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DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF ANTIFUNGAL DRUGS IN BULK AND PHARMACEUTICAL DOSAGE FORM BY CHROMATOGRAPHIC METHOD USING HPLC

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Keywords:

Itraconazole, Terbinafine, Estimation, Antifungal, HPLC

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ABSTRACT: A new simple, accurate, rapid, selective and robust high-pressure liquid chromatography (HPLC) method was developed and validated for simultaneous estimating itraconazole and Terbinafine in bulk and marketed formulation. Acetonitrile and double distilled water was used as a mobile phase for chromatographic separation and estimation on C_{18} Targetsil column with dimensions 250×4.6 mm in the ratio of 90:10 v/v at flow rate of 1.0 ml/min. The detection was carried out with U.V. Visible detector (Shimadzu SPD10A) at 225 nm. The retention time for itraconazole (6.1 min) and for Terbinafine (9.8min) was obtained. The linearity range for itraconazole and Terbinafine was 1-8µg/ml with a coefficient of linear regression 0.996 and 0.992, respectively. The method was validated in accordance with the requirements of the International Conference on Harmonization (ICHQ2 (R1) 2005) guidelines for accuracy, precision, LOD & LOQ, linearity and robustness.

INTRODUCTION: Antifungal drugs usually treat systemic and superficial fungal infections. Antifungal agents commonly work through mechanisms of action as these agents inhibit the synthesis of ergosterol, a major component of fungal cell, and modify the cell membrane permeability by binding to the phospholipids of fungal cell membranes [1, 2]. Itraconazole is a triazole derivative with 706 g/mol molecular weight [3], an orally active antifungal agent with broad spectrum potency against many fungal infections [4]. Itraconazole shows inhibition of ergosterol synthesis by binding fungal cytochrome P-450.

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Ergosterol is a necessary part of the cell membrane, which plays a significant role in the growth of fungal and yeast colonies along with perturbation of membrane-bound enzyme function and membrane permeability [5]. Itraconazole is metabolized [6, 7, 8] using CYP3A4 enzymatic system to form three active metabolites *viz.* hydroxy itraconazole, keto-itraconazole and N-desalkyl itraconazole.

Terbinafine is an allylamine derivative having a broad spectrum against fungal infections [9]. Squalene epoxidase is an enzyme involved in ergosterol synthesis, inhibited by Terbinafine, Terbinafine can be used for fungistatic or fungicidal purpose [10]. Recently, Terbinafine is used to treat refractory and systemic fungal infections caused by *Aspergillosis* sp. [11] and is possibly useful to treat other systemic fungal infections [12]. A new, simple, and rapid reversed-phase high-performance

liquid chromatography (HPLC) method was developed and validated to determine Itraconazole and Terbinafine in pharmaceutical dosage forms.

MATERIAL AND METHODS:

EXPERIMENTAL WORK:

Development of RP-HPLC Method:

Selection of Solvent: The ideal properties of the solvent to be used in UV-Visible Spectrophotometry includes (a) Drugs should show solubility in the solvent used, (b) Drugs should show stability in the selected solvent, (c) Drugs should obey Beer-Lambert's Law over an appropriate range of analytical Concentrations and (d) The solvent should be to the extent, be economical. After taking the above considerations on various solvents, Analytical Grade Methanol was selected as a solvent of analysis.

Selection of Wavelength: Dissolve 10mg of drugs (Itraconazole and Terbinafine) in methanol to produce 100 ml. 1 ml of the stock solution was taken and was further diluted with methanol up to 10 ml to produce a conc. of 10µg/ml.

It was scanned on a UV-Visible spectrophotometer wavelength ranges 200 nm to 400 nm.

Optimization of chromatographic conditions for Itraconazole and Terbinafine: Many preliminary trials were conducted to select and optimize mobile phase and flow rate at ambient temperature.

The composition of mobile phase, ratio, and observed retention time are mentioned in **Table 1.**

TABLE 1: OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR 1PPM DRUG

Mobile Phase Components	Compositions	Retention Time Itraconazole	Retention Time Terbinafine
ACN: Water	50:50	9.827	13.574
ACN: Water	70:30	7.492	11.573
ACN: Water	90:10	6.102	09.782
ACN: Methanol: Water	50:40:10	12.309	14.213
ACN: Methanol: Water	60:30:10	9.982	12.143
ACN: Methanol: Water	70:20:10	10.275	11.683

The flow rate of the mobile phase was varied in the range of 0.5 to 1.2 ml/min and different injection volumes in the range of 20µl were tried. Optimized mobile phase selected comprised of acetonitrile (ACN): water (90:10). Optimized chromatographic conditions are tabulated in below **Table 2.**

TABLE 2: OPTIMIZED CHROMATOGRAPHIC CONDITIONS FOR HPLC ANALYSIS STANDARD

Parameters	Itraconazole	Terbinafine
Mobile phase	Acetonitrile:water (90:10)	Acetonitrile:water (90:10)
Flow rate	1.0 ml/ min	1.0 ml/ min
Detection wavelenght	225 nm	225 nm
Injection volume	20 µl	20 µl

Validation of RP-HPLC Method: The method development and establishment phase define the chemical assay. The fundamental parameters for analytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Typical method development and establishment for an analytical method include determination of (1) selectivity, (2) accuracy, precision, recovery, (3) calibration curve and (4) stability of analyte in spiked samples.

Accuracy: The degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed trueness. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LOQ, which should not deviate by more than 20%. The mean deviation from the true value serves as the measure of accuracy.

Recovery: The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the method's sample extraction and processing steps. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability.

Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing analytical results for extracted samples at 3 concentrations (low, medium and high) with unextracted standards representing 100% recovery.

Precision: The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended.

The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LOQ, which should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents and laboratories.

Reproducibility: Reproducibility is the precision between two laboratories. It also represents precision of the method under the same operating conditions over a short period. The precision assessment during initial method validation often applies to the first two of these: repeatability and intermediate precision. Reproducibility is usually determined during method transfer or crossover to another laboratory or location. Precision is often expressed by a data set's standard deviation (SD) or relative standard deviation (RSD). If a set of n measurements is defined as:

$$x = \frac{\sum_{i=1}^{n} xi}{n}$$

Where, "x" is individual measurement of the sample. Standard deviation of these data given as

$$SD = \sqrt{\frac{\sum_{i=1}^{n} (X_i - \bar{X})^2}{n-1}}$$

The relative standard deviation (RSD) or coefficient of variation (CV) is:

$$RSD (\%) = 100\ SD / x$$

Linearity: The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurement at several analyte concentrations. The data are then processed using a linear least-squares regression. The resulting plot slope, intercept, and correlation coefficient provide the desired information on linearity. The numerical value of the slope and intercept will depend on the response measured, but intercepts greater than 2% (relative to the target level response) aretypically expected with well-designed HPLC methods for major component analysis. A linearity correlation coefficient above 0.999 is acceptable for most methods.

Range: The range of the method can be defined as the lower and upper concentrations for which the analytical method has adequate accuracy, precision, and linearity. The range of concentrations examined will depend on the type of method and its use. For a major component assay, concentrations of standards should be measured at or near the expected target measurement level.

Limit of Detection (LOD): The lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise called limit of detection.

Lower Limit of Quantification (LLOQ): Lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy called LLOQ.

Upper Limit of Quantification (ULOQ): The highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy is called the upper limit of quantification.

Specificity: Specificity can be defined as the ability to measure accurately the concentration of an analyte in the presence of all other sample materials; the determination of method specificity can be achieved in two ways; first and most desirable, all potential interfering compounds can be tested to demonstrate their separation from the peak (S) of interest with a specified resolution (usually $R_S \geq 2$) a second method for achieving a specificity is the use of selective detectors especially for coeluting compounds. For example, a selective detector (e.g. electrochemical and radioactivity) will respond to some compounds but not to others.

Ruggedness: Method ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals and solvents and so on.

Robustness: Robustness measures the analytical procedure's capability to remain unaffected by small but deliberate variations. Such testing should be performed during the development of the analytical procedure and the data discussed and/or submitted. Representative instrument output (e.g., chromatograms) should be submitted when an effect is observed. e.g. Mobile phase ratio, concentration of buffer, flow rate.

System Suitability: It can be defined as tests to ensure that the method can generate acceptable accuracy and precision results. The USP defines parameters that can be used to determine the system's suitability before analysis.

These parameters include plate no. (n), tailing factor, *k* and/or α , resolution (Rs), and relative standard deviation (RSD) of peak height or peak area or repetitive injections.

Selectivity: Selectivity is the ability of the analytical method to measure and differentiate the analytes in the presence of components that may be expected to be present.

These could include impurities, degradants, or matrix components. Selectivity should be ensured at the lower limit of quantification (LLOQ).

RESULT AND DISCUSSION:

Development of RP-HPLC Method:

Preparation of Mobile Phase: Mobile phase preparation by mixing Acetonitrile (HPLC grade) and water (HPLC grade) in selected proportions. The prepared Mobile phase was taken separately and filtered through membrane nylon filters of size 4.5µ, to the filtered and mixed solutions, then sonicated for 15 minutes and filtered through membrane nylon filters of size 4.5µ.

Selection of Wavelength of Itraconazole and Terbinafine: The provided standard drug samples, namely Itraconazole and Terbinafine at a concentration of 1 ppm working solution, was used to scan in the range from 300 nm to 190 nm wavelength to find out the λmax values. The λmax value of the 10 ppm diluted standard stock of a given drug/analyte are reported to be 229 nm for Itraconazole with an absorbance value of 2.688; while λmax value for Terbinafine was observed to be 223 nm with an absorbance value of 2.568.

Method Optimization:

Mobile Phase Optimization: During mobile phase optimization and considering the system suitability parameters like Rt, Tailing factor, No. of theoretical plates and HETP, the mobile phase phosphate buffer: acetone (90:10), pH was 3.0 at λmax 235nm was found satisfactory. After mobile phase selection, the effect of pH and flow rate was observed. It was found that pH = 3 and 1.0 ml/min. is suitable for the drug.

Mobile Phase Optimization for Itraconazole: Fig. 1-6.

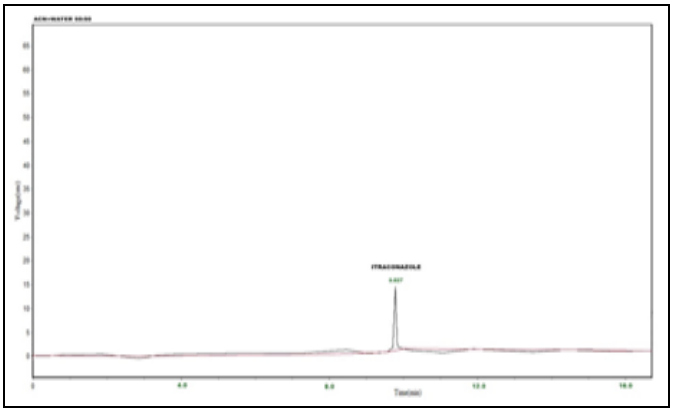

FIG. 1: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+WATER 50:50 V/V)

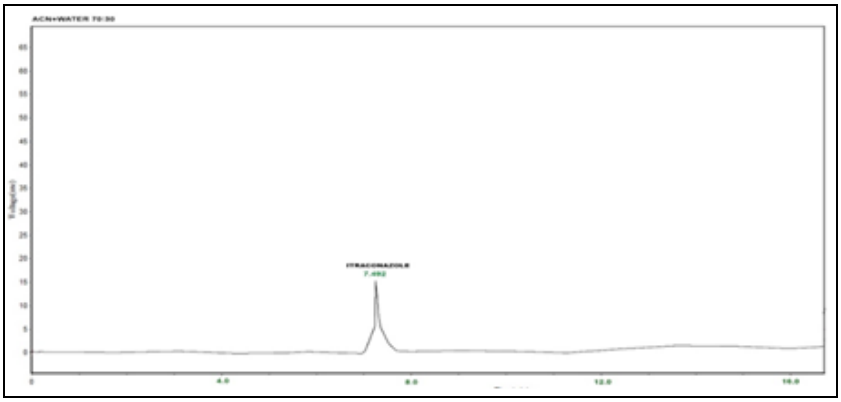

FIG. 2: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+WATER 70:30 V/V)

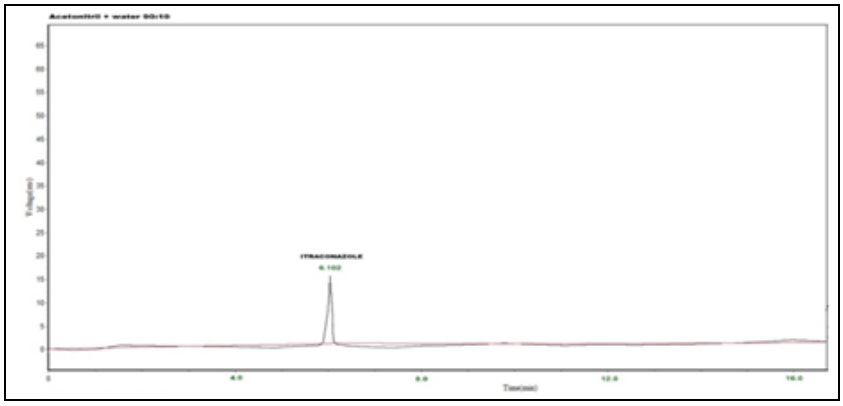

FIG. 3: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+WATER 90:10 V/V)

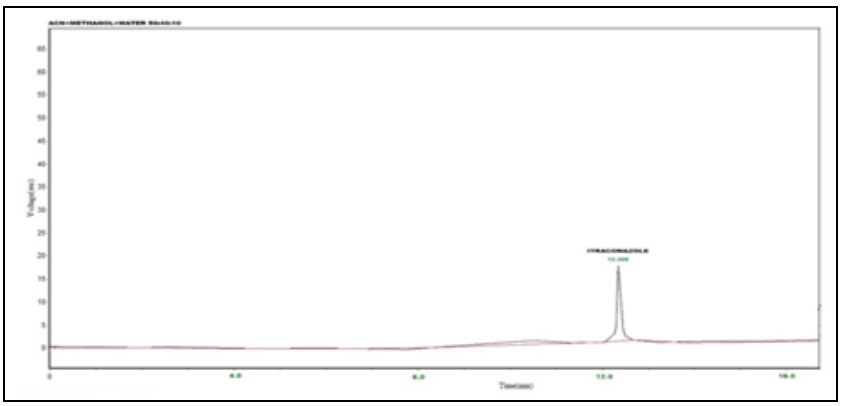

FIG. 4: CROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT OF ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 50:40:10 V/V)

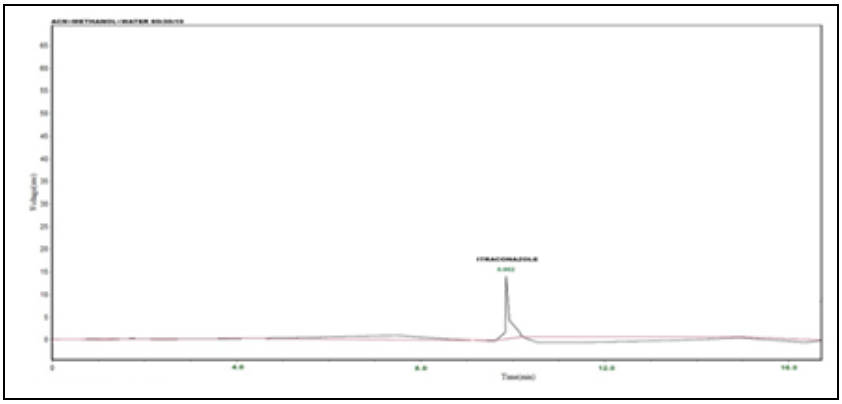

FIG. 5: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 60:30:10 V/V)

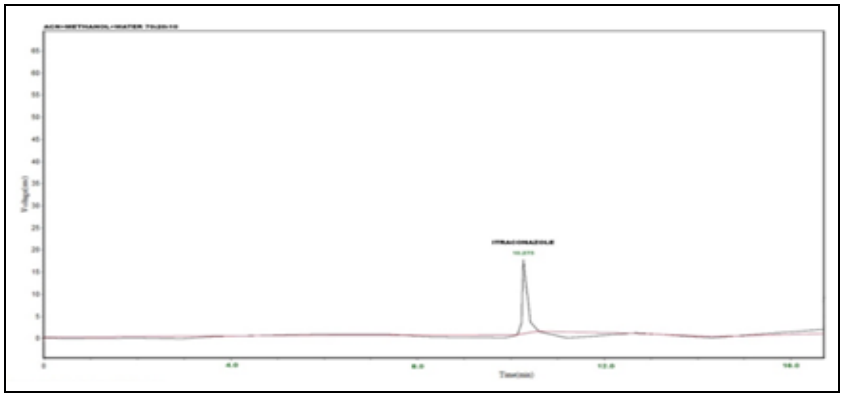

FIG. 6: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR ITRACONAZOLE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 70:20:10 V/V)

Mobile Phase Optimization for Terbinafine: Fig. 7-12.

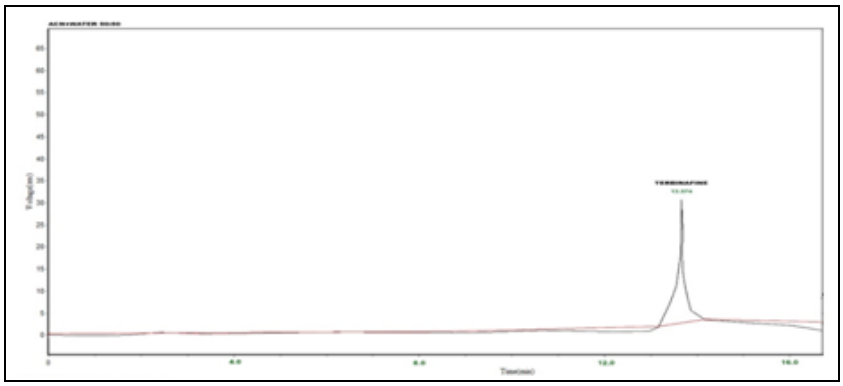

FIG. 7: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR TERBINAFINE IN MOBILE PHASE (ACETONITRIL+WATER 50:50 V/V)

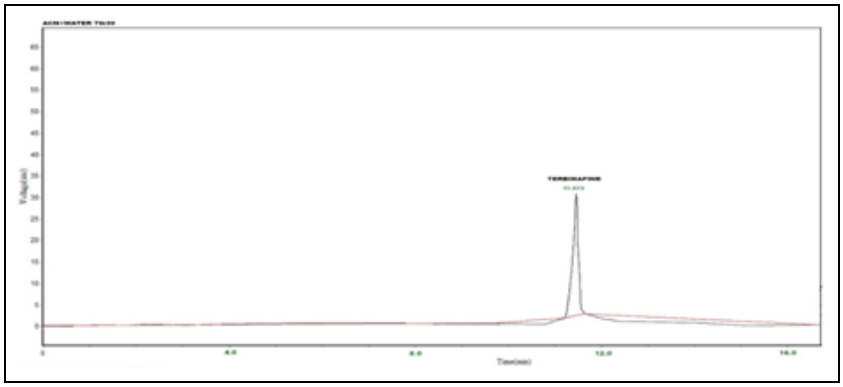

FIG. 8: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR TERBINAFINE IN MOBILE PHASE (ACETONITRIL+WATER 70:30 V/V)

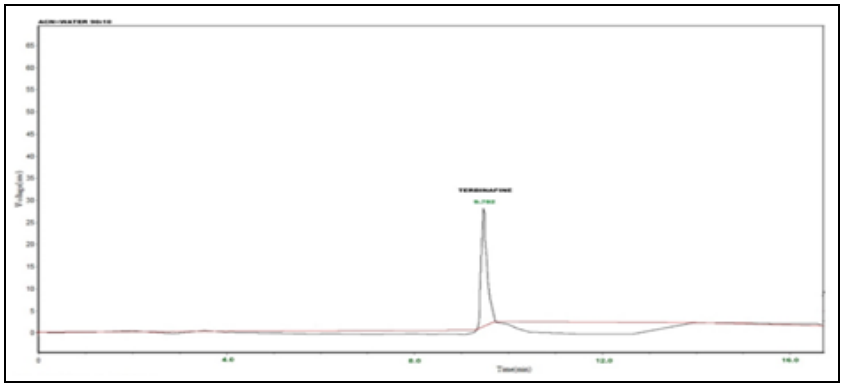

FIG. 9: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR TERBINAFINE IN MOBILE PHASE (ACETONITRIL+WATER 90:10 V/V)

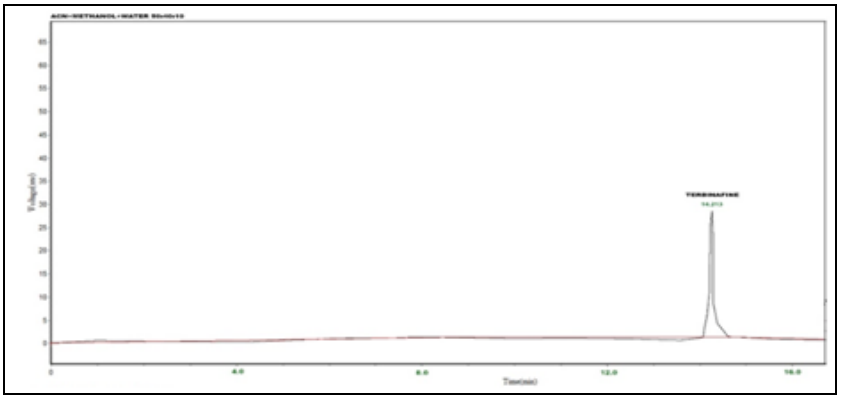

FIG. 10: CROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT OF TERBINAFINE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 50:40:10 V/V)

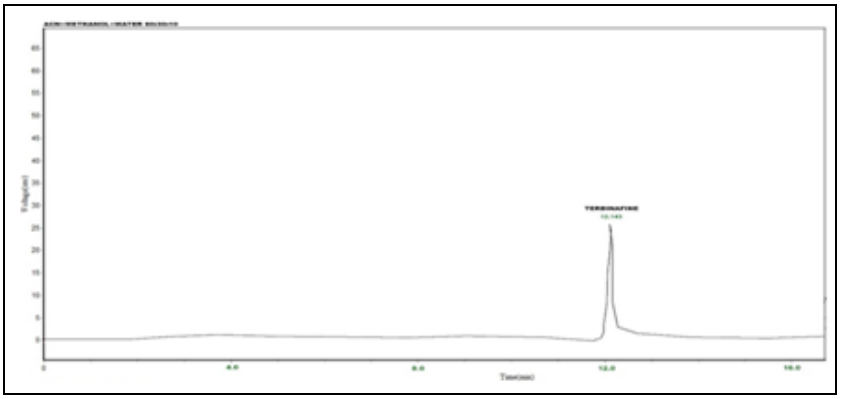

FIG. 11: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR TERBINAFINE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 60:30:10 V/V)

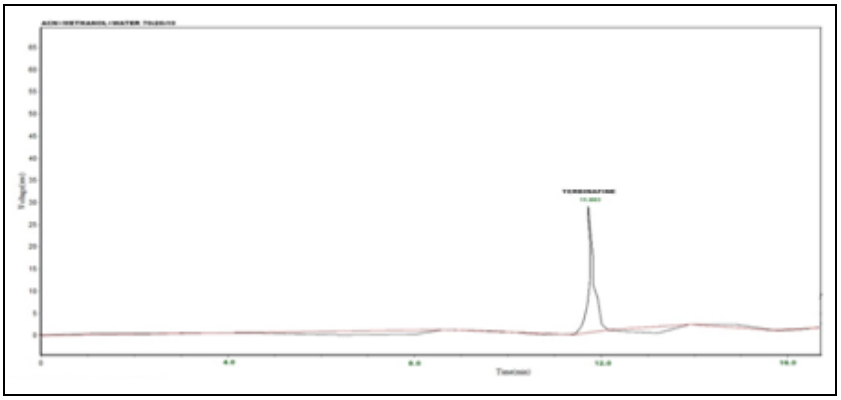

FIG. 12: CHROMATOGRAM OF ANALYTICAL METHOD DEVELOPMENT FOR TERBINAFINE IN MOBILE PHASE (ACETONITRIL+METHANOL+WATER 70:20:10 V/V)

Optimized Chromatographic Conditions: The terbinafine and Itraconazole samples was analysed by a double beam UV-Vis Spectrophotometer model of Lab Science (model: LS-2704) with 1cm matched quartz cells was used to determine the λmax of standard marker compound "Itraconazole and Terbinafine" which was utilized for estimation of marker compound in given sample. The Shimadzu LC10 HPLC system (Shimadzu Ltd, Japan) was used consisted of a pump (Shimadzu LC10AT), a U.V. Visible detector (Shimadzu SPD10A), a reverse phase C_{18} Targetsil column with dimensions 250× 4.6 mm, & particle size 5µm (Wesley Technologies, USA) and N2000 software for chromatography data analysis (Science Technology, Hangzhou. The mobile phase was Actonitrile: water (90:10) with a flow rate of 1ml/mn at room temperature using 225 nm detection wavelength, the retention time of Itraconazole and Terbinafine was 6.208 min and 9.849 min, respectively.

Determination of Retention Time: Retention for Itraconazole and Terbinafine under analysis at

optimized chromatographic conditions in actual sample was 6.208 ± 0.067 min and 9.849 ± 0.0542 min, respectively.

Validation of RP-HPLC Method for Itraconazole & Terbinafine:

Accuracy: The accuracy of the method was done by addition of standard drug solution to pre-analyzed sample solution at three different levels 80, 100 and 120 %.

Mean percentage recovery was determined. The given formula calculated % recovery:

% Recovery = Amount recover / Total present amount × 100

TABLE 3: RECOVERY STUDIES AND STATISTICAL VALIDATION FOR ACCURACY OF FORMULATION

Statistical Validation	Itraconazole			Terbinafine		
Level of Addition (%)	**80%**	**100%**	**120%**	**80%**	**100%**	**120%**
Conc. Present	10	10	10	10	10	10
(µg/ml)	10	10	10	10	10	10
	10	10	10	10	10	10
Conc. of Std. Added (µg/ml)	8	10	12	8	10	12
	8	10	12	8	10	12
	8	10	12	8	10	12
Conc. Recovered	7.993	9.949	11.986	7.946	9.912	11.922
	7.949	9.998	11.932	7.994	9.984	11.958
	7.979	9.948	11.969	7.99	9.942	12.000
% Recovery	99.91	99.49	99.88	99.32	99.12	99.35
	99.37	99.98	99.43	99.92	99.84	99.65
	99.74	99.48	99.74	99.89	99.42	100.0
Mean Recovery	99.675	99.6533	99.6867	99.71	99.461	99.66667
SD	0.27672	0.28733	0.22816	0.338083	0.361501	0.325320
%RSD	0.27762	0.28833	0.22888	0.339066	0.363461	0.326408

Precision: The method's precision was established by analyzing the compound based on intra-day and inter-day analysis. Precision was calculated by the relative standard deviation (% RSD), the percentage Coefficient of Variance (%CV).

TABLE 4: INTRA-DAY PRECISION DATA

Drug Sample	% Drug Determined at Period of Time						Mean	SD	% RSD
	1 h	2 h	3 h	4 h	5 h	6 h			
Itraconazole	99.6	99.8	98.7	98.9	99.7	99.8	99.41667	0.487510	0.49037
Terbinafine	99.7	99.7	99.8	99.6	99.9	99.7	99.73333	0.103279	0.10355

TABLE 5: INTER-DAY PRECISION DATA

Drug Sample	% Drug Determined at Period of Time			Mean	SD	% RSD
	1st Day	2nd Day	3rd Day			
Itraconazole	99.8	98.9	98.6	99.1	0.624499	0.630171
Terbinafine	99.9	99.8	99.9	99.87	0.057735	0.057812

Linearity and Calibration Curve:

Optimization of Chromatographic Conditions: The flow rate of the mobile phase was 1 ml/min with sample injection volumes of 20 µl was used. Optimized mobile phase selected solvents comprised of acetonitrile (ACN): water (90:10). Optimized chromatographic conditions are tabulated in the below table for both Itraconazole and Terbinafine markers.

TABLE 6: CALIBRATION CURVE FOR ITRACONAZOLE AND TERBINAFINE AT DIFFERENT CONCENTRATION

Level	Concentration (ppm)	No. of Reading	Itraconazole		Terbinafine	
			Retention Time	Peak Area	Retention Time	Peak Area
1	1ppm	3	6.102	23674	9.782	89362
2	2ppm	3	6.199	37197	9.810	176978
3	4ppm	3	6.271	94894	9.912	385674
4	8ppm	3	6.261	199564	9.891	674563

Correlation Coefficient (r^2)	0.996	0.992
Linearity Equation	Y = 25797X - 7907	Y = 83751X + 17576

Limit of Detection (LOD) and Limit of Quantification (LOQ):

TABLE 7: LOD & LOQ FOR ITRACONAZOLE AND TERBINAFINE

Sample Name	LOD	LOQ
Itraconazole	0.03961 X 10^{-3}	0.12 X 10^{-3}
Terbinafine	0.01221 X 10^{-3}	0.036978 X10^{-3}

Specificity:

TABLE 8: SPECIFICITY REPRESENTATION

S. no.	Peak name	Retention Time
1	Diluent	No peaks are observed at retention time of main peak
2	Placebo	No peaks are observed at the retention time of main peak
4	Main Peaks	13.103, 14.201

Ruggedness:

TABLE 9: RUGGEDNESS OF HPLC METHOD

Analysts	Itraconazole			Terbinafine		
	Conc. (µg/ml)	Conc. found (µg/ml)	(%) Drug Determined	Conc. (µg/ml)	Conc. found (µg/ml)	(%) Drug Determined
1	10	9.9485	99.485	10	9.934	99.344
2	10	9.999	99.99	10	9.955	99.55
3	10	9.996	99.955	10	9.964	99.64
Mean	10	9.9812	99.81	10	9.951	99.511
SD	0	0.02833	0.282002	0	0.015395	0.151741
% RSD	0	0.2838	0.282539	0	0.154708	0.1524866

6.4.5 6.4.7 Robustness:

TABLE 10: ROBUSTNESS OF HPLC METHOD

Method Variables		Itraconazole (4ppm)			Terbinafine (4ppm)		
		Rt	Area	Tailing	Rt	Area	Tailing
pH	2.8	6.142	94862	1.121	9.814	385722	1.133
	3.2	6.274	93781	1.139	9.653	385139	1.163
	Mean	6.208	94321	1.130	9.734	385431	1.148
	S.D.	0.0933	764.38	0.0127	0.1138	412.24	0.0212
	%R.S.D.	1.503	0.811	1.124	1.169	0.107	1.847
Temp. °C	25 °C	6.352	93643	1.131	9.812	379912	1.134
	35 °C	6.207	94211	1.122	9.723	376215	1.152
	Mean	6.279	93927	1.127	9.768	378064	1.143
	S.D.	0.1025	401.64	0.0064	0.0629	2614.17	0.0127
	%R.S.D.	1.633	0.4276	0.5679	0.6439	0.6915	1.111
Flow Rate (ml/min)	0.8	6.243	92523	1.111	9.821	377222	1.152
	1.2	6.371	94282	1.122	9.945	379347	1.128
	Mean	6.307	93403	1.117	9.883	378285	1.14
	S.D.	0.0905	1244.8	0.0078	0.0877	1502.60	0.0169
	%R.S.D.	1.435	1.3327	0.698	0.8874	0.3972	1.483

System Suitability

TABLE 11: DESCRIPTION OF SYSTEM SUITABILITY

S. no.	Parameters	Itraconazole	Terbinafine
	Resolution (Rs)	6.7243	6.3458
2	Capacity Factor (k´)	4.567	4.348
3	Theoretical Plate	385416.4571	445516.6657
4	HETP	0.13202	0.1131
5	Tailing Factor	1.0672	1.0719
6	Retention time(RT)	6.103	9.801
7	Asymmetry	1.041	1.105

Assay of Drugs in Marketed Formulation:

Estimation of Itraconazole & Terbinafine in Marketed Drug: The chromatograms of HPLC analysis for simultaneous detection and estimation of Itraconazole and Terbinafine in marketed tablet sample Gpitro-TR used in the present study through optimized mobile phase and chromatographic conditions as mentioned in **Table 1** of this report are as depicted in **Fig. 4, 5, 6** and **Table 7** ahead in this section;

For analysis, the tablet samples were crushed into fine powder in mortar pastel and dissolved in HPCL grade methanol, then suitably diluted to prepare the sample for HPLC analysis at a concentration of 1ppm, 2ppm & 4ppm. The prepared samples were sonicated and filtered through a 0.45-micron dissociable syringe filter before injecting into the HPLC machine for analysis.

TABLE 12: SIMULTANEOUS ESTIMATION OF ITRACONAZOLE AND TERBINAFINE AT OPTIMIZED CHROMATOGRAPHIC HPLC CONDITIONS IN GIVEN MARKETED TABLET GPITRO-TR

S. no.	Tablet concentration	Analyte of Interest	Corresponding Retention Time	Area Under Peak	Estimated Concentration of Drug
1	1 µg/ml	Itraconazole	6.179 min	583	Erroneous
		Terbinafine	9.717 min	42365	0.321 µg/ml
2	2 µg/ml	Itraconazole	6.177 min	1365	Erroneous
		Terbinafine	9.861 min	68365	0.629 µg/ml
3	4 µg/ml	Itraconazole	6.177 min	4939	0.511 µg/ml
		Terbinafine	9.861 min	114658	1.178 µg/ml

The weight of each single tablet in a strip of 10 tablets is different from each other and the average weight of each tablet can be considered 0.774±0.016 gram. Since, the amount of Itraconazole and Terbinafinein 0.774±0.016 gram weight tablet was mentioned as 100 mg and 250 mg respectively in each tablet, thus the estimation of these analytes must be in the range of approx. 12.9% and 32.3%, respectively.

Hence, the analytes were attempted to estimate by optimized & validated HPLC method in given tablet samples, it was observed that estimation of Terbinafine was successfully possible at both 1 µg/ml and 2 µg/ml concentration of tablet used for analysis, but at these concentrations analysis of Itraconazole was observed to be erroneous, however, their peak retention time was in acceptable range, but the area under peaks doesn't fit well in standard curve plot for simultaneous estimation of both analytes.

However, the simultaneous estimation of Itraconazole and Terbinafine at their corresponding retention time could be made when the test sample tables of 0.774±0.016 gram weight containing 100 mg and 250 mg of Itraconazole and Terbinafine, respectively, at optimized and validated chromatographic conditions which is 0.511 µg/ml and 1.178 µg/ml respectively which approximately corresponds to their mentioned concentration in tablet samples in the range of approx. 12.9% and 32.3%, respectively.

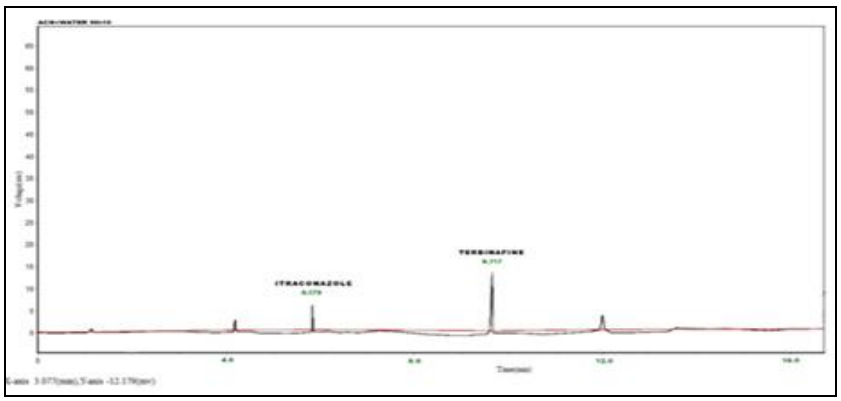

FIG. 13: CHROMATOGRAM FOR ESTIMATION OF ITRACONAZOLE AND TERBINAFINE IN SAMPLE TABLET AT ITS 1µG/ML CONCENTRATION USED FOR HPLC ANALYSIS

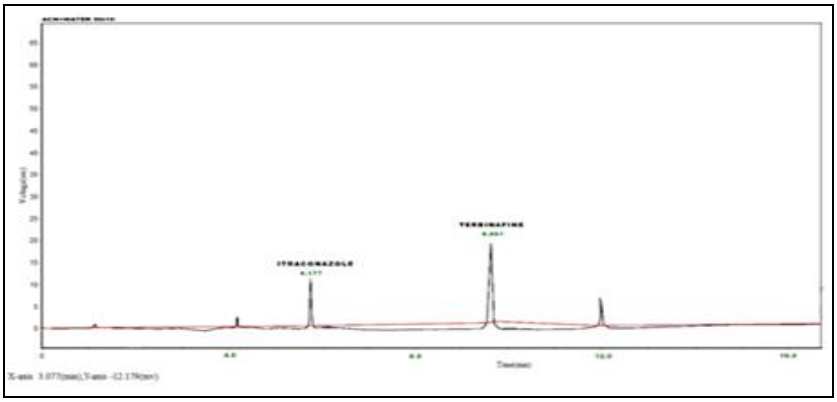

FIG. 14: CHROMATOGRAM FOR ESTIMATION OF ITRACONAZOLE AND TERBINAFINE IN SAMPLE TABLET AT ITS 2µG/ML CONCENTRATION USED FOR HPLC ANALYSIS

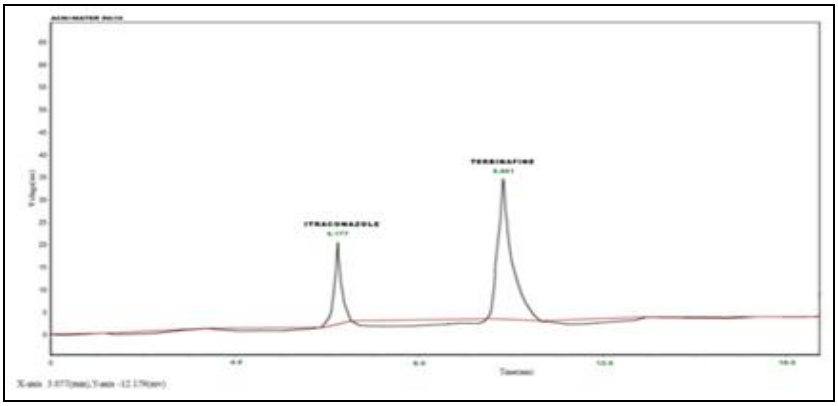

FIG. 15: CHROMATOGRAM FOR ESTIMATION OF ITRACONAZOLE AND TERBINAFINE IN SAMPLE TABLET AT ITS 4µG/ML CONCENTRATION USED FOR HPLC ANALYSIS

A simple, precise, accurate, reproducible, highly sensitive and effective RP-HPLC method was developed and validated for simultaneous estimation of Itraconazole and Terbinafine in sample. The method was validated under the different parameter like accuracy, precision, specificity and linearity. The developed method is LOD and LOQ values are in the range. In this study, the high recovery and low relative standard deviation confirm the suitability of the method for determination of Itraconazole and Terbinafine in pharmaceutical dosage forms. In conclusion, this method can be used for the routine simultaneous determination of Itraconazole and Terbinafine in pure and Tablet sample

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