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Original Research Article

A stability-indicating LC–MS/MS method for zidovudine: Identification, characterization and toxicity prediction of two major acid degradation products

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ABSTRACT

Zidovudine (AZT) is a nucleoside analogue reverse transcriptase inhibitor (NRTI), a class of 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 C_{18} column using 10 mM ammonium acetate: acetonitrile as the 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) 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. One of the degradation products, DP-1, was isolated by semi-preparative high performance liquid chromatography (HPLC) using Waters XBridge Prep C_{18} (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).

1. Introduction

According to the 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 the drugs from class of nucleoside analogue reverse-transcriptase inhibitor (NRTI). It has a crucial role as a component of a multidrug combination regimen for the treatment of adult and pediatric HIV-1 infection. AZT is the most effective in the prevention of mother-to-child HIV-1 transmission [3]. AZT is phosphorylated to its active 5′-triphosphate metabolite, zidovudine triphosphate (AZT-TP), intracellularly. The principal mechanism of the action of AZT-TP is the 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 an inevitable part of drug development cycle to get useful information within short span of time [4]. It is vital in evaluating 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 for finding the probable degradation products, the likely degradation pathways and the intrinsic stability of the molecule [5]. Stress study aims to understand the effects 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 is useful in the development of stable formulation [6]. Evaluation of toxicity of degradation products is vital as ICH Q3 guidelines include stringent reporting, identification, characterization and qualification thresholds [7,8].

A few chromatographic literatures are available for AZT alone and

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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]. A couple of HPLC stability indicating methods are available in literature as well [13,14] and there are many methods offering separation of AZT with combination drugs by HPLC with UV detection [15–25]. However, there is no study available for characterization of major degradation products obtained from forced degradation study of AZT. Hence, the objective of the current study was to develop a 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 separation of degradation products of AZT was performed on LCMS-2020 system (Shimadzu, Japan). The system comprised LC-20 CE 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, and liquid handler was used to isolate 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°C ± 5°C/75% ± 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. Conditions of stress study

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 the mixture of acetonitrile and water (1:1, v/v) as solvent. Each stock solutions of AZT was diluted with acid, base and water in 1:1 (v/v) ratio. Acidic, basic and neutral hydrolytic degradation studies 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 of AZT was diluted with 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 exposed to 1.2×10^6 lx h of fluorescent light and 200 W h/m^2 UV light in a photo stability chamber. The same photo stability study was performed with the stock solution. Powdered AZT was poured in amber bottle with 2 mm height and 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, and 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 with a ratio of 1:1 (v/v) before analysis by HPLC.

2.4. Method development for stressed samples

Several trials were taken over the whole pH range of mobile phase for separation of drug and degradation products. However, after multiple trials, a better and simpler separation of the drug and its degradation products was achieved on XBridge C_{18} (150 mm×4.6 mm, 3.5 µm) (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, and 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 °C, heat block temperature 200 °C, detector temperature 1.1 kV, and 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 using Waters XBridge Prep C_{18} (250 mm×10 mm, 5 µm) with the same mobile phase which was 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 were 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 the supernatant upper layer was taken off. 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. The 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 0.0–1.0 for submitted structures. Value from 0.0 to 0.3 is considered as non toxic, 0.3–0.7 is indeterminate and 0.7–1.0 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 by adhering to ICH guideline Q2 (R1) [28].

System suitability test was used to verify the repeatability and resolution of critical parameter of the system. System suitability

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

Spiked concentration (ng/mL)	Found concentration (ng/mL, Mean ± SD)	RSD (%)	Recovery (%)
10	10.10 ± 0.193	1.91	101.0
30	29.81 ± 0.493	1.65	99.4
50	50.32 ± 0.650	1.29	100.6

Table 2
Precision study of the developed method for AZT (n=3).

Concentration (ng/mL)	Intra-day precision		Inter-day precision	
	Found concentration (ng/mL, Mean ± SD)	RSD (%)	Found concentration (ng/mL, Mean ± SD)	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

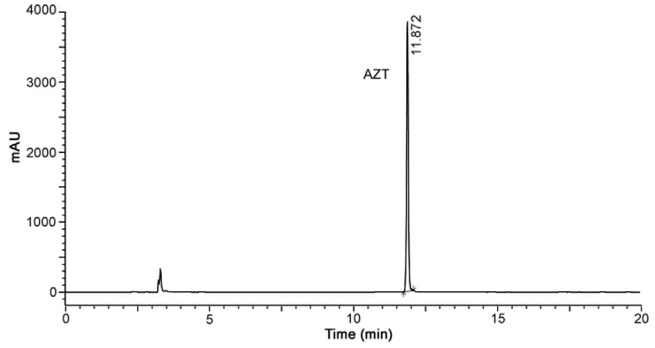


Fig. 1. Chromatogram of AZT (2 mg/mL).

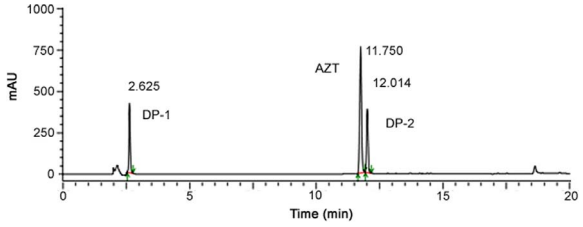
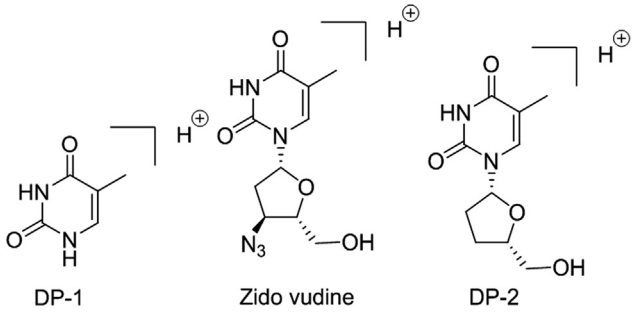


Fig. 2. Chromatogram of acid degradation products.



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

solution was prepared by spiking 20 ng/mL of AZT to a previously acid degraded solution. Resolution between AZT and its degraded impurity was 2.02 ± 0.04 for six individual preparations. PDA detector was used to evaluate peak purity of AZT and its degradation products for determination of method specificity and LC/MS was also used to confirm the same. The MS detector showed the purity of the 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 area 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 were spiked. Each solution was injected in triplicate and the percentage recovery range and % RSD value were found to be 99.4–101.0 and < 2%, respectively (Table 1).

The intra-day precision (repeatability) and inter-day precision (reproducibility) of the developed method for the determination of AZT and its degradation products were measured. 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 of intra-day and inter-day precisions of the method for AZT and the results showed that the method was 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 the method was robust.

3.2. Degradation profile of AZT

MS detector and PDA detector were used in line with HPLC to access the degradation behavior of AZT under various forced degradation conditions. Sufficient degradation was observed only under acidic condition whereas in other conditions it was found to be stable. The chromatograms of AZT alone (2 mg/mL) and its stressed degradation products in acidic condition are shown in Fig. 1 and Fig. 2, respectively. Two degradation products were identified and characterized by using LC–ESI/MS/MS and accurate mass measurements. The proposed structures of degradation products and their elemental compositions are shown in Scheme 1 and Table 3.

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

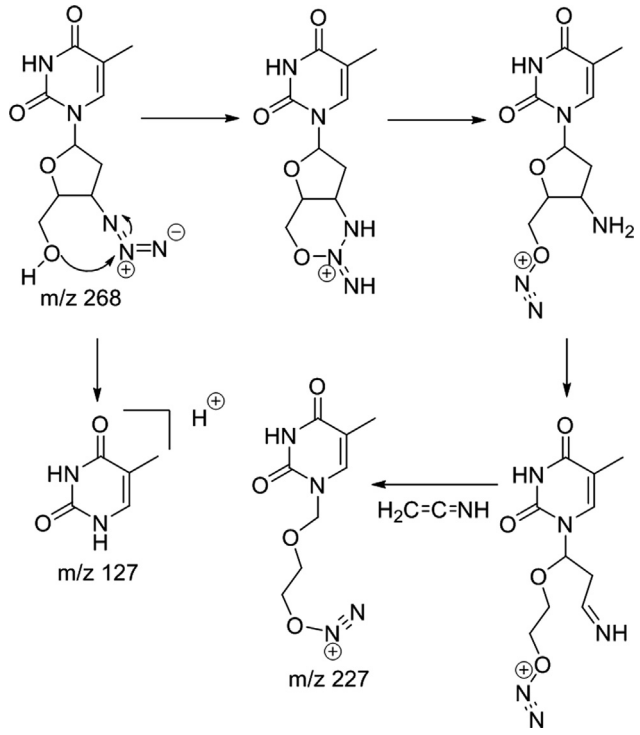
Oxidation, photolytic and thermal degradation sample showed no formation of major degradation products.

3.3. MS/MS of AZT

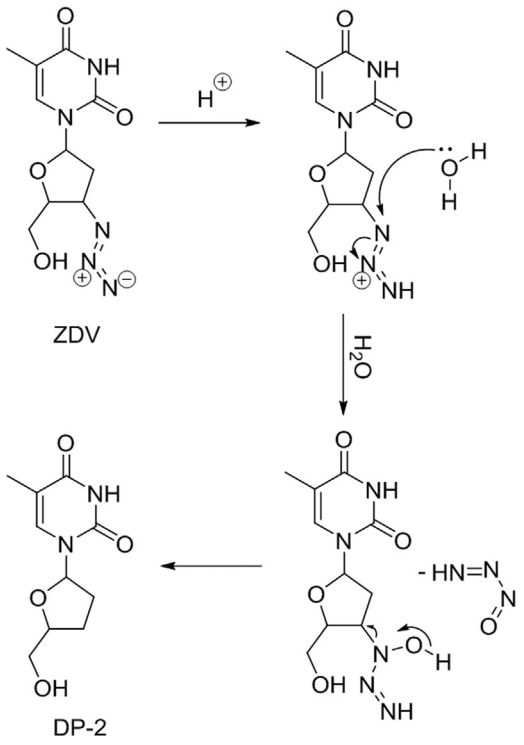
The MS/MS spectra of protonated AZT (Retention time (Rt) =11.8 min; m/z 268) display product ions at m/z 227 (loss of H_2C=C=NH) and m/z 127 (protonated 5-methylpyrimidine-2, 4 (1*H*, 3*H*)-dione) (Scheme 2 and Fig. 3). It can be noted that m/z 127 presents the presence of pyrimidine group in AZT. The elemental compositions of all these fragment ions were confirmed by accurate mass measurements (Table 3).

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



Scheme 2. Proposed fragmentation pathway of protonated AZT.



Scheme 3. Probable mechanism of formation of degradation product 2 (*m/z* 227).

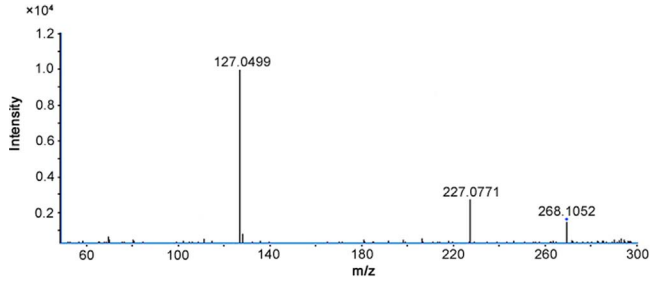
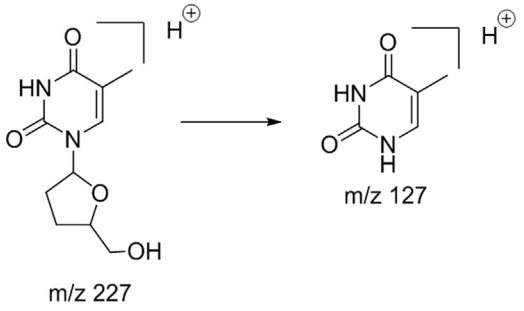


Fig. 3. ESI/MS/MS spectrum of AZT.



Scheme 4. Proposed fragmentation pathway of protonated degradation product 2 (*m/z* 227).

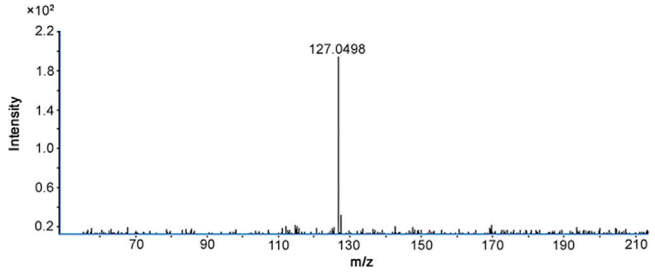


Fig. 4. ESI/MS/MS spectrum of degradation product 1 (*m/z* 127).

3.4. MS/MS of degradation products

MS/MS experiments were performed to characterize the degradation products and to identify the most probable structures based on the *m/z* values of product ions.

The ESI/MS/MS spectrum of $[M+H]^+$ ion (*m/z* 127) was identified as DP-1, eluting at Rt of 2.6 min (Fig. 4). A mass difference of 141 Da between mass of DP-1 and mass of the drug suggests that DP-1 was 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

was confirmed by accurate mass measurements (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 DP-2 was formed by the loss of N_3 from AZT and elemental composition of DP-2 was confirmed by the accurate mass measurements (Table 3 and Scheme 3). The ESI/MS/MS spectrum of $[M+H]^+$ ion of DP-2 (*m/z* 227, Rt=12.0 min) displays product ion at *m/z* 127 (loss of (2, 3-dihydrofuran-2yl) methanol) which is compatible with the structure 1–5-(hydro methyl) teterahydrofuran-2-yl)-5-methylpyrimidine-2, 4 (1*H*, 3*H*)-dione (Scheme 4 and Fig. 5). The elemental compositions of DP-2 and its fragment ions were confirmed by accurate measurements (Table 3).

3.5. ^1H NMR study

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

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

3.6. In-silico toxicity prediction

Table 4 shows TOPKAT predicted toxicity profile of AZT and its degradation products. The toxicity of degradation products was compared and calculated with AZT in different models. Degradation products showed higher carcinogenicity potential in different models such as 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.

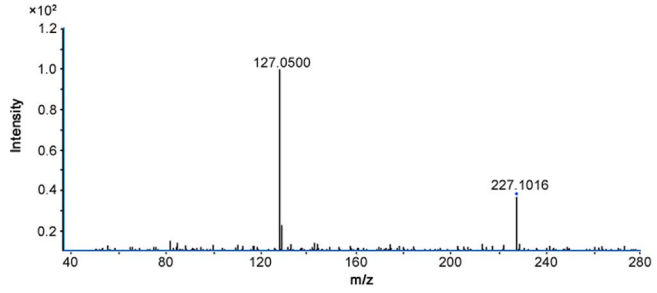


Fig. 5. ESI/MS/MS spectrum of degradation product 2 (*m/z* 227).

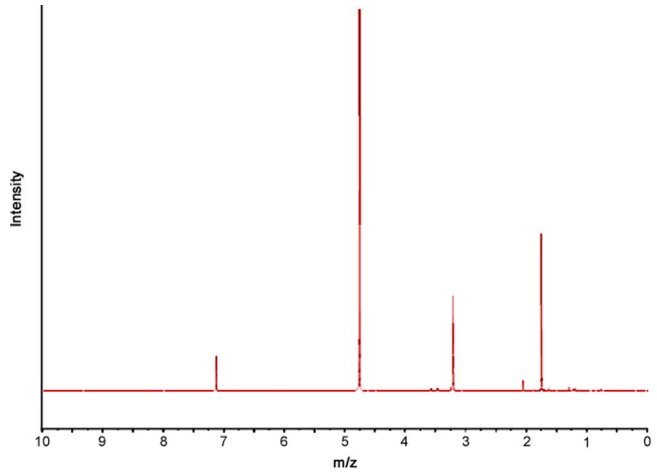


Fig. 6. ^1H NMR of degradation product 1 (*m/z* 127).

Table 4
Probable values of degradation products in different toxicity models by TOPKAT analysis.

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)	6.7 mg/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	39.7 g/L

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

4. Conclusion

A 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 supported by accurate mass measurements. A major degradant DP-1 was isolated and characterized by ^1H NMR. In-silico toxicity profile predicted carcinogenic possibilities of both degradation products using TOPKAT software. The results showed that degradation products have higher carcinogenicity potential in different models, and the DP-1 showed toxicity in Ames Mutagenicity and Aerobic Biodegradability (v6.1) model.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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