WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 12, Issue 6, 643-652. Research Article ISSN 2277– 7105

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF BIFONAZOLE AND IT'S STABILITY STUDY BY USING SOPHISTICATED RP-HPLC METHOD

Santosh R. Prajapati*, Atul R. Nagar and Dr. Purnima D. Hamrapurkar

Department of Pharmaceutical Analysis, Prin. K. M. Kundnani College of Pharmacy, Mumbai, Maharashtra, India.

Article Received on
22 Feb. 2023,

Revised on 13 March 2023,
Accepted on 03 April 2023

DOI: 10.20959/wjpr20236-27616

***Corresponding Author**
Santosh R. Prajapati
Department of Pharmaceutical Analysis, Prin. K. M. Kundnani College of Pharmacy, Mumbai, Maharashtra, India.

ABSTRACT

A simple precise and sensitive method has been developed and validated for the quantification of Bifonazole. The aim of the presented work was to develop a precise and accurate method. The column used was Haemochrom C18(150x4.6mm x5µ) and the mobile phase consisted of acetonitrile and 0.05% TFA (20:80) with a flow rate of 1ml/min. retention time was found to be 6.871minute, wavelength 256 nm detection was carried out using PDA detector. Linearity was studied in the range 10 µg/ml - 50 µg/ml with coefficient correlation R2=0.9991. The developed method was also evaluated for various system suitability parameters and validated for accuracy, precision, LOD, and LOQ. The Method was successfully applied for stability indicating assay for the estimation of Bifonazole ie. cream.

KEYWORDS:- Bifonazole, RP-HPLC, Validation, Column, Force Degradation.

INTRODUCTION

Bifonazole is a antifungal imidazole substituted agent that is structurally similar with other agent of this class. bifonazole chemically (1-{[1,1'-biphenyl]-4-yl phenyl methyl}-1 H imidazole. it has a broad spectrum activity against fungal cell and some other gram positive bacteria, yeast, dermatophaytes. Effectiveness of bifonazole against superficial fungal infection is good and also well tolerated. It will provide better compliance to the patient in terms of application by topical route once In a day in compare to other topical antifungal agents.[1]

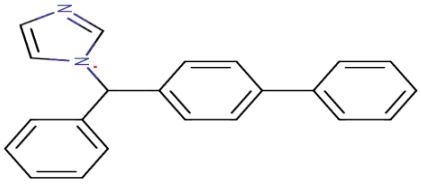

Fig. No. 1: Structure of bifonazole.

The literature survey reveals that there were few methods[2-8] reported for the estimation of Bifonazole. However[2-4] were carried out by using UV-Visible spectrophotometer. An attempt method is simple sensitive rapid using fewer solvents and fully validated RP-HPLC method, and method is also applied on degradation studies.

MATERIAL AND METHODS

Reagents

Bifonazole received as gift sample from vital laboratory Mumbai India. HPLC grade Acetonitrile (Finar) and AR Grade trifluroacetic acid (Merck), were procured from Ultra-Pure Analytics, Goregaon, Mumbai, India. A 1% bifonazole cream containing 2% benzyle alcohol as a preservative (Mycospore) marketed by Bayer. 0.05% trifluroacetic acid solution made in HPLC grade water were used for the preparation of mobile phase.

Equipments/Instruments

RP-HPLC analyses were performed on an Waters Alliance HPLC system 2695 Quaternary Gradient HPLC pump, Empower Pro software as an integrator, equipped with vacuum degasser, autosampler, column compartment and variable wavelength Waters 2996 Photodiode Array Detector. Also, analytical balance (Mettler Toledo), pH meter (Lab India), and a sonicator (Spectralab) were used. The column used for the separation of Bifonazole was Haemochrom C18 C18 (150x4.6mm x5µ).

Standard solution preparation

In a 10 ml volumetric flask, 10 mg of Bifonazole was accurately weighed and transferred. Drug was dissolved and diluted with Acetonitrile to 10 ml to give a (Solution A) (1000 µg/ml). 1ml of solution A was taken in 10 ml volumetric flask and was diluted with solvent to 10 ml (Solution B) (100 µg/ml). Solution B was used as working solution to make further dilutions. For validation of method 5 different concentration solutions in range 10, to 50 µg/ml of Bifonazole were made and calibration curve found by plotting the graph ratio of area against the drug concentration.

Chromatography parameters

The analytical method for Bifonazole was developed and validated using a state-of-the-art instrument like High-Performance Liquid Chromatography (HPLC), Separations were made on a Haemochrom C18 (150x4.6mm x5µ) using 0.05% TFA (trifluoroacetic acid) solution: acetonitrile as a mobile phase with gradient elution in a proportion of (80:20) at pH 2.5. Wavelength was detected at 256 nm with the help of a PDA detector. The elution was performed at a flow rate of 1 ml/min.

Assay of marketed formulation BIFONAZOLE cream

1gm of Bifonazole cream (equivalent to 10mg of Bifonazole) was accurately weighed. It was then transferred to a 10 ml volumetric flask. The volume was made upto 10ml with diluent (ACN) and was sonicated. The Solution was then filtered through nylon syringe filter. This is 1000µg/ml (Solution A). 1 ml from solution A was pipetted out and transferred to 10ml volumetric flask. The volume made upto 10ml with diluent. This is 100µg/ml (Solution B). 3ml of solution B was pipetted out and diluted to 10ml to give 30µg/ml (Solution C) 10µl of solution C injected onto the column.

Force degradation studies

Forced degradation studies were conducted out to justify the method's stability-indicating nature by subjecting the drug to the following conditions:[9]

1. Acid hydrolysis
2. Base hydrolysis
3. Oxidative Degradation
4. Thermal degradation
5. Photolytic degradation

1. **Acid hydrolysis:** 2 ml of solution A (1000µg/ml) was pipetted out in a 10ml volumetric flask. The solution was treated with 1 N HCl and kept at room temperature for 24 hrs which was then neutralized with 1 N NaOH (Base-Stabilizer) and made up the mark with diluent to give a solution of 200µg/ml. Finally, the solution was loaded into HPLC and the corresponding chromatogram was recorded.

2. **Base hydrolysis:** 2 ml of solution A (1000µg/ml) was pipetted out in a 10ml volumetric flask. The solution was treated with 1N NaOH and kept at room temperature for 24 hrs which was then neutralized with 1 N HCl (Acid-Stabilizer) and made up the mark with

diluent to give a solution of 200µg/ml. Finally, the solution was loaded into HPLC and the corresponding chromatogram was recorded.

3. **Oxidative degradation:** 2 ml of solution A (1000µg/ml) was pipetted out in a 10ml volumetric flask. The solution was treated with 1ml of 3% Hydrogen peroxide solution and kept at room temperature for 24 hrs which was then made up to the mark with the diluent. Finally, the solution was loaded into HPLC and the corresponding chromatogram was recorded.

4. **Thermal degradation:** 2ml of the solution A (1000µg/ml) was pipetted out in a 10ml volumetric flask. The solution was kept in water bath at 60℃ for 30 hr. The volume was made up with the diluent to give a solution of 200µg/ml. Finally, the solution was loaded into HPLC and the corresponding chromatogram was recorded.

5. **Photolytic degradation:** 2 ml of solution A (1000µg/ml) was pipetted out in a 10ml volumetric flask. The solution was kept in sunlight for 24 hr minutes. The volume was made up with the diluent to give a solution of 200µg/ml. Finally, the solution was loaded into HPLC and the corresponding chromatogram was recorded.

RESULTS AND DISCUSSION

Development of chromatographic method for Bifonazole using HPLC

In the method which developed for the analysis of Bifonazole carried out under gradient elution, the mixture of 0.05% TFA and acetonitrile was used as a mobile phase. And haemochrom C18 was used as a stationary phase during the work. Regarding the location and resolution of analytical peaks, the different flow rates of 0.5,1.0, and 1.5 ml/min were tested and finally, 1.0 ml/min was decided as the best. With the help of a PDA detector wavelength of Bifonazole was detected at 256 nm and the above chromatographic conditions described that the method allows better resolution of Bifonazole.

Validation of developed method

Validation of Bifonazole was carried out for parameters like System suitability, Specificity, Limit of detection (LOD), Limit of quantification (LOQ), Linearity, Range, Accuracy, and Precision.[10]

Table 1: Optimized chromatographic conditions.

Parameter	Specification
HPLC	Waters Alliance HPLC system 2695 Quaternary Gradient HPLCpump
Detector	2996 PDA detector
Software	Empower pro software
Mobile Phase	Acetonitrile:0.05% TFA (Trifluoroacetic Acid) in water (20:80)
Column	Hemochrom C18 (150 mm*4.6mm*5um)
Flow rate	1.0 ml/min
Injection Volume	10µl
Column Oven Temperature	28° C
Wavelength	256nm
Mode	Gradient

System suitability

Bifonazole standard solution of 30 µg/mL was injected in six replicates. The mean of system suitability parameters was obtained. Table 2 shows the Data of system suitability of Bifonazole.

Table 2: Data of system suitability of bifonazole.

Sr. No	System suitability parameters	Observations	Acceptance Criteria
1	Bifonazole standard solution	30 µg/ml	---
2	Area % RSD	0.75%	NMT 2%
3	Retention Time	6.836	---
4	NTP	33794	NTP>2000
5	Symmetry Factor	1.202	0.8 to 2

Specificity

The test for specificity is conducted to check the identification, interference and peak purity of the drug. Blank (Diluent) and Bifonazole standard solution (30µg/ml) were injected and the representative chromatograms for specificity are shown Fig No 2.

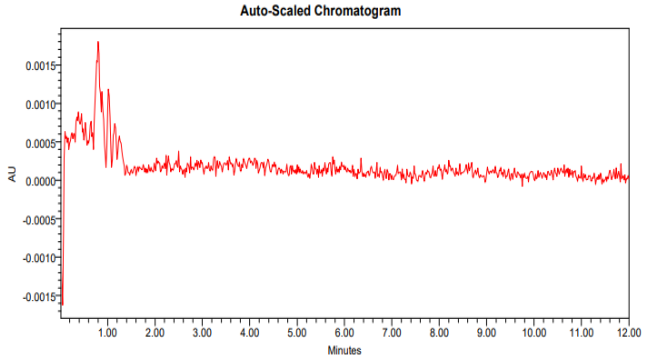

Fig. No. 2: Chromatogram for blank.

Linearity and Range

Linearity was evaluated in six replicates (10 µg/ml to 50 µg/ml) for Bifonazole. The correlation coefficient (R2) was found to be **0.9991** and the equation of the line was found to be **y = 45276x – 19432** from the calibration curve. Thus, the data shows that the response was found to be linear. This clearly indicates that an excellent correlation existed between the peak area and concentration of the analyte. Table 3 shows the Linearity data of Bifonazole.

Table 3: Linearity data of bifonazole.

Concentration (µg/ml)	Peak Area						
	Inj 1	Inj 2	Inj 3	Inj 4	Inj 5	Inj 6	Mean
10	446240	446006	452618	448286	454006	449618	449462
20	865659	868864	871620	866711	878602	868214	869945
30	1360066	1352726	1341436	1344409	1330716	1342406	1345293
40	1740939	1783471	1754443	1759617	1785070	1752341	1762646
50	2262438	2267427	2271589	2267151	2260407	2272509	2266920

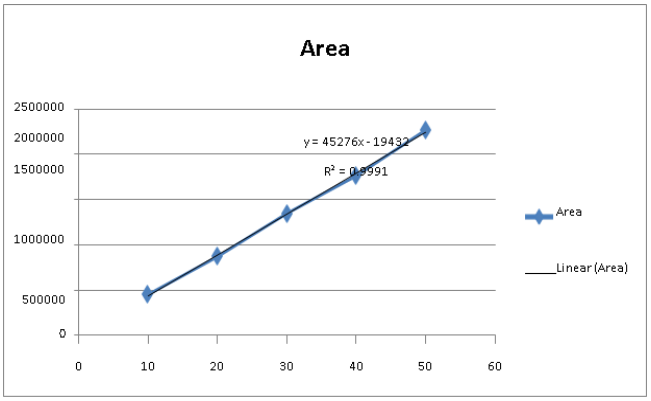

Fig. No. 3: Calibration curve for bifonazole.

Precision

Intraday Precision (Repeatability) of Bifonazle was determined by taking six replicates of 10µg/ml, 30µg/ml and 50µg/ml concentration at different time intervals and Inter-day Precision was performed using the same concentration levels and injecting six replicates on two consecutive days. The % RSD values for Intraday and Inter-day precision of three concentration levels was found to be 0.81% and 0.78% respectively which meets the acceptance criteria.

Accuracy

%Recovery study was performed using a minimum of 3 concentration levels, each in triplicates. % Recovery was carried out by spiking 50%, 100% and 150% of the working level concentration to blank in triplicates. % Recovery was found to be 100.10% and %RSD

was found to be 0.38% which are in the acceptance criteria. Table 4 shows the Accuracy data of Bifonazole.

Table 4: Accuracy data of bifonazole.

S. no.	Conc. (µg/ml)	Peak area			Mean	SD	RSD	Recovered conc.	% Recovery
		Injec.1	Injec.2	Injec.3					
1.	10	440831	443891	442031	442251.0	4572.80	1.01%	10.19	101.09%
1.	30	1323926	1326061	1325517	1325168	1109.46	0.08%	29.69	98.99%
3.	50	2251526	2248989	2249321	2249945	1378.92	0.06%	50.12	100.24%

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The Limit of Detection (LOD) of an analytical procedure is the lowest amount of analyte in the sample which can be detected (signal-to-noise ratio of 3:1) but not necessarily quantified as an exact value. The Limit of Quantification (LOQ) is the lowest concentration of the analyte which gives a response that can be accurately quantified (signal-to-noise ratio of 10:1).

The LOD for Bifonazole was found to be 0.2 µg/ml and S/N ration was found to be 3.39. The LOQ for Bifonazole was found to be 0.6 µg/ml and S/N ratio was found to be 10.53 which meets the acceptance criteria. This indicate the developed method is sensitive.

Solution stability

The stability of drug solution was evaluated for 3 different concentrations i.e. 10 µg/ml, 30 µg/ml and 50 µg/ml. The analysis was performed at initial, 1 hr, 3 hrs, 9 hrs, 24 hrs, and 48 hrs. The stability solution test results indicated that the drug solutions were stable up to 48 hours.

Table 5: Statistical data of validation.

S no.	Validation Parameter	Bifonazole
1	System suitability	NTP- 33794 %RSD- 0.75% Symmetry factor- 1.202
2	Specificity	No interference was found at the RT of the analyte
3	Linearity	R^2 = 0.9991 (Linear), y = 45276x – 19432
4	Accuracy	100.0%
5	Intraday precision(%RSD)	0.81%
6	Inter-day precision(%RSD)	0.78%
7	LOD	0.2 µg/ml
8	LOQ	0.6 µg/ml
9	Solution Stability(%RSD)	0.80%

Assay of marketed formulation by HPLC method

The developed and validated RP-HPLC method was applied to quantitatively estimate Bifonazole from marketed formulation Mycospore cream containing Bifonazole 1%. The differences between the amount claimed and those measured were very low and the RSD values were within the acceptance criteria mentioned by pharmacopoeias. The mean percentage recoveries obtained after three repeated experiments were 99.28% with a RSD of 0.80 % for BIF, indicating that the results are accurate and precise and there is no interference from the common excipients used in the cream. The chromatogram is shown Fig No 4.

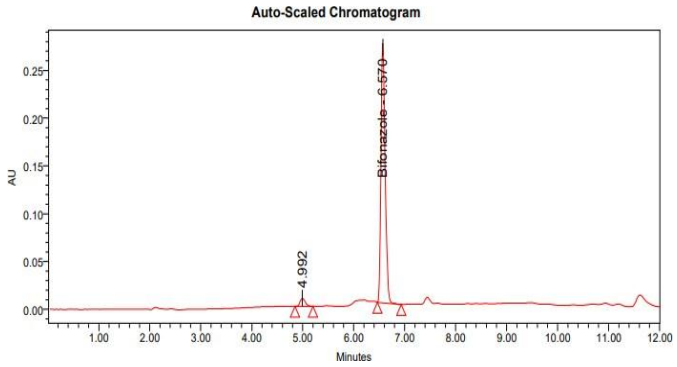

Figure 4: Chromatogram of bifonazole in marketed formulation.

Stability indicating method of bifonazole HPLC

To prove the stability indicating nature of the method, forced degradation studies were carried out by exposing the stock solution of the Bifonazole to the following conditions: Acid hydrolysis, Base hydrolysis, Oxidative Degradation, Thermal degradation & Photolytic degradation, to suggest forced degradation behavior.

The forced degradation studies indicate that the drug is susceptible to oxidative degradation. The degradation observed for oxidative degradation was found to be 13.65%. The representative chromatograms for forced degradation studies reveal that the method is a stability indicating method and can be used for the routine analysis of drug in bulk form. The chromatogram of oxidative degradation is shown Fig No 5.

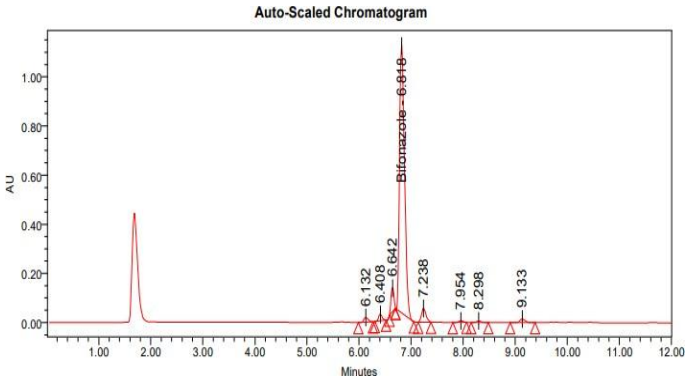

Fig. 5: Chromatogram of peroxide degradation.

CONCLUSION

The RP-HPLC assay method developed for the determination of Bifonazole was found to be sensitive, accurate, precise, and specific as proven in the validation results. The developed method was also proven to be stability indicating. This developed and validated method can be used for the routine analysis drug in bulk form.

Acknowledgement

The authors are grateful to Vital Laboratory Mumbai for providing the gift sample of the API. The authors would also like to thank Dr. Kiran Desai sir and the staff members of Ultra Pure Analytics Mumbai for providing the required facilities which helped in the completion of the project.

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