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Design of experiment based formulation optimization of chitosan-coated nano-liposomes of progesterone for effective oral delivery

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ABSTRACT

The aim of this research was to design and develop chitosan-coated nano-liposomes of progesterone for its safe and effective oral delivery through the vesicular system providing sustained drug release, enhanced drug stability in gastro-intestinal (GI) fluid and improved drug absorption leading to better patient compliance. The aqueous solubility of progesterone (poorly soluble drug) was enhanced by hydroxy-propyl-beta-cyclodextrin complexation and the drug-loaded liposomes were prepared by ethanol injection method followed by surface coating with chitosan. Design of experiment-based formulation optimization was performed using Box-Behnken design selecting lipid, cholesterol, and drug content as formulation factors (independent variables) and mean particle size (MPS), polydispersity index (PDI), zeta potential (ZP), entrapment efficiency (EE), drug loading (DL) and cumulative % drug release (CDR) as evaluation parameters (response variables). The optimized formulation was prepared and evaluated for all preferred critical quality attributes which showed 168.3 nm MPS, 0.307 PDI, 24 mV ZP, 53% EE, 7.2% DL, and 76.4% CDR at 24 hours. *In-vitro* GI drug stability of chitosan-coated liposomes was studied in simulated gastric fluid and simulated intestinal fluid which exhibited 2.12 and 77.3 fold extended half-life, respectively. The *ex-vivo* GI-drug absorption study demonstrated two-fold rise in progesterone absorption from liposomal formulation. The chitosan-coated liposomes of progesterone which showed sustained drug release following Higuchi model kinetics was found to be a better alternative for oral delivery of progesterone overcoming drawbacks of conventional dosage forms.

INTRODUCTION

Oral administration of therapeutic drugs is one of the oldest and most preferred approaches of medication because, it is non-invasive, inexpensive, self-administrable, and provides controlled dosing frequency resulting in high patient compliance and therefore proved to be a promising route of administration for both natural and synthetic drugs (Alqahtani *et al*., 2021). However, oral administration of conventional dosage forms such as tablet, capsule, syrup, suspension, etc. also encounters the problems like gastrointestinal (GI) instability of drugs, the effect of GI fluid enzymes, poor pharmacokinetic profile, and limited GI drug absorption leading to low oral bioavailability (Homayun *et al*., 2019). One of the major causes of low oral drug bioavailability is the drug's poor water solubility and rate of dissolution in GI/biological fluid, hence their effective oral delivery remains a challenge because about 70% of new drugs are practically insoluble in water (Ghassemi *et al*., 2018). Therefore, novel drug carrier systems such as liposomes, niosomes, polymeric micelles, nanocrystals, nanoparticles as well as drug-cyclodextrin complex are being widely explored for effective drug delivery eliminating drawbacks associated with conventional dosage forms (Babadi *et al*., 2021; Cagdas *et al*., 2014; Torchilin, 2005). Among all these, liposomal nano-drug carriers are extensively being reported

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showing desired/controlled drug release profile, improved uptake across biological membranes including GI absorption, prolonged half-life, and drug action, hence enhanced drug bioavailability with reduced side effects (Torchilin, 2005).

Liposomes are defined as spherical-shaped vesicles containing drug-loaded aqueous compartment that is enclosed by one or more concentric lipidic bilayers of 25–2,500 nm size range (Akbarzadeh *et al*., 2013; Liu *et al*., 2019). The water-soluble drug entraps in the aqueous compartment and water-insoluble drug intercalates in the lipophilic bilayer (Stanczyk *et al*., 2013). The essential components of the liposomal vesicle are phospholipid and cholesterol and they are considered biocompatible and biodegradable due to natural occurrence of these components in the biological membrane (Large *et al*., 2021).

As per literature review and regulatory reports, there is no marketed oral liposomal product available due to the limitations associated with their oral administration, e.g., liposomal instability in the gastric/intestinal fluid and leakage of the encapsulated drug resulting in low oral bioavailability (Lee, 2020). Various novel formulation approaches in liposome development such as modification of lipid components, liposomal surface modification, inner bilayer thickening, and enhanced absorption by mucoadhesion have been investigated to overcome the limitations associated with oral administration of liposomes (He *et al*., 2019). As compared to conventional liposomes, the chitosan-coated nano-liposomal formulation is considered a better approach for effective and safe oral drug delivery because chitosan coating protects liposomes from destruction in gastric/intestinal fluid and facilitates oral absorption of drugs having poor water-solubility. The chitosan-coated positive charged (cationic) liposomes readily interact with the negatively charged biological membrane and stabilizes the drug encapsulated in liposomes resulting into enhanced and safe drug permeation by opening tight junctions between the cells to promote drug transportation (Elsayad *et al*., 2021; Nguyen *et al*., 2014).

Progesterone is a female hormone and widely used in hormonal replacement therapy in conditions such as endometrium hyperplasia and dysmenorrhea. The bioavailability of progesterone on oral administration is less than 5% and unlike other drugs, micronized progesterone also has only 8.6% bioavailability (Simon *et al*., 1993) due to its low water-solubility which disfavors the oral administration of progesterone. Various formulation techniques have been used to enhance drug solubility such as inclusion-complex with cyclodextrins, pH adjustment, particle size reduction, etc. Inclusion-complex with cyclodextrins/hydroxy-propyl-beta-cyclodextrin (HP-β-CD) has been widely reported for enhancing the solubility and stability of drugs along with reducing their toxicity (Jansook and Loftsson, 2009; Lahiani-Skiba *et al*., 2006). Modification of drugs with complexing agents has been widely reported to increase the encapsulation efficiency of water-soluble as well as water-insoluble drugs in the liposomes (Kulkarni *et al*., 1995). Further development of chitosan-coated nano-liposomes may prove to be a better alternative and remedy to many limitations of oral drug delivery as conventional dosage forms.

The current study reports the formulation designing and design of experiments (DoE) based optimization of chitosan-coated nano-liposomes of progesterone with HP-β-CD complex. The Box-Behnken experimental design was used selecting different formulation factors as independent variables and critical quality attributes (CQAs) of liposomal products as the response variables. The developed liposomal formulation was characterized for different physico-chemical properties and evaluated for different *in-vitro* and *ex-vivo* performance parameters.

MATERIALS AND METHODS

Materials

Progesterone was procured as a free sample from M/s. Encube Ethicals Pvt. Ltd. (Mumbai, India). Hydrogenated soy phosphatidylcholine (HSPC) and cholesterol (CH) were purchased from Sigma-Aldrich (India). HP-β-CD was received from Ningbo Hi-Tech Biochemicals (China). Low molecular weight chitosan (CS) was purchased from Himedia (Mumbai, India). All other chemicals and solvents used in this work were of analytical grade.

Inclusion complexation of progesterone with HP-β-CD

The HP-β-CD complex of progesterone was prepared by simply dissolving progesterone in the aqueous solution of HP-β-CD. Accurately weighed 1 g of HP-β-CD was dissolved in 10 ml of purified water to prepare 0.07 M HP-β-CD solution. Accurately weighed progesterone (5 mg/ml) was dissolved in this solution by vortexing for 10 minutes. The stability of this inclusion complex as shown in Figure 1 was determined by phase solubility analysis of progesterone and HP-β-CD (Lahiani-Skiba *et al*., 2006; Soni and Saini, 2019).

Preparation of progesterone loaded nano-liposomes

Progesterone-loaded nano-liposomes were prepared by ethanol injection technique as schematically shown in Figure 2. The accurate amounts of HSPC and cholesterol were dissolved in ethanol and maintained at 50°C to form an ethanolic phase. Progesterone was dissolved in 0.07 M HP-β-CD solution and kept on a magnetic stirrer at 50°C to prepare the aqueous phase. The ethanolic phase was injected into the pre-heated aqueous phase kept stirring at 500 rpm resulting in translucent liposomal dispersion which was further stirred for 60 minutes for removal of ethanol. Resultant liposomal dispersion (large vesicles) was sonicated by a probe sonicator (Sonics, VCX 500) for 2 minutes for size reduction (Gouda *et al*., 2021; Jaafar-Maalej *et al*., 2010).

Chitosan coating of drug loaded nano-liposomes

Chitosan coating of liposomal vesicles was done using 0.2% (*w*/*v*) solution of low molecular weight chitosan in 0.1% (*v*/*v*) acetic acid. The prepared liposomal dispersion was added with the help of a dropper into an equal volume of chitosan solution

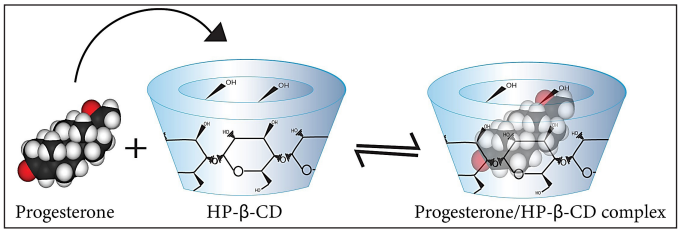

Figure 1. Schematic illustration of progesterone/HP-β-CD inclusion complex.

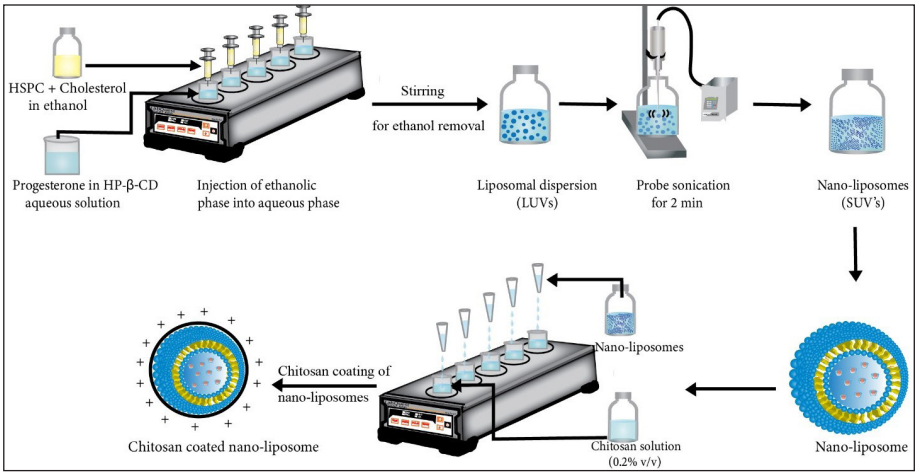

Figure 2. Schematic representation of preparation of chitosan-coated nano-liposomes of progesterone by ethanol injection method.

kept stirring at 100 rpm for 1 hour to form chitosan-coated nano-liposomes (Elsayad *et al*., 2021) as shown in Figure 2.

DoE optimization of progesterone loaded nano-liposomes

Experimental design

Design Expert 12 (Stat-Ease Inc., Minneapolis) was used in the formulation optimization of progesterone-loaded nano-liposomes, selecting a three-factor, three-level Box-Behnken design (BBD). The amount of formulation components i.e., HSPC (F1), cholesterol (F2), and progesterone (F3) at low, medium, and high level were selected as formulation factors (independent variables). Mean particle size (MPS), polydispersity index (PDI), zeta potential (ZP), % entrapment efficiency (EE), % drug loading (DL), cumulative % drug release (CDR) at 0.5, 1, 3, 6, 9, 12 and 24 hours were selected as evaluation parameters (response variables) for optimization studies (Soni and Saini, 2021a) and are shown in Table 1.

Statistical analysis

The 15 experimental runs (optimization batches) suggested by software with the proposed composition were prepared and evaluated for 12 response variables (R1–R12). The observations (response data) as shown in Table 2 were provided to the software for the statistical fitting into different models i.e., linear, 2FI, quadratic, and cubic. After statistical justification by analysis of variance, the software suggested the best fit linear model for MPS, PDI, ZP, % CDR at 1, 12, 24 hours; quadratic model for % EE, % DL, % CDR at 0.5 hours; and 2FI model for % CDR at 3, 6, and 9 hours as shown in Table 3. The *p*-value (<0.05) was regarded as statistically significant (Weng and Tong, 2020).

Response surface analysis and optimization standards

The effect of independent variables on each response variable was studied by plotting 3D response surface graphs for each response variable (Figs. 3–10). For finding the optimal composition of chitosan-coated progesterone-loaded nano-liposomes, the optimization goals for independent variables were fixed as, HSPC content (F1) in range, cholesterol content (F2) in range, and progesterone content (F3) was targeted to 50, while the response variables were set to minimum MPS, minimum PDI, maximum ZP, maximum %EE, maximum %DL, maximum CDR at 0.5, 1, 3, 6, 9, 12 and 24 hours. The above-mentioned optimization goals for both independent and response variables as shown in Table 1 were fed into the software. Consequently, the software predicted an optimized composition having maximum desirability value out of many alternative compositions.

Characterization of chitosan-coated nano-liposomes of progesterone

Particle size, PDI and ZP

The MPS, PDI, and ZP of all prepared batches of chitosan-coated nano-liposomes of progesterone were determined by Nanopartica SZ-100 (Horiba Scientific) particle size analyzer. The principle involved in the determination of MPS and PDI was dynamic light scattering, while in ZP measurement it was laser Doppler electrophoresis. Recorded observations are shown in Table 2.

%EE and %DL

The EE of chitosan-coated progesterone-loaded nano-liposome batches was determined by centrifugal ultra-filtration method (Soni and Saini, 2021b). Accurate 0.5 ml of each liposomal formulation was taken in the centrifugal concentrator tubes (Microcon® Ultracel YM-100) and centrifuged for 45 minutes at 10,000 rpm using refrigerated centrifuge (Eppendorf, Germany). The concentrated liposomes remained on the upper part of filter and the filtrate (containing unentrapped drug) was collected in the bottom part tubes which were then suitably diluted with ethanol for estimation of free drug content by UV spectrophotometer (Shimadzu 1700, Japan) at 241 nm. The %EE was calculated using the given formula (Panwar *et al*., 2010).

$$EE\ (\%) = \frac{\text{Total drug added} - \text{Free drug}}{\text{Total drug added}} * 100$$

The concentrated liposomes retained on upper filter tube were carefully collected and volume was accurately measured. Drug-loaded liposomes were lysed by adequate amount of ethanol with vortexing. The solution was analyzed for entrapped drug at

Table 1. Independent and response variables for optimization of chitosan-coated progesterone loaded nano-liposomes.

Independent variables	Unit	Level		
		Low	Medium	High
F1: HSPC	mg	200	350	500
F2: Cholesterol	mg	50	100	150
F3: Progesterone	mg	50	55	60
Response variables	**Unit**			**Desired constraint**
R1: Particle size	nm			Minimize
R2: Polydispersity index	-			Minimize
R3 : Zeta potential	mV			Maximize
R4: Entrapment efficiency	%			Maximize
R5 : Drug loading	%			Maximize
R6: Cumulative drug release at 0.5 hours	%			Maximize
R7: Cumulative drug release at 1 hour	%			Maximize
R8: Cumulative drug release at 3 hours	%			Maximize
R9: Cumulative drug release at 6 hours	%			Maximize
R10: Cumulative drug release at 9 hours	%			Maximize
R11: Cumulative drug release at 12 hours	%			Maximize
R12: Cumulative drug release at 24 hours	%			Maximize

241 nm on UV spectrophotometer. The DL (%) was calculated using formula (Arafat *et al*., 2017) as below.

$$DL\ (\%) = \frac{\text{Amount of drug entrapped in liposomes}}{\text{Total amount of liposomes}} * 100$$

In-vitro drug release study

In-vitro drug release of chitosan-coated nano-liposomes of progesterone was studied by dialysis method using dialysis membrane (Himedia, India) with 12,000–14,000 Da molecular weight cut off. Prior to use, the dialysis membranes were activated as per previously reported method (Soni and Saini, 2021a). An accurately measured 1 ml of liposomal dispersion was introduced in a dialysis membrane bag and then was closed in both ends using closure clips. It was then immersed into 250 ml volume of 3% *w/v* sodium lauryl sulfate solution in 0.1 N HCl kept at 37°C ± 0.5°C temperature and stirred at 75 rpm. At different time intervals, i.e., 0.5, 1, 3, 6, 9, 12, and 24 hours, the release media were taken out. The amount of drug release was estimated by a UV spectrophotometer (Shimadzu 1700) at 245 nm. The drug release was calculated and reported in Table 2 and graphically shown in Figure 11.

Optimized formulation of chitosan-coated nano-liposomes of progesterone

The design expert predicted the optimized chitosan-coated nano-liposomes of progesterone containing, 205.6 mg of HSPC, 108.1 mg of cholesterol, and 50 mg of progesterone with a maximum desirability value of 0.775. The predicted highest desirability in the optimized of chitosan-coated nano-liposomes of progesterone has been represented in the 3D response plot and the 2D contour plot as shown in Figure 12. The optimized formulation was prepared and experimentally evaluated for each response variable to perform the validation of software prediction.

Evaluation of optimized formulation

Microscopic evaluation

The microscopic examination of the optimized formulation was performed under the optical microscope (Leica DM 1000) at a magnification of 100× under the oil immersionlens. The microscopic view is depicted in Figure 13.

Particle size, PDI, and ZP study

The MPS, PDI, and ZP of optimized batch were analyzed using Nanopartica SZ-100 (Horiba Scientific) particle size analyzer. The observations of particle size analysis are recorded as data in Table 4 and presented graphically in Figure 14.

EE and DL study

%EE and %DL were determined by the centrifugal ultrafiltration technique as discussed in the previous sections and has been recorded in Table 4.

In-vitro drug release profile and drug release kinetics

In-vitro drug release of optimized chitosan-coated nano-liposomes of progesterone was studied according to previously described dialysis method and the amount of drug release was estimated by UV spectrophotometer (Shimadzu 1700) at 245 nm. The observed % drug release at different time intervals is reported in Table 4.

The drug release kinetics of optimized liposomal formulation was assessed by statistical fitting of drug release data in different kinetic models, like zero order, first order, Korsmeyer-Peppas, Higuchi, and Hixon-Crowel (Fig. 16).

DSC study

The lyophilized form of chitosan-coated progesterone loaded nano-liposomes, progesterone drug sample, HSPC, cholesterol, HP-β-CD, chitosan was analyzed for thermal property using DSC (Perkin Elmer 6000, Waltham, MA). Approximately, 3 mg weighed samples were individually placed and sealed in an aluminum pan and kept against a blank aluminum pan as the reference. The thermograms as shown in Figure 17 were recorded when it was further heated from 50°C to 300°C at of 40°C/minute heating rate under the purging of nitrogen (inert) gas with 20°C/minute flow rate (Sharma *et al*., 2017).

Ex-vivo drug permeation study

The drug permeation study of optimized formulation of progesterone-loaded liposomes and prepared suspension of marketed tablet product were performed using non-everted chicken intestine (ileum) segment. A freshly excised complete lower GI tract of a healthy chicken was procured from a nearby slaughter house. To perform the study, the ileum was isolated and cut into 6 cm pieces and then rinsed with phosphate buffer saline (PBS) pH 6.8. One end of ileum segment was tied and 1 ml of each formulation was filled in different ileum segments and then the other end was also closed using thread. The formulation holding intestinal segments were immersed into 250 ml of PBS pH 6.8 kept in a beaker at 37°C and stirred at 100 rpm. Then samples were taken out at pre-determined time intervals and the fresh buffer was replaced for maintaining the sink condition (Hasan *et al*., 2020; Ma *et al*., 2014). The % drug permeation was estimated by UV

Table 2. Composition of progesterone loaded nano-liposomes and their observed responses.

S. No.	Independent variable			Response variable										
	F1: HSPC in mg	F2: Cholesterol in mg	F3: Drug content in mg	R1: Particle size in nm	R2: PDI	R3: ZP in mV	R4: EE in %	R5: DL in %	R6: CDR at 0.5 hours in %	R7: CDR at 1 hour in %	R8: CDR at 3 hours in %	R9: CDR at 6 hours in %	R10: CDR at 9 hours in %	R11: CDR at 12 hours in %	R12: CDR at 24 hours in %
CCNL1	500	150	55	275.0	0.490	18.0	72.2	5.6	4.4	6.31	16.24	30.56	36.18	38.07	42.50
CCNL2	350	100	55	201.0	0.392	23.0	66.5	7.3	4.9	7.90	20.90	34.70	45.70	56.70	62.70
CCNL3	200	100	50	143.8	0.292	26.6	48.8	6.0	1.7	8.90	27.90	48.58	65.89	77.70	95.80
CCNL4	350	50	60	175.5	0.342	24.0	67.4	8.8	5.7	8.10	22.50	37.80	47.80	54.30	66.00
CCNL5	200	100	60	148.8	0.299	26.3	49.0	8.1	2.9	5.90	17.84	36.55	49.64	61.60	71.60
CCNL6	500	100	50	269.0	0.488	19.0	70.6	5.4	3.5	7.40	18.10	31.30	40.50	48.20	52.10
CCNL7	500	100	60	270.0	0.489	18.5	68.0	6.1	2.4	6.60	15.70	28.60	41.30	49.70	53.10
CCNL8	500	50	55	268.0	0.487	21.0	70.5	6.4	2.9	4.90	17.30	29.60	39.70	45.60	50.60
CCNL9	350	100	55	202.0	0.393	23.2	66.3	7.3	4.9	7.92	20.82	34.65	45.65	56.65	62.85
CCNL10	350	150	60	267.0	0.468	22.0	69.5	7.4	3.9	5.90	15.80	29.60	41.60	44.80	53.70
CCNL11	350	150	50	250.0	0.431	22.8	69.4	6.3	4.4	12.8	29.70	42.60	48.70	53.70	55.80
CCNL12	350	50	50	166.6	0.317	24.5	53.0	5.8	7.3	9.90	20.70	33.10	47.10	52.20	66.20
CCNL13	350	100	55	200.0	0.391	23.6	66.8	7.2	4.8	7.94	20.88	34.68	45.68	56.68	62.69
CCNL14	200	150	55	152.6	0.302	26.0	51.0	6.9	3.5	8.07	21.60	33.30	48.60	57.80	66.90
CCNL15	200	50	55	138.9	0.256	28.0	46.8	8.4	5.3	11.6	31.50	50.40	68.50	76.90	97.50

% CDR = Cumulative % drug release.

spectrophotometer at 247 nm and the graphical presentation is given in Figure 18. The apparent permeability coefficient (Papp) and steady-state drug permeation flux (Jss) of progesterone from optimized liposomal formulation was calculated by below given formula and it was compared with prepared suspension of marketed tablet product (drug content 2.5 mg/ml).

$$\text{Papp} = \frac{dQ}{dt * C_{\circ} * A * 60}$$

$$\text{Jss} = Papp * C_{\circ}$$

where dQ/dt is the equilibrium state permeation rate in the media; A is surface area of the intestinal segment; while $C_{\circ}$ expresses initial drug concentration. (Cylindrical-shaped ileum segments had a 6 cm length and 0.55 cm inner diameter and thus, calculated surface area was 10.84 cm^2 in each segment.)

In-vitro GI stability study

In-vitro GI stability of progesterone-loaded chitosan-coated nano-liposomes was studied in simulated gastric fluid (SGF:pH 1.2) and simulated intestinal fluid (SIF:pH 6.8). Accurate 1 ml liposomal dispersion was added to the 20 ml of each biological fluid. The solutions were homogenized and vortexed for 15 minutes, then incubated at 37°C for 2 hours. The study samples were taken out at 5, 15, 30, 60, 90, and 120 minutes time intervals and centrifuged for 15 minutes duration at 10,000 rpm. Then the sample was diluted with each simulated fluid, and estimation of progesterone content was performed spectrophotometrically. The degradation rate constant (K) of progesterone and degradation half-life ($t_{½}$) for both GI conditions were calculated (Braga Emidio *et al*., 2021; Wang *et al*., 2017). A similar study was performed with progesterone drug solution for comparison.

RESULTS AND DISCUSSION

Inclusion complexation of progesterone

Complex formation of the drug showed enhanced aqueous solubility of progesterone. The presence of hydrophobic inner cavity along with a hydrophilic external surface in cyclodextrins contributed to accelerate the aqueous solubility of the drug (Loftsson *et al*., 2005). The highest drug solubility was found in HP-β-CD, accordingly, it was selected from amongst the different types of cyclodextrins for complexation of progesterone. Non-covalent attraction between drug particles and inner hydrophobic cavity of cyclodextrin is responsible for complex generation (Shimpi *et al*., 2005). The optimum concentration of HP-β-CD (0.07 M) was finalized for further development to meet the desired solubility of progesterone in the formulation.

Preparation of chitosan-coated nano-liposomes of progesterone

Selection of HSPC as phospholipid was done considering its phase transition temperature (T_c), i.e., approximately 50°C and high stability in GI fluids because lipids having phase transition temperature less than 37°C, gets readily degraded in gastric fluids (He *et al*., 2019). Cholesterol was used

Table 3. BBD's statistical data and model fitting of response variables for optimization of progesterone loaded nano-liposomes.

Response variable	Model	Sequential *p* value	Lack of fit *p* value	Adjusted R^2 value	Predicted R^2 value	Remarks
R1, MPS	Linear	<0.0001	0.0027	0.8903	0.8198	Suggested
	2FI	0.9942	0.0018	0.8506	0.5636	-
	Quadratic	0.9053	0.0010	0.7843	−0.2322	-
	Cubic	0.0010	-	0.9996	-	Aliased
R2, PDI	Linear	<0.0001	0.0016	0.9253	0.8745	Suggested
	2FI	0.8535	0.0011	0.9063	0.7160	-
	Quadratic	0.9800	0.0006	0.8551	0.1720	-
	Cubic	0.0006	-	0.9999	-	Aliased
R3, ZP	Linear	<0.0001	0.3412	0.9790	0.9667	Suggested
	2FI	0.7485	0.2765	0.9750	0.9327	-
	Quadratic	0.0556	0.5532	0.9901	0.9638	-
	Cubic	0.5532	-	0.9898	-	Aliased
R4, % EE	Linear	0.0004	0.0024	0.7448	0.6115	Suggested
	2FI	0.5513	0.0021	0.7263	0.3391	-
	Quadratic	0.0849	0.0035	0.8712	0.2655	Suggested
	Cubic	0.0035	-	0.9992	-	Aliased
R5, % DL	Linear	0.0009	0.0002	0.7009	0.5293	-
	2FI	0.2390	0.0002	0.7503	0.3767	-
	Quadratic	0.0491	0.0005	0.9066	0.4664	Suggested
	Cubic	0.0005		0.9999	-	Aliased
R6, % CDR at 0.5 hours	Linear	<0.0001	0.0065	0.9666	0.9536	-
	2FI	0.2747	0.0069	0.9710	0.9527	-
	Quadratic	0.0181	0.0223	0.9928	0.9596	Suggested
	Cubic	0.0223		0.9997	-	Aliased
R7, % CDR at 1 hour	Linear	0.0601	0.0001	0.3332	−0.1217	Suggested
	2FI	0.2131	0.0001	0.4609	−0.6388	-
	Quadratic	0.3881	0.0001	0.5052	−1.8270	-
	Cubic	0.0001	-	0.9999	-	Aliased
R8, % CDR at 3 hours	Linear	0.0141	0.0001	0.4958	0.1524	-
	2FI	0.0174	0.0003	0.7918	0.3698	Suggested
	Quadratic	0.6024	0.0002	0.7632	−0.3531	-
	Cubic	0.0002	-	0.9999	-	Aliased
R9, % CDR at 6 hours	Linear	0.0122	<0.0001	0.5094	0.1806	-
	2FI	0.0039	<0.0001	0.8628	0.6085	Suggested
	Quadratic	0.8682	<0.0001	0.8076	−0.0993	-
	Cubic	<0.0001	-	1.0000	-	Aliased
R10, % CDR at 9 hours	Linear	0.0007	<0.0001	0.7152	0.5297	-
	2FI	0.0406	<0.0001	0.8533	0.5912	Suggested
	Quadratic	0.5229	<0.0001	0.8446	0.1119	-
	Cubic	<0.0001	-	1.0000	-	Aliased
R11, % CDR at 12 hours	Linear	0.0005	<0.0001	0.7315	0.5513	=Suggested
	2FI	0.2107	<0.0001	0.7836	0.3548	-
	Quadratic	0.0948	<0.0001	0.8916	0.3805	-
	Cubic	<0.0001	-	1.0000	-	Aliased
R12, % CDR at 24 hours	Linear	<0.0001	0.0001	0.8003	0.6656	Suggested
	2FI	0.0914	0.0002	0.8722	0.6179	-
	Quadratic	0.2152	0.0002	0.9102	0.4870	-
	Cubic	0.0002	-	1.0000	-	Aliased

% CDR = Cumulative % drug release.

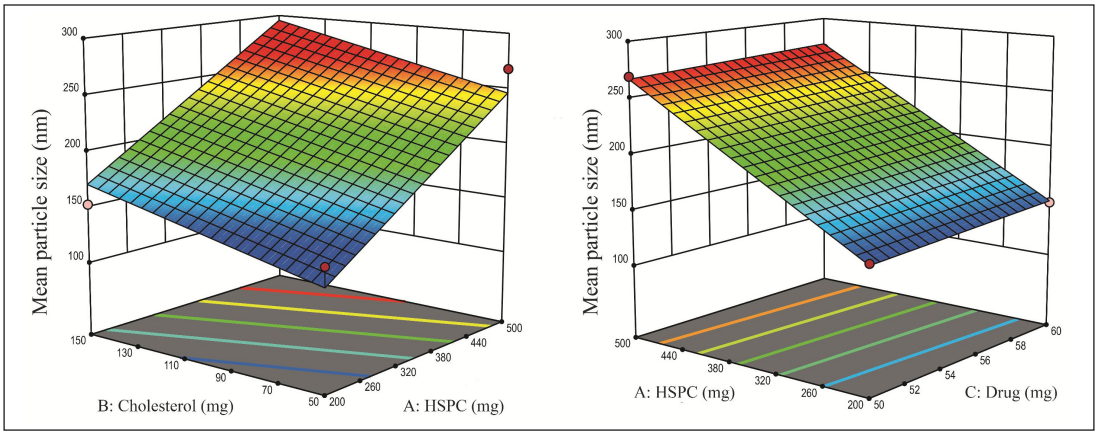

Figure 3. 3D surface plots showing the effect of HSPC, cholesterol and drug on MPS.

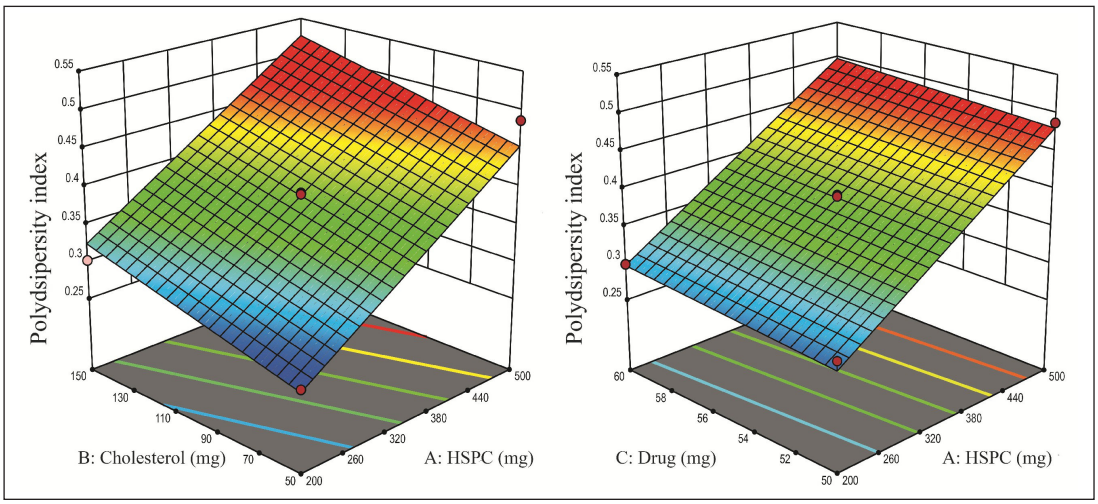

Figure 4. 3D surface plots showing the effect of HSPC, cholesterol and drug on PDI.

to improve the vesicular stability and avoid drug leakage from the vesicles (Vemuri and Rhodes, 1995). Aqueous and organic phase temperature of ethanol injection method was selected to be 50°C because cholesterol shows lowering effect on the phase transition temperature (Lombardo and Kiselev, 2022; Schwendener and Schott, 2010). As the ZP of plain (uncoated) liposomes was anionic (i.e., −1.82 mV), it was planned to impart a positive charge on liposomes by surface coating. The use of chitosan for liposomal coating was an important factor in their surface modification to enhance the drug stability in GI fluid (Nguyen *et al*., 2016) and therefore, 0.2% (*w*/*v*) concentration of chitosan solution was selected for surface modification of progesterone nano-liposomes.

Optimization of progesterone loaded nano-liposomes

Formulation optimization was required to achieve the desired quality attributes in the progesterone-loaded nano-liposomes. As a result of optimization studies, the final formulation was developed with all the desired CQAs such as minimized MPS (nm) and PDI and maximized ZP (mV), EE (%), DL (%), CDR (%). The effect of independent variables on each response variable was analyzed by response surface methodology and is as discussed below.

Formulation factors versus MPS

The optimization software using response surface methodology exhibited a correlation between independent variables (formulation factors) and MPS (R1) by following a linear process order equation.

$$R1 = 208.55 + 62.24A + 24.45B + 3.99C$$

where A is HSPC, B is cholesterol and C is drug content. In this equation, the positive value of factors signifies their direct proportionality to the response variable. The MPS of various optimization batches was found between 138.9 and 275 nm (Table 2). The 3D surface plot (Fig. 3) represented the effect of lipid, cholesterol, and drug on the MPS which goes on increasing with a rise in lipid (HSPC) content and cholesterol amount, whereas, there was no significant effect of the drug observed on MPS.

Formulation factors versus PDI

The optimization software using response surface methodology exhibited a correlation between independent variables (formulation factors) and PDI (R2) by following a linear process order equation.

$$R2 = 0.3891 + 0.1006A + 0.0361B + 0.0088C$$

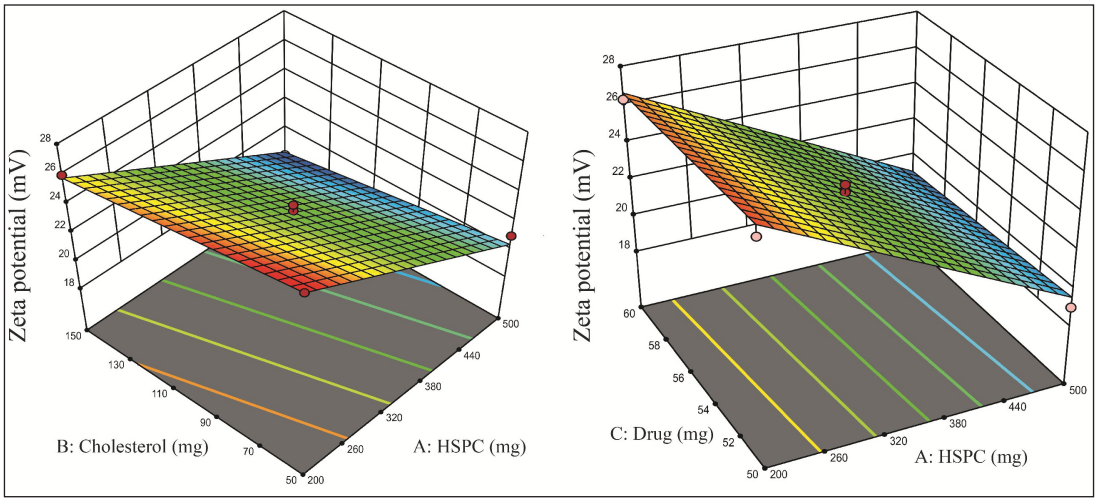

Figure 5. 3D surface plot showing the effect of HSPC, cholesterol and drug on ZP.

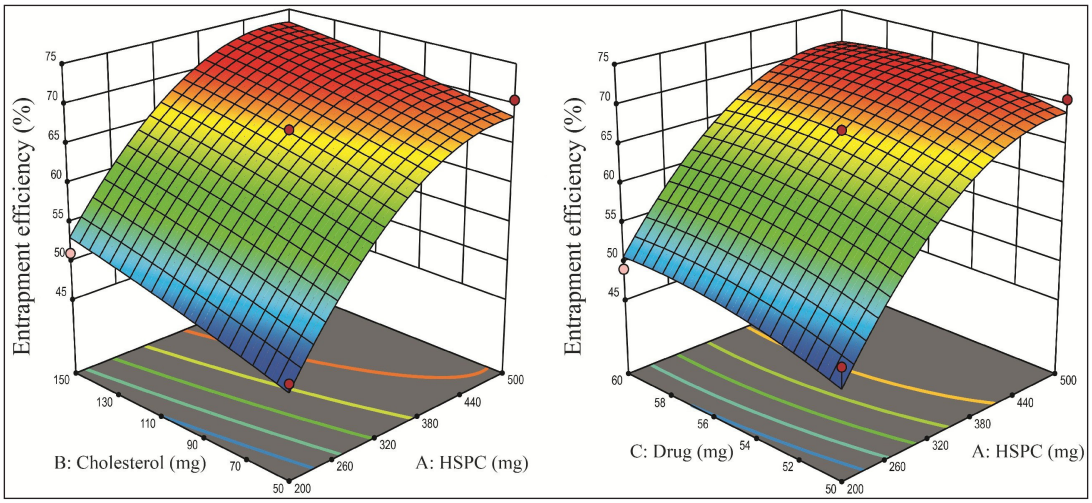

Figure 6. 3D surface plot showing the effect of HSPC, cholesterol and drug on % EE.

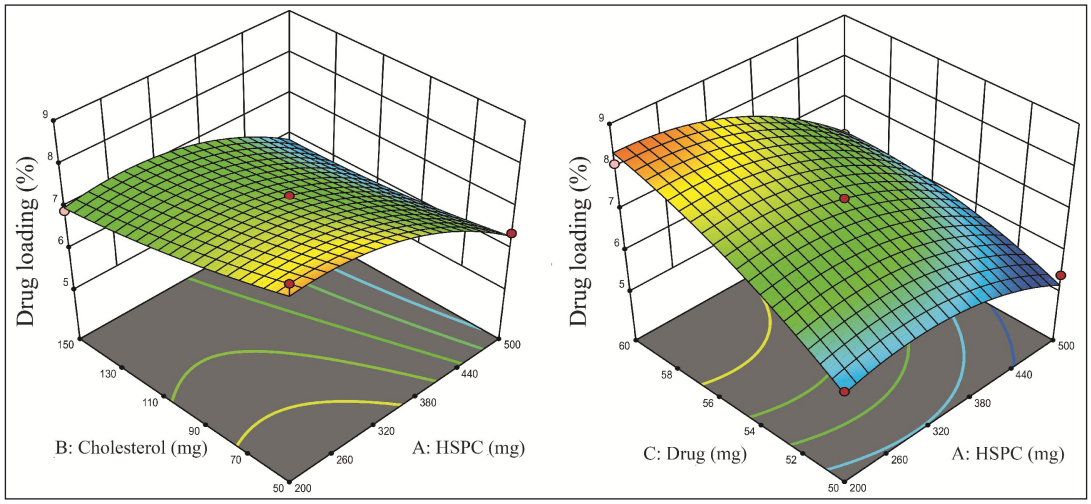

Figure 7. 3D surface plot showing the effect of HSPC, cholesterol and drug on %DL.

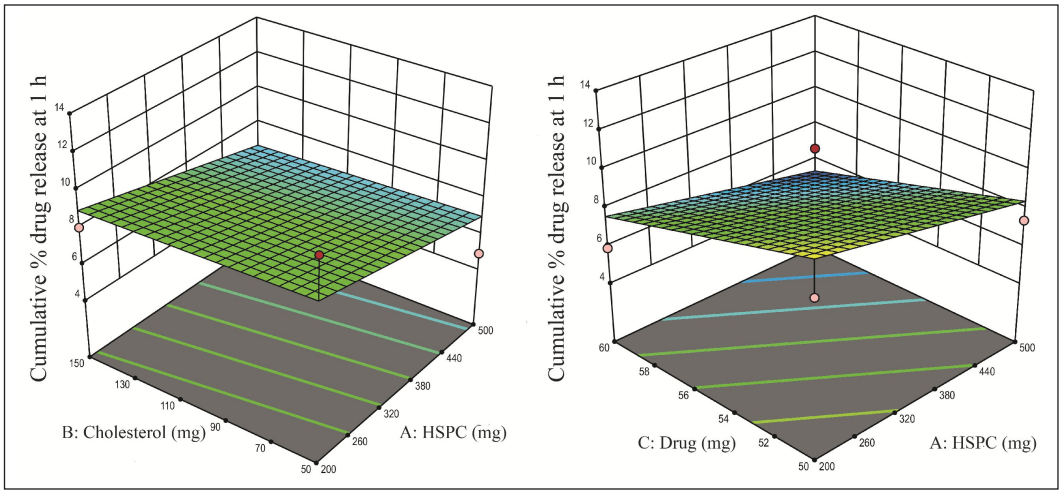

Figure 8. 3D surface plot showing the effect of HSPC, cholesterol and drug on CDR at 1 hour.

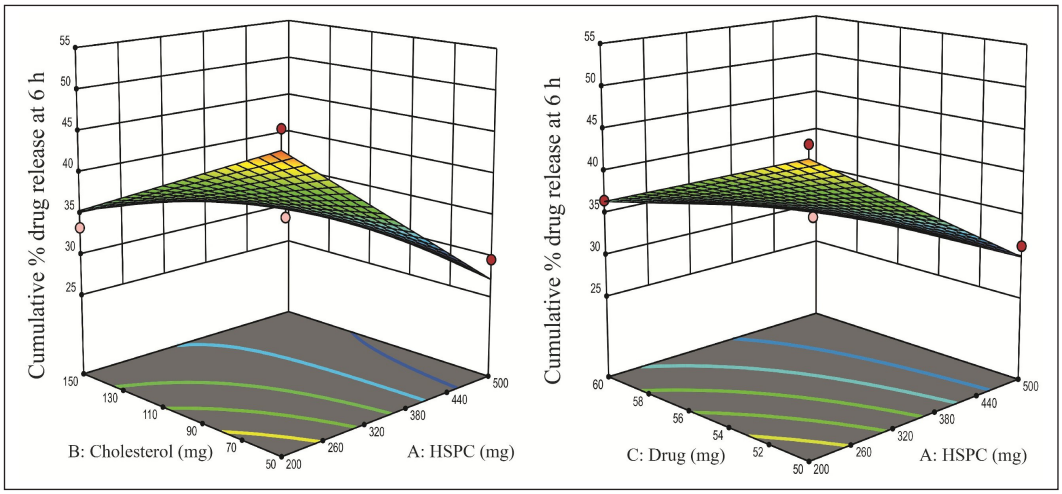

Figure 9. 3D surface plot showing the effect of HSPC, cholesterol and drug on CDR at 6 hours.

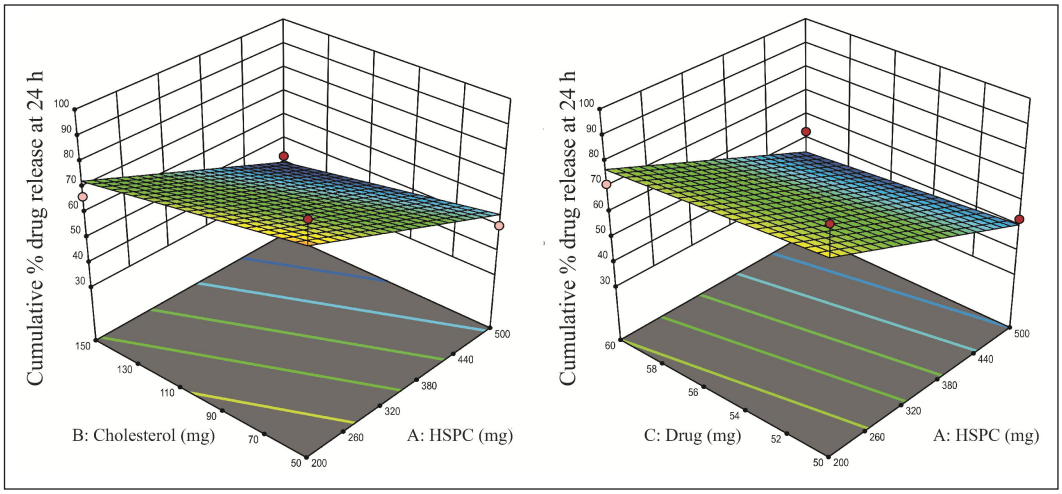

Figure 10. 3D surface plot showing the effect of HSPC, cholesterol and drug on CDR at 24 hours.

The PDI of optimization batches was observed from 0.256 to 0.490 (Table 2). The 3D surface plots (Fig. 4) showed that when lipid (HSPC) content and cholesterol content were increased the PDI was also increased, whereas no remarkable effect of the drug was seen on PDI.

Formulation factors versus ZP

The optimization software using response surface methodology exhibited a correlation between independent variables (formulation factors) and ZP (R3) by following a linear process order equation.

$$R3 = 23.10 - 3.80A - 1.09B - 0.2625C$$

The negative value of independent variables here signifies the inverse proportionality to the ZP. The ZP of optimization batches was found between 18 and 28 mV (Table 2). The 3D surface plots (Fig. 5) exhibited that as the lipid (HSPC) content and cholesterol content increases the ZP decreases because the surface charge of cholesterol is negative, whereas, no significant effect of the drug was seen on the value of the ZP.

Formulation factors versus % EE

The optimization software using response surface methodology exhibited a relationship between independent variables (formulation factors) and EE (R4) by following a quadratic process order equation.

$$R4 = 66.59 + 10.70A + 3.06B + 1.51C - 0.6584AB - 0.7108AC - 3.60BC - 6.08A^2 - 0.3560B^2 - 1.36C^2$$

The % EE of liposomes was observed to be from 46.2% to 72.2% (Table 2). The 3D surface plots (Fig. 6) showed that the % EE increases with rise in lipid (HSPC) and cholesterol content.

Formulation factors versus % DL

The optimization software using response surface methodology exhibited a correlation between independent variables (formulation factors) and % DL (R5) by following a quadratic process order equation.

$$R5 = 7.29 - 0.7471A - 0.04032B + 0.8556C - 0.1755AB - 0.3500AC - 0.4440BC - 0.5823A^2 - 0.1317B^2 - 0.3124C^2$$

The % DL of optimization batches was found in the range of 5.4%–8.8% (Table 2). The 3D surface plots (Fig. 7) confirmed that % DL noticeably increased with rise in drug content, whereas, it did not show a significant effect of lipid (HSPC) and cholesterol content on % DL.

Formulation factors versus CDR

The optimization software using response surface methodology exhibited a correlation between independent variables and % CDR at 0.5 hours (R6), 1 hour (R7), 3 hours (R8), 6 hours (R9), 9 hours (R10), 12 hours (R11), 24 hours (R12) by following equations.

$$R6 = 4.89 - 2.6A - 1.01B - 0.04355C - 0.02126AB - 0.1902AC + 0.2881BC + 0.4103A^2 + 0.1623B^2 + 0.3096C^2$$

$$R7 = 8.01 - 1.16A - 0.1768B - 1.56C$$

$$R8 = 21.17 - 3.94A - 1.08B - 3.07C + 2.21AB + 1.91AC - 3.92BC$$

$$R9 = 35.80 - 5.97A - 1.858B - 2.75C + 4.52AB + 2.58AC - 4.42BC$$

$$R10 = 47.50 - 9.37A - 3.50B - 2.73C + 4.10AB + 4.26AC - 1.95BC$$

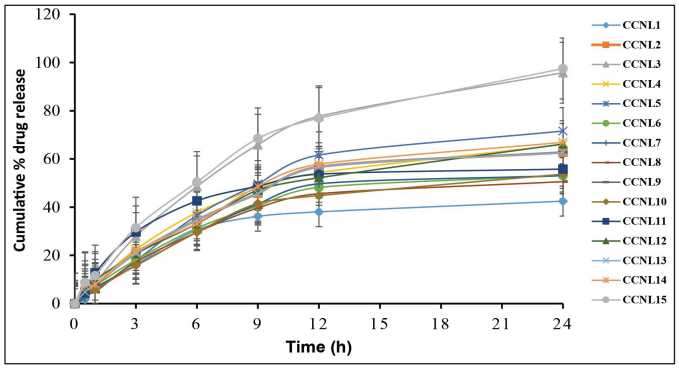

Figure 11. CDR profile of optimization batches of chitosan-coated nano-liposomes of progesterone (Mean ± SD; $n = 3$).

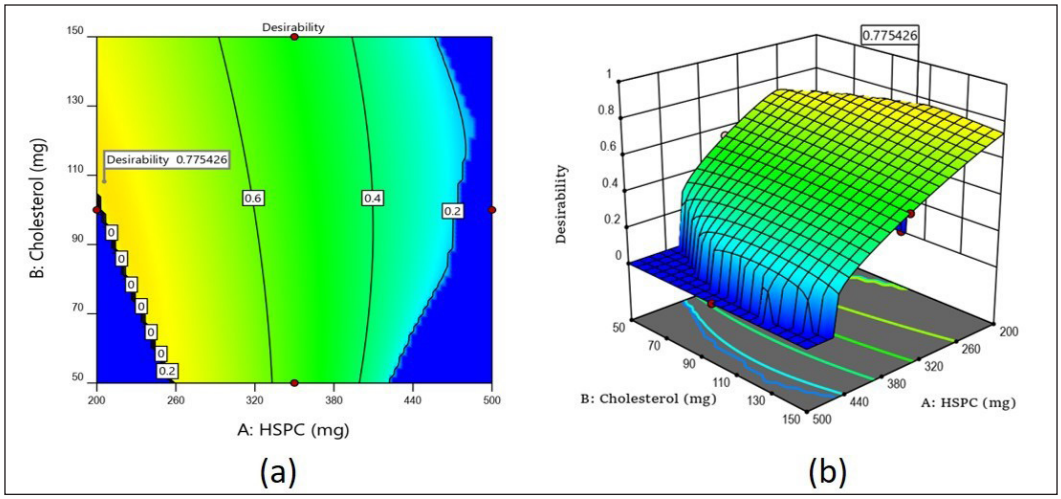

Figure 12. 2D-contour and 3D-surface plots presenting maximum desirability of developed formulation.

$$R11 = 55.37 - 11.55A - 4.33B - 2.68C$$
$$R12 = 64.07 - 16.56A - 7.68B - 3.31C$$

The % CDR of batches was found in the range of 1.7%–7.3% at 0.5 hours, 4.9%–12.8% at 1 hour, 15.7%–31.5% at 3 hours, 29.6%–50.4% at 6 hours, 36.1%–68.5% at 9 hours, 38.0%–77.7% at 12 hours and 42.5%–97.5% at 24 hours (Table 2). The 3D surface plots (Figs. 8–10) showed that % CDR got decreased with rise in lipid (HSPC) and cholesterol content.

Optimized formulation of chitosan-coated nano-liposomes of progesterone

After goal setting of different independent variables as HSPC (in range), cholesterol (in range) and drug (target = 50) whereas the response variables MPS and PDI (minimum) and rest all other responses (maximum), the design expert finally suggested composition of lipid content (HSPC), cholesterol and drug as 205.6, 108.1 and 50 mg, respectively for the predicted optimized batch with the maximum desirability (0.775). The 3D-response and 2D-contour graphs as shown in Figure 12 displayed the highest desirability value of the optimized batch. Optimized formulation was prepared as per suggested composition by software and evaluated for all response variables to validate the predicted values as recorded in Table 4.

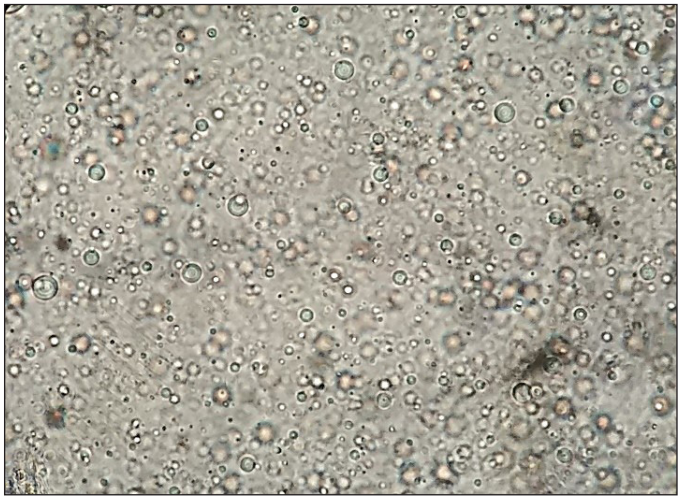

Figure 13. Microscopic view of optimized liposomal formulation.

Evaluation of optimized formulation

Microscopic evaluation

The microscopic study of the optimized chitosan-coated nano-liposomal formulation shown in Figure 13 confirmed the uniform, homogenous, spherical-shaped liposomal structures. The liposomes illustrated a high volume of aqueous core encapsulated in liposomal bilayers with entrapped drug.

Particle size, PDI and ZP analysis

The particle size and PDI directly affect the drug diffusion through the biological membranes. It was reported that liposomal particles smaller than 200 nm readily crosses the GI mucosal barrier; whereas drug transportation through the mucin was limited for particles larger than 500 nm (Bajka *et al*., 2015; Luo *et al*., 2021). After statistical analysis, the software predicted MPS was 148.6 nm whereas, the practically observed value of the optimized batch was observed to be 168.3 nm, and was relatively very close to the expected value (Fig. 14).

Physicochemical properties including size distribution affect the accumulation of nano-vesicles in the target tissue hence it requires homogenous dispersion. Generally, the PDI value ranges from 0.0 (indicates perfect sample for acceptance) to 1.0 (indicates multiple-size distribution). For lipid-based nano-vesicles, PDI of 0.3 or <0.3 is considered monodispersed (Danaei *et al*., 2018). The PDI of the optimized batch was found in the range of 0.256–0.490 and then the goal was set to a minimum value. After the analysis by software, the predicted PDI was 0.289 whereas the practically observed PDI of the optimized batch was found to be 0.307 which was relatively close to the value expected.

The ZP of liposomes depends on the surface charge of the dispersant and affects the stability of the formulation. Generally,

Table 4. Software predicted and practically observed response variable of optimized formulation.

Optimized formulation composition		Measured responses		
Component (CCNL-F)	**Quantity**	**Dependent variable**	**Software predicted value**	**Experimentally observed value**
F1: HSPC	205.6 mg	MPS (nm)	148.6	168.3
F2: Cholesterol	108.1 mg	PDI	0.289	0.307
F3: Drug content	50 mg	ZP (mV)	26.8	24.0
		EE (%)	48.2	53.01
		DL (%)	5.95	7.28
		% CDR at 0.5 hours	8.21	7.76
		% CDR at 1 hour	10.65	10.9
		% CDR at 3 hours	29.98	28.5
		% CDR at 6 hours	46.49	44.7
		% CDR at 9 hours	62.45	58.4
		% CDR at 12 hours	68.40	67.5
		% CDR at 24 hours	82.07	76.4

% CDR = Cumulative % drug release.

it is considered that a value in the range of −30 to +30 mV is the most acceptable for nano-dispersions. As the ZP of optimization batches was found between 18 and 28 mV and so the goal was set to maximum. The software predicted ZP was 26.84 mV and the practically observed value was found to be 24 mV, which was quite close to the value expected.

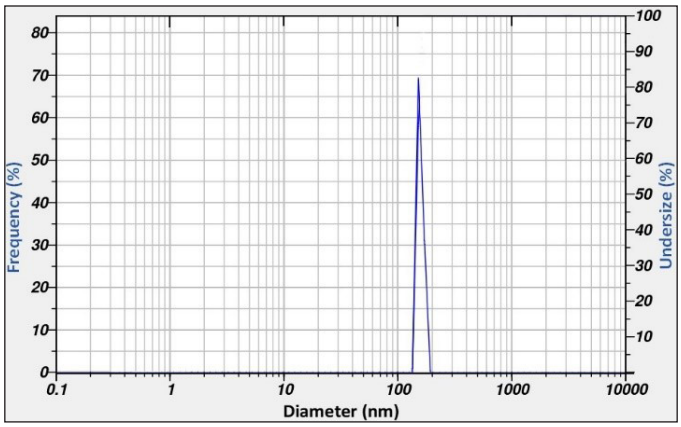

Figure 14. Particle size distribution graph of optimized formulation.

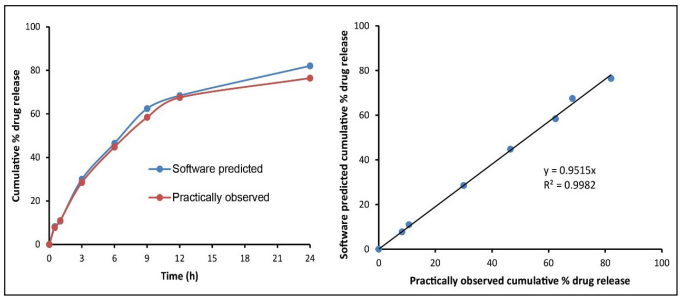

Figure 15. Correlation between software predicted and practically observed data of *In-vitro* drug release (a) Overlay curve (b) regression curve.

EE and DL

As %EE of optimization batches was found in the range of 46.2%–72.2% and the goal was set to a maximum value, so the software on statistical analysis predicted the % EE to be 48.29%, whereas, the practically observed % EE of the optimized batch was found to be 53.0%. The drug:HP-β-CD complexation was proved to be an excellent method for increasing the EE of progesterone for its effective use.

The % DL of optimization batches was observed to be from 5.4% to 8.8% and the goal was set to maximum. After statistical analysis software predicted the % DL of optimized batch to be 5.95%, whereas, the practically observed value was found to be 7.0%, which was better than the value expected. Higher DL would facilitate in achieving desired therapeutic effect on the administration of lower (small) dose volume of the formulation (Sur *et al.*, 2014).

In-vitro drug release study

The CDR of optimization batches was found in the range of 1.7%–7.3% at 0.5 hours, 4.9%–12.8% at 1 hour, 15.7%–31.5% at 3 hours, 29.6%–50.4% at 6 hours, 36.1%–68.5% at 9 hours, 38.0%–77.7% at 12 hours and 42.5%–97.5% at 24 hours and then the goal was set to maximum. After the statistical analysis, the software predicted % CDR were 8.2% at 0.5 hours, 10.65% at 1 hour, 29.98% at 3 hours, 46.49% at 6 hours, 62.45% at 9 hours, 68.45% at 12 hours and 82.06% at 24 hours. Whereas, practically observed % CDR of the optimized batch was found to be 7.76%,

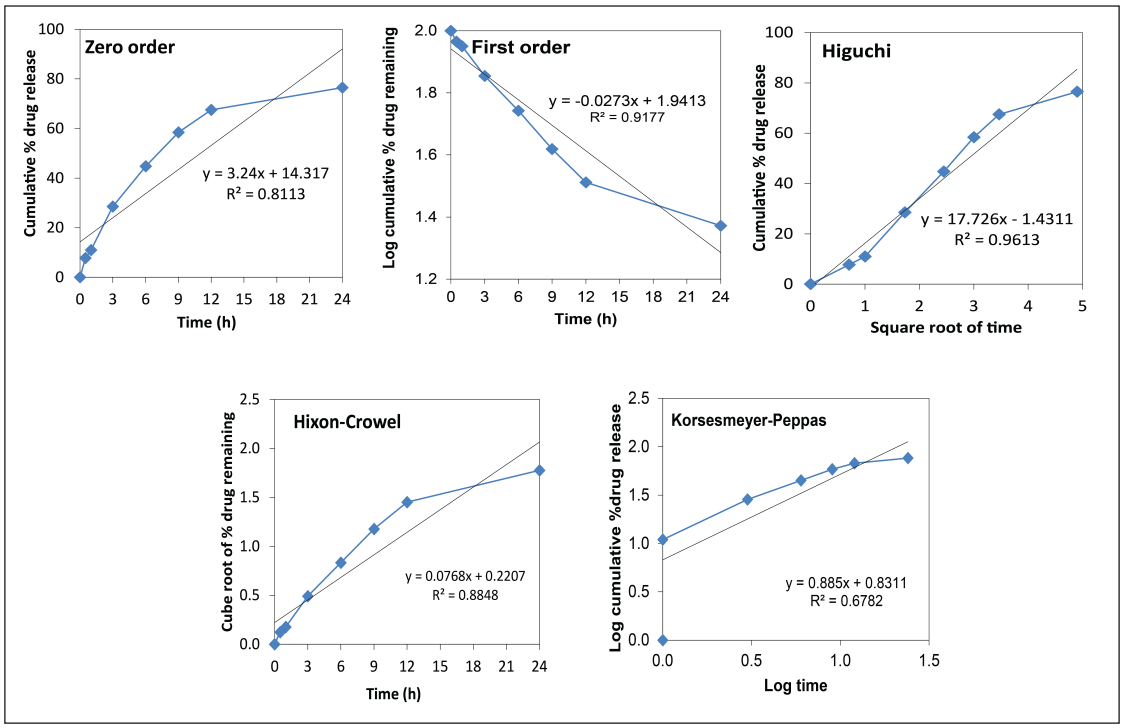

Figure 16. Drug release kinetics model plots of optimized formulation of chitosan- coated nano-liposomes of progesterone.

Table 5. Drug release kinetic profile and model fitting summary of progesterone loaded nano-liposomes.

Drug release kinetic model	Equation	R^2	K
Zero-order	$Q_0 - Q_t = k_0 t$	0.811	3.24
First order	$\log Q = \log Q_0 - kt/2.303$	0.917	−0.02
Higuchi	$Q_0 - Q_t = kt^{1/2}$	0.961	17.26
Hixon-Crowel	$Q_0^{1/3} - Q_t^{1/3} = kt$	0.884	0.076
Korsmeyer-Peppas	$\log (Q_0 - Q_t) = \log k + n \log t$	0.678	0.886

Where Q_0 = initial drug amount, Q_t = remaining drug amount, k_0 = rate constant, t = time.

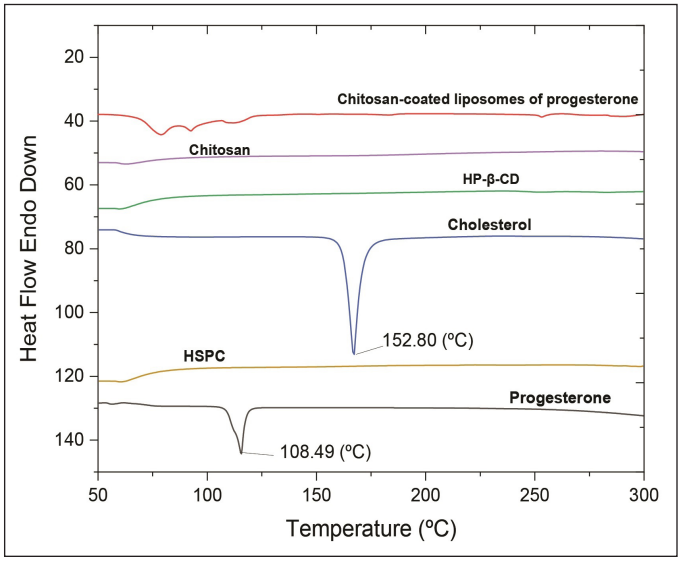

Figure 17. DSC thermograms of progesterone loaded nano-liposomes, progesterone, HSPC, cholesterol, HP-β-CD, and chitosan samples.

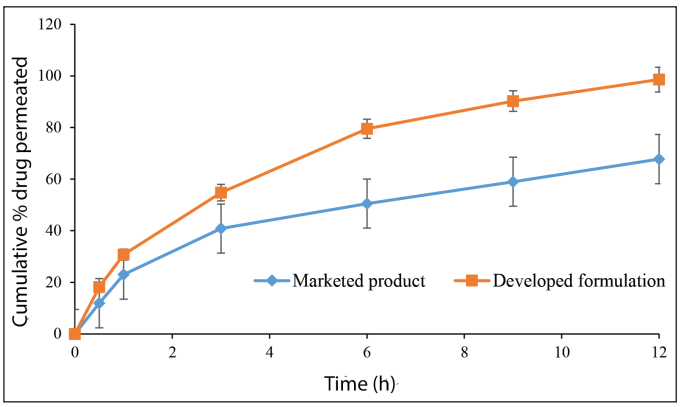

Figure 18. Cumulative % drug permeation profile of progesterone in developed formulation versus marketed product (Mean ± SD; n = 3).

10.9%, 28.5%, 44.7%, 58.4%, 67.5%, and 76.4%, which was relatively close to the value expected as shown in Figure 15.

Drug release kinetics study

The significant information related to desired drug release profile is provided by release kinetics (Weng and Tong, 2020). Different kinetic models, i.e., zero order, first order, Hixon-Crowel, Higuchi, and Korsmeyer-Peppas model were studied and graphs were plotted as shown in Figure 16. The regression coefficient values from the plotted model as shown in Table 5 were found to be 0.811, 0.917, 0.961, 0.884, and 0.678, respectively. It was concluded that Higuchi kinetic model was being followed on *In-vitro* drug release study as it exhibited the highest regression coefficient (R^2) as shown in Table 5.

Table 6. *Ex-vivo* drug permeation data of progesterone loaded nano liposomes versus marketed product.

Formulation sample	P_{app} (cm.minute^{-1})	Jss (mg.cm^{-2}.minute^{-1})
Marketed product	1.8×10^{-2}	4.6×10^{-2}
Developed formulation	3.6×10^{-2}	9×10^{-2}

Table 7. *In-vitro* GI stability data of developed liposomal formulation versus plain drug solution.

GI-Condition	Plain drug solution (2.5 mg/ml)		Developed liposomal formulation	
	K	$t_{1/2}$ (hours)	*K*	$t_{1/2}$ (hours)
SGF	1.3×10^{-2}	8.85	6.1×10^{-3}	18.800
SIF	2.6×10^{-2}	4.32	3.4×10^{-4}	334.34

Differential scanning calorimetry (DSC)

The DSC analysis was performed to study the nature of drug and excipients, and also study the drug-excipient interaction (Chadha and Bhandari, 2014). The DSC thermograms of progesterone, HSPC, cholesterol, HP-β-CD, chitosan, and lyophilized chitosan-coated progesterone-loaded nano-liposomes were plotted (Fig. 17). An intense endothermic peak of progesterone at 108.49°C and cholesterol at 152.80°C confirmed their crystalline nature (Jin *et al.*, 2013; Sharma *et al.*, 2017). Whereas the absence of sharp endothermic peaks in the case of other excipients indicates their amorphous nature (Zafar *et al.*, 2021). The effective complexation of the drug with HP-β-CD and complete entrapment of progesterone in nano-liposomes was confirmed by the absence of a sharp endothermic peak in the case of liposomal formulation.

Ex-vivo drug permeation study

The drug permeation across non-everted chicken intestinal segment was studied for prediction of the *in-vivo* drug absorption. The cumulative % drug permeation data of optimized nano-liposomal formulation was compared to prepared suspension of marketed tablet and are depicted in Figure 18. The apparent permeability coefficient of progesterone in optimized nano-liposomes and suspension of marketed tablet was found to be 3.6×10^{-2} and 1.8×10^{-2} cm.minute^{-1}, respectively. Their respective steady-state drug permeation flux was found to be 4.6×10^{-2} and 9×10^{-2} mg.cm^{-2}.minute^{-1} as shown in Table 6. Results of *ex-vivo* permeation study are indicating the higher drug permeation from the developed nano-liposomes as compared to conventional formulation. Therefore, it was confirmed that the chitosan coating of liposomes showed a positive effect on the drug permeation by intimate contact and interaction with negatively charged GI mucosa and epithelium.

In-vitro GI stability study

The drug degradation rate constant and half-life of the developed formulation were calculated for both GI conditions, i.e., SGF and SIF. The *In-vitro* stability assessment of chitosan-coated nano-liposomes was performed to estimate the *in-vivo* GI stability of progesterone. The degradation rate constant (K) and half-life ($t_{1/2}$) of developed chitosan-coated liposomes and plain drug solution were calculated and recorded in Table 7. The observed results evidently confirmed that the chitosan-coated nano-liposomes exhibited significantly low drug degradation rate as compared to plain drug solution resulting in 2.12 and 77.3 fold extended half-life in SGF and SIF, respectively.

CONCLUSION

Formulation development and optimization of chitosan-coated nano-liposomes of progesterone were successfully accomplished by response surface methodology using BBD. The aqueous solubility of progesterone was enhanced by HP-β-CD complexation which also lead to high EE in liposomes. The developed liposomes possessed all the desired physico-chemical properties (CQAs) in the acceptable range. The drug release data confirmed that the developed formulation exhibits sustained drug release profile following Higuchi's kinetic model which would be helpful in prolonged therapeutic action. The *ex-vivo* drug permeation study exhibited approximately twofold higher drug permeation in developed formulation in comparison to prepared suspension of marketed tablet. The stability data in GI fluid, i.e., SGF and SIF also confirmed that the chitosan-coated nano-liposomes of progesterone had better GI stability showing 2.12 and 77.3 fold longer half-life in SGF and SIF, respectively. It can be concluded that the chitosan-coated nano-liposomes of progesterone can be a better alternative for its effective and safe oral delivery even in the presence of GI fluids. The *in-vitro* and *ex-vivo* evaluation studies confirmed that the present formulation approach significantly enhanced the drug permeation/absorption which would lead to improved drug bioavailability and better progesterone hormonal therapy via oral route eliminating the limitation of conventional dosage forms and improving the patient compliance.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not associate with experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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