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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODOLOGY FOR BIFONAZOLE ESTIMATION: DEVELOPMENT AND VALIDATION INSIGHTS

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ABSTRACT

The objective of the present study was to develop a simple, sensitive, specific and reliable high performance liquid chromatography method for the determination of Bifonazole (BFN) in pharmaceutical formulations. Separations were made by using C18- column (4.6 × 250mm i.d., particle size 5µm) HSS (High Strength Silica). The mobile phase used was a mixture of Acetonitrile: Water in the ratio of 70:30 v/v isocratic at 40º C. The mobile phase flow rate was 1mL/min with injection volume of 10µL. Sample detection was done by PDA detector at 254 nm with a retention time of 6.747 minute. Developed HPLC method was validated in compliance to ICH guideline Q2 (R1) and various validation parameters Specificity, Linearity, Accuracy, Precision, Limit of detection (LOD), Limit of quantification (LOQ), Robustness were determined. The developed method was independent of pH adjustment and use of buffers. The method was successfully employed in determination of BFN in pharmaceutical formulations and can be a speedy and cost-effective quality control means for routine quantitative analysis of BFN.

Keywords: Method development, Validation, High performance liquid chromatography, BFN

INTRODUCTION:

BFN is an imidazole derivative with broad spectrum activity. It is from the azole group of antifungals and structurally related to molecules such as clotrimazole, econazole and miconazole belonging to the same group **[1]**. BFN is chemically 1-[biphenyl-4-yl-(phenyl) methyl] imidazole and is official in the European Pharmacopoeia **[2]**. BFN is used primarily for the treatment of fungal infections **[3]**. Its antifungal activity can be attributed to inhibition of the cell membrane formation. Azole group of antifungals are being extensively used for treatment of mycoses in patients especially with decrease immunity e.g. AIDS, organ transplantation, etc. **[4]**.

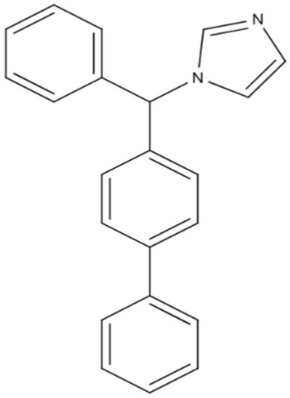

Figure 1: Structure of BFN

Literature survey divulges that few analytical techniques have been established for BFN. Cudina *et al* reported an HPLC method with isocratic elution using mobile phase methanol: ammonium acetate (pH =2 adjusted with ortho-phosphoric acid, 65mM) at a ratio 65:35 v/v (pH= 3.6) **[5]**. Another study by Prajapati *et al* investigated gradient elution with mobile phase comprising 0.05% trifluoroacetic acid solution: acetonitrile in a proportion of 80: 20 at a pH of 2.5 wherein the detection was done at 256nm **[6]**. Wahab et al reported an HPLC method for BFN wherein the mobile phase used was a mixture of methanol: 0.1M sodium acetate in the ratio of 70: 30 ratio at pH 3 with detection at 252 nm **[7]**. G. Popovic et al reported an HPTLC method for BFN using: n-hexane: ethyl-acetate: acetone: di-ethylamine (4.5:4.5:1:0.4; v/v/v/v), as a mobile phase **[8]**. One of the UV-Spectrophotometric methods investigated was based on the charge transfer complexation reaction of BFN with 2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone resulting in the formation of coloured complex **[9][10]**.

The present study reports the development of RP-HPLC method which uses a combination of acetonitrile: water as mobile phase which did not require any pH adjustment and use of buffers.

MATERIALS AND METHODS:

Materials and reagents:

BFN was purchased from Yarrow Chem Products, Mumbai, Maharashtra. HPLC grade methanol and acetonitrile [were purchased from Rankem chemical (Avantor brand), Mumbai, Maharashtra and Fisher Scientific-Qualigens, Mumbai, Maharashtra. HPLC grade water was sourced form Milli-Q (EQ 7008) lab water purification machine (Merck Millipore water purification system).

Instruments:

The Shimadzu-i - Series LC- 2050C 3D system (Shimadzu, Japan) is used for the HPLC method development equipped with a Shimadzu Photodiode Array Detector (PDA detector) and an autosampler. LabSolutions software version 5.106 is used for the chromatographic data analysis. The other instruments used are Electronic Weighing Balance (Mettler Toledo- ME204), ultrasonic sonicator bath (Analytical Technologies Limited) and vacuum pump.

Selection of wavelength of detection: The wavelength for detection of BFN was selected from preliminary studies of BFN using UV spectrophotometry.

Chromatographic conditions:

Separations were made by using C18-column (4.6 × 250mm i.d., particle size 5µm) HSS (High Strength Silica) operated at 40º C using a column oven. The mobile phase used was a mix of acetonitrile: water in the ratio of 70:30 v/v with the mobile phase flow rate set at 1mL/min. The volume of injection was 10µL. PDA detector was set at 254 nm for UV identification of the analyte. The sample run time was 15min. Quantitative assessments were done by using peak area.

Preparation of standard solution:

10mg of BFN drug is solubilized in 10 mL of HPLC grade methanol to get the stock solution of 1000 ppm. 1mL from the above solution was withdrawn and the volume as made up to 10mL of HPLC grade methanol to get stock solution of 100ppm. The 100ppm stock solution was serially diluted to prepare 10ppm, 15ppm, 20ppm, 25ppm and 30ppm BFN solutions in 10mL calibrated volumetric flask. All the dilutions were done with HPLC grade methanol.

Preparation of sample solution:

Methanolic extract of the BFN formulation was prepared by sonicating the formulation with HPLC grade methanol for 5 min at 40º C. The extract was then filtered through a micro-separation filter (0.45µm, 25mm) attached with a syringe. The extract is further diluted to prepare solution equivalent to 20 ppm BFN. All the standard and sample solutions were transferred into 1mL HPLC vial and loaded in the HPLC rack.

Method validation:

The method was validated according to the International Council on Harmonization (ICH) guidelines **[11]**. The validation parameters were: specificity, linearity, accuracy, precision, LOD, LOQ, robustness in accordance with ICH guideline Q2(R1). Specificity of the method was checked by observing if there was any interference from the formulation excipients. Linearity was obtained from the calibration curve made by plotting concentration of solution against peak area in the range of 10 ppm – 30 ppm. The accuracy of the method was found out by performing the recovery studies. Samples were injected 3 times in a single day as well as on 3 different days to compare the recovery and retention time for estimating precision. The peak areas were recorded and %RSD were calculated for both Inter-day and Intra-day variations. For the determination of LOQ and LOD, the linearity of the standard was performed in triplicate to obtain the SD of the intercept and slope of the regression equation (S). LOD & LOQ were determined by using the following formulae: LOD = 3.3 × SD/S; LOQ = 10 × SD/S. Robustness was analysed by varying the flow rate.

RESULT AND DISCUSSION:

The present study discusses the validated HPLC method devlopment for estimation of BFN in pharmaceutical formulations.

Assay specificity:

Specificity is a significant parameter for finalizing the HPLC analytical method. Hence, blank, placebo, standard, drug loaded formulation were prepared as per the optimized test procedure and injected into HPLC. The chromatograms did not show any interference at retention time of BFN **(Figure 2)**. The peak purity was also acceptable. Result indicated the significant specificity of the developed HPLC method to determine BFN in pharmaceutical formulations.

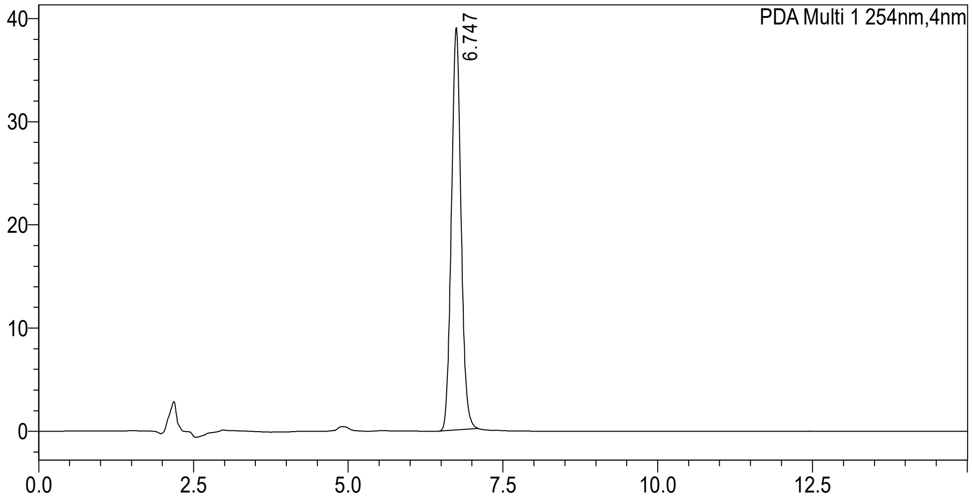

Figure 2: Optimized Chromatogram

Linearity:

Calibration curves were constructed for the standard solutions using concentration against area. The correlation coefficient was determined for the range of standard solutions explored and it was calculated to be 0.9904. The overlay **(Figure 3)** is the collection of all the sample concentration range from 5ppm- 30ppm.

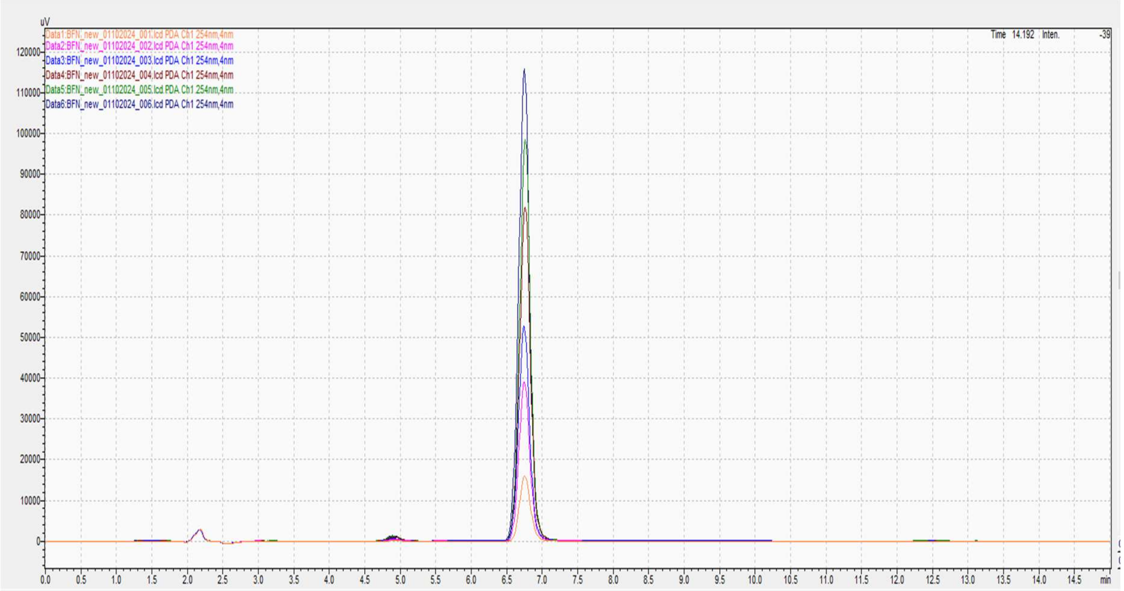

Figure 3: Overlay graph of the standard drug solutions

Accuracy:

The accuracy was assessed using the suggested method for the analysis of placebo in known quantities of BFN to obtain solutions at concentration of 12ppm, 15ppm and 18ppm of BFN. The selected concentration covers the linear range of the method and are equivalent to 80%, 100% and 120% nominal levels. Recovery results were found in the range from 100.957% to 101.263% of BFN using the proposed HPLC method which indicates the trueness of the present method. The accuracy results are compliant with the acceptance criteria.

Table 1: Recovery studies of BFN

LEVEL	STD. CONC. (ppm)	CONC. FOUND (ppm)	%RECOVERY	MEAN ± SD (%)	% RSD
80%	**12**	**11.85**	**101.26**	**101.263 ± 0.60**	**0.59**
	12	**11.92**	**100.67**		
	12	**11.88**	**101.86**		
100%	**15**	**14.85**	**101.01**	**101.0333±0.65**	**0.64**
	15	**14.94**	**100.4**		
	15	**14.75**	**101.69**		
120%	**18**	**17.85**	**100.84**	**100.9567± 0.79**	**0.78**
	18	**17.78**	**100.23**		
	18	**17.68**	**101.8**		

Precision:

The method precision was assessed by Intra-day precision and Inter-day precision. The Intra-Day precision was verified on the same day at three different time (T1, T2 and T3). Intra-day precision for concentrations 10ppm, 15 ppm and 20 ppm and the % RSD was found to be 0.16%, 0.24% and 0.26% respectively. The Inter-Day precision was performed on different days. The Inter-Day %RSD was observed as 0.34%, 0.35% and 0.36% respectively. This demonstrates that the present method is consistent with good precision and in accordance with ICH guidelines Q2(R1).

Table 2: Intra-Day Precision of BFN

Concentration (ppm)	Time	Area	Mean± SD (%)	%RSD
10	**T1**	**438019**	**437465.3 ± 697.5**	**0.16**
	T2	**436682**		
	T3	**437695**		
15	**T1**	**603678**	**604815.7 ± 1434.4**	**0.24**
	T2	**606427**		
	T3	**604342**		
20	**T1**	**896800**	**897305.3 ±2313.8**	**0.26**
	T2	**899830**		
	T3	**895286**		

Table 3: Inter-Day Precision of BFN

Concentration (ppm)	Day	Area	Mean± SD (%)	%RSD
10	**Day-1**	**438019**	**436717.7± 1496.8**	**0.34**
	Day-2	**437052**		
	Day-3	**435082**		
15	**Day-1**	**603678**	**604443 ± 2143.8**	**0.35**
	Day-2	**602786**		
	Day-3	**606864**		
20	**Day-1**	**896800**	**893229.7± 3222.99**	**0.36**
	Day-2	**892354**		
	Day-3	**568935**		

LOD and LOQ:

The limit of detection is the lowest amount of analyte concentration in the sample which can be detected. The limit of quantification is the lowest analyte concentration which gives a response that can be quantified accurately. LOD was calculated as 3.13 ppm and LOQ as 9.5 ppm.

Robustness:

In HPLC, robustness refers to the method's ability to remain unaffected by variation in parameters like flow rate. The flow rate was varied by 20% (0.8mL/min., 1mL/min. and 1.2mL/min) to check any variation in the peak area. The %RSD was determined as 0.23% and falls within the acceptance criteria indicating that the developed method was robust.

Table 4: Robustness

Parameter	Variable	Condition	Area	Mean ± SD (%)	%RSD
Flow rate	**0.2%**	**0.8 mL/min.**	**603678**	**604256 ± 1385.6**	**0.23**
		1 mL/min.	**605837**		
		1.2 mL/min.	**603253**		

Table 5: Summary of Validation parameters data of BFN

Parameter	Results
Linearity range (ppm)	10-30
Retention time (min.)	6.747
Run time (min.)	15
Correlation Coefficient (R^2)	0.9904
Theoretical plate	8252 ±38.7
Tailing factor	1.0192 ± 0.039
LOD (ppm)	3.13
LOQ (ppm)	9.5
Accuracy (%)	0.50 – 0.64
Precision (%RSD)	
Intra-day (n=3)	0.16 – 0.26
Inter-day (n=3)	0.34 - 0.36
Robustness (%RSD)	0.22
Resolution	16.8

The validation parameters are summarized in **Table 5** which shows the successful development of a RP-HPLC method for estimation of BFN.

CONCLUSION

The HPLC method developed in this study was optimized for the accurate, precise, and robust quantification of BFN in pharmaceutical formulations. The method demonstrated excellent linearity, sensitivity, and selectivity across the desired concentration range. Through systematic optimization of key parameters, the final method provided efficient separation and a short analysis time. Validation of the method confirmed its reliability with respect to parameters like precision, accuracy, robustness, LOD, and LOQ, making it suitable for routine analysis in [application area, e.g., pharmaceutical quality control, environmental monitoring, etc. Overall, the developed HPLC method represents a significant contribution to pharmaceutical analysis, offering a robust and reproducible tool for the analysis of BFN.

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