Nano-Carrier for Accentuated Transdermal Drug Delivery

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Abstract
This research objective was to design a nano-carrier for Glibenclamide (GBD) by loading it in to nano-transfersomes to provide an accentuated transdermal drug delivery for Non-Insulin Dependent Diabetes Mellitus (NIDDM) The nano-transfersomes were prepared by sonication method and optimized using a statistically three-factor three-level Factorial Design (Box-Behnken design). A second order equation (polynomial) was originated and construct contour plots (2-D) for prediction the responses and characterized by various parameters (i.e. entrapment efficiency, vesicle shape & size, zeta potential, degree of deformability, permeation and skin irritation study). The skin permeation of optimized formulation (GNTs2) was found to be significantly higher (enhancement ratio is 10.44) than the drug in solution and also further confirmed by fluorescence microscopy using due i.e. Rhodamine B. Hence, drug loaded nano-transfersomes accentuates its transdermal flux and can be used as a nano-vehicle for NIDDM.

Keywords: Diabetic mellitus; Glibenclamide (GBD); Design; Nano-transfersomes; Transdermal delivery

Introduction
Transdermal Drug Delivery System (TDDS) is an alternative to conventional delivery by lower the problems associated with the oral and parenteral administration of drugs. TDDS also bypasses the first pass metabolism effect that proved it suitability for low bioavailability drugs, to achieving a constant/controlled release of drug (especially for drugs having narrow therapeutic window) with minimize side effects attained by using variety of polymers (applied as a nano-carrier for microspheres, nanoparticles, gels etc. [1-5]). In TDDS route, drug molecules achieve a therapeutic amounts at their target site and skin application (limited due to the effective barrier properties of intact skin, primarily associated with the outermost layers of the epidermis, namely the stratum corneum) [6-10]. Also, there are numerous approaches have been adopted to overcome permeation problem associated with these routes, one such approach involves encapsulation of drug in a vesicular system i.e. a novel nano-carrier “nano-transfersomes” which is capable to improved transdermal delivery of drugs [11] which are ultra-deformable lipid supra-molecular aggregates. They are capable to penetrating across the intact mammalian skin when applied non-occlusive by using a surface active agent added in a proper ratio [sublytic concentrations provided certain degree of flexibility to the vesicle membrane]. NTs claimed to be able to squeeze through channels one-tenth their diameter, allowing them to spontaneously penetrate the stratum corneum due to the flexibility. They penetrate across the skin by osmotic gradient (driving force), which is caused by the difference in water content between the relatively dehydrated skin surface (~20% water) and the aqueous viable epidermis. A lipid suspension when applied to the skin is subjected to evaporation [in order to avoid dehydration], NTs penetrates to the deeper tissues and hence squeeze through stratum corneum lipid lamellar regions penetrating deeper to follow the osmotic gradient [12,13].

A mathematical and statistical technique response surface methodology (RSM-factorial design: central composite design, Box-Behnken design, and D-optimal design are the various types of designs) is available for modeling, optimization and analysis of problems influenced by several variables and evaluation of the relationship of a set of controlled experimental factors and observed results of the formulations [14,15]. Based on the principles of factorial designs; the methodology involves the use of Box-Behnken designs for generating polynomial mathematical relationships and mapping the response over the experimental domain for selecting the optimum formulation. The design is independent quadratic designs which have the treatment combinations at the mid-points of the edges of the process space and at the center. For exploration, quadratic response construct a polynomial model which helping in optimizing of a process using small number of experimental domain [16-18].

Non-Insulin Dependent Diabetes Mellitus (NIDDM) is one of the most common diseases nowadays and characterized by moderate non reduction in β cell mass and generally low, normal or even high level of insulin in circulation, no β cell antibody is demonstrable and high degree of genetic disposition [19]. Glibenclamide (GBD: a BCS-II drug has low oral bioavailability & highly lipophilic) an oral hypoglycemic agent belonging to the category of sulphonylureas, on oral administration produces remarkable hypoglycemia, frequent GI side effects such as nausea, vomiting, heartburn, anorexia, increased appetite etc. and may even cause hyper-insulinemia, major risk factor for atherosclerosis. In order to counteract the shortcomings associated with oral therapy of GBD, transdermal delivery system can be developed, which in addition also provides an ease of termination of therapy on manifestation of serious side effects. GBD have already been proven to be effective in management of NIDDM on transdermal administration [20-23]. For this, the main aim of the study was to develop a novel nano formulation of the model drug i.e. GBD loaded nano-transfersomes for enhanced its transdermal delivery for NIDDM and its optimization using Box Behnken design.

Materials and Methods
The model drug (GBD) was purchased from Suraksha Pharma Pvt Ltd. [Nagarjuna Nagar, Hyderabad]. Sodium Deoxy-cholate (SDC)
and Rhodamine B were purchased from Control Drug House Pvt. Ltd, (New Delhi). Phospholipon 90G was a kind gift sample received from Lipoid Gmbh (Ludwigshafen, Germany).

Animals

The used Albino rats in the experiments were approved by the CPCSEA (Control and Supervision on Experiments on Animals, protocol approval no.: PDM/CPCSEA/RES/2012/10, PDM College of Pharmacy, India) committee.

Preparation of GBD Loaded Nano-Transfersomes (GNTs)

Nano-transfersomes were prepared by sonication method using lipid (phospholipon 90G), surfactant (SDC) and ethanol (7% v/v) as hydrating medium. Precisely lipid, surfactant and model drug (GBD) mixture was dissolved in chloroform: methanol (1:1 v/v), then organic solvent was removed by rotary evaporation (Rotary Evaporator, Multitech Instrument Co. Pvt. Ltd. Delhi, India). The final traces of solvent were removed under vacuum overnight and deposited lipid film was hydrated with ethanol by rotation (60 rpm) for sixty minutes at room temperature. Resulting, vesicles were allowed to swell at room temperature (2 hr) to get LMLVs (large multi-lamellar vesicles) and film was hydrated with ethanol by rotation (60 rpm) for sixty minutes. The used Albino rats in the experiments were approved by the CPCSEA (Control and Supervision on Experiments on Animals, protocol approval no.: PDM/CPCSEA/RES/2012/10, PDM College of Pharmacy, India) committee.

Experiments design

To explore the “quadratic response surface” and constructing a second order polynomial model using Design Matrix Expert i.e. three factor-three level Box-Behnken Design (Design Expert®, Version 8.07) comprising of experimental runs [22] was constructed and the non-linear computer generated quadratic model is defined as;

\[ Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 \]

Where, Y is the dependent variable; b is the intercept; b to b are the regression coefficients from the experimental values of Y (dependent variables; entrapment efficiency (Y1), particle size (Y2) and transdermal flux (Y3)), and X, X, X (i=1, 2, or 3) independent variables (lipid to drug ratio (X1), lipid to surfactant ratio (X2) and sonication time (X3) represents the interaction and quadratic terms respectively. The dependent and independent variables with low, medium & high levels are illustrated (Table 1). The amount of dependent variable’s (X=ratio of lipid to surfactant; X=weight of lipid to surfactant and X=sonication time) were used to prepared different batches of the drug loaded nano-transfersomes (GNTs) and their experimental responses (Y=entrapment efficiency, Y=particle size and Y=transdermal flux) are summarized in Table 1.

Characterization of GNTs formulations

Entrapment efficiency: By ultra-centrifuged (14000 rpm) nano-transferosomal suspension for 30 min. After that supernatant was diluted with methanolic HCl (0.01M), measured the absorbance (UV-Vis spectrophotometer (Jasco, V-630, Japan)) at 229 nm and entrapment efficiency calculated using formula as:

\[ EE = \frac{WT - WF}{WT} \times 100 \]

Where, EE=entrapment efficiency; W=total amount of the drug in nano-transferosomal suspensions; W=free amount of the drug.

Vesicle size and zeta potential: Particle size and zeta potential were measured by zeta sizer (Zeta nano series ZS90, Malvern instruments). The system was used in auto measuring mode. The Polydispersibility Index (PI), a measure of homogeneity, was also determined by the same

<table>
<thead>
<tr>
<th>Code</th>
<th>X1 (w/w)</th>
<th>X2 (w/min)</th>
<th>X3 (min)</th>
<th>Y1 (%) mean ± SD, n=3</th>
<th>Y2 (nm) mean ± SD, n=3</th>
<th>Y3 (µg/cm²/hr) mean ± SD, n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNTs1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>69.10 ± 0.74</td>
<td>145.24 ± 3.48</td>
<td>53.39 ± 4.76</td>
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<tr>
<td>GNTs2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68.80 ± 0.56</td>
<td>142.24 ± 1.89</td>
<td>54.10 ± 3.63</td>
</tr>
<tr>
<td>GNTs3</td>
<td>+1</td>
<td>+1</td>
<td>0</td>
<td>59.96 ± 1.21</td>
<td>180.06 ± 5.56</td>
<td>36.65 ± 5.30</td>
</tr>
<tr>
<td>GNTs4</td>
<td>-1</td>
<td>0</td>
<td>+1</td>
<td>48.95 ± 1.23</td>
<td>156.15 ± 4.98</td>
<td>40.10 ± 3.81</td>
</tr>
<tr>
<td>GNTs5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68.90 ± 1.67</td>
<td>152.76 ± 3.80</td>
<td>53.40 ± 4.76</td>
</tr>
<tr>
<td>GNTs6</td>
<td>+1</td>
<td>0</td>
<td>+1</td>
<td>51.18 ± 2.56</td>
<td>166.58 ± 1.24</td>
<td>46.04 ± 2.48</td>
</tr>
<tr>
<td>GNTs7</td>
<td>0</td>
<td>+1</td>
<td>-1</td>
<td>86.88 ± 1.33</td>
<td>184.67 ± 3.43</td>
<td>42.78 ± 5.44</td>
</tr>
<tr>
<td>GNTs8</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>50.40 ± 0.75</td>
<td>186.32 ± 5.60</td>
<td>47.30 ± 7.20</td>
</tr>
<tr>
<td>GNTs9</td>
<td>0</td>
<td>-1</td>
<td>+1</td>
<td>72.68 ± 2.23</td>
<td>151.33 ± 4.69</td>
<td>48.52 ± 5.09</td>
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<tr>
<td>GNTs10</td>
<td>+1</td>
<td>-1</td>
<td>0</td>
<td>42.60 ± 1.32</td>
<td>140.04 ± 2.35</td>
<td>41.57 ± 9.36</td>
</tr>
<tr>
<td>GNTs11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>69.10 ± 0.98</td>
<td>142.31 ± 1.36</td>
<td>53.39 ± 4.76</td>
</tr>
<tr>
<td>GNTs12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>68.80 ± 3.34</td>
<td>147.68 ± 3.50</td>
<td>54.10 ± 3.63</td>
</tr>
<tr>
<td>GNTs13</td>
<td>+1</td>
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<td>-1</td>
<td>51.17 ± 2.35</td>
<td>170.24 ± 4.21</td>
<td>36.56 ± 5.49</td>
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<td>GNTs14</td>
<td>0</td>
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<td>-1</td>
<td>74.61 ± 1.13</td>
<td>175.09 ± 1.25</td>
<td>41.40 ± 3.21</td>
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<td>160.78 ± 2.12</td>
<td>39.94 ± 4.27</td>
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<td>GNTs16</td>
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<td>+1</td>
<td>86.20 ± 4.39</td>
<td>191.23 ± 3.22</td>
<td>38.31 ± 5.04</td>
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<tr>
<td>GNTs17</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>45.59 ± 0.87</td>
<td>154.33 ± 3.89</td>
<td>43.50 ± 4.58</td>
</tr>
</tbody>
</table>

Table: Box-Behnken design variables and results.
Diffusion coefficient were calculated using following formulas: 

\[
K_p = \frac{J_{ss}}{C_d}
\]

Where, \(K_p\) = permeability coefficient; \(J_{ss}\) = steady-state flux; \(C_d\) = concentration of drug in donor compartment.

**Vesicle shape and morphology:** For morphological characterization, transmission electron microscopic (TEM) studies using Phosphorthungastic Acid (PTA) as a negative stain were performed (Moragagni 268D FEI, The Netherlands). A drop of the sample was placed on a carbon-coated copper grid to leave a thin film on the grid, drop of the staining solution (PTA (1%) was added to the film, and the excess of the solution was drained off with a filter paper. The grid was allowed to thoroughly dry in air, and samples were viewed under a transmission electron microscope (Olympus, DX31, and Japan).

**Elasticity of vesicles:** The elasticity of DNTs was determined by nano-transferosomal suspension extruded through filter membrane (pore diameter 100 and 200 nm, using a stainless steel filter holder) applying a pressure of 2.5 bar using extrusion method. The quantity of suspension extruded was measured and particle size after extrusion was measured using Zeta Sizer (Zeta Nano series Z590, Malvern Instruments) [24-26]. The elasticity value of vesicles was calculated by formula:

\[
\text{Elasticity} = \frac{1}{\text{J}} \times (r_v^2 - r_p^2)
\]

Where, \(J\) = amount of suspension extruded in 5 minutes; \(r_v\) = vesicle size; \(r_p\) = pore diameter

**Drug release and permeation profile**

The abdominal and dorsal skin of sacrificed rat was removed using scalpels and scissors, wrapped in aluminium foil and stored at a temperature (~20°C). On the day of experiment, skin was thawed, then hairs were removed at room temperature and subcutaneous fat was removed by cotton soaked in isopropyl alcohol [27]. Thus prepared skin was cut into pieces according to the area of diffusion cell and ex vivo permeation profile of GBD from nano-transferosomes was done [28,29]. The receptor medium was continuously transferred using nano-transfersomal suspension. The receptor medium was continuously and then embedded in paraffin. The five micrometer thick sections of skin were cut using microtome and mounted on slide using commercial baker's mounting fluid. The paraffin was removed by warming the slide gently, until wax melted, and then washed with xylene followed by washing with absolute alcohol and water. The sections were stained with hemato-xylin eosin to determine gross histopathology. The slides were analyzed by optical microscope at 400X magnification [31].

**Fluorescence microscopy**

Drug loaded nano-transfersomes (GNTs) with Rhodamine B (provide the fluorescence labeling) was prepared as described method in section 2.2. The vesicle-skin interaction studied to confirm the better skin penetration using two rats (Wistar rats (weighing 120-160 g)); first rat received the application of aqueous solution of Rhodamine B alone (control) and applied GNTs with Rhodamine B on the dorsal skin surface of second one. After six hour of application, the rats (skin was removed, cut into small pieces and fixed) were sacrificed and examined under a fluorescence microscope [11,32].

**Skin irritation study**

For the skin irritation study, the dorsal side of rat (weighed around 230-250 gm) present hairs were removed using scalpel and the optimized formulation was applied. Any sign of irritation i.e. erythema\ redness for a period of 24 hrs was observed.

**Stability studies**

The Optimized NTs formulation was packed in amber colored glass bottle and stored at 5 ± 3°C (storage condition) and 25 ± 2°C/75 ± 5%RH (for accelerated stability study) [33] for a period of three months. The formulation was periodically evaluated for entrapment efficiency and visual changes. The shelf life of the GNTs2 was obtained using software Sigma Plot, version 12.5.

**Result and Discussion**

**Entrapment efficiency (EE)**

The percentage fraction of total drug incorporated (EE) into the NTs was obtained in range between 45.59 ± 0.87 to 86.88 ± 1.33 (Table 1). The entrapment efficiency usually increases with increase in lipid to surfactant ratio (higher EE of GNTs) further increase in the surfactant concentration showed a decrease in the entrapment efficiency. The initial increment in drug entrapment in the presence of low concentrations of surfactant may be due to the growth in vesicle size owing to the incorporation of more amount of drug. The entrapment efficiency was found to decrease with an increase in concentration of surfactant (>15 % w/w). This is due to the fact that at lower concentration (<15% w/w) the surfactant molecules get associated with the phospholipid bilayer. Above a certain concentration, some surfactant molecules lead to increased permeability of the vesicle membrane by generating pores thereby making the membrane leaky, and hence resulting in decreased entrapment efficiency. Also, when the concentration of surfactant in the bi-layer is increased beyond its critical micellar concentration (beyond 15% w/w), mixed micelles with lower entrapment efficiency were formed [34,35].

**Vesicle Size and Zeta Potential**

The particle size and zeta potential of the nano-transferosomal formulation (DNTs) was found to be in range 140.04 ± 2.35 to 191.23 ± 3.22 nm and -29.7 ± 1.03 to -18.9 ± 1.56 (Tables 1 and 2) respectively. Particle size increases with increase in lipid to surfactant ratio (X1) and vice-versa with sonication time (X3). The greater stability to GNTs due to more negative value of zeta potential and also poly-dispersibility index.
Degree of deformability

The elasticity of nano-transfersomes vesicles is most considerable parameter of nano-transfersomal formulations because this parameter differentiates it from other vesicular carriers and helping it to pass through narrow pores. Deformability index increased significantly with increase in the lipid concentration and beyond a certain concentration it decreases which may be attributed to rigidization effect observed at higher concentration of lipid (Table 2). It was found to be increase with increase in concentration of surfactant (up-to 15% w/w) but when concentration is further increased (beyond 15%), degree of deformability decrease was observed (owing to the formation of mixed micelle, which bears rigid membrane). Due to high flexibility, drug loaded nano-transfersomes pass through much smaller pores than their diameter and risk of skin rupture completely minimizing.

Drug release study

The amount of drug permeated determined the amount of drug which is available for absorption, so cumulative drug release, and permeation coefficient obtained from nano-transfersomal formulation at the end of 24hr (Figure 1). The value is substantially higher than that which is typically driven by transdermal concentration gradients. The higher permeation rate from nano-transfersomes is due to the capability of lipid to spontaneously penetrate across the skin due to transdermal hydration gradient. Xerophobia [11], affinity to avoid dry environs, causes the surface of skin dehydration resist which results drug transport from the dry skin surface to better hydrated skin.

Data fitting

All responses (\(Y_1, Y_2, \) and \(Y_3\) from prepared seventeen formulations) were fitted to first order, second order and quadratic models designs. Resulting, quadratic was the best-fitted model and the proportional values of \(R^2\), SD, and %CV are given along with each response regression equation with statistically significant (p<0.05) coefficients.

Equation analysis

A positive and negative value indicates; the effect that favors the

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Zeta potential (mV) mean ± SD, n=3</th>
<th>PDI mean ± SD, n=3</th>
<th>Extruded volume (ml)</th>
<th>Degree of deformability</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNTrs1</td>
<td>-28.9 ± 2.31</td>
<td>0.212 ± 2.30</td>
<td>7.8 ± 1.30</td>
<td>15.37</td>
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<tr>
<td>GNTrs2</td>
<td>-29.7 ± 1.03</td>
<td>0.174 ± 1.45</td>
<td>8.1 ± 0.90</td>
<td>15.70</td>
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<tr>
<td>GNTrs3</td>
<td>-25.0 ± -0.70</td>
<td>0.167 ± 3.12</td>
<td>5.3 ± 2.67</td>
<td>16.07</td>
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<td>GNTrs4</td>
<td>-22.3 ± -0.35</td>
<td>0.204 ± 0.98</td>
<td>8.0 ± 1.11</td>
<td>17.10</td>
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<tr>
<td>GNTrs5</td>
<td>-22.3 ± -0.35</td>
<td>0.204 ± 0.98</td>
<td>7.6 ± 2.93</td>
<td>15.23</td>
</tr>
<tr>
<td>GNTrs6</td>
<td>-20.5 ± 2.34</td>
<td>0.182 ± 3.48</td>
<td>8.6 ± 1.09</td>
<td>21.62</td>
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<td>GNTrs7</td>
<td>-27.7 ± -1.20</td>
<td>0.194 ± 4.38</td>
<td>4.8 ± 1.87</td>
<td>15.79</td>
</tr>
<tr>
<td>GNTrs8</td>
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<td>0.135 ± 0.98</td>
<td>7.8 ± 1.15</td>
<td>18.04</td>
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<td>GNTrs9</td>
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<td>5.8 ± 1.89</td>
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<td>GNTrs10</td>
<td>-20.7 ± 1.04</td>
<td>0.134 ± 1.89</td>
<td>6.5 ± 3.62</td>
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<td>0.147 ± 3.26</td>
<td>7.3 ± 1.11</td>
<td>10.66</td>
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</table>

Table 2: Data of all formulations.

Figure 1: The cumulative drug releases of all formulations at the end of 24 hr.
optimization and inverse relationship between factors and responses. It is plain that independent variables (lipid to drug ratio \(X_1\), lipid to surfactant ratio \(X_2\) and ST \(X_3\)) have interactive effects on the responses \((Y_1, Y_2, \text{and } Y_3)\).

**Entrapment efficiency: Response 1 \((Y_1)\)**

The following polynomial equation for entrapment efficiency of GBD purports by model: \(Y_1=68.14-1.45X_1-4.83X_2-0.81X_3+2.16X_1X_2+0.37X_1X_3+0.31X_2X_3+24.02X_1^2+4.17X_1X_3-2.90X_2X_3-4.42X_3^2; R^2=0.9999; \text{Adjusted } R^2=0.9998; \text{Predicted } R^2=0.9998; \text{S.D}=0.33; \%CV=0.31\)

Where \(Y_1\) is the entrapment efficiency of GBD, \(X_1\) is the ratio of drug to lipid, \(X_2\) is the ratio of lipid to surfactant and \(X_3\) is the sonication time. The Model F-values 8643.24 implies the model is significant \(p<0.0001\) and 2.52 implies the lack of fit is not significant. In this case \(X_1^2\) had a more prominent effect on entrapment efficiency of GBD than any other parameters. The desirable limit of an adequate precision with adequate signal ratio (295.103) indicates this model can be used to map the design space. The 2-D (contour) plots respectively (Figure 2) which show the effect of different independent variables on entrapment efficiency (EE; is expressed as fraction of drug incorporated in to nano-transfersomes relative to total amount of drug used) of GBD \((Y_1)\) for all batches was found to be in the range of 42.60 to 86.20%. A drug to lipid ratio \((X_1)\) at low level (-1) (61.0% lipid and 22.53% surfactant), found to be less as compared to the ratio of lipid to surfactant at high level (+1) (72.17 % lipid and 22.53% surfactant) when inferred from the experimental. At medium level [0] optimum value of EE was obtained [EE is bound to increase with increase in lipid, due to its solubilizing effect for lipophillic drug. The results also showed that EE depended on both the type and concentration of the surfactant in the bi-layer membrane [35,36] and sonication time \((X_3)\) was found a contrary relationship with entrapment efficiency of GBD.

**Effect on particle size of the vesicles: Response 2 \((Y_2)\)**

The model was projected polynomial equation for vesicles particle size is as follow; \(Y_2=269.51-20.49X_1-78.01X_2-36.14X_3+40.00X_1X_2+6.06X_1X_3+24.02X_2X_3-13.36X_1^2+64.95X_2^2+72.39X_3^2; R^2=0.9999; \text{Adjusted } R^2=0.9998; \text{Predicted } R^2=0.9998; \text{S.D}=10.1; \%CV=0.32\)

The model is significant \((F\text{-value}=8319.59; \text{p}<0.0001)\), lack of fit \((0.02)\) is not significant and 0.9998 values for predicted \(R^2\) in reasonable agreement with and adjusted \(R^2\). The \(X_1\) (sonication time) has negative coefficients proved that sonication time has inverse effect on particle size and observed (contour plots which show the effect of different independent variables on particle size \((Y_2)\) (Figure 3) that the size of the vesicles significantly increased with increasing concentration of lipid. At high level (+1) of lipid level, vesicle particle size larger due to inadequate drug molecules for inclusive scale of association with the lipids, while at equal concentration of the lipophilic drug into bi-layer of lipid which leads to considerable cohesion with hydrophobic portion of the membrane [11]. Also an increase in the phospholipids \((X_1)\) might increase the entrapment of GBD and repeal effect was observed with.

**Effect on transdermal flux of GBD: Response 3 \((Y_3)\)**

The model proposed constant, the regression coefficients and the statistical parameters for each response variable is as follows: \(Y_3=49.31-0.91X_1+10.84X_2+3.58X_3-0.34X_1X_2+4.17X_1X_3-2.90X_2X_3-6.72X_1^2+2.65X_2^2+5.48X_3^2; R^2=0.9999; \text{Adjusted } R^2=0.9998; \text{Predicted } R^2=0.9998; \text{S.D}=0.33; \%CV=0.72\)

Where, \(Y_3\) is the flux of GBD. The model was found to be significant \((F\text{-value}=688.96; \text{p}<0.0001)\). In this case \(X_1 \text{ and } X_2\) the lipid to surfactant ratio had a prominent effect on flux of GBD than any other parameters. The flux of GBD was found to be in the range of 36.65 µg cm\(^{-2}\) h\(^{-1}\) to 54.10 µg cm\(^{-2}\) h\(^{-1}\). Due to molecular mixing of phospholipids with lipids of skin, transdermal flux of GBD increases with increase in lipid to drug ratio from low (-1) to medium [0] level and then it starts decreasing (Figure 4). When nano-transfersomes come in contact with lipids of skin, resulting molecular mixing of nano-transfersomes with closely packed lipids of the stratum corneum will occur. As a result, a temporarily loosely-packed lipid structure formed which allows the drug to penetrate [11]. The effect of surfactant is also similar to that of lipid and optimum ratio of lipid to surfactant (medium level) was desirable to achieve maximum flux of GBD.

**Data validation**

The factors interaction affects on the responses by 2-D plots which...
are helpful in studying effects of two factors at one time on the response. For validation of RSM results, established high values of $R^2$ for all the three responses). The $R^2$ value for responses $Y_1$, $Y_2$, and $Y_3$ was found to be 0.9999, 0.9999 and 0.9974, respectively. Thus, a low error magnitude significant proved the high predictive ability of the RSM.

**Optimization**

The optimum formulation of GBD loaded nano-transfersomes was selected based on the criteria of attaining maximum value of percentage EE and transdermal flux, minimizing vesicle size by applying Design Expert prediction method. Upon “trading off” various variables and comprehensive evaluation of feasibility search and exhaustive grid search, GNTs2 was fulfilled the requisite of optimum formulation.

**Optimized GNTs2**

The optimized formulation was characterized by vesicle shape (morphology), vesicle size and size distribution, entrapment efficiency (%EE), transdermal flux, zeta potential, drug release and kinetics, permeation studies, vesicle skin interaction (fluorescence microscopy) and skin irritation study. GNTs2 showed high stability of the formulation due to high %EE: 68.80 ± 0.56; vesicle size: 142.24 ± 1.89 nm; transdermal flux: 54.10 ± 3.63 µg/cm²/hr and -29.7 value of zeta potential. The small poly-dispersibility index (PI=0.174) value proved narrow size distribution and dispersion homogeneity in the optimized formulation.

**Drug release**

GNTs transdermal flux is 54.10 ± 3.63 µg/cm²/hr, whereas as it is only 5.18 ± 2.66 µg/cm²/hr for the control (enhancement ratio=10.44), indicating much higher penetration capacity of drug entrapped in nano-transfersomes compared to the drug in solution. This can be attributed to better partitioning of drug entrapped in nano-transfersomes compared to drug in solution and high elasticity of the nano-transfersomal membrane. Moreover, at time 0.5 hr, the Cumulative Drug Release (CDR) of control (108.86 ± 1.44 µg/cm²) and GNTs2 (219.86 ± 6.38 µg/cm²) showing that nano-transfersomal formulation will show a quicker onset of action. CDR higher in GNTs2 at 0.5 hr is due to burst effect and higher flux obtained with the nano-transfersomes may be attributed to increase in thermodynamic activity, increased skin vehicle partitioning of drug, alteration in the barrier properties of the skin and elasticity of vesicle membrane [35].

**Skin histopathology**

The high power photomicrograph and fluorescent micrographs of...
untreated rat skin (control); showed dermis (Dx), epidermis (Ep) and stratum corneum (SC) (Figures 5 and 6). The corneal layer is quite well formed with the layers compacted. The epidermis is three to four cells thick with normal stratification. The skin photomicrograph treated with optimized formulation (GNTs2) for 6 hrs, the corneal layer is thinner and partly separated from the underlying epidermis which shows thinning of two to three layers, indicating the disruption of stratum corneum by nano-transfersomes. In case of skin treated for 24 hrs. (Figure 5), the corneal layer is mostly lost with some fragments of poorly stained corneal cells seen on the underlying epidermis and the epidermis here is also reduced in thickness with loss of basal layer cells, which shows further disruption of lipid bi-layer.

### Fluorescence microscopy

The skin treated with rhodamine B dye solution i.e. control and skin treated with dye loaded GNTs2; In the control skin, stratum corneum is intact and the dye almost remained confined to the stratum corneum with a minimal penetration [by the virtue of the dye lipophilicity] [38]. Whereas, in case of skin treated with dye loaded GNTs2, the stratum corneum was found to be disrupted, and nano-transfersomes appearing as clusters of particles showing fluorescence can be seen in the epidermis and in the deeper layers. Therefore, it can be concluded that nano-transfersomes penetrate across the skin by disrupting the stratum corneum, while remaining intact themselves, and reaches the deeper layers of the skin.

### Release kinetics

The ex vivo diffusion data was applied to zero order, first order, Higuchi kinetics and Korsmeyer Peppas models to find out the release kinetics of the GNTs2. The mathematical evaluation of order of release correlation regression was determined to know the order of drug release. The highest value of regression coefficient has been obtained for zero order kinetic models (R²=0.9862), indicating drug release from GNTs2 follows zero order kinetics, i.e. drug release first increases with time and then becomes constant. The value of n for the drug release was found to be 0.253, showing that Quasi Fickian drug release mechanism has been followed by the optimized formulation (GNTs2).

### Skin irritation study

A primary skin irritation test was conducted with rats to determine the potential of GNTs2 to produce irritation after a single topical application. There was no sign of irritation [no redness/erythema] was observed after 24 hrs of application of the optimized formulation. Therefore, the optimized formulation is safe on topical application.

### Stability studies

Optimized formulation (GNTs2) showed no significant changes in visual, entrapment efficiency and drug content at interval of 15, 30, 60 and 90 days (three months period), under both the conditions (accelerated and long term storage).

### Conclusion

In order to counteract the shortcomings associated with oral therapy of Glibenclamide (GBD), transdermal delivery system has been developed, which in addition also provides an ease of termination of therapy on manifestation of serious side effects. The present study was an attempt to enhance transdermal delivery of GBD, by loading it in novel approach (nano-transfersomes). The histopathology study showed loosening and disruption of stratum corneum indicated
penetration of GNTs2 across stratum corneum. The GNTs2 (flux was 10.44 more than control) has much higher penetration and its drug release followed zero order kinetics. During accelerated and long term storage, GNTs2 showed no significant changes, shelf life was 368.110 days and skin irritation study proved its safety for topical use.

Conflict Of Interest

The author’s confirmed that there is no conflict of content.

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