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A rapid stability indicating LC method for stavudine using RR-LC

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ABSTRACT

A simple, sensitive isocratic RR-LC method has been developed for the quantitative determination of Stavudine Related substances in bulk drug, used for the HIV Aids. The developed method is also applicable for the Assay determination. Efficient chromatographic separation was achieved on a C18 stationary phase with simple mobile phase combination delivered in a isocratic mode and quantification was carried out using ultraviolet detection at 265 nm at a flow rate of 0.5 mL min^{-1}. In the developed RRLC method the resolution between Stavudine and its two potential impurities was found to be greater than 4.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.9999 for Stavudine and it's all the two impurities. This method was capable to detect all two impurities of Stavudine at a level of 0.05 % with respect to test concentration of 0.5 mg mL^{-1} for a 20 µL injection volume. The inter day precision values for all four impurities and for Stavudine was found to be within 2.0 % RSD at its specification level. The method has shown good and consistent recoveries for Stavudine four impurities (93.9-103.9). The drug was subjected to stress conditions of exposure to acid hydrolysis, Oxidation and thermal degradation. Considerable degradation was found to occur in acid hydrolysis and Thermal stress conditions. The developed RR-LC method was validated with respect to linearity, accuracy, precision.

KEYWORDS

Column liquid chromatography;
Stavudine;
Forced degradation;
Validation.

INTRODUCTION

Stavudine : 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione (Figure 1) is a Anti retro viral product. The generic name of Stavudine is Zerit. Stavudine is a prescription medicine used in combination with other drugs to treat adults and children who are infected with HIV (the human immunodeficiency virus), the virus that causes AIDS. Stavudine belongs to a class of drugs called nucleoside reverse transcriptase inhibitors (NRTIs). By reducing the growth of HIV, Stavudine helps your body maintain its supply of CD4 cells, which are important for fighting HIV and other infections.

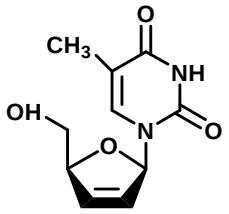
Indian pharmacopiea[1] has specifed method for determination of Impurities and Assay of Stavudine by reverse phase HPLC .The method is of isocratic and gradient elution and time consuming of 20 minutes. The proposed method is of 8 minutes which saves lot of

time and chemicals and is developed on Fast LC or RRLC. Analytical Time and money spent are important for Less value and high importance Antiretroviral drugs.

The present drug stability test guideline Q1A (R2) issued by International Conference on Harmonization (ICH)[2] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to separation of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability indicating and they should be fully validated.

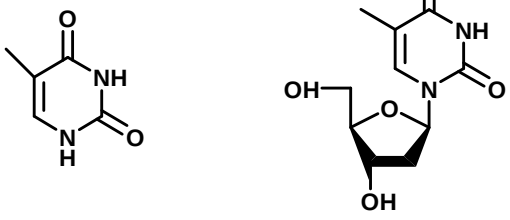
Accordingly, the aim of present study was to establish inherent stability of Stavudine through stress studies under a variety of ICH recommended test conditions and to develop a stability-indicating method.

Stavudine



1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione

Thymine **β-Thymidine**



5-methyluracil **deoxythymidine**

Figure 1 : Structures and labels of Stavudine and its impurities

EXPERIMENTAL

Chemicals

Samples of Stavudine were received from HETERO LABS Limited and its related impurities were received from Fluka. HPLC grade methanol and was purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system. All samples and impurities used in this study were of greater than 99.6% purity.

Equipment

The LC system, used for method development, forced degradation studies and method validation was Agilent 1200 RRLC. The output signal was monitored and processed using Chemistation software on Pentium computer (Digital equipment Co).

Chromatographic Conditions

The chromatographic column used was Symmetry C18 75x4.6 with 3.5 µm particles. The mobile phase contains a mixture of water and methanol in the ratio of 85:15 (*v/v*).

The flow rate of the mobile phase was 1.0 mL min^{-1}. The column temperature was maintained at 25 °C and the detection was monitored at a wavelength of 265 nm. The injection volume was 20 µL. mobile phase was used as diluent.

Preparation of Solutions

Preparation of Standard Solutions

A Stock solution of Stavudine (0.5 mg mL^{-1}) was prepared by dissolving appropriate amount in the diluent. Working solutions of 2.5 and 0.25 µg mL^{-1} were prepared from above stock solution for related substances determination and assay determination, respectively. A stock solution of impurities (mixture of Thymine and β-Thymidine) at a concentration of 0.05 mg mL^{-1} was also prepared in diluent.

Analytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity and system suitability.

Selectivity

Selectivity of the developed method was assessed by performing forced degradation studies. The terms selectivity and specificity are often used interchangeably. Selectivity is the ability of the method to measure the analyte response in the presence of its potential impurities. According to ICH[3] stress testing of the drug substance can help the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used. Photo stability testing should be

an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B[4]. The specificity of the developed LC method for Stavudine was determined in the presence of its impurities namely Thymine, β-Thymidine and degradation products. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), heat (80°C), acid hydrolysis (1N HCl). For heat and light studies, study period was 24 hrs where as for acid and oxidation it was 10 min. Peak purity of stressed samples of Stavudine was checked. The purity angle within the purity threshold limit obtained in all stressed samples demonstrates the analyte peak homogeneity. All stressed samples of Stavudine (heat (50°C), acid hydrolysis (1N HCl) and oxidation were analysed.

Analytical Method Validation

Precision

Precision was determined through repeatability (intra-day). The precision of the related substances method was checked by injecting six individual preparations of (500 μg mL^{-1}) Stavudine. The % RSD for percentage of each impurity was calculated.

Limit of quantification (LOQ)

The LOD and LOQ for Imp-1, Imp-2, Imp-3 and Imp-4were estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. LOQ for all the impurities is established at 0.05%.

The precision study was also carried out at the LOQ level by injecting six individual preparations of Thymine and Thymidine and calculated the % RSD for the areas of each impurity.

Linearity and Range

To establish linearity of the method, calibration solutions were prepared from stock solution at six concentration levels for chromatographic purity method-concentration levels ranging from LOQ to 150% (with respect to test concentration of 500 μg mL^{-1}, LOQ, 50, 80,100,120 and 150%) were prepared by diluting the impurity stock solution to the required concentrations of two impurities for Stavudine concentration levels ranging from LOQ to 150% (with respect to test concentration of 500 μg mL^{-1}, LOQ, 50, 80, 100,120 and 150) . Average peak area at each concentration level was subjected to linear regression analysis with the least square method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The residuals and sum of the residual squares were calculated from the corresponding predicted responses. The % y-intercept for related substances method was calculated. Analytical range of the method was established from the analysis of sensitivity curves. Upper and lower levels of range were also established.

Sensitivity

Sensitivity was determined by establishing Limit of quantification (LOQ) of Thymine, β-Thymidine and Stavudine estimated at a signal-to-noise ratio above 10 respectively, by injecting a series of dilute solutions with known concentration. The precision study was also carried out at the LOQ level by injecting six individual preparations of Thymine, β-Thymidine and Stavudine and calculated the % RSD for the areas of each impurity.

Accuracy

For determination of accuracy, recovery study was carried out by spiking analysis. A known amount of the impurity stock solutions were spiked to the previously analysed samples at LOQ,50,100 and 150% of the analyte concentration (500 μg mL^{-1}). The percentage of recoveries Thymine and β-Thymidine were calculated. Each concentration level was prepared for three times (TABLE 4).

TABLE 4 : Results of Accuracy study for Related substances

Added (μg/mL) (*n*= 3)	% Recovery of Thymine	% Recovery of β-Thymidine
0.25	103.9	93.9
2.5	99.3	101.8
5	99.7	99.6
7.5	100.2	100.1

***n* =3, Number of determinations**

Robustness

Robustness study was conducted by making small but deliberate changes in the optimized method parameters. Critical sources of variability in operating procedure such as percent organic strength was identified. By deliberate change in experimental conditions the resolution between Thymine and β-Thymidine was

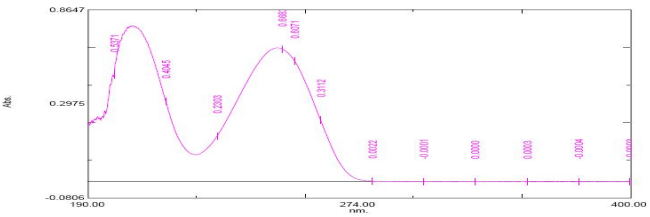
evaluated. The flow rate of the mobile phase was 1.0 mL min^{-1}.To study the effect of flow rate on the resolution, 0.2 units changed i.e 0.8 and 1.2 mL min^{-1}. In the above varied conditions, the components of the mobile phase were held constant.

RESULTS AND DISCUSSION

Method Development and Optimization

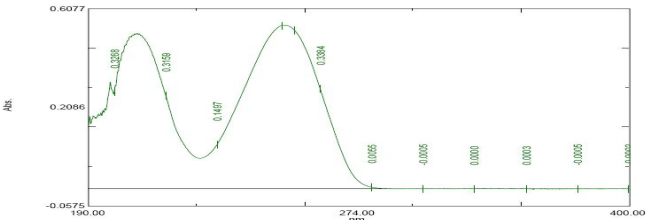
All the impurities and Stavudine solutions were prepared in diluent at a concentration of 1000 ppm and scanned in UV-visible spectrometer; all the 2 impurities and Stavudine were having UV maxima at around 265 nm. Hence detection at 265nm was selected for method development purpose. UV spectrums are shown in Figure 5

UV- Spectum of Thymine



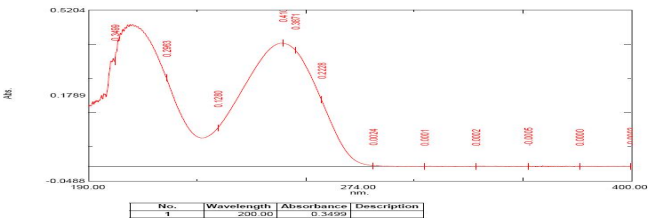
No.	Wavelength	Absorbance	Description
1	200.00	0.5371	
2	220.00	0.4045	
3	240.00	0.2303	
4	265.00	0.6693	
5	270.00	0.6071	
6	280.00	0.3112	
7	300.00	0.0622	
8	320.00	-0.0001	
9	340.00	0.0000	
10	360.00	0.0003	
11	380.00	-0.0004	
12	400.00	-0.0003	
13			

UV-spectrum of β-Thymidine



No.	Wavelength	Absorbance	Description
1	200.00	0.3268	
2	220.00	0.3159	
3	240.00	0.1497	
4	265.00	0.5506	
5	270.00	0.5355	
6	280.00	0.3364	
7	300.00	0.0055	
8	320.00	-0.0005	
9	340.00	0.0000	
10	360.00	0.0003	
11	380.00	-0.0005	
12	400.00	-0.0002	
13			

UV-Spectrum of Stavudine



No.	Wavelength	Absorbance	Description
1	200.00	0.3499	
2	220.00	0.2963	
3	240.00	0.1260	
4	265.00	0.4198	
5	270.00	0.3874	
6	280.00	0.2228	
7	300.00	0.0024	
8	320.00	0.0001	
9	340.00	0.0002	
10	360.00	-0.0005	
11	380.00	0.0000	
12	400.00	-0.0002	
13			

Figure 5 : UV-Spectrums of Stavudine and its impurities

Indian pharmacopeia has given a HPLC method for the determination of the impurities in Stavudine, but in that method the total run time keeping minimum about 30 min. to overcome time factor and chemical cost, a rapid resolution LC method was developed with in 8 min for the quantification of Stavudine and its related substances.

The primary target of this work was to develop a stability indicating chromatographic method for the determination of Stavudine. and its impurities Thymine and β-Thymidine. To get separation of Stavudine from its impurities, and degradation products.

The chromatographic separation was achieved on Symmetry C18 75x4.6mm with 3.5 µm particles). To decrease the interactions of Stavudine with stationary phase column (due to hydrophobicity) mobile phase was selected with higher percentage of water. Different ratios were tried to optimize the retention time of Stavudine and resolution between the impurities. Satisfactory results (retention time of Stavudine is ~2.821 min and the resolution between all the impurities is >4) were obtained with optimized conditions.

In the optimized conditions Stavudine, Thymine, β-Thymidine and Stavudine were well separated with a resolution of greater than 4 and the typical retention times of Thymine, β-Thymidine and Stavudine were about 1.366, 1.877 and 2.821 respectively, representative chromatogram is shown in Figure 2. The system suitability results were given in TABLE 1.

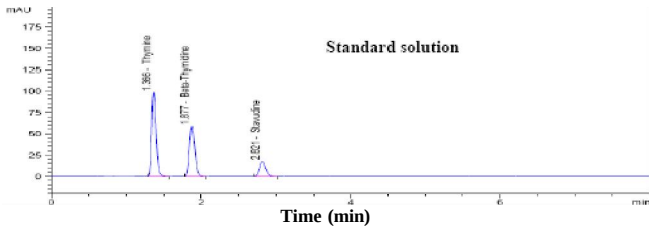


Figure 2 : Typical chromatogram of Stavudine standard solution with Impurities

TABLE 1 : System Suitability report

Compound	USP Resolution (R_S)	USP Tailing factor	No of theoretical plates USP tangent method (N)
Thymine	--	1.265	3135
β-Thymidine	4.617	1.165	3686
Stavudine	6.742	1.115	5197

Mobile phase composition ratio played a major role in achieving the separation between all four impurities and Stavudine.

Analysis was performed for different batches of bulk drug samples (n=3) Results were given in TABLE 2.

TABLE 2 : Batch analysis
(As per new developed method)

Batch No:	Thymine	β-Thymidine	Maximum single unknown impurity	Total Impurities	Assay by HPLC
B.No-01	0.03	ND	ND	0.03	99.8
B.No-02	0.01	ND	ND	0.01	99.6
B.No-03	0.01	ND	ND	0.01	99.7

Where ND = Not Detected

(As per IP method)

Batch No:	Thymine	β-Thymidine	Maximum single unknown impurity	Total Impurities	Assay by HPLC
B.No-01	0.03	ND	ND	0.03	99.7
B.No-02	0.01	ND	ND	0.01	99.8
B.No-03	0.02	ND	ND	0.02	99.5

Where ND = Not Detected

Method validation

Precision

The %RSD of area of Thymine and β-Thymidine in precision study were within 2.0 %. Confirming the good precision of the developed analytical method.

Sensitivity

The limit of quantification of of Thymine, β-Thymidine and Stavudine was 0.05 (of analyte concentration, i.e.500 μg mL^{-1}) respectively for 20 μL injection volume. The % RSD for area of Thymine, β-Thymidine and Stavudine were below 2.0% for precision at LOQ level.

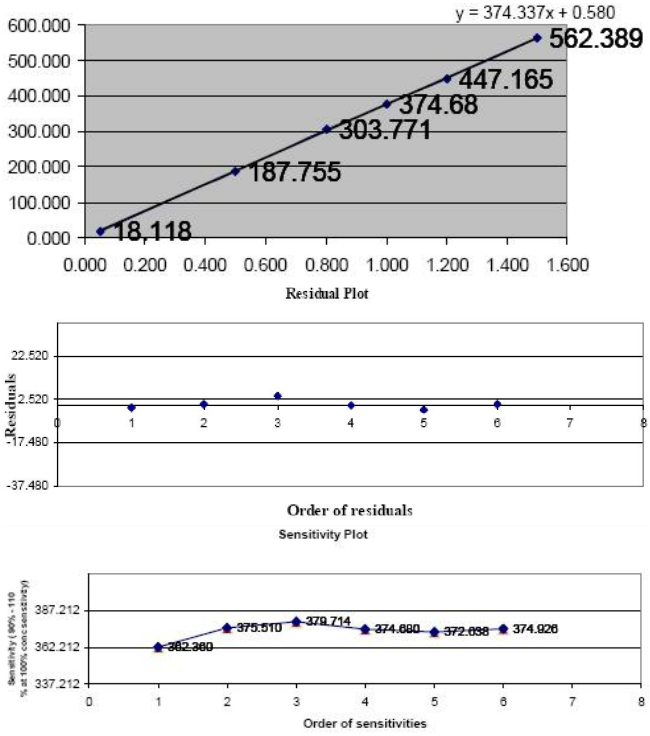
Linearity

Calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a regression coefficient of 0.9999 over the calibration ranges tested.

The results of linearity and range obtained for the four potential impurities were tabulated in the TABLE 3. Linear calibration plot for related substances method was obtained over the calibration ranges tested, i.e. LOQ to 1.50% for Thymine and β-Thymidine. And obtained over the calibration ranges tested, i.e. LOQ to 0.75% for Stavudine.The correlation coefficient obtained was greater than 0.9999 for all two impurities and Stavudine.

TABLE 3 : Linearity Results for Related substances estimation

	Thymine	β-Thymidine	Stavudine
Trend line equation	y =374.337x+0.580	y = 274.092x+1.330	Y203.544x0.397
Linearity Range	0.05%-1.50%	0.05%-1.50%	0.05%-0.75%
Regression Coefficient	0.9999	0.9999	0.9999
Slope	374.337	274.0921	203.544
Intercept	0.580	1.330	0.397
%Intercept	0.15	0.48	0.39
Residual sum of Squares	19.405	15.915	1.948



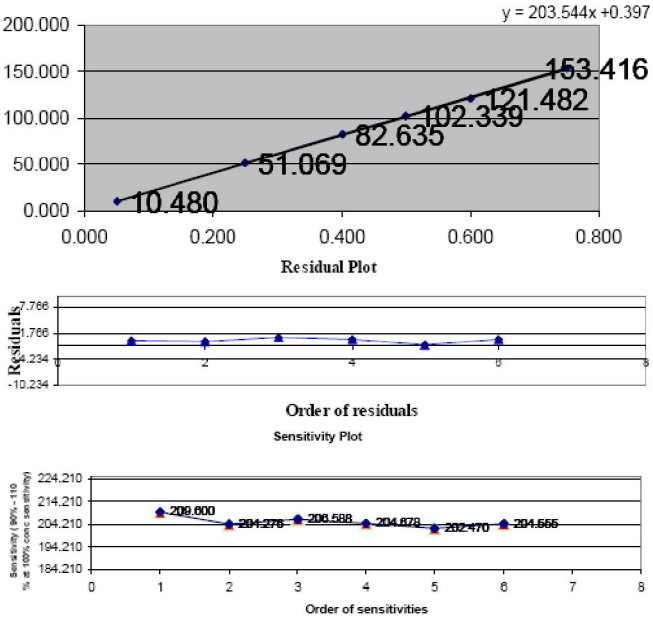
Linearity graphs for Thymine

Accuracy

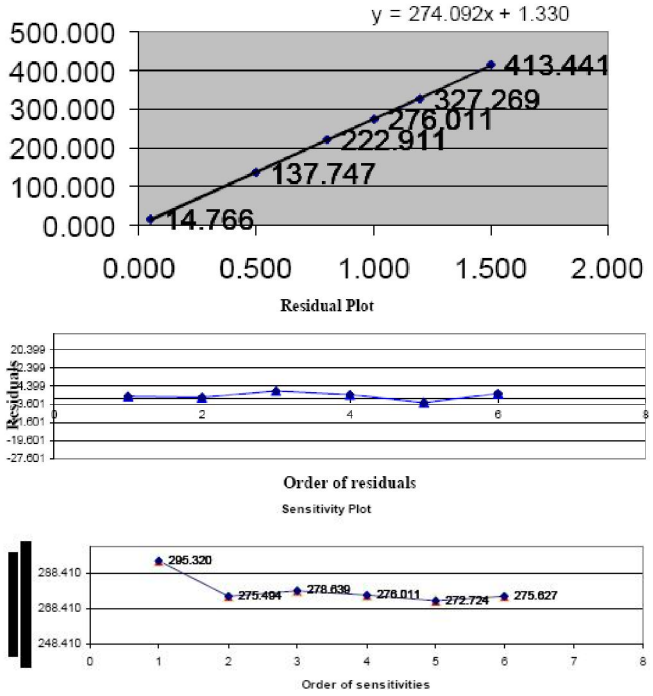
The percentage recovery of Thymine and β-Thymidine in bulk drug samples ranged from 93.9-103.9 (TABLE 5). HPLC chromatogram of spiked sample with all two impurities in Stavudine bulk drug sample is shown in Figure 3.

Robustness

Close observation of analysis results for deliberatly changed chromatographic conditions (flow rate) revealed that the resolution between closely eluting components, namely Thymine and Stavudine was greater



Linearity graphs for Stavudine



Linearity graphs for β-Thymidine

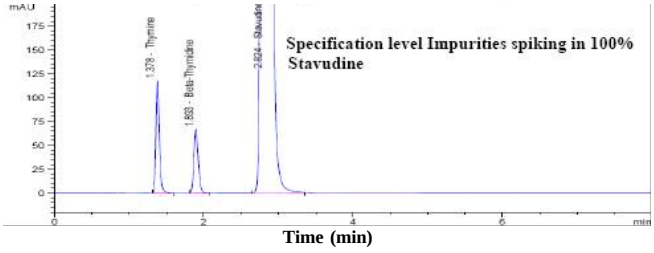


Figure 3 : Specification level Impurities spiking in 100% Stavudine sample

than 4.0, illustrating the robustness of the method (TABLE 5).

TABLE 5 : Results of robustness study

Parameter	Variation	Resolution (R_s) between Thymine & Stavudine
Flow rate (± 20% of the set flow)	0.80mL/min	4.653
	1.20mL/min	4.401

Results of Forced Degradation Studies

Degradation Behavior

Stress studies on Stavudine under different stress conditions suggested the following degradation behavior.

Degradation in Acidic solution

Stavudine is highly sensitive to bases and was degraded into Thymine and unknown impurities by acid hydrolysis in 1.0 HCl. The drug was exposed to 1.0N HCl at 50°C temperature for 10 min. Stavudine has shown significant sensitivity towards acid treatment. The drug undergone degradation with time and degraded into Thymine and unknown.

Degradation in Oxidative condition

Stavudine is sensitive to oxidizing and was degraded into Thymine by oxidation in 6% H_2O_2. The drug was exposed to 6% H_2O_2 at 50°C temperature for 10 min. Stavudine has shown significant sensitivity towards oxidation treatment. The drug gradually undergone degradation with time and degraded into Thymine.

Thermal Degradation

Stavudine is highly sensitive to the effect of tem-

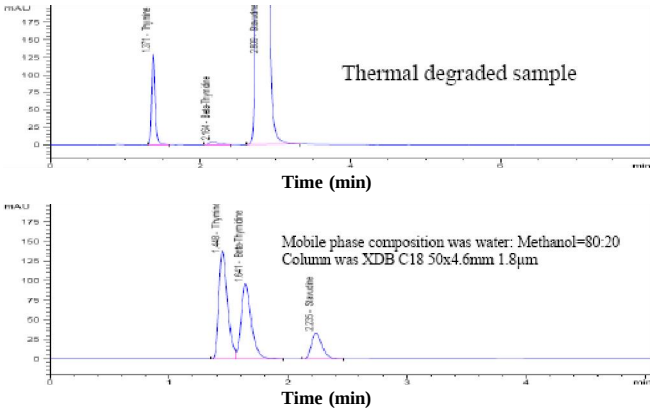


Figure 4 : Typical chromatogram of thermal degraded Stavudine sample

perature was degraded into Thymine by thermal degradation. The drug was exposed at 80°C temperature for 24 h. Stavudine has shown significant sensitivity towards thermal treatment. The drug gradually undergone degradation with time and degraded into Thymine. Representative chromatogram is shown in Figure 4.

CONCLUSION

The isocratic RR-LC method developed for quantitative and related substances determination of Stavudine in bulk drug is precise, accurate and specific. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of Stavudine samples.

ACKNOWLEDGEMENTS

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