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Validated Stability Indicating Rp-Hplc Method For Quantification Of Emtricitabine, Tenofovir And Dolutegravir In Active Pharma Ingridents And Pharmaceutical Dosage Form

MNB. Srinivas[1*], Dharmasoth Rama devi[2], S. Radha Krishna[3], K. Basavaiah[4]

[1*,4]Department of Chemistry, Andhra University, Visakhapatnam-530 003, India.
[1*,3]Laurus Labs Ltd., Visakhapatnam-531 021, India.
[2]Dr. Samuel George Institute of Pharmaceuticals Sciences, Markapur Andhra pradesh, India.
*E-mail : mnb_srinivas@yahoo.com, Mobile No.: 9989216535

***Corresponding Author:** MNB.Srinivas
*Address for Postal 404, B-Block, Fortune Heights, Sivaji Nagar, Kurmannapalem, Visakhapatnam -530046

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ABSTRACT

A simple, precise, accurate and robust reverse phase RP-HPLC method was developed and validated for the simultaneous estimation of Emtricitabine, Tenofovir and Dolutegravir in bulk and pharmaceutical dosage form. This method involves a simple isocratic mobile phase Acetonitrile: 0.01N Potassium dihydrogen orthophosphate (40:60) and a chromatographic separation using Ascentis column (C18 150x 4.6mm, 2.7µm) with PDA detection at 265nm, by using WATERS 2695 HPLC system. Diluent is 50:50 v/v of Acetonitrile: 0.01N Potassium dihydrogen orthophosphate. The average retention times of Emtricitabine, Tenofovir and Dolutegravir were found to be 2.243, 2.763 and 3.254 min respectively. The assay of Emtricitabine, Tenofovir and Dolutegravir were performed with tablets and the % assay were found to be 100.01%, 99.57% and 100.64% which shows that the method is useful for routine analysis. The linearity of Emtricitabine, Tenofovir and Dolutegravir were found to be linear with a R^2 of 0.999 for all the drugs, this shows that the method is able to produce good sensitivity. The %RSD for method precision for Emtricitabine, Tenofovir and Dolutegravir were 0.7, 1.4 and 0.6, which shows that the method is precise.

Keywords: Emtricitabine, Tenofovir, Dolutegravir, RP-HPLC, validation, forced degradation studies.

INTRODUCTION

Antiretroviral therapy has become the backbone for treating the patients suffering with immunodeficiency virus like HIV and AIDS. HIV belongs to retrovirus group where their RNA is enveloped as genetic material by using an enzyme called reverse transcriptase. This enzyme is a RNA- directed DNA polymerase that replicas the HIV-RNA genome into complimentary DNA strand to form a double-stranded DNA: RNA hybrid. And this formed hybrid is derived into double stranded DNA copy of the HIV genome, by an integrase enzyme. Once the HIV genome enters into human body, it is transmuted into new viruses by host cell. Anti-retroviral drugs act by inhibiting the reverse transcriptase enzyme, DNA chain termination and also by inhibiting the viral DNA synthesis. But for over period of time, drug resistance was observed for this antiretroviral drugs.

This resistance is mainly occurring through two type of mechanisms: the first is mutation of the residues that results in reduced incorporation of the NRTI into the growing DNA chain. While some of these mutations arise in the actual catalytic site of reverse transcriptase, a number of these mutations are actually proximal to the active catalytic site of reverse transcriptase but are still able to cause a conformational change in the enzyme that impairs binding of the drug to the active site. The second mechanism of resistance is associated with enhanced removal of drug from its site of attachment at the end of the DNA chain. These reverse transcriptase mutations allow ATP or pyrophosphate to bind at the active site adjacent to the bound nucleoside analog. The high energy ATP or pyrophosphate can then attack the bond that binds the drug to DNA, thereby liberating the drug and terminating its effect. To overcome this resistance complication, theretro viral treatment plan consists of two or more medication combinations from six different ARV groups.

Emtricitabine

Emtricitabine, a cytosine analogue acts as a nucleoside reverse transcriptase inhibitor (NRTI) in treatment and prevention of HIV infection and AIDS. It is a synthetic fluoro derivative of thiacytidine with potent antiviral activity. Administered dose of emtricitabine gets phosphorylated into its active triphosphate form, i.e., emtricitabine 5'-triphosphate, which binds into HIV reverse transcriptase of the viral DNA to prevent upcoming HIV DNA synthesis[1,2]. It also inhibits the formation of 3'-5' phosphodiester bond between the drug and the nucleoside triphosphates of the virus DNA, leading to termination of growing DNA of HIV virus. Usually NRTIs classification of drugs inhibits the HIV replication by blocking the reverse transcriptase enzyme, which is responsible for converting virus RNA into its respective DNA. Replication of HIV virus in human body is impossible without reverse transcriptase. By blocking the reverse transcriptase with NRTIs in the human body, leads to increase in CD4+ T-cell count and diminishes the viral accumulation proportionally.

Chemically it is: 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one[3]. Molecular formula: $C_8H_{10}FN_3O_3S$, molecular weight: 247.25

Tenofovir alafenamide is a lipophilic phosphonamidate prodrug of tenofovir, a synthetic acyclic nucleotide analog of adenosine 5-monophosphate and a nucleoside analog reverse transcriptase inhibitor[10], used for the treatment of chronic hepatitis B virus infection in adults which is a long term viral infection caused by hepatitis B virus[9] and potentially against HIV.

Tenofovir alafenamide is an alanine ester form considered for contribution low systemic levels as it is a novel prodrug of tenofovir and is developed to improve renal safety, when compared with Tenofovir disoproxil[11]. Due to poor absorptivity of tenofovir in systemic circulation it cannot permeates through cell membranes, but these prodrugs can enhance the cellular permeability and oral bioavailability of tenofovir[9]. These prodrugs were fashioned to cover the polar phosphonic acid group on Tenofovir by using uniqueoxycarbonyloxymethyl linkers to improve the oral bioavailability and intestinal diffusion[5,6,11]. Administered dose is taken by hepatocytes through passive diffusion and uptake transporters like organic anion transporting poly peptides 1B1 (OATP1B1) and 1B3 (OATP1B3). After diffusion, the tenofovir alafenamide is hydrolyzed and converted into tenofovir by carboxylesterase 1 (CES1)[12]. And in intracellular, tenofovir is phosphorylated into its pharmacologically active form known as tenofovir diphosphate. This tenofovir diphosphate enters into viral DNA and replaces the natural substrate like deoxyadenosine 5-triphsophate, leading to DNA chain termination, inhibition of HBV replication and diminishing of HIV replication[8,12]. Tenofovir alafenamide accumulates more in peripheral blood mononuclear cells compared to red blood cells[7]

Chemically it is (E)-but-2-enedioic acid; propan-2-yl (2S)-2-[[[(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-phenoxyphosphoryl]amino]propanoate.Molecular formula: $C_{25}H_{33}N_6O_9P$, Molecular weight: 592.5

Dolutegravir: Dolutegravir is an antiretroviral agent used in the treatment of HIV-1 infection. It is a HIV-1 integrase inhibitor that blocks the strand transfer step of the integration of the viral genome into the host cell (INSTI)[13]. This strand transfer step is essential in the HIV replication cycle.Dolutegravir inhibits this HIV integrase by binding to its active site and hindering the strand transfer step of retroviral DNA integration in the host celland resulting in the inhibition of viral activity. About 99% of the administered drug is bounded to the plasma proteins. Dolutegravir is highly metabolized through three main pathways, they are glucuronidation by UGT1A1, carbon oxidation by CYP3A4 and sequential oxidative defluorination and glutathione conjugations. Dolutegravir is used as a part of post exposure prophylaxis. It is used with combination of other anti-retro viral drugs for better efficacy.

Chemically it is (3S,7R)-N-[(2,4 difluorophenyl)methyl]-11-hydroxy-7-methyl-9,12-dioxo-4-oxa-1,8diazatricyclo[8.4.0.0^{3,8 }] tetradeca-10,13-diene-13-carboxamide. Molecular formula: $C_{20}H_{19}F_2N_3O_5$ and Molecular weight: 419.4

Emtricitabine, Tenofovir and Dolutegravir is available as an oral combination therapy under the brand name SPEGRA. This fixed-dose combination therapy was FDA-approved tablet. the chemical structures of Emtricitabine, Tenofovir and Dolutegravir is shown in (figure-1, 2, 3).

A thorough literature discovered that several analytical methods have been reportedin the literature, more economical methods were observed and there is no method reported for the estimation stability studies[15-27]. Hence a simple, cost-effective stability-indicating simultaneous estimation of Emtricitabine, Dolutegravir and Tenofovir by RP-HPLC in pharmaceutical dosage form has to be develop and validated as per the guidelines of ICH (Q2 specification)[24].

Materials and Reagents

Emtricitabine, Dolutegravir and Tenofovir pure drugs were obtained from spectrum pharma research solutions. The combination tablet Emtricitabine, Dolutegravir and Tenofovir (SPEGRA) was brought from local market,Hyderabad. All the chemical and buffers used in this estimation are procured from Rankem, India.

Instrumentation

WATERS HPLC, model: 2695 SYSTEM with Photo diode array detector was used for the development and method validation, with an automated sample injector with software Empower 2.

Chromatographic Conditions:

An Ascentis column (C18, 150x 4.6mm, 2.7μm) was used as a stationary phase, mobile phase was a mixture of buffer (0.01N Potassium dihydrogen Ortho phosphate, pH 4.0 adjusted) and ACN in the ratio of 60:40(%, v/v) with a flow rate of 1ml/min. Sample injection volume was 10μL and detection was carried out at 265nm. Total run time was 7 minutes. Diluent is 50:50 ratio of Buffer: ACN.

Preparation of Buffer

Preparation of 0.01N potassium dihydrogen phosphate Buffer: Weighed about 1.42gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask added 900ml of Milli-Q

water and degassed. Finally made the volume with water, then adjusted pH to 4.0 with dilute ortho phosphoric acid solution.

Preparation of Standard solution:

Standard Stock-I: Weighed about 50mg of Emtricitabine, 12.5mg of Dolutegravir and 6.25mg of Tenofovir working standards into a 50 ml volumetric flask. Added 25 ml of diluent and sonicated to dissolve. Diluted up to the mark with diluent and mixed. (Stock solution).

Standard Stock-II: Transferred 1ml of Standard Stock-I into a 10ml volumetric flask and made the volume to mark with diluent. (The final concentration of individual drugs were 100µg/ml Emtricitabine, 25µg/ml of Dolutegravir and 12.5µg/ml of Tenofovir).

Preparation of Sample solution: Determined the average weight of 10 Tablets, and powdered it. Weighed about 498 mg of sample and transferred into a 100 ml volumetric flask. Added 50 ml of diluent and sonicated to dissolve. Transferred 0.5 ml of solution into a 10 ml of volumetric flask and dilutedthe volume with the diluent and mixed. Filtered the solution through 0.45µm nylon membrane filter. (The final sample solution concentration was 100µg/ml Emtricitabine, 25µg/ml of Dolutegravir and 12.5µg/ml of Tenofovir).

Method Validation

The validation of HPLC method was carried out for the simultaneous estimation of Emtricitabine, Dolutegravir and Tenofovir drug substance as per the ICH guidelines to demonstrate that the method is proposed for the routine analysis.

System suitability:

The system suitability was performed for each validation parameters by injecting standard solution containing Emtricitabine 100 µg/ml, Dolutegravir 25 µg/ml and Tenofovir 12.5 µg/ml. System suitability chromatogram was shown in figure 4 and values are mentioned in the table 1.

Specificity (Selectivity):

Checking of the interference in the optimized method. There are no interfering peaks in blank and placebo at retention times of these drugs eluted in this method. So this method was said to be specific. Representative chromatogram is shown in Figure 5,6,7 and experimental data is given in Table 2.

Linearity: A series of linearity solutions were prepared containing the drug standards at different concentrations at 25 to 150 % of the specification limit. A series of solutions were prepared by quantitative dilutions of the stock solution of the main drug to obtain solutions at 25 to150 % of the sample concentration.Each solution was injected and the peak area was recorded. The Regression coefficient, slope and Y-intercept were calculated. The regression coefficient for all the three drugs were found above 0.999.The results indicate excellent linearity for emtricitabine tenofovir alafenamide and dolutegravir are shown in Table 3 and the graphs were shown in figure 8,9 and 10.

Accuracy: The accuracy of the method was determined by using solutions containing spiked samples of Emtricitabine, Tenofovir and Dolutegravir at 50%, 100% and 150% of the working

strength. All the solutions were prepared in triplicate and analyzed. The percentage recovery results obtained for each impurity was listed in Table 4.

System Precision: The system precision was performed by analyzing six replicate injections of standard solution at 100% of the specified limit with respect to the working strength of Emtricitabine, Tenofovir and Dolutegravir. Results of peak area are summarized in Table 5.
The % RSD for the peak areas of Emtricitabine, Tenofovir and Dolutegravir obtained from six replicate injections of standard solution were within the limit.

Method Precision: The precision of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir. (Six individual sample preparations). Data obtained is summarized in Table 6.
From the above results, the % RSD of method precision study were within the limit for Emtricitabine, Tenofovir and Dolutegravir.

Intermediate Precision: The precision of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir. (Six individual sample preparations). Data obtained is summarized in Table 7.
From the above results, the % RSD of intermediate precision study were within the limit for Emtricitabine, Tenofovir and Dolutegravir.

Robustness: Robustness of the method was evaluated by deliberately altering the method conditions from the original method parameters.

a) **Flow rate (-10%):** The robustness of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir with 0.9ml/min flow rate of mobile phase. (five individual sample preparations). The data obtained is summarized in Table 8.
b) **Flow rate (+10%):** The robustness of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir with 1.1 ml/min flow rate of mobile phase. (five individual sample preparations). The data obtained is summarized in Table 9.
c) **Mobile phase (-10%):** The robustness of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir with mobile phase ratio Buffer: ACN (65: 35%, v/v) (5 individual sample preparations). The data obtained is summarized in Table 10.
d) **Mobile phase (+10%):** The robustness of the method was determined by analyzing a sample of Emtricitabine, Tenofovir and Dolutegravir with mobile phase ratio Buffer: ACN (55: 45%, v/v)(5 individual sample preparations). The data obtained is summarized in Table 11.

Forced degradation studies[14]

Forced degradation is defined as degradation of new drug substance and drug product at conditions more severe than accelerated conditions. It is required to demonstrate specificity of stability indicating methods and also provides an insight into degradation pathways and degradation products of the drug substance and helps in elucidation of the structure of the degradation products. Forced degradation studies show the chemical behavior of the molecule which in turn helps in the development of formulation and package.

Base hydrolysis

To 0.5 mof standard stock solution Emtricitabine Tenofovir and Dolutegravir, 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60^{0}C. The resultant solution was diluted to obtain (100ppm,12.5ppm and 25ppm) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid hydrolysis

To 0.5ml of standard stock solution Emtricitabine, Tenofovir and Dolutegravir 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 600° C. The resultant solution was diluted to obtain (100ppm,12.5ppm and 25ppm) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidation (H_2O_2)

To 0.5 ml of stock solution of Emtricitabine, Tenofovir and Dolutegravir, 1 ml of 20% hydrogen peroxide (H_2O_2) was added. The solutions were kept for 30 min at 600° C. For HPLC study, the resultant solution was diluted to obtain (100ppm,12.5ppm and 25ppm) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Photodegradation

The photochemical stability of the drug was also studied by exposing the (2000ppm,250ppm and 500ppm) solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hour/ min photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (100ppm,12.5ppm and 25ppm) solutions and 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Thermal degradation

The standard stock solution was placed in oven at 105°C for 6h to study dry heat degradation. For HPLC study, the resultant solution was diluted to (100ppm,12.5ppm and 25ppm) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60° C. For HPLC study, the resultant solution was diluted to (100ppm,12.5ppm and 25ppm) solution and 10µl was injected into the system and the chromatograms were recorded to assess the stability of the sample.

From the results, no degradation was observed when the samples were exposed to acid, base, hydrolysis, thermal, light and water. According to the stress study, none of the degradant co-eluted with the active drug peaks formed and the details are capture in table 12. And also assay values of the emtricitabine, tenofovir and dolutegravir were tabulated in table 13.

Table 1: System suitability results

Peak Name	RT (min)	Area	Height	USP Resolution	USP tailing	USP Plate count
Emtricitabine	2.243	1623225	357938	NA	1.4	6358.4
Tenofovir	2.763	203765	38783	3.7	1.3	6865.2
Dolutegravir	3.254	342133	70349	4.2	1.2	10814.0

Table 2: Specificity data

Sample name	Retention time(min)	Area
Emtricitabine	2.233	1584968
Tenofovir	2.661	190684
Dolutegravir	3.257	345977

Table 3: Linearity

% Level	Emtricitabine		Tenofovir		Dolutegravir	
	Concentration	Area	Concentration	Area	Concentration	Area
25%	25	450206	3.125	51411	6.25	95195
50%	50	863305	6.25	99206	12.5	186957
75%	75	1273241	9.375	146500	18.75	275013
100%	100	1687186	12.5	195205	25	359653
125%	125	2076572	15.625	241108	31.25	449717
150%	150	2434617	18.75	281580	37.5	536110
R^2 value	**0.999**		**0.999**		**0.999**	

Table 4: Accuracy (%Recovery at 50%, 100%, 150%)

%Level	Recovery Data								
	Emtricitabine			Tenofovir			Dolutegravir		
	Amount added	Amount found	%Rec	Amount added	Amount found	%Rec	Amount added	Amount found	%Rec
50% Level	50	49.67	99.35	6.25	6.24	99.93	12.5	12.59	100.78
	50	49.64	99.29	6.25	6.25	100.12	12.5	12.40	99.27
	50	49.72	99.46	6.25	6.23	99.80	12.5	12.52	100.22
100%Level	100	99.79	99.80	12.5	12.37	98.99	25	24.90	99.64
	100	101.22	101.22	12.5	12.47	99.80	25	25.02	100.09
	100	100.67	100.67	12.5	12.40	99.24	25	24.87	99.51
150%Level	150	149.66	99.77	18.75	18.55	98.94	37.5	37.16	99.11
	150	150.42	100.29	18.75	18.93	100.98	37.5	37.15	99.07
	150	150.17	100.11	18.75	18.82	100.39	37.5	37.17	99.13
Mean%	**100.00**			**99.80**			**99.64**		

Table 5: System precision data

Injection	Emtricitabine	Tenofovir	Dolutegravir
1	1684938	197417	358056
2	1702094	204725	353886
3	1693875	198723	357143
4	1707355	202263	355350
5	1694678	204044	348176
6	1719728	202020	360078
Avg	1700445	201532	357115
Std dev	12158.9	2901.8	2206.0
%RSD	0.7	1.4	0.6

Table 6: Method precision data

Injection	Emtricitabine	Tenofovir	Dolutegravir
1	1700201	202768	360873
2	1707732	198704	358578
3	1699335	199693	360307
4	1709819	202676	361862
5	1707703	199222	360057
6	1699332	203353	359117
Avg	1704020	201069	360132
Std dev	4888.4	2077.7	1185.2
%RSD	0.3	1.0	0.3

Table 7: Intermediate precision data

Injection	Emtricitabine	Tenofovir	Dolutegravir
1	1447866	172211	361532
2	1504886	165036	358623
3	1482569	161847	357531
4	1457156	162241	360669
5	1439259	157667	359483
6	1451693	161653	360802
Avg	1463905	163442	359773
Std dev	24848.2	4898.5	1509.2
%RSD	1.7	3.0	0.4

Table 8: Flow rate of mobile phase (0.9 ml/min):

Injection	Emtricitabine		Tenofovir		Dolutegravir	
	RT	Area	RT	Area	RT	Area
1	2.396	1692689	2.780	201415	3.450	355676
2	2.398	1715476	2.781	202720	3.476	360404
3	2.399	1702595	2.783	199859	3.488	354687
4	2.400	1686280	2.785	199987	3.502	360077
5	2.400	1695766	2.786	198159	3.510	354959
Avg		1698561		200428		357161
Std dev		11130.0		1724.4		2837.0
%RSD		0.7		0.9		0.8

Table 9: Flow rate of mobile phase (1.1 ml/min):

Injection	Emtricitabine		Tenofovir		Dolutegravir	
	RT	Area	RT	Area	RT	Area
1	2.142	1687444	2.479	199362	3.070	355568
2	2.143	1692713	2.479	201470	3.076	362598
3	2.143	1670692	2.481	199908	3.109	356205
4	2.145	1695376	2.481	198733	3.109	360139
5	2.145	1677510	2.488	200399	3.125	359384
Avg		1684747		199974		358779
Std dev		10411.8		1041.3		2904.2
%RSD		0.6		0.5		0.8

Table 10: Mobile phase combination (Buffer: ACN (65: 35%, v/v)):

Injection	Emtricitabine		Tenofovir		Dolutegravir	
	RT	Area	RT	Area	RT	Area
1	2.217	1686293	2.562	199366	3.388	354292
2	2.218	1692396	2.563	201518	3.403	352724
3	2.220	1686818	2.564	200917	3.406	351774
4	2.220	1681784	2.565	201453	3.410	354617
5	2.221	1695390	2.566	198596	3.413	352356
Avg		1688536		200370		353153
Std dev		5372.4		1317.7		1241.1
%RSD		0.3		0.7		0.4

Table 11: Mobile phase combination (Buffer: ACN (55: 45%, v/v)):

Injection	Emtricitabine		Tenofovir		Dolutegravir	
	RT	Area	RT	Area	RT	Area
1	2.229	1673642	2.584	199244	3.069	355568
2	2.229	1668129	2.587	198551	3.070	362598
3	2.230	1670708	2.588	199012	3.086	356205
4	2.230	1662131	2.589	198457	3.096	360139
5	2.231	1686411	2.595	201178	3.097	359384
Avg		1672204		199288		358779
Std dev		9003.0		1105.0		2904.2
%RSD		0.5		0.6		0.8

Table 12: Degradation profile results for drugs

Degradation condition	Emtricitabine% found	Tenofovir % found	Dolutegravir % found
Acid	94.79	94.05	94.64
Base	95.23	95.46	95.50
Oxidation	95.58	96.05	95.89
Thermal	97.30	97.18	97.14
Photolytic	98.31	98.31	98.18
Hydrolytic	99.34	98.79	99.16

Table 13: Assay results for Emtricitabine, Tenofovir and Dolutegravir

Drug name	Label claim dose	%Assay
Emtricitabine	200	100.01
Tenofovir	25	99.57
Dolutegravir	50	100.64

Emtricitabine

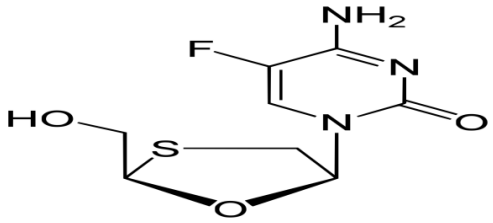

Figure-1: Structure of Emtricitabine

Dolutegravir

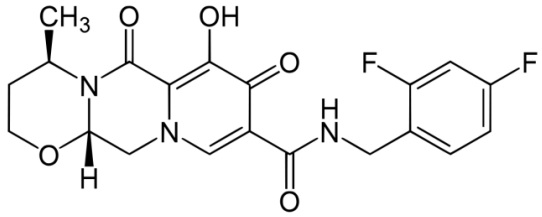

Figure-2: Structure of Dolutegravir

Tenofovir Alafenamide

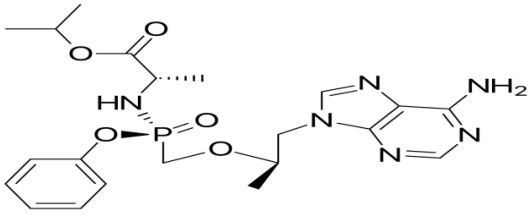

Figure-3: Structure of Tenofovir alafenamide.

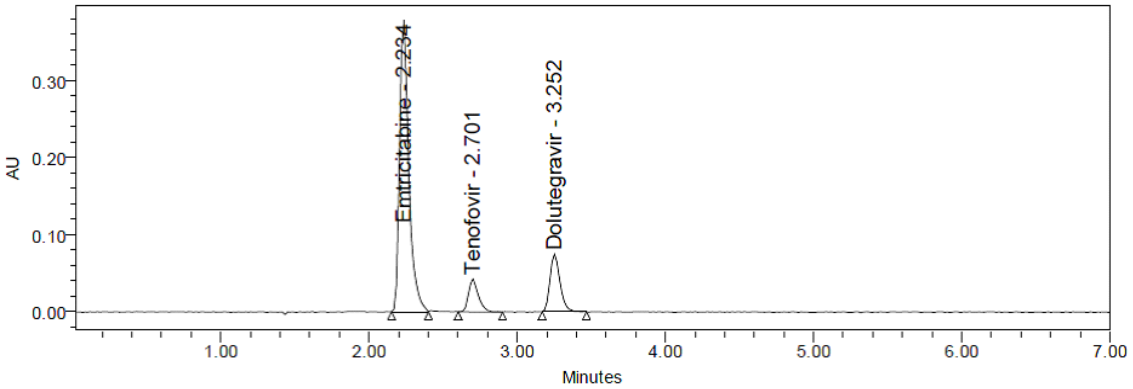

Figure 4: System suitability Chromatogram of Emtricitabine, Tenofovir and Dolutegravir.

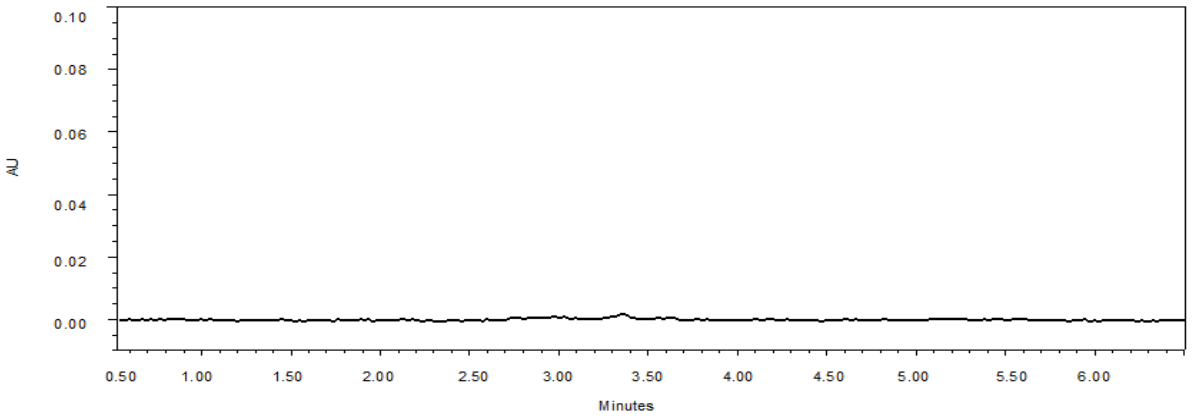

Figure-5: Specificity Chromatogram of blank

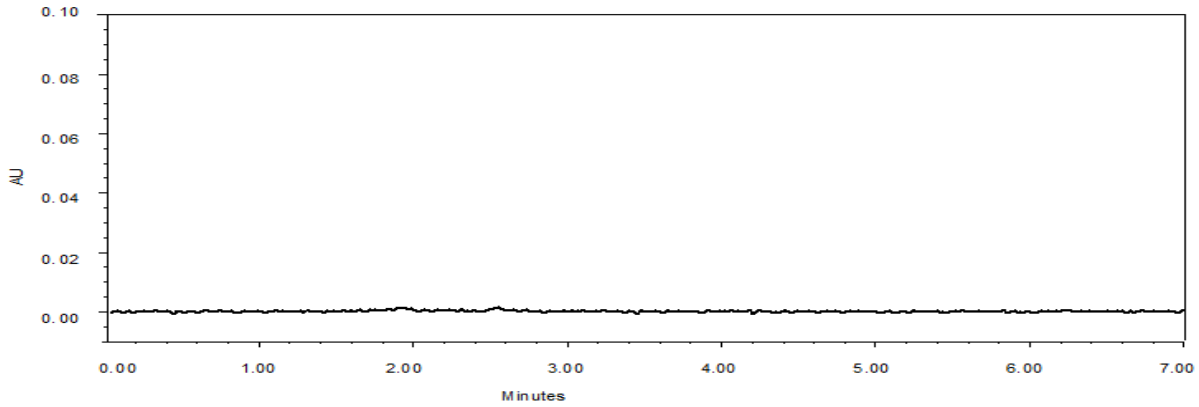

Figure-6: Specificity Chromatogram of placebo

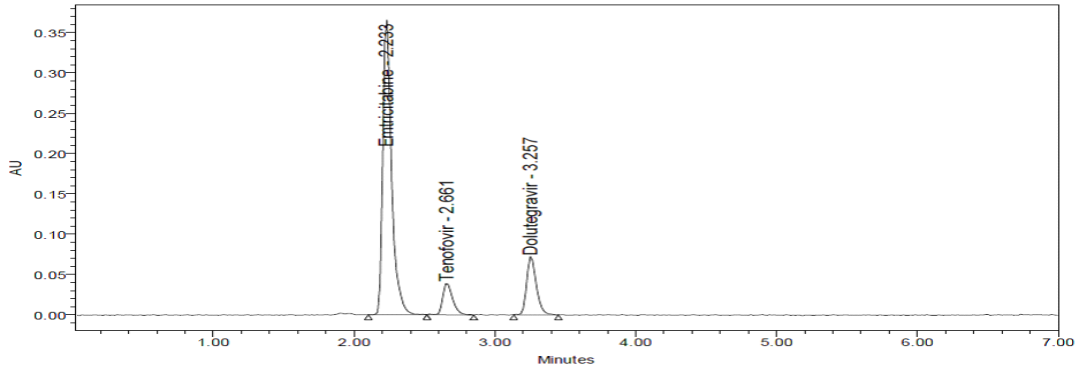

Figure-7: Specificity Chromatogram of Emtricitabine, Tenofovir and Dolutegravir.

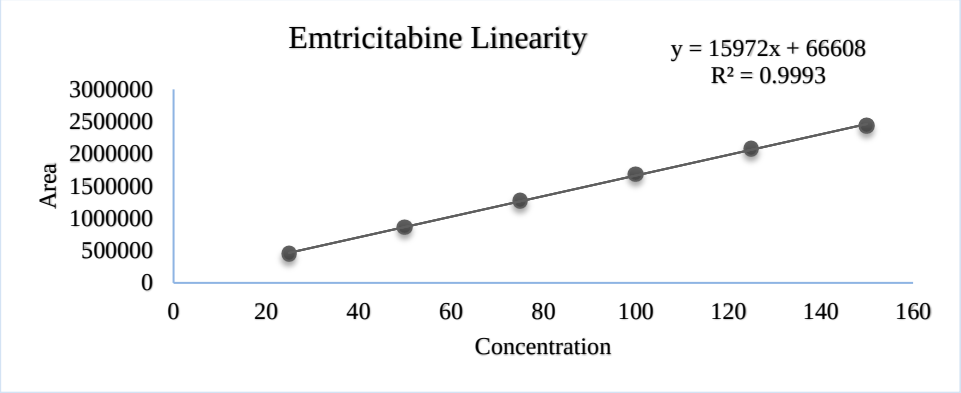

Figure-8: Linearity curve for Emtricitabine

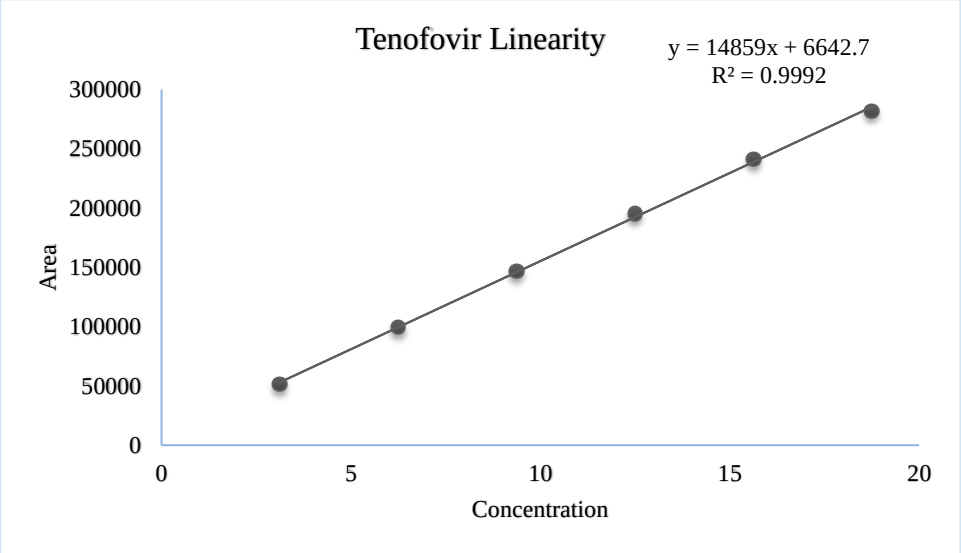

Figure-9: Linearity curve for Tenofovir

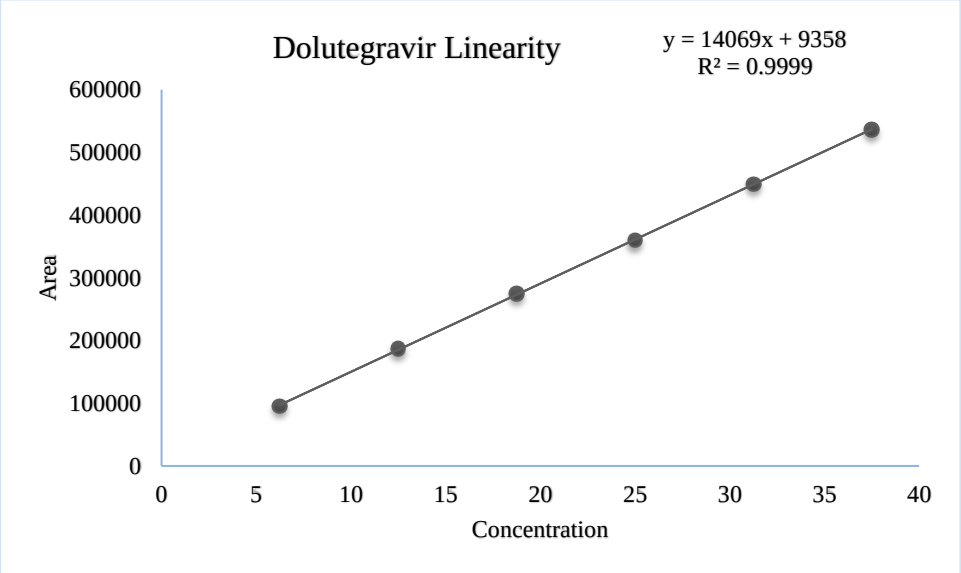

Figure-10: Linearity curve for Dolutegravir

CONCLUSION

A new stability indicating analytical method was developed and validated by RP-HPLC technique. The sample preparation is simple, consumes less amount of mobile phase and the required time for analysis is very short, the information given in the study will be very useful for the quality monitoring of Emtricitabine, Tenofovir and Dolutegravir in pharmaceutical dosage forms. The results of assay analysis of three drugs from a combined dosage form using this developed method were found to be close to 100 %. Recovery studies were satisfactory which shows that there is no interference of excipients.

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