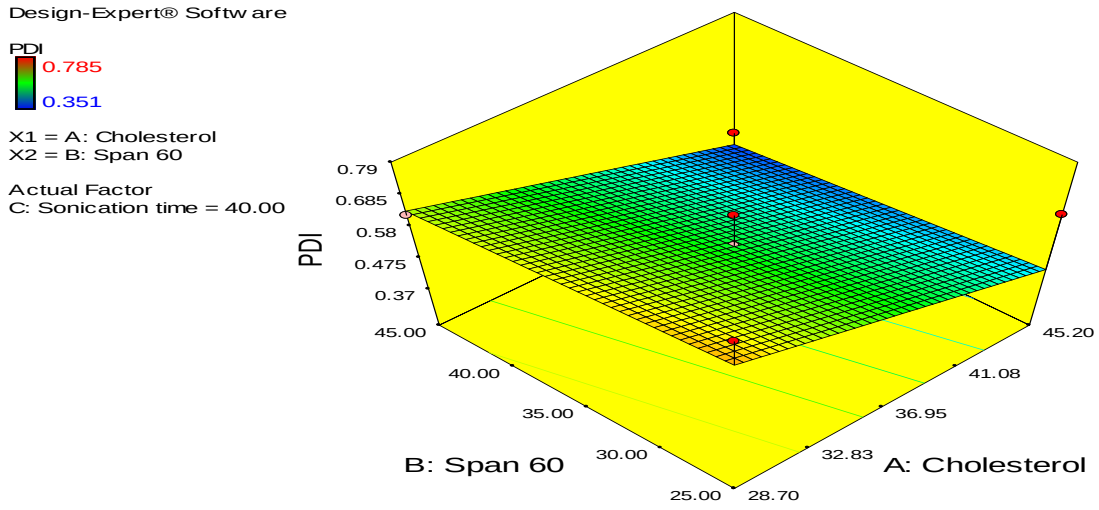


Fig 12: Response Surface plot showing interaction between Span 60 and Cholesterol

Optimum formula and checkpoint analysis

A numerical optimization technique using the desirability approach was working to prepared Maltodextrin based Clozapine Proniosomes with the required response constraint like minimize the particle size with maximum drug entrapment efficacy were set for achieve to establish the optimum situation of independent response variables. The optimized levels and predicted values of R1, R2 and R3 are shown in Table No.4. All three batches of obtained Clozapine Proniosomes were subjected to further characterization.

Table no.4 Optimized values obtained by the constraints apply on R1, R2 and R3

Independent Variable	Nominal value	Predicted value			Codes	Observed value		
		R1	R2	R3		R1	R2	R3
Cholesterol	36.13 mg	81.5 %	0.57	581 nm	CNP1	85.46	0.452	553.5
Span 60	35.49 mg				CNP2	80.25	0.351	423.8
Sonication time	35 minutes				CNP3	82.74	0.625	546.5

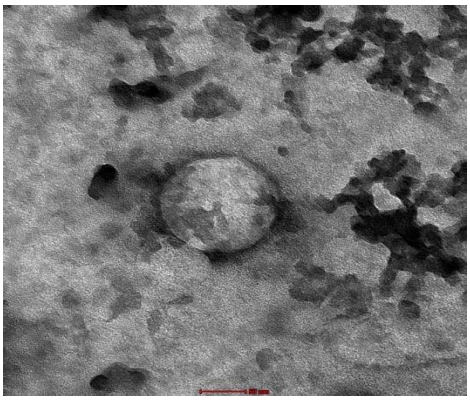
The mean vesicles size of diluted Clozapine Proniosomes are shown in above table No.4. It above range from 423.8 nm to 553.5 nm with good particle size distribution which favour drug delivery of Clozapine since the small particle size is advantage of improves penetration of particle in to tissue and decrease toxicity. Clozapine Proniosomes prepared using the optimum ration of surfactant (Span 60) and cholesterol demonstrated lamellar structure under compound microscope. Hydration of dry Proniosomes with pH 7.4 phosphate buffer solution.

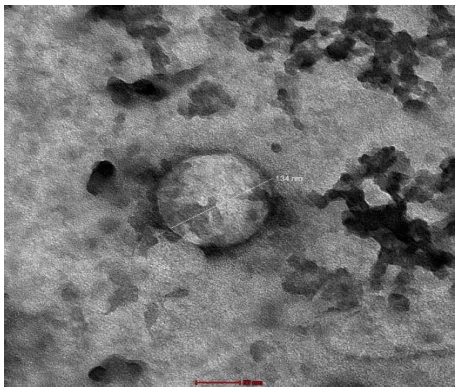
Table No.5 Optimized Batch code from Box-Benhken design

Codes	Drug Entrapment efficiency	Particle size	PDI Value
CNP1	85.46	0.452	553.5
CNP2	80.25	0.351	423.8
CNP3	82.74	0.625	546.5

Transmission Electron Microscope Proniosomes

The TEM micrographs of niosomes are given in Fig13. The TEM images confirmed the formation of Proniosomes. The shapes of the vesicles were spherical, and they were similar with the typical Proniosomes micrographs obtained in prior studies[13]





Conclusion

Optimization of a Proniosomes formulation is a compound process that required one to consider large numbers of variables and their interaction with each other. From above it conclude that Box Beheken design in optimization of Proniosomes formulations. The derived polynomial equation and counter plots give support to predicating the values of selected independent variable for the preparation of the optimum formulation with required properties.

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