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DEVELOPMENT OF A LIQUID ORAL *IN-SITU* GEL OF VORICONAZOLE FOR SUSTAINED RELEASE AND ENHANCED BIOAVAILABILITY: EQUIVALENCE TESTING USING EARTH MOVER'S DISTANCE

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ABSTRACT: Development of a sustained release liquid oral *in-situ* gel of Voriconazole with improved bioavailability has been the main aim of this project. Complexation of the drug with hydroxypropyl beta-cyclodextrin was done to improve the solubility and stability of the drug. The mucoadhesive polymer carbopol 934P and release retardant HPMC E50 were used as factors, and a two-square factorial design was employed. A simple mixing method was used for the formulation, and evaluation was done. The polymers had a significant effect on gelation time, mucoadhesive strength, and 8 h drug release. Carbopol showed a better- controlled release and mucoadhesive strength than HPMC E50. The experimental values for gelation time, mucoadhesive strength, drug release at 1 h, 8 h, 12 h, and gel strength were found to be 80 sec, 17453 dynes/cm, 21.10%, 62.98%, 90.54%, 55 sec respectively, which were close to the predicted values. Pharmacokinetic studies revealed a 12 h sustained drug release, plasma drug concentrations above 0.5µg/ml (MIC) and 6.76-fold increase in bioavailability. Thermal and photostability studies revealed a shelf life of 2 years. Equivalence testing proved that the prepared formulation was better than the reference as the Ɵtest (0.6835) < Ɵstd (0.7963). An elegant, needle-free liquid oral *in-situ* gel of Voriconazole could thus be successfully developed by statistical optimization techniques using mucoadhesive polymers to sustain the drug release up to 12 h, improve bioavailability and patient compliance.

INTRODUCTION: Voriconazoleis the second oral drug licensed for the treatment of invasive fungal infections such as aspergillosis. The present therapy to treat fungal infections includes systemic, topical administration and suspensions.

However, adverse effects and in many cases, less concentration of the drug at the site of infection have recently led to the development of various novel drug delivery systems such as *in-situ* gelling polymeric drug delivery systems, designed with the objective of retaining the drug in the gastrointestinal tract to achieve local as well as systemic effects in a controlled manner.

Mucoadhesion is widely used in many of the novel drug delivery systems for sustained delivery [1]. Oral *in-situ* gels or smart, environment-sensitive gels are often administered as low viscosity, solutions or

suspensions. Under sensitive environment or a combination of stimuli, the polymers undergo conformational changes to produce a gel which prolongs the contact time between the drug and the absorptive sites in the stomach, releasing the drug slowly and continuously [2].

An increasing number of liquid oral *in-situ* gelling systems have been reported in the literature for various biomedical applications, including drug delivery and tissue repair [3]. Such smart polymeric systems represent a promising means of delivering drugs. Administration as liquid orals gives better compliance, especially for pediatric and geriatric patients [4, 5]. Bioadhesive drug delivery systems have been reported for various drugs like ciclopirox olamine [6], sumatriptan [7], fluconazole [8], mebeverine hydrochloride [9] using natural polymers like chitosan, sodium alginate, pectin, synthetic polymers such as cellulose derivatives and carbopol.

Voriconazole (VCZ) is chemically a triazole used to treat invasive fungal infections seen in immunocompromised patients including invasive candidiasis, aspergillosis, and other emerging fungal infections [10]. Chronic cavitary pulmonary aspergillosis (CCPA) is characterized by a slowly progressive and symptomatic disease with multiple pulmonary cavities [11]. Voriconazole is rapidly and almost completely absorbed following oral administration, with a T_{max} of less than 2 h, the elimination half-life approximately 6 to 9 h at 3 mg/kg i.v. or 200 mg orally [12]. The present therapy to treat fungal infections includes systemic, topical administration, and suspensions are giving way to novel drug delivery systems [13]. Among all newly discovered chemical entities, about 40% of drugs are lipophilic and fail to reach the market due to poor aqueous solubility [14]. Voriconazole is a lipophilic, BCS Class II drug with a low pH-dependent aqueous solubility (0.71 mg/mL), a maximum solubility of 2.7 mg/mL at pH 1.2, 0.2 mg/mL at pH 3 and 0.61 mg/mL at pH 7 and also has photostability problems. Its low bioavailability is due to limited solubility in water.

Cyclodextrins (CDs) are hydrophilic, popular for their ability to form inclusion complexes that increase the aqueous solubility, chemical stability and provides the driving force for diffusion across the biological membrane for lipophilic drugs. Some chemically modified CDs such as hydroxypropyl-beta-cyclodextrin (HPβCD) have gained importance, because of their suitable cavity sizes and greater hydrophilicity [15, 16]. Carbopol is a well-known, pH-dependent polyacrylic acid (PAA) polymer that displays a sol-gel phase transition in aqueous solution as a result of raising the pH above its pKa of about 5.5. At high pH, the PAA swells, releasing drug molecules to the environment. Carbopol stays as a solution in acidic pH but forms a low viscosity gel at alkaline pH. It is extensively exploited for its mucoadhesive properties [17, 18]. HPMC is incorporated to enhance the viscosity of the carbopol solution while reducing its acidity, thus aiding in sustained drug delivery. Studies have been done using a carbopol-HPMC aqueous solution for plasmid DNA delivery [19].

The objective of the present study was to develop a liquid oral *in-situ* gelling system of Voriconazole using mucoadhesive polymers with pH-sensitive gelation, favorable rheological and drug release properties to give a needle-free, patient-friendly, elegant, sustained release dosage form with enhanced bioavailability. Encapsulation in a *β*-cyclodextrin derivative would increase its solubility and stability in aqueous solutions (the dose being 400mg / 10ml) while maintaining its lipophilicity and high permeability. Statistical optimization techniques would be employed to investigate the effect of the factors (polymer concentrations) on selected responses.

MATERIALS AND METHODS: Voriconazole was obtained as a gift sample from Dr. Reddy's Labs, Hyderabad, India. Carbopol-934P and HPMC E50 were purchased from Yarrow Chem products and all other chemicals used were of analytical grade.

Pre-formulation Studies Using FT-IR Spectroscopy: Compatibility studies were done by keeping physical mixtures of the drug and polymers at 40 °C for a period of one month. The spectra were scanned using the FTIR spectro-photometer (Model αE ATR module, BRUKER) between the wavelength range of 4000 to 400 cm⁻¹.

Design of Experiment: For the Design of experiment (DOE), the Design-Expert Software® (version 9.0.5.1), Stat-Ease Inc., Minneapolis,

USA) was used. A two-square factorial design was employed, where the two independent variables, *i.e.,* concentrations of carbopol-934 P (Factor A) and HPMC E50 (Factor B) were considered at two levels. The higher and lower values were coded as +1 and -1 and fed into the software. The dependent variables (responses) considered and evaluated were gelation time (R_1), mucoadhesive strength (R_2), drug release at 1 h (R_3), 8 h (R_4) and 12 h (R_5), gel strength (R_6) [20].

Preparation of a Liquid Oral Mucoadhesive *in-situ* Gelling System: The inclusion complex of VCZ (400 mg dose/10 ml) and HPβCD was first prepared in a 1:3 molar ratio by kneading method and then the drug solution was prepared by dissolving the complex in distilled water. 40-100 mg/ml of lactose (stabilizing and sweetening agent) was then added to it. Carbopol-934P (0.30-0.70% w/v) and HPMC E50 (0.1-0.25% w/v) were each added to deionized water with constant stirring using a magnetic stirrer and gentle heat until a uniform solution was obtained. 0.4% w/v NaOH was added to neutralize the free acid liberated from carbopol-934P.

Sodium metabisulphite (0.02% w/v as a preservative) was then added to it. The stabilized drug solution was added to the polymer solution with continuous stirring until a liquid *in-situ* gelling system was obtained. A batch minimum of 100 ml was prepared [21, 22].

Evaluation:
pH: The formulation pH was determined using a calibrated pH meter (EUTECH Instruments, pH Tutor). The readings were done in triplicate [23, 24].

Gelation Time: The time taken for the sol to convert to gel was determined by adding 5 ml of the sol to a phosphate buffer solution (pH 6) and noting the time taken for gelation [25].

Rheological Studies: Viscosity determination was carried out in triplicate using the Brookfield synchro electric viscometer (Brookfield, Massachusetts, USA). The formulation (250 ml) was poured into the small adaptor of the viscometer and the angular velocity was increased gradually from 10 to 50 rpm at 37 °C. The hierarchy of the angular velocity was then reversed and the average of both was taken to calculate the viscosity [26, 27].

Mucoadhesive Strength: The mucoadhesive strength of the formulation was determined by using a section of intestinal mucosa of a guinea pig, fixing it with the mucosal side out onto each glass vial using a rubber band. The vial was connected to a balance in an inverted position while the other vial was placed on a height-adjustable pan. The gel was added onto the mucosa of the first vial and evenly spread on the surface of the test membrane. Then the height of the latter vial was so adjusted that the mucosal surfaces of both the vials would be in close contact.

Two-minute contact time was allowed, and weight in the pan increased till the vials detached. The minimum weight required to detach the two vials was considered as the mucoadhesive force. The readings were done in triplicate after replacing with fresh mucosa each time [28].

Gel Strength: 50 gm of the gel was taken in a 100 ml graduated cylinder. A 20 gm weight was allowed to penetrate into the gel and the time taken by the apparatus to sink 5 cm through the gel was noted [29].

***In-vitro* Drug Release Studies:** *In-vitro*, drug release studies were carried out using the USP Type II (Paddle type) dissolution apparatus covered with a black sheet to protect the drug from light. 5 ml of the sol was taken in a small vial and immersed in the dissolution medium (pH1.2 buffer for 2h and pH 6 phosphate buffer for 10 h) at 37 ± 0.5 °C and 50 rpm. 5 ml aliquot was withdrawn and simultaneously replaced by 5 ml of a fresh buffer at time intervals of 30 min, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h. The samples were suitably diluted and measured spectrophotometrically at a wavelength of 255 nm. The experiments were done in triplicate using phosphate buffer as a blank and the average taken. The mechanism of drug release was also analyzed [30].

Drug Content: 100 mg of drug was taken dissolved in 1 drop of methanol and diluted with 10 ml of Millipore water. The solution was stirred for some time until the drug dissolved. From this solution, 1 ml was taken, diluted to 10 ml and analyzed using the UV spectrophotometer at 255 nm.

Regression Analysis: The response parameters were statistically analyzed using the ANOVA at 0.05 levels. The Linear model was used for evaluating each response parameter using the equation of multiple regression analysis:

$$R = b_0 + b_1A + b_2B + b_3AB + b_1A\ldots\ldots$$

Where, R was the level of the measured response, b0 the intercept of the arithmetic mean response of 'n' run s (here, n = 4 runs) and A and B the coded level of the independent variables.

Optimization: Optimization was done using the numerical optimization technique, and the desirability approach was employed to generate optimum settings for the formulation. The gelation time was kept at a minimum, mucoadhesive strength was kept at a maximum gel strength was kept at maximum, the drug release at 1 h was kept at maximum while 8 h and 12 h were kept in range.

Quantification of Plasma Voriconazole by HPLC: An Agilent HPLC system (LC Compact-1120 model, Germany) with a binary pump and EZ Chrome software was used for the study. The mobile phase used was a combination of acetonitrile and MilliQ water (7:3 v/v) with 1 ml/min flow rate. A standard graph was first prepared by taking appropriate dilutions of the VCZ stock solution (1g/l) in methanol. The samples were then vortexed and centrifuged (Spinwin, Tarsons, India) at 14000 rpm for 15 min. Plasma protein precipitation was carried out using perchloric acid. The filtered supernatant was passed through the stationary phase *i.e.*, a Waters X bridge column (250 × 4.6 mm, 5 µm) at 25 °C for the separation of the VCZ and UV detection was done at 254 nm [31].

***In-vivo* Pharmacokinetic Studies on New Zealand Male Rabbits:** The study protocol for the animal experiments approved by the Institutional Animal Ethics Committee (CPSCEA Registration no.: 1564/PO/a/11/CPSEA-23-11-2012) was followed as it complied with the Institutional Guidelines on Animal Experimentation. Two groups consisting of six healthy New Zealand white male rabbits each (having an average weight of 2.86 ± 0.12 kg) were taken. They were allowed to fast 18 h before and during the pharmacokinetic study. In the crossover study with one week apart as washout period, 5 ml

of the optimized formulation (test) containing an equivalent of 40 mg VCZ/kg was orally administered to one group using a stomach sonde needle while 5 ml of the pure drug solution (reference) was given to the other group. Blood samples (1.5 ml) were withdrawn *via* a cannula from the marginal ear vein of rabbits and collected in heparinized tubes at the time intervals of 0, 1, 2, 3, 4, 5, 8, 10 and 12 h. Quantification of VCZ in rabbit plasma was done using the HPLC method mentioned in the previous section [32]. The software for Pharmacokinetics/ Pharmacodynamics analysis, Kinetica 5.0 was utilized to calculate the pharmacokinetic parameters.

Equivalence Testing of Reference and Test Plasma Profiles based on Earth Mover's Distance: The population bioequivalence (PBE) statistical approach was recommended by the FDA to compare descriptors from the test and reference products to support product equivalence. The population bioequivalence approach considered both mean and variance information. Given two sets of profiles (reference *vs.* test), a reference center was first calculated by taking the grand average of all reference profile data.

The Earth Mover's Distance (EMD), a statistical metric was then applied to calculate the distance between the reference center and each individual reference profile. Similarly, the distance between the reference center and each individual test profile was also calculated using the EMD. The obtained two groups of EMD distances were then used as input to PBE for conducting a statistical test between the two groups to establish (in) equivalence between the test and reference [33, 34, 35].

Population Bioequivalence Approach: This approach was used to complement the average values. Bioequivalence (BE) method only explicitly controls the mean difference, by considering both the mean as well as the variability of the reference and test products. The criterion for population bioequivalence was summarized as follows:

$$PBC = \begin{cases} \dfrac{(\mu_T - \mu_R)^2 + (\sigma_T^2 - \sigma_R^2)}{\sigma_R^2} \leq \theta, \ if \ \sigma_R \geq \sigma_{T_0} \\ \dfrac{(\mu_T - \mu_R)^2 + (\sigma_T^2 - \sigma_R^2)}{\sigma_{T_0}^2} \leq \theta, \ if \ \sigma_R \leq \sigma_{T_0} \end{cases}$$

Eq. (1)

Where, μ_T and μ_R was the mean of the reference and test products, σ^2_T and σ^2_R the total variance of the test and reference drug products, σ^2_{To} a predefined scaling factor and m represented the Bioequivalence limit (1.25).

$$\theta = (ln\ (m))^2/(0.25)^2 \qquad \text{Eq. (2)}$$

Stability Studies: To establish the most appropriate storage conditions, stability studies were conducted as per ICH Q1AR guidelines. Long-term stability studies were done on the optimized formulation at $5°C \pm 3\ °C$ and $25°C \pm 2°C/60\%$ RH $\pm$ for 12 months. Accelerated stability studies were done for 6 months at $40°C \pm 2°C$ / 75% RH $\pm$ 5%. The optimized formulation was stored in tightly closed amber glass vials (shielded from light)in the humidity chamber and the clarity, pH and drug content periodically assessed (0, 3 weeks, 6 weeks, 6, 9 and 12 months). The experiments were done in triplicate [36, 37].

Photo Stability Studies: These studies were performed according to ICH Q1B guidelines using a Neutronic chamber in UV light and tube light (1.2 million lux hours) and an integrated near UV energy not less than 200 Watt/sq.m [38].

RESULTS AND DISCUSSION:

Compatibility Studies using FTIR: The drug-excipient interaction studies done using FTIR showed that the drug was compatible with all the excipients used in the formulations there were no extra peaks or shifting of peaks of the functional groups of Voriconazole (all peaks were within $\pm$ $5cm^{-1}$) in the spectra of binary mixtures of drug and excipients.

Design of Experiment: The concentration of the polymers (factors X_1, X_2) and the values obtained for selected responses (Y) are as seen in **Table 1**.

TABLE 1: FORMULATION CHART AS PER TWO FACTORIAL DESIGN ALONG WITH THE RESPONSES OBSERVED

Factors			Responses					
	Factor A	Factor B	R1	R2	R3	R4	R5	R6
Formulation code	Carbopol (g)	HPMC E50 (mg)	Gelation time (sec)	Mucoadhesive strength (dynes/cm^2)	% CDR 1 h	% CDR 8 h	% CDR 12 h	Gel strength (sec)
F1	30	10	128±0.41	13339±0.84	14.45±0.97	82.67±1.44	98.53±1.20	43±1.20
F2	70	10	60±0.28	18693±0.28	12.69±1.97	73.85±1.36	94.98±1.24	60 ±1.36
F3	30	25	116±0.24	14787±0.13	11.43±2.02	75.57±1.67	92.78±1.28	49±1.67
F4	70	25	51±0.18	20617±0.67	16.79±0.90	84.57±0.98	98.12±2.03	68±1.03

Fitting an empirical polynomial equation to these experimental results would help in optimizing the formula. The general polynomial equation considered was as follows:

$Y = B_0 + B_1 X_1 + B_2 X_2 + B_3 X_3 ……..+ B_{12} X_1X_2 + B_{13} X_1X_3 +…..$

pH: All the liquid oral formulations had gelation pH values of 4.37 ± 0.01.

Gelation Time: The gelation time of the formulations varied from 50-120 sec, decreasing with increased concentration of carbopol-934P. The constant and regression coefficient were as follows:

Gelation time (R1) = + 86.00 – 31.50A

The linear model was found to be significant for gelation time as the p-value was 0.0066. Factor A had a negative effect indicating that the gelation

time decreased as factor A increased. The 3-D response surface plot is seen in **Fig. 1** also showed the same.
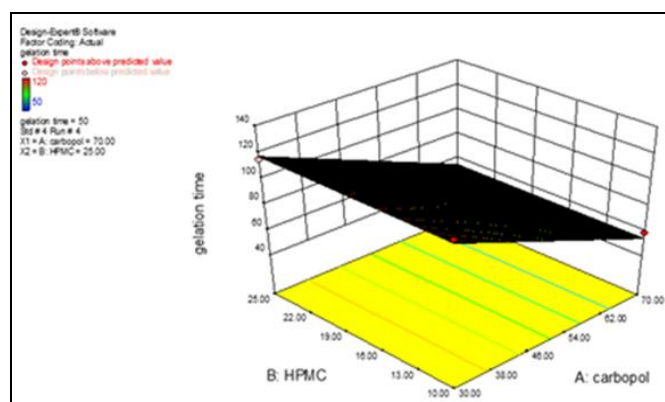

FIG. 1: 3-D RESPONSE SURFACE GRAPH SHOWING THE EFFECT OF POLYMER CONCENTRATIONS ON GELATION TIME

Rheological Studies: The formulations were sols at lower pH (indicating the ease of pourability and

swallowing) while they gelled at pH 6 (improving gastric residence time as the gels were also mucoadhesive thereby sustaining the release and improving bioavailability) as seen in **Fig. 2**. The viscosity of the sols varied from 92.65-129.93 Pa.son changing the rpm from 10 to 50 (at 37 °C).

They exhibited a decrease in viscosity with an increase in shear rate and such fluids are often preferred as liquid orals [39]. The apparent decrease in viscosity under shear was followed by gradual recovery when the shear was removed as seen in **Fig. 3**.

FIG. 2: THE FORMULATION REMAINED A SOL AT ACIDIC PH (A) BUT TURNED INTO A GEL (B) AT HIGHER PH VALUES (pH 6)
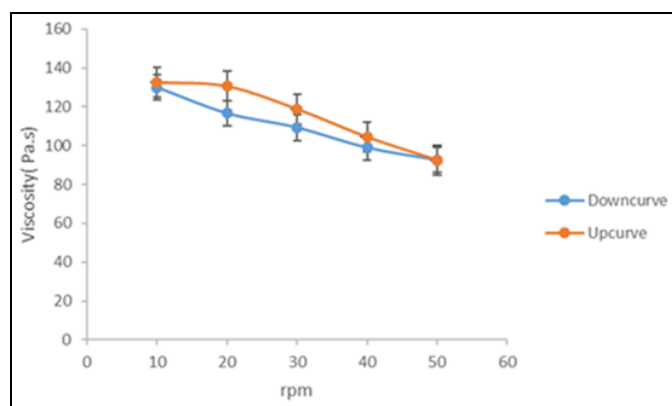
FIG. 3: RHEOLOGICAL BEHAVIOR OF THE OPTIMIZED FORMULATION

Mucoadhesive Strength: The mucoadhesive strength of the formulations varied from 13339 ± 0.84 to 20617 ± 0.67 dynes/cm^2. It was found to increase with an increase in carbopol-934 concentration. The constant and regression coefficient for R_2 are as follows:

Mucoadhesive strength $(R_2) = + 16829.50 + 2807.00A$

Carbopol-934P had a positive effect on this response and the 3D response surface graph **Fig. 4** also revealed that higher concentrations of this factor gave a higher R_2 and the effect was significant as ANOVA revealed a p-value of 0.0413.

Gel Strength: The gel strength varied from 43-68 sec and increased with the concentration of both carbopol-934P and HPMC E50. The equation for the regression coefficient is as follows:

Gel strength $(R_3) = + 55.270 + 9.27A + 3.24B$

Both the polymers had a positive effect on response R_3, and a p-value of 0.0265 indicated that the linear model was significant.
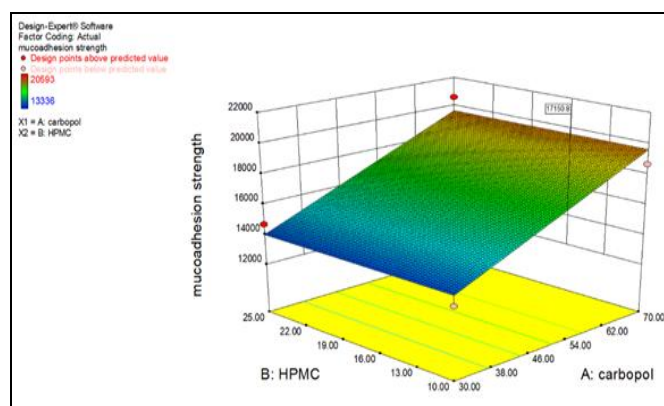

FIG. 4: 3-D RESPONSE SURFACE GRAPH SHOWING THE EFFECT OF POLYMER CONCENTRATIONS ON MUCOADHESIVE STRENGTH

***In-vitro* Drug Release Studies:** The graph of % CDR versus time for the prepared formulations are seen in **Fig. 5**. The rate of drug release was found to decrease as the polymer concentrations increased. The n values of the Korsmeyer-Peppas

equation strongly indicated that the diffusion mechanism was non-Fickian.

The effect of concentration of polymers on drug release can be explained by a mathematical equation in terms of coded factors as:

Drug release at 1 h = + 22.92 – 2.01A -1.62B

Carbopol-934P was found to decrease the drug release rate and the p-value of 0.0279 indicated that the effect was significant.

Drug release at 8 h = + 65.49 – 2.36A

Carbopol-934P had a significant negative effect on drug release at 8h as the p-value was 0.0463.

Drug release at 12 h = + 91.38 – 3.01A -1.23

Both polymers had an antagonistic effect on drug release at 12h.ANOVA revealed that the effect of carbopol was found to be significant (p<0.0372) while the effect of HPMC E50 was not significant (p<0.0924).
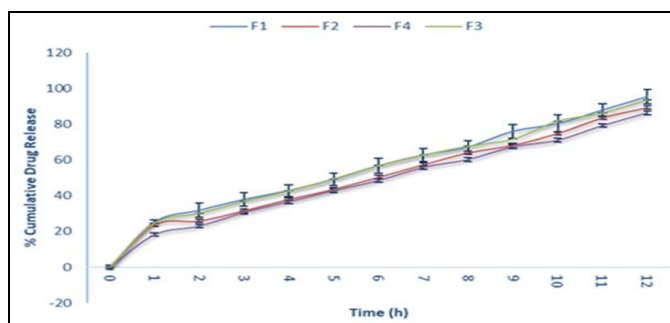

FIG. 5: COMPARISON OF *IN-VITRO* DISSOLUTION PROFILES OF THE PREPARED FORMULATIONS

Optimized Formula: The optimized formula obtained as per the 2-square factorial design consisted of 400 mg of Voriconazole, 52.35 mg of carbopol-934P and 10 mg of HPMC E50. It was prepared according to the prediction profiler and evaluation were done.

In-vivo **Pharmacokinetic Studies:** The HPLC method used for quantification of VCZ in plasma showed that the drug was well separated, and the chromatogram obtained for the optimized formulation is seen in **Fig. 6**. The formulation was rapidly absorbed after the oral administration. The peak plasma concentration was achieved in 2 h and was found to be 2.98 ±1.7 mcg/ml. The $AUC_{0-\alpha}$, which reflected the total amount of active drug

which reached the systemic circulation, was found to be 117.87 ± 2.91 µgh/ml which was higher than that of the control 17.419 ± 1.37µgh/ml indicating a 6.76-fold improvement in bioavailability that was also statistically significant (*p<0.05).

The optimized formulation maintained relatively constant plasma drug levels within the therapeutic window (the provisional therapeutic range being 1-6 µg/mL) [40] and above the minimum inhibitory concentration (MIC) of 0.5 µg/mL *i.e.*, the lowest VCZ concentration that showed a prominent reduction (90%) of fungal growth for a period of 12 h. The reference (pure drug solution) on the other hand, showed fluctuations (peak and valley profile) in the plasma drug concentrations as seen in **Fig. 7** due and the plasma drug levels also dipped below the MIC after 6 h.
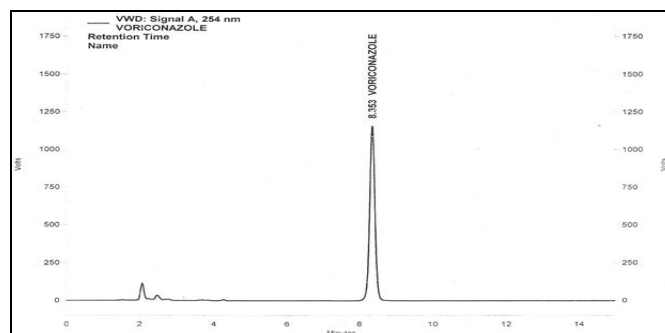

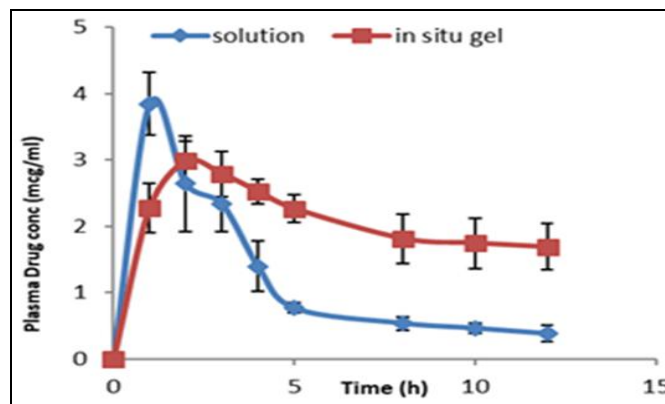**FIG. 6: CHROMATOGRAM OF PLASMA EXTRACT SHOWING THE RETENTION TIME OF VORICONAZOLE**

FIG. 7: COMPARISON OF PHARMACOKINETIC PROFILES OF PURE DRUG SOLUTION AND OPTIMIZED *IN-SITU* GELLING FORMULATION

Stability Studies: Stability studies revealed that there were no significant changes in the pH, clarity or drug content of the formulations. The formulation showed maximum stability for two years at room temperature. Photostability studies

also revealed that the optimized formulation was stable.

Equivalence Testing of Reference and Test Plasma Profiles based on Earth Mover's Distance: The Earth Movers Distance (EMD), the statistical metric used for bioequivalence testing to compare the plasma profiles of the reference (drug solution) and optimized (test) formulation was superior to the commonly used distance measures like Euclidean and Kolmogorov-Smirnov distances. The value of the Population Bioequivalence criteria (Θ) *i.e.*, Θtest (0.6835) < Θstd (0.7963) proved that the optimized formulation was better than the reference.

CONCLUSION: It can be concluded that a liquid oral *in-situ* gelling system of Voriconazole with improved bioavailability could be successfully developed using mucoadhesive polymers such as carbopol-934P and HPMC E50 (viscosity enhancer) to increase the gastric residence time, sustain the drug release for 12 h, thus enhancing bioavailability. This elegant, liquid oral sustained release formulation could pave the way for a successful, novel approach to antifungal therapy with better patient compliance especially for geriatric and pediatric patients because of the ease of administration of the liquid oral and reduced dosing frequency. Large doses of drugs can be easily incorporated into small volumes of liquid. The simple and economical method of preparation has positive implications on the ease of manufacture and reduced production costs.

CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest regarding the publication of this article.

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