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Research Article

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHOD FOR DISSOLUTION TESTING OF EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE IN TABLET DOSAGE FORM BY UPLC

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ABSTRACT

A simple, accurate and precise UPLC method was developed and validated for dissolution testing of Emtricitabine and Tenofovir disoproxil fumarate in tablet dosage form. The separation was achieved under optimized chromatographic condition on an Acquity UPLC HSS (50 mm X 2.1 mm, 1.7 µm) column with mobile phase consist of 20 mM Potassium dihydrogen phosphate buffer pH 3.5: Acetonitrile with gradient elution at a flow rate of 0.640 ml/min using 30°C column oven temperature with UV detection at 262 nm. The retention time for Emtricitabine and Tenofovir disoproxil fumarate were about 0.715 and 2.239 min respectively. The linearity was found to be in the concentration range of 20.14-120.86 µg/mL for Emtricitabine and 30.01-180.04 for Tenofovir disoproxil fumarate. The % recoveries at 20%, 100% and 120% were found to be within the limit of 98-102%. The method was validated as per ICH and USP guideline and the values were found to be within the limits. So, the proposed method was found to be simple, linear, accurate, precise, robust and specific.

Keywords: Emtricitabine, Tenofovir Disoproxil Fumarate, Dissolution, UPLC

INTRODUCTION

Emtricitabine4-amino-5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one is an NRTI used in the treatment of HIV infection and chronic hepatitis B virus (HBV) (Figure 1)[1]. Tenofovir disoproxil fumarate (TDF) {9-[(R)-2- [[bis [[isopropoxycarbonyl] oxy] methoxy] phosphonyl] methoxy] popyl] adenine fumarate} is a nucleotide analog reverse transcriptase inhibitor (NRTI) and is used for treating HIV infection in adults, in combination with other antiretroviral agents (Figure 1)[2]. Dissolution testing is a requirement for all solid oral dosage forms and is used in all phases of development for product release and stability testing. At early stages of development *in vitro* dissolution testing guides the optimization of drug release from formulations. The FDA guidance on dissolution testing for immediate release solid oral dosage forms includes the use of the Bio pharmaceutics Classification System (BCS) guidelines for bio relevant dissolution tests, which is based upon API solubility and permeability. *In vitro* dissolution specifications are established to ensure batch-to-batch consistency and to signal potential problems with *in vivo* bioavailability[3-6].

Ultra performance liquid chromatography (UPLC) takes advantage of technological strides made in particle chemistry performance, system optimization, detector design and data processing and control. Using sub-2 µm particles and mobile phases at high linear velocities and instrumentation that operates at higher pressures than those used in HPLC, dramatic increases in resolution, sensitivity, and speed of analysis can be obtained. This new category of analytical separation science retains the practicality and principles of HPLC while creating a step-function improvement in chromatographic performance[7].

During literature review multiple analytical methods have been identified for simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumarate in fixed dose combinations by HPLC[8], by UPLC[9] and by spectrophotometric[10] but not a single method is available for dissolution testing by UPLC. This article presents how to select dissolution media, dissolution parameters and what should be the criteria for product release.

Dissolution parameters were selected as per guidance for Industry-Dissolution Testing of Immediate Release Solid Oral Dosage Forms. This guidance is developed for immediate release (IR) dosage forms and is intended to provide general recommendations for dissolution testing; approaches for setting dissolution specifications related to the bio pharmaceutics characteristics of the drug substance; statistical methods for comparing dissolution profiles and a process to help determine when dissolution testing is sufficient to grant a waiver for an *in vivo* bioequivalence study[11].

MATERIALS AND METHODS

Chemicals/Reagents

Potassium dihydrogen phosphate, o-phosphoric acid, sodium acetate trihydrate, glacial acetic acid, hydrochloric acid (35%) and HPLC grade acetonitrile were purchased from Merck (India). Gift samples of analytical standards were provided by Piramal Enterprise ltd (India). Truvada tablet formulation purchased from market.

Instrument

The Waters UPLC with PDA detector equipped with Empower software was used for the method development and validation study.

Selection of dissolution medium

BCS solubility was performed to select suitable dissolution medium. Solubility of Emtricitabine and Tenofovir disoproxil fumarate was performed in different pH buffer.

Procedure

For BCS solubility highest dose of API needs to be dissolved in 250 mL of respective buffer media, but to avoid use of large amount of API, volume of buffer and API weights were scaled down to maintain same concentration as in 250 mL of buffer. Table 1 shows the actual amount and scaled down amount of API and volume of buffer medium. Samples were kept in thermo mixer at 900 RPM and 37°C temperature for 24 hours. After 24 hours' samples were centrifuged and suitable dilution carried out with respective buffer. Samples were analyzed using HPLC technique. Analytical standards were prepared in methanol due to high solubility of all API in methanol. All API were dissolved in water, 0.01N HCl, 0.1N HCl, pH-4.5 acetate buffer and pH-6.8 phosphate buffer.

Maximum solubility of Emtricitabine was in 0.01N HCl and Tenofovir disoproxil fumarate was in 0.1N HCl to achieve sink condition. However, during literature review it has been observed that Tenofovir disoproxil fumarate tends to degrade in 0.1 N HCl and generate monoester impurity. Hence, considering good solubility and stability in 0.01N HCl media, this media is selected as a dissolution medium. Table 2 shows the results of solubility study.

Selection of dissolution parameters

Dissolution parameters selected as per guidance for Industry-Dissolution Testing of Immediate Release Solid Oral Dosage Forms. All dissolution parameter mentioned in Table 3

Method development for dissolution testing of anti-HIV drugs

Waters Acquity UPLC was used with PDA detector and auto injector module to perform analysis of samples. 20 mM phosphate buffer pH-3.5 ± 0.05 was selected as a mobile phase-A and acetonitrile 100% as a mobile phase-B. Due to high difference in polarity of both actives, the gradient elution was performed for separation. Samples were injected on C18 column (UPLCHSS 50 × 2.1 mm; 1.8 µm) which was eluted at 0.640 mL/min. Injection volume kept 1 µL. UPLC column temperature was set to 30°C and auto sampler temperature kept ambient. Selected gradient was as follows: 0-0.50 min, isocratic 5% B 0.50-2.50 min, linear gradient 5-80% B; linear gradient 80-5%; 2.51-4.50 min.

Sample preparation

Individual tablets were weighed and transferred to each six individual dissolution bowl having a 900 mL of 0.01N HCl which was pre equilibrated at 37°C ± 0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point)' sample aliquots collected and filtered through 10 µm PVDF filters after discarding 5 mL of filtrate. Pipetted out 5.0 mL of filtered solution into a 10 mL volumetric flask and diluted up to the mark with dissolution media and mixed well.

Preparation of analytical standards

Accurately weighed and transferred about 15 mg Emtricitabine and 10 mg Tenofovir disoproxil fumarate into 25 mL of clean, dry volumetric flask. Added 5 mL of methanol and sonicated to dissolve; Diluted up to the mark with dissolution medium. Pipetted out 5.0 mL of this solution into a 20 mL volumetric flask and diluted up to the mark with dissolution media and mixed well.

Method optimization

Our finalized method for the chromatographic separation of anti-HIV is described further on in Section 2.8. The parameters that were optimized are described below.

Alternative chromatographic conditions

Dissolution samples were analyzed by UPLC equipped with a quaternary pump, auto sampler and column oven and photodiode array detector. Mobile phases and UPLC conditions tested were:

UPLC BEH column and mobile phase consisting of 20 mM phosphate buffer pH- 3.5 ± 0.05 (A), acetonitrile (B). Samples were eluted with a gradient of (B), 0-2.29 min, linear gradient 5-80% B 2.29-2.78 min, isocratic 80% B; 2.78-2.83 min, linear gradient 80-5% B; 2.83-4.72 min, isocratic 5% at a flow rate of 0.64 mL/min. Detection was achieved using wavelengths 262 nm. UPLC BEH column and mobile phase consisting of 20 mM phosphate buffer pH- 3.5 ± 0.05 (A), acetonitrile (B). Samples were eluted with a gradient of (B), 0-0.50 min, isocratic 5% B 0.50-2.50 min, linear gradient 5-80% B; 2.50-2.51 min, linear gradient 80-5% B; 2.51-4.50 min isocratic 5% at a flow rate of 0.64 mL/min. Detection was achieved using wavelengths 260 nm. UPLC HSS column and mobile phase consisting of 20 mM phosphate buffer pH- 3.5 ± 0.05 (A), acetonitrile (B). Samples were eluted with a gradient of (B), 0-0.50 min, isocratic 5% B 0.50-2.50 min, linear gradient 5-80% B; 2.50-2.51 min, linear gradient 80-5% B; 2.51-4.50 min isocratic 5% at a flow rate of 0.64 mL/min. Detection was achieved using wavelengths 260 nm.

Optimized method for analysis of dissolution samples

The final in-house method developed for dissolution testing of anti-HIV drugs in Tablet formulation. Individual tablets were weighed and transferred to each six individual dissolution bowl having a 900 mL of 0.01N HCl which was pre equilibrated at 37°C ± 0.5°C. RPM was set to 50 and dissolution was run. After 45 minutes (Q time point) sample aliquots collected and filtered through 10 µm PVDF filters after discarding 5 mL of filtrate. Pipetted out 5.0 mL of filtered solution into a 10 mL volumetric flask and diluted up to the mark with dissolution media and mixed well.

For chromatographic separation of the anti-HIV drugs, dissolution samples were analyzed by Waters Acquity UPLC equipped with a quaternary pump, auto sampler and column oven and photodiode array detector. The selected column was a UPLC HSS (50 × 2.1 mm; 1.8 µm) (Waters). Mobile phase consisted of 20 mM phosphate buffer pH- .5 ± 0.05 (A), acetonitrile (B). Samples were eluted with an increasing gradient of (B), 0-0.50 min, isocratic 5% B 0.50-2.50 min, linear gradient 5-80% B; 2.50-2.51 min, linear gradient 80-5% B; 2.51-4.50 min isocratic 5% at a flow rate of 0.64 mL/min. The total run time was 4.50 min. The

injection volume was 1 µL. The column temperature was held at 30°C. The PDA detection wavelength was 262 nm.

Method validation

The performance characteristics considered for validation of the optimized method were: specificity, linearity and working range, accuracy, precision and robustness.[12-13]

Specificity

Specificity was performed by checking interference from dissolution medium and placebo (excipients of formulation) at the retention time of both active in standard preparation. The placebo solution consisted of all the excipients without the drug as per test preparation.

Linearity and working range

Linearity was assessed visually. The working range was defined as the interval between the upper and the lower levels of the analytes within the calibration curve. A series of standard preparations were prepared over a range of 20% to 120 % of sample preparation.

Accuracy

Accuracy of analytical method was evaluated by recovery study. Known amount of API and placebo spiked in 1000 mL of dissolution medium at different level (20%, 100% and 120%). The accuracy is calculated as % recovery. Individual recovery, mean recovery and % RSD at each level are calculated and reported.

System precision

In order to optimize the efficiency of a chromatographic separation, the quality of the chromatography was monitored by applying the system suitability tests: % RSD of area response for five replicate injections and all bracketing injection of diluted standard for Emtricitabine and TDF peaks is NMT 2.0%

% **Method precision**

Six sample sets were injected to determine repeatability of analytical method.

Solution stability

Standard preparation and sample preparations were stored at room temperature and at 2-8°C for 2 days and analyzed against freshly prepared standards to check solution stability.

Robustness

Robustness of an analytical method was evaluated by changes in column oven temperature, detection wavelength and buffer pH. System suitability monitored during robustness study.

RESULTSAND DISCUSSION

Method Optimization

Alternative Chromatographic conditions

Optimization of the chromatographic separation of anti-HIV drugs was based on polarity of drugs and appropriate mobile phase techniques. Analyte peak identification was based upon retention time match with the reference standards.

The UPLC method was developed using Waters H-class Acquity equipment and based on sub 2 micron column chemistry, UPLC BEH (50 × 2.1 mm; 1.7 µm) was selected. High tailing factor was observed for Emtricitabine and TDF peaks. In further experiment, gradient program was modified to achieve better symmetrical peak of both actives, however there was no improvement observed in peak tailing. So, it was decided to select high strength silica (HSS) column for better peak symmetry than the BEH column with same mobile phase gradient program. Both columns were compared for peak symmetry. Figure 2 shows the differences in peak symmetry of Emtricitabine and TDF between BEH and HSS column.

Method Validation

Specificity

Specificity was performed by checking interference from dissolution medium and placebo (excipients of formulation) at the retention time of both active in standard preparation. There was no any interference found at retention time of Emtricitabine and TDF due to placebo and dissolution media. This indicated that method is specific.

Linearity and working range

A calibration curve was constructed for Emtricitabine and TDF. The detector response to both active was plotted against a series of concentrations (20.14-120.86 µg/mL for Emtricitabine and 30.01-180.04 µg/mL for TDF) and Linearity determined visually. Correlation coefficient of >0.999 achieved (Figure 3). The working range was defined as the interval between the upper and the lower levels of the analytes within the calibration curve (Table 4).

Accuracy

Table 6 and 7 summarizes the accuracy of analytical methodology. Accuracy is expressed as percent recovery. Accuracy performed in three sets and % RSD between three sets reported.

Method precision

Table 8 summarizes the precision of analytical methodology. Method precision is expressed as the RSD between six replicate measurements for Emtricitabine and TDF in as per dissolution condition.

Solution stability

Results of the stability experiments are summarized in Table 9 and Table 10. Standard and samples were considered stable at room temperature and at 2-8°C for 2 days.

Robustness

Results of the robustness experiments are summarized in Table 11 and Table 12. Robustness study was performed by changing column temperature (30 ± 5°C), Change in Wavelength (262 nm ± 2 nm), Change of pH in buffer (3.5 ± 0.2), change in column lot. Results were found within the range.

Figure 1: Structures of anti-HIV drugs. A, (-) Emtricitabine; B, (-) Tenofovir disoproxil fumarate

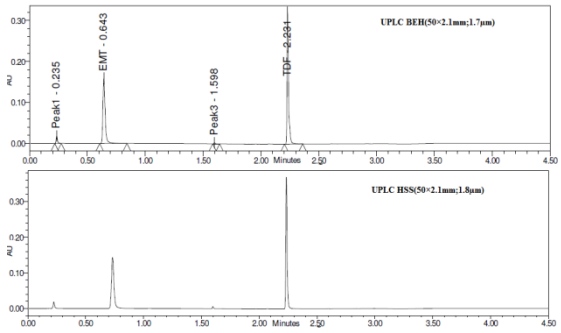

Figure 2: Comparison between columns for peak symmetry of Emtricitabine and TDF in standard preparation

Figure 3: Linearity curves of, A, (-) Emtricitabine; B, (-) Tenofovir disoproxil fumarate

Table 1: Actual amount and Scaled Down amount of Api and Buffer Volume

Name of API	Actual amount of API (mg)	Actual volume of buffer (mL)	Scaled down amount of API (mg)	Scaled down volume of buffer (mL)
Emtricitabine	300	250	12	10
TDF	300	250	12	10

Table 2: Results of Solubility Study

Buffer media	Solubility (mg/mL)	
	Emtricitabine	Tenofovir disoproxil fumarate
0.01N HCl	197.0	11.0
0.1N HCl	185.0	52.8
Water	191.5	7.5
pH-4.5 acetate buffer	133.6	9.3
pH-6.8 phosphate buffer	153.2	8.0

Table 3: Dissolution Parameters

Apparatus	USP Apparatus –II (Paddle)
Stirrer speed	50
Dissolution medium	0.01N HCl
Bath temperature	37°C ± 0.5°C
Media volume	900 mL
Sample volume	10 mL
Replenish volume	No replacement
Time points	45 minutes
Filters	10 µm full flow PVDF filters

Table 4: Linearity Data of Emtricitabine

Linearity Level (%)	Conc. (µg/mL)	Area	Response Factor
20	20.1400	42723	2121
40	40.2900	88143	2188
80	80.5700	171637	2130
100	100.7200	216785	2152
120	120.8600	260001	2151
Correlation Co-efficient		1.000	
y-Intercept		129.8	
Slope		2147.5	
% Y Intercept bias		0.1	
% RSD of Response factor		1.2	

Table 5: Linearity Data of TDF

Linearity Level (%)	Conc. (µg/mL)	Area	Response Factor
20	30.0100	58302	1943
40	60.0100	120358	2006
80	120.0300	233263	1943
100	150.0300	293939	1959
120	180.0400	352267	1957
Correlation Co-efficient		1.000	
y-Intercept		1043.6	
Slope		1949.4	
%Y Intercept bias		0.5	
%RSD of Response factor		1.3	

Table 6: Accuracy of Emtricitabine

Level	Sample ID	Amount Added (µg/mL)	Amount Recovered (µg/mL)	% Recovery	% Mean	% RSD
50%	Set 1	55.25	55.26	100.02	100.3	0.2
	Set 2	54.84	55.09	100.45		
	Set 3	54.72	54.92	100.37		
100%	Set 1	110.45	110.49	100.03	99.5	0.4
	Set 2	110.69	109.86	99.25		
	Set 3	110.59	109.86	99.34		
150%	Set 1	165.90	165.06	99.49	99.9	0.3
	Set 2	165.73	165.99	100.16		
	Set 3	165.86	165.83	99.98		

Table 7: Accuracy of TDF

Level	Sample ID	Amount Added (µg/mL)	Amount Recovered (µg/mL)	% Recovery	% Mean	% RSD
50%	Set 1	82.34	81.63	99.26	99.4	0.3
	Set 2	82.37	82.13	99.72		
	Set 3	82.71	82.14	99.28		
100%	Set 1	164.70	164.17	99.68	99.8	0.1
	Set 2	165.97	165.54	99.74		
	Set 3	165.54	165.31	99.86		
150%	Set 1	249.44	250.43	100.40	100.2	0.2
	Set 2	249.22	249.86	100.26		
	Set 3	249.52	249.63	100.04		

Table 8: Method Precision

S. No.	% Drug release	
	Emtricitabine	TDF
1	100	97
2	99	101
3	101	97
4	98	99
5	99	100
6	99	98
Mean	99	99
% RSD	1.0	1.7

Table 9: Solution Stability of Standards

Condition	Emtricitabine		TDF	
	% Assay	Absolute difference	% Assay	Absolute difference
Initial (T_0)	100.0	NA	100.0	NA
Room temperature Day-1 (15-25° C)	100.2	0.2	100.4	0.4
2-8° C Day-1	100.1	0.1	100.2	0.2
Room temperature Day-2 (15-25° C)	100.2	0.2	100.5	0.5
2-8° C Day-2	100.1	0.1	100.3	0.3

Table 10: Solution Stability of Sample

Condition	Emtricitabine		TDF	
	% Release	Absolute relative percent	% Release	Absolute relative percent
Initial (T_0)	100	NA	97	NA
Room temperature Day-1 (15-25° C)	101	101	98	101
2-8° C Day-1	100	100	98	101
Room temperature Day-2 (15-25° C)	101	101	98	101
2-8° C Day-2	100	100	98	101

Table 11: Robustness Data for Emtricitabine

Parameters	Condition	% RSD	Tailing Factor	Theoretical Plates	USP Resolution
Change in Column oven (30°C ± 5°C)	25°C	0.2	1.2	5835	17.1
	35°C	0.2	1.3	5857	15.7
Change in Wavelength (262 nm ± 2 nm)	260	0.2	1.2	5848	16.6
	264	0.2	1.2	5849	16.5
Change of pH in buffer (3.5 ± 0.2)	3.3	0.1	1.2	5645	14.9
	3.7	0.3	1.2	5580	17.2
Column lot change	NA	0.3	1.2	5620	15.7

Table 12: Robustness Data for TDF

Parameters	Condition	% RSD	Tailing Factor	Theoretical Plates	USP Resolution
Change in Column oven (30°C ± 5°C)	25°C	0.2	1.4	200797	33.0
	35°C	0.2	1.3	207985	33.8
Change in Wavelength (262 nm ± 2 nm)	260	0.1	1.4	201308	32.9
	264	0.5	1.4	200986	32.7
Change of pH in buffer (3.5 ± 0.2)	3.3	0.1	1.3	197696	32.7
	3.7	0.5	1.3	196453	32.8
Column lot change	NA	0.2	1.3	202284	33.3

CONCLUSION

A convenient, rapid, accurate and precise UPLC method was developed for the simultaneous determination and quantification of Emtricitabine and TDF in dissolution media from tablet formulation. This method can be said to be economical and time effective. The established method utilizes a HSS stationary phase with binary mobile phase consisting of 20 mM Phosphate buffer pH- 3.5 and acetonitrile using gradient program. The method was validated as per ICH guidelines and found to be specific, precise, linear, accurate, rugged and robust. The established dissolution conditions including dissolution medium i.e. 0.01 N HCl are suitable considering its sink condition and stability of both actives. The method suitable for the determination of Emtricitabine and TDF in tablet dosage form and hence can be used for routine quality control test.

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