Development and Validation of Stability-Indicating HPLC-DAD Method for Simultaneous Determination of Emtricitabine, Elvetegravir, Cobicistat and Tenofovir in their Tablet Dosage Forms

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ABSTRACT

A Simple, accurate, specific and rugged reverse phase liquid chromatographic method was developed for the simultaneous estimation of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir in bulk and tablet dosage form. A reverse phase gradient program has been developed to separate the all four active ingredients. The mobile phase consisting of 0.05M Phosphate buffer pH 3.0 (adjusted with dilute phosphoric acid) and Acetonitrile in the ratio 95:5 from 0 min to 4 minutes, further increased the Acetonitrile ratio from 5 to 50 from 4 min to 10 minutes, on a reverse phase C_{18} column (250x4.6mm, 5 µ) with a flow rate of 1.0 ml/min, monitored at 240nm. The mean retention times of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir were found to be 1.5, 5.4, 6.6 and 7.5 min respectively. The proposed method was validated in terms of Linearity, Range, Accuracy, Precision, Specificity, Robustness and Ruggedness and the method was successfully applied for the estimation of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir in combined tablet dosage form.

Key words: Emtricitabine, Elvetigravir, Cobicistat, Tenofovir, HPLC-DAD, Tablet.

INTRODUCTION

Emtricitabine (EMCB) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine (Figure 1). EMCB is the (-) enantiomer of thio analog of cytidine which differs from other cytidine analogs, in that it has a fluorine in 5th position. EMCB is an antiviral agent used for the prevention of perinatal HIV-1 reverse transcriptase.[1] It is also active against Hepatitis B virus.[2, 3]

Elvitegravir (ELVT) is a drug used for the treatment of HIV infection. It acts as an integrase inhibitor. Chemical name of Elvitegravir is 6-[(3- Chloro-2- fluorophenyl) methyl]-1-[(2*S*)-1- hydroxy-3- methylbutan-2 -yl]-7-methoxy- 4-oxoquinoline-3- carboxylic acid (Figure 1). Elvitegravir acts as an integrase inhibitor. According to the results of the phase II clinical trial, patients taking once-daily Elvitegravir boosted by ritonavir had greater reductions in viral load after 24 weeks compared to individuals randomized to receive a ritonavir-boosted protease inhibitor.[4]

Cobicistat (COB) is a licensed drug for use in the treatment of infection with the human immunodeficiency virus (HIV). Cobicistat acts as an HIV integrase inhibitor. Chemical name of Cobicistat is Thiazol-5-ylmethyl *N*-[1-benzyl-4-[[2-[[(2-isopropylthiazol-4-yl) methyl-methyl-carbamoyl] amino]-

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4-morpholinobutanoyl] amino]-5-phenyl-pentyl] carbamate (Figure 1). Cobicistat is the only other booster approved for use as a part of HAART, Cobicistat has no anti- HIV activity of its own. Cobicistat is a potent inhibitor of cytochrome P450 3A enzymes, including the important CYP3A4 subtype. It also inhibits intestinal transport proteins, increasing the overall absorption of several HIV medications, including atazanavir, darunavir and Tenofovir disoproxil fumarate alafenamide fumarate.[5-6]

Tenofovir disoproxil fumarate (TDF) belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs). Tenofovir disoproxil fumarate is a prodrug form of Tenofovir disoproxil fumarate. Chemical name of Tenofovir disoproxil fumarate is ({[(2*R*)-1-(6- amino-9*H*-purin-9-yl) propan-2-yl] oxy} methyl) phosphonic acid (Figure.1). Tenofovir disoproxil fumarate blocks reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections. Tenofovir disoproxil fumarate exhibits anti-HIV effects in humans when dosed by subcutaneous injection. Tenofovir disoproxil fumarate is indicated for the treatment of chronic hepatitis B in adults and pediatric patients 12 years of age and older.[7-9]

A survey of literature has revealed several analytical methods for the determination of Tenofovir and EMCB in combination with efavirenz in biological fluids and in pharmaceutical products. These include high-performance liquid chromatography (HPLC).[10-11] On the contrary, to the best of our knowledge, there is no method reporting the simultaneous determination of TDF, ELVT, COB, and EMCB in pharmaceutical formulation. In this paper, we report the very first reversed-phase-HPLC (RP-HPLC) method for the assay of EMCB, ELVT, TDF, and COB in fixed dosage form. The new method is capable of separating all four active ingredients present in the tablet. Validation of the current method will be performed according to the requirements of International Conference on Harmonization (ICH) for assay determination which include accuracy, precision, selectivity, linearity and range.

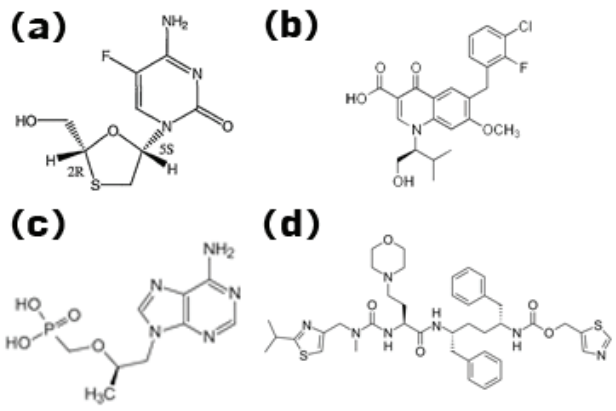

Figure 1: Chemical structures of a) Emtricitabine, b) Elvitegravir, c) Cobicistat , d) Tenofovir disoproxyl fumarate

Several HPLC methods are available in the literature for individual drugs and for a combination with other drugs for determination of TDF, FTC, and EFV, but no stability-indicting assay method (SIAM) has been reported.[12–27] Few RP-HPLC[28-29] methods are reported for estimation of FTC and TDF in pharmaceutical formulation. The literature survey revealed that there are a very few HPLC and spectroscopic methods available for the determination of Emtricitabine, Tenofovir disoproxil fumarate, Cobicistat, Elvitegravir in pure and combined dosage forms. The present study was aimed to develop a new HPLC method for simultaneous estimation of Emtricitabine, Tenofovir disoproxil fumarate, Cobicistat, Elvitegravir in combined pharmaceutical dosage form.

Experimental Materials

The Pharmaceutical grade working standards of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir were obtained as a gift from Richer Pharmaceuticals (Prasanthinagar, Hyderabad, India). Fixed dosage combination tablet containing 200 mg Emtricitabine, 150 mg Elvetigravir, 150 mg Cobicistat and 300 mg Tenofovir was purchased from local market Hyderabad, India. All the chemicals were HPLC grade purchased from SD Fine Chem., Mumbai. MilliQ water was used.

Chromatographic Conditions

Waters e 2695 series HPLC consisting pump, Auto sampler, Auto injector, VWD & photo diode array detector, thermostatic column compartment connected with Empower 2 software connected with a Hypersil BDS C_8 250x4.6mm, 5 µ, 100A.

Mobile phase

Accurately weighed 6.8 g of potassium dihydrogen orthophosphate in 1000ml of water, adjusted pH 3.0 with diluted phosphoric acid. Filtered the solution through 0.22 µ nylon filter and sonicated to degas it. The buffer was used as mobile phase preparation A, Acetonitrile used as mobile phase mobile preparation B, Emtricitabine, Elvetigravir, Cobicistat and Tenofovir were separated and eluted in a gradient program represented in Table 1. The flow rate of the mobile phase was maintained at 1.0ml/min. The column temperature was maintained at 30ºC and the detection was carried out at 240 nm with an injection volume of 20 µl.

Table 1: Gradient Table			
Time(mins)	**Mobile Phase A**	**Mobile Phase B**	**Mode**
0	95	5	Isocratic
4	95	5	Isocratic
10	50	50	Linear gradient
15	95	5	Linear gradient
20	95	5	Isocratic

Standard solution preparation

Weighed accurately working standards equivalent to 20 mg Emtricitabine, 15 mg Elvetigravir, 15 mg Cobcistat and 30 mg Tenofovir into 50 ml volumetric flask, added 30 ml of diluent and dissolved, further made the volume with the diluent. Further diluted 10 ml to 100 ml with mobile phase.

Sample preparation

Crushed to powder 20 tablets, weighed and transferred equivalent to 1 tablet powder into 500 mL volumetric flask added 300 mL of diluent, sonicated to dissolve for 10 minutes and diluted to volume with diluent. Further filtered the solution through 0.22 µ filter. Diluted 10 ml to 100 ml with diluent.

RESULTS AND DISCUSSION

Method development and optimization of chromatographic conditions

In order to achieve good separation between all the four components different buffer pH-conditions and different proportions of solvents like methanol, Acetonitrile and water tested binary and tertiary eluents. However, in 0.05 M phosphate buffer pH 3.0 adjusted pH with dilute phosphoric acid and Acetonitrile achieved good satisfactory results at a flow rate of 1.0 ml/minute measured at a détection of 240 nm. The chromatogram of optimized standard mixture chromatogram shown in Figure 2. The system suitability paramètres such as rétention time, assymetry, resolution and theoritical plates for optimized standard mixture chromatogram tabulated in Table 2.

Method Validation

System\ suitability

System suitability is an integral part of the method validation to evaluate the parameters like tailing factor, theoretical plates, resolution and %RSD for replicate injections. The results were within the limits and were presented in Table 2. Figure 2 shows the system suitability chromatogram.

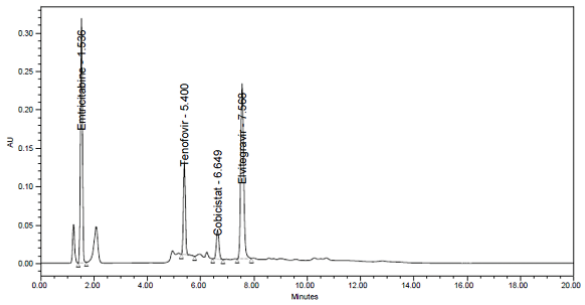

Figure 2. System suitability chromatogram

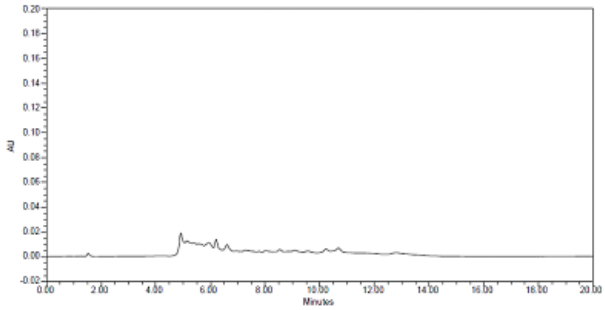

Figure 3: Blank chromatogram

Specificity

In the placebo chromatogram there were no peaks observed at the retention times of Emtricitabine, Elvetigravir, Cobicistat and Tenofavir, and also the degradation studies showed that there no interference with degradants and hence the method was specific. Figure 3 shows placebo chromatogram.

Accuracy

To determine the Accuracy of the proposed method, recovery studies were conducted; known amount of pure drug concentrations was spiked in placebo at three different levels, ie, 50%, 100% and 150% and was calculated. Accuracy was calculated as the percentage of recovery. The results were tabulated in Table 3.

Precision

The precision was evaluated at three levels, repeatability, reproducibility and intermediate precision each level of precision was investigated by six replicate injections of concentrations 40, 30, 30 and 60 mcg/ml Emtricitabine, Elvetigravir, Cobicistat and Tenofavir respectively. The result of precision was expressed as % of RSD and was tabulated in Table 4.

Linearity and range

The linearity was evaluated by measuring different concentrations (25% to 150%) of the standard solutions to Emtricitabine, Elvetigravir, Cobicistat and Tenofavir.

Table 2: System suitability results

Parameter	Results				Required limits
	Emtricitabine	Elvetigravir	Cobicistat	Tenofovir	
RSD of peak area	0.11	0.09	0.55	0.19	<2.0 for n≥6
RSD of retention time	0.13	0.07	0.05	0.03	<1.0 for n≥6
USP Tailing factor (T)	0.98	1.13	1.07	1.10	T < 2
USP Plate Count (N)	2985	23045	21851	22687	>2000
USP Resolution (R)	-	4.74	7.61	27.58	R > 2

Table 3: Accuracy data (Triplicate values at 50,100 &150 percent levels)

Parameter	Amount added(µg)	Amount Recovered(µg)	% of recovery	Mean % of Recovery
Emtricitabine				
50% level	20	19.992	99.96	99.54
100%level	40	39.888	99.72	
150%level	60	59.37	98.95	
Elvetigravir				
50% level	15	14.898	99.32	100.04
100%level	30	29.97	99.90	
150%level	45	45.41	100.91	
Cobicistat				
50% level	15	14.83	98.89	99.39
100% level	30	30.04	100.14	
150% level	45	44.61	99.14	
Tenofovir				
50% level	30	29.81	99.38	99.16
100% level	60	59.83	99.71	
150% level	90	88.54	98.38	

Table 4: Precision studies

Parameter	RESULTS			
	Emtricitabine	Elvitegravir	Cobicistat	Tenofovir
Repeatability				
Mean %RSD of Retention time	0.13	0.07	0.05	0.03
Mean %RSD of Peak Area	0.11	0.09	0.55	0.19
Mean % Assay	100.26	99.52	99.45	99.09
Reproducibility				
Mean %RSD of Retention time	0.12	0.06	0.15	0.34
Mean %RSD of Peak Area	0.25	0.65	0.06	0.21
Mean % Assay	100.63	99.52	99.21	99.21
Intermediate Precision				
Mean %RSD of Retention time	0.28	0.09	0.09	0.09
Mean %RSD of Peak Area	0.23	0.12	1.82	0.10
Mean % Assay	99.23	99.63	99.10	99.12

Table 5. Regression equation parameters				
Parameter	**Emtricitabine**	**Elvitegravir**	**Cobicistat**	**Tenofovir**
Linearity range(mcg/ml)	10 to 60	7.5 to 45	7.5 to 45	15 to 90
Correlation co-efficient	0.9992	0.9999	0.9999	0.9998
Slope	31567	39259	4807.9	7077.8
Y-intercept	361947	548607	113788	209511

Table 6: Assay Results			
Drug	**Labelled Amount (mg/tab)**	**Amount found (mg/tab)**	**% of Assay**
Emtricitabine	200	197.75	98.88
Elvitegravir	150	149.74	99.83
Cobicistat	300	150.6	100.38
Tenofovir	150	299.3	99.77

Table 7: Forced degradation and stability								
Condition	**Emtricitabine**	**%Rec**	**Elvitegravir**	**%Rec**	**Cobicistat**	**%Rec**	**Tenofovir**	**%Rec**
acid	1502999	94.09	1742470	99.12	248420	96.67	15271	2.39
base	1396666	87.43	1636263	94.96	246022	95.69	544608	85.22
perox	1566195	98.05	1796023	99.23	258272	100.45	593245	92.83
heat	1534048	96.03	1797641	99.32	260419	94.48	63946	10.06
UV	1494981	95.39	1728485	100.31	252313	98.14	595357	93.16

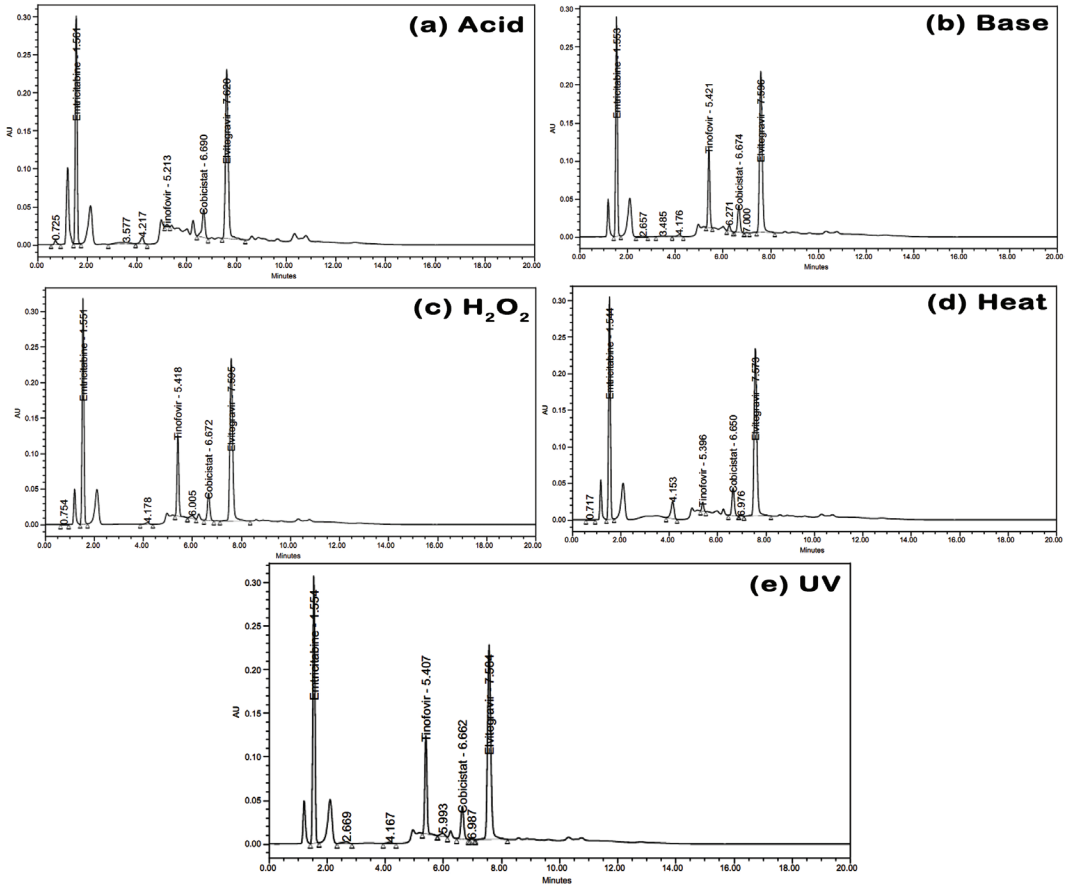

Figure 4: Degradation chromatograms of (a) Acid, (b) Base, (c) H_2O_2, (d) Heat and (e) UV

The calibration curve was constructed by plotting concentration of standard solutions against mean peak areas and the regression equation was computed. The summary of the parameters shown in Table 5.

Detection limit (DL) and quantitation limit (QL)

Estimation of DL and QL considered the acceptable signal-to-noise ratios 3:1 and 10:1 respectively. The limit of detection and quantitation to be determined 0.18 and 0.5455 µg/ml for Emtricitabine, 0.1239 and 0.3755 µg/ml for Elvetigravir, 0.9668 and 2.9298 µg/ml for Cobicistat and 0.5602 and 1.6975 µg/ml for Tenofovir respectively.

Robustness and ruggedness

The robustness of the method was unaffected when small, deliberate changes in the chromatographic conditions like, flow change, mobile phase composition, column temperature were performed at 100% test concentration.

The ruggedness of the proposed method studied under different columns, analyst, instrument, laboratories analysis of the same sample.

Standard solution stability

The stability of the standard solution was to test for an intervals 24 and 48 hours at room temperature. There were no significant changes observed in the system suitable parameters like theoretical plates, tailing factors, retention time and resolution. Hence the standard solution is stable up to 48 hours of room temperature.

Mobile phase stability

The stability of the mobile phase was observed for 24 and 48 hours at room temperature. There were no sinificant changes observed in peak areas, theoretical plates, tailing factors, retention time and resolution. Hence the mobile phase was stable up to 48 hours of room temperature.

Analysis of marketed sample

The proposed method was applied for the analysis of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir in tablet dosage forms, the results were found to be between 99 and 101%, the results were summarized in Table 6.

Forced degradation and stability-indicating tests

Stock solution

Weighed accurately working standards equivalent to 20 mg Emtricitabine, 15 mg Elvetigravir, 15 mg Cobcistat and 30 mg Tenofovir into 50 ml volumetric flask, added 30 ml of diluent and dissolved, further made the volume with the diluent. Results of Forced degradation was shown in Table 7. Figure 4 (a-e) shows the chromatograms of forced degradation studies.

Acidic degradation

10ml of 1N HCl added to 10 ml of stock solution and was kept at 80ºC for about 12 hours in water bath, cool made up the volume 100ml with mobile phase. Filtered the solution through 0.22 micron membrane filter.

Alkali degradation

10ml of 0.5N NaOH added to 10 ml of stock solution and was kept at 80ºC for about 48 hrs in water bath, cool made up the volume 100ml with mobile phase. Filtered the solution through 0.22 micron membrane filter.

Oxidative degradation

5ml of 3% H_2O_2 added to 10 mls of stock solution and was kept at 80ºC for about 24 hours in water bath, cool made up the volume 100ml with mobile phase. Filtered the solution through 0.22 micron membrane filter.

Thermal degradation

To 10 ml of stock solution and was kept at 70ºC for about 10 days, cool made up the volume 100ml with mobile phase. Filtered the solution through 0.22 micron membrane filter.

CONCLUSION

A simple, specific and reliable isocratic HPLC-DAD method was developed for the estimation of Emtricitabine, Elvetigravir, Cobicistat and Tenofovir in their pharmaceutical formulation. The four compounds were subjected to forced degradation applying several stress conditions. The proposed method was successfully separated all the compounds with degradants, estimate the active contents. The Proposed method is specific and stability-indicating power. Hence the developed method can be adapted to regular quality control analysis.

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