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Design and Development of Nano Lipid Carriers for Clozapine Using Central Composite Methodology

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Abstract:

This study aims to develop and characterize a solid lipid nanoparticle (SLN) formulation for the anti-psychotics drug clozapine to improve drug delivery efficiency. Using a modified high-speed homogenization and ultra-sonication method, the formulation was optimized through a central composite design, varying solid lipid weights, and surfactant amount. The optimized SLN formulation, consisting of 102.77 % glyceryl monosterate, 5.24 Compritol® 888%, and 3 % tween 80, resulted in particles with an average size of 275.30 nm, a PDI of 0.251±0.25, and a zeta potential of -19.2±0.11 mV. The entrapment efficiency was 97.15±0.25 %, and drug release was 98.06±0.14 % over 24 hours, showing an initial burst followed by sustained release. Scanning electron microscopy revealed spherical particles with porous surfaces. Kinetic analysis indicated a zero-order release mechanism with non-Fickian diffusion. Stability studies over 60 days under ICH guidelines showed no significant changes, confirming the formulation's stability.

KEY WORDS: solid lipid nanoparticle (SLN), Clozapine, entrapment efficiency, non-Fickian diffusion.

INTRODUCTION

In recent years, SLN’s have emerged as a hopeful drug delivery system for enhancing the therapeutic efficacy of poorly water-soluble drugs. Clozapine, primarily targets the dopamine D2 receptors, and has a high affinity for serotonin 5-HT2A receptors to treat the schizophrenia, presents inherent challenges due to its poor aqueous solubility (1, 2).

Encapsulating Clozapine within SLNs offers a compelling solution to address this limitation. By leveraging the encapsulation capabilities of SLNs, Clozapine's solubility can be significantly enhanced, thereby improving its dissolution rate and overall bioavailability (3). Additionally, SLNs allow controlled and sustained release of Clozapine, aligning well with its once-daily dosing regimen and ensuring prolonged therapeutic effects (4).

Moreover, SLNs can be tailored for targeted delivery, potentially concentrating Clozapine, at its site of action in the kidneys, thus maximizing its pharmacological effects while minimizing systemic side effects. Furthermore, the stability of Clozapine can be preserved through encapsulation within SLN's, safeguarding its potency during storage and transportation (5). Overall, the formulation of Clozapine, into SLNs represents a promising approach to enhance its therapeutic outcomes, offering improvements in solubility, bioavailability, sustained release, targeted delivery, and stability, thereby advancing the anti-psychotic treatments like schizophrenia (6, 7).

These SLN's are an innovative carrier system that offers an alternative to polymeric nanoparticles, liposomes, and oil-in-water emulsions (8-10). These aqueous dispersions are stabilized by suitable surfactants and composed of lipids that remain solid at both room and body temperatures. Compared to polymeric nanoparticles, SLNs present distinct advantages, particularly in topical and oral drug delivery, where the same lipids can serve as the matrix material. Additionally, the wide variety of surfactants and stabilizers used in conventional formulations can also be employed in SLNs, ensuring compliance with regulatory standards for excipients (11, 12).

Lipid nanoparticles have been widely researched for percutaneous drug delivery and offer notable advantages over other colloidal delivery systems. SLNs, in particular, stand out due to their biocompatibility, scalability, and capacity to modulate drug release, enhancing overall performance. These qualities make SLNs an attractive option for optimizing oral drug delivery (13, 14).

The literature contains limited reports on using SLNs to bypass first-pass metabolism. However, notable studies have shown significant improvements in oral bioavailability when drugs are loaded into SLNs. For example, the oral bioavailability of all-trans retinoic acid in rats increased four- to five-fold when delivered via SLNs compared to a suspension (15, 16).

In this study, Clozapine-loaded SLNs were prepared using glyceryl monosterate, Compritol® 888as the solid-lipids and Tween 80 as the surfactant, through a hot homogenization followed by ultra-sonication method. The SLNs were characterized, and the optimized formulation was evaluated for its potential to improve the oral bioavailability of Clozapine (17, 18).

MATERIALS AND METHODS

Materials

Clozapine was generously provided as a gift sample by Aurobindo Labs, Hyderabad. Gylceryl monosterate and Compritol® 888were procured from Sigma-Aldrich, Merck and Poloxamer-188 was acquired from Himedia, Mumbai. Chloroform was purchased from Qualigens, India, and methanol from Rankem, India. The dialysis membrane utilized in the study was sourced from Hi Media, Mumbai. All other reagents used were of analytical grade.

Screening of lipids

The solid lipids for formulating Clozapine were chosen based on the drug's maximum solubility. To determine the drug's solubility, it was tested in selected solid lipids, including,glyceryl monostearate,and Compritol® 888. One gram of each solid lipid was individually placed in a glass beaker and heated on a magnetic stirrer to a temperature exceeding 10 °C over the melting point of the lipid. The drug was then added gradually in specified amounts with small increments while maintaining constant stirring. The mixture was stirred continuously for 30 minutes after each addition to ensure the drug completely dissolved. The clarity and transparency of the mixture were monitored during this process. A loss of transparency indicated the drug's saturation point in the lipid (19).

Surfactant Screening for Size and Stability Assessment

In selecting the most appropriate surfactant, our focus centered on its impact on the formulation's size and stability. We conducted screening with various surfactants featuring different Hydrophilic-Lipophilic Balance (HLB) values. Tween 80 HLB value was found 15, along with Poloxamer 188, sodium deoxycholate, and Span 80, was selected for evaluation based on its HLB value. These surfactants were assessed for their impact on the size and stability of blank Nanostructured Lipid Carriers (SLNs). The stability was determined by monitoring phase separation/creaming or floccule formation in the placebo formulation. This systematic approach allowed for a comparative assessment of surfactants based on their ability to maintain the desired particle size and stability of the SLN formulation (20).

Experiment design (Response surface methodology)

Response Surface Methodology (RSM), specifically employing a three-level approach, was utilized for experimental design and formulation optimization of SLN's Clozapine intended for oral drug delivery. Design Expert® software (Version 13.0.5.0, State-Ease Inc., and India) was employed for this purpose. Based on previous literature and variables in the formulation, the most suitable design for analyzing quadratic response surfaces was found to be the central composite design over the linear responses, two-factorial interactions, and polynomial models (21). The central composite design, requiring only 19 runs with 5 replicated center points, facilitated process optimization. By utilizing a computer-generated nonlinear polynomial model quadratic equation, the three-factor three-level design was elucidated. This systematic approach enabled the optimization of SLN formulation parameters, focusing on desired characteristics for oral drug delivery (22).

Polynomial equation (Y)= $b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_{12} + b_{22}X_{22} + b_{33}X_3$----- (1)

In the developed model, Y represents the dependent variable, with b_0 representing the intercept and b_1 to b_{33} denoting the regression coefficients found from individual responses. The independent variables are represented by X_1 to X_3. The prefixed variables corresponded to coded levels in the central composite design. Specifically, X_1 represents the percentage weight of gylceryl monosterate, X_2 represents Compritol® 888, and X_3 represents Tween 80. Furthermore, terms like X_i^2 (where i = 1, 2, or 3) depict the interaction of independent variables, while the quadratic terms are denoted by X_i^2. Table 01 defines the encoded values and levels of the independent and dependent variables. This structured approach facilitate the interpretation and manipulation of variables in the regression analysis, aiding in the optimization of the SLN formulation process.

Preparation of SLNs Loaded with Clozapine

Clozapine-loaded SLNs were prepared via a sequential process involving hot homogenization followed by ultra-sonication. Table 02 shown the formulation composition overview. Initially, the required amount of gylceryl monosterate and Compritol® 888as the solid lipid and the emulsifier were dissolved in a 1:1 ratio of 10 ml mixture of methanol and chloroform (23). The organic solvents were completely removed using a rotary flash evaporator. The resulting lipid layer was melted by heating to approximately 5°C above the lipid's melting point (24).

The stabilizer (Tween 80) was dissolved in distilled water (1-3 % w/v) to prepare an aqueous phase, heated to match the oil phase's temperature. Subsequently, the hot aqueous phase was added to the oil phase, and homogenization was conducted for 5-10 minutes at 12,000 rpm using a homogenizer (model T-18 D, IKA T18, Ultra Turrax, Germany).The coarse oil-in-water emulsion obtained was subjected to sonication using a probe sonicator (Model No. HV-PRO-650) for 20 minutes. The Clozapine-loaded SLNs were obtained by allowing the hot nano emulsion to cool to room temperature. Subsequently, lyophilization was carried out until the nanoemulsion dried.

In this present work, Clozapine-loaded SLNs were fabricated using a Response Surface Methodology (RSM) approach. The study design employed a randomized, non-block, central composite, model quadratic with 3 factors of Non-dependent variables at three levels (-1, 0, and +1). The experimental data are summarized in Table 01.

Table 1: Clozapine-Loaded SLNs: Model Variables and Central Composite Design Coded Levels

Name of Variables			
In dependent (%w/v)	Glyceryl monosterate (X1)	Compritol® 888(X2)	Tween 80 (X3)
Dependent (Responses)	Particle in size nm (Y1)	Entrapment efficiency (%) (Y2)	*In-vitro* drug release (Y3)
Coded levels	Low	Centre	High
	-1	0	+1
Glyceryl monosterate(X1)	10	15	20
Compritol® 888(X2)	5	7.5	10
Tween 80 (X3)	1	2	3

Table 02 Experimental runs conducted employing Central Composite Design (CCD)

Run	Independent variable in mg		
	Glyceryl monosterate(X1)	Compritol® 888(X2)	Tween 80 (X3)
1	200	10	3
2	150	7.5	2
3	200	5	3
4	100	10	1
5	150	5	2
6	200	7.5	2

7	100	5	3
8	150	7.5	2
9	200	10	1
10	150	7.5	1
11	150	7.5	2
12	100	5	1
13	100	7.5	2
14	100	10	3
15	150	7.5	2
16	150	7.5	3
17	150	7.5	2
18	200	5	1
19	150	10	2

SLN's Characterization

FTIR studies for Drug- Excipient Interactions

Briefly, Fourier Transform Infrared Spectroscopy (FTIR) analysis was conducted to investigate potential interactions and compatibility between the drug and lipids used in Solid Lipid Nanoparticles (SLNs) preparation (25). An FTIR spectrometer from Bruker, India, was utilized for the analysis. Clozapine samples were blended with potassium bromide (KBr), and FTIR spectra were recorded for the pure drug, physical mixture of the drug and lipid, and surfactant mixture. Scanning was performed in transmission mode across a wavenumber range from 4000 to 400 cm^{-1} (26).

Differential Scanning Calorimeter (DSC) Characterization

Differential scanning calorimetry (DSC) analysis was performed using the Mettler-Toledo DSC 821e instrument in Columbus, OH, USA. DSC scans were conducted for all combinations of drug and lipid, employing a heating rate of 10°C/min over a temperature range of 50–250°C (27).

Measurement of Particle Size (nm), Poly dispersity Index (PDI), andZeta Potential (ZP) of SLN

Size, Polydispersity Index (PDI), and Zeta Potential (ZP) of Clozapine-loaded SLNs were evaluated utilizing a Malvern zetasizer (Nano ZS90, UK).Approximately 100 µL of the prepared SLN dispersion was diluted to 5 mL with double-distilled water before being subjected to analysis using the zeta sizer (28, 29).

Determination of Entrapment Efficiency (%)

To determine entrapment efficiency, free drug concentration in an aqueous medium was quantified using ultrafiltration with centrisart tubes (Sartorius, USA). After centrifugation, SLNs containing the drug remained in the outer chamber while the aqueous phase migrated to the recovery chamber. Clozapine concentration in the aqueous phase was analyzed using HPLC (30).

***In Vitro* Drug release studies**

In vitro release studies were conducted using the dialysis method with a Himedia membrane (India) featuring a pore size of 2.4nm and a molecular weight cut-off between 12,000 and 14,000 The membrane was pre-soaked overnight in double distilled water. Phosphate buffer pH 6.8 served as the release medium (31). The experimental setup included donor and receptor compartments. In the donor compartment, 1mL of SLN dispersion was placed in a boiling tube tied with the dialysis membrane. The receptor compartment, a 250mL beaker filled with 100mL release medium, was maintained at 37 ± 0.5 °C. Samples were withdrawn at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24-hour intervals, replaced with an equal volume of release medium. Samples were diluted and analyzed at 254 nm using a UV-visible spectrophotometer (SL-150, ELICO, and India) (32).

Drug release kinetics and mechanism analysis

To ensure the drug release kinetics and mechanisms of Clozapine-SLN's, we systematically evaluated the *In-vitro* dissolution profiles for optimized formulation. These profiles underwent analysis using a range of appropriate models, including zero-order kinetics, First-order kinetics, Higuchi's plot, and the Korsmeyer–Peppas (K-P) model. The statistical tool DD Solver software facilitated this analysis (33, 34).

In this assessment, crucial parameters such as the adjusted regression values (r^2 adjusted), the range of the Akaike Information Criterion (AIC), and the Model selection criterion (MSC) values played a pivotal role (35). These parameters generated distinct values

that formed the basis of comparison. By analyzing these values, it became possible to determine the best-fitting models that elucidate the release order behavior for the optimized formulation (36). Insights into the kinetics and mechanisms governing drug release from the selected formulations are provided by this systematic approach.

Physical Stability Studies

The optimized formulation of Clozapine-loaded solid lipid nanoparticles was stored at room temperature (25°C, 60 ± 5% RH) and refrigerator temperature was maintained at (4°C) for 60 days. The average size (nm), zeta potential (-mV), polydispersity index (PDI), and entrapment efficiency (%) were determined in triplicate.

Statistical analysis

All experiments were conducted in triplicate, and reported values represent the average of three measurements with standard deviation (± SD). Statistical analysis involved comparing various study groups using ANOVA for analysis of variance. Design Expert® (Version 13.0.5.0, State-Ease Inc., and India) and DDSolver 1.0 (Microsoft Corp., U.S.A.) were utilized for statistical analysis of different parameters. A significance level of P-value < 0.05 was considered.

RESULTS AND DISCUSSION

Lipid and surfactant Screening

We concluded that glyceryl monosterate, when combined with Compritol® 888 and Tween 80, constitutes the highly favorable solid lipid for drug-loaded SNPs. This conclusion stems from its high solubility, bio-compatibility, and stability. In this, the selected solid lipids plays a key role in stabilizing nanoparticles, facilitating the encapsulation of both in drugs has lipophilic and hydrophilic natures. Additionally, Tween 80 enhances drug release kinetics and prevents aggregation, thereby ensuring efficient drug delivery.

Physicochemical characterization

Employing FTIR analysis, potential interactions between drugs and solid lipid nanoparticle (SLN) components were investigated. The study focused on peak intensity and peak shifting to assess compatibility among pure drug, and excipients interactions in optimized Clozapine-SLN's. All the results were depicted in figure 01 and 02.

Pure Clozapine displayed characteristic peaks was measured at 3050.14 cm^{-1} aromatic C-H stretching, 1680.34 cm^{-1} carbonyl group, and C-N amine stretching 1220-1260cm^{-}

[1]Additionally, common peaks generated from Clozapine included a C-CL was found at 740cm^{-1}and N-H stretching (secondary amine) 3300-3400 cm^{-1}were recorded.

In Clozapine-SLN's (optimized formulation) spectra, characteristic peaks for aromatic C-H stretching was found 3070.25 cm^{-1}, carbonyl group was recorded1695.24cm^{-1}, and aromatic C-N amine stretching 1290.48cm^{-1} were identified. Furthermore, shifts in the peaks of lipid's C=O stretching recorded at 1740 cm^{-1}(ester bond) and methylene group in lipids was recorded at (C-H stretching) 2850-2920 cm^{-1}. Figure: 02,illustrates the FT-IR spectra of Clozapine (pure API), with physical mixture. Notably, the optimized Clozapine formulation's spectra contained all functional group peaks observed in the pure (Clozapine's) spectra, there with no additional peaks was detected. This indicates no interaction between the drug and excipients used in manufacturing Clozapine-loaded SLNs.

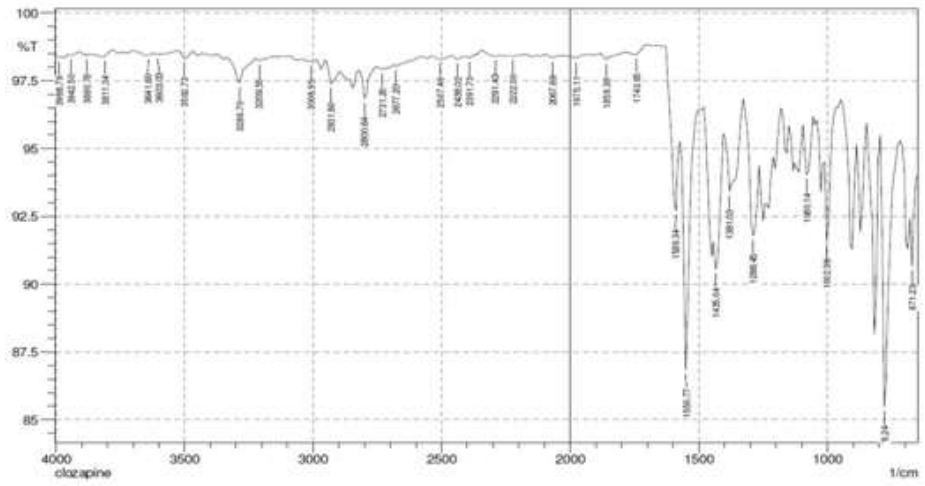

Figure: 01 FT-IR Spectra of pure Clozapine

Differential Scanning Calorimetry (DSC) thermograms were investigated for both pure API form of Clozapineand the optimized formulation of Clozapine loaded with SLNs containing the drug with selected lipids, and co-surfactants.

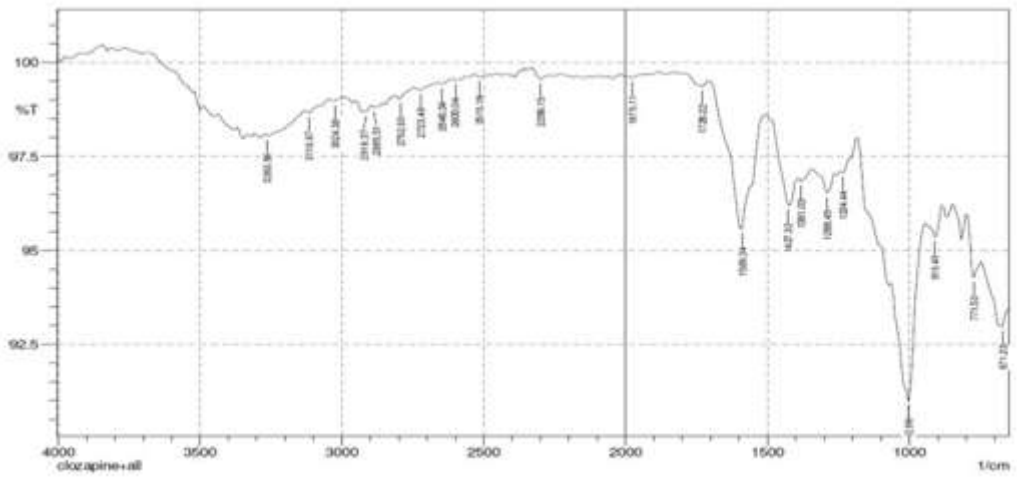

Figure: 02 FT-IR spectra of Clozapine SLN's optimized formulation

The DSC thermogram of the pure drug Clozapine, which exhibited a onset peak of endothermic at 183.18°C, indicating its melting point. This peak corresponds to the drug's crystalline nature and purity. In contrast, Figure:04 shown the DSC thermogram for the optimized formulation, which includes Clozapine, and all excipients. The thermogram reveals both wide and sharp endothermic peaks, with a significant one observed very close to 69.58°C. The observed endothermic peak corresponding to selected lipids melting point where incorporate in the clozapine loaded SLN' formulation and also It could may be shifted, broadened, or reduced in intensity, indicating that the drug is either in an amorphous state or dispersed within the lipid matrix.

XRD Crystallography

Powder-XRD analysis confirmed the molecular dispersion state of the drug using the established formulation method. It was additionally used to explore the polymorphic behavior and crystallinity of Clozapine.Figures 05 and 06 depict the diffraction patterns of the optimized SLNs compared to pure clozapine. The pure XRD spectra exhibited multiple distinct, intense peaks recorded at diffraction angles (2 theta scale) against d-spacing values.The peaks were namely 11 (d-28.31), 16.37 (d-15.02), 22.17 (d-11.74), 24.26 (d-8.51) and 26.07 (d-3.27 number of peaks were observed more.

In the case of Clozapine SLN's optimized formulation, the intensity of the XRD peaks pattern was obtained at 19.55 (d-12.07), 19.79 (d-8.17), 21.07 (d-5.61), 23.07 (d-2.17) and 23.89 (d-1.02) this would be significantly different from the pure APIand also numbers of

peaks were recorded at low with respect to the pure Clozapine. In addition to that, the model peaks disappeared as in the case of optimization formulation.

From the above observation, the optimized formulation of Clozapine resulted in a significant disruption of its crystalline nature, shifting it towards an amorphous stated due to the several reasons as like, This transformation may enhance drug solubility and dissolution kinetics within the lipid matrix of solid lipid nanoparticles (SLNs), potentially improving the formulation's efficacy.

The optimized formulation ofClozapine shifted its crystalline nature significantly towards an amorphous state. This transformation could be attributed to various factors, such as the lipid matrix, potentially high in the amorphous phase, played a role in disrupting Clozapine s crystalline structure or processing techniques like high-pressure homogenization or solvent evaporation applied mechanical or thermal stress on Clozapine crystals and also, this study includes the interactions between Clozapine and lipid components of the formulation might have contributed to the disruption. Incorporating Clozapine into the matrices of lipid could have altered the packing arrangement of drug molecules, prompting the transition from crystalline to amorphous.

Overall, the disruption of Clozapine’s crystalline nature and its shift towards an amorphous state in the optimized formulation suggest successful incorporation of the drug into the lipid matrix of the SLNs. This transformation may have implications for the formulation's drug release profile, bioavailability, and therapeutic efficacy.

Particle size (nm) PDI and Zeta Potential (mV) and Entrapment Efficiency (%)

All the experimental formulations (14) underwent comprehensive analysis to ascertain their particle size distribution, zeta potential, and PDI values, as outlined in Table03. The particle size across all formulations ranged from 275±0.14 nm to 302±0.24 nm, while PDI varied from 0.251±0.25 to 0.431±0.17, and zeta potential spanned from -19.2 ±0.11 to −25.5±0.19. Additionally, determining the entrapment efficiency (%) is crucial for characterizing solid lipid nanoparticles, analyzed for each formulation using HPLC.Based on the results obtained, all formulations demonstrated excellent entrapment efficiency, ranging from 85.01±0.11 to 97.54±0.25. Notably, all the recorded values were fall within acceptable ranges. Based on these findings, it was confirmed that the selected independent variables significantly influenced the characteristics of the solid lipid Nano formulations.

Consequently, the obtained results warranted further assessment to govern the optimized formulation through central composite design.

Table 3: Particle size, PDI, Zeta potential, and Entrapment efficiency (%) of Clozapine SLN's formulations

Runs	Particle size (nm)	PDI	zeta potential (mV)	Entrapment efficiency (%)
1	300±0.10	0.261±0.22	−19.5±0.11	87.11±0.11
2	289±0.14	0.302±0.13	−20.3±0.15	91.02±0.14
3	297±0.07	0.274±0.11	−20.6±0.24	89.12±0.13
4	280±0.13	0.403±0.15	−21.3±0.15	96.05±0.12
5	287±0.02	0.312±0.152	−23.4±0.19	92.13±0.01
6	297±0.04	0.270±0.21	−22.4±0.27	89.02±0.05
7	275±0.14	0.251±0.25	-19.2 ±0.11	97.15±0.25
8	282±0.03	0.271±0.33	−21.8±0.43	93.14±0.07
9	294±0.13	0.272±0.05	−21.4±0.73	90.15±0.11
10	280±0.05	0.405±0.15	−21.8±0.14	95.23±0.22
11	284±0.07	0.285±0.17	−19.9±0.54	93.12±0.03
12	280±0.02	0.314±0.19	−22.4±0.17	96.10±0.05
13	281±0.1.2	0.273±0.12	−23.5±0.24	95.14±0.03
14	287±0.04	0.413±0.22	−21.6±0.15	92.05±0.13
15	283±0.03	0.431±0.17	−24.7±0.54	94.02±0.03
16	280±0.04	0.421±0.24	−23.4±0.74	95.24±0.13
17	285±0.08	0.414±0.11	−24.7±0.54	93.21±0.08
18	302±0.29	0.417±0.23	−25.5 ±0.19	85.01±0.13
19	286±0.07	0.425±0.17	−24.9±0.64	92.13±0.14

(±S.D values n=3)

Application of Factorial design for optimization of Clozapine-SLN's formulations

The data was analyzed by fitting it into various models, resulting in actual, adjusted, and predicted regression coefficients. These coefficients were closely similar for each response variable. To select the best-fit model, we aimed to keep the difference between the adjusted r^2 value and the predicted r^2 value under 0.2.

The analysis revealed that a quadratic model was the most suitable representation for the responses. It showed that particle size, polydispersity index, and entrapment efficiency of SLN's were influenced by the levels of lipids and surfactant. These factors interactions also played a significant role, as indicated by the quadratic model.

To delve deeper, we proposed further assessment through ANOVA, contour plots, and response plots to understand the relationship between the responses and variables. The selected best-fit models for the responses showed significance, with *p-values* less than *0.001*.

Particle size (Y1)

We evaluated the impact of lipid and surfactant on particle size. Statistical analysis, ANOVA, was conducted to understand this relationship. The modelquadratic provided the best fit with a *p-value* < *0.001*, and a lack of fit non-significant test indicated that the model accurately represents the data. The ANOVA results revealed a significant influence of the selected variables, with high sum square values for the model and linear responses, indicating a strong overall factor influence. Interaction terms (AB, AC, and BC) also showed significant effects and high mean square values confirmed the substantial impact of the preferred variables on particle size.The polynomial equation for Particle size response is shown below.

Particle size (Y1)= +8.70 A + 0.60 B + 0.30 C – 2.12 AB -0.125 AC + 2.88 BC + 5.54 A^2 + 3.04 B^2 – 3.46 C^2-----2

Glyceryl mono stearate exhibited larger coefficient values of 8.70 for A and 5.54 for A^2 in the polynomial equation, indicating its substantial impression on particle size in SLNs. The coefficient sign positive, suggests that increasing glyceryl mono stearate concentration leads to proliferation in particle size. This phenomenon can be attributed to glyceryl mono stearate larger molecular size compared to other lipid components commonly used in SLNs. Its long hydrocarbon chains enable molecules to pack densely together. Consequently, higher concentrations of glyceryl mono stearate result in more of these larger molecules being integrated into the nanoparticles, leading to increased packing density and larger particle sizes overall. This efficient packing facilitates swift particle size increase, as depicted in Figures 03and 04.

The contour plot and 3D surface plot demonstrate that smaller particle sizes for Clozapine SLN lipid nanoparticles are achieved when lipid concentrations are from 102.78%, 5.24% w/v and surfactant concentrations range from 1 -3 %. These plots vividly illustrate the complex interactions between lipid-surfactant, lipid-co-surfactant, and surfactant-co-surfactant, offering valuable insights for optimizing formulations to achieve desired smaller particle sizes.

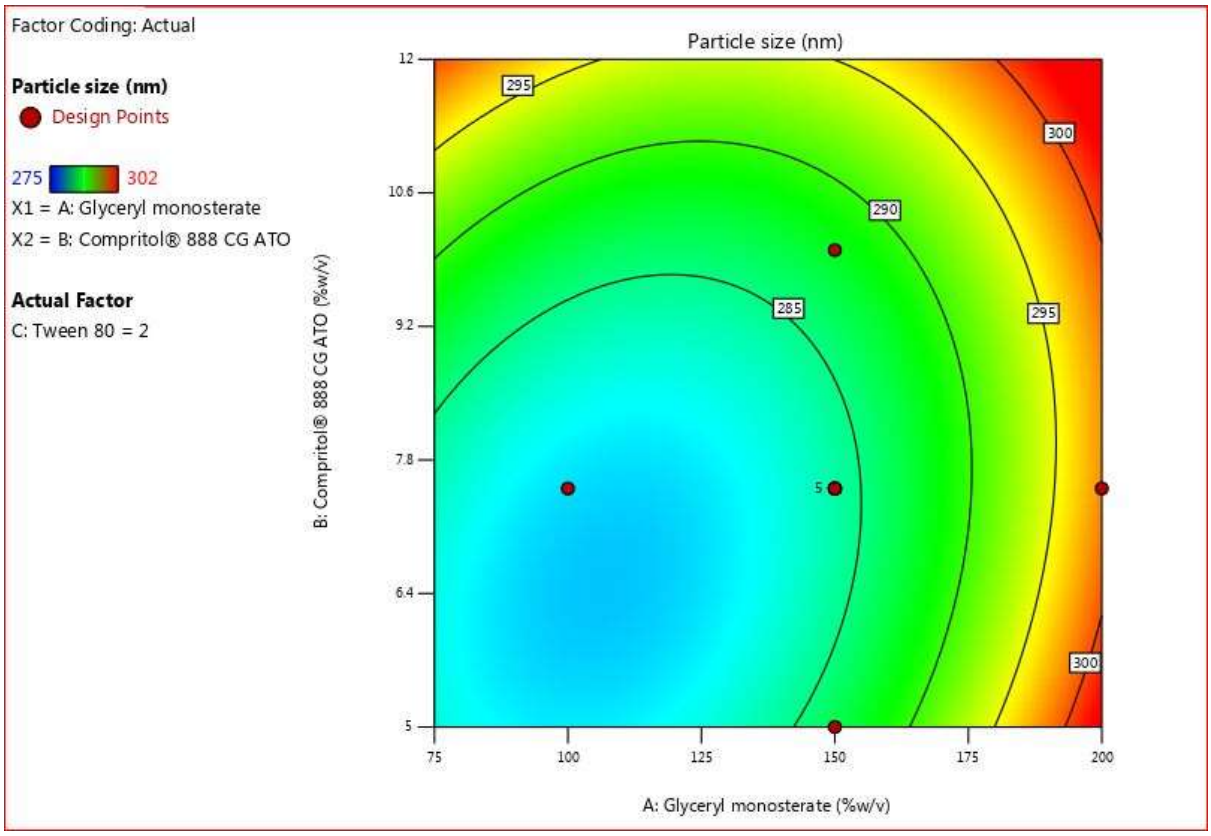

Figure 03: 2D Contour Plot of Independent Variables Influencing Clozapine SLN Particle Size

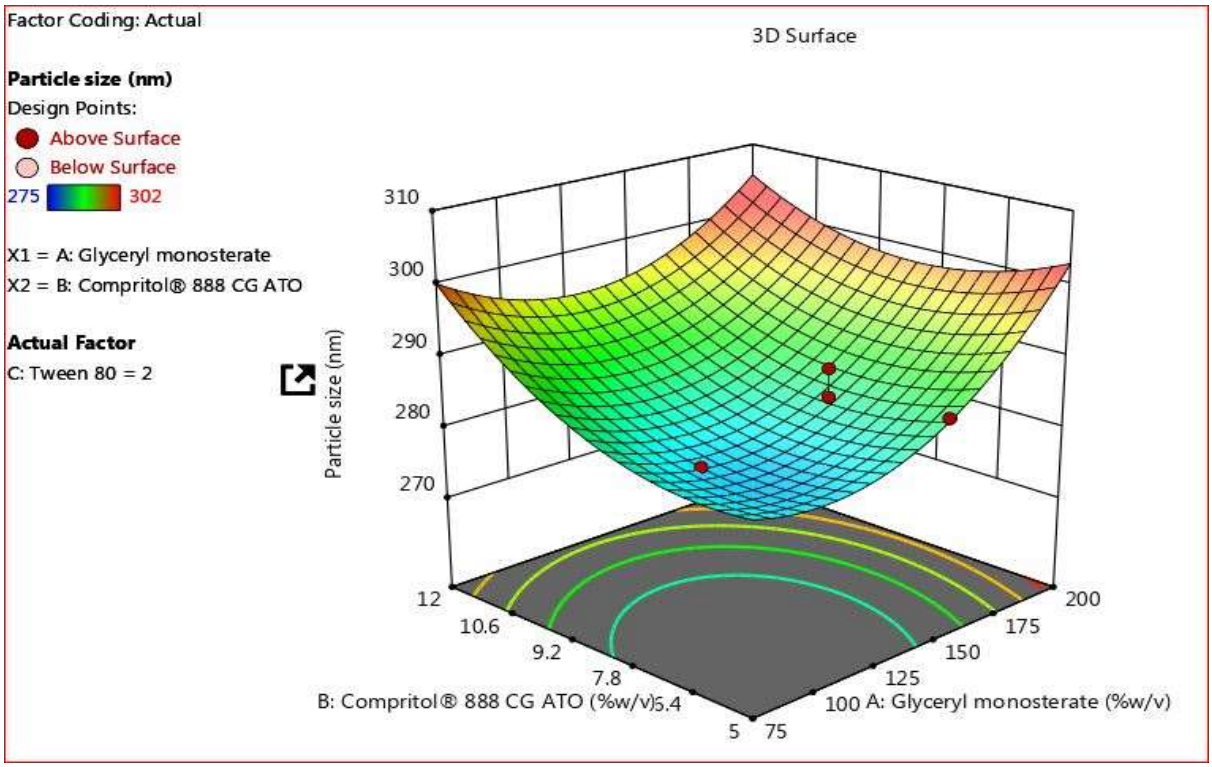

Figure: 04- 3D RSM Plot of Independent Variables Influencing Clozapine SLN Particle Size

Entrapment efficiency (%) Y2

The study systematically assessed the influence of independent variables—Lipid and surfactant—on Entrapment efficiency (%), followed by comprehensive statistical analysis to uncover key findings. ANOVA results, was conducted on the Entrapment efficiency (%) response from multiple experimental runs, revealing significant insights. The resulting ANOVA results summarizing the influence of lipid and surfactant on Entrapment efficiency are presented below.

Statistical analysis identified the quadratic model as the best fit *($p < 0.001$)*, confirming its accuracy in representing the data. ANOVA showed substantial influences from the overall factor (linear response) and interaction terms, with factor A being particularly significant (SS = 129.60, F = 147.86). Elevated mean square values further emphasized the strong impact on the dependent variable.

Based on the observations, it was concluded that factor A significantly influences entrapment efficiency, while factors B and C do not. Additionally, no significant interaction effects (except AB) BC, and AC were observed on entrapment efficiency. The relationship between stearic acid concentration and entrapment efficiency in nanoparticles can be attributed to several key factors. Higher stearic acid levels increase its ability to encapsulate the active ingredient, leading to improved entrapment efficiency. Optimal stearic acid concentrations also promote the formation of smaller nanoparticles, this provides increased surface area for encapsulation. Furthermore, combination of glyceryl mono sterate stabilizes and forms nanoparticles, ensuring efficient encapsulation, and its interactions with other formulation components also contribute to its role. Overall, higher glyceryl mono sterate concentrations generally result in better entrapment efficiency due to its vital roles in nanoparticle formation and encapsulation.

Entrapment efficiency (%) Y2 = 93.11+ 3.60 - 0.200 B – 0.200 C + 1.00 AB + 0.500 AC – 1.500 BC – 1.51 A2 – 1.51 B^2 + 1.149 C^2----------3

From the above polynomial equations, the components of A was shown significantly positive co efficient sign effect on entrapment efficiency. This positive coefficient recommended that as the amount of stearic acid increase, the entrapment of the core drug into the nanoparticle will increase. The results are depicted in Figures 05 and 06.

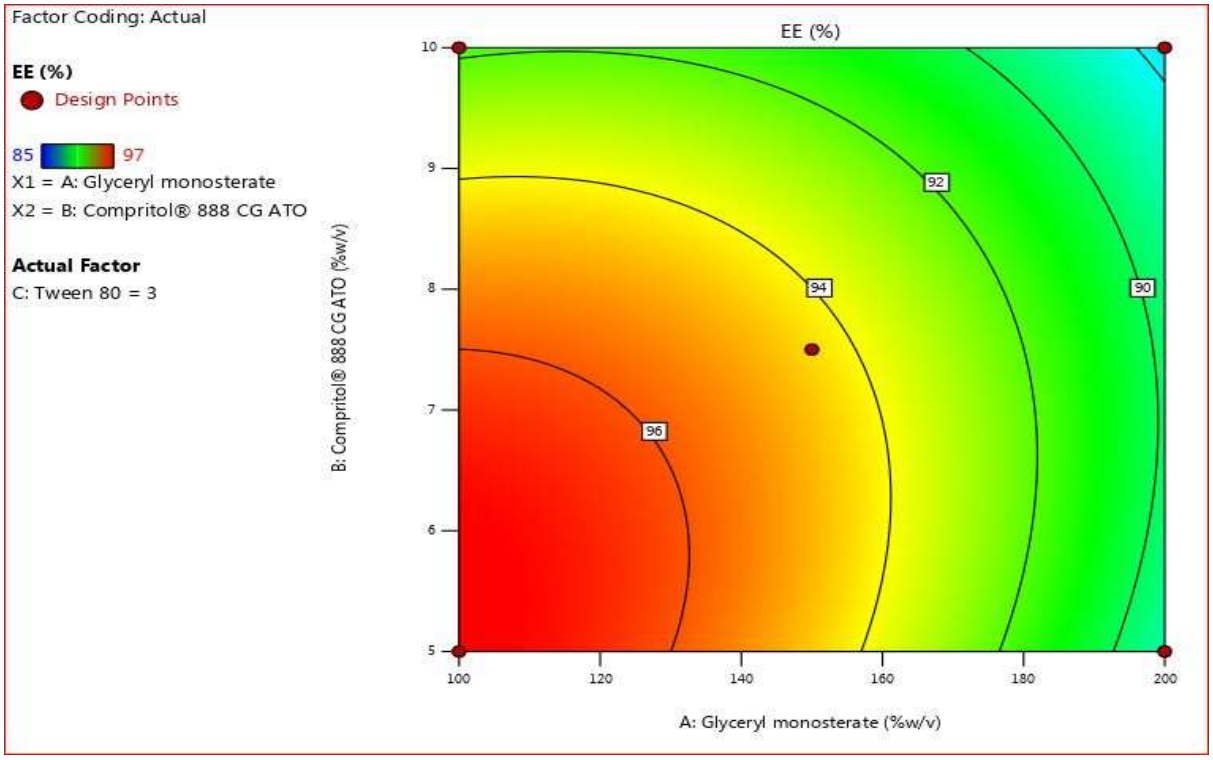

Figure 5: 2D Contour Plot of Independent Variables Influencing Clozapine SLN Entrapment efficiency (%)

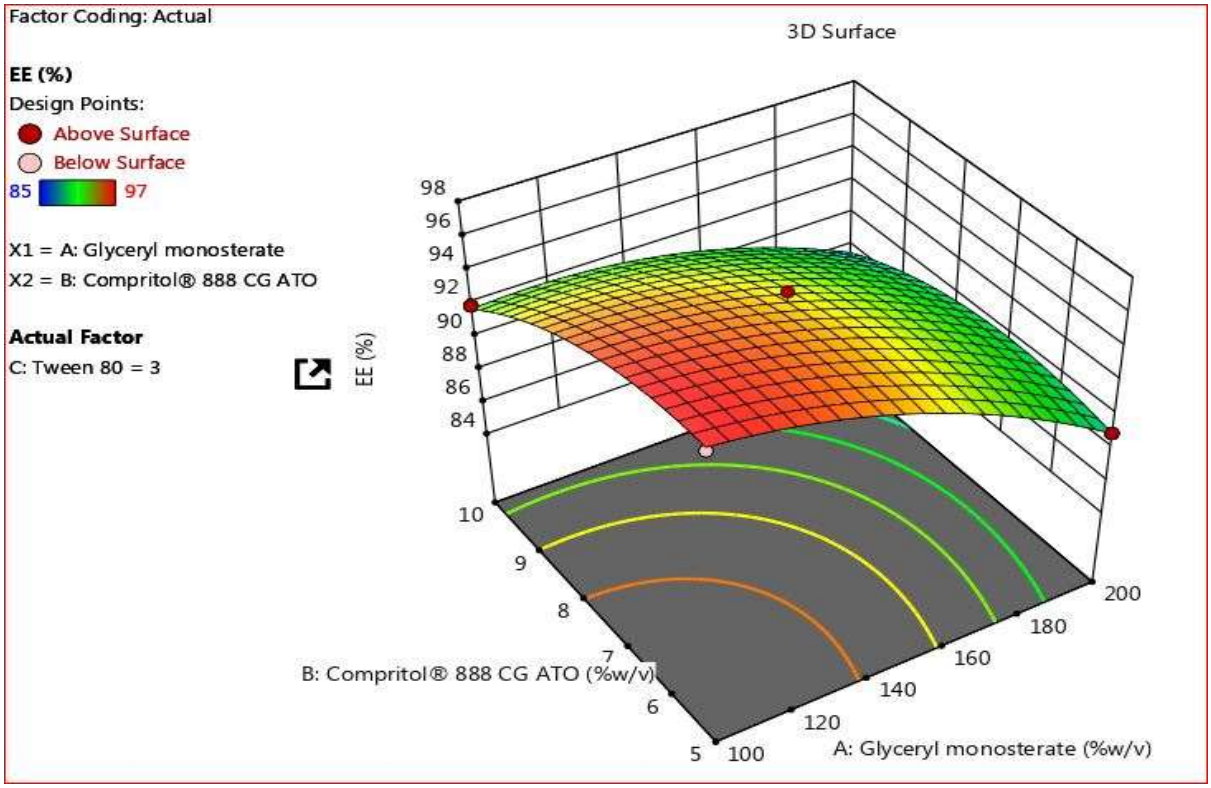

Figure 6: 3D RSM Plot of Independent Variables Influencing Clozapine SLN Entrapment efficiency (%)

***In-vitro* drug release (Y3)**

The analysis systematically evaluated the impact of independent variables lipid, surfactant, and surfactant on the *In-vitro* drug release, followed by rigorous statistical examination to extract key insights. ANOVA was performed on the *In-vitro* drug releaseresponse across multiple experimental runs, yielding significant findings. The quadratic model emerged as the most suitable fit for the data, supported by a highly significant *p-value (<0.001)*. A non-significant lack of fit test is preferred, indicating the model accurately represents the relationship between independent and dependent variables, facilitating reliable predictions and valid inferences.

ANOVA revealed significant influences of the overall factor (quadratic response) and interaction terms, particularly for A and BC, shown by the high sum of squares (SS) values. Elevated mean square values highlighted the strong impact of independent variables on the dependent variable. Specifically, surfactant and co-surfactant interactions significantly affected the polydispersity index, with high F-values for A (90.82) and BC interaction (8.01).

In-vitro drug release (Y3)= 92.28 - 4.50 A – 0.600 B - 0.600 C + 1.25 AB +0.750 AC – 1.50 BC -1.38 A^2 -2.88 B^2 + 3.12 C^2----------4

From the above polynomial equations, the components of A and BC were shown significantly negative sign effect on dependable response of drug release. This negative coefficient sign suggests that as the concentrations of glyceryl mono sterate and amount of surfactant ratios increases were leads to decreases the drug release of fabricated SLN's.

In general, Glyceryl monostearate (GMS) and Compritol influence the drug release of clozapine in solid lipid nanoparticles (SLNs) by creating a stable, rigid lipid matrix. GMS increases matrix density and crystallinity, slowing drug diffusion. Compritol forms a hydrophobic barrier, further reducing water penetration and drug release. Together, they provide controlled, sustained release of clozapine. Furthermore, their interaction with lipid components affects the structure and stability of nanoparticles. Optimizing their composition can lead to a more uniform particle size distribution and uniform drug release in controlled manner. The results are depicted in Figure 7 and 8.

Selection of optimized batch as a function of Desirability of all response variables

The selection of the best and most optimized formulation relied on the desirability response, a key function in Design Expert software. This approach played a crucial role in identifying the most reliable formulation. The desirability graph in the software combined

various responses from dependent variables, thus facilitating the creation of an ideal formulation with the desired physicochemical properties.

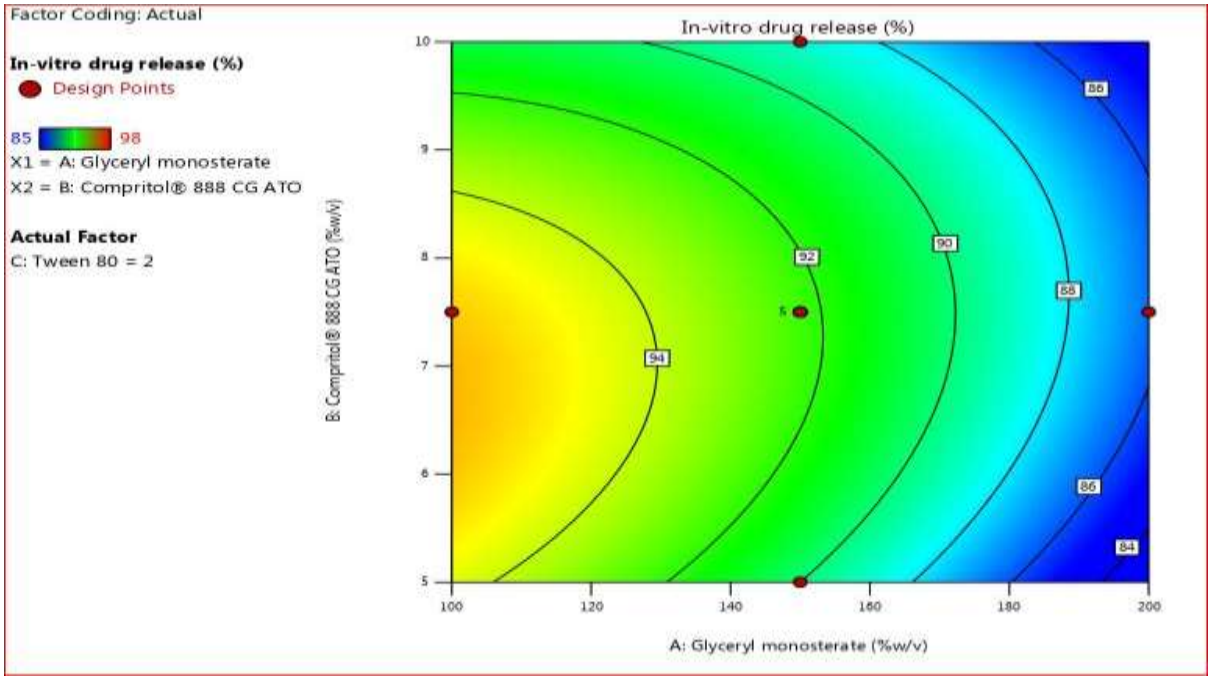

Figure 7: 2D Contour Plot of Independent Variables Influencing Clozapine SLN drug release

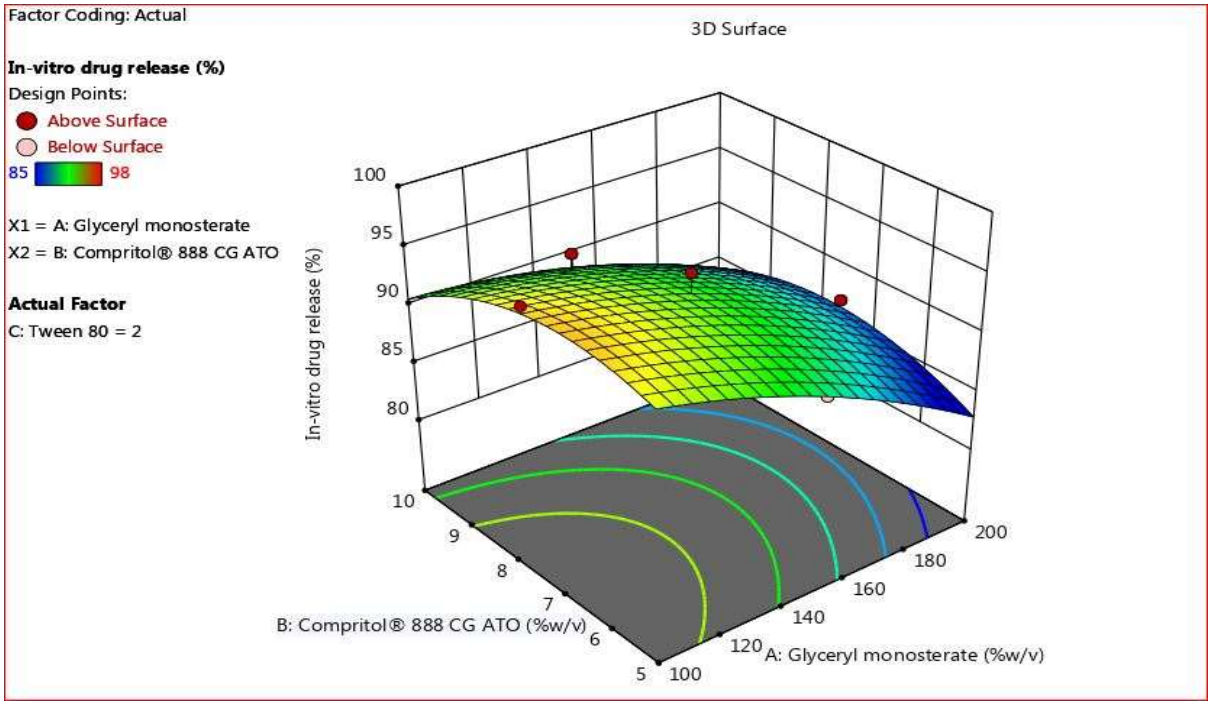

Figure 8: 3D RSM Plot of Independent Variables Influencing Clozapine SLN drug release

For optimization purposes, the desirability is scaled from 0 to 1. It aligned the values of individual response variables and was applied to evaluate each solution from 1 to 56 based

on considerations of the mentioned constraints depicted in Table no: 04, and to meet the objective of the present study, the desirability value close to zero indicated unfavorable and unacceptable conditions for the responses, rendering a formulation undesirable. Conversely, as the value approached one, the formulation became highly preferable and desirable.

Table 04: Constraints & Importance of Independent Variables

Name of the components	Goal	Limit (Low-Upper) in % w/v		Importance
A: Glyceryl monosterate	in range	10	20	+++
B:Compritol® 888	in range	5	10	+++
C: Tween 80	in range	1	3	+++
Particle size	Minimize	275±0.14	302±0.29	+++++
Entrapment efficiency (%)	Maximize	85.01±0.13	97.15±0.25	+++++
In-vitro drug release	Maximize	85.26±0.12	98.06±0.14	+++++

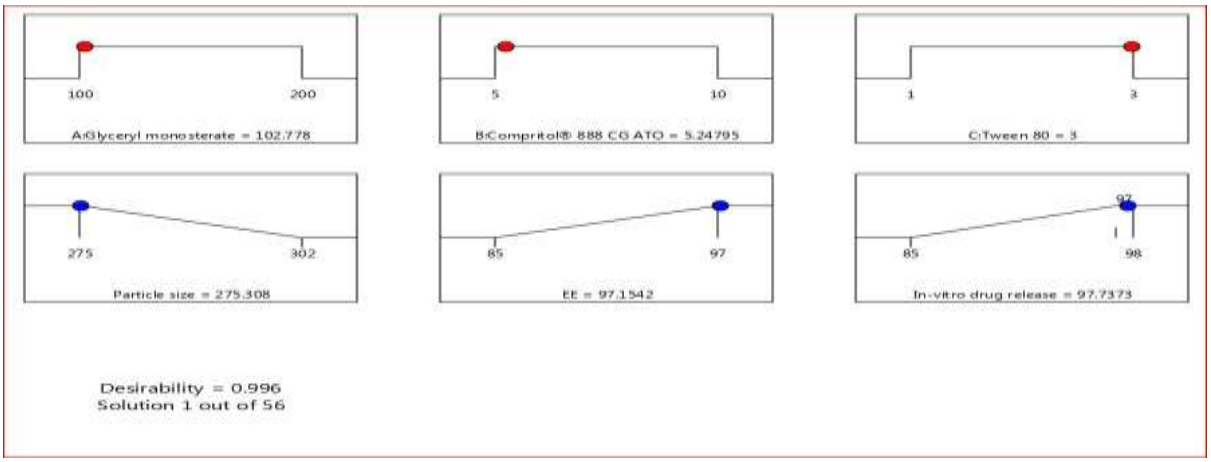

Figure 9: Independent Variable Components with Highest Desirability

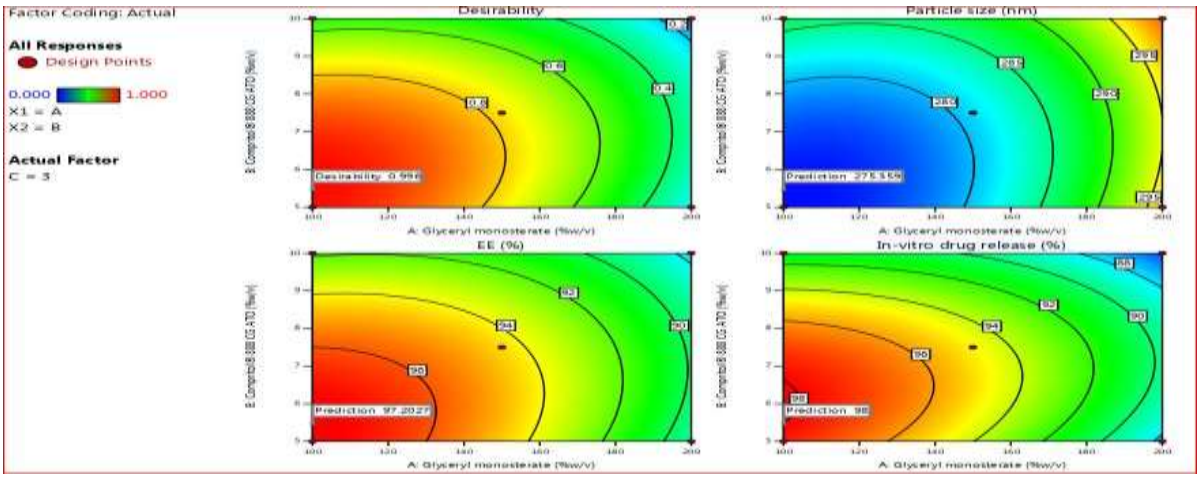

Figure 10: Desirability Plot for Particle Size, Entrapment Efficiency (%)and In-vitro drug release

In light of the results, the desirability of all solutions (56) was compared graphically using numerical optimization with software. This approach offered potential solutions for optimizing the Clozapine-loaded SLN's batch. The information was illustrated through desirability and overlay plots, showcased in Figures 12 and 13. Notably, from the desirability plot, it was evident that the percentage weight composition of glyceryl monosterate 102.78, Compritol® 8882.50 was 5.247 and Tween (80) was 3stood out as the most desirable formulation, given its highest desirability value of 0.996, highlighted by a yellow circle and composition of each i variables of present study were depicted in Figure 11.

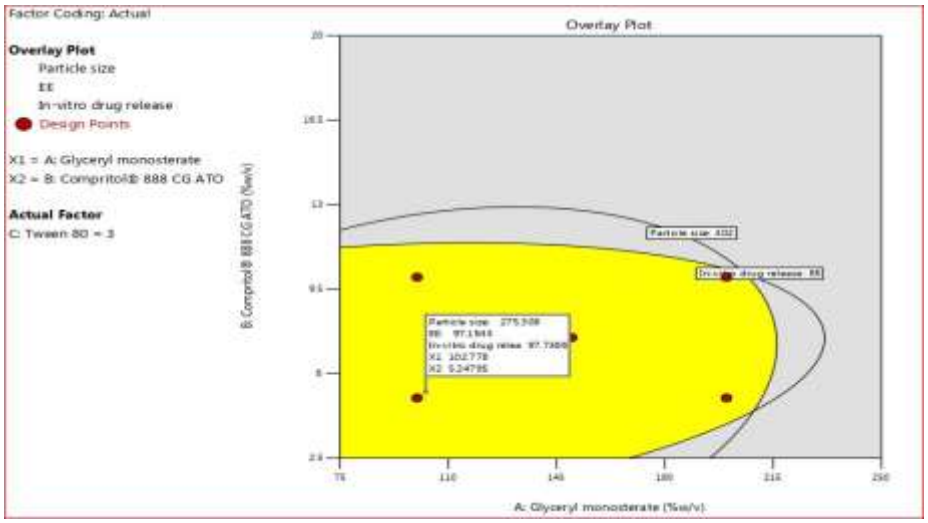

Figure11- Overlay plot for optimized formulation

***In vitro* Drug release studies**

This present work, aimed to develop a formulation capable of sustaining the release of Clozapine over a 24-hour period. The optimized Clozapine-loaded solid lipid nanoparticles (SLN's) were evaluated under specified conditions using 6.8 phosphate buffer as the medium for dissolution. This, release study demonstrated that the formulated SLN's successfully achieved this objective, exhibiting a remarkable drug release of 98.06±0.14 percent within the designated timeframe. Such an extended-release profile holds significant clinical value, particularly for drugs necessitating once-daily dosing regimens. This prolonged-release feature not only enhances patient convenience but also ensures the maintenance of therapeutic efficacy throughout the treatment duration.

Initially, during the first 4 hours, the release rate is relatively slow, ranging from 5.1±0.13 to 26.14±0.18 percentage, as Clozapine molecules diffuse through the lipid matrix. This gradual release is attributed to the sustained-release properties of stearic acid, which moderates the drug release, keeping it at a moderate level.

However, as time progresses, particularly beyond 6 hours, there is a notable increase in the release rate, reaching 48.18±0.13percentage. This acceleration in release is indicative of the onset of lipid matrix erosion or degradation. The gradual breakdown of the lipid matrix allows for a more rapid and sustained release of Clozapine over extended periods, contributing to the observed sustained release profile.

Between the 6 to 12-hour time intervals, the percentage of drug release escalated from 48.18±0.13to 77.14±1.02. During this period, the erosion or degradation of glyceryl monosterate intensified, leading to a consistent rise in drug release. As the lipid matrix continued to break down, more drug molecules became exposed to the dissolution medium, further contributing to the increasing drug release.

From 12 hours to 24 hours, the drug release increased from 77.14±1.02to98.06±0.14. This gradual progression release of drug in specified time intervals influenced by several factors. These factors include decreased surface area, increased lipid matrix density, diffusion resistance, limited solubility of Clozapine, and potential polymerization or cross-linking of lipid matrix components. Together, these factors contribute to the sustained and controlled release of Clozapine over the extended release period, ultimately achieving the desired 24-hour release profile.

In conclusion, the developed Clozapine SLN formulation shows promise for continuous Clozapine release, supported by robust in vitro release profiles and favorable physicochemical characteristics. This formulation holds potential for enhancing patient compliance and therapeutic outcomes in conditions requiring prolonged drug delivery. All results were summarized in Figure 14.

Release order kinetics

The release orders for the Clozapine-SLN’s optimized formulation were assessed using the DD Solver model software. This software served as a powerful tool for deciphering the intricate release patterns. By utilizing a range of mathematical models, the study yielded insightful results were shown in Table 05.

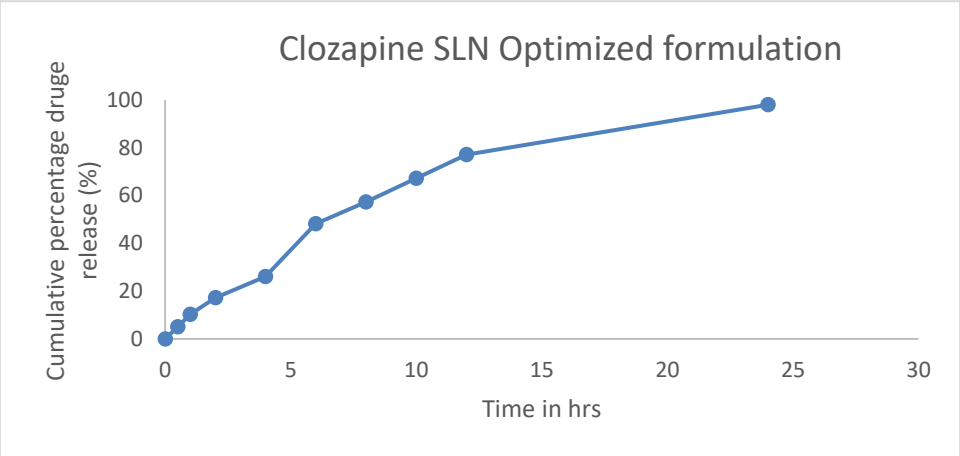

Figure: 12 *In-vitro* release profile of Clozapine-SLN's optimized formulation

From the zero-order model, the optimized formulation exhibited a notable r^2 value of 0.996, indicating a robust correlation between the model and the observed data. Correspondingly, the AIC value was calculated as 29.14, while the MSC value reached 6.214. In the context of first-order studies, the model-optimized formulation resulted in a higher r^2 value of 0.962, confirming the model's strong fit. The recorded AIC value was 40.27, and the MSC value stood at 3.214.

Shifting focus to the Higuchi Model, selected formulation demonstrated an r^2 value of 0.979, signifying the model's excellent alignment with the experimental data. The AIC value was determined as 30.25, while the MSC value reached 5.270. Notably, the inclusion of Hixon-Crowell release order analyses revealed that for formulation F1, the r^2 value was 0.964, AIC value was 3519, and MSC value was 4.517.

Extending the investigation, the Korsmeyer–Peppas equation provided deeper insights into optimized formulation. The model suggested an r^2 value of 0.985, in strong agreement with the data. The calculated AIC value was 36.27, while the MSC value was 5.142. Notably, the exponent release (n) value stood at 0.657, indicating a Non- Fickian diffusion-type mechanism governing the release.

Table 05: Summary Data on Release Kinetics of Optimized Clozapine-SLNs from DD Solver Software

Release order parameters	Zero-order (k0)	First order (k1)	Higuchi model (kH)	Hixon-Crowell (kHC)	Korsmeyer-Peppas (kKP)	n

Adjusted r^2	0.997	0.962	0.979	0.964	0.985	0.657
r^2	0.996	0.962	0.979	0.964	0.985	
AIC	29.14	40.27	30.25	35.19	36.27	
MSC	6.214	3.241	5.270	4.517	5.142	

Where, r^2 is regression co-efficient, Akaike Information Criterion (AIC) and Model selection criterion etc.

Physical Stability Studies of optimized formulation during Storage.

Clozapine-loaded solid lipid nanoparticles were stored for 60 days at both room temperature (25°C/60 ± 5% RH) and refrigerated temperature (4°C).The polydispersity index, zeta potential, average size, and entrapment efficiency (%) were measured.

Stability studies were performed for the optimized formulation, exhibiting improved size, PDI, zeta potential, and EE. Triplicate samples were analyzed, and the results are summarized in Table 06. The statistical t-test was applied, revealing a significant change only in the size of SLNs, with no notable differences observed in EE, PDI, and ZP throughout the storage period. Consequently, the optimized SLN preparation remained stable for a two-month period at both room temperature (RT) and 4°C.

Table 06: Stability studies of Optimized Clozapine-loaded SLNs under varied storage conditions

At room temperature (27°C)					At refrigerator (4°C)			
Time period	Particle size in (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)	Particle size in nm	PDI	Zeta potential (mV)	Entrapment efficiency (%)
Initial	275±0.14	0.251±0.25	−19.21±0.19	97.54±0.25	275±0.14	0.251±0.25	−19.21±0.19	97.54±0.25
I^{st} month	276±0.21	0.263±0.17	−20.25±0.34	96.35±0.34	275±0.11	0.268±0.33	−21.07±0.12	96.04±0.10
II^{nd} month	277.23±0.82	0.278±0.07	−21.44±0.07	95.08±0.21	276±0.21	0.269±0.11	−22.31±0.17	95.17±0.18

The statistical analysis of the data was conducted using an unpaired t-test in Excel (version 2013), with significance set at a *p-value of 0.05*. A significant difference was observed in the size of the solid lipid nanoparticles (SLN's) *(p = 0.69)*. However, no significant differences were found in terms of polydispersity Index (PDI), zeta potential, and entrapment efficiency (EE) during storage on the 1st, 30^{th}, and 60^{th} days.

CONCLUSION

The study successfully designed and optimized Clozapine-loaded solid lipid nanoparticles (SLN's) for potential Anti-psychotic (schizophrenia) therapy. The SLN's were prepared through high-pressure homogenization followed by ultra-sonication, with formulation optimization could done with central composite design type and modelresponse surface methodology. The aim was to develop a cost-effective, biodegradable, and stable nanocarrier with enhanced drug entrapment and prolong release over 24 hours. The optimized formulation exhibited a particle size of 275±0.14nm, within the optimal range for oral delivery. Additionally, it demonstrated high entrapment efficiency (97.54±0.25%) and a extended drug release profile, with an initial burst followed by prolonged release (98.06±0.14%). The release kinetics followed a zero-order equation, suggesting a Non-Fickian diffusion-type mechanism with an exponent diffusional coefficient (n) value of 0.657. These results underscore the potential of SLNs as an effective delivery system for Clozapine, offering improved treatment outcomes for schizophrenia actions. Further, In vivo studies and clinical trials are warranted to validate these promising *In vitro* findings and establish the clinical efficacy and safety of Clozapine-loaded SLNs.

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