Development and in vitro characterization of niosomal formulations of immunosuppressant model drug

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Abstract: Among immunosuppressive agents cyclosporine A is drug of unique importance. This drug has a low therapeutic index, and it has many toxic effects. After oral administration its absolute bioavailability is variable due to poor absorption. Niosomes are new and versatile carriers to deliver drug. The bioavailability of immunosuppressant drug cyclosporine A can be increased by niosomal drug delivery system. So our basic theme was to prepare niosomes of immunosuppressant drug using cholesterol, span 60 and tween 60 etc. Niosomes were characterized for zeta potential, size, poly dispersivity index (PDI), entrapment efficiency and In vitro release profiles. Six niosomal formulations (F₁–F₆) were successfully developed using thin film hydration technique. Among various formulations F₂ showed the highest entrapment efficiency 77.29 %. The DSC thermograms of physical mixtures and niosomal formulations indicated the presence of drug in crystalline form. In vitro drug release study demonstrated higher drug release values as compared to drug aqueous dispersion. Niosomal formulations were capable of releasing drug in sustained manner. The overall results demonstrated that developed niosomal carriers are competitive candidates for improving dissolution profile of cyclosporine A leading to increased bioavailability at the site of action.

Keywords: Immunosuppressive agents, Cyclosporine A, Niosomes, thermogram, Nonionic surfactants.

INTRODUCTION

Immunosuppressive agents suppress the immune function by one of several mechanisms of action which suppress cell mediated immune reactions. They cause depletion of T lymphocytes, blocks calcineurin, blocks IL-2 synthesis and some agents blocks cytokine stimulated cell proliferation (Harvey and Champe, 2009). Among immunosuppressive agents cyclosporine A is very important drug whose source is soil fungus. It is used to prohibit rejection of bone marrow, kidney and cardiac allogeneic transplants (Walker et al., 2007). Therapeutic index of cyclosporine A is very low and it has many toxic effects like hepatotoxicity, neurological problems and nephrotoxicity (Katzung et al., 2009).

Now a days, niosomes as novel carrier attained considerable attention, due to their ability to circumvent disadvantages associated with liposomes and greater stability. Low costs of material in niosomes make them suitable for industrial manufacture and development. Many nonionic surfactants can be used in niosomes which provide diversified options for tailoring niosomal drug delivery system as per requirements. Niosomes also have tendency to entrap both hydrophilic and hydrophobic active ingredients (El-Ridy et al., 2011).

According to biopharmaceutics classification system cyclosporine A comes in class II, its solubility is limited with high permeability. After oral administration its absolute bioavailability and pharmacokinetics is markedly variable due to poor absorption. In oral formulations it has highly variable absorption due to its poor dissolution characteristics (Chiu et al., 2003). In the gut wall and liver cyclosporine A is metabolized which reduces its bioavailability. The research is in progress to further improve the bioavailability of cyclosporine A. (Guan et al., 2011). There is no any previous report for increasing bioavailability of cyclosporine A using niosomal technology.

The primary focus of current study to report here was to prepare and evaluate niosomal vesicles of cyclosporine A by thin film hydration method. Further, cyclosporine A loaded niosomes were thoroughly assessed for physicochemical aspects like entrapment efficiency, zeta potential, zeta size, and stability profile etc. Dialysis bag was used to study the release of cyclosporine A for in vitro circumstances. So it was expected that niosomes will improve the solubility, thereby increasing bioavailability and dissolution profile of cyclosporine A with reduced side effects and a sustain release effect. The cost of this formulation is relatively low and development methods are easy.

MATERIALS AND METHODS

Materials
Cyclosporine A was provided by Xi’an Lyphar Biotech Co., Ltd China. Polysorbate 80 also known as Tween 80,
Sorbitan olate also known as Span 80 and Sorbitan monostearate also known as Span 60 and Polysorbate 60 also known as Tween 60 were acquired from Daejuung chemicals & metals Co., Ltd. 1-Hexadecyl pyridinium chloride monohydrate and cholesterol were attained from Alfa Aesar GmbH & Co. Germany. Methyl alcohol and Chloroform were acquired from Daejuung chemicals & metals co., Ltd. Korea. Distilled water was prepared in the laboratory of Faculty of Pharmaceutical Sciences, GC University Faisalabad.

**Preparation of niosomes**
Thin film hydration method was used to prepare niosomes of immunosuppressant drug (cyclosporine A) (Ravalika and Sailaja 2017). 500 µmol of each surfactant including Tween 80, Span 80, Tween 60 and Span 60 and cholesterol in different molar ratios were incorporated in twenty ml of mixture of chloroform and methanol in 2:1. 2.5% of 1-Hexadecyl pyridinium chloride monohydrate was added to each formulation. 25 mg (21 µmoles) of cyclosporine A was incorporated into each formulation. Then the organic solution was rotary evaporated at 60°C. So a thin film was formed on the wall of flask. Phosphate buffer saline (PBS) of pH 7.4 (20ml) was used to hydrate the thin film for one hour, in water bath with gentle shaking at 58°C. Then the formulations were left aside at 24°C overnight, so that niosomes can get mature (Abdelkader et al., 2010; El-Ridy et al., 2011). Then formulations were placed in refrigerator at 4°C. The detail of ingredients of niosomal formulation is given in table 1.

**Physical mixture 1 (PM1)** was prepared by mixing cholesterol and cyclosporine A, Span 60, Polysorbate 60 (Tween 60) in equimolar amounts (1:1) in pestle and mortar. For physical mixture 2 (PM2) cyclosporine A, cholesterol, Span 80 and Polysorbate 80 (Tween 80) were taken. Then these were mixed in mortar for about 10 to 15 minutes until a homogenous mixture was formed (Khan et al., 2015).

**Thermal analysis**
Cyclosporine A, niosomal formulations, physical mixtures and other ingredients were subjected to differential scanning calorimetry (DSC) (TA Instruments SDT-Q600) Simultaneous TGA / DSC). The ingredients were weighed accurately (3-5 mg) in aluminum pans and covered with aluminum foil. Thermograms were taken in atmosphere of nitrogen. The purging rate was taken at 50 ml min⁻¹. Samples were heated from 10°C to 250°C and the scan rate was taken at 10°C min⁻¹ (Li et al., 2005).

**Determination of drug content in the niosomes**
Cyclosporine A loaded in niosomes was partitioned by ultracentrifugation method at 12000 × g. Niosomes were centrifuged for half an hour at 4°C. Niosomes were disrupted using methanol (Abdelkader et al., 2011). High pressure liquid chromatography (HPLC) was used to calculate the amount of cyclosporine A in niosomes (Gao et al., 1998). Acetonitrile and water in 70:30, v/v was used as mobile phase. Membrane filter was used to filter the mobile phase, the size of filter was 0.45-µm. The column used was Nucleosil C18. The rate of flow was taken 1.2 ml/min and the effluents were checked at 210 nm (El-Shabouri, 2002; Nikouei et al., 2011).

**Vesicle size and zeta potential**
The size of niosomal vesicles, polydispersivity index (PDI) and zeta potential were determined at 24°C by using Malvern zetasizer, version 7.11. The niosomal formulation (50 micro liter) was diluted with 10 ml milliQ water for analysis. All readings were taken three times and then its mean value is taken (Khan et al., 2016; Rehman et al., 2018).

**Determination of stability**
To determine the stability of niosomes the formulations were preserved at temperature (4-8°C) and at normal room temperature (24°C±2) in transparent vials. Specimens were taken at proper time interval, and entrapment efficiency was determined to characterize the stability profile. Visual examination was also done to evaluate any color changes in formulation. (Arafa et al., 2015; Khan et al., 2015)

**Drug release studies**
To study the In vitro drug release of niosomes of immunosuppressant drug cyclosporine A (Fi – F2) and plain cyclosporine A aqueous suspension, dialysis membrane method was used. Drug release study was conducted at gastric pH 1.2 and intestinal pH 7.4. Tests of drug release were conducted under sink condition. Dialysis membrane was soaked in distilled water over night. Then 1 ml of formulation was added in dialysis membrane. Dialysis membrane was clamped from both side and placed in a beaker having one hundred ml of phosphate buffer saline (PBS) of pH 1.2 and pH 7.4. The temperature was taken at 37 °C, and the stirring speed was adjusted at one hundred rpm. The dialysate samples were collected at 0, 0.25, 0.50, 0.75, 1, 1.25, 1.75, 2.5, 4, 5.5,8,12,16,20 and 24 hours. Same amount of fresh PBS was added when sample was collected. The samples were assayed by High pressure liquid chromatography (HPLC) method as explained above. (Wagh et al., 2014; El-Ridy et al., 2014). The release study of cyclosporine A was assessed by using distinct kinetic models like zero order, first order, higuchi model and korsmeyer-pepaa model. (Costa et al., 2001)

**STATISTICAL ANALYSIS**
One way analysis of variance (ANOVA) was used to compare the mean values of size of niosomes and entrapment efficiency of six niosomal formulations. The significance level was set to p<0.05. ANOVA was conducted at 95% confidence interval by Graph Pad Prism 6 software.
RESULTS

Preparation of niosomes
Cyclosporine A niosomes were successfully prepared with all the ratios of surfactants and cholesterol given in table no 1 by thin film hydration method. Results of size, poly dispersivity index, zeta potential and percentage entrapment efficiency are given in table 2. The size of niosomal vesicles was in the range of 415.2×10⁻³ to 1049.2 × 10⁻³ µm. The value of zeta potential of niosomes was from 23.8 to 32.1 mV. F₃ formulation provided the highest % entrapment efficiency of 77.29%.

Stability of niosomes
For stability studies of the cyclosporine A loaded niosomes the entrapment efficiency was determined. As shown in table 3 the entrapment efficiency at temperature 4-8°C and 24°C are given at 0, 1, 2 and 3 months interval. It was concluded that at refrigerated temperature 4-8°C the amount of drug retained in niosomes was greater than at 25°C. Visual examination showed no significant color change after three months in formulations at 4-8°C, but at 25°C the color of formulation slightly faded or became a bit light yellow. So these niosomes can be an effective formulation with good stability but it is better to store them at 4-8°C.

DISCUSSION

Niosomes of the immunosuppressant drug cyclosporine A were prepared by thin film hydration method. Previous studies showed no formation of niosomes without cholesterol, it is responsible for integrity and cement effect of niosomal vesicles. Thus in this study we used cholesterol concentration ranging from 30-50% in formulations F₁, F₂ and F₃ and surfactants used were polysorbate 60 and span 60. Similarly in formulations from F₄, F₅ and F₆ the cholesterol concentration was 30 to 50 % and surfactants used were tween 80 and span 80. The total micromolar concentration of nonionic surfactant and cholesterol was kept at 500µmoles. Hexadecyl pyridinium chloride monohydrate was used as positive charge inducing agent in 2.5% molar ratio, as it stabilizes the niosomes (Junyaprasert et al., 2008).

Vesicle size is highly dependent on hydrophilic-lipophilic balance (HLB) value of surfactants. If the value of HLB is less, the size of vesicles will be smaller. The average HLB value of mixed surfactant system yielded rigid vesicles with smaller particle size. Size of the formulation F₁ to F₆ are shown in table 2, these values were significantly different from each other (p<0.05).
Table 1: Composition of niosomal formulations in (F₁-F₆)

<table>
<thead>
<tr>
<th>S No</th>
<th>Code of Formulation</th>
<th>Molar ratio (Surfactant: CHO)</th>
<th>Tween 60 (mg)</th>
<th>Span 60 (mg)</th>
<th>Cholesterol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F₁</td>
<td>7:3</td>
<td>229</td>
<td>75.35</td>
<td>57.99</td>
</tr>
<tr>
<td>2</td>
<td>F₂</td>
<td>6:4</td>
<td>196.35</td>
<td>64.58</td>
<td>77.33</td>
</tr>
<tr>
<td>3</td>
<td>F₃</td>
<td>5:5</td>
<td>163.62</td>
<td>53.84</td>
<td>96.66</td>
</tr>
<tr>
<td>4</td>
<td>F₄</td>
<td>7:3</td>
<td>229.25</td>
<td>75.35</td>
<td>57.99</td>
</tr>
<tr>
<td>5</td>
<td>F₅</td>
<td>6:4</td>
<td>196.50</td>
<td>64.58</td>
<td>77.33</td>
</tr>
<tr>
<td>6</td>
<td>F₆</td>
<td>5:5</td>
<td>163.75</td>
<td>53.84</td>
<td>96.66</td>
</tr>
</tbody>
</table>

Table 2: Average size, zeta potential, polydispersivity index and Entrapment efficiency (%) of different niosomal formulations

<table>
<thead>
<tr>
<th>S No</th>
<th>Parameters</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
<th>F₄</th>
<th>F₅</th>
<th>F₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Size (µm)</td>
<td>436.1±0.10³</td>
<td>1049.3±0.11³</td>
<td>534.6±0.216</td>
<td>415.2±0.312</td>
<td>424.1±0.186</td>
<td>673.8±0.137</td>
</tr>
<tr>
<td>2</td>
<td>Poly dispersivity index</td>
<td>0.452±0.03</td>
<td>0.435±0.15</td>
<td>0.473±0.08</td>
<td>0.356±0.11</td>
<td>0.481±0.17</td>
<td>0.572±0.21</td>
</tr>
<tr>
<td>3</td>
<td>Zeta potential (mV)</td>
<td>23.8±1.3</td>
<td>32.1±1.5</td>
<td>25.7±2.7</td>
<td>24.3±1.8</td>
<td>30.7±2.1</td>
<td>26.4±2.5</td>
</tr>
<tr>
<td>4</td>
<td>% Entrapment efficiency</td>
<td>26.87±0.43</td>
<td>77.29±0.35</td>
<td>52.07±0.38</td>
<td>63.85±0.41</td>
<td>56.13±0.52</td>
<td>43.67±0.39</td>
</tr>
</tbody>
</table>

This data is mean along with standard deviation. In all niosomal formulations, size of the niosomes and entrapment efficiency was significantly different from each other p <0.05.

Table 3: Stability studies of cyclosporin a niosomes at different temperatures.

<table>
<thead>
<tr>
<th>S No</th>
<th>Month</th>
<th>Temperature 4-8 °C</th>
<th>Temperature 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Entrapment Efficiency</td>
<td>% Entrapment Efficiency</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>26.88</td>
<td>77.28</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>26.65</td>
<td>77.06</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>26.33</td>
<td>76.97</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>26.28</td>
<td>76.86</td>
</tr>
</tbody>
</table>

The PDI of niosomes was found in range of 0.23 to 0.51 which showed homogenous nature of formulations. The charge on surface of niosomes plays a significant part in the behavior and stability of niosomes. Charged niosomes show good stability. It prevents niosomes from aggregation. (Seleci et al., 2016). The zeta potential of niosomal formulations was found in the range of 23.8 mV to 32.1 mV. Generally niosomes possessing zeta potential value close to thirty mV are considered stable, due to presence of electrical repulsive forces among the vesicles (Ali et al., 2014; Nikouei et al., 2011). PDI and zeta potential values are given in table 2.

It is well documented that as the concentration of cholesterol rises up to some extent i.e. 30% to 50% entrapment efficiency also raises (Teja et al., 2018). The optimum formulation F₂ in which surfactants to cholesterol ratio was (6:4) showed highest entrapment of 77.28%. Statistical analysis of data of nonionic surfactants to cholesterol ratio in the formulations F₁ to F₆ in table 2 depicts that % entrapment efficiency is significantly dependent on it (ANOVA; p<0.05).

The quantity of cholesterol required to form niosomal vesicles depends on HLB value of surfactant. (Kumar and Rajeshwarrao, 2011). When HLB value increases above 10, greater amounts of cholesterol are needed for niosomes development. Niosomes of minoxidil has high entrapment with Brij 76 (HLB 12.4) and higher quantity of cholesterol. But no substantial raise in entrapment efficiency using Brij 52 (HLB 5.3) with higher quantity of cholesterol. So after a certain increase of cholesterol quantity entrapment efficiency is decreased, may be due to decrease in volume diameter (Critical packing parameter or CPP<0.05) (Kumar and Rajeshwarrao, 2011). In present study we used mixed surfactant system.
in formulations from F1 to F6 as shown in table 2. F2 showed maximum entrapment efficiency 77.28 percent among developed formulations. It has surfactants to cholesterol ratio was 6:4, and on further increasing the amount of cholesterol as in F1 the entrapment efficiency does not increased further. In F3 and F4 having maximum cholesterol concentration (50%), less values of entrapment efficiency were achieved. The cholesterol concentration was maximum up to 50%. The reason for this fact can be higher concentration of cholesterol caused damage to niosomes which result in leakage of active ingredient (Mali et al., 2013). The data given in table 2 depicts that % entrapment efficiency is dependent on nonionic surfactant to lipid level.

DSC thermograms of cholesterol, cyclosporine A and span 60 depicted the peculiar endothermic melting peaks at 148, 150 and at 52 °C respectively, which showed their crystalline nature. The DSC thermograms of physical mixtures and formulations F1 to F6 showed the melting range of ingredients and cyclosporine A. The results of DSC revealed that there is no significant interaction among the active and inactive ingredient used in this niosomal formulation. (Lakshmana et al., 2009). DSC thermograms of niosomal formulations showed a broadened endothermic peak which showed improved solubility and dissolution and the sustained release nature of formulations (Deb et al., 2015). In stability studies of cyclosporine A, leakage at high temperature was may be due to high fluidity of lipid bilayers at high temperature (Nadzir et al., 2017).

For all niosomal formulations the percentage drug release was significantly higher as compared to drug aqueous dispersion at pH 1.2 and 7.4 as shown in fig. No 2 and 3. This shows the solubilization effect of cyclosporine A in the surfactant vesicles, which in turn increased the drug release. In all the formulations dissolution profile was improved. At pH 1.2 the percentage drug released in 24 hours by F1, F2, F3, F4, F5 and F6 was 62%, 83%, 76%, 87%, 89% and 68% respectively. And at pH 7.4 the percentage drug released in 24 hours by F1, F2, F3, F4, F5 and F6 was 46%, 67%, 62%, 69%, 72% and 54% respectively. In vitro release studies show sustained release behavior in all formulations (F1-F6). At low pH the cumulative drug release was higher as compared to at high pH, this might be due to effect of pH on cyclosporine A release. So the release study results shows that developed niosomal carriers are competitive candidates for increasing the solubility of cyclosporine A and can result in improved bioavailability.

The data of in-vitro release was applied to distinct kinetic models to predict the mechanism of drug release of niosomal formulation F1 to F6. From the slope of appropriate plots the release constant and regression coefficient \( r^2 \) were determined. Higuchi model and korsemeyer–Peppas model showed good linearity for the selected F2 formulation. The korsemeyer–Peppas model was applied to all niosomal formulations and n value was determined. The release exponent (n) suggest drug transport mechanism is non-fickian (anomalous) transport i.e combination of both erosion and diffusion processes (Costa and Lobo, 2001). The results showed that the zero order \( r^2 \) values for all formulations were greater than the first order values, so these formulations follow zero order release and can release active ingredient in sustained manner.

CONCLUSION

By using thin film hydration method niosomal formulations of immunosuppressant drug cyclosporine A were developed. The niosomal formulation F2 shows maximum entrapment of cyclosporine A 77.29 %. In vitro release studies showed improved dissolution profile of cyclosporine A. Moreover sustained release behavior was seen in all niosomal formulations. The release exponent (n) suggest drug transport mechanism is non-fickian (anomalous) transport, and the niosomal formulations followed zero order release, which release drug in a sustained manner. Hence these niosomes have potential to act as useful carriers for effective delivery of cyclosporine A.

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REFERENCES


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