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ORIGINAL ARTICLE

Factorial design studies of antiretroviral drug-loaded stealth liposomal injectable: PEGylation, lyophilization and pharmacokinetic studies

Beeravelli Sudhakar · Mylangam Chaitanya Krishna · Kolapalli Venkata Ramana Murthy

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Abstract The aim of the present study was to formulate and evaluate the ritonavir-loaded stealth liposomes by using 3^2 factorial design and intended to delivered by parenteral delivery. Liposomes were prepared by ethanol injection method using 3^2 factorial designs and characterized for various physicochemical parameters such as drug content, size, zeta potential, entrapment efficiency and in vitro drug release. The optimization process was carried out using desirability and overlay plots. The selected formulation was subjected to PEGylation using 10 % PEG-10000 solution. Stealth liposomes were characterized for the above-mentioned parameters along with surface morphology, Fourier transform infrared spectrophotometer, differential scanning calorimeter, stability and in vivo pharmacokinetic studies in rats. Stealth liposomes showed better result compared to conventional liposomes due to effect of PEG-10000. The in vivo studies revealed that stealth liposomes showed better residence time compared to conventional liposomes and pure drug solution. The conventional liposomes and pure drug showed dose-dependent pharmacokinetics, whereas stealth liposomes showed long circulation half-life compared to conventional liposomes and pure ritonavir solution. The results of statistical analysis showed significance difference as the p value is (<0.05) by one-way ANOVA. The result of the present study revealed that stealth liposomes are promising tool in antiretroviral therapy.

Keywords Stealth liposomes · Opsonization · Stearic stability · PEG-10000 · DSPE · Statistical optimization and mean residence time

Abbreviations

AIDS	Immunodeficiency syndrome
HIV	Human immunodeficiency virus
DSPE	1,2-Distearoyl-snglycero-3-phosphoethanolamine
SUV	Small unilamellar vesicles
PDI	Polydispersity index
%EE	Percent entrapment efficiency
MLRA	Multiple linear regression analysis
CV	Coefficient of variation
R^2	Coefficient of determination
Adjusted R^2	Adjusted coefficient of determination
FTIR	Fourier transform infrared spectrophotometer
DSC	Differential scanning calorimeter
PVDF	Filters polyvinylidene difluoride filters
df	Degrees of freedom
SS	Sum of squares
MS	Mean sum of squares
F	Fischer's ratio
S	Significant
NS	Nonsignificant

Introduction

In the field of pharmacy, cascades of improvements are taking place to overcast the obsolete ones to render the greatest comfort to patient. Drug delivery systems are

B. Sudhakar (✉) · M. C. Krishna · K. V. R. Murthy
Department of pharmaceutical Technology,
A.U.College of pharmaceutical sciences, Andhra University,
Visakhapatnam 530003, Andhra Pradesh, India
e-mail: sudhakarelixir@gmail.com

being renovated by the ceaseless efforts made by the formulators in achieving the drug action with minimal side effects, low dosing and reduction in frequency of dosing and retention of drug in the blood compartment releasing in a sustained manner. Vesicular drug delivery systems are developing at a rapid pace to circumvent the inconveniences associated with conventional drug delivery approaches. Vesicular systems are playing great role for effective management of HIV and prevent its progression toward AIDS. Vesicles are water-filled colloidal particles. In general, vesicles made of natural or synthetic phospholipids along with/without cholesterol are called liposomes. The liposomal vesicular dispersions are structurally similar to the biological membranes with alternating layers of lipid and aqueous portions (Li et al. 1987; Poznansky and Juliano 1983; Teewodros et al. 2010).

Human immunodeficiency virus (HIV) infection, which leads to acquired immunodeficiency syndrome (AIDS), remains a serious worldwide health problem. In 2012, there were 35.3 million (32.2–38.8 million) people living with HIV. Based on the profound knowledge gained about the HIV replication cycle, several drug targets have been identified over the years and effective treatment options are currently available. The discovery of HIV protease inhibitors introduced new and effective first-line therapies for HIV/AIDS (Puneet and Sanjay 2010).

Various HIV protease inhibitors are in clinical use. Among them, Ritonavir is one of the antiretroviral protease inhibitor and the half-life ($t_{1/2}$) is 3–4 h. The oral bioavailability of ritonavir is variable due to its poor aqueous solubility. Its oral absorption is dissolution rate limited and it requires enhancement in solubility and dissolution rate for increasing its bioavailability. Therefore, novel drug delivery systems that may safely enhance the bioavailability of protease inhibitors are needed. Hence ritonavir was selected as a drug to load into the stealth liposomes using polyethylene glycol 10000 and delivered by parenteral route. In the present study, vesicles were prepared by ethanol injection method using 3^2 factorial designs and their surface was modified by polymer coating method using polyethylene glycol 10000 (Josbert 2007; Lledo et al. 2007).

Materials and methods

1,2-distearoyl-snglycero-3-phosphoethanolamine (DSPE) and cholesterol were procured from Lipoid Pvt Ltd from Germany. Ritonavir and stearic acid gift samples were purchased from Hetero drugs Pvt Ltd, Hyderabad, India; polyethylene glycol 10000 was purchased from sigma Aldrich, India.

Experimental design

A 3^2 randomized full factorial design was used in this study and two factors were evaluated, each at three levels; experimental trials were performed at all nine possible combinations. Amount of lipid (DSPE) was taken as the first independent variable (X_1) and amount of cholesterol (X_2) was selected as the second independent variables for liposomes. These variables varied at three levels, low level (−1), medium level (0), and high level (+1). All the calculations were done at milligram level. Amount of stearic acid (20 mg), amount of ritonavir (20 mg) and final formulation volume 10 mL were kept constant. Vesicle size (nm) (Y_1), % entrapment efficiency (Y_2) and 100 % in vitro drug release at 24 h (Y_3) were selected as dependent variables. Values of variables and batch codes are shown in Tables 1 and 2 Design Expert® DX 8.0.7.1 trial version software was used for the generation and evaluation of statistical experimental design (Karimunnisa and Atmaram 2010; Karimunnisa et al. 2010; Krishnam Raju et al. 2014).

Preparation of vesicular dispersions

Ethanol injection method was used to prepare liposomes (Karimunnisa and Atmaram 2010; Karimunnisa et al. 2010; Krishnam Raju et al. 2014). An alternative method for producing single unilamellar vesicles (SUV) that avoids both sonication and exposure to high pressure is the ethanol injection technique described by Batzri and Korn. Accurately weighed amounts of DSPE, cholesterol, stearic acid and drug were taken in a beaker and dissolved in 2 mL ethanol with slight heating on a hot plate with temperature not exceeding 50° C. The monophasic ethanolic drug lipid mixture was injected at the rate of 0.25 mL/min using the 14-gauge needle into 10 mL pH 7.4 phosphate buffer (the aqueous phase) at 60° C under stirring at 500 rpm (Remi magnetic stirrer) using a Teflon-coated bead. The aqueous phase immediately turned milky indicating the vesicles' formation. The system was kept under stirring up to 1–2 h to facilitate the removal of ethanol. The vesicular

Table 1 3^2 full factorial design: factors, factor levels and responses

Factors (independent variables)	Factor levels used		
	Low (−1)	Medium (0)	High (+1)
Amount of lipid/surfactant	30	60	90
Amount of cholesterol	15	30	45
Responses (dependent variables)			
Y_1 = particle size (nm)			
Y_2 = % entrapment efficiency (%)			
Y_3 = 100 % in vitro drug release at 24 h			

Table 2 Compositions of different batches of ritonavir-loaded liposomes

Batch code	Amount of lipid (X_1)	Amount of cholesterol (X_2)
L1 (−1, −1)	30	15
L2 (−1, 0)	30	30
L3 (−1, +1)	30	45
L4 (0, −1)	60	15
L5 (0, 0)	60	30
L6 (0, +1)	60	45
L7 (+1, −1)	90	15
L8 (+1, 0)	90	30
L9 (+1, +1)	90	45

dispersions were made to 10 mL with the pH 7.4 phosphate buffer and transferred to vials. The dispersion was refrigerated for 2 h for effective vesicle sealing. They were assigned batch numbers from L1 to L9 and stored at 2–8 °C. The volume of liposomal dispersions prepared in each trial was 10 mL to contain 20 mg of ritonavir.

Characterization of vesicular systems

The prepared vesicular systems were characterized for percent drug content, pH, particle size, polydispersity index and zeta potential, percent entrapment efficiency and in vitro drug release studies.

Determination of percent drug content

One milliliter of suspension was pipetted from the ritonavir-loaded liposomal and lysed with methanol. It was further diluted with pH 7.4 phosphate buffer and the samples were analysed spectrophotometrically at 210 nm for ritonavir.

Determination of pH of vesicular dispersions

The pH of the vesicular dispersions was measured by pH meter (Elico pH meter LI 127).

Determination of particle size, polydispersity index and zeta potential (ζ)

The vesicles after dilution (1:100) with distilled water were taken in the cuvette. The cuvette was placed inside the sample holder of the instrument (Malvern Nano ZS90, Malvern, UK) for measurement of size (Prabhakar and Kishan 2011). The principle of photon correlation spectroscopy was used for determining the hydrodynamic diameter of the vesicle via Brownian motion. The observations for vesicle size were recorded at 90° light scattering angle and at 25° C. The ζ was measured based on the electrophoretic mobility of vesicle which used the Helmholtz–Smoluchowski equation.

Determination of percent entrapment efficiency (%EE)

The %EE of the vesicles was determined using ultracentrifugation technique (Karimunnisa and Atmaram 2010; Karimunnisa et al. 2010). 4 mL volume of vesicular dispersion was centrifuged at 15,000 rpm for 3 h at a controlled temperature of 4 °C (Remi cooling centrifuge). Supernatant containing unentrapped drug was withdrawn and measured UV spectrophotometrically at 210 nm against pH 7.4 phosphate buffer. All the determinations were made in triplicate. The amount of drug entrapped in liposomes was determined by Eq. 1.

$$\%\mathrm{EE} = [(C_\mathrm{d} - C)/C_\mathrm{d}] \times 100 \quad (1)$$

where C_d is the concentration of total drug and C is the concentration of entrapped drug.

In vitro drug release studies by dialysis

The In vitro release studies were performed by dialysis process (Prabhakar and Kishan 2011; Narashimhan et al. 2012). Cellulose dialyzing membrane (dialysis membrane 60 from HiMedia, Mumbai, India whose molecular cut-off is 6000 D) was soaked in pH 7.4 phosphate buffer overnight. 2 mL of vesicular dispersion equivalent to 4 mg of ritonavir was added by tying one end of the dialysis membrane and hanging into a beaker containing 500 mL of pH 7.4 phosphate buffer maintained at 37 ± 0.5 °C on a temperature-controlled magnetic stirrer and stirred at 400 rpm with Teflon-coated bead. 5 mL aliquots were withdrawn at predetermined time intervals from the beaker and were replaced with an equal volume of fresh buffer maintained at the same temperature. The samples were analysed spectrophotometrically at 210 nm for ritonavir.

Statistical analysis of the data and optimization

Response surface modelling and evaluation of the quality of fit of the model for the current study were performed employing Design Expert® DX 8.0.7.1 trial version software (Dhiman et al. 2008; Huang et al. 2005; Patil and Sawant 2008). Polynomial models including linear, interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA). A second-order polynomial equation that describes the effect of independent factors on the response is expressed in the following forms:

$$\text{Linear model: } Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 \quad (2)$$

$$\text{2FI (interaction) model: } Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad (3)$$

$$\text{Quadratic model} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (4)$$

where Y is the dependent variable; β_0 is the arithmetic mean response of the nine runs and $\beta_i(\beta_1, \beta_2, \beta_{12}, \beta_{11}$ and $\beta_{22})$ is the estimated coefficient for the corresponding factor X_i (X_1, X_2, X_1X_2, X_1X_1 and X_2X_2). The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X_1X_2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are included to investigate nonlinearity. The equations enable the study of the effects of each factor and their interaction over the considered responses. The polynomial equations were used to draw conclusions after considering the magnitude of coefficients and the mathematical sign they carry, i.e. positive or negative. A positive sign signifies a synergistic effect, whereas a negative sign stands for an antagonistic effect. The best fitting mathematical model was selected based on the comparisons of several statistical parameters, including the coefficient of variation (CV), the coefficient of determination (R^2), adjusted coefficient of determination (Adjusted R^2) and the predicted residual sum of square (PRESS), provided by Design Expert software. Among them, PRESS indicates how well the model fits the data and for the chosen model it should be small relative to the other models under consideration. Level of significance was considered at $p < 0.05$. Mathematical relationships in the form of polynomial equations are generated using multiple linear regression analysis (MLRA) and used to find out the relative influence of each factor on the response. Analysis of variance (ANOVA) for the responses was performed to identify significant effect of factors on responses and the model parameters were obtained. The relationship between the dependent and independent variables was further elucidated using contour and response surface plots. Two-dimensional contour plots and three-dimensional response surface plots resulting from equations were obtained by the Design Expert software. These plots are very useful in study of the effects of two factors on the response at one time and predict the responses of dependent variables at the intermediate levels of independent variables. Subsequently, a numerical optimization technique by the desirability and graphical optimization technique by the overlay plot approach were used to generate the new formulation with the desired responses. An optimized formulation was developed by setting constraints (goals) on the dependent and independent variables. To validate the chosen experimental design, the resultant experimental values of the responses were quantitatively compared with those of the predicted values and calculated the percent relative error by the following Eq. 5.

$$\%\text{Relative error} = \frac{(\text{Predicted value} - \text{Experiment value})}{\text{Predicted value}} \times 100 \quad (5)$$

Preparation of stealth vesicular dispersions

One milliliter 10 % w/v of polyethylene glycol 10000 polymeric aqueous solution was used for the preparation of stealth liposomes. Preparation was done by injecting 1 mL of 10 % w/v of polyethylene glycol 10000 to the vesicular dispersions that was being stirred at 500 rpm slowly to ensure uniform coating of PEG around the vesicles (Minghuang et al. 2009; Yang and Guangji 2008; Yang et al. 2007).

Characterization of stealth vesicular dispersions

The prepared stealth vesicles were characterized for percent drug content, pH, particle size, %EE, zeta potential and in vitro drug release by dialysis as discussed above. The in vitro drug release profile of stealth vesicular dispersions was fitted to release kinetic models.

Transmission electron microscopic studies of stealth vesicles

A drop of stealth vesicular dispersions was applied on a carbon film-covered copper grid. Excess dispersion was blotted from the grid with filter paper to form a thin film specimen. The sample was then stained with 2 % uranyl acetate, air dried and examined under transmission electron microscope (Hitachi, H-7500) at a magnification of 60,000× (John and Lonnie 1998).

Fourier transform infrared spectrophotometer (FTIR) studies

Appropriate amounts of ritonavir, lipid (DSPE), cholesterol, stearic acid, and stealth liposomes were studied for interaction studies with the functional groups of the drug and other used excipients. KBr pressed pellet technique was used in the preparation of pellet (Karimunnisa and Atmaram 2010; Karimunnisa et al. 2010; Shilpi et al. 2010). The resultant pellet was kept in the IR chamber and

IR spectra of the mixtures were recorded on a Bruker FTIR spectrophotometer equipped with Opus software.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a frequently used thermoanalytical technique that generates data on melting endotherms and glass transitions. The DSC curve of the pure drug and stealth liposomes was obtained using a differential scanning calorimeter (Mettler Toledo STAR eSW 8.10, Model DSC 822e) at a heating rate of 10° C/min from 25 to 150° C. An empty aluminium pan was served as reference. Nitrogen was used as a purge gas, at the flow rate of 20 mL/min for all the studies (Chen and Yu 2002).

Stability studies

Stability studies were conducted for conventional liposomes (L5) and stealth liposomes for a period of 6 months. Stability studies were carried out on selected formulations packed in screw-capped high-density polyethylene (HDPE) amber colour bottles which were charged for long-term stability studies according to ICH guidelines (2–8 ± 2 °C/60 ± 5 %RH) and (25 ± 2 °C/60 ± 5 %RH) for a period of 6 months in stability chambers. The samples were withdrawn at time intervals of 0, 3 and 6 months and evaluated for drug content (%), particle size, zeta potential and %EE (Karimunnisa and Atmaram 2010; Karimunnisa et al. 2010).

Lyophilization cycle

Conventional liposomes (L5) and stealth liposomes were selected for lyophilization using Christ Alpha 1–2 LD Freeze Dryer. 5 % w/v mannitol solution was used as cryoprotecting agent. The selected vesicular systems were initially filtered through 0.22 µm filter under aseptic conditions in sterile area into a glass vial and 10 mL of each vesicular system in pH 7.4 phosphate buffer was added with 10 mL 5 % w/v mannitol solution in water and thoroughly mixed. The process involves two steps. The sample was cooled to −60 °C by adjusting the condenser temperature with a pressure of 200 Torr. This primary drying process was continued for 8 h during which unbound water was removed completely by sublimation. The secondary drying process for removing the bound water was continued for 4 h at a temperature of 40° C. The entire process of lyophilization was carried out under aseptic conditions. The product was sealed and used for in vivo studies (Sanyog et al. 2013; Ghanbarzadeh et al. 2013).

In vivo pharmacokinetic study

The study was conducted in male Wistar rats weighing in the range of 200–250 g following parenteral administration. The selected conventional and stealth vesicular systems were tested against the pure ritonavir solution. Before administration, the selected vesicular formulations were subjected to two-step sterilization process of filtration under aseptic conditions using sterile grade 0.22 µm PVDF filters, followed by lyophilization. The conditions used in the lyophilization process were established by initial trials. The lyophilized formulations were reconstituted with previously sterilized pH 7.4 phosphate buffer saline before administration to rats by tail vein. The pure ritonavir drug solution was subjected for sterilization using sterile grade 0.22 µm PVDF filters under aseptic conditions. The animals were divided into three groups, each group containing four animals. All groups were intravenously administered via tail vein of a rat and study was carried out for a period of 24 h. Group 1 was given pure ritonavir solution; Group 2 and Group 3 were administered with conventional liposomes and stealth liposomes. The animal dose was calculated as 2 mg/250 g for rat for current study. Serial blood samples 0.25 mL aliquots of blood samples were obtained from the rat's retro-orbital sinus and stored in microcentrifuge tubes containing dipotassium ethylene diamine tetraacetic acid. Samples were taken at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h post dose. Plasma was immediately separated by centrifugation at 7,500 rpm for 15 min from the blood samples and stored in frozen conditions at −20 °C with appropriate labelling of subject code number, study date, and collection time prior to analysis. The concentration of ritonavir in rat plasma samples was measured by the reverse-phase high-performance liquid chromatography (HPLC) (Lledo et al. 2007; Kageyama et al. 2005).

Analysis of the ritonavir in rat plasma by HPLC

Prior to injection of the sample, ritonavir was extracted from the plasma by precipitating the plasma proteins as per the following procedure: 0.1 mL of plasma was transferred into the Eppendorf microcentrifuge tubes. To this, 0.1 mL of internal standard (didanosine) and 0.2 mL of methanol were added and vortexed for 5 min in Remi cyclomixer (R-24, Rimek, Mumbai, India) followed by centrifugation for 20 min at 7,500 rpm. After centrifugation, supernatant was collected and 20 µL of the supernatant was injected into the HPLC by Hamilton microsyringe (RZ07939-01, Mumbai, India). Peak area and height were computed and the concentration of the ritonavir in the sample was determined by the standard graph (peak area ratios of ritonavir to didanosine versus concentration of ritonavir).

Table 3 Physicochemical characterization of ritonavir vesicles

Batch code	% Drug content	Size (nm)	PDI	Zeta potential (mV)	%EE	pH
L1	97 ± 0.5	202 ± 0.1	0.196 ± 0.0	−24 ± 1.2	36 ± 1.5	7.4 ± 0.1
L2	101 ± 0.9	160 ± 0.2	0.310 ± 0.1	−30 ± 0.1	48 ± 1.7	7.4 ± 0.3
L3	98 ± 0.1	180 ± 0.6	0.204 ± 0.1	−20 ± 0.1	32 ± 3.0	7.4 ± 0.1
L4	97 ± 0.3	90 ± 0.1	0.242 ± 0.0	−26 ± 1.1	78 ± 2.6	7.4 ± 0.2
L5	98 ± 0.5	49 ± 0.3	0.171 ± 0.1	−33 ± 0.4	93 ± 1.7	7.4 ± 0.6
L6	98 ± 0.6	114 ± 0.1	0.302 ± 0.2	−24 ± 0.2	71 ± 1.5	7.4 ± 0.9
L7	101 ± 0.7	221 ± 0.0	0.367 ± 0.1	−28 ± 0.3	52 ± 2.0	7.4 ± 0.2
L8	97 ± 0.8	202 ± 0.1	0.354 ± 0.0	−32 ± 0.4	66 ± 1.3	7.4 ± 0.1
L9	98 ± 1.0	228 ± 0.0	0.315 ± 0.1	−22 ± 0.2	44 ± 0.9	7.4 ± 0.4

A sensitive HPLC method was developed and validated in the present study to estimate the plasma concentration of drug (Lledo et al. 2007). Samples were analysed using Grace Smart Altima C-18 column (150 × 4.6 mm, 5 µ) with mobile-phase acetonitrile: phosphate buffer pH 6.9 (55:45 % v/v) at a flow rate of 1 mL/min and detection wavelength was 235 nm using a Shimadzu SPD M10 AT VP model Photo Diode Array (PDA) detector (Shimadzu Corporation, Kyoto, Japan). The mobile phase was filtered through a 0.45-µm membrane filter before its use. The KINETICA 4.4.1 software (Thermo Electron Corporation, UK) was used to calculate the pharmacokinetic parameters. Pharmacokinetic parameters were statistically analysed using one-way analysis of variance (ANOVA). All tests were performed at a level of significance of ($p < 0.05$).

Results and discursions

The present work focused on the development of ritonavir-loaded stealth liposomal vesicular dispersions for parenteral delivery to enhance the circulation enhancement. Generally liposomes are prepared with various types of lipids (natural and synthetic)/and sterols. But in the present study, we selected the synthetic lipid DSPE which has high-phase transition temperature (74 °C) and short acyl chain length. Ethanol injection method was used as satisfactory and reproducible results were obtained. It was not evaluated earlier for its applicability in the liposomes for parenteral delivery.

The clinical use of ritonavir is very high in anti HIV therapy but the bioavailability of ritonavir is variable due to its poor water solubility. The major challenge in formulation of poorly soluble drugs for parenteral administration is to find an efficient preparation method resulting in a sufficiently high dose. Developments of parenteral formulations of such drugs are considerably limited in comparison to oral formulations. Parenteral administration of ritonavir is necessary in medication of HIV patients, who are incapable of receiving oral therapy for instance if they are unable to swallow due to health and physical conditions

The percent drug content of all the liposomes is tabulated in Table 3. The liposomal percent drug content was found to be in the range of 97–101 %. Percent drug content indicated that the ritonavir was uniformly distributed in vesicular dispersions and percent drug content near to 100 % indicated no loss of the material during the preparation. The pH of liposomal vesicular dispersions was found to be around pH 7.4. As pH was within the range of blood pH (7.35–7.45), these formulations were suitable for parenteral drug delivery and no irritation was expected. No significant difference was found in pH of different formulations. This is because of dilution of the final vesicular systems with pH 7.4 phosphate buffer. The results are shown in Table 3.

The mean vesicle size of ritonavir-loaded liposomes was found in the range of 49–228 nm and the polydispersity index (PDI) was in the range of 0.171–0.367. The result indicated a profound effect of DSPE and cholesterol on the liposomal vesicles' size. The vesicle size and PDI results of all the nine batches of liposomes are tabulated in Table 3. Vesicles were all in the nanometer range with low polydispersity indicating the homogeneity of the particle size. It was strongly affected by the selected variables. The above result indicated that cholesterol has more predominant effect on liposomal vesicles' size. The behaviour of vesicles completely depended on the amount of cholesterol. Lower concentrations of cholesterol allowed uptake of water in the aqueous compartment of the liposomal vesicles increasing the aqueous volume and consequently increase the vesicle size whereas the high lipophilicity produced by higher levels of cholesterol prevented water uptake across the bilayer causing reduction in size.

The zeta potential values of liposomal dispersions were found to be in the range of −20 to −32 mV, whereas for

any liquid dosage form surface charge is essential for its stability. Vesicular systems exhibited a higher zeta potential value of −32 mV due to the surface charge imparting nature of stearic acid. The values of zeta potential showed that vesicles had sufficient charge to inhibit aggregation of vesicles due to electric repulsion. The liposomal zeta potential values are given in Table 3.

The percent entrapment efficiency (%EE) of vesicles was determined using ultracentrifugation method. The liposomal %EE varied from 32 to 93 %. The values are shown in Table 3 and Fig. 1. Assuming that the core and membrane of vesicles were saturated with medium, this would allow ritonavir to distribute throughout the vesicle. A proportional relation between the %EE of ritonavir and the vesicle size was observed. Significant and continuous improvement in %EE was seen with increase in cholesterol content but higher concentrations of cholesterol lead to rigidity in the vesicles which in turn decreased the %EE. Important considerations in the entrapping efficiency of vesicles are the lipid surface charge, vesicle size, and aqueous volume of the vesicles. Basically ritonavir is a poorly water-soluble drug so it required high ratios of lipid to aqueous space.

The vesicular drug release mechanism was influenced by various physicochemical factors such as lipid/cholesterol composition, lamellarity, dispersion medium and method of preparation. The modified dialysis process was used for drug release. The study revealed that the release of the drug from the formulations depended on the relative amounts of lipid and cholesterol present.

The in vitro drug release profiles of liposomes are shown in Fig. 2. The percent drug release increased with increased concentration of lipid and at a certain level the percent release is retarded above that and the release was decreased at higher levels of cholesterol. This is because cholesterol at higher levels makes the lipid bilayers more rigid and retards the release of the drug. This was evident by the higher cholesterol concentration of vesicles showed around 50 % of the release except (L6) formulations. The L5 formulation found to have 99 % release at 24 h. At lower concentration of lipid/cholesterol, the drug release was very less due to formation of stagnant layer.

All the vesicular systems followed first-order release kinetics, except L5 liposomal batch that followed zero order. The drug release mechanism was determined by fitting the drug release data to Higuchi and Korsmeyer–Peppas equations. It was found that all the prepared liposomal vesicular dispersions followed diffusion mechanism. Plots of log fraction of ritonavir released versus log time of all the liposomal were found to be linear. The mechanism of drug release was followed by diffusion as observed from the higher '*r*' value (0.9911) of Higuchi plot for selected liposomes (L5). Liposomes followed Super case II transport diffusion as indicated by '*n*' value of Peppas plot (0.956) (Fig. 3).

Statistical analysis of the data and optimization

The data of the particle size and entrapment efficiency and 100 % drug release at 24 h were fitted to quadratic polynomial model. The equations obtained are (Figs. 4, 5):

$$\text{Particle size}\,(Y_1) = +52.54 + 39.88X_1X_1 + 118.87X_2X_2$$

$$\%\text{EE}\ (Y_2) = +92.13 - 16.87\,X_1X_1 - 34.37\,X_2X_2$$

$$100\%\text{in vitro drug release at 24 h}\,(Y_3) = +96.96 - 16.38X_1X_1 - 27.37\,X_2X_2.$$

From the ANOVA data, the *F* value of all three responses for liposomes (87.70, 842.99 and 43.57) indicated that the model is significant. The *p* value is <0.05 for all the response factors indicating that the models are significant. The liposomal response observation for particle size is X_1, X_1^2, X_2^2, %EE, X_1^2, X_2^2 and for 100 %

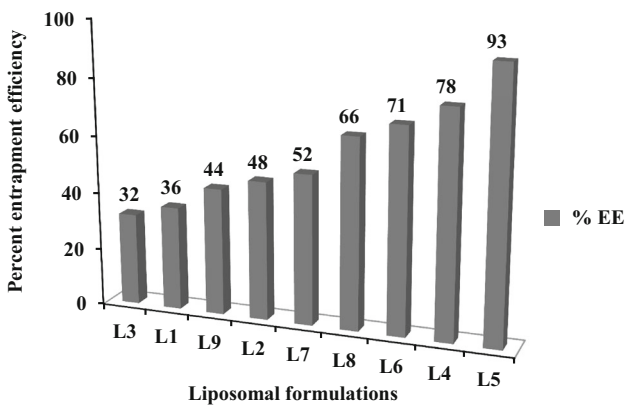


Fig. 1 Liposomal percent entrapment efficiency

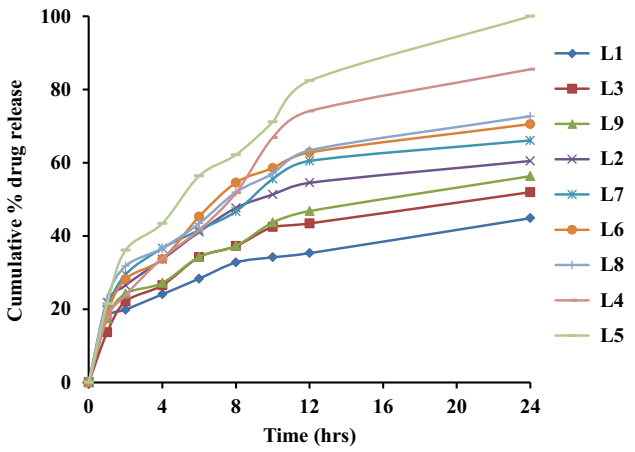


Fig. 2 Dissolution profiles of ritonavir released from liposomes

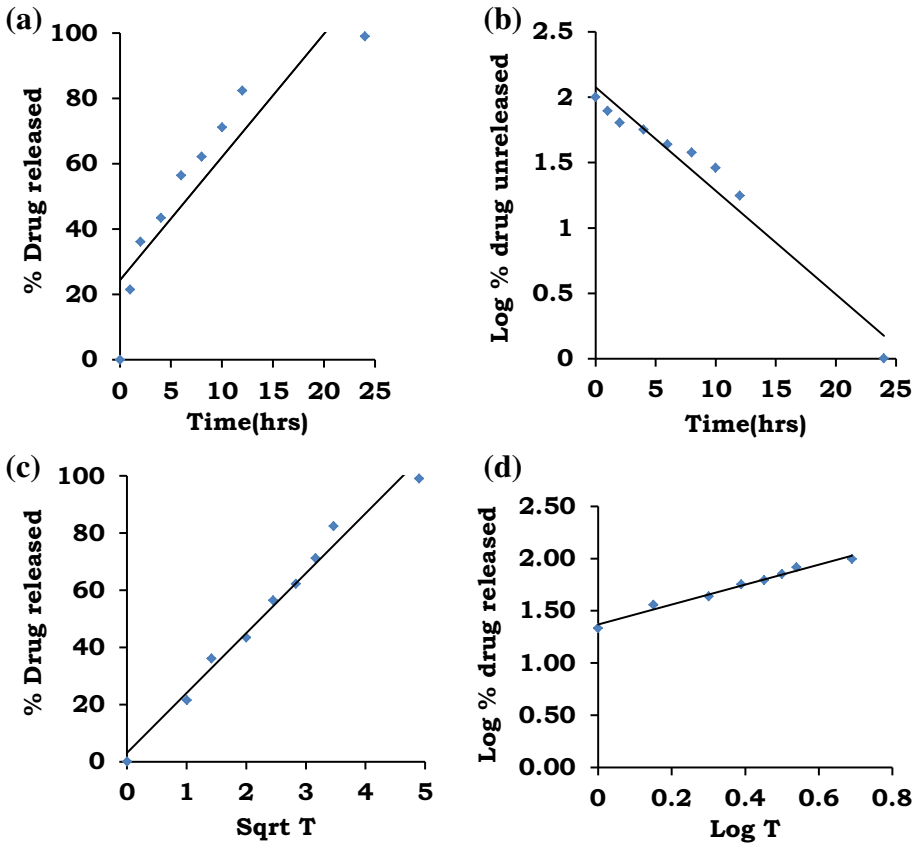


Fig. 3 Drug release kinetics and mechanism plots for L5 liposomes **a** zero order, **b** first order, **c** higuchi, **d** Korsmeyer–Peppas

drug release X_1, X_2, X_1^2, X_2^2 were found to be model significant terms. The predicted R^2 values of liposomes were in reasonable agreement with the Adjusted R^2 value. The desirability and counter plots were constructed and the optimized formulae were predicted using the constraints on the dependent variables. The liposomal desirability and overlay plots are shown, respectively, in Fig. 6. The desirability function was found to be higher (near to 1) for the optimized formula indicating the suitability of the formulations. The composition of the predicted formulations was matching with L5 liposomes. However, the experiment was repeated to reconfirmed result. The % relative error was calculated. The maximum % relative error was found to be 2.34. However, the values were found to be <5 % and hence it confirmed the suitability of experimental design followed for this study (Tables 4, 5).

Characterization of stealth vesicular dispersions

The selected conventional liposomes' (L5) batch was subjected for PEGylation using polyethylene glycol 10000 by polymer-coating method. It is an alternative method to prepare stealth liposomes. It is a characteristic of the polymer coating method that the manner of coating is very simple, just mixing a vesicular dispersions and polymer solution, without chemically linking the polymers to the lipid molecules (Fig. 7).

The percent drug content of stealth liposomes was found to be 94.12 %. The mean vesicle size of stealth liposomes was found to be 116.6 ± 0.11 nm, respectively. The stealth liposomes' size was slightly enhanced compared to conventional liposomes due to PEG-10000 forming thick surface layer on surface of the vesicles.

Zeta potential values of stealth liposomes were found to be −43.6 mV. The values of zeta potential showed that stealth liposomes had sufficient charge to inhibit aggregation of liposomes due to electric repulsion. When compared to conventional vesicle, stealth liposomes show more zeta potential values, due to the effect of polyethylene glycol 10000. The %EE of stealth vesicular dispersions was slightly enhanced when compared to the conventional liposomes. PEG-10000 enhances the solubility of the drug leads to enhance the %EE of stealth liposomes. The pH of stealth liposomes was also found to be around pH 7.4. The polyethylene glycol 10000 does not change the pH of the stealth vesicular dispersions and is suitable for parenteral drug delivery. The results are shown in Table 6.

The in vitro drug release profile of stealth vesicular dispersions was studied by the same method described earlier using dialysis membrane. The stealth vesicular

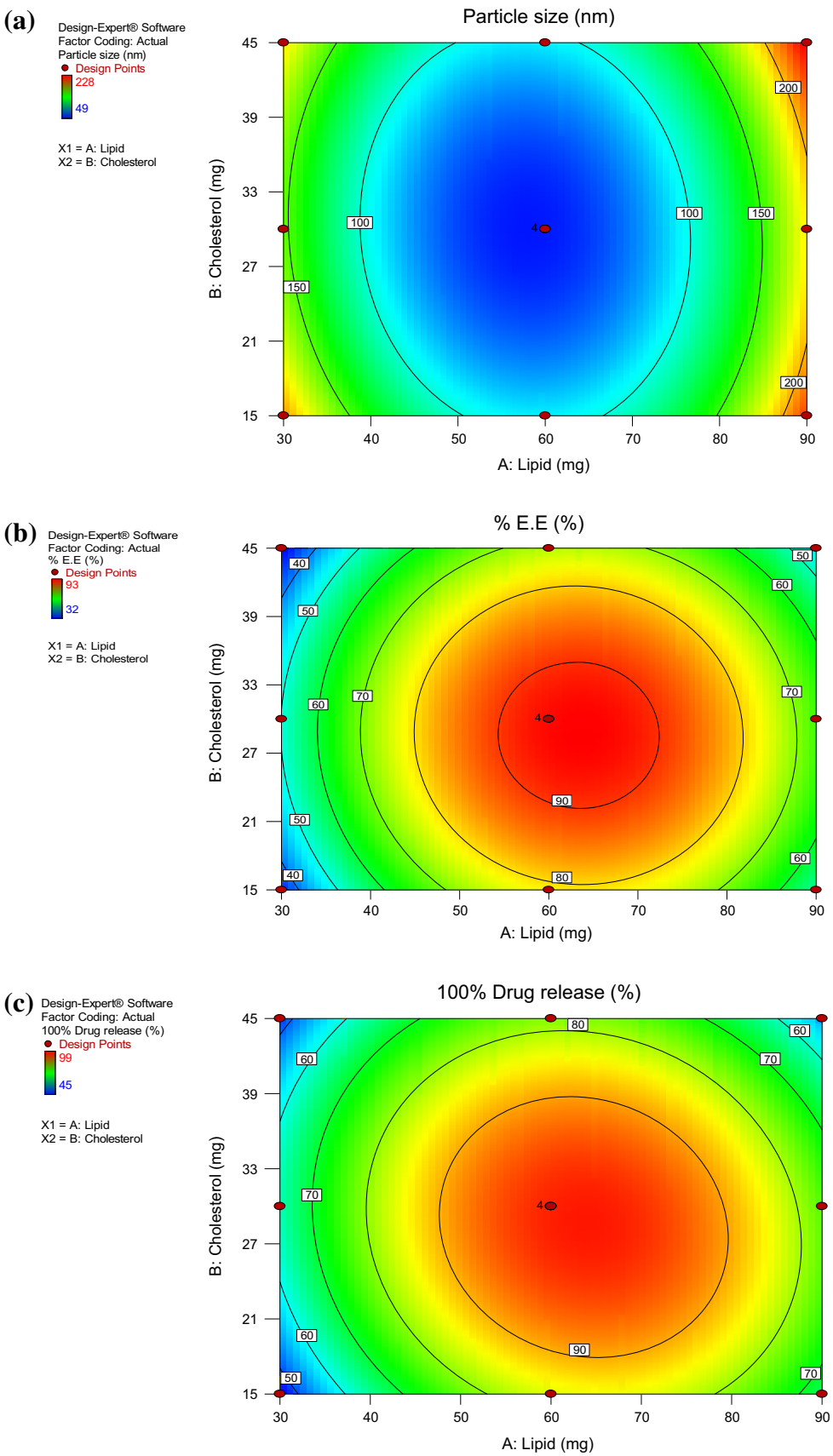


Fig. 4 Contour plots for the amount of lipid (DSPE) (X_1) and amount of cholesterol (X_2) in liposomal formulations **a** on particle size (Y_1), **b** on %EE (Y_2), **c** on 100 % in vitro drug release at 24 h (Y_3)

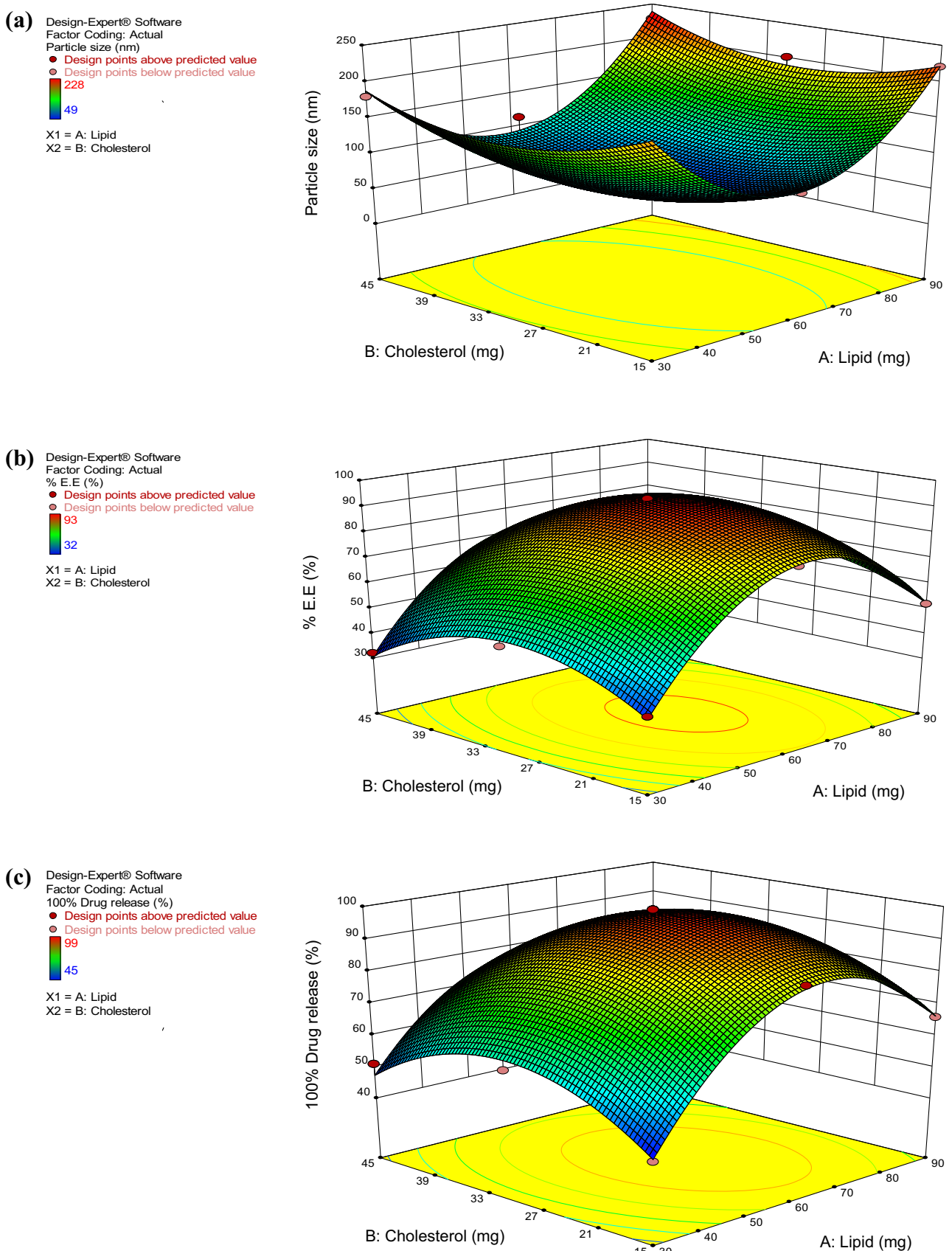


Fig. 5 Response surface plots for the amount of lipid (DSPE) (X_1) and amount of cholesterol (X_2) in liposomal formulations **a** on particle size (Y_1), **b** on %EE (Y_2), **c** on 100 % in vitro drug release at 24 h (Y_3)

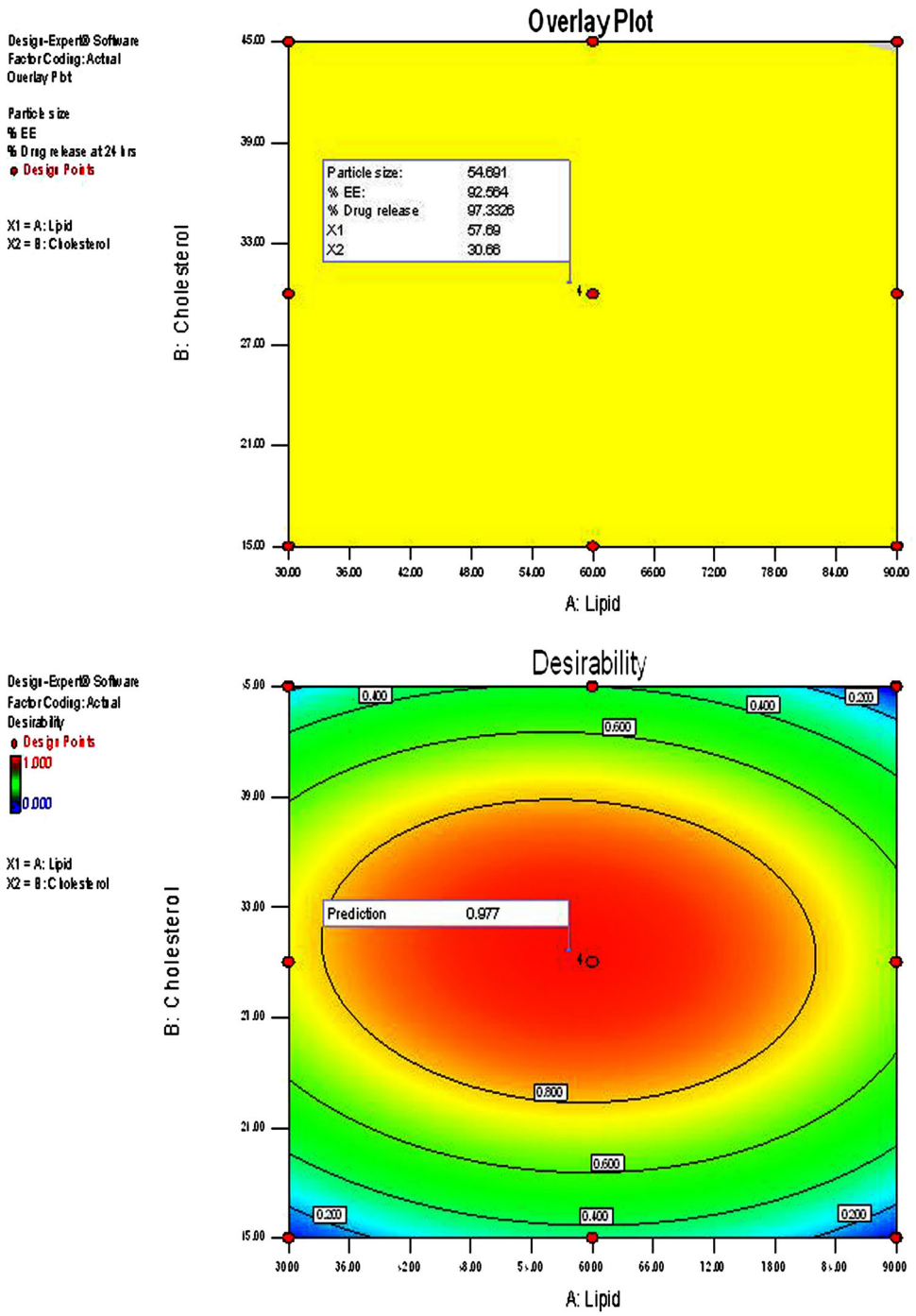


Fig. 6 Liposomal desirability and overlay plots

dispersions showed burst release within 1 h that is 17 % and afterwards release was followed by diffusion manner. Due to the effect of higher molecular weight of PEG-10000 prolongs the percent drug release up to 34 h. This is because the hydrophilic coat on the liposomes retards the drug release from stealth vesicles. Stealth liposomal dispersions followed zero-order kinetics as indicated by higher r values and followed non-Fickian diffusion mechanism (Fig. 8).

FTIR studies

Fourier transform infrared spectrophotometer (FTIR) studies were performed to detect the possible interactions between drug and the excipients. The FTIR spectra of pure drug ritonavir, DSPE, cholesterol, stearic acid, PEG-10000 and stealth liposomes are shown in Fig. 9. The characteristic C–H aliphatic stretching, C–H or C=C–H aromatic stretching, NH stretching of secondary amine, C=C

Table 4 ANOVA for model parameters (ANOVA) for the dependent responses of liposomes

Parameters	SS	*df*	MS	*F*	*p*	*S*
Response (Y_1)						
Model	5,8867.46	5	1,1773.49	87.70	<0.0001	*S*
Response (Y_2)						
Model	6,000.46	5	1,200.09	842.99	<0.0001	*S*
Response (Y_3)						
Model	4,316.04	5	863.21	43.57	<0.0001	*S*

Table 5 Validation of predicted and optimized liposomal batch

Response	Observed Liposomes	Predicted	% Relative error
Y_1	48.9 nm	50.06 nm	2.31
Y_2	94 %	95.02 %	1.07
Y_3	98.99 %	99.84 %	0.85

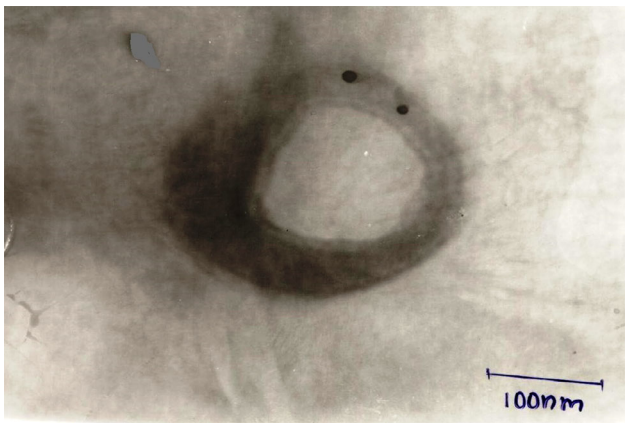


Fig. 7 Surface morphology of stealth liposomes by TEM

Table 6 Physicochemical characterization stealth vesicles

Parameters	Stealth liposomes	Stealth niosomes
Drug content (%)	94.12 ± 0.29	96.24 ± 0.47
size (nm)	116.6 ± 0.1	122.9 ± 0.3
PdI	0.193 ± 0.08	0.204 ± 0.12
Zeta potential (mV)	−43.6 ± 1.8	−51.6 ± 1.2
%EE	96.4 ± 1.8	98.1 ± 2.7
pH	7.4 ± 0.6	7.4 ± 0.2

Each value represents mean ± SD ($n = 3$)

aromatic stretching, C=O amide stretching, C=O ester stretching of pure drug were observed at 2,963–2,869, 3,024–3,098, 3,357, 1,458–1,526, 1,715 and 1,644 cm^{-1} The characteristic peaks confirmed the structure of

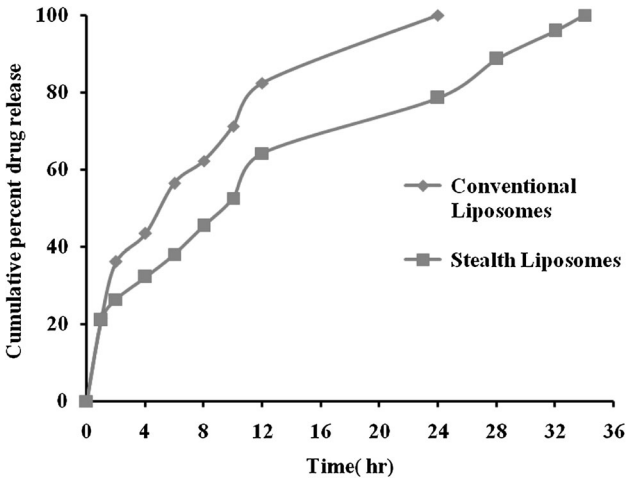


Fig. 8 In vitro comparative release profile of conventional and stealth liposomes

ritonavir. The FTIR spectrum of DSPE showed ester C=O at 1,793.18 cm^{-1} and aliphatic CH 2,955.93–2,849.53 cm^{-1}. The FTIR spectrum of cholesterol showed C–O alcoholic stretching vibration at 1,022.58 cm^{-1}, O–H stretching vibration at 3,398.38 cm^{-1}, aliphatic C–H stretching vibration at 2,933.42–2,849.37 cm^{-1} and C=C stretching vibration at 1,467.47 and 1,375.42 cm^{-1}. The FTIR spectrum of stearic acid showed C–H aliphatic stretching vibration at 2,955 cm^{-1} and C=O (acid) stretching vibration at 1,703 cm^{-1}. The FTIR spectrum of PEG-10000 C–H aliphatic, C–O–C aliphatic ester, and OH group of PEG-10000 was observed at 2,917–2,695, 1149, and 3,451 cm^{-1}. All the characteristic peaks of the drug corresponding to the C–H aliphatic stretching, C–H or C=C–H aromatic stretching, NH stretching of secondary amine, C=C aromatic stretching, C=O amide stretching, C=O ester stretching were present in the stealth liposomal formulations. The major peaks for the pure drug and the excipients are well in support with the theoretical prediction with respect to the functional groups. Presence of DSPE, cholesterol, PEG-10000, and steric acid did not produce any major shift in principal peaks of ritonavir and the presence of one ingredient did not produce shift in the peaks of other ingredients. This indicates that there is no interaction between drug and the excipients used in the study. Hence, FTIR spectral analysis proved the compatibility of the drug and excipients used in the study.

Differential scanning calorimeter

DSC is used to study the thermal behaviour of lipids in vesicular dispersions. Whenever these types of excipients undergo a change in physical state, such as transition from gel to liquid crystalline form, heat is absorbed. The

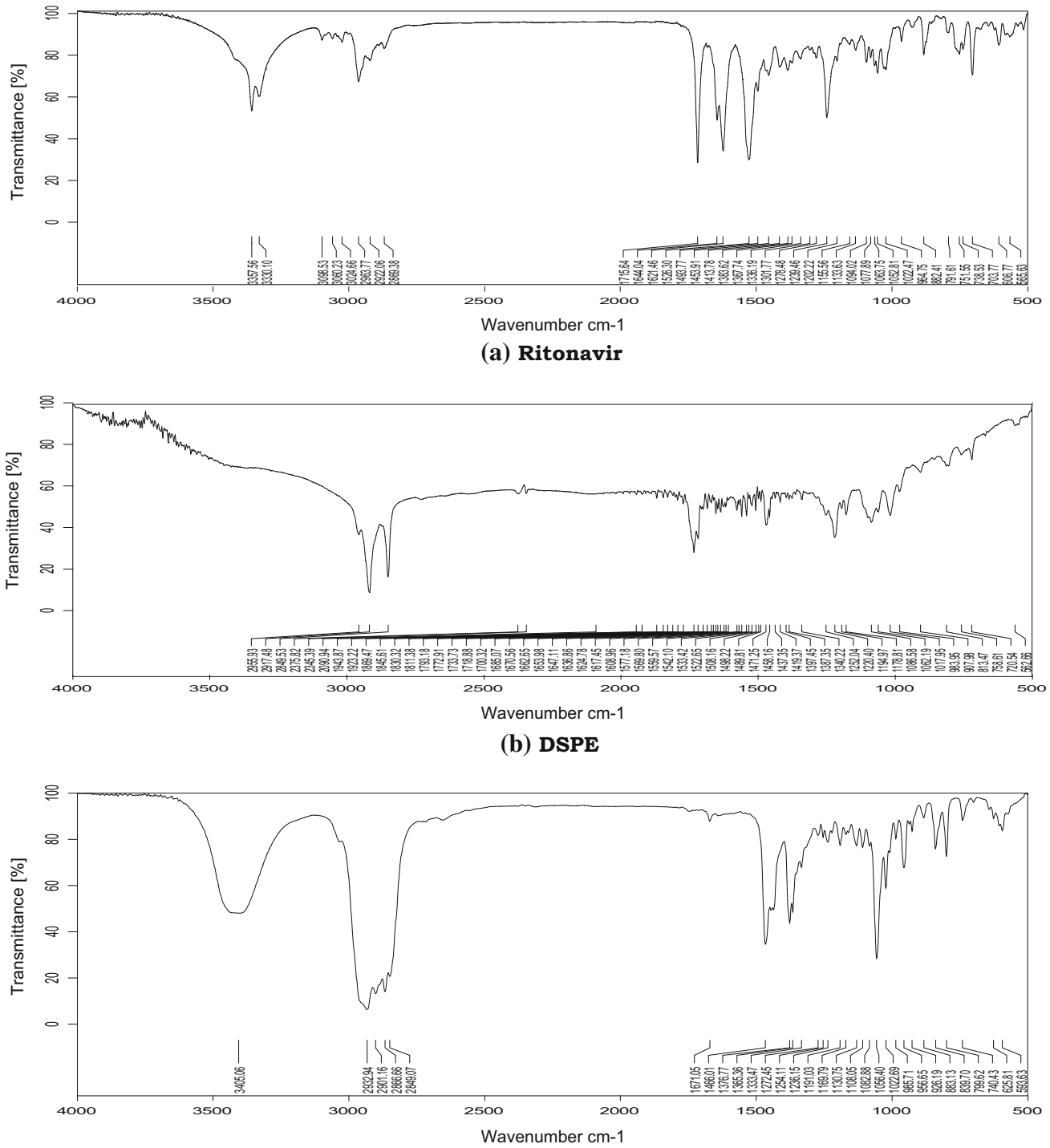
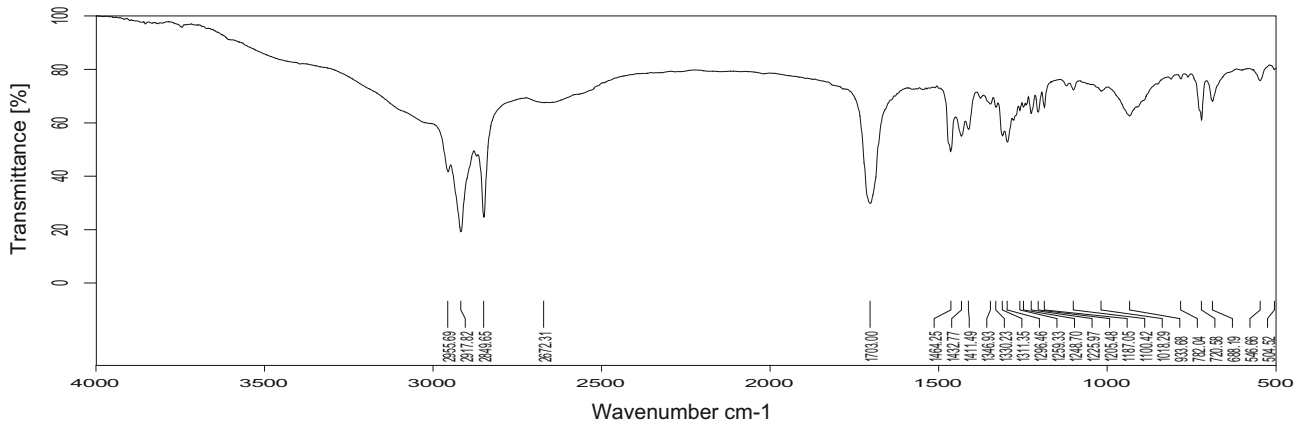
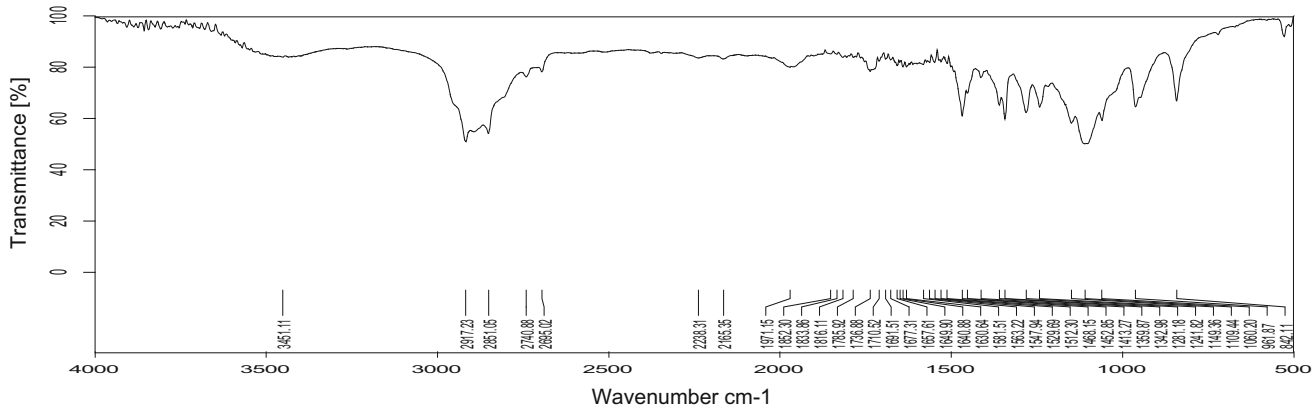


Fig. 9 **a** Ritonavir, **b** DSPE, **c** cholesterol, **d** stearic acid, **e** polyethylene glycol 10000, **f** stealth liposomes

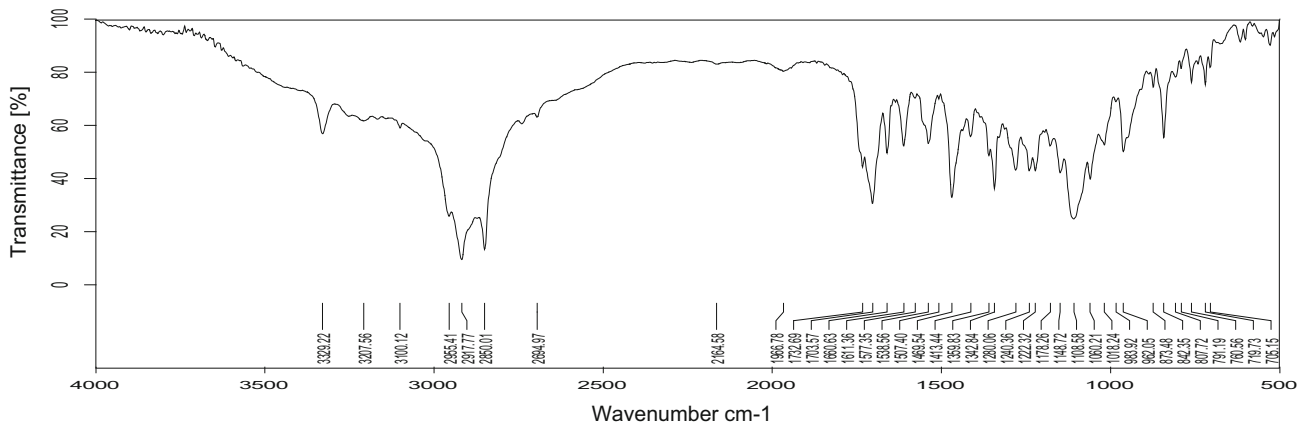
apparatus can be used to measure heat capacity and the purity of the lipid samples. A sharp DSC peak exhibited at 124.7° C was correspondence to the pure drug ritonavir. A broad peak observed at 66.2 °C was correspondence to the PEG-10000 in stealth liposomal formulation. From the DSC thermogram, it can confirm that drug was completely converted into amorphous state. So the drug peak was not observed in stealth liposomal formulation (Fig. 10).



(d) Stearic acid



(e) Polyethylene glycol 10000



(f) Stealth liposomes

Fig. 9 continued

Stability

The stability study indicated that there are major changes observed. All the evaluated parameters were changed from the initial values. Comparatively stealth liposomes showed the better stability profile than conventional liposomes profile due to the polyethylene glycol 10000 because it enhances the surface charge of the liposomes leads enhances the stability. Aggregation and fusion of liposomes cause shift in mean size and size distribution of liposomes towards higher value and cause destabilization of vesicle, so the liposomes size therefore is a useful marker for

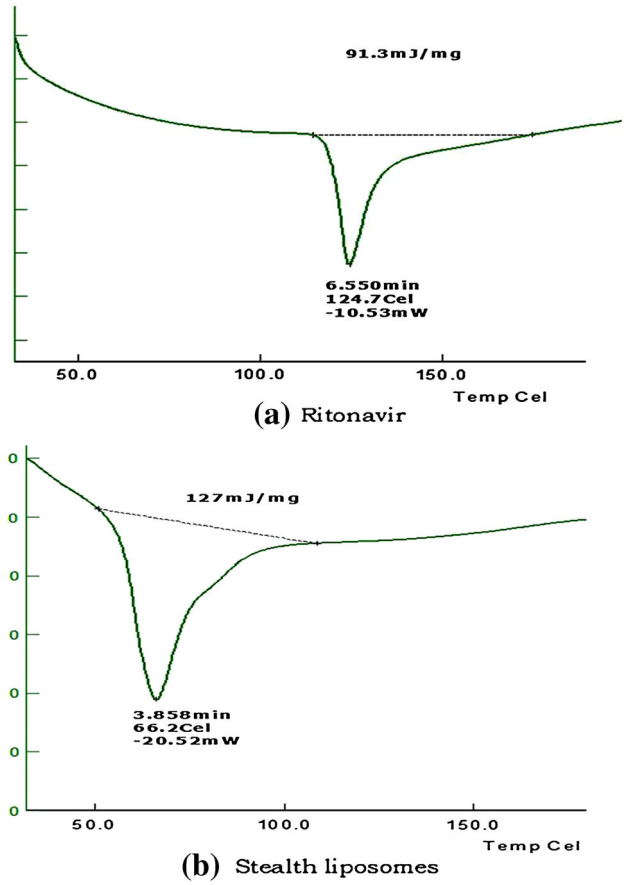


Fig.10 DSC thermograms of pure drug ritonavir and stealth liposomes

indicating changes in physical stability of vesicles. The stability studies revealed that temperature played major role, because all the vesicular dispersions are more stable at 2–8 ± 2 °C than 25 ± 2 °C/60 ± 5 %RH.

In the present stability studies, results indicated that conventional liposomes are more susceptible to hydrolysis and oxidation than stealth liposomes. The values obtained revealed three main conclusions viz., firstly, there was notable difference between the stability of conventional liposomes and stealth liposomes. The stability in terms of % drug content, particle size, zeta potential and %EE was found to be in the following stealth liposomes > conventional liposomes.

In vivo pharmacokinetics

The selected formulations were subjected for the in vivo pharmacokinetic evaluations on male Wistar rats weighing around 200–250 g. The selected conventional and stealth vesicular systems were tested against the pure ritonavir solution. The selected vesicular formulations were subjected to two-step sterilization process of filtration under aseptic conditions using sterile grade 0.22 μm PVDF filters, followed by lyophilization. The lyophilized products in the sealed vials were found to be free flowing without any cake formation and adherence to the wall of the container. pH 7.4 phosphate buffer saline sterilized by autoclaving was used for the reconstitution of the lyophilized vesicular systems for parenteral administration to Wistar rats. The reconstitution time was found to be 25–30 s for all the products resulting in uniform dispersion of the systems with ease of flow through syringe needle. No visual damage was observed for the vesicular systems after lyophilization

From Table 7, the AUC_{0-24} and $AUC_{0-\infty}$ of pure ritonavir solution show higher value than conventional liposomes (L5) and stealth liposomes. There was no significant difference for AUC_{0-t} and $AUC_{0-\infty}$ among conventional and stealth liposomes. When compared to conventional liposomes (L5), stealth liposomes showed slightly higher values due to the effect of PEG-10000 stearic properties which greatly enhance long systemic circulation. The elimination rate constant (K_{el}) and volume of distribution (V_d) were found to be lower for stealth liposomes compared to conventional L5 and pure ritonavir solution. The K_{el} and V_d values further reflect the longer

Table 7 Pharmacokinetic parameters of tested products in Wistar rats

Parameters	Units	Pure ritonavir solution	L5	Stealth liposomes
K_{el}	h^{-1}	0.412 ± 0.019	0.4299 ± 0.09	0.058775 ± 0.005
$t_{½}$	HR	1.79 ± 0.28	1.68 ± 0.36	11.75 ± 1.24
$AUC_{0-24\ h}$	ng h/mL	3,687.1 ± 200.71	2,887.3 ± 209.9	2,850.748 ± 65.40
$AUC_{0-\infty}$	ng h/mL	3,799.9 ± 233.71	2,934.07 ± 220.5	3,381.72 ± 175.75
$AUMC_{0-24}$	ng h/mL	12,048.7 ± 793.7	9,067.7 ± 1,461.001	17,248.98 ± 824.22
$AUMC_{0-\infty}$	ng h/mL	48,406.3 ± 704.6	9,989.7 ± 1,623.5	34,834.18 ± 5,030.56
MRT	h	3.5 ± 0.12	3.4 ± 0.2	11.58 ± 1.40
Bioavailability	%	100 ± 0.00	77.16 ± 1.1	89.28 ± 7.2
Volume of distribution	L/ng	0.219 ± 0.01	0.223 ± 0.04	0.0306 ± 0.002
Clearance	L/h	0.527 ± 0.03	0.522 ± 0.03	0.522 ± 0.02

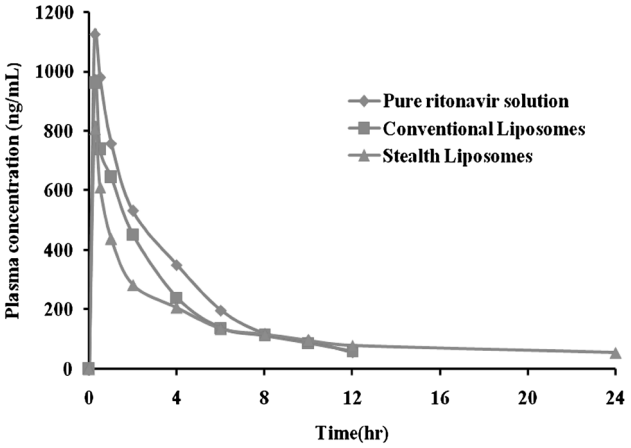


Fig. 11 Plasma concentrations of tested products in Wistar rats

blood circulating nature of stealth liposomes compared with rapidly clearing nature of conventional liposomes (L5) and pure ritonavir solution. In comparison with pure ritonavir solution, the half-life of the stealth liposomes was increased 6.65-fold and in comparison with conventional liposomes (L5) the half-life of stealth liposomes increased 6.9-fold. The lower half-life values of conventional vesicles and their pure ritonavir solution may be due to opsonization process. The Kupffer cells which are present in the liver and reticuloendothelial system (RES) are responsible to removed from the systemic circulation. The mean residence time (MRT) was found to be 11.58 h for stealth liposomes, while the conventional liposomes (L5) and pure ritonavir solutions found in the range of 3–3.5 h. The MRT of stealth liposomes has been increased 3.39-fold compared to conventional liposomes (L5) and pure ritonavir solution (Fig. 11).

The relative percent bioavailability (F_{rel}) was found to be 100 %, 77.1 ± 1.1 and 89.2 ± 7.2 % for pure ritonavir solution, conventional liposomes (L5) and stealth liposomes, respectively. Compared to pure ritonavir solution, the conventional and stealth liposomes bioavailability has been decreased because it may be the conventional liposomes rapidly cleared from systemic circulation but the stealth liposomes showed slightly higher values of relative percentage bioavailability compared to conventional liposomes (L5) due to their long systemic circulation time. The in vitro drug release of conventional and stealth vesicles showed 20 % of drug release within 1 h because of burst release. It may be one of the reasons for the higher initial plasma drug concentration of conventional and stealth vesicles.

Based on MRT values and half-life values of the drug we can confirm that the circulation time of the drug was improved from the stealth vesicular systems compared to conventional liposomes (L5), and pure ritonavir solution. Significant difference was observed for MRT and half-life of the drug from stealth liposomes as compared to conventional liposomes (L5) and pure ritonavir solution. These in vivo pharmacokinetic results confirmed the improvements in circulation time of drug from stealth vesicular systems in the rat body.

This might be due to the effect of sterical stabilization of PEG-10000. The polyethylene glycol possessing a flexible chain occupies the space immediately adjacent to the vesicles' surface and it tends to exclude other macromolecules from this space. Consequently access and binding of blood plasma opsonins to the vesicles surface are hindered thereby inhibiting the interactions of MPS macrophages with such vesicles are inhibited. The ability of the hydrophilic shell of polyethylene glycol to avoid aggregation between vesicles and to decrease the extent of vesicle–protein interaction in biological fluids is not only due to the molecular mass of the bound polymer and its uniformity ("molecular cloud") but also due to its considerable conformational flexibility. In the present study, PEG-10000 has been used to increase the drug solubility and stability, lower the toxicity and increase the circulation time by decreasing the clearance. The ANOVA data of selected pharmacokinetic parameters K_{el}, $t_{1/2}$, and $AUC_{0-\infty}$ showed statistically significant difference between the three formulations ($p < 0.05$).

Several factors have influenced the in vivo fate of the vesicles after intravenous dosing such as particle size, surface charge, method of preparation and lipid composition. The PEGylation increased the solubility of the drug and increased its in vivo stability. The PEG-10000 shows optimum sterical stabilization property, which enhanced the systemic circulation of the ritonavir-loaded stealth vesicles and reduced the accumulation of unwanted drug actions causing toxicity. The PEGylation increased the prolong release profile of vesicles at the site of action and it leads increased the patient compliances.

Discussions

A 3^2 full factorial design has been employed to produce ritonavir-loaded vesicular systems using ethanol injection method. The statistical optimization reduced the number of experiments to be carried out for obtaining formulations with desired properties. The derived polynomial equations, response and contour plots helped in predicting the values of selected independent variables for preparation of optimum vesicular systems with desired properties. Liposomes showed optimum particles size, higher %EE, and prolong drug release for 24 h. Among all the formulations, L5 liposomal batch showed desired optimum results such as lowest particle size, highest %EE and maximum drug release of 99 and 96 % at 24 h. Based on optimum particle

size, highest %EE and maximum percent drug release, L5 batch liposomes were selected for the PEGylation. PEG-10000 offers the versatile properties of flexibility, hydrophilicity, and biocompatibility. Stealth vesicular liposomes were prepared by polymer coating method. The physicochemical properties and drug release time of stealth vesicular dispersions showed better result compared to the conventional vesicular dispersions. The drug excipients' characterization parameters revealed no drug–excipients interaction.

The above in vivo studies indicated that stealth vesicular systems are suitable for lipophilic antiretroviral agent and this has been found to be suitable drug delivery systems for antiretroviral therapy. Ritonavir is a highly lipophilic antiretroviral agent and it has limited clinical use due to its poor solubility. The stealth liposomes approach altered the pharmacokinetic profile of ritonavir resulting in reduced plasma clearance and increased systemic circulation time of the drug comparatively conventional liposomes and pure ritonavir solution. The relative bioavailability was improved with longer $t_{1/2}$ and higher MRT and AUC values for stealth liposomes when compared to conventional liposomes. These results suggest that the ritonavir-loaded stealth liposomes could be an effective parenteral carrier for ritonavir delivery.

Conclusion

Hence the present study confirmed that the stealth vesicular systems that were prepared with various lipids/cholesterol can be used for enhanced circulation time of the drug and thereby significantly improved the drug efficiency for AIDS treatment.

Conflicts of interest None.

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