ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF EBERCONAZOLE NITRATE BY HPLC.

Purva Jadhav*[1], Amit J Kasabe[1], Pravin Kamble[2]

[1]*Departement of Pharmaceutical Quality Assurance, PDEA's Shankarrao Ursal College of Pharmaceutical Science and Research Center, Kharadi, Pune-411014.*

[2] *R&D Center, Healing Hands & Herbs Pvt. Ltd., 101, Mangalmurti Complex, CTS No. 990, Shukrawar Peth, Tilak Road, Pune - 411002. Maharashtra, India.*

***Correspondence to*: Purva Jadhav**

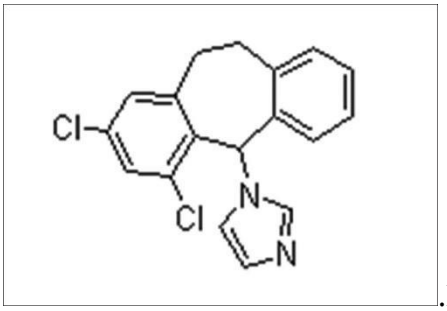
purvajadhav56@gmail.com

Abstract: A simple, precise and economical HPLC method was developed and validated for the determination of Eberconazole nitrate in Active pharmaceutical ingredients. The method involves the use of easily available inexpensive laboratory reagents. The separation was achieved on the Hi Q SilC_{18} column, (250 x 4.6 mm, 5µm) column with the isocratic flow. The mobile phase at a flow rate of 1 ml/min, consisted of Methanol: 10mM Sodium dihydrogen Phosphate buffer (90:10 v/v), pH adjusted to 5.0 with orthophosphoric acid. The UV detection was carried out at 261 nm. A linear response was observed over the concentration range of 5-30 µg/ml of Eberconazole nitrate. The method was successfully validated in accordance with ICH guidelines acceptance criteria for specificity, linearity, accuracy, precision, ruggedness and system suitability. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and rugged.

Keywords: Eberconazole, HPLC, validation.

Introduction:

Eberconazole nitrate (RS)-1-(2,4-Dichloro-10,11-dihydro-5H-dibenzol[α, d]-5cycloheptenyl)-1H-imidazole nitrate (Figure 1), is an Antifungal drug. Eberconazole nitrate is official in IP 2018.



.HNO_3

Figure 1: Structure of Eberconazole Nitrate

A literature survey [1,2] revealed that HPLC methods were developed for other drugs. This required us to develop simple, rapid, accurate, economical and sensitive analytical method development and Validation of Eberconazole by HPLC.

High-performance liquid chromatography, also referred to as high-pressure liquid chromatography (HPLC), is essentially a type of column chromatography in which the stationary phase is made up of small particles (3-50 μ) packed inside a column with a small bore (2–5 mm), one end of which is connected to a source of pressurized liquid eluant (mobile phase). Due to significant improvements in liquid chromatographic instruments that offer superior qualitative and quantitative results, reproducibility, high detection sensitivity, and unmatched reliability, HPLC is one of the most popular analytical techniques used today among various chromatographic procedures. [3]

1. Modes of HPLC [4]

1. Reverse phase or normal phase analysis of small (<2000 Da) organic molecules

2. Ion chromatography-analysis of ions

3. Size exclusion chromatography for separation of polymers

4. Chiral HPLC- determination of enantiomeric purity

2. Quantitative analysis by HPLC

The two most common techniques for quantitative analysis are. They are the internal standard and the external standard methodology.

i. External standard method

The majority of quantitative assays employ external standardisation techniques. To calibrate (standardise) an HPLC system, solutions with known concentrations of analyte reference standards are needed. For greater precision, bracketed standards that are injected before and after the samples are desired.

ii. Internal standard method

For bioanalytical analysis of medicines in physiological fluids or complicated samples needing substantial sample work-up to make up for losses occurring during preparation, internal standardisation procedures are frequently used. The internal standard should be added before the sample work-up and have a structure identical to that of the analysts. The internal standard needs to be distinguished from any potential sample components for UV detection. Isotopically labelled analysts, such as deuterated analysts, are frequently utilized in the majority of bioanalytical LC/MS assays.

3. General requirements of mobile phase [5]

- High solubility for the sample components
- Noncorrosive to HPLC system components
- High purity, low cost, UV transparency
- Low toxicity, non-flammability, and low viscosity are further desirable qualities.

Experimental work:

Instrumentation: Model PU 2080 Plus Intelligent HPLC pump having Borwin chromatography software (version 1.50) with Rheodyne sample injection port with 50µl loop, Hi Q SilC_{18} column, (250 x 4.6 mm, 5µm), JASCO UV-2075 UV-VIS detector. Shimadzu (model AY-120) Electronic weighting balance. Sonicator: PRAMA solutions for laboratory. Extra pure lab link water purification system. Electronic pH meter. Calibrated Glassware.

Reagents and chemicals: Eberconazole Nitrate was supplied by Central Drugs Testing Laboratory, Mumbai, India. Methanol (HPLC Grade), Sodium Hydrogen Phosphate (AR Grade) HPLC grade water all chemicals and reagents that Methanol, Phosphoric acid, were purchased from LOBA CHEMIE PVT. LTD., Mumbai.

A. Selection of mobile phase and chromatographic conditions: Chromatographic separation studies were carried out on the working standard solution of Eberconazole 10 µg/ml. Initially, trials were carried out using methanol and buffer in various proportions of varying pH, to obtain the desired system suitability parameters. After a few trials, methanol: 10 mM sodium dihydrogen phosphate buffer (pH 5) in the ratio of

90:10 v/v was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

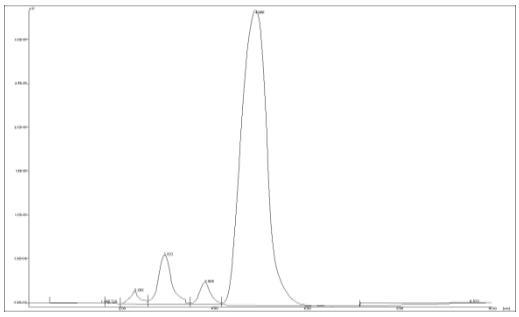
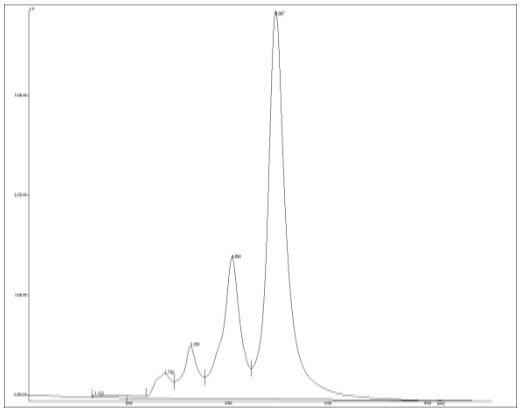
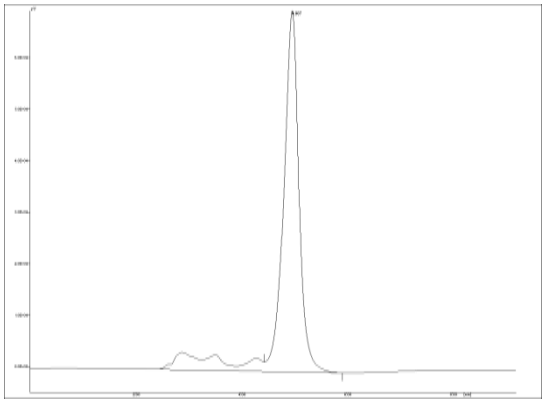
B. **Preparation of Sodium Hydrogen Phosphate buffer and mobile phase:** Sodium dihydrogen Phosphate buffer (10 mM) was prepared by dissolving 1.2 g of Sodium dihydrogen Phosphate in 1000 ml of HPLC grade water and pH was adjusted to 5 by orthophosphoric acid. The mobile phase was prepared by mixing methanol and Sodium dihydrogen Phosphate buffer in a ratio of 90:10 v/v. It was then filtered through 0.45 µm membrane filter paper using filtration assembly and then sonicated on an ultrasonic water bath for 15 min.

C. **Preparation of Standard stock solution:** Standard stock solution of the drug was prepared by dissolving 10 mg of the drug in 10 ml of methanol to get the concentration of 1000 µg/ml (A). From the corresponding standard stock solution, a working standard solution was prepared to contain 100 µg/ml of Eberconazole in methanol (B). From this further dilution was made in methanol to get the final solution of Eberconazole (10 µg/ml).

D. **Preparation of sample solution: (API)** API about 10 mg of Eberconazole was weighed and powdered. and transferred to a 10 ml volumetric flask and volume was made up with methanol to get concentration (1000 µg/ml) and was sonicated for 10 min. The solution was filtered, and from this solution, 1 ml of the drug was taken in a 10 ml volumetric flask and the volume was made up with methanol. Further dilution in methanol was done to get a final concentration of 10 µg/ml.

Result and Discussion:

Table 1: Trials of mobile phase for HPLC method development of Eberconazole:

Sr. No	Mobile phase	Observations	Chromatogram
1.	MeOH: water (70:30 v/v)	The peak shape was not proper. RT = 4.900 min.	
2.	MeOH: 10 mM Sodium Hydrogen Phosphate Buffer, pH-6.8 (90:10 v/v)	The peak shape was not proper. RT = 4.500 min.	
3.	MeOH: 10 mM Sodium dihydrogen Phosphate Buffer, pH-5 (90:10 v/v)	Good Peak Shape, RT at 4.966 min.	

Selection of Detection Wavelength: From the standard stock solution further dilutions were done using methanol and scanned over the range of 200 - 400 nm and the spectra were obtained (Fig. 1). It was observed that the drug showed considerable absorbance at 261 nm.

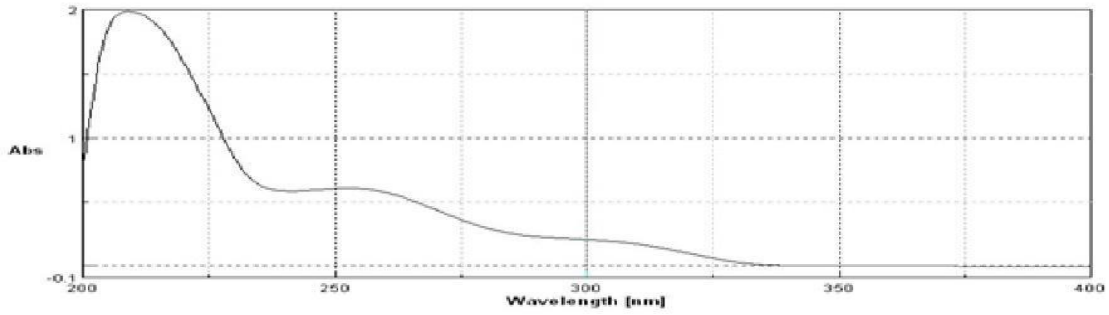
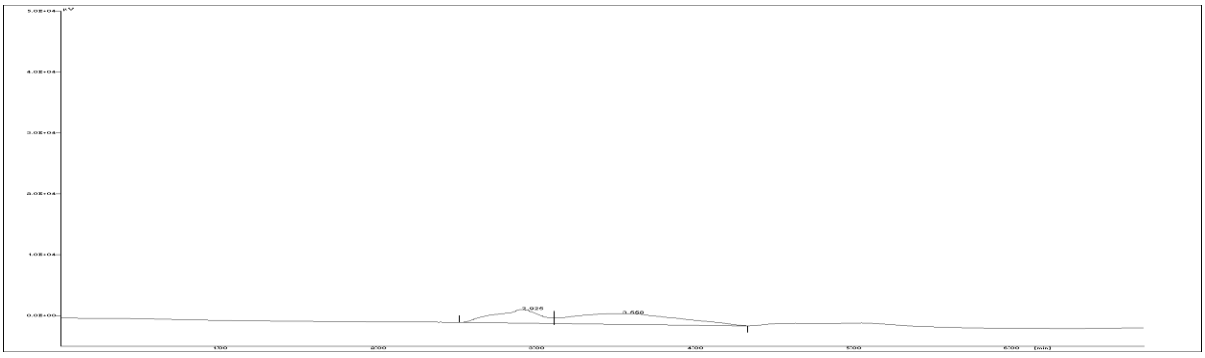


Fig 1: UV-VIS Spectra of Eberconazole (10 µg/ml)

Chromatogram and system suitability parameter of the drug: The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). The working standard solution of the drug (10 µg/ml) was injected into the system. The retention time for the drug was found to be 4.906 min.

The Chromatogram of Eberconazole shown in Fig. 2

A): Blank



B): Eberconazole (10 µg/ml)

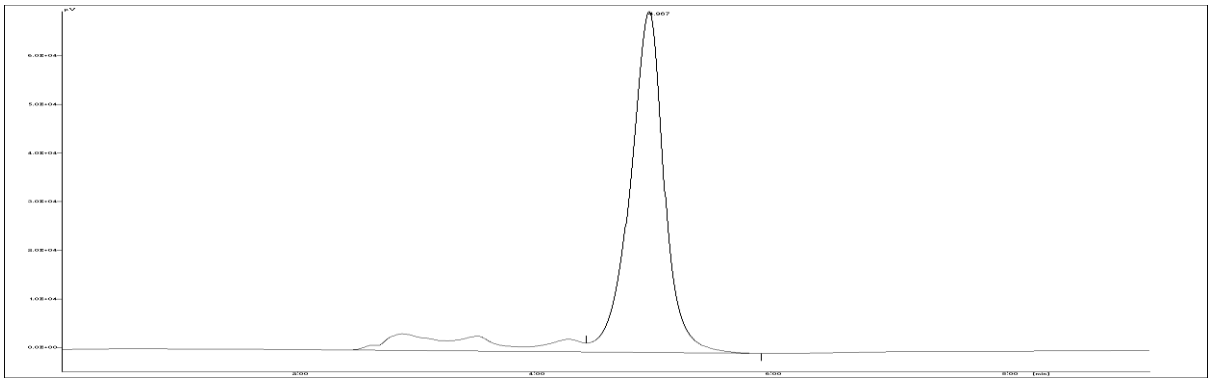


Fig 2: Chromatogram of A) Blank B) Eberconazole (10 µg/ml)

Table 2: System suitability parameters for Eberconazole

Drug	Concentration (µg/ml)	RT ± SD (Min)	Area	Plates	Asymmetry
EBERCONAZOLE	10	4.906 ± 0.055	1506562.99	2136.92	0.98

Summary of Chromatographic parameters selected:

Table 3: Summary of Chromatographic Parameters

Sr. No.	Parameter	Conditions Used for Analysis
1	Column	Hi Q SilC_{18} column ,(250 x 4.6 mm, 5µm)
2.	Mobile phase	Methanol: 10 mM Sodium dihydrogen Phosphate buffer (pH – 5) (90:10 v/v)
3.	Flow rate	1 ml/min
4.	Detection Wavelength	250 nm
5.	Sample injector	50 µl loop
6.	Column temperature	Ambient

Validation of Analytical Method

A. Linearity

From the standard stock solution (1000 µg/ml) of Eberconazole, a solution was prepared to contain 100 µg/ml of Eberconazole with methanol. This solution was further used to prepare a range of solutions containing six different concentrations. The linearity (relationship between peak area and concentration) was determined by analyzing six solutions over the concentration range of 5-30 µg/ml. The results obtained are shown in Table 4. The linearity curve of Eberconazole is shown in Fig. 3 and the calibration curve is shown in Fig. 4.

Replicates	**Concentrations of Eberconazole**					
	5 µg/ml	**10 µg/ml**	**15 µg/ml**	**20 µg/ml**	**25 µg/ml**	**30 µg/ml**
	Peak Area					
1	739172.8	1506562.99	2047492.07	2573762	3036488.15	3654048.18
2	734344.34	1501947.09	2065765.22	2584585.83	3030172.55	3687545.15
3	729374.2	1503484.33	2042307.17	2525993.74	3080920.42	3645740.21
4	735126.9	1501127.52	2047492.24	2520333.04	3024869.24	3617796.66
5	731859.27	1509430.19	2001504.84	2556506.53	3085330.48	3666642.68
6	735516.03	1575184.07	2080105.24	2572265.63	3024869.24	3662422.42
Mean	734232.25	1516289.36	2047444.463	2555574.462	3047108.347	3655699.21
Std. Dev.	3349.680	29016.506	26605.345	26716.612	28259.346	23313.669
%RSD	0.456	1.914	1.299	1.045	0.927	0.638

Table 4: Linearity study of Eberconazole

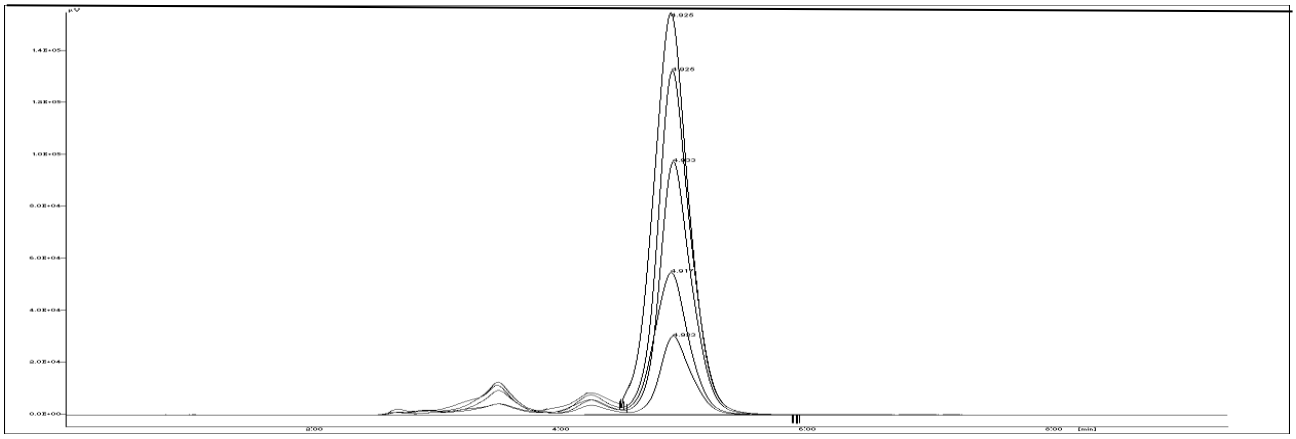


Fig 3: Linearity curve of Eberconazole (5-30 µg/ml)

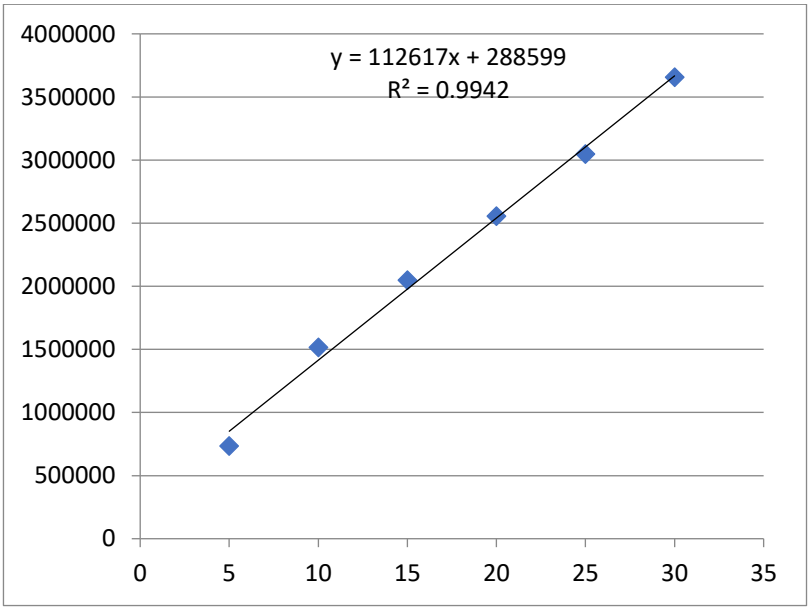


Fig 4: Calibration curve of Eberconazole

B. Range:

5-30 µg/ml.

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

LOD and LOQ are calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S} \qquad LOQ = \frac{10\ \sigma}{S}$$

Where,

σ = standard deviation of Y-intercept = 22265.29

S = slope of the calibration curve = 112616.67

LOD = 0.652 µg/ml

LOQ = 1.977 µg/ml

D. Precision:

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3 different concentrations were analyzed in a day and percentage RSD was calculated. For the inter-day variation studies, 3 different concentrations were analyzed on 3 consecutive days and percentage RSD was calculated. The results obtained for Intraday and Inter day variations are shown in Table 5 and Table 6, respectively.

Table 5: Intra-day Precision Results

Conc. (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
10	1413430.19	9.988	99.881	100.694	0.571	0.567
10	1422582.89	10.069	100.694			
10	1410184.07	9.959	99.593			
20	2563761.89	20.203	101.013	99.219	1.269	1.279
20	2523351.84	19.844	99.219			
20	2578556.87	20.334	101.670			
25	3085154.67	24.832	99.330	99.022	0.634	0.640
25	3076488.15	24.755	99.022			
25	3110821.41	25.060	100.241			

Table 6: Inter-day Precision Results

Conc (µg/ml)	Area	Amount recovered (µg/ml)	% Recovery	Average % Recovery	SD	%RSD
10	1412445.79	9.979	99.794	99.763	0.149	0.149
10	1412096.29	9.976	99.763			
10	1409381.038	9.952	99.522			
20	2534307.62	19.941	99.706	101.099	0.960	0.950
20	2565701.83	20.220	101.099			
20	2524234.73	19.852	99.258			
25	3114242.98	25.091	100.363	100.339	0.368	0.367
25	3102893.60	24.990	99.960			
25	3123578.29	25.174	100.695			

E. Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 994, indicating no interference of any other peak of degradation product, impurity, or matrix. (Table 7).

Table 7: Peak purity of Eberconazole

Drug	Purity tail	Purity front
Eberconazole	994.578	996.926

A. Accuracy

To check the accuracy of the method, recovery studies were carried out by spiking the standard drug to the sample solution, at three different levels around 50, 100 and 150 %. Basic concentration of the sample solution chosen was 10 µg/ml. The % recovery was determined from the linearity equation. The results obtained are shown in (Table 9)

Table 9: Recovery studies of Eberconazole

Level	Conc. of Sample solution (µg/ml)	Conc. of Standard solution spiked (µg/ml)	Area	Amount recovered (µg/ml)	Mean % recovery ± RSD
50%	10	5	1976627.83	14.988	99.924 ± 0.233
			1981105.24	15.028	
			1972265.63	14.949	
100%	10	10	2562436.23	20.190	100.641 ± 0.997
			2530212.26	19.904	
			2573761.89	20.291	
150%	10	15	3085114.64	24.831	99.906 ± 0.649
			3121154.67	25.151	
			3098172.55	24.947	

B. Robustness

The robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, detection wavelength, and flow rate were altered and the effects on the area were noted. The results obtained are shown in Table 10.

Table 10: Robustness study

% RSD Found for Robustness Study (peak area)								
MP COMPOSITION (± 2% Composition)			**DETECTION WAVELENGTH (± 1 nm)**			**FLOW RATE (± 0.05 ml/min)**		
92:8	**90:10**	**88:12**	**249**	**250**	**251**	**0.95**	**1.0**	**1.05**
0.745	0.561	0.600	0.971	0.496	0.358	1.521	1.015	0.806

Summary of validation study:

Table 11: Summary of validation study by HPLC method

Sr. No.	Validation Parameter	Eberconazole
1.	Linearity	y = 112617x + 288599 R^2 = 0.9942
2.	Range	5-30 µg/ml
3.	Precision	(% RSD)
	A) Intraday precision	0.567 – 1.279
	B) Interday precision	0.149 – 0.950
4.	Assay ± %RSD	100.048 ± 0.611
5.	Accuracy	Mean % recovery ± %RSD
	50 %	99.924 ± 0.233
	100 %	100.641 ± 0.997
	150 %	99.906 ± 0.649
6.	Limit of Detection	0.652
7.	Limit of Quantitation	1.977
8.	Robustness	Robust
9.	Specificity	Specific

A simple, specific, rapid, reliable and reproducible method for the estimation of Eberconazole has been developed and validated. The linearity range in the concentration range of 5-30 µg/ ml (R^2= 0.9942). It indicated that the concentrations of Eberconazole had good linearity. The LOD and LOQ were found to be 0.652µg/ ml and 1.977µg/ ml respectively.

Further, the precision of the method was confirmed by the repeatable analysis of the solution. From the robustness study, it is clear that the system suitability criteria meet the acceptance limit. Hence the method is robust. The % RSD was found to be 0.567 – 1.279 and 0.149 – 0.950 for intraday and interday precision respectively. It indicated that the method has good precision. The percentage recovery was found to be in the range of 5-30 µg/ml.

Conclusion: It can be concluded from the results that the proposed method was simple, rapid, reliable, accurate, precise and most economical for the determination of Eberconazole in bulk. This method can be used as a more convenient and efficient option for the analysis of Eberconazole to establish the quality of the drug substance during routine analysis with consistent and reproducible results.

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