Research Paper

Analytical Method Development and Validation for Estimation of Emtricitabine in Tablet Dosage Form by Reverse Phase High Performance Liquid Chromatography

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The aims of research is to develop and validates appropriate ultraviolet spectroscopy and reverse phase high performance liquid chromatography method for analyzing Emtricitabine in a dosage form which are accurate, sensitive and reproducible methods. For the ultraviolet spectroscopy we use wavelength of 280 nm using methanol as solvent and we also validate parameters such as linearity, accuracy, limit of detection, limit of quantification were studied, similarly for reverse phase high performance liquid chromatography method we using methanol (high performance liquid chromatography grade):water (high performance liquid chromatography grade) in the ratio of (60:40) and various validation parameters are evaluated for Emtricitabine by reverse phase high performance liquid chromatography which are determined according to International Council for Harmonization Q2B guidelines. Both the regression coefficient (r^2) was found to be 0.99 and 0.99 for ultraviolet and reverse phase high performance liquid chromatography respectively. The proposed method are effective, highly sensitive, precise and accurate and hence used for determination of Emtricitabine.

Key words: Emtricitabine, ultraviolet spectroscopy, reverse phase high performance liquid chromatography, validation, regression coefficient, International Council for Harmonization

4-Amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] Emtricitabine is a drug. A Nucleoside Reverse Transcriptase Inhibitor (NRTI) called Emtricitabine is used to treat Human Immunodeficiency Virus (HIV) infection in adults. Emtricitabine is a cytidine analogue. Emtricitabine aids in the inhibition of HIV reverse transcriptase, an enzyme produced by your body that is necessary for HIV replication. When treating HIV-positive patients, Emtricitabine is almost always combined with other anti-HIV medications. Emtricitabine functions by preventing the enzyme reverse transcriptase 1 from converting HIV RNA into new viral DNA. A synthetic analogue of cytidine's nucleoside is Emtricitabine. Emtricitabine 5'-triphosphate 2, which is formed when it is phosphorylate by cellular enzymes, inhibits HIV-1 reverse transcriptase[1-5].

Literature survey reveals few chromatographic methods in which different gradient are used for their method validation studies. Highly Sensitive, Selective, Rugged stability indicating High Performance Liquid Chromatography (HPLC) method will be very useful for the estimation of Emtricitabine in pharmaceutical formulations. The purpose of this study was to develop sensitive, simple, precise, accurate method for Emtricitabine by Ultraviolet (UV) and Reverse Phase High Performance Liquid Chromatography (RP HPLC). For that we use the simple mobile phase which are less expensive and easily available than other solvents[6-12].

In the present work, efforts have been made for Emtricitabine and its pharmaceutical dosage form. Several trials have been made with respect to the mobile phase composition, columns, as well as UV

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Accepted 13 February 2024
Revised 31 May 2023
Received 04 August 2022
Indian J Pharm Sci 2024;86(1):294-301

detector's wavelength to develop a suitable and fast method for the analysis of all the three drugs, simultaneously[3,13].

MATERIALS AND METHODS

UV-Spectroscopy method:

Chemicals, reagents and solutions: Emcure Pharmaceutical, Pune kindly gift sample Emtricitabine API. Methanol is analytical reagent grade was used as the solvent for the UV spectroscopic method. For chromatographic analysis methanol of HPLC grade and water of HPLC grade was procured from Merck, Mumbai. Double distilled water was prepared at the laboratory.

Instrument and software: Shimadzu UV-1900 double beam spectrophotometer connected to a computer loaded with Shimadzu UV probe 2.10 software was used for all the spectrophotometric measurements. HPLC is of Agilent Technologies 1120 Compact LC.

Preparation of standard stock solution: About 10 mg of Emtricitabine tablet was weighed and is dissolved in methanol and the final volume was adjusted to 100 ml to obtain stock solution (100 µg/ml) 100 ml. According to that take (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 ml) stock solution to prepared (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ml) solution respectively

Determination of analytical wavelength: About 1 ml is taken from stock solutions and volume adjusted to 10 ml using methanol and the samples were scanned to get good results. The wavelength selected should be such that at wavelength the absorbance of component should be as large as possible. So the wavelength chosen was 280 nm for Emtricitabine (λ_{max}).

Preparation of working standard solution: Appropriate aliquots were pipette out from the standard stock solution into a series of 10 ml volumetric flasks. The volume was made up to the mark with water to get a set of solutions for each drug having the concentration 2, 4, 6, 8, 10 µg/ml. The absorbance of each of these solutions were measured at the selected wavelength 280 nm and plotted against concentration.

UV method validation:

Linearity and range: The linearity was determined by using working standard solutions between 2-10 µg/ml. The absorbance of these solutions was recorded. Calibration curve of absorbance *vs.* concentration plotted on excel sheet linear regression was performed. The correlation coefficient, regression equation of Emtricitabine was calculated by using list square method and it was found that the method was linear at the desired concentration range.

Precision: The reproducibility of proposed method was determined by performing tablet assay at different time intervals (3 h interval) on same day (Intra-day precision) and on 3 consecutive d (Inter-day precision).

Limit of Detection (LOD) and Limit of Quantification (LOQ): LOD and LOQ of Emtricitabine by the proposed methods were determined using calibration standards. LOD and LOQ values were calculated as 3.3×SD/D and 10×SD/D respectively, where D is the slope of the calibration curve and SD is the standard deviation.

Accuracy: The accuracy was determined by standard addition method. Three different levels (80 %, 100 % and 120 %) of standards were spiked to commercial tablet in triplicate. The mean of percentage recoveries and the percentage Relative Standard Deviation (% RSD) was calculated.

HPLC method:

Chemicals, reagents and solutions: HPLC grade methanol and HPLC grade water were purchased from Merck Mumbai, India.

Selection of stationary phase: Luna 5 µ 4.6×250 mm C18 column was selected for analysis.

Selection of mobile phase: HPLC grade Methanol:HPLC grade water (60:40) which was filtered through 0.22 µm membrane filter and sonicate on ultrasonic bath for 15 min.

Selection of wavelength: Emtricitabine standard solution was prepared by adding accurately weighted quantity about 10 mg of Emtricitabine to 100 ml volumetric flask. Add 70 ml of diluents, sonicate to dissolve and dilute up to the mark with diluents and mixed. The standard solution were scanned separately between 400 to 200 nm. From the spectrum wavelength selected as 280 nm. At this spectrum shows high absorbance.

Emtricitabine standard stock solution was prepared by transferring 10 mg of Emtricitabine working standard into a 100 ml volumetric flask, 25 ml of

methanol (HPLC grade) was added and sonicate for 20 min. The volume was made up to 100 ml with HPLC grade water to get concentration of 100 µg/ml.

Preparation of sample solution: Take Tablet 0.5 ml from stock solution (100 ppm), dilute it with 10 ml, it will become 5 ppm. According to that take (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 ml) stock solution to prepared (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ml) solution respectively.

Procedure: Inject 20 µl of the standard, sample into the chromatographic system and measure the areas for the Emtricitabine.

HPLC method validation:

Linearity: The correlation coefficient (r^2) was calculated, and it was between 0.98 to 1.00 which is well within the acceptance criteria. The results are shown below. The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 0.2 to 0.6 mg/ml as shown in below

Precision: The standard solution was injected for three times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits

Accuracy: The accuracy of the RP HPLC method was evaluated by selecting three different concentrations Lower Quantitation Limit (LQC), Middle Quantitation Limit (MQC), and Higher Quantitation Limit (HQC). In each concentration, a minimum of 3 injections were given and the amount of the drugs present, percentage recovery and related standard deviation were calculated.

LOD and LOQ: The LOD and LOQ of Emtricitabine was determined by injecting progressively lower concentration of the standard solutions into the HPLC column using the optimized chromatographic conditions in accordance with 3.3 s/n and 10 s/n criteria, respectively, where s/n indicates signal/noise ratio. LOD= 3.3×SD/S; LOQ=10×SD/S

System suitability: Three replicate injections of system suitability solution were injected. the retention time, areas, theoretical plates, peak symmetry and resolutions were calculated for standard solution.

Robustness: For demonstrating the robustness of the method, slight variations in the optimized conditions were done and the standard solution was injected. The variation made were ±5 % in the ratio of methanol:water in the mobile phase ±0.1 ml/min in the flow rate, ±5° in the column temperature, and ±1 nm in the wavelength. The separation factor, retention time and peak asymmetry were calculate.

RESULTS AND DISCUSSION

The maximum absorption observed at the wavelength of 280 nm hence the wavelength for Emtricitabine is 280 nm hence the wavelength complies with the standard (fig. 1). The linearity was determined by using working standard solutions between 2-10 µg/ml. The absorbance of these solutions were recorded (Table 1). The correlation coefficient, regression equation of Emtricitabine was calculated by using list square method and it was found that the method was linear at the desired concentration range (fig. 2). LOD and LOQ values were calculated as 3.3 SD/D and 10 SD/D respectively, where D is the slope of the calibration curve and SD is the standard deviation (Table 2). Accuracy studies were carried out by standard addition method. Pure Emtricitabine was added at different levels i.e., 80 %, 100 % and 120 % to drug sample present in tablet dosage form (Table 3).

To evaluate the intermediate precision of the method, precision was performed on different day within the laboratory. The standard solution was injected for six times and measured the area for all six injections in HPLC. The % RSD for the area of six replicate injections was found to be within the specified limits (Table 4).

The correlation coefficient (r^2) was calculated, and it was between 0.98 to 1.00 which is within the acceptance criteria. The results are shown in Table 5. The concentration was found to be proportional to the area, and the response of the detector was determined to be linear over the range of 0.2 to 0.6 mg/ml as shown in fig. 3 and fig. 4.

The standard solution was injected for three times and measured the area for all five injections in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits (Table 6). % Recovery at each level was calculated and reported along with % mean recovery. Mean % recovery at each concentration level should be 98.0 % to 102.0 %. RSD of % recovery at each level should not be more than 2.0 (Table 7).

LOD=3.3×SD/S; LOQ=10×SD/S (Table 8).

The separation factor, retention time and peak asymmetry were calculated and shown in Table 9. System suitability was done to verify the repeatability of HPLC method. Theoretical plate, repeatability of retention time and peak area were determined and compared (Table 10).

In the present study suitable UV spectroscopic and RP HPLC method were developed and validated as per International Council for Harmonization guidelines for determination of Emtricitabine in bulk and combined dosage formulation. It was found that the proposed methods were linear, accurate, reproducible, repeatable, precise, selective, cost effective and specific providing the reliability of the methods. The developed methods are recommended for routine and quality control analysis of Emtricitabine in bulk or combination. The amounts found from the proposed methods were in good agreement with the label claim of the formulation. The developed method can also be conveniently adopted for dissolution testing and *in vivo* drug release studies in various pharmaceutical formulations for Emtricitabine.

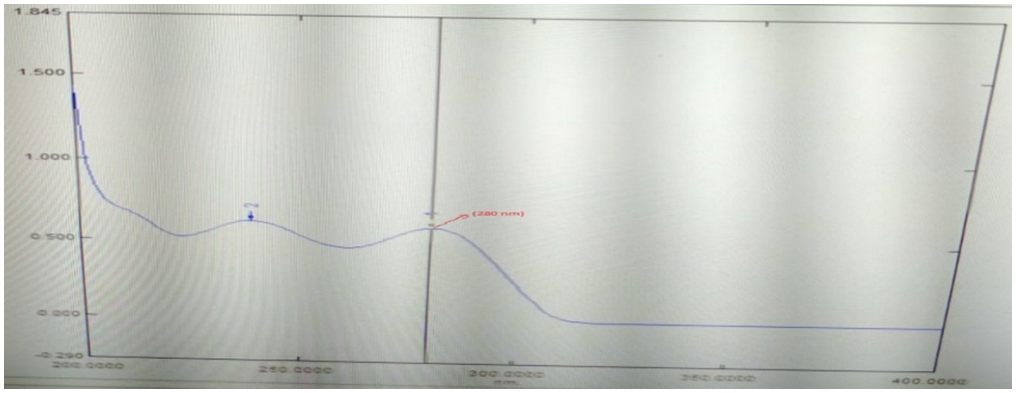

Fig. 1: UV spectra of Emtricitabine
Note: Arrow shows the maximum wavelength for drug Emtricitabine

TABLE 1: LINEARITY AND RANGE (UV)

S no	Concentration (ug/ml)	Absorbance
1	0.2	0.145
2	0.4	0.236
3	0.6	0.344
4	0.8	0.458
5	1	0.569
6	1.2	0.659
	r^2	0.998

Note: Each concentration gives a proper absorbance. as concentration increases the absorbance is also increases

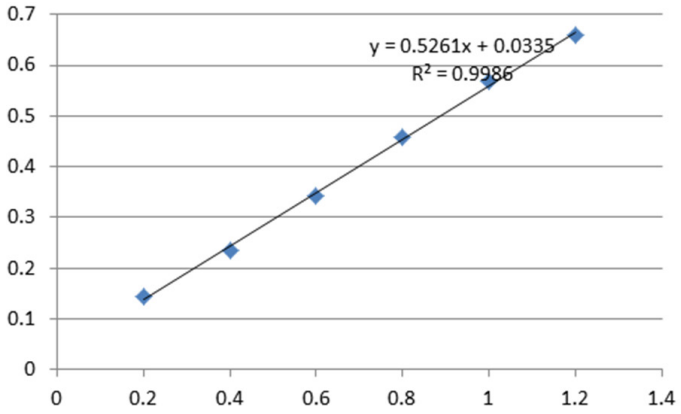

Fig. 2: Calibration curve of Emtricitabine
Note: (◆): Ads and (—): Liner (ads)

TABLE 2: LOD AND LOQ

	Emtricitabine
LOD	1.82
LOQ	2.16

Note: LOD and LOQ is in acceptance criteria

TABLE 3: ACCURACY OF EMTRICITABINE

S no	% level recovery	Amount added (ug/ml)	Amount found (ug/ml)	% recovery	Mean
1	80	4	3.92	99.4	99.3
2	80	4	3.87	98.85	
3	80	4	3.89	99.64	
4	100	5	4.91	99.2	99.45
5	100	5	4.93	98.6	
6	100	5	4.96	99.2	
7	120	6	5.87	99.64	99.6
8	120	6	5.98	99.75	
9	120	6	5.88	99.6	

TABLE 4: PRECISION (INTRA-DAY AND INTER-DAY)

Analyte	Absorbance		
	0 h	3 h	6 h
Intra-day precision (UV)			
Mean	0.5066	0.5006	0.3756
SD	0.0128	0.0126	0.0043
% RSD	0.6813	0.3021	1.189
Inter-day precision (UV)			
Mean	0.5066	0.3833	0.3664
SD	0.0128	0.0042	0.0026
% RSD	0.6813	1.0756	0.7923

TABLE 5: LINEARITY AND RANGE (HPLC)

S no	Concentration (µg/ml)	Area (linearity)
1	20	18682616
2	40	33965464
3	60	52114116
4	80	68062528
5	100	82323281
6	120	96154876
	Correlation coefficient (r^2)	0.9974

Note: Correlation coefficient (r^2) is in acceptance range

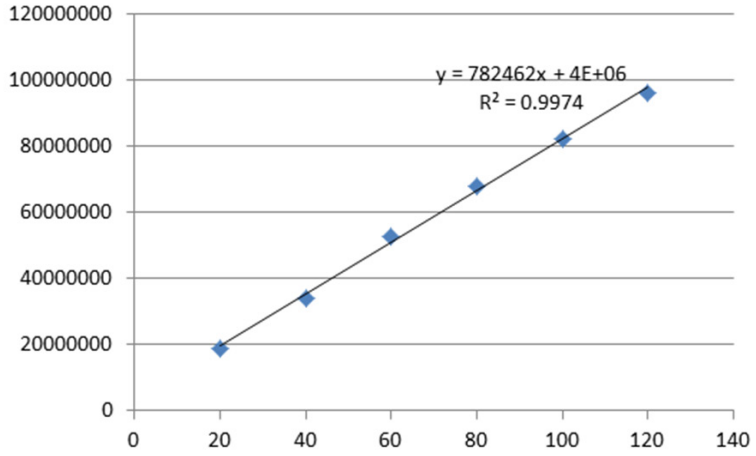

Fig. 3: Calibration curve of Emtricitabine by HPLC
Note: (◆): Area and (—): Linear (area)

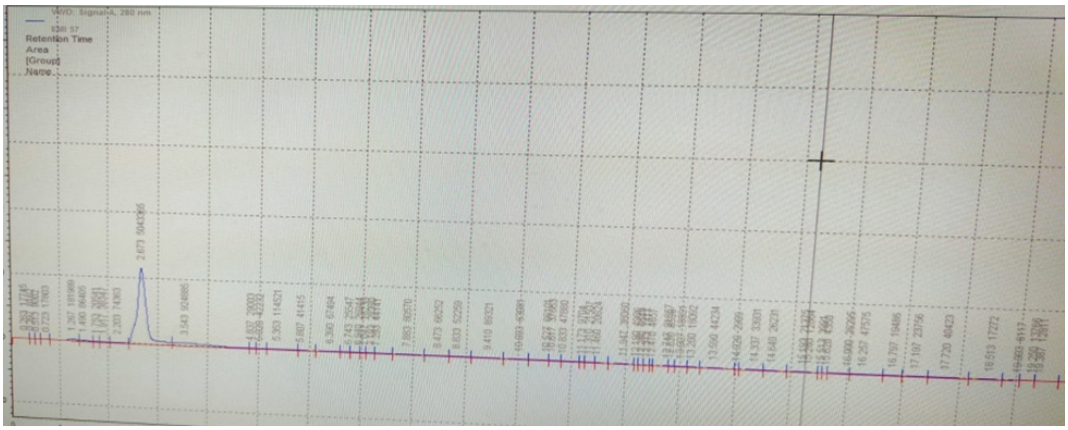

Fig. 4: Chromatogram for the drug Emtricitabine

TABLE 6: PRECISISON (INTRA-DAY AND INTER-DAY BY HPLC)

Analyte	Intraday precision			Inter day precision (Area)		
	2 h	4 h	6 h	1st d	2nd d	3rd d
Mean	33641905	33376096	33078930	34253263	34641344	36141484
Standard deviation	394621.32	428768.11	352469.38	439332.04	444061.68	364598.83
Relative standard deviation	1.10 %	1.20 %	1.00 %	1.20 %	1.20 %	1.00 %

Note: Above values of relative standard deviation is in within the range

TABLE 7: ACCURACY BY HPLC

S no	Concentration (ppm)	% level recovery	Amount added (ug/ml)	Amount found (ug/ml)	% Recovery	Mean
1	30	80	24	23.77	99.04	
2	30	80	24	23.82	99.25	99.6
3	30	80	24	22.76	98.91	
4	30	100	30	29.54	98.46	
5	30	100	30	29.5	98.33	98.7
6	30	100	30	29.82	99.4	
7	30	120	36	35.54	98.72	
8	30	120	36	35.3	98.05	98.7
9	30	120	36	35.72	99.22	

Note: Result given in the data is within the acceptance criteria

TABLE 8: LOD AND LOQ

S No	Parameter	Value
1	LOD	1.53 ug/ml
2	LOQ	4.64 ug/ml

Note: The value of LOD and LOQ are within the acceptance criteria

TABLE 9: ROBUSTNESS (CHNGE IN FLOW RATE, MOBILE PHASE RATIO AND WAVELENGTH)

	Area	USP plate count	USP tailing
Flow rate			
0.9	32962397	2774	1.14
1	33250323	2714	1.18
1.2	33694546	2792	1.17
Mean	33302422	2760	1.16
SD	368844.5	40.84	0.02
% RSD	1.1	1.47	1.78
Mobile phase ratio			
55:45:00	32163279	2708	1.14
60:40:00	33004141	2730	1.18
65:35:00	37171130	2778	1.17
Mean	32446183	2738.66	1.16
SD	483221.5	35.79	0.02
% RSD	1.48	1.3	1.78
Wavelength			
280	32759441	2652	1.21
281	33523350	2620	1.19
282	32692397	2590	1.18
Mean	32991729	2620.6	1.19
SD	461615.8	31	0.015
% RSD	1.39	1.18	1.28

Note: % RSD is within the acceptance criteria

TABLE 10: SYSTEM SUITABILITY

Injection	RT (min)	Peak area	USP plate count (N)	USP tailing (T)
1	2.55	38545477	2556	1.43
2	2.46	38411765	2550	1.46
3	2.51	38516654	2521	1.44
Mean	2.5	38491299	2542.33	1.44
SD	0.036	57456.6	18.71	0.01
% RSD	1.46	0.149	0.73	1.05

Acknowledgements:

The authors would like to thank Emcure Pharmaceutical, Pune for providing Emtricitabine (gift sample).

Conflict of interest:

The authors confirm that this article's content has no conflict of interest.

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