Research article

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FORMULATION AND OPTIMIZATION OF LIPOSOMES INCORPORATING CYCLODEXTRIN– CLOPIDOGREL BASED GEL

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ABSTRACT

In this work, we present a formulation of Clopidogrel-β-cyclodextrin lipid carrier which was developed for efficient incorporation and persistent release. A 3-factor, 2-level Box-Behnken design was used to optimize the process parameters including Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (1:3) (C). Six dependent variables globule size, refractive index, cumulative drug release after 12 hour, viscosity, gel strength and spreadability were measured as responses. Mathematical equations and response surface plots were used to relate the dependent and independent variables by using Design-Expert software (DX11).

Keywords: Clopidogrel-β-cyclodextrin lipid carrier, dependent variables, responses, Mathematical equations

INTRODUCTION

Liposomes have been established as a promising novel drug delivery vehicle in several different basic sciences. [1] Because of structure similarity between lipid bilayer & cell membrane, liposome can penetrate effectively deliver drug to such that a free drug would not penetrate.[2]Cyclodextrins (CDs) are cyclic oligosaccharides, consisting of glucopyranose units, which are able to form host-guest inclusion complexes with lipophilic molecules. [3]The ability of CD to increase drug solubility may be used to increase drug entrapment in the aqueous compartment of liposomes and liposomes can protect CD/drug inclusion complexes until drug release. [4] The principle objective of formulation of lipid-based drugs is to enhance their bioavailability. [5] Lipid-based drug delivery systems (LBDDS) are one of the emerging technologies designed to address challenges like the solubility and bioavailability of poorly water-soluble drugs. Furthermore, lipid-based formulations have been shown to reduce the toxicity of various drugs by changing the biodistribution of the drug away from sensitive organs. [6]

MATERIALS AND METHODS

Materials:

Clopidogrel was purchased from SM Pharmaceutical,Malaysia, β-cyclodextrin was purchased from (HiMedia Laboratories Pyt Ltd), Poloxamer 407 was purchased from (Merck), Span 80 was purchased from (QuickLab), Gellan Gum was purchased from (HiMedia Laboratories Pyt Lts), Cholesterol was purchased from (Sigma Aldrich, Germany) and diethyl ether was purchased from (Merck).

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METHODS

Scheme of clopidogrel-β-cyclodextrin loaded liposome as shown in figure 1.

Scheme of clopidogrel-β-cyclodextrin loaded liposomal gel as shown in figure 2.

IN-VITRO EVALUATION

Globule size analysis:

Globule size of Clopidogrel-β-cyclodextrin loaded liposomes was determined using Malvern particle size analyzer (ZETASIZER 4000S, Japan).

Refractive index determination:

The refractive index of the different formulations was measured by using Abbe refractometer. 1 to 2 drops of the formulation sample was put in between the illuminating prism and measuring prism. Prior administration, the sample prism should clean with distilled water carefully by simple wiping. Then, light source was adjusted until clear scale was determined. Rotating knob was used to adjust the shadow boundary so that it was bright on the upper half and dark on the lower half. The scale needle was moved until get the half dark half bright of the background colour by observing through the eyepiece of the Abbe Refractometer. The readings were noted by counting the scale number.

Drug diffusion studies:

Franz diffusion cell method was applied using

phosphate buffer (pH 7.4) at room temperature for in vitro drug release studies. A cellophane membrane was used to carry out the study and soaked overnight in phosphate buffer at room temperature to be prepared. The membrane was then placed between donor and receptor compartment of diffusion cell with an exposed membrane surface area of 2.97 cm2 to the receptor compartment. The receptor compartment was filled with 16.4 ml of freshly prepared phosphate buffer (pH 7.4) maintained at 37 ± 0.5°C with constant stirring using a teflon coated magnetic stir bead. At appropriate time intervals, 2 ml aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution to maintain sink conditions.

The amount of drug released from liposomal gel was determined by HPLC method.

The method employed a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. The LC solution version 1.25 was used for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 1.0 ml/min using a mobile that is phase constituted of acetonitrile, 10mm AA (35:65, v/v), and detection was made at 240 nm. The mobile phase was prepared daily, filtered through a 0.45µm membrane filter (Millipore) and sonicated before use. A Thermo C18 column (25cm × 4.6mm i.d., 5µ) was used for the separation.

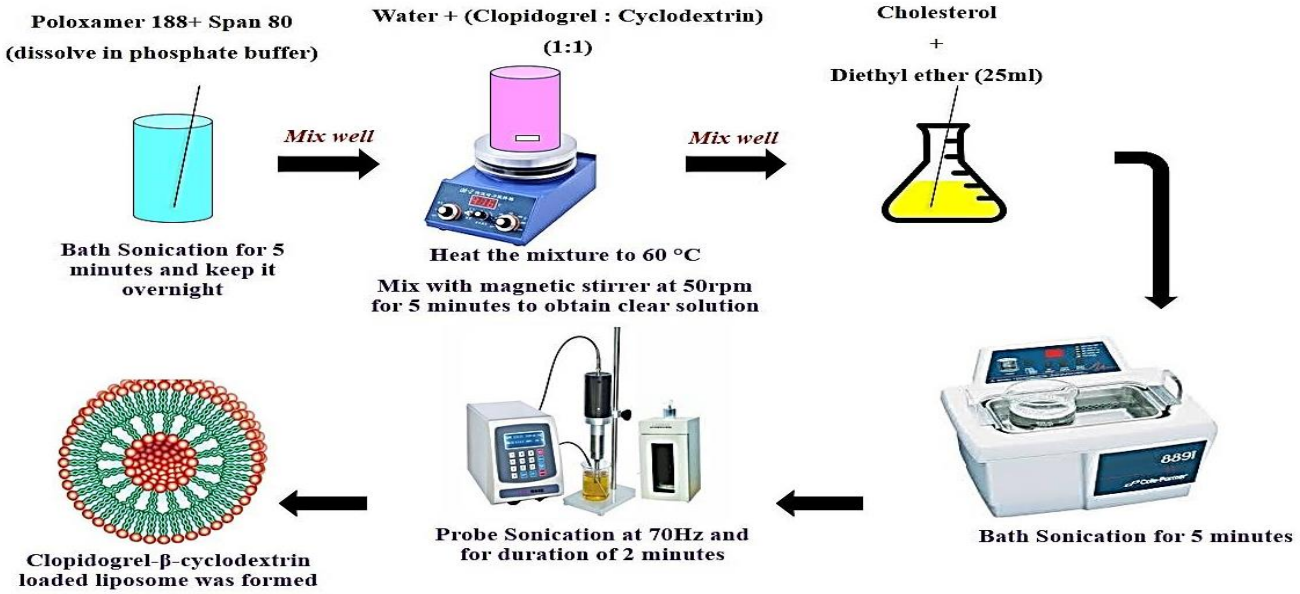

Figure-1: Showing the preparation of clopidogrel-β-cyclodextrin loaded liposome

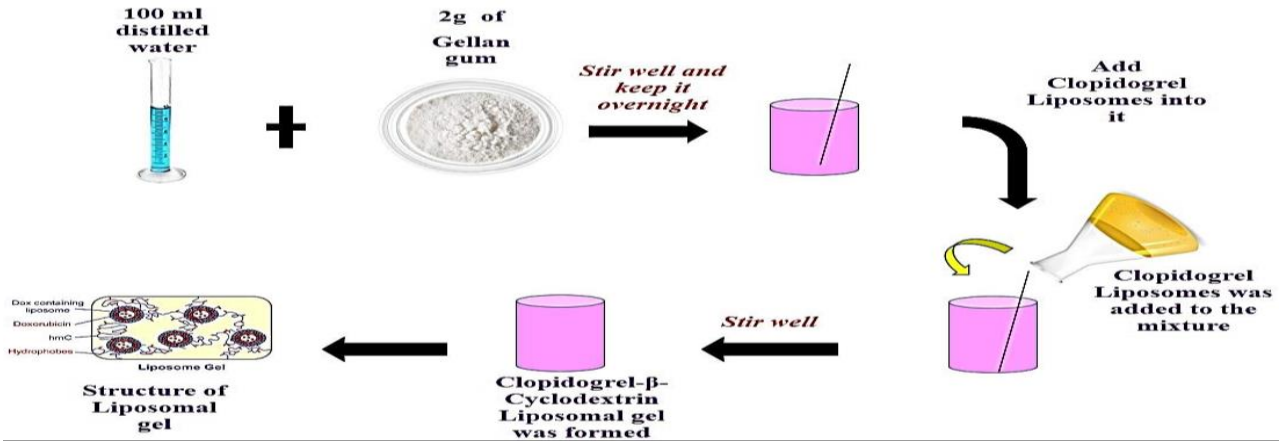

Figure-2: Showing the preparation of clopidogrel-β-cyclodextrin loaded liposomal gel

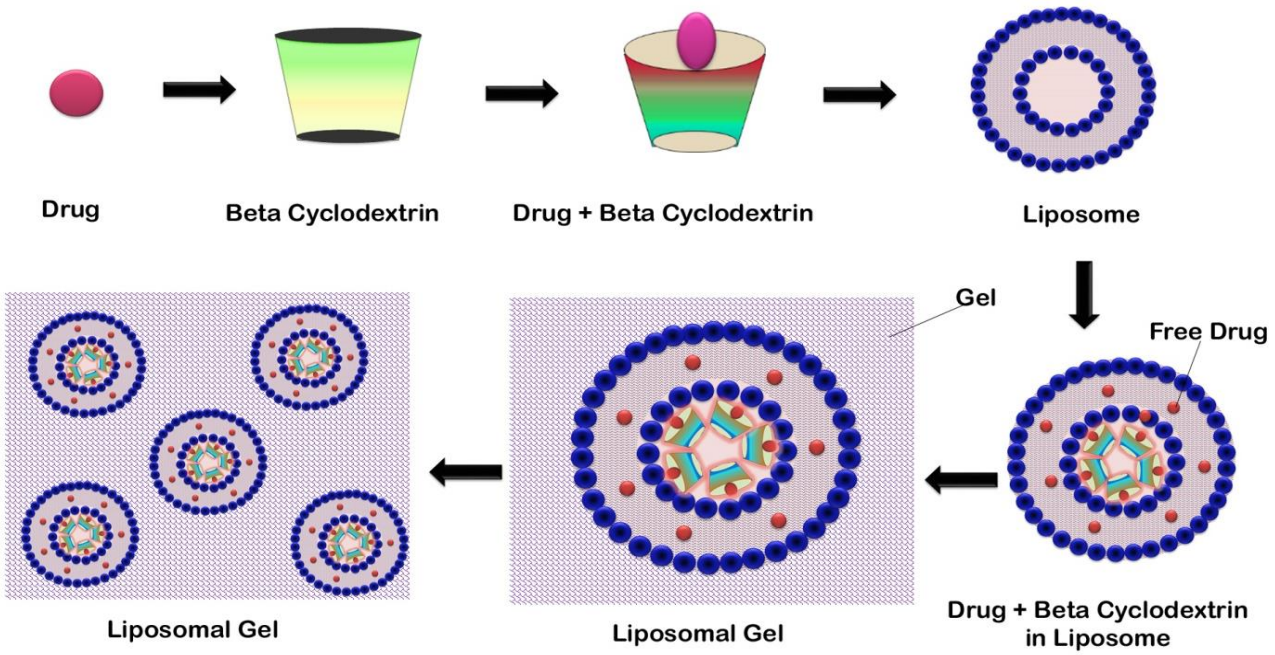

Figure 2a: Schematic diagram of clopidogrel: β-cyclodextrin loaded Liposomal gel

Viscosity determination:

The rheological studies of different formulations were performed by using Brookfield viscometer (DVII+ Model pro II type-USA. The spindle was immersed into the sample. Air bubbles were not allowed to be formed by tilting slightly while immersing. The spindle should not touch the bottom or sides of the container and should be centered. The process of selecting a spindle number and speed were based on trial and error. The motor was turned on and it was allowed for the indicated reading to stabilize. The time required for the stabilization depends on the characteristics of the sample fluid and the speed of the viscometer. The reading was obtained from the screen.

Gel strength determination:

2 marks were made on a 25ml measuring cylinder, an upper mark at 25th ml level and lower mark at 13th ml level. A metal rod with metal discs on both ends and metal cap through its body was set. The 25 ml measuring cylinder was filled with the formulation. The metal rod was then placed in the 25ml measuring cylinder and it was allowed to sink. The time taken for the metal rod to travel from the upper mark to the lower mark was recorded.

Spreadability determination:

For the determination of the spreadability, excess of sample was applied in between two glass slides and was compressed to uniform thickness by placing 1kg weight for 1-2 minutes on the glass slide. 50g of weight was added to the pan. The time in which the upper glass slides move over to the lower plate was taken as measure of spreadability (S).

pH determination:

The pH of all the formulations was determined by using Hanna instruments pH211 Microprocessor pH meter. Electrode of pH meter was dipped into different formulations. The pH of corresponding formulations was displayed on pH meter. In between each formulation, the electrode was washed with distilled water before next formulation was tested.

RESULTS AND DISCUSSION

Experimental Design:

In this work, we report the successful effect on the formulation of Clopidogrel-β-cyclodextrin loaded liposomal gel. Through preliminary experiments the Cholesterol (A), Diethyl ether (B) and Poloxamer 188 (C) were identified as the most significant variables influence the globule size, refractive index, controlled drug release, viscosity, gel strength and spreadability of Clopidogrel-β-cyclodextrin loaded liposomal gel. Among various design approaches, the Box-Behnken (BBD) has good design properties, little collinearity, rotatable or nearly rotatable; some have orthogonal blocks, insensitive to outliers and missing data. Does not predict well at the corners of

the design space. Use when region of interest and region of operability nearly the same. This Box-Behnken design is appropriate for exploring quadratic response surfaces and constructing second order polynomial models. The BBD consists of simulated center points and the set of points lying at the midpoint of each edge of the multi-dimensional cube. Twenty runs were essential for the response of surface methodology based on the BBD. Based on the experimental design, the factor combinations produced different responses as presented in Table 1. These results clearly indicate that all the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among the 15 runs. Data were analyzed using Stat-Ease Design-Expert software (DX11) to obtain analysis of variance (ANOVA), regression coefficients and regression equation.

The normality of the data could be proved through the normal % probability plot of the externally studentized residuals. If the points on the plot lie on a straight line, the residuals are normally distributed as confirmed in Figure.3a, b, c, d, e and f.

The assumption of constant variance was tested by plotting externally studentized residual versus predicted values as illustrated in above figures. The studentized residuals are located by dividing the residuals by their standard deviations. According to evident from this figure 4a, b, c, d, e and f, the points are scattered randomly between the outlier detection limits -3.5 to +3.5 and -4.5 to +4.5.

The Residuals vs. Predicted (figure 4a-f) and Residuals vs. Run (figure5a-f) were scattered randomly. From the results it can therefore be seen that the model is suitable for use and can be used to identify the optimal parameters. R1, R2, R3, R4, R5 and R6 results are quite satisfactory. Also, a high correlation between observed and predicted data indicates their low discrepancies.

Table-1: List of Independent variable and Dependent variables in Box-Behnken design

Independent variable		**Levels**					
Variable	Name	Units	Type	Changes	Std. Dev.	Low	High
A	Cholestrol	mg	Factor	Easy	0	100	120
B	Diethyl ether	ml	Factor	Easy	0	25	30
C	Poloxamer188/ Span80 (1:3)	mg	Factor	Easy	0	4000	5000

Dependent variable

Variable	Name	Units	Type	Std. Dev.	Low	High
R1	Globule Size	µm	Response	0.329492	1.16	2.8
R2	Refractive Index		Response	0.00134849	1.331	1.336
R3	Drug Loading Efficacy	%	Response	1.04413	86.16	95.66
R4	Viscosity	cps	Response	2105.98	31493	42891
R5	Gel Strength	seconds	Response	17.3737	6.39	87.42
R6	Spreadability	g.cm/sec	Response	10.5929	8.7	60.25

Table-2: Factorial design of Clopidogrel formulation

Run	A: Cholesterol mg	B: Diethyl ether ml	C: Poloxamer 188/Span80 (1:3) mg	Globule Size µm	Refractive Index	After 12th hour-CDR %	Viscosity cps	Gel Strength Seconds	Spreadability g.cm/sec
1	120	25	5000	2	1.385	82.14	38132	6.39	60.25
2	120	30	5000	1.4	1.386	80.64	38632	12.41	22.87
3	100	25	5000	2	1.331	84.32	38052	7.41	52.43
4	100	30	5000	1.32	1.334	83.61	37432	24.74	19.64
5	110	31.70	4500	2.8	1.343	78.22	37792	15.66	25.79
6	100	30	4000	2.32	1.334	75.36	36752	33.83	8.7
7	120	25	4000	1.36	1.38	75.19	37252	16.94	35.33
8	100	25	4000	2.48	1.335	76.85	35052	54.5	19.34
9	110	27.5	4500	1.48	1.343	77.34	36412	46.2	24.75
10	110	27.5	4500	1.48	1.323	77.58	36411	46.3	24.74
11	110	27.5	4500	1.48	1.332	76.52	36413	46.1	24.72
12	110	23.29	4500	1.64	1.333	77.89	36792	16.05	19.19
13	110	27.5	4500	1.48	1.344	77.52	37412	46.1	24.73
14	120	30	4000	2	1.335	76.24	37891	87.42	29.16
15	110	27.5	5340.9	2.08	1.344	85.76	36571	16.38	12.53
16	110	27.5	4500	1.48	1.333	77.61	36413	46.2	24.71
17	93.18	27.5	4500	1.16	1.334	77.69	34913	12.26	16.77
18	110	27.5	4500	1.48	1.341	78.61	36411	46.4	24.72
19	110	27.5	3659.1	1.76	1.342	70.41	35332	28.76	25.58
20	126.8	27.5	4500	1.16	1.432	77.68	37493	11.32	30.55

The plot of predicted response versus actual responses performs the same function, albeit graphically and also helps to detect the points where the model becomes inadequate to predict the response of the system. This is the simplest graph which shows that the selected model is capable of predicting the response satisfactorily within the range of data set as shown in the Figure 6a, b, c, d, e and f.

The transformation parameter, λ, is chosen such that it maximizes the log-likelihood function. The maximum likelihood estimate of λ agrees to the value for which the squared sum of errors from the fitted model is a minimum. This value of λ is determined by fitting a numerous value of λ and choosing the value corresponding to the minimum squared sum of errors. t can also be chosen graphically from the Box-Cox normality plot. Value of λ= 1.00 indicates that no transformation needed and produces result identical to original data shown in Figure 7a, b, c, d, e and f.

This is a plot of the residuals versus factor A (Cholesterol) for response 1-response 6 as illustrated in Figure 8a, b, c, d, e and f. It checks whether the variance not accounted for by the model is different for different levels of a factor. If all is okay, the plot should exhibit a random scatter. Pronounced curvature may indicate a systematic contribution of the independent factor that is not accounted for by the model. The residuals are well distributed as confirmed in Figure 8a-f.

Cook's distance is a measure of how much the entire regression function changes when the i^{th} point is not included for fitting the model. It is essentially the sum of differences in predictions at every point caused by leaving a point out for fitting the model. Relatively large values are associated with cases with high leverage and large studentized residuals.

Cases with large Di values relative to the other cases should be investigated. A large value in D may be due to large r, large leverage, or both. An equivalent interpretation of D is as a standardized weighted distance between the vector of regression coefficients obtained from the full model and the vector obtained after deleting the i^{th} case. If the value of D is substantially less than 1, deleting the i^{th} case will not change the estimates of the regression coefficients very much. Cook's distance graph are as illustrated in Figure 9a, b, c, d, e and f.

Leverage is a measure of how much each point influences the model fit. If a point has a leverage of 1.0, then the model exactly fits the observation at that point. That point controls the model. Leverage Limits **is** a run with leverage greater than 2 times the average is generally regarded as having high leverage. Such runs have few other runs near them in the factor space. The average leverage is the number of terms in the model divided by the number of runs in the design. Leverage of a point varies from 0 to 1 and indicates how much an individual design point influences the model's predicted values. A leverage of 1 means the predicted value at that particular case will exactly equal the observed value of the experiment, i.e., the residual will be 0. The maximum leverage an experiment can have is 1/k, where k is the number of times the experiment is replicated. The Leverage vs. Run graph are shown in figure 10a, b, c, d, e and f.

DFFITS is a measure of how much the prediction changes at the i^{th} point when the i^{th} point is not included for fitting the model. DFFITS measures the influence the i^{th} observation has on the predicted value. The DFFITS vs. Run graph are as shown in figure 11a, b, c, d, e and f.

DFBETAS is a measure of how much a coefficient estimate changes when the i^{th} point is not used to fit the model. There are separate DFBETA plots for each term in the model. This statistic is calculated for each coefficient at each run. The influence tool has a pull-down to pick which term's graph is shown. Shows the influence the i^{th} observation has on each regression coefficient. The DFBETAS, i is the number of standard errors that the i^{th} coefficient changes if the i^{th} observation is removed. The DFBETAS vs. Run graph are as shown in figure 12a, b, c, d, e and f.

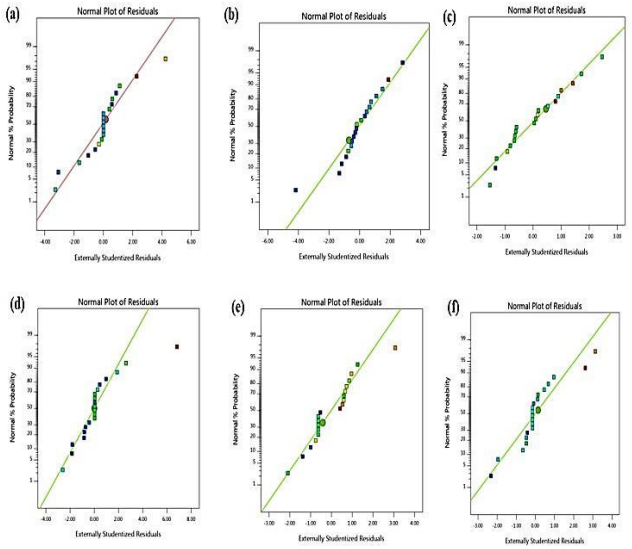

Figure-3: (a) Normal % probability plot of the externally studentized residuals (R1). (b) Normal % probability plot of the externally studentized residuals (R2). (c) Normal % probability plot of the externally studentized residuals (R3). (d) Normal % probability plot of the externally studentized residuals (R4). (e) Normal % probability plot of the externally studentized residuals (R5). (f) Normal % probability plot of the externally studentized residuals (R6).

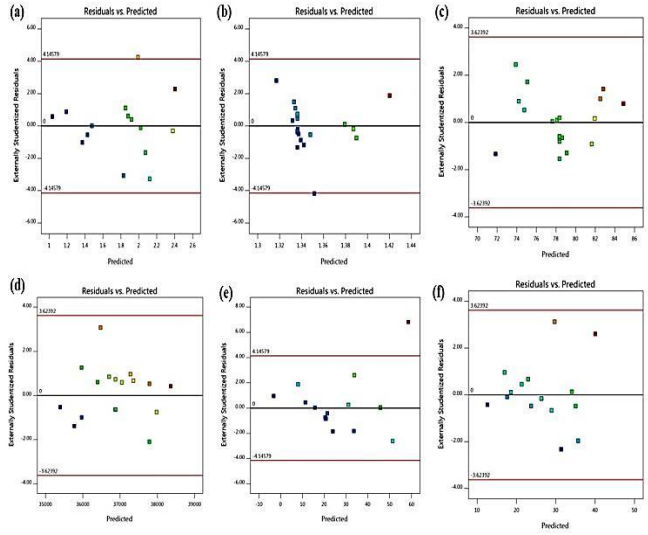

Figure-4: (a) Residuals vs. Predicted (R1). (b) Residuals vs. Predicted (R2). (c) Residuals vs. Predicted (R3). (d) Residuals vs. Predicted (R4). (e) Residuals vs. Predicted (R5). (f) Residuals vs. Predicted (R6)

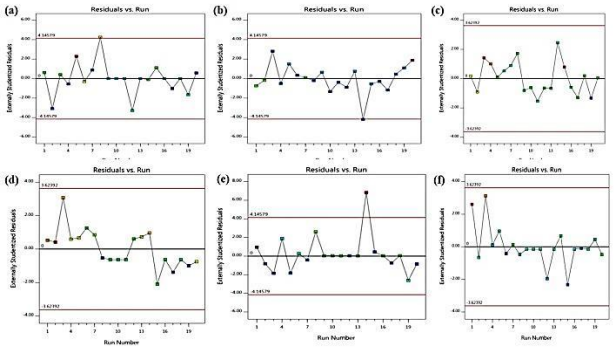

Figure-5 : (a) Residuals vs. Run (R1). (b) Residuals vs. Run (R2). (c) Residuals vs. Run (R3). (d) Residuals vs. Run (R4). (e) Residuals vs. Run (R5). (f) Residuals vs. Run (R6).

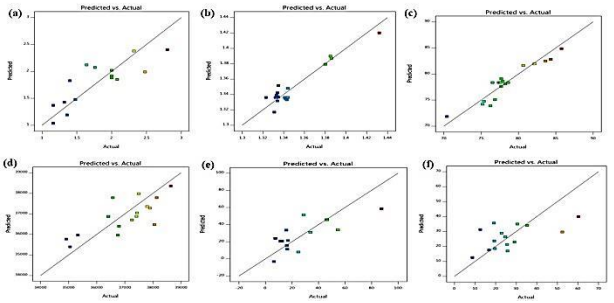

Figure-6: (a) Predicted vs. Actual (R1). (b) Predicted vs. Actual (R2). (c) Predicted vs. Actual (R3). (d) Predicted vs. Actual (R4). (e) Predicted vs. Actual (R5). (f) Predicted vs. Actual (R6).

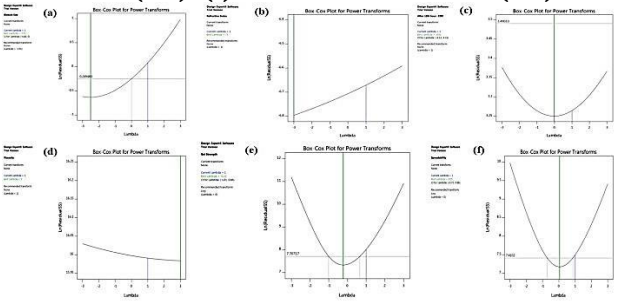

Figure-7: (a) Box-Cox Plot (R1). (b) Box-Cox Plot (R2). (c) Box-Cox Plot (R3). (d) Box-Cox Plot (R4). (e) Box-Cox Plot (R5). (f) Box-Cox Plot (R6).

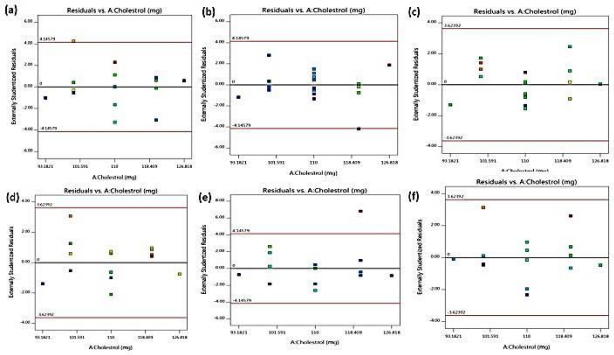

Figure-8: (a) Residual vs. Cholesterol (R1). (b) Residual vs. Cholesterol (R2). (c) Residual vs. Cholesterol (R3). (d) Residual vs. Cholesterol (R4). (e) Residual vs. Cholesterol (R5). (f) Residual vs. Cholesterol (R6).

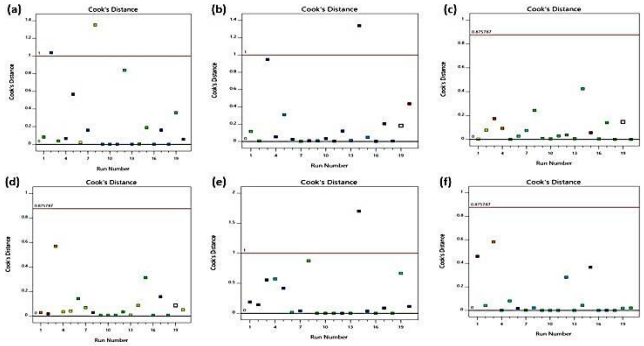

Figure-9: (a) Cook's Distance (R1). (b) Cook's Distance (R2). (c) Cook's Distance (R3). (d) Cook's Distance (R4). (e) Cook's Distance (R5). (f) Cook's Distance (R6).

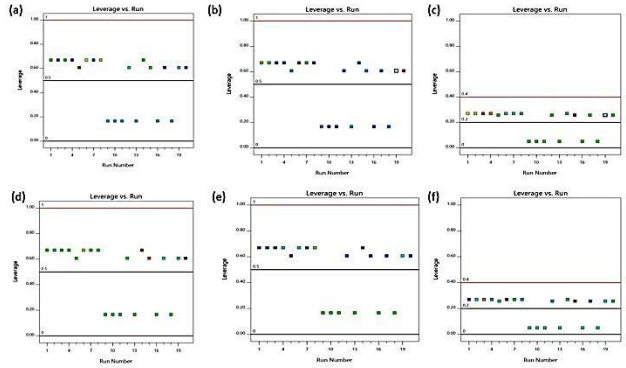

Figure-10: (a) Leverage vs. Run (R1). (b) Leverage vs. Run (R2). (c) Leverage vs. Run (R3). (d) Leverage vs. Run (R4). (e) Leverage vs. Run (R5). (f) Leverage vs. Run (R6).

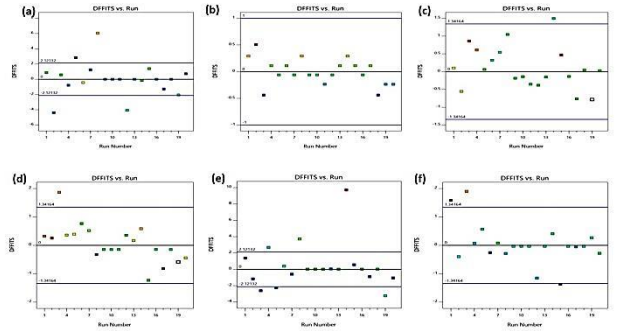

Figure-11: (a) DFFITS vs. Run (R1). (b) DFFITS vs. Run (R2). (c) DFFITS vs. Run (R3). (d) DFFITS vs. Run (R4). (e) DFFITS vs. Run (R5). (f) DFFITS vs. Run (R6).

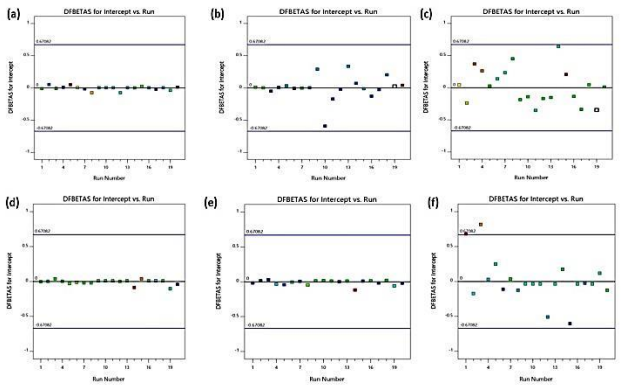

Figure-12: (a) DFBETAS vs. Run (R1). (b) DFBETAS vs. Run (R2). (c) DFBETAS vs. Run (R3). (d) DFBETAS vs. Run (R4). (e) DFBETAS vs. Run (R5). (f) DFBETAS vs. Run (R6).

Globule size analysis of Clopidogrel-β-cyclodextrin loaded liposomes was found to be in the range of 1.16-2.80 μm as shown in table 2. The factorial equation for globule size exhibited a good correlation coefficient (1.000) and the Model F value of 2.82 which implies the model is not significant. Values of "Probe>F" less than 0.0500 indicate model terms are significant. The Model F-value of 2.82 implies there is a 6.09% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case B^2 is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better. Adel Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 5.877 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the globule size was clarified using the perturbation and 3D response surface plots. Main effects of each A, B and C on globule size are revealed in figure 13a. All of the variables possess interactive effects on the response R1. For illustrating the effects of interaction among independent variables of the response R1, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R1 are presented in figure 13b, c and d.

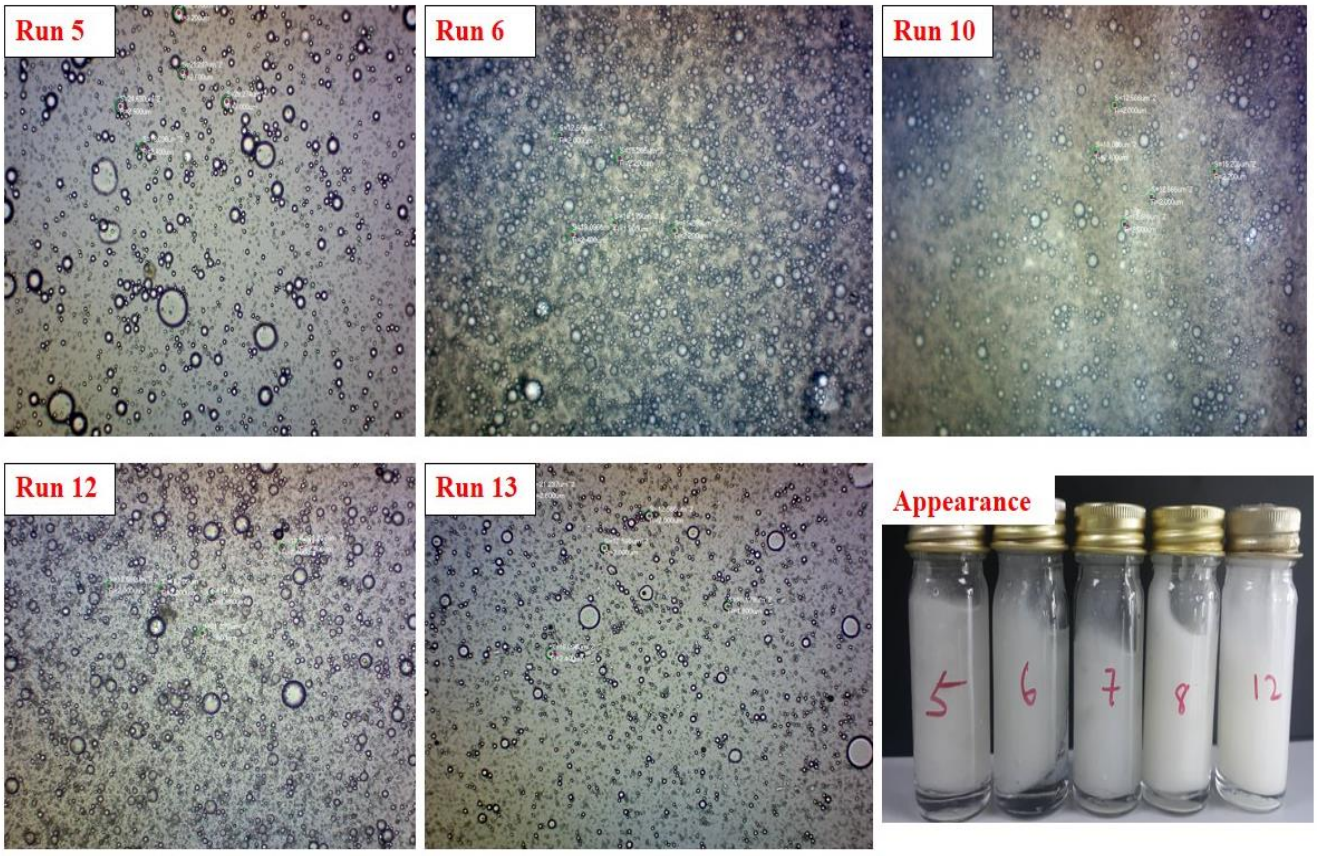

Figure-12(g): Globule size analysis of Clopidogrel-β-cyclodextrin loaded liposomal gel

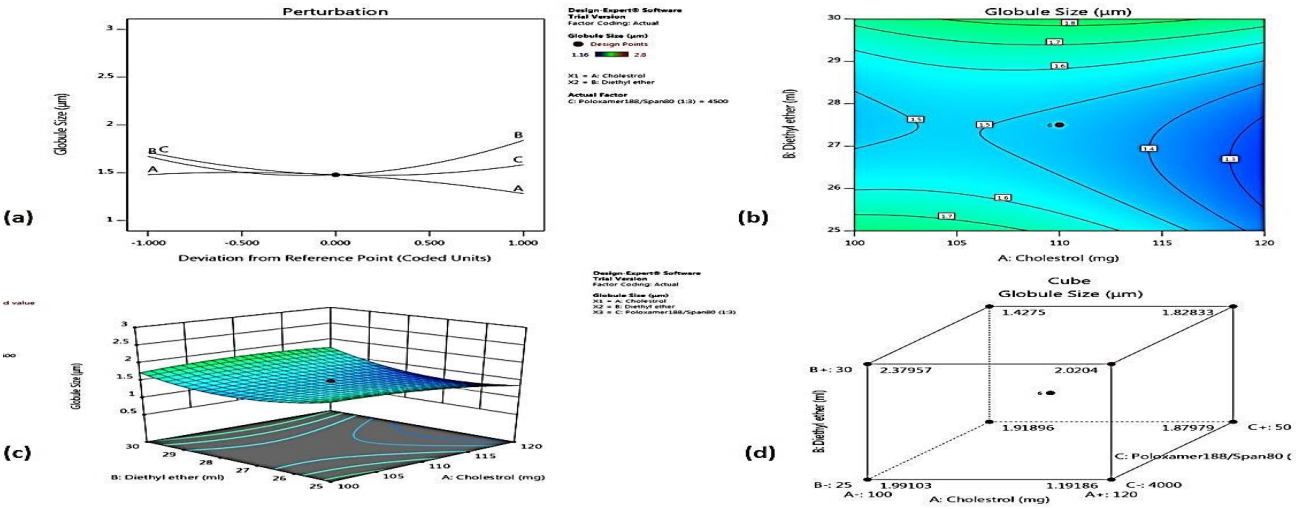

Figure-13: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on globule size (R1). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the globule size (R1). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the globule size (R1). (d) 3D cube plot of Box-Behnken design (R1).

The accurate model produced for refractive index of Clopidogrel-β-cyclodextrin loaded liposomal gel (R2) was found to be significant with F-value of 10.82 ($p<0.0001$) and R^2 value of 0.9069. The Model F-value of 10.82 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, A^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 2.82 implies the Lack of Fit is not significant relative to the pure error. There is a 14.01% chance that a Lack of Fit F-value this large could occur due to noise. The Predicted R^2 of 0.4173 is not as close to the Adjusted R^2 of 0.8230 as one might normally expect; i.e. the difference is more than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 12.956 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the refractive index was clarified using the perturbation and 3D response surface plots. Main effects of each A, B and C on refractive index are revealed in figure 14a. All of the variables possess interactive effects on the response R2. For illustrating the effects of interaction among independent variables of the response R2, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R2 are presented in figure 14b, c and d.

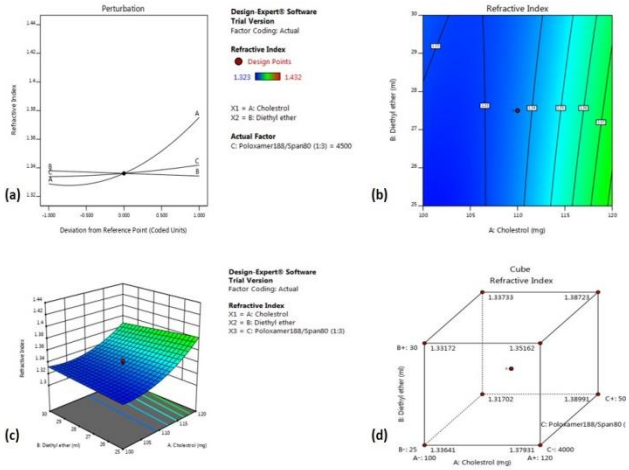

Figure-14: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on refractive index (R2). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the refractive index (R2). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the refractive index (R2). (d) 3D cube plot of Box-Behnken design (R2).

The accurate model produced for R3 was found to be significant with F-value of 42.34. The Model F-value of 42.34 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case C is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 4.88 implies the Lack of Fit is significant. There is only a 4.65% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad, we want the model to fit. The Predicted R^2 of 0.8125 is in reasonable agreement with the Adjusted R^2 of 0.8671; i.e. the difference is less than 0.2. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 22.775 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the CDR at 12th hour was clarified using the perturbation and 3D response surface plots. Main effects of each A, B and C on CDR at 12th hour are revealed in figure 16a. All of the variables possess interactive effects on the response R3. For illustrating the effects of interaction among independent variables of the response R3, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R3 are presented in figure 16b, c and d.

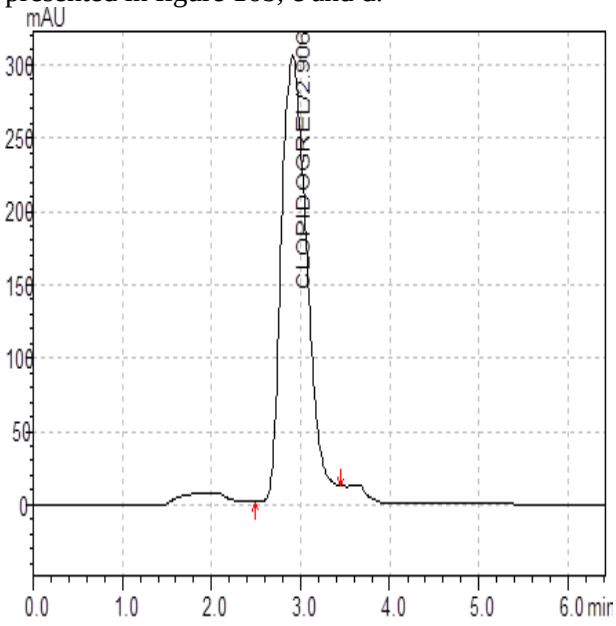

Figure-15: Typical HPLC chromatogram

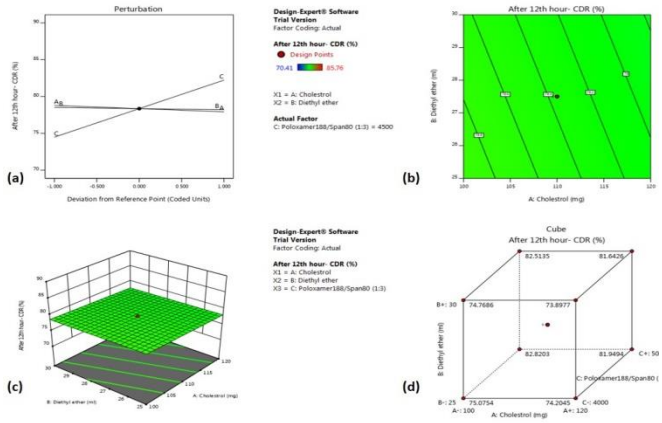

Figure-16: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on CDR (R3). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the CDR (R3). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the CDR (R3). (d) 3D cube plot of Box-Behnken design (R3).

The accurate model produced for R4 (viscosity) was found to be significant with F value of 6.65 and p-value of 0.0040. The Model F-value of 6.65 implies the model is significant. There is only a 0.40% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 4.35 implies there is a 5.85% chance that a Lack of Fit F-value this large could occur due to noise. Lack of fit is bad, we want the model to fit. This relatively low probability (<10%) is troubling. The Predicted R^2 of 0.2291 is not as close to the Adjusted R^2 of 0.4716 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.936 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the viscosity was clarified using the perturbation and 3D

response surface plots. Main effects of each A, B and C on viscosity are revealed in figure 17a. All of the variables possess interactive effects on the response R4. For illustrating the effects of interaction among independent variables of the response R4, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R4 are presented in figure 17b, c and d.

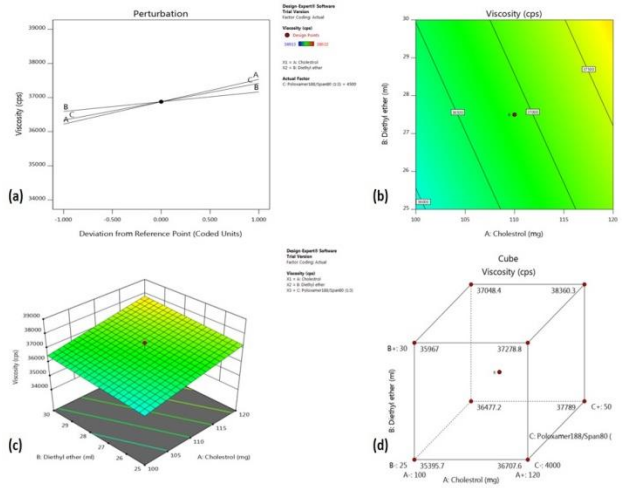

Figure-17: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on viscosity (R4). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the viscosity (R4). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the viscosity (R4). (d) 3D cube plot of Box-Behnken design (R4).

The accurate model produced for R5 (gel strength) was found to be not significant with F-value of 1.94 and p-value of 0.1579. The Model F-value of 1.94 implies the model is not significant relative to the noise. There is a 15.79% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case C is a significant model term. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The Lack of Fit F-value of 44171.65 implies the Lack of Fit is significant. There is only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad, we want the model to fit. A negative Predicted R^2 implies that the overall mean may be a better predictor of your response than the current model. In some cases, a higher order model may also predict better. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 5.025 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the gel strength was clarified using the perturbation and 3D response surface plots. Main effects of each A, B and C on gel strength are revealed in figure 18a. All of the variables possess interactive effects on the response R5. For illustrating the effects of interaction among independent variables of the response R5, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R5 are presented in figure 18a,b, c and d.

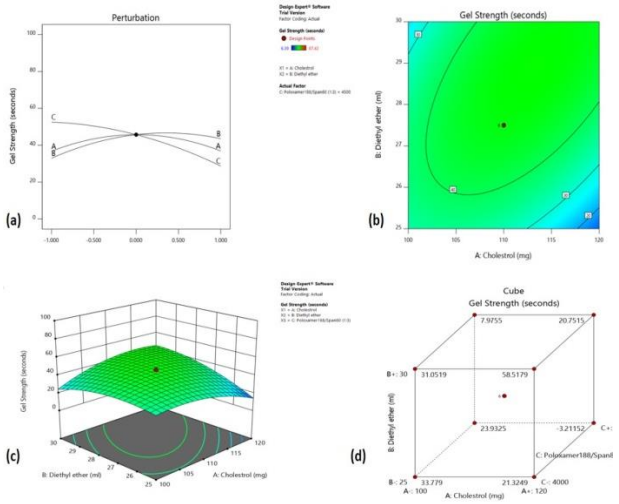

Figure-18: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on gel strength (R5). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the gel strength (R5). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the gel strength (R5). (d) 3D cube plot of Box-Behnken design (R5).

The accurate model produced for spreadability (R6) was found to be significant with F-value of 2.70 and p-value of 0.0805. The Model F-value of 2.70 implies there is a 8.05% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support

hierarchy), model reduction may improve your model. The Lack of Fit F-value of 753287.43 implies the Lack of Fit is significant. There is only a 0.01% chance that a Lack of Fit F-value this large could occur due to noise. Significant lack of fit is bad, we want the model to fit. The Predicted R^2 of 0.5739 is not as close to the Adjusted R^2 of 0.8239 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 5.789 indicates an adequate signal. The effect of the main and interactive consequences of independent variables on the spreadability was clarified using the perturbation and 3D response surface plots. Main effects of each A, B and C on spreadability are revealed in figure 19a. All of the variables possess interactive effects on the response R6. For illustrating the effects of interaction among independent variables of the response R6, the 2D response surfaces, 3D contour plots, 3D cube plot and 2D Interaction plot of the response R6 are presented in figure 19b, c and d.

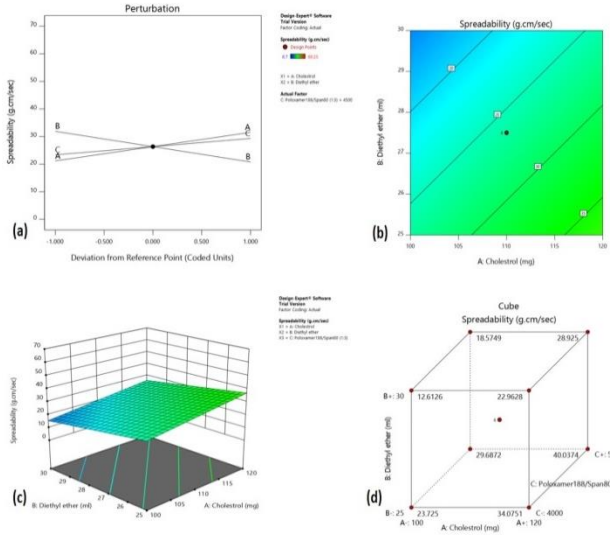

Figure-19: (a) Perturbation plot showing the main effect of Cholesterol (A), Diethyl ether (B) and Poloxamer 188/Span 80 (C) on spreadability (R6). (b) 2D response surface plot presenting the interaction between the Cholesterol and Diethyl ether affecting the spreadability (R6). (c) 3D response surface plot presenting the interaction between the cholesterol and diethyl ether affecting the spreadability (R6). (d) 3D cube plot of Box-Behnken design (R6).

R9, R10 and R16 batches code of Clopidogrel-β-Cyclodextrin enriched liposomal gel were formulated according to the optimized levels after the polynomial equations relating the independent and dependent variables was constructed. The conditions of optimization were acquired by setting constraints on both the independent and dependent variables. The observed values were close to the expected values of the optimized process. This was described in Table 3.

Table-3: Optimized values obtained by the constraints applies on R1 to R6

Independent variables	Values	Predicted values						Code	Observed values					
		Globule Size	Refractive index	CDR After 12 hours	Viscosity	Gel Strength	Spreadability		Globule size	Refractive index	CDR After 12^{th} hours	Viscosity	Gel Strength	Spreadability
Cholesterol	110	1.4397	1.34227	77.6831	36868.9	44.7329	28.3847	9	1.48	1.343	77.34	36412	46.2	24.75
Diethyl ether	27.5							10	1.48	1.323	77.58	36411	46.3	24.74
Poloxamer 188/Span 80 (1:3)	4500							16	1.48	1.333	77.61	36413	46.2	24.71

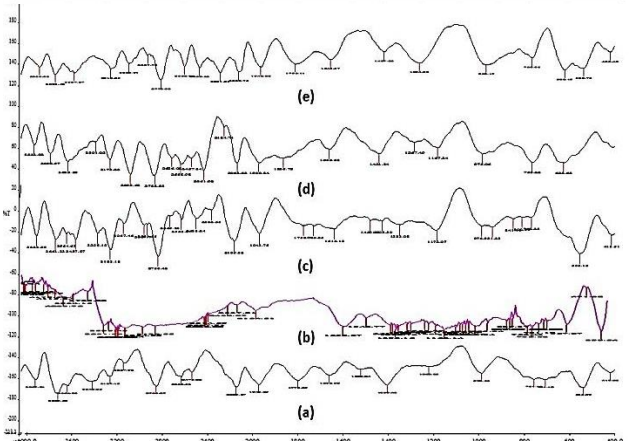

Figure-20: (a) FTIR spectrum of Clopidogrel. (b) FTIR spectrum of β-Cyclodextrin. (c) FTIR spectrum of Cholesterol (d) FTIR spectrum of P 407. (e) FTIR spectrum of Clopidogrel + β-Cyclodextrin + Cholesterol + P 407

Figure 20 shows the FT-IR spectra of pure Clopidogrel, Cholesterol, P 407, Cholesterol + P407, Clopidogrel + β-Cyclodextrin + Cholesterol + P 407. The spectrum of pure clopidogrel showed principal peaks at wavenumbers (cm^{-1}) of 3837.01, 3639.66, 3554.03, 3342.25, 3187.19, 3067.98, 2785.27, 2566.53, 2475.00, 2089.67, 1945.29, 1775.69,

1638.50, 1502.04, 1389.43, 1206.25, 978.56, 751.13, 701.12, 535.99 and 419.63. The spectrum of pure cholesterol showed principal peaks at wavenumbers (cm^{-1}) of 3823.99, 3661.33, 3564.63, 3487.87, 3295.19, 3183.18, 3067.46, 2887.74, 2861.15, 2768.42, 2665.82, 2563.07, 2438.24, 2302.65, 2105.32, 1943.75, 1750.01, 1706.05, 1616.13, 1459.18, 1408.30, 1333.08, 1172.07, 976.13, 931.23, 841.19, 802.76, 762.28, 552.16 and 413.51. The spectrum of pure P 407 showed principal peaks at wavenumbers (cm^{-1}) of 3833.92, 3690.57, 3536.59, 3301.02, 3173.20, 2995.68, 2788.53, 2636.08, 2558.98, 2467.34, 2361.92, 2184.71, 2068.62, 1939.84, 1835.75, 1635.68, 1421.34, 1267.49, 1167.34, 975.06, 759.22 and 623.68.

The spectrum of Clopidogrel + β-Cyclodextrin + Cholesterol + P 407 showed principal peaks at wavenumbers (cm^{-1}) of 3836.85, 3695.48, 3527.87, 3212.65, 3050.91, 2887.72, 2770.90, 2562.92, 2429.66, 2251.05, 2089.75, 1948.00, 1795.11, 1643.37, 1407.28, 1254.28, 960.17, 760.95, 620.19, 536.73 and 420.40.

Clopidogrel-β-Cyclodextrin enriched liposomal gel formed the polymer active with no disturbance in the functional group. Hence, a polymerized active constituent has no change in effect after polymerization.

CONCLUSION

In this study, neat Clopidogrel-β-Cyclodextrin enriched liposomal gels were successfully prepared. Microscopic images showed uniform morphology of the globule size with an average diameter range of 1.16-2.80 μm. Clopidogrel-β-Cyclodextrin enriched liposomal gel were fabricated and analyzed. The parameters examined were globule size, refractive index, CDR at 12^{th} hour, viscosity, gel strength and spreadability. Optimization was done based on the results obtained and the results of the prepared Clopidogrel-β-Cyclodextrin enriched liposomal gel coincide with the expected values of various parameters.

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