A New Stability Indicating Analytical Method Development And Validation For The Quantitative Determination of Emtricitabine And Lamivudine By RP-HPLC

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ABSTRACT
A novel stability indicating, precise, accurate and ecofriendly reverse phase high performance liquid chromatographic method was developed and validated for the quantitative determination of Emtricitabine and Lamivudine in pure and pharmaceutical dosage forms. Estimation of drugs in this combination was done with a C18 column (Kromasil C18 column. 5μm, 4.6×250 mm) using mobile phase of composition Methanol and phosphate buffer (40:60 v/v, pH 4). The flow rate was 1.0 ml/min and the effluents were monitored at 261 nm. The retention time of Lamivudine and Emtricitabine were 2.810 min and 4.727 min respectively. The linearity was found to be 40-80μg/ml for Lamivudine and 40-80μg/ml for Emtricitabine. The stability parameters were evaluated by injecting the stressed sample and it was proved that there were no degradants. The established method was validated according to ICH guidelines.

Key words: Emtricitabine, Lamivudine, RP-HPLC, Stability and Method Validation.

INTRODUCTION
Emtricitabine is chemically designated as 5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Emtricitabine is a nucleoside analogue and reverse transcriptase inhibitor used in combination with other agents for treatment and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). That is soluble in water, methanol, acetone, formaldehyde, and ethyl acetate. It is official drug in Martindale, Merck index and Indian Pharmacopoeia 2007 and 2014. 1, 3.

Fig 1: chemical structure of Emtricitabine

Lamivudine is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination. Lamivudine enters cells by passive diffusion and is phosphorylated to its active metabolite, lamivudine triphosphate. Lamivudine triphosphate competes with deoxycytidine triphosphate for binding to reverse transcriptase, and incorporation into DNA results in chain termination. Lamivudine has very low affinity for human alpha and omega DNA polymerases, moderate affinity for beta DNA polymerase, and higher affinity for gamma DNA polymerase, having the IUPAC name 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one. 1, 3.

Fig 2: chemical structure of Lamivudine

After literature survey it was proved that there were few HPLC methods reported for the estimation of selected drugs of interest. 4-8 So in the present investigation we tried to establish a novel, stable and sensitive chromatographic method for the estimation of selected drugs.

MATERIALS AND METHODS

Equipment used [4-8]
The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20μl fixed loop. A reverse phase C18 (Kromasil ODS UG 5 column, 250mm × 4.5mm) was used. Elico SL-210 double beam UV visible spectrophotometer and Axis AGN204-P0 electronic balances were used for

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spectrophotometric determinations and weighing purposes respectively.

Reagents and chemicals
Pharmaceutical grade pure Lamivudine and Emtricitabine gift samples were procured from Mylan Laboratories, Hyderabad. Marketed formulation Tablets with dose of 300mg of Lamivudine and 200mg of Emtricitabine were procured from local market. (Mfd by Emcure® Pharmaceuticals ltd). HPLC grade Methanol and Water were procured from Merck specialties private limited, Mumbai.

Chromatographic conditions [9-12]
Kromasil C18 (2) column 5μm [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 261 nm. Mobile phase of composition Methanol and Phosphate buffer pH 4in a ratio of 40:60 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 1.0 ml/min and the injection volume was 20μl.

Selection of Mobile Phase
The standard solutions containing Lamivudine and Emtricitabine were injected into the HPLC system and run in different solvent systems. By studying literature survey, different mobile phases in different proportions and different pH were tried in order to find the best conditions for the separation. It was found that methanol and water gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. Finally, the optimal composition of the mobile phase was obtained in the ratio of phosphate buffer pH (4.0): Methanol (70:30) with a flow rate of 1.0 ml/min.

Preparation of Mobile Phase
Phosphate buffer pH 4 was prepared by dissolve 0.504gm of disodium hydrogen phosphate and 0.301gm of Potassium dihydrogen phosphate of HPLC grade water and adjusts the pH to 4.0 with glacial acetic acid and sufficient water was added to produce100 ml filtered through 0.45 μm membrane filter and sonicated for 10 minutes.

Preparation of Standard Stock Solution
The separate stock solutions of Lamivudine and Emtricitabine were prepared by accurately weighing 25 mg each into a separate 25ml volumetric flasks A and B and made up to the volume with mobile phase to get 1000μg/ml respectively. From the above standard stock solutions 0.8ml from volumetric flask A and 0.8ml from volumetric flask B was transferred to a 10 ml volumetric flask and made up to the volume with same mobile phase to get 80μg/ml and 80μg/ml Lamivudine and Emtricitabine (Working stock solution).The stock solution was filtered through 0.45μm Millipore membrane filter, sonicated and degassed.

Selection of Analytical Wavelength
By appropriate dilution of each standard stock solution with mobile phase, various concentrations of Lamivudine and Emtricitabine were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer between the range of 200nm to 400nm and their spectra were overlaid. From the overlain spectra shown in figure 14 of Lamivudine and Emtricitabine, 261nm was selected as analytical wavelength for Multicomponent analysis using HPLC method.

Optimized Chromatographic Conditions
Mobile phase consisting of Phosphate buffer PH (4.0): Methanol (70:30 v/v) was used in isocratic mode. The mobile phase was initially filtered through 0.45μm Millipore membrane filter and sonicated for 10 min before use. The flow rate was maintained at 1ml/min and the injection volume was 20μl. UV detection was performed at 261 nm and the separation was achieved at ambient temperature.

Selection of Analytical Concentration Range and Construction of Calibration Curve for Lamivudine and Emtricitabine[13-15]
Appropriate aliquots ranging from 0.4 ml to 0.8 ml and 0.4 ml to 0.8 ml were pipetted out from the working stock solution (1000μg/ml of Lamivudine and Emtricitabine) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 40-80μg/ml of Lamivudine and 40-80μg/ml of Emtricitabine. Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20μl of each concentration of the drug were injected into the HPLC system three times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Peaks areas were recorded for all the peaks and a standard calibration curve of area against concentration was plotted as concentration of the drug Vs peak area (figure 4 and 5). The results were shown in table 2. Both the drugs follow the same chromatographic conditions as described above.

Analysis of Tablet Formulation
The tablets (Lamivudine or Emtricitabine) were initially powdered and an amount equivalent to 300mg of Lamivudine and 200mg of Emtricitabine was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 15 minutes. The solution was then filtered through 0.45μm millipore membrane filter. Final stock containing 150μg/ml and 100μg/ml of Lamivudine and Emtricitabine respectively was prepared by subsequent dilution with the same mobile phase. 20μl of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard. A typical chromatogram of test solution containing 150μg/ml of Lamivudine and 100μg/ml of Emtricitabine was shown in figure 6. The results were shown in table 7.

Method Validation[16-20]
The method was validated according to ICH Q2 B guidelines for validation of analytical procedures in order to determine...
system suitability, linearity, sensitivity, precision, accuracy and robustness for the analytes.

**Specificity and Selectivity**

The specificity of the RP-HPLC method was determined by complete separation of Lamivudine and Emtricitabine with parameters like retention time (Rt), resolution (Rs) and tailing factor (Tf). Here tailing factor for peaks of Lamivudine and Emtricitabin was less than 2% and resolution was also more than 2%. The average retention time and standard deviation for Lamivudine and Emtricitabine were found to be satisfactory for six determinations of sample solution containing 80µg/ml of Lamivudine and 80µg/ml of Emtricitabine. The peaks obtained for Lamivudine and Emtricitabine were sharp and have clear baseline separation as none of the excipients interfered with the analytes of interest. The chromatogram to represent specificity was shown in figure 3 and the results were given in table 1.

**Linearity and Range**

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample within a given range. Linearity of the method was determined by means of calibration curve using different concentration of the drugs. Linearity was evaluated by visual inspection of a calibration curve shown in figure 4 and 5. The linearity of the method was determined in concentration range of 40-80µg/ml for Lamivudine and 40-80µg/ml for Emtricitabine. Each solution was injected in triplicate. The slope, intercept was reported as required by ICH which were given in table 2.

**Accuracy**

To confirm the accuracy of the proposed method, recovery experiments were performed by standard addition technique. In this method a known quantity of pure drug was added at three different levels i.e. 50%, 100% and 150% to pre-analyzed sample solutions and calculated the recovery of Lamivudine and Emtricitabine for each concentration. The results of recovery studies by proposed method were validated by statistical evaluation and were given in table 4.

**Precision**

The precision of an analytical method was studied by performing intraday and inter day precision.

**Intraday Precision**

Variation of results within the same day was analyzed. Intraday precision was determined by analyzing a set of six combined standard solutions of Lamivudine (80µg/ml) and Emtricitabine (80µg/ml) in linearity range as 100% concentration at three different time intervals on same day. and the results were given in table 3.

**LOD and LOQ**

The LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S (Where, σ is the standard deviation of the responses and S is mean of the slopes of the calibration curves). The results were given in table 2.

**Robustness**

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different column temperate, different analytical wavelength, different flow rate. The solution containing 80µg/ml of Lamivudine and 80µg/ml of Emtricitabine was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate (±0.2ml/min) and detection wavelength (± 2 nm).

**Ruggedness**

The evaluation of ruggedness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters like different instruments, analysts, laboratories, reagents, days etc. The solution containing 80µg/ml of Lamivudine and 80µg/ml of Emtricitabine was injected into HPLC three times under different parameters like different analysts for change in analysts and the results were given in table 5.

**System Suitability: [21-25]**

The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for Lamivudine and Emtricitabine retention times and peak areas.

System suitability was carried out by injecting 100% concentration (sample having 80µg/ml of Lamivudine and 80µg/ml of Emtricitabine) into the HPLC system. This was repeated for six times under similar condition. The tailing factor (T) and no. of theoretical plates (N) obtained were shown in figure 3 and the results were given in table 1.

**Stability studies:**

1. **Acid degradation studies**

Prepared each 1mg/ml stock solution of Lamivudine and Emtricitabine by using mobile phase as solvent, and then filtered through 0.45µm membrane filter paper. Stock solutions of 0.8 ml and 0.8ml of Lamivudine and Emtricitabine were transferred into 10ml volumetric flask and added 1 ml of 0.1N HCL and diluted to volume with mobile phase. The resultant solution was injected into the system; there was no acid degradation products were found.

2. **Alkaline degradation studies**

20% 1 of Lamivudine and Emtricitabine was injected having concentrations of 80% g/ml Premixed with 1ml of 0.1N NaOH solution then filtered through 0.45mm membrane filter paper. Each which were Stock solutions of 0.8ml and 0.8ml of Lamivudine and Emtricitabine were transferred into 10ml volumetric flask and added 1 ml of 0.1N NaOH and diluted to volume with mobile phase.

3. **Oxide degradation studies**

Lamivudine and Emtricitabine were prepared by dissolving 25mg/25ml of mobile phase in two different 25ml volumetric flask then filtered through 0.45µm membrane filter paper. Stock solutions of 0.8ml and 0.8ml of Lamivudine and Emtricitabine was transferred into 10ml volumetric flask and added 1 ml of H2O2 and diluted to volume with mobile phase.
In this investigation no identifiable oxidative degradants were found.

4. Thermal degradation studies
From the primary stock solutions of 1mg/ml 80 µg/ml Lamivudine and Emtricitabine was prepared in 10ml volumetric flask and diluted to volume with mobile phase and kept for 60min at 60°C in hot air oven. 20 µl of above solution was injected in to system and from the obtained chromatogram it was proved that the selected samples were stable against thermal degradation.

RESULTS AND DISCUSSION
After a number of trials with mobile phases of different composition, Methanol, Phosphate buffer pH 4.0 in the ratio 40:60 v/v was selected as mobile phase because of better resolution and symmetric peaks. Lamivudine and Emtricitabine were found to show appreciable absorbance at 261nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Lamivudine and Emtricitabine at different Rs was shown in figure 3. System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Lamivudine and Emtricitabine at 2.8min and 4.7min respectively without any interference. The parameters were given in table 1. Concentration range of 40-80µg/ml for Lamivudine and 40-80µg/ml of Emtricitabine were found to be linear with correlation coefficients 0.999 and 0.999 respectively. The results were given in table 2. The limits of detection for Lamivudine and Emtricitabine were found to be 1.1µg/ml and 3.63µg/ml respectively and the limit of Quantitation were 2.7µg/ml and 8.91µg/ml respectively. Values were represented in table 2. The proposed method was found to be precise and reproducible with %RSD of 0.65 and 0.9 for Lamivudine and Emtricitabine respectively. %RSD was reported in table 3. Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.2% for Lamivudine and 98.3% for Emtricitabine. This indicates that the method was accurate. Values obtained were given in table 4. The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in table 5. The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 98.1% for Lamivudine and 98.3% for Emtricitabine. The typical chromatogram for assay of marketed formulations was shown in figure 6 and Values obtained were given in table 6.

FORCED DEGRADATION STUDY
Degradation studies indicated the specificity of developed method in presence of degradation products. Degradation was carried out in combination of two drugs and purity of drug peaks was confirmed by purity angles. Their combination drug products were exposed to acid, base, oxidative and thermal stress conditions. Then found to be no degradable substances presence and proved that the proposed method was stable towards acid, alkali, peroxide and thermal conditions. The obtained values were reported in table 8.

CONCLUSION
The proposed liquid chromatographic method allows a specific and rapid quantitative estimation of Emtricitabine and Lamivudine in bulk and marketed formulations. It was proved that all the validation parameters were fall in acceptance limits as per ICH guidelines. The established and validated method was proved to be sensitive and selective for the determination of drugs of present investigation. This was a most accurate, specific, precise and stable under different degradation conditions. Hence it can be utilized in the routine estimations of the dosage forms.

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FIGURES AND TABLES

**Fig 3:** Optimized chromatogram of Lamivudine and Emtricitabine

**Fig 4:** Calibration plot of Lamivudine
Fig 5: Calibration plot of Emtricitabine

Figure 6: Chromatogram for assay of marketed formulation

Fig 7: Chromatogram of acid degradation

Fig 8: Chromatogram of alkaline degradation

Figure 9: Chromatogram of Hydrogen peroxide degradation

Table 1: System Suitability Parameters

| Parameters                  | Lamivudine | Emtricitabine |
|-----------------------------|------------|---------------|
| Retention time (min)        | 2.8        | 4.7           |
| Theoretical plates (N)      | 8596       | 9542          |
| Tailing factor (T)          | 1.2        | 1.1           |
| Resolution (Rₛ)             | 1.9        |               |

Tab 2: Results for Linearity

| Parameters                  | Lamivudine | Emtricitabine |
|-----------------------------|------------|---------------|
| Slope                       | 28995      | 45641         |
| y intercept                 | 13581      | 15870         |
| Correlation coefficient r²  | 0.999      | 0.999         |
| Regression Equation         | y = 28995x + 13581 | y = 45641x + 15870 |
| Linearity range             | 40-80 µg/ml | 40-80 µg/ml   |
| LOD                         | 1.1 µg/ml  | 2.7 µg/ml     |
| LOQ                         | 3.63 µg/ml | 8.91 µg/ml    |

Tab 3: Results of Precision

| Drug           | Intraday Precision (%RSD) | Interday Precision (%RSD) |
|----------------|--------------------------|---------------------------|
| Lamivudine     | 1.12                     | 0.65                      |
| Emtricitabine  | 0.97                     | 0.9                       |

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Table 4: Results for Accuracy

| Recovery level | Amount Added (µg/ml) | Amount Found (µg/ml) | % Recovery | Amount Added (µg/ml) | Amount Found (µg/ml) | % Recovery |
|----------------|----------------------|----------------------|------------|----------------------|----------------------|------------|
| 50%            | std 10              | test 20              | 97         | std 10              | test 20              | 96.3       |
| 100%           | 40                   | 20                   | 98.3       | 40                   | 20                   | 99         |
| 150%           | 70                   | 20                   | 99.3       | 70                   | 20                   | 99.6       |
| Mean recovery  | 98.2%               |                      |            | 98.3%               |                      |            |

Table 5: Results for Robustness

| Parameters (n=3) | %RSD |
|------------------|------|
|                  | Lamivudine | Emtricitabine |
| Detection wavelength at 263nm | 0.31       | 0.39       |
| Detection wavelength at 259nm | 0.25       | 0.64       |
| Flow rate 0.8ml/min | 0.74       | 0.85       |
| Flow rate 1.2ml/min | 0.89       | 0.99       |

Table 6: Results for Ruggedness

| Parameters (n=3) | %RSD |
|------------------|------|
|                  | Lamivudine | Emtricitabine |
| Analyst-1        | 0.46       | 0.20       |
| Analyst-2        | 0.99       | 0.27       |

Table 7: Results for Assay of Marketed formulation

| Drug          | Label claim (mg/tab) | Amount recovered | % Amount found in drug |
|---------------|----------------------|------------------|------------------------|
| Lamivudine    | 300                  | 294.5            | 98.1%                  |
| Emtricitabine | 200                  | 196.7            | 98.3%                  |

Table 8: Results for Stability studies

| Parameters                 | % of degradation |
|----------------------------|------------------|
|                            | Lamivudine       | Emtricitabine    |
| Acid degradation           | 0.21             | 0.14             |
| Alkali degradation         | 0.5              | 0.78             |
| Peroxide degradation       | 0.1              | 0.31             |
| Thermal degradation        | 0.32             | 0.6              |