ORIGINAL ARTICLE

Proniosomal gel-mediated topical delivery of fluconazole: Development, *in vitro* characterization, and microbiological evaluation

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ABSTRACT

The aim of this study was to explore the potential of proniosomal gel for topical delivery of fluconazole, an antifungal drug used in fungal infections caused by pathogenic fungi. Fluconazole-loaded proniosomal gels were prepared by the coacervation phase separation method using different nonionic surfactants (spans and tweens). The prepared fluconazole proniosomal gels were evaluated for various parameters such as particle size (PS), drug entrapment efficiency percentage (EE%), and *in vitro* drug release. The experimental results showed that the EE% for the prepared formulae are acceptable (85.14%–97.66%) and they are nanosized (19.8–50.1 nm) and the diffusion from the gels gave the desired sustaining effect. F4, which was prepared from span 60, tween 80 (1:1), and cholesterol showed highest EE% and gave slow release (40.50% ± 1.50% after 6 h), was subjected to zeta potential (ZP) test, transmission electron microscopy as well as microbiological study. The results showed a well-defined spherical vesicle with sharp boundaries with good physical stability of fluconazole within the prepared gel. Moreover, F4 showed an excellent microbiological activity represented by a greater zone of inhibition (5.3 cm) compared to control gel (fluconazole in 2% hydroxy propyl methyl cellulose (HPMC) gel formula) (4.2 cm) and plain gel with no drug (0 cm) against *Candida albicans*. This study showed the suitability of the proniosomal gel in attaining the desired sustainment effect for topical delivery of fluconazole for the management of fungal infection. The physical stability study showed that there was no significant change in EE%, PS, and ZP of fluconazole proniosomal gel after storage for 6 months.

Key words: Antifungal drugs, fluconazole, proniosomal gel, provesicular drug delivery system

INTRODUCTION

Fluconazole is a first-generation triazole antifungal medication. It differs from earlier azole antifungals (such as ketoconazole) in that its structure contains a triazole ring instead of an imidazole ring.[1] The imidazole antifungals are mainly used topically; however, fluconazole and certain other triazole antifungals are used for systemically treatment because there are safe drugs and have good oral absorption.[2,3] It acts by interacting with lanosterol14-α demethylase, a cytochrome P-450 enzyme

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necessary to convert lanosterol to ergosterol, as ergosterol is an essential component of the fungal cell membrane. The inhibition of its synthesis results in increased cellular permeability causing leakage of cellular content.[4] Vesicular drug delivery systems using colloidal particulate carriers, such as liposomes or niosomes, have distinct advantages over conventional dosage forms. Proniosomal gels are structurally similar to liposome and niosome having a bilayer; however, the materials used to prepare proniosomes make them more stable and offer many more advantages over liposome and noisome.[5] Proniosomes reduce the physical stability problems of niosomes such as leaking, fusion, and aggregation and provide additional convenience in transportation, distribution, storage, and dosing.[6] Proniosomes can entrap both lipophilic and hydrophilic drugs either in aqueous layer or vesicular membrane and present low toxicity because of their nonionic nature.[7] Proniosomes are dry formulations of that can be measured as needed and rehydrated by brief agitation in hot water.[8]

MATERIALS AND METHODS

Materials

Fluconazole was provided as a gift sample from Sedico, Egypt. Spans (20 and 60) were purchased from Al-Rowad Chemicals 6th of October, Egypt. Tween 80 was purchased from Sedico, Egypt. Cholesterol extrapure was purchased from Mekkawy chemicals (Giza, Egypt). Ethanol was purchased from Delta Company for chemical industries, Egypt. All other chemicals and solvents were of analytical grade and obtained from El-Nasr Company for Pharmaceutical Chemicals, Cairo, Egypt.

Methods

Formulation of fluconazole proniosomal gels

Proniosomes were prepared using coacervation phase separation method[9] with some modifications using different surfactants from Span and Tween series, for topical application. The composition of different proniosomal formulations is summarized in Table 1. In a beaker, the surfactant and cholesterol were mixed with 0.5 ml of absolute ethanol, and then 50 mg of fluconazole was added. Then, the beaker was covered with a lid to prevent the loss of solvent and the beaker was warmed in a water bath (55°C–60°C) for 5 min while shaking until the complete dissolution of cholesterol. Then, about 0.16 ml of hot distilled water (55°C–60°C) was added while warming in the water bath for 3–5 min till a clear or translucent solution was produced.[10] The mixture was allowed to cool at room temperature until the dispersion was converted to gel. The obtained gels were stored in the same closed beaker for further characterization.

Evaluation of the prepared formulations

Organoleptic properties

Proniosomal gels were characterized for appearance, color, and homogeneity by visual inspection.

Optical microscopy

One drop of the formed gel was spread on a glass slide and examined for the vesicle structure using ordinary light microscope with varied magnification powers (×10 and ×40).[11] Photomicrographs were taken using a digital camera (Sony Cybershot DSCw55 7.2 megapixel, Tokyo, Japan).

pH measurement

The pH of the gel was determined by digital pH meter (Model 420, ORION, USA). A sample of 0.1 g of gel was dissolved in 10 ml of distilled water and the electrode was then dipped into gel formulation and constant reading was noted.[12] The readings were taken for an average of three times.

Determination of drug entrapment efficiency

A sample of 0.2 g of proniosomal gel was taken in a glass tube, and 10 ml of phosphate buffer (pH 7.4) was added. This aqueous suspension was sonicated in a sonicator bath (Rolex, India), followed by centrifugation at 9.000 rpm at 20°C for 30 min (Ultra centrifuge 5417R, Eppendorf, Hamburg, Germany). The supernatant was collected and assayed by using ultraviolet (UV) method (Shimadzu UV spectrophotometer (2401/PC), Japan) for unentrapped fluconazole content at 260 nm.[11] The percentage of drug encapsulation (entrapment efficiency percentage [EE%]) was calculated by the following equation:

EE% = (Total amount of drug − Unentrapped drug/Total amount of drug) ×100.

In vitro release study

The release of fluconazole from proniosomal gels was determined using membrane diffusion technique. The proniosomal gel equivalent to 25 mg of fluconazole was placed in a glass tube having a diameter 2.5 cm with an effective length of 8 cm that was previously covered with soaked osmosis cellulose membrane with a molecular weight cutoff 12,000 Daltons, which acts as a donor compartment. The glass tube was placed in a beaker containing 100 ml of phosphate buffer pH 5.5, which acts as a receptor compartment. The whole assembly was fixed in such a way that the lower end of the tube containing gel was just touched (1–2 mm deep) the surface of diffusion medium. The temperature of receptor medium maintained at 37°C ± 100°C, and the medium was agitated at 100 rpm speed using magnetic stirrer. Aliquots of 3 ml sample were withdrawn periodically and replaced with equal volume to maintain the volume constant of the receptor's phase. The collected samples were analyzed for the drug containing at 260 nm absorbance against a reagent using the UV spectrophotometer.[13]

Particle size analysis of fluconazole proniosomes

The particle size (PS) and Polydispersity Index (PDI) of proniosomes were measured using a Zeta sizer 3000

Table 1: Composition of different fluconazole proniosome formulations

Code	Fluconazole (mg)	Span 60 (mg)	Span 20 (mg)	Tween 80 (mg)	Cholesterol (mg)	Ethanol (ml)	Water (ml)
F1	50	450	-	-	50	0.5	0.16
F2	50	-	450	-	50	0.5	0.16
F3	50			450	50	0.5	0.16
F4	50	225		225	50	0.5	0.16
F5	50		225	225	50	0.5	0.16
F6	50	150		300	50	0.5	0.16
F7	50	300		150	50	0.5	0.16
F8	50		150	300	50	0.5	0.16
F9	50		300	150	50	0.5	0.16

PCS (Malvern Instr., England) equipped with a 5 mW helium–neon laser with a wavelength output of 633 nm. Measurements were made at 25°C, angle 90, and runtime at least 180 s.[14] The proniosomal gels were appropriately diluted with distilled water before measurements. PDI was determined as a measure of homogeneity. Small values of PDI (<0.1) indicate a homogeneous population, while PDI values >0.3 indicate high heterogeneity.[15]

Zeta potential analysis

Charge on drug-loaded vesicles surface was determined using zeta potential (ZP) analyzer (A Brookhaven Instrument Corp). Analysis time was kept for 60 s, and average ZP and charge on the proniosome preparation after hydration with phosphate buffer saline pH 7.4 were determined at 25°C and three runs were carried out.[16]

High-resolution transmission electron microscopy

The selected and prepared fluconazole proniosomal gel was characterized for its shape by transmission electron microscopy (JEOL Model - JEM 2100–200KV, Tokyo, Japan), using a 300 mesh carbon-coated copper grid and phosphotungstic acid (1%; w/v) as a negative stain. After being stained, the samples were allowed to dry at room temperature for 10 min for investigation.[17]

Physical stability studies

The selected fluconazole proniosomal gel (F4) was evaluated for their stability by storing and sealing in well-closed containers in the refrigerator at 4°C ± 1°C for 6 months. The stability study was performed according to different parameters, including physical appearance, %EE, PS, and ZP.[13] The changes of %EE, PS, and ZP against storage time were monitored.

Microbiological study of fluconazole proniosomal gel

The *in vitro* antifungal efficacy of fluconazole pronisomal gel was determined by performing agar-cup diffusion assay.[18] The assay was performed using cultures of *Candida albicans* (ATCC 60193) (0.1%), in Sabouraud dextrose agar. The strain was inoculated in sterile 0.85% NaCl tube in a ratio of 1:9. The culture was subjected to further dilution in a sterile 0.85% NaCl to get 106 CFU/ml. Sterile swab was dipped into the culture suspension and then placed on the edge of the agar plate and moved across to the other sides. Cups were made in the seeded agar plates of 6-mm diameter.[19] Cups were filled with 0.5 ml of the proniosomal gel and an equivalent weight of control and plain gel. The Petri dishes were then incubated at 37°C. The effectiveness of the prepared gel was compared with plain gel contains 0% of fluconazole and the control. The zones of growth inhibition were measured for all the tested samples. Each type of samples was tested in triplicate. The inhibition zone of growth of *C. albicans* was measured in mm after 48 h and the mean inhibition zone was then calculated.[20]

Statistical analysis

Statistical analysis of the results was performed using one-way analysis of variances to determine the significance of differences between groups; $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Organoleptic properties

The physical appearance results are shown in Table 2. It was found that the formulae 1, 4, 6, and 7 were white creamy gel; formula 2 was viscous gel; formula 3 was pale yellow gel; and the formulae 5, 8, and 9 were pale yellow viscous gel.

Optical microscopic examination

The photomicrograph of fluconazole (F4) is shown in Figure 1. It showed the presence of homogenous population of vesicles with spherical shape.

pH measurement

The pH of proniosomes was in the acceptable limits for topical application as they were ranged from 5.2 to 6.9 as shown in Table 2.

Fluconazole entrapment efficiency in proniosomes

The entrapment efficiency of fluconazole in different proniosomal gels is summarized in Table 2. F4 and F1 prepared using span 60 and tween 80 (1:1) and span 60 alone showed the highest entrapment efficiency

Table 2: The entrapment efficiency percentage, particle size, Polydispersity Index, and physical appearance of fluconazole in different proniosomal gels

Code	EE%±SD	Size±SD (nm)	PDI±SD	Physical appearance	pH
F1	93.37±1.09	19.80±1.10	0.24±0.023	White creamy gels	5.2
F2	91.64±0.98	35.47±1.07	0.11±0.004	Viscous gel	5.6
F3	87.43±1.99	50.10±1.63	0.28±0.150	Pale yellow gels	6.2
F4	97.66±1.24	25.41±0.51	0.17±0.070	White creamy gels	6.1
F5	89.56±1.94	33.20±0.67	0.23±0.056	Pale yellow viscous gel	5.9
F6	90.24±1.02	31.40±0.94	0.21±0.014	White creamy gels	6.9
F7	92.21±1.32	21.52±0.57	0.13±0.021	White creamy gels	6.4
F8	85.14±1.07	30.45±0.74	0.26±0.028	Pale yellow viscous gel	5.5
F9	89.74±1.54	35.54±0.41	0.22±0.019	Pale yellow viscous gel	5.9

EE%: Entrapment efficiencWy percentage, PS: Particle size (nm), SD: Standard deviation, PDI: Polydispersity Index,

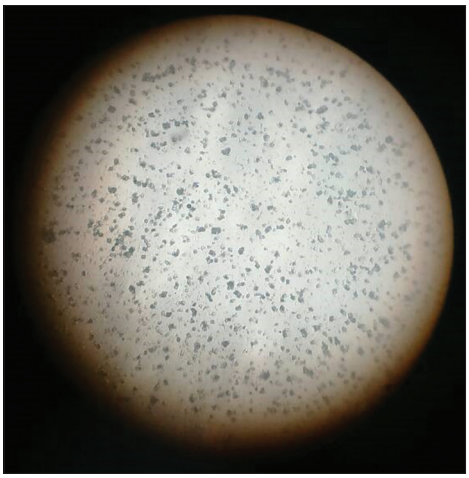

Figure 1: Microscopical examination of proniosomal gel F4

(97.66% and 93.37%, respectively). From these results, it was found that formulations containing span 60 alone showed higher entrapment efficiency than formulations containing span 20 and tween 80. The formulation containing tween 80 alone showed the least entrapment efficiency. This can be explained by many facts as follows:

a. The hydration temperature used to make niosomes should usually be above the gel–to-liquid phase transition temperature of the system that results in niosomes that are less leaky and have high entrapment efficiency.[21] Span 60 has highest phase transition temperature (50°C) and entrapment efficiency % as compared to both span 20 and tween 80[22]
b. The longer alkyl chain influences the hydrophilic lipophilic balance (HLB) value of the surfactant mixture which in turn directly influences the drug entrapment efficiency.[23] The lower the HLB of the surfactant, the higher will be the drug entrapment efficiency and stability as in the case of proniosomes prepared using span 60[24]
c. This could also be attributed to the structure, orientation, and packing behavior of the surfactants. Span 60 has longest saturated chain length and shows highest entrapment.[25]

Zeta potential and particle size results

Proniosomes (F4) that contain fluconazole, cholesterol, span 60, and tween 80 (1:1) as surfactants were subjected to appropriate dilution using ionized water where niosomes are derived and its dispersion was detected utilizing the ZP analyzer. ZP was found to be-32.9 mV with a negative charge. Wen *et al*. stated that the high value of ZP (more than ± 30 mV) showed greater repulsion between charged particles, therefore, reducing aggregation or flocculation and electrically stabilized the colloidal particles.[26] Junyaprasert *et al*. obviated that the preferential adsorption of hydroxyl ions at the vesicle surface was responsible for the negative surface charge of vesicles made from nonionic surfactants.[27]

The result showed a good physical stability of the fluconazole within the prepared gel was obtained, as ZP increased, also the repulsion between the vesicles has increased which prevent their reaggregation and provided electrical stability of the system.[28,29] The PS of all the prepared pronisomal gel was in the nanosize range (19.80–50.12 nm). The vesicles were discrete and separate with no aggregation or agglomeration [Figure 2]. It was observed that the vesicles were smaller with the presence of span 60 as compared to tween 80 alone (F1–F3). As the ratio of span 60 increased in the formulation (F4, F6, and F7), the vesicle size also decreased followed by span 20 (F5, F8, and F9) which is in an agreement to the findings of Pankaj *et al*.[30] Alsarra *et al*. have reported that spans are more hydrophobic, so as to minimize its surface free energy, it forms smaller vesicles, whereas tweens are hydrophilic; hence, the water intake of these bilayers increased and resulted in larger niosomes.[25]

In vitro release of fluconazole from proniosomal gel

Figure 3 showed that the rate of release of fluconazole from proniosomal gel prepared from span 20 (F2) was lower than span 60 proniosomal gel (F1); however, proniosomes prepared from a mixture of span 60 and tween 80 showed a greater decrease in the *in vitro* release of fluconazole. The least amount of fluconazole released was observed with proniosomal formula containing only tween 80 (F3), and it may be attributed to the hydrophilic nature of surfactant. The proniosomal gel formulation (F4) which prepared from

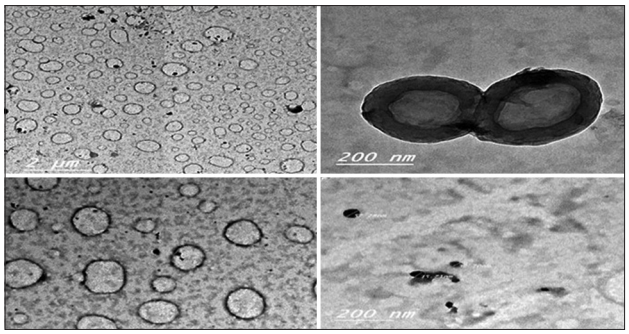

Figure 2: Transmission electron micrograph of fluconazole-loaded proniosome (F4)

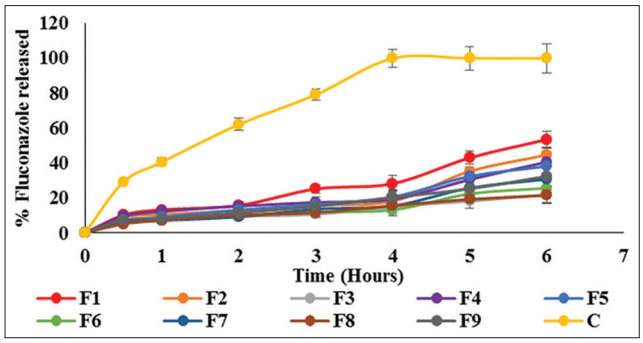

Figure 3: *In vitro* release profile of fluconazole-loaded proniosomes

span 60, tween 80 (1:1), and cholesterol showed the highest EE% and a significant *in vitro* prolonged release of drug at $P < 0.05$, and it was chosen as best formula. The slower release of fluconazole from proniosomal gel formulations compared to control gel may be due to drug encapsulation into vesicles providing prolonged drug release rate. The proniosomal gel (F3) gave a significant lowest drug release at $P < 0.05$ among the tested formulations (about 21.85% ± 5.32% after 6 H). The selected proniosomal gel (F4) showed a significant prolonged drug release at $P < 0.001$ which was 40.52% ± 8.25% after 6 h, in comparison to the control which released 99.8% ± 5.11% of fluconazole within 4 h [Figure 3]. This could be related to the condensed vesicular structure composed of span 60, tween 80, and cholesterol, which is considered a great barrier to drug diffusion and a retardant to its release. Thakur *et al*. obviated that the delayed release of proniosomal gel formulations is due to the slow release of drug from proniosomes and this may be attributed to the need of proniosomes for a time to be hydrated to form niosomal vesicles before starting release of drug across the cellophane membrane.[31] Thus, the formulation exhibited zero-order release over this period. Gupta *et al*. attributed this fact as the molecules of spans 60 and 20 are in an ordered gel state at the *in vitro* permeation condition of 25°C.[9]

Transmission electron microscope

High-resolution transmission electron micrographs showed that the investigated proniosomal formula (F4) was nanosized, as shown in Figure 2. Micrographs showed that the proniosomal particles were smooth, spherical, and homogenous nanovesicles and revealed the formation of well-defined spherical vesicles with sharp boundaries. They formed an enclosed bilayer because it has amphiphilic nature with physical agitation and some energy such as instance heat which facilitates to form this structure.[32]

Physical stability studies

In the present study, stability studies were performed on the selected formulation. It was stored at 4°C ± 1°C for 6 months and observed for the change in %EE, average PS of the vesicles, and ZP. It was observed that there was no change in the physical appearance as there was neither change in the formulation consistency nor aggregation of vesicles. Furthermore, there was no significant change at $P > 0.05$ in EE%, PS, and ZP of fluconazole proniosomal gel after the storage for 6 months [Table 3]. Our findings were in accordance with the study done by Sandeep *et al*. who explained the nonsignificant change in size and EE% in span 60 surfactant-based formulation due to its high phase transition temperature and low permeability.[11] This result showed the high stability and suitability of proniosomal gel for the topical fluconazole delivery.

Microbiological study of fluconazole proniosomal gel

The *in vitro* antifungal efficacy of fluconazole pronisomal gel was determined by performing agar-cup diffusion assay. The microbiological study was carried out for (F4) proniosomal gel, plain (proniosome gel without drug) and control (fluconazole in 2% hydroxy propyl methyl cellulose (HPMC) gel formula). The diameter of zone of inhibition (ZI) obtained with the three groups was shown in Figure 4. F4 showed ZI of 5.3 cm compared to ZI of 4.2 cm obtained for the control gel while there were no ZI for the plain gel after 48 h. The explanation for the significant increase of ZI at ($P < 0.05$) of F4 is that ZI largely depends on the solubility and diffusion of the fluconazole through the agar media and exert its fungistatic effect against *C. albicans*, by inhibiting the cytochrome P-450-dependent enzyme lanosterol demethylase which is required for the conversion of lanosterol to ergosterol.[33] The obtained results revealed that the developed proniosomal gel was more efficient when compared to the control (2% HPMC gel). Figure 5 shows a comparison between the mean values of inhibition zones of fluconazole proniosome gel (F4), control and plain gel after 48 h. Results indicate that F4 exhibits maximum antifungal activity after 48 h. This proniosome gel acts as carriers for drug delivery to the particular site of action; the antifungal activity is created by the drug incorporated in the proniosome gel.

CONCLUSION

Fluconazole-loaded proniosomal gel was prepared successfully by coacervation phase separation method. The prepared proniosomal gel exhibited an entrapment efficiency of fluconazole ranged from 97.6 to 85.1 and PSs

Table 3: Particle size, zeta potential, and percentage entrapment efficiency determined for the selected fluconazole proniosome formulation when fresh and after storage in the refrigerator for 6 months at 4°C

Form	EE%			PS			ZP		
	Initial	At 2 months	At 6 months	Initial	At 2 months	At 6 months	Initial	At 2 months	At 6 months
F4	97.66±1.24	96.4±0.95	95.05±1.12	25.41±0.51	26.51±1.51	28.14±1.64	−32.9	−32.9	−31.45

EE%: Entrapment efficiency percent, ZP: Zeta potential, PS: Particle size (nm)

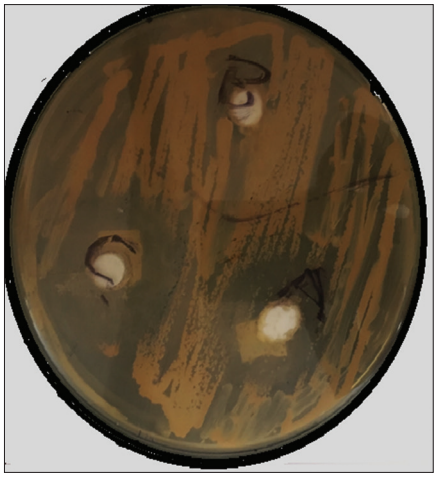

Figure 4: Zone of inhibition of fluconazole (a), control gel, (b) plain, and (c) proniosome gel (F4) after 48 h

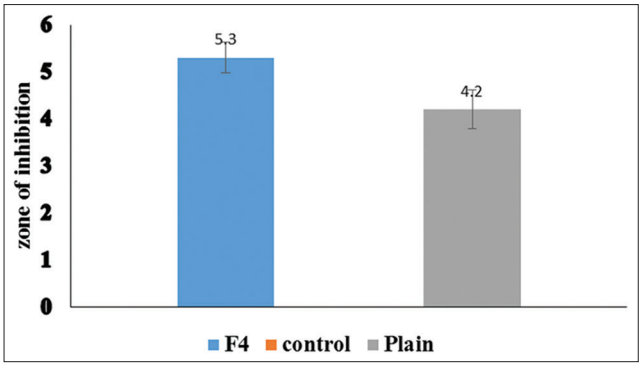

Figure 5: Comparison between the mean values of inhibition zones of fluconazole proniosome gel (F4), control, and plain gel after 48 h

were in the nanosize range (21. 4–50.12 nm). It was found that ZP of F4 which chosen as a best formula according to entrapment efficiency, PS, and *in vitro* release study was found to be (−32.9 mV), that showed its stability. Micrographs revealed the formation of smooth and round niosomal vesicle after hydration. It was found that F4 exhibits maximum antifungal activity after 48 H which revealed that the developed proniosomal gel was more efficient when compared with the control (2% HPMC gel).

It was found that F4 which contains a mixture of span 60 and tween 80 (1:1) is the most appropriate surfactant mixture for the preparation of proniosomes. *In vitro* release studies proved that the prepared proniosomal gel contains fluconazole considered to be a successful topical drug delivery system and provide a sustained release of encapsulated drug, which can be further proved by the future *in vivo* and bioequivalence studies. Furthermore, there was no significant change in EE%, PS, and ZP of fluconazole proniosomal gel after storage for 6 months.

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Conflicts of interest

There are no conflicts of interest.

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