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A Rapid Stability Indicating RP-HPLC Method and the Degradation Kinetics Data for the Simultaneous Estimation of Tenofovir Disoproxil Fumarate and Emtricitabine

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ABSTRACT

A gradient stability indicating assay method was developed and validated according to the ICH guidelines for simultaneous estimation of Emtricitabine (FTC) and Tenofovir Disoproxil Fumarate (TDF) in marketed tablet formulation. The resolution between the drugs and their degradants was achieved on HiQ Sil C18 HS HPLC column with mobile phase consisting of ammonium acetate buffer (pH 5.5) and methanol at a wavelength of 260 nm. Drugs were subjected to forced hydrolytic, oxidative, photolytic and thermal degradation conditions. FTC showed degradation in alkaline (0.5 N NaOH) and oxidative condition (3% H_2O_2) at room temperature and acidic degradation (0.1 N HCl) under reflux conditions at 80^0C. Complete degradation was observed for TDF in alkaline medium (0.1 N NaOH) at room temp. TDF also showed degradation in acidic medium (0.1 N HCl) when refluxed at 80^0C. The described stability indicating assay method was used to study degradation kinetics of TDF and FTC under different forced degradation conditions as specified above. The kinetic parameters like rate constant (K), half life ($t_{1/2}$), shelf life (t_{90}) were calculated and order of reaction was determined. The best fit corresponding to the first order kinetics was observed in the studies of the degradation kinetics of FTC and TDF.

INTRODUCTION

Emtricitabine (FTC) chemically is 4-amino-5-fluoro-1-[(2S,5R)-2-(hydroxylmethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one **(Figure 1)**. It is a nucleoside reverse transcriptase inhibitor (NRTI), a fluorinated derivative of lamivudine (3TC), an analog of deoxycitidine, which is phosphorylated to its active metabolite, emtricitabine 5'-triphosphate. It is active against HIV-1, HIV-2 and hepatitis B virus.

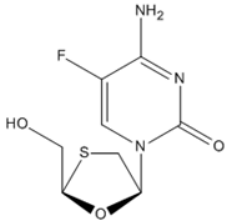

Figure 1. Chemical structure of FTC

Tenofovir Disoproxil Fumarate (TDF) chemically is 9-[(R)-2[[bis[[(isopropoxycarbonyl) oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate **(Figure 2)**, a nucleotide reverse transcriptase inhibitor (NtRTI), the ester prodrug of tenofovir which is hydrolyzed to tenofovir intracellularly and phosphorylated to the active metabolite, tenofovir diphosphate . Tenofovir is a nucleotide analogue of deoxyadenosine monophosphate, with activity against HIV-1, HIV-2 and Hepatitis B virus (HBV). Tenofovir disoproxil fumarate-emtricitabine (TDF-FTC) is a once-daily, fixed-dose NtRTI/NRTI combination that has demonstrated efficacy in well-designed clinical trials with good follow up[1-3].

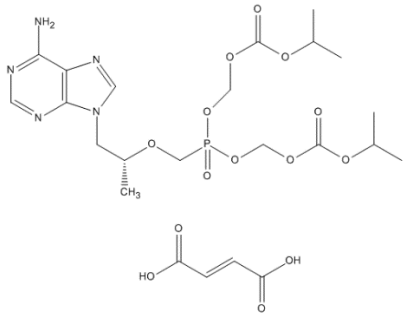

Figure 2. Chemical structure of TDF

As a part of the overall analytical control strategy, current regulations require stability indicating assay methods (SIMs) to demonstrate product integrity until the re-test period of drug substances or throughout the shelf life of the drug products. Forced degradation studies show the chemical behaviour of the molecule which in turn helps in the development of formulation and package. The degradation kinetics study reveals the rate of instability of drug molecules and serves as guidelines during preformulation stage with the appropriate storage recommendations. The kinetics data also help in predicting the shelf–life of the product and to solve problems during formulation and storage[4].

The analysis of Tenofovir Disoproxil Fumarate and Emtricitabine as combination in tablets is official in Indian Pharmacopoeia (I.P) 2010[5]. Literature survey revealed RP-HPLC[6-11], HPTLC[12-14], UV- spectrophotometric[15,16], LC-MS[17], LC-MS-MS[18,19] methods of analysis for determination of both the drugs TDF and FTC alone or in combination. To the best of our knowledge, no stability indicating assay method by HPLC for the simultaneous estimation of TDF and FTC was found to be reported. The data pertaining to the degradation kinetics of both TDF and FTC were also not reported. So, the aim of the work was to develop a stability indicating assay method for the simultaneous estimation of TDF and FTC by HPLC, and to carry out forced degradation studies and degradation kinetics study on the individual drugs that can help to solve the instability problems during the product development stage.

MATERIALS AND METHODS

Chemicals and Reagents

The standard drugs Emtricitabine (FTC) and Tenofovir disoproxil fumarate (TDF) were procured as gift samples from Cipla Ltd, Vikhroli, India. Marketed tablet formulation of TDF and FTC in combination (TENVIR-EM) was purchased from local pharmacy. Methanol and acetonitrile (HPLC grade) from Merck Chemical Company (India) and o-phosphoric acid, ammonium acetate (analytical grade) from S.D Fine Chemicals (Mumbai, India) were used for the study. Double distilled water was obtained in-house from Quartz double distillation plant of Lab-Sil instruments, India, Model No. LQD-5 and was filtered through 0.45 µm membrane filter for the HPLC analysis.

Optimised HPLC Chromatographic Conditions

The chromatographic system consisted of JASCO HPLC system with the JASCO-PU 2080 PLUS intelligent HPLC pump, and JASCO UV-2075 plus intelligent UV-Vis detector. The software used was Borwin software, Varian star 800 MODULE INTERFACE. A 20 µl Rheodyne injector port was used for injecting sample solutions. A HiQ Sil C18HS (250×4.6 mm; 5 µm particle size) column maintained at ambient temperature was used for the analysis. The gradient method consisting of mobile phase -20mM Ammonium acetate (pH 5.5 ± 0.2): methanol in a composition of 85:15 (0-20 min), 20: 80 (20-22 min), 20:80 (22-30 min), 85:15 (30-32 min), 85:15 (32-37 min) was found to be the most optimum method for separation of both the drugs TDF and FTC, and all their degradants in combination. The pH of buffer was adjusted with o-phosphoric acid. The mobile phase was filtered through 0.45 µm membrane filter followed by sonication for 10 min using bath sonicator. The optimum wavelength selected for quantification was 260 nm with a total run time of 35 min.

Preparation of solutions

Preparation of standard stock solution of drugs

Standard stock solution of both drugs FTC and TDF of concentration of 1000 µg/ml (1000 ppm) was prepared in methanol by dissolving 10 mg of each drug in 10 ml of methanol. The standard stock solutions were diluted with the mobile phase to get solutions in concentrations ranging from 0.1 µg/ml to 100 µg/ml.

Analysis of marketed tablet formulation

Preparation of test solution

Twenty marketed tablets of FTC and TDF in combination (TENVIR-EM, Cipla Ltd) were weighed and crushed to give a fine powder. Tablet powder equivalent to weight of one tablet (1.0172 g) was weighed and transferred to a 100 ml volumetric flask. Both drugs were extracted using a mixture of methanol and water in the ratio of 70:30 and were kept for bath sonication. The volume was made up to 100 ml with methanol and water mixture. The solution was filtered through 0.45 µm membrane filter. 1 ml of the filtered tablet solution

was diluted to 10 mL with mobile phase. Then 1 ml of this solution was further diluted to 10 ml with mobile phase to give test solution concentration of 20 µg/mL of FTC and 30 µg/mL of TDF. The peak areas of standard and sample solutions were compared and percentage assay was calculated.

Forced degradation samples of FTC and TDF

For all hydrolytic and oxidative degradation conditions, 15 mg of each drug was weighed and dissolved in 15 ml of acid (0.1 N, 0.2, 0.5, 1 N HCl), base (0.1 N, 0.2, 0.5, 1 NaOH), distilled water and hydrogen peroxide (3 % v/v and 10 % v/v) respectively. The reactions were monitored for 24 hr at room temperature and for 8 hr under reflux condition at 80ºC using TLC. In case of acid and base degradation, reaction mixture was neutralized by using same strength of acid and base respectively. The photolytic degradation study was carried both in solid and solution form by exposing 100 mg and 1mg/ml methanolic solution of each drug respectively to direct sunlight daily for 4 hr and was studied for 15 days. Dry heat degradation for TDF was carried out by subjecting 100 mg of drug to dry heat in hot air oven at 60ºC for 24 hr, and for FTC by subjecting 100 mg of drug to dry heat in hot air oven at 100ºC for 5 hr. For HPLC analysis of degradants, final dilutions of the samples were done in mobile phase.

Degradation kinetics study on FTC and TDF

The oxidative degradation kinetics study on FTC was carried out using 3 % H_2O_2 at room temperature. Samples were withdrawn at time interval of 0, 0.25, 0.5, 1, 2, and 3 hr. The degradation kinetics under acidic and basic conditions for FTC was studied using 0.1 N HCl at reflux conditions and 0.5 N NaOH at room temperature for 5 hr respectively. The degradation kinetics for TDF was studied under acidic degradation using 0.1 N HCl at reflux conditions (80ºC) for 5 hr. The degradation kinetics for TDF in basic condition was not studied as drug showed complete degradation on addition of 0.1 N NaOH. The order of reaction was determined by a plot of log % remaining versus time. The degradation rate constant (K), half-life or time left for 50% potency ($t_{1/2}$), shelf life or time left for 90% potency (t_{90}) for each stress condition were calculated by using the slope of the straight line for each degradation condition, Equation (1), and Equation (2) respectively.

Equation (1): $t_{1/2}$= 0.693/K

Equation (2): t_{90} = 0.105/K

Method Validation

The method was validated in terms of linearity, range, precision, accuracy, specificity, limit of detection, limit of quantitation and robustness as per ICH guidelines[20]. The system suitability parameters were evaluated as specified in Indian Pharmacopoeia 2010.

Linearity:

Six different concentrations of each drug (10, 20, 30, 40, 50 and 60 µg/mL) were prepared from standard stock solution of 1000 µg/mL of FTC and TDF respectively and were analysed in triplicate. The peak areas were plotted against the corresponding concentrations to obtain a linearity plot.

Precision:

The precision was evaluated with respect to both repeatability and intermediate precision. Repeatability was evaluated by injecting six replicate injections of test solution of the drugs FTC (20 µg/ml) and TDF (30 µg/ml). The studies were repeated for three different days to determine intermediate precision. Peak areas of the drugs were determined and % RSD was calculated.

Accuracy:

The accuracy of the method was assessed by the recovery studies at three different concentrations (corresponding to 80%, 100% and 120% of the test solution concentration for both FTC and TDF) by the addition of known amount of standard to the test solutions. The % recovery was calculated by slope and intercept of the linearity plot of drugs.

Limit of detection (LOD) and Limit of Quantitation (LOQ):

The limit of detection was calculated based on visual evaluation and S/N ratio (3: 1) and the limit of quantitation was calculated based on visual evaluation and S/N ratio (10: 1).

Specificity:

This was evaluated by injecting degradation sample solutions. The resolution between the drugs and degradation products was evaluated.

Robustness:

Robustness was evaluated by deliberate variation in parameters like pH and molar concentration of buffer. The pH of the mobile phase was varied by 5.5 ± 0.1 units and molar concentration was varied by 20 ± 5 mM. Robustness was studied at a concentration of 30 µg/mL for TDF and 20 µg/mL for FTC.

RESULTS AND DISCUSSION

A representative chromatogram showing the resolution between FTC, TDF and degradation products is shown in the **Figure 3.**

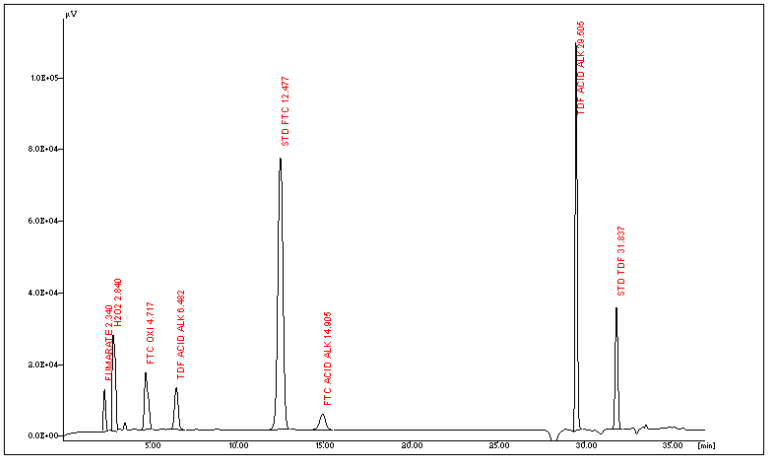

Figure 3. HPLC Chromatogram of combined degradants of TDF and FTC

H_2O_2- Hydrogen peroxide peak, FTC OXI- oxidative degradant of FTC, STD FTC- Standard drug peak, FTC ACID/ALK- acidic and alkaline degradant of FTC, TDF ACID/ALK- acidic and alkaline degradant of TDF, STD TDF- Standard drug peak

Forced degradation studies

FTC showed acidic degradation after 3 hr in 0.1 N HCl under reflux conditions at 80ºC, alkaline degradation after 1 hr in 0.5 N NaOH at room temperature, and oxidative degradation in 3% v/v H_2O_2 after 15 min at room temperature. Forced degradation studies on FTC showed that the drug is more susceptible for oxidation at room temperature. The drug was found to be stable under forced neutral hydrolysis, thermal and photolytic degradation conditions. TDF showed degradation in acidic medium (0.2 N HCl) under reflux condition at 80ºC after 1 hour and immediate complete degradation in alkaline medium (0.1 N NaOH) at room temperature. TDF was found to be very susceptible to alkaline hydrolysis. TDF was found to be stable under neutral hydrolytic, oxidative, photolytic and thermal degradation conditions.

The % degradation was calculated by comparing the peak areas of standard drug with peak areas of drug under degradation conditions. The % degradation for FTC in acidic, basic and oxidative degradation conditions was found to be 8.57%, 5.93% and 13.49% respectively. The % degradation for TDF in acidic and basic degradation conditions was found to be 68.54% and 100% respectively. The results are tabulated in the **Table 1.**

Table 1. Forced degradation studies of FTC and TDF

Degradation condition	% Degradation	
	FTC	TDF
Acid Hydrolysis (Reflux at 80ºC) 0.1 N HCl	8.57% (1 degradation product)	No degradation observed.
0.2 N HCl	68.54% (2 degradation products)	No degradation observed.
Base Hydrolysis (Room temperature) 0.1 N NaOH	No degradation observed.	100% (2 degradation products)
0.5 N NaOH	5.93% (1 degradation product)	No degradation observed.
Neutral Hydrolysis (Room temperature and Reflux condition)	No degradation observed.	No degradation observed.
Oxidation (Room temperature) 3% H_2O_2	13.49% (1 degradation product)	No degradation observed.
Photolytic Degradation (Exposure to sunlight for 15 days)	No degradation observed.	No degradation observed.
Thermal degradation (Exposure to dry heat at 60ºC and 100 ºC)	No degradation observed.	No degradation observed.

Degradation kinetics study

A plot of log % remaining versus time showed decrease in concentration of drugs with increasing time intervals under all degradation conditions, which indicates a first order degradation kinetics **Figure 4** and **Figure 5.**

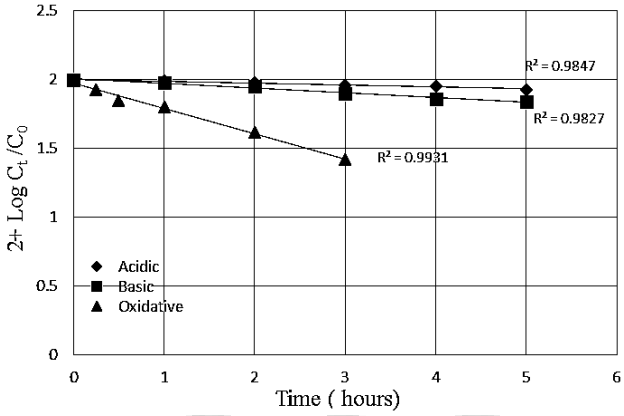

Figure 4. First order plots for degradation of FTC under different conditions

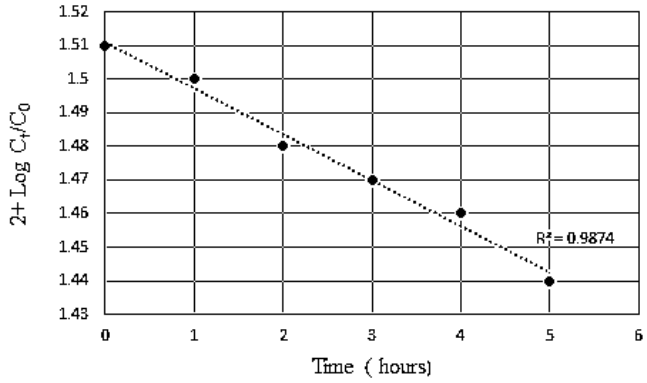

Figure 5. First order plot for TDF under acidic degradation condition

Extensive degradation was observed in oxidative condition for FTC as K value was found out to be highest in oxidative condition amongst the all tested conditions. The half-life ($t_{1/2}$) and shelf life (t_{90}) were found to be lowest for oxidative condition and highest for acidic condition. The $t_{1/2}$ values for FTC were found to be decreased in the order acidic > basic > oxidation. The kinetic parameters; degradation rate constant (K), half-life ($t_{1/2}$), shelf life (t_{90}) for the drugs FTC and TDF are presented in **Table 2** and **Table 3** respectively.

Table 2. Summary of FTC degradation kinetics

Degradation condition	K(hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
Acidic (0.1 N HCl reflux at 80ºC)	0.032	21.65	3.28
Basic (0.5 N NaOH at room temperature)	0.079	8.70	1.31
Oxidation (3% H_2O_2 at room temperature)	0.421	1.64	0.25

Table 3. Summary of TDF degradation kinetics

Degradation condition	K(hr^{-1})	$t_{1/2}$ (hr)	t_{90} (hr)
Acidic (0.2 N HCl reflux at 80ºC)	0.0315	22	3.33

Method Validation

Linearity

A linear response for FTC and TDF in combination was obtained in concentration range of 10 to 70 µg/ml. Linearity was studied using six different concentrations of each drug (10, 20, 30, 40, 50 and 60 µg/mL). The linear regression equation and correlation co-efficient (r^2) obtained for FTC were y= 26272x+68494 and 0.999 respectively and for TDF were y= 22264x+42617 and 0.9991 respectively.

Precision

The % RSD for intraday and inter day precision was less than 2%, which is within the acceptable limits, indicating that the method is precise.

The results are presented in the **Table 4.**

Table 4. Inter-day and Intra-day precision of standard FTC (20 µg/mL) & TDF (30 µg/mL) in combination

Drug	Intra-day precision		Inter-day precision	
	Mean peak area ± SD (n=6)	% RSD	Mean peak area ± SD (n=6)	% RSD
FTC	615591 ± 4726.021	0.76	615498 ±4367.426	0.70
TDF	755660 ±6877.844	0.90	762878 ±6341.785	0.83

Accuracy

The results obtained for accuracy are presented in the **Table 5**. The % recovery for FTC ranged from 98.36-101.34%, whereas for TDF the % recovery ranged from 99.66-101.75% indicating that the proposed method is accurate.

Table 5. Percent recovery of FTC and TDF in combination in marketed tablets

Level (%)	Concentration of drug added (µg/mL)	Total concentration of drug (µg/mL)	% RSD	% Recovery
FTC				
80	**16**	36	0.24	98.36
100	**20**	40	0.05	98.92
120	**24**	44	0.15	101.34
		Mean	0.158	99.54 %
TDF				
80	**24**	54	0.07	99.67
100	**30**	60	0.01	99.66
120	**36**	66	0.11	101.75
		Mean	0.3	100.36%

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The **LOD** and **LOQ** for FTC were found to be 0.05 µg/mL and 0.1 µg/mL respectively. The **LOD** and **LOQ** for TDF were found to be 0.04 µg/mL and 0.08 µg/mL respectively.

Specificity

Degradation peaks were well resolved from the drugs peak, which indicates that the method is specific. The chromatogram for the same is shown in **Figure 1.**

Robustness

The results were found to be unaffected by small changes in pH of mobile phase and molar concentration of buffer. The results for the same are represented in following **Table 6.**

Table 6. Robustness for analysis of TDF and FTC in combination

Parameters	**Level**	**Retention time (min)**		**Asymmetry**		**Peak area**	
		FTC	**TDF**	**FTC**	**TDF**	**FTC**	**TDF**
pH of the mobile phase	5.4	11.8	31.83	1.08	0.99	624353	735006
	5.5	11.7	31.83	1.06	1.01	610653	736564
	5.6	11.5	31.83	1.06	1.02	626855	745692
Mean±SD		**11.6±0.15**	**31.83±0**	**1.06±0.01**	**1.0±0.01**	**620620±8722**	**739087±5772**
Molar concentration of buffer (mM**)**	15	11.8	31.83	1.01	0.99	612695	746382
	20	11.7	31.84	1.02	1.02	614005	736662
	25	11.6	31.83	1.01	1.02	614183	746845
Mean±SD		**11.7±0.1**	**31.83±0.05**	**1.01±0.05**	**1.01±0.01**	**613628±812**	**743296±5749**

System suitability

System suitability parameters like number of theoretical plates, tailing factor, resolution were evaluated by injecting six replicate injections of the drugs at concentration of 20 µg/ml of FTC and 30 µg/ml of TDF. The number of theoretical plates observed for TDF was 538821 and for FTC it was 9470. The asymmetry values obtained for TDF and FTC were 1.09 and 1.01 respectively. The resolution from peak left of TDF and FTC was found to be 14.96 and

16.12 respectively. The resolution obtained for peak right of FTC was 5.05. The results were found to be within the acceptable limits as mentioned in I.P 2010.

Assay of marketed tablets

The marketed tablets were found to contain 198.65 mg (99.32% of label claim) of FTC and 299.63 mg (99.875% of label claim) of TDF when assayed. The results obtained in the assay were found to be within the limits as specified for the assay of Tenofovir Disoproxil Fumarate and Emtricitabine in I.P.

CONCLUSION

The forced degradation studies showed the presence of one degradation product of FTC under reflux at 80^{o}C each in acid, alkaline and at room temperature on subjecting to oxidation. The drug was found to be stable under forced neutral hydrolysis, thermal and photolytic degradation conditions. TDF showed two degradation products each in the acidic medium refluxed at 80^{o}C and 100% degradation at room temperature in alkaline medium. TDF was found to be stable under neutral hydrolytic, oxidative and thermal degradation conditions. The best fit corresponding to the first order kinetics was observed in the studies of the degradation kinetics of FTC in acidic medium, alkaline medium, and on subjecting to oxidation. Similarly for degradation of TDF in acidic media, the best fit was obtained for first order kinetics. The forced degradation and kinetics study results indicate that the drug FTC is more susceptible to oxidation at room temperature and the drug TDF demonstrates poor stability in alkaline medium. Hence these factors must be considered during formulation of these drugs and their storage conditions. The proposed stability indicating assay method for simultaneous estimation of TDF and FTC successfully resolves the degradation peaks and excipients from the drugs. The proposed method has a total run time of only 37 min and can be considered as an alternate method for monitoring the assay of stability samples of both the drugs in combination.

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"The authors declare that there is no conflict of interests regarding the publication of this paper."

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