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RESEARCH ARTICLE

Analytical Method Development and Validation for Simultaneous Estimation of Clotrimazole and Tinidazole by RP-HPLC

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ABSTRACT

An isocratic reverse phase High Performance Liquid Chromatographic (RP-HPLC) method was developed and validated for simultaneous determination of Clotrimazole and Tinidazole in combined pharmaceutical dosage form. The successful separation of Clotrimazole and Tinidazole was achieved using Purospher® C18 (250 mm x 4.6 mm i.d., 5 µm) column, with mobile phase consisting Phosphate buffer (pH 3.5): Acetonitrile (45:55). The mobile phase flow rate was 1.0 ml/min and the detection wavelength was 240 nm. The developed RP-HPLC method was validated according to ICH guidelines with respect to linearity, accuracy, precision, specificity and robustness and also the LOD and LOQ values were determined.

KEYWORDS

Clotrimazole, Tinidazole, RP-HPLC, Validation

INTRODUCTION

Clotrimazole (1-[(2-Chlorophenyl) (diphenyl) methyl] -1H-imidazole) is an antifungal agent used in local treatment of oropharyngeal candidiasis and vaginal yeast infections. Clotrimazole interacts with yeast 14-α demethylase, a cytochrome P-450 enzyme that converts lanosterol to ergosterol, an essential component of the membrane. In this way, Clotrimazole inhibits ergosterol synthesis, resulting in increased cellular permeability. Molecular formula of Clotrimazole is $C_{22}H_{17}ClN_2$and molecular weight is 344.8 g/mol (figure 1)[1,2,3].

Tinidazole (1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole) is an anti-amoebic drug used in the treatment of amebic liver abscess and intestinal amebiasis caused by *Entamoebahistolytica*, giardiasis caused by *Giardia duodenalis*. The nitro group of Tinidazole is reduced by a ferredoxin-mediated electron transport system. The free nitro radical generated as a result of this reduction is believed to be responsible for the antiprotozoal activity. It is suggested that the toxic free radicals covalently bind to DNA, causing DNA damage and leading to cell death. Molecular formula of Tinidazole is$C_8H_{13}N_3O_4S$ and Molecular mass is 247.27 g/mol (Figure 2)[4,5].

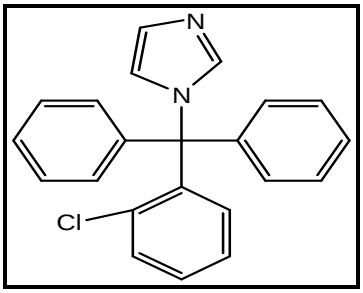

Figure 1: Structure of Clotrimazole

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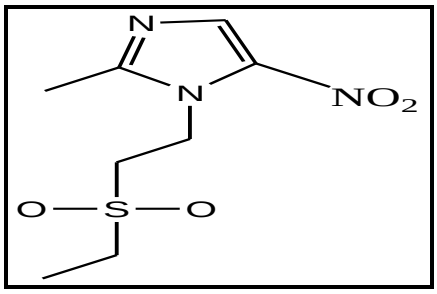

Figure 2: Structure of Tinidazole

Clotrimazole and Tinidazole are simultaneously utilized for gastrointestinal infections and available in tablet dosage form for this therapeutic purpose. Few analytical methods like simultaneous equation method and absorbance ratio methods by UV spectroscopy[6], High Performance Thin Layer Chromatography (HPTLC)[7] and RP-HPLC[8] methods are reported for simultaneous estimation of these two drugs. So the objective of the work is to develop a novel, simple, precise, accurate and validated RP-HPLC method for simultaneous estimation of these two drugs in tablet dosage form.

EXPERIMENTAL

Chemicals and Instrumentation

Clotrimazole API was obtained as gift sample from Amneal Pharmaceuticals, Ahmedabad and Tinidazole API was obtained as gift sample from Vaibhav laboratories, Ahmedabad. The tablets were procured from local pharmacy. Young Lin 9101 HPLC System [YL9110 Quaternary solvent delivery Pump], a YL9160 photodiode array (PDA) detector, a Rhenodyne autoinjector were used for the method development. Phosphate Buffer[9] was prepared by pharmacopeoial method, by using anhydrous disodium hydrogen phosphate and citric acid monohydrate, pH of solution was adjusted to 3.5 with orthophosphoric acid. Water and acetonitrile used were of HPLC grade.

Stock and Working Standard Solutions

Accurately weighed quantity of APIs, Clotrimazole and Tinidazole, 10 mg were transferred into separate 10 ml volumetric flask, dissolved and diluted up to mark with methanol (1000 µg/ml). From this, 100 µg/ml solution was prepared by withdrawing 1 ml of above solution into 10 ml volumetric flask and dilute it up to the mark with methanol.

Working standard solutions of Clotrimazole were prepared by transferring 0.2, 0.4, 0.6, 0.8, 1, 1.2 ml of stock solution to 10 ml volumetric flask. The volume was then adjusted with methanol to prepare a series of solutions ranging from 2-12µg/ml concentration of Clotrimazole. Working standard solutions of Tinidazole were prepared by transferring 0.5, 1, 1.5, 2, 2.5, 3 ml of stock solution to 10 ml volumetric flask. The volume was then adjusted with methanol to prepare a series of solutions ranging from 5-30 µg/ml concentration of Tinidazole.

Method Validation

After method development, validation of the chromatographic method was performed in accordance with ICH guidelines[10].

Linearity and Range

Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity. The linearity for Clotrimazole and Tinidazole were assessed by analysis of combined standard solution in range of 2-12µg/ml and 5-30 µg/ml respectively. The area at each concentration was determined and calibration curve was constructed by plotting peak areas versus concentrations, and the regression equation was calculated.

Accuracy

Accuracy of analytical method is the closeness of test results to the true value. Accuracy was determined over the range from 50 % as lowest sample concentration to 150 % as highest sample concentration. Triplicate preparations for each level were prepared and injected. The total amount found, Mean and % recovery were calculated.

Precision

The precision of the analytical method represents closeness of agreement between a series of

measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. Repeatability was checked by repeatedly (n = 6) injecting Clotrimazole solution (8 µg/mL) and Tinidazole (20 µg/mL) and recording the chromatogram. Intra-day and inter-day precisions of the method was determined by measuring the corresponding responses on the same day and on different days for 3 different concentration of Clotrimazole (6, 8 and 10 µg/mL) and Tinidazole (15, 20 and 25 µg/mL). The results were reported in terms of relative standard deviation.

Limit of Detection and Limit of Quantification

The linearity curve obtained in linearity study for Clotrimazole and Tinidazole were used to determine Standard deviation (SD) and slope. The following formulas were used to calculate LOD and LOQ.

LOD = 3.3 x (SD / Slope)

LOQ = 10 x (SD / Slope)

Robustness

Robustness study was carrying out by changing the minor parameters of the chromatographic conditions. The assay was performed by change in the flow rate by 10%, minor change in the ratio of mobile phase and minor change in pH by taking three replicates and % RSD was calculate.

Specificity

Specificity of an analytical method is its ability to measure the analyte accurately and specifically in presence of component that may be expected to be present in the sample matrix and through resolution factor of the drug peak from the nearest resolving peak and also among all other peaks. It was confirmed through peak purity data using a PDA detector.

System Suitability

System suitability parameters were calculated at the start of study of each validation parameter. The values of system suitability results obtained during the entire study are recorded. System Suitability values are from the first injection of six replicates of standard and are derived from YL clarity software.

Assay of Marketed Formulation

To determine the content of Clotrimazole and Tinidazole simultaneously in tablet, twenty tablets were accurately weighed and average weight was calculated. The tablets were then crushed to fine powder; an accurately weighed quantity of tablet powder equivalent to about 10 mg of Clotrimazole and 25 mg of Tinidazole was transferred to 10.0 ml volumetric flask and dissolved with shaking in methanol for 15 min. The volume was made up to the mark with methanol and the solution was mixed, filtered through 0.45 µm membrane filter and further diluted to get final concentration of 8 µg/ml of Clotrimazole and 20 µg/ml of Tinidazole.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The main objective of the chromatographic method development was to separate Clotrimazole and Tinidazole peaks. Several trials were carried out for accurate and precise method development, suitable column chemistry and good peak shape were obtained with Purospher® C18 (250 mm x 4.6 mm i.d., 5 µm) chromatographic column and Phosphate buffer (pH 3.5): Acetonitrile (45:55) as mobile phase.

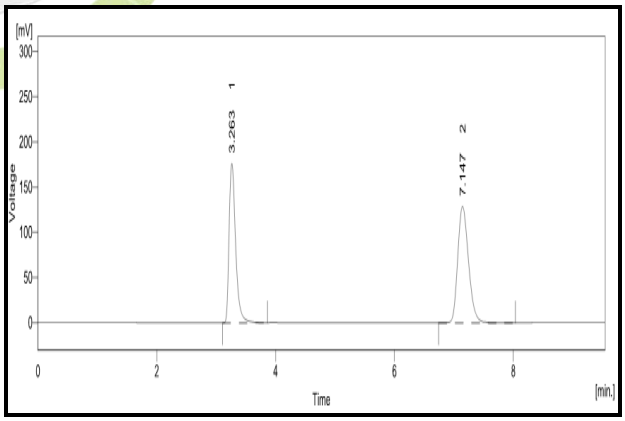

Figure 3: Chromatogram of Tinidazole (Rt- 3.26) and Clotrimazole (Rt-7.14) in Phosphate buffer (pH 3.5): Acetonitrile (45:55 v/v) (Flow rate-1.0 ml/min)

Detection was carried out at 240 nm wavelength, flow rate was 1.0 ml/min and column temperature was kept ambient. In optimized chromatographic conditions, Clotrimazole and

Tinidazole were separated with good resolution, typical retention time was 7.14 and 3.26 min respectively (figure 3). The system suitability results are given in Table 4 and the developed method was found to be specific.

Method Validation

Linearity and Range

Correlation co-efficient for calibration curve Clotrimazole and Tinidazole was found to be 0.997 and 0.999 respectively. The areas obtained are directly proportional to the concentration of analyte in the sample. The method is linear in the specified range (figure 4 and 5).

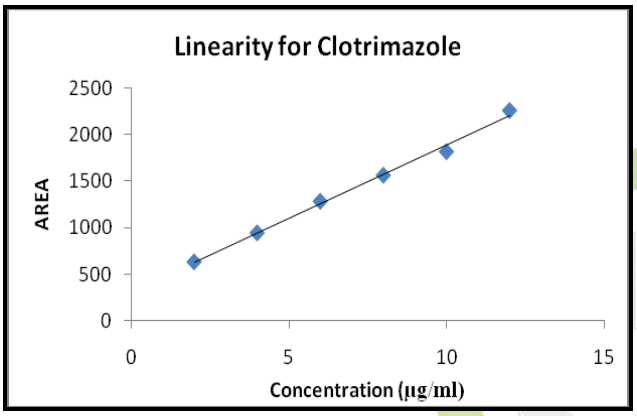

Figure4: Calibration Curve of Clotrimazole (2-12 µg/ml)

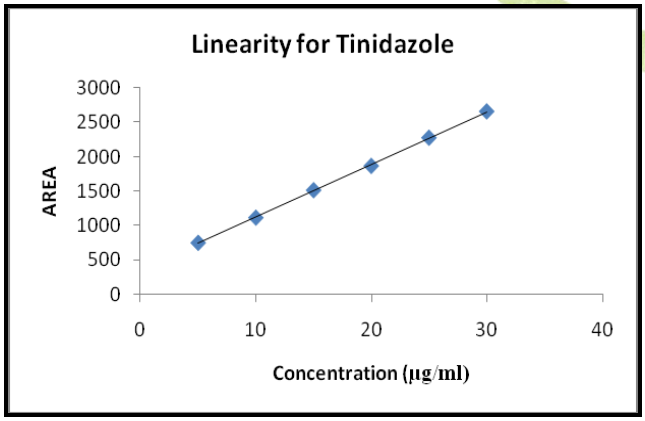

Figure 5: Calibration Curve of Tinidazole (5-30 µg/ml)

Accuracy

Percentage recovery for Clotrimazole was 98.68-99.84 %, while for Tinidazole it was found to be in range of 99.01-100.30 %. (Table 1)

Table 1: Accuracy results for Clotrimazole and Tinidazole

Drug	Concentration level	% Mean Recovery ± S.D
Clotrimazole	50 %	98.68±0.73
	100 %	99.84±1.09
	150 %	99.76±0.64
Tinidazole	50 %	99.01±0.45
	100 %	100.30±0.68
	150 %	99.45±0.36

Precision

In repeatability study, the % RSD for Clotrimazole and Tinidazole was found to be 1.143 and 1.053 respectively. Intraday and interday precision also exhibited % RSD less than 2 (Table 2). So the developed method is precise for its use.

Limit of Detection and Limit of Quantification

Limit of Detection for Clotrimazole and Tinidazole was found 0.58 µg/ml and 0.56 µg/ml respectively. Limit of Quantitation for Clotrimazole and Tinidazole was found 1.76 µg/ml and 1.72 µg/ml respectively.

Robustness

With the minor modifications in the chromatographic conditions i.e. Flow rate, ratio of mobile phase and pH of mobile phase, the % assay was not affected and % RSD was found to be less than 2 % (Table 3).

Specificity

The developed analytical method was found to be specific as the peak purity index for Clotrimazole and Tinidazole were 999.31 and 999.11 respectively in their tablet dosage form.

Table 2: Precision data of Clotrimazole and Tinidazole

Precision	Clotrimazole			Tinidazole		
	Conc. (µg/ml)	Mean Area	% RSD	Conc. (µg/ml)	Mean Area	% RSD
Repeatability	8	1560.014	1.143	20	1866.916	1.053
Intraday precision	6	1283.292	1.115	15	1526.342	0.593
	8	1557.902	0.492	20	1881.961	0.665
	10	1818.269	0.470	25	2277.328	0.645
Interday precision	6	1284.487	1.048	15	1514.095	0.800
	8	1564.642	0.392	20	1882.369	0.649
	10	1814.757	0.579	25	2275.295	0.476

Table 3: Robustness study for Clotrimazole and Tinidazole

Parameter	Variation	Clotrimazole		Tinidazole	
		% Assay ± SD (n=3)	% RSD	% Assay ± SD (n=3)	% RSD
Standard	-	98.88±0.51	0.524	99.56±0.18	0.185
Flow rate	+ 0.1 ml	99.14±0.72	0.732	99.23±0.61	0.620
	- 0.1 ml	98.90±0.10	0.109	98.80±0.21	0.216
Mobile phase (% v/v)	Phosphate buffer (pH 3.5):Acetonitrile (43:57)	98.73±0.15	0.153	98.85±0.21	0.217
	Phosphate buffer (pH 3.5):Acetonitrile (47:53)	98.41±0.16	0.170	99.02±0.30	0.308
pH	3.3	98.71±0.05	0.056	98.99±0.14	0.149
	3.7	98.95±0.24	0.250	99.17±0.28	0.284

System Suitability Test

Table 4: Results for system suitability test

Parameters	Data observed	
	Clotrimazole	Tinidazole
Theoretical plates per column	7316	4335
Retention time	7.14	3.16
Symmetry factor/Tailing factor	1.680	1.370
Resolution	14.586	

Assay of Marketed Formulation

Table 5: Assay of marketed formulation

Drugs (Tablets)	Labeled claim (mg)	Amount found (mg) ± S.D. (n=3)	% Label Claim ± S.D. (n=3)
Clotrima-zole	200	196.46±1.77	98.23±0.89
Tinida-zole	500	494.95±3.10	98.99±0.62

CONCLUSION

A novel and simple RP-HPLC method has been developed and validated for the estimation of Clotrimazole in combination with Tinidazole from tablet dosage form. All validation parameters are well within the acceptable criteria. The method is found to be specific as the peak purity index shows that peak is pure. The proposed method is found to be simple, accurate, precise, sensitive and robust. Hence, it can be used successfully for the routine analysis of Clotrimazole and Tinidazole in tablet dosage forms.

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