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Research Article

Design, Development and Characterization of Hyaluronic Acid Based pH Sensitive Liposomal *In Situ* Gel for the Treatment of Keratoconjunctivitis Sicca

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Abstract

The recent advances in ocular drug delivery have been successful in surpassing conventional therapy, owing to its considerable limitations. This study aims to develop and optimize *in-situ* liposomal ocular system to design once-daily liquid preparation to treat Keratoconjunctivitis sicca. Liposomes of Hyaluronic acid were prepared using soya lecithin by a thin film hydration method. Eight trial formulations were made by keeping drug as fixed ratio and varying the concentration of cholesterol and soya lecithin. The optimized formulation was selected for the formulation of liposomal *in-situ* hydrogel. Optimization was carried out by Central Composite Design (CCD) studying the factors, amount of cholesterol and soya lecithin and its effects on the responses, % In-vitro drug release (R1) and Percent Entrapment Efficiency (% EE) (R2). The best predicted model for R1 was the Quadratic model and, for R2 was Two-Factor Interaction model (2FI) without any significant lack of fit. Optimum formulation was found to be at 1:1:4 (drug: cholesterol: soya lecithin) showed sustained drug release of 31.28% in 8 hours with highest %EE of 47.36%. Liposomal *in-situ* gel preparation retained better stability throughout the study period when stored at refrigerator temperature and displayed prolonged action (76.44%) when compared to liposomal formulation (68.72%).

Keywords: Liposomes, Hyaluronic acid, Optimized formulation, CCD, sustained drug release, *in-situ* hydrogel.

INTRODUCTION:

Ophthalmic drug delivery is one among the foremost fascinating and difficult endeavors faced by pharmaceutical scientists. The challenge to the formulation is to bypass the protecting barriers of the attention while not inflicting any permanent tissue injury [1]. Liposomes are small artificial vesicles which are defined as defined as spherical vesicles with particle sizes ranging from 30 nm to several micrometers that can be produced from natural non- toxic phospholipids and cholesterol. Based on their size, properties and biocompatibility, liposomes are capable systems for ocular drug delivery [2].

Hyaluronic acid is a ubiquitous biomaterial used in ocular drug delivery systems owing to its biocompatibility, viscoelasticity [3]. Amount of cholesterol and soya lecithin are critical in the preparation and stabilization of liposomes. Absence of cholesterol shows non rigidity and irregular shape.

Dry eye disease is outlined as a "multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and sub-acute inflammation of the ocular surface [4]. DES is characterized by chronic dryness of the cornea and conjunctiva which is caused by unstable tear film associated with abnormality of the lipid, protein, and mucin profiles [5]. Changes in tear composition resulting from lacrimal dysfunction, increased evaporation, and/or poor clearance have proinflammatory effects on the ocular surface [6]. Typical symptoms of DES include burning, stinging, and photophobia. Additionally, patients with chronic, uncontrolled disease may complain about blurred vision, decreased ability to produce tear, and intolerance to contact lenses [7].

pH triggered mechanism was used to convert liposomes in to liposomal ocular *in-situ* gel formulation using carbopol 940 and Hydroxy propyl methyl cellulose viscous polymer. Carbopol is an important class of ocular bio adhesiveness and it is widely used in ophthalmology to enhance pre-corneal retention. It is synthetic polymer composed of 62 % of carboxyl polymers with high molecular weight formed by repeating units of acrylic acid. As the concentration of carbopol increases in the vehicle, its acidic nature may cause stimulation to the eye tissues and also causes lachrymation and hence combination of polymers was used. Hydroxy propyl methyl cellulose was used along with the carbopol in order to improve ocular bioavailability.

However, muco-adhesive polymers or stimuli sensitive polymers when used in combination with vesicular system may provide vesicles with necessary site adherence and site retention to achieve carrier and drug targeting in topical ocular therapy and endow them with the ability to muco-adhesive nature [8,9].

MATERIALS AND METHODS:

Materials:

HA was purchased from S.I.Shekels Pvt. Ltd. Vitamin A (USV Private limited), Omega 3- fatty acid (Cu-V-Kar Genetic Medicine (P) Ltd), Carbopol 940 (Sigma Aldrich), HPMC K 15M(Low) (Sigma Aldrich), Sodium chloride (Spectrum reagents and chemicals, Pvt. Ltd), Cholesterol (LobaChemie Pvt. Ltd), Soya lecithin (Hi-Media Laboratories Pvt. Ltd., Mumbai) were used in the study. All other chemicals and solvents were of analytical or pharmacopoeial grade.

Methods:

Formulation of liposomes of hyaluronic acid by thin film hydration technique:

Liposomes of hyaluronic acid were prepared by thin film hydration method using different ratio of soya lecithin and cholesterol with drug. Eight trial formulations (F1 to F8) were carried out and given in table 1.

Accurately weighed quantity of soya lecithin and cholesterol in different ratio concentration were dissolved in chloroform and methanol (2:1v/v) solvent mixture and stirred until it gets completely dissolved. Then it was vortexed in a round bottom flask at temperature 55ºC to remove the solvent under reduced pressure in the rotary flask evaporator at 100 rpm for 30-40 minutes. After evaporation of solvents, cholesterol and soya lecithin leads to formation of thin film on the inner sides of round bottom flask (RBF). Thin film was hydrated with aqueous phase containing drug (200 mg) in 10ml of phosphate buffer pH 7.4 for 30 minutes at temperature 55ºC to obtain yellowish white dispersion of liposomes. The above white dispersion of liposomes was cooled in an ice bath and then sonicated using probe type ultrasonicator for 3 minutes at 150V. The resultant vesicles of liposomes were stored at 4ºC in a refrigerator for further studies [10,11].

Characterization Studies of Liposomes of Hyaluronic Acid:

Optical microscopy study:

The particle size of the liposomal suspension was determined by optical microscopy. A drop of liposomal suspension was placed on a glass slide. A cover slip was placed over the liposome suspension and the average vesicle size was measured by an optical microscope (Motic digital microcope) and by using a pre-calibrated ocular eye piece micrometer. The prepared vesicles were studied under 40 X magnification to observe the formation of vesicles.

Transmission Electron Microscopy (TEM) study:

The sample of liposomes (5-10µl) was dropped onto carbon coated copper grid at one side. After complete drying, sample was stained using 2% w/v phosphotungstic acid. Digital micrograph and soft imaging viewer software were used to perform the image capture analysis, including particle sizing. The stained grid was air dried and observed. Image was visualized on screen under the electron microscope (Technai T20) and photographed.

Estimation of entrapment efficiency:

The Entrapment efficiency of liposomes was estimated by ultra-centrifugation method where the liposomal dispersions were centrifuged. The clear supernatant was diluted by using phosphate buffer pH 7.4 and analyzed for the drug concentration spectrophotometrically. The percentage encapsulation efficiency (EE %) was calculated using following equation[12].

$$EE\ (\%) = \frac{\text{Total drug - Diffused drug}}{\text{Total drug}} \times 100$$

Drug content analysis:

The amount of drug in the formulation was determined after lyzing the liposomes using 50% n-propanol and shaken well for the complete lysis of vesicles. After suitable dilution with the phosphate buffer saline pH 7.4, the absorbance of the solution was measured at 208 nm in the UV visible spectroscopy (Perkin Elmer) using empty liposomes as blank [13].

In vitro drug release studies of liposomes:

In vitro drug release study of liposomal formulation was studied by membrane diffusion technique. *In vitro* diffusion cell was made using cellophane membrane as a semi-permeable membrane. The diffusion cell consists of a beaker, magnetic stirrer with temperature control and test tube with both ends open. One end of the test tube was closed using treated cellophane membrane as a semi-permeable membrane and the other end was kept open to introduce liposomal formulation. The diffusion medium was freshly prepared phosphate buffer saline pH 7.2 of 100 ml equilibrated at 37ºC ± 0.5ºC. The liposomal formulation 5 ml was placed inside the diffusion cell through open end of the test tube on the cellophane membrane. The diffusion medium used was freshly prepared 100 ml of phosphate buffer pH 7.2. It was placed inside the beaker in such a way that the lower surface of the cellophane membrane was made contact with the buffer. The temperature of the buffer solution was maintained at37ºC ± 0.5ºC and stirred with magnetic stirrer throughout the study period. Aliquots (10 ml) of medium were withdrawn periodically and replaced with fresh diffusion medium of pH 7.2 buffer to maintain constant volume (sink condition). The samples were analyzed spectroscopically at 208 nm for concentration of Hyaluronic acid [14].

Experimental design:

To reduce the no of trials and obtain highest information, screening was carried out using CCD. A polynomial equation was generated for each of the dependent variables to systematically study the influence of independent variables on the dependent variables. The values obtained were compared with the predicted value obtained from transformed polynomial equation and evaluated statistically by ANOVA. The study was carried out using statistical software package Design Expert version7.0.0; Stat-Ease, Inc., Minneapolis, Minnesota, USA.

Optimization study:

After the selection of most favorable interactions, RSM (CCD) was used to obtain optimum levels of variables. The CCD has three groups referring to the variables namely, factorial points (+1, -1), axial points (–α, +α) and the center point. The alpha value is 1.41421. As per the matrix created by DoE software, total of 8 runs were generated. Using this design, best model was selected among the linear, two-factor interaction model and quadratic model due to the analysis of variance (ANOVA) *F*-value [15]. Predicting the response through the full second-order polynomial equation is as shown,

$$Y = \beta_{0+} \beta_1 X_1 + \beta_2 X_2 + {}_+ \beta_{11} X_1^2 + \beta_{22} X_2^2 + + \beta_{12} X_1 X_2$$

Y- predicted response(s)
β_0 - linear coefficients
β_{11}, β_{22}, - squared coefficients
β_{12} - interaction coefficients

Using this equation, an appropriate model is suggested based on the significance and the interactive effects of independent variables on responses are recorded accordingly in table 1 & 2.

Table 1: Central composite design in various runs with in-vitro drug release and Entrapment efficiency as responses

	Factor 1	Factor 2	Response 1	Response 2
Run	A:cholesterol	B:soya lecithin	invitro drug release	EE
	mg	mg	%	%
1	1	4	33.28	47.36
2	0	1	39.67	22.38
3	0.5	8.24264	41.54	31.28
4	1.20711	4	37.81	35.26
5	0	7	41.98	26.23
6	1	7	36.56	27.22
7	-0.207107	4	38.41	22.08
8	0.5	-0.242641	44.83	41.02

Table 2: The coded and actual values of the variables used in central composite design

	Name	Units	Low	High	-alpha	+alpha
A	cholesterol	mg	0	1	-0.207107	1.20711
B	soya lecithin	mg	1	7	-0.242641	8.24264

Formulation and evaluation of liposomal in situ gel of HA:

Among Eight formulations of liposomes prepared by thin film hydration technique i.e (F1- F8) based on the entrapment efficiency and in vitro drug release, the optimized formulation containing 4:1 ratio of SL and CL was found to be effective and that formulation was selected as best formulation for the conversion of liposomal *in situ* gel formulation. Formulation of in situ gel was carried out by dissolving weighed quantities of polymers carbopol 940 and cellulose derivatives (HPMC K 15 low viscous) in phosphate buffer pH 7.2 and they were allowed to hydrate. To this β-cyclodextrin, sodium chloride and benzalkonium chloride were added. Liposomal dispersion of 10 ml was added to the above under constant stirring until a uniform solution was obtained. Final volume was adjusted by using buffer. It was then filled in vials under aseptic conditions, sterilized in the autoclave at 121° C and 15 psi, for 20 minutes and was evaluated for further studies [16]. The composition of liposomal *in situ* gel was given in table 3.

Table 3: Composition of liposomal *in situ* formulation

Composition	Quantity
Lipsosomal Dispersion (ml)	10
Carbopol 940% w/v (gm)	0.25
HPMC K 15M	0.25
(Low viscous) (gm)	
β-cyclo dextrin (gm)	0.1
Benzalkonium chloride (gm)	0.002
Sodium chloride (gm)	0.9
Phosphate Buffer p H 7.2 (ml)	100

Characterization of Liposomal in-situ gel of Hyaluronic Acid:

Visual appearance:

Visual appearance was observed for the presence of any particulate matter.

Clarity:

The clarity was observed before and after gelling by visual examination of the formulations.

pH

pH was measured by using pH meter. The pH was noted by bringing the electrode near the surface of the formulation and allowing it to equilibrate for one minute and the values were measured in triplicate.

Drug content analysis:

Drug content of liposomal *in situ* gel was determined by adding 50% n-propanol to the formulation for the lysis of vesicle. About 1ml of liposomal *in situ* gel was then diluted to 100ml with simulated tear fluid of pH 7.4. The drug content was estimated spectroscopically at 208 nm and values were measured in triplicate [17].

Determination of viscosity:

The viscosities of the developed liposomal *in situ* gel formulation was measured by the Brookfield Viscometer DV2T model. The formulation was taken in the sampling tube and analyzed in the room temperature. The spindle 18 was connected to the viscometer in such a position and the samples were measured at 15 rpm for duration of 5 minutes. Finally, obtained values were measured in units as centipoises (cP) [18,19].

Gelling Capacity:

The determination of *in vitro* gelling capacity was done by visual method by using 1% amaranth dye solution in water and mixed with developed formulation. Placing 5ml of the simulated tear fluid pH 7.4 in glass tube, to this 20 µl-50 µl of colored formulation solution was added with the help of pipette. As the solution comes in contact with STF fluid 7.4, it gets converted into stiff gel. The gelling capacity of solution was evaluated on the basis of stiffness of gel formed and remains as such for a long time. The *in vitro* gelling capacity was graded in two categories on the basis of gelation time and time period for which the formed gel remains.

Effect of Osmotic Shock:

The effect of osmotic shock on liposomal *in situ* gel formulation was investigated by monitoring the change in vesicle diameter after incubation of liposome suspensions in media of different tonicity such as 1.6 % sodium chloride (hypertonic), 0.9% sodium chloride (normal) and 0.5% sodium chloride (hypotonic). Suspensions were incubated in these media for 3 hours and the change in vesicle size was measured by optical microscopy with a calibrated eyepiece micrometer.

Kinetic models for in vitro drug release data:

The data obtained from *in vitro drug* release study of liposomal *in situ* gel formulations were fitted to different kinetic models. Zero order (percentage drug release versus time), first order (logarithmic of percentage drug remaining to be released versus time), Higuchis model (percentage of drug release versus square root of time),Hixoncrowell and KorsmeyerPeppas equation was used to study the drug release mechanism by analyzing (n) as the diffusion exponent [20].

Sterility test:

The sterility testing of liposomal *in situ* gel was performed for the aerobic, anaerobic bacteria and fungi by using alternative thioglycolate medium and soya bean casein digest medium. The medium was prepared by dissolving 500 mg of peptic digest of animal tissue (such as bacteriological peptone) or its equivalent in water to make 100 ml and the pH was adjusted to 7.1 ± 0.2. The medium was filtered or centrifuged to clarify and dispersed into flasks of 10 ml quantities and sterilized at 121º C for 20 minutes. The positive control (growth promotion) and negative control (sterility) test were also carried out. *Bacillus subtilis*, *Bacteriodes vulgatus* and *Candida albicans* were used as test organisms which are aerobic, anaerobic bacteria and fungi, respectively. Incubation was carried out in all cases and growth was observed [21].

Ex-vivo Trans corneal permeation study:

Ex-vivo transcorneal permeation study was carried out on freshly excised goat cornea. A fresh, whole eye balls of the goat were obtained from local slaughter shop and stored in cold condition at 4º C in normal saline. The cornea was then carefully excised along with 2- 4 mm of surrounding scleral tissue and was washed with normal saline until the washings were free from proteins. The excised cornea was fixed between the clamped donor and receptor compartments of glass modified franz diffusion cell in which epithelial surface faced the donor compartment. The corneal area available for diffusion was 0.50 cm^2. The receptor compartment was filled with 10 ml of freshly prepared simulated tear fluid pH 7.4 and all air bubbles were expelled from the compartment. Aliquot 1 ml of the prepared liposomal *in situ* gel was placed on the cornea and the opening of the donor cell was sealed in a glass cover slip. The receptor fluid was kept at 37 ºC with constant stirring using a teflon coated magnetic stirrer beads. The permeation study was carried out and the samples were withdrawn from the receptor and analyzed for drug concentration by measuring absorbance at 208nm in a UV-VIS spectrophotometer [22].

In vitro drug release of liposomal in situ gel:

In vitro release studies were carried out using franz diffusion cell and the temperature was adjusted to 37º C ± 5º C. Dialysis membrane was soaked overnight in STF fluid. The sample was applied on to the membrane and the membrane was placed in between donor and receptor compartment of the cell consisting of STF fluid pH 7.4. Samples were withdrawn at periodic intervals for 8 hour and replaced with fresh buffer to maintain sink condition. The drug content was analyzed using UV visible spectrophotometer at 208 nm using STF fluid as blank [23].

Stability studies:

The optimized liposomal *in situ* gel was placed in a amber glass vials and sealed with aluminium for short term accelerated stability study at 25º± 2 ºC, 60 ± 5 % RH and 5º± 3 ºC at refrigerator condition as per modified International Conference on Harmonization guidelines for 3 months. Samples were analyzed every 30 days for appearance, pH, gelling studies and drug content [24,25].

RESULTS AND DISCUSSION:

Preparation of liposomes of hyaluronic acid by thin film hydration technique:

Liposomes were prepared by thin film hydration technique as described in the procedure earlier. In this, eight formulations of Hyaluronic acid, liposomes were prepared by using hyaluronic acid (200 mg), cholesterol (200 mg) and soya lecithin(800mg) in the ratio 1:1:4 showed optimized results on the basis on two responses R_1 and R_2 screened using design expert software.

Fitting response surface model:

The effect of amount of cholesterol, soya lecithin on the in-vitro drug release and entrapment efficiency is determined using Response Surface Methadology. The model was analyzed statistically and the best fit models for both the responses were obtained. The regression coefficients (R2), regression value (p-value) and derived equations for R_1 and R_2 are shown in table 4

Table 4: Regression coefficients and probability values for the final reduced model.

Responses	R2 value	p value
In vitro drug release	0.9994	p<0.0001
Entrapment efficiency	0.9334	p<0.0001

Central composite response surface methodology:

Analysis of variance (ANOVA) was used to assess the significance of the quadratic

polynomial model and 2F1 model developed for R_1 and R_2 respectively. The large F-value and small p-value of all terms in the models indicated significant influence on the response variables.

The results of fitting the polynomial equation to the data, when *in-vitro* release is the response, are shown in Table 5. As can be seen from Table 5, the model is highly statistically significant ($P < 0.05$) mathematical model describing the relationship between variables (A, B) and response (Y1) could be reduced to that shown in Equation 1.

$$Y_1 = 28.96 - 0.2363A - 1.23B - 2.45AB + 4.55A^2 + 7.09B^2 \quad \cdots\cdots 1$$

where Y1 is *in-vitro* release the and A and B are cholesterol and soya lecithin amount respectively.

The negative coefficients of A and B indicate that in-vitro drug release decreases with the increase in the composition of cholesterol.

Table 5: ANOVA results for *in-vitro* as the response (Y1).

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	89.61	5	17.92	677.07	0.0015	significant
A-cholesterol	0.3350	1	0.3350	12.66	0.0707	
B-soya lecithin	9.06	1	9.06	342.29	0.0029	
AB	14.40	1	14.40	544.01	0.0018	
A^2	12.43	1	12.43	469.71	0.0021	
B^2	35.48	1	35.48	1340.37	0.0007	
Residual	0.0529	2	0.0265			
Cor Total	89.67	7				

ANOVA- Analysis of variance

Using entrapment as the response, the two interaction equation is the best model, which has been fitted to the data according to the ANOVA *F*-value calculated by Design-Expert software. The results of the fit model summaries are shown in Table 6. As can be seen, the ANOVA *F*-value of the model indicated that the model *p*- value is less than 0.05 and considered to be significant.

The following two-interaction equation can be fitted to the data appropriately, as shown in Equation 2.

$Y_2 = 34.53 + 9.03A - 4.95B - 8.38AB$ 2

where Y_2 is the entrapment efficiency

Table 6: ANOVA results for loading efficiency as the response (Y2)

Source	Sum of square	df	Mean square	F-value	p-value	
Model	688.02	3	229.34	18.68	0.0081	significant
A-cholesterol	598.86	1	598.86	48.77	0.0022	
B-soya lecithin	151.38	1	151.38	12.33	0.0246	
AB	176.79	1	176.79	14.40	0.0192	
Residual	49.12	4	12.28			
Cor Total	737.14	7				

It is observed that increase in soya lecithin results in decrease in entrapment efficiency and vice versa with cholesterol composition. The 3D response of these factors on the response is given in fig 1.

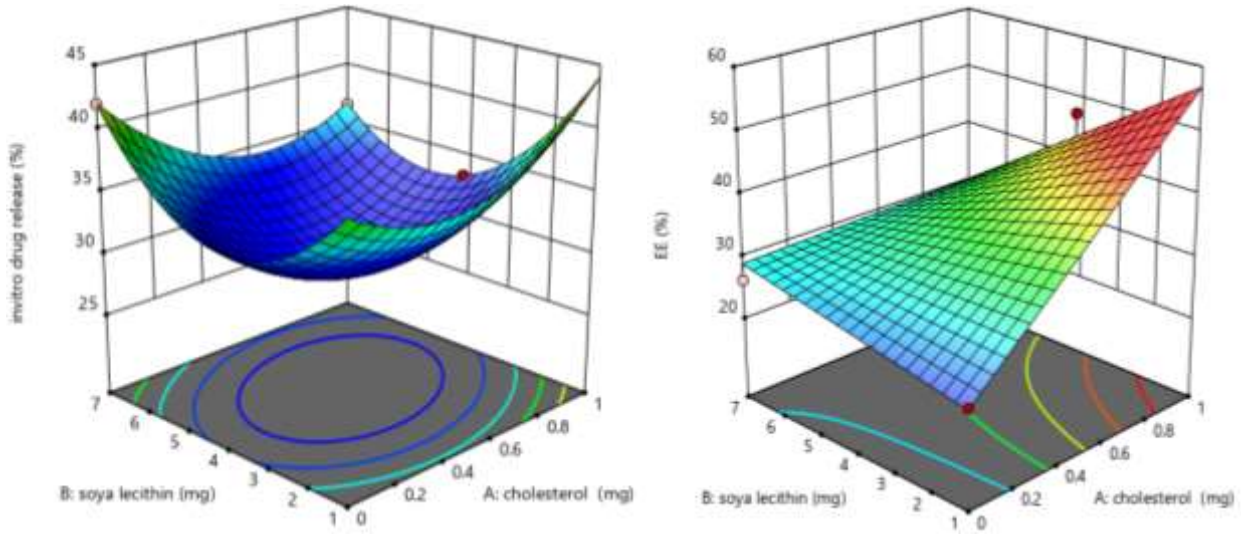

Figure 1: Response surface plots showing the effect of interaction between Cholesterol and Soya lecithin composition on the (a) in-vitro drug release, (b) Entrapment Efficiency

Optimization:

By solving equation 1 and 2 it was found that the amount of cholesterol and soya lecithin was found to be 200 mg and 800 mg. the predicted in-vitro drug release and entrapment efficiency was calculated to be 33.28% and 47.36% amongst the 8 trial formulations.

Optical microscopy study:

The optical microscopic images of the vesicles were spherical in shape. Liposomes prepared using Soya lecithin having ratio of (1:1:4) were larger in size compared to the other liposomal formulations. This can be attributed to the fact that vesicles with high drug entrapment are large and mean size of liposomes increases proportionally with decrease in the concentration of soya lecithin, after which higher concentration of soya lecithin vesicles are disconnected and form aggregates as shown in fig 2.

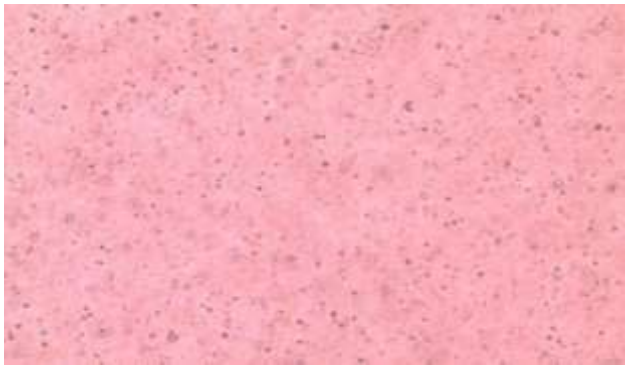

Figure 2: Optical microscopy image of optimized formulation (F4) under 40 x magnification.

Transmission Electron Microscopy (TEM) study:

The morphology of liposomal vesicles was determined by TEM. It gives information about the internal morphology, structure and size of liposomes. The optimized formulation (F4) was found to be spherical in shape, uniform and discrete in shape. The particle sizes exhibited in the range of 50-150 nm as shown in fig 3.

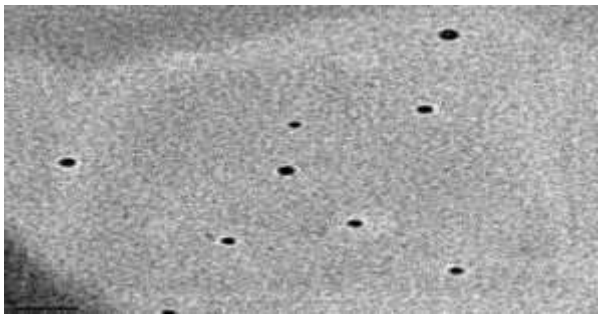

Figure 3: TEM images of optimized liposomal formulation (F4).

Entrapment efficiency:

The entrapment efficiency of the liposomal formulations was measured by centrifugation method. Low HLB and high transition temperature increase the entrapment efficiency. Among all the formulations, F4 showed maximum entrapment efficiency when compared with other formulations as shown in the Figure. It was due to its low HLB value and high transition temperature. Cholesterol has the capability to strengthen the leaking space in the bilayer membranes. Beyond certain level, increase in cholesterol content starts disrupting the regular bilayer structure thereby decreases the drug entrapment. Hence the optimized concentration of cholesterol (200 mg) was fixed and used in the formulation.

Drug content:

Drug content was analyzed spectrophotometrically at 208nm. All the formulations exhibited fairly uniform drug content. This ensures intended delivery of drug to the site after administration of the developed formulation. Results revealed that drug content to fall developed formulations were in the range of 95.45% - 98.81% as given in the Table 7.

Table 7: Drug content analysis of liposomal formulation

FC	Drug content
F1	98.26 ± 0.15
F2	97.38 ± 0.26
F3	98.14 ± 0.78
F4	98.67 ± 0.74
F5	97.78 ± 0.12
F6	98.81 ± 0.64
F7	96.20 ± 0.42
F8	95.45 ± 0.45

In vitro drug release of Liposomes of Hyaluronic acid:

The *in vitro* release of all developed liposomal formulations was carried out by diffusion method. The studies revealed that the rate of drug release depends on the percentage of drug entrapment efficiency. Among all the eight developed formulations, formulation ratio of CL/SL:1:4 showed maximum entrapment efficiency and sustained drug release of 33.08 ± 0.78 % in 8 hours when compared with other formulation as shown in the figure 4.

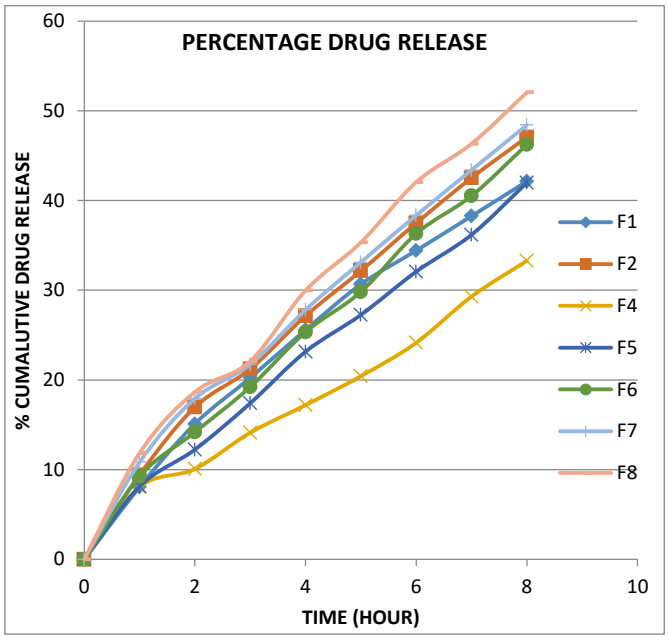

Figure 4: Percentage cumulative drug release of liposomal formulations.

FORMULATION AND EVALUATION OF LIPOSOMAL *IN SITU* GEL OF HYALURONICACID

Preparation of liposomal in situ gel of Hyaluronic acid:

Optimized formulation F6 was selected for formulation of liposomal *in-situ* gel. This formulation contained an addition of pH sensitive polymers carbopol 940 and HPMC K 15M.

Evaluation of liposomal in situ gel of Hyaluronic acid:

Visual appearance:

Visual appearance of developed formulations was found to be milky white dispersion.

Clarity:

Developed formulations were found to be clear and there were no particles or aggregates observed.

pH:

pH of developed formulation was measured in triplicate values and they were in the range of 5.72 ± 0.24 to 5.80± 0.62.

Drug content:

Drug content was measured in triplicate and they were found to be in the range of 92.67±0.44 to 94.63 ± 0.68 %.

Viscosity:

The viscosity of the developed liposomal *in situ* gel formulation was analyzed by Brookfield viscometer DV2T model by using S64 spindle at 10 rpm for duration of 5 minutes. Viscosity was found to be 983 cP and suitable for instillation. As the concentration of polymer increased, viscosities of the formulations were also found to be increased.

Gelling capacity:

Gelation studies were measured by the immediate gelation, immediate and stiff gelation for extended period of time. Optimized formulation exhibited immediate and stiff gelation for extended period of time as indicated. An immediate stiff gelation was found in 5.46 mins as shown in fig 5.

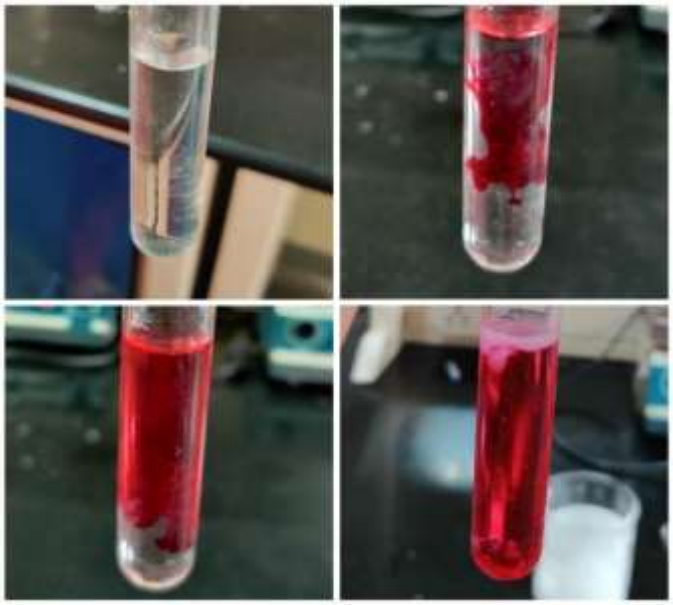

Figure 5: Gelling capacity

In vitro drug release study of Liposomal in situ gel:

The prepared gel formulations showed cumulative drug release of **IN4-31.58 %,** at 8 h. It is obvious that incorporation of liposomes in to structured vehicle resulted in a sustained release of drug. Also this is due to the presence of polymer in the preparations showed mucoadhesive properties of the gel, leading to higher viscosity of liposomal *in situ* gel which provides an extra barrier for release of hyaluronic acid. This may also be due to the high gelling capacity of the formulation Further the formulation showed immediate and stiff gelation for extended period of time as revealed in the gelling capacity studies.

Kinetics study and mechanism of drug release:

The in vitro drug release data of all developed liposomal *in situ* gel were fitted in to various kinetic models. From the studies it was revealed that the optimized formulation obeyed first order release kinetics (R2 value = 0.9965) and followed by Hixoncrowell model with (R2 value = 0.9949) and Non Fickian diffusion mechanism as the n value was 0.773 as mentioned in the table 8.

Table 8: Kinetic release studies data profile of the formulations

Kinetics Model	Parameters	IN4
Zero	**R2**	0.9296
	K0	3.403
	SS	320.1071
First	**R2**	0.9965
	K1	0.048
	SS	16.0796
Higuchi	**R2**	0.8909
	KH	11.725
	SS	495.696
Korsemeyer-Peppas	**R2**	0.9866
	KKP	6.173
	SS	60.8093
	N	0.773
Hixson-Crowell	**R2**	0.9949
	KHC	0.015
	SS	23.0988

Sterility test:

The sterility test was performed for seven days for the optimized formulation. Results revealed that the test tube containing negative control in which only the growth medium was used, showed no signs of precipitation indicating no growth of microorganisms. The test tube containing positive control in which the microorganisms were inoculated in the growth medium showed significant growth in the form of milky white precipitate. The test tube containing the formulation IN4 and the growth medium was found to be sterile and free from microorganism as there were no such precipitates found in the test tube. Results are shown in the Table 9.

Table 9: Results of sterility test for optimized liposomal formulation (IN4)

Sterility test	Results obtained		
	Negative control	Test sample	Positive control
Test for aerobic bacteria (Bacillus subtilis)	-	-	+
Test for Anaerobic bacteria (Bacteriodesvulgatus)	-	-	+
Test for Fungi (Candidaalbicans)	-	-	+

(- indicates: Sterile; + indicates: Non sterile)

Ex vivo trans corneal permeation study:

Finally, at the end of the experiment, each cornea, freed from sclera, was weighed, soaked in1ml of methanol, dried over night at 90ºC and reweighed. From the difference in weights corneal hydration was calculated.

P_{app} = ΔQ/Δt . 1/ (A.C_0.60)Where, ΔQ/Δt (µg/ml) is the flux across the corneal tissue.

A is the area of diffusion (cm^2), C_0 (µg/cm^3) is the initial concentration of drug in donor compartment and 60 is taken as the factor to convert minute into second.

The flux across the cornea was obtained from the slope of the regression line obtained from the linear part of the curve between the amount permeated (Q) Vs time (t) plot. Thus the calculated value for the liposomal *in situ* gel formulation was found to be Kp = 0.0074 cm/h. Images of goat cornea and franz diffusion tube are shown in figure 6.

Stability studies data:

The stability studies of liposomal in situ gel were performed at 5º ± 2º C and 25º ± 2º C / 60 ± 5% Relative Humidity (RH) for 3 months as per modified ICH guidelines. The optimized formulation was examined visually for any precipitation, drug content, pH and gelling capacity for every 30 days for 3 months. It was observed that there was no change in the physical appearance of the formulation. The drug content was analyzed and there was marginal difference between the formulations kept at different temperature as shown in Table 10 and 11. Liposomal *in situ* gel preparation retained good stability throughout the study period when stored at refrigerator temperature.

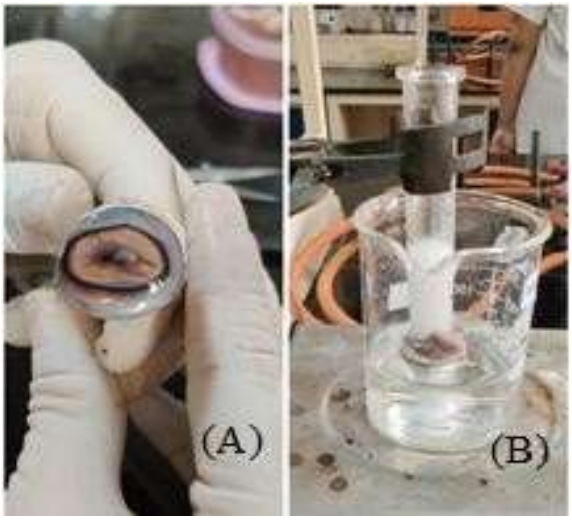

Figure 6: Images of (A) goat cornea and (B) Franz diffusion tube with cornea membrane

Table No.10: Stability data for optimized formulation (IN4) at 25 ± 2ºc/ 60±5% RH at short term accelerated condition

Months	0	1	2	3
Appearance	Milky white dispersion	Milky white dispersion	Milky white dispersion	Milky white dispersion
pH	6.02 ± 0.26	6.04 ± 0.32	6.03 ± 0.18	6.04 ± 0.54
Drug content	98.67 ± 0.44	98.41 ± 0.39	98.54 ± 0.12	98.16 ± 0.63
Gelling studies	+++	+++	+++	+++

Table No.11: Stability data for optimized formulation (IN4) at 5 ± 2ºc stored at refrigerator condition

Months	0	1	2	3
Appearance	Milky white dispersion	Milky white dispersion	Milky white dispersion	Milky white dispersion
pH	6.03 ± 0.28	6.05 ± 0.56	6.04 ± 0.33	6.07 ± 0.68
Drug content	99.62 ± 0.41	99.28 ± 0.19	99.82 ± 0.48	99.18 ± 0.87
Gelling studies	+++	+++	+++	+++

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DECLARATION OF INTEREST STATEMENT:

The authors report there are no competing interests to declare.

CONCLUSION:

The developed novel ocular Liposomal in-situ gel of hyaluronic acid was found to be sterile, non-irritant and provided sustained release with improved ocular residence time by reducing dosage frequency and hence from the above research work, it was concluded that liposomal in-situ gel system is a viable alternative when compared with conventional drops by virtue of its ability to enhance bioavailability through its longer pre corneal residence time, ability to sustain drug release. Also, it is important in case of administration affords, by decreasing the frequency of administration and resulted in better patient acceptance. Hence, it is a future magic targeted delivery in the field of ocular therapeutics.

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