

Development and Characterization of Liposomal Vaginal Gel Loaded With Spironolactone For The Treatment of PCOS/PCOD

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ABSTRACT

Oral spironolactone is mainly used in the treatment of polycystic ovarian disease (PCOD) but it has concerns high first pass metabolism and systemic side effects. The current study optimized a localized liposomal vaginal gel of spironolactone for sustained release and reduced systemic exposure. Liposomes were formulated by ether injection method and optimized by using 3² factorial designs (soya phosphatidylcholine: cholesterol ratios). Liposomes were converted into a gel matrix by using gelling agent like Carbopol 934P. The optimized liposomes (L2) showed vesicle size, zeta potential and entrapment efficiency of 198.7 nm, -30.1 mV and 91.75%, respectively. The final vaginal gel (G8) showed a high drug content (95.22%), excellent spreadability and shows zero-order sustained release (93.34% in 24h). The formulation was found to be physically and chemically stable for 3 months as per standard ICH conditions (30°C/65% RH). This novel localized delivery system provides the targeted anti-androgenic therapy and a safer and more effective way for the management of PCOD.

KEYWORDS: Polycystic ovarian disease (PCOD), Nanotechnology, Vaginal gel, Sustained release therapy.

1. INTRODUCTION

One of the most frequent hormonal and metabolic disorders is PCOD, impacting millions of women during the reproductive age [1-4]. Disorder is associated with hormonal abnormalities causing numerous small fluid-filled cysts to develop in the ovaries and irregular ovulation cycle, as well as symptoms such as irregular periods, high levels of testosterone, insulin resistance, obesity, and severe acne, hirsutism and hyperpigmentation of the skin that make life unmanageable [5-8]. The spherical vesicles (vesicles) with a diameter of nano size are composed of one or more phospholipid bilayers enclosing an aqueous core containing buffer solution, and can contain both lipophilic and hydrophilic drugs and protect the lipophilic drugs from enzymatic degradation, have strong biocompatibility and biodegradability, provide a sustained or targeted release over long time will further decrease the dose frequency, reduce the systemic adverse effect, and are valuable in cancer therapy, antifungal therapy, vaccines, gene delivery, cosmetic and nutraceutical formulations, etc [9-21]. Vaginal gels are the semi-solid mucosal drug delivery systems formulated having bioactive polymers, which are used for delivering drug to gynaecological area with high local concentration, prolonged persistence in the area, eliminating hepatic first-pass metabolism and minimum systemic side effects, and widely used for the management of gynaecological conditions like bacterial vaginosis, fungal infection, contraception, hormone replacement, and now, PCOS/PCOD with the advancement in the formulation of nano-vesicular carriers [22-27]. Although the oral bioavailability of spironolactone and its hepatic first-pass metabolism and side effects of hyperkalaemia and menstrual disturbances are limitations, effective, site-specific treatments are being developed such as liposomal gels to improve the site-specific therapeutic effect while reducing systemic exposure.

2. MATERIALS AND METHODS

2.1. Materials

Spironolactone and Soya Phosphatidylcholine purchased from Dhamtec Pharma (Navi Mumbai, India). Cholesterol, Carbopol 934P, Diethyl ether, Propylene glycol, Triethanolamine, Methyl paraben was collected from St. Wilfred's Institute of Pharmacy, Panvel also phosphate buffer (pH 7.4) and analytical distilled water were made. Magnetic stirrer (Remi 1MLH), Aczet Weighing Balance, Bath Sonicator, Optical Compound Microscope (Labline), UV-Visible Spectrophotometer (Shimadzu UV-1900i), and Fourier Transform Infrared Spectrophotometer (Shimadzu IR Affinity-1S CE) were used for analytical characterization and formulation A pH meter (Hanna Instruments HI2215 / Equitronics EQ 610), Brookfield Viscometer (DV2T), Zeta Potential Analyzer (Malvern Instruments), Transmission Electron Microscopy system (Tecnai 12 G2 Spirit BioTWIN),

custom self-assembled Spreadability Apparatus, Glass Franz Diffusion Cell, Centrifuge Machine (Remi C-24), Stability Chamber (Labline), LG Refrigerator, and standard analytical glassware were used. All chemicals and reagents were analytical grade without purification.

2.2. Pre-formulation Studies

The spectroscopic studies were performed to determine the identity, purity, and drug-excipient compatibility of Spironolactone. Because of its lipophilic nature, methanol was used as the primary solvent to prepare the standard stock solution (100 µg/mL) and subsequent serial dilutions were made. UV-Visible spectrophotometry across the 200–400 nm range provides the maximum absorption wavelength (λ_{max}) enabling the construction of a standard calibration curve of concentration vs absorbance for identification of compound. Furthermore, Fourier Transform Infrared (FTIR) spectroscopy (4000–400 cm^{-1}) was conducted on both the pure drug and 1:1 physical mixtures with selected excipients involved in the experiment. Comparison of the resulting spectra verified structural integrity and confirmed the absence of chemical interactions, supporting the drug's suitability for formulation design [28].

2.3. Preparation of Spironolactone-Loaded Liposomes

Spironolactone-containing liposomes were prepared by the ether injection technique. A full 3^2 factorial design, was used to study the effect of the independent variables, such as Soya Phosphatidylcholine (X1) and Cholesterol (X2), on the formulation characteristics. The quantities of Spironolactone, Soya Phosphatidylcholine and Cholesterol were carefully weighed and then dissolved in an organic solvent of diethyl ether. This organic phase was gently added dropwise via micro needle into a hot aqueous phase, which was stirring under a magnetic stirrer at 60 ± 2 °C. Evaporation of the organic solvent resulted in the spontaneous formation of liposomal vesicles. The dispersion obtained was sonicated to obtain uniform size vesicles and stored at 4 °C for further evaluation [29-30]. The detailed composition of liposomal batches (L1-L9) produced from 3^2 factorial design is summarized in Table 1 and scale up quantity of the drug to convert the concentration from 0.2% to 1% liposomal dispersion is summarized in Table 2.

Table 1: Optimization of SPC and Cholesterol quantity.

Batch	SPC (mg) (X ₁)	Cholesterol (mg) (X ₂)	Spironolactone (mg)	Diethyl Ether (ml)	Phosphate Buffer pH 7.4 (ml)
L ₁	150 (-1)	50 (-1)	20	10	10
L ₂	150 (-1)	75 (0)	20	10	10
L ₃	150 (-1)	100 (+1)	20	10	10
L ₄	225 (0)	50 (-1)	20	10	10
L ₅	225 (0)	75 (0)	20	10	10
L ₆	225 (0)	100 (+1)	20	10	10
L ₇	300 (+1)	50 (-1)	20	10	10
L ₈	300 (+1)	75 (0)	20	10	10
L ₉	300 (+1)	100 (+1)	20	10	10

Table 2: Scale-up the drug quantity to convert the concentration from 0.2% to 1% of liposomal dispersion

Formulation Code	SPC (mg)	Cholesterol (mg)	Spironolactone (mg)	Lipid- Drug Ratio	% Liposomal Dispersion
L ₂	150	75	20	11.25:1	0.2%
L _{2.1}	750	375	100	11.25:1	1%

2.4. Preparation of Liposomal Vaginal Gel

The Spironolactone liposomal gel was prepared by direct incorporation of the Carbopol 934P and Propylene Glycol in the optimized liposomal dispersion (Scale up batch L2.1). The 3^2 full factorial design was used to optimize the gel matrix with respect to two independent variables: Carbopol 934P concentration (X1) and Propylene Glycol concentration (X2). The optimized liposomal dispersion was prepared by adding the specified amounts of both Carbopol 934P (0.5%-1.5% w/w) and Propylene Glycol (0.5%-1.5% w/w), as optimized in the experimental design matrix, to the optimized liposomal suspension under gentle magnetic stirring. It was supplemented with preservative methylparaben (0.1 w/w). The mixture was left in stand (24 hours) at room

temperature to ensure the complete polymer hydration and swelling in the continuous phase (water). Then dropwise addition of Triethanolamine (TEA) was slowly and continuously added to the formulation to adjust the pH to the physiological range of the vagina (pH 4.5-5.0) [31]. Table 3 shows the complete experimental matrix (Batches G1- G9) of liposomal gel formulation.

Table 3: Formulation table of Vaginal gel

Batch	Liposomal Dispersion (%)	Carbopol 934P (mg) (X ₁)	Propylene glycol (mg) (X ₂)	Triethanolamine	Methyl Paraben (%)
G ₁	1	50 (-1)	50 (-1)	q. s	0.1
G ₂	1	50 (-1)	100 (0)	q. s	0.1
G ₃	1	50 (-1)	150 (+1)	q. s	0.1
G ₄	1	100 (0)	50 (-1)	q. s	0.1
G ₅	1	100 (0)	100 (0)	q. s	0.1
G ₆	1	100 (0)	150 (+1)	q. s	0.1
G ₇	1	150 (+1)	50 (-1)	q. s	0.1
G ₈	1	150 (+1)	100 (0)	q. s	0.1
G ₉	1	150 (+1)	150 (+1)	q. s	0.1

(Water adjusted to maintain total 10 gm, small amount of TEA used for neutralization)

2.5. Characterisation of Liposomes Loaded with Spironolactone

2.5.1. Entrapment Efficiency, Vesicle Size, Polydispersity Index, Zeta Potential and Surface Morphology

The entrapment efficiency of Spironolactone in the liposomal formulations was determined using the ultracentrifugation method. A known volume of the dispersion was centrifuged at and for 15 minutes using a cooling centrifuge to separate the drug from the untrapped (free) drug. The clear supernatant containing untrapped Spironolactone was collected, diluted appropriately with solvent and analysed using a UV-Spectrophotometer at the predetermined maximum absorbance wavelength. The percentage entrapment efficiency was calculated using the following equation:

$$\%EE = [(Total\ drug - Free\ drug) / Total\ drug] \times 100.$$

The average hydrodynamic vesicle size, size distribution (Polydispersity Index, PDI) and surface charge (Zeta Potential) of the optimized Spironolactone liposomes were evaluated by Dynamic Light Scattering. The liposomal suspension was diluted 1:100 with distilled water to prevent scattering effects before analysis.

Morphology and spherical shape of vesicles were studied by Transmission Electron Microscopy. A drop of dispersion was placed on a carbon-coated copper grid allowed to adhere and the excess liquid was blotted off with filter paper. The sample was negatively stained with a solution of phosphor- acid air-dried at room temperature. Observed under the TEM [32].

2.6. Characterization and Evaluation of Spironolactone Liposomal Vaginal Gel

2.6.1. Visual Inspection, Homogeneity, pH, Drug Content, Viscosity and Spreadability

The prepared liposomal vaginal gels (G1–G9) were observed for clarity, colour, texture, phase separation, and presence of any foreign particles present in it. A small amount of gel compressed between two glass slides to identity homogeneity of vaginal gel containing liposomes loaded with Spironolactone. pH was measured using a digital pH meter calibrated with buffer solutions (pH 4.0 and 7.0). To measure the stable pH at room temperature, one gram of gel was taken and the electrode was directly immersed.

The liposomal vaginal gel loaded with spironolactone were evaluated for % drug content, flow properties and spreadability. The % drug content of was studied using well calibrated UV spectrophotometer at maximum absorbance of wavelength (λ_{max}) 238 nm by dissolving 1 g of gel in 100 ml of methanol with bath sonication for 15 minutes to break the liposomal vesicles and extract the drug then required dilutions was made until absorbance become 0.2 - 0.9. The non-Newtonian, shear thinning (pseudoplastic) nature of the gel base was studied by studying the flow properties and viscosity using a Brookfield Viscometer (Spindle No. 64/65) at different speeds of rotation (10 to 100 rpm) at 25 ± 0.5 °C. Spreadability was studied using a parallel-plate glass slide apparatus ($S = \frac{M \times L}{T}$), where 1 g of gel was taken and compressed between two glass slides under a 500 g

load for 5 minutes, and the time (in seconds) required for the upper slide of length (in cm) to detach horizontally under a applied weight (in g) was recorded.

2.6.2. In Vitro Drug Release Studies

In vitro release studies of Spironolactone from the liposomal vaginal gel were conducted using a Franz Diffusion Cell apparatus across a cellulose acetate dialysis membrane/ Cellophane membrane (molecular weight cut-off: 12,000–14,000 Da). The dialysis membrane was standardised in Simulated Vaginal Fluid (SVF, pH 4.2–4.5) for 24 hours prior to the experiment.

The membrane was placed between the donor and receptor chambers. 1 g of liposomal vaginal gel (Batch G8) was added into the donor compartment and the receptor compartment with 45 mL of fresh SVF at and under constant magnetic stirring at 50 rpm for the definite interval of time (0.5, 1, 2, 4, 6, 8, 12, and 24 hours), 1 mL aliquots were withdrawn from the receptor compartment and immediately replaced with an equal volume of fresh, pre-warmed medium to maintain sink conditions. Samples were analysed via UV-Vis Spectrophotometer at λ_{max} , and the cumulative percentage of drug released was plotted against time.

2.6.3. Stability Studies

As per ICH guidelines an Accelerated stability testing (25°C/60% RH or 30°C/65% RH) for 3 months helps us to predict the shelf life of dosage forms [33–35].

2.6.4. Statistical Optimization and Analysis

The experimental data obtained from the 3^2 full factorial design were analyzed using Design-Expert / Stat-Ease software. Analysis of Variance (ANOVA) was performed to determine the statistical significance of the linear, quadratic, and interaction terms of the model ($p < 0.05$). Response surface 3D plots and contour plots were generated to visualize the interactive effects of the independent variables on the dependent variables and establish the optimum formulation space [36].

3. RESULT AND DISCUSSION

3.1. Pre-formulation Studies

3.1.1. Organoleptic Properties and Melting Point

Organoleptic testing confirmed that the Spironolactone sample was a white to off-white crystalline/amorphous, odourless solid powder that met material specifications. Drug melting point was 207°C–208°C (mean 207°C) by capillary method. API purity and identity are confirmed without structural degradation.

3.1.2. Spectroscopic Analysis and Calibration Curve

FT-IR Compatibility: The FT-IR spectrum of pure Spironolactone displayed characteristic absorption bands explained in Fig 1 and Table 4

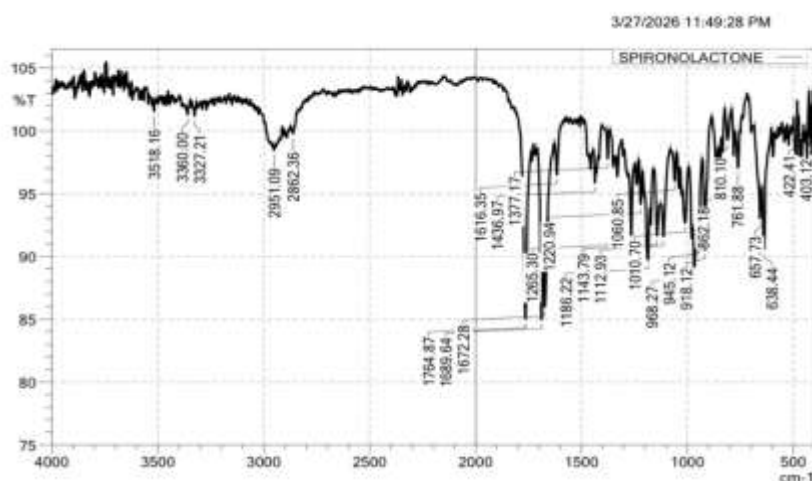


Fig 1: FT-IR spectra of Spironolactone

Table 4: FT-IR Correlation Table of Spironolactone

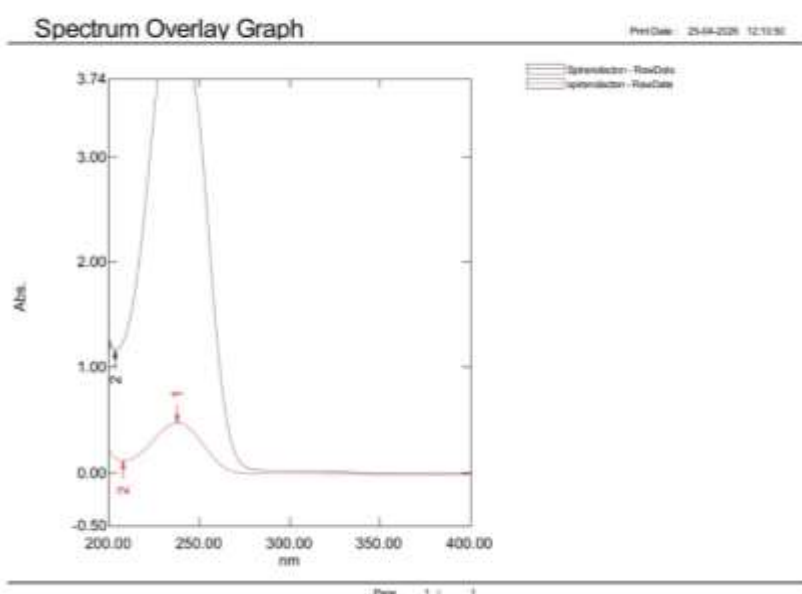
Functional Group	Peak Position on FTIR (cm ⁻¹)	Wavenumber (cm ⁻¹) Observed
C—H (Alkane)	2850-2950	2951.09, 2862.36
C=O (Lactone)	1735-1780	1764.87
C=O (Conjugated)	1680-1715	1689.64, 1672.28
C=C (Alkene)	1600-1650	1616.35
C—O (Ester/Lactone)	1000-1300	1265.30, 1220.94, 1186.22, 1143.79, 1112.93, 1060.85, 1010.70
C—H (Bending)	1350-1470	1436.97, 1377.17
O—H (Stretching)	3200-3600	3518.16, 3360.00, 3327.21

UV Spectrophotometric Calibration:

In Methanol: The λ_{max} was observed at 238 nm. The linear regression equation obtained across concentrations (5–25 µg/mL) was $y = 0.0408x + 0.0485$ with a correlation coefficient (R^2) of 0.9931. Absorbance of spironolactone in methanol at different concentration shown in Table 5 and its spectra and calibration graph figure is presented in Fig. 2 and Fig 3 respectively.

Table 5: Absorbance of Spironolactone in Methanol

Concentration in ug/ml (PPM)	Absorbance at 238 nm
5	0.235
10	0.488
15	0.649
20	0.861
25	1.024

**Fig 2: UV Spectroscopy of Calibration graph Spectra of Spironolactone by using Methanol**

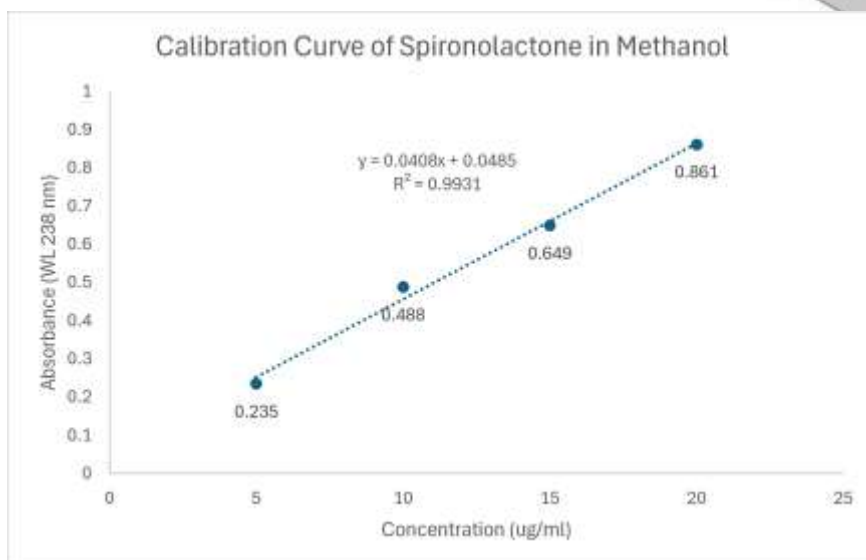


Fig 3: Calibration graph of the drug Spironolactone by using Methanol

In Phosphate Buffer Solution (PBS) pH 4.5: The λ_{max} shifted slightly to 242.5 nm. The standard calibration curve yielded the linear equation $y = 0.0385x + 0.0792$ with $R^2 = 0.9955$, demonstrating compliance with Beer-Lambert's law across the analytical range. Absorbance of spironolactone in Phosphate buffer solution of pH 4.5 at different concentration shown in Table 6 and its spectra and calibration graph figure is presented in Fig. 4 and Fig 5 respectively

Table 6: Absorbance of Spironolactone in PBS of pH 4.5

Concentration in ug/ml (PPM)	Absorbance at 242.5 nm
5	0.248
10	0.497
15	0.656
20	0.849
25	1.035

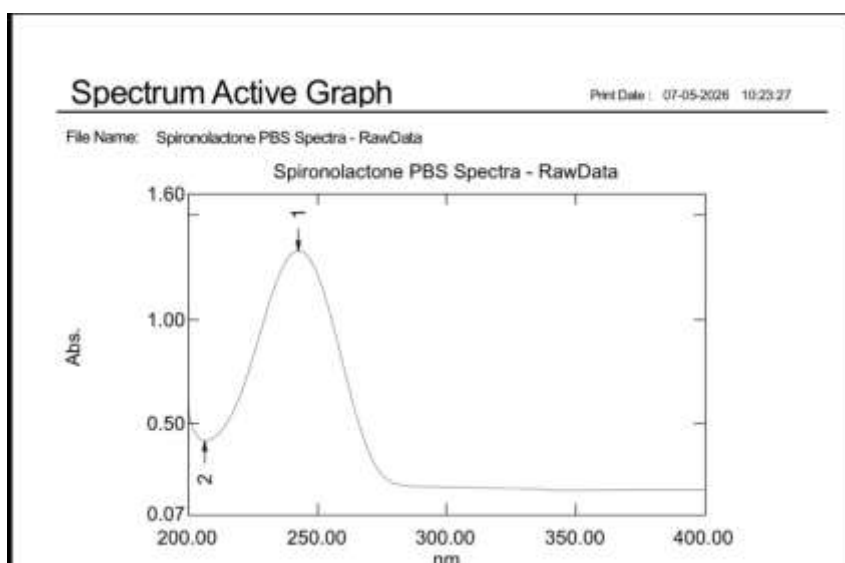


Fig 4: UV Spectroscopy of Calibration graph Spectra of Spironolactone by using PBS of pH 4.5

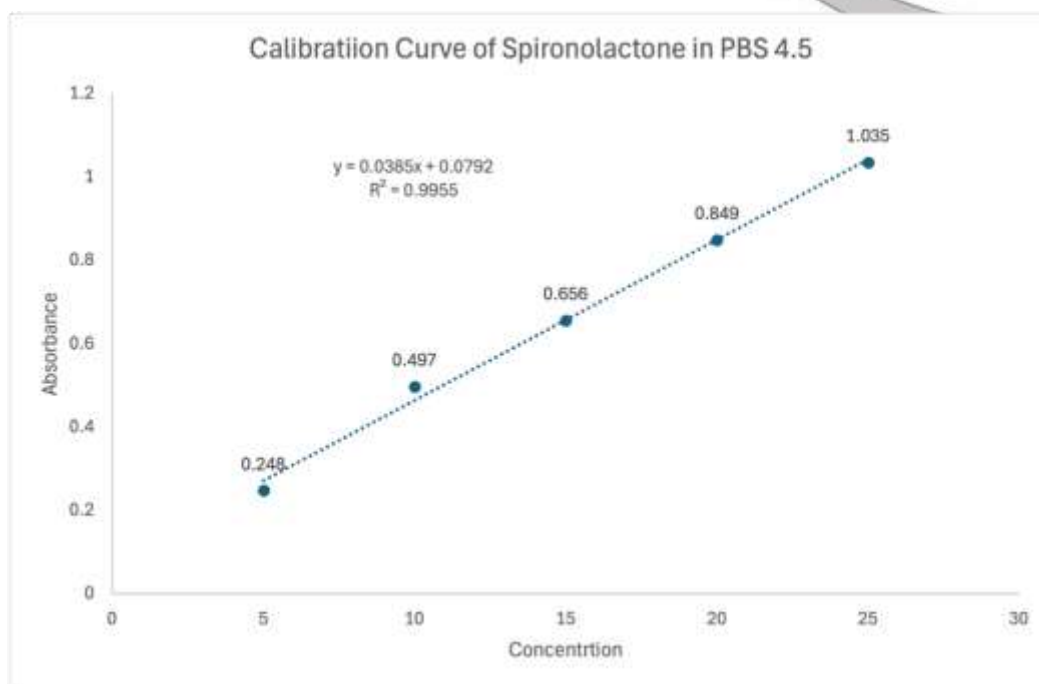


Fig 5: Calibration graph of the drug Spironolactone by using PBS 4.5

3.2. Characterisation and Optimisation of Liposomes

3.2.1. Particle Size, Polydispersity Index (PDI), Zeta Potential and Transmission Electron Microscopy (TEM) of Optimised liposomal dispersion

The % entrapment efficiency (%EE) of formulations L1–L9 ranged from 57.90–91.75%. Batch L2 (SPC:Cholesterol, 11.25:1 lipid to drug ratio) showed the highest % E. E. (91.75%). Higher cholesterol or lipid ratios decreased encapsulation of drug into the vesicles (e.g. L6: 57.90%) due to bilayer rigidity or drug displacement. Formulation L2 had a hydrodynamical diameter of 198.7 nm, PDI of 0.5 and strong colloidal stability with a zeta potential of -30.1 mV, which prevented the vesicle aggregation via electrostatic repulsion. Transmission electron microscopy (TEM) confirmed the discrete spherical vesicular nanostructures with core diameters of 90–100 nm; the larger hydrodynamic size measured by DLS is due to the hydration layer present in aqueous media compared to dried TEM sample. The % entrapment efficacy, particle size, PDI and zeta potential are explained in Table 7 while Transmission electron microscopic image showed in Fig 6.

Table 7: Entrapment Efficacy, Particle size and PDI values of Liposomes loaded with Spironolactone

Sr No.	Formulation Code	Free Drug (mg)	Entrapment Efficacy (%)	Particle Size (nm)	PDI Value	Zeta Potential (mV)
1.	Liposome L1	2.81	85.95	198.6 nm	0.3	-22.1
2.	Liposome L2	1.65	91.75	198.7 nm	0.5	-30.1
3.	Liposome L3	4.47	77.65	201.9 nm	0.4	-13.2
4.	Liposome L4	3.83	80.85	209.1 nm	0.6	-17.4
5.	Liposome L5	3.59	82.05	203.2 nm	0.7	-12.2
6.	Liposome L6	8.42	57.90	200.6 nm	0.7	-10.5
7.	Liposome L7	6.12	69.40	199.6 nm	0.6	-17.5

8.	Liposome L8	2.81	85.95	199.3 nm	0.6	-20.3
9.	Liposome L9	3.27	83.65	199.1 nm	0.5	-21.0

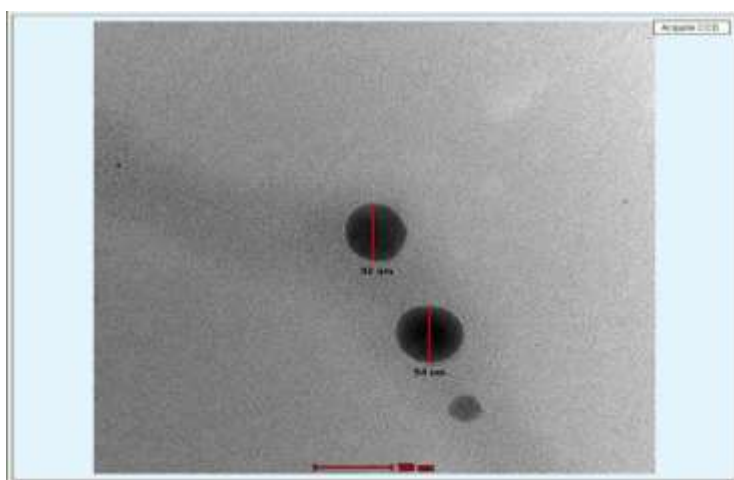


Fig 6: TEM of Liposome L2

3.3. Characterisation of Liposomal Vaginal Gel

3.3.1 Physical Properties, Homogeneity and pH

The prepared liposomal vaginal gel (batches G1–G9) was found to be a smooth, white, odourless semi-solid matrix devoid of any lumps or aggregates. Triethanolamine (TEA) adjustment resulted in a uniform pH of 4.5 for all batches, which is perfectly in line with the physiological vaginal pH 4.5 to prevent mucosal irritation.

3.3.2. FT-IR Compatibility in Final Formulation

The FT-IR spectrum of optimized formulation of liposomal gel exhibited the characteristic peaks of spironolactone along with a slight shifting and broadening of the peaks. The peaks of the spironolactone did not disappear and no new peaks were formed, suggesting that there was no incompatibility between the drug and excipients (SPC, Cholesterol, Carbopol) and that the drug was successful incorporated into the liposomal gel system. The absolute infrared tracking spectrum obtained for the final batch is presented in Fig 7 while the comparative physical peak overlap trace is presented in Fig 8 and FT-IR correlation table of optimized formulation shown in Table 8.

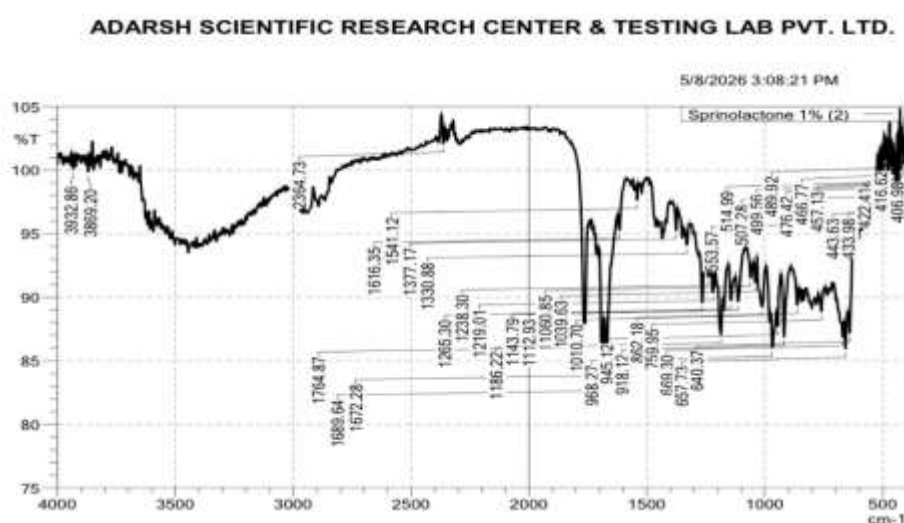


Fig 7: FT-IR Spectrum of Final Formulation

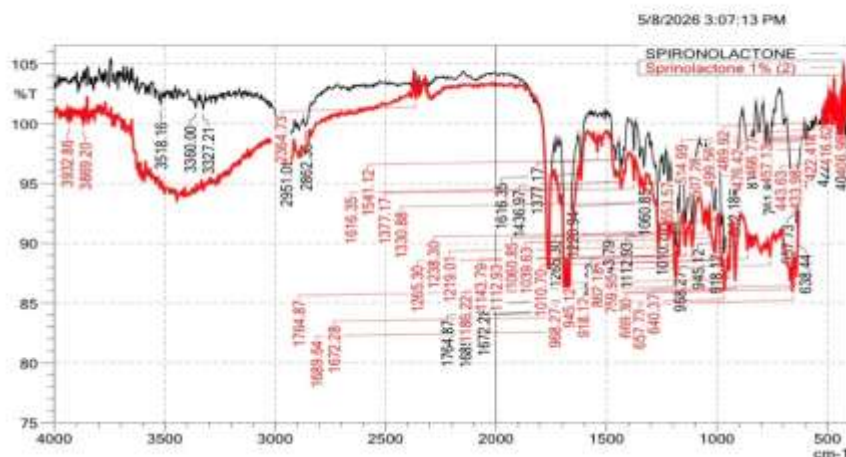


Fig 8: Comparative IR spectrum overlay of drug and final formulation

Table 8: FT-IR Correlation Table of Optimized Formulation

Observed Peaks (cm ⁻¹)	Functional Groups	Interpretation
3518-3327	O—H stretching	Broad peak due to moisture, Carbopol and phospholipid interaction
2951	C—H stretching	Aliphatic asymmetric stretching vibration
2862	C—H stretching	Symmetric stretching of CH ₂ groups
2364	CO ₂ absorbance	Atmospheric carbon dioxide interference
1764	C=O stretching	Characteristic ester/lactone carbonyl group of spironolactone
1689-1672	Carbonyl stretching	Characteristic ketone/lactone vibration
1616	C=C stretching	Skeletal vibration of spironolactone structure
1541	C=C/C—C vibration	Ring skeletal vibration
1377-1330	C—H bending	Aliphatic deformation vibration
1265-1219	C—O stretching	Ester functional group vibration
1186-1039	C—O and phospholipid vibration	SPC and formulation excipient contribution
968-918	C—H out of plane bending	Characteristic fingerprint region of drug
862-638	Fingerprint region	Drug and excipient characteristic peaks

3.3.3. Spreadability, Viscosity, and Drug Content

Spreadability ranged between 15.55 ± 1.21 g · cm/s (G8) and 26.00 ± 0.78 g · cm/s (G3). Lower spreadability values in higher polymer batches correlated directly with increased structural consistency and residence potential. Viscosity measured at 20 s⁻¹ shear rate, viscosity ranged from 2412.9 ± 98 cP (G2) to 5921.1 ± 96 cP (G8). Drug Content Uniformity ranged from 81.40% to 95.22%. The highest drug content of (gel) was recorded in batch G8 (Carbopol 934P, Propylene Glycol) which indicates homogeneous entrapment of the liposomal suspension in the hydrogel network. Spreadability, viscosity and drug content were reported in Table 9.

Table 9: Spreadability, Viscosity, and Drug Content of Optimized Formulation

Formulation Code	Spreadability (g · cm/sec)	Viscosity at 20 s ⁻¹ cP	Drug Content (mg)	% Drug Content (%)
Vaginal Gel G1	25.50 ± 1.86	2684.7 ± 23	8.140	81.40

Vaginal Gel G2	18.85 ± 0.98	2412.9 ± 98	8.762	87.62
Vaginal Gel G3	26.00 ± 0.78	3162.3 ± 45	8.140	81.40
Vaginal Gel G4	20.41 ± 0.87	3788.2 ± 55	7.920	79.20
Vaginal Gel G5	21.22 ± 0.97	4290.5 ± 56	9.470	94.70
Vaginal Gel G6	22.00 ± 1.11	4471.7 ± 25	9.375	93.75
Vaginal Gel G7	17.71 ± 1.03	4924.7 ± 88	8.419	84.19
Vaginal Gel G8	15.55 ± 1.21	5921.1 ± 96	9.522	95.22
Vaginal Gel G9	21.05 ± 1.35	4991.7 ± 79	9.279	92.79

3.3.4. In-Vitro Drug Release Kinetics

The in vitro drug release profile of optimized formulation G8 through a dialysis membrane in Franz diffusion cells (PBS pH 4.5) demonstrated controlled, sustained release kinetics initial release was 4.067% release at 30 min while progressive release reached 13.767% at 3 h, 34.536% at 8 h, and achieved 93.338% cumulative drug release over 24 hours. Kinetic model fitting evaluated against mathematical models demonstrated that the drug release profile best fitted Zero-Order Kinetics (R^2 approaching linearity), confirming a steady, concentration-independent drug release pattern from the liposomal gel depot over 24 hours. The diffusion study data were calculated and reported in Table 10, release kinetics plot of optimized formulation is shown in Fig 9.

Table 10: In-Vitro Drug Diffusion Study of Optimized Formulation

Time (Min)	SQRT	Log time	Abs	Conc	Conc*DF	CCD (ug)	%CDR	Log %CDR	log (100-%CDR)
0	0	0	0	0	0	0	0	0	0
30	5.477	1.477	0.114	0.903	9.038	406.753	4.067	0.609	1.981
60	7.745	1.778	0.119	1.033	10.337	474.233	4.742	0.675	1.978
120	10.954	2.079	0.139	1.553	15.532	718.337	7.183	0.856	1.967
180	13.416	2.255	0.194	2.981	29.818	1376.727	13.767	1.138	1.935
240	15.491	2.380	0.207	3.319	33.194	1558.493	15.584	1.192	1.926

300	17.320	2.477	0.237	4.098	40.987	1942.337	19.423	1.288	1.906
360	18.973	2.556	0.287	5.397	53.974	2567.740	25.677	1.409	1.871
420	20.493	2.623	0.312	6.046	60.467	2913.922	29.139	1.464	1.850
480	21.908	2.681	0.353	7.111	71.116	3453.610	34.536	1.538	1.816
1440	37.947	3.158	0.850	20.020	200.207	9333.818	93.338	1.970	0.823

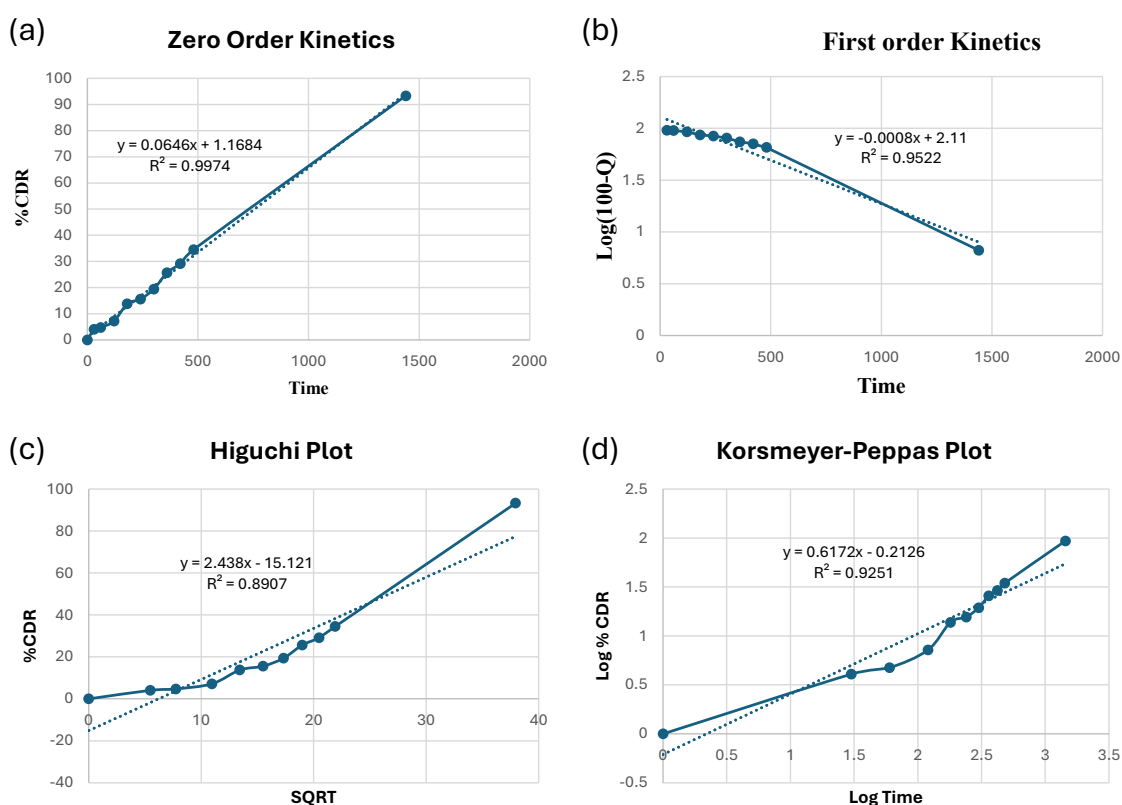


Fig 9: Release Kinetics of Optimized Formulation

3.3.5. Stability Studies

Accelerated stability testing of optimized batch G8 under ICH conditions ($30^\circ\text{C} \pm 2^\circ\text{C}$ / $65\% \pm 5\%$ RH) for 3 months demonstrated minimal physicochemical changes and the results confirm physical integrity, chemical stability, and negligible drug leakage throughout the 3-month storage period. Stability data of optimized formulation were reported in Table 11.

Table 11: Stability Data of Optimized Batch

Parameters	Initial	1 Month	2 Months	3 Months
pH	4.5	4.5	4.5	4.5
Drug Content (%)	95.22	95.18	95.18	95.15
Viscosity (cP)	5921.1 ± 96	5981.5 ± 83	5992.5 ± 67	5995.5 ± 65

3.4. Statistical Analysis and Characterisation (Design-Expert / Stat-Ease 360)

3.4.1. Factorial Characterisation of Liposomes (Response: % Entrapment Efficiency)

A 3² full factorial design evaluated Soya Phosphatidylcholine (Factor A) and Cholesterol (Factor B) on Entrapment Efficiency (%EE). Model Evaluated: sequential sum of squares selected a Quadratic model ($R^2 = 0.7255$, Adjusted $R^2 = 0.2679$, Model F-value = 1.59, $p = 0.3739$). Numerical vs. Empirical Selection states the software predicted a theoretical maximum %EE of 70.69% (172.78 mg SPC, 98.86 mg Cholesterol). However, empirical evaluation overruled the theoretical prediction, as Batch L2 (150 mg SPC, 75 mg Cholesterol) experimentally demonstrated the highest %EE of 91.75% and was selected for gel incorporation. ANOVA data, fit summary of liposome were reported in Table 12 and Table 13. Contour plot and 3D surface model graph are showed in Fig 13 and Fig 14 respectively.

ANOVA for Quadratic model

Response 1: Entrapment Efficacy

Table 12: Data of ANOVA of Liposomes by using Design Expert

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	602.70	5	120.54	1.59	0.3739	not significant
A-SPC	44.55	1	44.55	0.5860	0.4997	
B-Cholesterol	48.17	1	48.17	0.6335	0.4842	
AB	127.13	1	127.13	1.67	0.2866	
A ²	154.59	1	154.59	2.03	0.2491	
B ²	228.27	1	228.27	3.00	0.1816	
Residual	228.09	3	76.03			
Cor Total	830.79	8				

Sequential Model Sum of Squares

Response 1: Entrapment Efficacy

Table 13: Data of Fit Summary of Liposomes by using Design Expert

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	56826.61	1	56826.61			
Linear vs Mean	92.72	2	46.36	0.3769	0.7012	
2FI vs Linear	127.13	1	127.13	1.04	0.3545	
Quadratic vs 2FI	382.85	2	191.43	2.52	0.2281	Suggested
Cubic vs Quadratic	224.13	2	112.06	28.25	0.1319	Aliased
Residual	3.97	1	3.97			
Total	57657.41	9	6406.38			

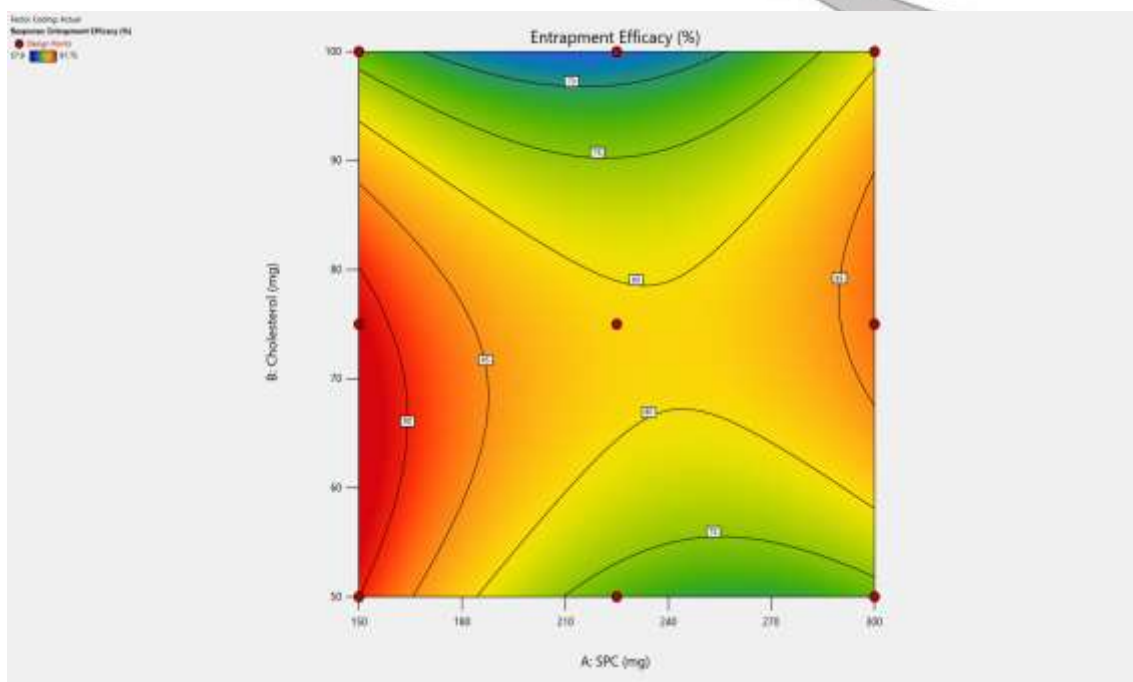


Fig 13: Contour plot of Liposomes by using Design Expert

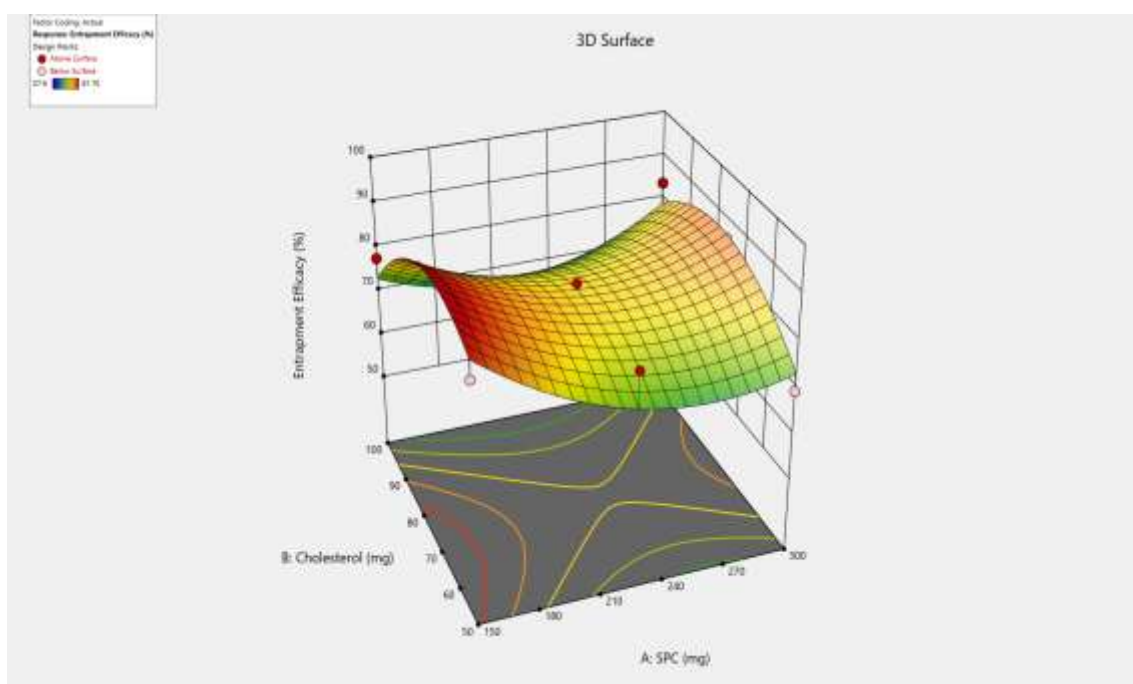


Fig 14: 3D Surface model graph of Liposomes by using Design Expert

3.4.2. Factorial Optimisation of Vaginal Gel (Response: Drug Content)

Carbopol 934P was selected as factor A, and Propylene Glycol was selected as factor B, and were optimised against Drug Content (%) using Stat-Ease Design Expert software. Model Fit Summary of vaginal gel loaded with optimised liposomal dispersion containing spironolactone suggests that the Quadratic model yielded $R^2 = 0.8901$ (Adjusted $R^2 = 0.7070$, Adequate Precision = 5.6298). Linear terms for Carbopol 934P ($p = 0.0839$) and Propylene Glycol ($p = 0.0731$) alongside the quadratic term B^2 ($p = 0.0644$) affects drug loading response trends. Selected optimised gel's Contour and 3D response surfaces demonstrated maximum drug content at higher polymer/co-solvent levels. Numerical optimisation predicted 95.43% drug content for 150 mg Carbopol 934P and 100 mg Propylene Glycol. Experimental validation of Batch G8 matched this prediction with 95.22% drug content, confirming G8 as the optimal gel formulation. ANOVA data and the fit summary of the vaginal gel

were reported in Table 14 and Table 15. Contour plot and 3D surface model graph are shown in Fig. 15 and Fig. 16, respectively.

ANOVA for Quadratic model

Response 1: Drug Content

Table 14: Data of ANOVA of Vaginal Gel by using Design Expert

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	295.44	5	59.09	4.86	0.1117	not significant
A-Carbopol 934P	79.06	1	79.06	6.50	0.0839	
B-Propylene Glycol	89.32	1	89.32	7.35	0.0731	
AB	18.49	1	18.49	1.52	0.3053	
A ²	8.93	1	8.93	0.7348	0.4544	
B ²	99.64	1	99.64	8.20	0.0644	
Residual	36.47	3	12.16			
Cor Total	331.91	8				

Fit Summary

Response 1: Drug Content

Table 15: Data of Fit Summary of Vaginal Gel by using Design Expert

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.1196		0.3431	-0.1023	
2FI	0.4609		0.3008	-0.9231	
Quadratic	0.1261		0.7070	-0.3200	Suggested
Cubic	0.1932		0.9672	0.2526	Aliased

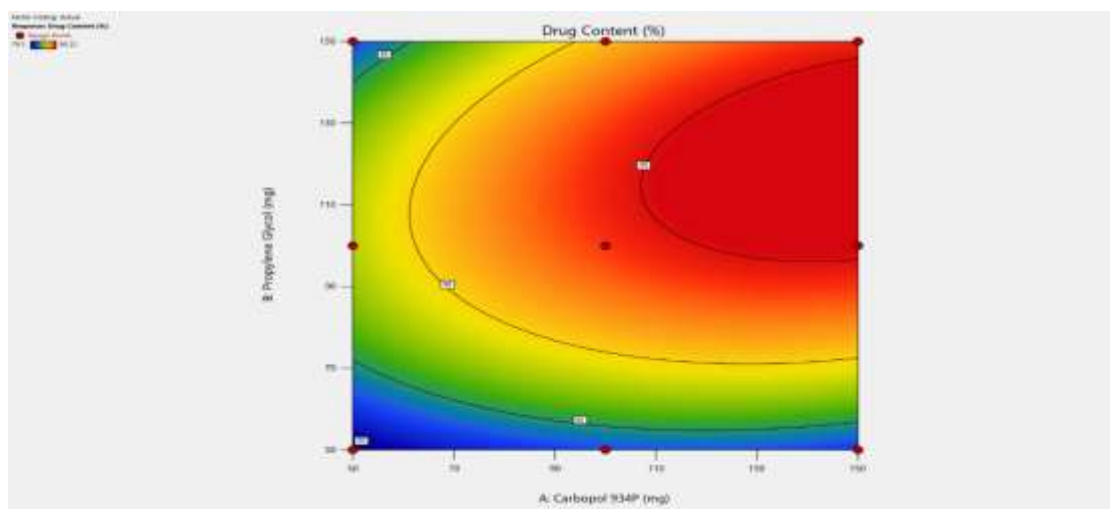


Fig 15: Contour plot of Vaginal Gel by using Design Expert

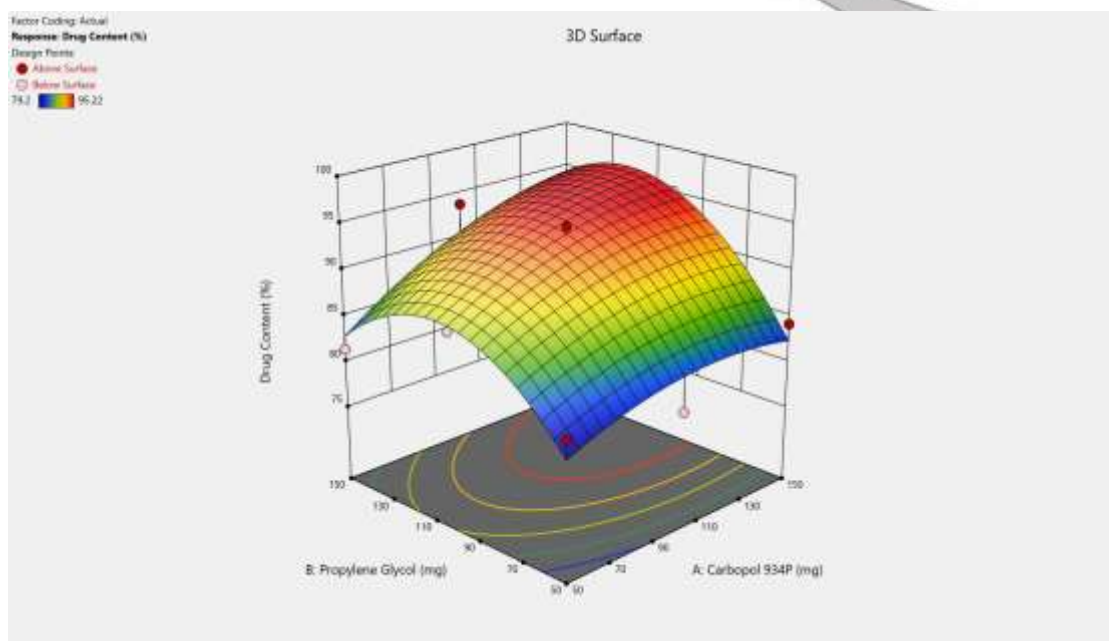


Fig 16: 3D Surface model graph of Vaginal Gel by using Design Expert

3.5. Discussion

The optimised spironolactone-loaded liposomal gel displayed favourable physicochemical characteristics essential for sustained transdermal drug delivery. The inverse relationship observed between cholesterol concentration and entrapment efficiency in batch L6 (%EE: 57.90%) compared to L2 (%EE: 91.75%) can be attributed to cholesterol-induced bilayer rigidification, where excessive cholesterol competes with the lipophilic spironolactone molecules for space within the hydrophobic phospholipid tail region, resulting in drug displacement during hydration. The variance is analysed between the hydrodynamic diameter obtained in dynamic light scattering (198.7 nm) and the smaller size observed in transmission electron microscopy (90–100 nm), as DLS measures in a dilution medium; due to this, the vesicles hydrates along with their surrounding aqueous solvent, whereas TEM captures dehydrated, collapsed vesicles under vacuum conditions. The sustained released profile (93.33% over 24 hours) follows Zero-Order kinetics ($R^2 = 0.9934$) which exhibits controlled-release mechanism. Spironolactone must first partition out of the liposomal lipid bilayer into the surrounding polymer matrix and subsequently diffuse through the cross-linked Carbopol 934P, effectively overcome initial burst release and maintaining a constant release rate over prolonged period of time.

4. CONCLUSION

This research provides a successful development, statistical optimization and characterization of a novel Liposomal vaginal gel to deliver spironolactone for localized treatment of hyperandrogenism associated with PCOS. Using an optimized method of injection (L2) into the liposomes with the aid of a 3^2 full factorial design resulted in a desired nanoscale dimension (198.7 nm) and an acceptable entrapment efficiency of 91.75%, which allowed for strong entrapment of the hydrophobic drug within the lipid bilayer. It was found that these vesicles could be successfully incorporated into gel matrix of Carbopol 934P (G8), which gave the best formulation with optimum rheological characteristics, biocompatible pH 4.5 and highly uniform drug content (95.22%). Importantly, in vitro diffusion study showed that the liposomal gel network is an effective depot and the drug concentration is independent of the release rate and it is sustained for 24 hours following zero-order release kinetics with a release percent of 93.338%. Additionally, the formulation exhibited good physical and chemical stability for three months under accelerated ICH conditions where there was minimal change in pH, viscosity and drug content. Finally, this liposomal vaginal gel represents a novel local delivery system and represents a very good alternative to the extensive first-pass metabolism and systemic side effects seen with oral spironolactone administration. Although these in vitro and physicochemical data are highly encouraging, additional studies to be performed in ex vivo permeation, muco-adhesion, and in vivo pharmacokinetic studies will be crucial to provide the ultimate translation of this targeted delivery system to the clinical arena as a therapeutic option for women with PCOS.

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