ORIGINAL ARTICLE

Formulation Design and Development of Anti-EGFR-BSA-CYP-SLNs *In Situ* Gel for Nasal Administration

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Abstract

Aim: A comprehensive approach was taken in the modern era to develop new distinguished *in situ* gels, as it has good desirability, sustainability, self-administrative approach and bypassing first pass metabolism. In this context, we developed and designed nasal administrable *in situ* gel containing anti-epidermal growth factor receptor-body surface area-CYP-solid lipid nanoparticle for glioma treatment. **Materials and Methods:** After multiple screenings, gellan gum (0.25-0.75%), carbopol 934 (0.20-0.60%), and poloxamer 188 (0.20-0.40%) was taken as developing polymers. Box-Behnken design was used for formulation designing. Almost all the formulation (F1-F17) shown good results. **Result and Discussion:** The various evaluation parameters for all the formulations such as, viscosity (231 ± 1.22 to 656 ± 1.11 CPs), gelling strength (85 ± 0.9 to 180 ± 0.6 s), pH (5.7 ± 0.06 to 6.2 ± 0.08), gelling temperature (31.34 ± 0.78 to 37.34 ± 0.45°C), gel melting temperature (51.06 ± 0.23 to 55.23 ± 0.65°C), % drug content (94.12 ± 0.4 to 98.87 ± 0.1), spreadability (7.28 ± 0.23 to 10.84 ± 0.45 cm), and mucoadhesive strength (4619.56 ± 0.56 to 6501.86 ± 0.22 dyne/cm^2) was been recorded. However, F8 turns out to be an optimized formula as it possess good dissolution profile (86.89% at 12th h), zero order kinetics (R^2 = 0.9971), maximum desirability factor (D = 0.9921), minimum permeation (9.23% CDP after 720 min study), and maximum skin deposition (91.76%). Further F8 formulation was undergone for stability studies as per ICH Q1A (R^2) guideline for 30 days. **Conclusion:** The results were satisfactory and signify good stability for F8 after 30 days of formulation development. However, further correlative *in vivo* studies were warranted for more conclusive outcome.

Key words: Box-Behnken design, cumulative percentage drug release, gelation temperature, gellan gum, *in situ* gel, viscosity

INTRODUCTION

Compare to conventional dosage form, *in situ* gels maintain more drug absorption peak and prolong drug residence time. The nasal route is the most significant route of administration as it permits all the compounds than the gastrointestinal tract due to less pH and less enzymatic activity in the nasal cavity.[1-5] During past decades extensive research on nasal research significantly enhanced due to its numerous advantages. In nasal administration, drug passes through nasal epithelium which helps to pass drugs to systematic circulation than to target site. On the other hand, an olfactory region of nasal cavity passes drugs to cerebrospinal fluids and directly in the brain.[1,6-8] As my research formulation is specifically designed for a brain tumor, this *in situ* nasal administration would be a promising approach toward formulation. Recently, it was confirmed that nasal administration is promising for certain hormones, steroids and low molecular enzymes due to complete absorption within the cavity. Hence, it significantly emphasizes the utilization of nasal route for both local and systematic use. Low viscous *in situ* gels when sprayed or dripped in the nasal cavity, the polymer reacts with the nasal mucosa to form more viscous

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gels which forms tighten appearance with nasal skin, by which sustain and prolong release is possible. *In situ* drug delivery also helps in improving local and systematic bioavailability, reduce dose requirements and patient safety and expectancy.

MATERIALS AND METHODS

In housed prepared anti-epidermal growth factor receptor (EGFR)-body surface area (BSA)-CYP-solid lipid nanoparticle (SLNs) were used for this study.

Materials used for in-house preparation of anti-EGFR-BSA-CYP-SLNs

Cyclophosphamide model drug was obtained as a gift sample from Emcure Pharmaceuticals Ltd. Ahmedabad, India. Glycerol monostearate (product No: 17145) purchased from astron chemicals, Ahmedabad, India. Soya lecithin (Product no: 88993) purchased from astron chemical, Ahmedabad, India. Poloxamer 188 purchased from balaji drugs, Surat-India. Dichloromethane from chemdyes corporation–Rajkot. Brij 78 obtained from chemdyes corporation–Rajkot. Tween-80 procured from fine star industry. Carbon tetrachloride procured from chemdyes corporation–Rajkot. Methanol (acetone free) was purchased from chemdyes corporation–Rajkot. Polyvinyl alcohol and lactose monohydrate from astron chemicals, Ahmedabad, India. Ultipor N56 Nylon 6, 6 membrane filter-0.45 mm, procured from Pall life science. Phosphate buffer from J&K Scientific Ltd. Ethylene diamine, 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimidehydrochloride was purchased from thermo fisher scientific, bovine serum albumin purchased from Sigma Aldrich (CAS Number 9048-46-8). Dimethyl sulfoxide (CAS Number 67-68-5) purchased from Sigma Aldrich, anti-EGFR1-Tyr 1175 (0.1 µg/ml) antibody was purchased from Santa Cruz USA. Glutaraldehyde solution 0.25% (100 fold molar excess) purchased from Fisher Chemicals. N-Hydroxysuccinimide (CAS Number: 6066-82-6) purchased from Sigma Aldrich. N, N′ dicyclohexylcarbodiimide (CAS Number 538-75-0) purchased from Sigma Aldrich. Dialysis bag (MWCO 12-14kDa) (Gift sample from Ganpat university).

Materials used for preparing anti-EGFR-BSA-CYP-SLNs incorporated *in situ* gels

Lyophilized powder of anti-EGFR-BSA-CYP-SLNs, poloxamer 188 from Balaji Drugs, Baroda. HPMC K4M, carbopol 934 were obtained from Research Chem., Mumbai. Gellan gum obtained from Gujarat chemicals, Baroda. All other chemicals were used of analytical grade.

Preparation of anti-EGFR-BSA-CYP-SLNs

The formulation of SLNs of cyclophosphamide was prepared by melting dispersion using selected ingredients followed by a high share homogeneous technique using POLYTRON® PR 2500E, and ultrasonication (Analab Scientific). The lyophilized product was then conjugated with bovine serum albumin using standard procedures. This conjugated product was further lyophilized and conjugated with anti-EGFR1-Tyr 1175 (0.1 µg/ml) antibody. The desired product was witness impel purification using dialysis (SIGMA-dialysis membrane 12 kDa) and centrifugation at 22000 RPM while maintaining 4°C for 20 min and were stored at 4°C. The final product was lyophilized (pressure of 600 m Torr for 2 h, then at 400 m Torr for 10 h, then at 250 m for 10 h, and 150 motors for 2 h) for 24 h at +20°C temperature ampoules and −110°C condenser temperature. The prepared product was named as anti-EGFR-BSA-CYP-SLNs and ancillary used for making *in situ* gel.

Development of loaded anti-EGFR-BSA-CYP-SLNs based gel formulation

Optimization of type of gelling agent

Different gelling agents (carbopol 934, xanthan gum, sodium alginate, gellan gum,[9] guar gum, poloxamer 188 and HPMC K4M, pectin, carrageenan) were used for the preparation of the *in situ* based gel. The suitable gelling agent was selected on the basis of their ability to form a good gel and that provide ease of spreadability.

Selection of concentration of polymer for in situ gel

After selecting polymers as a gelling agent, various concentrations of polymers ranging from 0.20% to 0.70% w/v were taken and all the formulations containing various amounts of polymers were evaluated for viscosity and gel forming capacity, from the evaluation parameter concentration range, were selected for further studies.

Preparation of in situ gel formulation

The "cold method" (Schmolka, 1972) was used with slight modification. Accurately weighted 2 mg of lyophilized anti-EGFR-BSA-CYP-SLNs powder was dissolved in sufficient quantity of double distilled water in aseptic condition, which is premix with required quantity of glycerin (humectant) and benzalkonium chloride (preservative). Individually, the varying concentration of polymeric solutions ranges from 0.20% to 0.70% w/v was prepared and kept separately in aseptic condition for 24 h. Further polymeric solutions were added to drug solution proportionately and mix all the excipients (4°C) maintaining constant stirring. The solution was transferred into amber color bottle and refrigerator until a clear solution was formed (6-12 h) [Figure 1].

Experimental design

Plackett and Burman Design was employed. The levels and factors were calculated and experiments were performed. The dependent variables are viscosity (Y_1), gelling strength (Y_2), and drug release in 1 h (Y_3), drug release in 6 h (Y_4), and drug release in 12 h (Y_5).

Figure 1: The *in situ* gel of lyophilized anti-epidermal growth factor receptor-body surface area-CYP-solid lipid nanoparticles powder

Dependent variables

I. Viscosity (Y_1)
II. Gelling strength (Y_2)
III. Drug release at 1 h (Y_3)
IV. Drug release at 6 h (Y_4)
V. Drug release at 12 h (Y_5).

EVALUATION PARAMETER

Drug excipient compatibility studies

Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (thermo scientific) was employed to find out the compatibility between prepared SLNs and selected polymers. During compatibility study, gellan gum, carbopol 934, poloxamer 188 were used with lyophilized SLNs.

Differential scanning calorimetry (DSC) study

DSC study of drug and optimized formulation was achieved using Shimadzu DSC 60 analyzer.

Clarity test

In situ gel was evaluated for clarity against dark and white background in the presence of light.

pH evaluation

The prepared *in situ* gel (1 ml) was diluted into 25 ml of double distilled water and pH of prepared *in situ* gels solution was recorded using the pH meter (Mettler instrument, Germany) attaining equilibrium for 2 min. Results were reproduced for three times and the average was taken.

Gelling time

By using Shydo model 100, gel timer with stirrer the freshly prepared *in-situ* gels, gelling time was been detected. In this technique recommended ingredients were placed in a wax free cup and engraved of this cup was fit with gel timer disk. The wire of the disk was adjusted with the motor shift. Start the gel timer by adjusting the stirrer from preventing it from touching in bottom or side of the prepared gel containing cup. After gel timer stopped record the reading on the counter as B.

For gelling time estimation, following formula is inspected.

Gel timing in minutes or second (G) =Mixing or weighing time in second or minutes (A) + Readings from the gel timer in minutes or seconds (B)

Gelation temperature

Gelation temperature is the prerequest for measuring the gelling capacity of *in situ* gels. Here, modified Miller and Donovan's technique was used. Gelation temperature can be measured by heating the formulations in a 30 ml borosilicate glass tube using stable water bath where per minute temperature was recorded. Required quantities of test tubes were taken and 4 ml of instantly formulated *in situ* solutions were drained insides of these test tubes, further heat, and gentle stirring is considered untilled solution transformed to gels. Gel formation was considered at a point of temperature when the flow of the prepared gel was fused when test tubes were tilted.[10] The meniscus of gels maintained 90°during invasion. Sol-Gel transformation took place due to higher concentration of polymers in *in situ* gels.

Gel melting temperature

Gel melting temperature is defined as the temperature at which the slicked gels in 30 ml test tubes again transform to solution on further increase of temperature. This gel-sol occurrence is important to understand the behavior of *in situ* gel inside of our body.

Drug content study

1 ml *in situ* gel was diluted in 10 ml of double distilled water. From that solution, 1 ml was withdrawn and poured into the 10 ml volumetric flask. 0.5 ml ferric chloride solution, 0.5 ml of ammonium molybdate solution and 1 ml of hot stannous chloride solution was added which results in the formation of deep blue-colored phosphomolybdate complex. The further solution was diluted up to 10 ml using double distiller water. The colored solution was estimated using SHIMADZU-1880 UV-VIS spectrophotometer at 722 nm.

Viscosity measurement

The viscosity of *in situ* gel was measured using Brookfield Digital Viscometer (LVDV III Ultra, Brookfield Engineering Laboratories, USA). The final *in situ* gel was taken into a beaker.[11] The viscosity was measured at 37°C using spinal number 62, at 100 rpm. The final viscosity was been

estimated after 15 min of initiation during cooling period. All the reading was taken in triplicates.

Spreadability study

10 cm length and 4 cm width rectangular glass slide clips with wooden block apparatus. The thread was used to tie up sheep serosal side contains mucous membrane. This everted skin along with this apparatus kept in a hot air oven maintaining 37°C. Two drops of *in situ* Sol-Gel was place on the mucosal surface at an angel of 120°. The relative distance travelled by the Sol-Gel before its get converted into gel was been measured in cm. Average of three readings was recorded.

Gel strength

50 g f *in situ* Sol-Gel was taken into 100 ml measuring cylinder. The temperature was maintained up to 37°C which helps accelerated gelation.[12] The relative strength of the gel was determined by measuring the time taken by a weight of 35 g to sink 5 cm inside of this gel.

Mucoadhesive strength

The *ex vivo* mucoadhesive strength is defined as the force required to detach the formulation from nasal everted mucosal tissue. The everted mucosal membrane was isolated from underlying fats and soft tissues. Further, the membrane had been cleaned using double distilled water and pH 6.4 phosphate buffer solutions. This membrane cuts into two fragments with a diameter of 1 cm^2 each. These two fragments were glued and attached in two of the modified glass slides. In one of the slides, 50 mg *in situ* gel was spread using glass rod. Slowly inverted slide was placed on the first slide considering inverted and second slide was attached with a modified weighing balance with a thread. The attachment between two slides allows for 2 min. Slowly weights were increased in aright pan of the modified weighing balance, which generates tension within the two slides.[13] The amount of weight or force required to detach the second slide from the first slide is consider the mucoadhesive strength of the prepared *in situ* gel.

The minimal weight requires to detached mucosal tissues from surface of formulation is called mucoadhesive strength (dyne/cm^2) =mg/A,

Where,
m = Weight required for detachment in gram,
g = Accelerated due to gravity (980 cm/see^2)
A = Total surface area where mucosa exposed.

In vitro drug release and kinetic study

Dialysis membrane was used for diffusion study. This membrane (LA-393-Mol. Wt. 12, 000-14,000 Daltons, Hi-media, average flat width 29.31 mm, and average diameter 17.5 mm) before mounting in the USP apparatus type II (paddle), the membrane was soaked in ultra-pure boiling distilled water for at least 12 h. The dissolution temperature has to be maintained 37°C ± 0.5°C. The optimized conjugated anti-EGFR-BSA-CYP-SLNs *in situ* gel (each contents 0.2% w/w of cyclophosphamide) was kept in different bags of dialysis membrane.[9] The dissolution medium was continuously stirred at a speed of around 50 rpm at 37°C ± 0.5°C. With every 1-h interval 5 ml of the sample was withdrawn and 5 ml of fresh phosphate buffer solution was placed inside in receptor compartment (Glass dissolution bowl). Withdrawn 5 ml of each sample was analyzed using SHIMADZU-1880UV-VIS Spectrophotometer at 722 nm (Table 8, Figure 12).

Concentration of drug (µg/ml) = (slope × absorbance) ± intercept

Y=0.0344X-0.0082 (As per linearity curve of cyclophosphamide) $R^2 = 0.9991$

$$\text{Amount of drug release in } (\text{mg/ml}) = \frac{\text{Concentration} \times \text{dissolution bath volume} \times \text{dilution factor}}{1000}$$

$$\text{Cumulative percentage release } (\%) = \frac{\text{Volume of sample withdrawn } (\text{ml}) \times P\,(t-1) + Pt}{\text{Bath volume } (v)} \times 100$$

Where, Pt = Percentage release at time t
Where, P (t–1) = Percentage release previous to "t."

Dissolution studies were determined by a best fitting method using Higuchi and Korsmeyer-Peppas plots.[14] With the used of linear regression analysis using Microsoft 2010, n and rate constant k were calculated. Coefficient studies (R^2) were used for evaluating the accuracy of the fit model.[15,16]

Ex vivo permeability studies

In-house modified Franz–diffusion cell apparatus was used to study the *ex vivo* diffusion. The aqueous drug solution of lyophilized SLNs, BSA-CYP-SLNs *in situ* gel, optimized formula (anti-EGFR-BSA-CYP-SNLs *in situ* gel), were studied for the permeation through gout everted nasal skin. The receptor area cross section was found to be 4.32 cm^2. Which is actually filled with double distilled water? The prepared *in situ* gel placed uniformly on the dorsal side of gout everted nasal skin. Each 1 h of interval 0.5 ml of the sample was removed and immediately replaced with equal volume of double distilled water. The amount of the drug diffused out to the receptor compartment can be determined by SHIMADZU-1880 UV-VIS Spectrophotometer at 722 nm.

Skin deposition study

Immediately after permeation study, the Franz diffusion cell was dismantled after a period of 720 min. The gout nasal

everted skin was carefully removed from the diffusion cell. The formulation which stacked into cell membrane mopped properly using phosphate buffer (pH 6.8) and methanol. This cleaning procedure was repeated thrice to ensure no traces of formulation particles left onto the skin surface. The skin was then chopped into pieces and extracted out with methanol for 48 h. Then, it was analyzed by SHIMADZU-1880 UV-VIS Spectrophotometer at 722 nm. The standard calibration curve equation was used to determine how much amount of drug is deposited in the skin [Figure 2].

In vitro gelation study

The *ex vivo* gelling capacity leveled in three categories on the basis of gelation time and time period
for which the formed gel remains.

Figure 2: The Franz diffusion cell and skin deposition experiment

- + - Gels after few minutes dispersed rapidly
- ++ - Gelation immediate remains for few hours consistently
- +++ - Gelation immediate remains for prolong period.

Accelerated stability studies

As per the International Conference on Harmonization (ICH) Q1A (R^2) guideline, 1 month stability studies were carried out on prepared and optimized anti-EGFR-BSA-CYP-SLNs *in situ* gel. An adequate quantity of *in situ* gel was placed inside of a nasal spray bottle and was stored in a small desiccator (Sabar scientific, India) and exposed it in refrigerator maintaining 5°C ± 3°C temperature. Further, the sample was withdrawn periodically for conducting initial, 15 days, 21 days, and 30 days investigation on appearance, pH, gelation temperature, gel melting temperature, % drug content, viscosity, spreadability, and *in vitro* gelation studies.

RESULT AND DISCUSSION

FT-IR spectroscopy

Compatibility studies of drug polymers are very essential before formulation design. Here, we took lyophilized anti-EGFR-BSA-CYP-SLNs as a drug. FT-IR spectra of lyophilized particles shown characteristic peaks on 3472/cm for N-H stretch, 1484/cm for methylene C-H bend, 1030/cm for C-N primary amine, 715/cm for analiphatic C-Cl stretch, and 895/cm for vinylidene C-H out of the plane bend [Figure 3]. The physical mixture of the drug with various polymers such as gellan gum, carbopol 934, and poloxamer 188 was evaluated [Figure 4]. The various characteristic peaks were 3645/cm for tertiary alcohol O-H stretch, 3480/cm normal polymeric O-H stretch, 2327/cm

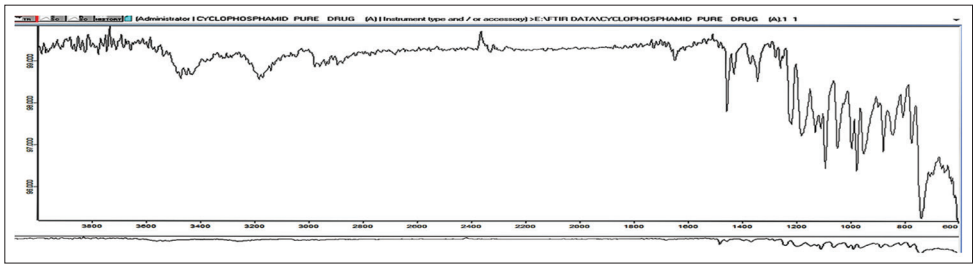

Figure 3: Fourier transform infrared spectra of lyophilized anti-epidermal growth factor receptor-body surface area-CYP-solid lipid nanoparticles

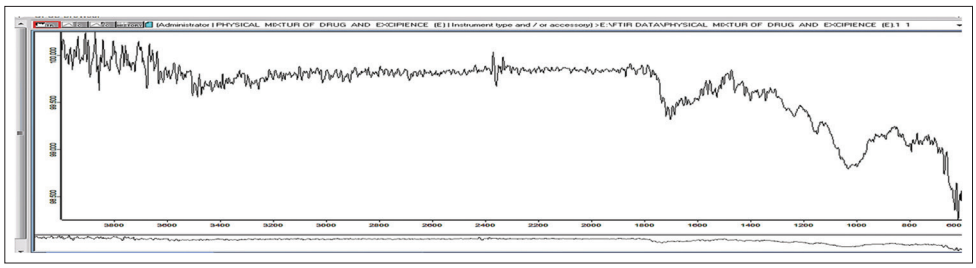

Figure 4: Physical mixture of selected polymers and lyophilized anti-EGFR-BSA-solid lipid nanoparticles

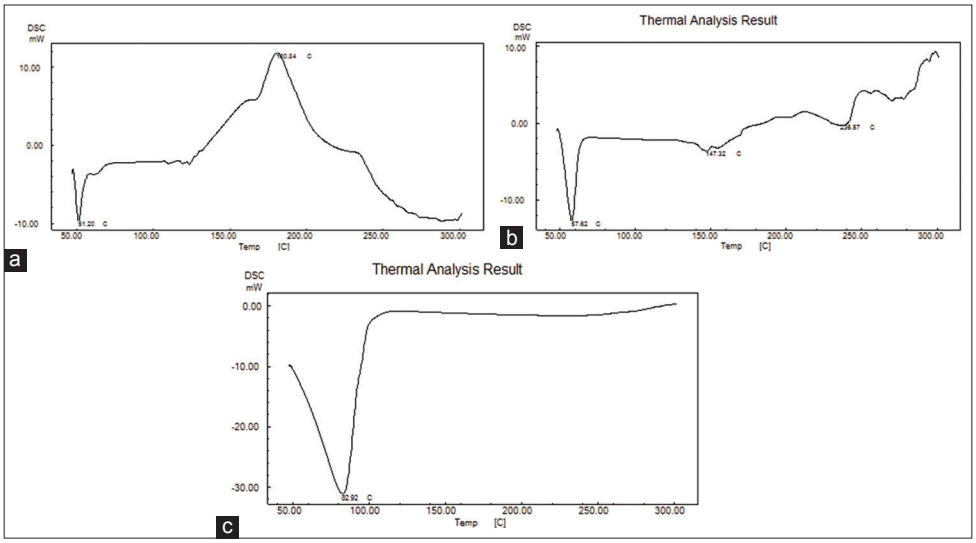

Figure 5: Differential scanning calorimetric analysis of (a) lyophilized anti-epidermal growth factor receptor-body surface area-CYP-solid lipid nanoparticles (b) The physical mixture of drug and selected polymer (c) prepared *in situ* gel

Table 1: Results of preliminary study on polymer

Polymers	Gelling behavior
Carbopol 934	Gel formed in moderate concentration
Xanthan gum	Gel not formed
Sodium alginate	Lucid gel formed
Guar gum	Gel not formed
Gellan gum	Gel formed in less concentration
Poloxamer 188	Gel formed in higher concentration
HPMC K4M	Gel formed
Pectin	Gel not formed
Carrageenan	Gel not formed

for S-H stretch, 1702/cm carboxylic acid (RCOOH), 1556/cm for aliphatic $–NO_2$, 1028/cm for C-O strong absorption, and 793/cm for C-Cl stretch. The drug spectra and drug and physical mixture spectra show maximum equivalence in respect of web numbers and no significant interaction observed, hence it can be concluded that lyophilized anti-EGFR-BSA-CYP-SLNs were stable with selected polymers, such as gellan gum, carbopol 934, and poloxamer 188.

DSC study

DSC helps to provide useful information about crystallite and amorphism of the prepared sample. The thermal curve of lyophilized anti-EGFR-BSA-CYP-SLNs (drug) shown characteristic peaks on 51.20°C and at 160.84°C. The physical mixture of drug and selected polymer shows thermal peaks on 57.62°C, 147.32°C, and 236.57°C. On the other hand, prepared *in situ* gel shows characteristic thermal peak at 82.92°C. No significant interaction was observed within drug and prepared *in situ* gel [Figure 5].

Plackett and Burman design output

For designing, 17 batches were taken. The various dependent variables are viscosity (Y_1), gelling strength (Y_2), %CDR (1 h)-Q1 (Y_3), %CDR (6 h)-Q6 (Y_4), %CDR (12 h)-Q12 (Y_5) shows distinct results from 287-656 CPS, 85-180 s, 12.43-44.76%, 43.57-78.34%, 83.81-105.23%. The multiple regression was performed and shown in the Tables 5 and 6. The value of $P < 0.05$ indicates models terms were significant itself.

Polynomial equation

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3 +\beta_{23}X_2X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2 \quad (1)$$

Where, Y is predicted response or dependent variables, X_1, X_2, and X_3 are independent variables. β_0 consider as intercept. β_1, β_2, β_3 are linear coefficients. β_{12}, β_{13}, β_{23} is considered as interaction coefficients and β_{11}, β_{22}, β_{33} consider as squared coefficients.

Responses 1 (Y_1): Viscosity

Effect of deigned factors on viscosity: After contour plot and 3D surface plot, it was confirmed, that all the factors have significant effects on viscosity.

Final equation in terms of coded factors

$$\text{Viscosity } (Y_1)=+541.40+159.75X_1+69.63X_2+23.63X_3 +8.25X_1X_2+11.75X_1X_3+45.00X_2X_3 -27.20X_1^2-78.95X_2^2-63.95X_3^2 \quad (2)$$

Table 2: Factor and levels for Plackett and Burman design

Independent variable	Actual value (%)			Coded value		
	Low	Medium	High	Low	Medium	High
Concentration of Gellan gum (X_1)	0.25	0.50	0.75	−1	0	+1
Concentration of Carbopol 934 (X_2)	0.20	0.40	0.60	−1	0	+1
Concentration of Poloxamer 188 (X_3)	0.20	0.30	0.40	−1	0	+1

Table 3: Box-Behnken design for three factors

Batch number	Coded value			Actual value (%)		
	X_1	X_2	X_3	X_1	X_2	X_3
F1	−1	0	−1	0.25	0.40	0.20
F2	0	0	0	0.50	0.40	0.30
F3	0	0	0	0.50	0.40	0.30
F4	0	−1	1	0.50	0.20	0.40
F5	1	0	−1	0.75	0.40	0.20
F6	0	1	−1	0.50	0.60	0.20
F7	−1	1	0	0.25	0.60	0.30
F8	1	0	1	0.75	0.40	0.40
F9	0	0	0	0.50	0.40	0.30
F10	0	1	1	0.50	0.60	0.40
F11	0	0	0	0.50	0.40	0.30
F12	−1	0	1	0.25	0.40	0.40
F13	−1	−1	0	0.25	0.20	0.30
F14	1	−1	0	0.75	0.20	0.30
F15	0	0	0	0.50	0.40	0.30
F16	1	1	0	0.75	0.60	0.30
F17	0	−1	−1	0.50	0.20	0.20

Table 4: Response of experimental design formulation

Batch number	Mean±SD				
	Viscosity in CPs (Y_1)	Gelling strength (second) (Y_2)	%CDR 1 h (Y_3) (Q1)	% CDR 6th h (Y_4) (Q6)	%CDR 1 h (Y_5) (Q12)
F1	302±1.23	105±0.7	38.12±1.34	68.48±2.22	98.78±2.89
F2	541±2.12	147±0.4	26.65±1.45	58.94±2.56	90.87±2.15
F3	540±0.23	146±0.1	26.12±1.11	58.93±1.23	90.45±2.78
F4	306±2.23	107±0.7	38.43±1.67	69.03±2.33	100.34±1.55
F5	590±2.24	141±0.6	14.65±2.12	47.25±2.21	86.19±1.20
F6	401±2.45	131±0.4	30.21±1.78	63.45±1.11	96.67±1.77
F7	312±1.03	115±0.7	37.34±1.45	67.11±2.34	98.01±1.98
F8	622±0.23	154±0.8	13.23±1.89	45.89±2.11	86.89±2.67
F9	542±1.97	147±0.5	25.45±2.23	57.34±3.53	89.35±2.55
F10	577±1.34	154±0.5	19.56±2.22	50.67±2.09	88.45±2.47
F11	542±1.45	147±0.5	26.87±1.56	58.89±2.62	90.67±2.39
F12	287±1.34	102±0.7	40.23±1.55	71.76±1.03	102.87±2.11
F13	231±1.22	85±0.9	44.76±1.78	78.34±1.98	105.23±1.06
F14	542±1.45	148±0.5	26.34±1.89	58.67±1.56	92.45±0.34
F15	542±1.78	148±0.9	26.34±1.67	58.74±1.32	90.01±0.20
F16	656±1.11	180±0.6	12.45±1.92	43.57±1.64	83.81±1.97
F17	310±1.34	110±0.4	37.89±2.11	67.45±2.87	99.28±1.46

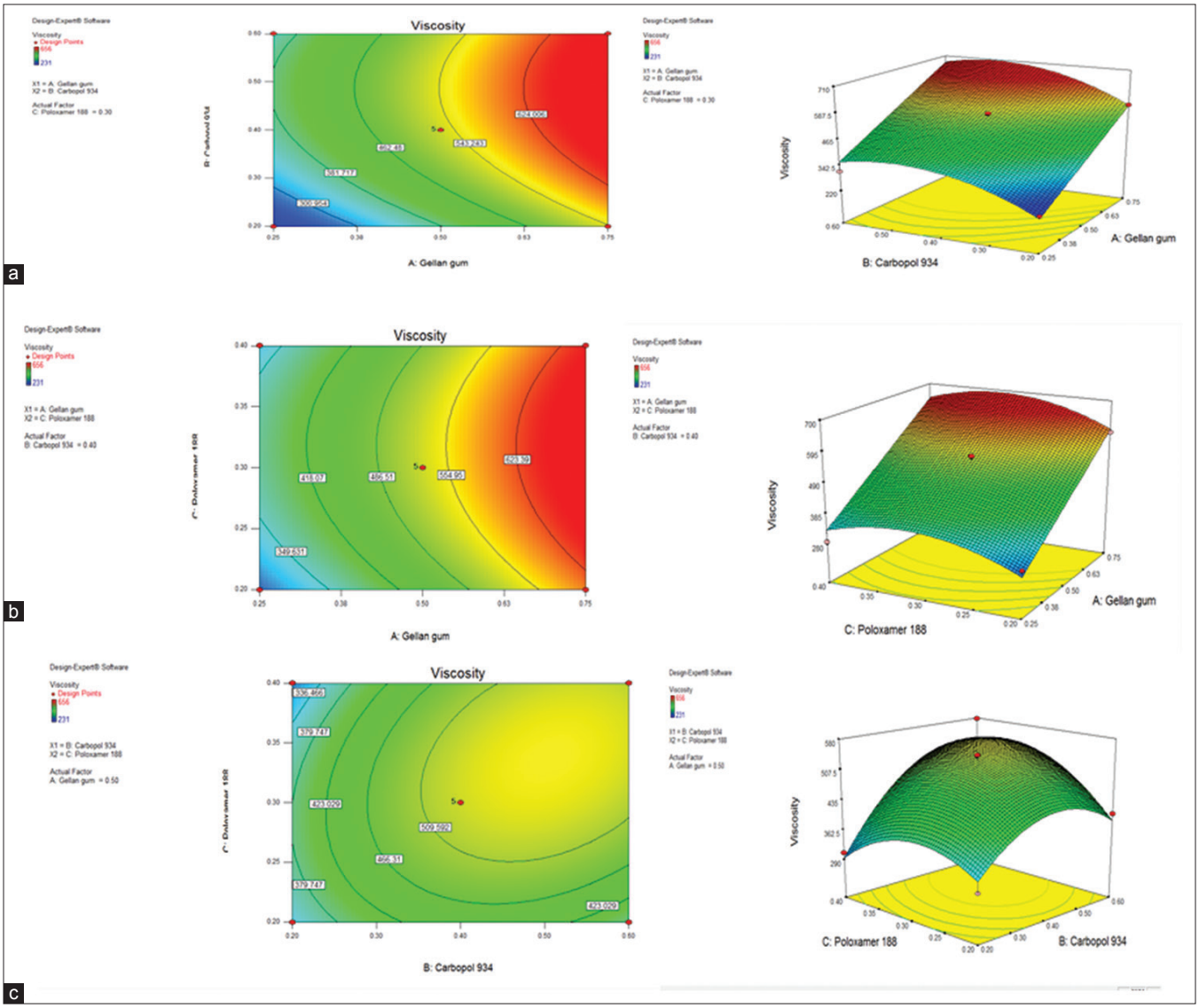

Figure 6: Response surface and contour plots showing effect of ([a] Concentration of gellan gum and carbopol-934, [b] Concentration of gellan gum and poloxamer-188 and [c] Concentration of carbopol 934 and poloxamer-188) on viscosity

Table 5: Polynomial coefficient of all five responses

Coefficient	Viscosity (Y_1)		Gelling strength (Y_2)		%CDR-Q1 (Y_3)		%CDR-Q6 (Y_4)		%CDR-Q12 (Y_5)	
	FM	RM	FM	RM	FM	RM	FM	RM	FM	RM
b_0	541.40	529.95	147.00	147.00	26.29	26.41	58.57	58.47	90.27	90.71
b_1	159.75	159.75	27.00	27.00	−11.72	−11.72	−11.29	−11.29	−6.94	−6.94
b_2	69.63	69.63	16.25	16.25	−5.98	−5.98	−6.09	−6.09	−3.79	−3.79
b_3	23.63	23.63	3.75	3.75	−1.18	−1.18	−1.16	−1.16	−0.30	−0.30
b_{12}	8.25	-	0.50	-	−1.62	-	−0.97	-	−0.36	-
b_{23}	11.75	-	4.00	-	−0.88	-	−1.16	-	−0.85	-
b_{13}	45.00	45.00	6.50	6.50	−2.80	-2.80	−3.59	-3.59	−2.32	−2.32
b_{11}	−27.20	-	−7.50	−7.50	−0.51	-	−0.48		1.05	-
b_{22}	−78.95	−80.38	−7.50	−7.50	4.45	4.47	3.83	3.82	3.55	3.61
b_{33}	−63.95	−65.38	−14.00	−14.00	0.79	-	0.25	-	2.36	2.42

Table 6: Calculation for testing the model in portions

Regression	Viscosity in CPS (Y_1)				
	DF	**SS**	**MS**	**R^2**	**P value Prob>F**
FM	9	3.074E+005	34154.38	0.9789	<0.0001
Residual	7	6620.45	945.78		
Total	16	3.140E+005			
RM	6	2.803E+005	46720.05	0.8927	0.0002
Residual	10	33689.59	3368.96		
Total	16	3.140E+005			
Regression	**Gelling strength in seconds (Y_2)**				
	DF	**SS**	**MS**	**R^2**	**P value Prob>F**
FM	9	9722.88	1080.32	0.9780	<0.0001
Residual	7	219.00	31.29		
Total	16	9941.88			
RM	7	9657.88	1379.70	0.9714	<0.0001
Residual	9	284.00	31.56		
Total	16	9941.88			
Regression	**%CDR-Q1(Y_3)**				
	DF	**SS**	**MS**	**R^2**	**P value Prob>F**
FM	9	1529.65	169.96	0.9835	<0.0001
Residual	7	25.62	3.66		
Total	16	1555.27			
RM	5	1512.52	302.50	0.9725	<0.0001
Residual	11	42.75	3.89		
Total	16	1555.27			
Regression	**%CDR-Q6 (Y_4)**				
	DF	**SS**	**MS**	**R^2**	**P value Prob>F**
FM	9	1450.16	161.13	0.9815	<0.0001
Residual	7	27.29	3.90		
Total	16	1477.45			
RM	5	1439.86	287.97	0.9746	<0.0001
Residual	11	37.58	3.42		
Total	16	1477.45			
Regression	**%CDR-Q12 (Y_5)**				
	DF	**SS**	**MS**	**R^2**	**P value Prob>F**
FM	9	615.21	68.36	0.9687	0.0002
Residual	7	19.86	2.84		
Total	16	635.07			
RM	6	607.18	101.20	0.9561	<0.0001
Residual	10	27.89	2.79		
Total	16	635.07			

Reduced model for viscosity

For viscosity (Y_1) coefficient β_{12}, β_{13}, β_{22} was found to be insignificant as P value were more than 0.05, hence they were omitted from the full model to generate the reduced model. The high value of correlation coefficient for viscosity designates a good fit for design. On the other hand, calculated F value was found to be less than the tabulated F value which suggested no significant difference between full and reduced model [Figure 6].

Final equation in terms of coded factors after reduced model

$$\text{Viscosity } (Y_1)=+529.95+159.75X_1+69.63X_2+23.63X_3+45.00X_2X_3-80.38X_2^2-65.38X_3^2 \quad (3)$$

A polynomial equation of viscosity was directly indicating that all the three factors have an effect on the viscosity. The viscosity of F1-F17 formulations was found to be increased with increased concentration of polymers. It was noticed that viscosity varies from 231-656 cps for all the formulations. Maximum viscosity was observed in F16, as it is consisting of a maximum concentration of gellan gum and carbopol-934, and minimum viscosity observed in F13 as it comprising less concentration of gellan gum and carbopol-943.

Response 2 (Y_2): Gelling strength

Effect of designed factors on gelling strength: After contour plot and 3D surface plot, it was confirmed, that the all the factors have significant effects on gelling strength.

Final equation in terms of coded factors

$$\text{Gelling strength } (Y_2)=+147.00+27.0X_1+16.25X_2+3.75X_3+0.50X_1X_2+4.00X_1X_3+6.5X_2X_3-7.50X_1^2-7.50X_2^2-14.00X_3^2 \quad (4)$$

Reduced model for gelling strength

For gelling strength (Y_2) coefficient β_{12}, β_{13} was found to be insignificant as P value was more than 0.05, hence they were omitted from the full model to generate the reduced model. The high value of correlation coefficient for gelling strength designate a good fit for design. On the other hand, calculated F value were found to be less than the tabulated F value which suggested no significant difference among full and reduced model [Figure 7].

Final equation in terms of coded factors-reduced model

$$\text{Gelling strength } (Y_2)=+147.00+27.00X_1+16.25X_2+3.75X_3+6.50X_2X_3-7.50\ X_1^2-7.50X_2^2-14.00X_3^2 \quad (5)$$

A polynomial equation of gelling strength was directly indicating that all three factors have an effect on the gelling time. The gelling strength of these F1-F17 formulations was found to be increased with increased concentration of polymers. It was noticed that gelling strength varies from 85-180 s for all the formulations. Maximum gelling was observed in F9, as it is consisting of a maximum concentration of gellan gum and carbopol-934, and minimum gelling strength is observed in F13 as it comprising less concentration of gellan gum and carbopol-943.

Response 3 (Y_3): %CDR at 1st h

Effect of deigned factors on Q1: After contour plot and 3D surface plot, it was confirmed, that all the factors have significant effects on %CDR at 1st h.

Final equation in terms of coded factors

$$\text{CDR at 1st h}= +26.29-11.72X_1-5.98X_2-1.18X_3-1.62X_1X_2-0.88X_1X_3-2.80X_2X_3-0.51X_1^2+4.45X_2^2+0.79X_3^2 \quad (6)$$

Reduced model for %CDR at 1st h (Y_3)

For coefficients, β_{12}, β_{13}, β_{11}, β_{33} was found to be insignificant as *P* values were more than 0.05, and hence, they were omitted from the full model to generate the reduced model. The high values of correlation coefficients for viscosity designate a good fit of design. Moreover, calculated F values were found to be less than the tabulated F value which suggested no significant difference between full and reduced model [Figure 8].

Final equation in terms of coded factors

$$\text{CDR at 1st h } (Y_3)=+26.41-11.72X_1-5.98X_2-1.18X_3-2.80X_2X_3+4.47X_2^2 \quad (7)$$

Polynomial equation Y_3 was indicated that all three factors have a negative effect on 1st h dissolution studies. F1-F17 formulations were found to decrease with increase concentration of polymer. It was observed that Y_3 varies from 12.45 % (F16) to 44.76 % (F13) for all formulations. It indicates immediate drug release was due to the burst effect. Furthermore describe that at initially, proper gelation did not occur and the drug was diffused rapidly through the matrix.

Response 4 (Y_4): %CDR at 6th h

Effect of designed factors on Q1: After contour plot and 3D surface plot, it was confirmed, that all the factors have significant effects on %CDR at the 6th h.

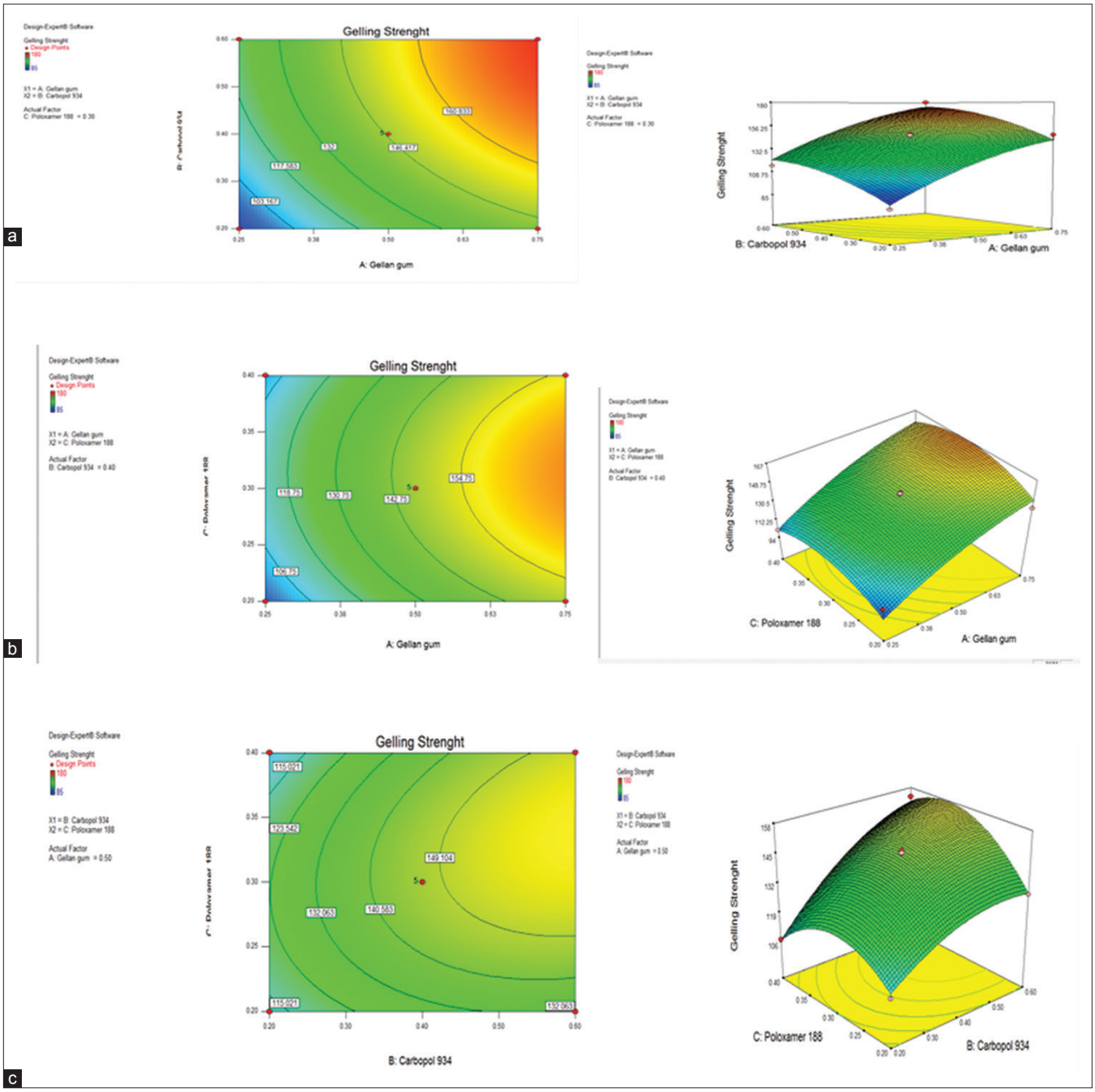

Figure 7: Response surface and contour plots showing effect of ([a] Concentration of gellan gum and carbopol-934, [b] Concentration of gellan gum and poloxamer-188 and [c] Concentration of carbopol 934 and poloxamer-188) on gelling strength

Final equation in terms of coded factors

$$\text{CDR at } 6^{th}\text{ h} = +58.57 - 11.29X_1 - 6.09X_2 - 1.16X_3 - 0.97X_1X_2 - 1.16X_1X_3 - 3.59X_2X_3 - 0.48X_1^2 + 3.8X_2^2 + 0.25X_3^2 \quad (8)$$

Reduced model for Y_4

For Y_4 coefficients, β_{12}, β_{13}, β_{11}, β_{33} was found to be insignificant as P values were more than 0.05, and hence, they were omitted from the full model to generate the reduced model. The high values of correlation coefficients for viscosity designate a good fit of design. Moreover, calculated F values were found to be less than the tabulated F value which suggested no significant difference between full and reduced model [Figure 9].

Final equation in terms of coded factors

$$\%\text{CDR at } 6^{th}\text{ h } (Y_4) = +58.47 - 11.29X_1 - 6.09X_2 - 1.16X_3 - 3.59X_2X_3 + 3.82X_2^2 \quad (9)$$

Effect of design factor on Y_4

The experimental design results, contour plot, and 3D surface plot for the 6th-h %CDR of F1-F17 formulations

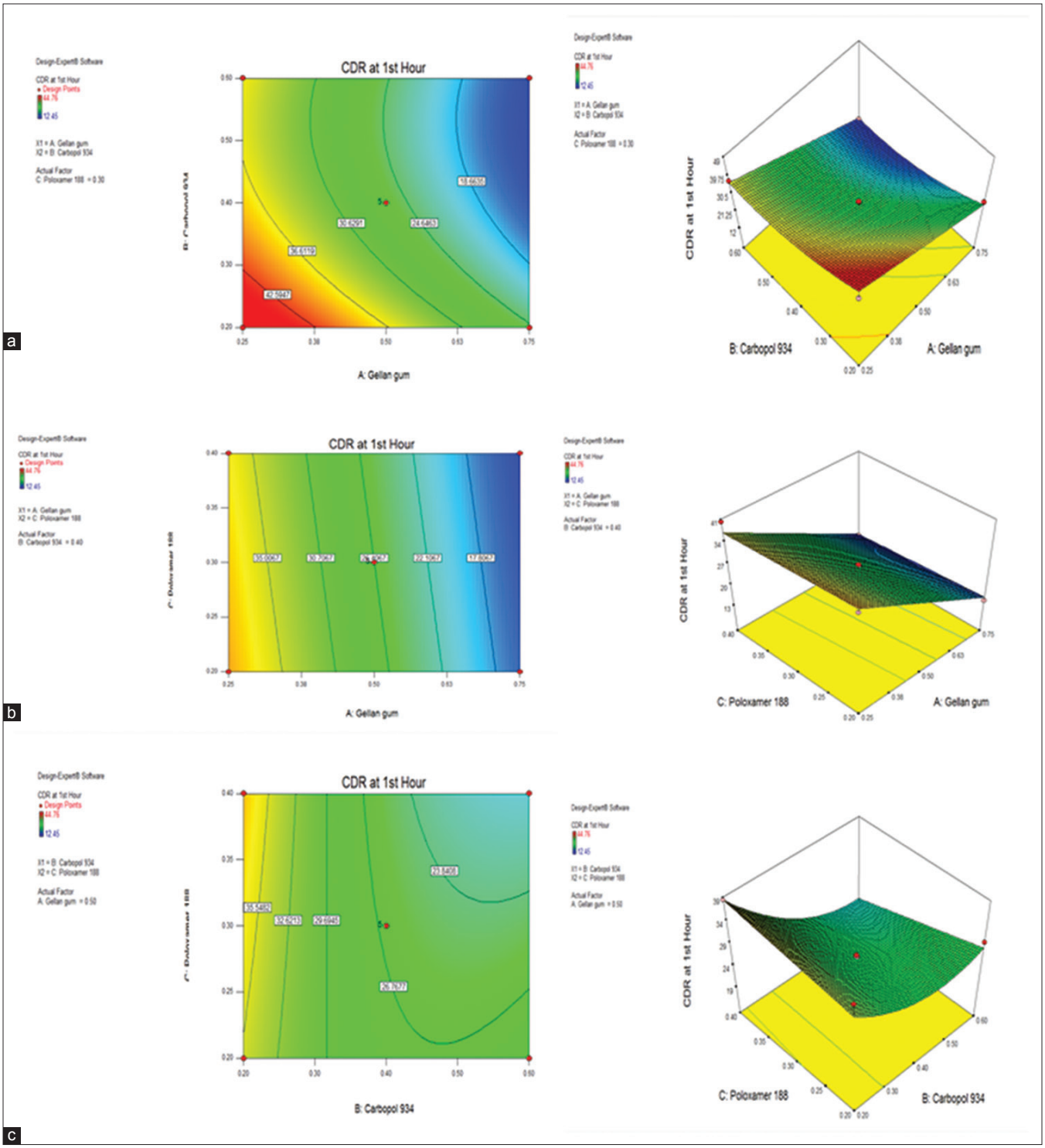

Figure 8: Response surface and contour plots showing effect of ([a] Concentration of gellan gum and carbopol-934, [b] Concentration of gellan gum and poloxamer-188 and [c] Concentration of carbopol 934 and poloxamer-188) on %CDR at 1st h

showed a strong effect of all three factors. A polynomial equation of Y_4 has indicated that all three factors have a negative effect on F1-F17 formulations were found to decrease with increase concentration of polymer. It was observed that varies from 43.57% (F16) to 71.76% (F12) for all formulations. It indicates that at the 6th h of dissolution, gelation occurred properly and formed matrix structure from that the drug cannot diffuse easily from the formulation.

Response 5 (Y_5):%CDR at 12th h

Effect of deigned factors on Y_5 after contour plot and 3D surface plot, it was confirmed, that all the factors have significant effects on %CDR at the 12th h.

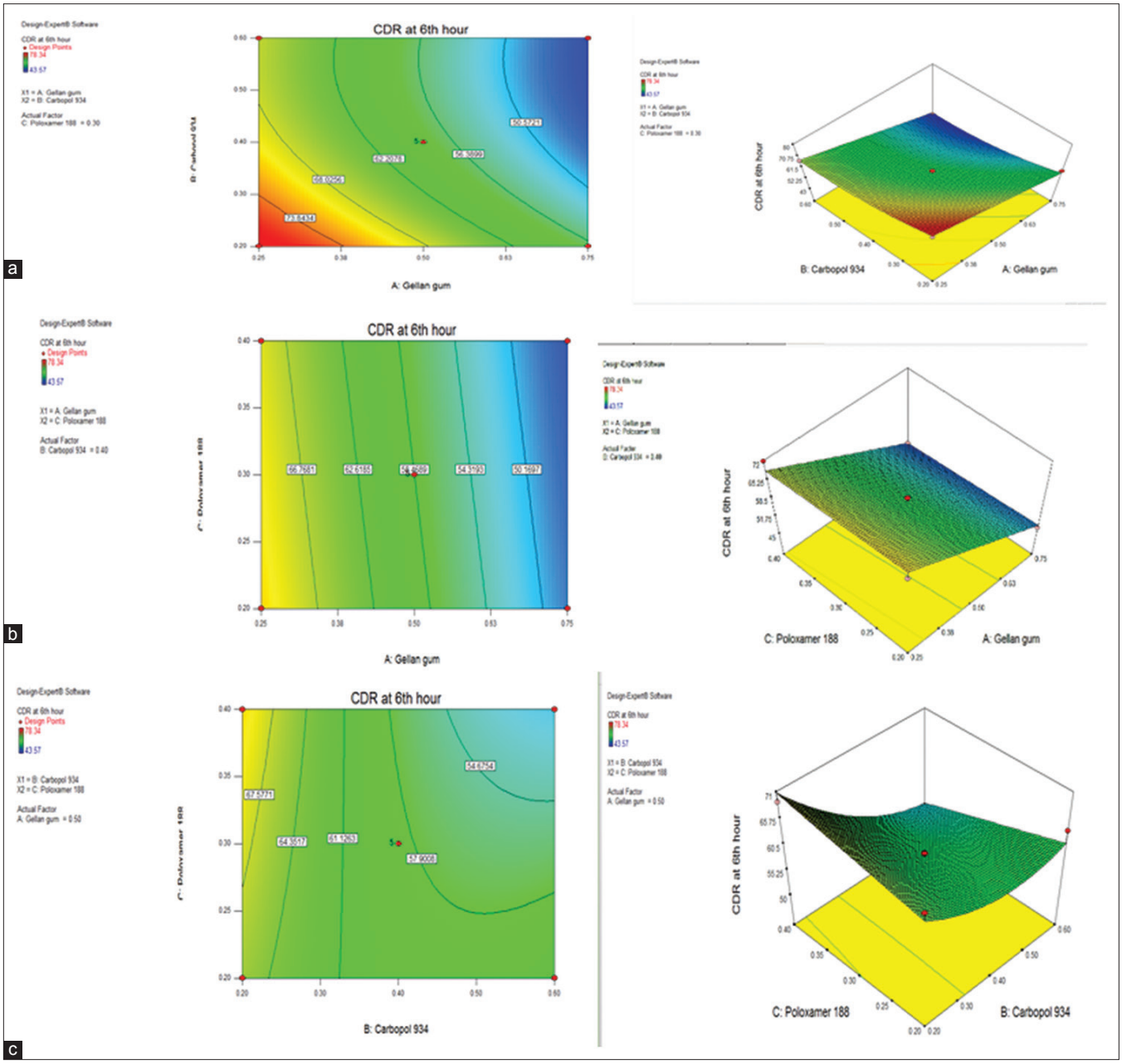

Figure 9: Response surface and contour plots showing effect of ([a] Concentration of gellan gum and carbopol-934, [b] Concentration of gellan gum and poloxamer-188 and [c] Concentration of carbopol 934 and poloxamer-188) on %CDR at 6th h

Final equation in terms of coded factors

$$\text{CDR at } 12^{th}\text{ h} = +90.27 - 6.94X_1 - 3.79X_2 - 0.30X_3 - 0.36X_1X_2 - 0.85X_1X_3 - 2.32X_2X_3 + 1.05X_1^2 + 3.55X_2^2 + 2.36X_3^3 \quad (10)$$

Reduced model for Y_5 (%CDR at 12th h)

For Y_5 coefficients, β_{12}, β_{13}, and β_{11} were found to be insignificant as *P* values were more than 0.05, and hence, they were omitted from the full model to generate the reduced model. The high values of correlation coefficients for viscosity designate a good fit of design. Moreover, calculated F values were found to be less than the tabulated F value which suggested no significant difference between full and reduced model [Figure 10].

Final equation in terms of coded factors

$$\%\text{CDR at } 12^{th}\text{ h} = +90.71 - 6.94X_1 - 3.79X_2 - 0.30X_3 - 2.32X_2X_3 + 3.61X_2^2 + 2.42X_3^2 \quad (11)$$

Effect of design factor on (Y_5) %CDR at 12th h

The experimental design results, contour plot and 3D surface plot for %CDR at 12th h of F1-F17 formulations showed a strong effect of all three factors. Polynomial equation Y_5 has indicated that all three factors have a negative effect on dissolution. F1-F17 formulations was found to decrease with increase concentration of polymer. It was observed that Y_5 varies from 86.19 % (F5) to 105.23% (F13) for all

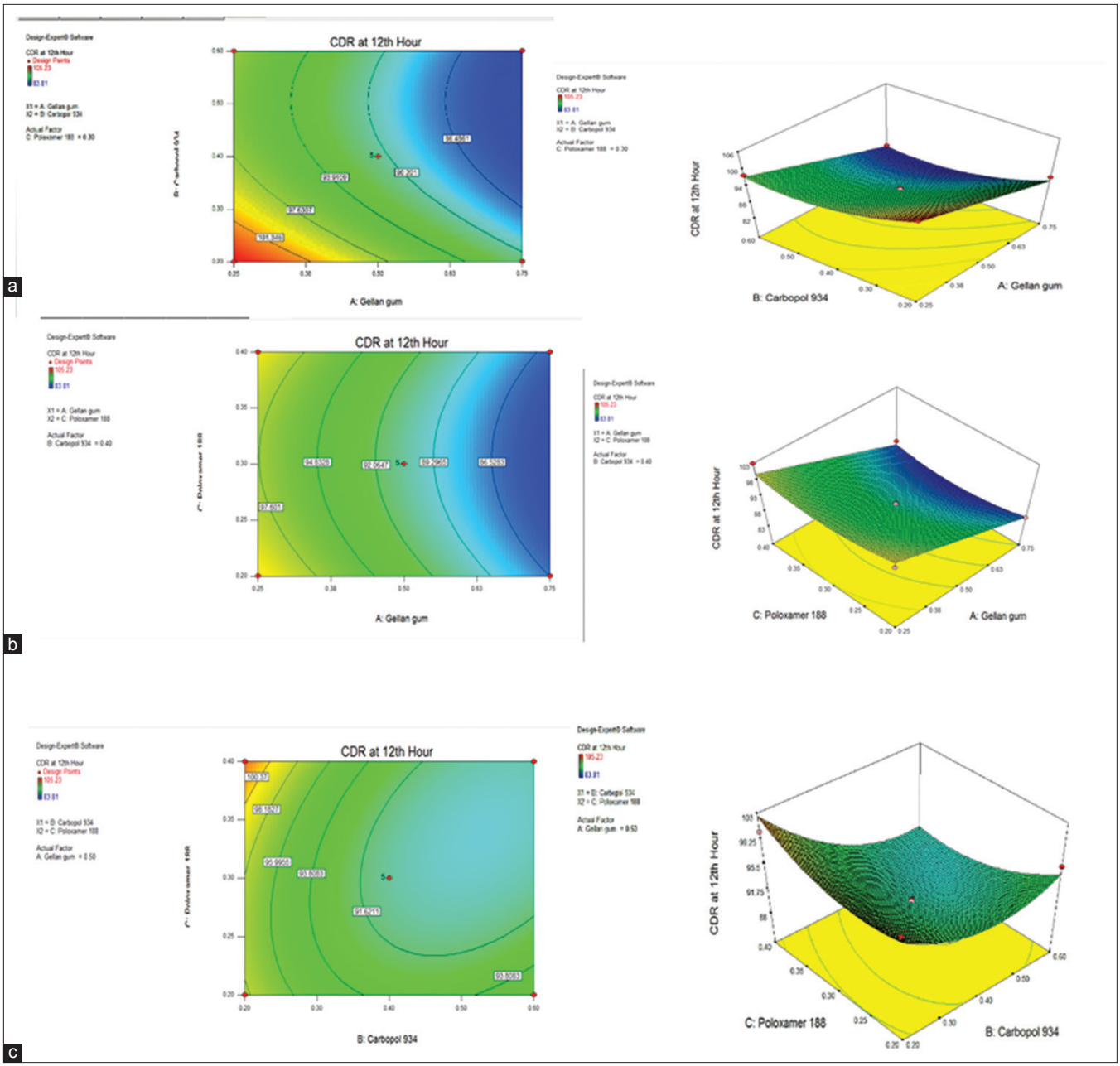

Figure 10: Response surface and contour plots showing effect of ([a] Concentration of gellan gum and carbopol-934, [b] Concentration of gellan gum and poloxamer-188 and [c] Concentration of carbopol 934 and poloxamer-188) on %CDR at 12th h

formulations. It indicates that in 12th h dissolution more than 80% amount of drug was dissolved and hear after drug release was decreased gradually. That indicates, there was a little amount of the drug is present in the formulation.

Experimental design

For designing, we took 17 batches. The various dependent variables are viscosity, gelling strength, %CDR (1 h)-Q1, %CDR (6 h)-Q6, %CDR (12 h)-Q12 and it shows distinct results from 287-656 CPS, 85-180 s, 12.43-44.76 %, 43.57-78.34%, 83.81-105.23%. The multiple regression was performed and shown in table number. The value of $P < 0.05$ indicates models terms were significant itself.

Optimized batch analysis

Contour plots of all dependents variables were overlapped to locate the area of common interest. The optimized batch was selected on the basis of following criteria: Minimum viscosity, minimum gelling strength, and in range drug release. The optimized batch was selected using DESIGN EXPERT trial version 8.0.5 (Stat-Ease. Inc. Minneapolis, USA) and overlay plot was generated [Figure 11]. To confirm the validity of design, the optimized batch was performed and % relative error was calculated which was found to be less than the 9% [Table 9] indicate goodness of fit in the model [Figure 11].

Desirability function used to determine optimized batch

To produce the desired product, the formulations responses during optimization, has to combine. It gives us predicting optimum level for independent variables. To produce one desirability function, individual desirability has to be calculated. The optimized parameter to be considered was viscosity, gelling strength, %CDR at a 1st h, %CDR at the 6th h, and %CDR at the 12th h. The best part of this study is no need of specific requirement for gelation temperature of the optimized formulation.

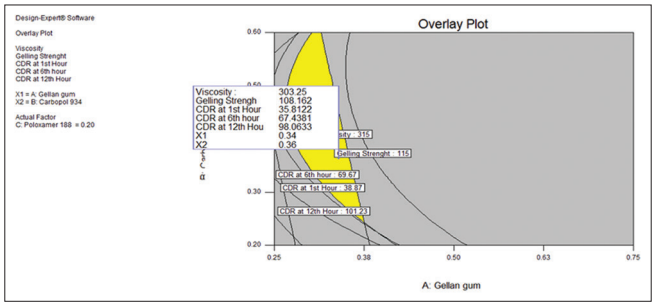

Figure 11: Overlay plot of optimized batch

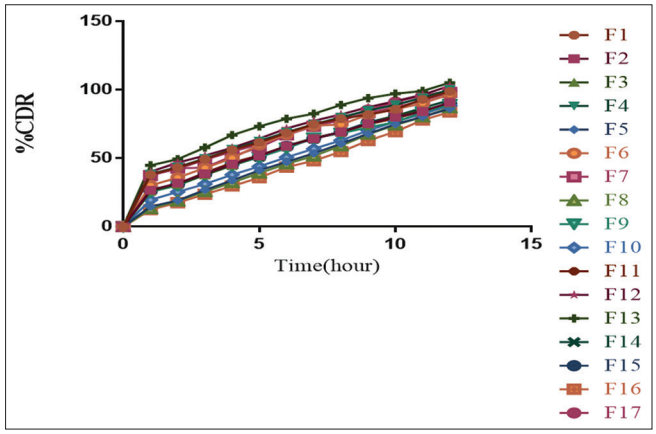

Figure 12: *In vitro* dissolution profile of all the *in situ* formulations

Our target is to find desirability for minimum viscosity for the formulation, hence the following equation to be followed:

$$d_1=\{(U-y)/(U-T)\} \quad (12)$$

Where, U=Upper limit of all the formulation viscosity (656CPs), y=Individual viscosity T=Targeted viscosity as per control chart (303.25 CPs), when y<T, T≤ y ≤U, y>U.

Our next target was to find desirability for minimum gelling strength (d_2), hence formula (1) was applied again. Here, U=180 s T= 108.162 s.

%CDR at 1st h was found to be maximum release before its get maintained.

Desirability factor for %CDR at 1st h (d_3) = {(y-L)/(T-L) (13)

When, y<L, L≤ y ≤T, y>T

Where, y=Individual percentage cumulative drug release, L=Lower limit (12.45%)

T=Targeted %CDR at 1st h (35.812%)

Desirability factor for %CDR at 6th h (d_4) depends on the maximum release of drug from the formulation. Hence, equation 2 was considered for calculation of d_4. Where L=Lower limit (43.57%) T=Targeted %CDR at 6th h (67.43%). Desirability factor for %CDR at the 12th h (d_5), again depends on the maximum release of drug from the formulation. Hence, equation 2 was considered for calculation

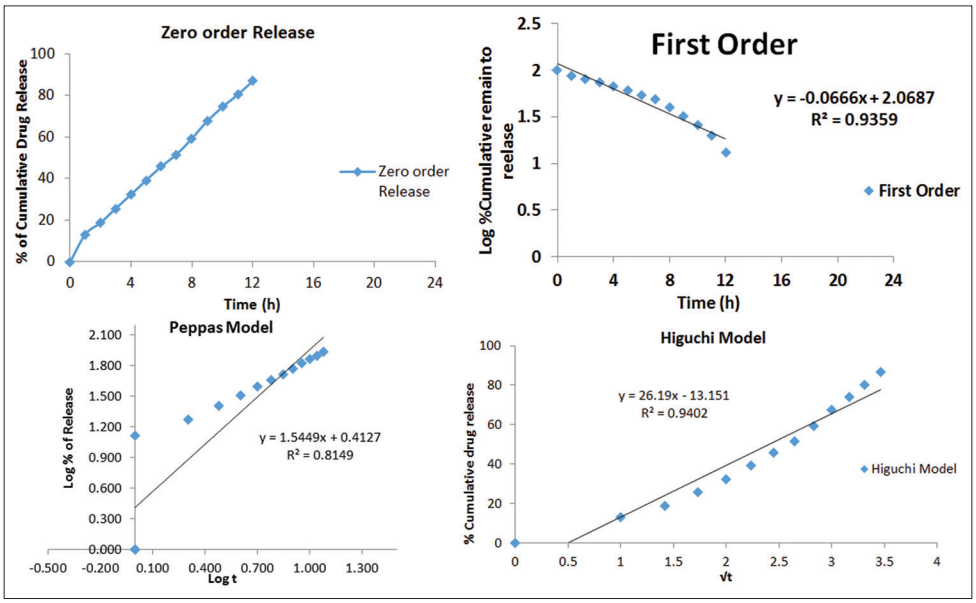

Figure 13: Kinetic profile of F8 formulation

Table 7: Result of checkpoint batch

Response	Predicted value	Experimental value*	Percentage relative error
Viscosity (CPS)	303.25	310±1.34	2.31%
Gelling strength (Sec)	108.16	110±0.40	1.18%
%CDR (Q1)	35.81	37.89±2.11	5.81%
%CDR (Q6)	67.43	67.45±2.87	0.029%
%CDR (Q12)	98.06	99.28±1.46	1.24%

*All results were shown in mean±SD (n=3). SD: Standard deviation

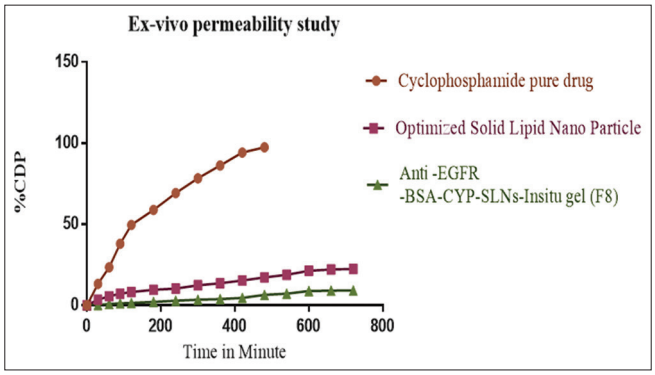

Figure 14: *Ex vivo* permeability study profile of pure drug, optimized nanoparticle and F8 formulation

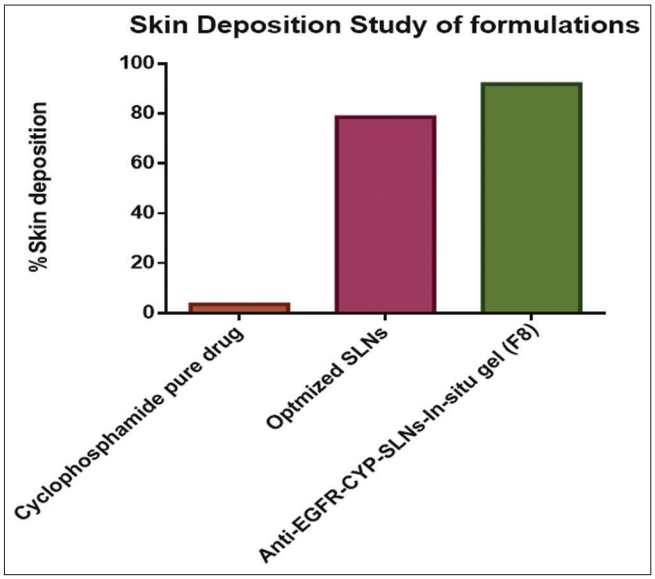

Figure 15: Skin deposition study of pure drug, optimized solid lipid nanoparticle and F8

of d_5. Here, L=Lower limit (83.81%) T=Targeted %CDR at 12th h (98.063%).

The overall desirability of prepared *in situ* gel was calculated for all the 17 batches using following equation:

$$\text{The overall desirability } (D) = (d_1 \times d_2 \times d_3 \ldots\ldots d_m)^{1/m} \qquad (14)$$

Where, m is the number of responses. The overall desirability value should be below 1 as the range is within 0-1, but the maximum value was to be considered for optimizing batch.

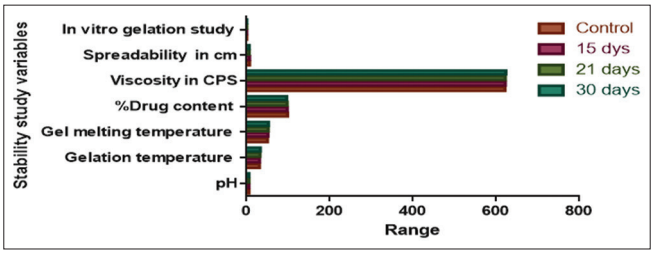

Figure 16: P-test on stability batch

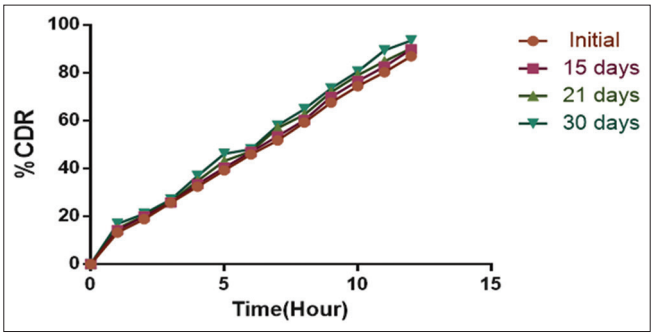

Figure 17: Dissolution profile of F8 during stability study

The optimized batch was found to be F8 as it produces maximum D value, that is 0.9921. Hence, optimized polymer concentration are: 0.75% gellan gum, 0.40% carbopol 934, and 0.40% poloxamer 188 (Table 9).

Kinetics of drug release

The obtained data from dissolution studies was fitted to various kinetic studies. The purpose of this study is to find the proper kinetic model for optimized batch (F8) and rest of the others.

The coefficient of regression and release rate constant values for zero, first order, Higuchi's and Korsmeyer–Pappas models were compared.

After kinetic studies, it was confirmed that F8 batch maintains proper zero order kinetics, hence F8 was concluded to be the optimized batch (Figure 13, Tables 10 and 11)

Ex vivo permeability study

The best formulation should give minimum permeation and maximum skin deposition. After 720 min permeation study,

Table 8: *In vitro* dissolution studies of F1-F17 *in situ* formulations

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	38.12	26.65	26.12	38.48	14.65	30.21	37.34	13.23	25.45	19.56	26.87	40.23	44.76	26.34	26.34	12.45	37.89
2	43.13	31.87	30.89	43.56	19.23	35.78	42.65	18.78	30.22	25.49	31.36	46.78	49.37	31.22	31.34	17.57	42.27
3	49.25	38.67	37.87	49.78	26.76	42.98	43.15	25.67	37.98	31.23	39.46	51.89	57.87	38.96	38.54	23.67	48.89
4	56.24	45.67	44.98	56.57	33.78	50.12	51.45	32.34	44.82	37.98	46.87	57.67	66.93	45.11	45.88	29.67	54.89
5	61.56	51.67	51.65	62.56	41.23	59.78	58.12	39.23	50.87	44.11	52.11	64.34	73.34	51.08	51.45	35.81	60.22
6	68.48	58.94	58.93	69.03	47.25	67.45	67.11	45.89	57.34	50.67	58.89	71.76	78.76	58.67	58.74	43.57	67.45
7	74.87	64.34	64.78	74.78	53.56	73.78	73.47	51.67	64.87	56.88	64.22	77.33	82.55	64.22	64.55	48.14	73.29
8	78.89	69.22	68.93	79.89	60.67	74.65	77.78	59.23	68.34	63.12	69.01	82.12	88.92	69.23	69.34	54.78	79.34
9	81.98	75.34	74.88	84.89	67.98	81.86	83.34	67.56	72.56	70.34	74.87	87.34	93.92	75.98	76.22	62.98	85.34
10	85.87	80.01	81.22	89.01	74.89	85.81	87.34	74.34	76.34	76.78	79.18	91.67	97.23	80.88	80.99	69.39	91.09
11	93.12	84.23	84.87	94.23	80.34	90.22	92.12	80.23	83.67	82.45	84.34	96.45	99.23	86.84	84.34	77.89	95.86
12	98.78	90.87	90.45	100.3	86.19	96.67	98.01	86.89	89.35	88.45	90.07	102.8	105.23	92.45	90.01	83.81	99.28

Table 9: Individual and overall desirability of prepared *in situ* gel

Formulation code	d_1	d_2	d_3	d_4	d_5	D
F1	1.003	1.044	1.098	1.044	1.050	0.1641
F2	0.326	0.459	0.607	0.644	0.495	0.7623
F3	0.328	0.473	0.585	0.643	0.465	0.7671
F4	0.992	1.016	1.112	1.067	1.159	0.1503
F5	0.187	0.542	0.094	0.154	0.166	0.9751
F6	0.722	0.682	0.760	0.833	0.902	0.4259
F7	0.975	0.904	1.065	0.986	0.996	0.2132
F8	0.096	0.361	0.033	0.097	0.216	0.9921
F9	0.323	0.459	0.547	0.577	0.388	0.8050
F10	0.223	0.361	0.304	0.297	0.325	0.9247
F11	0.323	0.459	0.617	0.642	0.481	0.7629
F12	1.046	1.085	1.189	1.181	1.337	0.0954
F13	1.2048	1.32	1.383	1.457	1.502	0.0292
F14	0.323	0.445	0.594	0.632	0.606	0.7474
F15	0.323	0.445	0.594	0.632	0.435	0.7814
F16	0	0	0	0	0	0
F17	0.980	0.974	1.088	0.992	1.085	0.1823

Table 10: Model used in kinetics studies

Model name	Model equation	Graphs
Zero order	$Qt=Q_0-K_0t$	Time versus drug release
First order	$InQt=InQ_0-t$	Time versus Log% drug remaining
Higuchi's	$Qt=K_ht_{1/2}$	SQRT time versus drug release
Korsmeyer–Peppas	Log Qtvs Log t	Log time versus Log% drug release

Q_t=Cumulative amount of the drug release at time t; Q_0=Initial amount of the drug present in the *in situ* gel membrane; K_0=Zero order release rate constant, K1=First order release rate constant K_h=Diffusion rate constant

it is concluded that cyclophosphamide pure drug produces less permeation as it releases almost 101 % of drug within 480 min. Whereas, anti-EGFR-CYP-SLNs *in situ* gel (F8) produces minimum permeation (9.23% CDP after 720 min study) and maximum skin deposition. Hence, skin deposition study has to be performed.

Skin deposition study results and discussion

The % drug deposition profile showing anti-EGFR-BSA-CYP *in situ* gel (F8) highest skin deposition compare with the pure drug and optimized SLN. The plain cyclophosphamide showed less accumulation. As per mandatory requirement of ideal formulation, maximum

Table 11: Kinetics study of drug released profiles of formulation batch

Formulation code	Zero	First	Higuchi	Pappas	K_1	Best fit model
F1	0.9021	0.8051	0.9889	0.6640	0.264	Higuchi
F2	0.9561	0.9542	0.9900	0.7133	0.171	Higuchi
F3	0.9594	0.9607	0.9887	0.7186	0.173	Higuchi
F4	0.9081	0.8219	0.9901	0.6658	-	Higuchi
F5	0.9951	0.9500	0.9500	0.8038	0.152	Zero order
F6	0.9361	0.9070	0.9932	0.7010	0.228	Higuchi
F7	0.9212	0.8616	0.9826	0.6725	0.251	Higuchi
F8	0.9971	0.9359	0.9402	0.8149	0.153	Zero order
F9	0.9559	0.9569	0.9897	0.7186	0.161	Higuchi
F10	0.9860	0.9423	0.9659	0.7584	0.160	Zero order
F12	0.9522	0.9607	0.9919	0.7110	0.167	Higuchi
F13	0.9007	0.8633	0.9883	0.6603	-	Higuchi
F14	0.9631	0.9363	0.9861	0.7176	0.184	Higuchi
F15	0.9565	0.9667	0.9901	0.7155	0.170	Higuchi
F16	0.9967	0.9918	0.9297	0.8210	0.138	Zero order
F17	0.9206	0.8094	0.9880	0.6708	0.310	Higuchi

Table 12: *Ex vivo* permeability studies of optimized formulation

Time in minute	Cyclophosphamide pure drug	Optimized solid lipid nanoparticle	Anti-EGFR-BSA-CYP-SLNs *in situ* gel (F8)
0	0.00	0.000	0.000
30	13.24±1.23	3.450±1.09	0.230±2.11
60	23.46±1.412	5.520±0.45	0.760±3.67
90	37.98±1.87	7.230±3.23	1.230±3.12
120	49.56±1.45	8.134±1.45	1.450±3.45
180	58.78±2.23	9.560±1.44	2.120±2.45
240	69.23±1.43	10.345±1.67	2.860±1.34
300	78.34±2.67	12.340±1.22	3.430±0,56
360	86.21±2.45	13.560±1.56	3.860±1.04
420	94.13±1.78	15.340±1.33	4.560±0.23
480	97.46±3.23	17.240±1.34	6.430±1.34
540	-	18.870±1.67	7.230±2.45
600	-	21.250±2.34	8.830±1.34
660	-	22.030±1.45	9.012±3.34
720	-	22.450±1.78	9.234±2.34

deposition of drug in the squamous epithelium skin and minimum penetration throughout skin is able to release the drug for a prolonged period of time after nasal application. Hence, anti-EGFR-CYP-SLNs *in situ* gel (F8) was found to be the best candidate for the topical treatment of glioma (Figure 15).

Cyclophosphamide pure drug	Optimized SLNs	Anti-EGFR-CYP-SLNs *in situ* gel (F8)
3.54	78.55	91.76

RESULT AND DISCUSSION FOR PH, GELATION TEMPERATURE, GEL MELTING TEMPERATURE, PERCENTAGE DRUG CONTENT, VISCOSITY, SPREADABILITY, MUCOADHESIVE STRENGTH IN VITRO GELATION STUDY

The pH was tested for all formulations. The obtained pH was within the range of 5.8 ± 0.04 to 6.2 ± 0.08. Gelling temperature obtained within the range of 31.34 ± 0.78

Table 13: Results of experimental design batches

Formulation code	Mean±SD						Mucoadhesive strength in dyne/cm²	*In vitro* gelation study
	pH	Gelation temperature	Gel melting temperature In °C	% drug content	Viscosity in CPS	Spread ability in cm		
F1	5.9±0.02	36.56±0.22	54.38±0.49	98.87±0.1	302±1.23	10.84±0.45	4708.67±0.24	++
F2	5.8±0.04	34.89±0.34	53.09±0.89	96.12±0.8	541±2.12	8.98±0.11	6012.23±0.11	+++
F3	5.9±0.01	34.71±0.91	53.00±0.12	98.23±0.5	540±0.23	8.67±10.33	5923.34±0.12	+++
F4	5.9±0.06	36.56±0.23	54.53±0.49	97.19±0.6	306±2.23	10.01±0.10	4715.11±0.10	++
F5	5.8±0.07	33.56±0.56	52.12±0.30	96.55±0.2	590±2.24	7.80±0.45	6213.22±0.23	+++
F6	6.2±0.08	35.86±0.45	54.15±0.39	94.12±0.4	401±2.45	9.02±0.90	5109.31±0.45	+++
F7	6.0±0.02	36.34±0.36	54.43±0.45	96.88±0.9	312±1.03	10.34±0.13	4721.45±0.24	++
F8	5.8±0.05	31.34±0.78	51.06±0.23	98.79±0.4	622±0.23	7.67±0.23	6402.98±0.11	+++
F9	5.7±0.06	35.74±0.33	54.34±0.39	98.81±0.1	542±1.97	8.67±0.34	6114.23±0.45	+++
F10	5.8±0.01	34.89±0.67	53.44±0.34	97.23±0.5	577±1.34	8.32±0.22	6239.04±0.35	+++
F11	5.9±0.03	35.78±0.65	55.23±0.56	95.39±0.7	542±1.45	8.50±0.29	6187.03±0.11	+++
F12	6.0±0.05	37.01±0.45	55.22±0.45	97.12±0.3	287±1.34	8.01±0.22	4638.32±0.55	++
F13	6.1±0.01	37.34±0.23	53.51±0.38	96.18±0.1	231±1.22	11.23±0.11	4619.56±0.56	++
F14	6.1±0.08	34.02±0.36	53.13±0.56	97.15±0.5	542±1.45	8.71±0.01	6108.23±0.18	+++
F15	5.8±0.04	34.23±0.45	53.22±0.30	97.01±0.2	542±1.78	8.77±0.13	6110.33±0.11	+++
F16	5.8±0.05	31.34±0.79	51.98±0.34	98.80±0.1	656±1.11	7.28±0.23	6501.86±0.22	+++
F17	5.9±0.07	36.11±0.33	54.98±0.31	98.68±0.7	310±1.34	11.24±0.09	4712.56±0.23	++

Table 14: 1-month stability studies of formulation F8 as per ICH Q1A (R^2) guideline

Time period of sample in (5°C±3°C) as per ICH Q1A (R^2) guideline				
Evaluation parameters	**Initial**	**15 days**	**21 days**	**30 days**
pH	5.80±0.05	5.80±0.78	5.90±0.24	6.00±0.35
Gelation temperature	31.34±0.78	31.85±0.34	32.52±0.21	33.51±0.44
Gel melting temperature	51.06±0.23	52.06±0.11	52.80±0.33	53.01±0.91
%Drug content	98.79±0.04	98.34±0.34	98.01±0.36	97.08±0.04
Viscosity in CPS	622.00±0.23	623.00±0.44	623.00±0.01	624.00±0.41
Spreadability in cm	7.67±0.11	7.64±0.09	7.01±0.15	6.90±0.15
In vitro gelation study	+++	+++	+++	+++

Table 15: *In vitro* dissolution profile of stability batch

Time (min)	Initial	15 days	21 days	30 days
0	0	0	0	0
1	13.23	14.230	14.86	16.89
2	18.78	19.980	20.26	21.01
3	25.67	25.780	26.12	27.01
4	32.34	33.450	34.76	36.89
5	39.23	40.330	43.12	46.09
6	45.89	46.889	47.11	48.02
7	51.67	53.450	56.87	57.93
8	59.23	60.230	62.48	64.77
9	67.56	69.890	71.87	73.45
10	74.34	76.560	78.98	80.56
11	80.23	82.560	84.78	89.39
12	86.89	89.760	90.23	93.45

to 37.34 ± 0.23, which indicates gelling starts with an exposure of body temperature. The gel-sol transition (melting temperature) of this prepared *in situ* reaches a higher degree of temperature (51.98 ± 0.34 to 54.98 ± 0.31), which means a good quality of mucoadhesion can be expected. Drug contents of the prepared formulations were found to be within the range of 94.12 ± 0.4 to 98.87 ± 0.1, which indicates uniformity of drug contents and good quality of formulation development. Viscosity increases or decreases with the variation of polymer concentrations. F13 formulation with a minimum concentration of gellan gum and carbopol 934 possess less viscosity (231 ± 1.22 Cps), whereas, F16 with an optimum concentration of gellan gum and carbopol 934 satisfied with a higher viscosity (656 ± 1.11 Cps). The spreadability of the formulation was decreased with the increase of viscosity (4619.56 ± 0.56 for F13 to 6501.86 ± 0.22 for F16). As far as mucoadhesive properties are concerned, it was found that increased

gellan gum concentration enhanced mucoadhesion and dissipation property. Good mucoadhesion circumvents first pass metabolism and enhance prolong release activity. *In vitro* gelation study indicates that decrease polymer concentration can produce an *in situ* gel of less physically stable formulations. However, optimized formulation (F8) can extend the physical stability of the formulations for a longer period.

Stability studies

As per ICH Q1A (R^2) guideline, 1-month stability study concluded to the optimized finish product-F8. It is maintaining good stability in refrigerator storage. The paired *t*-test results show P value satisfy the level of significance (<0.001), hence it was concluded that *in situ* gel is stable in refrigerator storage (Figures 16 and 17, Tables 14 and 15).

CONCLUSION

The novelty of this work was to design antiepidermal growth factor and bovine serum albumin conjugated lyophilized nanoparticles of cyclophosphamide in various *in situ* Sol-Gel formulations. *In vitro* studies have shown the optimized formulation (F8) dignifies all the evaluation parameters and also passed the stability studies. Further correlative *in vivo* studies were warranted for more conclusive outcome.

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