REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION AND FORCED DEGRADATION STUDIES OF EMTRICITABINE, RILPIVIRINE, AND TENOFOVIR ALAFENAMIDE 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 emtricitabine (EMT), rilpivirine (RIL), and tenofovir alafenamide (TAF) in combined dosage forms and its API.

Methods: Chromatographic separation was achieved on Waters ACQUITY RP-HPLC with PDA detector having Zodiac C18 Column (250×4.6×5µ) using mobile phase mixture of phosphate buffer: acetonitrile in the ratio of 40:60 v/v at 262 nm.

Results: The assay was performed with tablets, and the % assay was found to be 100.104 for EMT, 99.74 for RIL, and 102.41 for TAF which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999, which shows that the method is capable of producing good sensitivity. The retention time (RT) of EMT, RIL, and TAF using optimum conditions was found to be 2.517, 3.273, and 6.697 min. Forced degradation studies (FDS) were performed on sample using acid, base, thermal, photolytic, and peroxide degradation.

Conclusion: Due to its simplicity, rapidness, high precision, and low RT value, this method was successfully applied to the estimation of EMT, RIL, and TAF combined dosage form. The drugs were found to be stable at FDS, and the net degradation was found to be within the limits.

Keywords: Rilpivirine, Emtricitabine, Tenofovir alafenamide, Reverse-phase high-performance liquid chromatography.

INTRODUCTION

Emtricitabine (EMT) [1-3] is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults which works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA, resulting in early chain termination. Its chemical name is 5-fluoro-1-{(2R, SS)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl}cytosine and the molecular formula is C9H15FN5O5S.

Tenofovir alafenamide (TAF) [4-8] is an NRTI and a novel ester prodrug of the antiretroviral tenofovir. Its chemical name is ((2R)-1-[(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methyl) phosphoric acid and the molecular formula is C23H29FN7O6P.

Rilpivirine (RIL) [9] is non-NRTI (NNRTI) which is used for the treatment of HIV-1 infections in treatment-naive patients. RIL is a non-competitive NNRTI that binds to reverse transcriptase. Its chemical name is 4-(((4-[(1E)-2-cyanoeth-1-en-1-yl]-2,6-dimethyl[phenyl]amino)pyrimidin-2-yl)amino)benzonitrile and the molecular formula is C21H19N5O2P.

According to literature survey, there was no official method for the simultaneous estimation of EMT, RIL, and TAF, but only few reverse-phase high-performance liquid chromatography (RP-HPLC) [10-12] methods have been described in the literature for individual or in combination with other drugs for the estimation which were found to have high retention time (RT) and more total run time for analysis. There was no stability indicating analytical methods reported for simultaneous estimation of EMT, RIL, and TAF. The aim of the present work deals with the development of RP-HPLC method along with forced stability studies which was found to be simple, precise, accurate, and shorter RT which makes this method good for routine analysis in research institutions which justify that the developed method is advantageous over the existing method as per the ICH as shown in Fig. 1.

METHODS

Chemical and reagents

Pure samples were obtained from Hetero Pharma Ltd., Hyderabad, India; marketed formulation of combination was purchased from local market; tetrahydrofuran and acetonitrile (ACN) were obtained from Rankem, India Co. Ltd., methanol, water, and ammonium acetate were obtained from LiChrosolv (Merck), and potassium dihydrogen orthophosphate (ODP) was obtained from Mochchem.

Buffer and mobile phase (MP) preparation

17 g of ammonium phosphate was taken in a volumetric flask and add 90 ml of water in it and mix well and make up the volume to 100 ml with water which was used as buffer.

The mixture of 40 volumes of 0.1N ODP buffer and 60 volumes of ACN (40:60 v/v) was prepared and sonicated for 10 min which was used as MP.

Standard and sample preparation

Weigh accurately 13 mg of EMT, 1.62 mg of RIL, and 20 mg of TAF in 100 ml of volumetric flask and dissolve in 10 ml of MP and make up the volume with MP. From that, 13 µg/ml of EMT, 1.62 µg/ml of RIL, and 20 µg/ml of TAF was prepared by diluting 5.3 ml–10 ml with MP which was used as stock solution.

5 tablets were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 34.62 mg and dissolved. Further dilutions were prepared in five replicates of 13 µg/ml of EMT, 1.62 µg/ml of RIL, and 20 µg/ml of TAF which were
made by adding 5.3 ml of stock solution to 10 ml of MP which was used as sample solution.

**Instrumentation**

The separation was carried out on Waters Acquity RP-HPLC with PDA detector having Empower 2 software with Zodiac C18 Column (250×4.6×5 µ). Nicolet Evolution 100 UV/visible, METSAR pH meter, POWERSONIC 405 sonoicator, Afoset er-200a analytical balance and pipettes, beakers, and burettes made of borosil were used.

**Method validation [13-15]**

The analytical method was validated with respect to parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery, and ruggedness.

**Forced stability studies**

**Preparation of solution**

Weight equivalent to 1 tablet, i.e., 200 mg of EMT, 25 mg of RIL, and 25 mg of TAF into 50 ml capacity standard volumetric flask. The contents in the flask were dissolved using methanol and sonicate it and diluted up to the mark with methanol.

**Acid degradation condition**

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 2.5 ml of 0.1N HCl was added. The flask was refluxed at 60°C for 30 min using evaporator and then allowed to cool. Then neutralize with 0.1N NaOH solution. Using MP, finally volume was made up to the mark and percentage of degradation was calculated.

**Alkali degradation condition**

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 2.5 ml of 0.1N NaOH was added. The flask was refluxed at 60°C for 30 min using evaporator and then allowed to cool. Then neutralize with 0.1N HCl solution. Finally, volume was made up to the mark with MP, and percentage of degradation was calculated.

**Thermal induced degradation condition**

200 mg of EMT, 25 mg of RIL, and 300 mg of TAF were weighed accurately and transfer into four different Petri dishes and kept in a hot air oven for 8 h at 105°C. The content in the flasks was dissolved using methanol and sonicate it and diluted up to the mark with methanol.

**Photolytic degradation condition**

A 5 ml aliquot of above stock solution was exposed to sunlight for about 6 h and then the sample diluted with 5 ml of MP, and percentage of degradation was calculated.

**Peroxide degradation condition**

Accurately 5.0 ml aliquot of above stock solution was transferred into a 50 ml round bottom flask, and 3.0 ml of 3% H₂O₂ was added. The flask was kept at room temperature for 30 min then allowed to cool. Finally, volume was made up to the mark with MP, and the percentage of degradation was calculated.

**RESULTS AND DISCUSSION**

For selecting column chiral columns of OD52546 and SCDP52546, Inertsil was chosen to separate EMT, RIL, and TAF by injecting system suitability solution with the MP at 1.0 ml/min individually. Various solvents including water, ACN, triethyl amine, ammonium acetate, 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 RT of the drug samples. After performing several trials with various combinations of Methanol, ACN and buffer, an sharp and well resolved peaks were obtained using MP of 0.01 N phosphate buffer (pH:4)-ACT in the ratio of 40:60 V/V. Under above-described experimental conditions, all the peaks were well defined and free from tailing.

**System suitability**

The RT of EMT, RIL, and TAF using optimum conditions was 2.517, 3.273, and 6.697 min, respectively. The peak symmetry were <1.5, theoretical plates were >2000, and % relative standard deviation (RSD) was <2 as shown in Table 1.

**Specificity**

The specificity of the method was evaluated using placebo solution and a blank solution. Optimized chromatogram of EMT, RIL, and TAF is shown in Table 2 and Figs. 2-3.

| Parameter | EMT | RIL | TAF |
|-----------|-----|-----|-----|
| Peak area | 1012865 | 1105605 | 1118501 |
| Theoretical plates | 2862.66 | 6433 | 6402.16 |
| Retention time (min) | 2.517 | 3.273 | 6.697 |
| Tailing factor | 0.96 | 1.22 | 1.335 |

EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

**Table 2: Results of assay of EMT, RIL, and TAF**

| Injection | EMT | RIL | TAF |
|-----------|-----|-----|-----|
| Average area | 1079.485 | 1087.21 | 1744.953 |
| Label claim (mg) | 200 | 25 | 25 |
| Amount found (mg) | 200.08 | 24.93 | 25.60 |
| Assay (%) | 100.04 | 99.74 | 102.41 |

n=6; EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

![Fig. 1: Chemical structure of (a) emtricitabine, (b) rilpivirine, and (c) tenofovir alafenamide](image1)

![Fig. 2: Optimized chromatogram of emtricitabine, rilpivirine, and tenofovir alafenamide](image2)

![Fig. 3: Assay chromatogram of emtricitabine, rilpivirine, and tenofovir alafenamide](image3)
Linearity
Weight accurately 13 mg of EMT, 1.62 mg of RIL, and 20 mg of TAF in 100 ml of volumetric flask and dissolve in 10 ml of MP and make up the volume with MP. From the above stock solution, 13 µg/ml of EMT, 1.62 µg/ml of RIL, and 20 µg/ml of TAF were prepared by diluting 5.3 ml–10 ml with MP as shown in Table 3 and Figs. 4-6. The correlation coefficient for linear curve obtained between concentration and area for standard preparations of EMT, RIL, and TAF is 0.997, 0.993, and 0.995.

System precision
The system precision of the proposed method was determined by analyzing the corresponding responses for three different days over a period of 1 week. One dilution of all the drugs in six replicates was injected into HPLC system and was analyzed as shown in the Table 4.

LOD and LOQ
LOD values for EMT, RIL, and TAF were 0.75, 0.253, and 0.253 µg/ml with signal-to-noise ratios of 3:1. LOQ values for EMT, RIL, and TAF were 2.254, 0.74, and 2.524 µg/ml with signal-to-noise ratios of 10:1.

Method precision
Precision was expressed as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Prepare sample preparations of EMT, RIL, and TAF as per test method and inject 6 times into the column as shown in Table 5.

Ruggedness
The ruggedness of the method was studied by the determining the analyst-to-analyst variation by performing the assay by two different analysts. The % RSD of assay values between two analysts should not be >2.0%. Results were found within the acceptance limits (RSD <2) as shown in Tables 6.

Accuracy
Accuracy of the method was determined by recovery studies. To the formulation (pre-analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, and 150%. The recovery studies were carried out 3 times and the percentage recovery and percentage mean recovery were calculated for drug as shown in Table 7. The percentage mean recovery of EMT, RIL, and TAF is 100%, 101%, and 99%, respectively. The results are given in Table 7.

Robustness
To demonstrate the robustness of the method, prepare solution as per the test method and inject at different variable conditions using different conditions such as temperature and wavelength. System suitability parameters were compared with that of method precision. The result of the robustness study of the developed assay method is established in Table 8.

Forced stability studies
The stability studies were determined by applying the physical stress to the product. It was observed that there was marked degradation in the chromatograms. Results of forced degradation studies are shown in Table 9 and blank for control is recorded. Degradation studied was performed under different conditions, and in each condition, it was

| Concentration (µg/ml) | Peak area | Concentration (µg/ml) | Peak area | Concentration (µg/ml) | Peak area |
|----------------------|-----------|----------------------|-----------|----------------------|-----------|
| 24                   | 631.586   | 3                    | 659.236   | 30                   | 1229.584  |
| 32                   | 907.713   | 4                    | 919.393   | 40                   | 1482.509  |
| 40                   | 1091.004  | 5                    | 1086.050  | 50                   | 1750.266  |
| 48                   | 1339.312  | 6                    | 1348.518  | 60                   | 2124.626  |
| 56                   | 1549.123  | 7                    | 1552.332  | 70                   | 2413.579  |

n=5; EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

Table 3: Linearity data of EMT, RIL, and TAF.
Table 4: System precision data of EMT, RIL, and TAF

| S. No | EMT | RIL | TAF |
|-------|-----|-----|-----|
|       | Retention time (min) | Peak area | Retention time (min) | Peak area | Retention time (min) | Peak area |
| Average | 2.5190 | 1078.909 | 3.273 | 1088.830 | 6.700 | 1748.044 |
| SD     | 0.002 | 14.836 | 0.003 | 14.708 | 0.10 | 1.35 |
| % RSD  | 1.39 | 1.39 | 0.10 | 1.35 | 0.12 | 1.50 |

n=3; EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide. RSD: Relative standard deviation

Table 5: Method precision data of EMT, RIL, and TAF

| S. No | EMT | RIL | TAF |
|-------|-----|-----|-----|
|       | Retention time (min) | Peak area | Retention time (min) | Peak area | Retention time (min) | Peak area |
| Average | 2.5190 | 1078.909 | 3.273 | 1088.830 | 6.700 | 1748.044 |
| SD     | 0.0024 | 14.836 | 0.003 | 14.708 | 0.10 | 1.35 |
| % RSD  | 0.10 | 1.38 | 0.10 | 1.35 | 0.12 | 1.30 |

n=6; EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide. RSD: Relative standard deviation

Table 6: Ruggedness data of EMT, RIL, and TAF

| Sample | EMT         | RIL         | TAF         |
|--------|-------------|-------------|-------------|
| Analyst 1 | 100.86     | 100.479884 | 100.723731 |
| Analyst 2 | 99.97565   | 100.51467  | 99.1048846 |

EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

Table 7: Recovery data of EMT, RIL, and TAF

| Drug | Sample (%) | Amount (mg) | Area       | % Mean | % Average |
|------|------------|-------------|------------|--------|-----------|
| EMT  | 50         | 32          | 930.06     | 102.87 | 100.19    |
|      | 100        | 40          | 1050.7     | 99.6   |           |
|      | 150        | 48          | 1300.2     | 98.1   |           |
| RIL  | 50         | 4           | 930.02     | 102.52 | 101.3     |
|      | 100        | 5           | 1085.1     | 99.08  |           |
|      | 150        | 6           | 1380.5     | 102.5  |           |
| TAF  | 50         | 40          | 930.02     | 99.21  | 99.7      |
|      | 100        | 50          | 1085.1     | 101.28 |           |
|      | 150        | 60          | 1380.5     | 98.13  |           |

n=3; EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

Table 8: Robustness results of EMT, RIL, and TAF

| Parameter | EMT | RIL | TAF |
|-----------|-----|-----|-----|
|           | Rt (min) | Tf | Rt (min) | Tf | Rt (min) | Tf |
| Flow rate | 1.0 ml/min | 2.987 | 1.338 | 3.880 | 1.676 | 7.893 | 1.525 |
|           | 1.4 ml/min | 2.167 | 1.758 | 2.810 | 1.354 | 5.700 | 1.550 |
| Wavelength | 260 nm | 2.513 | 1.704 | 3.260 | 1.310 | 6.617 | 1.600 |
|           | 264 nm | 2.490 | 1.769 | 3.240 | 1.310 | 6.627 | 1.565 |

Rt: Retention time, Tf: Tailing factor, EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

Table 9: Robustness results of EMT, RIL, and TAF

CONCLUSION

A simple, rapid, accurate, and precise stability-indicating HPLC analytical method had been developed and validated for the routine simultaneous estimation of EMT, RIL, and TAF in API and tablet dosage forms. The RT of EMT, RIL, and TAF using optimum conditions was 2.517, 3.273, and observed that no interference of degradants with the analyte peak as shown in Figs. 7-11.
Table 9: Stability studies results of EMT, RIL, and TAF

| Condition | EMT | RIL | TAF |
|-----------|-----|-----|-----|
|           | Area | % Degraded | Area | % Degraded | Area | % Degraded |
| Control   | 1078.909 | - | 1088.830 | - | 1748.044 | - |
| Acid      | 988.107 | 6.25 | 1638.986 | 6.04 | 1229.216 | 6.70 |
| Base      | 985.109 | 5.08 | 1649.683 | 5.19 | 1224.892 | 5.76 |
| Peroxide  | 985.537 | 7.05 | 1641.595 | 6.99 | 1227.220 | 7.55 |
| Thermal   | 985.109 | 3.42 | 1649.646 | 2.54 | 1224.892 | 3.59 |
| Photo     | 985.023 | 1.85 | 1649.683 | 1.16 | 1224.559 | 2.32 |

EMT: Emtricitabine, RIL: Rilpivirine, TAF: Tenofovir alafenamide

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AUTHORS’ CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTEREST

The authors declared that they have no conflicts of interest.

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