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Short communication

Establishment of inherent stability of stavudine and development of a validated stability-indicating HPLC assay method

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Abstract

The present study describes degradation of stavudine under different stress conditions (hydrolysis, oxidation, photolysis and thermal stress), and establishment of a stability-indicating reversed-phase HPLC assay method. The drug was found to hydrolyse in acidic, neutral and alkaline conditions and also under oxidative stress. The major degradation product formed under various conditions was thymine, as evidenced through comparison with the standard and spectral studies (NMR, IR and MS) on the isolated product. Separation of drug, thymine and another minor degradation product was successfully achieved on a C-18 column utilising water–methanol in the ratio of 90:10. The detection wavelength was 265 nm. The method was validated with respect to linearity, precision (including intermediate precision), accuracy and specificity. The response was 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 24256 (±0.679) and 0.9994 (±0.0265), respectively. The R.S.D. values for intra- and inter-day precision studies were <0.210 and <1%, respectively. The recovery of the drug ranged between 99.7 and 101.5% from a mixture of degraded samples. The method even proved to be affective on application to a stressed marketed capsule formulation.

Keywords: Stavudine; Stress testing; Stability-indicating method; HPLC; ICH

1. Introduction

The aims of the present study were to: (i) establish inherent stability of stavudine through stress testing under a variety of conditions [1] recommended by the International Conference on Harmonisation (ICH) [2], and (ii) develop a validated stability-indicating assay. Stavudine, is a synthetic thymidine analog (2′,3′-didehydro-3′-deoxythymidine; D4T, Fig. 1). It is active against HIV-1 and HIV-2 [3].

The only information known in literature is that the drug degrades to thymine in the pH range 1–9 [4] and also in oxidative conditions [5]. The literature is silent on development of a stability-indicating assay method, though a number of reports exist on procedures for its determination from biological fluids, such as plasma and urine [6–13].

2. Experimental

2.1. Materials

Stavudine was received gratis from Cipla Ltd. (Mumbai, India) and was used as-received. Pure thymine was purchased from Lancaster (Morecambe, UK). Sodium hydroxide and hydrochloric acid were purchased from Ranbaxy Laboratories (SAS Nagar, Punjab) and LOBA Chemie Pvt. Ltd. (Mumbai, Maharashtra), respectively. Hydrogen peroxide was procured from S.D. Fine-Chem Ltd. (Boisar, Maharashtra). HPLC grade methanol was purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained from

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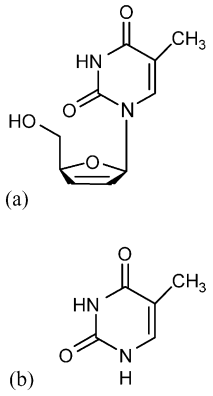

Fig. 1. Structure of (a) stavudine and (b) thymine.

ELGA (Bucks, UK) water purification unit. All other chemicals were of analytical reagent grade.

2.2. Instrumentation

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for acidic and neutral degradation studies. Dri-Bath (model DB28120-26, Thermolyne, Iowa, USA) was used for alkaline hydrolysis. Stability studies under accelerated conditions were done in a humidity chamber (KBF 760, WTB Binder, Tuttlingen, Germany). Photodegradation was carried out in a photostability chamber (KBF 240, WTB Binder, Tuttlingen, Germany) equipped with a light bank consisting of a combination of two blacklights (OSRAM L73) and four white fluorescent (OSRAM L20) lamps. The light system complied with option 2 prescribed in the ICH guideline Q2A [14]. The samples were placed at a distance of 9 in. and both UV and vis lamps were put on simultaneously. At any given time, UV energy at the point of placement of samples was ∼0.7 W/m^2 (tested with a calibrated radiometer, model 206, PRC Krochmann GmbH, Berlin, Germany) and visible illumination was ∼6000 lx (tested using a calibrated lux meter, model ELM 201, Escorp, New Delhi, India). The chamber was set at accelerated condition of 40 °C/75% RH during the studies. For thermal stability studies, a dry air oven (NSW Limited, New Delhi, India) was used. Other equipments used were a rotary evaporator (model R-114, Buchi Laboratechnik, Flawil, Switzerland), an aspirator (Eyela A-3S, Tokyo Rikakkai Co., Tokyo, Japan) and a freeze dryer (model DW8-85, Heto Holten, Allerød, Denmark).

Separation studies were carried out using an HPLC system from Waters (Milford, USA). The instrument consisted of a 600 E quaternary gradient pump, a 996 photo-diode array (PDA) detector, a 717 autoinjector, and a degasser module. The data were acquired and processed by use of Millennium software ver. 3.1. The second HPLC system used for robustness testing was 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). Reversed phase C-18 columns of 250 mm × 4.6 mm i.d. size and containing 5 μm stationery phase were purchased from Merck (Darmstadt, Germany).

IR spectra were obtained on an Impact 410 spectrometer (Nicolet, MD, USA). NMR spectra were recorded on Avance DPX 300 spectrometer (Bruker, Fallanden, Switzerland). Direct mass was taken on gas chromatograph–mass spectrophotometer (QP 5000, Shimadzu, Kyoto, Japan) in chemical ionisation (CI) mode.

2.3. Degradation studies

Decomposition studies were performed in solutions containing drug at a concentration of 1 mg ml^{-1}. Samples were withdrawn at suitable time intervals and subjected to HPLC analysis, after suitable dilution. The stress conditions were as follows:

(1) Hydrolysis
Solutions in water, 0.1 M HCl and 0.1 M NaOH were heated at 80 °C for 3 h, 1 h and 3 d, respectively.
(2) Oxidation
Drug was stored initially in 3% H_2O_2 for 24 h and subsequently in 30% H_2O_2 for 48 h. These studies were carried out at room temperature.
(3) Photolysis
Drug solutions in water, 0.1 M NaOH and 0.01 M HCl were exposed in a photostability chamber for 15 d. Also, solid drug was spread in a petri plate in 1 mm thickness and exposed in the photostability chamber for 2 months. Suitable controls were maintained under dark conditions.
(4) Thermal stress
Bulk drug was subjected to dry heat at 50 °C for 3 months.
(5) Pure drug was also subjected to accelerated condition of 40 °C/75% RH for 3 months.

2.4. Isolation and characterisation of major degradation product

A solution of stavudine in water (10 mg ml^{-1}) was heated at 80 °C for 4 h to allow complete decomposition of the drug. The reaction mixture was freeze-dried and extracted with dichloromethane (DCM), previously treated with anhydrous sodium sulphate. The DCM extract was completely evaporated on rotary evaporator resulting in a white to off white compound. The purity of the compound was verified by HPLC, where a single peak was obtained. The compound was subsequently characterised using MS, IR and NMR. Confirmatory studies were also carried out by spiking with the standard.

2.5. Separation studies

HPLC studies were carried out first on stressed solutions individually, and then on a mixture of those solutions in which

decomposition was observed. Satisfactory separation of the components of the mixture was achieved using a mobile phase composed of water–methanol in the ratio of 90:10. The injection volume was 10 μl and flow rate was 1 ml min^{-1}. The detection wavelength was 265 nm.

2.6. Validation of the method

2.6.1. Linearity and range

Drug solutions were prepared in the concentration range of 25–500 μg ml^{-1}. The solutions were injected in triplicate into the HPLC column using water–methanol (90:10) as the mobile phase and keeping the injection volume constant (10 μl).

2.6.2. Precision

Six injections, of three different concentrations (25, 250 and 500 μg ml^{-1}), were given on the same day and the values of relative standard deviation were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision. Intermediate precision was established through separation studies on another chromatographic system by a different analyst.

2.6.3. Accuracy

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.

2.6.4. Specificity

Specificity was established by determination of purity of the drug peak using a PDA detector. Also, the resolution factor of the drug peak was determined with respect to the nearest resolving peaks.

2.7. Analysis of stressed marketed capsules

Stavudine capsules were purchased from market and subjected to accelerated test condition of 40 °C/75% RH for 2 months. The product was also subjected to thermal stress at 50 °C for the same period of time.

For analysis, capsules were emptied into 100 ml volumetric flask. The contents were sonicated for 30 min in small quantity of methanol and volume was made to 100 ml. Five hundred microliters of this solution was diluted with mobile phase to yield drug concentration of 100 μg ml^{-1}. The solution was subjected to HPLC analysis after filtration through 0.22 μm nylon filter.

3. Results and discussion

3.1. Development and optimisation of the stability-indicating method

Initial method development was done on pure drug. A trial was made using water–methanol (20:80) as the mobile phase. With this mobile system, the drug resolved around 4.5 min and shape of the peak was also good. But considering that polar degradation products could interfere at such low retention time, methanol concentration was reduced to 10%, as a result of which the drug resolved at 12.5 min. This method was then used to analyse individual stressed samples. There was satisfactory resolution. Hence the method was applied to a mixture of stressed reaction solutions, particularly those in which sufficient decomposition was observed. The resultant chromatogram is shown in Fig. 2. It indicates that the method was successful in separation of drug and all chromophoric degradation products. Therefore, it was directly subjected to validation studies.

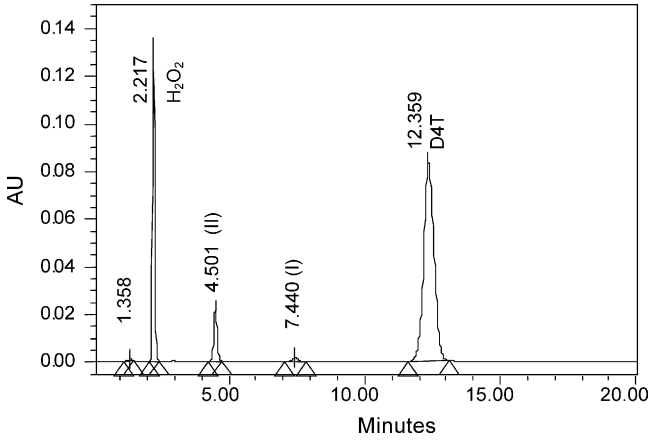

Fig. 2. Chromatogram showing separation of stavudine and degradation products in a mixture of reaction samples using water–methanol (90:10) as the mobile phase on a C-18 column. (I) Photolytic product in solid state; (II) oxidative, hydrolytic and photolytic degradation product. H_2O_2: hydrogen peroxide.

3.2. Validation of the developed stability-indicating method

With water–methanol (90:10) solvent system, the response for the drug was strictly linear in the concentration range between 25 and 500 μg ml^{-1}. The mean (±R.S.D.) values of slope and correlation coefficient were 24256 (±0.679) and 0.9994 (±0.0265), respectively. Intra- and inter-day precision data are given in Table 1. The R.S.D. values in the two cases were <0.21 and <1%, respectively which confirmed

Table 1
Reproducibility and precision data evaluated through intra-day and inter-day studies

Actual concentration (μg ml^{-1})	Measured concentration ± S.D. (μg ml^{-1}); R.S.D. (%)	
	Intra-day (n = 6)	Inter-day (n = 3)
25	24.712 ± 0.050; 0.202	24.479 ± 0.234; 0. 954
250	249.986 ± 0.287; 0.115	247.164 ± 2.356; 0.953
500	513.735 ± 1.000; 0.195	509.900 ± 4.196; 0.823

Table 2
Recovery studies ($n = 3$)

Actual concentration ($\mu g\,ml^{-1}$)	Calculated concentration ± S.D. ($\mu g\,ml^{-1}$); R.S.D. (%)	Recovery (%)
25	24.937 ± 0.250; 1.003	99.7
250	251.454 ± 1.850; 0.736	100.6
500	507.754 ± 4.061; 0.800	101.5

that the method was sufficiently precise. During intermediate precision testing, similar separation behaviour was observed when the method was run on another instrument by a different analyst. As shown from the data in Table 2, excellent recoveries were made at different fortified concentrations, despite the fact that the drug was added to a mixture containing degradation products. The PDA studies indicated that the method was even sufficiently specific. Fig. 3 shows the purity plot for the drug. The purity angle value was less than the threshold angle, indicating that the drug peak was pure by nature. The resolution factors were also calculated and it was >7 for the drug peak relative to nearest resolving peak.

3.3. Degradation behaviour

The following degradation behaviour of drug was observed during the above-mentioned HPLC studies:

3.3.1. Acidic condition

The drug was found to be highly labile to hydrolysis. On heating the drug solution in 0.1 M HCl at 80 °C for 30 min, 78% degradation was seen with simultaneous rise in degradation product at R_T 4.5 min. Almost complete degradation of the drug was observed in 1 h.

3.3.2. Neutral (water) condition

Upon heating the drug in water for 1 h, 73% fall in the original drug peak area was observed. At the end of 3 h, almost complete degradation was observed, with the corresponding rise in degradation peak at R_T 4.5 min.

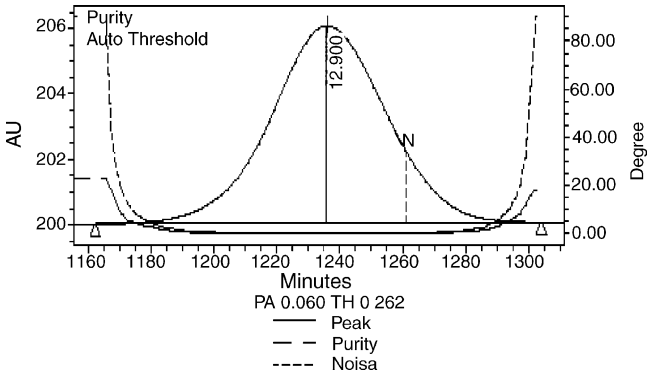

Fig. 3. Purity plot for stavudine in a mixture of stressed samples.

3.3.3. Degradation in alkali

Stavudine underwent alkali hydrolysis, but the rate of hydrolysis was slower as compared to that in acid or water. It took 60 h for the drug to decompose by 50%.

3.3.4. Oxidative conditions

The drug showed lability to hydrogen peroxide at room temperature. It decomposed to an extent of 16% in 30% H_2O_2 in 24 h. The degradation increased to 30% in 48 h. The degradation product had R_T 4.5 min, same as the one formed under hydrolytic decomposition. An additional small peak formed at 7.4 min, when the duration of exposure was increased to 72 h.

3.3.5. Photolytic conditions

The samples in 0.01 M HCl and water showed the same extent of degradation whether kept in light or dark. On the other hand, drug solution in 0.1 M NaOH showed no degradation in light as well as in the dark, indicating that light had no effect on the drug.

3.3.6. Solid-state study

Studies on solid drug showed that it was stable to the effect of temperature, and combined effect of temperature and humidity. No decomposition of drug was observed on subjecting it either to dry heat at 50 °C or 40 °C/75% RH for 3 months. Also, solid drug exposed to the combined conditions of light, temperature and humidity showed only 3% decomposition in 2 months.

3.4. Identification of major degradation product

The major degradation product was characterised to be thymine, based on spiking using a standard compound, and also through isolation and characterisation. Spectral data for the isolated compound is given below.

IR ($CHCl_3$) (cm^{-1}): 3355 (NH), 2924 (CH), 1714 (CO) and 1600 (NH)
^1H NMR (MeOD): δ 1.75 (3H, s) and 7.14 (1H, s)
MS (CI): 126 (M^+)

The data matched with pure thymine.

3.5. Applicability of the developed method to stressed marketed capsule formulation

Fig. 4 shows the chromatogram for control sample (a) and those for stavudine capsules subjected to accelerated conditions of temperature/humidity (40 °C/75% RH) and thermal heat for 2 months. The chromatograms, (b) and (c), show that drug degraded to some extent in the formulation environment, with thymine being the major degradation product.

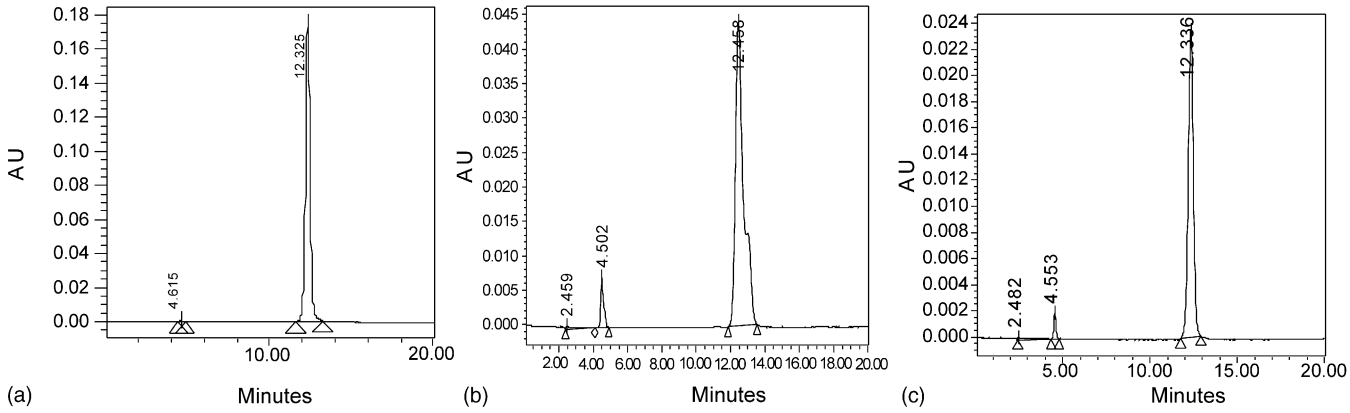

Fig. 4. Chromatographic separation of stavudine capsules subjected to thermal stress and accelerated conditions. (a) Control capsules; (b) capsules subjected to dry heat at 50 °C; and (c) capsules subjected to 40 °C/75% RH.

4. Conclusions

This study is a typical example of development of a stability-indicating assay, established following the recommendations of ICH guidelines. The developed method proved to be simple, accurate, precise and specific. The major degradation product, thymine, was well separated from the drug peak and same was the case even when the method was applied to marketed formulation subjected to thermal stress and accelerated stability testing. No revalidation was necessary for extending the method to the commercial product.

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