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Advances in Bioresearch

ORIGINAL ARTICLE

RP-UPLC Method for the Simultaneous Determination of Tenofovir Alafenamide, Emtricitabine and Dolutegravir

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ABSTRACT

A simple, accurate and precise method was developed for the simultaneous estimation of the EMTC, DLTG and TNFA in Tablet dosage form by RP-UPLC technique. Retention times of EMTC, DLTG and TNFA were found to be 1.328 min,1.765 min and 2.135 min respectively. Chromatographic elution was processed through a Std BEH C18 (50 x 2.1 mm, 1.8µ) reverse phase column and the mobile phase composition of buffer 0.1% orthophosphoric acid (2.2 pH) and acetonitrile in the ratio of 60:40 was pumped through a column at a flow rate of 1.0 mL/min. Repeatability of the method was determined in the form of %RSD and findings were 0.9, 0.6 and 1.0 for EMTC, DLTG and TNFA respectively. LOD, LOQ values obtained from regression equations of EMTC, DLTG and TNFA were 0.34, 1.03 µg/mL, 0.36, 1.09 µg/mL and 0.25, 0.76 µg/mL respectively. Three analytes were subjected for acid, peroxide, photolytic, alkali, neutral and thermal degradation studies and the results shown that the percentage of degradation was found between 0.29% and 6.41%. Retention times and total run time of two drugs were decreased and the developed method was simple and economical. So, the developed method can be adopted in industries as a regular quality control test for the quantification of EMTC, DLTG and TNFA.

Keywors : Tenofovir Alafenamide, Emtricitabine, Dolutegravir, Braf Inhibitor, Validation, Precision, Accuracy, Linearity.

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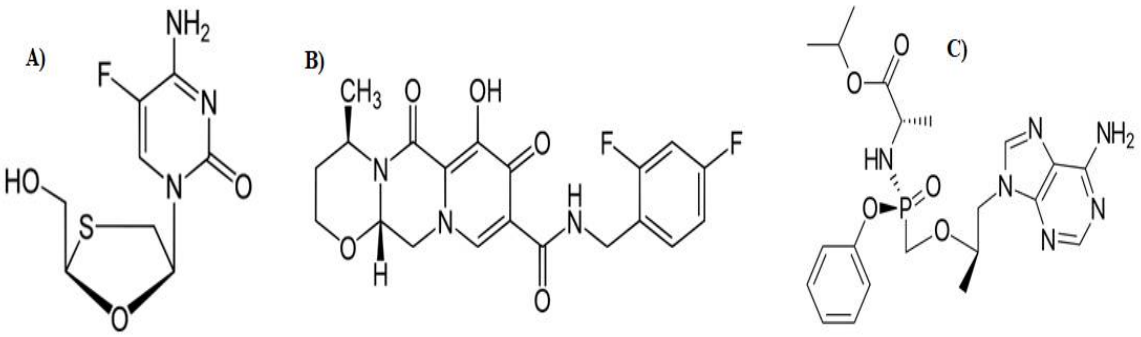
INTRODUCTION

Emtricitabine has the chemical formula 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan5-yl].Pyrimidine-2-one belongs to the class of nucleoside reverse transcriptase inhibitors, which includes anti-HIV-1 drugs. This powder is white to off-white in color, dissolves in water at a rate of around 112 mg/mL at 25°C, and has the chemical formula C8H10FN3O3S with a molecular weight of 247.244 g/mol[1]. Figure 1A shows that emtricitabine has a pKa of 2.65 and a log P of -0.43. Tenofovir alafenamide fumarate2 (TAF) is a novel ester prodrug that inhibits nucleotide reverse transcriptase and is an antiviral medication. Its chemical name is propan-2-yl (2S).C21H29N6O5P is the chemical formula for -2-[[[(2R)-1-(6- aminopurin-9-yl)propan-2-yl]oxymethyl-phenoxyphosphoryl]amino]propanoate. It is a solid powder that dissolves in water at a rate of 4.86 mg/mL [2]. The log P for this medication is 1.6 and its pKa is 3.96 (Figure 1B). Efavirenz3 is (4S), chemically speaking. -6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3,1- benzoxazin-2-one is one of the medications of the HIV-1Non-Nucleoside Analog Reverse Transcriptase Inhibitor family that are used to treat HIV. Its chemical formula is C14H9ClF3NO2, and its weight is 315.68 g/mol. It is a white to slightly pink crystalline powder that dissolves in water at a rate of around 0.093 mg/L at 25 °C. The log P for this medication is 4.6, and its pKa is 10.2, 12.52 (Figure-1C).

Emtricitabine, tenofovir alafenamide, and efavirenz alone as well as in combination with other medications have all been determined using a variety of analytical techniques, including spectroscopy [4]

and chromatography [5]. As per ICH guidelines [6], the author has attempted to develop and validate a simple, fast, precise, and accurate UPLC method for the simultaneous determination of emtricitabine, tenofovir alafenamide, and efavirenz in combined tablet dosage form, since there is currently no available UPLC method for this purpose.



A **B** **C**

Fig. 1: Chemical structures of A) emtricitabine, B) dolutegravir and C) tenofovir.

MATERIAL AND METHODS

Chemicals and Reagents

API of EMTC, DLTG and TNFA were obtained from spectrum Pharma Research Solutions, Hyderabad. HPLC-grade methanol and acetonitrile were procured from Merck chemical division, Mumbai, India, Potassium dihydrogen ortho phosphate, orthophosphoric acid, sodium dihyrogen ortho phosphate and HPLC-grade water were bought from Rankem, avantor performance material India limited. EMTC, DLTG and TNFA tablets were obtained from local pharmacy.

Method development

During the method development various mobile phase compositions consisting of methanol, acetonitrile, water, phosphate buffers and different stationary phases were executed to get fine chromatographic conditions like theoretical plates, resolution, tailing and peak shape. The processed trials were mentioned below:

Optimized Chromatographic Conditions

Liquid chromatographic UPLC system of Waters equipped with PDA (photodiode array detector), auto-sampling unit and Std BEH C18 (50 x 2.1 mm, 1.8µ) reverse phase column. The mobile phase composition of buffer 0.1% orthophosphoric acid (2.2 pH) and acetonitrile in the ratio of 70:30 was pumped through a column at a flow rate of 1.0 ml/min. Column oven temperature was maintained at 30°C and the detection wavelength was processed at 275 nm. Integration of output signals were monitored and processed by waters Empower software-2.0.

Diluent

Depending up on the solubility of the drugs, diluent was optimized. Initially dissolved in methanol and diluted with acetonitrile and water (50:50).

Preparation of Standard Stock Solutions

Exactly weighed 100mg of EMTC and 25mg of DLTG and 12.5 mg of TNFA poured in to three 50ml volumetric flasks alone. 10mL of diluent was added and vortexed for 20 min. Flasks were made up with water and acetinitrile (50:50) and marked as standard stock solution 1, 2 and 3 (2000µg/mL of EMTC, 500µg/mL DLTG and 250 µg/mL of TNFA). 1ml from each stock solution was pipetted out and taken into a 10mL volumetric flask and made up with diluent to get 200µg/mL of EMTC, 50µg/mL DLTG and 25 µg/mL of TNFA.

Preparation of Sample Stock Solutions

5 tablets were weighed and the average weight of each tablet was calculated. The weight equivalent to 1 tablet was transferred into a 100ml volumetric flask and 25 ml of diluent was added and sonicated for 25 min. Further the volume was made up with diluent and filtered through 0.45 µ filter (2000µg/mL of EMTC, 500µg/mL DLTG and 250 µg/mL of TNFA). 2ml of the resultant solution was poured in to a 10ml volumetric flask and made up with diluent (200µg/mL of EMTC, 50µg/mL DLTG and 25 µg/mL of TNFA).

Preparation of Buffer

0.1% orthophosphoric acid Buffer was prepared by diluting 1ml of concentrated orthophosphoric acid with water to up to 1000mL.

Method Validation

The developed method for EMTC, DLTG and TNFA was subjected for validation for the parameters like system suitability, linearity, robustness, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy as per the guidelines of ICH[6-9].

RESULTS AND DISCUSSION

Method Development and Optimization

With different mobile phase compositions and stationary phases three different trials were executed and fourth trail was optimized. In all the 3 trials there was no base line separation in trial-1, merged peaks were observed in trail and there was poor resolution in the trial -3. Optimized chromatographic peaks were shown in Fig.2

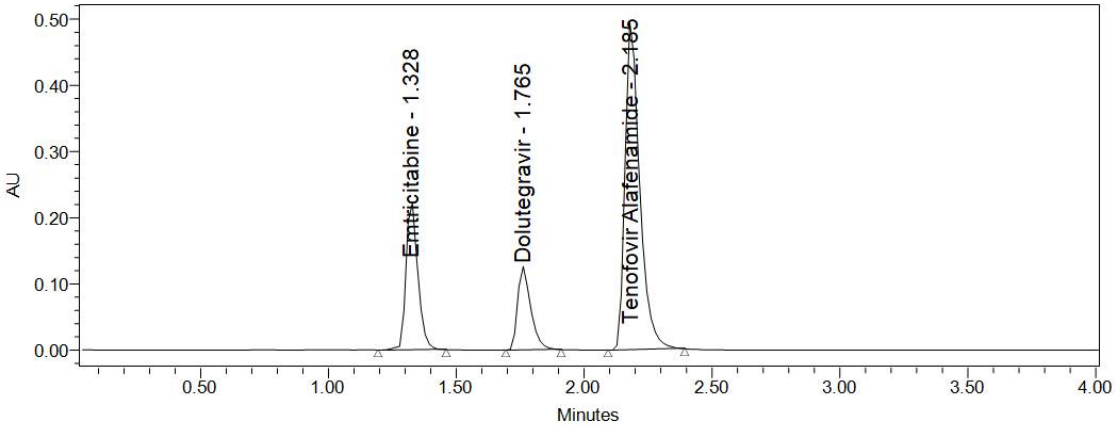


Fig 2: Representative chromatogram of optimized trial

Method Validation

System Suitability

The system suitability variables were estimated by preparing standard solutions of EMTC, DLTG and TNFA and the same were injected 6 times in to the chromatographic system. The variables like peak tailing, resolution and USP plate count were estimated[10-12]. The results were shown in Fig. 1 and Table 1. In the system suitability studies, plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits.

Table.1: System suitability parameters for EMTC, DLTG and TNFA

S. No	EMTC			DLTG				TNFA			
	RT (min)	USP Plate Count	Tailing	RT (min)	USP Plate Count	Tailing	USP Resolution	RT (min)	USP Plate Count	Tailing	USP Resolution
1	1.328	2958	1.34	1.765	4239	1.37	4.6	2.135	5185	1.22	4.7
2	1.327	2821	1.34	1.764	3953	1.27	4.3	2.136	4991	1.16	4.7
3	1.327	2758	1.34	1.765	4241	1.31	4.5	2.135	5058	1.16	4.5
4	1.328	2957	1.35	1.764	4326	1.19	4.5	2.135	5001	1.16	4.7
5	1.328	2534	1.35	1.765	3822	1.19	4.4	2.136	4987	1.15	4.7
6	1.327	3049	1.17	1.764	4108	1.23	4.5	2.135	5121	1.13	4.7

Specificity

Method specificity was determined by infusing the blank, placebo, standard and sample solutions in to a chromatographic system and the resulting chromatograms were evaluated for interference with the excipients, degradants and other components may expected to be present [13-18]. Blank, standard, formulation and placebo chromatograms were represented in Fig. 4.8. We did not found any additional peaks in blank and placebo at retention times of these drugs in this technique. So, this technique was said to be specific.

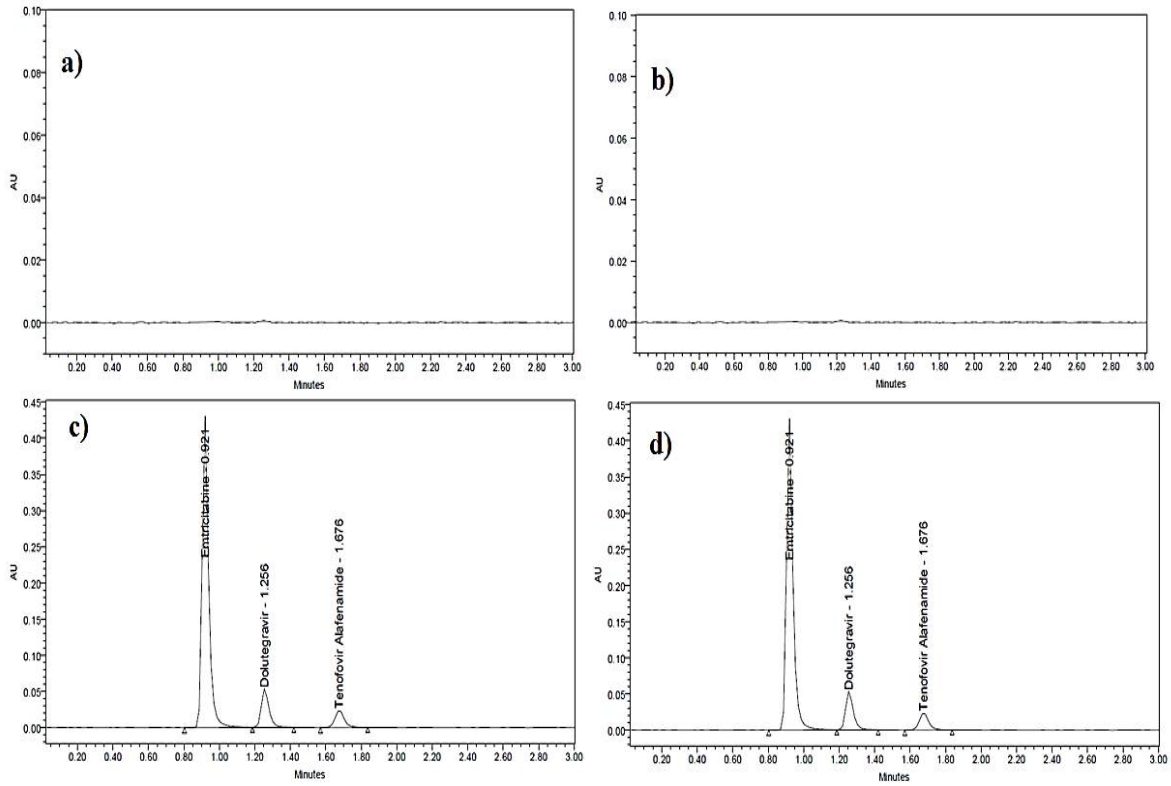


Fig.3: Chromatograms of a) Blank, b) Placebo, c) Standard and d) Sample.

Precision

Precision of the method was evaluated in terms of method precision and intermediate precision. The method precision (repeatability) was estimated by infusing 6 standard solutions and 6 sample solutions. Intermediate precision was evaluated by infusing 6 standard solutions and 6 sample solutions on different days by different employees on different chromatographic systems[15]. The peak responses of all the chromatograms were taken and standard deviation, % RSD (relative standard deviation) and percentage assay of sample solutions were calculated. The findings were represented in Tables 2 and 3. As the limit of precision was < 2(%RSD) and both the precisions were passed in this analysis process.

Table.2: Repeatability results of EMTC, DLTG and TNFA

S. No	Area of EMTC	Area of DLTG	Area of TNFA
1.	1308399	141741	79450
2.	1319874	143789	79650
3.	1334424	144058	77850
4.	1330955	142021	79069
5.	1323162	142845	78499
6.	1339892	143049	79860
Mean	1326118	142917	79063
SD	11347.2	924.2	764.1
%RSD	0.9	0.6	1.0

SD: standard deviation; RSD: relative standard deviation.

Table.3: Intermediate precision results of EMTC, DLTG and TNFA

S. No	Area of EMTC	Area of DLTG	Area of TNFA
1.	1309347	142451	78860
2.	1314245	143412	77412
3.	1322739	144424	78424
4.	1316867	143803	79803
5.	1331431	143584	77984
6.	1326250	144311	78311
Mean	1065590	143664	78466
SD	8167.3	715.1	813.9
%RSD	0.8	0.5	1.0

SD: standard deviation; RSD: relative standard deviation.

Accuracy

Method accuracy was estimated at three variable concentrations of 50%, 100%, and 150% level by spiking the known amount of the drug analytes[17]. The % recovery at each level was calculated and the findings were represented in Table 4.

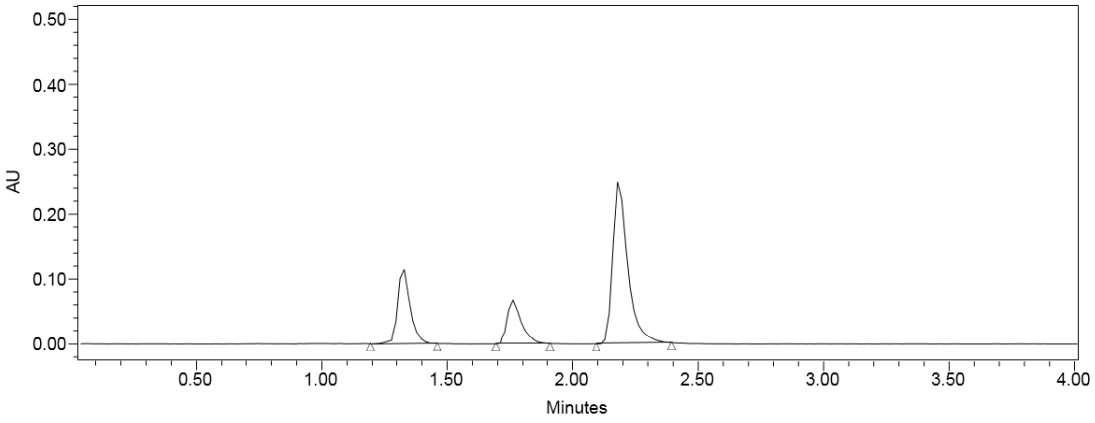


Fig.4: Chromatogram showing accuracy 50%injection

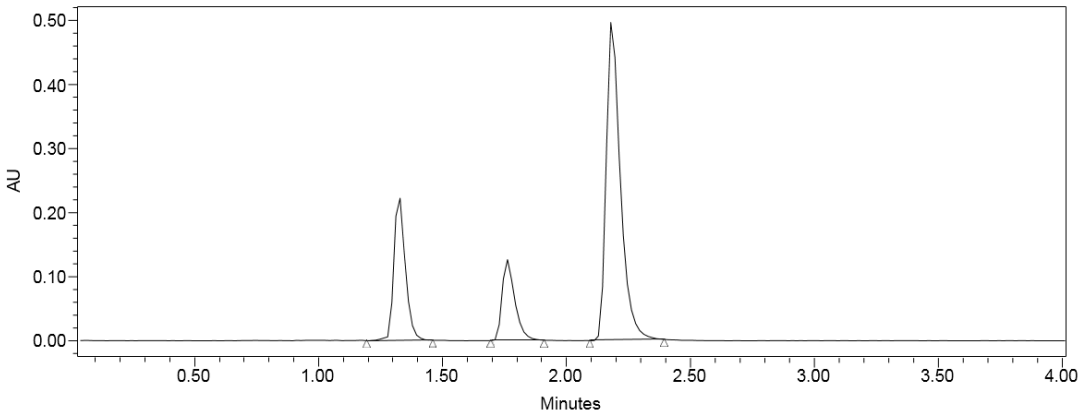


Fig 5: Chromatogram showing accuracy 100%injection

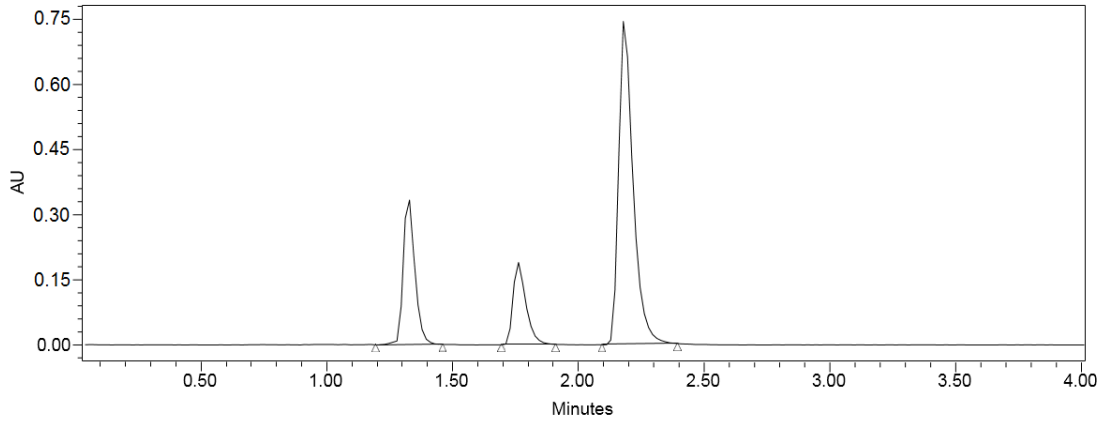


Fig.6: Chromatogram showing accuracy 150%injection-1

Table.4: Percentage recovery results of EMTC, DLTG and TNFA												
	EMTC				DLTG				TNFA			
Spiked level	spiked (µg/mL)	recovery (µg/ mL)	% recovery	Mean % recovery	spiked (µg/ mL)	recovery (µg/ mL)	% recovery	Mean % recovery	spiked (µg/ mL)	recovery (µg/ mL)	% recovery	Mean % recovery
50%	100	98.85	98.84	99.92	25	24.66	98.65	99.86	12.5	12.54	100.35	99.98
	100	99.35	99.35		25	24.78	99.10		12.5	12.36	98.89	
	100	99.95	99.95		25	25.38	101.53		12.5	12.44	99.53	
100%	200	200.10	100.05		50	50.15	100.30		25	25.01	100.06	
	200	200.50	100.25		50	49.66	99.31		25	24.83	99.33	
	200	201.53	100.77		50	50.09	100.18		25	25.22	100.86	
150%	300	300.54	100.18		75	75.78	101.04		37.5	37.67	100.46	
	300	300.77	100.26		75	74.38	99.18		37.5	37.09	98.74	
	300	298.90	99.64		75	74.571	99.43		37.5	38.09	101.57	

Linearity

Linearity of the developed method was evaluated by processing 6 different concentration levels of EMTC, DLTG and TNFA analytes over the concentration of50-300 µg/mL, 12.5- 75 µg/mL and 6.25- 37.5µg/mL. Each concentration level was processed in triplicates[11, 16] The linearity plots were acquired by plotting peak response (on X-axis) versus concentration (on Y-axis). The results of the linearity were represented in Fig. 7, 8, 9 and Table 5.

Table.5: Linearity table for EMTC, DLTG and TNFA

EMTC		DLTG		TNFA	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
0	0	0	0	0	0
50	338151	12.5	36858	6.25	18523
100	656048	25	73309	12.5	36822
150	993933	37.5	108797	18.75	54548
200	1332816	50	145292	25	72489
250	1656248	62.5	179487	31.25	91830
300	1978059	75	216518	37.5	109557

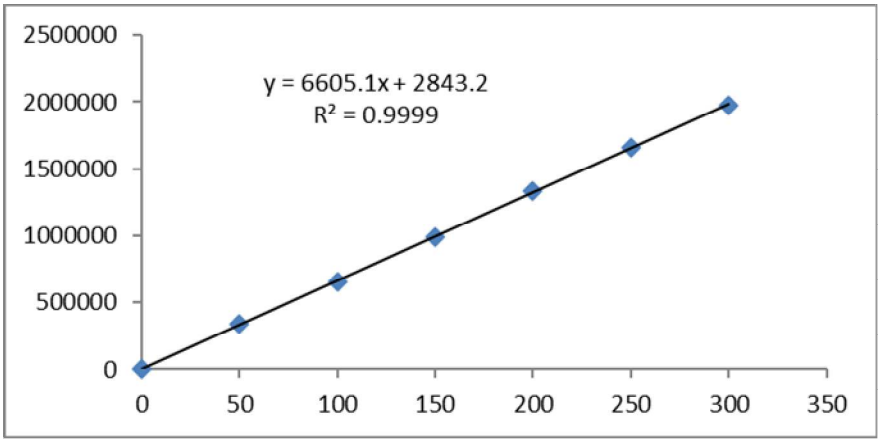


Fig. 7: Calibration curve of EMTC

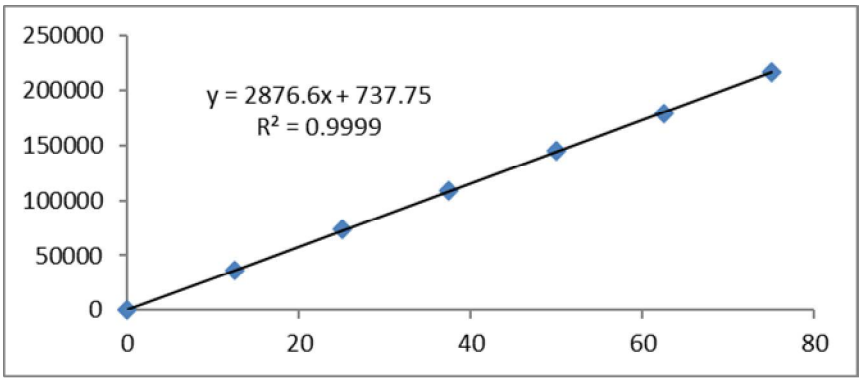


Fig. 8: Calibration curve of DLTG

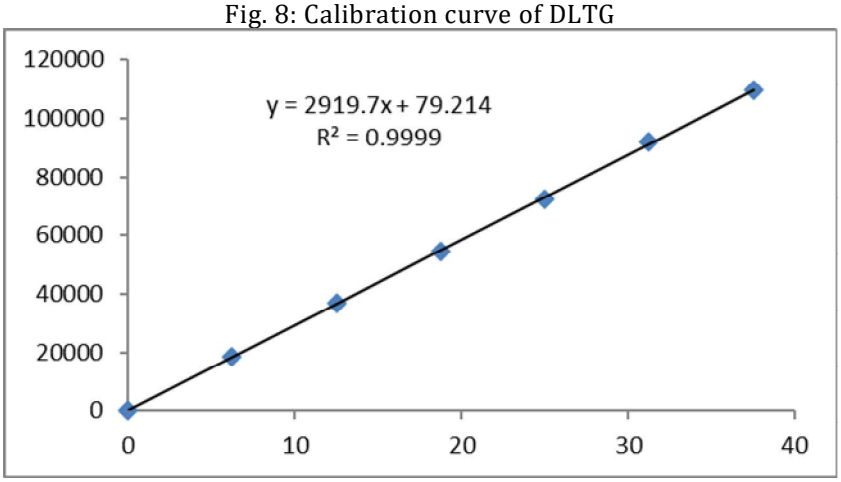


Fig. 9: Calibration curve of TNFA

LOD and LOQ

LOD is lowest quantity of drug in a sample that can be identified but cannot be quantify exactly. LOQ is the lowest quantity of a drug in an analyte which can be quantitatively estimated with a suitable accuracy and precision. The LOD and LOQ values were calculated from the linearity data by utilizing standard deviation and slope of the curve[13, 17]. The resulting LOD and LOQ findings were represented in Table 6.

Table 6: LOD and LOQ values of EMTC, DLTG and TNFA

Analyte	LOD (µg/mL)	LOQ (µg/mL)
EMTC	0.84	2.53
DLTG	0.22	0.67
TNFA	0.05	0.16

Robustness

The method robustness was processed by introducing small variation in the optimized LC conditions such as organic phase in mobile phase (±5%), flow rate (-0.27 and +0.33 mL/ min) and column temperature (±5°C). The findings were shown in the Table 7.

Table.7: Robustness data for EMTC, DLTG and TNFA

S.No	Variation in LC conditions	EMTC % RSD	DLTG % RSD	TNFA % RSD
1	Flow rate (-) 0.27ml/min	1.2	0.9	1.1
2	Flow rate (+) 0.33ml/min	1.0	1.1	0.7
3	Organic phase -5%	0.9	1.1	0.9
4	Organic phase + 5%	1.2	1.0	1.2
5	Temperature at 25°C	1.3	0.7	1.3
6	Temperature at 35°C	0.8	0.9	1.2

Degradation Studies

Alkali Degradation Studies

To 1 mL of each stock solution of EMTC, DLTG and TNFA, 1 mL of 2N NaOH was added in to a 10 mL volumetric flask and kept at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

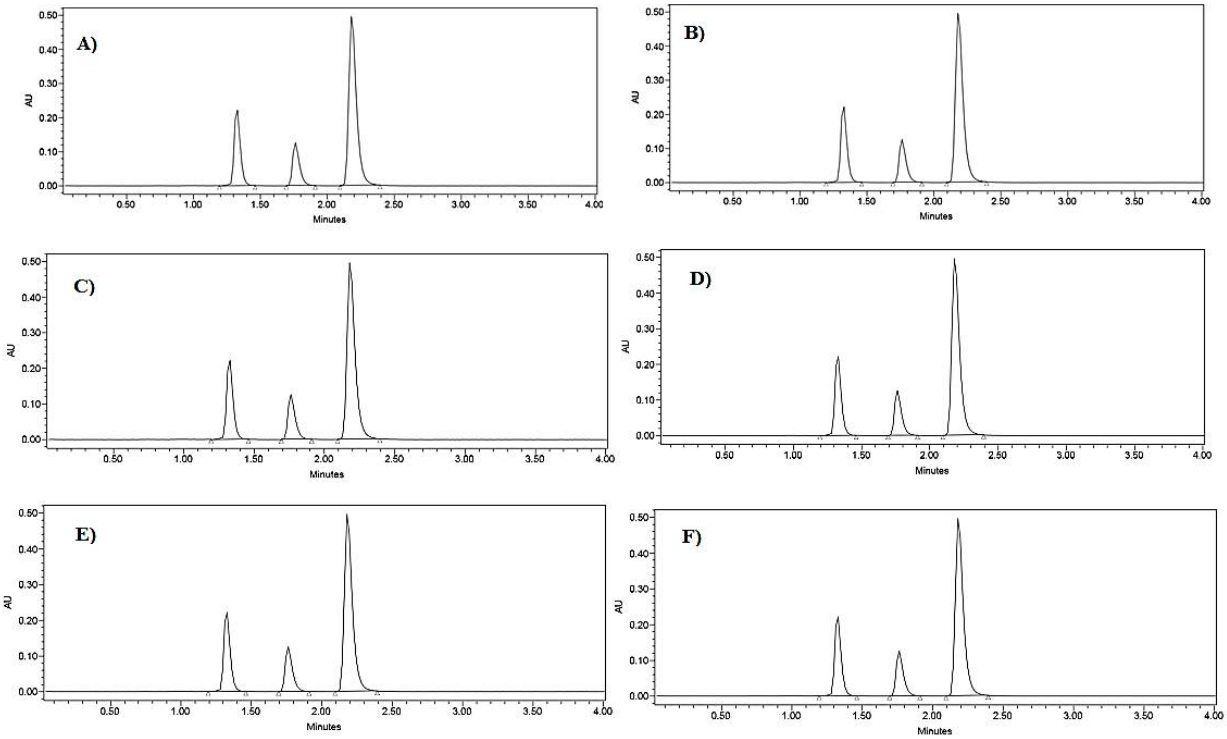


Fig.10: Chromatogram for A)Alkali B) UV-degradation C) Acid D) Neutral E) Peroxide and F) Thermal degradation study.

Table.8: Degradation data of EMTC, DLTG and TNFA

Type of degradation	EMTC			DLTG			TNFA		
	Area	%Recovered	% Degraded	Area	%Recovered	% Degraded	Area	%Recovered	% Degraded
Acid	1247541	96.96	3.04	133985	96.55	3.45	73202	98.67	1.33
Alkali	1296461	96.90	3.10	137837	97.69	2.31	74792	98.15	1.85
Peroxide	1268193	94.08	5.92	135238	97.33	2.67	73699	93.59	6.41
Thermal	1288527	97.04	2.96	139240	97.28	2.72	75870	97.61	2.39
UV light	1296788	98.41	1.59	140195	98.08	1.92	76434	97.06	2.94
Neutral	1324073	99.71	0.29	142991	99.19	0.81	78240	99.35	0.65

Photolytic Stability Study

For the photolytic stability study, EMTC 2000µg/mL, DLTG 500µg/mL and TNFA 250 µg/mL solutions were exposed to UV-light by placing the solutions in UV cabinet for 1day or 200 Watt hours/m2 in photo stability chamber. The resulting solutions were combined in a 10 volumetric flask and made up to the mark with diluent to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8

and Fig. 10.

Acid Degradation Studies

To 1 mL of each stock solution of EMTC, DLTG and TNFA, 1 mL of 2N Hydrochloric acid was added in to a 10 mL volumetric flask and refluxed at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

Neutral Degradation Studies

To 1 mL of each stock solution of EMTC, DLTG and TNFA, 5 mL of water was added in to a 10 mL volumetric flask and kept for refluxing at 60°C for 1 h. Further, the resulting solution was made up to the mark to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

Oxidation

To 1 mL of each stock solution of EMTC, DLTG and TNFA, 1 mL of 20% hydrogen peroxide (H2O2) were added in to a 10 mL volumetric flask and kept at 60°C for 30 min. Further, the resulting solution was made up to the mark to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

Dry Heat Degradation Studies

To a 10 mL volumetric flask add 1mL each stock solution of EMTC, DLTG and TNFA and monitored at 105°C for 1 h in an hot air oven to perform the dry heat stability study. Further, the resulting solution was made up to the mark to get 200µg/mL, 50µg/mL and 25 µg/mL concentrations of EMTC, DLTG and TNFA respectively. From that 0.50 µL of solution was infused in to an UPLC system and the resultant chromatograms were analysed for the stability of analytes. The findings were represented in Table 8 and Fig. 10.

CONCLUSION

A simple, accurate and precise method was developed for the simultaneous estimation of the EMTC, DLTG and TNFA in Tablet dosage form by RP-UPLC technique. Retention times of EMTC, DLTG and TNFA were found to be 1.328 min,1.765 min and 2.135 min respectively. Chromatographic elution was processed through a Std BEH C18 (50 x 2.1 mm, 1.8µ) reverse phase column and the mobile phase composition of buffer 0.1% orthophosphoric acid (2.2 pH) and acetonitrile in the ratio of 60:40 was pumped through a column at a flow rate of 1.0 mL/min. Repeatability of the method was determined in the form of %RSD and findings were 0.9, 0.6 and 1.0 for EMTC, DLTG and TNFA respectively. LOD, LOQ values obtained from regression equations of EMTC, DLTG and TNFA were 0.34, 1.03 µg/mL, 0.36, 1.09 µg/mL and 0.25, 0.76 µg/mL respectively. Three analytes were subjected for acid, peroxide, photolytic, alkali, neutral and thermal degradation studies and the results shown that the percentage of degradation was found between 0.29% and 6.41%. Retention times and total run time of two drugs were decreased and the developed method was simple and economical. So, the developed method can be adopted in industries as a regular quality control test for the quantification of EMTC, DLTG and TNFA.

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