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Ciprofloxacin Liposomes as Vesicular Reservoirs for Ocular Delivery: Formulation, Optimization, and In Vitro Characterization

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Management of extraocular diseases is mainly limited by the inability to provide long-term drug delivery without avoiding the systemic drug exposure and/or affecting the intraocular structures and poor availability of drugs, which may be overcome by prolonging the contact time with the ocular system, for instance with liposomes. Development and optimization of reverse phase evaporation ciprofloxacin (CPF) HCl liposomes for ocular drug delivery was carried out using a 2^5 full factorial design based on five independent variables. The effects of the studied parameters on drug entrapment efficiency (EE), particle size, and percentage of drug released after 1 and 10 h were investigated. The results obtained pointed out that the molar concentration of cholesterol was the predominant factor that increased the EE% of the drug and the particle size responses. The percentage of drug released after 1 h was significantly controlled by the initial CPF concentration while that after 10 h was controlled by molar cholesterol concentration. The designed liposomes had average particle sizes that ranged from 2.5 to 7.23 μm. In addition, liposomes revealed a fast release during the first hour followed by a more gradual drug release during the 24-h period according to Higuchi diffusion model.

Keywords reverse phase evaporation liposomes; vesicular system; ocular delivery; optimization; factorial design

INTRODUCTION

One of the most attractive areas of research in drug delivery is the design of vesicular systems that are able to deliver drugs to its specific target, with appropriate pharmacokinetic properties concerning drug levels and dosage timing (Dillen, Vandervoort, Mooter, Verheyden, & Ludwig, 2004).

Drugs are commonly applied to the eye for a localized action on the surface or in the interior of the eye (Davies, 2000). A major problem in ocular therapeutics is the attainment of an optimal drug concentration at the site of action. Poor bioavailability of drugs from ocular dosage form is mainly because of the precorneal loss factors, which include tear dynamics, nonproductive absorption, transient residence time in the cul-de-sac, and the relative impermeability of the corneal epithelial membrane (Kaur & Kanwar, 2002; Le Bourlais et al., 1998; Shell & Baker, 1974). Because of these physiological and anatomical constraints, only a small fraction of the administered drug, effectively 1% or even less of the instilled dose, is ocularly absorbed (Burstein & Anderson, 1985; Shell, 1984). Consequently, the clinician is obligated to recommend a frequent pulse-type dosing at an extremely high concentration, which results in several side effects of ophthalmic products.

Many attempts have been made to improve the precorneal residence time and/or penetration ability of the active ingredient via development of controlled drug delivery systems to maintain drug concentration at an effective level for a prolonged period. These systems include ocular inserts (Baeyens et al., 2002; Sasaki et al., 2003), mucoadhesive polymers (Kaur & Smitha, 2002), nanoparticles, nanocapsules (Alonso, 2004), and colloidal particles (Mainardes et al., 2005). Thus, they avoid the discomfort that is combined with the application of sticky preparations as ointment, the later preparations lead to a total blurred vision if they are properly utilized. Inserts, on the other hand, are difficult to administer, or if they are designed as nondissolving inserts, they are even more difficult to remove, especially by elderly patients (Zimmer & Kreuter, 1995).

Other approaches include vesicular drug delivery systems, such as liposomes (Budai et al., 2007; Cortesi et al., 2006; Sun, Wang, Huang, Liang, & Liu, 2006), that have been used in diagnostic and therapeutic approaches for ocular anterior segment abnormalities. In vitro and in vivo researches of these agents have elucidated many advantages of liposomal intercalation in the context of tear dynamics, corneal adhesion and penetration, and ocular pharmacokinetics (Ebrahim, Peyman, & Lee, 2005). Active ingredients encapsulated in liposomes allow not only an improved solubility and transport through the cornea, but liposomal delivery of drugs is also a tool for prolonged and controlled delivery. Moreover,

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liposomes offer the convenience of an ophthalmic drop and confinement of the action at the site of administration (Mainardes et al., 2005).

Liposomes (lipid vesicles) are microscopic vesicles consisting membrane-like lipid bilayers surrounding an aqueous medium. They are formed spontaneously when phospholipids are hydrated in aqueous media. Because of their structure, chemical composition, and colloidal size, liposomes exhibit several properties, which may be useful in various applications (Wen, Choi, & Kim, 2006). Because of their amphiphilic character, liposomes have the ability to entrap both hydrophilic and hydrophobic compounds in the aqueous compartments or within the lipid bilayers, respectively.

In pharmaceutical technology, development and optimization of different pharmaceutical dosage forms present a high number of factors influencing the formulation. Therefore, complex, expensive, and time-consuming formulation studies are often necessary for the development of a product with the required properties (Lewis, Mathieu, & Phan-Tan-Luu, 1999). The application of experimental design methodology in pharmaceutical technology would provide an effective and economical method to acquire the necessary information to understand the relationship between the independent variables and the performance (the dependent parameters) as well as its interaction (Seham, Sabry, & Alia, 1996).

In this study, we attempted to develop and optimize the formulation of reverse phase evaporation (REV) liposomes, containing ciprofloxacin (CPF) HCl, by trying to determine the factors that influence the physicochemical properties of the liposomes formed. Among these properties are the following: drug entrapment efficiency (EE), which should be maximized, particle size, which controls the residence time at the eye surface and is an important parameter considering endocytosis, and drug release kinetics, which should be optimized for the purpose. The factors examined were the molar concentration of cholesterol, initial concentration of CPF HCl, pH of the aqueous phase, the concentration of dicetyl phosphate (surface charge), and finally, sonication time. A 2^5 full factorial design was used to plan and perform the experimentation. This methodology allows the determination of the influence of the different factors on the liposomes properties.

MATERIALS AND METHODS

Materials

The phospholipid, Lipoid E 80, was purchased from Lipoid Company (Ludwigshafen, Germany), and CPF HCl, CPF, was purchased from Bayer Co. (Leverkusen, Germany). Cholesterol 99% was purchased from Winlab (Leicestershire, UK), dicetyl phosphate from Sigma-Aldrich (Steinheim, Switzerland), uranyl acetate-2-hydrate from Allied Signal (Riedel-Dehaen, Germany), and Spectrapore® 2 dialysis membrane form Spectrum laboratories Inc. (Houston, TX, USA). All other solvents and materials used were of analytical grade.

Methods

Preparation of Ciprofloxacin HCl-Loaded Liposomes

Liposomal dispersions were prepared by REV according to the method of Szoka and Papahadjopoulos (1978). Phospholipids and cholesterol with or without dicetyl phosphate were weighed and dissolved in chloroform : diethyl ether (1:1). Aqueous phase (buffer solution containing CPF HCl at molar ratios of 0.1552 or 0.7776:1 lipid) was added such that the organic–to–aqueous phase ratio was 6:1. The mixture was sonicated at 40°C for either 5 or 10 min in an ultrasonic bath (Julabo sonicator, model USR-3; Julabo Labortechnik, Ceelbach, Germany). A stable emulsion was produced, from which the organic solvent was slowly removed at reduced pressure at 45°C by a rotary evaporator (Rotavapor, type R110; Buchi Company, Postfacn, Switzerland). Liposomal dispersions formed were maintained for 1 h at a temperature exceeding the phospholipid transition temperature (40°C).

Experimental Design of Experiments: A 2^5 Full Factorial Design

Systematic optimization procedures were carried out by selecting an objective function, finding the most contributing factors and investigating the relationship between responses and factors. Objective function for present study was selected as maximizing the encapsulation efficiency percentage and minimizing the burst release.

Five independent factors and their influence on the liposomes properties were evaluated by utilizing 2^5 full factorial design composed of five variables, which are set at two levels each. The five factors investigated were the molar concentration of cholesterol, initial concentration of CPF, pH of the aqueous phase, sonication time, and finally the type of surface charge. For each factor, the lower and higher value of the lower and upper level can be represented by a 1 or –1 sign as shown in Table 1.

A design matrix comprising of 32 experimental runs (Table 2) was constructed using Design Expert® (Version 7.3.1, State-Ease Inc., Minneapolis, MN, USA), for which the interactive statistical first-order computer-generated equation is defined as

$$Y = \beta_0 + \beta_1 X_1 + \ldots + \beta_5 X_5 + \beta_{12} X_1 X_2 + \ldots + \beta_{123} X_1 X_2 X_3 + \ldots \beta_{12,345} X_1 X_2 X_3 X_4 X_5, \quad (1)$$

where Y is the measured response associated with each factor level combination (dependent variable); β_0 is the intercept ; β_1–$\beta_{12,345}$ are the regression coefficients computed from the observed experimental values of Y from experimental runs; and X_1, X_2, X_3, X_4, and X_5 are the coded levels of independent variables (factors). The terms X_1X_2 to $X_1X_2X_3X_4X_5$ represent the interaction terms.

TABLE 1
Variables and Responses in 2^5 Full Factorial Design

Factors	Levels Used, Actual (Coded)	
	Low (–1)	High (+1)
Independent variables (factors)		
X_1 = molar cholesterol concentration (μmole %)	20.0	33.34
X_2 = initial drug concentration (mg/100 mL)	20.0	100.0
X_3 = pH of the medium	4.50	7.40
X_4 = sonication time (min)	5.0	10.0
X_5 = concentration of dicetyl phosphate (molar ratio)	0.0	0.50
Dependent variables (responses)		
Y_1 = entrapment efficiency (%)		
Y_2 = particle size (μm)		
Y_3 = initial burst effect (indicated by % drug released at 1 h)		
Y_4 = percentage of drug released after 10 h (%)		

Separation of Free Unentrapped Ciprofloxacin HCl from Liposomes

To separate encapsulated liposomal CPF from nonencapsulated liposomal CPF, ultracentrifugation using centrifuge (Sigma laboratory refrigerated centrifuge, model 3K-30, Germany) of the prepared dispersions was carried out at 15,600 rpm for 60 min at 4°C. The supernatant was removed, the liposomal pellet was washed, and resuspended in the corresponding buffer, and recentrifugation was carried out again as described above. This cycle is repeated twice. The final liposomal pellet was resuspended with 1 mL isotonic phosphate buffer, pH 7.4 (Liang, Levchenko, & Torchilin, 2004).

Characterization of Ciprofloxacin HCl-Loaded Liposomes

Determination of Ciprofloxacin HCl Entrapment Efficiency

The supernatant (free CPF) was collected and filled to 5 mL in a volumetric flask; the UV absorbance using spectrophotometer (Perkin Elmer Lambda 3B; Perkin Elmer, New York, NY, USA) of the solution was measured at the predetermined λ_{max} of 272 nm (pH 7.4) and 277 nm (pH 4.5) using the corresponding buffer as a blank. For each determination, triplicate runs were made.

The entrapped CPF HCl (EE%) could be defined as the percent fraction of the total input drug encapsulated in the lipid bilayers and/or aqueous compartments in the liposome structure; it was calculated using the following equation:

$$\%\,\text{Entrapment efficiency} = \frac{\text{entrapped ciprofloxacin}}{\text{total ciprofloxacin}} \times 100. \quad (2)$$

Morphological Evaluation of the Prepared Liposomes

CPF-loaded liposomes were morphologically studied by optical (Euromed, Holland) and transmission electron microscopes (TEMs, model JEM-100S; Joel, Tokyo, Japan) to identify its shape and lamellarity. Negatively stained samples were prepared by applying a drop of liposomal dispersion to copper-coated grids. A saturated uranyl acetate aqueous solution was used as a staining agent. The samples were analyzed using TEM at 80 kV (Seth & Misra, 2002).

Particle Size Analysis

The mean size and size distribution of the prepared liposomes were studied using laser diffractometer (CilasL100, model 1064 liquid; Quantachrom, France). The polydispersity of the preparations were expressed by Span Index, which was calculated from the following equation:

$$\text{Span Index} = \frac{D_{(v,90)} - D_{(v,10)}}{D_{(v,50)}}, \quad (3)$$

where $D_{(v,10)}$, $D_{(v,50)}$, and $D_{(v,90)}$ are the equivalent volume diameters at 10, 50, and 90% cumulative volumes, respectively.

In Vitro Release of Ciprofloxacin HCl from Liposomal Dispersion

Membrane diffusion technique was used for the in vitro release study using modified Franz diffusion cell with 1 cm^2 surface area available for release: dialysis membrane was clamped between the donor and the receiving compartments. The upper compartment (donor) was exactly fitted on the surface of the lower compartment (receiving); the receptor phase was phosphate buffer saline, pH 7.4. The procedure was carried in a thermostatically controlled water bath (M.B.H. & Co., Staufen, Germany) at a temperature of 34 ± 0.5°C and shaken at 25 rpm. One milliliter of each liposomal preparation or solution control was placed into the cap on the membrane; 6 mL of freshly prepared phosphate-buffered saline equilibrated at 34 ± 0.5°C was used as release medium. Samples

TABLE 2
Composition of Different Liposomal Dispersions Prepared by Reverse Phase Evaporation Method According to 2^5 Full Factorial Design

Formula	Cholesterol Concentration (X_1)	Ciprofloxacin Concentration (X_2)	Sonication Time (X_3)	pH of the Medium (X_4)	Dicetyl phosphate Concentration (X_5)
F1	20.00	20	5	4.5	0
F2	20.00	20	10	4.5	0
F3	33.34	20	5	4.5	0
F4	33.34	20	10	4.5	0
F5	20.00	100	5	4.5	0
F6	20.00	100	10	4.5	0
F7	33.34	100	5	4.5	0
F8	33.34	100	10	4.5	0
F9	20.00	20	5	4.5	0.5
F10	20.00	20	10	4.5	0.5
F11	33.34	20	5	4.5	0.5
F12	33.34	20	10	4.5	0.5
F13	20.00	100	5	4.5	0.5
F14	20.00	100	10	4.5	0.5
F15	33.34	100	5	4.5	0.5
F16	33.34	100	10	4.5	0.5
F17	20.00	20	5	7.4	0
F18	20.00	20	10	7.4	0
F19	33.34	20	5	7.4	0
F20	33.34	20	10	7.4	0
F21	20.00	100	5	7.4	0
F22	20.00	100	10	7.4	0
F23	33.34	100	5	7.4	0
F24	33.34	100	10	7.4	0
F25	20.00	20	5	7.4	0.5
F26	20.00	20	10	7.4	0.5
F27	33.34	20	5	7.4	0.5
F28	33.34	20	10	7.4	0.5
F29	20.00	100	5	7.4	0.5
F30	20.00	100	10	7.4	0.5
F31	33.34	100	5	7.4	0.5
F32	33.34	100	10	7.4	0.5

were withdrawn at predetermined time intervals and analyzed spectrophotometrically at λ_{max} using phosphate buffer saline (pH 7.4) as a blank (Arnardottir, Sveinsson, & Kristmundsdottir, 1996; Maffei, Lombardi Borgia, Sforzini, Bergamante, & Ceschel, 2004).

After each sample, equal volume of fresh receptor fluid was replaced into the receptor chamber to maintain a constant volume. Kinetics of CPF HCl release from the prepared liposomal dispersions were examined based on the magnitude of correlation coefficients obtained after application of zero-order, first-order, and Higuchi diffusion models.

Optimization Data Analysis and Model Validation

Analysis of variance (ANOVA) provision available in the software was used to establish the statistical validation of the linear equations generated by Design Expert®. ANOVA, *F* ratios, and correlation coefficients are the criteria used for the validation of the model. In all cases, $p < .05$ was accepted to denote significance.

In addition to statistical validation, the reliability of the model could be challenged by comparing the predicted values of responses with the observed (actual) ones. To validate the agreement between the predicted values and the

actual data, both the ratio between actual and predicted values and bias were examined.

$$\text{Bias} = \frac{\text{predicted value} - \text{experimental value}}{\text{predicted value}} \times 100. \quad (4)$$

RESULTS AND DISCUSSION

The EE%, initial burst effect (% of drug released at 1 h), and % of drug released after 10 h were computed and listed for the different liposomal dispersions prepared by REV (Table 3).

Entrapment Efficiency of Liposomes

Drug loading level of REV liposomes was expressed as EE%. The percentage of CPF entrapped into the prepared liposomes ranged from 22.15 to 71.4%.

ANOVA revealed that EE% of liposomes was significantly affected by concentration of cholesterol, initial drug concentration, and pH of the medium ($p < .0001$). From regression equation after correcting for insignificant terms (Table 4), it could be deduced that the effect of cholesterol concentration was the main positive effect among the other significant terms as seen from its higher regression coefficient (4.59). The increase in entrapment efficiencies attributed to the fact that it increased the rigidity of

TABLE 3
Observed Responses in 2^5 Full Factorial Design for Ciprofloxacin HCl-Loaded REV Liposomes

	Dependent Variables				
Formula (F)	Y_1* (% wt/vol)	Y_2 (μm)	Y_3 (% wt/vol)	Y_4 (% wt/vol)	Span Index
1	40.4 ± 1.041	4.25	9.39	70.41	1.601
2	40.5 ± 1.520	4.29	6.78	69.7	2.072
3	50.11 ± 1.466	6.53	5.2	58.46	1.494
4	51.2 ± 1.362	5.82	5.61	55.27	1.931
5	45.6 ± 0.263	2.5	9.34	69.4	2.753
6	41.9 ± 1.591	3.34	11.67	70.08	2.641
7	50.32 ± 0.857	5.68	4.9	56.75	1.908
8	51.8 ± 2.007	6.39	7.83	57.15	1.622
9	60.85 ± 2.163	5.4	18.5	65.16	1.28
10	56.54 ± 0.355	5.1	16.37	64.29	1.848
11	67.8 ± 0.487	7.23	19.2	54.21	1.564
12	66.3 ± 0.909	7.1	18.76	55.2	1.381
13	63.8 ± 1.085	5.68	18.89	64	1.905
14	60.15 ± 0.878	5.03	18.5	63.1	1.937
15	70.31 ± 1.195	6.88	19.8	54.03	1.368
16	71.4 ± 0.247	6.67	20.4	52.35	1.517
17	44.7± 0.823	2.61	10.09	72.63	1.453
18	44.07 ± 0.500	3.47	6.33	72.1	2.363
19	53.22 ± 0.538	6.23	8.25	62.9	1.536
20	53.1 ± 0.842	6.01	12.05	62.06	2.408
21	45.73 ± 0.576	2.72	9.5	71.2	1.859
22	49.32 ± 0.816	4.09	7.19	70.8	1.645
23	55.46 ± 1.123	6.03	8.78	61.64	1.796
24	54.77 ± 0.486	6.32	12.63	60.77	1.454
25	22.34 ± 1.315	4.97	15	66.39	1.704
26	22.15 ± 1.070	4.88	14.72	67.45	1.842
27	36.13 ± 0.333	6.39	15.08	59.13	1.622
28	33.6 ± 1.222	6.88	15.26	59.79	1.377
29	26.37 ± 0.537	5.25	16.34	68.62	1.829
30	29.06 ± 0.698	4.72	13.25	69.32	1.849
31	37.19 ± 0.432	6.73	13.8	60.35	1.729
32	37.5 ± 0.640	6.79	13	60.35	1.302

*Each value was the mean ± SD., n = 3.

TABLE 4
Summary of Results of Regression Analysis for Responses Y_1, Y_2, Y_3, and Y4

Linear Model	R^2	Adjusted R^2	Predicted R^2
Y_1	.9820	.9853	.9881
Y_2	.9267	.9301	.9107
Y_3	.9223	.9197	.9073
Y_4	.9539	.9594	.9398

Regression equations of fitted model[a]

$Y_1 = +47.93 + 4.59 \times B + 1.49 \times C - 7.63 \times E - 9.42 \times D \times E$

$Y_2^{2.5} = +74.67 + 33.14 \times B + 16.48 \times D$

$Y_3 = +12.58 + 4.10 \times D - 1.50 \times D \times E - 0.95 \times B \times D \times E$

$Y_4 = +63.28 - 5.13 \times B - 1.80 \times D + 2.06 \times E$

[a]Only terms with statistical significance are included.

the phospholipid bilayer, reduced its permeability, and provided more physical stability against ultrasonication, thus increasing the loading of the drug which was in accordance with Cortesi, Romagnoli, Menegatti, and Esposito (2004). In addition, initial drug concentration played a positive role, as CPF is a hydrophilic compound mainly present in the aqueous compartment of liposome, so the increased initial drug concentration (from 20 to 100 mg/100 mL) resulted in 18.7% [(71.4 − 60.8/60.8) × 100] increase in EE, when other factors were kept constant.

On the other side, pH of the medium and its interaction term with the concentration of dicetyl phosphate had obvious negative effect on EE; CPF is an amphoteric molecule with two potential ionizable groups having $pK_{a,\,COOH} = 6.0$ and $pK_{a,\,N4}{}^{-} = 8.8$ (Hernandez-Borrell & Montero, 2003). Therefore, four different microspecies can be found in the solution (neutral, Zwitterions, positively, and negatively charged) depending on the pH of the solution; in fact, the uncharged and zwitterionic microspecies predominated at neutral pH, whereas the positive microspecies predominated at acidic pH (Hernandez-Borrell & Montero, 2003). At pH 4.5, an electrostatic attraction between the negatively charged bilayer as a result of incorporated dicetyl phosphate and the cationic CPF could be the factor that promoted entrapment of the drug, whereas at pH 7.4, such electrostatic attraction did not occur.

Response surface plots were generated (Figure 1). Contour lines serve as a two-dimensional representation of a three-dimensional surface Figure 1A. Contour plot revealed the significance of the interaction between pH of the aqueous phase and concentration of dicetyl phosphate. This interaction was reflected by the lack of parallelism between the lines of entrapment efficiencies. Contour lines represented points of equal response. When the pH of the aqueous phase was in the range of 5.22–4.5 and the concentration of dicetyl phosphate was more than 0.38 molar ratio, the EE showed the highest value (58.96%). Twisting in the three-dimensional plot, Figure 1B also demonstrated the interaction between these two independent variables.

Particle Size Analysis

The particle size is an important parameter, as the biopharmaceutical properties of a liposomal formulation can be influenced by its physicochemical properties. The size can also play an important role in endocytosis possibilities of liposomes.

The particle size and size distribution measured in terms of Span Index is shown in Table 3. Liposomes mean size diameter ranged from 2.5 to 7.23 μm. Low values of Span Index ranging from 1.302 to 2.753 indicated a narrow distribution of size and low polydispersity (unimodal). Optical and transmission microscopical images of the prepared liposomes (Figure 2) revealed that the RVE method produced uniform single-walled vesicles of bilayer enclosing an aqueous compartment (large unilamellar vesicles). This was in agreement with the finding reported by Perugini et al. (2000).

Transformation is a mathematical conversion of response values, which is used to satisfy the assumption required for the ANOVA. The Box–Cox plot is a tool to help in determining the most appropriate power transformation to be applied on response data (Design Expert, version 7.1.3).

The Box–Cox plot of the liposomes particle size showed that the 95% confidence interval did not include one suggesting power transformation of data. Model adequacy was checked on the power-transformed data. ANOVA revealed that the particle size of liposomes was significantly affected by the concentration of cholesterol and the presence of dicetyl phosphate ($p < .0001$; Table 4). Regression equation after correcting for insignificant terms demonstrated that cholesterol concentration was the main effect as seen from its higher regression coefficient (33.14). Structure features of cholesterol and its lipid solubility were the reason for its influence on the particle size, that is, it filled in empty spaces among the phospholipids molecules and thus increased the particle size of the prepared vesicles (Al-Muhammed, Ozer, & Hincal, 1996). This result was in accordance with the work done by Santo et al. (2004). Based on the same theory, dicetyl phosphate presence slightly increased the liposomes particle size through its lipid solubility. No interaction was observed between dicetyl phosphate and cholesterol concentration. A more pronounced effect of dicetyl phosphate presence was illustrated by Nagarsenker, Londhe, and Nadkarni (1999) because of the difference in the lamellarity of the liposome.

Concerning sonication time, it had insignificant effect as shown from ANOVA, which was attributed to the fact that droplet size of the emulsion formed during preparation deceased with increasing sonication time, but during the evaporation of organic solvent, coalescence of droplets occurred.

Burst Release from Liposomes

Percentage of CPF HCl released at 1 h (burst effect) ranged from 4.9 to 20.4% (Table 3). This wide range indicated the influence of the chosen variables on drug release pattern. ANOVA revealed that the burst effect (% of drug released at 1 h) from liposomes was significantly affected by the

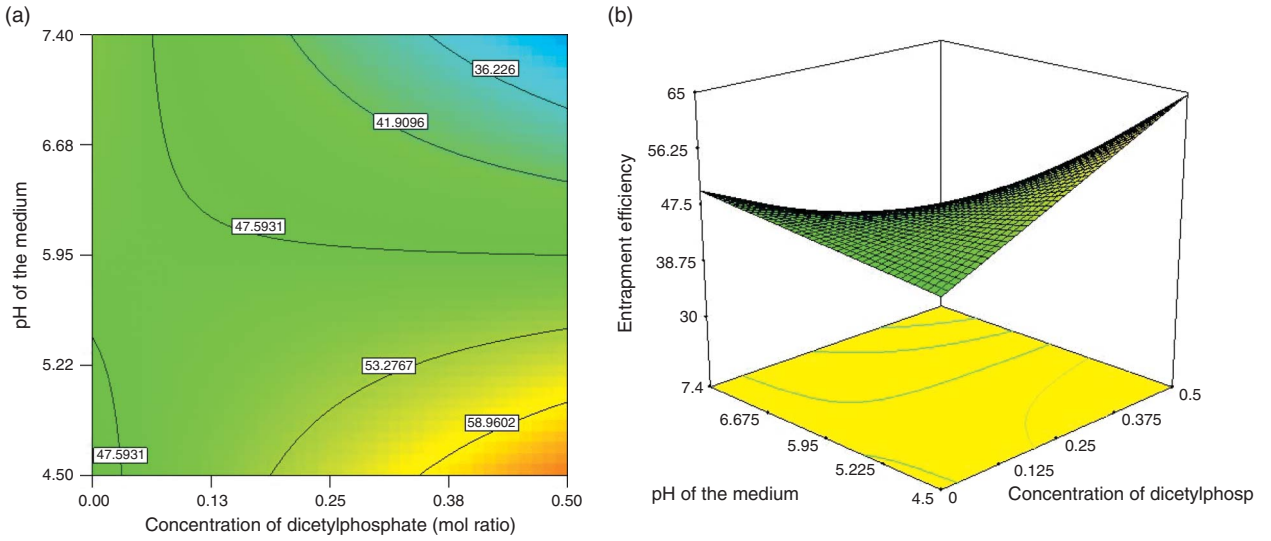

FIGURE 1. Two (A) and three-dimensional (B) plots for the multifactor effect of dicetyl phosphate concentration and pH of aqueous phase on the entrapment efficiency response.

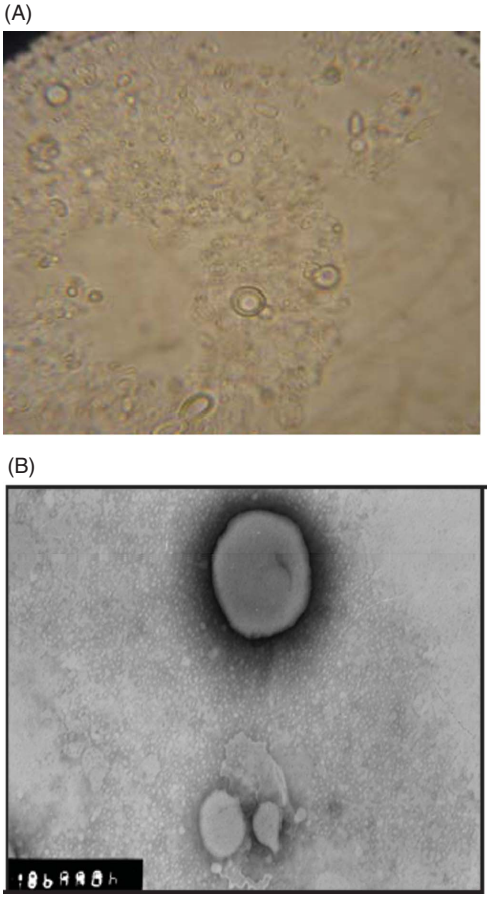

FIGURE 2. Optical photographs (A) and transmission electron microscopical photomicrographs (B) of ciprofloxacin HCl-loaded liposomes composed of phosphatidylcholine : cholesterol : dicetyl phosphate (2:1:0.5, mol/mol) prepared by reverse phase evaporation method.

concentration of dicetyl phosphate and its interaction with pH of the medium ($p < .0001$).

From the regression equation after correcting for insignificant terms (Table 3), it could be deduced that the variable having the most significant effect on the burst response was the dicetyl phosphate concentration in the prepared liposomes. The positive sign of the coefficient implied a positive effect. An increase in the concentration of dicetyl phosphate was accompanied with an increase in the percentage of CPF released at 1 h. The initial rate of release could be explained by the drug desorption mechanism from the liposome surface. This was independent on drug release from the liposome interior, as the molar concentration of cholesterol had no significant effect. As a consequence, presence of dicetyl phosphate, which imparted a negative charge to the liposome surface enhanced CPF adsorption via ionic interaction, thus increased the initial burst effect. This mechanism was predominated at pH 4.5 where the drug was cationic (Hernandez-Borrell & Montero, 2003).

Percentage of Drug Released After 10 h

The cumulative amount (%) of drug released as a function of time from selected liposomal dispersions into isotonic phosphate buffer (pH 7.4) at 34 ± 0.5°C was shown in Figure 3. All liposomal dispersions released CPF at a lower rate than that of the drug solution (control) which releases 92.62% within the first 2 h while F 10, F 14, F 26, and F 30 released 23.38, 26.10, 21.03, and 21.74%, respectively. This reduction in the rate of release was previously observed for multilamellar vesicles (MLVs) by Budai et al. (2007). Through ANOVA, it could be emphasized that the % of drug released after 10 h was significantly influenced by the concentration of cholesterol, dicetyl phosphate concentration, and by the pH of the medium ($p < .0001$).

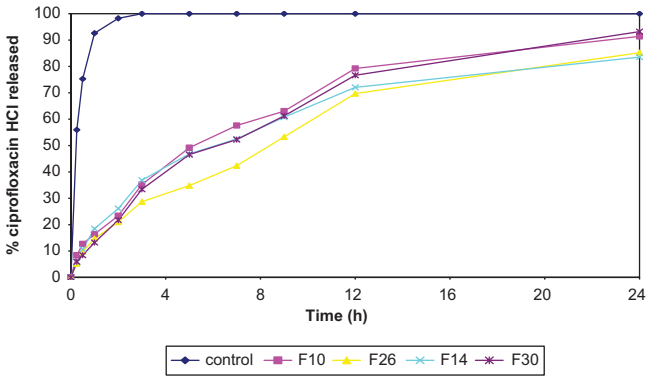

FIGURE 3. In vitro release profiles of ciprofloxacin-loaded REV liposomes into isotonic phosphate buffer (pH 7.4) at 34 ± 0.5°C (F 10, F 14, F 26, and F 30).

From regression equation (Table 4), it was observed that the effect of cholesterol concentration was the main negative effect among the other significant terms as seen from its higher regression coefficient (5.13). The reduction in the cumulative amount of the drug released was explained by cholesterol inclusion. Cholesterol caused a reduction of the density of the head group at the interfacial region of the bilayer and an increase in the package of the phospholipid tails in the middle of the bilayer, thereby reducing their permeability to encapsulated compound (Jedlovsky & Mezei, 2003). Moreover, the presence of cholesterol in liposome preparations reduced the leakage of the encapsulated material by decreasing the membrane fluidity (Peschka-Suss, Dennehy, & Szoka, 1998). This result was in agreement with those reported by Verly et al. (2008).

On the other side, change in pH of the medium from 4.5 to 7.4 was accompanied with a positive effect on the cumulative amount of the drug released. It was believed that only neutral species and probably the zwitterions could permeate freely through the bilayer (Furet, Deshusses, & Pechere, 1992). Diffusion of CPF from liposome depended on the proportion of each microspecies present. For instance, at pH 7.4, the neutral and zwitterionic microspecies predominated while the positive-charged microspecies predominated at acidic pH (Hernandez-Borrell & Montero, 2003). At pH 4.5, increasing the drug hydrophilic character and protonation, a stronger interaction between the drug and the phospholipid head groups occurred, consequently slower release was achieved. The same results for other fluoroquinolones were observed by Puglisi, Fresta, Mazzone, Furneri, and Tempera (1995).

Concentration of dicetyl phosphate had a negative effect on the cumulative amount of the drug released after 10 h. As dicetyl phosphate imparted a negative charge to the phospholipid bilayer, it induced an electrostatic attraction between negatively charged bilayer and cationic CPF HCl, which gave rise to a slower release rate (Law and Hung, 1998). Also it could be explained on the basis that the charged lipids served to tighten the molecular packaging of the vesicle bilayer resulting in decreasing drug release from charged liposomes compared with the neutral ones (El-Gazayerly & Hikal, 1997).

Kinetic Analysis of In Vitro Release

To investigate the mode of release of CPF from different liposomal dispersions, the release data were analyzed with the following mathematical models: zero-order kinetic, first-order kinetic, and square root of time equation (Higuchi equation).

Based on the correlation coefficient (R^2), the results of kinetic study showed that the best fit was achieved with Higushi model for the most of prepared liposomes, which indicated that drug release mechanism from liposomes was diffusion. Furthermore, the predominance of diffusion was confirmed by applying Kopcha equation (Koppcha, Lordi, & Tojo, 1991; Liu, Desai, Tang, & Chen, 2006).

In all formulations, diffusion coefficient was greater than erosion coefficient, giving a ratio of more than one, demonstrating that diffusion of the drug was the rate limiting step of its release from liposomes (data not shown).

Optimization

The optimum formulation of CPF-loaded REV liposomes microscopical reservoir systems was selected based on the criteria of attaining the maximum value of encapsulation efficiency. Upon trading of various response variables, the formulation composition with 33 mol% cholesterol and 100 mg/mL CPF concentration dissolved in pH 4.5 and carrying a negative charge was found to fulfill requisites of an optimum formulation. The optimized formulation (F 16) has the encapsulation efficiency of 71.4% with mean particle size of 6.67 μm and 20.4% burst release. The optimized formulation showed a diffusion-controlled release mechanism.

Validation of the Statistical Model

In addition to the close agreement between the predicted and the adjusted R^2 values for each response, the value of R^2 was also greater than .9 for all regression equations generated, suggesting the statistical validity and significance of these equations for optimization of liposomal systems (Table 4).

Linear correlation plots between actual and predicted response variables (Figure 4) showed the scatter of the residuals versus actual values to better represent the spread of the dependent variables under present experimental setting. Also they demonstrated high values of R^2 indicating excellent goodness of fit ($p < .0001$). Thus, the significant values of R^2 in the present investigation prove the high prognostic ability of the factorial design.

CONCLUSION

In this study, the potential of liposomes as drug carriers for ocular delivery was investigated. CPF HCL, a broad spectrum fluoroquinolone antibacterial agent used in the treatment of ocular infections, was successfully formulated in the form of liposomal reservoir system and the formulation was optimized by statistical screening design considering the molar concentration of

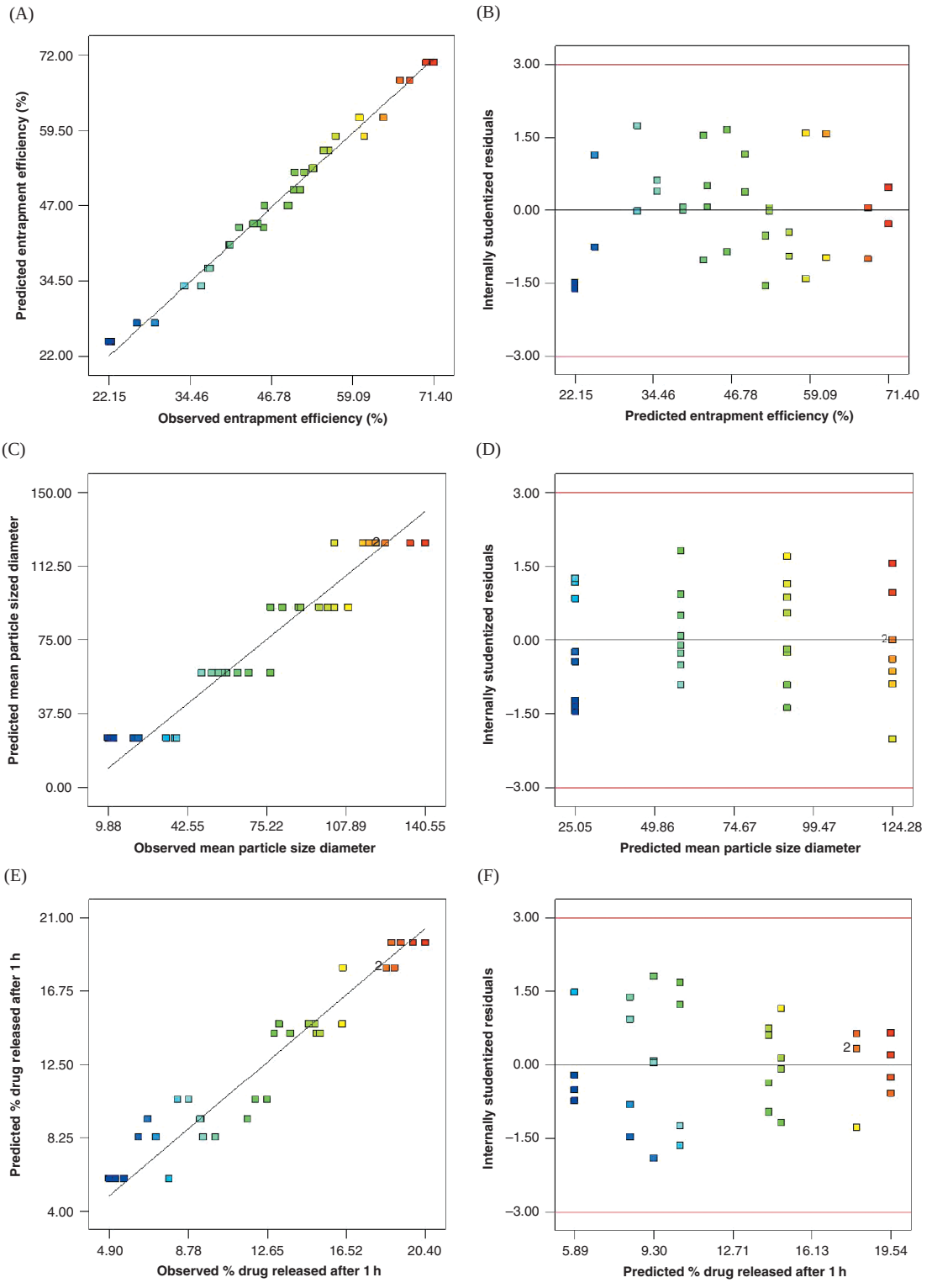

FIGURE 4. Linear correlation plots (A, C, E, and G) between actual and predicted values and the corresponding residual plots (B, D, F, and H) for various responses.

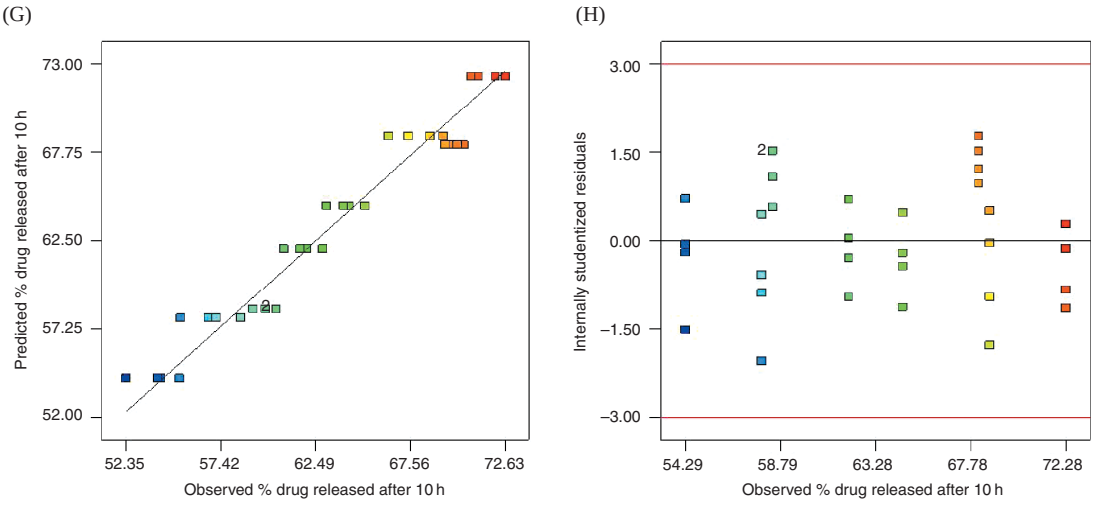

FIGURE 4. (*Continued*)

cholesterol, initial concentration of CPF HCl, pH of the aqueous phase, concentration of dicetyl phosphate (charging inducing agent), and the sonication time used during emulsification phase as independent variables. In vitro release studies showed that the drug was released from the optimized formulation over a period of 24 h in a sustained release manner, primarily by diffusion mechanism. This formulation is a valuable alternative to conventional eye drops by virtue of its ability to sustain the drug release, for its ease of administration because of the reduced dosing frequency resulting in better patient compliance.

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