The ICH Guidance in Practice: Stress Degradation Studies on Stavudine and Development of a Validated Specific Stability-Indicating HPTLC Assay Method

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Abstract

A sensitive, selective, precise, and stability-indicating high-performance thin-layer chromatographic (HPTLC) method for the analysis of stavudine both as a bulk drug and in formulations is developed and validated. The solvent system consisted of toluene–methanol–chloroform–acetone (7.0:3.0:1.0:1.0, v/v/v/v). Densitometric analysis of stavudine is carried out in the absorbance mode at 270 nm. This system is found to give compact spots for stavudine (retention factor value of 0.45 ± 0.05) following development of chromatoplates with the mobile phase. Stavudine is subjected to acid and alkali hydrolysis, oxidation, dry-heat and wet-heat treatment, and photo and UV degradation. The drug undergoes degradation under acidic and basic conditions, oxidation, and wet-heat degradation. Linearity is found to be in the range of 30–1000 ng/spot with a significantly high value of correlation coefficient. The linear regression analysis data for the calibration plots show a good linear relationship with r^2 = 0.9997 ± 0.05 in the working concentration range of 300 to 1000 ng/spot. The mean value of slope and intercept are 0.10 ± 0.06 and 22.12 ± 1.08, respectively. The method is validated for precision, robustness, and recovery. The limits of detection and quantitation are 10 and 30 ng/spot, respectively. The proposed HPTLC method is utilized to investigate the kinetics of the acid degradation process. Arrhenius plot is constructed and activation energy is calculated.

Introduction

Stavudine (Figure 1) is chemically 2',3'-didehydro-3'deoxythymidine, which is a thymidine nucleoside with in vitro and in vivo inhibitory activity against the reverse transcriptase of human immunodeficiency virus (HIV) (1–6). It is active at concentrations that are generally 100-fold below those which are cytotoxic. Antiretroviral treatment of infected women during gestation and delivery is an important factor for reducing the risk of HIV transmission to the infant (7,8). Therefore, the drug is useful for reducing the risk of maternal–infant HIV transmission (9–11).

Several methodologies for the individual determination of stavudine in biological fluids have been previously reported in the literature (12–21). These assays utilized a variety of techniques including radioimmunoassay (17), high-performance liquid chromatography (HPLC) using reduced sample volume (19), liquid chromatography–tandem mass spectrometry (LC–MS–MS) (20), and sensitive cartridge-radio immunoassay method (21). Simultaneous determination of stavudine along with other antiviral agents in serum using HPLC has been reported (22). Moore et al. (23) have described a sensitive LC–MS–MS method for the simultaneous measurement of the intracellular nucleoside 5'-triphosphate anabolites of zidovudine, lamivudine, and stavudine in peripheral blood mononuclear cells.

To our knowledge, no article related to the stability-indicating high-performance thin-layer chromatographic (HPTLC) determination of stavudine in pharmaceutical-dosage forms has ever been mentioned in literature. The International Conference on Harmonization (ICH) guideline entitled *Stability Testing of New Drug Substances and Products* requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance (24). Suscepti-

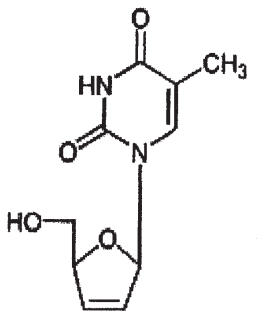

Figure 1. Chemical structure of stavudine.

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bility to oxidation is one of the required tests. Also, hydrolytic and photolytic stability are required. An ideal stability-indicating method is one that quantitates the drug, per se, and also resolves its degradation products. TLC, which is one of the oldest chromatographic methods, is commonly used in medical-biochemical, food, and environmental-pollutant analysis. Because of recent progress in plate technology and instrumentation, modern TLC is comparable in terms of accuracy, precision, and sensitivity to other chromatographic techniques and can be performed in full compliance with good laboratory practice. K. Ferenczi-Fodor et al. (25–27) explained basic acceptance criteria for the evaluation of validation experiments based on practical experience for planar chromatographic procedures, which may be used at different levels in qualitative identity testing, assays, semiquantitative limit tests, or quantitative determination of impurities. The parameters for robustness testing of given procedures and quality assurance of quantitative planar chromatographic testing have been described as per ICH guidelines. The European Pharmacopoeia (28,29) prescribes instrumental TLC as an official method for quantitative analysis. According to the European Pharmacopoeia (30), the profile of the impurities has been defined in relation to the sources of drug identified. The impurities detected by HPTLC are limited to 0.1%. The limits for these impurities have been fixed at the minimum level permitted by the analytical method in accordance with the requirements laid down in system conformity.

In recent years, there seems to be a resurgence of interest in modern TLC instrumentation, starting from the application of samples onto the plate to their elution and qualitative or quantitative analysis. This has opened up new avenues for the rapid and reliable measurements of various analytes, especially for samples that normally require cumbersome clean-up procedures. Complex samples can be screened on HPTLC plates using modern TLC scanners with relatively high resolution and sensitivity (31). Today HPTLC is becoming a routine analytical technique because of its advantages of low operating cost, high sample throughput, simplicity, speed, and need for minimal sample clean-up (32,33). The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase—unlike HPLC—thus lowering analysis time and cost per analysis. The main application of TLC in the pharmaceutical industry is intermediate quality control during the development and production of pharmaceutically active substances and testing of optically pure substances (34). Although TLC is mainly used as a drug screening and confirmation tool (35), quantitative pharmaceutical analysis by TLC has recently attracted considerable interest because of improved technologies with HPTLC. In recent years, the HPTLC technique has been improved to incorporate the following features: HPTLC-grade stationary phase, automated sample application devices, controlled development environment, automated developing chamber, computer-controlled densitometry and quantitation, and fully validated procedures. These features result in methods that are not only convenient, fast, robust, and cost efficient, but also reproducible, accurate, and reliable (36). Further optimization of all aspects of the separation process in TLC are HPTLC plates for quantitative determination because these new layers require smaller sample sizes and shorter development distances to reveal their separation potential and to provide faster separation and better resolution (37). Applications of HPTLC to the quantitative analysis of drug substances in biological and formulation matrices have been reported (33).

The aim of the present work is to develop an accurate, specific, repeatable, and stability-indicating HPTLC method for the determination of stavudine in the presence of its degradation products and related impurities for the assessment of purity of bulk drug and stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines (38,39) and its updated international convention (40). Acid-induced degradation kinetics were investigated by quantitation of the drug by a validated stability-indicating HPTLC method.

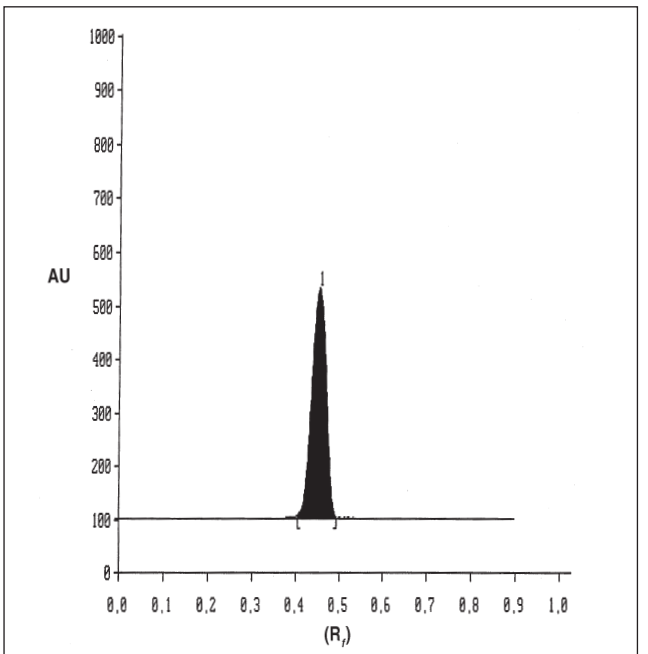

Figure 2. Densitogram of standard stavudine (1000 ng/spot). Peak 1 (R_f = 0.45 ± 0.05), mobile phase toluene–methanol–chloroform–acetone (7.0:3.0:1.0:1.0, v/v/v/v).

Table I. Linear Regression Data for the Calibration Curves*

Linearity Range (ng/spot)	*r* ± SD	Slope ± SD	Confidence limit of slope†	Intercept ± SD	Confidence of intercept†
300–1000	0.9997 ± 0.05	0.10 ± 0.06	0.52–0.148	22.12 ± 1.08	21.26–22.98
30–100	0.9988 ± 0.64	0.22 ± 0.54	0.21–0.65	24.41 ± 1.85	22.93–25.89

* $n = 6$.
† 95% confidence limit.

Experimental

Materials

Pharmaceutical-grade stavudine was kindly supplied as a gift sample (batch no: 2113-020801, vk 300696) by Cipla Ltd (Mumbai, India) and was used without further purification and certified to contain 99.75% (w/w) on dried basis. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, (Mumbai, India).

TLC

The samples were spotted in the form of bands of 6-mm width with a Camag 100-µL sample (Hamilton, Bonaduz, Switzerland) syringe on TLC silica gel 60 F 254 on aluminium, 10- × 20-cm (cut from a 20- × 20-cm) 200-µm thickness column (catalog no. 1.05554.0001, E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai, India) using a Camag Linomat IV (Muttenz, Switzerland). The plates were prewashed by methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.1 µL/s was employed and the space between two bands was 5 mm. The slit dimension was held at 5 × 0.45 mm, and 10-mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm with K 320 cut-off filter, each track was scanned thrice, and baseline correction was used. The mobile phase consisted of toluene–methanol–chloroform–acetone (7.0:3.0:1.0:1.0, v/v/v/v) and 15 mL of mobile phase was used per chromatographic run. Linear ascending development was carried out in a 20- × 10-cm twin trough glass chamber (Camag) (dimensions: length × width × height = 12 × 4.7 × 12.5 cm). It was saturated (lined on the two bigger sides with filter paper that had been soaked thoroughly with the mobile phase) and the chromatoplate development was carried out in the dark with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25°C ± 2°C) at a relative humidity of 60% ± 5%. The length of the chromatographic run was 9 cm and approximately 30 min. Subsequent to the development, the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Densitometric scanning was performed on a Camag TLC scanner III in the reflectance-absorbance mode at 270 nm for all measurements and operated by CATS software (V 3.15, Camag). The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

Table II. Robustness Testing*

Parameter	SD† of peak area	%RSD†
Mobile phase composition	1.89	1.48
Amount of mobile phase	1.74	1.33
Temperature	1.09	0.96
Relative humidity	1.95	1.32
Plate pretreatment	0.65	0.41
Time from spotting to chromatography	0.46	0.38
Time from chromatography to scanning	0.37	0.29

* $n = 6$.
† Average of three concentrations 400, 600, and 800 ng/spot.

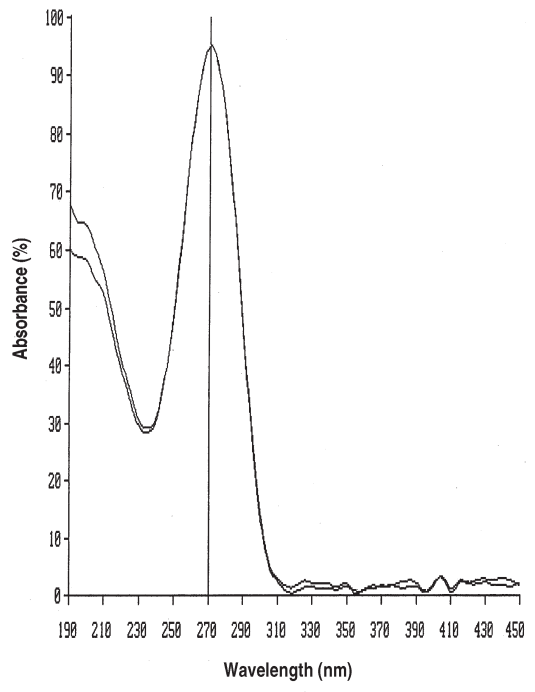

Figure 3. In situ spectrum of standard and sample stavudine measured from 190 to 450 nm ($r = 0.9998$).

Calibration curves of stavudine

A stock solution of stavudine was prepared in methanol at (100 µg/mL). One milliliter of stock solution was quantitatively transferred into a 100-mL volumetric flask and made to

Table III. Summary of Validation Parameters

Parameter	Data
Linearity range	300–800 ng/spot
Correlation coefficient	0.9997 ± 0.05
Limit of detection	10 ng/spot
Limit of quantitation	30 ng/spot
Recovery ($n = 6$)	98.89 ± 0.68
Precision (%RSD)	
Repeatability of application ($n = 7$)	0.75
Repeatability of measurement ($n = 7$)	0.33
Interday ($n = 6$)	1.45
Intraday ($n = 6$)	1.31
Robustness	Robust
Specificity	Specific

volume with methanol. Standard solutions were prepared by dilution of the diluted stock solution with methanol to give solutions containing stavudine in the concentration range of 0.03–1.0 µg/mL. One microliter from each standard solution was spotted on the TLC plate to obtain a final concentration range of 30–1000 ng/spot. Each concentration was spotted six times on the TLC plate.

Method validation

Precision

Precision of the method was determined with the product. An amount of the product powder equivalent to 100% of the label claim of stavudine was accurately weighed and assayed. System repeatability was determined by six replicate applications and the sample solution was measured six times at the analytical concentration of 800 ng/spot. The repeatability of sample application and measurement of the peak area for the active compounds were expressed in terms of relative standard deviation (%RSD) and standard error (SE) and found to be less than 2%. Method repeatability was obtained from the RSD value by repeating the assay six times on the same day for intraday precision. Intermediate precision was assessed by the assay of two, six sample sets on different days (interday precision). The intra- and interday variation for determination of stavudine was carried out at three different concentration levels: 400, 600, and 800 ng/spot.

Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition of toluene–methanol–chloroform–acetone (6.5:3.5:1.0:1.0, v/v/v/v), (6.5:3.0:1.5:1.0, v/v/v/v), (6.5:3.0:1.0:1.5, v/v/v/v), (7.5:2.5:1.0:1.0, v/v/v), (7.0:2.5:1.5:1.0, v/v/v/v), (7.0:2.5:1.0:1.5, v/v/v/v), (7.5:3.0:0.5:1.0, v/v/v/v), (7.0:3.5:0.5:1.0, v/v/v/v), (7.0:3.0:0.5:1.5, v/v/v/v), (7.5:3.0:1.0:0.5, v/v/v/v), (7.0:3.5:1.0:0.5, v/v/v/v), and (7.0:3.0:1.5:0.5, v/v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature, and relative humidity was varied in the range of ± 5%. The plates were prewashed by methanol and activated at 60°C ± 5°C for 2, 5, and 7 min, respectively, prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40, and 60 min. Robustness of the method was carried out at three different concentration levels: 400, 600, and 800 ng/spot.

Limit of detection and quantitation

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated

Table IV. Applicability of the HPTLC Method for the Analysis of the Pharmaceutical Formulations*

Drug	Label claim	Drug content (%)	RSD (%)	SE	*t*	*F*	*t*†	*F*†
Stavudine	40 mg	98.67 ± 1.45	1.24	0.98	1.35	3.56	2.44	9.27

* $n = 6$
† Theoretical values for *t* and *F*.

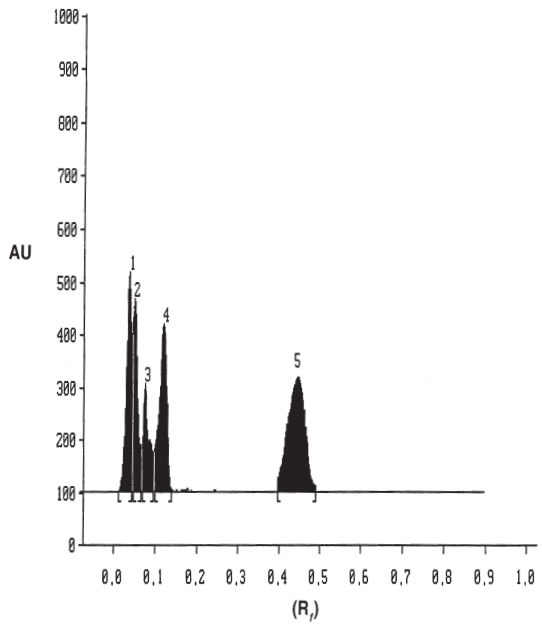

Figure 4. Densitogram of acid (1N HCl, reflux for 2.0 h, temperature 80°C) treated stavudine (1000 ng/spot): peak 1, degraded (R_f = 0.02); peak 2, degraded (R_f = 0.05); peak 3, degraded (R_f = 0.07); peak 4, degraded (R_f = 0.10); and peak 5, stavudine (R_f = 0.45).

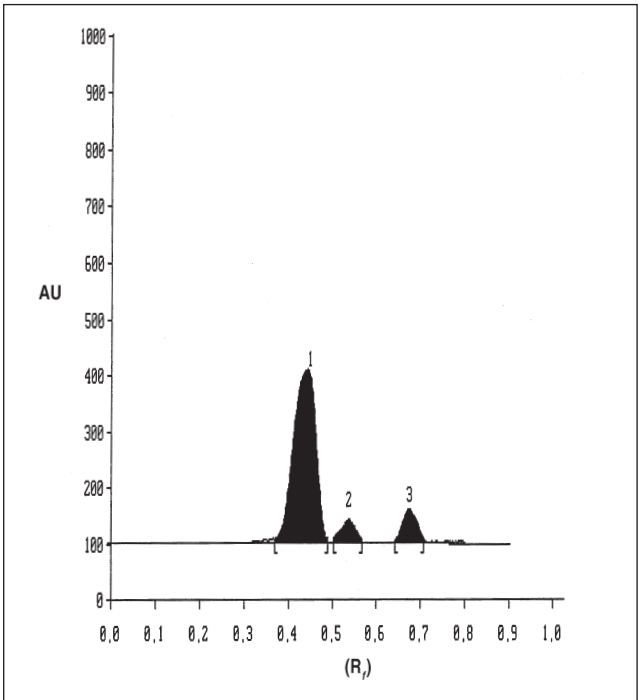

Figure 5. Densitogram of hydrogen peroxide (6.0% w/v, reflux for 2.0 h, temp 80°C) treated stavudine (1000 ng/spot): peak 1, stavudine (R_f = 0.45); peak 2, degraded (R_f = 0.53); and peak 3, degraded (R_f = 0.68).

as an exact value. The quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOQ is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities or degradation products (or both). In order to estimate the LOD and LOQ, blank methanol was spotted six times following the same method as explained in the TLC section. The signal-to-noise ratio (s/n) was determined.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The spot for stavudine in sample was confirmed by comparing the retention factor (R_f) and spectra of the spot with that of standard. The peak purity of stavudine was assessed by comparing the spectra at three different levels: peak start, peak apex, and peak end positions of the spot.

Recovery studies

Recovery studies were carried out by applying the method to a drug sample to which a known amount of stavudine (corresponding to 80%, 100%, and 120% of label claim) had been added. Six determinations were performed at each level of the amount.

Analysis of the marketed formulation

To determine the content of stavudine in capsules (label claim: 40 mg/capsule), the contents of 20 capsules were weighed, their mean weight was determined, and they were finely powdered. An equivalent weight of the capsule content was transferred into a 100-mL volumetric flask containing 50 mL methanol, sonicated for 30 min, and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000

Table V. Degradation of Stavudine

Condition	Time (h)	Recovery (%)	R_f value of degradation products
Acid 1N HCl, ref* (80°C)	1.0	74.4	0.02, 0.05, 0.07, 0.10
Acid 1N HCl, ref* (80°C)	2.0	36.9	0.02, 0.05, 0.07, 0.10
Base 5N NaOH, ref	1.0	75.6	–
Base 5N NaOH, ref	2.0	67.5	–
Phosphate buffer (pH 8)	1.0	80.3	–
Phosphate buffer (pH 8)	2.0	62.8	–
H_2O_2 6% w/v, ref	1.0	86.9	0.53, 0.68
H_2O_2 6% w/v, ref	2.0	82.5	0.53, 0.68
Dry heat (80°C)	6.0	100	–
Wet heat, ref (80°C)	2.0	38.2	–
Day light (25°C)	48.0	100	–
UV light	48.0	100	–

* Refluxed.

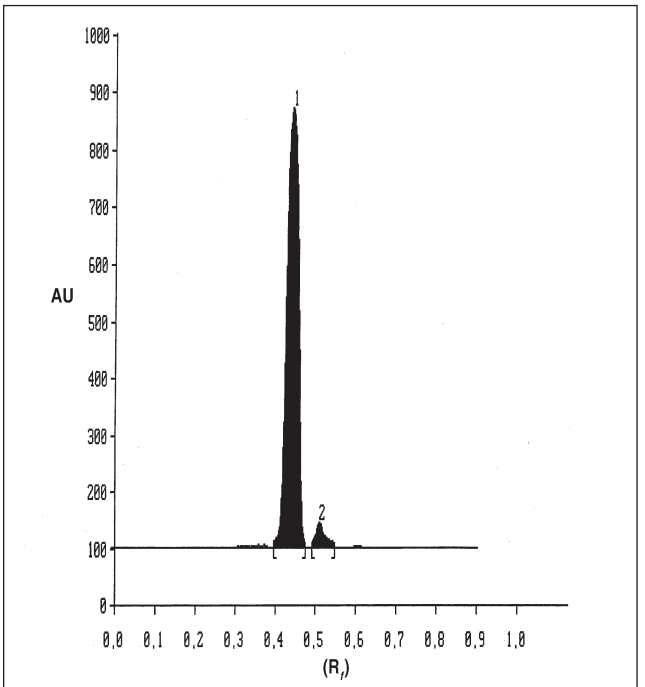

Figure 6. Densitogram of stavudine and its impurity: peak 1, stavudine (R_f = 0.45); and peak 2, impurity (R_f = 0.53).

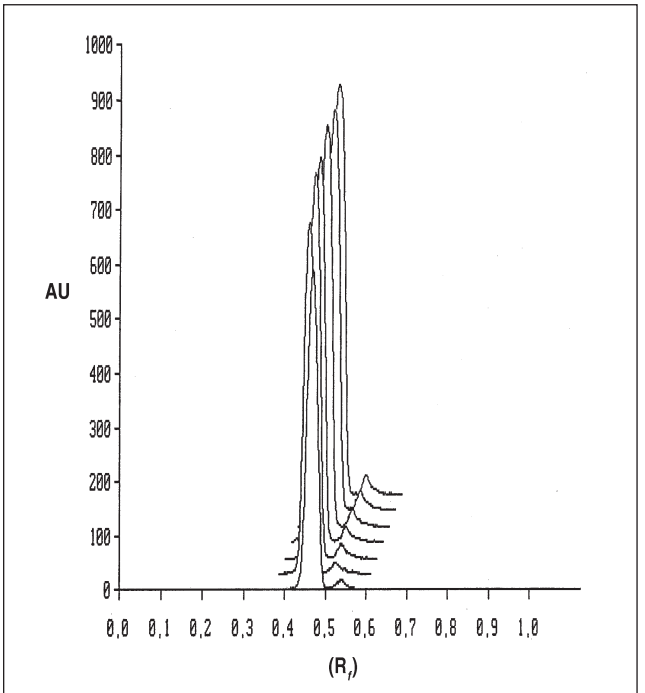

Figure 7. Three-dimensional densitogram of multiple runs of increasing concentration of stavudine applied on different tracks of TLC plate, showing the increase in area of its related impurity.

rpm for 5 min, and the supernatant was analyzed for drug content. Two microliters of the filtered solution (800 ng/spot) was applied on the TLC plate followed by the development and scanning as described in the TLC section. The analysis was repeated in triplicate. The possibility of excipient interference in the analysis was studied.

Forced degradation of stavudine

A stock solution containing 50 mg stavudine in 50 mL methanol was prepared. This solution was used for forced degradation.

Preparation of acid- and base-induced degradation product

For acid and alkaline degradation studies, to 10 mL of methanolic stock solution, 10 mL each of 1N HCl and 1N NaOH were added separately. These mixtures were refluxed for 2.0 h at 80°C. To study the degradation of drug in phosphate buffer pH 8.0, 10 mL of buffer solution was added to 10 mL of methanolic stock solution. It was refluxed at 80°C for 2.0 h. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. Two microliters of the resultant solutions (1000 ng/spot) were applied on the TLC plate, and the chromatograms were run as described previously.

Preparation of hydrogen peroxid-induced degradation product

To 10 mL of methanolic stock solution, 10 mL of hydrogen peroxide 6.0% w/v was added. The solution was heated in a boiling water bath for 10 min to completely remove the excess of hydrogen peroxide and then refluxed for 2.0 h at 80°C. Two microliters of the resultant solution (1000 ng/spot) was applied on the TLC plate and the chromatograms were run.

Dry- and wet-heat degradation product

The powdered drug was stored in an oven at 80°C for 6.0 h to study dry-heat degradation, and the stock solution was refluxed at 80°C for 2.0 h in a water bath for wet-heat degradation. One microliter (1000 ng/spot) of both solutions was applied on TLC plates.

Photochemical and UV degradation product

The photochemical and UV stability of the drug was also studied by exposing the stock solution to direct sunlight and UV radiation for 48 h, respectively. One microliter of both solutions (1000 ng/spot) was applied on a TLC plate and chromatograms were run. In all degradation studies, the average peak area of stavudine after application of seven replicates was obtained.

Detection of the related impurities

The related impurities were determined by spotting higher concentrations of the drug so as to detect and quantitate them. Stavudine (600 mg) was dissolved in 100 mL of methanol, and this solution was termed as sample solution (6 mg/mL). One milliliter of the sample solution was diluted to 100 mL with methanol, and this solution was termed as standard solution (0.06 mg/mL). One microliter of both the standard (60 ng/spot) and the sample solution (6,000 ng/spot) were applied on the TLC plate and the chromatograms were run.

Study of acid-induced degradation kinetics

Accurately weighed drug (100 mg) was dissolved in 100 mL methanol. Twenty milliliters of this standard solution was transferred into a 100-mL double-neck round-bottom flask. To this, 20 mL of 1N HCl was added to get final concentration of 500 µg/mL and refluxed at different temperatures (40°C, 50°C, 60°C, 70°C, and 80°C). At specified time intervals, the contents of the flask (100 µL) were quantitatively transferred to 10-mL volumetric flasks with the help of a microsyringe. Then, 2 µL were spotted to achieve the final concentration of 1000 ng/spot and estimated by the HPTLC method by one-point standardization using external standard. The experiment was carried out in triplicate. The concentration of the remaining drug was calculated for each temperature and time interval. Data was further processed, and degradation kinetics constants were calculated.

Results and Discussion

Development of the optimum mobile phase

The TLC procedure was optimized for the purpose of developing a stability-indicating assay method. Both the pure drug

Table VI. Related Impurities

Concentration of drug (ng/spot)	R_f value	Area
60	0.45	795
6000	0.45	78510
Related impurity		
6000	0.53	425

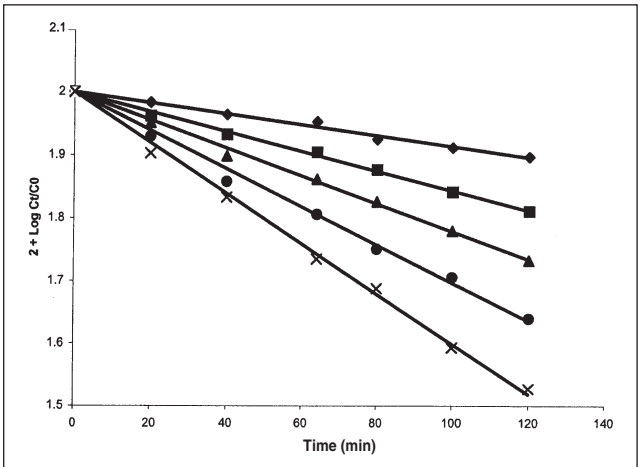

Figure 8. Pseudo first-order plots for the degradation of stavudine with 1N HCl at various temperatures using the HPTLC method. Key: 80°C (×), 70°C (●), 60°C (▲), 50°C (■), 40°C (◆), concentration at time *t* (C_t); and concentration at time zero (C_0).

and the degraded products were spotted on the TLC plates and run in different solvent systems. Initially, toluene–methanol (5.0:5.0, v/v) was tried in varying ratios. The spots after development were diffused and distorted. Then, 1 mL of chloroform was included to the previously mentioned mobile phase. After development, the spots were compact but peak symmetry was not good. Then 1.0 mL of acetone was added to obtain gaussian peak symmetry. Finally, the optimized mobile phase toluene–methanol–chloroform–acetone (7.0:3.0:1.0:1.0, v/v/v/v) gave good resolution with an R_f value of 0.45 for stavudine having typical peak nature (sharp and symmetrical). It was observed that prewashing of TLC plates with methanol (followed by drying and activation at 110°C for 5 min) and presaturation of the TLC chamber with the mobile phase for 30 min at room temperature ensured well-defined spots of stavudine with improved spot characteristics, good reproducibility, and peak shape (Figure 2).

Calibration curves

The calibration graph was found to be linear, that is, adherence of the system to Kubelka Munk's theory, which relies on the idea that light is traveling in all directions simultaneously within the precoated TLC plate. This is approximated as a flux of light traveling upwards and a flux traveling downwards at any depth in the plate. When this flux passes through a thin layer of material, some of it passes through, some of it is scattered backwards, and some of it is absorbed. Linearity was evaluated by determining six standard working solutions containing 0.03–0.1 µg/mL and 0.3–0.8 µg/mL of stavudine in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data (Table I) shows a good linear relationship over the lower concentration range of 30–100 ng/spot as well as over a higher concentration range of 300–1000 ng/spot. The higher concentration was selected as working range because of two reasons: the high value of the correlation coefficient (r^2 = 0.9997) and because it allows the main analyte to remain present after degradation, which allows a better study of its chromatography characteristics in the presence of degradation products. The linearity of calibration graphs and adherence of the system to Kubelka Munk theory was validated by a high value of correlation coefficient, and the standard deviation (SD) for the intercept value was less than 2%. No significant difference was observed in the slopes of standard curves (analysis of variance: $p < 0.05$).

Table VII. Degradation Rate Constant (K_{obs}), Half-Life ($t_{1/2}$), and t_{90} for Stavudine in presence of 1N HCl

Temperature (°C)	K_{obs} (h^{-1})	$t_{1/2}$ (h)	t_{90} (h)
40	0.0021	5.50	0.83
50	0.0037	3.12	0.47
60	0.0051	2.26	0.34
70	0.0069	1.67	0.25
80	0.0092	1.26	0.19

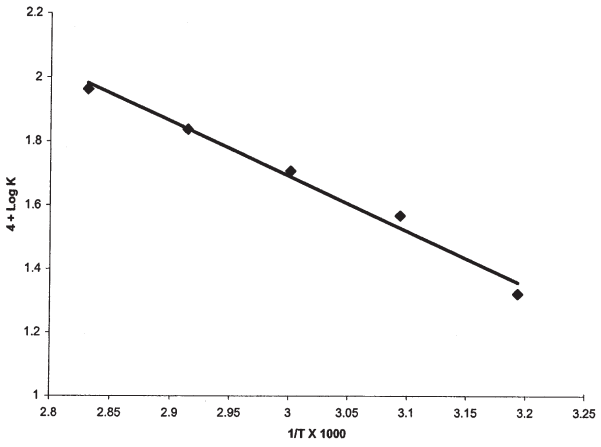

Figure 9. Arrhenius plot for the degradation of stavudine in presence of 1N HCl and its extrapolation for predicting the degradation of stavudine at room temperature (25°C).

Validation of the method

Precision

The repeatability of sample application and measurement of peak area at 800 ng/spot were expressed in terms of %RSD and found to be 0.75 and 0.33, respectively. The mean %RSD (and SE) values were found to be 1.31 ± 1.61 (0.62) and 1.45 ± 1.75 (0.67), respectively, for intraday and interday variation of stavudine at three different concentration levels: 400, 600, and 800 ng/spot.

Robustness of the method

The SD of peak areas was calculated for each parameter and %RSD was found to be less than 2%. The low values of %RSD as shown in Table II indicated the robustness of the method.

LOD and LOQ

The LOD and LOQ were separately determined at s/n of 3 and 10. LOD and LOQ were experimentally verified by diluting known concentrations of stavudine until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations. The s/n 3:1 and 10:1 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 10 and 30 ng/spot, respectively.

Specificity

The peak purity of stavudine was assessed by comparing the spectra at peak start, peak apex, and peak-end positions of the spot [i.e., r (start, middle) = 0.9995 and r (middle, end) = 0.9992]. Good correlation (r = 0.9998) was also obtained between standard and sample spectra of stavudine (Figure 3).

Recovery studies

The proposed method, when used for the extraction and subsequent estimation of stavudine from a pharmaceutical-dosage form (after spiking with additional drug) afforded a recovery of 98–102%. Mean recovery for stavudine from the marketed formulation was found to be 98.89% with %RSD

and SE values of 1.69 and 1.27, respectively. The data of summary of validation parameters are listed in Table III.

Stability in sample solution

Solutions of two different concentrations (400 and 800 ng/spot) were prepared from sample solution and stored at room temperature for 0.5, 1.0, 2.0, 4.0, and 24 h, respectively. They were then applied on the same TLC plate. After development, the chromatogram was evaluated for additional spots (if any) and %RSD and SE was found to be 1.74 and 1.41, respectively. There was no indication of compound instability in the sample solution.

Spot stability

The time that the sample is allowed to stand on the solvent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation (41). Two-dimensional chromatography using the same solvent system was used to find out if any decomposition is occurring during spotting and development. In case decomposition occurs during development, peaks of decomposition products shall be obtained for the analyte both in the first and second direction of the run. No decomposition was observed during spotting and development.

Analysis of the marketed formulation

A single spot at R_f of 0.45 was observed in the chromatogram of the drug samples extracted from capsules. There was no interference from the excipients commonly present in the capsules. The drug content was found to be 98.67 ± 1.45 with a %RSD of 1.24. It may therefore be inferred that degradation of stavudine had not occurred in the marketed formulations that were analyzed by this method, as shown in Table IV. The low %RSD value indicated the suitability of this method for routine analysis of stavudine in pharmaceutical-dosage form.

Stability-indicating property

Acid- and base-induced degradation product

The chromatogram of the acid degraded sample for stavudine showed four peaks at R_f value of 0.02, 0.05, 0.07, and 0.10, respectively (Figure 4). The areas of the degraded peaks were found to be less than the area of the standard drug concentration (1000 ng/spot), indicating that stavudine undergoes degradation under acidic condition. The chromatogram of the base- and phosphate buffer-degraded sample showed no degraded peaks, though the typical drug peak nature was missing (reduction in height as well as in area and broader peak base) without a corresponding rise in a new peak, and peak dragging was observed. This indicates that the drug was hydrolyzed under basic conditions to nonchromophoric products.

Hydrogen peroxide-induced degradation product

The sample degraded with hydrogen peroxide (Figure 5) showed two additional peaks at R_f value of 0.58 and 0.68, respectively. The spots of degraded product were well resolved from the drug spot.

Dry- and wet-heat degradation product

The samples degraded under dry- and wet-heat conditions did not show an additional peak. But the drug peak under wet-heat condition was reduced in height as well as in area. The indication is that the drug is degraded in wet-heat conditions also to nonchromophoric products.

Photochemical and UV degradation product

The photo- and UV-degraded sample showed no additional peak when drug solution was left in daylight and in UV light for 48 h, respectively. This indicates that the drug is susceptible to acid–base hydrolysis, oxidation, and wet-heat degradation. The lower R_f values of acid-degraded products indicated that they were more polar than the analyte itself. The higher R_f value of hydrogen peroxide-induced degradation products indicated their lesser polarity than the standard. The results are listed in Table V.

Detection of the related impurities

The spot other than the principal spot (stavudine) from the sample solution was not as intense as the spot from the standard solution. The sample solution showed one additional spot at R_f of 0.53 (Figures 6 and 7). However, the area of the additional spots were found to be much less when compared with the standard solution, as indicated in Table VI. Figure 7 indicates the increase in area of related impurity with increasing concentration of standard solution applied on different tracks of the TLC plate during multiple runs, thereby showing the reproducibility and specificity of the established method. Interestingly, the R_f value of impurity exactly matches with the R_f values of first hydrogen peroxide-induced degradation product. Therefore, it might be possible that during processing, transaction, or storage the drug may have undergone little oxidative degradation.

Degradation kinetics

In acidic medium, a decrease in the concentration of drug with an increase of time was observed. The influence of temperature on the degradation process in acid medium is shown in Figure 8. At the selected temperatures (40°C, 50°C, 60°C, 70°C, and 80°C), the degradation process followed pseudo first-order kinetics. Apparent first order degradation rate constant and half-life were obtained from the slopes of the straight lines at each temperature (Table VII). Data obtained from first-order kinetics treatment was further subjected to fitting in Arrhenius equation:

$$\text{Log } K = \text{Log } A - E_a / 2.303\ RT \qquad \text{Eq. 1}$$

where K is rate constant, A is frequency factor, E_a is energy of activation (Kcal degree^{-1} mole^{-1}), R is gas constant (1.987 cal/degrees mole), and T is absolute temperature (°K). A plot of $(2 + \log K_{obs})$ values versus $(1/T \times 10^3)$ the Arrhenius plot was obtained (Figure 9), which was found to be linear in the temperature range 40–80°C. The activation energy was calculated to be 7.91×10^{-3} Kcal degree^{-1} mole^{-1} and the Arrhenius frequency factor to be 960.16. The method of accelerating testing of pharmaceutical products based on principles of chemical

kinetics was used to obtain a measure of the stability of the drug under said conditions (42,43). The degradation rate constant at room temperature ($K_{25}°$) is obtained by extrapolating the resulting equation in Arrhenius plot at 25°C (where 1000/T = 3.354) and was found to be 2.26×10^{-5} h^{-1} and calculated $t_{1/2}$ and t_{90} are 21 and 3 days, respectively.

Conclusion

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance (44,45). This powerful and adaptable technology now occupies a pivotal position ensuring the identity, purity, concentration conformity, and physicochemical stability studies of various pharmaceutical dosage forms. The HPTLC CAMAG device is now one of the cornerstones of our quality assurance system. This analytical tool represents an undeniable contribution to accreditation and certification procedures to which quality control organizations are now committed in order to improve the quality of analytical method development.

The developed HPTLC technique is precise, specific, and accurate. Statistical analysis proves that the method is repeatable and selective for the analysis of stavudine as a bulk drug and in pharmaceutical formulations without any interference from the excipients. It is one of the rare studies in which forced decomposition was carried out under all different suggested conditions. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities and also in stability studies.

The results showed the suitability of the proposed method for an acid-induced degradation kinetic study of stavudine. The degradation rate constant, half-life, and t_{90} of stavudine can be predicted. In acidic medium at room temperature, the time to obtain 90% and 50% potency of the drug is calculated to be 3 and 21 days, respectively. It may be extended for quantitative estimation of said drug in plasma and other biological fluids. The method, however, is not suggested to establish material balance between the extent of drug decomposed and formation of degradation products. As the method separates the drug from its acid and hydrogen peroxide-induced degradation products, it can be employed as a stability-indicating one.

The presented method is an affirmation of both the effectiveness and ecological quality of modern instrumental TLC. As a result of the timely combination of a traditional method and spectrometry, computer-aided technologies, and qualitative as well as quantitative modern planar chromatography are rapidly gaining acceptance throughout the laboratories. A new finding of this study is that the drug is considerably stable in almost all conditions because the rate of acid hydrolysis of drug at room temperature is quite low.

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