International Journal for Pharmaceutical Research Scholars (IJPRS)

V-5, I-4, 2016 ISSN No: 2277 - 7873

RESEARCH ARTICLE

Formulation and Optimization of Novel Elastic Nano-Vesicular Carrier of Vancomycin Hydrochloride for Enhanced Corneal Permeability

Meenakshi Chauhan, Shipra Malik*

Department of Pharmaceutics, Delhi Institute of Pharmaceutical Sciences & Research, DPSARU, Pushp Vihar, Sector-3, New Delhi-110017.

Manuscript No: IJPRS/V5/I4/00147, Received On: 31/10/2016, Accepted On: 04/11/2016

ABSTRACT

The aim is to entrap vancomycin hydrochloride used topically for endophthalmitis, in a novel nanovesicular carrier system named spanlastics® to ensure the sustained and intravitreal delivery of the drug in eye over a period of 24 hours to overcome the problem of frequent dosing. To optimize the levels of different process parameters influencing size, polydispersity index (PDI) and encapsulation efficiency (EE %), an experimental design of 34 runs containing 5 central points was selected according to the Box-Behnken statistical design for three factors X_1 – Span: tween 80 ratio, X_2 – amount of drug, X_3 – stirring speed at three levels with one categorical factor, X_4 – type of span at two levels. The Design Expert software suggested an optimized formula to be prepared whose overall desirability was 0.950 for span 40. The optimized formulation resulted in entrapment efficiency of 88.12%, vesicle size of 180.5 nm and PDI of 0.21. In-vitro release study of the optimized spanlastics® suggested extended release for 24 hours and followed higuchi release model with R^2 value of 0.9963. Further ex-vivo release study showed better corneal permeability and sustained release for 24 hours. Ocular safety of the surfactant based vesicular carrier was also established by draize irritancy test.

KEYWORDS

Ocular Delivery, Spanlastics®, Span 40, Span 60, Tween 80

INTRODUCTION

Endophthalmitis is an inflammatory reaction of intraocular fluids or tissues. When caused by microbial organisms, infectious endophthalmitis often results in severe visual loss. Infectious endophthalmitis is classified by the events leading to the infection and by the timing of the clinical diagnosis. The broad categories include postoperative endophthalmitis (acute, delayed-onset, conjunctival filtering-bleb associated), post-traumatic endophthalmitis and endogenous endophthalmitis.

***Address for Correspondence:**
Shipra Malik,
Department of Pharmaceutics, Delhi Institute of Pharmaceutical Sciences & Research, DPSARU, Pushp Vihar, Sector-3, New Delhi-110017, India.
E-Mail Id: Shipra.malik92@gmail.com

Postoperative endophthalmitis is the most frequent category, accounting for greater than 70% of cases. Antibiotics can be delivered to the eye by several routes, including direct intraocular injection, systemic administration, periocular injection, and topical application. Intraocular vancomycin (1 mg) has been a commonly used regimen for the initial empiric treatment of presumed bacterial endophthalmitis. Significant intraocular levels of antibiotics can be achieved with frequent administration of highly concentrated solutions. Dose of vancomycin hydrochloride eye drops is 25-50 mg/mL, which needs to be instilled every hour so that concentration of the drug permeating cornea remains above minimum inhibitory concentration. Topical and intraocular

administration is generally well tolerated, although corneal epithelial toxicity is common. When the drug is used topically in a 5% concentration, patients generally complain of severe burning on administration; this can be alleviated by reducing the concentration to 1.5% and 2.5%.[1]

Traditional route of topical drug administration is also associated with several other complications like extensive pre-corneal drug loss due to high tear fluid turn over, non-productive absorption, drainage through nasolacrimal duct, impermeability of corneal epithelium, transient pre-corneal residence time and metabolism of drug by posterior segment enzymes.

Recently the use of colloidal drug delivery systems such as nanoparticles, nanoemulsions and liposomes, has received much attention as a way to enhance bioavailability of drugs both topically and systemically. Liposomes as a drug carrier system can target the retina when administered topically in form of eye drops.[2]

Van den Bergh *et al.* (2001) introduced a second generation of elastic vesicles mainly consisting of non-ionic surfactants. Extrusion measurements indicated an increase in elasticity of vesicle bilayers as the molar content of PEG-8-L was increased from 10 to 90%[3].

Kakkar and Kaur *et al.* (2011) introduced the term Spanlastics® to express the surfactant based elastic vesicles[4]. Spanlastics® of Ketoconazole were developed to enhance their corneal permeation and for targeting topical administered drug to the posterior segment of eye. Fluorescent vesicles labeled with 6-carboxyfluorescin when applied topically to the rabbit eye were observed in vitreous and internal eye tissue post 2 hours of topical administration.

They were also proved to be safe in terms of genotoxicity (Ames test) and cytotoxicity (MTT assay). ElMeshad and Mohsen (2014) developed Spanlastics® of Itraconazole, which is a triazole antimycotic agent with poor solubility and low corneal permeability, to prolong the retention time of the formulation in eye and enhance corneal permeation[5].

MATERIALS AND METHODS

Vancomycin Hydrochloride was gifted by Enaltec labs Pvt. Ltd., Maharashtra, India. Other materials used in the study are span 40 (Lobachemie Pvt Ltd, Mumbai), span 60 (Lobachemie Pvt Ltd, Mumbai), tween 80 (Lobachemie Pvt Ltd, Mumbai), sephadex G-50 (Sigma Aldrich, USA), absolute ethanol (Merck Synthesis Grade, Mumbai), methanol and orthophosphoric acid (Lobachemie Pvt Ltd, Mumbai). All other ingredients were of pure analytical grade.

Preparation of Vancomycin loaded Spanlastics®

Span 40 and span 60 were used as non-ionic surfactants and tween 80 as edge activator at different span: tween ratio using different concentrations of vancomycin hydrochloride to obtain the optimized formulation. Spanlastics® were prepared by *ethanol injection method*. Tween 80 was accurately weighed and dissolved in 10 mL of distilled water heated to the temperature of 80^0C. Spans were accurately weighed and dissolved in 4 mL of ethanol and sonicated for 5 minutes at 50^0C to obtain a clear solution, which was added drop wise using a 30 gauze syringe at a constant rate (1 mL/min) to the aqueous tween solution containing different concentration of drug which was being stirred at different speeds. Stirring was continued for 30 minutes at 80^0C then another 30 minutes at room temperature. Finally, formulation was made up to 10 mL using distilled water.[5]

Box-Behnken Design

In the present study, spanlastics® of vancomycin hydrochloride were optimized using response surface methodology (Design-Expert® trial version 9 software). A box-behnken statistical design with 3 numeric factors at 3 levels and one categorical factor at 2 levels was selected for optimization. It gave 17 runs for 3 factors varied at 3 levels and for each level of a categorical factor runs were duplicated as shown in table 2. Independent factors, X_1: span: tween 80, X_2: amount of drug, X_3: stirring speed were varied at 3 levels coded as -1, 0 and +1 along with one

categorical factor i.e. type of span X_4 which was varied at 2 levels and three response parameters were selected, entrapment efficiency Y_1, PDI (Poly Dispersity Index) Y_2 and size Y_3, as dependent variables as described in table 1.

Table 1: Design parameters and experimental conditions for Box-Behnken design

	Levels		
	-1	0	1
Independent Variables			
X_1 = Span: Tween 80 weight ratio	7:3	8:2	9:1
X_2 = Amount of drug	1 mg/mL	2 mg/mL	3 mg/mL
X_3 = Stirring Speed	800	1200	1600
	Categorical Factor		
X_4 = Type of Span	Span 40	Span 60	
Dependent Variables	**Target**		
Y_1 = Entrapment Efficiency	Maximum		
Y_2 = PDI	Minimum		
Y_3 = Size	In Range		

This design was suitable for exploring quadratic response surfaces and constructing second-order polynomial models with Design Expert software (Design-Expert® trial version 9 software). The polynomial equation generated by this experimental design is given as,

$$Y_0 = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where Y_0 is the dependent variable, b_0 is the intercept, b_1 to b_{33} are the regression coefficients computed from the observed experimental values of Y, X_1, X_2 and X_3 are the coded levels of independent variables. The terms XiXj (i, j=1, 2, 3, 4), and Xi2 (i = 1, 2, 3 or 4) represent the interaction and quadratic terms, respectively.[6,7,8]

Shape and Size Distribution

The vesicle size (Z-average) and size distribution i.e., polydispersity index (PDI) of the prepared formulations were determined by Dynamic Light Scattering technique (DLS), using a computerized inspection system (Malvern Zetasizer, Nano-ZS, Malvern, U.K.) with DTS (Nano) software, at a wavelength of 633 nm and at a scattering angle of 173^0, at a room temperature of 25^0C, after diluting the sample (0.1 mL made up to 1 mL using double distilled water). The value of Z-average diameter that is referred to as the harmonic intensity-weighted average hydrodynamic diameter of the vesicles was reported as mean droplet size of vesicles. All the measurements were repeated three times. The PDI was a dimensionless measure of the width of size distribution calculated from the cumulant analysis ranging from 0 to 1. A small value of PDI indicates a monodispersed population, while a large PDI indicates a broader distribution of droplet size.

Entrapment Efficiency

Spanlastic® formulation of vancomycin hydrochloride (0.2 mL) was poured in already prepared Sephadex G-50 column with pipette and it was then centrifuged for 2-3 min at 1500 rpm. Another 0.2 mL of formulation was again poured in column and centrifuged for another 2-3 min at 1500 rpm. After this, 0.2 mL of normal saline was poured 3 times in the column, and centrifuged each time for approximately 3 min. Thus, 1 mL was obtained in test tube which was then lysed with 500 µL of methanol and sonicated for 30 minutes in a bath sonicator.

Table 2: Box-Behnken experimental design

Run	X_1 S:T Ratio	X_2 Drug (mg/mL)	X_3 Stirring speed (rpm)	X_4 Span type	Run	X_1 S:T Ratio	X_2 Drug (mg/mL)	X_3 Stirring speed (rpm)	X_4 Span type
1	7:3	2	1600	Span 60	18	7:3	1	1200	Span 40
2	9:1	2	1600	Span 40	19	8:2	2	1200	Span 40
3	8:2	1	1600	Span 60	20	8:2	2	1200	Span 40
4	8:2	2	1200	Span 60	21	8:2	2	1200	Span 40
5	8:2	1	1600	Span 40	22	7:3	3	1200	Span 40
6	8:2	3	800	Span 60	23	9:1	3	1200	Span 60
7	8:2	3	800	Span 40	24	9:1	1	1200	Span 40
8	7:3	2	800	Span 40	25	9:1	2	1600	Span 60
9	8:2	2	1200	Span 40	26	9:1	1	1200	Span 60
10	7:3	1	1200	Span 60	27	8:2	3	1600	Span 40
11	8:2	2	1200	Span 60	28	9:1	2	800	Span 60
12	7:3	2	800	Span 60	29	8:2	2	1200	Span 60
13	9:1	2	800	Span 40	30	7:3	3	1200	Span 60
14	8:2	3	1600	Span 60	31	8:2	2	1200	Span 40
15	8:2	2	1200	Span 60	32	8:2	1	800	Span 40
16	9:1	3	1200	Span 40	33	7:3	2	1600	Span 40
17	8:2	1	800	Span 60	34	8:2	2	1200	Span 60

Then the volume was made up to 6 mL with distilled water and again sonicated for 15 minutes in a bath sonicator. This same procedure was repeated with all 34 runs of optimization, taking n=3. Final dilutions were analyzed by UV spectrophotometer at wavelength of 279 nm taking suitable volume of blank spanlastics® formulation as blank. Loaded drug amount was obtained using equation of straight line obtained from the standard curve. It was then used to obtain the entrapment efficiency using the formula:

Entrapment Efficiency = (Practical drug content)/(Theoretical drug content)

Zeta Potential

The zeta potential of particles (a measurement of the electrostatic repulsion between particles) is a good predictor of the stability of the formulation. A low zeta potential increases the probability for aggregation within a short time. Zeta potential of the optimized Spanlastics® formulation was estimated on the basis of electrophoretic mobility under an electric field (Zetasizer Nano ZS, Malvern Instruments, UK). The measurements were performed after diluting the formulation (100µL dispersed into 1 mL millipore water) at 25ºC.

Transmission Electron Microscopy

The prepared Spanlastics® were visualized by TEM at 200 kV (JEOL 2100F, USA). Samples were obtained by dispersing the formulation (0.1 mL) up to 1 mL of double distilled water. A drop of prepared dispersion was stratified over a carbon-coated copper grid for approximately 15 min. Excess dispersion was removed with a filter paper. The sample was allowed to air dry and observed under transmission electron microscope.

Elasticity Measurement

Spanlastics® possess the unique property of deformability[5], which was measured by extruding the vesicle through a polycarbonate membrane of 100 nm pore diameter (rp), at a constant pressure. After 5 min extrudate was collected and weighed (J). The average vesicle size after extrusion (rv) was obtained by using photon correlation spectroscopy. Elasticity of vesicular membrane (D) was calculated using the following equation:

$$D = J \times (r_v/r_p)^2$$

ATR

Infrared spectra of optimized formulation was obtained in the frequency range of 400 to 4000 cm-1 with a resolution of 2 cm-1 using ATR (Bruker, USA). In ATR, the sample was directly kept on to the ZnSe ATR crystal without need of any previous preparation and subsequently spectrum was recorded.

In vitro Release Kinetics

In vitro release kinetics was evaluated by reverse equilibrium dialysis method and quantification was carried out by UV-Visible Spectroscopy (UV 1800, Shimadzu, Japan). Spanlastics® (5 mL) were filtered through the Sephadex G-50 column to remove the unentrapped drug. Filtered formulation was added to phosphate buffer pH 7.4 outside of the dipped cellophane bag. Since vancomycin hydrochloride is water soluble, so aqueous reservoir can be considered a perfect sink. Samples (3 mL) were collected at known intervals from the cellophane bag and were replenished by PBS pH 7.4.[9]

Corneal Permeability Studies

Freshly excised goat cornea free from adhering sclera was first fixed between clamped donor and acceptor compartments of an all glass modified franz diffusion cell in such a way that its epithelial surface faced the donor compartment and the endothelial surface faced the receptor compartment. The corneal area available for diffusion was 0.50 cm^2. The receptor compartment was filled with 7 ml of freshly prepared phosphate buffer solution (pH=7.4) and all the air bubbles were expelled from the receptor compartment. The receptor fluid was kept at 37°C using a Teflon-coated magnetic stirred bead. Formulation (0.5 mL) was filtered to remove unentrapped drug and added to the donor compartment (n=3). Samples (200 µL) were withdrawn from the receptor compartment at regular intervals and were analyzed for vancomycin hydrochloride content by HPLC. Similarly, aqueous solution of vancomycin hydrochloride was also used (n=3) to study transcorneal permeation across goat cornea to obtain comparative results.[10,11]

Apparent Permeability coefficient of the preparations was determined using the following equation:

$$Papp = \Delta Q/(\Delta t.60.A.C)$$

Papp : Apparent permeability coefficient

$\Delta Q/\Delta t$: Steady-state slope of the linear portion of the plots of the amount of drug in the receiving chamber (Q) versus time (t)

A : Exposed corneal surface area

C : Concentration of drug in donor chamber

Ocular Safety Study

The ocular safety of administered delivery system is based on the draize irritancy test. The draize rabbit test, developed in the 1940s, is the only eye toxicity test officially accepted in the Organization for Economic Cooperation and Development (OECD) Guidelines (2002) for regulatory purposes in the classification of slightly and moderately irritating chemical. Approval for the use of animals in the study was obtained from the Institutional Animal Ethics Committee, DIPSAR, New Delhi, India (DIPSAR/IAEC/2014-II/Prot. No. 04). Albino male rabbits of weight 2-3 Kg were used for safety studies. The rabbits were housed singly in restraining cages, allowed food and water ad libitum and kept under 12 hour light/dark cycle during the experiment.[12,13]

Albino rabbits are typically used in the Draize tests because they have less tear flow than other animals, and the lack of eye pigment makes the effects easier to visualize.

RESULTS AND DISCUSSION

Statistical Analysis of Box-Behnken Design

To identify the optimum levels of different process parameters influencing size, polydispersity index (PDI) and encapsulation efficiency (EE %), an experimental design of 34 runs containing 5 central points was selected according to the Box-Behnken statistical design for three factors at three levels with one categorical factor at two levels. The individual and interactive effects of these process variables were studied. The results of experimental data are listed in table 3. Polynomial equations involving the main effect and interaction factors were determined based on estimation of statistical parameters such as multiple correlation coefficient, adjusted multiple correlation coefficient, and the predicted residual sum of squares generated by Design-Expert software. The statistical validation of the polynomial equations was established by ANOVA provision available in the software. Therefore, the optimum values of the variables were determined according to the obtained experimental data using the Design-Expert software, based on the constrained criterion of desirability presented in table 1.[15]

Effect on Entrapment Efficiency (EE %, Y_1)

The suggested model by the software was quadratic as its p-value was <0.0001 and r2= 0.8986 which showed that the suggested model was significant and the further analysis can be carried out on this basis. ANOVA results show that the span: tween 80 ratio (X_1), amount of drug (X_2) and type of span (X_4) had a significant effect on the EE %. The model proposes the following polynomial equation for EE %

$$Y_1 = +78.66 + 4.77X_1 - 14.19X_2 - 2.68X_3 - 4.06X_4 + 1.08X_1X_2 + 1.60X_1X_3 + 1.01\ X_1X_4 + 0.038X_2X_3 + 2.36\ X_2X_4 - 2.79X_3X_4 - 12.15X_1^2 - 13.20X_2^2 - 0.029X_3^2$$

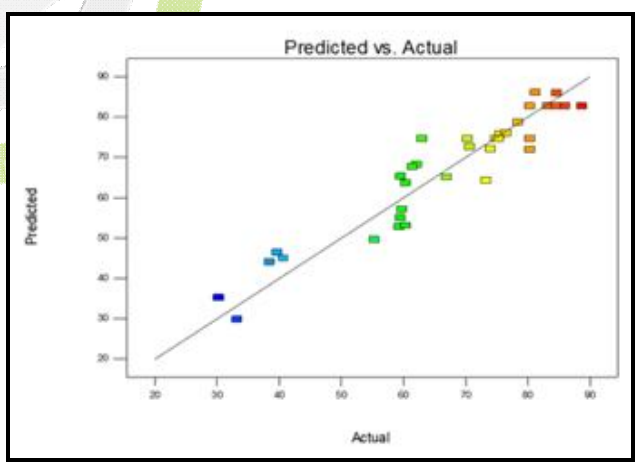

Figure 1: Predicted vs. Actual value curve for Y_1 (Entrapment Efficiency) response

To justify the validity of the equation, values of X_1, X_2 and X_3 were substituted in the equation to obtain the predicted values of Y_1. Figure 1 represents the predicted and actual values of the response in graphical form. The predicted and observed values were found to be in good agreement.

The interaction terms (X_1X_2, X_1X_3, X_2X_3, X_1X_4, X_2X_4, X_3X_4, X_{12}, X_{22} and X_{32}) show how the entrapment efficiency (EE %) changes when two variables are simultaneously varied. Positive coefficients of X_1, X_1X_2, X_1X_3, X_2X_3, X_1X_4, and X_2X_4 indicate the synergistic effect on entrapment efficiency (Y_1 response, EE %) while negative coefficients of X_2, X_3, X_4, X_3X_4, X_{12}, X_{22} and X_{32} indicate antagonistic effect on entrapment efficiency (EE %). On increasing the value of X_1 (span: tween 80), entrapment efficiency (EE %) increases while reverse happens if we increase X_2 (amount of drug) as maximum entrapment was achieved before the second level of X_2. Effect of factors X_1, X_2 and X_3 on EE % for span 40 (X_4) levels is shown in Figure 2 and Figure 3 as entrapment was found to be more in span 40.

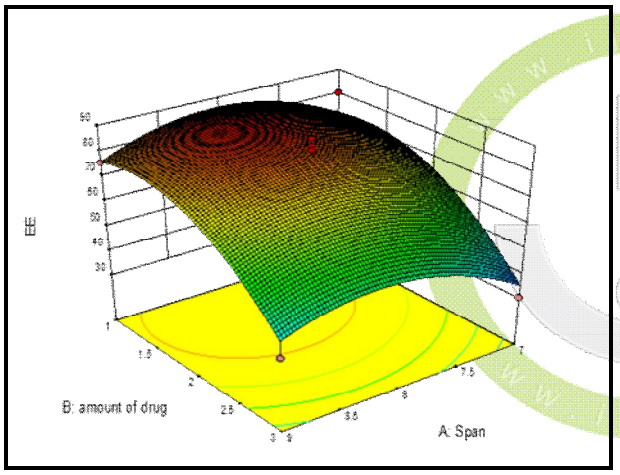

Figure 2: Response 3D plot for effect of X_2 and X_1 on EE%

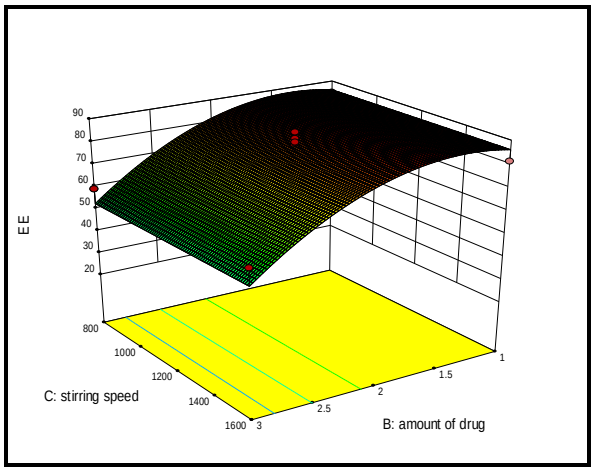

Figure 3: Effect of X_2 and X_3 on EE%

Effect of Formulation Variables on PDI (Polydispersity Index, Y_2)

The suggested model by the software was quadratic as its p-value was <0.0001 and r2= 0.8815, which showed that the suggested model was significant and further analysis can be carried on this basis. ANOVA results show that the span: tween 80 ratio (X_1), amount of drug (X_2) and stirring speed (X_3) had a significant effect on the PDI. The model proposes the following polynomial equation for PDI:

$$Y_2 = 0.216 - 0.009375\ X_1 + 0.041875X_2 + 0.0075X_3 + 0.00294118X_4 - 0.0025X_1X_2 + 0.00125X_1X_3 - 0.006875X_1X_4 - 0.01125X_2X_3 + 0.001875X_2X_4 + 0.0075X_3X_4 + 0.0445X_1^2 + 0.037X_2^2 + 0.02825X_3^2$$

To justify the validity of the equation, values of X_1, X_2 and X_3 were substituted in the equation to obtain the predicted values of Y_2. Figure 4 represents the predicted and actual values of the response in graphical form. The predicted and observed values were found to be in good agreement.

Positive coefficients of X_2, X_3, X_4, X_3X_4, X_2X_4, X_{12}, X_{22} and X_{32} in equation II indicate the synergistic effect on PDI, while negative coefficients of X_1, X_1X_2, X_2X_3 and X_1X_4 indicate the antagonistic effect on PDI. Minimum the value of PDI more uniform is the size distribution of the vesicles in the formulation. X_2, X_2X_3, X_{12}, X_{22} and X_{32} are the significant factors affecting the response Y_2 according to ANOVA data.

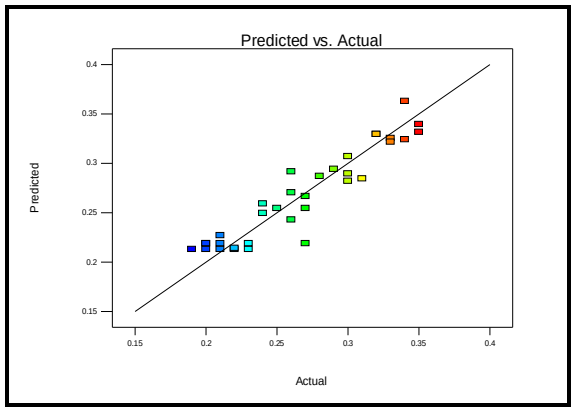

Figure 4: Predicted vs. Actual value curve for Y2 (PDI) response

Both the factors X_2 and X_3 have a positive coefficient. On decreasing these factors there will be an increase in the uniformity of the vesicular size which is quantified as low PDI value. Now, studying the interactive factors X_2 has a negative interaction with $_1$1 and X_3, hence on increasing the interaction PDI will be lowered. Figure 5 illustrates the response surface plots for the effects of the span: tween 80 ratio (X_1), amount of Drug (X_2), and stirring speed (X_3) on the PDI (Y_2).

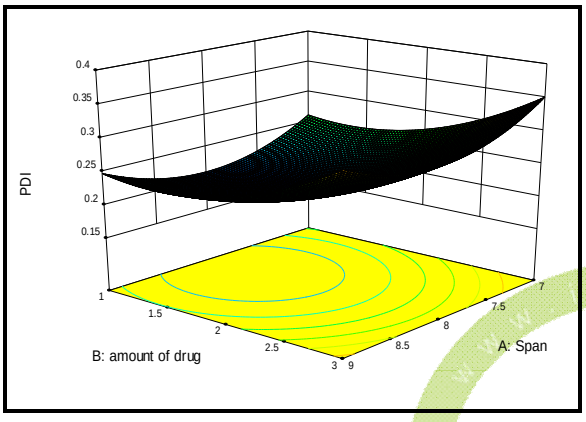

Figure 5a: Effect of X_2 and X_1 on PDI

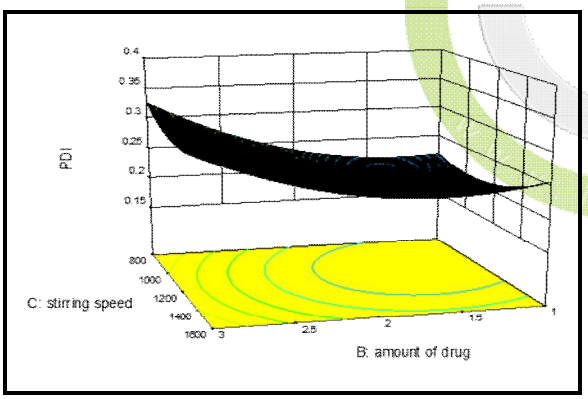

Figure 5b: Effect of X_2 and X_3 on PDI

Effects of formulation variables on Size (Y_3)

The suggested model by the software was quadratic as its p-value was <0.0001 and r2= 0.9501, which showed that the suggested model was significant and further analysis can be carried on this basis. ANOVA results show that the span: tween 80 ratio (X_1), amount of drug (X_2) and stirring speed (X_3) had a significant effect on the PDI. The model proposes the following polynomial equation for size (Y_3):

$$Y_3 = +217.00 - 20.78X_1 + 169.81X_2 - 21.72X_3 - 17.17X_4 - 22.88X_1X_2 - 3.56X_1X_3 - 19.85X_1X_4 - 5.14X_2X_3 - 31.81X_2X_4 + 28.13X_3X_4 + 78.67X_1^2 + 120.33X_2^2 + 18.46X_3^2$$

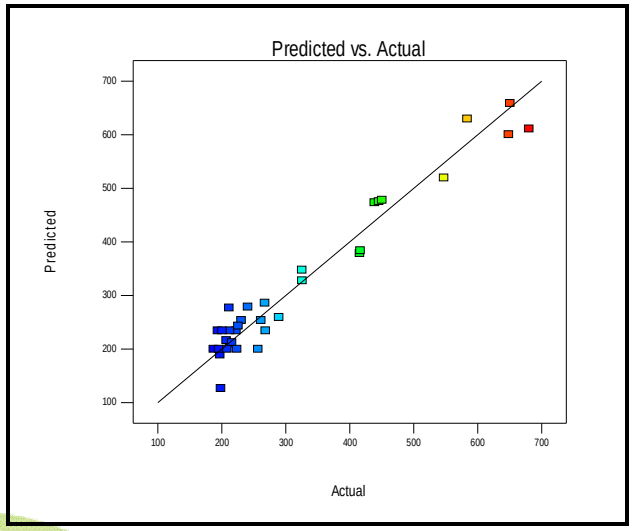

Figure 6: Predicted vs. Actual value curve for Y3 (size) response

To justify the validity of the equation, values of X_1, X_2 and X_3 were substituted in the equation to obtain the predicted values of Y_3. Figure 6 represents the predicted and actual values of the response in graphical form. Positive coefficients of X_2, X_3X_4, X_{12}, X_{22} and X_{32} in equation III indicate the synergistic effect on size, while negative coefficients of X_1, X_3, X_4, X_1X_2, X_2X_4, X_2X_3, X_1X_3 and X_1X_4 indicate the antagonistic effect on size. X_2, X_4, X_2X_4, X_3X_4, X_{12}, X_{22} and X_{32} are the significant factors affecting the response Y_3 according to ANOVA data.

Factor X_2 has a significant positive coefficient. On decreasing this factors there will be a decrease in vesicular size. X_1 and X_3 have negative coefficients, so increase in these factors will result in reduced size of vesicles. Now, studying the interactive factors X_2 has a negative interaction with X_1, X_3 and X_4, hence on increasing the interaction, size will be lowered. Figure 7(a) and (b) illustrates the response surface plots for the effects of the span: tween 80 ratio (X_1), amount of drug (X_2), and stirring speed (X_3) on the size (Y_3) for span 40.

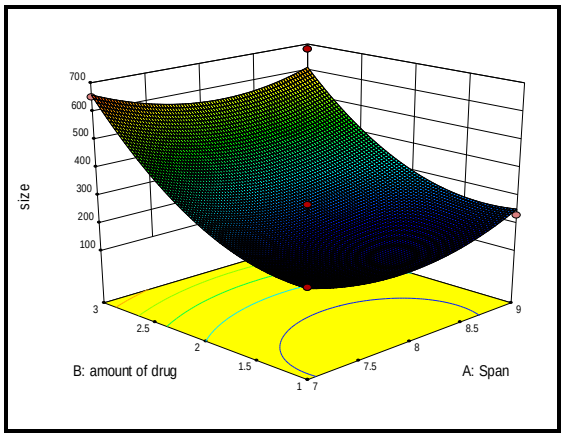

Figure 7 (a): Effect of X_1 and X_2 on size

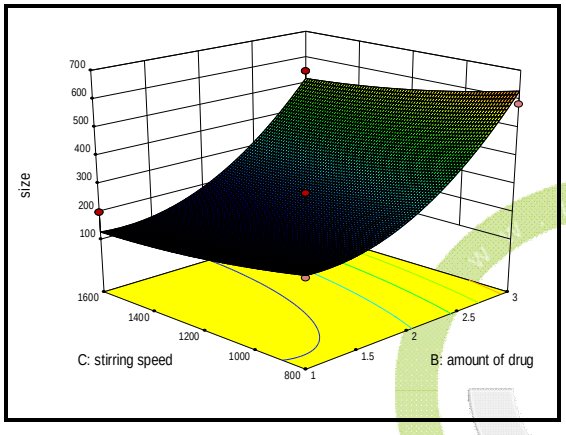

Figure 7(b): Effect of X_3 and X_2 on size

Optimization of Spanlastics®

After studying the effect of the independent variables on the responses, the levels of these variables that give the optimum response were determined. The optimum formulation was selected based on the criteria of attaining the maximum value of EE %, size value to be in range and minimizing the PDI. After applying constraints, the Design Expert software suggested an optimized formula to be prepared whose overall desirability was 0.950 for span 40. The suggested formula had a span 40: tween 80 ratio of 8.09:1.91, amount of drug as 1.53 mg/mL and stirring speed of 1056 rpm. The suggested formula was prepared and evaluated, and the residual between the predicted and observed responses was small demonstrating the validity of the optimization process. Values for the optimized formula are shown in table 3. Hence, the optimized formula was selected for further investigations.

Table 3: Predicted and observed responses for the optimized formulation

Factors	**X_1 (Span40: Tween80)**	**X_2 (amount of drug)**	**X_3 (Stirring speed)**
Optimized level	8.09:1.91	1.53 mg/mL	1056
Response	**Observed**	**Predicted**	**% Error**
Y_1 (EE %)	88.12±1.87	87.64	0.54%
Y_2 (PDI)	0.21±0.02	0.204	2.9%
Y_3 (Size)	180.5±3.58	187.4	-3.6%

Characterization of Optimized Spanlastics®

Size distribution

Optimized formulation was characterized for the vesicle size (Z-average) and size distribution i.e., polydispersity index (PDI) by Dynamic Light Scattering technique (DLS), using a computerized inspection system (Malvern Zetasizer, Nano-ZS, Malvern, U.K.) with DTS (Nano) software, at a wavelength of 633 nm and at a scattering angle of 173^0, at a room temperature of 25^0C. Size distribution is shown in Figure 8 was found to be uniform. PDI and Z-average Size of optimized formulation (n=3) was obtained as 0.21±0.02 and 180.5±3.58 (mean ± standard deviation).

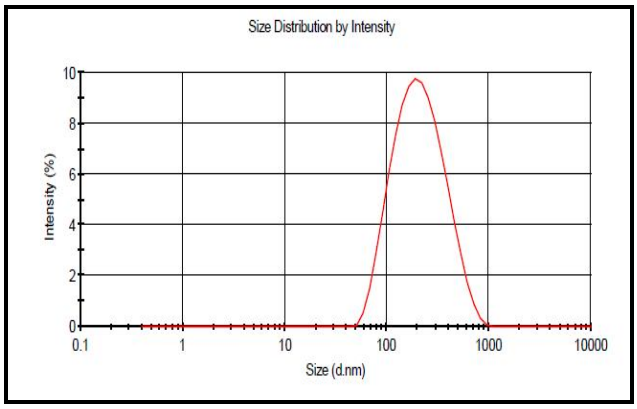

Figure 8: Size distribution of optimized formulation

Zeta Potential

High zeta potential values, either positive or negative, are expected to render stability to the system due to strong electrostatic repulsion. Many non-ionic surfactants like tween 80 exhibit a negative zeta potential, leading to repulsion between the vesicle bilayers. Zeta potential of optimized formulation was obtained as -30.2 as shown in Figure 9.

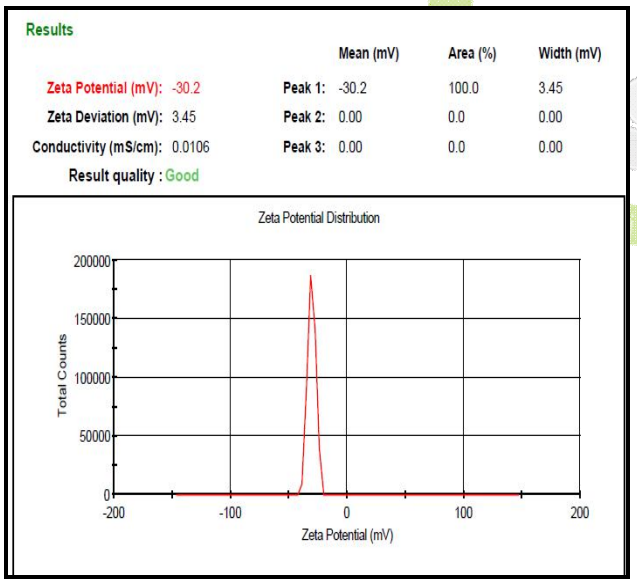

Figure 9: Zeta potential of optimized formulation

Attenuated Total Reflectance (ATR)

The IR spectra of pure drug and optimized liquid formulation are shown in Figure 10 and Figure 11 respectively. Significant peak at 1655 cm^{-1} observed in IR spectrum of pure drug is due to C=C stretching which is also observed in liquid formulation at 1639 cm^{-1}. Another peak at 1048 cm-1 obtained in formulation is similar to peak of drug at 1058 cm-1. Peak at 3372 cm-1 is due to O-H stretching in water molecules as formulation is aqueous based. No interaction between drug and other excipients is observed as significant peaks of drug are present in formulation as well.

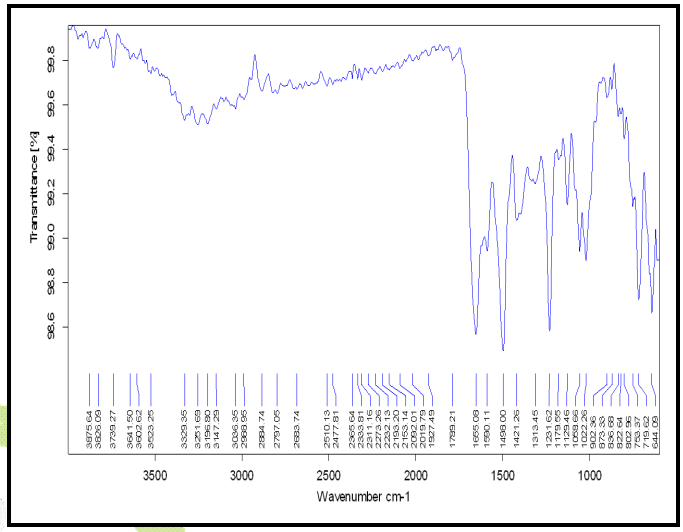

Figure 10: IR Spectrum of Vancomycin Hydrochloride

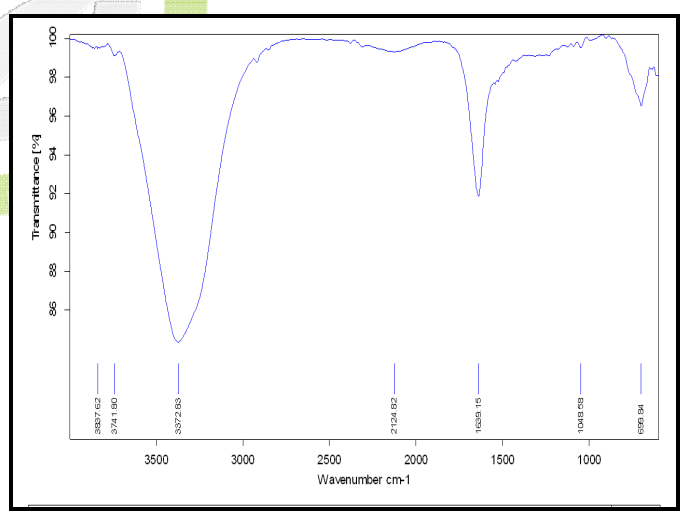

Figure 11: IR Spectrum of vancomycin hydrochloride entrapped in spanlastics®

Transmission Electron Microscopy (TEM)

The optimized formulation was characterized for its shape and surface morphology by TEM. Representative TEM image of spanlastics® demonstrate spherical and discrete vesicles of <200 nm which is consistent with the Dynamic

light Scattering (DLS) of formulation. The TEM image of formulation is shown in Figure 12.

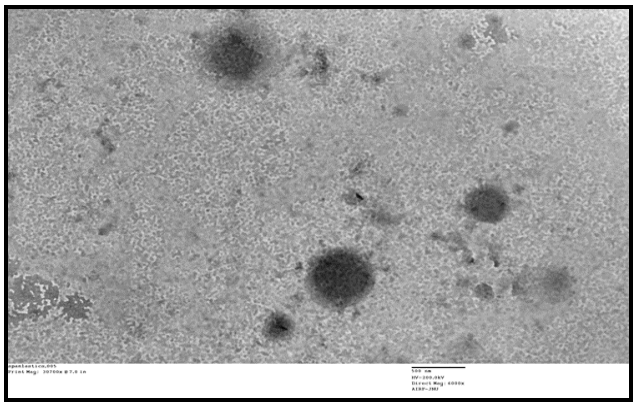

Figure 12: TEM image of optimized spanlastics® formulation

Elasticity Measurement

Deformability was found to be 24.8. Spanlastics® showed only 11.3 % change in size of vesicles after extrusion through 100 nm polycarbonate membrane. Thus optimized Spanlastics® formulation is highly elastic in nature. This deformability characteristic permits the elastic vesicles to penetrate spontaneously through the biological membranes, minimizing the risk of complete vesicle rupture while squeezing through.

In vitro Drug Release Study

In vitro release pattern of optimized Vancomycin Hydrochloride loaded Spanlastics® is shown in table 4 and Figure 13.

Table 4: *In vitro* release profile of optimized Spanlastics®

Time (hours)	% Cumulative Drug Release
0.5	15.80±1.6
1	24.38±0.8
2	32.76±1.9
4	42.02±2.7
6	51.82±1.1
12	67.86±1.6
24	89.28±0.9

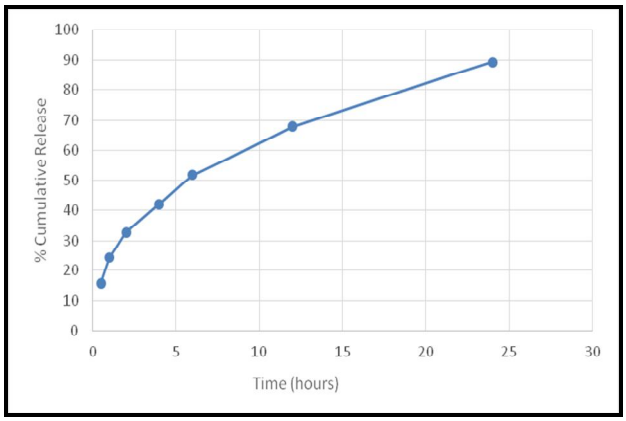

Figure 13: *In vitro* drug release pattern of optimized spanlastics®

Release study behavior of Spanlastics® shows biphasic release where the first rapid phase of release lasted for 6 h, followed by a slow diffusion order rate of release for the next 24 h. The first phase of release may be due to dissolution and diffusion of the drug that was poorly entrapped in the spanlastics®, while the slower and continuous release may be attributed to the diffusion of the drug localized in the core of the spanlastics®.

Ex vivo Release Study

Freshly excised goat cornea was carefully dissected along with 2-4 mm of surrounding sclera tissue from the eyeball and washed with cold saline to remove any adhering pigments as shown in Figure 14.

Figure 14: Freshly excised goat cornea

The results obtained for percentage cumulative drug release from optimized formulation and control vancomycin hydrochloride drug solution are shown in Figure 15.

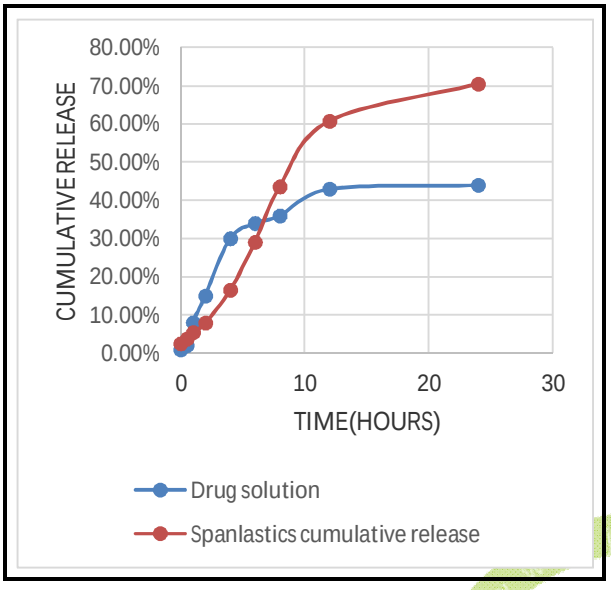

Figure 15: Graph showing cumulative drug release profile of vancomycin hydrochloride from spanlastics® and aqueous drug solution through goat cornea

Apparent Permeability Coefficient of Spanlastic formulation was found to be 5.92 x 10-6 cm/s, which was greater than the apparent permeability coefficient of the aqueous solution of drug i.e. 3.4 x 10-6 cm/s. Extent of permeation of total drug was higher in Spanlastics® formulation than the aqueous drug solution.

Ocular Safety Study

0.1 mL of formulation was instilled into the right eye of the rabbit and observations were noted according to the scoring scale of draize test, mentioned in table 5.

Table 5: Observations of draize irritancy test

Time (h)	Single Insult Challenge	Repeat Insult Challenge
1	0	0
6	0	2
12	0	2
24	0	0

A score of 0 indicates formulation is non-irritating. After repeat insult challenge, redness was observed at 6 hours observation which means practically non-irritating. In fact, being a controlled-release dosage form, spanlastics® would not be instilled in eyes following the same time regime used in the irritation study (repeated insult challenge). The frequency of administration would be less, so fear of causing inflammation is much diminished.

CONFLICT OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. Commonly Used Ophthalmic Antibiotics. American Academy of ophthalmology, accessed from: http://www.aao.org/focalpointssnippetdetail.aspx?id=f71ff617-5c78-4b288177a 7549fc766bc
2. Hironaka, K., Inokuchi, Y., Tozuka, Y., Shimazawa, M., Hara, H., & Takeuchi, H. (2009). Design and evaluation of a liposomal delivery system targeting the posterior segment of the eye. *Journal of Controlled Release*, *136*(3), 247-253.
3. van den Bergh, B. A., Wertz, P. W., Junginger, H. E., & Bouwstra, J. A. (2001). Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. *International Journal of Pharmaceutics*, *217*(1), 13-24.
4. Kakkar, S., & Kaur, I. P. (2011). Spanlastics—A novel nanovesicular carrier system for ocular delivery. *International Journal of Pharmaceutics*, *413*(1), 202-210.
5. ElMeshad, A. N., & Mohsen, A. M. (2014). Enhanced corneal permeation and antimycotic activity of itraconazole against Candida albicans via a novel nanosystem vesicle. *Drug Delivery*, 1-9.
6. Abdelbary, A. A., & AbouGhaly, M. H. (2015). Design and optimization of topical methotrexate loaded niosomes for enhanced management of psoriasis: Application of

Box–Behnken design, in-vitro evaluation and in-vivo skin deposition study. *International Journal of Pharmaceutics*, *485*(1), 235-243.

7. Gannu, R., Yamsani, V. V., Yamsani, S. K., Palem, C. R., & Yamsani, M. R. (2009). Optimization of hydrogels for transdermal delivery of lisinopril by Box–Behnken statistical design. *AAPS PharmSciTech*, *10*(2), 505-514.
8. Yasir, M., & Sara, U. V. S. (2013). Preparation and optimization of haloperidol loaded solid lipid nanoparticles by Box–Behnken design. *Journal of Pharmacy Research*, *7*(6), 551-558.
9. Phillips, D. J., Pygall, S. R., Cooper, V. B., & Mann, J. C. (2012). Overcoming sink limitations in dissolution testing: a review of traditional methods and the potential utility of biphasic systems. *Journal of Pharmacy and Pharmacology*, *64*(11), 1549-1559.
10. Chidambaram, N., & Burgess, D. J. (1999). A novel in vitro release method for submicron-sized dispersed systems. *AAPS PharmSci*, *1*(3), 32-40.
11. Rojanasakul, Y., & Robinson, J. R. (1989). Transport mechanisms of the cornea: characterization of barrier permselectivity. *International Journal of Pharmaceutics*, *55*(2), 237-246.
12. Organization for Economic Cooperation and Development (OECD). *Guidelines for the testing of chemicals No. 405*: acute eye irritation/corrosion, Paris, France. 2002; 1–14.
13. Draize, J. H., Woodard, G., & Calvery, H. O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics*, *82*(3), 377-390.

