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# A new stability indicating method was developed and validated for the simultaneous estimation of Lamivudine and Abacavir in tablet dosage form by RP-HPLC.

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**ABSTRACT**

A simple and new isocratic RP-HPLC method was developed and validated for the estimation of lamivudine and abacavir in pharmaceutical dosage form. The chromatographic separation was performed on Agilent Zorbax column (150×4.6mm, 5µm), mobile phase used for the analysis was prepared by the combination of 32.5 parts of methanol and 67.5 parts of 0.1% ortho phosphoric acid to prepare 32.5 : 67.5 (v/v) mixture. The run time for the separation was fixed at 7min and the flow rate was maintained at 0.8 ml/min with the detection wave length of 257nm. The column temperature was maintained at 28°c±5 and performed the hplc analysis. The retention times found to be 3.25 min and 2.17 min for abacavir and lamivudine respectively. Under these optimized conditions the respective drugs were shown symmetrical peaks with low tailing factor and high peak area without interference of any expients. The proposed method was validated under the ICH guidelines and this method can be successfully used for the routine quality control analysis of combined dosage form.

**Keywords**: Abacavir, Lamivudine, RP-HPLC, method development and validation.

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## INTRODUCTION

Human immunodeficiency virus (HIV) infection is recognized as a chronic illness. Antiretroviral drugs (ARV) are the class of drugs which are mainly used for the treatment of HIV. These drugs do not completely cure HIV, but they can minimize the amount of virus, and can keep the virus away from destroying immune system of the patient's body. Today, more than 20 antiretroviral drugs are approved to treat HIV [1]. A clear understanding of the viral replication and its interaction with host cell factors has led to the development of a large number of effective antiretroviral drugs (ARVs) [2].

Abacavir is an antiretroviral agent and is used in many HAART (highly active antiretroviral therapy) regimens [3]. Abacavir is a deoxy-guanosine base and is metabolized into carbovir triphosphate. It is an active intracellular agent and a nucleoside analogue reverse-transcriptase inhibitor of HIV type 1 (HIV-1) replication. It has similar in vitro potency to other nucleoside analogues [4, 5]. Use of abacavir in combination with ⩾3 antiretrovirals can be part of a successful salvage therapy as well [6 to 8]. Abacavir is Chemically known as [(1S, 4R)-4-[2-amino-6-(cyclopropylamino)purin-9-yl]cyclopent-2-en-1-yl]methanol [9].

Lamivudine (3TC), the negative enantiomer of 2'-deoxy-3'-thiacytidine, is a dideoxynucleoside analogue used in combination with other agents in the treatment of HIV-1 infection and as monotherapy in the treatment of hepatitis B virus (HBV) infection [10]. Lamivudine is a promising reverse transcriptase inhibitor. It is well tolerated, produces a rapid and profound decrease in serum HBV DNA levels in patients with chronic hepatitis B [11], and induces histologic improvement [12]. Lamivudine Chemically known as "4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one" [13]. Literature survey reveals that only few reports are available for the determination of abcavir and lamivudine in mixed dosage form. Hence we planned to develop a new, simple, precise, accurate, and stability indicting method for the simultaneous estimation of abacavir and lamivudine. Accordingly the authors made several attempts to optimize the conditions and validated for documenting the capabilities of the proposed method.

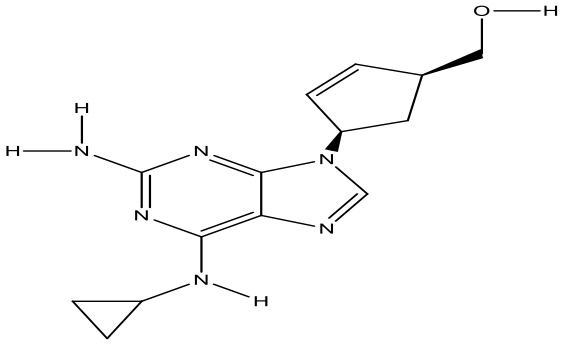

**Figure 1: Molecular structure of the Abacavir**

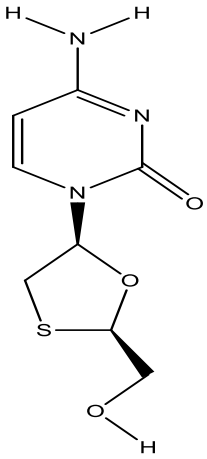

**Figure 2: Molecular structure of the Lamivudine**

**Assay procedure**

Weighed accurately 20 tablets of the drugs and made in to fine powder by crushing with motor and pestle. Now from these drugs powder 10 mg of abacavir and 27mg of lamivudine were taken and transferred in 250ml volumetric flask separately. Add 100ml of methanol to dissolve the content and make the flask filled with the mobile phase. It is sonicated for 30min and then passed through 0.45µm PVDF filter to remove the impurities.5ml of this solution of each flask was taken ad again added 100ml of diluent. Now, taken 20µl of this solution for 8 times and injected to the HPLC system. Observed the peaks obtained and % recovery of these drugs was calculated fro their regression equations of the respective linear curves.

## MATERIALS

**Instrumentation:**

**Equipment**

The chromatographic separation was carried out on an LC – 10 – ATVP HPLC system. The equipment of the system as follows.

| | |
|---|---|
| Instrument | Shimadzu class VP |
| Binary pump | LC – 10 ATVP |
| Auto sampler | SIL- 10 ADVP |
| Column Oven | CTO- 10 AVP |
| Detector | SPD – 10 AVP |
| System controller | SCL – 10 |
| Software | LC solutions |

**Chromatographic conditions:**

HPLC separation was tested by using Agilent Zorbax column (150×4.6mm,5µm). The mobile phase consists the mixture of 32.5 : 67.5 % (v/v) methanol and 0.1% ortho phosphoric acid (pH adjusted to 3.0 with acetic acid) operated on isocratic mode and it was filtered through 0.45 micro membrane. The flow rate is 0.8 ml/min with detection wavelength of 257 nm at 25ºC and the injection volume is 20 µL.

**Preparation of standard solution:**

To prepare standard stock solutions of the respective drugs weigh 10mg of abacavir and 27mg of lamivudine in to a seperate100 ml volumetric flasks, dissolved with 32.5:67.5%v/v of mobile phase and milli Q water and solicited until the solutions were completely dissolves. Calibration curve is linear, containing 6 non-zero standards were prepared by using diluents in the concentration range of 5.062 - 50.57 µg/ml and 2.50 - 24.98 µg/ml for lamivudine and abacavir respectively. The mixture was solicited for 5 min.

**Preparation of sample solution:**

To prepare test sample solutions of the drugs weigh 20 tablets of the respective drugs. The tablets were made in to a fine powder by using pestle and mortar. Now these drug powdered samples were transferred into a separate 100 ml volumetric flasks and made into homogeneous solution by using diluents. From this stock, quality control samples were prepared at 3 concentration levels: LQC (12.64 and 6.24 µg/mL), MQC (25.29 and 12.49 µg/mL), HQC (37.93 and 18.73 µg/mL) for lamivudine and abacavir to obtain low, median and high concentration quality control samples. Linearity curve has been calibrated and evaluated by using quality control samples.

**Method Development and Validation:**

To get the ideal separation of the drugs we made an effort by changing the concentration of the solvent, selection of solvent, pH strength with different buffers as ammonium formate, and ortho phosphoric acid, di- potassium hydrogen orthophosphate, in combination with acetonitrile, methanol and tetra hydro furan, type of columns such as like Hypersil- BDS- C18, Symmetry C18, Ymc-pack C18, Ymc-packpro; Spheris

orb C18, Phenomenex C18, different combinations for the preparation of mobile phase mixture. After variety of combinations, we fixed the mobile phase as the mixture of 32.5 parts of methanol and 67.5 parts of ortho phosphoric acid. Agilent Zorbax column (150×4.6mm,5µm) was used for the chromatographic separation at a temperature of 27°c±5, the flow rate was maintained at 0.8ml/min with the concentration ranges of 5.06-50.57 µg/ml and 2.50-24.98 µg/ml for lamivudine and abacavir respectively. The detection wave length was fixed at 257nm. By these optimized conditions the separation of the drugs was shown symmetrical peaks with low tailing factor and high peak area. Whereas by changing the flow rate an unacceptable tailing factor and poor peak shape was found. The chromatogram showing the separation of the drugs is given in figure 3.

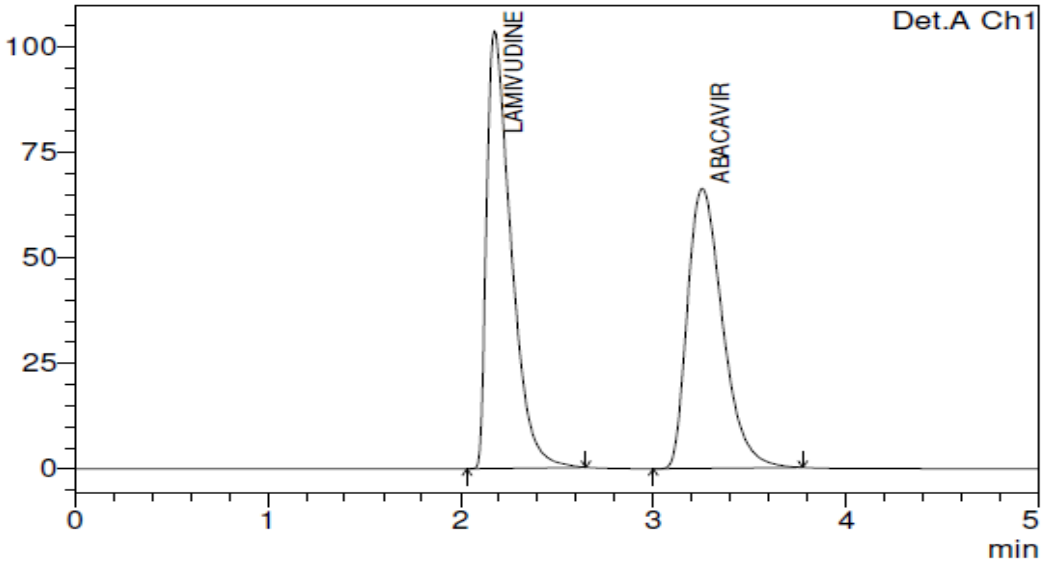


**Figure 3: Chromatogram showing the separation of drugs**

## RESULTS AND DISCUSSION

**System suitability:**

System suitability tests are the integral part of the chromatographic system. It is mainly used to check that the reproducibility of the system is suitable or not for the analysis performed [14]. It can be measured by injecting six replicate injections of standard drug samples of abacavir and lamivudine to the HPLC system for the analysis and then inject six injections of the sample drugs of the respective drugs. Now compare the chromatograms obtained in both the cases. System suitability parameters were calculated as the percentage of relative standard deviation of retention time and area, number of theoretical plates and resolution. The data observed from the analysis was given in table 1.

**Table 1: System suitability data of abacavir and lamivudine**

| Sample ID | Abacavir | | Lamivudine | |
|---|---|---|---|---|
| | Peak Retention Time | Peak Area | Peak Retention Time | Peak Area |
| 1 | 3.25 | 839440 | 2.17 | 909550 |
| 2 | 3.24 | 831997 | 2.17 | 908710 |
| 3 | 3.21 | 831831 | 2.15 | 906889 |
| 4 | 3.22 | 793839 | 2.15 | 865971 |
| 5 | 3.22 | 778875 | 2.14 | 851280 |
| 6 | 3.07 | 738296 | 2.00 | 817408 |
| MEAN | 3.20 | 802379.67 | 2.13 | 876634.67 |
| STDEV | 0.07 | 39623.64 | 0.06 | 38188.85 |
| %CV | 2.07 | 4.94 | 3.04 | 4.36 |

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**Specificity:**

The peak purity of the drugs (lamivudine and abacavir) can be measured by comparing the retention times of the standard and sample solutions of the respective drugs. Initially we prepared the blank solution which is diluent used for the analysis with expeints, and then prepared the sample solution of the drugs. Now transfer these two solutions through 0.45µ membrane, performed the analysis and compare the peaks obtained. There is no extra peaks were observed along with the drug peak, i.e; a good correlation was found between the standard and sample solutions of lamivudine and abacavir. The corresponding chromatograms were given in figures 4, 5 and 6.

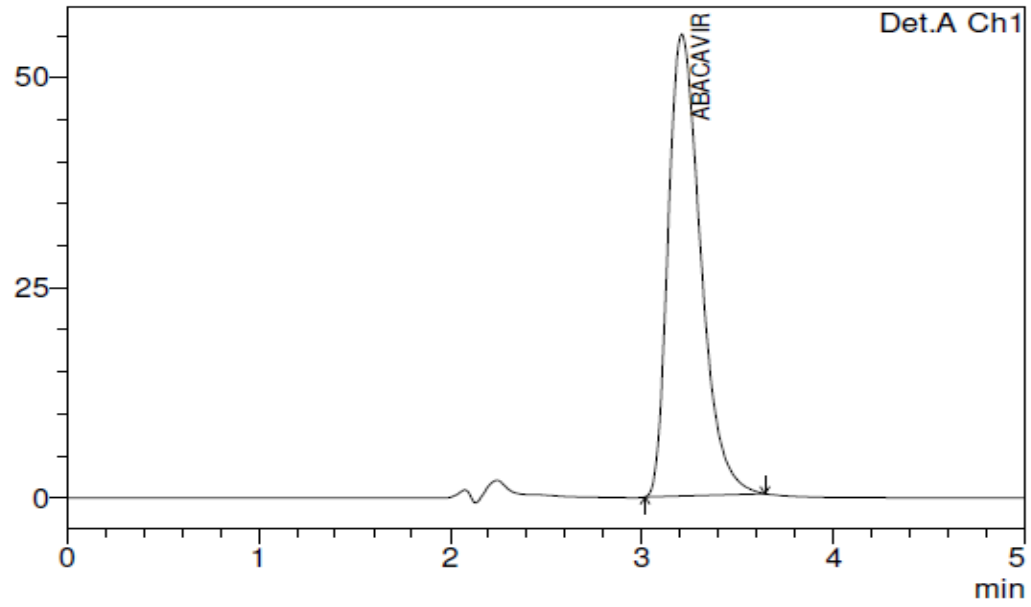


**Figure 4: Specificity of Abacavir**

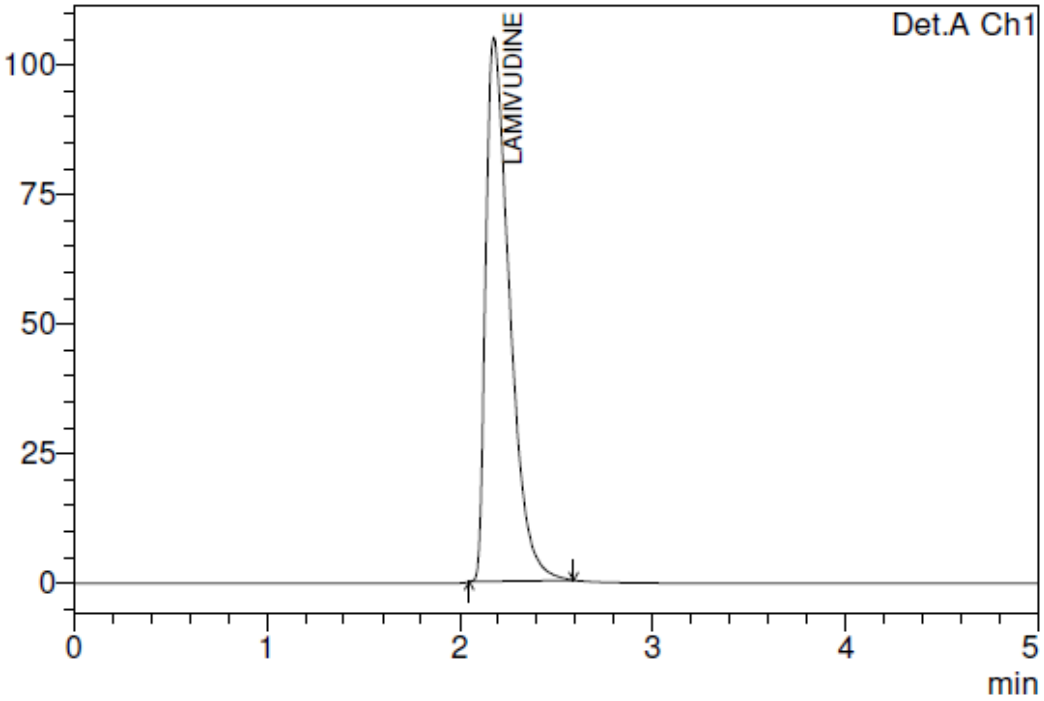


**Figure 5: Specificity of Lamivudine**

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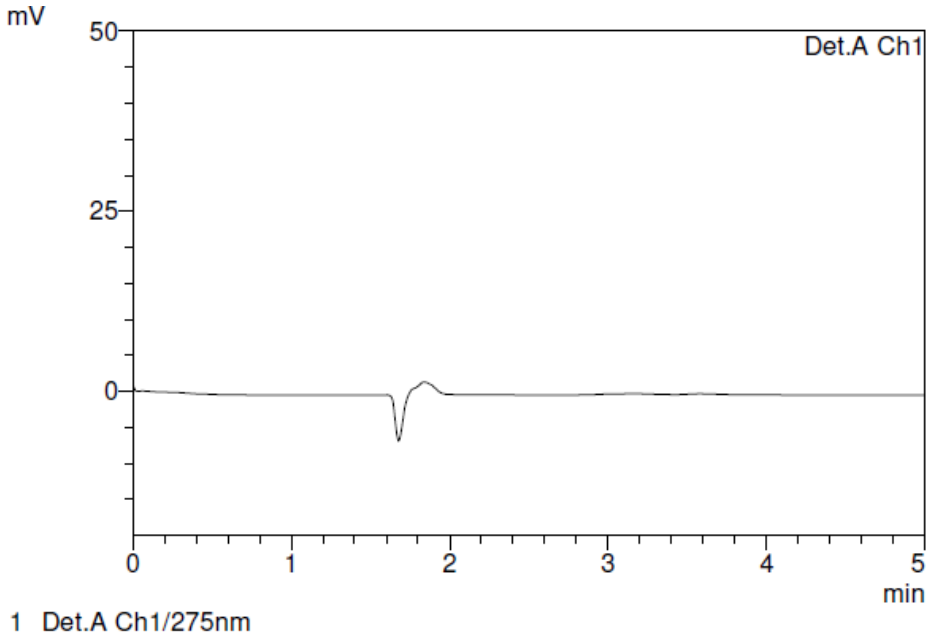


**Figure 6: Specificity of Blank**

**Linearity:**

Linearity can be measured by using external standard regression method [15]. It can be accomplished by preparing the standard solutions of abacavir and lamivudine from their stock at different concentration levels including the main concentration levels. 20 µL of each sample was injected to the HPLC system for the analysis at the detection wavelength of 257 nm. The chromatograms obtained were observed and from this data the linearity curves were plotted for the respective drugs and the regression of the plots were computed. The correlation coefficients of abacavir and lamivudine are 0.9997 and 0.9993 respectively. The corresponding linearity curves were shown in the figures 7 and 8.

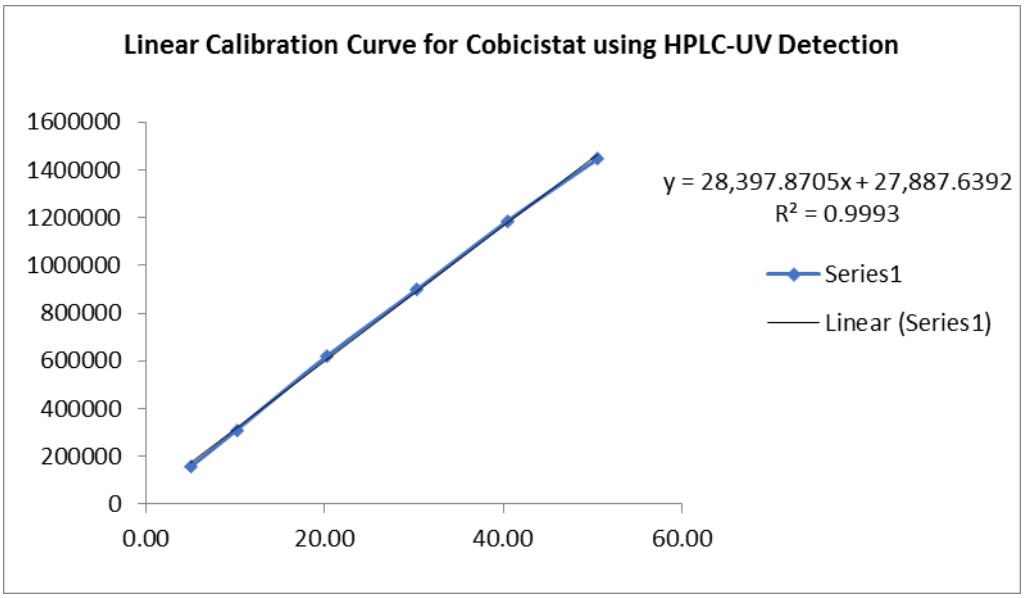


**Figure 7: Linearity of Lamivudine**

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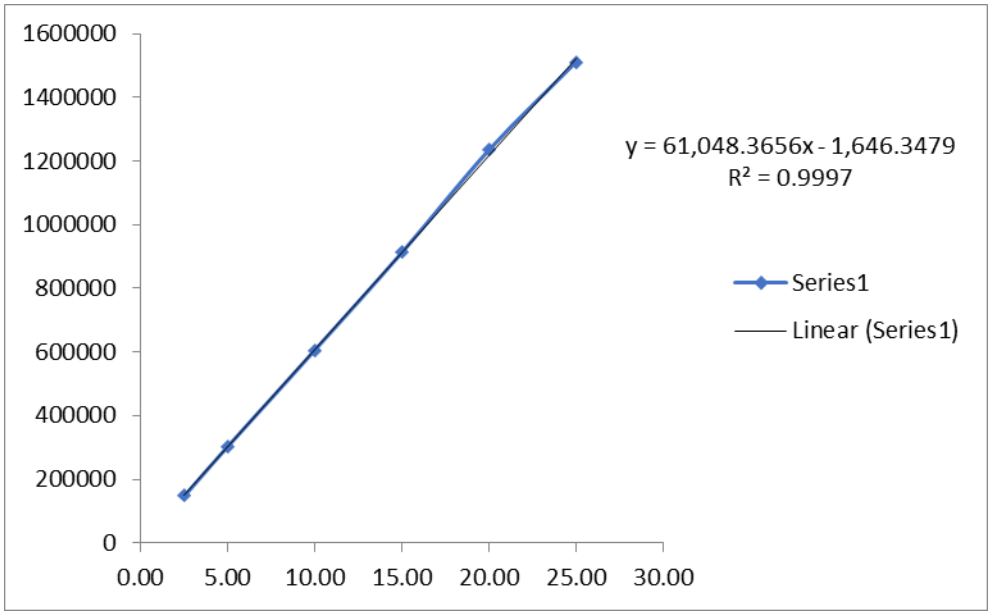


**Figure 8: Linearity of Abacavir**

**Precision:**

Precision is the degree of repeatability. It can be measured by repeating the experiment for three times in the same day (intraday precision), and each time in three different days (interday precision) of the same sample solution. It can be expressed in terms of standard deviation and relative standard deviation. The data observed was given in the tables 2 and 3.

**Accuracy:**

Accuracy is a measure of degree of closeness of the experimental value with the actual value, and it can be expressed in terms of percentage of recovery. Weigh tablet powder of the drugs accurately, transferred into 100ml of volumetric flask and make up the flask with the diluents upto the mark. It is sonicated for 10 min with handshaking. Recovery of the drug can be studied by increasing the concentration of the pure analyte 80%, 100%, and 120% of three different concentrations and it is added to the pre determined working standard solution of the drug. Performed the analysis, standard deviation and relative standard deviation of all the percentage recovery values were measured. These values are listed in the tables 2 and 3.

**Table 2: Data study for inter and intra-day accuracy & precision of abacavir by HPLC**

| Sample ID | LQC | MQC | HQC |
|---|---|---|---|
| Nominal Concentration (µg/mL) | 6.24 | 12.46 | 18.73 |
| Day 1 | | | |
| Mean Concentration (µg/mL) | 6.28 | 12.46 | 18.70 |
| SD | 0.03 | 0.04 | 0.03 |
| % CV | 0.00 | 0.00 | 0.00 |
| Day 2 | | | |
| Mean Concentration (µg/mL) | 6.24 | 12.52 | 18.78 |
| SD | 0.04 | 0.02 | 0.01 |
| % CV | 0.00 | 0.00 | 0.00 |
| Day 3 | | | |
| Mean Concentration (µg/mL) | 6.30 | 12.44 | 18.70 |
| SD | 0.03 | 0.02 | 0.02 |
| % CV | 0.00 | 0.00 | 0.00 |

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**Table 3: Data study for inter and intra-day accuracy & precision of lamivudine by HPLC**

| Sample ID | LQC | MQC | HQC |
|---|---|---|---|
| Nominal Concentration (µg/mL) | 12.64 | 25.29 | 37.96 |
| Day 1 | | | |
| Mean Concentration (µg/mL) | 12.68 | 25.23 | 37.96 |
| SD | 0.03 | 0.1 | 0.04 |
| % CV | 0.02 | 0.00 | 0.00 |
| Day 2 | | | |
| Mean Concentration (µg/mL) | 12.72 | 25.33 | 37.93 |
| SD | 0.04 | 0.02 | 0.01 |
| % CV | 0.03 | 0.00 | 0.00 |
| Day 3 | | | |
| Mean Concentration (µg/mL) | 12.60 | 25.28 | 37.98 |
| SD | 0.03 | 0.03 | 0.03 |
| % CV | 0.02 | 0.00 | 0.00 |

**Ruggedness:**

Ruggedness means reproducibility of the results from different analysts by different systems under specified test conditions. A mixture of MQC was injected to the HPLC system for three times by two different persons with two different systems. The results obtained from two analysts were correlated and the results were listed in the tables 4 and 5.

**Table 4: Ruggedness results of abacavir and lamivudine by analyst-1**

| Sr NO | Abacavir | | | Lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.23 | 810118 | 1.26 | 2.17 | 884041 | 1.72 |
| 2 | 3.41 | 817622 | 1.25 | 2.37 | 889807 | 1.72 |
| 3 | 3.21 | 826071 | 1.26 | 2.18 | 902090 | 1.75 |
| MEAN | 3.28 | 817937.00 | 1.26 | 2.24 | 891979.33 | 1.73 |
| ST DEV | 0.11 | 7981.16 | 0.01 | 0.11 | 9218.51 | 0.02 |
| % CV | 3.35 | 0.98 | 0.46 | 5.03 | 1.03 | 1.00 |

**Table 5: Ruggedness results of abacavir and lamivudine by analyst-2**

| Sr NO | abacavir | | | lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.18 | 813232 | 1.26 | 2.16 | 895046 | 1.75 |
| 2 | 3.17 | 814111 | 1.25 | 2.16 | 902185 | 1.75 |
| 3 | 3.15 | 821986 | 1.25 | 2.14 | 901337 | 1.74 |
| MEAN | 3.17 | 816443.00 | 1.25 | 2.15 | 899522.67 | 1.75 |
| ST DEV | 0.02 | 4820.46 | 0.01 | 0.01 | 3900.02 | 0.01 |
| % CV | 0.48 | 0.59 | 0.46 | 0.54 | 0.43 | 0.33 |

**Robustness:**

Robustness is a measure of reproducibility of the results obtained by varying the test conditions like changing the composition of the mobile phase, $P^H$ strength, and flow rate values etc. Changing the test conditions doesn't vary the results like retention time, tailing factor, and theoretical plates etc. Thus the proposed method is said to be robust.

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**Table 6: Actual mobile phase (87.5:32.5) results (Robustness)**

| Sr NO | Abacavir | | | Lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.18 | 813232 | 1.26 | 2.16 | 895046 | 1.75 |
| 2 | 3.17 | 814111 | 1.25 | 2.16 | 902185 | 1.75 |
| 3 | 3.15 | 821986 | 1.25 | 2.14 | 901337 | 1.74 |
| MEAN | 3.17 | 816443.00 | 1.25 | 2.15 | 899522.67 | 1.75 |
| ST DEV | 0.02 | 4820.46 | 0.01 | 0.01 | 3900.02 | 0.01 |
| % CV | 0.48 | 0.59 | 0.46 | 0.54 | 0.43 | 0.33 |

**Table 7: mobile phase variation (30:70) results (Robustness)**

| Sr NO | Abacavir | | | Lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.73 | 807430 | 1.26 | 2.16 | 874581 | 1.85 |
| 2 | 3.73 | 789823 | 1.28 | 2.16 | 844574 | 1.81 |
| 3 | 3.72 | 827854 | 1.27 | 2.16 | 884432 | 1.85 |
| MEAN | 3.73 | 808838.50 | 1.28 | 2.16 | 864503.00 | 1.83 |
| ST DEV | 0.01 | 26891.98 | 0.01 | 0.00 | 28183.86 | 0.03 |
| % CV | 0.19 | 3.32 | 0.55 | 0.00 | 3.26 | 1.55 |

**a) Variation of Mobile Phase:**

The composition of the mobile phase was changed from 87.5 : 32.5 to 30 : 70 for abacavir and lamivudine, and the results obtained were listed in the tables 6 and 7.

**b) Variation flow rate:**

The flow rate of the analysis was changed from 0.8 ml/min to 0.7 ml/min for abacavir and lamivudine. The results are given in the tables 8 and 9.

**Table 8 study of actual flow rate 0.8 ml/min**

| Sr NO | Abacavir | | | Lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.17 | 829811 | 1.69 | 2.17 | 928565 | 1.27 |
| 2 | 3.21 | 825643 | 1.24 | 2.15 | 927281 | 1.72 |
| 3 | 3.24 | 831997 | 1.27 | 2.17 | 908710 | 1.73 |
| MEAN | 1.41 | 829150.33 | 1.4 | 6.49 | 921518.66 | 1.2 |
| ST DEV | 0.03 | 3228.1 | 0.25 | 2.16 | 41788.02 | 0.01 |
| % CV | 0.02 | 0.00 | 0.1 | 0.33 | 0.04 | 0.85 |

**Table 9: Study of variation of flow rate 0.7 ml/min**

| Sr NO | Abacavir | | | Lamivudine | | |
|---|---|---|---|---|---|---|
| | Retention Time | Peak Area | Tailing Factor | Retention Time | Peak Area | Tailing Factor |
| 1 | 3.70 | 921085 | 1.26 | 2.49 | 104511 | 1.75 |
| 2 | 3.72 | 951554 | 1.26 | 2.49 | 1036364 | 1.79 |
| 3 | 3.72 | 956819 | 1.27 | 2.49 | 1017922 | 1.73 |
| MEAN | 3.71 | 943152.67 | 1.26 | 2.49 | 1033099.00 | 1.76 |
| ST DEV | 0.01 | 19291.62 | 0.01 | 0.00 | 13836.50 | 0.03 |
| % CV | 0.31 | 2.05 | 0.46 | 0.00 | 1.34 | 1.74 |

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**LOD and LOQ:**

These limits are normally applied to related substances in the drug product. Lowest detection limit (LOD) is the lowest amount of the analyte that we can detect in the sample under the proposed conditions.

Limit of quantification (LOQ) means the lowest amount of the analyte that we can measure in the sample at specified precision and accuracy under the proposed conditions. LOD and LOQ of the drugs can be detected by diluting their concentration and that would yield signal to noise ratio 3:1 for LOD and 10:1 for LOQ.

$$LOD = 3.3 \frac{\sigma}{S}$$

$$LOQ = 10 \frac{\sigma}{S}$$

Where $\sigma$ is the standard deviation of the response and 'S' is the slope of the linearity plot.

For the study of LOD and LOQ 6 replicate injections of the analyte of lowest concentrations were injected to the HPLC system for the analysis in the calibration range. The results obtained were studied and tabulated in the tables 10 and 11.

**Table 10: LOD and LOQ data of Abacavir**

**LOD**

| Sr NO | DRUG | |
|---|---|---|
| | Retention Time | Peak Area |
| 1 | 3.31 | 9995 |
| 2 | 3.31 | 9506 |
| 3 | 3.31 | 9618 |
| MEAN | 3.31 | 9706.33 |
| ST DEV | 0.00 | 256.19 |
| % CV | 0.00 | 2.64 |

**LOQ**

| Sr NO | DRUG | |
|---|---|---|
| | Retention Time | Peak Area |
| 1 | 3.30 | 15355 |
| 2 | 3.31 | 10867 |
| 3 | 3.31 | 18304 |
| MEAN | 3.31 | 14842.00 |
| ST DEV | 0.01 | 3744.95 |
| % CV | 0.17 | 25.23 |

**Table 11: LOD and LOQ data for Lamivudine**

**LOD**

| Sr NO | DRUG | |
|---|---|---|
| | Retention Time | Peak Area |
| 1 | 2.23 | 15494 |
| 2 | 2.22 | 15232 |
| 3 | 2.22 | 15545 |
| MEAN | 2.22 | 15423.67 |
| ST DEV | 0.01 | 167.94 |
| % CV | 0.26 | 1.09 |

**LOQ**

| Sr NO | DRUG | |
|---|---|---|
| | Retention Time | Peak Area |
| 1 | 2.20 | 23133 |
| 2 | 2.20 | 16435 |
| 3 | 2.21 | 23571 |
| MEAN | 2.20 | 21046.33 |
| ST DEV | 0.01 | 3999.53 |
| % CV | 0.26 | 19.00 |

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**Degradation studies:**

For the drug product the active components which are impurities, exciepients, placebo components, and residual substances may be present during the manufacturing process or from the container or closure system. These exciepients should not show any extra peaks along with the drug peaks. The chromatograms should be legible, stable, and symmetric.

**Stress studies:**

Stress studies were conducted under acidic, alkaline, oxidation, and photolytic (UV) conditions. In the proposed method abacavir and lamivudine were reasonably stable under the above stress conditions except in alkaline medium. Both the drugs showed some degradation criteria in the alkaline medium. The respective chromatograms were shown in the figures from 9 to 18.

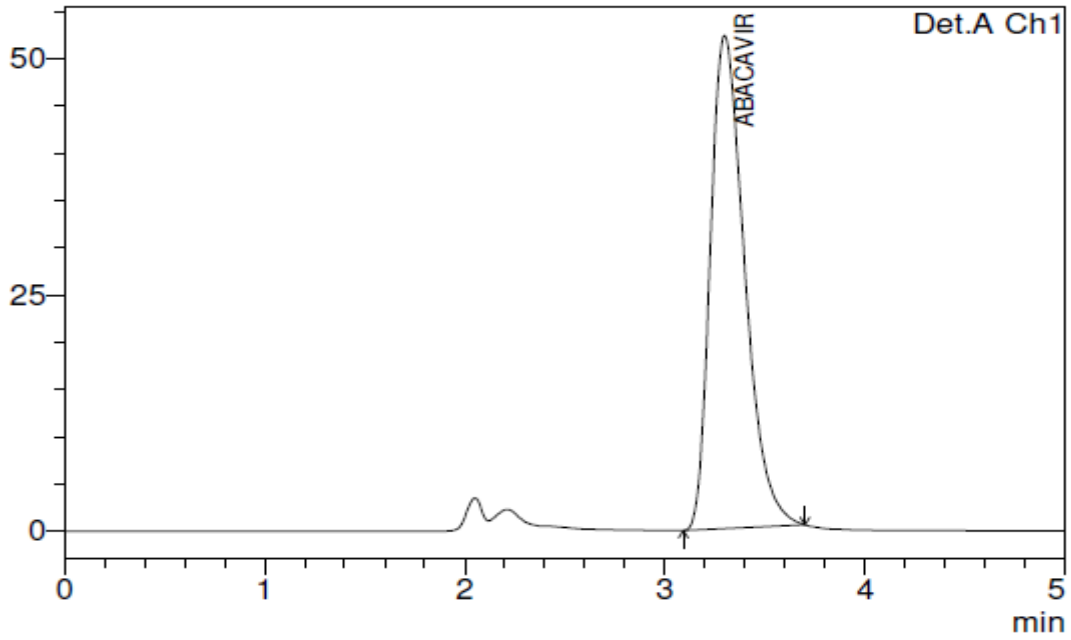


**Figure 9: Chromatogram of fresh sample of Abacavir**

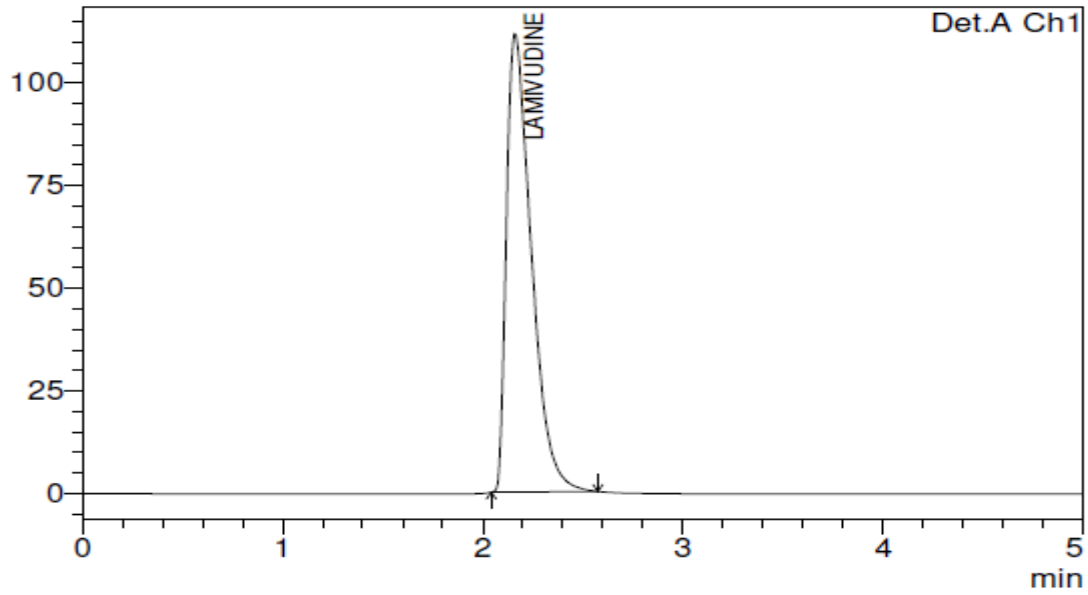


**Figure 10: Chromatogram of fresh sample of Lamivudine**

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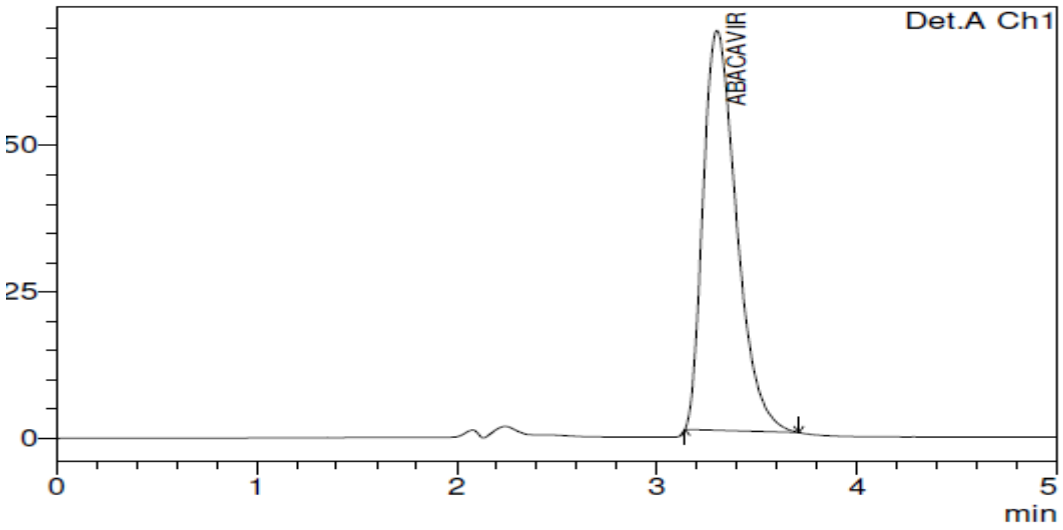


**Figure 11: Chromatogram of Abacavir by acidic stress**

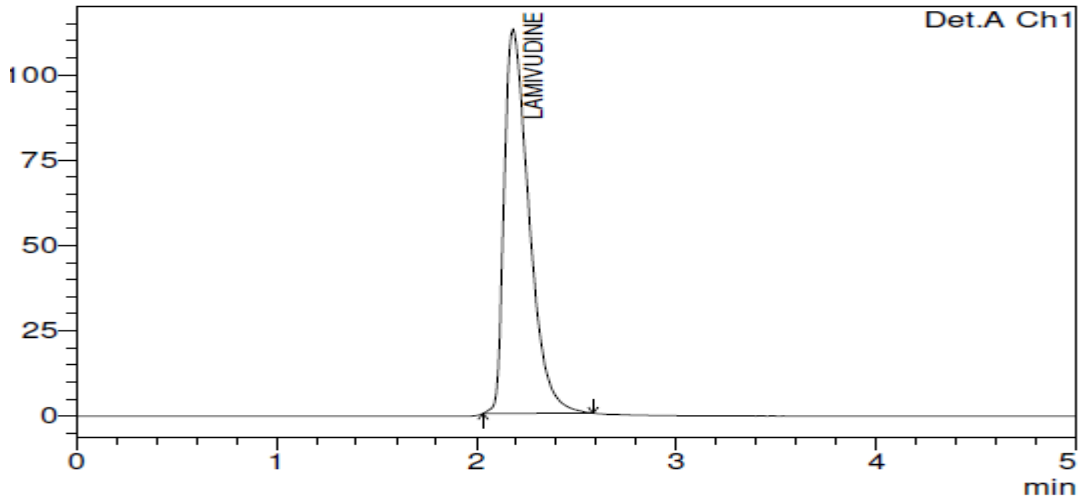


**Figure 12: Chromatogram of Lamivudine by acidic stress**

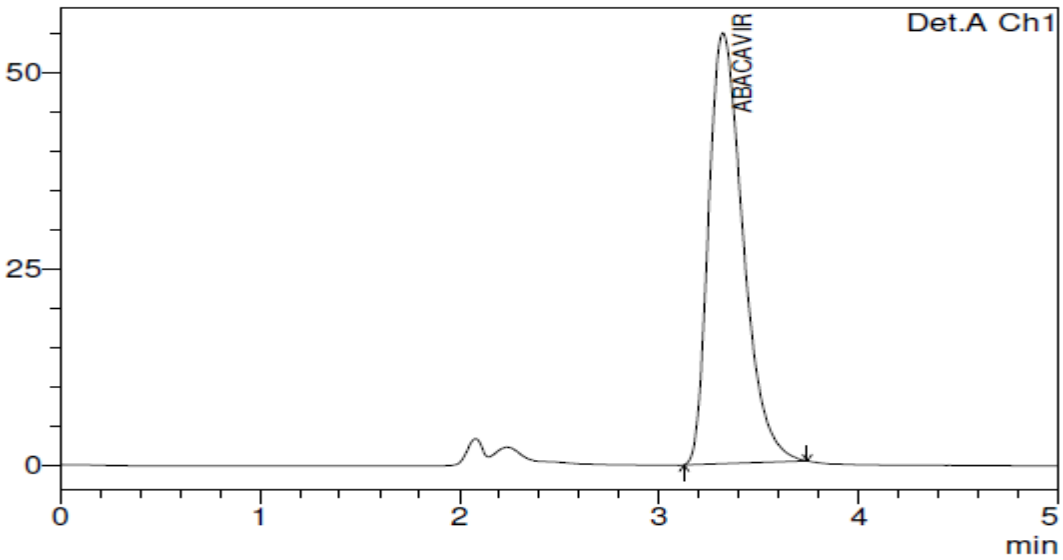


**Figure 13: Chromatogram of Abacavir by oxidative stress**

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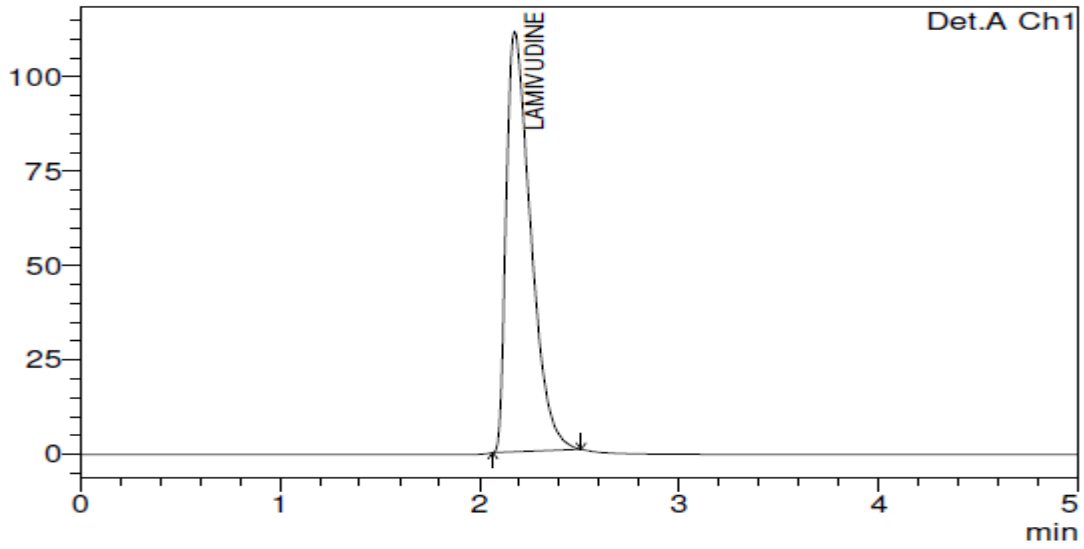


**Figure 14: Chromatogram of Lamivudine by oxidative stress**

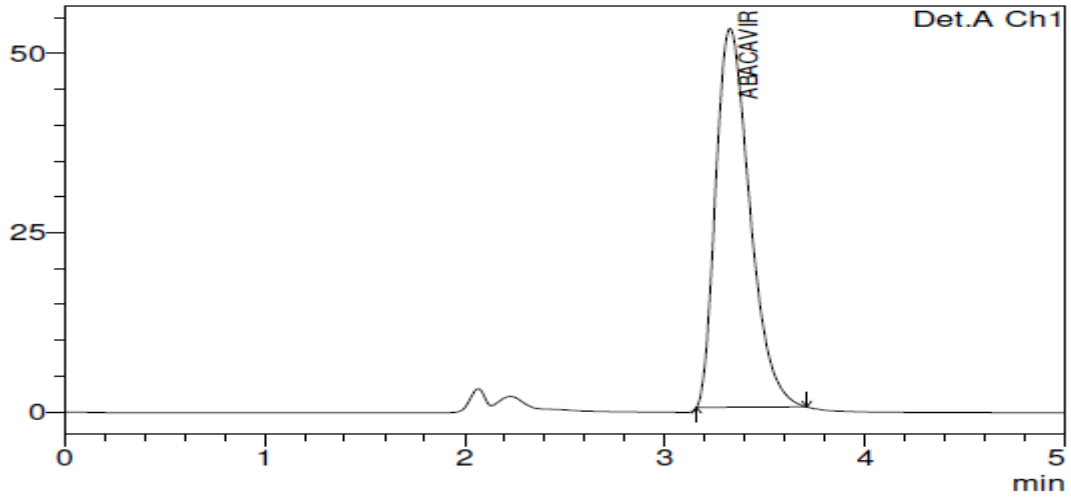


**Figure 15: Chromatogram of Abacavir by photolytic stress**

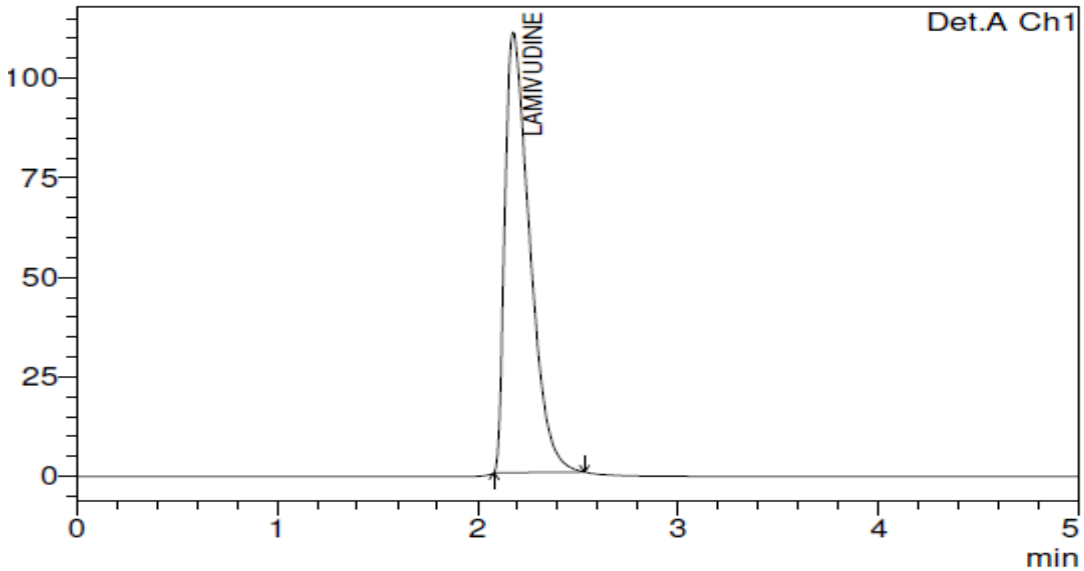


**Figure 16: Chromatogram of Lamivudine by photolytic stress**

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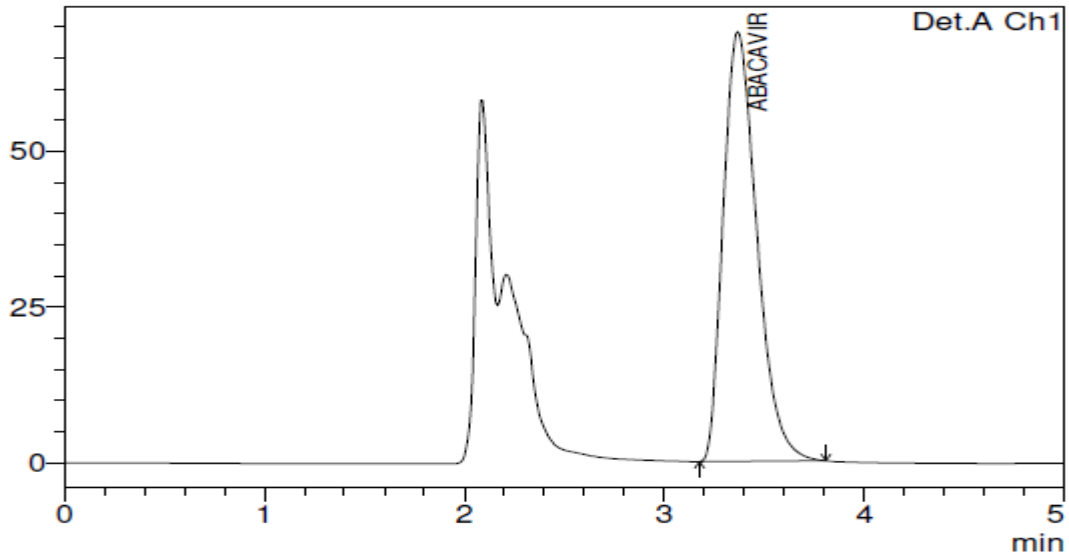


**Figure 17: Chromatogram of Abacavir by degradation by alkaline stress**

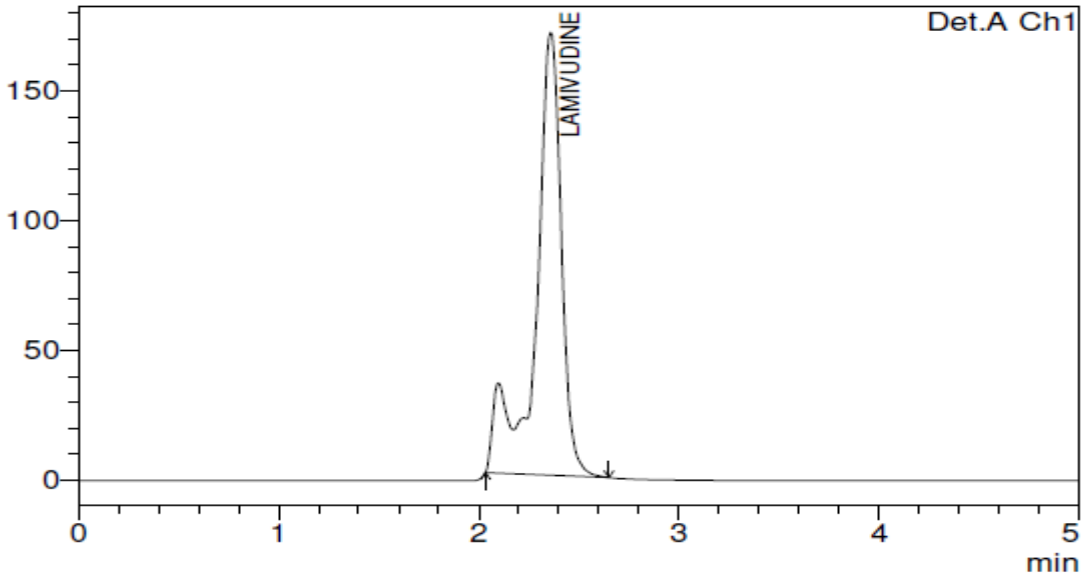


**Figure 18: Chromatogram of Lamivudine by degradation by alkaline stress**

## CONCLUSIONS

This method was validated on the report of precision, accuracy, specificity, linearity. In all the cases the method was stable with acceptance criteria followed by ICH guidelines. Mobile phase used in this method was very commonly available and it is sufficient for the quantification analysis of abacavir and lamivudine either in single dosage or in combined form of formulations in many pharmaceutical laboratories. These drugs were separated in less than 6 min with low tailing factor and good resolution without any interference of exciepients. This demonstrates the proposed method was simple, fast, accurate, specific with good retention time, and low cost. Thus the developed method is suitable for the routine quality control analysis of the drugs in bulk and tablet dosage form.

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