Bull. Pharm. Sci., Assiut University, Vol. 31, Part 2, 2008, pp. 293-311.

LIPOSOMES AS AN OCULAR DELIVERY SYSTEM OF FLUCONAZOLE: *IN-VITRO* STUDIES

F. S. Habib, E. A. Fouad and Dina Fathalla

Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut, Egypt

تم تحضير الليبوزومات لعقار الفلوكونازول بطريقة التبخير وإنعكاس الوسط وكذلك بتقييم الليبوزومات المحضرة بإستعمال المسح الحراري التفاضلي. وقد تم الإختبار المعملي لإنطلاق العقار من الليبوزومات بإستعمال طريقة الديلزة وقد تبين بطء معدل انطلاق العقار من الليبوزومات بزيادة نسبة الكوليسترول حتي يصل إلي نسبة معينة وبعدها يزداد وكذلك يقل معدل انطلاق العقار بإضافة شحنة موجبة أو شحنة سالبة. كما تم دراسة بعض عوامل الثبات الفيزيائي لليبوزومات الفلوكونازول المحضرة علي شكل قطرات سائلة للعين كالأس الهيدروجيني ومعامل اللزوجة وحجم الليبوزومات وتعيين نسبة العقار المتحوصلة داخل الليبوزومات بعد تخزينها في الثلاجة عند درجة حرارة ٥ درجة مئوية ومقارنتها بتخزينها عند درجة حرارة ٢٥°م لمدة ٢٤ أسبوعاً. وقد أوضحت النتائج أن تخزين الليبوزومات عند درجة حرارة ٥°م يعطي أعلي نسبة ثبات للعقار داخل الليبوزومات وأن الليبوزومات سالبة الشحنة قد أظهرت أعلي ثبات فيزيائي.

The purpose of this study was to formulate topically effective controlled release ophthalmic fluconazole liposomal formulations. Reverse-phase evaporation technique was used for the preparation of fluconazole liposomes consisting of phosphatidylcholine (PC) from soyabean and cholesterol (Ch) in weight ratios of (9:1), (7:2), (7:3), (7:4), (6:4), (7:6) and (5:5) with or without stearylamine (SA) or dicetyl phosphate (DP) as positive and negative charge inducers, respectively. The prepared liposomes were evaluated for their in-vitro release. The release mechanism was found to follow Higuchi and first order kinetics. Increasing cholesterol weight ratio in the prepared liposomal formulations progressively decreased the release of fluconazole from the vesicles. The positively charged liposomes showed slower rate of drug release compared to neutral ones. Negatively charged liposomes showed slight increase in the release rate and extent of fluconazole from the liposomal formulations 5:5:0.25 and 5:5:0.5; in comparison with neutral ones. Further increase in the amount of dicetyl phosphate 5:5:1 resulted in a significant decrease in the release rate.

Received in 9/9/2008, Received in revised form in 6/12/2008 & Accepted in 13/12/2008

Four fluconazole liposome eye drops were prepared. Physical stability study including, visual appearance, particle size and amount of drug leakage from liposome eye drops were studied. Approximately 82.82%, 76.55%, and 70.90% of fluconazole was retained in negative, positive and neutral liposomal ocular formulations up to a period of 24 weeks at 5°C.

INTRODUCTION

Liposomes are lyotropic liquid crystals consisting of one or more concentric spheres of amphiphilic lipid bilayers separated by aqueous phase compartments[1]. Liposomes have been widely used in the recent years as a drug delivery system for many drugs including antimicrobial agents[2]. In this regard, liposomes have been used as carriers and as a potentially valuable system for the controlled delivery of therapeutic agents. Encapsulation of pharmaceutically active drugs within liposomes seems to offer a simple and potent means of modifying and controlling the pharmacology of a variety of drugs[3]. Liposomal structures can encapsulate lipophilic and hydrophilic drugs, so they have the ability to control the rate of release of the encapsulated drugs, to protect the drugs from metabolic enzymes present at tear/corneal epithelium interface. Moreover, they prolong contact with corneal surface thereby improving the bioavailability and therapeutic efficacy of the drug through the cornea[4]. Liposomes offer advantages over most ophthalmic delivery systems in being completely biodegradable and relatively non-toxic. Another potential advantage of liposomes is their ability to come into intimate contact with the corneal and conjunctival surfaces, thereby, increasing the probability of ocular drug absorption[5].

In the previous study different liposomal formulations were prepared, characterized, and tested for their encapsulation efficiency. The *in-vitro* antifungal activity was also studied[6]. Table 1 summarizes fluconazole liposomal formulations reported in the previous study[6].

The objective of this work was to study the release of fluconazole from these liposome formulations and to characterize their vesicle size. Physical stability study including, visual appearance, vesicle size change and drug leakage from liposomes were also investigated for drug liposome eye drops prepared from selected liposome formulations.

EXPERIMENTAL

Materials

- Fluconazole, (Kindly provided by CIDCo (Cairo, Egypt).
- Phosphatidylcholine from Soyabean (PC), Cholesterol (Ch), Stearylamine (SA), and Dicetyl phosphate (DP) were purchased

from Sigma Chemical Co., (St. Louis, USA,).

- Methyl alcohol was obtained from BDH Ltd (Poole, U.K.).
- Chloroform, diethyl ether, sodium hydrogen phosphate, disodium hydrogen phosphate and sodium chloride were purchased from Adwic, El-Nasr Pharmaceutical Co., (Cairo, Egypt). All reagents were of analytical grade and 99% pure.
- Double distilled water, boiled and cooled was used through out the experiments.
- Disposable syringe filter 0.22 & 0.45 µm uni FlO®-25 Schleicher & Schuell Inc., Keene NH 03431 USA.
- The Spectra/Por® dialysis membrane 12000 to 14000 molecular weight cut off was obtained from Spectrum Laboratories Inc. (USA).

Equipment

- Rotavapor, type R 110 [Buchi, Switzerland].
- Water bath, Buchi 462 [Buchi, Switzerland].
- Brookfield Dv+II model Lv viscometer [Brookfield. Engineering laboratories, Inc., Stoughton, MA 02072 USA].
- Sensitive electric balance [Precisa 205A Super Bal-Series Swiss Quality].
- Digital pH meter [Janway Ltd., Felsted, dunmow, Essex, M63LB, U.K.].
- Ultraviolet Spectrophotometer [Jenway 6305 Single Beam spectrophotometer, U.K].
- Magnetic stirrer with hot plate [Sybron/ Thermolyne Co. Dubuque Iowa,USA]
- Sonicator, Model 275T [Crest Ultrasonics Corp., Trenton, USA].
- DSC-50 Differential Scanning Calorimeter (Shimadzu) from [Shimadzu, Seisakusho Ltd., Kyoto, Japan].
- Refrigerated centrifuge, Model 8880 [Centurion Scientific Ltd., W. Sussex, U.K].
- Laser diffraction particle size analyzer (Master Sizer X) Model MAMSOOO [Malvern Instrument Limited, Worcester shire, U.K].
- Hot oven [Model WST 5020 Germany].

Methodology

Preparation of liposomes

All the steps were performed under aseptic conditions. All glassware were sterilized by heating in hot air oven over 120°C for 2 hours. Boiled double distilled water was passed through a 0.22-µm disposable syringe filter (bacterial filter), and the entire procedures were performed in a laminar air flow hood in presence of flame. Fluconazole liposomes were prepared using the reverse-phase evaporation technique[7].

The lipid components (phosphatidylcholine and cholesterol either alone or mixed with charge inducing agent such as stearylamine or dicetyl phosphate) expressed as weight ratios[8&9] are represented in Table 1.

Table 1: Fluconazole liposomal formulations expressed as weight ratios of lipid components and their encapsulation efficiency[6].

Liposome formulae	Phosphatidyl choline (PC)	Cholesterol (Ch)	Stearylamine (SA)	Dicetyl phosphate (DP)	Encapsulation efficiency % ± S.D.
1	9	1	-	-	41.29 ± 0.67
2	7	2	-	-	45.34 ± 1.83
3	7	3	-	-	58.83 ± 1.86
4	7	4	-	-	41.80 ± 5.78
5	6	4	-	-	48.97 ±0.46
6	7	6	-	-	33.12 ± 1.78
7	5	5	-	-	43.68 ± 2.71
8	5	5	0.25	-	35.83 ± 2.8
9	5	5	0.50	-	47.37 ± 4.8
10	5	5	-	0.25	45.57 ± 1.8
11	5	5	-	0.50	50.44 ± 2.7
12	5	5	-	1	54.25 ± 1.3

The different liposomal products equivalent to 50 mg, of lipid components were weighed into 250 ml long-necked quick fit round bottom flask and dissolved in 10 ml chloroform. The organic solvent was slowly evaporated under reduced pressure, using a rotary evaporator, at 40°C to produce a thin lipid film. The lipid film was redissolved in 10 ml ether, and the fluconazole solutions in 10 ml acetone together with 5 ml distilled water were added. The mixture was sonicated for one minute, swirled by hand, and resonicated for another minute. The organic solvents were evaporated on the rotary evaporator under reduced pressure. The liposomal suspension was kept in the refrigerator to mature overnight (5°C).

Differential scanning calorimetry (DSC) measurements

Differential Scanning Calorimetry (DSC) experiments were performed with differential scanning calorimeter calibrated with indium. Samples of fluconazole, cholesterol, empty and drug loaded liposomes composed of PC:Ch (7:3) weight ratios were submitted to DSC analysis.

The analyses were performed on 50-µl or 1-mg samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate 10°C/min. Each sample was scanned between 20 and 160°C.

***In-vitro* release of fluconazole from liposomes**

In order to study the drug release from liposomes with different compositions, dialysis method was

applied using Spectra/Por® dialysis membrane of 12,000-14,000 molecular weight cut off. This membrane assures the permeation of the drug and at the same time retains liposomal forms.

An accurately weighed amount of liposomal formulations, each equivalent to 4mg fluconazole (as drug content) based on the mean percent of encapsulation, was suspended in a glass cylinder having the length of 10 cm and diameter of 2.5 cm. This cylinder was fitted, before addition of liposomal suspension, with presoaked membrane (Spectra/Por® membrane) and placed in a beaker (100 ml) containing 40 ml (drug free) Sørensen's modified phosphate buffer (pH= 7). The whole set is placed on a magnetic stirrer adjusted to constant speed (150 rpm) at 25°C. Sink condition is fulfilled since the saturated solubility was previously determined to be 0.84551±0.19%.

At predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6 and 12 hr); 5 ml aliquot of the release medium were withdrawn for analysis and replaced with equal volume of fresh Sørensen's modified phosphate buffer solution to maintain constant volume. The absorbance of the collected samples were measured spectrophotometrically at λ 261 nm using Sørensen's modified phosphate buffer as blank[10]. The results are the mean values of three release experiments.

Preparation and stability studies of fluconazole liposome eye drops formulations

Based on release results, four formulae were selected for the preparation of fluconazole liposome eye drops. Formulae number 4, 7, 9 and 12 were prepared aseptically as eye drops using Sørensen's modified phosphate buffer pH 7 containing 0.01% benzalkonium chloride as preservative so that, the eye drops contain the equivalent amounts of 0.2% of the drug.

Physical stability study of fluconazole liposome eye drops was carried out on samples stored in tightly sealed 20 ml glass vials in refrigerator at temperature (5°C) and at room temperature (25°C) for 24 weeks.

Samples of liposomal dispersions were regularly tested at time intervals of 0, 4, 8, 12 and 24 weeks for the following attributes at each temperature:

i) Signs of sedimentation, and change in color of the dispersions.
ii) The formulae were analyzed using laser diffraction particle size analyzer.
iii) Extent of leakage:

At predetermined time intervals of 2, 4, 6, 8, 10, 12 and 24 weeks, an aliquot (1 ml) of the stored samples of each formulation was centrifuged in a cooling centrifuge at 25000 rpm for one hour to separate the free drug that may have leaked out of the liposome during the storage period. Free drug in the supernatant was measured spectrophotometrically at λ 261 nm.

Viscosity and pH measurements of fluconazole-loaded liposome eye drops

The viscosity and pH values were measured at both temperatures (5&25°C) directly after preparation and at the end of the storage period (24 weeks). Results were compared with those of a fluconazole 0.2% w/w solution prepared using Sørensen's modified phosphate buffer pH 7 containing 0.01% benzalkonium chloride as preservative.

Statistical analysis

Results were expressed as mean value of three readings ± standard deviation (SD). Statistical analysis was carried out employing One-way ANOVA test followed by two-tailed paired Student's t test. Difference at $P< 0.05$ was considered as minimal level of significance.

RESULTS AND DISCUSSION

Differential scanning calorimetry (DSC) measurements

In order to investigate possible interaction between fluconazole and the used phospholipids in liposome formulations, Differential Scanning Calorimetry (DSC) measurements were conducted. The DSC thermograms of fluconazole, empty and drug-loaded liposomes composed of PC:Ch (7:3) weight ratios are illustrated in Figure 1.

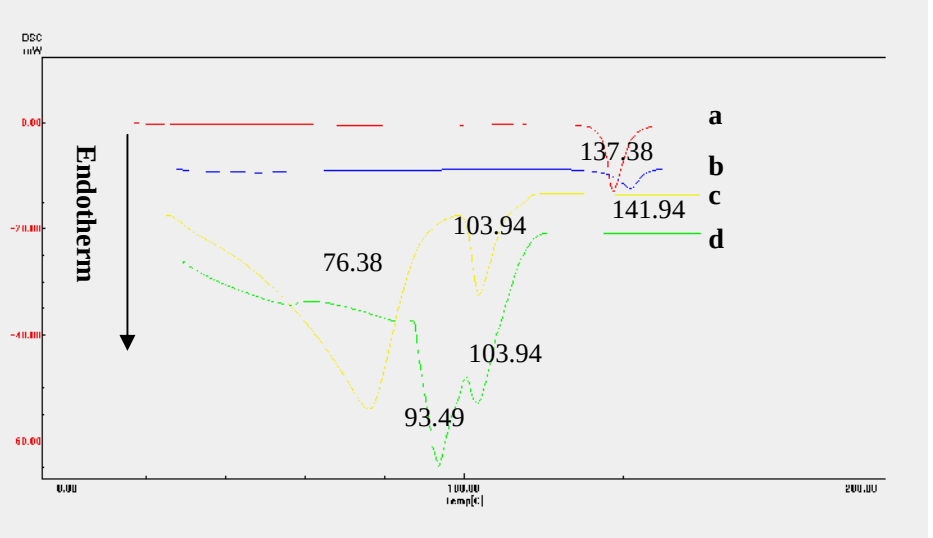

Fig. 1: Differential scanning calorimetry (DSC) thermograms of (a) fluconazole, (b) cholesterol (c) empty liposome and (d) drug loaded liposomal formulation at weight ratio (PC:Ch; 7:3).

DSC thermogram of fluconazole showed a melting endotherm at 137.38°C. DSC thermogram of empty aqueous liposomal dispersion containing (PC:Ch; 7:3) showed major endotherm at 76.38°C, corresponding to the phase transition temperature of phosphotidylcholine (from Soyabean). The melting endotherm of cholesterol was absent at 141.94°C and may have been shifted to 103.75°C, suggesting possible lipid components interaction while forming the lipid bilayer.

DSC thermograms of fluconazole liposomal dispersion, interestingly exhibited endotherms at 93.49°C and 103.94°C (Fig. 1). The incorporated fluconazole associated with the lipid bilayers, may have interacted with and perturbed them. The endotherm at 93.49°C suggested an increase in phase transition temperature of liposomes upon loading with fluconazole. Absence of the melting endotherm of fluconazole suggested either absence of crystalline drug due to its solubility in the lipid bilayer or interaction of drug with bilayer structure.

The above observation is concordant with the finding that, the interaction of the encapsulated drug with the lipid components of liposomes may alter the physicochemical properties of liposomes, which in turn would influence the drug transfer from the liposomes[1].

***In-vitro* release of fluconazole from liposomes**

Effect of cholesterol weight ratio

The effect of incorporating cholesterol, known to influence liposome stability within the lipid composition of liposomes on fluconazole release, was determined by varying the cholesterol contents from 10% to 50%, (Figs. 2 and 3).

From the release profiles, it is clear that, increasing cholesterol weight ratio in the prepared liposomes progressively decreased the release of fluconazole from the vesicles. On the basis of the weight ratio of the lipid content (Phosphatidylcholine : Cholesterol), the extent of drug released after 6 hours for the neutral liposomal preparations can be arranged in the following decreasing order 9:1 > 7:2 > 7:3 > 7:4 > 6:4 >7:6 corresponding to 93.08%, 88.25%, 87.80%, 77.31%, 74.73% and 65.33%, respectively. Differences between them were significant ($p= 4.54x10^{-9}$). The results can be explained by the presence of cholesterol in the bilayers above the phospholipids T_c, which modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains, reducing bilayer permeability[11] and decreasing the efflux of the encapsulated drug, resulting in prolonged drug retention[12]. Cholesterol in these weight ratios gave hydrophobicity that decreased the formation of the transient hydrophilic holes responsible for drug release through liposomal layers[13].

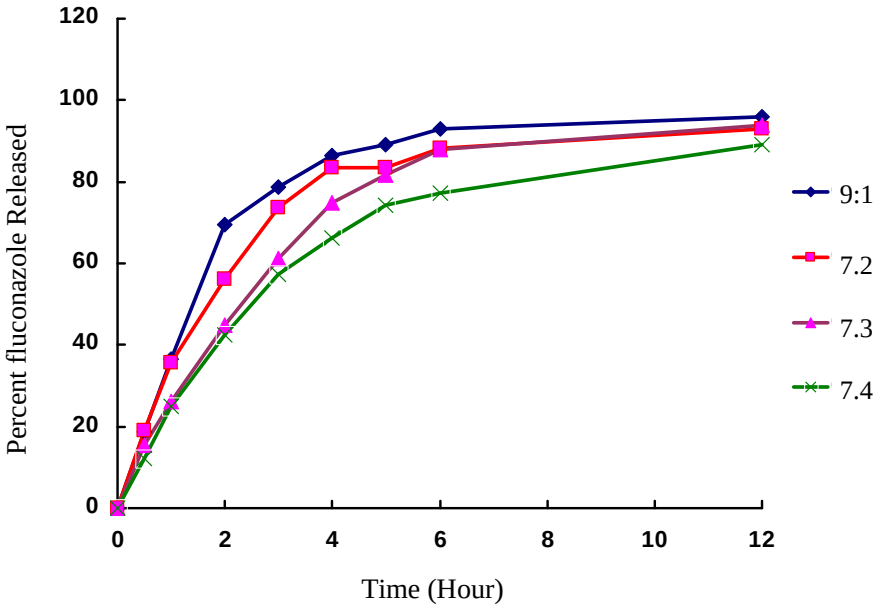

Fig. 2: Release profile of fluconazole from liposomal formulae composed of Phosphatidylcholine : Cholesterol; 9:1, 7:2, 7:3 and 7:4 weight ratios.

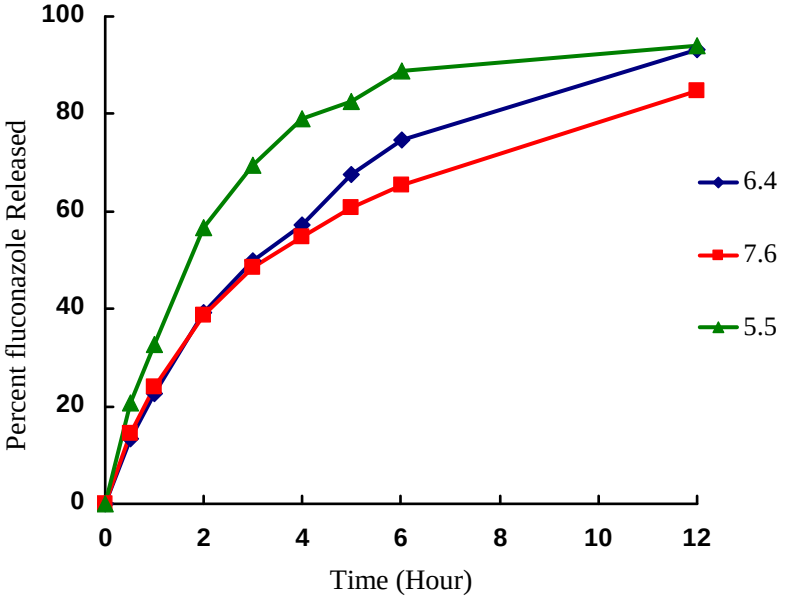

Fig. 3: Release profile of fluconazole from liposomal formulae composed of Phosphatidylcholine : Cholesterol; 6:4, 7:6 and 5:5 weight ratios.

Figure 3 shows that the release of fluconazole significantly increased by increasing the amount of cholesterol in the formulae (PC:Ch 5:5), because increasing cholesterol beyond a certain concentration can disrupt the regular linear structure of the liposomal membrane[14&15].

Effect of liposomal charge

Concerning the effect of charge-inducing agents (stearylamine and dicetyl phosphate) on the drug release from liposomal formulations, Figure 4 illustrates the release profile of fluconazole from neutral and positively charged liposomes having the composition of 5:5:0, 5:5:0.25 and 5:5 :0.5 (PC:Ch:SA) in Sørensen phosphate buffer at 25°C.

From the release profile, it is clear that, the positively charged liposomes showed slower rate of drug release compared to neutral one. The percent released after 6 hours was 75.62% and 73.75% for 5:5:0.25 and 5:5:0.5 respectively, while that of the neutral liposomes 5:5 was 88.79%.

One-way ANOVA revealed that, differences were significant (p= 0.00032) in contrast to neutral one but insignificant effect was observed between the two formulae containing two different concentrations of stearylamine. These results were unexpected. It was expected that, increasing the amount of stearylamine will lead to an increase in the electrostatic repulsion between the drug and the positively charged lipid and consequently the release rate increases. But, a decrease in the drug release was actually found. This finding coincides with Finkelestein and Weismann who reported that, the charged lipids served to tighten the molecular packing of the vesicle bilayer, and this has more pronounced effect[16].

Figure 5, illustrates the release profile of fluconazole from neutral and negatively charged liposomes with lipid content PC:Ch:DP in the weight ratio 5:5:0, 5:5:0.25, 5:5:0.5 and 5:5:1 in Sørensen phosphate buffer at 25°C.

It was clear that, negatively charged liposomes showed slight increase in the release rate and extent of fluconazole after 6 hours. The percent fluconazole released values were 91.24% and 92.93% for the liposomal formulations 5:5:0.25 and 5:5:0.5 respectively in comparison with neutral one which was 88.79%.

Although an apparent difference in the extent of drug release at the ratio 5:5:0.5 in comparison to that of the neutral ones was noticed, these differences are, however, statistically insignificant (p= 0.22). Further increase in the amount of dicetyl phosphate 5:5:1 resulted in a significant decrease in the release rate. The lowest amount of fluconazole released after 6 hours was 82.33% (p= 0.019).

This decrease in the release rate of negatively charged liposomal formula 5:5:1 may be attributed to the charge interaction between fluconazole molecules and the negatively charged liposomes leading to an increase in the drug partition into the lipid

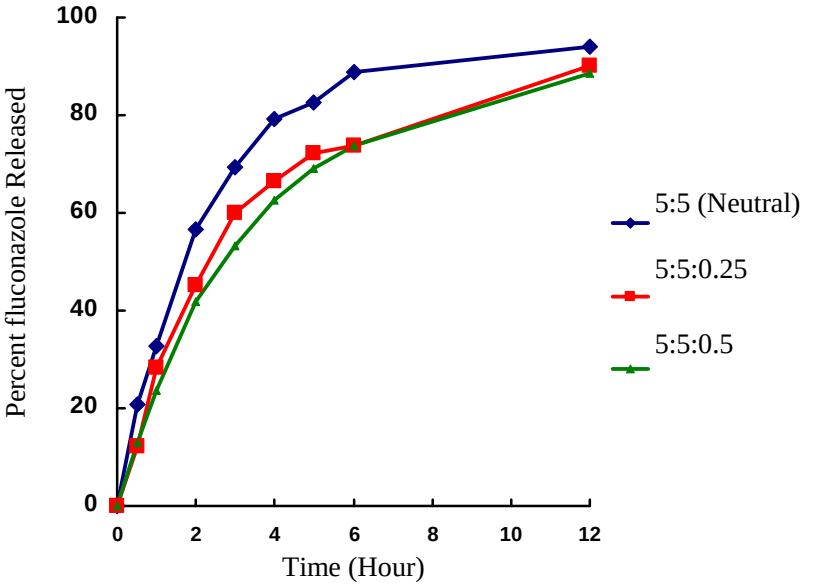

Fig. 4: Release profile of fluconazole from positively charged liposomal formulae composed of Phosphatidylcholine : Cholesterol: Stearylamine in weight ratios: 5:5:0, 5:5:0.25 and 5:5:0.5.

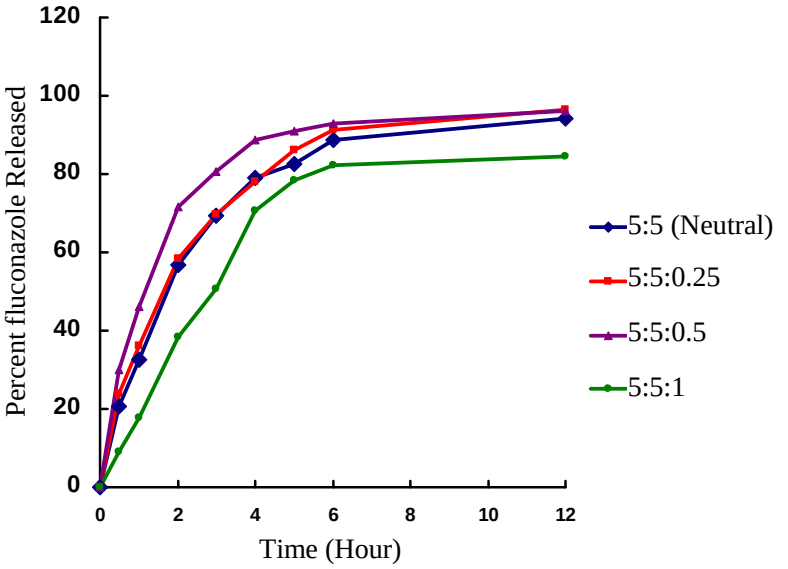

Fig. 5: Release profile of fluconazole from negatively charged liposomal formulae composed of Phosphatidylcholine : cholesterol : Dicetyl phosphate in weight ratios: 5:5:0, 5:5:0.25, 5:5:0.5 and 5:5:1.

bilayers[17] or could be due to decreased ability of the drug possessing positive centers, to cross the negatively charged phospholipids layers. The previous effect was not noticed in the other two ratios 5:5:0.25 and 5:5:0.5, where, the amount of dicetyl phosphate was, probably, not enough for charge interaction with fluconazole.

Linear regression analysis of the release data fitted into zero-order, first-order and Higuchi diffusion-controlled model equations[18] was applied to all *in-vitro* fluconazole release results. In case of similarity in coefficient of determination (r^2) between first-order and Higuchi diffusion-controlled model, Schwartz slope was used to differentiate between them. Deviation of Schwartz slope from 0.5 declines the Higuchi diffusion-controlled model. The drug released from liposomes followed diffusion controlled or first order mechanism as seen in Table 2.

The obtained results are in good agreement with previous studies[19-23].

Based on release data, four fluconazole liposome formulations (formulae 4, 7, 9 and 12 in Table 1) were selected for the preparation of fuconazole eye drops. The release results of the selected formulae are marked bold in Table 2.

Table 2: Kinetic parameters of the release data of fluconazole from liposomes.

Liposomal composition (phosphatidylcholine: cholesterol weight ratio)	Order of release	Intercept	Rate constant K	t1/2
(9:1)	Diffusion	10.07	31.81 % $hr^{-1/2}$	2.46 hr
(7:2)	Diffusion	8.11	30.61 % $hr^{-1/2}$	2.66 hr
(7:3)	First	1.918	0.247 hr^{-1}	2.79 hr
(7:4)	**First**	**1.923**	**0.1905 hr^{-1}**	**3.63 hr**
(6:4)	First	1.991	0.222 hr^{-1}	3.12 hr
(7:6)	Diffusion	0.407	25.85 % $hr^{-1/2}$	3.74 hr
(5:5)	**Diffusion**	**7.109**	**30.67 % $hr^{-1/2}$**	**2.65 hr**
(5:5:0.25) PC:Ch:SA	First	1.922	0.191 hr^{-1}	3.62hr
(5:5:0.5) PC:Ch:SA	**First**	**1.938**	**0.181 hr^{-1}**	**3.81 hr**
(5:5:0.25) PC:Ch:DP	First	1.879	0.286 hr^{-1}	2.42 hr
(5:5:0.5) PC:Ch:DP	First	1.753	0.277 hr^{-1}	2.49 hr
(5:5:1) PC:Ch:DP	**Diffusion**	**2.539**	**30.29 % $hr^{-1/2}$**	**3.72 hr**

Stability studies of fluconazole liposome eye drops

Viscosity and pH measurements of fluconazole-loaded liposome eye drops

The viscosity and pH were determined for both fluconazole solution (0.2% w/w), and fluconazole-loaded liposome eye drops having different weight ratios and surface charge directly after preparation and at the end of the storage period (24 weeks).

The pH values and the viscosity were measured at different temperature and summarized in Table 3.

The viscosity of liposomal formulae was 2-fold as viscous as that of fluconazole solution. The pH of both solution and liposomal formulae were approximately the same and equal to 7.03. The drug and/or the lipids didn't affect the pH of the buffer in both cases. By the end of the storage period, a slight decrease in the pH was noticed in the extent that produces no harm to the eye (close to neutral or very weak acid).

The viscosity of the liposome eye drops increased. This increase in viscosity may be due to the enlargement of the vesicles as a result of some coalescence by time. Negative liposomes had the least increase in the viscosity value among the other formulae.

Monitoring change in visual appearance, in vesicle size and measurement of drug leakage

The liposomal dispersions were sampled at regular intervals of 0, 4, 8, 12 and 24 weeks and examined for signs of sedimentation, creaming if any, and change in color of the dispersions.

The observations revealed that, the aqueous liposomal formulations stored at 5°C showed slight sedimentation which, on slight shaking, redispersed easily. Slight color change up to 24 weeks was observed for all liposomal preparations except for negative one. On the other hand, the liposomal formulations stored at room temperature showed sedimentation and yellow discoloration after 12 weeks except for negative liposomes.

These results are in agreement with what has been previously recommended that liposomal suspensions should be kept refrigerated to achieve the best stability[24].

Monitoring the particle size of a liposomal drug delivery system is an integral part of long-term stability studies. Particle size analysis of neutral, positively and negatively charged liposomes stored at different temperatures (5°C and 25°C) was determined using diffraction particle size analyzer and results are shown in Tables 4-7.

With regard to the change of particle size of the liposomes in buffer the following results were obtained:

- Neutral liposomes (PC:Ch; 7:4) showed an increase in particle size at the end of storage period at both temperatures (5°C and 25°C).

Table 3: Viscosity (η) and pH values of (0.2% w/w) fluconazole solution and fluconazole liposomes eye drops either freshly prepared or stored refrigerated at (5°C) or at room temperature (25°C) for 24 weeks.

Dosage form	Composition and weight ratios	η (cp)			pH		
		Fresh	5˚C	25˚C	Fresh	5˚C	25˚C
Solution	0.2%w/w fluconazole	2.16	2.16	2.16	7.02	6.77	6.74
Neutral Liposomes	PC : Ch 7 : 4	4.61	10.28	10.36	7.07	6.53	6.51
Neutral Liposomes	PC : Ch 5 : 5	4.62	8.56	9.32	7.05	6.78	6.76
Positive liposomes	PC: Ch: SA 5 : 5 : 0.5	4.63	7.01	7.29	7.03	6.76	6.69
Negative liposomes	PC : Ch : DP 5 : 5 : 1	4.62	6.22	6.50	7.03	6.79	6.76

Table 4: Particle size (μm) distribution of neutral liposomes composed of PC:Ch (7:4) stored for 24 weeks at (5°C) and at room temperature (25°C).

	Storage times (Weeks)								
	0	4		8		12		24	
Distribution percentiles		5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
10%	0.18	0.20	0.19	0.52	0.25	0.54	0.30	0.57	0.38
20%	0.24	0.26	0.25	0.59	0.41	0.63	0.40	0.69	0.49
50%	0.52	0.55	0.63	0.77	0.81	0.80	0.76	0.93	0.85
80%	0.80	0.84	1.03	0.88	1.05	0.90	1.02	0.98	1.03
90%	0.90	0.90	1.11	0.92	1.11	0.93	1.09	1.02	1.09
Distribution Modal Sizes (μm)	1.01	0.88	1.08	0.85	1.06	0.88	1.04	0.97	1.04

Table 5: Particle size (μm) distribution of neutral liposomes composed of PC:Ch (5:5) stored for 24 weeks at (5°C) and at room temperature (25°C).

	Storage times (Weeks)								
	0	4		8		12		24	
Distribution percentiles		5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
10%	0.20	0.24	0.19	0.27	0.20	0.35	0.21	0.36	0.52
20%	0.25	0.33	0.24	0.36	0.26	0.44	0.28	0.46	0.57
50%	0.52	0.63	0.45	0.64	0.48	0.69	0.49	0.69	0.69
80%	0.90	0.97	0.89	0.96	0.84	0.97	0.81	0.97	0.90
90%	1.04	1.07	1.04	1.06	1.01	1.06	0.98	1.06	1.02
Distribution Modal Sizes (μm)	1.04	1.05	1.05	1.04	1.04	1.03	1.03	1.03	1.03

Table 6: Particle size (μm) distribution of positively charged liposomes composed of PC:Ch:SA (5:5:0.5) stored for 24 weeks at (5°C) and at room temperature (25°C).

	Storage times (Weeks)								
	0	4		8		12		24	
Distribution percentiles		5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
10%	0.30	0.32	0.26	0.35	0.29	0.40	0.32	0.44	0.43
20%	0.48	0.48	0.36	0.52	0.40	0.56	0.42	0.61	0.52
50%	0.70	0.95	0.69	0.95	0.68	0.95	0.68	0.99	0.74
80%	1.02	1.29	1.02	1.29	0.99	1.28	0.98	1.30	1.00
90%	1.09	1.37	1.09	1.37	1.08	1.37	1.07	1.38	1.08
Distribution Modal Sizes (μm)	1.06	1.33	1.05	1.33	1.05	1.32	1.04	1.33	1.04

Table 7: Particle size (μm) distribution of negatively charged liposomes composed of PC:Ch:DP (5:5:1) stored for 24 weeks at (5°C) and at room temperature (25°C).

	Storage times (Weeks)								
	0	4		8		12		24	
Distribution percentiles		5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
10%	0.22	0.25	0.21	0.26	0.22	0.21	0.21	0.21	0.21
20%	0.34	0.35	0.28	0.35	0.28	0.27	0.27	0.28	0.26
50%	0.66	0.66	0.49	0.63	0.48	0.50	0.45	0.50	0.46
80%	1.00	0.99	0.82	0.96	0.78	0.87	0.72	0.83	0.74
90%	1.07	1.08	1.03	1.06	0.96	1.03	0.88	0.99	0.91
Distribution Modal Sizes (μm)	1.04	1.05	0.55	1.04	0.56	1.04	0.52	1.03	0.53

- Neutral liposomes (PC:Ch; 5:5) showed significant increase in particle size when stored at room temperature (25°C), while insignificant increase in the size of those stored in refrigerator at (5°C).
- Positively charged liposomes (PC:Ch:SA; 5:5:0.5) showed an insignificant increase in the liposomes size at both temperatures.
- Negatively charged liposomes (PC:Ch:DP; 5:5:1) showed decrease in the liposomes size during the storage period at both temperatures. This decrease in size may be attributed to the electrostatic attraction between the positive cation of the drug and the negative charge of the lipid bilayer in the same liposomal particle.

Liposomes can be arranged in descending order according to their stability as follow, negatively charged liposomes (PC:Ch:DP; 5:5:1) > positively charged liposomes (PC:Ch:SA; 5:5:0.5) > neutral liposomes (PC:Ch; 5:5) > neutral liposomes (PC:Ch; 7:4).

Drug leakage from liposomes was evaluated at definite time intervals[25]. The results of the stability study of fluconazole liposomes stored in refrigerator at 5°C for 24 weeks are illustrated graphically in Figure 6 in terms of percent fluconazole retained in liposomes.

The percent of fluconazole retained in liposomal formulations after 24 weeks were 64.77%, 70.90%, 76.55% and 82.82% for neutral (7:4), neutral (5:5), positively and negatively charged liposomes respectively. Negatively charged

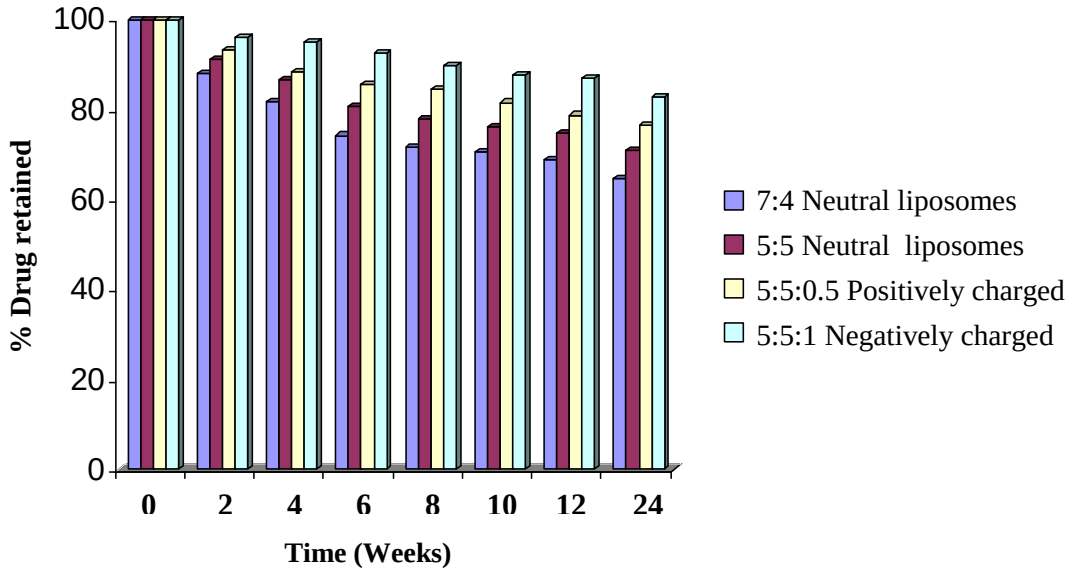

Fig. 6: Histogram representation for the change in the percent of fluconazole retained in liposomes stored at (5°C) for 24 weeks.

liposomes showed better stability, manifested in higher drug retention followed by the positively-charged liposomes then the neutral ones. These results can be explained by the fact that charge on lipids (either positive or negative) is an important parameter influencing liposomal behavior. High surface potential might contribute to liposome physical stability by reducing the rate of aggregation and fusion of liposomes during storage[26].

The results of the stability study of fluconazole-loaded liposomes stored at room temperature (25°C) are graphically depicted in Figure 7. Fluconazole retained in liposomal formulations after 24 weeks was 55.68%, 66.5%, 71.52 and 76.76% for neutral (7:4), neutral (5:5), positively and negatively charged liposomes respectively.

It is evident that, positively charged and negatively charged liposomes exhibited lower leakage rate compared to neutral liposomes revealing that, the charged liposomes are more stable due to reduced rate of fusion and aggregation[27].

It is obvious from the results that, despite the partial hydrolysis that might occur to the phosphatidylcholine, the liposomes are sufficiently stable under refrigerator storage, and the advantages of the lipid membrane were retained[28].

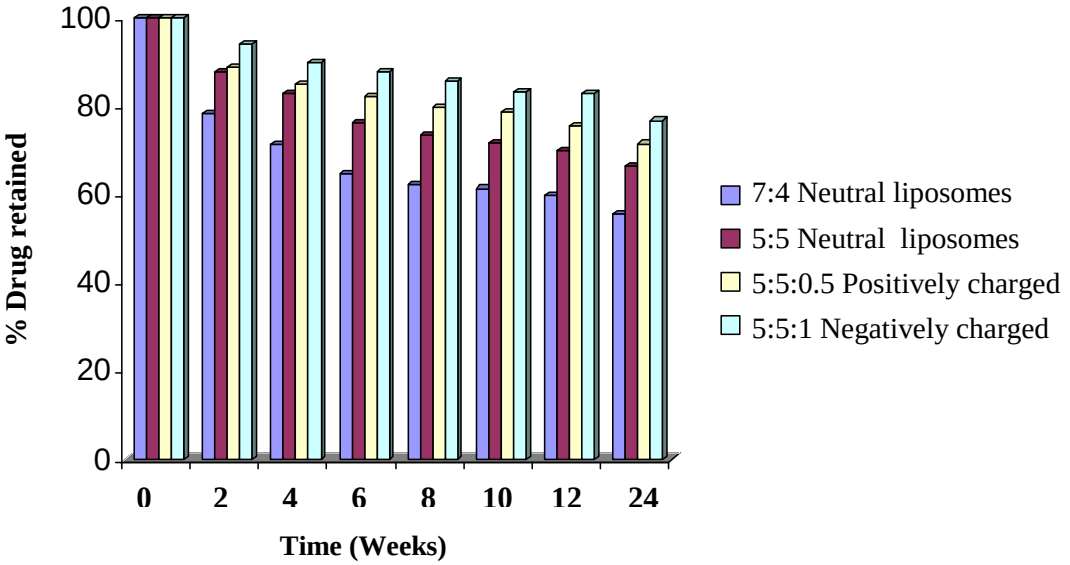

Fig. 7: Histogram representation for the change in the percent of fluconazole retained in liposomes stored at room temperature (25°C) for 24 weeks.

Comparing the two neutral liposomal formulations (7:4 and 5:5), the leakage of the drug was less with liposomes prepared with equal amounts of PC and Ch, and increased with decreasing the ratio of cholesterol.

The leakage from all investigated liposomes was slightly higher at room temperature. Drug leakage at elevated temperatures may be due to chemical degradation (oxidation and hydrolysis) of lipids in the bilayers, leading to defects in membrane packing. Earlier reports of the low-temperature stability of liposomes may be attributed to the deformation of gel-state lipid membranes that help in holding drug molecules in place[9].

Negatively charged liposomes (PC:Ch:DP; 5:5:1) were found to be reasonably stable in terms of aggregation, fusion and/or vesicle disruption tendencies, over the studied storage period either in refrigerator or at room temperature. This may be attributed to the inclusion of dicetyl phosphate (DP) as a negatively charged agent into the lipid layers which could impede the aggregation and fusion of vesicles so as to maintain their integrity and uniformity[9].

The improvement in the liposome-specific features (entrapment, vesicle uniformity, and stability) may be attributed to dicetyl phosphate's ability to incorporate into lipid-layer

domains imparting the polarity (negative charge). These properties facilitate the vesiculation process and pronounced uniformity in size distribution because the desired polar and non-polar interactions are reached[9].

REFERENCES

1- M. S. Nagarsenker and A. A. Joshi, Drug Dev. Ind. Pharm., 23, 1159 (1997).
2- D. J. A. Crommelin and H. Schreier, "Liposomes", In: "Colloidal Drug Delivery Systems", Kreuter, J. (Ed.), Marcel Dekker Inc., New York, Basel, Hong Kong, 1994, pp. 73-190.
3- L. J. Kirby and G. Gregoriadis, "Liposomes", In: "Encyclopedia of Controlled Drug Delivery, Mathiowitz, E (ed.), Vol. 1, New York, John Wiley and Sons, Inc., 1996, pp. 461-492.
4- H. A. El-Ghamry and R. R. Abdalla, Zagazig J. Pharm, Sci., 11, 85 (2002).
5- H. E. Schaeffer, J. M. Breitfeller and D. L. Krohn, Invest. Ophthalmol. Vis. Sci., 23, 530 (1982).
6- F. S. Habib, E. A. Fouad and D. Fathalla, Egypt. Pharm. J. (NCR), 7, 11 (2008).
7- F. Suzoka and D. Papahadjopoulos, Proc. Natl. Acad. Sci., USA, 75, 4194 (1978).
8- A. Bhatia, J. Pharm. Sci., 7, 252 (2004).
9- R. Agarwal O. P. and Katare, Pharmaceutical Technology, Nov. 2, 48 (2002).
10- A. M. Aly and E. A. Fouad, Egypt. J. Pharm. Sci., 1, 39 (2003).
11- M. S. Nagarsenker and V. Y. Londhe, Int. J. Pharm., 251, 49 (2003).
12- R. Peschka, C. Dennehy and F. C. J. Szoka, J. Control. Rel., 56, 41 (1998).
13- M. Cocera, O. Lopez, L. Coderch, J. L. Parra and A. De la Maza, Colloids Surf. A: Physicochem. Eng. Aspects, 221, 9 (2003).
14- V. B. Patel, A. Misra and Y. Marfatia, Pharm. Dev. Techn., 5, 455 (2000).
15- R. R. C. New, "Liposomes: A Practical Approach", Oxford, IRC, 1990, pp. 21, 92, 256.
16- M. C. Finkelestein and G. Weismann, Biochim. Biophys. Acta., 587, 202 (1979).
17- S. L. Law and H. Y. Hung, Int. J. Pharm., 161, 253 (1998).
18- T. Higuchi, J. Pharm. Sci., 50, 874 (1961).
19- M. S. El-Samaligy, N. N. Afifi and E. A. Mahmoud, Int. J. Pharm., 308, 140 (2006).
20- J. Al-Muhammed, A. Y. Ozer, M. T. Ercan and A. A. Hincal, J. Microencasul., 13, 293 (1996).
21- M. Glavas-Dodov, K. Goracinova, K. Mladenovska and E. Fredro-Kumbaradzi, Int. J. Pharm., 242, 381 (2002).

22- R. M. Hathout, S. Mansour, N. D. Mortada and A. S. Guinedi, A.A.P.S. Pharm. Sci. Tech., 8, E1 (2007).
23- M. S. El-Samaligy, O. N. El-Gazayerly and S. Mansour, Bull. Fac. Pharm. Cairo Univ., 42, 57 (2004).
24- S. Vemuri and C. Rhodes, Drug Dev. Ind. Pharm., 21, 1353 (1995).
25- V. Patel and A. Misra, J. Microencapsul., 16, 357 (1999).
26- X. Armengol and J. Estelrich, ibid., 12, 525 (1995).
27- A. Sharma and U. S. Sharma, Int. J. Pharm., 154, 123 (1997).
28- B. Pietzyk and K. Henschke, ibid., 196, 215 (2000).