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Identification, isolation, and characterization of potential degradation products in a triple combination lamivudine, zidovudine, and nevirapine tablet for oral suspension

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An unknown impurity (degradation product) present at a level below 0.1% in the initial sample was increased to 0.25% in 50 °C 3 M stability samples of lamivudine, zidovudine and nevirapine tablets for oral suspension, as detected by gradient reverse phase HPLC. This degradation product was isolated using reverse phase preparative HPLC. Based on the spectral data, the structure of this degradation product is characterized as 1-[5-hydroxymethyl-4-(5-methyl-2,3-dihydro-[1,2,3]triazole-1-yl)-tetrahydrofuran-2-yl]-5-methyl-1*H*-pyrimidine-2,4-(1*H*,3*H*)dione. Structural elucidation of this degradation product was carried out using MS, ¹H NMR, ¹³C NMR, DEPT and IR spectral data. The formation of this impurity and its mechanism are discussed.

1. Introduction

Lamivudine, zidovudine and nevirapine are well established antiretroviral agents used for the treatment of AIDS (Merck Index 14th ed., 2006). They are chemically (2*R*, 5*S*)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1*H*)-pyrimidinone, 1-(3-azido-2,3-dideoxy-β-D-ribofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione and 11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-*b*:2',3'-*e*][1,4]diazepin-6-one respectively. A fixed dose combination of these three drugs is commonly used to prevent the development of resistant viral strains. Some analytical methods have been reported in the literature for quantification of these drugs in human plasma and in combination dosage forms (Fan et al. 2002; Nandini et al. 2007; Indian Pharmacopoeia 2007). A number of impurities and degradation products have been reported for the drug substance and fixed dose combination of these three drugs (Indian Pharmacopoeia 2007; United States Pharmacopoeia 2009; European Pharmacopoeia 2009). However to the best of our knowledge there is no reported method for the identification, isolation and characterization of the light/thermal sensitive degradation product. According to regulatory requirements, an impurity profile study has to be carried out for any final product (ICH Guideline 2008; ICH Guideline 2006).

This paper describes the identification isolation, and characterization of an unknown impurity (degradation product) formed in the lamivudine, zidovudine and nevirapine combination product when exposed to accelerated stability studies carried out according to ICH guidelines. The various process related impurities and degradation products known in the Pharmacopoeias are listed in Fig. 1.

2. Investigations, results and discussion

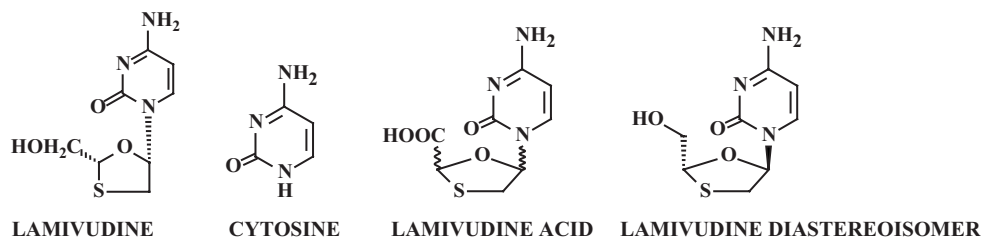
2.1. Detection of impurity

The 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 strength is a pediatric formulation available as tablets for oral suspension. In a fixed dose combination product, regulatory agencies emphasize the reporting of any unknown impurity against the lowest dose component of the combination product. In the 3 month stability test at 50 °C (PEPFAR condition) an unknown impurity was observed at a level of 0.25% when calculated against lamivudine which is the low dose component of the formulation. Since 0.25% is above the identification/qualification threshold, an attempt was made to isolate, characterize and identify this thermal degradation impurity. An analytical LC chromatogram of a laboratory batch of lamivudine, zidovudine and nevirapine tablets spiked with all known impurities is shown in Fig. 2 (a). The chromatogram of lamivudine, zidovudine and nevirapine tablets for oral suspension spiked with the unknown impurity is shown in Fig. 2(b). All the impurities were well resolved from the lamivudine, zidovudine and nevirapine peaks. the relative retention time of the degradation product with respect to lamivudine is 0.93. Chemical structures of lamivudine, zidovudine and nevirapine and the degradation product are shown in Fig. 2.

2.2. Structural elucidation of impurity (degradation product)

The electro spray ionization (ESI) mass spectrum of lamivudine, zidovudine, nevirapine and the degradation product

(a) Lamivudine and its impurities



(b) Nevirapine and its impurities

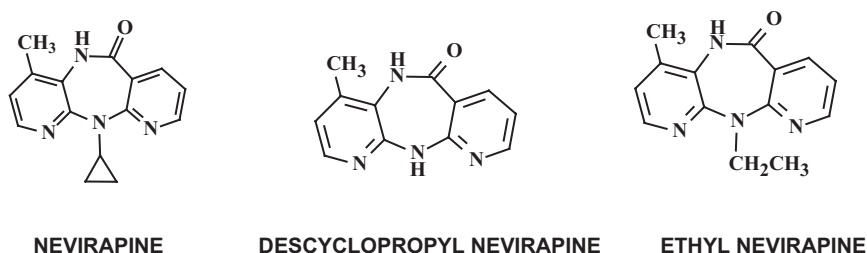


Fig. 1: Chemical structures of lamivudine, zidovudine and nevirapine and their impurities

shows molecular ion peaks at m/z 230, 268, 267 and 308 amu $[(MH)^+]$ respectively in positive ion mode. The mass spectra of zidovudine and the degradation product showed two major similar fragment ions with m/z 127 amu and 225 corresponding to the thymine moiety and azide group eliminated from zidovudine, confirming that this impurity is related to zidovudine. The fragmentation patterns for all four products are shown in Scheme 1. The degradation product has an additional mass of 41 units more than zidovudine that can be accommodated by a three carbon moiety coming from the sweetening agent acesulfame potassium as shown in Fig. 4. Moreover in 1H -NMR and ^{13}C -NMR spectra of this degradation product, the majority of protons and carbons show chemical shift values similar to those in zidovudine. However, in 1H NMR two additional signals, CH_3 [15- CH_3 ; 2.33(s,3H)] and CH [13- CH ; 7.55(s,1H)] were observed. In ^{13}C -NMR three additional carbon signals, CH_3 [15- CH_3 ; 8.6 ppm], CH [13- CH ; 133.3 ppm] and C [14- C ; 134.5 ppm] are due to the three carbon moiety from acesulfame potassium as shown in Scheme 2. In comparison to zidovudine NMR signals, no shift was observed in the signals corresponding to the thymine and sugar moieties, apart from an up field shift observed in ^{13}C NMR of [12- CH] from δ 61.0 ppm to δ 57.8 ppm and an appreciable down field shift in 1H NMR from δ 4.40 ppm, to δ 5.13 ppm, supporting the presence of a triazole ring. In the IR (KBr) absorption spectrum of the degradation product, the characteristic absorption bands corresponding to major functional groups are similar to zidovudine. IR absorption bands for the 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}C NMR also support our proposed structure. The proposed mechanism for the formation of the degradation product is shown in Fig. 4. 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-1-yl)-tetrahydro-furan-2-yl]-5-methyl-1H-pyrimidine-2,4-(1H,3H)-dione with molecular formula $C_{13}H_{17}N_5O_4$ and molecular weight 308.

The 1H and ^{13}C NMR chemical shift values of zidovudine and its degradation product are given in the Table 1.

2.3. Mechanism of formation of degradation product

This impurity was observed during stability testing of lamivudine, zidovudine and nevirapine tablets for oral suspension. It originated from the heat or light-induced interaction of the azide group of zidovudine with one of the excipients, namely acesulfame potassium present in the combination product. The proposed degradation pathway is shown in Fig. 4.

3. Experimental

3.1. Samples and chemicals

The investigated samples of lamivudine, zidovudine and nevirapine were formulated at the APL Research Centre (a unit of Aurobindo Pharma Limited, Hyderabad, India). All known impurities were synthesized in the Chemical Research Department of the APL Research Centre. Reagents used for analysis, i.e., ammonium acetate (GR Grade), glacial acetic acid and methanol (HPLC Grade), were procured from Merck (India) Limited. Milli-Q grade water was used for the analysis and isolation.

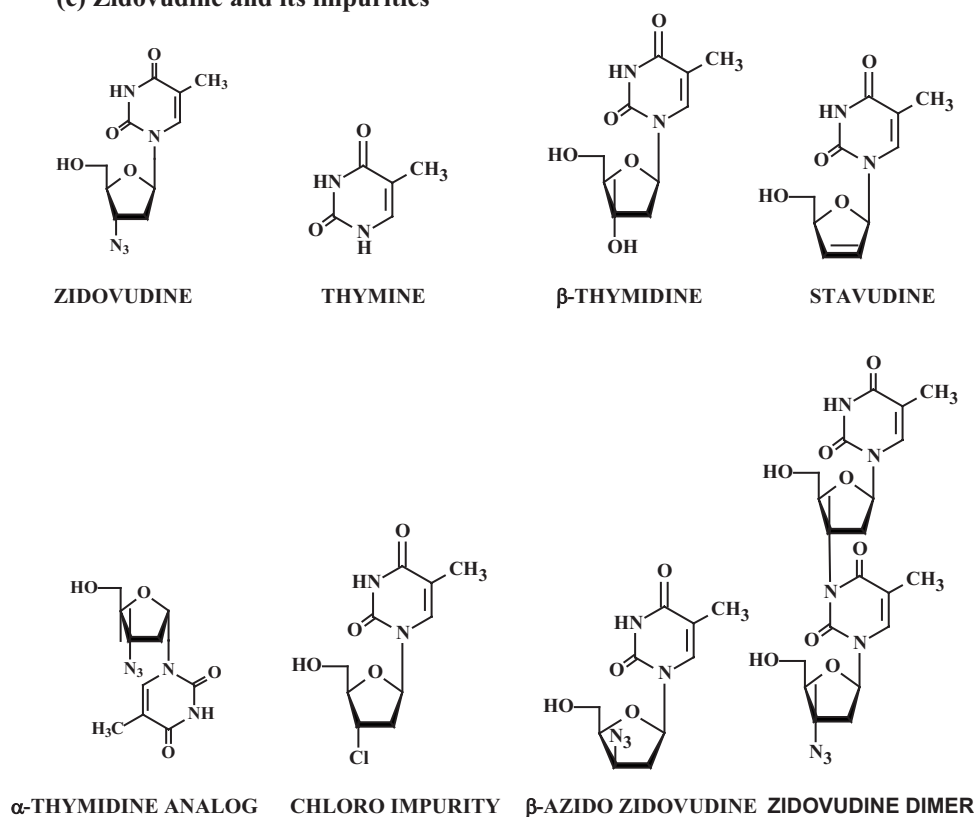
3.2. High performance liquid chromatography

A Waters 2695 separation module equipped with a 2996 photodiode array detector with an Empower pro data handling system (Waters Corporation, Milford, MA, USA) was used, and the analysis was carried out on a YMC-Pack ODS-AQ, 250 mm long, 4.6 mm id, 5 μm particle diameter column. Mobile phase A was prepared by dissolving 9.64 g of ammonium acetate in 1000 ml of water, adjusting pH to 4.2 with glacial acetic acid and filtering through a 0.45 μm size fine porosity membrane filter. Mobile phase B was degassed methanol. UV detection was carried out at 270 nm and flow rate was kept at 0.8 ml/min. Column oven temperature was maintained at 45 $^{\circ}C$. Data acquisition time was 50 min. Pump mode was Gradient and the program was as follows: time (min)/A(v/v):B(v/v); $T_{0.01}/95:05$, $T_{05}/95:05$, $T_{10}/85:15$, $T_{35}/40:60$, $T_{40}/40:60$, $T_{41}/95:05$, $T_{50}/95:05$.

3.3. Preparative liquid chromatography

A Shimadzu LC-8A preparative liquid chromatograph equipped with an SPD-10A VP, UV-Visible detector (Shimadzu Corporation, Analytical

(c) Zidovudine and its impurities



(d) Numbering of the structures

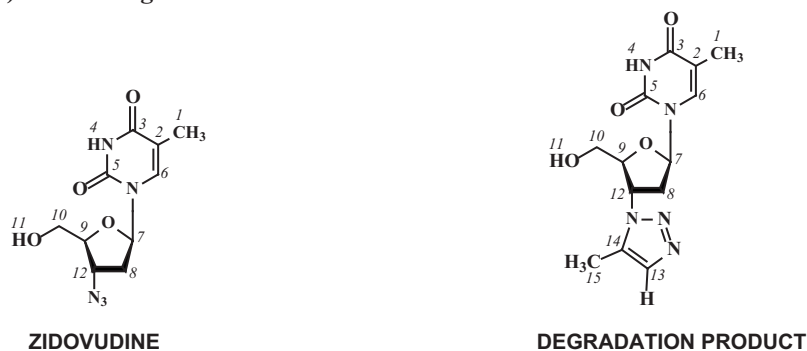


Fig. 1: (Continued)

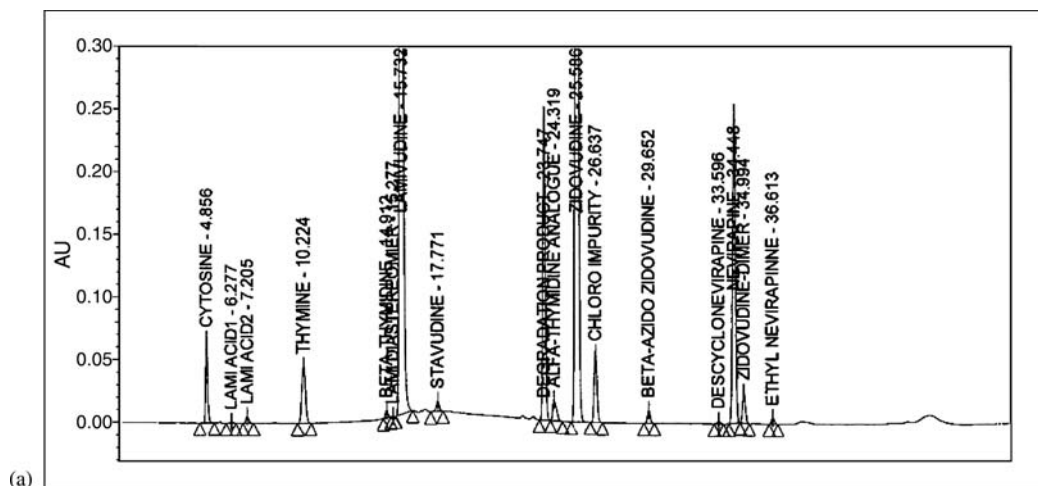


Fig. 2: (a) LC chromatogram of lamivudine, zidovudine and nevirapine tablets spiked with known impurities. (b) LC chromatogram of Lamivudine, zidovudine and nevirapine spiked with degradation product

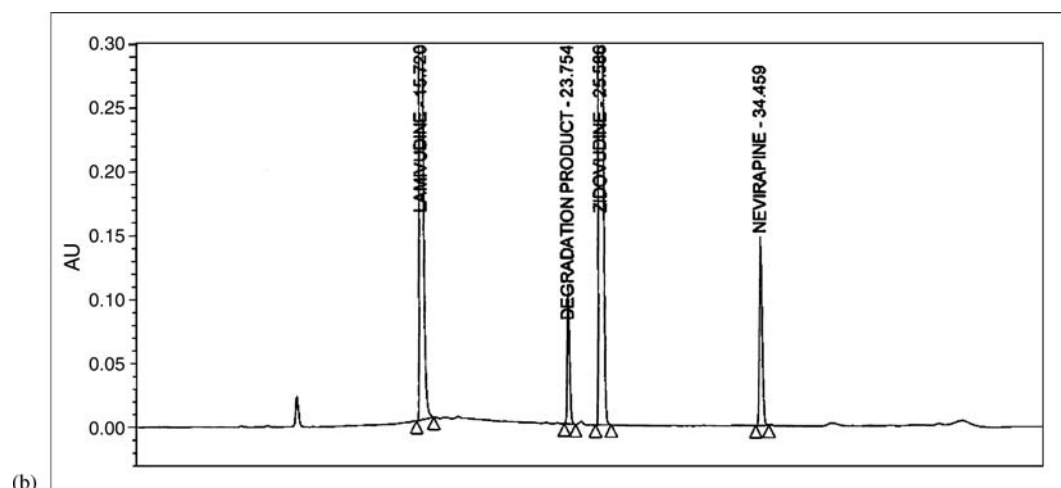
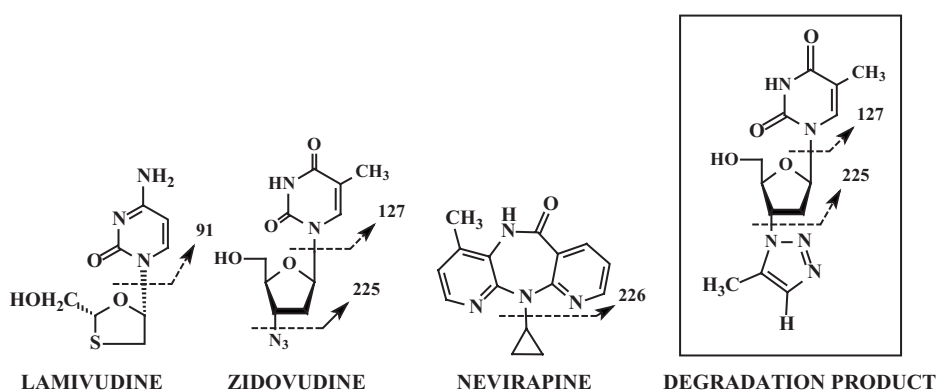


Fig. 2: (Continued)



Scheme 1: Mass fragmentation pattern of lamivudine, zidovudine, nevirapine and degradation product

Instruments Division, Kyoto, Japan) was used. A Hyper prep HS C₁₈ (500 mm × 30 mm id) preparative column packed with 10 μm particles was employed for isolation of the degradation product. The mobile phase consisted of (A) 0.2% trifluoro acetic acid solution and (B) methanol. UV detection was carried out at 270 nm. Flow rate was kept at 35 ml/min. Pump mode was Gradient and the program was as follows: time(min)/A(v/v):B(v/v); T_{0.01}/100:00, T₂₀/95:05, T₄₀/90:10, T₆₀/85:15, T₈₀/70:30, T₉₀/80:20.

3.4. NMR spectroscopy

The ¹H NMR, ¹³C NMR (proton decoupled) and DEPT experiments were performed on a Bruker 300 MHz nuclear magnetic resonance spectrom-

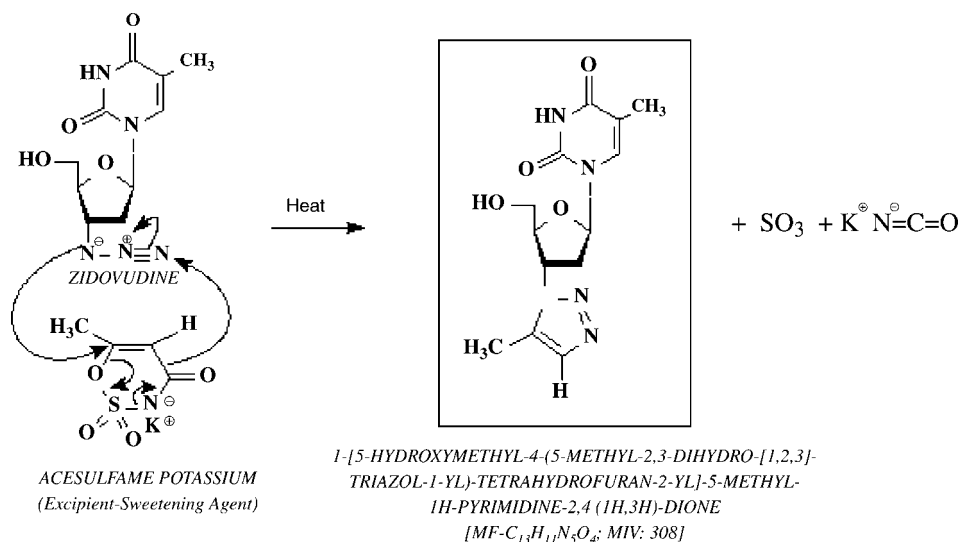
eter using DMSO-d₆ as solvent and tetramethylsilane (TMS) as internal standard.

3.5. Mass spectrometry

Mass spectra were recorded on a PE SCIEX-API 2000 triple, quadrupole mass spectrometer equipped with a turbo ion spray interface at 375 °C. Detection of ions was performed in electro spray ionization positive ion mode.

3.6. FT-IR spectroscopy

FT-IR spectra were recorded as KBr pellet on a Perkin-Elmer instrument model-spectrum one.



Scheme 2: Proposed mechanism for formation of degradation product

Table 1: Comparative ^1H , ^{13}C and DEPT NMR assignments for zidovudine and its degradation product

zidovudine				degradation product		
Position	^1H NMR δ (ppm)	^{13}C -NMR δ (ppm)	DEPT	^1H NMR δ (ppm)	^{13}C -NMR	DEPT
1	1.78(s,3H)	13.0	CH_3	1.82(s,3H)	13.09	CH_3
2	—	110.0	C	—	110.5	C
3	—	167.0	C	—	164.5	C
4	11.32(brs,1H)	—	—	11.38(brs,1H)	—	—
5	—	152.0	C	—	151.1	C
6	7.68(s,1H)	136.9	CH	7.81(s,1H)	137.0	CH
7	6.10(t,1H)	84.2	CH	6.50 (t,1H)	85.2	CH
8	2.27–2.36(m,2H)	38.0	CH_2	2.61–2.69(m,2H)	37.5	CH_2
9	3.81(m,1H)	84.8	CH	4.20(m,1H)	85.5	CH
10	3.62(m,2H)	62.0	CH_2	3.67(m,2H)	61.8	CH_2
11	5.22(t,1H)	—	—	—	—	—
12	4.40(m,1H)	61.0	CH	5.13(m,1H)	57.8	CH
13	—	—	—	7.55(s,1H)	133.3	CH
14	—	—	—	—	134.5	C
15	—	—	—	2.33(s,3H)	8.6	CH_3

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

DEPT: Distortionless enhancement by polarisation transfer

^aRefer to chemical structures in Fig 1.d for numbering of zidovudine and its degradation product

3.7. Enrichment of degradation product

Lamivudine, zidovudine and nevirapine tablet powder was kept in an autoclave at 121 °C/15-16 psi pressure/30 min for three cycles. Autoclaved samples were analyzed by the HPLC method mentioned in section 3.2. The degradation product was enriched to about 3.0% by area normalization.

3.8. Isolation of impurity (degradation product) by preparative HPLC

Lamivudine, zidovudine and nevirapine tablet powder equivalent to 6.0 g of zidovudine drug was taken into a beaker, about 600 ml of water added and mixed thoroughly and sonicated for 2 h. The same procedure was repeated. The mixture was filtered under vacuum using a Buchner funnel. Then the filtrate was loaded into the preparative column using the conditions mentioned in section 3.3. The fractions collected were analyzed by analytical HPLC under the conditions mentioned in section 3.2. Fractions of >90% were pooled together, and concentrated by rotavapour to remove solvent mixture. Concentrated fractions were passed through the preparative column using water: acetonitrile (50:50) as the mobile phase to remove the buffer used in isolation. Again the eluate was concentrated using a rotavapour to remove acetonitrile. The aqueous solution was lyophilized using a freeze dryer (Virtis Advantage 2XL). The impurity was obtained as an off-white powder and the chromatographic purity was 92.0%, determined by the HPLC method mentioned in section 3.2.

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