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Development and Validation of Stability-indicating HPLC-DAD method for simultaneous determination of Emtricitabine, Rilpivirine, and Tenofovir Alafenamide in bulk and their Pharmaceutical dosage forms

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Abstract : A simple and rapid high performance liquid chromatographic method was developed and validated for simultaneous estimation of Emtricitabine(EMT), Rilpivirine (RPV), and Tenofovir alafenamide fumarate(TAF)in bulk active pharmaceutical ingredients & its tablet formulation. The method was established using Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column, mobile phase consisting of 0.1%Formic acid: Acetonitrile (65:35%, v/v) at a flow rate of 1 mL/min with isocratic elution, injecting 20 µL sample into the chromatographic system. The eluted compounds were detected by using PDA detector at detection wavelength of 250 nm and temperature was maintained at 30 °C. Retention times of Reference Standard Emtricitabine, Rilpivirine, and Tenofovir alafenamide was found to be 2.90, 4.34, 6.58mins respectively. The calibration curve was plotted over the concentration range 4-20 μg/mL for EMT, 2-10 μg/mL of RPV and 1-5 μg/mL of TAF.The recoveries for EMT, RPV and TAF were found to be 99.12, 99.38, and 99.18%, respectively. All the validation parameters results were obtained with in acceptance limit.Developed method was subjected to forced degradation studies under specified conditions, which meets the required criteria. The present method was specific, sensitive, reproducible, precise, rapid and simple.
Keawords : RP-HPLC, Emtricitabine, Rilpivirine, and Tenofovir alafenamide, stability studies.

Introduction

Around 33.4 million people were living with HIV in year 2008 and around 2 million people have died in the same year. Highly active antiretroviral therapy (HAART) has brought new hope for those people who live with HIV/AIDS by decreasing the morbidity and mortality among people infected with HIV. Highly active antiretroviral therapy also has improved the quality of life among the people who live with HIV/AIDS.

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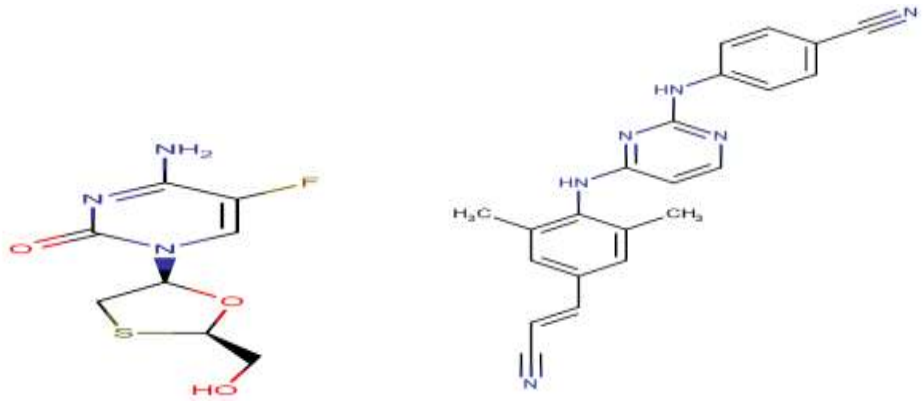
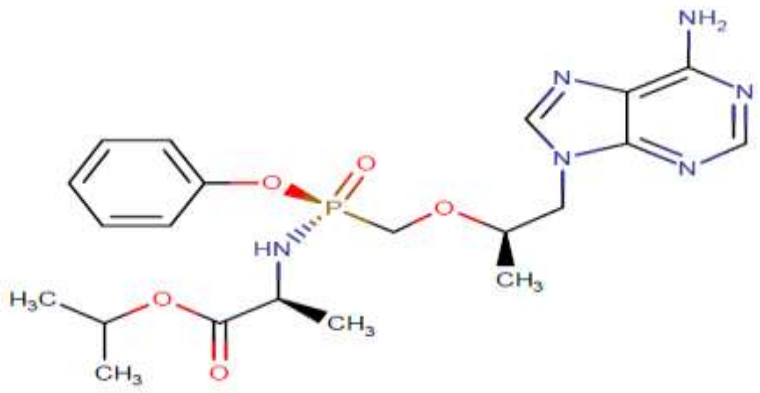
Combination therapy is preferred to be the gold standard for the treatment of AIDS so as to maximize potency, minimize toxicity, and diminish the risk for resistance development and reduction of pill burden to once-daily dosing so as to optimize the patient's compliance and reduce the treatment costs. The nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors as multidrug combinations are effective in the therapy of human immunodeficiency virus (HIV) infection and are used as a part of highly active antiretroviral Therapy, for the treatment of HIV [1, 2]. Literature indicates spectrophotometry [5-11], HPLC [12–15], HPTLC [16] and LC/MS/MS [17] methods for determination of TDF individually and in combination with other drugs in pharmaceutical formulations, drug substance, and biological matrices. Similarly for EMT individually and in combination with other drugs by UV [18, 19], HPLC in pharmaceutical formulations, drug substance and biological matrices [20–25], HPTLC, LC/MS/MS [26] and stability indicating liquid chromatographic methods [27] were reported. A detailed literature survey for RPV revealed that few analytical methods are available using spectrophotometric [28], HPLC [29] and HPTLC [30], individually. Literatures are available to show the existence of HPLC method for the triple drug combination of TDF, EMT, and RPV as well [3, 4].



(a) **(b)**



(c)

Fig. 1: Chemical structures of (a) Emtricitabine (EMT) (b) Rilpivirine (RPV) (c) TenofovirAlafenamide (TAF)

Emtricitabine (EMT), Rilpivirine (RPV), and TenofovirAlafenamide (TAF) combination is used in the treatment of HIV-1 as initial therapy as a part of anti retroviraltherapy. This combination was approved in March 2016 by US-FDA. Literature surveys indicate no HPLC –PDA (stability indicating) method for this new fixed dose Alafenamide derivative combination. As the Tenofovirdisproxilfumarate causing the release of formic acid with frequent administration in HIV-I patients, which ultimately cause GI disturbances, henceforth alternative analogue that is TenafovirAlafenamide was proved alternative drug to that of previous analogue, The current work describes the stability indicating accurate, Precise HPLC method for quantifying Emtricitabine, Rilpivirine, TenofovirAlafenamide in bulk drug &its formulation by using PDA detector.

Experimental

Chemicals and reagents

Working standard of Emtricitabine, Rilpivirine, Tenofovir Alafenamide was obtained as gift sample from Hetero Laboratories, Hyderabad, India.Reagents used were 0.1%Formic acid, HPLC graded acetonitrile Qualizines India, and Triple distilled water for the entire study.

Instrumentation

The method development and validation was carried out by using HPLC Agilent separation module model, it was equipped with auto-sampler with injection volume 20 µL, column used was Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column and data recorded using EZ chrome elite software.

Preparation of stock solution

Accurately weighed quantity of Emtricitabine (EMT), Rilpivirine (RPV) ,Tenofovir Alafenamide (TAF) were transferred to 10 ml volumetric flask then add small quantity of diluent and finally diluted up to the mark with mobile phase during the day of analysis (concentration :1000 μg/mL of EMT, RPV, and TAF).

Preparation of standard working solution

Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the day of analysis. The standard solution prepared for the optimization procedure constituted EMT, RPV, and TAF at 12, 6, and 3μg/mL respectively.

Preparation of mobile phase

Mobile phase was prepared by mixing 0.1% Formic acid: Acetonitrile (65:35%, v/v).The mobile phase was filtered through 0.2 μm membrane filter and degassed before use.

(Thediluent was prepared by mixing 650 ml of 0.1% Formic acid, 350 ml Acetonitrile (HPLC Grade), filtered through 0.45 μm membrane filter and degassed before use).

Sample Preparation.

Twenty tablets of Odefsey (containing 200 mg of Emtricitabine, 25 mg of Rilpivirine and 25 mg of Tenofovir alafenamide) were weighed and finely powdered. An amount of tablet powder equivalent to 20 mg of EMT and 2.5 mg of RPV with 2.5 mg of TDF was accurately weighed and transferred into a 50mL volumetric flask and small amount of diluents was added. This mixture was subjected to sonication for 10min and the final volume was made same to obtain solution. The mixture was then filtered through nylon 0.2 μm membrane filter. The above solution was suitably diluted with mobile phase to obtained final concentrations of 16 μg/mL, 2 μg/mL and 2 μg/mL of EMT, RPV, and TAF, respectively.

Chromatographic conditions:

The method was established using Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column, mobile phase consisting of 0.1%Formic acid: Acetonitrile (65:35%, v/v) at a flow rate of 1 mL/min with isocratic elution, injecting 20 µL sample into the chromatographic system. The eluted compounds were detected by using PDA Detector at detection wavelength of 250 nm and temperature was maintained at 30 °C.

Forced Degradation Studies:

Forced degradation studies are undertaken to degrade the active drug deliberately. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products without interference. Samples or drug product (spiked placebo) and drug substance are exposed to acid, base, oxidizing agent, reducing agent, and water. The degraded samples were then analyzed using the method to determine if there are interferences with the active. Thus, stability-indicating property was evaluated.

Acid stresscondition:

Solutions for acid degradation studies were prepared in mobile phase and 5N hydrochloric acid at 80^0C temperature for 2hrs and mixture was neutralized and the resultant solutions were filter, and inject into the system under optimized chromatographic condition.

Alkaline stress condition:

Solutions for base degradation studies were prepared in mobile phase and 5N sodium hydroxide at 80^0Ctemperature for 2hrs and mixture was neutralized and the resultant solutions were filter, and inject into the system under optimized chromatographic condition.

Oxidative degradation:

Solutions for use in oxidation studies were prepared in mobile phase and 6% hydrogen peroxide at 30^0C temperature for 2hrs and the resultant solutions were filtered and inject into the system under optimized chromatographic condition.

Neutral Hydrolysis:

Solutions for neutral degradation studies were prepared in mobile phase and water and the resultant solutions heated on a water bath at 50◦C for 48hrs and the resultant solutions were filter, and inject into the system under optimized chromatographic condition.

Results and Discussion

Optimized Chromatographic conditions

After a number of trials with mobile phases of different composition, 0.1%Formic acid: Acetonitrile (65:35%, v/v) was selected as mobile phase because of better resolution more no. of Theoretical plates and symmetric peaks. In order to achieve good separation between all the three components of Emtricitabine, Rilpivirine, and Tenofovir alafenamide were found to show in chromatogram in fig 2**.** And the optimized chromatographic conditions were shown in table 1. Retention times of Reference Standard Emtricitabine, Rilpivirine, and Tenofovir alafenamide was found to be 2.90, 4.34, 6.58 respectively, developed method was subjected to forced degradation studies under specified conditions, which meets the required criteria. The present method was specific, sensitive, reproducible, precise, rapid and simple.

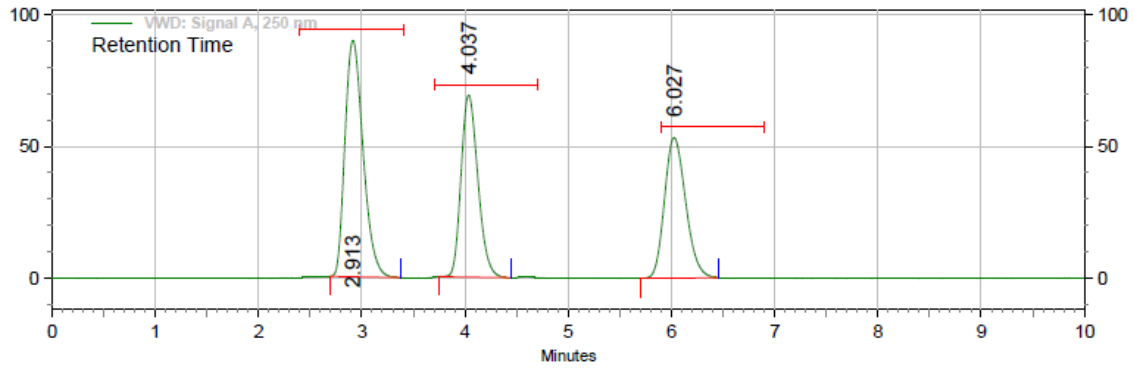


Fig. 2: Optimized Chromatogram of Emtricitabine, Rilpivirine, and Tenofovir alafenamide

Tab.1: Optimized chromatographic conditions

Column	Agilent, C18(250 X 4.6mm, 5µ)
Mobile phase	0.1%Formic acid: Acetonitrile (80:20%, v/v)
Flow rate	1ml/min
Column temperature	30^0C
Injection volume	10µl
Detection Wavelength	250nm
Run time	10 min
Retention time	Emtricitabine: 2.90 min Rilpivirine: 4.34 min Tenofoviralafenamide :6.58 min

Analysis of marketed sample

The proposed method was applied for the analysis of Emtricitabine, Rilpivirine and Tenofovir alfenamide in tablet dosage forms, the results were found to be between 99 and 101%, and the results were summarized in table 2.

Tab.2: Assay results of marketed formulation

Brand name	**Drug**	**Labelled amount (Mg/tab)**	**Amount found (Mg/tab)**	**% of assay**
Odefsey	Emtricitabine	200	199.76	99.88
	Rilpivirine	25	24.89	99.56
	Tenofovir alafenamide	25	25.13	100.52

Method validation

The optimized HPLC method for the analysis of fresh quality control samples was validated in accordance with the ICH Q2 (R1)[31] guidelines and reported.

System suitability:

System suitability is an integral part of the method validation to evaluate the parameters like tailing factor, theoretical plates, resolution and %RSD for replicate injections. The results were within the limits and were presented in Table 3.

Tab. 3: System suitability parameters

Parameters	**Emtricitabine**	**Rilpivirine**	**Tenofovir alafenamide**
Retention Time	2.90 min	4.34 min	6.58 min
Theoretical plates (USP)	2379	2879	4007
Resolution (USP)	0.00	2.713	4.246
Tailing factor	1.37	1.06	1.02

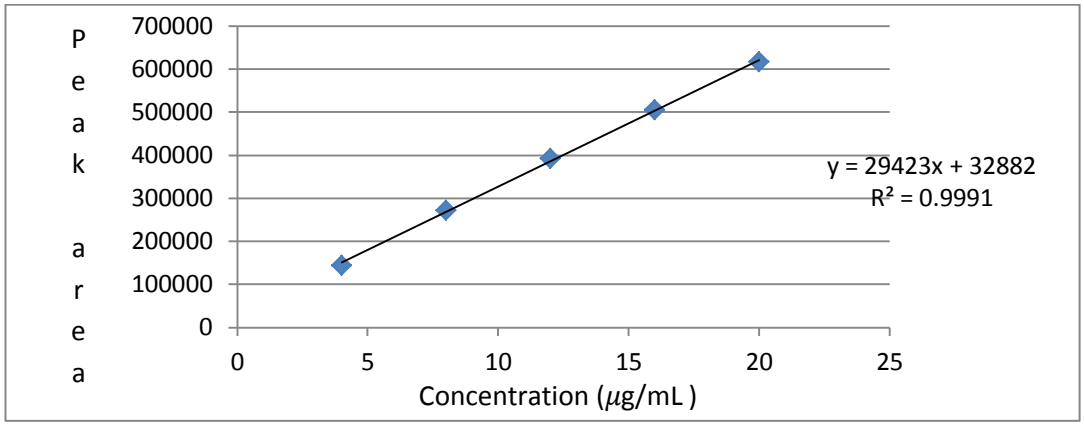
Selectivity and Specificity:

The selectivity of the method is depicted by the three sharp well resolved peaks for Emtricitabine, Rilpivirine, and Tenofovir alafenamide obtained at their retention times 2.90, 4.34, 6.58 mins respectively.

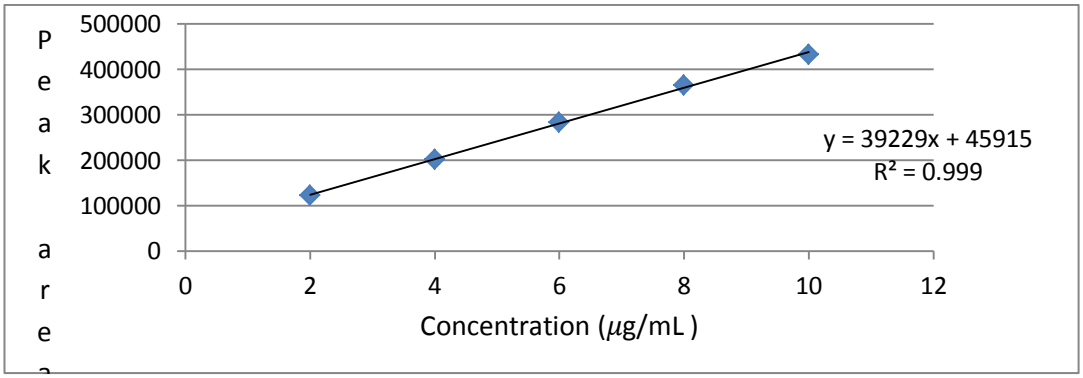
The specificity of the method was checked by comparison of chromatograms obtained from standard, sample and corresponding placebo. The retention time of the drug standard and the drugs from sample solutions were identical, conforming specificity of the method. The method was also selective because there was no interference observed from any of the excipients in the tablets formulation tested.

Linearity and range

The linearity was evaluated by measuring different concentrations of the standard solutions to Emtricitabine, Rilpivirine, and Tenofovir alafenamide. The calibration curve was plotted over the concentration range 4-20 μg/mL for EMT, 2-10 μg/mL of RPV and 1-5 μg/mL of TAF. Aliquots (20 μl) of each solution were injected under the operating chromatographic conditions describe above. Calibration curve was constructed by plotting peak area against concentration of EMT, RPV and TAF solutions, and the regression equation was calculated.The calibration curves of (a) Emtricitabine (EMT) (b) Rilpivirine (RPV) (c) Tenofovir Alafenamide (TAF) shown in fig.3.and table 4.



(a)



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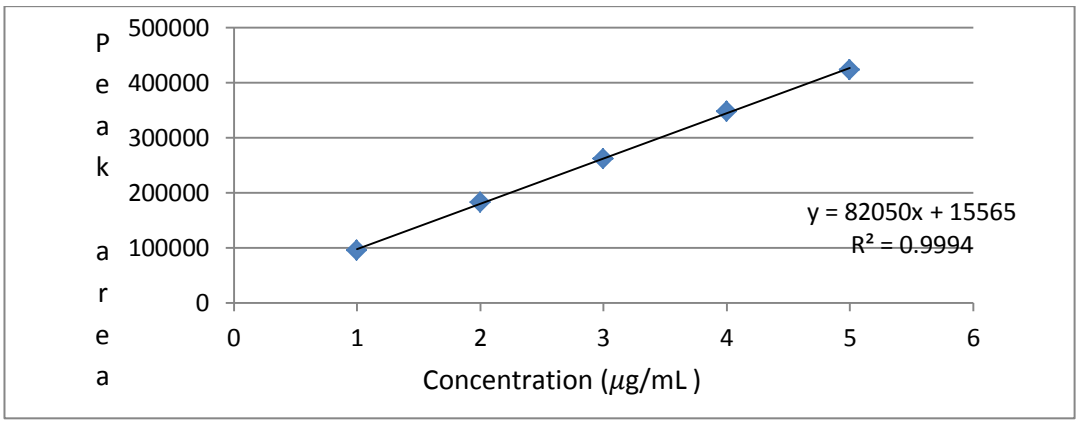

(c)

Fig. 3: Calibration curves of (a) Emtricitabine (EMT) (b) Rilpivirine (RPV) (c) Tenofovir Alafenamide (TAF)

Tab. 4: Regression equation parameters

Parameters	**Emtricitabine**	**Rilpivirine**	**Tenofovir Alafenamide**
Linearity range (μg/mL)	4-20	2-10	1-5
Correlation co-efficient	0.999	0.999	0.999
Slope	29423	39229	82050
Y-intercept	32882	45915	15565

Accuracy:

To determine the Accuracy of the proposed method, recovery studies were conducted; known amount of pure drug concentrations was spiked in placebo at three different levels, ie, 50%, 100% and 150% and was calculated.

Results:

The recoveries for EMT, RPV and TAF were found to be 99.12, 99.38, and 99.18%, respectively, which were within acceptable ranges of 100 ± 2%. Accuracy was calculated as the percentage of recovery. The results were tabulated in Table 5.

Tab. 5: Recovery studies for EMT, RPV, and TAF.

Recovery levels (%)	**Amount added**			**Amount recovered**			**% of recovery**		
	EMT	**RPV**	**TAF**	**EMT**	**RPV**	**TAF**	**EMT**	**RPV**	**TAF**
50	6	3	1.5	5.95	2.98	1.48	99.16	99.33	98.66
100	12	6	3	11.90	5.95	2.97	99.16	99.16	99.0
150	18	9	4.5	17.83	8.97	4.45	99.05	99.66	98.88
Mean % of recovery							99.12	99.38	99.18

Precision:

The repeatability of the method was verified by calculating the %RSD of six replicate injections of **100%** concentration (8 μg /ml of EMT, 4 μg /ml of RPV and 2μg /ml of TAF) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were shown in Table6.

Results:

The % RSD values for the intraday and interday precision were ≤2% confirming that the method was sufficiently precise. The results are presented in Table 6.

Tab. 6: Precision studies for EMT, RPV, and TAF.

Drug	**Intraday Precision (%RSD)**	**Interday Precision (%RSD)**
Emtricitabine	0.71	0.29
Rilpivirine	0.64	0.53
Tenofoviralfenamide	0.42	0.91

Robustness:

Robustness of the method was checked by small deliberate changes made in the method parameters such as wavelength (±2nm), flow rate (±0.1 mL), but these changes did not affect the method results.

Results:

The method parameters such as wavelength (±2nm), flow rate (±0.1 mL), but these changes did not affect the method results. The %RSD for the tablets were <2, indicating the robustness of the analytical methodology. The results are presented in Table 7.

Tab. 7: Results of Robustness by variation in flow rate and wavelength

Parameters	Retention time			Peak area			% Recovery		
	EMT	RPV	TAF	EMT	RPV	TAF	EMT	RPV	TAF
Flow rate Minus (0.8)	3.660	5.267	7.627	23024529	16367799	14943864	100.3	100.5	100.2
Flow rate Plus(1.2)	2.447	3.520	5.093	19893955	13882330	12802919	100.7	100.3	100.3
Wave length +5	2.933	4.220	6.107	21863522	17605822	39001312	100.5	100.4	100.2
Wave length -5	2.943	4.230	6.153	26435355	16946067	34412784	100.1	100.3	100.3

Limit of detection and Limit of quantification

LOD:

It is lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated, experimental conclusions. The detection limit is usually expressed as the concentration of analyte. The standard deviation and response of the slope.

LOD = 3.3*standard deviation (σ)/s

LOQ:

The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The standard deviation and response of the slope and the results obtained.

LOQ= 10* standard deviation (σ)/s

The obtained results were shown in table 8.

Tab. 8: Results of LOD and LOQ for EMT, RPV, and TAF.

Drug name	LOD (μg/mL)	LOQ(μg/mL)
Emtricitabine	0.31	0.81
Rilpivirine	0.16	0.41
Tenofoviralafenamide	0.11	0.29

Ruggedness.

A study was conducted to determine the effect of variation in analyst to analyst, lab to lab, and instrument to instrument in triplicate measurement as per the assay method. %RSD was calculated for each condition.

Forced Degradation Studies:

In acidic condition more degradation was observed in tenofovir alfenamide (99.35%) when compared with Emtricitabine (30.17%), Rilpivirine (1.94%).

In alkali condition more degradation was observed in Rilpivirine (99.56%), when compared with tenofovir alfenamide (31.88 %), Emtricitabine (0.52 %).

In peroxide condition more degradation was observed in Rilpivirine (99.97%), when compared with tenofovir alfenamide (60.15 %), Emtricitabine (65.71 %).

In hydrolytic condition no degradation were observed in Emtricitabine (0.52 %), Rilpivirine (2.80 %). Tenofovir alfenamide (1.88 %).

The obtained stress studies results and chromatograms were shown in table 9 & fig.4.

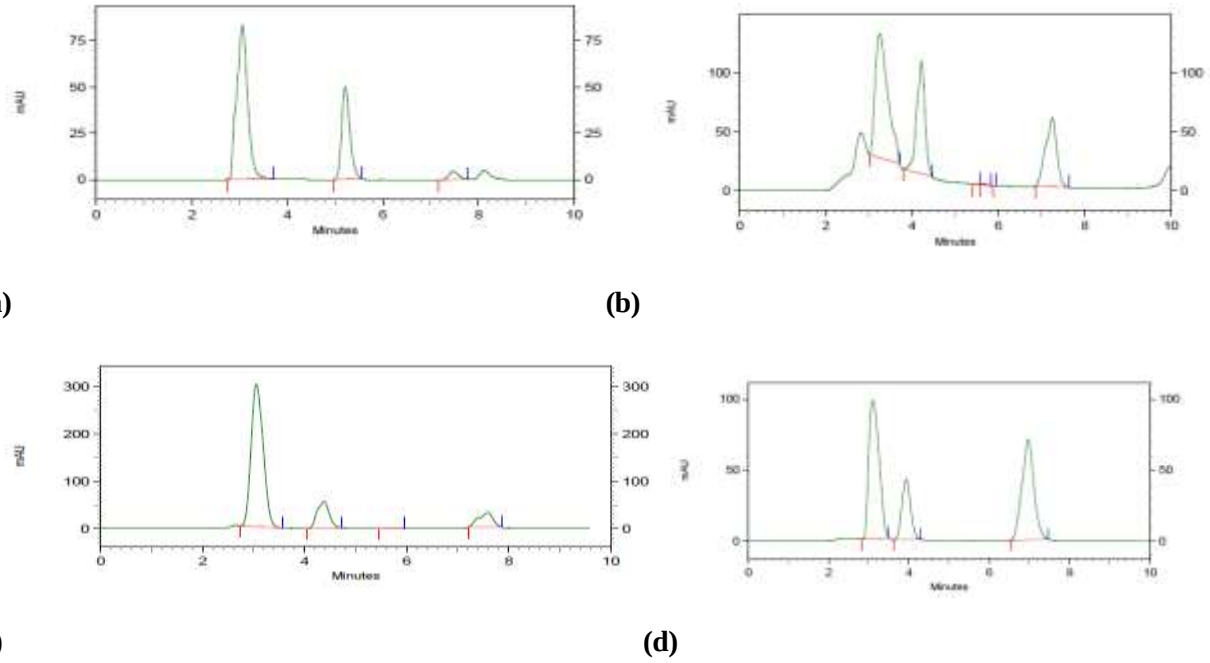

Fig. 4: Degradation chromatograms of (a) acidic condition (b) alkaline condition (c) Oxidative degradation (d)

Tab. 9: Results of Forced Degradation Studies

Stress conditions	**Emtricitabine, (% of degradation)**	**Rilpivirine (% of degradation)**	**Tenofovir alafenamide (% of degradation)**
Acid stress condition	30.17	1.94	99.35
Base stress condition	0.52	99.56	31.88
Peroxide stress condition	65.71	99.97	60.15
Hydrolysis condition	1.53	2.80	1.88

Conclusion

A simple, specific and reliable isocratic HPLC-DAD method was developed for the estimation of Emtricitabine, Rilpivirine, and Tenofovir alafenamide in bulk and their pharmaceutical formulation. The three compounds were subjected to forced degradation applying several stress conditions. The proposed method was successfully separated all the compounds with degradants, estimate the active contents. The method can be applied even to the analysis of stability samples obtained during accelerated stability experiments, as no interference was found with the degradants formed under various stress conditions. Hence, this method can simply and suitably take up for regular quality control analysis of Emtricitabine, Rilpivirine, and Tenofovir alafenamide in pure and its pharmaceutical dosage forms.

Conflict of Interest : None

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