Development of Oral Lipid Based Nano-formulation of Dapagliflozin: Optimization, *in vitro* Characterization and *ex vivo* Intestinal Permeation Study

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Abstract: The oral route is the most prevalent route of drug administration among various routes. Dapagliflozin is an oral hypoglycemic drug used for lowering the blood glucose level. The objective of this work is to developed and optimized dapagliflozin loaded nanostructured lipid carriers (DG-NLCs) for the improvement of oral delivery. DG-NLCs were prepared by a high-pressure homogenization method (hot) and optimized by Box-Behnken design software using lipid, surfactant, and homogenization cycle as an independent variable. DG-NLCs were further evaluated for particle size (Y₁), entrapment efficiency (Y₂), drug release (Y₃). The DG-NLCs were further evaluated for morphology, thermal and X-ray diffraction analysis, *ex-vivo* intestinal permeation, and stability study. Particle size (nm), entrapment efficiency (%) and drug release (%) of all seventeen formulations were found in the range of 113.71-356.22 nm, 60.43-96.54% and 63.44-83.62% respectively. Morphology of optimized formulation exhibited spherical in shape confirmed by transmission electron microscopy. Thermal and X-ray diffraction analysis of NLCs showed the drug was solubilized and lost the crystallinity. DG-NLCs-opt exhibited dual release pattern initial fast and later sustained-release (90.01±2.01% in 24 h) whereas DG-dispersion showed 31.54±1.87% release in 24 h. Korsmeyer-Peppas model was found to be the best fit model (R²=0.999). The DG-NLCs-opt exhibited significant-high (p<0.05, 1.293 µg/cm²/h) flux than DG-dispersion (0.2683 µg/cm²/h). Apparent permeation coefficient of DG-NLCs-opt was found to be significantly higher (p<0.05, 4.14×10⁻⁵ cm/min) than DG-dispersion (8.61×10⁻⁶ cm/min). The formulation showed no significant changes (p<0.05) on six months of storage study at 25±2°C/60±5%RH. The finding concluded that quality by design (QbD) based lipid nanocarrier for oral delivery could be a promising approach of dapagliflozin for the management of diabetes.

Key words: diabetes, nanostructured lipid carrier, Box-Behnken design, *ex vivo* intestinal permeation study, stability study

1 Introduction

Diabetes (type 2, diabetes mellitus) is a group of metabolic disease in which the blood glucose level increase (≥200 mg/dL) for a long period. Due to high blood glucose level, many other symptoms appeared simultaneously like more frequent urination, weight loss, increased hunger, dehydration, as well as the alteration in lipid profile, liver function test and kidney function test. The associated complication may occur like heart attack, kidney failure and damaged eye in chronic and serious condition. In worldwide approx. 422 million peoples were affected and about 1.6 million deaths occurred in the low and middle-income countries in every year¹. Various oral dosage form available in the market to reduce the blood glucose level (hyperglycemia) like pioglitazone, dapagliflozin (DG), metformin, glimepiride, glipizide, nateglinide with a different mechanism of action². In which DG is a new therapeutic molecule has good antidiabetic activity. Chemically it is (2S,3R,4R,5R,6R)-2-[4-Chloro-3-(4-ethoxybenzyl)phenyl]-6-hydroxymethyl (tetrahydro-2H-pyranyl)-3,4,5-triol. It lowers the blood glucose level by inhibiting the sodium-glucose transporter-2 (SPT-2) of the kidney (proximal tube of nephron) and glucose was excreted through the urine³. Low solubility is the main problem associated with new therapeutic molecules. It is white crystalline powder and has shown poor solubility (0.173 mg/mL). The oral route is a most common route for the administration of therapeutics but have major problem associated with poor or spar-
ingly soluble therapeutic molecule because it directly affects on bioavailability and dose proportionality\(^4\). In those cases, permeability/dissolution are the rate-limiting step for drug absorption.

Now a day’s nanotechnology-based drug delivery system are more attractive and attentive technology for improvement of solubility and bioavailability of poorly/sparsely soluble therapeutics. This technology includes that, semi-nanoemulsifying system of dapagliflozin\(^5\), polymeric nanoparticles of insulin\(^6\) micelles delivery of repaglinide\(^7\), solid lipid nanoparticle of pioglitazone\(^8\) and nanostructured lipid carrier of\(^9\) and showed the acceptable result.

Now from the past few years, lipid-based nanoformulation has shown more attention because it contains natural or synthetic lipid. The lipids are compatible with bio-lipids, biodegradable, non-toxic in nature and can provide the extended (control/sustained) release of the drug\(^10\). The lipid formulation (SLN, NLCs) may improve oral absorption of the drug by enhancement of dissolution in the intestine, diminish gastric emptying rate, prevent the effect of food, amplify mucosal permeability, prevent degradation of drug by enzyme, and endorse the lymph flow rate\(^11\). So lipid-based nanosystems can increase oral absorption to systemic circulation through lymphatic system and granted the comprehensive mechanism of therapeutics enrichment in gastrointestinal tract.

Along with lipid nanoformulation (NLCs) is a novel second-generation colloidal delivery system composed of solid as well as liquid lipid. NLCs system has overcome the problem associated with SLN like low drug loading, aggregation of particle, escaping of the drug on long term storage stability and crystallization of lipid\(^12\).

NLCs formulation also exhibited sustained and prolonged release of a drug than SLN. There are various previous work reported of NLCs for oral delivery of poorly/sparsely soluble drug for the improvement of drug load, sustained release and bioavailability i.e., NLCs delivery of ezetimibe\(^13\), saquinavir\(^14\) hydroxymethyl-nitrofurazone\(^15\), baicalin\(^16\) and exhibited the remarkable response. A few works were reported for DG i.e., solid self nano emulsifying system\(^17\), magnetic nanoparticle\(^18\), and sustained-release tablet\(^19\) for improvement of drug delivery.

The objective of this work is to formulate the DG loaded NLCs using solid, liquid lipid and surfactant and optimized by using quality by design (Box-Behnken design) software. Box-Behnken design is a novel tool for optimization of formulation and determines the influence of formulation variable like concentration of lipid, surfactant and others over the responses like particle size, entrapment efficiency(%) and drug release(%)\(^20\), etc. Further, formulation was evaluated for in vitro characterization, ex vivo permeation and stability study.

2 Material and Method

2.1 Material

Dapagliflozin was procured from Jamjoom pharmaceutical (Jedda KSA). Oleic acid, Castor, Peanut, Olive and Soyabean oil were procured from SD-fine (Mumbai, India). Tween 20 and Tween 80 were obtained from Sigma Aldrich (St Louis, Missouri USA). PEG200, Limonene, were procured from across organic, India. Labrasol, miglyol 812, Capmul MCM and Cremophore EL were obtained from Gattefosse Pvt Ltd (Mumbai, India). HPLC grade acetonitrile, methanol and water were obtained from Sigma Aldrich (St Louis, Missouri USA). Dialysis bag (MW cut off 12000-140000 Da, 2.4 nm pore size) procured from Himedia (Mumbai, India). All other chemicals used for the experiment were of analytical grade.

2.2 Method

2.2.1 Selection of appropriate lipids

Selection of solid and liquid lipids was done on maximum solubility of DG in it. 1 mL of liquid lipid(Oleic acids, Castor oil, Peanut oil, Olive oil, Soyabean oil, Labrasol, miglyol 812, Capmul MCM) into 2 mL of Eppendorf and excess amount of DG was added and vertex it for 15 min then stand for 72 h into orbital shaker. Same ways the excess amount of DG added in 1 g of melted solid lipid(5°C above the melting point, Glyceryl Monostearate stearic acid, Myristic acid, Palmitic acid) under continuous stirring (Magnetic stirrer). When transparency of the mixture was loosed, indicated the saturation solubility of DG. The mixture was centrifuged (5000 rpm, 15 min, Remi-centrifuge). The supernatant was separated and concentration of DG was analyzes by UV-Vis-spectrophotometer (Shimadzu 1800, Kyoto, Japan) at 235 nm after appropriate dilution.

2.2.2 Selection of appropriate surfactant

Transfer 1 mL of surfactant (Tween 20, Tween 80, PEG200, Limonene, and Cremophore EL) into an Eppendorf and the excess amount of DG was added and vertex it for 15 min. The mixture was stand for 72 h in an orbital shaker. The mixture was centrifuged at 5000 rpm for 15 min and then supernatant was carefully separated. The concentration of DG was measured by UV-Vis-spectrophotometer (Shimadzudzu, 1800, Tokyo, Japan) at 235 nm after appropriate dilution.

2.2.3 Determination of binary phase miscibility

Miscibility of binary system was determined by mixing of solid and liquid lipid to each other. The solid and liquid lipid fused at 90°C (water bath) in the different ratio(9:1, 8:2, 7:3) with continuous stirring (magnetic stirrer). The fused mixture was stand for 3 h for complete crystallization and observe visually of any phase separation, and breaking. The fused mixture showed only one phase system selected as best binary system.
2.2.4 Development of NLCs of DG

The NLCs of DG (DG-NLCs) was developed by the most commonly used high-pressure homogenization method (hot)\(^2\). Before passing of formulation into a high-pressure homogenizer, the melted selected solid, liquid lipid and drug mixed was added dropwise into an aqueous solution of selected surfactant (hot) under continuous stirring to form the primary emulsion. The developed primary emulsion passed into high-pressure homogenizer (Homogenizer, FPG12800, Harlow Essex, CM19, UK) at different cycle at the same pressure for the formation of the NLCs.

2.2.5 Optimization by statistical experimental design

Three factors and three levels of Box-Behnken statistical design was applied for optimization of DG-NLCs because it gives an appropriate number of experimental run. Lipid design was applied for optimization of DG-NLCs because it gives an appropriate number of experimental run. Lipid ratio, surfactant concentration and homogenization cycle were used as independent variable whereas particle size (PS, nm, \(Y_1\)), entrapment efficiency (EE, \(Y_2\)) and drug release (\(Y_3\)) selected as the dependent variable. Seventeen experimental runs with five repeated formulation were obtained from the software. All experiment runs were performed and the dependent variable data fits into the software. All responses (\(Y_1\), \(Y_2\), & \(Y_3\)) data were fitted into different model i.e., linear, second-order, cubic, and quadratic for identification of best fit model. Analysis of variance (ANOVA) and statistical regression analysis was analyzed for all models. Three dimensional (3D) and contour plot were plotted for determination of interaction effect of factors over the individual response.

2.3 Characterization of DG-NLCs

2.3.1 Particle size, polydispersibility index (PDI)

PS and PDI of DG-NLCs were analyzed by zeta sizer (Malvern Zetasizer, HAS3000, Grovewood, Malvern, UK). The diluted sample was filled into cuvette and measured at 90° scattered angle against water as a solvent.

2.3.2 Morphological examination

Morphological examination of optimized DG-NLCs (DG-NLCs-opt) formulation was performed by transmission electron microscopy (TEM, Philip Cm-10, Eindhoven, Holland) as per the previously prescribed method\(^3\).

2.3.3 Entrapment efficiency (EE)

% EE of DG in DG-NLCs was analyzed by ultracentrifugation technique\(^4\). Formulation was centrifuged at 18000 rpm (Remi 24-Mumbai, India) and concentration of DG in the supernatant was analyzed by UV-Vis spectroscopy at 235 nm. % EE of DG in NLCs was calculated by the following equation.

\[
EE(\%) = \frac{\text{Initial drug added} + \text{(Drug in supernatant + Drug in washing liquid)}}{\text{Initial drug added}} \times 100
\]

2.3.4 Thermal analysis

Thermal analysis of DG, GM and DG-NLCs-opt were done by using differential scanning calorimetry instrument (DSC, Pyris 6, Perkin Elmer, Norwalk, CT, USA). The appropriate amount of sample was packed in DCS aluminium pan and blank aluminium pan was taken as blank. Sample was scanned between 0 to 300°C at 10°C heating rate. The nitrogen gas was flow continuously at 0.2 mL/min. The re-crystallization index (RI) was calculated by given formula\(^5\).

\[
RI = \frac{\text{Enthalpy of fusion NLC}}{\text{Enthalpy of fusion GM} \times \text{fraction of lipid phase}} \times 100
\]

2.3.5 X-ray diffraction analysis (XRD)

Crystallinity of DG, GM and DG-NLCs-opt was analyzed by using XRD instrument (Ultima 1V, Auto-sampler, Diffractometer, Rigaku’s, Texas, USA). Appropriate quantity of sample place in a sample holder and sample was scanned at 0.5 degree/min in range of 3-60° (2tetha). The X-rays were applied using Cu/40 kV/40 mA in step mode.

2.3.6 In vitro release study

Dialysis bag technique was used for in vitro release of DG from DG-NLCs. 1 mL of NLCs formulation (equivalent to 5 mg of DG) was filled in a dialysis bag (MW cut off 12000-140000 DA, 2.4 nm pore size), sealed and kept into a 100 mL of released media (Phosphate buffer, pH6.8). Media was continuous stirring (75 rpm, Magnetic stirrer) and maintained at 37±0.5°C during whole study. At a predetermined time interval (0.5, 1, 2, 3, 4, 5, 6, 8, 12, and 24 h), 5 mL of sample was taken and replaced simultaneously same volume with fresh release media to maintained the sink condition (concentration gradient). The concentration of DG was analyzed by UV-Vis-spectrophotometry at 235 nm. The release of DG form NLCs in release media was statically analysed\(^5\). The in vitro release of pure DG-dispersion was performed as comparative analysis. Study was conducted in triplicate.

2.3.7 Kinetic release study

In vitro release profile of DG-NLCs-opt was fitted into different kinetic release model like zero, first-order, Higuchi, Korsmeyer-Peppas and Hixon-Crowell. The regression coefficient parameter was used for selection of best fit kinetic release model.

2.3.8 Ex vivo intestinal permeability study

Intestinal permeability of DG-NLCs-opt and pure DG-dispersion was performed by a previously reported method\(^5\). The rat intestinal sac was taken immediately after sacrificed. The DG-NLCs-opt and DG suspension (5 mg) were filled into mucosal side of the intestinal sac. The sac was tightly closed on both end and immersed into Krebs solution in a beaker. The complete system maintained at 37°C throughout study with continuous stirring and airation (95% oxygen, up to 1 h). 2 mL of aliquot was withdrawn at a definite time interval (0, 10, 20, 40, 60 min) and filtered by a membrane filter (0.25 μm). The concentration was determined using previous developed validated HPLC\(^5\). Flux was calculated through the plot between permeated amount Vs time. The apparent permeability co-

\[
J. Oleo Sci. 69, (11) 1389-1401 (2020)
\]

1391
efficient (APC) was calculated through the following formula.

\[
\text{APC} = \frac{\text{Flux}}{\text{Area} \times \text{initial conc of drug}}
\]

2.3.9 Stability study

Stability study of DG-NLCs-opt was performed as per ICH guideline at 25°C/60% RH for 180 days. The sample was placed in borosilicate glass vial into a stability chamber. At a definite time interval (0, 30, 60, 90, 180 days) sample was withdrawn and analyzed for physical appearance (phase separation, caking) PS, PDI, and EE (%).

2.3.10 Statistical analysis

The data were expressed in mean ± SD. Box-Behnken design was used for optimization. Graph pad prism was used for statistical analysis. \( P < 0.05 \) was considered as significant level.

3 Results and Discussion

3.1 Selection of appropriate lipids

In order to select solid and liquid lipids for development of NLCs was done on the basis of maximum solubility of DG. Solubility profile of DG in various lipids is depicted in Figs. 1A-B. Order of DG solubility in various liquid lipids is Labrasol > Miglyol 812 > Capmul MCM > Soyabean oil > Olive oil > Castor > Oleic acids > Peanut oil, and for solid lipids is Glyceryl Monostearate > Stearic acid > Palmitic acid > Myristic acid. Maximum solubility of DG was found in Labrasol (liquid lipid, 25.84 ± 1.02 mg/g) and in Glyceryl Monostearate (GM, solid lipid, 38.54 ± 2.08 mg/g).

3.2 Selection of surfactant

The screening of surfactant was done on the basis of DG solubility. Solubility profile of DG in various surfactants was expressed in Fig. 1C. Order of solubility of DG in surfactant is Tween 20 > Tween 80 > PEG200 > Cremophore EL > Limonene. Maximum solubility of DG (23.14 ± 2.65 mg/g) was found in Tween-20 and selected for development of NLCs. Tween-20 is a non-ionic surfactant and physiologically non-toxic for the human body (GRASS). It is a hydrophilic type (HLB = 16.7) of surfactant and having high emulsification capacity.

3.3 Optimization

Preliminary prepared DG-NLCs were optimized by QbD software (Box-Behnken design). All seventeen formulations with five centre point* and their responses were given in Table 1. Data of all responses of each were fitted into various models like linear, 2\textsuperscript{nd} order and quadratic. The quadratic model was found to best fit for all responses because it has a high value of regression coefficient than other. The experimental and predicted value of each response was found to very close to each other, it indicated high value of regression coefficient and well fitted and it proved by previously published research\textsuperscript{24}. ANOVA of best-fitted model (quadratic) of every response (PS, EE and drug release) were calculated and expressed in Table 2. \( P < 0.0001 \) was found for a quadratic model of all responses indicated that model was significant. The lack of fit of model was found to be non-significant \( (p > 0.05) \), indicated...
Table 1  Formulation composition of NLCs and actual and predicted value of responses.

| Standard Order | Total lipid ratio (%) | Surfactant (%) | Homogenization cycle | Particle size (nm) | EE (%) | Drug release (%) |
|----------------|----------------------|---------------|----------------------|-------------------|--------|------------------|
|                |                      |               |                      | Actual Value      | Predicted Value | Actual Value | Predicted Value | Actual Value | Predicted Value |
| 1              | 7.00                 | 1.00          | 4.00                 | 285.69            | 285.54           | 68.35       | 68.18           | 76.16       | 76.03           |
| 2              | 9.00                 | 1.00          | 4.00                 | 356.22            | 356.13           | 80.71       | 80.89           | 63.44       | 63.25           |
| 3              | 7.00                 | 5.00          | 4.00                 | 146.72            | 146.81           | 89.02       | 88.84           | 80.63       | 80.83           |
| 4              | 9.00                 | 5.00          | 4.00                 | 159.33            | 159.48           | 96.54       | 96.72           | 76.83       | 76.96           |
| 5              | 7.00                 | 3.00          | 2.00                 | 238.53            | 238.53           | 71.98       | 72.12           | 72.12       | 72.15           |
| 6              | 9.00                 | 3.00          | 2.00                 | 275.17            | 275.11           | 87.54       | 87.32           | 68.32       | 68.41           |
| 7              | 7.00                 | 3.00          | 6.00                 | 177.74            | 177.80           | 91.35       | 91.57           | 83.06       | 82.97           |
| 8              | 9.00                 | 3.00          | 6.00                 | 224.51            | 224.50           | 97.12       | 96.97           | 70.09       | 70.05           |
| 9              | 8.00                 | 1.00          | 2.00                 | 336.78            | 336.93           | 60.43       | 60.46           | 67.93       | 68.04           |
| 10             | 8.00                 | 5.00          | 2.00                 | 180.96            | 180.87           | 78.03       | 78.07           | 79.31       | 79.08           |
| 11             | 8.00                 | 1.00          | 6.00                 | 292.79            | 292.88           | 74.43       | 74.38           | 75.83       | 76.06           |
| 12             | 8.00                 | 5.00          | 6.00                 | 113.71            | 113.56           | 93.31       | 93.27           | 83.62       | 83.52           |
| *13            | 8.00                 | 3.00          | 4.00                 | 230.54            | 231.63           | 88.65       | 88.29           | 78.56       | 78.57           |
| *14            | 8.00                 | 3.00          | 4.00                 | 231.87            | 231.63           | 88.35       | 88.29           | 78.96       | 78.57           |
| *15            | 8.00                 | 3.00          | 4.00                 | 231.98            | 231.63           | 88.29       | 88.29           | 78.34       | 78.57           |
| *16            | 8.00                 | 3.00          | 4.00                 | 231.87            | 231.63           | 88.05       | 88.29           | 78.15       | 78.57           |
| *17            | 8.00                 | 3.00          | 4.00                 | 231.87            | 231.63           | 88.12       | 88.29           | 78.87       | 78.57           |

*Centre point: having same composition.

Table 2  ANOVA results of optimized quadratic model of all responses.

| Result (ANOVA) | Particle size (nm, Y₁) | Entrapment efficiency (%) (Y₂) | Drug release (%) (Y₃) |
|----------------|------------------------|---------------------------------|----------------------|
| Regression     |                        |                                 |                      |
| Sum of squares | 67045.51               | 1682.22                         | 505.44               |
| Degrees of freedom (df) | 9                      | 9                               | 9                    |
| Mean squares   | 7449.50                | 186.91                          | 56.16                |
| F-value        | 32325.99               | 2638.43                         | 548.00               |
| P              | < 0.0001               | < 0.0001                         | < 0.0001             |
| Lack of fit tests |                        |                                 |                      |
| Sum of squares | 0.13                   | 0.28                            | 0.25                 |
| Degrees of freedom (df) | 3                      | 3                               | 3                    |
| Mean squares   | 0.043                  | 0.092                           | 0.082                |
| F-value        | 0.12                   | 1.68                            | 0.70                 |
| P              | 0.9457                 | 0.3081                          | 0.6012               |
| Residual      |                        |                                 |                      |
| Sum of squares | 1.61                   | 0.50                            | 0.72                 |
| Degrees of freedom (df) | 7                      | 7                               | 7                    |
| Mean squares   | 0.23                   | 0.071                           | 0.10                 |
less variation in actual and predicted value and model is well fitted as well as independent factors are extensive effect on responses. Regression value ($R^2$) of all applied models were given in Table 3 and found to be maximum for quadratic ($R^2 = 0.9999$). The polynomial equation (Table 3), 3-D and contour plots for each response were generated and expressed in Figs. 2A-C. It expressed the contribution of variables over individual response and expressed by Supplementary Figure (Figs. SF1A-C) and have high value of regression coefficient ($R^2 = $ very close to one), indicating both values not have significantly different ($p > 0.05$, lack of fit nonsignificant). It represents the magnitudinal difference between an observed value and predicted value. 

3.3.1 Effect of independent variables on response particle size ($Y_1$)

Influences of factors i.e., lipid ratio ($A$), surfactant ($B$) and homogenization speed ($C$) on PS ($Y_1$) was mathematically expressed by a software-generated polynomial equation of quadratic model given bellow.

$$Y_1 = +231.63 + 20.82A - 83.85B -27.84C - 14.48AB + 2.53AC - 5.82BC - 4.28C^2$$

Table 3  Regression coefficient of all applied and suggested model obtained of design expert software.

| Model     | $R^2$ | Adjusted $R^2$ | Predicted $R^2$ | SD  | % CV | Remark  |
|-----------|-------|----------------|-----------------|-----|------|---------|
| **Response ($Y_1$)** |       |                |                  |     |      |         |
| Linear    | 0.9829| 0.9790         | 0.9644          | 9.37| -    | -       |
| 2F1       | 0.9978| 0.9966         | 0.9893          | 3.76| -    | -       |
| Quadratic | 0.9999| 0.9999         | 0.9999          | 0.48| 0.21 | Suggested |
| **Response ($Y_2$)** |       |                |                  |     |      |         |
| Linear    | 0.7736| 0.7213         | 0.5757          | 5.4133| -    | -       |
| 2F1       | 0.7915| 0.6665         | 0.1354          | 5.9224| -    | -       |
| Quadratic | 0.9997| 0.9993         | 0.9972          | 0.2661| 0.32 | Suggested |
| **Response ($Y_3$)** |       |                |                  |     |      |         |
| Linear    | 0.7654| 0.7112         | 0.5782          | 3.0222| -    | -       |
| 2F1       | 0.8526| 0.7642         | 0.53069         | 2.7313| -    | -       |
| Quadratic | 0.9985| 0.9968         | 0.9907          | 0.3201| 0.42 | Suggested |

Fig. 2A  3D response surface and contour plot of particle size for interaction effect.

J. Oleo Sci. 69, (11) 1389-1401 (2020)
The positive and negative sign represents the favour and unfavoured effect on PS. In this case, A, B, C, AB, AC, BC, $A^2$, $B^2$, $C^2$ are significant model terms because it has $p < 0.05$. The F-value of the model from ANOVA analysis was found to be 32325.99, revealed that the model is significant. $R^2$ of particle size is 0.9999 for the quadratic model and it significantly greater than other models represented that quadratic is a suggested model. The lack of fit for suggested model is nonsignificant $p > 0.05$, F-value-0.12 confirm that model was well fitted into given data. The adequate precision was fond to be 658.831 indicates an adequate signal. The PS of all seventeen formulations was found in the range of 113.71-356.22 nm, respectively. It was found that concentration of lipid increases the PS of NLCs increase because it increases the viscosity of solution, decreases emulsifying efficiency of surfactant, increased the interfacial tension leads to particle agglomeration. This result was agreed with previously published work like optimization of lurasidone NLCs for brain delivery, topical NLCs delivery of itraconazole, and oral NLCs delivery of telmisartan. Moreover, surfactant showed the negative effect on the PS, mean increases surfactant concentration, decreases PS of NLCs significantly $p < 0.05$. It is due to decrease the interfacial tension between the two phases, increases emulsification efficiency and showed well harmony with previous reported work. Also number of homogenisation cycle increases, the PS decreased, due to the high homogenization pressure. Figure 2A represents the 3D and contour plot of PS expressed interaction effect of more than one variable AB, AC, BC over the individual

Fig. 2B  3D response surface and contour plot of entrapment efficiency (%) for interaction effect.

Fig. 2C  3D response surface and contour plot of drug release (%) for interaction effect.
response. The difference between observed and predicted value of EE was depicted in Supplementary Figure (Fig. SF1A).

3.3.2 Influence of independent variable over entrapment efficiency

Influence of lipid ratio (A), surfactant (B) and homogenization speed (C) over the EE (Y2) was mathematically expressed by the software-generated polynomial equation given below.

\[ Y_2 = +88.29 + 5.15A + 9.12B + 7.28C - 1.21AB - 2.45AC + 0.32BC + 2.91A^2 - 7.54B^2 - 4.20C^2 \]

The model fisher’s ratio (F) was found to be 2638.43 from ANOVA analysis, implies that model is significant. The model term i.e., A, B, C, AB, AC, BC, A^2, B^2, C^2 are significantly affected on the response (p<0.05). The lack of fit was found to be nonsignificant (F=1.68, P=0.3081, p>0.05), clearly confirm that model was well fitted into given data31. The model R^2 is 0.9999 (close to unity) indicated that well-fitted. The predicted R^2 of 0.9972 is in reasonable agreement with the adjusted R^2 of 0.9993. The adequate precision (signal to noise ratio) of model is 178.86 (>4), indicted that model was well fitted31. The% EE of drug of all seventeen formulations was found in the range of 60.43 to 96.54% (Table 1). The polynomial equation showed that all independent variables showed a positive effect on EE (synergistic effect) but interaction showed negative (AB, & AC) and positive (BC) effect on EE. Interaction effect is less prominent than the individual variable. It was observed that increased lipid concentration, the EE increases because of more space available for housing as well as minimized escaping of drug to external phase. This result agreed to previously published work i.e., optimization of oral NLCs delivery of isradipine38, iloperidone39 and carvedilol7. On increasing surfactant concentration the EE increased, because viscosity of dispersion increases and thickness of lipid core increases. The surfactant (B) showed positive effect on drug release, means increases surfactant concentration increases drug release (%). It is due to decrease PS, increased the surface area and increases miscibility with release media and same result was found on increasing the number of homogenization cycle38,39. Interaction effect of formulation variable (AB, AC, BC) on drug release (%) was expressed by 3D and contour plot (Fig. 2C) and differences between the observed and predicted value was expressed by Supplementary Figure (Fig. SF1C).

3.3.4 Point prediction optimization

Optimized formulation was selected from point prediction of Box-Behnken software. The lipid ratio (8:2), surfactant (3%) and homogenization cycle (4 cycles) was found to full of our target goal and selected as an optimized formulation (DG-NLCs-opt). Optimized formulation was prepared and found to be PS of 231.9 ± 1.64 nm, EE of 88.32 ± 0.65% and drug release of 78.69 ± 1.34% respectively. The predicted value of optimized formulation from software was PS of 231.62 nm, EE of 88.29% and drug release of 78.58%. The DG-NLCs-opt formulation showed 100.11%, 100.03% and 100.13% of prediction of predicted value of responses (Y1, Y2, & Y3). The DG-NLCs-opt was used for further study.

3.4 Particle size, Pdi and morphology

PS of all formulations determined by Malvern zeta sizer and found in the range of 113.71 to 356.22 nm (Table 1). PS of DG-NLCs-opt is 231.9 ± 1.64 nm and PS-distribution graph was depicted in Fig. 3A. The Pdi of DG-NLCs-opt was found to be 0.223 (<0.5) indicated that uniform distribution of PS. Surface morphology of DG-NLCs-opt was examined and found to be spherical in shape (Fig. 3B).
Oral Lipid Based Nano-formulation of Dapagliflozin

J. Oleo Sci. 69, (11) 1389-1401 (2020)

3.5 Thermal analysis

Supplementary Figure (Fig. SF2) expressed thermal spectra of DG, GM and DG-NLCs-opt. Thermal spectra of DG exhibited a characteristic endothermic peak at 74°C (melting point), insured that crystallinity of DG (Fig. SF2A). The GM showed endothermic peak at its melting point (65°C) (Fig. SF2B). The DG-NLCs-opt showed a single asymmetric endothermic peak at 58.5°C but no marked of DG peak. DG-NLCs-opt formulation exhibited low melting point than GM, moreover a noticeable melting over 50°C, it confirmed that solid-state of lipid at room temperature (Fig. SF2C). Shifting of DG-NLCs-opt melting point may take place due to nanosize range of NLCs, or interaction between solid, liquid lipid and surfactant.

3.6 X-ray diffraction study

The spectra of GM showed one characteristic sharp peak at 20 angle value of 19.1 confirming that crystalline structure of GM (Fig. SF3A). The XRD spectral of DG showed characteristic many sharp peaks at 20 angle value of 17.0, 18.9, 20.4, 21.4, and 37.8 respectively (Fig. SF3B). Moreover, the spectra of DG-NLCs-opt not showed any characteristic peak at 20 angle (Fig. SF3C). The diminished or absent of characteristic peaks in DG-NLCs-opt may be due to nanosize range of NLCs, or interaction between solid, liquid lipid and surfactant.

3.7 In vitro release study

In vitro drug release profile of all developed DG-NLCs and DG-dispersion were carried by using dialysis bag in simulated intestinal fluid as release media (phosphate buffer, pH 6.8). The drug release of all DG-NLCs was found in the range of 63.44 to 83.62% (Table 1). The release profile of DG-NLCs-opt and DG-dispersion were depicted in Fig. 4. The DG-NLCs-opt exhibited dual release pattern, initial fast release (21.3 ± 3.12% in 1 h) followed by slow and prolonged release (90.01 ± 2.01%) whereas DG-dispersion showed 31.54 ± 1.87% release in 24 h. Initial fast release is due to presence of DG on NLCs surface and slow is due to erosion or degradation of lipid core matrix. The release data were fitted into different kinetics model and graphical representation was depicted in Supplementary Figure (Fig. SF4). The order of R² of models are Korsmeyer-Peppas model (0.9774) > first order (0.9744) > Higuchi (0.9516) > Hixon-Crowell model (0.9383) > zero-order (0.8283). Maximum R² was found for Korsmeyer-Peppas model and selected as best fitted kinetic model. The ‘n’ value for Korsmeyer-Peppas model is 0.4711 (0.45-0.85) indicating that drug release takes place with diffusion and swelling control mechanism.

3.8 Ex vivo intestinal permeability study

Figure 5A showed ex vivo permeability of DG-NLCs-opt and DG-dispersion in intestinal sac. DG-NLCs-opt exhibited significantly (p < 0.05) high permeation (79.76 µg/cm) than DG-dispersion (16.67 µg/cm). Flux was measured from slop.
of amount of drug permeated vs time. DG-NLCs-opt (1.293 µg/cm²/h) have 4.8-fold higher flux than DG-dispersion (0.2683 µg/cm²/h). The APC for DG-NLCs-opt and DG-dispersion was found to be 4.14 x 10⁻⁵ and 8.61 x 10⁻⁶ cm/min respectively. The high permeation of NLCs is due to colloidal size, which increases surface area and more energy contact between DG, lipid and surfactant as well as due to permeation enhancing capacity lipid and surfactant. It changing tight junction opening of intestinal sac due to high hydrostatic pressure and possess of drug through paracellular transport. Also, surfactant diminished intestinal efflux of the formulation by inhibiting Pgp efflux pump of enterocytes as well as reducing uptake of the reticuloendothelial system.  

3.9 Stability study  
Stability of optimized DG-NLCs performed at 25°C/60 ± 5%RH for six months and result was depicted in Fig. 5B and Supplementary Table. The formulation did not show any caking and phase separation during whole six-month stability study. No significant (p < 0.05) variation was found in PS, and EE (Fig. 5B). There was no remarkable (p < 0.05) variation was found in PdI (<0.5), indicated that uniform distribution particle. The finding concluded that DG-NLCs formulation was agreeable stable over an entire period of stability.

4 Conclusion  
Oral route is most prevalent route for drug administration among various routes. DG-NLCs was successfully prepared using lipids (solid and liquid lipid) and surfactant as well as statistically optimized by using Box-Behnken design software. The various influencing factor on responses was statistically evaluated. Formulation has high entrapment efficiency and uniform distribution of particles. Thermal analysis and XRD study exhibited that drug was amorphous into lipid core system. The NLCs system exhibited a sustained release profile over an extended period (24 h) with Korsmeyer-Peppas kinetic model. DG-NLCs showed high flux and apparent permeability coefficient as compare to DG-dispersion. The formulation was stable over six months of the storage stability. The finding concluded that QbD based lipid nano-carrier for oral delivery could be a promising approach of dapagliflozin for management of diabetes.

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Conflict of interest
The author declares no conflict of interest.

Supporting Information
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