Formulation and evaluation of ethosomes for transdermal delivery of clonazepam

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Key words: Clonazepam, Ethosome, Liposome, Skin Permeation.

http://dx.doi.org/10.12692/ijb/6.5.308-316 Article published on March 14, 2015

Abstract

The aim of this investigation was to compare ethosomal carriers containing clonazepam as an anti epileptic agent with classic liposomes and hydro alcoholic solutions for transdermal drug delivery. Clonazepam loaded ethosomes were prepared and then the vesicular shape and surface morphology, vesicular size, entrapment efficiency, stability, in vitro rat skin permeation in a non-occlusive condition were evaluated. Results indicated that the formulation which contains 3% phospholipid and 40% ethanol had the greatest entrapment efficiency (65.7%) with the nanometric size of 173±10 nm. This formulation was selected for further study. The stability profile of the prepared system was assessed for 120 days; it revealed very low aggregation and growth in vesicular size. Clonazepam loaded ethosomal carriers also provided an enhanced transdermal flux of 5.66 μg/cm²/h. These results suggested that ethosomes are an effective carrier for dermal and transdermal delivery of clonazepam.

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Introduction

Transdermal drug delivery provides a mean to obtain constant systemic drug levels and high patient compliance because of non-invasive nature. Despite many advantages of the skin as a site of drug delivery, the success of delivery through this route remains limited because the main barrier of the skin is located in the outermost layer of the skin - the stratum corneum (SC) (Scheuplein, 2011). To overcome the stratum corneum barrier, many techniques have been assessed to disrupt and weaken the highly organized intercellular lipids in an attempt to enhance the percutaneous drug penetration (Barry, 2001, Schreier & Bouwstra, 1994).

Novel drug delivery system (NDDS) has revolutionized the method of medication. The NDDS should ideally fulfill two prerequisites. Firstly, it should deliver the drug at a rate directed by the needs of the body, over the period of treatment. Secondly, it should channel the active entity to the site of action. Sincere attempts have been made to achieve them through various novel approaches in drug delivery. Several technological advances have been made over many years. One such technique is vesicular systems which shows a great promise and opens up new market for pharmaceutical and cosmetic industry for drug delivery through skin.

The ethosomes are an innovative vesicular system that exhibit attractive features associated with high deformability. Ethosomes can transport active agents through the stratum corneum into deeper layers of the skin more effectively than traditional liposomes (Touitou et al., 2000). The ethosomal vector has been shown to be capable of enhancing intracellular delivery of both hydrophilic and lipophilic molecules (Touitou et al., 2001), and to increase the permeation of an antibiotic peptide (Godin & Touitou, 2004). Ethanol, an important adjuvant in the ethosomal formulation, permeates rapidly through the human skin with a steady state flux of approximately 1 mg/cm²/h (Berner et al., 1989) and therefore has been used to enhance the flux of several drugs through the rat skin, including levonorgestrel, estradiol, and 5-fluorouracil (Friend et al., 1988, El maghraby et al., 2000, El maghraby et al. 2001). Propylene glycol (PG), a widely used vehicle and a well-accepted adjuvant, has also been demonstrated to work synergistically with many permeation enhancers (Williams, 2003). PG as a stand-alone penetration enhancer, has been shown to improve skin permeation as a result of structural changes (Yamane et al., 1995) and has already been investigated as a component of lipid vesicles by Elsayed (El sayed, 2007), who tested PG-liposomes as carriers for skin delivery of cinchocaine.

Different authors evidenced the potential advantages of conventional dosage forms of the transdermal administration of different kinds of benzodiazepines (Nokhodchi et al., 2003, Balaguier-Fernandez et al., 2010, Soler et al., 2012). Among these, clonazepam is an anticonvulsant benzodiazepine which is widely used in the treatment of epilepsy, particularly in children (Fuerst et al., 1986). This drug is considered a very interesting candidate for transdermal administration, due to its pharmacological characteristics, such as high first-pass metabolism, wide blood level oscillations, low dose sizes and need for long-term treatment, poor absorption and bioavailability (Ogiso et al., 1989).

On the basis of all the above considerations, and in continuation of previous studies on transdermal delivery of clonazepam (Corti et al., 1998, Mura et al., 2000, Mura et al. 2014), the present work was aimed at the development of a new innovative and effective transdermal delivery system of clonazepam. With this purpose, we investigated the effectiveness of ethosomes, as a potential carrier for transdermal delivery of clonazepam through rat abdominal skin, with the aim of the maximizing drug flux. In order to evaluate the feasibility of benzodiazepine transdermal administration, we evaluated the in vitro percutaneous permeation through excised rat skin for clonazepam loaded ethosomes in comparison with liposome, and hydroalcoholic solutions. To the best of our knowledge, clonazepam containing ethosomes have not previously been prepared.
Material and method

Materials
Phosphatidylcholine was purchased from Lipoid GmbH (Ludwigshafen, Germany) and contained 75% phosphatidylcholine (Lipoid S75). Clonazepam was purchased from Sobhan Darou Co. (Rasht, Iran). HPLC-grade methanol and tetrahydrofuran, ethanol, propylene glycol, potassium dihydrogen phosphate, hydrochloric acid, anhydrous dibasic ammonium phosphate, phosphoric acid and sodium hydroxide were from Merck (Germany). HPLC grade water used in the analysis was prepared by reverse osmosis and passed through a 0.45 µm millipore filter (Millipore Company, France) before use. Other chemicals used in the investigation were of analytical grade with no additional purification.

Preparation of liposome
Liposomes were prepared according to the thin-film hydration method. Phosphatidylcholine for final concentration of 3% (w/v) and clonazepam (for final concentration of 0.05% (w/v), were dissolved in 60 ml chloroform with 30 g small spherical glass (for an increased surface area) in a 250 ml round bottomed flask. The mixture was evaporated in a rotary evaporator (Buchi, R-124, Switzerland) at 30°C. The film was hydrated with distilled water for 30 min. Sample was sonicated at 4°C using probe sonicator in 3 cycles of 5 min with 5 min rest between the cycles, quickly sealed and store at a temperature of 4°C (Lopez-Pinto et al., 2005).

Preparation of ethosomes
Ethosomes were prepared from phosphatidylcholine (for concentration of 2, 3% (w/v)), ethanol (30, 40% (v/v)) and the drug (0.05% (w/v)) as shown in Table 1. Briefly, phosphatidylcholine and the drug were dissolved in ethanol. Distilled water was added slowly in a fine stream at the constant rate in a well-seal container with constant mixing by a magnetic stirrer at 700 rpm. Mixing was continued for additional 5 min. The system was kept at 30°C throughout the preparation (Dayan & Touitou, 2000, Elsayed et al., 2006).

HPLC assay for clonazepam quantification
The concentration of clonazepam in the receptor compartment of Franz cells was determined by reverse-phase HPLC using a Merck Hitachi 655A liquid chromatography system equipped with a 653A Merck Hitachi variable wave-length UV monitor, a L-5000 Merck-Hitachi LC controller and a D-2000 Merck Hitachi chromatointegrator. Separations were carried out using a RP-8 column. Clonazepam was detected at 254 nm with a mobile phase containing a mixture of ammonium phosphate buffer (0.01M, pH 8): methanol: tetrahydrofuran (60:52:13) at a flow-rate of 1 ml/min.

Vesicle characterization
Visualization by transmission electron microscopy (TEM)
TEM (Zeiss CEM902A Electron Microscope, Germany) was used as a visualizing aid for ethosomal vesicles. Samples were dried on carbon-coated grid and negatively stained with aqueous solution of phosphotungstic acid (Dubey et al., 2007). After drying, the specimen was viewed under the microscope at 30-250 k-fold enlargements at an accelerating voltage of 80 kV.

Vesicular size, size distribution and zeta potential
Particle size and zeta potential were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetasizer, Nano Zs, Malvern UK). The polydispersity index (PI) was used to determine parameters of the size distribution.

Determination of entrapment efficiency
The entrapment capacity of clonazepam by ethosomes and liposomes was measured by the centrifugation method. Vesicular preparations containing 0.05% w/v clonazepam were centrifuged at high speed in a centrifuge (Sigma, laborzentrifugen GMBH, 3K30, Germany) at 5°C for two cycle at 23000 rpm for 1 h each (Elsayed et al., 2006). Clonazepam was assayed in the supernatant. The entrapment efficiency of clonazepam was calculated from the equation [(T-C)/T] ×100, where T is the total amount of clonazepam that is the theoretical amount of
clonazepam that was added, and C is the amount of clonazepam detected only in the supernatant.

**Stability measurement**
Drug retention in the vesicles was assessed by keeping the ethosomal suspensions at 4 ± 2° C for 120 days. The vesicular suspensions were kept in sealed vials (10 ml capacity) after flushing with nitrogen. The stability of ethosomes was also assessed quantitatively by monitoring size and morphology of the vesicles over time using DLS and TEM.

**In vitro permeation and skin deposition studies**
Abdominal skin from rat of 150 to 200 g was used. Rats were sacrificed, shaved with shaving machine in order to remaining integrity of skin and then the skin was cut. A preliminary wash of the skin was done with the normal saline solution; Skin was used directly without storage. Experiments were run in Franz diffusion cells having a receptor compartment volume of 30 ml. The in vitro study design used in the present study was similar to that described by El Maghraby et al. (El Maghraby et al., 1999, El Maghraby et al., 2000). Experiments were performed in two stages. The first stage determined drug permeation in to the skin. In this stage pH 7.4 isotonic phosphate buffer was used (Beall & Sloan, 2001, Kanikkannan et al., 2001, Sloan et al., 1991), as the receptor medium. Skin membranes were mounted, with the stratum corneum side up, open to the atmosphere and floated on receiver solution for 24 h. The receiver content was then replaced by a fresh medium. Test formulations (4 ml non-occluded open application) were applied to the skin surface, which had an available diffusion area of 3.799 cm². Clonazepam solution (0.05%, w/v) in 30, 40% (v/v) alcohol in distilled water were used as controls (n = 3). 1ml samples of the receptor were removed at appropriate intervals for HPLC assay and immediately replaced with fresh medium. At the end of this stage (24 h), the donor compartment and the skin surface were washed five times with warm (45° C) receptor medium. The second stage determined amounts of the drug deposited into the skin. Receptor contents were completely removed and replaced by 50% (v/v) ethanol in distilled water and kept for a further 12 h. All samples were quantitatively analyzed by HPLC. This receiver solution was suggested to diffuse through the skin, disrupting any structure and extracting deposited drug from skin, thus evaluating levels of skin deposition (El Maghraby, et al., 1999, El Maghraby, et al., 2000, El Maghraby et al., 2001). The receptor medium was kept at 37±1° C throughout experiments in order to maintain the skin surface at a temperature of 32° C.

**Statistical analysis**
Results are reported as mean ± SD. Data obtained was compared using one-way ANOVA. Differences between the treatments were assumed to be significant at p < 0.05. Statistical analysis of all data was performed using IBM® SPSS® Statistics, v19, 2010.

**Results and discussion**
Clonazepam loaded ethosomes were prepared using different concentration of phosphatidyylecholine and alcohol and then examined by TEM. They appeared as unilamellar vesicles predominantly spherical in shape (Fig.1).

### Table 1. Composition of all formulations.

<table>
<thead>
<tr>
<th>Batches</th>
<th>Ethanol (%)</th>
<th>PG%</th>
<th>PC%</th>
<th>Drug (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>30</td>
<td>--</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>S2</td>
<td>40</td>
<td>--</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>S3</td>
<td>--</td>
<td>30</td>
<td>--</td>
<td>0.05</td>
</tr>
<tr>
<td>E4</td>
<td>30</td>
<td>--</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>E5</td>
<td>30</td>
<td>--</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>E6</td>
<td>40</td>
<td>--</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>E7</td>
<td>40</td>
<td>--</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>E8</td>
<td>--</td>
<td>30</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>E9</td>
<td>--</td>
<td>30</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>L10</td>
<td>--</td>
<td>--</td>
<td>3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*PG: propylene glycol
*PC: phosphatidyl choline.
Our results show that the average particle size of ethosomes was less than the liposomal formulation (p <0.05) (Table 2). Amounts of ethanol and phospholipid, used for the ethosome preparation, were found to have an influence on the vesicle size. Our results showed that the size of the vesicles increased with increasing the phospholipid concentration (2-3%) significantly (p< 0.05) (Table 2). Also it was observed that the size of the vesicles decreased significantly (p<0.05) with increasing ethanol concentration (30-40%). These data are in agreement with previous finding. The largest vesicles were observed in the formulation E5 (211±14 nm), while the smallest vesicles were observed in the formulation E6 (106±9 nm). As shown in Table 2, the size distribution of clonazepam loaded ethosomes, as measured by dynamic light scattering, showed one narrow peak, and the polydispersity index was below 0.3, indicating that the vesicle population was relatively homogenous in size. The entrapment efficiency of ethosomes have increased with the increase in the ethanol and phospholipid concentration (p<0.05). One possible reason for this result could be that according to biopharmaceutical classification system (BCS), clonazepam is a class II compound, which means that it has a poor water solubility and high permeability, so clonazepam entrapment increased with increasing the phospholipid and ethanol concentration. Maximum entrapment efficiency was observed with E7 formulation (65.7±2). The values of entrapment efficiency for ethosomes are higher than conventional liposome. This can be explained by the presence of ethanol, which increases clonazepam solubility in the polar phase of the colloidal of ethosomes.

<table>
<thead>
<tr>
<th>Batches</th>
<th>Particle size a (nm)</th>
<th>PI</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4</td>
<td>137±11</td>
<td>0.103 ± 0.017</td>
<td>58.3±2.1</td>
</tr>
<tr>
<td>E5</td>
<td>211±14</td>
<td>0.119 ± 0.023</td>
<td>61.4±2.6</td>
</tr>
<tr>
<td>E6</td>
<td>106±9</td>
<td>0.110 ± 0.019</td>
<td>60.2±2.2</td>
</tr>
<tr>
<td>E7</td>
<td>173±10</td>
<td>0.104 ± 0.020</td>
<td>65.7±2.0</td>
</tr>
<tr>
<td>E8</td>
<td>152±8.5</td>
<td>0.110 ± 0.020</td>
<td>59.1±2.2</td>
</tr>
<tr>
<td>E9</td>
<td>185±12</td>
<td>0.116 ± 0.018</td>
<td>62.3±2.4</td>
</tr>
<tr>
<td>L10</td>
<td>362±21</td>
<td>0.108 ±0.015</td>
<td>46.0±2.0</td>
</tr>
</tbody>
</table>

Values represent mean ±SD (n=3).

Table 3. Stability of ethosomes: vesicle size (nm) and zeta potential (mV).

<table>
<thead>
<tr>
<th>Days after preparation</th>
<th>Vesicle size (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>173±10</td>
<td>-40±1.9</td>
</tr>
<tr>
<td>120</td>
<td>191±9</td>
<td>-36±1.5</td>
</tr>
</tbody>
</table>

Table 4. Flux (J), % permeation (p), % in skin and Lag-time of Clonazepam after 24 hr of application.

<table>
<thead>
<tr>
<th>Batches</th>
<th>J (microg/h cm2)</th>
<th>P %</th>
<th>In skin %</th>
<th>Lag-time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1.27±0.25</td>
<td>10.61±1.42</td>
<td>3.94±0.43</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>2.40±0.08</td>
<td>13.45±0.52</td>
<td>1.35±0.31</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>2.07±0.38</td>
<td>11.24±1.90</td>
<td>3.5±0.62</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>E4</td>
<td>4.01±0.10</td>
<td>20.55±0.60</td>
<td>3.8±0.43</td>
<td></td>
</tr>
<tr>
<td>E5</td>
<td>4.39±0.03</td>
<td>23.0±0.31</td>
<td>5.0±0.52</td>
<td></td>
</tr>
<tr>
<td>E6</td>
<td>5.26±0.10</td>
<td>27.0±0.94</td>
<td>4.4±0.55</td>
<td></td>
</tr>
<tr>
<td>E7</td>
<td>5.66±0.01</td>
<td>31.35±0.35</td>
<td>9.0±1.80</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>4.84±0.13</td>
<td>24.30±0.84</td>
<td>2.1±0.52</td>
<td>1.09±0.25</td>
</tr>
<tr>
<td>E9</td>
<td>4.76±0.05</td>
<td>26.12±0.50</td>
<td>2.8±0.43</td>
<td>0.80±0.09</td>
</tr>
<tr>
<td>L10</td>
<td>2.70±0.05</td>
<td>14.19±0.39</td>
<td>1.7±0.70</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean ±SD (n=3).
In case of zeta potential no fixed correlation was observed with variables (ethanol and phospholipid concentration). Also it was observed that the differences in zeta potential between different formulations were very small and statistically insignificant. This might be due to the reason that phospholipid was the major excipient and the charge contributor in all the ethosomes. Therefore, there was no major variability in zeta potentials of different ethosomes (data not shown). Liposome exhibited a zeta potential of -$5.3\pm 0.2$ mV, addition of alcohol induced a transition in the charge of the vesicles to more negative. Optimized formulation (E7) exhibited a zeta potential of -$40\pm 1.9$ mV.

**Fig. 1.** Visualization of ethosomes by transition electron microscopy by magnification 30.00 k-fold.

In order to evaluate clonazepam permeation and deposition in the skin, in vitro skin permeation study across hairless rat skin were conducted using vertical Franz diffusion cells at 37 °C. The results of the skin permeation profiles are shown in Figs. 2, while the skin permeation parameters are listed in Table 3. A statistically significant increase in the permeation rate (flux) ($p < 0.05$) from ethosomes was observed with the initial increase in phospholipid and ethanol concentration. The in vitro skin flux of clonazepam in E7 was the highest among the formulations ($5.66 \pm 0.01 \mu g/h/cm^2$), and thus the cumulative amount of clonazepam permeated for 24 h was significantly higher when it was delivered from E7 compared to that of the other formulations. At the end of the 24 h permeation experiments, the amount of clonazepam deposited in the skin of hairless rat skin was determined (Table 3). Formulation E7 also led to better skin drug deposition ($9\pm1.8\%$), possibly due to the combined effect of ethanol and phospholipid thus providing a mode for dermal and transdermal delivery of clonazepam. These findings are in good agreement with previous observation of melatonin (Dubey, et al., 2007), ammonium glycyrrhizinate (Paolino et al., 2005) and cannabidol loaded ethosomes (Lodzki et al., 2003) which produced significant accumulation of these drugs in the skin. Also with the change of alcohol from ethanol to propylene glycol in ethosomal formulations, cumulative amount of clonazepam that permeated from the skin and flux increased but skin drug deposition decreased because propylene glycol enters the skin, increase partitioning properties for clonazepam and thus increase the flux. This process can also explain a lag time for these formulations.

**Fig. 2.** Amount of clonazepam permeation from solution formulations and liposome.

The stability of ethosomes was tested using DLS method. Table 4 shows that during three months of storage at 4± 2° C, particle size of formulation E7 resulted in a slight change (only 10.4 % size increase) after storage. These data suggest that there is a
stabilizing effect of ethanol in the formulation, in terms of aggregation of vesicles by providing a net negative charge on the surface, thus avoiding aggregation. These findings are in agreement with data on melatonin-loaded ethosomes (2% PC, 30% ethanol) (Dubey, et al., 2007). TEM photomicrography also revealed a non-aggregated, stable state of stored ethosomes (data not shown).

Fig. 4. Comparison of permeation of clonazepam from ethanolic and PG ethosomes.

Conclusion
Ethosomes have been studied as a possible vehicle for transdermal delivery of clonazepam, an anti epileptic agent. This study confirmed that ethosome is a very promising carrier for the transdermal delivery of drug clonazepam as revealed from an enhanced transdermal flux, higher entrapment efficiency and optimal nanometric size compared to conventional liposome and hydroalcoholic solutions.

Acknowledgment
The authors report no conflict of interest in this work. The authors gratefully acknowledge the financial support granted by the Research Deputy of Tehran University of Medical Sciences (Iran). We are also grateful to the Laboratory of Electron Microscopy of College of Science, University of Tehran and TEM equipment.

References


