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RESEARCH ARTICLE

Development and Optimization of Liposomes Containing 5 Fluorouracil and Tretinoin for Skin Warts: 3^2 Experimental Design

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Development and Optimization of Liposomes Containing 5 Fluorouracil and Tretinoin for Skin Warts: 32 Experimental Design

SUMMARY

Tretinoin (TTN) is indicated for treatment of acne vulgaris, melasma, photoaging, follicular keratosis and disorders of keratinization. 5 Fluorouracil (5FU) is a medication which is used in the treatment of cancer. It belongs to the family of drugs called the antimetabolites. The objective of the research work was to design novel liposomes containing 5FU and TTN and optimization of liposomes was done by 32 full factorial design. The effect of independent variables was determined on dependent variables. Liposomes were prepared by ethanol injection method and evaluated by Transmission Electron Microscopy (TEM), entrapment efficiency (EE), Fourier Transform Infrared Spectroscopy (FTIR), zeta potential and in vitro drug release. Optimized formulation was subjected to stability studies at 40C, 250C and 600C temperatures. FTIR spectrums of the pure drug, soya lecithin, cholesterol and their physical mixtures was performed and noted that there was no possible interactions between drugs and the other ingredients. No drug crystals were visible in TEM-images, regardless of the preparation technique or the loaded drug. The average percent drug entrapment efficiency of F9 showed a maximum drug entrapment of 72.86% and 69.70% for 5FU and TTN respectively. When phospholipid concentration was increased from 40 to 60 mg/ml, the encapsulation efficiencies of formulation increased. High amount of drug release (30.6 to 67.42%) was observed at 2 h. from formulations F1 to F6. Drug release profile of F9 showed a best fit to the desired control release of drug. Stability study showed that liposomes were not stable at high temperature but formulations were most stable when stored at lower temperature i.e. 4oC. Thus, in the liposomes both hydrophilic and hydrophobic drugs were successfully incorporated and it can be further used for formulation development.

Key Words: *5-Fluorouracil, tretinoin, liposomes, in vitro drug release, 32 experimental design, stability*

Cilt siğilleri için: 32 Deneysel tasarımı için 5 Fluorourasil ve Tretinoin İçeren Lipozomların Geliştirilmesi ve Optimizasyonu

ÖZ

Tretinoin (TTN), acne vulgaris, melasma, fotoyaşlanma, foliküler keratoz ve keratinizasyon bozukluklarının tedavisinde kullanılır. 5-Florourasil (5FU) kanser tedavisinde kullanılan bir ilaçtır ve antimetabolitler sınıfında yer almaktadır. Araştırmanın amacı yeni 5FU ve TTN içeren lipozomların tasarımlanması olarak belirlenmiş ve 32 tam faktöriyel tasarımı optimizasyonu ile yapılmıştır. Bağımsız değişkenlerin etkisi bağımlı değişkenlere göre belirlenmiştir. Lipozomlar etanol enjeksiyon yöntemi ile hazırlanmıştır ve Transmisyon Elektronu Mikroskopi (TEM), tuzak verimliliği (EE), Fourier Dönüşümü Kızılötesi Spektroskopisi (FTIR), zeta potansiyeli ve in vitro ilaç salımı ile değerlendirilmiştir. Optimize edilmiş formülasyonun, 40, 250 ve 600 ° C'lerde stabilite çalışmaları yapılmıştır.Saf ilacın FTIR spektrumları, soya lesitin, kolesterol ve fiziksel karışımları yapılmıştır ve ilaçlar ile diğer bileşenler arasında olası bir etkileşim olmadığı belirlenmiştir. Hazırlık tekniği veya yüklü ilaç ne olursa olsun TEM görüntülerinde ilaç kristalleri görülmemiştir. F9'un maksimum ortalama yüzde ilaç tutma etkinliği 5FU ve TTN için sırasıyla % 72.86 ve% 69.70'dır. Fosfolipid konsantrasyonu 40'tan 60 mg / ml'ye kadar arttırıldığında, formülasyonun kapsülleme verimleri artmıştır. Yüksek miktarda ilaç salınımı (% 30.6 ila 67.42) F1 ila F6 arasındaki formülasyonlardan 2 saatte gözlenmiştir. F9'un istenen kontrol salımı profili en iyi sonucu vermiştir. Satbilite çalışması gösterdi ki lipozomlar yüksek sıcaklıkta stabil değildir, ancak, formülasyonlar 4oC gibi düşük sıcaklıkta depolandığında kararlıdır. Böylece, lipozomarın içinde hem hidrofilik hem de hidrofobik ilaçları başarıyla birleştirmiştir ve ayrıca ileri formülasyon gelişimi için kullanılabilir.

Anahtar Kelimeler: *5-Florourasil, tretionin, lipozom, in vitro ilaç salımı, 32 deneysel tasarım, stabilite*

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INTRODUCTION

Liposomes are vesicles made of phospholipid bilayers. These phospholipid bilayers surround an aqueous core. Liposomal size is directly related to the method of preparation and can range from 50 nm to several microns. They form spontaneously when these lipids are dispersed in aqueous media. Vesicles can be constructed of natural constituents such that the vesicle membrane forms a bilayer structure which is principal identical to the lipid portion of natural cell membrane. Their ability to mimic the behaviour of natural membranes and also to be degraded by the same pathways, makes vesicles a very safe and efficacious vehicle for medical applications. Vesicles can be composed even of entirely artificial components, chosen for their improved chemical properties (e.g. fatty acids, double chain secondary amines, cholesterol derivatives). Moreover, liposomes may entrap both hydrophilic and lipophilic molecules; and be used as drug carrier for both types of drug molecules (Tiwari et al., 2018). Vesicle membranes are semi-permeable membranes, in that the rate of diffusion of molecules and ions across the membrane varies considerably. For molecules with high solubility in both organic and aqueous media, a phospholipid membrane clearly constitutes a very tenuous barrier, but polar solutes and higher molecular weight compounds pass across the membrane only very slowly. Release rate of different types of drug molecules from liposomes is dependent on the type of drug applied (Cevc 1990). Cholesterol may be included to improve bilayer characteristics of vesicles, increase microviscosity of the bilayers, reduce the permeability of the membrane to water-soluble molecules, stabilize the membrane and increase the rigidity of the vesicles (Egbaria et al., 1992). Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains. 5FU is most often prescribed for actinic keratoses and Bowen disease. It destroys sun-damaged skin cells so the skin appears smoother and more youthful. It works best on face and scalp, and is less effective on other areas. TTN enhances the effect of 5FU by peeling off the top layer of skin. It reduces the time required for the course of 5FU treatment.

The present study was planned to formulate and evaluate a new combination of 5FU and TTN for topical administration. The main aim of the study is to attain effective drug concentration at the intended site of action for a sufficient period of time to elicit the response.

MATERIALS AND METHODS

Materials

Tretinoin (TTN) and 5 Fluorouracil (5FU) were kindly provided by Curetech Skincare, Baddi, Himachal Pradesh and Shalaks pharmaceuticals, New Delhi respectively. Cholesterol and Soy Lecithin were purchased from the Central drug house, New Delhi. Cellophane membrane (molecular weight cut off 12,000-14,000) was purchased from Qualigens Fine Chemicals, Mumbai, India. Ethanol and all other chemicals used were of analytical grade.

Fourier Transform Infrared Spectroscopy (FTIR)

To identify the compatibility of drugs and excipients, FTIR study was performed. The IR Spectra of all physical mixtures were obtained with FTIR Spectrophotometer, Perkin Elmer Spectrum at the end of two weeks of storage (50^0C). The scanning range was 4000-400 cm^{-1} at a scan period of 1 minute (Epstein et al., 2008).

Experimental design

A 3^2 randomized full factorial design was adopted to optimize the variables. In the design two factors were evaluated, each at 3 levels and experimental trials were at all nine possible combinations using Design Expert Software 10 (State-Ease, inc., Minneapolis, USA). In the present investigation, the concentration of TTN (X1) and concentration of phospholipid (X2) were selected as independent variables. The entrapment efficiency (EE; R1), and cumulative drug release (%DR; R2) were selected as dependent variables (Table 1). The polynomial equation was generated for the dependent variables that were reduced by removing non-significant coefficients by applying one way ANOVA. To demonstrate graphically the influence of each factor on responses, the response surface plots were generated. The value of $p<0.05$ was considered to be significant (Mayer et al., 1986).

Table 1. 3^2 Factorial design for preparation of liposomal formulations

Formulation Code	Independent Variables		Dependent Variables
	X1	X2	
F1	-1	-1	%EE (R1)
F2	0	-1	
F3	+1	-1	%DR (R2)
F4	-1	0	
F5	0	0	
F6	+1	0	
F7	-1	+1	
F8	0	+1	
F9	+1	+1	
X1	Concentration Ratio of TTN		
X2	Ratio of phospholipid		
Coded Values	**Actual Values**		
	X1 (mg/ml)	**X2 (mg/ml)**	
-1	0.050	20	
0	0.075	40	
+1	0.100	60	
All batches contained 500 mg of 5FU, 4mg/ml cholesterol.			

Formulation of drug loaded liposomes

Preparation of blank liposomal particle

Liposomes were prepared by modified ethanol injection method with certain modifications. Lecithin and cholesterol were dissolved in ethanol. The organic phase was slowly added to the aqueous phase which was previously heated to the 45± 2^0C and the mixture was stirred for 30 minutes at 500- 700 rpm under magnetic stirring. Spontaneous liposome formation occurred as soon as the ethanolic solution was in contact with the aqueous phase.

Method for drug loaded liposomes development

Liposomes were prepared by a modified ethanol injection method (Tiwari et al., 2017). The required amounts of phospholipids (20, 40 and 60 mg/ml) and cholesterol (4 mg/ml) were dissolved in ethanol and the lipophilic drug (TTN) was added to the organic phase. The resulting organic phase was injected by means of a syringe pump to the 45± 2 °C aqueous phase (The hydrophilic drug (5FU) was added to the aqueous phase) under magnetic stirring (Çoban et al., 2013). Spontaneous liposome formation occurred as soon as the ethanolic solution was in contact with the aqueous phase. The liposome suspension was then kept under stirring for 1h at room temperature to remove the traces of solvent. The unloaded drug was removed by ultracentrifugation of liposome suspension (Beckman, Miami, Florida, USA) at 60000 rpm for 1 hour and stored at 4°C.

Evaluation of liposomes

Morphological study by transmission electron microscopy (TEM)

Liposome suspensions were imaged by using TEM (Philips CM120; Eindhoven, The Netherlands). A drop of the liposome suspension was placed onto a carbon-coated copper grid, forming a thin liquid film. The films were negatively stained with 2% phosphotungstic acid solution for 1 minute. The excess of phosphotungstic solution was removed with a filter paper and stained samples were characterized by using an accelerating voltage of 80 kV (Batzri et al., 1973).

Entrapment efficiency

Five ml of liposome formulation was taken and transferred to a 100 ml volumetric flask containing 25 ml of the mobile phase, then sonicated using an ultrasound bath for few minutes and filtered through a 0.45µm membrane filter. The filtrate was finally diluted with pH 6.8 and appropriate dilutions were made and the absorbance was recorded by Shimadzu 1700 UV visible spectrophotometer (Study was done in triplicate and standard deviation was found to be less than 0.02 %) at 266 and 340 nm respectively and the amount of 5FU and TTN were determined (Batzri et al., 1973). Percentage of drug entrapped calculated by using following formula.

Where, % EE is the drug entrapment efficiency, WT is the total amount of Allopurinol in transferosomal suspensions, WF is the free amount of Allopurinol that was found in the supernatant (Agardan et al., 2018). The mean percentage of drug entrapment was measured after performing the experiment in triplicate.

***In vitro* drug release**

The liposomal suspension (equivalent to 2 mg) was placed in a dialysis bag, suspended in 250 ml of phosphate buffer (pH 6.8) and stirred at 100 rpm. Two milliliters of sample was withdrawn from the receptor compartment and analyzed spectrophotometrically at 266 and 340 nm for 5FU and TTN respectively. An equal volume of fresh phosphate buffer (pH 6.8) was added after each sampling to maintain the sink conditions (Egbaria et al., 1992).

Mathematical modeling of release kinetics

The kinetics of drug release from the matrix tablets was determined by fitting the appropriate drug release data to zero order, first order, Higuchi equation, Hixson-Crowell equation and the Korsemeyer-Peppas model (Batzri et al., 1973).

$Q = Q_0 + k_0 t$ (Zero Order)

$\ln Q = \ln Q_0 + k_1 t$ (First Order)

$Q = k_H t^{1/2}$ (Higuchi Model)

$Q_0^{1/3} - Q_R^{1/3} = k_s t$ (Hixson Crowell Model)

$Q/Q_T = k_{kp} t^n$ (KorsemeyerPeppas Model)

Where Q is amount of drug release at time t, Q_0 is

the initial amount of drug, Q_R is the amount of drug remaining at time t, and Q_T is the total amount of drug release. k_0, k_1, k_H, k_s and k_{kp} are the kinetic constants for zero order, first order, Higuchi, Hixson-Crowell and Korsemeyer-Peppas models respectively, and n is the release exponent.

Selection of optimized formulation

A numerical optimization technique using the desirability approach was employed to select optimized formulation with the desired responses. Constraints, like maximizing entrapment efficiency and % drug release at the end of 8 hours as well as minimizing the particle size, were set as a goal to select the optimized formulation using Design expert software version 10 (Stat-Ease, Inc., Minneapolis, USA).

Validation of experimental design

The formulation developed was evaluated for the response and the experimental values obtained were compared with those predicted by the mathematical models generated.

Stability study

The purpose of the stability study was to provide evidence of the quality of a drugs which varies with time under the influence of a various factors such temperature, humidity and light. Formulations F3, F6 and F9 were sealed in vials and stored at different temperatures such as 4±2°C, 25±2°C and at 60±2°C and relative humidity 75±5% RH as per ICH guidelines for 1 month. Results were expressed in terms of Drug content (Tiwari et al., 2018).

RESULTS AND DISCUSSION

FTIR spectrophotometric study

At the end of two weeks, the physical mixtures were observed for IR spectra. In the spectrum of 5FU, the strong stretching vibration of the -C=O was at 1728 cm^{-1}. Peaks at 1238 cm^{-1} and 1645 cm^{-1} correspond to the -C-F and -C=C stretching vibrations respectively. Similar peaks of DAP were found in the spectra of physical mixtures (Figure 1). In the spectrum of TTN -C=O peak shown at 1710.20 cm^{-1}, -OH peak at 2855.30 cm^{-1} and -C=C (Ar-H) peak at 1677.9 cm^{-1}. All vibrations respectively with the similar peaks of TTN were found in the spectra of physical mixtures, which revealed that there was not any type of chemical reaction between drug-drug and drug-excipients. The spectra of phospholipids showed -C=O bands at 1735.8 cm^{-1}, -C-O bands at 1075.55 cm^{-1} and (-CH) alkene stretching band at 2822.7 cm^{-1}. Similar peaks were found in the spectra of physical mixtures. From IR spectra, it was noted that there was no possible interactions between drugs and the other ingredients within the formulation and the presence of functional groups are within the range, so this may not affect the formulation stability during its shelf life (Fang et al., 2001).

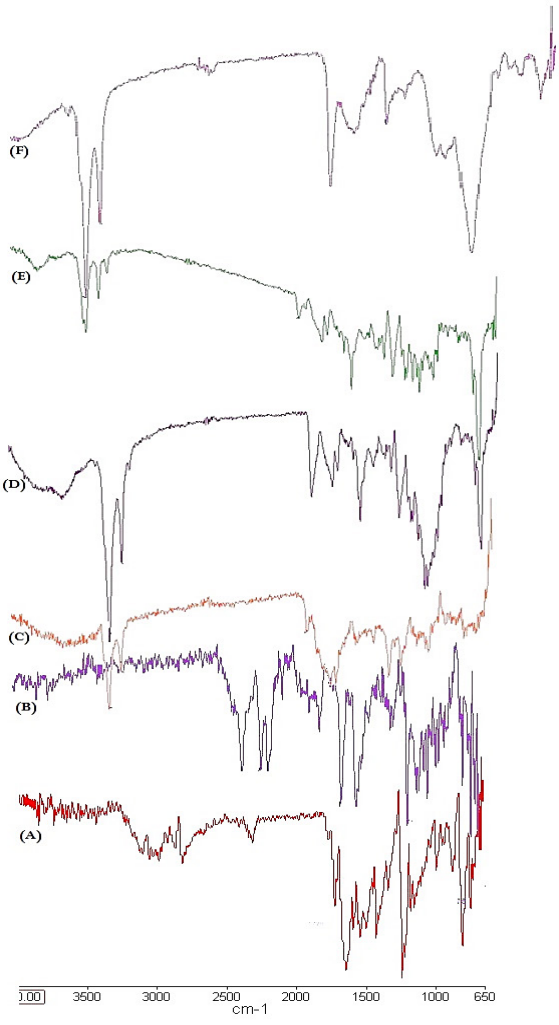

Figure 1. FTIR spectra of (A) 5FU, (B) TTN, (C) Physical mixtures of 5FU+TTN, (D) Soy lecithin, (E) Cholesterol, (F) TTN+5FU+Cholesterol+Soy lecithin

Evaluation of prepared liposomes

Liposome morphology was studied by TEM (Figure 2). Negative-strain TEM images showed that liposomes obtained were spherical shaped, which could have an impact on drug-release. No drug crystals were visible in TEM-images, regardless of the preparation technique or the loaded drug (Hitzman et al., 2006). The average vesicle size of the liposomes was found between 117-200 nm.

A £ value > ± 30 mV is essential for effective stability and to inhibits aggregation. As surface charge is chiefly generated by liposome constituting phospholipids, which were the same in all formulas, no significant difference was found between the different drug-loaded batches (detailed data are not given).

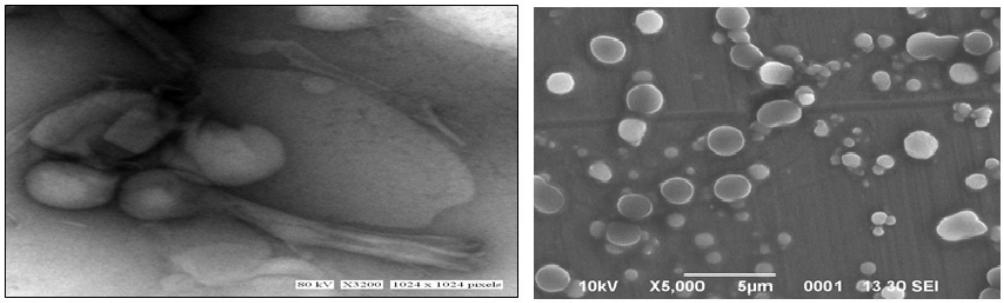

Figure 2. TEM images of formulations (A) F9, (B) F6.

All liposomes were negatively charged and zeta-potential values varied between −21.42 and −25.61 mV, which is considered as an optimal potential for assuring particle stability. It was observed that £ potential of prepared liposomes has sufficient charge to inhibit aggregation of vesicles (Juliano et al., 1998). The average percent drug entrapment efficiency of formulations ranges from 34.29 to 72.86% and 42.42 to 69.70% of 5FU and TTN respectively, where the Formulation F9 showed a maximum drug entrapment of 72.86% and 69.70% for 5FU and TTN respectively.

In Vitro release study of liposomes

An *in vitro* release study was performed on nine different formulations of drug-loaded liposomes. The studied formulas were selected in order to investigate the impact of phospholipid and cholesterol concentration on drug-release profile, these being the main parameters that influenced size and encapsulation efficiencies. *In vitro* drug release studies of drug-loaded liposomes were conducted for a period of 8 h using dialysis method, entire formulations were performed in skin pH to evaluate the release. The release profiles of liposomes of entire formulations were shown in (Figure 3). As the concentration of phospholipid was increased from 20 to 60 mg/ml, using the same cholesterol percentage with respect to phospholipid amount, the cumulative released amount of drugs decreased. The amphiphilicity confers phospholipids with self-assembly, emulsifying and wetting characteristics. It has been stated that the release of lipophilic agents from liposomes is delayed because of their location within the lipid bilayers. However, water-soluble drugs show relatively fast release. By performing the drug release studies for all nine formulations and from the data obtained it was found that the formulation F9 showed good release pattern. From the drug release profile of formulations F2, F5, F8 it was observed that drug release was found to be increased within 2 h and very less amount of drug was left for further release. Release profile of formulation F9 showed that almost entire drug was released within 7h. The higher amount of phospholipid in F9 leads to a time-dependent increase in diffusion coefficient is a highly lipophilic drug, should be entrapped within the phospholipid bilayers. Hence, the release mechanism involves slow diffusion through the liposome wall. No sudden release occurred during the release study, indicating that no liposome disintegration had taken place. *In vitro* dissolution studies showed that as the concentration of phospholipid was increased, drug release rate was decreased. Dissolution profiles of formulations F1 to F6 were not good because high amount of drug release (30.6 to 67.42%) at 2 h. The drug release profile of F9 showed a best fit to the desired control drug release profile among all the for-

Table 2. Release kinetics of 5FU and TTN

Formulations	Zero order (r^2)		First Order (r^2)		Hixson Crowell (r^2) 5FU		Higuchi (r^2)		Korsemeyer- peppas			
							TTN					
	5FU	TTN	5FU	TTN	5FU	TTN	5FU	TTN	(r^2)	n	(r^2)	n
F1	0.994	0.997	0.928	0.974	0.968	0.987	0.963	0.951	0.995	1.10	0.997	1.10
F2	0.975	0.994	0.985	0.974	0.993	0.984	0.978	0.944	0.984	1.10	0.993	1.23
F3	0.983	0.989	0.965	0.968	0.980	0.978	0.953	0.931	0.988	1.19	0.997	1.32
F4	0.983	0.966	0.969	0.935	0.989	0.947	0.972	0.996	0.989	1.17	0.974	1.37
F5	0.987	0.961	0.975	0.934	0.990	0.946	0.980	0.876	0.992	1.03	0.965	1.35
F6	0.970	0.968	0.986	0.952	0.988	0.960	0.974	0.899	0.983	1.11	0.976	1.41
F7	0.957	0.967	0.975	0.957	0.971	0.963	0.961	0.909	0.981	1.13	0.953	1.33
F8	0.997	0.964	0.975	0.928	0.986	0.942	0.958	0.878	0.996	1.11	0.985	1.31
F9	0.988	0.985	0.963	0.951	0.976	0.970	0.937	0.932	0.985	1.16	0.991	1.46

mulations. The results of drug release profile of the F9 showed the release of 32.48% of drug during initial 2h. While within the first 4 h 61.1% of drug was released and the remaining drug was released during last 4 h. The formulation F7 also showed controlled release pattern but significantly less amount of drug was released from them, so batch F7 was not considered for further study. After evaluating all the parameters the liposomal formulations were tested for their stability studies by subjecting them to different temperatures for a period of one month and were found to be more stable at 4^0C and destabilized at high temperature. Thus, liposomes were successfully incorporated both hydrophilic and hydrophobic drugs and it can be further used for formulation development.

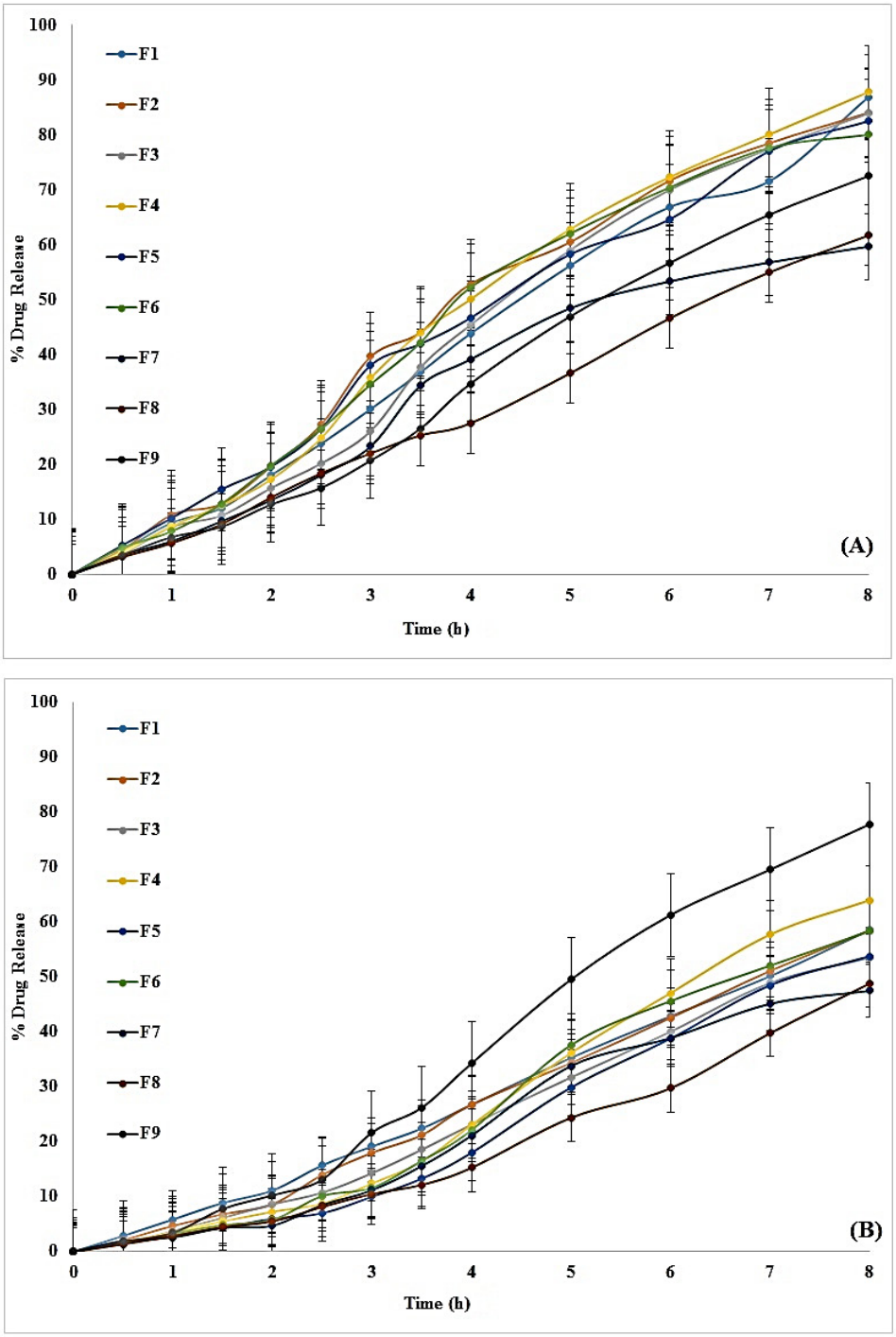

Figure 3. Drug Release profiles of (A) 5FU and (B) TTN

Evaluation of release kinetics parameters

The *in vitro* drug release data from different formulations were evaluated kinetically using various mathematical models like zero order, first order, Higuchi, and Korsemeyer–Peppas to know the mechanism of release from liposomes. Highest correlation coefficient (r^2) value was judged to be the more appropriate model for dissolution data. The results of the curve fitting into these above mentioned mathematical models were given in (Table 2). According to the result obtained by release kinetics liposomal optimized formulation from F9 followed zero order release pattern and showed super case-II transport mechanism. This suggested the development of pores and channels and thus facilitating solvent penetration and elevation of drug release and it was reported that zero order release is ideal for transdermal drug delivery as it provide constant release of drug over an extended period of time and reflect improvement in therapeutic index (Tiwari et al., 2018).

Influence of phospholipid concentration on vesicle size and encapsulation efficiency

Phospholipid concentration showed a positive effect on liposome encapsulation efficiencies. The influence of the phospholipid concentration on encapsulation efficiency and vesicle size has been studied. It was found that when phospholipid concentration was increased from 40 to 60 mg/ml, 5FU encapsulation efficiencies were nearly doubled (from 34.29 to 72.86 %) (Figure 4). Similar encapsulation results have been obtained for the TTN efficiency of liposomes was found to be in the range of 42.42 to 69.70%. The positive effect of phospholipid concentration on the encapsulation efficiency of hydrophilic drugs has been previously reported (Kesisoglou et al., 2005). Whereas their mean vesicle size ranged from 117 to 200 nm. Encapsulation results may indicate the high association of the drug with phospholipid bilayers of liposomes with the increase of lipid surface and bilayer number. It has been reported that the thickness of the lipid surface of liposome increases with the increasing amount of phospholipid used in the formulation (Ali et al., 2007). Similar results have been obtained for other lipophilic agents (Fang et al., 2001).

Optimization of liposome formulation using 3^2 full factorial design

Statistical analysis was done by Design expert software version 10 (Stat-Ease, Inc., Minneapolis, USA) and the second order polynomial equations were derived. A 3^2 full factorial design was constructed to study the effect of TTN concentration (X1) and phospholipid concentration (X2) on the drug release from liposomal preparation. The percentage entrapment efficiency at pH 6.8 (R1) and percentage drug release (R2) was selected as dependent variables. The main effects (X1 and X2) represent the average result of changing one factor at a time from its low to high value. The statistical model incorporating interactive and polynomial terms was utilized to evaluate the responses. The interaction terms (X1, X2) showed how the response changes when 2 factors are changed simultaneously. The full Equation (equation containing only statistically significant terms) is then used for drawing plots to visualize the impact of changing variables at a glance. The optimum point may be identified from the plot.

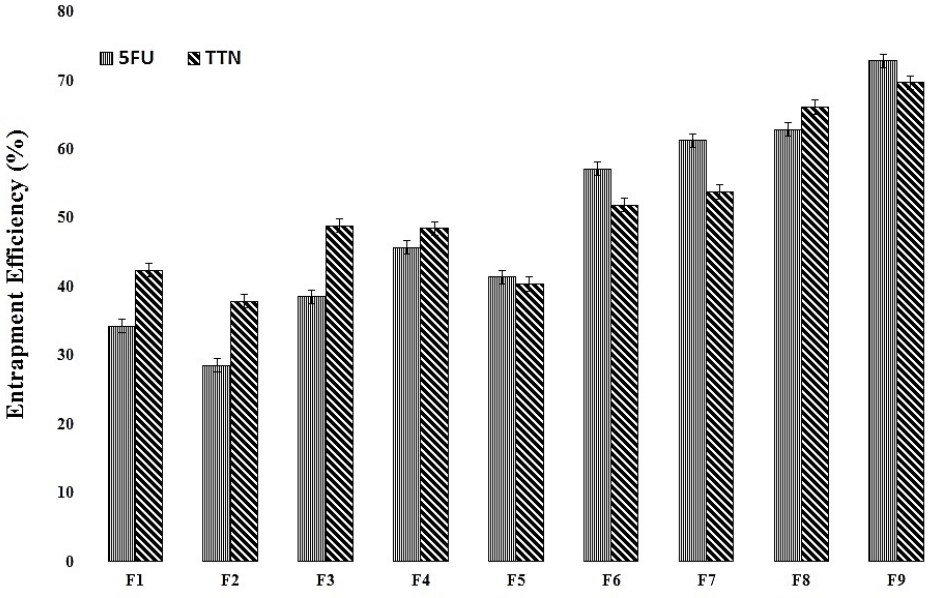

Figure 4. Entrapment efficiency of formulations F1-F9.

The 3D surface plot clearly depicted the effects of independent variables i.e. %EE (R1) and %DR (R2). The Response Surface linear model generated for X1 and X2 was found to be significant with an F-value of 2.50 and 6.80 (P<0.0500) respectively (Figure 5). The factorial equation for R1 (Eq 1) and R2 (Eq 2) was found to be:

$$R1 = 57.87 + 0.47x1 + 0.47x2 + 0.67x1\text{-}2 + 0.67x1 + 0.67\,x2^2 \quad \text{Eq 1}$$

$$R2 = 51.07 + 0.47x1 + 0.47x2 + 0.67x1x2 + 0.67x1 + 0.67x2^2 \quad \text{Eq 2}$$

The coefficient of X1 and X2 was found to be positive indicated that predicted values could be obtained when the concentration of TTN and phospholipid increased. The P value for variable X1 and X2 was 0.0231 and 0.0112 respectively (P<0.0500) indicated that both the independent variables show a significant effect on dependent variable i.e. R1 and R2.

The results of experimentally observed responses and those predicted by mathematical models along with the percentage prediction errors were compared. The prediction error in the response parameters ranged between 0.47 and 0.79% to the value of the absolute error of 0.90±0.70% (Figure 6). The low values of error indicate the high prognostic ability of factorial equation and counter plot methodology. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 7.56 indicates an adequate signal. Thus, this model can be used to navigate the design space (Tiwari et al., 2017). Figure 7 showed the interaction between X1 and X2. From the plot, the spread of points on the right side of the graph (where X1 is low) is larger than the spread between the points at the left side of the graph where X1 is high. In other words, the effect of X2 is less significant where X1 is high (Gregoriadis et al., 1979). Therefore, at a very high X1 value, the effect of phospholipid concentra-

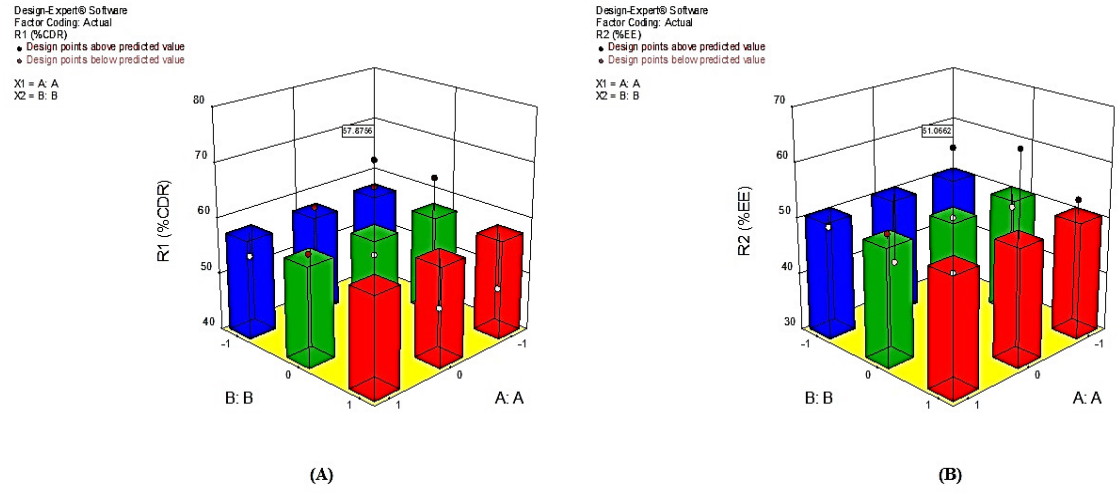

Figure 5. 3D desirability plot of (A) R1:%CDR (% cumulative drug release), (B) R2:%EE (% Entrapment efficiency).

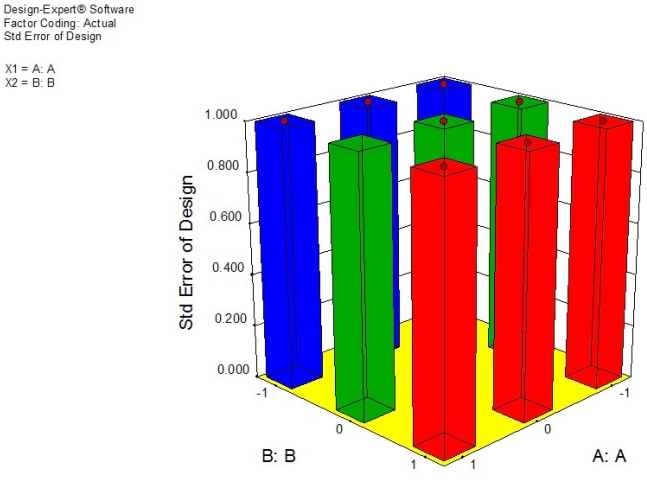

Figure 6. 3D desirability plot for standard error

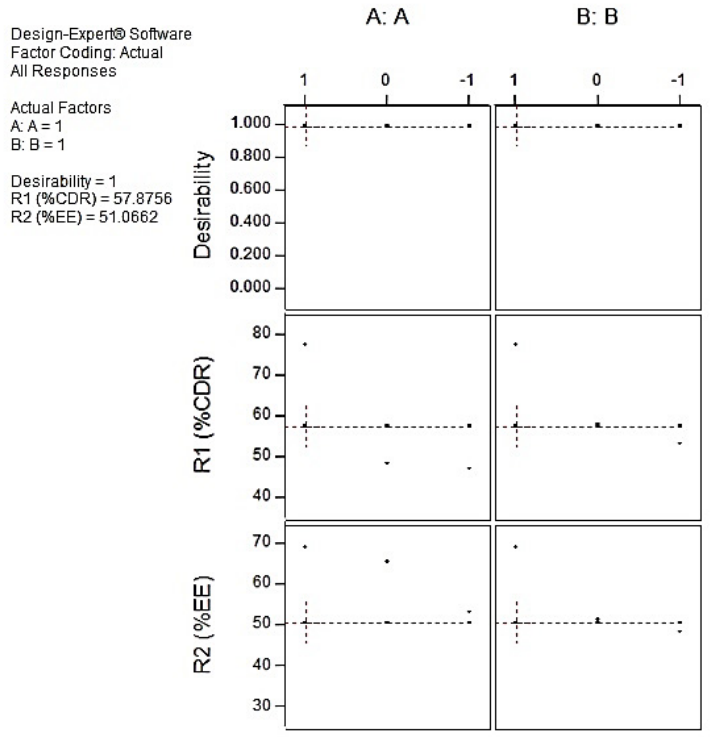

Figure 7. Interaction plot between R1, R2.

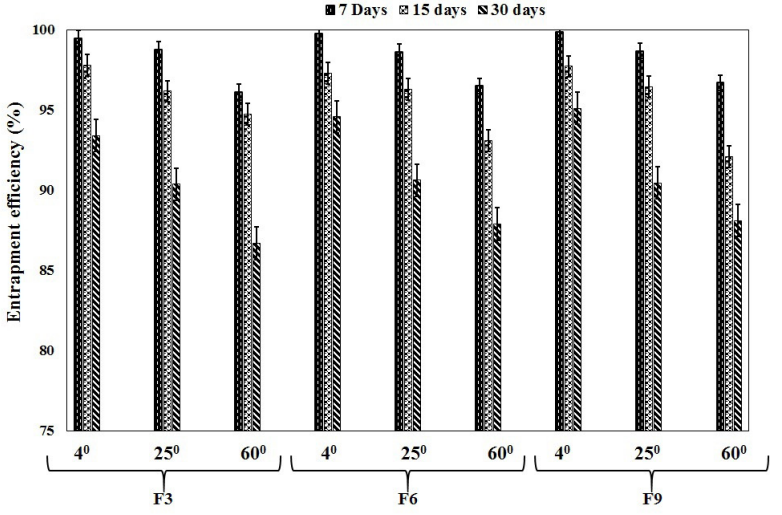

Figure 8. Stability study of F3, F6 and F9.

tion can be significantly reduced, thus reducing the R1 and R2. Also, there exists generic increase in R1 and R2 as phospholipid significantly decreases. The 3D plots (Figure 5) indicated that response R1 increases with a decrease in X1 and X2. Higher X1 and X2 reduced response R1. Simultaneous effect of interactive factors X1 and X2 was observed in the 3D plot for response R2. Formulations with high X1 and X2 showed highest desirability factor of 1.00 with highest R1 and R2 was selected as optimized formulation (Figure 7). Ramps report clearly supported optimized formulation. After generating the polynomial equation for the dependent and independent variables, the combination was optimized for responses. Upon comprehensive evaluation of feasibility search and subsequently grid searches, the formulation composition with high fulfilled the maximum desired requisites. The prepared liposome formulation of F9 showed R1 as 31.49% and R2 as 98.85%.

Stability study

A stability study was performed on randomly selected three liposome formulations, in which phospholipid amounts were varied. Liposomes were stored under static conditions 4^0C, (room temperature) 25^0C and at high temperature 60^0C in the glass over a period of one month. Results were expressed in terms of entrapment efficiency. The entrapment of all formulations at 4^0C does not show any significant decrease, followed by the formulations kept at 25^0C whereas the formulations kept at 60^0C showed a significant loss in the drug entrapment (Figure 8). A study showed that liposomes were not stable at high temperature and caused loss of drug content. As a result of stability studies, it can be concluded that the formulations are most stable when stored at lower temperature i.e. 4^0C (Tiwari et al., 2017).

CONCLUSION

Liposomal carriers were found to be stable at room temperature and advocated as an efficient topical delivery system to deliver 5 FU and TTN with higher entrapment efficiency than reference liposomal vesicles. *In vitro* release study confirmed the uniform and sustained release of drugs via liposomal carriers. The liposomal phospholipids (also one of the natural constituent of skin lipids) helped generating and retaining the required physico-chemical state of the skin for enhanced permeation of the 5FU and TTN. Further, the phospholipid-rich domains of vesicles might have helped to produce the depot effect for drug molecules. The latter has been reflected as higher amount of drug retention within the skin layers in case of liposomal formulations.

Thus, liposomal formulations, with desired characteristics for topical administration, could be successfully prepared and optimization was advocated by 3^2 factorial design and maximum desirability value. F9 liposomes was optimized and have shown an appreciably enhanced skin permeation as well as release of drug molecules in the skin.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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