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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF FLUCONAZOLE AND TINIDAZOLE IN TABLET DOSAGE FORM

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ABSTRACT
Pharmaceutical Analysis plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and on finished products. RP-HPLC method was developed and validated as per ICH guidelines for the estimation of Fluconazole and Tinidazole. Simultaneous Estimation of Fluconazole and Tinidazole were carried out by RP- HPLC using Ortho phosphoric acid buffer (P^H 4.0): Methanol (70:30) and column Phenomenex Luna C-18 (250*4.6 mm, 5um) as a stationary phase and peak was observed at 210 nm which was selected as a wavelength for quantitative estimation. After the development of the method, it was validated for specificity, linearity, precision, accuracy, robustness and ruggedness studies. Theoretical plate for Fluconazole was found to be 7040, for Tinidazole it was found to be 7677. From the linearity studies, the specified range for Fluconazole and Tinidazole was found to be 50% to 150%. it was found that recovery value of pure drug were between 99 % to 101% which indicates that the method is accurate and also reveals that commonly used excipients and additives present in the pharmaceutical formulations were not interfering in the proposed methods. The ruggedness of the method was checked by different analysts and found that the results were nearly same which indicates that the method is rugged. Based on the results observed, it was concluded that proposed method can be used for routine analysis of Fluconazole and Tinidazole.

KEYWORDS: Fluconazole, Tinidazole, specificity, linearity, precision, accuracy, robustness and ruggedness.

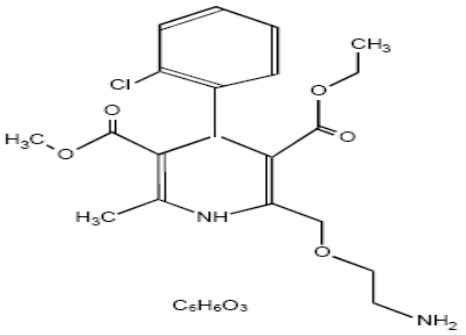
INTRODUCTION
Pharmaceutical Analysis plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and on finished products. It also plays an important role in building up the quality products through in process quality control. Pharmaceutical analysis is the application of principles of analytical chemistry to drug analysis. The analytical chemistry may be defined as the science of developing sensitive, relative and accurate methods for determining the composition of materials in terms of elements or compounds which they contain (D.A.Skoog 1998).

The most important aspect of analysis is quantitative chemical analysis. In the present age, the physical, chemical and biological analysis, Involve computerized techniques to facilitate better result.

CHROMATOGRAPHY
Chromatography **(**from Greek: *chroma*, colour and: "grafein" to write) is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated (P. D. Sethi, 2001). Chromatography defined as amethod of separating a mixture of components into individual components through equilibrium distribution between two phases (Gurdeep R Chatwal& Sham K.Anand 2002).

The objective of this work is to develop and the estimation of Fluconazole and Tinidazole in formulations. So an attempt was made to develop and validate a simple, precise, accurate, linear and rapid. RP-HPLC method is done as per as ICH guidelines for the estimation of Fluconazole and Tinidazole in pure pharmaceutical dosage forms and to apply the developed method to determine the validation of compounds. Hence, the present study work to develop the simple, rapid, accurate, precise and validated methods for the estimation in combination dosage forms.

DRUG PROFILE
FLUCONAZOLE
STRUCTURE



CHEMISTRY
- m-[(2,4-Diflurophenyl)-m-(1H-1,2,4-triazol-yl-methyl)-1H-1,2,4-trizol-1-ethanol)

a) Molecular formula: $C_{13}H_{12}F2N_6O$ b) Molecular weight: 306.27.

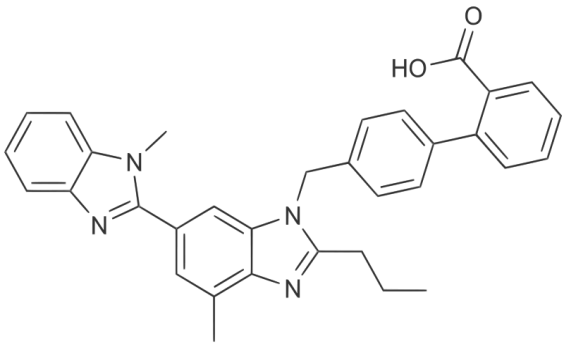
APPEARANCE: It is odorless, white crystalline powder.

SOLUBILITY: It is slightly soluble in water and sparingly soluble in ethanol, soluble in methanol, and acetonitrile.

CATEGORY: Triazole Antifungal.

MECHANISM OF ACTION
Fluconazole Found effective in several systemic fungal infections especially in candidiasis and cryptococcosis associated with immune suppression as in AIDS or Cancer therapy. Succeptible pathogens include Aspergillus spp, Blastomyces dermatitis, candida spp, coccidiodes immitis, dyptococcus neoformans and Histo plasma capsulatum.

TINIDAZOLE
STRUCTURE



CHEMISTRY
Tinidazole is chemically 1-[2-(-ethyl sulphonyl) ethyl]-2-methyl-
5-nitroimidazole.

a) Molecular formula:$C_8H_{13}N_3O_4$ b) Molecular weight: 247.3
APPEARANCE: Tinidazole is a pale yellow crystals or a crystalline powder.

MELTING POINT: 125-128^{0}C.

SOLUBILITY: It is practically insoluble in water, soluble acetone and in methylene chloride, sparingly soluble in methanol.

CATEGORY: Anti- protozoal and anti- bacterial.

MECHANISM OF ACTION
It is Highly active against Entamoeba histolytica, Trichomonas vaginalis, Giardia lambila and an aerobic bacteria such as Gardnerella vaginalis, clostridium species. Tinidazole is converted to the active metabolite by reduction at the 5-nitro position. The active metabolite causes DNA damage in pathogens.

MATERIALS AND METHODS
Drug samples
Fluconazole and Tinidazole were generously given by, Mankind Pharma Ltd. New delhi.

Tablets used: Brand: **NUFORCE-KIT,** each tablet contains: Fluconazole –150mg and Tinidazole 1000mg.

Chemicals and solvents used: Water for HPLC, Ortho phosphoric acid, methanol AR grade, distilled water.

Instruments used: Shimadzu electronic balance, Elico pH meter, Soltec ultra sonicator, Millipore – solvent filtration unit, Labindia-3000, UV-visible spectrophotometer, 1 cm matched quartz cells. Shimadzu Isocratic HPLC system with following configurations, LC-10AT Vp series, Isocratic solvent delivery system (pump). Rheodyne 7725i injector with 20 µl loop. spinchrome data station. Analytical column: Phenomenex – Luna, C_{18} (250 x 4.6 mm i.d., 5µ). UV-Visible – SPD 10AVp series detector.

PREPARATION OF MOBILE PHASE
Preparation of Buffer
1 ml of ortho phosphoric acid into 1000 ml of beaker dissolve and diluted volume with distilled water. Mobile-phase 700 ml of Buffer and 300 ml of Methanol were added in a beaker to give 1000ml. Mobile-Phase Ratio Buffer: Methanol (70: 30 % V/V) The method development by RP-HPLC method for the estimation of Fluconazole and Tinidazole is Development of suitable mobile phase, Optimization of the chromatographic conditions, Selection of suitable detection of wavelength. Preparation of standard calibration curve of Fluconazole and Tinidazole Assay of pure mixed standards and formulation, validation of the developed method, the chromatographic parameters for the development and optimization for the Fluconazole and Tinidazole.

SOLUBILITY
Solubility of drugs in different solvents at different pH conditions which is useful while selecting diluents for standard solution and extraction solvents for test solutions. According to Indian pharmacopoeia and literature review collected Fluconazole and Tinidazole are very Slightly soluble in water, Acetonitrile. Sparingly soluble in alcohol. Then it was checked for different dilutions of Methanol and Ortho phosphoric acid buffer and the mixture was chosen for present work.

STABILITY

Stability of drug with storage conditions. This helps to adopt suitable and adequate precaution while handling drug substances and its solutions.

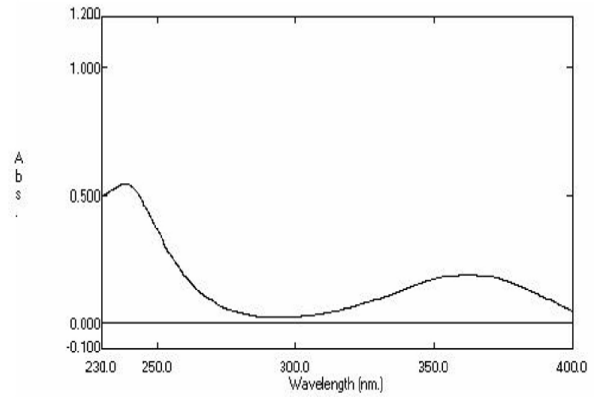
SELECTION OF CHROMATOGRAPHIC METHOD FOR SEPARATION

Depending on nature of sample (ionic or neutral molecule), its solubility and molecular weight, chromatographic method is to be selected. The drugs are polar in nature and so reverse phase chromatographic technique were selected for the present study.

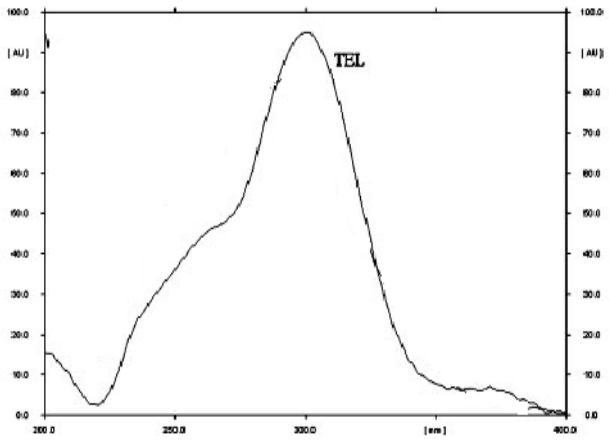
DETECTION OF WAVELENGTH (λmax)

Selection of detection of wavelength is a critical step in the analytical method. The conditions for the development of the assay method for the choice of detection wavelength was based on the scanned absorption for Fluconazole and Tinidazole The spectrum was scanned over the range of 200 – 400 nm and was obtained by measuring the absorption of 0.01mg/ml solution of Fluconazole and Tinidazole and in methanol prepared from stock solution. The spectrum was obtained by using 1cm quartz cell using water as reference solution. λmax of Fluconazole was 361 nm and λmax of Tinidazole was 225nm. Hence for simultaneous estimation 210nm was selected.

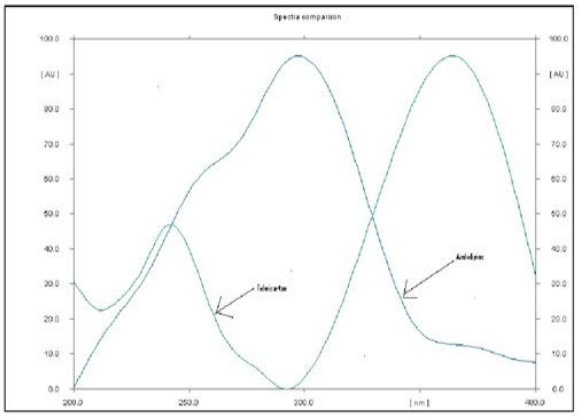
UV Spectrum of Fluconazole



UV Spectrum of Tinidazole



UV Spectrum of Fluconazole **and** Tinidazole



METHOD DEVELOPMENT TRIALS

Five trials on composition of buffer and organic phase were done to decide the ultimate composition of mobile phase.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Stationary phase : Phenomenex Luna C_{18} (250 × 4.6 mm, 5µm)
Mobile phase : Ortho phosphoric acid buffer: Methanol (70:30% v/v).
P^H : 4
Flow rate : 1 ml/min.
Column Temperature : 30.
Volume of Injection : 10µl.
Detection of Wavelength : 210 nm.
Run time : 8 min.
Mode of Operation : Isocratic.

METHOD VALIDATION

After the method development the method was validated in terms of parameters like Accuracy, Precision, Specificity, Linearity, Ruggedness, and Robustness, stability etc.

SYSTEM SUITABILITY PARAMETERS

For system suitability, five replicates of standard solutions of Fluconazole and Tinidazole were injected and studied the suitability parameters like Plate number (N), Resolution (R) and Relative retention time (α), and Peak symmetry of samples (As) were studied with the help of standard chromatograms.

Table No: System suitability Parameters.

System Suitability Parameters	Fluconazole	Tinidazole
Resolution	8.02	
Tailing Factor	1.0	1.1
Number of theoretical Plates	7040	7677
Retention time	5.4	3.7

LINEARITY AND RANGE: The linearity of calibration curves (analyte to peak area ratio V_s concentration) in pure solution was checked over the concentration ranges of 50-150µg/ml for the Fluconazole and 50-150 µg/ml for Tinidazole respectively. The linearity was evaluated by linear regression analysis, using least squares method.

The calibration curves were linear in the studied range and equations of the regression analysis obtained for Fluconazole and Tinidazole Y = 27252X + 29970(r = 0.999), and Y = 45065X + 37029 (r = 0.999) respectively. The mean ± standard deviation (SD) for the slope, intercept and correlation coefficient of standard curves were calculated.

The slope, intercept and correlation coefficient values for Fluconazole were found to be 27252, 29970 and 0.999 respectively. The slope, intercept and correlation coefficient values for Tinidazole were found to be 45065, 37029 and 0.999 respectively.

Accuracy expresses the closeness of agreement between the value, which was accepted either as conventional true value or and accepted reference value (International standard e.g pharmaceutical standard) and the value found (mean value) obtained by applying the test procedure a number of times.

To study reliability, suitability and accuracy of the method, recovery studies were carried out, by adding a known quantity of the standard to the pre analysed sample and recovery study were done. The recovery was carried out at 50%, 100%, 150% level and the contents were determined from respective chromatogram. From the results obtained we conclude that the method was accurate.

ACCURACY

Recovery studies for Fluconazole and Tinidazole

S.I No:	Inj. Sample	Spike level	Amount Present	Amount Recovered	% Recovered
1	**Fluconazole**	50 %	38.89857	38.3608	99%
2		100 %	74.42867	75.6458	101%
3		150 %	111.5671	110.3353	99%
4.	**Tinidazole**	50 %	48.6632	48.19807	99%
5		100 %	100.026	100.5271	101%
6		150 %	150	152.0698	101%

PRECISION

REPEATABILITY OF INJECTION

The Precision of test method was done by performing assay on five replicate determination of sample preparation at test concentration level (as per method of analysis) and calculated relative standard deviation of assay results.

Five injections from standard solutions were injected and the peak areas were obtained and %RSD was calculated. System precision and method precision were determined.

Table No 6: System Precision Fluconazole and Tinidazole.

S.NO:	Area of Fluconazole (mV)	Area of Tinidazole (mV)
1	804.202	4664.222
2	806.602	4665.943
3	816.968	4562.585
4	815.694	4663.066
5	817.332	4664.558
Mean	812.1596	4642.075
S.D	6.256531	44.65221
R.S.D	0.770357	0.961902

ROBUSTNESS

For demonstrating the robustness of the developed method experimental conditions were purposely altered and evaluated. The method must be robust enough to withstand such slight changes in chromatographic conditions and allow routine analysis of the sample. Effect of column temperature, and Effect of buffer P^H were carried out and standard was injected. There was no change in system suitability parameters.

Table No: 8.

Effect	Retention time of Fluconazole	Retention time of Tinidazole
Flow rate (0.8ml/min)	6.825	4.723
Flow rate (1.2ml/min)	4.579	3.167
Temp(25ºc)	5.504	3.801
Temp(35ºc)	5.498	3.797

RUGGEDNESS

Defined by USP, The Ruggedness is the degree of reproducibility of test results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory and from analyst to analyst.

Table no: 9.

Analysts	Area of Fluconazole	Area of Tinidazole
Analyst 1	1113829	1710222
Analyst 2	1113819	1710212

LIMIT OF DETECTION

Limit of detection is the lowest concentration of the analyte that can be detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the formula.

The lowest concentration of Fluconazole that can be detected was determined from standard curve was 0.000815 µg/ml.

The lowest concentration of Tinidazole that can be detected was determined from standard curve was 0.000466 µg/ml.

LIMIT OF QUANTITATION

Limit of quantitation is the lowest concentration of the analyte in a sample that can be estimated quantitatively by injecting decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method. Limit of quantitation can be obtained from linearity curve by applying the following formula.

LOQ = 10 SD/ slope.

Table No: 10.

Sample	LOD	LOQ
Fluconazole	0.000815µg	0.00271 µg
Tinidazole	0.000466µg	0.001555µg.

The lowest concentration at which peak can be quantified is called LOQ. It was found to be 0.00271 µg for Fluconazole and for Tinidazole was found to be 0.001555 µg.

STABILITY STUDIES

Stability of the sample and standard used in HPLC method is required for a reasonable time to generate reproducible and reliable results. The stability of the sample spiked with drug was subjected to short term stability at room temperature (Initial & after 8 hours).

ASSAY RESULTS

PROCEDURE

Separately Blank, Standard and test preparation was injected into liquid chromatogram and the areas for major peaks were recorded by using the following formula.

Calculation

Calculated the amount of Amlodipine Besylate and Telmisartan using the formula.

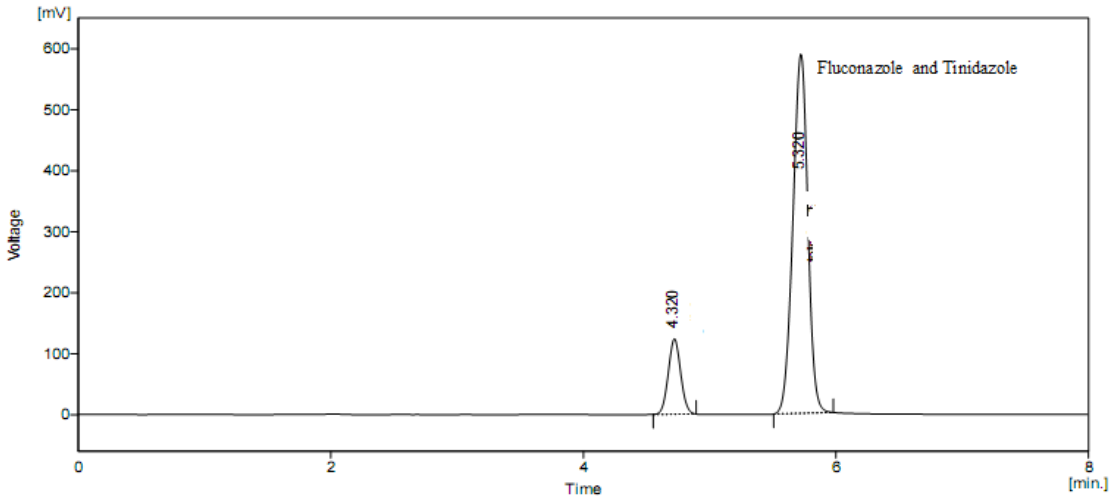
Sample area × sample dilution × purity of working standard × Average weight×100

Standard area × standard dilution × Label claim

RESULT: The mean recovery for assay results was found to be 98 – 101% for Fluconazole as well as Tinidazole.

Chromatogram

Standard chromatogram Fluconazole as well as Tinidazole.



RESULTS AND DISCUSSION

After several trails with various solvents, mobile phase system composed of Methanol and Ortho phosphoric acid buffer of P^H 4.0 in the proportion of 70: 30 v/v. respectively was chosen for the simultaneous estimation of Fluconazole and Tinidazole and in combined dosage form by RP-HPLC. This mobile phase composition offered maximum resolution for the drug at the detection wavelength of 210nm. The column used was C_{18} phenomenex luna (250 × 4.6mm) with flow rate of 1.0 ml/min and UV Detection was carried out.

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. The tablet shows percentage purity values ranging from 99.80 % for Fluconazole and 99.65% for Tinidazole respectively.

The Linearity for the both drugs**,** From the calibration curve constructed by plotting concentration $v_{s,}$ peak area, it was found that there exists a linear relationship in the concentration range 50 to 150 µg/ml and 50 to 150 µg/ml for Fluconazole and, with 0.999 Tinidazole and 0.999 as the value of correlation coefficient for the both drugs respectively as (Table 2) & (Fig 3). these are within Limit.

System suitability studies were carried out in which the resolution between the peaks, tailing factor and number of theoretical plates was found and are presented.(Table 1).

For System Precision studies, the standard solution was prepared at working concentration and analysis was carried for five replicated injections. The percentage relative standard deviation (% RSD) was calculated for the peak

areas for Fluconazole and Tinidazole and it was found and presented in (Table 6). and it is not more than 2.0%.

The acceptance criterion of method precision was found to be RSD NMT 2.0% and the Method Precision for Fluconazole and Tinidazole shows 0.9949 and 0.9619.

For the Accuracy of the method was determined by performing recovery studies at 50%, 100%, 150%. The recovery study was carried out and results were expressed in terms of the percentage recovery range found to be within the limit and the data was presented in (Table 4 and 5).

The Robustness of the method was by changing the parameters like buffer pH and changing the column temperature and the result was found to be within limit. The method shows no in the system suitability and precision parameters.

Ruggedness was determined by preparing six individual samples in replicate injections using different analyst, different column, and different system.

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) Of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The detection limit (LOD) was found to be 0.000815 µg/ml for Fluconazole and 0.000466 µg/ml for Tinidazole respectively.

The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). The quantitation limit (LOQ) was found to be 0.00271 µg/ml for Fluconazole and 0.001555 µg/ml for Tinidazole respectively.

The Stability studies was carried on sample and standard solutions on initial day and after 8 hours for the estimations of Fluconazole and Tinidazole and were considered stable for 8 hrs.
The Linearity, precision, accuracy, specificity, repeatability of measurement of peak area as well as repeatability of sample applications are validated as per ICH guidelines and the results are shown in the table below.

S.NO	Parameter	Limits	Observation	
			Fluconazole	Tinidazole
1	Specificity	No interference	No interference	No interference
2	System precision Method precision	RSD NMT 2.0%	0.7703 0.9949change	0.9619 0 .9619change
3	Linearity range	Correlation coefficient NMT –0.999	0.999	0.999
4	Accuracy	% Recovery range 98 – 10 2%	99-101	99-101
5	Limit of Detection	Signal noise ratio should be more than 3:1	0.000815	0.000466
6	Limit of Quantitation	Signal noise ratio should be more than 10:1	0.00271	0.001555
7	Asymmetry factor	NMT 2%	1.85	1.06
8	Number of Theoretical Plates	NLT 2000	7040	7677
9	Robustness Change in column temperature, Change in buffer pH		No effect on system suitability parameters	No effect on system suitability parameters

SUMMARY AND CONCLUSION

RP-HPLC method was developed and validated as per ICH guidelines for the estimation of Fluconazole and Tinidazole.

Simultaneous Estimation of Fluconazole and Tinidazole were carried out by RP- HPLC using Ortho phosphoric acid buffer (P^H 4.0): Methanol (70:30) and column Phenomenex Luna C-18 (250* 4.6mm, 5um) as a stationary phase and peak was observed at 210 nm which was selected as a wavelength for quantitative estimation. After the development of the method, it was validated for specificity, linearity, precision, accuracy, robustness and ruggedness studies.

The system suitability parameter also reveals that the values within the specified limit for the proposed method. Theoretical plate for Fluconazole was found to be 7040, for Tinidazole it was found to be 7677.

The precision of the System and Method were checked and found to be within limits. This indicates that the method is precise.

From the linearity studies, the specified range for Fluconazole and Tinidazole was found to be 50% to 150%. It was evaluated by the visual inspection of the plot of Peak area vs. Concentration.

From the results shown in the accuracy table, it was found that recovery value of pure drug were between 99% to 101% which indicates that the method is accurate and also reveals that commonly used excipients and additives present

in the pharmaceutical formulations were not interfering in the proposed methods.

The ruggedness of the method was checked by different analysts and found that the results were nearly same which indicates that the method is rugged.

The robustness of the method was checked by varying P^H change in buffer solution, variation in temperature and found that the system suitability parameters were within limit at all variable conditions, hence the method is robust.

Based on the results observed, it was concluded that proposed method can be used for routine analysis of Fluconazole and Tinidazole.

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