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Identification, isolation and characterization of new process related impurities in Tenofovir

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ABSTRACT

Tenofovir disoproxil fumarate (DF) is a nucleotide analogue, which acts against to retroviruses, including HIV-1, HIV-2 and hepadnaviruses. In vivo Tenofovir disoproxil fumarate is rapidly converted to tenofovir, which is metabolized intracellularly to its active anabolite tenofovir diphosphate, which is a competitive inhibitor of HIV-1 reverse transcriptase and terminates the growing DNA chain. The chemical name of Tenofovir disoproxil fumarate is 9-[(R)-2-[[bis[[(isopropaxycarbonyl)oxylmethoxylphosphinyl] methoxy]propyl]adenine fumarate (1:1). During routine monitoring of samples by HPLC, two unknown impurities were observed other than those specified in monograph. The molecular weights of the impurities were determined by LC-MS. The structures were postulated to be (R)-5-[2-6-(Isopropyloxycarbonylamino)-9H-purin-9yl-)-1-methylethoxy]-methyl]-2,4,6,8-tetraoxa-5 phosphanonanedioic acid bis-(1-methylethyl)ester5-oxide [Impurity/Impurity-I] and (R)-5-[[2-6(Isopropoxymethylamino)-9H-purin-9yl)-1-methylethoxy]-methyl]-2,4,6,8-tetraoxa-5-phosphanonane-dioicacidbis-(1-methylethyl) ester-5-oxide [Impurity-II]. These impurities were analysed by mass, IR and NMR. Their presence was confirmed by spiking into Tenofovir sample and carrying out HPLC analysis.

Key words: Tenofovir, Impurities, Isolation, Characterization, LC-MS, NMR, IR.

1. INTRODUCTION

Tenofovi chemically known is 9-[(R)-2-[bis[[(isopropaxycarbonyl)oxylmethoxylphosphinyl] methoxy]propyl]adenine fumarate, is a nucleotide reverse transcriptase inhibitor used for the treatment of HIV infection. It is given orally as the disoproxil fumarate ester. Literature available is mainly regarding determination of Tenofovir and or its metabolites in human plasma and Pharmacokinetics of formulated drug product. To date, no mention is available regarding impurity-I and impurity – II in literature to the best of our knowledge.

During the analysis of different laboratory batches of tenofovir, two new process related impurities were observed whose area percentage ranged from 0.05% to 0.1%. This paper deals with identification, isolation, characterization and also the formation of two impurities. A thorough study has been undertaken to characterize these impurities, by spectroscopic techniques. In wake of regulatory requirements to control impurities to ≤ 0.1% level, impurity profile has to be carried out for any final product. All the unknown impurities above 0.05% need to be identified, and characterized [1-11].

2. EXPERIMENTAL

2.1. Sample, Chemicals and reagents

Tenofovir was synthesized in CRD department of APL Research Centre (A Division of Aurobindo Pharma Ltd.) (Bachupally, Quthubullapur, Hyderabad-90, INDIA). Ammonium acetate (BDH grade), Acetic acid (Fluka grade), Aacetonitrile (HPLC grade) and, Distilled water was prepared by using Milli-Q water purification system (Millipore, Bedford, MA).

2.2. High Performance Liquid Chromatography (Analytical)

Chromatographic separation was performed on High Performance Liquid Chromatography system with Shimadzu binary gradient system with SCL-10At Vp pumps, SIL-10AD Vp auto injector and Class-VP Software for instrument control and data acquisition [Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan]. Analysis was carried out on Zorbax SB Phenyl with dimensions of 250 mm*4.6 mm i.d packed with 5 µm particle size was employed for separations. Mobile phase A was 0.01M Ammonium acetate in water pH adjusted to 4.5 with Acetic acid. Mobile phase B was acetonitrile. UV detection was at 262 nm and flow rate was kept at 1 ml/min. data acquisition

time was 60 min. pump mode was gradient and the program was as follows, 0-25 min: 85% A - 15% B; 25-40 min. 70% A-60% B; 40-50 min. 40% A- 80% B; 50-60 min. 20% A–80% B;60-62min.20%A-15%B; 62-70min.85%A-15%B.

2.3. High Performance Liquid Chromatography (Preparative)

A Shimadzu LC-8A Preparative Liquid Chromatograph equipped with SPD-10A VP, UV-Vis detector [Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan] was used. Hyper Prep HS C18 (500 mm long x 30 mm i.d.) preparative column packed with 10 µm particle size [Thermo Electron Corporation, UK] was employed for isolation of impurity. The mobile phase consisted of (A) 0.01M Ammonium acetate in water pH adjusted to 4.5 and (B) acetonitrile. Flow rate was kept at 40 ml/min and detection was carried out at 262 nm. The gradient program was as follows, 0.01 – 40 min.: 85% A-15% B; 40 – 70 min.: 70% A – 60% B; 70 – 90 min.: 40%A – 80%B; 90 – 120 min.: 20% A-80% B; 120 – 122 min 20% A – 15% B, 122 – 140 min 85% A – 15% B.

2.4. LC-MS/MS Analysis

ESI mass spectra were recorded on Perkin Elmer triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with shimadzu HPLC equipped with SPD 10 A VP UV-VIS detector and LC 10 AT VP pumps. Analyst software was used for data acquisition and data processing. The turbo ion spray voltage was maintained at 5.5 Kv and temperature was set at 375°C. The auxiliary gas and sheath gas used was high pure Nitrogen. Zero air was used as Nebuliser gas. LC-MS spectra were acquired from m/z 105-1200 in 0.1 amu steps with 2.0 sec dwell time. LC-MS analysis of the sample was carried out using Zorbax SB Phenyl with dimensions of 250 mm*4.6 mm i.d packed with 5 µm particle size was employed for separations. Mobile phase A was 0.01M Ammonium acetate in water pH adjusted to 4.5 with Acetic acid. Mobile phase B was acetonitrile. UV detection was at 262 nm and flow rate was kept at 1 ml/min. data acquisition time was 60 min. pump mode was gradient and the program was as follows, 0-25 min: 85% A - 15% B; 25-40 min. 70% A-60% B; 40-50 min. 40% A-80% B; 50-60 min. 20% A–80% B;60-62min.20%A-15%B; 62-70min.85%A-15%B.

2.5. NMR Spectroscopy

The ^1H experiments were performed on a Bruker Avance DPX-300 MHz NMR spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard.

2.6. Mass Spectrometry

A Mass spectrum was recorded on Perkin Elmer triple quadrupole mass spectrometer (API 2000, PE SCIEX) with Electro spray ionization.

2.7 IR spectroscopy

The IR spectra were recorded in the solid state as KBr pellet using PerkinElmer instrument, model-spectrum one [PerkinElmer Ltd., Beaconsfield, UK]

3. RESULTS AND DISCUSSIONS

3.1. Detection of impurities

Sample solution equivalent to 1 mg/ml of Tenofovir prepared in mobile phase was injected into the analytical LC using the solvent system as described in section 2.2. Two impurities about 0.05% and 0.10% eluted at relative retention times (RRT) 1.17, and 1.19 respectively while the retention time of Tenofovir is about 31.0 minutes. A typical chromatogram is shown in Figure.1. The same samples were subjected to LCMS analysis using conditions as described in section 2.4 to identify the mass of the impurities. The mass of the impurities recorded in positive ion mode was same i.e 606 [M+H] for Impurity-I eluted at RRT-1.17, and 592 [M+H] for Impurity - II eluted at RRT-1.19 respectively. The chemical structures of these impurities are given in Figure 2.

Fig-1: Typical Chromatogram of Tenofovir

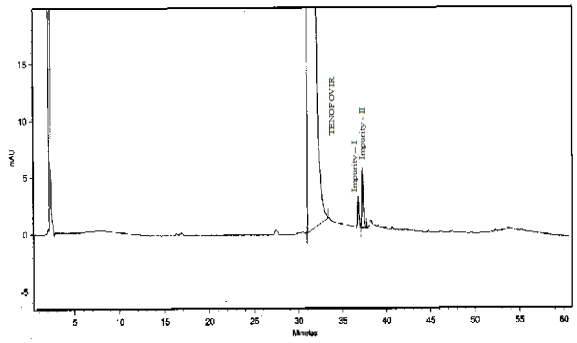
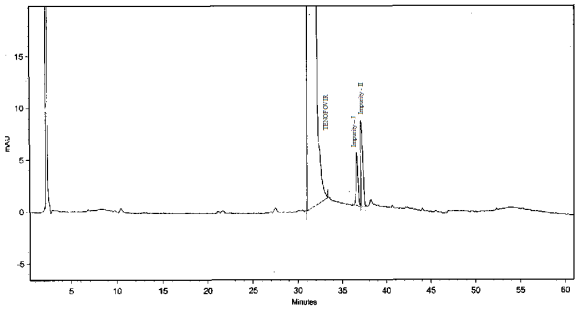


Fig-2: Typical chromatogram of Tenofovir spiked with Impurities I and II



3.2. Isolation of impurities by preparative HPLC

Crude samples was subjected to preparative HPLC as per the conditions given in section 2.3.Fractions collected were analyzed by analytical chromatographic conditions given in section 2.2.Fractions of more than 90% were pooled together concentrated on rotavapour to remove acetonitrile. Tenofovir impurity-I was obtained as an off-white powder with chromatographic purity of 95%, and Tenofovir impurity-II was obtained white powder with chromatographic purity of 94%.

3.3. Structural elucidation

3.3. 1.Tenofovir impurity-I

ESI mass spectrum of Tenofovir impurity-I is in positive ion mode showed a molecular ion peak at m/z 606 $[(MH)^+]$ indicating the molecular weight of Tenofovir impurity-I as 605.In the ^1H NMR spectrum of this impurity all the signals corresponding to tenofovir protons. The signals corresponding to purine attached NH protons(10.28ppm as exchangeable singlet).Additional signals observed at 4.9ppm(CH) and 1.27ppm($(-CH_3)_2$) signals corresponding to purine attached amine and it is concluded from the ^1H NMR and ^{13}C NMR values that chloromethyl isopropyl carbamate which is used as reagent in the synthesis of tenofovir. And the formation of Tenofovir impurity-I due to excess quantity of chloromethyl isopropyl carbamate react with tenofovir in presence of water and temperature .The above spectral data confirms the impurity as ((R)-5-[2-6-(Isopropyloxycarbonylamino)-9H-purin-9yl-)-1-methylethoxy]-methyl]-2,4,6,8-tetraoxa-5-phosphanonanedioicacidbis-(1-methylethyl) ester5-oxide) impurity-I Molecular formula is $C_{23}H_{36}N_5O_{12}P$ with molecular weight 605 and formation of tenofovir impurity-I is given in Figure-3.

Fig-3: Mechanism for the formation of Impurity – I

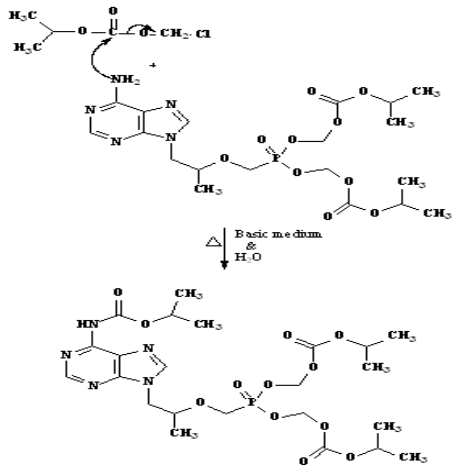


Table -2: NMR Data of Tenofovir and its impurities

	TENOFAVIR		
	^1H NMR (ppm)	^{13}C	DEPT
1	1.23 (d, 12H)	22.1	4*CH_3
2	4.32 (m,2H)	73.7	2CH
3	--	153.4	2C
4	5.53 (m, 4H)	85.0,85.1	$2CH_2$
5	3.99(m,2H)	61.8,64.0	CH_2
6	3.99(m,1H)	76.7,76.8	CH
7	1.06(d,3H)	17.5	CH_3
8	4.32(ABq,2H)	47.4	CH_2
9	8.03(s,1H)	142.2	CH
10	--	119.1	C
11	--	150.6	C
12	8.14 (s, 1H)	153.1	CH
13	--	156.7	C
14	7.23(s,2H)	--	--
	IMPURITY - I		
	^1H NMR (ppm)	^{13}C	DEPT
1	1.24 (d, 12H)	21.4	4*CH_3
2	4.81 (m, 2H)	73.0	2CH
3	--	152.7	2C
4	5.51(m,4H)	84.2,84.3	$2CH_2$
5	4.01(ABq, 2H)	60.9,63.1	CH_2
6	3.98 (m,1H)	75.80,75.95	CH
7	1.10 (d, 3H)	16.7	CH_3
8	3.98 (ABq, 2H)	47.0	CH_2
9	8.31 (s, 1H)	144.7	CH
10	--	122.9	C
11	--	152.6	C
12	8.59 (s, 1H)	151.60	CH
13	--	152.2	C
14	10.28 (Brs, 1H)	--	--
	--	151.8	C
	4.90 (m, 1H)	68.6	CH
	1.27 (d, 6H)	21.90	$2CH_3$
	--	--	--

	IMPURITY – II		
	^{1}H NMR (ppm)	^{13}C	DEPT
1	1.24 (d, 12H)	22.0	4*CH_3
2	4.83 (m, 2H)	73.7	2CH
3	--	152.8	2C
4	5.57(m,4H)	84.9,85.0	2CH_2
5	4.25,4.30 (ABq, 2H)	61.2,63.4	CH_2
6	4.01 (m, 1H)	76.6,76.8	CH
7	1.15 (d,3H)	17.33	CH_3
8	4.01(m, 2H)	46.9	CH_2
9	8.13 (s, 1H)	142.7	CH
10	--	118.9	C
11	--	150.0	C
12	8.30(s, 1H)	153.0	CH
13	--	154.3	C
14	8.44 (Brs, 1H)	--	--
	--	--	--
	3.86 (m,1H)	68.6	CH
	1.09 (d, 6H)	23.1	2CH_3
	5.12 (Brs, 2H)	67.3	CH_2

3.3.2. Tenofovir impurity-II

ESI mass spectrum of Tenofovir impurity-II is in positive ion mode showed a molecular ion peak at m/z 592 [$(MH)^+$] indicating the molecular weight of Tenofovir impurity-II as 591.In the ^{1}H NMR spectrum of this impurity all the signals corresponding to tenofovir protons. The signals corresponding to purine attached NH protons(8.44ppm as exchangeable singlet). Additional signals observed at 3.86ppm(CH),1.09ppm(($-CH_3$)$_2$) and 5.12ppm(CH_2)signals corresponding to purine attached amine and it is concluded from the ^{1}H NMR and ^{13}C NMR values that chloromethyl isopropyl carbamate which is used as reagent in the synthesis of tenofovir. And the formation of Tenofovir impurity-II due to excess quantity of chloromethyl isopropyl carbamate is converting to chloromethyl isopropyl ether which is reacting with tenofovir in presence of water, temperature and basic medium. The above spectral data confirms the impurity as ((R)-5-[[2-6(Isopropoxy methylamino)-9H-purin-9yl)-1-methylethoxy]-methyl]-2,4,6,8-tetr aoxa-5-phosphanonane-dioicacidbis-(1-methylethyl) ester-5-oxide) impurity-II Molecular formula is $C_{22}H_{34}N_5O_{12}P$ with molecular weight 591 and formation of tenofovir impurity-II is given in Figure-4.

Table -1: FTIR Data of Tenofovir and its impurities

	TENOFOVIR		IMPURITY - I	IMPURITY – II
1	OH- stretch	3223		
2	CH- stretch	2986,2941,2837	3003,2944	3165,3003,2945
3	C=O- stretch	1760&1682	1826,1759	1763,1699
4	C=N- stretch	1623,1506,1471	1632	1632
5	Gem-dimethyl groups	1389,1378	1444,1376	1444,1376
6	C-N- stretch	1269	1243	1272
7	C-O-C- stretch	1034	1040	1040
8	CH out-of –plane bend	893,834	958,918	952,919

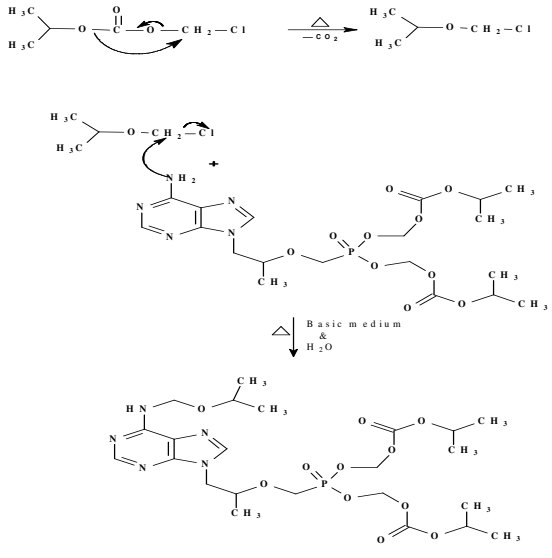
3.4. Origin of impurities

Chloromethyl isopropyl carbamate is used as raw material in the synthesis of tenofovir. There is possibility for range of impurities in the material evident from its preparation. The raw material used in the synthesis was evaluated for its purity by Gas chromatography.

3.4.1. Formation of impurities

Tenofovir is synthesized by carrying out with the Chloromethyl isopropyl carbamate. The presence of chloromethyl isopropyl ester and chloromethyl isopropyl ether impurities due to high temperature and water in the reaction to give corresponding impurities in the final product thus giving Impurity-I and Impurity-II. Control of these impurities are in the final product is important. The mechanism for formation of impurities is given in Figure-3 and 4.

Fig-4: Mechanism for the formation of Impurity - II



4. CONCLUSION

Two unknown process impurities, observed during regular monitoring by HPLC in tenofovir bulk drug samples were identified by LC-MS. These impurities were isolated and characterized by spectroscopic techniques viz., IR, NMR, and MS. The structures for these impurities were confirmed based on the spectral data. The origin of these impurities, formation of these impurities and structure elucidation of these impurities by spectral data has been discussed in detail.

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