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Development and Validation of Spectrophotometric method for determination of Emtricitabine and Tenofovir Disoproxil Fumarate in Bulk and Tablet dosage form

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Abstract: Three simple Spectrophotometric methods have been developed for simultaneous estimation of Emtricitabine and Tenofovir Disoproxil Fumarate from tablet dosage form. Method A is Least Square method, involves the measurement of Emtricitabine and Tenofovir Disoproxil Fumarate at their λmax at 281.0 nm and 260.5nm respectively. Method B is First order derivative spectroscopy, wavelength selected for quantitation were 234.5nm for Emtricitabine (zero cross for Tenofovir Disoproxil Fumarate) and 281.0nm for Tenofovir Disoproxil Fumarate (zero cross for Emtricitabine). Method C is Area under Curve method, AUC in the range of 278.0-283.0nm (for Emtricitabine) and 258.0- 262.0nm (for Tenofovir Disoproxil Fumarate) were selected for the analysis. The linearity lies between 5-25µg/ml and 10–50µg/ml for Emtricitabine and Tenofovir Disoproxil Fumarate respectively for method A, B and C. The accuracy and precision of the methods were determined and validated statically. All the methods showed good reproducibility and recovery with low % RSD.

Key Words: Emtricitabine, Tenofovir Disoproxil Fumarate, Least Square Method, First order derivative spectroscopy, Area under curve method.

INTRODUCTION

Emtricitabine (FTC) is a nucleoside reverse transcriptase inhibitor (NRTIs). Chemically it is 5-fluoro-1-(2R, 5S)-[2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] cytosine (Fig 1). FTC is the (-) enantiomer of thio analogue of cytidine which differs from other cytidine analogues, in that it has fluorine in 5th position. FTC inhibits reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. FTC is used for the prevention of perinatal HIV-1 reverse transcriptase (1). It is also active against Hepatitis B virus (2, 3).

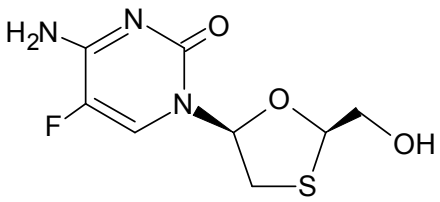

Figure 1: Structure of Emtricitabine (FTC)

Tenofovir disoproxil Fumarate (TDF), acyclic phosphonate nucleotide analogue, is a fumaric acid salt of the bis iso propoxy carbonyl oxy methyl ester derivative of tenofovir. Chemically it is [[(1*R*)-2-(6-Amino-9*H*-purin-9-yl)-1-methylethoxy] methyl] phosphonic acid (1) (Fig 2). It is the first nucleotide reverse transcriptase inhibitor (NtRTIs) approved for use in combination with other antiretroviral agents in the treatment of HIV-1 infection in the United States. Unlike the nucleoside reverse transcriptase inhibitors, which must undergo three intracellular phosphorylation steps for activation, nucleotide analogues such as Tenofovir require only two such steps. This reduction in the phosphorylation requirement has the potential to produce more rapid and complete conversion of the drug to its pharmacologically active metabolite (4).

Different methods have been reported in the literature for monitoring plasma levels of TDF and FTC individually. Rezk et al. have reported a simultaneous method for the estimation of TDF and FTC in human plasma using a validated HPLC method (5) with a rather long run time. Some other techniques used in individual analysis of TDF from plasma include HPLC with mass spectrometric (6, 7) Spectrofluorometric (8) and simple UV detection (9). King et al. quantified TDF alone from human peripheral blood mononuclear cells using a validated LC–MS/MS method (10). Pruvost et al. reported a LC-positive ESI-MS/MS method for the measurement of nucleotides supported by its application in determining carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate simultaneously in human peripheral blood mononuclear cells (11). Takahashi et al. also report a conventional LC–MS method for quantifying TDF individually from human plasma (12). Sparidans et al reported a liquid chromatographic method for determination of TDF in plasma after derivatization with chloroacetaldehyde (13). Shirkhedkar et al. reported a UV method for estimation of TDF in tablets (14). FTC has been quantified in presence of other antiretroviral drugs, by Notari et al. (15) and Rebiere et al. (16). Sparidans et al. have quantified FTC individually in human plasma using a validated LC–MS/MS method (17). FTC is reported to be analysed by various spectroscopic methods (18-20), HPLC (21, 22) and HPTLC (23). The present methods are developed to quantify FTC and TDF in combined or single dosage form in cost effective way. These methods can be used for the quality assurance of these drugs in bulk and dosage forms.

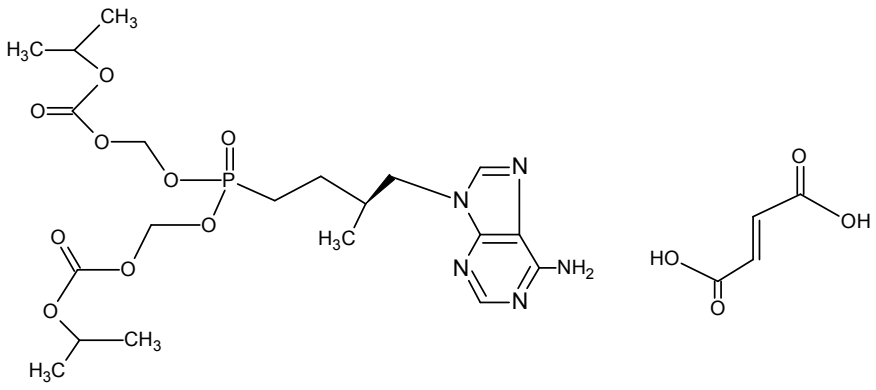

Figure 2: Structure of Tenofovir Disoproxil Fumarate (TDF)

EXPERIMENTAL

INSTRUMENTATION

A double beam JASCO UV –Visible spectrophotometer, V-630, with spectral band width of 1.5nm and wavelength accuracy ± 0.5nm with a pair of 1cm matched quartz cells was used for measuring the absorbance of the resulting solution.

CHEMICALS AND REAGENTS

A standard drug of FTC (Emtricitabine) was procured from Cipla Pvt. Ltd (Mumbai, India) and TDF (Tenofovir Disoproxil Fumarate) was collected from Matrix Laboratories (Hyderabad, India). Fixed dosage forms of FTC and TDF (**Tenvir – EM** and **Tavin – EM** containing 300mg TDF and 200mg FTC, manufactured by Cipla Pvt. Ltd, Goa and Hetero Drugs Ltd, Hyderabad respectively) were purchased from the local market. Methanol and double distilled water in the ratio 1:1(v/v) was used as the solvent. The methanol was of analytical grade procured from Merck, India.

PREPARATION OF STANDARD SOLUTION AND SAMPLE SOLUTION OF TABLET

Standard drug solution of 100µg/ml of FTC and TDF were prepared in mixture of methanol and double distilled water (1:1, v/v). Working dilutions of FTC and TDF were prepared and scanned from 200 – 400nm for obeyance of Beer's law. FTC follows the Beer's law in the range of 5 - 25 µg/ml, whereas TDF follows the Beer's law in the range of 10 - 50 µg/ml. For preparation of sample solution 20 tablets were weighed accurately and a quantity of tablet powder equivalent to 30mg of TDF (20mg of FTC) was weighed and dissolved in 75ml of solvent mixture of methanol and double distilled water, sonicated in ultrasonic bath for 30 mins. The resulting solution was filtered through Whatman filter paper No. 41. To a 100ml volumetric flask and the final volume was made up to 100ml with solvent mixture. The sample solution was further diluted with solvent mixture to get a dilution containing 30 µg/ml of TDF and 20 µg/ml of FTC.

METHODS

METHOD A: LEAST SQUARE METHOD

For this method, 10µg/ml solution of standard drug FTC and TDF were prepared in solvent mixture and scanned from 400nm to 200nm. From the spectra of both the drugs, the λmax were found to be 281nm and 260.5nm for FTC and TDF respectively (**Fig 3).** Serial dilutions of both the drugs were prepared and FTC was found to follow the Beer's law in the range of 5 - 25 µg/ml at 281nm and TDF follows in the range of 10 - 50 µg/ml at 260.5nm. The calibration curves were plotted by absorbance Vs the concentration of both the drugs.

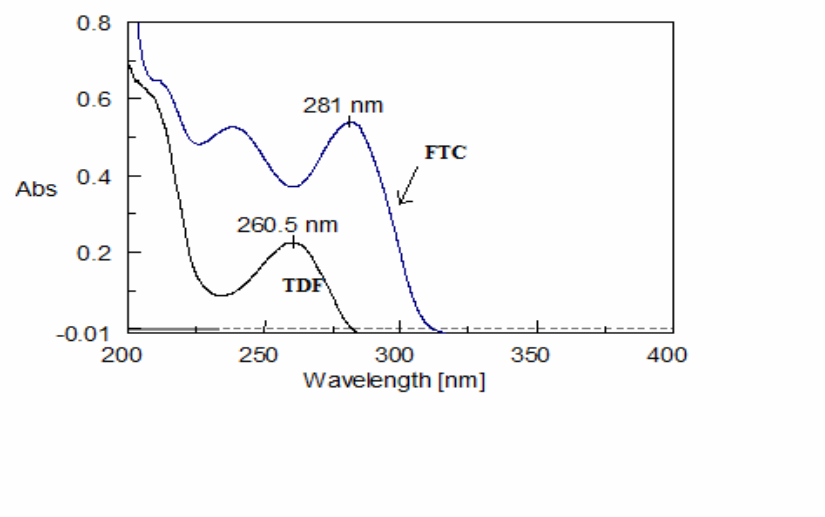

Figure 3: Overlain spectra of FTC and TDF

METHOD B: FIRST ORDER DERIVATIVE METHOD

In this method, 10µg/ml solution of standard drug FTC and TDF were prepared in solvent mixture and scanned from 400nm to 200nm. The absorption spectra thus obtained were derivatized from first order to fourth order. First order derivative spectra were selected for analysis of both drugs. From the overlain spectra of both the drugs (**Fig 4),** wavelength selected for quantification were 234.5nm for FTC (zero cross of TDF) and 281nm for TDF (zero cross of FTC). The calibration curve for FTC and TDF were plotted in the concentration range of 5 - 25µg/ml at 234.5nm and 10 - 50µg/ml at 281nm respectively.

METHOD C: AREA UNDER CURVE METHOD

In this method, 10µg/ml solution of standard drug FTC and TDF were prepared in solvent mixture and scanned from 400nm to 200nm.From the overlain spectra of both the drugs (**Fig 5**), area under the curve in the range of 278 – 283nm ($\lambda_1 - \lambda_2$ for FTC) and in the range of 258 – 262nm ($\lambda_3 - \lambda_4$ for TDF) were selected for analysis. The calibration curve for FTC and TDF were plotted in the concentration range of 5 - 25µg/ml and 10 - 50µg/ml with respect to their respective area under the curve. The 'X' values for both the drugs were determined at the selected AUC range. The 'X' is the ratio of area under the curve at selected wavelength ranges with the concentration in gm/lit. The 'X' values were the mean of five independent determinations. A set of two simultaneous equations obtained by using mean 'X' values are given below.

$A_1 = 132.88\ C_{FTC} + 9.58\ C_{TDF}$ (at $\lambda_1 - \lambda_2$) -------- (1)
$A_2 = 75.41\ C_{FTC} + 89.85\ C_{TDF}$ (at $\lambda_3 - \lambda_4$) ------ (2)

Where A_1 and A_2 were area under curve of samples at the wavelength range 278 – 283nm ($\lambda_1 - \lambda_2$) and 258 – 262nm ($\lambda_3 - \lambda_4$) respectively. 132.88 and 75.41 were 'X' values of FTC in the $\lambda_1 - \lambda_2$ and $\lambda_3 - \lambda_4$ respectively. Similarly 9.58 and 89.85 were 'X' values of TDF in the $\lambda_1 - \lambda_2$ and $\lambda_3 - \lambda_4$ respectively. C_{FTC} and C_{TDF} were concentrations of FTC and TDF respectively. The concentrations of FTC and TDF in sample were determined by using equation (1) and (2).

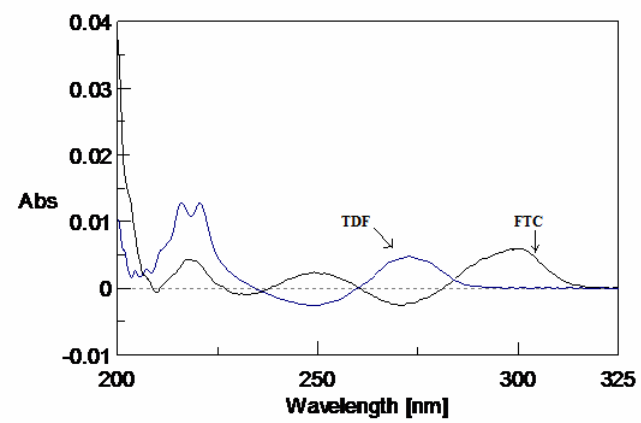

Figure 4: Overlain of First order derivative spectrum of FTC and TDF

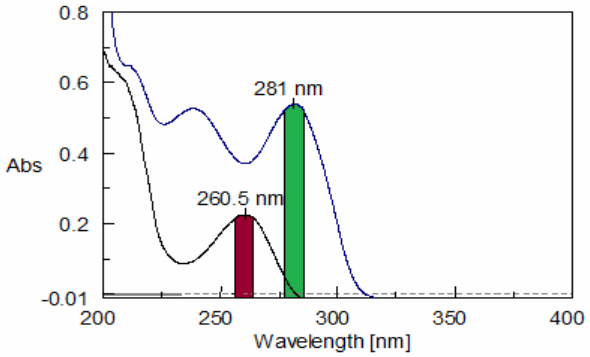

Figure 5: Overlain spectra of FTC and TDF for Area under Curve method

APPLICATION OF THE PROPOSED METHODS FOR THE DETERMINATION OF FTC AND TDF IN DOSAGE FORM

For the estimation of FTC and TDF in commercial formulations, twenty tablets from each brand (Tenvir – EM and Tavin - EM) were weighed and average weight was calculated. The tablets were crushed and tablet powder equivalent to 30mg of TDF (20mg FTC) was transferred to a 100ml volumetric flask and fractions of solvent mixture were added up to 75ml with 15mins shaking. The solutions were sonicated in ultrasonic bath for 15mins. The volume was made up to 100ml with the solvent mixture and filtered through Whatman filter paper (No. 41).From the filtrate 10ml was transferred to a 100ml volumetric flask and volume was made up by solvent mixture to obtain 30µg/ml of TDF and 20µg/ml of FTC. In method A, the concentration of FTC and TDF were determined by measuring the absorbance of the sample at their λmax (281nm for FTC and 260.5nm for TDF respectively) and calculated from standard calibration curve. For method B, the absorbance of the sample solution were measured at 234.5nm (for FTC) and 281nm (for TDF) in first order derivative mode and concentration was calculated against the calibration curve. For method C, the concentration of both FTC and TDF were determined by measuring area under curve in the range of 278 – 283nm (for FTC) and 258 – 262nm (for TDF) and values were substituted in the respective equation (Equation 1 and 2) to get the concentration present in the sample solution.

VALIDATION OF THE METHODS

The methods were validated with respect to linearity, accuracy, precision and selectivity.

LINEARITY: In case of all the three methods both the drugs FTC and TDF follow the Beer' law from 5 µg/ml - 25 µg/ml and 10 µg/ml - 50 µg/ml respectively.

ACCURACY: In all the three methods the accuracy was tested by recovery study by standard addition method. The standard drugs were added to the pre - analysed marketed dosage form at three different levels i.e. 25%, 50% and 100%.

PRECISION: The precision of the methods were tested in terms of repeatability and reproducibility. The precision of the proposed methods were performed by intra-day and inter-day assay. In intra-day assay the same sample was assayed with time interval for 6times with in the same day. In inter-day assay the assay of the samples were done on 3 consecutive days. The precision of the methods were expressed in terms of %RSD.

LOD AND LOQ: The LOD and LOQ was determined by using equation (1) and (2) respectively

$LOD = 3.3\ \sigma / S$ ---------- (3)

$LOQ = 10\ \sigma / S$ ----------(4)

Where 'σ' is the standard deviation of y-intercept and 'S' is the slope of calibration curve.

SELECTIVITY AND SPECIFICITY: The methods were performed in presence of excipients, but there were no interference of the excipients which indicates the selectivity of the methods.

RESULTS AND DISCUSSION

I) Optimization of solvent: As FTC is soluble in water and sparingly soluble in methanol and TDF is sparingly soluble in water and soluble in methanol, various solvent mixtures containing water, methanol, chloroform and acetone were tried for the solubility test. Methanol and double distilled water in the ratio 1:1(v/v) was used as the solvent.

II) Assay of marketed formulations: All the three methods are suitable for the simultaneous estimation of FTC and TDF accurately. In all the methods the content of both the compounds were found within the limits of IP and the lower values of Relative Standard Deviation suggests the precision of the methods (Table 1).

Table 1: Assay of Formulations.

Name of the formulation	Label claim (mg)		Amount found (%) Mean* ± S.D., R.S.D		
			Method A	Method B	Method C
Tenvir – EM	FTC	200	99.97 ± 0.64, 0.67	99.65 ± 0.38, 0.38	99.21 ± 0.83,0.84
	TDF	300	99.24 ± 0.75, 0.75	100.02 ± 0.17, 0.17	99.65 ± 0.27, 0.27
Tavin -EM	FTC	200	100.08 ± 0.29,0.29	99.26 ±0.45,0.46	99.78 ± 0.63, 0.63
	TDF	300	99.84 ± 0.45, 0.45	99.89 ± 0.63, 0.63	100.5 ± 0.55, 0.55

* Mean of five determinations

Table 2: Accuracy of the methods (Recovery study)

Name of the formulation	Name of the compound	Amount of sample taken (µg/ml) + amount of standard added (µg/ml)	Mean*(%) ± S.D., R.S.D.		
			Method A	Method B	Method C
Tenvir – EM	FTC	12 + 3	100.31 ± 0.62, 0.61	99.36±0.15, 0.15	100.22 ± 0.72, 0.72
		12 + 6			
		12 + 12			
	TDF	18 + 4.5	100.5 ± 0.55, 0.55	100.8 ± 0.63, 0.63	101.09 ± 0.84, 0.83
		18 + 9			
		18 + 18			
Tavin -EM	FTC	12 + 3	100.53 ± 0.22, 0.22	100.22±0.02, 0.02	99.4 ± 0.15, 0.15
		12 + 6			
		12 + 12			
	TDF	18 + 4.5	100.23 ± 0.73, 0.73	100.32 ± 0.62, 0.61	99.94 ± 0.5, 0.5
		18 + 9			
		18 + 18			

* Mean of five determinations

Table 3: Precision of the methods

Name of the formulation	Name of the compound	Intra-day precision (n =6) Mean*(%) ± S.D, R.S.D.			Inter-day precision (n = 3) Mean*(%) ± S.D, R.S.D.		
		Method A	Method B	Method C	Method A	Method B	Method C
Tenvir – EM	FTC	99.63± 0.18 0.18	100.54± 0.02 0.02	100.22± 0.02 0.02	99.39± 0.07 0.07	99.65± 0.16 0.16	99.03± 0.34 0.34
	TDF	99.42± 0.36 0.36	99.59 ± 0.38 0.38	100.01± 0.46 0.46	99.76± 0.32 0.32	99.16± 0.7 0.7	99.03± 0.6 0.6
Tavin - EM	FTC	99.8± 0.7 0.7	99.90± 0.7 0.7	99.77± 0.84 0.84	99.63± 0.4 0.4	99.64± 0.27 0.27	100.08± 0.62 0.62
	TDF	99.44± 0.24 0.24	99.96± 0.66 0.66	100.14± 0.47 0.47	99.71± 0.52 0.52	99.5± 0.34 0.34	99.83± 0.61 0.61

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Table 4: Optical characteristics of Method A, B and C

Parameter	Emtricitabine			Tenofovir Disoproxil Fumarate		
	Method A	Method B	Method C	Method A	Method B	Method C
Wavelength (nm)	281	234.5	278 - 282	260.5	281	258 - 262
Beer - Lambart's Law (µg/ml)	5 - 25	5 - 25	5 - 25	10 - 50	10 - 50	10 - 50
LOD (µg/ml)	0.5617	1.189	0.439	1.706	1.4507	1.0471
LOQ (µg/ml)	1.702	3.6	1.3312	5.17	4.396	3.17
Molar absorptivity (l mol^{-1} cm^{-1})	$6.65x10^3$	$1.64x10^4$	$1.57x10^4$	$6.4x10^3$	$1.02x10^4$	$2.56x10^4$
Sandell's sensitivity (µg cm^{-2}/ 0.001 absorbance unit)	0.0376	0.0152	0.0159	0.0446	0.0282	0.0111
Regression equation [Y = mX +c]						
Slope (m)	0.028	0.044	0.139	0.0243	0.0228	0.093
Intercept (c)	0.014	0.2183	0.067	0.0529	0.1337	0.082
Correlation coefficient (r^2)	0.9996	0.9946	0.999	0.9972	0.992	0.999

Validation of the methods

i) Linearity: In all the methods, at the selected range of λmax, both the drug solutions follow the Beer's Law in the concentration range of 5 - 25µg/ml (for FTC) and 10 - 50 µg/ml (for TDF). The values of coefficient of correlation were found to nearly equal to 0.999 (Table 4).

ii) Accuracy: The accuracy of the developed methods was tested by standard addition method at the level of 25%, 50% and 100%. The percentage of recovery, lower values of standard deviation and relative standard deviation (< 2) indicates the accuracy of the three methods (Table 2).

iii) Precision: The intra-day and inter-day assay of the formulations by the proposed methods were found to be suitable with very low values of standard deviation. This justifies the reproducibility and repeatability of the proposed methods (Table 3)

iv) LOD and LOQ: LOD and LOQ were determined by using the equation 3 and 4. (Table 4).

v) Selectivity and specificity: All the formulations were assayed in presence of the excipients by the proposed methods. It was found that there is no interference of the excipients which justifies the selectivity of both the drugs for the proposed methods. By changing slightly the experimental conditions like changing the λmax, changing the solvent ratio alters the results, which suggests the specificity of the methods.

CONCLUSION

All the three proposed methods are accurate, precise, rapid, reproducible and economical and can be employed for routine quality control of Emtricitabine and Tenofovir Disoproxil Fumarate in combined dosage formulation.

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