Preparation and Evaluation of Proliposomes Containing Clotrimazole

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Clotrimazole (CT)-containing proliposomes were prepared by penetrating an ethanol solution of CT and Egg phosphatidylcholine (PC) into microporous sorbitol particles, followed by vacuum evaporation of the solvent. As a result, CT proliposomes with free-flowing flowability were obtained. On contact with water, the proliposomes were rapidly converted into a liposomal dispersion, in which a certain amount of CT was entrapped by the liposomes. The result in scanning electronic micrograph confirmed the formation of liposomes structures from proliposomes, and the particles revealed round or ellipse. The ratio of drug to total lipid, ratio of PC to cholesterol and ratio of lipid to sorbitol affected the entrapment efficiency (EE%). The EE% of optimized formulation (CT 10 mg, 0.1 g total lipid, PC/CH ratio is 60 : 40 and 1 g sorbitol) in this investigation was 96.2 ± 1.5%. The proliposomes system can provide sustained release in simulated vaginal fluid at 37±1°C for 24 h. In-vivo performance of blank proliposomes, a physical mixture of sorbitol and drug, clotrimazole proliposomes and commercial tablets were evaluated using antifungal activity test. At 7 d post-dose, the c.f.u. of C. albicans decreased in proliposomes-treated groups than ointment and the physical mixture (t-Student, p<0.05). The results indicated that CT-containing vaginal proliposomes prolonged drug release and may increase amount of drug retention into the mucosa to result in more antifungal efficacy. In addition, CT-proliposomes did not affect the morphology of vaginal tissues. Therefore, the dosage form might be further developed for safe, convenient, and effective treatment of vaginal candidiasis with reduced dosing interval.

Key words proliposome; clotrimazole; vaginal drug delivery

Clotrimazole (CT), which is an imidazole derivative, is widely and effectively used for the treatment of vulvovaginal candidiasis. Unfortunately, oral use of CT is unacceptable due to the severe side effects. Thus, topical administration of CT is recommended. However, it is limited by its very low water solubility resulting in the essential to incorporate CT into a suitable vehicle. Commercially conventional CT vaginal delivery systems, such as creams, foams, and gells, are considered to reside for a relatively short period of time at the targeted site. The entrapment of drug in vesicles is viewed to help in the localized delivery of the drug and an improved solubility and availability of the drug at the site will reduce the dose.

As can be known, liposomes are starting to be widely investigated in topical applications for the skin and oral administration of CT is unacceptable due to the severe side effects. Thus, topical administration of CT is recommended. However, it is limited by its very low water solubility resulting in the necessity to incorporate CT into a suitable vehicle. Commercially conventional CT vaginal delivery systems, such as creams, foams, and gells, are considered to reside for a relatively short period of time at the targeted site. The entrapment of drug in vesicles is viewed to help in the localized delivery of the drug and an improved solubility and availability of the drug at the site will reduce the dose.

In present study it was to investigate the feasibility of proliposomes to formulate the vaginal administration of CT. Proliposomes are formed by penetrating organic solution of CT and phosphatidylcholine (PC) into microporous sorbitol particles, followed by vacuum evaporation of the solvent method. Formulations composed of egg phospholipid, cholesterol compositions have been characterized by encapsulation efficiency. In addition, we are investigating the optimized resultant liposomes in vitro release study. Furthermore, we describes an attempt to achieve the antifungal activity more efficiency followed by a prolonged delivery of CT via a vaginal application of appropriate dosage forms of CT. A physical mixture of CT and sorbitol was also prepared and compared for antifungal efficiency with proliposomes following vaginal application in rats. Further, proliposomes system was evaluated by tolerability on tissue level in rat.

Experimental

Materials Clotrimazole (CT) and Egg phospholipids (PC) (>98%) were the generous gifts of Xi’an Libang Liposomes Pharmaceutical Company. Cholesterol (Chol) and sorbitol were brought from Sigma Company. Cellulose nitrate membrane filters (0.22 μm, Whatman, Maidstone, U.K.); Dialysis bag (cut off 12000—14000) was purchased from Sigma Company. Acetate buffer (pH 4.5) was made of 0.2 M CH3COONa (430 ml) and 0.2 M CH3COOH (570 ml). All other reagents used in the study were of analytical grade and commercially available.

Preparation of CT Proliposomes CT-containing proliposomes were prepared by penetrating an ethanol solution of CT and PC into microporous sorbitol particles, followed by vacuum evaporation of the solvent. Precisely, a 50 ml round-bottom flask containing 1 g of sorbitol (mean diameter of 180—400 μm) was attached to the rotary evaporator. Formulations containing different compositions with PC, cholesterol were dissolved in ethanol solution and were introduced into the round-bottom flask on the rotary evaporator by sequential powder. During the spraying period, the rate of reagent used in the study were of analytical grade and commercially available.

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of lipid solution had been applied. After addition of the final aliquot, evapo-
ration was continued until the powder was completely dry. The material was
further dried in a desiccator under vacuum at room temperature overnight.
This dry preparation is referred to as “proliposomes” and was used for
preparations and for further study on powder properties.

Proliposomes-driven liposomal dispersions were obtained by hydrating the
proliposome (10 mg) preparation with 35 °C distilled water 1 ml and vor-
texes mixing for a while till no residual substance in the bottom of vial. The
resulting liposome dispersion was used for the determination of the entrap-
ment efficiency and morphological studies. The entrapment efficiency of CT
in liposomes was used as a criterion for the evaluation of the tested formul-
tions.

Scanning Electron Microscopy (SEM)  The surface morphology of pro-
liposomes and liposomes from proliposomes was examined by SEM after
coating them with platinum in an ion sputter coater (Eilo IB-5, Japan), and
was photographed using a HITACHI scanning electron microscope (HI-
TACHI S-2500, Japan).

Drug-Entrapment Efficiency  The entrapment liposomes were sepa-
rated by dialysis. Dialysis was applied to separate unentrapped drugs from
hydration liposomes containing drugs. The procedure was as follows: sam-
ple of liposome suspension was placed in a tube (MW cut off 12000—
14000) and extensively dialysed against the buffer solution for 5 h. One mil-
litre of clotrimazole liposomal suspension each of which before and after
dialysis were withdrawn at definite volume intervals with methanol in a 10-ml volu-
metric flask, and the amount of drug was determined by HPLC. The HPLC
system consisted of Gold Nouveau software workstatation, a Beckman 126
NM solvent delivery system, Beckman 508 autosampler with a 100-μl loop,
and Beckman 168 NM PDA detector. The column used was Beckman C18
dp 5 μm, 4.6 mm×25 cm (Beckman, U.S.A.). The mobile phase consisted of
methanol and H2O (pH 3.0) (95 : 5, v/v). The flow rate was 0.8 ml/min. The
chromatogram was monitored at a wavelength of 220 nm.

The regression equation for clotrimazole content (μg/ml) in methanol
ranging from 10 to 500 μg/ml was:

\[ C = 1801.34 - 28.537 \times \text{R}^2 = 0.9995 \]

where C (μg/ml) and \( \text{R} \) represented the concentration and peak area of
clothrimazole, respectively. The mean recovery was 98.50±1.73 (n=3). The
precision assay showed that relative standard deviations within 1 d and
among every other day were all below 2%. This method was validated in
terms of specificity, linearity and reproducibility. The limit of quantification
was 1 μg/ml. The exact amounts of clotrimazole were used for the calibration
curve.

Entrapment efficiency (EE%) could be achieved by the following equa-
tion:

\[ \text{EE%} = \frac{\text{content of CT in post-dialyzed liposomes}}{\text{content of CT in pre-dialyzed liposomes}} \times 100\% \]

In present study, dialysis method was applied in entrapment efficiency de-
determination. Recovery of drug was determined for all samples and was be-
tween 94.5% and 96.2% of the amount taken into preparation.

Angle of Repose  Because proliposomes are a dry powder, further pro-
cessing is possible. To provide convenient unit dosing, the present prolipo-
osomes powder may be processed to make tablets or capsules for vaginal drug
delivery. Angle of repose measurements indicated that the fluidity of proli-
posomes dry powder is equal to or better than that of sorbitol powder, so fur-
ther processing of proliposomes powder should be straightforward. The
angle of repose of dry proliposome powder was measured by a funnel
method.

Release Study  In order to investigate the formulations release profile in
simulated physiological condition, the EE% change of liposomes were tested in
each culture solution (acetic acid buffer (pH 4.5) and PBS at 37±1 °C). We chose the formul-
tion: CT-proliposomes using 0.1 g total lipid, 1 g sorbitol, PC/CH ratio was
60 : 40. The drug release percent from vesicles results are shown in Fig. 3.
Briefly, dialysis tubes containing 2.5 ml of CT liposomes were placed into a
flask containing 125 ml of vaginal fluid or PBS (pH, 7.4) at 37 °C. The bag
was secured with two knots at each end and the air spaces minimized as
much as possible. These flasks were placed in a shaker (HZQ-C, Har’er bin,
China) and shaken at 100 rpm at a temperature of 37 °C. The samples from
liposomes were withdrawn at definite time intervals; the residual amount of
drug in vesicles was determined as described mentioned above.

Antifungal Efficacy Studies  A major problem in studies of vaginals
absorption in rodent species is the variable structure of the vaginal epithel-
ium at different stages of the oestrous cycle. In order to standardize the
thickness of the vaginal epithelium, the animals used in the present study
were ovariectomised. Ovariectomised female Sprague–Dawley rats, 200±10 g
(body weight), receiving subcutaneous administration of estradiol benzoate
(25 mg/kg) every 2 d during the experiment, were selected and housed in
individual cages and received food and water ad libitum. The animals were
infected by intravaginal inoculation of C. albicans (CMCC(B) 98001) sus-
pended in sterile saline containing 106 c.f.u./ml. A vaginal smear was taken
2 d after the challenge to confirm the establishment of infection.

Female rats were randomly divided into 4 groups (4 in each group).
Group 1, vaginal administration of proliposomes without drug, as negative
control; group 2, vaginal administration of a physical mixture of sorbitol and
drug (25 mg/kg); group 3, vaginal administration of commercial CT oint-
ment; group 4, vaginal administration proliposomes (25 mg/kg).

The rats were fasted for 24 h prior to the experiments. Ovariectomised rats
were anaesthetized by intraperitoneal injection of 60 mg/kg pentobarbitone
sodium (Beijing Chemical Reagents Company, Beijing). The proliposomes
powder was inserted into the vaginal tract through a plastic tube at a CT
of 25 mg/kg once a day for 3 consecutive days starting 24 h after challenge
(day 0). For control experiments, blank proliposomes and the physical mix-
ture of sorbitol with free CT was similarly administered, the commercial CT
ointment were applied into the vaginal tract of rats at a CT dose of 25 mg/kg
using a stomach sondle needle. For the vaginal colony counts, an analysis of
variance was done on the log_{10} colony counts for each day of vaginal culture
each drug formulation from days 4 to 7. Vaginal lavage samples were
collected with 100 μl saline by washing the fluid three times up and down in
the vagina. The fluid was then plated onto sabouraud dextrose agar and incu-
bated for 48 h at 37±1 °C and c.f.u. values were recorded.

Morphology Study of Vaginal Tissues after Application of CT-Proli-
posomes  Oophorectomized female Sprague–Dawley rats weighing
200±10 g were used after a recovery period of at least 7 d. CT-proliposomes
were administered into the vagina of the rats at a CT dose of 25 mg/kg, the
vaginal tissues of the blank sorbitol treated rats (A) and CT-proliposomes
rats (B) were isolated, fixed in 10% neutral carbonated-buffered formalde-
hyde, embedded in paraffin, and cut into slices. After hemotoxylin–eosin
staining, the slides were observed under a light microscope.

Data Analysis  Data were analyzed statistically by the one way ANOVA
analysis using the Microsoft Excell 2000 and by the r-Student test (level of
significance for p<0.05).

Results and Discussion

Microscopic Appearance  Scanning electron microscopy of uncoated sorbitol and dry proliposomes powder (Fig. 1a) reveals that there appears to be a slight difference on the appearance of the surfaces. The powder in (Fig. 1b) appears to be smoother and to have fewer “fine feature” such as whiskers and sharp corners.

The morphology of the resultant liposome vesicles was evaluated. Figure 2 shows the shape of the resultant lipo-
osomes entrapping with the drug clotrimazole. It was evident that the particles investigated revealed round or ellipse.

Effect of CT Content  With the total concentration of lipid kept constant at 0.1 g, the ratio of PC to Chol was 6 : 4

CT proliposomes formulation: CT 10 mg, 0.1 g total lipid, PC/CH ratio is 60 : 40, 1 g sorbitol.
17) As can be seen, partitioning of drug molecules to the bilayer membrane, the oriented toward the aqueous surface and the aliphatic chain could be inserted into the membrane with its hydroxyl group into the phospholipid bilayers. Since it is amphipathic, Chol itself form the bilayer structure but it can be incorporated into the liposome preparations, as well as liposomes. Cholesterol does not provide limited entrapment capacity.

The entrapment efficiency of CT showed no significant difference, remaining at about 95% when CT content is below 3 mol% of total lipid. The result supported the statement made by Payne16) that the proliposomes system is ideally suited for lipophilic actives, where after suitable optimization, the majority of drug partitions into the liposomal lipid phase.

Effect of Total Lipid-to-Sorbitol Ratio The concentration of sorbitol has no measurable effect on EE% of CT, based on comparison of total lipid: sorbitol ratios from 1 : 10 to 1 : 20. However, from the point of preparation, it is difficult to prepare the proliposomes when the total lipid-to-sorbitol ratio is higher than 1 : 10. Because only a very small volume of the solution of membrane-forming components can be introduced and sprayed onto the limited amount of sorbitol each time, the spraying-evaporating process becomes much time consuming. Because a higher sorbitol concentration did not improve the formation, 1 : 10 sorbitol was used for the formulation of proliposomes.

Effect of PC-to-Chol Ratio The percentage drug entrapment efficiencies of CT proliposomes prepared at various amount ratios of PC to Chol are shown in Table 2, which reveals that incorporation of more Chol would yield higher EE (%) at a constant molar ratio range. The content of Chol is one of the important parameters in the design of proliposomal preparations, as well as liposomes. Cholesterol does not itself form the bilayer structure but it can be incorporated into the phospholipid bilayers. Since it is amphipathic, Chol could be inserted into the membrane with its hydroxyl group oriented toward the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. But, when the Chol content is over a range, it might lower the partitioning of drug molecules to the bilayer membrane, the degree of encapsulation would decrease.15) As can be seen, the entrapment efficiencies of all proliposomal formulations were found to be significantly enhanced with the increasing Chol content (p<0.05) when PC-to-Chol ratio is lower than 50 : 50. It was observed Chol crystal when PC-to-Chol ratio was higher than 5 : 5. The enhancing effect of Chol on the entrapment efficiency may be attributed to the rigidifying effect in the fluid crystal state, facilitating the complete formation of the vesicles with the bilayer-bound drug during the process of proliposomes hydration.

Effect of PC/CH Ratio on CT Entrapment Efficiency (%,

<table>
<thead>
<tr>
<th>Molar ratio PC/CH ratio</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 : 10</td>
<td>70.2±2.5</td>
</tr>
<tr>
<td>80 : 20</td>
<td>89.1±2.5</td>
</tr>
<tr>
<td>70 : 30</td>
<td>90.1±1.5</td>
</tr>
<tr>
<td>60 : 40</td>
<td>95.2±3.6</td>
</tr>
<tr>
<td>50 : 50</td>
<td>93.1±1.9</td>
</tr>
<tr>
<td>30 : 70</td>
<td>73.1±2.1</td>
</tr>
</tbody>
</table>

Table 2. Effect of PC/CH Ratio on CT Entrapment Efficiency (%,

The angle of repose of dry proliposome powder increases slightly, more closely approaching the angle measured for pure sorbitol. A higher proportion of sorbitol to lipid is increased, the angle of repose of proliposome powder is smaller than that of pure sorbitol. This is consistent with the scanning electron microscopic observation of proliposome powder, in which it was observed that the proliposomes surface was smoother. If the proportion of sorbitol to lipid is increased, the angle of repose of dry proliposome powder increases slightly, more closely approaching the angle measured for pure sorbitol.

Table 3. Angle of Repose of Dry Proliposome Powder

<table>
<thead>
<tr>
<th>Preparation angle</th>
<th>Sorbitol</th>
<th>Proliposome</th>
<th>Proliposome (2×)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.19±0.75</td>
<td>34.66±2.10</td>
<td>37.65±1.07</td>
<td></td>
</tr>
</tbody>
</table>

a) The mass of sorbitol was doubled, but the mass of lipid was kept constant.
higher drug vaginal mucosa retention, creation of reservoir effect for drug in mucosa due to deposition of other components of resultant liposomes from proliposomes hydration contacting with vaginal fluid with drug into the mucosa and thereby increasing the drug retention capacity into the mucosa. The significantly higher mucosa retention of the liposomes drug resulting in higher partitioning of the drug into the mucosa may be responsible for prolonged and enhanced antifungal activity.

**Tolerability of Clotrimazole CT-Proliposomes in Tissue Level**

CT-proliposomes did not alter the morphology of vaginal tissues. Figure 4 shows the histopathology of the vaginal mucosa after intravaginal application of CT-containing proliposomes powder. As compared to the control with no treatment, the CT-proliposomes-treated group showed no visible sign of inflammation or necrosis. CT-proliposomes did not affect the morphology of vaginal tissues, which indicated such drug delivery systems are safety for vaginal delivery.

**Conclusion**

The result confirmed the formation of liposomes structures from proliposomes, and the particles revealed round or ellipse. Compared with conventional liposomes, the preparation of liposome dispersions from these proliposomes is much more convenient. Apparently due to the great surface area of the lipid film that form on the surface of sorbitol, the hydration of the proliposomes and the formation of the liposome dispersion is very easy. Because the lipid is all coated on the soluble sorbitol, there is little risk of material loss. The hydration of proliposomes progressed sufficiently rapidly as to be evident at times as short as 30 s after contact with water, suggesting a progressive and rapid conversion to liposomes on contact with physiological fluids (such as vaginal fluid) in the body. The proliposomes system can provide

Fig. 4. Morphology of Vaginal Tissues after Application of Sorbitol and CT-Proliposomes Powder were Administered into the Vagina of the Rats at a CT Dose of 25 mg/kg

The vaginal tissues of the blank sorbitol treated rats (A) and proliposomes powder-treated rats (B) were isolated, fixed in 10% neutral carbonate-buffered formaldehyde, embedded in paraffin, and cut into slices. After hematoxylin–eosin staining, the slices were observed under a light microscope (×100).

**Table 4. Antifungal Efficacy of the Prepared Proliposomes (Concentrations of C. albicans log c.f.u./ml) (n=4)**

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Blank</th>
<th>Control</th>
<th>Standard</th>
<th>Proliposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.88±0.23</td>
<td>5.01±0.33</td>
<td>4.93±0.54</td>
<td>4.98±0.50</td>
</tr>
<tr>
<td>4</td>
<td>4.91±0.52</td>
<td>1.71±0.23*</td>
<td>1.03±0.14*</td>
<td>1.09±0.12*</td>
</tr>
<tr>
<td>5</td>
<td>4.93±0.57</td>
<td>1.83±0.25*</td>
<td>1.81±0.25*</td>
<td>1.45±0.14** ***</td>
</tr>
<tr>
<td>6</td>
<td>4.92±0.43</td>
<td>3.95±0.31*</td>
<td>2.93±0.28***</td>
<td>1.52±0.12** ***</td>
</tr>
<tr>
<td>7</td>
<td>4.96±0.26</td>
<td>4.84±0.27</td>
<td>4.83±0.28</td>
<td>2.26±0.25** ***</td>
</tr>
</tbody>
</table>

Blank: proliposomes without drug; control: a physical mixture of sorbitol and drug; standard: commercial clotrimazole ointment. *p<0.05; compared to the day 0; **p<0.05, compared to the mixture control; ***p<0.05, compared to the standard.
sustaining release in simulated vaginal fluid at 37±1 °C for 24 h. *In-vivo* performance of blank proliposomes, a physical mixture of sorbitol, clotrimazole proliposomes and marketed ointment were evaluated using antifungal activity test. At 7 d post-dose, the c.f.u. of *C. albicans* decreased in proliposomes-treated groups than ointment and the physical mixture (*t*-student, *p*<0.05). The results indicated that CT-containing vaginal proliposomes prolonged drug release and may increase amount of drug retention into the mucosa to result in more antifungal efficacy. In addition, CT-proliposomes did not affect the morphology of vaginal tissues. Therefore, the dosage form might be further developed for safe, convenient, and effective treatment of vaginal candidasis with reduced dosing interval.

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