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Research Article

Development and Validation of a New Stability Indicating RP-UPLC Method for the Simultaneous Estimation of Anti-Viral Drugs: Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir (Tablets)

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Abstract

Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium are anti-HIV drugs. The authors have developed a new stability indicating RP-UPLC method for the simultaneous determination of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium in tablets on gradient mode using a mixture of mobile phase A (0.1% Tri ethyl amine pH adjusted to 4.5 ± 0.05 with ortho phosphoric acid) and mobile phase B (0.1% Tri fluoro acetic acid in acetonitrile) with 1.0 mL/min flow rate are the chromatographic conditions for the entire study. Shimadzu NexeraX2 Model UPLC system with PDA detector Zorbax column (100 mm × 4.60 mm, 3.5 µ) was employed for the present study. Beer-Lambert's law was obeyed over the concentration range 2-600, 2-900 and 1-150 µg/mL with linear regression equation y = 1226.8x - 160.82 (R^2 = 0.9999), y = 1200.3x - 1540.5 (R^2 = 0.9999) and y = 3214x + 693.63 (R^2 = 0.9999) for Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium and the method was validated as per ICH guidelines. The LOQ values were found to be 1.8231, 1.9014 and 0.9243 µg/mL and that of LOD values as 0.6002. 0.6259 and 0.3039 µg/mL for Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium respectively. The combination of Tenofovir Disoproxil Fumarate, Dolutegravir sodium and Emtricitabine was exposed to various stress conditions and the stability of the proposed method was carried out at UV detection 260 nm. The proposed RP-UPLC method is simple, precise, accurate, robust and used for the routine analysis of tablet dosage forms.

Keywords: UPLC; Stability Indicating; Emtricitabine; Tenofovir Disoproxil Fumarate; Dolutegravir Sodium

Introduction

Tenofovir disoproxil fumarate (CAS No. 202138-50-9) is chemically bis (1-methylethyl) 5-{[(1R-2-(6-amino-9H-purin-9-yl)-ethylethoxy] methyl}-5-oxo-2, 4, 6, 8-tetraoxa-5-λ5-phosphanonanedioate (ester) hydrogen (2E)-but-2-enedioate [1] with molecular formula, $C_{19}H_{30}N_5O_{10}P$. $C_4H_4O_4$ and molecular weight 635.52 g/mol. It is an antiretroviral agent acts as a reverse Transcriptase inhibitor.

Emtricitabine (CAS No. 143491-57-0) is chemically 4-Amino-5-fluoro-1- [(2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidine [2] with molecular formula, $C_8H_{10}FN_3O_3S$ and

molecular weight 247.247 g/mol. Emtricitabine acts by inhibiting the reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. It is a synthetic nucleoside analogue of cytidine. Emtricitabine inhibits the activity of HIV-1 reverse transcriptase both by competing with the natural substrate deoxycytidine 5'-triphosphate and by its incorporation into viral DNA.

Dolutegravir sodium (CAS No. 1051375-19-9) is chemically 2H-Pyrido [1',2':4, 5] pyrazino [2,1-b][1,3]oxazine-9-carboxamide,N-[(2,4-difluorophenyl)methyl]-3,4,6,8,12,12a-hexahydro-7-hydroxy-4-methyl-6,8-dioxo-, sodium salt (1:1), (4R,12aS) with molecular formula, $C_{20}H_{18}F_2N_3NaO_5$ and molecular weight 441.367 g/mol. Dolutegravir is a HIV Integrase Inhibitor [3,4]. Dolutegravir is an orally bioavailable integrase strand-transfer inhibitor with activity against HIV-Type 1 infection.

The chemical structures of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium were shown in figure 1. Analytical methods such as spectrophotometric [5], LC/MS/MS [6], HPTLC [7] and HPLC [8] methods were developed for the simultaneous estimation of Tenofovir and Emtricitabine in tablets. Liquid chromatographic methods [9-12] were developed for the simultaneous estimation of Tenofovir disoproxil, Lamivudine and Efavirenz in tablets. Liquid chromatographic methods [13-16] were developed for the simultaneous estimation of Tenofovir disoproxil fumarate, Lamivudine and Dolutegravir in tablets and their potential impurities. HPLC methods [17-19] were developed for the simultaneous estimation of Tenofovir disproxil, Emtricitabine and Efavirenz in tablets.

Till now there is not even a single stability indicating RP-UPLC method for the combined dosage forms of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir. In the present study, the authors have proposed a new stability-indicating RP-UPLC method for the simultaneous estimation of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir in tablet dosage forms and the method was validated as per ICH guidelines.

Materials and Methods

HPLC grade acetonitrile, AR grade HCl, NaOH, H_2O_2, Triethylamine, Trifluoroacetic acid, dibasic potassium hydrogen phosphate and *o*-phosphoric acid were procured from Merck and used without further purification. Mobile phase A was prepared by using 0.1 % of Tri ethyl amine in water and by adjusting the pH to 4.5 ± 0.05 with the help of ortho phosphoric acid whereas mobile phase B was prepared by using 0.1 % Tri fluoro acetic acid in acetonitrile and were used after mixing well. A diluent consisting of phosphate buffer (adjusted the pH 3.0 ± 0.05 with ortho phosphoric acid) and acetonitrile in 50: 50 ratio was used for the chromatographic study. Phosphate buffer (pH 3.0) was prepared by dissolving 4.40 g of dibasic potassium hydrogen phosphate anhydrous (K_2HPO_4) in 1000 ml of water, adjust pH to 3.0 ± 0.05 with Ortho phosphoric acid.

Figure 1: Chemical structures of anti-HIV drugs.

Instrumentation and chromatographic conditions

Shimadzu NexeraX2 Model UPLC system with PDA detector Zorbax column (100 mm × 4.60 mm, 3.5 µ) was employed for the present study. The injection volume was 1 µL and the total run time was 5 mins. A mixture of mobile phase A (0.1% Tri ethyl amine pH adjusted to 4.5 ± 0.05 with ortho phosphoric acid) and mobile phase B (0.1% Tri fluoro acetic acid in acetonitrile) with 1.0 mL/min flow rate are the chromatographic conditions.

Preparation of standard solutions of tenofovir disoproxil fumarate, emtricitabine and dolutegravir sodium

Stock solution of Dolutegravir sodium was prepared by transferring 50 mg accurately to a 50 ml volumetric flask and dissolved in acetonitrile and dilutions were made as per the requirement with the diluent. Stock solution of Emtricitabine was prepared by transferring 100 mg accurately to a 100 ml volumetric flask and dissolved in acetonitrile and dilutions were made as per the requirement with the diluent. Stock solution of Tenofovir disoproxil fumarate was prepared by transferring 250 mg accurately to a 25 ml volumetric flask and dissolved in acetonitrile and dilutions were made as per the requirement with the diluent. All these solutions were filtered through 0.45µm nylon filter before injecting in to the system.

Method validation [20]

Linearity, precision and accuracy

A series of Emtricitabine (2-600 µg/mL), Tenofovir disoproxil fumarate (2-900 µg/mL) and Dolutegravir sodium (1-150 µg/mL) solutions were prepared from the stock solution (1000 µg/mL) and diluted with the diluent and 1.0 µL each of these solutions were injected into the UPLC system three times and the average peak area was calculated from the respective chromatograms. A calibration graph was drawn by plotting the concentration of the drug solutions on the x-axis and the corresponding mean peak area on the y-axis. The intraday precision studies were conducted on the same day at different equal time intervals and the interday precision studies were conducted on three successive days (Day 1, Day 2 and Day 3) and the data was analysed. Accuracy studies were performed by spiking the formulation solution with 50, 100 and 150% of API solution and % recovery was calculated. The percentage relative standard deviation was calculated in all the validation parameters.

Assay of combined dosage forms of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir (Tablets)

The tablet dosage form consisting of Emtricitabine (200 mg), Tenofovir disoproxil fumarate (25 mg) and Dolutegravir (50 mg) are available with brand name, Spegra from Emcure Pharmaceuticals Ltd (India). 20 Tablets containing Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir were weighed, powdered and tablet powder equivalent to Emtricitabine (200 mg), Tenofovir disoproxil fumarate (25 mg) and Dolutegravir (50 mg) was first extracted with acetonitrile and sonicated for 30minutes. The contents were filtered and required concentrations were prepared using the diluent and 1 µL of these solutions were injected in to the UPLC system and the chromatogram was recorded. The percentage purity was determined from the peak area of the chromatogram obtained.

Stress degradation studies [21]

Stress degradation studies of Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir were performed to identify the specificity of the method.

Acidic hydrolysis

Acid hydrolysis was carried out by treating the drug solution containing Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir with 5 ml of 2N HCl and allowed to stand at room temperature for 1hour and then neutralized with 10 mL of 1N NaOH in a 250 ml volumetric flask and made up to volume with the diluent. 5 ml of this resulting solution was further diluted in a 50 ml volumetric flask and 1 µl of this was injected in to the UPLC system after filtering through 0.45µm nylon filter and the peak area of each of Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir was noted from the respective chromatogram.

Alkaline hydrolysis

Alkaline hydrolysis was carried out by treating the drug solution containing Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir with 5 ml of 1N NaOH and allowed to stand at room temperature for 1hour and then neutralized with 5 mL of 1N HCl in a 250 ml volumetric flask and made up to volume with the diluent. 5 ml of this resulting solution was further diluted in a 50 ml volumetric flask and 1 µl of this was injected in to the UPLC system after filtering through 0.45µm nylon filter and the peak area of each of Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir was noted from the respective chromatogram.

Oxidative degradation

Oxidative degradation was carried out by treating the drug solution containing Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir with 5 ml of 3% H_2O_2 and allowed to stand at

room temperature for 1hour and in a 250 ml volumetric flask and made up to volume with the diluent. 5 ml of this resulting solution was further diluted in a 50 ml volumetric flask and 1 µl of this was injected in to the UPLC system after filtering through 0.45µm nylon filter and the peak area of each of Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir was noted from the respective chromatogram.

Thermal degradation (Dry heat)

Thermal degradation was carried out by keeping the weighed amount of solid drug substances, Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir in hot air oven for 1hour at 80°C and cooled to room temperature, then transferred in to 250 mL volumetric flask and made up to volume with the diluent. 5 ml of this resulting solution was further diluted in a 50 ml volumetric flask and 1 µl of this was injected in to the UPLC system after filtering through 0.45 µm nylon filter and the peak area of each of Tenofovir Disoproxil Fumarate, Emtricitabine and Dolutegravir was noted from the respective chromatogram.

Results and Discussion

A new stability indicating RP-UPLC method for the simultaneous determination of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium in tablets on gradient mode using a mixture of mobile phase A (0.1% Tri ethyl amine pH adjusted to 4.5 ± 0.05 with ortho phosphoric acid) and mobile phase B (0.1% Tri fluoro acetic acid in acetonitrile) with 1.0 mL/min flow rate (Detection wavelength 260 nm) are the chromatographic conditions for the entire study. Shimadzu NexeraX2 Model UPLC system with PDA detector Zorbax column (100 mm × 4.60 mm, 3.5 µ) was employed for the present study. The total run time was 5 minutes and the gradient program was given in Table 1. The chromatogram obtained was shown in Figure 2 in which Emtricitabine was eluted at 20153 min, Tenofovir disoproxil fumarate at 3.444 min and Dolutegravir sodium at 4.081 min with system suitability parameters within the acceptance criteria (Table 2).

Table 1: Gradient program.

Time (min)	Mobile phase A	Mobile phase B
0.0	97.0	3.0
2.34	50.0	50.0
4.0	50.0	50.0
4.5	97.0	3.0
5.0	97.0	3.0

Table 2: System suitability parameters.

Drug	Emtricitabine (EM)	Tenofovir DF (TDF)	Dolutegravir sodium (DT)	Acceptance criteria
Retention time (min)	2.153	3.428	4.063	>2.0
Theoretical plates	74435	102154	37178	>2000
Tailing factor	1.38	1.27	1.12	<2.0
Resolution	-	34.18	10.05	>2.0

Figure 2: Representative chromatograms of the combination of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir.

Linearity, precision and accuracy

Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium obey Beer-Lambert's law over the concentration range 2-600, 2-900 and 1-150 µg/mL (Table 3) with linear regression equation y = 1226.8x - 160.82 (R^2 = 0.9999) (Figure 3), y = 1200.3x - 1540.5 (R^2 = 0.9999) (Figure 4) and y = 3214x + 693.63 (R^2 = 0.9999) (Figure 5). The LOQ values were found to be 1.8231, 1.9014 and 0.9243 µg/mL and that of LOD values as 0.6002. 0.6259 and 0.3039 µg/mL for Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium respectively.

Table 3: Linearity.

Conc. (µg/mL)	*Mean peak area		
	EM	TDF	DT
0	0	0	0
1	-	-	3325
2	2424	2221	6664
5	6065	5564	16527
10	12121	11048	33017
20	24259	22127	65211
40	48416	45224	131152
50	62278	60152	162118
60	70354	72034	192145
80	96451	96251	258377
100	124531	120085	324250
120	140613	144022	384113
150	186659	176289	482638
200	248567	230152	-
300	371211	353581	-
400	491548	479018	-
500	615629	601251	-
600	731158	724982	-
700	-	840127	-
800	-	949008	
900	-	1083537	-

*Mean of three replicates.

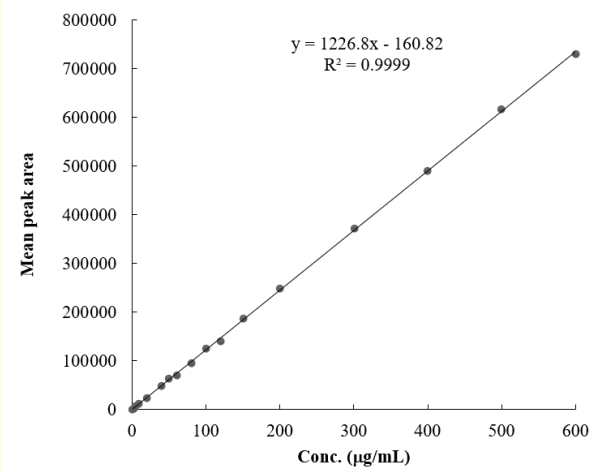

Figure 3: Calibration curve of Emtricitabine.

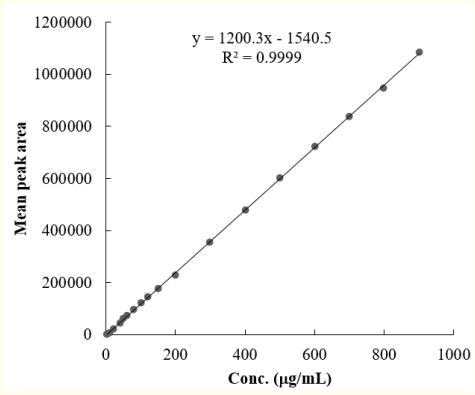

Figure 4: Calibration curve of Tenofovir disoproxil fumarate.

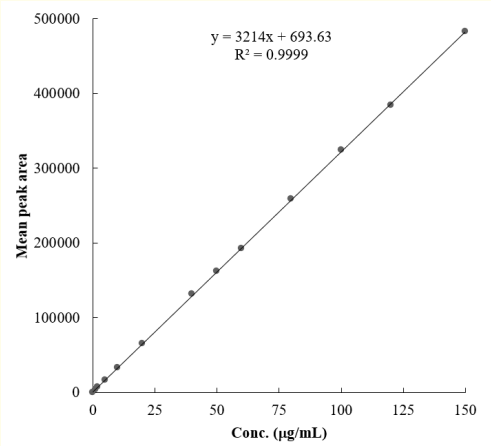

Figure 5: Calibration curve of Dolutegravir sodium.

Precision study was performed by injecting the mixture of EM, TDF and DT six times into the UPLC system and the chromatographs were recorded. The mean peak area, standard deviation and the relative standard deviation were calculated from the respective linear regression equations. The % RSD in intraday precision was found to be 0.2655, 0.3188 and 0.2149 whereas for interday precision it was found to be 0.1693, 0.0804 and 0.1195 for Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir sodium respectively (Table 4) which was found to be less than 2.0% demonstrating that the method is precise. In the accuracy study the % RSD was found to be 0.31-0.92 for Emtricitabine, 0.27-1.09 for Tenofovir disoproxil fumarate and 0.72-1.02 for Dolutegravir sodium respectively which is less than 2.0 demonstrating that the method is accurate (Table 5).

Table 4: Precision study.

Intraday precision study						
S. No.	R_t (min)			*Peak area		
	EM	TDF	DT	EM	TDF	DT
1	2.160	3.436	4.073	481788	671232	323371
2	2.163	3.439	4.076	482639	673008	323282
3	2.161	3.439	4.073	479517	668137	322221
4	2.166	3.443	4.077	481019	669729	322563
5	2.157	3.433	4.071	479728	667435	321784
6	2.159	3.433	4.068	482061	671523	323453
Mean	-	-	-	481125.33	670177.33	322779.00
SD	-	-	-	1277.2133	2136.3599	693.6561
% RSD	-	-	-	0.2655	0.3188	0.2149
Interday precision study						
Day				**EM**	**TDF**	**DT**
Day 1				241515	350793	166792
Day 2				240807	350527	167168
Day 3				241514	351091	167096
Mean				241278.67	350803.67	167018.67
SD				408.4756	282.1513	199.5729
% RSD				0.1693	0.0804	0.1195

*Mean of three replicates.

Table 5: Accuracy study.

Level %	Spiked conc. (µg/mL)			Formulation (µg/mL)			% Recovery* (% RSD)		
	EM	TDF	DT	EM	TDF	DT	EM	TDF	DT
50	100	12.5	25	200	25	50	98.29 (0.31)	98.83 (0.52)	99.91 (0.72)
100	200	25	50	200	25	50	98.67 (0.92)	99.17 (0.27)	99.46 (0.91)
150	300	37.5	75	200	25	50	99.28 (0.54)	99.69 (1.09)	99.78 (1.02)

*Mean of three replicates.

Assay of combined dosage forms of emtricitabine, tenofovir disoproxil fumarate and dolutegravir (Tablets)

The combination of Dolutegravir (50 mg), Emtricitabine (200 mg) Tenofovir disoproxil fumarate (25 mg) is available as tablets with brand name, Spegra from Emcure Pharmaceuticals Ltd. The proposed RP-UPLC method was applied for the tablet dosage forms with the optimized chromatographic conditions. The percentage of purity of Emtricitabine, Tenofovir and Dolutegravir was found to be 98.94 (Emtricitabine) 99.95 (Tenofovir disoproxil fumarate) and 99.87 (Dolutegravir). The representative chromatogram of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir was shown in Figure 2C.

Stress degradation studies

The combination of Dolutegravir (50 mg), Emtricitabine (200 mg) Tenofovir disoproxil fumarate was exposed to stress conditions such as acidic hydrolysis, alkaline hydrolysis, Oxidation and thermal degradation with the optimized chromatographic conditions. During the acidic hydrolysis EM, TDF and DT were eluted at Rt 2.143, 3.409 and 4.034 min respectively with theoretical plates more than 2000 and tailing factor less than 1.5 with % recovery 96.97 (EM), 89.65 (TDF), 99.59 (DT) with resolution 34.19 and 10.01 which were greater than 2.0 were observed.

During the alkaline hydrolysis EM, TDF and DT were eluted at Rt 2.163, 3.431 and 4.055 min respectively with theoretical plates more than 2000 and tailing factor less than 1.5 with % recovery 94.64 (EM), 92.29 (TDF), 97.65 (DT) with resolution 35.11 and 10.19 which were greater than 2.0 were observed.

During the oxidative degradation EM, TDF and DT were eluted at Rt 2.151, 3.416 and 4.041 min respectively with theoretical plates more than 2000 and tailing factor less than 1.5 with % recovery 94.37 (EM), 95.89 (TDF), 99.92 (DT) with resolution 34.83 and 10.24 which were greater than 2.0 were observed.

During the oxidative degradation EM, TDF and DT were eluted at Rt 2.159, 3.427 and 4.053 min respectively with theoretical plates more than 2000 and tailing factor less than 1.5 with % recovery 97.19 (EM), 92.59 (TDF), 99.75 (DT) with resolution 35.01 and 10.62 which were greater than 2.0 were observed.

In all degradation studies the system suitability parameters were within the acceptable criteria (Table 6) and the corresponding chromatograms obtained during the stress degradation studies were shown in figure 6.

Figure 6: Typical chromatograms of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir during the stress degradation studies.

Table 6: Stress degradation studies.

	R_t (min)			% Recovery* (%Decomposition)			Theoretical Plates (Tailing factor)			Resolution (>2)		
Drug	**EM**	**TDF**	**DT**	**EM**	**TDF**	**DT**	**EM**	**TDF**	**DT**	**EM**	**TDF**	**DT**
Acidic hydrolysis 2N HCl/1hour	2.143	3.409	4.034	96.97 (3.03)	89.65 (10.35)	99.59 (0.41)	74593 (1.39)	102152 (1.27)	37281 (1.10)	-	34.19	10.01
Alkaline hydrolysis 1N NaOH/1hour	2.163	3.431	4.055	94.64 (5.36)	92.29 (7.71)	97.65 (2.35)	74982 (1.21)	102261 (1.31)	37112 (1.13)	-	35.11	10.19
Oxidative degradation 3 % H_2O_2 /1hour	2.151	3.416	4.041	94.37 (5.63)	95.89 (4.11)	99.92 (0.08)	75136 (1.42)	102228 (1.19)	37193 (1.23)	-	34.83	10.24
Thermal degradation 80°C/1hour	2.159	3.427	4.053	97.19 (2.81)	92.59 (7.41)	99.75 (0.25)	74657 (1.11)	102893 (1.29)	37184 (1.19)	-	35.01	10.62

*Mean of three replicates.

Conclusion

The proposed new stability indicating RP-UPLC method for the estimation of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir is simple, accurate, precise and robust. The method is useful for the routine analysis of Emtricitabine, Tenofovir disoproxil fumarate and Dolutegravir tablets in pharmaceutical industries. The method is specific and there is no interference of excipients during the study.

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