Production and characterization of newly developed alcohol-free topical liposome-gel transdermal drug delivery systems containing estradiol (E2)/estriol (E3) for post-menopausal women

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ABSTRACT: The aim of the research was to develop alcohol-free and relatively safe liposome-gel formulations containing Estradiol (E2)/Estriol (E3) combinations for menopausal women. Hereewith, we purposed to solve some of the transdermal products containing ethanol by preparing liposome-gel formulations. The purpose of this research was the develop of liposome-gel formulations including Estradiol (E2) /Estriol (E3) hormones. Mean particle size, zeta potential, FT-IR spectrum, rheological behaviour studies were evaluated in transdermal non-invasive formulation. The optimum formulation (LHG6) was concluded to be the negatively charged liposomes, which exhibited high physical characteristics, and relatively optimum particle size for transdermal penetration as 153.3 nm ± 1.1. Mean particle size distribution of empty liposome dispersion was smaller than E2/E3 loaded liposomes (LH6) because of encapsulation of them. So, the best E2/E3 loaded formulation (LH6) was selected according to the mean particle size distribution analysis, PDI values (< 0.5 PDI value) PDI value was found as 0.371 ± 0.01 for LH6. Moreover, since the zeta potential was found to be -54.9 mV± 0.25, it is predicted to be more stable than other E2/E3 loaded liposomes according to DLVO theory. When U21 and U30 used as gelling agent were compared, it was observed that U21 showed more stable rheological behavior with approximately 25500 cP at skin pH. For this reason, U21 was preferred as a gelling agent for liposome dispersions in the second step. Considering the liposome-gel formulations, the LHG6 formulation, which is not excessively viscous in skin application compared to the other three formulations in terms of ease of transdermal application, was found to have a value of 34500 cP and was selected as the optimum liposome-gel formulation. Formulation and characterization studies supported that liposome gel delivery system is suitable for topical applications. All results supported that liposome-gel delivery system is more appropriate for transdermal applications. Since the liposome-gel transdermal drug delivery system is suitable and safer than oral administration in all characterization studies, efficacy studies of E2/E3 loaded liposome-gel formulations will soon be possible with in-vivo studies in human volunteers.

KEYWORDS: Liposome; transdermal; estradiol; estriol; menopause.

1. INTRODUCTION

Liposomes are microscopic spherical structures consisting of membrane-shaped lipid sheets with hydrophilic head parts. The lipid sheets are basically composed of phosphatidylcholine, which are amphiphilic due to their hydrophilic head and hydrophobic tail. Liposomes are commonly used in drug delivery systems, cosmetics, tumor diagnosis and imaging, and enzyme replacement therapy. Liposome systems, one of the most important colloidal carriers, were discovered by Alec. D. Bangham in the 1960s during biological membrane modeling studies in the 1960s. It was later discovered that these artificial cell-like structures could be used as drug delivery systems [1, 2]. Liposome carrier systems, one of the most widely studied topics today, are mostly spherical-shaped vesicles consisting of nonpolar lipid layers and a hydrophilic region between these layers. Particle sizes of liposomes...
vary between nanometers and micrometers [3, 4]. Liposomes can be considered as artificial versions of natural membranes. Liposomes containing phospholipids, cholesterol, surface charge donors, and some cargo donors in their structure are very similar to the cell membrane structure and are therefore highly compatible with biological systems [5]. According to their physicochemical properties, liposomes can carry molecules used in the pharmaceutical industry and food in different regions. Similar to cells in biological systems, liposomes can hold hydrophilic drug active ingredients in their internal regions and highly fat-soluble active ingredients and amphiphilic molecules in their membrane parts [6, 7].

Liposomal carrier systems used in both therapeutic and diagnostic fields are preferred due to their many advantages. These advantages are as follows. In addition to being biodegradable, these carrier systems are bio-permeable and biocompatible [8, 9]. Their high degree of biocompatibility is due to their content in the structure of biological membranes. They have low systemic toxicity and cytotoxicity due to their high degree of biocompatibility. Thanks to their hydrophilic and lipophilic regions, they allow the transport of both hydrophilic and lipophilic drug active ingredients [10, 11]. It is possible to create long and controlled drug release with phospholipid-containing drug carrier systems [12]. In addition, thanks to controlled drug release, plasma drug concentration can be maintained at the desired level for the desired period. When considered therapeutically, extended-release liposomal systems not only extend the dosing interval and drug half-life but also reduce toxic effects [13, 14]. They can be produced in various sizes and surface properties. In this way, they allow active and/or passive targeting of drug-active ingredients to the site of action. Liposomal drug delivery systems can be administered by many alternative delivery routes such as oral, topical, nasal, pulmonary, intramuscular, intravenous, subcutaneous, and ocular routes [15, 16].

Menopause is explained as a stopping of ovarian function that results in permanent amenorrhea. During menopause, hormonal changes include an increase in follicle-stimulating (FSH) and luteinizing hormone (LH), on the contrary, a decrease in the production of estradiol occurs which leads to complex changes, mainly bone loss, in the entire body of a woman. The estradiol's discontinuation is associated with vasomotor symptoms like hot-flashes and vaginal dryness which are one of the annoying symptoms [17, 18].

A healthy woman has an average of 150 pg/mL free E2 in her blood. If the E2 level is around 5 pg/mL in blood, it is understood that menopause has been confirmed [19, 20].

The treatment of menopause with Estradiol (E2) and Estriol (E3) administered orally in drinking water are very effective in reducing symptoms of menopause such the bone loss [21]. However, it is worthy of note that women taking oral E2 medications confront with common side effects such as increased risk of cardiovascular problems [22]. Generally, transdermal routes are safer and less strict than intravenous ways [23]. Estradiol (E2) plays important role in the treatment of postmenopausal hormonal problem, which may lead to atherosclerosis, fractures, and osteoporosis. Oral use of estradiol may create some side effects in postmenopausal women.

It has extensive first-pass metabolism. E2 has highly first-pass effect in the liver to estrone (E1). For the reason topical use of estradiol may improve the systemic bioavailability and maintain a favorable estradiol/estrone (E1) ratio in the blood. The transdermal route has been used especially for the treatment of postmenopausal issues. It has been administered different pharmaceutical dosage forms [24].

Ethanol applied topically as a skin absorption enhancer can facilitate the transdermal absorption of many active substances and even xenobiotics including carcinogenic contaminants in formulations [25]. The use of ethanol is included in many commercial products containing hormones.

After regular application of ethanol to the skin, relatively low levels of its metabolite acetaldehyde may be formed in the blood, although these are below acutely toxic levels and there is a risk of percutaneous toxicity, especially when applied to skin with compromised epidermal integrity [26]. Ethanol-containing topical hormone products on the market were given in Table 1.

Table 1. Ethanol-containing transdermal hormone preparations on the market [27].

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testim®</td>
<td>Testosterone (1%), ethanol, carbomer 980, carbomer 1342, propylene glycol, glycerol, macrogol 1000, trometanol, pentadecalactone, purified water</td>
</tr>
<tr>
<td>Androgel®</td>
<td>Testosterone (1%), ethanol, carbomer 980, isopropyl myristate, sodium hydroxide, purified water</td>
</tr>
<tr>
<td>Tostrex®</td>
<td>Testosterone (2%), ethanol, carbomer 1382, isopropyl alcohol, propylene glycol, oleic acid, trolamine, butylhydroxytoluene, purified water</td>
</tr>
<tr>
<td>Androtiv®</td>
<td>Testosterone (2 %), alcohol, water, carbomer, tea, phenoxyethanol, methyl/paraben, ethyl/paraben, propyl/paraben, butyl/paraben</td>
</tr>
<tr>
<td>Estreve®</td>
<td>Estradiol hemihydrate, alcohol (ethanol), isopropyl alcohol, propylene glycol</td>
</tr>
</tbody>
</table>
Formulation of certain delivery systems, such as E2 liposomes in optimized vehicle bio-adhesive hydrogels is a promising challenge for the treatment of postmenopausal symptoms [12]. Although the endpoint of the above-mentioned problems and their solutions seem obvious, there is no research article about the development of alcohol-free hormonal liposome gel formulations. Therefore, the first aim of this study was to develop new liposome-gel formulations containing E2/E3 hormone combinations for menopause treatment. To assess the benefits of the topical liposome-gel formulations, the E2/E3 was appropriately gelled and evaluated by various characterization parameters including determination of particle size, zeta potential, polydispersity index, and FT-IR analysis.

2. RESULTS

2.1. Physicochemical properties of the liposome formulations

Results of mean particle size and size distribution, polydispersity index, and zeta (ζ) potential of liposomes were shown in Table 2. The mean particle size of all liposome formulations was between 100–400 nm. Polydispersity indexes were determined as 0.200-0.500. Zeta (ζ) potential is a function of the overall charge of a particle, and changes in size reflect aggregation or separation. Zeta (ζ) potential values of liposomes were found between -40 mV to -100 mV.

Table 2. Particle size, Zeta (ζ) potential, and polydispersity index result of liposomes (n=3).

<table>
<thead>
<tr>
<th>Code</th>
<th>Composition</th>
<th>Molar ratio</th>
<th>Average diameter (nm)</th>
<th>(Poly Dispersity Index) PDI</th>
<th>Zeta (ζ) potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH 1</td>
<td>SPC:DCP:CHOL</td>
<td>7:1:2</td>
<td>120.7 ± 0.3</td>
<td>0.287 ± 0.01</td>
<td>-55.6 ± 0.07</td>
</tr>
<tr>
<td>LH 2</td>
<td>SPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL)</td>
<td>7:1:2</td>
<td>170.1 ± 13.5</td>
<td>0.295 ± 0.08</td>
<td>-45.2 ± 3.25</td>
</tr>
<tr>
<td>LH 3</td>
<td>SPC:DCP:CHOL</td>
<td>10:1:4</td>
<td>129.1 ± 0.4</td>
<td>0.256 ± 0.01</td>
<td>-57.4 ± 0.75</td>
</tr>
<tr>
<td>LH 4</td>
<td>SPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL)</td>
<td>10:1:4</td>
<td>374.8 ± 13.6</td>
<td>0.468 ± 0.09</td>
<td>-48.3 ± 1.20</td>
</tr>
<tr>
<td>LH 5</td>
<td>HSPC:DCP:CHOL</td>
<td>7:1:2</td>
<td>153.3 ± 1.1</td>
<td>0.229 ± 0.01</td>
<td>-103 ± 0.61</td>
</tr>
<tr>
<td>LH 6</td>
<td>HSPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL)</td>
<td>7:1:2</td>
<td>175.2 ± 1.9</td>
<td>0.371 ± 0.01</td>
<td>-54.9 ± 0.25</td>
</tr>
<tr>
<td>LH 7</td>
<td>HSPC:DCP:CHOL</td>
<td>10:1:4</td>
<td>143.7 ± 0.7</td>
<td>0.252 ± 0.01</td>
<td>-61.4 ± 0.58</td>
</tr>
<tr>
<td>LH 8</td>
<td>HSPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL)</td>
<td>10:1:4</td>
<td>260.1 ± 9.5</td>
<td>0.400 ± 0.04</td>
<td>-44.3 ± 1.70</td>
</tr>
</tbody>
</table>

2.2. Molecular characterization of liposome components and hormones by FT-IR

The molecular structures of DCP, SPC, HSPC, cholesterol, estradiol, and estriol samples were given in Figure 1, and also the molecular structures of liposome formulations were shown in Figure 2.
Figure 1. FT-IR spectrums of DCP, SPC, HSPC, cholesterol, estradiol, and estriol.

Figure 2. FT-IR spectrums of liposome dispersions (LH1-LH8.)

2.3 Rheological result of the gel and liposome-gel formulations

Rheological diagrams of liposome-gel formulations were in Table 3 and Figure 3.
Table 3. Rheological result of the gel bases and liposome-gel formulations of liposomes.

<table>
<thead>
<tr>
<th>Code</th>
<th>Composition</th>
<th>Volume Ratio (Liposome/Gel)</th>
<th>Viscosity (cP)</th>
<th>Shear Stress (dyne/cm²)</th>
<th>Shear Rate (1/s)</th>
<th>Torque (%)</th>
<th>Speed (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U21</td>
<td>Acrylates/C10-30 Alkyl Acrylate Crosspolymer (0,5 % w/v) gel base</td>
<td>-</td>
<td>25500</td>
<td>1,275</td>
<td>5000</td>
<td>25.5</td>
<td>20</td>
</tr>
<tr>
<td>U30</td>
<td>Carbomer(0,5% w/v) gel base</td>
<td>-</td>
<td>22700</td>
<td>1,135</td>
<td>5000</td>
<td>22.7</td>
<td>20</td>
</tr>
<tr>
<td>LGH 2</td>
<td>SPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL) + Polymer (U21 gel base)</td>
<td>1/1</td>
<td>58400</td>
<td>2,920</td>
<td>5000</td>
<td>58.4</td>
<td>20</td>
</tr>
<tr>
<td>LGH 4</td>
<td>SPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL) + Polymer (U21 gel base)</td>
<td>1/1</td>
<td>59100</td>
<td>2,955</td>
<td>5000</td>
<td>59.1</td>
<td>20</td>
</tr>
<tr>
<td>LGH 6</td>
<td>HSPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL) + Polymer (U21 gel base)</td>
<td>1/1</td>
<td>34500</td>
<td>1,725</td>
<td>5000</td>
<td>34.5</td>
<td>20</td>
</tr>
<tr>
<td>LGH 8</td>
<td>HSPC:DCP:CHOL+ E2/E3(0,2mg-0,8 mg/mL) + Polymer (U21 gel base)</td>
<td>1/1</td>
<td>66500</td>
<td>3,325</td>
<td>5000</td>
<td>66.5</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 3. Rheological behaviour results of gel and liposome-gel formulations: a) U21, b) U30, c) LGH2, d) LGH4, e) LGH6, and f) LGH8.

3. DISCUSSION

All results were discussed respectively the obtained from data about E2/E3 liposome and liposome-gel formulation. It was proven that all ingredients and hormones that were used for the preparation of liposome-gel samples were successfully integrated in the formulations according to the results of FT-IR. Results were evaluated from Figure 1 and Figure 2. DCP is confirmed by the in-plane P–O–H stretching bands at 1387 cm⁻¹, while the out-of-plane bending vibration is observed at about 893 cm⁻¹. The strong bands at about 1120, 1060, and 984 cm⁻¹ are assigned to PO3 asymmetric stretching modes [28]. The P–O–C bond of HPC is about 1070 cm⁻¹ [29].
The characteristic band of HSPC is seen at 1690 cm\(^{-1}\) corresponding to C=O stretching vibration, and absorbance maxima are seen around 2922 and 2862 cm\(^{-1}\) due to the C-H bands [30]. The cholesterol spectrum has a characteristic weak broadband at 3180-3450 cm\(^{-1}\) corresponding to O-H stretch [31]. Estradiol has some characteristic peaks belonging to a broad intense O-H stretching peak at 3397 and 3465 cm\(^{-1}\), the free hydroxyls and the intermolecular hydrogen bonds in the range of 1360-1220 cm\(^{-1}\), stretching C-H vibrations in CH\(_3\), CH\(_2\), and CH groups in the range of 2990-2812 cm\(^{-1}\), skeletal C-C vibrations of the whole aromatic ring at 1616 and 1582 cm\(^{-1}\) [32].

Estriol also has some characteristic peaks belonging to symmetric and asymmetric CH2 peaks at 3510 nm and 3445 cm\(^{-1}\), C-H stretching at 3000-3100 cm\(^{-1}\), stretching C-H vibrations in CH3, CH2, and CH groups at 2936-2818 cm\(^{-1}\), skeletal vibrations of the aromatic C-C bonds at 1609-1501 cm\(^{-1}\), polar C-O bond in phenols at 1231-1148 cm\(^{-1}\), C-O stretching vibrations of secondary alcohols at 1125-1030 cm\(^{-1}\) [33].

The bioidentical hormones used in the study were preferred because they are safer than synthetic types. In addition, the E2 / E3 ratio was studied as 1/4, since the E3 hormone minimizes the risk of breast cancer [34, 35]. All liposome-gel formulations are alcohol-free and relatively safe for women [24, 25].

The mean particle size of all liposome formulations was in the range of 100-4000 nm (Table 2). The best formulation (LH5) was concluded to be the negatively charged liposomes, which exhibited high physical characteristics, and relatively optimum particle size for transdermal penetration as 153.3 nm ± 1.1. According to the results, the mean particle size distribution of empty liposomes was smaller than E2/E3 loaded liposomes (LH6) because of encapsulation of them. So, the best E2/E3 loaded formulation (LH6) was selected according to the mean particle size distribution analysis, and PDI values (<0.5 PDI value) PDI value were found as 0.371 ± 0.01 for LH6. Moreover, since the zeta potential was found to be -54.9 mV ± 0.25, it is predicted to be more stable than other E2/E3 loaded liposomes according to DLVO theory. Hydrogenated soy phosphatidylcholine sourced liposomes are known to have better phase transition temperature and stability than conventional soy phosphatidyl choline liposomes in topical applications. As a result of the study, when the physicochemical properties were compared, HSPC liposomes were found to be more suitable than SPC liposomes in parallel with the literature [12].

When U21 and U30 used as gelling agent were compared, it was observed that U21 showed a more stable rheological behavior with approximately 2500 cP at skin pH (Table 3). For this reason, U21 was preferred as a gelling agent for liposome dispersions in the second step. Considering the liposome-gel formulations, the LHG6 formulation, which is not excessively viscous in skin application compared to the other three formulations in terms of ease of transdermal application, was found to have a value of 34500 cP and was selected as the optimum liposome-gel formulation.

4. CONCLUSION

Liposomes are commonly used for medical and non-medical areas. Ethanol-free transdermal application has been used especially for the treatment of postmenopausal problems by using liposomes. It was clear that the individual characteristics of the E2/E3-loaded topical liposome-gel systems significantly affected transdermal uses. Non-hormonal liposomal drug delivery systems also will be affected by women’s health diseases. The results obtained in this study confirm that alcohol free liposome-gel containing E2/E3 formulations are relatively safer than the other commercial E2/E3 products. Formulation and characterization studies supported that the liposome gel delivery system is suitable for topical applications.

Herewith, we purposed to solve problems some of the transdermal products containing ethanol by preparing liposome-gel formulations. The purpose of this research was the develop of liposome-gel formulations including Estradiol (E2) /Estriol (E3) hormones. Since the liposome-gel transdermal drug delivery system is suitable and safer than oral administration in all characterization studies, efficacy studies of E2/E3 loaded liposome-gel formulations will soon be possible with in-vivo studies in human volunteers.

5. MATERIALS AND METHODS

5.1 Materials

In this research, Lipoid P100 (Soybean Phosphatidylcholine-SPC) and Lipoid P100-3 (Hydrogenated Soybean Phosphatidylcholine-HSPC) were provided by Lipoid AG company (Steinhausen, Switzerland). Other raw materials; Dicetyl phosphate (DCP), Cholesterol (CHOL) (Sigma-Aldrich Co., Germany), and E2 (Estradiol)/E3 (Estriol) (Biosynth, United Kingdom) were purchased before the study. All reagents were of analytical grade.
5.2 Preparation of E2/E3 liposome and liposome-gel formulations

E2 and E3 containing 8 different liposome formulations were prepared by using thin film, sonication, and extrusion methods, respectively. Briefly, liposome was prepared by dissolving the 100 µmol mL⁻¹ of soybean and hydrogenated soybean phospholipids in 50 mL chloroform in a bottom flask. The chloroform was evaporated using a rotary evaporator under pressure to form a thin film over the wall of the flask. The dried film was then hydrated over a water bath with distilled water. Then SUV liposomes were prepared by ultrasonication and extrusion process. Free E2/E3 was separated by ultracentrifugation three times at 17,500 rpm for 45 min for each of them. Formulation details were given in Table 2. Four different E2/E3 loaded liposome dispersions (LH2, LH4, LH6, LH8) were incorporated into the gel base containing 0.5 % (w/v) Acrylates/C10-30 Alkyl Acrylate Crosspolymer at pH 5.5, then we finalized four liposome-gel formulations as LHG2, LHG4, LHG6, LHG8. Gel bases and liposome-gel formulation components were given in Table 3.

5.3 Physicochemical characterization of liposomes

5.3.1 Particle Size Distribution (PSD) and Polydispersity Index (PDI)

0.1 ml of liposome dispersions were diluted with 0.9 ml of distilled water after liposome preparation. The size distributions of the liposomes were measured by dynamic light scattering (DLS) using a Particle Sizer. The mean particle size and size distribution and polydispersity index results were obtained as the average of 3 experiments.

5.3.2 Zeta (ζ) potential

Zeta (ζ) potential was measured by using a Zetasizer (Malvern Nano ZSP, United Kingdom) and each result was the mean of 10 measurements. All the measurements were performed at 25°C and an angle of 173°.

5.4 Molecular characterization of liposome components and hormones by Fourier-transform infrared spectroscopy (FTIR)

FTIR (Jasco, FT/IR 4700, USA) was used to evaluate the chemical structures and contents of liposome components, raw materials, and samples. It was evaluated whether DCP, SPC, HSPC, cholesterol, estradiol, and estriol were loaded successfully in the liposome formulations. Measurements were obtained between 400 and 4000 cm⁻¹ wavenumbers with a resolution of 4 cm⁻¹ at room temperature. OPUS Viewer version 6.5 software was used during the analysis.

5.5 Rheological characterization of liposome-gels

The rheological properties of the liposome-gels were measured using a rheometer (Brookfield DVNext Rheometer, United Kingdom). The measuring system used was concentric cylinders, with an inner diameter of 25 mm, an outer diameter of 27 mm, and a 32 mm height. The cylinders are surrounded by a double jacket with an electric resistance and the whole unit can be heated. The double jacket is connected to a liquid nitrogen reservoir and the gel can be cooled. The temperature is determined by a thermocouple that was connected to the inner cylinder. 10 ml of cold polymer gel and liposome gel were transferred to the cylinders. To measure the linear viscoelastic properties, the instrument was used in the oscillatory mode, in which the outer cylinder performs dynamic oscillations at a given frequency. To measure the shear steady-state properties, the same geometry was used; in this case, the outer cylinder rotates at a given angular velocity (ω), which produces a shear rate (γ) gradient through the gap between the two cylinders. The linear viscoelastic properties measured were the complex moduli, \( G'(\omega) \), and the complex viscosity, \( \eta^*(\omega) \). \( G'(\omega) = G'' + iG' \), where \( G' \) is the storage modulus and \( G'' \) is the loss modulus. \( G' \) is related to the storage of energy during the cycle or elastic energy, while \( G'' \) is related to the dissipation of energy during the cycle or viscous energy. \( \eta^*(\omega) = \eta' - i\eta'' \), where \( \eta' \) is the dynamic viscosity and \( \eta'' \) is the imaginary viscosity. The shear steady-state property measured was the shear rate-dependent viscosity, \( \eta(\gamma) \).
5.6. Statistical analysis

Data from the physicochemical, and rheological studies were statistically analyzed with the aid of the Statistical Analysis Software (SPSS version 22. Inc.). One-way analysis of variance (ANOVA) was performed for comparison (P<0.05).

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Conflict of interest statement: The authors declared no conflict of interest.

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