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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF LAMIVUDINE AND ZIDOVUDINE IN TABLET

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ABSTRACT

A rapid and sensitive stability indicating analytical method was developed for the estimation of Lamivudine and Zidovudine drug by RP-HPLC. The chromatographic separation was achieved on Inertsil (250*4.6 mm, 5 µm) at 35˚C. The separation is achieved employing a Mobile Phase Methanol: Water (65:35) % v/v. The flow rate was 0.80 ml/min. and detection was carried at 254 nm. The individual absorption maxima was found at 246 nm and 278 nm for Lamivudine and Zidovudine respectively. The average retention time was found to be 3 min. for Lamivudine and 4.6 min for Zidovudine. The proposed method was validated for precision, linearity, accuracy, specificity, robustness. All validation parameter were within the acceptable limit. The assay method was found to be linear from 50% to 150% for Lamivudine and Zidovudine. Forced degradation studies on the formulation were conducted by adopting the proposed method to assess the stability of analyte under acid, alkaline, peroxide, thermal and photolytic condition and suitability of method to resolve degradation product.

KEYWORDS: Lamivudine, Zidovudine, RP-HPLC method, Methnol: Water.

INTRODUCTION[1-3]

The human immunodeficiency virus (HIV) is a Lentivirus (A subgroup of retrovirus) that

causes HIV Infection and over time acquired immune deficiency syndrome (AIDS). AIDS is a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic diseases and cancer to thrive. Without treatment, average survival time after infection with HIV is estimated to be 9 to 10 years, depending on the HIV subtype. Infection with HIV occurs by the transfer of blood, pre-ejaculate fluid, semen, vaginal fluid, or breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells.

HIV infects vital cells in the human immune system such as helper-T cells (specifically CD4+ T cells), macrophages and dendritic cells. HIV infection leads to low levels of CD4+ T cells through a number of mechanisms, including pyroptosis of infected T cells, apoptosis of uninfected cells, direct viral killing of infected cells and killing of infected CD4+ T cells by cytotoxic lymphocytes that recognize infected cells. When CD4+ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections like Pneumonia and Tuberculosis.

Lamivudine

The chemical name is 4- Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. The Molecular formula is $C_8H_{11}N_3O_3S$. Lamivudine is soluble in water, sparingly soluble in Methanol, Slightly soluble in Ethanol.

Pharmacokinetic Study: Oral bioavailability of Lamivudine is high and plasma half life is 6-8 hours. Intracellular half life is even longer (More than 12 hours). It is excreted mainly in urine in unchanged form.

Mechanism of Action: This deoxycytidine analogue is phosphorylated intracellularly and inhibits HIV reverse transcriptase as well as Hepatitis B virus. Its incorporation into DNA results in chain termination. Most human DNA polymerases are not affected and systemic toxicity of Lamivudine is low.

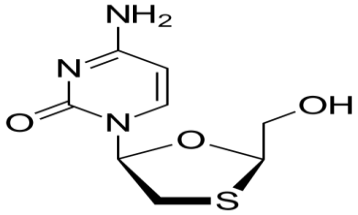

Fig. 1: structure of lamivudine.

Zidovudine

The chemical name is 1-[(2R,4S,5S)-4-Azido-5-(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dion. The Molecular formula is $C_{10}H_{13}N_5O_4$. Zidovudine is soluble in water, sparingly soluble in methanol, Slightly soluble in Ethanol.

Pharmacokinetic Study: Oral bioavailability of Zidovudine is 65%.It is cleared by hepatic glucuronidation (half life of 1 hour); 15-20% off unchanged drug along with metabolite is excreted in urine.

- **Mechanism of Action:** Zidovudine (Prototype of NRTI) is a thymidine analogue. After phosphorylation in host cell, Zidovudine triphosphate selectively inhibits viral reverse transcriptase (RNA-dependent DNA polymerase) in presence of cellular DNA polymerase. It prevents conversion of single stranded viral RNA to double stranded viral DNA.

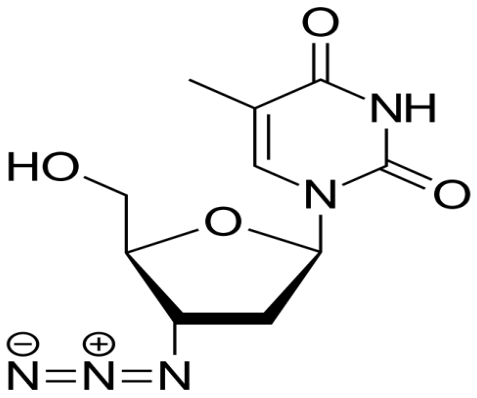

Fig 2: structure of zidovudine.

MATERIALS AND METHOD

Instrumentation

Materials: Lamivudine and Zidovudine pure API obtained from Cipla Pharmaceuticals Ltd., Methanol and Water are HPLC grade obtained from Finar Chemicals.

Optimized chromatographic method

The sample separation was achieved on Inertsil (250 mm*4.6 mm, 5 µm) column, aided by mobile phase mixture of Methanol: Water (65:35 %V/V) that was filtered and degassed prior to use. The flow rate was 0.8 ml/min. And detection wavelength was 254 nm. The run time was 10 min. The injection volume was 20 µl and 35°C temperature.

Preparation of mobile phase

Accurately measure 500 ml of Methanol and 500 ml of HPLC grade water, Mix thoroughly and degassed by sonication to make 50:50 %v/v.

Diluent used is Methanol.

Preparation of solutions

Preparation of Lamivudine standard stocks solutions (150 µg/ml): weigh accurately 15 mg of Lamivudine and transfer it to 100 ml volumetric flask. Add 50 ml of Methanol, shake well to dissolve and dilute upto the mark with Methanol and mix them thoroughly.

Preparation of Zidovudine standard stocks solutions (300 µg/ml): weigh accurately 30 mg of Zidovudine and transfer it to 100 ml volumetric flask. Add 50 ml of Methanol shake well to dissolve and dilute upto the mark with Methanol and mix them thoroughly.

Working standard solution of Lamivudine and Zidovudine (15:30 µg/ml): Pipette out 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution into 10 ml volumetric flask and dilute up to the mark with diluents and mixed thoroughly.

Preparation of Forced degradation solutions[4-5]

Preparation of sample stock solution: Crushed 5 tablets and weigh powder equivalent to 150 mg Lamivudine and 300 mg Zidovudine. 50 ml of Methanol was added in 100 ml volumetric flask shake well to dissolve and sonicated and filtered and dilute upto the mark with Methanol and mix them thoroughly.

ACID DEGRADATION

Working standard solution preparation: 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution were transferred in 10 ml volumetric flask. 1 ml of 0.1N HCl was added and reflux and keep for 8 hours. Cooled to room temperature and added 1 ml of 0.1N NaOH and make up the volume upto the mark with diluent and mixed. Inject into HPLC system. Similarly solutions were prepared for respective conditions.

Preparation of working Sample Solution: Take 1 ml from sample stock solution in 10 ml volumetric flask and 1 ml of 1 N HCl solution in it and reflux and keep for 8 hours then add 1 ml of 1N NaOH to neutralize it and make volume upto mark with methanol.

ALKALI DEGRADATION

Working standard solution preparation: 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution were transferred in 10 ml volumetric flask. 1 ml of 0.1N NaOH was added and reflux and keep for 8 hours. Cooled to room temperature and added 1 ml of 0.1N HCl and make up the volume upto the mark with diluent and mixed. Inject into HPLC system. Similarly solutions were prepared for respective conditions.

Preparation of working Sample Solution: 1 ml from standard sample solution was taken in 10 ml volumetric flask and 1 ml of 1 N NaOH solution in it and reflux and keep for 8 hours then add 1 ml of 1N HCl to neutralize it and make volume upto mark with methanol.

PEROXIDE DEGRADATION

Preparation of working standard solution: 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution were transferred in 10 ml volumetric flask. 1 ml of 3% H_2O_2 was added and reflux and keep for 6 hours. Cooled to room temperature and make up the volume upto the mark with diluent and mixed. Inject into HPLC system. Similarly solutions were prepared for respective conditions.

Preparation of working Sample Solution: 1 ml from sample stock solution was taken in 10 ml volumetric flask and 1 ml of 3% H_2O_2 was added and refluxed for 6 hours. Cooled to room temperature and make up the volume upto the mark with diluent and mixed. Inject into HPLC system.

THERMAL DEGRADATION

Preparation of working standard solution: 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution were transferred in 10 ml volumetric flask. Heated for 2 hours at 80ºC temperature. Cooled to room temperature, diluted up to the mark with diluent and mixed Inject into HPLC system. Similarly solutions were prepared for respective conditions.

PHOTOLYTIC DEGRADATION

Preparation of working standard solution: 1 ml of Lamivudine standard stock solution and 1 ml of Zidovudine standard stock solution were transferred in 10 ml volumetric flask. Exposed to 1.2 X 10^6 Lux hr exposures, diluted up to the mark with diluent and mixed Inject into HPLC system. Similarly solutions were prepared for respective conditions.

Method Validation[6]

Precision

- Method precision was demonstrated by making six samples set as per the method representing the single batches. Assay of these sample were determined and precision of method was evaluated by computing the % RSD of the assay results.

Repeatability

- Repeatability expresses the precision under the same operating conditions over a short interval of time. Six repeated injections of standard solutions were made and percentage RSD was calculated.
- Concentration of 15 µg/ml for Lamivudine and 30 µg/mL for Zidovudine was repeated for 6 times and %RSD should be determined.

Intraday

Combined solution containing the mixture of Lamivudine (12, 15, and 18 µg/ml) and Zidovudine (24, 30 and 36 µg/ml) were analyzed for 3 times on the same day, peak areas were determined and %RSD was calculated.

Interday

Combined solution containing the mixture of Lamivudine (12, 15, and 18 µg/ml) and Zidovudine (24, 30 and 36 µg/ml) were analyzed for 3 different day, peak areas were determined and %RSD was calculated.

Linearity and Range

The Linearity of peak area responses versus concentration was demonstrated by linear least square regression analysis. Good linearity (correlation coefficient $r^2 > 0.9996$) was observed for Lamivudine and (correlation coefficient $r^2 > 0.9996$) was observed for Zidovudine over the Concentration range of 7.50 to 22.5 µg/ml and 15 to 45 µg/ml, respectively.

The calibration curve data of Lamivudine and Zidovudine are represented in below tables represents the calibration plots of Lamivudine and Zidovudine, respectively. Correlation coefficients (r^2) and linear Regression equations (slope and intercept) are also presented in below table.

Specificity

Specificity was checked by comparison of chromatogram of diluent, chromatogram of mobile phase, chromatogram of placebo, chromatogram of standard solution and chromatogram of sample solution.

Accuracy

Accuracy was carried out by the recovery studies at 3 different levels of concentration (50%, 100%, and 150%). To a fixed amount of pre-analyzed sample of Lamivudine (7.5 µg/ml) and Zidovudine (15 µg/ml), an increasing amount of the standard stock solution of mixture of Lamivudine and Zidovudine were added at a concentration (50%, 100%, 150%) of pre analyse sample.

Limit of detection

The detection limit of individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The Limit of detection (LOD) for both the drugs was determined based on the standard deviation of the response and slope as per equation designated by International conference of harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$

Where, σ = standard deviation of the response

S = slope of the calibration curve

Limit of quantification

The quantification limit of individual analytical procedure is the lowest amount of analyte in a sample which can be determined with the suitable precision and accuracy. The Limit of quantification (LOQ) for both the drugs was determined based on the standard deviation of the response and slope as per equation designated by International conference of harmonization (ICH) guidelines.

$LOD = 10 \times \sigma/S$

Where, σ = standard deviation of the response S = slope of the calibration curve.

Robustness

The robustness was studied by analysing the samples of Lamivudine and Zidovudine by deliberate variation in the method parameters. The change in the response of Lamivudine and

Zidovudine were observed.

Robustness of the method was studied by flow rate ± 0.02 mL/minutes, mobile phase composition ± 2 ml and change in wavelength ± 2nm. The changes in the response of Lamivudine and Zidovudine were noted and compared with the actual results.

Parameters for robustness were changed as per below:- Flow rate 0.78 ml/minutes and 0.82 ml/minutes.

Mobile phase composition

Methanol: Water (63:37,v/v) and (67:33,v/v) Change in wavelength ± 2nm. (252 and 256 nm).

RESULTS

The spectrum of Lamivudine and Zidovudine was taken in the UV spectrophotometer. Lamivudine 50 µg/mL solution is prepared in methanol. Taken UV spectra 200-400nm using methanol as blank. Wavelength maxima found at 246 nm. Zidovudine 50 µg/mL solution is prepared in methanol. Taken UV spectra 200-400 nm using methanol as blank. Wavelength maxima found at 278 nm. And out of all those wavelength, At 254 nm both the drugs showed good response in HPLC, so 254 nm was selected as the detection wavelength.

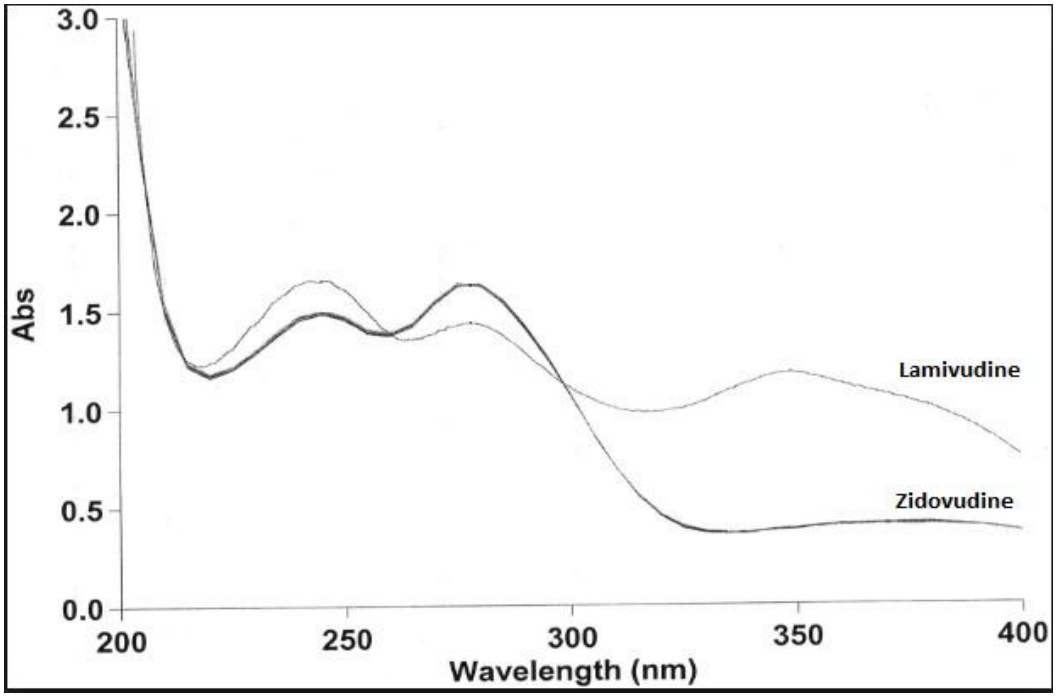

Fig. 3: UV spectra of lamivudine and zidovudine.

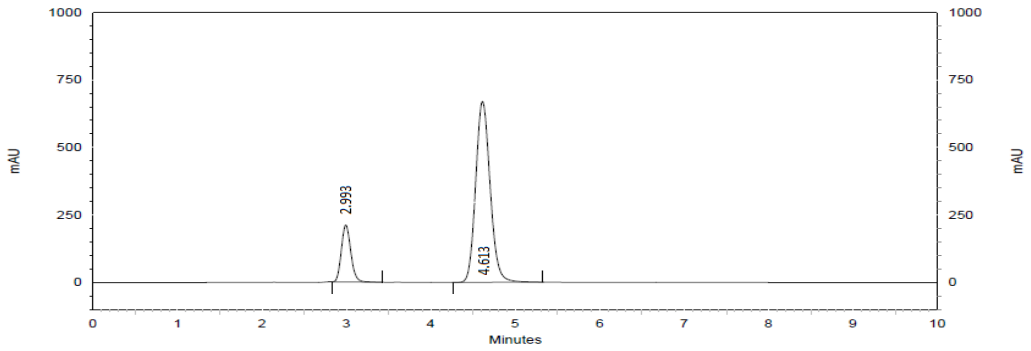

Fig. 4: Chromatogram of lamivudine and zidovudine.

Table 1: Optimized Chromatographic Conditions.

Stationary phase (Column)	**Inertsil, (250x 4.6 mm, 5µm)**
Mobile Phase	Methanol : Water (65:35 %V/V)
Detection wavelength	254 nm
Injection volume	20 µl
Flow rate	0.8 ml/min
Column temperature	Room temperature
Run time	10 Minutes

Table 2: Forced degradation condition.

Stress type	**Condition**
Acid Degradation	1 ml 1 N HCl for, Reflux for 12 hrs.
Alkali Degradation	1 ml of 2 N NaOH Reflux for 24 hrs
Peroxide Degradation	1 ml 6% H_2O_2 reflux for 6 hrs.
Thermal Degradation	6 hours at 80ºC
Photolytic Degradation	2 X 10^6 Lux for 24 hrs.

Table 3: Forced Degradation study of Lamivudine and Zidovudine.

Degradation method	**Optimized condition**	**% Degradation**	
		Lamivudine	**Zidovudine**
Acidic	1 ml 1 N HCl for reflux for 12 hours	12.3%	6.08%
Basic	1 ml of 2N NaOH reflux for 24 hours	7.2%	4.9%
Peroxide	1 ml 6% H_2O_2 reflux for 6 hours	11.03%	10.06%
Thermal	6 hours at 80ºC	4.6%	1.5%
Photolytic	2 X 10^6 Lux for 24 hours	0.32%	0.21%

Table 4: Repeatability data for Lamivudine and Zidovudine.

Lamivudine		Zidovudine	
Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
15	2768183	30	11663320
15	2778083	30	11661849
15	2754314	30	11624086
15	2782331	30	11685897
15	2778694	30	11652345
15	2778490	30	11691312
Mean	2773349	Mean	11663135
SD	10460.45	SD	24310.444
RSD	0.377177	RSD	0.2084383

Table 5: Intraday data for Lamivudine and Zidovudine.

Lamivudine			Zidovudine		
Conc. (µg/mL)	Area Mean ±SD (n=3)	% RSD	Conc. (µg/mL)	Area Mean ±SD (n=3)	% RSD
12	2230335.3 ± 12621.04	0.565881	24	97302233 ± 364628.5	0.374738
15	2778344 ± 16545.59	0.59552	30	11901574 ± 57091.72	0.479699
18	3347959.7 ± 20147.03	0.60177	36	14283927 ± 73349.93	0.513514

Table 6: Interday data for Lamivudine and Zidovudine.

Lamivudine			Zidovudine		
Conc. (µg/mL)	Area Mean ±SD (n=3)	% RSD	Conc. (µg/mL)	Area Mean ±SD (n=3)	% RSD
12	2319131.7 ± 4938.526	0.212947	24	96187107 ± 18619.11	0.019357
15	2782116.3 ± 15136.26	0.544056	30	11833797 ± 96683.72	0.817013
18	3341737 ± 22595.97	0.676174	36	14289841 ± 51670.16	0.361587

Table 7: Data for the linearity and range for Lamivudine.

Sr. No.	Lamivudine			
	Conc.In (µg/ml)	Average area (n=3)	SD	RSD
1	7.5	1525203	5879.53323	0.385491
2	12	2223093	7327.501279	0.329608
3	15	2738541	14127.96137	0.515893
4	18	3165351	9919.992255	0.313393
5	22.5	3884746	8944.566451	0.230248

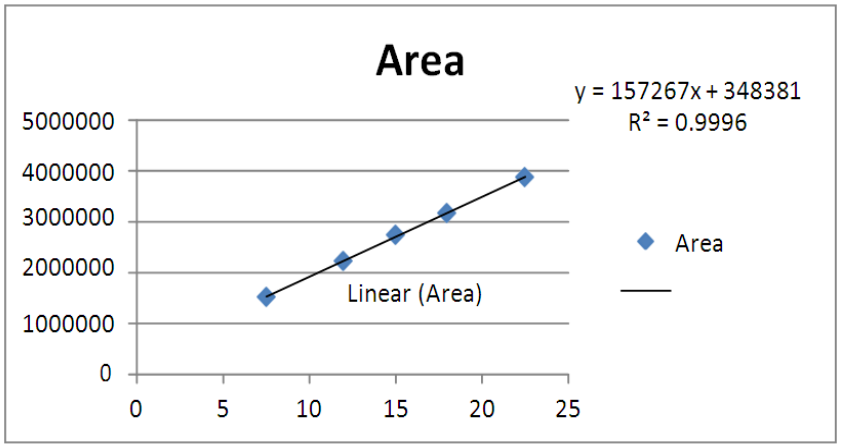

Fig 5: Linearity graph of lamivudine.

Table 8: Data for the linearity and range for Zidovudine.

Sr. No.	Zidovudine			
	Conc. In (µg/ml)	Average area (n=3)	SD	RSD
1	15	7031575	22333.03	0.318778
2	24	9815214	47531.81	0.483566
3	30	11624086	22239.28	0.190899
4	36	13668015	29447.94	0.215229
5	45	16244980	55444.67	0.339968

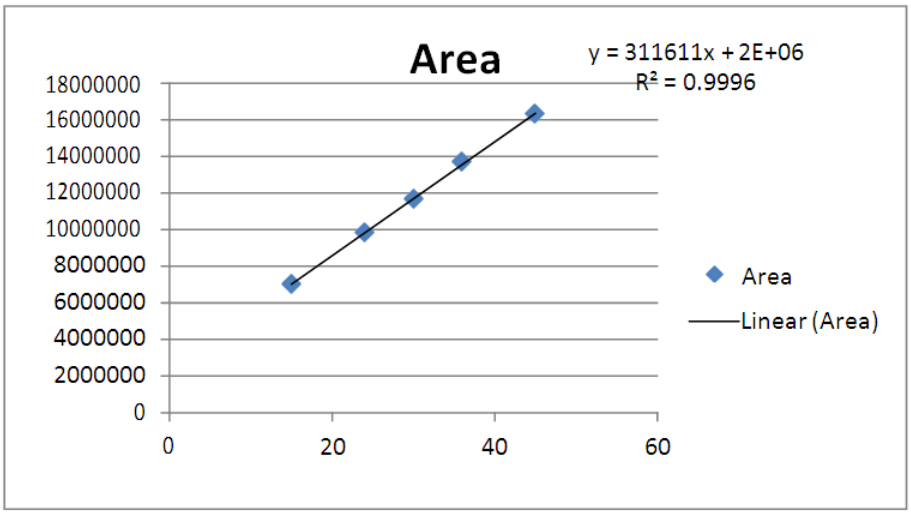

Fig. 6: Linearity graph of Zidovudine.

- **Specificity**

➢ Chromatogram shows that there no interference of diluent with a peak of standard drugs. So it is concluded that method is specific for Lamivudine and Zidovudine.

Accuracy

Table 9: Accuracy Study for Lamivudine.

% accuracy level	Amount of Lami taken (75 µg/ml)	Amount of standard Lami added (150 µg/ml)	Total amount of Lami (µg/ml)	Amount of Lami found (µg/ml) ±SD (n=3)	Mean % recovered ±SD (n=3)
50	7.5	3.75	11.25	11.23 ± 0.07	99.83 ± 0.15
100	7.5	7.5	15	14.98 ± 0.11	99.73 ± 0.20
150	7.5	11.25	18.75	18.73 ± 0.05	99.76 ± 0.20

Table 10: Accuracy Study for Zidovudine.

% accuracy level	Amount of Zido taken (150 µg/ml)	Amount of standard Zido added (300 µg/ml)	Total amount of Zido (µg/ml)	Amount of Zido found (µg/ml) ±SD (n=3)	Mean % recovered ±SD (n=3)
50	15	7.5	22.5	22.47 ± 0.20	99.76 ± 0.11
100	15	15	30	29.97 ± 0.10	99.70 ± 0.21
150	15	22.5	37.5	37.46 ± 0.12	99.66 ± 0.20

Table 11: LOD and LOQ of Lamivudine and Zidovudine.

Parameter	Lamivudine (µg/ml)	Zidovudine (µg/ml)
Limit of detection (LOD)	0.296453	0.235517
Limit of quantification (LOQ)	0.898342	0.713687

Robustness

Table 12: Robustness of Lamivudine and Zidovudine.

Parameter		Area (n=3)	
		Lamivudine	Zidovudine
Flow rate (± 0.02 ml/minutes)	0.78 ml	2731120.33	12075449
	0.8 ml	2737496.33	11900133
	0.82 ml	2705771	11916193
	Mean ± SD	2724795.889 ± 16781.63109	11963925 ± 96915.471
	% RSD	0.615885805	0.8100642
Mobile phase composition Methanol : Water (± 2 ml)	63:37 %	2735569.67	11934223
	65:35 %	2724163	11904136
	67:33 %	2698010.67	11959475
	Mean ± SD	2719247.778 ± 19255.88682	11931277 ± 29780.471
	% RSD	0.708132851	0.2496
wavelength (± 2nm)	252	2735584.33	11934112
	254	2724130	11913467
	256	2698010.67	12125675
	Mean ± SD	2719241.667 ± 19257.9064	11991085 ± 117015.14
	% RSD	0.708208712	0.9758512

Table 13: Optical regression characteristics and validation parameters.

Parameter	Lamivudine	Zidovudine
Detection wavelength	254 nm	
Calibration range (µg/ml)	7.50 to 22.5	15 to 45
Regression equation (y=mx+c)	y = 157267x + 348381	y = 311611x + 2E+06
Slope (m)	157267	311611.978
Intercept (c)	348381	2 X 10^6
Correlation coefficient (r^2)	0.9996	0.9996
Limit of Detection (µg/ml)	0.296453	0.235517
Limit of quantification (µg/ml)	0.898342	0.713687

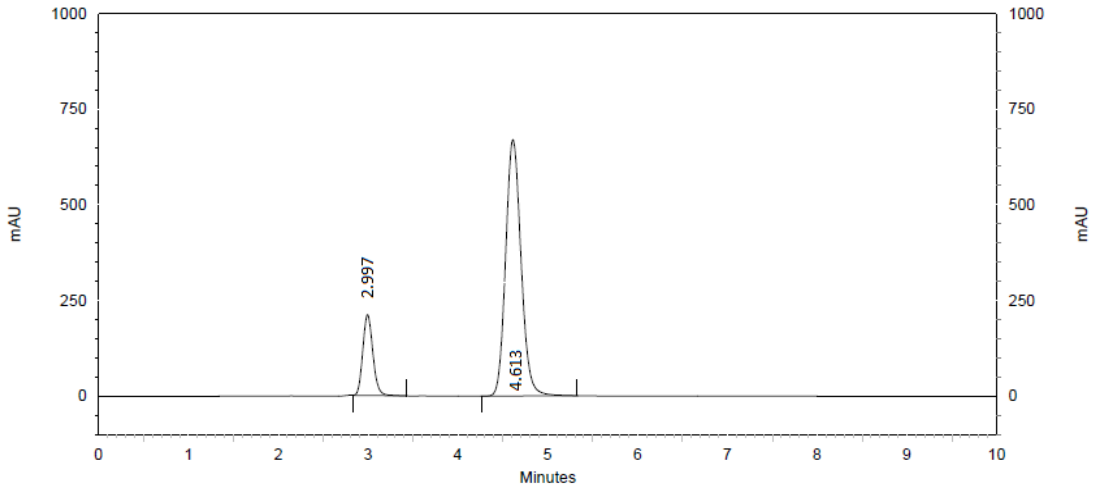

Fig. 7: Chromatogram of std. solution of acid degradation.

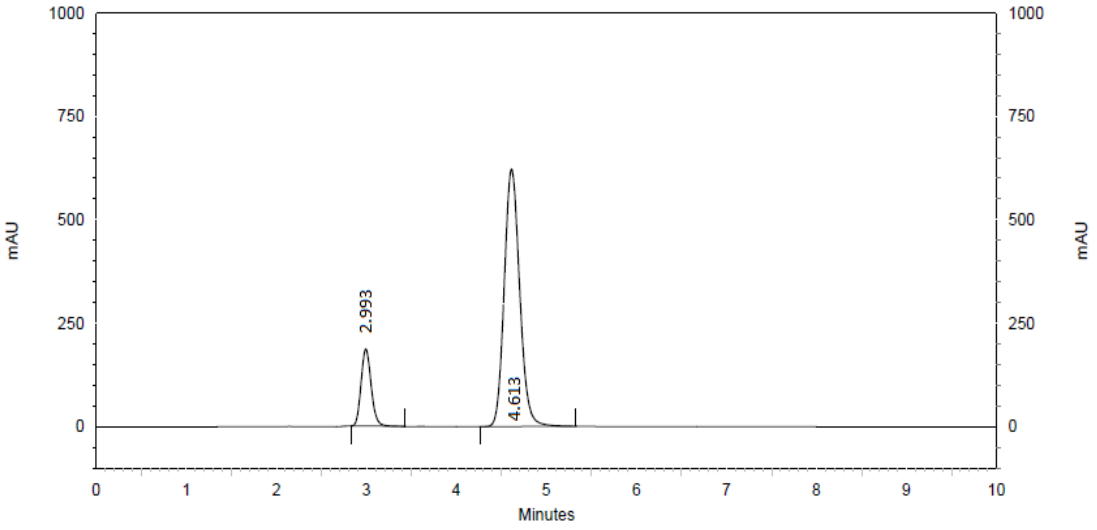

Fig 8: Chromatogram of sample solution of acid degradation.

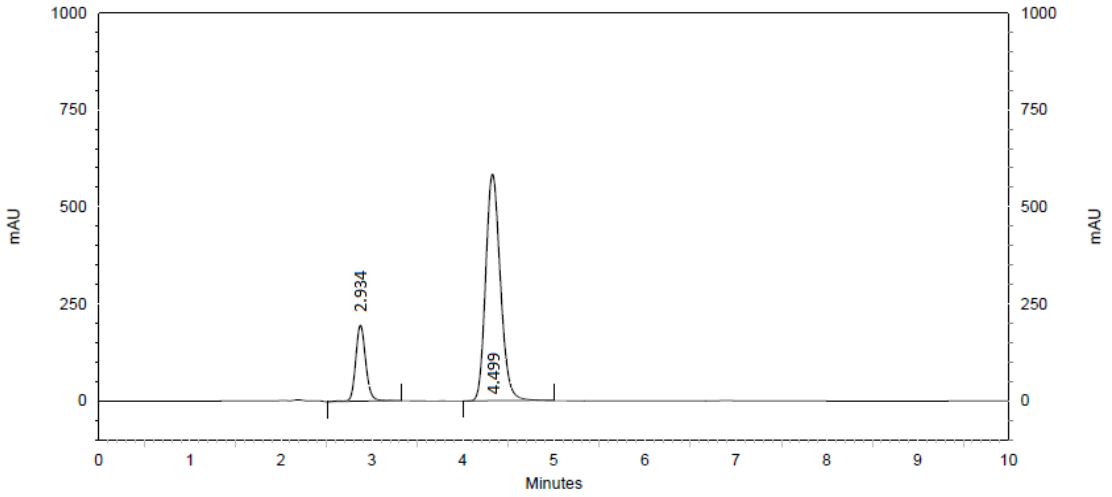

Fig. 9: Chromatogram of std. solution of alkali degradation.

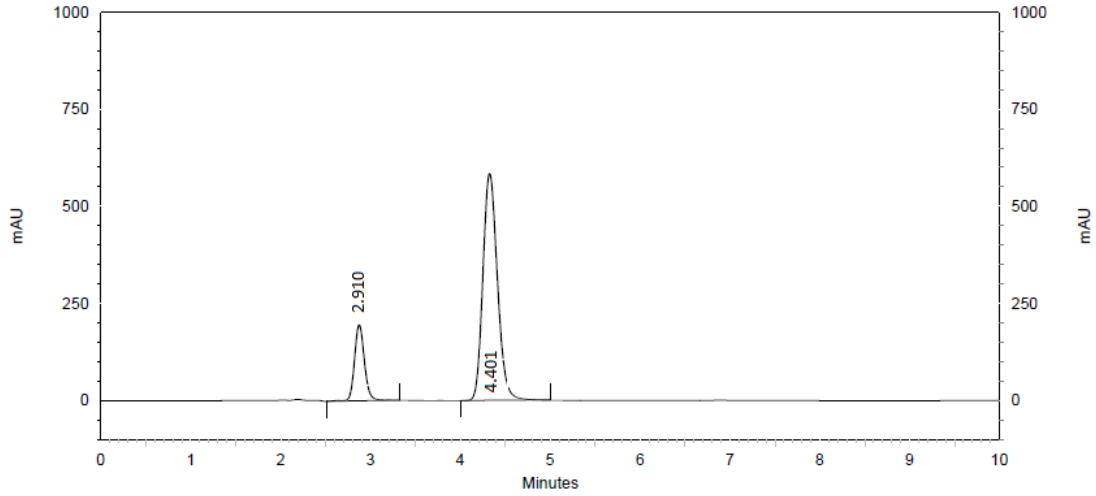

Fig. 10: Chromatogram of sample solution of alkali degradation.

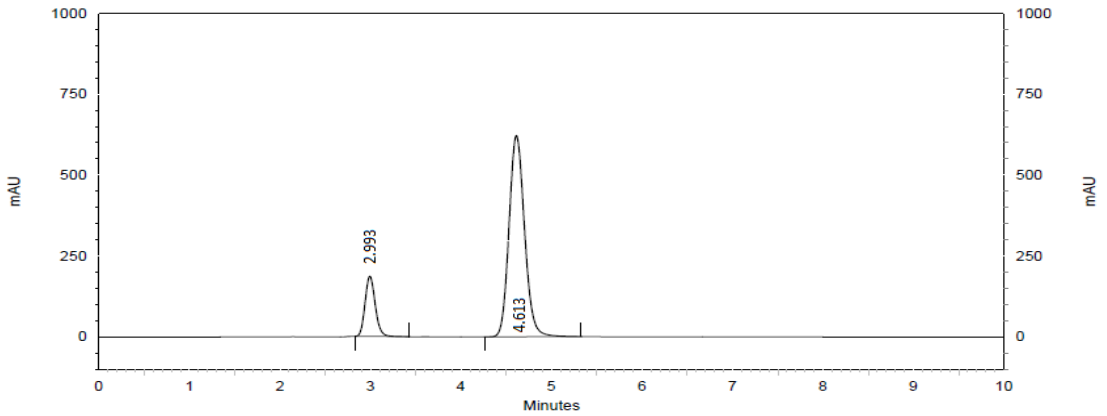

Fig. 11: Chromatogram of std. solution of peroxide degradation.

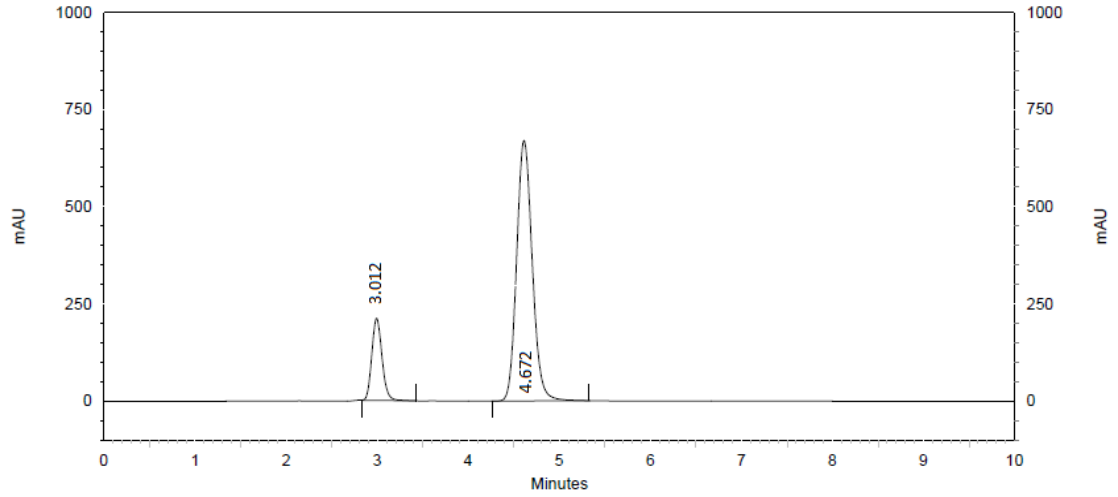

Fig. 12: Chromatogram of sample solution of peroxide degradation.

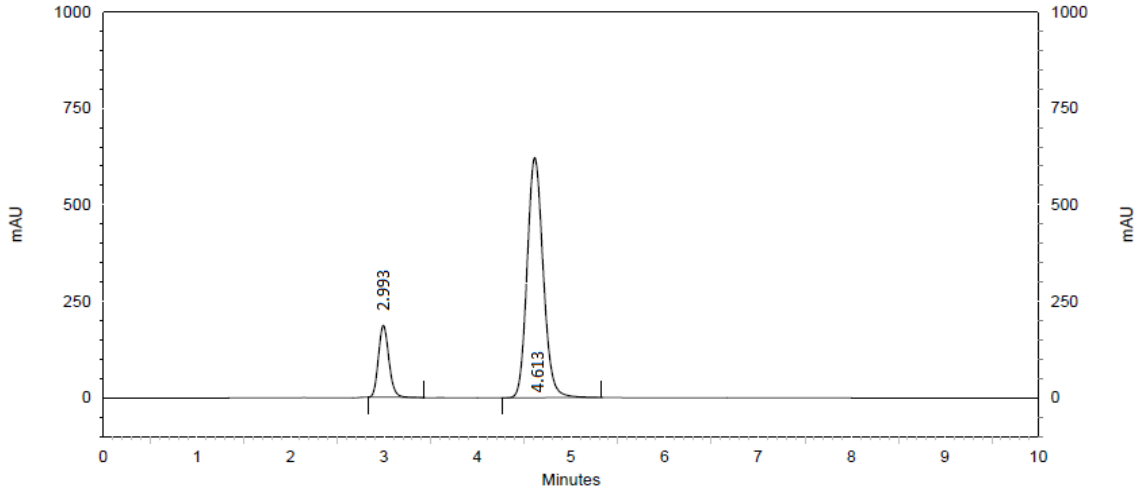

Fig. 13: Chromatogram of thermal degradation of std. solution.

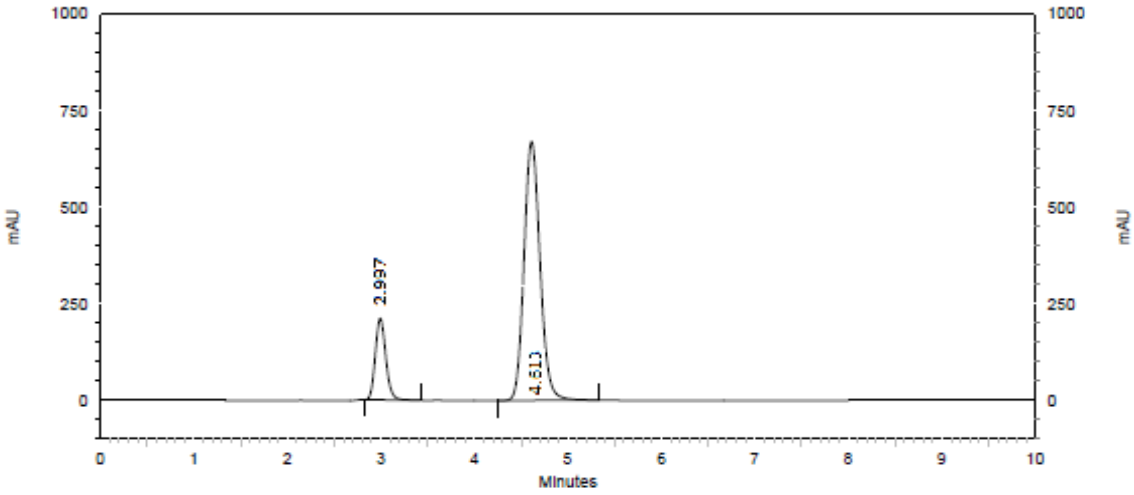

Fig. 14: Chromatogram of thermal degradation of sample solution.

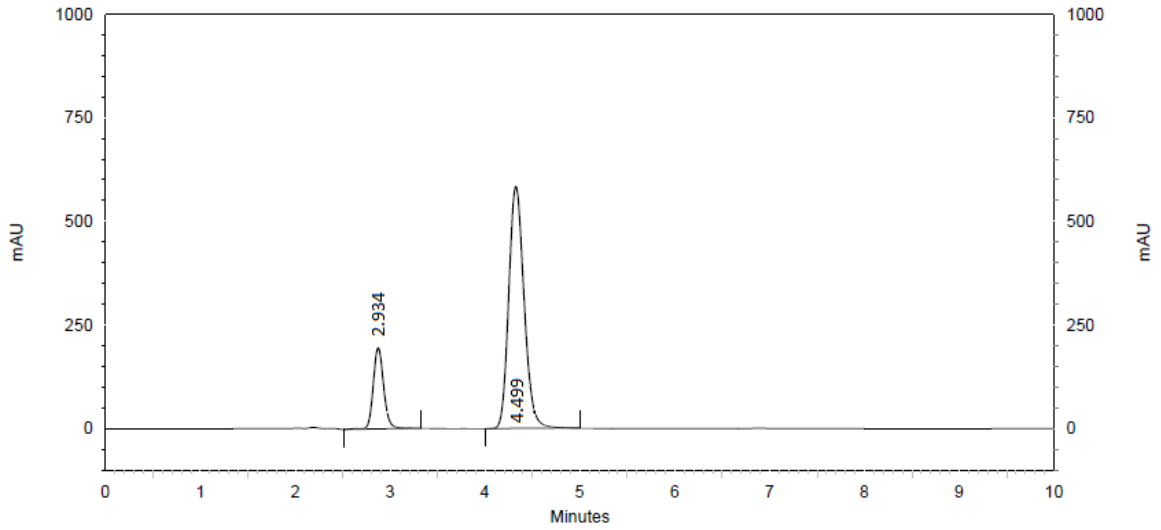

Fig. 15: Chromatogram of photolytic degradation of standard solution.

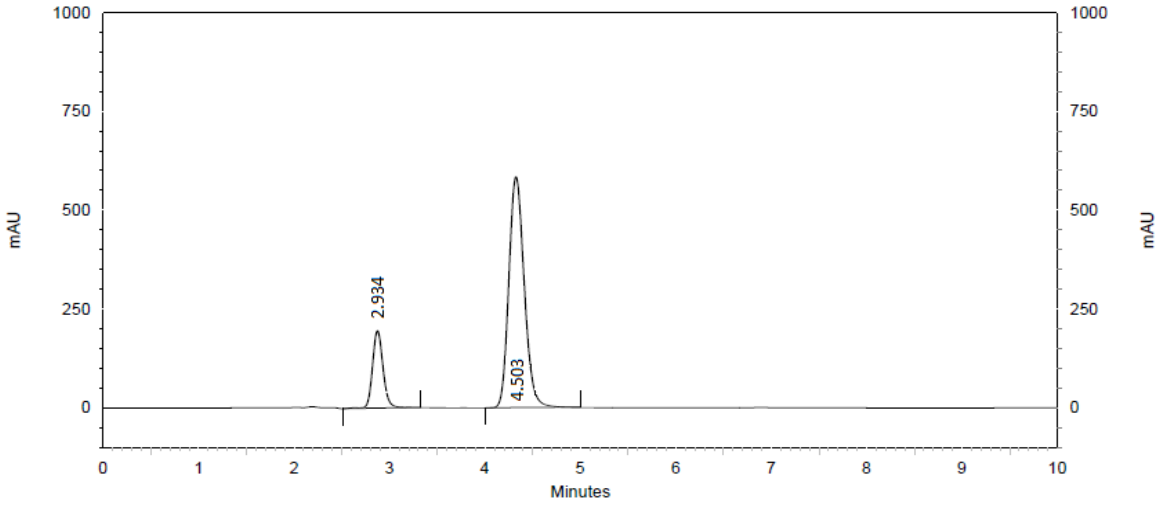

Fig. 16: Chromatogram of photolytic degradation of sample solution Specificity.

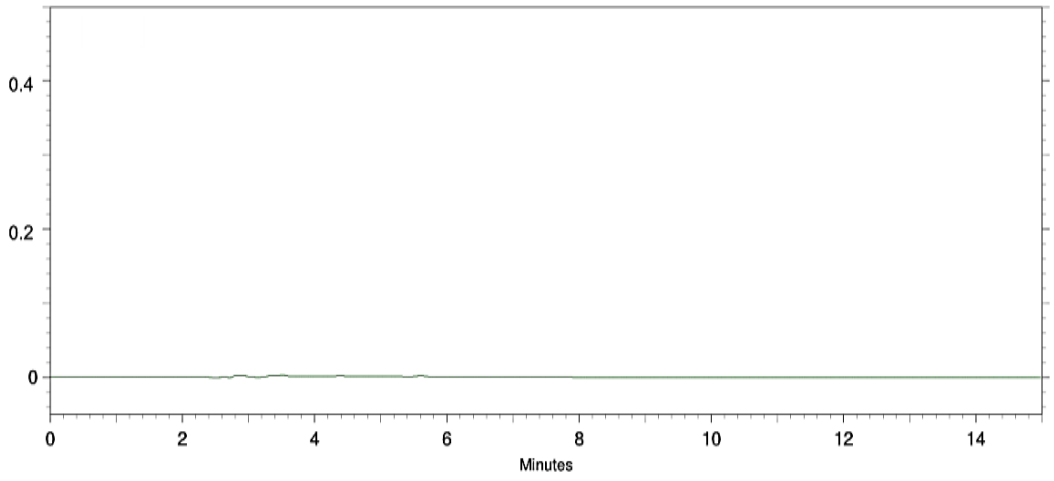

Fig. 17: Chromatogram of blank.

Specificity Chromatogram of mobile phase

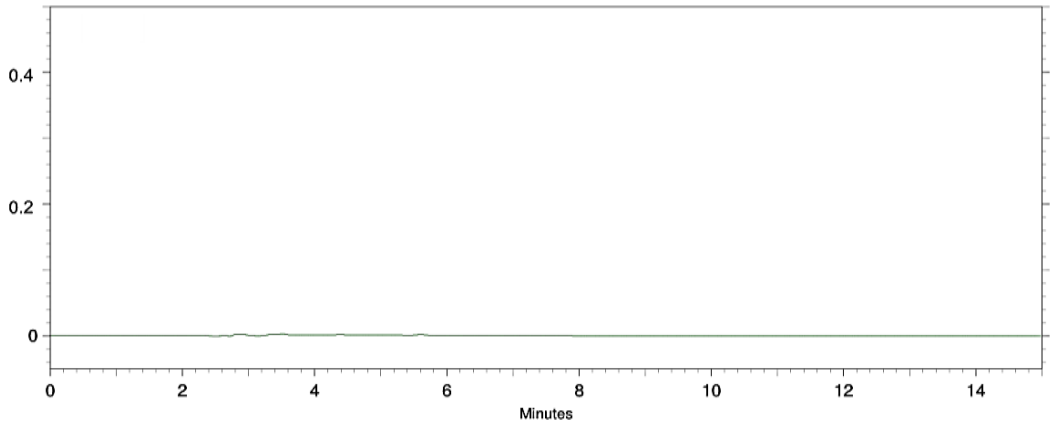

Fig. 18 Chromatogram of mobile phase Specificity Chromatogram of placebo.

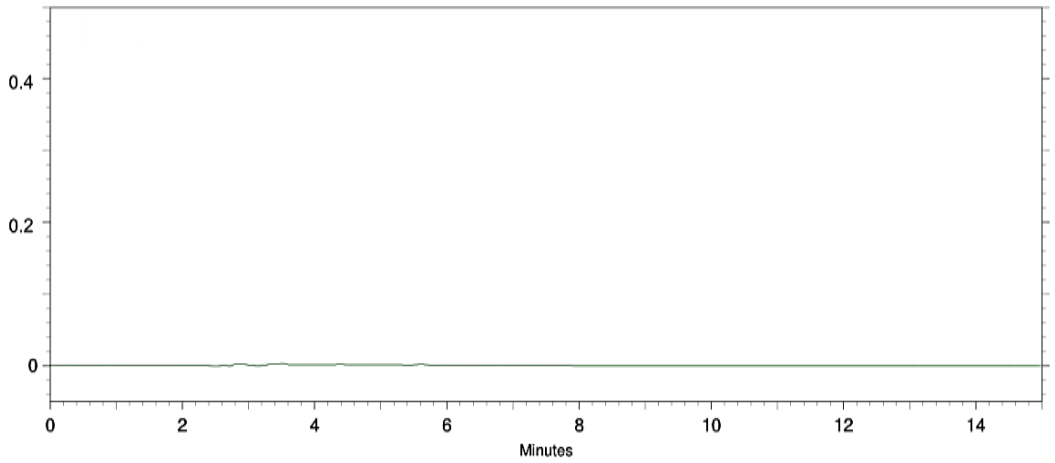

Fig. 19: chromatogram of placebo Specificity Chromatogram of standard solution.

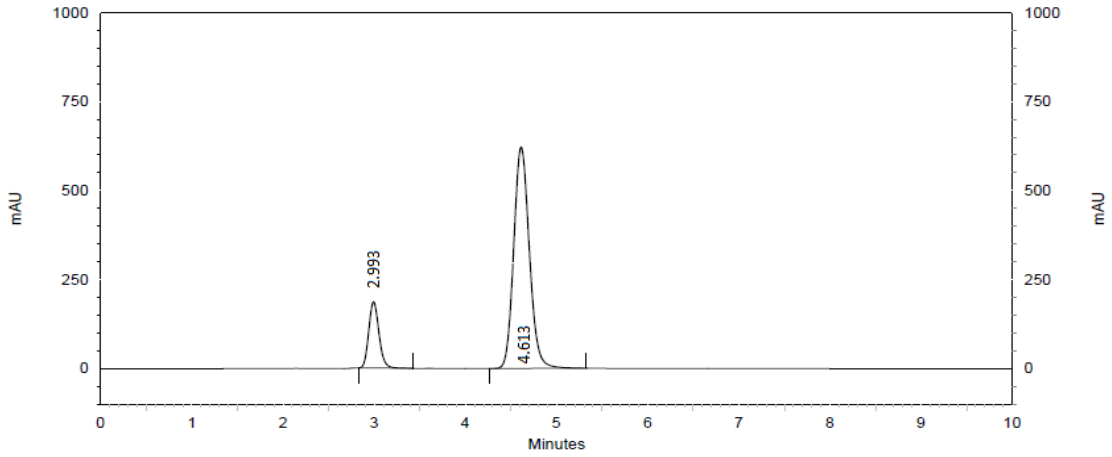

Fig. 20: Chromatogram of standard solution.

Specificity Chromatogram of sample solution of Lamivudine

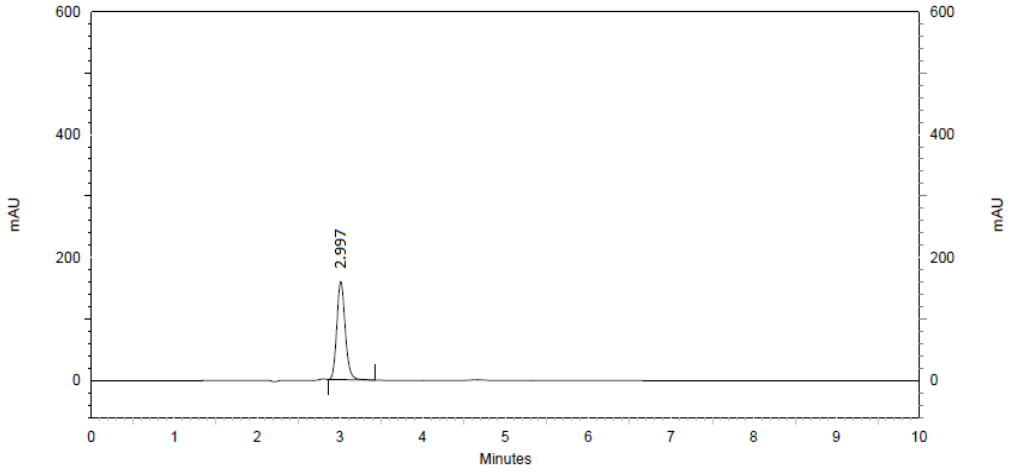

Fig. 21: Chromatogram of sample solution of Lamivudine.

Specificity Chromatogram of sample solution of Zidovudine

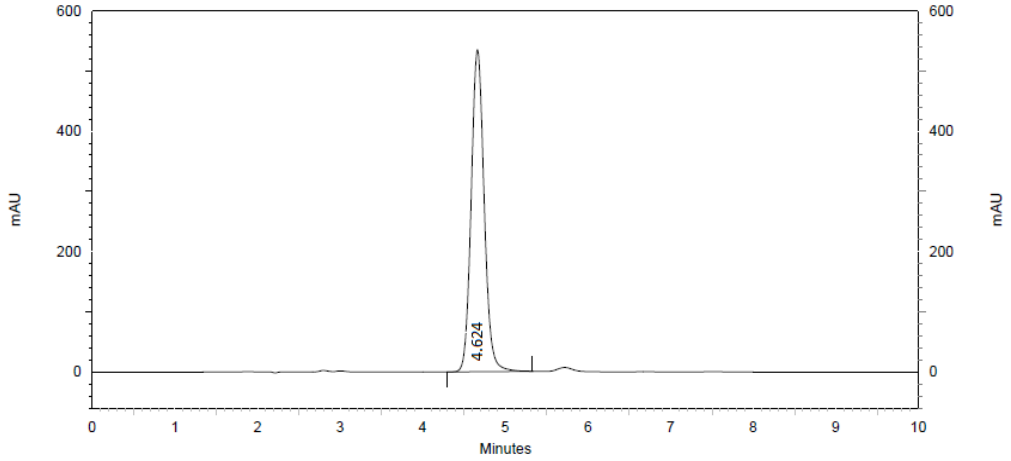

Fig 22: Chromatogram of sample solution of Zidovudine.

DISCUSSION

A Novel stability indicating RP-HPLC method was developed and validated for the estimation of Lamivudine and Zidovudine in Tablet Dosage form. Forced Degradation study was conducted to know the stability of analyte under specified conditions.

CONCLUSION

The combination therapy is very effective for treating HIV disease. The combination is used to prevent transfer of HIV from infected mother to foetus. Thus a simple, precise and stability indicating method was developed for estimation of Lamivudine and Zidovudine in combination.

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