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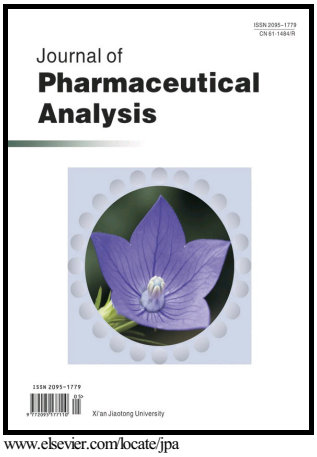

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A stability indicating HPLC method of zidovudine: validation, characterization and toxicity prediction of two major acid degradation products.

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ABSTRACT

Zidvovudine (AZT) is a nucleoside analogue reverse transcriptase inhibitor (NRTI), a class from Anti-retroviral drug. A stability indicating assay method for AZT was developed in line with ICH guideline. Successful separation of AZT and its degradation products was achieved by gradient elution mode on reverse phase C18 column using 10 mM ammonium acetate: acetonitrile as a mobile phase at 0.8 mL/min flow rate, 25 µL injection volume, 30°C column temperature and 285 nm detection wavelength. Two major acid degradation products were identified and characterized by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS/MS) experiments and accurate mass measurements. The probable mechanisms for the formation of degradation products have been identified based on a comparison of the fragmentation pattern of the $[M + H]^+$ ions of zidovudine and its degradation products. One of the degradation products, DP-1 was isolated by semi preparative high performance liquid chromatography (HPLC) using Waters XBridge Prep C18 (250mm X 10 mm, 5 µm). Degradation products were showing higher toxicity compared to drug in few 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).

Keywords: Stability study; Isolation; Characterization; In-Silico toxicity prediction; Degradation pathway

1. Introduction

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 [1]. Zidovudine (AZT) was the first agent approved by U. S. Food and Drug Administration (USFDA) for treatment of HIV disease in 1987 [2]. 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 pediatric HIV-1 infection. AZT is most effective in the prevention of mother-to-child HIV-1 transmission which has been demonstrated in several studies [3]. AZT is phosphorylated to its

active 5′-triphosphate metabolite, zidovudine triphosphate (AZT-TP), intracellularly. The principal mechanism of action of AZT-TP is inhibition of reverse transcriptase (RT) via DNA chain termination after incorporation of the nucleotide analogue. AZT-TP is a weak inhibitor of the cellular DNA polymerases α and γ and has been reported to be incorporated into the DNA of cells in culture [3].

Forced degradation study is inevitable part of drug development cycle to get useful information within short time of span [4]. These studies are vital to evaluate the shelf life period in which the drug would retain its desired quality, safety and efficacy. The purpose of stability testing is to provide evidence on how the quality of active pharmaceutical ingredients (API) or formulations varies with time by various phenomena such as hydrolysis, oxidation and photolysis as per ICH guidelines. Stress testing of the drug substance or products is useful to find the probable degradation products, the likely degradation pathways and the intrinsic stability of the molecule [5]. Stress study is understanding effect of severe conditions such as heat, moisture, pH, oxidation and light on molecules. However, identification and characterization of degradation products by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in combination with high-resolution mass spectrometry (HRMS) is useful in the process of development of stable formulation [6]. Evaluation of toxicity of degradation product is vital as ICH Q3 guidelines included stringent reporting, identification, characterization and qualification thresholds [7, 8].

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 [9, 10] and with the help of ion pair HPLC [11, 12]. Couple of HPLC stability indicating methods [13, 14] are available in literature as well and there are many methods [15-25] 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. Hence, the objective of this current paper was to develop of stability indicating assay method for AZT; to identify and characterize major degradation products formed; to propose most probable degradation pathways; and to predict toxicity of major degradation products.

2. Experimental

2.1 Chemicals and reagents

AZT was procured from Sigma Aldrich, Bangalore, 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.

2.2 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).

All weighing tasks were done on a Sartorius balance (CPA225D, Germany) and pH was measured using pH tutor (Eutech Instruments, Singapore).

Photolytic degradation process was carried out by photo-stability chamber (Osworld OPSH-G-16-GMP series, India) preset as 40^{o}C ± 5^{o}C/ 75%RH ± 3%RH and consisting of a combination of two UV lamps and four fluorescent lamps compliant with two options suggested in the ICH guideline Q1B [26].

In-Silico toxicity study was performed by using TOPKAT (Discovery Studio 2.5, USA) software.

2.3 Establishing stress conditions

Forced degradation studies were carried out on AZT as per ICH guidelines Q1A (R2) [27]. AZT stock solution was prepared at 2 mg/mL by using mixture of acetonitrile and water (1:1) as solvent. Each stock solutions of AZT was 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 10^6 lux h of fluorescent light and 200W h/m^2 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.

2.4 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, 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, drying gas flow 15 L/min, DL temperature 250℃, heat block temperature 200℃, 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, 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 analyzed by proton nuclear magnetic resonance (^{1}H-NMR).

2.5 In-Silico toxicity evaluation

The potential toxicity of AZT and its degradation products were evaluated by using TOPKAT (Komputer Assisted Technology) software. Software estimates the toxicity of a compound quantitatively using structural, electronics, topological and electro-topological molecular descriptors. TOPKAT gives probable value of toxicity from scale of 0.0 to 1.0 for submitted structures. Value from 0.0 to 0.3 is considered as non toxic, 0.3 to 0.7 is indeterminate and value from 0.7 to 1.0 is considered as toxic.

3. Results and discussion

3.1 Analytical method validation

The stability indicating assay method was validated for linearity, precision, accuracy and specificity, adhere to ICH guideline Q2 (R1) [28].

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 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 the same. 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. 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 0.999 in the concentration range of 10-100 ng/mL.

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 1).

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 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.

3.2 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 (2mg/mL) and stressed degradation AZT in acidic condition are given in Fig.1 and Fig.2, 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 in Scheme 1 and Table 3.

3.2.1 Hydrolysis

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.2). In 2 M NaOH and neutral condition, drug was found to be stable.

3.2.2 Oxidation, photolytic and thermal degradation

Oxidation, photolytic and thermal degradation sample showed no formation of major degradation products.

3.3 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 $H_2C{=}C{=}NH$) and m/z 127 (protonated 5-methylpyrimidine-2, 4 (1*H*, 3*H*)-dione) (see Scheme 2; Fig.3). 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 (see Table 3).

3.4 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, eluting at Rt of 2.6 min (see Fig.4). 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 (see Table 3). 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/z 227) indicates that the DP-2 is formed by the loss of N_3 from AZT and elemental composition of DP-2 has been confirmed by the accurate mass measurements (see Table 3 and Scheme 4). 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 Scheme 3 and Fig.5). The elemental compositions of DP-2 and its fragments ions have been confirmed by accurate measurements (see Table 3).

3.5 ^{1}H-NMR study

The DP-1 was isolated by semi preparative HPLC. The isolated peak was concentrated and submitted for ^{1}H-NMR. The ^{1}H NMR details of the DP-1 is as follows:

DP-1: ^{1}H NMR (CD_3OD, 500MHz), δ 7.22 (s, 1H), 1.85 (s, 3H) (see Fig.6).

AZT: ^{1}H NMR (CD3OD, 500MHz), δ 7.80 (s, 1H), 6.17 (t, 1H, J = 6.4 Hz), 4.36 (dd, 1H, J = 12.05, 5.18 Hz), 3.93-3.89 (m, 1H), 3.84 (dd, 1H, J = 12.20, 3.35 Hz), 3.74 (dd, 1H, J = 12.20, 3.35 Hz), 2.45-2.34 (m, 2H), 1.88 (s, 3H).

3.6 In-Silico toxicity prediction

Table 4 shows TOPKAT predicted toxicity profile for AZT and its degradation products. The toxicity of degradation products were compared and calculated with AZT in different models. Degradation products showed higher carcinogenicity potential in different model for example, NTP Carcinogenicity Call (Male Rat) (v3.2), NTP Carcinogenicity Call (Male Mouse) (v3.2), FDA Carcinogenicity Male Rat Single vs Mult (v3.1), FDA Carcinogenicity Female Mouse Non Vs Carc (v3.1) and FDA Carcinogenicity Female Mouse Single vs Mult (v3.1). However the DP-1 showed toxicity in Ames Mutagenicity and Aerobic Biodegradability (v6.1) model.

4. 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 ^{1}H-NMR. In Silico toxicity profile was predicted carcinogenic possibilities for both degradation products using TOPKAT software.

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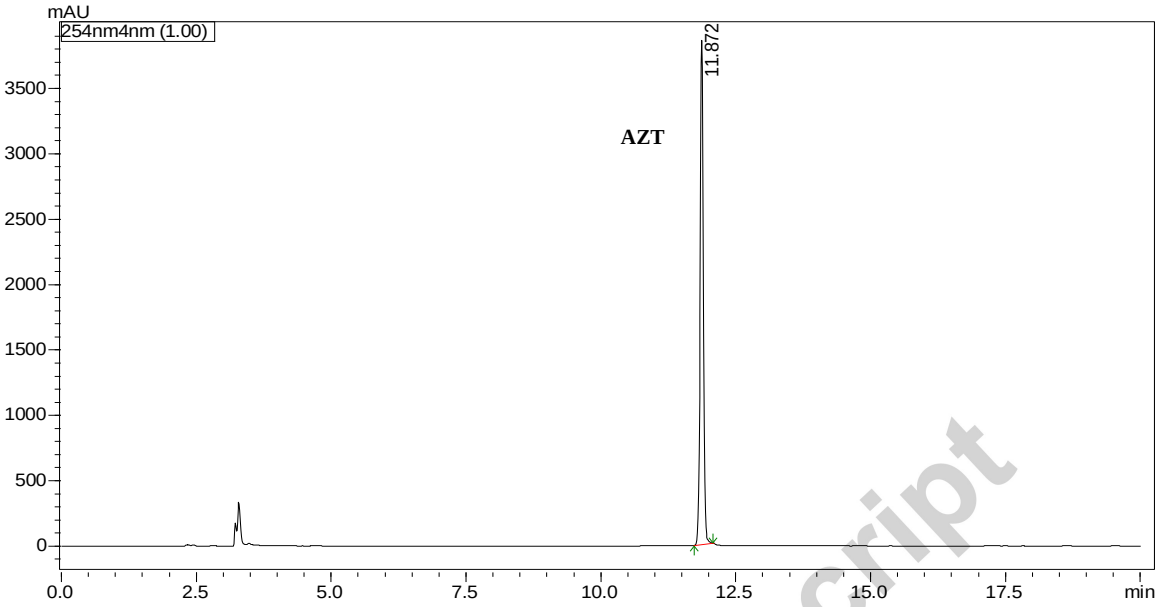


Figure 1. Chromatogram of AZT (2mg/mL)

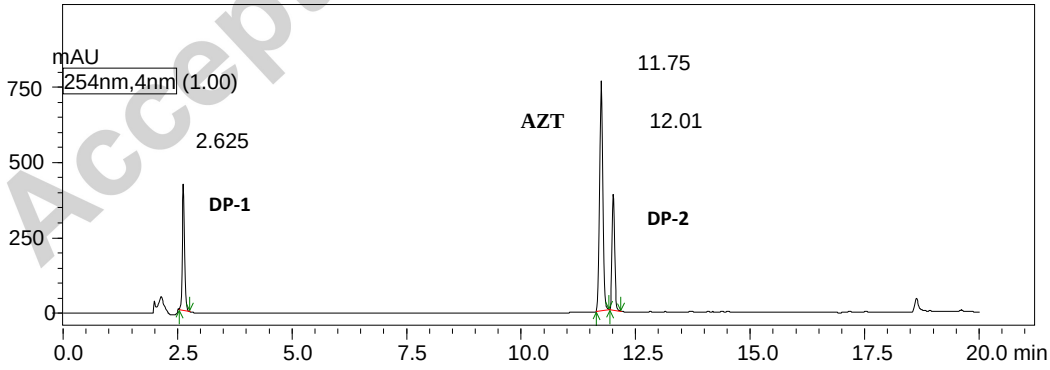


Figure 2. Chromatogram of acid degradation products

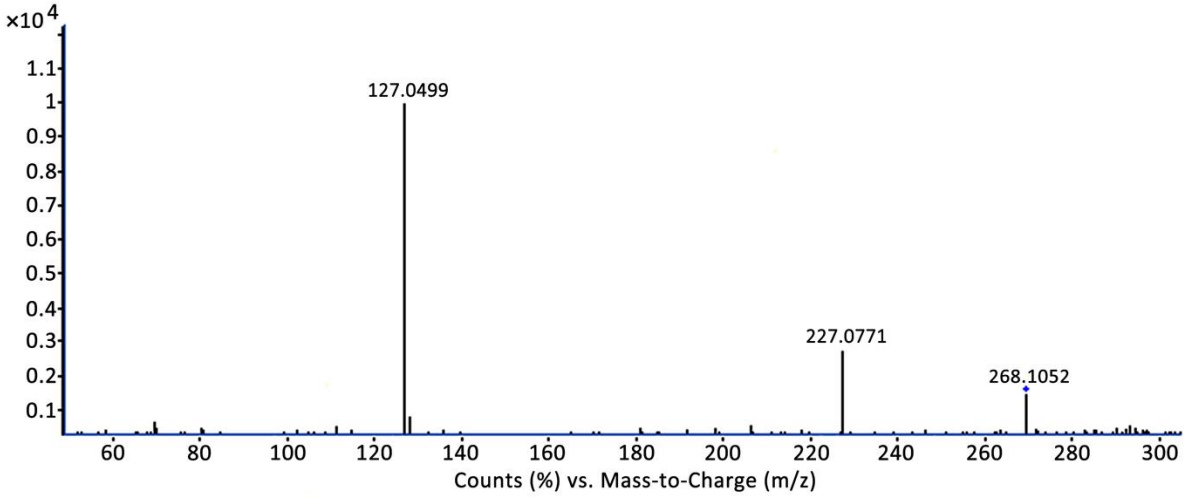


Figure 3. ESI/MS/MS spectrum of zidovudine

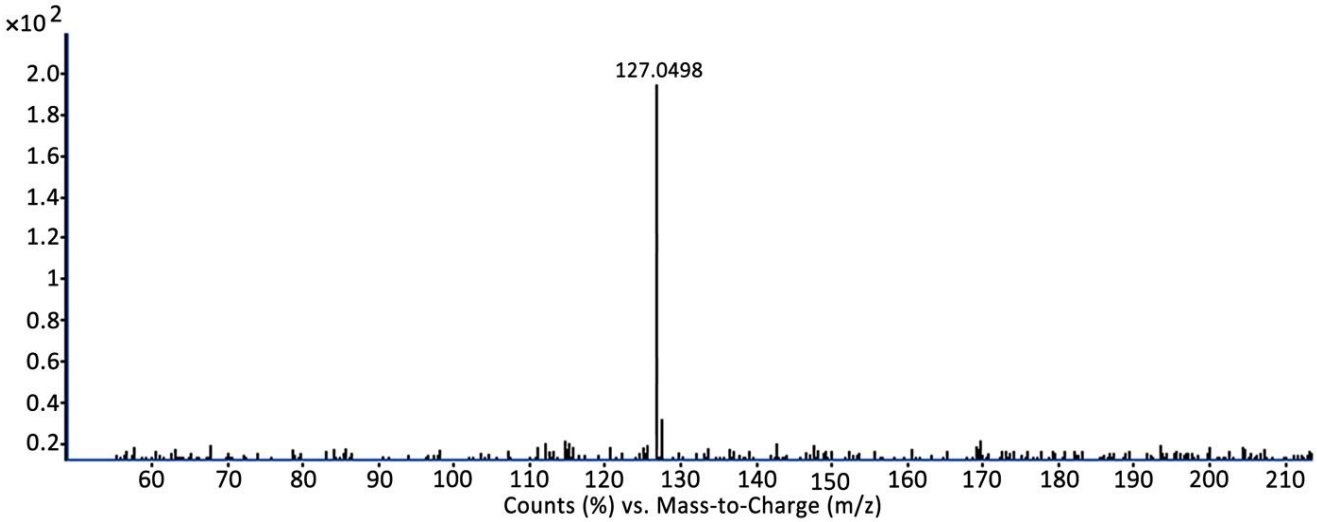


Figure 4. ESI/MS/MS spectrum of degradation product 1 (*m/z* 127)

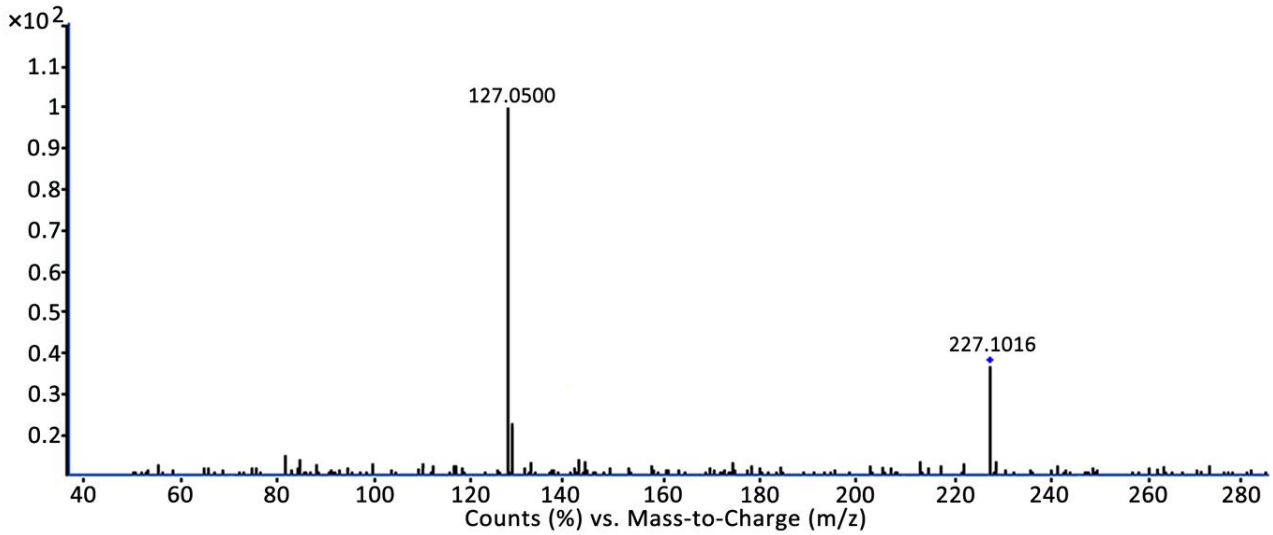


Figure 5. ESI/MS/MS spectrum of degradation product 2 (*m/z* 227)

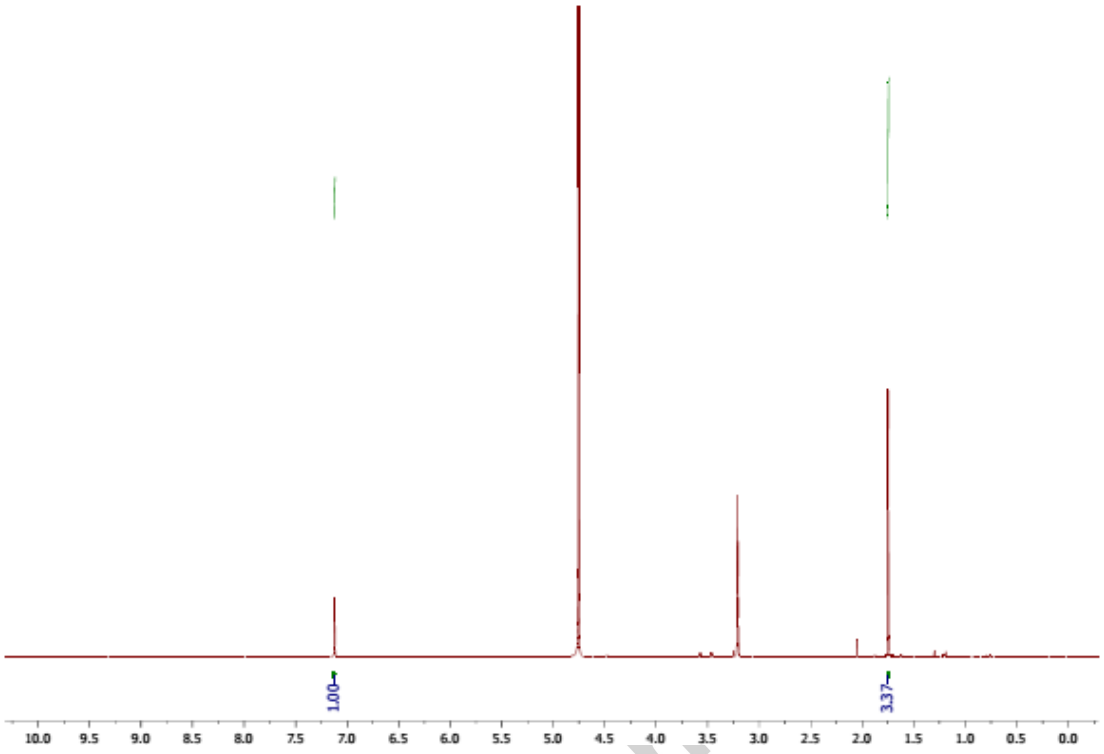
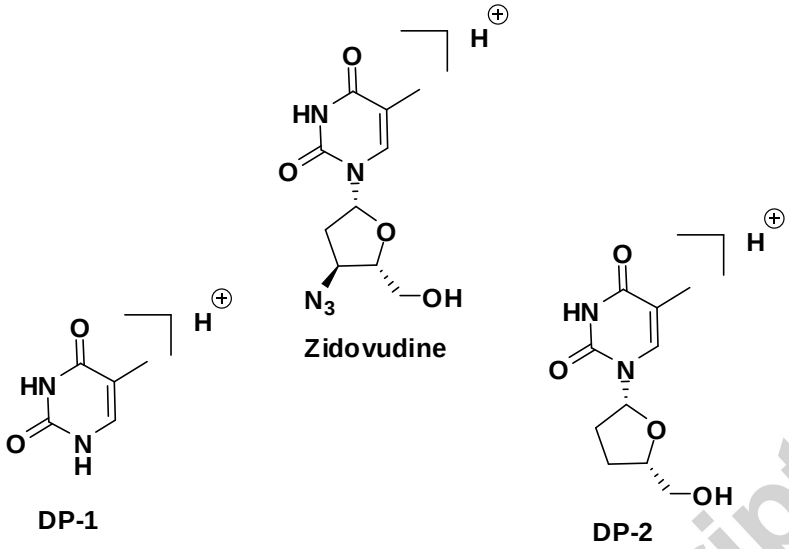
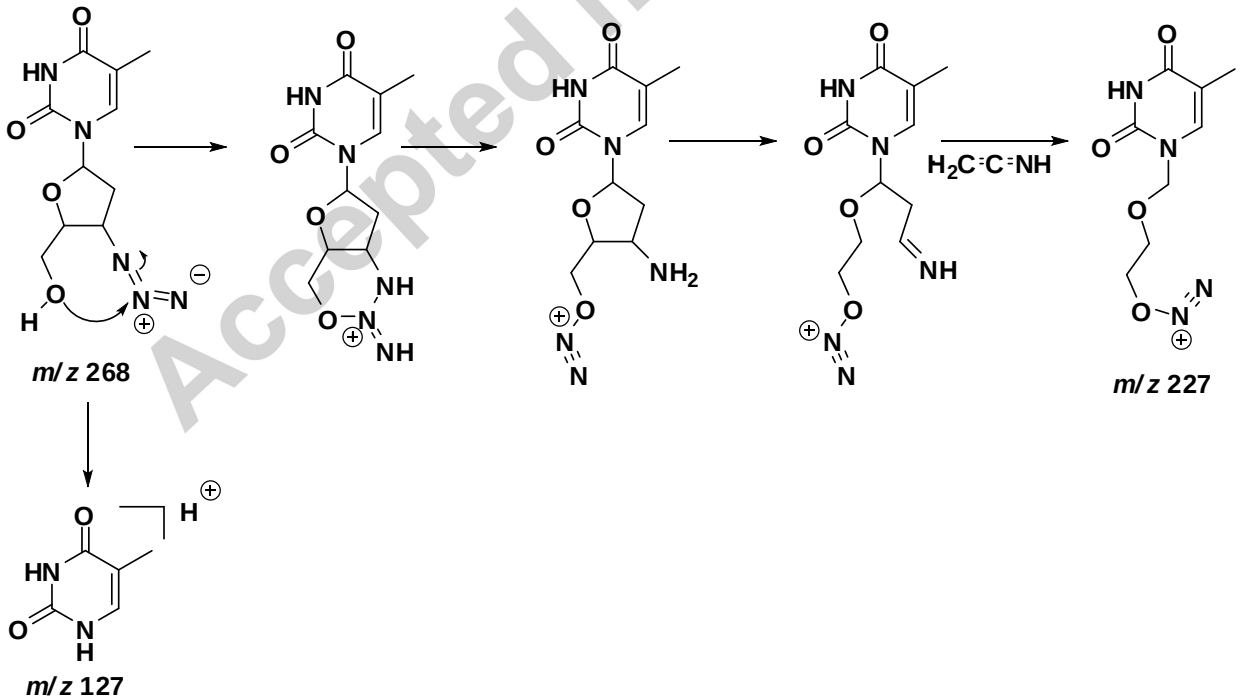


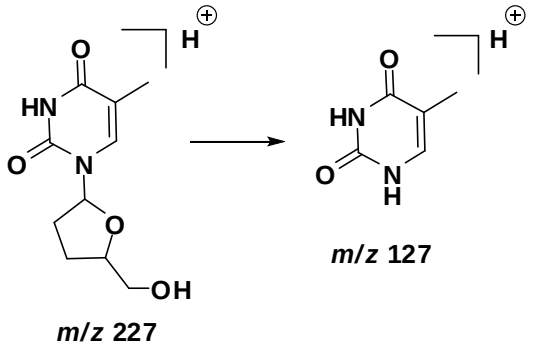
Figure 6. ^{1}H NMR of degradation product 1 (m/z 127)



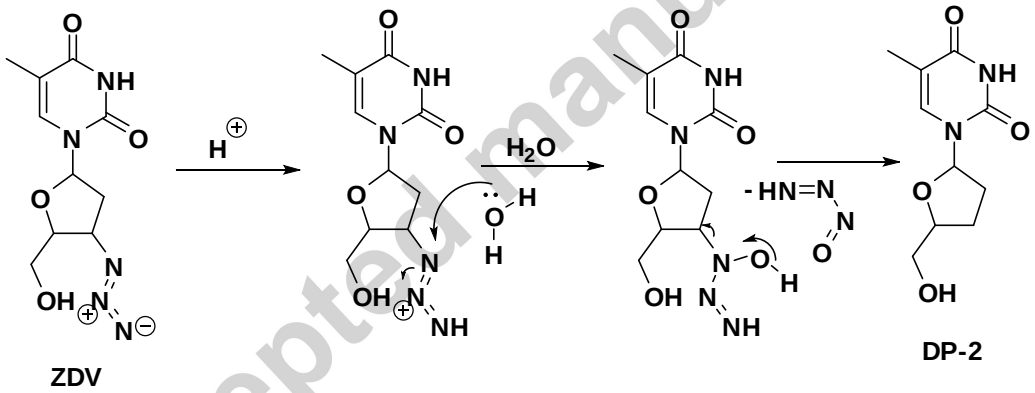
Scheme 1. Proposed structures of protonated degradation products of AZT formed under various stress conditions



Scheme 2. Proposed fragmentation pathway of protonated AZT



Scheme 3. Proposed fragmentation pathway of protonated degradation product 2 (m/z 227)



Scheme 4. Probable mechanism of formation of degradation product-2 (*m/z* 227)

Table 1. Recovery data of AZT (n=3)

Spiked concentration (ng/ml)	Concentration found (Mean ± SD, ng/ml)	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

Table 2. Precision Study (n=3)

Concentration (ng/ml)	Intra-day precision		Inter-day precision	
	Concentration found (Mean ± SD, ng/ml)	RSD (%)	Concentration found (Mean ± SD, ng/ml)	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. Elemental compositions of AZT and its Degradation Products

Degradation product	Retention time (min)	Molecular formula $[M+H]^+$	Calculated *m/z*	Observed *m/z*	Error (ppm)	MS/MS fragment ions

AZT	11.7	$C_{10}H_{14}N_5O_4^+$	268.1040	268.1052	-4.5	227, 127
DP-1	2.8	$C_5H_{17}N_2O_2^+$	127.0502	127.0498	3.1	127
DP-2	12.0	$C_{10}H_{15}N_2O_4$+	227.1026	227.1016	4.4	127

MODEL	**DP-1**	**DP-2**
NTP Carcinogenicity Call (Male Rat) (v3.2)	**1.00**	**1.00**
NTP Carcinogenicity Call (Female Rat) (v3.2)	0.063	0.004
NTP Carcinogenicity Call (Male Mouse) (v3.2)	**1.00**	**1.00**
NTP Carcinogenicity Call (Female Mouse) (v3.2)	0.00	0.040
FDA Carcinogenicity Male Rat Non vsCarc (v3.1)	**0.970**	0.000
FDA Carcinogenicity Male Rat Single vsMult (v3.1)	**1.00**	**1.00**
FDA Carcinogenicity Female Rat Non vsCarc (v3.1)	0.001	0.00
FDA Carcinogenicity Female Rat Single vsMult (v3.1)	**0.914**	0.001
FDA Carcinogenicity Male Mouse Non vsCarc (v3.1)	0.002	0.004
FDA Carcinogenicity Male Mouse Single vsMult (v3.1)	0.000	**0.955**
FDA Carcinogenicity Female Mouse Non vsCarc (v3.1)	**0.997**	**0.960**
FDA Carcinogenicity Female Mouse Single vsMult (v3.1)	**0.990**	**0.997**
Weight of Evidence Carcinogenicity Call (v5.1)	**0.935**	0.303
Ames Mutagenicity (v3.1)	**0.955**	0.000
Developmental Toxicity Potential (DTP) (v3.1)	**0.999**	**0.875**

Rat Oral LD 50 (v3.1) (g/kg)	1.8	391.0
Rat Maximum Tolerated Dose - Feed/Water (v6.1)	6.7mg/kg	4.4 g/kg
Rat Inhalational LC 50 (v6.1) ($g/m^3/H$)	4.7	10
Chronic LOAEL (v3.1) (mg/kg)	891.2	292.9
Skin Irritation (v6.1)	0.422	0.954
Skin Sensitization NEG v SENS (v6.1)	**1.000**	**1.00**
Skin Sensitization MLD/MOD v SEV (v6.1)	0.002	0.000
Ocular Irritancy SEV/MOD vs MLD/NON (v5.1)	0.046	0.000
Ocular Irritancy SEV vs MOD (v5.1)	0.000	0.000
Ocular Irritancy MLD vs NON (v5.1)	0.150	0.000
Aerobic Biodegradability (v6.1)	**1.000**	0.000
Daphnia EC50 (v3.1)	147.8 mg/l	39.7g/l

Table 4. Probability values of different toxicity models of degradation products by TOPKAT analyses

Note: Data in bold indicates the severity of toxicity for both degradation products in respective model.