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A REVIEW OF METHOD DEVELOPMENT AND METHOD VALIDATION OF FLUCONAZOLE BY RP-HPLC METHOD

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ABSTRACT: For the simultaneous detection of fluconazole in bulk and pharmaceutical dose forms, a simple, quick, precise, accurate, and sensitive reverse phase liquid chromatographic approach has been established. Waters ODS C18 RP column, 250 mm 4.6 mm, particle size 5m i.e., UV detection at 274 nm, and a flow rate of 1.0 ml/min were used to standardize the chromatographic procedure. Both bulk and pharmaceutical dose forms of fluconazole were satisfactorily quantified using the suggested approach. Fluconazole was reported to have a linearity range of 6- 16% g/ml. It was determined that the correlation coefficient was 0.995. For fluconazole, 2.90 g/ml was determined to be the LOQ. The lowest detectable concentration of fluconazole was 0.90 g/ml. Research into deterioration was also created. Guidelines from the International Council for Harmonization (ICH) were also used to calculate precision, accuracy, robustness, and other analytical metrics.

KEYWORDS: Fluconazole, HPLC, linearity, accuracy, precision, degradation studies.

INTRODUCTION: As a synthetic triazole derivative antifungal drug, fluconazole has been demonstrated to be effective against a broad variety of systemic and superficial fungal infections after oral and intravenous treatments. Its chemical name is 2-(2,4-difulrophenyl)-1,3-bis(1H1,2,4-triazole-1-yl)-2-propanol. Some of the techniques used to analyze fluconazole include gas chromatography (GC) and high performance liquid chromatography (HPLC) for the evaluation of fluconazole in biological fluids; HPLC for eye drops and creams; UV spectrophotometry for syrups, capsules, and intravenous solution; and microbiological test for capsules.

The weight of one mole of fluconazole is 306.271 grams. C13H12F2N6O is the chemical formula for this compound. The chemical makeup of fluconazole is

Figure No.1: structure of fluconazole.

The fluconazole is affected by the reduction in renal function. Therefore, the dose of fluconazole needs to be reduced in patients with impaired renal function. If creatinine clearance is ≤ 50 ml/min, the clinician must reduce the dose to 50%. The plasma halflife of fluconazole is approximately 30 hours.

EXPERMINENTAL

Apparatus

The liquid chromatography equipped with water OSD column C_{18} 250mm × 4.66mm and the particle size is 5 μm.

Reagents and materials

The drugs used were of AR grade. The tablets used is diflucon 150 mg

S.NO.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Table No.1: list of chemicals used

SOLUBILITY

Solubility studies

SOLVENT	SOLUBILITY
Ethanol	Soluble
DMSO	Soluble
Dimethyl formamide	Soluble
Aqueous Buffers	Sparingly soluble
Methanol	Soluble
Water	Slightly Soluble
Ethyl Acetate	Soluble

Table No.2: list of solvents

Chromatographic conditions

The chromatographic conditions were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Symmetry ODS RP C_{18},5m, 15mm x 4.6mm i.d.	Water :ACN = 50 : 50	0.8 ml/min	274nm	Low response	Method rejected

Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.	Methanol : Water = 60 : 40	1.0 ml/min	274nm	Very low response	Method rejected
Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.	ACN: Methanol = 70 : 30	1.0 ml/min	274nm	Tailing peak	Method rejected
Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.	ACN: Phosphate buffer = 45:55	1.0 ml/ min	274nm	Broad Peak	Method rejected
Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.	ACN : Phosphate buffer (pH=3.2) =35:65	1.0 ml/ min	274nm	Tailing peak	Method rejected
Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.	ACN : Phosphate buffer (pH=3.2) =20:80	1.0 ml/min	274nm	Good Peak	Method accepted

Table No.3: summary of process optimization

Drug Name	Rt	Peak Area	Tailing Factor	Theoretical Plates
Fluconazole	3.544	2812469	1.01	1733

Table No.4: peak results of optimized conditions

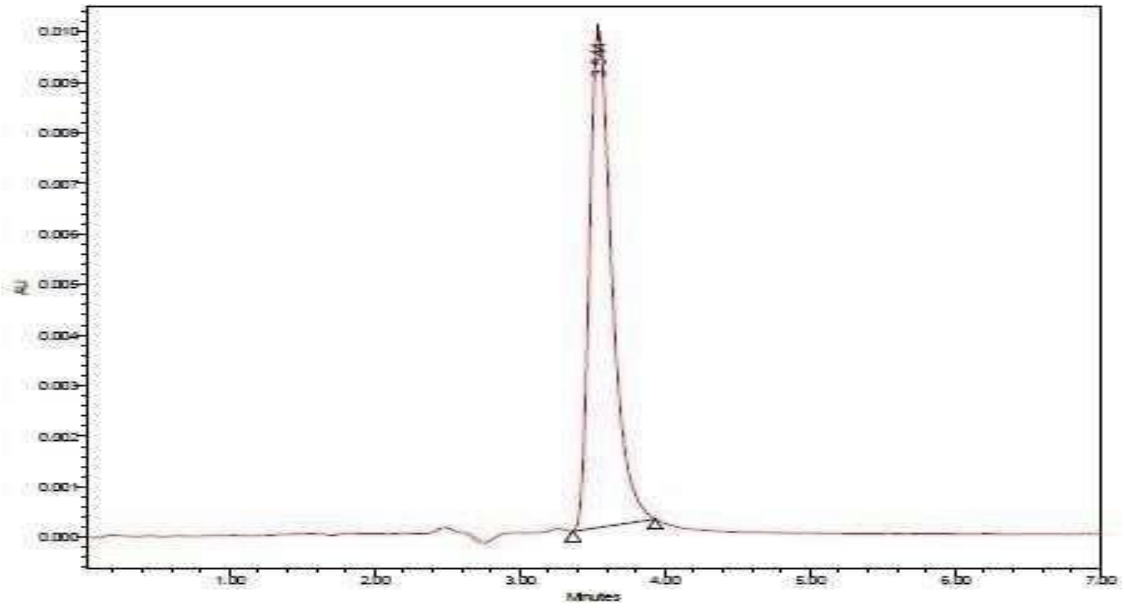

Fig No.2: chromatogram of fluconazole in optimized conditions

METHOD DEVELOPMENT

Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Fluconazole, so that the same wave number can be utilized in HPLC UV detector for estimating the Olmesartan. The scanned UV spectrum is attached in the following page

Sample & standard preparation for the UV-Spectrophotometer analysis

10g of fluconazole standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with mobile phase.

Further dilution was done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

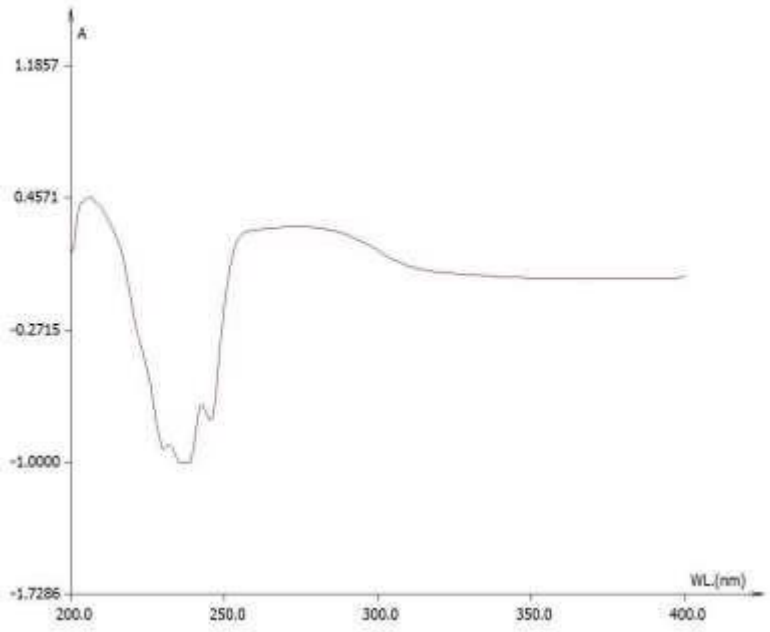

Fig No.3: UV spectrum for fluconazole

Observation: while scanning the fluconazole solution we observed the maxima at 274nm. The UV spectrum has been recorded on T60-LAB INDIA make UV-VIS spectrophotometer model UV-2450.

Preparation of mobile phase

800ml of phosphate buffer and 200 ml of HPLC grade water were mixed well and degassed in ultrasonic water bath for 15 minutes. The solutions was filtered through 0.45µm filter under vacuum filtration.

Final result & discussion: the selected and optimized mobile phase ACN : phosphate buffer (p.H=3.2) = 20:80 and conditions optimized were flow rate (1.0 ml/minute), wavelength (274nm), run time was 7 minutes. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry. The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Estimation of fluconazole in tablet dosage form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25ml volumetric flask, make and solutions was sonicated for 15 minutes, there after volume was made up to 25 ml with solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45μ m) sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

Brand name of Tablets	**Labeled amount of Drug (mg)**	**Mean(SD) amount (mg) found by the proposed method (n=6)**	**Assay % (SD)**
Flucanazole	150	149.98 (0.498)	99.9(0.343)

Table No.5: recovery data for estimation fluconazole, diflucon 150 mg of fluconazole

ACCURACY

To determine the accuracy of the proposed method, recovery studies were carried out by pure drug of fluconazole taken and added to pre-analysed formulation of concentration 10µg/ml. From that percentage recovery values were calculated.

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Sample ID	**Concentration (g/ml)**		**Peak Area**	**% Recovery of Pure drug**	**Statistical Analysis**
	Amount Added	Amount Found			
S_1 : 80 %	8	8.069	485317	100.862	Mean= 100.5413% S.D. = 0.947606 % R.S.D.= 0.942503
S_2 : 80 %	8	7.958	478751	99.475	
S_3 : 80 %	8	8.103	487312	101.287	

S_4 : 100 %	10	10.048	601947	100.48	Mean= 100.2367% S.D. = 0.650103
S_5 : 100 %	10	10.073	603395	100.73	
S_6 : 100 %	10	9.950	596176	99.50	% R.S.D.= 0.648568
S_7 : 120 %	12	11.985	716127	99.875	Mean= 100.3607% S.D. = 0.555257 % R.S.D. = 0.553262
S_8 : 120 %	12	12.116	723840	100.966	
S_9 : 120 %	12	12.029	718706	100.241	

Table No.6: Accuracy readings

PRECISION

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Fluconazole (API). The percent relative standard deviation was calculated for fluconazole are presented in below table.

HPLC Injection Replicates of Flucanazole	Retention Time	Peak Area
Replicate – 1	3.545	661022
Replicate – 2	3.537	683137
Replicate – 3	3.543	671941
Replicate – 4	3.538	682245

Replicate – 5	3.542	671941
Replicate – 6	3.550	692444
Average	3.5425	677121.7
Standard Deviation	0.004764	11046.13

Table No.7: repeatability readings.

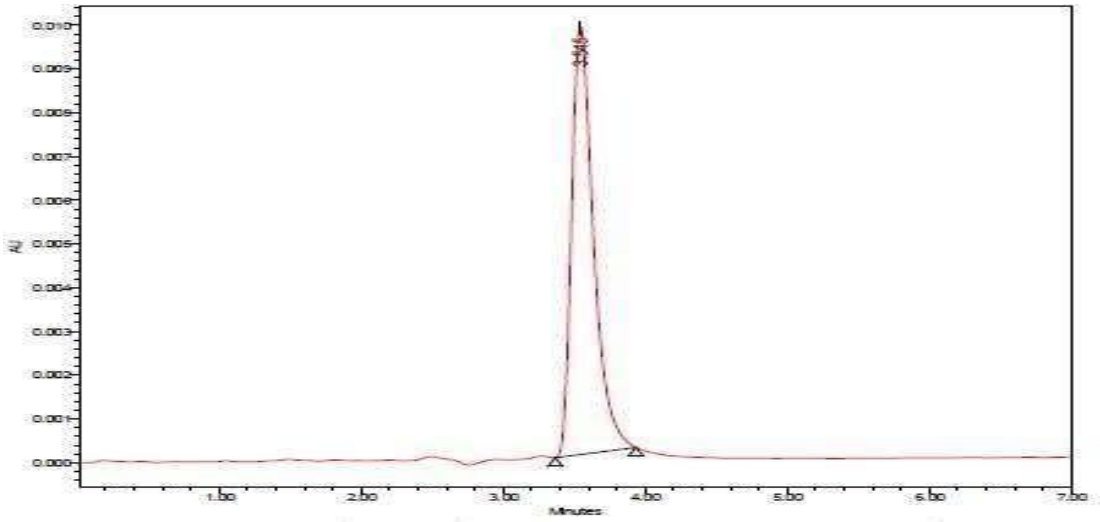

Fig-4: Chromatogram of Repeatability-1

Table-24: Results of Repeatability-1

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.545	661022	3169	1.09

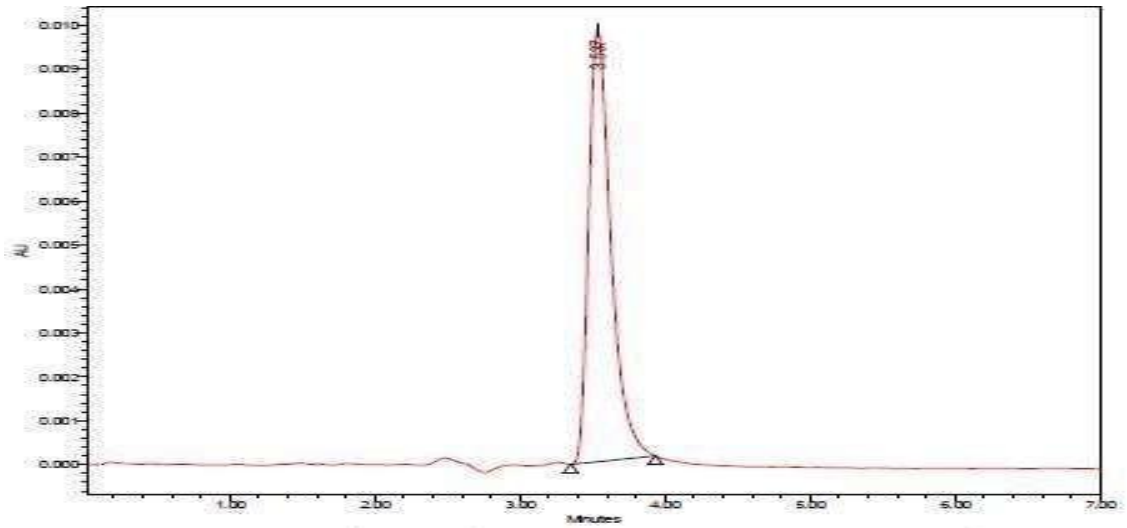

Fig-5: Chromatogram of Repeatability-2

Table-25: Results of Repeatability-2

b	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.537	683137	5214	1.02

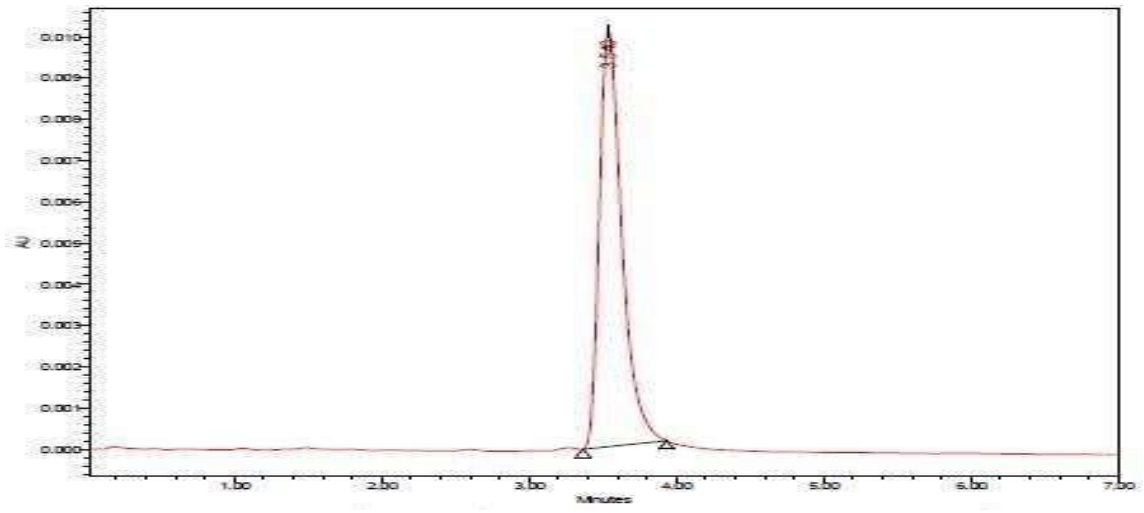

Fig-6: Chromatogram of Repeatability-3

Table-26: Results of Repeatability-3

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.543	671941	3368	1.13

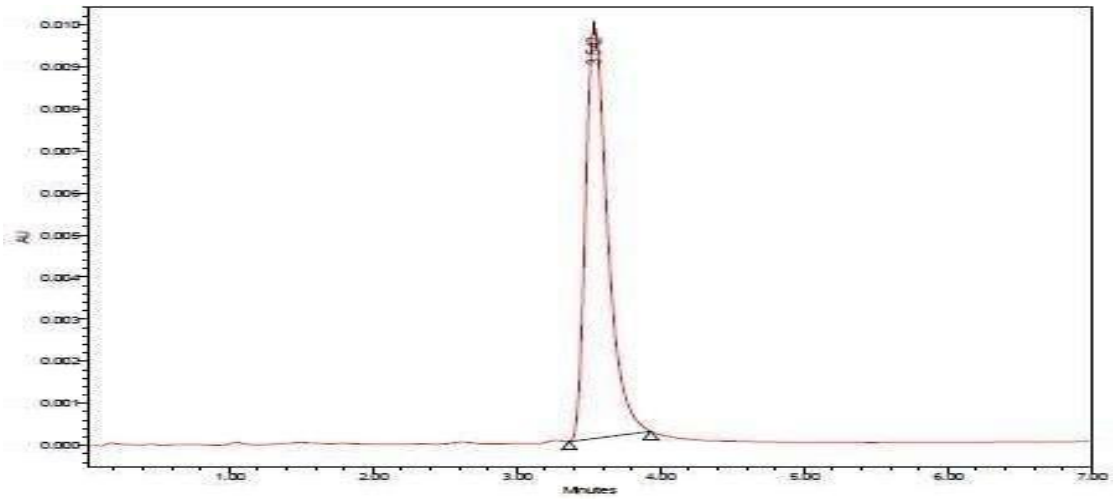

Fig-7: Chromatogram of Repeatability-5

Table-27: Results of Repeatability-5

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.542	671941	6254	0.99

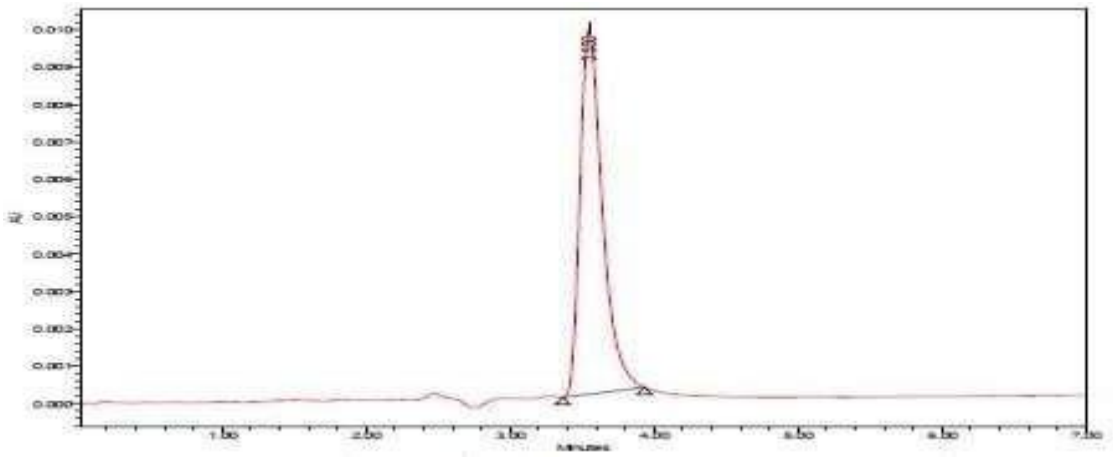

Fig-8: Chromatogram of Repeatability-6

Table-28: Results of Repeatability-6

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.550	692444	6524	1.25

Intermediate precision

Intra assay & inter assay

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (%RSD < 2%) within a day & day to day variations for fluconazole revealed that the proposed method is precise

Conc. Of Flucanazole (API) (µg/ml)	Observed Conc. Of Flucanazole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.76	0.82	8.28	0.98
10	10.16	0.42	9.59	0.23
12	11.68	0.13	12.19	0.33

Table No.8: results of intra assay & inter assay

LINEARITY AND RANGE

The calibration curve showed the range of 0-16µg/ml, for fluconazole (API) with coeffeicent of 0.999. a typical calibration curve has the regression equation of y = 58954x + 9634 for

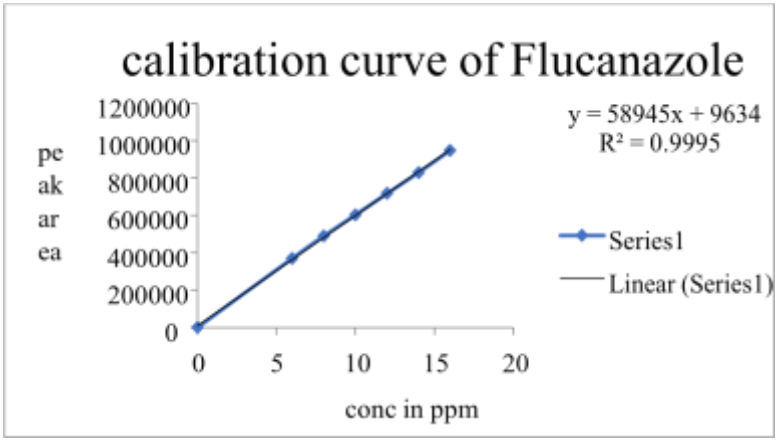

Fig No.10: calibration curve for fluconazole

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
6	370200
8	490231
10	602707
12	717538
14	829248
16	947852

Table No.9: linearity results

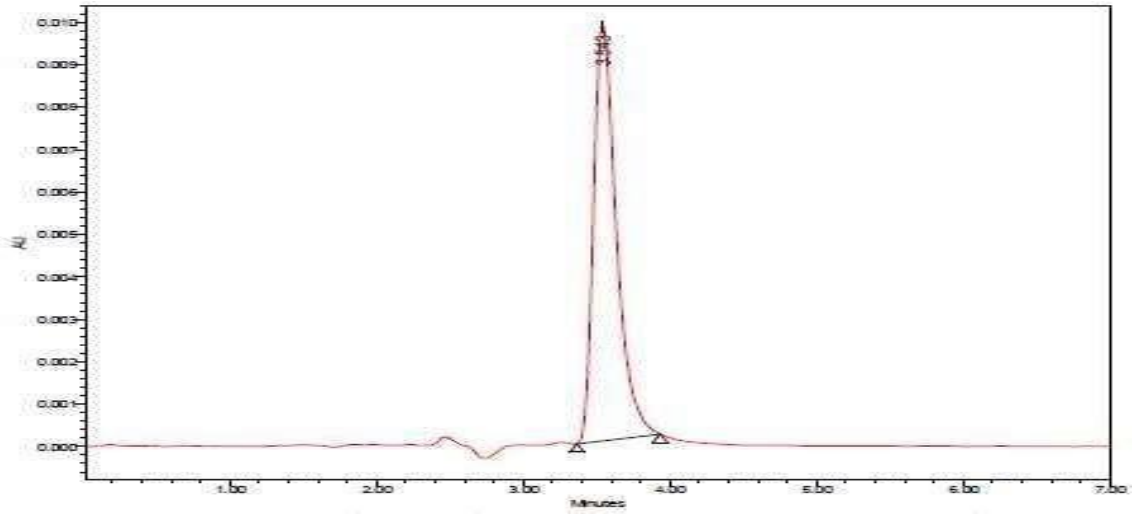

Fig-11: Chromatogram for Linearity-1 (6 ppm)

Table-32: Results of Linearity-1 (6 ppm)

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.543	370200	6854	1.17

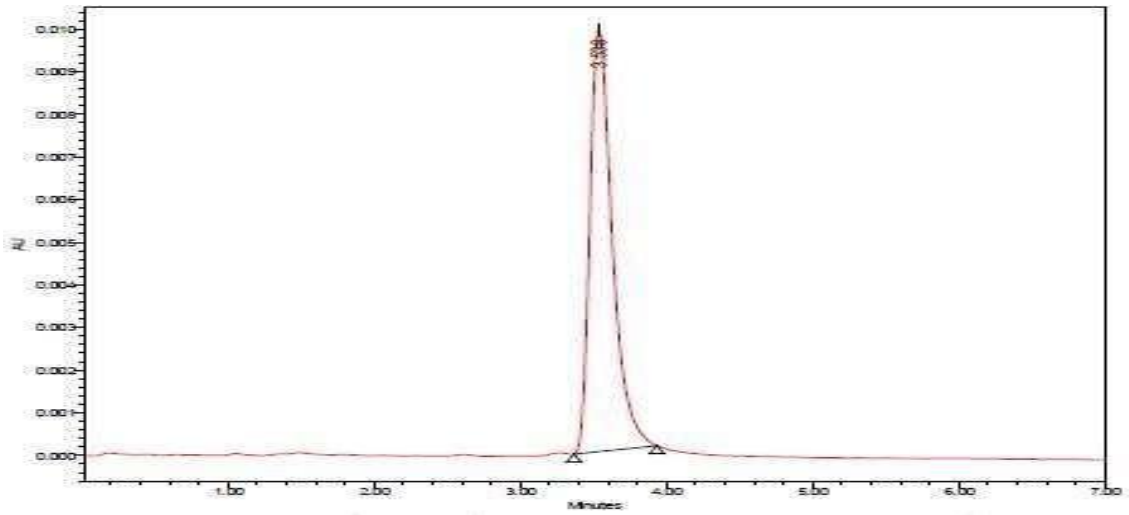

Fig-32: Chromatogram for Linearity-2 (8 ppm)

Table-33: Results of Linearity-2 (8 ppm)

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.538	490231	7458	1.21

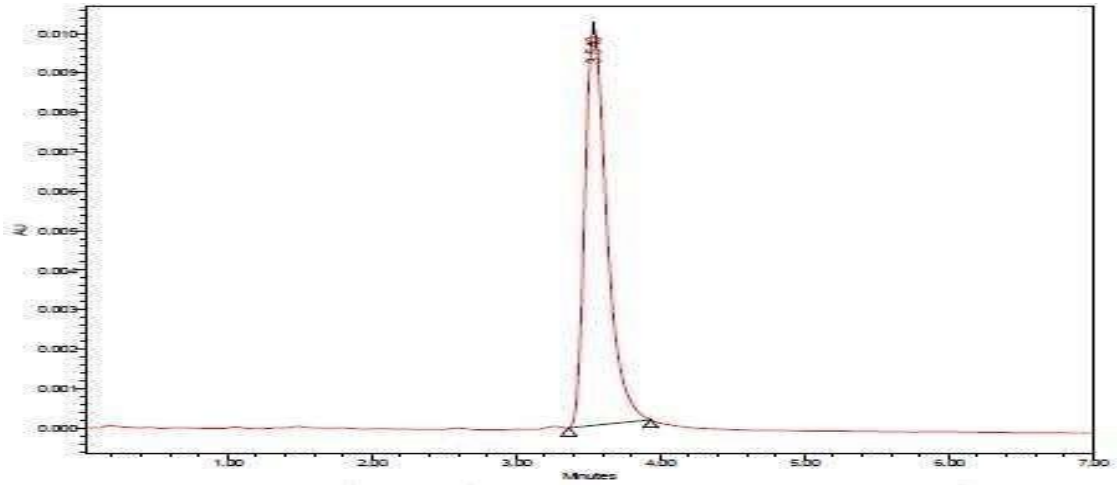

Fig-33: Chromatogram for Linearity-3 (10 ppm)

Table-34: Results of Linearity-3 (10 ppm)

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.543	602707	4286	1.20

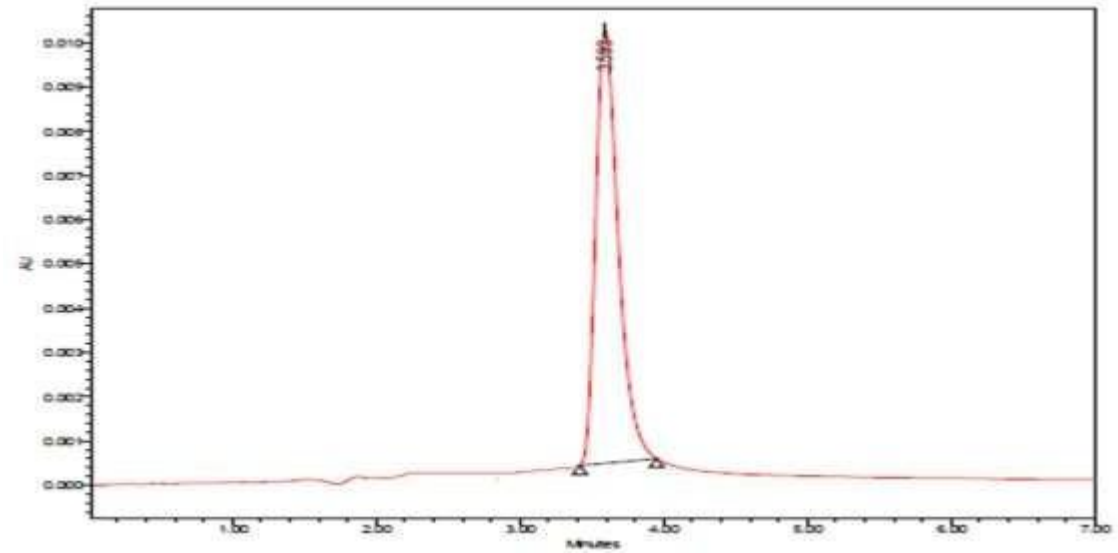

Fig-34: Chromatogram for Linearity-4 (12 ppm)

Table-35: Results of Linearity-4 (12 ppm)

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.593	717538	5896	1.26

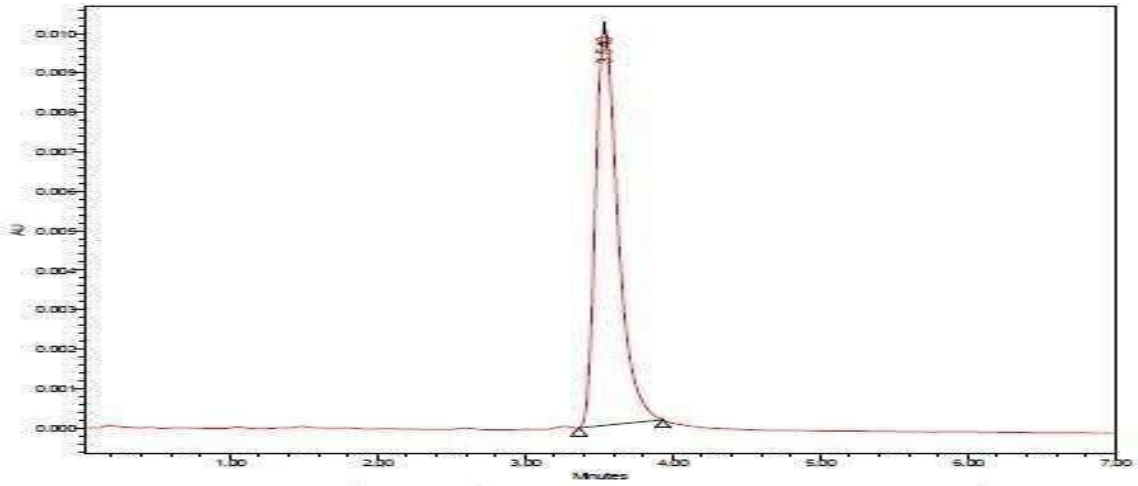

Fig-35: Chromatogram for Linearity-5 (14 ppm)

Table-36: Results of Linearity-5 (14 ppm)

S.No.	Rt	Peak Area	Theoretical Plates	Tailing factor
1	3.543	829248	5486	1.42

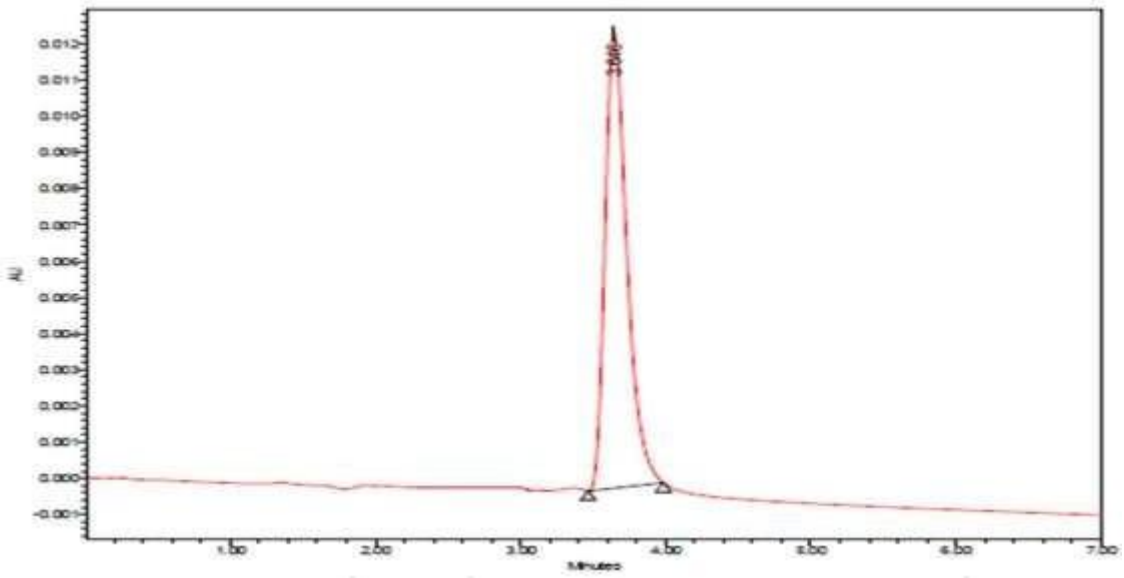

Fig-36: Chromatogram for Linearity-6 (16 ppm)

Table-37: Results of Linearity-6 (16 ppm)

S.No.	Rt	Peak Area(	Theoretical Plates	Tailing factor
1	3.646	947852	4265	1.12

ROBUSTNESS

Influence pf small changes in chromatographic conditions such as flow rate, temperature, wavelength of detection & Acetonitrile content in mobile phase studied to determine the robustness of the method are also in favour the developed RP-HPLC method for the analysis of fluconazole (API).

Change in parameter	% RSD
Flow (1.1 ml/min)	0.52
Flow (0.9 ml/min)	0.56
Temperature (27^0C)	0.52
Temperature (23^0C)	0.49
Wavelength of Detection (270 nm)	0.97
Wavelength of detection (266 nm)	0.98

Table No.10: results of method robustness

LOD & LOQ

The minimum concentration level at which the anlyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.90 & 2.90 μg/ml respectively.

RESULTS AND DISCUSSION

Summary

The optimum chromatographic conditions obtained from expermients can be summarised as below:

Mobile phase	ACN : Phosphate buffer (pH=3.2) =20:80
Column	Symmetry ODS RP C_{18},5 m, 15mm x 4.6mm i.d.

Flow rate	1.0 ml/ min.
Wavelength	274nm
Sampling System	Automatic
Temp. of Auto sampler	Ambient
Volume of injection	10µl
Run time	07 mins
Mode of Separation	Isocratic

Table No.11: summary of optimised chromatographic conditions

Conclusion

A sensitive and selective RP-HPLC method has been developed and validated for the determination of fluconazole in pure form and pharmaceutical dosage form. The UV detection was performed at 274 nm. The retention time for fluconazole was found to be 3.544 minutes. The percentage standard deviation(%RSD) of the fluconazole was found to be 1.63%. the detector response was linear in the concentration range of0-16µg/ml. The respective linear regression equation being Y=58945.x + 9634 with R2=0.999. % recovery values was found to be within range. Limit detection and limit quantitation was found to be 0.90µg/ml and 2.90µg/ml respectively. Further the process was rapid, accurate, precise. The rapid method was developed and validated according to the international council of harmonization (ICH) guidelines. The results of the study of fluconazole was found to be precise, accurate, simple, rapid which can be used for the routine determination of fluconazole in pure form and pharmaceutical dosage form.

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