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RESEARCH ARTICLE

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Formulation and characterization studies of inclusion complexes of voriconazole for possible ocular application

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ABSTRACT

In our study, Voriconazole (VOR) was selected as an active agent to be used for the treatment of ocular fungal infections. To overcome low aqueous solubility of VOR, inclusion complexes with α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), hydroxypropyl-cyclodextrin (HP-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) hydroxypropyl-γ-cyclodextrin (HP-γ-CD), methyl-β-cyclodextrin (M-β-CD) and sulfabutylether-β-cyclodextrin (SBE-β-CD) were formulated. Characterization studies revealed that inclusion complexes were formulated successfully with the lyophilization method. Aqueous solubility of VOR was enhanced up to 86 fold with the formation of the inclusion complexes. MTT analyses results revealed the safety of the complexes on 3T3 mouse fibroblast cell lines while Microbroth Dilution Method revealed the remarkable antifungal activities of the complexes. Analyses results revealed that inclusion complexes will overcome the poor ocular bioavailability of VOR resulting inefficient treatment of severe ocular fungal infections.

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Voriconazole; inclusion complexes; lyophilization; antifungal activity

1 Introduction

Conventional drug delivery systems like eye drops constitute the majority of ocular drug applications, however, it is very difficult to ensure therapeutic levels on the target site depending on the unique structure of the eye (Başaran and Yazan 2012; Al Khateb et al. 2016). Treatment approaches differ considering the location of the infections whether anterior and posterior regions are involved (Tiwari et al. 2021). Although most of the bacterial and fungal infections are related to the anterior region of the eye, severe infections can lead to total loss of vision therefore, ocular infections need to be treated effectively in a short time (Boateng and Popescu 2016). Previous studies had shown that basically two approaches emerge in order to increase the very low ocular bioavailability (5–10%) with the topical application; increasing corneal permeability and prolonging the duration of stay in the eye (Morsi et al. 2016). Benzalkonium chloride, cetylpyridinium chloride, capric acid, EDTA, nonionic surfactants and bile salts are frequently used as penetration enhancers, however frequent installation may lead to toxicity and permanent corneal damages therefore care has to be taken upon their use (Mahaling and Katti 2016). The second approach used to increase ocular bioavailability is to increase the contact time with the cornea either with electrostatic interactions or with increased viscosities (Tundisi et al. 2021).

Among ocular drug delivery systems, inclusion complexes with cyclodextrins are one of the most studied drug delivery systems considering their superior ability to increase the aqueous solubility of the lipophilic drugs which was regarded as the main reason for the low ocular bioavailability of the active agents (Zhang et al. 2016; Qin et al. 2017). Cyclodextrins are truncated-cone-shaped structures that consist of oligosaccharide (α-1-4)-α-D glucopyranose units, with hydrophobic internal cavities while outer parts of the hydrophilic structure (Jambhekar and Breen 2016). Cyclodextrins are synthesized by enzymatic hydrolysis of starch and have biocompatible, nontoxic structures with high chemical and physical stability performances (Savic et al. 2019). The most commonly used natural cyclodextrins are α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD) cyclodextrins with 6, 7 and 8 glucopyranose structures (Jambhekar and Breen 2016). In addition to natural cyclodextrins, hydrophilic (methylated, hydroxyalkylated and branched), hydrophobic (ethylated), ionic (sulfated) cyclodextrin derivatives have also been synthesized (Cal and Centkowska 2008; Agüeros et al. 2009). They have high molecular weights (1000–2000 Da and greater) and can form true solutions depending on the formation of micellar-like structures (Jóhannsdóttir et al. 2015). Due to their special structures, they can increase the aqueous solubility of BCS Class II and Class IV substances and result in higher bioavailability rates. They also ensure that the incorporated active substances are protected against oxidation, light and heat by settling in the inner space of the structure (Demirel et al. 2011). Cyclodextrins also act as penetration enhancers in ocular applications while carrying the active agents in solution form to the corneal surface (Elmotasem and Awad 2020; Jansook et al. 2021). They show less toxic properties than benzalkonium chloride increases their potential in ocular applications (Loftssona and Järvinen 1999; Jambhekar and Breen 2016).

Voriconazole (VOR) is the second-generation synthetic derivative of fluconazole. Attachment of the methyl group to the propyl

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chain and the replacement of the triazole structure with the fluoropyrimidine structure results in more efficient molecules (Pawar et al. 2013). It is mostly used in the treatment of fungal infections that are not sensitive to other antifungal agents (Marangon et al. 2004). VOR is a lipophilic substance and has low aqueous solubility, therefore there is no commercial ocular formulation available in the pharmaceutical market yet (Pawar et al. 2013; de Sá et al. 2015). Although many side effects were mentioned in the former studies, extremely good results were also obtained after topical, intrastromal and systemic application, especially in the treatment of fungal keratitis (de Sá et al. 2015). In our study inclusion complexes of VOR were prepared with different types of cyclodextrins in order to enhance the aqueous solubility of the active substance. *In vitro* characterization studies were studied in detail as well as the toxicological evaluations of the complexes were carried out with MTT analyses on 3T3 mouse fibroblast cell lines (Cao et al. 2007). VOR is very effective against a wide variety of yeasts, molds, even the fluconazole-resistant *Candida* strains as well as many species of *Aspergillus*. Therefore antifungal activities of the formulations were determined on *Candida albicans* (*C. albicans)*, *Candida krusei (C. krusei)*, *Candida glabrata (C. glabrata)* and *Candida parapsilopsis* (*C. parapsilopsis)* for better evaluation of the efficacy of the complexes (Breit et al. 2005; Kernt and Kampik 2009)

2 Materials and methods

2.1. Materials

Voriconazole (VOR) was donated by Deva İlaç. A. Ş. (İstanbul, Turkey). Hydroxypropyl-cyclodextrin (HP-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were the products of Roquette (Lestrem, France) and were donated as free samples from Barents Food Chemistry Trade. Ltd. Şti. (İstanbul, Turkey). Sulfabutylether-β-cyclodextrin (SBE-β-CD; Captisol®, CA, United States) was provided as a free sample. α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), methyl-β-cyclodextrin (M-β-CD) and Pluronic® F-127 were the products of Sigma-Aldrich (Steinheim, Germany). Poloxamer® 407 was from Sigma-Aldrich, (MO-America) while the acetic acid, acetonitrile, dimethylsulfoxide, ethanol and methanol (HPLC grade) were the products of Merck (Darmstadt, Germany). All other chemicals used were in analytical grade.

2.2. Methods

2.2.1. Analytical method validation

The high performance liquid chromatography (HPLC) method was used for the determination of VOR (Kumar and Sinha 2014). Analyes were performed by Shimadzu 20-A (Japan) HPLC system (automatic injection and PDA detector) with C18 column (column diameter: 4.6 mm, column length: 25.0 cm, particle diameter: 5 µm and particle size: 100 Å) at 255 nm wavelength with 40 °C column temperature. Analyses were carried out using Methanol: Acetic acid solution (0.1%) (v/v) (70:30; pH: 4) as mobile phase with a flow rate of 1 ml min^{-1}. Validation studies were performed according to ICH guidelines (ICH Q2 R1 I 2020).

2.2.2. Formulation of inclusion complexes

In our study, inclusion complexes with eight different natural and synthetic cyclodextrins; α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), hydroxypropyl-cyclodextrin (HP-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) hydroxypropyl-γ-cyclodextrin (HP-γ-CD), methyl-β-cyclodextrin (M-β-CD) and sulfabutylether-β-cyclodextrin (SBE-β-CD) were formulated.

2.2.2.1 Determination of equilibrium times. Determination of the equilibrium times at which the solubility of the active substance is reached its highest point is a very important step prior to the formulation studies of the complexes (Liu et al. 2018). In our study cyclodextrin solutions at 5 mM constant concentration were prepared using distilled water and transferred to 50 ml Eppendorf tubes. An excess amount of VOR was added to the cyclodextrin solutions and was started to be agitated at the highest agitation speed (225 strokes min^{-1}) in a horizontal shaker (MLW-THYS-2, Germany). The starting time was recorded and samples were taken at specified time intervals, filtered with a polyamide membrane filter (pre-saturated, 0.45 µm, Sartorius, Germany) and were analyzed by HPLC. This process was continued until the point where the solubility of VOR remained unchanged.

2.2.2.2 Phase solubility studies. Stoichiometric values of the complexes are mostly determined using solubility phase diagrams (Loftsson et al. 2019). Cyclodextrin solutions were prepared in distilled water within the 0–30 mM concentration range and an excess amount of VOR was added to the solutions. The prepared mixtures were agitated in a horizontal shaker with a shaking speed of 225 strokes min^{-1} during the determined equilibrium time. At the end of the shaking period, the suspensions were filtered with a pre-saturated 0.45 µm polyamide membrane filter and the amount of VOR was determined by HPLC analyses (Pralhad and Rajendrakumar 2004). In our study, solubility phase diagrams of α-CD, β-CD, γ-CD, HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD were plotted and were evaluated along with the stability constants of the complexes (Loftsson and Brewster 2010; Savic-Gajic et al. 2016).

2.2.2.3 Preparation of the complexes. Considering the avoidance of heat exposure as well as the possibility to achieve higher yields of products, lyophilization was selected as the preparation method for the inclusion complexes (Ren et al. 2020). With this method, briefly, VOR and cyclodextrins in calculated molar ratios were dissolved in ethanol: distilled water system. The system was agitated at room temperature, at a constant shaking rate during equilibrium time, as being protected from direct light exposure. At the end of this period, ethanol was evaporated from the system under constant stirring at 200 rpm (WiseStir, Daihan, Korea) at room temperature overnight and the resulting system was frozen at $-80\,^{\circ}C \pm 1\,^{\circ}C$ and lyophilized (Labconco Freezone 6 plus, America). The complexes were collected as dry powders and were stored in well-closed containers until being analyzed.

2.2.3. Characterization studies

2.2.3.1 Morphological analyses. Morphological properties of the prepared complexes were analyzed using scanning electron microscopy (SEM; Carl Zeiss SUPRA 50VP, Oberkochen, Germany).

2.2.3.2 Zeta potential and pH analyses. Zeta potential (ZP) analyses of the prepared complexes were performed using Malvern Nano ZS (Zetasizer Nano Series, Worcestershire, UK). The pH values of the complexes were determined by the WTW Profi Lab (pH597, Weilheim, Germany) pH meter. Analyses were performed after dispersing the constant amount of complexes in distilled water and being vortexed for 2 min for complete dissolvation.

2.2.3.3 Thermal analyses. Differential scanning calorimetry (DSC) analyses were carried out on DSC-60 (Shimadzu, Tokyo, Japan) at a temperature range of 30 °C–300 °C against the empty cell as a reference. Analyses were performed under a nitrogen atmosphere with a flow rate of 50 ml min^{-1}, (with an increase of 10 °C min^{-1}). Thermograms of pure VOR and pure cyclodextrins were used as references for the evaluation of the analyses results of the complexes.

2.2.3.4 X-Ray diffraction analyses. X-ray diffraction (XRD) analyses were performed using D/Max-3C (Rigaku Corporation, Tokyo, Japan) in the range of 2°–40° (2Θ) at a speed of 2° min^{-1}. XRD diffractograms of pure VOR and pure cyclodextrins were used as references for the evaluation of the analyses results of the complexes.

2.2.3.5 Fourier transform infrared analyses. Fourier transform infrared spectroscopy (FT-IR) analyses of the formulations were carried out using the Shimadzu-IR Affinity-1S (Tokyo, Japan) device in the range of 4000 cm^{-1} and 400 cm^{-1}. FT-IR spectra of pure VOR and pure cyclodextrins were used as references for the evaluation of the analyses results of the complexes.

2.2.3.6 Nuclear magnetic resonance analyses. Proton nuclear magnetic resonance (^{1}H-NMR) analyses of the formulations were performed using the Bruker Fourier 300 (Germany). ^{1}H-NMR spectra of pure VOR and cyclodextrins were used as references for the evaluation of the analyses results of the complexes.

2.2.3.7 Determination of voriconazole. In order to determine the amount of VOR of the complexes, 10 mg of the complex was dissolved in 1 ml of distilled water and was analysed by HPLC after proper dilutions. Each analysis was repeated 3 times.

2.2.3.8 Solubility enhancement of voriconazole. In order to investigate the effect of the complex formation on the solubility of VOR in water, the initial solubility of VOR in water was determined. In this study, an excess amount of VOR was added to 2 ml of distilled water and was shaken in a horizontal shaker at room temperature of 25 °C ± 2 °C for 1 h. At the end of the period the dispersion was filtered through a polyamide membrane filter (pre-saturated, 0.45 μm), and the supernatant was analyzed by HPLC for the determination of water solubility of VOR. Analysis was repeated 3 times. And for the determination of the influence of complex formation on the water solubility of VOR, an excess amount of complex was added to 1 ml of distilled water, and dispersion was shaken in a horizontal shaker at room temperature of 25 °C ± 2 °C for 1 h. At the point where the precipitation was observed, the addition of the complex was stopped. The dispersion was filtered through a polyamide membrane filter (pre-saturated, 0.45 μm), and the supernatant was analyzed by HPLC for the determination of water solubility of VOR after complex formation. Analysis was repeated 3 times.

2.2.4. MTT Analyses

The cytotoxic properties of the prepared complexes were analyzed by MTT test using 3T3 mouse fibroblast cells (Eidi et al. 2015). In this method, 3T3 mouse fibroblast cells were grown in 96-well plates in DMEM solution containing 10% fetal bovine serum albumin, HEPES (25 mM), NaHCO3 (3.7 g L^{-1}) and penicillin (100 units mL^{-1}) at a density of 2×10^4 cells. 100 μL^{-1} and left to incubate at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. At the end of the period, the growth medium was removed from each well and the fresh medium which contained different concentrations of VOR solution and dispersions of pure substances and VOR loaded complexes was applied to cells to determine dose-dependent cytotoxicity. The stock VOR solution was prepared by dissolving 2 mg VOR in 250 μL DMSO and medium up to 2 ml. VOR solutions with different concentrations (25, 50, 75 and 100 μg 100 μL^{-1}) were prepared with proper dilutions with the medium before the addition on to the cells. Prior to treating the cells, VOR and cyclodextrin amounts were taken into consideration for complexes and placebo formulations respectively. The samples were applied to the cells and were incubated at 37 °C (5% CO_2 and 95% air) for 48–72 h. After the incubation period, the medium on the cells was removed from each well and replaced with 20 μL (5 μg μL^{-1}) MTT dye and the plates were incubated at the same conditions for another 4 h to transform the MTT dye into formazan crystals. By adding 200 μL DMSO and following 30 min of incubation, quantification of dissolved formazan crystals was performed with a spectrophotometric microplate reader (BIOTEK Brand Cytation5 Model, US) at 570 nm (Büyükköroğlu et al. 2016; Kaur et al. 2020). Each formulation was tested three times and results were expressed as the percentage of absorbance of control cells.

2.2.5. Microbroth Dilution analyses

The antifungal activity of the formulations was evaluated with the CLSI M27-A2 protocol (CLSI 2008). All of the formulations and standard antibiotics (VOR and Ketoconazole (KET)) were dissolved in DMSO within the concentration of 4000–0.0152 μg mL^{-1} 100 μL of formulations, standard antibiotics and gel formulations were transferred to the 96 – well plates. Fresh *C. albicans* (ATCC 90028), *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 90030) and *C. parapsilopsis* (ATCC 22019) cultures were adjusted to 1×10^6 cfu mL^{-1} by McFarland 0.5 standard in Sabouraud Dextrose Broth and inoculated at the concentrations of 100 μL. Positive (KET) and negative control groups were used as references. At the end of this step, all plates were incubated for 24 h at 35 °C. After the incubation period, resazurin was added to the plates at the concentration of 20 μL. The plates were observed and the minimum inhibitory concentration (MIC) was evaluated as the lowest concentration that inhibits microbial growth. The experiments were carried out in 3 replicates.

3 Results and discussion

3.1. Analytical method validation

The linearity studies were performed in the range of 5–100 μg mL^{-1} with r^2 value of 0.9993 ($y = 4235.6x + 69475$). All analyses were repeated six times ($n = 6$). The repeatability values were valued as 2.49, 3.20 and 0.74 while reproducibility values were determined as 3.42, 2.94, 1.25 for the concentrations of 5 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}, respectively in precision studies. The accuracy values were 99.94% ± 0.55–100.74 ± 2.28 ($n = 6$). The limit of detection (LOD) of the method was calculated as 1.6 μg mL^{-1}, while the limit of quantitation (LOQ) was calculated as 3.4 μg mL^{-1} which were both below the linearity studies initial concentration. In the method used, the retention time of VOR with a constant sample volume of 20 μL was recorded at 5.9 min.

3.2. Formulation of inclusion complexes

3.2.1. Determination of equilibrium times

As a result of the studies, the equilibrium time for VOR was determined as 72 h for all inclusion complexes and in all subsequent

studies, the shaking time was kept constant as 72 h at a speed of 225 strokes min^{-1}.

3.2.2. Phase solubility studies

In our study, phase solubility studies were carried out with eight different cyclodextrins; α-CD, β-CD, γ-CD, HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD. Due to the solubility problem of α-CD, 0.5 mM was the highest concentration that could be achieved. Solubility phase diagrams were drawn with determined VOR amount against cyclodextrin concentrations and diagrams obtained were evaluated according to the Higuchi and Connors solubility phase diagram definitions (Loftsson and Brewster 2010). The diagrams obtained in the 0–30 mM concentration range were presented in Figure 1. As a result of the phase solubility studies, it was very clear that α-CD which was determined to have a B_I phase-type diagram had no effect on the solubility of the active agent. β-CD and γ-CD both conformed to the B_S phase-type in the 0–30 mM concentration range also did not have a significant effect on the solubility of VOR therefore the studies envisaged to be carried out with α-CD, β-CD and γ-CD have been terminated

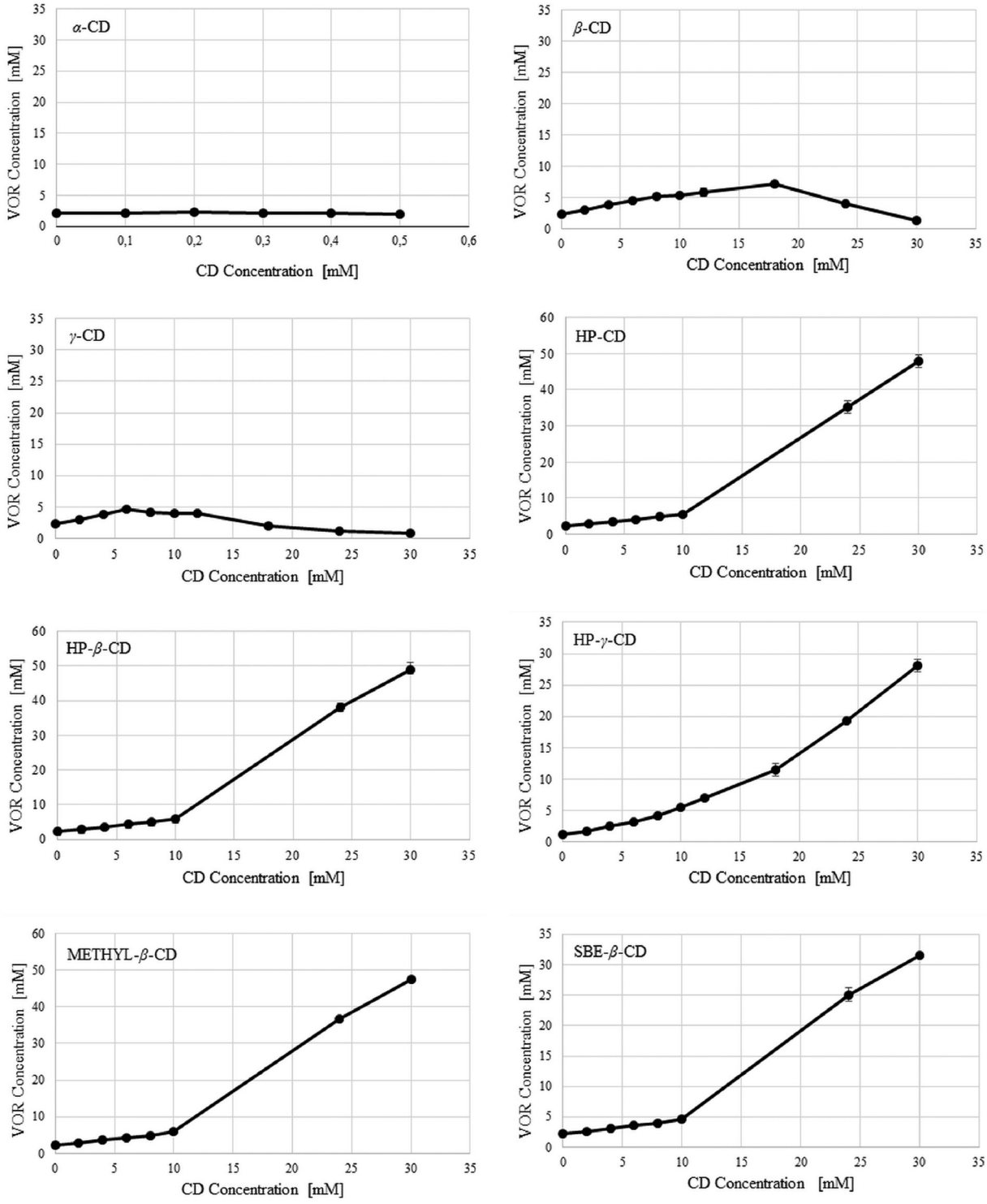


Figure 1. Solubility phase diagrams within the 0–30 mM concentration range (Mean ± SE, $n = 3$).

(Figure 1). HP-CD, HP-β-CD, M-β-CD and SBE-β-CD which formed linear profiles in the 0–10 mM concentration range (A_L type), have transformed to profiles that fit the A_P type solubility phase diagram in the 0–30 mM concentration range (Figure 1). HP-γ-CD preserved its initial 0–10 mM concentration range profile and maintained a profile matching the A_P type solubility phase diagram in the 0–30 mM concentration range (Figure 1). Higuchi and Connors define the A_L type phase diagrams with active substance:CD molar ratio of [1: 1] while [1: 2] for A_P type and [2: 1] for A_N type phase diagrams (Loftsson and Brewster 2010). When the solubility phase diagrams were evaluated, depending on the fact that HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD have A_P type phase diagrams in the 0–30 mM concentration range the molar ratio of VOR: cyclodextrin was determined as [1: 2]. The stability constant of the [1: 2] complexes ($K_{1:2}$, M^{-1}) were calculated using the phase-solubility data according to Equation 1 where the square brackets were the equilibrium concentrations (Savic-Gajic et al. 2016).

$$\mathrm{VOR} + 2\mathrm{CD} = \mathrm{VOR(CD)_2}, \quad K_{1:2} = \left[\mathrm{VOR(CD)_2}\right]/[\mathrm{VOR}][\mathrm{CD}]^2 \qquad (1)$$

The apparent stability constants were valued as 2.95×10^3 M^{-1}, 2.65×10^3 M^{-1}, 0.36×10^3 M^{-1}, 2.53×10^3 M^{-1} and 1.75×10^3 M^{-1} for HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD respectively showing that HP-CD showed more stable complex formations among the cyclodextrins studied (Sadaquat and Akhtar 2020).

3.2.3. Preparation of the complexes

Briefly, VOR and cyclodextrins were dissolved in ethanol: distilled water system in calculated [1: 2] molar ratios. The system was shaken at room temperature (25 °C ± 2 °C) during equilibrium time (for 72 h) with a constant agitation speed (225 strokes min^{-1}) being protected from light (Ren et al. 2020). At the end of this period, ethanol was evaporated overnight under constant stirring of 200 rpm at 25 °C ± 2 °C, and the system was frozen at −80 °C ± 1 °C, then lyophilized. Complexes were collected as dry powders and were stored in tightly closed containers until being analyzed.

3.3. Characterization Studies

3.3.1. Morphological Analyses

SEM analyses were performed in order to evaluate the surface morphology after complex formations. The physical mixtures demonstrated the original morphology of each component. The crystalline structure of VOR disappeared during complex formations and smooth-surfaced, rough, blocklike uniform structures were achieved showing the alteration of the individual morphology of the components revealing successful inclusion complex formation (Figure 2) (Xu et al. 2021).

3.3.2. Zeta potential and pH analyses

In ocular applications, when the route is topical application, the corneal surface is regarded as the main absorption site. Considering the anionic feature of the corneal surface due to sialic acid residues, cationic drug delivery systems tend to be formulated for prolonged contact time with the corneal surface resulting in enhanced ocular bioavailability (Chowhan and Giri 2020). ZP analyses results showed that even the values were not very high the complexes prepared in our study had anionic properties (Table 1). Therefore, no electrostatic interactions were expected between the corneal surface and the complexes however since the inclusion complexes will be incorporated into *in situ* gelling systems prior to the *in vivo* studies, enhanced bioavailability will be achieved by the enhanced residence time on the corneal surface due to the increased viscosity of the formulations (Chowhan and Giri 2020). The analyses results of the pH analyses were valued in the range of 5.25 ± 0.00–5.55 ± 0.02. Despite the reported ocular pH being 7.2–7.4, the eye has the ability to compensate a wide range of pH therefore no adjustments were performed (Table 1) (Başaran et al. 2017).

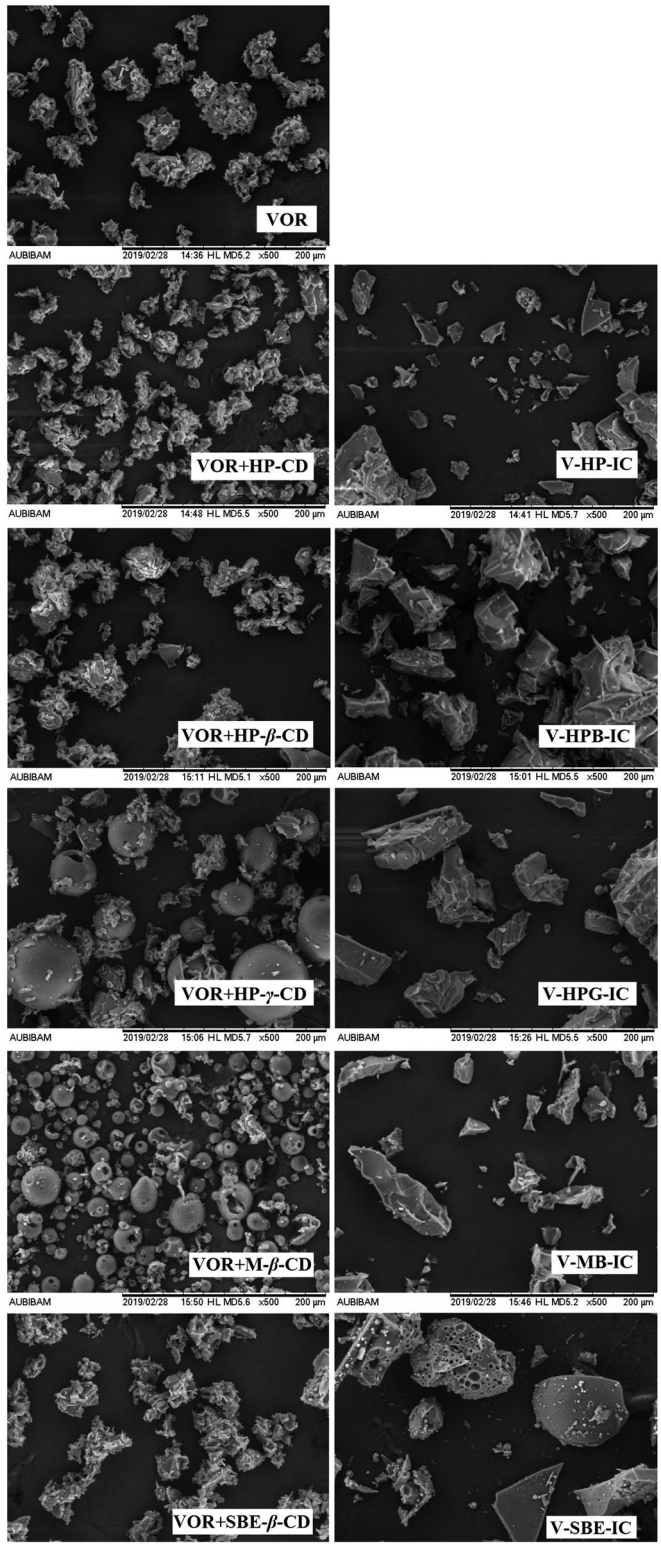


Figure 2. SEM micro-images of VOR, physical mixtures and inclusion complexes.

Table 1. ZP, pH analyses results and VOR content of the inclusion complexes prepared (Mean ± SE; $n = 3$).

Code	Type	ZP (mV)	pH	VOR (mg in 1 mg Complex)	VOR (T%)	VOR (P%)
V-HP-IC	HP-CD	−3.1 ± 0.9	5.3 ± 0.1	0.089 ± 0.002	10.5	8.9 ± 0.2
V-HPB-IC	HP-β-CD	−4.2 ± 0.9	5.2 ± 0.0	0.098 ± 0.005	11.0	9.8 ± 0.5
V-HPG-IC	HP-γ-CD	−6.4 ± 0.2	5.6 ± 0.0	0.079 ± 0.001	9.9	7.9 ± 0.0
V-MB-IC	M-β-CD	−7.1 ± 0.8	5.3 ± 0.1	0.103 ± 0.002	11.5	10.3 ± 0.2
V-SBE-IC	SBE-β-CD	−6.8 ± 1.0	5.5 ± 0.0	0.074 ± 0.003	10.9	7.4 ± 0.3

T%: Theoretical %; P%: Practical %

3.3.3. Thermal analyses

Thermal behaviours and structural properties of the complexes were evaluated by DSC analyses. DSC explains the thermal behavior of active agents before and after complex formation. Absence or a shift to different temperatures of the of endothermic peak of the active agent states the modification in melting, boiling, sublimation points and crystal lattice formations which enlightens the successful complex formations (Sadaquat and Akhtar 2020). Therefore in our study, thermograms of pure ingredients were used as references. An endothermic melting peak at 134 °C was recorded for VOR showing the crystalline structure of the active agent while cyclodextrins showed no remarkable signals due to their amorphous structures (Figure 3). The thermograms of the physical mixtures showed that VOR was present in the mixtures as in crystalline form (marked with arrows). The amorphous structures of the complexes were revealed since no clear melting peak of VOR was revealed in the thermograms of the complexes (Figure 3) (Cui et al. 2012).

3.3.4 X-Ray diffraction analyses

In the process of characterization studies of the cyclodextrins, one of the most important feature is to evaluate the changes in crystalline forms during complexation processes or production methods like lyophilization (Conceição et al. 2019). The crystal or amorphous form transitions can be easily analyzed by both DSC and XRD analyses (Dinesh Kumar et al. 2015). For XRD analyses single crystal XRD analysis is one of the most effective method revealing the formations and structures of crystal hydrates and formation of inclusion complexes of cyclodextrins. However it is very time consuming method therefore powder XRD analyses are mostly referred to get brief results of structural transformations of solid inclusion complexes during the processes (Dang et al. 2011).

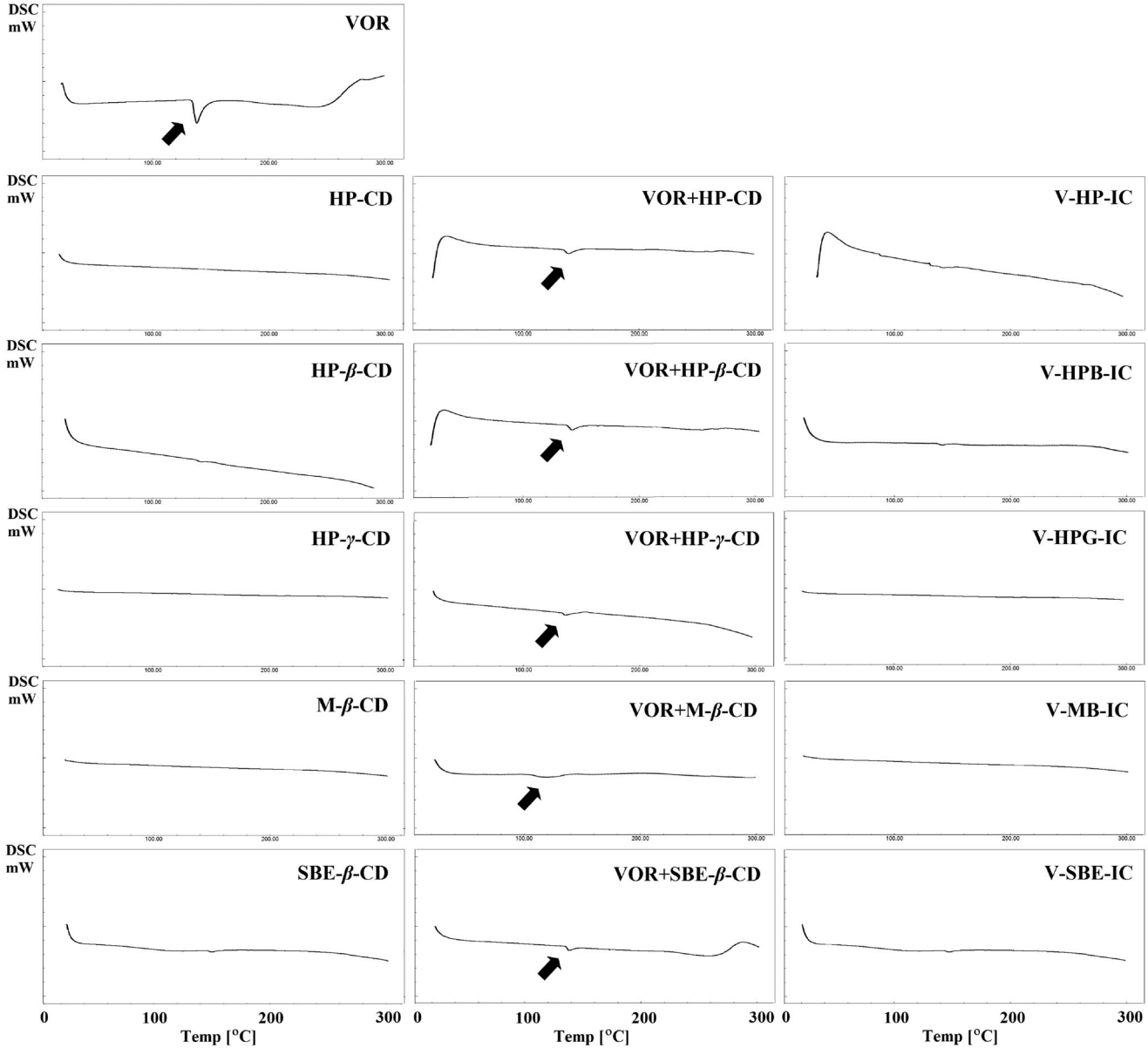


Figure 3. DSC thermograms of VOR, pure cyclodextrins, physical mixtures and inclusion complexes (Endothermic melting peaks of VOR were marked with arrows).

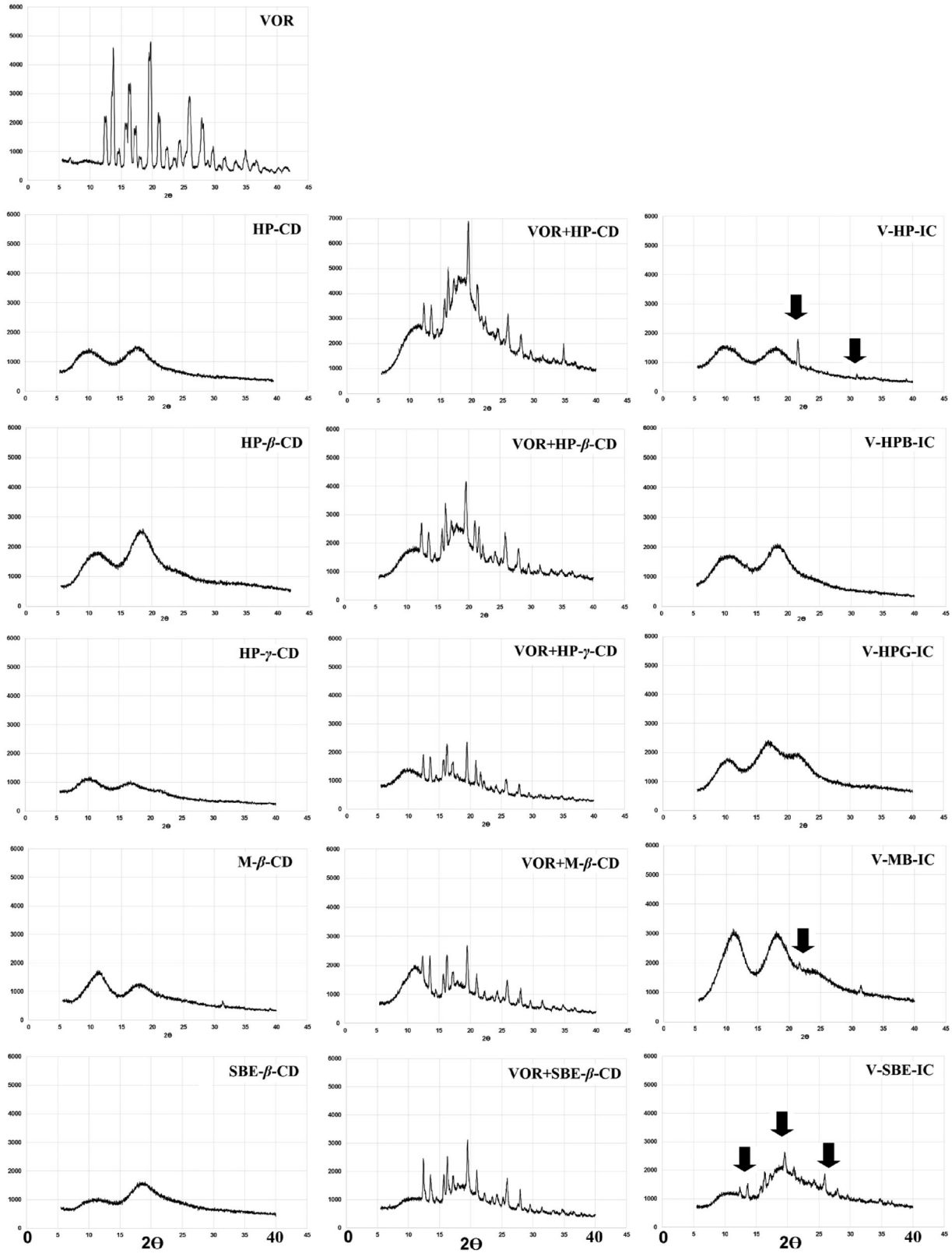


Figure 4. XRD diffractograms of VOR, pure cyclodextrins, physical mixtures and inclusion complexes (Specific signals of VOR crystals were marked with arrows).

In our study in order to determine the structural properties of the prepared complexes as well as the effects of the formulation steps on the crystalline structure of VOR and its complexation, XRD analyzes were performed (Figure 4) (Dinesh Kumar et al. 2015;

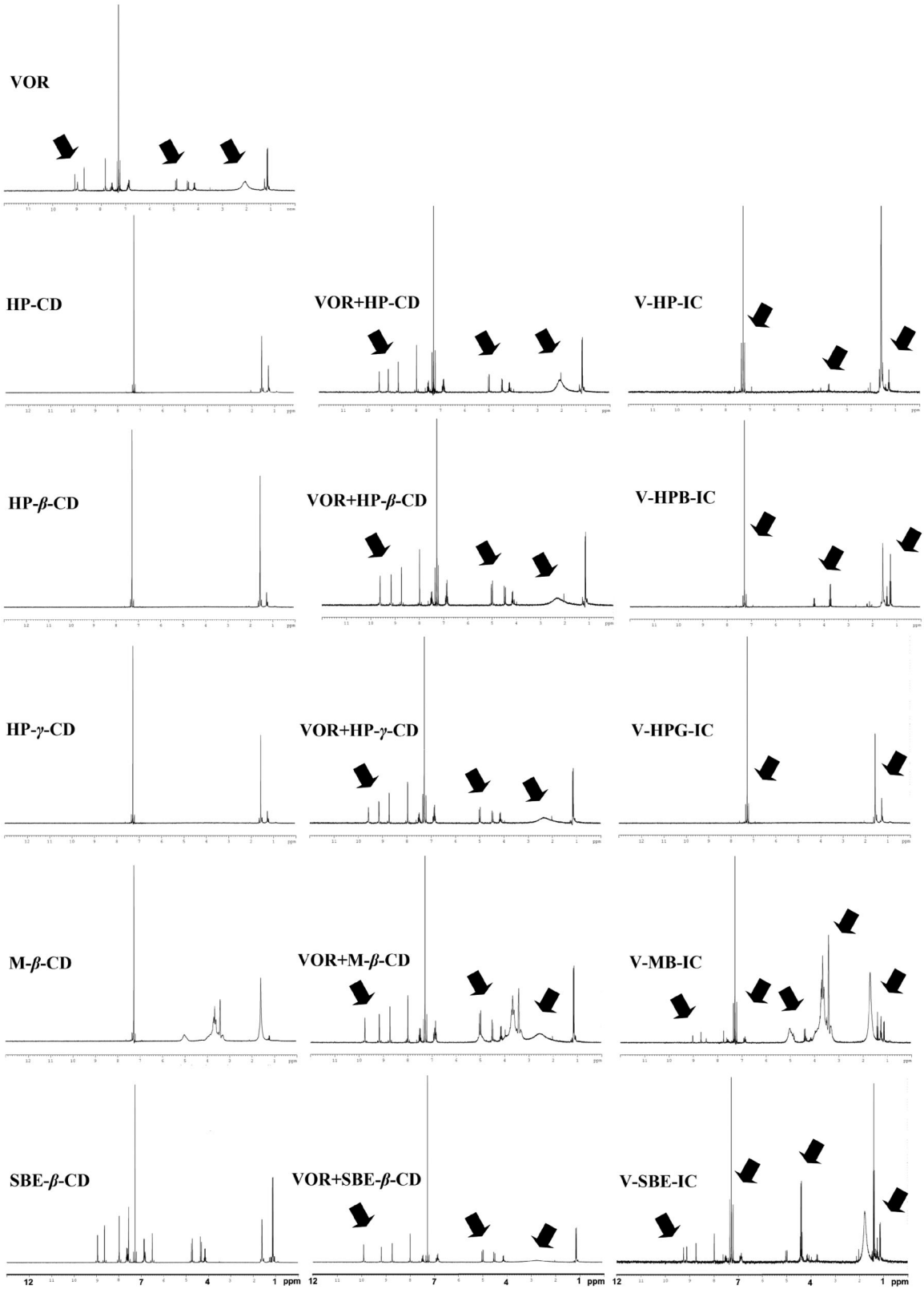


Figure 6. 1H-NMR spectra of VOR, pure cyclodextrins, physical mixtures and inclusion complexes (Specific signals of VOR were marked with arrows).

Conceição et al. 2019). Analyses results revealed the crystalline structure of pure VOR, while all cyclodextrins used were in amorphous state in accordance with DSC analyses (Figure 4). The signals of crystalline structure of VOR was also revealed in the physical mixture diffractograms. However complexation has decreased the crystallinity of VOR and therefore most of the

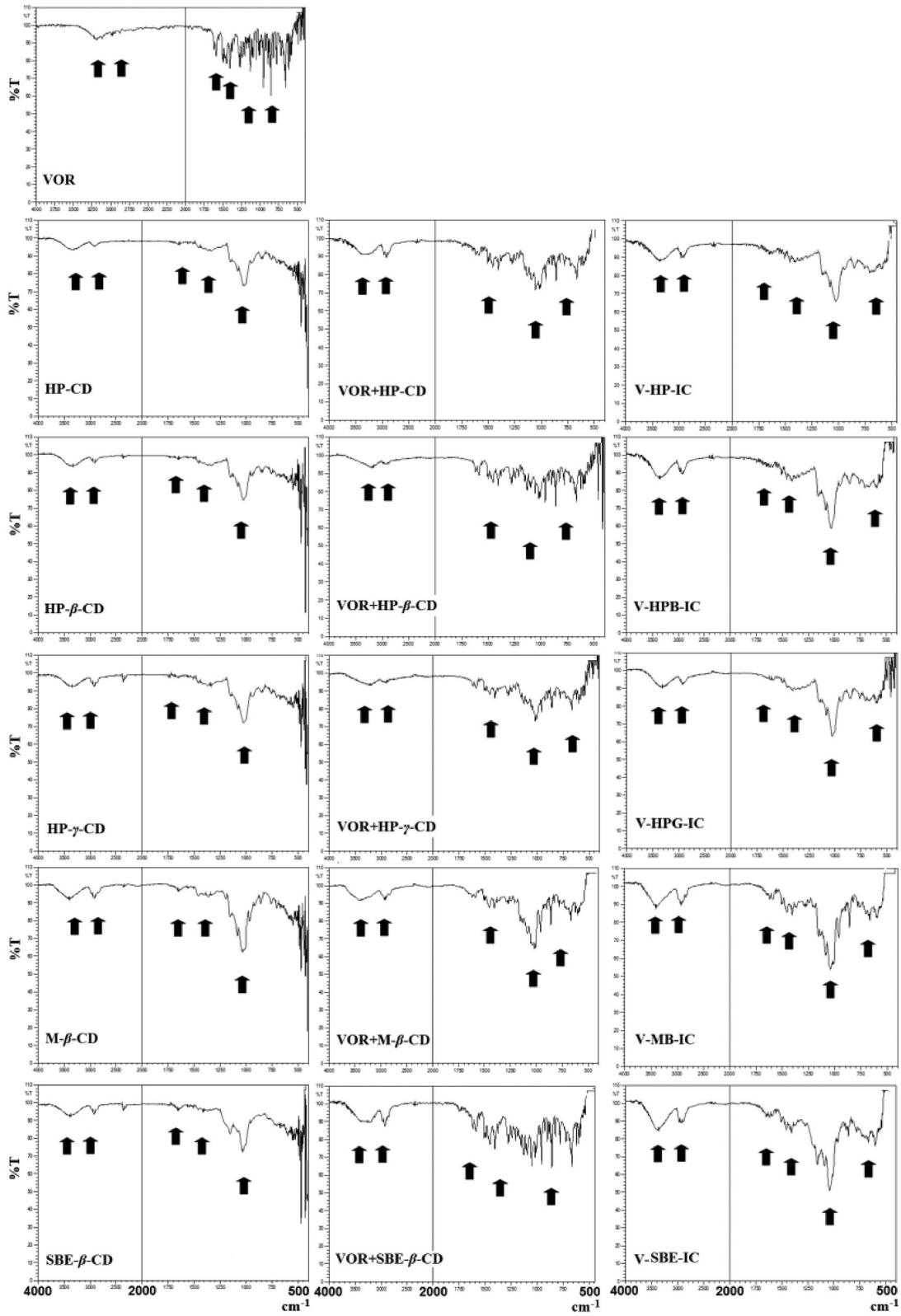


Figure 5. FT-IR spectra of VOR, pure cyclodextrins, physical mixtures and inclusion complexes (Specific signals were marked with arrows).

signals of VOR were dissapeared in the diffractograms of the complexes (Figure 4).

3.3.5. Fourier transform infrared analyses

Chemical interactions between VOR and cyclodextrins were determined with FT-IR analyzes within the wavelength range of 400–4000 cm^{-1} (Figure 6) (Naik and Raval 2016). The characteristic stretching vibrations at 3196 cm^{-1} of -OH, 1585 cm^{-1} of -CO, 1406 cm^{-1} of -CF, 1267 cm^{-1} of -CN and 958 cm^{-1} of C = C were observed for pure VOR and also in physical mixtures. Some of the specified signals for VOR were disappeared in the spectra of the complexes and characteristic signals of pure substances

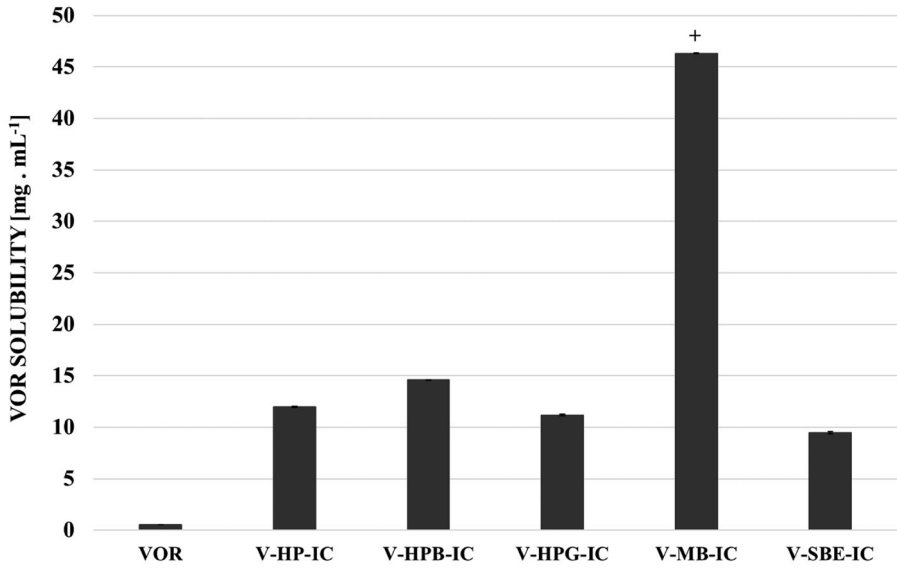


Figure 7. Aqueous solubility values of VOR after inclusion complex formations (Mean ± SE; $n = 3$).

slightly shifted towards lower wavenumbers due to the complexation. The characteristic peaks around 3253 cm^{-1} (stretching vibration of phenolic OH), 1618 cm^{-1} (stretching vibrations of C = O and C = C), 1585 cm^{-1} (stretching vibration of benzene ring skeleton), 1506 cm^{-1} (stretching vibration of aromatic C = C), 1406 cm^{-1} (bending vibration of C–H) and 1018 cm^{-1} (stretching vibration of C–O–C) were recorded for the complexes primarily belong to cyclodextrins (Figure 5) (Wang et al. 2020).

3.3.6. Nuclear magnetic resonance analyses

NMR spectroscopy is well described analysis method for cyclodexrin based inclusion complexes since both guest and host molecules can be simultaneously observed at the atomic level with the determination of complex stoichiometries, association constants, and conformations and to obtain information on their symmetry and dynamics of the complex components (Recio et al. 2018; Melo C de et al. 2020). Signals at 2 ppm, 4–5 ppm and 6.9–7.9 ppm and 8.6–9.1 ppm of pure VOR were clearly revealed in the spectrum of the physical mixture samples (marked with arrows) (Figure 6). Incorporation of VOR within 2 molecules of cyclodextrins [1: 2] hinders some –H signals and especially the signals within 8.7–9.2 ppm were dissappeared in the spectra of V-HP-IC, V-HPB-IC and V-HPG-IC inclusion complexes. Downfield and upfield shifts were also recorded in the spectra of inclusion complexes suggesting the successfull formation of inclusion complexes with all types of cyclodextrins used in our study (Figure 6) (Chakraborty et al. 2019).

3.3.7. Determination of voriconazole

Depending on the molar weight of the ingredients, the amount of active ingredient in each 1 mg complex will differ from each other. The VOR amounts of the complexes were determined by the validated HPLC method and the analyses results revealed the successfull incorporation of VOR in the inner cavities of cyclodextrins (Table 1).

3.3.8. Solubility enhancement of voriconazole

The initial solubility of VOR in water was determined as 0.54 ± 0.02 mg mL^{-1}. In order to investigate the effect of the complexation on the aqueous solubility of VOR, an excess amount of complexes were added to 1 ml of distilled water and the complexes were shaken for 1 h to achieve complete dissolvation. Dispersions were filtered with 0.45 μm polyamide membrane filter and the amounts of VOR in the supernatants were determined by HPLC analyses (Figure 7).

Analyses results clearly stated that complexation with cyclodextrins has enhanced the aqueous solubility of VOR in great extend. In the study approx. 250 mg of complex was sufficient for solubility studies in 1 ml distilled water however for V-MB-IC formulation despite the addition of 782 mg complex to 1 ml distilled water it required more complex to be added. Since the clear difference was achieved compared to other complexes, no more complex was added to the solution and marked as "+" in the graph (Figure 7). With complex formations aqueous solubility of VOR enhanced 22.1, 27.0, 20.7, 85.8 and 17.6 times for V-HP-IC, V-HPB-IC, V-HPG-IC, V-MB-IC and V-SBE-IC respectively (Figure 7).

3.4. Mtt Analyses

In this study, toxicological evaluations of the complexes were carried out by the MTT Test analysis method. The method briefly enables us to gain information about cell viability based on the reduction of the annular structure of tetrazolium salts by the mitochondria of living cells and the transformation of the initially light yellow color to purple due to the formation of formazan crystals (Cao et al. 2007). When evaluating the MTT test results performed on 3T3 mouse fibroblast cells, it was determined that both the active substance and the formulations did not reduce the cell viability below 70% after 24 and 48 h, at selected doses (0.042, 0.084, 0.126, 0.168, 0.210 and 0.252 μg mL^{-1}). None of the formulations at the specified concentrations showed toxic properties, therefore the study was repeated by increasing the applied concentrations up to 25, 50, 75 and 100 μg 100 μL^{-1} for the determination of the toxicity limits at 48 and 72 h (Figure 8).

Considering the MTT analyses results, pure VOR showed dose and time dependant toxicity. The effect of the lowest dose (25 μg 100 μL^{-1}) on cell viability at the end of 48 h was determined as 82.13% and it was consistent with the preliminary studies. And the toxicity has incresed and cell viability was recorded as 53.08% after 72 h (Figure 8). In the 48-h evaluation of pure HP-CD, it was observed that except the highest dose all other does increased cell viability showing that HP-CD has no

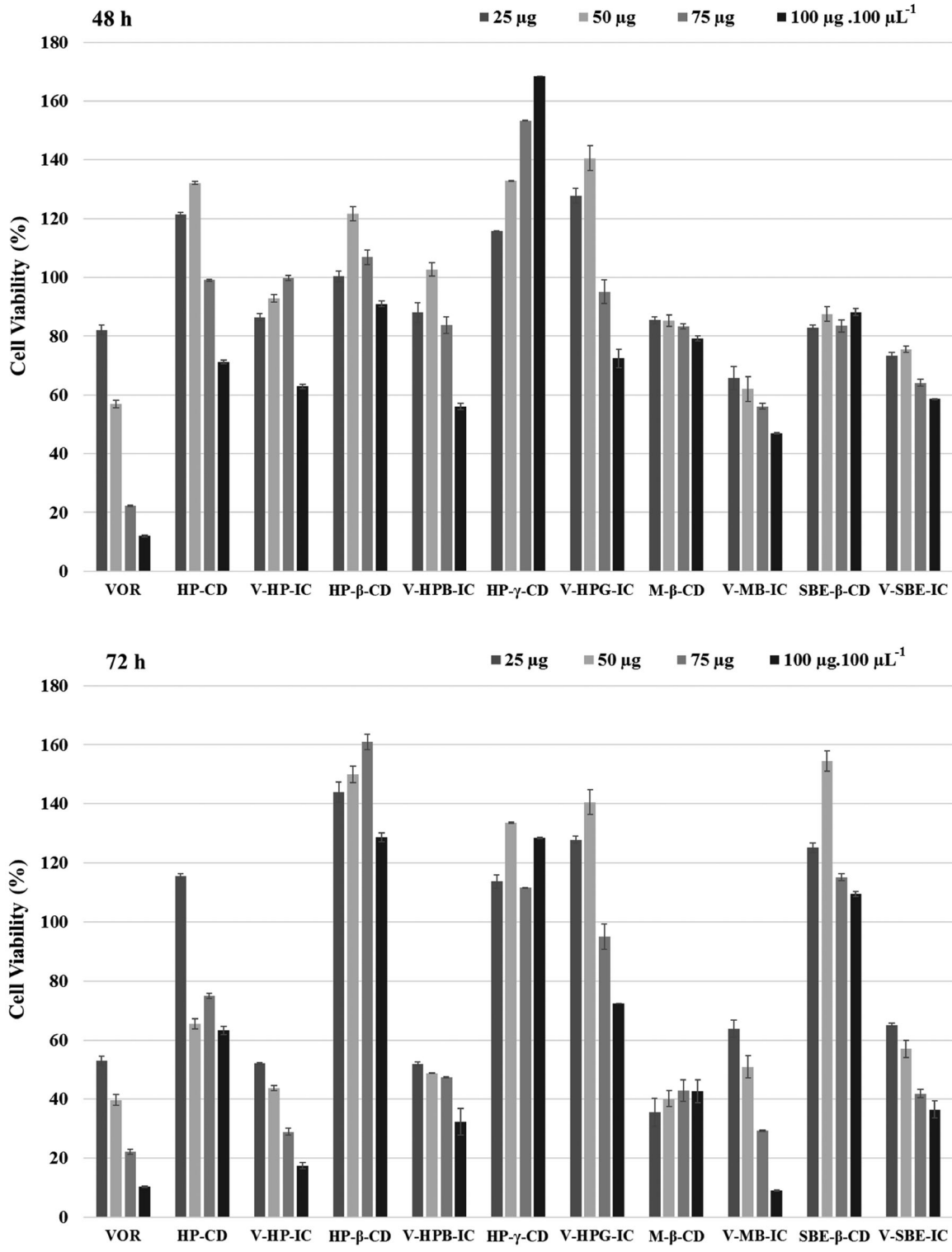


Figure 8. MTT analyses results of pure VOR, pure cyclodextrins and the inclusion complexes (Mean ± SE; $n = 3$).

toxicity on the 3T3 mouse fibroblast cell lines after 48 h. However after 72 h, it was determined that the first dose showed the ability to increase cell viability while other doses reduced cell viability to 60%. For V-HP-IC complex it was determined that the highest dose reduced cell viability to 62% at the end of 48 h. And after 72 h, it was observed that all doses decreased cell viability below 60% (52.1, 43.8, 29.0 and 17.5% for 25, 50, 75 and 100 µg 100 µL^{-1} respectively) (Figure 8). Pure HP-β-CD enhanced the cell viability in the first three doses (to max 160.8%) with respect to the time and dose. For the V-HPB-IC, toxicity was observed only with the highest dose at 48 h. After 72 h, cell viability decreased to 50% for all doses and toxicity was observed (Figure 8). Pure HP-γ-CD has increased the cell viability (max 168.5%) depending on the increasing dose in both time periods. For V-HPG-IC, cell viability was over 70% in all time periods and at all doses, and even an increase in cell viability was observed for the 1st and 2nd doses ($\sim$ 120% and 140%) (Figure 8). Pure M-β-CD showed no toxicity during 48 h at all

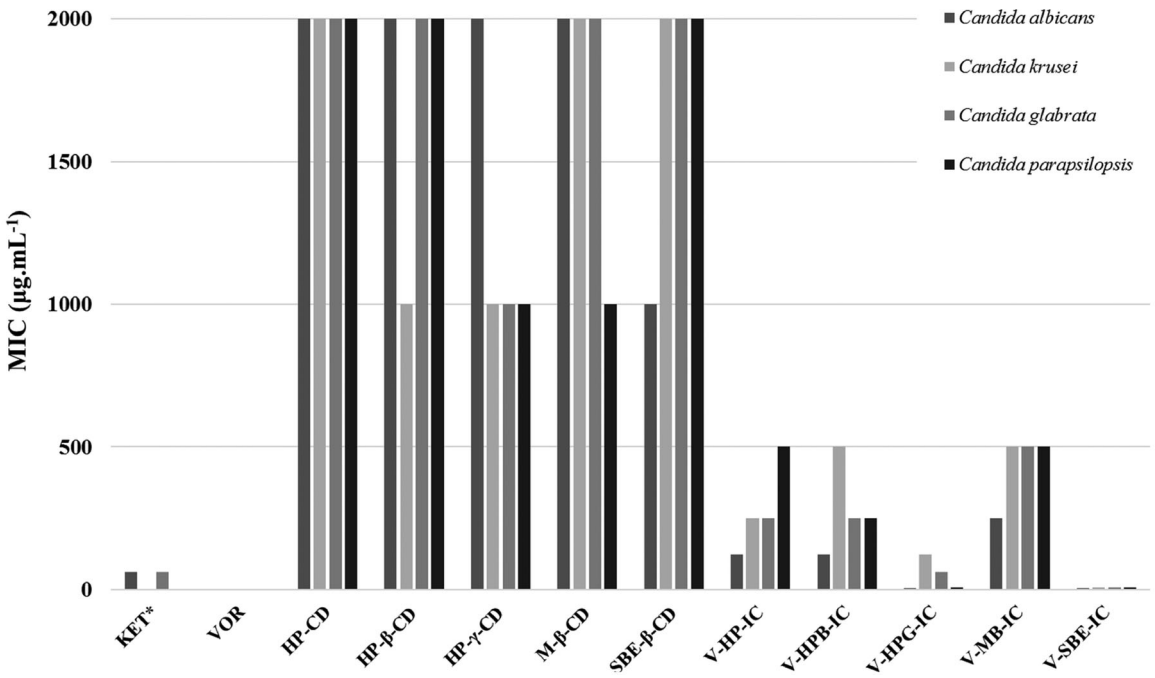


Figure 9. The MIC values of pure VOR, pure cyclodextrins and the inclusion complexes (*= + control Ketoconazole).

doses, however, toxicity was observed after 72 h at all doses. For V-MB-IC the highest toxicity levels were recorded among the complexes studied with cell viability values below 70% for all doses after 72 h (63.9, 50.9, 29.2 and 9.1% for 25, 50, 75 and 100 µg 100 µL^{-1} respectively) (Figure 8). And for pure SBE-β-CD no toxicity was recorded at all doses and on the contrary, an enhanced cell viabilities were achieved even after 72 h. For V-SBE-IC, cell viabilities were decreased below 70% (65.2, 57.0, 41.9 and 36.5% for 25, 50, 75 and 100 µg 100 µL^{-1} respectively) in accordance with other complexes after 72 h (Figure 8). Our results showed that the toxicity of pure VOR was higher than all the complexes showing that complexation has potential to provide toxicity-reducing effects of the active agents which will enhance the ocular safety of the formulations by topical application while maintaining the antifungal activity.

3.5. Microbroth Dilution analyses

In vitro antifungal activities of the complexes were evaluated by Microbroth Dilution Method (Başaran et al. 2017). Highest MIC values, were associated with the lowest bactericidal power of the applied formulation (de Almeida Magalhães et al. 2020). Analyses results, revealed the higher efficacy of VOR in comparison with positive control KET showing that VOR has very high potential for the treatment of severe KET resistant *Candida* related ocular infections (Figure 9). None of the pure cyclodextrins showed antifungal activity against *Candida* strains by gaining the highest MIC values (Figure 9). Complex formations increases the solubility of lipophilic drugs and also decreases the drug efflux from the cells maintaining higher intracellular drug levels for efficient therapeutic effect (Elmotasem and Awad 2020). In accordance with the literature, all of the inclusion complexes were found to be active against all selected strains of *Candida* showing the efficacy of the formulations with enhanced solubility of incorporated VOR (Figure 9). Depending on the cyclodextrin type, the efficacy of the formulations also differentiated on different types of Candida strains. V-SBE-IC has shown the most remarkable activity even at the lowest concentrations when compared with other formulations also with better results than positive control KET on both selected Candida strains (Figure 9). Therefore, regarding low MIC values, all the inclusion complexes prepared have potentials for the treatment of severe ocular fungal infections.

Conclusion

Corneal penetration is regarded as the main penetration route via topical application. Therefore the attems of enhancement of ocular bioavailability focused on the elavated transportation of the drug from corneal layers. Depending on the unique structure of cornea it acts as a barrier to both hydrophilic and lipophilic compounds. Therefore the first attemt to enhance the poor ocular bioavailability of the applied drug (mostly lypophilic drugs) is to enhance water solubility of the active compound. In our study inclusion complexes with α-CD, β-CD, γ-CD, HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD were formulated with lyophilization method for the enhancement of aqueous solubility of VOR. Solubility phase diagrams were plotted and analyses results revealed that α-CD, β-CD and γ-CD showed no significant effect on the solubility of VOR while HP-CD, HP-β-CD, HP-γ-CD, M-β-CD and SBE-β-CD showed A_p type phase diagramms [1: 2] within the 0–30 mM concentration range. *In vitro* characterization studies were peformed in detail with SEM, DSC, XRD, FT-IR and ^{1}H-NMR analyses. Successfull complex formations were revealed with structural analyses. With complex formations, aqueous solubility of VOR was enhanced 22.1, 27.0, 20.7, 85.8 and 17.6 times with V-HP-IC, V-HPB-IC, V-HPG-IC, V-MB-IC and V-SBE-IC respectively. Safety of the formulations were determined on 3T3 mouse fibroblast cell lines according to the MTT analyses results. Remarkable antifungal activities of the complexes were revealed with Microbroth Dilution Analyses on *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilopsis* strains. Our study stated that, incorporation of VOR into inclusion complexes will enhance the ocular bioavailability of VOR with enhanced aqueous solubility while providing safe and effective treatment of severe ocular fungal infections.

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Disclosure statement

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