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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF MICAFUNGIN IN BULK AND INJECTABLES FORMULATION

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ABSTRACT

Micafungin is an antifungal drug. It belongs to the antifungal class of compounds known as echinocandins and exerts its effect by inhibiting the synthesis of 1, 3-beta-D-glucan, an integral component of the fungal cell wall. A reversed-phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the estimation of micafungin in bulk and injectables formulation. The separation was achieved on Thermo C_{18} analytical column (250 mm × 4.6 mm i.d., 5.0 μm) using 20mM KH_2PO_4: acetonitrile (pH 3.0 with orthophosphoric acid) in the ratio 20:80 v/v as mobile phase and at a flow rate of 1.0 ml/min. Detection was carried out using a UV detector at 264 nm. The total chromatographic analysis time per sample was about 7.0min with micafungin eluting at retention time of about 4.569±0.3min. The method was validated for accuracy, precision, specificity, linearity and sensitivity. Validation studies demonstrated that this HPLC method is simple, specific, rapid, reliable and reproducible. The standard curve was linear over the concentration range of 5-25μg/ml with r^2 close to one (0.999). The limit of detection (LOD) and limit of quantitation (LOQ) obtained for micafungin were 0.32μg/ml and 1.05μg/ml respectively. The developed and validated method was successfully applied for the quantitative analysis of mycamine 50mg injection. The high recovery and low relative standard deviation confirm the suitability of the proposed method for the determination of micafungin in injectables formulation.

Keywords: Analytical method development, Reversed phase HPLC method, ICH guidelines, Tablet dosage forms, Accuracy and precision

INTRODUCTION

Fungal infections are caused by microscopic organisms that can invade the epithelial tissue. The fungal kingdom includes yeasts, molds, rusts and mushrooms. Fungi, like animals, are heterotrophic, i.e. they obtain nutrients from the environment and not from the endogenous sources. Some of these fungi are pathogenic and can produce mild to severe fungal infections. An antifungal agent is a drug that selectively eliminates fungal pathogens from a host with minimal toxicity to the host [1]. They can be categorized in to several categories according to different pharmacophores and different mechanisms. Polyene antifungal drugs like amphotericin B, nystatin interacts with sterols in the cell membrane to form channels through which small molecules leak from the inside of the fungal cell to the outside. Azoles

like fluconazole, itraconazole, ketoconazole, clotrimazole, voriconazole, posaconazole etc. inhibit cytochrome P450-dependent enzymes (particularly C14-demethylase) involved in the biosynthesis of ergosterol, which is required for fungal cell membrane structure and function.

Allylamines like naftifine, terbinafine inhibit ergosterol biosynthesis at the level of squalene epoxidase. Antimetabolites like 5-Fluorocytosine act as an inhibitor of both DNA and RNA synthesis via the intracytoplasmic conversion of 5-fluorocytosine to 5-fluorouracil. Echinocandins like anidulafungin, caspofungin and micafungin are used for systemic fungal infections in immunocompromised patients and they inhibit the synthesis of glucan in the cell wall via the enzyme 1, 3-β glucan synthase [2, 3]. Analytical method Development and validation for newly introduced pharmaceuticals is of importance, as drug or drug combination may not be official in pharmacopoeia and so analytical method for quantification is not available. To check and ensure the quality standards of drug molecules and their formulation various analytical methods are employed. Most of the drugs in single or multi component dosage forms can be analyzed by HPLC method because of the associated advantages like speed, greater sensitivity, improved resolution, specificity, accuracy, precision, reusable columns and ease of automation in this method [4, 5, 6]. Micafungin (trade name Mycamine) [5-[(1S,2S)-2-[(3S, 6S, 9S, 11R, 15S, 18S, 20R, 21R, 24S, 25S, 26S)-3-[(1R)-3-amino-1-hydroxy-3-oxopropyl]-11, 20, 21, 25-tetrahydroxy-15-[(1R)-1-hydroxyethyl]-26-methyl-2, 5, 8, 14, 17, 23-hexaoxo-18-[[4-[5-(4-pentoxyphenyl)-1,2-oxazol-3-yl]benzoyl]amino]-1, 4, 7, 13, 16, 22-hexazatricyclo [22.3.0.09,13] heptacosan-6-yl]-1,2-dihydroxyethyl]-2-hydroxyphenyl] hydrogen sulfate (Fig. 1). Micafungin is a cyclic semisynthetic derivative of the echinocandin-like lipopeptide FR-901379 isolated from the culture broth of *Coleophoma empetri*, a plant pathogen associated with postharvest fruit rot in cranberries [7,8]. It has an empirical formula of $C_{56}H_{70}N_9NaO_{23}S$, a molecular weight of 1292.26 g/mol [9]. Micafungin selectively inhibit 1,3-β-D-glucan, which is required for fungal cell wall synthesis. It has been approved for the treatment of esophageal candidiasis and for prophylaxis of candida and aspergillus infections in patients undergoing hematopoietic stem cell transplantation [10, 11]. The drug was first launched in Japan in December 2002 and then also approved by US food and drug administration in March 2005 [12, 13]. Several methods have been employed for the estimation of micafungin alone and combination with other drugs by UV and RP-HPLC methods in bulk drug and plasma samples [14-17]. Shengsheng et al reported stability indicating HPLC method for determination of micafungin and related substances [18] and Joshi et al reported RP-UPLC method for determination of micafungin and related substances [19]. But there is no simple and easy method for the analysis of micafungin. Hence, it is necessary to develop a rapid, accurate and validated RP-HPLC method for the determination of micafungin in bulk and injectables formulation. This paper describes the development and validation of reliable, simple, robust, time and money saving

reversed phase HPLC method, using PDA detection, for the estimation of micafungin in bulk and injectables formulation. The developed method validated according to ICH guidelines [20].
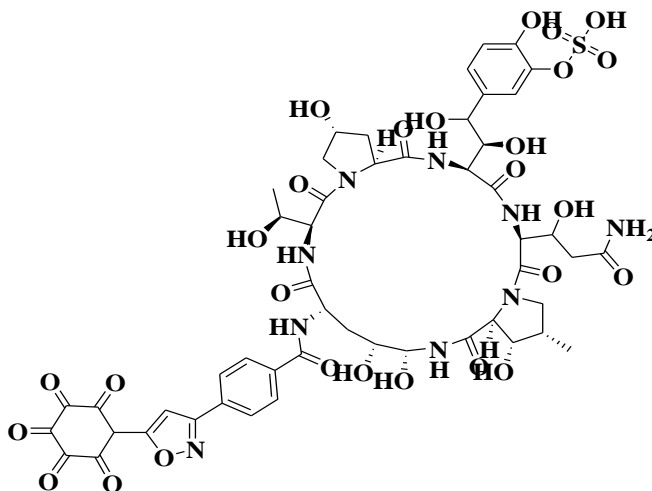

Figure 1 Chemical structure of micafungin

Materials and Methods

Instrumentation

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 binary pump for constant flow and constant pressure delivery and UV-Visible detector connected to software Data Ace for controlling the instrumentation as well as processing the generated data. Weighing was done on a Digital Micro Balance (CX-265) manufactured by Citizen Scale (I) Pvt. Ltd.

Reagents and chemicals

Analytically pure sample of micafungin was a generous gift from Glaxo SmithKline Pharmaceuticals Ltd, Mumbai along with their analytical reports. Potassium di hydrogen phosphates (AR grade), disodium hydrogen phosphate (AR grade), OPA and acetonitrile (HPLC Grade) was purchased from E. Merck Ltd. Worli, Mumbai, India. All other chemical used were of analytical grade. Triple distilled water was used for whole experiment was generated in house. Mycamine 50 mg injection Glaxo SmithKline Pharmaceuticals Ltd, Mumbai, India was purchased from local market.

Diluents

A mixture of 20mM KH_2PO_4: acetonitrile (pH 3.0 with orthophosphoric acid) in the ratio 20:80 v/v was used in RP-HPLC as diluents.

Selection of mobile phase

Initially to estimate micafungin simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was 20mM KH_2PO_4:

acetonitrile (pH 3.0 with orthophosphoric acid) in the ratio 20:80 v/v run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by Sonication. Flow rate employed for analysis was 1 ml/min.

Chromatographic conditions

The isocratic mobile phase consisted of 20mM KH_2PO_4: acetonitrile (pH 3.0 with orthophosphoric acid) in the ratio 20:80 v/v, flowing through the column at a constant flow rate of 1.0 ml/ min. The mobile phase was filtered through nylon 0.22 μm membrane filters and was degassed before use (30 min). A Thermo (C-18) column (5 μm, 250mm x 4.60mm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for drugs, 264.0 nm was selected as the detection wavelength for UV-Visible detector.

Standard preparation

Standard stock solution

Accurately weighed 10 mg of micafungin was transferred into 10 ml volumetric flask, dissolved in 5ml of diluents and volume was made up to 10ml with diluents to get concentration of solution 1000 μg/ml (Stock-A).

Working standard solution

From stock solutions of micafungin, 1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0, 1.5, 2.0, 2.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with diluents, gives standard drug solution of 5, 10, 15, 20, 25 μg/ ml concentration.

Sample preparation

For analysis of the injectables formulation, weight equivalent to weight 10 mg of micafungin was transferred to 10 ml volumetric flask and dissolved in diluents. The solution was shaking vigorously for 20 min and filtered through Whattman filter paper no.41, then volume was made up to mark with diluents. From the above solution 1 ml of solution was taken and diluted to 10 ml with diluents to get a solution containing 100μg/ml. From the above solution 1 ml of solution was taken and diluted to 10 ml with diluents to get a solution containing 10 μg/ml of micafungin. The amounts of micafungin in injection were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with formulation.

Results and discussion

Chromatography

The mobile phase was chosen after several trials with methanol, isopropyl alcohol, acetonitrile, water and buffer solutions in various proportions and at different pH values. A mobile phase consisting of 20mM KH_2PO_4: acetonitrile (pH 3.0 with orthophosphoric acid) in the ratio 20:80 v/v was selected to

achieve maximum separation and sensitivity. Flow rates between 0.5 and 1.5 min were studied. A flow rate of 1 ml/min gave an optimal signal-to-noise ratio with a reasonable separation time. Using a reversed-phase C_{18} column, the retention times for micafungin was observed to be 4.569±0.3min. Total time of analysis was less than 7 min. The maximum absorption of micafungin was detected at 264nm and this wavelength was chosen for the analysis Fig. 2
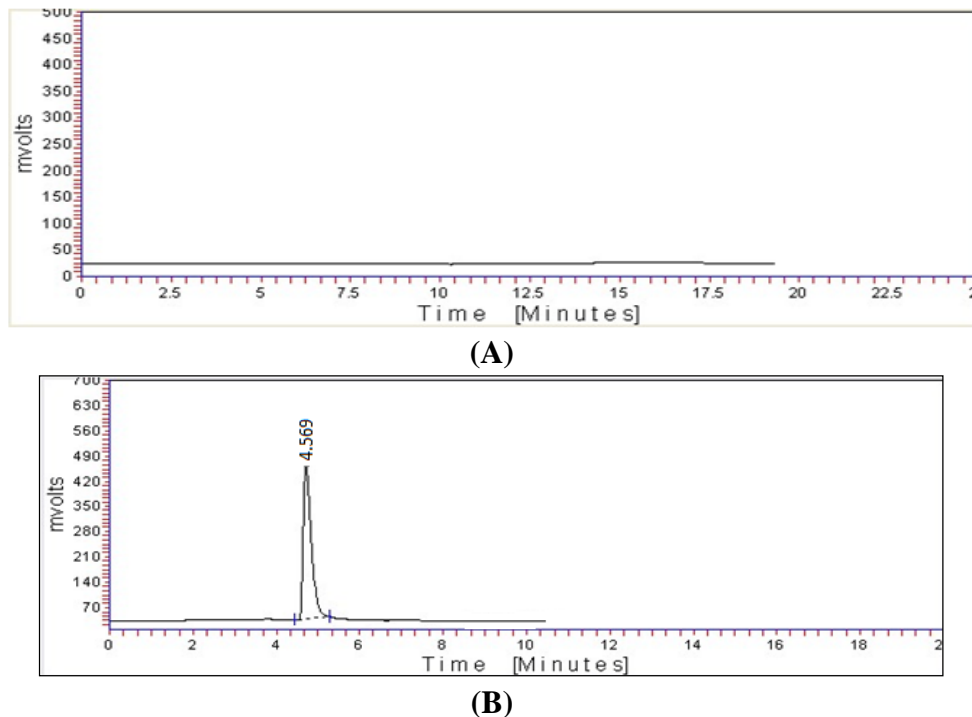

(A)

(B)

Figure 2 Chromatograms of (A) Blank mobile phase (B) micafungin (10µg/ml) as reference substances

System suitability

System suitability parameters such as number of theoretical plates, HETP and peak tailing are determined. The results obtained are shown in Table 1. The number of theoretical plates for micafungin was 2127.

Table 1 Results of system suitability parameters

Parameters	Micafungin
AUC*	971.4593
No. of Theoretical Plates	2127
Tailing Factor*	0.945
Retention time*	4.569
Calibration range (µg/ml)	5-25

*Each value is the mean ± SD of six determinations

Linearity

The calibration curve was linear over the concentration range of 5-25µg/ml for micafungin. The linearity was represented by a linear regression equation as follows:

Y (micafungin) = 65.52conc-0.821 (r^2 = 0.999)

Accuracy

Recovery studies were performed to calculate the accuracy of developed method to preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed. The value of percentage RSD was found less than 2 (0.647, 0.659 and 0.438) show good recovery at all three level 80, 100 and 120% respectively. Each level was made in triplicate Table 2.

Table 2 Results of recovery study

% Level	% Mean±SD* Micafungin
80%	99.167
100%	99.800
120%	99.944

* Value of three replicate and three concentrations.

Precision

Repeatability

Five dilutions in three replicates were analyzed in the same day for repeatability and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Intermediate precision

Five dilutions in three replicates were analyzed on two different days and by two analysts for day-to-day and analyst-to-analyst variations and results were found within acceptable limits (RSD < 2) as shown in Table 3.

Robustness

As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, 20mM KH$_2$PO$_4$: acetonitrile (20:80 % v/v), to (25: 75% V/V) and method is found robust as RSD is again found < 2.0 Table 3.

Table 3 Statistical data for precision and robustness

Statistical parameter	Esomeprazole		
	Mean*	S.D*	R.S.D*
Repeatability	99.70	0.068	0.273
Intermediate Precision (I) (A day to day)	99.72	0.035	0.139
(II) Analyst to Analyst	99.22	0.166	0.670
Robustness	99.44	0.064	0.545

*Mean of 15 determinations (three replicates at five concentration level)

Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve Table 4.

Table 4 LOD and LOQ

Name	LOD (µg/ml)	LOQ (µg/ml)
Micafungin	0.32	1.05

Analysis of marketed formulation

The assay value of drugs was close to 100, SD and % RSD are less than 2 indicate the no interference of excipients in the estimation of drug Table 5.

Table 5 Assay of tablet formulation

S. No.	Parameter	Micafungin
1.	**Mean**	99.12
2.	**S. D.**	0.215
3.	**% RSD**	0.265

Mean of nine determinations

Conclusion

The proposed HPLC method was validated as per the International Conference on Harmonisation (ICH) Q2B Guidelines, and was found to be applicable for routine quantitative analysis of micafungin by HPLC in pharmaceutical dosage form. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of micafungin with no interference from other formulation excipients. The proposed method was highly reproducible, reliable, rapid, robust and specific. Therefore, a high percentage of recovery and the run time of less than seven minutes allow its application for the routine determination of micafungin in the pharmaceutical dosage form.

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