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METHOD DEVELOPMENT, VALIDATION AND FORCE DEGRADATION STUDY OF EMTRICITABINE AND TENOFOVIR ALAFENAMIDE IN THEIR PHARMACEUTICAL DOSAGE FORMS USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract: A study has been conducted to develop and validate a rapid and highly selective stability-indicating LC-MS/MS method, and to identify a degradation product for Emtricitabine and Tenofovir alafenamide. Emtricitabine and Tenofovir alafenamide are nucleoside reverse transcriptase inhibitors (NRTIs) used to treat HIV infection in adults and children.Shimadzu LC-20 AT equipped with mass spectrometer ABScix API 200 was used for LC-MS/MS. The chromatographic analysis were performed on Agilent, Zorbax, C18 (150mm x 4.6mm, 5µm) column using 10mM ammonium acetate buffer in water having pH 5.0 and Methanol (50:50, v/v) as a mobile phase. The flow rate was kept at 1 mL/min with a short run time of 10 minutes and the sample injection volume was 20 µL. MRM scans have been performed for m/z products 248.300 (daughter ion 130.000) for Emtricitabine and 477.200 (daughter ion 346.500) for Tenofovir alafenamide. A force degradation study has been performed using LC-MS for Emtricitabine and Tenofovir alafenamide at different stress levels(acidic, alkaline, oxidative, photolytic and thermal) to demonstrate the stability-indicating ability of the developed LC-MS/MS method. Emtricitabine and Tenofovir alafenamide were unstable in acidic, basic as well as oxidation conditions whereas stable in thermal and photo-degradation conditions. The developed method is validated as per ICH guidelines and found to be linear, precise, accurate, and robust. As a result of all the validation parameters, we can conclude that the developed method can be used for routine therapeutic drug monitoring with the desired precision and accuracy.

Keywords: Emtricitabine, Force Degradation, Liquid chromatography-tandem mass spectrometry, Nucleoside Reverse Transcriptase Inhibitor, Tenofovir Alafenamide.

1. Introduction

Emtricitabine, (4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one), with trade name Emtriva, is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults and children [1]. As a component of antiretroviral therapy, Emtricitabine (EMTR) effectively reduces and/or maintains suppression of viral load in antiretroviral-naive adults or experienced adults switching from stable combination regimens. It is also a preferred choice for patients coinfected with HIV and hepatitis B virus when combined with tenofovir, which also has anti-hepatitis B activity [2].

Tenofovir alafenamide, propan-2-yl (2R)-2-[[[(2S)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-phenoxyphosphoryl]amino]propanoate[3], is also a nucleotide reverse transcriptase which blocks reverse transcriptase, an enzyme crucial for the viral production in HIV-infected individuals[4]. Tenofovir alafenamide (TENO) is a prodrug of tenofovir (TFV) that is approved for HIV treatment in combination with other antiretroviral drugs [5].Tenofovir alafenamide shows high antiviral efficacy and improved renal and bone safety compared with tenofovir disoproxil fumarate when used for HIV treatment. Emtricitabine and tenofovir alafenamide had more favorable effects on bone mineral density and biomarkers of renal safety [6].

The literature survey reveals several Spectrophotometric and HPLC methods [7 - 11] but very few LC-MS methods are available for the determination of Emtricitabine and Tenofovir Alafenamide in plasma [12 - 16]either alone or combined with other antiviral drugs.According to the literature survey, the force degradation study of Emtricitabine and Tenofovir Alafenamide were carried out using HPLC/ UV Spectrophotometric methods only [17,18].The present work describes the quantitative analysis of the Emtricitabine and Tenofovir alafenamide after exposure to different types of force degradation conditions by LC-MS/MS. The aim was to establish a rapid and highly selective method to determine both the drugs by Liquid chromatography-tandem mass spectrometry. The method was extensively validated and subsequently applied for the force degradation studies for using LC-MS/MS. This study will be useful to determine the self-life of the Emtricitabine and Tenofovir alafenamide in different conditions. The stability-indicating method was validated as per International Conference on Harmonization (ICH) guidelines [19]. The force degradation study was performed to assess the stability of Emtricitabine and Tenofovir alafenamide in drugs and their products.

2. Materials and methods

2.1. Materials

Emtricitabine and Tenofovir Alafenamide were obtained from Par Drugs Pvt Ltd(Baroda, Gujarat, India) and Avantika Medix Pvt Ltd (Ahmedabad, Gujarat, India), respectively. Acetonitrile (J.T. Baker), Water (Aquarch), and Methanol (J.T. Baker)were ofLC-MS grade. The other chemicals used for this study, such as Ammonium acetate, Acetic acid, Hydrogen Peroxide, and Hydrochloric acid were of analytical grade.

2.2. Instrumentation

The LC-MS/MS instrument used was Shimadzu LC-20 AT equipped with mass spectrometer ABScix API 2000 equipped with the auto-sampler, auto-injector, column oven,ESI - electron spray ionizer with Q1 and collision energy. The chromatographic separation was carried out using Agilent, Zorbax, C18 (150mm x 4.6mm, 5µm) column. Weighing balance of Shimadzu ATX-224, ultra sonicator of Frontline 1870, pH meter of Analab Scientific Private Ltd, Hot Air Oven of Kesar Control System, and Photo-stability Chamber of Kesar Control System were used for the present study.

2.3. Standard and Sample Preparation

Water and Methanol in the ratio of 50:50(v/v) was used as a diluent in the preparation of analytical solutions. Standard Stock Solution of Emtricitabine was prepared by accurately weighing and transferring about 20.0mg of Emtricitabine into a 100mL volumetric flask and making up the volume with diluent(200µg/mL). Standard Stock Solution of Tenofovir Alafenamide was prepared by accurately weighing and transferring about 25.0mg of Tenofovir Alafenamideinto a 100mL volumetric flask and making up the volume with diluent (250µg/mL). Working Standard Solution was prepared by taking 1mL from Emtricitabine stock solution and 1mL from Tenofovir Alafenamidestock solution into a 100mL volumetric flask and making up the volume with diluent. (EMTR-2.0µg/mL and TENO-2.5µg/mL)

2.4. LC-MS/MS analysis

The LC-MS/MS analysis was carried out for method development, validation and degradation study under the following conditions: Prepare 10mM ammonium acetate buffer in water having pH 5.0 and Methanol in the ratio of 50:50 and used as a mobile phase. The flow rate of the mobile phase 1.0 mL/min was used throughout the analysis. The total run time was 10 min and the sample injection volume was 20 µL. Column oven temperature was kept at 35 °C. For mass spectrometer, MRM scan study of Emtricitabine and Tenofovir Alafenamidewas carried out usingpositive Electrospray Ionization (ESI+) mode. Where, Curtain gas 20psi, Ion spray voltage 5000Kv, source temperature 400°C, Ion source gas 1 and 2 were 50psi, Declustering potential 30 V, entrance potential 10 V, and Focusing Potential 300 V. The scan time selected was 1-10 min. The MRM scan for m/z product 248.300 (daughter ion 130.000) for Emtricitabine and m/z product 477.200 (daughter ion 346.500) for Tenofovir Alafenamidehas been carried out. The selected transitions and fragmentation pathways for all compounds are summarized in this study. Further, the method was validated by testing parameters like LOD, LOQ, linearity, Precision, Accuracy, ruggedness, and Robustness, etc.

2.5. Method Validation

The following method has been validated using the below-mentioned validation parameters as per the ICH Q2 (R1) guidelines specifications [19].

2.5.1. Linearity and Range:

The range for Emtricitabine and Tenofovir Alafenamide was taken from 50% to 150% of the standard preparation. The standard stock solution of Emtricitabine was prepared by diluting 20mg of Emtricitabine ina 100mL volumetric flask with diluent (200µg /mL) whereas the standard stock solution of Tenofovir Alafenamide was prepared by diluting 25mg of Tenofovir Alafenamide in a 100 mL volumetric flask with diluent (250µg/mL). Linearity of any method declares a proportional relationship between input concentrations and the obtained output peak area responses [20]. Linearity solution preparation is shown in Table.1.

Table.1 Standard solutions preparation for Linearity

Linearity Level (%)	Std stock soln of Emtricitabine (mL)	Std stock soln of Tenofovir Alafenamide(mL)	Make up with diluent (mL)	Conc of Tenofovir Alafenamide (µg /mL)	Conc of Emtricitabine (µg /mL)
50	0.50	0.50	100	1.25	1.0
75	0.75	0.75	100	1.87	1.5
100	1.00	1.00	100	2.50	2.0
125	1.25	1.25	100	3.12	2.5
150	1.50	1.50	100	3.75	3.0

2.5.2. Precision

Intraday precision was performed on same day for 3 concentrations of the given range (n=3) and Interday precision was performed on three consecutive days for 3 concentrations of the given range (n=3). For Emtricitabine standard preparation, range of Lower concentration- 50% (1.0 µg /mL), Middle concentration- 100% (2.0 µg /mL) and higher concentration- 150% (3.0 µg /mL) were taken whereas for Tenofovir Alafenamide standard preparation, range of Lower concentration- 50% (1.25 µg /mL), Middle concentration- 100% (2.5 µg /mL) and higher concentration- 150% (3.75 µg /mL) were taken.

2.5.3. Repeatability

Repeatability was performed for 1 concentration (n=6) at 100% level. For Emtricitabine and Tenofovir Alafenamide standard preparation, Middle concentration- 100% (For Emtricitabine: 2.0 µg /mLand TenofovirAlafenamide: 2.5µg /mL) was taken for the study.

2.5.4. Recovery

The standard stock solution of Emtricitabine was prepared by diluting 20.0mg of Emtricitabine in 100mL volumetric flask with diluent (200µg /mL)whereas the standard stock solution of Tenofovir Alafenamidewas prepared by diluting 25.0mg of Tenofovir Alafenamidein 100mL 100mL volumetric flask with diluent (250µg /mL). Recovery solution preparation is shown in Table.2.

Table.2 Recovery solution preparation

Recovery Level (%)	Solution 1(mL)	Std stock soln of Emtricitabine(mL)	Std stock soln of Tenofovir Alafenamide (ml)	Diluted with diluents(mL)
80	0.5	0.4	0.4	10
100	0.5	0.5	0.5	10
120	0.5	0.6	0.6	10

2.5.5. Robustness:

The robustness study was accomplished to assess the effect of small but deliberate variation in the chromatographic condition.Robustness was performed by infusing working standard preparation for different flow rates and different mobile phase compositions. (Flow rate: ±0.2mL/min, Solvent % in mobile phase: ±2% solvent).

2.5.6. Assay for marketed formulation

Standard Stock Solution of Emtricitabine was prepared by accurately weighing and transferring about 20.0mg of Emtricitabine into a 100mL volumetric flask and making up the volume with diluent (200µg /mL). Standard Stock Solution of Tenofovir Alafenamide was prepared by accurately weighing and transferring about 25.0mg of Tenofovir Alafenamide into a 100mL volumetric flask and making up the volume with diluent (250µg /mL). Working Standard Solution was prepared by taking 1mL from Emtricitabine stock solution and 1mL from Tenofovir Alafenamide stock solution into a 100mL volumetric flask and making up the volume with diluents (EMTR-2.0µg /mL and TENO-2.5µg /mL). Pharmaceutical Formulation Label claims of Emtricitabine was 20.0mg and Tenofovir Alfenamide was 25.0mg. Sample Stock Solution for the assay was prepared by accurately weighing 20mg of EMTR/25mg of TENOinto a 100mL volumetric flask and 60 mL of diluents was added followed by sonication for15 min. Then the volume was made-up with diluent to

100mL and was filteredwith Whatman filter paper no-1. Sample Working Solution for the assay was prepared by taking 1mL from sample stock solution into a 100mL volumetric flask and made-up with diluent(EMTR-2.0µg /ml and TENO-2.5µg /ml).

2.6. Force Degradation Studies

In order to establish the stability of the Emtricitabine and Tenofovir Alafenamide, research has been conducted under a variety of stress conditions, including acidic, basic, oxidation, thermal, and photolytic. Forced degradation studies were conducted according to Q1 guidelines of ICH [19].

2.6.1 Forced Degradation solution preparation to find out Degradant Compound from Standard 2.6.1.1 Emtricitabine Degradation Procedure

1) Acid Degradation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 1N HCL and the flask was kept for 2 hours in a water bath at 60℃. After 2 hours it was diluted to make up the volume with diluent(2.0µg /mL).

2) Base Degradation: 1.0mL of standard stock solutionwastransferred into a 100mL volumetric flask followed by the addition of1.0mL 1N NaOH and the flask was kept for 4 hours in a water bath at 60˚C.After 4 hours it was diluted to make up the volume with diluent(2.0µg /mL).

3) Oxidation Degradation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 5 hours in a water bath at 60℃.After 5 hours it was diluted to make up the volume with diluent(2.0µg /mL).

4) Thermal Degradation:50.0mg of Emtricitabine standard was taken in a petri-dish and placed in ahot air oven at 105°C for 5 days. After 5 days,20.0 mg of the above compound was weighed and transferred into a 100mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).

5) Photo Degradation: 50.0 mg of Emtricitabine standard was taken in a petri-dish and placed into a photo-stability chamber for 5 days.After 5 days, 20.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).

2.6.1.2 Tenofovir Alafenamide Degradation Procedure

1) Acid Degradation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 1N HCL and the flask was kept for 4 hours in a water bath at 60℃. After 4hours it was diluted to make up the volume with diluent(2.5µg /mL).

2) Base Degradation: 1.0mL of standard stock solution was transferred into a 100 mL volumetric flask followed by the addition of1.0mL 1N NaOH and the flask was kept for 3 hours in a water bath at 60˚C.After 3 hours it was diluted to make up the volume with diluent(2.5µg /mL).

3) Oxidation Degradation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of 1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 6 hours in a water bath at 60℃.After 6 hours it was diluted to make up the volume with diluent(2.5µg /mL).

4) Thermal Degradation:50.0 mg of Tenofovir Alafenamide standard was taken in a petri-dish and placed in a hot air oven at 105°C for 5 days. After 5 days, 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).

5) Photo Degradation: 50.0 mg of Tenofovir Alafenamide standard was taken in a petri-dish and placed into a photo-stability chamber for 5 days.After 5 days 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).

Note: The forced degradation preparation solution of Emtricitabine and Tenofovir Alafenamide is infused into the mass spectrometer to identify the degradation product of Emtricitabine and Tenofovir Alafenamide.

2.6.2 Forced Degradation Solution preparation for LC-MS/MS

Sample Stock Solution for forced degradation:

25mg of TENO/20mg of EMTR were accurately weighed and transferred into a 100mL volumetric flask and 60 mL of diluents was added followed by sonication for15 min. Then the volume was made-up with diluent to 100mL and was filtered with Whatman filter paper no-1.1ml of this solution was transferred intoa 100mL volumetric flask and diluted up to the mark with diluent (EMTR-2.0µg /mL andTENO-2.5µg /mL).

2.6.2.1 Emtricitabine Degradation Procedure

1) Acid Degradation:Standard Preparation:1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of 1.0mL 1N HCL and the flask was kept for 2 hours in a water bath at 60℃. After 2 hours it was diluted to make up the volume with diluent(2.0µg /mL).
Sample Preparation: 1.0mL of sample stock solution was transferred into a 100mL volumetric flask followed by the addition of 1.0mL 1N HCL and the flask was kept for 2 hours in a water bath at 60℃. After 2 hours it was diluted to make up the volume with diluent(2.0µg /mL).

2) Base Degradation:Standard Preparation: 1.0mL of standard stock solution was transferred into a 100 mL volumetric flask followed by the addition of 1.0mL 1N NaOH and the flask was kept for 4 hours in a water bath at 60˚C.After 4 hours it was diluted to make up the volume with diluent(2.0µg /mL).
Sample Preparation: 1.0mL of sample stock solution was transferred into a 100 mL volumetric flask followed by the addition of 1.0mL 1N NaOH and the flask was kept for 4 hours in a water bath at 60˚C.After 4 hours it was diluted to make up the volume with diluent(2.0µg /mL).

3) Oxidation Degradation: Standard Preparation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of 1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 5 hours in a water bath at 60℃. After 5 hours it was diluted to make up the volume with diluent(2.0µg /mL).
Sample Preparation:1.0mL of sample stock solution was transferred into a 100mL volumetric flask followed by the addition of 1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 5 hours in a water bath at 60℃. After 5 hours it was diluted to make up the volume with diluent (2.0µg /mL).

4) Thermal Degradation: Standard Preparation: 50.0 mg of Emtricitabine standard was taken in a petri-dish and placed in a hot air oven at 105°C for 5 days. After 5 days, 20.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).
Sample Preparation: Put about 50.0 mg of Emtricitabine sample was taken in a petri-dish and placed in a hot air oven at 105°C for 5 days. After 5 days, 20.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).

5) Photo Degradation: Standard Preparation: 50.0 mg of Emtricitabine standard was taken in a petri-dish and placed into a photo-stability chamber for 5 days. After 5 days, 20.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).

Sample Preparation: 50.0 mg of Emtricitabine sample was taken in a petri-dish and placed into a photo-stability chamber for 5 days. After 5 days, 20.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.0 µg /mL).

2.6.2.2 Tenofovir Alafenamide Degradation Procedure

1) Acid Degradation: Standard Preparation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 1N HCL and the flask was kept for 4 hours in a water bath at 60℃. After 4hours it was diluted to make up the volume with diluent(2.5µg /mL).
Sample Preparation: 1.0mL of sample stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 1N HCL and the flask was kept for 4 hours in a water bath at 60℃. After 4hours it was diluted to make up the volume with diluent(2.5µg /mL).

2) Base Degradation: Standard Preparation: 1.0mL of standard stock solution was transferred into a 100 mL volumetric flask followed by the addition of1.0mL 1N NaOH and the flask was kept for 3 hours in a water bath at 60˚C.After 3 hours it was diluted to make up the volume with diluent(2.5µg /mL).
Sample Preparation: 1.0mL of sample stock solution was transferred into a 100 mL volumetric flask followed by the addition of1.0mL 1N NaOH and the flask was kept for 3 hours in a water bath at 60˚C.After 3 hours it was diluted to make up the volume with diluent(2.5µg /mL).

3) Oxidation Degradation: Standard Preparation: 1.0mL of standard stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 6 hours in a water bath at 60℃. After 6 hours it was diluted to make up the volume with diluent (2.5µg /mL).
Sample Preparation: 1.0mL of sample stock solution was transferred into a 100mL volumetric flask followed by the addition of1.0mL 30.0% Hydrogen Peroxide and the flask was kept for 6 hours in a water bath at 60℃. After 6 hours it was diluted to make up the volume with diluent (2.5µg /mL).

4) Thermal Degradation: Standard Preparation: 50.0 mg of Tenofovir Alafenamide standard was taken in a petri-dish and placed in a hot air oven at 105°C for 5 days. After 5 days, 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).

Sample Preparation: 50.0 mg of Tenofovir Alafenamide sample was taken in a petri-dish and placed in a hot air oven at 105°C for 5 days. After 5 days, 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).

5) Photo Degradation: Standard Preparation: 50.0 mg of Tenofovir Alafenamide standard was taken in a petri-dish and placed into a photo-stability chamber for 5 days. After 5 days 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).
Sample Preparation: 50.0 mg of Tenofovir Alafenamide sample was taken in a petri-dish and placed into a photo-stability chamber for 5 days. After 5 days 25.0 mg of the above compound was weighed and transferred into a 100 mL volumetric flask and diluted to make up the volume with diluent. Further, the above 1.0mL solution was transferred into a 100mL volumetric flask and diluted to make up the volume with diluent (2.5 µg /mL).

3. Result and Discussion

3.1. Method development

The literature search showed that many LC-MS methods were available for the determination of individuals and the combination of two drugs. Based on the literature, efforts were made to develop a simple method that had less retention time and higher selectivity. The developed method enables the quantification of EMTR and TENO by liquid chromatography coupled with tandem mass spectrometry. The chromatographic analysis were performed on Agilent, Zorbax, C18 (150mm x 4.6mm, 5µm) column using 10mM ammonium acetate buffer in water having pH 5.0 and Methanol (50:50, v/v) as a mobile phase. The flow rate was kept at 1 mL/min with a short run time of 10 minutes and the sample injection volume was 20 µL. After injection of Emtricitabine and Tenofovir Alafenamide in LC-MS/MS, it gives a chromatogram with a single peak at the retention time of 1.77 min and 6.80 min respectively, as shown in Fig.1-(A) and the Mass spectrum of Emtricitabine and Tenofovir Alafenamide shown in Fig.1-(B) and Fig.1-(C) respectively.

A

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	emtri-teno std for stability	emtri-teno\emtri-teno std for stability.wiff	41021.298	1.77	248.300/130.000 Da
2	emtri-teno std for stability	emtri-teno\emtri-teno std for stability.wiff	17667.843	6.80	477.200/346.500 Da

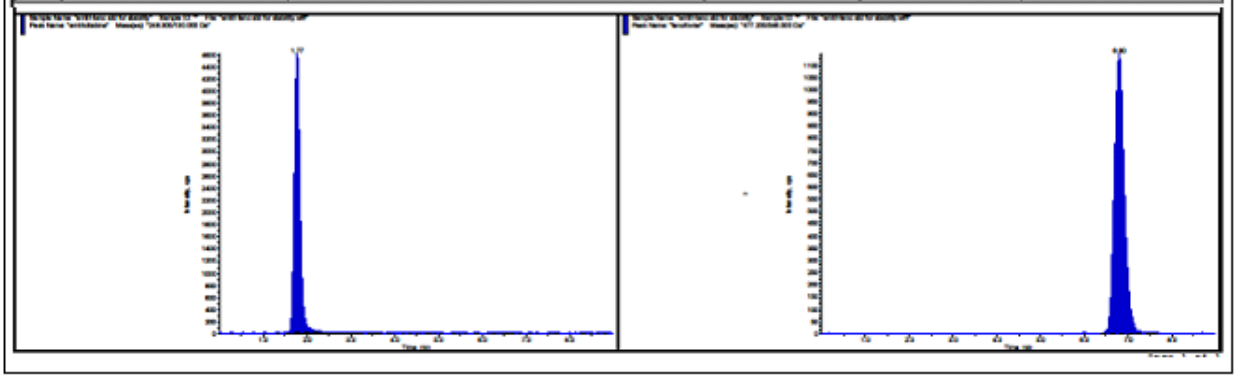

Figure 1- (A) Chromatogram of Emtricitabine and Tenofovir Alafenamide

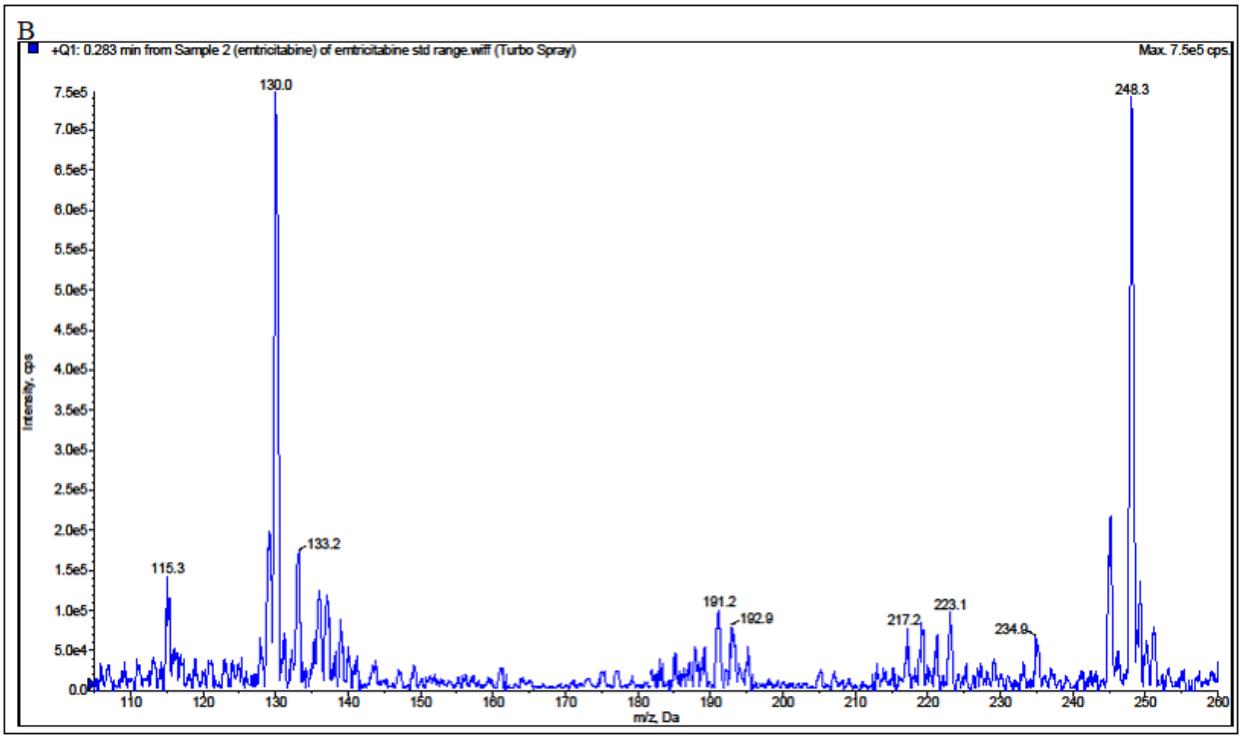

Figure 1- (B) Mass spectrum of Emtricitabine

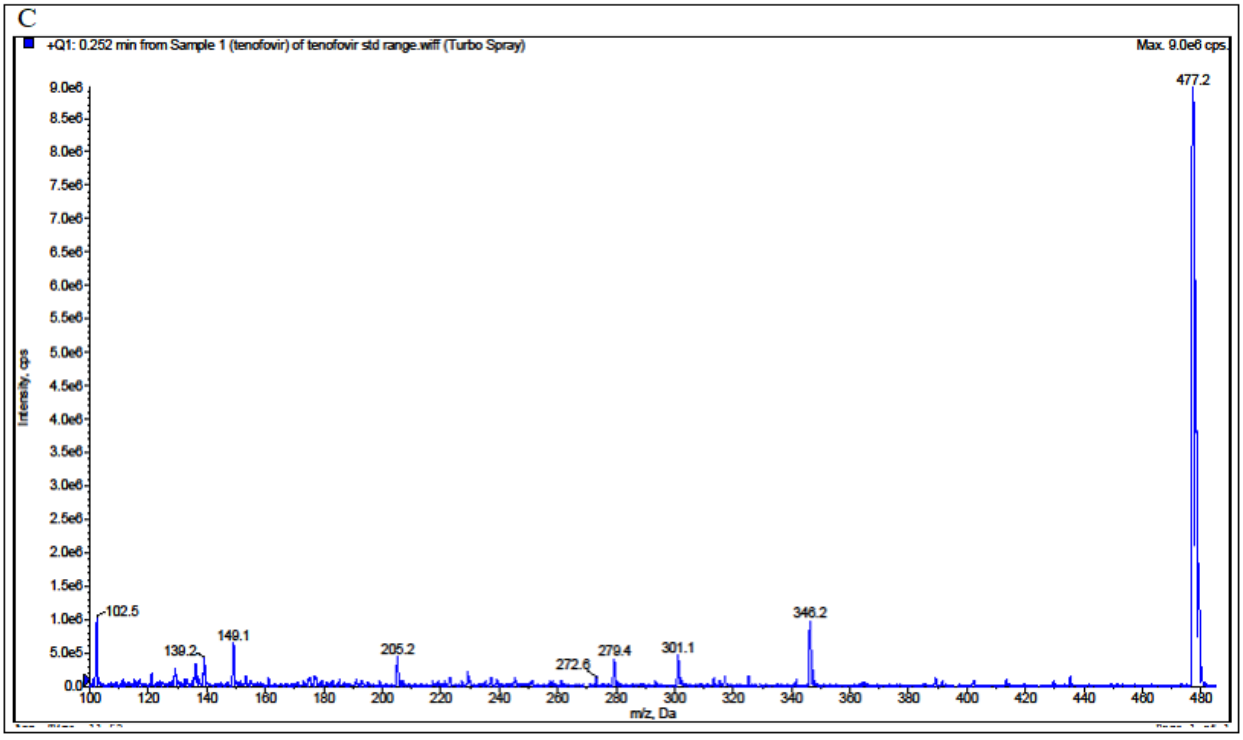

Figure 1- (C) Mass spectrum of Tenofovir Alafenamide

3.2. Method validation

After optimization, the studies for the limit of detection (LOD)and limit of quantitation(LOQ)were conducted, which showed that LOD for Emtricitabine and Tenofovir Alafenamidewere 7.311(µg /mL) and 26.300(µg /mL),respectively, whereas LOQ for Emtricitabine and Tenofovir Alafenamide were 22.153(µg /mL) and 79.698(µg /mL), respectively. Drug response was found to be linear between 50% and 150% for both drugs. As shown in Fig.2-(A) and Fig.2-(B), the calibration curves were linear with correlation coefficients of 0.999 for EMTR and 0.997 for TENO. An intraday and the interday precision study were conducted. A study was conducted at 80%, 100%, and 120% percent recovery. A 98% to 102% recovery rate was recommended. By changing the Flow rate and Mobile phase, a robustness study was performed. These conditions include the change in Flow rate: ±0.2ml/min and Solvent % in the mobile phase: ±2% solvent in the mobile phase. The results of Precision, Repeatability, Recovery, Robustness, and Assay for Emtricitabine and Tenofovir Alafenamide are shown in Table.3 to Table.7-B, respectively.

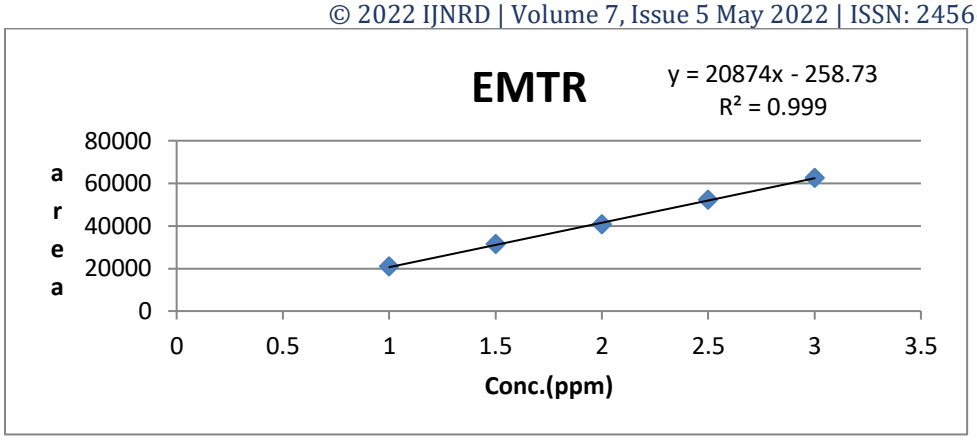

Figure 2-(A) Calibration curve Emtricitabine

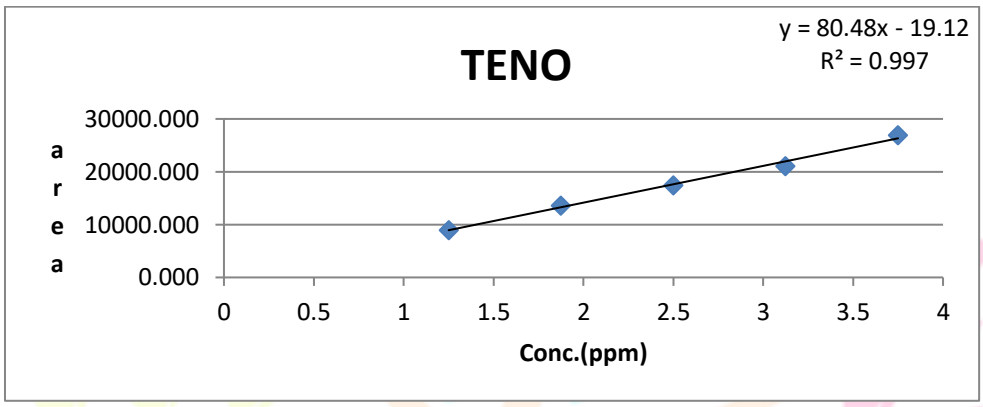

Figure 2-(B) Calibration curve Tenofovir Alafenamide

Table.3 Results of Precision study for Emtricitabine and Tenofovir Alafenamide (n=3)

		Concentration (µg/mL)	Intraday Precision			Interday Precision		
			Concentration measured		%RSD	Concentration measured		%RSD
			AVG	SD		AVG	SD	
EMTR	50%	1.00	21944.325	142.306	0.648	23192.645	290.440	1.252
	100%	2.00	40068.320	587.417	1.466	41891.307	284.864	0.680
	150%	3.00	62413.596	872.219	1.397	63147.586	298.983	0.473
TENO	50%	1.25	8824.823	34.168	0.387	9808.267	153.216	1.562
	100%	2.50	18875.773	224.927	1.192	17937.276	292.735	1.632
	150%	3.75	27121.654	493.404	1.819	28831.413	296.552	1.029

Table.4 Results of Repeatability study for Emtricitabine and Tenofovir Alafenamide

Std	Area	
	EMTR	**TENO**
1	40982.609	17631.481
2	41635.981	16987.361
3	40972.364	17558.314
4	41581.647	17086.364
5	41559.341	16979.364
6	40227.728	17194.613
Avg	41159.945	17239.583
%RSD	1.331	1.665

Table.5 Results of Accuracy (%Recovery) study for Emtricitabine and Tenofovir Alafenamide

	Amounted added in concentration level (%)	amount added(µg/mL)	amount recovered(µg/mL)	%recovery	AVG	%RSD
EMTR	80	0.8	0.786 0.806 0.810	98.280 100.783 101.283	100.116	1.607
	100	1.0	1.005 1.015 0.998	100.464 101.490 99.794	100.583	0.849
	120	1.2	1.193 1.222 1.188	99.445 101.822 98.992	100.086	0.519
TENO	80	1.0	1.008 1.002 0.983	100.847 100.168 98.257	99.757	1.347
	100	1.25	1.227 1.267 1.263	98.127 101.393 101.011	100.177	1.783
	120	1.5	1.525 1.525 1.483	101.696 101.693 98.892	100.761	1.606

Table.6 Results of Robustness study for Emtricitabine and Tenofovir Alafenamide (Average of three replicates)

		EMTR (2µg/mL)		TENO (2.5µg/mL)	
Factors	**Level**	**Peak Area**	**RSD (%)**	**Peak Area**	**RSD (%)**
Flow rate	+0.2	33879.210	0.828	13548.229	1.026
	-0.2	45845.189	0.589	20191.383	1.669
Mobile phase	+2	37182.769	0.941	15324.106	1.067
	-2	42291.928	1.032	18158.974	1.717

Table.7-A Results of Assay for Emtricitabine and Tenofovir Alafenamide

EMTR		TENO	
Area of std	**41531.366**	**Area of std**	**17522.253**
Area of samples	**%Assay**	**Area of samples**	**%Assay**
41634.303 41876.721 40897.672	100.248 100.832 98.474	17692.347 17829.543 17294.672	100.971 101.754 98.701
AVG Assay	99.851	AVG Assay	100.475
%RSD of assay	1.230	%RSD of assay	1.578

Table.7-B Results of Assay for Emtricitabine and Tenofovir Alafenamide (Lable claim)

	Label claim(w/w)	Result(w/w)	%Assay	AVG %Assay	%RSD
EMTR	10	10.025	100.248	99.851	1.230
	10	10.083	100.832		
	10	9.847	98.474		
TENO	20	20.194	100.971	100.475	1.578
	20	20.351	101.754		
	20	19.740	98.701		

3.3. Force degradation Study

3.3.1. Acidic condition:

Changes in the peaks were noted for both, Emtricitabine and Tenofovir Alafenamide under acidic conditions. Peaks were found to be degraded for both drugs. Thus, it proves that the drug is unstable in an acidic condition. Fig.3-A and Fig.3-B show the mass spectra for the acid degradation of Emtricitabine and Tenofovir Alafenamide, respectively. There was the degradation of Emtricitabine at a retention time of 5.98 min for the standard (Fig.3-C) and 5.92 min for the sample (Fig.3-D), where tenofovir Alafenamide at a retention time of 1.63 min for the standard (Fig.3-E) and 1.60 min for the sample (Fig.3-F).

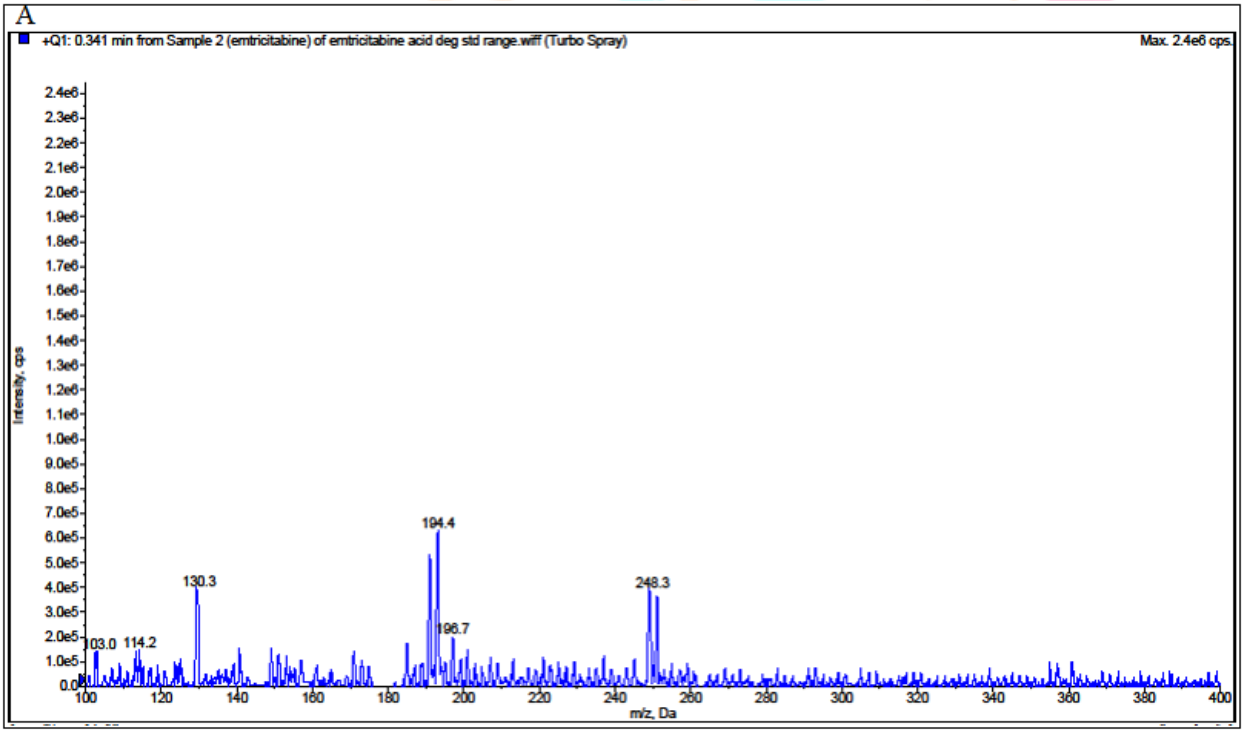

Figure 3- (A) Emtricitabine acid degradation mass spectra

B

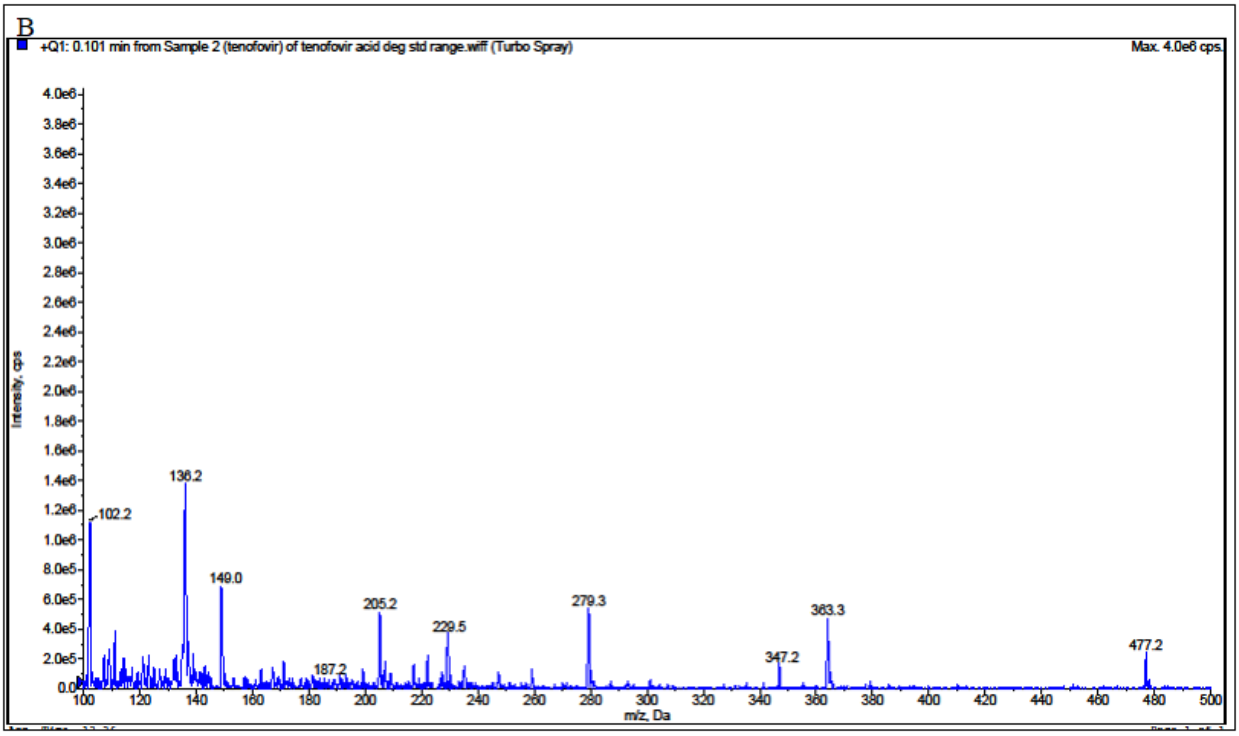

Figure 3- (B) Tenofovir Alafenamide acid degradation mass spectra

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	emtri acid deg std DP1	emtri-teno\emtricitabine acid deg std DP1.wiff	1580.745	5.98
2	emtri acid deg std DP1	emtri-teno\emtricitabine acid deg std DP1.wiff	13337.363	1.74

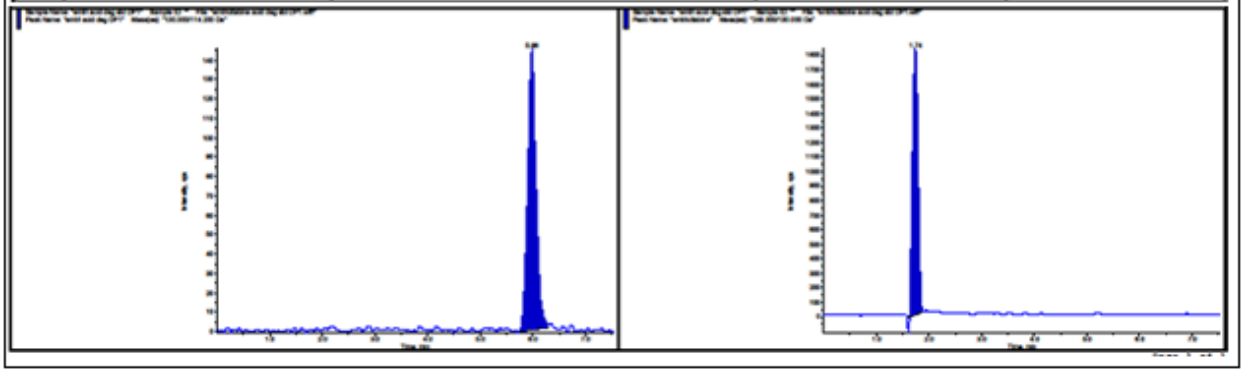

Figure 3- (C) Chromatogram of Emtricitabine degraded product in acid (Standard)

D

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	emtri acid deg smp DP1	emtri-teno\emtricitabine acid deg smp DP1.wiff	1405.361	5.92
2	emtri acid deg smp DP1	emtri-teno\emtricitabine acid deg smp DP1.wiff	13245.627	1.78

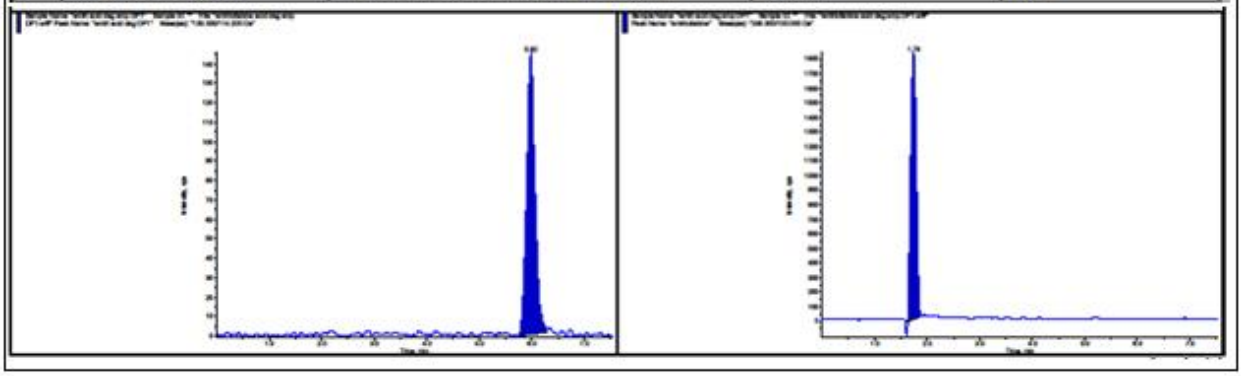

Figure 3- (D) Chromatogram of Emtricitabine degraded product in acid (Sample)

E

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	tenofovir acid deg std DP1	emtri-teno\tenofovir acid deg std DP1.wiff	26434.469	1.63	363.300/229.500 Da
2	tenofovir acid deg std DP1	emtri-teno\tenofovir acid deg std DP1.wiff	5773.996	6.69	477.200/346.500 Da

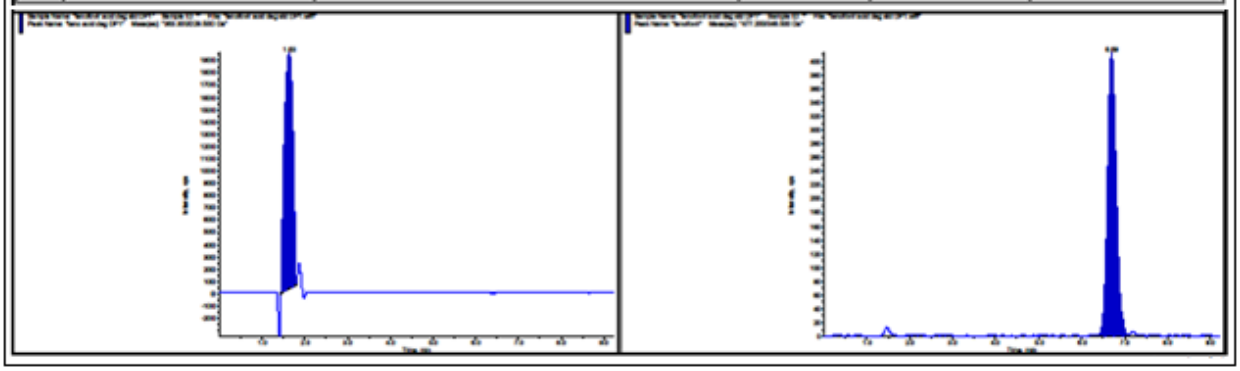

Figure 3- (E) Chromatogram of Tenofovir Alafenamide degraded product in acid (Standard)

F

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	tenofovir acid deg smp DP1	emtri-teno\tenofovir acid deg smp DP1.wiff	26258.317	1.60	363.300/229.500 Da
2	tenofovir acid deg smp DP1	emtri-teno\tenofovir acid deg smp DP1.wiff	5825.361	6.62	477.200/346.500 Da

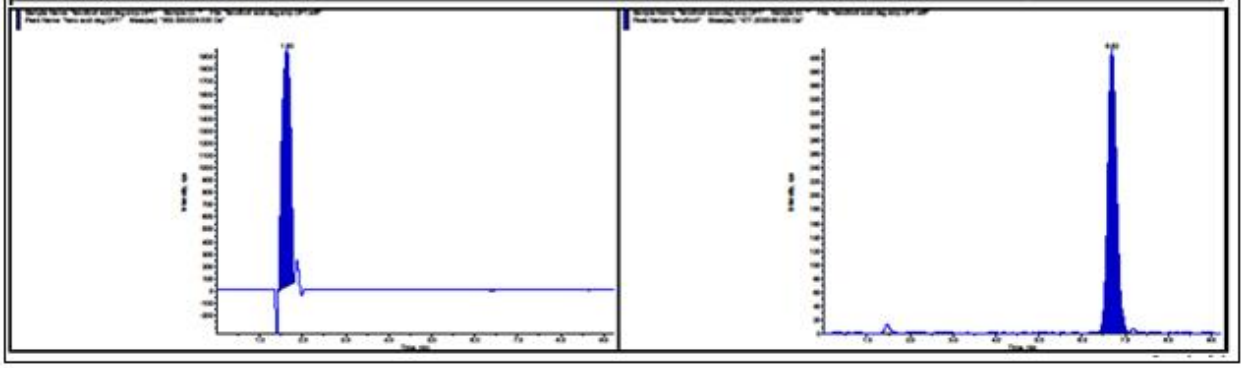

Figure 3- (F) Chromatogram of Tenofovir Alafenamide degraded product in acid (Sample)

3.3.2. Basic condition:

Both Emtricitabine and Tenofovir Alafenamide undergo degradation in alkaline conditions. This confirms that the drug is unstable under basic conditions. Fig.4-A and Fig.4-B shows the mass spectra for Emtricitabine and Tenofovir Alafenamide,respectively in a basic environment. Emtricitabine shows degradation at a retention time 0.0 min for the standard (Fig.4-C) and 0.0 min for the sample (Fig.4-D) whereas tenofovir Alafenamide shows degradation at the retention time of 2.51min for the standard (Fig.4-E) and 2.49 min for the sample (Fig.4-F).

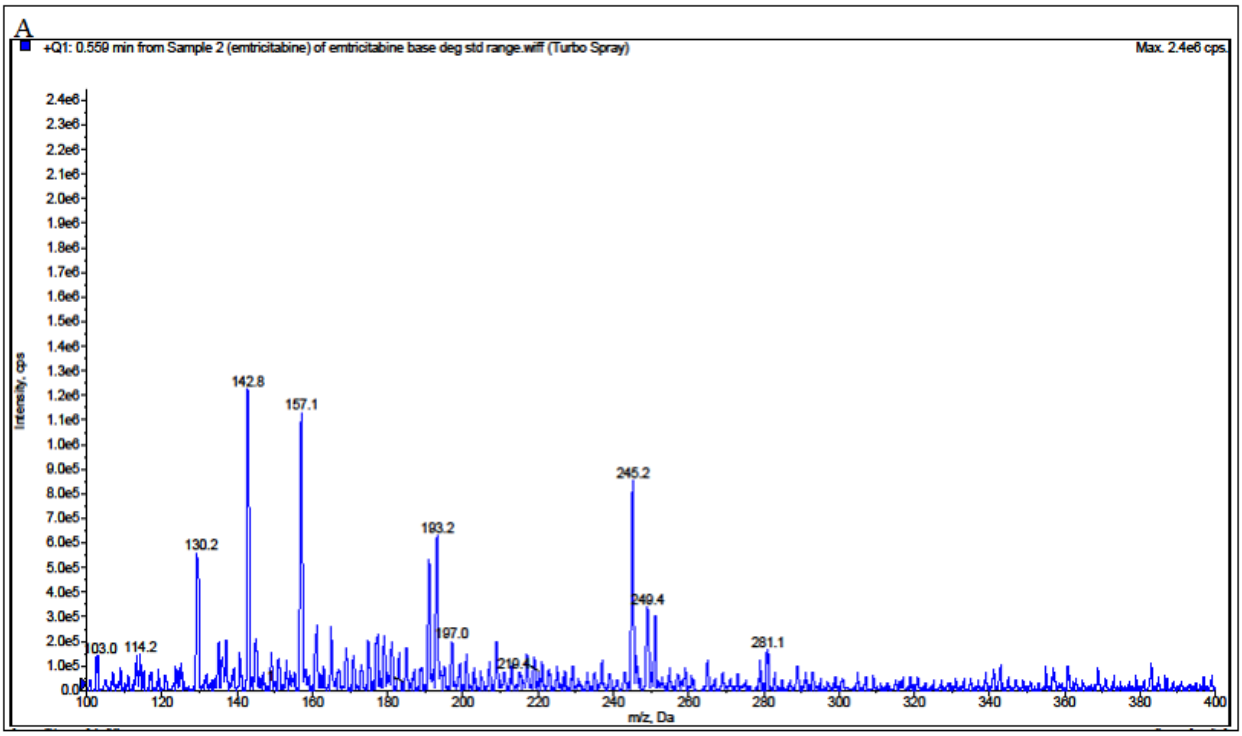

Figure 4- (A) Emtricitabine base degradation mass spectra

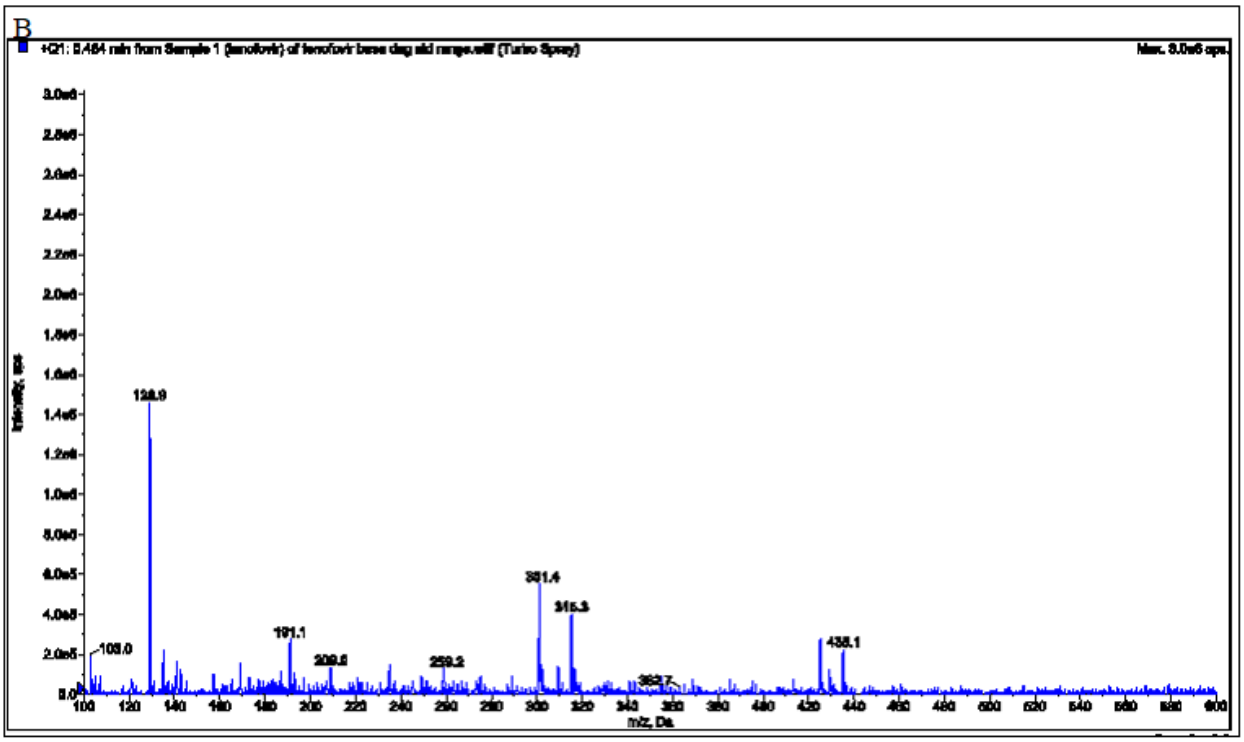

Figure 4- (B) Tenofovir Alafenamide base degradation mass spectra

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	emtri base deg std DP2	emtri-teno\emtricitabine base deg std DP2.wiff	5437.565	1.77	299.400/130.200 Da
2	emtri base deg std DP2	emtri-teno\emtricitabine base deg std DP2.wiff	0.000	0.00	248.300/130.000 Da

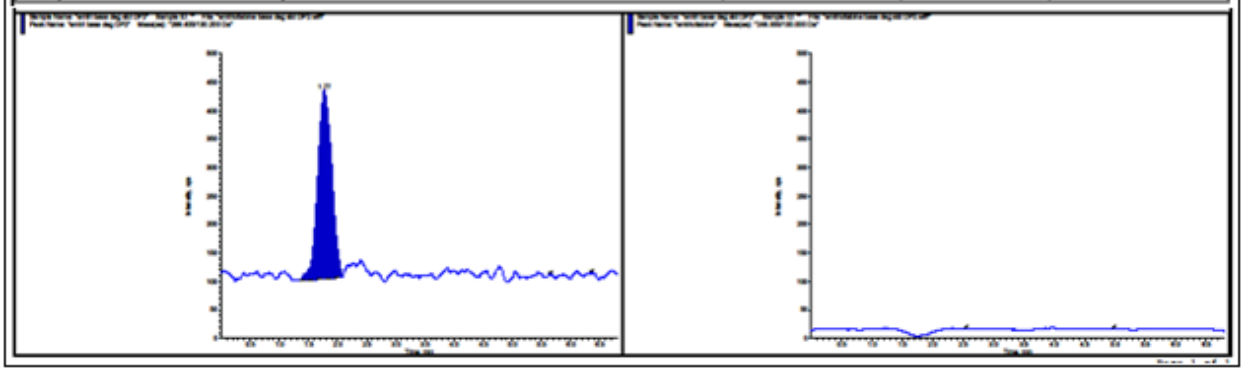

Figure 4- (C) Chromatogram of Emtricitabine degraded product in base (Standard)

D

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	emtri base deg smp DP2	emtri-teno\emtricitabine base deg smp DP2.wiff	5385.853	1.79	299.400/130.200 Da
2	emtri base deg smp DP2	emtri-teno\emtricitabine base deg smp DP2.wiff	0.000	0.00	248.300/130.000 Da

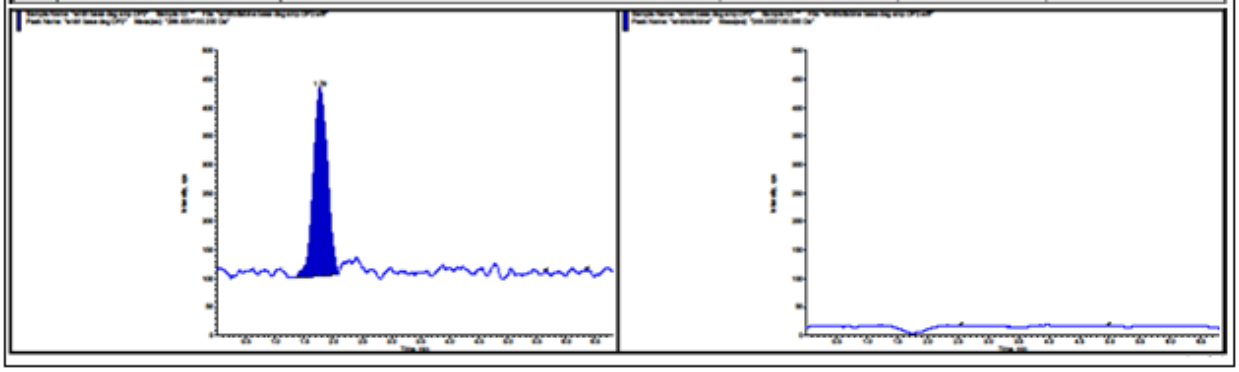

Figure 4- (D) Chromatogram of Emtricitabine degraded product in base (Sample)

E

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	teno base deg std DP2	emtri-teno\tenofovir base deg std DP2.wiff	1668.550	6.81
2	teno base deg std DP2	emtri-teno\tenofovir base deg std DP2.wiff	18960.158	2.51

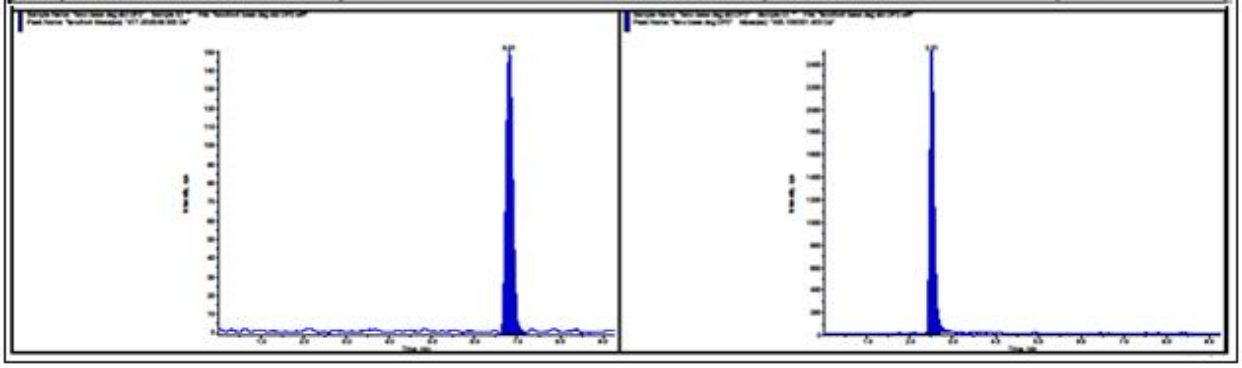

Figure 4- (E) Chromatogram of Tenofovir Alafenamide degraded product in base (Standard)

F

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	teno base deg smp DP2	emtri-teno\tenofovir base deg smp DP2.wiff	1542.631	6.88
2	teno base deg smp DP2	emtri-teno\tenofovir base deg smp DP2.wiff	19154.371	2.49

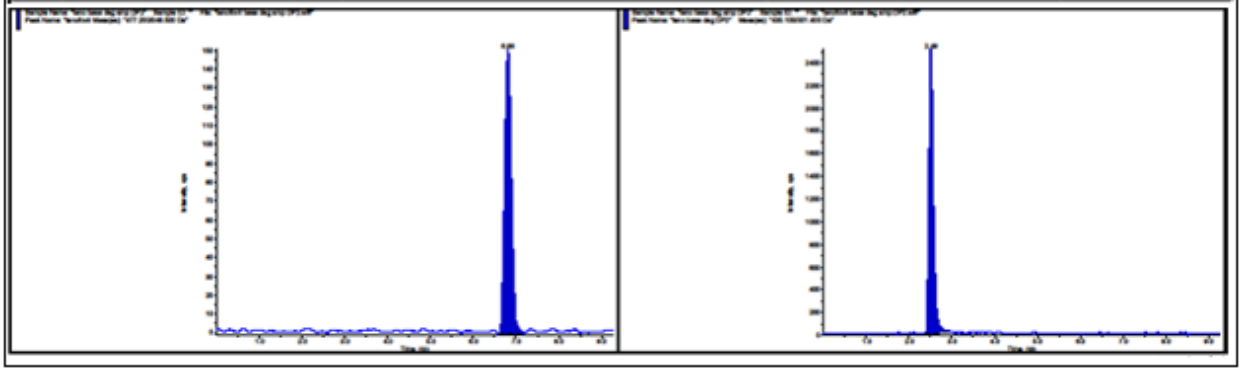

Figure 4- (F) Chromatogram of Tenofovir Alafenamide degraded product in base (Sample)

3.3.3. Oxidation condition:

In the hydrogen peroxide solution, degradation was observed. The mass spectrum of Emtricitabine and Tenofovir Alafenamide for oxidation degradation is shown in Fig.5-A and Fig.5-B. Emtricitabine observed degradation at the retention time of1.62 min for the standard (Fig.5-C) and 1.58 min for the sample (Fig.5-D). Tenofovir Alafenamide observed degradation at the retention time of6.77 min for the standard (Fig.5-E) and 6.70 min for the sample (Fig.5-F). So, both the drugs were unstable in the oxidation condition also.

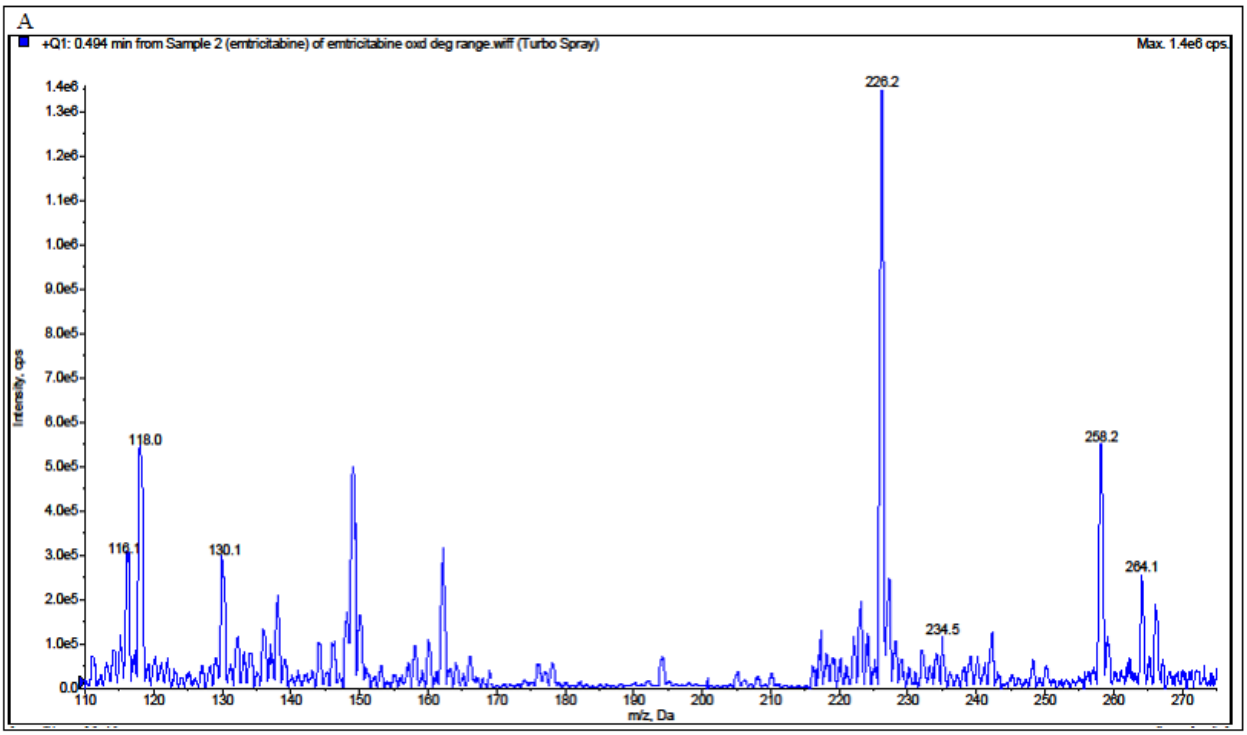

Figure 5- (A) Emtricitabine oxidation degradation mass spectra

B

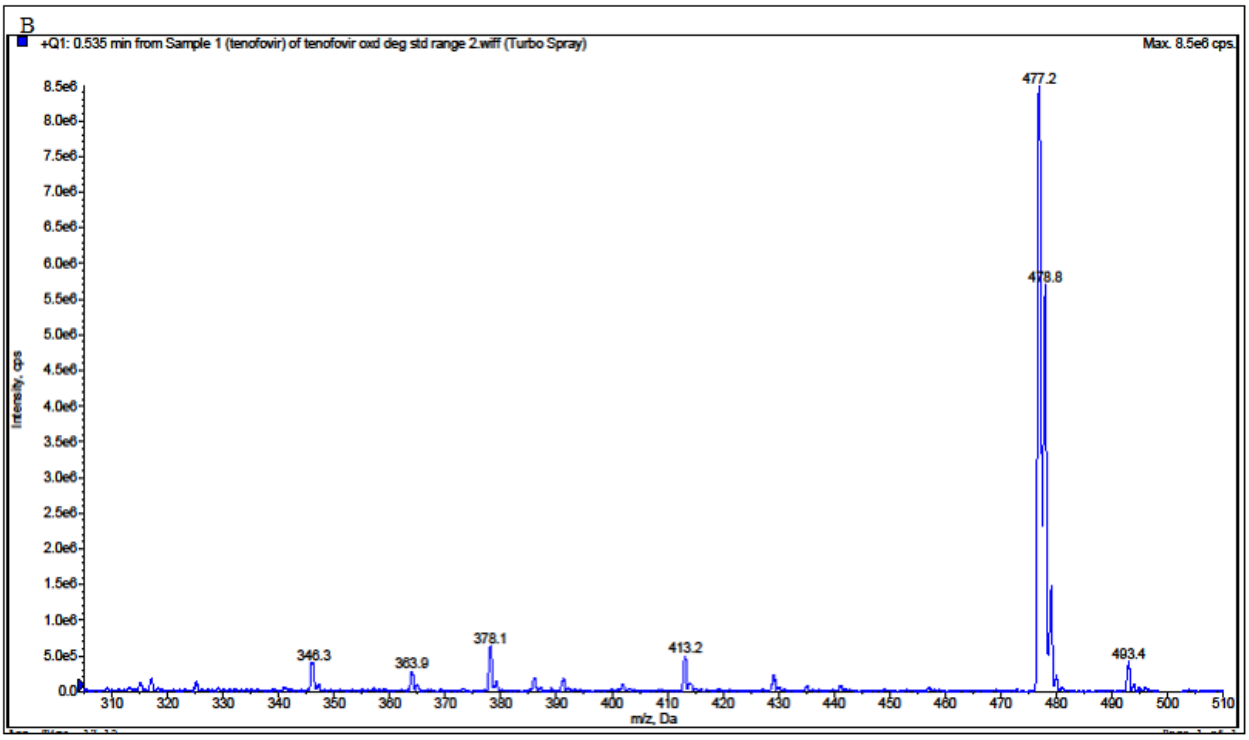

Figure 5- (B) Tenofovir Alafenamide oxidation degradation mass spectra

C

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	emtri oxd deg std DP3	emtri-teno\emtricitabine oxd deg std DP3.wiff	1135.651	1.62
2	emtri oxd deg std DP3	emtri-teno\emtricitabine oxd deg std DP3.wiff	23256.722	1.74

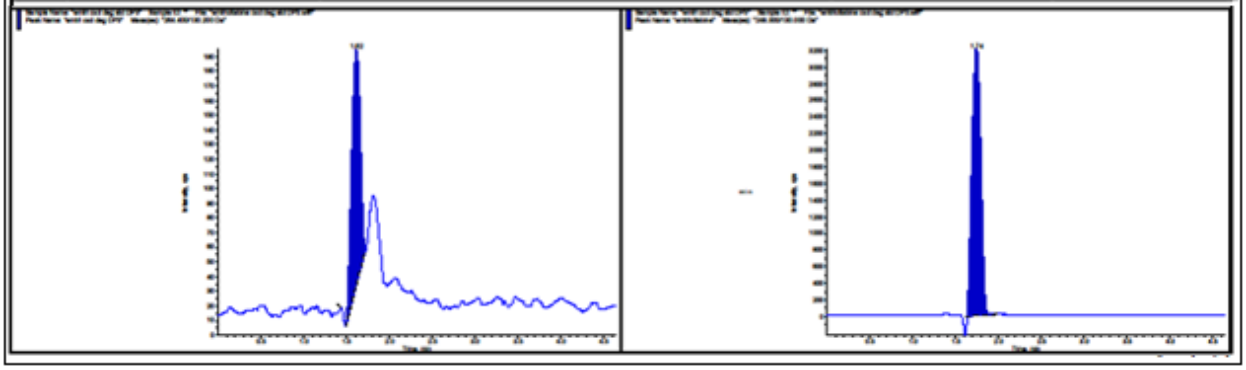

Figure 5- (C) Chromatogram of Emtricitabine degraded product in oxidation (Standard)

D

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)
1	emtri oxd deg smp DP3	emtri-teno\emtricitabine oxd deg smp DP3.wiff	1045.321	1.58
2	emtri oxd deg smp DP3	emtri-teno\emtricitabine oxd deg smp DP3.wiff	23110.321	1.76

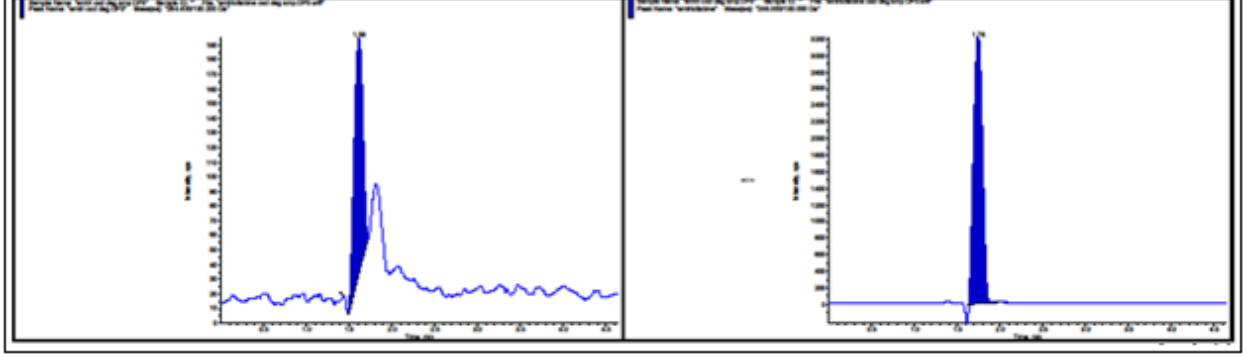

Figure 5- (D) Chromatogram of Emtricitabine degraded product in oxidation (Sample)

E

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	tenofovir oxd deg std DP3	emtri-teno\tenofovir oxd deg std DP3.wiff	4351.402	6.77	493.400/478.800 Da
2	tenofovir oxd deg std DP3	emtri-teno\tenofovir oxd deg std DP3.wiff	4645.617	6.76	477.200/346.500 Da

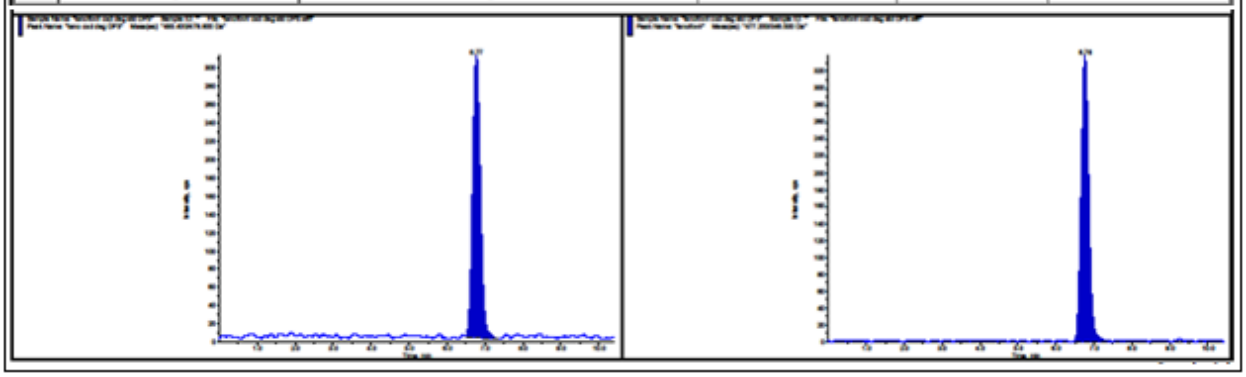

Figure 5- (E) Chromatogram of Tenofovir Alafenamide degraded product in oxidation (standard)

F

	Sample Name	File Name	Analyte Peak Area (counts)	Analyte Retention Time (min)	Analyte Mass Ranges (Da)
1	tenofovir oxd deg smp DP3	emtri-teno\tenofovir oxd deg smp DP3.wiff	4452.364	6.70	493.400/478.800 Da
2	tenofovir oxd deg smp DP3	emtri-teno\tenofovir oxd deg smp DP3.wiff	4510.427	6.80	477.200/346.500 Da

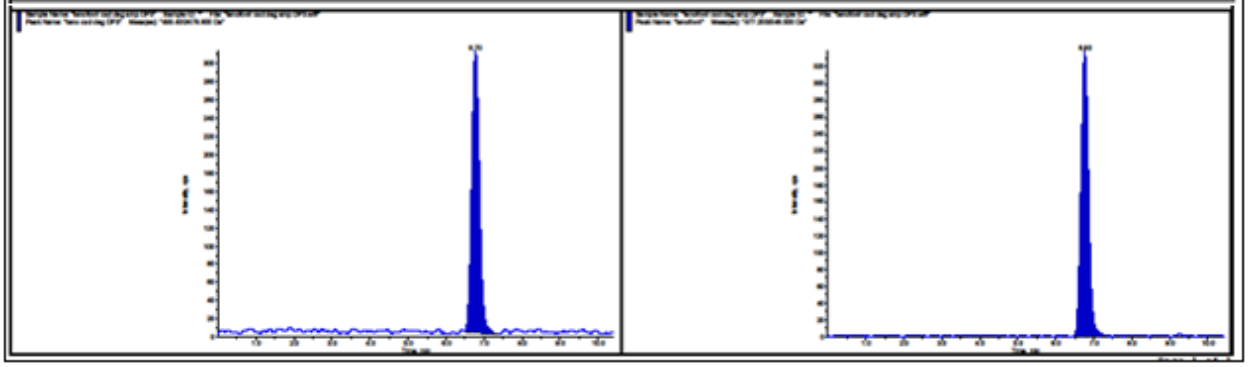

Figure 5- (F) Chromatogram of Tenofovir Alafenamide degraded product in oxidation (Sample)

3.3.4. Thermal condition:

No degradation was observed for the Emtricitabine and Tenofovir Alafenamide under thermal conditions. Both the drugs were stable in thermal conditions. The mass spectrum of Emtricitabine and Tenofovir Alafenamide for Thermal degradation is shown in (Fig.6-A and Fig.6-B) respectively.

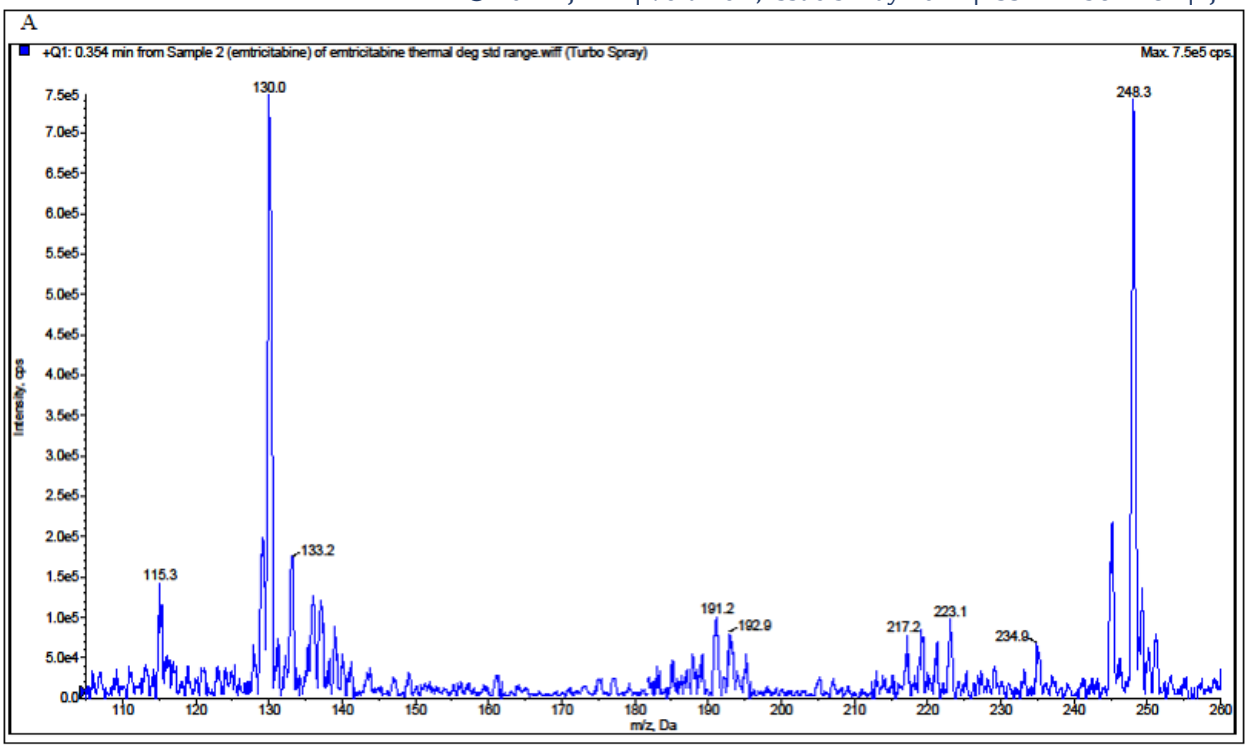

Figure 6- (A) Emtricitabine thermal degradation mass spectra

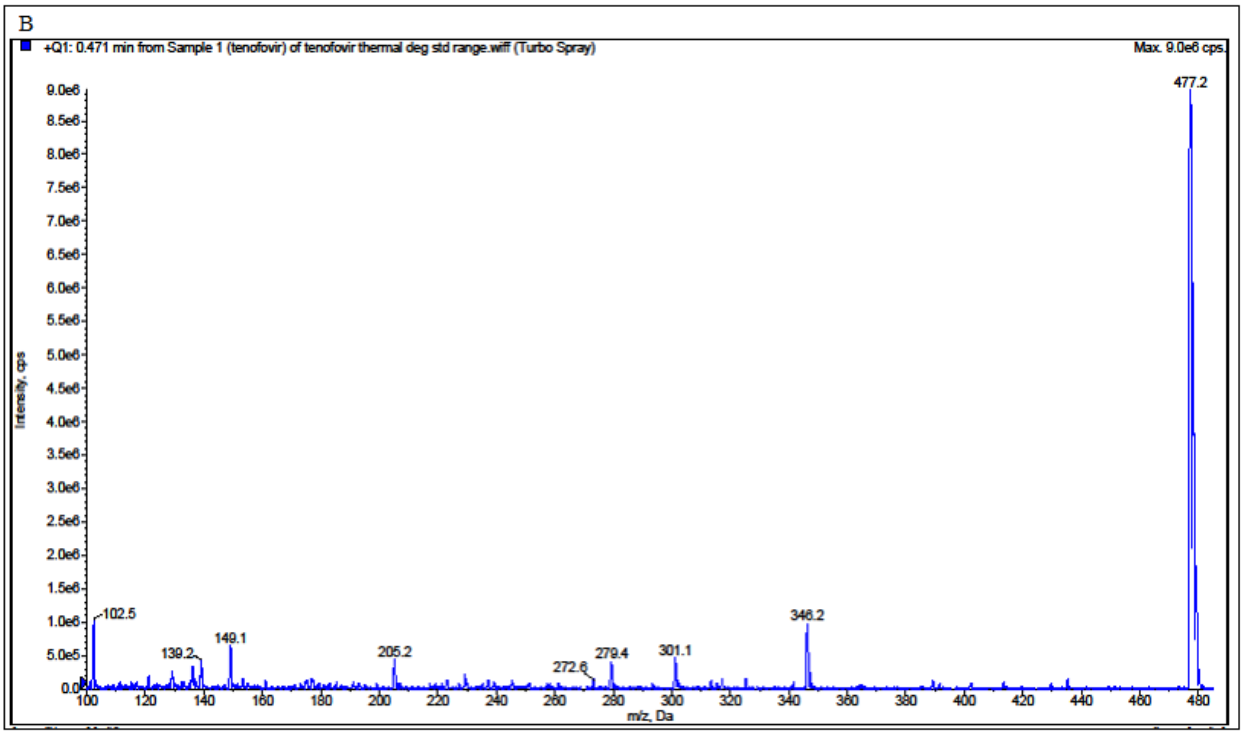

Figure 6- (B) Tenofovir Alafenamide thermal degradation mass spectra

3.3.5. Photo condition:

No degradation was observed for the Emtricitabine and Tenofovir Alafenamide in photo condition. So, Emtricitabine and Tenofovir Alafenamidearebothwerestable in photo condition. Mass spectrum of Emtricitabine and Tenofovir Alafenamide for photo-degradation is shown in (Fig.7-A and Fig.7-B), respectively.

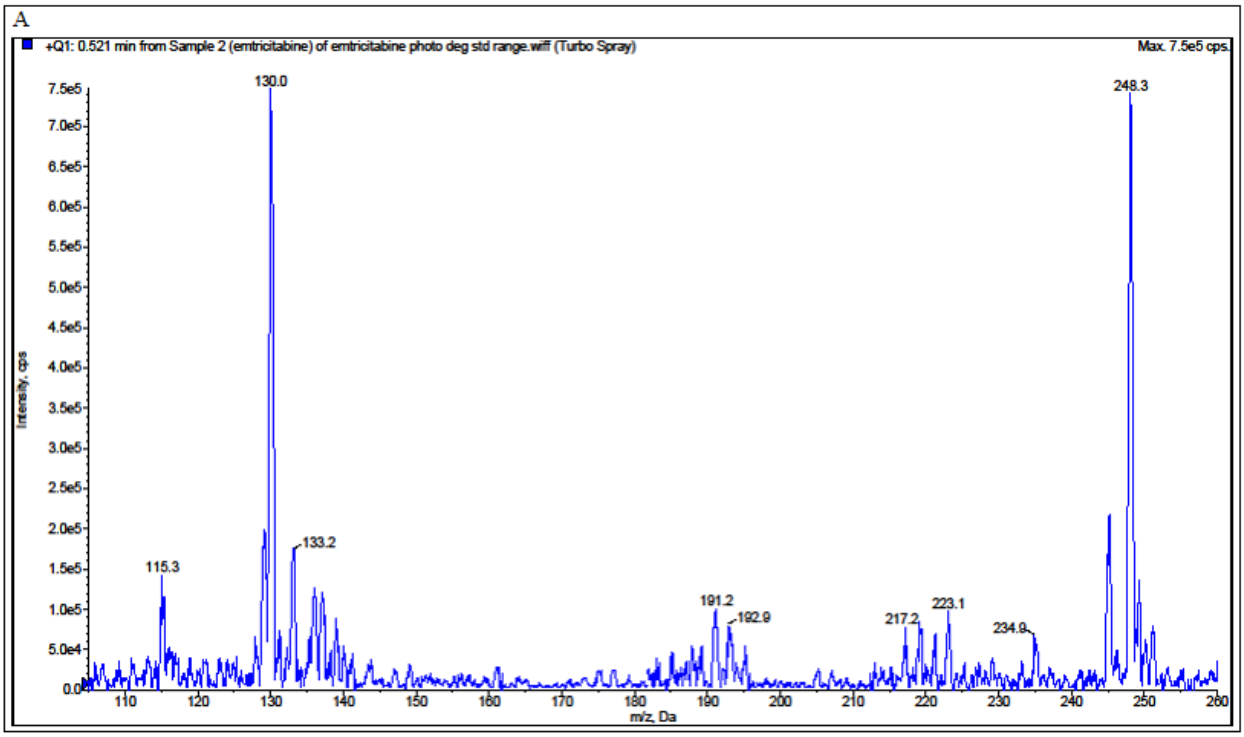

Figure 7- (A) Emtricitabine photo degradation mass spectra

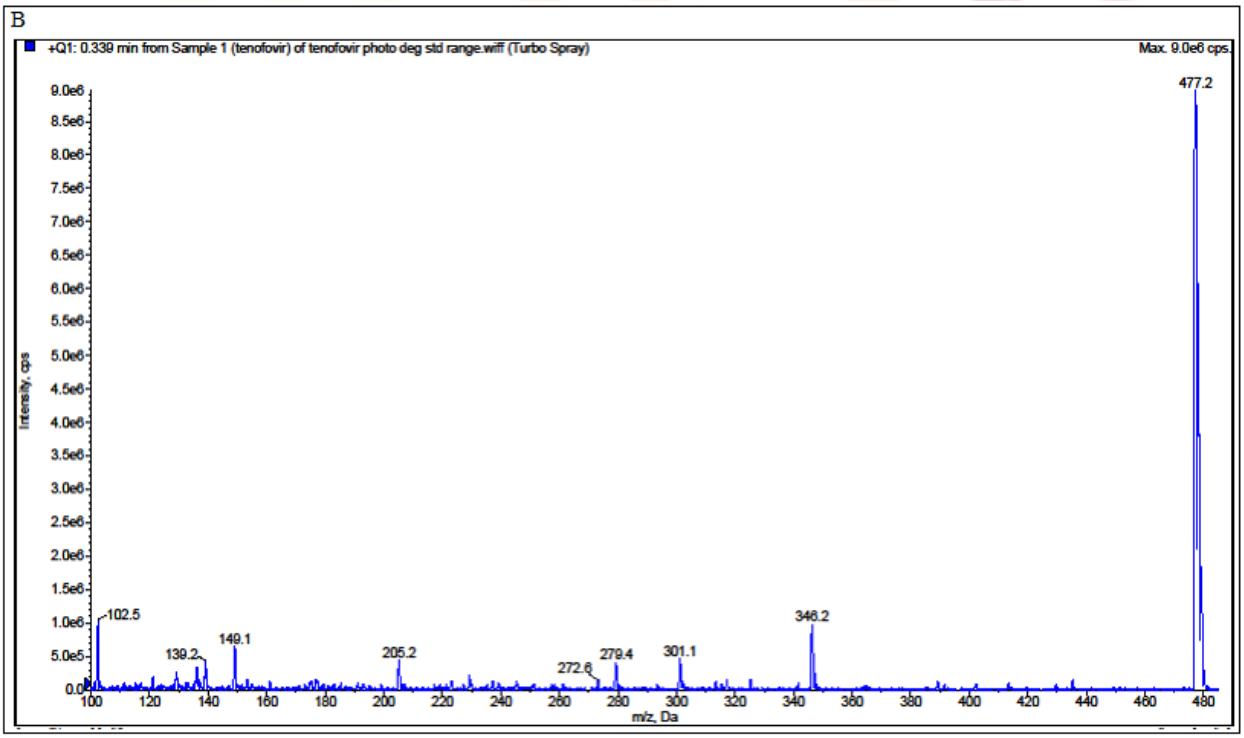

Figure 7- (B) Tenofovir Alafenamide photo degradation mass spectra

3.4. Fragmentation pathway

The analysis of the degradation products was carried outby LC and LC-MS. The degradation products were subjected to MRM studies in ESI+ mode to determine their molecular ion peaks and to establish their fragment profile. Fig. 8-A to 8-D and 9-A to 9-D lists the m/z values of product ions and its fragmentations forEmtricitabine and Tenofovir Alafenamide,respectively. Fragmentation of Emtricitabine [MRM:(Q1)248.300 Da and (Q3) 130.000 Da], EMTRDP1[MRM:(Q1)130.300 Da and (Q3) 114.200 Da], EMTR DP2[MRM:(Q1)299.400 Da and (Q3) 130.200 Da], EMTRDP3[MRM:(Q1)264.400 Da and (Q3) 130.200 Da] and Fragmentation of Tenofovir Alafenamide [MRM:(Q1)477.200 Da and (Q3) 346.500 Da], TENO DP1[MRM:(Q1)363.300 Da and (Q3) 229.500 Da], TENODP2[MRM:(Q1)435.100 Da and (Q3) 301.400 Da], TENO DP3[MRM:(Q1)493.400 Da and (Q3) 478.800 Da] as shown in Fig. 8-A to 8-D and 9-A to 9-D, respectively.

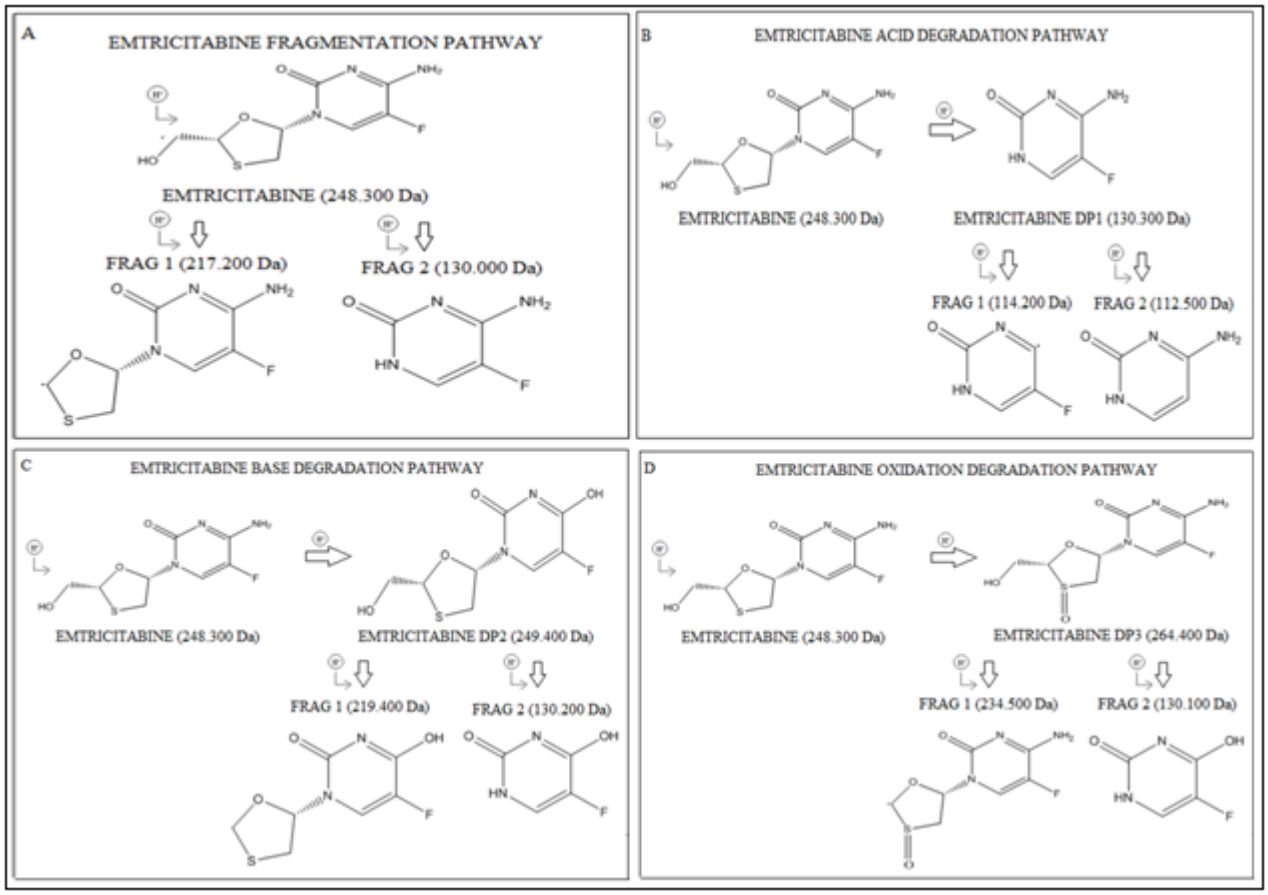

Figure 8-(A, B, C, D) Emtricitabine Fragmentation

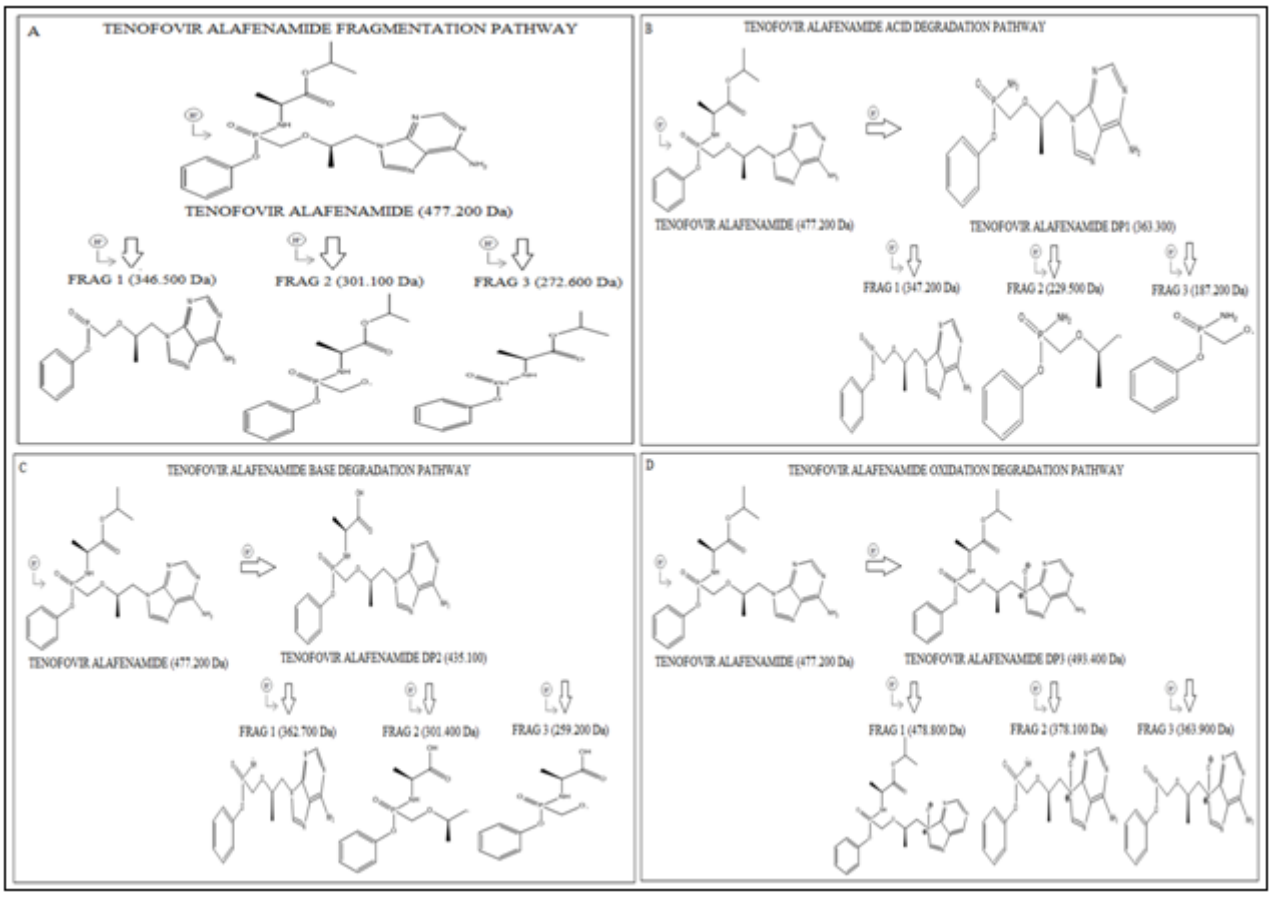

Figure 9-(A, B, C, D) Tenofovir Alafenamide Fragmentation

4. Conclusion

Emtricitabine and Tenofovir Alafenamide degradation products were identified and validated by the study using the stability-indicating LC-MS/MS method. As a result, the developed method is accurate, precise, simple, sensitive, stable, and suitable for routine analysis of drugs in their formulations. The results demonstrated excellent performance in terms of analysis time (10.0 min) which is beneficial for high-throughput applications and is economical as well as environmentally friendly. Force degradation studies using LC-MS/MS were not reported till now and the method proved to be more

sensitive and selective toward the determination of degradation products than previously reported methods.Emtricitabine and Tenofovir Alafenamide were found to be unstable in acidic, basic, and oxidation conditions but stable in thermal and photo-degradation conditions.This study would help in the development of a stable formulation with suitable storage conditions.

5. Acknowledgment

The authors thank Molecule Laboratory (Ahmedabad, Gujarat) for providing the necessary facilities and gratefully acknowledges Dr. Rutesh Shah, Principal of KKSJ Maninagar Science College, Ahmedabad, Gujarat, for whole heartedly supporting this work.

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