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Development and Validation of Microbiological Analytical Method for Determination of Potency of Luliconazole Cream

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ABSTRACT

A new microbiological method was developed for analysis of luliconazole cream using *Candida albicans* as test organism. The diffusion agar method was optimized by using different media organisms and conditions. The purpose of this research is to establish a microbiological analytical method for the estimation of the pure and pharmaceutical dosage type (cream) of luliconazole and to validate the method as per the ICH guidelines. As a comparative tool for luliconazole estimation, high performance liquid chromatography (HPLC) was selected. Acceptable linearity (0.9965), accuracy (percent Relative Standard Deviaton □2) and accuracy (mean percent recovery = 100.12) were seen by a prospective validation of the process. The statistics of the microbiological and HPLC methods were correlated with the student t-test, and there was a considerable link among both the luliconazole content determined by both methods. The developed microbiological analytical method provides a true indication of biological activity and can be used for routine analysis of the quality control of luliconazole in drug formulations. The microbiological approach is a procedure which does not make use of organic solvents and advanced equipment for its study poses little worry for chemical waste and is cost-effective in contrast with instrumental methods such as HPLC and UV-spectrophotometry. According to the literature, when measured using a physicochemical procedure, measuring the content of an antimicrobial agent by its potency will produce altered result.

INTRODUCTION

Luliconazole is one of the most active and wide-spectrum topical prescription antifungal agents known to induce 90 percent of onychomycosis against dermatophytes (Trichophyton rubrum and Trichophyton mentagrophytes). This is a topical antifungal agent that works through unknown mechanisms, but is believed to include altering the synthesis of the fungi's cell membrane. This was approved in November 2013 by the FDA (USA), and is sold under the Luzu brand name.[1] There is uncertainty about the mechanism of action against dermatophytes, luliconazole acts by inhibiting the enzyme lanosterol demethylase tends to inhibit ergosterol synthesis.Inhibition results in reduced levels of ergosterol, a fungal cell membrane component, and subsequent amassing of lanosterol.[2] Luliconazole chemical structure is shown in Figure 1.

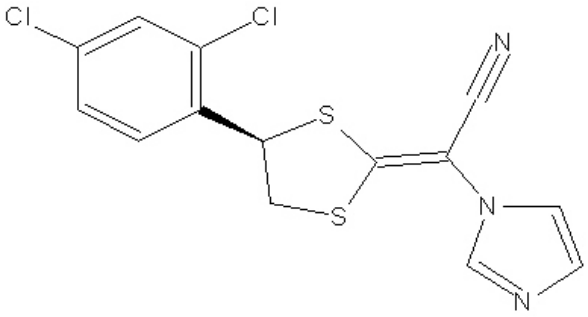

Fig. 1 : Structure of Luliconazole

MATERIAL AND METHODS

Chemicals

Luliconazole reference standard was obtained from Hetero

labs (Hyderabad, India), luliconazole cream (1%) was obtained from local market with brand name Lulifin, methanol of AR grade from RCP (India), dibasic potassium phosphate and sodium hydroxide of AR grade from SDFCL (Thane, India), monobasic potassium phosphate of AR grade from RANKEM (India), hydrochloric acid of AR grade from FCL (India), distilled water of HPLC grade from SDFCL (Thane, India), acetonitrile of HPLC grade from RANKEM (India). Sabouraud dextrose agar medium, Malt extract glucose yeast extract peptone medium (MGYP), potato dextrose agar medium were obtained from HIMEDIA (India)

Preparation of buffer solution

2g of dibasic potassium phosphate and 8g of monobasic potassium phosphate were weighed accurately, transferred to 1000ml volumetric flask, volume was made up to mark with water. The pHof the prepared buffer solution was adjusted with 0.1N NaOH or 0.1N HCl to 6.0±0.05 using a pHmeter.

Preparation of Luliconazole standard solutions

10 mg luliconazole was weighed diluted with methanol to attain a solution of concentration 1000µg/ml. Aliquots of 0.1, 0.2, 0.4, 0.8 and 1.6ml of this solution were transferred to 10ml volumetric flasks and diluted with buffer to obtain concentrations of 10, 20, 40, 80, and 160µg/ml, named as S1, S2, R, S4 and S5 respectively.

Preparation of Luliconazole sample solution

The cream (Lulifin) 1g was weighed and diluted with methanol, sonicated for 5min and the volume was made up to the mark with methanol to obtain a solution with a concentration of 10000µg/ml. 1ml was taken from the above solution in a 50ml volumetric flask and a volume of 200µg/mL was developed up to the mark to obtain a solution. Aliquots of 0.5, 1, 2ml of this solution were transferred to 10ml volumetric flask and diluted with buffer to obtain concentrations of 10, 20, 40µg/ml named as T1, T2, and T3 respectively.

Preparation of Sabouraud dextrose agar medium

40grams of dextrose, 10g of peptone, and 20g of agar were weighed and transferred to a 1000ml volumetric flask, and volume was made up to 1000ml with distilled water. The medium was dissolved by boiling. Sterilization was done at 15lbs pressure 121^{0}C for 15minutes by autoclaving technique.

Preparation of inoculum

The strain of *Candida albicans* was cultivated, inoculated in Malt Extract Glucose Yeast Extract Peptone medium (MGYP), and incubated for 48hrs at 30^{0}C±2^{0}C.

Development of Microbiological assay (Diffusion method)

25ml of sabouraud medium was taken and added to petri plate, allowed to solidify at room temperature for 10 to 15 minutes. Wells with a diameter of 5mm were bored after solidification at six points on each plate. The bores were filled with 50µl standard solution and were incubated at 30^0±2^{0}C for 24h. After the incubation period, the Petri dishes were observed and the diameters of the inhibition zone of microorganism's growth were measured using an antibiotic growth scale. The potency of luliconazole cream was determined by Hewitt equation. The assay was performed and analyzed statistically by the linear parallel model and using regression analysis of variance.

Method Validation

According to ICH (International Council for Harmonization) Q2 R(1) guidelines, the microbiological technique was validated by analysis of linearity, precision and accuracy.

Linearity

It was performed within the specified range as per guidelines. Replicates of the drug substance and the drug product were assessed to evaluate the linearity of the methods. For microbiological assay, the calibration curves were obtained with 5 replicates of each standard solution and sample solution, i.e. at 10, 20, 40, 80, 160µg/ml.

Precision

The repeatability was performed by with six replicates of luliconazole solutions, at 100% test concentration (40µg/ml). In the same way, inter-day precision was assessed on three different days at three concentrations of 32, 40 and 48µg/ml respectively. Luliconazole concentration was calculated in the cream samples and the relative standard deviation (RSD) was measured.

Accuracy

It was performed by adding known amount of voriconazole reference standard (40µg/ml) to a sample solutions (32, 40 and 48µg/ml) at the commencement of the analysis, equivalent to 80, 100 and 120% of the test concentration. Solutions were prepared in triplicates at each stage and added to the plate assay and the microorganism growth inhibition zone (mm) diameters were calculated using the antibiotic growth scale. The results of the bioassay were compared to the HPLC system.

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 comprised of water and acetonitrile (60:40v/v).The chromatographic separation was done using Enable C18 analytical column (250 × 4.6 mm; 5µm) at a flow rate of 1.2ml/min. The volume of the injection is 20µl. The peak areas were defined as analytical signs, with detection at 296nm.The peak areas were identified at 296nm.18 According to the guidelines of the International Conference on Harmonization (ICH) Q2 R(1), this method was optimised and validated.

Comparison of Methods

The results of the analysis obtained in this study using the microbiological method and the chromatographic method were statistically compared using the student t-test, with a significance level of 5% using MS Excel.

RESULTS

The experimental conditions were standardized to get reproducible results. Incubation temperature (30^{0}C) and period (24h) were optimised. A large number of strains of *Candida* and *Aspergillus* were used as test organisms for screening antifungal activity. In this study *Candida albicans* NCIM3471 (National Collection of Industrial Microorganisms) was used and found to be adequate for luliconazole activity testing.

An experimental design was developed for determination of luliconazole in cream by agar diffusion method. A strain of *Candida albicans* NCIM3471 was selected as test microorganism for quantitation of luliconazole under appropriate conditions. The

Table 2 : Overview of the linearity data obtained forluliconazole

S. no	Concentration (µg/ml)	*Zone of inhibition (Drug Substance) mm	*Zone of inhibition (Drug Product) mm
1	10	21.667	22
2	20	23	24
3	40	25	25.333
4	80	26.333	27
5	160	28	28.667
Slope		5.4266	5.3153
Correlation coefficient		0.9971	0.9965

*Average of three determinations

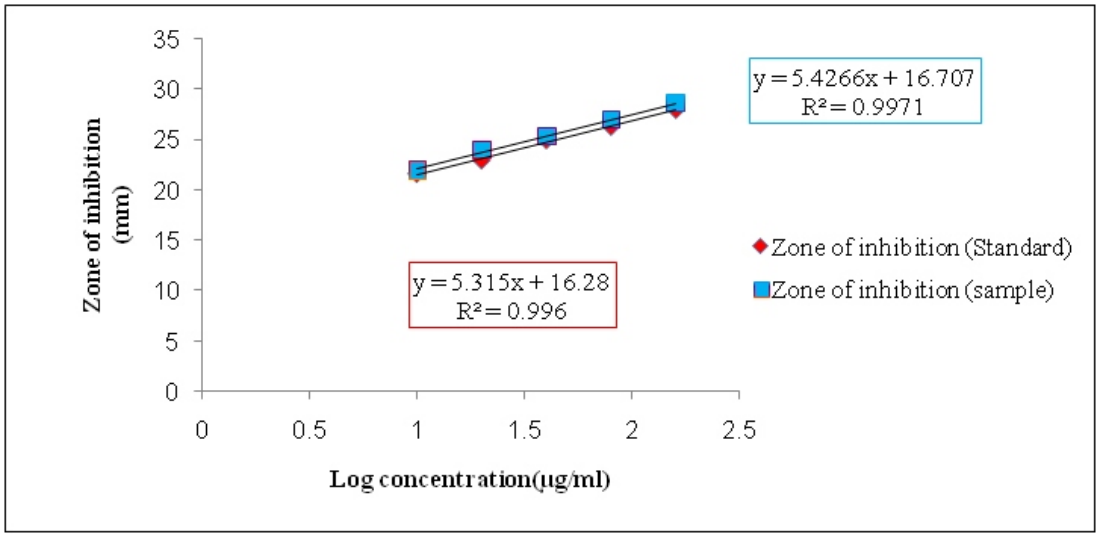

Fig. 2 : Comparison of linearity graphs for Luliconazole

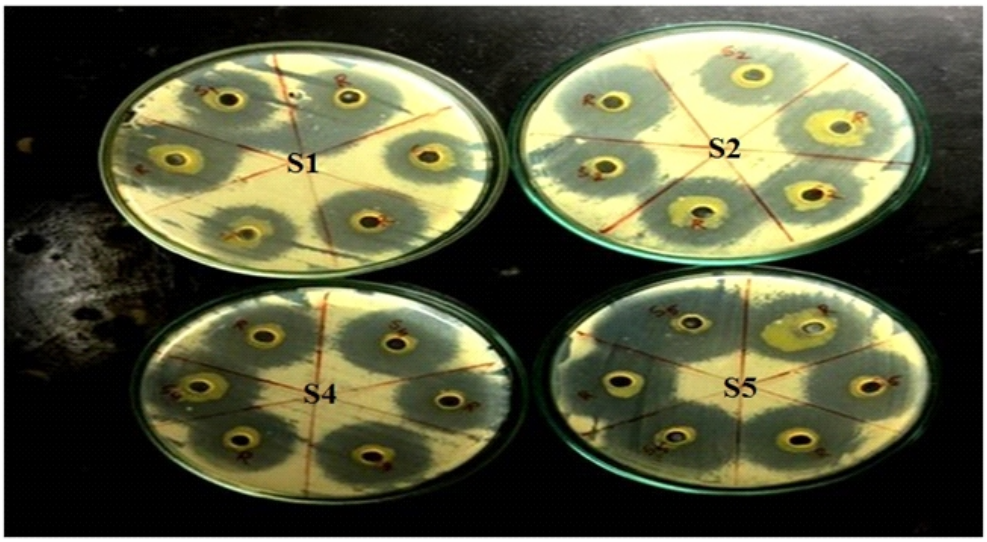

Fig. 3 : Linearity for drug substance

antibiotic potency can be determined by comparing the growth inhibition of the microorganisms produced by the determined concentrations of antibiotics to be tested and the reference standard. In a laminar air flow cabinet, both tests were performed and the test specimen was disinfected and destroyed. The analytical curve was developed from an average of three curves collected over three different days. The knowledge derived from the analytical curve was assessed by the least squares, and to check linearity and parallelism, the variance analysis (ANOVA) was used. The linearity data for luliconazole drug substance and product values were listed in Table No. 1 and calibration curve with image was given in Figure 2 and Figure 3.

The calibration curve was developed by plotting log concentration versus zone of inhibition and showed good linearity between 10-160µg/ml. The linearity and parallelism of the two curves found in the results obtained did not deviate.

The repeatability and interday method precision was seen as a percentage of 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.

At 80, 100 and 120% of the range, the precision of the method was assessed, which showed a mean accuracy of 100.12 percent. This indicates the system's ability to accurately determine the concentration of luliconazole and reveals that the effects of the bioassay were close to the true sample concentration. The experimental values obtained for the determination of luliconazole in samples are presented in Table No. 2 and the findings are shown in Figure 4.

The voriconazole calibration curve was obtained by plotting the drug concentration against absorbance using HPLC method. The curve was linear in the concentration range of 2.4-5.6µg/ml, with regression coefficient of 0.9945 and a linear regression equation of y = 20062x + 22293 shown in Figure 5. The accuracy of the assay was calculated and listed in Table 3, the mean recovery was found to be 100.61%.

The data of microbiological method and HPLC method were statistically compared by student t-test at a significance level of 5% (Table 4 and Figure 6). This showed a difference between two methods which is consider to be statistically significant at a level of 5%, indicating rejection of null hypothesis.

Table 2 : Recovery of luliconazole for accuracy evaluation by Microbiological method

Concentration (µg/ml)	Conc. of standard added (µg/ml)	Conc. of standard found (µg/ml)	% Recovered
32	40	71.70	99.58
40	40	79.68	99.60
48	40	89.04	101.18

*Average of three determinations

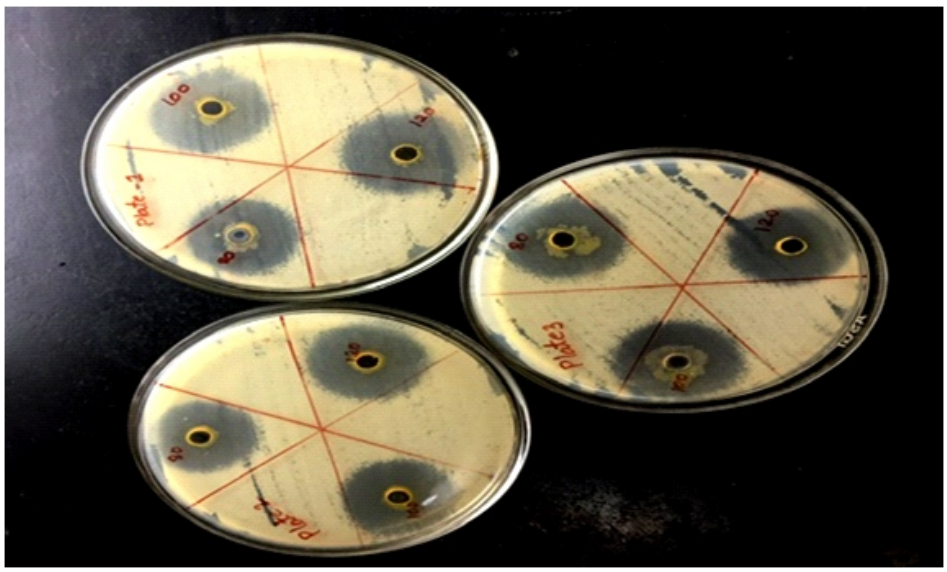

Fig. 3 : Accuracy image for 80, 100, and 120%

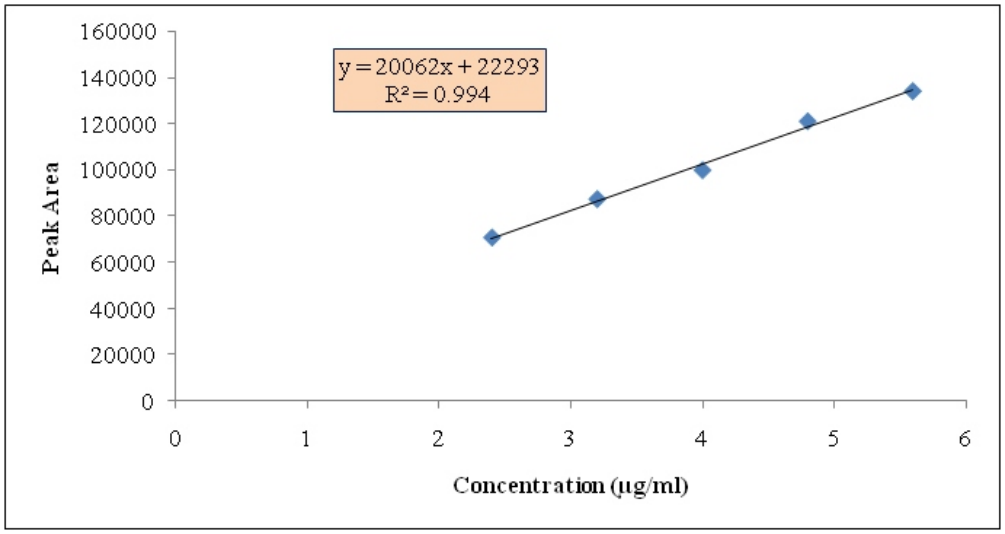

Fig. 5 : Linearity of Luliconazole by HPLC

Table 3 : Comparison of both the methods

Concentration (µg/ml)	Conc. of standard added (µg/ml)	Conc. of standard found (µg/ml)	% Recovered
3.2	4.0	7.19	99.89
4.0	4.0	8.12	101.53
4.8	4.0	8.84	100.41

Table 4 : Luliconazole contents in cream samples obtained by bioassay and HPLC methods

S. no	Luliconazole content %	
	Microbiological method	HPLC
1	98.731	97.964
2	98.569	97.536
3	98.432	97.826

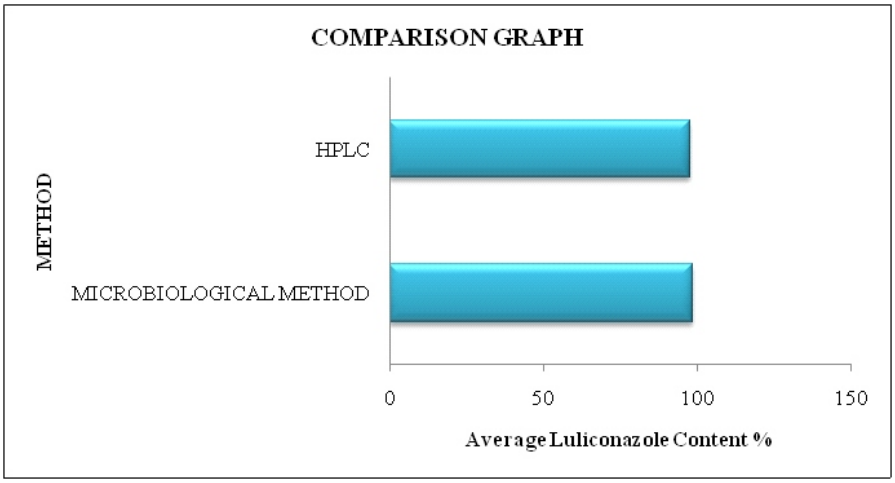

Fig. 6 : Comparison of both the methods

DISCUSSION

Luliconazole is an antifungal categorized as azole. The cup-plate and the turbidimetry are two most popular microbiological methods. The cylinder-plate method (Method A) relies primarily on the diffusion from the vertical cylinder of the antibiotic, the growth of the introduced micro-organism is entirely prevented in the area around the cylinder containing the antibiotic solution. The turbidimetric method (Method B) is based on the inhibition of microorganisms culture growth in a uniform antibiotic solution in a fluid medium that favours fast growth in the lack of the antibiotic.[3,4]

The literature review reveals that several UV (Ultraviolet) spectrophotometric [5-8], HPLC [9-12], HPLC - UV [13], HPLC and microbiological assay [14], microbiological assay [15-18] methods for estimating luliconazole have been published to date. A microbiological analytical procedure to assess the potency of luliconazole in cream was developed and validated. The antibacterial activity results were compared with the results obtained by studying HPLC.

The aim and objective of the study was to develop and validate the microbiological analytical method for estimation of luliconazole in pure and pharmaceutical dosage form (cream) according to ICH Q2 R(1) guidelines. [19]

Luliconazole was validated for microbiological analytical method with different parameters. The linearity was found in the range of 10-160 µg/ml, shown in Table 1. The r^2 value was found to be 0.9971, shown in the Figure 2 for microbiological method. The linearity was found in the range of 2.4-5.6 µg/ml and the r^2 value was found to be 0.9945, shown in the Figure 5 for HPLC method. The recovery studies revealed that the proposed method was accurate to determine small change in concentration of the solution. Precision was determined by studying the repeatability and interday precision and was found to be less than 2%.

The data of microbiological method and HPLC method were statistically compared by student t-test at a significance level of 5% (Table 4 and Figure 6). This showed a difference between two methods which is consider to be statistically significant at a level of 5%, indicating rejection of null hypothesis.

While statistical analysis has shown that HPLC and microbiological approaches have shown statistically close findings with respect to the pharmaceutical determination of luliconazole, it should be noted that there is a distinction between these approaches. The HPLC technique is adaptive and is suitable for the measurement of degradation products and impurities in the analysed matrix.

However, in addition to the use of vast amounts of organic solvents as a mobile phase, it involves the use of expensive instruments, solvents and analytical columns, which make management of the procedure expensive and lead to environmental and occupational contamination.

CONCLUSION

A microbiological analytical method for assessing the potency of luliconazole cream was developed and validated using *Candida albicans*. Although quantifying antibiotic components utilizing instrumental methods suchlike HPLC and UV spectrophotometry is accurate, they do not give accurate result for biological activity and demonstrated sufficient linearity, precision and accuracy when tested in compliance with ICH guidelines.

Although the biological test methods are highly variable, the results obtained have shown that the planned method is helpful in evaluating luliconazole in pharmaceutical dosage forms as a choice of method for the study of luliconazole quality control.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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