Original Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANIOUS ESTIMATION AND FORCED DEGRADATION STUDIES OF LAMIVUDINE AND RALTEGRAVIR IN SOLID DOSAGE FORM

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ABSTRACT

Objective: A stability indicating reverse phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the estimation of the combined tablet formulation of lamivudine (LAM) and raltegravir (RAL) in dosage forms and its API.

Methods: Chromatographic separation was achieved on inertsil ODS C18 5 μm (4.6 X 150 mm) using a mobile phase (MP) consisting of a mixture of mixed orthophosphoric acid (OPA): acetonitrile (ACN) in the ratio 50:50 v/v which was determined at 242 nm respectively.

Results: The assay of LAM and RAL was performed with tablets, and the % assay was found to be 100.12 and 99.89 which shows that the method is useful for routine analysis. The linearity of LAM and RAL was found to be linear with a correlation coefficient of 0.998 and 0.999, which shows that the method is capable of producing good sensitivity. The retention time of LAM and RAL was 1.99 min and 4.34 min respectively; linearity range was found to lie from 15 μg/ml to 75 μg/ml for LAM, 30 μg/ml to 150 μg/ml for RAL with a correlation coefficient of 0.999 respectively. Forced degradation studies were conducted in acidic, basic, thermal, photolytic and peroxide where all the degradation peaks were monitored.

Conclusion: The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of LAM and RAL in bulk and tablet dosage form. Thus the validated economical method was applied for forced degradation study of LAM and RAL tablet.

Keywords: lamivudine, Raltegravir, Acetonitrile, Stability

INTRODUCTION

Human immunodeficiency virus (HIV) is a retrovirus that gradually attacks the immune system, which protects the human body against illness. Currently, there is no cure for HIV, but with early diagnosis and effective antiretroviral (ARV) treatment, people with HIV can live a long and normal, healthy life. Therefore, it is important to take the correct treatment regularly.

Lamivudine (LAM) [1-4] belongs to a group of anti-HIV medicines called nucleoside reverse transcriptase inhibitors (NRTI). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. Its chemical formula is 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydro pyrimidin-2-one and the molecular formula is $C_8H_{11}N_3O_3S$.

Raltegravir (RAL) [5-13] is an antiretroviral compound belongs to the class of organic compounds known as pyrimidine carboxylic acids and

derivatives. RAL potassium is a pyrrolidinone derivative and an HIV integrase inhibitor that is used in combination with other anti-HIV agents for the treatment of HIV infection. Inhibition (INH) of integrase prevents insertion of HIV DNA into the human DNA genome, thus blocking HIV replication [7, 8]. Its chemical formula is N-[2-[4-[(4-fluorophenyl)methylcarbamoyl]-5-hydroxy-1-methyl-6-oxopyrimidin-2-yl]propan-2-yl]-5-methyl-1,3,4-oxadiazole-2-carboxamide and the molecular formula is C20H20FKN6O5.

The previous established methods were found high Rt and increased the total run time for analysis. Literature search reveals that only few analytical methods were reported for simultaneous estimation of LAM and RAL using HPLC [14, 15] method according to the international council on harmonization (ICH). There was no stability indicating analytical methods reported for simultaneous estimation of LAM and RAL. The present study was aimed to develop a simultaneous estimation of LAM and RAL along with forced stability studies which were found to be simple, precise, accurate and shorter retention time which makes this method good for routine analysis in research institutions which justify that the developed method is advantageous over the existing method. The structures were given in fig. 1.
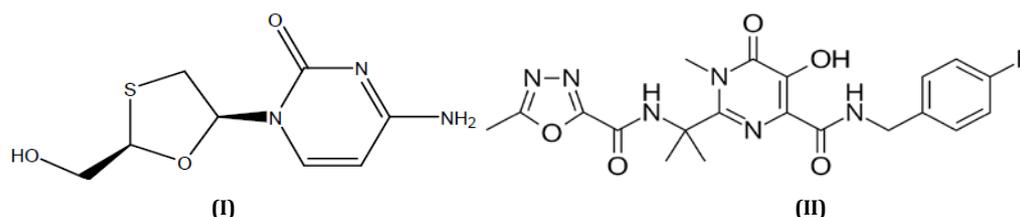

Fig. 1: Structure of LAM (I) and RAL (II)

MATERIALS AND METHODS

Chemical and reagents

Pure samples were obtained as a gift from hetero pharma ltd, hyderabad, india. OPA was obtained from finar chemical ltd. methanol and ACN were obtained from rankem india ltd.

Preparation of buffer and mobile phase

Pipette out 1 ml of 0.1% OPA in 900 ml HPLC water in it. Mix well and makeup the volume to 1000 ml with water used as a buffer. Mix a mixture of above buffer 500 ml (50%) and 500 ml ACN HPLC (50%) in 50:50 v/v ratio and degas in ultrasonic water bath for 5 min.

Preparation of standard and sample solutions

Accurately transfer 15 mg of LAM and 30 mg of RAL working standard into a 10 ml clean dry volumetric flask and sonicate. (Stock solution) Further pipette 1 ml of the above stock solution into a 10 ml volumetric flask and dilute. Further pipette 3 ml of LAM and RAL of the above stock solution into a 10 ml volumetric flask and dilute with diluents.

Accurately transfer 15 mg of LAM and 30 mg RAL equivalent weight of the sample into a 10 ml clean volumetric flask, add about 70 ml of diluents and sonicate. (Sample solution) Further pipette 1 ml of the above solution into a 10 ml volumetric flask and dilute. Further pipette 3 ml of LAM and RAL of solution into a 10 ml volumetric flask and dilute with diluents.

Preparation of mixed standard solution

Accurately transfer 15 mg of LAM and 30 mg of RAL working standard into a 10 ml 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 1 ml of LAM and RAL of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. Further pipette 3 ml of LAM and RAL of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents.

Instrumentation

The proposed method was carried out on inertsil ODS C18 5 μm (4.6 X 150 mm) and the mobile phase consisted of OPA (pH: 3): ACN (50:50 v/v) the column temperature was set 25 °C. From the UV spectrum wavelength selected as 242 nm.

Assay procedure

Inject 20 μL of the standard, sample into the chromatography system and measure the assay of LAM and RAL was performed with tablets.

Method validation

The analytical method was validated with respect to parameters such as linearity, LOQ, LOD, precision, accuracy, selectivity, recovery and ruggedness and was applied for forced degradation studies as per the ICH guidelines. [16-18]

Forced degradation studies

Acid degradation condition

Accurately 3.0 ml of stock solution into a 10 ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60 °C for 6 h and then neutralized with 0.1 N NaOH and makeup to 10 ml with diluent. The solution was filtered through 0.45μ filter, and then filtrate was injected into system and percentage of degradation was calculated.

Alkali degradation condition

Accurately 3.0 ml of stock sample into a 10 ml volumetric flask and add 3 ml of 0.1N NaOH was added. Then, the volumetric flask was kept at 60 °C for 6 h and then neutralized with 0.1N HCl and makeup to 10 ml with diluent. The solution was filtered through 0.45μ filter, and then the filtrate was injected into the system and percentage of degradation was calculated.

Thermal-induced degradation condition

3 ml of stock sample was taken in petri dish and kept in hot air oven at 110 °C for 24 h. The samples were then placed in a desiccator till reaching the room temperature. The content in the flasks was dissolved using methanol and diluted up to the mark. Then the sample was taken and diluted with diluents and injected and percentage of degradation was calculated.

Photolytic degradation condition

Accurately 3.0 ml of stock sample was exposed to sunlight for about 6 h and then the sample diluted with 5 ml of mobile phase and percentage of degradation.

Oxidative degradation condition

Accurately 3.0 ml of stock sample into a 10 ml volumetric flask, 1 ml of 3.0 ml of 3% H_2O_2 was added and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. The solution was filtered through 0.45μ filter and then filtrate was injected into the chromatography system and the percentage of degradation was calculated.

RESULTS AND DISCUSSION

There is no official method for this combination so far. However, few methods have been reported in either of one or two in this combination with some other drugs. For selecting column chiral columns of OD52546 and SCDP 52546 inertsil was chosen to separate LAM and RAL by injecting system suitability solution with the mobile phase at 0.9 ml/min individually. Various solvents including water, ACN, phosphate buffer, OPA, and methanol were used in different combinations to get good peaks resolutions and lesser runtime. Different flow rates from 0.4 to 1 ml/min in gradient mode have been studied to achieve a good peak resolution. The column temperature was set at 25°, 30° and 35 ° C for optimizing according to its effect on peak resolutions and retention times of the drug samples. After several initial trials with mixtures of methanol, ACN and different buffer in various combinations, a trail with a mobile phase mixture of OPA: ACN (50:50 v/v) with 25 °C at 242 nm the flow rate was 0.9 ml/min in gradient elution and the injection volume was 20 μg/ml of mixed standard solution and the % assay was found to be 100.48 and 98.84 which shows that the method was useful for routine analysis under the described experimental conditions, all the peaks were well defined and free from tailing and % recovery values were shown in table 1 and fig. 2.

System suitability

It is defined to measure that can generate the result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation was completed. The retention time of LAM and RAL using optimum conditions were 1.996 min and 4.336 min respectively. Resolution between two drugs must be not less than 2. Theoretical plates must be not less than 2000. Tailing factor must be not more than 2 as shown in table 2.

Table 1: Assay and % recovery of LAM and RAL

Drug	Label claim (mg)	Estimated claim (mg)	Average area	% Purity	% Recovery (%)			
					80	100	120	Mean
LAM	150	150.72	42117.66	100.48	100.58	99.18	100.60	100.12
RAL	300	296.52	215547.33	98.84	98.94	99.67	101.07	99.89

LAM: lamivudine; RAL: raltegravir
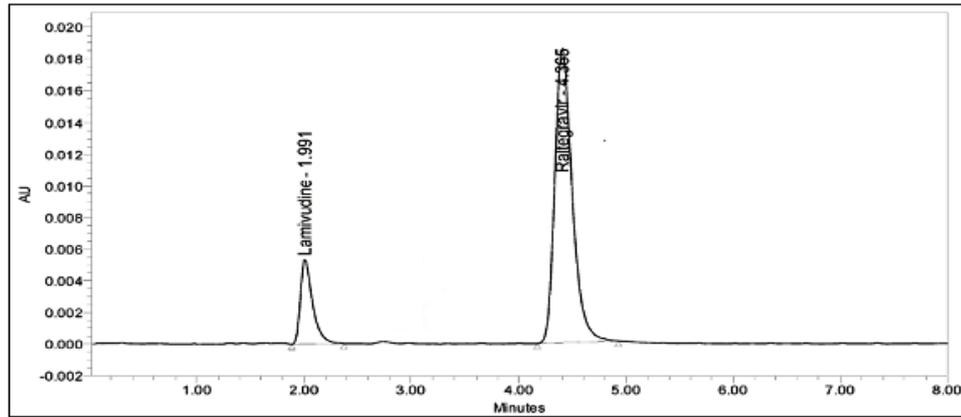
Fig. 2: Assay chromatogram of LAM and RAL

Table 2: System suitability results of LAM and RAL

Parameter	LAM	RAL
Peak area	42115	215502
Theoretical plates (N)	2559.08	3511.35
Retention time (min)	1.996	4.336
Tailing factor (T)	1.65	1.35

LAM: lamivudine; RAL: raltegravir

Specificity

The specificity of the method was evaluated in a placebo solution and a blank solution were also prepared. In practice, this can be done by spiking the drug substance or product with appropriate levels of excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. Optimized chromatogram of LAM and RAL are shown in fig. 3.
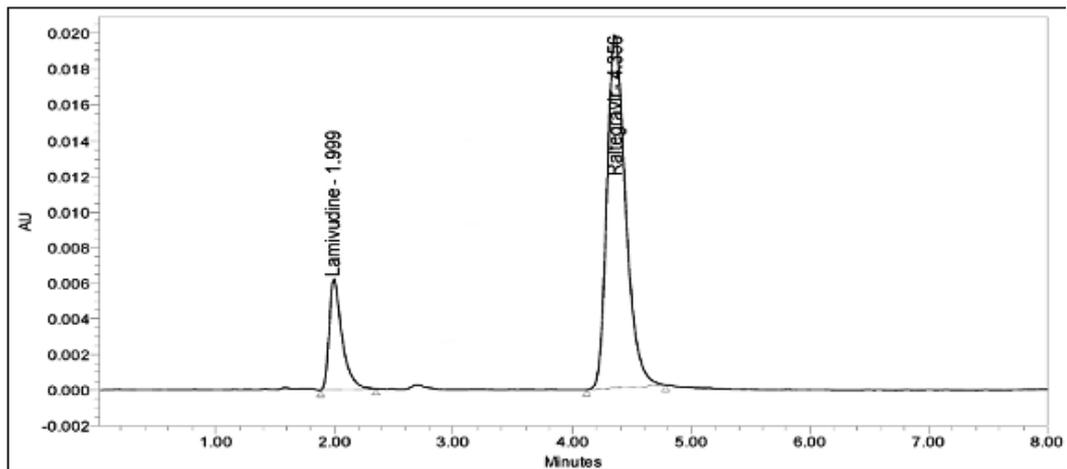

Fig. 3: Specificity chromatogram of LAM and RAL

Linearity

From the stock solution, inject each level into the chromate-graphy system and measure the peak area. Plot a graph of peak area versus concentration and calculate the correlation coefficient. The correlation coefficient obtained was 0.99 which is in the acceptance limit. The linearity range was found to lie from 15 μg/ml to 75 μg/ml of LAM 30 μg/ml to 150 μg/ml of RAL and chromatograms were shown below as shown in fig. 4-5 and table 3.

Table 3: Linearity data of LAM and RAL

LAM		RAL	
Concentration (μg/ml)	Peak area	Concentration (μg/ml)	Peak area
15	14891	30	67496
30	30568	60	151923
45	43243	90	223324
60	59103	120	304753
75	71989	150	374626

LAM: lamivudine; RAL: raltegravir
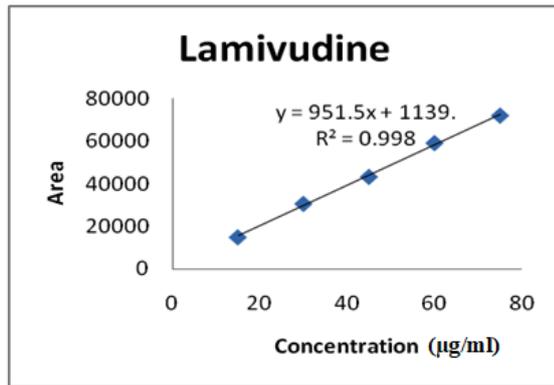
Fig. 4: Linearity graph of LAM
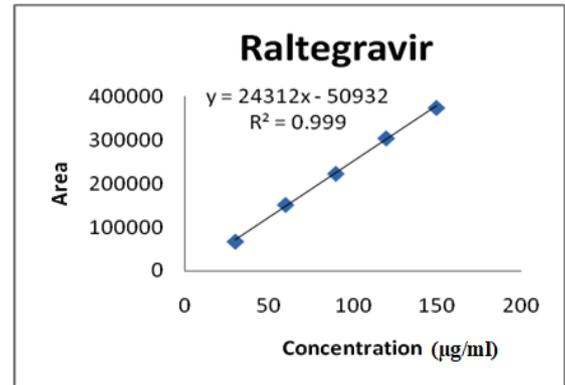
Fig. 5: Linearity graph of RAL

Table 4: Precision data of LAM and RAL

Parameter	LAM		RAL	
	Retention time (min)	Peak area	Retention time (min)	Peak area
1	1.991	48270	4.365	218798
2	1.984	46977	4.385	216905
3	1.989	46914	4.362	216459
4	1.991	46722	4.359	221802
5	1.995	47013	4.366	218749
Average (min)	1.984	47179.2	4.355	21852.6
SD	0.002	620	0.002	2106.8
% RSD	1.32	1.31	0.92	0.96

n=5; LAM: lamivudine; RAL: raltegravir; SD: standard deviation; RSD: relative standard deviation

Precision

The standard solution was injected for five times and measured the area in HPLC. % RSD for sample should be NMT 2. One dilution of all the drugs in six replicates was injected into HPLC system and was analyzed and the results were shown in the table 4.

LOD and LOQ

LOD and LOQ for LAM and RAL were estimated and as the amounts for the signal to noise ratios were found to be 3:1 and 10:1 respectively. LOD values for LAM and RAL were 2.96 and 2.95 s/n ratio. LOQ values for LAM and RAL were 9.96 and 9.98 s/n ratio. Signal to noise ratio shall be 3 for LOD and 10 for LOQ solution.

Method precision

The standard solution was injected for five times and measured the area for all five injections. The precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results were below as table 5.

Table 5: Method precision data of LAM and RAL

Parameter	LAM		RAL	
	Retention time (min)	Peak area	Retention time (min)	Peak area
1	2.004	48270	4.374	218798
2	1.999	46977	4.356	216905
3	1.993	46914	4.358	216459
4	1.992	46722	4.356	221802
5	1.990	47013	4.354	218749
Average (min)	1.994	47179.2	4.358	218542.6
SD	0.004	620	0.005	2106.8
% RSD	0.31	1.31	0.96	0.96

n=5; LAM: lamivudine; RAL: raltegravir; SD: standard deviation; RSD: relative standard deviation

Table 6: Ruggedness data for a sample of LAM and RAL

Parameter	LAM		RAL	
	Retention time (min)	Peak area	Retention time (min)	Peak area
1	1.996	45921	4.382	213936
2	2.001	47289	4.382	219057
3	1.992	46249	4.374	218423
4	1.992	47758	4.75	222496
5	1.990	47193	4.61	219155
Average (min)	1.992	46838.17	4.352	218181
SD	0.004	694.5	0.005	2935.6
% RSD	0.31	1.48	0.96	1.35

n=5; LAM: lamivudine; RAL: raltegravir; SD: standard deviation; RSD: relative standard deviation

Ruggedness

To evaluate the ruggedness of the method, precision was performed on a different day within the laboratory. %RSD of five different sample solutions should not more than 2. There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation. The standard solution was injected for five times and measured the area for all five injections as shown in tables 6.

Accuracy

For accuracy determination, three different concentrations were prepared separately, i.e. 50%, 100% and 150% for the analyst and chromatograms were recorded for the same. Calculate the amount found and amount added for LAM and RAL and calculate the individual recovery and mean recovery values. The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate. The results were given in tables 7.

Table 7: Recovery data of LAM and RAL

Drug	Sample	Amount added (μg/ml)	Amount found (μg/ml)	Area	% Mean	% Average
LAM	50%	7.5	7.62	22056	100.58	100.12
	100%	15	14.85	43140	99.18	
	150%	22.5	22.52	66628	100.60	
RAL	50%	15	14.83	106479	98.94	99.89
	100%	30	29.88	214516	99.67	
	150%	45	45.47	326302	101.07	

n=3;LAM: lamivudine; RAL: raltegravir

Robustness

Standard solution 45 μg/ml and 90 μg/ml of LAM and RAL was prepared and analysed using the varied flow rate and mobile phase composition along with the actual mobile phase composition in the method. System suitability parameters were compared with that of method precision. The retention time, plate count, tailing factor obtained for a change of flow rate, variation in mobile phase was found to be within the acceptance criteria. Hence the method is robust. The result of the robustness study of the developement assay method was established in table 8.

Table 8: Robustness for LAM and RAL

Drug	Flow rate (ml/min)	System suitability USP		Change in mobile phase	System suitability USP	
		Plate	Tailing		Plate	Tailing
LAM	0.8	2736.08	1.53	10% less	2732.24	1.54
	0.9	2559.08	1.65	actual	2559.08	1.65
	1.0	2540.88	1.53	10% more	2865.60	1.66
RAL	0.8	3910.92	1.31	10% less	3884.75	1.29
	0.9	3511.35	1.35	actual	3511.35	1.35
	1.0	3456.84	1.29	10% more	4002.13	1.36

n=3;LAM: lamivudine; RAL: raltegravir

Table 9: Degradation studies results for LAM and RAL

| Condition	LAM		RAL	
	Area	% Degraded	Area	% Degraded
Control	45921	-	213936	-
Acid	45623.56	5.25	213754.25	5.04
Alkali	45589.54	5.06	213624.87	5.17
Peroxide	45423.69	5.87	213542.24	6.25
Thermal	45752.84	3.23	213564.36	2.54
Photo	45856.56	1.15	213896.21	1.13

LAM: lamivudine; RAL: raltegravir
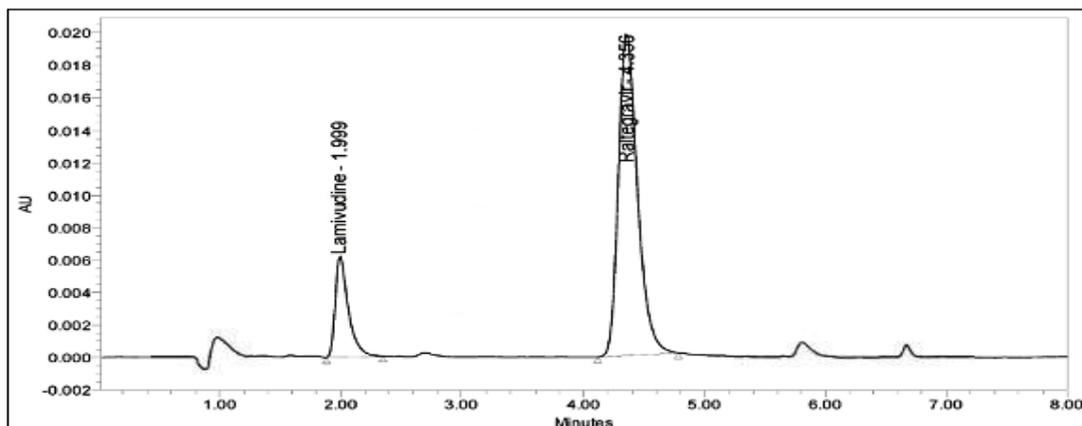
Fig. 6: Acid degradation of LAM and RAL

Forced degradation studies

The stability studies were determined by applying the physical stress to the product. Results in forced degradation were shown in table 9 and blank was recorded. The results of forced degradation studies for the simultaneous estimation of LAM and RAL were in limits, and respective chromatograms were represented. It was observed that the drug degrades as shown by the decreased areas in the peaks when compared to peak areas of the same concentration of the non-degraded drug, with additional degradation peaks. Percent degradation was calculated by comparing the areas of the degraded peaks at each degradation condition with the corresponding areas of the peaks of both the drugs under non-degradation condition. Degradation studied were performed and it was observed that no interference of degradants as shown in fig. 6-10.
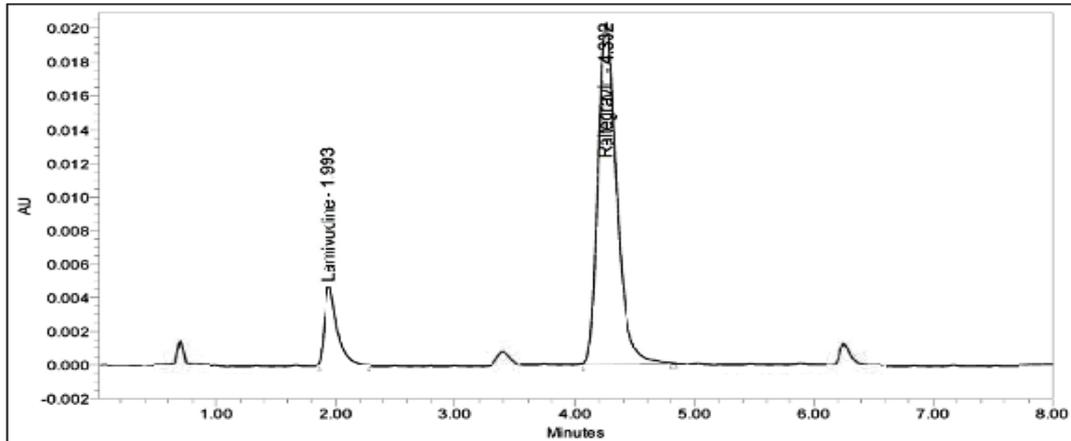

Fig. 7: Alkali degradation of LAM and RAL
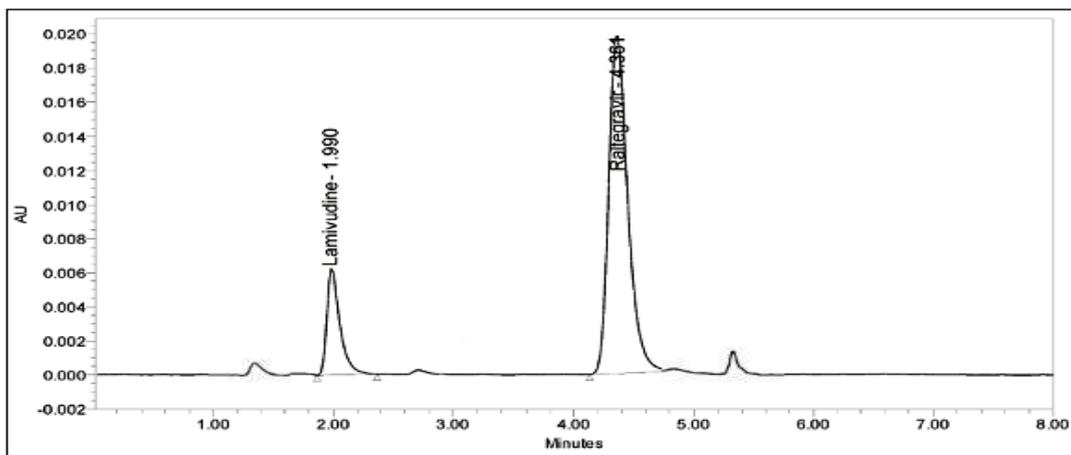
**Fig. 8: Thermal degradation of LAM and RAL**
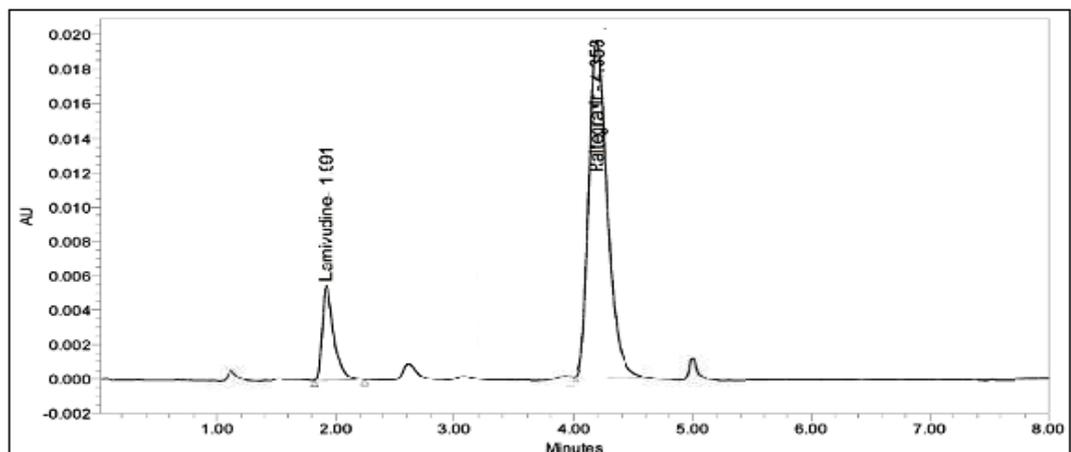
**Fig. 9: Photolytic degradation of LAM and RAL**
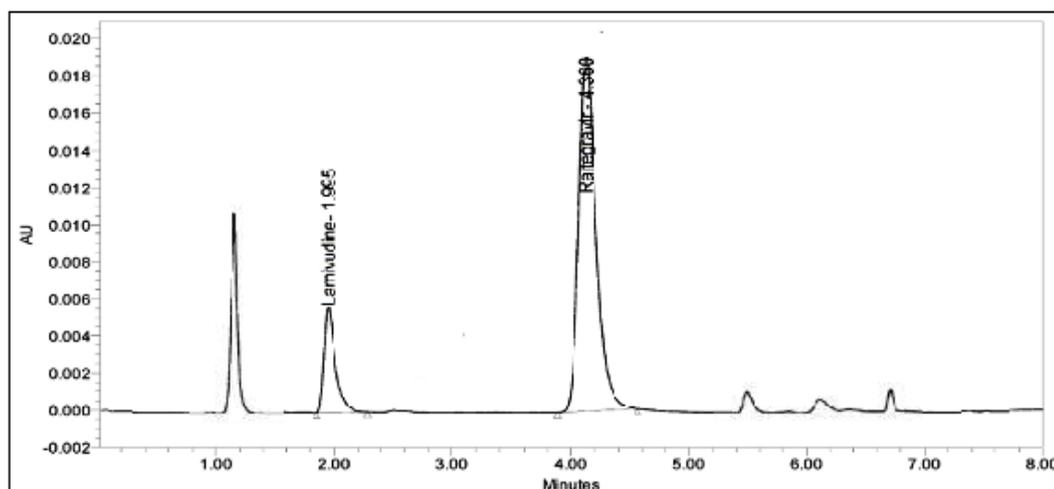
Fig. 10: Oxidative degradation of LAM and RAL

CONCLUSION

A simple and selective RP-HPLC method was described for the determination of LAM and RAL dosage forms. The retention time of LAM and RAL was 1.99 min and 4.34 min respectively. The linearity of LAM and RAL was found to be linear with a correlation coefficient of 0.998 and 0.999. The acceptance criteria of precision were RSD should be not more than 2.0% and the method shows precision 1.31 and 0.96 for LAM and RAL which shows that the method was precise. The results of forced degradation studies for the simultaneous estimation of LAM and RAL were in limits, and respective chromatograms were represented. Hence the method was successfully applied for degradation studies, and HPLC method for simultaneous estimation of LAM and RAL was novel, simple, precise, accurate, robust and cost-effective method.

AUTHORS CONTRIBUTION

All the authors have contributed equally

CONFLICTS OF INTERESTS

Declare none

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