INTRODUCTION

Felodipine (ethyl methyl 4-(2,3-dichlorophenyl)-1, 4-dihydro-2, 6-dimethyl-3,5 pyridine dicarboxylate) is a calcium channel antagonist most commonly used to treat hypertension and angina pectoris.[1] Commercialised as tablet dosage form (2.5-10 mg) for oral administration it displays 15-20% bioavailability, owing to extensive hepatic first pass metabolism with an additional problem of dose dependent adverse effect that proportionally augments with increase in dose size (doubled with twice dose size).[2] This demanded search of alternatives that enable to deliver felodipine in more safer and patient compliant fashion. Several workers approached to eliminate the hepatic first pass metabolism by opting transdermal route and developed conventional, as well as modified drug delivery systems. Transdermal route for drug administration manifests definite advantages in the context of being user friendly and noninvasive, but its utilization has been hampered by naturally occurring protective barrier properties of the skin, especially the stratum corneum. Hence, the major emphasis of research related to transdermal delivery of felodipine has focussed on overcoming the permeation difficulties utilizing different approaches.

Ren et al. developed solid lipid nanoparticles (SLN) for transdermal delivery of felodipine.[3] This system is proven to be more effective for localized topical therapy rather than transdermal systemic delivery.[4] In addition, these carriers exhibit low drug payload, drug expulsion during storage and high water content of SLN dispersions.[5] Wang et al. developed transdermal patches of felodipine and metoprolol for maintenance therapy.[6] The designed system exhibited increased bioavailability, but with higher lag time. Similarly, Diez et al. developed transdermal patch[7] of felodipine incorporated with the penetration enhancer (d-limonene) that lowered the lag time from 9 h to 1.5 h. A lag time of 1.5 h is too high that can limit the efficacy of the designed patches, moreover the use of permeation enhancer can be irritant and immunogenic to skin on prolonged use.[8] To refine the delivery...
of felodipine, the strategy of using deformable lipid vesicles for transdermal delivery has been considered.

Transfersomes are self-optimising ultra-adaptable lipid and edge activator based vesicular carriers with an inheritable transgressing ability through skin to provide enhanced transdermal delivery. Several reports propose their effectiveness to permeate intact skin, transport drugs in therapeutic concentrations when applied to an open biological barrier under nonocclusive conditions. The key factor involved in improved permeation across skin through transfersomes is the liquid-state vesicle system with highly deformable lipid bilayer that allows them to permeate easily even through very narrow constrictions under the influence of hydration gradient. The presence of edge activator enables the vesicle to deform without losing their structural integrity. The aptness of edge activators to cumulate at highly stressed sites furnishes series of stress-dependent adaptations that minimize hindrance to their motion through narrow channels and allows transport of drugs noninvasively.

The present work is focussed to enhance the bioavailability of felodipine in safe fashion using transfersomes by avoiding extensive hepatic first pass metabolism, adequately retaining the drug onto the skin for large time duration and systemically deliver the drug to achieve therapeutic blood levels.

**MATERIALS AND METHODS**

Felodipine was generous gift of Astra Zeneca Pharma India Ltd., (Karnataka, India). Soya lecithin and egg lecithin was procured from Himedia Laboratories Pvt. Ltd., (Mumbai, India). Tween-80, span 80 and chloroform was purchased from SD Fine-Chem Ltd., (Mumbai, India). Rhodamine red was procured from New Drug House (P) Ltd., (New Delhi, India). Methanol, ethanol and isopropyl alcohol was purchased from Qualikems Fine Chemical Pvt. Ltd. (New Delhi, India). Millipore nylon membrane filters were purchased from Merck specialties Pvt. Ltd. (Mumbai, India). All other reagents used were of analytical reagent grade.

All animal experiments have been conducted in full compliance with the institutional ethical and regulatory principles and as per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care and International expectations for animal care and use/Ethics Committees. The investigations were performed after obtaining approval by the Institutional Animal Ethical Committee of Rajiv Academy for Pharmacy, Mathura, India (IAEC No: IAEC/RAP/3648a). The study has been classified in four different sections namely preliminary trials, formulation development, dosage form design and in vivo pharmacokinetic study.

**Preliminary trials**

**Optimization of vortexting and sonication time**

The ratio of lipids and edge activator (95:5) was taken on the theoretical basis and loaded with felodipine (1% w/w). The formulation was vortexed for different time intervals of 15, 30, 45, and 60 min and entrapment efficiencies calculated. Sonication time was optimized on the basis of the vesicle size obtained by zetasizer (Malvern Mastersizer, Malvern Co., Worcestershire, UK). The time point is producing vesicles of optimal size with low polydispersity index (PDI) values was selected.

**Optimization of loading drug concentration**

The lipid:edge activator composition (95:5) was exposed to variable felodipine concentrations (0.25, 0.50, 0.75, 1.00, 1.25 and 1.50% w/w) and the effect of different loading concentration was evaluated in terms of entrapment efficiency (EE). The felodipine concentration at which the maximal drug entrapment occurred was selected. Drug entrapment was determined by centrifugation method described in the latter section.

**Optimization of lipid:edge activator ratio**

To optimize the lipid:edge activator ratio, transfersomes were prepared using lipids and edge activators at three different levels (Table 1) by vortexing sonication method considering all the preoptimized processing conditions and coded as F1-F12 preliminary formulations. The method involved incorporation of lipid, edge activator, felodipine (1% w/w) and 10 ml phosphate buffer (pH 6.8) in a vortexing tube followed by vortexing for 30 min. The resulted in milky suspension was sonicated using bath sonicator (PCI Analytics, Mumbai, India) for 30 min at 33 ± 3 KHz with input voltage of 170-250 V AC and then extruded through a series of Millipore nylon membrane filters of 450, 200 and 100 nm pore size and stored at 4°C.

**Characterization of preliminary formulations**

**Determination of vesicle size, polydispersity index and zeta potential**

The vesicle size, PDI and zeta potential of the prepared transfersomes were determined by light scattering using the Malvern Master sizer by appropriately diluting the sample with water as dispersant.

**Deformability studies**

The vesicle suspension was extruded through Millipore nylon membrane filter of 50 nm at constant pressure of 1.2 MPa for 10 min. The deformability was reported as deformability index calculated by following equation:

\[ \text{Deformability index} = j \times \left( \frac{r_v}{r_p} \right)^2 \]

Where, jis the weight of the suspension, rv the size of the vesicle and rp the pore size of the membrane.

**Determination of entrapment efficiency and loading efficiency**

Purified transfersomal suspensions were centrifuged at 10,000 rpm at 4°C for 10 min in a cooling centrifuge (REMI Instrument Ltd., C-24BL/CPR24 Vasai, India) to obtain sedimented pellet. The pellet was washed with distilled water to remove free felodipine and placed in a test tube for disruption of vesicle by using 50% v/v
isopropyl alcohol. Further, it was vortexed, filtered and analyzed spectrophotometrically (Shimadzu Corp., Pharmaspec 1700, Kyoto, Japan) at 237 nm. The EE and loading efficiency was calculated by the equations:

\[
\% \text{ EE} = \frac{\text{Amount of drug present in vesicles}}{\text{total drug incorporated}} \times 100 \tag{2}
\]

\[
\% \text{ Loading efficiency} = \frac{\text{Amount of drug present in vesicles}}{\text{total amount of lipid incorporated}} \times 100 \tag{3}
\]

**Ex vivo skin permeation**

The rat skin was isolated, shaved and separated from the underlying tissues. The excised skin was mounted on Franz diffusion cell of internal area 2.03 cm\(^2\). Defined volume of transfersomal suspension containing equivalent amount of felodipine was placed on dorsal side of the skin (donor compartment side). The receptor compartment was filled with 15 ml phosphate buffer, pH 6.8 maintained at 37°C ± 0.5°C and stirred at 100 rpm. Aliquots of 1 ml were withdrawn from the receptor compartment at different intervals of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h and replaced with similar volume of fresh media. The cumulative amount of felodipine permeated across the skin per square surface area was plotted against time to calculate the steady state flux (Jss),

\[
\text{Jss} = \frac{\text{Amount of drug permeated}}{\text{time} \times \text{area of permeation}} \tag{4}
\]

**Skin deposition**

The skin obtained after the ex vivo permeation experiment (after 24 h) was used to determine the amount of felodipine within the skin. The surface of the skin was washed 5 times with phosphate buffer pH 6.8 maintained at 45°C, cut into small pieces, homogenized by sonicating it with ethanol (95% v/v) and placed for 6 h at room temperature. The resulting suspension was centrifuged for 10 min at 8000 rpm, the supernatant was separated and analyzed by validated high performance liquid chromatography (HPLC) method described in the latter section.

**Formulation development**

The selected preliminary compositions (F2, F5, F8 and F11) containing lipid: Edge activator (95:5) were modified by Rotary evaporation sonication method. The lipid, edge activator (95:5) and felodipine (1% w/w) was dissolved in ethanol: Chloroform (3:2) and placed in 250 ml round bottom flask that was attached with rotary vaccum evaporator (Macro Scientific Works Pvt. Ltd., New Delhi, India). The solution was continuously rotated and dried under vaccum to obtain a thin film. The dried film was hydrated using 30 ml phosphate buffer, pH 6.8 maintained at 45°C with continuous stirring of 100 rpm. The vesicular suspension was set aside for 2 h, sonicated using bath sonicator and extruded to obtain transfersomal suspension(s) that were coded as MF2, MF5, MF8 and MF11, respectively [Table 2].

The formulations were characterized for physical (vesicle size, PDI, zeta potential, entrapment and loading efficiency) and performance characteristics (ex vivo permeation studies, flux determination and skin deposition) by the methods discussed earlier.

**Transmission electron microscopy**

A drop of the selected transfersomal formulation (MF8) was placed on a carbon coated copper grid for 2 min, adsorbed with

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**Table 1: Composition details of (a) preliminary formulations (F1-F12), (b) modified formulations (MF2, 5, 8 and 11) and (c) developed dosage form: Gel (s) (TG and CG)**

(a)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Formulation code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1</td>
</tr>
<tr>
<td>Felodipine (mg)</td>
<td>10</td>
</tr>
<tr>
<td>Soya lecithin (%)</td>
<td>98</td>
</tr>
<tr>
<td>Egg lecithin (%)</td>
<td>—</td>
</tr>
<tr>
<td>Tween 80 (%)</td>
<td>2</td>
</tr>
<tr>
<td>Span 80 (%)</td>
<td>—</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Selected formulation code</th>
<th>Modified formulation code</th>
<th>Drug (% w/w)</th>
<th>Soya lecithin (% w/w)</th>
<th>Egg lecithin (% w/w)</th>
<th>Tween-80 (% w/w)</th>
<th>Span-80 (% w/w)</th>
<th>Ethanol: chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2</td>
<td>MF2</td>
<td>1</td>
<td>95</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>6:4</td>
</tr>
<tr>
<td>F5</td>
<td>MF5</td>
<td>1</td>
<td>95</td>
<td>—</td>
<td>5</td>
<td>—</td>
<td>6:4</td>
</tr>
<tr>
<td>F8</td>
<td>MF8</td>
<td>1</td>
<td>—</td>
<td>95</td>
<td>—</td>
<td>5</td>
<td>6:4</td>
</tr>
<tr>
<td>F11</td>
<td>MF11</td>
<td>1</td>
<td>—</td>
<td>95</td>
<td>—</td>
<td>5</td>
<td>6:4</td>
</tr>
</tbody>
</table>

(c)

<table>
<thead>
<tr>
<th>Gel code</th>
<th>Drug (mg)</th>
<th>Carbopol-934P (% w/v)</th>
<th>Triethanolamine (% v/v)</th>
<th>Ethanol (% w/v)</th>
<th>Menthol (% w/w)</th>
<th>Double distilled water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>MF8 Eq. to 10 mg of drug</td>
<td>1</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>CG</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

TG: Transfersomal gel, CG: Control gel
Table 2: Comparative data for characterization of preliminary formulations (F1-F12)

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Vesicle size (nm)</th>
<th>PDI</th>
<th>Zeta potential</th>
<th>Entrapment efficiency (%)</th>
<th>Loading efficiency (%)</th>
<th>Deformability index</th>
<th>Cumulative drug permeation% (24 h)</th>
<th>Skin deposition (%)</th>
<th>Transdermal flux ($J_{\text{max}}$) (µg/h/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>132.4±5.4</td>
<td>0.430</td>
<td>-39.6</td>
<td>71.62±2.42</td>
<td>1.432±0.11</td>
<td>68.97</td>
<td>63.02±1.98</td>
<td>3.38±0.54</td>
<td>16.25±0.34</td>
</tr>
<tr>
<td>F2</td>
<td>118.4±8.9</td>
<td>0.458</td>
<td>-39.7</td>
<td>79.44±2.3</td>
<td>1.588±0.17</td>
<td>121.70</td>
<td>75.06±1.89</td>
<td>5.40±0.37</td>
<td>17.63±0.41</td>
</tr>
<tr>
<td>F3</td>
<td>157.8±7.2</td>
<td>0.508</td>
<td>-34.0</td>
<td>73.21±1.97</td>
<td>1.464±0.09</td>
<td>139.29</td>
<td>67.28±2.98</td>
<td>3.58±0.72</td>
<td>16.82±0.16</td>
</tr>
<tr>
<td>F4</td>
<td>262.2±9.6</td>
<td>0.615</td>
<td>-38.7</td>
<td>68.57±2.95</td>
<td>1.391±0.10</td>
<td>67.26</td>
<td>60.48±1.65</td>
<td>2.96±0.62</td>
<td>15.10±0.27</td>
</tr>
<tr>
<td>F5</td>
<td>287.3±12.4</td>
<td>1.000</td>
<td>-39.4</td>
<td>78.97±1.6</td>
<td>1.578±0.22</td>
<td>74.12</td>
<td>67.15±1.75</td>
<td>3.41±0.71</td>
<td>16.77±0.59</td>
</tr>
<tr>
<td>F6</td>
<td>222.0±11.2</td>
<td>0.313</td>
<td>-33.4</td>
<td>70.24±2.21</td>
<td>1.404±0.15</td>
<td>95.19</td>
<td>62.56±1.20</td>
<td>3.08±0.97</td>
<td>15.62±0.36</td>
</tr>
<tr>
<td>F7</td>
<td>128.4±18.1</td>
<td>0.244</td>
<td>-35.6</td>
<td>72.22±1.24</td>
<td>1.440±0.13</td>
<td>84.27</td>
<td>67.98±1.64</td>
<td>3.40±0.82</td>
<td>16.99±0.21</td>
</tr>
<tr>
<td>F8</td>
<td>111.5±8.5</td>
<td>0.277</td>
<td>-44.0</td>
<td>80.14±3.1</td>
<td>1.602±0.18</td>
<td>109.97</td>
<td>78.94±0.99</td>
<td>7.90±0.85</td>
<td>19.73±0.82</td>
</tr>
<tr>
<td>F9</td>
<td>152.9±5.7</td>
<td>0.269</td>
<td>-30.7</td>
<td>70.26±1.90</td>
<td>1.404±0.16</td>
<td>129.42</td>
<td>69.42±1.88</td>
<td>3.88±0.74</td>
<td>17.35±0.42</td>
</tr>
<tr>
<td>F10</td>
<td>238.7±7.9</td>
<td>0.541</td>
<td>-33.8</td>
<td>69.24±1.21</td>
<td>1.385±0.12</td>
<td>81.37</td>
<td>70.03±1.87</td>
<td>4.32±0.27</td>
<td>17.50±0.33</td>
</tr>
<tr>
<td>F11</td>
<td>260.8±11.4</td>
<td>0.902</td>
<td>-23.2</td>
<td>73.51±1.89</td>
<td>1.470±0.11</td>
<td>85.62</td>
<td>72.59±1.22</td>
<td>4.71±0.93</td>
<td>18.49±0.61</td>
</tr>
<tr>
<td>F12</td>
<td>109.2±10.8</td>
<td>0.420</td>
<td>-36.9</td>
<td>70.20±2.31</td>
<td>1.404±0.08</td>
<td>102.49</td>
<td>71.52±2.01</td>
<td>3.65±0.28</td>
<td>17.88±0.57</td>
</tr>
</tbody>
</table>

PDI: Polydispersity index

Dosage form design

Formulation MF8 was developed as transdermal gel (TG) by dispersion method and compared with control gel (CG). Carbopol 934P was soaked in distilled water (1% w/v) for 2 h in order to form its aqueous dispersion. The transdermal suspension (equivalent to 10 mg) and pure felodipine solution (10 mg) was separately incorporated in gel base to obtain two different compositions [Table 1]. The pH of gel(s) was measured by digital pH meter (Hanna Instrument Ltd., Italy) and viscosity was determined by Brookfield viscometer R/S-CPS (Brookfield Engineering Laboratories, Inc., USA) using T-spindle S-93 at 20 rpm (22.36 × 10⁻⁷ g). Drug content was determined spectrophotometrically to assess content uniformity. In vivo skin permeation study with gels was conducted to determine the steady state flux (Jss) by using rat skin mounted over Franz diffusion cell. The study was performed for 24 h at 37°C ± 0.5°C.

Confocal laser scanning microscopy

The study was conducted using Wistar rats, divided into two groups namely control and treatment groups (n = 2/group). The animals were fasted overnight with free access to water. Subsequently the animals of the control group and treatment group were applied with 1 g of rhodamine loaded CG and transfersomal gel, respectively on the dorsal side of skin (area = 2 cm²), cleared of the hairs by shaving. After 6 h of the study one animal each from the treatment and control group was sacrificed by heart puncture and the dorsal skin was excised, washed and placed on aluminum foil. The skin samples were then sliced to 10-15 µm (Microtome India, Spence, New Delhi, India). Rest of the two animals were sacrificed after 12 h and processed as described. The prepared and fixed skin was subjected to confocal laser scanning microscopy (CLSM) studies. The slide was irradiated with laser of 540 nm and observed at magnification of ×100. The micrographs obtained were recorded to analyze the corresponding depth transversed by rhodamine loaded TG and CG respectively, in 6 and 12 h.

Pharmacokinetic studies

Study design

Wistar rats (100-150 g) were divided into two groups (n = 4/group) and designated as control and test group. The rats were individually housed, fasted overnight. Water was allowed ad libitum. Animals in control group were administered an oral suspension of felodipine (0.15 mg/kg) with 2 ml of water and test group animals were treated with TG of equivalent dose size.

Sample collection, preparation and chromatographic condition

Blood sample (0.5 ml) was collected using a 26-Gx 11/4 in (0.7 mm × 30 mm) 2 ml syringe (Dispo Van, HMD, Faridabad, Haryana, India) at 0, 1, 2, 4, 6, 12, 24 and 48 h. The collected samples were stored in 5 ml heparinized plastic plasma tubes (150 USP Units of sodium heparin spray coated, 13 mm × 7.5 mm, with BD hemogaurd closer, BD and Co. NJ, and USA). The collected samples were centrifuged at 3500 rpm for 15 min to separate the plasma and the separated plasma was transferred into micro centrifuge tubes (Sigma Aldrich, USA).
to obtain cellular debris free plasma by further centrifugation at 12,000 rpm. The supernatant was collected and stored at −20°C until analysis. The felodipine plasma level in the supernatant was determined by a validated HPLC assay method\[10\] using HPLC (Waters, 2695, USA) with variable wavelength UV detector (Aguity T UV Ch A) operated at 237 nm. Column used was C18 (Water, BEH, C18) of dimension 150 mm × 4.6 mm and a flow rate of 0.8 ml/min was maintained for the mobile phase. The mobile phase was acetonitrile: Water (80:20 v/v) filtered through 0.45 μ nylon membrane filter and degassed in sonicator for 15 min.

**Sample analysis**

Calibration standards in plasma were prepared by spiking 100 μL blank rat plasma with a stock solution of felodipine. Stock solutions of felodipine (100-μg/ml) and nifedipine (10-μg/ml) as internal standard were prepared in 30% v/v methanol. The secondary standard solutions were prepared by diluting the stock solutions with 30% v/v methanol. The working standard solutions were prepared by diluting the secondary solutions with blank plasma. Eight calibration standards of final concentration of 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 ng/ml were prepared. 50 μL nifedipine solution was added to each plasma sample as an internal standard and analyzed by HPLC.

**Pharmacokinetic analysis**

The pharmacokinetic parameters (Cmax, Tmax, area under the curve [AUC0-∞], K, Kα, t1/2, t½) were estimated from the plasma concentration versus time profile. The relative bioavailability (Fr) of felodipine was calculated as AUC0-α (transdermal) in comparison to AUC0-∞ (oral). The unpaired Student’s t-test was used to test the differences between the calculated parameters. Statistical differences yielding P > 0.05 were considered as significant.

**RESULT AND DISCUSSION**

**Optimization of process variables**

**Optimization of vortexing and sonication time**

The process of vortexing subjects the sample to high shear forces that cause repeated cavitations in the lipid lamellae resulting in a reduction of vesicle size. Results indicated that 15, 30, 45 and 60 min of vortexing resulted entrapment efficiencies of 61.1 ± 1.22, 79.44 ± 1.47, 75.55 ± 1.09 and 75.0 ± 0.98 respectively. The results suggested that when the composition was vortexed at slow rate and for longer duration, higher EE and smaller vesicle size was occurred. It is attributed to slow annealing of vesicles that allowed larger contact time between the vesicle bilayer and the felodipine led to higher entrapment.\[13\]

On the contrary, application of excessive vortexing exposed the vesicles to high shear stress that reduced the vesicle size greatly and led to generation of rigid vesicles of smaller size with lower EE. On the basis of results obtained, a vortexing time of 30 min at which the EE was found to be maximum (79.44% ± 1.47%) was optimized for the preparation of transfersomes by vortexing sonication method. Sonication provides a rearrangement of the initial multilamellar organization into smaller vesicles that necessitates sonication of hydrated vesicles. The transfersomal suspensions were sonicated for 15 and 30 min and results indicated that sonication time of 15 min yielded vesicles of 2184 nm, whereas a sonication time of 30 min downsized the vesicle to 102.3 nm. Thus, a sonication time of 30 min was used.

**Optimization of loading drug concentration**

On increasing the felodipine concentration from 0.25% to 1.0% w/w there was a significant increase in the drug entrapment (P < 0.02), but at concentration beyond 1.0% w/w, considerable decrease in the drug entrapment was documented. The increase in the drug entrapment observed at high drug loading concentration can be attributed to the drug lipid ratio. It has been suggested\[17\] that when the drug:lipid ratio is high the loading efficiency of the vesicular system is low because the amount of drug exceeds the vesicular loading capacity (overloading). This overloading may damage the vesicular membrane leading to drug leakage and lowering the final drug entrapment, hence a drug concentration of 1.0% w/w was selected.

**Pharmacokinetic analysis**

The pharmacokinetic parameters (Cmax, T max, area under the curve [AUC0-∞], K, Kα, t1/2, t½) were estimated from the plasma concentration versus time profile. The relative bioavailability (Fr) of felodipine was calculated as AUC0-α (transdermal) in comparison to AUC0-∞ (oral). The unpaired Student’s t-test was used to test the differences between the calculated parameters. Statistical differences yielding P > 0.05 were considered as significant.

**Effect on vesicle size polydispersity index and zeta potential**

The size of soya lecithin vesicles ranged from 82.9 ± 5.7 nm to 157.8 ± 7.2 nm while that of egg lecithin vesicles ranged from 109.3 ± 10.8 nm to 287.3 ± 12.4 nm [Table 2]. Comparative results indicate significant differences in the size of transfersomes composed of different lipids (P < 0.02). This is in contrary to the findings of vesicle size analysis reported by El Maghraby et al.\[18\] and Jain et al.\[19\] who suggested similarity in sizes of vesicles prepared by same method irrespective of lipid type. The variation observed in the vesicle size in our case may be attributed to the differences in the intrinsic chemical compositions and packing behavior of the lipids.

The effect of edge activator type suggested that the nature of the surfactant has a profound impact on the size of vesicles. Again the results are contrary to the findings of Duangit et al.\[20\] who suggested that vesicle size is unaffected by lipid composition and surfactant. Our results indicated that use of edge activator with lower hydrophilic lipophilic balance HLB resulted in vesicles with smaller size. Hence, vesicles made with span 80 were smaller in size than those obtained with hydrophilic edge activator (Tween-80). The inverse relationship between vesicle size and HLB of edge activator can be attributed to a decrease in surface energy with increasing hydrophobicity thus resulting
in a smaller vesicle. These results are consistent with reports of Yoshioka et al.\[21\] and El Zaafarany et al.\[21\]

The PDI value varied from 0.228 to 0.902 revealed that except formulation F7, F8 and F9, rest of the formulations were heterogeneous. Generally PDI values lower than 0.3 is preferred for homogeneity in the vesicle size of dispersive system. The zeta potential for all the vesicular formulations ranged between $-23.2\text{ mV}$ and $-44.0\text{ mV}$, while the dividing line between stable and unstable suspensions is generally taken at either $+30\text{ mV}$ or $-30\text{ mV}$ for good physical stability hence all the formulation showed acceptable stability except F11. The net negative charge observed was due to the lipid composition in the formulations. The isoelectric point of soya and egg lecithin is 6.2, while the pH of hydration media was 6.8 that was higher than the isoelectric point, so the vesicles carried a net negative charge. Felodipine and Tween-80 and span 80 are nonionic hence did not contribute in charge development.

**Effect on entrapment and loading efficiency**

The entrapment and loading efficiency narrowly ranged between 69.24 ± 1.21 – 80.14 ± 3.1 and 1.602 ± 0.18 – 1.385 ± 0.12 respectively. Maximum drug entrapment was observed with soya lecithin and span 80 containing formulation having lipid:edge activator in 95:5 ratio while minimum values were observed with vesicle composed of egg lecithin [Table 2]. The effect can be ascribed to intrinsic entrapment ability of the lipid, nature of the drug and their interaction with the edge activator. According to various literature reports, the EE of egg lecithin vesicles is higher than soya lecithin vesicles which is specifically applicable to hydrophobic drugs.\[23\] As felodipine is a lipophilic drug, the interaction of lipids with edge activators can be considered as a key factor for the difference in the EE. The decreased EE of egg lecithin vesicles as compared to soya lecithin agrees with the work of Memoli et al.\[24\] It also describes that for a given surfactant concentration soya lecithin vesicles is higher than egg lecithin vesicles which is specifically applicable to hydrophilic drugs.\[23\]

The drug entrapment was also affected by the type of edge activator. Vesicles containing span 80 provided higher EE as compared to the vesicles of Tween-80 [Table 2]. The difference can be explained on the basis of HLB values of 4.3 and 16.7, respectively. Lower the HLB value higher will be the lipophilicity of the edge activator that will facilitate its interaction with the lipid bilayer leading to growth of vesicle and increased drug entrapment. The variation in the ratio of lipid:edge activator also indicated marked differences in EE. The formulations containing lipids at higher level and edge activator at lower level (98:2), exhibited low EE irrespective of the type of lipid used. On the other hand, on increasing the amount of edge activator (95:5), the EE increased significantly ($P < 0.002$). Further increase in the edge activator concentration to 10% w/w led to a decrease in EE [Table 2]. The results obtained are consistent with the results of Ahad et al.\[25\] and can be justified by three-step model of surfactant/edge activator interaction with the lipid bilayer. Initially at low concentration, surfactant monomers are incorporated within the lipid bilayer according to partition equilibrium between aqueous and lipid phase but the growth of the vesicle is less that restricts the drug entrapment. While increasing surfactant concentration induced vesicle growth and imparted fluidity to membrane bilayer resulting in increased EE. Further increase in the edge activator concentration results in phospholipids solubilization into mixed micelles\[26\] that coexist with surfactant-saturated vesicles and pore generation\[27\] within the vesicles. The mixed micelles formed are rigid structure with low EE. Additionally, the pore generation due to high concentration of edge activator leads to leakage of the vesicles resulting in high drug loss hence low drug entrapment.

**Effect on deformability index**

The membrane deformability is a result of combination of at least two different amphiphiles (lipid + edge activator) that have totally different packing characteristics into a single bilayer. The deformability index of preliminary formulations ranged widely between 67.26 and 139.29. Deformability index of soya lecithin vesicles was higher than egg lecithin vesicles that might be a result of higher interaction ability of soya lecithin with the surfactant/edge activator as compared to egg lecithin. As a well-documented fact, the deformability of transfersomes is due to the intercalation of edge activator within the membrane bilayer, which imparts flexibility and ability to form edges so that they can deform. However, the phenomena is practically applicable up to a certain limit of surfactant concentration above which mixed micelles are formed that are rigid vesicles with less/no deformability. Deformability was also affected by the type of edge activator as evident by the results [Table 2]. It was higher in Tween-80 containing vesicles rather than span 80 containing vesicles. This may be attributed to the hydrophobic property of Tween-80 by virtue of which transient hydrophilic holes were generated that increased the amphiphilic property of membrane bilayer responsible for fluidity. Other parameter that affects the deformability is the chemical structure of the edge activator. Edge activators with high bulky carbon chain substitution have lesser deformability as shown by span 80. Flexible and nonbulky carbon chain substitution imparts higher fluidity to the membrane bilayer\[28\] as observed with Tween-80. The dependency of deformability on lipid:edge activator concentration showed a definite pattern. At a ratio of 98:2 the vesicles were less deformable as compared to the 95:5 ratio that further increased as the edge activator concentration (90:10) increased, but committantly the EE got lowered either due to the formation of pores within the vesicles or generation of mixed micelles.

**Ex vivo skin permeation**

The amount of felodipine permeated though rat skin [Figure 1a] from F1-F12 was found in the range of 60.48 ± 1.65 (F4) to 78.94 ± 0.99 (F8) in 24 h. The experimental flux of all the formulations was compared with the theoretical target flux (24.28 μg/cm²/h) to screen out formulations that were considerably closer to the target flux. The flux value [Table 2] varied with the minimum value of 15.10 ± 0.27 μg/cm²/h to
the highest value of 19.73 ± 0.82 μg/cm²/h correlatable to the formulation F4 and F8 respectively. The results also indicated that felodipine permeation rates were lower for egg lecithin vesicles than the soya lecithin vesicles that satisfied earlier description made in the context of vesicle size, drug entrapment and relative deformability that were not optimal with egg lecithin vesicles.

The skin deposition ranged between 2.98 ± 0.62% and 7.90 ± 0.85%. The deposition of the drug within the skin was highly variable that may be attributed to the differences in the physical properties of transferosomes such as vesicle size and deformability. Maximum drug deposited in the skin was 7.90% ± 0.85% for F8 that may be attributed to the ability of transferosomes to permeate the skin under nonocclusive conditions and their interaction with the lipid. The variation in lipid:edge activator ratio also documented potential effect on the drug release rates. When the edge activator concentration was very low (98:2) the transdermal flux was low. On increasing to 5% w/w (95:5) maximal transdermal flux was recorded and at 10% w/w (90:10), flux decreased due to formation of mixed micelles that exhibit low EE with no deformability or it may be a consequence of pore formation that induces higher drug leakage from the vesicles. The pattern was observed with all the formulations irrespective of the type and nature of lipid or edge activator but the extent of permeation varied in accordance of type and nature of lipid or edge activator. Mixed micellar system offers small resistance to permeation as transfersomes but fails to elicit higher flux due to their inability to respond the transepidermal hydration gradient so they are unable to open the tight junctions between the cells to cross stratum corneum.[29] The characterization of preliminary formulations concluded that the transferosomal formulations containing lipid and edge activator in ratio of 95:5 have favorable properties in terms of physical and performance characteristics than other ratios of 98:2 and 90:10 irrespective of type of lipid or edge activator used. However, cumulative drug permeation, permeability coefficient and flux value of vesicles composed with 95:5 ratio was too low when compared to the target flux. This may be attributed to large vesicle sizes with higher PDI values due to improper hydration of the lipid by vortexing sonication method. This method was employed for screening purpose due to its cost effectiveness, feasibility and rapidness in results. In order to reduce vesicle size the formulations made with 95:5 ratio were subjected to method manipulation to improve the product.

**Formulation development**

The selected formulations (F2, F5, F8 and F11) were modified by preparing them by rotary evaporation sonication method and coded as MF2, MF5, MF8 and MF11 respectively and compared with original formulations. These formulations showed significant ($P < 0.002$) improvement in the vesicle size (75 ± 3.7 – 123.3 ± 4.2 nm) and PDI (0.228-0.285), while insignificant increase in zeta potential was observed in comparison to original formulations [Table 3]. The improvisation in the vesicle size is a result of proper hydration of the thin lipid film formed by rotary evaporation of the solvent resulting in large surface area exposed for hydration that conferred easy hydration of the lipid film and homogenization of vesicles. The zeta potential changed insignificantly ($P > 0.002$) as it depends on the formulation composition that remained unaltered. Similarly, marked improvement in both entrapment and loading efficiencies was recorded in comparison to the original formulation. This was the result of cumulation of numerous factors like increased contact points due to large surface area of the lipid film and slow annealing of vesicles for larger duration. Deformability index that depends on the proper intercalation of edge activators within the lipid improved on the method manipulation.
On similar lines, ex vivo permeation studies indicated significant improvement ($P < 0.002$) in the felodipine permeation and the transdermal flux of the modified formulations [Figure 1b]. Among the modified formulations, maximum permeation was achieved by formulation MF8 (94.91%) with a transdermal flux of 23.72 μg/cm²/h, while the minimum permeation was recorded for MF5 (76.82%, flux = 19.22 μg/cm²/h). The increased permeation and flux is a result of a combination of factors such as an increase in EE, deformability index and decreased vesicle size. Efficient entrapment confers the vesicle to carry a higher amount of drug though the skin while the smaller size of the vesicle synergizes the enhanced drug permeation as observed with the MF8. However, the deformability of MF8 was lower than MF2 that resulted from differences in the nature of the edge activators as explained in earlier sections. In spite of lower deformability index, MF8 was able to achieve target flux that compensated low deformability, was adjudged as best formulation and characterized for vesicle shape and thermal behavior by DSC.

**Vesicle shape**

Transmission electron micrograph of MF8 showed spherical vesicles with lesser number of oval vesicles [Figure 2]. The structural appearance confirmed a lighter core surrounded by denser outline that sealed the core perfectly. When a thin film of lipid gets hydrated it tends to form enclosed vesicular structure with shape ranges from spherical to oval in order to attain thermodynamic stabilization by reducing total free energy of the system. No disruptions of vesicular structure confirmed vesicle integrity, even after application of various mechanical stresses such as sonication and extrusion.

**Differential scanning calorimetry**

Differential scanning calorimetry of pure felodipine exhibited a sharp melting endothermic peak at 148.2°C [Figure 3a] that was ascribed to drug melting. DSC of pure soya lecithin exhibited endothermic peaks at 43.2°C, 104.1°C and 205.9°C. In the thermogram, the state below the advent of first endothermic peak at 43.2°C of soya lecithin defines more ordered lamellar gel phase in which the acyl chains remain closely packed and in trans configuration.[31] The peak observed at 43.2°C [Figure 3b] indicated the transition temperature (Tm) at which the gel phase got converted to liquid crystalline state and above this temperature (Tm) trans/gauche rotational isomerism takes place along the acyl chain that expands laterally. As a result, the thickness and density of bilayer decreases and lipid becomes more fluid.[31]

Transition temperature (Tm) is important, while developing vesicles as above Tm, vesicle formation is easy so in the present research hydration temperature of 45°C was selected to obtain liquid crystalline vesicles. The peak observed at 209.5°C may be attributed to the isotropic liquid phase of the lipid. The presence of bound water in soya lecithin that could not be completely eliminated at normal storage conditions raised some difficulties in the thermal analysis and may explain some disturbances in thermogram hence an exothermic peak at 251ºC is probably associated with the release of the bound water and is irreversible once the sample is heated.[32] The exothermic nature of the peak suggests that crystallization may occur as the water is released. Thermogram of lecithin + span 80 showed mild reduction [Figure 3c] in Tm (40.8°C) and broadening of the peak due to the presence low melting span. Span being lipophilic had higher affinity for the lipid thus producing considerable effects on Tm. DSC thermogram of the physical mixture of felodipine + soya lecithin + edge activator [Figure 3d] showed single broad peak at 119.4°C that is suggestive of a homogenous mixture of all the ingredients. Interestingly, similar compound peak was observed for MF8 [Figure 3e] that indicates solubilization and dilution of the drug in the molten lipid at higher temperature during the

**Table 3: Comparative results of different characterization parameters of selected versus modified formulations**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Vesicle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>Entrapment efficiency (%)</th>
<th>Loading efficiency (%)</th>
<th>Deformability index</th>
<th>Percent cumulative drug permeated</th>
<th>Transdermal flux (μg/cm²/h)</th>
<th>Skin deposition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2</td>
<td>118.4±8.9</td>
<td>0.458</td>
<td>–39.7</td>
<td>79.44±2.3</td>
<td>1.58±0.17</td>
<td>121.71</td>
<td>70.06±2.37</td>
<td>17.63±0.41</td>
<td>5.40</td>
</tr>
<tr>
<td>MF2</td>
<td>94.71±4.7</td>
<td>0.285</td>
<td>–42.5</td>
<td>84.16±1.72</td>
<td>1.683±0.12</td>
<td>127.59</td>
<td>88.5±1.65</td>
<td>21.63±0.52</td>
<td>6.97</td>
</tr>
<tr>
<td>F5</td>
<td>287.3±9.5</td>
<td>1.00</td>
<td>–39.4</td>
<td>78.97±1.6</td>
<td>1.578±0.22</td>
<td>74.12</td>
<td>67.15±1.92</td>
<td>16.77±0.59</td>
<td>3.41</td>
</tr>
<tr>
<td>MF5</td>
<td>123.3±6.2</td>
<td>0.228</td>
<td>–40.9</td>
<td>83.21±1.24</td>
<td>1.664±0.23</td>
<td>87.54</td>
<td>76.82±1.20</td>
<td>19.20±0.36</td>
<td>4.21</td>
</tr>
<tr>
<td>F8</td>
<td>111.5±5.6</td>
<td>0.277</td>
<td>–44.0</td>
<td>80.14±3.10</td>
<td>1.602±0.18</td>
<td>109.97</td>
<td>78.94±3.76</td>
<td>19.73±0.82</td>
<td>7.90</td>
</tr>
<tr>
<td>MF8</td>
<td>75.71±5.4</td>
<td>0.255</td>
<td>–49.9</td>
<td>85.14±1.39</td>
<td>1.702±0.17</td>
<td>119.68</td>
<td>94.91±1.88</td>
<td>23.72±0.64</td>
<td>8.15</td>
</tr>
<tr>
<td>F11</td>
<td>260.8±7.6</td>
<td>0.902</td>
<td>–23.2</td>
<td>73.51±1.89</td>
<td>1.470±0.11</td>
<td>85.62</td>
<td>73.99±3.21</td>
<td>18.49±0.61</td>
<td>4.71</td>
</tr>
<tr>
<td>MF11</td>
<td>102.3±4.9</td>
<td>0.288</td>
<td>–33.7</td>
<td>78.24±1.92</td>
<td>1.564±0.39</td>
<td>99.86</td>
<td>82.57±1.64</td>
<td>20.64±0.27</td>
<td>4.92</td>
</tr>
</tbody>
</table>

PDI: Polydispersity index

Figure 2: Transmission electron micrograph of MF8

Figure 3a: Differential scanning calorimetry of pure felodipine exhibited a sharp melting endothermic peak at 148.2°C; Figure 3b: The peak observed at 43.2°C in the thermogram of soya lecithin defines more ordered lamellar gel phase; Figure 3c: Thermogram of lecithin + span 80 showed mild reduction in Tm (40.8°C) and broadening of the peak due to presence low melting span; Figure 3d: Single broad peak at 119.4°C that is suggestive of a homogenous mixture of all the ingredients; Figure 3e: Compound peak observed for MF8 that indicates solubilization and dilution of the drug in the molten lipid at higher temperature during the
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heating of the sample.[33] Felodipine being lipophilic drug was molecularly dispersed within the lipid matrix.

Stability
Temperature dependent study of MF8 indicated that the rate of drug loss at 4°C was 0.071/day and % drug retained was 93.25% after 90 days compared to 25°C where the rate of drug loss was 0.154/day and % drug retained was 85.23%. Increased drug leakage at higher temperature may be associated with the gel to liquid transition of the vesicle. At lower temperature, the vesicles remain in a gel state and exhibits good packing characteristic hence the loss of drug was low. As the temperature increased the gel state vesicles got converted to liquid crystalline state that rendered them more flexible due to loosening of membrane packing making them leakier. The effect of storage time on vesicle size was also evaluated and an increase from 75.71 nm to 155.2 nm in 90 days, was observed. This may be attributed to aggregation and fusion of small vesicles that led to the enlargement of the vesicle size.[34] A simple model for aggregation describes that the isolated vesicle initially tends to flocculate resulting in increment in size. However, flocculation of vesicles is a reversible process, and they can be redispersed to their original vesicular state by mild shearing. Coagulation and fusion that may occur in later stage are irreversible and lead to permanent increment in vesicle size.[35] This behavior was observed for MF8 when stored for longer duration (90 days) in suspension state. In order to delay coalescence the transferosomal suspension can be designed as gel wherein the gelling agent can provide a physical barrier to coalescence of vesicles. Thus the development of transferosomal gel was undertaken.

Dosage form design
An effective transdermal dosage form design should fulfill several demanding expectations in respect to feasibility, stability, sensitivity, therapeutic elegance as well as patient compliance. MF8 was developed as gel (coded as [TFG]), characterized for various

Figure 3: DSC thermograms of (a) felodipine, (b) soya lecithin (c) soya lecithin and span 80 (d) felodipine, soya lecithin and span 80 (e) MF8
parameters such as pH, viscosity, drug content, ex vivo drug permeation and compared with the control transdermal gel (CG).

**pH, viscosity and drug content**
Physical examination of gels revealed them as transparent products with a faint smell of raw materials used. The pH of TFG and CG was 6.9 ± 0.16 and 6.8 ± 0.21 respectively, that was within the specified range of physiological skin pH of 3-9, and rendered the formulations safe and nonirritating to the skin. Viscosity of TFG and CG was found to be 37584.2 ± 7.23 cp and 35945.3 ± 5.97cp that will facilitate the retention of gel on the skin as well as provide easy extrusion of the dosage form from the package. Carbopol 934P was present in both the gels in the same strength, and equal volume has been maintained, still the viscosity of TFG was found to be slightly higher than the CG. This may be attributed to the presence of lipid vesicles in TFG that increased viscosity of the gel. Drug content of TFG and CG was determined and found to be 97.12 ± 1.76 and 98.57 ± 1.91% indicating homogenous dispersion of drug in the gel matrix.

**Ex vivo skin permeation**
The maximal permeation from TFG was 2.6 times higher (93.91 ± 1.88%) as compared to CG (35.97 ± 1.31%) at 24 h [Figure 1c]. The transdermal flux achieved by the CG was 2.7 times lower (8.90 ± 0.09 μg/cm²/h) than the target flux (24.28 μg/cm²/h) and 2.6 times lower than TFG (23.40 ± 0.19 μg/cm²/h). Low permeation of the drug though CG might have resulted from poor aqueous solubility, which in spite the presence of permeation enhancers and hydrophilic environment, was unable to generate sufficient concentration gradient to permeate across the skin. On the other hand, increase in the drug permeation of TFG was consequence of increased association of drugs within lipid bilayers that increased partitioning of vesicles into the stratum corneum and ultraflexibility that permitted rapid permeation of intact vesicles. Different kinetic equations were applied to for interpreting the release kinetics of drug from the gels. TFG and CG obeyed zero order kinetics with correlation coefficient value of 0.999 and 0.975 respectively. The value of diffusion exponent ‘n’ dictated the mechanism of drug release as Case-II transport mechanism as the values of diffusion exponent (n) was 1.125 and 1.272 respectively.

**Confocal laser scanning microscopy**
The study was focused on determining the depth of penetration using dermatomed rat skin, and also for demonstrating the possible mechanism of permeation though the skin. The thickness of rat stratum corneum is 18 μm that is close to the human stratum corneum 17-20 μm. On viewing CLS micrographs after 6 h of application of TFG [Figure 4], the fluorescence in the stratum corneum region was very low as compared to CG. This can be attributed to the ultraflexibility of the transfersomes that facilitated intact permeation though stratum corneum. However, a faint fluorescence in this region characterized by the interaction of stratum corneum lipids with the vesicles is suggestive of permeation enhancing effect by lipid system. On the other hand, higher fluorescence intensity with CG indicated confinement of the tracer predominantly in the stratum corneum. The depth of penetration of tracer increased gradually for both TFG and CG, but the fluorescence intensity for TFG was higher than CG in viable epidermal region. The fluorescence intensity of CG started fading beyond the depth of 38.09 μm while in case of TFG the intensity was maintained till the depth of 109.6 μm. Beyond this intensity got decreased but fluorescence was detectable till 165.6 μm that signifies penetration of tracer to upper dermal region via vesicular system. In comparison to TFG, CG remained confined on the upper region of a viable epidermis with few traces of fluorescence observed to the maximum depth of 49.2 μm. These results confirm deep and fast permeation behavior via TFG that can improvise pharmacokinetic behavior of felodipine.
Pharmacokinetics
The plasma concentration-time profile [Figure 5] showed that TFG exhibited higher plasma drug concentration at all-time points than CG. The rapid appearance of drug in plasma is attributed to the high permeation ability of TG when applied under nonoccluded conditions. The peak plasma concentration (C_{max}) displayed by TFG was 8.05 ± 0.42 ng/ml while control oral formulation attained 2.31 ± 0.47 ng/ml in 6.0 ± 1.9 and 4.85 ± 2.3 h (t_{max}) respectively.

Considering the data beyond the C_{max}, followed first order kinetics, the elimination rate (Ke), was insignificantly different (P > 0.05) for TFG and oral control formulation (0.043 ± 0.03 and 0.0602 ± 0.02 h^{-1} respectively). The absorption rate constant (Ka) was 0.391 ± 0.04 for TFG and 0.267 ± 0.02 h^{-1} for oral control formulation. The higher absorption rate of TFG was a result of the fast permeation ability of ultradeformable vesicular system, which made felodipine rapidly available for absorption. Higher absorption coupled with lower t_{max} is expected to improve bioavailability that was verified by AUC determination. AUC_{0-\infty} that directly reflects bioavailability was 45.27 ± 6.34 ng.h/ml and 162.26 ± 4.97 ng.h/ml for oral control formulation and TFG. Consequently, the relative bioavailability (Fr) of TFG was 358.42%. The increase in bioavailability may be attributed to the avoidance of hepatic first pass metabolism of felodipine and supports the ex vivo permeation data that suggested rapid drug permeation with negligible lag time.

CONCLUSION
The outcomes of the present analysis established transfersomes as highly effective transdermal carrier for passive transport of lipophilic and practically water insoluble felodipine. The research also demonstrates the effects of type of lipid and edge activators on the physical and performance characteristic of transfersomes. The combination of soya lecithin and span provided most optimal results and carried maximal drug across the skin. The results of in vivo pharmacokinetic studies and confocal laser scanning microscopic studies depicted rapid permeation of felodipine loaded transfersome to achieve high drug plasma levels with enhanced bioavailability. The research concludes that transfersomes rapidly and noninvasively permeated across the skin to obtain rapid therapeutic drug levels in plasma at lower dose and successfully avoided the hepatic first pass metabolism of felodipine.

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Figure 5: Plasma concentration versus time profile of TG and control oral formulation.