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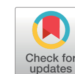
Improving therapeutic efficacy of voriconazole against fungal keratitis: Thermo-sensitive *in* situ gels as ophthalmic drug carriers



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A R T I C L E I N F O

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A B S T R A C T

The aim of this research was to evaluate the potential use of *in situ* gel formulations for voriconazole ocular delivery as fungal keratitis treatment. The *in situ* gelling system was applied to increase residence time and the bioavailability of voriconazole in the ocular mucosa. Temperature-triggered *in situ* gel formulations were prepared by cold method. Poloxamer 188, poloxamer 407 and carboxymethylcellulose were used for the preparation of thermosensitive *in situ* ocular gel. Voriconazole concentration in formulations was 0.1% (w/w). The prepared gels were evaluated for clarity, sol-gel transition temperature, gelling capacity, pH, viscosity, FT-IR and drug content. The gelation temperatures of all the formulations were within the range of 29–34 °C. All formulations exhibited fairly uniform drug content. Furthermore, sterility, antifungal activity, stability, *in vitro* drug release, *ex vivo* permeation, and penetration and *in vivo* study of these formulations were also examined. Drug release results indicated that all formulations showed sustained release properties. Irritation studies showed that no ocular damage or clinically abnormal signs were observed in the cornea, conjunctiva or iris upon administration of the formulation. In conclusion, voriconazole loaded *in situ* gels could be offered as a promising strategy as ocular carriers for the treatment of fungal keratitis.

1. Introduction

The anatomy, physiology, and biochemistry of the eye render this organ exquisitely impervious to foreign substances such as active ingredients. Consequently, compared with conventional routes of administration (oral, intravenous etc), ocular drug delivery has to overcome significant problems [1–3]. Conventional eye drops could generate eye irritation due to the shape and particle size while eyes different barriers such as lachrymal fluid-eye and blood-ocular barriers might induce lachrymation, nasolachrimal drainage system, enzymatic metabolism [4,5]. The extent of ocular drug absorption is seriously limited by physiological constraints. Ocular absorption is mainly restricted by the relatively impermeable corneal barrier. It has been well referred that cornea is comprised from three membranes: the epithelium, the endothelium, and inner stroma which act as main absorptive barriers. The epithelium facing the tears with lipophilic cellular layers plays the role of a barrier to ion transport. The tight junctions of the corneal epithelium act as the barrier for small molecules preventing the diffusion of macromolecules via the paracellular route. In further, 90% of the cornea consists of the stroma beneath the epithelium which is a highly hydrophilic layer. The corneal endothelium is responsible for maintaining normal corneal hydration [6–8].

Fungal infections, generally occurring in both animals and plants, are often observed in immunocompromised eyes after corneal trauma, surgery, or topical corticosteroid treatment [9]. Fungal keratitis is the major cause of ocular morbidity and blindness. Although fungal infections are distributed worldwide, they are more frequently found in tropical and subtropical regions [10,11]. In previous reports, the most common organism isolated from culture-proven fungal endophthalmitis was the yeast species *Candida albicans*, followed by molds such as *Aspergillus* species. A limited number of *Colletotrichum* species have been reported to induce infection in humans. More specifically, *Colletotrichum dermatium* and *Colletotrichum truncatum* were responsible for keratitis with few endophthalmitis cases [12]. In fungal keratitis, early

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diagnosis and antifungal therapy are essential to prevent further complications such as hypopyon formation, endophthalmitis, or loss of vision. Several drugs have been applied against fungal infections with oral, intravenous or topical eye solution of voriconazole to win the race for clinicians [13]. The majority of the reports noted that 1% w/v voriconazole eye drops is efficacious for the management of ophthalmic fungal keratitis, though the final voriconazole concentration achieved in the eye was not high enough to treat all types of fungal keratitis [11,14].

Voriconazole (VCZ) (UK-109,496) is a potent new triazole derivative with a broad spectrum of antifungal activity against many opportunistic fungal pathogens including *Candida*, *Cryptococcus*, and *Aspergillus* species [15–18]. The *in vitro* susceptibility opposed to various fungal isolates associated with keratitis and endophthalmitis has been found nearly 100%. Moreover, various studies indicated a superior efficacy of VCZ against several ocular mycoses following topical administration [11]. It should also be mentioned that the clinical results which have been achieved following VCZ treatment in cases of fungal keratitis and endophthalmitis caused by *Aspergillus* spp., *Scedosporium apiospermum*, *Paecilomyces lilacinus*, *Candida* spp., and *Fusarium* spp. are outstanding [19,20].

Clinicians, so as to treat eye infections, usually choose topical administration [1,21] since the 90% of the marketed ophthalmic formulations are in the form of eye drops. Among others, features like patient compatibility, tolerability, the easier production method, and economical cost make eye drops acceptability wider. However, the meager bioavailability (< 5%) of conventional eye drops (suspensions, solutions, etc.) is one of the major concern for pharmaceutical technologists and companies [22]. This low bioavailability is mainly due to rapid drainage of the molecules by tears and corneal epithelium membranes impermeability. Additionally, the temporary residence in the conjunctival fornix and non-productive absorption in the nasal cavity are also pledged for the low drug dose in intraocular tissues. Several research groups suggest that innovative systems such as suspensions, ointments, inserts, hydrogels, polymeric micelles, and lipid-based nanocarriers could be effective on meeting the challenges resulted from ocular delivery [1,23–25].

Generally, ocular ointments were studied for their excellent drug bioavailability due to the improved contact time, minimum tear removal as well as their resistance on nasolacrimal drainage. On the contrary, ointments could provoke blurred vision, limiting their use during the night or against infections on the outside and edges of the eyelids [26]. Controlled drug delivery systems using non-erodible non-toxic polymers seems to be the solution for the ocular problems since the primal ophthalmic solutions, suspensions, and ointments dosage forms are insufficient in ophthalmic diseases management [2].

In situ gelling systems, involving various polymers have been researched because they present prolonged contact time onto the ocular surface and improved drug corneal permeability [27–31]. *In situ* activated gel-forming systems can be described as viscous liquids which by changing physiological conditions (pH, temperature, ionic strength, UV) can shift to a gel phase [32]. *In situ* gels, in contrast to already gelled formulations, are advantageous formulations considering that can be accurately administered in reproducible quantities, promoting precorneal retention. For the production of *in situ* gels several both natural and synthetic polymers have been applied [33]. Poloxamers are triblock copolymers between polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO) which have the ability to form optical clear gels in aqueous media. Their high water content which leads to the swellable hydrogels have a positive effect on drugs absorption. Their reversed thermal gelation behavior (solution in 3–4 °C shifting to gel under physiological conditions) in addition to their mucomimetic properties classified poloxamers as optimal candidates for ophthalmic delivery gels systems [8,34,35]. Poloxamer 407 (P407) and Poloxamer 188 (P188) are used for several pharmaceutical products due to their non-toxicity, safety [36] and suitability as controlled-release agents [37]. In order to achieve better mucoadhesive properties and controlled diffusion, hydrophilic polymers, such as water-soluble derivatives of cellulose, polyacrylic acid and chitosan are incorporated in gels [38,39]. Carboxymethylcellulose (CMC) is water-soluble derivative of cellulose, sensitive in ionic strength and pH. It is widely known for its ionization and mucoadhesiveness because its -CH_2COOH groups are substituted on the glucose units of the cellulose chain through an ether linkage [40].

The aim of this work was to develop new ocular *in situ* gel formulations containing VCZ for the treatment of fungal keratitis and to evaluate them as effective topical ocular delivery. For this purpose, physicochemical characterization, rheological studies, stability, *in vitro* release, microbiological studies, *ex vivo* and *in vivo* studies of ocular *in situ* gels were assessed.

2. Materials and methods

2.1. Materials

VCZ was purchased from Sigma-Aldrich, Germany. Poloxamer 407 and poloxamer 188 were kindly gifted from BASF, Turkey. Carboxymethyl cellulose (CMC) was purchased from ZAG, Turkey. Benzalkonium chloride (BZC) was supplied from Sigma-Aldrich, Germany. High-pressure liquid chromatography (HPLC) grade acetonitrile (Sigma, Germany) was used for HPLC experiments. All the other solvents and chemicals were of analytical or HPLC grade. Dialysis membrane (Spectra/por 4, diameter 16 mm, the molecular weight of 12–14 kDa) was purchased from Spectrum. Distilled water was used throughout the study.

For microbiological studies, fluid thioglycollate medium was purchased from Merck Millipore Corporation, Darmstadt Germany, soyabean casein digest medium (CM0129) purchased from Oxoid, Thermo Fisher Scientific Inc. was used. Roswell Park Memorial Institute (RPMI 1640) medium was purchased from Sigma-Aldrich, Germany. Dimethyl sulfoxide purchased from Merck Millipore, Germany. 96 well microtiter plates were purchased from LP, Italiana. The McFarland standard was acquired from Biomerieux. While Mueller-Hinton agar and D-glucose were purchased from BD Difco, methylene blue dye was obtained from SSI Diagnostica, Hillerød, Denmark.

2.2. Preparation of in situ gel formulations

The polymeric solutions were prepared by dispersing the required quantity of P407 and P188 in water using a magnetic stirrer until the poloxamers completely dissolve. Aqueous solutions were stirred for about two hours by using magnetic stirrer [33].

In situ gels were selected according to pH values and gelling temperatures of formulations. For the preparation of ocular *in situ* gel, CMC, VCZ, BZC as well as sodium chloride were incorporated in aqueous solutions containing P407, P188, and distilled water. BZC (0.02% w/w) was added as a preservative to the solutions. Sufficient amount of sodium chloride (0.9% w/w) was added to the mixture to maintain the isotonicity. Voriconazole concentration was 0.1% (w/w).

2.3. Characterization of in situ gels

2.3.1. Determination of sol-gel temperature ($T_{sol-gel}$)

20 g of cold sample solution was put into a beaker and placed in a temperature-controlled stirrer. A thermometer (JG-220 Digital Thermometer (−50 + 260 °C), ± °1C accuracy) was immersed in the sample solution for constant monitoring. The solution was heated at the rate of 2 °C/min with the continuous with stirring at 200 rpm. The temperature at which the magnetic bar stopped moving due to gelation was reported as the gelation temperature. The maximum limit for gelation was checked up to 60 °C.

2.3.2. Gelling capacity

The gelling capacity of the prepared formulation was determined by placing a drop of the formulation in a beaker at 32 ± 0.5 °C and it was visually observed for gelling time [2,41].

2.3.3. Determination of pH

pH is one of the most important parameters involved in ophthalmic formulations. The pH of the gel was measured using calibrated pH meter (Mettler Toledo, Switzerland). Determination of pH was carried out in triplicate and the average of these determinations was taken as the pH of the prepared gels.

2.3.4. Drug content

0.125 mL of the developed formulations (IS-1-8-VCZ) were dissolved in 25 mL mobile phase followed by HPLC estimation of the aliquot to determine drug concentration [2].

2.3.4.1. HLPC analysis. The HPLC system consisted of a gradient pump and a UV detector supplied by Agilent 1100. C18 column (5 µm, 150 × 4.6 mm) was used. The samples were analyzed at 256 nm with 1 mL/min flow rate at 25 °C. The mobile phase was a mixture of acetonitrile: ultrapure water (50:50). The retention time of VCZ was 4.098 min (Üstündağ Okur et al., 2016b). The method was validated for linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision, accuracy and specificity, selectivity and stability. The linearity between peak area and concentration was analyzed using calibration curve obtained from standard solutions of VCZ (1–30 µg/mL). The accuracy of an analytical method is the closeness of test results obtained by the method to the true value and is defined recovery. The prepared standard solutions were injected five times at different levels as a test sample. Eight µg/mL solution was injected ten times in order to evaluate method precision, standard deviation (SD) and coefficient of variation. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analytes.The used method for VCZ analysis was found to be linear. The standard deviation of the slope and intercept were low. It is well known that if the standard deviation is less than the acceptance criteria (2%), the analysis system for the determination of assay is to verify.

2.3.5. Spreadability of VCZ loaded in situ gels

To determine spreadability of VCZ loaded *in situ* gels, 0.1 g of VCZ loaded *in situ* gels were transferred to the center of a glass plate (10 cm × 10 cm), which this glass plate temperature 32 ± 0.5 °C and this glass plate was compressed under another glass plate of the same size. Thus, the gel was spread out in between the plates. After one minute, the weight was removed and the diameter of the spread area (cm) was measured. The measurement was performed in triplicate [42].

2.3.6. Stability of VCZ loaded in situ gels

In order to check physical stability, VCZ *in situ* gels were stored at 4 ± 1 °C in the refrigerator for 3 months. After 3 months storage visual appearance, clarity, pH, gelation time of *in situ* gels and VCZ content were investigated. The experiments were repeated three times.

2.3.7. FT-IR spectroscopy of in situ gels

In situ gels were characterized via FT-IR spectroscopy using FTIR—2000 spectrometer (Perkin Elmer, Germany). KBr disks (thickness of 500 µm) technique was applied to check the possible interactions between drug and excipients. The spectra were recorded from 4000 to 400 cm^{-1} (Resolution of 2 cm^{-1} was applied, 32 co–added scans). Baseline corrected and converted to the absorbance mode spectra are shown herein.

2.3.8. Rheological studies

The viscosity measurements were carried out using Brookfield viscometer LVDV-E model with a cylindrical spindle. The *in situ* gel formulations were placed in the sample tube. The samples were analyzed with 50 rpm at 4 ± 0.5 °C by a circulating bath connected to the viscometer adaptor prior to each measurement [2].

2.4. In vitro drug release studies

In vitro release study of *in situ* gel solution was carried out in simulated tear fluid at 50 rpm. Simulated tear fluid (composition: sodium chloride 0.68 g, sodium bicarbonate 0.22 g, calcium chloride dihydrate 0.008 g, potassium chloride 0.14 g, and distilled deionized water to 100 mL) was used as the medium for *in vitro* release study [1,43]. The temperature was maintained at 32 ± 0.5 °C to mimic eye temperature. 5 g of formulations were separated from release media using dialysis membrane (Spectra/por, MW of 12–14 kDa) and capped with closures. 1 mL of sample was withdrawn at a predetermined time interval of 30 min to 12 h and the same volume of fresh medium was replaced. The samples were analyzed with HPLC for the drug content.

2.5. Microbiological studies of in situ gel

2.5.1. Sterility studies

Pure *in situ* gels as well as VCZ *in situ* gels were prepared at Laminar air flow Cabinet (Haier HR40-IIA2). To check the sterility of the prepared ocular formulations sterility control testing were performed. Sterility testing of the *in situ* gel formulations with or without voriconazole was carried out under aseptic conditions according to the international pharmacopoeia. For anaerobic bacteria, fluid thioglycollate medium was used. For fungi and aerobic bacteria, soya-bean casein digest medium was used. 1 mL of formulation solution was added to each medium and incubated in BINDER GmbH incubator at 35 °C for bacteria and 25 °C for fungi for 14 days. No growth of the microorganisms occurred.

To check the suitability of the used mediums for the sterility testing promotion test were performed. For growth promotion test of aerobes, anaerobes, and fungi, fluid thioglycollate media (using a separate portion of media for each microorganism) were inoculated with 100 CFU of *Staphylococcus aureus* ATCC 6538, *Clostridium sporogenes* ATCC 19404 and *Candida albicans* ATCC 10231. Media were incubated at 35 °C for 48 h. Both microorganisms showed visible growth.

2.5.2. Determination of MIC of VCZ

The broth microdilution test was done in accordance with CLSI guidelines for filamentous fungi (CLSI document M38-A2) and yeasts (CLSI document M27-A3). VCZ was dissolved in dimethyl sulfoxide, final dilutions of formulations were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 M MOPS buffer [3-(N-morpholino) propanesulfonic acid] and final concentrations were 250–0.125 µg/mL. Using the spectrophotometric method of inoculum preparation, a suspension with a 0.5 McFarland standard was utilized for yeasts and 0.4–5 × 10^4 spores/mL for moulds, and RPMI 1640 medium buffered with MOPS was used. *C. albicans* ATCC 10231, *C. tropicalis* RSKK 2412, *A. fumigatus* ATCC 204305, *A. niger* ATCC 10864, *A. terreus* ATCC 20542 and *A. flavus* ATCC 204304 which could be possible causes of fungal keratitis were used to evaluate antifungal activity of developed formulations [44,45]. A 0.1 mL inoculum was added to each well of the microdilution trays. The MICs were determined after 48 h of incubation. The plates were shaken before the comparison of growth in wells. With an aid of a reading mirror, growth in each well was compared with the voriconazole free growth control well. The MIC endpoints were evaluated for the lowest drug concentration that showed a prominent reduction (50%) of the growth in the control well.

2.5.3. Disk diffusion testing

Disk diffusion testing was performed according to CLSI standard M44-A2 for yeasts and M51-A for filamentous fungi. Mueller-Hinton

agar supplemented with 2% w/v glucose and 0.5 μg/mL methylene blue dye was used. Blank disks that were 12.1 mm in diameter were impregnated with 20 μL of formulations at the final concentration of 1 μg/disk and allowed to dry at room temperature in aseptic conditions. *C. albicans* ATCC 10231 and *C. tropicalis* RSKK 2412, *A. fumigatus* ATCC 204305 *A. niger* ATCC 10864, *A. terreus* ATCC 20542 and *A. flavus* ATCC 204304 were used to evaluate antifungal activity of developed formulations. The mold inocula were prepared at optical densities ranging from 80 to 82% and a suspension with a 0.5 McFarland standard was utilized for yeasts. The plates were incubated at 35 °C and inhibition zone (in millimeters) diameters were read by using a digital ruler at 24 and 48h.

2.6. Ex vivo studies (permeation and penetration studies)

Vertical diffusion cells with an effective area of 0.63 cm^2 were used for the corneal permeation and penetration experiments The excised goat corneas were clamped between the donor and the receptor chamber of cells. An exact amount of 0.5 mL *in situ* gels and 9 mL simulated tear fluid were filled into the donor and receptor chamber, respectively. The cells temperature was maintained at 32 ± 2 °C and magnetic stirring was also applied. The samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h. Sink conditions were maintained in the receptor compartment during *ex-vivo* permeation studies. To ensure the sink condition 1 mL Tween 80 was used.

After 24 h of contact time, each cornea was washed for penetration study. Each cornea was placed in 10 mL of methanol for 24 h in a mixer to determine the amount of penetration VCZ. Penetrated drug and permeated drug were assayed by means of HPLC. The experiment was performed in triplicate.

2.7. In vivo studies

2.7.1. Animals

New Zealand albino rabbits ranging in weight from 2.5 to 3.5 kg and with no signs of ocular inflammatory were used for *in vivo* studies. The *in vivo* experimental protocol was approved by the Ethical-Scientific Committee of Bezmialem Vakıf University (approval number: 2018/118). All animal experiments comply with ARRIVE guidelines and were carried out in accordance with the UK animals and associated guidelines such as EU directive 2010/63/EU. Rabbits were housed in a room maintained at 22 ± 1 °C and humidity in air-conditioned chambers with an alternating 12 h light-dark cycle was used. Animals had free access to a pellet diet and water ad libitum. During the *in vivo* studies, the rabbits were placed in boxes, and their heads and eyes movements were not restricted.

2.7.2. Determination of the VCZ in tear

Six rabbits were used in the study and, during the experiments, the rabbits were allowed to move their heads freely, and their eye movements were not restricted. Schirmer Tear Test Strip (ERC®) was used for the take tears. Fifty microliters of sterilized VCZ loaded *in situ* gel formulation (IS-1-VCZ) was administered in the lower conjunctival sac of the right eye only one time. The same amount of suspension of VCZ was administered to the lower conjunctival sac of the left eye only one time. After the formulations were applied to the eye, tears were collected at 0.5, 1, 2, 3, 4, 6, 8, and 24 h using tear strip. During the samples were taken, the tear strip placed into the conjunctival sac of the eye and marked tear strip was expected to reach a certain level. Strips were immediately placed in eppendorf tubes with 2 mL methanol and stored at + 4 °C until HPLC analyses. The HPLC was used to determine drug amount in tears [35,46].

2.7.3. Ocular irritation test

The possible ocular irritancy and/or damaging effects of VCZ loaded *in situ* gels were evaluated according to the modified Draize test. The *in situ* gel formulation (0.01 mL) was instilled directly onto the cornea of the right eye every 30 min for 6 h (12 treatments). Left eye served as control and was treated with 0.9% w/v sodium chloride. The congestion, swelling, discharge, and redness of each rabbit eyes were graded on a scale from 0 to 3, 0 to 4, 0 to 3, and 0 to 3, respectively. Irritation and corneal opacity were graded on a scale from 0 to 4 [47].

2.7.4. Statistical data analysis

Statistical data analysis was performed using the student's t-test with $P < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Preparation of in situ gel formulations

Temperature sensitive *in situ* gels were successfully prepared by the cold method using poloxamer 407, poloxamer 188 and CMC. Cold method is one of the preferred methods because it provides clear solution for *in situ* gel and it does not form polymer lumps as it has been reported when hot process is applied [48]. The prepared formulations were coded as IS-1-8. Amount of 0.1% VCZ (w/w) was loaded to *in situ* gel formulations and 0.3% CMC was added in poloxamer solutions (in case of IS-5-8) with continuous stirring until complete dissolution while as a preservative to the solutions, BZC 0.02% (w/w) was added. Sufficient amount of sodium chloride was added to the solution to maintain isotonicity. The final concentration of VCZ in formulations was 0.1% (w/w). The dispersion was kept in a refrigerator for 48 h to get a clear solution. The components of ocular *in situ* gels absence or presence of CMC are shown in Table 1.

3.2. Characterization of in situ gel formulations

Physicochemical characterization of *in situ* gel formulations is an important issue to be considered in the formulation stage, especially when the formulations intended for ocular administration. The physicochemical parameters of *in situ* gels without VCZ are reported in Table 2 and physicochemical parameters of *in situ* gels with VCZ are seen in Table 3. Clarity is one of the most crucial factors of ophthalmic preparations. All developed formulations were assessed for clarity by visual observation against a black and white background, demonstrating that the clarity of all the formulations was sufficient. Gelation temperature of prepared *in situ* gel formulations without VCZ was changed as about 30.27 ± 0.15 °C to 33.83 ± 0.06 °C and gelation temperature of prepared *in situ* gel formulations with VCZ was changed as about 29.73 ± 0.21 °C to 34.13 ± 0.32 °C. It can be safely concluded that mucoadhesive polymers (like CMC) played a reverse role on gelation temperature [49,50]. This indicates that the formulations can be converting to gel when they installed the eye surface. Between 32 and 34 ± 2 °C, the solutions are converted into gels with high viscosity. The gelling capacity data of prepared formulations with and without VCZ are presented in Tables 2 and 3, respectively. It was

Table 1
Formulation codes (FC) and components.

Composition of the prepared formulations						
FC	P 407 (g)	P 188 (g)	CMC (g)	VCZ (g)	BZC (% w/w)	Physiological Saline (q.s) (g)
IS-1-VCZ	15	25	–	0.1	0.02	100
IS-2-VCZ	18	22	–	0.1	0.02	100
IS-3-VCZ	20	5	–	0.1	0.02	100
IS-4-VCZ	20	20	–	0.1	0.02	100
IS-5-VCZ	15	25	0.3	0.1	0.02	100
IS-6-VCZ	18	22	0.3	0.1	0.02	100
IS-7-VCZ	20	5	0.3	0.1	0.02	100
IS-8-VCZ	20	20	0.3	0.1	0.02	100

Table 2
Physicochemical properties of neat *in situ* gels.

FC	Gelling temperature (°C)	pH	Clarity	Gelling capacity (sec)	Spreadability (cm)	Viscosity (cP)
IS-1	33.63 ± 0.38	6.79 ± 0.21	+ + +	1.27 ± 0.06	1.92 ± 0.08	446.33 ± 0.58
IS-2	31.13 ± 0.35	6.74 ± 0.29	+ + +	1.03 ± 0.06	1.30 ± 0.09	620.33 ± 3.51
IS-3	30.27 ± 0.15	6.64 ± 0.01	+ +	1.50 ± 0.10	1.80 ± 0.20	75.00 ± 1.00
IS-4	30.66 ± 0.29	6.82 ± 0.02	+ + +	0.80 ± 0.10	1.31 ± 0.02	647.33 ± 2.52
IS-5	33.83 ± 0.06	7.22 ± 0.03	+	1.07 ± 0.06	1.28 ± 0.07	460.00 ± 5.00
IS-6	31.56 ± 0.21	7.24 ± 0.01	+	0.97 ± 0.15	1.23 ± 0.06	533.67 ± 0.58
IS-7	30.43 ± 0.42	7.27 ± 0.01	+	1.13 ± 0.15	1.63 ± 0.30	79.67 ± 2.02
IS-8	31.20 ± 0.10	7.45 ± 0.02	+	0.80 ± 0.10	1.30 ± 0.10	611.33 ± 0.58

Table 3
Physicochemical properties of VCZ loaded *in situ* gels.

FC	Gelling temperature (°C)	pH	Clarity	Gelling capacity (sec)	Spreadability (cm)	Viscosity (cP)	Drug content (%)
IS-1-VCZ	34.13 ± 0.32	6.80 ± 0.03	+ + +	1.77 ± 0.20	1.6 ± 0.08	533.33 ± 5.77	92.55 ± 0.19
IS-2-VCZ	31.83 ± 0.25	6.85 ± 0.02	+ + +	1.27 ± 0.25	1.48 ± 0.07	668.33 ± 7.64	92.64 ± 0.22
IS-3-VCZ	30.00 ± 0.10	6.63 ± 0.01	+ +	1.90 ± 0.10	1.81 ± 0.06	71.67 ± 7.64	106.87 ± 0.19
IS-4-VCZ	30.77 ± 0.80	6.81 ± 0.01	+ + +	1.03 ± 0.06	1.40 ± 0.13	613.33 ± 7.64	93.69 ± 0.53
IS-5-VCZ	33.73 ± 0.20	7.22 ± 0.01	+	1.17 ± 0.29	1.21 ± 0.09	371.67 ± 7.63	90.96 ± 0.07
IS-6-VCZ	31.20 ± 0.26	7.24 ± 0.01	+	0.80 ± 0.10	1.23 ± 0.06	401.67 ± 2.89	92.88 ± 0.11
IS-7-VCZ	30.06 ± 0.15	7.13 ± 0.01	+	1.37 ± 0.23	1.28 ± 0.15	78.33 ± 1.44	97.78 ± 0.08
IS-8-VCZ	29.73 ± 0.20	7.24 ± 0.01	+	0.73 ± 0.20	1.32 ± 0.07	568.33 ± 7.64	93.30 ± 0.22

Table 4
Stability of VCZ loaded *in situ* gels for drug content and pH at 4 ± 1 °C.

FC	Drug content (% w/w)	Drug content (% w/w) (after 3 months)	pH	pH (after 3 months)
IS-1-VCZ	92.55 ± 0.19	93.97 ± 0.38	6.80 ± 0.03	6.90 ± 0.02
IS-2-VCZ	92.64 ± 0.22	95.56 ± 0.34	6.85 ± 0.02	6.83 ± 0.04
IS-3-VCZ	100.88 ± 0.19	61.55 ± 0.09	6.63 ± 0.01	6.72 ± 0.01
IS-4-VCZ	93.69 ± 0.53	94.08 ± 0.41	6.81 ± 0.01	6.84 ± 0.01
IS-5-VCZ	90.96 ± 0.07	92.36 ± 0.16	7.22 ± 0.01	7.04 ± 0.02
IS-6-VCZ	92.87 ± 0.11	91.25 ± 0.23	7.24 ± 0.01	7.07 ± 0.02
IS-7-VCZ	97.78 ± 0.08	81.84 ± 0.35	7.13 ± 0.01	7.30 ± 0.08
IS-8-VCZ	93.30 ± 0.22	90.59 ± 0.42	7.24 ± 0.01	7.27 ± 0.01

established that the formulations had immediate gelation existing for two seconds.

The pH of an ophthalmic formulation is significant for patient compliance considering that if pH value is beyond 4–8 which condoned by eye, the patient could feel discomfort. In further, the eye could be irritated, and drug bioavailability can be further decreased because of increased tearing [26]. The pH of the prepared formulations without VCZ ranged between 6.64 ± 0.01 and 7.45 ± 0.02 and pH of the prepared formulations with VCZ ranged between 6.63 and 7.24 which is appropriate for ocular delivery since they were iso-hydric. This fact reveals the non-irritancy of the formulation in the ocular mucosa. When a formulation is administered to the eye, it stimulates the flow of tears. Tear fluid is capable of quickly diluting and buffering small volumes of added substances, thus the eye can tolerate a fairly wide pH range. Ophthalmic solutions may range from 6.5 to 8.5 [51,52]. Among others, the formulation should present an optimum viscosity, which would allow its instillation into the eye as a liquid, which afterward would undergo rapid sol-gel transition due to temperature change. When the *in situ* solutions were installed the 32–34 ± 2 °C surface temperature, the solutions were converted to gel form after only a few seconds. Additionally, so as to facilitate sustained release of the drug to the ocular tissue, the gel formed *in situ* should preserve its integrity without dissolving or eroding for a prolonged period of time. Tables 2 and 3 shows the gelling capacity of all formulations. The gelling capacity increases by increasing the concentration of the gelling agent. Similarly to our study, Patil et al. prepared norfloxacin loaded *in situ* gels for the treatment of conjunctivitis which evaluated for their gelling

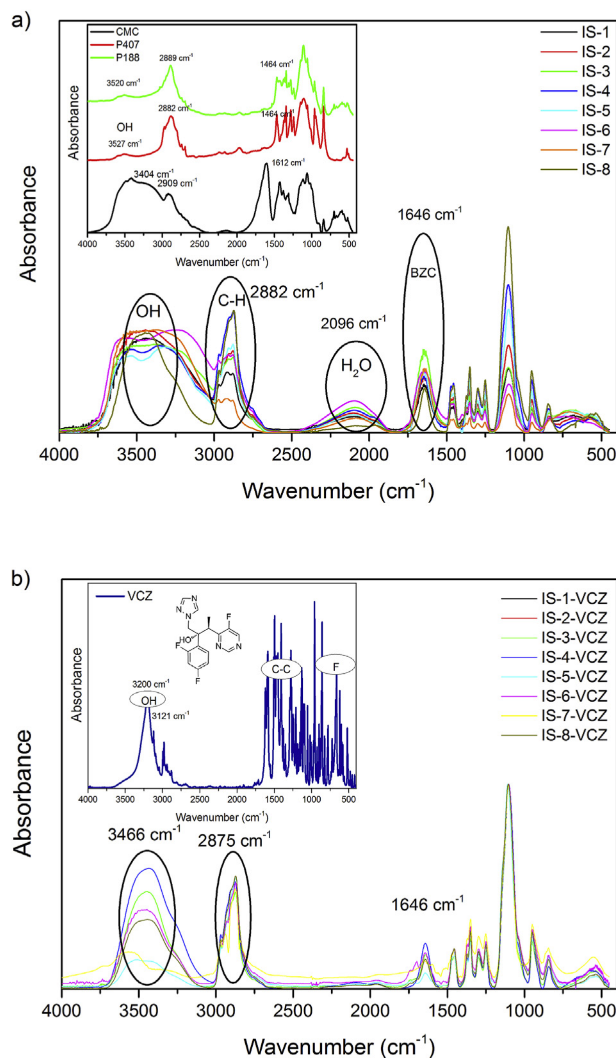

Fig. 1. a) FT-IR spectroscopy of unloaded *in situ* gels (inset: P407, P188, and CMC neat polymers) and b) loaded with VCZ *in situ* gels (inset: pure VCZ).

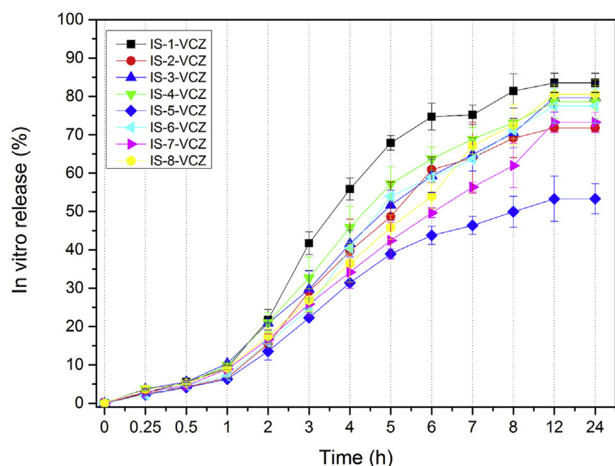

Fig. 2. *In vitro* release studies of VCZ from the prepared *in situ* gels (n:3, ± STD).

capacity. They found analogous results with our study as gelling capacity depends on polymer conglutination of the formulations [53]. Moreover, spreading diameter of the developed formulations demonstrated that is similar for all formulations. However, IS-1 and IS-3 spreadability is higher than the other formulations. This could be explained by their increased viscosity since Chaudhary et al. found that by increasing the concentration of the polymer, viscosity of the solution was also improved but the spreadability of the formulation was reduced [54].

3.2.1. Stability

The stability studies were carried out at 4 ± 1 °C, for a period of 3 months using the refrigerator. The samples were analyzed periodically on every month, and it was found that there are no changes in visual appearance, clarity, and gelation time. After 3 months, pH values of IS-1-VCZ–IS-8-VCZ formulations were established as 6.73 ± 0.01–7.30 ± 0.08, respectively.

Drug content was analyzed with HPLC using a validated method. The analytical method has good linearity, accuracy, precision, selectivity, and stability. The determination coefficient R^2 for the regression line is 0.999 with the slope of 13.764x and y-intercept of -0.656 for standard solution of VCZ. The percentage of recovery was almost 100% and the standard deviation was less than the acceptance criteria. Moreover, the LOD was 0.022 μg/mL, LOQ was 0.065 μg/mL and the assay exhibited a linear range of 1–30 μg/mL. The drug entrapment was high in all tests ranging from 90 to 100% whereas 61–95% of initial drug content of formulations was detected after the studied period of 3 months (Table 4). No indication of aggregation/precipitation or pH change was observed over a period of 90 days. This fact revealed that the formulations were stable after such period.

3.2.2. FT-IR spectroscopy of in situ gels

FT-IR spectroscopy belongs to the most useful techniques of pharmaceutical formulations characterization given that it can provide information concerning interactions between carriers and active molecules as well as compatibility of drug and excipients [55–58]. In this work, various polymers were used for the preparation of *in situ* gels. P407 and P188 are copolymers of poly(propylene oxide) and poly (ethylene oxide). IR spectra of poloxamer 188 and 407 are characterized by principal absorption peaks at 3400 (hydroxyl groups), 2889 and 2891 cm^{-1} (C-H stretch aliphatic), 1348.15 cm^{-1} and 1339 cm^{-1} (in-plane O-H bend), as well as at 1108 cm^{-1} (C-O stretch), respectively. IR spectrum of CMC exhibits a broad absorption band at 3432 cm^{-1}, due to the –OH group stretching. The observed peak at 2909 cm^{-1} is assigned to C–H stretching vibration while the strong absorption band at 1603 cm^{-1} indicates the presence of COO^- group. The bands at 1423 and 1325 cm^{-1} are attributed to $–CH_2$ scissoring and –OH bending vibration, respectively whereas at 1061 cm^{-1} is depicted the $CH–O–CH_2$ stretching (Fig. 1-a) [59–61].

In the case of unloaded *in situ* gels, the presence of water [62] and BZC is depicted by the main peaks at 2096 and 1646 (alkenes) cm^{-1}, respectively (Fig. 1-a). The other peaks attributed to poloxamers are similarly observed with small changes, identifying their compatibility. The addition of CMC in IS-5-8 formulations did not affect the spectra since it was in low content. The broadening of OH groups band could be due to the formulation method. Observing Fig. 1b, it can be said that VCZ spectrum exhibits broad peaks at 3200 and 3121 cm^{-1} corresponds to the hydroxyl groups and aromatic rings, respectively. Aromatic C–C stretch peaks are seen at 1615 and 1586 cm^{-1} while the presence of (Fluoro) groups is indicated by sharp peaks from 690 to 515 [17]. In most studies found in literature, similar *in situ* gels do not exhibit new peaks after the addition of drug in gels [58]. According to this fact the drugs and excipients are compatible (Fig. 1-b). Similarly, in our research, the absence of newly recorded peaks proves the compatibility of the gels [18]. However, it can be concluded that there are some interactions between the polymers and VCZ due to the shift in lower wavenumbers of some peaks [1].

Table 5
Inhibition zone diameters (mm) of blank and VRZ loaded *in situ* gels against several types of microorganisms (Candida albicans, Candida tropicalis, Aspergillus flavus, Aspergillus fumigates, Aspergillus terreus, Aspergillus niger).

Formulations	*Microorganisms*					
	C.tropicalis	*C.albicans*	*A.fumigatus*	*A.flavus*	*A.niger*	*A.terreus*
IS-1	19	0	0	0	0	0
IS-2	20	0	0	0	0	0
IS-3	0	0	0	0	0	0
IS-4	19	0	0	0	0	0
IS-5	15	0	0	0	0	0
IS-6	19	0	0	0	0	0
IS-7	20	0	0	0	0	0
IS-8	17	0	0	0	0	0
IS-1-VCZ	38	26	59	54	52	57
IS-2-VCZ	41	22	58	55	54	55
IS-3-VCZ	42	25	56	55	52	56
IS-4-VCZ	43	23	54	57	50	60
IS-5-VCZ	41	19	60	54	56	55
IS-6-VCZ	43	24	61	59	55	59
IS-7-VCZ	45	26	59	54	53	57
IS-8-VCZ	42	23	54	56	57	58

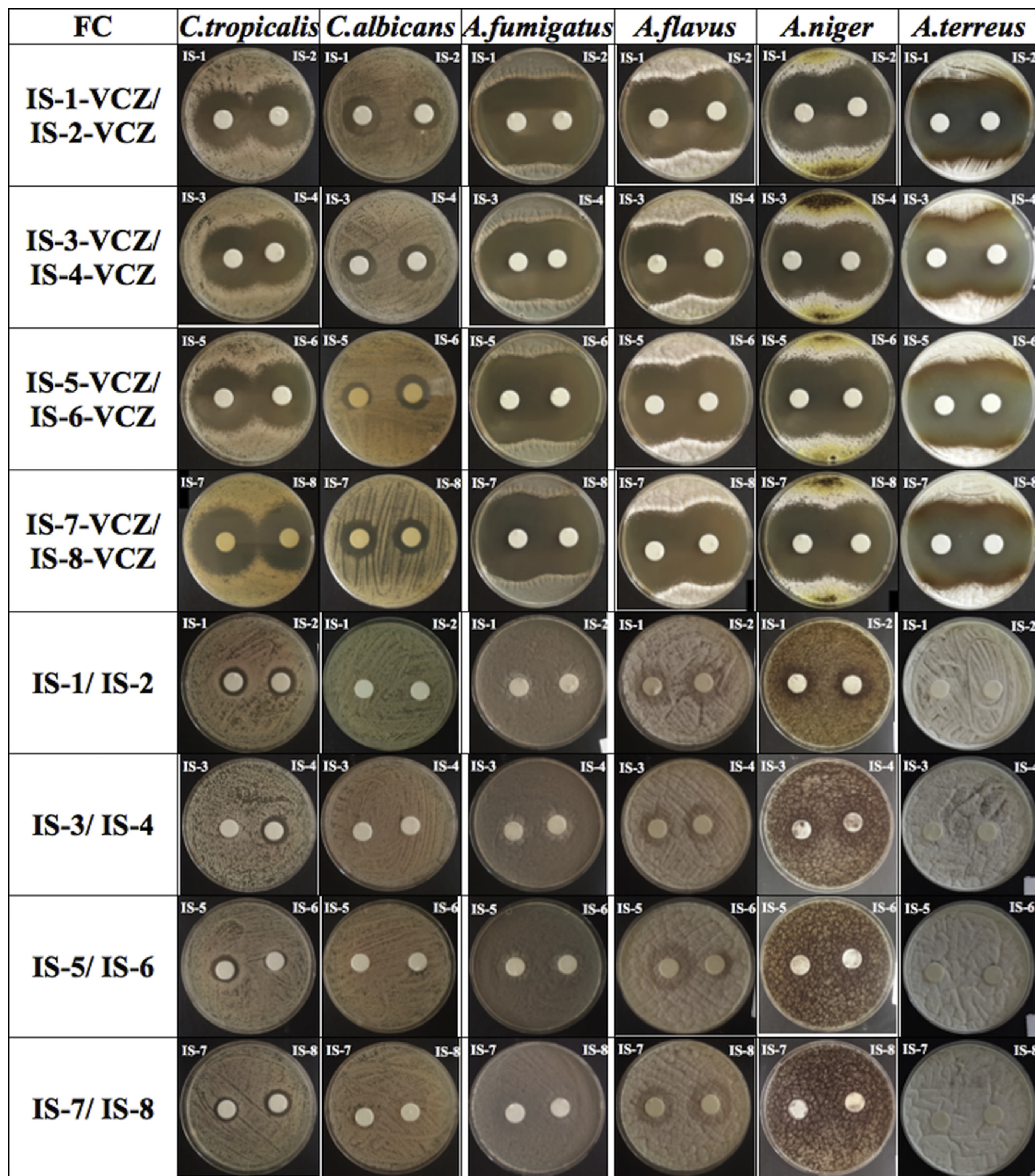

Fig. 3. Zone inhibition diameters of VCZ loaded and unloaded formulations.

Table 6
Ex vivo permeation and penetration studies of IS-1-VCZ and IS-5-VCZ.

Formulations	% Permeation from the cornea (after 24 h)	% Penetration to the cornea
IS-1-VCZ	42.92 ± 6.81	4.99 ± 1.06
IS-5-VCZ	38.34 ± 2.55	7.57 ± 0.26

3.2.3. *In vitro* drug release studies

The prepared VCZ loaded *in situ* gel formulations IS-1-VCZ – IS-8-VCZ were evaluated for their *in vitro* release, using simulated tear fluid of pH 7.4 as release medium. *In vitro* drug release results of VCZ loaded *in situ* gels are shown in Fig. 2. VCZ API belongs to class II drugs on biopharmaceutics classification system while its low solubility in gastric fluids has already been reported in our previous studies [17,18]. This

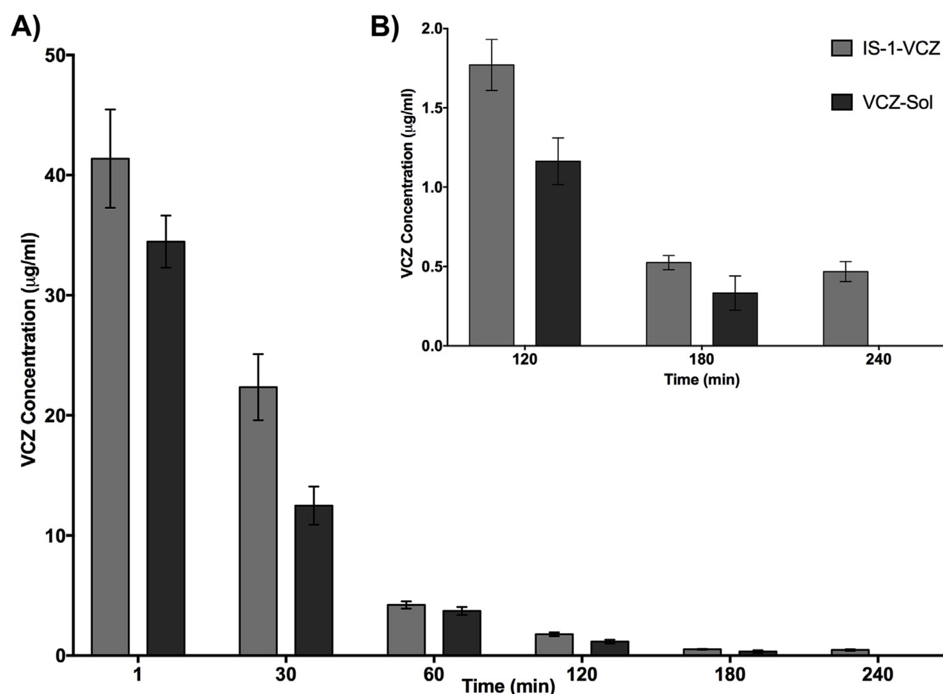

Fig. 4. VCZ concentrations of VCZ loaded *in situ* gel (IS-1-VCZ) and VCZ solution in the tear fluid A) at 1, 30, 60, 120, 180 and 240 min, B) at 120, 180 and 240 minutes

problem is a challenge for pharmaceutical technologists, nonetheless, in this study, VCZ release was effectively improved. Probably, the possible interactions found in FTIR spectroscopy could have lead to drug efficient dissolution or the drug was dispersed in small molecules. More specifically, all formulations showed sustained drug release for a period of 12 h with the absence of burst phenomenon (Fig. 2). Within 1st hour of the experiment only 5–10% of the drug has been released providing that gels can control the release of VCZ. After 60 min a progressive release rate can be seen. In general, it seems that viscosity plays an important role for *in vitro* release of *in situ* gels by lowering the release [63]. However, in this work viscosity is not the leading cause of slowing release rate.

At the end of the 12 h, *in vitro* VCZ release from IS-1-VCZ, IS-8-VCZ, IS-4-VCZ, and IS-6-VCZ formulations was found as 83.5%, 80.5%, 78.6%, 77.4%, respectively ($p > 0.05$). IS-5-VCZ formulation demonstrated slower release than the other formulations which can be attributed to the higher concentration of both P407 and CMC. In addition, all the formulations containing CMC present lower release rates. Mandal et al. prepared moxifloxacin hydrochloride loaded *in situ* gel using sodium alginate and hydroxypropyl methyl cellulose as polymers. They found that when sodium alginate and hydroxypropyl methyl cellulose concentration was increased, the release rate was decreased [2]. IS-1-VCZ presents the higher release at 83.5% after 24 h and for this reason, it is selected as the optimal carrier for *in vivo* studies.

3.4. Microbiological studies

The prepared *in situ* gels containing VCZ were tested in terms of their antifungal ability. Several fungal cultures were used as it can be seen (Table 5, Fig. 3). In order to check the sterility of the prepared formulations sterility control testing in fluid thioglycollate medium for anaerobic and soya-bean casein digest medium for fungi and aerobic bacteria were used and after incubation period for 14 days, no growth of any microorganism was seen. To check the suitability of the used mediums for the sterility testing, promotion test was performed.

For disk diffusion testing, yeasts such as *C. albicans, C. tropicalis* and filamentous fungi such as *A. fumigatus, A. terreus, A. niger* and *A. flavus* were used as they are the common organisms causing ocular fungal infections [44,45]. Above mentioned microorganisms were inoculated to media and after incubation period both microorganisms showed visible growth. The MIC endpoints were evaluated for the lowest drug concentration that showed a prominent reduction (90% and 50%) of the growth in the control well. MIC_{90} values of VCZ against *C. albicans, C. tropicalis, A. fumigatus, A. niger, A. terreus,* and *A. flavus* were 2, 1, 0.5, 0.5, 0.5 and 0.5 µg/mL respectively and MIC_{50} values were 0.25 µg/mL for all of the microorganisms.

Antifungal results of the VCZ loaded and unloaded *in situ* gels are given in Table 5. Clear zones of inhibition were obtained. *In situ* gels were found to be more effective on filamentous fungi than yeasts. While most of the test organisms were not affected by the blank gels, a minor inhibition zone was seen in *C. tropicalis* when blank gels except IS-3 were applied to it. This fact can reveal that some of the gel components could affect the growth of *C. tropicalis*. Since the interpretive breakpoints of VCZ for susceptible species is ≥ 17 mm and for resistant is ≤ 13 mm, it can be said that all of the organisms are susceptible to the *in situ* gels loaded VCZ formulations [64].

The antifungal study shows that VCZ retained its antifungal efficacy against the selected microorganisms, when formulated as a gel-forming ophthalmic system. It can be concluded that according to microbiology data, IS-1-VCZ and IS-5-VCZ are the best candidates to be applied on the eye. More specifically, physicochemical properties as gelling temperature, pH, clarity, gelling capacity, spreadability, viscosity and drug content parameters of formulations IS-1-VCZ and IS-5-VCZ are optimal and therefore formulations IS-1 and IS-5 have chosen for *ex vivo* studies.

3.4. Ex vivo studies (permeation and penetration studies)

The prepared *in situ* gelling formulations of VCZ, IS-1-VCZ, and IS-5-VCZ, were evaluated for their *ex vivo* permeation and penetration studies, using simulated tear fluid of pH 7.4 as the medium. *Ex vivo* permeation and penetration results of VCZ loaded *in situ* gels, IS-1-VCZ and IS-5-VCZ are shown Table 6. As it was stated at previous section, IS-1-VCZ and IS-5-VCZ formulations were chosen as the optimal candidates to use for *ex vivo* studies considering their characterization parameters

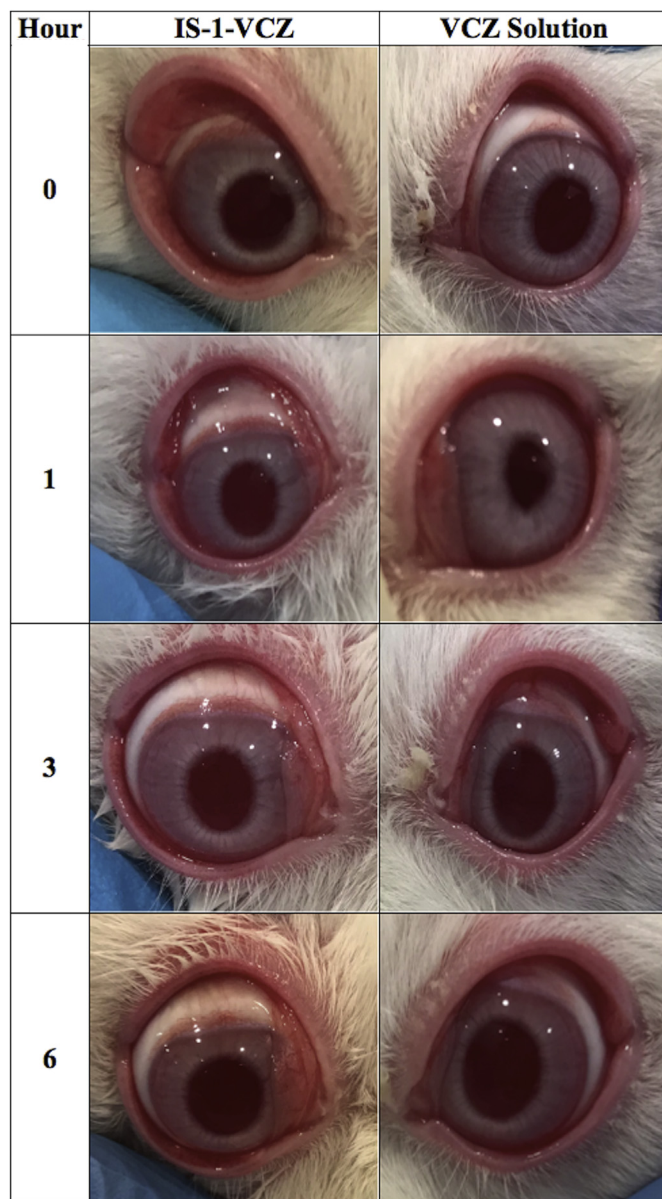

Fig. 5. Results of irritation test at 0, 1, 3 and 6 h after the administration of IS-1-VCZ and VCZ solution.

and *in vitro* release. Goat corneas were used for permeation and penetration studies.

Table 6 shows VCZ % diffusion and cumulative VCZ % remaining in the cornea after 24 h. It was ruled out that afte 24 h the amount of VCZ permeated through the cornea from IS-1-VCZ and IS-5-VCZ was detected as 42.92 ± 6.81%, 38.34 ± 2.546%, respectively. So, IS-1-VCZ formulation depicts improved permeation from cornea compared to IS-5-VCZ formulation due to the absence of CMC. Similarly, Paavola et al. studied lidocaine loaded poloxamer gel using poloxamer 407, CMC, HPMC and dextran. It was found that cellulose additives prolong the release [65]. In further, when the penetration to the corneal tissue was examined, it was seen that the amount of VCZ penetrated, in case of IS-1-VCZ and IS-5-VCZ were 4.99 ± 1.06% and 7.57 ± 0.26%, respectively. Finally, it is indicated that IS-5-VCZ formulation provides more penetration to the cornea, due to the presence of CMC which presents mucoadhesive properties. In a similar way, Sassi et al. prepared topical halofuginone loaded gel formulations using different concentrations of CMC and they found that by increasing the concentration of CMC effect increases the penetration of the drug [66].

3.5. *In vivo studies*

3.5.1. *Determination of the VCZ in tear*

IS-1-VCZ was selected in order to be studied for *in vivo* studies. This fact was the result of the obtained characterization data. Although, IS-5-VCZ presents desirable physicochemical characteristics as well as permeation and penetration ability, its low release rate (50%) after 24 h, can limit its application for further studies. Therefore, IS-1-VCZ was chosen for *in vivo* studies due to its improved release rate, as well as permeation and penetration studies.

Fig. 4 depicts VCZ concentration in the tear fluid as *in vivo* studies demonstrated. After instillation of 50 μL of IS-1-VCZ and VCZ solution to the eye, the samples were taken from tear using Schirmer tear strip. In the initial samples (at first minute), the amount of VCZ established as 41.370 ± 10.05 μg/mL and 34.465 ± 5.269 μg/mL for IS-1-VCZ and VCZ solution, respectively. After detecting the first drug concentration, tear samples have been taken at 0.5, 1, 2, 3, 4, 6, 8 and 24 h. At 30 min, the cumulative concentration of VCZ was detected as 22.344 ± 6.19 μg/mL and 12.488 ± 3.88 μg/mL from IS-1-VCZ and VCZ solution, respectively. At the end of the 4th hour, VCZ was detected in case of *in situ* gel treated eyes, whereas VCZ could not be detected in the VCZ solution treated eyes. In further, after 4 h, the higher amount of VCZ is found in the gel applied eye than VCZ solution applied eye. Finally, at the end of the 6th hour, no VCZ has been detected to both *in situ* gel applied eyes and in the VCZ solution applied eyes. To conclude, the maximum concentration of VCZ obtained from *in situ* gel (IS-1-VCZ) instead of neat VCZ solution. This is a quite rational result given that the gel is stable after hours.

3.5.2. *Ocular irritation test*

It has been reported that *in situ* gels, in most cases, do not show irritability or undesirable effects on eye [67,68]. Ocular contact is one of the most probable routes of human exposure. Therefore, the determination of the eye irritating potential is a rational basis for risk assessment in man. The common method assessing the ocular irritation potential of substances is the *in vivo* Draize Rabbit Eye Test [69]. Consequently, the above mentioned test was chosen to study the ideal formulation herein (IS-1-VCZ). New Zealand white rabbits are most commonly used in this test, as they have large eyes with a well-described anatomy and physiology, are easily handled, readily available and are relatively inexpensive [70]. In this research, no ocular damage or clinically abnormal signs were observed in the cornea, conjunctiva or iris upon administration of IS-1-VCZ and VCZ solution. Only grades 0 and occasionally 1 were recorded, according to modified Draize test. No difference between control and treated eyes for each group of rabbits were observed (Fig. 5). The eye irritation scores for all groups were less than 1, indicating the excellent ocular tolerance of IS-1-VCZ. No irritation was seen after application of VCZ loaded *in situ* gel to healthy rabbit eyes. The hydrogel properties of *in situ* gels can be criticized as suitable for application to the eye.

4. Conclusion

In the present study, the potential of VCZ loaded *in situ* gels as drug carriers for ocular delivery was evaluated. It was ruled out that *in situ* gels of VCZ can be successfully prepared with the cold method. The clarity, pH, gelation time and drug content of all formulations were found to be satisfactory. The developed *in situ* gels demonstrated antifungal activity against *C. albicans, C. tropicalis, A. fumigatus,* and *A. flavus*. In addition, the formulations were found to be stable for 3 months. FT-IR spectroscopy studies did not reveal undesirable effects and provide useful information such as compatibilty of drug and excipients. Further, the formulations showed sustained drug release for a period of 8 h, which is satisfying in order to treat ocular diseases. According to their physicochemical properties, formulations IS-1-VCZ and IS-5-VCZ were criticized as the most suitable for eye applications.

Ex vivo permeation and penetration studies, revealed that both of the carriers diffuse the drug. However, IS-5-VCZ exhibited low release rate and for this reason, IS-1-VCZ was applied for *in vivo* studies. Determination of the VCZ in tear study showed that maximum concentration of VCZ obtained from *in situ* gel (IS-1-VCZ) which was higher in comparison to neat VCZ solution. Finally, from *in vivo* Draize Rabbit Eye Test no ocular damage or clinically abnormal signs were observed in the cornea, conjunctiva or iris upon administration of IS-1-VCZ and VCZ solution. In conclusion, this research can open up a window on ophthalmic field since *in situ* gels loaded with VCZ, found to be a better alternative to conventional eye drops in the treatment of fungal keratitis.

Declaration of interest

The authors declare no conflict of interest.

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