4. MATERIAL AND METHODS

4.1. Materials

PPK was isolated from dried seeds of ripened fruits of MC by the procedure reported by Khanna (2004). CRM, cotton seed oil, Poly ethylene glycol (PEG) 200, 400, 600, and 800, PG, Tween 20, 60 and 80, span 20, 40, 60 and 80, Pluronic F-68, sesame oil, peanut oil, sunflower oil, soyabean oil, mustard oil, oleic acid, olive oil, eucalyptus oil, castor oil, fumed silica [Aerosil® 200], Polyvinyl alcohol (PVA) and sodium carboxy methyl cellulose (Na-CMC) were purchased from Central Drug House (CDH), New Delhi, India. Polyglycolyzed glycerides like Labrafac, Labrafir® M1944 CS (oleoyl polyoxyl-6 glycerides), Labrafir® M2125CS (oleoyl polyoxyl-6 glycerides), Labrasol, Lauroglycol FCC, Maisine 35-1, Capryol 90 [propylene glycol monocaprylate (type II)] and Transcutol® P (diethylene glycol monoethyl ether) were received as gift sample from M/s Gattefosse, Mumbai, India. Capmul MCM was obtained from M/s Abitec Corp., Ohio, USA. Miglyol 812N was received from Cremer Oleo GmbH & Co. KG, Germany. Triacetin and soya phosphatidyl choline were procured from Sigma Aldrich, USA. Ethanol was procured from Changshu Yangyuan Chemicals, China. Cithrol GMS 90 was obtained from Croda India Company Pvt. Ltd, India. Egg phosphatidyl choline was obtained from Lipoid GmbH, Germany. Microcrystalline cellulose PH102 (MCC PH102) was gifted by Colorcon Pvt. Ltd., Mumbai, India. Magnesium stearate (MS) was purchased from SD Fine Chemicals Ltd. Mumbai, India. Livvon Diabegard® 15 mg tablets were gifted by Pushpa Jyoti Herbal, Delhi, India. Mesoporous silicas, Syloid 244FP (SFP) and Syloid XDP 3150 (SXDP), were gifted by Grace Materials Technologies, Discovery Sciences, Pune, India. All other chemicals and reagents used were of analytical grade. Streptozotocin was purchased from Sigma, St. Louis, MO, USA. Spray dryer, SprayMate, Jay Instruments and systems, Navi Mumbai, India was used in the study. In order to quantify the drug, UV-Visible double beam spectrophotometer (UV-1800, Shimadzu, Japan) was used. Magnetic Stirrer, REMI, India was used for mixing of solutions. Dissolution apparatus DS8000, Lab India, Mumbai, India was used to carry out release studies. Design-Expert Dx 9.0.3 software, Stat-Ease, USA was used to conduct the DOE study.
4.2. Isolation of PPK from ripened seeds of MC

The ripened fruits of MC were procured and seeds were taken out. To start with, 500g of dried seeds of MC were taken and washed 3-4 times thoroughly with plenty of water to remove the debris. The water was drained off, seeds were air dried and then were kept in hot air oven at 45 °C for 2-3 h. The seeds were then grinded in grinder to a fine powder. Powder was mixed with 800 mL of hexane in a 1000 mL beaker. The mixture was stirred well with glass rod and then kept for 24 h (for de-oiling of seeds) with intermittent stirring. The slurry was then filtered, solvent from the filtrate was evaporated using rotary evaporator. The defatted seed powder was then soaked in 800mL of ammonium hydroxide solution of pH 9.5. It was stirred well with glass rod and then kept aside for next 24 h with intermittent stirring. After 24 h, the slurry was filtered and the filtrate was collected in a beaker. The marc was also pressed and filtered and both the filtrates were mixed together. To the filtrate, 1 N sulphuric acid solution was added drop wise so as to precipitate the protein present in it (5 mL acid required to precipitate the complete protein in the filtrate). The filtrate was set aside for few hours for complete precipitation of PPK. The precipitated filtrate was then centrifuged at 3000-4000 rpm to collect the protein precipitates at the bottom of the centrifuge tubes. The supernatant was discarded and the precipitates were collected and given thorough washings with 90 % acetone solution to remove any organic impurities. The precipitates were washed and dried in hot air oven at about 50 °C. The dried precipitates were then triturated well in pestle-mortar to get a fine powder (Garg et al., 2017a, 2017b; Kaur et al., 2016a; Khanna, 2004).

4.3. Characterization of isolated PPK

4.3.1 Morphological analysis of PPK

The powder of PPK was tested for their color, odor and taste.

4.3.2 Determination of melting point of the drug using DSC

About 2.5 mg of isolated PPK and its marker each sample was heated in pierced aluminium pan from 0 to 300 °C at a heating rate of 10 °C/min under a stream of nitrogen at a flow rate of 50 mL/min (Kumar et al., 2018; Rajesh et al., 2018).
4.3.3  **Fourier transform infrared spectroscopy (FTIR)**

For the structural analysis and authentification of the isolated PPK, FTIR was used. The potassium bromide (KBr) sample discs were prepared using isolated PPK and marker were compressed individually into discs under pressure of 10,000 pounds per square inch. The infrared spectrum was recorded in the wave number range of 4000-400 cm\(^{-1}\) using FTIR spectrophotometer and the characteristic bands of isolated PPK were compared with that of the marker (Pavia et al., 2007).

4.3.4  **Thin layer chromatography (TLC)**

For TLC analysis, a 10% solution of isolated PPK as well as marker was prepared in 10% v/v formic acid. The precoated plates were cut into 10 × 10 cm dimension. The markings (four spots) were made exactly 1cm above the one side of the precoated TLC plate. About 20µL solution of PPK and marker were spotted of the markings on the TLC plate and then kept in the TLC chamber presaturated with n-butanol, acetic acid and water in the ratio of 12:5:2, respectively. The developed plates were dried at room temperature and sprayed with 0.25% ninhydrin in acetone (Bhawani et al., 2012).

4.3.5  **Determination of partition coefficient (Log P)**

In a separating funnel, 12.5 mL of octanol and 12.5 mL of water were taken and mixed homogenously. To this, accurately weighed 50 mg of the PPK (drug) was added and then solution was shaken by alpha shaking for 30 min in order to achieve uniform mixing. The two layers were allowed to separate. The lower and upper layer was collected separately. Both solutions were filtered and analyzed (EUR-Lex - 31992L0069.Commission Directive 92/69/EEC of 31 July 1992; Streng, 2001).

4.3.6  **Compatibility study**

Compatibility study of PPK and CRM was done via FTIR. FTIR septra of PPK, CRM and PPK-CRM was taken as per the procedure given in section 4.3.3 (Athira and Jyoth, 2014; Pavia et al., 2007).
4.4 Development and validation of UV spectroscopic method for PPK

4.4.1. Development of calibration curve of PPK

100 mg of PPK was dissolved in 100 mL solution of 10 % v/v formic acid to get 1mg/mL stock solution. From the stock solution, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL aliquots were withdrawn and transferred to 10 mL volumetric flask. Volume was adjusted to 10 mL using distilled water in order to get concentration of 50, 100, 150, 200, 250 and 300 µg/mL. The mean absorbance was recorded at absorption maxima of PPK (ICH Q2 (R1)).

4.4.2. Stability study of PPK in solution

The stability of PPK standard stock solution in 10% v/v formic acid at a concentration of 200 µg/mL was investigated at different time intervals at 25 °C.

4.4.3. Linearity and range

In order to find out linearity, solutions of 50, 100, 150, 200, 250 and 300 µg/mL were prepared and analyzed. All these dilutions were prepared five times and mean data was recorded. From the calibration plot regression equation and coefficient of regression were calculated (ICH Q2 (R1)).

4.4.4. Specificity studies

Specificity was evaluated by analysis of the UV spectrum of A) Placebo L-SNEDDS, B) Pure PPK solution, C) Pure CRM solution and D) PPK SNEDDS E) CRM SNEDDS F) PPK-CRM SNEDDS G) PPK-CRM H) Placebo S-SNEDDS. Their spectrum was scanned in the range of 200 to 600 nm. The scanning was carried out in order to prove the absence of any spectral interference (ICH Q2 (R1); Kaur et al., 2016b).

4.4.5. Accuracy

The accuracy of method was determined by measuring the reference standard recovery in triplicate at three levels, 80 %, 100 % and 120 % of the method concentration (200 µg/mL). The dilutions were made at three different levels i.e. lower quantified concentration [LQC (80%)], medium quantified concentration [MQC (100%)] and high quantified concentration [HQC (120%)] of method concentration (i.e. 200 µg/mL).

A standard stock solution 1 mg/mL of PPK was prepared. In volumetric flasks of 10 mL, aliquots of 1.6, 2.0 and 2.4 mL of this solution (which would yield
concentrations of 160, 200 and 240 µg/mL, respectively) were combined with 1mL of the 600 µg/mL sample solution (This added the concentration of 60 µg/mL to the standard solution). The final concentrations were adjusted to 220, 260 and 300 µg/mL, which correspond to 80, 100 and 120 % of the target concentration, respectively. The mean recoveries expressed in terms of the percentage recovery and relative standard deviation (% RSD), were determined (Kaur et al., 2016b).

4.4.6. **Precision**

Precision was evaluated with respect to both repeatability and intermediate precision. Repeatability was tested by seven determinations at three levels, 80 %, 100 % and 120 % of the method concentration of the same day and under the same experimental conditions. Intermediate precision was evaluated by performing the analysis on three different days (interday) on the same levels as mentioned above and by three analysts performing the analysis in the same laboratory and under the same experimental conditions (between analysts). Seven replicates at a concentration of 160, 200 and 240 µg/mL were assayed at 272 nm. The percentage of relative standard deviation (% RSD) of the analytical responses was calculated (ICH Q2 (R1)).

4.4.7. **Limit of detection (LOD) and Limit of quantification (LOQ)**

Estimation of LOD and LOQ were determined by standard deviation of response (σ) and slope of calibration curve (S). Standard deviation of Y intercepts of regression line was used as standard deviation. Equations 7 and 8 for LOD and LOQ respectively are as follow.

\[
    \text{LOD} = 3.3 \sigma / S \tag{7}
\]

\[
    \text{LOQ} = 10 \sigma / S \tag{8}
\]

4.4.8. **Robustness**

The robustness of analytical method is a measure of its ability to remain unaffected by small changes. In the present study robustness was calculated by varying the concentration of formic acid solution. The sample solution (PPK: 15 mg) was prepared in 8, 10 and 12 % v/v formic acid. The absorbance was recorded at 272 nm. Moreover, the robustness of the method was determined by analyzing a change of 2 nm in the wavelength of analysis. Seven replicates of sample solution were prepared
at the same concentration (200 µg/mL), and assayed at 270, 272 and 274 nm. The % RSD was calculated (ICH Q2 (R1)).

4.5. Method development and validation of UV spectroscopic method for CRM

4.5.1. Development of calibration curve for CRM

In order to prepare calibration curve of CRM, 10 mg of CRM was accurately weighed and transferred to 100 mL of volumetric flask. To this flask, 20 mL of ethanol was added to dissolve CRM and then the volume of flask was adjusted to 100 mL using ethanol. This has provided 100 µg/mL of CRM stock solution. From this, 10 mL of solution was withdrawn and transferred to another 100 mL volumetric flask and volume of the flask was adjusted to 100 mL in order to achieve a concentration of 10 µg/mL. From this suitable aliquotes of 2, 4, 6 and 8 mL were withdrawn from above solution and transferred to 10 mL of standard volumetric flask separately and volume was adjusted to 10 mL. The serial dilutions of 2, 4, 6 and 8 µg/mL were prepared. The spectrum of CRM was scanned in the range of 200-600 nm on a UV-Visible double beam spectrophotometer (UV-1800, Shimadzu, Japan) (ICH Q2 (R1); Kaur et al., 2015).

4.5.2. Stability study of CRM in solution

The stability of CRM solution in ethanol at a concentration of 6 µg/mL was investigated at different time intervals (ICH Q2 (R1)).

4.5.3. Linearity and range

Linearity was accessed by calibration curve in which solutions of 2, 4, 6, 8 and 10 µg/mL were prepared 5 times as per the procedure mentioned in section 3.4.4.

4.5.4. Specificity study

PPK was used as another active ingredient in the formulation as antidiabetic drug along with CRM, hence the absorption spectra of PPK was overlapped with spectrum of CRM. The detailed procedure is given below.

For specificity study eight samples were prepared, A) Placebo L-SNEDDS, B) Pure PPK solution, C) Pure CRM solution and D) PPK S-SNEDDS E) CRM S-SNEDDS F) PPK-CRM S-SNEDDS G) PPK-CRM H) Placebo S-SNEDDS. Their spectrum was scanned in the range of 200 to 600 nm. The scanning was carried out in order to prove the absence of any spectral interference (ICH Q2 (R1)).
4.5.5. **Accuracy**

The accuracy of method was determined by recovery studies in triplicate. In order to carry out this, suitable aliquots of 4.8, 6 and 7.2 mL were withdrawn from 10 µg/mL of standard stock solution and transferred individually into 10 mL volumetric flask in order to achieve concentration of 4.8, 6 and 7.2 mg/mL, which corresponds to 80, 100 and 120 % of method concentration. The mean, % recovery and % RSD were recorded (ICH Q2 (R1)).

4.5.6. **Precision**

Precision was evaluated at LQC, MQC and HQC levels of CRM as per the procedure mentioned in section 3.4.7. The mean data was recorded (ICH Q2 (R1)).

4.5.7. **Robustness**

The robustness of analytical method is a measure of its ability to remain unaffected by small changes. The robustness of the method was determined by analyzing a change of 2 nm in the wavelength while taking UV absorbance. Seven replicates of the working standard solution were prepared at the same concentration (6µg/mL), and assayed at 418, 420 and 422 nm. The % RSD was calculated (ICH Q2 (R1)).

4.5.8. **LOD and LOQ**

Estimation of LOD and LOQ were determined as per the procedure mentioned in section 4.4.7.

4.6. **Development of UV method for PPK using the principle of standard addition method (in presence of CRM)**

It was observed that there was no interference of PPK absorption spectra on the absorption spectra of CRM. However, CRM shown little absorbance on the absorption spectra of PPK at 272 nm. Hence the calibration curve of PPK was also developed by using standard addition method.

The calibration curve of PPK was prepared in the range of 50-250 µg/mL as described earlier and to all these dilutions, 10µg/mL of standard CRM was added (Ravishankar, 2001; Saxberg Bo E. H., and Kowalski, 1979; Watson, 2015).

The stock solution of 1000 µg/mL of PPK was prepared in 10 % v/v formic acid as mentioned earlier. Similarly, 100 µg/mL solution of CRM was prepared in ethanol as mentioned earlier. Suitable aliquots of 0.5 mL, 1 mL, 1.5 mL, 2 mL and 2.5
mL were withdrawn from stock solution of PPK and each were transferred to different 10 mL volumetric flasks. To each of flasks 1 mL of CRM solution was transferred. The volume of all the flasks was adjusted to 10 mL using 10 % formic acid. Hence, the final concentration achieved for PPK was 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, 250 µg/mL and the concentration of CRM in each solution was 10 µg/mL. All the solutions were scanned at 272 nm (ICH Q2 (R1); Ravishankar, 2001; Saxberg Bo E. H., and Kowalski, 1979; Watson, 2015).

The solutions of LQC, MQC and HQC to carry out accuracy and precision were prepared in similar way as described earlier for PPK. However, in these CRM (10 µg/mL) solution was also added (ICH Q2 (R1); Saxberg Bo E. H., and Kowalski, 1979; Watson, 2015).

4.7. Solubility Studies of PPK and CRM, in Various Oils, Surfactants And Co-Surfactants

In order to select the best oil, surfactant and co-surfactant for the formulation of L-SNEDDS, solubility studies were performed for PPK and CRM in oils (Castor oil, Sesame oil, Coconut oil, Peanut oil, Sunflower oil, Eucalyptus oil, Cotton seed oil, Oleic acid, Sunflower oil, Labrafac, Olive oil, Labrafil® M1944 CS, Capmul® MCM, Labrafil® M2125, Soyabean oil, Capryol® 90, Lauroglycol® FCC, Maisine® 35-1, Miglyol® 812N, Mustard oil, Triacetin® and Cithrol GMS®), surfactants (PEG 400, PEG 200, PEG 600, PEG 800, PG, Tween 20, Tween 60, Tween 80, Span 20, Span 40, Span 60, Span 80, Egg phosphatidyl choline [1% w/v in water: ethanol mixture (50:50 v/v)], soya phosphatidyl choline [1% w/v in water: ethanol mixture (50:50 v/v)] and Labrasol®) and co-surfactants (Transcutol® P, ethanol). To 1mL of each oil, surfactant and co-surfactant, an amount equivalent to 200 mg of PPK and 200 mg of CRM were taken separately in 5 mL clean glass vials and vortexed (CM 101 CYCLO MIXER, REMI, India) for 2 min for proper mixing of PPK and CRM with the vehicle. The vials were stoppered and agitated for 48 h at 37 ± 0.2°C in a shaking water bath. Upon equilibration all the samples were centrifuged at 10000 rpm for 15 min to remove the undissolved PPK and CRM from saturated solutions (Beg et al., 2012; Garg et al., 2017b; Inugala et al., 2015; Kumar et al., 2018). The supernatants were accurately measured and appropriately diluted with ethanol and
PPK/CRM concentration was estimated by UV-Visible spectrometer at 272 and 420 nm, respectively.

4.8. **Preparation of L-SNEDDS**

Based on the results of solubility studies (As discussed in section no 5.4) Labrafil® M1944 CS was selected as oil, Tween 80, PEG 200 and PEG 600 as surfactants and Transcutol® P and ethanol as co-surfactant. A total of 486 SNEDDS prototypes (162 for each drug) were prepared by varying the ratios of oils, surfactants and co-surfactants. The concentration of Labrafil® M1944 CS (oil) was varied from 10 to 90 % and mixture (S mix) of surfactants (i.e. PEG 600, PEG 200 and Tween 80) and co-surfactants (i.e. Transcutol® P, ethanol) was varied from 10 to 90% in the ratio of 1:1, 2:1 and 1:2, respectively. PPK (30 mg), CRM (2.75 mg) and combination of both were added individually to all the prepared formulations separately in small increments with continuous vortex mixing to form a monophasic system. The prepared L-SNEDDS were stored in screw capped clean glass vials at room temperature until further evaluation (Garg et al., 2017b; Inugala et al., 2015).

4.8.1. **Construction of ternary phase diagram and pre-screening**

In order to establish the stable spontaneous self-emulsification zone, various ratios of selected oil, surfactant and co-surfactant were plotted on a ternary-phase diagram. In order to assess the self-emulsification properties of prepared L-SNEDDS, a visual test reported by Craig et al., 1995 and Inugala et al., 2015 with minor modification was performed (Craig et al., 1995; Garg et al., 2017b; Inugala et al., 2015). Ternary phase diagram was constructed by considering the factors like tendency to form emulsion, clarity, phase separation, coalescence of droplets and drug precipitation. The prepared L-SNEDDS (200 µL) were dropped in 500 mL distilled water (maintained at 37±0.2°C) in a glass beaker, which was continuously stirred using magnetic stirrer at 100 rpm. The resulting emulsions were observed visually for the relative turbidity. Then the stability of formed emulsions was determined by visual observations such as extemporary emulsification, phase separation, drug precipitation, cracking of the emulsion on storage (48 h) at room temperature. Poor or no emulsion formation with immediate coalescence of droplets with phase separation and drug precipitation indicates formation of unstable emulsion (Craig et al., 1995; Garg et al., 2017b; Inugala et al., 2015).
Ternary phase diagrams using above method were prepared for PPK, CRM and PPK-CRM.

Pre-screening study was conducted to select the levels of DOE. Emulsion time, thermodynamic stability and cloud point was used for pre-screening.

4.8.2. Thermodynamic stability studies and cloud point measurement of L-SNEDDS formulations

Stability of the selected L-SNEDDS formulations was evaluated at various stress conditions by heating cooling cycles (4°C and 40°C) and freeze thaw cycles (-21°C and +25°C) with storage at specified temperature for 48 h. In order to carry out centrifugation stress study, 1 mL of each formulation was diluted to 100 mL with distilled water and centrifuged at 10000 rpm for 20 min and visually observed for any phase separation (Craig et al., 1995; Garg et al., 2017b; Inugala et al., 2015; Kallakunta et al., 2012). The cloud point temperature of the diluted L-SNEDDS formulation (10 mL) was determined by gradual heating on a water bath and the temperature at which cloudiness appears was noted using thermometer (Craig et al., 1995; Garg et al., 2017b; Inugala et al., 2015; Zhang et al., 2008a, 2008b).

4.8.3. DOE

From the pseudo ternary phase diagram, nanoemulsion region was selected. The results revealed that formulations wherein Labrafil® M1944 CS, Tween 80 and Transcutol® P were used as oil, surfactant and co-surfactant, in varying ratios exhibited the largest nanoemulsion area. Hence, these three excipients were selected for further formulation development and considered as independent variables. A set of experiments with BBD were adopted to develop the L-SNEDDS of PPK, CRM and PPK-CRM. All these variables were operated at three levels (+1, 0 and -1), shown in Table 17 and 18. Design-Expert®Dx 9.0.3 software was used to conduct the study. A total of 17 experiments were designed by the software with 5 center points (in order to allow the estimation of pure error) for each of the L-SNEDDS formulations (Table 19-21). Experiments were run in random order to increase the predictability of the model. The amount of PPK and CRM added to formulations was 30 mg and 2.75 mg, respectively, and kept constant. Moreover, type of oil (Labrafil® M1944 CS), type of surfactant (Tween-80) and co-surfactant (Transcutol® P) were also kept constant for all the experiments. However, concentration of Labrafil® M1944 CS, Tween 80 and
Transcutol® P was varied. The design was used to statistically optimize the independent variables: concentration of oil (20-40%), surfactant (30-40%) and co-surfactant (30-40%) and evaluate the main effects and interaction effects of these formulation ingredients on emulsion droplet size (Y1, Y5, Y9), polydispersity index (PDI) (Y2, Y6, Y10), % drug loading (Y3, Y7, Y11, Y12) and zeta potential (Y4, Y8, Y13) (Garg et al., 2017b). The study design is shown in Table 19 for PPK L-SNEDDS, Table 20 for CRM L-SNEDDS and Table 21 for PPK-CRM L-SNEDDS (Garg et al., 2017b; Rajesh et al., 2018).

Table 17
Variables for BBD

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<tr>
<th>Independent Factors</th>
<th>Design Level</th>
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<tr>
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<td>Coded</td>
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<td>Tween 80</td>
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</tr>
<tr>
<td>Transcutol® P</td>
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</tr>
<tr>
<td></td>
<td>30 0</td>
</tr>
<tr>
<td></td>
<td>40 +1</td>
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<td></td>
<td>35 0</td>
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Table 18
Formula composition of L-SNEDDS system

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity/batch (PPK)</th>
<th>Quantity/batch (CRM)</th>
<th>Quantity/batch (PPK-CRM)</th>
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<tbody>
<tr>
<td>PPK (mg)</td>
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<td>CRM (mg)</td>
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<tr>
<td>Transcutol® P (% w/w)</td>
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Table 19
Factor level and response data of PPK L-SNEDDS for BBD

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<thead>
<tr>
<th>Run</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Response 1</th>
<th>Response 2</th>
<th>Response 3</th>
<th>Zeta potential (mV) (Y4)</th>
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<tr>
<td>A:</td>
<td>Labrafil® M1944 CS (%) (X1)</td>
<td>B: Tween 80 (%) (X2)</td>
<td>C: Transcutol® P (µL) (X3)</td>
<td>Mean droplet size (nm) (Y1)</td>
<td>PD (Y2)</td>
<td>Drug loading (%) (Y3)</td>
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<tr>
<td>1</td>
<td>30 (0)*</td>
<td>40 (+1)</td>
<td>40 (+1)</td>
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<td>35 (0)</td>
<td>35 (0)</td>
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<td>35 (0)</td>
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<td>35 (0)</td>
<td>35 (0)</td>
<td>57.6</td>
<td>0.146</td>
<td>80.2</td>
<td>-19.4</td>
</tr>
<tr>
<td>Run</td>
<td>Factor 1A: Labrafil® M1944 CS (%) (X1)</td>
<td>Factor 2B: Tween 80 (%) (X2)</td>
<td>Factor 3 C: Transcutol® P (µL) (X3)</td>
<td>Response 1: Mean droplet size (nm) (Y5)</td>
<td>Polydispersity index (PDI) (Y6)</td>
<td>Drug loading (PPK) (%) (Y11)</td>
<td>Drug loading (CRM) (%) (Y12)</td>
</tr>
<tr>
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Table 21
Factor level and response data of PPK-CRM L-SNEDDS for BBD

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor 1A: Labrafil® M1944 CS (%) (X1)</th>
<th>Factor 2B: Tween 80 (%) (X2)</th>
<th>Factor 3 C: Transcutol® P (µL) (X3)</th>
<th>Response 1: Mean droplet size (nm) (Y9)</th>
<th>Response 2: Polydispersity index (PDI) (Y10)</th>
<th>Response 3: Drug loading (PPK) (%) (Y11)</th>
<th>Response 3: Drug loading (CRM) (%) (Y12)</th>
<th>Zeta potential (mV) (Y13)</th>
</tr>
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<td>1</td>
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<td>79.0</td>
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</tr>
<tr>
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<td>0.158</td>
<td>60.2</td>
<td>69.1</td>
<td>-28.3</td>
</tr>
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</table>
4.9. Validation of optimized batches

All the three optimized formulations i.e. PPK L-SNEDDS, CRM L-SNEDDS and PPK-CRM L-SNEDDS were characterized for mean droplet size, zeta potential, PDI and drug loading. The obtained results were correlated with the theoretical values of responses that were obtained through BBD.

4.10. Characterization of L-SNEDDS

4.10.1. Calculation of drug loading

SNEDDS were prepared by adding PPK/CRM/PPK-CRM to each batch containing 1 mL mixture of Labrafil® M1944 CS, Tween-80 and Transcutol® P as per the design mentioned in DOE (Table 3, 4 and 5). These were vortexed using vortex mixer for 15 min and then added to 500 mL of double distilled water being stirred at 500 rpm at a temperature of 37 ºC. Sample (5 mL) was withdrawn and centrifuged at 10000 rpm for 15 min for removal of the un-dissolved drug. The supernatants were accurately measured and appropriately diluted using distilled water and PPK/CRM concentration was estimated. It is important to note that for amount of PPK and CRM present in their individual L-SNEDDS sample, their absorbance was recorded at their individual wavelength (i.e. 272 nm for PPK and 420 nm for CRM). However, the amount of PPK present in mixture of PPK-CRM SNEDDS was calculated by using standard addition method where the volume of sample was kept constant and known concentration of CRM 2, 4, 6 8 and 10 µg/mL was spiked and graph was plotted between absorbance on Y axis and concentration of CRM on X axis. The plot was extrapolated to the X coordinate and the concentration of PPK was recorded (Ravishankar, 2001; Saxberg and Kowalski, 1979; Watson, 2015). The percentage drug loading was calculated as per the formula given in equation 9 (Garg et al.,
% Drug Loading = \frac{\text{Amount of drug in known amount of L-SNEDDS}}{\text{Initial drug loading}} \times 100

4.10.2. Emulsion droplet size and zeta potential analysis

Measurement of droplet size, PDI and zeta potential were determined by Photon Correlation Spectroscopy (PCS) using Malvern zeta sizer nano ZS90 (Malvern Instruments Ltd., UK) with a 50 mV laser at a fixed angle of 90°. Measurements were carried out at 25°C using disposable polystyrene cells and disposable plain folded capillary cells after dilution. L-SNEDDS sample (100µL) was diluted with 100mL double distilled water. The low interparticle space between droplets results in multiple light scattering that may lead to false results, hence, dilution is necessary. Each run underwent 12 sub-runs for a period of 2 min (Beg et al., 2012; Kumar et al., 2018; Rajesh et al., 2018).

4.11. Solidification of optimized batch of L-SNEDDS

4.11.1. Oil adsorption capacity

In order to enhance the stability of L-SNEDDS formulations, it was further solidified by using array of porous carriers. Both hydrophobic like Aerosil® 200 (A 200), Syloid244FP (SFP), Syloid XDP 3150 (SXDP), Magnesium stearate (MS), Micro Crystalline Cellulose (MCC) PH102 and hydrophilic carriers like PVA, Na-CMC and HP-ß-CD, were used. In order to achieve better flow and compaction, the oil adsorption capacity (OAC) of carriers should be high, hence, selected carriers were subjected for estimation of their OAC. Gravimetric method was used to carry out OAC, where the amount of porous carrier required to transform the unit dose of oily liquid formulation into the free-flowing powder was calculated (Kumar et al., 2018; Modasiya et al., 2009; Malaysia 2012).

4.11.2. Preparation of S-SNEDDS using spray drying

Different batches of S-SNEDDS including PPK S-SNEDDS, CRM S-SNEDDS and PPK-CRM S-SNEDDS were formulated using spray dryer (SprayMate, Jay Instruments and systems, Navi Mumbai, India). The hydrophobic carriers (1g) such as Aerosil® 200, SFP, SXDP, MCC PH 102 and MS, were individually
suspended in 100 mL ethanol. Furthermore, hydrophilic carriers (1g) PVA, Na-CMC and HPβCD, were separately dissolved in 100 mL water. The L-SNEDDS (1 mL) was added to these solutions with constant stirring and the solution was continuously stirred at room temperature for 15 min to obtain good suspensions. Each suspension was delivered through the nozzle (0.7 mm diameter) at a flow rate of 16 mL/min using a peristaltic pump, dried at inlet temperatures of 70°C and 100°C and recorded outlet temperatures of 35 and 50°C, for ethanol and water, respectively. The atomization air pressure of the spray was 4 kg/cm$^2$. The flow rate of the drying air was maintained at an aspirator setting of 10, which indicated that the pressure of the aspirator filter vessel was - 25 mbar. The direction of air flow was the same as that of the sprayed product (Kumar et al., 2018; Rajesh et al., 2018).

4.1.2. Flow properties of developed S-SNEDDS formulations

The S-SNEDDS powders were further subjected for true, bulk, and tapped density, flow rate, angle of repose, Carr's compressibility index.

4.12.1. Flow rate and angle of repose

The flow rate of the powders was determined as the ratio of mass (g) to time (s) using glass funnel with an orifice diameter of 10 mm (n = 3). The angle of repose was determined using fixed funnel and free-standing cone method (Kaur et al., 2015a). On a flat horizontal surface, a graph paper was placed and a funnel was clamped above a graph paper by maintaining about 7 cm gap between paper and tip of funnel. Accurately weighed powders were poured through the funnel until the apex of the cone, thus formed, just reached the tip of the funnel. Average diameters of the base of the powder cones were determined and tangent of the angle of repose was calculated using Eq. (10) (Garg et al., 2017b; Kaur et al., 2015; Rajesh et al., 2018):

$$\tan \alpha = \frac{2h}{D}$$

Here, h = Height of the heap of powder; D = Diameter of the base of the heap of powder

4.12.2. True density

The study was carried out as reported by Beg et al., (2016). True density for blend of S-SNEDDS powder prepared by using different carriers was determined by liquid displacement method. Ethanol was used as the immiscible solvent. The study
was carried out in triplicate and mean data was recorded. True density \((\rho_T)\) was calculated as per formula given in Eq. (11) (Beg et al., 2016):

\[
\rho_T = \frac{W_1}{W_2 + W_1 - W_3 \times S_g}
\]  

(11)

Here, \(W_1\) = Weight of powder; \(S_g\) = Specific gravity of the solvent; \(W_2\) = Weight of bottle and solvent, and \(W_3\) = Weight of bottle along with solvent and powder.

### 4.12.3. Bulk density

A graduated measuring cylinder was taken and accurately weighed powder of S-SNEDDS was poured through it and bulk density was calculated by the formula given in Eq. 12 (Garg et al., 2017b; Kaur et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).

\[
\rho_b = \frac{M}{V_b}
\]

(12)

Here, \(\rho_b\) = Bulk density; \(V_b\) = Bulk volume; \(M\) = Weight of powder

### 4.12.4. Tapped density

Accurately weighed S-SNEDDS powder was taken in a measuring cylinder and the cylinder was tapped 100 times. Tapped density \((\rho_t)\) was calculated using the following formula Garg et al., 2017b; Kaur et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).

\[
\rho_t = \frac{M}{V_t}
\]

(13)

Where, \(V_t\) = Minimum volume occupied by the blend in the cylinder; \(M\) = Weight of the blend.

### 4.12.5. Compressibility index

Carr’s compressibility index (CI) was calculated using the formula given in Eq. (14) Garg et al., 2017b; Kaur et al., 2015; Kumar et al., 2018).

\[
CI = \frac{\text{Tap density} - \text{Bulk density}}{\text{Tap density}} \times 100
\]

(14)

### 4.13. Preparation of S-SNEDDS tablets

Based on the flow properties and compaction characteristics of used porous carrier, selected batch of S-SNEDDS were compressed into tablets. Wet granulation of prepared blend was carried out using 3 % w/v gelatin solution and kept for drying at 60°C. Subsequently, each of the prepared blend was compressed into tablet using 12 mm flat circular punch in a tablet compression machine with hydraulic pressure at
8 Kg/cm² (Trover Pharma Mach, India) (Garg et al., 2017b; Kaur et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).


The formulated tablets were subjected to various quality control tests as per the procedures described for tablets in pharmacopoeia (Beg et al., 2016; Garg et al., 2017b; Kumar et al., 2018; Indian Pharmacopoeia 2014; US Pharmacopoeia, 2015a, b).

Weight variation test was done by following procedure. Twenty tablets were weighed individually and their mean weights were recorded (Beg et al., 2016; Indian Pharmacopoeia, 2014). Percentage weight variation for each tablet from their mean weight was then calculated. In order to evaluate hardness, six tablets were taken from each of the prepared formulations and subjected to hardness testing (M/s Electrolab Pvt. Ltd., Mumbai, India), and the mean applied pressure (Kg/cm²) for crushing the tablet was determined (Beg et al., 2016; US Pharmacopoeia, 2015b; Rajesh et al., 2018). For friability testing, ten tablets were weighed and placed in a friability tester (M/s Electrolab Pvt. Ltd., Mumbai, India) (Beg et al., 2016; US Pharmacopoeia, 2015a). Further, the tester was rotated for 4 min at 25 rpm. After completion of rotations, dusting of friabilator was done and weight of the tested tablets was recorded. Afterwards, their percent friability was calculated.

Standard disintegration tester (Electrolab Pvt. Ltd., Mumbai, India) was used to evaluate disintegration time for the tablets. Distilled water (900 mL) maintained at 37 ± 0.5°C was used for the testing of tablets. One tablet was placed in each of six tubes of the basket and instrument was run by lifting up and lowering down the basket in water at a constant frequency rate between 29 and 32 cycles per min, through a distance of not less than 53 mm and not more than 57 mm and the tablets were observed for complete disintegration (Garg et al., 2017b; Rajesh et al., 2018).

4.15. Characterization of optimized batch of S-SNEDDS of PPK, CRM and PPK-CRM

4.15.1. Droplet size, zeta potential, percentage drug loading

Droplet size, zeta potential, percentage drug loading and thermodynamic stability of S-SNEDDS were carried out as discussed above for characterization of L-SNEDDS.
4.15.2. *In vitro* dissolution studies

PPK (30 mg), PPK-L-SNEDDS (equivalent to 30 mg PPK), CRM (equivalent to 2.75 mg), CRM-L-SNEDDS (equivalent to 2.75 mg CRM), PPK-CRM (equivalent to 30 mg PPK and 2.75 mg CRM), PPK-CRM-L-SNEDDS (equivalent to 30 mg PPK and 2.75 mg CRM) selected batch of S-SNEDDS powder and its tablets for PPK (equivalent to 30 mg PPK), CRM (equivalent to 2.75 mg CRM) and PPK-CRM (equivalent to 30 mg PPK and 2.75 mg CRM) having desirable disintegration results, were also subjected for *in vitro* dissolution studies in USP type II dissolution apparatus employing 900 mL of simulated gastric fluid (SGF) (pH 1.2) maintained at 37 ± 0.5°C, at a stirring speed of 50 ± 4 rpm. PPK, CRM, PPK-CRM, S-SNEDDS powder and L-SNEDDS were weighed and filled into size “0” hard gelatin capsules and kept in sinker (Stainless steel capsule sinker with 6 spirals, 21.3 x 9.4 mm capacity) and then subjected to dissolution apparatus (type II). Samples (5 mL) were withdrawn after 5, 10, 15, 30, 45 and 60 min, filtered using a 0.2 µm membrane filter. Filtered solutions were then centrifuged at 10000 rpm for 15 min. Supernatant was collected and analyzed for drug content. The study was carried out using six tablets and mean data (± s.d.) was recorded. It is important to note that for amount of PPK and CRM present in their individual SNEDDS sample, their absorbance was recorded at their individual wavelength (i.e. 272 nm for PPK and 420 nm for CRM). However, the amount of PPK present in mixture of PPK-CRM SNEDDS was calculated by using standard addition method where the volume of sample was kept constant and known concentration of CRM 2, 4, 6 8 and 10 µg/mL was spiked and graph was plotted between absorbance on Y axis and concentration of CRM on X axis. The plot was extrapolated to the X coordinate and the concentration of PPK was recorded (Rajesh et al., 2018; Ravishankar, 2001; Saxberg and Kowalski, 1979; Watson, 2015).

4.15.2.1. *Release kinetics of the SNEDDS formulations*

Different kinetic models were used to examine the release of the drugs from their L-SNEDDS and S-SNEDDS formulations. The model that was most suitable to define the release mechanism was selected on the basis of regression coefficient ($R^2$) value. The major benefit of this was to analyse dissolution properties using statistical
and mathematical methods (Shoaib et al., 2006). The different types of kinetic models available at the disposal of a researcher are:

i. Zero order kinetic model
ii. First order kinetic model
iii. Korsmeyer-Peppas model
iv. Hixson-Crowell model
v. Higuchi model

Zero order kinetic model

Drug release by zero order is shown when the drug release is independent of its concentration. This method is adopted to achieve pharmacologically prolonged action.

\[ C = K_0 t \]  

(15)

Where, \( k_0 \) = zero order rate constant; \( t \) = time

The graphical representation is between concentration and time.

First order kinetic model

This kinetic model is used to describe absorption and/or elimination of drug and can be expressed by the following equation:

\[ \log C = \log C_0 - K_1 t/2.303 \]  

(16)

This logarithm of drug release vs time curve will be graphically linear. This dissolution profile is generally expected for water-soluble drugs in a porous matrix and drug release is proportional to the amount of drug remaining within the matrix.

Korsmeyer-Peppas model

This simple method was developed by Korsmeyer in 1973 and it relates the drug release to the time elapsed.

\[ \frac{M_t}{M_\infty} = K t^n \]  

(17)

Where, \( K \) = drug release constant
\( t \) = release time
\( \frac{M_t}{M_\infty} \) = fraction of drug release
\( n \) = diffusion constant
**Hixson-Crowell model**

This model describes drug release with the change in surface area or particle diameter.

\[ W_0^{1/3} - W_t^{1/3} = \kappa t \]  

(18)

Where, 

- \( W_0 \) = Initial amount of drug in the formulation
- \( W_t \) = Amount of drug left after time \( t \)
- \( \kappa \) = Surface-volume relation constant
- \( t \) = time in h

**Higuchi model**

Higuchi model is used to identify drug release from inert matrices. The model describes diffusion as drug release process based on Fick’s law.

\[ f_t = K_H t^{1/2} \]  

(19)

Where, \( f_t \) = Amount of drug released in time \( t \)
- \( K_H \) = Higuchi dissociation constant

**4.15.3. Ex vivo diffusion studies by modified everted goat sac**

**4.15.3.1. Preparation of transport buffer solution**

Simulated intestinal fluid (SIF) (pH 6.8) was used. Buffer solution was prepared by dissolving calcium chloride (0.132 g), magnesium chloride (0.2438 g), disodium hydrogen phosphate (0.3406 g), sodium di hydrogen phosphate (0.0624 g), sodium bi carbonate (2.1002 g), potassium chloride (0.3726 g), sodium chloride (6.6716 g) and glucose (0.8970 g) in 900 mL distilled water (Kumar et al., 2018).

**4.15.3.2. Preparation of everted goat sac**

Permeation study was carried out using freshly isolated goat intestinal sacs, collected from local slaughter house. The experimental goat was fasted for 24 h in the slaughter house and then sacrificed to isolate its intestine. The isolated goat intestine was preserved in transport buffer. Further, the intestine was cut into 2 pieces, each about 4 cm; approximate diameter of intestine was 0.8 cm. One end of the intestine was tied up and everted and other end of the intestine was connected to a cannula in order to form a pouch. The tissue was kept alive by the supply of oxygen with the help of aerator and buffer solution; the temperature was maintained at 37±0.5 °C.
After eversion, the mucosal side was kept outwards and serosal side was kept inwards (Kumar et al., 2018; Rajesh et al., 2018).

4.15.3.3. Experimental procedure

Organ baths were taken containing buffer solution. To each of the organ baths, everted intestinal sacs were placed individually and attached to the bath. Oxygen supply was maintained through an aerator and temperature was maintained using thermostatically controlled heater. The stirrer was placed for the agitation to get the effect similar to peristaltic moments. L-SNEDDS (of PPK, CRM and PPK-CRM), selected S-SNEDDS powder (of PPK, CRM and PPK-CRM), S-SNEDDS tablet (of PPK, CRM and PPK-CRM), containing PPK (equivalent to 30 mg PPK), CRM (equivalent to 2.75 mg CRM) and PPK-CRM (equivalent to 30 mg PPK and 2.75 mg CRM) were placed separately in each organ bath containing 900 mL buffer solution. For comparison, PPK (30 mg) and CRM (2.75 mg) were suspended separately in 1 mL solution of 0.1% w/v carboxy methyl cellulose (CMC), prepared in buffer and placed separately in two organ baths containing 900 mL buffer solution. Plain buffer was placed inside the intestinal sac, so that buffer solution containing drug/formulations was present outside (mucosal side) and plain buffer was present inside (serosal side) the sac. The study was carried out for 90 min and sample (1 mL) was collected 6 times at an interval of 15, 30, 45, 60, 75, and 90 min, from the receptor compartment (serosal side of sac). Withdrawn samples were filtered through 0.2 µm membrane filter and analyzed for drug concentration. Each study was carried out in triplicate and mean data was recorded. It is important to note that for amount of PPK and CRM present in their individual SNEDDS sample, their absorbance was recorded at their individual wavelength (i.e. 272 nm for PPK and 420 nm for CRM). However, the amount of PPK present in mixture of PPK-CRM SNEDDS was calculated by using standard addition method where the volume of sample was kept constant and known concentration of CRM 2, 4, 6 8 and 10 µg/mL was spiked and graph was plotted between absorbance on Y axis and concentration of CRM on X axis. The plot was extrapolated to the X coordinate and the concentration of PPK was recorded (Ravishankar, 2001; Saxberg and Kowalski, 1979; Watson, 2015). The permeation profile was constructed by plotting % drug permeated through sac versus time (min) (Kumar et al., 2018; Rajesh et al., 2018).
4.15.4. TEM

TEM studies were performed in order to detect the droplet morphology of the selected S-SNEDDS formulation (PPK S-SNEDDS, CRM S-SNEDDS and PPK-CRM S-SNEDDS). The instrument used for scanning was H-7500, Hitachi, Japan. The procedure was carried out as reported by Inugala, et al., 2015. Selected S-SNEDDS formulation (20 mg) was diluted with 10 mL of distilled water. For negative staining of sample, a drop of emulsion was placed onto a carbon-coated copper grid to leave a thin film and excess of solution was drained off by using filter paper. After 10 min, a drop of phosphotungstic acid (PTA) solution (2 % w/v) was dripped on the copper grid for about 1 min and excess of solution was drained. The grid was allowed to air dry thoroughly and sample was viewed using TEM (Garg et al., 2017b; Inugala et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).

4.15.5. SEM analysis of S-SNEDDS

The surface morphology of the PPK, CRM, PPK-CRM, Aerosil® 200, physical mixture, PPK S-SNEDDS, CRM S-SNEDDS and PPK-CRM S-SNEDDS were visualized through SEM. SEM was carried out as reported in Kaur et al., 2015 and Renuka et al., 2014. A metallics tub with double-sided conductive tape of 12 mm diameter was taken and the samples were fixed over it. A Supra 35VP (Oberkochen, Zeiss, Germany) data station with an acceleration voltage of 10.00 kV and a secondary detector was used in the study (Kaur et al., 2015; Renuka et al., 2014; Rajesh et al., 2018).

4.15.6. DSC analysis

The thermal characterization was carried out for PPK, CRM, PPK-CRM, Aerosil® 200, Labrafil® M1944 CS, Tween 80. Transcutol® P and PPK S-SNEDDS, CRM S-SNEDDS, PPK-CRM S-SNEDDS. About 1 mg of each drug and optimized formulations were crimped separately in an aluminium pan. Each sample was heated from 0 to 300°C at a heating rate of 10°C/min under a stream of nitrogen at a flow rate of 50 mL/min. An empty aluminium pan was used as reference. The melting points (T_m) were determined using TA-Universal Analysis 2000 software (version 4.7A). DSC tracings were recorded in DSC (DSC Q200 TA, Universal. V24.4 SOFTWARE) (Kaur et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).
4.15.7. **PXRD studies**

The PXRD pattern of PPK, CRM, PPK-CRM, Aerossil® 200, PPK S-SNEDDS, CRM S-SNEDDS and PPK-CRM S-SNEDDS was recorded using an X-ray diffractometer (Bruker axs, D8 Advance) with Cu line as the source of radiation. Standard runs using a 40-kV voltage, a 40-mA current, at a scanning rate of 0.010°min⁻¹ over a 2θ range of 3–45° were used (Garg et al., 2017b; Kaur et al., 2015; Kumar et al., 2018; Rajesh et al., 2018).

4.16. **Stability Study**

4.16.1. **Thermodynamic stability**

Thermodynamic stability of S-SNEDDS was done as discussed above in section 4.8.2, based on cloud point.

4.16.2. **Robustness to dilution and pH change**

Dilution stability study was conducted in four different media - water, simulated gastric fluid (SGF) (pH 1.2), acetate buffer (pH 4.5), and simulated intestinal fluid (SIF) (pH 6.8), in order to evaluate the effect of dilution on droplet size of optimized batches of S-SNEDDS of PPK, CRM and PPK-CRM. S-SNEDDS sample (1 mL) was diluted to 10, 100, 250, 500 and 900 times with each of the medium. The diluted samples were subjected to mean droplet size analysis. Each study was carried out in triplicate and mean data was recorded (Garg et al., 2017b; Rajesh et al., 2018).

4.16.3. **Stability studies of S-SNEDDS**

The stability studies were carried out for selected optimized S-SNEDDS of PPK, CRM and PPK-CRM powder and tablets in refrigerator at 5°C ± 3°C, 25°C ± 2°C/65 % R.H. ± 5% R.H., and 40°C ± 2°C/75 % R.H. ± 5% R.H., in stability chamber (Remi electro technique, Mumbai, India) for 180 days. The aged samples were analyzed and compared with results of freshly prepared S-SNEDDS for their droplet size analysis (after dilution in 500 mL SGF (pH 1.2), drug precipitation, assay, hardness, and friability at the end of 7th, 30th, 60th, 90th, 120th and 150th and 180th days. The results of their freshly prepared powder and tablets were considered as time zero and used as a standard to evaluate various parameters of stability studies (Garg et al., 2017b; Rajesh et al., 2018).
The dissolution profiles of fresh and aged optimized S-SNEDDS that were kept at three different temperatures, were compared at the end of six month (180th day) in 900 mL of SGF (pH 1.2) as dissolution medium. The procedure to carry out dissolution has been discussed above in previous section 4.15.2. Each study was carried out six times and mean data (± sd) was recorded.

4.17. **In vivo studies**

The results of dissolution studies revealed no significant difference in the drug release profile of L-SNEDDS, S-SNEDDS powder and S-SNEDDS tablets. Hence, in order to have ease of dose administration in rats, S-SNEDDS powders were used for in vivo studies.

4.17.1. **Experimental animals**

Albino Wistar male rats 7-8 weeks old, weighing 150–200 g, were purchased from National Institute of Pharmaceutical Education and Research (NIPER), Mohali, India for the present study. Rats were housed in polypropylene cages lined with husk in standard environmental conditions (temperature 25±2°C, relative humidity 55±10% and 12:12 light: dark cycle). The animals were fed with standard pellet diet and water ad libitum. The experimental protocol was subjected to the scrutiny and ethical clearance was obtained from Institutional Animal Ethics Committee of School of Pharmaceutical Sciences, Lovely Professional University (LPU/LSPS/IAEC/CPCSEA/Meeting No 5/2014/2015 Protocol No. 11. and LPU/LSPS/IAEC/CPCSEA/Meeting No 2/2016/2017 Protocol No. 1.) before beginning of the experiment (Garg et al., 2017b).

4.17.2. **Study design**

The experimental groups were comprised of non-diabetic control rats, diabetic control rats and diabetic rats treated with: I) Non-diabetic control rats administered with cold 0.1 M sodium-citrate buffer (pH 4.5). II) Diabetic control rat injected with 55 mg/kg Streptozotocin (STZ) in cold 0.1 M sodium-citrate buffer (pH 4.5).III) STZ induced diabetic rats treated with PPK S-SNEDDS formulation at low dose (400 mg/Kg). IV) STZ induced diabetic rats treated with PPK S-SNEDDS formulation at high dose (800 mg/Kg).V) STZ induced diabetic rats treated with naïve PPK suspended in 0.5% carboxy methyl cellulose (CMC) at low dose (400 mg/Kg).VI) STZ induced diabetic rats treated with naïve PPK suspended in 0.5% CMC at high
dose (800 mg/Kg). VII) Placebo of S-SNEDDS formulation. VIII) Glipizide (0.5 mg/Kg) suspended in 0.5% CMC. IX) STZ induced diabetic rats treated with naïve CRM suspended in 0.5% CMC at low dose (40 mg/Kg). X) STZ induced diabetic rats treated with naïve CRM suspended in 0.5% CMC at high dose (80 mg/Kg). XI) STZ induced diabetic rats treated with naïve PPK suspended in 0.5% CMC at low dose (800 mg/Kg). XII) STZ induced diabetic rats treated with CRM S-SNEDDS formulation at low dose (40 mg/Kg). XIII) STZ induced diabetic rats treated with CRM S-SNEDDS formulation at high dose (80 mg/Kg). XIV) STZ induced diabetic rats treated with PPK-CRM S-SNEDDS formulation at low dose (PPK: 400 mg/Kg; CRM; 40 mg/kg). XV) STZ induced diabetic rats treated with PPK-CRM S-SNEDDS formulation at half of low dose (PPK: 200 mg/Kg; CRM; 20 mg/kg). The study design is shown in Table 22.

4.17.3. **Experimental induction of diabetes**

After overnight fasting, diabetes was induced by intraperitoneal (i.p.) injection of STZ which was dissolved in 0.1 M cold sodium citrate buffer (pH 4.5), at a dose of 55 mg/kg. Blood samples were collected before the administration of STZ and after seven days of its administration. Animals with fasting blood glucose above 250 mg/dL were considered as diabetic and were included in the study (Garg et al., 2017b).

| Table 22 |
| Study design for *in vivo* antidiabetic activity in rats |

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Treatment (doses)</th>
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<tbody>
<tr>
<td>I</td>
<td>Non-diabetic control rats administered with cold 0.1 M sodium-citrate buffer (pH 4.5)</td>
<td>N</td>
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<tr>
<td>II</td>
<td>Diabetic control rat injected with 55 mg/kg Streptozotocin (STZ) in cold 0.1 M sodium-citrate buffer (pH 4.5)</td>
<td>EC (D)</td>
</tr>
<tr>
<td>III</td>
<td>STZ induced diabetic rats treated with SNEDDS-PPK formulation at low dose (400 mg/Kg)</td>
<td>PPK SNEDDS-L+(D)</td>
</tr>
<tr>
<td>IV</td>
<td>STZ induced diabetic rats treated with SNEDDS-PPK formulation at high dose (800 mg/Kg)</td>
<td>PPK SNEDDS-H+(D)</td>
</tr>
<tr>
<td>V</td>
<td>STZ induced diabetic rats treated with naïve PPK suspended in 0.5% carboxy methyl cellulose (CMC) at low dose (400 mg/Kg)</td>
<td>Naive PPK -L+(D)</td>
</tr>
<tr>
<td>VI</td>
<td>STZ induced diabetic rats treated with naïve PPK suspended in 0.5% CMC at high dose (800 mg/Kg)</td>
<td>Naive PPK -H+(D)</td>
</tr>
<tr>
<td>VII</td>
<td>Placebo of SNEDDS-PPK formulation</td>
<td>Placebo SNEDDS+(D)</td>
</tr>
<tr>
<td>VIII</td>
<td>Glipizide (0.5 mg/Kg) suspended in 0.5% CMC</td>
<td>Glipizide+(D)</td>
</tr>
<tr>
<td>IX</td>
<td>STZ induced diabetic rats treated with naïve CRM suspended in 0.5% CMC at low dose (40 mg/Kg)</td>
<td>Naive CRM L-(D)</td>
</tr>
<tr>
<td>X</td>
<td>STZ induced diabetic rats treated with naïve CRM</td>
<td>Naive CRM H -(D)</td>
</tr>
</tbody>
</table>
suspended in 0.5% CMC at high dose (80 mg/Kg)

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
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<tbody>
<tr>
<td>XI</td>
<td>STZ induced diabetic rats treated with naïve PPK suspended in 0.5% CMC at low dose (800 mg/Kg) Naive PPK –CRM L-+(D)</td>
</tr>
<tr>
<td>XII</td>
<td>STZ induced diabetic rats treated with SNEDDS-CRM formulation at low dose (40 mg/Kg) CRM S-SNEDDS- L -+(D)</td>
</tr>
<tr>
<td>XIII</td>
<td>STZ induced diabetic rats treated with SNEDDS-CRM formulation at high dose (80 mg/Kg) CRM SNEDDS- H -+(D)</td>
</tr>
<tr>
<td>XIV</td>
<td>STZ induced diabetic rats treated with SNEDDS-PPK-CRM formulation at low dose (PPK: 400 mg/Kg; CRM; 40 mg/kg) PPK- CRM SNEDDS L -+(D)</td>
</tr>
<tr>
<td>XV</td>
<td>STZ induced diabetic rats treated with SNEDDS-PPK-CRM formulation at low dose/2 (PPK: 200 mg/Kg; CRM: 20 mg/kg) PPK-CRM SNEDDS--L/2-+(D)</td>
</tr>
</tbody>
</table>

4.17.4. Drug administration

After seven days of STZ induction, the formulations were administered orally through intra-gastric tube at different doses as described in Table 7. In order to achieve low dose of S-SNEDDS formulation (i.e. 80 mg/day), an amount of powder equivalent to 20 mg of PPK was suspended in 15 mL of 0.5% w/v CMC solution and administered 4 times a day through intra gastric tube. Similarly, for achieving high dose of S-SNEDDS formulation (i.e. 160 mg/day), an amount of powder equivalent to 40 mg of PPK was administered 4 times a day, using the same procedure. Similarly dose of CRM was calculated.

Overnight fasted animals were analyzed weekly for their body weights and blood glucose level. After completion of the experimental duration, the animals were fasted for overnight and blood was collected for various biochemical estimations. Terminal sacrifice was carried out by cervical decapitation and pancreas was dissected out. The dissected pancreas was immediately rinsed in ice cold saline and paraffin sections of pancreatic, tissues were prepared. These were stained with hematoxylin and eosin to observe histopathological changes (Chandran et al., 2016).

4.17.5. Biochemical determinations

Rats were anaesthetized by using diethyl ether and blood was withdrawn by puncturing their retro-orbital plexus. In order to develop haematogram (hemoglobin estimation), whole blood was collected into pre-filled ethylene diamine tetra-acetic acid (EDTA) bottles whereas, for biochemical analysis, blood samples were collected in the plain Eppendorf tubes. Blood was allowed to clot and centrifuged at 1000 rpm for 10 min to collect serum. Collected serum was stored in deep freezer at -20°C till
Liver function test was performed using serum biomarkers such as; alkaline phosphatase (ALP), aspartate aminotransferase (AST), Alanine transaminase (ALT). Lipid profile was carried by estimation of serum triglycerides (TG), total cholesterol (TC) and high density lipoprotein (HDL). All estimations were done using kits of Erba Diagnostics, India (Arunachalam and Parimelazhagan, 2013; Garg et al., 2017b). Total haemoglobin was determined using whole blood by commercially available kits.

**4.17.6. Estimation of in vivo antioxidants**

The kidneys were isolated, weighed and washed in ice-cold saline for removal of blood. Isolated kidneys were sliced into pieces and homogenized (Glass-Teflon pot-ter homogenizer, Thomas Scientific, USA) using buffer (0.025 M Tris–HCl buffer of pH 7.5). Tissue homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was separated out and used for various antioxidant enzyme estimations (Garg et al., 2017b). Protein concentrations were estimated by the method of Lowry et al. (1951).

Liver homogenates were prepared in ice-cold 10% (w/v) potassium chloride solution, levels and activities of various markers were measured. These include: catalase (CAT) (Sinha et. al., 1972), lipid peroxidation (LPO) (Ohkawa et al., 1979), and reduced glutathione (GSH) (Ellman et al., 1959).

**4.17.7. Histopathological studies**

Paraffin sections of pancreas tissues were made and stained with haematoxylin and eosin to observe histopathological changes (Garg et al., 2017b).

**4.18. Statistical analysis**

All the experimental data expressed as mean ± standard deviation (sd). Statistical analysis of obtained data was carried out either by analysis of variance or Tukey’s multiple comparison test using GraphPad Prism version 7.0 (GraphPad Software Inc., CA, USA). A value of P < 0.05 indicated significant difference in the obtained results. The dissolution profiles of various formulations were compared using model independent analysis (F<sup>2</sup> comparison) as discussed by Shah et al., 1998 (Garg et al., 2017b; Shah et al., 1998).