ORIGINAL RESEARCH

Ketoconazole Nanocrystals Fortified Gel for Improved Transdermal Applications

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Abstract: Ketoconazole (KTZ) is a commonly prescribed antifungal drug used to treat several tropical and systemic fungus infections. The topical bioavailability of KTZ is limited due to extremely low water solubility and high molecular weight. The present investigation aimed to develop a ketoconazole nanocrystals formulation to improve the biomimetic attributes. The ketoconazole nanocrystals were prepared using a top-down media milling technique with a size of less than 800 nm and a narrow polydispersity index (PDI) of 0.434. The simplex lattice design was used to further investigate the effect of change in the proportion of different stabilizers on critical product attributes such as particle size and PDI. The result of the in vitro drug release and anti-fungal study proved the superiority of the optimized KTZ nanocrystal in inhibiting fungal infection and suspension of pure drug. The optimized nanocrystals entrapped within Carbopol-934P gel had higher permeability through cellulose membrane compared, to the marketed product (2 % ketoconazole % w/w Ketodoc Cream®) with sustained release for 24 h. Moreover, the release profile indicated diffusion-controlled release from gel following Korsmeyer-Peppas. The present investigation successfully demonstrated the application of simplex lattice design and desirability function in optimizing ketoconazole nanocrystals to improve its transdermal application.

Keywords: Ketoconazole; Nanocrystals; Simplex lattice design; Antifungal; Gel; Diffusion.

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1. INTRODUCTION

Ketoconazole, an imidazole derivative, is a highly effective broad-spectrum antifungal drug used to treat a variety of superficial and systemic mycoses. Ketoconazole anti-fungal activity can inhibit the synthesis of ergosterol, an essential component of the fungal cell membrane (Wilson, 2004). Moreover, for superficial and localized infections, topical application is preferred over oral administration to avoid systemic side effects and restrict the therapeutic effect to the affected area. KTZ is a weakly basic compound that possess two pKa values - 2.94 and 6.15. KTZ has an excellent water solubility below pH 3; however, solubility drastically reduced above pH 6 (around 10-25 µg/mL at 25 °C). Additionally, it also has a high molecular weight of 531.43 g/mol and a high log P of 4.31 (Jacobs *el al.*, 2016). The bioavailability of KTZ from currently available market formulations such as cream and gels is very poor as the individual particles do not permeate very efficiently into the skin due to their extremely low water solubility and high molecular weight. Other formulations such as

soaps and shampoos are applied for a very short time on the skin and thus do not give sufficient time to reach the required therapeutic concentration (Patel *el al.*, 2011).

Nanoparticles improve drug dissolution by increasing the specific surface area and solubility, however, their tendency to agglomerate after dispersion hinders their full potential. Chemical strategies such as modifying drug molecules or forming soluble salts, prodrugs, or cyclodextrin complexes have been effective in enhancing dissolution and should be considered alongside nanotechnology (Chi Lip Kwok & Chan, 2014). Nanocrystal-based topical formulations have the potential to improve drug delivery through sustained release and localized effects. The morphology and barrier nature of the skin can be overcome by nanocrystals to enhance drug penetration pathways. The use of nanocrystals in topical drug delivery showed promising breakthroughs in addressing issues of solubility, bioavailability, and toxicity (Patel *el al.*, 2018). Conventional topical antifungal treatments have certain limitations such as frequent applications and side effects. To overcome these issues, researchers are exploring the use of nanomedicines. KTZ-loaded PLGA nanoparticles and AgNPs were incorporated into a gel for topical application. The objective was to sustain the release of KTZ at the site of infection and reduce systemic absorption. The gel formulation was evaluated in a rat model for its efficacy against a resistant strain of Candida albicans. The results showed promising outcomes in treating the skin infection (Sadozai *el al.*, 2022). Ramzan and *el al.*, (2022) aimed to evaluate and investigate optimized and novel KTZ-loaded solid lipid nanoparticles (KTZ-SLNs) for enhanced permeation across the skin. The results showed that the optimized KTZ-SLN formulation exhibited a suitable particle size and high entrapment efficiency. The nanoparticles showed improved penetration into the viable epidermis and demonstrated photostability and long-term stability. In another investigation, Souto *el al.*, (2005) prepared ketoconazole-loaded lipid nanoparticles using compritol-888 ATO (glyceryl behenate) as lipid material for improving the topical application. Moreover, Singh demonstrated that liquisolid compact techniques could be an important technique to improve the solubility of KTZ and thus improve the therapeutic efficacy (Singh *el al.*, 2015). Additionally, in a recent study, the researcher designed KTZ nanocrystal-based cryopelets combining the strengths of freeze-dried powders and pellets that potentially improve the nanocrystals redispersibility, compared with other drying techniques while facilitating the downstream processing (Touzet *et al.*, 2020).

Despite having various promising therapeutic properties, all the above-mentioned formulation approaches suffer from some critical inherent limitations associated with them. For example, the preparation of nano/microemulsion requires a high concentration of surfactants to keep the drug in solubilized form. Such a high concentration of surfactants may cause skin irritation due to their ability to solubilize lipid membranes (Effendy & Maibach, 1995). Moreover, the preparation of solid lipid nanoparticles requires expensive instruments such as rotary vacuum evaporators or spray dryers, however different critical process parameters associated with such processes can be optimized skillfully to ensure a product with consistent characteristics (Müller *el al.*, 2000).

Therefore, compared to the above-listed techniques, the fabrication of nanocrystals is simple, easy to scale, and an effective technique to improve the solubility and dissolution rate of poorly soluble drugs, resulting in better permeation and retention of drugs when applied topically (Pelikh *el al.*, 2018; Yu *el al.*, 2018). Nanocrystals are pure drug particles, stabilized by suitable excipients (such as surfactants or long-chain polymers) with no matrix material, and have an average diameter below 1µm (typically within 200-800 nm (Müller *el al.*, 2011). In addition, nanocrystals improve the dermal penetration of the drug by following mechanisms: An increase in saturation solubility of the drug results in an increased concentration gradient between the formulation layer and the skin, which increases the drug's diffusion through the skin (Muller *el al.*, 1999). Increase in dissolution velocity due to the high surface area per unit volume of the drug particles. Nanosized drug particles demonstrate better adhesiveness to the skin, compared to their coarse counterparts (Müller *el al.*, 2002). Nanoparticles have better penetration capacity into the hair follicles, reaching deeper functional structures of the skin, where they can be stored as a reservoir to have a prolonged effect (Lademann *el al.*, 2007). However, one of the critical issues associated with the development of drug nanocrystals is the propensity of drug nanocrystals to aggregate and enlarge due to high surface energy which can destabilize the system so nowadays the current scenario is to entrap the drug nanocrystals in viscous hydrogel

formulation to have better stability and easier application. It was also proved that suspended solid particles can penetrate the skin more pronouncedly and deeper compared to the formulations where the active drug is been dissolved in the vehicle.

Carbopol 934P was selected as the gelling polymer in the current investigation. Firstly, Carbopol 934P is a highly efficient gelling agent, capable of forming viscous gels at relatively low concentrations. This allows for the creation of stable gel formulations with desirable rheological properties suitable for transdermal delivery. Moreover, Carbopol 934P is a pH-responsive polymer, meaning its gelation and swelling behavior can be modulated by adjusting the pH of the formulation. This property enables fine-tuning of drug release rates from the gel matrix, offering flexibility in controlling the delivery profile. Another crucial advantage is the bioadhesive nature of Carbopol 934P. Its ability to adhere to the skin surface can enhance the residence time of the gel formulation, potentially increasing the extent of drug absorption and bioavailability. Additionally, Carbopol 934P is a non-ionic polymer, which minimizes the risk of skin irritation or adverse reactions, making it a safe choice for transdermal formulations.

Lastly, Carbopol 934P exhibits compatibility with a wide range of active pharmaceutical ingredients (APIs), enabling its use in formulating diverse transdermal drug products.

In light of the above-mentioned attributes, the present investigation aimed to formulate and optimize KTZ nanocrystal-loaded hydrogel for improved transdermal delivery.

2. MATERIALS AND METHODS

Ketoconazole was received as a generous gift from Torrent Pharma Ltd. HPMC E-15, HPMC E-5, Carbopol 934P were purchased from Lobachemie, Mumbai. Poloxamer 407 was procured from Sigma Chemicals, Mumbai, India. All other reagents used were of analytical grades.

2.1. Preliminary studies

The preliminary studies were conducted using OFOT (one parameter/factor at a time approach) to fix different process parameters such as milling time and bead size based on their efficiency in reducing the particle size of the drug. In the OFOT approach, only one parameter is adjusted at a time, while all other parameters are kept constant. For example, while fixing the milling time, other parameters such as drug, polymer ratio, suspension, bead ratio, and bead size were kept constant. The particle size was analyzed after specific time intervals (1 h, 2 h, 4 h, etc.) until no substantial change in particle size was observed. Subsequently, All the simplex lattice design batches were milled up to this specific time only. The same way screening was carried out for zirconium bead size (0.7 mm and 1 mm) as well where all other factors except the bead size were kept constant.

2.2. Fabrication of ketoconazole nanocrystals

The ketoconazole nanocrystals were prepared using a top-down media milling technique. The appropriate quantity of stabilizers considering simplex lattice design was pre-dissolved in distilled water (50 mL) under sonication (FS-750, Frontline Electronics and M/C Pvt. Ltd., Ahmedabad). This polymeric dispersion was added to a round vial (50 x 98 mm) in which subsequently drug (25 mg = 0.5 % w/w), beads with a size of 1 mm, and a magnetic stirrer were placed (Remi Elektrotechnik, India). Later the vial was sealed and placed over a magnetic stirrer. Stirring was performed for 16 h with a stirring speed of about 800 rpm (Singh, Srinivasan *el al.*, 2011, Salazar, Ghanem *el al.*, 2012).

2.3. Preparation of simplex lattice design batches

The simplex lattice design was applied to investigate the effect of change in the proportion of different stabilizers (HPMC E5, HPMC E15, and Poloxamer 407) on the critical properties of nanocrystals such as particle size and PDI. The composition of simplex lattice design batches according to simplex lattice design is presented in Table 1.

2.4. Evaluation of simplex lattice design batches

2.4.1. Particle size, PDI, and zeta potential

Malvern Zetatrac Instruments (Malvern, UK) was used to measure the average particle size of ketoconazole nanocrystals. Samples were directly measured without dilution. A particle size analyzer measures particle size by analyzing fluctuations in

the intensity of scattered light due to the Brownian motion of the particles. Each sample was measured in triplicate.

2.4.2. In-vitro dissolution

In-vitro dissolution was performed using the USP apparatus-2 (paddle) apparatus (Electrolab, India). The suspension containing the equivalent of 25 mg of pure drug or nanocrystals was directly placed in a dissolution apparatus containing 250 mL pH 7.4 phosphate buffer solution, maintained at 37 ± 0.5°C (50 RPM). The samples were periodically withdrawn and filtered through a 0.45 µm membrane filter. The concentration of the drug in the filtrate was measured by using a UV-visible spectrophotometer (model and make of instrument) at 226 nm.

2.5. Statistical analysis of design batches

Regression analysis was performed to study the relationship between change in the independent variable (proportion of stabilizer) and its effect on critical response variables such as particle size and PDI. The common polynomial equation for simplex lattice design is given below:

$$Y = \beta1X1 + \beta2X2 + \beta3X3 + \beta12X1X2 + \beta13X1X3 + \beta23X2X3 + \beta123X1X2X3$$

2.6. Formation of nanocrystals loaded hydrogel

To prevent the agglomeration and the growth of nanocrystals induced by the Ostwald ripening, KTZ nanocrystals were incorporated into a viscous hydrogel. In addition to inhibiting Ostwald ripening, the gel formulation is also considered to be more suitable for transdermal administration compared to the liquid formulation. Carbopol 934P, a polyacrylic acid derivative, has a solution-to-gel transition depending on the pH of the medium (solution at pH 1-6 and gel at 6-8). Carbopol 934P (500 mg) was sprinkled slowly on 50 mL on an optimized batch of ketoconazole nanosuspension (0.5% w / w ketoconazole) with constant stirring by using a magnetic stirrer until a uniform dispersion was formed. Once Carbopol 934P was added, the dispersion was allowed to hydrate for 12 h. The prepared dispersion was neutralized by adding triethanolamine in a dropwise manner until the transparent gel was formed (6.0 to 6.5 pH).

2.7. Compatibility through differential scanning calorimetry analysis

DSC studies were performed using a Shimadzu DSC-60 instrument. Samples of the ketoconazole and the optimized nanocrystal formulation, each weighing 2-5 mg, had been accurately weighed and hermetically sealed in aluminum pans. The sample chamber had been purged with dry nitrogen gas at a flow rate of 50 mL/min to maintain an inert atmosphere.

The heating rate had been set to 10°C/min for the temperature program, which had been defined to range from 25°C to 300°C, exceeding the expected melting point. Before the heating cycle, the sample and reference pans had been equilibrated at 25°C.

2.8. Evaluation of KTZ-nanocrystals incorporated gel

2.8.1. Antifungal activity

Antifungal activity of pure drug suspension and optimized nanosuspension were tested against Candida albicans (MTCC No 183) as reported (Li *el al.*, 2018). Briefly, 25 mL of Sabgroud Dextrose Agar was added to the petri plate and subsequently sterilized in an autoclave at 121 °C for 15 min to prevent the growth of undesirable microorganisms. After 2 h, 100 µL suspension of candida albicans was spread uniformly on the surface of the Petri plate by using a sterilized spreader rod. Subsequently, two wells of approximately 100 µL volumes were made into it. The wall in one petri dish was poured with 100 µL of ketoconazole pure drug suspension (0.5 mg/mL) and another was poured with 100µL of optimized nanosuspension (equivalent to ketoconazole 0.5 mg /mL). The plates were incubated for 72 h at 30 °C and the zone of inhibition (mm) was measured.

2.8.2 Physical appearance and spreadability

The prepared gel was assessed for color and homogeneity of dispersion. Furthermore, the spreadability of the gel was measured in terms of the “slip” and “drag” characteristics of the gel in a modified spreadability apparatus as reported (Reddy *el al.*, 2006). In brief, the excess gel (about 2 g) was placed on a glass slide that was fixed at the bottom (ground slide). Another slide having the same dimension was placed on top of the gel in such a way that the gel layer got sandwiched between two slides. Later

100 gm weight was placed on the upper slide for 5 min to remove the entrapped air bubble and to make a uniform film of the gel between the two slides. The excess gel was scraped from the edges of the slides by using a spatula. The upper glass slide was attached to the hook with the help of a thin string. The hook was pulled by putting a specific weight on it. The weight was gradually increased, and the time required for the top slide to move 6.5 cm was measured. The spreadability was calculated using the equation.

$$\text{Spreadability} = \frac{ML}{t}$$

Where M is the weight in grams tied to the upper slide, L is the distance the upper glass slide has moved in cm, and t is the time in seconds to move L cm distance.

2.8.3. Rheological and pH measurement study

Viscosity was determined at 37 °*C* by using a Brookfield viscometer (RV, Brookfield Engineering Laboratories, Inc). The spindle no. 7, rotating at 20 rpm, was used to measure the viscosity of nanocrystal-loaded hydrogel. The mean of three measurements was considered for calculating the final viscosity of the gel. Furthermore, 1 gm gel was accurately weighed and dispersed in 100 ml of distilled water using a magnetic stirrer. The pH of this dispersion was measured by using a digital pH meter (DP-505, Digital Instruments Corporation, India).

2.8.4. Drug content

Approximately, 1 gm of gel was transferred to a 100 mL volumetric flask containing methanol. The drug was extracted by shaking it overnight on a shaker incubator. The extracted methanolic solution, 5 mL extract was filtered through a 0.45 µm membrane filter, and the drug content in the filtrate was analyzed by UV-visible spectrophotometer at 226 nm (UV-1800, Shimadzu, Japan).

2.8.5. In-vitro drug diffusion

Franz diffusion cell (with an effective diffusion area of 3.14 cm2 and 20 mL receptor compartment volume) was used for the in-vitro permeation study (EMFDC-06, Orchid Scientific, India) with slight modification as reported (Ochi *el al.,* 2014). Briefly, 1 gm ketoconazole nanocrystals incorporated gel, marketed product (Ketodoc Cream® by Dr. Morepen, ketoconazole 2% w/w), or alone ketoconazole pure drug-loaded gel was placed over the donor compartment. Semi-permeable cellulose nitrate membrane (molecular weight cut off 12,000-14,000 Daltons; pore size: 2.4 nm) was kept between donor and receptor compartments. The receptor compartment was filled with freshly prepared phosphate buffer (7.4 pH) solution, maintained at 32 ± 2 °C. The solution in the receptor compartment was stirred continuously by a magnetic stirrer (20 rpm) to uniformly distribute the drug. one mL sample was collected at a specific time and filtered through a syringe filter (0.45 µm). The filtrate was collected and analyzed for drug content by UV visible spectrophotometer at 226 nm after appropriate dilutions. Different mathematical models such as zero order, first order, Higuchi, Hixon-Crowell, and Korsmeyer-Peppas, etc., were fitted to find the best-suited model to analyze the drug release from formulation.

2.9. Stability study

The optimized nanocrystal-loaded gel was stored at accelerated storage conditions (40 ºC ± 2ºC/75% RH ± 5% RH) for 60 days. After the stability period, the formulations were evaluated for particle size, drug content, pH, clarity, and viscosity.

3. RESULTS AND DISCUSSION

3.1 Results of the preliminary studies

No substantial change in particle size was observed after 16 h of milling time, hence all the subsequent simplex lattice design batches were milled for 16 h. Moreover, the results showed that 1 mm zirconium beads were more effective in reducing the particle size of the drug, compared to 0.7 mm beads therefore in subsequent studies 1 mm beads were used.

3.2. Evaluation of batches designed for ketoconazole nanocrystals

3.2.1. Particle Size, polydispersity index, and zeta potential

The particle size, PDI, and zeta potential value of simplex lattice design batches are presented in Table 1. The formulated batch F-3 fabricated using

poloxamer 407 had the lowest particle size among all the simplex lattice design batches containing a single polymer as a stabilizer (F-1, F-2, and F-3). Poloxamer 407 is a triblock copolymer consisting of a central hydrophobic polypropylene glycol block attached to two hydrophilic polyethylene glycol blocks. This polymer is a hydrophilic non-ionic surfactant used for improving the solubility of poorly soluble drugs (Bodratti & Alexandridis, 2018). Thus Poloxamer-407 can stabilize the nanocrystals by following two mechanisms: firstly, it acts as a surfactant that can reduce the surface energy of the nanocrystals hence improving wetting and solubilization (Kolašinac *el al.*, 2011), and secondly due to long-chain molecule within Poloxamer 407 that can get adsorb on the surface of the nanocrystals, providing a physical barrier to particle agglomeration (Dai *el al.*, 2008). The formulated batches were two stabilizers in their composition such as batches F-5 and F-6 containing poloxamer-407 had smaller particle sizes than batch F-4, which had unincorporated poloxamer-407. This result proved the better efficacy of formulations incorporated poloxamer-407 to stabilize nanocrystals, compared to HMPCE-5 and HPMC E-15. The zeta potential value for designed batches was between 0.73 to 13.28 (mV), indicating a high aggregation tendency of prepared nanocrystals; however, in the present investigation, the final intended dosage form was not a liquid system, it is a semisolid viscous gel. Therefore, the highly viscous internal matrix of gel is capable of restricting the mobility of nanoformulations and thus prevents their aggregation (Ontong *et al.*, 2023; Chittasupho *et al.*, 2022; Chittasupho *et al.*, 2023).

Batch	Coded value			Transformed value			Mean particle size (nm)	PDI	Zeta Potential (mV)
	X_1 HPMC E5	X_2 HPMC E15	X_3 Poloxamer 407	X_1 (%w/w HPMC E5)	X_2 (%w/w HPMC E15)	X_3 (%w/w Poloxamer 407)			
F-1	1	0	0	0.5	0	0	421±41	0.653	4.18
F-2	0	1	0	0	0.5	0	447±31	0.716	4.05
F-3	0	0	1	0	0	0.5	307±45	0.423	13.28
F-4	0.5	0.5	0	0.25	0.25	0	443±40	1.24	5.51
F-5	0.5	0	0.5	0.25	0	0.25	346±36	0.693	0.76
F-6	0	0.5	0.5	0	0.25	0.25	205±27	0.567	3.34
F-7	0.33	0.33	0.33	0.16	0.16	0.16	776±36	0.434	0.73

Table 1. Coded and transformed value for simplex lattice design batches with mean particle size, polydispersity index, and zeta potential.

3.2.2. In-vitro dissolution

The formulations designed using factorial statistics released more than 80 % of the drug in less than 5 min and almost 100 % drug in less than 10 min. The particle size analysis proved that all the design batches had particle sizes of less than 450 nm (except F-7, which had 776 nm). These nano-sized crystals have a very high effective surface area and high surface energy, resulting in the complete release of the incorporated drug in less than 10 min. Nano-sized crystals, with dimensions in the nanometer range, possess a remarkably high surface area-to-volume ratio, compared to larger particles of the same polymeric material. This large effective surface area resulted in a significantly higher proportion of molecules exposed on the particle surface, leading to increased surface energy (Xi *et al.*, 2013). The high surface energy of nanocrystals acts as a driving force for dissolution, as it develops a thermodynamic instability that favors the transition of molecules from the solid state to the dissolved state. Consequently, nanocrystals exhibit an enhanced dissolution rate, rapidly achieving a saturated solution in the surrounding medium.

According to the Noyes-Whitney equation or the Hixson-Crowell model, which describes the dissolution kinetics of solid particles, the rate of dissolution is directly proportional to the surface area available for dissolution (Hintz *et al.*, 1989; Koutsoukos *et al.*, 2006). Thus, the immense surface area of nanocrystals facilitates rapid dissolution, leading to near-complete drug release within a short timeframe, such as 10 min or less. Furthermore, the small size of nanocrystals eliminates

diffusion barriers that can hinder drug release from larger particles. Once in solution, the dissolved drug molecules can readily diffuse away from the particle surface, maintaining a high concentration gradient that drives continued dissolution until complete drug release is achieved. However, compared to that only 40 % of pure was were able to dissolve in 30 minutes (Fig. 1).

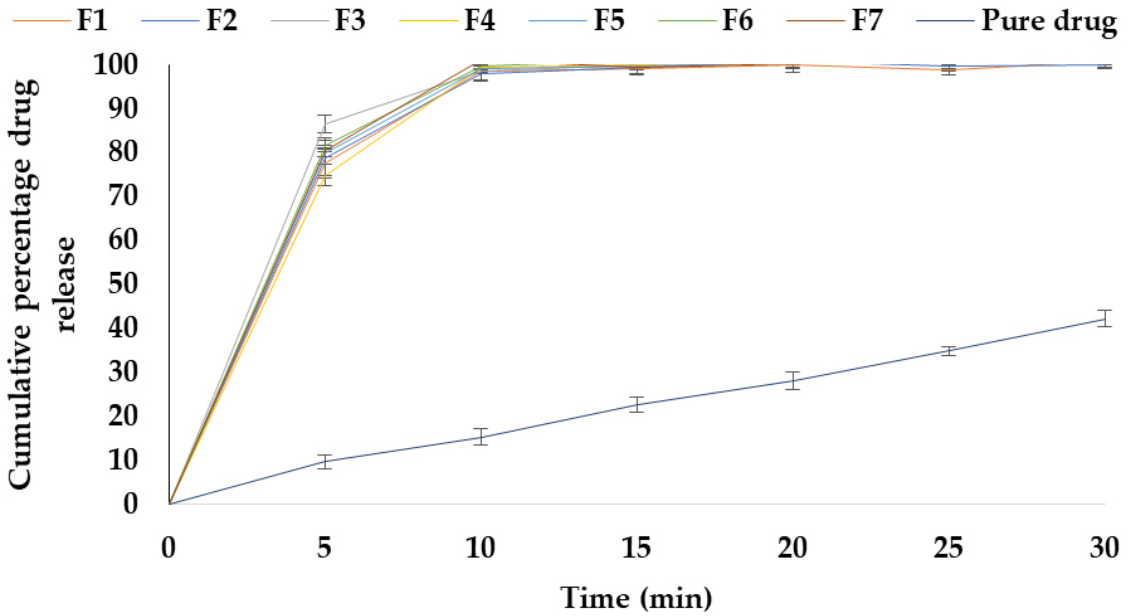

Figure 1. In-vitro dissolution profile of drug nanocrystals and pure drug.

3.3. Statistical analysis of simplex lattice design batches

The statistical analysis of the design batches is presented in Table 2. For response variable Y_1 (particle size), the terms X_1, X_2, X_3, X_{13}, X_{23}, and X_{123} had a significant p-value < 0.05, indicating their significant effect on the response variable Y_1. For response variable Y_2 (PDI), the terms X_1, X_2, X_3, X_{12}, X_{23}, and X_{123} had a significant p-value < 0.05. The R2 value, obtained by the regression analysis of both the selected response variables was greater than 0.9 which proved the good predictability of the model. The F value is another statistical parameter that can indicate the fitness of the model. For all responses, the $F_{calculated}$ >>> $F_{tabulated}$ confirmed the significant effect of all the independent variables on response variables. Response surface plots were generated to graphically represent the effect of independent variables on response variables. The 3-D response surface plot and contour plot for particle size and PDI are given in Fig. 2a,b, respectively.

Mean Particle size (Y_1)

Response	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{123}
Coefficient	421	457	307	36	–688	–72	12983
p Value	0.001	0.001	0.001	0.061	0.003	0.030	0.001
R^2 value	**0.9998**						

PDI (Y_2)

Response	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{123}
Coefficient	0.653	0.716	0.423	2.378	–0.01	0.62	–13.44
p Value	0.0006	0.0006	0.001	0.0009	0.212	0.003	0.0009
R^2 value	**0.9996**						

Table 2. Summary of regression analysis for design batches.

3.4. Statistical analysis

The polynomial equations generated by the regression analysis are given below. Here the terms having p-values, greater than 0.05 were omitted from the equation.

$$Y_1 = 421X_1 + 457X_2 + 307X_3 - 688X_2X_3 - 72X_1X_3 + 12983X_1X_2X_3$$

$$Y_2 = 0.653X_1 + 0.716X_2 + 0.423X_3 + 2.378X_1X_2 + 0.62X_2X_3 - 13.44X_1X_2X_3$$

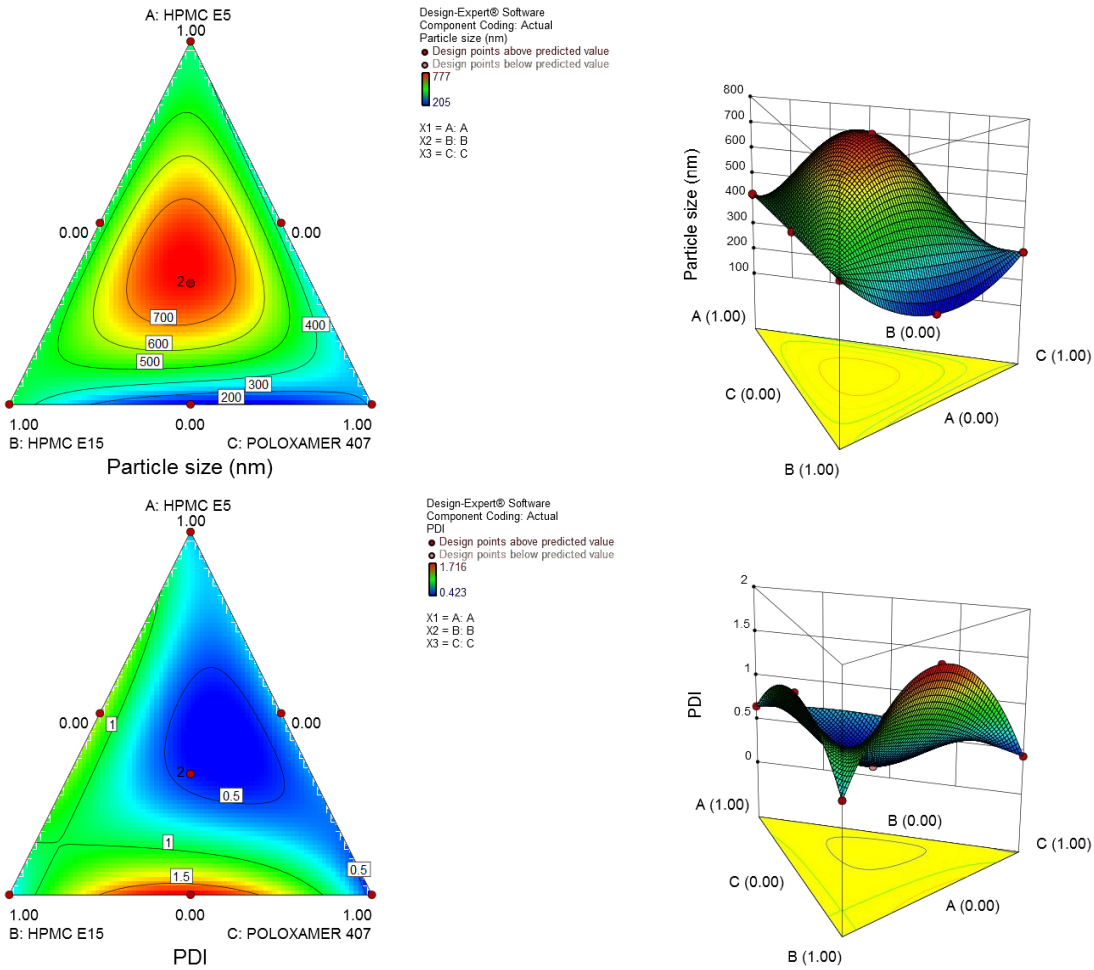

Figure 2. Contour plot and 3-D surface plot depicting the effect of the independent variable on response variable Y_1 (particle size) (a) and contour plot and 3-D surface plot depicting the effect of the independent variable on response variable Y_2 (PDI) (b).

A positive value of the coefficient indicated direct proportionality between the independent variable and response variables, while a negative value indicated an inverse relationship between them. All the stabilizers when used alone (F-1, F-2, and F-3), had a positive effect on particle size and PDI. The X_2 had a higher effect on particle size (Y_1) compared to X_1 and X_3. This means in fixed-weight formulation, the change in the proportion of HPMC E15 had a higher impact on particle size compared to the change in the proportion of HPMC E5 and poloxamer-407. In the regression equation, the coefficient with more than one term represents interaction terms. It is possible that, when more than one factor is changed simultaneously, they can have completely different effects on the response variables. Among all the interaction terms, only X_{12} had no significant effect on particle size (Y_1). The interaction terms X_{13} had a negative effect on particle size. This meant that the particle size decreased when the proportion of X_1 and X_3 increased simultaneously.

The terms X_1, X_2, and X_3 had a positive effect on PDI (Y_2). Among all the interaction terms, only interaction term X_{13} had no significant effect on Y_2. As depicted in the graph (Fig. 2a,b), as the proportion of X_3 increased in fixed weight formulation, the particle size and PDI decreased significantly. This might be due to the stabilizing effect provided by poloxamer-407 by the following mechanisms 1. Ability to act as a solubilizing agent by reducing the surface free energy of nanocrystals 2. Providing steric hindrance against particle aggregation.

3.5. Desirability function for the selection of optimized batch

All responses were assigned a value between 0 to 1 depending upon the desirability of a response. 0 for the least desirable ones and 1 for the most desirable ones. The software calculated the desirability for each response and gave a composite desirability value for each batch by taking the mean of the desirability values for all the responses. The constraint values for selected response variables along with set goals are given in Table 3.

Response	Constraints		
	Minimum	Maximum	Goal
Y_1 Mean particle size (nm)	205	776	Minimize
Y_2 PDI	0.423	1.279	Minimize

Table 3. Constraints selected for the optimization.

	Coded value			Predicted Value		Experimentally obtained value		Desirability
Solution –1	X_1	X_2	X_3	Y_1 (nm)	Y_2	Y_1 (nm)	Y_2	
	0	0.46	0.54	205	0.56	220	0.59	0.919

Table 4. Predicted and obtained value for optimized batch with the solution suggested by Design Expert® software.

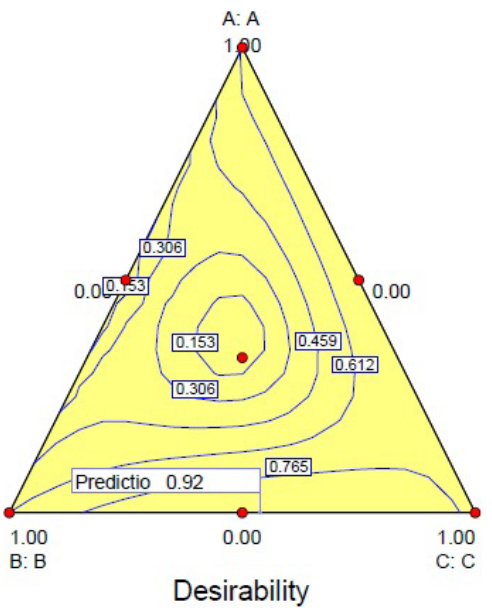

Figure 3. Overlay plot for desirability function suggesting the optimized batch composition.

The software suggested a single solution for the optimized formulation based on data given to it (Fig 3). The suggested values for independent variables are given in Table 4. As depicted in Table 4, the percentage bias between the predicted values and practically obtained values was around 5 percent only which proved the validity of the obtained model. Fig 4 depicts the particle size analysis results of the optimized batch (Formulation as shown in Table 5).

Sr. No.	Variable	Optimized Value
1	Ketoconazole	0.5 % w/w
2	HPMC E15	0.29 % w/w
3	Poloxamer 407	0.27 % w/w
4	Stirring time	16 h
5	Stirring Speed	800 rpm

Table 5. Composition of optimized batch (OP).

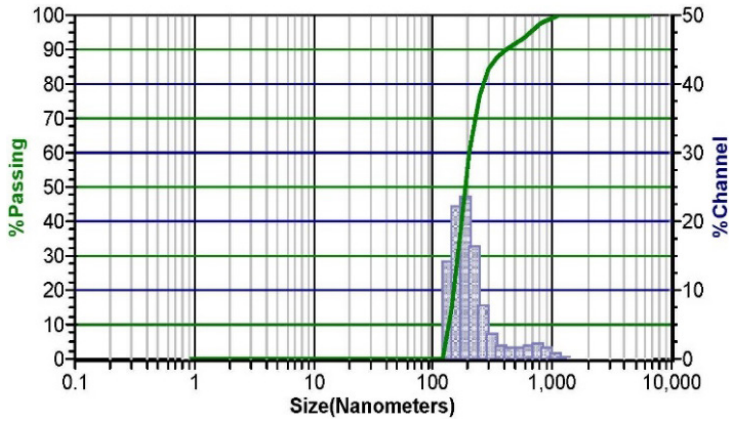

Figure 4. Particle size analysis of optimized batch (OP1).

3.6. Compatibility study

The DSC thermogram of pure drug (KTZ) and optimized nanosuspension batches are presented in Fig. 5a,b, respectively. Both graphs displayed a sharp endotherm peak at around 153 °C. The result of the DSC study proved that the drug was still in the crystalline state after passing through a media milling process. The DSC analysis also indicated a decrease in the crystallinity of the pure product upon conversion to nanocrystals. Medial milling is a high-energy process that can destabilize the crystal lattice and can produce defects in crystals at weak sites, leading to the conversion of some proportion of crystalline drug into amorphous one (Dong *el al.*, 2009). This resulted in the reduced intensity of the endothermic peak for the optimized batch.

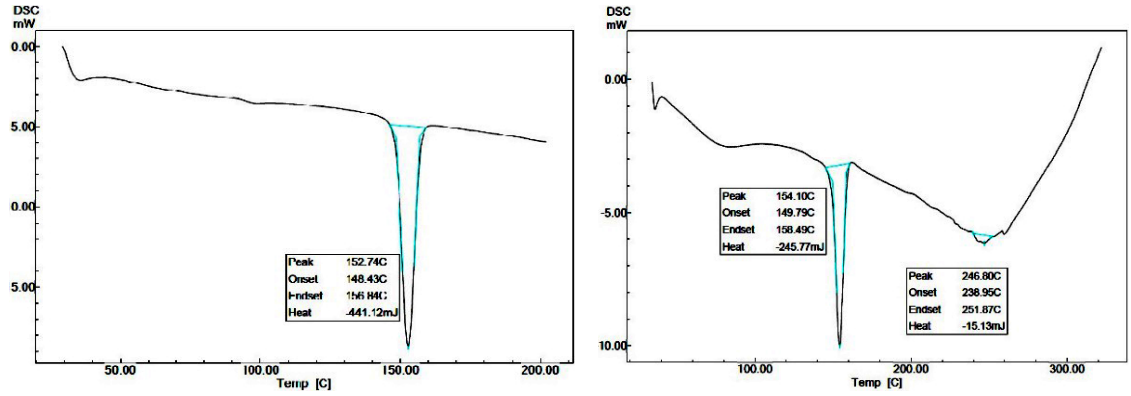

Figure 5. DSC thermograph of the pure drug (a) and optimized batch (b).

3.7. Evaluation of nanocrystal-fortified gel

3.7.1. Antifungal activity

The mean value for the zone of inhibition for pure drug suspension was around 30.12 ± 0.98 mm, compared to that of optimized nanosuspension, which was 37.98 ± 0.45 as presented in supplementary Fig. 1. The results of this study proved that the optimized nanosuspension was able to diffuse in a larger area, compared to alone pure drug suspension. This was due to the high saturation solubility of the drug nanocrystals, compared to pure drugs, resulting in a high concentration gradient and diffusion in a larger area. In a similar study, KTZ microemulsion fortified with various surfactants demonstrated minimum inhibitory concentration between 16 - 0.0078 µg/mL, while alone KTZ and surfactant showed a bit higher than those of microemulsion formulations indicating effective improvement in the antifungal activity (Nisha *et al.*, 2018). Additionally, a cocrystal formulation of KTZ indicated significant improvement in the solubilization of drug (Hiendrawan *et al.*, 2015), suggesting an overall improvement in solubility can improve the KTZ antifungal activity.

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3.7.2. Physical appearance, spreadability, rheology, pH, and drug content analysis

The prepared gel was hazy and white in appearance. The spreadability of the nanocrystal-loaded gel was between 10.33 to 13.67 gm.cm/sec suggesting good spreadability of gel even at low shear stress. The measured viscosity range for nanocrystal-loaded gel was between 22,500 cps to 24,700 cps. There is no specific acceptable viscosity range given in any official standards for topical gel formulations; however, the resulting viscosity range for prepared gel formulation corroborated with results published in similar research articles (Reddy *el al.*, 2006). The pH of nanocrystal loaded gel was between 6.67 to 7.05, indicating its suitability for topical formulation. The drug content of nanocrystal-loaded gel was between 97.33 % to 98.97 %, which proved a uniform distribution of nanocrystals throughout the gel.

3.7.3. In-vitro drug diffusion

However the marketed product (Ketodoc®) demonstrated about 65 % in vitro drug release, which was higher than the pure drug-loaded gel; however, lower than the nanocrystal-loaded gel as presented in Fig 6. An efficient moderate drug release from the marketed product was possible might be due to w/o a base system that aids the drug molecules to permeate through the membrane. Ketoconazole nanocrystals loaded gel was able to release more than 80 % drug within 24 h, compared to the drug release from pure drug-containing gel was about 30 %. This was due to the high concentration gradient generated by high saturation solubility and high dissolution velocity of the nanocrystals. The high concentration gradient of nanocrystals generates a high flux for drug molecules to permeate through the semipermeable membrane (Ochi, Kawachi *el al.*, 2014). Since most of the drug permeates through the skin by passive diffusion, better apparent solubility, and dissolution of the drug lead to a high amount of drug permeation. Another contributing reason for the improvement in drug release was the ability of the hydrogel to maintain the supersaturation state of the drug due to the viscous internal matrix. The viscous matrix restricts the movement of nanocrystals and prevents their aggregation thus maintaining the super-saturation state. In addition to that, nanocrystals also facilitate penetration of the drug into the hair follicles which can serve as a reservoir to replenish the absorbed drug (Patzelt *el al.*, 2011). Since the lower follicular orifice lacks the stratum corneum barrier, the hair follicle provides a better permeable site compared with the skin surfaces (Mittal *el al.*, 2013). Moreover, nanocrystal also improves the retention time of the drug on the skin surface due to its better adhesive capacity.

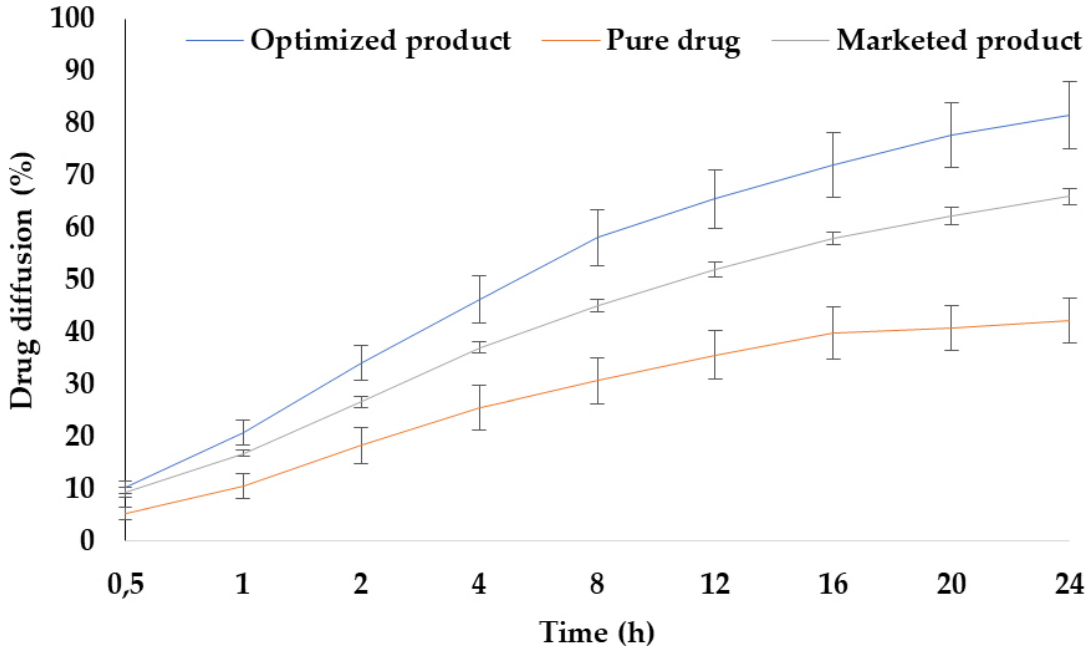

Figure 6. Drug diffusion study of nanocrystal fortified formulation, marketed product (Ketodoc®), and pure drug-containing gel.

Batch	Zero	First	Higuchi	Korsmeyer-Peppas		Hixon Crowell	Best fit
B4	R^2 0.4003	R^2 0.8391	R^2 0.9421	R^2 0.9762	n 0.397	R^2 0.7424	Korsmeyer-Peppas

Table 6. Model fitting for optimized batch.

The R^2 value for various predicted models is given in Table 6. The best-fit model was found to be the Korsmeyer-Peppas model with R^2 value of 0.9762, indicating diffusion-controlled drug release from the gel. The diffusion coefficient "n" indicates the mechanism of the drug release from the gel. The "n" value of 0.397 indicated a Fickian diffusion mechanism for drug release from the gel. It meant that the Fickian diffusion is the rate-limiting step for the drug to come out from the gel (Siepmann & Siepmann, 2008). The Fickian diffusion follows Fick's first law, which means the amount of drug that gets diffused in unit time per unit area is directly proportional to the concertation of the drug. The nanocrystals increased the saturation solubility of the drug and thus enhanced the diffusion of the drug from the gel.

3.8. Stability study

Nanocrystals may start to agglomerate and enlarge due to the Ostwald ripening phenomena during storage. Such kinds of systems lose the advantages of high saturation solubility and high dissolution velocity. The nanocrystals entrapped in high-viscosity gel have very limited mobility due to a continuous network of polymers which reduces particle aggregation and enlargement. No significant change was observed in the size of drug nanocrystals after 60 days of storage period (from 220 nm to 242 nm) as presented in Fig. 7, compared to the result presented in Fig. 4. This was possible because of the high resistance provided by the carbopol 934P gel network for the movement of nanocrystals. No significant change was observed in viscosity, clarity, and drug content in stored gel. The results proved the physical and chemical stability of ketoconazole-loaded nanocrystal gel during storage conditions.

4. CONCLUSIONS

The ketoconazole nanocrystals were successfully developed using the top-down media milling technique, which showed acceptable particle sizes with narrow PDI. Moreover, the regression analysis suggested a significant effect of independent variables on response variables. The prepared nanocrystal displayed a better zone of inhibition, compared to the pure drug in invitro anti-fungal study. Additionally, nanocrystals indicated improved in-vitro dissolution and in-vitro permeation of the drug through the cellulose membrane with significant antifungal efficacy against tested Candida albicans. The gel formulation maintained the physical and chemical integrity of nanocrystals during the stability period encouraging further animal study. Overall ketoconazole nanocrystal fortified gel has the potential to improve the antifungal treatment

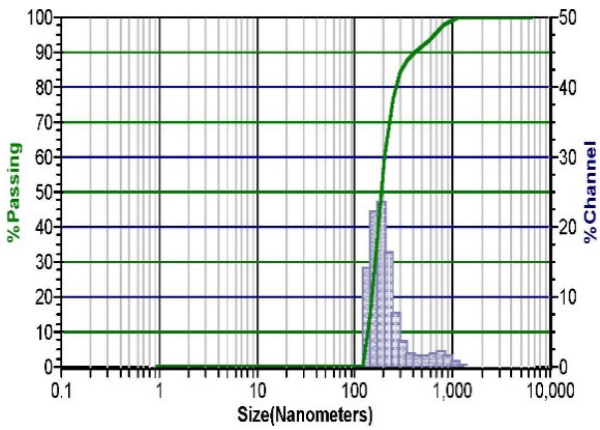

Figure 7. Particle size distribution of optimized batch after storage in stability studies.

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by offering more effective and patient-friendly options. In future clinical trials, this innovative formulation and its comparison with the currently marketed formulation would add more authenticity to the research work.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: MD and BGP; Writing original draft preparation: BV and KD; Writing review and editing: SS, AK, and SS; supervision: MD and BGP. All authors have read and agreed to the published version of the manuscript.

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