Trade Science Inc.

January 2008 Volume 7 Issue 2

Analytical CHEMISTRY

An Indian Journal

Full Paper

ACAIJ, 7(2) 2008 [95-102]

Spectral characterization of itraconazole impurities in the drug product•

Nirmala Munigela[1], J.Moses Babu[1]*, Anjaneyulu Yerramilli[3], Sudhakar Parthasarathy[1], Vyas Krishnamurthy[1], K.N.Reddy[2]

[1]Department of Analytical Research, Discovery Research, Dr. Reddy's Laboratories, Bollaram Road, Miyapur, Hyderabad-500 049, (INDIA)

[2]IPD-Unit-IV Dr. Reddy's Laboratories, Bollaram Road, Miyapur, Hyderabad-500 049, (INDIA)

[3]Jawaharlal Nehru Technological University, Kukatpally, Hyderabad-500 072 (INDIA)

Tel.: +91 40 23045439x258; Fax: +91 40 23045438.

E-mail : mosesbabuj@drreddys.com

•DRL Publication No. 566

Received: 13th July, 2007 ; Accepted: 18th July, 2007

ABSTRACT

Two unknown impurities, Impurity-1 and Impurity-2 in itraconazole drug product were detected using a simple isocratic reversed-phase high performance liquid chromatography (HPLC or LC). These impurities were enriched by heat stress of the drug product and isolated from degraded sample by column purification followed by isolation through preparative high performance liquid chromatography (Prep HPLC). Based on the 2 dimensional -nuclear magnetic resonance spectroscopy (2D-NMR) data and mass spectral (MS) the Impurity-1 and 2 were characterized as N-[4-(1-sec-Butyl-5-oxo-1, 5-dihydro-[1,2,4]triazol-4-yl)-phenyl]-N-[2-({4-[2-(2,4-dichloro-phenyl)-2-[1,2,4]triazol-1-yl-methyl [1,3] dioxolan-4-ylmethoxy]-phenyl}-formyl-amino)-ethyl formamide and N-{2-[4-(1-sec-Butyl-5-oxo-1,5-dihydro-[1,2,4]triazol-4-yl)-phenyl amino]-ethyl}-N-{4-[2-(2,4-dichlorophenyl)-2-[1,2,4]triazol-1-yl-methyl-[1,3]dioxolan-4-ylmethoxy]-phenyl}-formamide respectively. The formation of these impurities is explained.

KEYWORDS

Isolation;
Spectral characterization;
Itraconazole impurities.

INTRODUCTION

Itraconazole belongs to the class of azoles. It is used in the treatment of fungal infections, such as aspergillosis, blast mycosis, histoplasmosis and those localized to the toenails and fingernails (onychomycosis) without side effects. The examples of other drugs belonging to this class are ketoconazole and fluconazole[1]. Sporanox is the brand name of itraconazole and is available in the form of 100mg capsules. Itraconazole works by inhibiting the fungal enzymes that produce "ergosterol," an important component of the fungal cell wall. Without adequate ergosterol, the fungal cell becomes weak, leaky and ultimately dies.

To our knowledge, degraded impurities of itra conazole have not been reported; hence we isolated and characterized two impurities in the drug product of itraconazole which were recorded to the extent of 0.2% by HPLC. The aim of the present study was to isolate and characterize the two major degradation impurities, as any impurity present at the level of 0.1% has to be characterized[2]. The drug product as such was degraded and it was found to have both impurities. The formation of the impurities has been explained in the present study.

Full Paper

The impurities were enriched by degradation of the drug product and then subjected to column purification.

After preparative HPLC the impurities were identified using liquid chromatography-mass spectroscopy (LC-MS) and NMR[3]. Identification of two degradation related impurities are described[4] in which piperazine ring is oxidized of an azole containing moiety which has a related structure to that of itraconazole during the stability studies on the drug substance unlike the drug product described in the present work.

EXPERIMENTAL

Materials

The investigated samples of itraconazole capsules (B.No. H001) were obtained from Dr. Reddy's Laboratories Ltd. Bulk Actives-III, Hyderabad, India. The materials used for HPLC analysis viz., ammonium acetate(AR grade) and acetonitrile(gradient grade) were purchased from SD fine chemicals, India and Ranbaxy Laboratories, India respectively. Water used was purified using Milli-Q plus purification system(Make: Milford, USA).

Heat stress of itraconazole drug product

About 50 capsules of itraconazole containing active pharmaceutical ingredient(API) 100mg (B.No. H001) were taken and the contents from capsule were ground using a pestle and mortar and the ground sample was transferred into a petridish covered with aluminum foil and stored in an oven maintained at 150^{0}C for 5 days. The heat stressed drug product became a brown chunky mass and was then subjected to column purification.

Column chromatography

The heat stressed drug product became a brown chunky mass. Two grams of this mass was dissolved in chloroform and methanol($CHCl_3$: MeOH) (90:10% v/v) and filtered, the filtrate was collected and concentrated. This concentrate was mixed with 4gm of 60-120 mesh silica in a flask and evaporated to dryness on a rotavapor.

The above evaporated product was purified using column chromatography to remove all the excipients. About 1g of the evaporated product was loaded and the column was filled with a slurry of silica gel mesh 60-120 as adsorbent and eluted with $CHCl_3$ initially, followed by $CHCl_3$: MeOH (98:2 % v/v), (97:3 % v/v) and (96:4 % v/v). Fractions were collected and those exhibiting similar thin layer chromatography (TLC) profiles were combined and evaporated on a rotavapor. HPLC purity of the column purified material was about 60%. To get good spectral data the column purified material was subjected to preparative high performance liquid chromatography.

Preparative high performance liquid chromatography (Prep HPLC)

A Shimadzu Prep HPLC (Make: Shimadzu, Japan) equipped with LC-8A pump, SCL-8A system controller, SPD-6AV UV-VIS detector, FCV-100B Fraction collector and Rheodyne Injector Model 7725I with a 2.0mL loop was used.

An in-house Prep HPLC method was developed for the separation of impurity 1 and impurity 2. The following conditions were employed for the Prep. HPLC method. Using hyperprep C18, 250×10mm I.D., 8µ particle size column (Thermocorp UK). Mobile phase consisting of ammonium acetate (0.025M), acetonitrile in the ratio of 55:45%v/v was used with UV detection at 225nm and a flow rate of 10mL/min. The data was recorded using CR-7A chromatopac software. The column purified sample was injected into the semi-preparative column and impurities were collected and evaporated.

To confirm the retention times of the isolated impurities, they were analysed by analytical HPLC. The 2D NMR and MS data of the isolated fractions of impurity-1 and impurity-2 after thorough drying of the solvents were recorded.

High performance liquid chromatography (analytical)

A Waters Model Alliance 2695 Separations module(Make: Waters corporation, USA) equipped with a Waters 2996 photo diode array UV detector was used. A simple in-house isocratic LC method was developed for the analysis of itraconazole drug product and its degradation products, where a C_{18} column, Hypersil BDS C18, 250×4.6mm I.D., 5µ (Thermocorp UK) with a mobile phase consisting of a mixture of

ammonium acetate (0.025M) and acetonitrile in the ratio of 55:45(v/v) was used with UV detection at 225nm at a flow rate of 1.5mL/min for the resolution of both impurities. The itraconazole drug product was extracted into methanol and sonicated the sample; the same was filtered with 0.22µ syringe filter and injected at a concentration of 1mg/mL into HPLC. The impurities were dissolved in methanol, sonicated and injected at a concentration of 0.5mg/mL after filtering through 0.22µ syringe filter before injecting into HPLC. The data was recorded using Waters Empower software Build 1154.

Electrospray ionization(ESI-MS/MS)

The MS/MS experiments were performed on a PE-SCIEX triple quadrupole MS(Model API 3000, Make: MDS SCIEX, Vendor name: Applied biosystems). The sample was introduced through a turbo ion spray interface in positive ionization mode using infusion pump. The nebuliser and curtain gases used were zero air and nitrogen respectively, ion spray voltage was maintained at 4500V. Focusing potential, declustering potential and entrance potential were kept at 180 V, 70 V and 10 V respectively.

NMR

The proton NMR(^{1}H NMR), carbon-13(^{13}C NMR), distortionless polarization transfer (DEPT) and 2D NMR experiments(gDQCOSY, gHSQC and gHMBC) for itraconazole, impurities 1 and 2 were performed in $CDCl_3$ solvent(Procured from Cambridge Isotopic Labs, USA) using Varian Mercury plus 400 MHz FT NMR spectrometer(Varian, Germany) equipped with a 5-mm ID probe at 298 K. The data were collected and processed by Varian NMR 6.1C Version software running on SUN ULTRA-10 PC with microsoft windows^{xp}. For the ^{1}H- NMR analysis, 24 transients were acquired with a 5-s relaxation delay using 32K data points. The 45° pulse duration was of 4µs and spectral width of 8800 Hz. The digital resolution was 0.25 Hz and line broadening was 0.2. The ^{1}H-^{1}H bond correlations were confirmed by gDQCOSY experiments. The protonated carbon positions were confirmed by gHSQC experiments. The non-protonated carbons were confirmed by a gHMBC experiments. The ^{1}H chemical shifts are reported in ppm with reference to tetramethyl silane(δ=0.0ppm). The ^{13}C NMR experiments were carried out with a spectral width of 59880 Hz using 60 K data points, chemical shift values were reported relative to $CDCl_3$(δ=77.00ppm) and DMSO-d_6(δ=39.50 ppm) as internal standards respectively.

LC-MS

LC-MS experiments on the degraded drug product of itraconazole were performed on a PE SCIEX API 3000(Make: MDS SCIEX, Vendor name: Applied Biosystems) Q trap LC-MS/MS mass spectrometer using an Agilent 1100 series LC pump. The mobile phase used was A: Ammonium acetate(0.01M) pH=7.0 with liquor ammonia (Qualigens, India); B: Acetonitrile using the following gradient program(T/%B: 0/50, 16/100) with a flow rate of 1.0mL/min and monitored at a wavelength of 225nm. A Hypersil BDS C18(250× 4.6mm) 5µ was used. The concentration of samples was 1mg/mL with an injection volume of 20µL. Zero air was used for nebulization and high pure nitrogen was used as collision gas. The following MS conditions were used for data acquisition: Nebulizer 8.00psi, Curtain gas 8.00 psi, Ion spray voltage 4500V, Temperature 0^{0}C, Declustering potential 70V, Focusing potential 180V, Entrance potential 10V.

High resolution mass

All samples were analysed on the Micromass LCT Premier mass spectrometer(Waters Corporation, UK) equipped with an(Electro spray ionization) ESI Lock spray source for accurate mass values. Leucine enkephalin was used as an internal reference compound which was introduced via the Lock spray channel using the Waters reagent manager. The mass range was calibrated with the cluster ions of sodium formate using a fifth order polynomial fit. Data were acquired using the W mode.

The mass spectrometer was equipped with a Waters Acquity system. Itraconazole capsule was dissolved in methanol at a concentration level of 1mg/mL, sonicated for 10 minutes and centrifuged for 6 minutes at 16000 rpm. The supernatant was diluted 1:10 with methanol and introduced to the mass spectrometer via a HPLC column. The chromatographic conditions used were same as LC-MS.

Full Paper

RESULTS AND DISCUSSION

The analytical HPLC chromatograms of itraconazole capsule(Figure 1a), itraconazole impurity-1, after isolation(Figure 1c), itraconazole impurity-2, after isolation(Figure 1b) and itraconazole capsule heat stressed sample are shown in(Figure 1d). The itraconazole eluted at a retention time(RT) of 44.08min(Figure 1a). In the chromatogram of the stressed sample two prominent impurities 1 and 2 eluted at 12.04min and 16.50 min respectively in addition to the itraconazole peak at 44.08 min (Figure 1d).

To get further structural insight, the LC-MS analysis was carried out on the stressed sample. The mass spectra of stressed sample thus obtained showed the protonated molecular ions of the impurities -1 and 2 at m/z 735 and 707 respectively, whereas the itraconazole displayed protonated molecular ion at m/z 705. Thus the impurity-1 and impurity -2 has 30 atomic mass units (amu) and 2 amu more than the molecular ion of itraconazole. The results of LC-MS and LC are given in TABLE 1.

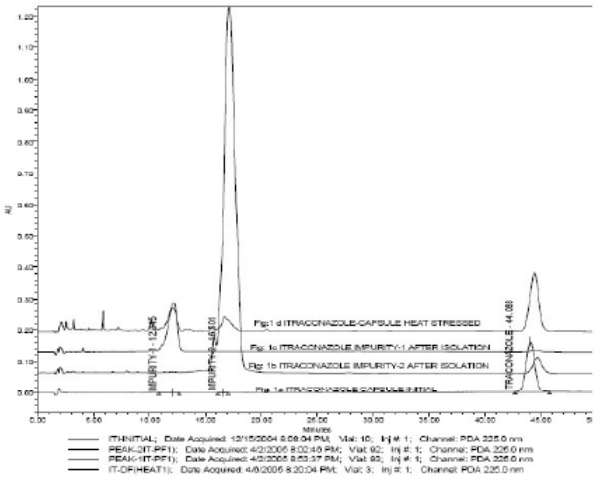


Figure 1: Overlay of HPLC chromatograms of itraconazole capsule initial, itraconazole capsule heat streesed, impurity-1 and impurity-2 after prep HPLC isolation

As the molecular ion information is not sufficient to arrive at the structure, the drug product was stressed and subjected to purification by Prep HPLC so as to isolate more quantities of impurity-1 and impurity-2 for

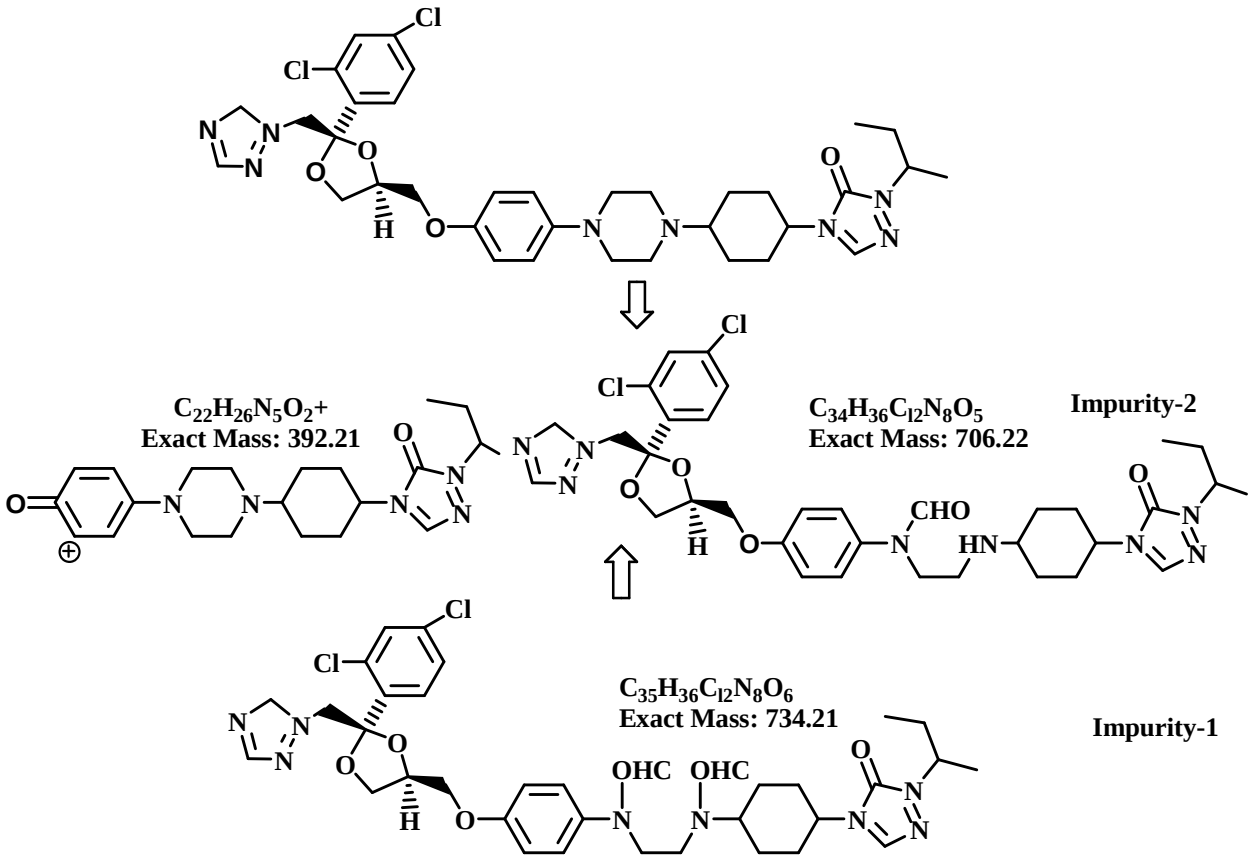
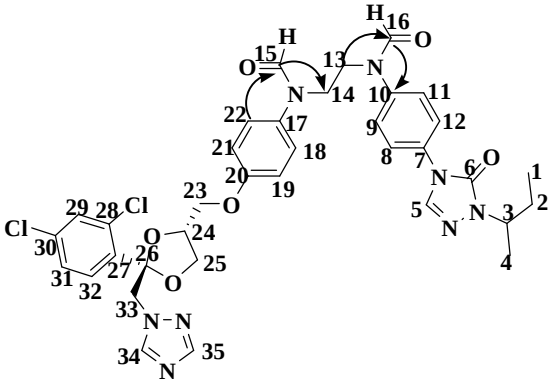
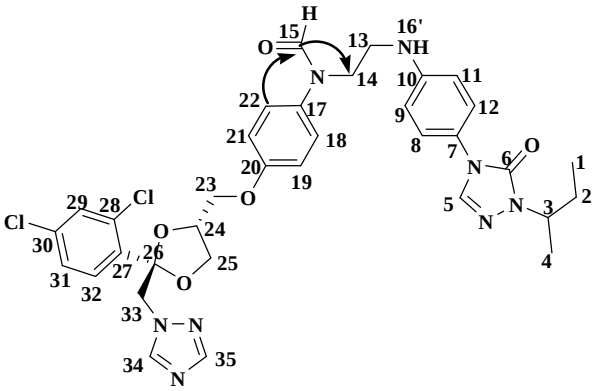
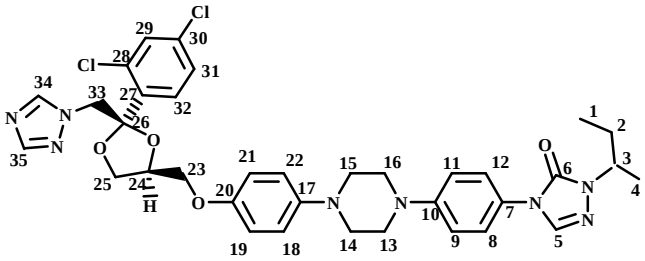


Figure 2 : Fragmentation schemes of Itraconazole, impurity-1 and impurity-2

TABLE 1: LC-MS data and HPLC data of Itraconazole impurities

S.no	HPLC RT(min)	LC-MS RT(min)	Structure/Molecular weight	Remarks
1	12.04	7.72	 Mol.Wt.734	Impurity-1 (Degradation related)
2	16.50	8.52	 Mol.Wt.706	Impurity-2 (Degradation related)
3	44.09	11.26	 Mol. Wt.704	API in drug product

spectroscopic investigation. The NMR data of the isolated impurities were collected and the structures of impurities have been assigned with the help gradient double quantum correlation spectroscopy (gDQCOSY or DQCOSY), gradient single quantum correlation spectroscopy (gHSQC or HSQC) and gradient hetero multiple bond correlation spectroscopy (gHMBC or HMBC) data.

Structure elucidation of impurity 1

The ^{1}H NMR data was compared with those of itraconazole. The spectral data was similar, but the resonances of the piperazine ring in itraconazole are absent. In itraconazole the four methylenes of piperazine resonate at δ 3.37 and 3.24ppm each integrating for two protons with their corresponding ^{13}C resonances at δ 50.27 and 48.94 ppm respectively. On the other hand, impurity 1 has only two methylenes at 3.97 and 4.03 with their connected carbons resonating at δ 43.6 and 43.3 respectively. In addition, two singlets at δ 8.22 and 8.35ppm each integrating for one proton has been observed. These are connected to carbonyl resonances at δ 162.50 and 162.96ppm indicating the presence of aldehyde functionality. To identify the location of these new aldehydic groups in the structure of impurity 1, long range proton carbon experiment (gHMBC) was

Full Paper

TABLE 2: ^{1}H and ^{13}C NMR assignments for itraconazole, impurity-1 and impurity-2

Position	Itraconazole				Impurity-1				Impurity-2			
No.	1H	ppm/J	13C	DEPT	1H	ppm/J	13C	DEPT	1H	ppm/J	13C	DEPT
1	3H	0.91/t7.4	10.56	CH_3	3H	0.91/t7.4	10.87	CH_3	3H	0.90/t7.6	10.99	CH_3
2	2H	Ha1.72/m, Hb1.87/m	28.18	CH_2	2H	Ha1.74/m, Hb1.87/m	28.60	CH_2	2H	Ha1.71/m, Hb1.85/m	28.48	CH_2
3	1H	4.30/m	52.41	CH	1H	4.30/m	52.90	CH	1H	4.20/m8.0	52.96	CH
4	3H	1.39/d6.8	19.01	CH_3	3H	1.40/d6.8	19.80	CH_3	3H	1.38/d6.8	19.30	CH_3
5	1H	7.65/s	133.69	CH	1H	7.60/s	131.67	CH	1H	7.60/s	123.40	CH
6	-	-	151.77	-	-	-	151.69	-	-	-	152.37	-
7	-	-	152.35	-	-	-	157.40	-	-	-	133.50	-
8	1H	6.95/d8.8	118.16	CH	1H	7.02/d8.8	126.15	CH	1H	7.27/m	124.55	CH
9	1H	6.81/dd8.8	115.07	CH	1H	6.85/d8.8	115.70	CH	1H	6.60/d8.8	113.06	CH
10	-	-	145.80	-	-	-	151.56	-	-	-	147.67	-
11	1H	6.81/d8.8	115.07	CH	1H	6.85/d8.8	116.11	CH	1H	6.60/d8.8	113.06	CH
12	1H	6.95/d8.8	118.16	CH	1H	7.02/d8.8	126.15	CH	1H	7.27/m	124.55	CH
13	2H	3.24/br	48.94	CH_2	2H	3.97/m	43.60	CH_2	2H	3.30/m	42.39	CH_2
14	2H	3.37/br	50.27	CH_2	2H	4.03/m	43.30	CH_2	2H	4.00/m	45.43	CH_2
15	2H	3.37/br	50.27	CH_2	1H	8.35/s	162.50	CH	1H	8.40/s	163.75	CH
16	2H	3.24/br	48.94	CH_2	1H	8.22/s	162.96	CH	-	-	-	-
16′	-	-	-	-	-	-	-	-	1H	8.20br	-	NH
17	-	-	125.71	-	-	-	139.70	-	-	-	157.62	CH
18	1H	7.40/d2.0	123.22	CH	1H	7.65/d8.8	123.51	CH	1H	6.86/d8.8	115.78	CH
19	1H	7.03/d2.4	116.35	CH	1H	7.23/d8.8	125.14	CH	1H	7.08/d8.4	127.09	CH
20	-	-	150.23	-	-	-	132.96	-	-	-	136.50	-
21	1H	7.03/d2.4	116.35	CH	1H	7.23/d8.8	125.14	CH	1H	7.08/d8.4	127.09	CH
22	1H	7.40/d2.0	123.22	CH	1H	7.65/d8.8	123.51	CH	1H	6.86/d8.8	115.78	CH_2
23	2H	Ha 3.80/m H_b4.00/m	67.47	CH	2H	Ha3.50/m, Hb3.80/m	67.60	CH_2	2H	Ha 3.51/m H_b3.78/m	67.63	CH_2
24	1H	4.20/m	74.50	CH	1H	4.40/m	74.70	CH	1H	4.38/m	74.65	CH_2
25	2H	Ha 3.20/m H_b 3.82/m	67.17	CH	2H	Ha 3.94/m Hb3.83/m	67.40	CH_2	2H	Ha 3.93/m H_b 3.83/m	67.35	CH_2
26	-	-	107.39	-	-	-	107.90	-	-	-	107.95	-
27	-	-	135.74	-	-	-	136.29	-	-	-	136.50	-
28	-	-	132.88	-	-	-	133.19	-	-	-	133.37	CH
29	1H	7.48/d1.6	131.14	CH	1H	7.49/d2	131.88	CH	1H	7.48/d2	131.70	CH
30	-	-	133.92	-	-	-	133.97	-	-	-	134.10	-
31	1H	7.60/d0.8	129.39	CH	1H	7.28/dd2,8	127.72	CH	1H	7.57/d8.4	134.56	CH
32	1H	7.24/d	126.99	CH	1H	7.60/d8.8	129.93	CH	1H	7.59/m	129.93	CH_2
33	2H	4.80/q16.0	53.37	CH_2	2H	4.81/q16.0	53.30	CH_2	2H	4.81/q14.8	53.66	CH_2
34	1H	7.80/s	144.69	CH	1H	7.92/s	151.90	CH	1H	7.91/s	151.66	CH
35	1H	8.20/d5.6	151.10	CH	1H	8.22/s	145.26	CH	1H	8.18/s	144.32	CH

Refer TABLE 1 for numbering: J- This column gives the ^{1}H-^{1}H coupling constant, (s) Singht, (d) Doublet, (t) triplet, (m) Multiplet, (br) broad

performed. Interestingly these two aldehydic carbons C-15 and C-16 at δ 162.50 & 162.96ppm are connected to the two methylenes mentioned above viz., at δ 43.60 and 43.30 ppm respectively through long range bond correlation and also with aromatic methine(C-22) at δ 123.51ppm and aromatic quaternary carbon at δ 151.56ppm(C-10) respectively. These interactions are shown in the structure(TABLE 1). Thus the formation of impurity 1 can be explained by the opening of the piperazine ring, followed by the oxidation of two methylenes to form two aldehyde groups.

The mass spectral fragmentation of itraconazole and impurity -1 were compared. The data indicates that the diagnostic fragment in itraconazole is at m/z 392 whereas the MS/MS data displayed a diagnostic fragment at m/z 475(Figure 2) with characteristic isotopic abundance for two chlorine atoms.

In itraconazole also the isotopic abundance for chlorine atoms is seen this indicates that there is no change in ring attached to triazole ring. The HR MS data showed

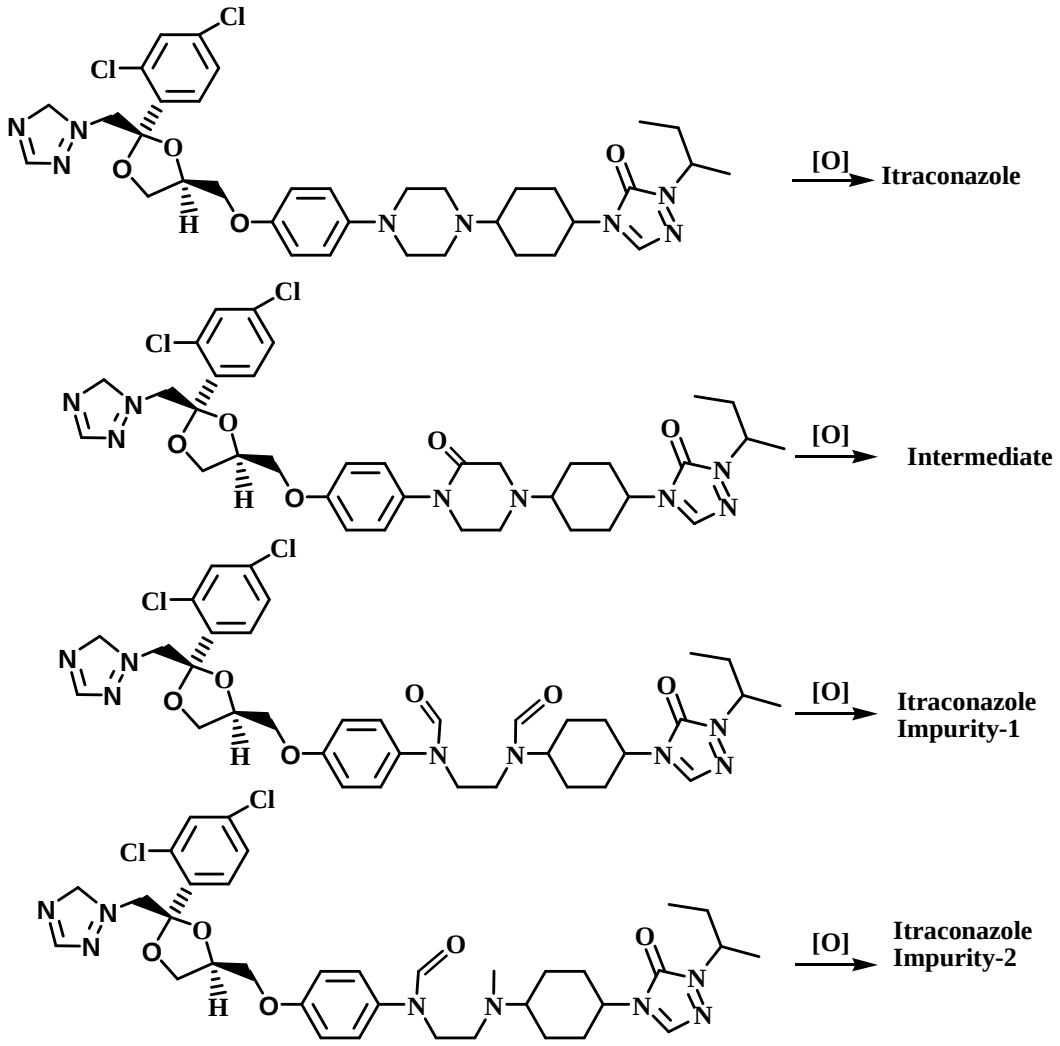


Figure 3 : Probable reaction mechanism for formation of impurities on oxidation

exact mass of the protonated molecular ion at m/z 735.2211(Cal. 735.2213 for $C_{35}H_{36}N_8O_6Cl_2$) which corresponds to the molecular formula $C_{35}H_{35}N_8O_6Cl_2$. Thus structure of the impurity-1 was characterized as N-[4-(1-sec-Butyl-5-oxo-1,5-dihydro-[1,2,4]triazol-4-yl)-phenyl]-N-[2-({4-[2-(2,4-dichloro-phenyl)-2-[1,2,4]triazol-1-yl-methyl-[1,3]dioxolan-4-yl-methoxy]-phenyl}-formyl-amino)-ethyl formamide. The structure is shown in TABLE 1 along with numbering scheme and the NMR assignments are tabulated in TABLE 2.

Structure elucidation of impurity 2

The ^{1}H NMR data of impurity 2 was compared with those of both itraconazole and impurity 1. The spectral data was similar but the resonances of the piperazine ring were absent. In itraconazole the four methylenes of piperazine resonate at δ 3.37 and 3.24ppm each integrating for two protons with their corresponding ^{13}C resonances at δ 50.27 and 48.94ppm respectively. On the other hand, impurity 2 has only two methylenes at δ 3.30 and 4.00ppm with their connected carbons resonating at δ 42.39 and 45.43ppm respectively. In addition a singlet at δ 8.40 ppm connected to carbonyl resonance at δ 163.75ppm and a broad multiplet at δ8.20ppm having no connected carbon, each integrating for one proton were observed. This clearly indicates the presence of only one aldehydic group in impurity 2.

To identify the location of the aldehydic group in the structure of impurity-2 long range proton-carbon experiment(HMBC) was performed. The aldehydic

Full Paper

proton at H-15(δ 8.40ppm) shows long range through bond correlation with methylene at C-14(δ 45.43ppm) and aromatic methine at C22(δ 115.78ppm). These interactions are shown in TABLE 1. Thus the impurity-2 structure can be explained as the opening of the piperazine ring and one methylene attached to nitrogen being oxidized to form an aldehyde group and the loss of the second methylene attached to other nitrogen.

The mass spectral fragmentation of itraconazole and impurity 2 were compared. The data indicates that the diagnostic fragment in itraconazole is at m/z 392 whereas the MS/MS data of impurity 2 displayed a diagnostic fragment at m/z=475 with characteristic isotopic abundance for two chlorine atoms. Thus the fragmental data lends support to the proposed structure of impurity 2. Similar to impurity-1 the product ion at m/z 475 was observed showing the evidence for the opening of the center piperazine ring(Figure 2). The MS/MS experiments also confirm the proposed structure by NMR giving a conclusive evidence for the structure of impurity 2. The HR MS data of impurity 2 showed exact mass of the protonated molecular ion at m/z 707.2270 (Cal.707.2264 for $C_{34}H_{36}N_8O_5Cl_2$) which corresponds to the molecular formula $C_{34}H_{35}N_8O_5Cl_2$.

Thus structure of impurity 2 was characterized as N-{2-[4-(1-sec-Butyl-5-oxo-1,5-dihydro-[1,2,4] triazol-4-yl)-phenyl amino]-ethyl}-N-{4-[2-(2,4-dichlrophenyl)-2-[1,2,4]triazol-1-ylmethyl-[1,3] dioxolan-4-ylmethoxy]-phenyl}-formamide. The structure is shown in TABLE 1 along with numbering scheme and the NMR assignments are tabulated in TABLE 2.

Formation of impurities

Both impurity 1 and impurity 2 were formed due to the heat stress of itraconazole drug product. However, stressing the isolated impurity 1 does not generate impurity 2. The probable reaction mechanism is shown in figure 3; both impurities are formed on oxidation of the drug product. Impurity 1 is formed due to the cleavage of the piperazine ring and the impurity 2 is obtained by the loss of carbonyl group from impurity 1.

ACKNOWLEDGMENTS

The authors wish to thank the management of Discovery Research, Dr. Reddy's Laboratories Ltd. for supporting this work. Cooperation from colleagues of Analytical Research Department of Discovery Research is appreciated.

REFERENCES

[1] William E.Dismukes; Clin.Infect.Dis., **30**, 653-657 **(2000)**.
[2] Q3B (R), ICH Guidance for Industry., **(2002)**.
[3] Karen M.Alsante, Peter Boutros, Michel A. Couturier, Robert C.Friedmann, Jeffrey W. Harwood, George J.Horan, Andrew J.Jensen, Qicai Liu, Linda L.Lohr, Ronald Morris, Jeffrey W.Raggon, George L.Reid, Dinos P.Santafianos, Thomas R.Sharp, John L.Tucker, Glenn E.Wilcox; J.Pharm.Sci., **93(9)**, 2296-2309 **(2004)**.
[4] Wenqing Feng, Haiying Liu, Guodong Chen, Rodney Malchow, Frank Bennett, Elizabeth Line, Birendra Pramanik, Tze-Ming Chan; J.Pharm.Biomed.Anal., **25**, 545-557 **(2001)**.