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Novel Reverse Phase HPLC Method development and validation of Fluconazole and Tinidazole in a combined tablet dosage form

Chiranjeevi Bodepudi*, Swati Bantu, Kalyan Obula Reddy M, P.Shanmugasundaram, M.Vijey Aanandhi

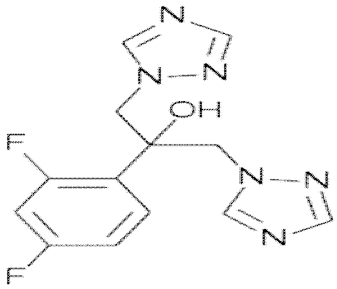
School of pharmaceutical sciences, Vels University, Zameen Pallavaram, Chennai – 600117,India.

Corres.author: chiru.bodepudi@gmail.com
Ph no: 8099713480, 9092562015.

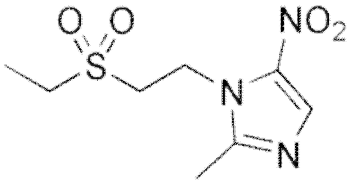
Abstract: A precise and feasible high-performance liquid chromatographic (HPLC) method for the analysis of the Fluconazole and Tinidazole in a combined tablet dosage form has been developed. The analysis was carried out on a Kromasil stainless steel C_{18} (250 x 4.6 mm, 5 µ) reversed-phase column, using a mixture of Acetonitrile: Water (55:45%v/v) as the mobile phase using a low pressure gradient mode with flow rate at 1ml/min. The injection volume was 20µl..The retention time of the drug was 2.5 for Fluconazole and 3.1 for Tinidazole. The method produced linear responses in the concentration range of 10 to 50µg/ml for both Fluconazole and Tinidazole. The Tailing factors of Fluconazole and Tinidazole were found to be 1 and 1.3 respectively. The method was found to be applicable for determination of the drug in tablets.
Key words: HPLC, Validation and quantification, Fluconazole and Tinidazole combined tablet dosage form.

Introduction:

Fluconazole [1, 2] is chemically 2-(2, 4-difluorophenyl)-1, 3- bis (1H-1, 2,4-triazol-1-yl)-2-propanol, a synthetic triazole derivative antifungal agent that has been shown to be effective against a wide range of systemic and superficial fungal infections, following both oral and intravenous administration**.** Tinidazole is a 1-[2-(ethyl sulphonyl) ethyl] – 2- methyl – 5- nitro 1H- imidazole, derivative used as antiprotozoal/antibiotic and antibacterial [3]



Chemical structure of Fluconazole



Chemical structure of Tinidazole

Literature survey revealed a few UV[3] methods for the estimation of Fluconazole in tablet form, HPLC[4, 5, 6, 7] methods for the determination of fluconazole and Tinidazole in combined tablet dosage form and separately, HPLC[8, 9, 10, 11] methods for estimation of Tinidazole in combination with other drugs, No UV method for simultaneous estimation of these two drugs has been reported till date. In the present study, an attempt has been made to develop a method for the simultaneous estimation of two drugs- Fluconazole (I), Tinidazole (II). It can also be applied for routine analysis of either one or of any combinations of in these drugs dosage forms.

Materials and Methods:

Chemicals and Reagents

a. Milli-Q-water
b. Acetonitrile - HPLC Grade
c. Water -HPLC Grade

Instrumentation

The LC system, used for the method development and validation was from Shimadzu LC-2010CHT series consists quaternary gradient pump, auto sampler, column oven and PDA detector. The out output signal was monitored and processed using CLASS-VP software on Pentium computer.

Preparation of mobile phase, standard and sample solutions of Fluconazole and Tinidazole

Mobile phase:

A mixture of 55 volume of Acetonitrile, 45 volume of Water (HPLC grade) and was prepared. The mobile phase was sonicated for 10min to remove gases.

Standard solution of Fluconazole and Tinidazole:

Accurately weigh and transfer 10 mg of Fluconazole and Tinidazole working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent (mobile phase) and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 5ml of Fluconazole and Tinidazole the above stock solution into a 50ml volumetric flask and dilute up to the mark with diluent. Further pipette 5ml of Fluconazole and Tinidazole above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample solution of Fluconazole and Tinidazole:

Accurately weigh and transfer tablet powder equivalent to 10 mg of Fluconazole and Tinidazole sample (Flucoty tablets) into a 10mL 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. (Stock solution).Further pipette 5ml of Fluconazole and Tinidazole of the above stock solution into a 50ml volumetric flask and dilute up to the mark with diluent. Further pipette 5ml of Fluconazole and Tinidazole the above stock solution into a10ml volumetric flask and dilute up to the mark with diluent.

Diluent

Prepared a mixture of Acetonitrile: Water in the ratio of 55: 45 which was used as diluent for dilution of standard stock solution.

Chromatographic conditions:

Instrument : Shimadzu pump LC – 2010HT
Detector : PDA detector
Column **:** kromasil Stainless steel Column C_{18} (250 X 4.6 mm, 5µ) packed with ODS chemically bounded porous silica particles.
Temperature : 40°C
Flow rate : 1 ml/min
Wave length : 260nm
Runtime : 5 min
Sample size : 20µl
Diluent : Acetonitrile: Water (55: 45)
Sample retention time:
Fluconazole RT (minutes): 2.5 ± 0.05
Tinidazole RT (minutes) : 3.1 ± 0.05

Method validation [12]

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 10 mg of Fluconazole and Tinidazole working standard into a 10mL 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.(Stock solution). Further pipette 5ml of Fluconazole and Tinidazole the above stock solution into a 50ml volumetric flask and dilute up to the mark with diluent.

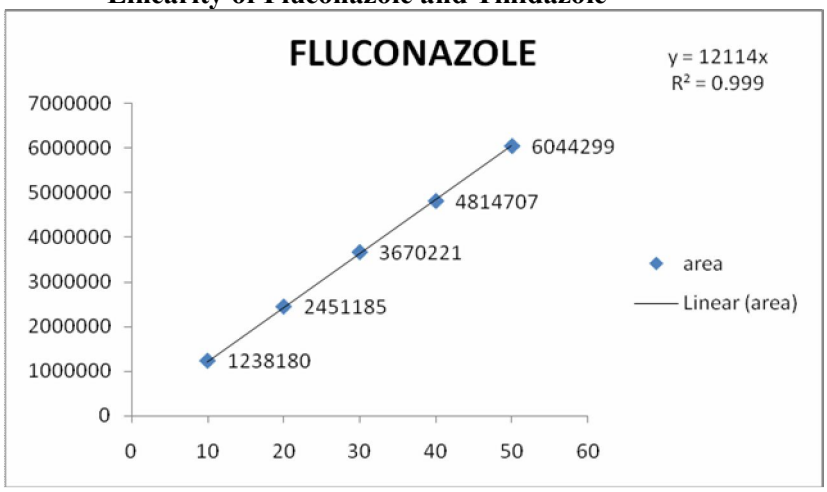
From this prepare 10, 20, 30, 40 and 50µg/ml of Fluconazole and Tinidazole concentrations.

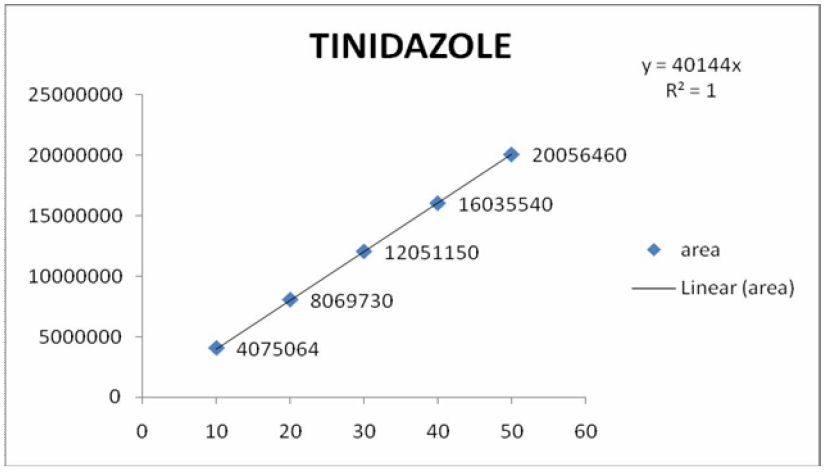
Procedure:
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results shown in Table-1.

Table1: Linearity

S.NO	CONCENTRATION µg/ml		AREA	
	FLU	TIN	FLU	TIN
1	10	10	1238180	4075064
2	20	20	2451185	8069730
3	30	30	3670221	12051150
4	40	40	4814707	16035540
5	50	50	5844259	20056460

Linearity of Fluconazole and Tinidazole





Parameters	Results observed Fluconazole	Results observed Tinidazole
Slope	12114	40144
Intercept	5599.1	21099
Correlation	0.999	1

Assay:

Assay of formulation available in the market (Flucoty tablets) was carried by injecting sample corresponding to equivalent weight into HPLC system. And percent purity was found out by following formulae. Calculate the percentage purity in tablet using the formula..

Assay % =

AT WS DT P Avg. Wt
----- x -----x ---- x ------x ---------- X 100
AS DS WT 100 Label Claim

AT = average area counts of sample preparation.
As= average area counts of standard preparation.
WS = Weight of working standard taken in mg.
P = Percentage purity of working standard
LC = label claim

Twenty tablets were weighed and powdered. A quantity equivalent to 100 mg of Tinidazole and 50 mg Fluconazole (by standard addition) were weighed and transferred to 100 ml volumetric flask and dissolved on about 100 ml of Mobile phase. The solution was ultrasonicated for 10 minutes and filtered through Whatmann filter paper No.41 and final volume made up to mark with same solvent. Appropriate dilutions were prepared from the above solution to 20µg/ml and 40µg/ml and the amount of drug was determined. The results were shown in Table 2**.**

Table2: Assay

Drug	Label claim (mg/tab)	Standard addition (mg)	Amount estimated (mg/tab)	%amount estimated	%RSD
Fluconazole	75	75+425	501.25 (75.18)	100.25	0.56
Tinidazole	1000	1000	999.5	99.5	1.20

Figure 1: Chromatograms of standard Fluconazole

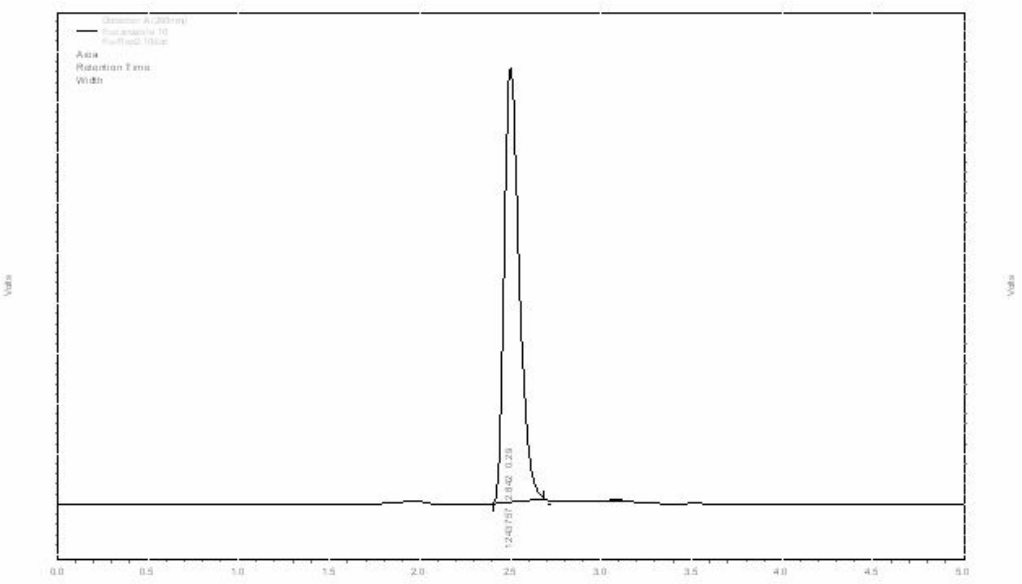


Figure 2 Chromatograms of standard Tinidazole

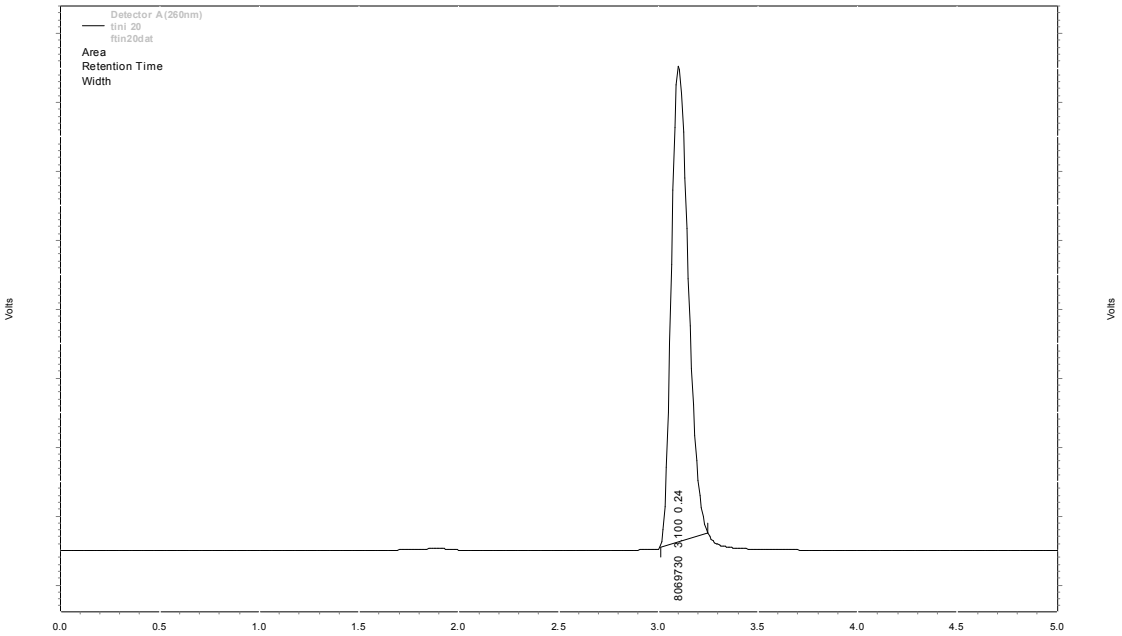


Figure 3: Chromatogram of sample of Fluconazole and Tinidazole

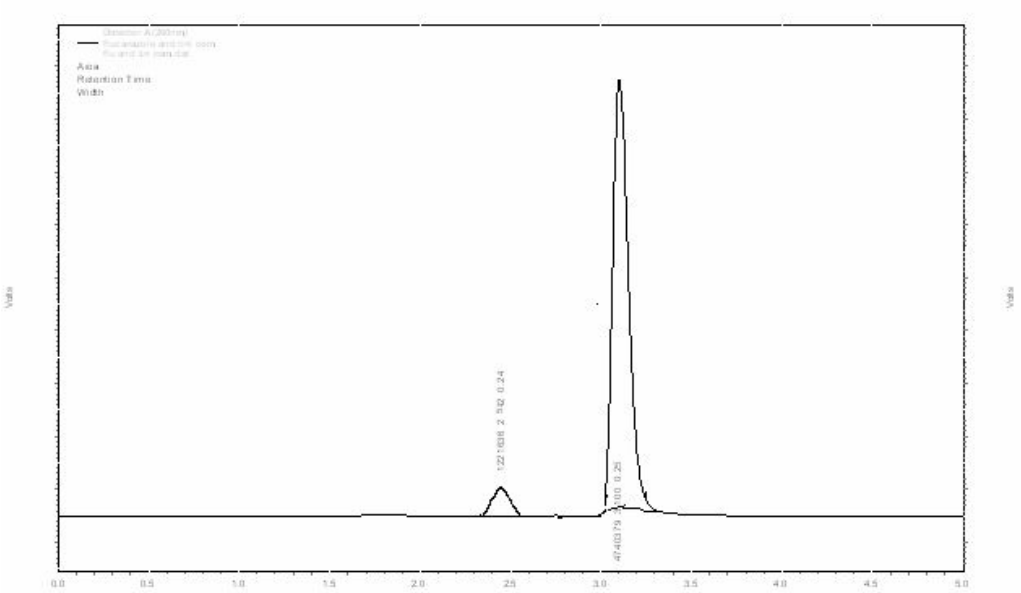


Figure 4: Chromatogram of mixed standards of Fluconazole and Tinidazole

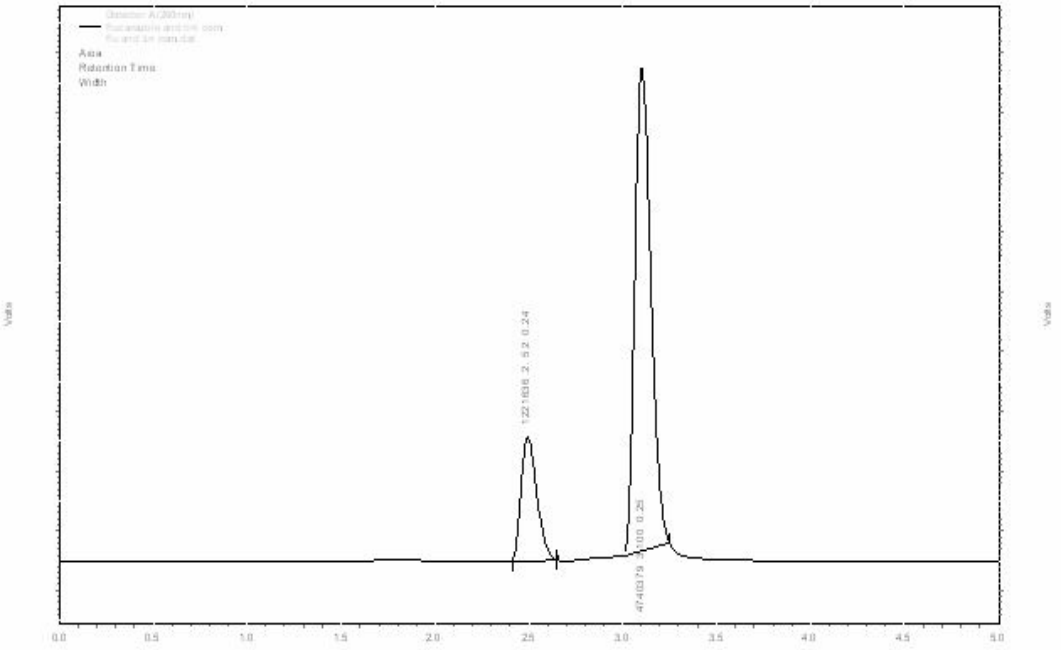


Table 3: Accuracy

Drug	Label claim (mg)	sample conc (µg/ml)	Amount added in (µg/ml)	Amount Recovered* in (µg/ml)	% Recovery*	Average recovery (%)	%RSD
FLU	75	40	20	20.05	102.5	100.64	1.91
			40	40	100.01		
			60	59.92	98.66		
TIN	1000	80	40	40.5	101.5	100.35	1.85
			80	80.05	100.05		
			120	120.1	100.1		

Accuracy as recovery

The recovery studies were carried out at 80, 100 and 120 % of the test concentration as per ICH guidelines. The results of the recovery studies and its statistical validation data are given in Table 3**.**

Acceptance criteria

The mean % recovery of the Fluconazole and Tinadazole at each level should be not less than 97.0% and not more than 103.0%.

Precision

Three samples were Prepared and analyzed as per the test method on 3 different days and calculated the % RSD for Assay of five preparations. Results are shown in Table-4.

Table4: precision

S.no	Concentration µg/ml	Intraday		Inter day	
		S.D	%RSD	S.D	%RSD
1	10	1734.81	0.63	1245.94	0.459
2	30	5666.9	0.70	1694.06	0.21
3	50	3064.8	0.23	2430.10	0.18

Limit of detection and limit of quantitation: The parameters LOD and LOQ were determined on the basis of Signal by Noise ratio.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 44 µV

Signal Obtained from LOD solution (0.9% of target assay concentration): 148 µV

S/N = 148/44 = 3.36

Limit of detection of Tinidazole

Signal Obtained from LOD solution (0.16% of target assay concentration): 141 µV

S/N = 141/44 = 3.20

Limit of Quantification:

Limit of Quantification of Fluconazole:

Calculation of S/N Ratio:

Signal Obtained from LOQ solution (0.29% of target assay concentration): 436µV

S/N = 436/44 = 9.90

Limit of Quantification of Tinidazole:

Signal Obtained from LOQ solution (0.49% of target assay concentration): 434µV

S/N = 434/44= 9.86

Table5 System Suitability Results of Fluconazole.

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	2071.2	1.3
2	1.0	2123.4	1.3
3	1.2	2142.7	1.3

Table6 System Suitability Results of Tinidazole.

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	4001.1	1.0
2	1.0	3935.2	1.0
3	1.2	4032.4	1.0

Table7 Results of system suitability parameters

Parameters	Data of Fluconazole	Data of Tinidazole
No. of Theoretical plates	2249.2	4678.5
Resolution	2.5	2.5
Retention Time	2.51	3.10

Table 8: Specificity parameters of Fluconazole and Tinidazole

Component	Observation
Diluent	No interference at RT of analyte peak.
Sample	No interference at RT of analyte peak.

Specificity parameters

The specificity of the method was predicted by preparing diluent, sample , and excipients as placebo sample and injected into the HPLC system. The results were calculated shown in table-8.

Results and Discussion

RP-HPLC Method

An effort has been made to develop a simple, specific and accurate method for the estimation Fluconazole and Tinidazole in bulk and formulation by using RP-HPLC.

The λ_{max} of Fluconazole and Tinidazole in mobile phase was found to be 260nm. The different combination of mobile phase was employed for the analysis.

Optimization of the method for the mobile phase of Acetonitrile: Water (55:45) was carried by changing the various flow rates.

From the above studies the mobile phase consisting of Acetonitrile: Water (55:45) and flow rate 1ml/min was selected from the system suitability parameters within the limit. The chromatogram of the standard was shown in fig-6.

Assay

The method developed is sensitive and specific for the quantitative determination of Fluconazole and Tinidazole and also subjected to validation for different parameters, hence has been applied for the estimation of drug in pharmaceutical dosage form. The amount of Fluconazole was found to be 100.5% and retention time was found to be 2.51min. The amount of Tinidazole was found to be 99.98% and retention time was found to be 3.1min.results are shown in table2, fig-3, and 4.

Method validation

The linearity was tested for the concentration range of 10-50µg/ml and the calibration curve was shown in table1, constructed was evaluated by correlation coefficient. The analyte response was plotted against its concentration and peak area.

The accuracy of the method was predicted by performing by recovery studies. The standard drug as added to the sample and the analysis method was carried out same as per assay. The results were calculated in terms of percentage of recovery, for Fluconazole and Tinidazole and it was shown in table3.

The intraday and inter-day variations of the method were determined using five replicate injections of three concentrations and analyzed on the same day and three different days over a period of two weeks. The result revealed the precision with %RSD (0.26% and 0.19%) respectively for intraday and inter day. Results were shown in table 4.

The limit of detection and limit of quantification were determined from the linearity studies. The limit of detection was found to be with in limits are mentioned.

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