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Original Article

Development and Validation of Microbiological Analytical Method for Determination of Potency of Voriconazole Tablets

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ABSTRACT

A new microbiological method was developed for analysis of voriconazole tablets using *Candida albicans* as test microorganism. Various media, species and conditions were used to optimize the diffusion test. A prospective validation of the system showed adequate linearity (0.995), accuracy (RSD<2%) and consistency (mean recovery= 101.77%). High performance liquid chromatography was selected as a tool of comparison for evaluating voriconazole. Results of both the microbiological and HPLC methods have been compared with the student t-test and the voriconazole content determined by both methods has shown strong correlation. The developed microbiological analytical method provides true indication of biological activity and can be used in dosage forms for routine quality control analysis of voriconazole.

Key words: *Candida albicans*, Comparison, Diffusion method, HPLC, Method validation, Voriconazole.

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INTRODUCTION

Voriconazole is a second generation synthetic diazole with a broad-spectrum of activity offering the potential to treat life-threatening fungal infections. It is used to treat invasive fungal infections that are generally seen in patients who are immune compromised.[1] It is an imidazole antifungal derivative and used for the treatment of local and systemic fungal infection. These fungal infections include invasive candidasis, invasive aspergillosis, fusarium infections and febrile neutropenia not responding to antibacterial therapy. Voriconazole has over 95% of oral bioavailability. It acts by inhibiting CYP450- dependent 14-alpha sterol demethylase which is a vital step in cell membrane ergosterol synthesis by fungi. It binds to cytochrome P-450 enzyme lanosterol 14-alpha demethylase, which prevents the conversion of lanosterol to ergosterol. This results in cell membrane depletion and cell death.[2]

Voriconazole and itraconazole had the lowest MICs for *Candida albicans* isolates. Overall, voriconazole showed more potency than fluconazole or itraconazole for most candida isolates studied. Voriconazole demonstrated low MICs for all aspergillus species tested but appeared to be most active against *Aspergillus fumigates*.[3]

Voriconazole is available commercially for oral administration in tablets in brand name of voritek and vfend. Voriconazole determination in pharmaceutical formulation by bioassay are scarce, where the majority of papers are related to its study include voriconazole determination in agar plate using *sacharomyces cerevisiae*[4] and voriconazole in plasma levels.[5,6]

The most commonly described assay methods for voriconazole in tablets are UV Visible[7-13] and high performance liquid chromatography (HPLC)[14-29] may be also quantified by bioassay for its activity assessment. The purpose of this study was to develop and validate a microbiological analytical method to determine the potency of voriconazole in tablets. The bioassay results were compared to those obtained by HPLC analysis. The chemical structure of Voriconazole is s Figure 1.

MATERIALS AND METHODS

Standards and Reagents

Voriconazole reference standard was obtained by yarrow chem. products, Mumbai. Voriconazole tablets (200mg) were obtained from local market with brand name voritek. Methanol, sodium chloride, dibasic and mono basic potassium phosphate were used for analysis along with distilled water, acetonitrile of HPLC grade.

Preparation of buffer solution

2 g of dibasic potassium phosphate and 8 g of monobasic potassium phosphate were weighed accurately, transferred to 1000mL volumetric flask and volume was made up to 1000mL with distilled water. The pH of the prepared buffer solution was adjusted with 18N phosphoric acid or 10N potassium to 6.0±0.05 using a pH meter.

Preparation of voriconazole standard solutions

For the preparation of voriconazole reference standard stock solution, 15.0 mg equivalent of voriconazole RS was weighed and then it was transferred to a 50 mL volumetric flask and the volume was adjusted with methanol to obtain a solution with a concentration of 300µg/mL. Aliquots of 0.2, 0.4 and 0.8mL of this solution were transferred to 10 mL volumetric flasks and diluted with buffer in order to obtain working solutions with concentrations of 6.0, 12 and 24µg/mL voriconazole, respectively named as S1, S2 and S3.

These standard solutions were used in bioassay of voriconazole.

Preparation of voriconazole sample solutions

10 tablets of voriconazole (voritek) were weighed and powdered. An accurately weighed portion of the tablet powder equivalent to about 15.0mg of drug was transferred to a 50 mL volumetric flask, to this 15mL of methanol was added, shaken for 10 min in sonicator and the volume

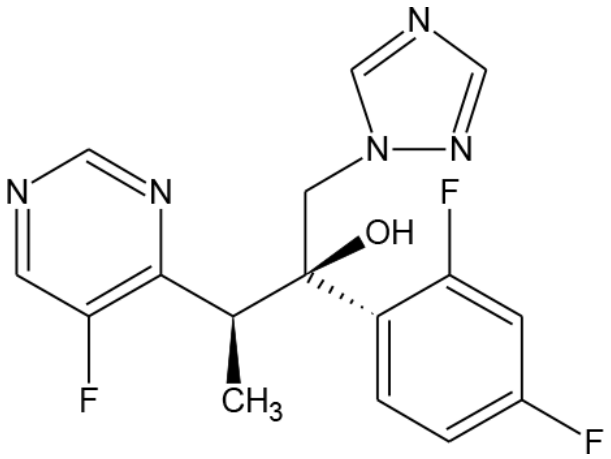


Figure 1: Structure of Voriconazole.

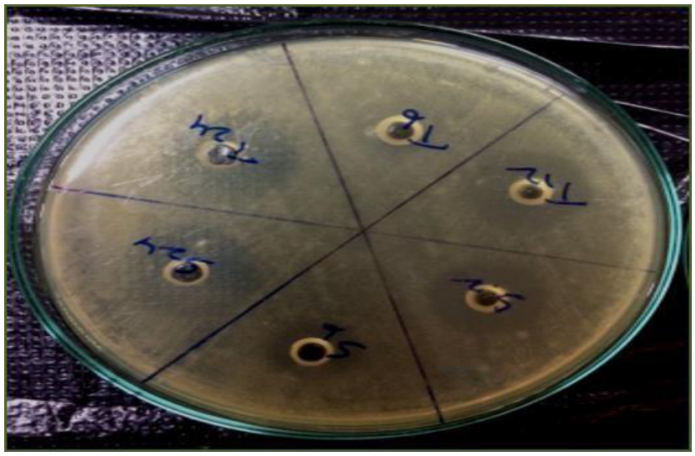


Figure 2: Microbiological assay $S_{6'}$ S_{12} S_{24} are standard solutions and $T_{6'}$ T_{12} T_{24} are test sample solutions with concentrations 6.0, 12 and 24µg/mL.

was adjusted with methanol to obtain a solution with a concentration of 300µg/mL. Aliquots of 0.2, 0.4 and 0.8mL of this solution were transferred to 10 mL volumetric flasks and diluted with buffer in order to obtain working solutions of sample with concentrations of 6.0, 12 and 24µg/mL, respectively named as T1, T2 and T3 which were used in the bioassay.

Preparation of sabouraud dextrose 2% agar medium

40 g of dextrose, 10 g of peptone and 20 g of agar were accurately weighed and transferred to 1000mL volumetric flask and volume was made up to 1000mL with distilled water. The medium was dissolved by boiling and pH was adjusted to 5.6±0.2. Sterilization was done at 15 lbs pressure (121°C) for 15 min by autoclaving technique.

Microorganism and Innoculum

Candida albicans NCIM 3471 is used as microorganism for analysis. The medium used was sabouraud dextrose agar medium. The strain of *Candida albicans* NCIM was cultivated, inoculated on sabouraud dextrose slant agar and incubated for 24hrs at 30°C±2°C. After this period, organism was suspended in saline solution (0.9% NaCl) and the transmittance was adjusted to 85% at a wavelength of 520 nm, which measures 1-5×106 CFU/mL[8]. For the biological assay of voriconazole, 1mL of this suspension was added to 100mL sabouraud 2% agar, kept at 48°C and used as inoculated layer.

Development of Microbiological assay (Diffusion method)

A 20mL amount of sabourand 2% agar was poured into petri dishes for base layer. After its solidification, a 5mL portion of inoculated sabouraud 2% agar was poured onto the base layer. The agar was allowed to gel at room temperature for 10 to 15 min. After solidification, 5mm- diameter wells were bored at six points in each plate. Three alternated cylinders were filled with 50µl standard solution and other three with the sample solutions. The plates were then incubated at 30°C±2°C for 24 hr. After incubation period, the petri dishes were observed (Figure 2) and the diameters of inhibition zone of the microorganism growth (mm) were measured using antibiotic growth scale. Six assays were performed in the same manner using three plates in each one (2 assays each day).

Potency calculation

The potency of voriconazole tablets was calculated by the Hewitt equation.[30] The assay was treated statistically by the linear parallel model and by linear regression analysis. Analysis of variance (ANOVA) was used to verify the validity of the assay.

Method Validation

The microbiological method was validated by evaluation of linearity, precision and accuracy, according to ICH Q2 R (1) guidelines.[31]

Linearity: Linearity was performed within the specified range as per guidelines. To assess the linearity of the method, replicates of drug substance and drug product were evaluated on 3 different days. A calibration curve for log concentration of voriconazole versus inhibition zones was plotted and the obtained data were subjected to regression analysis using the Least Squares Method.

Precision: The intraday precision was evaluated by analyzing six replicates of voriconazole solutions, at 100% test concentration (12µg/mL). Similarly, the inter-day precision was evaluated on three different days, at three concentrations levels of 6.0,12 and 24µg/mL. The concentration of voriconazole in tablet samples was determined and the relative standard deviation (RSD) was calculated.

Accuracy: It was determined by adding known amount of voriconazole reference standard (12µg/mL) to a sample solutions (9.6, 12 and 14.4µg/mL) at the beginning of the analyses, corresponding to 80, 100 and 120% of the test concentration. At each level, solutions were prepared in triplicate and applied to the plate assay described above. The recovery percentage of voriconazole was determined. In addition, the bioassay results were compared to with that of a second known HPLC method.

Chromatographic analysis

The analysis of voriconazole tablet was performed on a Schimadzu 20AT binary gradient system with UV detector and LC solutions software. The HPLC analysis was done in gradient mode using the mobile phase consisted of acetonitrile and water (60:40v/v).The chromatographic separation was carried out on an Enable C_{18} analytical column (250 × 4.6 mm; 5 mm) at a flow rate of 1 mL/min. The volume of the injection is 20µL. The peak areas were defined as analytical signs, with detection at 256nm. This method was optimized and validated according to ICH Q2 R (1) guidelines.

Comparison of Methods

The results of the analysis obtained by the microbiological method in this study and chromatographic method were compared statistically using student t- test, at a level of significance of 5% using graph pad prism software.

RESULTS AND DISCUSSION

In this work, microbiolgical assay method was developed and validated. The experimental conditions were standardized to get reproducible results. Incubation temperature (30°C) and period (24hr) were optimised. A large number of strains of *Candida* and *Sacharomyces* were used as test organisms for screening antifungal activity. In this study *C. albicans* NCIM3471 was used and found to be adequate for voriconazole activity testing. The inocula concentration of 1-5 x105CFU/mL provided the best visualization of the inhibition zones compared to the inocula with 103 and 104CFU/mL, for which zones were not clearly delineated.

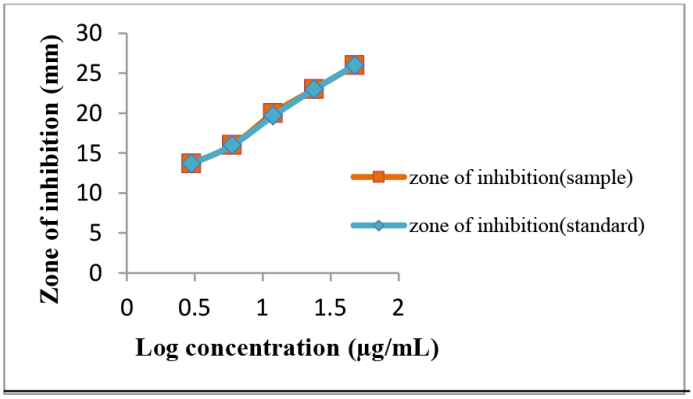
An experimental 3×3 design was developed for determination of voriconazole in tablets by agar diffusion method. A strain of *C. albicans* NCIM 3471 was selected as test microorganism for quantitation of voriconazole. The potency of an antibiotic may be demonstrated under appropriate conditions by comparing the growth inhibition of sensitive microorganisms generated by known antibiotic concentrations to be examined and a reference standard. All assays were performed in a laminar air flow cabinet; the material was decontaminated and discarded. The analytical curve was constructed from average of three curves obtained on three different days. The data obtained from the analytical curve were evaluated by the least squares and the study of variance (ANOVA) was used to check linearity and parallelism.

The calibration curve for voriconazole values are listed in Table 1

The calibration curve was developed by plotting concentration log (g/mL) versus zone diameter (mm) and showed good linearity between 3.0–48µg/mL ranges. The experimental values obtained for the determination of voriconazole in samples are presented in Table 2. As no deviation in the linearity and parallelism of two curves was observed in obtained results.

Table 1: Overview of the linearity data obtained for voriconazole.

S.no	Conc (µg/mL)	*Zone of inhibition± SD (drug substance) mm	*Zone of inhibition± SD (drug product) mm
1	3.0	13.6667±0.882	13.6667±0.882
2	6.0	16±0	16±0
3	12	19.6667±0.882	20±0
4	24	23±0	23±0
5	48	26±0	26±0
Slope		10.51	10.51
Correlation coefficient		0.996	0.995



The method precision in terms of repeatability and interday was shown as %RSD that was less than 2%. This verified the method's capacity to generate reproducible results with the same sample, with low variability in the response in separate assays.

The accuracy of method was evaluated at 80, 100 and 120% of the range which shown a mean accuracy of 101.77% (Table 2). This confirms the ability of method to accurately determine the concentration of voriconazole in tablets and shows that the results obtained from bioassay were close to the true concentration of sample.

In the HPLC method, the calibration curve of voriconazole was obtained by plotting the drug concentration against absorbance. The curve was linear in the concentration range of 3.0-48µg/mL, with regression coefficient of 0.999 and a linear regression equation of y=19244x+6014. The accuracy of the assay was studied, the mean recovery was found to be 104.39%.

The data obtained by microbiological method and HPLC method were statistically compared by student t-test at a significance level of 5% (Table 3). This showed a difference between two methods which is consider to be statistically significant at a level of 5%, indicating rejection of null hypothesis.

Although the statistical analysis has shown that the HPLC and microbiological methods presented statistically similar results in relation to the determination of voriconazole in pharmaceutical form, it is necessary to highlight that there are difference between these methods. The HPLC method is selective, being suitable for the determination of degradation products and impurities in the matrix analyzed.

However, it needs the employment of expensive instrumentation, solvents and analytical columns, in addition to using large volumes of organic solvents as mobile phase, which makes the maintenance of the technique costly and leads to occupational and environmental contamination.

Table 2: Recovery of voriconazole for accuracy evaluation.

Sample conc (µg/ml)	Conc of standard added (µg/ml)	Conc of standard found (µg/ml)*	% Recovered
1	21.6	20.83	96.45
2	24	23.777	99.07
3	26.4	28.988	109.80

*average of three determinations

Table 3: Voriconazole contents in tablet samples obtained by bioassay and HPLC methods.

Sample	Voriconazole Content (%)	
	HPLC	Bioassay
1	93.64	93.61
2	98.665	100.0
3	104.850	107.35

CONCLUSION

A microbiological analytical method was developed and validated for determination of potency of voriconazole tablets by using microorganism *Candida albicans*. The developed diffusion agar method provide true indication of biological activity and showed adequate linearity, precision and accuracy when validated as per ICH guidelines. Microbiological methods do not require any specialized equipment and are cost effective.

Although the biological assay methods have a high variability, the obtained results demonstrated that the proposed method can be useful

for determination of this drug in pharmaceutical dosage forms, as an acceptable alternative method for the quality control analysis of voriconazole.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

RSD: Relative Standard Deviation; **HPLC:** High Performance Liquid Chromatography; **CYP:** Cytochrome; **MIC:** Minimum Inhibitory Concentration; **UV:** Ultra Violet; **ICH:** International Council for Harmonization; **SD:** Standard Deviation.

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