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A novel validated RP-UPLC for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, Cobicistat and Elvitegravir in tablet dosage form

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ABSTRACT

The present study provides us a single analytical tool for the determination of elvitegravir, cobicistat, emtricitabine and tenofovir disoproxil fumarate in dosage forms. This method helps us to separate and detect the four active pharmaceutical ingredients in a single run. The objective is to develop and validate a simple Ultraphase liquid chromatography method for the simultaneous determination of emtricitabine, tenofovir disoproxil fumarate, elvitegravir and cobicistat in dosage tablets. For this method, an Agilent 1200, high performance liquid chromatography system, Acquity Ethylene Bridged Hybrid technology (BEH) C18, 1.7µ. 100 x 2.1 mm column was used. The gradient program was adjusted at 0.3 ml/min flow rate and 10 µl injection volumes were maintained. Detection wavelength of 260 nnm is found to specific and could able to provide precise area counts for each drug. Calibration curves depicting a proper linearity response from concentration versus area observed for each drug with correlation coefficients of greater than 0.99. The eluted compounds were monitored at 240 nm. The column oven temperature was maintained at 30°. The developed chromatographic method was validated for selectivity, linearity, precision, accuracy, sensitivity, robustness, and system suitability.

Keywords: *performance, method, specific*

INTRODUCTION

Elvitegravir (EL) /Cobicistat(CO)/ Emtricitabine(EM)/ Tenofovir Disoproxil fumarate (TDF) tablets are available in FDC drug under the brand name STRIBILD. In other names, it is also called as QUAD. STRIBILD is approved by United States Food and Drug administration (USFDA) for treatment in adults for HIV infection. Each tablet contains 150 mg of EL, 150 mg of CO, 200 mg of EM and 300 mg of TDF(equivalent to 245 mg of Tenofovir Disoproxil) respectively. EL is a HIV medicine known an integrase inhibitor(1-3). Chemical name is "6-(3-Chloro-2- fluorobenzyl)-1-[(2S)-1-hydroxy-3methylbutan-2-yl]-7-methoxy-4-oxo-1,4- dihydroquinoline-3-carboxylic acid" with a molecular weight of 447.9 g.mol-1 and molecular formula of C23H23ClFNO5. CO is a pharmacokinetic enhancer, which would be useful to increase

the effectiveness of EL. CO has a chemical name of "1,3- thiazol-5-ylmethyl [(2R,5R)-5-{[(2S)-2- [(methyl{[2-(propan-2-yl)-1,3-thiazol-4- yl]methyl} carbamoyl)amino]-4-(morpholin-4-yl) butanoyl] amino}-1,6-diphenylhexan-2-yl]carbamate" with a molecular weight of 776.0 g.mol-1 with an molecular formula of C40H53N7O5S2(4-6).

EM is defined as a Nucleoside Reverse Transcriptase Inhibitor. The chemical classification of EM is Nucleoside Analog. EM forms "Emtricitabine 5'- triphosphate" within the cell by phosphorylation. The action of the metabolite is to inhibits the activity of HIV reverse transcriptase both by competing with the natural substrate "deoxycytidine 5'-triphosphate" and by incorporation into viral DNA causing a termination of DNA chain elongation. EM has a chemical name of "5-fluoro-1- (2R,5S)-[2-(hydroxymethyl)-1, 3- oxathiolan-5-yl]cytosine". It is a thio analog of cytidine with (-) enantiomer with a molecular weight of 247.24 g.mol-1 and molecular formula of C8H10FN3O3S. TDF is a pro-drug and exists as fumaric acid salt form of tenofovir. The chemical category of TDF is a nucleoside reverse transcriptase inhibitor analog of adenosine. It is mainly prescribed to treat not only for HIV and also for hepatitis B virus for chronic conditions in adults in combination with other antiviral therapeutic agents. TDF has a chemical name of "9-[(R)- 2[[bis[[(isopropoxy carbonyl)oxy]methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1)" with a molecular weight of 635.52 g.mol-1 and a molecular formula of C19H30N5O10P • C4H4O4 (Olin Jacqueline et.al., 2012;Perry et.al., 2014;Arribas et.al., 2014) .

Survey of different research articles, journals and publications indicate that procedures are present for quantification of EL, CO, EM and TDF in FDC products by UV Spectrophotometric, HPLC and UPLC. Literature is also cited for estimation of some of the components along with other drugs. Publications are also cited for impurity profiling for some of the components in the FDC product. From the above literature review it is evident that publication for quantification of this FDC product by UPLC for estimation of the constituents in dissolution method is not reported. Hence short and specific method is developed and validated for estimation of EL/CO/EM/TDF in FDC product (QUAD) by using dissolution conditions as proposed in Office of Generic Drugs (OGD) recommended medium i.e. 0.01 N HCl with 2% Polysorbate 80 . The summary of literature review of EL/CO/EM/TDF in FDC tablet is summarized below: (Refer Table 3.1)

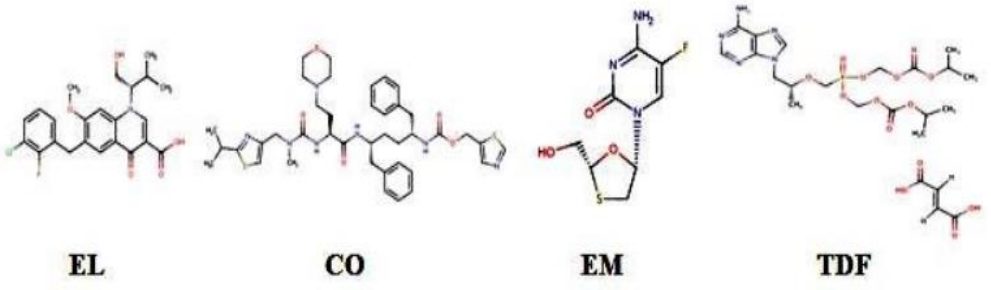
TABLE 3.1: Summary of literature review for EL/CO/EM/TDF Tablets

Sr.no	Author Name	Technique	Estimation
1	Harini et al., 2018	UV spectrophotometric	Simultaneous determination of EL/CO/EM/TDF -Assay method
2	Nagasarapu et al., 2012 Babu et al., 2014 Raghuram et al., 2014 Purnachandra et al., 2014 Raveendra et al., 2014 Khaleel et al., 2015 Chinnalalaiah et al., 2016 Mallikarjuna et al., 2016 Gummaluri et al., 2016	HPLC	Simultaneous estimation of EL/CO/EM/TDF -Assay method
3	Revathi et al., 2016 Uttam et al., 2016	UPLC method	Simultaneous estimation of EL/CO/EM/TDF -Assay method

Since all drugs are having different polarity, it is difficult to fix a common chromatographic method with short run time. While performing dissolution profiles during drug product development, it is very difficult to conclude the results if it runs for longer run time. Hence to avoid such practical problems, method was targeted to develop using simple volatile buffer which is compatible with low micron ID columns with RP- UPLC. The advantage of using volatile buffers is to increase the longevity of column's life when compared against organic buffers.

Validated test procedure is specific with respective to dissolution medium and placebo. Hence developed procedure can be claimed as stability demonstrating method and meets the ICH requirement parameters. Method validation was performed and found to be suitable for quantification of dissolution profiles required for EL, CO, EM and TDF in FDC product by using dissolution conditions as proposed in Office of Generic Drugs (OGD) recommended media for QUAD(6-9). 0.01 N HCl with 2% Polysorbate 80 is used as Dissolution medium, USP Apparatus type II (Paddle) with a stirring speed of 100 rpm, proposed dissolution volume is 1000 mL. The specified time points are 5, 10, 15, 20 and 30 minutes respectively. Chemical structures of EL, CO, EM and TDF have been illustrated in Figure 3.a.

Figure 3.a: Chemical structures of EL, CO, EM and TDF



3.1MATERIALS AND METHODS

3.1.1 Instrumentation

Waters-Acquity UPLC of waters make is used which contains binary mode gradient pump system, Auto sampler, temperature controlled column oven compartment and PDA detector for detection. Empower 2 software was used as interphase. Dissolution profiling was performed using Distek dissolution Apparatus type II system. Acquity Ethylene Bridged Hybrid technology (BEH) C18, 1.7µ. 100 x 2.1 mm column was used.

3.1.2 Chemicals

EL, CO, EM and TDF standardized pure substances, Stribild tablets from Gilead Sciences, Inc. were taken from Aurobindo Pharma limited. Ultra-pure Acetonitrile, Hydrochloric acid of GR grade and Polysorbate 80 of GR grade were taken from Merck chemicals. Ultrapure water has taken from Evoqua water purifier.

3.1.3 Development along with optimization of UPLC technique

The intention of the current paper is to reproduce, precise and accurate results for Dissolution profiling using shortest run time in EL, CO, EM and TDF tablets. QUAD is not official or cited in any compendial monographs. There is no RP-UPLC method being published so far for dissolution profiling test. EL, CO, EM and TDF are having different polarities. The pKa values observed for EL is about 6.6, for CO is about 6.4, for EM is about 2.65 and for TDF is about 3.75 respectively. The amine groups in the structural moieties of EM and TDF may tends to pose peak tailing due to silanol effect. To avoid this it is preferred to choose acidic Elution phase for development activity.

The OGD recommended dissolution medium contains 0.01 N HCl with 2% w/w Polysorbate 80(8-9). Hence care must be taken during optimizing chromatographic conditions. Due to viscous nature of the medium, there could be a probable chance to accumulate back pressure after repeated number of injections, which may reduce the life of the column. Especially in UPLC applications, this practical problem can be addressed in two ways i.e., by keeping column oven temperature at higher side or usage of volatile buffers which do not give much column back pressure. By considering these issues, method parameters were optimized accordingly. Perchloric acid is strongly acidic and volatile in nature and also acts as small ion pair reagent. It completely dissociates in water and provides true ion exchange selectivity when interacted with different drugs especially present in FDC products. Hence for Elution phase preparation purpose 0.1% perchloric acid was selected and considered as Elution Phase~A along with acetonitrile was chosen as Elution Phase~B. Since drugs are having different polarity, to get shorter run time method it was recommended to prefer step mode gradient elution by keeping moderate flow rate at 0.3 mL per minute. The column thermostat was maintained at 40°C to have low back pressure from column.

Ethylene bridged hybrid (BEH) technology bonded phase present in Waters Acquity column work on hydrophilic interaction, thus produces a versatile robust separation between the compounds and also can operate at wider usable pH range(10-12). Since the buffer used in Elution phase preparation contains acidic pH, it was preferred to keep reverse phase column with C18 chemistry using BEH technology. For trial purpose, Waters Acquity UPLC (BEH) C18, 1.7µ. 100 x 2.1 mm column was opted and found appropriate for optimum separation between all drugs present in QUAD. The wavelength maxima observed for EL is about 259nm, for CO is about 240nm, for EM is about 288nm and for TDF is about 260nm (Spectral data has been mentioned) Figure 3.b. To quantity all drug components, 260 nm is optimized for detection as TDF response decreases with increase or decrease of its maximal wavelength. Using 2µL injection volume all drugs has shown reproducible area counts which are found to be suitable for drug profiling at 260 nm.

Upon taking several logical gradient trials using 0.1% perchloric acid along with acetonitrile as Elution phase A & B. Resolution between the components is found to be more than 3.0 in the optimized chromatographic conditions. Flow rate was finalized at 0.3mL per minute. The column oven is controlled at 45°C in order to keep column backpressure under control in the optimized parameters. In all robustness conditions, resolution is seen more than 2.5 between every component and tailing factor is observed to be less than 1.2 for all the constituents. This indicates in optimized chromatographic conditions, quantification of drugs shall not be altered for minor changes that are likely to occur during continuous run of the system.

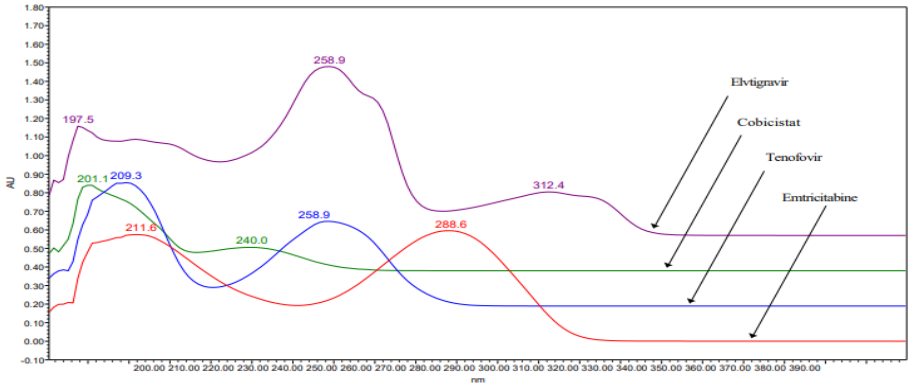


FIGURE 3.B: Spectral characteristics of EL, CO, EM and TDF

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3.1.4 Method optimized chromatographic conditions

The finalized chromatographic conditions are given in (Table 3.2). The Typical retention times observed for EM, TDF, CO and EL in the finalized chromatographic conditions are about 0.89, 1.42, 2.01and 2.77 minutes respectively.

TABLE 3.2: Finalized chromatographic conditions

Column	Waters Acquity UPLC (BEH) C18, 1.7µ. 100 x 2.1 mm.			
Detection	260 nm (PDA Detector).			
Column temperature	45°C.			
Inj. volume	2 µL.			
Elution phase~A	1mL of perchloric acid in 1000 mL of water.			
Elution phase~B	Degassed acetonitrile			
Diluent	10mLof acetonitrile followed by dissolution medium for preparation of Standard and sample preparation is performed in dissolution medium only.			
Step gradient programme	Time (minutes)	Flow (mL)	% Elution Phase~A	% Elution Phase~B
	0.0	0.3	60	40
	1.5	0.3	30	70
	3.2	0.3	30	70
	3.3	0.3	60	40
	4.0	0.3	60	40

3.1.5 Preparation of Solutions

3.1.5.1 Standard solution Preparation

Standard stock solution of EL, CO, EM and TDF were prepared at 0.9mg/mL, 0.9mg/mL, 1.2mg/mL and 1.2mg/mL respectively was prepared by dissolving in 10mL of acetonitrile and later diluted with dissolution medium. This stock solution was further diluted to obtain a concentration of 36µg/mL, 48µg/mL, 36µg/mL and 72µg/mL respectively using dissolution medium.

3.1.5.2 Dissolution Test Conditions

The dissolution profiling test was conducted for QUAD tablets as per OGD recommended dissolution medium of 2.0% polysorbate 80 in 0.01N HCl, using USP type II apparatus (Paddle) with 100 rpm stirring speed. Dissolution medium volume is 1000 mL which was maintained at 37°C (± 0.5°C) in dissolution vessels. Samples of about 10 mL were withdrawn from the dissolution bowl at specified time points of 5, 10, 15, 20 and 30 minutes respectively. After each sampling, about 10 mL of dissolution medium which is maintained at 37°C is replaced into each dissolution vessel. Sample solutions are filtered using syringe filters.

3.2 Analytical Method Validation

Stribild tablets are available in FDC with 150mg of EL, 150mg of CO, 200mg of EM and 300mg of TDF(14-16). The same label claim tablets were considered for method validation purpose. Validations parameters covered for System suitability evaluation, Specificity, Precision, intermediate precision, Linearity, Accuracy/Recovery, solutions stability, suitability of Filter papers and Robustness parameters as per compendia recommendation.

3.2.1 System suitability evaluation

Standard solution was prepared and injected for five replicate injections and observed for peak area of EL, CO, EM and TDF. Theoretical plate count, tailing factor, Resolution and

%RSD were evaluated. Suitability of chromatographic system Values observed are represented in Table 3.3

TABLE 3.3: Suitability of chromatographic system Values

Component	Plate count	Tailing	Resolution	%RSD
EM	362	1.14	-	0.50
TDF	2081	1.06	3.43	0.58
CO	4201	0.94	4.73	1.33
EL	7290	0.98	5.87	0.62

3.2.2 Specificity

Equal proportions of excipients are mixed as per QUAD formula. This placebo powder which is equivalent to individual tablet weight is transferred to dissolution vessel which contains dissolution medium. Rotation of the paddle is maintained at 100 rpm for 60 minutes. Placebo solution is withdrawn from dissolution vessel and filtered through 0.22µ PVDF filter paper and analyzed in UPLC system.

3.2.3 Precision

STRIBILD tablets were tested for precision of the method for intra and inter day precision for six individual preparations. All test samples were analyzed after subjecting it in dissolution vessels as per proposed time intervals. Measured % dissolution at every time interval and % RSD is determined for same values at every time point.

3.2.4 Linearity

Linearity study was assessed by preparing the test solutions ranging from 10 % - 120 % level using concentrated standard stock solutions for each drug. Linearity curves were plotted using concentration (µg/mL) against area of the peak for each component. Method of least squares was used to calculate the regression line.

3.2.5 Accuracy

Known amounts of EL, CO, EM and TDF reference substances were transferred to dissolution bowls at 10%, 80%, 100% and 120% levels along with tablets placebo. Dissolution was run for the samples as per OGD recommended dissolution test conditions. Triplicate preparations are made at each level.

3.2.6 Solution Stability

To establish solution stability, standard and sample solutions were periodically injected at different time intervals. Values at different time points were extrapolated from initial freshly injected solutions of standard and sample.

3.2.7 Filter evaluation

To demonstrate the filter paper interference, standard along with sample, solutions were filtered using 0.45µ PVDF, Nylon membrane filters by initially discarding 2-3 mL of aliquots from the filters. The filters were presaturated with dissolution medium prior to filtration. Results are compared against centrifuged sample areas.

3.2.8 Robustness

Robustness study was assessed by making deliberate changes in the optimized chromatographic conditions and impact was noted for USP plate count, Tailing factor and resolution between each drug. Accordingly conditions were modified for flow rate of 0.3 mL (± 10%), wavelength of 260 nm (± 5nm), temperature of 45°C (± 5°C) and Organic ratio in gradient elution (±2% absolute). For each robustness experiment, one parameter was modified and remaining chromatographic conditions were kept as such.

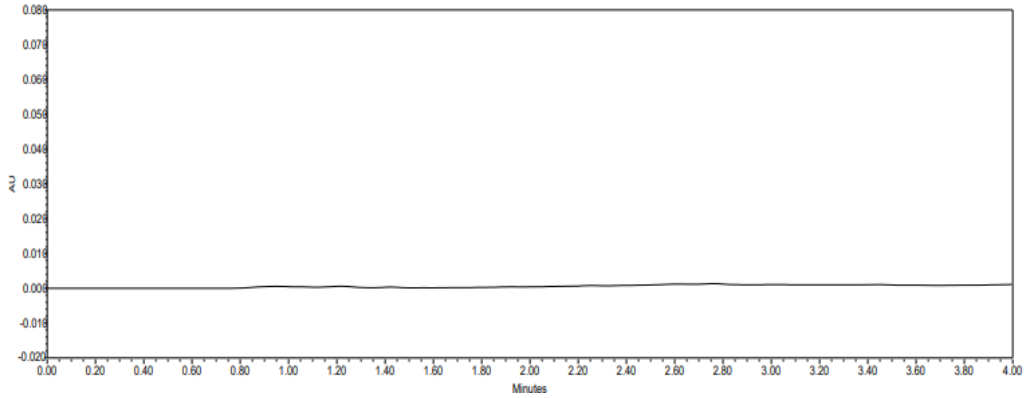
3.3 RESULTS AND DISCUSSION

3.3.1 Specificity

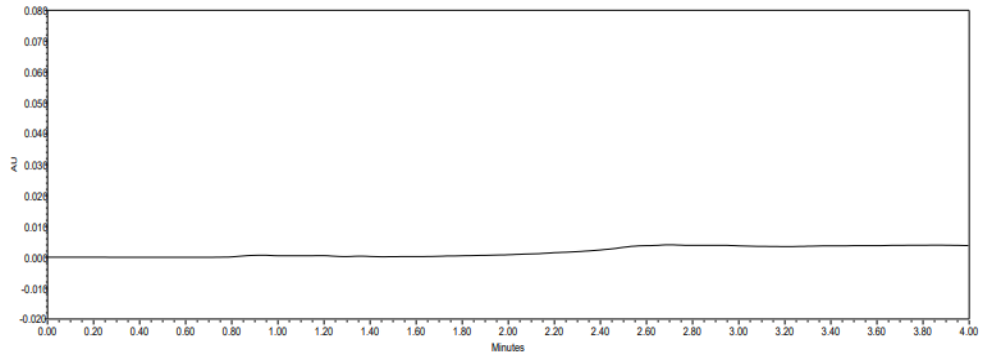
Placebo chromatograms were assed in RP UPLC method to check interference in chromatographic data. From the placebo chromatograms it is evident to see no interference was observed from placebo mixture being used for tablet fabrication at the retention times of EL, CO, EM and TDF. Hence the developed UPLC method is found specific to quantify the drugs of EL, CO, EM and TDF in pharmaceutical formulation using standard reference solution. For chromatograms refer Figure 3.c to 3.g.

Figure 3.c to 3.g: Chromatogram of Diluent, Placebo, Standard chromatogram, Sample chromatogram and Individual chromatograms of EM, TDF, CO and EL.

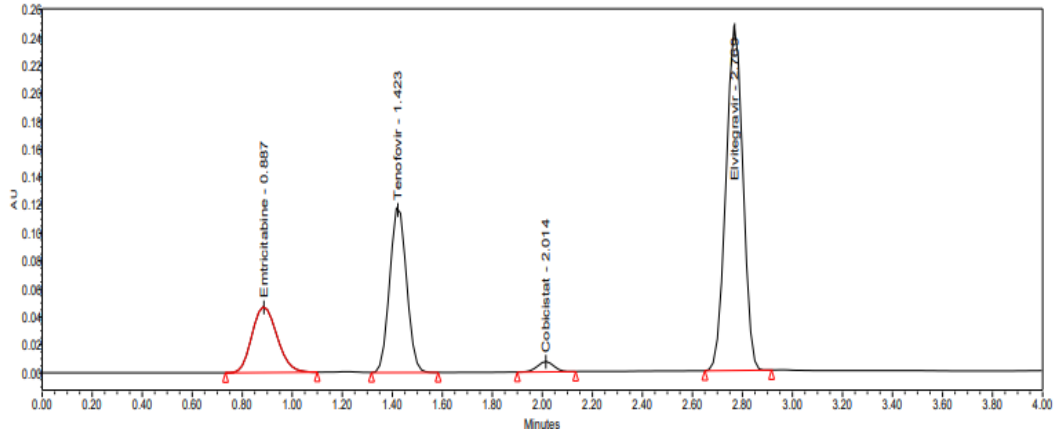
3.c Diluent chromatogram



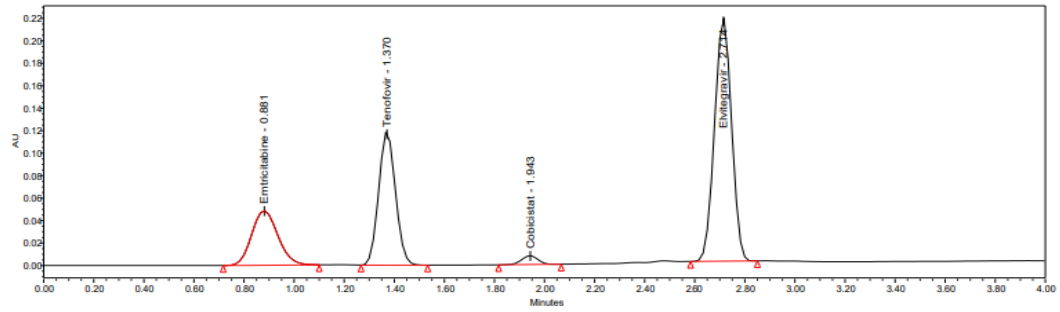
3.d Placebo chromatogram



3.e Standard Chromatogram

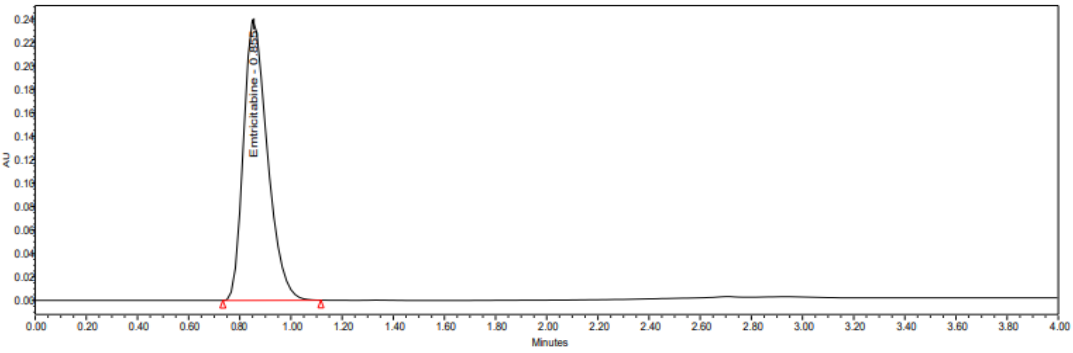


3.f Sample Chromatogram (Stribild tablets - 150mg of EL/ 150mg of CO/ 200mg of EM/ 300mg of TDF)

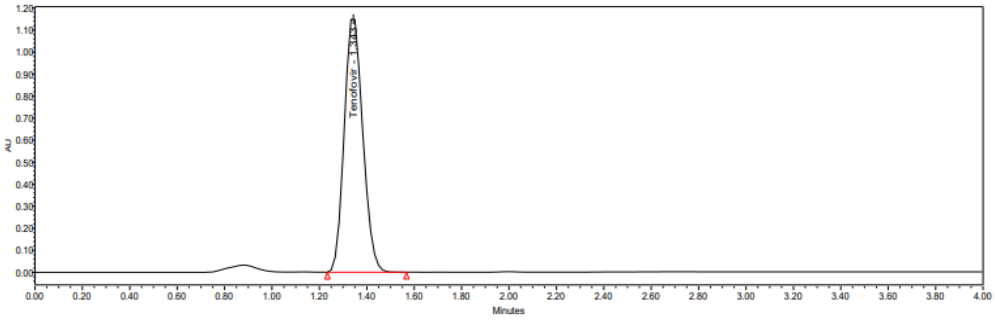


3.g Individual chromatograms of EM, TDF,CO and EL

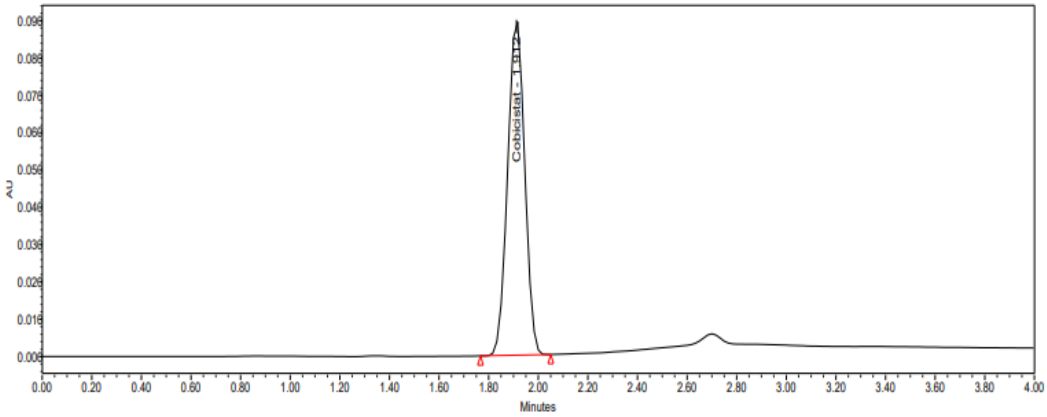
Emtricitabine (EM) Chromatogram



Tenofovir Disoproxil (TDF) Chromatogram



Cobicistat (CO) Chromatogram



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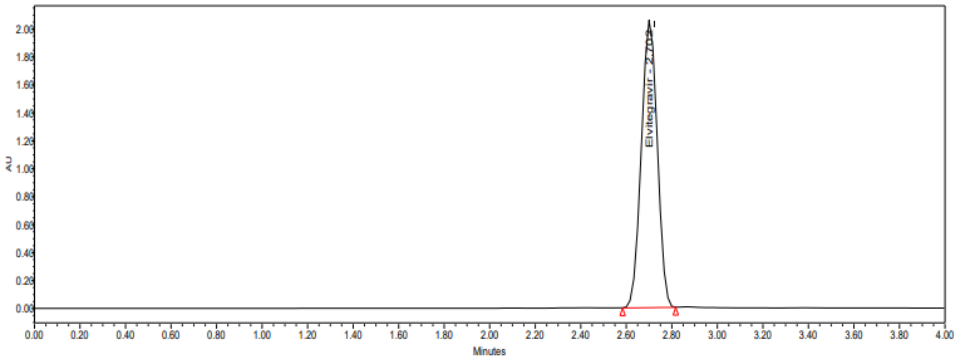
Elvitegravir (EL) Chromatogram



3.3.2 Precision

The results obtained from precision along with intermediate precision shows that the percentage RSD did not exceed 5% especially after initial release at 5 minutes. This demonstrates the precision of the method Table 3.4 and Table 3.5.

TABLE 3.4: Method precision results

For EL									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	77	72	74	77	75	74	75	2.59
2	10	85	91	84	85	91	84	87	3.89
3	15	93	90	91	93	91	90	91	1.50
4	20	96	95	93	96	95	93	95	1.44
5	30	100	100	98	100	101	98	100	1.22

For CO									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	95	80	86	94	86	82	87	7.06
2	10	96	94	94	96	98	95	96	1.58
3	15	96	99	99	99	97	95	98	1.80
4	20	100	99	96	102	99	99	99	1.96
5	30	101	98	98	102	98	101	100	1.86

For EM									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	97	91	94	97	94	94	95	2.38
2	10	98	98	101	99	98	100	99	1.28
3	15	99	98	101	100	98	100	99	1.22
4	20	100	99	101	101	99	102	100	1.21
5	30	101	99	101	101	99	101	100	1.03

For TDF									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	95	90	89	95	94	89	92	3.22
2	10	98	99	96	99	99	95	98	1.79
3	15	100	98	96	100	98	96	98	1.83
4	20	101	99	96	101	100	97	99	2.12
5	30	101	100	96	101	100	97	99	2.16

TABLE 3.5: Intermediate precision results

For EL									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	76	71	75	77	75	74	75	2.75
2	10	86	92	84	86	91	84	87	4.01
3	15	94	90	90	94	91	90	92	2.15
4	20	96	96	94	96	96	94	95	1.09
5	30	100	100	98	101	101	100	100	1.10

For CO									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	79	78	83	84	84	82	82	3.15
2	10	98	98	92	96	95	91	95	3.12
3	15	96	94	96	101	94	96	96	2.67
4	20	103	97	98	100	101	100	100	2.14
5	30	99	99	97	98	100	100	99	1.18

For EM									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	95	89	93	95	92	92	93	2.42
2	10	97	97	99	97	96	99	98	1.25
3	15	98	96	99	98	97	100	98	1.44
4	20	99	98	101	100	98	101	100	1.38
5	30	100	98	101	100	98	101	100	1.37

For TDF									
Sr. No	Time (min)	% Release						Average % Release	% RSD
		1	2	3	4	5	6		
1	5	95	90	89	95	93	88	92	3.34
2	10	99	99	96	99	98	96	98	1.50
3	15	100	99	96	100	99	97	99	1.66
4	20	101	101	97	101	100	97	100	1.97
5	30	101	100	98	102	100	98	100	1.60

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3.3.3 Linearity

Linearity curves were assessed for EL, CO, EM and TDF by checking the concentration versus area observed that ranges from 10%-120%. From the calibration curvesextrapolated, the values for slope, coefficient of correlation and y-intercept for each drug were assessed. The obtained data shows a linear relationship to all drug components with a satisfactory coefficient of correlation more than 0.995 on tested concentration range. Linearity graphs for EL, CO, EM and TDF have been depicted in Figure 3.h. Statistical summary of Linearity values are given in Table 3.6.

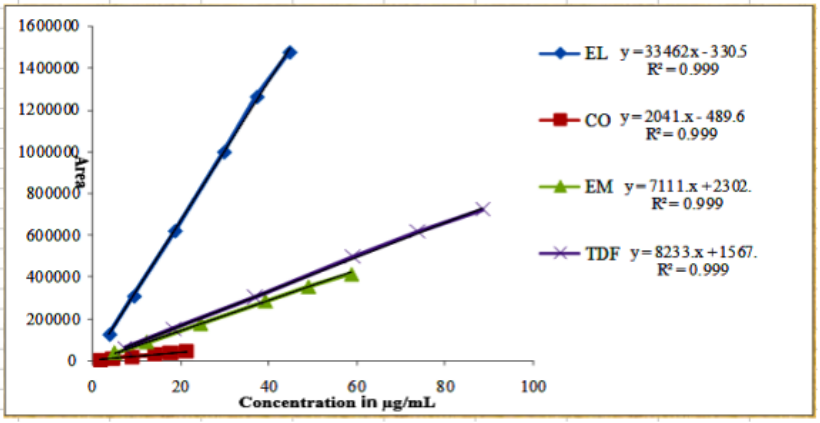

FIGURE 3.H: Linearity curve of EL, CO, EM and TDF

TABLE 3.6: Statistical summary of Linearity data

Component	Regression line equation	Linear range(r)	y- intercept	Coefficient of correlation
EL	y = 33462x-331	3.713-44.559	-331	0.99977
CO	y = 2041.x-490	1.769-21.233	-490	0.99978
EM	y = 7111.x + 2303	4.895-58.742	2303	0.99968
TDF	y = 8233.x + 1567	7.367-88.399	1567	0.99968

3.3.4 Accuracy

ICH guidelines or USP general chapter requirement for validation of compendial procedures <1225>, the recovery of dissolution results shall be in the range between 95– 105%. The % recovery was found within acceptable range in all specified ranges and found acceptable Table3.7.i and Table 3.7.ii.

TABLE 3.7.I: Accuracy results for EL and CO

Spike level	EL					CO				
	Spiked (mg/mL)	Obtained mg/mL)	% Recovery	Avg.	% RSD	Spiked (mg/mL)	Obtained mg/mL)	% Recovery	Avg.	% RSD
10	3.74	3.82	102.1	101.8	0.9	3.17	3.31	104.4	100.2	3.7
	3.74	3.77	100.8			3.17	3.14	99.1		
	3.74	3.83	102.4			3.17	3.08	97.2		
	30.04	30.46	101.4			30.14	28.73	95.3		

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50	30.04	30.71	102.2	101.8	0.4	30.14	29.64	98.3	97.1	1.6
	30.04	30.55	101.7			30.14	29.46	97.7		
100	37.55	38.8	103.3	103.9	0.5	37.67	37.33	99.1	99.2	1.5
	37.55	39.12	104.2			37.67	37.94	100.7		
	37.55	39.09	104.1			37.67	36.83	97.8		
120	45.05	45.8	101.7	101.5	0.4	45.21	43.74	96.7	96.7	1.0
	45.05	45.82	101.7			45.21	44.15	97.7		
	45.05	45.49	101.0			45.21	43.31	95.8		

mg/mL = milli gram/milli liter, % RSD = Percentage Related Standard Deviation

TABLE 3.7.II: Accuracy results for EM and TDF

Spike level	EM					TDF				
	Spiked (mg/mL)	Obtained (mg/mL)	% Recovery	Avg.	% RSD	Spiked (mg/mL)	Obtained mg/mL)	% Recovery	Avg.	% RSD
10	5.43	5.39	99.3	98.5	1.5	7.48	7.75	103.6	102.7	1.1
	5.43	5.25	96.7			7.48	7.59	101.5		
	5.43	5.4	99.4			7.48	7.7	102.9		
50	40.71	41.46	101.8	100.8	0.8	60.25	62	102.9	102.3	0.5
	40.71	40.89	100.4			60.25	61.49	102.1		
	40.71	40.84	100.3			60.25	61.41	101.9		
100	50.88	51.64	101.5	102.1	0.7	75.31	77.78	103.3	103.7	0.6
	50.88	51.86	101.9			75.31	77.88	103.4		
	50.88	52.37	102.9			75.31	78.63	104.4		
120	61.06	61	99.9	99.5	0.6	90.38	91.66	101.4	101.1	0.5
	61.06	60.94	99.8			90.38	91.63	101.4		
	61.06	60.41	98.9			90.38	90.8	100.5		

mg/mL = milli gram/milli liter, % RSD = Percentage Related Standard Deviation

3.3.5 Solution Stability

No significant changes were observed in the area of EL, CO, EM and TDF when both standard and samples were analyzed at room temperature of 25°C. Both standard and samples solutions of each drug is found to be stable up to 24 hours. % degradation is found to be less than 2 for all drugs. Since there is no issue observed at room temperature, solution stability at cooler temperature of 2-8°C was not established.

3.3.6 Filter evaluation

The results obtained for filter paper evaluation is clearly indicating that there is no drug absorption is seen for any compound when analyzed for both standard and sample solutions. The absolute difference between the area of unfiltered standard versus filtered standard solutions and centrifuged sample versus filtered samples were within 98–102%. This indicates that the absence of EL, CO, EM and TDF absorption by the filters used for study i.e. PVDF and Nylon membrane filters.

3.3.7 Robustness

Results from robustness study shows retention times for EL, CO, EM and TDF are not altering much. Also there is no considerable change being observed for system suitability parameters such as USP plate count, tailing factor and resolution between each drug. The critical attribute of USP resolution in all parameters is found to be more than 2.6 between each drug, which shows the optimized chromatographic parameters are robust in nature over tested conditions. For

Robustness study chromatogram refer Figure 3.i).

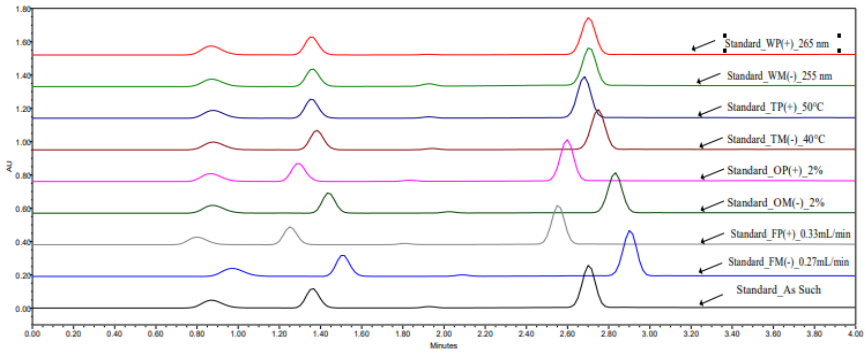

FIGURE 3.I: Robustness study chromatogram

3.4 Comparison Of Existing Methods And Present Method

For comparison of existing methods and present method refer Table 3.8 Table 3.8 Comparison of existing methods and present method

Reference	**Khaleel et al.**	**Revathi et al.**	**Present-Work**
Identification	Estimation of EL/CO/EM/TDF in Assay method	Estimation of EL/CO/EM/TDF in Assay method	Estimation of EL/CO/EM/TDF in Dissolution method
System	HPLC	UPLC	UPLC
Mobile Phase	0.1% OPA Buffer and Acetonitrile taken in the ratio 55:45%v/v.	0.1% TFA in acetonitrile and 0.1% TFA in water	1.0 mL/L of perchloric acid in water and Acetonitrile
Pump mode	Isocratic	Gradient	Gradient
Column	Kromasil C18, 250×4.6mm, 5µm	ACE C18, 50 x 3mm, 5µ	Waters Acquity UPLC BEH C18, 100 x 2.1mm, 1.7□
Flow rate	1.0 mL.min-1	0.4 mL.min-1	0.3 mL.min-1
Detection	240 nm	240 nm	260 nm
Column Temperature	30°C	30°C	45°C
Run time	10 minutes	8 minutes	4 minutes
Pros and cons	Quantification of FDC components in assay method.	Quantification of FDC components in assay method.	Quantification of FDC components in dissolution method.

3.5 Summary Along With Conclusions

Publications are available for quantification of EL/CO/EM/TDF FDC product by assay method. Research papers are not available for quantification of EL/CO/EM/TDF components in the FDC product by dissolution method. Method was validated as per ICH general requirement for Dissolution test procedure. The result obtained from specificity experiment is showing that there is no placebo interference

observed at the retention times of EL, CO, EM and TDF. Detection wavelength of 260 nnm is found to specific and could able to provide precise area counts for each drug. Calibration curves depicting a proper linearity response from concentration versus area observed for each drug with correlation coefficients of greater than 0.99. Recovery/Accuracy results confirming that satisfactory drug recovery is seen on proposed test concentrations for each drug.

Dissolution is generally performed on six units on about minimum of 6-8 time points at R&D and quality control department. During initial development of formulation, trails are taken repeatedly to match the drug release inline with innovator samples for generic companines. Hence the analytical department needs to perform dissolutions on initial and stability samples charged in various conditions. The developed method is more appropriate for simultaneous estimation of the four components in a short run time of 4 minutes. Hence the projected method could be useful to pharmaceutical analytical laboratories to release the profiling results at faster rate.

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