MICROBIOLOGICAL ANALYTICAL METHOD FOR DETERMINATION OF SERTACONAZOLE NITRATE IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Sertaconazole nitrate is a third generation synthetic broad-spectrum, benzothiophene imidazole antifungal agent. A new microbiological method was developed for analysis of sertaconazole tablets using candida Albicans as test microorganism. The diffusion assay method was optimized by using different media, organisms and conditions. Prospective validation of the method showed adequate linearity (r = 0.9964), precision (% RSD<2%) and accuracy (mean recovery = 98.26%). High-performance liquid chromatography was chosen as a comparison method for voriconazole determination. Results of both the microbiological and HPLC methods were compared with student t-test and the contents of voriconazole determined by both methods, showed a strong correlation. High-performance liquid chromatography was chosen as a comparison method. Results of both the microbiological and HPLC methods were compared with student t-test and the contents of sertaconazole determined by both methods showed a strong correlation. The microbiological analytical method which was developed gives true indication of biological activity and can be used for routine quality control analysis of sertaconazole nitrate in dosage forms.

***Key words**: Sertaconazole nitrate, t-test, Candida albicans, Diffusion method, HPLC, Method validation.*

INTRODUCTION

Sertaconazole nitrate is a newer imidazole agent that possesses both fungicidal and fungistatic properties to eradicate existing infections. In addition to antifungal properties, it exhibits anti-inflammatory and antipruritic effects. Chemically, sertaconazole contains a benzothiophene ring which makes it unique from other imidazole antifungals. Sertaconazole topical (for the skin) is used to treat tinea pedis (athlete's foot) in adults and children. Controlled clinical trials have confirmed its safety, tolerability, and efficacy in participants with tinea pedis [1]. Sertaconazole nitrate inhibits fungal cytochrome P-450 sterol C-14 alpha demethylase and was approved by the Food and Drug Administration in 12/10/2003 (Ertaczo cream). Sertaconazole demonstrated high MICs for all Aspergillus species when compared with other antifungal drugs. Such activity usually can be known by microbiological methods [2].

Chemically sertaconazole nitrate is a (Rs)-1-{2-[(7-chloro-1-benzothiophen-3-yl) methoxy]-2-(2,4dichlorophenyl) ethyl}- 14imidazole with Empirical formula $C_{20}H_{15}Cl_3N_2OSH\ NO_3$. Sertaconazole nitrate is a white or almost white powder [3-5]. It is practically insoluble in water, soluble in methanol, sparingly soluble in alcohol, and in methylene chloride. It has a molecular weight of 500.78300g/mol. The chemical structure of sertaconazole is shown in (Figure 1).

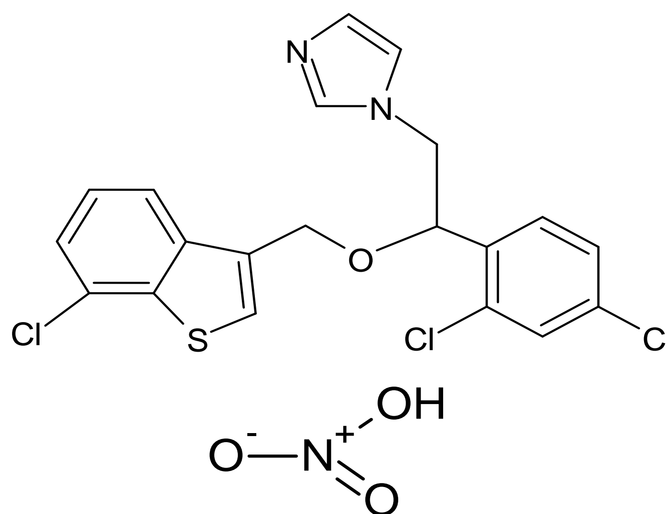

Figure 1. Chemical structure of sertaconazole nitrate

Different UV- spectrophotometric and HPLC methods are available for the estimation of sertaconazole nitrate in pure and pharmaceutical dosage forms in the literature. Microbiological assays by the agar diffusion method were reported for the determination of others fluoroquinolones in pharmaceutical formulations, such as norfloxacin [6], sparfloxacin [7], ofloxacin [8], enrofloxacin [9], lomefloxacin [10], gatifloxacin [11] and orbifloxacin [12]. From the literature review, it was concluded that different UV-spectrophotometric and HPLC methods [13-22] are available for estimation of sertaconazole nitrate in pure and pharmaceutical dosage forms. There are no Microbiological analytical methods developed either by diffusion or turbidimetric method for the estimation of sertaconazole nitrate in pure and pharmaceutical dosage forms. The quantification of anti-infective agents by instrumental methods such as HPLC and UV methods are precise, but they cannot provide a true indication of biological activity. The present work is planned to develop a new microbiological analytical method to estimate the activity of sertaconazole nitrate in the dosage form.

EXPERIMENTAL

Chemicals and Reagents

A pure drug sample of sertaconazole nitrate was obtained as a gift sample from Optimus pharma, Hyderabad. Sertaconazole nitrate tablet- (Onabet V1, Glenmark pharmaceuticals Ltd) was purchased from a local pharmacy store. Disodium potassium phosphate, monobasic potassium phosphate, methanol, sodium chloride, Acetonitrile, and distilled water (HPLC Grade) were procured from SD Fine Chemicals Limited (SDFCL), Mumbai.

Instruments

UV-Visible Spectrophotometer (Shimadzu UV1800; UV probe software, Japan), Digital Balance (Shimadzu BL-220H, Japan), and Ultra Sonic Bath Sonicator (PCI Analytics 6.5 li200H, India) are the

instruments used in the research work. All weighing was done on an analytical balance (Contech Instruments ltd). Calibrated glass wares were used throughout the work.

Preparation of standard stock solution

For the preparation of Sertaconazole nitrate reference standard (RS) stock solution, 10 mg equivalent of Sertaconazole nitrate RS was weighed, and then it was transferred to a 10 ml volumetric flask and the volume was adjusted with methanol to obtain a solution with a concentration of 1000µg/ml. Aliquots of 0.1, 0.2, 0.4, 0.8, and 1.6 ml of this solution were transferred to 10 ml volumetric flasks and diluted with buffer in order to obtain working solutions with concentrations of 10, 20, 40, 80, and 160 µg/ml Sertaconazole nitrate respectively named as S1, S2, S3, S4, and S5. These standard solutions were used in the microbiological analytical method development of Sertaconazole nitrate.

Preparation of sample solution

10 tablets of Sertaconazole nitrate (Onabet V1) were weighed and powdered. An accurately weighed portion of the tablet powder equivalent to about 10 mg of drug was transferred to a 10 ml volumetric flask, to this10ml of methanol was added, shaken for 10 min in a sonicator and the volume was adjusted with methanol to obtain a solution with a concentration of 1000µg/ml. Aliquots of 0.1, 0.2, 0.4, 0.8, and 1.6 ml of this solution were transferred to 10 ml volumetric flasks and diluted with buffer in order to obtain working solutions of a sample with concentrations of 10, 20, 40, 80, and 160 µg/ml, respectively named as T1, T2, T3, T4 and T5 which were used in the development and optimization of bioassay.

Microorganism and Inoculum

The strain of Candida albicans NCIM was cultivated, inoculated on sabouraud dextrose slant agar medium, and incubated for 24hrs at 300C±20C. After this period, organism was suspended in saline solution (0.9% NaCl) and the transmittance was adjusted to 85% at a wavelength of 520nm, which measures 1-5×106 CFU/ml. For the biological assay of Sertaconazole nitrate 1ml of this suspension was added to 100ml sabouraud 2% agar, kept at 48^0C, and used as an inoculated layer.

Microbiological Assay (Diffusion Method)

A 20 ml of sabouraud 2% agar was poured into petri dishes for the 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 the other three with the sample solution. The plates were then incubated at 30^0C±2^0C for 24 hours. After the incubation period, the petri dishes were observed and the diameters of the inhibition zone of the microorganism growth (mm) were measured using an antibiotic growth scale. Six assays were

performed in the same manner using three plates in each one (2 assays each day). Note that all these operations must be done in a laminar airflow chamber in order to avoid cross-contamination.

Potency Calculation

To calculate the activity of sertaconazole in tablets the Hewitt equation was used. The assay was statistically analysed by the linear parallel model and by means of regression analysis of variance.

METHOD VALIDATION

The method was validated according to ICH Q2 R (1) guidelines. The following parameters were determined: linearity, precision, and accuracy. According to the ICH guidelines, the limits of detection and quantification are not required for microbiological assay of drug substances [23].

Linearity

Linearity was performed within the specified range as per guidelines. To assess the linearity of the methods, replicates of drug substance and drug product were evaluated on 3 different days. For the microbiological assay, the calibration curves were obtained with 5 replicates of each standard solution and sample solution at concentration levels, 10, 20, 40, 80, and 160 µg/ml.

Precision

The precision of the method was evaluated at two levels, repeatability (intra-assay) and intermediate precision (inter-assay). Method repeatability was studied by analysing samples of tablets, at the same concentration, within the day and under the same experimental conditions. The intermediate precision was evaluated by comparing the assays on 3 different days.

Accuracy

The accuracy of a method expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Three replicates of each sample were prepared. A series of sample solutions were prepared in the range of 80% -120% of test concentrations.

HPLC ANALYSIS

A previously developed and validated HPLC method was selected as a comparative method for the determination of sertaconazole nitrate in tablets. The HPLC analysis was done in gradient mode 0.01 M monobasic sodium phosphate and acetonitrile in a ratio of 20:80 % v/v as the mobile phase. The chromatographic separation was carried out on an Enable C18 analytical column (250 × 4.6 mm; 5 mm) at a flow rate of 1.2 ml/min. The volume of the injection is 20 µL. The room temperature was maintained

at 35 ± 2°C. The peak areas were defined as analytical signs, with detection at 260 nm. This method was modified, optimized and partially validated.

Comparison of methods

The results of the analysis obtained by the microbiological method and HPLC method were compared statistically using the student t- test, at a level of significance of 5% ($\alpha = 0.05$). This test was mainly based on two hypotheses. Null Hypothesis and Alternative hypothesis. Null hypothesis states that there is no significant difference between specified methods whereas alternative hypothesis states that there is significant difference between two methods. The basic criteria to accept null hypothesis is t-calculated = t-critical, and for alternative hypothesis is t-calculated > t-critical or t-calculated < t-critical. The t value was calculated by using graph pad prism software version 7 whereas t critical value was found from t- table at a level p=0.05.

RESULTS AND DISCUSSION

A new microbiological analytical method was developed for quantitative analysis of sertaconazole nitrate in tablets using the agar plate diffusion method. The activity of antimicrobial agents may be demonstrated under suitable conditions by their inhibitory effect on microorganisms. (USP 25, 2002). The microbial activity of sertaconazole has been tested against different microorganisms. These studies have shown that sertaconazole nitrate has potent activity against Candida albicans. In this work, experimental conditions were evaluated by using five dose levels for each standard and sample. The conditions tested to establish the parameters for the microbiological assay are given in (Table 1). The calculation procedures normally assume a direct relationship between the observed zone diameter and the logarithm of the applied dose. The results of the growth inhibition zone diameter of the sertaconazole nitrate reference substance and sample are presented (Table 2).

Table 1: Conditions tested to establish the parameters for microbiological assay

Parameters	Conditions
Solvents	Methanol and phosphate buffer (6.0±0.05)
Concentrations (µg/ml)	10, 20, 40, 80 and 160 µg/ml
Inoculum	Candida albicans
Medium	Sabouraud dextrose agar medium
Method	Diffusion agar method
Regression equation(y)	Y=10.365x – 1.5654
Coefficient of regression (r^2)	0.9979

Table 2. Zone of inhibition values of standad and sample

S.No	Concentration (µg/mL)	Zone of inhibition (drug substance)mm	Zone of inhibition (drug product)mm
1	10	8.6	8.2
2	20	12	12.23
3	40	15.2	15.22
4	80	18.4	18.2
5	160	21	21.33
Slope		10.365	10.743
Correlation coefficient		0.9979	0.9964

*Mean of three determinations

The calibration curves were constructed by plotting zone diameter (mm) versus log of concentrations (g ml−1) and showed good linearity in the 10-160 g ml−1 range (Figure.2). The representative linear equation was $y = 11.24178+9.40043 \ln x$ ($n = 3$, $r = 0.99998$, $r2 = 0.99996$). The analytical curve was constructed from the average of three curves obtained on three different days. The data obtained from the analytical curve were analyzed by the least squares and the verification of linearity and parallelism was done by the analysis of variance (ANOVA). Linearity data obtained were analysed by the least squares and parallelism was done by the analysis of variance (ANOVA). The value of the correlation coefficient (r), 0.999, is considered highly significant for this method. For this research, a parallel-line model has been chosen, in which two curves are constructed, one of them for sertaconazole nitrate RS and the other for the sample of the tablet, and these two curves must be parallel and linear over the working range chosen. These parameters must be verified by validity tests, considering a given probability, which is usually $p = 0.05$. The tests performed in this study were $y = 10.743x - 2.1535$ $R^2 = 0.9964$ & $y = 10.365x - 1.5654$ $R^2 = 0.9979$. Zone of inhibition (Standard) Zone of inhibition (sample) validated through the analysis of variance (ANOVA). Through this analysis, it was found that there was no deviation in the linearity and parallelism of two curves ($p < 0.05$), i.e., $p < 0.0001$.

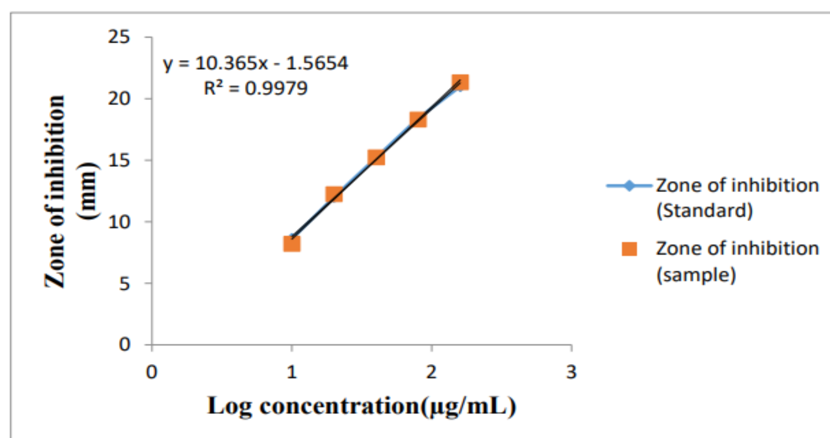

Figure 2. Standard graph of Sertaconazole nitrate (drug substance) by diffusion method using Candida albicans

The inter-day precision was evaluated by comparing the linear regressions of the three standard plots on three different days in a 2 months period, the coefficient of correlation was 0.99998 and the coefficient of variation of the slope of the three lines was 1.14%. The experimental values obtained for the determination of sertaconazole in samples are present in (Table 3). According to Brazilian (1988) and European Pharmacopoeia (2002), if a parallel-line model is chosen, the two log dose-response lines of the preparation to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions were verified by a validity test for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as described in these official codes already cited in this paragraph. There are no deviations from parallelism and linearity with the results obtained here ($P < 0.05$).

Interday precision

The inter day precision results given in table 3 obtained for three replicates of three concentrations of sertaconazole nitrate. The zone of inhibition values of each sample solutions was used for calculation of % RSD.

Table 3: Statistical validation of Interday precision

Concentration (μg/mL)	Mean zone of inhibition (mm)			Mean+SD	%RSD
	Day 1	Day 2	Day 3		
20	12.2	12.23	12	12.143±0.125	1.02
40	15.22	15.2	15	15.14±0.121	0.80
80	18	18.33	18.333	18.221±0.191	1.05

*Mean of three determinations

The inhibition zone diameters are almost same on three days, indicating the interday precision of the method with optimized conditions. The calculated %RSD values are less than 5.0% indicating that the method was precise.

Accuracy

Accuracy of the method was determined at three different concentration levels. The accuracy of the method was confirmed by reporting the % recovery and the data calculated are shown in (Table 4).

Table 4. Statistical validation of recovery studies

Sample concentration (μg/mL)	Concentration of standard added (μg/mL)	Concentration of standard found (μg/mL)	% Recovered	Mean % recovery
32	40	71.39	97.32	98.26
32	40			
32	40			
40	40	79.82	99.20	
40	40			
40	40			
48	40	88.24	101.3	
48	40			
48	40			

Comparison of methods

The results obtained with the diffusion agar method assay were comparable with declared amounts and with those obtained by HPLC. ANOVA indicated no significant differences.

Table 5. Assay results of both methods

Parameters	Average sertaconazole nitrate content%	Mean
HPLC	97.02	98.116
	99.37	
	100.02	
Microbiological method	99.42	99.736
	101.36	
	98.32	

The difference between the microbiological method and HPLC was considered to be extremely statistically significant at a level of 5%, indicating rejection of the null hypothesis. Although the statistical analysis has shown that the HPLC and microbiological methods presented statistically similar results in relation to the determination of sertaconazole nitrate in pharmaceutical form, it is necessary to highlight that there are differences between these methods. The HPLC method is selective, being suitable for the determination of degradation products and impurities in the matrix analyzed. However, it requires the use of costly equipment, 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.

CONCLUSION

A microbiological analytical method was developed and validated for the determination of the potency of sertaconazole nitrate tablets by using the microorganism Candida albicans. The developed diffusion agar method provides a true indication of biological activity and indicated that the proposed method demonstrated good linearity, precision, and accuracy when validated as per ICH guidelines. The microbiological method is a technique that does not use organic solvents for its analysis and does not require any specialized equipment, therefore causes no concern about chemical waste and is cost-

effective compared to instrumental methods such as HPLC and UV spectrophotometry. Although the biological assay methods have high variability, both methods enable a quantitative determination of sertaconazole nitrate in pharmaceutical preparations and can be used in routine analysis.

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