CHAPTER-2
IDENTIFICATION, ISOLATION AND CHARACTERISATION OF DEGRADATION IMPURITY IN MULTICOMPONENT DOSAGE FORM LAMIVUDINE ZIDOVUDINE AND NEVIRAPINE TABLETS FOR ORAL SUSPENSION

2.1. Introduction

Lamivudine, Zidovudine and Nevirapine are antiretroviral drugs are medications for the treatment of AIDS\(^1\), primarily HIV. Lamivudine is a potent reverse transcriptase inhibitor. It belongs to the class
nucleoside analog reverse transcriptase inhibitor (NARTI). It is an
analogue of cytidine.

Fixed dose combination of Lamivudine and Zidovudine was
approved by the Food and Drug Administration in 1995. Chemical
Name (IUPAC) 4-amino-1-((2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-
yl)pyrimidin-2(1H)- one. molecular formula C$_8$H$_{11}$N$_3$O$_3$S and a molecular
weight of 229.3 and structural formula (1).

Zidovudine is a pyrimidine nucleoside analogue active against
HIV-1. The molecular formula is C$_{10}$H$_{13}$N$_5$O$_4$. The chemical name of
zidovudine is 3' azido-3'-deoxythymidine, molecular weight 267.24 it
has the following structural formula (2).
Chemically Nevirapine is a dipyridodiazepinone. Its molecular weight is 266.30 and the molecular formula C₁₅H₁₄N₄O. The chemical name of nevirapine is 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido [3,2-b:2',3'-e][1,4] diazepin-6-one. Nevirapine has the following structural formula (3).

Lamivudine, Zidovudine and Nevirapine tablets is a fixed dose combination product available in the strengths of 150 mg, 300 mg, 200 mg and 30 mg, 60 mg, 50 mg. The 30 mg, 60 mg and 50 mg is a pediatric formulation available as tablets for oral suspension. combination of these three drugs is commonly used to prevent the development of resistant viral strains.

2.2. Literature and Aim of the present work

A number of methods were reported in the literature reveal with estimation and determination of these drugs by HPLC (2-3). Drug substances are official in Indian Pharmacopoeia, United States pharmacopoeia and European pharmacopoeia (4-12). There is also an official monograph for estimation of assay, dissolution and Related
substances in Indian Pharmacopoeia. The methods and monographs in the literature were about estimation of lamivudine, zidovudine and nevirapine and some impurities. The purity requirements for the customer for the commercial point of view demands that all the unknown impurities (above threshold levels) in the final product must be identified and characterized as per the regulatory requirements. The degradation product developed during forced degradation studies involving light and heat has not been identified and characterized. In this chapter an attempt was made to address the issue of characterization of unknown impurity developed during stability studies of this fixed dose combination. The various degradation products and related impurities are Pharmacopoeias (4-15)

**Chemical structures of Lamivudine, Zidovudine and Nevirapine related impurities**

(a) Lamivudine Related Impurities

![Chemical structures](image)

(b) Nevirapine related Impurities
DESCYCLOPROPYL NEVIRAPINE (7)  ETHYLNEVIRAPINE (8)

c) Zidovudine related Impurities

THYMINE (9)  β-THYMIDINE (10)  STAVUDINE (11)

α-THYMIDINE ANALOGUE (12)  CHLORO IMPURITY (13)  β-AZIDO ZIDOVUDINE (14)
2.3. Experimental

2.3.1. Materials

Fixed dose combination of LZN was obtained from APL Research center (A unit of Aurobindo Pharma Limited, Hyderabad, India). The impurity standards were obtained from Chemical Research Department of APL Research Centre. Reagents and solvents were obtained from Merck and were of HPLC grade. Filter paper used for sample preparation are PVDF & nylon 0.45µ pore size.

2.3.2. Equipment

1. Chromatographic system:

   (a) For identification: 2695 separation module with diode array detector (Waters Corporation) Column: 25 cm X 0.46 cm YMC ODS AQ (5 um particle size)
(b) For isolation: LC-8A preparative liquid chromatography with UV visible detector (Shimadzu, Japan) Column: 50 cm X 3 cm Hyper preparative HS C18 (10 um particle size)

2. NMR and DEPT studies: The H-NMR and the C13-NMR as also the DEPT study were carried out on Bruker 300 MHz NMR spectrometer.

3. Mass Spectra studies: PE SCIEX API 2000 Mass spectrophotometer equipped with turbo ion spray interface was used to record the mass spectra. The spectra were recorded at 375 deg C.

4. FT-IR: Perkin Elmer FT IR spectrophotometer was used to record the FTIR. The KBr pellet method was used.

2.3.3 High Performance Liquid Chromatography

2.3.3.1 Preparation Of mobile phase:
Mobile Phase A -Acetate buffer (pH 4.2): This was prepared by dissolving 9.64 g of Ammonium Acetate 1000 ml of water and pH was adjusted to 4.2 with glacial acetic acid. This was filtered through using Millipore vacuum pumpMobile Phaase B: Methanol

2.3.3.2 Diluent
Milli Q Water

2.3.3.3 Preparation Of sample solution.
Tablets powder equivalent to about 100 mg of Zidovudine was weighed into a 100 mL clean dry volumetric flask, added 60 mL of diluent and
sonicated for about 20 minutes at room temperature with intermittent shaking. Made up to the volume with diluent and filtered.

### 2.3.3.4. HPLC conditions

The sample solutions prepared above were injected into a waters HPLC system. The analysis was carried out on. Mobile phase flow rate was maintained at 0.8 mL/min and Column oven temperature at 25°C. Column eluent was monitored at wavelength of 270 nm. Data acquisition time was 50 min. Pump mode was gradient and the programme was as follows.

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Mobile Phase A (v/v)</th>
<th>Mobile Phase B (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0.01</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>T0.05</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>T0.10</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>T0.35</td>
<td>40</td>
<td>60</td>
</tr>
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<tr>
<td>T0.41</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>T0.50</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

### 2.3.4. LC-MS/MS-analysis

#### 2.3.4.1. Preparation of mobile phase

0.77 g of ammonium acetate was dissolved in 1000 ml of water, pH adjusted to 4.2 with acetic acid. This solution was filtered through membrane filter and used as a mobile phase A.

Methanol was used as mobile phase B.

#### 2.3.4.2. Preparation of diluent

For analysis is purified milli Q water.
2.3.4.3. Preparation of sample solution

50mg of sample was weighed and transferred into a 50ml clean dry volumetric flask, 30ml of diluent was added and sonicated of 20minutes to dissolve. Made up to volume with diluent and filtered.

2.3.4.4. LC-MS/MS Conditions

The above prepared sample solution was subjected to LC-MS/MS analysis using the mobile phase as described under section 2.3.3.1. Detection was carried out at wave length of 270nm and flow rate was at 0.8 ml/min. sample Acquisition time 50 min. The pump mode was gradient and the programme was as below.
One unknown impurity with a mass of 307 [(MH)⁺] was detected in addition to 229 [(MH)⁺](Lamivudine), 267 [(MH)⁺](Zidovudine) and 266 [(MH)⁺](Nevirapine) in the laboratory batch sample. So this sample was subjected to preparative HPLC to isolate the impurity in pure form.

### 2.3.5. **Enrichment of Degradation Product:**

Lamivudine, Zidovudine and Nevirapine tablet powder was kept in an autoclave at 121°C/15-16 psi pressure/30min for three cycles. Autoclaved samples were analyzed by the HPLC method mentioned above. The degradation product was enriched was 3.0% by area normalization.
2.3.6. Preparative Liquid Chromatography

2.3.6.1. Preparation of mobile phase

0.2 ml of Triflouroacetic acid solution was added to 1000 ml of water and this solution was filtered through PVDF membrane filtered, used as mobile phase A. Methanol was used as mobile phase B.

2.3.6.2. Preparation of sample solution

Lamivudine, Zidovudine and Nevirapine tablets powder that is equivalent to 6.0 g of Zidovudine drug was taken into a beaker, added about 600 ml of water and mixed thoroughly and sonicated for 2 hours. The same procedure was repeated again. The mixture was filtered under vacuum using Buchner funnel. Then the filtrate was loaded into the preparative column using the conditions mentioned in section 2.1.2.3.

2.3.6.3. Preparative HPLC conditions

The sample solutions prepared above were loaded into a Shimadzu preparative liquid chromatography Hyper prep HS C\textsubscript{18} (500 mm × 30 mm id) preparative column packed with 10 µm particle size was employed for isolation of impurities. Flow rate was kept at 35 ml/min
and detection was carried out at wavelength of 270 nm. The gradient programme was as follows.

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Mobile Phase A (v/v)</th>
<th>Mobile Phase B (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀₀₁</td>
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<td>0</td>
</tr>
<tr>
<td>T₂₀</td>
<td>95</td>
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<td>T₄₀</td>
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<td>30</td>
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<tr>
<td>T₉₀</td>
<td>80</td>
<td>20</td>
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</table>

2.4. Results and Discussions
2.4.1. Detection of impurity

In our regular analysis of lamivudine Zidovudine and Nevirapine tablets for oral suspension we have observed one unknown impurity (degradation product) in initial sample below 0.1% (Fig-2.1). This unknown impurity was increased to 0.25 % in the 3 months stability condition of 50°C PEPFAR condition (President emergency plan for AIDS relief) when calculated against Lamivudine which is the low dose component of the formulations (Fig-2.2).
We have started our experiment to find out which factor is responsible for the formation of this impurity. This is not coming due to diluent (Fig 2.3) and plain placebo (Fig 2.4), it is coming only during stability of 3m 50°C. We have analyzed autoclaved placebo with individual API's of lamivudine, zidovudine and nevirapine (Fig 2.5,2.6,2.7). The unknown impurity was observed in the chromatogram of zidovudine and placebo (Fig 2.8). Then we have analysed zidovudine and individual excipients microcrystalline cellulose, sodium starch glycolate, povidone, carbomer, magnesium stearate, pineapple flavour, pipperrment 501500, colloidal silicon dioxide, acesulfame potassium sweetening agent. We have observed
the unknown impurity peak in the chromatogram of zidovudine with
sweetening agent acesulfame potassium (Fig-2.8). With this we got
confirmation this unknown impurity was coming from sweetening
agent and zidovudine.

Fig-2.3.LC Chromatogram of diluent

Fig-2.4-LC Chromatogram of placebo
Fig-2.5 - Placebo with lamivudine API

Fig-2.6 Placebo with Zidovudine API

Fig-2.7- Placebo with Nevirapine API
Fig-2.8. Acesulfamepotassium + Zidovudine

To check the resolution with other known impurities, an analytical LC chromatogram of a laboratory batch of Lamivudine, Zidovudine and Nevirapine sample (Fig-2.9) and all known impurities spiked with unknown impurity (degradation product) is shown in (Fig. 2.10). All impurities were separated from Lamivudine, Zidovudine and Nevirapine peaks. Relative retention time of the degradation product with respect to zidovudine is 0.93.

Fig-2.9. LC Chromatogram of Lamivudine  Zidovudine and
Neverapine TFOs spiked with degradation product.

Fig-2.10. LC Chromatogram of Lamivudine Zidovudine and Neverapine all known impurities spiked with degradation product.

In a fixed dose combination product agency emphasize the reporting of unknown impurity against the lowest dose available in combination product. Since 0.25 level is above the identification/qualification threshold, an attempt was made to isolate, characterize and identify this thermal degradation impurity.

2.4.2. Isolation of Impurity (Degradation Product) By Preparative HPLC

Fractions of impurity above 85% was isolated using procedure given in section 2.3.5. Analytical conditions mentioned in section 2.3.3 was used to analyse the pooled fractions which were then concentrated using rotavapour. Excess buffer was removed by passing concentrated fractions through preparative column with acetonitrile and water as mobile phase. Acetonitrile was also removed subsequently from the eluate by rotavapour. The aqueous concentrate was lyophilised using
freeze dryer. The chromatographic purity of isolated compound is 92% analyzed by method under section 2.3.5.3. This was then subjected for further characterization.

2.4.3. Structural elucidation of impurity (degradation product)

The mass spectrum of Lamivudine, Zidovudine, Nevirapine and the degradation product shows molecular ion peaks at m/z 230, 268, 267 and 308 amu [(MH)+] respectively in positive ion mode (Fig 2.11-2.14). In the mass spectra of Zidovudine and the degradation product two major similar fragment ions m/z 127 amu and 225 corresponding to Thymine moiety and Azide group eliminated Zidovudine moiety confirming that this impurity is related to Zidovudine. The fragmentation pattern for all these four products is shown in (16-19).

The degradation product has additional mass of 41 units more to Zidovudine that can be accommodated by a three carbon moiety coming from sweetening agent Acesulfame potassium as shown in scheme 2.1. Moreover in 1H-NMR (Fig.2.15) and 13C- NMR(Fig.2.16) spectra of this degradation product, the majority of protons and carbons chemical shift values (Table-2.1) are similar to those present in Zidovudine (Fig.2.17-2.18). But in 1H-NMR two additional signals, CH3 [15-CH3; 2.33(S,3H)] and CH [13-CH; 7.55(s,1H)] were observed. In 13C-NMR three additional carbon signals, CH3[15-CH3; 8.6 ppm], CH[13-CH,133.3 ppm] and C[14-C,134.5 ppm] are due to three carbon moiety coming from sweetening agent Acesulfame potassium as shown in Scheme-I. In comparison to Zidovudine NMR (Fig -2.17)
signals, there is no shift observed in the signals corresponding to thymine and sugar moieties. Except a up field shift observed in $^{13}\text{C}$-NMR of [12-CH] from $\delta$ 61.0 ppm to $\delta$ 57.8 ppm and an appreciable down field shift in $^1\text{H}$-NMR from $\delta$ 4.40 ppm to $\delta$ 5.13 ppm supporting the presence of triazole ring. Major functional groups characteristic of Zidovudine (Fig2.20) was observed in the IR absorption scan of the degradation compound (Fig 2.19).

IR Absorption bands for degradation product (cm$^{-1}$) are 1694(C=O stretch), 1472(aliphatic CH bend), 1104(C-N stretch). The absence of azide absorption in IR (2080-2115) and the presence of CH$_3$ and CH signals in $^{13}\text{C}$ -NMR also recommend our proposed structure. The proposed mechanism for the formation of degradation product is shown in scheme-2.1. From the above spectral data the structure of this impurity is confirmed as 1-[5-Hydroxymethyl-4-(5-methyl-2,3-dihydro-[1,2,3]triazol-1yl)-tetrahydro-furan-2-yl]-5methyl-1H-pyrimidine-2,4-(1H,3H)-dione with molecular formula C$_{13}$H$_{17}$N$_5$O$_4$ and molecular weight 308.

Mass fragmentation pattern of Lamivudine(16), Zidovudine (17), Nevirapine (18) and degradation product (19)
Lamivudine (16) Zidovudine (17) Nevirapine (18) Degradation product (19)

Zidovudine Numbering (20) Degradation Product Numbering (21)

Table 2.1 Comparative $^1$H, $^{13}$C and DEPT NMR assignments for Zidovudine and degradation product

<table>
<thead>
<tr>
<th>Position</th>
<th>ZIDOVUDINE $^1$H-NMR δ (ppm)</th>
<th>ZIDOVUDINE $^{13}$C-NMR δ (ppm)</th>
<th>DEPT</th>
<th>DEGRADATION PRODUCT $^1$H-NMR δ (ppm)</th>
<th>DEGRADATION PRODUCT $^{13}$C-NMR δ (ppm)</th>
<th>DEPT</th>
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<tbody>
<tr>
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<td>1.82 (s, 3H)</td>
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<td>C</td>
<td>-</td>
<td>110.5</td>
<td>C</td>
</tr>
<tr>
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<td>-</td>
<td>167.0</td>
<td>C</td>
<td>-</td>
<td>164.5</td>
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</tr>
<tr>
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<td>-</td>
<td>11.38 (brs, 1H)</td>
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<tr>
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<td>-</td>
<td>2.33 (s, 3H)</td>
<td>8.6</td>
<td>CH$_3$</td>
</tr>
</tbody>
</table>

s, singlet; t, triplet; m, multiplet; brs, broad singlet;

DEPT: Distortionless Enhancement by Polarisation Transfer.
Position of numbers for Zidovudine and degradant is referred in structures 20 and 21 respectively.

2.5. Mechanism of Formation of Degradation Product

This impurity was observed during the stability of Lamivudine, Zidovudine and Nevirapine tablets for oral suspension. This was originated due to heat-or light induced interaction of azide group of Zidovudine with one of the excipient namely Acesulfame Potassium present in combination product of Lamivudine, Zidovudine and Nevirapine tablets for oral suspension. The proposed degradation path way is shown in scheme-2.1

Scheme-2.1. Formation of Degradation product with Mechanism
2.6. Conclusion

An unknown impurity (degradation product) present at a level threshold limit in initial sample increased to a % above threshold limit in 50°C 3M stability samples of Lamivudine, Zidovudine and Nevirapine tablets for oral suspension which was detected by reverse phase HPLC. This thermal degradation impurity was identified, isolated and characterized by using HPLC (analytical and preparative) mass, NMR (\(^{1}\text{H}\) and \(^{13}\text{C}\)) and IR techniques. The spectral data, conforms the structure of this degradation product as 1-\([5\text{-Hydroxymethyl-4-(5-methyl-2,3-dihydro-[1,2,3]triazole-1yl]-tetrahydro-furan-2-yl]-5-methyl-1H-pyrimidine-2,4-(1H,3H)-dione}\).