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Isolation By Preparative HPLC And Characterization Of Process-Related Impurities In Tebuconazole

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ABSTRACT

Two unknown impurities in tebuconazole bulk material, at levels ranging from 0.07% to 0.3% were detected by a gradient RPHPLC and gas chromatography (GC). The impurity at 0.3% level was isolated from a crude sample of tebuconazole using reverse phase preparative LC. The level of the other impurity in crude sample of tebuconazole was less than 0.1% and hence it was not isolated. Rather this impurity has been characterized by LC-MS/MS and GC-MS experimental data. The impurity isolated by preparative LC was characterized by NMR, MS experimental data. Based on the results obtained from different spectroscopic experiments, these impurities have been characterized as 3-((4H-1,2,4-triazol-4-yl)methyl) methyl)-1-(4-chlorophenyl)-4,4-dimethylpentan-3-ol. and 3-((1H-1,2,4-triazol-1-yl)methyl)-4,4-dimethyl-1-phenylpentan-3-ol. Formation of these impurities also discussed in details.

KEYWORDS

Tebuconazole;
Preparative HPLC;
Impurities;
Identification and characterization.

INTRODUCTION

Tebuconazole, 1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol, is a potent antifungal agent. High performance liquid chromatographic(HPLC) method was cited in the literature for testing the enantiomeric purity of tebuconazole bulk material and its enantiomeric impurities[1]. Even the CIPAC method reported, deals with the assay of tebuconazole technical material by gas chromatography[2] which is not useful for the determination of potential impurities of tebuconazole technical material. The stringent purity requirement from regulatory agencies that all the individual impurities,

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which are =0.1%, must be identified and characterized, this paper aims at the identification and characterization of two impurities in tebuconazole active ingredient by LC-MS/MS and NMR in tebuconazole. To the best of our knowledge, these impurities are novel, and are not reported in literature. A thorough study has been undertaken to isolate, wherever possible, and characterize these impurities by chromatographic and spectroscopic techniques.

EXPERIMENTAL

Samples and chemicals

The samples of bulk tebuconazole were received from Excel Crop Care Ltd, Mumbai, India. HPLC grade acetonitrile was obtained from Merck Co., Mumbai, India. HPLC grade acetone was obtained form Rankem, Mumbai, India. Ultra pure water was collected from Elix Millipore Water Purification System. HPLC grade dichloromethane used for liquid/liquid extraction was purchased from Spectrochem, Mumbai, India. Nitrogen, hydrogen and zero air used were of ultra pure grade(99.999%).

HPLC

An Agilent HPLC system equipped with 1200 series low pressure quaternary gradient pump along with pulse dampener, photo diode array detector and auto liquid sampler handling system has been used for the analysis of samples. The data was collected and processed using Agilent 'Chemstation' software. An Inertsil ODS-3V(250* 4.6mm, 5-Micron, GL Sciences, Japan) column was employed for the separation of impurities from tebuconazole. The column eluent was monitored at 220nm. A linear gradient program was optimized for the separation of impurities from tebuconazole active ingredient where the initial mobile phase ratio was a mixture of water and acetonitrile in a ratio of 45:55, v/v for 15 min. Subsequently the percentage of acetonitrile in the mobile phase was increased from 55 to 90 up to 30 min. The same ratio was held for 20min, and brought back to initial condition within 5 min. The column was allowed to get equilibrated for 10min before performing the next injection. Chromatography was performed at room temperature using at a flow rate of 1.0ml min^{-1}

Gas chromatography(GC)

An Agilent 6890N series GC system equipped with FID(Flame Ionization Detection) detector, auto sampler has been used. The data was collected and processed using Agilent 'Chemstation' software. A capillary column, HP-1(30m×0.32mm i.d. ×0.12μm film thickness) was employed for the separation. The FID detector and injector temperatures were set at 320^0C and 290^0C respectively. Initial temperature of oven was set at 200^0C for 3 min followed by increasing temperature to 280^0C at the rate of 30^0C min^{-1} and held for 17 min. The constant flow of the column was set at 4 ml min^{-1}. Injection volume was 2μl. Sample was dissolved in acetone and injected in split mode using a split ratio of 1:5.

HPLC(preparative)

An Agilent preparative HPLC system equipped with 1200 series pump, photo diode array detector, auto liquid sampler handling system fitted with 900μL loop and 1200 series preparative fraction collector has been used. The data was collected and processed using agilent 'Chemstation' software. A Zorbax C18 column (50×50mm, 5-Micron, Agilent Technologies) was employed for loading the sample. An analytical method was developed in isocratic mode separately to resolve these impurities, followed by scaling up the same method for prep LC to collect the required impurity fractions. The mobile phase consisted of water acetonitrile in the ratio of 25:75 (v/v). The flow rate was set at 25ml min^{-1}. Detection was carried out at 220 nm. Approximately 200mg ml^{-1} of sample was prepared using acetonitrile and water as diluent in the ratio of 50:50, v/v to load on to the column.

Mass spectrometry(GC-MS)

Mass spectra were run on HP 5989 using ionization electron beam energy of 70 eV. The sample was introduced into the source by connecting the capillary GC column. Initial temperature of oven was set at 200^0C for 3 min followed by increasing temperature to 300^0C at the rate of 30^0Cmin^{-1} and held for 20min. The source manifold and quadrupole temperatures were maintained at 230^0C and 150^0C respectively.

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Mass spectrometry(LC-MS/MS)

LC-MS/MS analysis has been performed on API 2000, mass spectrometer(Applied Biosystems). The analysis was performed in positive ionization mode with turbo ion spray interface. The parameters for ion source voltage IS=5500 V, declustering potential, DP=70 V, focusing potential, FP=400 V, entrance potential, EP=10 V were set with nebulizer gas as air at a pressure of 40 psi and curtain gas as nitrogen at a pressure of 25 psi. An Inertsil ODS-3V(250×4.6 mm, 5-Micron, GL Sciences, Japan) column was used for the separation. The mobile phase is a mixture of water, acetonitrile in a ratio of 45:55(v/v). The analysis was performed at a flow rate of 1.0 ml min^{-1}.

NMR spectroscopy

The ^{1}H and ^{13}C NMR experiments of one of the impurities were carried out at a precessional frequencies of 200 MHz and 50MHz respectively, in $CDCl_3$ at 25^0C temperature on a Bruker Avancedpx-200 FT NMR spectrometer. ^{1}H and ^{13}C chemical shifts are reported on the δ scale in ppm, relative to tetra methyl silane(TMS) δ 0.00 and $CDCl_3$ at 77.0 ppm in ^{13}C NMR respectively. D_2O exchange experiment was performed to confirm the exchangeable protons.

FT IR spectroscopy

IR spectra of one of the impurities of tebuconazole was recorded in the solid state as KBr dispersion using perkin elmer spectrum 100 FT-IR spectrophotometer with DRS(Diffuse Reflectance Sampler) accessory.

RESULTS AND DISCUSSIONS

Detection of impurities by HPLC

Typical analytical LC chromatogram of tebuconazole and its impurities obtained by using the LC method discussed, is shown in figure 1. The targeted impurities under study are marked as I and II eluted at retention times of about 7 min and 9 min respectively, while tebuconazole eluted at about 14 min. Crude sample of tebuconazole was also injected to check the levels of impurities.

Detection of impurities by GC

Typical GC chromatogram of tebuconazole and its impurities obtained by using the GC method discussed, are shown in figure 2. The targeted impurities under study are marked as I and II with retention times of about 8 and 17min, respectively, while tebuconazole eluted at about 9min. Interestingly in GC analysis the impurity I(marked in LC chromatogram) eluted after the tebuconazole. There was a significant increase in the area percentage of the other impurity in GC analysis when compared to that of analytical LC. The difference in the response of this impurity on HPLC and GC may be attributed to the detection mode(UV detection in HPLC and FID in GC).

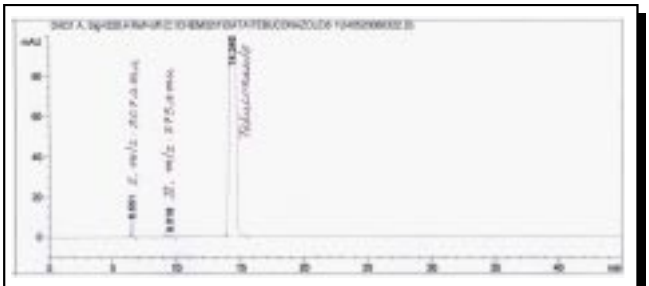

Figure 1: A typical LC chromatogram of tebuconazole bulk material

LC-MS/MS analysis

LC-MS/MS analysis of crude sample of tebuconazole was performed using the solvent system as described. Results of LC-MS/MS analysis revealed that first impurity exhibited molecular ion at m/z(M+1) 308 amu while the second impurity exhibited molecular ion at m/z 274(M+1) amu. Based on this fact it was assumed that the first impurity was similar to that of tebucona-zole which might be a positional isomer.

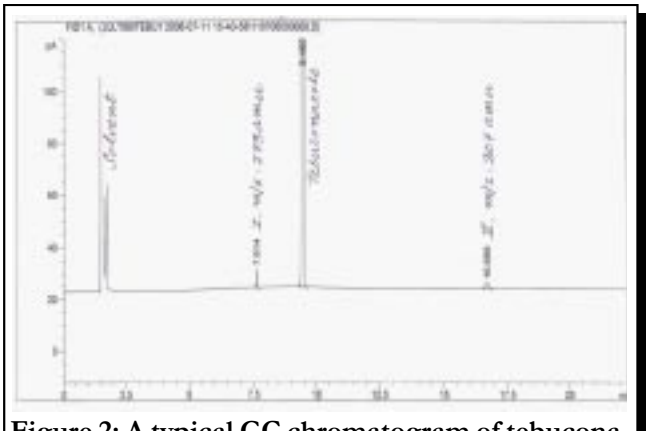

Figure 2: A typical GC chromatogram of tebuconazole bulk material

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The second impurity was assumed to be a deschloro derivative of tebuconazole based on the absence of chlorine isotopic abundance in the MS profile. To unambiguously confirm the structure of first impurity(m/z 308, M+1), this impurity was isolated by preparative LC in a pure form. Isolation of second impurity was not taken up since its area percentage was less in the technical as well as crude samples.

GC-MS analysis

GC-MS analysis of tebuconazole was performed using the method as described. Interestingly in GC the first impurity observed in HPLC eluted after the tebuconazole peak. Electronic impact ionization of impurity eluting before tebuconazole exhibited molecular ion at m/z 273(M+) amu where the chlorine abundance was found to be absent. This profile matched with the profile of second impurity which eluted in HPLC analysis. Fragmentation pattern also matched in LC-MS/MS and GC-MS for this impurity. The other impurity which eluted after the tebuconazole exhibited molecular ion at m/z 307(M+) amu which matched with the first impurity eluted in HPLC analysis. It was quite interesting to note that the fragmentation pattern of this impurity was exactly matching in both LC-MS/MS and GC-MS analysis.

Isolation of the impurity(m/z; 307) by preparative HPLC

A simple reverse phase solvent system discussed, was used for isolating the impurity with m/z 307 (M+1)amu. In this solvent system, tebucona-zole eluted at about 8.5 min whereas the impurity eluted at about 5.0 min. The impurity fractions isolated were concentrated at room temperature under high vacuum on a Buchii rotavapour model R124. The aqueous layer containing this impurity was extra-cted into methylene chloride. These fractions were concentrated at room temperature under high vacuum on a rotavapour. Purity of this impurity was tested in analytical mode and was found to be 99.4% before carrying out spectroscopic experiments.

Structural elucidation of impurity(273amu)

LC-MS/MS and GC-MS spectral data of impurity was compared with the spectral data of tebuconazole. This impurity exhibited molecular ion at m/z 273(M+) and 274(M+1) in GC-MS and LC-MS/MS analysis respectively. This molecular ion is exactly 35.5 amu less than that of tebuconazole. Interestingly the isotopic abundance due to chlorine was found to be absent in mass spectral data of LC-MS/MS and GC-MS analysis. Fragmentation pattern also matched for this impurity in LC-MS/MS and GC-MS analysis. This clearly indicated that this impurity did not have chlorine atom. Based on these results it was confirmed that this impurity was a deschloro compound of tebuconazole. The above data can be rationalized in terms of this impurity having the molecular formula $C_{16}H_{23}N_3O$ and the same was characterized as 3-((1H-1,2,4-triazol-1-yl)methyl)-4,4-dimethyl-1-phenylpentan-3-ol. Major fragments obtained in LC-MS/MS and GC-MS analysis for tebuconazole and impurity are given in TABLE 1.

TABLE 1: Major mass fragments of tebuconazole and impurities

Compound	Major fragments(amu)
Tebuconazole	307(M+), 250, 165, 151, 139, 125
Impurity(m/z:307amu)	307(M+), 250, 165, 151, 139, 125
Impurity(m/z:273amu)	273(M+), 256, 131, 117, 105, 91

Structural elucidation of impurity(307 amu)

The spectral data of this impurity was compared with the spectral data of tebuconazole. LC-MS/MS and GC-MS analysis exhibited molecular ion for this impurity at m/z 308(M+1) and 307(M+) respectively. Fragmentation patterns of tebuconazole and impurity were also exactly matching. The ^{1}HNMR spectrum of tebuconazole exhibited two separate signals for the protons on the triazole ring which indicated that these two protons on the triazole ring were chemically non-equivalent. It was quite interesting to note that the ^{1}H NMR spectrum of this impurity exhibited a single signal integrating for two protons attached to the triazole ring. The ^{1}HNMR spectrum is shown in figure 3. This observation indicated that the two protons attached to triazole ring were in chemically equivalent environment.^{13}C NMR and DEPT-NMR experiments were also performed on this impurity. The two carbon atoms in this triazole ring of this impurity resonated at the same frequency when compared to that of tebuconazole. This observation further confirmed that two carbon atoms of this impurity in the triazole ring were chemically

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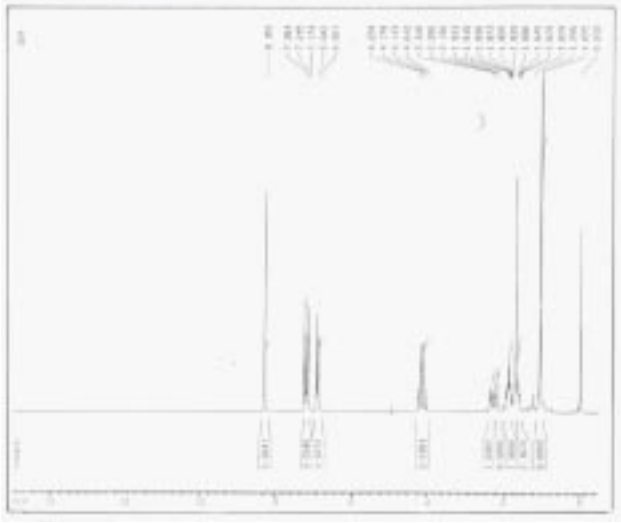

Figure 3: ^{1}H-NMR spectrum of tebuconazole impurity(m/z: 307amu)

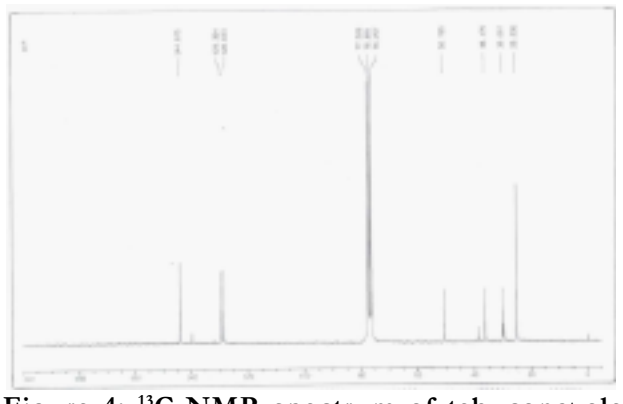

Figure 4: ^{13}C-NMR spectrum of tebuconazole impurity(m/z:307amu)

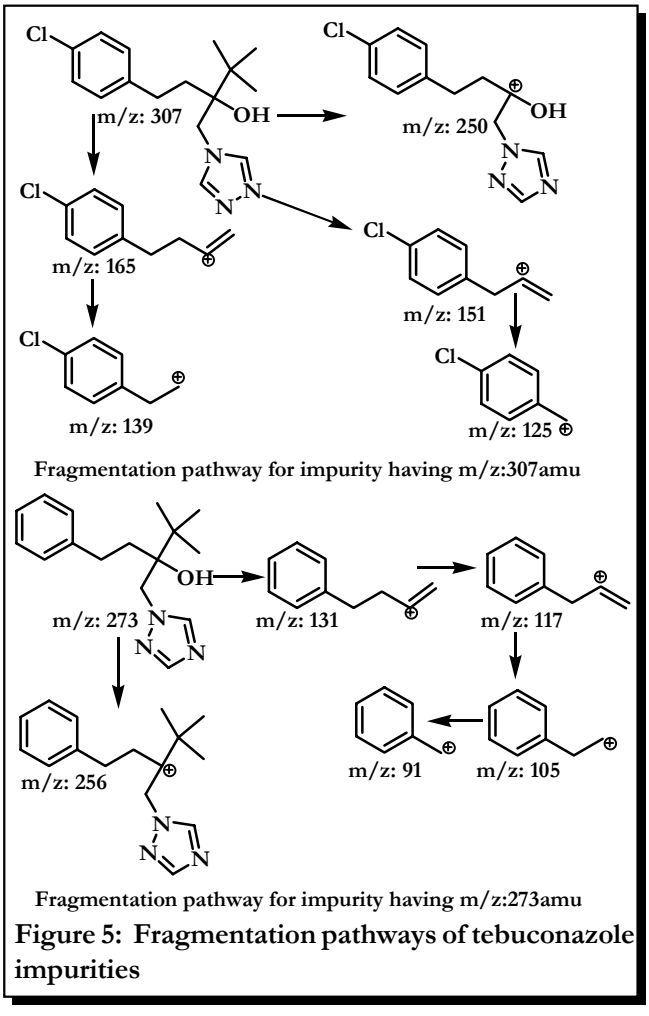

Figure 5: Fragmentation pathways of tebuconazole impurities

equivalent. The ^{13}C NMR spectrum is shown in figure 4. Based on these spectral results, it was confirmed that the impurity having the molecular formula $C_{16}H_{22}ClN_3O$ and the same was characterized as 3-((4H-1,2,4-triazol-4-yl)methyl)methyl)-1-(4-chlorophenyl)-4,4-dimethylpentan-3-ol.

Chemical structures of tebuconazole and its impurities are shown in figure 5. Fragmentation pathways for impurities are shown in figure 6. Major fragments obtained in LC-MS/MS and GC-MS analysis for tebuconazole and impurity are given in TABLE 1.

Formation of impurities

1. Formation of impurity(m/z 273)

Synthesis of tebuconazole involved 4-chloro benzaldehyde as one of the starting materials. This 4-chloro benzaldehyde may also contain trace amounts of benzaldehyde. This will also undergo the similar reactions as that of 4-chloro derivative and leads to

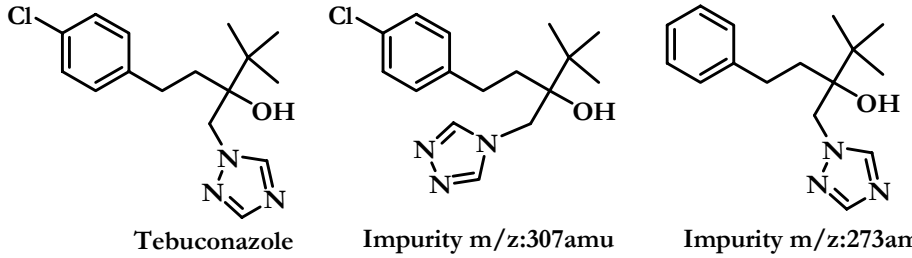

Figure 6: Chemical structures of tebuconazole and its impurities

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the formation of des chloro derivative of tebuconazole.

2. Formation of impurity(m/z 307)

During the prefinal stage of tebuconazole, 1H 1,2,4-triazole was used to reduce the epoxide ring to obtain tebuconazole. The 1H 1,2,4-triazole may also contain 4H-1,2,4-triazole which leads to the formation of this impurity during the final step of the synthesis of tebuconazole.

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