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Original Article

Execution of Quality by Design Approach in the Formulation of Fluconazole Inclusion Complex Based Suppository for Vaginal Candidiasis

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ABSTRACT

Aim: The present work was carried out to improve the aqueous solubility of Fluconazole by formulation of inclusion complex using β-cyclodextrin and then loaded into the suppository for the treatment of vaginal candidiasis. **Materials and Methods:** Fluconazole (FOZ) Inclusion Complex (INC) with β-Cyclodextrin (βCD) was formulated first after optimization of the D-Optimal mixture design for the molar ratio. Then INC was prepared (1:1 molar ratio) with different methods (kneading, coprecipitation and microwave irradiation) and was studied for FTIR, DSC and XRD studies. **Results:** Microwave irradiation (MW) was the best method for the preparation of INC with the maximum % drug content (98.96±1.78%) and solubility (3.14±0.85 mg/mL). The QbD approach was employed to select the best ratio of the base and plasticizer for the suppository. The suppository was prepared using PEG 6000 (base) and PEG 400 (plasticizer) and an optimized inclusion complex was incorporated. The D-Optimal mixture design was applied to optimize a combination of PEG 6000 and PEG 400 by studying responses such as hardness, melting time and drug release from the suppository. The *in vitro* release study showed that higher drug release was achieved from the FOZ MW INC based suppository (99.40±1.43%) compared to the plain drug suppository (71.41±2.19%). **Conclusion:** The prepared FOZ suppository had higher drug release and enhanced antifungal activity. Therefore, it would be beneficial in the treatment of Vaginal Candidiasis.

Keywords: D-Optimal mixture design, Fluconazole, Inclusion complex, Solubility enhancement, Vaginal Candidiasis, β-cyclodextrin.

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INTRODUCTION

Up to 75% of women will experience vaginal candidiasis at least once in their lifetime. It is a frequent ailment. Most patients with vaginal candidiasis react well to oral and local therapies, which are primarily caused by *Candida albicans.*[1] Imidazole is the most often recommended medication for vulvovaginal candidiasis. Commercial versions of fluconazole are available as tablets, capsules, injections and ocular drops.[2] The mainstay of care for practically all types of susceptible infections caused by Candida is Fluconazole (Figure 1). Furthermore, using FOZ to treat vaginal candidiasis is significantly more successful than treating infections at other sites. Fluconazole at a dose of 400 mg/day is given to the patients for long term in case of fungal infections. Due to the high dose of drug, various side effects are observed.[3] When the medication is taken orally or parenterally, it can cause many side effects, including headache, nausea, vomiting and abdominal pain. It is a white, crystalline powder that dissolves very slightly in water (5 mg/mL at 37°C). This reduced aqueous solubility led to a decrease in systemic absorption.[4-7] Its mechanism is the inhibition of ergosterol biosynthesis, which makes it difficult for the fungus to maintain the integrity of its cytoplasmic membrane.[2]

Numerous technical approaches, including nanonization,[8,9] liquisolid compact,[10] solid dispersions[11-14] etc., have been documented in the literature as ways to improve the solubility and dissolution properties of poorly water-soluble antifungal drugs. Nevertheless, the commercial application of these systems is severely limited by the typical procedures utilized to create them, which frequently result in physical instability of the solid dispersion during storage, grinding issues, or challenges in eliminating the hazardous organic solvent. The most promising method among the many that have been tried to increase a drug's solubility and rate of dissolution is complexation with cyclodextrins.[15]

Cyclodextrin and its derivatives are increasingly being used as polymers of choice in the pharmaceutical industry because

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of the capability of forming complexes with a wide range of medicinal molecules. The cyclic oligosaccharide family known as Cyclodextrins (CDs) has a lipophilic center chamber and a hydrophilic outer surface. They typically combine with different medications that have solubility issues to form inclusion complexes that significantly improve pharmacological and solubility characteristics, as well as bioavailability, dissolution rate and sometimes even palatability, without impairing the drug's inherent lipophilicity or pharmacological qualities. Out of the three cyclodextrin forms (α, ß and γ-cyclodextrin), ß-cyclodextrin is considered as far advantageous due to its ease of complex formation and cost-effectiveness in comparison to other versions.[4]

Because of its configuration, the cyclodextrin can accept a guest molecule inside the cavity. Since, cyclodextrins have increased solubility and enhanced chemical and physical stability, they can be used to prepare inclusion complexes with a range of medicinal molecules, which will mainly improve dissolution and bioavailability.[6,7,15]

Pessaries, sometimes referred to as vaginal suppositories, are the most suitable of them because of their uniform dose, little drug leakage, shape, volume and consistency that are ideal for administration into the vagina.[16] Some benefits of a vaginal suppository include the ability to maintain dose homogeneity, the ability to enter the medication inside the vagina without irritating and the fact that a significant amount of dissolving fluid is not needed for the active ingredient to be released. This is why the current study was started to formulate a vaginal suppository-a pharmaceutical formulation intended for local administration.[17-19]

Experimental design is used to arrange, carry out and analyze test results through carrying out only a handful of trials. Scientists employ experimental design when producing their products or processes to understand independent variables. With the emergence of user-friendly software tools, design ideas such as Design of Experiments (DoE) are implemented in the design space and adherence to regulatory norms is promoted. A pharmaceutical product manufacturing procedure called "Quality by Design" (QbD) aims to analyze product performance and optimize and improve process efficiency while meeting crucial quality requirements. A tried-and-true method for organizing and carrying out elucidative experiments for formulation optimization is statistical experimental design, or DoE.[20]

A DoE application can serve a variety of purposes, such as in-depth response studies that are especially beneficial in optimization, mixture designs, etc., or studies to determine the most influential factors affecting the product or process under study. To ensure that the product retains within the specification, robustness is used as the final assessment prior to the product release and optimization is utilized to obtain a combination of parameters connected to the response. Determining a relationship between variables that are independent (factors) as well as dependent variables (outcomes, results) within the experimental setting is the main objective of any study including experiments.[21,22]

Mixed design is one of the major categories of DoE. The impact of altering the components of the mixture on its qualities is investigated in this design. A mixture has the property that all of its constituent parts add up to 100%. This indicates that while the proportions of these elements, or mixture factors, range from 0 to 1, they cannot be altered independently of one another. Therefore, in experimental work, this design takes into account all factors at once. D-optimal design makes multivariate analysis with few experiments simple to accomplish. Compared to other factorial designs, the D-optimal design is a sort of mixture design that will reduce the generalized variance of the predicted regression coefficients and optimize the independent factors with the fewest number of runs. After measuring the dependent response for the design on each trial, a quadratic, interactive, or basic linear model is fitted.[20,21]

Fluconazole based tablets,[12,23,24] nanoformulation,[25,26] film,[27] scaffold,[28] gel,[29] and insert[30] have been used previously. None of the literature has reported the use of FOZ inclusion complex suppositories for improving drug solubility and enhanced antifungal activity. An inclusion complex would help in the dose reduction of almost half of the oral dose when applied vaginally. Also, the undesirable side effects of the oral FOZ administration can be surpassed by the suppository given through the vaginal route. A higher drug concentration can be achieved in the intravaginal cavity by the application of a FOZ suppository. Hence, the drug concentration at the site of infection would be higher and that would benefit during the candidal infection and result in complete clearance of the fungal colonies. Hence, the present work may help in an economic product that could be easy to manufacture from the industrial point of view through the application of the DoE. The inclusion complex could enhance the drug aqueous solubility which would lead to improvement in the dissolution rate and antifungal action of the drug.

To enhance the physicochemical features of FOZ, attempts were made in this study to prepare the inclusion complex of FOZ utilizing βCD and some techniques, including kneading, coprecipitation and microwave irradiation methods.

MATERIALS AND METHODS

FOZ and β-Cyclodextrin were generously gifted by Unijules Ltd., Nagpur and Jaychem marketing Ltd., Mumbai respectively. DoE approach was used for the preparation and optimization of the FOZ inclusion complex. For the cause-and-effect analysis, the software used was Minitab (Minitab, Philadelphia) which assisted in the construction of the Ishikawa fishbone diagram. This diagram helped to understand the different characteristics that could affect the Critical Quality Attributes (CQAs) of the FOZ Inclusion Complex (INC).

Preparation and optimization of ratio for inclusion complex

The inclusion complex was prepared using different ratios of drug and β-Cyclodextrin by using a D-optimal mixture design through the kneading method. The 2 factors were drug and β-Cyclodextrin and responses were *in vitro* drug release and solubility. Table 1 gives the factors that are categorical and continuous in nature. The Design Expert® Software 7 was utilized to develop a level II coordinate exchange type of design. The level II design was employed for the design because it helped in the reduction of average prediction of variance over the experimental trials. There was a reduction in the quadratic and impacts after 5 trial runs. Design expert 7 had the statistical tool for the prediction of the linkage between the dependent (factors) and independent (responses) variables. The responses obtained in the polynomial model (such as linear, quadratic or cubic) were transformed into the mathematical equations that described the relationship between the factors and the responses. The reliable tool of Design expert® software such as analysis of variance that includes p-value was used to estimate the significance of the model. These models of the responses were further used to identify the results using the desirability function.[31]

The D-optimal mixture design was used as the complex contained two components in various ratios (molar ratio). The two components included Fluconazole and β-cyclodextrin which were the factors and the responses included the drug release and solubility. The Design Expert® software was used to augment the optimization process of the inclusion complex. The two-level design was selected due to the advantage of reduced numbers of runs. The statistical tool in the software provides the analysis of the polynomial equation in the form of two-component graphs that help to understand the relationship between the factors and the responses. The statistical tool of ANOVA includes R^2, *p*-value and accuracy to qualify for a significant model for each of the responses based on the input experimental values.

Analysis of phase solubility

Using phase solubility as described by Higuchi and Connors, the FOZ-βCD-complex ratio in the dissolved phase was examined. A solution of βCD 1-20 mM containing AVF pH 4.1 buffers was used to suspend 10 mg of FOZ.[32] For 48 hr at 37°C, the samples were continuously shaken on an orbital shaker set at 200 rpm. Using spectrophotometry, the absorbance was determined at 261 nm after 48 hr. The apparent stability constant was obtained from the phase solubility diagram's slope using Equation (1).

$$Ks = \frac{Slope}{So(1-Slope)} \quad (1)$$

Where, Ks is the apparent stability constant and so is the drug's solubility in the absence of cyclodextrin.[33]

Preparation of FOZ INC

FOZ INC was prepared through physical mixing, Kneading (KN), Coprecipitation (COPPN) and Microwave irradiation (MW). The following methods of preparation for each type of INC is given below.[34]

Physical mixture

In the mortar, the drug and the βCD was added after weighing according to 1:1 molar ratio. The mixture was triturated and lastly stored in a glass vial for further use.

Kneading method

In the mortar, the drug and the βCD was added after weighing according to 1:1 molar ratio. Then the solution of ethanol: water 1:1 v/v was added to the mixture powder and kneaded for 25-30 min. The solution was sufficiently added to get a wet mass and then this mass was dried for 48 hr at 45±1°C in a hot air oven (Spectra Equipments, Hyderabad). The mass dried was scrapped out and pulverized, then screened through sieve no. 50 and finally stored in glass vial for further use.

Coprecipitation method

The FOZ was dissolved in ethanol solution and βCD was dissolved in 200 mL of distilled water. FOZ solution was poured dropwise into the βCD solution. The obtained mixture was allowed to stir for 24 hr using a hot-plate magnetic stirrer (Remi equipment, India) at room temperature and then filtered. The resulting filtrate was evaporated for collection of the precipitate and the obtained precipitate was dried for 12 hr at 50°C.[3]

Microwave irradiation

A domestic microwave (IFB, 17 PM-MEC1) was used for the inclusion complex preparation. The drug and βCD were dissolved

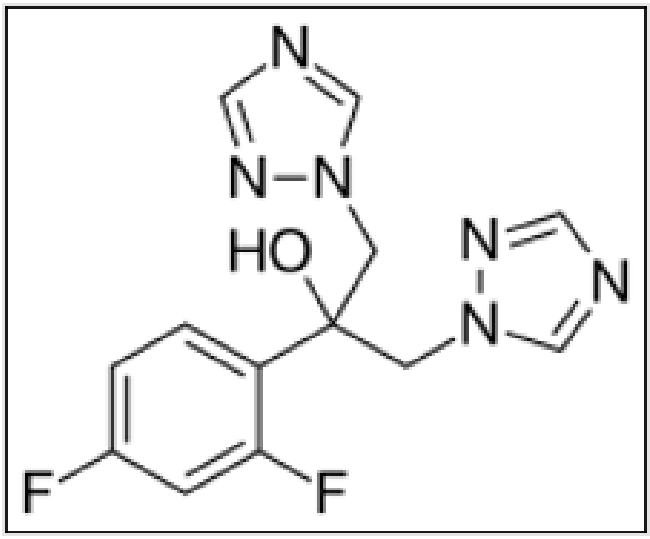

Figure 1: Molecular structure of Fluconazole.

in a minimum quantity of 1:1 ethanol: water v/v mixture. The solution containing the Petri plate was kept in the microwave for 3 min and then stopped for 1 min interval and further kept for 3 min. After this step, the obtained product was kept in vacuum overnight. The obtained product was scrapped out and stored until further use at room temperature.

Characterization of FOZ INC

Percent practical yield

The percent practical yield gives the efficiency of the formulated INC. The yield for all types of FOZ INCs were calculated by using the following equation (2):[31]

$$Practical\ yield = \frac{Practical\ mass\ of\ INC}{Theoretical\ mass} \times 100 \quad (2)$$

Drug content

The solid powder of inclusion complexes was taken equivalent to 20 mg and added into a volumetric flask of 25 mL. Then methanol 10 mL was added and the flask was sonicated for 30 min. Then the volume was made up with methanol and filtered through filter paper (pore size 0.22 µm). The filtrate solution was properly diluted with methanol and the UV spectrophotometric analysis was performed (Shimadzu, 1800, Japan) at 261 nm. The drug content % formula used is given below in equation (3):

$$Drug\ content = \frac{FOZ_{act}}{FOZ_{theor}} \times 100 \quad (3)$$

Where FOZ_{act} is the content in 20 mg complex and FOZ_{theor} is the amount of FOZ 20 mg.

Saturation solubility study

An excess quantity of FOZ INC was weighed and added in 10 mL capacity glass vials and filled with AVF pH 4.1. The sample solutions were subjected to sonication using ultrasonicator (PCi, Mumbai, India) for a duration of 20 min at ambient condition. Then the vials were placed in an orbital shaker incubator (RS-24BL, Remi Instruments Ltd., Mumbai, India) for 48 hr at 37±0.1°C with 100 rpm. The sample solutions were kept at 10,000 rpm for centrifugation (C-24 Plus, REMI Electrotechnik Ltd., Vasai, India) for 30 min. The solutions were filtered through 0.45 µm membrane filter and analysis was performed spectrophotometrically at 261 nm.[34]

Characterization of the FOZ INC

FTIR study

The FTIR study was performed to verify the formation of the inclusion complex as well as to understand the interaction between the drug and βCD. The samples of plain FOZ, βCD, PM (FOZ+βCD) and FOZ INCs were triturated with KBr separately and then placed in the sample cell and scanned using FTIR spectrophotometer (Shimadzu, Japan) in the range of 4000 to 800 cm^{-1} wavelength.[11,12]

DSC study

The DSC study was performed for the samples of plain FOZ, βCD, PM (FOZ+βCD) and FOZ INCs and thermograms were obtained by using DSC (DSC 60, Shimadzu, Japan). Sample of 2 mg was properly weighed, added in the aluminum pan, sealed hermetically and the DSC scanning was recorded from 30°C to 300°C with the heating rate of 10°C/min. The purging was made of nitrogen gas having flow rate of 100 mL/min. For the reference an empty pan was employed for the study.[35]

XRD study

The samples of FOZ, βCD and all the FOZ INC complexes were subjected to powder XRD study using a diffractometer (Ultima-III, Rigaku, Japan) using the monochromatic Cu Kα radiation. The diffraction angle range used for the analysis was 10° to 70°.[36]

Table 1: Preparation of inclusion complex based on experimental design using D-Optimal mixture design for optimization.

Run	A: FOZ	B: βCD	Solubility (mg/mL)	*In vitro* drug release (%)
1	1	1	2.8973	96.52
2	1.5	0.5	1.4234	69.47
3	0.5	1.5	0.7382	62.27
4	1.25	0.75	2.7339	85.89
5	0.75	1.25	2.1139	82.72

Table 2: Drug content of FOZ-βCD complexes.

Inclusion type	% yield	% drug content	Aqueous solubility (mg/mL)
Physical mixture	97.74±1.72	99.75±1.28	1.73±0.49
Kneading method	92.62±2.13	94.35±1.79	2.14±0.57
Coprecipitation method	95.71±1.27	96.98±1.67	2.87±0.67
Microwave irradiation method	96.84±2.48	98.96±1.78	3.14±0.85

Each value represented the mean±S.D. (n=3).

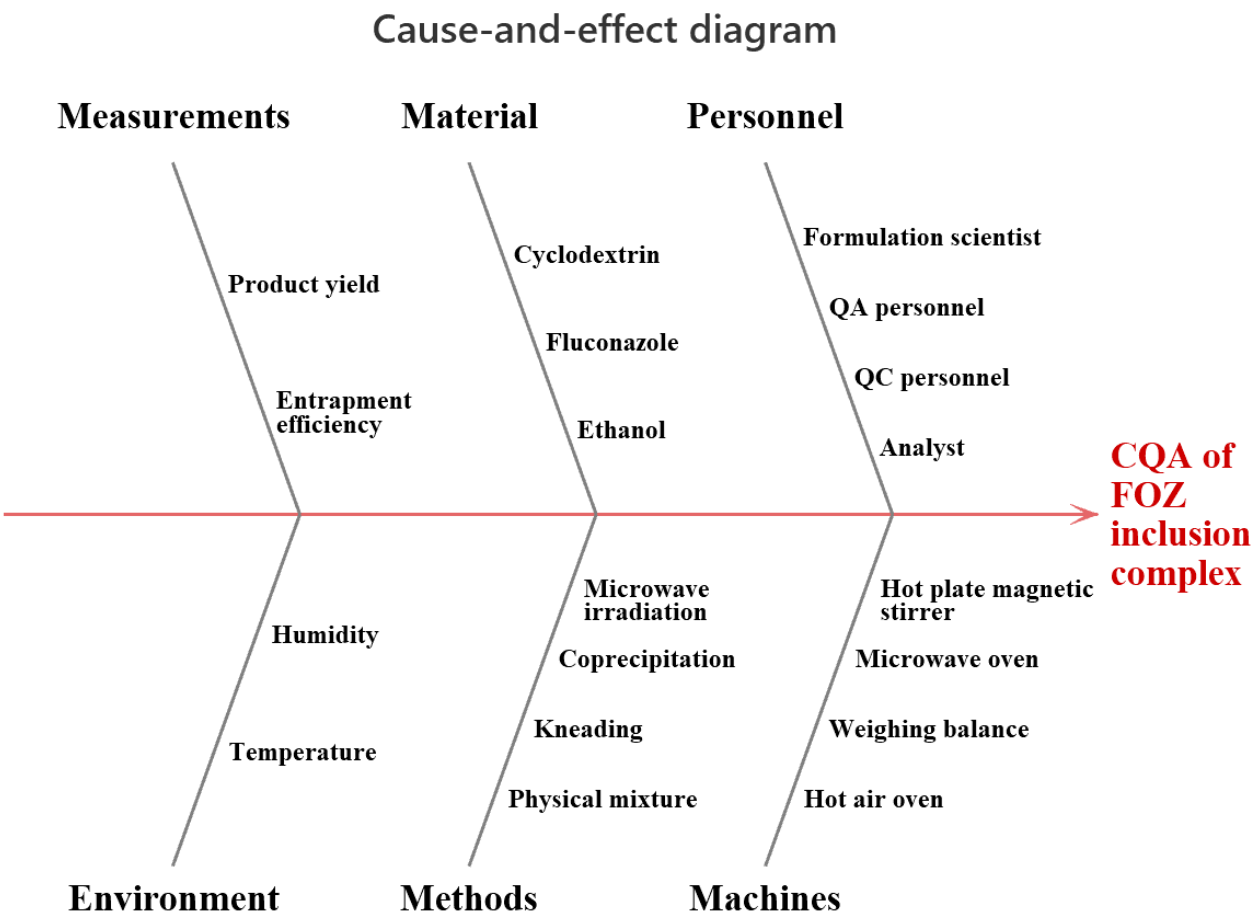

Figure 2: The Ishikawa Cause effect diagram having the different types of factors that has impact on the CQAs of the FOZ INC complex.

SEM study

The SEM study examined the surface morphology of powder samples, including FOZ, βCD and optimized FOZ INC. All the samples were positioned over the aluminum stubs using double-sided carbon tape. Subsequently, the stubs were coated with gold sputter and inserted again into the scanning electron microscope's vacuum chamber (Hitachi, S-3700N). After correctly adjusting the magnification to produce a high-resolution image, the picture was taken.[11,12]

Preparation of suppository

The suppository batches were obtained by the fusion method that included melting of the base and plasticizer at 70-80°C and then FOZ INCs were added and the molten mass was poured into the suppository mould and allowed to cool at room temperature. After cooling, the suppositories were taken out of mould.[17] For the evaluation of the best ratio of PEG 6000 and PEG 400 in the first part, the inclusion complex of kneaded method was loaded into the suppository for the evaluation of the best suppository base ratio and in the second part, all the different inclusion complex prepared were loaded into the optimized base and suppositories were prepared.

Characterization of suppository

Physical evaluation

The suppositories were evaluated for color, surface texture and presence of cracks and fracture.[17]

Weight variation

Prepared suppositories were checked for weight variation as per the British Pharmacopoeia. All the suppositories were weighed individually using a digital weighing balance (Mettler Toledo, AG135, Switzerland) and the average weight was calculated. The obtained average weight was used for checking the deviation of 5% and 7%. It was checked that not more than 2 suppositories should exceed 5% and 7% of the average weight.[19]

Hardness or breaking strength

From all the batches, suppositories (n=6) were taken at random and tested for hardness or breaking strength. The thick portion was chopped from the suppository by slicing the tapering part and then by using a hardness tester (Icon Instruments, New Delhi), the breaking strength was determined by taking the average of 6 suppositories.[19]

Melting time

The time required for the suppository to melt after the application into the vaginal cavity is considered the melting time. A small length wire was introduced within the suppository while the base was in the molten stage and then allowed to solidify. These wire-containing suppositories were used for the evaluation of the melting time. The suppository was introduced into the AVF pH 4.1 and the temperature of the buffer was slowly increased until there was removal of the molten base from the wire. Thus, this point is noted as the melting time of the suppository.[33]

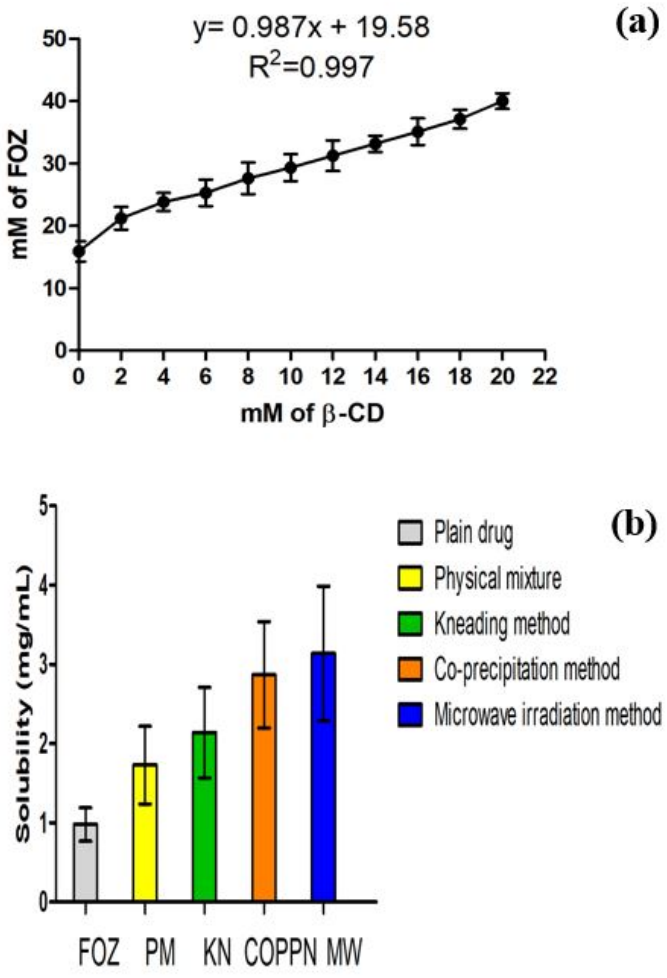

Figure 3: (a) Phase solubility curve of the FOZ-βCD in solution and (b) Saturation solubility of FOZ-βCD complexes (Data presented as mean±S.D.).

Drug content in suppository

The suppository was added into a volumetric flask of 25 mL. Then AVF pH 4.1 10 mL was added and the flask was sonicated for 30 min. Then the volume was made up with AVF pH 4.1 and filtered through filter paper (pore size 0.22 µm). The filtrate solution was properly diluted with AVF pH 4.1 and the UV spectrophotometric analysis was performed using (Shimadzu, 1800, Japan) at 261 nm. The drug content % formula used is given below in equation (4):[33]

$$Drug\ content = \frac{FOZ\ SUPPO_{act}}{FOZ_{theor}} \times 100 \quad (4)$$

In vitro release study and release kinetics

In vitro release study was performed in AVF pH 4.1 using USP dissolution apparatus II (Electrolab, India). The assembly was equilibrated at 37±0.5°C before the start of the experiment. Then, in the dissolution vessel the suppositories in the dissolution medium (AVF pH 4.2) of volume 100 mL.[14] The stirring speed was aligned at 50 rpm. The sampling was done at the appropriate interval by the withdrawal of the sample and the sample was analyzed spectrophotometrically (Shimadzu, UV-1800, Japan) at 261 nm. The vessel was replenished with the same amount of medium simultaneously into the flask. The release data was directed to various release kinetic models to understand the process of drug release from the suppository. Thus, the data were fitted into zero order, first order, Higuchi and Korsmeyer-Peppas models to check the correlation coefficient (R^2) and verify the best model among these.[11,12]

Microbiological screening

The agar well diffusion assay was utilized to conduct the microbiological test for the various suppositories against *Candida albicans*. The suppository was dissolved in AVF pH 4.1 buffer and the resulting solution served as the study's test sample. Following the inoculation of the candida in Sabouraud dextrose broth, the culture was standardized to 0.5 McFarland (10^8 CFU/mL). After pouring the sterilized media of Sabouraud dextrose agar to the sterile petri plate and allowing it to solidify completely, the culture was applied to the agar surface using a sterile swab. Next,

Table 3: D-Optimal mixture design with 5 runs for the optimization of suppository base and plasticizer.

Batch	Ratio of PEG 6000: 400	Average weight (g)	Melting time (s)	Hardness (kg)	*In vitro* release (%)
F1	70:30	2.43±0.14	191±12	2.12±0.3	87.83±3.67
F2	65:35	2.40±0.18	166±20	2.01±0.1	86.34±2.93
F3	60:40	2.41±0.31	161±15	1.87±0.3	83.76±2.74
F4	55:45	2.47±0.23	105±11	1.65±0.1	81.98±2.44
F5	50:50	2.11±0.22	65±5	1.15±0.2	80.84±1.27

Each value represented the mean±S.D. (n=3).

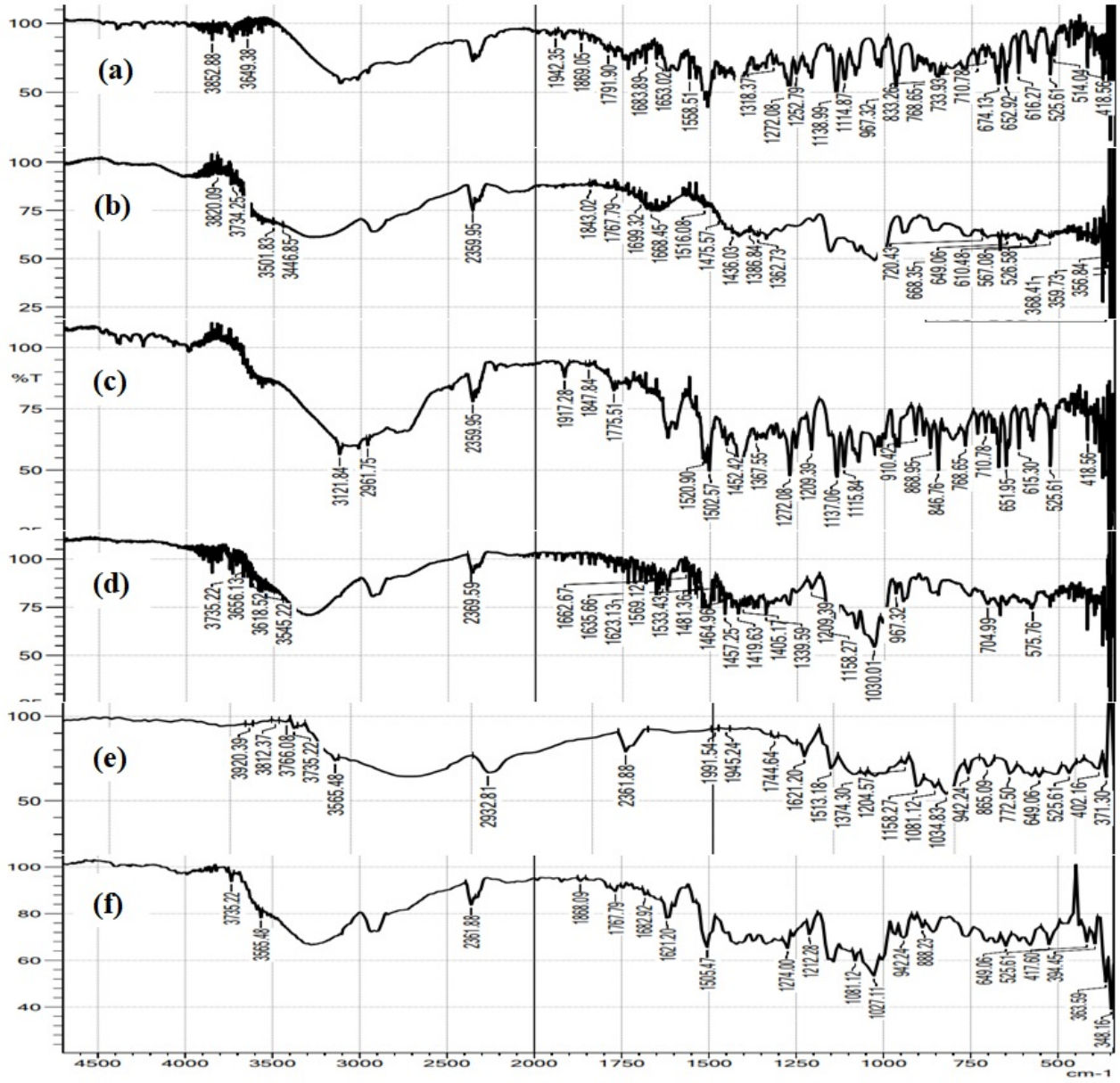

Figure 4: FTIR spectra of (a) FOZ, (b) βCD, (c) FOZ+βCD, (d) KN INC, (e) COPPN INC and (f) MW INC.

a sterile borer was used to drill holes in the agar plate and 50 µL test samples (FOZ INC suppository and FOZ plain suppository) were transferred inside the holes. The incubator was set to 37°C and the agar plates were loaded in the incubator. Following the incubation interval, the inhibition zones were precisely measured in millimeters. Zones were reported as mean±S.D. after the experimentation was done thrice.[8-12]

HET-CAM Test (Hen's Egg Chorioallantoic Membrane Assay)

The vaginal formulation may cause irritation after application and hence it is necessary to check the irritation potential before the application. The HET-CAM assay is used to study the irritation potential of vaginal formulation. The eggs after fertilization are kept for 9 days for embryo formation. The fertilized eggs that have been checked under light for embryo formation are used for the test. On the 9th day, the eggs were taken out from the incubator and placed on the egg tray with the broader/ovoid part faced upside and the CAM was exposed by removing the shell. The thin membrane was carefully removed and the samples were applied and observed for 5 min. The test samples applied are 0.1 N NaOH, AVF pH 4.1 and FOZ suppository dissolution sample. The irritation signs (hemorrhage, vasoconstriction and coagulation) are recorded for the sample. To classify the samples using computed values for 0.9% NaCl and 0.1 M NaOH, the Irritation Score (IS) was used as a guide from earlier research. As a result, the formulation's suitability was determined by checking

its severity.[37,38] The equation for the determination of the severity of the irritation is given in the equation (5) below:

$$Irritation\ score = \left(5 \times \frac{301 - hemorrhage\ time}{300}\right) + \left(\frac{301 - vasoconstriction\ time}{300}\right) + \left(\frac{301 - coagulation\ time}{300}\right) \quad (5)$$

Stability Study

To check the stability of the optimized inclusion complex loaded suppository and to study the changes such as physical (presence of crack, mottling, or color change) or chemical parameters that change during storage, the sample was studied for 3 months for stability. The suppositories were packed in aluminum foil stored in a glass vial and kept at 8°C. The samples were removed after 3rd month and analyzed for *in vitro* drug release and drug content.[17]

RESULTS

The INC was prepared for the prior optimization of the ratio of drug and βCD. Thus, a D-Optimal mixture design was applied for the optimization of the ratio of FOZ INC and therefore, the same, with the responses such as solubility and *in vitro* release, were taken under consideration. The different ratios prepared of the FOZ INC are given in Table 1.

Optimization for the preparation of FOZ INC

The QbD methodology was applied through the investigation of variable parameters in the development of analytical techniques. Critical parameters were determined using principal component analysis and data observation as shown in Figure 2. Additionally, each strategy was verified following ICH Q2 (R1) specifications.[31] Improving the formulation of the FOZ βCD complex through optimization is one of the essential steps in boosting the FOZ's bioavailability. To do it, the right ratio of βCD to FOZ must be optimized. The D-optimal mixture design was used to optimize the ratio of the FOZ and βCD and determine the desired outcomes. Two independent variables were chosen: the quantity of FOZ and βCD. The design allowed for 5 experimental runs of each of the chosen independent variables. Two responses were chosen: *in vitro* release and solubility for the study. Table 1 lists the values of these responses. The two-component mix graph for both responses is given in Figure S1.

Effect of independent variables on the solubility

One of the crucial factors to consider when formulating a dosage form with poorly soluble FOZ is its solubility. The drug's bioavailability at the spot of action improves with increasing solubility. The link between the independent variables on solubility is displayed in Equation (6). The same effect is given in the two-component mix graph as shown in Figure S1(a).

$$Solubility = -1.76450 * Drug - 2.56123 * Beta\ cyclodextrin + 7.20472 * Drug * Beta\ cyclodextrin \quad (6)$$

The terms "+" and "-" in the equation stand for the factors' respective effects of antagonism and synergism. The presence of the drug and βCD caused an increment in the drug solubility. Hence, the interaction between the drug and βCD caused improved FOZ solubility.

Table 4: ANOVA summary of the response variables.

Source	Sum of squares	d_f	Mean square	F value	*p* value	Remark
Hardness						
Model	0.58	2	0.29	88.54	0.0112	significant
Linear mixture	0.53	1	0.53	161.00	0.0062	
AB	0.053	1	0.053	16.08	0.0569	
Residual	6.571E-003	2	3.286E-003			
Cor Total	0.59	4				
Melting time						
Model	9796.90	1	9796.90	39.59	0.0081	significant
Linear mixture	9796.90	1	9796.90	39.59	0.0081	
Residual	742.30	3	247.43			
Cor Total	10539.20	4				
***In vitro* dissolution**						
Model	58.90	1	58.90	21.65	0.0187	significant
Linear mixture	58.90	1	58.90	21.65	0.0187	
Residual	8.16	3	2.72			
Cor Total	67.06	4				

p value <0.05 was considered to have significant terms and values >0.100 indicated the non-significance of the terms.

Effect of independent variables (FOZ and βCD) on *in vitro* drug release

One of the crucial factors to take into account while making a complex with another substance is drug release. The mathematical model proposes a standard equation for establishing the correlation between the independent variables and the chosen response. The typical equation between the independent variable and % Drug release (selected response) is presented in Equation (7). The same phenomenon is presented in the two-component mix graph as illustrated in Figure S1(a).

In vitro drug release=+0.49603*Drug-6.54455*Beta cyclodextrin+95.98105*Drug*Beta cyclodextrin (7)

The terms "+" and "-" in the equation indicate the components' synergistic and antagonistic effects, Drug is the amount of FOZ and Beta cyclodextrin is the concentration of βCD. The interaction between the drug and βCD resulted in improvement in the drug release.

The two-component mix plots were used to illustrate how both independent factors affected the chosen replies. Drug release reached its peak when the ratio of FOZ to βCD was 1:1. The percentage of drug release falls as the ratio shifts in both directions.

Figure S1 presents the information on the simultaneous influence representation of both variables, namely FOZ and βCD, as a two-component mix plot. As seen in the two-component mix plot, the complex reached its maximum solubility enhancement at a ratio of 1:1 and began to decrease on both sides.

Desirability function application and validation of the model

The optimal values of FOZ and βCD that aid in reaching the predefined target profile were found using the desirability component of the Design Expert software. The key intention of the current work was to increase the solubility and dissolution, absolute oral bioavailability and therapeutic effectiveness of FOZ, by using FOZ βCD, a non-toxic, biocompatible, customized cyclic oligomer of glucose. In the current study, FOZ was changed and made more soluble by employing a molecular inclusion technique. The constraints applied during the optimization of the inclusion complex were drug from 0.5 to 1.5, βCD from 0.5 to

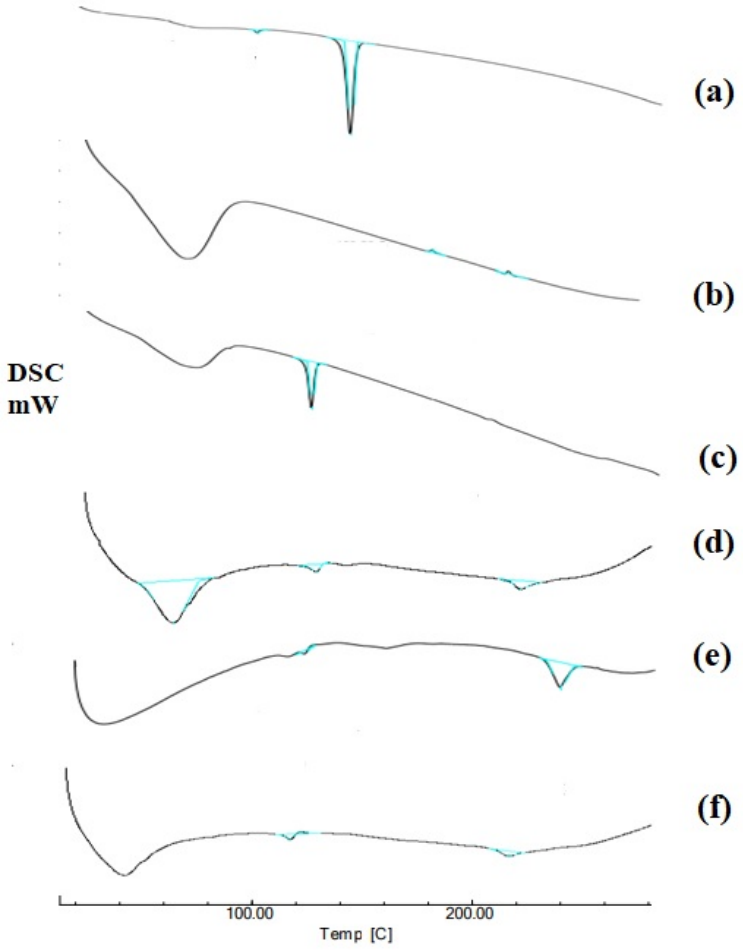

Figure 5: DSC thermograms of (a) plain FOZ, (b) βCD, (c) FOZ+βCD, (d) KN INC, (e) COPPN INC and (f) MW INC.

1.5, solubility from 2.5 to 2.89 mg/mL (maximize) and *in vitro* dissolution from 75-89.52% (maximize). The reason for keeping the constraints of solubility and *in vitro* release to maximum was to have the optimized inclusion complex with higher solubility and higher *in vitro* drug release. The desirability was found to be 1 for two solutions out of which one was chosen that showed the results nearer to the 1:1 INC batch. Hence, this was further taken for the study. The ramp for the desirable solution gave the values of ratio for FOZ (1.066) and βCD (0.934) along with the magnitude of the solubility (2.90 mg/mL) and *in vitro* release (89.97%). This suggested that a 1:1 ratio was optimum for the preparation of FOZ INC.

Statistical analysis of design for the FOZ inclusion complex

Using statistical analysis, the correlation between the chosen independent variable was found. The various ANOVA parameters, including the p-value, R^2 value, sufficient R^2 and F value, were ascertained with the aid of Design Expert software. Table S1 displayed several ANOVA parameters for each of the two responses. The p-values for the two responses (Solubility and *in vitro* dissolution) were determined to be 0.0098 and 0.0007, respectively, indicating the significance of the constructed model. A *p*-value of less than 0.005 is required. The responses such as solubility and *in vitro* release had model F-values of 100.99 and 1365.61 respectively. For both responses i.e., solubility and *in vitro* dissolution, the variation in the adjusted R^2 (0.9902 and 0.9993 respectively) and predicted R^2 (0.8295 and 0.9947 respectively) was determined to be less than 0.2, which is crucial for the experimental model to be considered significant. The values for R^2 were 0.9902 and 0.9993 respectively. The signal-to-noise ratio is determined by the model precision and if it is greater than 4, it is deemed significant. It was discovered that the adequate precision for each response was 22.394 and 80.108 respectively. Following the above-discussed investigation of the ANOVA's numerous parameters, the generated model was determined to be significant.

Phase solubility

FOZ solubility in the aqueous phase at various βCD concentrations at 37°C is illustrated in Figure 3(a). FOZ solubility increases as the concentrations of βCD rise. This linear graph is categorized as A_L-type and is thought to be suggestive of the ligand (the βCD) and substrate (FOZ) forming soluble complexes.

Preparation of FOZ INC

The inclusion complex of FOZ was prepared by different methods such as Kneading (KN), Coprecipitation (COPPN) and Microwave irradiation (MW) method after the optimization of the drug: βCD ratio from the D-optimal mixture design by taking the responses of solubility and *in vitro* dissolution.

Saturation solubility

The saturation solubility study was performed for the different types of INCs prepared including physical mixture and plain drug. The results of the saturation solubility are demonstrated in Figure 3(b). The plain drug had a solubility of 0.98±0.21 mg/mL. The maximum solubility was obtained for MW INC (3.14±0.85 mg/mL) followed by COPPN INC (2.87±0.67 mg/mL) then KN INC (2.14±0.57 mg/mL) while the PM INC had 1.73±0.49 mg/mL. The plain drug had poor solubility in AVF pH 4.1. After the formation of the inclusion complex, the FOZ solubility enhanced drastically with MW INC showing a 3.2 times increment.

FTIR analysis

FTIR study is helpful in the identification of the interaction between the drug and excipients and especially in understanding the inclusion complex formed between the drug and the βCD. The vibrational mode changes that occurred during the host-guest interaction are thus revealed through the FTIR studies. Evidence of the formation of the inclusion complex is obtained using the FTIR spectroscopic bands of the FOZ characteristic functional groups. The FTIR spectra of pure FOZ, βCD, PM (FOZ+βCD) and inclusion complexes prepared by different methods (kneading, coprecipitation and microwave irradiation) are presented in Figure 4. The spectrum of FOZ indicated characteristic bands at (O-H stretching) 1653.02 cm^{-1} (C-F stretch), 1558.51 cm^{-1} (aromatic C-C stretch), 1272.08 cm^{-1} aromatic C-H stretch, 1138.99 cm^{-1} and 967.32 cm^{-1}. The βCD indicated the characteristic peak for O-H stretching at 3330 cm^{-1}. These values for FOZ and βCD are concordant with the previous literature. In general, the drug peaks were partially present in the case of all the inclusion complexes and the intensity of the appeared band of the drug peaks was reduced in comparison

Table 5: Evaluation parameters of different types of suppositories.

Batch name	Average weight	Hardness (kg)	Melting time (s)	Drug content (%)	*In vitro* release (%)
Plain drug	2.41±0.12	2.41±0.12	187±13	96.77±2.56	71.41±2.19
PM	2.46±0.11	2.35±0.21	182±14	96.98±2.14	79.49±1.44
KN	2.47±0.17	2.42±0.17	185±11	95.49±2.37	85.39±2.23
COPPM	2.42±0.15	2.44±0.15	192±17	97.66±1.42	94.42±2.34
MW	2.43±0.14	2.36±0.13	194±18	99.75±1.22	99.40±1.43

Each value represented the mean±S.D. (*n*=3).

to the pristine drug. The kneaded complex had a more intense peak of FOZ as compared to COPPM and MW complexes. In the case of KN and COPPN complex, the shift of the drug band from 1138.99 to 1158 cm^{-1}, while in the case of MW from 1272.05 to 1274 cm^{-1} was observed.

DSC study

DSC thermograms for pure FOZ, pure βCD, Physical Mixture (PM) and FOZ/βCD complexes obtained by different methods of preparation are presented in Figure 5. Pure FOZ showed a sharp melting endotherm at 139.47°C with heat enthalpy of -200.03 mJ or -100.01 J/g. The physical mixture indicated a sharp endotherm at 138.90°C by an enthalpy of -48.62 mJ or -24.31 J/g. Pure βCD exhibited no event of melting and no sharp peak that showed the sample obtained was amorphous. A distinct endothermal event occurred, ranging between 50 to 170°C corresponding to the dehydration as evidenced by the previous literature[6]. In the case of FOZ KN, FOZ COPPN and FOZ MW the melting event occurred at 135.37°C (-7.0 mJ or -3.52 J/g), 140.86°C (-0.42 mJ or -0.21 J/g) and 135.44°C (-2.5 mJ or -1.25 J/g) respectively. The vanishing of the sharp endotherm belonging to the drug was observed in all the cases.

XRD study

Powder XRD is a very powerful study for understanding the physical state of any substance with a solid nature. Pure FOZ has a characteristic diffractogram with sharp peaks of crystallinity as shown in Figure S2. The characteristic peaks observed for the FOZ were 15.335°, 16.591°, 20.003° and 25.586° which revealed the crystalline status of the drug. In the case of βCD, there were

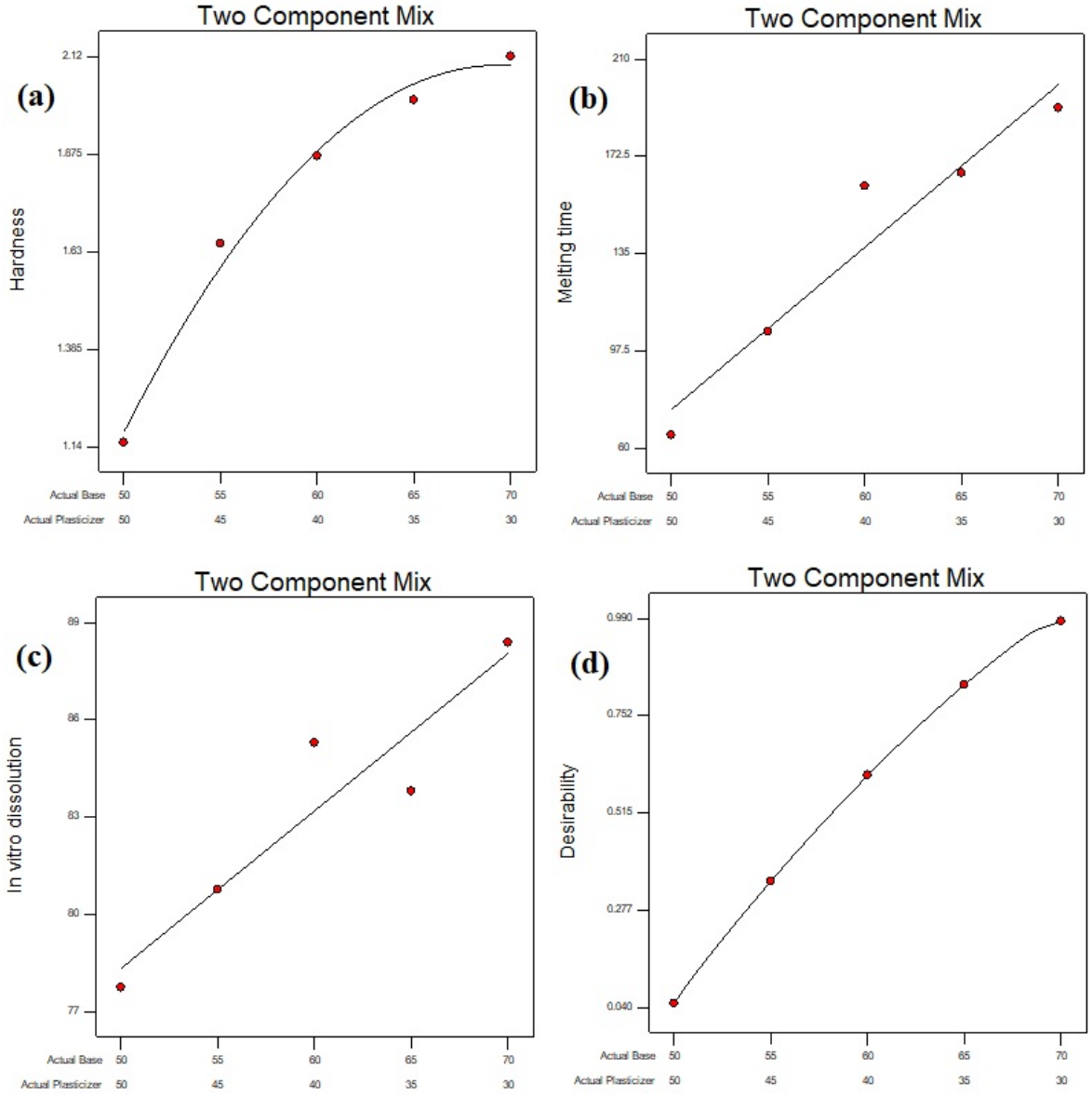

Figure 6: Two-component mix graphs for (a) *In vitro* dissolution, (b) Hardness and (c) Melting time of suppository with (d) desirability plot.

four major peaks of crystallinity at 12.488°, 15.367°, 16.599° and 20.011°. The physical mixture and kneaded complex had a closer resemblance. From the diffractogram of the kneaded complex, it was suggested that the complex formed has a slight reduction in crystallinity. In the diffractograms of coprecipitated and microwave complex, the drug 2θ peaks disappeared and the amorphic humps were observed. As compared to the kneaded complex, both the other complexes had much-reduced crystallinity. The reason could be the method of preparation that caused the change in the powder characteristics.

SEM study

The SEM micrograph of the plain FOZ showed the crystalline solid powder with crystals as elongated needles as shown in Figure S3. The βCD crystals were irregular in shape with the absence of sharp edges. The optimized inclusion complex had a completely different morphology than the individual components. This confirmed the formation of FOZ INC. Due to the formation of FOZ INC, the drug solubility may increase which could enhance the dissolution rate of the drug.

Drug content

The INC formed with different methods was evaluated for the drug content. The results are given in Table 2. The MW INC had the highest % drug content followed by COPPN INC and then finally KN INC.

Optimization of the suppository base and plasticizer

The results of all the responses of the five batches of suppositories are given in Table 3. The high and low level of the base and plasticizer ratio along with all the responses of the suppositories is given in Table S2.

Hardness

The hardness of the suppositories was studied and the higher value of hardness was observed for F1 (2.12±0.3 kg) followed by F2 (2.01±0.1 kg) and then F3 (1.87±0.3 kg). Lower hardness was obtained for F4 and F5 batches with values of 1.65±0.1 kg and 1.15±0.2 kg respectively. The relation between the response and factor is given in equation (8):

$$\text{Hardness}=-0.20857*\text{Base}-9.72286*\text{Plasticizer}+24.57143*\text{Base}*\text{Plasticizer} \quad (8)$$

The effect of PEG 6000 and PEG 400 concentration on the hardness is presented in the two-component mix graph as shown in Figure 6(a). The increased concentration of PEG 400 caused a reduction in the hardness while the increased concentration of PEG 6000 caused an increment in the hardness of the suppositories.

Melting time

F1, F2 and F3 displayed melting times of 191±11, 166±9 and 161±7 sec, respectively; F4 and F5 displayed reduced melting times (105±8, 65±6 sec, respectively) (Table). When PEG 6000 was added in greater amounts, F1, F2 and F3 displayed higher melting points; however, when PEG 400 was added in greater amounts, the suppository's melting point decreased. As a result, melting time increased when PEG 400 concentration decreased and climbed as PEG 6000 concentration increased. Equation (9) illustrates how independent variables serve to further elucidate both the separate and combined impacts of melting point.

$$\text{Melting time}=+388.00000*\text{Base}-238.00000*\text{Plasticizer} \quad (9)$$

The overall impact of PEG 6000 and PEG 400 levels is displayed by the melting time in the two-component mix graph as shown in Figure 6(b). As a result, it was discovered that the suppository's melting time increased as PEG 6000 content increased. PEG 400, on the other hand, demonstrated the opposite impact on melting time.

In vitro release study

The release of drug for F1 was found to be highest 87.83±3.67% followed by F2 which was 86.34±2.93% then F3 83.76±2.74%. Lower drug release was observed for F4 and F5 which was 81.98±2.44% and 80.84±1.27% respectively. Equation (10) indicated the rise in drug release as PEG 6000 concentration increased and PEG 400 concentration also increased.

$$\textit{In vitro}\ \text{dissolution}=+102.61200*\text{Base}+54.07200*\text{Plasticizer} \quad (10)$$

The two-component mix graph as shown in Figure 6(c), which indicated a link between two factors, helped to better clarify *in vitro* release (%).

Table 6: *In vitro* release kinetic modeling for various ratios of different suppositories of FOZ.

Batch	Zero order R^2	First order R^2	Higuchi plot R^2	Hixson Crowell plot R^2	Korsemeyer-peppas plot	
					R^2	n
F1	0.8764	0.9644	0.9844	0.9631	0.9657	0.4637
F2	0.9830	0.9933	0.9684	0.9995	0.9956	0.8854
F3	0.9873	0.9771	0.9434	0.9909	0.9873	1.1848
F4	0.9782	0.9953	0.9705	0.9976	0.9922	0.8559
F5	0.9044	0.9824	0.9818	0.9637	0.9664	0.7338

Statistical analysis of the design

To obtain the best formulation utilizing the data produced by the design of experiment software, the mathematical relationship between the factor and the variables was utilized. To maximize the response variables, a D-optimal mixture design was employed. To obtain the fastest possible antifungal activity of the drug in the vagina, an optimized formula was developed with a high level of drug release, appropriate hardness and appropriate melting time. As recommended by the software, the optimized batch had a desirability value of '1' in the design space and the same is presented in Figure 6(d). Among 5 batches, batch F1 has traits very similar to the solution given by the software and hence this batch was considered as optimized one.

The relevance and strength of the effects of the independent variables and how they interact with the dependent variables of the formulation were assessed using an ANOVA and the summary is given in Table 4. The acquired regression model was employed to examine how the independent components interacted with one another. The fit statistics summary was obtained which prompted that the responses i.e., hardness, melting time and *in vitro* dissolution, the variation in the adjusted R^2 (0.9777, 0.9061 and 0.8377 respectively) and predicted R^2 (0.8130, 0.8074 and 0.7545 respectively) was determined to be less than 0.2, which is crucial for the experimental model to be considered significant. The values for R^2 were 0.9888, 0.9296 and 0.8783 respectively. The signal-to-noise ratio is determined by the model precision and if it is greater than 4, it is deemed significant. It was discovered that the adequate precision for each response was 20.720, 12.585 and 9.307 respectively. Following the above-discussed investigation of the ANOVA's numerous parameters, the generated model was determined to be significant.

Evaluation of suppository

The suppositories were checked visually for physical properties like color, surface texture and presence of cracks or fractures. All the suppository batches had smooth and shiny surfaces with no fractures or cracks. The color of all the batches appeared as creamy buff white due to the presence of PEG 6000. The smooth and shiny appearance could be due to the addition of PEG 6000. PEG 400 provides the plasticizing effect. The smoothness of the suppositories ensures assistance during the administration into the vaginal cavity. The lubrication is, therefore, provided through the surface of the suppositories. A weight variation test was performed as per the British Pharmacopoeia and the results were within the limits. From Table 5, the weight of the suppository batches did not deviate from the limits of 5% and 7.5%. The assessment of hardness helps to check the resistance to the mechanical shock without undergoing any damage. The pharmacopoeial limit of the hardness is within 1.8-2 kg pressure. Thus, it was evident from the hardness results that all the batches met the hardness criteria and none of the batches was below the hardness limit. Faster dissolution of the suppository batches resulted due to the presence of the liquid plasticizer PEG 400 causing quick melting followed by dissolution. It took a longer time to dissolve the suppository batches and as a result of higher amount of high molecular weight solid base PEG 6000 and lower concentration of PEG 400.

The results of the physicochemical characteristics of the suppositories prepared after optimization of the ratio of inclusion complex are given in Table 5.

Drug content

The drug content for the different suppository batches is given in Table 5.

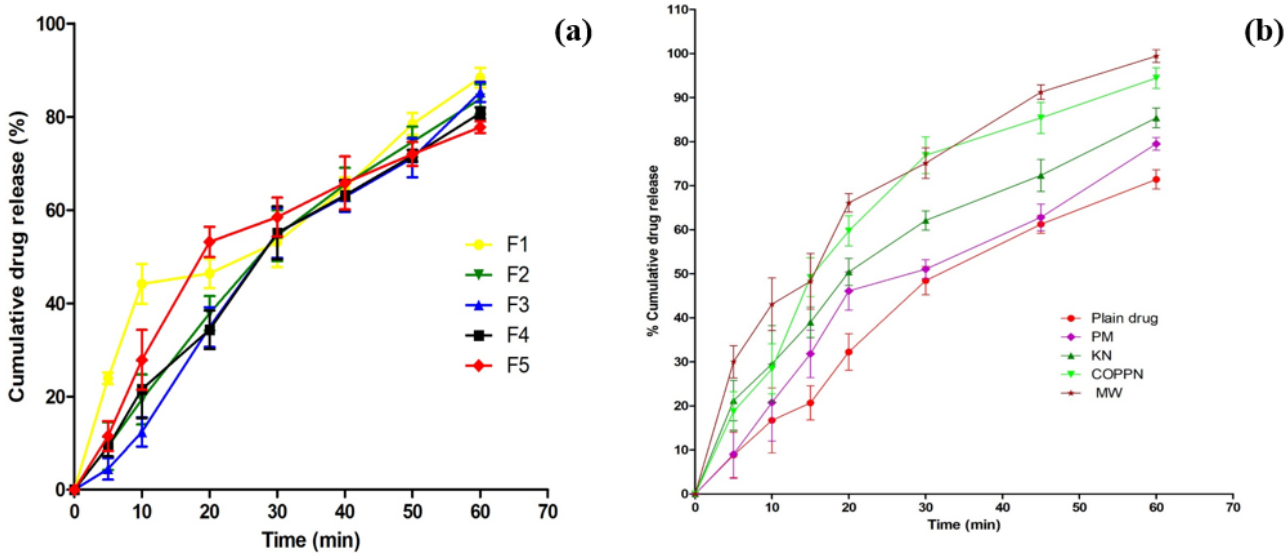

Figure 7: (a) *In vitro* dissolution profile of different types of FOZ suppositories made with various ratios of PEG 6000 and PEG 400 and (b) *In vitro* dissolution profile of different types FOZ suppositories.

In vitro release study

The dissolution study showed the release of the FOZ suppositories for 60 min. From the dissolution profile graph as exposed in Figure 7(a), the % cumulative release was observed in suppositories with FOZ inclusion complex (prepared by kneading method) with different suppository base ratios. The drug release from different bases was observed. The greater release of drug was seen from the base with a (batch F1) 70:30 ratio of PEG 6000: PEG 400 (88.37±2.12 %) followed by (batch F2) 60:40 (85.29±2.14 %), (batch F3) 65:35 (83.80±3.21 %), (batch F4) 55:45 (80.77±1.23 %) while least was seen for the (batch F5) 50:50 ratio (77.75±1.25%). To grasp the impact of the INCs prepared by different methods on FOZ release, dissolution studies of FOZ inclusion complex suppositories were compared to those of plain FOZ suppositories. The dissolution data of the same were modeled into different kinetic models and the results are presented in Table 6. The batches F2, F3 and F4 had R^2 values of 0.9995, 0.9909 and 0.9976 respectively. All these suppositories followed the Hixson-Crowell model. The batch F1 followed Higuchi model (R^2=0.9844) and F5 followed first order model (R^2=0.9824).

The finalized suppository base that gave good dissolution property was chosen as 70:30 PEG 6000: PEG 400. Further, the inclusion complex prepared by Kneading (KN), Coprecipitation (COPPN) and Microwave irradiation (MW) method was loaded into the final suppository base and dissolution was studied. The results of the *in vitro* dissolution of the different suppositories are shown in Figure 7(b). The plain drug suppository had the lowest drug release of 71.41±2.19 % while in the case of the physical mixture, there was the release of drug greater than the plain drug suppository at 79.49±1.44%. The KN, COPPN and MW-based suppositories showed the drug release of 85.39±2.23%, 94.42±2.34% and 99.40±1.43% respectively. The plain drug suppository had lower release due to poor solubility of FOZ while the PM had higher release due to the presence of βCD. The INC-based suppositories had much higher drug release than the PM suppository. Therefore, significantly higher release was seen as a consequence of the formation of the INC of the drug with βCD that affected the solubility and dissolution. The microwave-irradiated complex-based suppository had the highest drug release over all the suppositories. The reason could be the formation of a porous inclusion complex that had greater amorphous characteristics than other complexes.The release data was mounted in the dissolution kinetic models to understand the release mechanism of FOZ from the formulations. The release kinetics of the formulations is given in Table 7. The plain drug and physical mixture suppository both had first-order release kinetics with the R^2 value of 0.9966 and 0.9890 respectively. The KN-kneaded suppositories depicted the Korsemeyer-Peppas model with an R^2 value of 0.9954. The COPPN-based suppository revealed first order (R^2=0.9955) and the MW suppository followed the Higuchi matrix model (R^2=0.9950). This suggested n value between 0.45-0.89 to have the anomalous diffusion or non-Fickian diffusion.

Microbiological screening

Candida albicans locally resides in the vagina during candidal infection and at the infection site the drug is required to be present at a higher concentration, then the antifungal effect is enhanced extensively. The antifungal screening was done against *C. albicans* for the plain drug suppository and FOZ INC suppository respectively. Figure S4 shows the inhibition zones for the control solvent and FOZ INC suppository respectively. A significantly higher inhibition zone was achieved with the FOZ INC suppository (38±1 mm) as compared to the plain drug suppository (31±2 mm).

HET-CAM assay

The HET-CAM *in vitro* test is a quick and economical method for assessing the excipients' potential to cause irritation in a formulation. Similar symptoms for vaginal irritation are replicated by the vascular damage, which is accompanied by hemorrhage, lysis, or coagulation that is seen in the chorioallantoic membrane following exposure to the test sample. The developing embryo with fully formed blood vessel tissue makes up the CAM, which serves as a reliable stand-in for the study. The inflammatory event seen in the rabbits' vagina is paralleled by the CAM response. Figure S5 displays the HET-CAM study's outcome. Table S3 presents the findings of the study. The findings displayed no irritation after applying the normal saline 0.9% NaCl and FOZ INC suppository solution. Hemorrhage resulted in blood vessel lysis in the 0.1 N NaOH-treated CAM. The results suggest that there was absence of irritation after application of the FOZ INC suppository and therefore, it would be suitable for vaginal application.

Table 7: *In vitro* release kinetic modeling for various suppositories of FOZ.

Batch	Zero order R^2	First order R^2	Higuchi plot R^2	Hixson Crowell plot R^2	Korsemeyer-peppas plot	
					R^2	n
PM	0.9458	0.9890	0.9701	0.9864	0.9765	0.8410
KN	0.8872	0.9941	0.9952	0.9805	0.9954	0.5750
COPPN	0.8768	0.9955	0.9808	0.9864	0.9765	0.6788
MW	0.8133	0.9433	0.9950	0.9918	0.9923	0.4957
Plain FOZ	0.9787	0.9966	0.9521	0.9959	0.9936	0.8707

Table 8: Stability study of optimized suppository.

Evaluation parameter	0 day at room temperature	3 months at room temperature
Color and appearance	White	White
Surface texture	Smooth	Smooth
In vitro release (%)	99.40±1.43	98.97±1.67
Drug content (%)	98.75±2.25	98.65±2.11

Each value represented the mean±S.D. (n=3).

Stability study

A stability study was performed for three months and the various suppository parameters such as color appearance, surface texture and % cumulative drug release were studied at 8±2°C. Table 8 shows the result of the stability study with the different evaluation parameters. The suppository was clear and smooth afterwards the storage duration. There was no noteworthy variation in drug content and FOZ release. Thus, the suppositories exhibited acceptable parameters and assured stability for 3 months.

DISCUSSION

The phase solubility study was performed for the FOZ and βCD using Higuchi-Connor's method using AVF pH 4.1 at 37°C. An A_L-type graph shows that, depending on the βCD's aqueous solubility, the FOZ solubility increased in a linear pattern as the βCD concentration increased. The $K_{1:1}$ value observed was 3.88 mM^{-1}. It was observed that as the βCD concentration was increased, the FOZ solubility also increased. Hence, from the phase solubility study, it was clear that the βCD could help to improve the aqueous solubility of FOZ. Similar results were observed with linear graph in the case of previous literature.[15]

The D-optimal mixture design was applied to optimize the ratio for the preparation of the INC. The optimization of the INC was done by studying the responses such as solubility and *in vitro* drug release. The constraints that were applied for the optimization included solubility and *in vitro* release to be maximized. The solution given by the design expert had the desirability of 1 which suggested that the inclusion complex of ratio 1:1 was the optimized one for further work. This batch was then taken further and the INC was prepared by three different methods. The results of the fit statistics also displayed a good correlation after the ANOVA treatment. Both factors were significant and hence the model developed was valid.

The saturation solubility was performed for inclusion complexes prepared by different methods such as KN, COPPN and MW. The saturation solubility study resulted in improvement in drug solubility through inclusion complex formation. The reason was the encapsulation of FOZ within the cyclodextrin hollow space that assisted in the solubilization of the drug in the AVF pH 4.1. The highest solubilization of the drug was seen with MW INC. The improvement in FOZ solubility after the inclusion complex formation was 3.2-fold.

The solubility of guest molecules can be increased by βCD molecules, as has been widely documented in the literature. Table 2 displays the drug's water solubility concerning several FOZ/βCD complex methods of manufacture used in this study. Regarding solubility tests conducted at 37°C, it was shown that the addition of βCD increased the solubility of FOZ. The degree of drug solubility increase was directly correlated with the drug's interaction with βCD, indicating that FOZ has a stronger affinity for the non-polar cavity of βCD. Two factors have been identified as responsible for the notable increase in solubility observed with COPPN INC and MW INC: solid-state complexation and the highly dynamic amorphous phase/ decrease in crystallinity that follows complexation. These results corroborated with the previous literature.[6-7,15]

It is clear from earlier research that cyclodextrins promote water solubility by forming non-covalent inclusion complexes that are soluble in water. The higher drug water solubility contributes to the dosage form's improved bioavailability and, consequently, greater therapeutic efficacy. Additionally, there is no making or breaking of the covalent bonds during the formation of INC. As a result, the complex dissociates easily in an aqueous solution, releasing the drug molecule and maintaining equilibrium between the free drug and the drug molecules bound within the cyclodextrin complex.[5]

The main techniques used in conventional INC preparation are coprecipitation, kneading and physical mixing. While co-precipitation and freeze-drying techniques produce actual ICs, they invariably come with some drawbacks. The usage of organic solvents and the lengthy processing time are the main disadvantages. As an alternative, microwave-assisted chemistry has become more popular recently because it completes the reaction quickly and is less harmful to the environment because it uses fewer solvents, produces fewer byproducts and lowers the reaction temperature.[34] Thus, the results suggested that MW INC was superior in the context of preparation and reduced the time in preparation with having a higher % drug content.

For the understanding of the occurrence of the interaction between the drug and βCD, the FTIR study was performed. There was a shifting of the drug peaks, absence of the peaks, reduction in the peak intensity and presence of the new peak.[6,7,11,12] All these events were observed in the FTIR spectra of the complexes. The results thus suggested that the kneading method, coprecipitation and microwave method both were able to form the inclusion complex. These results also suggested the amorphization of the formed complex or the possible drug and βCD interaction.

It has been observed that cyclodextrin complexes can be characterized using thermal analysis. The melting, boiling, or sublimation points of guest molecules that are introduced into

the βCD cavity typically change or vanish within the temperature range where the βCD dehydrates or breaks down. The drug's incorporation in the cyclodextrin cavity is strongly supported by the lack of the drug's characteristic peak.[6,7,11,12,15] The thermogram of the FOZ INC showed the absence of FOZ melting event that corresponded to the formation of the INC. This suggested that the drug molecule was encapsulated within the cyclodextrin cavity.

The nature of the powder is analyzed by the XRD studies. Loss or reduction in the drug peak intensity may be linked to probable drug-βCD interactions. The non-existence of the characteristic drug peak is convincing proof of the insertion of the guest (drug) into the host (cyclodextrin) void.[6] The plain FOZ had characteristic peaks of crystallinity. In the FOZ INC diffractogram, amorphization of the complexes could be observed even though the βCD that was used initially had several peaks of crystallinity. A high degree of amorphous nature was seen for the microwave-irradiated complex. Thus, it was concluded that the crystalline drug was transformed into an amorphous solid.

To understand the powder morphology after the inclusion complex formation, SEM studies were performed. The different morphology of the inclusion complex could be signified by the fusion of the drug and βCD with uniform-size particles having sharp edges resulting in numerous pores in the structure of the single particle. This could help in the improvement of aqueous solubility of the drug and result in a higher dissolution rate. The FOZ INC powder had more uniformity in size, spherical in shape and was homogenous.

Initially, just the PEG 6000 suppository was tried without the use of a plasticizer (PEG 400). Only blank PEG 6000 when used resulted in a suppository that was too hard. The addition of an increased amount of PEG 400 results in a sticky suppository that oozes out the undesirable liquid. Thus, by trial and error, the ratio was taken from 70:30 to 50:50 (PEG 6000: PEG 400) which gave the desirable suppository characteristics. This could also have caused a problem in the application into the vaginal cavity. The presence of the plasticizer provides the lubrication effect that assists in the insertion into the vaginal cavity.[17]

For the preparation of the suppository, the PEG base and plasticizer were required to optimize and for the optimization of the same D-optimal mixture design with 5 runs were studied with responses such as hardness, melting time and *in vitro* drug release. The best batch was found to be 70:30 PEG 6000 and PEG 400. The finalized suppository for the different types of inclusion complex such as KN, COPPN and MW INC was then prepared using the same ratio composition of PEG 6000 and PEG 400.

For the optimization of the suppositories, constraints were placed on the design layout to get the best batch of suppositories with optimum levels of hardness, melting time and *in vitro* drug release. These constraints included hardness between (maximize), melting time (maximize) and *in vitro* drug release (maximize). As the suppository should have good hardness, the hardness parameter was kept to a maximum. It ensures that during the shipment or transport the suppositories would resist the mechanical shock and remain sturdy. If the suppository melts too quickly then there are chances of leakage which is undesirable from the intravaginal cavity. Hence to avoid such incidences, the melting time was taken to be longer so that the suppository melts slowly and helps to retain the drug effectively. The *in vitro* drug release was kept maximum because the drug concentration at the fungal infection site needs to be maximum and therefore, increased dissolution allows more drug molecules to have closer contact with the vaginal tissue.

For the optimization, the different evaluation parameters were studied that included melting time, hardness and *in vitro* dissolution for the different ratio-based suppositories of PEG 6000 and PEG 400. Also, in addition, a weight variation study was performed. The weight of the suppository batches was found to be within the limits as defined by the B.P. The hardness of the suppositories was within the limit of 1-2 kg. The melting time of the suppository batches was also favorable for the suppository to release the drug.

In the *in vitro* drug release study of different suppositories performed in AVF pH 4.1, there was an improvement in the dissolution rate of the drug after complexation as compared to the plain drug. The reason could be that complexes dissolve more quickly due to their high-energy amorphous state. Since cyclodextrins enhance the apparent solubility and dissolving rate of drugs, they also reduce the crystallinity of drugs upon complexation. Moreover, βCD possesses characteristics similar to those of a surfactant, which can lower the tension at the interface between the drug that is insoluble in water and the dissolution media, resulting in a faster rate of dissolution. It was discovered that the kind of preparation procedure used affects the solubility enhancement caused by FOZ/βCD complexes. The high energy amorphous state, inclusion complex formation and interactions between βCD and the active agent were identified as the contributing factors for the greater release of the active agent from βCD complex-prepared when compared to plain drug. The results corroborate with the previous literature.[7-6]

The antifungal study was carried out against *C. albicans* for plain drug suppository and INC-based suppository by agar well diffusion assay. It was remarked that there was not at all negotiation in fungal inhibition potential after encapsulation of the drug inside the cyclodextrin and no unconstructive effect was encountered with inclusion complex. Thus, the results suggested the eligibility of FOZ INC for enhancing the antifungal action. The enhanced activity may be obtained due to the formation of INC that resulted in adequate accessibility of the FOZ to diffuse through the agar pore channels gel and prevent colony growth. The smaller zone obtained for the plain FOZ suppository may be due to inadequate solubility of the drug and less drug solubility

led to decreased fungal inhibition as depicted from the zones. Similar results were obtained in the previous studies.[11,12]

HET-CAM assay was performed for the estimation of the irritation of the vaginal tissue. Upon application of the formulated suppository solution to the CAM, no occurrence of hemolysis and coagulation indicated the absence of the signs of irritation. Thus, the suppository can be tolerated well if applied vaginally. Previous studies produced similar results.[9,10]

The stability studies were conducted for 3 months and the evaluation parameters studied were physical appearance, *in vitro* drug release and drug content. The results suggested no significant changes in the physical appearance, *in vitro* drug release and drug content after the duration of 3 months at 8°C.

CONCLUSION

Vaginal candidiasis is treated employing azole drugs but due to the poor aqueous solubility, there is suboptimal therapeutic efficacy. The oral treatment with Fluconazole leads to various side-effects and thus to overcome the systemic side-effects and to improve the aqueous solubility, the Fluconazole inclusion complex loaded vaginal suppository was proposed. The INC with β-cyclodextrin was prepared by kneading and coprecipitation methods. The complex formation was confirmed by FTIR, DSC, XRD and SEM studies. The physicochemical parameters of the suppository were studied such as breaking strength, melting time and *in vitro* release. The dissolution studies showed improvement in the dissolution rate and good release from the inclusion of complex-based suppository than the plain FOZ suppository. The optimized suppository selected was the one with a microwave irradiation-based inclusion complex in it. The *in vitro* antifungal activity against *C. albicans* showed a larger inhibition zone for the INC suppository than the control suppository. Thus, there was an improvement in the antifungal effect due to an increase in the solubility of FOZ by the INC formation. The HET-CAM study was performed to examine the irritability of the formulation. The stability study confirmed that the suppositories were stable for three months at 8±2°C. Further, preclinical studies can be performed in future to understand the performance of the prepared suppository and the feasibility of scaling up at the industrial level of manufacturing. Thus, the study established the potential of Fluconazole-based inclusion complex-loaded suppository in the therapy of vaginal candidal infection as an efficient dosage form for vaginal delivery.

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CONFLICT OF INTEREST

The authors declare that there is no significant competing financial, professional, or personal competing interest that might have influenced the performance or presentation of the work described in this manuscript.

ABBREVIATIONS

FOZ: Fluconazole; **βCD:** Beta cyclodextrin; **INC:** Inclusion complex; **AVF:** Artificial vagina fluid; **PM:** Physical mixture; **KN:** Kneading; **COPPN:** Coprecipitation; **MW:** Microwave irradiation; **FTIR:** Fourier transform infrared spectroscopy; **DSC:** Differential scanning calorimetry; **XRD:** X-ray diffraction; **SEM:** Scanning electron microscopy; **PEG:** Polyethylene glycol; **HET-CAM:** Hen's Egg Chorioallantoic Membrane Assay.

SUMMARY

Fluconazole is the primary drug and the first line treatment for Vaginal Candidiasis given orally. But it has various oral side effects. Hence vaginal delivery of Fluconazole inclusion complex in suppository was proposed. In the first step, the inclusion complex of Fluconazole was prepared with βCD and the ratio of the inclusion complex was optimized by using D-Optimal mixture design. The molar ratio of 1:1 was found to be the optimized batch and hence it was taken further for the study. The inclusion complex was prepared by different methods such as kneading, coprecipitation and microwave irradiation technique. The confirmation of the inclusion complex formation was done by FTIR, DSC, XRD and SEM studies. Then, for the preparation of suppository, the base (PEG 6000) and plasticizer (PEG 400) ratio was optimized by again using D-Optimal mixture design in which the responses taken were hardness, melting time and *in vitro* drug release were studied as responses. The best batch was found to be 70:30 ratio of PEG 6000 and PEG 400. Then into this ratio of suppository base, the different inclusion complexes prepared were added. The highest drug release was obtained from microwave irradiated inclusion complex loaded suppository. The antifungal activity against *C. albicans* showed higher activity for inclusion complex-based suppository than the plain drug suppository. For the evaluation of the irritation, the HET-CAM assay was performed and no event of irritation was evidenced with the inclusion complex-based suppository. The stability study for the optimized inclusion complex showed stability for three months.

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SUPPLEMENTARY DATA

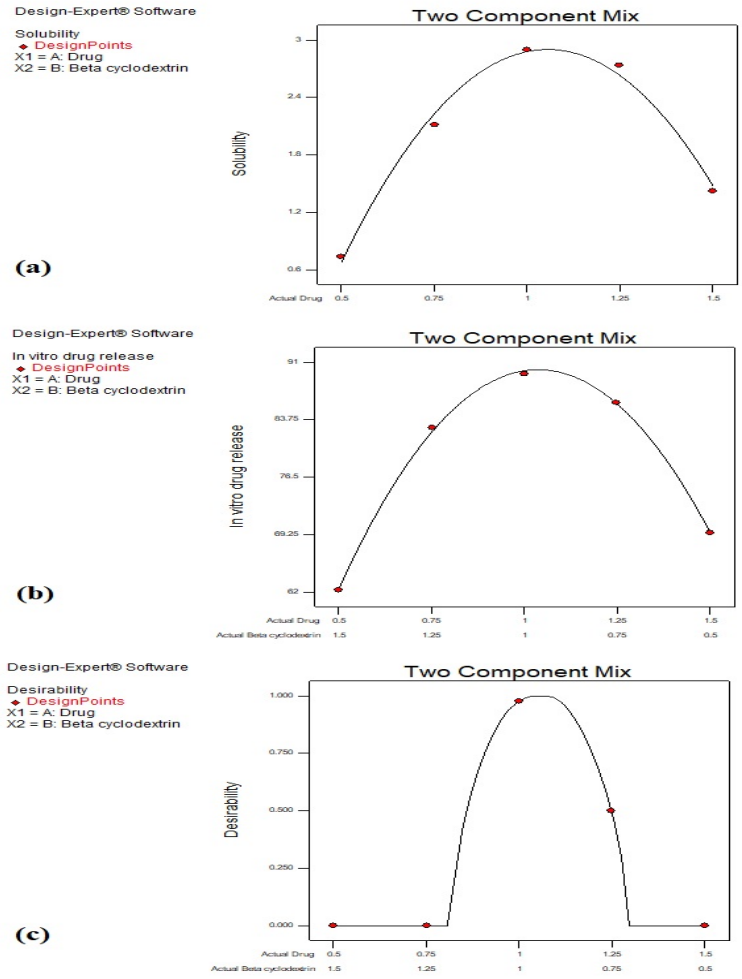

Figure S1: Two-component mix plot for solubility and *in vitro* release study for FOZ INC.

Table S1: ANOVA parameters for the response of inclusion complex.

Source	Sum of squares	d_f	Mean square	F value	*p* value	Remark
Solubility						
Model	3.25	2	1.62	100.99	0.0098	significant
Linear mixture	0.40	1	0.40	24.60	0.0383	
AB	2.85	1	2.85	177.38	0.0056	
Residual	0.032	2	0.016			
Cor Total	3.28	4				
***In vitro* release**						
Model	537.04	2	268.52	1365.61	0.0007	significant
Linear mixture	30.88	1	30.88	157.05	0.0063	
AB	506.16	1	506.16	2574.17	0.0004	
Residual	0.39	2	0.20			
Cor Total	537.43	4				

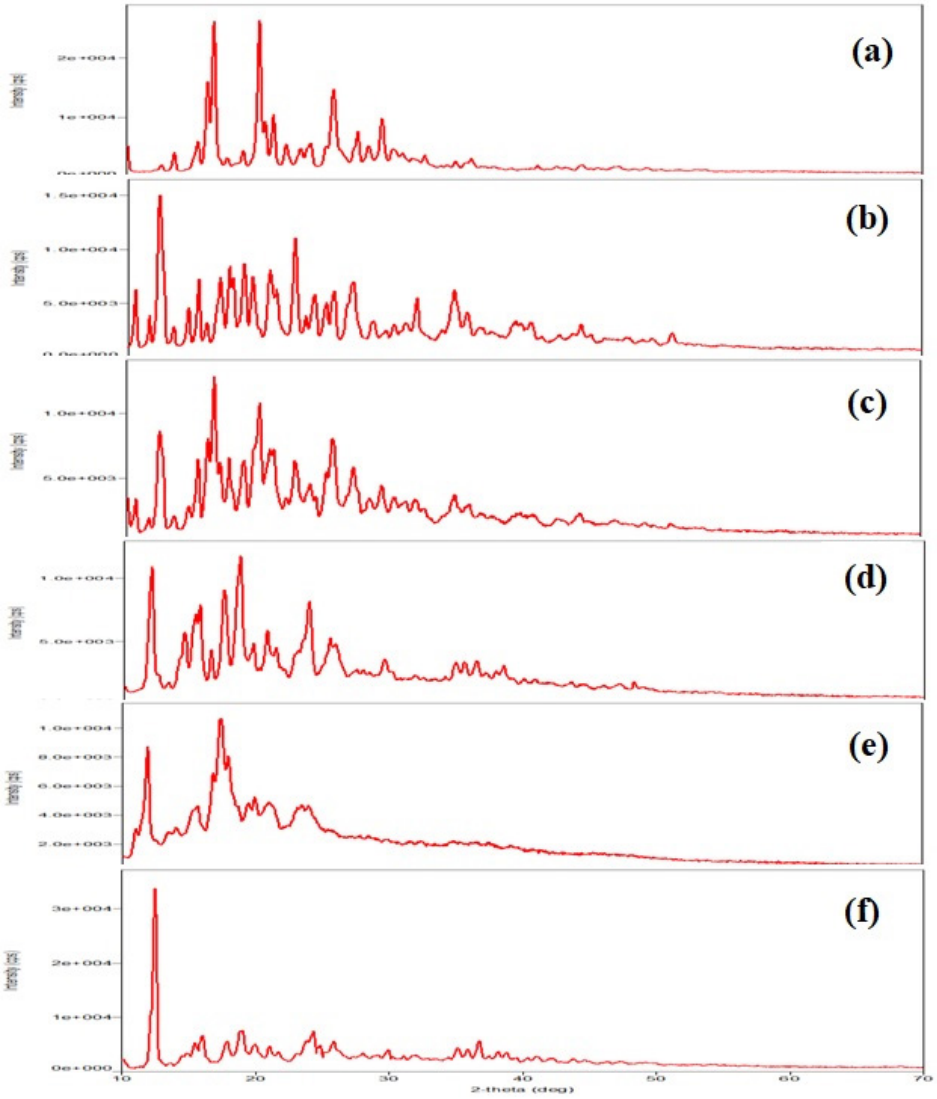

Figure S2: XRD diffractograms of (a) plain FOZ, (b) βCD, (c) FOZ+βCD, (d) FOZ KN, (e) FOZ COPPN and (f) FOZ MW.

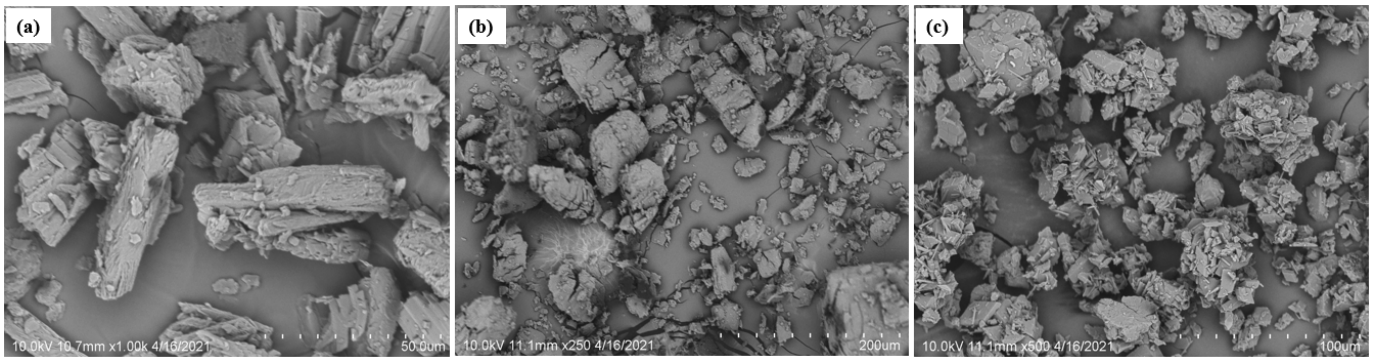

Figure S3: SEM micrographs of (a) FOZ, (b) βCD and (c) FOZ INC.

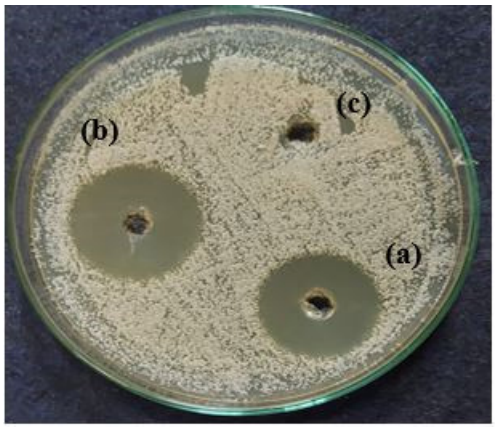

Figure S4: Zone of inhibition of (a) Plain FOZ suppository (32±2) and (b) FOZ IC suppository (40±1) and (c) Solvent control.

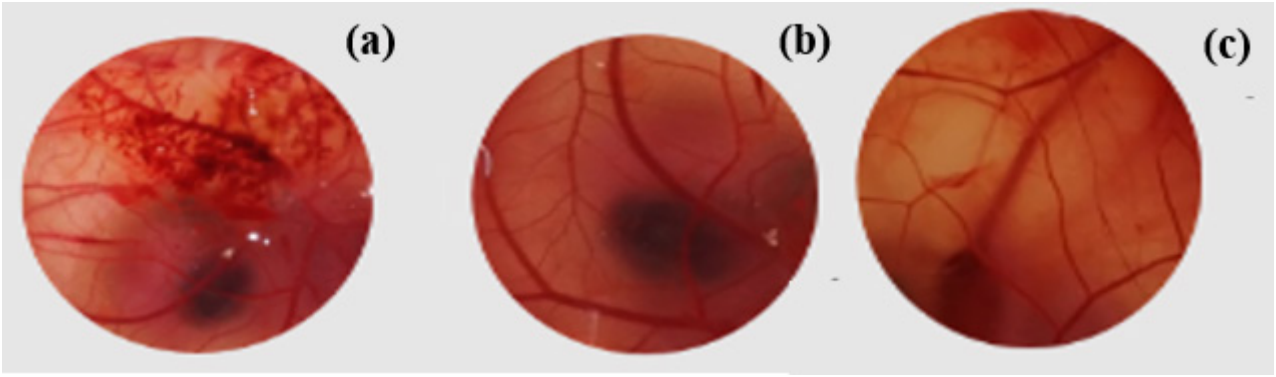

Figure S5: Images showing the vascular effects of samples applied on the chorioallantoic membrane over a period of 5 min. (a) 0.1 M NaOH (b) Normal saline (0.9% w/v) and (c) FOZ INC suppository dissolution media.

Table S2: Variables of the D-Optimal mixture design.

Independent variables	Low level	High level	
Base (A)	50	70	
Plasticizer (B)	30	50	
Dependent variables	Low limit	High limit	Goal
Melting time (sec)	65	191	Maximize
Hardness (kg)	1.15	2.12	Maximize
Drug release (%)	77.75	88.37	Maximize

Table S3: Irritation scores of the different samples.

Ingredients/ Formulation	Irritation Score (IS)	Irritation Severity (Mean)	Result
Normal saline (0.9% w/v)	0	0	Non-irritant.
0.1 M NaOH	17.83	3	Strong irritant.
FOZ INC suppository	0	0	Non-irritant.