Simple HPLC Method for Simultaneous Estimation of Fluconazole and Tinidazole in Combined Dose Tablet

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Abstract

A rapid and sensitive reversed-phase high-performance liquid chromatographic method is developed for simultaneous estimation of fluconazole, an orally active triazole anti-fungal agent, and tinidazole, which belongs to the group of 5-nitroimidazoles in combined dose tablet. Chromatographic separation was on an ODS Hypersil C_{18} column using 0.05 M potassium dihydrogen phosphate buffer (pH 3.25, adjusted with orthophosphoric acid) and acetonitrile (82:18, v/v) as the mobile phase at a flow rate of 1.5 mL/min with detection at 210 nm. The asymmetry factors are 1.36 ± 0.04 for fluconazole and 1.26 ± 0.07 for tinidazole with a total run time of less than 7 min. The calibration curves were linear in the range 6–14 µg/mL for fluconazole and 80–190 µg/mL for tinidazole. The method was validated with respect to linearity, precision, accuracy, and specificity. The mean recovery for fluconazole and tinidazole is 99.65 ± 0.84 and 99.34 ± 0.70, respectively. The utility of the procedure is verified by its application to the market formulation that was subjected to various stressed conditions. Two potential degradation products of tinidazole on exposure to alkaline stressed condition are well-resolved. The method separated the two target drugs and degradation products well. No chromatographic interference is observed.

Introduction

Fluconazole (FLZ, α-(2, 4-Diflurophenyl)-α-(1*H*-1,2,4-triazol-1-yl-methyl)-1*H*-1,2,4-trizol-1-ethanol) (Figure 1A) is a broad spectrum antifungal agent and recommended for the treatment and prophylaxis of disseminated and deep organ candidiasis.

Tinidazole (TNZ, 1-[2-(ethylsulphonyl) ethyl]-2-methyl-5-nitroimidazole) (Figure 1B) belongs to the group of 5-nitroimidazoles, which are used in the chemotherapy of infectious diseases such as amoebiasis, giardiasis, and trichomonasis and against anaerobic bacteria. 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.

Literature survey has revealed various methods for estimation of FLZ in biological fluids and in pharmaceutical formulations, such as IR spectroscopic (1), UV spectrophotometric (2–6), and microbiological methods (7).

Similarly, literature survey has revealed various colorimetric and spectrophotometric methods for the estimation of TNZ. High-performance liquid chromatography (HPLC) (8–9) methods reported in literature have shown similar degradation pattern in basic conditions. Though literature reports various methods for simultaneous estimation of TNZ with other drugs, there currently is no method cited for simultaneous estimation of TNZ and FLZ from tablet dosage forms.

The present paper describes simultaneous estimation of TNZ and FLZ from tablet dosage form by HPLC. The HPLC method developed is rapid and sensitive and allows simultaneous determination of the target analytes in the presence of degradation products of TNZ.

Experimental

Materials and reagents

FLZ was obtained as a gift sample from Zim Labs (Nagpur, India), and TNZ was kindly supplied by Suven (Chennai, India). HPLC-grade methanol and acetonitrile was purchased from Merck (Mumbai, India). All other chemicals and solvent were of analytical-grade and purchased locally. Flucoti tablet (labeled claim: TNZ 1000 mg and FLZ 75 mg) manufactured by Fourrts Pvt. Ltd. (Chennai, India) were purchased from a local pharmacy.

Instrumentation and operating parameters

HPLC determinations were performed with a Shimadzu (Kyoto, Japan) model 10 ATVP LC system consisting of in-built degasser unit, an SPD 10A UV detector, a column oven, and a Rheodyne 7725i manual injector with final volume loop of 20 µL. The experimental parameters of the HPLC system, including monitoring wavelength, flow rate, concentration of the mobile phase, and column temperature, were directly controlled by the control panel of each instrument unit. The separation was per-

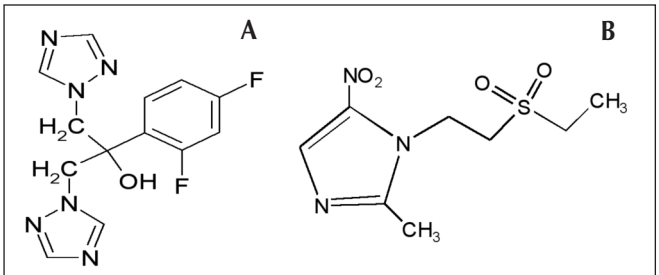

Figure 1. Structure of FLZ and TNZ.

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formed on ODS Hypersil C_{18} column (Thermo Scientific, 4.6 × 250 mm, particle size 5 µm). The mobile phase was a mixture of 0.05 M potassium dihydrogen phosphate buffer (pH 3.25, adjusted with orthophosphoric acid) and acetonitrile (82:18, v/v). The flow rate was set at 1.5 mL/min. The column effluent was monitored at 210 nm with UV detection. The column temperature was regulated at 40°C.

Preparation of standards and system suitability

Concentrated stock solution of each drug and mixed stock standard solution (containing FLZ 100 µg/mL and TNZ 1.33 mg/mL) were prepared separately in methanol and stored at 4°C. Mixed stock standard solution was further diluted to 10.0 mL with mobile phase to study system suitability parameters.

Sample preparation

Twenty tablets were accurately weighed, and the average weight was calculated. The tablets were then crushed to fine powder. An accurately weighed quantity of tablet powder equivalent to about 67 mg of TNZ was shaken with 25 mL methanol for 15 min, and the volume was made up 50.0 mL with methanol. The solution was mixed and filtered, and 1.0 mL of the filtrate was diluted to 10.0 mL with mobile phase.

Validation of the developed method

The proposed method was validated in terms of linearity, accuracy, precision, and specificity. Accurately measured volume (12.5 mL) of mixed stock standard solution was diluted to 25.0 mL with mobile phase. Aliquot portions of the resulting solution (1.2, 1.6, 2.0, 2.4, and 2.8 mL) were further diluted to 10.0 mL with mobile phase (concentrations 6–14 µg/mL FLZ and 80–190 µg/mL TNZ). Peak areas were recorded for each injected concentration of drugs and calibration curves, concentration vs. peak areas were constructed for both the drugs. Concentration vs. peak areas were constructed for both the drugs. Repeatability studies were performed by five time injections of the two drugs on the same day. The studies were also repeated on different days to determine intermediate precision.

Accuracy of the method was evaluated by spiking the standard drugs at different concentrations in preanalyzed tablet powder. Accurately weighed quantities of preanalyzed tablet powder equivalent to 46 mg of TNZ (equivalent to about 70% of the prescribed sample weight as per the proposed method) were transferred to three different 50.0-mL volumetric flasks; and 1, 3, and 5 mL of mixed standard solution (concentrations 0.50 mg/mL FLZ and 6.67 mg/mL TNZ) were added to them followed by methanol. The flasks were shaken for 30 min, and the volumes were made up to the mark with methanol. The drug content in different flasks represent 80–120% of labeled claims with constant amount of excipients. The solutions were mixed and filtered, and 1.0 mL of each of the clear filtrate was diluted to 10.0 mL with mobile phase.

Specificity of the method towards the drugs was established by attempting deliberate degradation of the two drugs with exposure to stress conditions like acidic (0.1 M HCl), alkali (0.1 M NaOH), oxidizing agent (3% H_2O_2), heat (60°C), and UV rays. After 24 h the samples were diluted with methanol, and they were analyzed by proposed methods.

Applicability of the developed method to marketed formulation

The developed method was applied for the estimation of two drugs in tablet formulations.

Result and Discussion

Method development and optimization

The purpose of this study was to investigate the feasibility of the HPLC method for the determination of the antifungal agent FLZ and TNZ in the presence of two degradation products of TNZ. Method development was concentrated on the sensitivity and resolution of two degradation products of TNZ from FLZ and TNZ itself. In order to affect proper resolution of all the components, the mixture of methanol or acetonitrile with water or different buffers in different combinations were assayed as the mobile phase using ODS Hypersil C_{18} column as the stationary phase. Binary mixture of 0.05 M potassium dihydrogen phosphate buffer–acetonitrile (82:18, v/v) (pH 3.25, adjusted with orthophosphoric acid) proved to be better for the separation because the chromatograms were well-defined, resolved, and almost free of tailing. Among several flow rates tested (0.8–2.0 mL/min), the rate of 1.5 mL/min was the best with respect to location and resolution of analytical peaks. A 210 nm wavelength was selected as it gave reasonably high absorption for both the drugs when used in proportions usually found in marketed formulation. Using a UV detector at 210 nm, the previously described chromatographic conditions allow a resolution between TNZ and FLZ with a reasonable time of 4.30 and 5.38 min, respectively (Figure 2). Similarly sample solution chromatographed under optimized conditions exhibited similar retention pattern as observed in chromatograms obtained from standard solution, thus indicating the specificity of the method.

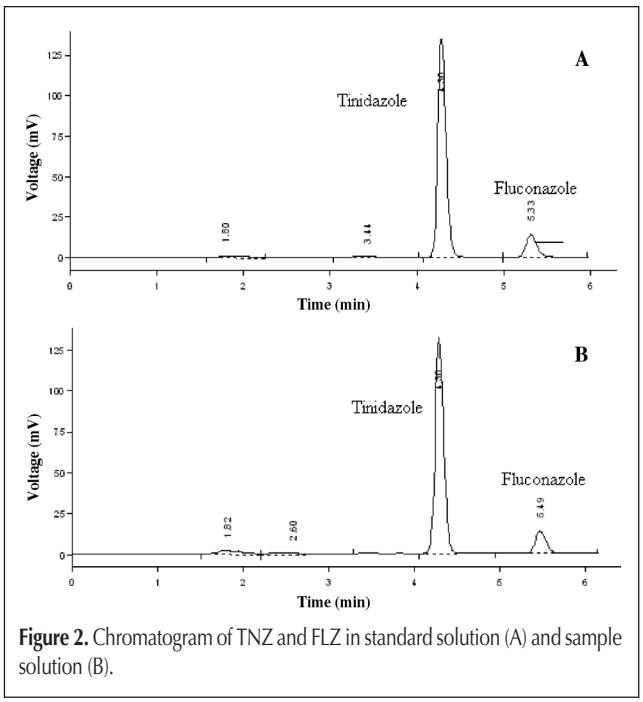

Figure 2. Chromatogram of TNZ and FLZ in standard solution (A) and sample solution (B).

Under these conditions separation of the two degradation products of TNZ from FLZ and TNZ lasted no longer than 6 min, which is better than compared to the two reported methods (8–9).

A system suitability test was applied to a representative chromatogram of freshly prepared standard stock solution of TNZ and FLZ to check various parameters, such as resolution, capacity factors, and asymmetry. The results of system suitability parameters are given in Table I. The results are in concurrence with U.S. Pharmacopeia requirements.

The peak area of TNZ and FLZ exhibited linear relationship with their concentrations. The characteristics of linear regression analysis are given in Table II. The limit of detection (LOD) and limit of quantitation (LOQ) of the method are also shown in Table II, which were calculated according to the 3 *s/m* and 10 *s/m* criterions, respectively, where *s* is the standard deviation of peak areas (four injections) and *m* is the slope of the corresponding calibration curve (10).

The intra- and inter-day variations of the method were determined using three replicate injections at three different concen-

Table I. System Suitability Parameters

Parameter	Results	
	TNZ	FLZ
Retention Time	4.30 ± 0.18	5.38 ± 0.14
Resolution	–	4.66 ± 0.60
Capacity Factor	3.30 ± 0.02	4.33 ± 0.16
Asymmetry	1.26 ± 0.07	1.36 ± 0.04
Theoretical plates (per meter)	71199 ± 123.55	80371 ± 47.44
Repeatability (%RSD) (*n* = 6)	0.99	0.90

Table II. Characteristics of the Linear Regression Analysis of Tinidazole and Fluconazole

Parameter	Results	
	TNZ	FLZ
Linearity range (µg/mL)	79.98–186.62	6–14
Slope	6.21	11.31
Intercept	52.10	6.69
Correlation coefficient	0.9926	0.9944
RSD of slope	1.07	1.18
RSD of intercept	0.26	0.41
LOD (µg/mL)	4.39	0.30
LOQ (µg/mL)	14.64	0.99

Table III. Intra-day and Inter-day Precision of TNZ and FLZ Standard Solutions

Comp.	Theoretical conc. (µg/mL)	Intra-day measured* conc.		Inter-day measured† conc.	
		Mean	%RSD	Mean	%RSD
TNZ	106.64	106.32	0.39	106.12	0.62
	133.3	132.95	0.34	132.63	0.52
	159.96	159.26	0.41	158.95	0.78
FLZ	8	7.89	0.47	7.76	0.57
	10	9.86	0.43	9.81	0.68
	12	11.92	0.42	11.82	0.82

* Mean value represents three different sample standards for each concentration at µg/mL.
† Inter-day reproducibility was determined from three different runs for each concentration over a twp-week period.

Table IV. Recovery Studies of TNZ and FLZ (*n* = 3)

Comp.	Amount of standard drug added (mg)	% level of Drug added	Amount of standard drug recovered, RSD (%)	Rec. (%)
TNZ	6.67	80	6.63, 1.03	99.35
	20.01	100	19.70, 0.91	98.47
	33.35	120	33.11, 0.76	99.29
FLZ	0.50	80	0.502, 1.4	100.55
	1.5	100	1.509, 1.06	100.58
	2.5	120	2.477, 0.77	99.06

Table V. Results of Specificity Studies

Sr. No.	Sample	Percent label claim	
		TNZ	FLZ
1.	Normal ± SD	99.34 ± 0.33	100.82 ± 0.92
2.	Alkali	23.16	98.81
3.	Acid	98.49	98.88
4.	UV chamber	99.96	98.71
5.	Oxide	98.02	99.20
6.	Heat	100.22	100.13

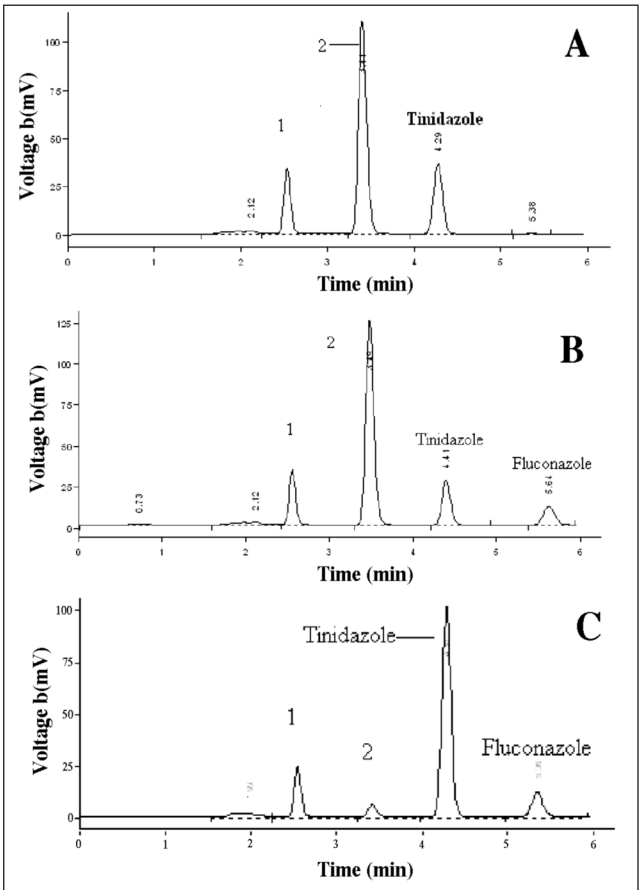

Figure 3. (A) Chromatogram of standard TNZ exposed to alkaline stress for 24 h at 50°C; (B) Chromatogram of TNZ and FLZ (sample) exposed to alkaline stress for 24 h at 50°C; (C) Chromatogram of TNZ and FLZ (sample) exposed to alkaline stress for 24 h at room temperature.

trations, which were prepared and analyzed on the same day and on three different days over a period of two weeks, respectively (Table III). These data indicate a considerable degree of precision and reproducibility of the method during one analytical run and different runs. Sample solutions analyzed after 48 h did not show any appreciable change in the assay value.

As shown from the data in Table IV, good recovery of the two drugs in the range of 98.47–100.55% at various added concentrations indicates accuracy of the method.

The results of specificity studies have shown that the estimated content of drugs under different stress conditions were different in certain cases as compared to normal samples. Results of specificity studies are summarized in Table V. In the case of the alkali-exposed sample, TNZ content was found to be much lower compared to normal. The chromatogram showed two extra peaks: one at 2.56 min and other at 3.49 min. In order to ascertain which of the drugs has undergone degradation, pure drugs (TNZ and FLZ) were exposed to alkaline conditions similar to that of tablet powder. It was observed that chromatogram of TNZ depicted similar degradation pattern as observed in case of alkali-exposed tablet powder. The chromatograms for alkaline stress condition of TNZ pure drug and sample are shown in Figure 3A and 3B. Further, it was observed that degradation of TNZ also depends on temperature. When tablet powder was exposed to similar alkaline conditions at room temperature for 24 h, it was observed that the degradation of TNZ was less. Further the amount of degradation product no. 1 obtained was similar to that at 50ºC, but the amount of degradation product no. 2 obtained at 50ºC was 90% more than that obtained at room temperature (Figure 3C). Thus, it can be concluded that degradation of TNZ to degradation product no. 2 is temperature-dependent and that degradation of TNZ to degradation product no. 1 is not temperature-dependent. The degradation pattern is comparable to that mentioned in reference no. 8 and 9 on the basis of number of degradation products formed. The present method enables determination of both the target analytes in presence of degradation products of TNZ. The method described in reference 8 and 9 helps determination of TNZ in formulations containing only TNZ because simultaneous determination has not been carried out in that work. The present method hence serves the purpose of simultaneous determination of FLZ and TNZ in the presence of degradation products of TNZ.

However, FLZ was found to be stable under alkaline stress condition applied in this method. In the case of acid, UV, oxide, and heat exposed samples, the estimated contents of both the drugs were similar to that of normal samples, which indicate their stability against the said conditions. The results of specificity studies indicate that both the drugs can be specifically estimated in the presence of the degradation products and that the mobile phase is able to resolve the target analyte from degradation products.

Analyses of the tablet sample using the developed method showed that there was no chromatographic interference from the common excipients used in the tablet formulation. The assay values for determination of FLZ and TNZ were 100.82% and 99.34%, respectively. The percent relative standard deviation (%RSD) values were 0.91 and 0.33 for FLZ and TNZ, respectively.

Conclusion

Thus, it can be concluded that the proposed method is sufficiently sensitive, reproducible, and specific in the analysis of TNZ and FLZ in combined dose tablet within a short analysis time (< 7 min). The proposed HPLC method was validated by evaluation of the validation parameters. The LOD, LOQ values, relative standard deviation of slope and intercept, correlation coefficient, inter- and intra-day reproducibility, resolution, and capacity factors for this technique were obtained. Assay parameters used in this study showed better resolution of the target analyte with proper symmetry. The method is also able to separate the degradation products from the target analyte and thus allow estimation in their presence.

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