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SIMULTANEOUS ESTIMATION OF EMTRICITABINE, TENOFOVIRDISOPROXILFUMARATE AND RILPIVIRINE IN PHARMACEUTICAL DOSAGE FORM BY USING RP-HPLC

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Abstract
The study describes method development and subsequent validation of RP-HPLC method for simultaneous estimation of emtricitabine, tenofovir disoproxil fumarate and rilpivirine in combined tablet dosage forms. Chromatographic separation was achieved on a hypersilBDSC18 column (250 mm x 4.6 mm, 5 µm) using a mobile phase consisting of (45:55 v/v) buffer: acetonitrile at a flow rate of 1 mL/min. The detection wavelength is 280 nm. The retention times of emtricitabine, tenofovirdisoproxil fumarate and rilpivirine were found to be 2.692, 4.402 min and 5.725 min respectively. The developed method was validated as per ICH guidelines. The developed and validated method was successfully used for the quantitative analysis of emtricitabine,tenofovirdisoproxilfumarate and rilpivirine in tablet dosage forms.

Key words: HPLC, emtricitabine,tenofovirdisoproxilfumarate, rilpivirine, Validation.

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Introduction

Emtricitabine [1,2] chemically**,** 4-amino-5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one(Figure 1) and is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults and children. Tenofovirdisoproxil fumarate [3, 4] chemically, Bis{[(isopropoxycarbonyl)oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl) phosphonate (Figure 2) belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors. Rilpivirine [5, 6] (Figure 3) chemically**,** 4-{[4-({4-[(*E*)-2-cyanovinyl]-2, 6-dimethylphenyl} amino) pyrimidin-2-yl]amino}benzonitrile and is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA.

Detailed survey of literature revealed that, few methods were reported based on UV- Spectrophotometric methods, [7-8] and Liquid Chromatography [9-13], for Emtricitabine alone as well as in combination with other drugs using pharmaceutical formulations. Literature for Tenofovir revealed several methods which include Spectrophotometric method, RP-HPLC, HPTLC,and Liquid Chromatography in biological fluids for Tenofovir alone as well as with other drugs in different pharmaceutical formulations [14-19].

The present research work is aimed to develop a simple, rapid, accurate, sensitive and stability indicating method for simultaneous estimation of the above mentioned three drugs for routine analysis with ashorter run time. The proposed method is optimised and validated as per the International Conference on Harmonization (ICH) guidelines [20].

Materials and Methods

Equipment

Separation was carried out by using Waters 2695 system equipped with Waters 2996 Photo Diode Array (PDA) detector and the peak areas were integrated by using Empower-2 software. Analysis was carried out on hypersilBDS C18 (250 mm x 4.6 mm, 5 µm) column.

Chemicals and reagents

HPLC grade water, acetonitrile and analytical grade orthophosphoric acid, Potassium dihyrogen orthophosphate, triethylamine were obtained from M/s. Rankem Chemicals Ltd, Mumbai, India.

Preparation of solutions

Preparation of buffer solution (pH 4.0)

1.36 g of Potassium dihydrogen orthophosphate was accurately weighed and taken into a 1000 mL volumetric

flask and 900 mL of Milli Q water was added and the solution was mixed until the contents were dissolved completely and then the volume was made up to 1000 mL. Further, 1 mL of Triethylamine was added and the pH of the solution was adjusted to 4.0 with diluteOrthophosphoric acid solution. Buffer solution was filtered through 0.45 µ membrane filter and sonicated for 10 min.

Preparation of Mobile phase

The contents of the mobile phase were Buffer and Acetonitrile, mixed in the ratio of 40:60. The prepared mobile phase was passed through 0. 45 µ membrane filter under vacuum and was degassed before use.

Preparation of mixed standard solution

Standard stock solution of the drug was prepared by dissolving 20 mg of Emtricitabine, 30 mg of Tenofovir and 2.5 mg of Rilpivirinestandard drugs into a 10 mL volumetric flask containing 7mL of diluent (Acetonitrile: Water 50:50). The above contents were sonicatedfor 30 minutes to ensure complete solubility of the drugand then volume was made upto 10 mL with diluent to obtainstandard stock solution containing 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir and 250 µg/mL of Rilpivirine.

Preparation of Sample Stock solution

Twenty tablets ofComplera (Emtricitabine -200 mg, Tenofovir – 200 mg, Rilpivirine – 25 mg) in a combined dosage form were weighed and groundinto a fine powder. Tablet powder with weight equivalent to 1000 mg of Emtricitabinewas weighed accuratelyand mixed with 260 mL of diluent (Acetonitrile: Water 50:50) in a 500mL volumetric flask. The mixture was sonicated for 30 min to ensure complete solubility of the drug and the volume was madeupto 500 mL with diluent. The drug concentrationswere 2000 µg/mL of Emtricitabine, 3000 µg/mL of Tenofovir and 250 µg/mL of Rilpivirine in the sample stock solution.The prepared solution wasfiltered through a 0.45 µ membrane filter to remove impurities and the excipients which remained undissolved in the solution.

Chromatographic conditions

A reverse phase column hypersilBDS C18 column (250 mm x 4.6 mm, 5 µm particle size), equilibrated with mobile phase (buffer: acetonitrilein the ratio of 45:55 v/v) was used. Mobile phase flow rate was maintained at 1 mL/min and effluents were monitored at 280 nm. The sample was injected using 20 micro litre manual sample injector and run time was 10 min.

Procedure: Under optimized chromatographic conditions 20 µl of each standard of linearity range was injected and chromatograms were recorded. Typical chromatogram showing separation of emtricitabine,tenofovirdisoproxilfumarate and rilpivirine is given in Figure 4.

Method Validation

System suitability

The system suitability studies were done for parameters like theoretical plates, tailing factor, retention time, resolution by injecting the standard solution in to the optimized chromatographic system for six times and the results are given in the Table 1.

Linearity

Linear calibrations plots of the proposed method were obtained over concentration ranges of 50-300µg/mL for Emtricitabine, 75 µg/mL-450 µg/mL for Tenofovir and 6.25 µg/mL-37.5 µg/mL for Rilpivirine. Each solution was prepared in triplicate. Regression coefficient was found to be 0.999 for all three the drugs (Figure 5- 7).Standard curve had a reliable reproducible over the standard concentrations across the calibration range. All back calculated concentrations did not differ from the theoretical value as no single calibration standard point was dropped during the validation.

Accuracy

The standard addition method was used to demonstrate the accuracy of the proposed method. For this purpose, known quantities of emtricitabine, tenofovirdisoproxilfumarate and rilpivirine were supplemented to the previously analysed sample solution and then experimental and true values compared. Three levels of solutions were made corresponding to 50, 100 and 150 % of nominal analytical concentration (500 µg/mL of Emtricitabine, 0.2 µg/mL of Tenofovir and 2 µg/mL of Rilpivirine). Standard preparation & sample preparation was injected into the HPLC and % RSD foremtricitabine,tenofovirdisoproxilfumarate and rilpivirine peaks in standard preparation was calculated and tabulated in Table 2. The mean recovery values of emtricitabine,tenofovirdisoproxilfumarate and rilpivirine were found to be 100.28, 99.84 and 99.83%respectively.

Precision

For precision same concentration solution i.e. 500 µg/mL of Emtricitabine, 0.2 µg/mL of Tenofovir and 2 µg/mL of Rilpivirine was injected 6 times and observed for any peculiar change in the areas and % RSD was calculated for each drug.The standard deviation values of peak area were found to be 33512.1, 4245.86and 14039.23 for emtricitabine, tenofovirdisoproxilfumarate and rilpivirineand the % RSD values were 0.67, 0.65 and 0.68 for emtricitabine, tenofovirdisoproxilfumarate and rilpivirine and the results are tabulated in the Table 3.

Robustness

Robustness is generally done by changing the parameters like flow rate, organic phase of the mobile phase and column temperature.The results are shown in the **Table 4-6.**

Limit of detection (LOD)

The LOD for this method was found to be 0.0128 µg/mL, 0.380 µg/mL and 0.0016 µg/mL for emtricitabine,tenofovirdisoproxilfumarate and rilpivirinerespectively.

Limit of quantitation (LOQ)

The LOQ for this method was found to be 0.039µg/mL, 1.154 µg/mLand 0.0049 µg/mL for emtricitabine, tenofovirdisoproxilfumarate and rilpivirinerespectively.

Results and Discussion

To develop a new RP-HPLC method, several mobile phase compositions were tried. A satisfactory separation with good peak symmetry was obtained with hypersilBDS column.In the present study, a new simple, precise and accurate HPLC method was developed and validated for the simultaneous estimation of emtricitabine, tenofovirdisoproxilfumarate and rilpivirinein tablet dosage forms. In this method, a hypersilBDS C18 (250 x 4.6 mm; 5 µm) column using mobile phase containing buffer and acetonitrile (45:55 v/v) at a flow rate of 1 mL/min. Quantification was achieved with UV detection at 280 nm based on peak area. The retention time for emtricitabine, tenofovirdisoproxilfumarate and rilpivirine were found to be 2.692, 4.402 min and 5.725 min respectively. The optimized method was validated as per ICH guidelines. The System suitability parameters observed by using this optimized conditions were reported. A linearity range of 50-300µg/mL with correlation coefficient 0.999 was established for emtricitabine, 75-450µg/mL with correlation coefficient 0.999 was established for tenofovirdisoproxilfumarate and 6.25-37.5µg/mL with correlation coefficient 0.999 was established for rilpivirine. The precision of the proposed method was carried in terms of the repeatability and the % RSD values of emtricitabine was found to be 0.67 %, of tenofovirdisoproxilfumarate was found to be 0.65% and of rilpivirine was found to be 0.68 % and reveal that the proposed method is precise. The LOD and LOQ values for emtricitabine were0.0128 and 0.039 µg/mL respectively, for tenofovirdisoproxilfumarate were found to be 0.380 and 1.154µg/mL respectively and for rilpivirine were found to be 0.0016 and 0.0049µg/mL respectively. The study of robustness in the present method shows no significant changes in the peak area. The results of analysis of commercial formulation indicated that there is no interference due to common formulation excipients with the developed method. Therefore, the proposed method can be used for routine analysis of these three drugs in their combined pharmaceutical dosage form.

Conclusion

The proposed method was found to be simple, precise, accurate and rapid for simultaneous determination of emtricitabine, tenofovirdisoproxilfumarate and rilpivirine from pure and its combined dosage forms. The mobile phase is simple to prepare and economical. The sample recoveries in the formulation were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. Hence, this method can be easily and conveniently adopted for routine analysis of emtricitabine, tenofovirdisoproxilfumarate and rilpivirine in pure form and its combined dosage form.

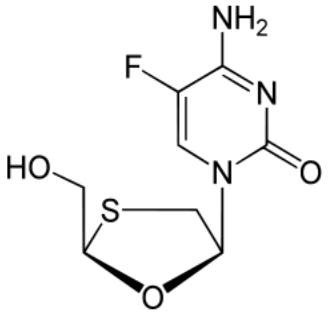

Figure 1: Chemical structure of Emtricitabine

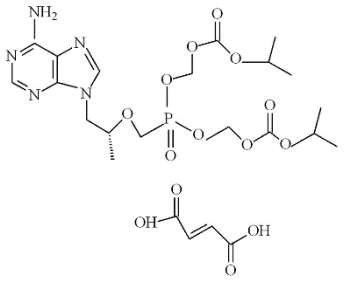

Figure 2: Chemical structure of Tenofovirdisoproxilfumarate

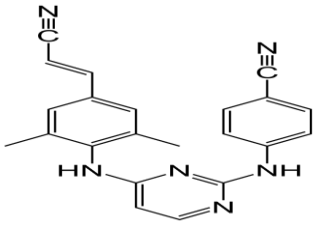

Figure 3: Chemical structure of Rilpivirine

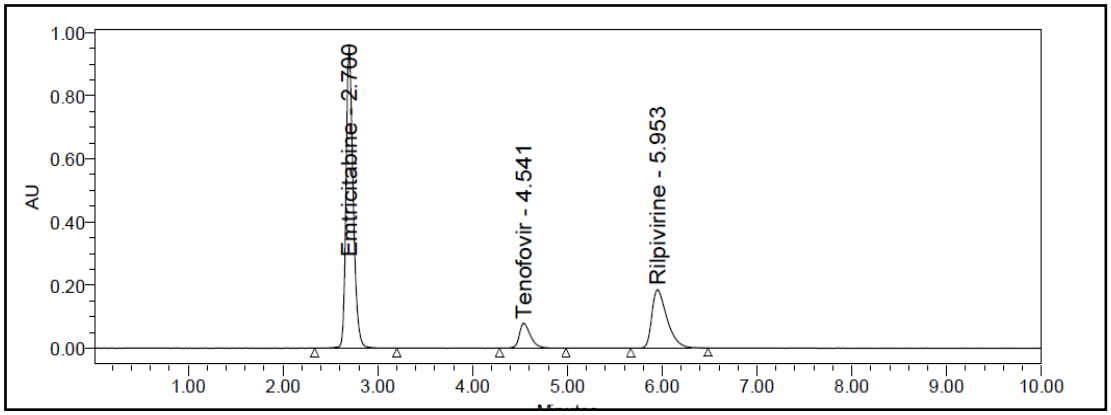

Figure 4: Chromatogram showing separation of emtricitabine,tenofovirdisoproxilfumarate and rilpivirine

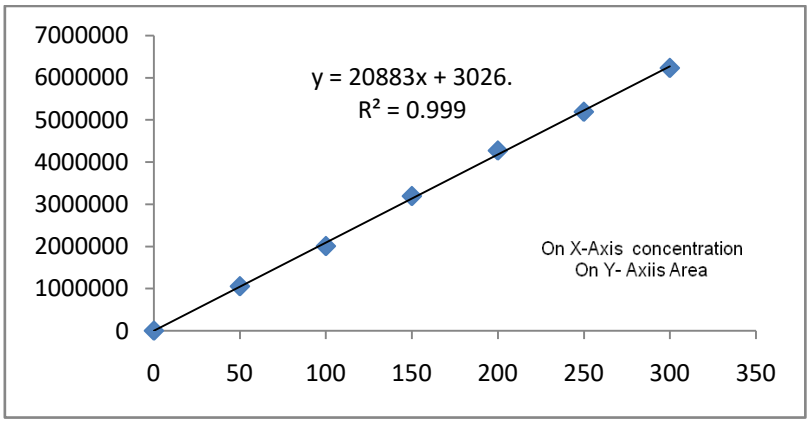

Figure 5: Standard Calibration Curve of Emtricitabine

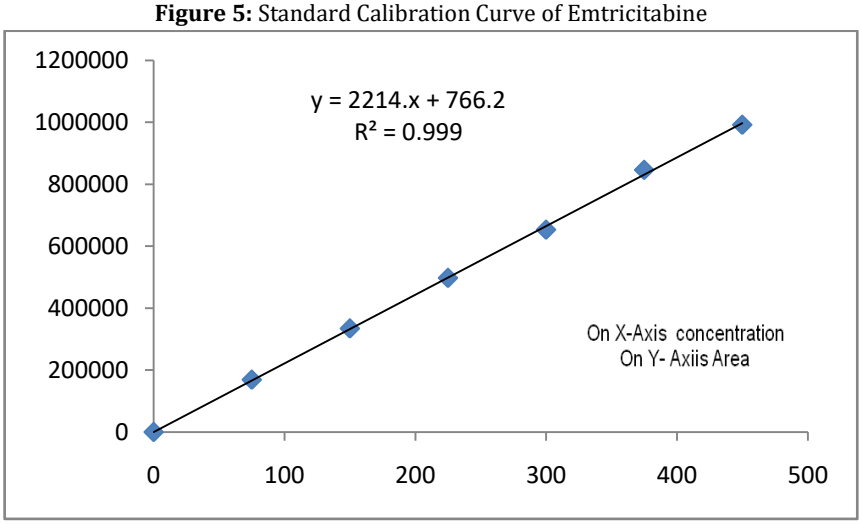

Figure 6: StandardCalibrationCurve of Tenofovir

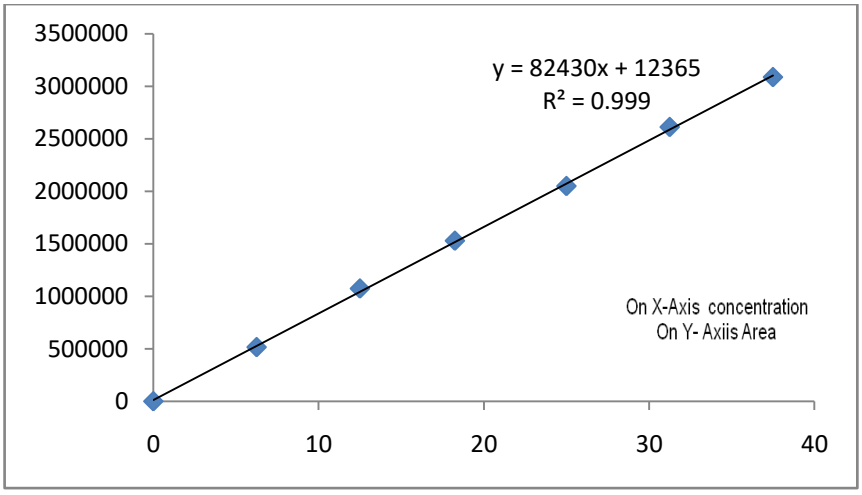

Figure 7: Standard Calibration Curve of Rilpivirine

Table 1: System suitability study

S. No	Parameters	Emtricitabine	Tenofovir	Rilpivirine	Limits
1	Relative retention time (min)*	2.692	4.402	5.725	--
2	% RSD of Retention Time	0.147	1.225	1.558	Not more than 2
3	Peak Area*	4956585	648376	2040033	--
4	% RSD of Peak area	0.676113	0.654	0.688	Not more than 2
5	Theoretical plates	5539	6990	6479	More than 2000
6	Tailing factor	1.27	1.29	1.54	Less than 2
7	Resolution	-	> 2	>2	More than 2

* Mean of six determinations

Table 2: Results of recovery experiments

Drug/ Parameters	Emtricitabine			Tenofovir			Rilpivirine		
Concentration in %	50	100	150	50	100	150	50	100	150
Concentration of sample in µg/mL	100	200	300	150	300	450	12.5	25	37.5
Concentration of standard in µg/mL	200	200	200	300	300	300	25	25	25
Total Amount after spiking	300	400	500	450	600	750	37.5	50	62.5

in µg/mL									
Total amount recovered in µg/mL*	299.79	401.44	500.98	450.04	603.06	747.63	37.49	49.89	62.49
% recovery*	99.79	100.72	100.33	100.03	100.03	99.47	99.92	99.58	99.98
% RSD of Recovery	0.463			0.769			0.261		

* Mean of three determinations

Table 3: Results of precision study

Injection	**Emtricitabine**		**Tenofovir**		**Rilpivirine**	
	Retention Time in Min	**Peak Area**	**Retention Time in Min**	**Peak Area**	**Retention Time in Min**	**Peak Area**
1	2.69	4943873	4.438	644753	5.788	2048637
2	2.695	4934143	4.443	649559	5.792	2035496
3	2.696	4911523	4.542	646315	5.962	2051722
4	2.698	4993370	4.546	655746	5.962	2055805
5	2.7	4960648	4.546	644399	5.962	2022212
6	2.701	4995953	4.555	649485	5.984	2026325
Mean	2.696	4956585	4.511	648376.2	5.908	2040033
Std. dev	0.00	33512.1	0.05	4245.86	0.09	14039.23
%RSD	0.14	0.67	1.22	0.65	1.55	0.68

Table 4: Results of robustness by variations in flow rate, columntemperature and Mobile phase composition of Emtricitabine

S.No	**Parameter**	**Used**	**Peak Area**	**Retention Time**	**Plate count**	**Tailing Factor**
Optimised Conditions		1.0 mL/min; 30 0C; Phosphate buffer (pH 4): Acetonitrile (40:60);	4936742	2.689	6032	1.27
1	Flow Rate (±0.1 mL/min)	0.9 mL/min	3699782	2.985	6698	1.24
		1.1 mL/min	4315222	2.447	6671	1.24

2	Column Temperature (±50C)	250C	4994809	2.691	6978	1.25
		35 0C	4994030	2.686	7043	1.24
3	Mobile phase composition	45:55	2266221	2.691	6940	1.26
		35:65	3350456	2.700	6756	1.25

* Mean of three determinations, *Retention time in min

Table 5: Results of robustness by variations in flow rate, column temperatureand Mobile phase composition of Tenofovir

S.No	Parameter	Used	Peak Area	Retention Time	Plate count	Tailing Factor
Optimised Conditions		1.0 mL/min; 30 0C; Phosphate buffer (pH 4): Acetonitrile (40:60);	648585	4.507	7280	1.29
1	0.9 mL/min 1.1 mL/min	0.9 mL/min	735412	4.878	7818	1.32
		1.1 mL/min	577164	3.980	7504	1.30
2	250C 35 0C	25ºC	659027	4.351	8014	1.28
		35 ºC	659340	4.305	8134	1.27
3	45:55	45:55	370312	4.307	8148	1.25
		35:65	665550	4.483	7567	1.33

Table 6: Results of robustness by variations in flow rate, column temperature and Mobile phase composition of Rilpivirine

S.No	Parameter	Used	Peak Area	Retention Time	Plate count	Tailing Factor
Optimised Conditions		1.0 mL/min; 30 0C; Phosphate buffer (pH 4): Acetonitrile (45:55)	2051395	5.918	6569	1.54
1	Flow Rate (±0.1 mL/min)	0.9 mL/min	849093	6.344	11443	1.03
		1.1 mL/min	1821382	5.158	6752	1.56
2	Column Temperature (±50C)	25ºC	2070379	5.504	7230	1.52
		35 ºC	2070379	5.504	7230	1.52
3	Mobile phase composition	40:60	939732	5.555	7157	1.50
		50:50	1362362	5.851	7037	1.52

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Conflict of interest
Authors are decaled that no conflict of interest.

Author Contribution
All authors are contributed equally.

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