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Preparative isolation and structural elucidation of impurities in fluconazole by LC/MS/MS

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Abstract

Three impurities were detected in the LC/MS analysis of fluconazole bulk drug substance. Two of the impurities were unknowns having not been reported previously. Structural assignment of these impurities was carried out by LC/MS/MS using electrospray ionization source and an ion trap mass analyzer. Structural elucidation using nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy was facilitated by newly developed rapid preparative isolation method. These impurities were characterized as 1-(1-*H*-1,2,4-triazole-1-yl) propane-2,3-diol and *Z*-2-(2,4-difluorophenyl)-3-(1-*H*-1,2,4-triazole-1-yl)-2-propen-1-ol. Their formation and synthesis are discussed.
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Keywords: Fluconazole; Impurities; LC/MS/MS; Identification; Isolation; Preparative HPLC; IR; NMR

1. Introduction

Fluconazole, 2-(2,4-difluorophenyl)-1,3-bis-(1*H*-1,2,4-triazol-1-yl)-2-propanol (CAS 86386-73-4), is a triazole antifungal drug administered orally or an intravenous infusion. It is used for oseopharynageal, oseophageal, vaginal or systemic candiasis and for fungal skin infections. It may also be used as an alternative to amphotericin in the treatment of cryptococcosis [1]. Fluconazole has become the drug of choice for the treatment of coccidioidal meningitis because of lower morbidity than with intrathecal amphotericin B [2]. It is also effective in initial and maintenance therapy for osyptococcal meningitis in patients with AIDS [3].

A few chromatographic methods have been reported in the literature describing the analysis of fluconazole and its related substances using UV detection [4–6]. A liquid chromatographic method for fluconazole and an intermediate using mass spectrometric detection is reported by Creaser et al [7]. Spectral characterization of fluconazole was also carried out by Cyr et al. [8]. Some bioanalytical methods using mass spectrometry are also reported [9,10]. There are no reports available on the investigation using LC/MS/MS and isolation of related substances in fluconazole active pharmaceutical ingredient (API). The chromatographic conditions mentioned in European Pharmacopoeia (EP) [6] were found to be compatible with mass spectrometric detection. However, in order to get better chromatographic resolution and peak shape the method has been modified for use in the present investigation.

During the HPLC analysis of different laboratory batches of fluconazole, three impurities were detected. Examination of LC/MS data revealed that two of the three impurities were unknowns. A thorough investigation was undertaken to identify and elucidate the structures of the two impurities using LC/MS/MS techniques followed by isolation using preparative HPLC to facilitate spectroscopic characterization by IR and NMR spectroscopy.

It is pertinent to mention that in view of the stringent quality requirements of global regulatory authorities, it is mandatory to know structural details the threshold levels of impurities appearing at or above 0.1% in an active pharmaceutical ingredient (The ICH guidelines [11] specify an identification threshold of 0.10% for a maximum daily dose of 2 g/day of drug substance). The present paper describes structural elucidation of impuri-

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ties as well as their formation during synthesis of fluconazole [12].

2. Experimental

2.1. Materials and reagents

Samples of the API (Batch. No. FCZ-Pure and FCZ Crude) were obtained from Ipca Laboratories Ltd., Chemical Research Division, Mumbai, India. HPLC grade acetonitrile, ammonium formate and ammonium acetate were purchased from Merck India Limited. Chloroform-d_3 and dimethyl sulphoxide-d_6 (for NMR) were purchased from Aldrich Chemical Co., USA.

2.2. High performance liquid chromatography

Samples were analyzed on a Waters Alliance 2690 HPLC equipped with Waters 2487 UV detector. A Vydac C18 column (150 mm × 4.6 mm i.d., 5 μm particles and carbon coverage in excess of 4 μmol/m^2, 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 with UV detection at 260 nm. Sample solutions were prepared in mobile phase and 20 μl was injected. Column temperature was maintained at 40 °C.

2.3. Liquid chromatography–tandem mass spectrometry (LC/MS/MS)

The MS/MS studies were carried out on LCQ-Advantage (ThermoFinnigan, USA) ion trap system. The HPLC consisted of an Agilent-1100 series quaternary gradient pump with a degasser, an auto sampler and column oven. The chromatographic conditions described in Section 2.2 have been used for the analysis. The HPLC effluent was introduced into electrospray ionization (ESI) source of the mass spectrometer at 1 ml/min without split. The source voltage was maintained at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used as both sheath and auxiliary gas; mass range was kept at 150–500 amu. MS/MS studies were carried out by maintaining normalized collision energy at 35 with the range *m/z* 50–300 amu.

2.4. Preparative liquid chromatography

Impurities were isolated from the crude sample using Waters Auto purification system consisting of 2525 binary gradient pump, a 2487UV detector and 2767sample manager (Waters, Milford MA, USA). A Waters Symmetry C18 column (100 mm × 30 mm i.d., particle size 5 μm) was used for the separation. The mobile phase was consisted of a mixture of 0.01 M ammonium acetate and acetonitrile in the ratio of 85:15 and was pumped at flow rate 25 ml/min. The detection was monitored at 260 nm.

2.5. NMR

^{1}H and ^{13}C spectra of isolated impurities were recorded on Bruker 400 MHz instrument. The ^{1}H and ^{13}C chemical shift values were reported on δ ppm scale relative to CDCl3 (7.26 ppm). DEPT and nOe experiments were also conducted on the same instrument.

2.6. IR spectroscopy

The IR spectra of isolated impurities were recorded in the solid state as KBr powder dispersion using a Perkin Elmer FT-IR spectrometer.

3. Result and discussion

3.1. Detection of impurities by HPLC and LC/MS

HPLC analysis of samples of fluconazole drug substance was carried out using the method described in Section 2.2. By adapting the modified chromatographic conditions to a column stationary phase with higher carbon coverage, higher buffer concentration and sample preparation, chromatographic resolution and peak shape (USP tailing factor: 1.1, for principle peak) was found to be enhanced. Apart from the principle peak (eluting at 12.7 min) three impurities were detected at retention times 6.3 min (impurity I), 9.1 min (impurity II) and 16.7 min (impurity III), respectively. A typical chromatogram is shown in Fig. 1A.

Mass spectral data showed a protonated molecular ion peak at *m/z* 307 for fluconazole and impurity I, while peaks at *m/z* 256 and 238 were obtained for impurity II and impurity III, respectively.

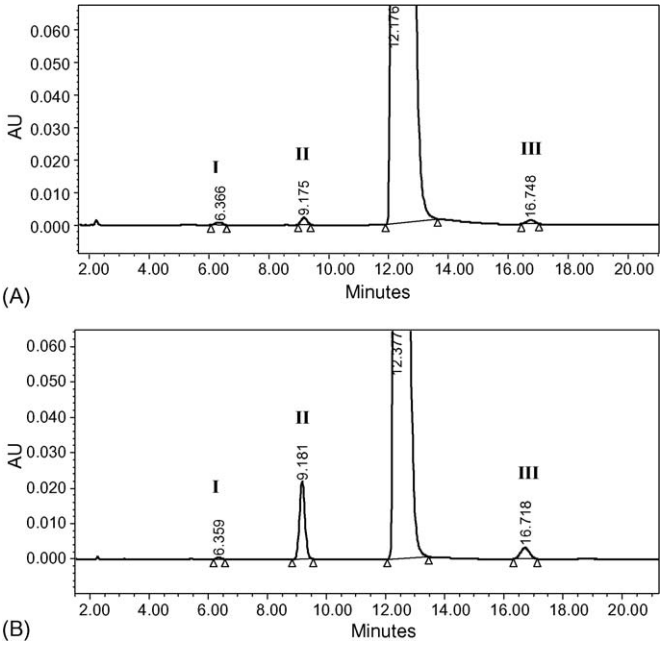

Fig. 1. (A) HPLC chromatogram of fluconazole sample. (B) HPLC chromatogram of fluconazole sample spiked with impurities II and III.

Identical protonated molecular ion peak obtained for both impurity I and fluconazole indicates that these compounds are isomers. On the basis of its relative retention time (RRT) with respect to fluconazole (as described in the European Pharmacopeia, EP), and the mass spectral data this impurity has been identified as, 2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-3-(4*H*-1,2,4-triazol-4-yl)-2-propanol, a positional isomer of fluconazole.

The mass spectrum obtained for the impurity II exhibited a protonated molecular ion peak at *m/z* 256 (Fig. 2B), which did not match with the identity assigned in the EP (on the basis of RRT) as 2-(2-fluorophenyl)-1,3-bis-(1*H*-1,2,4-triazol-1-yl)-2-propanol (molecular mass of 288).

The mass spectrum of impurity III exhibited a protonated molecular ion peak at *m/z* 238 (Fig. 2C). This value matches the expected protonated molecular ion peak for one of the known synthetic intermediates Ia. However, a discrepancy between the elution time of the compound labeled as impurity III (RT 17 min) and the elution time of the synthetic impurity Ia (RT 23 min when coinjected with the sample under investigation) clearly indicates that these two compounds are distinct.

Based on the above HPLC and LC/MS spectral data impurities II and III are inferred to be unknown.

3.2. LC/MS/MS analysis

Prior to characterization work on these two impurities, it is logical to generate LC/MS/MS data for fluconazole, the parent drug molecule. The mass spectrum of fluconazole exhibits a protonated molecular ion peak $[M+H]^+$ at *m/z* 307 (molecular mass of fluconazole is 306). The MS/MS spectrum obtained for the protonated fluconazole molecule showed three prominent peaks at *m/z* 289, 238 and 220 (Fig. 3B). The formation of product ion at *m/z* 289 can be attributed to the loss of H_2O (18 amu) from protonated fluconazole (*m/z* 307). The parent ion *m/z* 307 also eliminates triazole moiety (69 amu) to form the product ion at *m/z* 238 (inductive cleavage), which upon further loss of H_2O yields the product ion at *m/z* 220. These fragmentation mechanisms are depicted in Fig. 3C and D, respectively. These fragmentation pathways were used as a reference for examining spectral data acquired for the unknown impurities.

Since the molecular mass of impurity II was not in agreement with that expected for 2-(2-fluorophenyl)-1,3-bis-(1*H*-1,2,4-triazol-1-yl)-2-propanol (molecular mass 288), LC/MS/MS data was acquired to interrogate the unknown structure. The product ion spectrum of *m/z* 256 gave fragment peaks at *m/z* 169 and

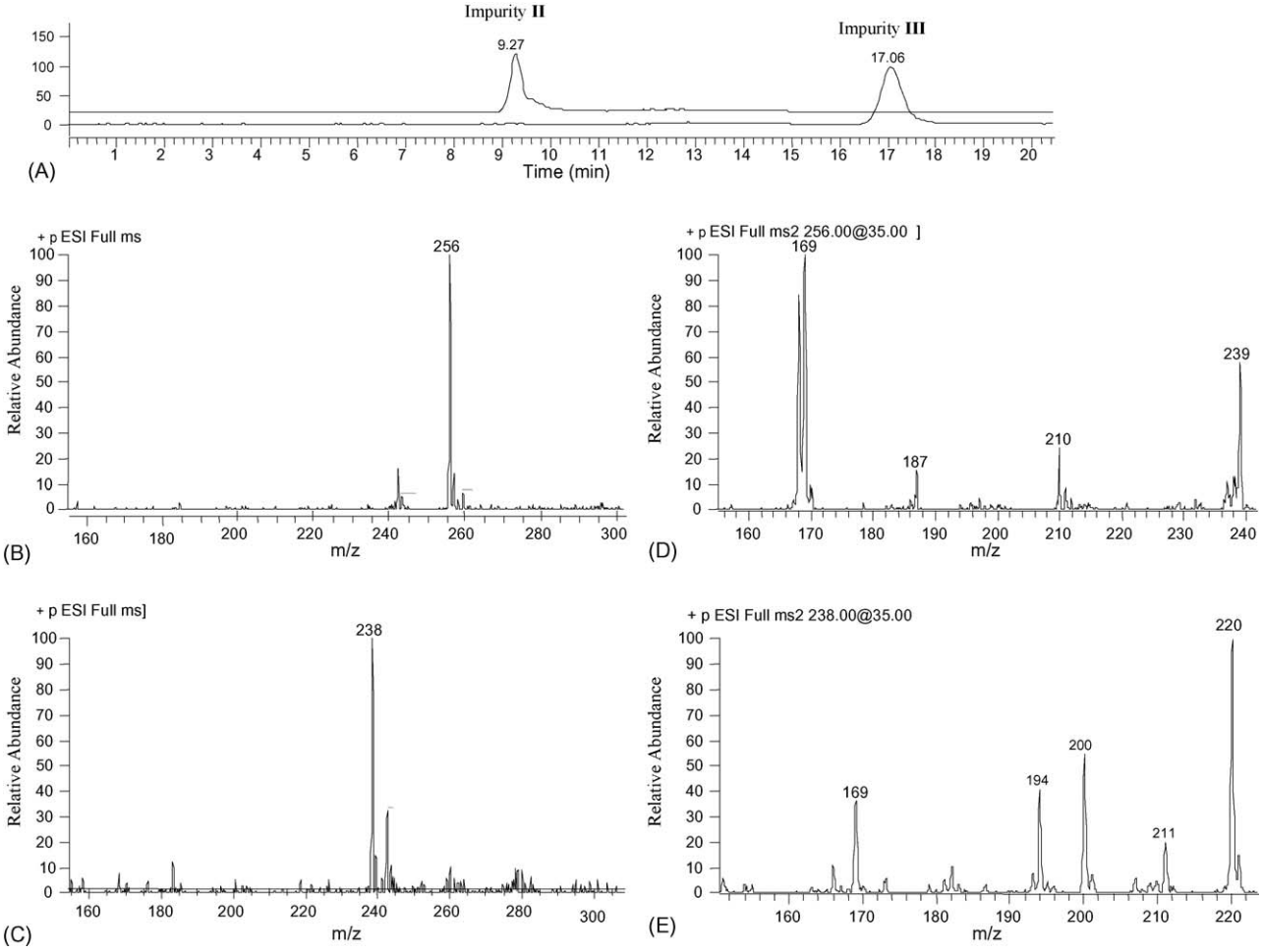

Fig. 2. LC/MS and LC/MS/MS data of impurity II and III. (A) Selected ion chromatograms for the $[M+H]^+$ ions for the two impurities, (B) mass spectrum of impurity II, (C) mass spectrum of impurity III, (D) product ion MS/MS spectrum of impurity II and (E) product ion MS/MS spectrum of impurity III.

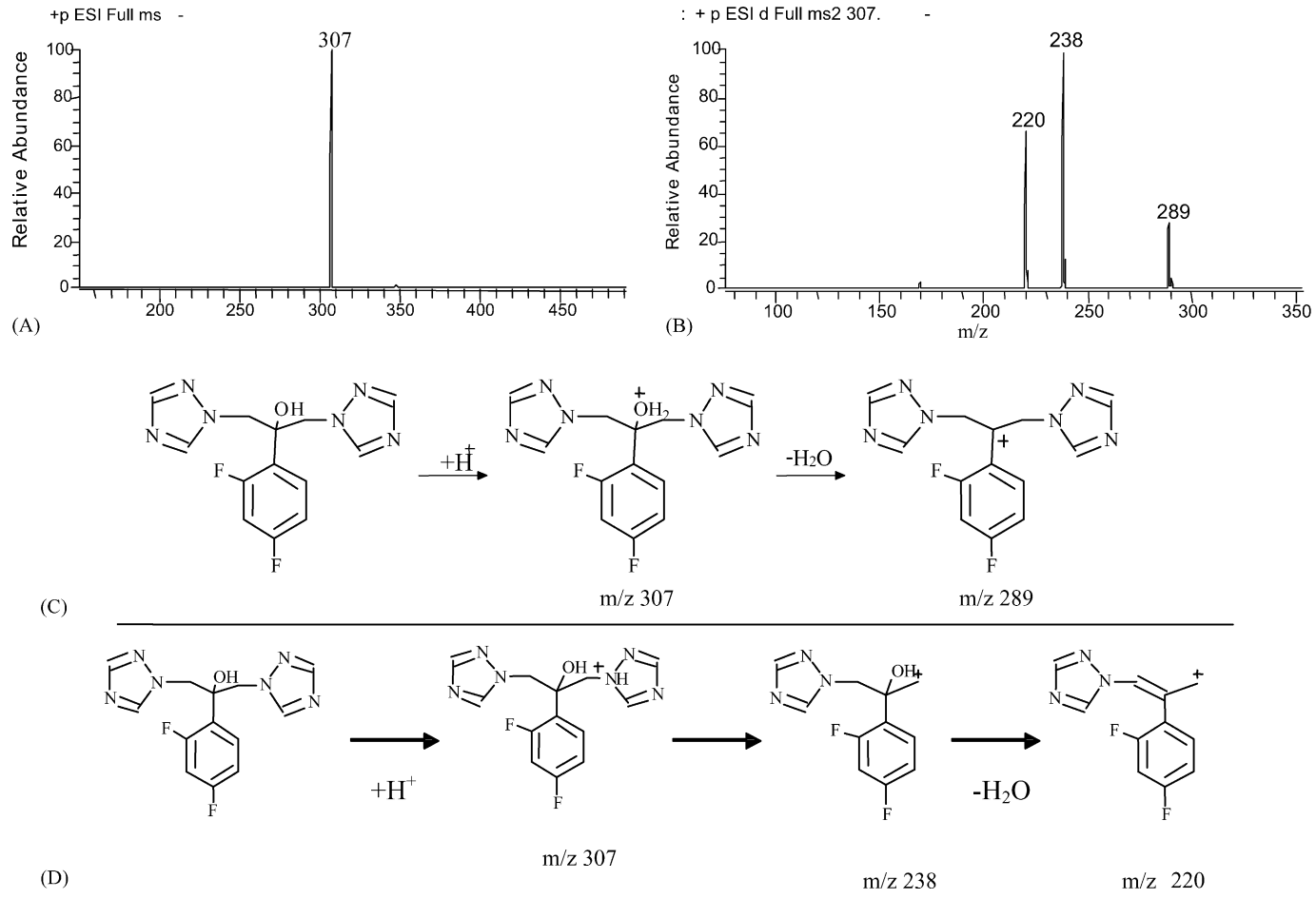

Fig. 3. LC/MS and LC/MS/MS data for fluconazole molecule. (A) Mass spectrum, (B) product ion MS/MS spectrum, (C) fragmentation mechanism for product ion with *m/z* 289 and (D) mechanism for formation of product ions with *m/z* 238 and 220.

187, as shown in Fig. 2D. Appearance of the ion *m/z* 187 from parent ion *m/z* 256 (protonated molecular ion) was attributed to the loss of triazole moiety and *m/z* 169 to the subsequent loss of a water molecule from the ion *m/z* 187. The mechanism for formation of these fragments can be rationalized from the structure 1-(1-*H*-1,2,4-triazole-1-yl) propane-2,3-diol (Fig. 4).

Impurity III and intermediate Ia exhibited protonated molecular ion peaks having the same mass-to-charge ratios, implying similar molecular formulae and possibly similar structures as well. The product ion MS/MS spectrum of the parent ion of the impurity III gave fragments at *m/z* 169 and 220. The formation of the product ion *m/z* 220 can be attributed to the loss of H_2O moiety from parent ion. The formation of another ion *m/z* 169 can be attributed to the loss of triazole moiety from the parent ion. The mechanism for this fragmentation can be rationalized from the structure 2-(2,4-difluorophenyl)-3-(1-*H*-1,2,4-triazole-1-yl)-2-propen-1-ol, and shown in Fig. 5.

3.3. Isolation of impurities by preparative HPLC

A newly developed isocratic reverse phase preparative chromatographic method (described in Section 2.3) was used for isolating impurities II and III. The retention times for impurity II, fluconazole and impurity III were observed at 2.3, 5.1 and

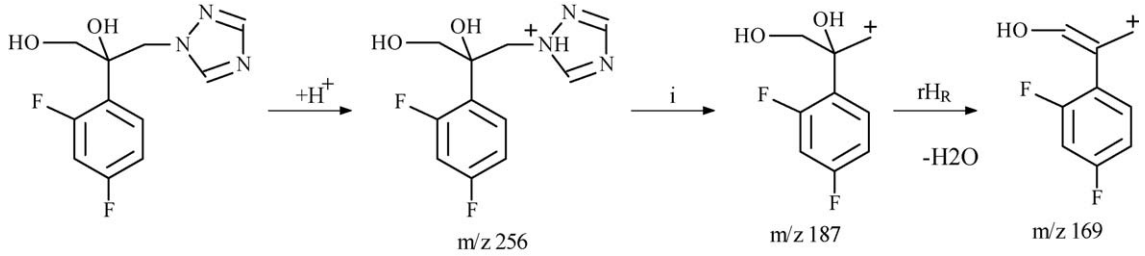

Fig. 4. Mechanism of formation for the fragment ions from impurity II.

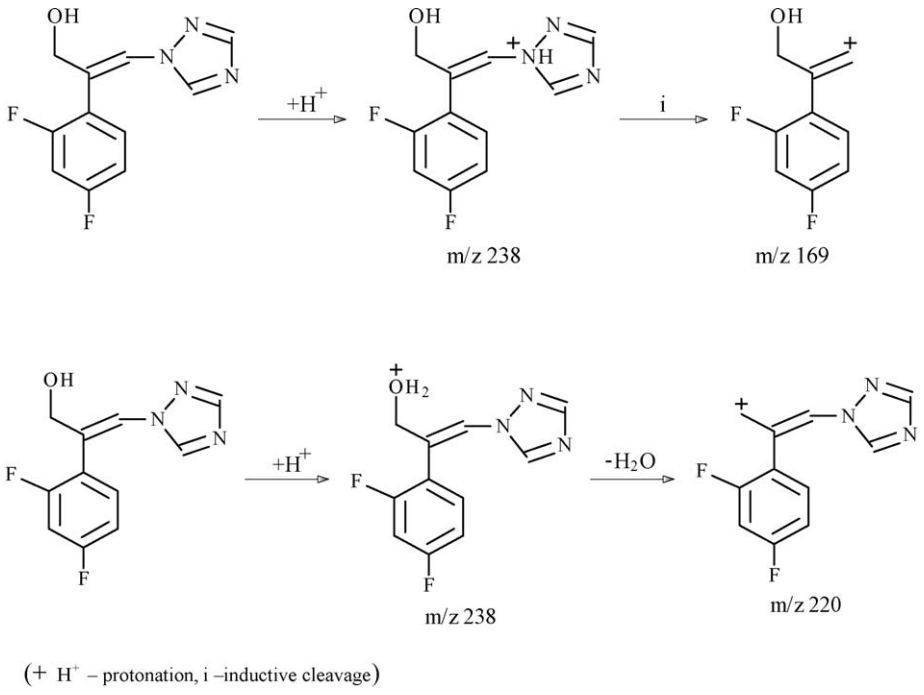

Fig. 5. Mechanism of formation for the fragment ions from impurity III.

7.3 min, respectively. About 100 mg of material was injected at a time. The shorter analysis time and higher sample loading resulted in the efficient recovery of impurity samples. The collected fractions were combined and concentrated to dryness under high vacuum using lyophilization. The chromatographic purity of these isolated impurity samples was determined by HPLC and found to be 97 and 99%, respectively. These samples were used without further purification for spectroscopic studies.

3.4. Structural elucidation of impurities

The infrared (IR) spectrum of impurity II exhibited a broad peak at 3239 cm^{-1}. Additionally, two signals in the proton NMR spectrum (5.12 and 5.80 ppm) were found to be exchangeable with D_2O. These observations indicated the presence of two hydroxyl groups in the proposed structure of impurity II. The multiplicity of the proton NMR signals (a triplet at 5.12 ppm and a singlet at 5.80 ppm) added further support to the conclusion of the former being a primary carbon (i.e. attached to $–CH_2–$ group) and the other to be of tertiary in nature (i.e. attached to tertiary carbon). The doublet at 3.5–3.7 ppm for $–CH_2–$ group confirmed to be flanked by hydroxyl group and quaternary carbon atom (for complete NMR details refer Table 1 and Fig. 6). Taken together, these data support the structure of impurity II that was proposed on the basis of MS/MS data.

The presence of broad peak at 3191 cm^{-1} in the IR spectrum and a signal at 2.7 ppm in the proton NMR spectrum (a broad

Table 1
NMR spectral assignments for impurity II

Position[a]	No. of protons (H)	Proton chemical shift δ (ppm)	J (Hz)[b]	^{13}C chemical shift	DEPT[c]
1	1H	7.15	m, 6.65, 2.5	104	CH
2	–	–	–	–	–
3	1H	6.9	m, 6.03, 2.5	111	CH
4	1H	7.38	m, 6.97, 2.03	130	CH
5	–	–	–	–	–
6	–	–	–	–	–
7OH	1H	5.8	s	75	CH
8	2H	4.57	s	54	CH_2
9	2H	3.53–3.77	d	66	CH_2
9OH	1H	5.12	t		–
10	1H	7.71	s	150	CH
11	1H	8.30	s	145	CH

[a] Refer the structural formula for numbering (Fig. 6).
[b] $^1H–^1H$ coupling constants.
[c] Hybridization (degree of bonding) of carbon atoms.

Table 2
NMR spectral assignments for impurity III

Position[a]	No. of protons (H)	Proton chemical shift δ (ppm)	J (Hz)[b]	^{13}C	DEPT[c]
1	1H	6.7–6.9	m, 2.5	105	CH
2	–	–	–		–
3	1H	6.7–6.9	m, 8.3, 2.5	112	CH
4	1H	7.05	m, 8.3	130	CH
5	–	–	–		–
6	–	–	–		–
7	–	–	–		–
8	1H	7.2	–	123	CH
9	2H	4.4	d, 4.13	65	CH2
9OH	1H	2.7	s		–
10	1H	7.8	s	151	CH
11	1H	7.6	s	142	CH

[a] Refer the structural formula for numbering (Fig. 7).
[b] ^{1}H–^{1}H coupling constants.
[c] Hybridization (degree of bonding) of carbon atoms.

hump that was found to be exchangeable with D_2O) of impurity III suggested the presence of one hydroxyl group in the structure. The singlet at 4.55 ppm in the ^{1}H NMR spectrum and a negative peak in DEPT spectrum were also found to be in agreement with the assigned structure on the basis of MS/MS data (for complete NMR details refer Table 2 and Fig. 7). In order to determine geometrical isomerism, a 1D nOe experiment was carried out [13]. Irradiation of protons at positions 10 and 11 resulted in an enhancement of intensity of protons at position 9, indicating the protons at position 9 are in proximity of triazolyl ring (Fig. 7). This observation indicated Z configuration for the impurity molecule.

3.5. Synthesis of impurities

Impurity II was synthesized by hydrolysis of intermediate Ia in aqueous NaOH solution at 60–80 °C. The reaction mixture was cooled to room temperature and extracted with dichloromethane. The organic layer was concentrated to dryness. The product obtained was recrystallised from ethanol.

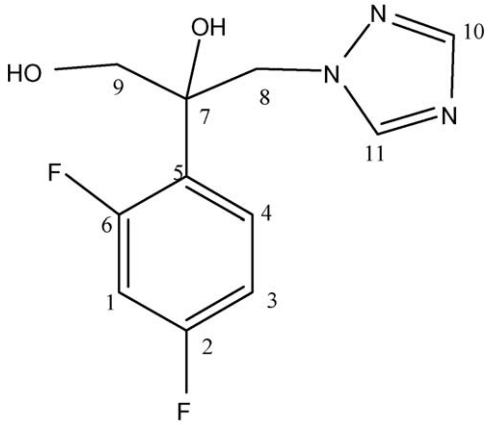

Fig. 6. Structural formula for impurity II of fluconazole.

To synthesize impurity III, the intermediate Ia was treated with NaH in anhydrous dimethylformamide at 0–5 °C. The reaction was quenched by addition of chilled water, followed by filtration through Celite. The filtrate was distilled under vacuum, and the solid obtained was recrystallised from ethanol.

3.6. Formation of impurities

During the synthesis of fluconazole, intermediate Ia was observed to react with traces of water present in reaction mixture leading to formation of impurity II, as shown in Fig. 8. Formation of impurity III can be explained through opening of an epoxide ring followed by a rearrangement (Fig. 8).

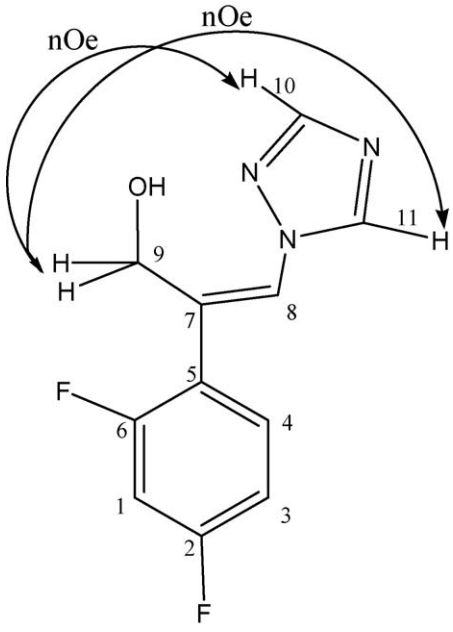

Fig. 7. Structural formula for impurity III of fluconazole, showing nOe effect.

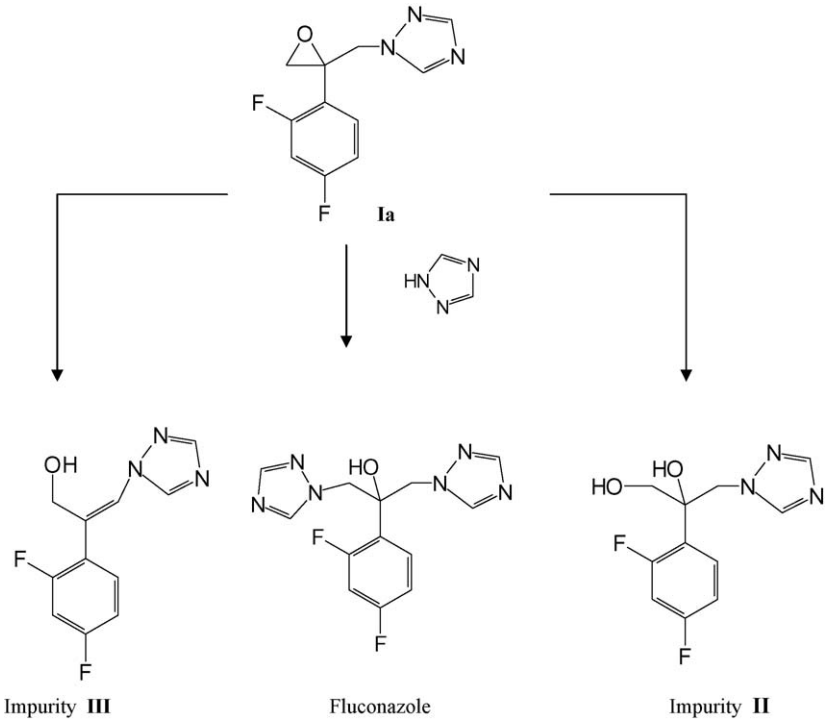

Fig. 8. Scheme for the synthesis of fluconazole showing the formation of impurities.

4. Conclusion

A study of the impurity profile of fluconazole has been carried out by LC/MS and LC/MS/MS. Preliminary structural assignments for two unknown impurities were made on the basis of mass spectral data. The complete characterization of the compounds was carried out by various spectroscopic studies after preparative chromatographic isolation. The structures were ultimately confirmed by synthesizing the two compounds.

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