

Method Development and Validation for the Simultaneous Estimation of Emtricitabine and Tenofovir in Pharmaceutical Dosage Forms by RP-HPLC

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Abstract

A Simple precise, rapid method has been developed for the simultaneous estimation of Tenofovir and Emtricitabine in pharmaceutical dosage form by RP-HPLC. The optimum wavelength for the determination of Tenofovir and Emtricitabine was selected at 265nm on the basis of isobestic point. Several trials were performed with dissimilar mobile phases in dissimilar ratios, but finally Sodium Phosphate Buffer pH 2.8: Acetonitrile: Methanol (60:20:20) %v/v/v) was selected as good peak symmetry and resolution between the peaks was observed. The Retention time of Tenofovir and Emtricitabine were found to be 1.953 and 3.733 min respectively.

The calibration curve was obtained by plotting peak area versus the concentration over the range of 100-300 µg/mL For Emtricitabine and 150-450 µg/mL for Tenofovir. From linearity the correlation coefficient R² value was found to be 0.999 for Emtricitabine and 0.999 for Tenofovir. The proposed HPLC method was also validated for method precision, system precision and system suitability. The percentage of recovery of Tenofovir and Emtricitabine were found to be 99.5 and 99.2 respectively, shows that the proposed method is highly accurate. Hence the proposed method is highly accurate, sensitive and precise and it successfully applied for the quantification of API in the commercial formulations of Tenofovir and Emtricitabine in Educational institutions and Quality control laboratories.

Keywords: Emtricitabine, Tenofovir, Validation, RP-HPLC.

Introduction

Pharmaceutical analysis simply means analysis of pharmaceuticals. Webster' dictionary defines a pharmaceutical is a medical drug. Research and development (R&D) play a very comprehensive role in new drug development and follow up activities to ensure that a new drug product meets the established standards is stable and continue to approved by regulatory authorities, assuring that all batches of drug product are made to the specific standards utilization of approved ingredients and production method becomes the responsibility of pharmaceutical analysts in the quality control (QC) or quality assurance department [1,2].

That drug substance and drug product meet two critical requirements.

- Established identity and purity.
- Established bio availability/dissolution.

Different types of chromatography

- Adsorption Chromatography

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- Partition Chromatography
- Ion Exchange Chromatography
- Molecular Exclusion Chromatography
- Affinity Chromatography

High performance liquid chromatography: basic principle of HPLC

• High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase.

• Under a certain dynamic condition, each component in a sample has difference distribution equilibrium depending on solubility in the phases and or molecular size.

• As a result, the components move at different speeds over the stationary phase and are thereby separated from each other.

• The column is a stainless steel (or resin) tube, which is packed with spherical solid particles.

Types of high performance liquid chromatography Based on modes of chromatography

• **Normal phase chromatography:** Normal-phase liquid-liquid chromatography uses a polar stationary phase and less polar mobile phase. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptanes. If the example is immovably held, the limit of the stationary stage should be extended, perhaps by including little measures of methanol or dioxane.

• **Reverse phase chromatography:** Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. The elution order of the classes of compounds in table is reversed (thus the name reverse-phase chromatography).

Based on principles of separation

- Adsorption chromatography
- Ion exchange chromatography
- Ion pair chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chairal phase chromatography

Based on elution technique

- Isocratic separation
- Gradient separation

Based on the scale of operation

- Analytical HPLC
- Preparative HPLC [3-7]

HPLC Instrument (Figure 1,2)

- Mobile part reservoir, filtering
- Pump

- Injector
- Column Oven
- Detector
- **Data system**

Materials and Methods

Preparation of phosphate bufferpH 4.5

1.19 gm of Sodium Phosphate Monobasic was weighed and dissolved in 1000 mL of water. then Adjust the pH to 2.8 ± 0.02 using diluted orthophosphoric acid. Buffer was filtered through $0.45 \mu\text{m}$ filters to remove all fine particles and gases.

Determination of working wavelength (λ_{max})

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

Preparation of standard solution

About 10 mg of Emtricitabine and 10mg of Tenofovir were weighed into a 50 mL volumetric flask, to this 50 mL of mobile phase was added, sonicated and the volume was made up to mark with the mobile phase [8].

Dilutions

Necessary dilutions are made from standard stock solutions to get the concentration range of $10 \mu\text{g/mL}$ of Emtricitabine and $10 \mu\text{g/mL}$ of Tenofovir. The wavelength of maximum absorption (λ_{max}) of the solution of the drugs in mobile phase were scanned using UV-Visible spectrophotometer within the wavelength region of 200–

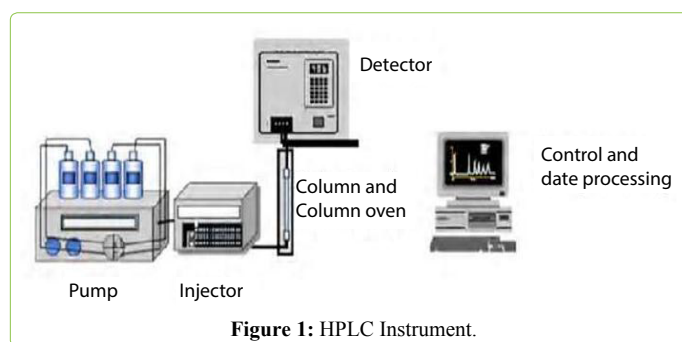


Figure 1: HPLC Instrument.

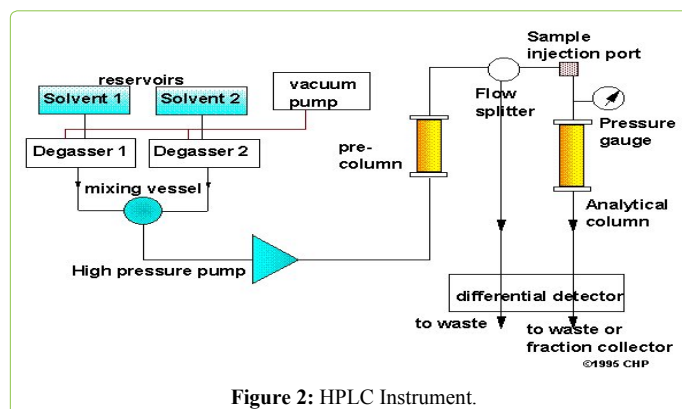


Figure 2: HPLC Instrument.

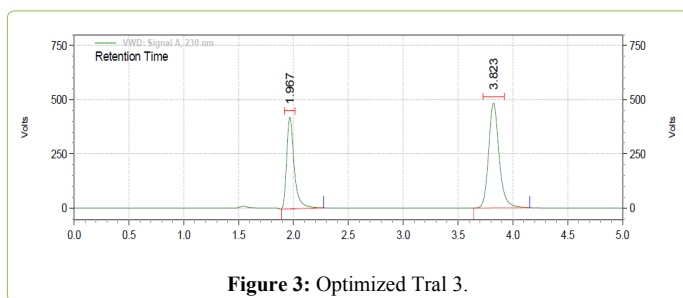


Figure 3: Optimized Trial 3.

400 nm against mobile phase as blank. The absorption curve shows characteristic absorption maxima at 261 nm for Emtricitabine, 249 nm for Tenofovir and at 265nm same absorbance for both the drugs, i.e., isobestic point. Thus 230nm was selected as detector wavelength for the HPLC chromatographic method [9,10].

Method development of Emtricitabine and Tenofovir

Various analytical development trails has been performed by using different chemicals and reagents, organic solvents at different pH ranges and strengths in differents proportions of buffer and organic solvents to separate the 2 peaks shape. Based on the observations and conclusions obtained from the no.of chromatographic trails performed on RP-HPLC, a particular set of chromatographic conditions were optimized to be suitable for estimation of the Emtricitabine and Tenofovir in the tablets.The optimized chromatographic conditions which are found to be suitable for the estimation of sofosbuvir and velpatasvir are given below.

Method Validation

System suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated [11-13].

Preparation of samples for Assay

Preparation of Standard solution

About 200 mg of Emtricitabine and 300mg of Tenofovir were weighed into a 200 mL volumetric flask, to this 70mL of mobile phase was added, sonicated and the volume was made up with the mobile phase. Pipette out 5 mL of the clear solution in to 25 mL volumetric flask and make up volume with mobile phase.

Preparation of Sample solution

Sample name : Tavin-EM TAB(200 mg of Emtricitabine and 300mg of Tenofovir)

Manufacture name: Emcure Pharmaceuticals

Crush more than 20tablets then weigh a quantity of powder equivalent to 200mg of Emtricitabine and 300mg of Tenofovir in 200 mL volumetric flask and add70mL of mobile phase then sonicated it for 30min intermit Tenofovir shacking after 30min make up volume with mobile phase. Pipetted 5 mL of the clear solution in to 25 mL volumetric flask and make up volume with mobile phase. Filter the

solution through 0.45µm filter paper. The resulting solution is used to record the chromatogram (Figure 3).

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Linearity and range

Preparation of standard stock solution

Standard stock solutions of Emtricitabine (2000µg/mL) and Tenofovir (3000mg/mL) were prepared by dissolving 200 mg of Emtricitabine and 300 mg of Tenofovir in 100 mL of mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min.

Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drugs.

Results and Discussion

Method development for assay (Optimized Trial)

Figure 3, table 1.

Optimized chromatographic conditions for assay

Table 2.

Assay results

Table 3.

System suitability

Tables 4 and 5.

Method precision

Table 6

Linearity and range

Tables 7,8,9 and 10

Table 1: Results for Trail 3.

S.No	Name	RT	Area	TP	TF	R _s
1	Emtricitabine	1.967	34194536	4303	1.51	-
2	Tenofovir	3.823	54108386	8013	1.20	12.7

Table 2: Optimised conditions.

Mobile phase	Sodium phosphate Buffer pH 2.8: Acetonitrile:Methanol (60:20:20) %v/v
Column	PhenomenexC18(50mm x2.1 mm ID) 1.8µm
Flow rate	0.5mL/min
Column temperature	30°C
Sample temperature	15°C
Wavelength	265 nm
Injection volume	10µL
Run time	5 min

Table 3: Results of assay.

Drug	Label claim(mg)	Amount found(mg)	% Assay
Emtricitabine	200	196.38	98.2
Tenofovir	300	297.94	99.3

Table 4: Results for system suitability of Emtricitabine.

Injection	RT	Peak area	Theoretical plates (TP)	Tailing factor (TF)
1	1.967	34194536	4362	1.46
2	1.967	34072012	5682	1.25
3	1.967	33941375	8753	1.17
4	1.963	34192398	8712	1.27
5	1.963	33902378	8624	1.25
6	1.960	33575783	8544	1.19
Mean	1.965	33979747	-	-
SD	0.003	232600	-	-
%RSD	0.2	0.7	-	-

Table 5: Results for system suitability of Tenofovir.

Injection	Tenofovir retention time	Peak area	Theoretical plates	Tailing factor	Resolution
1	3.823	54108386	8112	1.21	12.6
2	3.820	54202133	8763	1.14	11.5
3	3.817	54234358	8854	1.27	12.8
4	3.803	53936936	8561	1.30	13.1
5	3.783	54339791	8743	1.32	12.2
6	3.780	54112192	7895	1.28	11.8
Mean	3.804	54155633	-	-	-
SD	0.019	137275	-	-	-
%RSD	0.5	0.3	-	-	-

Table 6: Method precision results for Emtricitabine and Tenofovir.

Injection	Emtricitabine		Tenofovir	
	Area	% Assay	Area	% Assay
1	33942217	99.3	54335283	99.5
2	33512713	98.0	53884296	98.7
3	33507949	98.0	54091715	99.1
4	33519794	98.0	54660522	100.1
5	33564969	98.2	54144218	99.2
6	33367398	98.6	54219865	99.3
Average	-	98.2	-	99.3
SD	-	0.6	-	0.5
%RSD	-	0.6	-	0.5

Table 7: Linearity preparations.

Preparations	Volume from standard stock transferred in mL	Volume made up in mL (with mobile phase)	Conc. obtained (µg/mL)	
			Emtricitabine	Tenofovir
Preparation 1	1.0	20	100	150
Preparation 2	1.6	20	160	240
Preparation 3	2.0	20	200	300
Preparation 4	2.4	20	240	360
Preparation 5	3.0	20	300	450

Table 8: Linearity data of Emtricitabine

S.No	Concentration (µg/mL)	Area
1	100	15890101
2	160	25254326
3	200	32405343
4	240	38272765
5	300	47706707

Table 9: Linearity data of Tenofovir.

S.No	Concentration (µg/mL)	Area
1	150	25392026
2	240	40262613
3	300	51900805
4	360	61480613
5	450	76802892

Table 10: Observation for linearity.

S.No	Parameter	Emtricitabine	Tenofovir
1	Correlation coefficient	0.9994	0.9994
2	Slope	159586	172121
3	Intercept	11377	468474

Table 11: Results for Recovery of Emtricitabine.

%Recovery	Amount present (µg/mL)	Amount found (µg/mL)*	Percent recovery *	% Mean recovery
50%	100	99.64	99.6	99.5
100%	200	198.58	98.8	
150%	300	300.45	100.2	

* Mean of three observations

Table 12: Results for Recovery of Tenofovir.

%Recovery	Amount present (µg/mL)	Amount found (µg/mL)*	Percent recovery *	% Mean recovery
50%	150	150.31	100.2	99.2
100%	300	298.74	99.2	
150%	450	442.20	98.3	

Accuracy

Table 11,12

Limit of detection

$$LOD = \frac{3.3\sigma}{s} = (3.3) * (2801.69)/15958$$

$$= 0.57 \mu\text{g/ml (Emtricitabine)}$$

$$= (3.3) * (3150.14)/17212$$

$$= 0.60 \mu\text{g/ml (Tenofovir)}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Observation: The LOD for this method was found to be 0.57 µg/ml for Emtricitabine and 0.60 µg/ml for Tenofovir.

Limit of quantification (LOQ)

$$LOQ = \frac{10\sigma}{s}$$

$$= (10) * (2801.69)/15958$$

Table 13: Results for Robustness of Emtricitabine and Tenofovir.

Chromatographic changes		Theoretical Plates		Tailing factor		Resolution
		Emtricitabine	Tenofovir	Emtricitabine	Tenofovir	Between Emtricitabine and Tenofovir
Flow rate (mL/min)	0.4	4236	7185	1.18	1.48	11.9
	0.6	3579	6558	1.35	1.16	11.2
Temperature(°C)	25	3864	6541	1.39	1.18	11.4
	35	3939	6882	1.35	1.18	11.6

$$= 1.75 \mu\text{g/ml (Emtricitabine)}$$

$$= (10) * (3150.14)/17212$$

$$= 1.83 \mu\text{g/ml (Tenofovir)}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Observation: The LOQ for this method was found to be 1.75 µg/ml for Emtricitabine and 1.83 µg/ml for Tenofovir.

Robustness

Table 13

Ruggedness

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts Table 14.

Conclusion

A new precise, accurate, rapid method has been developed for the simultaneous estimation of Tenofovir and Emtricitabine in pharmaceutical dosage form by RP-HPLC. The optimum wavelength for the determination of Tenofovir and Emtricitabine was selected at 265nm on the basis of isobestic point. Various trials were performed with different mobile phases in different ratios, but finally Sodium Phosphate Buffer pH 2.8: Acetonitrile: Methanol (60:20:20) %v/v/v) was selected as good peak symmetry and resolution between the peaks was observed. The Retention time of Tenofovir and Emtricitabine were found to be 1.953 and 3.733 min respectively. The retention times for both the drugs were considerably less compared to the retention time obtained for the drugs in the other mobile phase.

The different analytical performance parameters such as linearity, precision, accuracy, and specificity were determined according to International Conference on Harmonization ICH Q2B guidelines. The calibration curve was obtained by plotting peak area versus the concentration over the range of 100-300 µg/mL For Emtricitabine and 150-450 µg/mL for Tenofovir. From linearity the correlation coefficient R² value was found to be 0.999 for Emtricitabine and 0.999 for Tenofovir. The proposed HPLC method was also validated for system suitability, system precision and method precision. The %RSD in the peak area of drug was found to be less than 2%. The number of theoretical plates was found to be more than 2000, which indicates efficient

Linearity of Emtricitabine

$$y = 159586x - 11377$$

$$R^2 = 0.9994$$

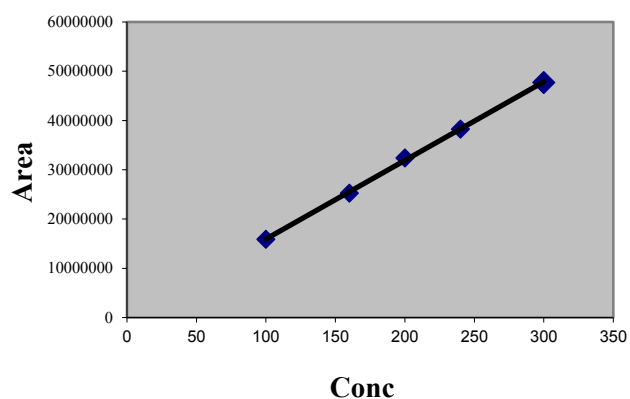


Figure 4: Graph for Linearity data of Emtricitabine.

Linearity of Tenofovir

$$y = 172121x - 468474$$

$$R^2 = 0.9994$$

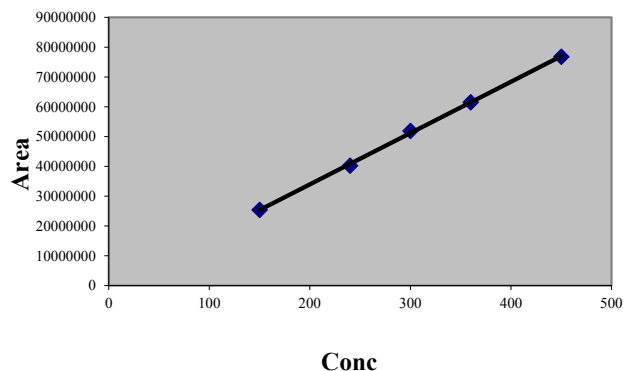


Figure 5: Graph for Linearity data of Tenofovir.

Table 14: Results for Ruggedness.

Emtricitabine	%Assay	Tenofovir.	%Assay
Analyst 01	100.4	Analyst 01	100.3
Analyst 02	99.9	Analyst 02	100.5
% RSD	0.55	% RSD	0.27

performance of the column. The percentage of recovery of Tenofovir and Emtricitabine were found to be 99.5 and 99.2 respectively shows that the proposed method is highly accurate Figure 4,5.

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