Formulation of tretinoin-loaded topical proniosomes for treatment of acne: *in-vitro* characterization, skin irritation test and comparative clinical study

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Abstract

Tretinoin (TRT) is a widely used retinoid for the topical treatment of acne, photo-aged skin, psoriasis and skin cancer which makes it a good candidate for topical formulation. Yet side effects, like redness, swelling, peeling, blistering and, erythema, in addition to its high lipophilicity make this challenging. Therefore, the aim of this study was the development of TRT-loaded proniosomes to improve the drug efficacy and to increase user acceptability and compliance by reducing its side effects. Nine formulae were prepared according to 3² factorial design and were evaluated for their morphology, vesicle size, entrapment efficiency (EE %), and % of drug released after 5h. Hydrogel of the candidate formula, N8G (proniosomes prepared with 0.025% TRT, and Span60: cholesterol molar ratio of 3:1 and incorporated in 1% carbopol gel) was developed and evaluated for skin irritation test and clinical study in acne patients compared to marketed product. Candidate formula showed higher efficacy and very low irritation potential when compared to marketed product in human volunteers.

Introduction

Tretinoin (TRT) is (a vitamin-A derivative) a widely used retinoid in the topical treatment of acne, photo-aged skin, psoriasis, skin cancer, cutaneous lupus erythematosus and other skin disorders (Laszlo & Fenske, 1998). Vitamin A is applied to the skin to improve wound healing, reduce wrinkles, and to protect the skin against UV radiation (Humphrey et al., 1996). Topical TRT regulates keratinization of the pilosebaceous unit and prevents obstruction of the follicular unit. Thus, TRT is most active against comedonal acne (Laszlo & Fenske, 1998). Although retinoids such as TRT (all-trans-retinoic acid and retinoic acid acidified form of vitamin-A) are very effective in the treatment of acne, their side effects to some people are not acceptable. Retinoids can irritate the skin, especially when they are first used. Excessive use results in redness, swelling, peeling, blistering in treated areas, erythema, burning and stinging. It may cause or aggravate eczema, particularly atopic dermatitis.

TRT has limited stability and loss of the product occurs during storage. The principal reason for this damage is the process of oxidation. TRT is sensitive to air oxygen, light and acids. (Arsić & Vuleta, 1999). TRT facing these two problems of skin irritation and instability and in addition to very low water solubility and poor percutaneous absorption is an ideal candidate for our research.

Formulation design can be a useful approach to solve the problems of these drug candidates. Newer developments in the formulation approach have raised hopes in making topical therapy more useful and acceptable (Katare et al., 2010). These new strategies, often called drug delivery systems (DDS) are based on delivery techniques that minimize toxicity, improve efficacy, offer great potential benefits to patients, and decrease side effects (Kapariassides et al., 2006). One of the novel delivery systems is the use of vesicular systems, such as niosomes, whose effectiveness depends on their physicochemical properties. Vesicular systems have been widely studied as vehicles for dermal and transdermal drug delivery (Gupta et al., 2009; Katare et al., 2010). Their benefits in enhancing drug permeation have been well established (Gupta et al., 2009). Vesicles can increase retinoic acid concentration in the skin while decreasing systemic absorption (Imbert et al., 1994). On the other hand, vesicles can protect compounds, like vitamins, from oxidation. Encapsulation of TRT into vesicular carriers as liposomes and ethosomes and nanoparticulate carriers as Solid lipid nanoparticles and, nanostructured lipid carriers (NLCs) offered enhanced photostability, skin transport and anti-psoriatic activity (Manconi et al., 2002, 2003; Razaa et al., 2013).
Nonionic surfactant-based vesicles (niosomes) are formed from the self-assembly of nonionic amphiphiles in aqueous media resulting in closed bilayer structures. The low cost, entrapping of more substances, ease of handling and storage and availability of prepared materials in pure form have led to the exploitation of these compounds as alternative to liposomes (Essa, 2010). Non-ionic surfactants have been widely used in pharmaceutical field as solubilizers and stabilizers of insoluble drugs due to their surface activity and low toxicity (Ji et al., 2011). The enhanced delivery of niosomes encapsulated drugs through the stratum corneum has been observed (Ning et al., 2011). They showed desired interaction with human skin when applied through topical preparation by improving the horny layer characteristics (Junginger et al., 1991). Unfortunately, niosomes suffer from some problems related to physical stability such as fusion, aggregation, sedimentation and leakage on storage that prevent many promising vesicular formulation from moving from the experimental scale to the market as stated by Ko & Lee (2010). Approaches to stabilize niosomal drug delivery system without affecting its properties of drugs have resulted in the development of the promising drug carrier, proniosomes. Proniosomes are dry formulation using suitable carrier coated with nonionic surfactants and can be converted into niosomes immediately before use by hydration. These proniosomes-derived niosomes are as good as or even better than conventional niosomes. Ease of transfer, distribution, dosing and storage make proniosomes a versatile delivery system. (Kakar et al., 2010; Abhinav et al., 2011; Bayindir & Nilufer, 2012). The proniosome approach minimizes these problems as it is a dry and free flowing product which is more stable during sterilization and storage (Solanki et al., 2007). So the aim of this study was the formulation of TRT-loaded proniosomes as an on demand prepared formula to improve TRT stability and to increase user acceptability and compliance by reducing its side effects. Candidate formula showing high entrapment efficiency and high % of drug released after 5h will be subjected to skin irritation test and comparative clinical study compared to marketed product in human volunteers.

The photostability of the produced candidate formula is currently under investigation. On reviewing the literature, we found no previous published date dealing with the use of TRT-loaded proniosomal gel in a clinical trial for acne patients.

Experimental

Materials

TRT was obtained as a present from Alkan pharma company (Cairo, Egypt), Span 60 and D-Sorbitol were purchased from Qualikem Fine Chem Pvt. Ltd. (New Delhi, India), Span 40 was purchased from Fluka (New York, USA), Cholesterol 95% stabilized, and Tween 20 were purchased from Acros Organics (NJ) Di-cetyl phosphate free acid, was purchased from MP Biomedical, LLC, (Paris, France), Dichloromethane HPLC grade was obtained from Fisher Scientific, (Loughborough, UK), Potassium di-hydrogen phosphate, analytical grade, was purchased from ADWIC (Kaliubeya, Egypt), Ortho-phosphoric acid, analytical grade, was purchased from (Sigma-Aldrich, Buchs, Switzerland).

Methods

Design of experiments

A complete 2^3 factorial design was used for the modification of the preparation of TRT proniosomes. The study design involves the investigation of the three independent variables namely; drug concentration, surfactant type and surfactant: cholesterol molar ratio at two different levels, on mean vesicle size, % entrapment efficiency and% of drug released after 5 h. The experimental design is presented in Table 1.

Preparation of proniosomes by slurry method

Accurately weighed cholesterol, surfactants and TRT (Table 2) were dissolved in 20 ml of dichloromethane. Sorbitol was weighed in ratio of (1) g/mol of total content (surfactant: cholesterol content). Powder sorbitol was placed in a 100 ml rotary evaporator flask and vacuum was applied until the powder is dry and free flowing. The organic solution (composed of calculated amount of TRT, surfactant and cholesterol dissolved in dichloromethane) was then added to the dry powder in the rotary evaporator flask. The water bath was kept at 40 ± 2 °C. The rotation speed was adjusted at 100rpm vacuum was applied until the dichloromethane was removed. The free flowing proniosomal powder was then removed and kept in the fridge at 8 °C. The niosome dispersion was then prepared by hydration of the proniosomes using 100 ml distilled water at 60 °C. (Patel et al., 2009; Mujoriya et al., 2011).

Characterization of the prepared hydrated TRT proniosomal dispersions

Transmission electron microscope (TEM)

Morphological characteristics of the vesicles were examined by the TEM (JEM-100 S, Jeol Ltd., Tokyo, Japan). Samples
were prepared by the negative staining technique. The samples were dispersed directly into bidistilled water, and then copper grid coated with collodion film was put into the solution for several times. After being stained by 2% (w/v) phosphotungestic acid solution and dried at room temperature, the sample was ready for the TEM investigation at 70 kV.

**Vesicle size analysis**

A sample of freshly prepared hydrated niosomal dispersion was diluted with distilled water by shaking to produce weak opalescence dispersion. Then diluted dispersion was used to characterize the vesicle size and size distribution using laser diffraction (LD analyzer) particle size distribution analyzer (Horiba-LAZER Scattering particle size distribution analyzer LA920, Japan). The vesicle size range was set between 0.1 and 20 μm. Samples were measured in distilled water 10% LD10, 50%LD50 and 90% LD90 were used as qualitative parameters to characterize dispersions. The LD values represent the percentage of the vesicles that is smaller than the given size were LD 90 means that 90% (volume distribution) of the measured vesicles below the given value.

**Entrapment efficiency**

Hydrated TRT proniosomal dispersions were separated from unentrapped TRT by centrifugation (Megafuge 1.0 R, Heraeus, Frankfurt, Germany) at 18 000 rpm for 60 min at 2 °C according to the method described by Flaten et al. and Keservani et al. (Flaten et al., 2004; Keservani et al., 2011). The supernatant was separated from precipitate. Unentrapped TRT was then assayed spectrophotometrically (UV-VIS spectrophotometer, Shimadzu UV 1601 PC, Kyoto, Japan) by measuring UV absorbance at λmax 355 nm using phosphate buffer pH 5.5 containing 1% Tween 20 as blank.

The entrapment efficiency was determined relative to the original drug added, applying the following equation (Jaafari et al., 2009):

\[
\text{Entrapment efficiency} = \frac{\text{total drug amount} - \text{unloaded drug amount}}{\text{total drug}}.
\]

**In-vitro release studies**

**In-vitro** release studies of hydrated TRT proniosomal dispersions were done using home-made static Franz glass diffusion cells. These cells consist of donor and receptor chambers separated by a cellulose membrane with molecular weight cut-off of 12 000–14 000 (Spectrum Medical Inc., Los Angeles, CA); the area of diffusion was 1.7 cm². The dialysis membrane was hydrated in the receptor medium, which consisted of a phosphate buffer pH5.5 containing 1% Tween 20, for 12 h before mounting into a Franz diffusion cell. A 2.5 ml hydrated TRT proniosomal dispersion was placed in the donor chamber and the receptor chamber was filled with 7.5 ml receptor medium and stirred continuously at 100 rpm at 37 °C after 1, 2, 3, 4 and 5 h, samples were withdrawn from the receptor chamber through a side-arm tube. After each withdrawal of sample, an equal volume of receptor medium was added to the receptor chamber to maintain a constant volume throughout the study. Samples were analyzed for TRT concentration using ultraviolet spectrophotometry at λmax 355 (Kumar & Prakash, 2004). Measurements were carried out in triplicate.

**Differential scanning calorimetry (DSC)**

DSC experiments were performed with differential scanning calorimeter (model TA-60, Shimadzu, Japan). Samples of pure TRT, Span60, cholesterol and drug-loaded proniosomes (N8) were submitted to DSC analysis. The analyses were performed on 5-mg samples sealed in standard aluminum pans. Thermograms were obtained at a scanning rate of 20 °C/min. Each sample was scanned between 0 °C and 290 °C.

**Data analysis**

Data are expressed as the means ± standard deviation (SD) of the mean and statistical analysis was carried out employing the one-way analysis of variance (ANOVA). A value of p < 0.05 was considered statistically significant.

**Formulation of TRT-based proniosomal hydrogel**

Based on the previously mentioned characterization, and the results of the main effects of the adopted factorial design a candidate formula N8 (containing 0.025% TRT) with adequate vesicle size, highest entrapment efficiency and high % of drug released after 5 h was selected. The selected N8 hydrated proniosomal formulation was formulated into hydrogel (N8G) by adding 1% (w/w) Carbopol 934 under magnetic stirring at 800 rpm. Stirring was continued until Carbopol was dispersed. The dispersions were neutralized using triethanolamine solution (Shah et al., 2007). Hydrogel formulation containing 0.025% TRT dispersion (TG) was prepared for comparison.

**Skin irritation test**

The study protocol and subject informed consent were approved by the institutional review board of Faculty of Pharmacy, Cairo University (IRB00007140) and the study was conducted according to the Declaration of Helsinki (Declaration of Helsinki–Current, 2013) and the International Conference on Harmonization of Technical requirements for Registration of Pharmaceuticals for Human Use Guidance for good clinical practice (Guidelines for Good Clinical Practice. European Medicines Agency, CPMP/ICH/135/95 July2002). Ten healthy subjects (aged from 23 to 40 years) participated in this study. The participants were briefed on the study procedures and a written informed consent was obtained from all subjects prior to conducting procedure. Each formulation (N8G, TG, and marketed product) was applied once to each volunteer, at a dose of 0.3 g on a surface area of 5 cm² on forearm. After 6 h, the test specimen was thereafter washed off by tap water and observed for any visible change such as erythema (redness). The mean erythema scores were recorded (ranging from 0 to 4) according to Draize (Campbell & Bruce, 1981), where 0 means no erythema, 1 slight erythema, 2 moderate erythema, 3 moderate to severe erythema, and 4 severe erythema.
Clinical evaluation of the selected formulae

Study subjects

The study was conducted in the dermatology outpatient clinic in Kasr Al-Aini Teaching Hospital, Cairo, Egypt. A total of 12 patients with clinically confirmed lesions of acne vulgaris in face area were selected for study inclusion based on the following criteria.

Inclusion criteria

Patients could be enrolled in this planned study follow-up visits based on the following criteria:

- Adult patients (>18 years old)
- Participants agreed to stop applying any topical product 14 days before the beginning of the study, and not to use/apply any topical intervention/product throughout the entire study period.
- Patients agreed to use the provided product and were ready to comply with the treatment and follow up procedures.

Study participants exclusion criteria

Subjects were excluded if:

- They had previously received oral antibiotics, benzoyl peroxide, TRT, and oral retinoids.
- Patients with endocrine disease, diabetes mellitus, or severe physical illnesses.
- Patients who were currently using oral contraceptives, or implantable contraceptives.
- Patients who were using any systemic medications likely to affect (flaring or healing it up) such as oral phenytoin, finasteride, spironolactone, flutamide, testosterone, or dietary body-building protein powders, and
- Patients who used topical, systemic, inhaled, or intravascular corticosteroids on within the past 4 weeks (Emanuele et al., 2012; Vender & Vender, 2012).

This study had been approved by the Research Ethics Committee Office of Faculty of Medicine, Cairo University, which followed the tenets of the Declaration of Helsinki. (Bolton & Bon, 2003).

Each participant was informed about the purpose of the study, and signed informed consent to be photographed before and after treatment.

Study design

The study was designed in a way that each subject is working as his/her own control, where the face of the selected subject was divided into two zones; the right side for the application of N8G once daily while the left side for the application of marketed TRT product (Acretin 0.025% TRT from Jamjoom Pharma, Saudi Arabia) once daily. During the baseline evaluation visit (Day 0), all the patients were instructed on how to apply the formulae over the acne areas on the face. All the patients were then assessed at follow-up visit every week for 4 weeks. The assessment included both the safety and the efficacy of the applied medication. Safety was assessed by interviewing the patients about any sign/symptom of adverse reactions (erythema, peeling, burning sensation, dryness and pruritus) using structured questionnaire at each visit.

The efficacy was determined by lesions counting. The counting process was carried out by a blinded and trained dermatologist. For each treatment an improvement was recorded when the number of papules, closed comedons and open comedons decreased at follow-up. The total number of lesions counted in the first visit was considered to be 100% and any decrease in the number of lesions was calculated accordingly and regarded as percentage reduction. Means and standard deviation of this percentage reduction were calculated in each group of patients every week and were used for further statistical analysis for every lesion type and for total lesion count. The significant difference between percent change of the marketed product and N8G were tested by using student t-test at $p < 0.05$.

Data were collected and analyzed using SPSS statistical package version 19 (SPSS Inc., Chicago, USA).

Results and discussion

Our objective in this study is to prepare TRT proniosomes formed of non-ionic surfactants, cholesterol, and sorbitol, which can be converted to a stable niosome dispersions by hydration. TRT is insoluble in water, so for entrapment efficiency and release studies, a phosphate buffer solution pH 5.5 containing 1% Tween 20 was used also the use of pH 5.5 is expected to give better simulation of in-vivo condition where it is similar to the pH of skin.

Characterization of the prepared hydrated proniosomes

Transmission electron microscope (TEM)

The TEM micrographs of formulations prepared using Span 40 (N4) and Span 60 (N8) are illustrated in Figure 1(A) and (B), respectively. It was observed that the vesicles of the niosomes formed by hydration of the proniosomes are almost spherical in shape and in nanometer size. This could be attributed to the fact that on niosome formation using Span, spherical shaped niosomes were obtained in order to minimize the surface free energy. The non-ionic surfactants form a closed bilayer vesicle in aqueous media based on its amphiphilic nature using some energy from instance heat, physical agitation to form this structure. (Masud et al., 2010). This is in agreement with what was observed by Das & Palei (2011).

Vesicle size analysis

The results revealed that all the prepared hydrated proniosomes showed a considerable small vesicle size with $\text{LD}_{90\%}$ less than 0.5 $\mu$m. The mean vesicle size of hydrated proniosome dispersions ranged from 0.29 ± (0.011) $\mu$m (N6) to 0.398 ± (0.04) $\mu$m (N3) (Table 3). The polydispersity index, PDI, as a characteristic parameter for the width of vesicle size distribution ranged from 0.46 to 0.55 (Table 3). These low values contributed to relatively narrow size distribution and homogenous distribution (Kakar et al., 2010; Das & Palei, 2011; Keservani et al., 2011). Needless to say that small
diameter is advantageous to decrease irritation and improve the penetration of the vesicles into skin. Statistical analysis showed that drug concentration, surfactant: cholesterol ratio and surfactant type had significant effects \((p < 0.05)\) on the vesicle size.

**Effect of drug concentration**

Higher concentration of TRT \((0.025\%)\) significantly increased the vesicle size compared to lower concentration \((0.01\%)\), \(p < 0.05\). This could be attributed to the lipophilicity of the drug that it was embedded in the bilayer within the surfactant molecules inside the vesicles competing for the spaces leading to increase in vesicular size. This was in accordance to Solanki et al., Essa & Keservani et al. who found that at low drug concentration vesicle size decreased significantly \((\text{Solanki et al., 2008; Essa, 2010; Keservani et al., 2011})\).

**Effect of surfactant type**

Hydrated proniosomes prepared using Span 40 HLB \((6.7)\) had significantly larger size than hydrated proniosomes prepared using Span 60 HLB \((4.7)\). This may be due to the decreased surface free energy with increased surfactant hydrophobicity on decreasing the HLB \((\text{The higher the surfactant hydrophobicity, the higher the water uptake into the bilayer})\). It was previously published that vesicle size was directly proportional to the surfactant monomer hydrophilicity \((\text{Uchegbu, 1994})\). Keservani et al. found that the observed relationship between the vesicular diameter of niosomes and sorbitan fatty acid ester hydrophobicity has been attributed to the decrease in surface free energy with increasing hydrophobicity, resulting in smaller vesicles \((\text{Keservani et al., 2011})\).

**Effect of surfactant/cholesterol molar ratio**

Low surfactant. Cholesterol molar ratio \((1.5:1\text{molar ratio})\) (i.e. high cholesterol content where the \% of cholesterol constituted 40\% of total weight of proniosomes) significantly increased vesicle size when compared to high surfactant: cholesterol molar ratio \((3:1)\) \((\text{cholesterol content constituted 25\% of total weight of proniosomes})\). This could be explained by the easier formation of the vesicle and/or the better accommodation of the surfactant in the vesicle structure \((\text{Shatalebi et al., 2010})\). Naggar stated that formulae containing no cholesterol might have the smallest particle size as cholesterol filled in empty spaces among the surfactant molecules and thus increased the particle size of the prepared vesicles \((\text{Naggar et al., 2012})\). Essa results indicated that niosomal vesicle size increased \((p < 0.05)\) linearly with increasing cholesterol ratio, at low cholesterol concentration, it was feasible to expect that cholesterol would have resulted in close packing of surfactant monomers with increasing curvature and reducing size. However, increasing cholesterol content, with its known lipophilic nature and consequently reducing nonionic surfactant content (within the vesicle), would have resulted in increased hydrophobicity of the bilayer membrane and may had imparted disturbance in the vesicular membrane, thus, increasing vesicle radius in a way to establish a more thermodynamic stable form. \((\text{Essa, 2010})\). This result was in accordance with the result stated by Keservani et al., who found that cholesterol increased particle size \((\text{Keservani et al. 2011})\). Negi et al. and Mujoriya et al. also stated that increased cholesterol concentration led to an increase in vesicle size \((\text{Mujoriya et al., 2011; Negi et al., 2011})\).

Table 3. Vesicle size distribution, mean vesicle size, polydispersity index (PDI) entrapment efficiency (E.E \(\%\)), of different tretinoin hydrated proniosomal dispersions.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>LD&lt;sub&gt;10%&lt;/sub&gt;</th>
<th>LD&lt;sub&gt;50%&lt;/sub&gt;</th>
<th>LD&lt;sub&gt;90%&lt;/sub&gt;</th>
<th>Mean vesicle size ((\mu m)) Mean ± SD</th>
<th>PDI</th>
<th>Entrapment efficiency mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>0.28</td>
<td>0.377</td>
<td>0.49</td>
<td>0.38 ± (0.012)</td>
<td>0.55</td>
<td>76.6 ± 0.012</td>
</tr>
<tr>
<td>N2</td>
<td>0.23</td>
<td>0.30</td>
<td>0.38</td>
<td>0.33 ± (0.046)</td>
<td>0.46</td>
<td>81.7 ± 0.011</td>
</tr>
<tr>
<td>N3</td>
<td>0.34</td>
<td>0.43</td>
<td>0.56</td>
<td>0.398 ± (0.04)</td>
<td>0.50</td>
<td>87.4 ± 0.041</td>
</tr>
<tr>
<td>N4</td>
<td>0.29</td>
<td>0.39</td>
<td>0.50</td>
<td>0.390 ± (0.0005)</td>
<td>0.52</td>
<td>91.9 ± 0.046</td>
</tr>
<tr>
<td>N5</td>
<td>0.39</td>
<td>0.30</td>
<td>0.23</td>
<td>0.32 ± (0.009)</td>
<td>0.50</td>
<td>84.5 ± 0.010</td>
</tr>
<tr>
<td>N6</td>
<td>0.22</td>
<td>0.27</td>
<td>0.35</td>
<td>0.29 ± (0.011)</td>
<td>0.46</td>
<td>82.4 ± 0.001</td>
</tr>
<tr>
<td>N7</td>
<td>0.28</td>
<td>0.37</td>
<td>0.48</td>
<td>0.377 ± (0.003)</td>
<td>0.52</td>
<td>90.6 ± 0.01</td>
</tr>
<tr>
<td>N8</td>
<td>0.23</td>
<td>0.30</td>
<td>0.38</td>
<td>0.33 ± (0.046)</td>
<td>0.46</td>
<td>94.15 ± 0.01</td>
</tr>
</tbody>
</table>
**Entrapment efficiency**

The entrapment efficiency of all hydrated proniosomal dispersions is shown in Table 3. The entrapment efficiency ranged from 76.6% ± 0.001 (N1) prepared using Span 40 to 94.15% ± 0.041 (N8) prepared using Span 60.

**Effect of drug concentration**

Concerning drug concentration, increasing drug concentration led to significant increase in %drug entrapment efficiency, \( p < 0.05 \). The increased entrapment efficiency with higher amount of drug used in the formulation could be due to the saturation of the media with the drug that forces the drug to be encapsulated into the hydrated proniosomes (Akhilesh et al., 2012). This is in accordance to the results stated by Aboelwafa et al. who found that on increasing the amount of Carvedilol added; an increase in entrapment efficiency was observed (Aboelwafa et al., 2010). Balakrishnan et al. also found that the entrapment efficiency of minoxidil was increased in Span 40 and Span 60 niosomes, as the drug concentration was increased from 20 to 25 mg (Balakrishnan et al., 2009).

**Effect of surfactant/cholesterol molar ratio**

It was found that higher surfactant/cholesterol molar ratio (3:1)(cholesterol content constituted 25% of total weight of proniosomes) significantly increased %entrapment efficiency of the TRT when compared to lower surfactant/cholesterol molar ratio (1.5:1) cholesterol content constituted 40% of total weight of proniosomes). This could be explained on the basis that cholesterol may compete with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into the vesicles (Balakrishnan et al., 2009). On the other hand, Singh et al. found that higher cholesterol concentration resulted in increased entrapment efficiency till certain concentration at which increase in cholesterol concentration resulted in decrease in entrapment efficiency (Singh et al., 2011).

**Effect of surfactant type**

Hydrated proniosomes prepared using Span 60 showed significantly higher entrapment efficiency \( (p < 0.05) \) than those prepared using Span 40. This may be correlated to the hydrophobicity of the alkyl chain of the sorbitan esters and the affinity of the hydrophobic drug (TRT) to surfactant with lower HLB (Span 60(4.7)) than to surfactant with higher HLB (Span 40(6.7)). The results were in accordance to results stated by Singh et al. who stated that entrapment efficiencies for niosomes prepared using Span 60 were superior to those prepared using Span 20 and 40 (Singh et al., 2011). Keservani et al. also found that entrapment efficiency of niosomes prepared by span 60 was higher than entrapment efficiency of niosomes prepared by Span 40 (Keservani et al., 2011). Abdallah et al. (2013) stated that the surfactant having the highest triglycerides produces the highest entrapment efficiency. On the other hand, Shatalebi et al. found that Span 60 showed lower entrapment than span 40 niosomes (Shatalebi et al., 2010).

**In-vitro release study**

The in-vitro release profile of TRT from the hydrated proniosomal dispersions was investigated over 5 h. In-vitro release studies give information about the product behavior in the in-vivo. The results are shown in Figure 2. During the first hour, the drug release was less than 10% probably because of the slow diffusion of drug from vesicles then it increased till the 5th hour. The % of TRT released from the hydrated proniosomes after 5 h varied between 43% ± 2 for N5 prepared using 0.01% TRT and Span 60: cholesterol molar ratio of 1:1.5 to 78% ± 1.5 for N4 prepared using 0.025% TRT and...
Span 40: cholesterol molar ratio of 1:3. The three factors under study namely, drug concentration, surfactant: cholesterol ratio and surfactant type were found to significantly affect the % of drug released from hydrated proniosomes after 5 h ($p < 0.05$).

Effect of drug concentration

Regarding the drug concentration, high TRT concentration 0.025% significantly ($p < 0.05$) enhanced the percent of drug released from the vesicles after 5 h when compared to lower concentration (0.01%). This might be due to higher entrapment of the drug in formulae prepared with high drug concentration.

Surfactant/cholesterol molar ratio

Concerning the surfactant: cholesterol ratio, cholesterol was found to significantly affect the percentage of TRT released after 5 h form the hydrated proniosomal formulae ($p < 0.05$). Increased cholesterol % of total surfactant content (surfactant: cholesterol molar ratio 1.5:1) led to significant decrease in the percentage of drug released which could be attributed to its membrane stabilizing ability against the diffusion of drug entrapped in the hydrophobic regions of the vesicles where cholesterol act as a cement material. Another explanation could be on the base that cholesterol is known to abolish the gel to liquid phase transition of niosomal system resulting in less leaky niosomes. This result could be explained by the decreased leakage and permeability of niosomal formulations in presence of cholesterol which led to lower drug elution from the vesicles (Shatalebi et al., 2010).

Effect of surfactant type

Regarding the effect of surfactant type, hydrated proniosomes prepared using Span 40 showed higher release than those prepared using Span 60 ($p < 0.05$). This may be due to that niosomes exhibit an alkyl chain length-dependent release and the higher the chain length, the lower the release rate. This can be attributed to the higher HLB value of Span 40 than Span 60, leading to the easier drug release. This also can probably be due to the higher fluidity of these vesicles.

Span 40 has a relatively lower phase transition temperature and therefore may render more fluidity and leakiness to the corresponding niosomes. Shatalebi et al. (2010) also found a relatively higher extent of release from Span 40 than Span 60.

Differential scanning calorimetry

DSC thermograms (Figure 3) of TRT and cholesterol showed endotherm at 178.86°C and at 150°C, respectively, while Span 60 showed 3 endotherms at 54.63, 122.96, and 271.2. DSC thermogram of N8 composed of TRT and Span60: cholesterol in 3:1molar ratio interestingly showed disappearance of the melting endotherm of TRT and a shift of the endotherm of cholesterol to 142°C. Similarly, the melting endotherms of Span60 were shifted signifying that all the lipid components might interact with each other to an extent while forming the lipid bilayer. Absence of the melting endotherm of TRT and shifting of the lipid bilayer components endotherm suggested enhanced entrapment of the drug. This was in accordance to results recorded by Patel et al. and Bayindir & Nilufer (Patel et al., 2009; Bayindir & Nilufer, 2012).

Irritation test

Based on the previously mentioned characterization, and the results of the main effects of the adopted factorial design a candidate formula N8 (containing 0.025% TRT) with small vesicle size (0.33 μm ± 0.046), highest entrapment efficiency (94.15% ± 0.041) and high% of drug released after 5 h (70% ± 2%) was selected and incorporated into 1% carbopol gel.

Results of skin irritation are shown in Table 4. Only very slight erythema (score = 0.143 ± 0.377) was observed for TRT proniosomal gel. Conversely, 0.025 % TRT gel showed

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Reaction in volunteers (Mean ± SD) $N = 10$</th>
</tr>
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<tbody>
<tr>
<td>Free TRT gel</td>
<td>1.70 ± 0.755</td>
</tr>
<tr>
<td>N8 Gel</td>
<td>0.143 ± 0.377</td>
</tr>
<tr>
<td>Marketed product</td>
<td>1.50 ± 0.354</td>
</tr>
</tbody>
</table>

Table 4. Results of skin irritation test of free tretinoin, tretinoin-loaded n8, and tretinoin marketed product.
significantly higher than the meloxicam suspensions. The efficacy of follicular penetration (Jung et al., 2006). in the range of the skin temperature or below have the highest increase the trans-follicular drug uptake. Niosomes that are hair follicle than standard formulations. Thus, niosomes could (Jaafari et al., 2009) epidermis and dermis by electron microscopy studies. proved this by the presence of intact niosomes in the through the SC, reach the epidermis and deep dermis, and was not able to diminish the irritation caused by topical application of TRT. This could be attributed to the the small vesicle size and the role of proniosomes in protecting the skin from direct contact with the drug which was embedded in the vesicles. Incorporation of TRT in vesicles would reduce the contact of the acidic group (−COOH) of TRT with the stratum corneum and allowed gradual delivery of TRT to epidermal, therefore resulting in reduced the erythematous events and improve skin tolerability (Shah et al., 2007). The small vesicle size and the high entrapment efficiency of TRT in this candidate formula could be beneficial to reduce the skin irritation.

Clinical study

The study population comprised 12 Egyptian patients aged >18 years (2 males and 10 females; with an average of 20 (± 4) years) with acne (papules, closed comedons and open comedons) on their face. Also, the overall lesion improvement during the whole study period was in favor of N8 G formula. These results could be attributed to the small vesicle size and enhanced penetration of the TRT across the stratum corneum. Although the improvement was clear, it was statically insignificant this may be attributed to the small sample size which limited the power of the results.

Jaafari et al. previously stated that, vesicles in the proper formulations and sizes have been shown to be able to pass through the SC, reach the epidermis and deep dermis, and also target the macrophages within the dermis. The author proved this by the presence of intact niosomes in the epidermis and dermis by electron microscopy studies. (Jaafari et al. 2009)

It has been proven that, vesicles penetrate deeper into the hair follicle than standard formulations. Thus, niosomes could increase the trans-follicular drug uptake. Niosomes that are in the range of the skin temperature or below have the highest efficacy of follicular penetration (Jung et al., 2006).

Duangjit et al. found that, the flux of meloxicam permeated through the skin in all vesicle formulations was significantly higher than the meloxicam suspensions. The vesicle systems were able to promote skin permeation of an active drug by a variety of mechanisms: (a) the free drug mechanism, (b) the penetration-enhancing process of the vesicle components, (c) vesicle adsorption to and/or fusion with the SC, and (d) intact vesicle penetration into and through the intact skin and the localization at the site of action. (Duangjit et al., 2011).

Fang et al. concluded that, transdermal permeation and skin partitioning of enoxacin from Span 40 and 60 niosomes were similar but much higher than that from free form. This could be attributed to the fact that, surfactant in formulation acts as a permeation enhancer. Another explanation is that niosomes can fuse at the interface of stratum corneum leading to the generation of high local drug concentration in the bilayers with high thermodynamic drug activity (Fang et al., 2001).

Conclusion

The encapsulation of TRT in proniosomes provided the advantage of overcoming solubility and skin irritancy problems. N8G (hydrated proniosomes prepared with 0.025% TRT; Span 60 and span60: cholesterol molar ratio of 3:1 and incorporated in 1% carbopol gel) showed superiority when compared to marketed product in decreasing the open and closed comedons, papules and the total number of acne lesions in acne patients which could be attributed to the enhanced penetration of the TRT across the stratum corneum. In conclusion, the results of this study emphasize the potential of TRT-loaded proniosomes as a topical drug delivery system for enhancing the acne treatment efficacy of TRT while reducing its side effects.

Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

References


Aboelwafa AA, El-Setouhy DA, Elmendash AN. (2010). Comparative study on the effects of some polyoxyethylene alkyl ether and sorbitan fatty acid ester surfactants on the performance of transdermal


Available at: http://www.aizonano.com/oars.asp.


