NOVEL NANOEMULSION AS VEHICLES FOR TRANSDERMAL DELIVERY OF CLOZAPINE: IN VITRO AND IN VIVO STUDIES

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ABSTRACT

Objective: The purpose of the present study is to develop and evaluate the potential of nanoemulsions for increasing the solubility and the in vitro transdermal delivery of clozapine.

Methods: Clozapine is practically insoluble in water and low (27%) bioavailability mainly due to first pass metabolism. An optimized oil-in-water nanoemulsion of Clozapine was prepared by the spontaneous emulsification method. Pseudoternary phase diagrams were constructed to obtain the nanoemulsion region. Oleic acid was chosen as the oil phase, Tween 20 and Transcutol-P were used as surfactant and cosurfactant respectively, on the basis of solubility studies, in formulation of nanoemulsion. The optimized nanoemulsion was characterized for pH, viscosity, droplet size, droplet shape, and refractive index. The formulations were evaluated for thermodynamic stability testing, in vitro rat skin permeation, skin irritation test and in vivo study.

Results: Significant difference in the steady state flux (Jss), permeability coefficient (Kp) and enhancement ratio (Er) was observed in nanoemulsion formulations and control (P< 0.05). The composition of optimized formulation F1 (Oleic acid 4.54% w/w) showed highest value of flux 391.3357 µg cm⁻² h⁻¹. At the 24 h post application plasma clozapine was increased 4.4 fold to marketed dosage form.

Conclusion: The study suggested that nanoemulsion significantly enhanced bioavailability of transdermally applied clozapine and eliminated the first pass metabolism.

Keywords: Clozapine, Nanoemulsion, Transdermal drug delivery, Surfactant.

INTRODUCTION

Poor bioavailability is possible due to poor solubility, degradation in GI lumen, poor membrane permeation and pre-systemic elimination [1]. By many estimates up to 40% of new chemical entities (NCEs) discovered by the pharmaceutical industry today and many existing drugs are poorly soluble or lipophilic drugs which leads to poor oral bioavailability, high intra and inter subject variability. In the treatment of diseases topical drug delivery is an appropriate strategy to restrict the therapeutic effect to the affected area and to reduce systemic incrimination on the other hand, systemic availability is the aim in transdermal delivery, which can be minimized or eliminated the first pass effect.

Nanoemulsions (NE) are a class of emulsions with very small and uniform droplet size, typically in the range of 20-500 nm [2]. Nanoemulsion formulation delivers the active substance at target organ with minimal discomfort and side effects. In this respect, transdermal route excels because of avoidance of hepatic first pass metabolism, typical peak through plasma profile, ease of administration [3,4]. However the improvement of drug permeability through the skin is always a difficult problem, because of barrier function of human skin epithelia to exogenous substances. Therefore, the major challenge in topical administration is to increase the drug penetration into the skin. Moreover, most of the pharmaceutical substances are lipophilic in nature. The clinical efficacy of such drugs is being impeded by their low aqueous solubility resulting in poor absorption and penetration mainly when they are designed for transdermal administration. A way is to use the skin penetration enhancers [5] and the other way is to develop appropriate vehicles to increase the solubility and the thermodynamic activity of the drug and then to increase the permeation [6,7]. In recent years nanoemulsions have attracted a great deal of attention because of their considerable potential to increase the transdermal delivery of a wide range of drug molecules [9]. Previous reports have confirmed that the incorporation of a lipophilic drug into the internal phase of an o/w nanoemulsion has become an attractive technique for the percutaneous administration of the drug due to high solubilizing capacity [10]. This approach favors high concentration gradient across the diffusion membrane, leading to an increase in the activity of the drug in the vehicle and thereby improving the drug diffusion rate. The dispersive phase acts as a reservoir which maintains a constant concentration gradient over the skin for a long period [11]. Oil/water (o/w) nanoemulsions are promising colloidal drug carrier systems for diverse therapeutic applications. Successfully developed, intravenous, oral and ocular delivery systems showed reduced side effects of various potent drugs and prolonged pharmacological effect of drug in these nanoemulsion formulations [12,13,14].

Nanoemulsions are thermodynamically stable, transparent (or translucent), dispersion of oil and water stabilized by an interfacial film of surfactant molecules having the droplet size in nanometer range. These system require relatively appropriate amount of surfactant to stabilize the large interfacial area created by the nano-droplets often addition of cosurfactant such as alcohol is also required to attain appropriate fluidity or viscosity of the interface. Nanoemulsion provides ultra-low interfacial tension and large o/w interfacial area. Nanoemulsion have a higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions, such emulsions and suspension, because they can be manufactured with little energy input (heat or mixing) and have a long shelf-life. However long term physical stability of nanoemulsion (which no apparent flocculation or coalescence) make them unique and they are sometime referred as "approaching thermodynamic stability" [15, 16].

Clozapine is an effective a typical antipsychotic drug applied to the treatment of resistant schizophrenia. Clozapine is used to suppress both positive and negative symptoms of schizophrenia and many neuroleptic responses. Clozapine undergoes extensive first pass metabolism with a bioavailability of only 27% [17]. Clozapine is extensively metabolized by hepatic microsomal enzymes (CYP1A2 and CYP3A4) and forms N-demethyl and N-oxide metabolites. Majority of the orally administered drugs absorbed into the systemic circulation via portal blood and undergo first pass metabolism, thus exhibit low oral bioavailability. Moreover, in cases of psychosis, patients are unable to take medication frequently. Hence, formulating clozapine for a controlled release offers potential advantage such as sustained drug content in blood, attention of
adverse effects, and improved patient compliance. The purpose of this study was to formulate transdermal delivery system for poorly water soluble clozapine and minimized or eliminate first pass metabolism. Nanoemulsion was prepared by spontaneous emulsification method with varying weight ratios of oil to surfactant along with cosurfactant. This can be useful in reducing the side effects, decrease the dose and frequency of administration and perhaps even the cost of the therapy.

MATERIALS AND METHODS

Clozapine was obtained as a gift sample from Torrent Pharmaceutical Ltd. (Ahmedabad, India). Oleic acid, Tween 20, eucalyptus oil, glycerol triacetate, Transcutol-P were purchased from Central Drug House (New Delhi, India), isopropyl myristate was purchase from Qualikems, Fine Chemical Pvt. Ltd (New Delhi, India), castor oil was purchase from LOBA CHEMIE, Pvt. Ltd (Mumbai, India), Capmul MCM, Capmul MCM C8 and Captex 355 were obtained from ABITEC Corporation, Janesville, WI. All other reagents used were of analytical grade.

Screening of component for nanoemulsion

The most important criterion for the screening of component for nanoemulsion formulation is the solubility of poorly soluble drug in oils, surfactants and cosurfactants. The solubility of clozapine in various oils (Oleic acid, IPM, castor oil, triacetin, eucalyptus oil, capmul MCM C8, Capmul MCM and captex 355), surfactants ( Tween 20 and trition X 100) and cosurfactant (Transcutol-P and propylene glycol) was determined by dissolving excess amount of Clozapine in 2 mL of each selected oils, surfactants and cosurfactants in 5 mL capacity stoppered vials separately and mixed using a vortex mixer. The mixture vials were kept at 25 ± 1.0 °C in an isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated sample were removed from the shaker and centrifuged at 5000 rpm for 15 min, the supernatant was taken and filtered through a 0.45 µm membrane filter. The concentration of clozapine was determined in each component by double beam UV-VIS spectrophotometer at λmax 294 nm after appropriate dilution with methanol. Solubility of clozapine in oils phase, surfactant and cosurfactant was shown in Table 1.

Table 1: It shows Solubility of clozapine in various oils, surfactants and cosurfacants

<table>
<thead>
<tr>
<th>Components</th>
<th>Solubility (mg/mL) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid</td>
<td>154.66 ± 2.14</td>
</tr>
<tr>
<td>Triacetin</td>
<td>21.21 ± 2.75</td>
</tr>
<tr>
<td>IPM</td>
<td>13.43 ± 1.22</td>
</tr>
<tr>
<td>Castor oil</td>
<td>37.27 ± 2.84</td>
</tr>
<tr>
<td>Eucalyptus oil</td>
<td>112.25 ± 3.12</td>
</tr>
<tr>
<td>Capmul MCM C8</td>
<td>22.69 ± 1.46</td>
</tr>
<tr>
<td>Capmul MCM</td>
<td>16.67 ± 2.68</td>
</tr>
<tr>
<td>Captex 355</td>
<td>13.22 ± 2.82</td>
</tr>
<tr>
<td>Tween 20</td>
<td>126.84 ± 3.12</td>
</tr>
<tr>
<td>Trition X 100</td>
<td>14.26 ± 3.43</td>
</tr>
<tr>
<td>Transcutol-P</td>
<td>22.72 ± 3.96</td>
</tr>
</tbody>
</table>

Pseudoternary phase diagram

On the basis of the solubility studies, oleic acid was selected as oil phase. Tween 20 and Transcutol-P were used as surfactant, cosurfactant and double distilled water used as an aqueous phase. For determination of existence of nanoemulsion, pseudoternary phase diagrams were constructed using water titration method (spontaneous emulsification method).Surfactant and cosurfactant (Smax) were mixed in different weight ratios (1:1, 1:0, 1:2, 2:1, 1:3, & 3:1) for each diagram, oil and specific Smax ratio was mixed thoroughly in different weight ratios from 1:9 to 9:1 in different glass vials. Sixteen different combinations of oil and Smax 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 3:7, 1:2, 1:1.5, 1:1, 1:0.7, 1:0.43, 1:0.25, and 1:0.1, were made so that maximum ratios were covered for the study to delineate the boundaries of phase precisely formed in the phase diagrams. Pseudo-ternary phase diagram were developed using aqueous titration method. Slow titration with aqueous phase was done to each weight ratio of oil and Smax and visual observation was carried out for transparent and easily flowable nanoemulsion. The physical state of nanoemulsion was marked on a pseudo-thermodynamic phase diagram with one axis representing aqueous phase, other representing oil and the third representing a mixture of surfactant and cosurfactant at fixed weight ratio [Smax ratio][18, 19].

Preparation of Nanoemulsion Formulation

Nanoemulsions were prepared by dissolved Clozapine in oleic acid and then it mixed with the mixture of tween 20 and Transcutol-P, followed by gentle mixing with double distilled water at room temperature until the system was transparent under vortexing. The monophasic formulations were formed spontaneously at room temperature.

Selection of formulation from phase diagrams

From the pseudoternary phase diagrams showing maximum nanoemulsion area, a number of nanoemulsion with different formula were selected covering the entire range of nanoemulsion occurrence in the phase diagram. For each phase diagram constructed, different formulation were selected from nanoemulsion region so that drug could be incorporated in the oil phase on the following bases.

(a) The oil concentration should be such that it solubilizes the drug (single dose) completely depending on the solubility of the drug in the oil. As per saturation solubility studies of clozapine in oils (oleic acid) around 160mg of the drug was solubilised per ml of oil so in this case 12.5 mg of clozapine will dissolve easily in oil phase.

(b) The minimum concentration of the Smax used for that amount of oil was taken.

(c) For convenience purpose, about 1 mL was selected as the nanoemulsion formulation, so that it can increase or decreased as per requirement in the proportions. The beauty of this system is the scale up of the proportions is easy, as the system is thermodynamically stable.

All the formulations having surfactant: cosurfactant ratio 1:1, 1:0, 2:1 and 3:1 were subjected to different thermodynamic stability tests.

Physical stability studies

To overcome the problem of metastable formulations physical thermodinamic stability testing were performed [20,21]. The selected formulation was subjected to centrifugation at 4000rpm for 30mins. The formulations that did not show any phase separations were taken for the heating and cooling cycle. Six cycles between refrigerator temperature 4 °C and 45 °C with storage at each temperature of not less than 48 h were carried out. Those formulations that were found stable were subjected to a freeze thaw cycle test. Those such cycles were done for the formulations between -20 °C and +25°C for 24 hours. After 24 hours the formulation were removed and kept at room temperature. The physically stable nanoemulsions were returned to their forms within 2 to 4mins.

The formulations that survived stability tests were selected for further studies and are shown Table no.2.

Transmission electron microscopy (TEM)

Morphology and structure of the nanoemulsion were studied using transmission electron microscopy (TEM) Morgani 268D SEL, USA operating at 200 KV capable of point to point resolution. Combination of bright field imaging at increasing magnification and diffraction modes was used to reveal the form and size of the occurrence in the phase diagram. For each phase diagram constructed, different formulation were selected from nanoemulsion region so that drug could be incorporated in the oil phase on the following bases.

Figure 7 shows the transmission electron microscope (TEM) micrograph of the nanoemulsion formulation showing uniform nanoemulsion droplet size distribution. The TEM micrograph (Fig 7) shows the size and shape of the nanoemulsion droplets. The TEM micrograph also shows the uniformity of the nanoemulsion droplets which is important for the formulation of a drug delivery system.
Table 2: It shows the composition of selected formulations

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Oleic acid (% w/w)</th>
<th>Smix (% w/w)</th>
<th>Water (% w/w)</th>
<th>Ratio of S/CoS</th>
<th>Clozapine (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>4.54</td>
<td>59.96</td>
<td>54.64</td>
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<tr>
<td>F2</td>
<td>5.12</td>
<td>45.11</td>
<td>48.71</td>
<td>0.01</td>
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<tr>
<td>F3</td>
<td>6.94</td>
<td>47.61</td>
<td>44.44</td>
<td>0.01</td>
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<tr>
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<td>52.43</td>
<td>39.94</td>
<td>0.01</td>
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<tr>
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<td>45.15</td>
<td>48.71</td>
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<td>7.27</td>
<td>49.9</td>
<td>41.81</td>
<td>0.01</td>
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<tr>
<td>F8</td>
<td>7.63</td>
<td>52.43</td>
<td>38.93</td>
<td>0.01</td>
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<td>F10</td>
<td>6.94</td>
<td>47.69</td>
<td>44.44</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

**Droplet size and size distribution**

Droplet size was determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles [22] using a Zetasizer (1000 HS, Malvern Instruments UK). The formulation (0.1 mL) was dispersed in 50 mL of water in a volumetric flask, mixed thoroughly with vigorous shaking and light scattering was monitored at 25 °C at 90° angle [23]

**Viscosity, Refractive index and pH determination**

The viscosity of the formulation was determined by using Brookfield DV III ultra v60 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) using spindle # CPE40 at 25 ± 0.5 °C. The software used for the calculations was RheoCal V2.59. The refractive index was determined for nanoemulsion formulation by using an Abbes type refractometer (Rajat scientific work) at 25°C in triplicate. Refractive index of nanoemulsion was shown in Table 3. The pH was determined for the optimized emulsion for using calibrated digital pH meter ([VSI-IB, VSI electronics, India])

**Optimized formulation**

To determine the penetration capacity of the formulations the solubility of clozapine was also determined in optimized nanoemulsion formulations by taking an excess amount of clozapine in 1 mL of optimized nanoemulsion formulation. More soluble drug will produce a greater concentration gradient towards the skin. The procedure used for solubility determination was the same as described in screening of component for nanoemulsion

**In vitro skin permeation studies**

Albino rats (Male) weighting 150 to 200 g were obtained from animal house of Bundelkhand University Jhansi for the in vitro permeation studies. The full thickness of rat skin was excised from the abdominal region and hairs were removed with an electric clipper. The subcutaneous tissue was removed surgically and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleaned skin was washed with distilled water and stored at -21°C until further use [24].

Franz diffusion cell with an effective diffusion area 1.36 cm² was used for in vitro permeation studies. The skin was brought to room temperature and mounted between donor and receiver compartments of the Franz diffusion cell were the stratum corneum side was facing the donor compartment and dermal side was facing receiver compartment. Initially, the donor compartment was empty and the receiver chamber was filled with methanolic phosphate buffer saline pH 7.4 (40:60 % v/v). The receiver fluid was stirred with a thermally controlled magnetic stirrer at 600 rpm and the assembled apparatus was placed in hot air oven where the temperature was maintained at 37 ± 0.5 °C. The buffer mixture solution was replaced with fresh buffer every 0.5 h to stabilize the skin. It was found that the receiver fluid showed negligible peak absorption after 5 h and beyond, indicating complete stabilization of the skin.

After complete stabilization of the skin, 1 mL nanoemulsion formulation (12.5 mg/mL clozapine) or control were placed into each donor compartment and sealed with paraffin film in order to provide occlusive conditions. Samples were withdrawn at regular intervals (viz. 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 & 24 h), filtered though a 0.45 µm membrane filter, and analyzed for drug content by double beam UV-VIS spectrophotometer at 294 nm. The cumulative amount of clozapine permeated through the rat skin (Q, µg/cm²) was plotted as function as a time (h). The drug flux (permeation rate) at study state (Jss, µg cm⁻² h⁻¹) was calculated from the slope of linear portion of the curve. Cumulative amount of drug permeated = Concentration (µg/mL) × dilution factor / Area (cm²)

Flux = slope of steady state portion of the plot between cumulative amount of drug permeated Vs time (µg cm⁻² h⁻¹)

Permeability coefficient (Pb) = Flux/dose concentration in donor compartment (µg/mL)

Enhancement ratio was calculated by dividing the Jss of the respective formulation by the Jss of the control formulation:

\[ Er = \frac{J_{ss}}{J_{ss,control}} \]

**Statistical analysis**

All the skin permeation studies were repeated three times and data were expressed as the mean value ± SD. Statistical data were analyzed by one-way analysis of variance. A multiple comparison test was used to compare different formulations and P < 0.05 was considered to be significant.

**Optimized formulation selection**

The nanoemulsion was insufficiently viscous and could be therefore quickly removed from the skin. Therefore, the optimized nanoemulsion (F1) was converted into nanoemulsion gel. Nanoemulsion gel was prepared by dispersing 1% w/w of Carbopol 934 in sufficient quantity of distilled water [25]. The dispersion was kept in dark for 24 h for complete swelling of carbopol 934. The skin permeation profile of nanoemulsion formulation was compared with nanoemulsion gel and control. The nanoemulsion gel was further evaluated for their permeation enhancement activity by adopting the same protocol that was used for the in vivo skin permeation of nanoemulsion formulation and control.

**Pharmacokinetic studies**

Approval to carry out in vivo study was obtained from Bundelkhand University Institutional Animal Ethics committee, CPCSEA, Institute of Pharmacy, (Registration no: 716/02/a/CPCSEA) and their guideline were followed for the studies. The nanoemulsion formulation (F1), which showed the highest release profile of drug which was converted into gel based on in vitro studies, was taken for in vivo studies. The animals used for in vivo experiments were adult albino rats (200-250 g). Two groups were made for the study, and six rats were kept in each group. The animals were kept under standard laboratory conditions, temperature at 25 ± 2 °C and relative humidity (55 ± 5%). The animals were housed in polypropylene cages, with free access to standard laboratory diet (Lipton feed, Mumbai, India), and water ad libitum. The formulations (nanoemulsion and marketed tablet) were given transdermally and orally.

The abdominal region of the rat was shaved 12 h before starting the experiment. Nanoemulsion gel was applied on the shaved abdominal region of all animals except in the control group. The rats were anesthetized using ether and formulation (10mg/kg dose level) was
administered [26]. Blood samples (0.5 mL) were withdrawn from retro-orbital vein of rat at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0 and 24 h, in vacutainer tubes, mixed and centrifuged at 2000 rpm for 20 min. The plasma was separated and stored at -20 °C until drug analysis was carried out using UV spectroscopy.

Skin irritating test
The skin irritating test was performed by previously mentioned method [27] to confirm the safety of the formulation. Skin irritating test was done on male albino rat, weight 200 to 250 g. The animals were housed in polypropylene cage, six per cage, with free access to standard laboratory diet (Liptom feed, India) and water. A single dose of 10 μL of the nanoemulsion formulation was applied to the left ear of the rat, with the right ear as a control [28]. The development of erythema, which is a manifestation of cutaneous vascular dilation, was monitored daily for 6 days.

RESULTS AND DISCUSSION

Solubility of clozapine in various excipients
The important criteria for selection of the materials were that components must be pharmacologically acceptable, nonirritant and non-sensitizing to the skin, and they should fall under GRAS (generally regarded as safe) category. Selection of an appropriate oily phase is very important as it influences the selection of other ingredients and nanoemulsion, mainly in case of O/W nanoemulsion. Usually the oil, which has maximum solubilising potential for the selected drug candidates, is selected as oil phase for the formulation of nanoemulsion. Safety is a major determining factor in choosing a surfactant, as large amount of surfactants may cause skin irritation non-ionic surfactants as less toxic than ionic surfactants and therefore Tween 20 were selected. An important criterion for selection of the surfactants is that the required hydrophilic lipophilic balance (HLB) value to form oil-in-water nanoemulsion is greater than 10. In the present study Tween 20 was selected as a surfactant having the HLB value 16.7. Transient negative interfacial tension and fluid interfacial film is easily achieved by the use of single surfactants usually necessitating addition of co-surfactants. The presence of co-surfactant decrease the bending stress of the interface and allows the interfacial film sufficient flexibility to take up different curvatures required to form nanoemulsion over a wide range of composition. Thus, the co-surfactant selected for the study was Transcutol-P with the HLB value of 4.2.

The most important criterion for screening of components is the solubility of a poorly soluble drug in oil, surfactants and cosurfactants. As the aim of this study is to develop a transdermal formulation, it is important to determine drug solubility in oils, surfactants and cosurfactants. Among the various oil evaluated the maximum solubility of clozapine as compared to other was found to be highest in oleic acid (154.66 ± 2.14 mg/mL). Hence oleic acid selected as the oil phase for development of optimal formulation. The maximum solubility of clozapine in surfactants was found with Tween 20. The maximum solubility of clozapine in cosurfactants was found with Transcutol-P.

These nanoemulsions were prepared by using oleic acid as oil phase. It has been reported oleic acid is a better enhancer for transdermal delivery [29]. It is increase the fluidity of the intercellular lipid barriers in the stratum corneum by forming separate domains which interface with the continuity of the multilamellar stratum corneum and induce highly permeable pathways in the stratum corneum [30]. Tween 20 and Transcutol-P as surfactant and cosurfactant respectively.

Pseudoternary phase diagram
On the basis of selected surfactant and cosurfactant, pseudoternary phase diagrams were constructed by phase titration in order to define the extent and nature of nanoemulsion region and surrounding two and three phase domains. Constructing phase diagram is time consuming, particularly when the aim is to accurately delineate a phase boundary. Care was taken to ensure that observations are not made on metastable system, although the free energy required to form an emulsion is very low or negative, the formation is thermodynamically spontaneous. The relationship between the phase behaviors of a mixture and its composition can be captured with the aid of a phase diagram. Different ratios of surfactant and cosurfactant were used to prepared nanoemulsions (1:0, 1:1, 1:2, 2:1, 1:3 and 3:1). [Fig. 1-6] Pseudoternary phase diagrams were constructed and the ratios that yielded clear and stable solutions were selected as nanoemulsions consisting of oleic acid as oil phase, Tween 20 as surfactant, Transcutol-P as cosurfactant, and double distilled water as aqueous phase. The influence of the weight ratio of surfactant:cosurfactant in the areas of o/w nanoemulsion region was investigated from the diagrams it was found that the region of microemulsion increased with the $S_{max}$ ratio 1:0 to 3:1. In the 3:1 ratio, the maximum region of nanoemulsion was formed, while in 1:0 ratio showed smaller area of nanoemulsion as the cosurfactant concentration was increased in $S_{max}$ (ratio 3:1), a higher nanoemulsion region was observed. When surfactant concentration was increased from 1:1 to 3:1 compared to cosurfactant, the nanoemulsion area decreased. In Figure 1(A) $S_{max}$ ratio 1:0, it can be observed when Tween 20 was used alone without cosurfactant, very low amount of oil could be solubilized at higher concentration of surfactant and observed nanoemulsion area was smaller. It has been reported that surfactant alone are unable the formation of a stable nanoemulsion. Medium chain length alcohols, which are commonly added as cosurfactant (e.g. Transcutol-P), further helps in the lowering of interfacial tension by increasing the fluidity at the interface, thereby increasing the entropy of the system. This increase in fluidity and entropy helps in formation of a thermodynamically stable nanoemulsion.

From the study it is suggested that large amount of surfactant causes skin irritation and toxicity related problem, and therefore minimum amount of surfactant and co-surfactant was used in the formulation. It is important to determine the surfactant concentration properly and use the optimum concentration of surfactant in the formulation from pseudoternary phase diagrams, the formulation in which the amount of oil phase completely solubilized the drug and which could accommodate the optimum quantity of $S_{max}$ and distilled water were selected for the study.
Physical stability studies

Nanoemulsions are considered to be thermodynamic stable systems that are formed at a particular concentration of oil, surfactant, cosurfactant and water, with no phase separation, creaming or cracking. Selected formulations F1-F10 from phase diagram were subjected to different stress stability testing like heating and cooling cycle, centrifugation and freeze thaw cycle. The results showed that all the formulations had a good physical stability. Very low interfacial tension between oil and water and small droplet size made these systems thermodynamically stable. It is the thermostability which differentiates nanoemulsion from emulsions that have kinetic stability and will eventually phase separate. The nanoemulsions were then evaluated for their permeation characteristics.

Transmission electron microscopy (TEM)

The TEM studies were carried out to get more insight about the morphology of the nanoemulsion systems. The nanoemulsion appears dark and bright surrounding and a positive image. From the results of TEM it was concluded that particle of optimized formulation were spherical in shape and the droplet size range in between 53.48 and 81.07 nm. The droplet size is in agreement with the result obtained from droplet size analysis using Zetasizer [Fig. 7].
Nanoemulsion droplet size analysis

The globule size analysis of the optimized formulations was done using Zetasizer. The globule size increases with increase in concentration of oil in the formulations. The droplet size of formulation F1, containing 4.5% of oil was 53.48 ± 1.44 nm, which was lower as compared to other formulations. The droplet size distribution of nanoemulsion was determined by Malvern Zetasizer (1000 HS, Malvern Instruments, UK). The mean size of droplets of nanoemulsion F1 was shown size distribution report was found 53.48 ± 1.44 nm Fig.8 All the formulations had droplets in the 50-100 nm range which is well evident from the low value of polydispersity. This signifies the uniformity of droplet size within the formulation.

Viscosity, refractive index and pH

The viscosity of the nanoemulsion formulation was very low as expected for o/w nanoemulsion (39.55 ± 2.04 cP). The low viscosity may be due to presence of low amount of Smix also the low concentration of oil [31]. Refractive index is the net value of the components of nanoemulsion and indicates isotropic nature of formulation. The mean value of the refractive index (Rajat Scientific Work, Moradabad U.P.) was found to be 1.401(Table 3). There was no significant change in the values of the refractive index of selected formulation of placebo formulation and drug loaded formulations, thus it can be concluded that the nanoemulsion formulation were not only physically stable but also chemically stable and remained isotropic in nature, without interaction between nanoemulsion components and drug. The apparent pH of the formulation was measured by pH meter (VSI-IIB, VSI electronics, India) triplicate at 25 ± 1 ºC and found to be 6.4 which are favorable for tropical application.

Table 3: It shows Refractive index of nanoemulsion

<table>
<thead>
<tr>
<th>Code</th>
<th>Drug loaded formulation</th>
<th>Placebo formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.401 ± 0.03</td>
<td>1.405 ± 0.03</td>
</tr>
<tr>
<td>F2</td>
<td>1.422 ± 0.01</td>
<td>1.429 ± 0.03</td>
</tr>
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<td>F3</td>
<td>1.431 ± 0.04</td>
<td>1.435 ± 0.05</td>
</tr>
<tr>
<td>F4</td>
<td>1.402 ± 0.07</td>
<td>1.407 ± 0.08</td>
</tr>
<tr>
<td>F5</td>
<td>1.391 ± 0.02</td>
<td>1.401 ± 0.04</td>
</tr>
<tr>
<td>F6</td>
<td>1.412 ± 0.03</td>
<td>1.417 ± 0.06</td>
</tr>
<tr>
<td>F7</td>
<td>1.411 ± 0.01</td>
<td>1.417 ± 0.05</td>
</tr>
<tr>
<td>F8</td>
<td>1.413 ± 0.05</td>
<td>1.417 ± 0.08</td>
</tr>
<tr>
<td>F9</td>
<td>1.414 ± 0.08</td>
<td>1.419 ± 0.09</td>
</tr>
</tbody>
</table>

Solubility of optimized formulation

The solubility of clozapine in optimized formulation F1 (46.2 mg/mL and F2 42.68 mg/mL) was determined. The increase in solubility of clozapine in nanoemulsion formulation F1 and F2 was found as compared to water content, which could be due to the presence of surfactant (Tween 20) and cosurfactant (Transcutol-P), thus indicates the enhancement permeation of clozapine though the skin.

In vitro skin permeation studies

In vitro skin permeation studies were performed to compared the release of drug from ten different nanoemulsion formulations (F1 to F10), nanoemulsion gel and control (Drug solution in phosphate buffer (PBS pH 7.4), all formulations having the same quantity of clozapine. The permeation parameters (cumulative amount of clozapine permeated, transdermal flux, permeability coefficients) of selected nanoemulsion and control formulation were presented in Table 4. The solubility of clozapine in PBS very low, therefore 40% v/v methanol was added into receiver compartment to increase clozapine solubility. This was done in order to maintain the sink condition. Cumulative amount of clozapine permeated through rat skin of nanoemulsions were shown in the Figure 9(A-B). Nanoemulsion F1 shows the highest Cumulative amount of clozapine permeated through rat skin. In vitro skin permeation was the highest in formulation F1 and lowest F3. The optimized nanoemulsion formulation F1 showing highest flux (391.3357µg·cm⁻²·h⁻¹) was converted into nanoemulsion gel (adding 1% carbopol994 and kept overnight) and its permeation profile assessed by in vitro study. The flux of all formulation was shown in figure 9(C). Nanoemulsion Gel showed high value of flux (344.7319µg·cm⁻²·h⁻¹) which is slightly less than the nanoemulsion formulation F1, but significantly very higher than control. The decrease in the flux (compared with nanoemulsion) might be due to change in water content. The maximum release in F1 and F2 could be due to the smallest droplet size and lowest viscosity compared to other formulation. To explain the probable mechanism by which nanoemulsion enhance the skin permeation of drug, the histological and histochemical structure of stratum corneum must be taken into consideration. Drug permeate stratum corneum though two micro pathways i.e., intercellular and intracellular pathways. The intercellular pathways play a major role in percutaneous uptake of drugs.
Fig. 9(A): It shows In-Vitro Skin Permeation studies from various Nanoemulsion formulation

Fig. 9(B): It shows Comparative In-Vitro skin permeation profile of Nanoemulsion gel, F1 and Control

Fig. 9(C): It shows Flux of Different formulations, control and Nanoemulsion gel

The small droplet size and low viscosity of the nanoemulsion make it an excellent carrier for enhancing permeation uptake of clozapine, since the number of vehicle than can interact on fixed area of stratum will increase when droplet size and viscosity decrease. Permeability parameters like steady state flux (Jss), permeability coefficient (Kp) and enhancement ratio (Er) significantly increased in nanoemulsion and nanoemulsion nanoemulsion gel contain permeation enhancer like oleic acid, Tween 20 and Transcutol-P. The permeability parameters of different formulations are given in Table 4.
Pharmacokinetic studies

Formulation F1 was converted into gel and selected for the pharmacokinetic studies due to highest drug release properties in terms of percentage drug release (70.17 % in 10.5 h). Pharmacokinetic parameters obtained with following transdermal administration of clozapine loaded conventional emulsion and nanoemulsion Gel to six rats (mean value ± S.D, n=6). Fig. 9: Shows the pharmacokinetics profiles of clozapine after topical application of plain drug aqueous solution, conventional emulsion and nanoemulsion Gel formulation. The AUC∞ of transdermally applied nanoemulsion gel formulation was approximately 3 fold and 3.4 fold higher compared to topically applied aqueous solution and conventional clozapine loaded conventional emulsion respectively, and statistic calculated to provide significantly difference between both values (P < 0.05). The nanoemulsion gel formulation showed the low Cmax and prolonged Tmax, which was due to barrier properties of the skin and slow partitioning of the drug into skin from nanoemulsion gel. The in vivo data, which have demonstrated significantly higher bioavailability of clozapine often transdermal administration though nanoemulsion gel was due to avoidance of substantial amount of hepatic first pass metabolism associated with oral administration.

Skin irritancy test

Various formulations, when applied topically, might elicit primary skin irritation. Rat skin irritation experiments were, therefore, conducted in order to assess the potential irritant effects of the developed nanoemulsion formulation. The skin irritancy test was performed to confirm the safety of the nanoemulsion gel formulation. According to the previous study a value of skin irritancy score between 0 and 9 indicates that the applied formulation is nonirritating and safe for human skin. The mean value of skin irritancy score for formulation nanoemulsion gel was found to be 0.28. From this can be concluded that the prepared nanoemulsion formulation was safe to be used as transdermal drug delivery system.

CONCLUSION

In the present study, the application of nanoemulsion system for transdermal delivery of clozapine was investigated and eliminated the hepatic first pass metabolism associated with oral administration. An appropriate combination of the oil, surfactant, cosurfactant, and water is a major formulation consideration in nanoemulsion preparation for the transdermal drug delivery. Based on higher drug release, optimum globule size, lower viscosity and lower surfactant concentration as well as higher bioavailability nanoemulsion formulation of clozapine containing oleic acid (4.54 % w/w), Tween 20 (4.049 % w/w), and Transcutol-P (13.66 % w/w) and double distilled water as oil, surfactant, cosurfactant and aqueous phase respectively. Studies also showed how nanoemulsion formulation can be optimized for delivery of hydrophobic compound with higher drug loading and minimum S/CoS concentration.

REFERENCES


