Chapter – V

Separation, identification, isolation and characterization of process related impurities in letrozole active pharmaceutical ingredient

5.1.0 Introduction

Letrozole, a nonsteroidal drug, is chemically known as 4,4’-((1H-1,2,4-triazol-1-yl)methylene) dibazonitrile. It has been approved by the FDA (food and drug administration) for the treatment of late-stage breast cancer in post-menopausal women [1-6]. It is a potent antineoplastic inhibitor of aromatase[1] (a rate limiting enzyme in the biosynthesis of estrogens) and is increasingly used to treat women with breast cancer[3]. Aromatase inhibitors, which potently suppress estrogen synthesis in post-menopausal women, are increasingly used in the treatment of early-stage breast cancer to prevent disease recurrence. Aromatase inhibitors reduce the plasma estrogen concentration by suppressing peripheral estrogen synthesis and might also suppress estrogen synthesized locally by aromatase in bone [4]. Breast carcinoma has become a major health problem over the past several years, affecting as many as one in eight women [5]. Even though the chemists globally are working continuously on development of a new medicine to treat breast carcinoma disease, approximately twenty five percent of women who suffer from breast carcinoma will eventually die from the disease [7]. The chemical structure of letrozole is:
It is mandatory for all the drug manufacturers that the impurity profile study should be carried out for any bulk drug to identify and characterize all the unknown impurities that are present at a level of above 0.05% [8]. A comprehensive study was undertaken to isolate these impurities by chromatography and characterize them by spectroscopic techniques since such a study has not been done by any group previously. The work presented in this chapter describes the separation, identification, isolation and characterization of two impurities in addition to the one known impurity that are present in the range of 0.08%-0.13% by peak area in the bulk drug of letrozole. Letrozole and one known impurity (letrozole metabolite) are officially mentioned in the USP [9] but the two impurities are not included in the monograph.

5.2.0 Experimental details

5.2.1 Samples and chemicals

The letrozole bulk drug sample was received from Cipla Ltd, India. HPLC grade acetonitrile and A.R-grade ammonium acetate was obtained from Merck Co., 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%).
5.2.2 High performance liquid chromatography (Analytical)

A Waters HPLC system (Waters corporation, USA) equipped with Alliances 2695 series low pressure quaternary gradient pump along with photo diode array detector and auto sampler was used for the analysis of samples. The data were collected and processed using Waters“Empower 2” software. An Inertsil ODS-3V (250 * 4.6 mm, 5-Micron, GL Sciences, Japan) column was employed for the separation of impurities from letrozole. The column eluent was monitored at 230 nm. A simple isocratic reverse-phase HPLC method was optimized for the separation of impurities from letrozole active pharmaceutical ingredient where the mobile phase was a mixture of 2 mM ammonium acetate and acetonitrile in the ratio, 50:50, (v/v). Chromatography was performed at room temperature (25 ±2°C) using a mobile phase flow rate of 1.0 mL min⁻¹. The chromatographic run time was 40 min.

5.2.3 High performance liquid chromatography (preparative)

An Agilent prep-HPLC system equipped with 1200 series pump, photo diode array detector, auto sampler fitted with 5000 µL loop and 1200 series preparative fraction collector was used. The data were collected and processed using Agilent “Chemstation” software. An inertsil C18 column (250 X 20 mm, 5-Micron) 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-HPLC to collect the required impurity fractions. The mobile phase consisted of 2 mM ammonium acetate and acetonitrile in the ratio, 50:50, (v/v). The flow rate was set at 25 mL min⁻¹. Detection was carried out at 230 nm. Approximately 100 mg mL⁻¹ sample solution was prepared using acetonitrile and water in the ratio, 70:30, (v/v) as diluent to load on to the column.
5.2.4 Mass spectrometry (GC-MS)

Mass spectra were recorded on Agilent 5973 series mass spectrometer (Agilent technologies, USA) 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 100°C for 3 min followed by increasing temperature to 300°C at the rate of 30°C min⁻¹ and held for 20 min. The source manifold and quadrupole temperatures were maintained at 230° and 150°C, respectively.

5.2.5 Mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was 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 air as nebuliser gas at a pressure of 40 psi and nitrogen as curtain gas 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 was a mixture of 2 mM ammonium acetate and acetonitrile in a ratio, 50:50(v/v). The analysis was performed at a flow rate of 1.0 mL min⁻¹ with splitting.

5.2.6 NMR spectroscopy

The \(^1\)H NMR, \(^{13}\)C NMR and DEPT (distortionless enhancement by polarization transfer) NMR experiments were carried out at precessional frequencies of 399.939 MHz (\(^1\)H NMR) and 100.574 MHz (\(^{13}\)C NMR and DEPT), respectively, in CDCl\(_3\) at 25°C temperature on a Varian-400 FT NMR spectrometer. The \(^1\)H and \(^{13}\)C
chemical shifts are reported on the $\delta$ scale in ppm, relative to tetra methyl silane (TMS) $\delta$ 0.00 ppm and CDCl3 at 77.0 ppm in $^{13}$C NMR respectively.

5.3.0 Results and discussion

5.3.1 Detection of impurities by HPLC

Typical analytical HPLC chromatogram of letrozole bulk drug and its impurities obtained using the HPLC method discussed in the section 5.2.2 is shown in Figure 5.1. The targeted impurities under study are marked as Impurity I and II eluted at retention times of about 2.5 min and 6.3 min respectively, while letrozole and known impurity (letrozole metabolite) were eluted at 5.4 min and 2.2 min, respectively.
Figure 5.1: A typical HPLC chromatogram of letrozole drug substance. The unzoomed and zoomed chromatogram is given separately for the better view of impurity peaks present in letrozole drug substance.
5.3.2 Isolation of the impurities by prep-HPLC

A simple reverse phase chromatographic system, discussed in the section 5.2.3 was used for isolating the impurities. In this chromatographic system, letrozole eluted at about 6.8 min whereas the impurity I eluted at about 3.1 min, and impurity II eluted at about 7.6 min. The impurity I fraction was collected between 2.7 min and 3.3 min, impurity II fraction was collected between 7.3 min and 8.2 min. The impurity fractions were concentrated by evaporating acetonitrile portion at room temperature under high vacuum on a Buchii Rotavapour Model R124. The concentrated aqueous layer containing individual impurity was extracted into methylene chloride. These fractions were concentrated by evaporating methylene chloride layer to get the residue at room temperature (25±2°C) under high vacuum on a Rotavapour. Purity of these impurities was tested in analytical mode and was found to be 96.8% and 98.1% (Figure 5.2) before carrying out spectroscopic experiments. The isolated impurities were spiked to the anastrazole bulk drug and analysed in analytical HPLC system to confirm the retention time of isolated impurities are matching with respect to the impurities present (Figure 5.3).
**Impurity-I**

**Impurity-II**

**Figure 5.2: HPLC chromatograms of isolated impurities I and II.**
5.3.3 LC-MS/MS analysis

LC-MS/MS analysis of letrozole bulk drug sample and impurity-I was performed using the chromatographic system as described in the section 5.2.5. Results of LC-MS/MS analysis revealed that impurity-I exhibited molecular ion at m/z (M+1)185.3 amu (Table 5.1), and fragmentation pattern of this molecular ion was also recorded. The peak at 2.2 min revealed the molecular ion at m/z (M+1) 235.2 which is the molecular ion of known impurity (letrozole metabolite) which already has been established [2]. The molecular ion and fragmentation pattern of peak at 2.2 min support to the structure of letrozole metabolite shown in Figure 5.5. Impurity-II was analyzed comfortably by GC-MS.
Table 5.1: Major mass fragments of Letrozole and its impurities

<table>
<thead>
<tr>
<th>Compound</th>
<th>Major fragments (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Letrozole</td>
<td>286.2 (M+1), 217.3, 190.2, 123.9</td>
</tr>
<tr>
<td>Impurity I (m/z:185.3amu)</td>
<td>185.3(M+1), 116.3, 89.3</td>
</tr>
<tr>
<td>Impurity II (m/z:121amu)</td>
<td>121 (M+), 102, 94, 63</td>
</tr>
<tr>
<td>Known impurity (metabolite) (m/z:235amu)</td>
<td>235.2(M+1), 218.2, 192.4, 165.2, 152.4</td>
</tr>
</tbody>
</table>

5.3.4 GC-MS analysis

GC-MS analysis of impurity II was performed using the method as described in the section 5.2.4. Electronic impact ionization of impurity II exhibited molecular ion at \( m/z \) 121 (M+) amu. Fragmentation pattern (Figure 5.8) also matched with GC-MS for this impurity (Table 5.1).

5.3.5 Structural elucidation of impurity I

The spectral data of this impurity was compared with that of letrozole spectral data. LC-MS/MS analysis exhibited molecular ion for this impurity at (M+1) \( m/z \) 185.3 amu and the fragmentation pattern (Figure 5.7) also conformed the structure given in Figure 5.5. The \(^1\)H NMR spectrum of letrozole exhibited two separate signals for the protons on the triazole ring at \( \delta \) 8.13 and \( \delta \) 8.71 ppm 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 two signals integrating for two protons attached to the triazole ring at \( \delta \) 8.02 and \( \delta \) 8.70. The \(^1\)H NMR spectrum is shown in Figure 5.6. This observation indicated that the two protons attached to triazole ring were in chemically non-equivalent environment. The
$^{13}$C NMR and DEPT-NMR experiments were also performed for this impurity. The two carbon atoms in the triazole ring resonated at two different frequencies $\delta$ 144.6 and $\delta$ 152.0 respectively compared to that of letrozole which contained two signals at $\delta$ 145.0 and $\delta$ 152.4. This observation further confirmed that two carbon atoms of this impurity in the triazole ring were chemically non-equivalent (see Table 5.2). The compound contained a singlet at $\delta$ 5.55 ppm which corresponds to two protons of (CH2-triazole), which was absent in letrozole. The signals corresponding to aromatic protons appeared as doublets at $\delta$ 7.42 and $\delta$ 7.84 ppm. Based on these spectral results, the impurity was assigned the molecular formula, C$_{10}$H$_8$N$_4$ and the same was characterized as 2,2’-((5-((4H-1,2,4-triazol-4-yl) methyl)-1,3-phenylene) bis (2-ethyl propane nitrile). Chemical structures of letrozole and the impurities are shown in Figure 5.5. Fragmentation pathways for impurities are shown in Figure 5.4. Major fragments obtained in LC-MS/MS analysis for letrozole and its impurities are given in Table 5.1.

Impurity I

![Diagram of Impurity I]

$m/z : 185.3$

$m/z : 116.3$

$m/z : 89.3$

Impurity II

![Diagram of Impurity II]

$m/z : 121$

$m/z : 102$

$m/z : 94$
Metabolite (Known impurity)

Figure 5.4: Proposed fragmentation pathways of impurities I, II, and metabolite (Known impurity).
<table>
<thead>
<tr>
<th>Position</th>
<th>Letrozole</th>
<th>Impurity</th>
<th>Impurity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1H / ppm</td>
<td>13C</td>
<td>DEPT</td>
</tr>
<tr>
<td>1</td>
<td>7.39</td>
<td>64.0</td>
<td>64.1</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>5</td>
<td>8.71</td>
<td>152.4</td>
<td>152.4</td>
</tr>
<tr>
<td>6</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>7</td>
<td>_</td>
<td>111.2</td>
<td>_</td>
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<tr>
<td>8</td>
<td>_</td>
<td>118.3</td>
<td>_</td>
</tr>
<tr>
<td>9</td>
<td>_</td>
<td>143.2</td>
<td>_</td>
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<tr>
<td>10</td>
<td>_</td>
<td>_</td>
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</table>

Note: For numbering refer Figure 5.5
5.3.6 Structural elucidation of impurity II

The mass spectrum of impurity II exhibited a molecular ion at (M+) 121 amu and the fragmentation pattern also conformed the structure given in Figure 5.5, which was 164 amu less than that of letrozole. The odd molecular ion of impurity II indicates the possible presence of odd number of nitrogen atoms. The major fragments obtained in GC-MS analysis for impurity-II (Figure 5.8) is given in Table 5.1. The $^1$H NMR spectrum of impurity II showed two multiplet signals at $\delta$ 7.42-7.50 and $\delta$ 7.94-8.00 ppm due to –CH- proton on the aromatic ring (Figure 5.6). This observation was supported by the appearance of signals at $\delta$ 7.42-7.50 and $\delta$ 7.94-8.00 due to fluorine presence in the letrozole impurity, which was not observed in the $^1$H NMR spectrum of letrozole. Further, the $^1$H NMR spectrum displayed signal at $\delta$ 7.39 ppm due to methylene proton in the letrozole, which was absent in the $^1$H NMR spectrum of impurity II. On the other hand, $^{13}$C NMR spectrum of letrozole shows signal at $\delta$ 64.0 ppm due to methylene group which was not observed in the $^{13}$C NMR spectrum of impurity II. In addition to this observation, the $^{13}$C spectrum displayed signals at $\delta$ 116.7 & 117.1 , $\delta$ 135.2 & 135.3 and $\delta$ 163.3 & 165.8 ppm as doublets due to fluorine coupling with the aromatic carbons in the impurity II, which was not observed in the letrozole. Based on the above spectral data, the structure of the impurity II was characterized as 2, 2’-(5-methyl-1, 3-phenylene) bis (2-methylpropanenitrile) and assigned the molecular formula as C$_7$H$_4$FN.
Figure 5.5: Chemical structures of letrozole and its impurities
Figure 5.6: $^1$H NMR spectrum of impurity – I and II
Figure 5.7: LC-MS spectrum of letrozole, impurity – 1 and metabolite (known impurity)
5.4.0 Conclusion

Letrozole is a potent aromatase inhibitor drug used in the treatment of cancers. The present research work describes a HPLC method for detection, separation of two process related impurities and one known impurity (letrozole metabolite) from letrozole drug substance and prep-HPLC method for isolation of the two impurities from the letrozole bulk drug. One known impurity (letrozole metabolite) was already reported [2] and the mass of this obtained by LC-MS/MS was matching with the reported one. Both the impurities detected were characterized using GC–MS, LC-MS/MS and NMR experimental techniques.
5.5.0 References


