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(Research Article)

Development and Validation of Stability Indicating HPLC Method for Lamivudine, Zidovudine and Abacavir in Tablet Dosage Forms

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ABSTRACT

A novel rapid, sensitive and reproducible mass compatible, ultra performance liquid chromatographic method was developed for quantitative determination of Lamivudine, Zidovudine and Abacavir in active pharmaceutical ingredients and its dosage forms. The synthetic nucleoside reverse transcriptase inhibitor analogues Abacavir, Lamivudine and Zidovudine form one of the fixed dosage combinations used in the effective management of HIV. It belongs to a group of anti-HIV medicines called non-nucleoside reverse transcriptase inhibitors (NNRTIs). The method is applicable to the quantification of related compounds of Abacavir, Lamivudine and Zidovudine form one of the fixed dosage combinations. Chromatographic separation of drugs from the possible impurities and the degradation products was achieved on an Inertsil ODS-3V 250 x 4.6 mm, 5.0µm column; the gradient elution achieved with in 90.0 min. Ammonium dihydrogen phosphate and Diammonium hydrogen phosphate buffers pH 3.9 as mobile phase A and methanol as mobile phase B . The flow rate was 1.0 mL/min, column temperature 50°C and the detection was done at 270 nm.

The above developed HPLC method was further subjected to hydrolytic, oxidative, photolytic and thermal stress conditions. The performance of the method was validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, and ruggedness.

Key Words: Abacavir, Lamivudine, Zidovudine, Force degradation studies, Validation, HPLC method

INTRODUCTION

Antiretroviral drugs like nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors, and protease inhibitors are essential in the management of HIV infection. The synthetic nucleoside reverse transcriptase inhibitor analogues Abacavir, Lamivudine and Zidovudine form one of the fixed dosage combinations used in the effective management of HIV[1-2]. Abacavir, chemically known as [(1R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl}-2-clopentene]-1-methanol, is a carbocyclic synthetic analogue. Its active metabolite carbovir triphosphate, an analogue of deoxyguanosine-5'-triphosphate (d GTP), inhibits the activity of HIV-1 reverse transcriptase both by competing with the natural substrate dGTP and by its incorporation into viral DNA. The active triphosphate metabolites Zidovudine (3- azido-3-deoxythymidine) and Lamivudine(4-amino-1-((2R,5S-2-hydroxymethyl)-1,3-oxathiolan-5-yl)pyrimidin-2-(1H)-one) act against HIV by inhibition of reverse transcriptase via DNA chain termination after incorporation of the nucleotide analogue.

A literature survey reveals the report of a few analytical methods for the determination of these drugs individually in serum samples and in their dosage forms. Methods for the simultaneous determination of Lamivudine and Zidovudine in biological samples and in pharmaceutical preparations were also reported [3-6].

A reported method for the determination of the Lamivudine, Zidovudine and Abacavir in tablets, human serum and in drug dissolution studies

employs a complex buffer system (methanol, water, phosphate buffer)[7]. The authors now propose for stability indicating HPLC method for simple, precise and accurate method for Lamivudine, ziduvudine and Abacavir in tablet dosage forms.

MATERIAL AND METHODS

Chemicals and Materials

Methanol (HPLC grade) and Ammonium dihydrogen phosphate, Diammonium hydrogen phosphate anhydrous, trifluoroacetic acid was purchased from Spectrochem and E-Merck Limited respectively. In-house purified water (USPgrade) was used throughout the study. Active pharmaceutical ingredients and its related impurities (Fig.1) were procured from Manus Aktteva, India, commercially available.

Equipments

The High performance liquid chromatography (Waters) used was equipped with Photo diode array detector with gradient elution capacity and an auto sampler with data handling system (Empower software) on lenovo computer.

Chromatographic conditions

The chromatographic separation was achieved using a gradient method on an Inertsil ODS-3V 250 x 4.6 mm, 5-µm column; the gradient Liquid chromatographic method employs solution A and solution B as mobile phase. The solution A contains 2.3 g of Ammonium dihydrogen phosphate and 1.32 g of Di ammonium hydrogen phosphate anhydrous into a beaker containing 1000 ml of water and mix. Adjust pH of the solution to 3.9±0.05 with 50% trifluoroacetic acid. Filter the solution through 0.22µm membrane filter. The solution B contains is HPLC grade methanol. The flow rate was 1.0 mL/min. The HPLC gradient program was set as Time/Mobile phase A/Mobile phase B. The column temperature was maintained at 50 °C, sample compartment temperature was maintained at 5 °C and the detection wavelength was 270 nm for identified and unidentified impurities. The injection volume of 20 µL.

Diluent : Mobile phase A

Gradient Program

Time (Minutes)	Mobile phase - A (%v/v)	Mobile phase - B (%v/v)
0	97	3
15	97	3
70	60	40
80	40	60
82	97	3
90	97	3

Standard Solutions

Preparation of Lamivudine resolution solution

Accurately weighed and transferred about 3 mg of the Lamivudine resolution mixture (containing Lamivudine and Lamivudine Diastereomer) into a 10 ml volumetric flask, added 5 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix.

Preparation of Lamivudine standard stock solution

Accurately weighed and transferred about 30 mg of Lamivudine working standard into a 200 ml volumetric flask. Added about 120 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 2.0 ml of the above solution into a 50 ml volumetric flask, dilute to volume with diluent and mix.

Preparation of Abacavir sulfate and Zidovudine standard stock solution

Accurately weighed and transferred about 35 mg of Abacavir sulfate working standard and 30 mg of Zidovudine working standard into a 200 ml volumetric flask. Added about 120 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 2.0 ml of the above solution into a 50 ml volumetric flask, dilute to volume with diluent and mix.

Preparation of Standard solution

Transfer 2.0 ml of Lamivudine standard stock solution and 4.0 ml of Abacavir sulfate and Zidovudine standard stock solution into a 20 ml volumetric flask, dilute to volume with diluent and mix.

Sample Solutions

Accurately weighed and transferred tablets powder equivalent to about 30 mg of Lamivudine into a 100 ml volumetric flask, added about 60 ml of diluent and sonicate for not less than 30 minutes with occasional shaking (maintain the sonicator temperature between 20 to 25°C). Dilute to volume with diluent and mix. Filter a portion of the solution through 0.45µm membrane filter and discard first few ml of the filtrate.

Degradation Studies

Specificity is the ability of method to measure the analyte response in the presence of its potential impurities and degradation products. The specificity of the developed RP-HPLC method of Lamivudine, ziduvudine and Abacavir was carried out in presence of its seven potential impurities, namely Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine

Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir. Forced degradation studies were performed on for Lamivudine, ziduvudine and Abacavir bulk drugs (Fig.2). Intentional degradation was attempted with stress conditions of UV light (254 nm), heat and humidity (60 °C at 70 % RH), acid (0.1 N HCl), base (0.1 N NaOH) and oxidation (3 % H2O2) to determine the ability of the proposed method to separate lamivudine, ziduvudine and Abacavir from its impurities and degradation products generated during forced decomposition studies . For heat and light studies, study period was 7 days where as for acid, base and oxidation it was 24 hrs. Peak purity test was carried out on the stressed samples by using PDA. Related compounds studies were carried out for stress samples against qualified reference standard. Related compounds were also calculated for bulk sample by spiking with its impurities at its specification level (0.1%).

Method Validation

System and Method Precision

The system precision is indicated by the repeatability of multiple injections and indicates the performance of the HPLC instrument under the prescribed chromatographic conditions. The variance of the values obtained is represented as the percent relative standard deviation (% RSD). A working standard solution of lamivudine, ziduvudine, Abacavir and its related compounds was consecutively injected six times under the same analytical conditions. The % RSD of peak areas, difference of retention times, tailing factor (T) column efficiency (N) and resolution (R) are calculated. The intermediate precision of the method was also evaluated using one unspiked sample and 6 independent sample preparations spiked with a 100 % of the target concentrations as defined by the method. The samples were injected using a different instrument and column.

Linearity

The linearity is determined by the ability of the method to obtain test results, which are directly proportional to the concentration of the compounds of interest in the sample. Stock solutions were serially diluted to produce solutions containing concentration levels from QL to 160% with respect to impurity specification limits of 0.1 %. The calibration curve was drawn by plotting the peak areas of Lamivudine, ziduvudine and Abacavir; Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine-carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir versus its corresponding concentrations. The % RSD value of the slope and Y intercept of the calibration curve was calculated.

Quantification limit (QL) and Detection Limit (DL)

The lower end of the linear range was considered to be the QL for the method. The QL concentrations were determined by injecting diluted standard solution to a level such that % RSD was not more than 10%. Precision study was also carried at the QL level by injecting six individual preparations of Lamivudine, Ziduvudine, Abacavir, Zidovudine Related Compound-C, Lamivudine Diastereomer, Lamivudine-carboxylic acid, Zidovudine Related compound-B, Zidovudine Related Compound-A, Cyclopropyldiaminopurine Abacavir, Escyclopropyl Abacavir and O-Pyrimidinyl Abacavir.

Accuracy

Lamivudine, Ziduvudine and Abacavir sample solution was spiked with impurity standard solutions containing Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine-carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir and O-Pyrimidinyl Abacavir at three concentration levels corresponding to QL 100 % and 160 % of analyte concentration. The % recovery is the amount of the compound of interest analyzed as a percentage of the theoretical amount present in the medium was calculated from the slope and the Y-intercept of the calibration curve.

Robustness

Deliberate variations in critical method parameters were done to assess the robustness of the related compounds method to evaluate method reliability. The flow rate of the mobile phase was 1.0 mL/min, to study the effect of flow rate on the resolution; it was changed by 0.1 unit from 0.9 to 1.1 mL/min. The effect of column temperature on resolution was studied at 45 and 55 °C instead of 50 °C.

Solution Stability

The stability of the analyte was established for standard and sample solutions under conditions as prescribed in the method. The purpose of this procedure was to determine the time during which the standard and sample solutions remain stable. In this validation three solutions were studied: Stock standard solution, Working standard solution and Sample solution.

RESULTS AND DISCUSSIONS

Method Development and Optimization

Lamivudine, ziduvudine and Abacavir drug substance has a reported pKa of 3.5 and logP value of 5.2 14. The main aim of the chromatographic method is to achieve the separation of precursors, intermediates and the main components Lamivudine, ziduvudine and Abacavir. From the UV profiling it was found that the suitable wavelength for the Lamivudine, ziduvudine and Abacavir drugs and its related impurities is 270 nm. Hence it was concluded anticipating the possible base line interferences at lower wavelength 270 nm was selected as the detection wavelength for the quantification of Lamivudine, ziduvudine and Abacavir, its identified and unidentified impurities. When developing a reversed phase method for basic compounds, like Lamivudine, ziduvudine and Abacavir, you can expect a more robust method when using acidic mobile phases. Based on the experimental data and the opted wavelength it was found ammonium hydrogen and a diammonium buffer is suiable.

The chromatographic separation was achieved on an Inertsil ODS-3V 250 x 4.6 mm, 5 um column. The gradient liquid chromatographic method employs solution A and Solution B as mobile phase. Mobile phase A contains 2.3 g of Ammonium dihydrogen phosphate and 1.32 g of Di ammonium hydrogen phosphate anhydrous into a beaker containing 1000 ml of water and mix, Adjust pH of the solution to 3.9±0.05 with 50% trifluoroacetic acid.and mobile phase B is HPLC grade methanol. The flow rate was 1.0 mL/min. The HPLC gradient program was set as Time / mobile phase A/ mobile phase B. The column temperature was maintained at 50 °C, sample compartment temperature is maintained at 5 °C and the detection wavelength was 270 nm for identified and unidentified impurities. The injection volume 10 µL. The peak shape of Lamivudine, ziduvudine and Abacavir were found to be symmetric and well separated by its potential process impurities and degradants. In the optimized conditions, Lamivudine, ziduvudine and Abacavir, Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir were well separated with a resolution greater than 3.5 and the typical retention times for Lamivudine, ziduvudine and Abacavir, Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir were about 19.4, 46.06, 56.34, 9.65, 17.43, 5.43, 48.04, 31.04, 32.93, 39.36 and 73.66 respectively. The system suitability results were tabulated and the developed method for Lamivudine, ziduvudine and Abacavir and its impurities was found to be specific (Table-1)

Forced Degradation

Forced degradation samples were analyzed with a sample concentration of 300 mg/mL of Lamivudine equivalent with above mentioned chromatographic conditions using a PDA detector to monitor the homogeneity and purity of the Lamivudine, ziduvudine and Abacavir peaks. Degradation was not observed under stress condition like, heat and humidity (60 °C and 70 % RH for 7 days) oxidative (3 % H2O2 at RT for 24 hours) and light exposure in solid state and liquid state. Very mild degradation of drug material was observed during acid hydrolysis (0.1 N HCl 24 hours at 80 °C) however the drug is more susceptible to base hydrolysis (0.1 N NaOH 24 hours at 60 °C) (Fig. 2a,2b,2c). The RS studies were carried out for the stress samples against a Lamivudine, ziduvudine and Abacavir qualified reference standard. The mass balance (%assay + % sum of all related compounds + % sum of all degradants) were calculated for all of the stressed samples and were found to be more than 95 %. Peak purity test results obtained from PDA confirm that the Lamivudine, ziduvudine and Abacavir peaks were homogeneous and pure in all analyzed stress samples, which confirms the stability indicating power of the developed method.

Method Validation

Precision

The injection (system) precision was evaluated by performing six replicate injections for its related compounds at 100 % working standard concentration. The % relative standard deviation of 6 injections was calculated, the % RSD for Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir were found to be 1.30, 1.30, 1.37, 1.81, 1.56, 0.39, 0.87 and 1.99% respectively. The RSDs of the % recovery values meet the requirement of not more than 10% for all impurities. (Table-2)

Linearity

For all eight impurities, a linear calibration curve was obtained ranging from QL to 0.16 %. The

analytical data and linearity results for Zidovudine Related compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir were tabulated in (Table-2).
The coefficient of determination (r^2) is 0.9975, 0.9990, 0.9978, 0.9999, 0.9997, 0.9997, 0.9980 and 0.9987 respectively, which meets the specification for the r^2 value of not more than 0.99, confirming the linearity of the method.

Quantification limit (QL) and Detection limit (DL)
The quantification limit (QL) and detection limit (DL) of its related impurities are tabulated (Table-2).

Accuracy
The related compounds of Lamivudine, ziduvudine and Abacavir can also be determined accurately over a concentration range varying from QL to 160 % of their respective target analyte concentrations when in Lamivudine, ziduvudine and Abacavir sample solution. The percentage recovery for the related compounds Zidovudine Related Compound-C, Lamivudine Diastereomer, Lamivudine- carboxylic acid, Zidovudine Related compound-B, Zidovudine Related compound-A, Cyclopropyldiaminopurine Abacavir, Descyclopropyl Abacavir, and O-Pyrimidinyl Abacavir were ranged from 83.24 to 118 (Table-2).

Robustness
In all the deliberate varied conditions (flow rate and column compartment temperature) the resolution between Lamivudine, lamivudine diastereomer and its impurities was greater than 1.5, illustrating the robustness of method.

Solution Stability
The Stock standard solution, Working standard solution and Sample solution were prepared as per the method, after dispensing an amount for the testing of initial time , the solutions were stored in volumetric flasks and kept in refrigerator (5 ±3 °C) prior to the testing at each time interval of 1st week, 2nd week, 3rd week and 4th week for Stock standard solution and 24 hours, 48 hours, 72 hours and 7th day for Working standard solution and Sample solution, the flasks were taken out of the refrigerator, allowed to equilibrate to room temperature before use.
The % recovery of each analyte meets the requirement of 90 to 110 % after 4th week for Stock standard solution; however working standard is stable up to 7th day. No extra peaks detected, no peaks disappeared and no peak areas are increased or decreased by more than the respective QL level after 72 hours in case of sample solution. Therefore sample solution was found to be stable for 72 hours. However working standard and sample stored at room temperature showed a stability of 24 hours.

CONCLUSION

A stability indicating HPLC related compounds method was developed for the quantification of Lamivudine, Ziduvudine, Abacavir and its potential impurities in active pharmaceutical ingredients and its dosage forms. The developed method is specific, precise, accurate, linear and robust for Lamivudine, ziduvudine, Abacavir and its impurities. Degradation products formed during forced decomposition studies were very well separated from analyte peak, which demonstrates that the developed method was specific and stability indicating. The run time of 7 minutes indicates excellence of sub 2µ particle size in terms of speed and selectivity. This method can be used to carry out the analysis of Lamivudine, Ziduvudine and Abacavir drug product in regular quality check and stability samples.

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Table 1: Forced degradation results

Impurities	Stress Condition					
	HCl	NaOH	H2O2	UV	Thermal	Humidity
Zidovudine Related compound-C (imp 1)	0.104	0.021	0.014	0.101	0.591	0.013
Lamivudine Diastereomer (imp 2)*	ND	0.013	ND	ND	ND	ND
Lamivudine	26.55	22.85	16.94	27.46	28.70	26.63
Lamivudine- carboxylic acid (imp 3)*	0.020	0.018	0.014	0.019	0.028	0.019
Zidovudine Related compound-B (imp 4)	ND	ND	ND	ND	0.116	ND
Zidovudine Related compound-A (imp 5)	ND	ND	ND	ND	ND	ND
Cyclopropyldiaminopurine Abacavir (imp 6)	4.810	0.101	0.020	0.018	1.770	0.022
Descyclopropyl Abacavir (imp 7)	0.013	0.044	0.019	0.013	0.034	0.008
Zidovudine	39.67	41.93	41.76	35.96	30.91	42.09
Abacavir	28.33	33.42	32.25	36.29	32.92	31.07
O-Pyrimidinyl Abacavir (imp 8)	ND	ND	ND	ND	ND	ND

ND: Not detected, *Process impurities

Table 2: Summary of method validation results

Validation Parameter	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5	IMP 6	IMP 7	IMP 8
System Precision (% RSD of peak area)	1.30	1.30	1.37	1.81	1.56	0.39	0.87	1.99
% Difference of Retention time (Last two std.)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
% Difference of Retention time (Last std and check std.)	0.000	0.001	0.000	0.001	0.000	0.001	0.001	0.000
Resolution Tailing Factor Column efficiency	- 0.94 44198	2.5 0.93 27616	- 1.06 8215	- 1.14 189902	- 1.04 29895	- 1.02 129093	- 1.10 245422	- 1.12 12970
Linearity Slope Intercept r^2 RRF	 798785 390 0.9975 1.000	 9258796 -212 0.9990 1.159	 6902752 80 0.9978 -	 7024056 95 0.9999 0.879	 8279906 27 0.9997 1.037	 8362019 -112 0.9997 1.047	 5739805 34 0.9980 0.719	 4984011 -41 0.9987 0.624
Quantitation limit(µg/mL)	0.0214	0.0216	0.0218	0.0318	0.0214	0.0208	0.0309	0.04128

Detection limit(µg/mL)	0.0071	0.0072	0.0073	0.0106	0.0071	0.0069	0.0103	0.01376
Accuracy Mean % Recovery at QL	NA	91.3 %	97.8 %	96.4 %	84.2 %	91.2 %	94.7 %	112.6 %
100 %		91.1 %	95.5 %	100.2 %	91.3 %	93.7 %	103.8 %	116.9 %
160 % of target		91.9 %	97.7 %	96.3 %	94.3 %	94.4 %	101.5 %	112.2 %
Intermediate Method Precision (% RSD)	1.25	1.48	1.60	1.82	1.55	1.28	1.22	2.38
Stability of Solutions								
Stock Standard Solution (5±3°C)	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks
Working Standard Solution (5±3°C)	7 days	7 days	7 days	7 days	7 days	7 days	7 days	7 days
Working Standard Solution (Room temp)	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours
Sample Solution (5±3°C)	72 hours	72 hours	72 hours	72 hours	72 hours	72 hours	72 hours	72 hours
Sample Solution (Room temp)	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours	24 hours

SD: Standard Deviation, RSD: Relative Standard Deviation, NA: Not Applicable

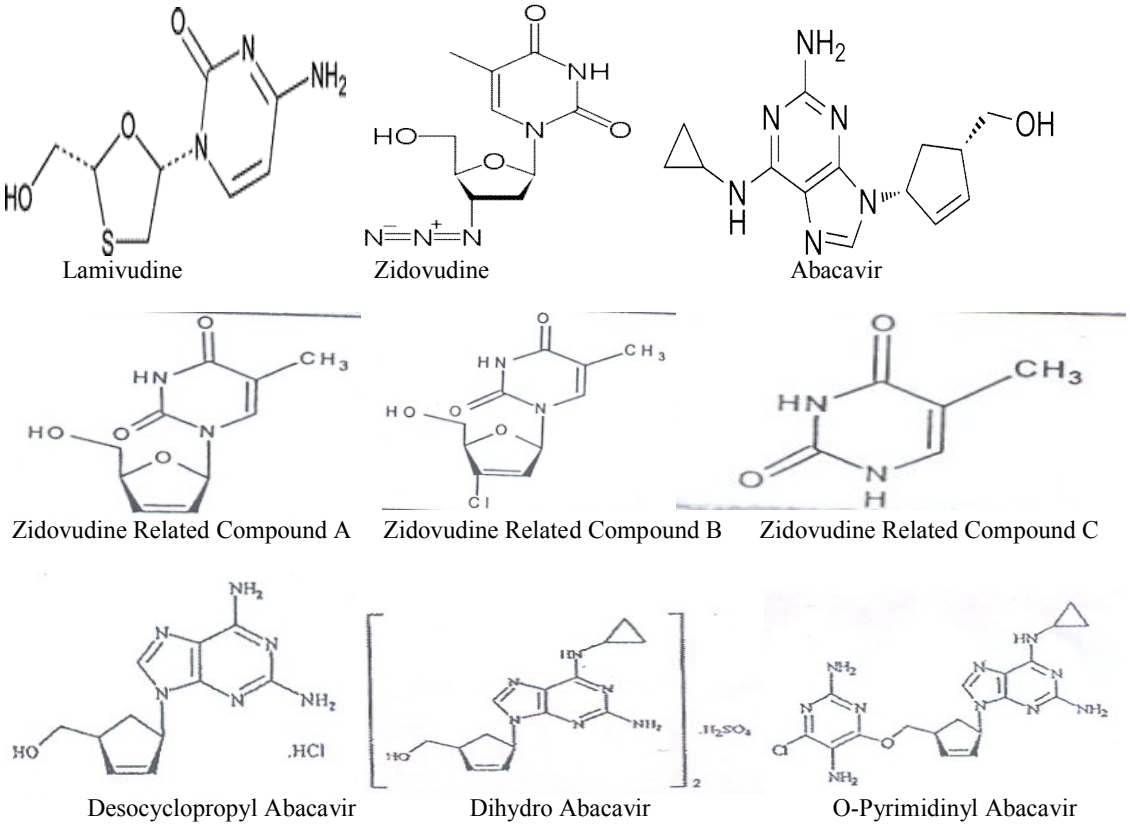

Fig. 1: Lamivudine, Zidovudine, Abacavir and its related compounds structures.

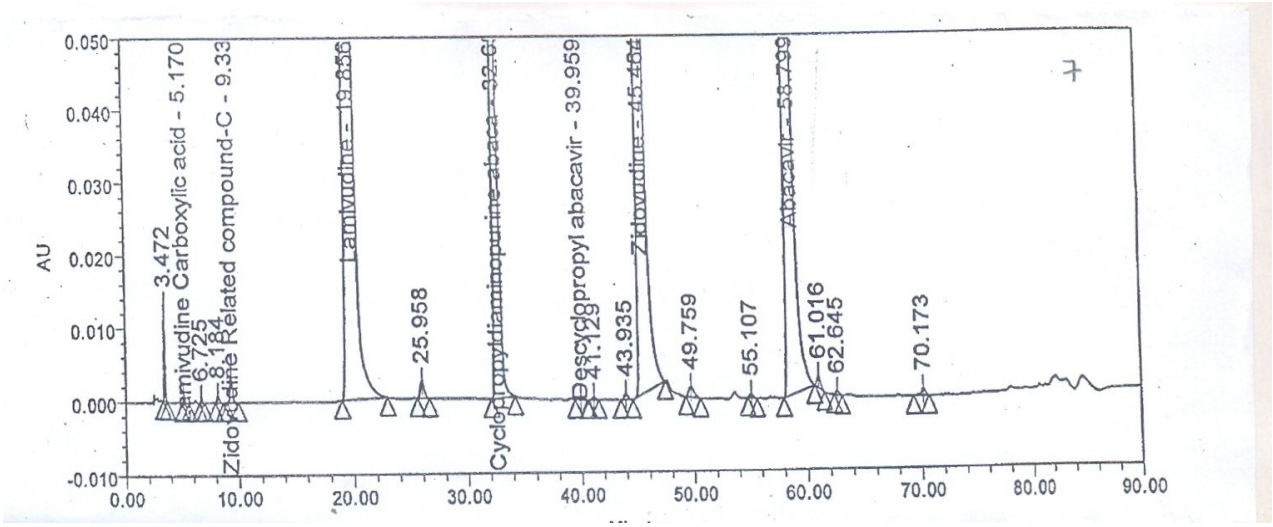

Fig. 2a :Sample solution-Acid degradation chromatogram

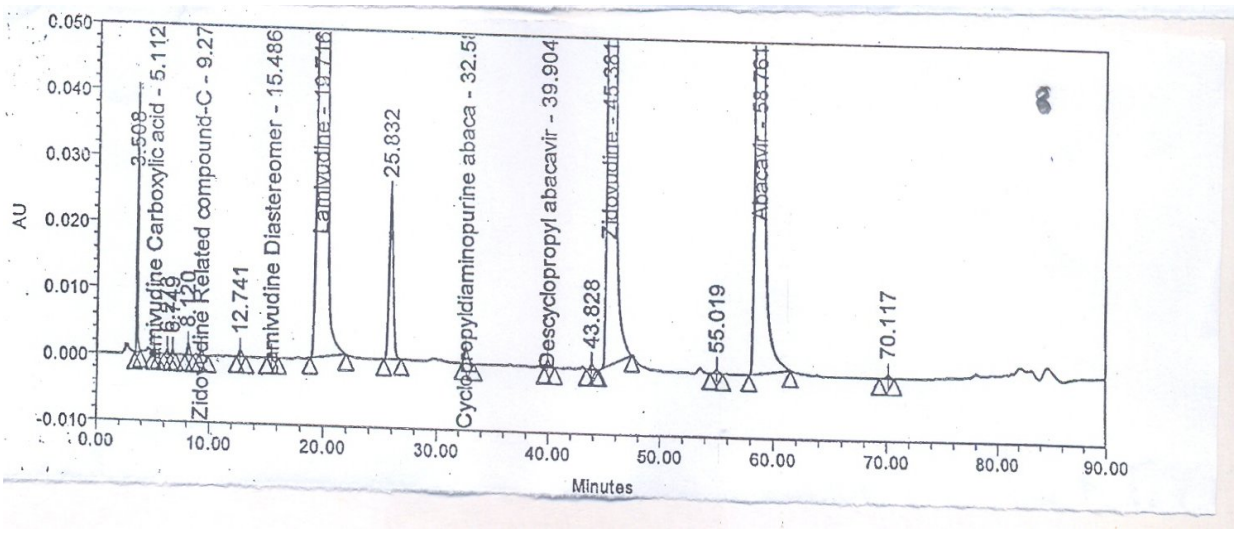

Fig. 2b:Sample solution-Base degradation chromatogram

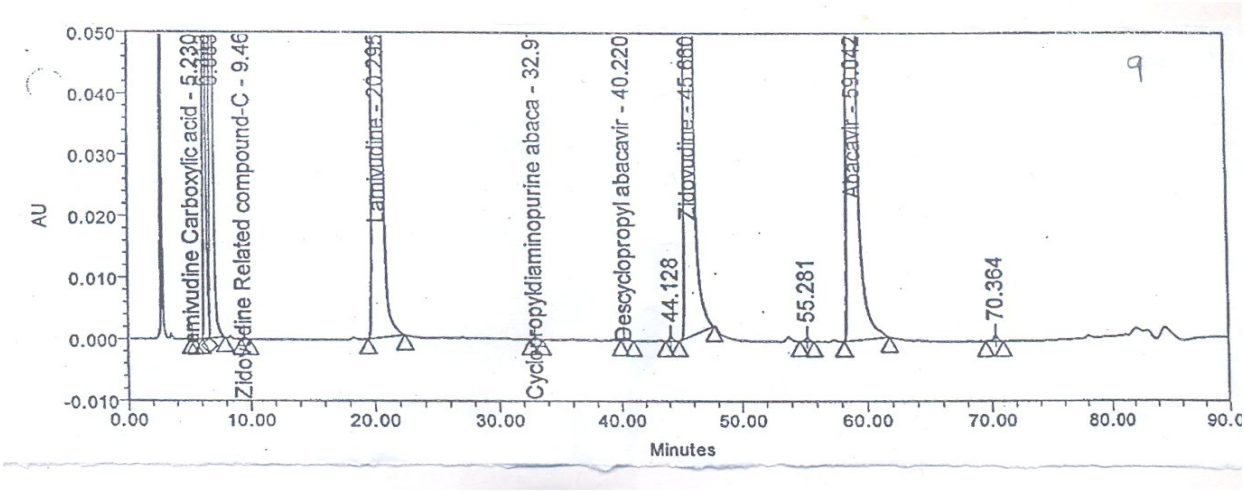

Fig. 2c :Sample solution-Peroxide degradation chromatogram

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