International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page:www.iscientific.org/Journal.html

Method development and validation of simultaneous estimation of lamivudine and raltegravir in bulk and pharmaceutical dosage forms by RP-HPLC

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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 a Inertsil ODSC$_{18}$ column (250mmX4.6mm, 5.0µ) with a Acetonitrile: Water: Methanol (60:20:20v/v) mixture as a mobile phase. The detection of the combined dosage form was carried out at 260 nm and flow rate employed was 1.0 ml/min. The retention times were 2.2±0.3 and 3.3±0.3min for Lamivudine and Raltegravil respectively. Linear was established in the concentration range of 20 to 60 µg/ml for LAM and 10 to 30 µg/ml for RAL with a correlation coefficient of both drugs for found to be 0.998 and 0.999. The recoveries obtained were 99.39-100.09% for LAM and 98.34-101.37% 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, Lamivudine, Raltegravir and Validation; Robustness and ICH Guidelines.

Full length article **Corresponding Author*, e-mail:drkumaraswamygandla@gmail.com

1. 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 inhibitor1,2. 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 inhibitor1,2. 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 RAL1. Literature survey indicates that various analytical methods like UV [3-10], HPLC [2,3,11-17], HPTLC3 [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. 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.

2. Material and methods

2.1 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 C18 column (150mm X 4.6 mm in diameter) with an average particle size of 5µ and the column was kept at an ambient

2.2 HPLC method development

2.2.1 Mobile Phase Optimization

Initially the mobile phase tried was Acetonitrile: Water and Acetonitrile: Sodium dihydrogen phosphate buffer with varying proportions. Finally, the mobile phase was optimized to Acetonitrile with Sodium dihydrogen phosphate buffer (pH 6.8), in proportion 20:80 v/v respectively.

2.2.2 Optimization of Column

The method was performed with various columns like C18 column, X- bridge column, Xterra, and C8 m, Make: Waters) was found to be ideal as it gaveµcolumn. Phenomenex Luna C18 (4.6mm x 250mm, 5 good peak shape and resolution at 1ml/min flow. Validation methods procedures followed as per ICH guidelines.

2.3 Method development and optimization of chromatographicconditions

2.3.1 Selection of chromatographic condition

Proper selection of the method depends upon the nature of the sample, its molecular weight and solubility. The drugs selected in the present study are polar in nature and hence reversed phase or ion-pair or ion exchange chromatography method may be used. The reversed phase HPLC was selected for the separation because of its simplicity and suitability.

2.3.2 Standard Preparation

Weigh accurately 10 mg Lamivudine Working Reference Standard and 10mg of Raltegravir Working Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark (200µg/ml). After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase (100µg/ml).Further pipette 0.5ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent (5µg/ml).

2.3.3 Optimized Chromatographic condition

Mobile phase: Acetonitrile: Water:Methanol (60:20:20v/v)
Column: Hypersil C18 (4.6×150mm, 5.0 µm)
Flow rate: 1 ml/min
Wavelength: 260 nm
Column temp: Ambient
Sample Temp: Ambient
Injection Volume: 10 µl
Run time: 7 minutes

2.3.4 Selection of mobile phase

Initially the mobile phase tried was methanol, acetonitrile and buffer and water in various proportions. Finally, the mobile phase was optimized to Buffer: acetonitrile in proportion 40:60 v/v respectively.

2.3.5 Optimization of flow rate

The method was performed with flow rates 0.8ml, 1.5ml and 1ml/min. Flow rate of 1ml/min was found to be ideal as it gave sharp peak.Based on the above study, the following chromatographic conditions were selected for the simultaneous estimation of drugs in multi component dosage forms.

2.3.6 Preparation of Buffer

About 7.0g of potassium dihydrogen orthophosphate was dissolved in 1000ml of HPLC grade water and pH 2.5 was adjusted with ortho phosphoric acid. It was filtered through 0.45µm nylon membrane filter and degassed with sonicator. It was used as a diluent for the preparation of sample and standard solution.

2.3.7 Preparation of mobile phase

Mobile phase consists of buffer: Acetonitrile of P^H 2.5 (40:60) was taken sonicated and degassed for 10min and filtered through 0.45 µm nylon membrane filter.

2.3.8 Preparation of samples for Assay Standard preparation

Weigh accurately 10mg Lamivudine Working Reference Standard and 20 mg of Raltegravir Working

Reference Standard is taken in to 100ml volumetric flask and then it was dissolved and diluted to volume with mobile phase up to the mark. After that 50ml of the above solution was taken into 100ml standard flask and made up with mobile phase. (Stock solution). Further pipette 1 ml of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

2.3.9 Content Uniformity

Twenty Tablets were weighed and calculate the average weight of each Tablet. **Brand Name: DUTREBIS, Merck co & Ltd., Film Coated Tablet** Lamivudine-150mg Raltegravir-300mg
Lamivudine and Raltegravir- 450mg. **Total Weight of 10 Tablets:** 6.942mg **Average Weight each Tablet:** 0.694mg **Limits:** 0.687-0.763mg
Uniformity of weight (UFW): It is within Limits.

2.3.10 Sample preparation

Twenty tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 10 tablets was transferred into a 100ml standard flask. A volume of 70ml of mobile phase was added and sonicate for 30min.Then the solution was cooled and diluted to volume with mobile phase and filtered through 0.45µm membrane filter. (Stock solution) Further pipette 1.0 ml of Lamivudine and Raltegravir of the above stock solution in to a 10ml volumetric flask and dilute up to the mark with diluent.

2.3.11 Method Development

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Lamivudine and Raltegravir was obtained and the Isobestic point showed at 260nm.

2.3.12 Assay procedure

20 mL of the standard and sample solutions of Lamivudine and Raltegravir were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the capsules were calculated using the peak areas. The results are shown on Table 01.and fig.2&3.

Amount of drug in tablet was calculated using following formula:

$$\% \text{ Label claim} = \frac{Asp}{Ast} \times \frac{DstA}{DspLc} \times P$$

Where,

Asp = Area for sample solution.

Ast = Area for standard solution.

Dst = Dilution factor for standard.

Dsp = Dilution factor for sample.

2.4 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**.**

2.4.1 Specificity

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

2.4.2 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.3,4, 5 & 6.

2.4.3 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.7 and 8.

2.4.4 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 20 to 60 µg/ml for LAM and 10 to 30µg/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.5 and 6 and it was found that correlation coefficient (R2) and regression analysis were within the limits.

2.4.5 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.9 and figure 8& 9.

2.4.6 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.10 & 11. 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.

2.4.7 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.

3. 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 (250mm X 4.6 mm X 5µm) column and with flow rate 1.0 mL/min and detection wavelength of 260 nm Column temperature was maintained at ambient. Injection volume is 10µL and runtime is for 10min. The mobile phase consists of Acetonitrile: Water: Methanol (60:20:20v/v) mixture as a mobile phase**.** was used. The retention times of Lamivudine and Raltegravir peaks are about 2.3±0.3 and 3.3±0.3 minutes respectively. Quantitative linearity was observed over the concentration range of 10 to 30µg/mL for LAM and 30 to 60 µg/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 2349.08 and 4321.39 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%.

3.1 Limitofdetection andlimitof quantification

3.2 Robustness

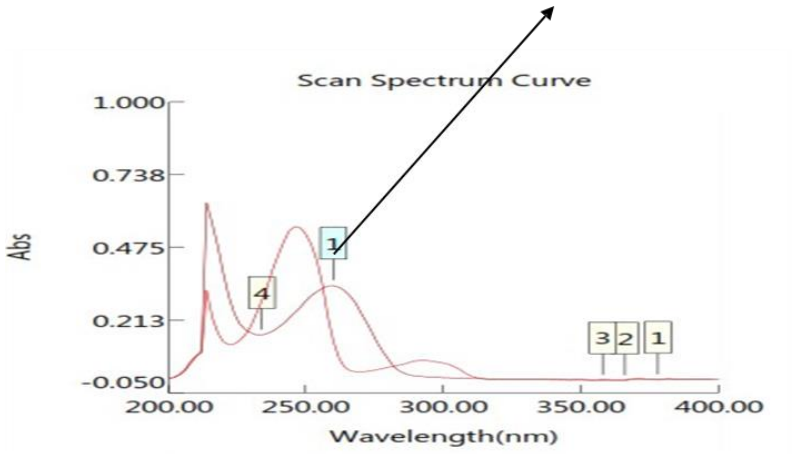

Figure 1: Over line Spectrum of Lamivudine and Raltegravir

Table 1: Peak results of Standard & Test Chromatograms for Assay

Parameters	Lamivudine	Raltegravir
Standard peak area	810802	681469
Test peak area (mean)	828933	687178
Average Weight	694.2mg	694.2mg
% Purity of Standard	99.50	99.58
Amt obtained	399.88 mg	150.10 mg
% Assay	99.77%	100.12%

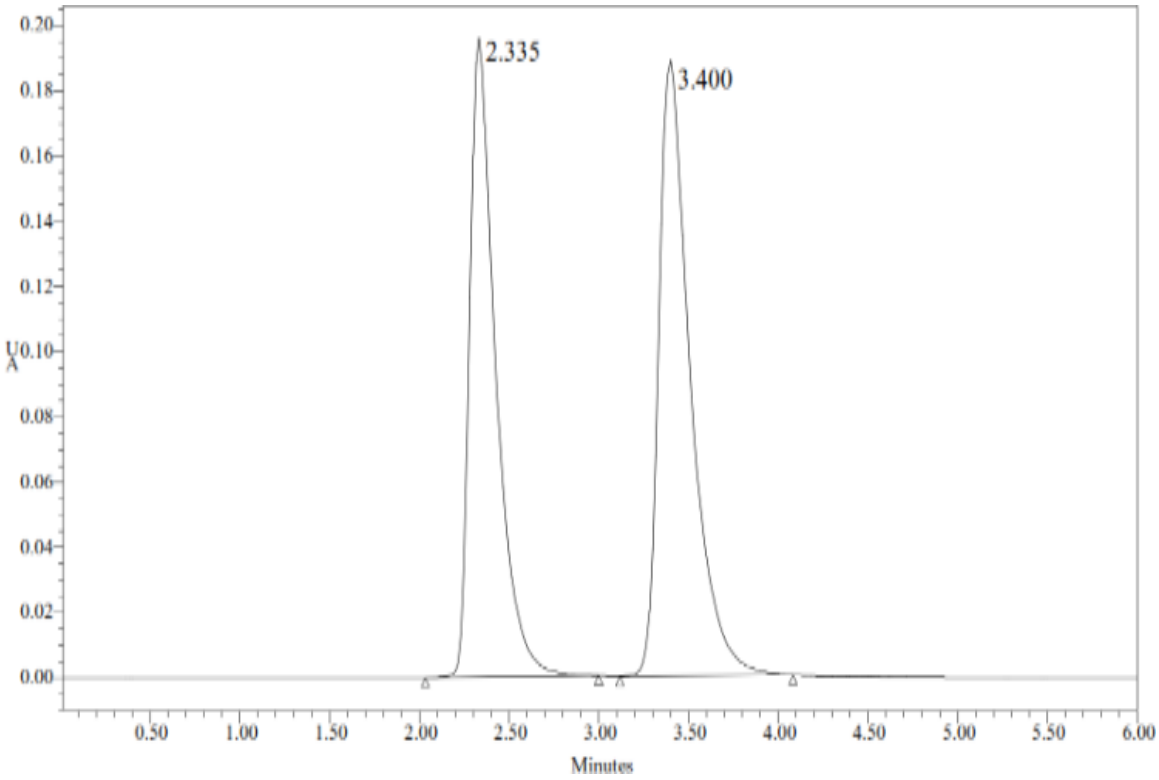

Figure 2: Chromatogram of standard

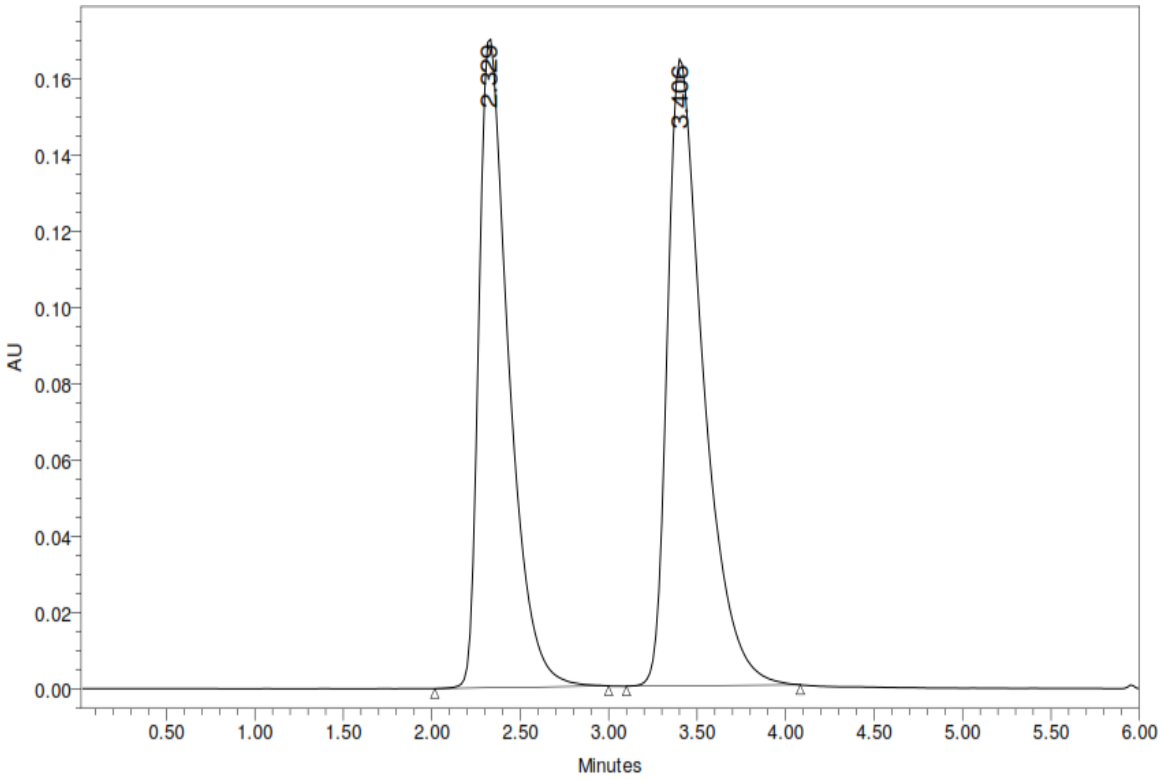

Figure 3: Chromatogram of Test

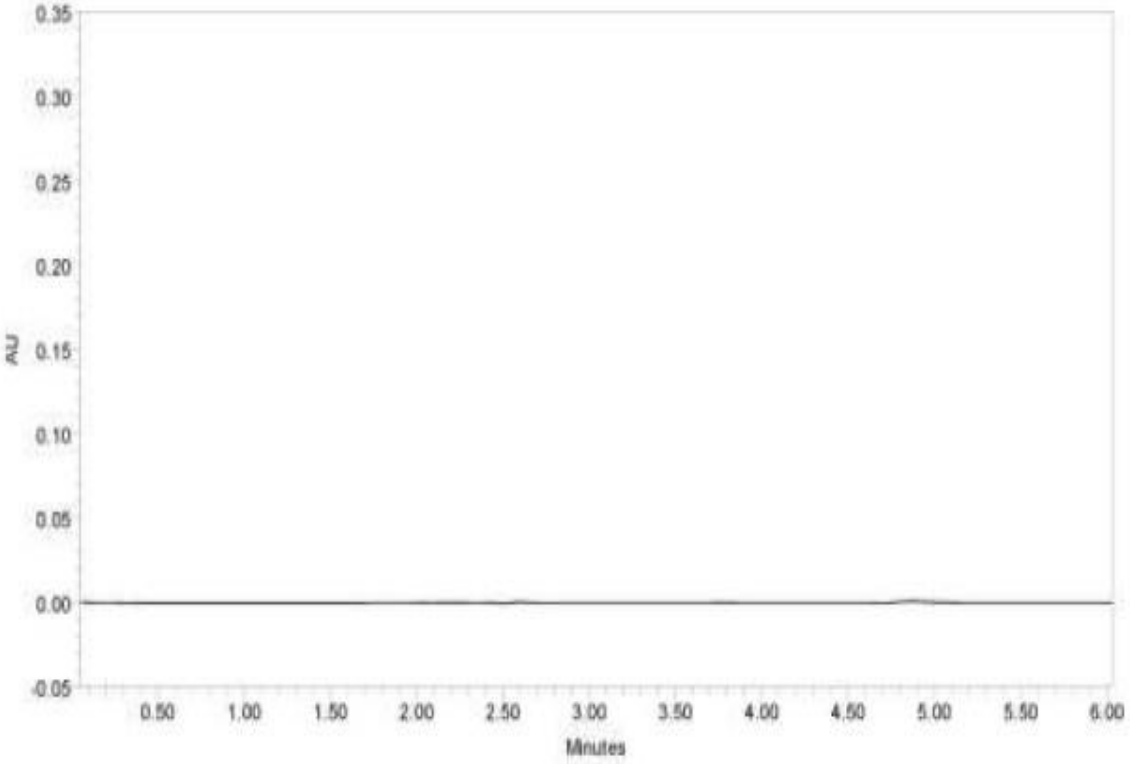

Figure 4: Chromatogram of Blank

Table 2: Linearity data of Raltegravir and Lamivudine

Sample ID	Raltegravir		Lamivudine	
	Concentration (mcg/ml)	Peak area	Concentration (mcg/ml)	Peak area
20% of operating concentration	20	226418	10	277182
40% of operating concentration	30	432920	15	521695
60% of operating concentration	40*	677256	20*	808274
80% of operating concentration	50	869825	25	1033875
100% of operating concentration	60	1095759	30	1285804
Correlation Coefficient			0.999	

Table 3: Precision Results for Lamivudine

S.NO	NAME	Retention Time	Peak Area
1.	Lamivudine	2.353	1963566
2.	Lamivudine	2.332	1964716
3.	Lamivudine	2.333	1965030
4.	Lamivudine	2.330	1960856
5.	Lamivudine	2.331	1966445
	Mean		1964123
	Standard deviation		2094.7
	% RSD		0.14

Table 4: Precision Results for Raltegravir

S.NO	NAME	Retention Time	Peak Area
1.	Ralitegravir	3.408	2304558
2.	Ralitegravir	3.408	2299453
3.	Ralitegravir	3.408	2296908
4.	Ralitegravir	3.408	2295001
5.	Ralitegravir	3.408	2299613
	Mean		2299631
	Standard deviation		3596
	% RSD		0.17

Table 5: Intermediate Precision Results for Lamivudine

S.NO	NAME	Retention Time	Peak Area
1.	Lamivudine	2.353	1984866
2.	Lamivudine	2.332	1985156
3.	Lamivudine	2.333	1985359
4.	Lamivudine	2.330	1987338
5.	Lamivudine	2.331	1984589
	Mean		1989356
	Standard deviation		8308.2
	% RSD		0.43

Table 6: Intermediate Precision Results for Raltegravi

S.NO	NAME	Retention Time	Peak Area
1.	Ralitegravir	3.413	2316755
2.	Ralitegravir	3.409	234487
3.	Ralitegravir	3.408	2314403
4.	Ralitegravir	3.406	2313639
5.	Ralitegravir	3.401	2313639

	Mean	2318434
	Standard deviation	8174.5
	% RSD	0.37

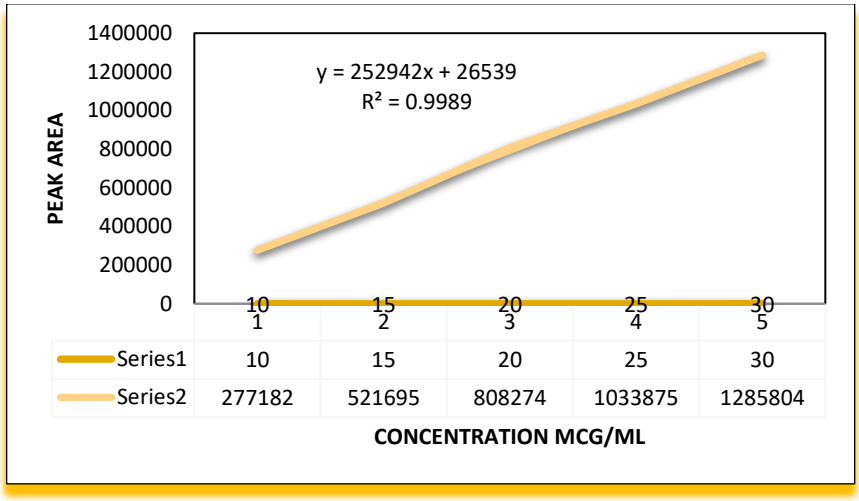

Figure 5: Linearity Graph of Lamivudine

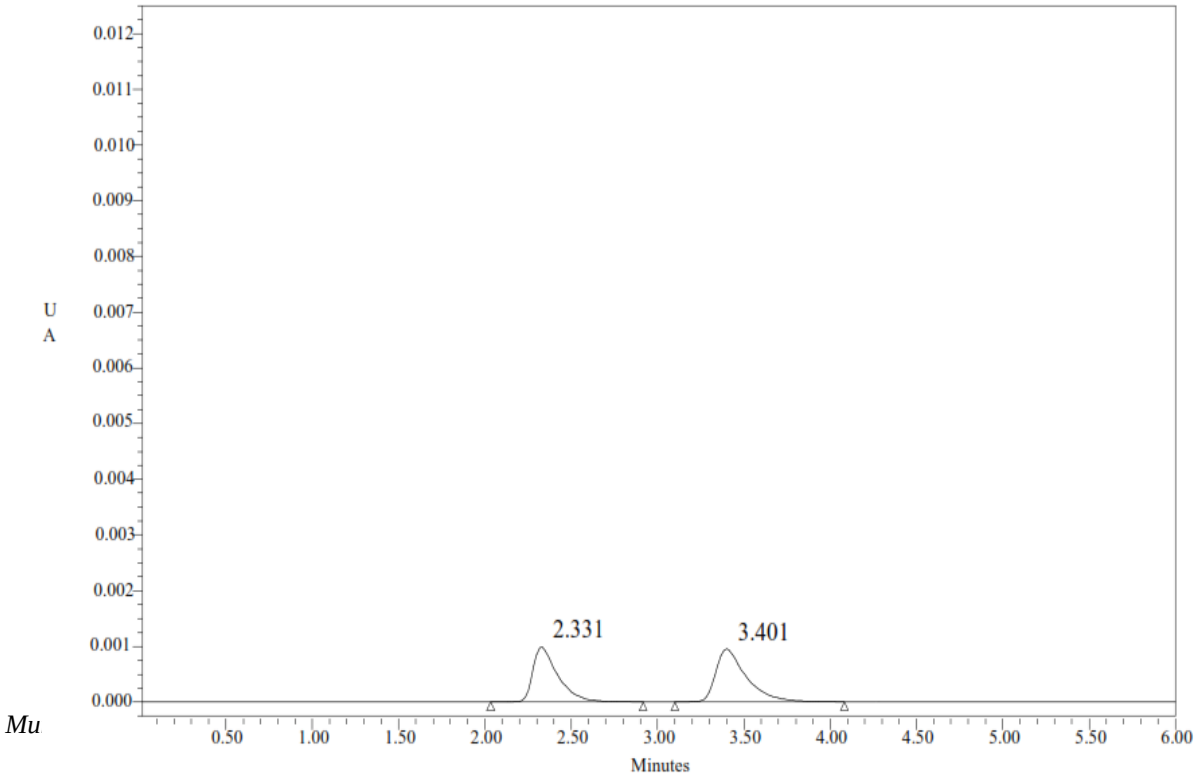

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Figure 6: Chromatogram of LOD

Table 7: Accuracy Study of Lamivudine

SampleId	Concfound (µg/ml)	Concn Obtained (µg/ml)	%Recovery	Mean recovery	Statistical Analysis
50%	5	5.01	100.2		
50%	5	4.96	99.2	99.73	
50%	5	4.99	99.8		**%RSD= 0.505**
100%	10	9.95	99.5		
100%	10	9.87	98.7	98.8	
100%	10	9.82	98.2		**%RSD=0.66**
150%	15	14.64	97.6		
150%	15	14.76	98.4	98.8	
150%	15	15.06	100.4		**%RSD=1.45**

Table 8: Accuracy Study of Raltegravir

SampleId	Conc (µg/ml)	Concn Obtained(µg/ml)	%Recoveryofdrug	Mean accuracy	%RSD
50%	5	4.92	98.0		
50%	5	4.96	99.2		
50%	5	5.02	100.4	**99.2**	**1.2**
100%	10	9.95	99.5		
100%	10	9.94	99.4		
100%	10	9.98	99.8	**99.5**	**0.2**
150%	15	14.78	98.6		
150%	15	14.94	99.6		**0.530**
150%	15	14.83	98.8	**99.0**	

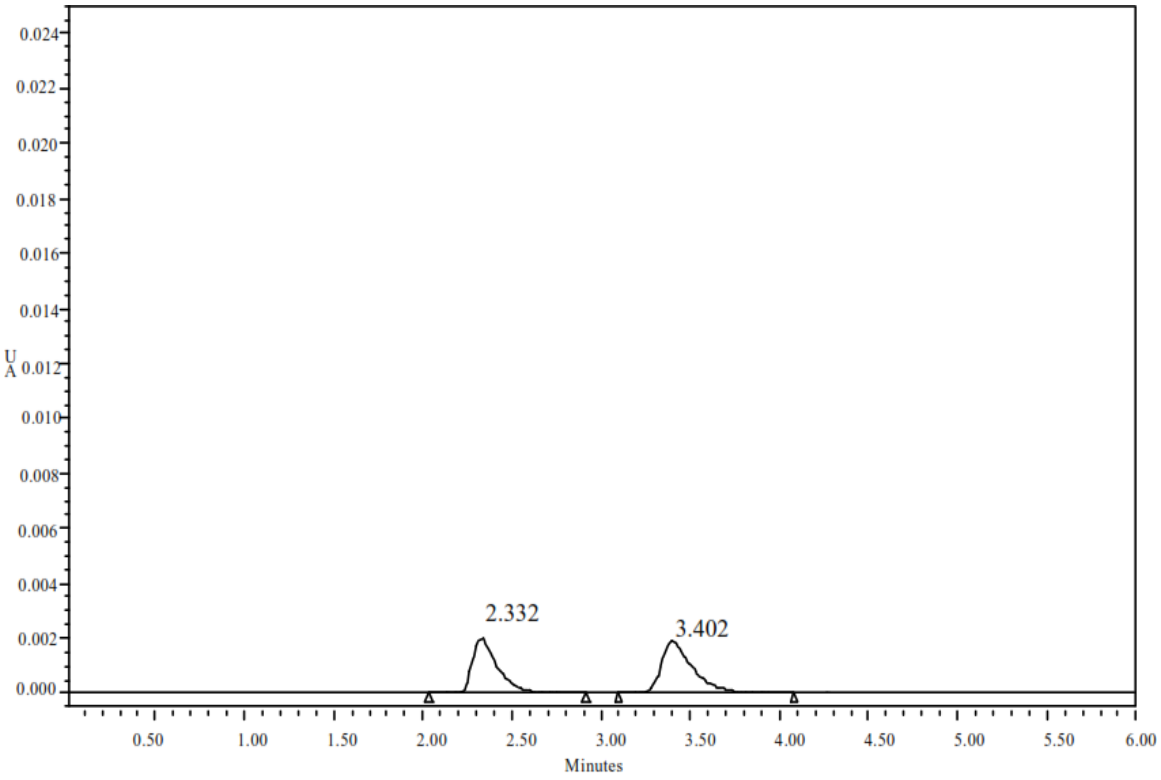

Figure 7: Chromatogram of LOQ

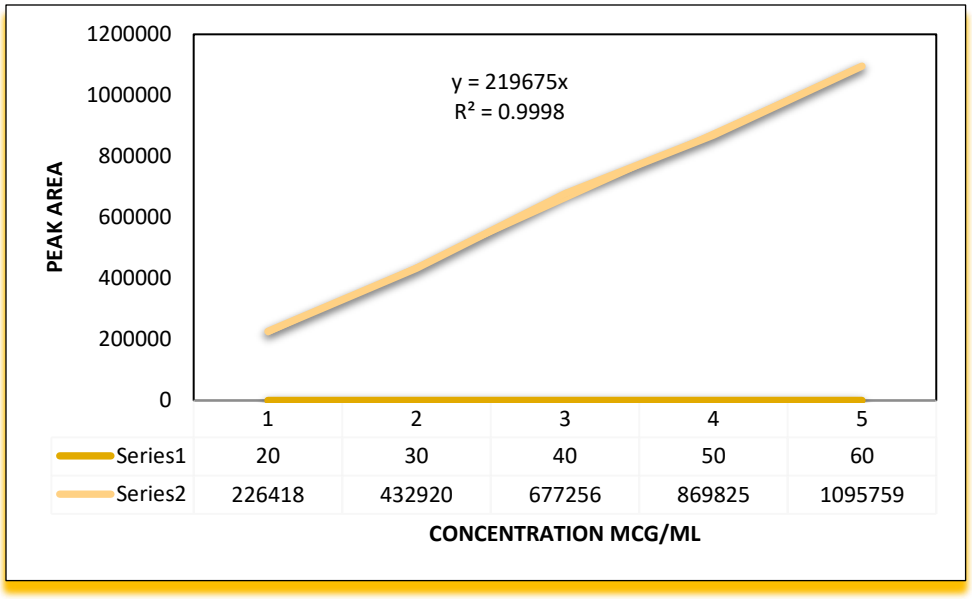

Figure 8: Precision Results for Lamivudine

Table 9: LOD and LOQ Data of Lamivudine and Raltegravir

Lamivudine			**Raltegravir**		
Conc.(x) (µg/ml)	**Peak Areas(y)**	**Statistical Analysis**	**Conc.(x) (µg/ml)**	**Peak Areas(y)**	**Statistical Analysis**
40	2004682	S =39092 c=618048 LOD:0.001µg/ml LOQ:0.004µg/ml	20	1184227	S =39092 c=369381 LOD:0.005 µg/ml LOQ: 0.015µg/ml
40	2004587		20	1186425	

Table 10: Robustness data for Lamivudine

Std. Replicate	**Variation in flowrate**		**Variation in Mobile phase composition**	
	Flow Rate 0.8ml/min	**Flow Rate 1.2ml/min**	**Buffer: Acetonitrile (40:60v/v)**	**Buffer: Acetonitrile (30:70v/v)**
Tailing factor	0.9	0.9	1.1	1.1
Theoretical plates	2690	2503	2707	2818

Table 11: Robustness data for Raltegravir

Parameter	**Variation in flowrate**		**Variation in Mobile phase composition**	
Standard	**Flow Rate 0.8ml/min**	**Flow Rate 1.2ml/min**	**Buffer: Acetonitrile (40:60v/v)**	**Buffer: Acetonitrile (30:70v/v)**
Tailing factor	0.9	0.9	1.0	1.0
Theoretical plates	2716	2685	3018	3107

4. Conclusion

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Lamivudine and Raltegravir in tablet dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims.

From literature review and solubility analysis initial chromatographic conditions Mobile phase ortho phosphoric acid buffer: Acetonitrile 40:60 was set (Buffer pH 2.45 adjusted with Triethylamine), Symmetry C18 (250×4.6mm, 5µm) Column, Flow rate 1.0 ml/min and temperature was ambient, eluent was scanned with PDA detector in system and it showed maximum absorbance at 260nm. As the methanol content was increased Lamivudine and Raltegravir got eluted with good peak symmetric properties. The retention times for Lamivudine and Raltegravir was found to be 2.335 min and 3.400 min respectively. System suitability parameters were studied by injecting the standard five times and results were well under the acceptance criteria. Linearity study was carried out between 50% to150 % levels, R^2 value was found to be as 0. 999.By using above method assay of marketed formulation was carried out, 100.7% was present. Full length method was not performed; if it is done this method can be used for routine analysis of Lamivudine and Raltegravir.

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