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# RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND RALTEGRAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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## ABSTRACT

A simple, specific, precise, accurate, rapid and reproducible efficient reversed phase HPLC method with PDA detector has been developed and validation for simultaneous estimation of Lamivudine (LAM) and Raltegravir (RAL) in pharmaceutical dosage form. Chromatography was performed on aInertsil ODSC$_{18}$column (150mmX4.6mm, 5.0µmparticlesize) with a 50:50v/v mixture of 0.1% orthophospharic acid buffer: acetonitrileasa mobile phase. The detection of the combined dosage form was carried out at 242nm and flow rate employed was0.9 ml/min. The retention times were 1.9±0.3 and 4.3±0.3min for Lamivudine and Raltegravil respectively. Linear was established in the concentration range of 15.0to75.0µg/ml for LAM and 30to150µg/ml for RAL with a correlation coefficient of both drugs for found to be 0.998 and 0.999. The recoveries obtained were 99.18-100.60% for LAM and 98.94-101.07% for RAL. Similarly the %RSD value for precision was also found to be within the acceptable limit. The method was validated according to international conference of harmonization guidelines in terms of accuracy, precision, specificity, robustness, linearity and other aspects of analytical validation. The results of the analysis were validated statistically and recovery studies confirmed the accuracy and precision of the proposed method. Developed method was rapid and convenient which could be successfully applied for the routine control of both the component.

## KEYWORDS

RP-HPLC, Lamuvidine, Raltegravir and Validation.

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## INTRODUCTION

Lamivudine (LAM) is chemically (2R, cis)-4-amino-1-(2-hydroxymethyl-1, 3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. It is an HIV-1 nucleoside analogue reverse transcriptase and HBV polymerase inhibitor[1,2]. Similarly, Raltegravir (RAL) is chemically N-[(4-Fluorophenyl) methyl]-1, 6-dihydro-5-hydroxy-1-methyl-2[1-methyl-1-[[(5-

methyl-1, 3, 4-oxadiazol-2-yl) carbonyl] amino] ethyl]-6-oxo-4 pyrimidine carboxamide mono potassium salt. It is a human immunodeficiency virus (HIV) integrase strand transfer inhibitor[1,2]. The chemical structure of LAM and RAL were shown in Figure No.1.

Recently, RAL (300mg) and LAM (150mg) a combined formulation was approved by FDA for the treatment of HIV-1 infection. The action of RAL (300mg) and LAM (150mg) in combination are showing equivalent action to that of individual doses of RAL (400 mg) and LAM (150 mg) taken simultaneously. In the combined formulation, content of RAL was less than that of single formulation of RAL with having similar action. Therefore because of the synergistic effect of RAL with LAM the intake of single formulation of RAL can be reduced by using combined formulation[1,2]. Presently, it is not commercially available in market. So the study was performed in the laboratory prepared binary mixture of LAM and RAL[1].

Literature survey indicates that various analytical methods like UV[3-10], HPLC[2,3,11-17], HPTLC[3,18,19] and LC-MS[20,21] are available for the estimation of LAM either individually or combined dosage form and biological sample. Similarly, for estimation of RAL, few analytical methods such as UV[22-25], HPLC[2, 26-31,35], UPLC[32], LC-MS[33-34]-and HPTLC[35] have been reported in either alone or combined dosage form and biological sample. To best of our knowledge one HPLC method for simultaneous estimation of LAM and RAL in bulk active pharmaceutical ingredient (API) dosage form has been recently published[2]. This reported method has not showing a systematic optimization procedure for the separation and quantitation of LAM and RAL. Although, these methods employed a time consuming trial and error approach for giving potential information concerning the sensitivity of the factors on the analytes separation. But it did not provide the information concerning interaction between factors[2].

Correspondingly, this manuscript described the optimization of an isocratic RP-HPLC method for the routine quality control analysis of LAM and RAL in laboratory prepared binary mixture. In spite of that Development and optimization of isocratic RP-HPLC method is a tedious process that involves instantaneous determination of several factors[37-40]. It is recognized to provide risk-based understanding of the analytical as well as major factors affecting the performance of analytical method[42,43]. Furthermore, it provided thorough understanding of the possible risk and associated with interaction among the method variables, respectively[45,46].

Therefore, the aim of present study was to develop, optimize and validate sensitive, and cost-effective RP-HPLC method for estimation of LAM and RAL in laboratory prepared binary mixtures.

## MATERIAL AND METHODS

### REAGENTS AND CHEMICALS

Pure drugs LAM (99.95%) and RAL (99.95%) were kindly supplied by Richer Pharmaceuticals (Prasanthinagar, Hyderabad, India) and Emcure Pharmaceuticals (Pune, India) respectively. Acetonitrile (HPLC grade) and Orthophospharic acid from Fischer scientific and triple distilled water. Mobile phase was filtered using 0.45µ nylon filters made by Millipore water, sonicated and degassed by using Ultra Sonicator bath. The Pharmaceuticals LAM and RAL (DYMISTA) were purchased from local pharmacy (Meda Pharmaceuticals).

### Instrumentation and chromatographic conditions

A Labindia HPLC system consist of LC-10AT-vp Solvent delivery system (pump), SPD – 10Avp – UV visible detector, Rheodyne injector with 20µL loop volume, Spinchrom CFR software was used for data collections and processing. The mobile phase was composed of 50% Orthophospharic acid (0.1%): 50% Acetonitrile: 10 % (0.05mM) phosphate buffer (at pH 3.0), in the various ratios with a flow rate of 1.2 ml/min. Separation was achieved using Intersil ODS $C_{18}$ column (150mm X 4.6 mm in diameter) with an average particle size of 5µ and the column was kept at an ambient

temperature. The column effluent was monitored at 242 nm by UV detection.

**Phosphate buffer solution ph 2.8**

Dissolve 7.8 g of sodium dihydrogen phosphate R in 900 ml of water R, adjust to pH 2.8 (2.2.3) with phosphoric acid R and dilute to 1000 ml with the same solvent.

**Preparation of mobile phase**

Mix a mixture of above buffer 500ml (50%) and 500ml of acetonitrile HPLC (50%) and degas in ultrasonic water bath for 5minutes. Filter through 0.45µ filter under vacuum filtration.

**Diluen**t

Use the same mobile phase as the diluent.

**Preparation of standard solution**

Accurately weigh and transfer 15mg of Lamivudine and 30mg of Raltegravir working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1ml of Lamivudine and Raltegravir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1ml of Lamivudine and Raltegravir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

**Sample preparation**

Accurately weigh and transfer equivalent to 15mg of Lamivudine and 30mg Raltegravir equivalent weight of the sample into a 10ml clean dry volumetric flask add about 70ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 1ml of Lamivudine and Raltegravir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 3ml of Lamivudine and Raltegravir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

## METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

The initial literature search indicated that many HPLC methods are available for individual drugs and their combination with different drugs. Based on literature search, attempts were made to develop a simple method which has less retention time and high selectivity, top priority was given for complete separation of Lamivudine and Raltegravir. Several mobile phase were tested until good resolution obtained between two drugs. In preliminary experiments all the two Lamivudine and Raltegravir were subjected to separation by reverse phase HPLC equipped with the Intersil ODS C-18 (150mm X 4.6 mm X 5µm) column and with flow rate 0.9mL/min and detection wavelength of 242nm. Column temperature was maintained at ambient. Injection volume is 10µL and runtime is for 10min. The mobile phase consists of Orthophospharic acid buffer and acetonitrile (40:60%v/v). These drugs were able to be separated on the chromatogram but failed in peak purity and peak shape was not good. The effect of mobile phase composition was checked. It improved peak purity. Finally a method developed with Orthophospharic acid buffer: acetonitrile (50:50%v/v). The chromatogram obtained was better than the previous one in all aspects with good peak shape, tailing factor, resolution and theoretical plate as per USP requirement. The retention times of Lamivudine and Raltegravir peaks are about 1.9±0.3 and 4.3±0.3 minutes respectively. The chromatograms were shown in the Figure No.3.

## METHOD VALIDATION

The method was successfully validated as per ICH guideline kQ2 (R1): validation of analytical procedures: text and methodology, international conference on harmonization, Food and Drug Administration, USA, November 2005. The method was validated and parameters were linearity, range, accuracy, precision, LOQ, LOD, and robustness.

**Specificity**

The method is found to be specific and there is no blank or placebo interference.

**Precision**

To check the system precision (repeatability) for peak response obtained with five replicates of standard at specified concentration. The %RSD found to be within 2.0%. To check repeatability (method precision) of the method six individual sample preparations form same batch were prepared and injected the % RSD with six samples found to be within 2.0%. The results obtained were presented in Table No.5.

**Accuracy**

The accuracy of an analytical method is established across its range. Accuracy is performed in three different levels for Lamivudine and Raltegravir. The known quantity of Lamivudine and Raltegravir at 50%, 100% and 150% level is analyzed for each level. The % recovery values for these drugs were found to be in between 99.67% to 101.07% and %RSD values were found to be less than 2.0%. The accuracy results were tabulated in the Table No.3 and 4.

**Linearity and range**

The Linearity of detector response to different concentration of these drugs was studied with a series of working standard solutions prepared by diluting the stock solution with diluents. The Standard plots were constructed between concentrations vs. peak area a linear response of peak area was observed over the concentration range of 15 to 75µg/ml for LAM and 30 to 150µg/ml for RAL. 10µl of each sample was injected under above chromatographic conditions and peak area was measured. The data of linearity curve was summarized in the Table No.2 and Figures No.4 and 5 and it was found that correlation coefficient ($R^2$) and regression analysis were within the limits.

**LOD and LOQ**

These methods were evaluated on the basis of signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. A typical signal-to-noise ratio required for LOQ is 10:1 According to a formula given by miller, the limit of detection (LOD) and limit of quantification (LOQ) were calculated. The resulted are given in Table No.3.

**Robustness**

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was done by changing the column temperature, flow rate and the mobile phase. The results were tabulated in Table No.4.

**Ruggedness**

This is to prove the lack of influence of operational and environmental variables of the test results by using the method. The average of the six preparations and % RSD for the six observations was calculated and recorded. The method precision was carried out as described above using different analyst, different column and different instrument. The % RSD for the six determinations shall be NMT 2.0%. The results are given in Table No.4.

## SYSTEM SUITABILITY

According to USP system suitability tests are an integral part of chromatographic method validation. The tests were used to verify that the reproducibility of the chromatographic system is adequate for analysis. To ascertain its effectiveness system suitability tests were carried out on freshly prepared standard solution. 10µL of solution was injected into the optimized chromatographic system. For system suitability six replicates of working standard samples were injected and the parameters like retention time (RT), theoretical plate (N), peak area, tailing factor and resolution of sample were calculated these results are presented in the Table No.1.

## RESULTS AND DISCUSSION

To optimize the mobile phase various proportions of buffers with acetonitrile were tested. Mobile phase composition was changed and the method development was started by Intersil ODS C-18 (150mm X 4.6 mm X 5µm) column and with flow

rate 0.9mL/min and detection wavelength of 242nm Column temperature was maintained at ambient. Injection volume is 10µL and runtime is for 10min. The mobile phase consists of 0.1% Orthophospharic acid buffer: acetonitrile (50:50 %v/v) was used. The retention times of Lamivudine and Raltegravir peaks are about 1.9±0.3 and 4.3±0.3 minutes respectively.

Quantitative linearity was observed over the concentration range of 15 to 75µg/mL for LAM and 30 to 150µg/mL for RAL. The regression equations of concentration of Lamivudine and Raltegravir are found to be y= 951.5x + 1139 and y= 24312 x+50932 respectively, where y is the peak area and x is the concentration of drugs (µg/ml).

The correlation coefficient of Lamivudine and Raltegravir was found to be 0.999 and 0.999 respectively.

The numbers of theoretical plates obtained were 2559.08 and 3511.35 for Lamivudine and Raltegravir respectively which indicates the efficiency of the column. The high percentage recovery indicates that the proposed method is highly accurate. There is no interference of filters with standard and sample solutions as the difference in responses is within the limit. The %RSD was found to be less than 2.0%.

**Table No.1: System Suitability data for Lamivudine and Raltegravir**

| S.No | Parameters | Lamivudine | Raltegravir |
|---|---|---|---|
| 1 | Retention time (min) | 1.9±0.3 | 4.3±0.3 |
| 2 | Theoretical plate | 2559±163.48 | 3511±163.48 |
| 3 | Tailing factor | 1.65±0.117 | 1.35±0.117 |
| 4 | Resolution | 9.4 | |

**Table No.2: Linearity data showing equation of regression line and coefficient of determination**

| S.No | Drug | Conc. Range (µg/ml) | Equation | $R^2$ |
|---|---|---|---|---|
| 1 | Lamivudine | 15 - 75 | y = 951.5.x + 1139 | 0.998 |
| 2 | Raltegravir | 30 – 150 | y = 24312x+50932 | 0.999 |

**Table No.3: The Accuracy results of Lamivudine**

| S.No | % Concentration (at specific level) | Amount Added (µg/ml) | Amount Recovered (µg/ml) | % Recovery | % RSD |
|---|---|---|---|---|---|
| 1 | 50 | 7.5 | 7.61 | 100.58 | 0.33 |
| 2 | 100 | 15 | 14.88 | 99.18 | 0.81 |
| 3 | 150 | 22.5 | 22.98 | 100.60 | 0.81 |

**Table No.3: Results of LOD and LOQ for Lamivudine and Raltegravir**

| S.No | Drugs | LOD(µg/ml) | LOQ(µg/ml) |
|---|---|---|---|
| 1 | Lamivudine | 1.69 | 2.96 |
| 2 | Raltegravir | 0.46 | 3.12 |

**Table No.4: The Accuracy results of Raltegravir**

| S.No | % Concentration (at specific level) | Amount Added (µg/ml) | Amount Recovered (µg/ml) | % Recovery | % RSD |
|---|---|---|---|---|---|
| 1 | 50 | 15 | 14.84 | 98.94 | 0.65 |
| 2 | 100 | 30 | 29.90 | 99.67 | 0.81 |
| 3 | 150 | 45 | 45.48 | 101.07 | 0.81 |

**Table No.4: Typical Robustness results of Lamivudine and Raltegravir**

| S.No | Conditions | Lamivudine | | | Raltegravir | | |
|---|---|---|---|---|---|---|---|
| | | USP plate count | USP tailing | %RSD | USP plate count | USP tailing | %RSD |
| 1 | Flow rate minus | 1596 | 1.07 | 0.6 | 1807 | 1.02 | 0.3 |
| 2 | Flow rate plus | 2641 | 1.08 | 1.2 | 2769 | 1.00 | 0.2 |
| 3 | Organic composition minus | 1569 | 1.09 | 1.4 | 1717 | 1.07 | 1.5 |
| 4 | Organic composition plus | 1681 | 1.07 | 0.34 | 1853 | 1.06 | 0.26 |
| 5 | Temperature minus | 2267 | 1.02 | 0.22 | 2283 | 1.04 | 0.72 |
| 6 | Temperature plus | 1765 | 1.00 | 0.23 | 1844 | 1.04 | 1.09 |

**Table No.5: Precision results were summarized for Lamivudine and Raltegravir**

| S.No | Injections | Lamivudine(Area) | Raltegravir ( Area) |
|---|---|---|---|
| 1 | 1 | 45921 | 213936 |
| 2 | 2 | 47289 | 219057 |
| 3 | 3 | 46249 | 218423 |
| 4 | 4 | 47758 | 222496 |
| 5 | 5 | 47193 | 219155 |
| 6 | 6 | 46619 | 216019 |
| 7 | Mean | 46838.17 | 218181 |
| 8 | SD | 694.5 | 2935.6 |
| 9 | % RSD | 1.48 | 1.35 |

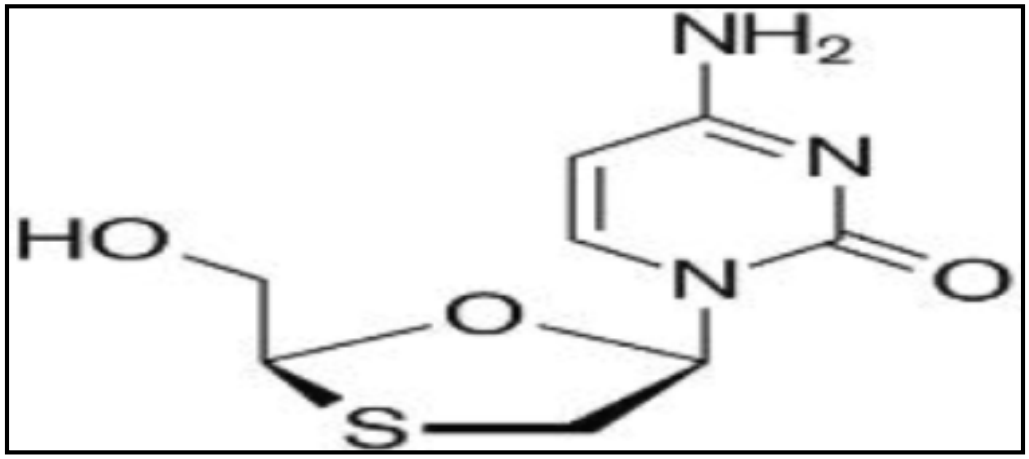


**Figure No.1: Structure of Lamivudine**

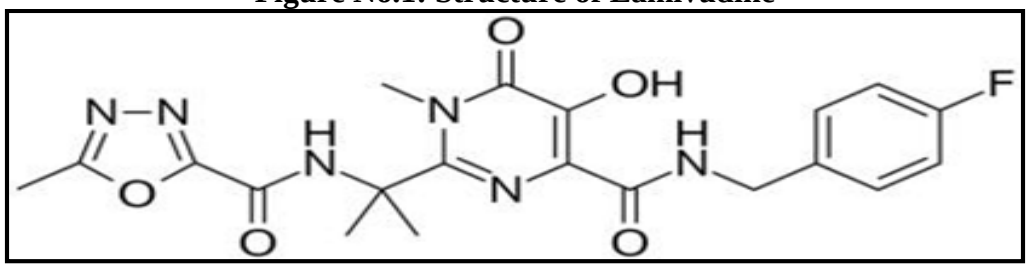


**Figure No.2: Structure of Raltegravir**

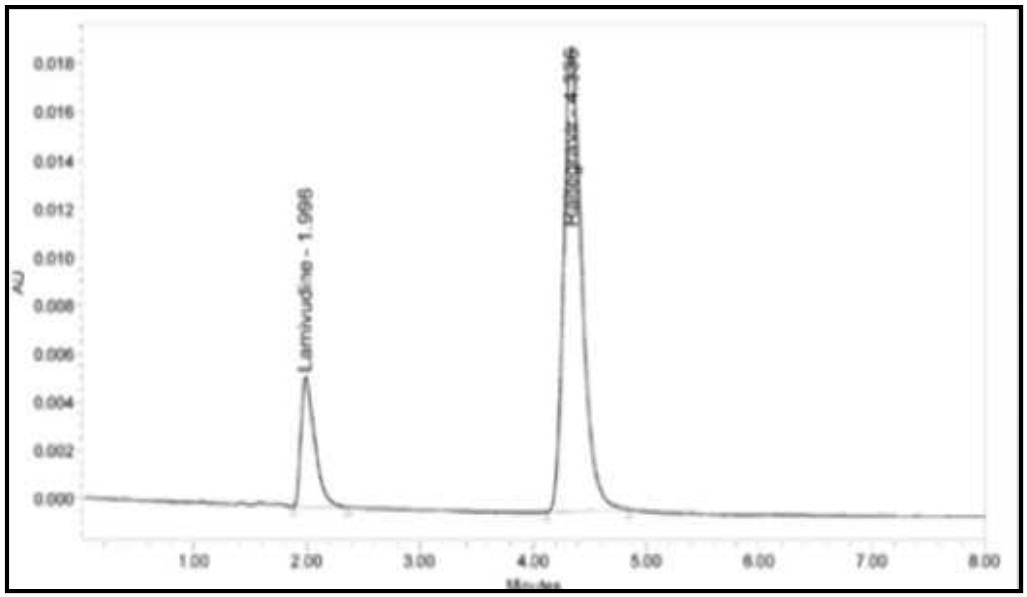


**Figure No.3: Standard chromatogram of Lamivudine and Raltegravir**

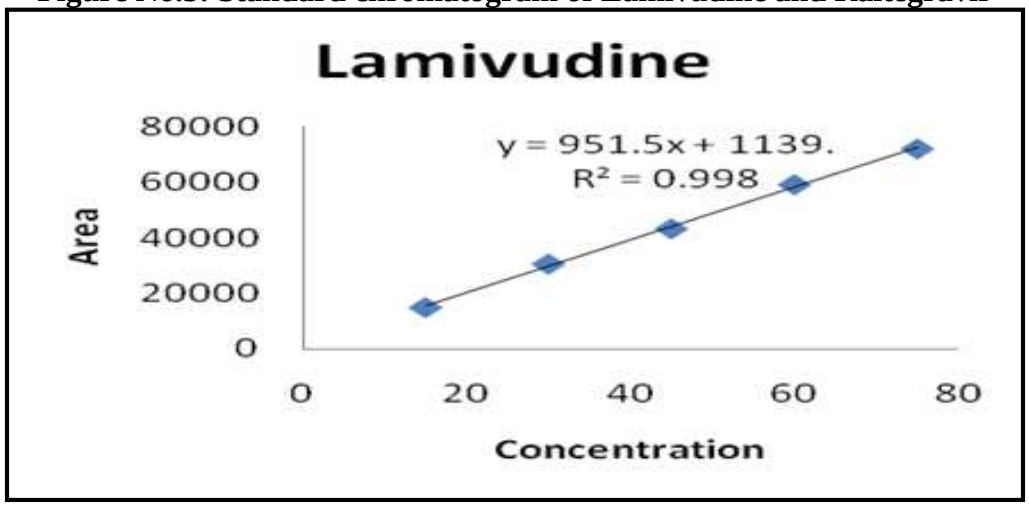


**Figure No.4: Linearity graph of Lamivudine**

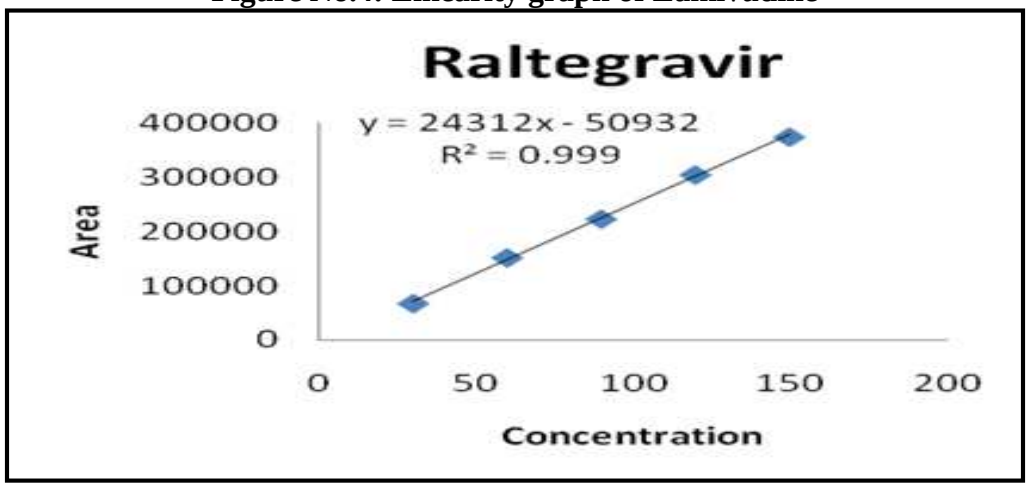


**Figure No.5: Linearity graph of Raltegravir**

## CONCLUSION

A simple, specific, accurate, precise, reproducible and efficient reverse phase high performance liquid chromatography (RP-HPLC) method has been developed and validated as per ICH Q2 (R2) for Lamivudine and Raltegravir in bulk and dosage form, which can be used accurately for quantitative estimation of Lamivudine and Raltegravir for routine analysis of individual and combination of drugs.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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