FORCED DEGRADATION STUDY OF LAMIVUDINE UNDER THE SCOPE OF GENOTOXIC IMPURITY

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ABSTRACT
Genotoxic impurities in drug substances or drug products are growing concern to ensure safety of the public health. Genotoxic impurity present in drug substances and drug products may be DNA reactive and posed significant problems for drug regulators and industry alike over the last decade. The principal concern relates to drug safety is the prolong exposure to compounds that can alter DNA, may ultimately produce a carcinogenicity. Therefore, the practical issue is that the conventional procedures should be there to identify DNA-reactive impurities in the shelf life of drug product. In the present study, lamivudine, an antiretroviral agent is used to evaluate degradation pathways under different stress conditions in order to identify degradation products as prescribed by ICH guidelines. Lamivudine was found to degrade under acidic, basic and oxidative conditions followed by formation of four degradation products. The degradation products were separated and identified by LC-MS to propose degradation pathways followed by evaluation of similarity with the structural alerts for genotoxic impurities. Finally, characterization of the genotoxic impurity by FT-IR, NMR and LC-MS.

KEYWORDS: Lamivudine; Forced degradation studies; HPLC; LC-MS; Genotoxic impurity.
INTRODUCTION

Genotoxic substances are those which impact on genetic material by means of mutations. Exposure to very low levels of a genotoxic may lead to cancer.[1] Therefore, it is important to identify genotoxic substances followed by monitoring and control to safeguard public health. It can be came from multiple sources like starting materials, reagents, intermediates unwanted side reactions during synthesis that carried over to final drug product. In addition, drug itself can decompose to form genotoxic impurities.[2]

Forced degradation is a process where the natural degradation rate of a drug or drug product is accelerated by the application of an additional stress.[3] Stress testing is designed to estimate degradation pathways and intrinsic stability of the drug molecule. Data generated from these studies provide to support identification of possible degradants. The identification and qualification of degradants in drug products is essential as impurities may cause unwanted effects on the patients and moreover, may have influence on quality, safety and efficacy of the drug products.

Lamivudine is a potent nucleoside reverse transcriptase inhibitor used for the treatment of HIV-1 infection. It is given orally up to 300 mg once daily or divided doses in combination with other antiretroviral agents. It is chemically 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. It has a molecular formula of C₈H₁₁N₃O₃S having molecular weight of 229.26 daltons.[4]

Although there are previously reported works on forced degradation study of lamivudine by using HPLC and LC-MS,[5–7] to date there is no reported method to identify degradants followed by characterization of the genotoxic impurity. Hence, the endeavor of our present investigation was to: (i) carry out the stress studies of lamivudine according to the ICH guidelines.[8–9]; (ii) separate the degradation products by HPLC; (iii) identify and establish the degradation pathway of all the degradation products with the help of LC-MS; (iv) characterize the genotoxic impurity by FT-IR, NMR and LC-MS.

EXPERIMENTAL

Materials and Reagents

Lamivudine (purity ≥ 99%) bulk drugs were obtained as gift sample from a renowned manufacturer. Ammonium acetate, glacial acetic acid (Analytical-reagent grade) was purchased from Merck Pvt. Ltd., whereas methanol (HPLC-grade) were purchased from
Sigma Aldrich. HPLC grade water having resistivity of 18.2 MΩ cm (Milli Q water purification system) was used throughout the analysis. All other reagents used like hydrochloric acid, hydrogen peroxide, and sodium hydroxide was of analytical grade (Merck Pvt. Ltd.)

**Instrumentation**

LC-MS study was performed using liquid chromatography system coupled with mass spectrometer with ESI source equipped with an autosampler, and diode array detector (Agilent Technologies). High-precision heating mantel (Remi) capable of controlling the temperature within ±1°C was used for generating hydrolytic degradation products. The solid state thermal stress studies were carried out in a dry-air oven (NSW Limited). Other equipment used were a pH meter and electronic weighing balance (Mettler Toledo).

**Stress degradation studies**

Stress degradation studies were performed as per ICH guidelines. Some practical aspects of stability indicating procedures of specific drugs were also found in literature.\(^{10-14}\) All stress decomposition studies were performed with control solution i.e. prepared and treated in similarly to the respective stress conditions without active component.

Acid degradation: 50mg of lamivudine was taken in 50 mL volumetric flask. Then the sample was dissolved in 5 mL of water and adds 5mL of hydrochloric acid solution (1N). The resulting solution was refluxed at 80°C for 30 min and then cooled to room temperature followed by neutralization with 5 mL of sodium hydroxide solution (1N).

Base degradation: 50mg of lamivudine was taken in 50 mL volumetric flask. Then the sample was dissolved in 5 mL of water and adds 5mL of sodium hydroxide solution (1N). The resulting solution was refluxed at 80°C for 30 min and then cooled to room temperature followed by neutralization with 5 mL of hydrochloric acid solution (1N).

Oxidative degradation: 50mg of lamivudine was taken in 50 mL volumetric flask. Then the sample was dissolved in 5 mL of water and adds 5mL of hydrogen peroxide solution (30%). The resulting solution was refluxed at 80°C for 30 min and then cooled to room temperature.

Thermal degradation: Degradation was also carried out in solid state by exposing about 200mg of lamivudine on dry heat at 80°C for 72hr in hot air oven. This sample was taken for analysis.
Sample Preparation for LC-MS analysis
Stress samples were collected and made up to volume with mobile phase whereas solid samples were directly dissolved and diluted with mobile phase. Sample concentration of 1000 µg/mL was used to conduct degradation studies. All the samples were filtered through 0.22 µm membrane filter and injected into LC-MS system.

LC-MS analysis
The LC system was coupled with QTOF mass spectrometer which was operated in both positive and negative modes with ESI source for mass spectrometric detection. Separation was achieved with Zorbax eclipse plus C18 (100 mm x 4.6 mm, 3.5 µm particle size; Agilent Technologies) column kept at 35°C with isocratic mobile phase consisting of a mixture of methanol: 0.19% w/v of ammonium acetate having pH 3.8 with glacial acetic acid (5:95, %v/v) at a flow rate of 0.3 mL/min with injection volume of 1 µL. Eluent was monitored at 277nm. The capillary voltage applied was 3500 V. The gas temperature was set at 310°C, using nitrogen as nebulizing gas and drying gas. Drying gas flow at 10 L/min, nebulizer pressure 40 psig and fragmentor 70. Mass spectra were acquired over an m/z range of 50-1000. All gas used in this experiment was generated from a Peak gas generator (Peak Scientific). The instrument was controlled and the data integration was performed with Mass Hunter software.

Characterization of genotoxic impurity
The genotoxic impurity formed in degradation study, was separated and characterized by FT-IR, NMR and LC-MS. The FT-IR spectrum was collected in the range of 400–4000 cm⁻¹ by potassium bromide disc method using FT-IR spectrophotometer instrument (Make: PerkinElmer, USA; Model: Frontier Optica with spectrum software). NMR spectra were recorded on NMR system (Agilent Technologies) operating at 500 MHz using DMSO-d₆. For LC-MS analysis, above mentioned method was adapted.

RESULT AND DISCUSSION
Lamivudine is official in Indian Pharmacopoeia. In the present study, forced degradation study was carried out by subjecting exaggerated conditions of acid, base, oxidation and heat. No major degradation products were found in the applied conditions for thermal degradations of drug substance. Whereas lamivudine degraded up to 39% and 58% during acid and base degradation, respectively followed by formation of three major degradation products (Degradant-I, Degradant-II and Degradant-III). On the other hand, it led to formation of one
degradation products (Degradant-IV) under oxidative stress condition. Data from stressed sample was compared to that of the respective controls to determine the formation of additional degradants. The percentage of degradation obtained under each condition is described in Table-1 and the respective chromatograms are shown in Figure-1.

Table-1: Results of forced degradation study

<table>
<thead>
<tr>
<th>Degradation study</th>
<th>Purity (% area normalization)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>API</td>
<td>99.7%</td>
<td>-</td>
</tr>
<tr>
<td>Acid degradation</td>
<td>71.1%</td>
<td>Degradation product I, II, and III observed</td>
</tr>
<tr>
<td>Base degradation</td>
<td>42.1%</td>
<td>Degradation product I, II, and III observed</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>54.7%</td>
<td>Degradation product IV observed</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>99.7%</td>
<td>No significant degradation observed</td>
</tr>
</tbody>
</table>

Figure-1: HPLC chromatograms of lamivudine's degradation products
Table-2: Characterization of degradation products by LC-MS

<table>
<thead>
<tr>
<th>Degradation products</th>
<th>Experimental mass</th>
<th>Best possible molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degradation product-I</td>
<td>111.99</td>
<td>C₄H₅N₃O</td>
</tr>
<tr>
<td>Degradation product-II</td>
<td>112.95</td>
<td>C₄H₄N₂O₂</td>
</tr>
<tr>
<td>Degradation product-III</td>
<td>230.03</td>
<td>C₈H₁₀N₂O₄S</td>
</tr>
<tr>
<td>Degradation product-IV</td>
<td>246.04</td>
<td>C₈H₁₁N₃O₄S</td>
</tr>
</tbody>
</table>

Figure-2: Mass spectrum of lamivudine's degradation products.
Figure-3: Chemical structure of lamivudine and its degradation products

Figure-4: Structural alerts for pharmaceutical impurities

Figure-5: FT-IR spectra of Degradant-IV.
The identification of degradation products was also very effective for knowing the pathways of degradation of drug substances or drug products. Therefore, the degradation products were subjected to LC-MS study to elucidate structural details. Results are tabulated in Table-2 and mass spectrums are shown in Figure-2. Mass spectrum observed for pure drug at m/z 230.12. Degradant-I, Degradant-II and Degradant-III with m/z 111.99, 112.95 and 230.03, respectively were obtained in acid and base degradation. Degradant-IV with m/z 246.04 was found in oxidative degradation. The tentative degradation pathway is also predicted based on this data. The possible structure of degradants was shown in Figure-3.
Structural alerts for pharmaceutical impurities are shown in Figure-4. Formations of degradation products IV which follow sulphoxides structural similarity. It’s an indication that there may be formations of genotoxic impurities.[16]

In FT-IR spectra, the principle peak obtained at wave number of 3434 cm\(^{-1}\) (broad) due to -OH and –NH stretching, 2925 cm\(^{-1}\) due to –CH\(_2\), 1752 cm\(^{-1}\) due to –C=O, 1626 cm\(^{-1}\) due to NH bending and 1074 cm\(^{-1}\) due to S=O (Figure-5).\(^{[17]}\) Therefore, results revealed that the functional group present in the compound follow the Degradant-IV chemical structure. Results of proton NMR spectra could be summarized with respect to chemical shift (δ, ppm) as follows: 3.00-3.03, 3.36-3.40 & 7.17-7.23 (doublet, 2H), 3.67-3.75 (multiplet, 2H), 5.14-5.16, 5.30-5.32 & 6.17-6.19 (triplet, 1H), 5.70-5.72 & 7.79-7.80 (doublet, 1H). No of protons present in the compound fully support the structure of Degradant-IV (Figure-6). Positive electrospray mass spectra of the compound shown an intense [M+H]\(^+\) ion at m/z 246.04. As Degradant-IV having the mass of 245.04, the mass obtained in LC-MS study corresponds to its molecular mass.

CONCLUSION

The degradation study indicated that lamivudine was found to be stable to thermal treatment while susceptible to degradation in acidic, basic and oxidative stress and. LC-MS study results revealed that possible degradants are C\(_4\)H\(_3\)NO\(_3\) (m/z 111.99), C\(_4\)H\(_4\)O\(_2\)N\(_2\) (m/z 112.95) and C\(_8\)H\(_{10}\)O\(_4\)N\(_2\)S (m/z 230.03) in both acidic and basic conditions whereas C\(_8\)H\(_{11}\)N\(_3\)O\(_4\)S (m/z 246.04) in oxidative stress conditions. Structural alerts for pharmaceutical impurities indicates the formations of sulphoxide which may be genotoxic impurity. Degradant-IV was characterized by FT-IR, NMR and LC-MS. These findings may have an impact on the further investigation for confirmation as genotoxic and thereby control and monitoring in drug products to be fit for human consumption.

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