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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF FLUCONAZOLE AND TINIDAZOLE IN BULK AND TABLET DOSAGE FORM BY RP-HPLC

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ABSTRACT

An analytical method consists of a detailed, stepwise list of instructions to be followed in the qualitative, quantitative or structural analysis of a sample for one or more analytes and using a specified technique. A facile and rapid isocratic reverse phase high performance liquid chromatography assay method has been developed for simultaneous estimation of fluconazole and tinidazole in bulk and tablet. A waters HPLC system with Empower software was used. Inertsil ODS Column with phosphate buffer of pH 3.0 and methanol in the ratio of (70:30 v/v) as mobile phase at flow rate of 1 ml/min was employed for the analysis. The column was maintained at ambient temperature (27 ^{0}C). The eluent was monitored using PDA detector at 210nm. The run time was found to be 8 min. The developed method was validated as per ICH (International Conference on Harmonisation) guidelines. The developed method was found to be linear over a workable drug concentration, accurate, precise and robust. This fast and inexpensive method is suitable for research laboratories as well as for quality control analysis in pharmaceutical industries.

Keywords: RP-HPLC, isocratic, validation, fluconazole, tinidazole, precision

INTRODUCTION

Pharmaceutical analysis plays a vital role in the Quality Assurance and Quality control of bulk drugs. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix. Analytical methods development plays important roles in the discovery, development and manufacture of pharmaceuticals. RP-HPLC is probably the most universal, most sensitive analytical procedure and is unique in that it easily copes with multi-component mixtures. While developing the analytical methods for pharmaceuticals by RP-HPLC, must have good practical understanding of chromatographic separation to know how it varies with the sample and with varying experimental conditions in order to achieve optimum separation. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed-phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method as it is able to handle compounds of a diverse polarity and molecular mass **[1-4]**.

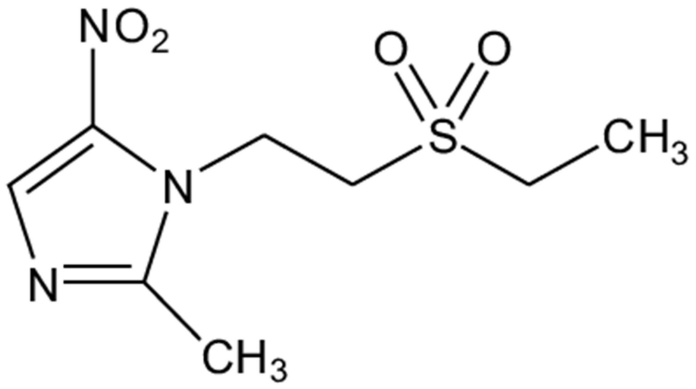

Figure 1: Chemical structure of tinidazole

Fluconazole is a highly selective inhibitor of fungal Cytochrome (CYP) P450 dependent enzyme lanosterol 14-α-demethylase. Chemically it is 2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl) propan-2-ol, recommended for the treatment and prophylaxis of disseminated and deep organ candidiasis. Tinidazole is a 1-[2-(ethyl sulphonyl) ethyl] -2-methyl-5-nitro-1H-imidazole, derivative used as anti-protozoal/anti-biotic and anti-bacterial. Tinidazole belongs to the group of 5-nitroimidazoles, which are used in the chemotherapy of infectious diseases such as amoebiasis, giardiasis, and trichomoniasis and against anaerobic bacteria **[5, 6]**. The nitro-group of tinidazole is reduced by cell extracts of

trichomonas. The free nitro-radical generated as a result of this reduction may be responsible for the anti-protozoal activity. Both the drugs are now used in treatment of systemic fungal infection either as two different tablets in a form of a kit or as combined dosage form tablet **[7, 8]**.

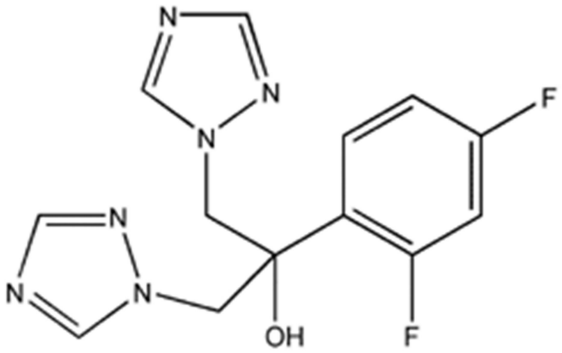

Figure 2: Chemical structure of fluconazole

The literature survey revealed that few methods have been reported for the estimation of fluconazole and tinidazole. The aim of this study is to develop RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution. The validated method would be applicable in both formulation development and routine quality control analysis **[9, 10]**.

This paper describes validated RP-HPLC for simultaneous estimation of fluconazole and tinidazole in combination using phosphate buffer of pH 3.0 and methanol in the ratio of 70:30. The column used was Inertsil ODS with flow rate of 1 ml/min using waters HPLC with auto-sampler and PAD or detector at 210nm.

MATERIALS AND METHODS

Table 1: Instruments used

S. No.	Instrument	Model
1	HPLC	Waters, software: Empower, 2695 separation module, PDA detector
2	UV/VIS-Spectrophotometer	Labindia UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing Machine	Afcoset ER-200A
5	Pipettes, Beakers and Burettes	Borosil

Table 2: Chemicals used

S. No.	Name of the chemical used	Brand name
1	Tinidazole	Crestor
2	Fluconazole	Ambact
3	Potassium dihydrogen orthophosphate	Finer Chemical Ltd.
4	Water and Methanol for HPLC	Lichrosolv (Merck)
5	Acetonitrile for HPLC	Molychem
6	Ortho phosphoric Acid	Merck

HPLC Method Development:

Preparation of Phosphate buffer: Accurately weighed 6.8g of potassium dihydrogen orthophosphate (KH_2PO_4) was taken in a 1000 ml volumetric flask, dissolved and diluted to 1000 ml with HPLC grade water and pH was adjusted to 3.0 with orthophosphoric acid.

Preparation of mobile phase: Accurately measured 300 ml (30%) of above buffer and 700 ml of methanol HPLC grade (70%) were mixed and degassed in an ultrasonic water bath for 10 min and then filtered through 0.45 µ filter under vacuum filtration.

Diluent preparation: The mobile phase was used as the diluent.

Preparation of the tinidazole and fluconazole standard & sample solution:

Standard solution preparation: Accurately weigh and transfer 10mg of fluconazole and 10mg of tinidazole working standards into a 10 mL & 100 mL clean dry volumetric flask add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. From stock solution, further pipette out 3 ml & 0.3 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation: Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 10mg of fluconazole and tinidazole (marketed formulation) sample into a 10 mL clean dry volumetric flask add about 7 mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. From stock solution, further pipette out 3 ml of tinidazole and fluconazole of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject 20 µL of the standard, sample into the chromatograph and measure the areas for fluconazole and tinidazole peaks and calculate the % assay by using the formulae.

RESULTS AND DISCUSSION

Estimation of fluconazole and tinidazole in tablet dosage forms by RP-HPLC method was carried out using optimized chromatographic conditions. The standard and sample solutions were prepared. The chromatograms were recorded. The peak area ratio of standard and sample solutions was calculated. The results of analysis shows that the amount of drugs was in good agreement with the label claim of the formulation.

Optimized Chromatographic Conditions

Instrument used : Waters HPLC with auto-sampler and PAD or detector

Temperature : Ambient

Column (4.6 x 150mm, 5µm)	:	Inertsil ODS
Mobile phase (pH=3.0) 70% methanol	:	30% buffer
Flow rate	:	1 ml per min
Wavelength	:	210nm
Injection volume	:	10 µl
Run time	:	8 min

From the chromatogram **(Figure 3)** it was observed that there are no interferences.

From the chromatogram **(Figure 4)** it was observed that the tinidazole and fluconazole peaks are well separated. Retention time of tinidazole and fluconazole were 2.057 min and 3.663 min respectively.

Retention time of tinidazole and fluconazole were 2.063 min and 3.646 min respectively **(Figure 5)**.

Method Validation

System suitability: Tailing factor for the peaks of Fluconazole and Tinidazole in Standard solution should not be more than 2.0. Theoretical plates for the fluconazole and tinidazole peaks in standard solution should not be less than 2000 **(Table 3)**.

Precision:

Preparation of stock solution: Accurately weigh and transfer 25mg of tinidazole and fluconazole working standard into a 10 ml clean dry volumetric flask add about 7ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. From stock solution, further pipette 3 ml of tinidazole and fluconazole of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent **(Table 4)**.

Procedure: The standard solution was injected for five times and measured the area for all five Injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits.

Acceptance criteria: The % RSD for the area of five standard injections results should not be more than 2%.

Intermediate precision: To evaluate the intermediate precision of the method Precision was performed on different day by using different make column of same dimensions.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits **(Table 6, 7)**.

Acceptance Criteria: The % RSD for the area of five standard injections results should not be more than 2%.

Accuracy: Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated **(Table 8, 9)**.

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

- The percentage recovery was found to be within the limit (97-103%).
- The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Linearity:

Preparation of stock solution: Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 10mg of fluconazole and tinidazole (marketed formulation) sample into a 10 mL clean dry volumetric flask add about 7mL of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. From the stock solution different concentrations of fluconazole and tinidazole were prepared as mentioned below.

Procedure: Inject each into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis peak area) and calculate the correlation coefficient **(Table 10, 11, 12; Figure 6, 7)**.

Acceptance criteria:

- Correlation coefficient (R^2) should not be less than 0.999
- The correlation coefficient obtained was 0.999 which is in the acceptance limit. The linearity was established in the range of 10% to 50% of Tinidazole and 5% to 25% of Fluconazole.

Limit of detection:

Tinidazole 300 µg/ml solution and 0.12 µg/ml solution were prepared. Fluconazole 45µg/ml solution and 0.015 µg/ml solutions were prepared **(Table 13, Figure 8)**.

Acceptance criteria: S/N Ratio value should be 3 for LOD solution.

Limit of quantification:

Tinidazole 300 µg/ml solution and 0.42µg/ml solution were prepared. Fluconazole 45µg/ml solution and 0.05 µg/ml solutions were prepared **(Table 14, Figure 9)**.

Acceptance Criteria: S/N Ratio value shall be 10 for LOQ solution.

Robustness:

As part of the robustness, deliberate change in the flow rate, mobile phase composition, temperature variation was made to evaluate the impact on the method.

a) Change in flow rate: Standard solution 300ppm of Tinidazole & 45ppm of Fluconazole was prepared and analysed using the varied flow rates along with method flow rate **(Table 15, 16)**.

b) Change in composition of Mobile phase:

Standard solution 300 µg/ml of Tinidazole & 45µg/ml of Fluconazole was prepared and analysed using the varied Mobile phase composition along with the

actual mobile phase composition in the method **(Table 17)**.

Acceptance criteria:

- Percentage RSD should be below 2.
- The % RSD obtained for change of flow rate, variation in mobile phase was found to be below 1, which is within the acceptance criteria. Hence the method is robust.

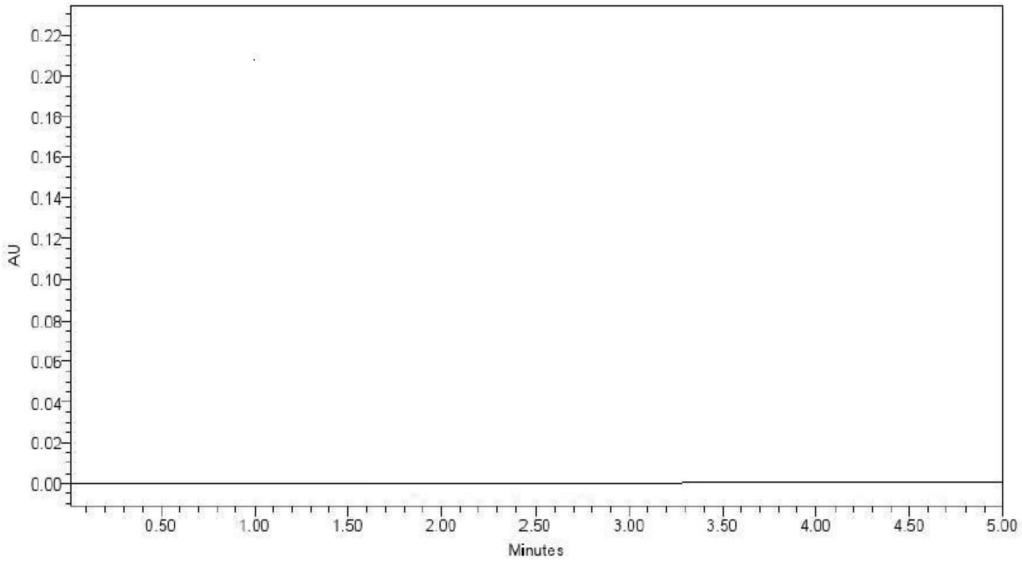

Figure 3: Chromatogram for blank

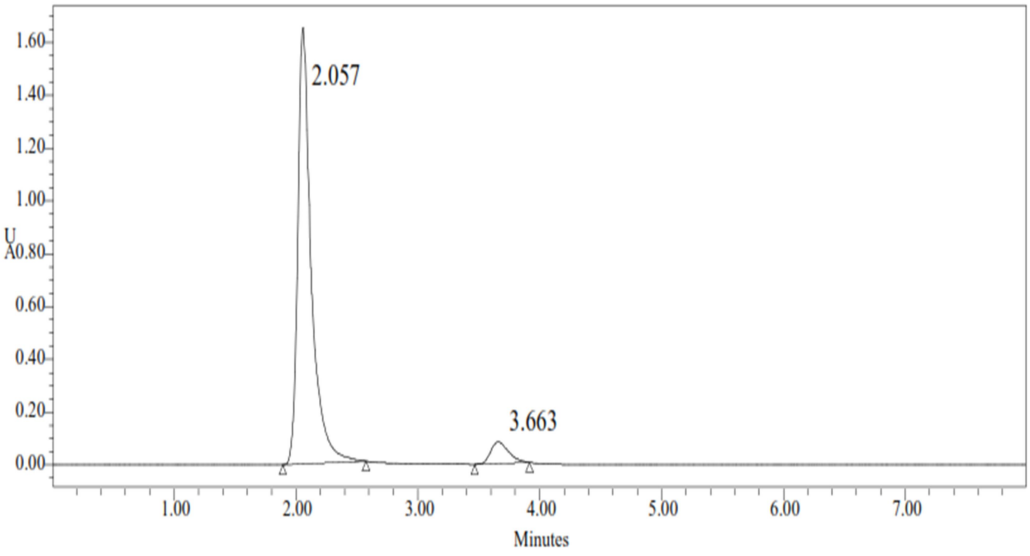

Figure 4: Chromatogram for tinidazole and fluconazole sample preparation

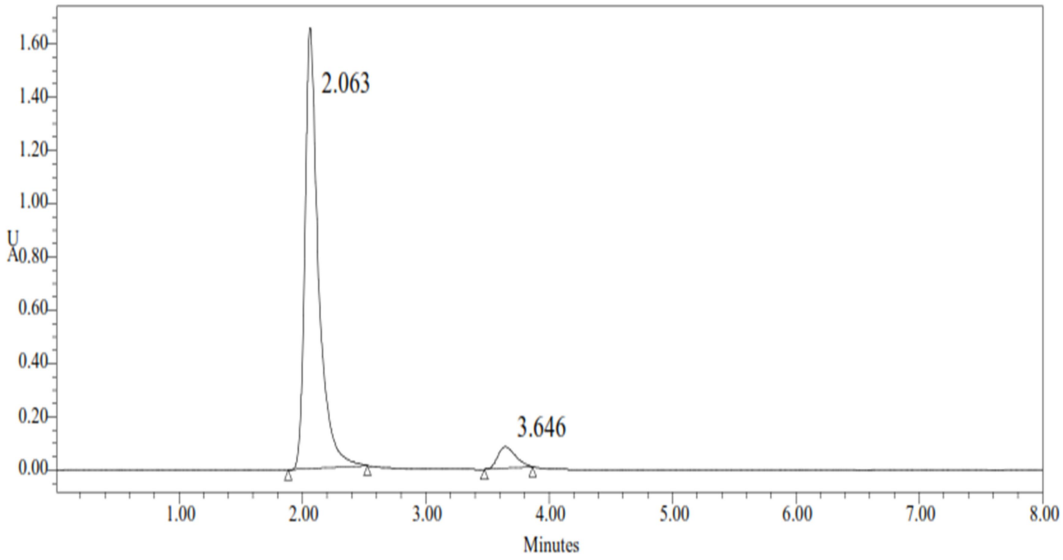

Figure 5: Chromatogram for tinidazole and fluconazole standard preparation

Table 3: Results of system suitability parameters for tinidazole and fluconazole

S. No.	Name	Retention time (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Tinidazole	2.5	124505	213642	4.6	1.2	4673.4
2	Fluconazole	3.9	1308495	154566	6.0	1.3	6090.3

Table 4: Results of method precession for tinidazole

Injection	Area
Injection-1	1302729
Injection-2	1302947
Injection-3	1303236
Injection-4	1303977
Injection-5	1309759
Average	1304529.8
Standard deviation	2961.1
% RSD	0.2

Table 5: Results of method precession for fluconazole

Injection	Area
Injection-1	123149
Injection-2	123766
Injection-3	124271
Injection-4	124691
Injection-5	124956
Average	124162.7
Standard deviation	725.6
% RSD	0.6

Table 6: Results of Intermediate precision for tinidazole

Injection	Area
Injection-1	1300148
Injection-2	1304520
Injection-3	1305937
Injection-4	1306476
Injection-5	130871
Average	1305070.2
Standard deviation	3061.8
% RSD	0.2

Table 7: Results of Intermediate precision for Fluconazole

Injection	Area
Injection-1	122487
Injection-2	122626
Injection-3	122632
Injection-4	122702
Injection-5	122962
Average	122681.8
Standard deviation	174.8
% RSD	0.1

Table 8: Accuracy (recovery) data for tinidazole

% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	656659.5	5.0	5.036	100.7%	99.84%
100%	1304258	10.0	10.003	100.0%	
150%	1854608	14.4	14.224	98.780%	

Table 9: Accuracy (recovery) data for fluconazole

% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean Recovery
50%	65800	5.3	5.34	100.8%	100.51%
100%	124353	10	10.10	100.01%	
150%	177940	14.2	14.45	99.68%	

Table 10: Area of different concentration of tinidazole

S. No.	Linearity Level	Concentration	Area
1	I	100ppm	447957
2	II	200ppm	885915
3	III	300ppm	1313873
4	IV	400ppm	1791831
5	V	500ppm	2189788
Correlation Coefficient			0.997

Table 11: Area of different concentration of fluconazole

S. No	Linearity Level	Concentration	Area
1	I	1ppm	41600
2	II	2ppm	82895
3	III	3ppm	124802
4	IV	4ppm	169545
5	V	5ppm	203659
Correlation Coefficient			0.999

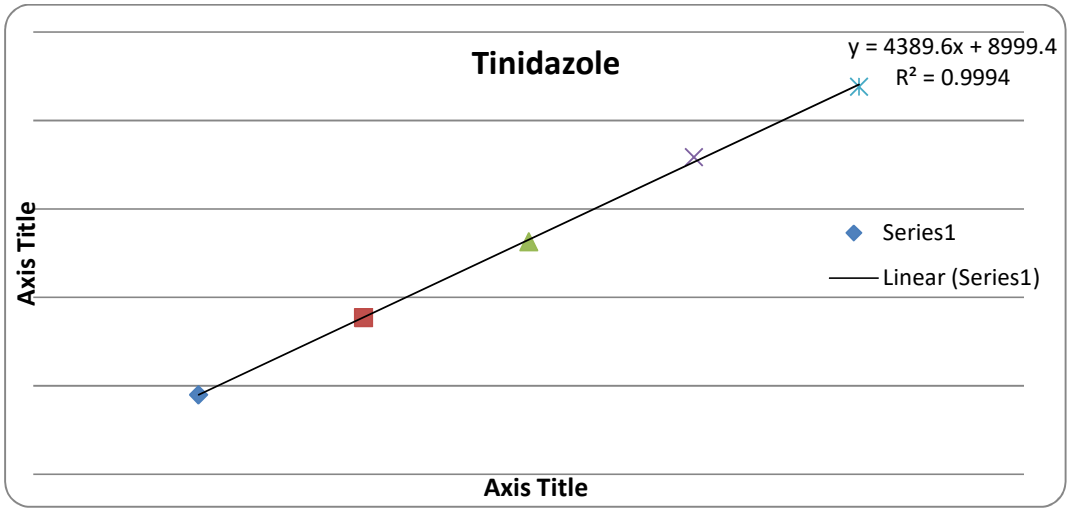

Figure 6: Calibration graph for tinidazole

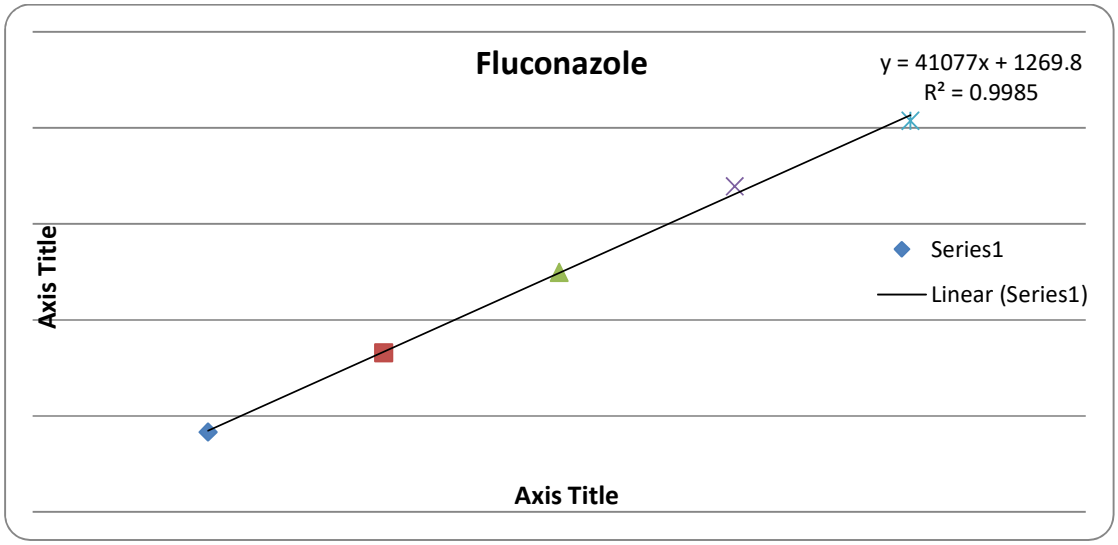

Figure 7: Calibration graph for fluconazole

Table 12: Analytical performance parameters of tinidazole and fluconazole

Parameters	Tinidazole	Fluconazole
Slope (m)	66574	12529
Intercept (c)	53592	50245
Correlation coefficient (R^2)	0.999	0.999

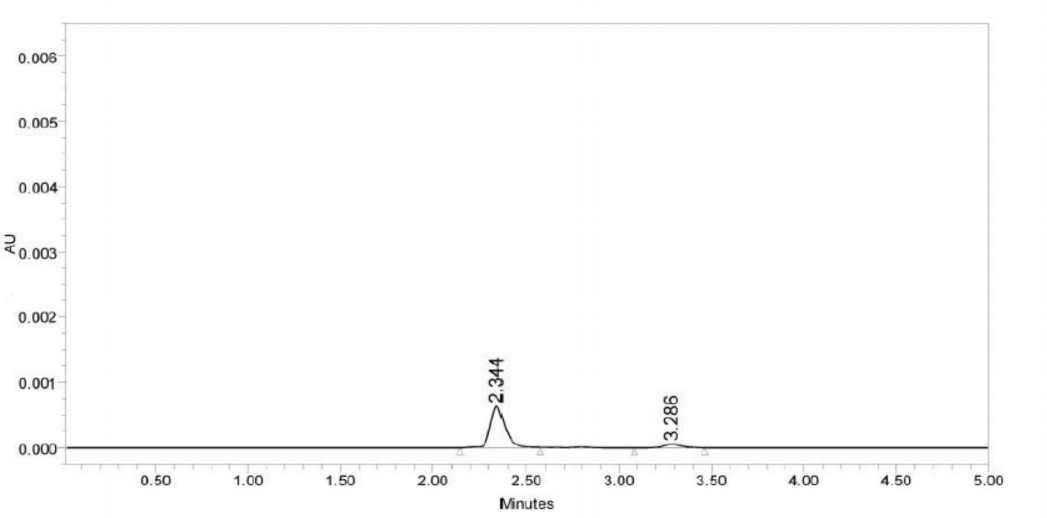

Figure 8: Chromatogram of tinidazole & fluconazole showing LOD

Table 13: Results of LOD

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Tinidazole	52	152	2.9
Fluconazole	52	156	3

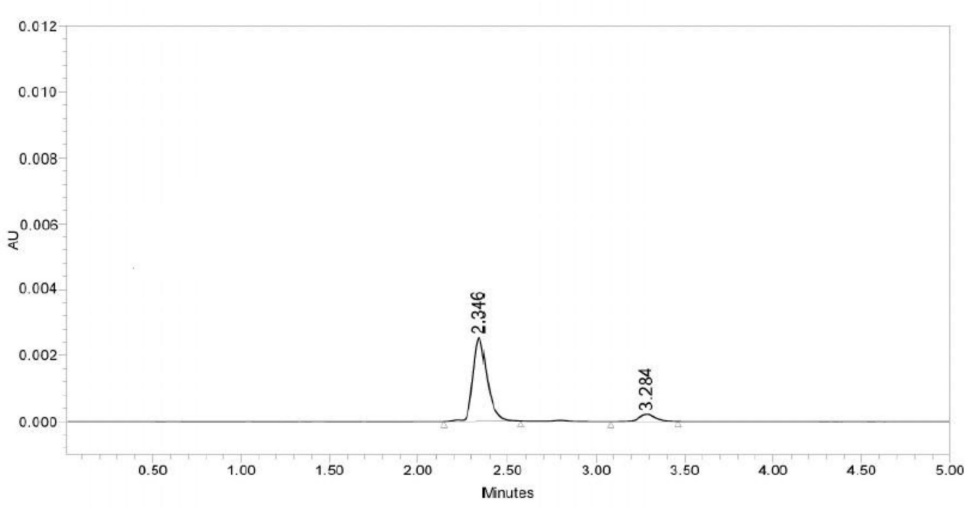

Figure 9: Chromatogram of tinidazole & fluconazole showing LOQ

Table 14: Results of LOQ

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
Tinidazole	52	522	10.03
Fluconazole	52	524	10.1

Table 15: Flow Rate (ml/min) data for tinidazole

S. No.	Flow rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.6	5339.9	1.4
2	0.8	4673.4	1.3
3	1.0	5216.0	1.4

Table 16: Flow rate (ml/min) data for fluconazole

S. No.	Flow rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	7063.3	1.3
2	1.0	6090.3	1.2
3	1.2	6998.0	1.3

Table 17: Change in organic composition in the mobile phase for tinidazole

S. No.	Change in organic composition in the mobile phase	System suitability results	
		USP plate count	USP tailing
1	10% less	4508.4	1.3
2	*Actual	4673.4	1.4
3	10% more	4318.1	1.3

Table 18: Change in organic composition in the mobile phase for fluconazole

S. No.	Change in composition of mobile phase	System suitability results	
		USP plate count	USP tailing
1	10% less	6387.7	1.2
2	*Actual	6090.3	1.2
3	10% more	6232.5	1.2

CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tools of the analysis. The estimation of tinidazole and fluconazole was done by RP-HPLC. The Phosphate buffer was pH 3.0 and the mobile phase was optimized with consists of methanol:phosphate buffer

mixed in the ratio of 70:30 % v/v. Inertsil C_{18} column (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 210nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of tinidazole and fluconazole were found to be from 100-500 µg/ml of tinidazole and 1-5 µg/ml of fluconazole. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of tinidazole and fluconazole. LOD and LOQ were found to be within the limit.

The results obtained on the validation parameters met ICH and USP requirements. The developed method was found to be simple, accurate, precise, linear and having suitable application in routine laboratory analysis with high degree of accuracy and precision.

Declarations

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Author contributions

All authors contributed to experimental work, data collection, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Competing interest statement

All authors declare that there is no conflict of interests regarding publication of this paper.

Additional information

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Ethical approval

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