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A New Gradient Liquid Chromatographic Method for Simultaneous Estimation of Tenofovir Disoproxil Fumarate, Cobicistat, Emtricitabine and Elvitegravir in Bulk Drug and Tablet Dosage Form

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A new gradient reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir. The chromatography was achieved on Hypersil BDS, C18 column, (100 × 4.6 mm, 5 m) with a mobile phase composed of phosphate buffer (pH 3) and acetonitrile taken in gradient mode at a flow rate of 0.8 mL/min. Detection was performed at 245 nm by using PDA detector. The retention times for tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were 2.1, 4.3, 5.2 and 8.5 min, respectively. This method was validated for parameters like system suitability, linearity, precision, specificity, accuracy and robustness. This method was successfully applied for the estimation of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir in bulk drug and tablet dosage form.

Keywords: Tenofovir disoproxil fumarate, Cobicistat, Emtricitabine, Elvitegravir, Gradient elution.

INTRODUCTION

Tenofovir is a nucleotide analog of deoxyadenosine monophosphate, with activity against HIV-1, -2 and Hepatitis B virus (HBV). The chemical name of tenofovir disoproxil fumarate (TDF) is 9-[(R)-2- [[*bis*[[(isopropoxycarbonyl)oxy]-methoxy] phosphinyl]methoxy]propyl] adenine fumarate[1,2]. The chemical name of cobicistat (CBT) is 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-4yl)butanoyl]amino}-1,6-diphenylhexan-2-yl]carbamate. Cobicistat is a pharmacokinetic enhancer, is a effective mechanism-based inhibitor of cytochrome P450 3A4, an enzyme that metabolizes medicinal compounds in the body. Inhibition of CYP3A-mediated metabolism by cobicistat enhances the systemic exposure of CYP3A4 substrates, mainly drugs like elvitegravir, where bioavailability is decreased and half-life is reduced by CYP3A-dependent metabolism[3]. Emtricitabine (ETC) is a fluorinated derivative of lamivudine, an analog of deoxycitidine. The chemical name of emtricitabine is 5-fluoro-1-[(2R,5S)-2(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Emtricitabine, a synthetic nucleoside analog of cytidine, is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate. Emtricitabine 5'-triphosphate inhibits the activity of the HIV-1 reverse transcriptase by competing with the natural substrate deoxycytidine 5'-triphosphate and by being incorporated into nascent viral DNA which results in chain termination[4]. Elvitegravir (EVG), the second integrase inhibitor used in treatment-naïve and treatment-experienced HIV-1 infected adults[24]. The chemical name of elvitegravir is 6-(3-chloro-2-fluorobenzyl)-1-[(2S)-1hydroxy-3-methylbutan-2-yl]-7-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid[5]. The chemical structures of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir are shown in Fig. 1.

Elvitegravir/cobicistat/emtricitabine/tenofovir disoproxil fumarate (Stribild), manufactured by Gilead Sciences, Inc, is a combination antiretroviral agent approved by the FDA as a complete regimen for the treatment of HIV-1 infection in adults who are antiretroviral treatment-naïve[6-9]. Various UV, HPLC and LC/MS/MS assay methods were reported in the literature for the estimation of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir individually and in-combination with other drugs. These methods include; UV spectroscopy method[10-13], Ion pair HPLC method[14], HPLC method[15-18], HPTLC method[19-20] and LC/MS/MS[21-23]. As per literature, there is no official method for the simultaneous estimation of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir by RP-HPLC in tablet dosage form. Hence, we planned to develop a new method for simultaneous estimation and validation of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir in bulk drug and pharmaceutical dosage form.

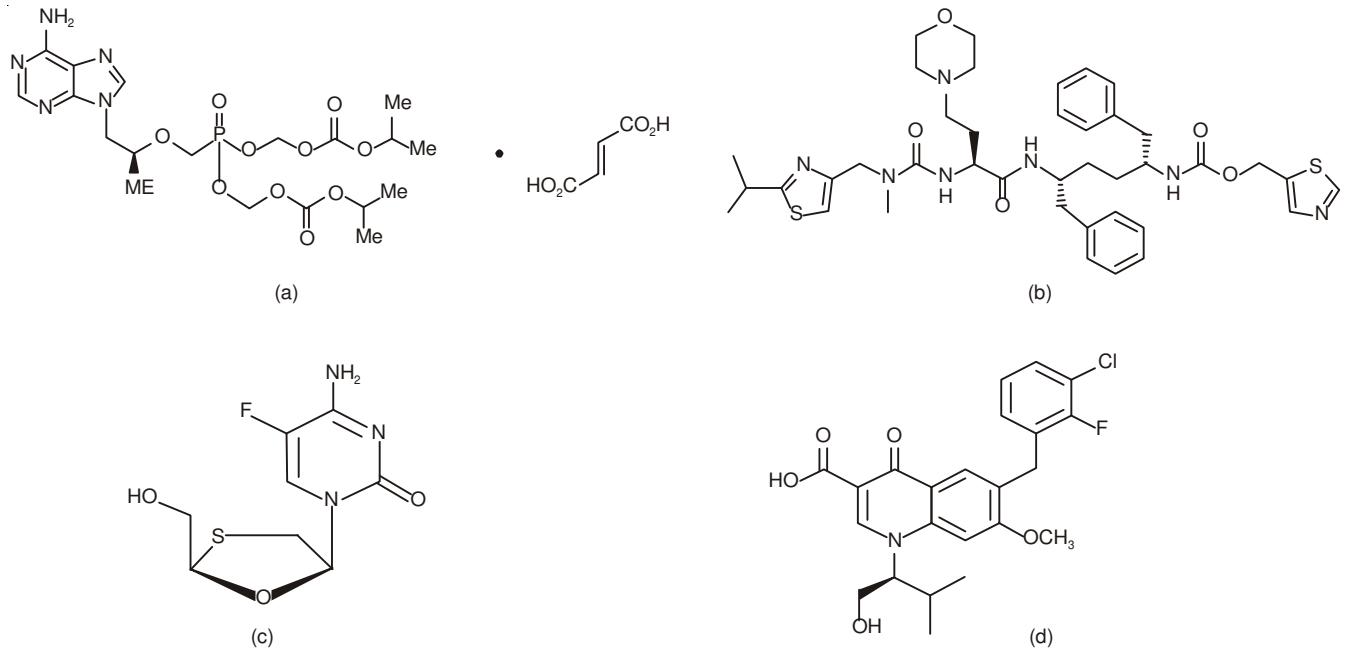

Fig. 1. Structure of (a) Tenofovir Disoproxil Fumarate (b) Cobicistat (c) Emtricitabine and (d) Elvitegravir

EXPERIMENTAL

The chromatography was performed on a Alliance WATERS 2695 with high speed auto sampler, column oven, degasser and 2996 PDA detector to provide a compact and convenient for LC with class Empower-2 software

Chromatographic condition: The mobile phase consists of potassium dihydrogen ortho phosphate buffer (pH 3) and acetonitrile were taken in gradient: T (min)/%buffer/% acetonitrile: 0.0/56/44, 5/60/40, 6/30/70, 10/30/70, 11/60/40 and 12/60/40 with flow rate of 0.8 mL/min. Hypersil BDS column (4.6 × 100 mm, 5 µ particle size) was used as the stationary phase.

Pharmaceutically pure sample of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were obtained from Spectrum Pharma, Hyderabad as gift samples along with their analytical reports. HPLC grade acetonitrile, methanol, water and all other chemicals were obtained from Merck chemical division, Mumbai. Commercial tablets of stribild were procured from local pharmacy store.

Preparation of standard stock solution: Accurately weighed and transferred weight equivalent to 300 mg of tenofovir, 200 mg of emtricitabine, 150 mg of elvitegravir and 150 mg of cobicistat working standards into a 100 mL clean dry volumetric flask, added 70 mL of diluent (water:acetonitrile = 20:80), sonicated for 0.5 h and make up to the final volume with diluents.

Preparation of working standard solutions: Aliquot of 0.125, 0.25, 0.375, 0.5, 0.625 and 0.75 mL were pipette out from stock solution into 10 mL volumetric flask separately and volume was made up to 10 mL with diluent. This gives the solutions of 37.5, 75, 112.5, 150, 187.5 and 225 µg/mL for tenofovir disoproxil fumarate, 18.75, 37.5, 56.25, 75, 93.75 and 112.5µg/mL for cobicistat, 25, 50, 75, 100, 125 and 150 µg/mL for emtricitabine and 18.75, 37.5, 56.25, 75, 93.75 and 112.5 µg/mL for elvitegravir, respectively.

Sample preparation: Twenty tablets were weighed and calculated the average weight of 20 tablets and then the weight equivalent to 5 tablets was transferred into a 500 mL volumetric flask, 300 mL of diluent added and sonicated for 0.5 h, further the volume made up with diluent and filtered. From the filtered solution 0.5 mL was pipeted out into a 10 mL volumetric flask and made upto 10 mL with diluent.

Method validation: Validation parameters like system suitability, linearity, accuracy, precision, solution stability, specificity, limit of detection, limit of quantification and robustness were performed as per ICH guidelines[25].

RESULTS AND DISCUSSION

Method development: Phosphate buffer (pH 3) and acetonitrile were taken in gradient: T (min)/%buffer/% acetonitrile: 0.0/56/44, 5/60/40, 6/30/70, 10/30/70, 11/60/40 and 12/60/40 with flow rate of 0.8 mL/min was employed. Hypersil BDS column (4.6 × 100 mm, 5 µ particle size) was used as the stationary phase to improve resolution and the tailing of four peaks was reduced considerably and temperature was set at 30 °C. The detection of these drugs was tried at various wavelengths from 215 to 280 nm. The wavelength at which tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir showed maximum absorption at 245 nm was selected as the detection wavelength for PDA detector. The retention times of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were found to 2.1, 4.3, 5.2 and 8.5 min, respectively. The chromatogram obtained is shown in the Fig. 2.

Method validation

System suitability: To ensure the suitability of the instrument, a system suitability test was established. Data from six injections of 10 µL of the working standard solutions of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were used for the evaluation of the system

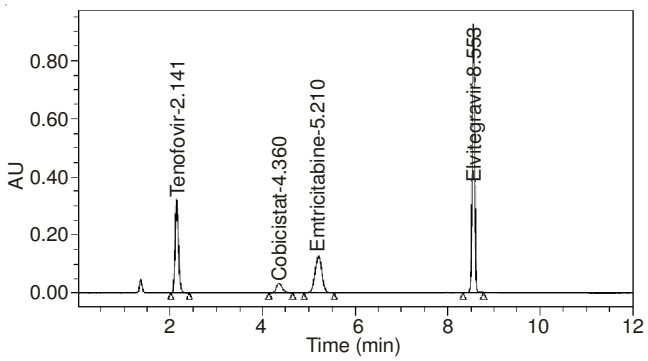

Fig. 2. Typical chromatogram of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir

suitability (n = 6). Parameters such as number of theoretical plates, average area and peak tailing were determined and all the parameters are within the limits. Results are shown in Table-1.

Linearity: By appropriate aliquots of the standard tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir solutions with the diluent phase, six working solutions ranging between 37.5-225, 18.75-112.5, 25-150 and 18.75-112.5 µg/mL were prepared and injected (n = 3). The mean peak areas were plotted against the concentration of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir to obtain the calibration curve and the results are shown in Fig. 3.

Accuracy: To the placebo solution, known amounts of standard tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir corresponding to 50, 100 and 150 % of 100 % of target concentrations were added. Mean recovery (%) for tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir are 99.99, 100.20, 99.89 and 100.17, respectively and these results are within acceptable limit of 98-102 %. The % RSD for tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir are 0.9, 0.7, 0.5 and 0.8, respectively and these results were within limit of ≤ 2. Hence the proposed method is accurate and the results are summarized in Table-2.

Precision: Repeatability and intermediate precision were determined in accordance with ICH guidelines. Determinations were performed on the same day as well as well as on consequent days (n = 6). Six replicate injections in same concentration were analyzed on two different days with different analyst and column for verifying the variation in the precision and the % RSD for tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir are found to be within acceptable limit of ≤ 2. Hence the method is reproducible on different days with different analyst and column and the results are summarized in Table-3.

Robustness: The robustness of the method was performed by changing the chromatographic conditions. The organic strength was varied by ± 5 %, column temperature was varied by ± 5 °C and the flow rate ± 0.1 mL. There were no significant

TABLE-1
SYSTEM SUITABILITY OF TENOFOVIR DISOPROXIL FUMARATE (TDF), COBICISTAT (CBT), EMTRICITABINE (ETC) AND ELVITEGRAVIR (EVG)

S.No.	TDF	CBT	ETC	EVG
No of theoretical plates	4737	5398	6210	118940
Tailing Factor	1.2	1.1	1.0	1.0
Average Area	1540504	293207	1286441	3378967
SD	3538.6	1179.6	2967.2	11429.5
%RSD	0.2	0.4	0.2	0.3

SD = Standard deviation RSD = Relative standard deviation

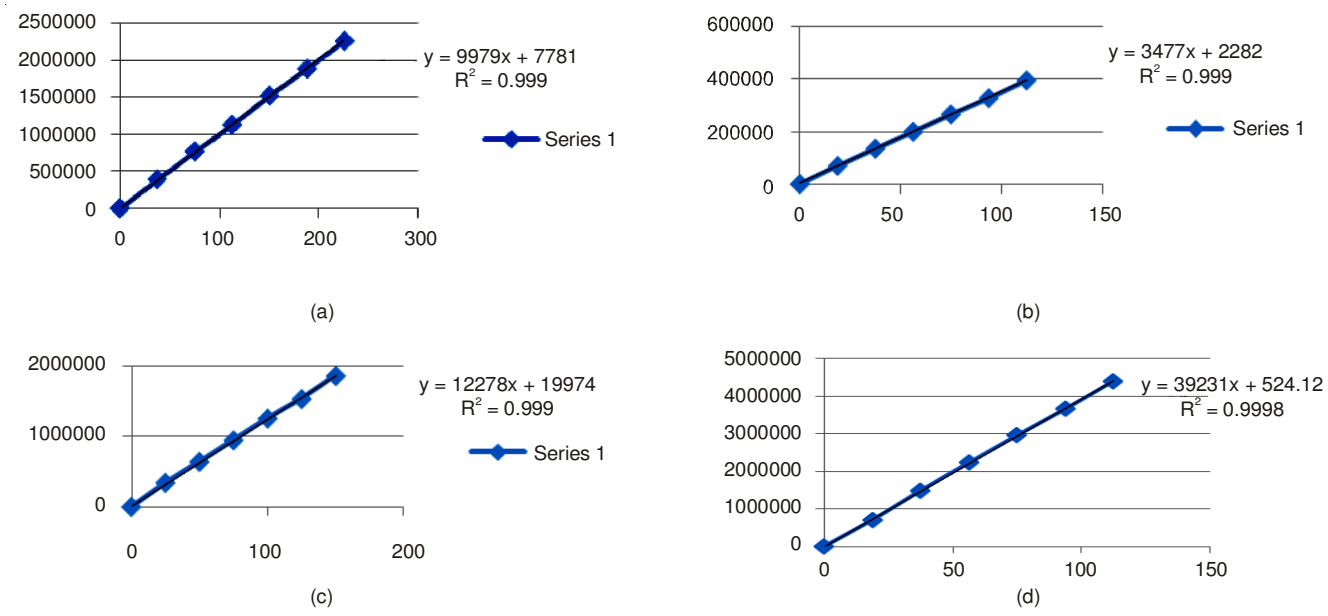

Fig. 3. Linearity graphs of (a) Tenofovir Disoproxil Fumarate, (b) Cobicistat, (c) Emtricitabine and (d) Elvitegravir

TABLE-2
RECOVERY EXPERIMENTS OF TENOFOVIR DISOPROXIL FUMARATE (TDF), COBICISTAT (CBT), EMTRICITABINE (ETC) AND ELVITEGRAVIR (EVG)

Standard Amount Spiked (µg/mL)				% Recovered			
TDF	CBT	ETC	EVG	TDF	CBT	ETC	EVG
75	37.5	50	37.5	99.13	100.48	99.60	100.99
				101.58	100.18	100.61	101.34
				100.80	100.13	99.90	100.36
150	75	100	75	99.57	101.60	99.38	99.97
				99.11	100.18	100.30	99.38
				99.18	100.08	99.14	99.05
225	112.5	150	112.5	99.67	100.35	99.84	100.88
				100.22	99.65	99.75	100.08
				100.61	99.16	100.48	99.49
			Average	99.99	100.20	99.89	100.17
			SD	0.871	0.658	0.495	0.788
			%RSD	0.9	0.7	0.5	0.8

SD = Standard deviation; RSD = Relative standard deviation

TABLE-3
PRECISION DATA OF TENOFOVIR DISOPROXIL FUMARATE (TDF), COBICISTAT (CBT), EMTRICITABINE (ETC) AND ELVITEGRAVIR (EVG)

	Repeatability (%Assay)				Day to Day (%Assay)			
	TDF	CBT	ETC	EVG	TDF	CBT	ETC	EVG
Sample 1	99.28	99.75	99.93	100.03	99.98	100.29	99.98	99.95
Sample 2	99.84	99.83	99.93	99.14	100.14	99.23	100.01	100.15
Sample 3	99.20	100.33	99.19	99.69	99.89	99.81	100.13	99.92
Sample 4	99.26	99.40	99.40	99.23	100.1	100.4	99.84	99.73
Sample 5	99.98	101.29	100.73	100.28	100.07	100.43	100.3	100.06
Sample 6	99.96	100.09	100.58	99.83	99.8	99.82	99.71	100.17
%Mean	99.59	100.12	99.96	99.70	100.00	100.00	100.00	100.00
SD	0.376	0.656	0.614	0.447	0.132	0.467	0.208	0.165
%RSD	0.4	0.7	0.6	0.4	0.1	0.5	0.2	0.2

SD = Standard deviation RSD = Relative standard deviation

changes in the chromatography pattern when the above modifications were made in the experimental conditions, showing that the method is robust.

Stability of sample solution: The sample and standard solution injected at 0 h and after 24 h and did not show any appreciable change in the assay.

Specificity: Specificity was established by injecting Samples of mobile phase, placebo, unspiked and spiked standard sample. No interference was found at room temperaure of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir in mobile phase and placebo and the assay result for spiked and unspiked sample are within limit of 98-102 % and also the assay result is unaffected by presence of excipients in spiked samples when compare to unspiked sample. Hence the proposed method is specific to the tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir.

Limit of detection and limit of quantification: Limit of detection (LOD) and limit of quantification (LOD) of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were determined by calibration curve method. Solutions of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were prepared in linearity range and injected (n = 3). Average peak areas were plotted against concentration. These were calculated by using following equations (ICH, Q2 (R1)).

$$\text{Limit of detection} = (3.3 \times \sigma)/S \text{ and}$$

$$\text{Limit of quantification} = (10 \times \sigma)/S$$

where s is the standard deviation of the response; S is the slope of the calibration curve.

The slope S estimated from the calibration curve of the analyte. Limit of detection and limit of quantification of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir were determined by calibration curve method and the results are shown in the Table-4.

Tablet analysis: Content of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir was found in the tablets by the proposed method and results were shown in Table-5.

TABLE 4
LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ) DATA OF TENOFOVIR DISOPROXIL FUMARATE (TDF), COBICISTAT (CBT), EMTRICITABINE (ETC) AND ELVITEGRAVIR (EVG)

Name	LOD (µg/mL)	LOQ (µg/mL)
TDF	0.058718	0.177932
CBT	0.210964	0.639286
ETC	0.132316	0.400959
EVG	0.036112	0.109429

Conclusion

A new, simple, sensitive RP-HPLC method was developed for simultaneous estimation of tenofovir disoproxil fumarate,

TABLE-5
RESULTS OF TABLET ASSAY (n = 6)

S. No.	Drug	Lable claim (mg)	Amount found (mg)	Assay (%)
1.	Tenofovir disoproxil fumarate	300	297.74	99.24
2.	Cobicistat	150	149.55	99.70
3.	Emtricitabine	200	199.72	99.86
4.	Elvitegravir	150	150.18	100.12

cobicistat, emtricitabine and elvitegravir in bulk drugs and pharmaceutical dosage forms. The validation parameters like system suitability, linearity, accuracy, robustness, solution stability, specificity, limit of detection and limit of quantification, were found to be within the limits. This method was successfully applied for estimation of drug content in pharmaceutical dosage forms. Hence, this method can be used in quality control of tenofovir disoproxil fumarate, cobicistat, emtricitabine and elvitegravir in pharmaceutical industries.

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