Design and optimization of surfactant-based nanovesicles for ocular delivery of Clotrimazole

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Abstract

The objective of this study was to develop an efficient ocular nanovesicular carrier providing a controlled delivery of Clotrimazole (CLT); a water insoluble antifungal drug. The nanovesicular carriers were formulated using Span 60 with one of the following edge activators (EA): Tween 80 (TW80), sodium cholate (SC) or sodium deoxycholate (SDC). A 3² full factorial design was used to study the effect of two independent variables, namely, the type of EA and the ratio of Span 60 to EA. The effects of these parameters on the mean particle size, entrapment efficiency (EE) and zeta potential (ZP) were investigated as dependent variables. Then, optimization was performed producing the best optimized formulation composed of SDC as an EA at the ratio of 90:10 (Span 60:EA) with an average diameter of 479.60 nm, EE of 87.92% and ZP of –33.7 mV. The optimized nanovesicular carriers appeared as spherical unilamillar vesicles with CLT in an amorphous state as evidenced by the differential scanning calorimetry study. The antifungal activity against Candida albicans compared to niosomal formulation as well as the CLT suspension was determined. CLT-loaded nanovesicular carriers displayed sustained antifungal effect over 12 h. The AUC of the optimized formulation was 3.09 times more than that of drug suspension with no sign of irritation after testing for ocular tolerance. Therefore, the present study showed the feasibility of using non-ionic surfactant nanovesicles as carrier systems for prolonged ocular delivery of CLT.

Keywords

Clotrimazole, edge activators, nanovesicles, ocular delivery

Introduction

Drug delivery in ocular therapeutics is a challenging problem and is a subject of interest to scientists working in the multidisciplinary areas pertaining to the eye. Current trends in ocular therapeutics and drug delivery suggest that the existing dosage forms will be replaced by novel drug delivery systems that offer improved biopharmaceutical properties (Kaur et al., 2012). Conventional eye drops currently account for more than 90% of the marketed ophthalmic formulations (Bourlais et al., 1998). However, after instillation of an eye drop, only a small amount of the applied drug penetrates the cornea and reaches the intraocular tissues. This is due to the rapid and extensive pre-ocular loss caused by drainage and high tear fluid turn-over. It has been previously reported that 90% of the dose was cleared within 2 min for an instilled volume of 50 μl (Ch’ng et al., 1985). Consequently, the ocular residence time of conventional solution is limited to few minutes, and the overall absorption of a topically applied drug is limited to 1%–10% (Lee & Robinson, 1986).

Colloidal carriers have been investigated as drug delivery systems for the past 30 years in order to achieve specific drug targeting, facilitate the drug transfer through biological membranes, improve bioavailability, control release characteristics, reduce or prevent side effects and protect the drug against enzymatic degradation therefore being ideal for long-term use of drugs ( Muller et al., 2000; Souto & Muller, 2006). However, their use in topical administration and especially in ocular delivery has only been studied for the last two decades (Bourlais et al., 1998; Järvinen et al., 1995). The use of colloidal drug delivery systems, such as nanoparticles and liposomes, is a suitable strategy to enhance the bioavailability of topically administered drugs (Calvo et al., 1996; Monem et al., 2000). Recent approaches in modulating vesicle compositions have been investigated to develop systems that are capable of carrying drugs and macromolecules to deeper tissues. These approaches have resulted in the design of two novel vesicular carriers, ethosomes and ultraflexible lipid-based elastic vesicles (Transfersomes) (El Zaafarany et al., 2010). A second generation of elastic vesicles, mainly consisting of non-ionic surfactants, was also introduced (van den Bergh et al., 1999, 2001). Elastic liposomes were developed showing a significant trans-ocular absorption of the antiviral drug ganciclovir (Shen & Tu, 2007). A recent development for increasing the corneal permeability of drugs is novel surfactant-based nanovesicular carriers composed of Span 60 as a non-ionic surfactant along with an edge activator (EA) (Kakkar & Kaur, 2011; Kaur et al., 2012).
Clotrimazole (CLT), (1-2-chlorophenyl-diphenylmethyl)-1-4-imidazole, is an azole-type antifungal agent that is known to have topical activity against pathogenic dermatophytes and yeasts (Sawyer et al., 1975). The clinical use of CLT has some practical disadvantages mainly due to its poor water solubility. Therefore, the development of novel carriers for CLT could lead to an improvement in its solubility and permeability (Winnicka et al., 2011). Many attempts have been made to improve the solubility of CLT comprising; cyclodextrins complexation (Prabagar et al., 2007), as well as different delivery systems such as microcapsules (Abdel-Moety et al., 2002), liposomes and niosomes (Ning et al., 2005).

Earlier literature lacks sufficient data about the use of CLT in an ophthalmic preparation for treatment of fungal keratitis. Thus, this work aims to optimize the ocular administration of CLT for treatment of fungal infections through loading into novel surfactant-based nanovesicles. The effect of using different concentrations of several EAs on vesicle characteristics is investigated employing a factorial design method. The in-vivo efficacy of this elastic system is examined, compared to conventional niosomes and free drug preparations.

**Materials and methods**

**Materials**

CLT was kindly supplied by Memphis Pharmaceutical Co., Cairo, Egypt. Sorbitan monostearate (Span 60), Polyoxyethylene sorbitan monoooleate 80 (Tweed 80 (TW 80)), sodium cholate (SC), sodium deoxycholate (SDC) and cholesterol (CH), minimum 99%, were purchased from Sigma Chemical Company, St. Louis, USA. Ethyl acetate and ethanol were obtained from El Nasr Pharmaceutical Chemicals Company, Cairo, Egypt.

**Methods**

**Preparation of CLT nanovesicular carriers**

CLT-loaded nanovesicles were prepared by the ethanol injection method (Kakkar & Kaur, 2011). Briefly, CLT was dissolved in ethanol in water bath at 70°C. The resultant solution was quickly injected into preheated aqueous phase containing the EA (TW 80, SC or SDC) which was stirred continuously on a magnetic stirrer at 1000 rpm for 15 min at 70°C. The obtained emulsion was allowed to cool at room temperature; yielding drug-loaded nanovesicular dispersion. Blank dispersions were prepared exactly in the same manner as the above process using the same procedure variables.

**Preparation of CLT niosomal formulation**

Niosomes containing CLT were prepared employing the chloroform film method (Baillie et al., 1986). Preparation took place using Span 60 along with CH at the ratio of (1:1). Accurately weighed quantities of CH, Span 60 and CLT were dissolved in 10 ml chloroform and slowly evaporated using rotary evaporator at 55 ± 2°C under reduced pressure to obtain a thin film on the wall of the flask. This thin film was hydrated with 10 ml distilled water at a temperature of 55 ± 2°C for formation of the niosomal dispersion.

**Characterization of CLT-loaded nanovesicles**

Table 1. Formulation of CLT nanovesicular carriers using 3^2 full factorial design.

<table>
<thead>
<tr>
<th>Factors (independent variables)</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>X_1: Type of EA</td>
<td>TW 80</td>
</tr>
<tr>
<td>X_2: Ratio of EA to Span 60</td>
<td>70:30</td>
</tr>
<tr>
<td>Constraints</td>
<td></td>
</tr>
<tr>
<td>Y_1: Particle size (PS)</td>
<td>400 ≤ Y ≤ 500 nm</td>
</tr>
<tr>
<td>Y_2: Entrapment efficiency (EE%)</td>
<td>80 ≤ Y ≤ 100%</td>
</tr>
<tr>
<td>Y_3: Zeta potential (ZP)</td>
<td>−30 ≤ Y ≤ −35 mV</td>
</tr>
</tbody>
</table>

Optimization of CLT-loaded nanovesicles using a 3^2 full factorial experimental design

CLT-loaded nanovesicles were prepared using a 3^2 full factorial experimental design in order to investigate the joint influence of formulation variables and experimental conditions using the Design-Expert® 8 software (Stat-Ease, Inc., Minneapolis, MN). In this design, two factors were evaluated, each at three levels and the experimental trials were performed at all nine possible combinations. The independent variables were the type of EA (X_1) and the ratio of EA to Span 60 (X_2) (Table 1). The particle size (PS) (Y_1), entrapment efficiency (EE%) (Y_2) and zeta potential (ZP) (Y_3) were selected as the dependent variables. An ANOVA test was performed to evaluate the level of significance of the tested factors on the selected responses as well as the interactions between these factors.

**Determination of CLT entrapment efficiency.** To determine the amount of encapsulated CLT, 1 ml of the investigated formulation was separated by ultra-centrifugation at 18 000 rpm and temperature −20°C for 1 h using a cooling centrifuge (Beckman, Fullerton, Canada). The vesicles were separated from the supernatant, mixed with ethyl acetate and sonicated for 15 min in order to obtain a clear solution. The amount of entrapped CLT was determined by measuring the concentration of CLT in ethyl acetate spectrophotometrically at a wavelength of 270 nm (Shimadzu UV Spectrophotometer, 2401/PC, Shimazu, Japan). The entrapment efficiency (EE)
percentage of CLT was calculated from the following equation:

\[ EE\% = \frac{\text{amount CLT entrapped} \times 100}{\text{total amount of CLT}} \]

Transmission electron microscopy. The morphologic examination of the optimum formulation as well as the niosomal dispersion was performed by a transmission electron microscope (TEM) operating at 80 kV (model JEM-1230, Jeol, Tokyo, Japan). One drop of the diluted vesicular dispersion was deposited on the surface of a carbon-coated copper grid, negatively stained with 2% phosphotungstic acid then allowed to dry at room temperature for 10 min for investigation.

Differential scanning calorimetry. The thermal analysis of pure materials and lyophilized optimum formulation were determined using a Shimadzu differential scanning calorimeter (DSC-50, Kyoto, Japan). Approximately 4 mg of each sample was heated in aluminum pans in a temperature range of 30 to 300 °C at a heating rate of 10 °C/min under inert nitrogen flow (25 ml/min).

In-vivo evaluation of CLT nanovesicular carriers

Microbiological assays

Candida albicans susceptibility to CLT. Candida albicans NRRL Y-477 was chosen as a test organism to be used as a challenging yeast in all experiments as it is a well-characterized and highly invasive strain that has been investigated in rabbit keratitis model. Test tubes containing 5 ml of Sabouraud dextrose broth (SDB) were inoculated with 10^6 CFU/ml. The inoculated 5 ml of SDB medium were incubated at 27 ± 0.5 °C for 24 h. Sterile 6 mm diameter filter paper discs (Whatman no. 1) were placed under the eyelid of rabbit for 1 min at specific time intervals (0.5–12 hr) following a single installation (50 μl) of the investigated formulae each in the conjunctival sac of the right eyes of six rabbits. The discs were then placed in the inoculated SDB tubes. The inoculated broth was then incubated at 27 ± 0.5 °C for 24 h. Percentage of inhibition, which is related to the level of drug in the eye tears following the topical application of tested drug formulae, was calculated using the SDB medium inoculated with C. albicans NRRL Y-477 as control. The area under the curve from 0 to 12 h (AUC(0-12h)) was estimated by the linear trapezoidal method and used to predict and compare the mean time for the antifungal effect of CLT in the eye tears obtained from the tested CLT-loaded nanovesicular carriers as well as CLT suspension.

Statistical analysis

Pairs of groups were compared by performing one-tailed Student’s t-test and multiple group comparison was conducted by the one-way analysis of variance (ANOVA) and then by LSD using the statistical software (SPSS-11, SPSS Inc., Chicago, IL). All data are presented as mean values with their standard deviation (mean ± SD). Differences were considered to be statistically significant when the p values were less than 0.05.

Results and discussion

Analysis of factorial design

The experimental runs, with independent variables and the measured responses are shown in Table 2. The table shows nine experimental runs (NV1–NV9) to accommodate two factors with three levels.

The effect of formulation variables on the particle size of CLT nanovesicular formulations using a full factorial design (3^2)

As shown in Table 2, nine nanovesicular carriers (NV1–NV9) were prepared, using three types of EAs, showing mean particle diameters less than that of the conventional niosomes (1240 nm), the PI of all measured formulations was acceptable at values <0.1, thus indicating a narrow size distribution of the measured dispersions and consequently a homogenous distribution (Essa et al., 2002). The smaller particle size of the developed nanovesicles compared to the niosomes might be attributed to the presence of EAs. Generally vesicles formulated using EAs are likely to be more spherical, having low aggregation tendency and consequently having
smaller particle sizes (Rosen, 2004). Unlike the nanovesicles, niosomes being formulated using only CH in combination with Span 60 lack the previously mentioned advantages of EAs and hence having larger particle size.

The ANOVA results showed that only the type of EA had a significant effect on the particle size of CLT nanovesicles \( (p < 0.0001) \). Figure 1(a) shows the effect of the type of EA on the particle size of CLT nanovesicles. The smallest particle size was observed in the case of nanovesicles prepared using TW 80 (NV1–NV3). The differences in HLB of these EAs may have certain impact in the explanation of these findings. The HLB values are 15, 16.7 and 16.7 for TW 80, SC and SDC, respectively. Generally, the use of EAs with lower HLB, results in vesicles with a smaller size (Yoshioka et al., 1994). Hence, TW 80 being the EA of the smallest HLB value gave rise to nanovesicles of the smallest particle sizes. Furthermore, in a previous study, elastic anionic niosomes prepared with various concentrations of SC and SDC as EAs showed larger vesicular sizes (Manosroi et al., 2010). Although, SC and SDC have the same HLB values, there is no available explanation for the difference in particle size among nanovesicles prepared using these EAs.

The effect of formulation variables on the EE of CLT nanovesicles using a full factorial design \( (3^2) \)

The EE% of all nanovesicles investigated ranged from 70.44% to 87.92%, clearly higher than that of conventional niosomes (67.705%). Higher EE% of nanovesicles was also observed in other studies comparing nanovesicles with other elastic vesicles (Kakk & Kaur, 2011). In a previous study, meloxicam transfersomes showed higher EE% than liposomes (Duangjit et al., 2011). The ANOVA results showed that the type and concentration of EA had a significant effect on the EE of CLT nanovesicles \( (p = 0.0469, p = 0.0011) \) respectively). Figure 1(b) shows the effect of type, and concentration of EA on the EE of CLT nanovesicles formulations.

By observing the effect of EA ratio on EE%, we can see that the EA ratio of 20% showed the highest EE% in case of TW 80, while a 10% ratio was optimum for drug entrapment in case of SC and SDC. However, further increase in the EA’s ratio lead to a consequent decrease in EE%. van den Bergh et al. suggested that further increase in the content of EA leads to pore-formation in vesicle bilayers and when the concentration reaches a certain threshold, micelles or mixed micelles are starting to be formed, leading to a decrease in EE% (2001). This was also previously reported in case of transfersomes (Jain et al., 2003). Moreover, a clear decrease in EE% of estradiol was observed with higher ratios of SC in ultra-deformable liposomes (El Maghraby et al., 2000).

When observing the effect of type of EA on EE% for each EA type, we can find that SC and SDC showed a higher EE% than TW 80 at an EA ratio of 10%. Upon increasing the EA ratio to 20% and 30%, the highest EE% was observed in case of TW 80. These findings are consistent with other results concerning ultra-deformable vesicles with TW 80 leading to higher EE% at a ratio of 15% and 25%, whereas both cholates showed higher EE% at EA ratio between 2% and 5% (El Zaafarany et al., 2010).

The effect of formulation variables on the ZP of CLT nanovesicles formulations using a full factorial design \( (3^2) \)

The ZP values of all vesicles prepared were negative, ranging from \(-23.65 \text{ mV}\) to \(-46.95 \text{ mV}\). The ANOVA results showed that both the type and concentration of EA had significant effect on the zeta potential of CLT nanovesicles formulations \( (p < 0.0001, p = 0.0026) \) respectively). Figure 1(c) showed the effect of type, and concentration of EA on the ZP of CLT nanovesicles formulations. The addition of EA to the nanovesicles led to a decrease in the ZP values compared to conventional niosomes \((-46.95 \text{ mV})\). This may be attributed to the fact that EAs investigated tend to reside on the surface of the vesicular bilayers because of their hydrophilicity (Wilson et al., 2008). This may have resulted in a shielding of the negative surface charge (Huang et al., 2011). Nanovesicles prepared using SC and SDC showed more negative ZP values, attributed to their anionic nature, compared to TW 80, which is a non-ionic surfactant. Nanovesicles prepared using SDC (NV7–NV9) showed more negative ZP values at the three ratios investigated, indicating better stability than vesicles prepared employing SC (NV4–NV6). This difference in ZP values between SC and SDC might be attributed to the presence of one additional OH group in SC compared to SDC, which might lead to the difference in the ZP values (Lee et al., 2005). The ratio of 30% showed the most negative ZP values in case of SC and SDC, which can be expected considering their anionic nature.

Optimization of CLT nanovesicular carriers

The aim of the optimization of pharmaceutical dosage formulations is generally to determine the levels of variables
from which a robust product with high quality characteristics may be produced. According to Zimmer et al., the particle size for ophthalmic application should not exceed 10 μm because with larger sizes a scratching feeling might occur (Gonzalez-Mira et al., 2010; Zimmer & Kreuter, 1995). Therefore, for an optimized formulation intended for ocular instillation, a reduction in particle size, PI as well as higher EE and good physical stability are prerequisites in order to improve the patient comfort during administration.

CLT nanovesicles were optimized for the responses $Y_1$, $Y_2$ and $Y_3$. The desirable range of these responses was restricted to $400 \text{ nm} \leq Y_1 \leq 500 \text{ nm}$, $80\% \leq Y_2 \leq 100\%$, and $-30 \leq Y_3 \leq -35 \text{ mV}$, respectively. The optimum values of the variables were obtained by graphical and numerical analyses using the Design-Expert® 8 software and based on the criterion of desirability (Basalious et al., 2010). Therefore, the optimized CLT nanovesicles formulation with the predicted levels of formulation variables was prepared to confirm the validity of the optimization procedure. The composition,

Figure 1. Effect of (a) type of EA on the particle size, (b) type, and concentration of EA on the EE% and (c) type, and concentration of EA on the ZP of CLT nanovesicular carriers.
The morphology of the optimized formulation as well as the niosomal dispersion were observed using transmission electron microscopy (TEM). The TEM micrographs of the optimized formulation showed unilamellar nanovesicles with uniform spherical shape compared to the niosomal vesicles showing multi-lamellar structure. The vesicles appeared non-aggregated characterized by smooth surface with narrow size distribution (Figure 3). The figure clearly shows that the diameters of the vesicles observed in the micrographs are in accordance with the data obtained by particle size analysis.

**In-vivo evaluation of CLT-loaded nanovesicular carriers**

**Irritancy test**

For a drug delivery system to be used safely as an ophthalmic drug carrier, it is important to be tested for its ocular tolerability. Therefore, the in-vivo ocular irritancy toward our optimized formulation as well as the niosomal dispersion was studied. The results showed that over the study period (48 h) none of the tested formulations showed any sign of redness, inflammation or increased tear production proving the safety of the used non-ionic surfactants to be applied topically in the eye.

**Susceptibility test**

The antifungal activity of CLT-loaded nanovesicular carriers was compared to that of the niosomal formulation and the drug suspension. The tested CLT-loaded nanovesicular carriers did not produce any discomfort to rabbit eyes and provided, to the eye surface, greater antifungal effect all over the study period (48 h) compared to the niosomal dispersion. However, the antifungal activity of CLT-loaded nanovesicular carriers showed a fairly constant antifungal effect all over the study period (12 h). However, the in-vivo test has evidenced that the

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**Table 3. Composition, predicted and observed responses of the optimized nanovesicular CLT formulation.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Values</th>
<th>Response</th>
<th>Predicted values</th>
<th>Observed values</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: Type of EA</td>
<td>SDC</td>
<td>Y1: PS</td>
<td>479.60 nm</td>
<td>579.05 nm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y2: EE%</td>
<td>87.92%</td>
<td>89.16%</td>
</tr>
<tr>
<td>X2: EA: Span 60</td>
<td>90:10</td>
<td>Y3: ZP</td>
<td>−33.70 mV</td>
<td>−31.20 mV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y4: C</td>
<td>31.20 mV</td>
<td>30.68 mV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y5: C</td>
<td>188.06 mJ/g</td>
<td>189.66 mJ/g</td>
</tr>
</tbody>
</table>

**Figure 2. DSC thermograms of (a) CLT, (b) SDC, (c) Span 60, (d) physical mixture and (e) optimized formula.**
AUC(0–12h) of CLT-loaded nanovesicular carriers was significantly higher ($p < 0.05$) compared to that of the niosomal formulation and the drug suspension (500.63, 426.84 and 161.50, respectively). This could be attributed to the smaller particle size of the nanovesicles prepared compared to that of the niosomal formulation, which resulted in an increase in the mean residence time of the drug on ocular surface, as previously reported (Kassem et al., 2007), making such vesicles more appropriate for ocular delivery.

Hence, CLT-loaded nanovesicular carriers fulfill the criteria for sustained dose maintenance for longer period (high retention time) when compared with the niosomal formulation and the drug suspension alone. The overall results signify the potential topical application of CLT-loaded nanovesicular carriers, which is in favor of sustaining drainage of drugs from the conjunctival sac of the eye, simultaneously without blinking difficulty.

**Conclusion**

A promising nanovesicular ophthalmic formulation of CLT made only of surfactants was successfully prepared. The optimized CLT-loaded nanovesicular carriers had a greater antifungal activity, and a longer mean residence time of the drug on the ocular surface compared to the drug suspension. The nanocarriers showed an intimate contact with the targeted organs comprising the corneal and conjunctival surfaces, thus...
eliminating systemic drug exposure and ensuring long-term drug levels. The study suggests that the optimized CLT formulation developed in this work can be a breakthrough for effective ocular delivery of CLT, with improved antifungal effect for the treatment of fungal keratitis.

Declaration of interest
The authors report no declarations of interest.

References