Improved Subcutaneous Delivery of Ketoconazole Using EpiDerm and HSPiP Software-Based Simulations as Assessed by Cell Viability, Cellular Uptake, Permeation, and Hemolysis In Vitro Studies

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ABSTRACT: Ketoconazole (KETO) is the drug of choice to control local, systemic, and resistant types of fungal infections. Subcutaneous (sub-Q) delivery offers several benefits. The present study investigated the sub-Q delivery of KETO using HSPiP software based on optimized concentrations of dimethylacetamide (DMA) in binary solvents (DMA + water), in vitro cellular uptake (J774A.1) assays, cellular toxicity (L929), and in vitro hemolysis studies. Results showed that the estimated permeation coefficient (9.6 × 10⁻³ cm/h) and diffusion coefficient (3.9 × 10⁻³ cm²/s) of KETO (22.3 mg) in KF3 (300 mg of DMA + water) across EpiDerm were relatively higher as compared to the other formulations [KF1 (11.2 and 150 mg as KETO and DMA, respectively) and KF2 [(22.3 and 300 mg as KETO and DMA, respectively)] due to the increased content of DMA and KETO. HSPiP simulated and predicted the impact of constant and variable diffusion coefficients on the percent drug absorption across EpiDerm and the time needed to achieve equilibrium. The concentration-dependent diffusion coefficient fed into HSPiP predicted that the drug absorption and permeation values were linearly dependent on the square root of time. The HSPiP predicted permeation flux values from KF3, KF2, and KF1 across the EpiDerm were 4.07 × 10⁻³, 4.01 × 10⁻⁶, and 1.1 × 10⁻⁶ g/cm²/s, respectively, at respective D range values. The selected K30G (324 mOsm/Kg) showed an optimal pH (6.9) and minimum drug loss (0.01%) over a period of 1 month at room temperature. KG30 was found to be less toxic to normal L292 cells and caused maximum cytotoxicity to candida cells residing within infected macrophage cells (J774A.1 incubated for 24 h), which was attributed to the slow diffusion of K30G compared to DS (the drug solution with an equivalent concentration). KG30 elicited substantial internalization with cellular uptake (J774A.1) assays, hemolysis studies (1 and 5 g/mL) corroborated the safety of K30G for sub-Q delivery. Therefore, this new formulation and approach for delivering KETO is a promising alternative to conventional products to control fungal infections and, thus, should be further studied in vivo.

INTRODUCTION

There are currently 40 million active cases of fungal infections and co-infections in the world; 20% of France’s 40 million population, ~26% of Germany, 5–15% of China, and ~19% of the Netherlands are infected by virus.1 In 2020, Heaney et al. reviewed and identified the common risk factors associated with fungal co-infections (coccidioidomycosis) with Covid-19 (Coronavirus disease 2019), which include old age, diabetes (an independent risk factor for Covid-19 and mucormycosis), race, ethnic minority status, working location (dust prone area), and immunosuppression resulting in similar symptoms of Covid-19 (which may delay coccidioidomycosis diagnosis and subsequent death in the USA).2 Moreover, progressive cases of rhino-orbital mucormycosis have been observed in India positively diagnosed with Covid-19 and two major fungi (Candida and Aspergillus), which are prime pathogens for co-infections in these patients.3 Notably, phycymycosis (now mucormycosis) is an aggressive, uncommon but fetal fungal infection caused by Rhizopus oryzae in humans (60% cases of mucormycosis and 90% cases of the rhino-orbital-cerebral form).3 In a 2019–2020 estimate, the prevalence of cases of mucormycosis in India and the world was 0.14 per 1000 and 0.005 per 1.7 million people, respectively, suggesting an 80 times higher prevalence in India (the highest cases in the world) as compared to developed nations.4–6

The study designed was based on the thorough understanding of theoretical and literature information. Ezati et al. reported the...
measurement of HSP values of normal human skin, psoriasis human skin, and various organic solvents. The study was conducted to determine the uptake across the skin.\(^7\) Hansen and Andersen attempted to investigate the affinity of organic solvent in the biological system using the HSP technique. The tendency of organic solvents to swell the skin or to be absorbed/preferential absorption can be estimated for the solvent, for which HSP is available.\(^8\) Therefore, we designed out work for using organic solvent possessing HSP parameters comparable to skin and the drug. Theoretically, HSP for a solvent and solute is considered soluble or miscible if the difference of any HSP parameter is close to zero or zero. Vay et al. applied HSP values to understand and predict the drug distribution in the microsphere.\(^9\) Hansen investigated the diffusion of a solute from a solution into the polymeric film and its dependency upon surface, surface thickness, and concentration. This concept was applied in this case, wherein the skin was considered as the biological barrier acting as a surface resistance and polymeric barrier.\(^10\) Thus, the drug diffusion (in terms of permeation) across skin may be affected with the drug concentration and surface area of application. Once sub-Q is injected, the drug was considered to be diffused immediately, being hydrophilic. However, since skin structure is of protein biopolymeric texture, the drug diffusion may be affected and should be investigated. Therefore, the concept was applied in this study using EpiDerm serving as artificial human skin.

Ketoconazole is a broad spectrum antifungal drug used to control local (cutaneous), systemic, and resistant types of fungal infections as a standalone drug or in combination with other medications. Poor aqueous solubility, gastric complications, hepatic metabolism, in vivo degradation, low absorption, and poor oral bioavailability limit its clinical applications for oral, parenteral, and topical delivery. The drug is associated with high oral dose (200–400 mg per day), high octanol–water partition coefficient (log \(P = 4.3\)), poor aqueous solubility, dose-dependent toxicity, and high molecular weight (531 g mol\(^{-1}\)).\(^11\) The drug is the first choice option to treat dermatitis, mucormycosis, and onychomycosis (nail infection) as well as opportunistic local (cutaneous, subcutaneous phycomycosis, deep dermal, anddermal) and systemic (chromomycosis, histoplasmosis, blastomycosis, and paracoccidioidomycosis) infections. In particular, two specific cases of phycomycosis were treated with KETO, where doctors initially prescribed an oral potassium iodide solution (30 mg/kg/day) to both 10 month old boys and 18 month old boys with no benefits. Later, clinicians switched to oral KETO (5 mg/kg/day) and resolved the issue within 2 months (for 10 month old patients) and 6 months (for 18 month old patients).\(^12\) Considering the physicochemical properties of the drug, disease condition, high oral dose, low adherence to topical delivery, and low patient compliance (parenteral or oral), subcutaneous delivery of KETO would be a promising strategy to control local (mucormycosis) and systemic fungal infection with reduced toxicity and side effects.

In fact, in our recently published article, we found maximum solubility of the drug in a biocompatible solvent like N,N-dimethylacetamide (DMA) followed by in silico simulation with a prediction for excellent in vivo performance using a subcutaneous route of administration.\(^13\) Here, we extended such studies by exploring the selection of an appropriate volume fraction (in term of concentration) of DMA in a binary mixture “DMA + water” system using EpiDerm as a dermal substitute of human skin. Based on experimental values of the permeation and diffusion coefficients, HSPIP software was used to simulate and predict the drug absorption across the artificial membrane and impact of diffusion coefficients (constant and variable) on the drug absorption (% C) and in vivo flux. Finally, the selected KF3 (containing 300 mg of DMA and 22.3 mg of KETO) was used to formulate various solutions (K30A-I) for subcutaneous delivery followed by a stability study and in vitro characterization. Then, an in vitro cell line was used to study uninfected and co-infected macrophages (J774A.1), and cellular uptake (L929) studies were carried out to confirm efficient interaction and therapeutic benefits using the explored binary mixture solution (2% w/w). Eventually, the explored K30G was negated for hemolysis using three different concentrations (1, 5, and 10 \(\mu g/mL\) for 6 h). Thus, although requiring in vivo confirmation, the optimized K30G was found to be promising for the sub-Q delivery of KETO with reduced side effects, cost-effectiveness, and high patient compliance.

**MATERIALS AND METHODS**

Materials. Ketoconazole was procured from Sigma-Aldrich, Mumbai, India. Ethanol and DMA (N,N-dimethylacetamide) were obtained from SD Fine, Chem. Ltd., Mumbai, India. Tween 80, PEG200 (polyethylene glycol 200), PEG400 (polyethylene glycol 400), butyl hydroxyl toluene (BHT), and NMP (N-methyl-2-pyrrolidone) were procured from Sigma-Aldrich, Mumbai, India. Arachis oil was obtained from a local shop. Oleic acid (>99.0% pure) was purchased from Sigma-Aldrich, Mumbai, India. Fluorescein isothiocyanate (FITC) (fluorescent marker) was procured from Sigma-Aldrich, Mumbai, India. Tris salt [(tris(2,4-dimethyl-5-sulfanatophenyl))phosphine trisodium salt] was obtained from Merck, Mumbai, India. *Candida albicans* (MTCC 4748) was procured from IMTECH (Institute of Microbial Technology, Chandigarh, Punjab, India). Macrophage (J774A.1) and L929 cell lines were procured and maintained as per M/s ATCC (American Type Culture Collection), Manassas, USA.

**METHODS**

**Solubility Study in Excipients.** The solubility (mole fraction solubility, \(X_e\)) of KETO was studied in tween 80, ethanol, dimethyl acetamide (DMA), N-methyl-2-pyrrolidone (NMP), polyethylene glycol 400 (PEG400), polyethylene glycol 200 PEG200, oleic acid, and arachis oil. The drug was precisely weighed and added to a glass vial containing the excipient to obtain the saturated solubility at 308 ± 0.2 K following the method that was previously reported.\(^13,14\) In brief, the weighed amount of KETO was transferred to a vial and subjected to constant stirring for 24 h using a water bath shaker (Remi Instruments, India). After 24 h, the mixture was centrifuged (8000 rpm for 15 min) to separate the undissolved drug. The supernatant was used to quantify the dissolved amount of KETO using the equations below. The study was repeated to obtain the mean and standard deviation values (\(n = 3\)).

Mole fraction solubility equation is given as follows

\[
X_e = \left( \frac{W_1/M_1}{W_1/M_1 + W_2/M_2} \right)
\]  

(1)

where \(W_1\) and \(W_2\) are the weight of the solute and the solvent, respectively. Similarly, \(M_1\) and \(M_2\) are the molecular mass of the solute and the solvent, respectively.

**KETO-Loaded Solutions (DMA–Water Co-solvent Binary Solution).** Based on the experimental mole fraction solubility, DMA was selected as the suitable co-solvent for sub-Q
delivery. Therefore, a specific amount of KETO (1.2, 11.1, and 22.3 mg/g of solution) was completely dissolved in the DMA–water co-solvent solution. Three formulations (KF1, KF2, and KF3) were prepared by varying DMA concentrations (5, 15, and 30% w/w) in the mixture. Based on the recommended maximum concentration of DMA (30.8%) as per the US-FDA (United State-Food and Drug Administration) for parenteral product administration (particularly for intravenous, intramuscular, and subcutaneous delivery), various concentrations of DMA (5, 15, and 30% w/w) were used to formulate the DMA–water co-solvent mixture. The solutions were inspected visually for any signs of physical instability (such as color change and precipitation) over 24 h. Each formulation was measured for final pH using a digital pH meter. These solutions were subjected for a simulated topical skin permeation study using the cultured human skin EpiDerm (Ashland, MA, USA).

**In Vitro Permeation Assessment Using EpiDerm.** The sterilized (UV irradiation) three formulations (KF1, KF2, and KF3) were used to determine the permeation parameters (specifically, the permeation coefficient, percent cumulative amount of drug release, permeation flux, and diffusion coefficient) using EpiDerm. Topical or sub-Q administration is best studied using cultured human epidermal keratinocytes (EpiDerm), which is as similar to human skin as possible. EpiDerm is an ideal formulation for such studies and was prewashed with phosphate buffer solution (PBS, pH 7.4) for 15 s, which may affect the outer layer and, thus, diffusion of molecules. The cultured skin was mounted over a Franz diffusion cell between the receptor and donor chambers. The receptor chamber was filled with release medium (PBS, pH 7.4) and stirred (300 rpm) using a rice bead (on the magnetic stirrer). The assembly was jacketed to control constant temperature (37 ± 1 °C). The inner portion (1.34 cm²) was in constant contact with the receiver medium, and the receiver medium was maintained at 32 ± 1 °C throughout the study. The test sample was loaded onto the surface exposed to a donor chamber. Sampling (1 mL) was performed at varied time points (1, 2, 4, 8, 12, and 24 h), and an equal volume was replaced with the same fresh medium. This study was repeated to obtain mean and standard deviation values (n = 3).

**Data Analysis for the Permeation Profile.** The drug permeation or release across the EpiDerm was estimated over 24 h, and data were plotted based on the total amount of the drug loaded as the cumulative amount of drug permeated versus time (using Fick’s first law). Similarly, the cumulative amount of the drug permeated versus square time was plotted to calculate the diffusion coefficient (Higuchi model). The flux (J) was calculated using the following eq 2 of Fick’s first law

\[ J = \frac{dQ}{At} = D \times \frac{dC}{dx} = D \times \frac{(C_1 - C_2)}{x} \tag{2} \]

where Q is the amount of KETO (mg), and J is the permeation flux (µg/cm²/h). D, A, x, and P are the diffusion coefficient, operational surface area, thickness (cm), and permeation coefficient (cm/h), respectively. dQ/dt is the fitted permeation of KETO against time. C is the concentration of KETO in the formulation loaded onto the donor chamber. Based on solubility, KF1, KF2, and KF3 contained 1.2, 11.1, and 22.3 mg/mL of KETO, respectively.

The permeability coefficient (P) is the permeation (velocity) of KETO that permeated through the cultured skin, which was calculated from the slope of percent (%) drug permeated versus time as

\[ P = \text{slope} \times \text{volume of receiver}/\text{applied area (A)} \tag{3} \]

On the other hand, the diffusion coefficient (D) (diffusivity) was estimated by plotting the content of KETO diffused per unit area (µg/cm²) against the square root of time (h) using eq 4

\[ D = \frac{J}{(\frac{dC}{dx})} \tag{4} \]

where J and “dC/dx” are the flux and the concentration difference (C₁−C₂) diffused across dx (0.2 cm as the thickness of EpiDerm), respectively.

**HSPiP Software-Based Simulation and Prediction.** This software was used to simulate the permeation rate (µg/cm²) obtained from the artificial EpiDerm-based permeation data. Moreover, several input parameters were used (Table 2) related to the physicochemical properties of KETO, experimental data, literature data, and default values in the software. These input parameters are required to predict the skin absorption of KETO after sub-Q administration or transdermal application. For this purpose, we obtained experimental values of lower and higher levels of diffusion coefficient after our in vitro EpiDerm permeation study as prime input parameters.

Here, we investigated the following: (a) the effect of the constant diffusion coefficient on percent drug absorption (permeation) across EpiDerm and (b) the effect of the varied diffusion coefficient on percent drug absorption. Consideration of the constant diffusivity in the Crank model could result in a lack of model fitting. Some models consider variable diffusivity and could transpose the lack of precision from the Crank model. Four data points were required to model diffusion exactly using Modeller diffusion in the HSPiP software.

The crank model is based on the second Fick diffusion law. These are (a) soluble concentration of the drug at the applied surface, (b) low value of the diffusion coefficient for the drug, (c) varied values of the diffusion coefficient due to variable concentrations, and (d) the diffusion modeller. In this study, three different concentrations of KETO were dissolved in the DMA–water co-solvent solution. Three solutions (specifically, 5, 15, and 30% of DMA in water) were used to dissolve 1.2, 11.1, and 22.3 mg/g of KETO, respectively. These three concentrations were used as the dosing solution on the epidermis (considering transdermal delivery) or a single sub-Q shot (0.12–2.23/100 mg per shot). The obtained values for the diffusion coefficients at different doses (low and high) were used to predict the time expected to obtain an equilibrium within the 0.2 mm thick EpiDerm (Tₑ), breakthrough time (Br), Tₑ/2, and L/Di (surface resistance to diffusion resistance ratio expressed as “B”) values. The maximum volume fraction was 0.3 (0.3 mL per mL) for the DMA–water co-solvent solution depending on the approved maximum concentration of DMA (30.1%) for sub-Q/parenteral delivery.

**Evaluation of the KETO Formulations for Sub-Q Delivery.** Various aqueous formulations of KETO were prepared in the DMA–water co-solvent solution intended for sub-Q delivery. Therefore, various required components such as polyethylene glycol 400 (as a stabilizer), tris salt (as a buffer), and butylated hydroxyl toluene (as a preservative) were incorporated at a constant ratio of DMA in water (30% in KF30). In brief, a constant amount (20 mg/mL) of KETO was dissolved in an aqueous solution of 30% DMA, BHT (0.4%) was used as preservative, followed by adding PEG400. The required quantity of PEG400 was added to maintain osmolality under...
The final pH of the formulations was set at approximately 7 using tris buffer. KETO is a weak dibasic compound, with two pKa values (6.51 and 2.9) exhibiting complete solubility at low pH (<2−3.0) and drug precipitation at pH > 5−6.0.24,25

The prepared formulations were evaluated for final pH, osmolality, and final drug content for 30 days under room temperature. The pH values were measured using a digital Hanna pH meter (Hanna Instruments, USA). The drug content was quantitatively assessed using a previously reported validated method.14 Osmolality was measured using a freezing point osmometer (Knaeur Semi-Micros Osmometer K-7400S). These experiments were replicated to obtain mean and standard deviation values (n = 3). These parameters were evaluated for

Figure 1. (A) Mole fraction solubility (10^3 × Xe) of KETO in solvent at 308.2 K temperature, (B) permeation rate across EpiDerm, and (C) diffusion coefficient over time across EpiDerm for KF1, KF2, and KF3 formulations (the binary solvent mixture containing KETO dissolved in DMA + water mixed system) (data are expressed as mean ± standard deviation taking n = 3 and p < 0.05 as significant (*) data compared to controls).
Fresh formulations and after 30 days of storage at room temperature (30 ± 1 °C). They were visually evaluated for clarity against a black background.

**In Vitro Studies.** Cellular Uptake Using Uninfected Macrophages. KF30G was selected as the most optimized formulation for sub-Q delivery based on the following evaluation parameters: pH, osmolality, and percent drug content after 30 days of stability study at room temperature. K30G was further diluted to lower concentrations (1, 5, and 10 μg/mL) using water for injection. Similarly, aqueous solutions (containing 5% v/v of dimethyl sulfoxide or DMSO) of KETO were prepared in the same concentration range for comparison studies.

This study was conducted based on previously established macrophages (J774A.1) cultured (1 million cells/well) in 50% Dulbecco’s modified essential medium (DMEM), 50% of medium supplemented with 10% fetal calf serum (FCS), and 10 ng/mL epidermal growth factor (EGF) for 2 days in a humidified incubator (Galaxy 170S, New Brunswick, Germany) at 37 °C in 5% CO₂. The percent viability of J774A.1 cells was assessed using 96-well microtiter plates (Greiner CELLSTAR Merck, Germany). For cell differentiation, the cell culture was incubated in RPMI 1640 (Sigma-Aldrich) media supplemented with 10% FCS and 50 ng/mL Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). Macrophages were then washed with 1640 medium to remove non-adherent cells. Adherent cells were incubated for 24 h in RPMI medium to free the cultures from PMA. The medium was replaced with fresh medium containing the formulations or the KETO solution at different concentrations followed by incubation for 24 h. After incubation, macrophages were treated with the MTT salt for 4 h, and then DMSO (5 mg/mL) was added to dissolve formazan crystals formed in the well. The developed purple color was measured using a multiwell reader spectrophotometer (CLARIOstar, BMG LABTECH, Germany) at 570 nm.

**Growth of Candida albicans and Co-infection with Macrophages.** A *candida* strain was grown in YPD (yeast extract peptone dextrose) medium containing peptone (1%), yeast extract (1%), glucose (2%), and agar (1%). The strain was incubated at 30 °C for 24 h. This culture was assessed for CFU (colony forming unit)/mL using standard methods. For this, the fungal strain was grown to 4 × 10⁵ CFU/mL. Then, J77A.1 macrophages (1 million cells/well) and the *candida* strain at 4 × 10⁵ CFU/mL were co-infected followed by incubation for 4 h at the same temperature. For the MTT assay, the infected macrophages were washed with a PBS (3 times) solution and seeded in 96-well plates for adherence. The medium was replaced with fresh medium containing the formulations or KETO solution at different concentrations followed by incubation for 24 h. After incubation, the MTT salt was added, and cells were exposed for 4 h for formazan crystal formation. DMSO was added to dissolve the developed crystals and absorbance was measured at 570 nm. The infected macrophages were treated separately with various concentrations of the K30G and KETO solution. Untreated cells served as a control group (100% viable). Percent cell viability was calculated and compared against the pure drug solution.

**Qualitative Cellular Toxicity Study.** L929 cell lines (the parent cell line derived from normal subcutaneous areolar adipose tissue) were grown on 96-well cell culture plates and exposed to fluorescein isothiocyanate (0.01% w/v) loaded formulations and an equivalent aqueous solution of fluorescein isothiocyanate (FITC solution) in culture medium for 2 h. They were treated separately and incubated at 37 °C in a CO₂ incubator (Galaxy 170S, New Brunswick Germany). Afterward, the cells were washed (three) with PBS (pH 7.4) to remove the excess medium containing the dye and were fixed with 4% paraformaldehyde. Images were acquired on a fluorescent microscope (Axio Vert.A1, ZEISS, Germany) with 60x magnification. Representative cells were selected, and images were captured in FITC channels to observe the internalization of the probed K30G (10 μg/mL of KETO), FITC-Sol, and probed KETO solution (10 μg/mL). Images were acquired on a BD FACSCanto II (BD Biosciences, USA) flow cytometer and analyzed with FlowJo 8.6.3 software (Tree Star, Inc., Ashland, OR). The data gathered were analyzed statistically by side scatter (SSC) parameters and then on FL-2 (RFP-BCG)-positive events versus SSC.

**In Vitro Hemolysis Study.** The study aimed to deliver KETO (2 mg) sub-Q (0.1 mL) for the management of local cutaneous and systemic fungal infection. Therefore, it was a prerequisite to assess preliminary toxicity at concentrations higher than plasma drug concentrations achieved after oral administration (200–400 mg tablet). The present formulation was, thus, tested for hemolysis (first safety concern) if it comes into contact with blood cells. Rat blood (heparinized tube) was centrifuged (SL8/8R Centrifuge, Thermo Scientific, Germany) for 10 min (5000 rpm) to separate erythrocytes (red blood cells, RBCs). RBCs were washed and suspended in saline to obtain a 4% hematocrit value. The RBC suspension (0.5 mL) and the sample (1.0, 5.0, and 10.0 μg/mL) were added to test tubes. The final volume was made up to 5 mL using PBS (phosphate buffer solution). Triton-X100 (0.1% v/v) and the saline solution served as positive and negative controls, respectively. The RBC suspension treated with the samples (individually) was incubated for interaction for 6 h at 37 ± 1 °C. Then, each treated tube was carefully inspected for the lysed cellular debris and intact cells (un-lysed) after centrifugation. The obtained supernatant was subjected for released hemoglobin (Hb) using a spectrophotometer (UV-1601, Shimadzu, GmbH, Germany) at 540 nm (λmax). Positive controls caused 100% hemolysis (n = 3 and p > 0.05 for insignificant lysis).

**RESULTS AND DISCUSSION**

**Solubility Study.** KETO was solubilized in various excipients, and solubility results are shown in Figure 1A. Specifically, ethanol (3.9 ± 0.08 mg/mL), PEG400 (8.2 ± 0.12 mg/mL), PEG200 (7.5 ± 0.3 mg/mL), NMP (8.7 ± 0.14 mg/mL), and DMA (3.2 ± 0.08 mg/mL) exhibited considerable solubility for sub-Q delivery. Commercial products containing 1% w/w of KETO are expected to deliver 1 mg after the application of 100 mg of cream. However, the expected drug (1 mg) cannot be delivered across the skin barrier using conventional cream to control subcutaneous mycosis sitting on the dermal deeper layer. Therefore, the DMA + water binary solvent mixture solubilized 1.2 mg of the drug in 0.1–0.5 mL of the sub-Q injection volume (which is a pain free volume as a pen injection). The drug dose in the optimized sub-Q volume (0.1 mL) is convincing to deliver directly to the infected site with high patient compliance. Chemically, KETO bears six hydrogen-bonding acceptor groups and zero hydrogen-bonding donor groups. PEG200 and PEG400 are related with three hydrogen-bonding acceptors and two hydrogen-bonding acceptor—donor groups. Increased solubility of the drug in PEG200 and PEG400 may be attributed to hydrogen bond interaction between the solute and solvent. NMP and DMA contain 2 and 1 hydrogen bonding acceptor groups (only), respectively, in the molecular
structure. These groups may be responsible for forming hydrogen bonding for improved solubility of the drug. In this study, oil and oleic showed least solubility. Thus, the improved solubility of the drug observed in these five solvents may be due to specific and nonspecific interactions between the solute and solvent (the excipient).29

**Binary Solvents Mixed Solution.** DMA was selected as the cosolvent for further formulation despite the relatively high solubility of KETO in NMP, PEG200, PEG400, and ethanol. This decision was based mainly on safety concern and osmolarity (comparable to blood). The PEG400- and PEG200-based binary mixed solutions were clear. However, these solutions showed high osmolality (>300 mOsm/Kg) as compared to DMA and ethanol based solutions containing KETO (data not shown). Moreover, ethanol can be toxic upon sub-Q delivery at high concentrations and result in fatal cases due to ethanol poisoning (blood level as 2.3 mg/mL).30,31 Therefore, DMA is well suited as a co-solvent used for parenteral and nasal products at as much as 30% and 0.1 mL, respectively.15,22 In this context, DMA was selected to formulate three formulations containing KETO, as shown in Table 1. In the current study, each mL of the aqueous binary formulation (KF1, KF2, and KF3) contained 50, 150, and 300 mg of DMA, respectively, as shown in Table 1. The drug content present in each binary mixed solution varied as per the content of DMA responsible to dissolve the drug and maintained drug stability at the basic optimal pH of 7.4.32 The developed solution was kept overnight for benchtop stability at room temperature to observe any sign of instability. However, the dissolved drug was completely soluble and clear (against a black background) for sub-Q delivery. The final pH was found in the range of 7.2–7.4, which is acceptable.

**In Vitro Permeation Assessment Using EpiDerm.** The three developed binary solutions containing KETO were tested for permeation parameters, and results are presented in Table 1. The permeation flux values across EpiDerm were found to be 5.98, 99.21, and 217.38 μg/cm²/h for KF1, KF2, and KF3, respectively. Similarly, the permeability coefficient (P) and diffusion coefficient (D) values were calculated by using these formulations increased with increasing content of DMA and KETO. The values for the diffusion coefficient (D) are presented in Table 1. The high diffusion coefficient of KF3 may be attributed to the higher rate of diffusion of KETO through the EpiDerm as a result of the higher concentration gradient developed by the DMA based increased solubilization.16

| code | DMA (mg) | drug content (mg) | flux (μg/cm²/h) | P (cm/h) | D (cm²/h) | D range (cm²/s) |
|------|----------|------------------|----------------|----------|-----------|----------------|
| KF1  | 50       | 1.2              | 5.98           | 1.2×10⁻⁴ | 2.1×10⁻⁵ | 2.2×10⁻⁶–1.08×10⁻⁴ |
| KF2  | 150      | 11.1             | 99.21          | 8.9×10⁻³ | 2.5×10⁻³ | 1.5×10⁻⁶–6.9×10⁻⁷ |
| KF3  | 300      | 22.3             | 217.38         | 9.6×10⁻² | 3.9×10⁻³ | 3.7×10⁻⁶–5.8×10⁻⁷ |

Note: P and D indicate permeability and diffusion coefficients, respectively. KF1, KF2, and KF3 represent a binary mixture of DMA + water carrying KETO at the volume fraction of 0.05 (5%w/w), 0.15 (15%w/w), and 0.3 (30%w/w), respectively, for sub-Q delivery.

Predicted the impact of dose and diffusion coefficients (constant and variable diffusion coefficients) on percent drug absorption (% C), equilibrium time of diffusion rate (T_e), permeation rate (g/cm²) or flux (g/cm²/s), breakthrough time (Br) = Time (or cut-off time), and the T_1/2 = half-life to obtain equilibrium over the studied concentration range (in terms of volume fraction, v = 0.05–0.3) (Figures 2A–I, 3A–I, Tables 3, and 4). The value of “h” as the mass transfer coefficient was set low (0.1) to investigate the impact of the surface or entry resistance (or tissue in case of in vivo conditions) condition on diffusion across the EpiDerm or physiological membrane on the diffusion process.

**FUTURE PERSPECTIVES.** The abovementioned values were used as the input parameters in HSPiP Hansen software for predicting the impact of constant and varied diffusion coefficients on % drug absorption after sub-Q delivery. It is obvious from Table 1 that permeability and diffusion coefficient values increased with an increase in DMA and KETO content in the formulation. Diffusion and permeation data obtained in the human skin would be the most relevant to establish an in vivo performance and mechanistic perspective.33,34 However, in vitro permeation and diffusion data generated using the EpiDerm membrane are still valuable to investigate impact of DMA and KETO content on permeation and diffusion rate from the sub-Q injected site. This would be a promising approach for the oral delivery of KETO at low toxicity, low drug degradation, low drug–drug/ drug–food interactions, and minimum side effects.35 KETO is a weak dibasic compound and is soluble in binary mixed solutions composed of DMA (basic solvent) and water. Enhanced drug permeation and diffusion can be maximized for the sub-Q delivery of KETO in the current binary system, which may be attributed to a non-protonated form of KETO existing at this pH.35

**HSPiP Software-Based Simulation and Prediction of In Vivo Absorption.** Using the experimentally obtained diffusion coefficient values of KF1, KF2, and KF3 across 0.2 mm of EpiDerm and other input parameters (Table 2), HSPiP software predicted the impact of dose and diffusion coefficients (constant and variable diffusion coefficients) on percent drug absorption (% C), equilibrium time of diffusion rate (T_e), permeation rate (g/cm²) or flux (g/cm²/s), breakthrough time (Br) = Time (or cut-off time), and the T_1/2 = half-life to obtain equilibrium over the studied concentration range (in terms of volume fraction, v = 0.05–0.3) (Figures 2A–I, 3A–I, Tables 3, and 4). The value of “h” as the mass transfer coefficient was set low (0.1) to investigate the impact of the surface or entry resistance (or tissue in case of in vivo conditions) condition on diffusion across the EpiDerm or physiological membrane on the diffusion process.

**Table 1. Summary of Applied Doses for KF1, KF2, and KF3 Loaded With KETO and Estimated Permeation Parameters Obtained Using EpiDerm**

| code | DMA (mg) | drug content (mg) | flux (μg/cm²/h) | P (cm/h) | D (cm²/h) | D range (cm²/s) |
|------|----------|------------------|----------------|----------|-----------|----------------|
| KF1  | 50       | 1.2              | 5.98           | 1.2×10⁻⁴ | 2.1×10⁻⁵ | 2.2×10⁻⁶–1.08×10⁻⁴ |
| KF2  | 150      | 11.1             | 99.21          | 8.9×10⁻³ | 2.5×10⁻³ | 1.5×10⁻⁶–6.9×10⁻⁷ |
| KF3  | 300      | 22.3             | 217.38         | 9.6×10⁻² | 3.9×10⁻³ | 3.7×10⁻⁶–5.8×10⁻⁷ |

**Table 2. Input Parameters for HSPiP Software-Based Simulation and Prediction**

| required input parameters | values |
|---------------------------|--------|
| diffusion coefficient range (cm²/sec) for KF1 | 2.2×10⁻¹–1.08×10⁻⁶ (low to high) |
| diffusion coefficient range (cm²/sec) for KF2 | 1.5×10⁻⁶–6.9×10⁻⁷ (low to high) |
| diffusion coefficient range (cm²/sec) for KF3 | 3.7×10⁻⁶–5.8×10⁻⁷ (low to high) |
| epiderm thickness (mm) | 0.2 |
| grid | 100 |
| solvent density (g/mL) | 0.94 |
| surface concentration (mg) | 1.2–22.3 |
| prediction mode | absorption |
| time to achieve equilibrium (min) | in minute |
| breakthrough (%) | 0.1 |
Using Concentration-Dependent Diffusion Coefficients

where \((EpiDerm)\). coefficient as a limiting factor due to innate surface resistance unable to penetrate across a membrane provides no diffusion

Table 3. Results Obtained From HSPiP-Based Predictions on Permeation Parameters (Drug Absorption and Equilibrium Time) Using Constant Diffusion Coefficients

\[
\text{Table 3. Results Obtained From HSPiP-Based Predictions on Permeation Parameters (Drug Absorption and Equilibrium Time) Using Constant Diffusion Coefficients}
\]

| parameters\(^{a}\) | KF1 | KF2 | KF3 |
|------------------|-----|-----|-----|
| \(D_i (cm^2/s)\) | \(2.2 \times 10^{-8}\) | \(1.1 \times 10^{-6}\) | \(1.5 \times 10^{-10}\) | \(6.9 \times 10^{-7}\) | \(3.7 \times 10^{-10}\) | \(5.8 \times 10^{-7}\) |
| \(T_e (min)\) | 4 h | 8 min | 500 h | 12 min | 400 h | 15 min |
| \(B (L/D_i)\) | \(1.8 \times 10^3\) | \(3.6 \times 10^3\) | \(2.7 \times 10^7\) | \(5.8 \times 10^7\) | \(1.1 \times 10^7\) | \(6.9 \times 10^7\) |
| \(T_{1/2} (min)\) | 13.1 min | 0.3 min | 31.1 h | 0.5 min | 13.1 h | 0.5 min |
| \(T_{1/2} (min)\) | 13.76 min | 0.28 min | 33.64 h | 0.4 min | 13.64 h | 0.52 min |

\(^{a}\)Note: \(D_i = \) Diffusion coefficient or diffusivity, \(T_e = \) time to attain equilibrium, \(B = \) diffusion resistance to surface resistance \((L/D_i)\), \(Br=\) breakthrough time = time (cut-off time), and \(T_{1/2} = \) half-life to attain equilibrium.

Note: \(D_i = \) Diffusion coefficient, \(T_e = \) time to attain equilibrium, \(B = \) diffusion resistance to surface resistance \((L/D_i)\), \(Br=\) breakthrough time = time (cut-off time), and \(T_{1/2} = \) half-life to attain equilibrium.

Table 4. Results Obtained From HSPiP-Based Predictions on Permeation Parameters (Drug Absorption and Equilibrium Time) Using Concentration-Dependent Diffusion Coefficients

\[
\text{Table 4. Results Obtained From HSPiP-Based Predictions on Permeation Parameters (Drug Absorption and Equilibrium Time) Using Concentration-Dependent Diffusion Coefficients}
\]

| parameters\(^{a}\) | KF1 | KF2 | KF3 |
|------------------|-----|-----|-----|
| \(D_i (cm^2/s)\) | \(2.2 \times 10^{-8}\) | \(1.0 \times 10^{-7}\) | \(1.1 \times 10^{-6}\) | \(1.5 \times 10^{-10}\) | \(6.9 \times 10^{-7}\) | \(3.7 \times 10^{-10}\) | \(8.0 \times 10^{-8}\) | \(5.8 \times 10^{-7}\) |
| \(T_e (min)\) | 30 | 50 | 50 | 120 | 120 | 120 |
| \(B (L/D_i)\) | \(1.8 \times 10^3\) | \(2.6 \times 10^7\) | \(1.1 \times 10^7\) | \(1.1 \times 10^7\) | \(1.1 \times 10^7\) | \(1.1 \times 10^7\) |
| \(Br (min)\) | 5.1 min | 11.1 min | 32.1 min | 32.1 min | 32.1 min | 32.1 min |
| \(T_{1/2} (min)\) | 1.05 min | 1.38 min | 3.51 min | 3.51 min | 3.51 min | 3.51 min |

\(^{a}\)Note: \(D_i = \) diffusivity, \(T_e = \) time to attain equilibrium, \(B = \) diffusion resistance to surface resistance \((L/D_i)\), \(Br=\) breakthrough = time (cut-off time), and \(T_{1/2} = \) half-life to attain equilibrium.

Diffusion is concentration-dependent. However, a molecule unable to penetrate across a membrane provides no diffusion coefficient as a limiting factor due to innate surface resistance \((EpiDerm)\). Therefore, an additional term “\(h\)” is included indicating the mass transfer coefficient in \(1/s\). The constant diffusion coefficient \((B)\), is thus mathematically defined as

\[
B = (h \times L/D)
\]

where \(h\) and \(L\) are the mass transfer coefficient and the membrane thickness \((0.2 \text{ mm})\), respectively. The term “\(D\)” indicates the diffusion coefficient. The surface resistance becomes visible in the graph (linear dependence over time rather than square root of time) when \(B\) approaches unity. This in vitro program could simulate and predict the in vivo performance of the drug after sub-Q injection of a binary solution for local and systemic effects (immediate and sustained delivery) in the human body. HSPiP predicted drug absorption \((\% \text{ C})\) from the site of sub-Q injection (as considered for this study), equilibrium time of the drug diffusion, and permeation flux over the explored volume fraction of DMA in the binary solution \((\text{KF1}, \text{KF2}, \text{and KF3})\). The results are presented in Figures 2A–I and 3A–I for constant and concentration-dependent diffusion coefficients, respectively.

In the case of constant diffusion coefficients over the volume fraction range of 0.05–0.3, the HSPiP software modelled diffusion coefficients versus volume fraction \((\text{Figure 2A})\), absorption with a linear dependence over the square root of time \((\text{min})\) \((\text{Figure 2B})\), and the drug concentration variation across the membrane \((EpiDerm)\) thickness \((0.2 \text{ mm})\) \((\text{Figure 2C})\). The percent concentration \((\% \text{ C})\) is defined as the concentration \(C\) (volume fraction) as specified as 100%. Therefore, \(C = 0.05\) (or 5% as the volume fraction) for a 100% concentration \((1.2 \text{ mg of KETO in KF1})\). Similarly, 0.15 and 0.3 as the volume fraction correspond to a 100% concentration of KF2 \((11.1 \text{ mg})\) and KF3 \((22.3 \text{ mg})\), respectively \((\text{Tables 1 and 2})\). The model predicted Fickian diffusion of the drug across EpiDerm at a constant diffusion coefficient (high \(D\) values) from KF1 to KF3 over a volume fraction of 0.05–0.3 with rapid equilibrium time \((8, 12, \text{ and } 15 \text{ min})\) corresponding to KF1, KF2, and KF3, as shown in Figure 2 and Table 3. These equilibrium values were predicted using experimentally obtained diffusion coefficient values such as \(1.1 \times 10^{-6} \text{ cm}^2/\text{s} \text{(KF1)}, 6.9 \times 10^{-7} \text{ cm}^2/\text{s} \text{(KF2)}, \text{ and } 5.8 \times 10^{-7} \text{ cm}^2/\text{s} \text{(KF3)}, \text{ respectively. The equilibrium of the diffusion process was achieved relatively soon at a high diffusion coefficient \((1.1 \times 10^{-6} \text{ cm}^2/\text{s})\) \((\text{Figure 2A})\). The \% \text{ C} or permeation value across the thickness was linearly dependent on the square root of time \((\text{Figure 2B,C})\). A similar pattern was predicted for KF2 and KF3 at \(6.9 \times 10^{-7} \text{ cm}^2/\text{s}, \text{ and } 5.8 \times 10^{-7} \text{ cm}^2/\text{s}, \text{ respectively (Figure 2D–I). Figure 2C,F,I illustrates the drug absorption over thickness of EpiDerm wherein the breakthrough time \((\text{Br})\) was found to increase with a decrease in diffusion coefficient over the explored volume fraction range of 0.05–0.03. These values were predicted as 0.3, 0.5, and 0.52 min at \(1.1 \times 10^{-6} \text{ cm}^2/\text{s} \text{(KF1)}, 6.9 \times 10^{-7} \text{ cm}^2/\text{s} \text{(KF2)}, \text{ and } 5.8 \times 10^{-7} \text{ cm}^2/\text{s} \text{(KF3)}, \text{ respectively (Tables 1 and 3). The values of half-life used to obtain equilibrium followed the same trend (Table 3), which may be due to the increased concentration of KF3 as compared to KF1.}

In case 2, Figure 3A–I demonstrates the predicted pattern of the percent drug absorption (permeation or diffusion) across EpiDerm based on the concentration dependent diffusion coefficient using HSPiP software. The percent drug absorption or permeation value was linearly dependent on the square root of time \((1.1 \times 10^{-6}, 1.0 \times 10^{-7}, \text{ and } 2.2 \times 10^{-7} \text{ cm}^2/\text{s}) \text{ for KF1 over the volume fraction range of 0.05–0.3 (Figure 3A–C). A similar pattern was obtained for KF2 and KF3. The diffusion coefficient values were concentration-dependent (linear up to a 0.3 volume fraction). Notably, the absorption concentration or permeation values were predicted as linear dependent over the square root of time \((\text{Figure 3B,E,H})\), with equilibrium times of 30 min \((\text{KF1})\), 50 min \((\text{KF2})\), and 120 min \((\text{KF3})\) \((\text{Table 4})\). Figure 3C–I illustrates that the drug absorption/diffusion across EpiDerm followed the mechanism between Fickian and case-II
type for KF1, KF2, and KF3. HSPiP predicted permeation flux values from KF3, KF2, and KF1 across the EpiDerm as $4.07 \times 10^{-6}$, $4.01 \times 10^{-6}$, and $1.1 \times 10^{-6}$ g/cm$^2$/s at respective D range values (Table 4). Thus, the maximum predicted value of KF3 indicated enhanced drug solubilization at a fraction volume 0.3 for augmented permeation as compared to the others (KF1 and KF2). Figure 3 shows a drug absorption concentration profile (case-II absorption with linear uptake) predicted by moving boundaries (variable diffusion coefficient) and the diffusion front will progress after reaching a critical concentration (the surface concentration increased slowly).

Conclusively, KF1, KF2, and KF3 obtained short equilibrium times (8−18 min) at constant diffusion coefficients as compared to concentration-dependent diffusion coefficients (30−50 min) across EpiDerm, which can be prudent to predict the in vivo performance (absorption/permeation/diffusion) of the drug after sub-Q delivery for local and systemic therapeutic benefits. The diffusion coefficient values were found to decrease with an increasing content of the drug in the formulations (KF1−KF3). KF1 (1.2 mg) obtained equilibrium at 8 min as compared to KF2 (11.1 mg at 14 min) and KF3 (22.3 mg at 18 min). Thus, rapid drug clearance/absorption/permeation (via the constant diffusion coefficient based formulation) of the sub-Q injected formulation is mandatory for immediate release as compared to sustained action (via the concentration-dependent diffusion coefficient). For prolonged action, the diffusion coefficient should be dependent on the concentration and involvement of surface resistance or entry resistance or diffusion resistance. In physiological conditions, all of these factors play a critical role in case-II (for the solvent concentration dependent diffusion) and super case-II (i.e., the diffusion rate is the combination of the concentration-dependent coefficient and involvement of surface resistance or entry resistance) types of drug diffusion.

The theoretical molar mass of DMA is 87.122 g mol, which is considered suitable for maximized absorption (estimated in the Hansen program using HSPiP software). In general, very small molecules (molar mass < 70 g mol) are preferably absorbed compared to large molecules (molar mass ~ 200 g mol). Therefore, molecules of average size could be appropriate for improved permeability across the dermal region for systemic drug access. Molecules with a relative energy difference (RED)
greater than unity (RED > 1) are difficult to permeate across the skin or dermal region or permeation is prolonged for sustained and controlled release (RED < 1 for small molecule and rapid permeation).

**KETO Formulations for Sub-Q Delivery and Evaluation.** Considering the results obtained from ex vivo permeation and HSPiP-based simulation prediction studies, KF3 was selected for developing a subcutaneous formulation of KETO as an alternative topical or oral formulation. The detailed compositions of several formulations (K30A-K30I) are shown in Table 5. To deliver a single shot of a sub-Q pen delivery, a 0.1 mL (2.0 mg of KETO) volume might be suitable for patient compatibility and high patient compliance. Tris salt was used to maintain the pH comparable to physiological conditions, as obtained from the HSPiP-based prediction for the effect of the varied diffusion coefficients on permeation parameters (% drug absorbed over time and skin thickness). (A) Diffusion coefficient was increasing with increase in volume fraction of DMA up to 0.3. Beyond volume fraction of 0.3, there was constant value of diffusion coefficient for KF1 across epiderm, (B) percent drug concentration absorbed vs the square root of time for KF1 across epiderm (exponential curve of absorption), (C) trend of % concentration of the drug absorbed into the varied depth of epiderm thickness (85% thickness of the epiderm covered for the drug absorption), (D) diffusion coefficient was increasing with increase in volume fraction of DMA up to 0.3. Initially, it was sharp increment (below volume fraction 0.1) followed by a linear progress till 0.3. Beyond volume fraction of 0.3, there was constant value of diffusion coefficient for KF2 across epiderm (E) Percent drug concentration absorbed vs the square root of time for KF2 across epiderm, (F) trend of % concentration of the drug absorbed into the varied depth of epiderm thickness (90% of the thickness covered) from KF2, (G) diffusion coefficient was increasing with increase in volume fraction of DMA up to 0.3. Initially, it was sharp increment (below volume fraction 0.1), followed by a linear progress till 0.3. Beyond volume fraction of 0.3, there was constant value of diffusion coefficient for KF3 across epiderm, (H) percent drug concentration absorbed vs the square root of time for KF3 across epiderm, and (I) trend of % concentration of the drug absorbed into the varied depth of epiderm thickness (90% thickness remained for absorption) from KF3.

**Table 5. Detailed Composition of KETO Loaded Formulations and Evaluation Parameters Intended for Sub-Q Delivery**

| code   | KETO | DMA  | PEG400 | Tris | BHT | pH  | Osm (mOsm/Kg) | clarity |
|--------|------|------|--------|------|-----|-----|-------------|---------|
| K30A   | 20   | 300  | 150    | 0.01 | 0.5 | 6.8 | 489         | clear   |
| K30B   | 20   | 300  | 150    | 0.02 | 0.5 | 8.1 | 501         | clear   |
| K30C   | 20   | 300  | 150    | 0.05 | 0.5 | 8.7 | 517         | clear   |
| K30D   | 20   | 300  | 150    | 0.01 | 1.0 | 7.0 | 508         | clear   |
| K30E   | 20   | 300  | 150    | 0.01 | 2.0 | 7.1 | 513         | clear   |
| K30F   | 20   | 300  | 150    | 0.01 | 3.0 | 7.1 | 523         | clear   |
| K30G   | 20   | 300  | 100    | 0.01 | 4.0 | 6.9 | 324         | clear   |
| K30H   | 20   | 300  | 50     | 0.01 | 4.0 | 7.0 | 249         | clear   |
| K30I   | 20   | 300  | 25     | 0.01 | 4.0 | 6.9 | 201         | clear   |

*Note: KETO = ketoconazole, DMA = dimethyl acetamide, PEG400 = polyethylene glycol 400, Tris = Tris salt for buffer pH, BHT = butylated hydroxytoluene, and oSm = osmolality. Each formulation was made to a final volume of 1 mL using water for injection.*
shown in Table 5. The content of BHT (as a preservative) and tris salt was optimized to maintain the desired osmolality (Osm) value (∼300 mOsm/kg). Therefore, K30G was suitable among them possessing an Osm value near blood with a clear solution. PEG400 worked as a co-solvent to maintain KETO soluble in the desired dosing volume. All formulations were stored in a clear glass vial to observe physical stability (any sign of instability such as drug precipitation, suspended particle, and color changes) for a month at room temperature. All formulations were chemically and physically stable (Tables 5 and 6).

| code  | pH  | mOsm | % DC  | pH  | mOsm | % DC |
|-------|-----|------|-------|-----|------|------|
| K30A  | 6.8 | 489  | 99.98 | 6.8 | 491  | 99.92 |
| K30B  | 8.1 | 501  | 99.6  | 7.9 | 498  | 99.91 |
| K30C  | 8.7 | 517  | 99.89 | 8.6 | 511  | 99.85 |
| K30D  | 7.0 | 508  | 99.99 | 6.9 | 510  | 99.94 |
| K30E  | 7.1 | 513  | 99.92 | 7.2 | 506  | 99.81 |
| K30F  | 7.1 | 523  | 99.95 | 7.0 | 524  | 99.94 |
| K30G  | 6.9 | 324  | 99.98 | 6.9 | 315  | 99.97 |
| K30H  | 7.0 | 249  | 99.98 | 7.1 | 254  | 99.95 |
| K30I  | 6.9 | 201  | 99.94 | 6.8 | 205  | 99.91 |
| K30J  | 6.8 | 489  | 99.97 | 6.8 | 484  | 99.89 |

**Cellular Uptake Using Uninfected and Infected Macrophages.** It was mandatory to investigate the cytotoxic behavior of the developed K30G formulation at diluted concentrations against uninfected J774A.1 cells. Results are demonstrated in Figure 4A,B for uninfected and infected cells, respectively. K30G exhibited cell viability as 97.5 ± 4.6, 89.6 ± 3.8, and 82.4 ± 2.7% at 1, 5, 10 μg/mL after 24 h of incubation, respectively. DS values were observed as 60.9 ± 2.1, 31.8 ± 1.5, and 18.5 ± 0.9% at 1, 5, 10 μg/mL after 24 h of incubation, respectively. It is apparent that K30G exhibited no significant growth rate of macrophages in a dose dependent manner as compared to DS (Figure 4A). The higher cytotoxicity caused by DS may be attributed to the presence of DMSO in DS. However, K30G could not exhibit such cytotoxicity due to the free form DMSO content and safe components in the formulation. The IC₅₀ value of KETO against J774A.1 cells was previously reported as 351 μg/mL, which is quite high as compared to our finding (17 μg/mL). Thus, the explored formulation could be promising to reduce fungal cells or amastigotes without causing normal cell toxicity.

Figure 4B shows the cellular uptake by the infected cells (candida) using various concentrations of K30G. Result showed that K30G caused preferentially higher cellular uptake by the infected cells as compared to uninfected cell lines. This can be correlated to maximized drug-cell internalization of the formulation in infected cells. Thus, K30G showed slowed cytotoxicity to normal cells (compared to DS), and this may be correlated to facilitated diffusion of soluble KETO into the infected cells (fungal cell mediated host membrane disruption) and subsequent internalization with residing candida cells. On the other hand, uninfected cells and host cell plasma membrane are normally healthy to hinder the entry to KETO to a toxic level. This also suggests a safe and compatible binary mixture based formulation as compared to DS causing no substantial impact on normal cells. The effectiveness of K30G can be correlated with fine-tuned solubility, low sub-Q dose, and a customized dosing volume (0.1 mL) in a binary system based formulation capable of improving permeation and drug properties.

**Qualitative Cellular Cytotoxicity Study.** The optimized formulation K30G, blank K30G, DS, and controls (untreated) were used to investigate cellular toxicity on dermal fibroblast L929 cells using the combined techniques CLSM and FCM. Cellular internalization of K30G was quantified and compared against DS and control groups, as shown in Figure 5A–D. It is obvious from the findings that K30G exhibited maximum cellular internalization (intense blue fluorescence by the candida cells and L929 cells) as compared to DS due to the fine-tuned solubility of KETO in K30G. Intense blue color as a result of maximized cellular uptake by the candida cells indicated the cytotoxic nature of K30G without affecting normal host cells. Controls (untreated) did not show significant fluorescence intensity as compared to K30G, whereas blank KF30 revealed relatively higher cellular uptake as evidenced by the high fluorescence intensity as compared to DS and intact cellular margin and integrity of fungal cells (Figure 3C,E). The result clearly shows that the blank K30G easily permeated across the fungal cell membrane without causing cytotoxicity to fungal hyphae/cells (as evidenced by the normal morphology of fungal cells after treatment), whereas the K30G-treated group revealed...
high cellular uptake and a subsequent cytotoxic effect to fungal cells (as evidenced with reduced cell size, condensed nuclear materials, and fragmented candida cells). DS showed limited intensity due to drug insolubility and limited dye permeation across the cell membrane. Notably, cellular uptake of K30G by the dermal L929 cells is particularly important to control local cutaneous fungal infections (intracellular candida cells) and systemic mycosis without causing normal host cell toxicity. Similar findings were obtained from a solid lipid-based nanocarrier for improved cellular uptake by dermal cells (L929) to control dermal fungal infection as reported earlier by our research group. In Vitro Hemolysis Study. The optimized K30G formulation was subjected to a hemolysis study at three different concentrations (1 and 5 μg/mL), as shown in Figure 6. The study was performed at different concentrations to estimate concentration-dependent hemolysis. The result was compared against negative control groups (PBS and saline). Hemolysis was monitored by estimating the released hemoglobin released after incubation of cells with the test sample. Results showed that the control saline and PBS had 8.9 and 9.2% hemolysis, respectively, whereas K30G showed 10–12% hemolysis over the investigated concentration range. Thus, the percent hemolysis caused by the optimized product was comparable to negative controls, suggesting a safe and biocompatible formulation. Moreover, this may be attributed to GRAS (generally regarded as safe grade) excipients in K30G for sub-Q delivery. However, a repeated dose toxicity study at various time points and various concentrations of K30G using an animal model would give more a reliable assurance for a long term treatment strategy (and will be determined in a future study). Maximum lysis caused by the positive control may be due to the toxic nature of triton X100 by inducing lysis and alteration in the cell membrane proteins to create micropores. Moreover, the issue of relevance to the in vitro findings of hemolysis to in vivo conditions is complex, and it does not reflect the tangible situation in vivo wherein several damaging factors play critical roles interacting with RBCs. Similar findings were achieved using KETO loaded solid lipid nanoparticle for transdermal delivery to control systemic and deep dermal fungal infections. Thus, although requiring further study, the product may be safe and biocompatible for sub-Q delivery due to the absence of ionic components and toxic organic solvents.
CONCLUSIONS

Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy. Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy. Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy. Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy. Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy. Considering physicochemical (highly insoluble in water and high molecular weight) and pharmaceutical (high dose and long-term dosing for chronic cases) properties as well as poor patient compliance (gastric complications and hepatic first pass effects) of KETO, sub-Q delivery offers a promising opportunity for patient compliance and cost-effective strategy.

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