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Statistical optimization of fluconazole - loaded vesicular systems for the treatmenet of skin fungal infection

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Abstract

The aim of the present study is to improve the bioavailability of fluconazole as a topical antifungal drug by developing an optimized formulation of fluconazole vesicular system for enhanced skin delivery.

Different fluconazole loaded vesicular systems were prepared and optimized using a 2^3 full factorial design considering three independent factors at two levels. In this design, three factors were evaluated; phosphatidyl choline (PC): cholesterol molar ratio (XA), amount of drug added (XB) and type of vesicles either liposomes or ethosomes (XC). The particle size of the vesicles, percentage entrapment efficiency (%EE) and the fluconazole release rate through synthetic membrane were chosen as dependent variables. The levels of the independent variables were chosen based on the preliminary experiments.

All fluconazole formulations showed particle size ranging from 794.2 to 5425 (μm), the drug entrapment efficiency ranged from 71.25 to 93.75% and the in vitro drug released ranged from 150.87 to 239.58 (μg/hr/cm^2). Screening the influence of the studied factors in the factorial design on the responses revealed that the particle size and %EE of liposomes and ethosomes were increased by decreasing both phosphatidyl choline: cholesterol molar ratio and the drug amount. The release rate of fluconazole through synthetic membrane was increased in both liposomes and ethosomes by increasing phosphatidyl choline: cholesterol molar ratio and the drug amount.

It was suggested that the optimized formulation (5:1 phosphatidyl choline: cholesterol molar ratio and 100 mg drug amount) was found to have particle size 5389.85μm, entrapment efficiency 99.5324% and release rate of fluconazole through synthetic membrane 119.906 μg/hr/cm^2.

Keywords: Fluconazole,Vesicular Systems, Full Factorial Design,Optimization, Antifungal Activity

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Introduction

Nanotechnology is widely used nowadays. Nanoparticles are used to carry or entrap drug within them. As a result of that, overcoming the problems of drug can be achieved to increase drug absorption and bioavailability.

Liposomes and ethosomes are types of nanoparticles that can be used to entrap drug within them. They can entrap either hydrophilic or hydrophobic drugs. Each of liposomes and ethosomes has different mechanisms of action. Liposomes have ability to encapsulate drug to protect it from external environment, and reducing toxicity also used to deliver drugs with low penetration through the skin [1][2][3][4]. Ethosomes which are elastic lipid vesicles composed of lipids and ethanol have ability deliver drug with large molecular weight to the site of action as proteins and peptides, the system of ethosomes is non-invasive and passive [5][6][7][8][9].

Fluconazole is a broad spectrum antifungal drug used for treatment of dermal infection. It has low penetration rate when applied topically and many oral side effects [10].

Materials and Methodology

Materials

Fluconazole was kindly provided by Alexandria Co. for pharmaceutical and chemical industries (Alexandria, Egypt), concentrated hydrochloric acid, SDFCL, Mumbai (India), carbopol, Zhong Tang (Dalian) Materials Co., Ltd., cholesterol, Research-lab fine chem. industries, Mumbai (India), and phosphatidyl choline, Lab M limited, UK. were supplied from Sigma Chemical Company (Saint Louis, MO). Disodium hydrogen phosphate, Oxford lab chem., India, dichloromethane, El Nasr pharmaceutical chemicals co, Egypt and ethanol absolute, Biochem., Egypt. were purchased from Al – Nasr pharmaceutical chemicals (Cairo, Egypt).All chemicals and solvents were of analytical grade.

Methodology

Preparation of fluconazole loaded liposomes and ethosomes suspension

Liposomes and ethosomes were prepared by thin film hydration method. Cholesterol and phosphatidyl choline were dissolved in a mixture of 2:1 methylene chloride/ methanol as an organic solvent. The solvent was removed by rotary evaporator (Heidolph 2, Germany) at 40ºC which allows a thin film of dry lipids to form on the inner wall of the flask. The dry lipid film that is formed was hydrated with fluconazole solution and rotary evaporator is used for one hour at room temperature. The obtained vesicles were sonicated for 5 minutes using bath type sonicator (Jiotech UC-10, china). The suspension was left overnight at 4ºC for maturation of vesicles and to ensure full lipid hydration [11].

Experimental Design and Statistical analysis

A 23 full factorial design was employed to evaluate the individual and combined effects of three formulation variables on liposomes and ethosomes performance and characteristics with duplication of each formula. In this design, three factors were evaluated; phosphatidyl choline (PC): cholesterol molar ratio (XA), amount of drug added (XB) and type of vesicles either liposomes or ethosomes (XC). The particle size of liposomes and ethosomes, percentage entrapment efficiency and the fluconazole release rate through synthetic membrane were selected as dependent variables. The levels of the independent variables were chosen based on the preliminary experiments [12].

Design-Expert 7.0.0 software (State-Ease Inc., USA) was used for generation and evaluation of the statistical experimental design. The matrix of the design including investigated factors is shown in Table 1.

Table1: 2^3 Full factorial design for different liposomes and ethosomes containing fluconazole

Code	Normalized Level of individual variables		
	PC:CH (molar ratio) (X_A)	Drug Amount (%mg) (X_B)	Vesicles (X_C)
F1	-1	+1	Liposome
F2	+1	-1	Liposome
F3	+1	+1	Liposome
F4	-1	-1	Liposome
F5	-1	+1	Ethosome
F6	+1	-1	Ethosome
F7	+1	+1	Ethosome
F8	-1	-1	Ethosome

Table2: Levels of investigated factors

Individual variables	High Level (1)	Low level (-1)
X_A PC:CH (molar ratio)	5:1	1:1
X_B Drug amount	200mg%	100mg%
X_C Vesicles' type	Liposome	Ethosome

A first-order polynomial regression equation was generated between the factors and responses as follows:

Y= bo+ b1XA+ b2XB+ b3XC+ b12 (XA XB) + b13 (XA XC) + b23 (XB XC) + b123 (XA XB XC) (1)

Where Y is the dependent variable (response), bo is the intercept representing the arithmetic averages of all the quantitative outcomes of all experimental runs; b1-b3 are the coefficient computed from the observed experimental value of Y; and XA ,XB and XC are the coded levels of factors. The term b12, b13, b23 and b123 represent the interaction terms. Coefficient with one factor represents the effect of that particular factor while the coefficients with more than one factor represent the interaction between those factors. The polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries. A positive sign in front of the terms indicates synergistic effect while negative sign indicates antagonistic effect of the factors. One-way ANOVA was applied to estimate the significance of the model ($P<0.05$) and individual response parameters.

Evaluation of fluconazole loaded vesicles

Particle size measurement: The samples were diluted with phosphate buffer saline for measuring the particle size using zeta sizer (Nano ZS® 90 Malvern,UK) [1]. The determination was performed in triplicate.

Determination of Fluconazole Entrapment Efficiency: Entrapment efficiency was determined by ultracentrifugation technique. All the formulations were centrifuged 48000xg (Megafuge 16R, Germany) for 1 hour followed by filtration. Phosphate buffer saline was added to the supernatant (1ml) of each formula till 10 ml in volumetric flask. The absorbance was assayed by UV spectrophotometrically at 260 nm. [13].The percentage of the drug was calculated from the following equation as follows:

% Entrapment efficiency = (amount of total drug- amount of free drug) / amount of total drug × 100 (2)

Preparation of liposomes and ethosomes gel: Liposomal and ethosomal gel are prepared by adding carbopol (0.5 gm) portion wise to 4 ml of the previously prepared suspension and stirred until a gel was formed [14].

In vitro drug release: In vitro release study was performed using vertical type Franz diffusion cell apparatus. Liposomal and ethosomal gel of different formulations was placed in the donor compartment [15]. Phosphate buffer saline (7 ml) at pH 6.8 was used as receptor medium. The receptor compartment was maintained at 37˚C ± 0.5 and stirred by a Teflon-coated magnetic bar at 1000 rpm for 2 minutes to remove any air bubbles and then adjusted at 500 rpm for the rest of the experiment period. The donor compartment was separated from the receptor compartment by cellophane membrane with molecular weight cut-off of 12,000-14,000 which was soaked in the receptor medium overnight. At predetermined time intervals (0.5, 1, 2, 3, 4, 5, 6 and 24 hour), 400 µl aliquots were withdrawn from the sampling port and were replaced with an equal volume of fresh solvent to maintain constant volume. The samples were analyzed spectrophotometrically at λ max 260 nm in reference with the constructed calibration curve. Constant sampling and replacement with buffer maintained sink conditions. The corrected concentration for fluconazole was determined using correction formulation. For each formulation, drug release was studied in triplicate at least, with keeping the sink conditions and the cumulative amount of drug released was determined [11][16].

Determination of Zeta potential: The samples were diluted with phosphate buffer saline (pH 6.8) for measuring zeta potential using zeta sizer on the optimized formulation [11].

Results and Discussion

Particle size measurement

The vesicle size is very important parameter in the liposomal and ethosomal preparation as it determines the ease of release and permeation of the drug from vesicular structures through the skin [17].

Regarding to the results, the particle size of all formulae were ranging from 794.2 to 5425(µm).The results of vesicles particle size are shown in table (3).

Table3: The particle size of liposomes and ethosomes formulae

Formulae	Particle size (μm)
F1	802.6
F2	1178
F3	5425
F4	794.2
F5	4046
F6	5425
F7	1178
F8	802.6

Equation in terms of coded factors

Particle size = +1807.77+ 878.98 XA- 273.13 XB+1074.37XC - 162.99 XAB +1010.38 XAC -182.23 XBC (3)

- For liposomes

Particle size of liposomes = +733.40- 131.40XA- 90.90XB - 162.993XAB (4)

(Where F=366.34, $P<0.05$ and R^2 =0.996)

- For ethosomes

Particle size of ethosomes = + 2882.13+1889.36XA - 455.36XB - 162.99XAB (5)

(Where F=366.34, $P<0.05$ and R^2 =0.996)

According to equation (4), the negative coefficient of XA reveals that increase in phosphatidyl choline: cholesterol molar will lead to a decrease in the particle size of liposomes. This could be attributed to the increase in the phosphatidyl choline amount which will lead to an increase in the hydrophobicity of the bilayers resulting in limitation of the water uptake to the vesicles core and decrease in the surface free energy, which finally will reduce the vesicular size. While with ethosomes, the increase in PH: CH leads to an increase in the particle size [17]

On decreasing the drug amount, the particle size of liposomes and ethosomes was observed to increase as can be deduced from the negative coefficient of XB, which could be due to the increase in the %EE by decreasing the drug amount which slightly increases the particle size of both liposomes and ethosomes.

The particle size of liposomes and ethosomes was observed to increase by decreasing both the Phosphatidyl choline: cholesterol molar ratio and the drug amount as can be deduced from the negative coefficient of XAB [17].

Equation (3) represented that particle size of both liposomes and ethosomes was observed to increase by the presence of liposomal and ethosomal vesicles as can be deduced from the positive coefficient of XC. Particle size of both liposomes and ethosomes was observed to be augmented by increasing phosphatidyl choline: cholesterol molar ratio and presence of liposomal and ethosomal vesicles as can be deduced from the positive coefficient of XAC.

The absence of liposomal and ethosomal vesicles decrease the drug amount lead to increase the particle size of both liposomes and ethosomes [17][18].

The particle size of liposomes and ethosomes is significantly affected by PC: CH molar ratio, amount of drug, type of vesicle, particle size, entrapment efficiency %EE and in vitro release of the drug as shown in table 4.

Table4: Sum of squares, degree of freedom, df, mean squares, F-values for the model coefficients estimated from the factorial study for the particle size of liposomes and ethosomes

Term	Sum of squares	d.f.	Mean squares	F value	P value
X_A	1.236E+007	1	1.236E+007	550.99	<0.0001
X_B	1.194E+006	1	1.194E+006	53.20	<0.0001
X_C	1.847E+007	1	1.847E+007	823.18	<0.0001
X_{AB}	4.251E+005	1	4.251E+005	18.95	0.0018
X_{AC}	14.633E+007	1	1.633E+007	728.05	<0.0001
X_{BC}	5.313E+005	1	5.313E+005	23.68	0.0009

Normal probability curve for the particle size of liposomes and ethosomes can be shown in figure 1 which shows the significant effects that are near and far from the normal curve.

Contour plots and 3D surface plots were obtained by fixing the XC factor using either liposomes or ethosomes and varying the XA and X B factors over the ranges used in the factorial study as represented in Figure 2

Entrapment Efficiency measurements (EE%)

Entrapment efficiency is the percent of the total drug incorporated into liposomes and ethosomes, 18,000 rpm was used which was effective for the separation process [18].

Regarding to the results, the drug entrapment efficiency for all formulae were ranged from 71.25 to 93.75% as shown in table 5

Figure 1: Normal probability plot of liposomes and ethosomes formulae for screening the influence of the particle size

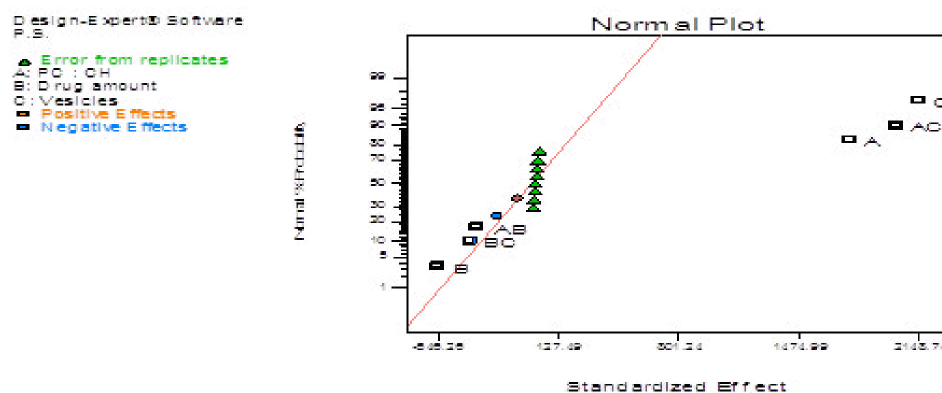


Figure 2: 3D (a and b) and surface contour plots (c and d) of particle size of liposomes and ethosomes

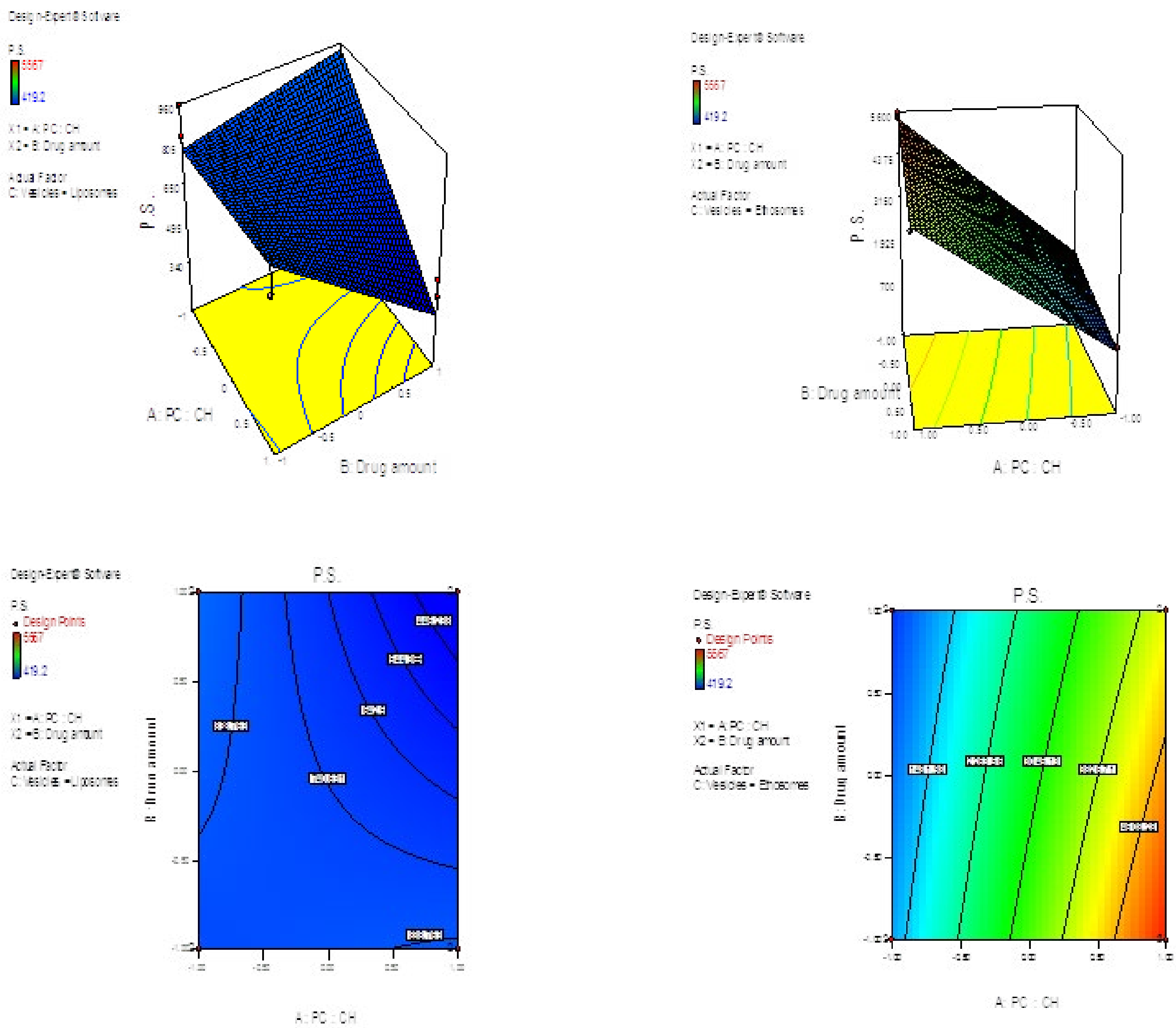


Table5: The percentage of entrapment efficiency of liposomes and ethosomes formulae

Formulae	EE%
F1	75.75%
F2	93.75%
F3	98.25%
F4	71.25%
F5	84.25%
F6	97.56%
F7	92.87%
F8	74.95%

Equation in terms of coded factors

%EE= +76.41+2.97XA -1.08XB+11.24XC -0.61XAB +0.34XAC - 6.88XBC (6)

- For liposomes

%EE= +65.168+2.636XA+ 5.801XB -0.613XAB (7)

(Where F=97.64, $P<0.05$ and R^2 =0.996)

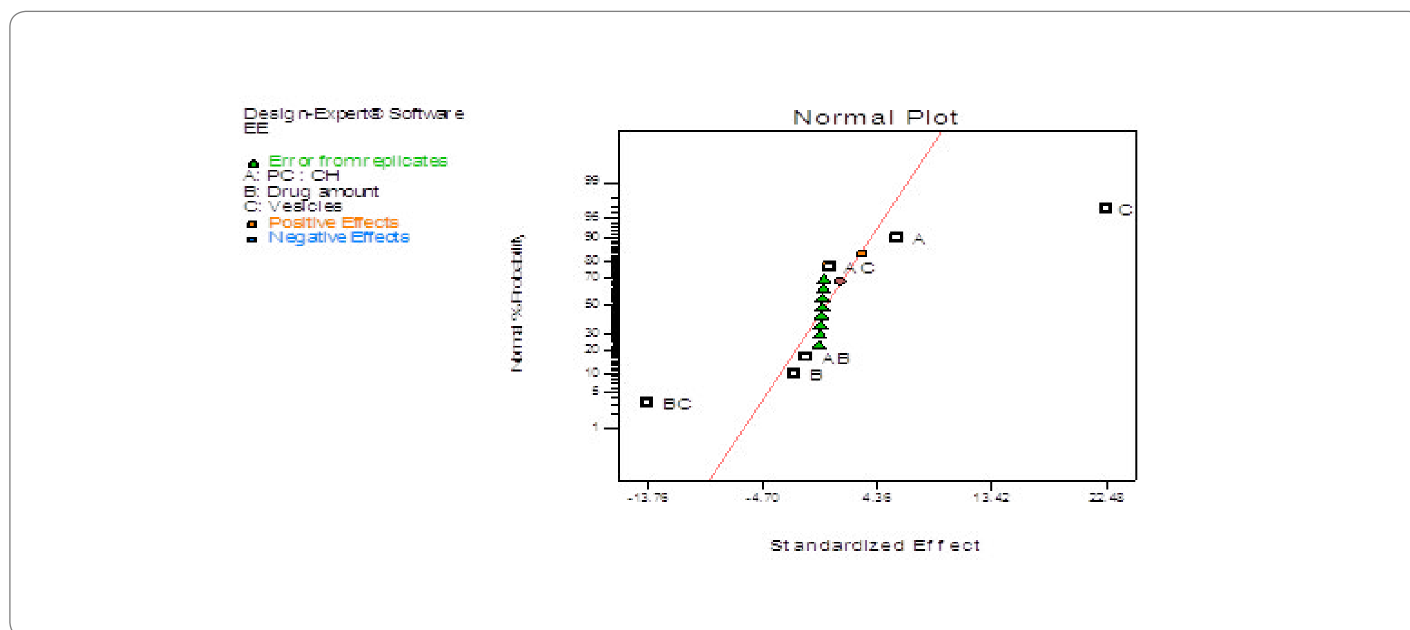
- For ethosomes

%EE= +87.648+3.311XA-7.958 XB-0.6137XAB (8)

(Where F=97.64, $P<0.05$ and R^2 =0.994)

As can be deduced from equations (7), (8), increase in PC: CH molar lead to increase in the entrapment efficiency of both liposomes and ethosomes. The reason of this is that by increasing phosphatidyl choline: cholesterol ratio, the formation of the bilayers increases and the drug holding capacity and multi-lamillarity of vesicles are increased.

Figure 3: Normal probability plot of liposomes and ethosomes formulae for screening the influence of the %EE



On increasing the drug amount, the %EE is observed to increase in liposomes as can be deduced from the positive coefficient of XB , while increasing the drug amount will decrease the %EE of ethosomes as can be deduced from the negative coefficient of XB. . The reason for increasing %EE of liposomes is that the drug is enforced to be encapsulated to the vesicles due to saturation of the medium with the drug. Decreasing %EE of ethosomes is caused because high concentration of ethanol makes the vesicle membrane leaky thereby decreases the drug entrapment [18].

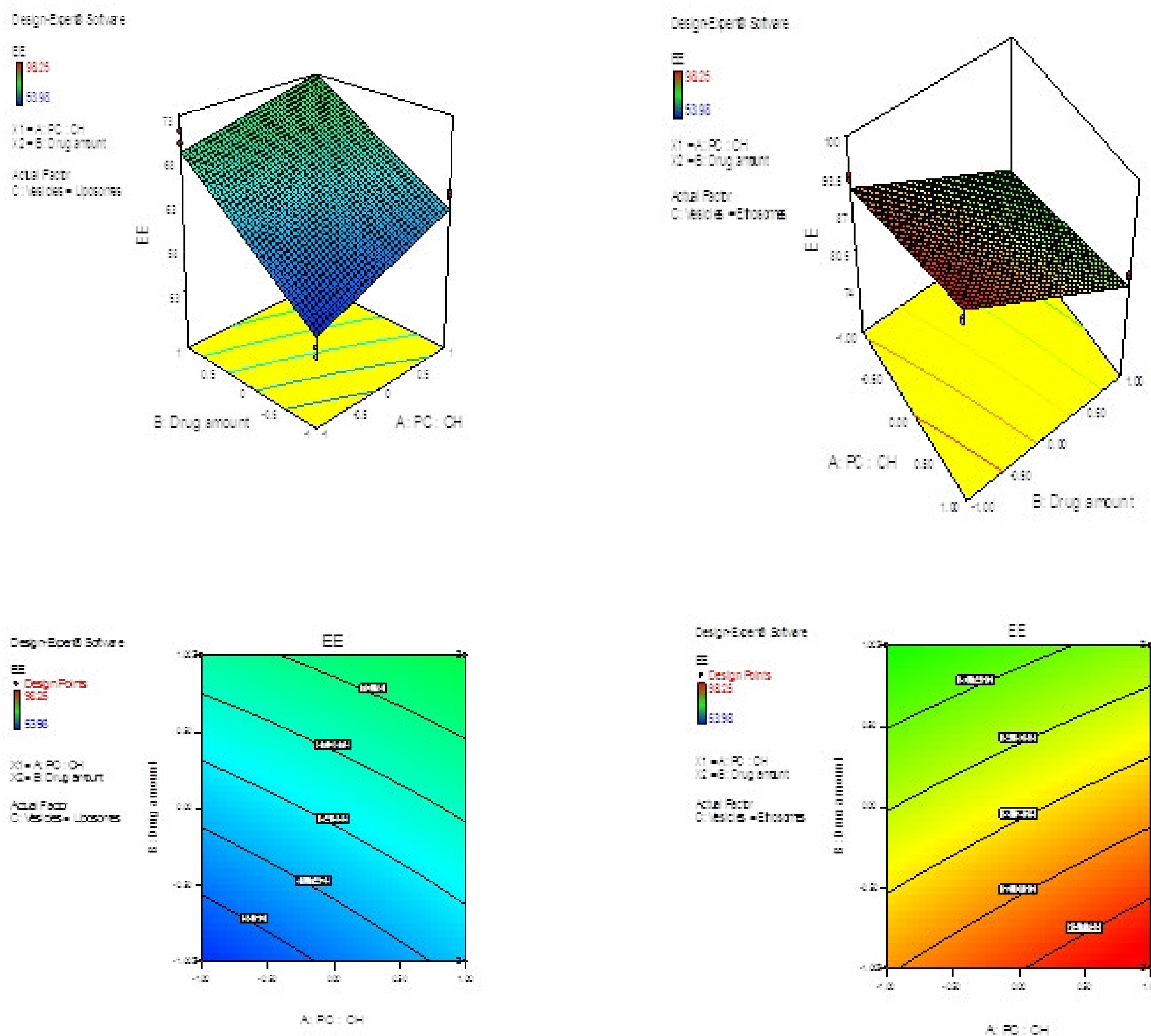
Equation (6) shown that %EE was observed to increase for both liposomes and ethosomes by the presence of liposomal and ethosomal vesicles as can be deduced from the positive coefficient of XC.

On decreasing the drug amount and absence of liposomal and ethosomal vesicles, %EE was observed to increase for both liposomes and ethosomes as can be deduced from the negative coefficient of XBC [18]. As shown from table (6), the entrapment efficiency is significantly affected by all the factors except (XAB, XAC) which were insignificant Contour plots and 3D surface plots were obtained by fixing the XC factor using either liposomes or ethosomes and varying the XA and XB factors over the ranges used in the factorial study as represented in Figure 4.

Table6: Sum of squares, d.f, degree of freedom, mean squares, F-values and P-values for the model coefficient estimated from the factorial study for the %EE of liposomes and ethosomes

Term	Sum of squares	d.f.	Mean squares	F value	P value
X_A	141.49	1	141.49	28.13	0.0005
X_B	18.62	1	18.62	3.70	0.0865
X_C	2021.40	1	2021.40	401.87	<0.0001
X_{AB}	6.03	1	6.03	1.20	0.3021
X_{AC}	1.82	1	1.82	0.36	0.5621
X_{BC}	757.35	1	757.35	150.57	<0.0001

Figure 4: 3D (a and b) and surface contour plots (c and d) of %EE.



In vitro drug release

The in vitro drug released for all formulae were ranged from 150.87 to 239.58 (µg/hr/cm2). As shown in Figures 5-6

Any factor that increases the formation of liposomes and ethosomes or increases its %EE, will lead to retardation in the release rate of fluconazole through synthetic membrane, as the drug passes through the bilayers of the vesicular structure leading to a decrease in the release rate of fluconazole through synthetic membrane [19].

Equation for the corrected concentration was employed as follows (ref 67)

C1n = Cn [VT / (VT- VS)] (C1n-1 / Cn-1) (9)

As observed in figures (5-6), the in vitro release profile of the drug from the formulae followed a biphasic release. This could be attributed to the presence of free drug together with the entrapped one, which is due to the limited capacity of the lipid to hold large amounts of the drug leading to disposition of the free drug at the surface. This will lead to an initial rapid release due to the presence of the free drug and the drug present in the aqueous core, followed by slower sustained release phase due to diffusion of the entrapped drug through the lipid bilayers of the vesicles, which is very effective in sustaining and controlling the release of fluconazole. These findings were in accordance with Elzaafarany et al. 2010, Aboelwafa et al., 2010, Gillet et al., 2009, Guinedi et al., 2005 and Jain et al., 2003 [20] [21].

Figure 5: In vitro release profiles of fluconazole from liposomes from formulae (F1-F4)

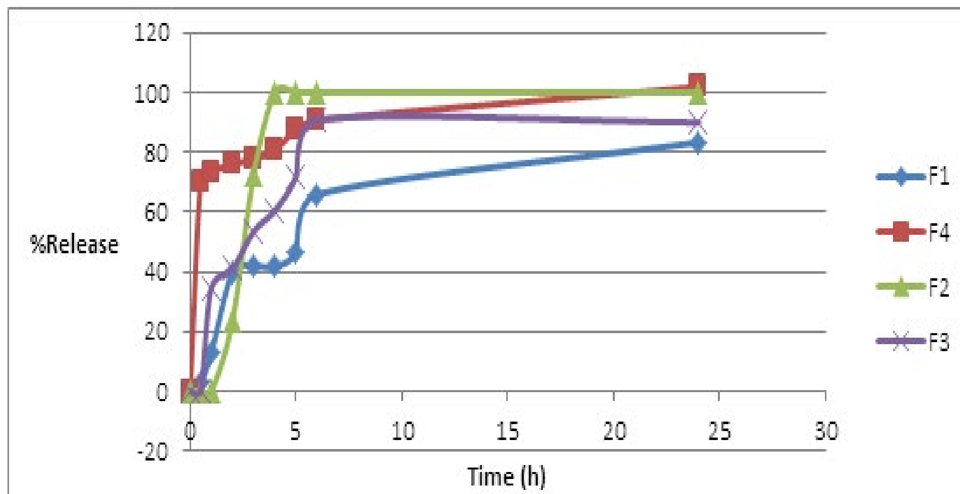
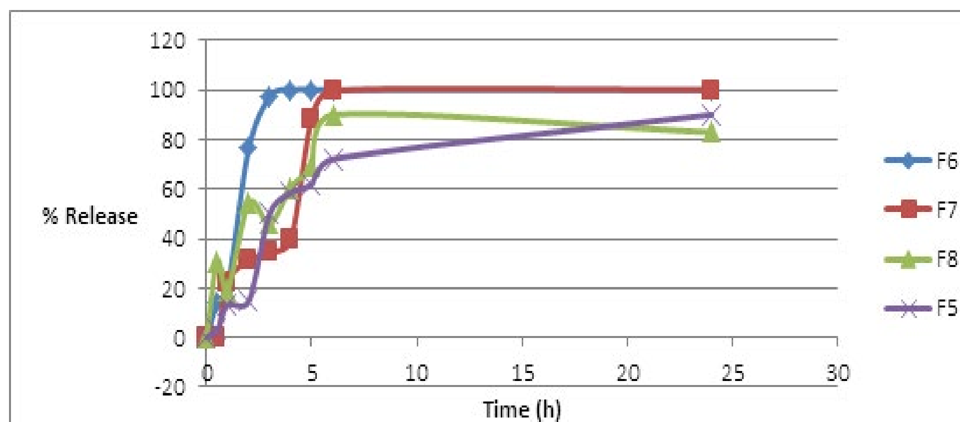


Figure 6: In vitro release profiles of fluconazole from ethosomes from formulae (F5-F8)



Equation in terms of coded factors

Release rate = +218.06-4.97XA+13.29XB -35.86XC +20.14XAB -23.89XAC -1.875E-003XBC (10)

- For liposomes

Release rate = +253.92+18.91XA+13.29XB+20.13XAB (11)

(Where F =3.59, P<0.05, R^2 =0.996)

- For ethosomes

Release rate = +182.20 -28.86XA+13.29XB+20.13XAB (12)

(Where F =3.59, P<0.05, R^2 =0.994)

An increase in the release is observed to occur as both of phosphatidyl choline: cholesterol molar ratio and the drug amount increase due to the positive coefficient of X_{AB} as can be observed from equation (11), (12).

According to equation (10), the release rate for liposomes and ethosomes is observed to decrease by the presense of liposomal and ethosomal vesicles due to the negative coefficient of XC, which could be attributed to the long pathway of the drug through the vesicles [18].

The release rate for liposomes and ethosomes is observed to decrease by increase phosphatidyl choline: cholesterol molar ratio and presence of liposomal and ethosomal vesicles as a result of the negative coefficient of XAC.

On increasing the drug amount and presence of liposomal and ethosomal vesicles, the release rate for liposomes and ethosomes is observed to decreased as can be deduced from the negative coefficient of XBC.

As shown in table (7), the release rate of fluconazole through synthetic membrane is significantly affected by all factors except XA and XB. Increase in the release is observed to occur as both of phosphatidyl choline: cholesterol molar ratio and the drug amount increase due to the positive coefficient of XAB.

Normal probability curve for the release rate of fluconazole through synthetic membrane can be noticed from figure (7) which shows the significant effects that are near and far from the normal curve.

Contour plots and 3D surface plots were obtained by fixing the XB factor at its high and low levels and varying the XA and XC factors over the ranges used in the factorial study as represented in Figure 8

Figure 7: Normal probability plot of liposomal and ethosomal formulae for screening of the influence of the release rate of fluconazole through synthetic membrane

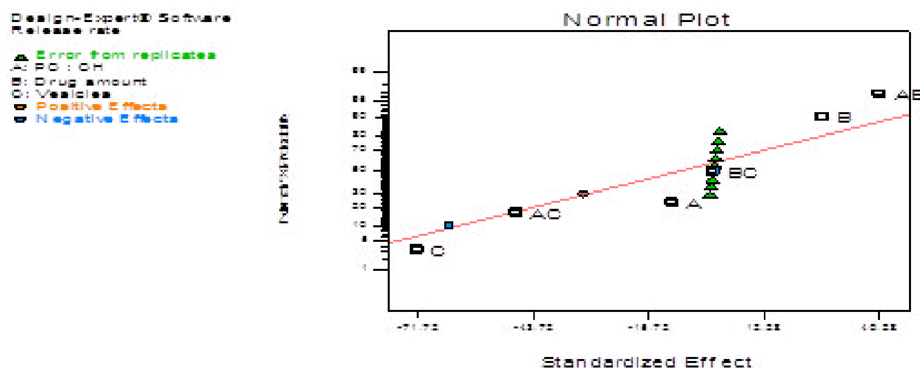


Table 7: Sum of squares, d.f,degree of freedom, mean squares, F-values and P-values for the model coefficient estimated from the factorial study for the drug release of liposomes and ethosomes

Term	Sum of squares	d.f.	Mean squares	F value	P value
X_A	395.91	1	395.91	0.22	0.6529
X_B	2826.78	1	2826.78	1.54	0.2453
X_C	20575.75	1	20575.75	11.24	0.0085
X_{AB}	6489.51	1	6489.51	3.55	0.0923
X_{AC}	9134.10	1	9134.10	4.99	0.0523
X_{BC}	5.625E-005	1	5.625E-005	3.074E-008	0.9999

Contour plots and 3D surface plots were obtained by fixing the XB factor at its high and low levels and varying the XA and XC factors over the ranges used in the factorial study as represented in Figure 8

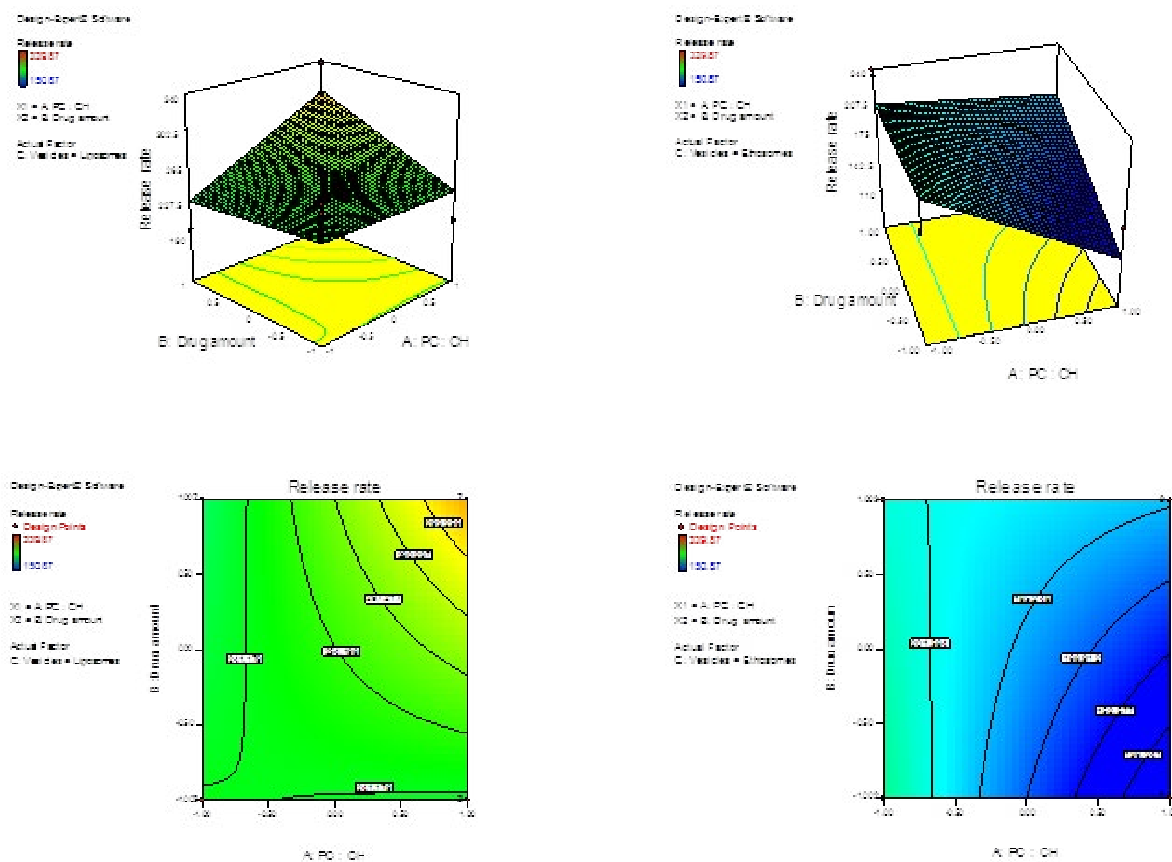


Figure 8: 3D (a and b) and surface contour plots (c and d) of release rate of fluconazole through synthetic membrane.

Optimization

The software Design Expert 7.0.0 suggested one ethosomal formula (F6). The values for the suggested formula and the predicted values are shown in tables (8 and 9).

From the obtained results, the %EE of fluconazole is higher in ethosomal formula six (F6) which contains high amount of phosphatidyl choline: cholesterol molar ratio and low amount of drug. The reason for increasing the %EE is by decreasing the drug amount; incorporation of the drug into ethosomal vesicle is more and easier.

In addition, increasing the amount of phosphatidyl choline: cholesterol molar ratio leads to raise the formulation of the bilayers, increase drug holding capacity and increase multilamillarity of vesicles [22].

Zeta potential measurement

Zeta potential is the measure of charge repulsion or attraction between particles and also affects the stability of vesicles. Its measurement gives details about the causes of aggregation, dispersion or flocculation. Optimized formula showed accepted zeta potential which lies in the range between -10.9 mV [11][19].

Table8: The coded values for the optimized ethosomal formula (F6)

Factor	Optimized level
X_A; Phosphatidyl choline : cholesterol molar ratio	+1
X_B; Drug amount	-1
X_C; liposomal or ethosomal vesicles	Ethosome

Table9: The observed and the predicted values for the optimized ethosomal formula (F6)

Response	Expected	Observed	Residual
Particle size (µm)	5425	5389.85	35.15
%EE	97.56%	99.5324%	1.9724
Release rate (µg/hr/cm2)	152.95	119.906	33.044

Conclusion

Liposomes and ethosomes are vesicular structures which are used to entrap fluconazole antifungal drug within them. Liposomes and ethosomes were prepared using thin film hydration method and full factorial design, 23, was carried out. Three main factors; Phosphatidyl choline: cholesterol molar ratio (XA), amount of drug added (XB) and types of vesicles either liposomes or ethosomes (XC) were used in this design each at two levels (low and high), where 8 formulae were suggested by Design Expert 7.0.0 software. The dependent responses selected were particle size of liposomes and ethosomes, %EE, and the in vitro drug release rate of fluconazole through synthetic membrane. All fluconazole formulae showed particle size ranging from 794.2 to 5425 (µm), the drug entrapment efficiency ranged from 71.25 to 93.75 % and the in vitro drug released ranged from 150.87 to 239.58(µg/hr/cm2). Screening the influence of the studied factors in the factorial design on the responses revealed that the particle size and %EE of liposomes and ethosomes were increased by decreasing both phosphatidyl choline: cholesterol molar ratio and the drug amount. The in vitro drug release rate of fluconazole was increased in both liposomes and ethosomes by increasing phosphatidyl choline: cholesterol molar ratio and the drug amount. The optimized formula (5:1 phosphatidyl choline: cholesterol molar ratio and 100 mg drug amount) was found to have particle size 5389.85µm, entrapment efficiency 99.5324% and release rate of fluconazole through synthetic membrane 119.906 µg/hr/cm2. The observed results were much closed to the expected results indicating validity of the design.

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