Enhanced oral bioavailability and anti-diabetic activity of canagliflozin through a spray dried lipid based oral delivery: a novel paradigm

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Abstract

Aim Canagliflozin (CFZ), a novel SGLT II antagonist, exhibits erratic absorption after oral administration. The current study entails development and evaluation of spray dried lipid based formulation (solid SMEDDS) for enhancing oral bioavailability and anti-diabetic activity of CFZ.

Methods Solid SMEDDS developed through spray drying containing Neusilin US2 as an adsorbent. The formed solid SMEDDS were characterized for physicochemical and solid state attributes. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were used to confirm the spherical morphology. In vitro dissolution, ex vivo permeability and in vivo pharmacokinetic studies were conducted to determine the release rate, permeation rate and absorption profile of CFZ, respectively. Pharmacodynamic studies were done as per standard protocols.

Results The optimized solid SMEDDS exhibited acceptable practical yield and flow properties and is vouched with enhanced amorphization, nanoparticulate distribution and acceptable drug content. The spherical morphology of solid SMEDDS and reconstituted SMEDDS were confirmed in SEM and TEM, respectively. In vitro dissolution studies revealed multi-fold release behavior in CFZ in various dissolution media, whereas, remarkable permeability was observed in jejunum segment of rat intestine. Pharmacokinetic studies of CFZ in solid SMEDDS demonstrated 2.53 and 1.43 fold enhancement in C_{max} and 2.73 and 1.98 fold in AUC_{0-24h} , as compared to pure API and marketed formulation, respectively. Pharmacological evaluation of solid SMEDDS revealed enhanced anti-diabetic activity of CFZ through predominant SGLT II inhibition in rats, as evident from evaluation of biochemical levels, urinary glucose excretion studies and SGLT II expression analysis.

Conclusion The current work describes significant improvement biopharmaceutical properties of CFZ in solid SMEDD formulation.

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Introduction

Canagliflozin (CFZ) belongs to a novel class of sodium glucose co-transporter (SGLT II) inhibitors and has been widely used for the management of type 2 diabetes mellitus (T2DM) through insulin independent mechanism [1]. SGLT II is a low capacity/high affinity Na^+/K^+ co-transporter predominantly located in proximal convoluted tubules (PCT) of kidneys which prevents reabsorption of glucose and thereby stimulating glucose excretion in the urine [2, 3]. This novel pathway of treating T2DM creates interest in developing various prototype inhibitors of SGLT II [3]. Despite promising anti-diabetic activity of CFZ, biopharmaceutical challenges like poor water solubility (< 0.003 mg/ml), poor permeation ($P_{app} < 2 \times 10^{-5}$ cm/s), cytochrome P450 enzymes mediated high first-pass metabolism and susceptibility to P-glycoprotein (P-gp) mediated efflux resulted in poor systemic

absorption [4, 5]. These biopharmaceutical challenges possess a notable obstacle to a successful oral drug product being developed. Besides various technological innovations of enhancing oral delivery, novel lipidic excipients are capable of overcoming the aforementioned barriers of erratic bioavailability and favor the lymphatic transportation of drugs. Such novel lipid excipients produce isotropic delivery systems called self-microemulsifying drug delivery system (SMEDDS) which possess mammoth potential in augmenting the oral bioavailability and biological activity of lipophilic drugs [5]. A systematic attempt had been made to enhance the solubility and bioavailability of CFZ through Liquid SMEDDS as a novel nano-carrier [4]. However, chemical and physical instability can pose formidable challenges in conventional oral lipid based formulations. Due to the complexity of oral absorption, the dispersion and digestion of liquid SMEDDS in vivo has to undergo various structural changes in the gastrointestinal (GI) tract, which causes higher chances of in vivo drug precipitation. Moreover, storage/handling issues, high production cost and polymorphism of lipid excipients in liquid formulation are noteworthy limitations which limit its clinical applications [6]. Solidification of liquid SMEDDS has widely been scrutinized to address the limitations of conventional micro particulate lipid based oral formulations [7]. The preparation of solid SMEDDS by specialized techniques like spray drying is laced with myriad benefits like scalability, robustness, stability profile, fewer chances of drug precipitation and improved patient compliance [8, 9]. It can be hypothesized that solubility/permeability of CFZ could be enhanced with the optimized proportion of lipids and effective adsorption through spray drying, thereby providing stable and effective formulation for oral delivery with better anti-diabetic effect. Hence, current investigation was aimed at developing novel spray dried lipid based nano-formulation for enhanced biopharmaceutical performance and anti-diabetic activity of CFZ.

Materials

Canagliflozin (purity >99%) was obtained as ex gratia sample from Zydus Cadila Healthcare Limited (India). Acetonitrile (HPLC Grade) was obtained from Finar Chemicals Pvt. Ltd. (India). Lauroglycol FCC was kindly gifted by Gattefosse (India). Tween 80 was obtained from Merck, India. Transcutol P and orthophosphoric acid was acquired from Sigma Aldrich and Thermo Fisher Scientific Pvt. Ltd. (India), respectively. Neusilin US2 as a solid adsorbent was obtained ex gratia from Sun Pharma Pvt. Ltd. (India). Sodium lauryl sulphate (SLS) was purchased from Himedia Laboratories Limited (India). Syringe filter papers of HPLC grade (0.22 μm) were purchased from Pall Corporation (India). Streptozotocin was obtained from Alfa Aesar Pvt.

Ltd. (India). Nicotinamide (Molecular biology grade) was purchased from Central Drug House Limited (India). SGLT II primary antibody (source: rabbit) was obtained from Alomone Labs (Israel). Secondary antibody (anti-rabbit) conjugated with horseradish peroxidase was obtained from Sigma Aldrich (India). All biochemical kits for biochemical estimations were obtained from Erba Diagnostics Limited (India). Marketed product of CFZ was purchased from local pharmacy. Deionized water (Lab Water Systems Pvt. Ltd. Rions India) was used throughout the study.

Methods

HPLC analysis of CFZ

The fully automated High Performance Liquid Chromatography (HPLC) system (Nexera X2, Shimadzu Asia Pacific Limited, Japan) along with 4.6×150 mm C-18 analytical column was utilized for analysis. The temperature of auto sampler and column oven was maintained at 8 °C and 45 °C, respectively. A low pressure binary gradient mode containing mobile phase (acetonitrile and 0.01 M orthophosphoric acid) at an optimized ratio of 50:50% v/v was used and analysis was carried out at a flow rate of 0.9 ml/min. The acquisition time of running the samples was set at 10 min. The analyte detection was carried out by the photodiode array (PDA) detector at a λ_{max} of 290 nm.

Development of CFZ loaded liquid SMEDDS

Based upon the preliminary studies, the optimized concentration of Lauroglycol FCC (320 mg) Tween 80 (1200 mg) and Transcutol P (480 mg) were mixed under stirring to form a homogenous blend. CFZ (400 mg) was incorporated into the homogenous mixture and the components were vortexed until CFZ was completely solubilized. The homogenous mixture was constantly stirred at 45 °C to prepare CFZ loaded liquid SMEDDS [4].

Preparation of spray dried solid SMEDDS

The optimized batch of liquid SMEDDS was spray dried using hydrophobic adsorbent Neusilin US2 with an objective to attain stable and acceptable nano-particulate formulation and to overcome the limitations of conventional adsorption technique [10]. Neusilin US2 (1.376 g) was dissolved in 100 ml ethanol under continuous stirring at room temperature until a solubilized state was achieved. Liquid SMEDDS (2 g) was suspended in adsorbent solution until a fine dispersion was formed. The formed dispersion containing hydrophobic adsorbent was spray dried through well calibrated spray nozzle (Lab Ultima, India) in atomizer having inlet and outlet

temperature of 50 °C and 35 °C, respectively with a feed rate of 1 ml/min [11]. The atomization pressure and feed pump speed were set at 3 kg/cm² and 25 RPM, respectively. The final composition of solid SMEDDS is depicted in Table 1.

Characterizations of solid SMEDDS

Physicochemical attributes of solid SMEDDS

Drug content of solid SMEDDS was determined by dispersing 0.1 g of formulation in acetonitrile (10 ml), sonicated and was filtered through 0.22 µm filter membrane. The filtrate was quantified by using optimized HPLC method. The particle size of solid SMEDDS was determined by Particle Size Analyzer (Microtrac S3500 Particle Size Nano Series, United States) [12]. Reconstitution properties were evaluated by determining the reconstitution rate by calculating the time required for dispersion of solid SMEDDS in deionized water under stirring. The Dynamic Light Scattering (DLS) analysis (globule size and zeta potential) of reconstituted microemulsion was evaluated through Malvern Particle and Zeta Sizer Analyzer (Zetasizer Nano Series, Worcestershire, UK). The reconstituted self-forming microemulsion was also subjected to optical clarity by determining the percent transmittance using UV Spectrophotometer at a λ_{max} of 540 nm (Blue Star-Au-2701) and pH was recorded in calibrated pH meter (SD Fine Chemicals Limited, India) [13]. Micromeritics studies were done by evaluating specific surface area measurement through Brunauer-Emmett-Teller (BET) Surface Area and Porosity System (Micromeritics ASAP 2020, USA). Angle of repose, tapped density, bulk density, Compressibility index and Hausner ratio were determined as per standard protocols [14].

Solid state attributes of solid SMEDDS

The crystallinity/amorphous pattern of CFZ, adsorbent, physical mixture and solid SMEDDS were determined through X-ray Diffractometer (Bruker D8 Focus X-ray Diffractometer, Germany). The test samples were scanned at a diffraction angle (2θ) of 10–80° θ with a scanning rate of 4°/min [15]. The data acquisition of X-ray diffractograms were interpreted using OriginPro 8.5.1 (Origin Lab, Northampton, USA). Differential

Scanning Calorimeter (Pyris 6, Perkin Elmer, United States) was used to investigate the thermal characteristics of test samples. The samples were packed in sealed aluminium pans and thermally exposed at a temperature range of 20 to 350 °C with a heating rate of 10 °C/min. Furthermore, Fourier Transform Infrared Spectroscopic (FTIR) studies were performed to ascertain any chemical interactions within the investigated samples. All test samples were homogeneously mixed with IR grade potassium bromide and pressed under hydraulic pressure (3843 psi) to form pellets. The pellets were placed in sample cell and analysis of spectra was carried out using Alpha FTIR spectrophotometer (Varian 600-IR Series, Germany) equipped with a diamond attenuated total reflectance (ATR) module and deuterated triglycine sulphate (DTGS) detector [16]. The spectrograms were scanned at the finger print range of 4000–400 cm⁻¹. Raman studies were carried out to study possible intermolecular interactions using a Raman Spectrophotometer (Renishaw Invia Microscopy, United Kingdom). The instrumental conditions involve an argon-ion laser of 514 nm, 50 mW excitation source fitted with an edge filter for Stokes spectra and a Peltier cooled charge coupled device (CCD) detector. The samples were run at a Raman shift ranges from 10 to 3500 cm⁻¹ in an unpolarized mode [17].

Scanning electron microscopy (SEM)

SEM was used to analyze the critical morphological characteristics of the powdered samples. Prior to imaging, samples were placed in brass stubs and gold coated in an Emitech 1030 sputter coater for 30 min at 20 mA ionizing radiation under high vacuum. The analysis of coated samples was carried out under a scanning electron microscope (SEM EVO-LS10, USA). The operating conditions for SEM were; a vacuum pressure of 7.19–7.52^{E-4} Pa, excitation source of 5 kV and a secondary electron detector. The surface of *solid* specimens was exposed to the beam of high energy electrons to generate a variety of signals including sample-electron interactions. The SEM micrographs of the samples obtained were captured and saved digitally.

Transmission electron microscopy (TEM)

The reconstituted optimized solid SMEDD formulation was subjected to globule morphology using TEM (JEM-1200 Ex, Joel, Japan). The optimized solid SMEDD formulation (0.1 g) was reconstituted 100 fold with deionized water to form microemulsion. A 100 µL sample was placed on a carbon coated grid in petri dish and allowed to air dry for about 1 h. The grids were placed in the specimen cabinet and beam of electrons focused by electron gun interacted with small aperture condensed lens. The transmitted samples were focused on charge coupled device (CCD) camera and images were generated at a magnification of 15,000 X with a fixed X-ray source of 100 cm.

Table 1 Composition of solid SMEDDS

Ingredients (mg)	Solid SMEDDS
CFZ	400
Lauroglycol FCC	320
Tween 80	1200
Transcutol P	480
Neusilin US2	1376.4

In vitro drug release studies

USP Type II Dissolution Apparatus (DS 8000, Lab India, India) was used for conducting in vitro drug release studies. The various dissolution media including deionized water, 0.75% sodium lauryl sulphate (SLS) in water, 0.1 N HCl (pH 1.2), phosphate buffer (PBS) having pH 6.8 and 7.4 were used for analyzing drug release profile. CFZ (Pure), marketed product and solid SMEDD formulation were added in the dissolution vessel preloaded with 900 ml dissolution media. The entire experiment was run at a revolution speed of 50 RPM and at a temperature of 37 ± 0.5 °C. At predefined time intervals, aliquots were collected in test tubes and filtered through a filter membrane of 0.22 μ m. After each sampling, the equal volume of fresh dissolution medium was filled to maintain sink conditions. The filtrate was appropriately diluted and drug released in the samples was quantified by using optimized HPLC method [4, 11]. The study was done in triplicate to validate the reproducibility of results.

Ex vivo permeability studies

Confocal laser scanning microscopy (CLSM)

CLSM was performed to determine in-depth permeability of active drug in tested formulations across medial jejunum segment using intestinal sac method. Wistar rats weighing 225 ± 5 g (previously abstained from fed state 12 h prior to the study), were firstly anesthetized and then sacrificed through cervical dislocation. The midline incision was performed in the abdomen and medial jejunum segment was expunged and dipped in physiological salt solution (PSS) before the tissue preparation, ligation and aeration. The jejunum segment was washed multiple times with fresh PSS for removal of any type of the adhered intestinal contents and mucus. One end of jejunum segment was ligated with thread and placed in receptor compartment containing 67 ± 0.5 ml of PSS with continues aeration and a temperature of 37 ± 0.5 °C in organ bath assembly (Biocraft Scientific Systems, India). Positive control i.e. 0.05% w/v Rhodamine 123 (Group A), CFZ (Group B), marketed product (Group C) and optimized solid SMEDDS (Group D) were added into the jejunum segment and were closed tightly with thread and placed in organ bath for 3 h at 35 ± 2 °C. At the end of the study, the residual samples from group A, B, C and D were cleaned and the sections were placed on glass slides. The sections were deblocked and in-depth fluorescence was observed in Confocal Microscope (Nikon Corporation, Japan) [18].

In vivo pharmacokinetic studies

Dosage regimen

The animal study was reviewed and accepted by Institutional Animal Ethics Committee (IAEC) having Approval no. 226/PO/Re/S/2000/CPCSEA/08. Hence, the regulations and ethical practices were done in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Rats were housed in central animal house and kept in cages containing husk and acclimatized at a temperature of 35 ± 2 °C, $55 \pm 5\%$ relative humidity and 12 h light/dark cycle. Before conducting in vivo study, all rats were fasted for about 12 h, but were allowable with free access to drinking water. Fifteen Wistar rats weighing 250 ± 15 g were equally divided ($n = 5$) for pure drug suspension, marketed product and solid SMEDDS. Each formulation was administered at a dose rate of 7.07 mg/kg orally in 0.1% w/v carboxymethyl cellulose (CMC) suspension. After administration of test formulations, the rats were anesthetized and blood was collected at predetermined time points of 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h from retro-orbital route [19]. Heparin (2500 IU, 10 μ l per sample) was added to the prepared blood samples and was subjected to centrifugation at 5000 RPM for 30 min to separate plasma.

Plasma extraction and analysis

300 μ l of plasma samples were subjected to solvent extraction and partitioned with acetonitrile (100% v/v) having plasma: acetonitrile ratio (1:3) and stored overnight in centrifuge tubes and then vortexed for 30 s. The precipitated phase in the vials was detached by centrifugation at 5000 RPM. The drug in the solvent phase was diluted with 500 μ l of acetonitrile and was filtered using 0.22 μ m filter membrane. The drug concentration in solvent phase was quantified using HPLC. The linear response of CFZ was observed in ranges between 100 and 30,000 ng/ml ($r^2 > 0.957$) with a retention time of 5.013 min (Fig. S1a and S1b, Supporting Information). For validation of bio-analytical method, the precision (interday and intraday) assay variations were found to be less than 2% relative standard deviation (RSD) and the observed recovery of CFZ was in range of 91.70–102.03%, which was in permissible limits.

In vivo pharmacodynamic studies

Study design

The anti-diabetic study was approved by the Board of IAEC with CPCSEA Approval no. 226/CPCSEA/2018/21. Forty rats with body weight 220 ± 20 g were equally allocated into five groups containing eight rats in each group. The equally allocated groups viz. Control group (I) and Streptozotocin

(STZ) + Nicotinamide (NAD) induced diabetic group (II). The III, IV and V group of STZ + NAD induced diabetic groups were treated with pure CFZ, marketed CFZ tablets and solid SMEDD formulation in the form of 0.1% w/v CMC suspension.

Induction of type 2 diabetes mellitus (T2DM)

STZ was dissolved in 0.1 M cold citrate buffer (pH 4.5) and physiological saline was used to dissolve NAD. In overnight fasted rats, NAD (110 mg/kg) was given as an intraperitoneal injection followed by STZ (50 mg/kg) *i.p* in rats. Since, STZ is able to induce fatal hypoglycemia due to massive release of pancreatic insulin, after 1 h of STZ + NAD administration, rats were given 5% w/v sucrose solution to prevent immediate hypoglycemia. During experimentation, no significant mortality was observed in diabetic groups. After 5 days of STZ + NAD administration, confirmation of diabetes was done by determining blood glucose levels [19]. Rats with levels above 250 mg/dl of fasting glucose were considered as animals induced with T2DM.

Drug administration and blood collection

After 5 days of STZ + NAD induction, the tested formulations were administered orally at a dose of 7.07 mg/kg (1.417 mg/rat) in 0.1% w/v CMC suspension through oral gavage daily. During experimentation, all the experimental animals were checked frequently for their weekly body weights and feed/water intake repeatedly after 3 days. After completion of the experimental model, the rats were subjected to overnight fasting and blood samples were collected to determine biochemical parameters.

Urinary glucose excretion (UGE) studies

The rats in aforementioned groups were housed in metabolic cages for collection of urine for determining glucose excretion in urine. Briefly, urine was collected at predefined time intervals and samples were analyzed for urine glucose concentration by glucose oxidase method and urine volume was measured gravimetrically. The UGE was calculated by following Eq. 1 [20].

$$UGE \left(\frac{\text{mg}}{\text{day}} \right) = \text{Urine concentration} \left(\frac{\text{mg}}{\text{dl}} \right) \times \text{Urine volume (ml)} \quad (1)$$

Biochemical estimations in T2DM

After 28 days experimental model, rats were anesthetized and sacrificed by cervical dislocation. By puncturing their retro-orbital plexus, the blood was withdrawn. For biochemical analysis, the collected blood samples were allowed to clot and centrifuge at 5000 RPM for 20 min to collect serum. The serum was subjected to blood glucose levels (BGL) and glycosylated hemoglobin (HbA1c) determination. Liver function tests were determined by evaluating aspartate transaminase (AST), alanine transaminase (ALT) and serum alkaline phosphatase (ALP) levels. Kidney function tests were carried out by determining serum creatinine, blood urea nitrogen (BUN). Urine samples were analyzed for microproteinuria. [20].

Histological analysis

The specimens of kidneys were prepared by fixing with 4% neutral buffered formalin and were embedded in paraffin. These 4 µm tissue sections were cut and stained with hematoxylin and eosin (H/E) and observed for any histological changes in rat kidneys.

SGLT II protein expression in rats

The isolated kidneys of all the groups were homogenized using extraction buffer [0.2 M Ethylenediaminetetraacetic acid (EDTA), 2 mM Ethyleneglycoltetraacetic acid (EGTA), 0.12 mM Dithiothreitol (DTT), 2 mM Phenylmethylsulfonyl fluoride (PMSF) and 0.5% w/v protease inhibitor cocktail]. The extracted samples were sonicated and centrifuged at 25000 RPM for 30 min and supernatant containing total protein was collected. The protein samples were run in sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE). After the SDS-PAGE separation, the gel was treated with transfer buffer (100 mM glycine, 25 mM Tris buffer, 20% v/v methanol and 0.1% SDS) for 30 min and then the gel was transferred onto a nitrocellulose (NC) membrane in a semi-dry blot chamber (TE70X Semi Dry Blotter, Hoefer Inc., USA). After transferring, the membrane was blocked with 5% bovine serum albumin (BSA) in PBS at 4 °C overnight. The membrane was kept at room temperature for 30 min and washed with 1X PBS and incubated with corresponding SGLT II primary antibody diluted in 1% BSA in PBS (SGLT II extracellular antibody, Alomone Labs, Israel) for 4 h at rocker. After incubation, NC membrane was washed thrice with 1X PBS with Tween 80 (PBST) (4 M NaCl, 2.3 M KCl, 1.4 M Na₂HPO₄, 0.5 M KH₂PO₄ and 0.025% w/v Tween 80) for 15 min. The NC membrane was then incubated with horseradish peroxidase conjugated secondary IgG antibody (Sigma Aldrich, India) for 2 h at rocker. Afterwards, the membrane was washed thrice with PBS and twice with PBST for 30 min and developed with diaminobenzidine (DAB) and

hydrogen peroxide (1 mg/ml DAB and 10 ml of 30% H₂O₂). The blot was developed in dark light and bands were quantified by Image J software (National Institutes of Health, USA) [21]. In this study, β -actin was taken as positive control and developed by the same procedure.

Accelerated stability studies

The optimized spray dried solid SMEDDS was closely packed in screw capped vials and placed at accelerated storage conditions (45 °C/75% RH) in stability chamber (SC 19 Plus, REMI, India) for a period of 6 months. After each month, test sample was withdrawn and evaluated for various critical quality attributes like physical intactness, flowability, powder rheology and drug content. Amorphization was confirmed through X-ray diffractograms [22].

Statistical analysis

The Statistical treatment of the data was evaluated using Graph Pad Software (Version: 7.0.1 Graph Pad Inc. San Diego, CA). One way ANOVA was applied using Tukey Post Hoc test to determine the statistical significance of the reported results.

Results and discussion

Physicochemical attributes of solid SMEDDS

The optimal critical process parameters of spray drying such as inlet and outlet temperature, atomization pressure and feed flow

rate have shown a drastic effect on the physicochemical properties and flowability of the final powder obtained [23]. The solid SMEDDS prepared through spray drying containing hydrophobic adsorbent (Neusilin US2) was subjected to various physicochemical characterization parameters and the results are tabulated in Table 2. The acceptable practical yield of optimized spray dried solid SMEDDS and drug content in solid SMEDDS suggest uniform encapsulation through spray drying using inert adsorbent. Spray drying using Neusilin US2 as adsorbent adsorbed liquid SMEDDS quite well and produced free flowing powder and observed with nano-particulate size distribution (Fig. S2, *Supporting Information*). Micromeritics studies revealed excellent flowability and enhanced specific surface area of solid SMEDD formulation that governs enhanced dissolution and absorption, as evident from BET evaluation (Table S1, *Supporting Information*). These observations might be due to highly porous nature, larger surface area and nano-particulate size of Neusilin US2 and were also observed with highest oil adsorption/desorption tendency (Table S2, *Supporting Information*). This mechanism favors possible enhancement in flow behavior which ensures sufficient filling into capsule dosage form, thus enhancing patient compliance [24]. Reconstitution studies were done to entail the stability of the formulation to mimic the in vivo processes in the GI tract. The optical clarity after reconstitution exhibited enhanced birefringence and reduced turbidity with quick rate of reconstitution, suggesting optimum stability of solid SMEDDS

Table 2 Characterization parameters of solid SMEDDS

Study type	Characterization parameters	Solid SMEDDS
Physiochemical properties	Powder Morphology	Free flowing
	Practical Yield (%)	54.67 ± 3.67
	Particle Size (nm) (Powder)	456.3 ± 2.43
	Aqueous Solubility (mg/ml)	89.38 ± 1.75
	Specific Surface Area (m ² /g)	345.98 ± 2.35
	Pore size distribution (nm)	6.78 ± 0.88
Micromeritics properties	Angle of Repose (°)	21.4 ± 4.98
	Bulk Density (g/ml)	0.768 ± 0.04
	Tapped Density (g/ml)	0.613 ± 0.05
	Compressibility Index	12.39 ± 2.38
	Hausner Ratio	1.18 ± 0.09
	Drug Content (mg/g)	93.34 mg/g
Reconstitution studies	Reconstituted Particle Size (nm)	168.44 ± 5.87
	Reconstituted Zeta Potential (nm)	−11.48 ± 2.91
	pH	6.88 ± 2.09
	Reconstitution Rate (Sec)	76.43 ± 4.09
	Percent Transmittance (%)	86.39 ± 2.38

obtained. Regarding droplet size and stability, solid SMEDDS yielded small droplet size (Fig. S3a, *Supporting Information*) with negative electrophoretic mobility (Fig. S3b, *Supporting Information*), indicating the formation of a stable nano-colloidal system.

Solid state characterizations of solid SMEDDS

X-ray diffractogram of CFZ typically consists of random distribution of crystalline solids which showed sharp diffraction peaks at various 2θ degrees of 15.38° , 19.37° , 20.37° , 22.67°

Fig. 1 Solid state characteristics viz. **a** XRD, **b** FTIR, **c** DSC and **d** Raman spectroscopy of CFZ, Neusilin US2, Physical Mixture and Solid SMEDDS

Fig. 1 (continued)

and 30.56°, respectively. Furthermore, solid adsorbent like Neusilin US2 exhibited minute diffraction peaks with sharp noise at a Bragg angle of 45.3°, 64.7° and 78.5°. Physical mixture detected with partial amorphization, whereas, optimized solid SMEDDS observed with a reduction in the crystallinity index indicating changing in overall geometry of crystalline to amorphous form, suggesting effective encapsulation of CFZ in solid dosage form matrix (Fig. 1a) [25]. Furthermore, the characteristic infrared diffractogram of CFZ revealed major stretching and bending vibrations at 3403 cm⁻¹ for -O-H group, -C-O group at 1213.9 cm⁻¹, -

C=C group at 1463.7 cm⁻¹ -CH₃ group at 2734 cm⁻¹ (symmetric stretch) and 2950 cm⁻¹ (asymmetric stretch), respectively, indicating the presence of strong prototype active groups. Neusilin US2 also indicated active frequency peaks of silicate tethered by magnesium and silicate ions at 3456.6 cm⁻¹, 2054.2 cm⁻¹ and 1083.45 cm⁻¹. No inherent interaction was seen in the physical mixture, whereas, solid SMEDDS showed broadened infrared spectra that might be due to partial loss of crystallinity or amorphous transformation of CFZ in the formulation (Fig. 1b). These findings showed that no definite chemical interaction was observed between

Fig. 2 Scanning electron micrographs of **a** CFZ, **b** Neusilin US2, **c** Physical mixture, **d** Solid SMEDDS (low magnification) and **e** Solid SMEDDS (high magnification)

drug and adsorbents and successfully be transformed into stable *in house* development of pharmaceutical dosage [26]. In DSC thermograms, CFZ gives a sharp endothermic peak at 170.56 °C which corresponds to its highly crystalline state and infers its sharp melting point. Neusilin US2 observed with an endothermic peak at around 124.26 °C. The reduction in the intensity of endothermic peak (174.62 °C) in physical mixture revealed partial reduction in crystallinity of CFZ, however, complete amorphous transition occurs as the disappearance of endothermic peak of CFZ was observed in the thermograms of solid SMEDDS suggesting the confirmation of molecularly dispersed state of CFZ in the formulation (Fig. 1c) [26]. Raman spectroscopy was performed to entail the possible drug-adsorbent intermolecular interactions and possible solubilization mechanism of CFZ in the formulation. CFZ has characteristics OH bending groups at a raman shift of 1600–1700 cm^{-1} , indicating the presence of hydrogen bond donor groups in CFZ. The sharp noise at a raman spectrum of Neusilin US2 justify the inert silicate groups present in the structure that is stabilized with oxygen atoms. The relative change in the peak intensities was noted in the physical mixture indicated the formation of partial hydrogen bonding between CFZ and Neusilin US2. Furthermore, reduction in the noise of relative OH bending region in solid SMEDDS indicated the formation of effective intermolecular hydrogen bonding (H—O—H) and active drug CFZ was homogeneously distributed onto the surface of solid SMEDDS (Fig. 1d). It

can be hypothesized that compounds interacted via proton donor moieties must form stable hydrogen bonding that do not break upon dispersion into aqueous medium [17, 27].

Fig. 3 Transmission electron micrograph of reconstituted microemulsion of optimized solid SMEDDS at a magnification of 15,000 X

This stable intermolecular interaction and homogenous distribution of drug are the major mechanisms responsible for amorphization of CFZ in solid SMEDD formulation.

Scanning electron microscopy (SEM)

Scanning electron micrographs of CFZ appeared as rectangular shaped crystalline solids with rough edges which were partially separated from each other (Fig. 2a). Neusilin US2 was witnessed as spherical particles with well-defined echo texture and pores without aggregation (Fig. 2b). Physical mixture appeared as partially crystalline particles with deep crevices (Fig. 2c). Solid SMEDDS presented with well-defined morphology because of spherical particles having smooth outer surface and effective encapsulation of pores (Fig. 2d). Furthermore, no CFZ crystals were seen on the surface of prepared solid formulation displaying effective encapsulation or perfectly adsorbed onto the surface of Neusilin US2. At higher magnification, a single particle of solid SMEDDS was captured and observed with uniform spherical morphology with smooth echo texture and perfect encapsulation of CFZ (Fig. 2e). These morphological outcomes revealed desired characteristics of solid SMEDDS containing hydrophobic adsorbent in the present work.

Transmission electron microscopy (TEM)

TEM was executed to determine the physical morphology of reconstituted microemulsion formed after dispersion in vitro. The formed microemulsion droplets appeared as darker dots with bright surroundings in carbon coated grids. The formed microemulsion droplets after in vitro dispersion were in spherical state with negligible aggregation, revealing the efficiency of solid SMEDDS prepared. Furthermore, no interference of any foreign particulate matter or CFZ crystals suggested effective encapsulation of CFZ or stability of solid SMEDDS prepared (Fig. 3). The desired outcome of TEM analysis was consistent with the average droplet size of microemulsion analyzed by DLS.

In vitro drug release studies

As evident from pH dependent solubility profile of CFZ (Fig. S4, *Supporting Information*), dissolution studies were performed in different dissolution media to mimic the in vivo conditions in GI tract [4, 11]. Pure drug and marketed product exhibited poor and fair release behavior which might be due to poor aqueous solubility of CFZ resulting in hindered dissolution. In vitro release assay revealed burst effect as about 50% of drug was released in 20 min period of time. Experimental observations indicate that solid SMEDDS eliminate the influence of pH variability of CFZ. It further substantiates the

Fig. 4 Dissolution profile of pure drug, marketed product, physical mixture and optimized solid SMEDDS in **a** Deionized Water; **b** 0.75% SLS; **c** 0.1 N HCl; **d** PBS 6.8 and **e** PBS 7.4 as dissolution media (Mean $\pm$ S.D., $n = 3$). * $p < 0.05$, vs. pure drug and marketed product

enhancement in release behavior of the drug in five different dissolution media and found statistically significant ($p < 0.05$) than pure drug and commercial product. It might be attributed to nano-globular size of solid SMEDD formulation and quick self-emulsification rate leading to immediate solubilization of drug which causes profound dissolution rate [28]. In the GI lumen, adsorbent hydration occurs in the inner core of spray dried particles. After hydration, lipophilic and amorphous domains of drug rich particles are released leading to rapid dissolution [28]. Solid SMEDDS exhibited variation in the drug release profile as it showed 61.76%, 65.68%, 80.99%, 92.73% and 97.31% in deionized water, 0.75% SLS, 0.1 N HCl, PBS 6.8 and PBS 7.4, respectively (Fig. 4a–e). The high surface

area, interparticulate void space and oil adsorbing/desorbing capacity favor the enhancement in release behavior of CFZ in solid SMEDD formulation. This release favors high systemic availability of drug in blood owing to its improved absorption and bioavailability.

Ex vivo permeability studies (CLSM)

CLSM is a highly prevalent tool to analyze in-depth fluorescence of various compounds to determine the permeability within live tissues. Due to the hydrophilic nature, Rhodamine 123 is freely permeable through the intestinal membrane, indicating moderate permeability

Fig. 5 CLSM images of **a** Rhodamine 123 (Positive control), **b** Pure Drug, **c** Marketed Product, **d** Solid SMEDDS and **e** Quantitative intensity plot of all tested formulations

Fig. 6 Absorption profile after oral administration of pure drug, marketed product and solid SMEDDS in Wistar rats (Mean $\pm$ S.D., $n = 5$). * $p < 0.05$ vs. pure drug and marketed product

(Fig. 5a) [29]. However, as manifested from hepatic first pass profile and p-glycoprotein (P-gp) mediated efflux profile of CFZ, the poor and fair permeability of pure drug and marketed product in jejunum segment was observed which was evident from minuscule and moderate fluorescence, respectively (Fig. 5b, c). The optimized solid SMEDDS exhibited remarkably higher intensity in fluorescence, as compared to positive control, thereby demonstrating that the formed complex between Rhodamine 123 and solid SMEDDS has stronger permeability through the intestine (Fig. 5d, e). Initially, the spontaneous formation of microemulsion from solid SMEDDS occurs in the GI lumen. Endogenous phospholipids, the chief constituents of intestinal membrane, are able to form mixed micelles by combining with microemulsion droplets and bile salts. The formed mixed micelles with good affinity for intestinal epithelial cells might transport poorly soluble drugs in their hydrophobic core to improve intestinal absorption. P-gp, an ATP-dependent multidrug efflux pump, has shown provincial difference in its activity and expression [30]. CFZ is preferably absorbed in the medial jejunum segment and is a high affinity substrate for P-gp mediated efflux. Hence, the inhibition of this efflux mechanism by solid SMEDD formulation in epithelial transport triggers the remarkable enhancement in CFZ

permeability, which was more obvious in jejunum segment. Different biological properties such as enzymatic activity, thickness of intestinal segment and microclimate pH may be subsidized in the GI micro flora for intestinal absorption of CFZ.

Pharmacokinetic studies

The ultra-liquid chromatograms of CFZ, blank plasma, blank plasma containing CFZ and plasma sample after oral administration to rats are given in Fig. S5, *Supporting Information*. The plasma concentration time profile of optimized formulation exhibited a quick rate of self-emulsification by optimized formulation in the GI lumen owing to its decreased T_{max} (Fig. 6). Enhanced AUC_{0-24h} (3.09 and 2.27 fold) and C_{max} (3.16 and 1.62 fold) as compared to pure drug and marketed product were observed for CFZ in solid SMEDDS (Table 3). On the contrary, the pharmacokinetic parameters for the optimized liquid SMEDD revealed lower AUC_{0-24h} (2.73 and 1.98 fold) and C_{max} (2.53 and 1.43 fold), as compared to pure drug and marketed product. These comparative results demonstrated that adsorption of the liquid SMEDDS by spray drying showed no significant changes in the in vitro release and ex vivo permeation, except a moderate enhancement in the values of C_{max} and AUC_{0-24h} for solid SMEDDS as compared to the optimized liquid SMEDD formulation. These observations of enhancing drug absorption through solid SMEDDS can be ascertained to enhanced solubilization, gut permeability of CFZ in isotropic mixture, nano-lipid droplets of formed microemulsion after dispersion and greater surface area governed by rapid desorption of Neusilin US2 in biological fluids [31]. The key molecular mechanism for absorption is combined micellization of endophospholipids with self-forming microemulsion that is primarily absorbed through transcellular absorption. The formed mixed micelles in the intestinal mucosa are rapidly uptaken and form chylomicrons which facilitate its absorption through lymphatic circulation. The lipid excipients are also reported to inhibit the P-gp, a transporter located in epithelial surface of the intestinal membrane, thus constrain the efflux of the substrate drug CFZ out of the cells resulting in increased concentration for therapeutic action. Results of pharmacokinetics revealed enhanced

Table 3 Pharmacokinetic parameters after oral administration of pure drug, marketed product and optimized solid SMEDDS in rats

Parameter	Pure drug	Marketed product	Solid SMEDDS
C_{max} (ng/ml)	692.13 $\pm$ 564.43	1551.32 $\pm$ 765.98	3522.37 $\pm$ 456.65
T_{max} (h)	2.11 $\pm$ 0.67	2.09 $\pm$ 0.56	1.05 $\pm$ 0.34
AUC_{0-24h} (ng/ml.h)	12,381.7 $\pm$ 1034.65	19,906.9 $\pm$ 1456.89	32,388.3 $\pm$ 1134.5
K_a (h^{-1})	0.97 $\pm$ 1.03	1.05 $\pm$ 0.98	1.45 $\pm$ 0.37
K_e (h^{-1})	0.029 $\pm$ 0.003	0.033 $\pm$ 0.004	0.05 $\pm$ 0.006
Relative Bioavailability (%)	56.35 $\pm$ 5.85	88.42 $\pm$ 9.56	172.54 $\pm$ 24.56

bioavailability of CFZ from solid SMEDDS by facilitating the drug into lymphatic circulation.

Pharmacodynamic studies

Chemically, STZ is 1-methyl-1-nitrosourea, commonly used compound to induce diabetes in animal models by selectively damaging β -cells in pancreatic islets [32]. This damage is mainly attributed to the damage in pyrimidine nucleotide junctions in pancreatic β -cells. Because of the alkylating nature of STZ, it activates poly-ADP-ribose synthetase and destroys nicotinamide adenine dinucleotide in the islets [33]. Hence, co-administration of NAD, a poly-ADP-ribose synthetase inhibitor is done to protect the islets functionality and reverse the

massive insulin secretion to prevent the damaging loss to pancreas [34]. This STZ-NAD combination contributes to the above mentioned features similar to T2DM.

Analysis of body weight, feed and water intake

The enhancement in body weight was observed in diabetic rats, as compared to control group (Fig. S6a, Supporting Information). Moreover, the feed and water intake in rats followed similar trend (Fig. S6b and S6c, Supporting Information). Notably, insulin facilitates conversion of glucose into glycogen and its storage into liver. During non-fed stages, glycogen is broken down to glucose and provides energy to body [35]. At cellular level,

Fig. 7 Urinary analysis viz. **a** Urine glucose concentration and **b** UGE profile of tested formulations in T2DM in rats (Mean $\pm$ S.D., $n = 5$). ^a $p < 0.05$ vs. control, ^b $p < 0.05$ vs. diabetic group, ^c $p < 0.05$ vs. diabetic group + pure drug.

Table 4 Biochemical estimations of control group, diabetic group, pure drug, marketed product and solid SMEDDS in T2DM in rats

Group	Blood glucose tests		Liver function tests			Kidney function tests			
	BGL (mg/dl)	HbA1c (%)	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine (mmol/L)	BUN (mg/dL)	Microproteinuria (mg/day)	
Control	110.53 ± 2.13	5.23 ± 0.13	126.73 ± 5.67	55.33 ± 5.33	114.51 ± 5.65	0.76 ± 0.13	20.27 ± 1.55	3.99 ± 1.65	
Diabetic rats	282.34 ± 12.24 ^a	7.53 ± 0.12 ^a	189.41 ± 9.54 ^a	90.41 ± 5.19 ^a	184.72 ± 7.53 ^a	1.32 ± 0.33 ^a	70.84 ± 5.64 ^a	10.37 ± 1.87 ^a	
Diabetic Rats + Pure Drug	170.42 ± 8.53 ^b	6.52 ± 0.21 ^b	156.42 ± 8.66 ^b	78.64 ± 7.42 ^b	153.84 ± 8.88 ^b	0.89 ± 0.26 ^b	59.61 ± 4.32 ^b	9.45 ± 2.45 ^b	
Diabetic Rats + Marketed Product	155.64 ± 9.41 ^b	6.43 ± 0.12 ^b	150.33 ± 10.44 ^b	72.57 ± 7.33 ^b	144.82 ± 9.43 ^b	0.87 ± 0.51 ^b	38.93 ± 3.19 ^b	6.34 ± 2.77 ^b	
Diabetic Rats + Solid SMEDDS	144.52 ± 5.31 ^c	5.73 ± 0.15 ^c	130.45 ± 11.55 ^c	62.58 ± 8.12 ^c	122.26 ± 4.42 ^c	0.82 ± 0.22 ^c	22.13 ± 4.35 ^c	5.22 ± 2.54 ^c	

^a $p < 0.05$ vs. control, ^b $p < 0.05$ vs. diabetic rats, ^c $p < 0.05$ vs. diabetic rats + pure drug

insulin facilitates entry of glucose into cells by promoting glucose transporters and thus, regulates its concentration in blood. T2DM is characterized by polydipsia (increased thirst) by polyphagia (increased hunger) and polyuria (increased urination) [36, 37]. Initial reduction in body weight (first week) followed by improvement in the weight gain (between 14th–28th day) was observed in diabetic rats treated with pure drug, marketed product and solid SMEDD formulation. Furthermore, feed and water intake closely mimicked the control rats in 3rd and 4th week of treatment with pure drug, marketed product and solid SMEDD formulation. The improvement in physio-metabolic attributes provides an indication of better control of hyperglycemia in T2DM.

UGE studies

UGE studies were conducted for T2DM in rats to compare excretion levels of glucose treated with various CFZ containing formulations [38]. Control and diabetic group observed with negligible and minimal glucose excretion, respectively. Our observations on tested formulations indicated that pure CFZ and marketed product indicated moderate glucose excretion due to the poor aqueous solubility and permeability. However, solid SMEDD formulation facilitated enhanced urinary glucose concentration through kidneys ($p < 0.05$), as compared to pure drug and marketed product (Fig. 7a, b). The urine volume was also observed to be enhanced and found directly proportional to the urine glucose concentration in diabetic rats (Table S2, Supporting Information). Notably, SGLT type II is low capacity/high affinity Na^+/K^+ co-transporter in glucose hemostasis and accounts for 90% glucose reabsorption in the PCT of kidneys [39]. The selective inhibition of SGLT II by CFZ in the S1 segment in the glomerulus of kidneys leads to reduced glucose reabsorption and improves glycemic levels in blood [40]. This selective antagonism provides an insulin-independent mechanism and is more responsible for regulating plasma glucose levels in the body with low potential of causing hypoglycemia [41].

Analysis of biochemical parameters in T2DM

The analysis of BGL, HbA1c and various biochemical estimations in liver and kidney is depicted in Table 4. The STZ + NAD treated diabetic groups observed with marked increase in BGL, HbA1c, liver and kidney parameters, as compared to control group. A significant reduction ($p < 0.05$) in all biochemical parameters in the treated groups was observed, as compared with diabetic group. The increasing numbers of glucose molecules stick with hemoglobin thereby causing its glycosylation and were remarkably higher in a diabetic group. Our results are in accordance with previous findings [42]. The increased levels of AST, ALT and ALP are well established markers to liver

Fig. 8 Histopathology of **a** Control, **b** Diabetic control, **c** Diabetic control + Pure drug, **d** Diabetic control + Marketed product and **e** Diabetic control + Solid SMEDDS

damage [43, 44]. Furthermore, chronic hyperglycemia damages the sub-micron blood vessels and glomeruli in kidneys thereby leading to increase protein content in urine and alters the functionality of kidneys profile [45, 46]. Long term complication in T2DM causes chronic kidney damage. All treated groups significantly normalized the biochemical profile, more particularly with solid SMEDD formulation. The increased anti-diabetic activity is in the order of solid SMEDDS > marketed product > pure drug. The decrease in biochemical levels treated with optimized solid SMEDD formulation might be due to increased solubility, permeability and oral bioavailability enhancement of CFZ. Consequently, increased blood concentration of CFZ prompts therapeutic efficacy.

Histological analysis

The H/E stained kidneys of rats in control group demonstrated integrated glomerulus and tubular structure. The kidneys of diabetic rats demonstrated glomerulus expansion, tubular degeneration and necrosis. The tested formulations i.e. pure drug, marketed product and solid SMEDDS witnessed with attenuation of structural abnormalities and provide Reno protection to the kidneys in T2DM (Fig. 8a–e).

SGLT II protein expression in rats

A single band of 72 KDa was seen which corresponds to the known molecular weight of SGLT II in rat and bind very precisely with known primary antibody of SGLT II. The expression of SGLT II in STZ + NAD induced diabetic rats was observed to be high, as compared to control group. In

hyperglycemic state of T2DM, proximal tubular cells are continuously exposed to higher concentrations of filtered glucose thereby, posing difficulty in its excretion through kidneys [47]. This mechanism increases renal intracellular glucose

Fig. 9 SGLT II expression viz. **a** Western blot and **b** Quantitative expression analysis of control group, diabetic group and tested formulations in T2DM in rats. ^a $p < 0.05$ vs. control, ^b $p < 0.05$ vs. diabetic group, ^c $p < 0.05$ vs. diabetic group + pure drug.

Table 5 Stability data of solid SMEDDS under accelerated storage conditions (45 °C/75% RH)

Solid SMEDDS								
Time (days)	Physical nature	Flow behavior	Reconstitution time (Sec)	Reconstituted particle size (nm)	Zeta potential (mV)	Drug content (%)	Percent yield (%)	Physical state
0	Intact	Excellent	73.11 ± 3.15	257.5 ± 2.86	−4.59 ± 1.52	93.4 ± 1.52	54.6 ± 0.43	Amorphous
30	Intact	Excellent	71.48 ± 4.87	249.2 ± 2.96	−4.27 ± 1.93	93.2 ± 1.52	53.8 ± 0.64	Amorphous
60	Intact	Excellent	88.36 ± 2.84	232.5 ± 2.47	−2.45 ± 1.92	93.5 ± 1.13	53.5 ± 1.65	Amorphous
90	Intact	Excellent	83.27 ± 3.98	244.9 ± 3.48	−1.97 ± 1.21	92.7 ± 1.82	53.6 ± 1.75	Amorphous
120	Intact	Excellent	87.13 ± 2.88	254.1 ± 2.87	−2.34 ± 0.70	92.9 ± 1.02	53.4 ± 1.09	Amorphous
150	Intact	Excellent	90.52 ± 4.12	256.2 ± 2.65	−3.54 ± 0.40	91.5 ± 0.87	53.2 ± 1.23	Amorphous
180	Intact	Good	98.32 ± 3.78	267.4 ± 2.43	−4.09 ± 0.52	91.7 ± 1.03	52.9 ± 1.54	Amorphous

levels which enhances SGLT II expression which up regulates reabsorption and further increased glycemic levels in blood [48, 49]. However, due to mild to moderate absorption of pure CFZ and marketed product, the UGE observed was mild to moderate through less number of CFZ molecules in blood to bind the specific receptor in the PCT of kidneys (Fig. 9a, b). Moreover, solid SMEDDS inhibit the SGLT II expression remarkably ($p < 0.05$) through the enhancement of solubility and biological absorption. The enhanced concentration of CFZ in blood hits the SGLT II molecules creating strong antagonism in the PCT of kidneys. The specific inhibition of SGLT II by the tested formulations was consistent with the UGE in rats. These observations indicated that the successful development of solid SMEDDS containing CFZ enhanced the anti-diabetic activity vis-à-vis pure drug and marketed product.

Accelerated stability studies

Results demonstrated no notable alteration in physicochemical properties of solid SMEDDS as all critical quality attributes are in acceptable limits during 6 months storage at 45 °C/75% RH (Table 5). The maintenance in physicochemical properties, nanoparticulate size, stability and amorphization signify the high robustness of nano-colloidal based system under highly stressful conditions.

Conclusion

The present investigation vouches the development and evaluation of optimized solid SMEDDS for a BCS class IV drug, CFZ. The enhanced in vitro dissolution, ex vivo permeability and in vivo absorption of CFZ in solid SMEDDS holds great promise in improving therapeutic performance. The remarkable enhancement in all of the biopharmaceutical and anti-diabetic attributes, therefore, ratified spray dried lipid based

formulation of CFZ as one of the promising alternative for oral delivery with suitable translational potential.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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