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Short communication

Identification and characterization of new impurity in didanosine

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Abstract

Didanosine is an antiviral drug. During the preparation of didanosine in our lab, six process related known impurities and one unknown impurity were detected in HPLC analysis at levels ranging from 0.05 to 0.8%. The same unknown impurity was also observed in commercial batches. This new impurity was isolated by preparative HPLC and co-injected with didanosine sample to confirm the retention times in HPLC. This impurity was characterized as, 9-(2,3,5-trideoxy-β-D-*glycero*-pentofuranosyl)-9H-purin-6-one (2′,3′,5′-trideoxyinosine). Structural elucidation of this impurity by spectral data (^{1}H NMR, ^{13}C NMR, MS and IR) has been discussed.
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Keywords: Didanosine; Impurities; Isolation; Preparative HPLC; Characterization

1. Introduction

Didanosine is a synthetic purine nucleoside analogue active against the Human Immuno Deficiency Virus (HIV) and designated as 9-(2′,3′-dideoxy-β-D-ribofuranosyl) hypoxanthine [1,2]. It is the second antiretroviral agent, after zidovudine, approved by the Food and Drug Administration (FDA). Didanosine is indicated for the treatment of adult and pediatric patients with advanced HIV infection who are intolerant to zidovudine therapy. Several analytical methods have been reported in the literature for the determination of didanosine [3–9].

During the preparation of didanosine in the laboratory as per the scheme given in Fig. 1, one unknown impurity was detected consistently in HPLC along with six known impurities, i.e., hypoxanthine, inosine, 2′-deoxyinosine, 3′-deoxyinosine, 2′,3′-anhydroinosine and 2′,3′-dideoxy-2′,3′-didehydroinosine. One of the impurities of didanosine, 2′,3′,5′-trideoxyadenosine was reported in the European Pharmacopoeia [10], however, there was no report of this new 2′,3′,5′-trideoxyinosine in the literature. The HPLC analysis of didanosine bulk drug has been performed as per the method mentioned in Section 2.2 that revealed the presence of seven impurities, up to 0.8%. The impurity profile study has to be carried out for any final product as per the regulatory requirements to identify and characterize all the unknown impurities [11]. This paper describes the identification, isolation and characterization of unknown impurity present in didanosine. Isolation and characterization of this new impurity was not reported till date to the best of our knowledge.

2. Experimental

2.1. Samples

The investigated samples of didanosine and known impurities were prepared in APL Research Centre (A unit of Aurobindo Pharma Limited, Hyderabad, India). Reagents used for analysis, i.e., ammonium acetate (AR grade), glacial acetic acid (AR grade), methanol (HPLC grade) and acetonitrile (HPLC grade) were procured from Merck (India) Limited. Water used was Milli-Q grade.

2.2. High-performance liquid chromatography (analytical)

A Water 2695 separation module equipped with 2996 photo diode array detector with Empower pro data handling system [Waters Corporation, MILFORD, MA01757, USA] was used.

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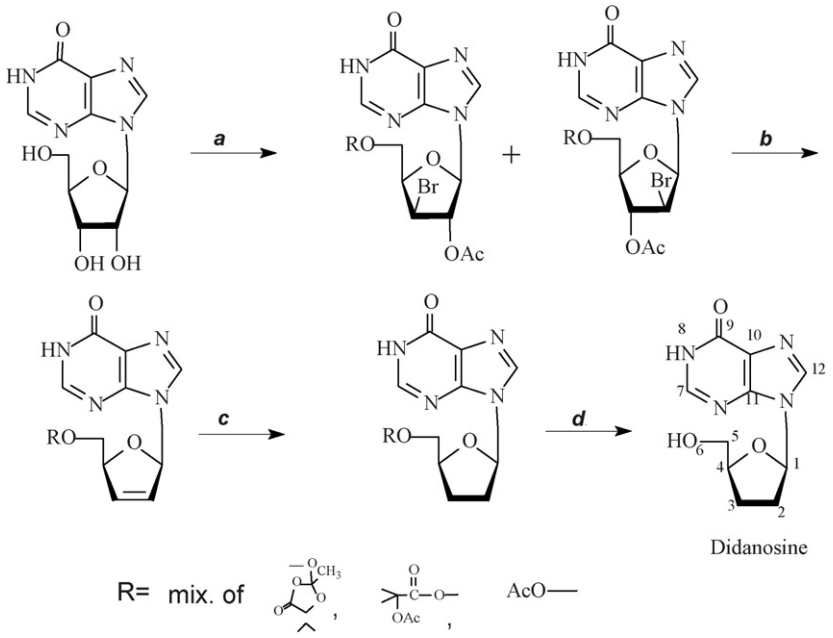

Fig. 1. Scheme for the synthesis of didanosine. Reagents: (a) 2-acetoxy isobutyryl bromide, (b) zn-cu couple, (c) Pd-c/H2 and (d) methyl amine.

The analysis was carried out on Hypersil BDS C8, 150 mm long, 4.6 mm i.d., and 5-μm particle diameter column. Mobile phase A was acetate buffer (pH 6.0 ± 0.05) and acetonitrile in the ratio of 97:3 [acetate buffer (pH 6.0), prepared by dissolving 3.85 g of ammonium acetate in 1000 ml of water, pH adjusted to 6.0 ± 0.05 with dilute acetic acid. Mobile phase B was acetonitrile and methanol in the ratio of 1:1 (v/v). UV detection was at 254 nm and flow rate was kept at 1.0 ml/min. Data acquisition time was 45 min. Pump mode was gradient and the program was as follows, 0–20 min.: 100% A; 20–25 min.: 90% A, 10% B; 25–30 min.: 85% A, 15% B; 30–40 min.: 60% A, 40% B; 40–45 min.: 60% A, 40% B; 45–46 min.: 100% A; 46–55 min.: 100% A.

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. Hypersil BDS C18 (250 mm long × 21.2 mm i.d.) preparative column packed with 8 μm particle size [Thermo Electron Corporation, UK] was employed for isolation of impurity. The mobile phase consisted of (A) 0.1 M ammonium acetate solution and (B) methanol. Flow rate was kept at 20 ml/min and detection was carried out at 254 nm. The gradient program was as follows, 0–0.01 min.: 100% A; 0.01–50 min.: 95% A, 5% B; 50–80 min.: 90% A, 10% B; 80–100 min.: 50% A, 50% B; 100–101 min.: 100% A; 101–110 min.: 100% A.

2.4. LC-MS/MS analysis

LC-MS/MS analysis was carried out using Perkin-Elmer triple quadrupole mass spectrometer (API 2000, PE SCIEX) coupled with a shimadzu HPLC equipped with SPD 10 A VP UV–vis detector and LC 10 AT VP pumps [Foster city, CA]. 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. High pure nitrogen gas was used as auxillary gas and curtain gas. Zero air was used as nebulizer gas. LC-MS spectra were acquired from m/z 100 to 1000 in 0.1 amu steps with 2.0 s dwell time. Didanosine sample was subjected to LC-MS/MS analysis. The analysis was carried out using Hypersil BDS, 250 mm × 4.6 mm column with 5-μm particle diameter with mobile phase consisting of a mixture of 20 mM ammonium acetate solution, pH adjusted to 7.0 with dilute acetic acid and methanol in the ratio of 88:12 (v/v). Flow rate was 1.0 ml/min. Seven impurities were detected in laboratory batch sample. The masses of detected peaks were identical to the values of the known impurities. Additionally, one impurity corresponding to mass of 220 was also observed in sample of didanosine.

2.5. NMR Spectroscopy

The ^{1}H, ^{13}C NMR (proton decoupled) spectra were recorded on Bruker 300 MHz spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard.

2.6. Mass Spectrometry

Mass spectra were recorded on Perkin Elmer PE SCIEX-API 2000 mass spectrometer equipped with a Turboionspray interface at 375 °C.

2.7. FT-IR Spectroscopy

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

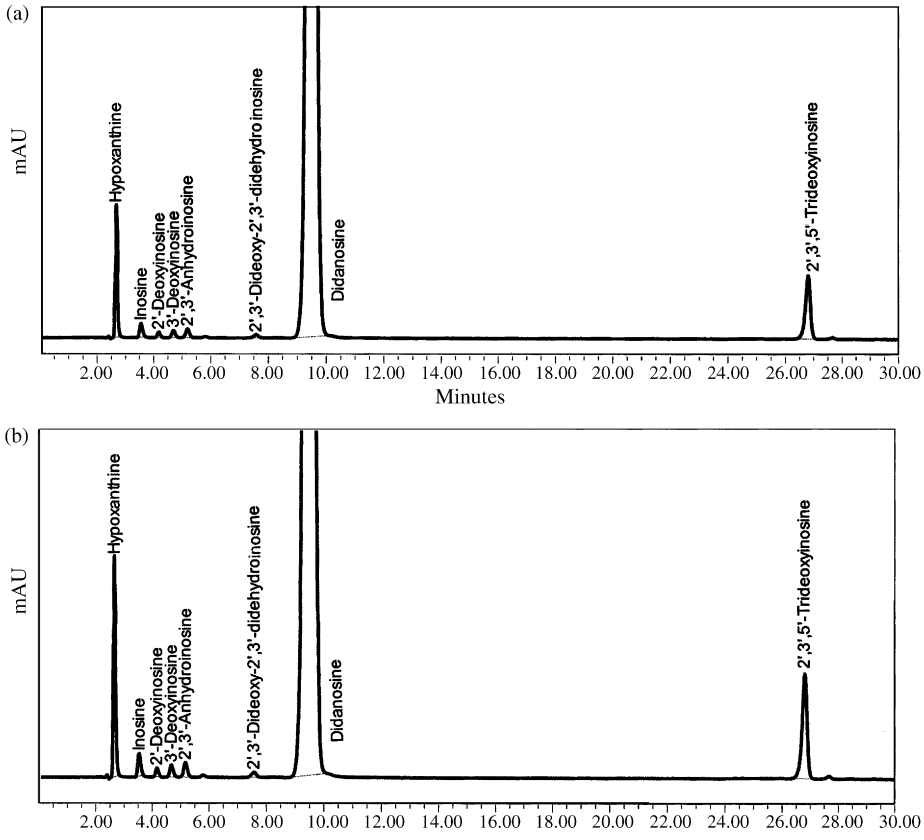

Fig. 2. LC-chromatogram of (a) didanosine sample and (b) didanosine sample spiked with 2′,3′,5′-trideoxyinosine.

2.8. Isolation of impurity (2′,3′,5′-trideoxyinosine) by preparative HPLC

The mother liquor collected during the isolation of didanosine was evaporated to yield dark brown viscous oily mass and impurity was present at about 6% level (analyzed as per the method given in Section 2.2). This oily mass was subjected to flash column chromatography by using the mobile phase dichloromethane and methanol (80:20). Collected fractions from flash column were concentrated on rotavapour and injected in the HPLC method mentioned in Section 2.2. The required impurity was enriched to 22% level and most of the

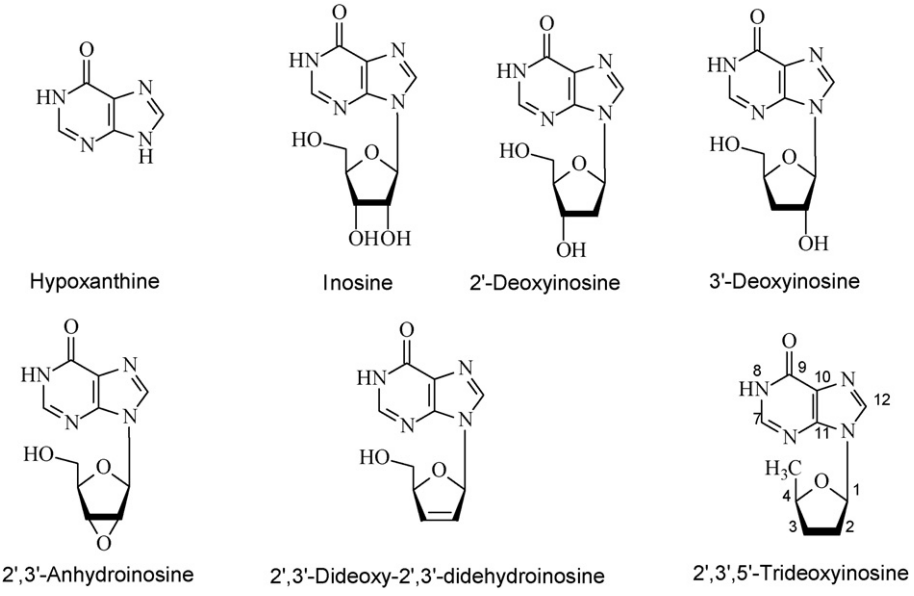

Fig. 3. Chemical structures of didanosine impurities.

Table 1
Comparative ^{1}H, ^{13}C (proton decoupled) and DEPT NMR assignments for didanosine and impurity 2′,3′,5′-trideoxyinosine

Position[a]	Didanosine			2′,3′,5′-Trideoxyinosine		
	^{1}H, δ (ppm) multiplicity	^{13}C, δ (ppm)	DEPT	^{1}H, δ (ppm) multiplicity	^{13}C, δ (ppm)	DEPT
1	6.20 (dd, 1H)	85.3	CH	6.15 (dd, 1H)	84.9	CH
2	2.35–2.51 (m, 2H)	33.0	CH_2	2.37–2.46 (m, 2H)	33.1	CH_2
3	1.99–2.06 (m, 2H)	26.3	CH_2	1.84 and 2.12 (2 m, 2H)	32.2	CH_2
4	4.10–4.13 (m, 1H)	82.9	CH	4.13–4.20 (m, 1H)	78.1	CH
5	3.53–3.62 (m, 2H)	63.5	CH_2	1.30 (d, 3H)	21.5	CH_3
6	5.0 (brs, 1H)	–	–	–	–	–
7	8.34 (s, 1H)	146.5	CH	8.20 (s, 1H)	146.6	CH
8	12.33 (brs, 1H)	–	–	12.28 (brs, 1H)	–	–
9	–	157.5	–	–	157.5	–
10	–	125.2	–	–	125.5	–
11	–	148.4	–	–	148.6	–
12	8.05 (s, 1H)	139.1	CH	8.05 (s, 1H)	139.0	CH

s, singlet; d, doublet; dd, doublet of a doublet; m, multiplet.
[a] Refer chemical structures Fig. 1 for numbering of didanosine and Fig. 3 for numbering of impurity.

colored matter got removed. This was subjected for isolation by following the method mentioned in Section 2.3. Fractions collected were analyzed by analytical HPLC as per the conditions mentioned in Section 2.2. Fractions of >95% were pooled together, concentrated on rotavapour to remove methanol. Concentrated fraction was passed through the preparative column using water as mobile phase to remove ammonium acetate and then compound was eluted with mixture of water and methanol (50:50). Again the eluate was concentrated using rotavapour to remove methanol and the aqueous solution was lyophilized using freeze dryer (Virtis advantage 2XL). This compound was obtained as a white powder with chromatographic purity of 96%.

3. Results and discussion

3.1. Detection of impurities

Laboratory batches of didanosine were analyzed for their impurities identification using the HPLC method described in Section 2.2. These samples were subjected to LC-MS/MS analysis using the method as described in Section 2.4. The known impurities and isolated 2′,3′,5′-trideoxyinosine impurity were co-injected with didanosine to confirm the retention times. All the impurities were well resolved from didanosine peak and the representative resolution mixture chromatogram was shown in Fig. 2. The chemical structures of impurities are given in Fig. 3.

3.2. Structural elucidation of Impurity 2′,3′,5′-trideoxyinosine

The molecular ion peak at *m/z*; 221.1 [$(MH)^+$] in positive ion mode by LC-MS analysis indicated a molecular weight of 220, which is 16 amu less than that of didanosine. Being less polar than didanosine, it was suggested that the loss of hydroxyl group happened to the molecule. Major fragmentation peak at *m/z* 137.2, which corresponds to purine moiety, also indicated that no change happened to the purine moiety and the peak at *m/z* 85.2 indicating the presence of methyl substituted glucose moiety. To confirm the retention time, pure 2′,3′,5′-trideoxyinosine was co-injected with didanosine sample in HPLC. It was observed that the retention time was matching with the retention time of 2′,3′,5′-trideoxyinosine. IR spectrum of the isolated impurity did not exhibit any charactereristic absorption band due to hydroxyl group stretching which indicated the absence of hydroxyl group. In ^{1}H NMR spectrum, a multiplet at 3.5 ppm corresponds to $-CH_2OH$ in didanosine was disappeared and an additional methyl doublet was observed at 1.30 ppm ($J = 6.0$ Hz). In ^{13}C NMR spectrum, signal at 63.5 ppm corresponds to $-CH_2OH$ carbon was disappeared and addition signal was observed at 21.5 ppm, confirmed as $-CH_3$ carbon by DEPT experiment. The structure of the isolated new impurity was also confirmed by the following spectral data. IR (KBr, cm^{-1}) 3106 (NH), 1703 (C=O), 1215 (C–N). Anal. Calcd. For $C_{10}H_{12}N_4O_2$ (220.23): C, 54.54; H, 5.49; N, 25.44. Found: C, 54.28; H, 5.65; N, 25.68. ^{1}H NMR, ^{13}C NMR and DEPT data for didanosine and impurity are given in Table 1. This impurity may be arising due to the deoxygenation in hydrogenation stage during the preparation of didanosine.

4. Conclusion

The process related new impurity in didanosine bulk drug was identified, isolated and characterized by using HPLC (analytical and preparative), Mass, IR and NMR (^{1}H and ^{13}C NMR) techniques.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2007.05.034.

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