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Simultaneous Determination and Method Validation of Fluconazole and its impurities by High Performance Thin Layer Chromatography Using Reflectance Scanning Densitometry

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Abstract

A selective method based on HPTLC using reflectance scanning densitometry was developed and validated for the simultaneous separation and quantification of Fluconazole in formulations and its structurally related impurities. The chromatographic separation was accomplished on TLC aluminum plates precoated with silica gel 60 F_{254} as the stationary phase using a saturated mixture of butanol: water: acetic acid (8:2:1 v/v). For visualisation, the plate was dipped in to a modified anisaldehyde reagent and heated at 120°C for 30 minutes in a drying oven. Densitometric quantification was performed at 254 nm by reflectance scanning. Fluconazole appeared as a brown spot (Rf 0.67±0.02) and resolved well from the two impurities. The standard fluconazole curve is linear (r =>0.9995) over a concentration range of 100-800ng/spot. Recovery from tablet formulation was statistically equal to 100% .The limits of detection and quantification were 91.39 and 304.66 ng/spot respectively. The precision of the method with respect to concentration is acceptable with a relative standard deviation of 0.73%. The proposed method is specific for fluconazole in the presence of its structurally related impurities and proved to be a valuable complimentary method for impurity profiling and quality control.

Keywords:

Densitometry, Fluconazole, Impurities, HPTLC, Method validation

1. Introduction

Fluconazole is a widely used bis-triazole antifungal agent [1-3]. It is used in the treatment and prevention of superficial and systemic fungal infections [4-7]. Like other imidazole and triazole-class antifungals, fluconazole affects the conversion of lanosterol into ergosterol by inhibiting cyctochrome p450 sterol 14α-dimethylase [8-9]. Inhibition of ergosterol synthesis leads to the disruption of fungal membranes, accumulation of phospholipids within the cell and ultimately cell death [10-11]. It is chemically described as 2-(2-4-dofluorophenyl)-1, 3-di (1H-1, 2, 4-Triazol-1-yl) propan-2-ol.

Samples of fluconazole may contain some structurally related impurities (Fig-1)

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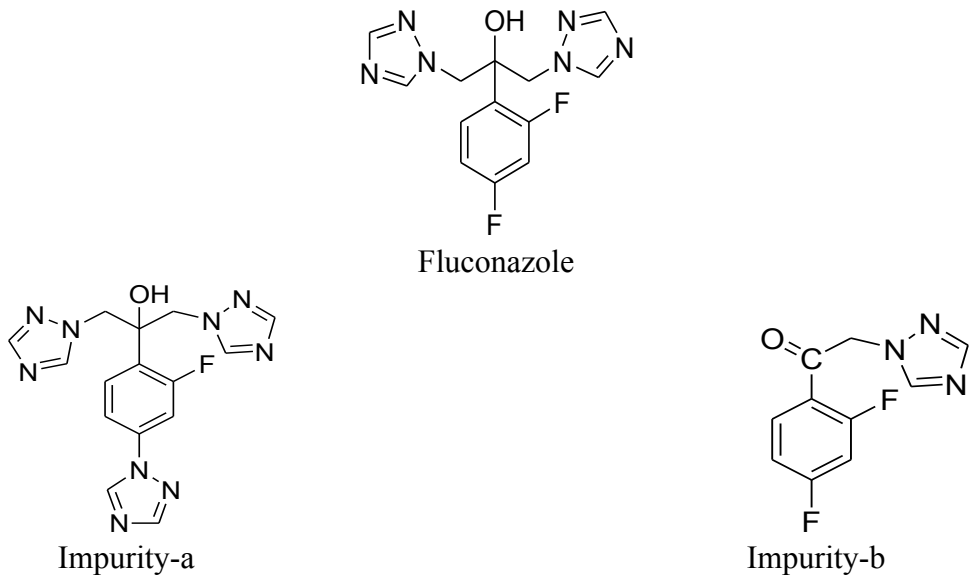

Figure 1. Structures of Fluconazole and its impurities.

derived from the manufacturing process, such as 2-(2-fluoro-4- (1H-1, 2,4-triazol-1-yl) phenyl)-1,3-di (1H-1, 2,4-triazol-1-yl) propan-2-ol (Impurity-a), 1-(2,4-difluorophenyl)-2-(1H-1, 2,4- Triazol-1-yl) ethanone (Impurity-b). An impurity is a component of a drug product, which is not the active drug substance but an excipient in the drug product, which should not be present beyond certain threshold limits as defined by ICH guidelines [12-13]. Therefore, were is exercised from initial stages of the development of a potential bulk drug to the quality control of a marketed pharmaceutical product to fulfill the specified requirements of regulatory agencies with respect to toxicity and safety aspects.

The literature survey reveals that various analytical methods have been reported for the analysis of fluconazole in pharmaceutical formulations. [14-17] However, to our knowledge no HPTLC method for the simultaneous separation of fluconazole from its structurally related impurities has been reported. HPTLC has a potential which meets the demands of a routine analytical technique due to its advantages of low operating cost, high sample throughput and need for minimum sample clean up. The major advantage is that, several samples can be run simultaneously using a small quantity of mobile phase, unlike HPLC, thus lowering the analytical run times and cost per analysis. In pharmaceutical laboratories, there is always a need for faster, simpler, cheaper and better performing analytical methods. Further, TLC and HPTLC in instrumentalized mode using scanning densitometry have been included as general methods in European pharmacopoeia, permitting the use of planar chromatography for quantification at different stages of pharmaceutical research, development and production. [18] Further, one of the main advantages of planar chromatography is its ability to facilitate separations which can be successfully utilized to evaluate very different drug molecules, their impurities and the metabolites in one run. Generally, the separations were discrete and very often complementary to other classified techniques such as HPLC and GLC. Therefore, HPTLC can be a viable alternative for impurity profiling, characterization of newer drugs and the unknown compounds.

Although, many reports were available for the determination of fluconazole, the impurities of this antifungal drug have not been analysed, separated and quantified till date. Hence, the objective of the present study is to develop a new HPTLC method for

simultaneous separation and identification of fluconazole and the impurities in bulk drug. The optimization of the method development, separation, evaluation and quantification of the process components of fluconazole in bulk drug and advantages of the HPTLC approach were studied. Here in, we describe the details of our investigative study and the potential utility of the method we have developed.

2. Experimental

2.1 Materials

HPTLC system (Camag, Muttenz, Switzerland) consists of Spectrodensitometer (Scanner 3), equipped with the software (winCATS) with band application device: Linomat 5, twin trough chromatographic chambers and HPTLC plates precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany).

2.2 Chemicals and reagents

Synergenic Active Ingredient Ltd, Hyderabad, India, gifted Fluconazole and its related impurities. All chemicals and reagents used were of analytical grade and were purchased from Merck.

2.3 Standard Solutions

Standard solutions of fluconazole and related impurities were prepared by dissolving each of the compounds in methanol to obtain a concentration of 1mg mL^{-1}. Now, 1 mL of the above solution was further dissolved in 10 mL of methanol and before analysis, the required concentrations of fluconazole (100-800 ng $spot^{-1}$) and related impurities (100-800 ng $spot^{-1}$) were prepared. The standard stock solutions of all the compounds were stored at 4°C until further analysis.

2.4 Chromatography

Chromatography was performed on 10cm×10cm HPTLC plates coated with 0.25 layer of silica gel 60 F_{254} (Merck, Darmstadt, Germany). Before use, the plates were washed with methanol and activated at 110°C for 5 min. Samples were applied as bands of 6mm wide and 10mm apart using Linomat 5 sample applicator equipped with a 100µl syringe. A constant application of 6µl s^{-1} was used. The mobile phase employed was butanol: water: acetic acid (8:2:1, v/v) and 11 mL of mobile phase was used for chromatography. Linear ascending development was performed in a Camag 10cm ×10cm glass twin-trough chamber. Before placing the plate, the chamber was saturated with mobile phase vapor for 20 min at room temperature (25±2°C) and relative humidity 60±5% by lining the TLC chamber on three sides with filter paper, also placed in the mobile phase. The development distance was 9cm and after development, the TLC plates were dried in a current of air by means of a hot air blower in a wooden chamber with adequate ventilation. Densitometric scanning was performed with a Camag TLC scanner 3 in reflectance/ absorbance mode at λmax 254nm controlled by winCATS software resident in the system. The slit dimensions were 5 × 0.45 mm and the scanning speed was 20 mm s^{-1}. The radiation source was a deuterium lamp emitting continuous UV radiation between 190 and 400nm. Concentration of the compounds chromatographed was determined from the intensity of diffusely reflected light and evaluation was carried out via peak areas.

The method was validated according to the ICH guidelines on the validation of analytical methods [19, 20]. All results were expressed as percentages, where 'n' represents the number of values. For the statistical analysis windows 2003 (Microsoft Office) was used.

3. Results and Discussion

Analysis is a measurement science, which involves the study of various parameters that contribute directly for the evaluation of molecules with respect to their structure, polarity, stability and ability to represent unique individual characteristic features to distinguish them from each other with the help of a suitable analytical instrumentation. With stringent international quality guidelines for globalization, the task is more challenging because every analytical parameter that is involved in the process of method development and validation is very well defined. Therefore, authenticity of the generated data that directly speaks of system suitability for chromatographic evaluation of any molecule under analysis is very important. Therefore, on the part of analyst, it is important to ensure that the generated data is accurate and the method adopted is suitable in terms of accuracy, precision and selectivity. Hence, the present method also has been validated according to ICH Guidelines.

3.1 Selection of the optimum mobile phase

Since there is no literature report of an HPTLC method for the separation of fluconazole and its impurities, the selection of mobile phase was carried out with different solvent systems on the basis of polarity. A solvent system that would give dense and compact spots with appropriate and significantly different R_f values for fluconazole and its impurities was desired. The mixture (fluconazole + impurities) was spotted on TLC plates and run in different systems. Among these, the solvent system butanol: water: acetic acid solution (8:2:1 v/v) offered compact spots for fluconazole from its impurity-b, which is clearly visible under UV light, but Impurity–a is inactive under UV light.

3.2 Identification of Impurity –A

Although, the above mobile phase facilitated very satisfactory separation of the main drug from its impurities, the impurity –a is characteristically not UV active and has not shown any spot under UV light but vividly visible when sprayed with anisaldehyde solution (6%). Encouraged by this observation, the plate after spraying was dried and and densitometrically scanned where by the quantitation of impurity-a was accomplished. The exercise was repeated several times to check the reproducibility. The video image of the same facilitated the complete picture of the separation (Fig-2).

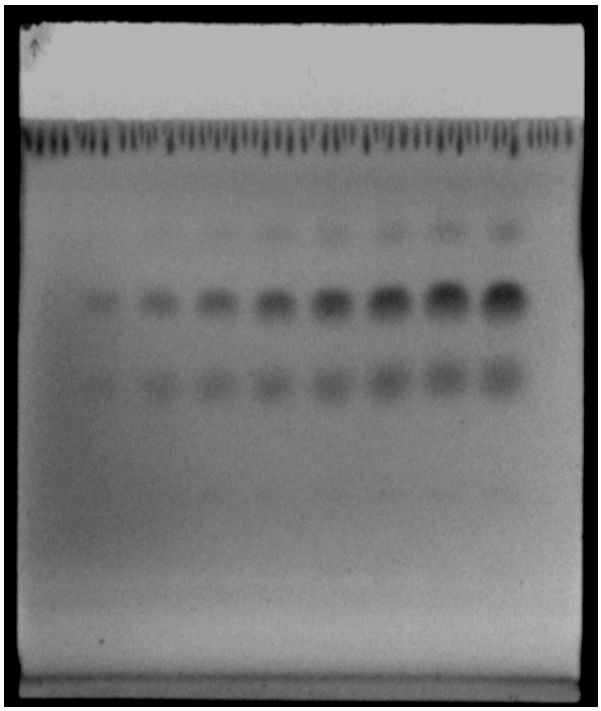

Fig. 2. Video image showing separation of Fluconazole and its impurities at higher concentrations.

The results were compared with quantitative conventional UV spectroscopy and the authenticity of the adopted procedure was confirmed.

It is evident that butanol: water: acetic acid (8:2:1 v/v) facilitated a sharp and well resolved peaks for Fluconazole (0.67±0.02), impurity-a (0.49±0.02) and impurity-b (0.79±0.02) respectively. R_f values and wavelengths of absorption maxima ($\lambda_{max)}$ were shown in Table 1. Scanning profiles of chromatographic separation were shown in Fig- 3 and 4.

Table 1. R_f and λ max values of Fluconazole and impurities

Substance	Rf	λ max
Impurity- a	0.49±0.02	295nm
Fluconazole	0.67±0.02	296nm
Impurity-b	0.79±0.02	294nm

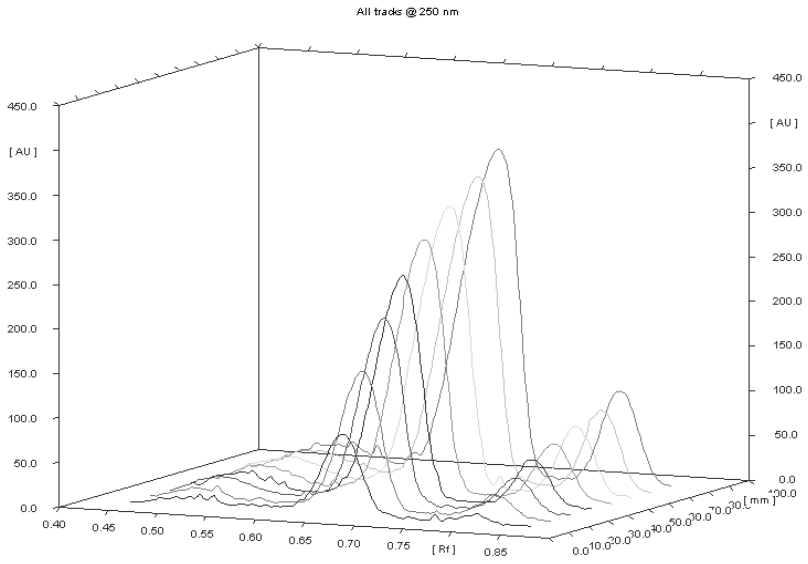

Fig. 3. Three dimensional densitogram showing all tracks of Fluconazole and its impurities.

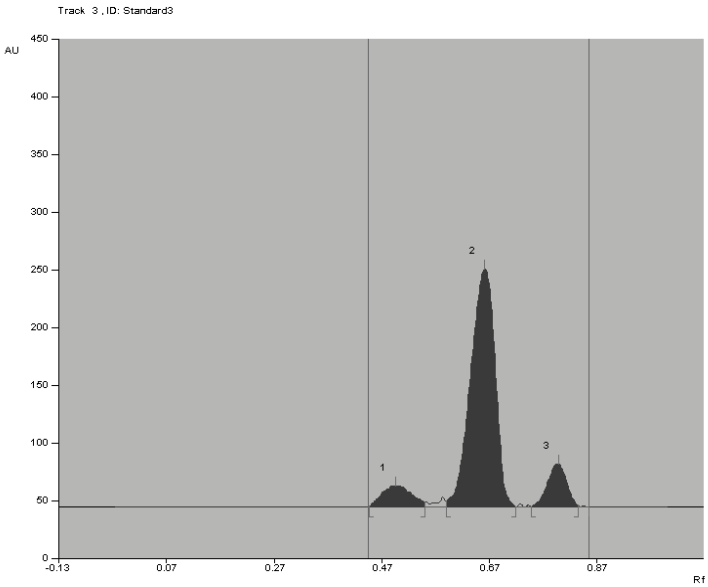

Fig. 4. Densitogram showing the separation of Fluconazole and its impurities using butanol: water: acetic acid (8:2:1 v/v) at 294 nm.
1= Impurity-a, 2 = fluconazole, 3=Impurity-b.

Further, the in situ spectra recorded for fluconazole and the impurities were measured from 190 to 400nm and presented in Fig-5.

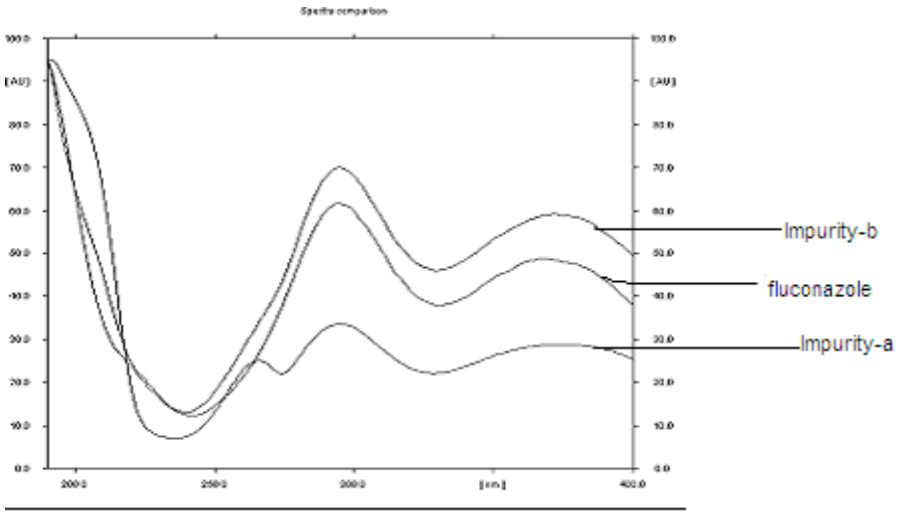

Fig. 5. In situ U.V Spectra of Fluconazole and impurities procured by HPTLC.

3.3 Method Validation

3.3.1 Linearity

Linear regression data for the calibration curves (n=3) as depicted in Table 2, showed a good linear relationship over the concentration range of 100-800ng per spot, 200-700ng per spot and 100-800ng per spot for fluconazole, impurity-a and impurity-b respectively with respect to area.

3.3.2 Limit of Detection and Limit of Quantification

The LOD and the LOQ were determined by using the equations LOD =3.3σ/b; LOQ = 10σ/b, where σ is the SD of the response and "b" corresponds to the slope obtained from the linearity study of the method. LOD and LOQ of fluconazole and the impurities were shown in Table 2.

Table 2. Linear regression data for the calibration curves (n=6)

Parameter	Fluconazole	Impurity-a	Impurity-b
Linear range (ng/spot)	100-800	200-700	200-800
Correlation Coefficient ±SD	0.99946 ±0.0022	0.99919 ±0.0003	0.99909 ±0.0004
Slope	1.86	4.44	1.79
Confidence Limit of slope[a]	1.88-1.83	4.45-4.42	1.80-1.77
Intercept	465.40	394.90	360.80
Confidence Limit of Intercept[a]	467-463	395-393	361-359
LOD (ng/spot)	91.00	26.00	34.00
LOQ (ng/spot)	304.66	89 .00	95.00

3.3.3 Accuracy

According to ICH guidelines, the accuracy of an analytical method expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found. [15, 16] The accuracy of the method was investigated by means of recovery experiments where analysis of standard fluconazole (200ng per spot), impurity-a (400ng per spot) and impurity-b (600ng per spot) was carried out six times to ensure that these quantities were accurately reflected in their peak areas. The results were listed in Table 3. A mean recovery (98.14% –100.68%) of each substance proved that the method facilitated accurate results.

3.3.4 Precision

The precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [15, 16]. The precision was investigated at two levels: the repeatability (precision under the same conditions over a short interval of time) and the intermediate precision (investigating the effect of performing the analysis on different days).

The repeatability and the intermediate precision were determined by performing six determinations of small (200ng/band), medium (400g/bnand), large (600ng/band) amounts of fluconazole, small (200ng/band), medium (500ng/band), large (700ng/band) amounts of impurity-a and small (200ng/band), medium (600ng/band), large (800ng/band) amounts of impurity-b. The CV values for repeatability (CV_r) and for intermediate precision (CV_i) for each substance were summarized in Table 3.

Table 3. Precision studies (n=6)

Compound	Amount [ng/band]	Mean area ± SD	CV_r [%]	Mean area ± SD	CV_i [%]
Fluconazole	200	1227±1.5	0.28	1223±2.9	0.89
	400	1318±3.7	0.51	1327±3.9	0.77
	600	1443±1.2	0.25	1445±1.8	0.54
Impurity-a	200	854±2.9	0.27	814±3.6	0.91
	500	1413 ±1.4	0.11	1412±1.5	0.36
	700	1504 ±3.6	0.07	1503±4.1	0.21
Impurity-b	200	713±1.5	0.41	812±1.8	2.04
	600	1241±2.5	0.50	1248±3.3	1.26
	800	1549±2.0	0.39	1532±3.7	1.60

3.3.5 Recovery

A synthetic mixture containing known quantities of all the two impurities and Fluconazole were analyzed again in triplicate by the proposed method to check recovery of different amounts. The results were listed in Table 4.

Table 4. Recovery studies (n=3)

Compound	Amount taken (ng)	Amount found (ng)	Recovery% ± S.D	RSD (%)
Fluconazole	200	201.5	100.12 ± 4.85	0.05
Impurity-a	400	397.3	98.14 ± 3.87	0.16
Impurity-b	600	602.1	100.68 ± 2.83	0.14

3.3.6 Ruggedness

Ruggedness is the measure of the reproducibility of a test result under normal, expected operating conditions from instrument to instrument and from analyst to analyst. Ruggedness was tested by analysis of 500 ng per band Fluconazole, 300 ng per band impurity-a and 300 ng per band impurity-b and the results were listed in Table 5.

Table 5. Ruggedness of the method (n=3)

Compound	Variable	Recovery [%] ±SD	RSD [%]
Fluconazole	Analyst I	100.8±2.93	0.36
	Analyst II	101.5±2.77	0.34
Impurity-a	Analyst I	101.7±3.00	0.73
	Analyst II	102.1±3.40	0.83
Impurity-b	Analyst I	101.5±3.14	0.77
	Analyst II	99.16±5.19	1.26

3.3.7 Robustness

Robustness was checked by the analysis of sample solutions after making small changes in mobile phase composition and development distance. Mobile phase of composition butanol:water:aceticacid 8:2:1 and 7.5:2:1.5 (v/v) were tried with two different development distances 9 and 8 cm for amount 200ng per band of the main compound fluconazole, 500ng per band impurity-a and 600ng per band impurity-b. The low values of % RSD obtained after introduction of the small changes (Table 6) were indicative of the robustness of the method.

Table 6. Robustness of the method**.**

Compound	Condition	Mean area ± SD	RSD [%]
Fluconazole	Mobile phase composition Bu+Water+ A.A (8+2+1	1237±4.6	0.69
	Bu+Water+ A.A (7.5+2+1.5)	1228±2.7	0.46
	Development distance 9 cm 8 cm	 1224±4.4 1223±3.7	 0.36 0.54
Impurity-a	Mobile phase composition BU+Water+ A.A (8+2+1) BU+Water+ A.A (7.5+2+1.5)	 1141±2.1 1184±3.5	 0.66 0.85
	Development distance 9 cm 8 cm	 1121±4.1 1160±1.2	 0.98 0.50
Impurity-b	Mobile phase composition BU+Water+ A.A (8+2+1) BU+Water+ A.A (7.5+2+1.5)	 1241±4.8 1248±1.5	 0.41 0.92
	Development distance 9 cm 8 cm	 1244±2.8 1245±3.7	 0.63 0.83

Bu=Butanol, Water, A.A=Acetic acid.

3.3.8 Specificity

According to ICH guidelines, the specificity of an analytical method is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products, excipients, etc [15, 16]. . In the present study, the specificity of the analytical method was ascertained by spiking experiments. Thus, fluconazole bulk drug was spiked with known quantities of related impurities where it was observed that all the impurities and the main compound were well resolved and did not interfere with the retention factor of fluconazole (Fig.4).

4. Conclusion

A densitometric thin layer chromatographic method was developed for the separation of Fluconazole from its structurally related impurities. The developed HPTLC method is suitable not only for separation and quantitative determination of active drug ingredient and the impurities to monitor the synthetic reactions, but also for quality assurance of Fluconazole in the presence of its structurally related impurities. The method was validated according to ICH guidelines and shown to be specific, selective, repeatable and accurate within the established ranges.

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