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Preparation, Optimization, and Characterization of Topotecan Loaded PEGylated Liposomes Using Factorial Design

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This study reports the development of liposomal system for a potent antitumor drug, topotecan. To achieve this goal conventional and PEGylated liposomes were prepared according to a factorial design by hydration method followed by extrusion. Parameters such as type of lipid, percentage of cholesterol, percentage of phosphatidylglycerols, percentage of polyethylene glycol (PEG)-lipids, and drug to lipid molar ratio were considered as important factors for the optimizing the entrapment and retention of topotecan inside the liposomes. The size and zeta-potential of the PEGylated and conventional liposomes were measured by particle size analyzer and zeta-potentiometer, respectively. The stability and release characteristics of PEGylated liposome loaded topotecan were compared with conventional liposomes and free topotecan.

The optimized PEGylated [distearoyl phosphatidylcholine (DSPC)/cholesterol/ distearoyl phosphatidylglycerol (DSPG)/ distearoyl phosphatidylethanolamine-PEG_{2000} (DSPE-PEG_{2000}); 7:7:3:1.28] and related conventional [DSPC/cholesterol/DSPG; 7:7:3] liposomes showed a narrow size distribution with a polydipersity index of 0.15 and 0.10, an average diameter of 103.0 ± 13.1 and 95.2 ± 11.10 nm, and with drug loading of 11.44 and 6.21%, respectively. Zeta-potential was −10 ± 2.3 and −22 ± 2.8 mV for PEGylated and conventional liposomes, respectively. The results of stability evaluation showed that the lactone ring of topotecan was notably preserved upon liposome encapsulation. PEGylated liposomes containing topotecan showed a significant decrease ($P < 0.001$) in release rate in comparison with conventional leptosomes. These results indicate the suitability of PEGylated liposomes in controlling topotecan release.

The prepared liposomes (especially PEGylated liposomes) as those described here may be clinically useful to stabilize and deliver topotecan for the treatment of cancer.

Keywords topotecan; PEGylated liposome; conventional liposome; factorial design; release kinetics

INTRODUCTION

Topotecan (TPT, 9-dimethylaminomethyl-10-hydroxy-camptothecin) (Figure 1), a hydrophilic analog of camptothecin (CPT) is one of the two CPT analogues currently approved for clinical use by the US Food and Drug Administration (Pizzolato & Saltz, 2003). Topotecan is used for the treatment of ovarian and small-cell lung cancer after failure of first line chemotherapy (Broom, 1996; Herzog, 2002), and is increasingly being combined with other standard chemotherapeutic agents for improved therapy (Dunton, 1997; Emerson, 2000). TPT is a cell cycle-specific drug (Hertzberg et al., 1989; Kingsbury, Boehm & Jakas, 1991) and therefore, it is advantageous to expose tumor cells to the drug for a prolonged period. Unfortunately, TPT is extremely unstable in physiological conditions and undergoes a pH dependent rapid and reversible hydrolysis from closed lactone ring to the inactive carboxylated form with loss of antitumor activity (Fassberg & Stella, 1992; Hochster, Liebes & Speyer, 1994).

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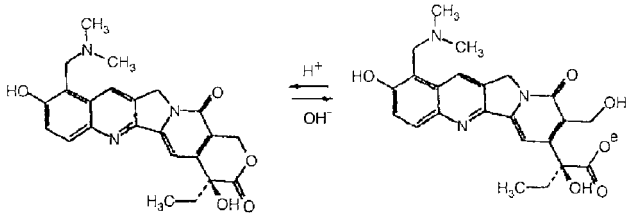

FIGURE 1. Chemical structure of the lactone and carboxylate forms of topotecan (Biloti et al., 2003).

Liposomes have previously been used as carriers for anticancer drugs, and they have been shown to reduce side effects, such as anthracycline-induced cardiomyopathy (Gabizon, Goren, Cohen & Barenholz, 1998). Liposomes can also provide slow release of an encapsulated drug, resulting in sustained exposure to tumor cells and enhanced efficacy (Chang et al., 1997; Lim, Masin, Madden & Bally, 1997; Webb, Harasym, Masm, Bally & Mayer, 1995). In the case of camptothecins including TPT, besides these advantages, the properties of drug, i.e. S phase-specific cytotoxicity and fast inactivation at physiological pH, make it worthwhile to develop liposomal formulation. Previous works have shown that complex of CPTs with lipid vesicles, composed of DMPC or DMPG, can stabilize its lactone moiety and thereby prevent drug inactivation (Burke, Mishra, Wani & Wall, 1993). Recent studies have demonstrated that specific type of liposomes including formulations containing a small fraction of polyethylene glycol (PEG) derivatized phospholipid can circulate in the blood for prolonged periods of time and may alter dramatically the release properties and pharmacokinetic properties of drugs (Daemen et al., 1997; Gabizon et al., 1994; Unezaki et al., 1995).

Hydration method followed by extrusion is quite simple, rapid, inexpensive and highly reproducible and therefore, is commonly used for liposome preparation. Concerning this preparation method parameters such as type of lipid, percentage of cholesterol, percentage of phosphatidylglycerols, percentage of PEG-lipids, and drug to lipid molar ratio have been widely considered as important factors for the optimizing the entrapment of drugs (especially hydrophilic and amphiphilic drugs) inside the liposomes (Gregoriadis, 1988).

Due to strong rational for preparation of topotecan liposomal formulation and considering its amphiphilic characteristics we attempted to optimize abovementioned variables for obtaining TPT liposomes with the best possible drug entrapment. One of the important parameters that were selected for this study was PEG-lipid percentage.

As a first step, by means of a factor analysis, we evaluated the possible synergic action of all of these parameters, present together and forming liposomes at two different levels. A factor analysis performed on the formulation parameters obtained for 16 different liposomes, could find the optimum combination useful for practical application. We also investigated the effect of PEG coating on stability and release characteristics of TPT liposomes.

MATERIAL AND METHODS

Chemicals

Topotecan was obtained from Ohua Pharmaceutical Technology (China); 1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine (DSPC), 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), 1,2-Distearoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DSPG), 1,2-Dimyristoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DMPG), and 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Carboxy(Polyethylene Glycol)2000] (DSPE-PEG_{2000}) was purchased from Lipoid GmbH (Switzerland). Cholesterol, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and TritonX-100 were supplied from Merck (Darmstadt, Germany). Cellulose dialysis tubing (12000 MWCO) was supplied by Biogen (USA).

Liposome Preparation

Small unilamellar vesicles (SUV) were prepared by hydration method (Hope, Bally, Webb & Cullis, 1985). Lipids containing DSPC and/or DMPC/cholesterol/DSPG and/or DMPG/DSPE-PEG_{2000} were dissolved in chloroform: methanol (5:1). The chloroform and methanol were removed under a high vacuum for 5 min at 60°C. Dried lipid films were hydrated with different concentration of TPT in PBS pH 5 at 60°C to achieve a final lipid concentration of 10 mg/ml. Following hydration the multilamellar vesicles (MLVs) were extruded 10 times through stacked polycarbonate filters with 0.1 μm pore size at 60°C using a water-jacketed Extruder™ (Northern lipids, Vancouver BC, Canada). Unencapsulated TPT was removed from the preparation by dialysis.

Experimental Design

Based on preliminary and literature study of the effect of parameters on the drug loading of liposomes, the experiments were performed by hydration method using a fractional factorial design. We used Design-expert version 6 software for this experiment. Five independent variables were taken at its two levels: low and high, which were represented by transform values of −1 and +1 respectively. The independent variables were type of lipid (X_1), molar ratio of cholesterol to main lipid (X_2), molar ratio of phosphatidylglycerol to main lipid (X_3), mole percent of DSPE-PEG_{2000} (X_4), and drug to lipid molar ratio (X_5). Values of these selected variables are shown in Table 1. The prepared batches were evaluated for percentage of drug entrapment (PDE), as dependent variable. Sixteen batches of liposomes were prepared according to experimental design shown in Table 2. Fitting a multiple linear regression model to

TABLE 1
Coded Units of Factorial Design for Preparation of Topotecan Liposomes by Hydration Method

	Levels	
Variables	Low	High
X_1 (type of lipid)	DMPC	DSPC
X_2 (molar ratio of cholesterol to the main lipid)	0.5 : 1	1 : 1
X_3 (molar ratio of phosphatidylglycerol to main lipid)	1 : 9	3 : 7
X_4 (mole percent of DSPE-PEG_{2000})	0	7
X_5 (drug to lipid molar ratio)	1 : 60	1 : 30
Transformed values	–1	1

the factorial design gave a predictor equation which was a first-order polynomial, having the form:

$$\begin{aligned} Y = {} & a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_5X_5 \\ & + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{15}X_1X_5 \\ & + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{25}X_2X_5 + a_{34}X_3X_4 \\ & + a_{35}X_3X_5 + a_{45}X_4X_5 \end{aligned} \quad (1)$$

where Y is the dependent variable (PDE) while a_i, and a_{ij} represent the regression coefficients for the first order polynomial and X_i represents the levels of the independent formulation variables.

Statistical analysis of results was performed using analysis of variance (ANOVA) and regression coefficients of all factors and interaction terms were calculated (El-Gibaly & Abdel-Ghaffar, 2005).

Characterization

Particle Size Analysis

Size distribution of liposomes was monitored by photon correlation spectroscopy using a Coulter Model N4SD submicron particle analyzer (Coulter Electronics, FL). The analysis was performed at a scattering angle of 90° and at a temperature of 25°C. Mean diameters and standard deviations were derived using software supplied by the manufacturer.

Zeta Potential Measurement

Zeta potentials were determined using 90 PLUS particle size analyzer with ZETA PALS system, Brookhaven Corp. (Hostville, NY) at 25°C. Liposomes were prepared in N-2-hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES) buffer without NaCl. The analysis was performed at 25°C using 40 µl of samples diluted with 1.3 ml HEPES buffer without NaCl.

Estimation of Entrapped Topotecan

Topotecan loaded within liposomes was estimated after removing un-entrapped topotecan by dialysis at 4°C for 24 h against 100 volumes of PBS pH 7.4 with two times changes the external buffer. Liposomes were disrupted by adding 0.1 ml acidic methanol (1% perchloric acid in methanol) and 10 µl of

TABLE 2
Experimental Design and Percent of Percent of Drug Entrapment (PDE)

Run	X_1: Coded Value, Mole	X_2: Coded Value, Mole	X_3: Coded Value, Mole	X_4: Coded Value, Mole	X_5: Coded Value	PDE
1	–1, 9	–1, 4.5	–1, 1	–1, 0	1	0.84
2	1, 9	–1, 4.5	–1, 1	–1, 0	–1	9.96
3	–1, 9	1, 9	–1, 1	–1, 0	–1	2.84
4	1, 9	1, 9	–1, 1	–1, 0	1	3.76
5	–1, 7	–1, 3.5	1, 3	–1, 0	–1	2.43
6	1, 7	–1, 3.5	1, 3	–1, 0	1	6.70
7	–1, 7	1, 7	1, 3	–1, 0	1	4.31
8	1, 7	1, 7	1, 3	–1, 0	–1	7.61
9	–1, 9	–1, 4.5	–1, 1	1, 1.09	–1	9.65
10	1, 9	–1, 4.5	–1, 1	1, 1.09	1	2.36
11	–1, 9	1, 9	–1, 1	1, 1.43	1	3.67
12	1, 9	1, 9	–1, 1	1, 1.43	–1	0.68
13	–1, 7	–1, 3.5	1, 3	1, 1.02	1	4.47
14	1, 7	–1, 3.5	1, 3	1, 1.02	–1	7.27
15	–1, 7	1, 7	1, 3	1, 1.28	–1	5.93
16	1, 7	1, 7	1, 3	1, 1.28	1	11.44

TritonX-100 20% to 0.1 ml of drug-loaded liposomes. The total concentration of topotecan was analyzed by our previously reported HPLC method (Vali, Shafaghi & Dadashzadeh, 2005). Briefly, the HPLC system consisted of a model 510 solvent delivery equipped with a model 474 fluorescence detector (Waters Assoc., MA). The analytical column was Novapack C_{18} column and the mobile phase was a mixture of 0.05 M ammonium acetate, acetonitrile and triethylamine (84:16:1.50, v/v) containing tetrabutyl ammonium hydrogen sulfate (2 mM) adjusted to pH 5 with hydrochloric acid. The amount of drug entrapment in liposomes was calculated using the following equation:

$$\begin{aligned}\%\ \text{Drug entrapment efficiency} = (&\text{amount of topotecan in liposomes} / \\ &\text{total amount of topotecan used in liposome preparation}) \times 100\end{aligned} \quad (2)$$

Here, the amount of initially added drug was regarded as that of total drug (Zhang et al., 2005) because the drug loss in the preparation process was negligible.

Stability of Liposomes

Physical Stability of Liposomes. The physical stability of liposome drug product is a function of the integrity and the size distribution of the lipid vesicles. Liposomes are susceptible to fusion and aggregation and drug leakage during storage. Small unilamellar vesicles are more susceptible to size changes than are multilamellar vesicles. Therefore, the physical stability of liposomal topotecan kept at 4°C was evaluated by monitoring drug leakage for 1 week by removing portions of liposomes from a pool stored at 4°C at various time point for 1 week and by monitoring particle size changes at two time points (time 0 and 14 days).

Topotecan Stability During Liposome Preparation. During liposome preparation, dried lipid film was hydrated with topotecan in PBS pH 5 at 60°C for 4 hours by agitation. After incubation of topotecan in PBS with dried lipid film at 60°C, aliquots of 50 µl were removed at different times (0, 1, 2, 3, and 4 h). Also, one sample was taken after extrusion. The liposome samples were disrupted as described previously and TPT was analyzed by the HPLC method.

Stability of Topotecan Lactone Form After Loading into Liposomes. To determine the effect of liposomal formulation on the stability of topotecan lactone form, the lactone ring opening rate for free topotecan and liposomal topotecan as a result of hydrolysis were evaluated. A drug concentration of 50 ng/ml as free topotecan and liposomal topotecan were prepared in plasma. At time points of 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h, 100 µl of aliquots were withdrawn and mixed with 100 µl of cold methanol and 10 µl of 20% TritonX-100. The mixture was diluted and injected directly into HPLC for analysis.

In Vitro Drug Release

The *in vitro* release of topotecan from liposomes and free drug (as control) was determined by dialysis method using Franz diffusion cell (Franz, 1968). Cellulose acetate membrane with a molecular weight cut-off of 12 KDa was used as dialysis membrane. Liposomes at a concentration of 10 mg/ml total lipid, were diluted in human plasma and/or PBS buffer pH 7.4. Diluted liposomal solutions (2 ml) were placed in donor chamber and dialyzed against 30 ml of PBS buffer pH 7.4 and/or human plasma at 37°C, under magnetic stirring. At specific time intervals (up to 48 h), 100 µl of medium was taken and replaced with the same volume of fresh PBS buffer pH 7.4 and/or plasma. For assay of total form of topotecan, the taken samples were treated with 1% perchloric acid and acidic methanol before HPLC analysis. The concentration of released topotecan was determined by HPLC. All dissolution tests were run in triplicate and mean values were reported.

Kinetic Analysis of Release Data

The release kinetics of topotecan from liposome formulations was investigated using data corresponding to the first 60 to 70% of drug released according to the following mathematical models:

Ritger–Peppas Model

This model is often applied to describe drug release from the systems of various geometries by using following simple exponential relation:

$$M_t / M_\infty = Kt^n \quad (3)$$

Where M_t/M_∞ denotes the fraction of drug released at time t, K the release rate constant characteristic of the controlled-release device and n the diffusional exponent, indicative of the mechanism of drug release. In spherical carriers, if $n \leq 0.43$, a Fickian diffusion, $0.43 \leq n < 0.85$, a non-Fickian transport and $n \geq 0.85$, a zero order drug release mechanism dominates (El-Gibaly & Abdel-Ghaffar, 2005; Ritger & Peppas, 1987).

Higuchi Model

The Higuchi model is described by following equation:

$$M_t / M_\infty = K_H\ t^{1/2} \quad (4)$$

where K_H is the Higuchi rate constant (Siepmann & Peppas, 2003).

The model parameters with their standard errors and descriptive statistics of regression for each model were estimated by the non-linear regression module of Sigma plot. The

derived parameters of the models were employed for the pair wise comparison of the profiles using one way ANOVA with Tukey post test.

RESULTS AND DISCUSSION

The liposome was adopted as a promising delivery system because its organized structure could accommodate drugs, depending on their solubility characteristics, in both the aqueous and lipid phases. Topotecan, a semisynthetic water-soluble camptothecin analogue, has shown promising antitumor activity in preclinical and clinical studies of ic solid tumors and leukemia (Santana et al., 2003). In order to increase antitumor efficacy, while reducing systemic side effects, TPT loaded liposomes were prepared and characterized in the present study. In addition, loading TPT in liposomes is expected to protect the active lactone form of the drug from rapid hydrolysis to inactive carboxylate form in physiological pHs (Biloti, Santana & Pessine, 2003).

Liposome Preparation

From a pharmaceutical point of view, optimum liposome preparations would avoid the use of organic solvents and detergents (which are difficult to remove), would yield well-defined and reproducible vesicles, and would be rapid and amenable to scale-up procedures (Cullis, Hope, Bally, Madden & Mayer, 1987). The introduced extrusion procedure after hydration method appears to satisfy most of these demands. The considerable advantages of this technique include: it is rapid (preparation time about 10 min), it works directly from MLV, it can be applied to all liquid crystalline bilayer lipids and lipid mixtures, and it yields a homogenously sized, reproducible product (Cullis et al., 1987).

The drug entrapment of hydrophilic drugs within liposomes, prepared by hydration method may be affected by different factors including lipid composition, percentage of each lipid component, and drug to lipid molar ratio (Cullis et al., 1987; Papahadjopoulos, 1999). Most of the early studies on the in vivo disposition of liposomes had used compositions of neutral phospholipids such as phosphatidylcholine or sphingomyelin, mixed with varying amounts of cholesterol and some times including a small percentage of an acidic phospholipid. Cholesterol at a high mole ratio was added to increase the stability of liposomes in the presence of plasma and the negative charge in order to avoid aggregation and also to increase encapsulation efficiency (Papahadjopoulos, 1999).

Because of abovementioned reasons, hydration method followed by extrusion was selected for liposome preparation. A fractional factorial design taking five prime selected formulation variables at their two different levels, affecting the eminence of liposomes was used to design the experimental batches for the preparation of topotecan liposomes. The selection of factors and levels in the design, which most affect on drug entrapment were based on the results of preliminary and literatures study (data not shown). Sixteen batches of different combination were prepared by taking values of selected variables: type of lipid (X_1), molar ratio of cholesterol to main lipid (X_2), molar ratio of phosphatidylglycerol to main lipid (X_3), mole percent of DSPE-PEG_{2000} (X_4), and drug to lipid molar ratio (X_5) at two levels as shown in Table 2. The prepared batches were evaluated for percentage of drug entrapment, a dependent variable and results are recorded in Table 2.

Mathematical modeling of the preparation of topotecan liposomes was carried out by using equation 1 to obtain a polynomial equation.

Transformed values of independent variables, X_1 to X_5, and its products as in equation 1 along with PDE values (dependent variable) were subjected to multiple regression to determine the coefficients (a_0, a_i, a_{ij}) and the P-values of each term of the equation. A polynomial equation was derived by substituting the values of a_0, a_i, and a_{ij} in equation 1. This equation represents a full model (Eq. 5).

$$\begin{aligned} Y = {} & 5.25 + 0.98X_1 - 0.21X_2 + 1.03X_3 \\ & + 0.44X_4 + 0.55X_5 - 0.13X_1X_2 + 1.01X_1X_3 \\ & - 1.22X_1X_4 - 0.39X_1X_5 + 1.27X_2X_3 - 0.04X_2X_4 \\ & - 1.32X_2X_5 + 0.57X_3X_4 - 1.01X_3X_5 - 0.35X_4X_5 \end{aligned} \quad (5)$$

Small values of coefficients of terms X_2, X_4, X_1X_2, X_1X_5, X_2X_4, and X_4X_5 (having $P > 0.05$) in Eq. (5) are regarded as least contributing in the preparation of topotecan liposomes by hydration method. Hence, these terms were neglected from full model considering non-significant and a reduced polynomial equation (equation 6) obtained following multiple regression of percent drug entrapment and significant terms ($P < 0.05$) of Eq. (5).

$$\begin{aligned} Y = {} & 5.25 + 0.98X_1 + 1.03X_3 + 0.55X_5 \\ & + 1.01X_1X_3 - 1.22X_1X_4 + 1.27X_2X_3 \\ & - 1.32X_2X_5 + 0.57X_3X_4 - 1.01X_3X_5 \end{aligned} \quad (6)$$

Results of ANOVA of full model and reduced model was carried out and then F-statistic was applied to check whether the non-significant terms can be omitted or not from the full model (Table 3). Based on the results of ANOVA, for the formation of high drug loading liposomes the optimum setting was significantly ($P < 0.001$) affected by type of lipid (X_1), molar ratio of phosphatidylglycerol to main lipid (X_3), drug to lipid molar ratio (X_5), interaction of type of lipid-molar ratio of phosphatidylglycerol to main lipid (X_1-X_3), type of lipid-mole percent of DSPE-PEG_{2000} (X_1-X_4), molar ratio of cholesterol to lipid-molar ratio of main lipid to phosphatidylglycerols (X_2-X_3), molar ratio of cholesterol to lipid-drug to lipid molar ratio (X_2-X_5), molar ratio of phosphatidylglycerol to main lipid-mole percent of DSPE-PEG_{2000} (X_3-X_4), and molar ratio of

TABLE 3
Analysis of Variance for a Fractional Factorial Design for Evaluation of the Effect of Dependent Variables on the Topotecan Loading in Liposomes

Source	*SS*	*df*[a]	*MS*	*F* Value	*p*-Value
Model	160.65	14	11.47	458.21	0.0366
X_1	15.31	1	15.31	611.44	0.0257
X_2	0.74	1	0.74	29.44	0.1160
X_3	16.82	1	16.82	671.64	0.0246
X_4	3.08	1	3.08	122.86	0.0573
X_5	4.85	1	4.85	193.56	0.0457
X_1X_2	0.29	1	0.29	11.58	0.1820
X_1X_3	16.23	1	16.23	648.14	0.0250
X_1X_4	23.98	1	23.98	957.62	0.0206
X_1X_5	2.47	1	2.47	98.50	0.0639
X_2X_3	25.68	1	25.68	1025.60	0.0199
X_2X_4	27.68	1	27.68	1105.20	0.0191
X_3X_4	5.16	1	5.16	206.18	0.0443
X_3X_5	16.38	1	16.38	654.09	0.0249
X_4X_5	1.98	1	1.98	79.04	0.0713
Residual	0.025	1	0.025		
Corrected Total	160.67	15			

The Model *F*-value of 458.21 implies the model is significant.
[a]degree of freedom.

phosphatidylglycerol to main lipid-drug to lipid molar ratio (X_3-X_5). The main effects of X_1, X_2, X_3, X_4, and X_5 represent the average result of changing one variable at a time from its low to high value. The interactions show how the percentage of drug entrapment changes when two or more variables were simultaneously changed.

Interaction graphs were established using reduced polynomial equation 6. Significant interaction between the independent variables in equation 6 is shown in Figures 2–6. In each Figure, two independent variables were computed at prefixed values of percent of drug entrapment and the other independent variables were fixed at level 0 for nominal variables and 1 and −1 for categorical variable (X_1).

The plots of the X_2X_5 and X_2X_3 interactions in Figures 2 and 3 show that in case of DSPC or DMPC, the PDE is sensitive to cholesterol percentage for both high and low level of lipid to drug molar ratio as well as PG to lipid molar ratio. It was elucidated from Figures 2 and 3 that at high level of lipid to drug molar ratio (Figure 2) and low level of PG to lipid molar ratio (Figure 3) maximum PDE could be obtained if the low level of cholesterol to main lipid molar ratio was used (0.5:1; $P < 0.01$). On the contrary, at low level of lipid to drug molar ratio and high level of PG to lipid molar ratio, the higher PDE value was resulted by increasing the amount of cholesterol ($P < 0.01$).

The X_1X_4 interaction graph is shown in Figure 4A. It was deduced that the solidity of the main lipid was very important for improving the PDE of conventional liposomes (without PEG), but did not have a major impact on the PDE value of the PEGylated liposomes.

As shown in Figure 4B, changing the acyl chain of lipid was important in combination with using high level of PG, but with using low level of PG in liposome formulation, changing the length of acyl chain had no impact on PDE. For DSPC based liposome formulations (Figures 4B and 5A), the maximum PDE was obtained by using higher amount of PG at either low or high level of drug to lipid molar ratio.

The plot of the X_3X_5 interaction graph in Figure 5B shows that for DMPC based liposome formulations containing low level of PG, using the high lipid to drug molar ratio had significant effect on PDE but for DMPC based liposomal formulations containing high level of PG, changing lipid to drug molar ratio had no impact on PDE.

The X_3X_4 interaction plotted in Figures 6A and 6B confirm that for both conventional and PEGylated liposomes prepared from DSPC as the main lipid (Figure 6A) PDE increases with using higher amount of PG in the formulation. For conventional liposomes containing DMPC as the main lipid, PDE was insensitive to the amount of PG used in this study ($P > 0.05$). For DMPC based PEGylated liposomes, PDE increased with using higher amount of PG ($P < 0.01$).

Thus, the results of the interaction graphs revealed the best possible composition and amount of lipids for preparation of topotecan liposomes by hydration method. It is evident from the results that plotted interaction graphs can be used in

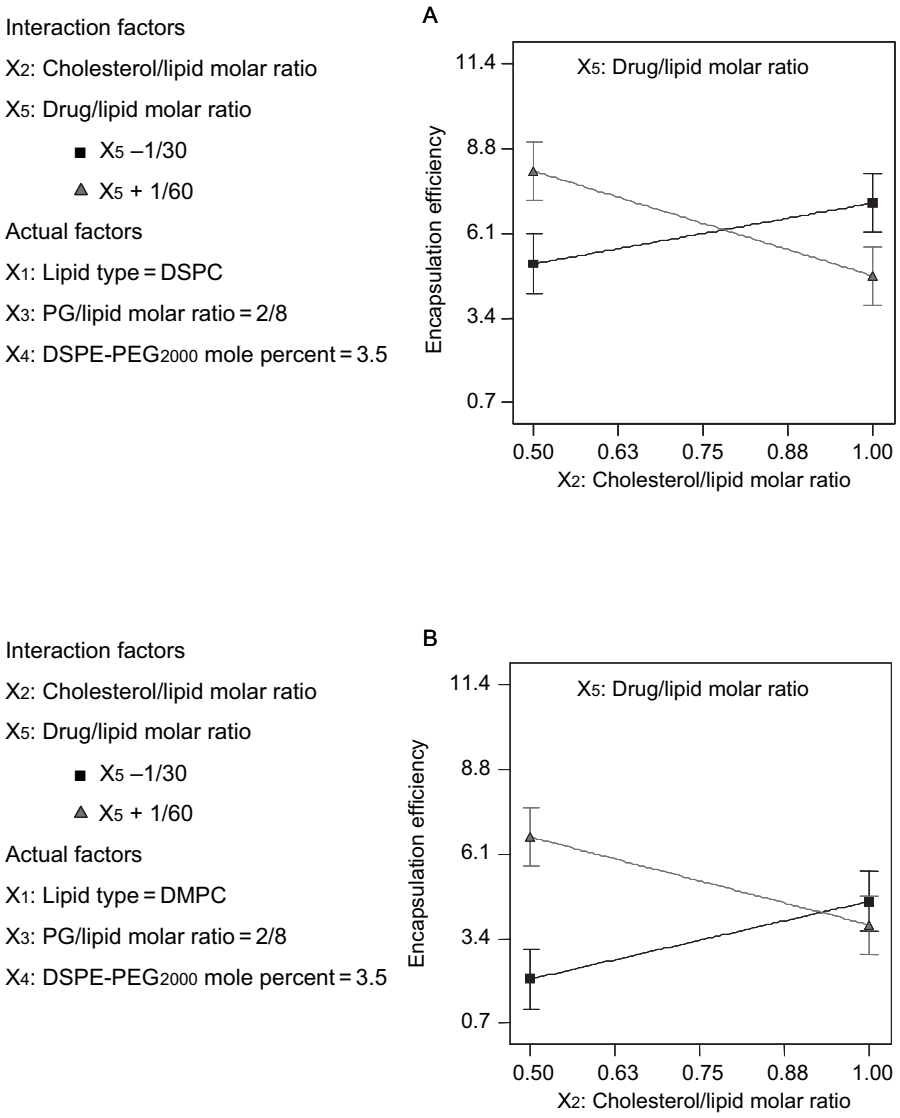

FIGURE 2. Interaction graph of X_2 and X_5 factors at 1 level of X_1, 0 level of X_3 and 0 level of X_4 (A); interaction graph of X_2 and X_5 factors at –1 level of X_1, 0 level of X_3 and 0 level of X_4 (B).

predicting the type of lipids, range and ratio of phosphatidylglycerol, cholesterol, DSPE-PEG_{2000}, and topotecan in a precise manner.

Topotecan liposome formulations, either conventional or PEGylated, were prepared by extruding multilamellar liposomes using different cholesterol, PG and DSPE-PEG ratios in phospholipid mixtures (Table 2). Hydration of the drug-lipid film, followed by 10 cycles of extrusion through 0.1-μm polycarbonate filters, was found to be a feasible preparation method for homogeneous small unilamellar vesicles.

Incorporation of 9.96% and 11.44% of topotecan was the upper limit in conventional and PEGylated formulations, respectively. Usually the hydrophilicity of drugs limits the loading efficiency in liposomes. Inclusion of lower amount of PG, and changing DSPC by DMPC, dramatically decreased encapsulation efficiency. It has been suggested that including some negatively charged lipids, such as phosphatidylglycerol, in the vesicles reduces the possible lipid aggregation shown after preparation of liposomes (Maruyama et al., 1994). In our case, no aggregation was occurred after liposome preparation.

Cholesterol in sufficient quantities could maximize the longevity of conventional liposomes in the blood (Nygren, Woodburn, Decker & Kessel, 1995; Sykes et al., 1994) and may reduce or prevent leakage of the encapsulated drug from the liposomes (Liebman et al., 1994; Rowinsky & Donehower, 1995) or decrease interaction of liposomes with plasma components (Sharma, Balasubramanian, & Straubinger, 1995; Weiss et al., 1990). The inclusion of cholesterol in

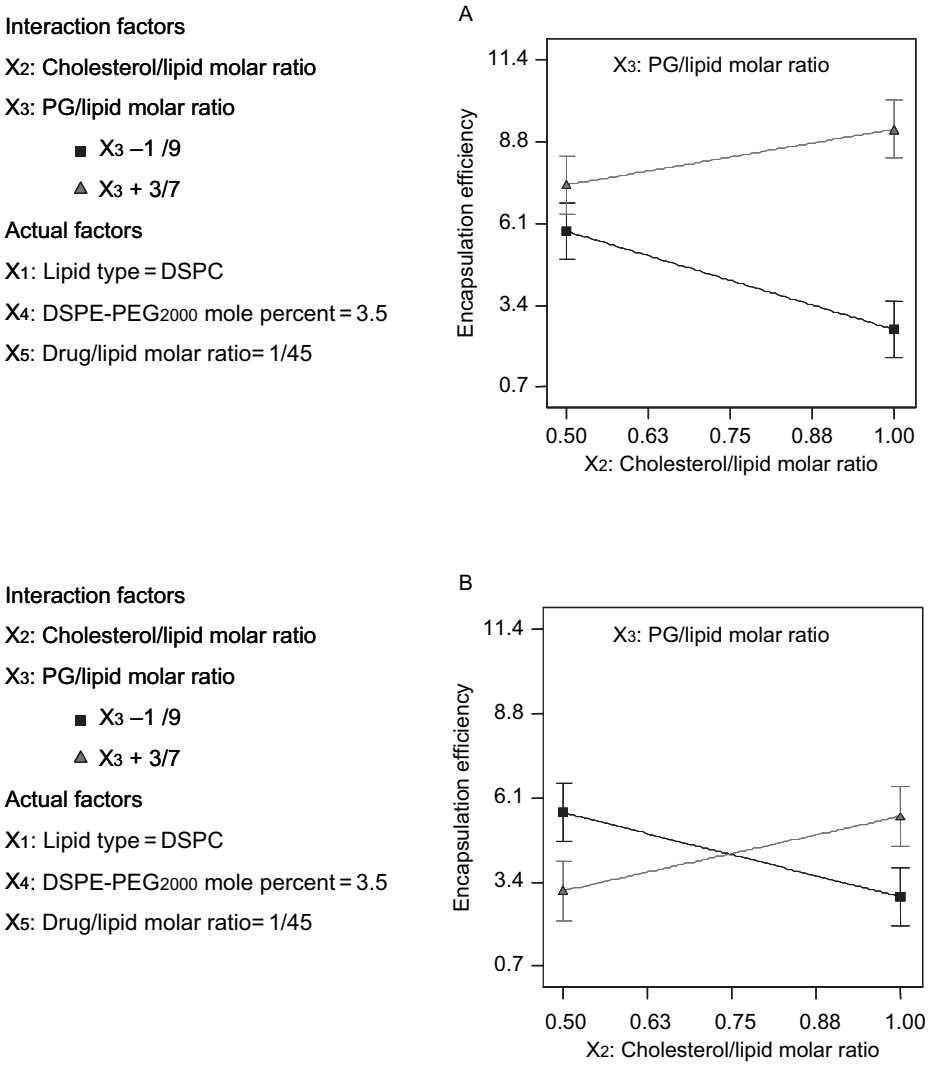

FIGURE 3. Interaction graph of X_2 and X_3 factors at 1 level of X_1, 0 level of X_4 and 0 level of X_5 (A); interaction graph of X_2 and X_3 factors at −1 level of X_1, 0 level of X_4 and 0 level of X_5 (B).

PEGylated liposomes, even at low concentrations, generally inhibit mixed micelle formation, by restricting the flexibility of the hydrocarbon chain and reducing PEG chain interaction and entanglement, and hence limiting the formation of phase separated lamellae (Sharma & Straubinger, 1994). The presence of cholesterol may also prevent fusion or aggregation between vesicles induced by the presence of PEG molecules (Sharma & Straubinger, 1994). We found that increasing the percentage of cholesterol in the topotecan liposomal formulations in some cases had a negative effect and in some cases had a positive effect on encapsulation efficiency of the drug (Figures 2 and 3).

It is reasonably well accepted that increases in membrane rigidity can increase drug retention in liposomes and this is exemplified by liposomal formulations utilizing sphingomyelin or DSPC (C_{18}) instead of egg phosphatidylcholine (EPC) or DMPC (C_{14}) as the bulk phospholipid component (Cullis & Bally, 1989; Mayer et al., 1993; Webb et al., 1995). In this context, changing the composition of the DSPC containing liposomes by replacing DSPC (C_{18}) with DMPC (C_{14}) made it more difficult to prepare and load the liposomes. It is acknowledged that changes in acyl chain composition improved the properties of liposomal topotecan formulation, but in some cases like idarubicin liposomes (Santos et al., 2005) increase in acyl chain length did not improve the properties of liposomes.

In order to further increase in the PDE value of formulaion 16 (Table 2) a liposome formulation composed of the same lipid composition (as formulation 16) but containing 1.6% mole of topotecan (mole drug : mole total lipids) was also prepared. (formulation 17). Although this formulation showed a good drug entrapment equal to 12.52% (drug concentration equal to 12.30 mg/ml), but related conventional

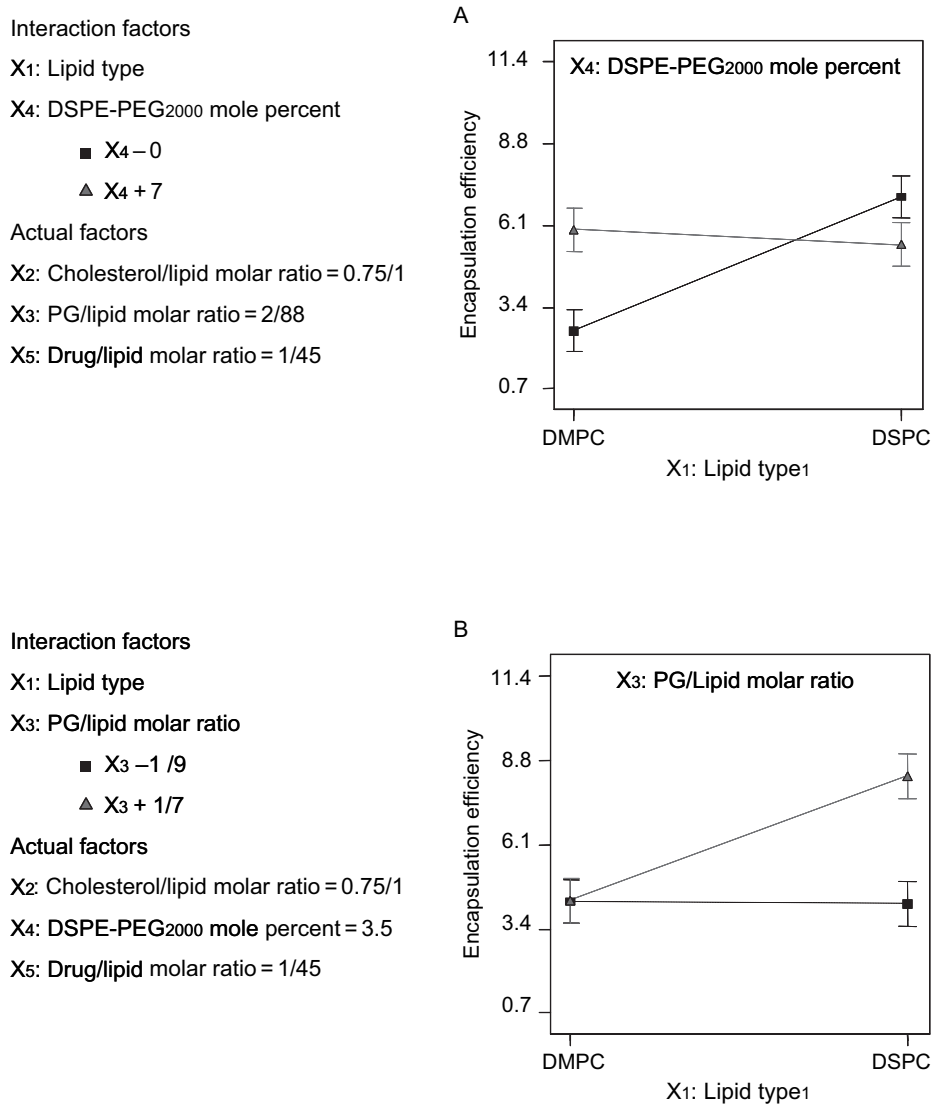

FIGURE 4. Interaction graph of X_1 and X_4 factors at 0 level of X_2, 0 level of X_3 and 0 level of X_5 (A); interaction graph of X_1 and X_3 factors at 0 level of X_2, 0 level of X_4 and 0 level of X_5 (B).

liposome formulation without any DSPE-PEG_{2000} (formulation 18; containing 1.6 mole% of topotecan) showed a lower drug entrapment (7.61%) with drug concentration of 9.27 mg/ml.

Using 3.34 mole% of topotecan in formulation 16 (composed of DSPC/cholesterol/DSPG/DSPE-PEG_{2000} with a molar ratio of 7:7:3:1.28) and also in the related conventional liposome formulation without DSPE-PEG_{2000} (formulation 19) increased drug concentration to 22.49 mg/ml (entrapment, 11.44%) and 15.14 mg/ml (entrapment, 6.21%) respectively. Therefore, because of higher concentration of topotecan and the well-known in vitro and in vivo stability of cholesterol-phospholipid formulations, conventional and long-circulating liposomes using 3.34 mole% of topotecan, composed of DSPC/cholesterol/DSPG/DSPE-PEG_{2000} (molar ratio; 7:7:3:1.28) (formulation 16) and DSPC/CHOL/DSPG (molar ratio; 7:7:3) (formulation 19), were selected for further characterization and release studies.

Characterization

Size Distribution of Liposomes

Mean particle size distribution of liposomes was determined by photon correlation spectroscopy using a Coulter Model N4SD submicron particle analyzer (Coulter Electronics, FL). Mean particle size for all liposome preparations were found to be in range of 95 to 103 nm with a polydispersity value of around 0.1 indicating limited variation in particle size (Table 4).

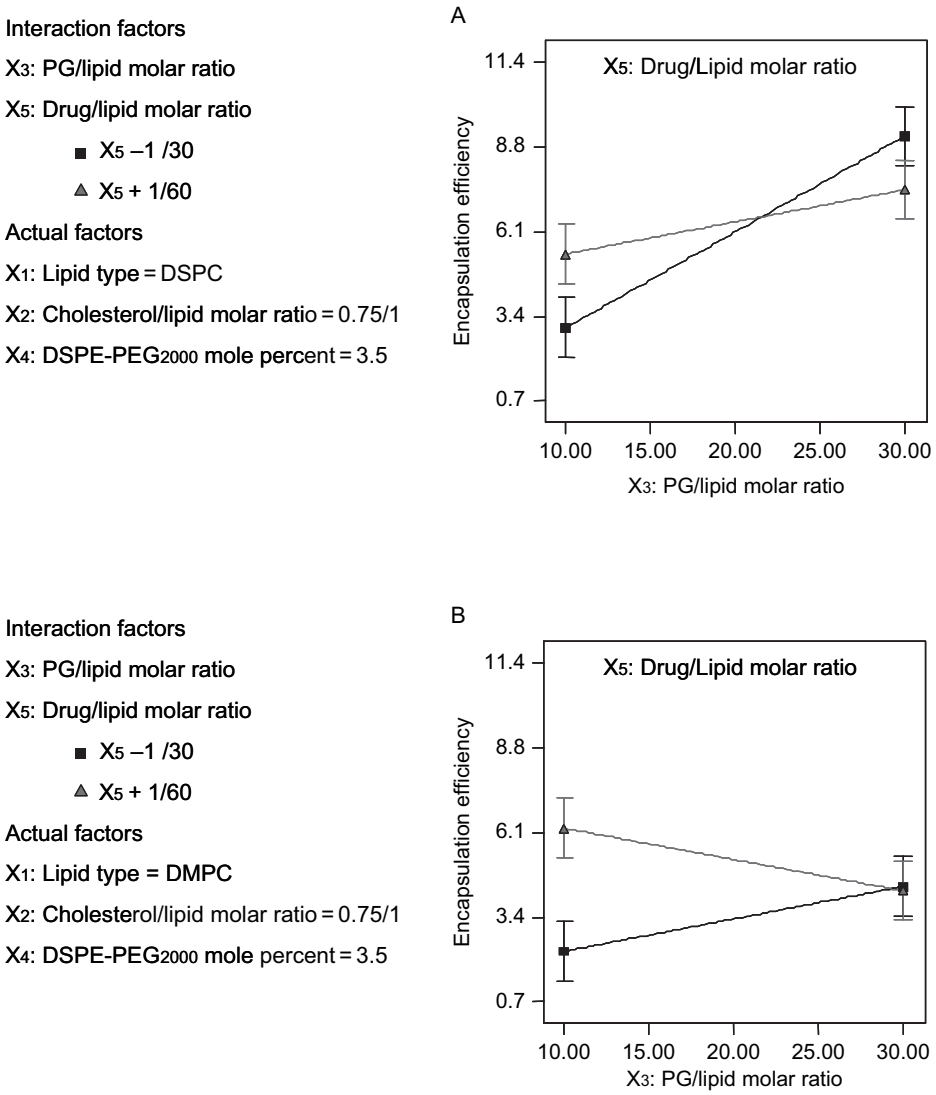

FIGURE 5. Interaction graph of X_3 and X_5 factors at 1 level of X_1, 0 level of X_2 and 0 level of X_4 (A); interaction graph of X_3 and X_5 factors at -1 level of X_1, 0 level of X_2 and 0 level of X_4 (B).

Zeta Potential

Values of the zeta-potential of liposomes indirectly reflect vesicle surface net charge and can therefore be used to evaluate surface coating of liposomes with PEG. Results in Table 4 show that liposome formulation containing DSPE-PEG_{2000} have less negative charge (mean zeta potential value of –10 mV) in comparison of liposome formulation without DSPE-PEG_{2000} (mean zeta potential value of –22 mV). This could be attributed to the masking of some of the anionic charges of DSPG by DSPE-PEG_{2000}.

Stability

Physical Stability of Liposomes. The physical stability of liposome drug products was evaluated in the hydrated state at 4°C for 14 days. PEGylated and conventional liposomes containing topotecan were physically stable for about 3 days and 1 day at 4°C, respectively, and retained at least 95% of their initial drug content over that period. Therefore, for every experiment the conventional and PEGylated liposomes were freshly prepared and used at the same day of manufacturing. During storage (14 days) no appreciable variation ($P > 0.05$) of liposome size was detected by photon correlation microscopy (Table 4) and no drug precipitation or liposome aggregation was observed.

Stability of Topotecan During Liposome Preparation. Topotecan was incubated with dried lipid film at 65°C by agitation, to determine the stability of topotecan during liposome preparation. Topotecan was stable at 65°C for 4 h during agitation and after extrusion ($P > 0.05$).

Stability of Lactone Ring Form of Topotecan After Loading into Liposomes. Based on Biloti's study (Biloti et al., 2003), the intraliposomal space of liposomes containing DSPC,

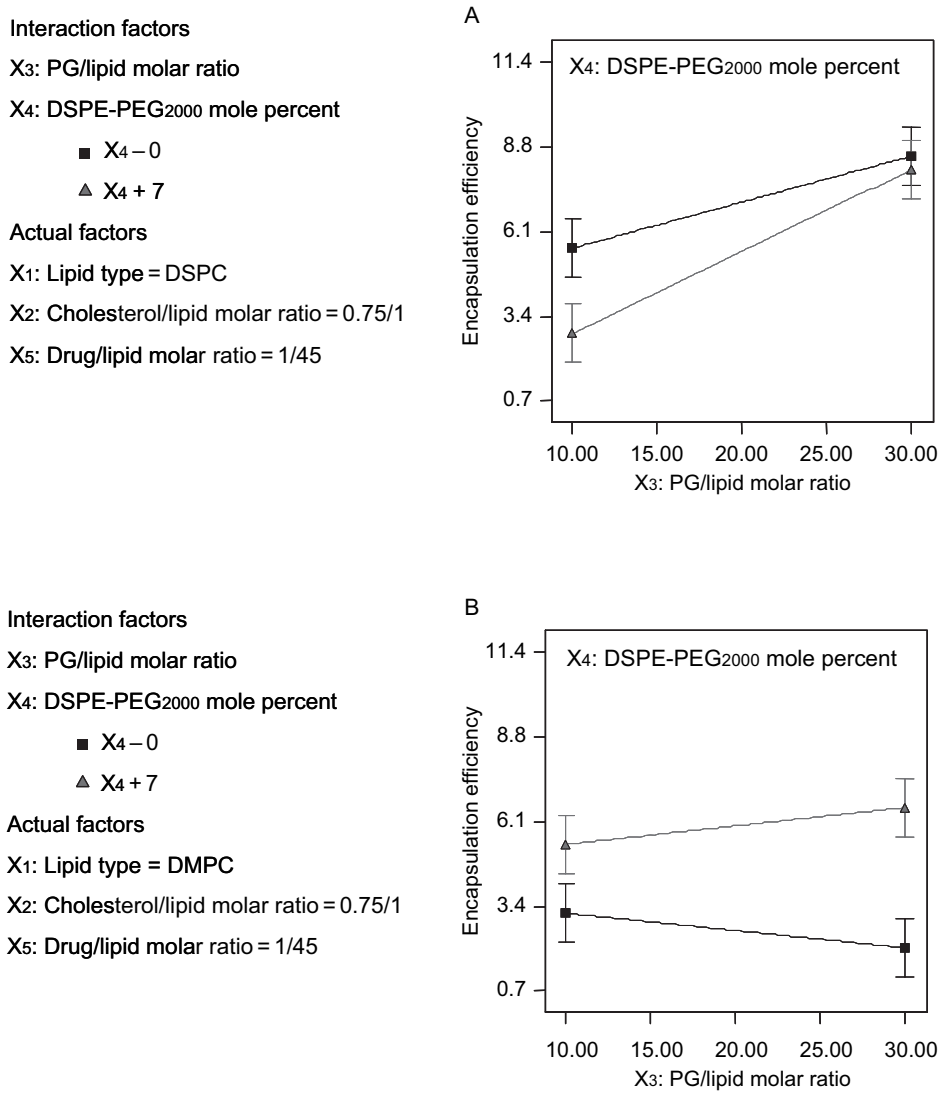

FIGURE 6. Interaction graph of X_3 and X_4 factors at 1 level of X_1, 0 level of X_2 and 0 level of X_5 (A), interaction graph of X_3 and X_4 factors at -1 level of X_1, 0 level of X_2 and 0 level of X_5 (B).

TABLE 4
Size Distribution, Zeta Potential, and Physical Stability of Prepared Liposomes (n = 3)

Liposome	Composition	Molar Ratio	Size (nm) $M \pm SD$ Day 0	Size (nm) $M \pm SD$ Day 14	Polydispersity Index	Zeta-Potential (mV) $M \pm SD$
PEG-liposome	DSPC/cholesterol /PG/DSPE-PEG	7:7:3:1.28	103.0 ± 13.10	107 ± 13.20	0.15	−10 ± 2.3
CL-liposome	DSPC/cholesterol/PG	7:7:3	95.2 ± 11.10	102.0 ± 16.8	0.10	−22 ± 2.8

DSPE-PEG_{2000}, DSPG and cholesterol could maintain a low pH environment after loading of topotecan in PBS pH 5. Therefore, the stability of lactone form is expected to be enhanced while topotecan retain within liposome.

Figure 7 depicts the changes of lactone percentage at physiological pH in plasma as a function of time. Stability data were fitted on two exponential decay equation. Half-lives were determined from the two exponential decay equation using non-linear regression module of Sigmaplot 9. Hydrolysis of free topotecan proceeded quickly with a short half-life ($t_{50\%}$ value) of about 21 minutes. In contrast, for liposomal topotecan, the stability of the lactone moiety was markedly enhanced by liposome encapsulation. The half-lives for converting of topotecan lactone form to carboxylate form as PEGylated and conventional (CL) liposome preparations were 5.58 and 4.31 h, respectively and the difference were not statistically significant. After 10 h incubation of CL liposomes and PEGylated liposomes with plasma at 37°C, 20 and 29 percent of topotecan remained in lactone form, respectively. Hydrolysis of topotecan in liposome form is partly because of release of topotecan from the liposomes and partly, because of H^+ diffusion out of liposomes. In overall the results of stability evaluation showed that the lactone ring of topotecan was notably preserved upon liposome encapsulation.

In Vitro Release of Topotecan Liposomes

The release profiles of topotecan from PEGylated and conventional liposomes in PBS pH 7.4 and human plasma and from free drug as control are shown in Figure 8.

About 50 and 68% of drug was released over a period of 10 h from PEGylated and CL liposomes respectively. In both cases the release profiles were followed by a prolonged release

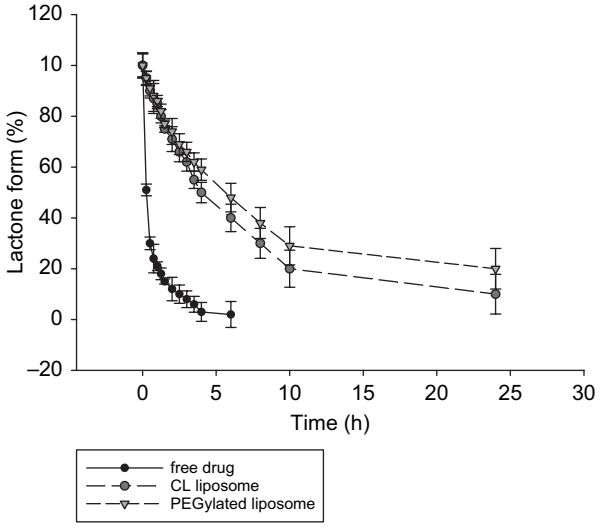

FIGURE 7. Stability of lactone form of topotecan loaded in PEGylated liposomes, conventional (CL) liposomes and free topotecan (control) in plasma at 37°C.

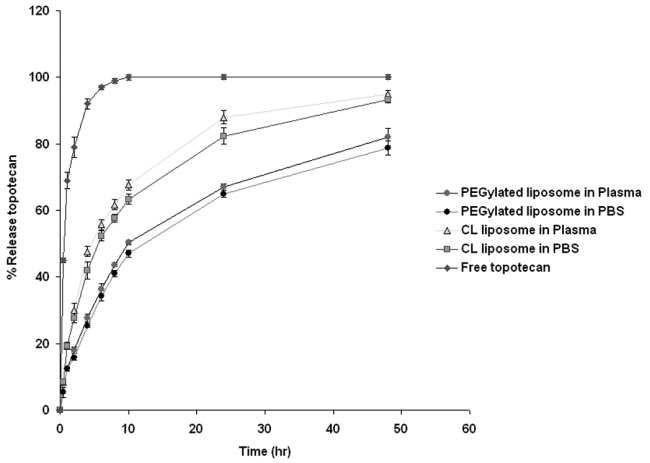

FIGURE 8. In vitro release profile of topotecan from PEGylated and conventional (CL) liposomes (in plasma and PBS pH 7.4) and from free drug (in PBS pH 7.4); Data represents $M \pm SD$ (n = 3).

TABLE 5
Release Half-Lives of Topotecan from Liposomes Derived from Higuchi Model (n = 3).

Liposome	Release Media	t_{50}% ($M \pm SD$)
PEGylated liposome	Human plasma	12.12 ± 1.22
PEGylated liposome	PBS pH 7.4	13.36 ± 0.94
Conventional liposome	Human plasma	5.31 ± 0.36
Conventional liposome	PBS pH 7.4	6.06 ± 0.48

Data were fitted on Higuchi model. Half-lives were determined from the Higuchi equation using non-linear regression analysis.

up to more than 48 h. The followed delayed release may be attributed to diffusion of the dissolved drug within the core of the liposome into the dissolution media.

The release of free topotecan was fast, indicating that drug penetration through dialysis membrane was not limiting step.

The release kinetics were evaluated by Higuchi equation as well as by Ritger-Peppas model (Ritger & Peppas, 1987) for further mechanistic evaluation. Considering the release data up to 60–70% the studied models were fitted properly ($r^2 > 0.993$) to the release data of both liposome formulations in PBS and human plasma. The calculated values of n, release exponent in Ritger-Peppas equation, for PEGylated and conventional liposomes were approximately 0.5 (0.507 and 0.524 respectively). This equation has two distinct physical meanings in the two special cases of n = 0.5, indicating a Fickian diffusion and n = 1, indicating a time independent release mechanism that creates a zero order kinetics. Values of n between 0.5 and 1.0 can be regarded as an anomalous transport (Ritger & Peppas, 1987). It

TABLE 6
Comparison of the Derived Model Parameter, K_H, and $t_{50\%}$ of the PEGylated and Conventional Liposomes in Plasma and PBS pH 7.4 by ANOVA with Tukey Post Test

		K_H[a]		$t_{50\%}$[b]	
Product		Mean Difference	*P* Value	Mean Difference	*P* Value
Conventional in plasma	PEGylated in plasma	–7.470	< 0.001	6.806	< 0.001
Conventional in PBS 7.4	PEGylated in PBS 7.4	–6.700	< 0.001	7.298	< 0.001
PEGylated in plasma	PEGylated in PBS 7.4	0.740	> 0.05	–1.242	> 0.05
Conventional in plasma	Conventional in PBS 7.4	1.510	> 0.05	–0.750	> 0.05

[a]Release rate constant based on Higuchi model.
[b]Release half-lives.

should be pointed out that the mentioned values are only valid for slab geometry. For spheres $n = 0.43$ and $n = 0.85$ are the two extreme values, therefore the obtained values for exponent n in the present study ($0.43 < n < 0.85$) suggest that drug diffusion from the liposome bilayers may not be the exact mechanism for the release of topotecan from the prepared formulations.

Time to 50% release ($t_{50\%}$) for selected formulations was calculated using Higuchi model and the results are shown in Table 5. The values for Higuchi release rate constant (K_H) and $t_{50\%}$ were compared using one way ANOVA with Tukey post test. Statistical evaluation showed that topotecan release rate constant for PEGylated liposomes in both human plasma and PBS pH 7.4 were significantly lower ($P < 0.001$) than related values for CL liposomes (Table 6). The relatively prolonged topotecan release from PEGylated liposomes may be attributed to the more bilayer rigidity. In general, PEGylated liposomes containing topotecan showed a significant decrease ($P < 0.001$) in release rate and an increase in resistance to release in comparison to CL liposomes. The effect of human plasma on the release profile appeared to be non-significant for both liposomes ($P > 0.05$) (Table 6).

CONCLUSION

In present study the preparation and characterization of liposomes of topotecan as a potent anticancer drug was carried out. A factorial experimental design was employed for the simultaneouse analyzing the influence of different factors on the properties of the liposomes and finding optimum formulations. The hydration method followed by extrusion utilized to prepare topotecan loaded liposomes is quite simple, rapid, inexpensive and highly reproducible. The optimized topotecan loaded PEGylated liposome formulation had relatively high drug entrapment showing a sustained-release behavior which is suitable for passive tumor targeting. The type of lipid, amount of DSPG, drug to lipid molar ratio and the interaction between these factors played a dominant role in encapsulating the drug. Presence of DSPE-PEG_{2000} in liposome formulation could successfully control the release of TPT up to more than 48 h.

In overall the results of present study revealed that the PEGylated liposomes loaded with topotecan may be considered as a promising carrier system for controlled release and targeted delivery and could be evaluated for clinical administration in future.

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