

# Characterization and Toxicity Prediction of two major acid degradation Products by LC Method

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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 C18 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 X Bridge 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).

## Keywords:

Zidovudine, Stability study, Degradation products, In-silico toxicity prediction LC-ESI/MS/MS, Degradation pathway

## 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  $\alpha$  and  $\gamma$  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 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. Material and Method**

### **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  $40^{\circ}\text{C} \pm 5^{\circ}\text{C}/75\% \pm 3\% \text{RH}$  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 solution 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  $80^{\circ}\text{C}$  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  $1.2 \times 10^6 \text{ lx h}$  of fluorescent light and  $200 \text{ W h/m}^2$  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  $80^{\circ}\text{C}$  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  $5^{\circ}\text{C}$  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 X Bridge C18 (150 mm  $\times$  4.6 mm, 3.5  $\mu\text{m}$ ) (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  $\mu\text{L}/\text{min}$ , 25  $\mu\text{L}$ , 30  $^{\circ}\text{C}$  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  $^{\circ}\text{C}$ , heat block temperature 200  $^{\circ}\text{C}$ , 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 C18 (250 mm $\times$ 10 mm, 5  $\mu\text{m}$ ) 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  $\mu\text{L}$ , 30  $^{\circ}\text{C}$  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 ( $^1\text{H}$  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 3.1 Recovery data of AZT (n=3)**

Spiked concentration ( $\text{ngmL}^{-1}$ )	Concentration found (Mean $\pm$ SD, $\text{ngmL}^{-1}$ )	RSD(%)	Recovery(%)
10	10.10 $\pm$ 0.193	1.91	101.0
30	29.81 $\pm$ 0.493	1.65	99.4
50	50.32 $\pm$ 0.65	1.29	100.6

Table 3.2 Precision Study (n=3)

Concentration (ng mL <sup>-1</sup> )	Intra-day precision		Inter-day precision	
	Concentration found (Mean ± SD, ng mL <sup>-1</sup> )	RSD (%)	Concentration found (Mean ± SD, ng mL <sup>-1</sup> )	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

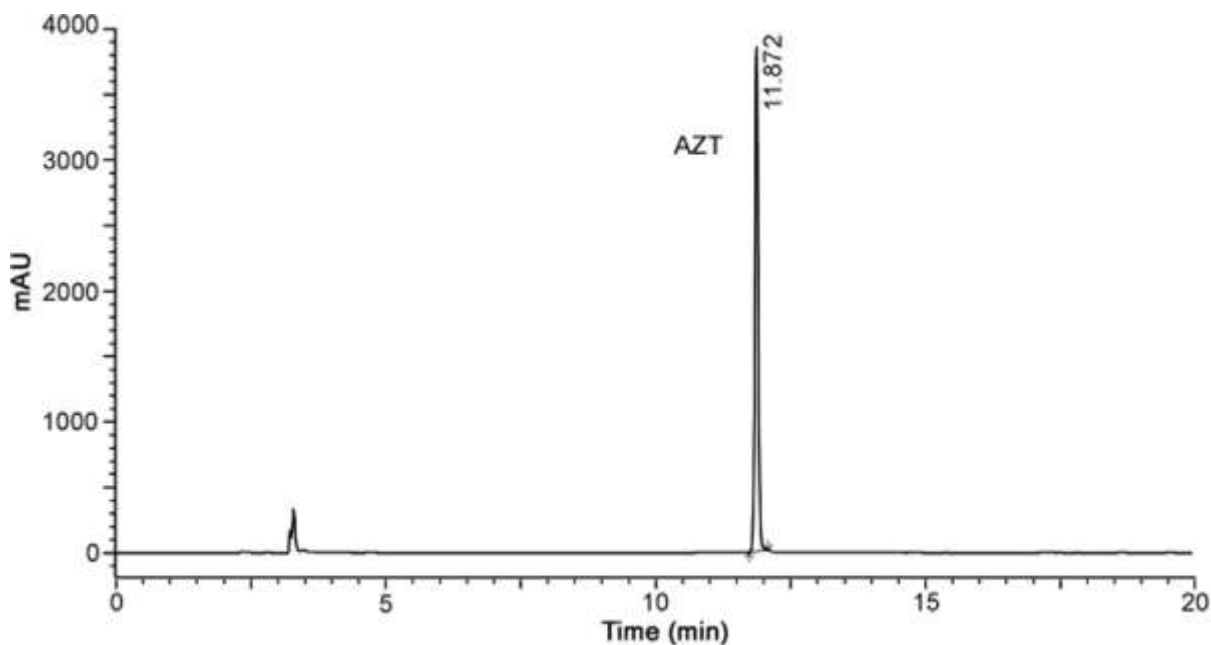


Fig. 1. Chromatogram of AZT (2 mg/mL).

solution was prepared by spiking 20 ng/mL of AZT to a previously aciddegraded solution. Resolution between AZT and its degraded impurity was  $2.02 \pm 0.04$  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

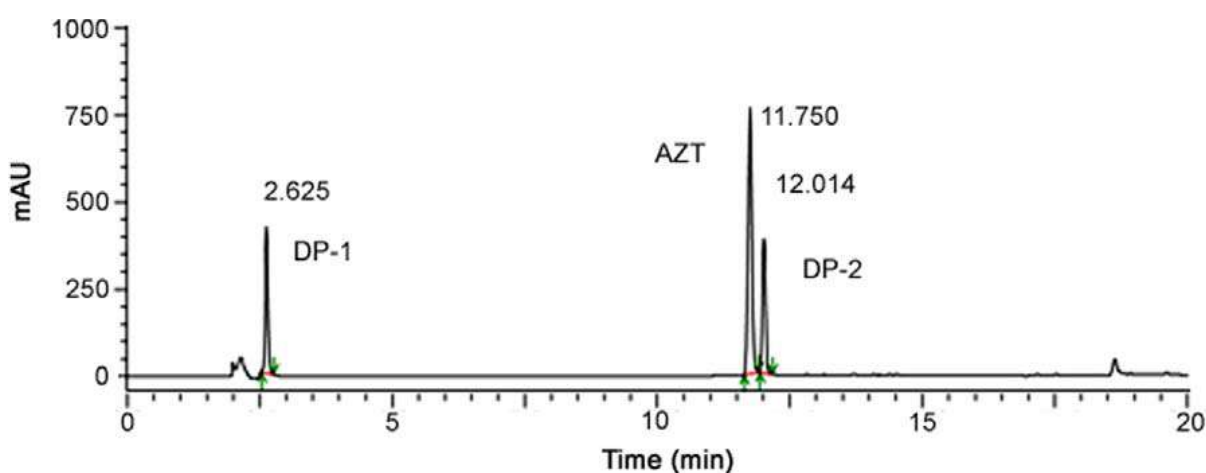
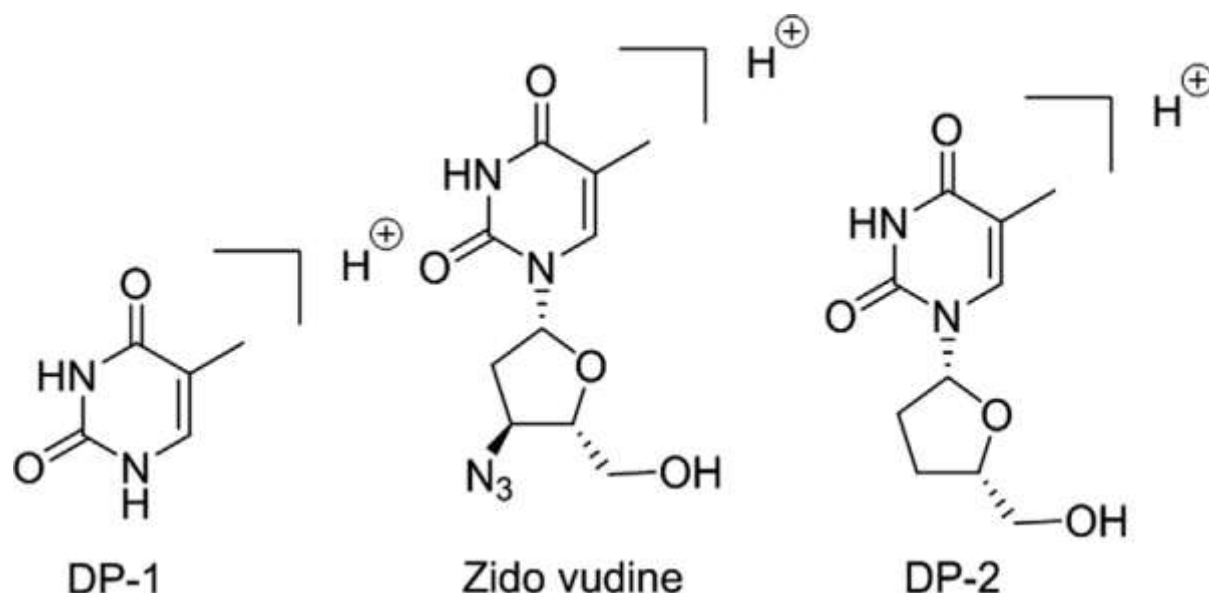


Fig. 2. Chromatogram of acid degradation products



Scheme 1.

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

#### 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 <sup>1</sup>H 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.

#### ACKNOWLEDGEMENT:

The authors are grateful to the authorities of Sunrise University Alwar Rajasthan for the facilities.



**CONFLICT OF INTEREST:**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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