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Original Article

MODIFIED CYCLODEXTRIN-BASED THERMOSENSITIVE IN SITU GEL FOR VORICONAZOLE OCULAR DELIVERY AGAINST FUNGAL KERATITIS

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ABSTRACT

Objective: Fungal keratitis is a severe corneal infection. The present study aims to design and formulate an inclusion complex of Voriconazole-Sulfobutyl ether-beta-cyclodextrin (V-SBECD) loaded thermosensitive *in-situ* gel to improve solubility, therapy efficacy, durability and reduce the dose-related side effect.

Methods: Poloxamer 407, a thermosensitive polymer along with hydroxypropyl methylcellulose (HPMC E 15), were used as gelling agents; the formulations with poloxamer (16% w/v) and HPMC E15 (1 and 1.5 % w/v) led to a consistent *in-situ* gel at 37 °C. The formulations were evaluated for drug content, pH, gelation temperature, viscosity, sterility test, antifungal studies, and cell lines studies.

Results: The molar ratio of the drug to SBECD (1:3), showing 42-fold increase in solubility, was chosen to prepare the inclusion complexes using the lyophilization method. The stability constant was found to be 721^{-m}. ATIR peaks, DSC thermograms and NMR spectra indicate the inclusion behavior of Voriconazole and SBECD. *In vitro* and *ex-vivo* studies demonstrated that optimized formulation sustained the drug release for over 12 h. Cellular cytotoxicity on Human corneal epithelial cells showed that V-SBECD formulations do not cause corneal epithelial damage after 24 h. *In-situ* gel and marketed formulation have shown a markable reduction in the growth of the *Aspergillus Niger*. The optimized SBECD-loaded *in-situ* gel formulation (F10) did not vary significantly in pH, drug content, viscosity, and % cumulative drug release, signifying stable formulations when tested at 4, 25, and 40 °C.

Conclusion: The research findings envisaged V-SBECD *in-situ* gel formulation as a concrete strategy to treat severe fungal keratitis.

Keywords: Human corneal epithelial cell lines, *In-situ* gels, Poloxamer, Sol-gel temperature, Voriconazole-sulfobutyl ether-beta-cyclodextrin

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INTRODUCTION

Fungal keratitis is an infection and inflammation of epithelial and stroma cells of the cornea. It can result in scarring and visual loss if left untreated. The current treatment strategies for fungal infections of the eye include oral antifungal therapy and administering antifungal eye drops hourly to bi-hourly [1].

Topical administration for treating ophthalmic infections, especially eye drops, remains the most potent approach. However, limited volume, corneal barriers, and tear turnover rate pose potential limitations for drug administration to the eye [2, 3].

In general, the systemic bioavailability of ocular delivery systems usually ranges from 0.1-5 %. Even though a combination of oral and ocular medications is used to treat fungal keratitis, the treatment regimen remains ineffective because of the low bioavailability of the drug.

Voriconazole is a Biopharmaceutical classification system (BCS) class-II and is available in the market as Voriconazole (oral) for eye infections, which includes Vfend, vorilex, and voritrol [4]. Previous studies reported extreme drying and scaling of the oral cavity with abdominal pain in corneal chemical burn patients when treated with 200 mg oral voriconazole and topical Voriconazole. The reason behind supplementing oral medication with eye drop therapy is the incredibly low bioavailability owing to the shape and limited capacity of the eye. However, topical Voriconazole was well tolerated. Hence, a strategy has to be devised that can have high systemic availability when administered solely by topical route. In severe fungal keratitis, patients admitted to hospitals are generally administered ophthalmic Voriconazole, which is a reformulate I. V. voriconazole; however, precipitation of the drug as needle-shaped crystals was noticed when instilled into the eye, leading to severe irritation, and burning, due to its low solubility in tear fluid [5, 6]. As the tear volume in the eye is meagre, a practical approach to upsurge the solubility of the drug in such a low volume is desirable, for which solid dispersion can be considered.

Even though cyclodextrin (CD) and derivatives will enhance the solubility, the low residence time of the instilled drug solution requires repeated administration, which increases the treatment cost and drug loss [7].

Of the various approaches to improve corneal permeation, bioavailability, and prolonged drug release, *in-situ* gels are fascinating, as they undergo a sol-gel transition after administration and have a satisfactory residence time.

Till date, no voriconazole topical inventions were approved by USFDA or EMA. VFEND®, a parenteral commercial of voriconazole, contains solubilizing agent-β (SBECD). Cyclodextrin facilitates the formulation of the solution and enhances its clinical properties. 5-15 % of SBECD has been successfully used for ocular drug delivery. Hence, we have employed a strategy of incorporating the SBECD-voriconazole inclusion complex into an *in-situ* gel.

MATERIALS AND METHODS

Materials

Voriconazole (M. W. = 349.3, purity>95% purity, Batch No.: VZ0300416; Expiry date: May 2023) was a gift from MSN Laboratories Pvt Ltd, Hyderabad, India. Sulfobutyl ether-beta-cyclodextrin (SBECD) was procured from Cydex Pharmaceuticals Inc, Kansas, U. S. Poloxamer 407 was obtained from Sigma Aldrich, Bangalore, India. Benzalkonium chloride, sodium chloride, and disodium hydrogen

orthophosphate were procured from SD Fine Chem Ltd, Mumbai, India. Sodium hydroxide and sodium bicarbonate were obtained from Finar Chemical Ltd, Ahmedabad, India. HPMC E15, calcium chloride, and potassium dihydrogen phosphate were obtained from Loba Chemie Pvt. Ltd, Mumbai, India. All the remaining reagents and solvents used for experimental work are of analytical grade.

Methods

HPLC method development of voriconazole

HPLC analysis for Voriconazole was carried out by an isocratic, reverse-phase high-performance liquid chromatography system (Waters 2996 HPLC system) with a Photodiode Array Detector (PDA) [8]. Chromatographic separation was done using Waters column (5 µm, 4.6 × 250 mm) maintained at room temperature, and the flow rate was 1 ml/min. A standard calibration curve was plotted in a 5-30 µg/ml concentration range at λ_{max} 255 nm.

Phase solubility studies

This study was conducted in triplicate to investigate the outcome of Sulfobutyl ether-beta-cyclodextrin (SBECD) on the solubility of Voriconazole, utilizing the method described by Higuchi and Connors [9, 10]. An aqueous solution of SBECD of different concentrations (1, 4, 8, 16, 20, 24 mmol) was added to an additional amount of Voriconazole and agitated (SI-300 test incubate shaker, Lab-companion.) at 25 °C for 72 h. After reaching the steady, the solution was filtered using a 0.45 µ filter, centrifuged, and diluted. Analysis was done chromatographically using a 2 µm (50×2.1 mm) C-18HT column with a transition flow of water: acetonitrile (95:5, v/v) and 0.05 % formic acid. The flow rate during analysis was 0.7 ml/min. The concentration (in mmol) of Voriconazole vs. concentration (mM) of the SBECD plot can be used to extrapolate the slope and stability constant calculations [11].

$$K_s = S_0(1 - \text{Slope})$$

Where: S_0 was the equilibrium solubility of Voriconazole in water.

Procedure for preparation of voriconazole *in-situ* gel (cold method)

In brief, the accurately weighed required quantity of poloxamer 407 was dissolved in 10 ml of cold double distilled water (4 °C) and kept in the refrigerator for 12 h [12]. Then, weighed amounts of drug and excipients were added to the soaked polymer solution and stirred well [13]. The pH of the formulation was adjusted to 7.4 with 0.1N sodium hydroxide (NaOH). The compositions of the various *in-situ* gels are given in table 1. All the *in-situ* gel formulations were subjected to sol-gel transition temperature using a cone and plate MCR 102 rheometer (Anton Paar) with a diameter of 40 mm. The compositions of *in-situ* gel whose transition temperature was close to ocular temperature (35-37 °C) were selected for the loading drug inclusion complex.

Preparation of voriconazole/SBECD inclusion complex loaded (V-SBECD) into an *In-situ* gel

Voriconazole and SBECD in 1: 3 Milli Molar quantities were dissolved separately in 10 ml of double distilled water. The resultant mixed solution was frozen at-20 °C. The frozen mixture was then freeze-dried using a freeze dryer (New Brunswick Scientific, Germany) at-52 °C to-54 °C under a vacuum for 8 h (Heto dry Winner, Germany). The resulting freeze-dried product was incorporated into the optimized *in-situ* gel systems. Table 1 shows different compositions of *in-situ* gels.

Table 1: Composition of Formulations excipients along with their concentrations used in voriconazole *in situ* gel

Ingredients	Concentration(%w/v)											
Formulation code	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**	**F11**	**F12**
Voriconazole	0.3									-	-	-
Lyophilized Inclusion Complex of Voriconazole: SBECD	-	-	-	-	-	-	-	-	-	1:3 mmol	1:3 mmol	1:3 mmol
Poloxamer 407	15	15	15	16	16	16	17	17	17	16	16	17
HPMC E15	0.5	1	1.5	0.5	1	1.5	0.5	1	1.5	1	1.5	0.5
NaCl	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Benzalkonium chloride	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Distilled water	q. s. to 10 ml											

Characterization of voriconazole SBECD inclusion complex loaded(V-SBECD) *In-situ* gel

Attenuated total reflectance-FTIR analysis (ATR-FTIR)

I. R. spectra of Voriconazole, SBECD, physical mixture (PM), and inclusion complex were noted on an I. R. spectrophotometer (Perkin-Elmer 1600, V-650 Jasco) in the range of 4000-400 cm^{-1} wavenumber to study interactions [14, 15].

Differential scanning calorimetry (DSC)

DSC analysis of Voriconazole, SBECD, PM, and inclusion complex was conducted (Mettler Toledo DSC 821). Dry nitrogen was pumped into the sample cell at 10 ml/min. Up to 10 mg samples were placed in pans with a pinhole and aluminium crimping, then scanned at 10 °C/min from 25-300 °C [16].

Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR experiments were performed using a Bruker MSL 400 with a high-power static probe and solenoid coil spectrophotometer operating at 400 MHz. NMR spectroscopy of Voriconazole, SBECD, PM, and inclusion complex was carried out. These samples were dissolved in Dimethyl sulfoxide (DMSO) in an unsealed NMR tube [17].

Scanning electron microscopy (SEM)

SEM (FEI Quanta 250 FEG, Netherlands) was used to scan the surface morphology of simple Voriconazole, SBECD, PM, and inclusion complex). Samples were mounted on an aluminum stub with Au using an ion sputter in a vacuum coater and inspected under a microscope with an Everhart–Thornley detector running at 5 kV at 10000–85000 X magnification [18].

Physico-chemical evaluation

The prepared formulations' parameters, like general appearance, color, and clarity, were visually evaluated. The prepared formulations' pH was measured using a calibrated pH meter.

Sol-gel transition temperature

Gelation temperature was performed according to Repka *et al.* [19]. 1 ml aliquots of prepared formulations were transferred in a test tube, and the test tube was sealed with a parafilm and immersed in a water bath at 4 °C The temperature of the bath was increased in increments 1 °C and left to equilibrate for 15 min at each new temperature setting. The samples were examined for gelation, which is considered to have occurred when the meniscus would no longer move when tilted more than 90°; that temperature was considered the gelation temperature of the formulation. All the experiments were performed in triplicate. The rheological properties of the prepared formulations were determined using a spinning cone and plate rheometer MCR 102 (Anton Paar) with a diameter of 0.05m. The experiment was performed by maintaining a constant gap of 0.08 mm between geometry and Peltier. The graph of viscosity vs. temperature was plotted for all prepared formulations at different

temperatures to determine the gelation temperature. A Peltier P-PTD 200 (Anton Paar) was used to regulate the temperature on the lower plate. An initial 1-2 min stabilization period was given to achieve constant temperature using a Peltier system to control the temperature. The sample was placed on the Peltier plate and analyzed at a temperature range of 15-45 °C [20].

Drug content

The drug content of voriconazole SBECD inclusion complex loaded (V-SBECD) *in-situ* gels was determined. A measured formulation volume was diluted for 300 µg/ml concentration. A completely dissolved formulation was used as a sample and was analyzed by HPLC.

Rheological studies

Rheological properties of the plain *in-situ* gel were determined using a spinning cone and plate rheometer MCR 102 (Anton Paar) with a 40 mm diameter. The experiment was performed by maintaining a constant gap of 0.08 mm between geometry and Peltier. The viscosity vs. shear rate graph was plotted for all *in-situ* gel formulations (F1 to F9). Peltier P-PTD 200 (Anton Paar) was installed at the lower plate to control the temperature. The sample was placed on the Peltier plate and analyzed at a shear rate of 0-100 rpm [21].

Sterilization of *in-situ* gel using autoclave

The optimized formulation was sterilized by autoclaving at 121 °C under a pressure of around 15 *psi* for 20 min. The effect of autoclaving on physicochemical properties was studied before and after autoclaving.

Preparation of simulated tear fluid (STF) (pH7.4)

The STF was prepared by dissolving 2.08 g of sodium bicarbonate, 6.78 g of sodium chloride, and 0.084 g of calcium chloride in 1000 ml milli-Q water and adjusting the pH to 7.4 with 0.1N HCl.

***In vitro* drug release study of voriconazole SBECD inclusion complex *in-situ* gel**

An in vitro drug release study of *in-situ* gels was carried out by placing a dialysis membrane soaked in STF (pH 7.4) between the donor and receptor compartment of a glass Franz diffusion cell. The receptor compartment was filled with 25 ml STF and deaerated. An aliquot of optimized formulation (1 ml) was placed on the dialysis membrane. The apparatus was maintained at 37 °C and stirred at 50 rpm using a magnetic stirrer. The study was conducted for eight hours. At respective time points (1,2,3,4,5,6, 7, 8 and 12 h), aliquots were withdrawn, replaced by an equal volume of dissolution medium, and analyzed by HPLC.

***Ex-vivo* corneal permeation study**

Ex-vivo corneal permeation studies were performed for the optimized formulation F10 and pure drug as per the approved protocol (NIP/01/2018/PE/262). The separation of goat cornea was followed as per the method reported by Nair A *et al.*, 2009. Goat eyeballs in their entirety were obtained from a local slaughterhouse, and the corneas were meticulously separated from the attaching tissues. It was immediately stored in STF at 4 °C prior to the experiment. The cornea (0.8 cm^2) was fixed between the donor and receptor compartment so the anterior surface faces the donor compartment. For drug analysis, the procedure adopted in the *in vitro* study was followed [22].

Release kinetics

The data obtained from *in vitro* drug release studies of optimized drug-inclusion complex loaded formulations was fitted to various mathematical models like zero-order, first-order, Higuchi, and Peppa's to find out the best fitting model and thereby predict the release pattern of *In-situ* gels [23].

Cytotoxicity studies

Cytotoxicity study was carried out on Human corneal epithelial cells [HCEP] for four formulations, i.e., V-SBECD *In-situ* gel formulation, plain Voriconazole, marketed formulation and Sulfobutyl ether-beta-cyclodextrin and observed till 24 h followed by harvesting to check for the viability rate of the cells.

Cell culture and drug treatment

In two 25 cm flasks, HCEP cultures were incubated in a 10% FBS-DMEM medium at 37 °C. When the HCEP cells were in the logarithmic phase (80% fullness), 10 % FBS-DMEM medium and the mentioned four viz. *In-situ* gel formulation, plain drug, marketed formulation, and SBECD were added to each flask, and the cells were grown in the same circumstances.

Morphological observations

HCEP cells were inoculated into 24-well plates and grown in 10% FBS-DMEM media at 37 °C in a humid environment with 5% CO_2 for morphological observations. An inverted phase-contrast microscope (EclipseTS100) was used to observe the state of growth and morphology of cells after 24 h.

Cell viability assay

MTT (methyl thiazolyl tetrazolium) assay was used to examine the vitality of cells at 0 and 24 h. In a nutshell, HCEP cells were injected at a density of $1x10^4$ cells per well into 96-well plates along with respective samples. Exactly 20 µl of 5 g/l MTT was added to each well at a 2-4 h interval, followed by a 4 h incubation at 37 °C in the dark. The liquid in each well was replenished with 150 µl DMSO to dissolve the formed formazan crystals. A Multiskan GO microplate reader (Thermo Fisher Scientific) was used to measure the absorption of each well at 490 nm, followed by IC_{50} estimates [24].

Antifungal activity test

The agar well diffusion method was employed to estimate the antifungal activity of formulations.

Four holes were aseptically punched on a cork borer on a sterilized and solidified potato dextrose agar plate. The underside of the Petri plate was marked to label the wells. Approximately 20 µl of the indicator organism (*Candida Albicans (ATCC 10231)* and *Aspergillus Niger (ATCC 16404)-Sourced from Himedia labs*) was aseptically spread onto the agar plate. The plate was left undisturbed for 5 min. Formulations of 10 µl concentration (stock solution of same was prepared in methanol) were filled in the appropriate wells. The plates were then incubated at 25 °C for 72-96 h. Zones of inhibition were measured in millimeters (mm) using a ruler on the underside of the plate.

Stability studies

Optimized sterile in-situ formulations were subjected to stability testing. As per ICH guidelines, formulations were kept at 4, 25 and and 40 °C for three months. Samples were withdrawn and estimated for visual appearance, pH, drug content, rheology, and in vitro release [25].

RESULTS AND DISCUSSION

Analytical method development

Standard calibration curve by HPLC

HPLC method development of Voriconazole was carried out using acetonitrile and phosphate buffer (1:1) as mobile phase. Voriconazole's retention time was 7 min at a flow rate of 1 ml/min. The results showed good linearity (R^2=0.999) within the scanned concentration range at λ_{max} 255 nm [26]. The data of concentrations and peak area is given in suppl table 1 and the calibration curve is shown in suppl fig. 1.

Phase solubility studies

Phase solubility data to evaluate Voriconazole in SBECD is given in suppl table 2. The results indicated a linear (r^2=0.983) association between voriconazole solubility and concentration of SBECD, as shown in fig. 1. Conferring to Higuchi and Connor's, the A. L. type (linear increased drug solubility as a function cyclodextrin concentration) phase solubility curve was observed [27]. The molar ratio of the drug to SBECD (1:3), showing a 42-fold increase in solubility, was chosen to prepare the inclusion complexes using the lyophilization method. The stability constant was found to be $721^{-m.}$

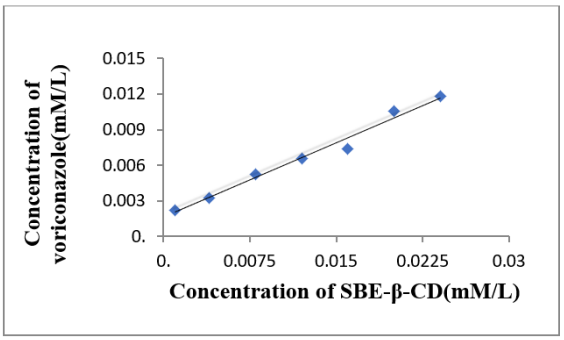

Fig. 1: Phase solubility graph of voriconazole with SBE-β-CD

Preparation of *in-situ* gel formulations

In-situ gels were prepared by soaking poloxamer 407 and HPMC E15 overnight in cold double distilled water. Voriconazole (0.3 % w/v) was added, with continuous stirring, into a beaker containing gel using a magnetic stirrer (200 rpm). The remaining excipients were added to the drug-loaded gel while stirring to form a homogenous system. The compositions of the prepared *in-situ* gels are mentioned in table 1. After *in-situ* gels(F1-F9) were prepared, sol-gel transition temperatures were measured using an Anton Paar rheometer. The *in-situ* gels whose transition temperature was close to ocular temperature (35-37 °C) were loaded with freeze-dried Voriconazole–SBECD inclusion complex (F10-F12).

Characterization and evaluation

Sol-gel transition temperature

Anton Paar rheometer was used to envisage sol-gel transition temperature. The data on sol-gel transition temperature is shown in table 2, and the rheogram is shown in fig. 2. All the formulations showed different transition temperatures based on the polymer concentration of poloxamer and HPMC E15. Among the various formulations, the formulations containing low concentrations of poloxamer (16 % w/v) and higher concentrations of HPMC E15 (1 and 1.5 % w/v) led to a consistent *in-situ* gel at 37 °C. The concentration of poloxamer (17 % w/v) with 0.5 % of HPMC E 15 also resulted in a consistent gel. The addition of HPMC E 15 had a significant impact on the mucoadhesive strength of the gel. At lower concentrations of poloxamers, the poly-propylene-oxide groups interact to form the core of the hydrophobic micelles, whereas the poly-ethylene-oxide groups occupy the micelle shell. At a temperature of 37 °C, it favors interaction between groups. An increase in temperature causes polymer desolvation and aggregation of micelles, forming a gel. This reversible process can be significantly exploited to formulate *in-situ* gel formulations [28]. The transition temperature study was performed at 15-45 °C. Formulation F10, F11, and F12 transition temperatures were like ocular surface temperature [29, 30]. Hence, these gels were selected for further loading with SBECD-drug complex instead of plain Voriconazole.

Table 2: Sol-gel transition temperatures of VCZ *in-situ* gels

Code	Transition temperature
F1	No gelation
F2	No gelation
F3	29.5±0.35 °C
F4	31.5±0.35 °C
F5	34.5±0.35 °C
F6	36.5±1.76 °C
F7	35.5±1.06 °C
F8	28.5±1.06 °C
F9	27±1.41 °C

*n=3, mean±SD

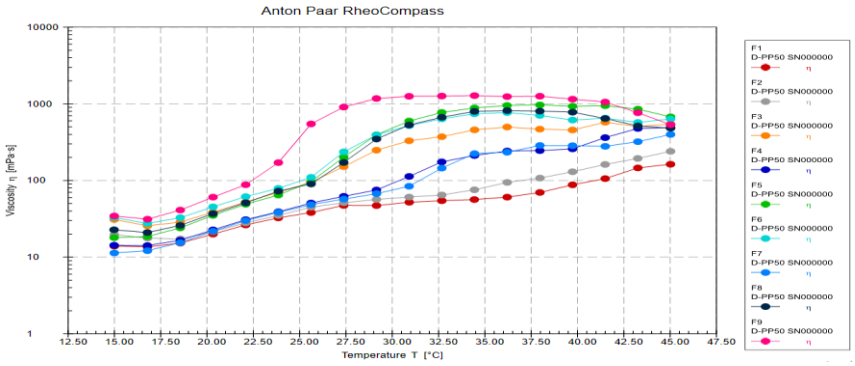

Fig. 2: Rheogram of voriconazole *in situ* gel

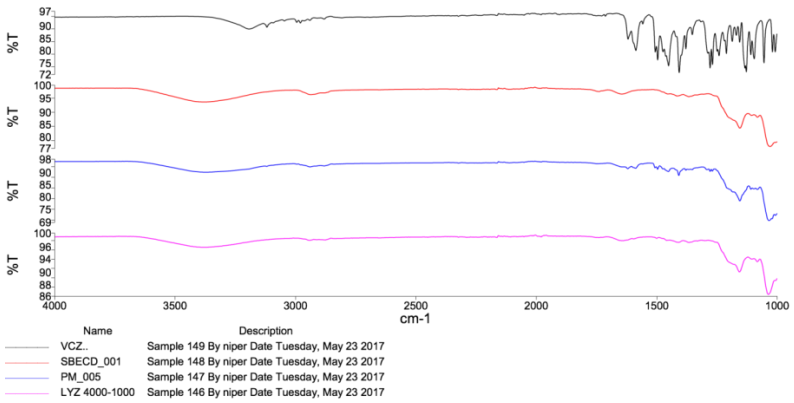

Fig. 3: ATR-IR spectra of voriconazole, SBE-β-CD, physical mixture, and inclusion complex

ATR-FTIR spectroscopy

The ATR-IR spectra of Voriconazole show characteristic peaks at OH (3200-3600 cm^{-1}), C-H alkane (2800-3000 cm^{-1}), C=C aromatic (1400–1600 cm^{-1}), and C-N aryl (1250-1360 cm^{-1}). The complex of the drug and SBECD did not show any noteworthy change in these characteristic peaks, indicating complexation [18]. The ATR-IR (Bruker, OPUS) peaks of Voriconazole, SBE β-CD, PM, and inclusion complex are shown in fig. 3.

Differential scanning calorimetry (DSC)

The addition of cyclodextrin molecules to a cavity may alter medicinal compounds' melting or decomposition points. DSC was used to investigate the melting temperatures of the drug, SBECD, and complex (fig. 4). Voriconazole thermograms showed a sharp endothermic peak at 134 °C, consistent with the drug's melting point. A broad endothermic peak was identified in the thermograms of SBECD at 80 °C, which could be attributed to dehydration, and was followed by a decomposition peak at 275 °C. The thermograms of the physical combination were created by superimposing endothermic peaks from various species. The endothermic peak at 134 °C had vanished, and the endothermic peak at 275 °C had moved to a broad peak between 220 and 256 °C, according to the thermograms of the inclusion complex [31].

Nuclear magnetic resonance spectroscopy (NMR)

Chemical shift variations in NMR spectroscopy indicate a change in the electronic settings of protons after the guest molecule is included in the cyclodextrin. Slight chemical shift variations (Δδ) in the complexes are due to non-covalent bonds between interiors of host molecules and Voriconazole rather than covalent chemical bonds [32–34]. The inclusion behaviour of Voriconazole and SBECD could be seen in ^1H NMR spectra of Voriconazole (fig. 5a), SBECD (fig. 5b) and complex of SBECD-voriconazole (fig. 5d). Chemical shifts were listed in suppl table 3.

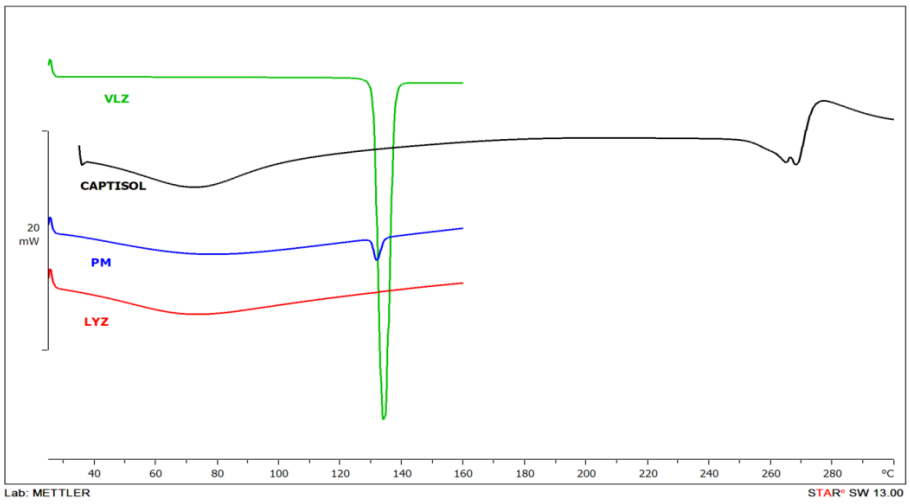

Fig. 4: DSC thermograms of voriconazole, SBE-β-CD (Captisol), physical mixture and inclusion complex

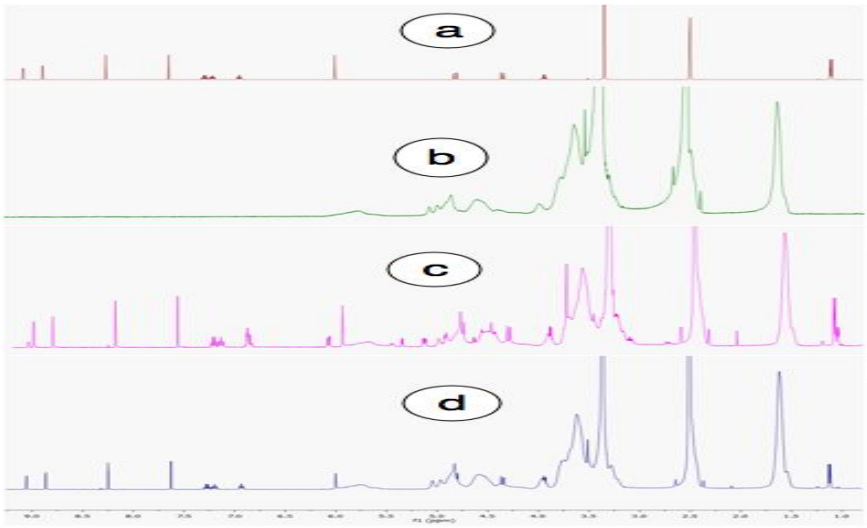

Fig. 5: ^1H NMR spectra of (a) VCZ(b) SBE-b-CD(c)PM and (d) Inclusion complex

Scanning electron microscopy (SEM)

To envisage deviations in the surface morphology of Voriconazole and SBECD when complexed, SEM images were studied as shown in fig. 6a. Pure antifungal voriconazole was crystal-shaped, while SBECD were different-sized spheres (fig. 6b). The PM upheld the unique morph of free species, with only adherence of drug crystals to the surface of SBECD (fig. 6c). However, an irregular block structure was seen with inclusion complexes (fig. 6d), distinct from free moieties, confirming the complexation [35].

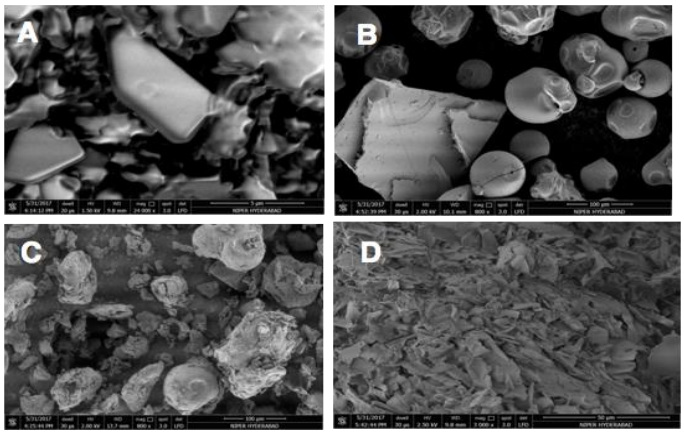

Fig. 6: SEM images of voriconazole, SBE-β-CD, physical mixture, and inclusion complex at 1000x

Evaluation of ophthalmic *in-situ* gel

ATR-IR spectroscopy

An interaction study was performed with an ATR-IR spectrophotometer ranging from 4000 cm^{-1} to 400 cm^{-1}. The characteristic peaks at 3195.66 (OH. group), 3392.3 (NH. group), and 1364 cm^{-1} (S=O group) were evident in fig. 7a and b. These characteristic peaks and the absence of new peaks in the formulation and PM indicate the absence of chemical interaction between the drug and excipients.

Physico-chemical evaluation

The prepared formulation's general appearance, color, and clarity were visually evaluated. All formulations were clear, colorless, and free from visible particles. A calibrated pH meter was used to read the pH values, ranging from 6.5 to 7.4 [36]. The data is shown in table 3.

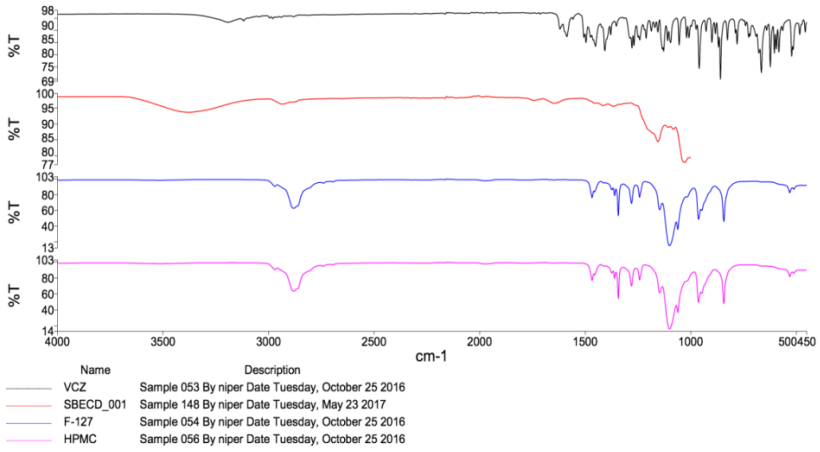

Fig. 7a: ATR-IR spectra of VCZ, SBE-β-CD, Pluronic F-127and HPMC

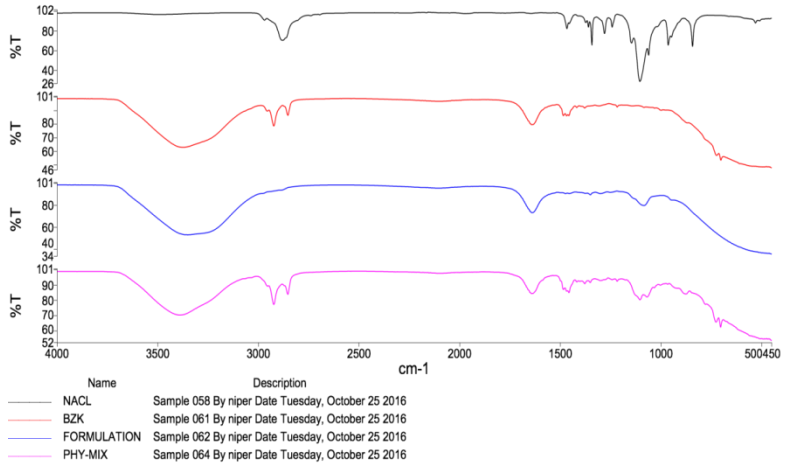

Fig. 7b: ATR-IR spectra of NaCl, benzalkonium chloride, *In-situ* formulation, and PM

Table 3: Physicochemical evaluation of various formulations

Code	Clarity	pH*
F1	Clear, colorless solution free from visible particles	7.23 (±0.12)
F2		7.37 (±0.74)
F3		7.24 (±0.14)
F4		7.43 (±0.05)
F5		7.36 (±0.45)
F6		7.26 (±0.16)
F7		7.46 (±0.11)
F8		7.34 (±.10)
F9		7.29 (±0.05)
F10		7.35 (±.12)
F11		7.38 (±.19)
F12		7.32 (±.0.9)

*n=3, mean±SD

Drug content

The drug content of the optimized formulation was done, and the data is given in table 4. The drug content ranges from 83% to 98%.

Rheological studies

Viscosity was determined by Anton Paar rheometer. All the formulations showed different viscosity based on polymer concentration. The viscosity study was performed in the shear rate range of 0-100 rpm, and the data is shown in table 4. An insight obtained from the data presented in table 5 is that the viscosity of formulations, especially those containing optimum amounts of poloxamer 407 and HPMC E15, show an intensification in viscosity with a rise in energy supplied to the system in the form of rpm. This might indicate sol-gel transformation because of energy supply to the system [37, 38]. At physiological temperatures, the viscosity of thermoresponsive *in-situ* gels increases due to aggregation between polyethylene oxide groups and polypropylene moieties to form micelles. When the concentration and temperature exceed critical micellar concentration (CMC) levels, hydration of surrounding polypropylene oxide groups occurs with dehydration of polyethylene oxide core groups. As a result of hydration, an increase in viscosity might be observed. The rheogram is presented in fig. 8 [39, 40].

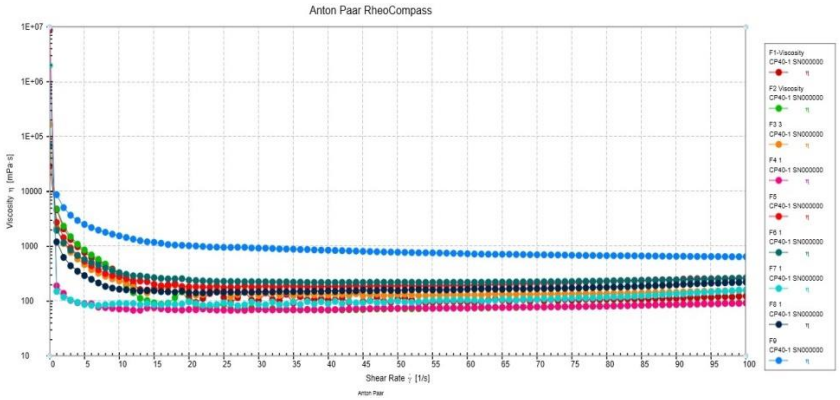

Fig. 8: Rheogram of voriconazole *in situ* gel

Table 4: Evaluation parameters (clarity, pH, and drug content) for optimized formulations

Code	Clarity	pH	Drug content (%)
F10	Clear	7.35(±.12)	83(±2.4)
F11	Clear	7.38(±.19)	90 (±3.2)
F12	Clear	7.32(±.0.9)	98(±1.53)

n=3, mean±SD

Table 5: Viscosity of *in-situ* gel formulations at different rpm

Formulations	The viscosity of in situ gel (at 50 rpm)	The viscosity of in situ gel (at 100 rpm)
F1	109 (±0.85)	123 (±0.75)
F2	72 (±0.93)	92 (±0.82)
F3	133 (±0.76)	157 (±0.96)
F4	73 (±0.58)	92 (±1.14)
F5	191 (±1.53)	265 (±1.23)
F6	221 (±0.78)	262 (±0.95)
F7	99 (±0.97)	159 (±1.1)
F8	154 (±1.34)	221 (±0.65)
F9	779 (±0.76)	790 (±1.24)
F10	174 (±0.68)	225 (±1.64)
F11	225 (±0.89)	250 (±1.22)
F12	110 (±0.55)	175 (±0.72)

N=3, mean±SD

Sterilization of *in-situ* gel using autoclave

The optimized formulations F10, F11, and F12 were autoclaved at 121 °C and 15 lb pressure for 20 min. The drug content and pH of formulations were examined before and after autoclaving. The data is given in table 6. It was found that the formulation's pH remained the same, and a nominal percentage reduced drug content. The slight decrease in the drug content might be due to drug hydrolysis that could have happened when the formulation is exposed to autoclaving conditions [41]. The studies indicated that *in-situ* formulations were stable regarding pH and drug content even after autoclaving at 121 °C and 15 lb pressure for 20 min.

Table 6: Data of pH and drug content before and after autoclaving sterilization

Formulation code	Before autoclaving (n=3)		After autoclaving(n=3)	
	pH	Drug content (%)	pH	Drug content (%)
F10	7.26(±0.35)	83(±2.4)	7.02(±0.35)	82.5(±1.5)
F11	7.36(±0.16)	90 (±3.2)	7.23(±0.16)	87.3(±0.9)
F12	7.34(±.40)	98(±1.53)	7.19(±.40)	86.9(±0.2)

*n, =3, mean±SD

***In vitro* drug release studies**

In vitro drug release study was performed using the Franz diffusion apparatus for plain drug and optimized complex-loaded *in-situ* gel formulations under simulated eye conditions using STF pH 7.4 at 35 °C and 200 rpm [42]. The drug released from plain drug was less than 25%, whereas the formulations loaded with drug-SBECD complexes released more than 70 % in 12 h. The drug release from formulations is considerably improved due to the inclusion of Voriconazole in the host SBECD, a potent solubility enhancer, and the process of freeze drying adopted in complexation. It was also noted that poloxamer facilitates the formation of transparent gels at physiologically relevant temperatures only in the 15-20 wt% range. The graphical representation of the release profile is shown in fig. 9.

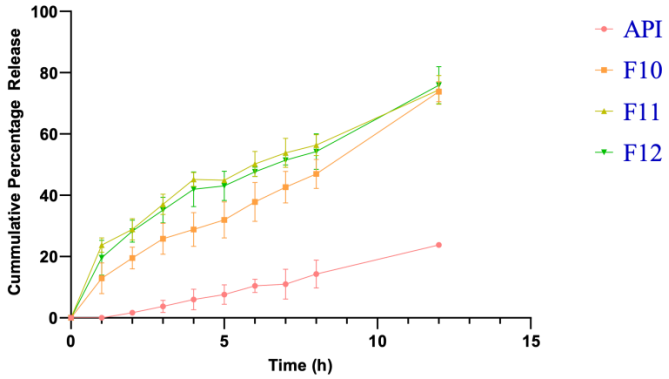

Fig. 9: *In vitro* drug release profile of plain drug, formulations F10, F11, and F12. Error bars indicate SD values of triplicate

***Ex-vivo* corneal permeation study**

To mimic the ocular environment, goat cornea was used for an eight-h permeation experiment [43, 44]. Formulation F 12 showed 82 % release, and the plain drug had 24% release at the end of 8 h. Compared to plain drug, *in-situ* gel formulation F 12 was more permeable in goat cornea, owing to the surfactant properties of poloxamer; the amount of drug permeable was represented in fig. 10. It was evident that drug permeation was three times higher in SBECD complex-loaded Voriconazole than Voriconazole. These results align with earlier reports [45], where the *in-situ* gels have tremendously improved the trans-corneal permeation of other drugs.

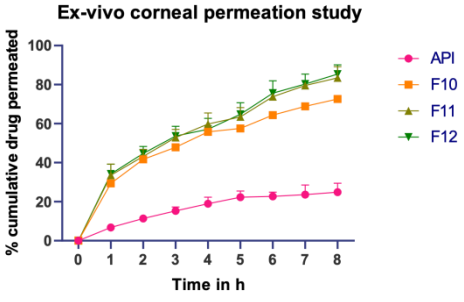

Fig. 10: Ex vivo corneal permeation study
Note: Error bars indicate SD values of triplicate

The release pattern from SBECD complex-loaded voriconazole gels predicted that the release followed the Higuchi mechanism, suggesting that the amount of drug released over time is proportional to the square root of time. The Higuchi release pattern suggests that drug release takes place in one dimension, and drug particles are much smaller than the matrix involved in the system [46].

Cytotoxicity and cell viability studies

Cytotoxicity study used Human corneal epithelial cells with *in-situ* gel formulation, Voriconazole-plain drug, marketed formulation, Sulfobutyl ether β-cyclodextrin. The cells were observed for 24 h, followed by harvesting to check for the viability rate, shown in fig. 11 and 12 [47-49]. Cytotoxicity study of *in-situ* gel of Voriconazole and other formulation components used for cell studies are given in table 7, and the MTT cell viability image is given as suppl fig. 2.

In-situ gel formulation contains 0.028 mg voriconazole and 0.528 mg Sulfobutyl ether-beta-cyclodextrin per 1 ml. The cell viability at this concentration of gel formulation was found to be 93.8%, which is slightly less than the pure form of Voriconazole (0.02 mg/ml–97%) as well as the pure form of Sulfobutyl ether-beta-cyclodextrin (0.2 mg/ml–95.9%) together. The marketed formulation (Voriconazole = 0.027 mg/µl and Sulfobutyl ether-beta-cyclodextrin = 0.426 mg/ml) showed 87.3% viability. This first run data suggests that the i*n-situ* gel formulation is similarly cytotoxic as that of the pure drug or marketed formulation. Voriconazole below 0.025 mg/ml was reported to have no cytotoxic effect on HCEP cell lines. Hence, from the study,

Voriconazole i*n-situ* gel using SBECD can be proved to be safe. However, *in vitro* cell line studies cannot be immediately transferred to humans as the drainage rate is 1-2µl/min and thereby, *In vitro-In vivo* correlation (IVIVC) may not be established [50].

Table 7: Percentage cell viability of control, *in-situ* gel, voriconazole pure drug, marketed formulation, and SBECD

Code	Composition/Compound	Concentration	Cell viability
A	Control	Medium	100 %
B	*In-situ* gel formulation	Voriconazole-0. 02 mg, SBECD-0.0558 mg	93.80%
C	Voriconazole	0.02 mg	97%
D	Marketed formulation	Voriconazole-0.027 mg, SBECD-0.426 mg	87.30%
E	Sulfobutyl ether-beta-cyclodextrin (SBECD)	0.2 mg	95.90%

Antifungal efficacy studies

The antifungal efficiency of the Voriconazole *in-situ* formulation was evaluated against organisms including *Candida Abicans* and *Aspergillus Niger* [51]. *In-situ* gel and marketed formulation have shown a markable reduction in the growth of the *Aspergillus Niger* (25 mm). In comparison, they have only shown meager antifungal activity against candida albicans (2.5 mm). Notably, the pure drug also has a similar kind of antifungal activity against *Aspergillus Niger* and *Candida Albicans*. The Zone of inhibition images is shown in fig. 13.

Stability studies

According to ICH guidelines, stability studies were performed at 4, 25, and 40 °C for the optimized SBECD-loaded *in-situ* gel formulation (F10). The samples were evaluated for visual appearance, pH, drug content, viscosity at 50 rpm, and % cumulative drug release at the end of 12 h, on the initial day, just after preparation, and up to three months [52, 53]. There was not much difference in pH, drug content, viscosity, and % cumulative drug release signifying stable formulations [54, 55]. The results obtained are tabulated in table 8.

Table 8: Stability study parameters (pH, drug content, viscosity and % drug release) of *in-situ* gel formulation F10 at 4 °C, 25 °C, and 40 °C

F10		Initial	On 45th day	On 90th day
4 °C	pH	7.36 (±0.45)	7.02 (±0.35)	7.2 (±0.35)
	Drug content (%)	94.3 (±4.53)	94.3 (±3.92)	93.5 (±3.86)
	Viscosity at 50 pm	195 (±8.33)	205 (±10.23)	210 (±12.35)
	% Cumulative drug release	46.97 (±4.23)	46.8 (±3.83)	46.3 (±4.42)
25 °C	pH	7.36(±0.45)	7.02(±0.35)	7.3 (±0.35)
	Drug content (%)	94.3(±4.53)	94.1(±3.92)	93.3(±3.86)
	Viscosity at 50 pm	193 (±9.23)	200 (±11.23)	208 (±12.42)
	% Cumulative drug release	48.15 (±4.53)	49.25(±4.3)	49.8 (±2.10)
40 °C	pH	7.36 (±0.45)	7.02(±0.35)	7.1 (±0.35)
	Drug content (%)	94.3(±4.53)	94.1(±3.92)	93.3(±3.86)
	Viscosity at 50 pm	190 (±10.23)	198 (±8.23)	205 (±13.15)
	% Cumulative drug release	48.15 (±4.53)	49.25(±4.3)	49.8 (±2.10)

n=3, mean±SD

CONCLUSION

In the present study, V-SBECD *in-situ* gels were successfully formulated as a novel topical alternative for treating fungal keratitis. The freeze-dried V-SBECD complexes were incorporated into thermo-reversible mucoadhesive *in-situ* gels formulated using poloxamer 407 as a gelling agent and HPMC E15 as a viscosity enhancer. The V-SBECD complex formation is based on the internalization of the drug's di fluoro phenyl ring into the cavity of the host-SBECD. The prepared formulations were evaluated for drug content, pH, gelation temperature, *in vitro* drug release, *ex-vivo* permeation studies, sterility tests, cell lines studies and for antifungal studies.

The formulation was stable for up to three months when stored at room temperature. *In vitro* studies demonstrated that SBECD-loaded voriconazole *in-situ* gels have sustained drug release. The cell line studies displayed that SBECD and voriconazole concentrations used in the formulations are not causing any corneal epithelial damage. However, long-term studies using human corneal endothelial cell studies and clinical studies are needed to prove the safety and efficacy of formulation that can help treat ocular fungal keratitis. Our study revealed that SBECD-loaded voriconazole i*n-situ* gels can be an alternative approach to conventional eye drops and ointments. Hence, developing an *in-situ* gel provides ease and decreases the frequency of administration, resulting in patient acceptance in the treatment of fungal keratitis.

AUTHORS CONTRIBUTIONS

Conceptualization, Sunitha Sampathi; methodology, Sunitha Sampathi and Ramdas Ramavath; data curation, writing-original draft preparation Sravya Maddukuri and Sunitha Sampathi; Pharmacological investigations, review and editing Sujatha Dodoala, review and editing Vijaya Kuchana. All authors have read and agreed to the published version of the manuscript.

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LIST OF ABBREVIATIONS

V-SBECD–lyophilized inclusion complex of Voriconazole-Sulfobutyl ether-beta-cyclodextrin

SBECD-Sulfo butyl ether-beta-cyclodextrin

FBS–DMEM-Fetal Bovine Serum-Dulbecco's Modified Eagle Medium

GIT–Gastrointestinal tract

HCEP–Human corneal epithelial cell line

NaOH–Sodium hydroxide

STF-Simulated tear fluid

AUTHORS CONTRIBUTIONS

All authors are contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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