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Poloxamer-188 co-polymer and HPMC-E15 Based Voriconazole Nanosuspension Loaded Topical In situ Gel using 3^2 Factorial Design

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Abstract
The present investigation aimed to develop novel voriconazole nanosuspension loaded topical *in situ* gel for the management of serious fungal infections by virtue of modified cold approach. The solvent evaporation approach was applied for generating the voriconazole nanosuspension and was assessed for particle-size, particle distribution index, zeta potential, entrapment efficiency, saturation solubility, FTIR, DSC. Then the loading of voriconazole nanosuspension to form *in situ* gel for topical release was effectively achieved employing 3^2 factorial design that optimized the process. Developed *in situ* gel was assessed for various parameters like appearance and pH, in vitro gelation, viscosity, sterility testing, skin permeation as well as retention analysis, in vitro antifungal activity, and stability. The poloxamer based gelling system was found to be clear and transparent with +++ gelation capacity with 240 cps viscosity (batch F8). Nanosuspension-loaded *in situ* gel encompassing poloxamer-188 and HPMC-E15 demonstrated enhanced antifungal activity against *Candida albicans* as compared to pure drug voriconazole. The present study concludes that voriconazole nanosuspension loaded *in situ* topical gel employing poloxamer-188 as well as HPMC-E15 act as a potential remedy for serious fungal infections.
Key words: Voriconazole, nanosuspension, topical *in situ* gel, poloxamer-188, HPMC-E15.

1. Introduction

The novel drug delivery system (NDDS), which blends more recent dosage forms and modern techniques, is more and more accepted as a means of overcoming the drawbacks of traditional administration methods and providing superior therapeutic outcomes.[1] It is frequently categorized as drug delivery system (DDS) which is targeted, controlled, and modified. Drug delivery has increased dramatically as a result of nanotechnology development.[2] Significant surface-to-mass ratios, absorption-related quantum-size effects, potential to transfer Active Pharmaceutical Ingredients, (API) and other unique features of nano-formulations let them to get over the issue of limited absorption of drugs and new chemical entities. Numerous investigations have employed nano-formulations to boost drug absorption, solubility, and duration of drug retention while lowering adverse outcomes.[3] Pharmaceutical nanosuspension is sub-micron colloidal dispersion of nanosized drug, stabilized by virtue of surfactants and polymers.[4] Small particle size confers significant surface area-to-volume ratio, leading to improved solubility as well as dissolution and are extensively employed in pharmaceutical and biotechnological applications for carrying API deficient in solubility, raising drug dissolution rates, along with ameliorating drug bioavailability. Since poor water solubility results in lower bioavailability and ineffective drug delivery, over 40% of newly developed drugs have poor drug absorption windows, which further reduces therapeutic action.[5] When a drug molecule has multiple restrictions, like being incapable of forming a salt or possessing a large molecular weight, dose, log P, or melting point, only option available is nanosuspension.[6] Nanosuspensions can address such particular drug delivery obstacles by maintaining API in a crystalline state while permitting higher drug loading during formulation development.[7]

Nanosuspension offers plenty of upper-hand advantages like high drug loading capacity, reduced risk of particle aggregation, scalability and manufacturing efficiency, controlled particle size, and homogeneous particle size distribution ensuring consistent performance in various applications, including drug delivery, where particle size distribution can affect drug release kinetics, stability, and bioavailability. The synthesis of multifunctional nanoparticles (NPs) with combined therapeutic, diagnostic, or imaging functionalities, offer versatility and customization in NPs design, allowing for the incorporation of various materials, drugs, and functional

components into nanosuspensions, thus exhibit enhanced drug solubility, dissolution rates, and bioavailability compared to conventional drug formulations.[8] In recent decades, the development of nanosuspension technology has surged due to these benefits. Boosting a drug's solubility, dissolution rate, and bioavailability through nanosuspension is a productive strategy for Biopharmaceutics Classification System (BCS) class II drugs.

Voriconazole, is (2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1,2,4-triazol-1-yl)butan-2-ol, a triazole derivative of fluconazole and is a water-insoluble drug that is inhibitor of cytochrome P450 2C9 (CYP2C9) as well as CYP3A4 along with superior broad-spectrum antifungal activity that is commercially accessible for oral as well as intravenous administration.[9] It performs a P450 inhibitory function. It serves to treat invasive fungal infections, which are typically encountered in immunocompromised people[10] as well as invasive candidiasis, invasive aspergillosis, as well as emerging fungal infections. Voriconazole's higher affinity for 14-alpha sterol demethylase renders it efficacious against certain organisms resistant to fluconazole. Voriconazole is primarily employed to relieve invasive pulmonary aspergillosis, esophageal candidiasis, and severe fungal infections brought on by *Fusarium* and *Scedosporium apiospermum* species. It demonstrates fungicidal efficacy in vitro, against every species of *Aspergillus*, moulds, particularly those linked to keratitis, *Scedosporium*, and *Fusarium*.[11]

When exposed to physiological conditions like temperature, pH, or ionic concentration, a topical in situ gel, a drug delivery system, transforms a liquid to a gel state. Topical *in situ* gel is composed of environmentally sensitive polymers that undergo structural alterations in response to minute variations in environmental parameters like pH, temperature, and ionic strength. Key characteristics of *in situ* gel include liquid-to-gel transition, regulated drug release, extended residence, and patient compliance. Topical *in situ* gel is used to deliver medications to the skin over extended period, minimizing need for frequent administration.

Voriconazole[12] is a class II drug in BCS that has a dissolution rate dependent absorption and strong permeability but poor aqueous solubility (25.82±2µg/ml). Owing to this, goal of present study was to ameliorate solubility through development of a nanosuspension of a poorly soluble model drug, voriconazole. Following this voriconazole nanosuspension-loaded topical *in situ* gel was then designed, developed, and characterized. Therefore, the current study uses a

combination strategy to boost solubility of the poorly soluble drug and form a topical *in situ* gel of voriconazole loaded nanosuspension.

2. Materials and methods

2.1 Materials

Voriconazole was purchased from Srini (India). HPMC-E15 and Poloxamer-188 were purchased from Loba Chemie Pvt Ltd. All experimental work was conducted using double-distilled water throughout the investigation. Analytical grade solvents and reagents were utilized in all other cases.

2.2 Preparation and optimization of voriconazole nanosuspension

Nanosuspension containing voriconazole was prepared as per 3^2 factorial design. Quantity of Poloxamer-188 (X_1) and HPMC-E15 (X_2) was independent variables used for optimization. Three levels (+1, 0 and -1) were selected and batches were prepared using different levels of variables. Coded values with its translation in actual unit and composition in actual formulations are shown in Table 1. Nine formulations are feasible based on the model[13]. Approach of solvent-evaporation was employed to produce voriconazole nanosuspension. Voriconazole was dissolved in ethanol (selected solvent) to form clear drug solution, which was then slowly injected at rate of 1ml per minute into 20 ml distilled water (anti-solvent) containing stabilizer combinations in various concentrations (-1, 0 and +1) to form coarse nanosuspension. Solid NPs immediately began to precipitate. Thereafter, formed nanosuspension was ultra-sonicated for 20 min in bath sonicator. Nanosuspension was placed later in magnetic stirrer at room temperature and stirred for hour to facilitate organic solvent evaporation.[14]

Table 1 Variable levels and actual composition of formulations according 3^2 factorial design

Batch Code	**Drug (% w/w)**	**Variable levels**		**Composition of different formulations**	
		X_1	X_2	Quantity of X_1 (mg)	Concentration X_2 (mg)
F1	1	-1	-1	25	120
F2	1	0	-1	50	120

F3	1	+1	-1	75	120
F4	1	-1	0	25	180
F5	1	0	0	50	180
F6	1	+1	0	75	180
F7	1	-1	+1	25	240
F8	1	0	+1	50	240
F9	1	+1	+1	75	240

2.2.1 Experimental design

To verify impact of formulation parameters on dependent variables *in situ* gels, preliminary selection experiments were conducted. Two most crucial formulation factors were concentration of Poloxamer-188 and HPMC-E15. 3^2 factorial design with three levels (-1, 0, and +1) was utilised to estimate as well as optimise selected formulation parameters via design of experiment. No changes were made to batch size or drug concentration.[15]

2.2.2 Optimization of formulations

Nanosuspensions of voriconazole were optimised by adjusting particle-size, particle distribution index, zeta potential along with entrapment efficiency. Nanoparticle Analyzer SZ-100 equipped with a Zetasizer measured particle size and zeta potential (Horiba Scientific, Japan).

3. Characterization of nanoparticles

3.1.1 Particle size, polydispersity index (PDI) and zeta potential

Using Zetasizer, particle size and zeta potential of NPs were ascertained. (Nanoparticle Analyzer SZ-100 & Zetasizer Horiba Scientific, Japan). 5 mg NPs were dispersed in 5 ml deionized water and subjected to Zetasizer analysis. Zeta cells were employed for zeta potential. All experiments were performed in triplicate.[16]

3.1.2 Entrapment efficiency (EE)

15 mg NPs were added to 30 ml phosphate buffer solution PBS (pH 7.4). Following that, mixture was centrifuged for 45 minutes at 5000 rpm. Supernatant was collected and analyzed with a UV

visible spectrophotometer[17] (Shimadzu UV spectrophotometer 1800, Japan) at a wavelength of 256 nm. EE was computed using following equation.

$$\% EE = \frac{Weight\ of\ drug\ in\ nanoparticles}{Weight\ of\ nanoparticles} \times 100$$

3.1.3 Drug content (DC)

NPs were dissolved in 1% v/v acetic acid solution followed by analysis through UV visible spectrophotometer (Shimadzu UV spectrophotometer 1800, Japan) at wavelength 256 nm. DC was computed using equation:

$$\% DC = \frac{Amount\ of\ drug\ in\ nanoparticles}{Amount\ of\ drug\ used\ in\ formulation} \times 100$$

3.1.4 Determination of saturated solubility

Solubility profile of voriconazole in its pure form as well as voriconazole nanosuspension was investigated in different media of DW and PBS 7.4. Voriconazole was added in excess to 20 ml of each medium, and mixture was shaken in water bath for 48 hours at 37℃. The samples were analysed following filtration spectrophotometrically (Shimadzu UV spectrophotometer 1800) at 256 nm. Concentration was then, assessed from corresponding calibration curve.[18-20]

3.1.5 Drug excipients compatibility study (FTIR)

Samples were analyzed along with KBr pellet method using IR spectrophotometer (Alpha T Bruker) in the standard region from 4000 to 400 cm-1 in wave number. FTIR spectra of pure voriconazole, poloxamer-188, HPMC-E15, along with voriconazole nanosuspension were recorded. Scans were interpreted for retention of principle peaks, shifting as well as appearance of new peaks. Figure 2 presents FTIR spectra of different excipients with API as well as final formulation.

3.1.6 Drug excipients compatibility study Differential scanning calorimetry (DSC)

DSC has been performed to study interaction of API with other additives particularly when incorporated into NPs. Samples of pure voriconazole, poloxamer-188, HPMC-E15, and voriconazole nanosuspension were heated from 30 to 300°C temperature by keeping constant rate (10°/min) in aluminium pan using differential scanning calorimeter (Mettler Toledo, Staresw 920). Thermal characteristics of pure voriconazole, HPMC-E15, poloxamer-188, along with voriconazole nanosuspension were assessed. Figure 3 presents DSC thermograms.[21]

3.1.7. Morphological examination

Morphological examination of NPs formulations was performed using Transmission Electron Microscope (TEM). Phosphotungstic acid (2% (w/v) was used to stain the NP samples. NPs suspension (5–10 μL) was positioned on copper grids with films for TEM (Hitachi H7500, Tokyo, Japan) observation. The image was captured employing imaging viewer program and a digital micrograph.

3.2 Preparation of *in situ* gel

A modified cold approach was employed to formulate the topical *in situ* gel based on poloxamer-188 and HPMC-E15. In short, poloxamer-188, thermosensitive polymers that gel at body temperature, and HPMC-E15, a thickening agent were gradually added to appropriate amount of cold acetate buffer (pH 6.5) holding voriconazole (0.5% w/v) with constant stirring until a clear solution was obtained. Poloxamer-188 solution that had partially dissolved was kept in refrigerator and occasionally stirred. Upon application to skin, temperature rise induces gelation.[22-23]

3.3 Characterization of *in situ* gel[24]

3.3.1 Appearance and pH

In situ gel was visually checked under fluorescent light with black as well as white background in cabinet holding sufficient light for clarity testing to assess for turbidity or presence of any transparent or coloured particle matter. Following standard protocol, pH was measured employing calibrated pH meter (Dolphin Autodelux) set at 25±0.5°C.[25]

3.3.2 In vitro gelation

Gelation of gel was ascertained employing polypropylene vial with simulated skin or mucosal fluid (SSF) as gelation solution with composition of sodium chloride (NaCl) 0.6 g, potassium chloride (KCl) 0.2 g, calcium chloride ($CaCl_2$) 0.1 g, urea 0.2 g, lactic acid to adjust pH to 5.5 (approximately 0.5 ml of 90% lactic acid solution), glycerol 0.1 g to simulate skin hydration, distilled water to make up to 1 Liter, equilibrated at 34±0.5°C employing water bath. Aliquot 100 µl were precisely transferred into separate vial followed by gradual addition of SSF 2 ml

employing a micropipette. Gelling potential was ascertained by visual inspection for gel formation and time needed for gelation and time required to dissolve gel formed. Based on 3 categories *in vitro* gelling potential time period was computed.[26]

1. (+) Gel forms within few min, disperses speedily.
2. (++) Immediately gelation occurs, persists for few hours.
3. (+++) immediately gelation occurs, lasts for extended time period.

3.3.3 Viscosity

The viscosity of *in situ* gels was assessed at different angular velocity (0.5 to 100 rpm) at 34 ± 1˚C employing Brookfield viscometer (Brookfield Ametek DVE). A typical run involved allowing spindle to rotate and reach a steady state. The viscosity value displayed on the viscometer was recorded.

3.3.4 In vitro release

Franz diffusion device (Dolphin 1475) was employed to carry dissolution test with PBS 7.4 to simulate simulated skin or mucosal fluid to investigate *in vitro* release of voriconazole from developed gels. Silicon membrane was set between donor and receptor compartments, ensuring it was properly sealed and no air bubbles were trapped and was secured with clamps. Receptor medium was filled inside the receptor compartment. Temperature was set and maintained, at 37±0.5°C with 50 rpm. *In situ* gel was applied to the membrane surface in donor compartment was spread evenly to cover the membrane. Donor compartment was sealed to impede evaporation. Receiver solution was blended at 50 rpm employing magnetic stirrer. At predetermined time, 1 ml receptor medium was withdrawn at 30 min, 1, 2, 4, 8, 12, 16, 20, 24 hours using a syringe and substituted with the equivalent amount of SSF held at the same temperature and was analysed spectrophotometrically (Shimadzu UV spectrophotometer 1800) to quantify amount of drug diffused through membrane. Cumulative amount of drug permeated was computed and was plotted against time to obtain diffusion profile. [27-29]

3.3.5 Sterility testing

Fluid thioglycolate medium (FTM) and soy-bean digest medium (SBDM) were prepared and sterilized.[30] All work was performed under aseptic conditions in a Laminar flow hood (Dolphin)

to prevent contamination. 10 ml *in situ* gel to be tested was transferred with sterile syringe directly into a sterile container of FTM and 10 ml was transferred to SBDM with ensuring well mixing of sample with both medium. FTM container was incubated at 30-35°C for 14 days to identify anaerobic microbial growth. SCDM container was incubated at 20-25°C for 14 days to identify aerobic microbial growth. Media containers were observed daily for signs of turbidity or microbial growth e.g., cloudiness, colour change.

3.3.6. Skin permeation and retention

3.3.6.1 Skin preparation

Studies were performed as per standard procedure and approved by the CPCSEA (Protocol approval no. 2114/PO/Rc/S/20/CPCSEA.26/11/2020). The healthy male Sprague Dawley rats excised skin was used to determine skin permeation. Rats were 3-4 months old and weighed between 200–250 g. Rats were sacrificed using the cervical dislocation technique. Rats ventral regions were shaved, with sharp blade, and their skin was surgically removed. Utilizing a surgical blade, the lipid layer that was affixed to the removed skin was separated and thereafter cleaned with a normal saline solution (0.9%). Following washing, the skin was wrapped in aluminum foil and then put in a freezer at -20°C to be used later.[31]

3.3.6.2 Skin permeation analysis

To ascertain skin permeation profile, PBS 7.4 was added to recipient chamber of Franz diffusion cell (Dolphin 1475). This guarantees the preservation of sink conditions for the duration of study, permitting absorption rate to proceed as it would ordinarily under *in vivo* circumstances with functioning circulatory system. Isotonic saline or buffered isotonic saline (pH 7.4) is thought to be a rational choice for maintaining a physiological environment. *In situ* gel formulation 1 g was applied on skin. Temperature of cells was held at 37±1°C. At predetermined intervals, 2 ml sample was taken out of recipient chamber and assessed employing a UV visible spectrophotometer. (Shimadzu UV spectrophotometer 1800). [32]

3.3.6.2 Skin retention analysis

Following skin penetration study, skin from Franz diffusion cell (Dolphin 1475) was taken off and gently washed with PBS 7.4, sliced into pieces and put in beaker with 20 ml fresh PBS 7.4.

After that, this was agitated for three hours using a magnetic stirrer. Then 5 ml methanol was added to extract drug and it was stirred again for 1 hour. After that, 3 ml stirred solution was collected, filtered, as well as analyzed on UV-visible spectrophotometer (Shimadzu UV spectrophotometer 1800). [32]

3.3.7 *In vitro* antifungal activity

In situ gel formulations antifungal activity was assessed using the cup-plate approach. The ingredients of Sabouraud dextrose agar broth (SDB) are 4 g dextrose, 1.5 g agar, and 1 g mycological peptone in 100 ml distilled water. Once dextrose and peptone were dissolved, agar was added to the solution. Solution was boiled until the agar was completely dissolved and pH was kept at 5.5. Prepared media was autoclaved for 15 min at 121°C. Three aseptic 93 mm glass petri plates were used, and medium was aseptically added to dishes aseptically and kept for solidification. A sterile cork borer was used to pierce the agar plate's surface once the agar medium had set. Agar medium surface was streaked to introduce *Candida albicans*. Each of three petri dishes had 8 mm-sized holes drilled into it, each one held 75 µL *in situ* gel and left for at least 30 min and were incubated for 24 hours at 25±1°C. Employing a Dolphin vernier caliper, the most consistent external diameter of inhibitory zone was noted after 24 hours and represented by radial dimensions. Zones diameter including diameter of well, was noted. Each assay was carried out in triplicate.

3.3.8 Surface response study and statistical optimization

Effect of various factors on response variables were evaluated statistically by one way ANOVA at P< 0.05 level employing Design-Expert® (Stat-Ease Inc.). To find out response surface curvature, evaluation of design was executed for suitable model with equation,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2 \quad \text{..... Eq (1)}$$

Where Y is response variable,

b_0 constant,

b_1, b_2, ... b_5 regression coefficient

X_1 and X_2 stand for main effect

X_1X_2 are interaction terms

3.3.9 Stability study

Stability study for factorial batch optimized through viscosity and permeation as evaluation parameters was carried out with stability chamber (Remi SC-19 Plus) by storing *in situ* gel in screw-capped glass bottles at accelerated and controlled temperatures 40°C and relative humidities (75%) for period of 6 months.[33-35] *In situ* gel was evaluated for multiple parameters like pH, *in vitro gelation*, viscosity, dissolution etc. at the end of every month.

4. Result and discussion

4.1. Characterization of nanoparticles

4.1.1. Particle size, PDI and zeta potential

Particle size of freshly prepared poloxamer-188 co-polymer and HPMC-E15 based voriconazole nanosuspension is given in Table 2 and found to be 102 nm to 300 nm. F1 exhibited larger size of NPs (300±10.17 nm), as insufficient stabilizer concentration can lead to larger particles or particle agglomeration due to inadequate stabilization. High stabilizers ratio in (F9, 102±14.30 nm) result in nanosuspensions with smaller particle sizes owing to decrease in surface tension and impedes particle agglomeration resulting in a nanosuspension with smaller particles.[17, 36] F8 exhibited optimal concertation of stabilizer, thus exhibited particle size (230±8.11 nm), which is appropriate dimensions for TDDS. PDI is unique marker of particle size distribution. When it comes to submicron particles, a score between 0.15 and 0.3 denotes size homogeneity; a number higher than 0.3 denotes heterogeneity.[37] PDI was from 0.198±0.02 to 0.337±0.05 suggesting narrow size distribution revealing higher stability of voriconazole NPs. Zeta potential is a crucial characterisation approach for assessing NP system's surface charge and potential stability. Zeta potential of NPs containing voriconazole of batch F8 is given in Figure 1. Since electrostatic repulsion impedes particles with same charge from aggregating, very large negative or positive zeta potential values are typically indicative of colloidal dispersion stability.[16,39-40] All formulations exhibited negative zeta potential and varied from −21.51±0.43 mV to −28.96±0.31 mV as per Table 2, which is closer to −30 mV, ensuring physical stability.

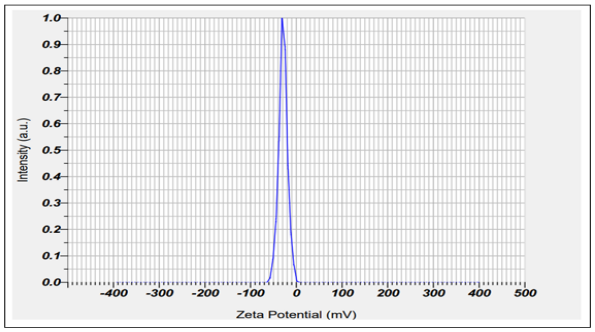

Figure 1 Zeta potential of NPs containing voriconazole of (batch F8).

Table 2 Physiochemical characterization of voriconazole NPs

Batch code	Average size (nm)	PDI	Zeta potential (mV)	% EE	% DC
F1	300±10.17 nm	0.198 ± 0.02	−22.81 ± 0.52	60.19 ± 3.04	61.21± 0.40
F2	150 ± 9.23 nm	0.292 ± 0.03	−21.51 ± 0.43	70.36 ± 0.08	70.11± 0.50
F3	155 ± 7.34 nm	0.361 ± 0.07	−25.72 ± 0.46	76.07 ± 0.03	78.95 ± 0.30
F4	160 ± 9.11 nm	0.381 ± 0.03	−22.73 ± 0.81	72.09 ± 0.02	76.95 ± 0.20
F5	172 ± 9.34 nm	0.231 ± 0.04	−27.86 ± 0.68	74.38 ± 0.04	79.94 ± 0.40
F6	189 ± 7.34 nm	0.347 ± 0.03	−25.46 ± 0.40	76.05 ± 0.02	78.05± 0.60
F7	202 ± 9.12 nm	0.288 ± 0.03	−27.83 ± 0.55	78.04 ± 0.03	86.05± 0.50
F8	230 ± 8.11 nm	0.337 ± 0.05	−28.96 ± 0.31	85.75±1.14	85.42± 0.63
F9	102 ± 14.30 nm	0.319 ± 0.03	−22.46 ± 0.60	59.18 ± 0.08	87.42± 0.53

4.1.2 EE

% EE of voriconazole NPs prepared by solvent evaporation, was satisfactory high and was from 59.18±0.08 % to 85.75±1.14 % Table 2. According to the data, drug EE rose considerably when the polymer concentration raised owing to its higher viscosity. However, a subsequent rise in polymer concentration (F10 having a higher polymer concentration than the others) revealed reduction in EE, which is essentially the result of a reduction in drug loading because EE is ratio of actual drug loading to theoretical drug loading.[41]

4.1.3 DC

All formulations were analyzed for their DC spectrophotometrically, shown in Table 2, and exhibited highest drug content. DC for voriconazole-loaded NPs fluctuated from 61.21±0.40% to 87.42±0.53%. As particle size increases, drug content was also increased.

4.1.4. Saturated solubility

Saturated solubility of pure voriconazole was studied in distilled water and PBS 7.4. As the drug holds weak basicity (pKa = 1.76) solubility was lower in basic media, 11.7±0.5 µg ml^{-1}. Drug has poor water solubility and was found to be 25.82±2 µg ml^{-1}.[42] Solubility of poloxamer-188 and HPMC-E15 based nanosuspension was increased tenfold in DW; and as well as in PBS 7.4 it was boosted by twelvefold. Reducing drug particles to the nanometer might be perfect reason for the solubility boost.[43] Its solubility was boosted by the high energy of interfacial tension owing to the amorphous structure of NPs, as opposed to the crystalline condition of pure voriconazole.

4.1.5. FTIR

To assess interaction between pure drug, poloxamer-188, and HPMC-E15 FTIR investigation was performed. Figure 2 displays FTIR of pure voriconazole, poloxamer-188, HPMC-E15 and poloxamer-188 and HPMC-E15 based voriconazole nanosuspension. FTIR spectrum of pure drug voriconazole displayed C-N aryl stretching at 1510.28cm^{-1}, OH stretching at 3460.38 cm^{-1}, C-F stretching at 1477–1425.4 cm^{-1}, and C-H alkane at 2883.32 cm^{-1}. FTIR spectrum of poloxamer-188 was characterized by principal absorption peaks at C-O stretching appeared at 1112 cm^{-1}, 2896.40 cm^{-1} owing to aliphatic C-H stretching, as well as O-H bending appeared at 1353.01 cm^{-1}. HPMC-E15, exhibited characteristic peaks at 1140.31 cm^{-1} due to C-O-C stretching vibration peak, 3400 cm^{-1} owing to OH stretching, 1749 cm^{-1} due to C=O stretching, as well as 1375-1450 cm^{-1} owing to C-H bending.[44] All these fundamental characteristic peaks were retained and did not shift in nanosuspension and no significant changes occurred indicative of no major interaction between voriconazole and stabilizers, poloxamer-188, HPMC-E15. Thus, it was concluded from FTIR investigation that pure drug and excipients poloxamer-188, HPMC-E15 were compatible with each other.

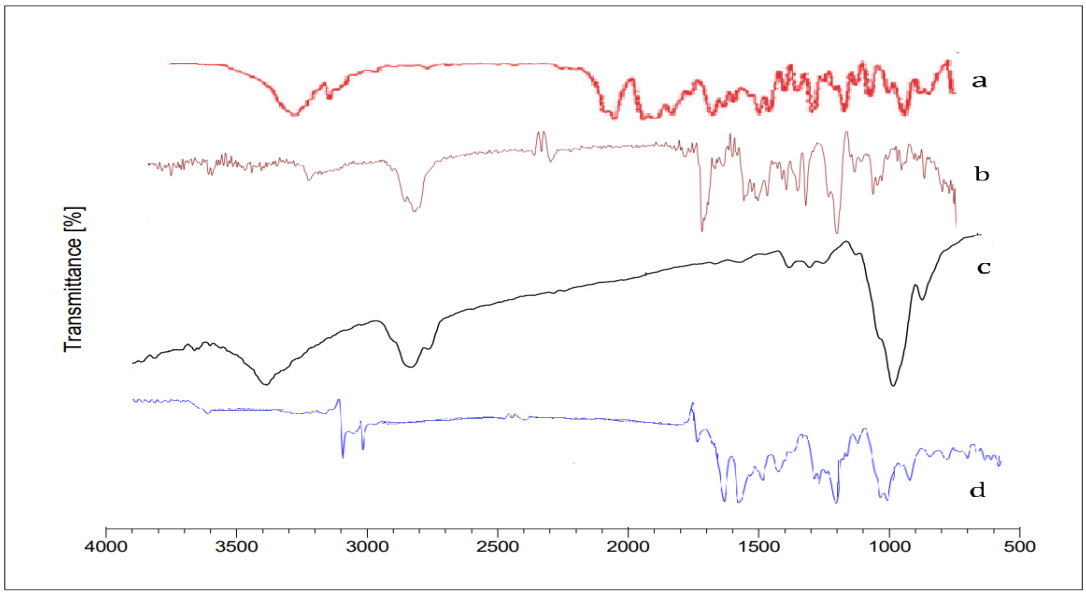

Figure 2 FTIR spectra of **a)** pure drug voriconazole **b)** Poloxamer-188 **c)** HPMC-E15 **d)** Poloxamer-188 and HPMC-E15 based voriconazole nanosuspension

4.1.6 DSC

DSC analysis was performed for pure voriconazole, poloxamer-188, HPMC-E15, poloxamer-188 and HPMC-E15 based voriconazole nanosuspension to verify compatibility with drug. DSC thermograms are displayed in Figure 3. Thermogram of voriconazole is characterized by sharp melting endotherm at 132.04°C. DSC thermogram of poloxamer-188 exhibited a broad endotherm at 57.64°C. DSC thermogram of HPMC-E15 displayed broad endothermic peak at 100.45°C, which might be attributed to loss of water molecules. DSC studies indicated all selected excipients were suitable for the preparation of nanosuspension. Poloxamer-188 and HPMC-E15 based voriconazole nanosuspension exhibited slight shifting of endotherm but there were no new peaks, this result revealed that there was no unexpected interaction between drug, poloxamer-188 as well as HPMC-E15.

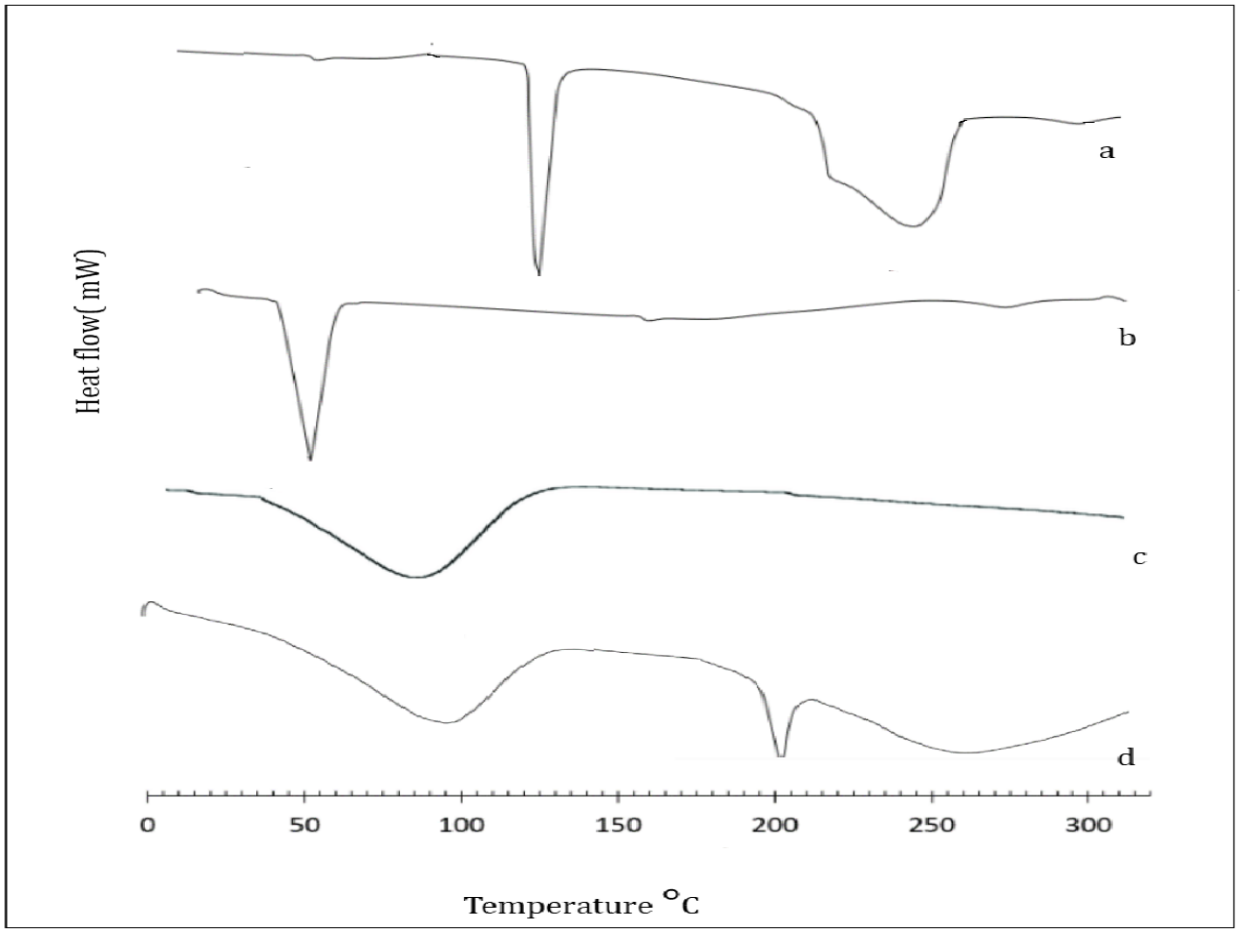

Figure 3 DSC thermograms of **a)** Pure drug voriconazole **b)** Poloxamer-188 **c)** HPMC-E15 **d)** Poloxamer-188 and HPMC-E15 based voriconazole nanosuspension

4.1.7. Electron microscope examination

TEM was employed to assess morphology of voriconazole nanosuspension. Figure 4 depicts TEM images of the voriconazole nanosuspension developed via the solvent evaporation. This demonstrated that all formulations had a smooth surface and spherical shape, suggesting possible stabilization of NPs. NPs possibly do not irritate skin since particles are small enough typically in nanometre range, they reduce mechanical irritation of skin because smaller particles are less likely to abrade skin or clog pores than larger particles as well as it has proved that particles with blunt angles along with edges cause less irritation than those with sharp angles and edges.

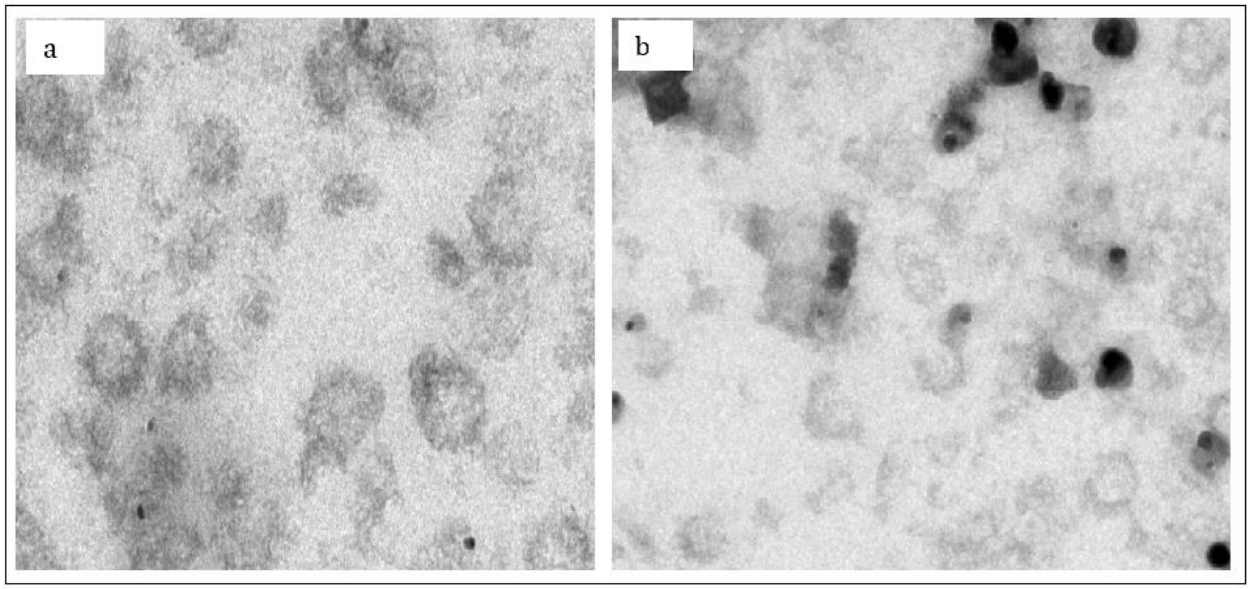

Figure 4 TEM images of **a)** *in situ* gel factorial batch (F8) 200 nm **b)** *in situ* gel factorial batch (F8) at higher magnification 0.5 μm

4.2 Characterization of *in situ* gels

4.2.1 Appearance and pH

Developed *in situ* gel formulation had pH value in range of 6.0 to 6.5 (Table 3), which is deemed appropriate to minimize irritation and ensure maximum drug stability and absorption while avoiding the danger of skin irritation upon application to skin as it resembles skin's natural pH. Developed in situ gel formulation was in clear in nature.

4.2.2 *In vitro* gelation

Viscosity as well as gelling capacity are two primary elements of a gelling system. *In situ* gel formulations gelled spontaneously on addition of SSF in a 1:1 ratio and extended for few hours as well as retained its integrity for extended period without eroding or dissolving enabling sustained release of drugs topically. Gelation time of *in situ* gel formulation was 58 seconds, as well as *in vitro* gelling capacity was +++, (Table 3) which means gelation occurs immediately and remains for an extended period.

4.2.3 Viscosity

Viscosity correlated precisely with the polymeric content and was found directly proportional to

polymeric content. Preliminary study data revealed that the viscosity raised as the concentration of HPMC-E15 raised. HPMC-E15 was crucial factor contributing for viscosity whereas poloxamer-188 was not directly related to viscosity context. Viscosity of *in situ* gel (Batch F8) was found to be 240 cps at 100 rpm. Evaluation parameters for factorial batches of *in situ* gel are mentioned in Table 3.

Table 3 Evaluation parameters for factorial batches of *in situ* gel

Batch code	pH	Viscosity (cps)	*In vitro* gelation (capacity)
F1	6.5	45	+
F2	6.4	75	+
F3	6.2	95	+
F4	6.1	90	++
F5	6.0	85	++
F6	6.3	100	++
F7	6.5	125	+++
F8	6.5	240	+++
F9	6.3	390	+++

+ gel forms within few minutes, disperses rapidly; ++ immediate gelation, remains for few hours; +++ immediate gelation, remains for extended period of time.

4.2.4 In vitro release

In vitro release profile was performed in PBS 7.4 for entire duration as *in situ* gel formulation supposed to adhere at skin mucosa and release as well as permeate through various layers of skin. Pure drug voriconazole exhibited only 35.14±1.56 drug release whereas drug release at 24 hours from *in situ* gel containing voriconazole was in range of 70.98±2.08 to 99.79±0.53%. Poloxamer-188 is thermosensitive polymers that undergo a sol-to-gel transition upon increase in temperature when applied to body (at body temperature), Poloxamer-188 transition from liquid to a gel, helps in maintaining the formulation at the application site and controlling the drug release. HPMC is a hydrophilic polymer that swell upon contact with water or body fluids and can create a gel-like matrix, which controls drug release by forming a barrier that drug must diffuse through. Additionally, HPMC-E15 may undergo gradual erosion, further contributing to drug release mechanism. Thus, drug was released in controlled and sustained manner, enhancing therapeutic efficacy and duration of action of *in situ* gel formulation. Thus, as concentration of one of the independent formulation variables, HPMC-E15 increases it causes decrease in

diffusion of drug molecules. At lower concentration of formulation variables corresponding % diffusion was more. Factorial batch F9 which contains higher level of both independent variables, showed 75.98±2.07 % drug release at 24 hours indicates how both variable act as a diffusion retarding agent, while formulation F1 which contains lower level of both independent variables, showed nearly complete diffusion of drug at 14 hours. Factorial batch F8 which contains optimal level of HPMC-E15 as well as higher Poloxomer-188 exhibit nearly complete diffusion of drug at 24 hours. Dissolution profiles of all batches are given in Figure 5.

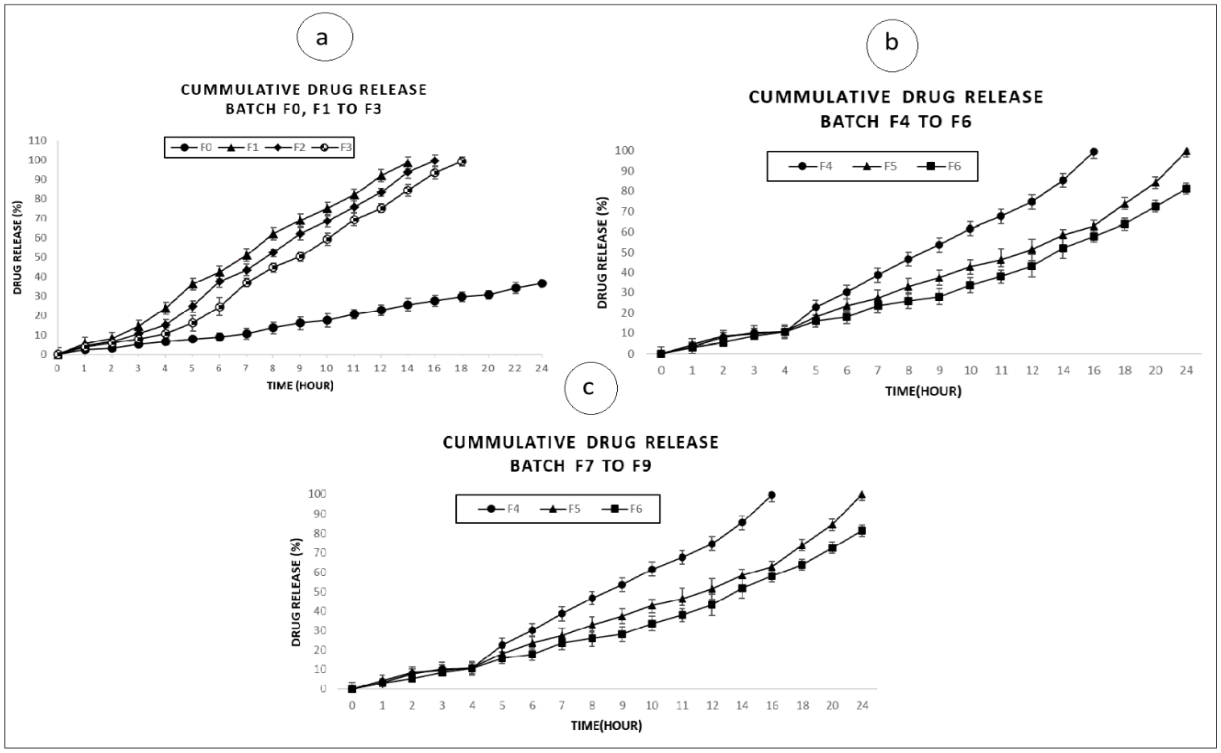

Figure 5 Dissolution profiles of factorial batches of *in situ* gel a) Pure drug voriconazole b) Batches F1 to F3 c) Batches F4 to F6 d) Batches F7 to F9

4.2.5 Sterility testing

Sterility testing for topical *in situ* gel ensures that the product is free from viable contaminating microorganisms, which is crucial for patient safety. No microbial growth was observed in media after incubation period; thus, sample was sterile. This meant that topical *in situ* gel complies the sterility criteria and was safe for use.

4.2.6 Skin permeation analysis

Interpreting skin permeation analysis data aids in assessing the efficiency and behavior of *in situ* gel in delivering drug through the skin. Poloxamer-188 gels at body temperature, enabling a steady and regulated release of drug. This unique feature is especially advantageous for topical formulations, as it ensures that the gel remains in place on skin. HPMC-E15 aids in forming a gel matrix that can encapsulate drug, tailors viscosity of *in situ* gel, as well as strengthens bioadhesive features of gel, ensuring it stays in place on skin for an extended period, and can form a thin film on skin, which can act as a secondary barrier, averting water loss and potentially boosting drug retention and absorption in skin. Higher concentrations of HPMC increase the viscosity of formulation. This creates a more viscous gel, which can slow down the diffusion of the drug molecules as well as HPMC can form a film on the skin surface, acting as a barrier that can reduce the rate of drug permeation. Thus, Batch F1 with lower concentrations of both polymers exhibited highest, 80.50% permeation while batch F9 with higher concentrations exhibited lowest 62.30% permeation whereas batch F8 with optimal concentration of both polymers exhibited 68.50% permeation. Compared to permeation of pure drug voriconazole which was 10.65%, *in situ* gel formulation exhibited higher in vitro permeation. In vitro permeation profile was calculated by plotting a graph showing quantity of drug permeated vs. time. In vitro average % permeation and drug retention of *in situ* gel formulations are given in Table 6.

Table 6 In vitro average % permeation and drug retention of *in situ* gel formulations

Sr. No	Batch code	% Permeation	Drug retention
1	F1	80.50	9.90±1.22
2	F2	78.20	13.80±1.74
3	F3	76.50	15.90±1.63
4	F4	74.40	9.80±1.44
5	F5	72.30	14.50±1.93
6	F6	70.10	15.70±1.53
7	F7	69.40	10.90±1.82
8	F8	68.50	17.63±1.29
9	F9	62.30	16.80±1.73

4.2.7 Skin retention analysis

Figure 7 depicts drug retention of poloxamer-188 and HPMC-E15 based voriconazole *in situ* gel as well as pure drug voriconazole. Skin drug retention of pure voriconazole was 9.90±0.99%

whereas voriconazole *in situ* gel exhibited 17.63±1.29% drug retention (Batch F8). Therefore, maximal drug retention can be accomplished when drug is formulated as *in situ* gel formulation.

4.2.8 *In vitro* antifungal activity

This microbiological investigation employed cup and plate method. The findings demonstrated that when compared to simple voriconazole (1% w/w), growth of *C. albicans* was substantially inhibited by voriconazole *in situ* gel encompassing poloxamer-188 and HPMC-E15, and its antifungal effect was boosted. Mean diameter of zones of inhibition against *C. albicans* was 8.36±1.49 mm for voriconazole (1% w/w), and 18.59±1.72 mm for voriconazole *in situ* gel (Batch F8). Voriconazole *in situ* gel exhibited grater zone of inhibition and this was owing to a synergistic effect of poloxamer-188 and HPMC-E15.

4.2.9 Statistical analysis

a) Effect of factors on viscosity Y_1

The surface linear model for response Y_1 was found significant with F value of 16.65 ($p<0.005$). In this case, all factors $X_1, X_2, X_1X_2, {X_1}^2, {X_2}^2$ found significant and factorial equation for response Y_1 can be written as,

$$Y_1 = 160.1111 + 91.0000X_1 + 69.83333X_2 \quad \text{…… Eq. (2)}$$

As amount of X_1 and X_2 increases corresponding viscosity also increases. Quantity of Poloxamer-188 (factor X_1) and HPMC-E15 (factor X_2) hold positive influence on viscosity Y_1, revealed by positive sign in equation means that increasing the concentration of X_1 as well as X_2 the Y_1 which is viscosity also increases. The surface linear model exhibits a significant fit, as evidenced by the reasonable agreement with the adjusted R^2. This would indicate a strong connection between the obtained and predicted values. Significance of the suggested model can be justified employing counter plot and 3D surface response plot as demonstrated in Figure 6.

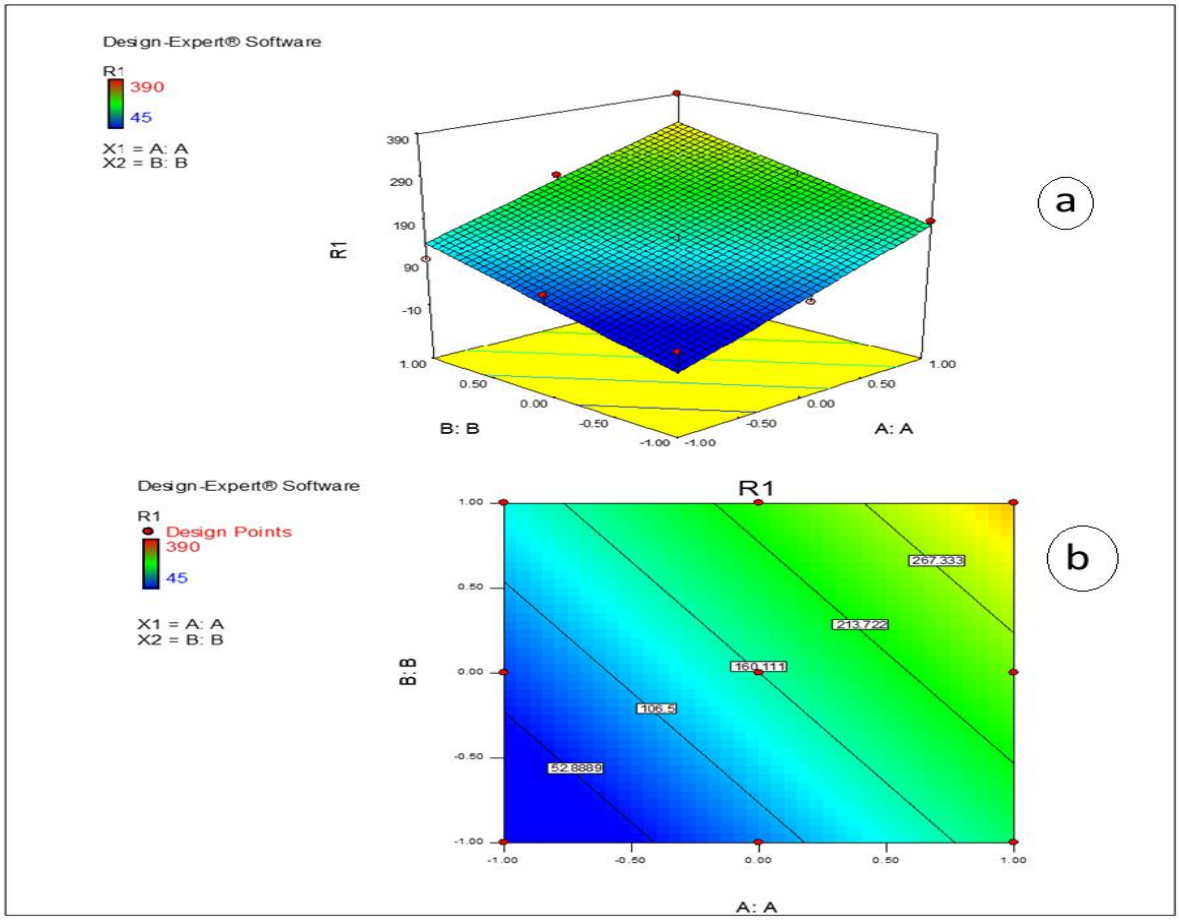

Figure 6 various surface response graphs for effect of factors on viscosity Y_1 including A) Counter plot B) 3D surface response plot

b) Effect of formulation variables on permeation *(Y_2)*

Linear model for response Y_2 was found to be significant with F value of 93.17 ($p<0.0001$). In this case, all factors X_1 and X_2were found to be significant and factorial equation for response Y_2 permeation can be written as:

$$Y_2 = 72.46667 - 2.56667X_1 - 5.83333X_2 \quad \text{..... Eq. (3)}$$

As amount of X_1 and X_2 increases Y_2 decreases. Equation's negative sign shows that Y_2 value is low for all high levels of X_1 and X_2. This behavior is brought on by independent variable, X_2, which promotes viscosity of formulation which slows down the drug's diffusion rate and forming network of gel-like consistency as a result of swelling. HPMC is often used as a

controlled-release agent. At higher concentrations, it can lead to a sustained or slower drug release, reducing the immediate amount of drug available for permeation. Another independent variable, X_1, Poloxamer-188, as one of the components of temperature-sensitive in situ gel, contributes very less for dependant variable Y_2. Figure 7 shows response surface counter plot and 3D plot.

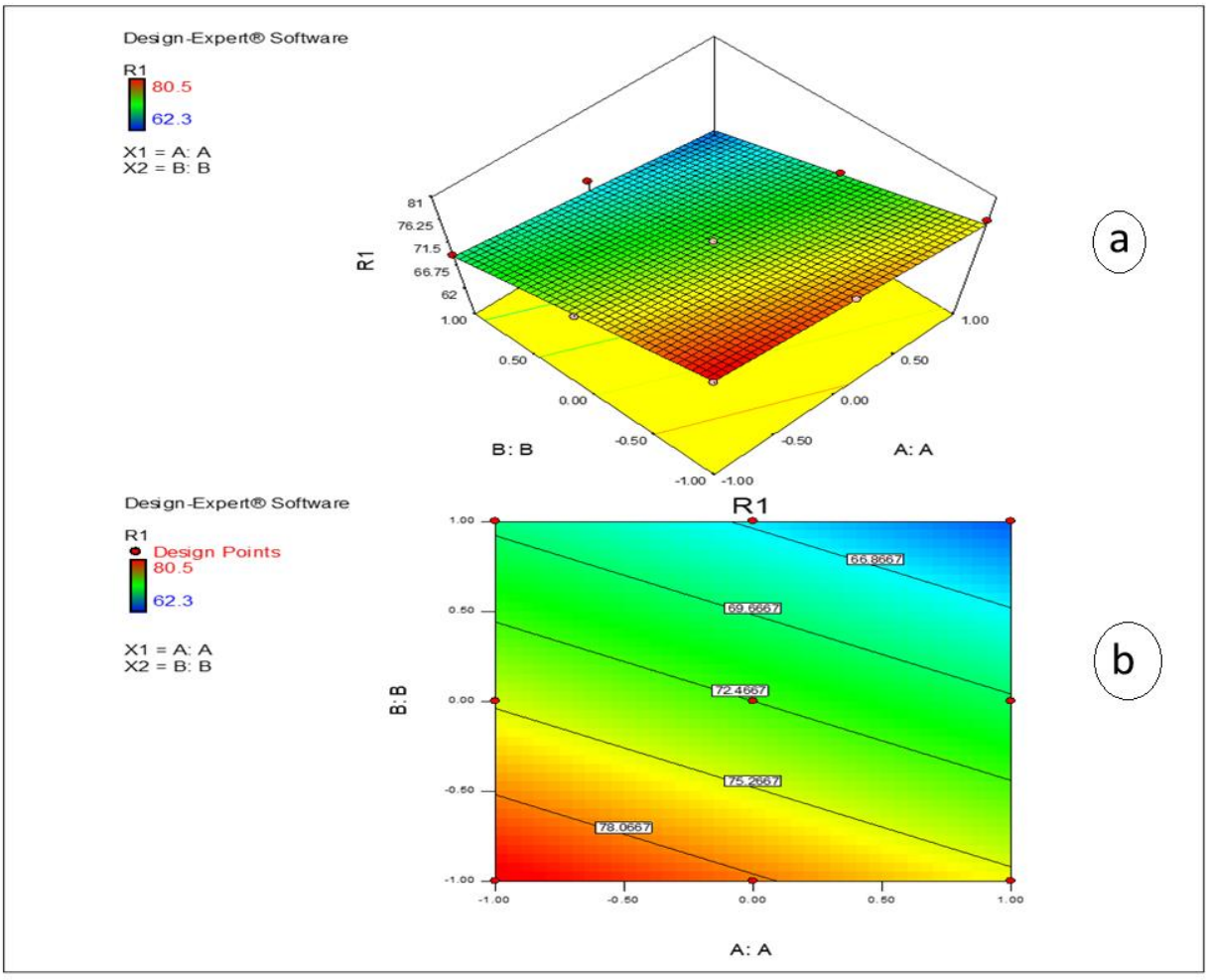

Figure 7 various surface response graphs for effect of factors on permeation Y_2 including A) Counter plot B) 3D surface response plot

4.2.10 Stability study

Stability assessment was conducted to assess various crucial parameters. The developed *in situ* gel was stable under accelerated and controlled conditions as demonstrated by stability data. Important assessment characteristics, such as pH, EE, in vitro gelation, and viscosity were also

not significantly altered, indicating that *in situ* gel proved stable at accelerated temperature settings. Results of different parameters studied after specific interval of time are mentioned in Table 7.

Table 7 Results of stability study

Parameter/ Time interval	**pH**	***EE (%)**	**In vitro gelation (time in seconds)**	**Viscosity (cps at 100 rpm)**
Initial	6.5	85.75±1.14	58	240
After 1 Month	6.2	85.55±1.31	57	240
After 2 Month	6.1	85.75±1.62	59	241
After 3 Month	6.2	85.85±1.59	58	239
After 6 Month	6.1	85.95±1.44	57	240

*Average of determinants (n=3)

Conclusion

Nanoparticles as pretreatment followed by formulation of *in situ* gel of voriconazole was successfully executed in the present investigation. Poloxamer-188 and HPMC-E15 as a stabilizer in nanoparticles was successfully assessed to address solubility concern of voriconazole and which is vital for drug release and ultimately a requirement for drug success. Poloxamer-188 and HPMC-E15 were also employed as crucial components in *in situ* gel. 3^2 factorial design was employed to formulate an optimal batch and optimized formulation of *in situ* gel exhibited release profile which fulfills requirement of ideal topical formulation. Owing to surfactant features of poloxamer-188 it aids in the solubilization of hydrophobic drug inside gel matrix. It additionally has the potential to avoid drug precipitation while boosting stability and uniformity of drug distribution throughout gel. High-viscosity polymer HPMC-E15 thickens formulation forming a stronger gel matrix. Boosted viscosity of gel aids in preserving its structural integrity by preventing it from flowing away from the application site. In this present investigation combined approach as pretreatment for solubility enhancement by virtue of formulation of nanoparticles of voriconazole followed by its formulation as *in situ* gel to achieve topical release

was successfully accomplished. Thus, developed topical *in situ* gel can be considered promising formulation for antifungal treatments.

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