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Research Article

Stability Indicating RP-HPLC Method Development and Validation for the Determination of Potential Degradation Impurities of Efavirenz, Emtricitabine and Tenofovir in Combined Pharmaceutical Dosage Form

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ABSTRACT

A simple, very accurate and precise stability indicating RP-HPLC gradient was developed to determine related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil from tablet dosage form.

The experiment was carried out on Zorbax SB-Phenyl, (250 mm X 4.6 mm), 5 µm column using the gradient composition of buffer pH 3.7 as mobile phase A and mixture of methanol, acetonitrile and tetrahydrofuran (50: 50: 2 v/v/v), degas as mobile phase B at flow rate 1.2ml/min and detection wavelength 265 nm

The new proposed method was simple, accurate, precise, linear and rugged. Method was validated as per ICH guidelines[1, 2, 3, 4] for simultaneous determination related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil in tablet dosage form hence can be used for routine analysis.

Key-words: Method Determination of related substances by stability indicating RP-HPLC gradient method of Efavirenz, Emtricitabin and Tenofovir disoproxil in tablet dosage form, Shimadzu HPLC with PDA detector, Zorbax SB-Phenyl, (250 mm X 4.6 mm), 5 µm,

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INTRODUCTION:

Efavirenz[5] (S)-6chloro (cyclopropylethylethynyl-1,4-(trifluoromethyl)-2H-1-benzoxazin-2-one) non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for treatment of human immunodeficiency virus (HIV). Emtricitabine[5] is chemically 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2-(1H)-pyrimidon a nucleoside reverse transcriptase inhibitor (NRTI). The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. Tenofovir[5] is [{1R)-2-(6-amino-9Hupurin-9-yl-1-methylethoxy} methyl] phosphonic acid. Tenofovir is a nucleoside analog reverse transcriptase inhibitor (NRTI).

Literature survey reveals that chromatographic methods were not reported for related combination dosage form of Efavirenz, Emtricitabine and Tenofovir disoproxil. Purpose of this study is to develop the stability indicating method for determination of related substances in bulk drug and combination drug dosage form of Efavirenz, Emtricitabin and Tenofovir disoproxil tablet in commercial tablet.

MATERIALS AND CHEMICAL

Chemical and Reagents

Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate were gifted from Macleods Pharmaceutical Ltd. Mumbai, India and were used with purity 99.3%, 99.9% and 98.5%, respectively. The combination drug dosage form of Efavirenz, Emtricitabin and Tenofovir disoproxil fumarate (600 mg / 200 mg / 300 mg) tablets used were gifted from Macleods Pharmaceutical Ltd. Mumbai, India.

Water (Milli-Q), Tetrahydrofuran (Rankem, HPLC), Methanol (Rankem, HPLC), 1-Hexane sulfonic acid sodium salt (Rankem, HPLC), Sodium hydroxide (Rankem), Acetonitrile (Rankem, HPLC), Orthophosphoric acid (Rankem, HPLC), Hydrochloric acid (Rankem)

Filter paper : GF/C (Glass Micro fiber, 25 mm, Whatman), 0.45 µm Nylon (25 mm and 47 mm, Advanced Micro devices Pvt. Ltd (Mdi)), 0.45 µm PVDF(25 mm, Advanced Micro devices Pvt Ltd (Mdi)) were purchased from reliable source and used at it is unless and until it was stated.

2.2 Instruments / Equipments

The HPLC with UV and PDA detector from Shimadzu LC-2010 CHT, Agilent, Analytical Balance (Sartorius), pH meter (Lab India), Hot air oven(Meta Lab), Photostability Chamber(Atlas (Suntest CPS+)), Humidity Chamber(Neutronics) and Column of Zorbax SB-Phenyl, with dimension 250 mm X 4.6 mm and particle size 5 µ were used.

3.0 OPTIMIZATION OF HPLC METHOD:

The HPLC procedure was optimized with view to develop the precise, accurate and simple method to determine related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate from dosage form. For optimization of HPLC method, different compositions of mobile phase composed of methanol and acetonitrile with or without adding tetrahydrofuran were tried. 20µL of standard solution was injected at the flow rate of 1.2 mL/min and using UV detection at 265nm, to obtain the good resolution and better separation between known impurities the method was further optimized by fine tunings in the gradient program with changing the ratio of methanol and acetonitrile and tetrahydrofuran. It was found that a buffer of pH 3.70 as mobile phase A and a mixture of methanol, acetonitrile and tetrahydrofuran (50: 50: 2 v/v/v) as a mobile phase at flow rate 1.2mL/min to provide the good separation of known impurities, plates and peak shape.

4.0 METHODOLOGY:

The separation of drug was achieved with gradient method on a reverse phase Zorbax SB-Phenyl, (250 mm X 4.6 mm), 5 µ column at wavelength 265nm, injection volume 20µL and column oven temperature was 30°C. The gradient program is of 135 mins for sample and 30 mins for diluted standard as mentioned in the table 1.

Table 1: Gradient program

Time (mins)	Mobile phase A (% v/v)	Mobile phase B (% v/v)	Program
0 → 20	100 → 95	0 → 5	Gradient
20 → 30	95 → 88	5 → 12	Gradient
30 → 60	88 → 55	12 → 45	Gradient
60 → 70	55 → 65	45 → 35	Gradient
70 → 80	65 → 60	35 → 40	Gradient
80 → 120	60 → 35	40 → 65	Gradient

120 → 125	35 → 100	65 → 0	Gradient
125 → 135	100	0	Re-equilibration

Preparation of buffer solution:
Buffer solution was prepared by diluting the 1 mL of orthophosphoric acid in 1000 mL of water and dissolved 0.2 g of 1-Hexane sulfonic acid sodium salt and finally adjusted the pH to 3.70 ± 0.05 with 10 % w/v sodium hydroxide solution. Filtered the solution through 0.45 µm nylon filter
Buffer having pH 3.70 used as Mobile phase A and mixture of methanol, acetonitrile and tetrahydrofuran (50: 50: 2 v/v/v) were used as Mobile phase B.
Diluent: Prepared a mixture of buffer and methanol (80: 20 v/v).
Placebo preparation (Placebo I):
Weighed accurately and transferred 255 mg of placebo to a 100 mL volumetric flask. Add 40 mL of methanol and sonicated for 10 mins and add about 30 mL of diluent and again sonicate for 15 mins. Allow to equilibrate at room temperature (RT) and dilute to volume with diluent. Filter the solution through 0.45 µm nylon filter (25 mm) by discarding first few mL of the filtrate.
Placebo containing Emtricitabine (Placebo II):
Weighed accurately and transferred 355 mg of placebo to a 100 mL volumetric flask. Add 40 mL of methanol and sonicated for 10 min and add about 30 mL of diluent and sonicate for 15 mins. Allow to equilibrate at RT and dilute to volume with diluent. Filter as mentioned previous and used.
Placebo containing Tenofovir (Placebo III):
Weighed accurately and transferred 405 mg of placebo to a 100 mL volumetric flask. Add 40 mL of methanol and sonicate for 10 min. Add about 30 mL of diluent and sonicate for 15 min. Allow to equilibrate to room temperature and dilute to volume with diluent, mix. Filter as mentioned previously and used
Placebo containing Efavirenz (Placebo IV):
Weighed accurately and transferred 555 mg of placebo to a 100 mL volumetric flask. Add 40 mL of methanol and sonicated for 10 mins, added about 30 mL of diluent and sonicate for 15 mins. Allow to equilibrate at RT and dilute to volume with diluent, mix. Filter as mentioned previously and used.
Impurity stock preparation:
Weigh accurately and transfer 2 mg of Fluorouracil analog impurity standard to a 20 mL volumetric flask. Add about 10 mL of diluent, mix and sonicate to dissolve. Allow to equilibrate at RT and dilute to volume with diluent and mix.
Resolution solution:
Weighed accurately and transferred 100 mg of Emtricitabine reference/ working standard to a 100 mL volumetric flask. Add 40 mL of methanol and sonicate to dissolve equilibrate at RT and add 10 mL of Fluorouracil analog impurity stock preparation, mix. Dilute to volume with diluent, mixed.
Diluted standard preparation:
Weighed accurately and transferred 25 mg of Emtricitabine reference/ working standard to a 100 mL volumetric flask. Add about 70 mL of diluent and sonicate to dissolve. Allow to equilibrate to room temperature and dilute to volume with diluent, mix. Dilute 2 mL of the solution to 200 mL with diluent and mix.
Sample preparation:
Weigh and crush 10 tablets to a fine powder. Weigh accurately and transfer tablet powder equivalent to about 100 mg of Emtricitabine to a 100 mL volumetric flask. Add 40 mL of methanol and sonicate for 10 min. Add about 30 mL of diluent and sonicate for 15 min. Allow to equilibrate to room temperature and dilute to volume with diluent, mix. Filter the solution through 0.45 µm nylon filter (25 mm), discarding first few mL of the filtrate. Use the filtrate.

5.0 EXPERIMENTAL:
The optimized method was validated for following parameter:
5.1 Specificity: Specificity is the ability of method to measure the analyte response in the presence of its potential impurities.
5.2 Forced degradation studies: Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule. Study was performed for following parameter.

- **Thermal Degradation (Forced degradation) :** Heated the crushed tablet powder at 80°C for 12 hours,

- **Photolytic degradation (Forced degradation with Photolytic degradation**): Powder covered with aluminum foil and directly exposed (without aluminum foil) in the photo stability chamber, as per ICH guidelines[8]
- **Thermal and Humidity degradation (Forced degradation):** Exposed the sample at 40°C/75%RH for 24 hrs
- **Acid Degradation (forced degradation):** Powder was kept in 0.2N HCl for 5 hrs at RT
- **Base Degradation (Forced degradation):** Powder was kept in 0.1M NaOH for 5 hrs at RT
- **Oxidative Degradation (Forced degradation):**:Powder was kept in 5 ml of 3% H_2O_2 heated for 2 hrs at 80°C on water bath

5.3 Solution Stability: Before challenging the validation parameter to proposed method its necessary to confirm solution stability of standard solution and sample solution to avoid the misinterpretation of result.

5.4 Filter compatibility: To confirm the filter compatibility for proposed analytical method the different filter were used. Filtration recovery experiment was carried out by sample filtration by Whatman GF/C filter paper (25 mm), 0.45µm PVDF filter and 0.45µm nylon (25 mm) filter and compared against the centrifuge sample.

5.5 Limit of Detection and Quantitation (LOD & LOQ) : To determine LOD and LOQ, a series of solutions were prepared by quantitative dilutions of the stock solution of Efavirenz, Efavirenz Impurity-I, Efavirenz Impurity-II, Emtricitabine, Fluorocytosine, Emtricitabine Sulfoxides, Emtricitabine 5- fluorouracil analog, Tenofovir Disoproxil , Adenine, (R) -9-[2-(phosphono methoxy)-propyl] adenine and Impurity-A standards to obtain solutions at 5 %, 10 %, 15 %, 20 %, 25 %, and 30 % with respect to the sample concentration. Results were presented in result table no. 4

5.6 Linearity and Range: A standards to obtain solutions at LOQ, 25%, 50 %, 100%, 150% and 250% of the specification limit with respect to sample concentration.
Each solution was injected and area was recorded; corrected concentration was calculated. A graph of peak area vs. corrected concentration in ppm was plotted for all. The slope, intercept, correlation coefficient of the regression line and residual sum of squares were determined.

5.7 Precision: The Precision of the method was determined by repeatability (Intraday) and intermediate precision (Inter-day).
Repeatability was carried out as described in the methodology on six samples prepared by spiking Efavirenz Impurity-I, Efavirenz Impurity-II, Fluorocytosine, Emtricitabine 5- fluorouracil analog, Adenine and (R) -9-[2-(phosphono methoxy)-propyl] adenine at 100% of specification level w.r.t sample concentration from the same homogeneous tablet blend of Efavirenz, Emtricitabine and Tenofovir Disoproxil Fumarate (600 mg / 200 mg / 300 mg) tablets and the % Impurities were determined.
Intermediate Precision (Inter-day): The analysis was carried out as described in the Repeatability exercise. A different day, using a different HPLC and different lot of column.
The % Impurities were determined. The results obtained were presented in result table no 6.

5.6 Accuracy / Recovery; Recovery solutions were prepared by spiking the stock solution. To obtain solutions at LOQ, 50%, 100%, 150% and 250% of specification level w.r.t sample concentration. The % recovery of Efavirenz, Efavirenz Impurity-I, Efavirenz Impurity-II, Emtricitabine, Fluorocytosine, Emtricitabine Sulfoxides, Emtricitabine 5- fluorouracil analog, Tenofovir Disoproxil , Adenine, R-9- Adenine, and Impurity-A was calculated for each of the recovery solutions,

5.8 Robustness: The analysis was carried out as described in the methodology and by making the following alterations in the chromatographic conditions

- Change in the column oven temperature (± 5°C)
- Change in flow rate (± 0.2)
- Change in the pH of buffer (± 0.2)

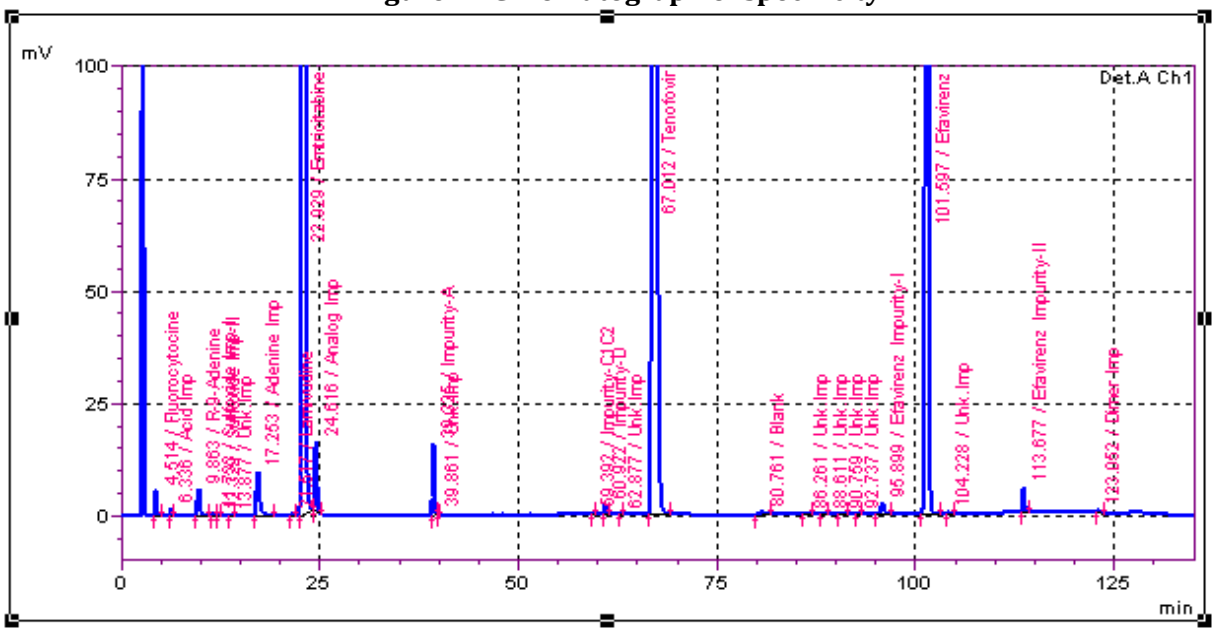
6.0 RESULTS AND DISCUSSION

6.1 Specificity: There was no interference observed due to blank, placebo and impurities at the same retention time as the peaks of Efavirenz, Emtricitabine and Tenofovir disoproxil fumarate in standard solution and sample solution chromatograms.

Table No. 2: Retention time obtained with spiked sample

Name of solution	Interference / R.T (Min)
Blank	No interference
Plain Placebo	No interference
Placebo with Emtricitabine	No interference
Placebo with Tenofovir	No interference
Placebo with Efavirenz	No interference
Emtricitabine standard solution	22.975 min
Efavirenz-I impurity Identification Solution	95.416 min
Efavirenz-II impurity Identification Solution	112.330 min
Fluorocytosine impurity Identification Solution	4.568 min
Adenine impurity Identification Solution	18.448 min
Emtricitabine Sulfoxides impurity Identification Solution	Sulfoxide I - 12.362 min & Sulfoxide II - 12.949 min
(R) -9-[2-(phosphono methoxy)-propyl] adenine impurity Identification Solution	10.523 min
Emtricitabine 5-fluorouracil analog Identification Solution	24.437 min
Impurity A Identification Solution	39.218 min
Efavirenz Identification Solution	100.529 min
Tenofovir Disoproxil Identification Solution	67.440 min

Figure 1 : Chromatograph of specificity



6.2 Forced degradation study:

Forced degradation of drug product was carried out under thermal, photolytic thermal & humidity, acid, base and oxidation stress condition.

Table No 3: Data of forced degradation study of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet

Parameter	Efavirenz		Emtricitabine		Tenofovir Disoproxil	
	% Degrad.	Peak Purity *	% Degrad	Peak Purity *	% Degrad	Peak Purity *
Thermal Degradation	No Degradation	0.990968	5.6	1.000000	6.6	1.000000
Photolytic degradation	No degradation	0.991228	5.7	1.000000	5.6	1.000000
Thermal & Humidity degradation:	No degradation	0.991056	7.5	1.000000	8.8	1.000000
Acid Degradation	No degradation	0.991389	4.9	1.000000	8.2	1.000000
Base Degradation	4.6	0.992735	4.1	1.000000	4.8	1.00000
Oxidative Degradation	0.6	0.990998	5.1	1.000000	0.7	1.00000

% Degrad: Percentage degradation, *in degradation sample

Figure 2 Chromatographs of Thermal Degradation.

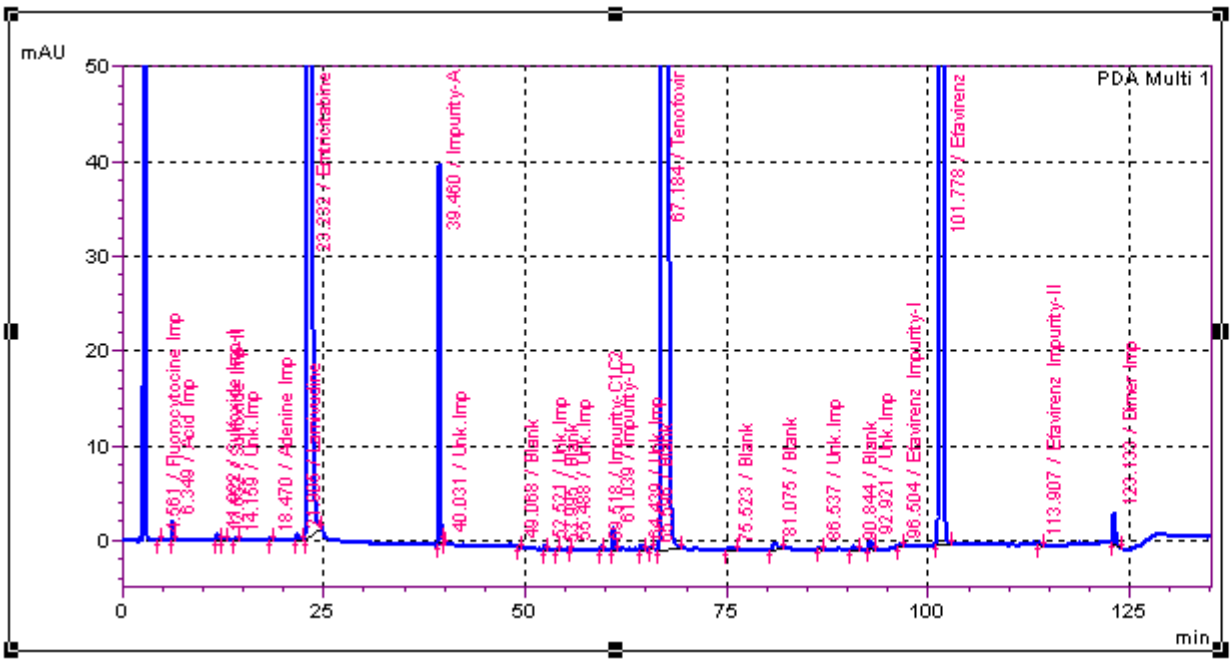
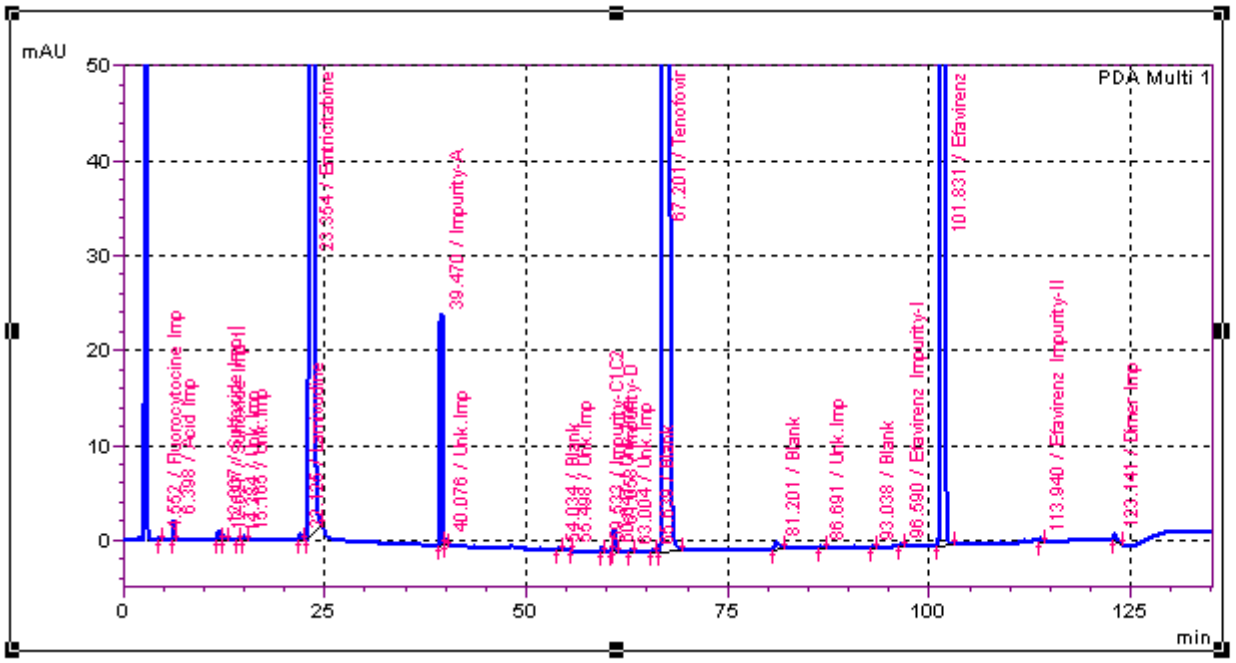


Figure 3 Chromatographs of Photolytic Degradation

(a) Exposed sample



(b) Control Sample :

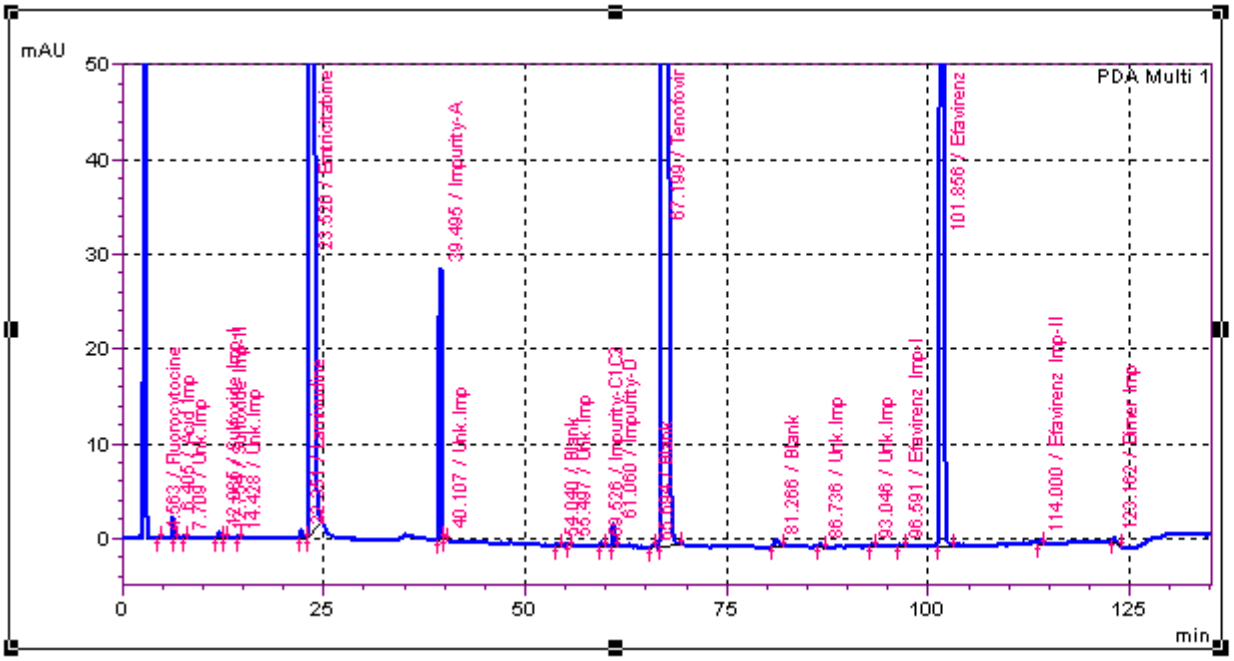


Figure 4 Chromatographs of Thermal and Humidity degradation.

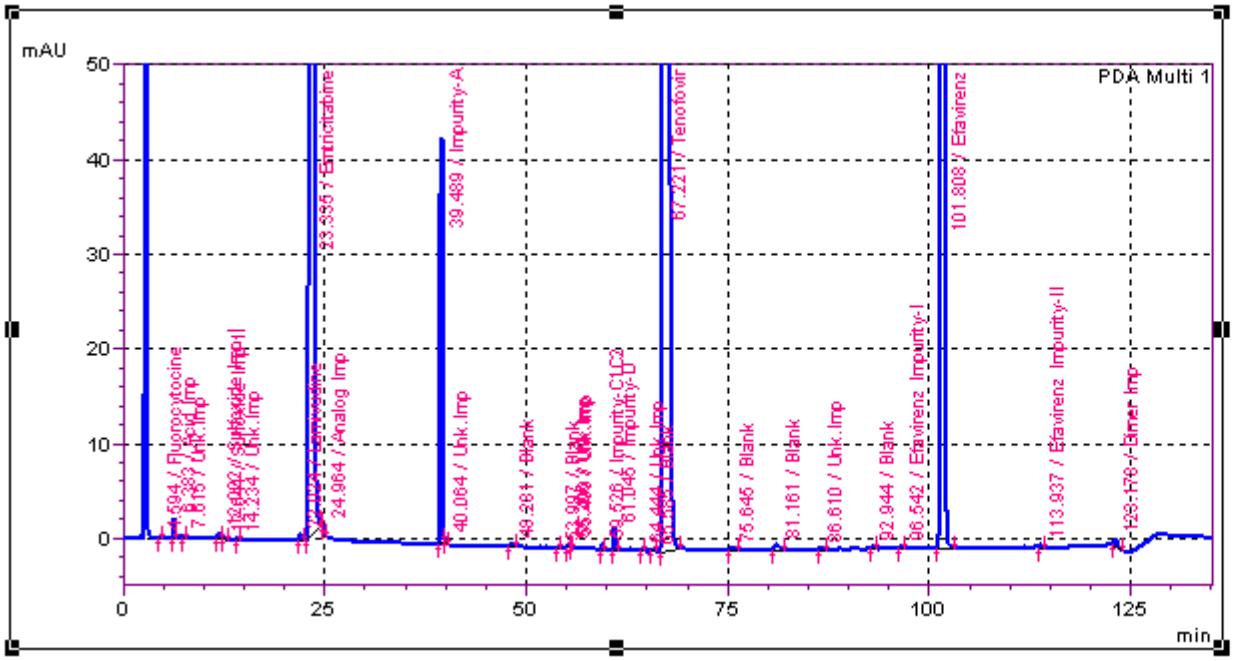


Figure 5 Chromatographs of Acid degradation.

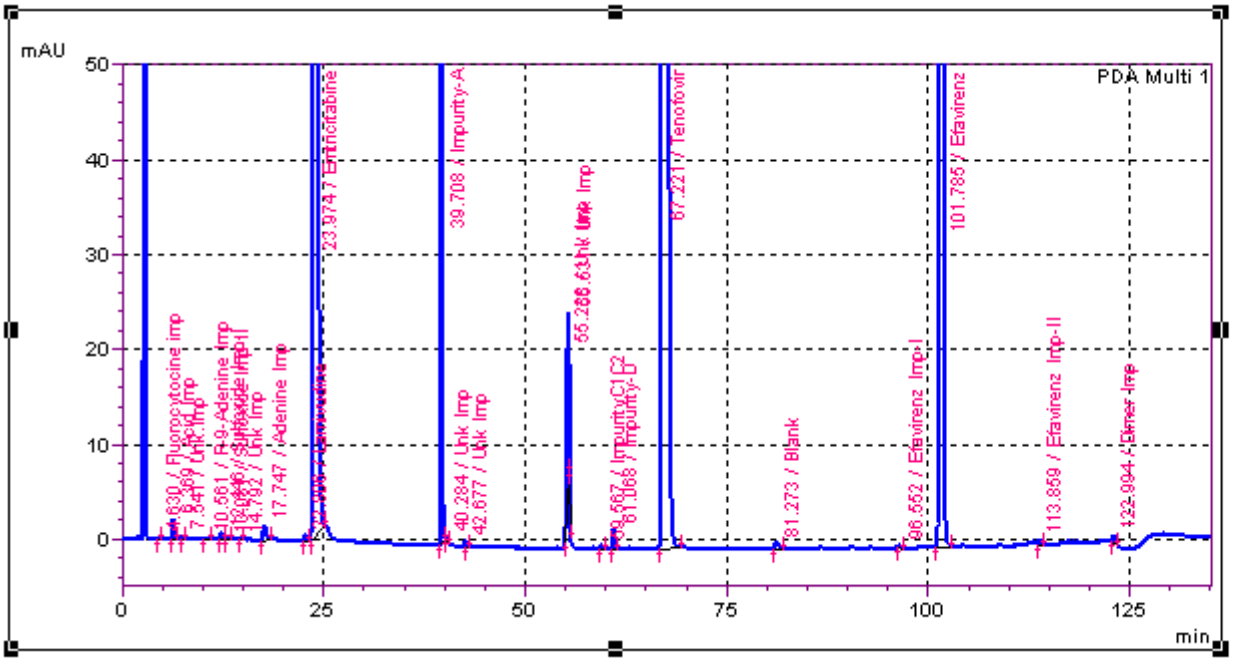


Figure 6 Chromatographs of base degradation.

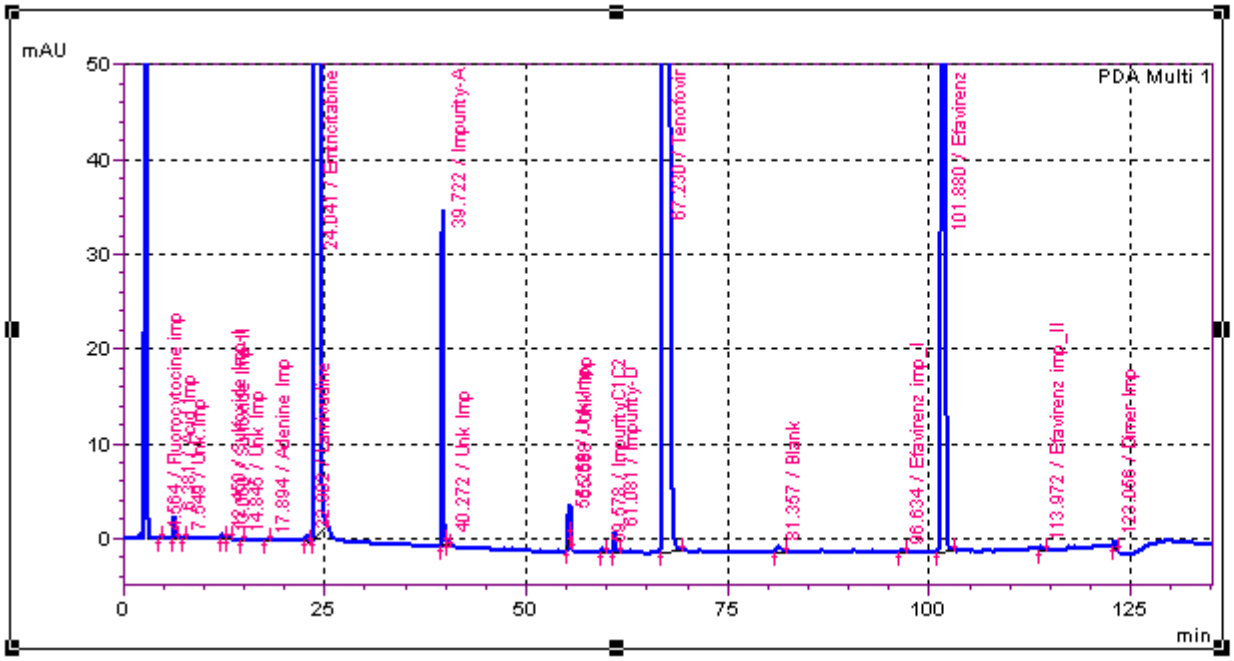
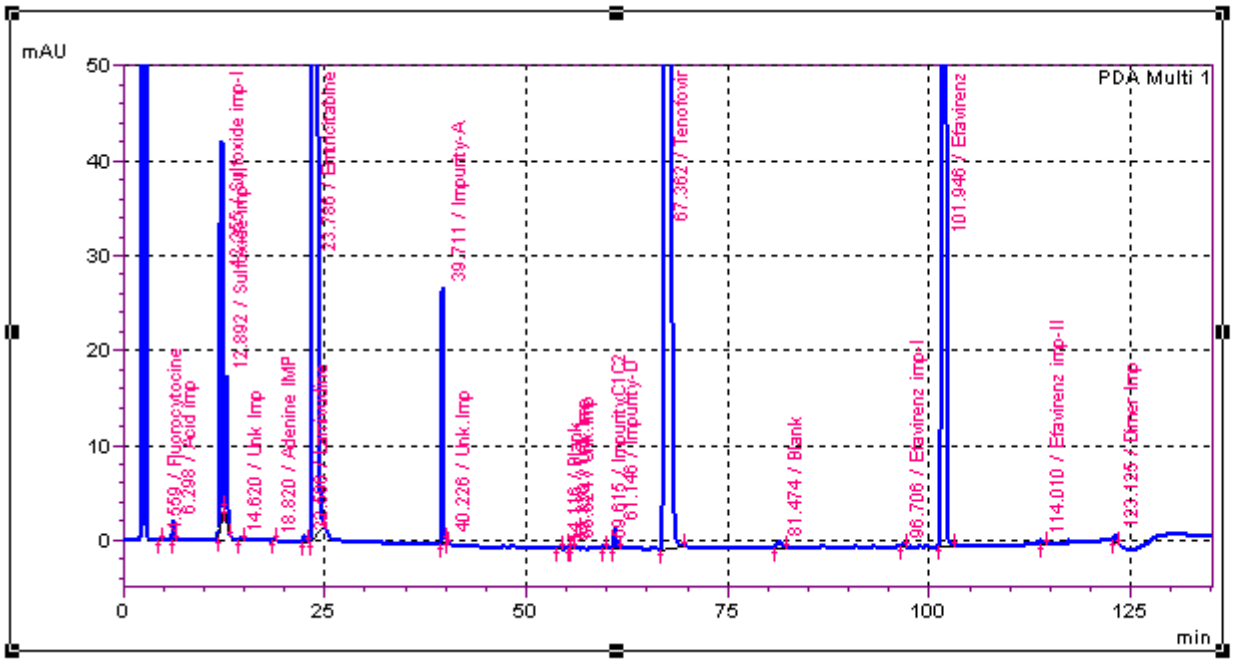


Figure 7 Chromatographs of Oxidative Degradation



6.3 Solution Stability: Solution stability demonstrated for standard solution at room temperature and 2-8°C and found the stable upto 61 hours. Solution stability demonstrated for sample solution at room temperature and sample 8 hours at 2-8°C experimented and found that known & unknown impurities were exceeds as compare with initial result hence sample solution needs to be prepared freshly.

6.4 Filter capability: The solution filter through different filter**,** Whatman GF/C filter paper (25 mm), 0.45µm PVDF filter and 0.45µm nylon (25 mm) filter. The unfiltered sample solution was centrifuged. The sample solution was not showing any significient difference in result with respect to centifiguge sample.

6.5 Limit of Detection and Quantitation (LOD & LOQ):

The LOD and LOQ were established by linearity method. Linearity was performed with concentration range of respective impurities given in table no 4.

Table No 4: LOD and LOQ precision data of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet

Name of Component	concentration rang (µg/ml)	LOD (%)	LOQ (%)	% RSD For six replicates of LOQ level
Efavirenz	0.3 to 1.8	0.006	0.020	9.62
Efavirenz Impurity-I	0.3 to 1.8	0.006	0.017	9.05
Efavirenz Impurity-II	0.3 to 1.8	0.009	0.026	9.68
Emtricitabine	0.1 to 0.6	0.002	0.020	2.78
Emtricitabine Uracil Analog	0.5 to 3	0.010	0.030	3.15
Sulfoxides I & II	0.2 to1.2	0.003	0.020	4.26
Fluorocytosine	0.1 to 0.6	0.002	0.010	3.40
Tenofovir DF	0.15 to 0.9	0.002	0.020	8.81
Adenine	0.15 to 0.9	0.002	0.005	3.91
(R) -9-[2-(phosphono methoxy)-propyl] adenine	0.15 to 0.9	0.002	0.010	1.81
Impurity- A	3.75 to 22.5	0.044	0.133	5.38

6.6 Linearity and Range

Each linearity solution was injected and area was recorded; and corrected concentration was calculated, Slope was determined and Relative response factor was determined for Efavirenz, Efavirenz Impurity-I, Efavirenz Impurity-II, Emtricitabine, Fluorocytosine, Emtricitabine Sulfoxides, Emtricitabine 5- fluorouracil analog, Tenofovir Disoproxil , Adenine, (R) -9-[2-(phosphono methoxy)-propyl] adenine and Impurity-A.

Table No 5: Linearity and range data for related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet

Name of Component	Linearity Range	Slope	Intercept	Correlation co-efficient	Relative Response Factor
Efavirenz	0.60 to 15.11	7467.41	1486.57	0.99970	0.25
Efavirenz Impurity-I	0.60 to 15.10	10823.16	-9854.31	0.99605	0.36
Efavirenz Impurity-II	0.60 to 15.13	18996.67	-5863.85	0.99994	0.63
Emtricitabine	0.21 to 5.17	30085.17	2040.74	0.99876	1.00
Emtricitabine Uracil Analog	0.30 to 25.48	35405.28	16025.40	0.99812	1.18
Sulfoxides I & II	0.20 to 10.24	22056.64	2205.24	0.99931	0.73
Fluorocytosine	0.10 to 5.05	40339.99	2460.92	0.99931	1.34
Tenofovir	0.30 to 7.50	23542.32	389.10	0.99920	0.78
Adenine	0.07 to 7.58	102426.99	3618.64	0.99965	3.40
(R) -9-[2-(phosphono methoxy)-propyl] adenine	0.16 to 7.88	53649.41	3124.13	0.99960	1.78
Impurity- A	2.04 to 186.82	33112.85	92853.86	0.99911	1.10

6.7 Precision

Repeatability(Intraday) with six samples and Intermediate Precision (Inter-day) with using a different HPLC, on different days and different lot of column established. On the baisis of obtained result ( ) concluded that method is Precise.

Table No 6: Method Precision data for related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet

Name of Component	% RSD (For 6 sample preparation) (Intra day)	% RSD (For 12 sample preparation) (Interday, different HPLC & different lot of column)
Efavirenz Impurity-I	1.91	8.00
Efavirenz Impurity-II	0.97	0.77
Fluorocytosine	0.44	0.53
Sulfoxide I & II	11.64	8.60
Fluorouracil Analog	1.42	2.34
(R) -9-[2-(phosphono methoxy)-propyl] adenine	1.52	1.70
Adenine	0.50	1.65
Impurity-A	5.14	3.96
Total	1.27	1.86

6.8 Accuracy (Recovery)

Accuracy was established by recovery methodology by spiking of Efavirenz, Efavirenz Impurity-I, Efavirenz Impurity-II, Emtricitabine, Fluorocytosine, Emtricitabine Sulfoxides, Emtricitabine 5- fluorouracil analog and Tenofovir Disoproxil , Adenine, (R) -9-[2-(phosphono methoxy)-propyl] adenine and Impurity-A standards to the placebo powder in triplicate. The % recovery at LOQ, 50 %, 100 %, 150 % and 250% of target concentration was well within the acceptance criteria of 80.0 % to 120.0 %.

Table No 7: Accuracy data for related substances of Efavirenz, Emtricitabine and Tenofovir disoproxil tablet

Name of Component	Parameter	LOQ	50%	100%	150%	250%
Efavirenz	Conc (µg/ml)	0.0655	0.3276	0.6553	0.9960	1.6513
	% Mean Recovery	113.7	104.83	95.17	91.13	88.67
Efavirenz Impurity-I	Conc (µg/ml)	0.0646	0.3231	0.6462	0.9822	1.6284
	% Mean Recovery	103.1	109.03	103.27	108.7	110.83
Efavirenz Impurity-II	Conc (µg/ml)	0.0659	0.3297	0.6594	1.0023	1.6617
	% Mean Recovery	111.5	83.7	93.13	91.17	92.7
Emtricitabine	Conc (µg/ml)	0.0206	0.128	0.2055	0.3124	0.5180
	% Mean Recovery	95.13	101.33	100.57	99.9	99.9
Fluorocytosine	Conc (µg/ml)	0.0101	0.1011	0.2021	0.3073	0.5094
	% Mean Recovery	105.3	97.83	97.63	95.9	95.83
Sulfoxides I & II	Conc (µg/ml)	0.0203	0.2029	0.4057	0.6167	1.0224
	% Mean Recovery	102.8	99.77	97.67	97.53	97.63
Fluorouracil Analog	Conc (µg/ml)	0.0290	0.4828	0.9657	1.4679	2.4336
	% Mean Recovery	106.0	99.53	98.87	97.97	98.17
Tenofovir Disoproxil	Conc (µg/ml)	0.0308	0.1541	0.3082	0.4684	0.7765
	% Mean Recovery	115.4	99.1	96.37	95.0	95.37
Adenine	Conc (µg/ml)	0.0065	0.1477	0.2954	0.4490	0.7444
	% Mean Recovery	85.13	96.57	97.17	96.7	96.93
(R) -9-[2-(phosphono methoxy)-propyl] adenine	Conc (µg/ml)	0.0154	0.1544	0.3089	0.4695	0.7784
	% Mean Recovery	97.2	98.67	98.4	97.53	97.87
Impurity- A	Conc (µg/ml)	0.2062	3.6827	7.3655	11.1955	18.5609
	% Mean Recovery	97.2	91.23	90.53	89.33	89.07

6.9 Robustness:

The robustness was demonstrated by varying the column oven temperature, flow rate and pH. The method was found robust with respect to column oven temperature, flow rate and pH without any changes in system suitability parameter and relative retention time of impurities in sample solution.

Table No 8 : Robustness data for System suitability :

Altered condition	Resolution Between Emtricitabine and Fluorouracil Analog peak	Tailing factor	Theoretical plates	% RSD
Unaltered (Repeatability)	3.34	1.09	25041	0.31
Column oven temp 25°C	3.08	1.12	24091	0.45
Column oven temp 35°C	2.41	1.06	23330	0.32
Buffer pH 3.5	2.78	1.09	21968	0.47
Buffer pH 3.9	2.62	1.08	21961	0.27
Flow 1.0 ml/min	3.04	1.02	27047	0.21
Flow 1.4 ml/min	2.31	1.06	21885	0.19

7. CONCLUSION

A stability indicating RP HPLC method was successfully developed for determination of related impurities in drug product of Efavirenz, Emtricitabine and Tenofovir in Combined Pharmaceutical Dosage Form and validated according to ICH guidelines. Established RRT and RRF values for known impurities can be useful to determine the related impurity. The satisfactory LOD, LOQ precision and accuracy are the main features of this method. The method was critically validated and statistically generated high quality data proves that the method is linear, sensitive, selective, specific and roubust. This method can be applied for in quality control laboratory.

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