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Isolation and structural identification of an impurity in fluconazole bulk drug substance

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Abstract

Four impurities in fluconazole API sample obtained from a recently proposed synthetic process were detected by HPLC. One of the impurities was unknown having not been reported previously. This less polar unknown impurity was isolated from the crude sample of fluconazole bulk drug using semi-preparative HPLC. Structure of impurity was elucidated as 2-(2-(dimethylamino)-4-fluorophenyl)-1,3-di(3*H*-1,2,4-triazol-1-yl)propan-2-ol by using NMR spectroscopy(^{1}H, ^{13}C, ^{19}F, ^{1}H–^{1}H, ^{1}H–^{13}C, HMBC and nOe) and mass spectrometry. The formation and synthesis of the impurity was discussed.

Keywords: Fluconazole; Impurities; Identification; Isolation; Semi-preparative HPLC; NMR; IR

1. Introduction

Fluconazole is a triazole antifungal drug used in the treatment and prevention of superficial and systematic fungal infections [1,2]. Like other imidazole and triazole class antifungals, fluconazole inhibits the fungal cytochrome P450 enzyme 14α-demethylase and can be used as a first line treatment for the indications like coccidioidomycosis, cryptococcosis, hystoplasmosis, and prophylaxis of candidiasis in immunocompromised people [3]. Fluconazole is also indicated for the prophylaxis of fungal infections where other antifungals have failed or are not tolerated, including candidiasis caused by susceptible strains of candida, tinea corporis, tinea cruris, or tinea pedis, onychomycosis, and cryptococcal meningitis [4]. It is also effective in initial and maintenance therapy for osyptococcal meningitis in patients with AIDS [5]. A few methods were reported in the literature for the analysis of fluconazole and its related substances using chromatographic and spectroscopic methods [6–12].

Organic impurities can arise during the manufacturing process and storage of the drug substances and the criteria for their acceptance up to certain limits are based on pharmaceutical studies or known safety data. As per regulatory guidelines, the pharmaceutical studies using a sample of the isolated impurity can be considered for safety assessment [13]. It is, therefore, essential to isolate and characterize unidentified impurities present in Active Pharmaceutical Ingredients (APIs).

Different methods of synthesis of fluconazole are reported in literature [14–16]. Fluconazole sample obtained from two synthetic routes were analyzed by HPLC. Three impurities were detected in the sample obtained form synthetic process reported by Narayanan et al. [16]. In a prior publication [17] detailed structural elucidation of these impurities was carried out after preparative chromatographic isolation. More recently, a new improved synthesis of fluconazole bulk drug has been proposed [18]. Four impurities were detected in the bulk drug sample obtained by this process. One of the impurities was found to be unknown having not been reported previously. Present paper describes the isolation and characterization of this fourth less polar impurity found in the fluconazole bulk drug sample.

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2. Experimental

2.1. Materials and reagents

Fluconazole API and intermediate samples were obtained from Chemical Research Division, Ipca Laboratories Ltd., Mumbai, India. HPLC grade acetonitrile was purchased from Merck India Limited. De-ionized water was prepared using Millipore milliQ plus purification system. Chloroform-d_3 and dimethyl sulfoxide-d_6 (for NMR) were from Aldrich Chemical Co., USA. Analytical reagent grade trifluoro acetic acid (TFA) was purchased from Lancaster, England. KBr was purchased from Merck KGaA, Germany.

2.2. Mass spectrometry

The LC–ESI/MS and MS/MS studies were carried out on LCQ-Advantage (Thermo Finnigan San Jose, USA) ion trap spectrometer. The source voltage was kept at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used as both sheath and auxiliary gas. Mass range was kept at *m/z* 150–500. MS/MS studies were carried out by maintaining normalized collision energy at 35% with the mass range *m/z* 50–350. The LC part was consisted of an Agilent 1100 series quaternary gradient pump with a degasser and an auto sampler. A Vydac C18 column (150 × 4.6 i.d. 5 μm, Denali 238, W.R. Grace, Davison division, USA) was used for chromatographic separation. The mobile phase consisting of a mixture of 0.02 M ammonium formate and acetonitrile in the ratio 86:14 (v/v) was used. The flow rate was maintained at 1 ml/min.

The EI-MS studies were carried out on Shimadzu QP 5050 mass spectrometer with ionization electron beam energy of 70 eV. The sample was introduced in the source with the help of direct inlet probe.

2.3. Semi-preparative HPLC

The impurity was isolated from the mother liquor sample of fluconazole using Waters Auto-purification system consisting of 2525 binary gradient pump, a 2487 UV detector and 2767 sample manager (Waters, Milford, MA, USA). A Waters XTerra C18 column (150 mm × 19 mm i.d., particle size 5 μm) was used for semi-preparative isolation. A mixture of water and acetonitrile in the ratio of 80:20 (v/v), at a flow rate of 18 ml/min was used as a mobile phase. The detection was carried out at 260 nm. The sample solution (25 mg/ml) was prepared

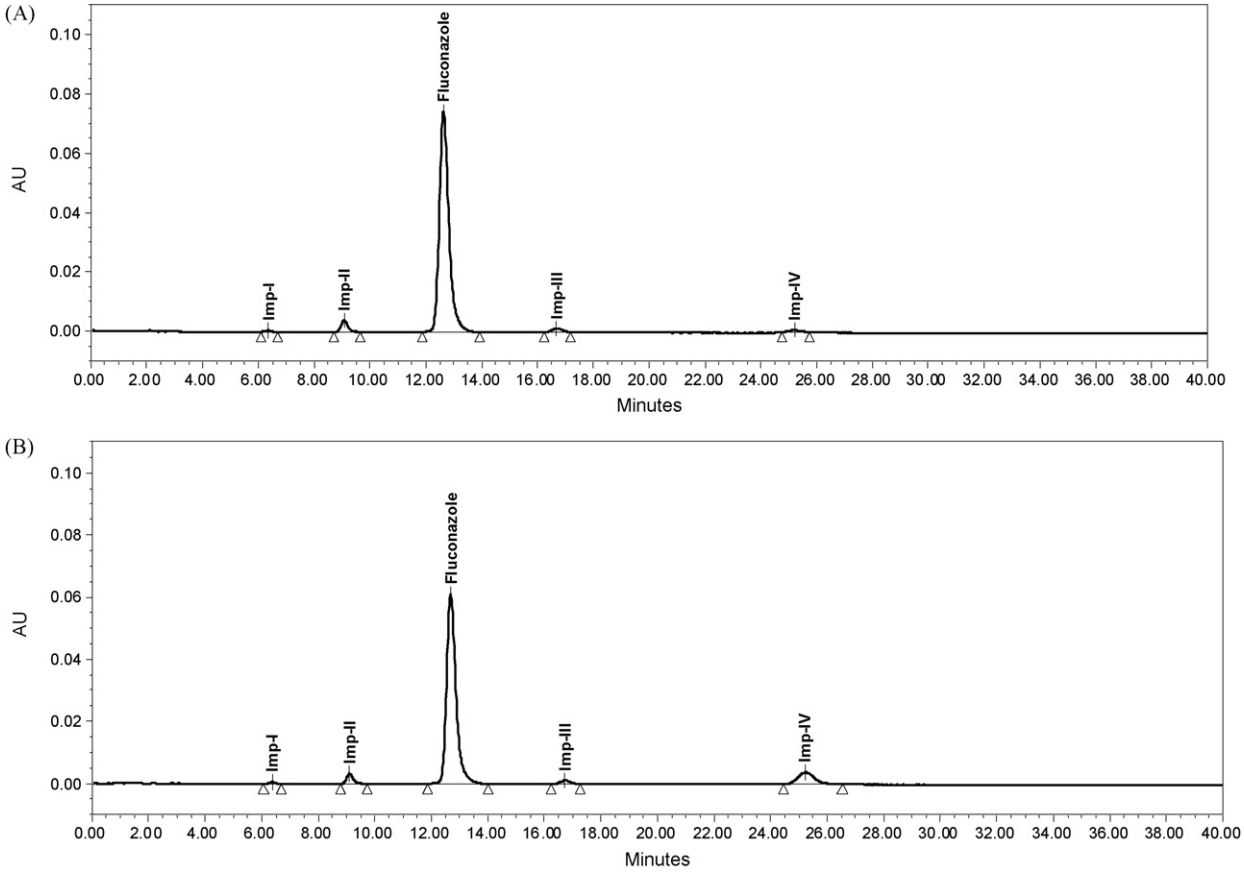

Fig. 1. (A) HPLC chromatogram of fluconazole sample. (B) HPLC chromatogram of fluconazole sample spiked with Imp-IV. HPLC conditions: mobile phase, −0.02 M ammonium formate and acetonitrile in the ratio (86:14); column, Vydac C18 column (150 mm × 4.6 mm), 5 μm particle size; flow rate, 1 ml/min; wavelength, −260 nm; column temperature, −40 °C.

by using mobile phase as a diluent. The injection volume was 1 ml.

2.4. NMR

The ^{1}H, ^{13}C, and ^{19}F NMR measurements of the isolated impurity were performed on a Bruker 400 MHz instrument at 25 °C. The DEPT, nOe and 2D experiments were also carried out at 25 °C using same instrument. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to DMSO (2.49 ppm) and $CDCl_3$ (77.00 ppm), respectively, while ^{19}F values relative to TFA (−76.55 ppm versus $CFCl_3$).

2.5. IR spectroscopy

The IR spectrum of isolated impurity was recorded in the solid state as KBr powder dispersion using a Perkin-Elmer (Spectrum-one) FT-IR spectrometer.

3. Results and discussion

3.1. Detection of impurity by HPLC and LCMS

An intermediate, 3-((2-(2,4-diflurophenyl)oxiran-2-yl)-methyl)-3*H*-1,2,4-triazole, was treated with triazole in alkaline medium using DMF as a solvent to obtain fluconazole [18]. The product obtained was analyzed by HPLC [17]. A typical chromatogram is shown in Fig. 1. The analysis revealed the presence of four impurities: Imp-I, Imp-II, Imp-III and Imp-IV. Imp-I: 2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)3-(4*H*-1,2,4-triazol-4-yl)-2-propanol, Imp-II: 1-(1-*H*-1,2,4-triazole-1-yl)propane-2,3-diol and Imp-III: 2-(2,4-difluorophenyl)-3-(1-*H*-1,2,4-triazole-1-yl)-2-propen-1-ol were identified by co-injecting the reference materials of respective impurity. The less polar impurity eluting at 25 min (Imp-IV) was found to be unknown. The level of this impurity was found between the range of 2–60% in crude and mother liquor samples. The impurity level in the purified drug substance

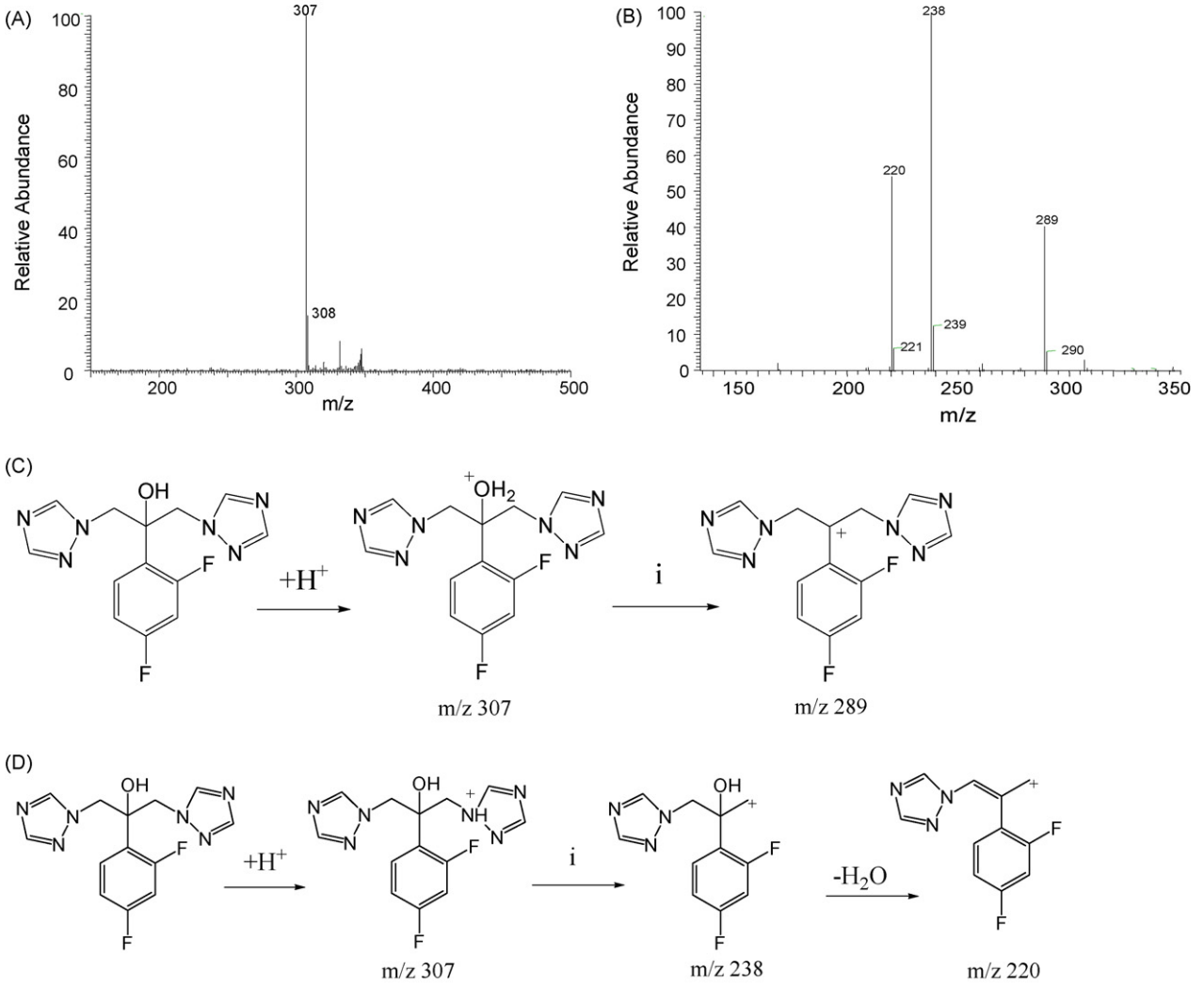

Fig. 2. MS and MS/MS data for fluconazole. (A) Mass spectrum of fluconazole, (B) MS/MS spectrum of product ion, (C) fragmentation mechanism for product ion with *m/z* 289 and (D) mechanism for formation of product ions with *m/z* 238 and *m/z* 220.

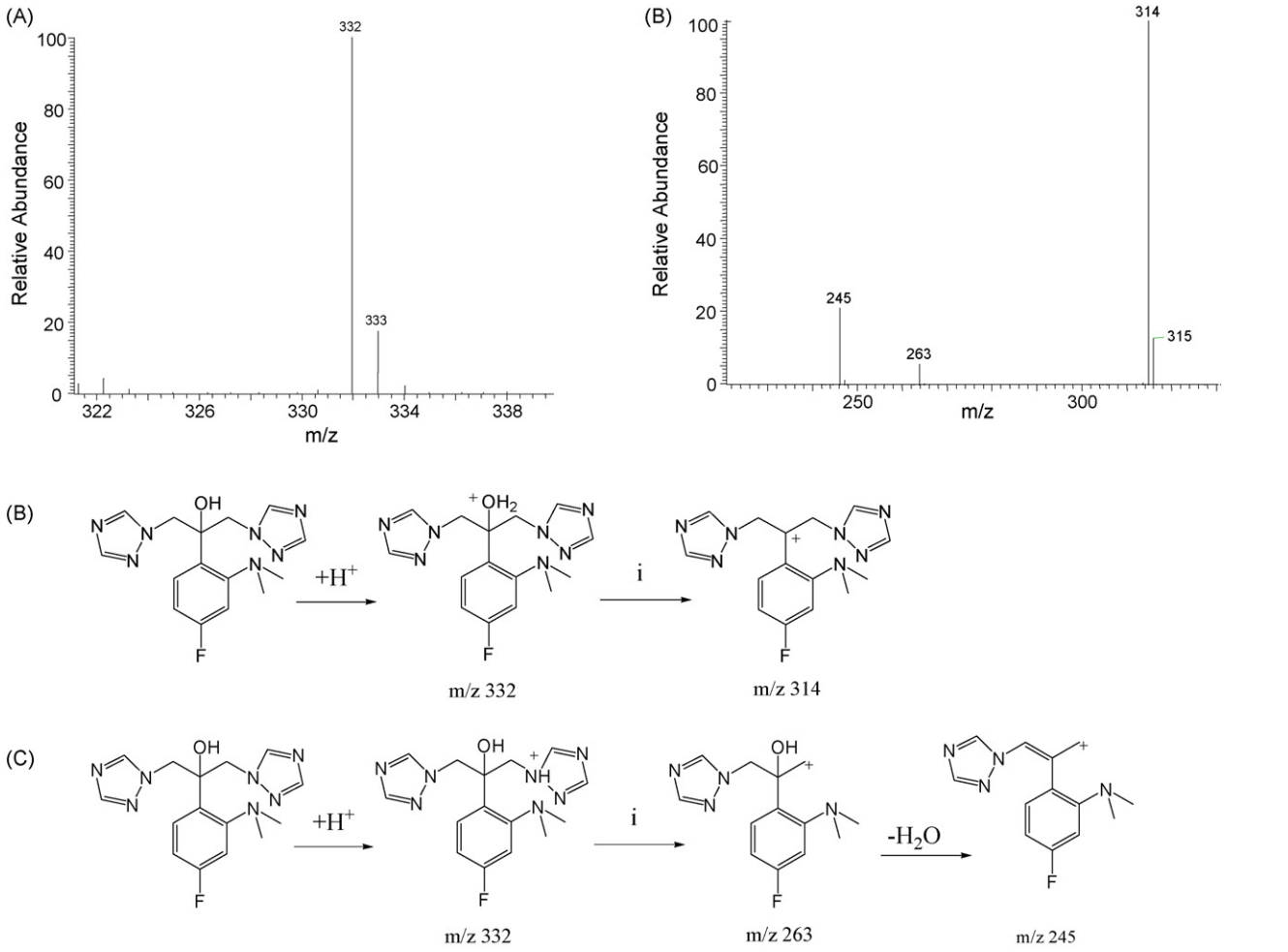

Fig. 3. MS and MS/MS data for Imp-IV. (A) Mass spectrum of Imp-IV, (B) MS/MS spectrum of product ion, (C) fragmentation mechanism for product ion with *m/z* 314 and (D) mechanism for formation of product ions with *m/z* 263 and *m/z* 245.

on laboratory as well as manufacturing scale was obtained in the range of 0.01–0.05%.

The crude API sample was subjected for LC/ESI/MS (positive mode) and MS/MS analysis. Mass spectral data showed protonated molecular ion peaks at *m/z* 307 and *m/z* 332 for fluconazole and the impurity (Figs. 2 and 3). MS/MS spectrum obtained for fluconazole showed three product ion peaks at *m/z* 289, *m/z* 238 and *m/z* 220. MS/MS study of the impurity showed daughter ion peaks at *m/z* 314, *m/z* 263 and *m/z* 245. Further MS^3 experiments showed no significant fragments of these peaks.

3.2. Isolation of impurity by semi-preparative HPLC

The sample obtained form the mother liquor was subjected for preparative isolation. All the fractions were checked for retention time and purity. The fractions showing the presence of impurity above 97% were mixed together. The targeted Imp-IV was isolated as described in Section 2.3. Fluconazole and the impurity were eluted at 2.65 min and 4.43 min, respectively (Fig. 4). The collected fractions were concentrated to dryness under high vacuum using lyophilization. The chromatographic purity of the isolated impurity sample was tested in analytical mode by HPLC and found to be 99%. This isolated solid obtained was used, without further purification to generate spectroscopic and spectrometric data.

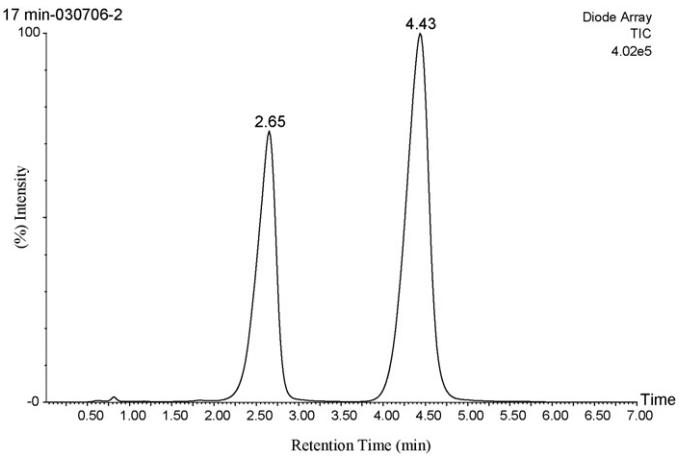

Fig. 4. Semi-preparative HPLC chromatogram chromatograph of crude fluconazole sample.

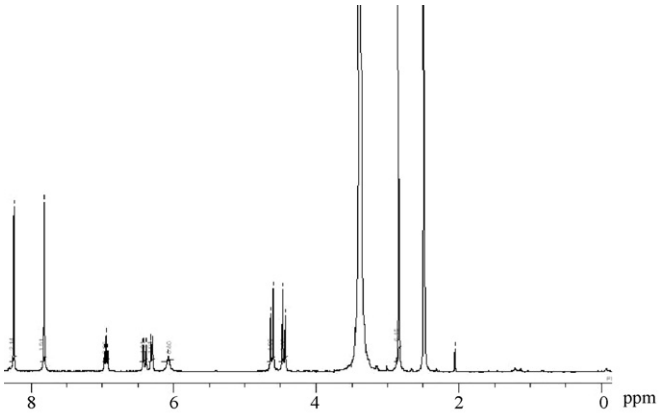

Fig. 5. ^{1}H NMR spectrum of Imp-IV.

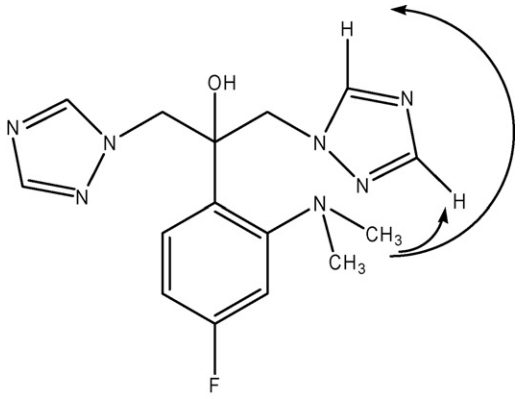

Fig. 7. Structural formula for Imp-IV of fluconazole, showing nOe effect.

3.3. Structural elucidation of Imp-IV

For structural elucidation of the impurity, it is logical to compare the NMR and mass spectral data obtained for fluconazole and the targeted impurity. Comparison of ^{1}H NMR revealed an extra signal integrating six protons at 2.9 ppm for Imp-IV (Fig. 5). The ^{13}C NMR of the impurity also showed an extra signal at 40.1 ppm. HETCOR experiment was performed to identify the ^{1}H–^{13}C coupling which correlated the ^{13}C nuclei at 40.1 ppm with protons obtained at 2.9 ppm for impurity. DEPT experiment showed attachment of three H atoms to this carbon atom, suggesting the presence of two equivalent CH_3 groups. The higher chemical shift values indicated the attachment of these methyl groups to the electronegative atom.

The mass spectral data indicated an odd molecular mass (331 amu) for impurity. This implies the existence of an odd number of nitrogen atoms [19]. The fluconazole molecule contains six nitrogen atoms belonging to two triazole rings. It is evident from the NMR spectral data that the impurity in question also contains the triazole rings. It is, therefore, noted that there must be an additional nitrogen atom which contributes to the odd molecular mass of the impurity. Additionally, the peak at 1370 cm^{-1} (strong –C–N stretching band) in the IR spectrum indicated the presence of a tertiary aromatic amine functional group in the impurity. In ^{19}F NMR spectrum two signals were obtained at 6.62 ppm and 2.16 ppm for fluconazole, while only one signal at 2.10 ppm for the impurity (Fig. 6). Taken together the above mass and NMR spectral data, it was concluded that the impurity contains a $–N(CH_3)_2$ group and a fluorine atom instead of two fluorine atoms as in case of fluconazole.

Signals at 6.62 ppm and 2.16 ppm in ^{19}F NMR of fluconazole were assigned for fluorine atoms at positions 6 and 2, respectively (Fig. 6B). Since the electron withdrawing group is attached to carbon at position 5, the fluorine atom at position 6 was appeared in deshielded region. The ^{19}F NMR spectrum of the impurity showed only one signal at 2.10 ppm. This indicated the attachment of $–N(CH_3)_2$ group to the carbon at position 6.

In order to confirm the above proposed positional isomerism, a 1D nOe experiment was carried out [20]. Irradiation of protons at positions 9 and 10 resulted in the enhancement of intensity of protons at position 11 (Fig. 7). Hence protons at position 11 are in proximity of a triazole ring which supports the proposed configuration. Based on above observations, the molecular formula of the impurity was confirmed as $C_{15}H_{18}ON_7F$ and the corresponding structure of the impurity was characterized as 2-(2-(dimethylamino)-4-fluorophenyl)-1,3-di(3*H*-1,2,4-triazol-1-yl)propan-2-ol.

The MS/MS data was found to be in agreement with the structure assigned on the bases of NMR and MS data. In the MS/MS

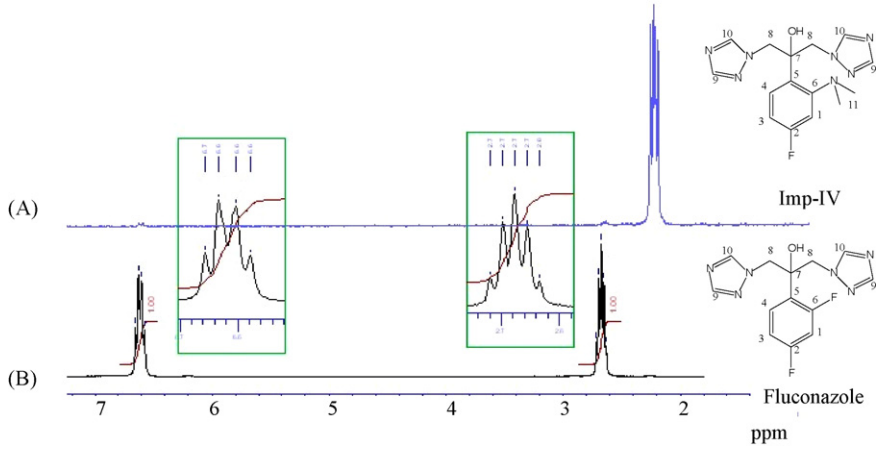

Fig. 6. Overlaid ^{19}F NMR spectra of (A) Imp-IV and (B) fluconazole.

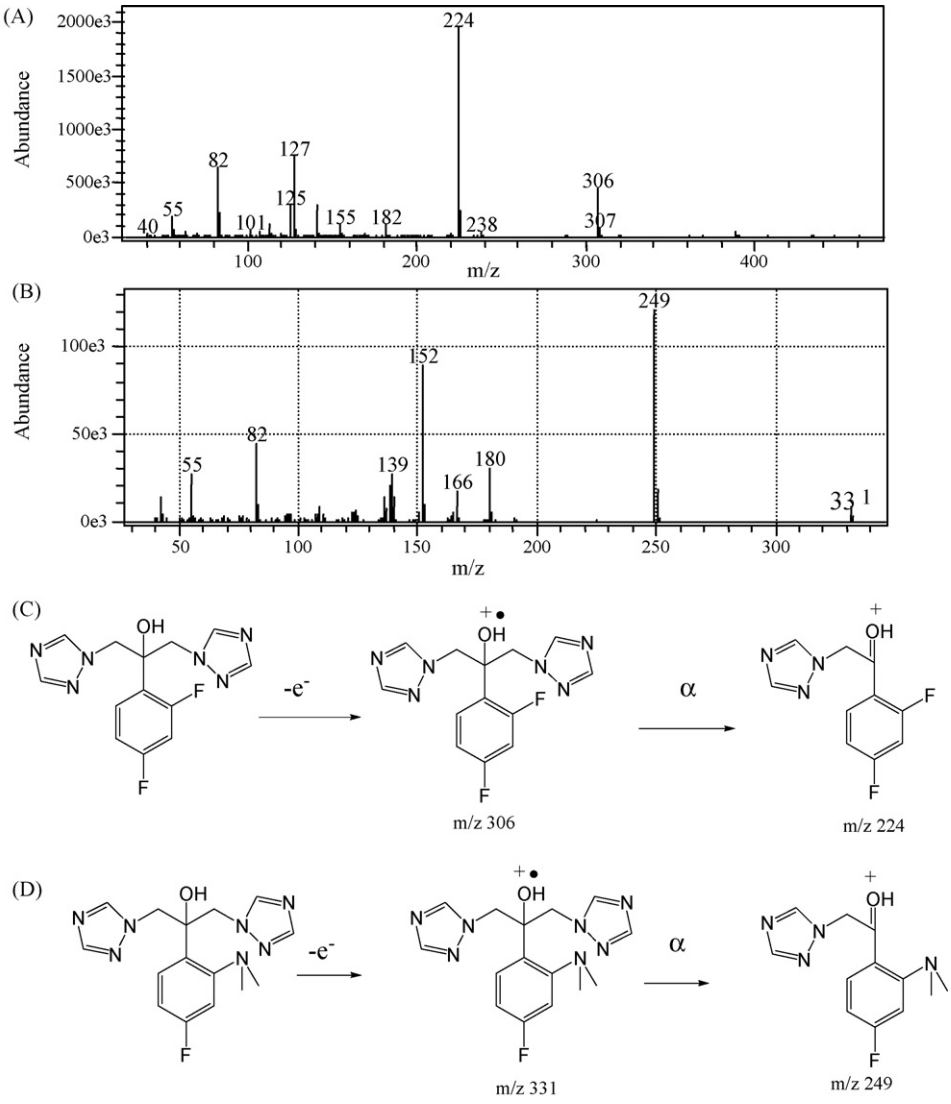

Fig. 8. EI-MS data of fluconazole (A) EI-MS spectrum for fluconazole, (B) EI-MS spectrum for Imp-IV, (C) mechanism of formation of fragment with *m/z* 224 and (D) mechanism of formation of fragment with *m/z* 249.

Table 1
NMR assignments for Imp-IV

Position[a]	^{1}H	δ (ppm)[b]	J (Hz)[b]	COSY	^{13}C	DEPT[c]	HETCOR
1	1H	6.31	dd, 8.8, 2.0	–	99	CH	1H
2	–	–	–	–	158	–	–
3	1H	6.41	dd, 16.0, 1.8	4H	108	CH	3H
4	1H	6.95	t, 9.1	3H	129	CH	4H
5	–	–	–	–	113	–	–
6	–	–	–	–	152	–	–
7	–	–	–	–	74	–	–
7OH	1H	6.08	s	–	–	–	–
8	2Ha	4.46–4.62	dd, 14.1, 14.4[d]	2Hb	55	CH_2	8Ha, 8Hb
	2Hb	4.46–4.62	dd, 14.1, 14.4[d]	2Ha	55	CH_2	8Ha, 8Hb
9	1H	7.82	14.1	–	152	CH	9H
10	1H	8.24	s	–	145	CH	10H
11	6H	2.85	s	–	40	CH_3	11H

[a] Refer the structural formula for numbering (Fig. 9).
[b] Refer Fig. 10.
[c] Hybridization (degree of bonding) of carbon atoms.
[d] Magnetically non-equivalent protons.

Table 2
HMBC Spectral assignment of Imp-IV

Proton/carbon[a]	1	2	3	4	5	6	7	7OH	8	9	10	11
C-1	Bonded	–	β	–	–	–	–	–	–	–	–	–
C-2	α	–	α	β	–	–	–	–	–	–	–	–
C-3	β	–	Bonded	α	–	–	–	–	–	–	–	–
C-4	–	–	α	Bonded	–	–	–	–	–	–	–	–
C-5	β	–	β	α	–	–	–	β	β	–	–	–
C-6	α	–	–	β	–	–	–	–	–	–	–	β
C-7	–	–	–	β	–	–	–	α	α	–	–	–
C-8	–	–	–		–	–	–	β	Bonded	–	β	β
C-9	–	–	–		–	–	–	–	–	Bonded	β	–
C-10	–	–	–	–	–	–	–	–	β	β	Bonded	–
C-11	–	–	–	–	–	–	–	–	–	–	–	Bonded

α and β are the positions denoting two- and three-bond coupling.
[a] Refer the structural formula for numbering (Fig. 9).

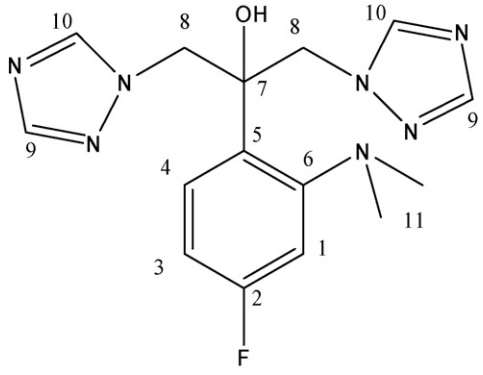

Fig. 9. Structural formula for Imp-IV of fluconazole.

study of fluconazole the formation of a daughter ion at *m/z* 289 is attributed to the loss of H_2O (18 amu) from parent ion peak at *m/z* 307. The parent ion also loses a triazole moiety (69 amu) to form another daughter ion at *m/z* 238, which on further loss of H_2O yields a product ion at *m/z* 220. The mechanism is given in Fig. 2. Similarly, the formation of daughter ion at *m/z* 314 for impurity can be attributed towards the loss of a H_2O molecule from parent ion (*m/z* 332). The parent ion also loses a triazole moiety (69 amu) to form another daughter ion at *m/z* 263, which on further loss of a water molecule (18 amu) yields a product ion at *m/z* 245. The mechanism is given in Fig. 3. In EI-MS study of fluconazole and the impurity, many fragments differing by 25 amu were observed, which is equivalent to mass difference between fluconazole and the impurity, i.e. 331 − 306 = 25 (Fig. 8).

The ^1H, ^{13}C NMR data, DEPT assignments, and 2D data of fluconazole and Imp-IV are listed in Table 1. The HMBC data is given in Table 2.

3.4. Formation and synthesis of impurity

An intermediate 2-(4-(dimethylamino)-2-fluorophenyl)-1,3-di(3*H*-1,2,4-triazol-3-yl) propan-2-ol reacts with triazole in basic condition to form fluconazole, which further reacts with

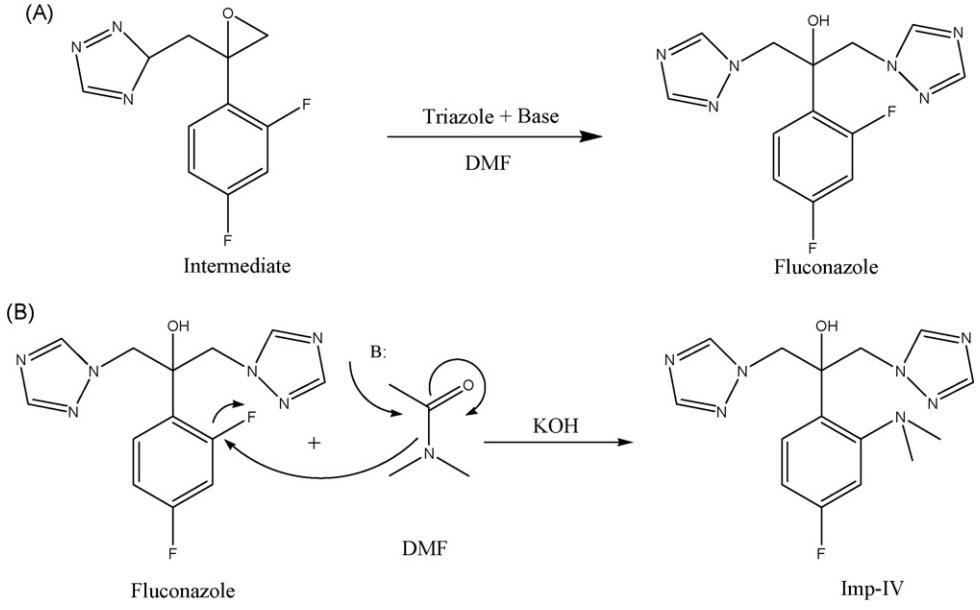

Fig. 10. (A) Scheme for the synthesis of fluconazole and (B) plausible mechanism of formation of Imp-IV.

dimethylformamide to form the impurity. The plausible mechanism is shown in Fig. 10. To confirm the proposed mechanism, the impurity was synthesized independently by treating fluconazole with DMF in presence of KOH.

4. Conclusion

Isolation of a new less polar impurity of fluconazole has been carried out by semi-preparative HPLC. The structural assignment of the isolated impurity was carried out by using spectroscopic and spectrometric analysis. The structures were confirmed by independent synthesis of the impurity.

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