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Short communication

Validated specific HPLC method for determination of zidovudine during stability studies

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Abstract

The objective of the current study was to develop a validated stability-indicating assay method (SIAM) for zidovudine (3′-azido-3′-deoxythymidine) after subjecting it to forced decomposition under hydrolysis, oxidation, photolysis and thermal stress conditions. The drug decomposed under hydrolytic stress upon refluxing, and also on exposure to light. It was stable to oxidation and thermal stress. The same major decomposition product could be seen in all the decomposed solutions, which was identified as thymine through comparison with the standard. Separation of drug from major and minor degradation products was successfully achieved on a C-18 column utilising water–methanol in the ratio of 77:23. The detection wavelength was 265 nm. The method was validated and response was found to be linear in the drug concentration range of 25–500 $\mu g\, ml^{-1}$. The mean values (±R.S.D.) of slope and correlation coefficient were 21,859 (±0.213) and 0.9995 (±0.00578), respectively. The R.S.D. values for intra- and inter-day precision were <0.9 and <1.6%, respectively. The method was established to have sufficient intermediate precision as similar separation was achieved on another instrument handled by a different operator. The recovery of the drug from a mixture of degraded samples ranged between 100.6 and 100.9%. PDA peak purity test confirmed the specificity of the method. The method was also successful in analysis of drug in marketed tablets subjected to stability testing under accelerated conditions of temperature, humidity, and to thermal and photolytic stress.
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Keywords: Zidovudine; Forced decomposition; Stress testing; Stability-indicating method; HPLC; ICH

1. Introduction

The International Conference on Harmonization (ICH) drug stability test guideline Q1A (R2) [1] requires that analysis of stability samples should be done through the use of validated stability-indicating analytical methods. It also recommends carrying out of stress testing on the drug substance to establish its inherent stability characteristics and to support the suitability of the proposed analytical procedure. The stress testing encompasses the influence of temperature, humidity, and light, oxidizing agents as well as susceptibility over a wide range of pH values.

The objective of the present study was to study degradation of zidovudine under different ICH recommended stress conditions, and to establish a validated stability-indicating HPLC method. Zidovudine is chemically 3′-azido-3′-deoxythymidine (Fig. 1a). It is active against human immunodeficiency virus (HIV-I and -II) and human T-cell lymphotropic virus (HTLV-I and -II) [2]. The drug is official in USP [3] and BP [4]. The drug is reported to exhibit acid and water catalysed degradation and remained stable in alkali [5]. Recently, its decomposition behaviour in hydrolytic and photolytic conditions was compared with stavudine [6]. Reports also exist on the stability of the drug in pharmaceutical dosage forms such as extemporaneous syrup and injection [7]. However, there is no report yet on the development of stability-indicating assay method for the drug employing

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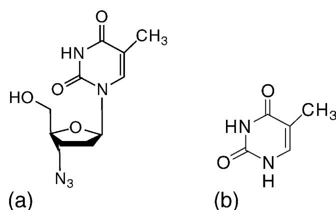


Fig. 1. Structure of zidovudine (a), and its degradation product thymine (b).

ICH suggested approach. Otherwise, a number of reports are available for the determination of drug in intracellular milieu and from biological fluids, such as plasma and urine [8–19].

2. Experimental

2.1. Materials

Zidovudine was received gratis from Ranbaxy Laboratories Ltd. (Gurgaon, India) and was used without further purification. Pure thymine was procured from Lancaster (Morecambe, England). Sodium hydroxide was purchased from Ranbaxy Laboratories (S.A.S Nagar, Punjab) and hydrochloric acid was procured from LOBA Chemie Pvt. Ltd. (Mumbai, Maharashtra). Hydrogen peroxide was procured from S.D. Fine-chem Ltd. (Boisar, Maharashtra). HPLC grade methanol was purchased from Merck (Darmstadt, Germany) and acetonitrile of the same grade was procured from J.T. Baker (Xalostoc, Mexico). All other chemicals were of analytical reagent grade. Ultra-pure water was obtained from ELGA (Bucks, UK) water purification unit.

2.2. Instrumentation

pH of the mobile phase was checked on a pH/ion analyser (MA 235, Mettler Toledo, Schwerzenbach, Switzerland). Refluxing of the drug in hydrolysis conditions was carried out in a round bottom flask-condenser assembly using a boiling oil bath. Stability studies were carried out in humidity chamber (KBF 760, WTB Binder, Tuttlingen, Germany) and a photostability chamber (KBF 240, WTB Binder, Tuttlingen, Germany), capable of controlling tolerances in temperature (±1 °C) and humidity (±3 °C), below the limits specified in the ICH guideline Q1A (R2) [1]. The photostability chamber was equipped with light sources defined under option 2 in the ICH guideline Q1B [20]. The light bank consisted of a combination of two blacklight OSRAM L73 and four white fluorescent OSRAM L20 lamps. Both UV and VIS lamps were put on simultaneously. The samples were placed at a distance of 9 inches from the light bank. The overall illumination at the point of placement of samples was 6000 lux, which was tested using a calibrated lux meter (model ELM 201, Escorp, New Delhi, India). The near UV energy at the same distance was 0.7 W/m^2 when tested using a calibrated radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany). Thermal stability study was performed in a dry air oven (NSW Limited, New Delhi, India).

The HPLC system consisted of a 600 E pump, a 996 photodiode array (PDA) detector, a 717 autoinjector, and a degasser module; data were acquired and processed using Millennium software version 2.1 (all equipment from Waters, Milford). The chromatographic separations were carried out on Merck (Darmstadt, Germany) C-18 column (250 mm × 4.6 mm i.d. with particle size of 5 μm). Robustness testing of the method was done on another HPLC system, equipped with a LC-$10AT_{VP}$ pump, a SPD-$10AV_{VP}$ UV–vis dual wavelength detector, a SIL-$10AD_{VP}$ autoinjector, and a DGU-14A degasser module; data were acquired and processed using CLASS-VP software (all from Shimadzu, Kyoto, Japan).

2.3. Degradation studies

All degradation studies in solution were carried out at a drug concentration of 1 mg ml^{-1}. Hydrolytic reactions were carried out in water, 0.1 M HCl and 0.1 M NaOH under refluxing for 5 days. Oxidative studies were conducted at room temperature in 3 and 30% H_2O_2 for 24 and 48 h, respectively. Photo-degradation studies were carried out in water, 0.1 M HCl and 0.1 M NaOH in a photostability chamber up to 15 days. Pure solid drug (in 1 mm thick layer in a petri plate) was also exposed in the same chamber for 60 days. Suitable controls were kept under dark conditions. Pure drug was additionally exposed to dry heat at 50 °C and separately to accelerated conditions of 40 °C and 75% RH in the stability chamber for 3 months. Samples were withdrawn periodically and analysed by HPLC after suitable dilution.

2.4. Identification of thymine as a major degradation product

The formation of thymine as a major degradation product in reaction solutions of zidovudine was checked by spiking with the standard. The suspicion that thymine could be formed during degradation of zidovudine was based on information in literature that stavudine, a closely related drug, was degraded to thymine in the pH range 1–9 [6,21].

2.5. HPLC method development

The stressed samples were initially analysed by HPLC using a RP 18 column and a mobile phase composed of water and methanol (85:15). As the separation and peak shape were not good, therefore, organic modifier concentration was changed from 15 to 25%, but no improvement was observed. Subsequent attempts were made by lowering of pH of the mobile phase (using acetic acid) and replacement of methanol by acetonitrile. In both cases, marked improvement was observed. Considering the low cost of methanol, it was retained as an organic modifier and further trials were carried out by varying the pH and simultaneously optimising ratio of buffer and methanol. Eventually, a mobile phase composition of

buffer (pH 4.5): methanol (77:23) gave the best results. During these studies, injection volume was 10 μl and the mobile phase flow rate was constant at 1 ml min^{-1}. The analytical wavelength was 265 nm.

2.6. Validation of the developed method

Linearity was established by triplicate injections of solutions containing drug in the concentration range of 25–500 μg ml^{-1}. Intra-day precision was established by making six injections of lowest, middle and highest concentration in the above range (25, 250 and 500 μg ml^{-1}) on the same day. These studies were also repeated on different days with different weightings to determine inter-day precision. Intermediate precision was established through separation studies on a different chromatographic system by a different operator. Accuracy was evaluated by fortifying a mixture of decomposed reaction solutions with three known concentrations of the drug. The recovery of added drug was determined. Specificity was established by determining peak purity for the drug peak in a mixture of stressed reaction solutions using a PDA detector. The resolution factor of the drug peak with respect to nearest resolving peaks was also determined.

2.7. Analysis of marketed tablets subjected to accelerated stability testing

Zidovudine tablets were purchased from market and subjected to accelerated test conditions of 40 °C/75% RH in stability and photostability chambers for 2 months. The tablets were also subjected to thermal stress at 50 °C for the same time period. For analysis, these were crushed and sonicated in methanol for 30 min. The contents were transferred to a 100 ml volumetric flask and the volume was made up to 100 ml with methanol. 500 μl of the solution was withdrawn and diluted five times with mobile phase to obtain a concentration of 100 μg ml^{-1}. The samples were filtered through 0.22 μm nylon membrane and subjected to analyses.

3. Results and discussion

3.1. Stress studies

HPLC studies on zidovudine under different stress conditions using water:methanol (77:23) as the solvent system suggested the following degradation behaviour.

3.1.1. Acidic condition

The drug on refluxing in 0.1 M HCl resulted in formation of three degradation products, one major and two minor. At the end of 24 h, around 32% fall in drug peak area was observed. After refluxing for 5 days, drug degraded by 90% with corresponding increase in concentration of the degradation products.

3.1.2. Neutral (water) condition

The drug was more stable in water, falling by only 16% upon refluxing up to 5 days.

3.1.3. Degradation in alkali

Similar to neutral condition, the drug was found to be relatively stable in alkaline condition. On refluxing the drug in 0.1 M NaOH for 5 days, again around 15% of the drug was only degraded.

3.1.4. Oxidative conditions

The drug was completely stable to oxidation. No degradation was seen in 3% and even 30% H_2O_2 at room temperature up to 2 days.

3.1.5. Photolytic conditions

Zidovudine proved labile on exposure to light in acid, alkali or neutral conditions. Under all the three conditions, the drug decomposed to the same major degradation product, which was also formed during hydrolysis. No degradation was observed in control samples in the dark chamber. Rate of photolysis was highest in acid, followed by base and water.

3.1.6. Studies on solid drug

No decomposition was seen on exposure of solid drug powder to either dry heat at 50 °C or 40 °C/75% RH for 3 months. The exposure of the drug in a photostability chamber for 2 months resulted in only 5% decomposition, forming the same major degradation product as produced on hydrolysis and on exposure of drug solution to light. The colour of the powder changed to yellow in 5 days and this got darkened as duration of exposure was increased.

3.2. Identification of major degradation product

Fig. 2 shows the results of the spiking study, which confirms that pure thymine was the major degradation product formed during hydrolysis and light exposure.

3.3. Establishment of a stability-indicating method for a mixture of stressed solutions

The mobile phase comprising of water–methanol in the ratio of 77:23 was first used to analyse individual stressed samples. It was then applied to a mixture of those stressed samples in which there was recognizable degradation and/or different degradation products were formed. The method was able to separate the drug as well as all major and minor degradation products. The resulting chromatogram is shown in Fig. 3a.

3.4. Validation of the method

The method was validated with respect to parameters like linearity, precision, accuracy, specificity and robustness.

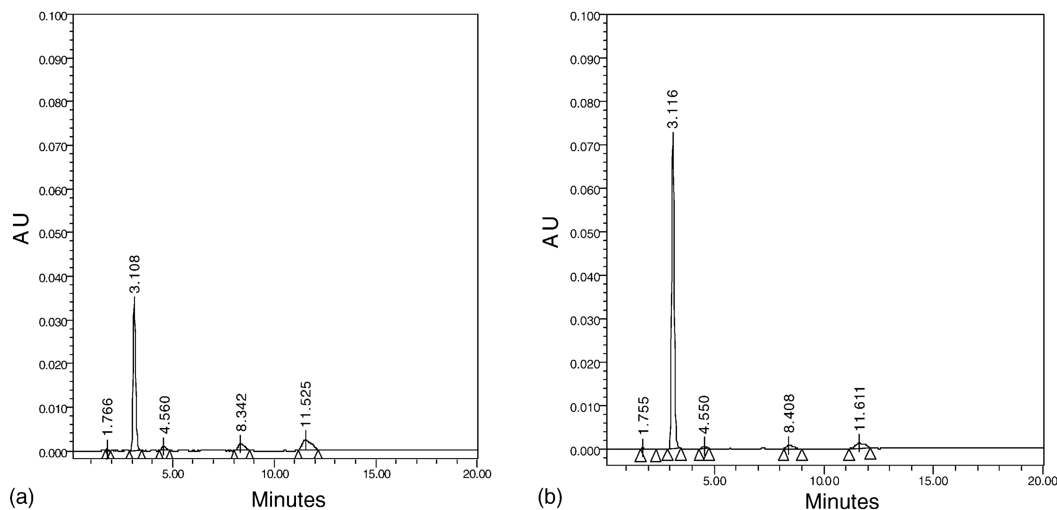
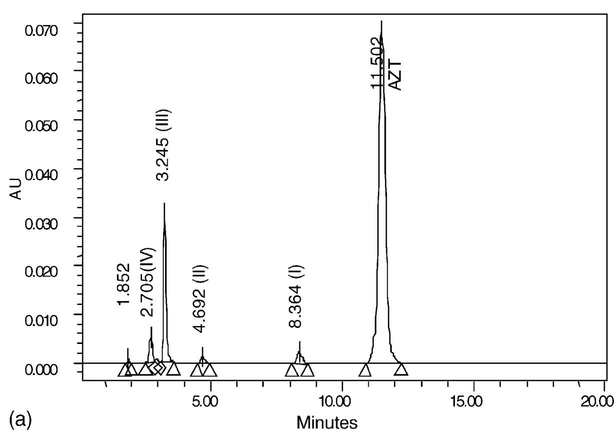


Fig. 2. Chromatograms showing (a) degraded solution of zidovudine before spiking and (b) after spiking with thymine. Based on this study, the peak at 3.1 min could be attributed to thymine.



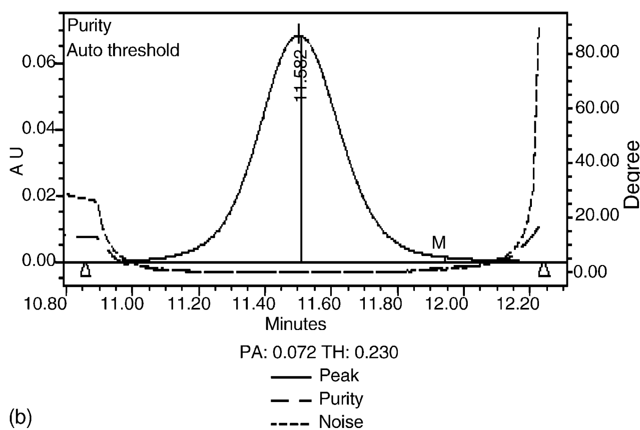


Fig. 3. Chromatogram (a) showing separation of zidovudine (AZT) and major and minor degradation products in a mixture of stressed samples. Key-I and -II: hydrolytic degradation products in acid; key-III: hydrolytic and photolytic degradation product in acid, base and water; key-IV photolytic degradation product in acid. (b) depicts the purity plot for the AZT peak in (a).

3.4.1. Linearity

The response for the drug was strictly linear in the studied concentration range ($r^2 = 0.9995$). The mean (±R.S.D.) values of slope and correlation coefficient were 21,859 (±0.213) and 0.9995 (±0.00578), respectively.

3.4.2. Precision

Table 1 provides data obtained from the precision experiments. The R.S.D. values for intra- and inter-day precision were <0.9 and <1.6%, respectively, thereby indicating that the method was sufficiently precise. A similar qualitative separation of the drug as shown in Fig. 3a was obtained even on analysis on a different chromatographic system on a different day (Fig. 4), indicating that the method has sufficient intermediate precision.

3.4.3. Accuracy

Percentage recovery was calculated from differences between the peak areas obtained for fortified and unfortified

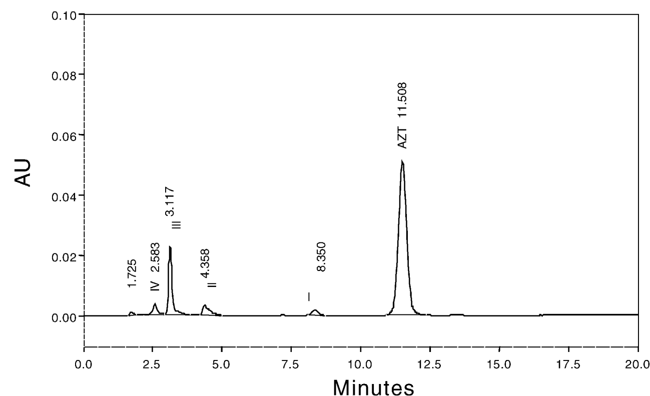


Fig. 4. Chromatogram showing resolution of zidovudine from degradation products when analysis was performed on a different chromatographic system by a different operator. Key: same as in Fig. 3a.

Table 1
Reproducibility and precision data evaluated through intra-day and inter-day studies ($n = 6$)

Actual concentration (μg ml^{-1})	Measured concentration ± S.D. (μg ml^{-1}), R.S.D. (%)	
	Intra-day	Inter-day
25	24.305 ± 0.202, 0.835	24.184 ± 0.225, 0.920
250	248.871 ± 1.598, 0.642	246.780 ± 3.903, 1.581
500	512.452 ± 1.930, 0.376	506.751 ± 4.481, 0.884

Table 2
Recovery studies ($n = 3$)

Actual concentration (μg ml^{-1})	Calculated concentration ± S.D. (μg ml^{-1}), R.S.D. (%)	Recovery (%)
25	25.191 ± 0.401, 1.593	100.8
250	251.539 ± 0.666, 0.265	100.6
500	504.617 ± 1.127, 0.223	100.9

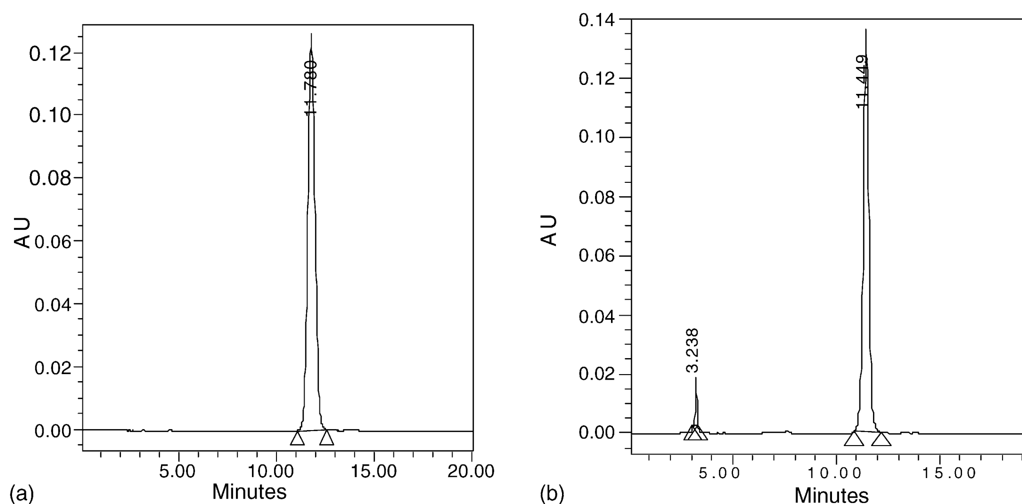


Fig. 5. Chromatograms showing separation behaviour of zidovudine in (a) control tablets and (b) tablets subjected to 40 °C/75% RH in light chamber for 2 months. The chromatograms for tablets subjected to dry heat at 50 °C and 40 °C/75% RH in dark chamber for 3 months were similar to control (a). This shows that thymine was the lone degradation product in the tested formulation and that too only on exposure to light. The minor degradation products formed under stress conditions (Figs. 3a and 4) were not formed on exposure of formulation to accelerated or dry heat conditions.

solutions. As shown from the data in Table 2, excellent recoveries were made at each added concentration, despite the fact that the drug was fortified to a mixture that contained drug as well as the degradation products (formed under various reaction conditions).

3.4.4. Specificity

The method was found to be specific to the drug. The PDA analyses proved that the purity-angle value for the drug peak in a mixture of stressed samples was less than purity-threshold value (as evident from the purity plot in Fig. 3b), thereby indicating that the drug peak was free from any coeluting peak. Moreover, the resolution factor for the drug peak (Fig. 3a) was 7.5 from the nearest resolving peak.

3.5. Applicability of the developed method to marketed formulation

No degradation was observed on HPLC analysis of marketed tablets subjected to thermal stress at 50 °C and separately to an accelerated condition of 40 °C/75% RH for 3 months. Fig. 5 shows the chromatogram for the tablets exposed to light at 40 °C/75% RH for 2 months, as compared to dark control. It is shown that a single degradation product ($R_T = 3.2$ min) was formed on exposure of the formulation to light (Fig. 5b). The study indicated that the method could be applied successfully even to stress marketed formulation.

4. Conclusion

A validated stability-indicating HPLC assay method was developed for zidovudine, using the stress-testing route suggested by ICH. The developed method is simple, accurate, precise, specific, and could separate drug from degradation products. It is suggested for use in analysis of samples generated during stability studies on zidovudine and its formulations.

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