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STUDY FOR VALIDATION OF TWO MAJOR ACIDS DEGRADATION PRODUCTS: HPLC STABILITY INDICATING METHODS

Aarti Bhati ,

Research Scholar, School of Pharmacy, Glocal University Saharanpur (U.P)

Dr. Chhater Singh,

Research Supervisor, School of Pharmacy, Glocal University Saharanpur (U.P)

ABSTRACT

One of the degradation products, DP-1, was isolated by semi-preparative high performance liquid chromatography (HPLC) using Waters XBridge Prep C18 (250 mm×10 mm, 5 µm). Degradation products showed higher toxicity compared to the drug in some models assessed by TOPKAT software. The method validation was performed with respect to robustness, specificity, linearity, precision and accuracy as per ICH guideline Q2 (R1).Two major acid degradation products were identified and characterized by liquid chromatography electrospray ionization mass spectrometry (LC–ESI/MS/MS) and accurate mass measurements. The probable mechanisms for the formation of degradation products were identified based on a comparison of the fragmentation pattern of the $[M^{+}H]^{+}$ ions of AZT and its degradation products.

INTRODUCTION

AZT is most effective in the prevention of mother-to-child HIV-1 transmission which has been demonstrated in several studies.The principal mechanism of action of AZT-TP is inhibition of reverse transcriptase (RT) via DNA chain termination after incorporation of the nucleotide analogue. A few chromatographic literatures are available for AZT alone and for combination of drugs. There are multiple bio-analytical methods available for AZT and combination drugs with quantification by high performance liquid chromatography (HPLC) with tandem mass spectrometry (Kenney *et al.*, 2000)and with the help of ion pair HPLC (Pereira *et al.*, 2000; Fan and Stewart, 2002).Couple of HPLC stability indicating methods (Fan and Stewart, 2001; Dunge *et al.*, 2005) are available in literature as well and there are many methods (Radwan,1994; Verweij-van Wissen *et al.*, 2005; Djurdjevic *et al.*,2004; Musa, 2014; Alnouti *et al.*, 2004; Lewis *et al.*, 2007; Kumar *et al.*, 2010;Uslu and Özkan, 2002; Kumar *et al.*, 2010; Sharma *et al.*, 2010; Dos Santos *et al.*, 2011) offering separation of AZT with combination drugs by HPLC with UV detection. However, there is no study available for characterization of major degradation product obtained from forced degradation study of AZT.According to World Health Organization (WHO), there were around 37 million people living with human immunodeficiency virus (HIV) at the end of 2014 with 2 million people becoming newly infected with HIV

in that year (WHO, 2016).Zidovudine (AZT) was the first agent approved by U. S. Food and Drug Administration (USFDA) for treatment of HIV disease in 1987 (FDA, 2016) AZT is chemically 3'-azido-3'-deoxythymidine, synthetic nucleoside analogue of a thymidine. It is one of drugs from class of nucleoside analogue reverse transcriptase Inhibitor (NRTI). It has crucial role as a component of a multidrug combination regimen for the treatment of adult and paediatric HIV-1 infection.

KEYWORDS:

Zidvovudine, Stability study, Degradation products, toxicity prediction LC

MATERIALS AND METHOD

Chemicals and reagents

AZT was procured from Sigma Aldrich, Bengaluru, India. Milli-Q-water was obtained by filtrating through a Millipore Milli-Q plus system (Millipore, USA).Analytical reagent grade Ammonium acetate was purchased from Finar Chemicals Pvt. Ltd. (Ahmedabad, India) whereas analytical reagent grade sodium hydroxide pellets, 37% hydrochloric acid, 30% hydrogen peroxide and Chromosolv HPLC grade acetonitrile were purchased from Merck, India.

Instruments and software

The liquid chromatographic system for separation of degradation products of AZT was performed on LCMS-2020 system (Shimadzu, Japan). The system comprised of LC-20 AD prominence pumps, auto sampler, solvent degasser, prominence photo diode array detector and temperature controlled column compartment. Semi preparative HPLC instrument (GILSON, USA) equipped with a binary pump, a column compartment, a photo diode array detector, a liquid handler was used to carry out isolation of degradation product 1 (DP-1).

Establishing stress conditions

Forced degradation studies were carried out on AZT as per ICH guidelines Q1A (R2) (ICH Q1A (R2), 2003). AZT stock solution was prepared at 2 mg mL-1 by using mixture of acetonitrile and water (1:1) as solvent. Each stock solutions of AZT were diluted with acid, base, and water in 1:1 ratio. Acidic, basic, and neutral hydrolytic degradation study were carried out by refluxing in 2 M hydrochloric acid (HCl), 2 M sodium hydroxide (NaOH) and water at 80° C for 72 h, respectively. The stock solution was diluted to 10% Hydrogen peroxide and kept at room temperature for 10 h for oxidative degradation. Drug was layered with 2 mm height in quartz petri dish and same was exposed to 1.2 X 106 lux h of fluorescent light and 200W h/m2 UV light in a photo stability chamber. Same photo stability study was performed with stock solution. Powdered AZT was poured in amber bottle with 2mm height was loaded in an oven at 80°C for 2 days to study thermal stability. All stressed solid samples and solutions were well protected covered with aluminum foil, kept in a refrigerator at 5°C until analysis.Solutions from each study were withdrawn after the mentioned specific time and diluted with acetonitrile and water mixture in a ratio of 1:1 (v/v) before analysis by HPLC.

Method development for stressed samples

Several trials were taken over whole pH range of mobile phase for separation of drug and degradation products. However after multiple trials better and simpler separation of the drug and its degradation products were achieved on XBridge C18 (150mm X 4.6 mm, 3.5µm) (Waters, USA). 10 mM ammonium acetate and acetonitrile were used as mobile phase in a gradient elution method as follows. (Time/% proportion of acetonitrile): 0-4 min/10, 8 min/30, 14 min/70, 18 min/90, 18.1-20 min/10. The flow rate, injection volume, column temperature and detection wavelength were 800 µL min^{-1}, 25 µL, 30°C and 285 nm, respectively. The typical MS scan operating source conditions in electrospray ionization (ESI) positive ion mode were reserved as follows: nebulizing gas flow 1.5 L min^{-1}, drying gas flow 15 L min^{-1}, DL temperature 250 ºC, heat block temperature 200ºC, detector temperature 1.1 kV, interface voltage 4.5 kV.MS/MS fragmentations of the drug and its degradation products were studied on a quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with an ESI source.Major degradation product was isolated by semi preparative HPLC by using Waters XBridge Prep C18 (250mm X 10 mm, 5 µm) with same mobile phase which used with analytical column. The gradient solvent program was set as follows. (Time/% proportion of acetonitrile): 0-4 min/20, 7 min/28, 20 min/75, 21-25 min/20. The flow rate, injection volume, column temperature and wavelength were 8.0 mL min-1, 250 µL, 30°C and 285 nm, respectively. Fractions of DP-1 was collected at particular retention time. Ethyl acetate was added to this isolated fraction. The solutions were kept under magnetic stirring for 10 min and centrifuged for 15 min at 2500 rpm and then took off supernatant upper layer.

Supernatant solution was evaporated on vacuum concentrator to acquire dry compound. The dry compound was dissolved in deuterated DMSO and analysed by proton nuclear magnetic resonance (1H-NMR).

RESULTS AND DISCUSSION

The stability indicating assay method was validated for linearity, precision,accuracy, and specificity, adhere to ICH guideline Q2 (R1) (ICH Q2A (R2),2003). System suitability test is used to verify that repeatability and resolution of critical parameter of system. System suitability solution was prepared by spiking 20 ng mL-1 of AZT to a previously acid degraded solution. Resolution between AZT and its degraded impurity is 2.02±0.04 for six individual preparations. PDA detector was used to evaluate peak purity of AZT and its degradation production for determination of method specificity and LC/MS was also used to confirm thesame. The mass detector has showed purity of drug and all degradation products.

Calibration curve for linearity was plotted by analysis of working standard solutions of AZT at six different concentrations in the range 10-100 ng mL-1.Calibration curve was plotted by taking peak are on Y axis versus nominal concentration of drug on X axis. Correlation coefficient of AZT was found to be 1.0000 in the concentration range of 10-100 ng mL-1 (see Table 3.3). Standard addition method was adopted for the determination of accuracy. To the previously degraded solution of AZT, known quantities of AZT have been

spiked. Each solution was injected in triplicate and the percentage recovery range and % RSD value were found to be 98%-100% and < 2%, respectively (see Table 3.1 and Fig. 3.1).

Table 3.1 Recovery data of AZT (n=3)

Spiked concentration (ng mL-1)	Concentration found (Mean ± SD, ng mL-1)	RSD (%)	Recovery (%)
10	10.10±0.193	1.91	101.0
30	29.81±0.493	1.65	99.4
50	50.32±0.65	1.29	100.6

Precision of the developed method for the determination of AZT and its degradation products was measured for intra-day precision (repeatability) and inter-day precision (reproducibility). Repeatability of the developed method was determined from the results of five solutions each in triplicate prepared at different concentrations. The method reproducibility was evaluated on consecutive days by analyzing five separate sample solutions at the same concentration of intra-day solution.Table 3.2 represents % RSD for intra-day and inter-day precision of method for AZT and results shows the method is precise. The robustness of the method was determined by deliberate slight change in flow rate, pH of buffer, column temperature and buffer concentration. There were no significant changes in assay value of the drug which showed that method was robust.

Table 3.2 Precision Study (n=3)

Concentration (ng mL^{-1})	Intra-day precision		Inter-day precision	
	Concentration found (Mean ± SD, ng mL^{-1})	RSD (%)	Concentration found (Mean ± SD, ng mL^{-1})	RSD (%)
10	9.83±0.05	0.51	9.80±0.10	1.02
20	19.70±0.10	0.51	19.80±0.10	0.51
40	39.69±0.21	0.53	39.84±0.16	0.40
80	79.87±0.22	0.28	79.65±0.10	0.13

100	99.87±0.29	0.29	99.74±0.22	0.22

Table 3.3 Linearity data of AZT (n=3)

Concentration (ng mL-1)	Concentration found (ng mL-1)
5	12.47
10	25.12
30	75.35
50	125.35
100	250.98

Degradation profile of AZT

MS detector and PDA detector was used in line with HPLC to access the degradation behavior of AZT under various forced degradation conditions. Sufficient degradation was observed in only acidic condition whereas in other condition it was found to be stable. The chromatograms of AZT alone (2 mg mL-1) and stressed degradation AZT in acidic condition are given in Fig. 3.7 and Fig. 3.8, respectively. A total two degradation products were identified and characterized by using LC/ESI/MS/MS experiments and accurate mass measurements. The proposed structures of degradation and their elemental compositions are given. Initially, AZT was found to be stable when refluxed in 0.5 M HCl and 0.5 M NaOH at 80° C for 24 h. While, two degradation products (DP-1 and DP-2) were formed in 2 M HCl at 80° C for 72 h (see Fig. 3.8). In 2 M NaOH and neutral condition, drug was found to be stable. Oxidation, photolytic and thermal degradation sample showed no formation of major degradation products.

MS/MS of AZT

The MS/MS spectrum of protonated AZT (Retention time (Rt) = 11.8 min; *m/z* 268) display products ion at *m/z* 227 (loss of H2C=C=NH) and m/z 127(protonated 5-methylpyrimidine-2, 4 (1*H*, 3*H*)-dione). It can be noted that m/z 127 is diagnostic for the presence of pyrimidine group in AZT .The elemental compositions of all these fragment ions have been confirmed by accurate mass measurements.

Table 3.6 Elemental compositions of AZT and its Degradation Products

AZT and DP	**Retention time (min)**	**Molecular formula [M+H]+**	**Calculated m/z**	**Observed m/z**	**Error (ppm)**	**MS/MS fragment ions**
AZT	11.7	$C10H14N5O4^+$	268.1040	268.1052	-4.5	227, 127
DP-1	2.8	$C5H17N2O2^+$	127.0502	127.0498	3.1	127
DP-2	12.0	$C5H17N2O2^+$	227.1026	227.1016	4.4	127

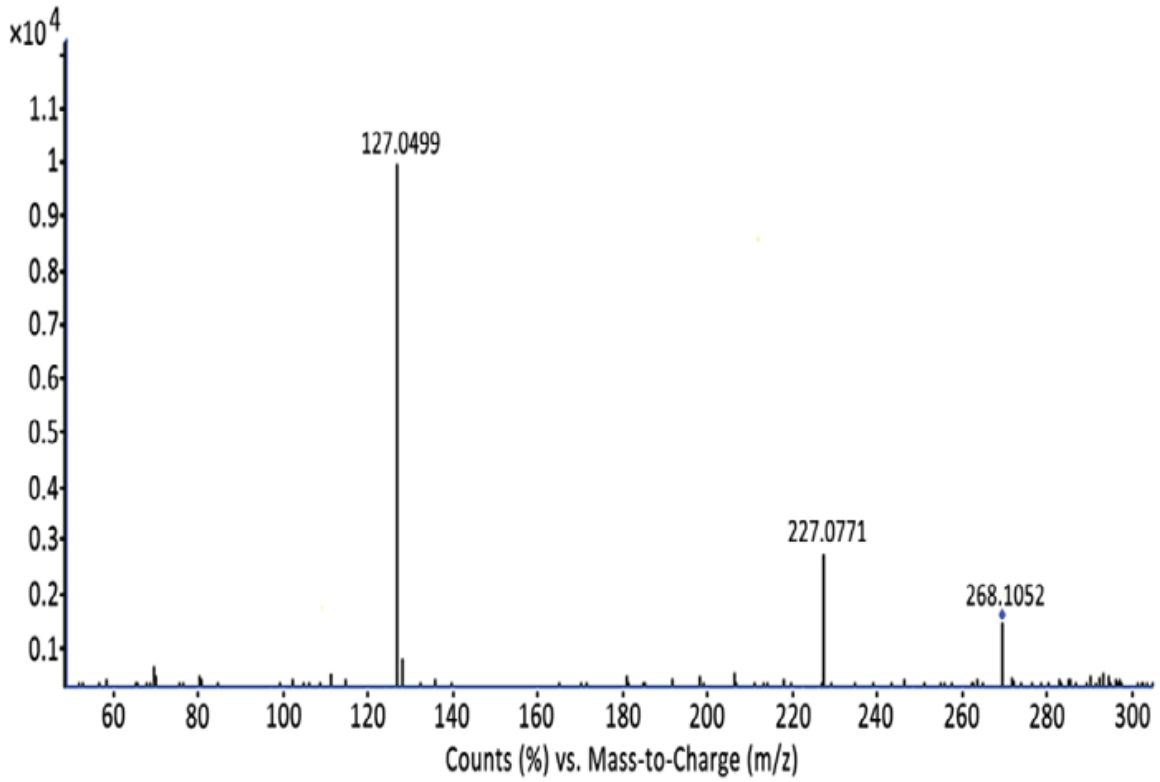

Fig. 3.10 ESI/MS/MS spectrum of zidovudine

MS/MS degradation products

MS/MS experiments were performed to characterize the degradation products and to identify most probable structure based on the m/z values of product ions.The ESI/MS/MS spectrum of [M+H] + ion (*m/z* 127) of DP-1 (see Fig. 3.11),eluting at Rt of 2.6 min. A mass difference of 141Da between mass of DP-1 and mass of the drug suggests DP-1 is formed by the loss of ((2S, 3S)-azido-2, 3-dihydrofuran-2-yl) methanol from AZT. The probable elemental composition of[M+H] + of DP-1 has been confirmed by accurate mass measurements. All these data indicate the proposed structure 5-methylpyrimidine-2, 4 (1*H*, 3*H*)-dione. A mass difference of 41 Da between mass of AZT and mass of DP-2 (m/z227) indicates that the DP-2 is formed by the loss of N3 from AZT and elemental composition of DP-2 has been confirmed by the accurate mass measurements.The ESI/MS/MS spectrum of [M+H] + ion of DP-2 (*m/z* 227, Rt=12.0 min) displays product ions at m/z 127 (loss of (2, 3-dihydrofuran-2yl) methanol) which are compatible with the structure 1-5-(hydro methyl) teterahydrofuran-2-yl)-5- methylpyrimidine-2, 4 (1*H*, 3*H*)-dione (see Fig. 3.12, Fig. 3.13 and Fig 3.14).

The elemental compositions of DP-2 and its fragments ions have been confirmed by accurate measurements.

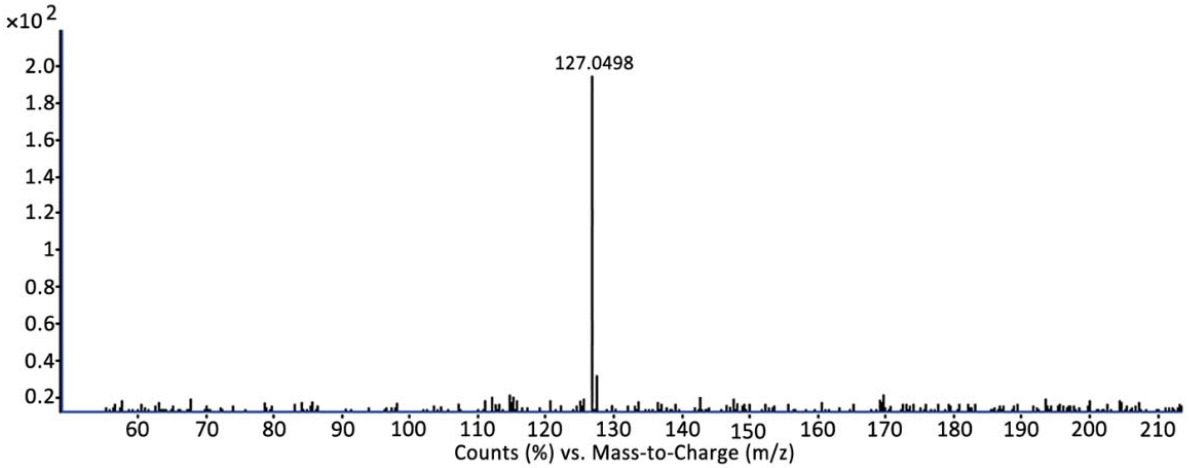

Fig. 3.11 ESI/MS/MS spectrum of degradation product 1 (m/z 127)

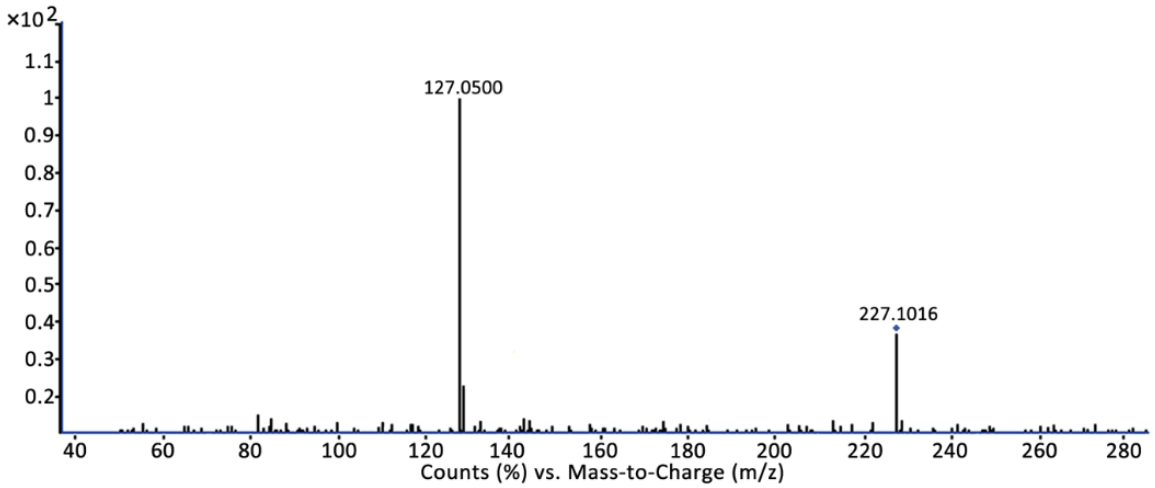

Fig. 3.14

ESI/MS/MS spectrum of degradation product 2 (m/z 227)

CONCLUSION

A selective validated stability indicating LC/MS/MS/ assay method was established to study the degradation pattern of AZT under hydrolysis, oxidation, photolysis, and thermal stress conditions. Two unknown degradation products were identified under acid degradation forced study and characterized using LC/ESI/MS/MS experiments supported by accurate mass measurements. A major degradant DP-1 was isolated and characterized by 1H-NMR. *In-silico* toxicity profile was predicted carcinogenic possibilities for both degradation

products using TOPKAT software.

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