Design, Development and Evaluation of Clozapine Loaded Mucoadhesive Microspheres using Natural Crosslinker for Brain Drug Delivery

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ABSTRACT:

Schizophrenia is a severe and most dangerous form of a mental illness. About 21 million people affected by this mental disorder worldwide. Proportion of Male to Female schizophrenia affected patients are 12 million to 9 million respectively. The chronic nature of schizophrenia revealed by some key characteristics such as disturbance in thought process, mental imbalance and emotional dysregulation[1]. Conventional dosage forms and treatments are available for schizophrenia but it was found that patients require high standard care during the treatment.

Keywords: Proportion, patients, require, affected

Introduction:

Schizophrenia is a severe and most dangerous form of a mental illness. About 21 million people affected by this mental disorder worldwide. Proportion of Male to Female schizophrenia affected patients are 12 million to 9 million respectively. The chronic nature of schizophrenia revealed by some key characteristics such as disturbance in thought process, mental imbalance and emotional dysregulation[1]. Conventional dosage forms and treatments are available for schizophrenia but it was found that patients require high standard care during the treatment.

A tricyclic dibenzodiazepine are also known as atypical antipsychotic agents. It binds with the various types of receptors and execute its pharmacological effect. The treatment of schizophrenia involves antipsychotic agents of two types i.e., first generation (typical) and second generation (atypical) medications. Typical agents include haloperidol, chlorpromazine and atypical antipsychotic agents includes olanzapine, clozapine, risperidone, aripiprazole etc. Second generation or atypical agents are more effective that first

generation agents to decrease relapse rate and treating the positive and negative symptoms of schizophrenia[2].

Clozapine is most commonly used to counteract the treatment resistant schizophrenia (TRS). It helps to reduce the suicidal behavior in patients who not responding to treatment of other drugs. Clozapine is superior in managing the both positive and negative symptoms in schizophrenic patients. Clozapine is of BCS class category showing high permeability and low solubility[3]. Poor solubility affects the absorption of drug via oral route. It shows poor drug bioavailability (<27%) due to hepatic first pass metabolism and delayed onset of action. Clozapine can be combined with other drugs as per the requirement of patient. For oral route of administration 100mg tablets of clozapine are available in the market, but these are strictly restricted and advised to take only when the patients not responding to other antipsychotic agents. There are some side effects associated with oral administration of clozapine such as weakness, constipation, nausea, cough, cold, bleeding, pale or yellowing skin, muscle stiffness, sedation, tremor etc[4]. Hence there is a need to develop a medication which can be administered by route other than enteral. On the other hand, intramuscular route also has some disadvantages. Intramuscular route is invasive and painful for drug delivery also there is a risk of inflammation and infection at the site of application. Intramuscular route requires simultaneous administration of oral administration of antipsychotic drugs for maintaining plasma drug concentration. By considering all these limitations and drawbacks associated with administration of antipsychotic drugs there is a need to develop newer dosage forms that can surpass all these barriers and easily delivers the drug at brain[5]. As a researcher we have to focus on improving the solubility of clozapine and its bioavailability also.

Intranasal route for drug delivery has gained specific attention because of its non-invasiveness. Intranasal route is most popular one and used for both local and systemic effect. Most commonly it is used for brain drug delivery. Intranasal route surpasses the blood-brain-barrier and able to target the central nervous system via olfactory and trigeminal nerve pathway. The highly vascularized nasal mucosa facilitates the drug absorption. Intranasal route offers rapid onset of action by avoiding hepatic first pass metabolism which can be occurred via oral route. Also eliminates the necessity of painful injections[6]. With this overview by considering drawbacks of oral route and limitations of intramuscular route we focused on development of intranasal drug delivery for clozapine. Nasal cavity has about 160cm^2 surface area largely extended by turbinate structure which serves as a surface for drug deposition and absorption[7].

In this research, we have developed mucoadhesive microspheres of clozapine by using natural excipients in order to avoid nasal irritation. Starch is biodegradable polymer. Its rigidity can be enhanced by crosslinking technique. Also, the degree of polymerization and molecular mass can be increased by crosslinking method. Crosslinked polymers lose its water solubility and thus become soluble in organic solvent. The hydroxyl groups of polysaccharides can be crosslinked with carboxylic acidic group. Citric acid is used as a natural agent for the crosslinking with starch. Citric acid has power to react with one or more hydroxyl groups of starch. Citric acid is GRAS agent and harmless to be used for nasal drug delivery. This crosslinking of starch with citric acid results in improved thermal stability and fluidity due to the formation of strong hydrogen bond in between starch and citric acid[8]. The objective of this research is to develop mucoadhesive microspheres of clozapine for intranasal administration using natural polymer starch which will be crosslinked with natural agent i.e., citric acid by single emulsion crosslinking technique.

Materials & Methods:

Clozapine was obtained as a gift sample from Om Sai Pharmaceuticals Limited, India. Starch, Citric acid and soluble starch were procured from Loba Chemicals, India. All other excipients

and solvents used in the experiment were of analytical grade.

A. Screening of excipients:

1. Cross-linking agent:

The microspheres of natural polymers are prepared by single emulsion crosslinking technique[9]. The natural polymers are dissolved in aqueous medium followed by dispersion in the non-aqueous medium (example- oil). In the second step, cross linking of the dispersed globule is carried out either by using chemical cross linkers. The chemical cross-linking agents used are gluteraldehyde, formaldehyde, sodium tri-metaphosphate, terephthaloyl chloride and citric acid etc[10]. By considering bio-safety, loading capacity, cross-linking time and temperature, suitable cross-linker was selected.

2. External phase of w/o emulsion:

Different organic phases (liquid paraffin, chloroform, acetone, cyclohexane) were tried for the preparation of w/o emulsion. Selection of external organic phase was done based on free-flowing nature of microspheres.

3. Surfactant:

There are different types of non-ionic surfactants such as different grades of tweens and spans. Depending upon their hydrophilicity, hydrophobicity and HLB value, suitable surfactant was chosen for the preparation of W/O single emulsion.

B. Preparation of clozapine loaded crosslinked starch microspheres (CSMs):

Clozapine loaded CSMs were prepared by single emulsion crosslinking method. Briefly, aqueous phase prepared by dissolving starch in water and heated to 60°C to form a gel and then it is allowed to cool at room temperature. Drug was added into the gel matrix. Drug loaded gel matrix was added drop wise using syringe into the external organic phase containing span 80 as a surfactant under constant mechanical stirring and temperature of the system was maintained about 40°C to form w/o emulsion. After complete homogenization of w/o emulsion, 1ml of citric acid solution as a cross-linker was added drop wise. Organic phase was allowed to evaporate completely and obtained microspheres were dried by lyophilisation technique and further characterized.

To optimize formulation variables employed in formulation of CSMs, Taguchi orthogonal array design was applied.

C. Optimization using Taguchi design:

The Taguchi orthogonal array (L9) design approach was used to study the effect of various formulation parameters on formation of CSMs. Statistical validity of the polynomials was established based on one-way ANOVA provisions of the Minitab software. To study the effect of different variables on the response, main effect plots were generated using the Minitab software.

D. Characterization of CSM's:

1. Particle size:

Particle size was measured by laser diffraction particle size analyzer (Malvern Mastersizer 2000 SM version 5.22, Malvern Instruments Corp., U.K) using dry assembly. The particle size was reported as d (0.9). The particle size distribution was also expressed in terms of SPAN factor determined as:

$$\mathbf{SPAN = d_{90} - d_{10} / d_{50}}$$

Where,

d_{10}, d_{50} and d_{90} are the diameter sizes and the given percentage value is the percentage of particles smaller than that size. A high SPAN value is indicative of a wide size distribution[13].

2. Scanning Electron Microscopy (SEM)

The SEM of pure CLZ & the optimized CSMs batch F7 sample were performed to investigate the surface morphology and homogeneity of the particles. Both pure drug & optimized microsphere sample was Sutter-coated with thin layer of platinum at room temperature prior examination to render the surface of particle electro conductive. The SEM analysis of the samples was done by Energy dispersive

spectrometer (JEOL JSM- 6360A) scanning electron microscope.

3. FTIR Study:

Compatibility between drug and excipients was studied using FTIR spectra. Samples were mixed with KBr in the ratio 9:1. FTIR spectra of pure CLZ, starch and CLZ loaded CSMs were scanned using JASCO V4100 FTIT (Tokyo, Japan) in transmission mode over wavenumber range of 4000-400 cm^{-1}.

4. Powder X-Ray diffraction (PXRD)

The powder X-ray diffraction patterns of pure CLZ, starch and optimized lyophilized CSMs (Batch F7) were recorded using an X-ray diffractometer (PW 1729, Philips, Netherlands), operated at a voltage of 40 kV and a current of 40 mA. The samples were exposed to a Cu- Kα radiation over a range of 2θ angles from 2 to 60°.

5. Differential Scanning Calorimetry (DSC)

The DSC thermograms of CLZ, starch and lyophilised CSMs of optimized batch (F7) were recorded in nitrogen environment using a METTLER TOLEDO Star 821e instrument with an intercooler (METTLER-Toledo, GmbH, Switzerland)[9]. The samples were accurately weighed (5–10 mg), hermetically sealed in aluminium pans and heated at a constant rate of 10 °C/min over a temperature range of 25- 300°C.

E. Evaluation of CSM's:

1. Percent Entrapment Efficiency:

CSMs containing appx. 5mg CLZ were accurately weighed and dissolved in 5ml of methanol. Shaken for 24 hours on rotary shaker at ambient temperature and then centrifuged at 10,000rpm for 15 min. at 25°C. The supernatant was filtered, further diluted using phosphate buffer pH 7.4 and drug content of this solution was determined using UV spectrophotometer at 259nm.

Entrapment efficiency (% EE) was calculated as in the following formula:

$$\% \text{ Entrapment Efficiency} = \frac{\text{Actual Wt. of CLZ in CSMs (mg)}}{\text{Theoretical Wt. of CLZ in CSMs (mg)}} * 100$$

2. % Mucoadhesive strength:

Falling Film technique used to determine percent mucoadhesive strength of prepared CSM's. Piece of nasal mucosa mounted on a slide which is tilted slightly at an angle appx. 45C. 100mg of CSM's spread on this mucosal surface. Effluent fluid was allowed to pass over the surface.

Effluent was collected on whatman filter paper and detached particles weighed. Percent Mucoadhesive strength was determined using following formula:

$$\% \text{ Mucoadhesion} = \frac{\text{Weight of CSMs Taken} - \text{Weights of Detached CSMs}}{\text{Weight of CSMs taken}} * 100$$

3. Percent yield:

Total amounts of microspheres obtained after lyophilisation were weighed and the percentage yield was calculated taking into consideration the weight of drug and polymer.

4. Equilibrium Swelling Degree

The equilibrium swelling degree (ESD) of CSMs was determined by swelling 5mg dried microspheres in 5ml phosphate buffer pH 7.4, overnight in a measuring cylinder. The ESD (mg/ml) was expressed as the ratio of the swollen volume to the mass of dried microspheres[14].

5. Surface pH:

Enough quantity of microspheres was taken on glass slide and moistened with appx. 1ml of water. Electrode was placed at the surface of microspheres and pH was recorded in triplicate and mean was calculated.

6. In-vitro drug release study:

Drug release was studied by using Franz diffusion cells (using artificial cellophane membrane) (Membra – Cel MD 34-14, cut-off 14kD). For this experiment a vertical Franz

diffusion cell having a surface area of 2.54 cm^2 and a reservoir capacity of 22 ml was used. The artificial membrane was securely placed between the two halves of the diffusion cell. The receptor fluid consisted of a mixture of phosphate buffer pH 7.4; its temperature maintained at 37 ± 1°C and stirred continuously using magnetic stirrer. CSMs equivalent to 5mg of CLZ were placed in a donor compartment over the membrane and covered with parafilm. At different time intervals from 0 to 24 hours, aliquot (2 ml) of release medium was withdrawn through a hypodermic syringe and filtered through a millipore filter (pore size 0.8µm) and replaced with the same volume of pre-wormed fresh buffer solution. The drug content in the withdrawn samples was determined UV spectroscopy at λmax 259 nm using a pre-constructed calibration curve[15].

7. Stability Study:

Stability studies were conducted to test the physical and chemical stability of the optimized CSMs batch at 25°C ± 60% RH and 40°C ± 75 % RH for 3 months. CSMs sample was characterized for particle size and drug entrapment at 1, 2, and 3month time intervals.

Result and Discussion:

A. Screening of excipients

1. Crosslinking agent:

Starch is a natural biodegradable polymer and it can be crosslinked with various crosslinking agents. In this experiment, starch microspheres crosslinked with three different crosslinkers such as epichlorhydrin, formaldehyde and citric acid (CA) etc.

Table 1: Screening of crosslinking agent.

Cross-linker	Drug Loading Capacity
Epichlorohydrin	62 %
Formaldehyde	77 %
Citric acid	85 %

These crosslinkers exhibited different loading capacity within 30 min at 40°C as shown in Table 1.

Starch can be cross-linked with different cross-linking agents such as epichlorhydrin, formaldehyde, glutaldehyde, sodium trimetaphosphate, terephthaloyl chloride and citric acid (CA) etc. Recently, CA has been proven convenient for crosslinking[15]. It is non-toxic, naturally occurring and hence, generally classified as a safe (GRAS) for application in pharmaceuticals.

As compared to other cross-linkers, CA cross-linked with starch at low temperature[16] showing higher loading capacity. Even if extra amount of CA remained in the system, then also it is nutritionally safe and it will act as a plasticizer for starch. By considering advantages of CA from the literature, CA was tried as a cross-linker for further development of CSMs.

2. Screening of External Phase:

Selection of external phase was done by achieving free flowing nature of microspheres. Table 2 shows the nature of microspheres using different external phases. Free flowing microspheres were obtained using chloroform, since it gets evaporated readily when meets air.

Table 2: Nature of Microspheres using different External Phases

Sr. No.	External Phase	Nature of CSM's
1	Liquid paraffine	Sticky powder

2	Cyclohexane	Yellow colored powder
3	Acetone	Not free flowing powder
4	Chloroform	Free flowing powder

Screening of surfactant:

Hydrophilic nature of Tweens makes it suitable for O/W type of emulsion[17]; hence hydrophobic spans were chosen for W/O type of emulsion. Single W/O emulsion was prepared using Span 80 having HLB 4.3 since its unsaturated oleyl chain has greater affinity for non-polar solvents.

B. Preparation of clozapine loaded crosslinked starch microspheres (CSMs):

Single emulsion crosslinking method is used to prepare CSM's. Initially blank CSM's (without drug) were prepared. While preparing drug loaded CSM's various factors influencing the formation of CSM's such as particle size and entrapment efficiency were identified and optimized using Taguchi Design. Optimization study reveals that, 10% polymer concentration, 0.2% surfactant and 6% cross-linker concentration results in formation of CLZ loaded CSM's having desired particle size (>10μm).

For the development of CLZ loaded CSM's 15% W/V starch was used as polymeric aqueous phase with 20mg of CLZ and chloroform used as organic phase. 0.2% of span 80 used as surfactant followed by addition of citric acid 6% W/V as a crosslinker. The temperature of system was maintained at 40°C.

C.
ptimization using Taguchi Design:

Taguchi orthogonal array (L9) design was employed to study the effect of various formulation (independent) variables such as polymer concentration (X1), surfactant concentration (X2) and cross-linker concentration (X3) on response variables i. e., entrapment efficiency (Y1) and mucoadhesive strength (Y2).

Table 3: S/N ratios obtained for responses for each experimental batch.

Batch	Y1 (%)	S/N ratio for Y1	Y2 (%)	S/N ratio for Y2
F1	48.56	33.72	47.53	33.82
F2	52.03	34.32	55.09	34.82
F3	56.45	35.03	59.87	35.54
F4	64.30	36.16	68.09	36.66
F5	68.55	36.72	79.94	38.05
F6	72.34	37.18	73.87	37.36
F7	**92.45**	**39.31**	**93.46**	**39.41**
F8	85.04	38.59	85.96	38.68
F9	88.95	38.98	87.48	38.88

Total 9 batches of CLZ loaded CSMs were prepared using three independent variables at three levels (-1, 0, +1). The results obtained for all the batches were summarized in Table 3. 'Larger-the-better' S/N ratio was by using Minitab software (version 15). The level of independent variable showing larger S/N ratio was considered as an optimized level. To analyse the data main effect plots were generated for both the response variables.

Characterization of CSM's"

Particle Size:

CSM's prepared by all the batches was found to be in the range of 58-92µm. All microspheres had low SPAN values indicating a narrow particle size distribution. These results indicate that prepared microspheres are suitable for deposition in the nasal cavity without the risk of passing to the lower respiratory tract.

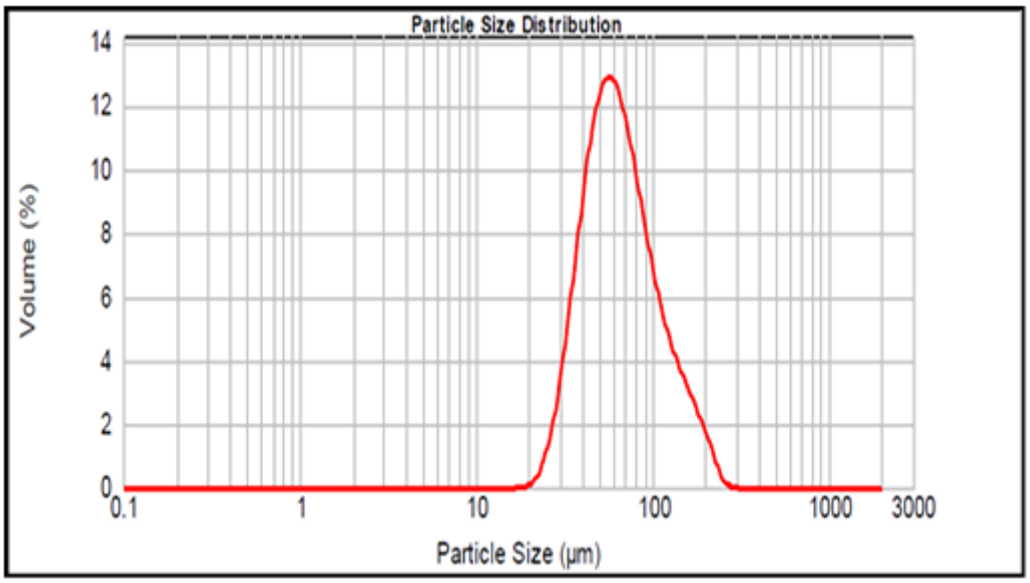

Figure 1: Particle Size Distribution of CSMs.

An optimum diameter for nasal microspheres was reported to be in the range of 10-250µm. It was observed that on increasing concentration of polymer particle size increases proportionally since amount of polymer in droplet and thus viscosity of droplet increased .

Scanning Electron Microscopy:

The surface morphology of CSMs captured under scanning electron microscope revealed spherical shaped particles with smooth surface as depicted in Figure 2.

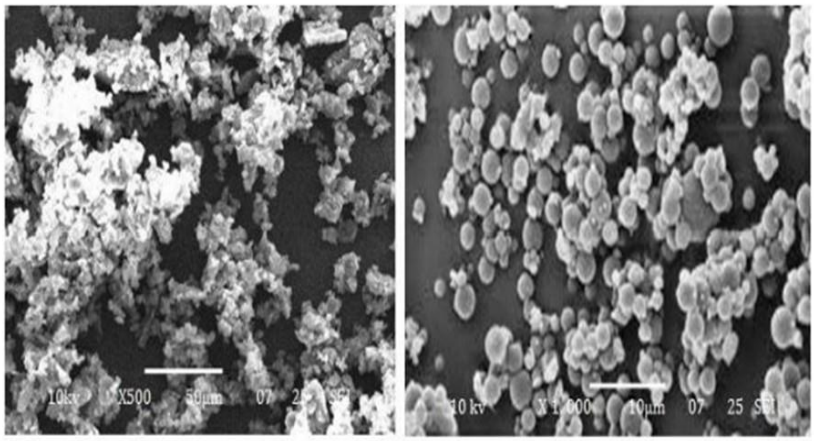

Figure 2: Surface Morphology of Pure drug clozapine and Optimized F7 Batch

1. FTIR:

FTIR spectra of CLZ, starch and lyophilized CSMs of optimized batch F7 were examined and are depicted in Figure 3.

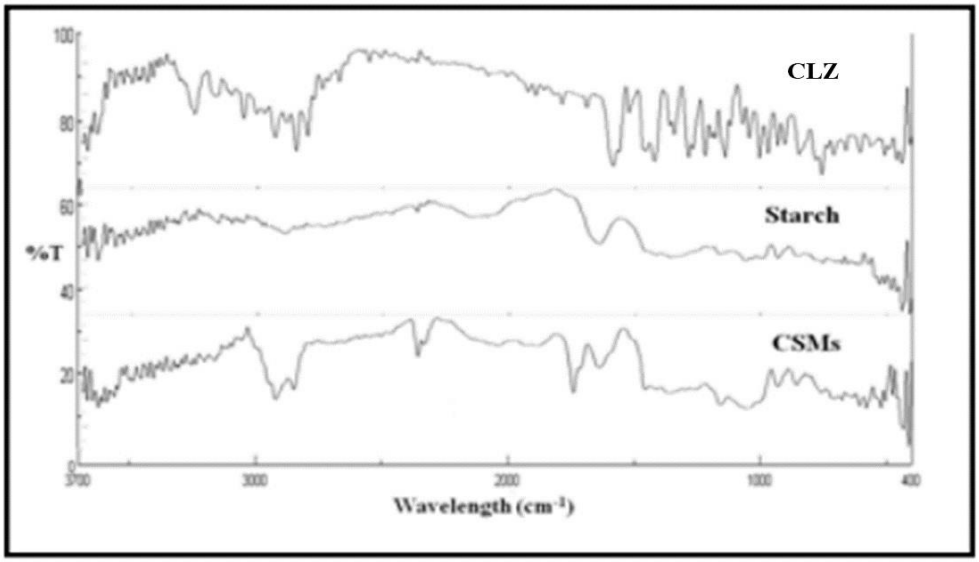

Figure 3: FTIR spectra of Clozapine, Starch and Batch F7

The FTIR spectrum of CLZ showed the characteristic peaks of aromatic tertiary alkyl amine N-CH3 at 1221 cm^{-1}, thiophene ring at 1587 cm^{-1}, C-CH3 bending at 1344 cm^{-1}, piperazine ring stretch at 3247 cm^{-1} and aromatic CH=CH at 1000-650 cm^{-1}. For starch IR absorption peaks were recorded. In the IR spectra of F7 batch shows characteristic ester peak at 1746 cm1 which confirms the starch had esterifies with citric acid successfully. The intensity of the CLZ peak however decreased in the F7 and this may be due to the drug being molecularly dispersed in the polymeric matrix.

2. Powder X-Ray diffraction (PXRD):

The physical state of CLZ incorporated in microsphere was identified by XRPD analysis. The X-ray diffraction patterns of CLZ, starch and lyophilized CSMs of F7 batch were displayed in Figure 4. No sharp peaks, characteristic of crystalline nature of CLZ were observed in lyophilized CSMs powder. Diffractogram of lyophilized CSMs revealed broad predominated but low intensity peaks which can possibly be attributed to the encapsulation of CLZ in polymer matrix. Intensity of sharp peaks of CLZ was reduced and also some of the peaks were disappeared in the CSMs diffractogram which suggested that CLZ was in amorphous form in the CSMs formulation.

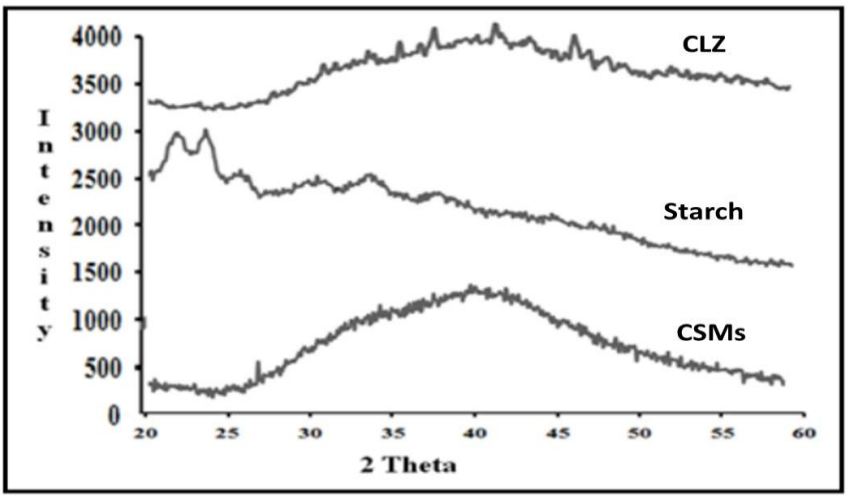

Figure 4: XRPD Study of Clozapine, Starch and Batch F7

Differential Scanning Colorimetry (DSC)

DSC thermogram of pure CLZ, starch and lyophilized CSMs batch F7 w depicted in Figure 5. DSC thermogram of CLZ and starch revealed endothermic peaks at 185.05°C and 278°C, corresponding to melting of CLZ and starch, respectively. Melting endotherm of the CLZ disappeared completely in F7 indicating that CLZ in starch was in amorphous state to form solid solution within the matrix of microspheres. Lyophilized batch F7 shows broad endothermic peak at 150°C which may be due to formation of starch citrate. For polymeric materials in general, Tg has been shown to increase with increasing molecular weight and crystallinity and to decrease with increasing degree of branching and flexibility of the chains which occurs on modification of starch. However, cross-linking has been shown to only slightly affect the Tg of starch. Addition of citric acid results in reduction of Tg of starch.

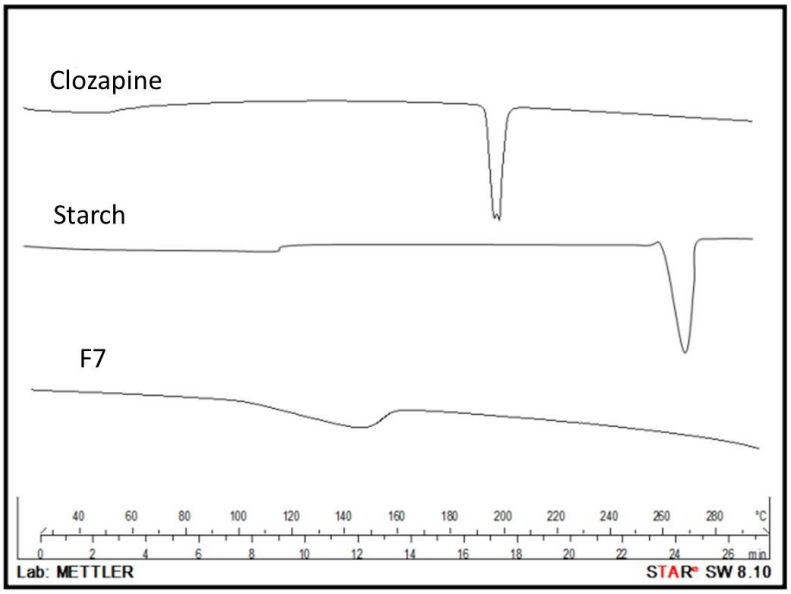

Figure 5: DSC thermogram of pure CLZ, starch and lyophilized CSMs batch F7

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Evaluation of Clozapine loaded CSMs:

1. Percent Entrapment Efficiency (%EE):

Entrapment efficiency is the percentage of the actual mass of drug entrapped in the polymeric matrix, relative to the initial amount of loaded drugs. The EE of CLZ in CSMs was found to be more than 90 %. Effect of independent variables on % EE was studied. The S/N response table for % EE was shown in Table 4.

Table 4: S/N response table for % EE (larger-is-better)

Level	Polymer	Surfactant	Cross-linker
1	34.36	36.40	36.50
2	36.69	36.55	36.49
3	38.96	37.07	37.02
Delta	4.60	0.67	0.53
Rank	1	2	3

In this table the average S/N ratio of each independent variable at each level and delta was computed along with the rank. Delta value is nothing but the difference between maximum and minimum S/N ratios, factor having higher delta value indicates it has more significant effect on % EE and according to that rank was given.

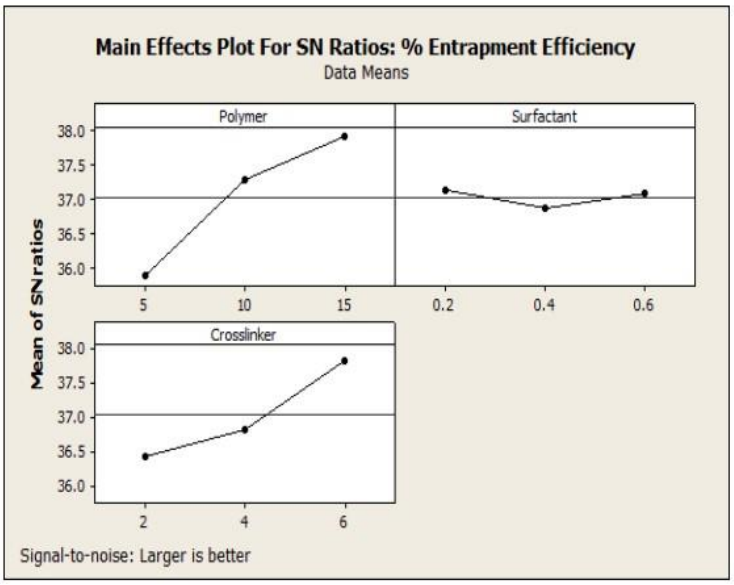

Figure 6: Effect of independent variables on average S/N ratios of % EE.

The factor polymer showing higher delta value (4.60) had more significant effect on % EE with first rank indicating greater influence than surfactant and cross-linker which was having second and third ranks respectively reveals that apart from polymer, surfactant affects secondly on % EE however, cross-linker had very least influence on % EE that means polymer and surfactant plays vital role in increasing % EE[18]. The optimum levels of independent variables for the preparation of CSMs was found to be polymer at level 3, surfactant at level 1, and cross-linker at level 3.

Table 5 shows the computed results of ANOVA with 95% confidence level. The values of fisher's ratio (F-ratio) indicate the percent contribution of formulation variable in the preparation of CSMs. The P value less than 0.05 indicate that the model terms are significant[21].

Table 5 ANOVA for %EE reveals that polymer concentration had significant effect on % EE. The 'P' value for polymer, surfactant and cross-linker was found to be 0.013, 0.441 and 0.458 respectively, elucidates that the polymer had most significant effect on CSMs and R^2 was found to be 0.9874.

Table 5: Analysis of variance (ANOVA) for % EE

Source	DF	Seq. SS	Seq. MS	F-ratio	P
Polymer	2	2004.26	1002.13	75.95	0.013*****
Surfactant	2	33.50	16.75	1.27	0.441
Crosslinker	2	31.22	15.61	1.18	0.458
Error	2	26.39	13.20	-	-
Total	8	2095.38	-	-	-
S=3.632 **R^2 = 0.9874*** R^2 (adj.) = 0.9496 R^2 (pred.) = 0.7450.					
*Significant at 95% confidence level					

A strong positive coefficient was observed for X_1 (Polymer concentration; $p < 0.05$) as compared to X_2 and X_3 ($p > 0.05$), which reveals that increase in concentration of polymer % EE increases, since there is increased polymeric environment made available for more

encapsulation. Also, the positive coefficient of cross-linker reveals that increase in amount of cross-linker leads to increase in degree of cross-linking which prevents leaching of drug out of the polymeric matrix contributed to increase in % EE. Surfactant had negative influence on % EE. As the concentration of surfactant increases % EE decreases. Due to hydrophobic nature of surfactant, it has higher affinity towards lipophilic drug molecule (CLZ has log P - 3) results in solubilization of drug in surfactant layer results in decrease in encapsulation efficiency.

2.
ercent Mucoadhesive Strength:

Mucoadhesive strength can be defined as the potency of adhesive bonds between two materials[22]. The % mucoadhesion of CLZ loaded CSMs were found to be more than 90 %. Effect of independent variables on % mucoadhesion was studied.

Table 6: S/N response table for % mucoadhesion (larger-is-better)

Level	Polymer	Surfactant	Cross-linker
1	34.63	36.54	36.53
2	37.36	37.19	36.79
3	39.00	37.27	37.67
Delta	4.37	0.73	1.14
Rank	1	3	2

The S/N response table for % mucoadhesion was shown in Table 6. The factor polymer showing higher delta value (4.37) with first rank had more influence on % mucoadhesion than surfactant and cross-linker which was having third and second ranks respectively which indicates that except polymer concentration, cross-linker had more influence on % mucoadesion whereas surfactant had negligible influence on % mucoadhesion.

Figure 7 displays optimal levels of independent variables along with average S/N ratios for each variable. The optimum levels of formulation variables for the preparation of CSMs was found to be polymer at level 3, surfactant at level 1, and cross-linker at level 3.

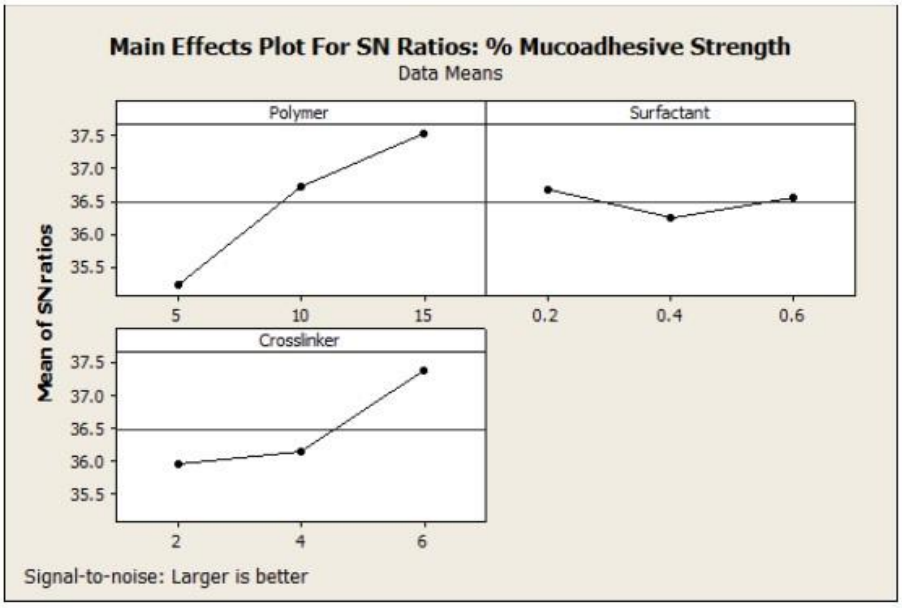

Figure 7: Effect of independent variables on average S/N ratios of % mucoadhesion.

ANOVA for % mucoadhesion was shown in Table 7, which reveals that the polymer concentration and cross-linker concentration had significant effect on % mucoadhesion. The P value for polymer, surfactant and cross-linker was found to be 0.007, 0.290 and 0.045 respectively, elucidates that the polymer and cross-linker had most significant effect on CSMs with $p < 0.05$ than surfactant concentration ($p>0.05$) and R^2 was found to be 0.9933 which was statistically significant at 95 % confidence level. The % mucoadhesion of CSMs ranged from 47.53 % to 93.46 % as shown in Table 3.

Table 7: Analysis of variance (ANOVA) for % Mucoadhesion.

Source	DF	Seq. SS	Seq. MS	F-ratio	P
Polymer	2	1845.10	922.55	134.82	0.007*
Surfactant	2	33.57	16.786	2.45	0.290
Crosslinker	2	130.51	65.257	9.54	0.045*
Error	2	13.69	6.843	-	-
Total	8	2022.87	-	-	-
S= 2.615 R^2 = 0.9933 R^2 (adj.) = 0.9729 R^2 (pred.) = 0.8630.					
*Significant at 95% confidence level					

A strong positive coefficient was observed for X_1 and X_3 ($P < 0.05$), as compared to X_2 ($P > 0.05$), which reveals that increase in concentration of polymer % mucoadhesion increases, since polymer itself has mucoadhesive properties.

The positive coefficient of cross-linker reveals that increase in amount of cross-linker leads to enhanced esterification reaction forming more ester linkages between starch and citric acid which may leads to produce strong cationic coat of starch citrate on drug particle, which has higher affinity for anionic mucosal surface results in strong electrostatic interaction between them contributed to increase in % mucoadhesion. Surfactant had negative influence on % mucoadhesion. As the concentration of surfactant increases % mucoadhesion decreases. Surfactant itself has higher affinity towards negatively charged mucoasal surface hence, on increase in concentration of surfactant it will hinder the adhesion between polymer and mucosal surface by adhering itself to mucosal surface results in decrease in mucoadhesion of CSMs.

3.

ercent Yield, Drug Content and loading Capacity of CSMs:

Percent yield, drug content and loading capacity of all the formulation batches were displayed in Table 8. The % yield for all the formulation batches were rages from 67.40 to 85.63 %. Drug content was found to be in the range of 3.41 to 4.72 mg/5ml.

Table 8: % yield, drug content and loading capacity of all the batches.

Batch	% yield ± S.D.	Drug content (mg/5ml) ± S.D.	Loading capacity (%) ± S.D.
F1	77.21 ± 0.72	3.41 ± 0.2	57.33 ± 0.05
F2	67.40 ± 0.82	3.78 ± 0.42	62.81 ± 0.19
F3	73.09 ± 1.02	4.02 ± 0.18	70.32 ± 0.23

F4	84.03 ± 0.75	4.53 ± 0.26	73.94 ± 0.17
F5	64.14 ± 1.03	4.23 ± 0.15	72.83 ± 0.09
F6	85.63 ± 0.88	3.98 ± 0.28	68.30 ± 0.07
F7	83.26 ± 0.30	4.72 ± 0.42	75.83 ± 0.11
F8	78.87 ± 0.92	4.08 ± 0.22	71.01 ± 0.25
F9	72.38 ± 1.04	4.22 ± 0.38	69.44 0.08

Mean ± S.D. (n=3)

The highest drug content was obtained for F7 batch. Loading capacity was found to be in the range of 57.33 to 75. 83%, and also the highest loading capacity were observed with batch F7 which was optimized one using statistical design.

Surface pH and Equilibrium Swelling degree (ESD):

Surface pH and ESD of all the batches were reported in Table 9. All the batches had pH in the range of 5 to 7 which indicates that CSMs has pH in the range of nasal mucus pH, reveals that prepared CSMs will not cause irritation at the mucosal surface and are safe to administer intranasally[23]. On increasing the concentration of cross-linker pH was found to be slightly decreasing but still it is in the range of mucosal pH. ESD of all the batches were found to in the range of 2.43 to 4.2. It was found that on increasing concentration of polymer ESD increase

Table 9: Surface pH and ESD of all the batches.

Batch	pH ± S.D.	ESD ± S.D.
F1	6.23 ± 0.04	2.43 ± 0.2
F2	5.81 ± 0.07	2.54 ± 0.42
F3	5.68 ± 1.02	2.85 ± 0.18
F4	6.04 ± 0.08	2.99 ± 0.26
F5	5.74 ± 0.05	3.57 ± 0.15
F6	6.43 ± 0.02	3.76 ± 0.28
F7	5.62 ± 0.04	4.02 ± 0.42
F8	6.50 ± 0.06	3.98 ± 0.22
F9	6.42 ± 0.03	4.25 ± 0.38

Mean ± S.D. (n=3)

In-vitro Drug Release:

Comparative drug release profiles of optimized CSMs batch (F7) and pure CLZ was depicted in Figure 8. At the end of 24 hours pure CLZ shows 39.86% drug release whereas F7 batch shows 91.31% drug release at the end of 24 hours. At 4th hour about 70% drug release was obtained with CSMs, and then the drug was released in controlled manner showing sustained drug release profile. The presence of hydrophilic polymer matrix results in enhancement of water solubility of drug contributed to increased drug release properties. Significant enhancement in the release rate from CSMs was observed due to the decreased particle size, which in turn increased the surface area, increased wettability and reduced diffusion layer thickness. Cross-linking of starch leads to delayed degradation of

starch and thus sustained drug release pattern was observed with CSMs.

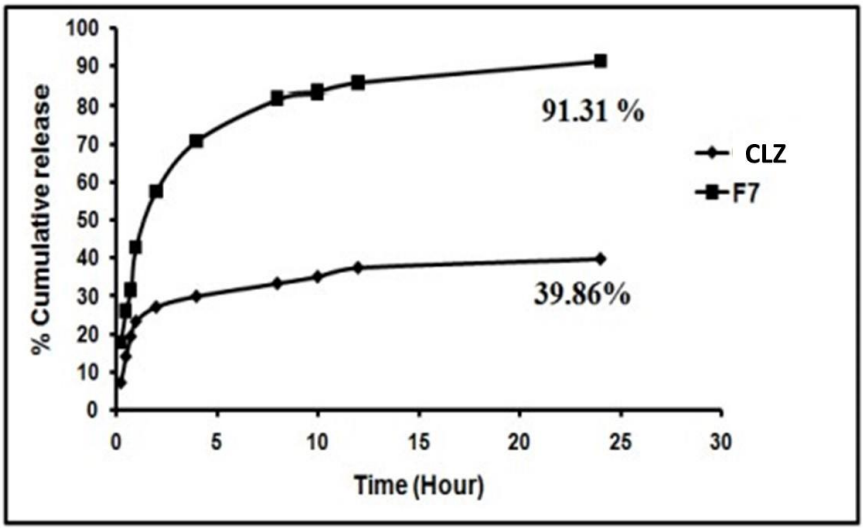

Figure 8: Drug release profiles of optimized CSMs batch (F7) and pure CLZ

The release data were fitted to various kinetic models in order to ascertain the release mechanism. Among the models tested, the drug release profile for CSMs was best fitted with Higuchi kinetic model with $R^2 = 0.975$.

Stability study of optimized F7 batch was carried out at room temperature and accelerated stability conditions of temperature and relative humidity (25°C ± 60% RH and 40°C ± 75 % RH) as per ICH guidelines is shown in Table 10.

Stability Studies:

Table 10: Influence of storage condition and storage duration on particle size and entrapment efficiency (%EE) of batch F7

Month	25°C (Room Temperature)		40°C (Accelerated Temp.)	
	Size (µm) ± SD	EE (%) ± SD	Size (µm) ± SD	EE (%) ± SD
0	88.44 ± 1.2	92.38 ± 2.1	88.44 ± 1.2	92.38 ± 2.1
1	89.21 ± 1.3	92.59 ± 0.86	89.2 ± 2.3	92.18 ± 1.2
2	89.82 ± 1.9	91.78 ± 1.4	90.02 ± 1.3	91.82 ± 1.8
3	90.45 ± 2.2	91.22 ± 1.7	90.12 ± 1.6	90.87 ± 1.5

There was increase in the particle size after 3 months from 88.44µm to 90.12µm and encapsulation efficiency decreased to 90.87% after 3 months when stored at accelerated condition.

High temperature (40°C) increased the kinetic energy of system, which could accelerate the collision of particles, and consequently increased the possibility of aggregation for microparticles.

As shown in Table 10, drug entrapment and particle size revealed no significant change at room temperature over a period of three months ($p > 0.05$) which ensures stability of the system.

Conclusion:

In the present research clozapine loaded CSMs was developed first time. Single emulsion crosslinking method were applied to develop the CSMs successfully. Batch optimization was done using Taguchi statistical model. Batch F7 was optimized showing spherical structure, high drug encapsulation capacity and long-term stability. In order to surpass the BBB and deliver the drug at CSF intranasal dose administration

chosen. Since intranasal route avoids hepatic drug degradation and offers improvement in the drug bioavailability. Development of CSMs shown increased bioavailability of BCS class II drug and able to deliver the drug at CSF via intranasal route. For brain targeting these nasal mucoadhesive microspheres has a great potential. Clinical potential can be established further by carrying out various clinical trials. Further investigations will definitely result in efficient drug product having desired therapeutic effects on human beings.

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