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Comparative evaluation of lyophilized voriconazole formulations marketed in European Union

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ABSTRACT: Voriconazole is an antifungal derived from the structure of fluconazole and is designed to increase the efficacy and spectrum of action of fluconazole. In addition to the original formulation of the lyophilized product, there are several approved formulations in Europe. The aim of this study is to compare three lyophilized formulations of voriconazole in terms of manufacturing problems, stability and cost. For this purpose, three approved formulations were prepared. Briefly, sulfobutyl ether β-cyclodextrin (SBE-β-CD) was used to prepare the reference product formulation. The second formulation was prepared using arginine (Arg) and hydroxypropyl beta-cyclodextrin (HP-β-CD) as excipients. And the last formulation was prepared using sodium chloride (NaCl) and HP-β-CD as excipients. The prepared formulations were lyophilized. After lyophilization, the structure of the cake, assay, in vitro antifungal activity and stability of the formulations were evaluated. It was found that the formulation containing HP-β-CD and Arg should be freeze-dried at a lower primary drying temperature compared to the other formulations. The assays and in vitro antifungal activities of the formulations were found to be similar. The accelerated stability results showed that the impurity increase of the finished products in the Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations was similar to that of the reference product. The impurity increase was slower in the Vor-HP-β-CD arginine formulation than in the others. The cost of Vor-SBE-β-CD was also higher than the others. Despite the advantages of SBE-β-CD, such as providing the same formula as the original product, HP-β-CD and NaCl/Arg-containing formulations can provide the targeted chemical quality at low cost.

KEYWORDS: Voriconazole; HP-β-CD; SBE-β-CD; Lyophilized Formulation; Arginine; Sodium Chloride

1. INTRODUCTION

In the 1990s, although many fungal infections were treated with fluconazole and itraconazole, the absorption problem of itraconazole and the limited spectrum of fluconazole were limiting factors for these two drugs [1]. Voriconazole is an antifungal agent derived from the structure of fluconazole and designed to increase the potency and spectrum of activity of fluconazole. Unlike fluconazole, voriconazole shows activity against molds such as *Aspergillus* [2]. Voriconazole is primarily used in life-threatening infections and therapeutic indications of voriconazole are listed as: treatment of invasive aspergillosis, treatment of candidaemia non-neutropenic patients, treatment of fluconazole-resistant serious invasive *Candida* infections, treatment of serious fungal infections caused by *Scedosporium* spp. and *Fusarium* spp. [3].

Tablet, suspension and lyophilized powder formulations of voriconazole are commercially available [4]. Among these, sulfobutylether β-cyclodextrin (SBE-β-CD) is used to dissolve and stabilize voriconazole in the reference lyophilized dosage form (VFEND 200 mg powder for solution for infusion). The use of this complex was described in the patent US6632803B1/EP1001813B1 and the protection period of this patent ended in Europe in 2018. In the patent numbered EP1001813B1, information was given that voriconazole has low solubility (0.2mg/ml at pH 3) and that it is not stable in water, and solution preparation information using SBE-β-CD is described [5].

On the other hand, when other Voriconazole 200 mg Powder for Solution for Infusion formulations approved in Europe are examined, it is seen that two more formulations are approved, different from the

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original product formulation. One of these formulations, used in Voriconazole Accordpharma 200 mg Powder for Solution for Infusion, contains arginine hydrochloride and hydroxypropyl beta cyclodextrin (HP-β-CD) as excipients [6]. Preparation method of this formulation with arginine (Arg) was also described in US20140275122A1 by Fresenius Kabi USA LLC [7]. The other formulation, Voriconazole 200 mg powder for solution for infusion, is marketed by Aspire Pharma Ltd. and Anfarm Hellas S.A. It is seen that sodium chloride (NaCl) is used in addition to HP-β-CD in this formulation [8].

This study aims to compare these three formulations, marketed in Europe, in terms of manufacturing difficulties, stability and cost. For this purpose, three approved formulations were prepared and lyophilized and evaluated with in vitro antifungal activity and accelerated stability tests. Finally, comparisons were performed based on determined criteria.

2. RESULTS

The reference product formulation which is containing 200 mg Voriconazole and 3200 mg SBE-β-CD in a vial is coded as Vor-SBE-β-CD. The second formulation containing 200 mg Voriconazole, 2660 mg HP-β-CD and 1000 mg Arg in a vial is coded as Vor-HP-β-CD-Arginine. The last formulation containing 200 mg Voriconazole, 2400 mg HP-β-CD and 225.6 mg NaCl in a vial is coded as Vor-HP-β-CD-NaCl. Preparation methods and details were given in Methods Section. Lyophilisation of formulations was carried out at -5 °C (Table 8), in order to evaluate the conditions of the three formulations in the same cycle. At the end of this lyophilisation cycle, the cake structure of the Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations was obtained properly, while puffing and melting were observed in the Vor-HP-β-CD-Arginine formulation (Figure 1). To obtain smooth cake structure for Vor-HP-β-CD-Arginine formulation, primary drying temperature was selected as -15 °C with a separate recipe (Table 9).

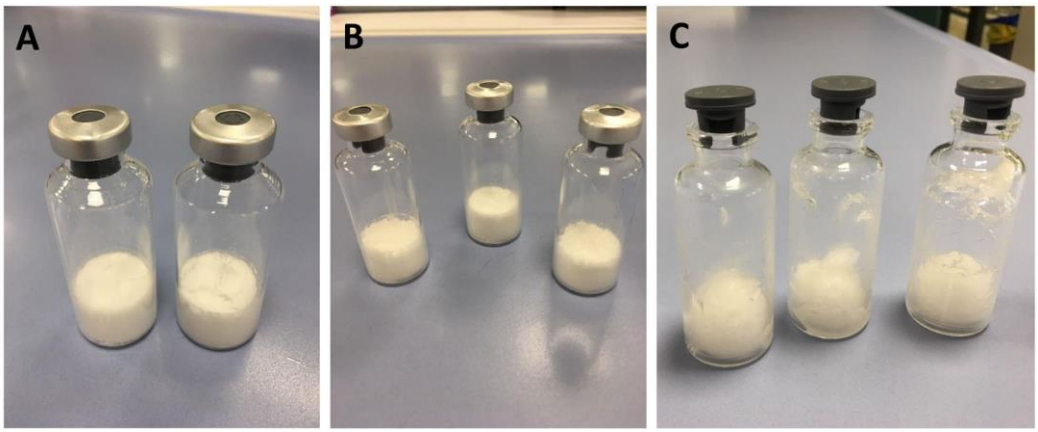

Figure 1. Cake appearance of formulations after lyophilisation cycle described in Table 8: (A) Vor-SBE-β-CD, (B) Vor-HP-β-CD-NaCl, (C) Vor-HP-β-CD-Arginine. *Because of melting in Vor-HP-β-CD-Arginine formulation, product was lyophilized as described in Table 9.

Karl Fischer Titration method was used for water content analysis. Water contents for Vor-SBE-β-CD, Vor-HP-β-CD-NaCl, Vor-HP-β-CD-Arginine formulations were found 1.86±0.94%, 0.65±0.04%, 0.97±0.23%, respectively (Table 1). The difference between humidity values did not reach a statistically significant level (for each comparison $p>0.05$). Reconstitution times for each formulation were determined. This analysis was carried out by adding 19 ml of water to 1 vial, as stated in the instructions for use of Vfend, and the time that the vial was completely dissolved was recorded. When the reconstitution times were examined, it was found as 39±2.1 seconds, 40±1.4 seconds, 37±1.4 seconds for Vor-SBE-β-CD, Vor-HP-β-CD-NaCl, Vor-HP-β-CD-Arginine formulations. The difference between the reconstitution times did not reach a statistically significant level (for each comparison $p>0.05$).

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Table 1. Analyses of lyophilized formulations

	Vor-SBE-β-CD	Vor-HP-β-CD-NaCl	Vor-HP-β-CD-Arginine
Water Content (%)	1.86±0.94	0.65±0.04	0.97±0.23
Assay (%)	102.7±0.2	102.6±0.2	102±3.8
Antifungal Activity against *C. krusei* (MIC value, µg/mL)	<0.97	<0.97	<0.97
Antifungal Activity against *C. glabrata* (MIC value, µg/mL)	>500	>500	>500

The resulting cake structures of all three formulations were acceptable. Assay values were calculated for all three formulations after lyophilization, 102.7±0.2%, 102.6±0.2% and 102±3.8% for Vor-SBE-β-CD, Vor-HP-β-CD-NaCl, Vor-HP-β-CD-Arginine formulations, respectively. The difference between the assay values did not reach a statistically significant level (for each comparison $p>0.05$). Antifungal activity analyses against *C. krusei* and C. *glabrata* bacteria belonging to 3 different formulations were studied. Antifungal activity results were similar for the three formulations. (Table 1)

Initial impurity analyses of 3 different formulations were also studied and compared with the original product, Vfend. In addition, 3 separate lyophilized products and the original product were stored at 40°C 75% RH, which is the accelerated stability condition, for 1 month, and the change in impurities of these products was investigated (Table 2-3).

Table 2. Impurity analyses of reference product and Vor-SBE-β-CD formulation after 1 month at 40°C 75 %RH

	VFEND (Reference Product)		Vor-SBE-β-CD	
	Beginning	**1 Month 40°C 75 %RH**	**Beginning**	**1 Month 40°C 75 %RH**
Impurity A (%)	0.07	0.15	0.01	0.08
Impurity B (%)	ND	ND	ND	ND
Impurity C (%)	0.04	0.08	0.04	0.03
Maximum unknown impurity (%)	0.03	0.07	0.03	0.05
Total impurity (%)	0.1	0.3	0.2	0.3

When the reference product was kept at 40°C 75% RH conditions for 1 month, an increase of 0.08% was observed in Impurity A value, 0.07% in Vor-SBE-β-CD formulation, 0.08% in Vor-HP-β-CD-NaCl formulation. An increase of 0.03% was observed in the HP-β-CD-Arginine formulation (Table 2-3). While the impurity increase rate in the Vor- HP-β-CD-Arginine formulation was found to be significantly slower than the other Vor-SBE-β-CD formulation ($p=0.016$), Vor-HP-β-CD-NaCl formulation ($p=0.007$), and the reference drug ($p=0.007$), the impurity increase rate in the reference drug and the other two formulations was found to be similar (for each comparison $p>0.05$).

Table 3. Impurity analyses of Vor-HP-β-CD-NaCl and Vor- HP-β-CD-Arginine formulations after 1 month at 40°C 75 %RH

	Vor-HP-β-CD-NaCl		Vor- HP-β-CD-Arginine	
	Beginning	**1 Month 40°C 75 %RH**	**Beginning**	**1 Month 40°C 75 %RH**
Impurity A (%)	0.01	0.09	0.01	0.04
Impurity B (%)	ND	ND	ND	ND
Impurity C (%)	0.004	0.05	0.005	0.01
Maximum unknown impurity (%)	0.03	0.05	0.03	0.03
Total impurities (%)	0.1	0.3	0.1	0.1

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3. DISCUSSION

Voriconazole is an active substance with low water solubility (0.2mg/ml at pH 3) [3, 5]. SBE-β-CD was used in order to make it soluble in water and to prepare its formulation that can be used parentally, and voriconazole was made soluble by forming a cyclodextrin complex. Voriconazole-cyclodextrin complex is available in lyophilized form, which is have limited stability in aqueous media [9]. Cyclodextrins are excipients that are frequently used without dissolving them by forming complexes with water-insoluble drugs, thanks to their hydrophobic cavity and hydrophilic outer surfaces. It seems that many different cyclodextrins are preferred according to the way of use of the drugs [10, 11].

Among the six cyclodextrins available on the market today, HP-β-CD and SBE-β-CD seem to be used in parenteral drugs. After IV administration, cyclodextrins are rapidly lost from the system and excreted by the kidneys. It appears that up to 16 grams of HP-β-CD (itraconazole) per day and 14 g of SBE-β-CD (voriconazole) per day are used in commercially available parenteral formulations. Taking the guideline published by EMA as reference, it states that 250 mg/kg/day for 21 days (HP-β-CD) or 6 months (SBE-β-CD) are found safe in humans older than 2 years [12].

When the posology of the reference product VFEND 200 mg powder for solution for infusion (VFEND) is examined, it is seen that the highest dose is in the group of Children (2 to <12 years) and young adolescents with low body weight (12 to 14 years and <50 kg). It seems that Loading Dose Regimen (first 24 hours) is 9 mg/kg every 12 hours and Maintenance Dose (after first 24 hours) is 8 mg/kg twice daily. From this point of view, the amount of cyclodextrin taken on the first day in a formulation containing 3200 mg of cyclodextrin versus 200 mg of voriconazole appears to be 288 mg/kg/day at a continuation dose of 256 mg/kg/day. Even if these values are slightly above the EMA recommendation, they are the amounts used by the original drug [9, 12]. SBE-β-CD is used to dissolve and stabilize VFEND 200 mg powder for solution for infusion voriconazole, and the use of this complex was described in the patent US6632803B1 / EP1001813B1 and the protection period of this patent expired in Europe in 2018[5]. Since the formulation was under patent protection until 2018, alternative formulations were developed before 2018. These alternative formulations have led to the development of equivalent drugs with HP-β-CD, another cyclodextrin that can be used parentally.

One of these equivalents is the formulation containing HP-β-CD and NaCl in addition to voriconazole commercialized by Aspire Pharma Ltd on the EMC site and commercialized by Aspen Pharma Pty Ltd in Australia [8, 13]. Each vial contains 2400 mg of HP-β-CD and additionally 225.60 mg of NaCl to increase the decreased osmotic pressure. In addition, it is stated that HCl is used to adjust the pH. Another equivalent containing HP-β-CD is the formulation commercialized in Europe by Fresenius Kabi and Accord Pharma. The formulation contains L-arginine (or HCl salt) as stabilizing agent and pH adjusting agents in addition to HP-β-CD. Related formulation preparation trials are described in the patent application US20140275122A1 of Fresenius Kabi [6, 7].

In this study, it was aimed to compare these three formulations currently licensed in Europe. For this purpose, an evaluation was made in terms of solution preparation step, lyophilisation step, accelerated stability and cost.

During the solution preparation process, it is seen that the reference product was filled into a 20 mL vial when analysed and filled with 1.13 ml (5.65%) overfill [14]. Since it was determined in our preliminary experiments that it was possible to prepare the solution for all three formulations in 10 mL, the total solution volume was determined as 10 mL and solutions were prepared with 5.65% overfill. In the Vor-HP-β-CD-Arginine formulation, it was observed that the pH increased due to the presence of arginine and caused degradation of voriconazole [15]. For this reason, arginine was dissolved first and the pH was adjusted to the range where voriconazole was stable, and then cyclodextrin and voriconazole were added to the solution to form a complex. The fact that the voriconazole solution is sensitive to basic conditions and arginine changes the pH significantly makes the solution preparation step of this formulation more difficult than the others. This obstacle could be overcome by using HCl salt of arginine. In the Vor-HP-β-CD-NaCl formulation, the dissolution rate was found to be slower than the Vor-SBE-β-CD formulation. The slower dissolution is clearly due to the fact that the amount of cyclodextrin is much less than the amount of SBE-β-CD.

One of the most critical steps in the production of a lyophilized product is the lyophilisation process of the product. The lyophilisation process basically consists of freezing, primary drying and secondary drying steps [16]. Among these steps, the primary drying temperature is the most critical step in obtaining the product in accordance with the desired properties, shortening the production process and eliminating many costs in production. When the lyophilisation processes for the voriconazol-cyclodextrin complex were

examined, it was stated that the products tested with the patent numbered US20140275122A1 HP-β-CD and SBE-β-CD were frozen at -45°C, primary drying was done at a temperature between -15 and -35°C, and secondary drying was done at 40°C. In this patent, there is no information about which process is used for which product and cake structures [7]. The patent numbered EP2409699B1 is aimed at increasing the stability of HP-β-CD and voriconazole complex by formulating them to contain glycine [17]. The collapse temperature of the prepared formulation was found in the range of -18 to -25 C. In the present invention, the lyophilisation process is sublimated in the range of -27 to -30, a temperature below this temperature. Based on this information, lyophilisation was carried out at -5 °C, a primary drying temperature higher than that specified in the patents, in order to evaluate the conditions of the three formulations in the same cycle. At the end of this lyophilisation cycle, the cake structure of the Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations was obtained properly, while puffing and melting were observed in the Vor-HP-β-CD-Arginine formulation (Figure 1) [18]. To obtain smooth cake structure for Vor-HP-β-CD-Arginine formulation, primary drying temperature was selected as -15 °C with a separate recipe. Then proper cake structure was obtained with Vor-HP-β-CD-Arginine formulation. These trials show that Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations can be lyophilized at higher temperatures than Vor-HP-β-CD-Arginine formulation and are more advantageous in terms of lyophilisation. Also, it was observed that the formulation change did not change the in vitro antifungal activity. (Table 1)

Another criterion, when the impurity increases rates of the finished products at 1 month 40°C 75 %RH were examined, it was found that increase rate of impurities in Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations was similar to the reference product. It was determined that this rate was lower in the Vor-HP-β-CD-Arginine formulation (Table 2, 3). The unit costs of the products are also calculated and expressed symbolically in Table 9 and the final comparison of the three formulations is summarized in Table 4.

Table 4. The final comparison of the three formulations

	Manufacturing Difficulties	Stability	Cost
Vor-SBE-β-CD	+++	++	€€
Vor-HP-β-CD-NaCl	+++	++	€
Vor- HP-β-CD-Arginine	++	+++	€

4. CONCLUSION

In this study, it was found that the formulation containing HP-β-CD and Arg should be lyophilized at a lower primary drying temperature compared to the other formulations. The accelerated stability results showed that the impurity increase rates of the finished products in the Vor-SBE-β-CD and Vor-HP-β-CD-NaCl formulations were similar to the reference product. This rate was lower in the Vor-HP-β-CD arginine formulation. The cost of Vor-SBE-β-CD was also higher than the others. In conclusion, despite the advantages of SBE-β-CD, such as the same formula as the original product, HP-β-CD and NaCl-containing formulations can provide the targeted chemical quality at low cost.

5. MATERIALS AND METHODS

5.1. Materials

Voriconazole was obtained from SynergeneAPI (India). HP-β-CD and SBE-β-CD were purchased from Shandong Binzhou (China). NaCl was purchased from Dominion Salt (New Zealand). Arg was obtained from Ningbo-Create-Bio Engineering (China). Type I glass was purchased from Schott AG (Germany) and teflon coated chlorobutyl stopper was purchased from Hualan (China). All other products were either chromatography grade or extra pure.

5.2. Determination of Formulations and Preparation of Solutions

The reference product formulation content was created with reference to SmPC information and patent information (EP1001813B1) [5, 9]. Only the total volume was set as 10 mL in order to shorten the lyophilisation time. The formulation is coded as Vor-SBE-β-CD and shown in Table 5.

Table 5. Formulation of Vor-SBE-β-CD

Ingredient	mg/vial	Function
Voriconazole	200	Active ingredient
SBE-β-CD	3200	Complexing agent/ solubilizing agent
Water for injection	to 10 ml	Solvent used and removed in lyophilisation

The preparation methods for Vor-SBE-β-CD were carried out as follows:

• With continuous mixing with a mechanical stirrer (IKA Eurostar 20 Digital) at 750 rpm, SBE-β-CD was added to 70% of the volume of water for injection and stirred at 25 °C until all SBE-β-CD was dissolved.

• Voriconazole was added and dissolved at 25 °C with stirring.

• The solution is made up to volume with water for injection.

• Considering the withdrawable volume, 10 ml of solution was filled into 30R Type 1 glass vials with 5.65% overfill, half-plugged with stopper, and made ready for lyophilisation.

The product containing HP-β-CD and Arg has been prepared as described in US20140275122A1[7]. The formulation is coded as Vor-HP-β-CD-Arginine and shown in Table 6.

Table 6. Formulation of Vor-HP-β-CD-Arginine

Ingredient	mg/vial	Function
Voriconazole	200	Active ingredient
HP-β-CD	2660	Complexing agent/ solubilizing agent
Arginine	1000	Stabilizing agent
Hydrochloric Acid	q.s.	for pH adjustment
Water for injection	to 10 ml	Solvent used and removed in lyophilisation

The preparation methods of Vor-HP-β-CD-Arginine were carried out as follows:

• At 25 °C, Arg is added to 40% of the water by stirring continuously with a magnetic stirrer (IKA Eurostar 20 Digital) at 750 rpm and dissolved by adding 30% (v/v) HCl solution.

• The pH is adjusted up to around 7 with concentrated hydrochloric acid. The pH is adjusted between 5.4 and 5.8, preferably with 5.6 or 0.1 N HCl.

• It is diluted to be 70% of the final water and the pH is measured.

• HP-β-CD is added and dissolved at 25 °C.

• Voriconazole is added and dissolved at 25 °C.

• The pH is measured, and if there is a change, it is adjusted to 5.6 with 0.1N hydrochloric acid.

• Complete to volume pH is measured.

• Considering the withdrawable volume, 10 ml of solution was filled into 30R Type 1 glass vials with 5.65% overfill, half-plugged with stopper, and made ready for lyophilisation.

The product containing HP-β-CD and NaCl was prepared with reference to the Australian Public Assessment Report file of the commercial product Vorcon offered by Aspen Pharma Pty Ltd. [13]. The formulation is coded as Vor-HP-β-CD-NaCl and shown in Table 7.

Table 7. Formulation of Vor-HP-β-CD-NaCl

Ingredient	mg/vial	Function
Voriconazole	200	Active ingredient
HP-β-CD	2400	Complexing agent/ solubilizing agent
NaCl	225.6	Stabilizing agent/ Tonicity agent
Hydrochloric acid	q.s.	for pH adjustment
Water for injection	to 10 ml	Solvents used and removed in lyophilisation

The preparation methods of Vor-HP-β-CD-NaCl were carried out as follows.

• With continuous mixing with a mechanical stirrer (IKA Eurostar 20 Digital) at 750 rpm, HP-β-CD was added to 70% of the volume of water for injection and mixing was continued at 25 °C until all was dissolved (IKA Eurostar 20 Digital) Voriconazole was added and at 30 °C. It was dissolved by stirring.

• The pH was adjusted to 5.6 with 0.1N hydrochloric acid.

• Added 225.60 mg NaCl after dissolving.

• The solution is made up to volume with water for injection.

• Considering the withdrawable volume, 10 ml of solution was filled into 30R Type 1 glass vials with 5.65% overfill, half-plugged with stopper, and made ready for lyophilisation.

5.3. Lyophilisation of Formulations

No details are given about the lyophilisation process of voriconazole in the patent number EP1001813B1[5]. When the patent numbered US20140275122A1 is examined, it is stated that the products tested with SBE-β-CD or HP-β-CD are frozen at -45°C, primary drying is done at a temperature between -15 and -35°C, and secondary drying is done at 40°C [7]. In this patent, there is no information about which process is used for which product and cake structures.

Based on this information, determined three formulations were lyophilized with lyophilisation cycle described in Table 8 (Christ Epsilon 2-60 LSC).

Table 8. Lyophilisation cycle using for lyophilisation of Vor-HP-β-CD-NaCl and Vor-SBE-β-CD and Vor-HP-β-CD-Arginine

Steps	Starting Temperature (^{0}C)	End Temperature (^{0}C)	Pressure (mbar)	Time (Minutes)
1	20	-45	1000	150
2	-45	-45	1000	180
3	-45	-5	0.1	280
4	-5	-5	0.1	2400
5	-5	40	0.06	155
6	40	40	0.06	480

When the cakes obtained were examined, it was determined that while the cake structures of Vor-HP-β-CD-NaCl and Vor-SBE-β-CD were suitable, the Vor-HP-β-CD-Arginine formulation was not suitable (Figure 1). So Vor-HP-β-CD-Arginine formulation was lyophilized with lyophilisation cycle described in Table 9.

Table 9. Lyophilisation cycle using for lyophilisation of Vor-HP-β-CD-Arginine

Steps	Starting Temperature (⁰C)	End Temperature (⁰C)	Pressure (mbar)	Time (Minutes)
1	20	-45	1000	150
2	-45	-45	1000	300
3	-45	-15	0.1	280
4	-15	-15	0.1	3300
5	-15	40	0.06	155
6	40	40	0.06	480

5.4. Analytical Methods

For quantification assay analyses of formulations, Inertsil ODS-3V, 150 x 4.6 mm, 5 µm column was used under isocratic conditions with a 1-mL/min flow rate at 35 °C column temperature. Analyses were performed at 256 nm with a UV detector (e2695, Waters). Injection volume was 10 µL. The mobile phase consists of Buffer solution: Methanol: Acetonitrile (400: 350: 250) (v/v/v). Buffer solution consists of 1.9 g of ammonium acetate and 1000 mL of deionized water and pH of buffer adjusted to pH 4.0 ± 0.05 with glacial acetic acid.

For impurity analyses of formulations, Inertsil ODS-3V, 150 x 4.6 mm, 5 µm column was used with a 1-mL/min flow rate at 35 °C. Analyses were performed at 256 nm with a UV detector (e2695, Waters). Injection volume was 60 µL. The mobile phase consists of Buffer Solution: Methanol: Acetonitrile. Buffer solution prepared as described above and gradient system details given in Supplement Table 1.

5.5. Stability Studies

For determination of stability in accelerated conditions, formulations and reference product was kept at 40°C 75% RH conditions (KBF 720, Binder). After a month, impurity A, impurity B, impurity C, maximum unknown impurity and total impurities were analysed.

5.6. Antifungal Activity Assays

Voriconazole formulations were screened on fungal strains according to the standard procedure of CLSI [19]. *Candida krusei* and *Candida glabrata* were used to test the antifungal activity of formulations. The Minimum Inhibition Concentration (MIC) test was performed by the microdilution method using 96-well microplates. The wells were prepared to contain finally 100 µl volume of Sabouraud Dextrose Broth (SDB) medium including voriconazole formulations at different concentrations. Using 1000 ppm stock Voriconazole solutions, the voriconazole concentration in the first well was prepared to be 250 µg/ml. Serial dilutions were made in a 1: 2 ratio and the concentrations of the next wells were adjusted to 125, 62.5, 31.25, 15.625, 7.1, 3.90, 1.95, and 0.97 µg/ml, respectively. The microbial suspension adjusted to a 0.5 McFarland standard was inoculated into wells. The same procedures were applied as the positive control, with Voriconazole and Fluconazole for fungal cells. Each microorganism was incubated for 48 hours following optimum growth conditions. Voriconazole and fluconazole were used against *Candida* sp. strains as standard reference drugs.

5.7. Statistical analysis

All of the measurements were performed at least in duplicates. Data were presented as the mean ± standard deviation. The comparison of more than two groups were investigated using ANOVA followed by Tukey post hoc test. Tests were performed with Minitab®16 (Minitab Inc.; State College, PA, USA).

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Conflict of interest statement: Adem Şahin, Göksu Yağ Çilingiroğlu, Tuğçe Çatak, Sakine Ulusoy and Sena Özlem Gündoğdu are employee of Centurion Pharma.

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