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,SS]-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one and the molecular formula is C8 H11N3 O3.

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.
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 make up the volume to 1000 ml with water used as a buffer. Mix a solution of 0.5 ml of 3% HCl and 500 ml ACN. The solution was filtered through 0.45μ filter and then neutralized with 0.1N NaOH 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.

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 with diluents.

Preparation of mixed standard solution
Accurately transfer 15 mg of LAM and 30 mg of RAL to a 10 ml clean volumetric flask and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (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 standard and sample solutions
Accurately transfer 15 mg of LAM and 30 mg of 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 and sonicate to dissolve it completely and make volume up to the mark with the same solvent and sonicate. (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) with 25 °C at 242 nm. 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 sample 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 the filtrate was injected into the system and percentage of degradation was calculated.

Alkal 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 sample was then placed in a desicator 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% H2O2 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 99.84 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.

| Drug | Label claim (mg) | Estimated claim (mg) | Average area | % Purity | % Recovery (%) |
|------|-----------------|---------------------|--------------|----------|----------------|
| LAM  | 150             | 150.72              | 42117.66     | 100.48   | 100.58         |
| RAL  | 300             | 296.52              | 215547.33    | 98.84    | 98.94          |

Table 1: Assay and % recovery of LAM and RAL

LAM: lamivudine; RAL: raltegravir
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.

Linearity

From the stock solution, inject each level into the chromatography 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  | 14091 | 30  | 67496 |
| 30  | 30568 | 60  | 151923 |
| 45  | 43243 | 90  | 223324 |
| 60  | 59103 | 120 | 304753 |
| 75  | 71989 | 150 | 374626 |

LAM: lamivudine; RAL: raltegravir
Precision

The standard solution was injected for five times and measured the area in HPLC. % RSD for sample should be ≤ 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 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 shown in the table 5.
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.

| 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 development assay method was established in table 8.

| Drug | Flow rate (ml/min) | System suitability USP | Change in mobile phase | System suitability USP |
|------|--------------------|------------------------|------------------------|------------------------|
|      |                    | Plate                  | Tailing                | Plate                  |
| LAM  | 0.8                | 2736.08                | 1.53                   | 2732.24                |
|      | 0.9                | 2559.08                | 1.65                   | 2559.08                |
|      | 1.0                | 2540.88                | 1.53                   | 2865.60                |
| RAL  | 0.8                | 3910.92                | 1.31                   | 3884.75                |
|      | 0.9                | 3511.35                | 1.35                   | 3511.35                |
|      | 1.0                | 3456.84                | 1.29                   | 4002.13                |

n=3; LAM: lamivudine; RAL: raltegravir

Table 9: Degradation studies results for LAM and RAL

| Condition | LAM Area | % Degraded | RAL 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       | 213096.21| 1.13       |

LAM: lamivudine; RAL: raltegravir

![Fig. 6: Acid degradation of LAM and RAL](image)
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 studies 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
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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