MATERIALS & METHODS
4. MATERIALS AND METHODS

4.1 Materials

The important materials employed in the research work are given in Table 11.

Table 11: List of important chemicals along with source.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Material name</th>
<th>Source of acquisition of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Talinolol</td>
<td>Gift sample from Pioneer Agro Industries, Thane, India</td>
</tr>
<tr>
<td>2.</td>
<td>Tizanidine</td>
<td>Gift sample from Panacea Biotec, Baddi, India</td>
</tr>
<tr>
<td>4.</td>
<td>Labrafac PG</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>5.</td>
<td>Plurol Oleique 479</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>6.</td>
<td>Lauroglycol 90</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>7.</td>
<td>Labrafil M 1944 CS</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>8.</td>
<td>Labrafil M 2125 CS</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>9.</td>
<td>Labrafac L WL-1349</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>10.</td>
<td>Transcutol</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>11.</td>
<td>Labrasol</td>
<td>Gattefosse India Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>12.</td>
<td>Cremophor EL/RH40</td>
<td>Abitec Corporation, Janesville, USA</td>
</tr>
<tr>
<td>13.</td>
<td>Triacetin</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>14.</td>
<td>Miglyol 812</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>15.</td>
<td>Castor oil</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>16.</td>
<td>Isopropyl myristate</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>17.</td>
<td>Soyabean oil</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>18.</td>
<td>Pluronic F68</td>
<td>HiMedia Laboratories Pvt. Ltd., Mumbai, India</td>
</tr>
<tr>
<td>19.</td>
<td>Olive oil</td>
<td>Loba Chemie Pvt. Ltd., Mumbai, India</td>
</tr>
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</table>

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<table>
<thead>
<tr>
<th>No.</th>
<th>Material</th>
<th>Supplier</th>
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<tbody>
<tr>
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<td>Polyethylene glycol 200</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>20</td>
<td>Polyethylene glycol 400</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>21</td>
<td>Span 20</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>22</td>
<td>Span 80</td>
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<td>Oleic acid</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>24</td>
<td>Propylene glycol</td>
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<td>25</td>
<td>Tween 20</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>28</td>
<td>Toluene</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>29</td>
<td>Acetone</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>30</td>
<td>Ammonia</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>31</td>
<td>Hexane</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
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<td>32</td>
<td>Potassium dihydrogen orthophosphate (AR)</td>
<td>RFCL Ltd., New Delhi, India</td>
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<tr>
<td>33</td>
<td>Sodium dihydrogen phosphate</td>
<td>RFCL Ltd., New Delhi, India</td>
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<td>34</td>
<td>Sodium hydroxide pellets</td>
<td>Sisco Research Labs., Pvt. Ltd., Mumbai, India</td>
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<td>35</td>
<td>Isopropyl alcohol (AR)</td>
<td>Thermo Fisher Scientific Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>36</td>
<td>Diethyl ether</td>
<td>RFCL Limited, New Delhi, India</td>
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<td>37</td>
<td>Dichloromethane (AR)</td>
<td>Sisco Research Labs., Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>38</td>
<td>Hydrochloric acid</td>
<td>HiMedia Laboratories Pvt. Ltd., Mumbai, India</td>
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<td>39</td>
<td>Hydrogen peroxide (AR)</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
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<td>40</td>
<td>Sodium chloride (AR)</td>
<td>Central Drug House Pvt. Ltd., New Delhi, India</td>
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<td>41</td>
<td>Calcium chloride</td>
<td>Central Drug House Pvt. Ltd., New Delhi, India</td>
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<td>42</td>
<td>Potassium chloride</td>
<td>Central Drug House Pvt. Ltd., New Delhi, India</td>
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<tr>
<td>43</td>
<td>Magnesium sulphate</td>
<td>Central Drug House Pvt. Ltd., New Delhi, India</td>
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<tr>
<td>44</td>
<td>Glucose</td>
<td>Central Drug House Pvt. Ltd., New Delhi, India</td>
</tr>
</tbody>
</table>

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45. Formaldehyde Loba Chemie Pvt. Ltd., Mumbai, India
46. Octanol E-Merck Ltd., Mumbai, India
47. HPLC grade acetonitrile E-Merck Ltd., Mumbai, India
33. HPLC grade methanol E-Merck Ltd., Mumbai, India
34. Sodium carboxy methyl cellulose Loba-Chemie, IndoAustranal, Mumbai, India
35. Heparin Gland Pharma Ltd., Mumbai, India
36. Double distilled water Milli-Q water purification system (Millipore, Billerica, MA)

4.2 Equipments

The important equipments used in the research work are given in Table 12.

Table 12: List of equipments used along with source.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Isothermal shaker</td>
<td>Remi, Mumbai, India</td>
</tr>
<tr>
<td>2.</td>
<td>RM 12 C micro centrifuge</td>
<td>Remi Instruments, Mumbai, India</td>
</tr>
<tr>
<td>3.</td>
<td>Digital weighing balance</td>
<td>Shimadzu, AUW220D, Japan</td>
</tr>
<tr>
<td>4.</td>
<td>HPLC LC-10 ADVP</td>
<td>Shimadzu, Japan</td>
</tr>
<tr>
<td>5.</td>
<td>C-18 RP (250 mm X 4.6 mm (i.d.), 5µm ODS-3) column</td>
<td>Inertsil, Japan</td>
</tr>
<tr>
<td>6.</td>
<td>HPLC Waters 2996 PDA</td>
<td>Waters, Milford, USA</td>
</tr>
<tr>
<td>7.</td>
<td>Dialysis membrane (MWCO 12-14 kD)</td>
<td>HiMedia Laboratories Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>8.</td>
<td>pH meter (L 1612)</td>
<td>Elico Pvt. Ltd., India</td>
</tr>
<tr>
<td>9.</td>
<td>Stability chamber (Humidity cum photo-stability chamber)</td>
<td>Thermolab Sciences Equipment Pvt. Ltd., Thane, India</td>
</tr>
<tr>
<td>10.</td>
<td>Brookfield DV-II+ pro Viscometer</td>
<td>Brookfield Engineering Laboratories, USA</td>
</tr>
<tr>
<td>11.</td>
<td>Digital conductivity meter</td>
<td>PICO Labs, India</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Melting point apparatus</td>
<td>Veego’s melting point apparatus</td>
</tr>
<tr>
<td>13</td>
<td>UV-Visible (Genesys 6) spectrophotometer</td>
<td>Thermospectronic, USA</td>
</tr>
<tr>
<td>14</td>
<td>Refractometer</td>
<td>Abbe, India</td>
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<tr>
<td>15</td>
<td>HPTLC - ATS4</td>
<td>Camag, Switzerland</td>
</tr>
<tr>
<td>16</td>
<td>Particle size analyzer (Meta Sizer 2000)</td>
<td>Malvern, Germany</td>
</tr>
<tr>
<td>17</td>
<td>LC-MS Q-TOF micro</td>
<td>Waters, UK</td>
</tr>
<tr>
<td>18</td>
<td>Zetasizer 2000 HS</td>
<td>Malvern Instruments Ltd., Worcestershire, UK</td>
</tr>
<tr>
<td>19</td>
<td>UV-1601, UV-Visible double beam spectrophotometer</td>
<td>Shimadzu, Tokyo, Japan</td>
</tr>
<tr>
<td>20</td>
<td>0.45 and 0.22 μ Nylon membrane filters</td>
<td>Millipore, Bangalore, India</td>
</tr>
<tr>
<td>21</td>
<td>TEM, H7500</td>
<td>Hitachi, Japan</td>
</tr>
<tr>
<td>22</td>
<td>FT-IR spectrum RX1 system</td>
<td>Perkin Elmer, USA</td>
</tr>
<tr>
<td>23</td>
<td>Magnetic stirrer</td>
<td>REMI Equipments, Mumbai, India</td>
</tr>
<tr>
<td>24</td>
<td>NMR spectrometer (II 400)</td>
<td>Bruker Avance, Switzerland</td>
</tr>
<tr>
<td>25</td>
<td>Vortex mixer</td>
<td>REMI Equipments, Mumbai, India</td>
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<tr>
<td>26</td>
<td>Dissolution apparatus (DISSO 2000)</td>
<td>LABINDIA Instruments Pvt. Ltd., Mumbai, India</td>
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<tr>
<td>27</td>
<td>DSC apparatus Q20</td>
<td>TA Instruments, USA</td>
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<tr>
<td>28</td>
<td>Vacuum oven</td>
<td>Narang Scientific Works, New Delhi, India</td>
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<tr>
<td>29</td>
<td>Vacuum pump</td>
<td>Crompton Parkinsons Ltd., India</td>
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<tr>
<td>30</td>
<td>Bath sonicator (Misonix sonicator S-3000)</td>
<td>Misonix Inc., USA</td>
</tr>
<tr>
<td>31</td>
<td>Refrigerator</td>
<td>LG, India</td>
</tr>
</tbody>
</table>

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4.3 Methods

4.3.1 Identification and characterization of drug

Drug samples of talinolol and tizanidine were characterized for their physiochemical properties such as ultra-violet spectroscopy, infra-red spectroscopy, nuclear magnetic resonance (NMR) analysis, differential scanning calorimetry (DSC), melting point and particle size analysis and matched with those reported in the literature.

4.3.1.1 Ultra-Violet spectra of talinolol and tizanidine

Stock solutions (1 mg/ml) of talinolol and tizanidine were prepared in methanol: water (5:95) and water, respectively. The samples were diluted to obtain a concentration of 50 µg/ml each and scanned in the UV-visible spectrophotometer in the region of 200 to 400 nm to determine the λ_max.

4.3.1.2 Infrared Spectrum

The chemical structural intactness of the pure drug was revealed from the Fourier Transform Infrared (FT-IR) spectral data. FT-IR spectrum was recorded for both the drugs on FTIR spectrophotometer (Perkin Elmer EM 360, USA) using potassium bromide pellets.

4.3.1.3 NMR Spectrum

The sample for NMR was prepared by making the solution of drug in CDCl₃. The samples were filtered and then the magnetic field was applied. Depending on the different types of protons present in the sample, the spectrum was obtained.

4.3.1.4 Differential Scanning Calorimeter (DSC)

Drug sample was sealed in the standard aluminium pans and subjected to heating at a scanning rate of 10°C/min using dry nitrogen flow of 25ml/min. DSC thermogram of drugs was obtained in the temperature range of 50°C to 350°C.

4.3.1.5 Melting Point

Melting point for the drugs was determined using Veego melting point apparatus. The purity of the drug sample can be inferred from melting point, closer the practical value of the melting point to theoretical value, more pure is the compound.

4.3.1.6 Particle size Distribution

Particle size analysis was done for the drug candidates by Laser diffractometry using Malvern Meta Sizer 2000. The volume particle size distribution was performed by means of University Institute of Pharmaceutical Sciences, Panjab University.
a dry sampling system with a robust standard operating procedure (measurement time: 8 s, vibration feed rate: 25%, refractive index: 1.52, dispersive pressure: 4bas). The particle size distribution was described using the mass median diameter (d [0.5]), that is, 50% of the sample was smaller and the rest 50% was larger and the size was measured in micron.

4.3.2 Development of analytical method for talinolol and tizanidine

4.3.2.1 Preparation of standard plot by UV method

Standard plots of the drugs in the concentration range of 2–20 µg/ml were prepared in distilled water, hydro-alcoholic solution, methanol, 0.1 N HCl, phosphate buffer pH 6.8, pH 7.4 and Kreb’s Ringer phosphate buffer (KRPB), in triplicate. The $E_{1%}^\lambda$ was calculated from the regressed line obtained from a plot between concentration and absorbance at respective $\lambda_{max}$.

4.3.2.1.1 Preparation of standard plot in water/hydro-alcoholic solution

Standard plots of talinolol and tizanidine were prepared in methanol: water (5:95) and water, respectively, by dissolving 10 mg of drug in small volume of alcohol and finally volume was made to 100 ml with distilled water. Concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and these were analyzed at wavelength of 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E_{1%}^\lambda$ was calculated.

4.3.2.1.2 Preparation of standard plot in methanol

Standard plots of talinolol and tizanidine were developed by dissolving 10 mg of drug in methanol and the volume was made to 100 ml. Concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and these were analyzed at wavelength of 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E_{1%}^\lambda$ was calculated.

4.3.2.1.3 Preparation of standard plot in 0.1N HCl

Standard plots of talinolol and tizanidine were prepared by dissolving 10 mg of drug in 0.1N HCl and the volume was made to 100 ml with the 0.1N HCl. Concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and were analyzed at wavelength of 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E_{1%}^\lambda$ was calculated. For the preparation of 0.1N HCl, accurately measured 8.6 ml of 11.5N HCl, added to 800 ml of distilled water, mixed and then volume was made to 1000 ml with distilled water.

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4.3.2.1.4 Preparation of standard plot in phosphate buffer pH 6.8

Standard plots of talinolol and tizanidine were prepared in 0.2M phosphate buffer of pH 6.8 by dissolving 10 mg of drug in 5 ml of methanol and distilled water for talinolol and tizanidine, respectively. Finally, the volume was made to 100 ml with the buffer of pH 6.8. Concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and these were analyzed at wavelength of 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E'_{1%m}$ was calculated. For the preparation of phosphate buffer pH 6.8, accurately weighed 76.02 g of 0.2M tri-basic sodium orthophosphate was dissolved in 1000 ml of distilled water. Then 250 ml of 0.2M tri-basic sodium orthophosphate was added to 750 ml of 0.1N HCl prepared as above. The pH was adjusted to 6.8.

4.3.2.1.5 Preparation of standard plot in phosphate buffer pH 7.4

Standard plots of talinolol and tizanidine were prepared in phosphate buffer of pH 7.4, by dissolving 10 mg of drug in 5 ml of methanol and distilled water for talinolol and tizanidine, respectively. The volume was made to 100 ml with the buffer of pH 7.4. From this stock solution of drugs (100 µg/ml), concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and analyzed spectrophotometrically at 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E'_{1%m}$ was calculated. For the preparation of phosphate buffer pH 7.4, accurately weighed 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride was dissolved in 1000 ml of distilled water. The pH was adjusted to 7.4.

4.3.2.1.6 Preparation of standard plot in Kreb’s Ringer phosphate buffer (KRPB)

Standard plots of talinolol and tizanidine were prepared in KRPB by dissolving 10 mg of drug in 5 ml of methanol and distilled water, respectively. The volume was made to 100 ml with KRPB. Concentrations ranging from 2 to 20 µg/ml were prepared in triplicate and these were analyzed at wavelength of 242 and 320 nm for talinolol and tizanidine, respectively. The value of $E'_{1%m}$ was calculated. For the preparation of KRPB (pH 7.4), accurately weighed 0.67 g of sodium chloride, 0.034 g of potassium chloride, 0.059 g of magnesium sulphate, 0.011 g of calcium chloride, 0.234 g of sodium dihydrogen phosphate and 0.18 g of glucose was dissolved in 100 ml of distilled water.

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4.3.2.2 Development of validated RP-HPLC method for talinolol

RP-HPLC method was developed and validated for the estimation of talinolol. Developed method was used for the solubility estimation of talinolol in various solvents used in the formulation development of SEDDS/nanoemulsions.

4.3.2.2.1 Chromatographic conditions

The HPLC conditions as performed in the analytical method are as follows:

- **HPLC instrument:** HPLC equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10 AVP UV–visible detector. Data acquisition was performed on Spinichrom software.
- **Column:** Inertsil ODS-3 C-18 column (250 mm x 4.6 mm (i.d.), 5μm).
- **Mobile phase:** The mobile phase was composed of a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer (pH 4.4) in a ratio of 27:73 (v/v).
- **Flow rate:** 1.0 ml/min.
- **Temperature:** 40°C.
- **Injection volume:** 20 μl.
- **Detector:** UV detector, λ<sub>max</sub> 242 nm.
- **Diluent:** Mobile Phase.

For the preparation of talinolol standard stock solution (1 mg/ml); accurately weighed 100 mg of talinolol powder was transferred to a 100 ml volumetric flask, to this 50 ml of methanol was added and the mixture was sonicated for 30 s. The final volume was made up with triple distilled water and the resulting solution was vortexed for 1 min. After suitable dilution with mobile phase, a range of concentrations (2-100 μg/ml) were prepared and the resulting solutions were injected after filtration using 0.22 μm nylon membrane filters, in triplicate into the column.

4.3.2.2.2 Validation of the method (BP, 2003; Sinha and Ghai, 2011; USP, 2007)

4.3.2.2.2.1 Linearity and range

Calibration curve of talinolol was prepared in the concentration range of 2-100 μg/ml for the establishment of linearity. The peak area was plotted against the corresponding

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concentration to obtain the calibration graph and the plots were subjected to linear regression analysis.

4.3.2.2.2 Accuracy

The accuracy of the method was assessed by analyzing spiked talinolol concentration in standard solutions. Talinolol was spiked into three levels, corresponding to 2, 50 and 100 µg/ml. The percentage recovery of the added drug was then calculated using the linearity plot.

4.3.2.2.3 Precision

Repeatability studies were performed for the determination of intra-day and inter-day precision. Intra-day precision studies were performed by injecting four different concentrations of talinolol i.e. 2, 10, 50 and 100 µg/ml in hexaplicate on the same day. While for inter-day precision studies, these concentrations were injected in triplicate on six different days. Drug concentrations were calculated using the area obtained from the linearity plots and the results were expressed as percent R.S.D.

4.3.2.2.4 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 3.3 σ/S and 10 σ/S, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation (n=6). LOD and LOQ of sample were determined by injecting each concentration in hexaplicate.

4.3.2.2.5 Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (ICH, 2000). The method specificity and stability-indicating of the analytical method were evaluated by the ability of the chromatographic conditions to separate the major degradation products from talinolol and by determination of the purity of the drug peak in the presence of its degraded sample using a PDA detector. Peak purity of the talinolol in stressed samples was verified using the PDA detector in wavelength range of 200-400 nm. Specificity testing was done on Waters (Milford, USA) Delta 600 HPLC equipped with a 600 controller pump, 2996 PDA detector and a degasser module. Empower 2 Software was used for data acquisition and processing.

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4.3.2.2.6 Robustness

For robustness, experimental conditions were purposely altered and the resolution between talinolol and its degraded products was evaluated. For this study the flow rate and column temperature were changed from 0.8 to 1.2 ml/min and from 35°C to 45°C, respectively, in order to study its effect on resolution of peaks.

4.3.2.2.7 System suitability test parameters

System suitability test parameters must be checked to ensure that the system is working correctly during the analysis. The following parameters were used for system suitability evaluation (BP, 2003; USP, 2007).

a. Capacity factor ($k'$).

Capacity factor (retention factor) is a measure of the retention time of a compound of the sample with a given combination of mobile phase at a given temperature and flow rate in a specified column. It is defined as $k'_{(A)} = (t_A - t_0)/t_0$ in which $t_A$ is the retention time of the compound and $t_0$ refers to retention time for an unretained compound. $t_0$ can be calculated by observing the initial baseline deflection of the trace above and below the baseline, caused by the difference in the composition of sample solutions as well as the mobile phase. An entail baseline deflection of this shape is found to be safe to assure that this correspond to $t_0$ (Snyder et al., 1997). In the present work, $t_0$ was 1.39 min. For an optimum separation, retention factor should be in the range of $0.5 < k' < 10$.

b. Selectivity factor ($\alpha$).

Selectivity parameter is a measure of separation of two compounds in the sample under given conditions. For two components A and B it is defined as $\alpha = k'_{B}/k'_{A}$ ($k'$ is the respective capacity factor). Therefore, it is the ratio of the relative retentions of the two compounds.

c. Resolution ($R_s$).

Resolution is a measure of the degree of separation between the adjacent peaks. For two compounds X and Y in a chromatographic run it is expressed as $R_s = 1.18 (t_{r,Y} - t_{r,X})/(w_{0.5,X} + w_{0.5,Y})$ in which $t_{r,X}$ and $t_{r,Y}$ = retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks, and $w_{0.5,X}$ and $w_{0.5,Y}$ = peak widths at half-height. A value of 1.5 for resolution implies a complete separation of two compounds.

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d. Number of theoretical plates (Column efficiency).

In a particular separation the number of theoretical plates or column efficiency refers to the performance of the stationary phase and implies to how well the column is packed. In the present study the number of theoretical plates was calculated using the following equation: 
\[ n = 5.54 \left( \frac{t_R}{w_{0.5}} \right)^2 \] in which \( w_{0.5} \) is the width of the peak at half-height and \( t_R \) is retention time along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component.

e. Symmetry factor.

Symmetry or tailing factor (T) refers to peak asymmetry. Many chromatographic peaks do not appear in the shape of normal Gaussian distribution. A tailing factor of 1 refers to a symmetric peak. Tailing factor was calculated using the following equation for chromatographic peaks: 
\[ T = \frac{w_{0.05}}{2d} \] in which \( w_{0.05} \) is the width of the peak at 5% of the peak height and \( d \) is distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

4.3.2.3 Development of RP-HPLC stability indicating assay method for talinolol

- Forced degradation studies

Stability is one of the most fundamental aspects of the product characteristics. Stability testing and forced degradation studies play a very crucial role during drug development to elucidate the intrinsic stability of the drug substance (Kommanaboyina and Rhodes, 1999). The term stability-indicating assay had been used to describe “a procedure which affords specific determination of a drug substance in the presence of its degradation products” (Taylor and Shivji, 1999). According to International Conference on Harmonization (ICH) guideline Q1A (R2) on the stability testing of new drug substances and products, the stability testing of the drug substance should be carried out under different stress conditions to validate the stability-indicating supremacy of the analytical methods used for the analysis of stability samples (ICH, 2003). In addition, the need for the stability studies of a drug candidate arises from the fact that the chemical integrity of the drug substance should be maintained until the compound is delivered to the intended site of action. Furthermore, a stability indicating assay method provides assurance on detecting changes in identity as well as purity potency of the product.
Identification of the degradation products, establishment of degradation pathways and determination of intrinsic stability of the drug molecules and validation of the analytical procedure are the goals achieved by stress testing (ICH, 2003). The various conditions specified for the forced degradation studies include extremes of pH, oxidation, photolytic degradation, and the effect of temperature (Bakshi and Singh, 2002; Bhinge et al., 2008; ICH, 1996; Rao et al., 2005; Singh and Bakshi, 2000; Sinha and Ghai, 2011; Sinha et al., 2007). Different stress conditions were used for the forced degradation studies of bulk drug and drug formulations. Zero time samples and placebo i.e. samples without drug were also prepared and analyzed for the comparison with the stressed samples. The chromatograms of the blank solutions consisting of stress agents with and without the drug and the zero time drug solutions together with stress agents were inspected in order to mark the peaks corresponding to stress agents and to distinguish them from the potential drug degradation products.

4.3.2.3.1 Hydrolysis
4.3.2.3.1.1 Acidic conditions

For acidic hydrolysis, hydrochloric acid of different strengths was used for the preparation of 1 mg/ml talinolol solution. 100 mg of talinolol in 100 ml volumetric flask was dissolved in 5 ml methanol and the volume was made up using 0.01N HCl and was kept at 25°C for 2 h, and at 40°C for 8 h. Further, the drug solutions were prepared similarly in 0.1N HCl and kept at 40°C for 24 h, and also refluxed for 2, 8 and 12 h. Samples were collected at specific time intervals, diluted 10 times and analyzed using the validated analytical RP-HPLC method. Identification of the degradation product was done after acidic hydrolysis of drug solution to complete degradation by refluxing for 12 h.

Subsequently, after complete degradation, the pH of the solution was adjusted to precipitate the degradation product of talinolol. The precipitates were filtered, washed and dried under vacuum and protected from air and light. The identification of the degradation products was done using IR, NMR and MS/ESI, by comparing spectra of degradation product with that of pure drug i.e. talinolol.
4.3.2.3.1.2 Alkaline conditions

Degradation studies at alkaline conditions were performed by preparing 1 mg/ml talinolol solution. 100 mg of talinolol in 100 ml volumetric flask was dissolved in 5 ml methanol and the volume was made up to 100 ml with 0.01N sodium hydroxide and was kept at 25°C for 2 h, and at 40°C for 8 h. Similarly, the drug solutions prepared in 0.1N NaOH were kept at 25°C for 2 h, at 40°C for 8 h, and refluxed for 2 and 8 h. Samples were collected at specific time intervals, diluted 10 times and analyzed using the validated RP-HPLC method. The degradation samples were subjected to mass spectroscopy to characterize the degradation product so formed and their structures were proposed by calculating mass units of molecular ion peaks obtained.

4.3.2.3.1.3 Neutral (water) conditions

For neutral hydrolysis, 100 mg of talinolol in 100 ml volumetric flask was dissolved in 5 ml methanol and the volume was made up with triple distilled water (1 mg/ml) and exposed to different conditions like at 25°C for 2 h, at 40°C for 8 h, and then refluxed for 12 and 24 h. Samples were collected at specific time intervals, diluted 10 times and analyzed using the validated RP-HPLC method. The degradation samples were subjected to mass spectroscopy to characterize the degradation product so formed and its structure was proposed by calculating mass units of molecular ion peaks obtained.

4.3.2.3.2 Thermal degradation studies

To investigate the susceptibility of talinolol under thermal stress conditions, the bulk drug (spread in a thin layer in a petri plate) and the drug solution (1 mg/ml in 0.01N HCl) were exposed to dry heat in a hot air oven at 70°C for 15 days. Samples were collected at specific time intervals, diluted 10 times and analyzed using the validated RP-HPLC method. For the identification, purification and analysis of major degradation products after thermal degradation, the solution was exposed to freeze drying and protected from air and light. The identification and analysis of the degradation products was done by IR, NMR and MS/ESI. By comparing spectra of degradation product obtained with that of pure drug, its structure was established.

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4.3.2.3 Oxidative conditions

Oxidative degradation studies were performed using hydrogen peroxide (H$_2$O$_2$) solution of different strengths at room temperature. Talinolol solution (1 mg/ml) was prepared in 3% H$_2$O$_2$ and exposed for 6, 10 and 24 h. A similar solution was prepared in 10% H$_2$O$_2$ and it was analyzed after 24 h. Studies were also carried out on drug solution in 30% H$_2$O$_2$ kept for 48 h at room temperature. Samples were collected at specific time intervals, diluted 10 times and analyzed using the validated RP-HPLC method.

4.3.2.3.4 Photo degradation studies

Photo degradation studies were carried out by exposing the drug solutions (1 mg/ml in 0.01N HCl, water: methanol mixture and 0.01N NaOH) as well as powdered drug in a photo-stability chamber. The powder was spread as thin layer in a petri plate. The overall illumination at the point of placement was 6000 lux fluorescent and 0.7 W/m$^2$ UV light. Samples were withdrawn after 15 days, diluted 10 times and analyzed using the validated RP-HPLC method. The samples of both solutions and powder form were kept in parallel in dark (wrapped) for the same period.

For the investigation of photo-stability of talinolol, stability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with light sources, as defined under option 2 of the ICH guideline Q1B was used (ICH, 1996). In this chamber, the combination of two black light OSRAM L73 lamps and four white fluorescent OSRAM L20 lamps formed the light bank. The spectral distribution of black light lamp was between 345 and 410 nm with a maximum at 365 nm, whereas the output of white fluorescent lamps was similar to that specified in ISO 10977 (1993). Both UV and visible lamps were put on simultaneously. The chamber was maintained at 40°C and 75% relative humidity (RH).

4.3.2.4 Preparation and characterization of degradation products

Q-TOF Micro (Waters, UK) equipped with an ESI source was used for the identification of degraded products of talinolol. The TOF/MS analysis worked in positive ion mode and the mass range was set at m/z 50 to 1200. Infusion experiments were carried out to optimize the instrument parameters for maximal generation of protonated molecules and capillary voltage was set to 36 V. Nitrogen gas set at 50 and 21 psi was used as nebulizing and drying gas, respectively. The API housing and drying gas was kept at 80 and 200°C.

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respectively. Protonated analyte molecules were subjected to collision induced dissociation using argon as the collision gas at 1.8 mTorr to yield product ions. The capillary voltage and sample cone voltage were set to 2500 V and 30 V, respectively. The pure drug as well as degraded samples were analyzed exactly at same MS conditions. The pure drug and degraded samples was also analyzed using IR and NMR spectrophotometer. The IR spectrophotometer used was a Perkin Elmer, Spectrum RX1 FT-IR System, USA. The NMR spectra were recorded on a Bruker Avance II 400 NMR spectrometer (200 mHz), Switzerland.

4.3.2.5 Development of validated HPLC method in plasma for talinolol

HPLC method was developed and validated for the estimation of talinolol in plasma. Developed method was used for the determination of talinolol in the in-vivo bioavailability samples for final nanoemulsion formulations.

4.3.2.5.1 Sample Preparation

Talinolol was extracted by a modified method from the plasma (Tubic et al., 2006b). 200 μl of plasma was transferred to 2 ml eppendorfs tubes followed by addition of 1 ml of dichloromethane: isopropanol (95:5) which was vortexed for 30 s, followed by centrifugation at 5,000 rpm for 10 min. Subsequent to this, the organic layer was separated and evaporated to dryness at room temperature. The residue was reconstituted with 500 μl of 0.1N HCl and 500 μl of mobile phase followed by vortexing for 5 min and analyzed using a modified HPLC method (Sinha and Ghai, 2011). All the samples were analyzed after filtration using 0.22 μ nylon membrane filters.

4.3.2.5.2 Chromatographic conditions

The HPLC conditions used in the development of analytical method for estimation of drug in plasma were as follows:

- **HPLC instrument:** HPLC (Waters 2695, alliance, US) instrument equipped with Waters 2996 PDA detector (Milford, USA). Data acquisition and processing was performed on Empower 2 software.
- **Column:** Inertsil ODS-3 C-18 column (250 mm x 4.6 mm (i.d.), 5μm).
- **Mobile phase:** The mobile phase was composed of a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer (pH 4.4) in a ratio of 27:73 (v/v).
- **Flow rate:** 1.0 ml/min.

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• **Temperature:** 40°C.
• **Injection volume:** 20 µl.
• **Detector:** UV detector, $\lambda_{\text{max}}$ 242 nm.
• **Run time:** 20 min.

### 4.3.2.5.3 Revalidation of the developed method

#### 4.3.2.5.3.1 Linearity and range

Calibration curve of talinolol was prepared in plasma in the concentration range of 200-25000 ng/ml for the establishment of linearity. For standard plot, stock solution of the drug (10 mg/ml) in 0.01N HCl was prepared. The suitable dilutions were prepared by mixing 200 µl of plasma with 200 µl of the drug solution and extraction was done as described above. The above mixture was vortexed and centrifuged. The organic phase was separated and evaporated to dryness. The resulting residues were dissolved in 500 µl of 0.1N HCl and 500 µl of mobile phase and injected after filtration in triplicate into the column. The peak area was plotted against the corresponding concentration to obtain the calibration graph and the plots were subjected to linear regression analysis.

#### 4.3.2.5.3.2 Accuracy

The accuracy of the method was assessed by analyzing spiked blank plasma samples with drug plasma samples using independent working standard solutions. Talinolol was spiked into three different levels; corresponding to 200, 500 and 1000 ng/ml. The percentage recovery of the added drug was then calculated using the linearity plots.

#### 4.3.2.5.3.3 Precision

Repeatability studies were performed for the determination of intra-day and inter-day precision. Intra-day precision studies were performed by injecting three different concentrations of talinolol i.e. 200, 500 and 1000 ng/ml in hexaplicate on the same day. While for inter-day precision studies; these concentrations were injected in triplicate on six different days. Drug concentrations were calculated using the area obtained from the linearity plots and the results were expressed as percent R.S.D.

#### 4.3.2.5.3.4 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 3.3 $\sigma$/S and 10 $\sigma$/S, respectively, where $S$ is the slope of the calibration curve and $\sigma$ is the standard deviation.
deviation of y-intercept of regression equation \((n=6)\). LOD and LOQ of sample were determined by injecting each concentration in hexaplicate.

4.3.2.5.3.5 Specificity

The method specificity of the analytical method was evaluated by the ability of the chromatographic conditions to separate the plasma peaks from talinolol. Peak purity angle and purity threshold of the peaks were verified using the PDA detector.

TIZANIDINE

4.3.2.6 Preparation of standard plot of tizanidine using validated HPTLC method

A previously reported HPTLC method was revalidated for the determination of tizanidine hydrochloride. This method was then used for the determination of drug in the solubility studies.

4.3.2.6.1 Chromatographic conditions

The HPTLC conditions as performed in the analytical method are as follows:

- **HPTLC instrument**: Camag Linomat IV HPTLC equipped with automatic sampler ATS4, scanner, and camera detector. Data acquisition was performed on Win CATS software.
- **Plate**: HPTLC plates (Merck #5548) precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm) with 250 mm thickness (E. Merck, Germany).
- **Mobile phase**: Toluene-acetone-ammonia in volume ratio of 5:5:0.1 (%v/v/v).
- **Development**: Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. The plate was developed up to 8 cm and air dried.
- **Sample application**: Bands of width 6 mm were applied with a Camag microlitre syringe at a constant application rate of 0.1 ml/s with the space of 5 mm between the two bands. The slit dimension was kept at 5 mm x 0.45 mm, and 10 mm/s scanning speed was employed.
- **Detector**: Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode between 200 and 400 nm. The plate was then scanned and quantified at 254 and 366 nm. The wavelength chosen for further quantification was 315 nm.

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The stock solution of 500 µg/ml was prepared in water. The spots of the stock solution were applied on aluminium backed TLC plates using automatic sampler and the plates were developed in the solvent system.

4.3.2.6.2 Method revalidation

4.3.2.6.2.1 Linearity and range

Linearity for HPTLC method was done by applying various bands in the range of 300-10000 ng/spot in triplicate. The linearity plot was constructed and linear regression was demonstrated.

4.3.2.6.2.2 Accuracy

For determination of accuracy, a mixture of stressed samples were mixed with three known concentrations (300, 1200 and 10000 ng/spot) of tizanidine and the recovery of the drug was determined for the developed analytical methods.

4.3.2.6.2.3 Precision

To determine intra-day and inter-day precision three different concentrations i.e. 300, 1200 and 10000 ng/spot were applied on the TLC plate in triplicate, thrice on the same day and were repeated for three consecutive days. Finally the values of percent R.S.D. was calculated for the developed analytical methods.

4.3.2.6.2.4 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated as 3.3 \( \sigma/S \) and 10 \( \sigma/S \), respectively, where S is the slope of the calibration curve and \( \sigma \) is the standard deviation of y-intercept of regression equation (n=3).

4.3.2.6.2.5 Specificity

Specificity of the method towards the drug was established using Win CATS software. The purity of the peak was established using purity correlation spectra so obtained.

4.3.2.7 Preparation of standard plot of tizanidine using validated HPLC method

A previously reported HPLC method was revalidated for the estimation of tizanidine hydrochloride (IP, 2007). This method was used for the estimation of tizanidine in the biological sample.

4.3.2.7.1 Chromatographic conditions

The HPLC conditions of analytical method were as follows:

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- **HPLC instrument:** HPLC (Waters 2695, alliance, US) instrument equipped with Waters 2996 PDA detector (Milford, USA). Data acquisition and processing was performed on Empower 2 software.
- **Column:** Inertsil ODS-3 C-18 column (250 mm x 4.6 mm (i.d.), 5µm).
- **Mobile phase:** Acetonitrile and phosphate buffer pH 6.6 in the ratio of 20:80 (v/v).
- **Flow rate:** 1.0 ml/min.
- **Temperature:** 40°C.
- **Injection volume:** 20 µl.
- **Detector:** UV detector, λmax 320 nm.

4.3.2.7.2 Preparation of standard plot of tizanidine by HPLC method in distilled water

Calibration curve of tizanidine was prepared in distilled water for the establishment of linearity. For standard plot, stock solution of the drug (1 mg/ml) was prepared in water. After the suitable dilution with mobile phase, a range of concentrations (100-5000 ng/ml) were prepared and the resulting solutions were injected (20 µl) after filtration through 0.22 µm nylon membrane filter, in triplicate into the column. The linearity plots were constructed and linear regression was applied on the data.

4.3.2.7.3 Preparation of standard plot of tizanidine by HPLC method in plasma

4.3.2.7.3.1 Preparation of plasma samples

Tizanidine was extracted from the plasma by method reported by Henney et al., 2008. 500 µl of plasma was separated and mixed with 100 µl of methanol. Then 2.5 ml of extraction solvent (diethyl ether: hexane, 60:40) was added, mixed for 5 min and centrifuged for 5 min at 3000 rpm. Subsequent to this, 2 ml of the organic layer was separated and evaporated at room temperature. The residue was reconstituted with 1 ml of the mobile phase followed by vortexing for 5 min and analyzed using the HPLC method (IP, 2007). All the samples were analyzed after filtration using 0.22 µ nylon membrane filters.

4.3.2.7.3.2 Revalidation of the method

4.3.2.7.3.2.1 Linearity and range

Calibration curve of tizanidine was prepared in plasma in the concentration range of 75-2500 ng/ml for the establishment of linearity. For standard plot, stock solution of the drug

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(1 mg/ml) in distilled water was prepared. The plasma samples were prepared and tizanidine was extracted as described above by mixing it with the suitable dilution of the stock solution. The above mixture was vortexed and centrifuged. The organic phase was separated and evaporated to dryness. The resulting residues were dissolved in 1 ml of mobile phase and injected after filtration in triplicate into the column. The peak area was plotted against the corresponding concentration to obtain the calibration graph and the plots were subjected to linear regression analysis.

4.3.2.7.3.2.2 Accuracy

The accuracy of the method was assessed by analyzing spiked blank plasma samples with drug plasma samples using independent working standard solutions, in triplicate. Tizanidine was spiked into three different levels; corresponding to 150, 800 and 1000 ng/ml. The percentage recovery of the added drug was then calculated using the linearity plots.

4.3.2.7.3.2.3 Precision

Repeatability studies were performed for the determination of intra-day and inter-day precision. Intra-day precision studies were performed by injecting three different concentrations of tizanidine i.e. 150, 500 and 1000 ng/ml in hexaplicate on the same day. While for inter-day precision studies; these concentrations were injected in triplicate on six different days. Drug concentrations were calculated using the area obtained from the linearity plots and the results are expressed as percent R.S.D.

4.3.2.7.3.2.4 Limit of detection and limit of quantification

LOD and LOQ were calculated as $3.3 \sigma/S$ and $10 \sigma/S$, respectively, where $S$ is the slope of the calibration curve and $\sigma$ is the standard deviation of $y$-intercept of regression equation ($n=3$). LOD and LOQ of the sample were determined by injecting each concentration in triplicate.

4.3.2.7.3.2.5 Specificity

The method specificity of the analytical method was evaluated by the ability of the chromatographic conditions to separate the plasma peaks from tizanidine. Peak purity angle and purity threshold of the peaks were verified using the PDA detector.
4.3.3 Development of SEDDS/nanoemulsion formulations

For the development of SEDDS/nanoemulsion formulations, the component selection is an important criterion need to be considered. The component selection was done on the basis of maximum drug solubility, which was further confirmed by measuring percentage transmittance. Further, in order to justify the stability and self-nanoemulsification ability of the developed SEDDS/nanoemulsion formulations, the thermodynamic and kinetic stability studies and dispersibility/self-emulsification ability tests were paramount for the delivery of drug in the form of nanoemulsion (Agatonovic et al., 2004; Azeem et al., 2009).

4.3.3.1 Solubility studies

For the selection of components of SEDDS/nanoemulsion, drug equilibrium solubility measurements of talinolol and tizanidine were carried out in different solvents; i.e. oils (Miglyol 812, castor oil, Labrafac PG, ethyl oleate, isopropyl myristate, soyabean oil, olive oil, oleic acid and triacetin), surfactants (Tween 20, Tween 80, Cremophor EL/RH40, Labrafac L WL-1349, Labrasol, Labrafil 1944, Labrafil 2125 and Brij 721), co-surfactants (ethanol, propylene glycol, polyethylene glycol 200 and polyethylene glycol 400, Plurol Oleique 479, Transcutol and Lauroglycol 90) and aqueous media (distilled water, buffer pH 1.2, phosphate buffer pH 6.8 and 7.4).

In brief, excess of drug was added to 5 ml of the solvent i.e. oil, surfactant, co-surfactant and aqueous phase in sealed tubes. The solubility was determined using shake flask method at room temperature for 72 h in a thermostatic water bath shaker. The samples (0.5 ml) were taken at specific time interval and centrifuged for 10 min in an Eppendorf centrifuge at 5000 rpm. The supernatant was filtered through 0.22 μm nylon membrane filters, diluted with methanol to a suitable concentration range and analyzed by a validated HPLC and HPTLC method for talinolol and tizanidine concentration, respectively.

4.3.3.2 Selection of components

4.3.3.2.1 Selection of oil

The selection of oil for the development of SEDDS/nanoemulsion formulations was done on the basis of drug solubility studies. The oils which could solubilize maximum amount of talinolol and tizanidine i.e. triacetin and oleic acid, respectively, were selected.
4.3.3.2 Selection of surfactants

The surfactants which showed maximum drug solubility were evaluated for their emulsification ability. The percent transmittance of different mixtures of selected oil and surfactant was compared in order to explicate the rational for nanoemulsion formulation (Date and Nagarsenker, 2007). Briefly, equal quantity of surfactant was added to the selected oil (triacetin and oleic acid for talinolol and tizanidine, respectively). The mixture was gently heated at 45°C-50°C for homogenizing the components. The 1 ml of this isotropic mixture was diluted to 250 ml using double distilled water in order to obtain a fine emulsion. The time taken for the formation of fine emulsion was also noted. The obtained emulsions were visually observed for their relative turbidity. These emulsions were then indorsed to stand for 2 h and the percentage transmittance was measured at 400-700 nm by UV-1601 UV-Visible double beam spectrophotometer using distilled water as the blank (Date and Nagarsenker, 2007; Sinha and Ghai, 2010).

4.3.3.2.3 Selection of co-surfactants

Similarly, the co-surfactants which gave maximum drug solubility were evaluated for their nanoemulsification ability. In order to measure the relative efficacy of the co-surfactant to increase the nanoemulsification ability of the surfactant, turbidimetric method was used. The selected surfactant and co-surfactant was mixed in 1:1 ratio to form Smix mixture. Then, the equal quantity of selected oil was added and it was homogenized at 45°C-50°C. The 1 ml of this isotropic mixture was diluted to 250 ml using double distilled water in order to obtain a fine emulsion. The time taken for the formation of emulsions was noted. The emulsions were then allowed to stand for 2 h and the percentage transmittance was measured at 400-700 nm by UV-1601 UV-Visible double beam spectrophotometer. Since the ratio of surfactant to co-surfactant is same in this case, the clarity or turbidity of developed nanoemulsion formulations will aid in evaluating the relative efficacy of the co-surfactant in order to increase the nanoemulsification ability of the selected surfactant for the selected oil.

4.3.3.3 Apparent partition coefficient

The octanol/water partition coefficient offers a thermodynamic measure of the propensity of a substance to have a preference for the non-aqueous or oily phase instead of water (the hydrophilic/hydrophobic balance). Thus, the partition coefficient is the ratio of the

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concentration of compound between the oil phase and the aqueous phase of nanoemulsion. This study reflects the affinity of the drug to one phase. The value of the log P determines the lipophilic nature of the drug. The octanol/water system is an established system for simulating the partitioning between biological membranes and their natural environment, particularly water saturated octanolic phase. Also, octanol is an amphiprotic solvent having hydrogen bond donor and acceptor properties. Water saturated octanol is a reasonably good solvent for many organic compounds while other organic solvents have a more limited range due to solubility problems. The partitioning study of talinolol was carried out in octanol/water, triacetin/water, triacetin/buffer pH 6.8 and triacetin-ethanol/water systems. Similarly, the partitioning study of tizanidine was done in octanol/water, oleic acid/water, oleic acid/buffer pH 6.8, castor oil/water and oleic acid/propylene glycol systems.

Firstly, the two phases in the ratio of 1:1 were kept in shaker for 24 h, so that the two phases become saturated with each other. These phases were then separated using separating funnel. Placed 5 mg of drug in 25 ml conical flask followed by addition of 10 ml each of pre-saturated water and organic phase. The conical flask was kept in water bath shaker for 24 h at 37±0.5°C and the compound was allowed to partition between both phases. At the end of 24 h, the two phases were separated using separating funnel. The aqueous layer was filtered using 0.22 µm nylon membrane filter. Suitable aliquots were diluted prior to drug assay by UV spectroscopy at 242 and 320 nm for talinolol and tizanidine, respectively, against suitable blank. The partition coefficient of drug was calculated using the formula:

\[ P = \frac{C_0 V_w}{C_w V_0} \]

Where, P is the Partition Coefficient, \( C_0 \) is the Concentration of drug in the organic phase, \( C_w \) is the Concentration of drug in the aqueous phase, \( V_o \) and \( V_w \) are volumes of organic and aqueous phase, respectively (10 ml).

Based upon the results of solubility studies and apparent partition coefficient, triacetin and oleic acid were selected as an oil phase for the development of talinolol and tizanidine loaded SEDDS/nanoemulsion formulations, respectively. Surfactant and co-surfactants were selected on the basis of their percentage transmittance measurement for the selected oil.

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4.3.3.4 Construction of pseudo ternary phase diagrams

Pseudo ternary phase diagrams of selected surfactant, co-surfactant and oil were constructed using aqueous titration method. Mixtures with changing oil and the Smix (surfactant+co-surfactant) concentrations were prepared by keeping one of these components constant each time at room temperature. The percentage limit of co-surfactant, surfactant and oil used was selected on the basis of the requirements stated by Pouton, (2000) for the spontaneously emulsifying systems (Table 2) and by considering their acceptable safe dose (Table 9).

The surfactant and co-surfactant were taken at different ratios (1:1, 1:2, 1:3 and 1:4, % v/v) in each tube and were vortexed for 30 s to prepare Smix. Ternary mixtures with varying compositions of Smix and oil were prepared, where the ratios of oil to Smix were varied from 9:1 to 1:9 (% v/v). Slow titration with aqueous phase was done to each weight ratio of oil and Smix mixture, with gentle stirring to allow equilibration. Visual observation was carried out for determining transparency and ease of formation of o/w nanoemulsion. For any mixture, the total of surfactant, co-surfactant, oil and aqueous phase concentration always added to 100% (v/v). Different pseudo ternary phase diagrams were constructed for the development of SEDDS/nanoemulsion formulations of talinolol and tizanidine, shown in Table 13 and 14, respectively.

Table 13: Composition of different combinations developed as pseudo-ternary phase diagrams for talinolol formulations.

<table>
<thead>
<tr>
<th>Phase diagram</th>
<th>Oil (O)</th>
<th>Surfactant (S)</th>
<th>Co-surfactant (CoS)</th>
<th>Smix ratio (S:CoS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL1 Triacetin</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>TL2 Triacetin</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>1:2</td>
<td></td>
</tr>
<tr>
<td>TL3 Triacetin</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>TL4 Triacetin</td>
<td>Tween 80</td>
<td>Ethanol</td>
<td>1:1</td>
<td></td>
</tr>
<tr>
<td>TL5 Triacetin</td>
<td>Tween 80</td>
<td>Ethanol</td>
<td>1:2</td>
<td></td>
</tr>
<tr>
<td>TL6 Triacetin</td>
<td>Tween 80</td>
<td>Ethanol</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>TL7 Triacetin</td>
<td>15% aqueous solution of Pluronic F68</td>
<td>Ethanol</td>
<td>1:1</td>
<td></td>
</tr>
</tbody>
</table>

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Table 14: Composition of different combinations developed as pseudo-ternary phase diagrams for tizanidine formulations.

<table>
<thead>
<tr>
<th>Phase diagram</th>
<th>Oil (O)</th>
<th>Surfactant (S)</th>
<th>Co-surfactant (CoS)</th>
<th>Smix ratio (S:CoS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZ1</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Propylene glycol</td>
<td>1:1</td>
</tr>
<tr>
<td>TZ2</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Propylene glycol</td>
<td>1:2</td>
</tr>
<tr>
<td>TZ3</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Propylene glycol</td>
<td>1:3</td>
</tr>
<tr>
<td>TZ4</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Propylene glycol</td>
<td>2:1</td>
</tr>
<tr>
<td>TZ5</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>1:1</td>
</tr>
<tr>
<td>TZ6</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>1:2</td>
</tr>
<tr>
<td>TZ7</td>
<td>Oleic acid</td>
<td>Labrasol</td>
<td>Transcutol</td>
<td>1:2</td>
</tr>
<tr>
<td>TZ8</td>
<td>Oleic acid</td>
<td>Labrasol</td>
<td>Ethanol</td>
<td>1:2</td>
</tr>
<tr>
<td>TZ9</td>
<td>Oleic acid</td>
<td>Tween 20</td>
<td>Ethanol</td>
<td>2:1</td>
</tr>
<tr>
<td>TZ10</td>
<td>Oleic acid</td>
<td>Tween 80</td>
<td>Ethanol</td>
<td>1:1</td>
</tr>
</tbody>
</table>
4.3.3.5 Thermodynamic and kinetic stability testing

Nanoemulsions are kinetically and thermodynamically stable systems with no phase separation, creaming or cracking. It was made ensure that the observations are not made on the metastable systems. To overcome the problem of metastable formulation which are not thermodynamically or kinetically stable and takes long time to separate, thermodynamic and kinetic stability tests are recommended (Agatonovic et al., 2004; Constantinides, 1995; Craig et al., 1995; Eccleston, 1994; Ghosh and Murthy, 2006; Shafiq et al., 2007; Shakeel et al., 2007). The formulations were exposed to diverse stresses such as heating cooling, freeze thaw cycle and centrifugation.

*Heating cooling cycle:* Heating cooling and freeze thaw cycles were performed to assess the thermodynamic stability of the formulations. Formulations were stored in sequence at different temperature conditions i.e. at room temperature, in refrigerator (4°C), at 25±0.5°C and at a higher temperature (45±0.5°C) for 48 h and the cycle was repeated six times. These formulations were evaluated for their physical stability as phase separation, pH and clarity, after each cycle. The formulations which were found stable at the end of this cycle were subjected to the next test i.e. freeze thaw cycle.

*Freeze thaw cycle:* A set of 6 sequential freeze thaw cycles, i.e. between -21°C and +25°C were studied with storage of formulations at these temperature for 48 h. The formulations which were found stable at the end of six cycles (i.e. thermodynamically stable) at these temperatures were carried forward for the assessment of their kinetic stability studies i.e. centrifugation test.

*Centrifugation test:* Thermodynamically stable formulations were centrifuged at 4500 rpm for 30 min. Those formulations which did not show any phase separation (i.e. kinetically stable) and were clear after 30 min were carried forward for further studies.

Those formulations which passed these stress stability tests were further taken for the dispersibility test for the self-emulsification efficiency assessment. However, unless any chemical reactions e.g. oxidation, pH variations occur, no specific testing is required during storage for the nanoemulsion.

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4.3.3.6 Dispersibility/self-emulsification ability

The dispersibility (self-emulsification ability) of the drug loaded (50 mg/ml and 4 mg/ml of talinolol and tizanidine, respectively) nanoemulsion formulations was determined. In brief, the USP rotating paddle apparatus (Type 2) was used to evaluate the self-emulsification efficiency of for different mixtures. The 1 ml of each mixture was added to 250 ml of distilled water with gentle agitation condition provided by a standard stainless steel dissolution rotating paddle at 50 rpm and maintained at a temperature of 37±0.5°C. The in-vitro process of self-emulsification was visually monitored for the rate of emulsification and for the appearance of the produced emulsions using the below grading systems (Agatonovic et al., 2004; Ghai and Sinha, 2011; Khoo et al., 1998; Pouton, 1985b, 1997; Shafiq et al., 2007).

- Grade A: Rapidly forming nanoemulsion i.e. within 1 min and having a clear, transparent appearance.
- Grade B: Rapidly forming slightly less clear nanoemulsion having a bluish white appearance.
- Grade C: Fine milky emulsion formed within 2 min having a white appearance.
- Grade D: Slowly formed dull grayish white emulsion i.e. longer than 2 min having slightly oily appearance.
- Grade E: Appearances of large oil globules on the surface i.e. phase separation.

The nanoemulsion formulations that passed thermodynamic and kinetic stability tests and also the dispersibility test under Grade A and Grade B were further selected and evaluated for size, shape and morphology.

4.3.3.7 Selection of SEDDS/nanoemulsion formulations

The selection criteria used for SEDDS/nanoemulsion formulations were as follows:

a. Thermodynamic and kinetic stability of the formulations (Constantinides, 1995; Eccleston, 1994; Ghosh and Murthy, 2006).

b. Dispersibility and self-emulsification ability of prepared formulations.

c. Minimum and safe concentration of surfactant(s) and co-surfactant(s) that produce nanoemulsion.

d. Mixture having the maximum drug solubilization.
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Following the above criteria, SEDDS/nanoemulsion formulations were selected from different phase diagrams to study their potential as drug delivery system. These selected formulations were evaluated for robustness to dilution, droplet size, transmission electron microscopy (TEM), pH, drug content, percentage transmittance, refractive index, conductivity, viscosity and in-vitro release determinations.

4.3.4 Evaluation of SEDDS/nanoemulsion formulations

4.3.4.1 Robustness to dilution

Robustness of drug loaded formulations to dilution was studied by drop-wise dilution of formulation to 50, 100 and 1000 times with distilled water (Date and Nagarsenker, 2007). The test was performed using a magnetic stirrer operating at 100 rpm at room temperature. Formulations were visually observed immediately after dilution for the stability on the basis of appearance i.e. phases separation, precipitation of drug and transparency (clarity). In addition, the diluted nanoemulsions were stored for 48 h and the same observation was made to assess the dilution stability.

4.3.4.2 Droplet size, polydispersity and zeta potential analysis

The droplet size, size distribution and polydispersity index was determined for nanoemulsion formulations by photon correlation spectroscopy (PCS) using a Zetasizer 1000 HS. PCS analyzes the fluctuations in light scattering due to Brownian motion of the particles. The formulation was placed in the cuvette in instrument and light scattering was measured at an angle of 90° at 25°C. The ratio of standard deviation to the mean droplet size gives the polydispersity index (PI) of the formulation. The particle size distributions of selected transparent compositions were measured to verify the existence of nano-sized globules. The zeta potential values of blank and drug loaded nanoemulsion formulations were measured with the Zetasizer. The zeta potential value was calculated based on the electrophoretic mobility (Shakeel et al., 2007).

4.3.4.3 Morphology of SEDDS/nanoemulsions by Transmission Electron Microscopy

For transmission electron microscopy (TEM) analysis, the drug loaded nanoemulsion samples were studied using transmission electron microscope H7500 operating at 100 kV capable of point-to-point resolution. A drop of formulation was directly placed on a Formvar-coated copper electron microscopy grid. The excess liquid was drawn off with Whatman.
filter paper. The samples were then negatively stained with 50 μl of 0.5% (w/v) phosphotungstic acid; the staining process was allowed to proceed for 5 minutes at room temperature. Again the excess liquid was drained off with Whatman filter paper and the nanoemulsion was observed after drying. The form and size of the nanoemulsions was observed by using combination of bright field imaging at increasing magnification.

4.3.4.4 pH Measurement

The pH of the formulations was measured using a pH meter (Elico L1-120).

4.3.4.5 Drug content estimation

The SEDDS/nanoemulsion formulations were assayed for the drug content. Firstly the formulation (0.1 ml) was diluted to 1 ml with methanol which was further diluted to a concentration of 500 μg/ml using methanol. This diluted sample was analyzed using UV spectrophotometer at λmax of 242 and 320 nm for talinolol and tizanidine, respectively, after sufficient dilutions using a suitable blank.

4.3.4.6 Percentage transmittance

The percentage transmittance of the nanoemulsion formulation was determined using UV-1601 UV-Visible double beam spectrophotometer (Shimadzu, Japan) as described above in section 4.3.3.2.

4.3.4.7 Refractive index determination

The refractive indices of formulations with and without drug were measured using Abbe refractometer. It consists of a pair of glass prisms with a film of liquid between them. The double-prism of the Refractometer was opened and both glass surfaces were cleaned with a filter paper, and then the double prism was closed. The refractive index was measured by placing a drop of the formulation in the space between the two prisms using a syringe. The refractometer scale knob was turned to get a clear interface between the illuminated and dark regions. The micrometric screw was used for the additional refinement of the scale, until the clear interface appeared and the refractive index was recorded from a graduated scale.

4.3.4.8 Conductivity measurement

The electrical conductivity, σ (μS/cm) was measured by means of a PICO digital conductivity meter (Labindia Instruments) operating at 50 Hz, equipped with platinum conductance electrode, having cell constant, k = 1.062. Temperature was kept at 30±0.5°C.

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and was maintained by a RE320 Ecoline thermostat. Conductivity determines the type of nanoemulsion and detect phase inversion phenomenon (Angelico et al., 1998; Mehta et al., 1999; Yu and Neuman, 1995). Electrical conductivity, a structure-sensitive property, is one of the frequently used techniques to explore structural changes and nanostructures based upon percolation theory (Gennes and Taupin, 1982).

### 4.3.4.9 Viscosity measurement

The viscosity measurements can specify the existence of worm/rod like reverse micelle (Angelico et al., 1998; Yu and Neuman, 1995). The viscosity of the developed formulations was evaluated using spindle number 21 at altered angular velocities at 25±0.2°C using a rotating-spindle Brookfield DV-II+ pro viscometer. A typical run involved first increasing (from 1 to 100 rpm) and then decreasing (from 100 to 1 rpm) the speed of the rotating spindle of viscometer with a similar period for equilibration at each speed. The values of shear stress and viscosity were recorded with a subsequent increase and decrease in shear rate. The rheological behavior of the disperse systems was studied via creating rheograms of shear stress versus shear rate. The rheograms so produced delivered valuable information about the flow properties of the developed formulations.

### 4.3.4.10 In-vitro release studies

In-vitro release experiments of developed selected drug loaded SEDDS/nanoemulsion formulations were carried out in comparison with conventional formulations i.e., oily drug solution, o/w emulsion and pure drug suspension. The drug was loaded at a dose of 50 mg/ml and 4 mg/ml for talinolol and tizanidine, respectively. In-vitro release studies were carried out in 500 ml of distilled water and phosphate buffer pH 6.8 for talinolol and tizanidine, respectively, using USP Type 2 apparatus rotating at a rate of 50 rpm and a temperature of 37±0.5°C, using an assembly described by Mishra et al., 1987. In this, a treated dialysis membrane as diffusional barrier was tied at both the sides with the help of dialysis clips, containing formulation inside the dialysis sachet (Grove et al., 2006a; Ghai and Sinha, 2011). A sinker was tied with each dialysis membrane sachet and was kept in the dissolution beaker. It was ensured that the dialysis membrane sachets were properly dipped in the release medium. During the study, 5 ml of aliquots were withdrawn at suitable time intervals (5, 15, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h) from the release medium and replaced with
equal volume of fresh buffer maintained at same temperature. The amount of drug released in the release medium was measured spectrophotometrically at a $\lambda_{\text{max}}$ of 242 nm and 320 nm for talinolol and tizanidine, respectively, after appropriate dilutions against suitable blank.

For the treatment of dialysis membrane, the membrane was washed in running water for 3-4 h to remove the glycerin. The tube was then treated with 0.3% w/v solution of sodium sulphide at 40°C for 1 min so as to remove sulphur compounds. After this the membrane was washed with distilled water at 60°C for 2 min followed by acidification with 0.2% v/v solution of sulphuric acid. Finally, the membrane was rinsed with hot water to remove the acid. The treated membrane was then stored in release media and washed with distilled water prior to use.

4.3.4.11 Ex-vivo permeation studies

All animal experiments were carried out after approval of the protocol by the Institutional Animal Ethics Care (IAEC) committee, Panjab University, Chandigarh, India and conducted according to the Indian National Science Academy (INSA) guidelines for the use and care of experimental animals. Male Wistar rats weighing between 175-230 g were used and housed at temperature of 24±2°C and 50%-60% relative humidity. Animals were acclimatized to laboratory conditions before the experiment (Ghai and Sinha, 2011). Porcine small intestine was procured from a local slaughterhouse. The tissue was stored in Kreb’s Ringer phosphate buffer (KRPB) at 4°C continuously aerated. To evaluate the effect of nano-sized oil globules upon improved drug permeation through biological barrier, ex-vivo permeation studies were performed using porcine small intestine and everted rat gut sac method.

4.3.4.11.1 Porcine small intestine permeation study

Porcine small intestine as diffusional barrier was used to investigate the drug permeation potential of SEDDS/nanoemulsions in comparison with conventional formulations. About a 10 cm length of the porcine small intestine was excised and it was washed with saline from both the sides and put on filter paper which was soaked with saline. The isolated intestinal tract was cut lengthwise with scissor and spread in the form of a sheet. The serosal membrane inside was set upward using a filter paper and the cut was made with the help of scalpel to remove the muscle layers using tweezer (Legen et al., 2005; Ghai and University Institute of Pharmaceutical Sciences, Panjab University.
Sinha, 2011). The membrane was then fixed at one end of the vertical glass tube, which acts as a donor compartment (mucosal side). The selected nanoemulsion formulation (50 mg/ml talinolol or 4 mg/ml tizanidine) was placed in the donor compartment. This tube was then suspended in a beaker containing 250 ml of phosphate buffer pH 6.8 at 50 rpm maintained at 37±0.5°C, which acts as a receptor compartment (serosal side). Further, the 1% w/v of sodium azide was added to the phosphate buffer pH 6.8 to maintain tissue viability.

Drug loaded formulation was added from the top of the tube at the mucosal side of the isolated porcine small intestine. The sample (2 ml) was collected from the serosal side buffer receptor at pre-determined time intervals (5, 15, 30, 45 min, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 h). Further, 2 ml of the freshly prepared phosphate buffer pH 6.8 (37±0.5°C) was added and supplemented into the serosal side after each sampling. The amount of drug diffused in the receptor compartment medium was determined using UV spectrophotometer at a λmax of 242 nm and 320 nm for talinolol and tizanidine, respectively after appropriate dilutions.

4.3.4.11.2 Everted rat gut sac method

Male Wistar rats (fasted for 15-19 h with free access to potable water until before the experiment) were anesthetized by excessive ether inhalation. Following a midline incision in the abdomen, the small intestine was excised. The entire length of the small intestine was carefully removed and prior to tissue preparation, placed in cold KRPB continuously aerated with the aid of an electrical aerator. Medial jejuna segments (~6 cm) were prepared and used for the permeation study (n=3). This segment was then ligated with silk thread to one end of a glass rod and carefully everted on the rod (Legen et al., 2005).

A weight of ~1 g was tied to the end of the everted gut segment to make it an empty gut sac and also to prevent peristaltic muscular contractions, which may otherwise alter the shape and internal volume of the sac (Mahomoodally et al., 2004). The gut sac filled with 2 ml of KRPB solution (serosal fluid), was placed inside the bath containing 50 ml of the test solution (mucosal fluid) continuously bubbled with atmospheric air at 15-19 bubbles/min and maintained at 37±0.5°C using an outer jacket. One ml of the formulation (50 mg/ml talinolol or 4 mg/ml tizanidine) was introduced to 50 ml of the mucosal fluid. An aliquot of 0.5 ml was withdrawn from the serosal compartment at different time point’s i.e. 15, 30, 45, 60, 75,
90, 105 and 120 min and immediately replaced with fresh KRPB solution maintained at 37±0.5°C to keep the volume of the serosal solution constant. The amount of drug permeated across the intestine from mucosal to serosal side was determined spectrophotometrically using suitable blank (Ghai and Sinha, 2011; Guo et al., 2004). Apparent permeability coefficient (Papp) of drug at 45 min was calculated from mucosal to serosal direction according to the equation.

\[
P_{\text{app}} \text{ (cm/s)} = \frac{(dQ/dt)}{(A*Co)}
\]

Where, the \(dQ/dt\) is the drug permeation rate from the tissue, \(A\) is the cross sectional area of the tissue and \(Co\) is the initial drug concentration in the donor compartment at \(t=0\) (Legen et al., 2005; Ghai and Sinha, 2011).

**4.3.4.12 Kinetics of drug release and permeation**

In order to propose the possible release and permeation mechanism, the release and permeation pattern was evaluated to check the goodness of fit for zero order, first order, Higuchi’s square root of time equation (Higuchi, 1963) and Korsmeyer-Peppas power law equation (Korsmeyer et al., 1983; Peppas, 1985). The goodness of fit was evaluated by correlation coefficient values (R^2) (Dash et al., 2010; Kalam et al., 2007; Sankalia et al., 2008).

**4.3.4.13 In-vivo bioavailability determination**

In-vivo bioavailability studies were performed for SEDDS/nanoemulsion formulations of talinolol and tizanidine and compared with pure drug. Final formulations were prepared one day prior to dosing. Wistar rats were divided randomly into groups comprising six animals in each group and fasted overnight but were allowed free access to water ad libitum. Single dose (10 mg/kg of talinolol and 0.357 mg/kg of tizanidine) of final formulations was administered orally to each rat, according to the weight of the animals. One group was also kept as control by administering dummy formulations.

After oral administration the rats were anaesthetized using diethyl ether and blood samples were collected at 0 (predose), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h post administration. Blood samples were collected from the retro orbital plexus into tubes containing heparin. Plasma was separated by centrifugation and stored at -20°C until
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analysis. Samples were extracted and assayed for talinolol (Section 4.3.2.5.1) and tizanidine (Section 4.3.2.7.3.1), respectively.

The in-vivo preclinical pharmacokinetic parameters like maximum plasma concentration (C\text{max}) and the time of its occurrence (T\text{max}) were determined directly from the individual plasma concentration versus time profiles. The area under the plasma concentration versus time curve (AUC\text{0-t}) was estimated by trapezoidal integration to the last sampling point (24 h). Further, AUC\text{0-\infty}, elimination half-life (t\text{1/2}), total elimination clearance (CL) and volume of distribution (V\text{d}) were also determined using WinNonlinTM version 3.3 (Pharsight Corporation, USA) software. The relative bioavailability of developed SEDDS/nanoemulsion formulations was calculated with respect to pure drug suspension using the equation.

\[ F_r (\%) = \left( \frac{\text{AUC}_{0-\infty} \text{ SEDDS}}{\text{AUC}_{0-\infty} \text{ Suspension}} \right) \times 100 \]

Where, \( F_r \) is the relative bioavailability, \( \text{AUC} \) is the area under the concentration-time curve. Statistical comparison of the various formulations was performed on the log transformed data using a one-way analysis of variance (ANOVA) and post hoc Tukey’s multiple comparisons test. P values of <0.05 were considered significant. All statistical calculations were performed using SigmaStat version 3.1 (Systat Software Inc., USA).

4.3.4.14 Toxicity study

The safety of Cremophor at 30, 40, 50 and 60% v/v strength was compared to that of placebo (oily drug solution) at a dose rate of 12.8 ml/kg/day. Male Wistar rats were divided into six groups, containing six animals in each group. Cremophor (1.6 ml/rat twice a day) was administered orally to \( n=6 \) unfasted rats by gavage (oesophageal intubation) in 4 groups, respectively, for 8 days. In addition, two control groups (\( n=6 \)) were maintained; control group 1 received 1.6 ml of oily drug solution (placebo) twice a day and control group 2 served as negative control (without any treatment). Evaluations and measurements based on mortality (daily), changes in appearance and behavior (daily), body weight (daily) and feed and water intake (daily) were performed. On completion of the study, overnight fasted rats of all groups were sacrificed and selected organs were weighed and preserved in 10% v/v buffered formalin and histomorphological examination of stomach, small intestine and large intestine were performed.

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4.3.4.15 Stability studies

Stability studies as per ICH Q1A (R2) guidelines were performed on final formulations for six months. The formulations were kept in air tight glass vials at temperature of 40±0.2°C and 75±5% RH. In addition, stability of the formulations was also determined at different temperature conditions i.e. in refrigerator (4°C) and at 25±5°C. Samples were withdrawn at the end of 0, 30, 60, 90, 120 and 180 days. The samples were examined for phase separation, refractive index, pH change, clarity, percentage transmittance and drug content.

4.3.4.16 Preparation of conventional formulations

Conventional formulations such as oily solution, o/w emulsion and suspension of talinolol and o/w emulsion of tizanidine were prepared for comparison with the developed SEDDS/nanoemulsion formulations. 50 mg/ml of oily drug solution of talinolol was prepared by adding drug directly to triacetin and vortexed. In order to prepare the pure drug suspension, talinolol was grinded and sieved by mesh 60. 0.5% of CMC-Na aqueous solution was prepared. It was mixed until it become uniform and made up to volume to prepare 50 mg/ml of pure drug suspension. For the preparation of conventional o/w emulsion the drug was incorporated in the oily phase by agitating at room temperature for 10 min. Further, 30 parts of oil (triacetin or oleic acid for talinolol or tizanidine, respectively) and 70 parts of water containing 15% v/v of surfactant was mixed vigorously in a glass vial.

4.3.4.17 Statistical analysis of data

Statistical comparison of in-vivo bioavailability of various formulations was performed on the log transformed data using a one-way analysis of variance (ANOVA) and post hoc Tukey’s multiple comparisons test. P values of <0.05 were considered significant. All statistical calculations were performed using SigmaStat version 3.1 (Systat Software Inc., USA).

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