Original Article

METHOD DEVELOPMENT AND VALIDATION STUDY FOR QUANTITATIVE DETERMINATION OF GENOTOXIC IMPURITY AND ITS PRECURSOR IN FLUCONAZOLE SAMPLE BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: The objective of this work is method development and validation study for quantitative determination of 1-[2-(2,4-difluorophenyl)-2,3-epoxypropyl]-1H-1,2,4-triazole, a genotoxic impurity and its precursor in a fluconazole drug sample by liquid chromatography–tandem mass spectrometry.

Methods: LC-MS/MS analysis of these impurities was performed on Hypersil BDS C18 (100 mm x 4.0 mm, 3 μm) column. 5 mmol ammonium acetate and acetonitrile in the ratio of 65:35 (v/v) was used as the mobile phase with a flow rate of 0.4 ml/min. The developed method was accomplished with a short run time of 10 min. Triple quadrupole mass detector coupled with positive electrospray ionization was used for the quantification of genotoxic impurities in multiple reaction monitoring (MRM).

Results: The method was validated as per International Conference on Harmonization (ICH) guidelines. The method was linear in the range of 0.30 μg/g to 11.37 μg/g for impurity A and 0.30 μg/g to 11.34 μg/g for impurity B with a correlation coefficient of 0.999. The accuracy of the method was in the range of 98.25 % to 100.53 % for both impurities.

Conclusion: A specific, selective, highly sensitive and more accurate analytical method using LC-MS/MS coupled with positive electrospray ionization has been developed for the quantification of genotoxic impurity (1-[2-(2,4-difluorophenyl)-2,3-epoxypropyl]-1H-1,2,4-triazole) and its precursor (1-(2,4-difluorophenyl)-2-[1,2,4]triazol-1-yl-ethanone) at 0.3 μg/g with respect to the 5.0 mg/ml of fluconazole.

Keywords: Fluconazole, Genotoxic Impurity, LC/MS/MS, Method Development, Validation

INTRODUCTION

Recently, genotoxic impurities manifestation in active pharmaceutical ingredient (API), as well as products of drugs, received more attention by industries and various governing agencies because of their toxic effect on human health [1]. Even lower concentration (<1 ppm) of genotoxic impurities causes hereditary transformations, chromosome disruptions and leads to oncological diseases. In general, the sources of organic genotoxic impurities in drugs are starting materials, reagents, intermediates, degradation products and by-products of the production of the drug. As per the recommendations of ICH, there is a need to remove the impurities or to reduce their content to a safe level. As per the guidelines of ICH M7, FDA and EMA, the acceptable level of genotoxic impurities is calculated based on the toxicological concern and is considered safe for lifetime use is 1.5 μg day −1 [2-4]. Hence there is a need for chemists to focus development of the manufacturing process of these drugs on minimizing such genotoxic impurities, as it is not possible to eliminate genotoxic impurities completely from the synthetic scheme.

Fluconazole is used for the treatment of systemic and superficial fungal infections, by inhibiting the action of fungal cytochrome enzyme [5-7]. Fluconazole active pharmaceutical ingredient was commercially prepared by reacting its intermediate 1-[2-(2,4-difluor-ophenyl)-2,3-epoxypropyl]-1H-1,2,4-triazole, genotoxic impurity] with triazole in the presence of a base [8]. The scheme of the synthesis of fluconazole is presented in fig. 1. From the scheme given in fig. 1, an intermediate of the fluconazole was prepared from 1-(2,4-difluorophenyl)-2-[1,2,4]triazol-1-yl-ethanone (precursor of intermediate, in the scheme it is represented as compound 3). As per the study of Muller *et al.* [9], this intermediate belongs to group 2 of genotoxic impurities and may cause the unwanted genotoxicity and carcinogenicity. According to the study of micronucleus assay by Fucic *et al.* [10], fluconazole was caused genome damage in young animals and in new-born pups. So, quantitative determination of this genotoxic impurity and its precursor in fluconazole at lower levels is important for organic chemists to control them during the synthesis of fluconazole. Here onwards the impurities, 1-(2,4-difluorophenyl)-2-[1,2,4]triazol-1-yl-ethanone (precursor of genotoxic impurity) and 1-[2-(2,4-difluorophenyl)-2,3-epoxypropyl]-1H-1,2,4-triazole (genotoxic impurity) are abbreviated as impurity A and impurity B in the manuscript.
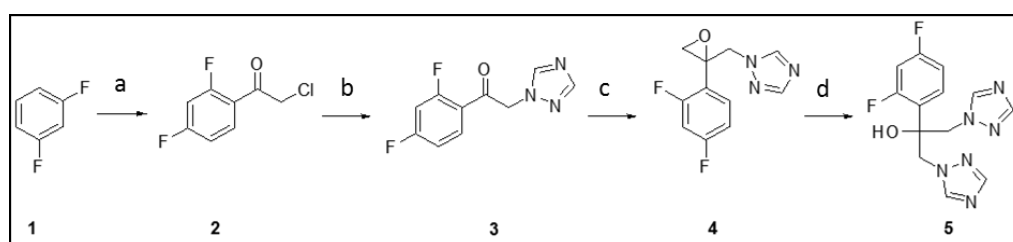

Fig. 1: Scheme of the synthesis of Fluconazole (5), (a) AlCl₃, Chloroacetyl chloride (b) EtoAc, TEA, triazole, reflux. (c) Toluene, NaOH, TMSOI, triazole, reflux (d) DMF, triazole, anhy. K₂CO₃, 90 °C, 2.5 h. genotoxic impurity (4) and precursor of genotoxic impurity (3)

As per the concern of regulatory agencies, the amount of genotoxic impurities in the drug substance should be limited to 7.5 μg/g by assuming of daily dosage as 200 mg/day dose. A method capable of detecting such a lower level is a great challenge for the analyst. Development of a method for the determination of genotoxic impurities at a lower level using conventional analytical instruments (ultraviolet-visible spectrophotometry, gas chromatography and high-performance liquid chromatography) is a difficult task in the pharmaceutical industry as the sensitivity of these instruments is low. Hence, the sensitivity of analytical equipment must be increased in order to determine impurities at lower levels (≤1ppm). This can be achieved by combining chromatography with mass spectrometry (MS). Because of reliability and high sensitivity of chromatography methods coupled with mass spectrometry (LC/MS/MS or GC/MS), recently many authors used these methods for the determination of organic genotoxic impurities [11-20]. The present study is an attempt to develop LC/MS/MS method for the determination of both the genotoxic impurity and its precursor. The literature survey revealed that some spectrophotometric and HPLC methods were developed for the determination of fluconazole in formulations as well as bio samples [21-28], but no method has been found for the determination of genotoxic impurities at low levels (μg/g).

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and ammonium acetate of HPLC grade were purchased from Merck (Mumbai, India). Analytical grade formic acid, trifluoroacetic acid and methanol were supplied by SD Fine Chemicals Limited (Mumbai, India). Purified water was collected through Milli-Q Plus water purification system (Millipore, Milford ford, MA, USA). Fluconazole API and reference substances of genotoxic impurities were obtained from A. R. Life Sciences Pvt. Ltd., (Hyderabad, India).

LC-MS/MS system conditions

The MS of LC-MS/MS system was an Applied Biosystems AB Sciex API 4000 model (Switzerland). The HPLC consisting an LC-20AD binary gradient pump, an SPD-10AVP UV detector, SIL-10HTC autosampler and a column oven CTO-10ASVP (Shimadzu Corporation, Kyoto, Japan) was used for method development and validation. Data acquisition and processing were conducted using the Analyst 1.5.1 software on a Dell computer (Digital equipment Co). Hypersil BDS C18 (100 mm x 4.0 mm, 3 μm) column was used for the analysis.

Operating conditions of LC/MS/MS

The analytical column used in LC/MS/MS was Hypersil BDS C18 (100 mm x 4.0 mm) 3 μm column (Thermo Co, USA) in isocratic mode. 5 mmol ammonium acetate and acetonitrile in the ratio of 65:35 (v/v) was used as a mobile phase. The flow rate was 0.4 ml/min, with the flow rate split down to 0.1 ml/min into the MS source. The column oven temperature was maintained at 40 °C; the sample cooler temperature was 5 °C and the wavelength were set at 260 nm. The injection volume was 10 μl. Positive ion electrospray ionization probe in multiple reaction monitoring (MRM) modes was used in MS method for quantification of impurities in fluconazole drug substance. In this method impurity-A was monitored with its molecular ion $[M+H]^+$ m/z 224.2 and daughter ion m/z 127.2 (224.2-127.2); impurity-B was monitored with its molecular ion $[M+H]+$m/z 238.2 (protonated) and daughter ion m/z 141.2 (238.2-141.2) and fluconazole was monitored with its molecular ion $[M+H]+$m/z 307. The ion spray voltage (V), declustering potential and entrance potential was kept as 4500 V, 50 V and 10 V respectively. The ion source gas 1 and ion source gas 2 nebulization pressures (psi) were maintained at 35, 30 and 14 respectively. This method is suitable for the quantification of impurity A and impurity B both in reaction monitoring samples as well as fluconazole active pharmaceutical ingredient.

Preparation of standard and sample solutions

A stock solution of 0.1 mg/ml was prepared by dissolving appropriate amount of impurity-A and impurity-B in methanol. Preparation of stock standard solution of 0.001 mg/ml was attained by dilution with methanol. Finally, desired concentration (7.5 μg/g) of standard solution with respect to the sample concentration of 5

mg/ml was prepared by the diluting standard stock solution to 10 ml with methanol. Fluconazole sample was prepared with the concentration of 5 mg/ml. The LOQ spiked sample was prepared by adding the impurities at LOQ level.

RESULTS AND DISCUSSION

Analytical method development

The main aim of the LC-MS/MS method in this study was to separate and quantify impurity-A and impurity-B in the fluconazole active pharmaceutical ingredient. The present method was developed by testing different stationary phases to achieve proper separation of analytes and drug substance. Various columns like Kromasil C-8, Hypersil BDS C8, Zorbax Rx C8 and Hypersil BDS C18 of different dimensions were evaluated. Hypersil BDS C8 and Zorbax Rx C8 were not found suitable as the response of analytes was less and the impurity peaks were not well resolved among them as well as from the drug substance peak. The resolution between fluconazole and impurities were poor with Kromasil C8 and Zorbax Rx C8 columns. On Hypersil BDS C18 column of dimension 100 mm × 4.0 mm, 3.0 μm, the separation and response for two impurities were found to be good. On this column, fluconazole, impurity A and impurity B were eluted at 2.31 min, 3.84 min and 4.75 min, respectively and separated from each other and from the active pharmaceutical ingredient. In addition, different mobile phases containing buffers such as formic acid, trifluoroacetic acid and ammonium acetate with different organic modifiers like acetonitrile and methanol have been tested. Trifluoroacetic acid in buffer was suppressed the ionization. Finally, good separation was achieved on a Hypersil BDS C18 (100 mm x 4.0 mm) 3 μm column (Thermo Co, USA) in an isocratic mode using 5 mmol ammonium acetate and acetonitrile in the ratio of 65:35 (v/v). The presence of ammonium acetate in mobile phase enhances ionization and detection. The flow rate was 0.4 ml/min, with the flow rate split down to 0.1 ml/min into the MS source. Here in this work methanol is used as diluent.

Method validation

Specificity

Solutions of fluconazole, impurity-A and impurity-B were prepared separately at a concentration of about 0.01 mg/ml in the diluent and injected. The corresponding chromatograms of fluconazole and its impurities are presented in fig. 2. The chromatograms in fig. 2 showed that fluconazole, impurity A and impurity B peaks were eluted at 2.31, 3.84 and 4.75 min, respectively. The peaks of impurities with fluconazole were well separated, and no interference was observed.

Determination of LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from S/N (signal to noise) ratios. In this process the concentrations were reduced sequentially, such that they yield S/N ratio as 9.7 and 10.1, respectively, for impurity-A and impurity-B. The LOQ of 0.3 μg/g is typical for the impurity-A and impurity-B, with a LOD of 0.1 μg/g approximately three times less than LOQ. The precision of six injections of a sample containing 0.3 μg/g of impurity A and impurity B (with respect to the API concentration 5 mg/ml) at LOQ level was below 1.5% RSD. The determined LOD, LOQ chromatograms and LOQ spiked chromatograms are shown in fig. 3-5. The found LOQ values are sufficient to quantify these impurities at ultra-level in the fluconazole API as per the limits defined by pharma regulating agencies. As per the literature survey, no high-performance liquid chromatography method has been found for the determination of these potential impurities in fluconazole active pharmaceutical ingredient. Al-Rimawi [23] described a high-performance liquid chromatography-ultraviolet (HPLC-UV) method for the detection of other related compounds at 260 nm which, unfortunately, is not sensitive enough to quantify the impurities in the μg/g range. But the present LC/MS/MS method offers great sensitive detection of impurities at 0.1 μg/g (0.5 ng/ml), when compared to a high performance chromatography technique of the detection limit of 100 μg/g (1 μg/ml). Existing methods such as HPLC with UV detection and GC with FID detection have some drawbacks as retention times can vary, uncertainty can arise as to whether a peak at a new retention time is a new impurity and these

methods certainly needed some other method to characterize the impurities online. But the current developed LC/MS/MS method can directly use to quantify impurities at lower levels without above mentioned difficulties.
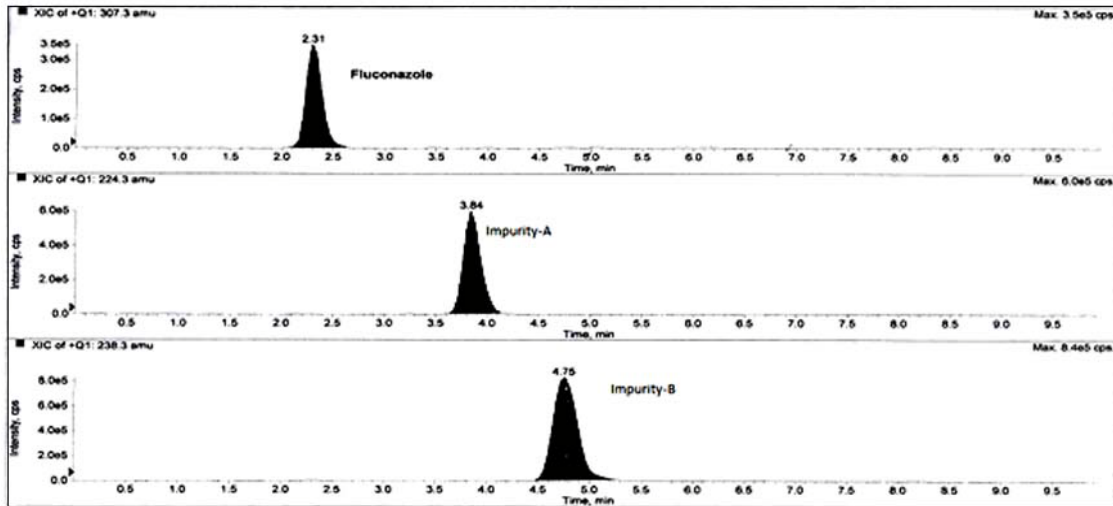

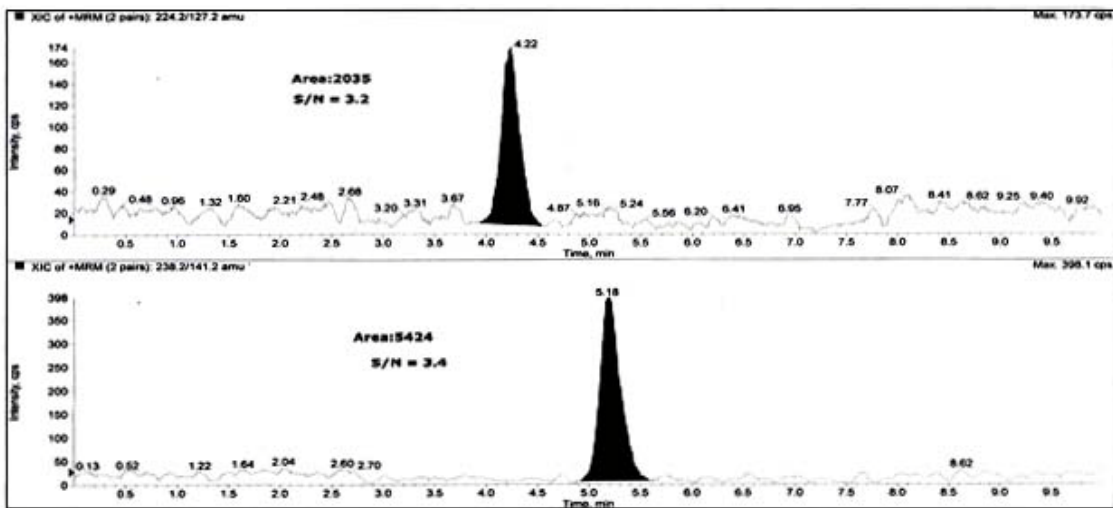Fig. 2: Chromatograms of simultaneous determination of fluconazole and its impurities

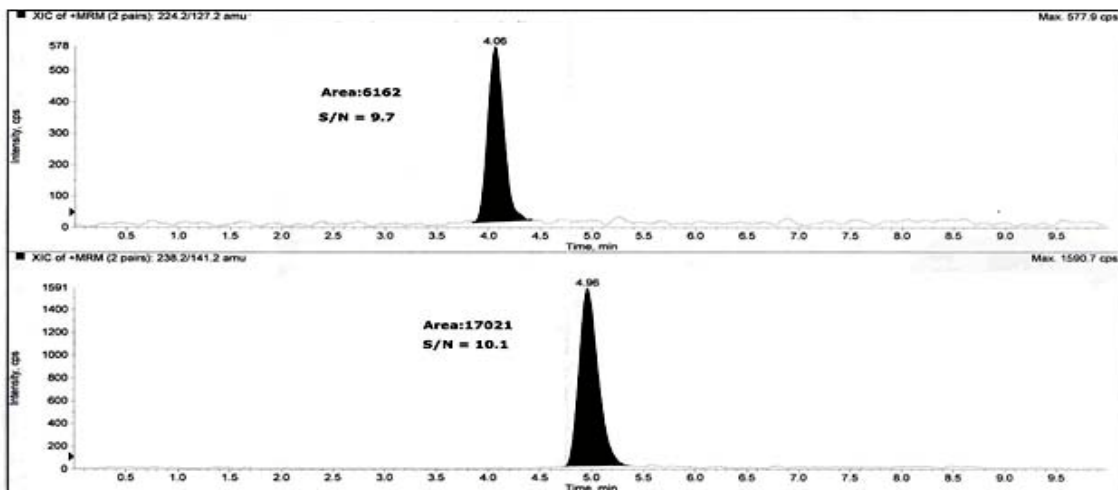Fig. 3: LOD chromatograms of impurity-A (above) and impurity-B (below)

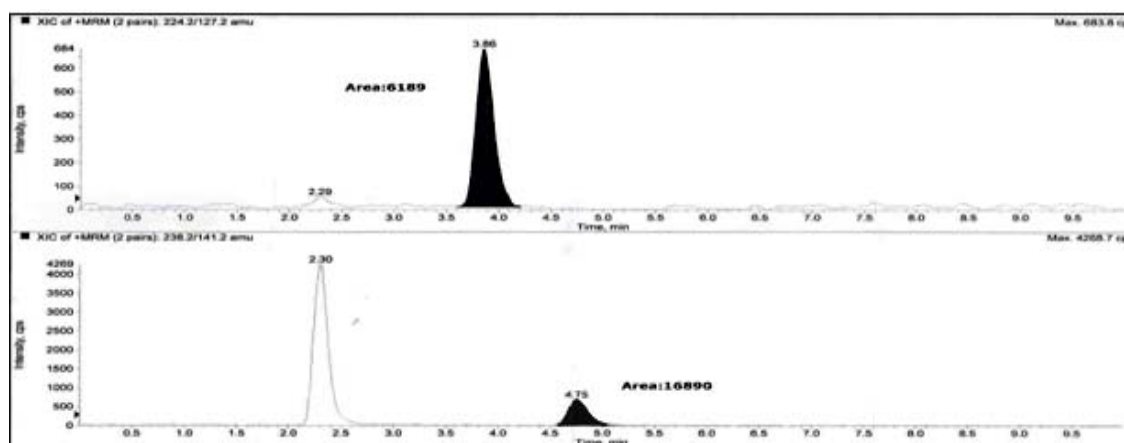Fig. 4: LOQ chromatograms of impurity-A (above) and impurity-B (below)

Fig. 5: LOQ spiked chromatogram of impurity A (above) impurity B (below)

Linearity

The linearity of genotoxic impurity and its precursor satisfactorily demonstrated with a six-point calibration graph between LOQ to 150% of analyte concentrations (60, 80, 100, 120 and 150%). The relation between impurity concentration (x) and its corresponding peak area (y) was expressed by the equation y = mx+b. The slope, intercept, and correlation coefficient values are derived from linear least-square regression analysis, and the data are presented in table 1. The linearity results tabulated in table 1 indicate that an excellent correlation exists between the peak areas and the concentration of impurity-A and impurity-B.

Recovery studies

The accuracy of the method was obtained by recovery studies. In this process, recovery studies were performed in triplicate at LOQ level, 50%, 100% and 150%. Then the percentage recoveries were calculated to evaluate the accuracy of the method and values are shown in table 2. Excellent recovery values were obtained for impurity-A (98.55-100.20%) and impurity-B (98.25-100.53%). Results of accuracy studies in table 2 confirmed that excellent recoveries were obtained with % RSD which is less than 1.2.

Precision and ruggedness

Method precision was determined from the results of six determinations by injecting six freshly prepared solutions containing 7.5 µg/g of each, impurity-A and impurity-B on the same day. The results of method precision are shown in table 3. Ruggedness was carried out such as method precision with different days, different columns and different lots of mobile phase and the results are shown in table 3. Results showed in table 3 confirm that both the precision and ruggedness of the developed method are good. Lower % RSD values of ruggedness indicate that the method was well suited for different laboratory conditions.

Table 1: Linearity of two impurities from LOQ level to 150%

% Level	Impurity-A concentration (µg/g)	Peak area	Impurity-B concentration (µg/g)	Peak area
LOQ	0.30	6215	0.30	17150
60%	4.51	93005	4.54	255125
80%	6.07	123445	6.05	342500
100%	7.58	152330	7.56	418125
120%	9.10	186554	9.07	516512
150%	11.37	231445	11.34	642125
	Slope	20332.1935	Slope	56620.8882
	Intercept	234.8026	Intercept	-1440.0798
	Correlation	0.9999	Correlation	0.9998

Table 2: Accuracy of the method for two impurities (n=3)

% Level	Impurity-A				Impurity-B			
	Added amount	Found amount mean (µg/g)±SD	Recovery (%) mean±SD	RSD (%)	Added amount	Found amount mean (µg/g)±SD	Recovery (%) mean±SD	RSD (%)
LOQ	0.30	0.30±0.002	99.27±0.648	0.6	0.30	0.30±0.0036	99.20±1.180	1.2
50	3.78	3.76±0.026	99.38±0.682	0.7	3.78	3.74±0.0137	99.13±0.362	0.4
100	7.56	7.55±0.041	99.70±0.540	0.5	7.56	7.52±0.0409	99.53±0.541	0.5
150	11.34	11.29±0.078	99.28±0.692	0.7	11.34	11.33±0.028	99.95±0.246	0.2

Robustness

The robustness of the method was studied by intentional changes in flow rate of the mobile phase and column temperature. The flow rate of the mobile phase was altered by 0.04 units, i.e. 0.36 to 0.44 ml/min. The effect of column temperature on the analysis was studied at 38 °C and 42 °C (temperature altered by 2 units). The robustness of the proposed method was evaluated by the changing composition of buffer solution with±5%. All the above intentional changes did not show any significant differences between the columns and the separation of the two impurities with fluconazole. The results revealed that these changes do not impact on chromatographic performance.

Solution stability

The solution stability of fluconazole and two impurities was tested by monitoring the peak areas and retention times over a period of 2 d. The results showed that the retention times and peak areas of fluconazole and two impurities were with relative standard

deviation of lower than two and that no significant degradation is observed within the given period, indicating that the solutions are stable. The solution stability study revealed that the stability of impurity-A and impurity-B was satisfactory up to 48 h.

Method application

This method is applied to the real fluconazole sample and chromatograms are shown in fig. 6 which indicate that no impurities are found in fluconazole sample.

Table 3: Method precision and intermediate precision for two impurities

| S. No.	Method precision				Ruggedness			
	Impurity A		Impurity B		Impurity A		Impurity B	
	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	7.52	99.13	7.52	99.42	7.53	99.93	7.45	99.30
2	7.57	99.78	7.57	100.12	7.52	99.76	7.55	100.65
3	7.6	100.20	7.49	99.06	7.53	99.95	7.68	102.34
4	7.59	100.12	7.46	98.71	7.56	100.32	7.66	102.18
5	7.53	99.29	7.49	99.04	7.6	100.79	7.63	101.72
6	7.57	99.78	7.63	100.87	7.62	101.08	7.66	102.09
Mean	7.56	99.72	7.53	99.54	7.56	100.31	7.61	101.38
SD	0.032	0.432	0.063	0.810	0.041	0.530	0.089	1.187
%RSD	0.42	0.4	0.83	0.8	0.55	0.5	1.17	1.2
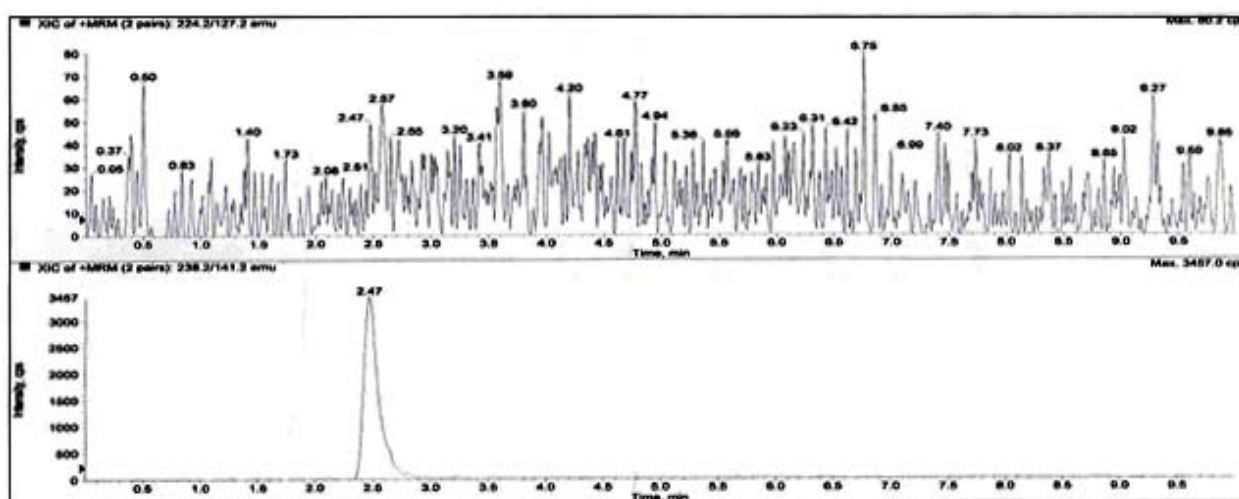
Fig. 6: Sample chromatogram

CONCLUSION

A specific, selective, highly sensitive and more accurate analytical method using LC-MS/MS coupled with positive electrospray ionization has been developed for the quantification of genotoxic impurity, 1-[2-(2,4-difluorophenyl)-2,3-epoxypropyl]-1H-1,2,4-triazole and its precursor, 1-(2,4-difluorophenyl)-2-[1,2,4]triazol-1-yl-ethanone at 0.3 µg/g in fluconazole API. The method has been validated for specificity, linearity, accuracy, precision, robustness and stability. This method is able to detect the impurities in presence of other impurities and main drug. The method is more linear in the range of 0.30 µg/g to 11.37 µg/g for impurity A and 0.30 µg/g to 11.34 µg/g for impurity B with a correlation coefficient not less than 0.999. The accuracy of the method is in the range of 98.25% to 100.53% for both impurities. It has been demonstrated that this method is sensitive with a limit of detection (LOD) of 0.1 µg/g of genotoxic impurity. The information presented here could be useful for monitoring of impurity-A and impurity-B in fluconazole sample. This method is useful for the genotoxic determination impurities during manufacture of fluconazole API.

CONFLICTS OF INTERESTS

Declared none

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