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(RESEARCH ARTICLE)

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Development and validation of a spectrofluorimetric method for the estimation of Tenofovir in bulk and formulation

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Abstract

A simple, accurate, precise, sensitive and cost-effective spectrofluorimetric method was developed and validated for the estimation of Tenofovir in bulk and formulation. The relative fluorescence intensity of Tenofovir was measured in Distilled Water: Methanol (40:60%v/v) at an excitation wavelength of 374 nm and an emission wavelength of 409 nm. Proposed method was found to be linear over the range of 50 to 1000 ng/ml with correlation coefficient 0.999. Proposed method was validated using different analytical method validation parameters viz. Accuracy, precision, LOD, LOQ, robustness and ruggedness using QC standards as per the ICH guidelines**.** The percentage recovery was found to be 100.29 % and percentage RSD values were found to be less than 2 for accuracy and precision studies. The detection and quantification limits for the proposed method were found to be 3.009ng/ml and 9.120ng/ml, respectively. A simple, accurate, precise, sensitive yet cost-effective spectrofluorimetric method was developed for the estimation of Tenofovir in bulk and formulation. The said spectrofluorimetric method was found to be economic as it comprises water as a solvent.

Keywords: Spectrofluorimetry; Tenofovir; Validation; Excitation; Emission

1. Introduction

Tenofovir is mainly known for BCS Class III antiretroviral drug which comes under acyclic Nucleoside Reverse Transcriptase Inhibitors (NTRI's) Drugs category. Tenofovir itself as a single agent used to treat Hepatatis B Virus (HBV) infection as well as Herpes Simplex Virus Type – 2 (HSV – II).[1-3] Tenofovir mainly shows its pharmacological activity at cellular targeted location is cytoplasm and cellular membrane.[2-6] It is an analogue of adenosine which is used in combination with other agent to treat Human Immunodeficiency Virus (HIV) by stopping/inhibiting the function of sexual transmission of HIV affected cells (Termination process of HIV virus affected cells) by help of reverse transcriptase in the body.[4-7]

Chemically Tenofovir IUPAC Name is *Bis{[(isopropoxycarbonyl)oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl)* phosphonate which is also known as Tenofovir Disoproxil has partition coefficient of -1.6 and value of pKa is 3.8 and 6.7 respectively.

Considering the physico-chemical as well as pharmaceutical properties of Tenofovir, an accurate, precise and cost-effective spectrofluorometric method was developed and validated. Developed method was successfully used for the estimation of tenofovir in the marketed formulation of Tenofovir i.e., Tenvir – Tenofovir Disoproxil Fumarate Tablets IP 300mg.

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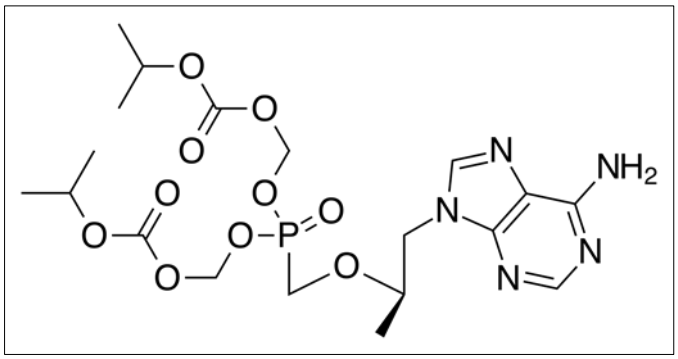


Figure 1 Chemical structure of Tenofovir Disoproxil (Tenofovir)

2. Material and methods

2.1. Material

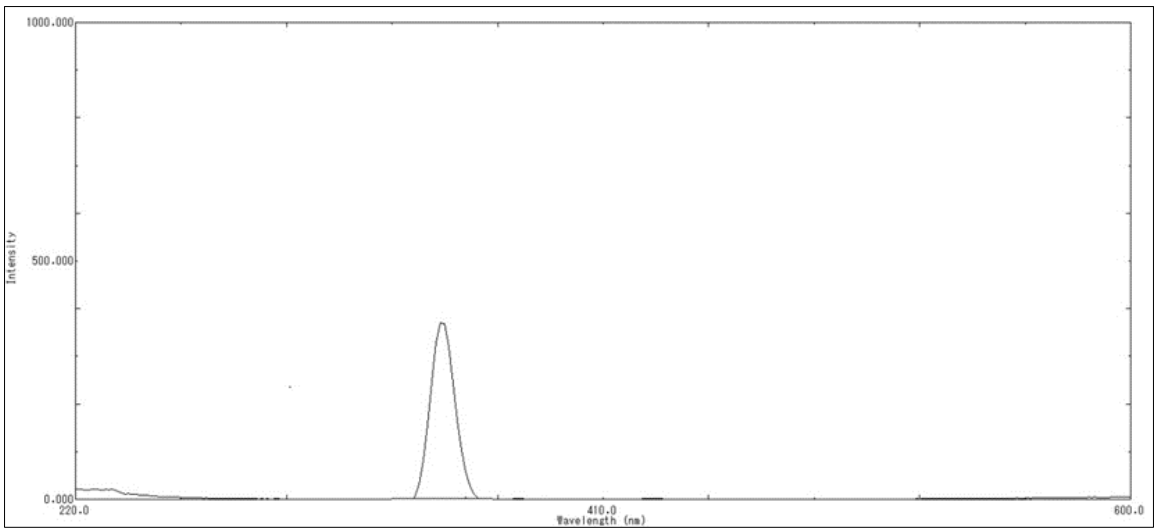
Tenofovir has been purchased from TCI Chemicals (India) Pvt. Ltd, Chennai. Methanol was purchased from Merck. All the chemicals of analytical grade were used for the proposed study.

2.2. Instruments used

The spectrofluorimetric study was carried out with a ShimadzuRF-5301 fluorimeter to determine levels of fluorescence in the Tenofovir. A Xenon 150w lamp was used as a light source. Quartz cells having 48mm height, 10mm path length with 0.5mm slit width were used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

2.3. Preliminary analysis

A preliminary analysis was carried out to determine the excitation and emission wavelength of Tenofovir. Various solvents like distilled water, methanol, acetonitrile and their combinations were used to determine appropriate media for Tenofovir. Tenofovir showed maximum fluorescence intensity in Methanol: Water (40:60%v/v) as a media. Initially, Tenofovir solution of 100ng/ml strength was prepared in methanol. Prepared solution was scanned spectrofluorimetrically to obtain the excitation and emission wavelengths. The scanning was performed over 220 nm to 600 nm range and excitation and emission wavelength were found to be 374nm and 409nm (figure.2) respectively.



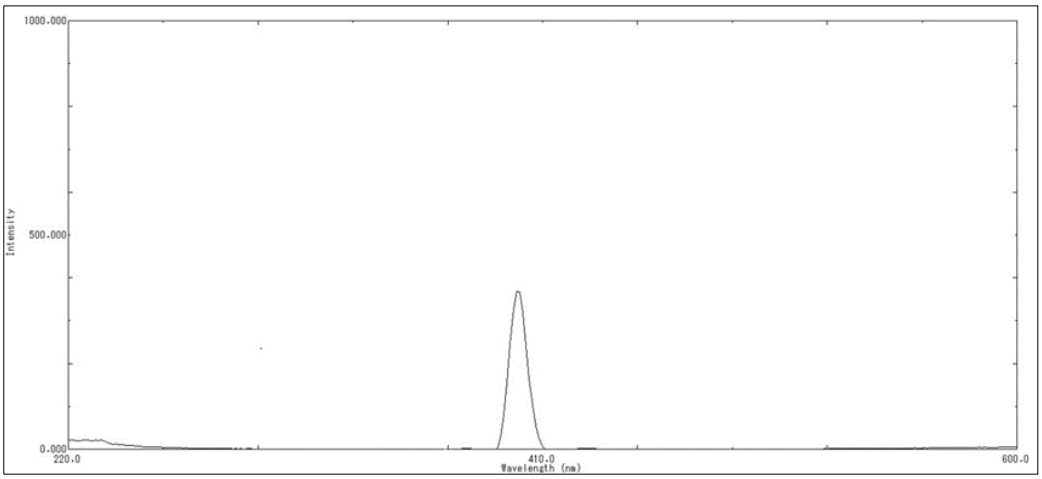


Figure 2 Excitation and Emission Spectra of Tenofovir

2.4. Preparation of Standard Stock Solution

Accurately weighed 5 mg of Tenofovir was transferred into the calibrated volumetric flask and dissolved in 10 ml water to achieve a stock solution of 1000 µg/ml (stock-I). Stock- I solution was suitably diluted with water to achieved further calibration standards.

2.5. Construction of Calibration Curve

Calibration curve was prepared by diluting the stock-I (1000 µg/ml) solution to achieve the seven different calibration standards representing CAL STD 1 (50ng/ml), CAL STD 2 (100ng/ml), CAL STD 3 (200ng/ml), CAL STD 4 (400ng/ml), CAL STD 5 (600ng/ml), CAL STD 6 (800ng/ml), CAL STD 7 (1000ng/ml) strength. The fluorescence intensity was measured at pre-defined excitation and emission wavelengths of 374 and 409 nm respectively. The calibration curve representing concentration vs. Fluorescence intensity was plotted using excel program of Microsoft office 2013. Above mentioned procedure was repeated three times, so that reproducible results can be obtained.

2.6. Spectrofluorimetric Method Validation

Validation is the process which provides a high degree of assurance, so as to produce a desired result and meeting its predetermined specifications and quality characteristics. Developed fluorimetric method for the estimation of Tenofovir was validated as per the ICH guidelines. Different validation parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were calculated using predefined calibration standards and or quality control standards as described below [11-12].

2.7. Linearity and Range

Linearity of the proposed spectrofluorimetric method was calculated by using seven different calibration standards. After analysis of calibration standards, calibration curves in terms of Concentration vs. Fluorescence intensity plots were developed and subjected to linear least square regression analysis. R^2 value was considered to be important factor for determining the linearity of the proposed method.

2.8. Accuracy

To determine the accuracy of the method, different quality control solutions were prepared independently from stock-I i.e., LQC: 60ng/ml, MQC: 500ng/ml and HQC: 950ng/ml and analyzed at level of 80%, 100% and 120% of its predefined concentrations to the predefined concentrations, different amounts of Tenofovir were added (standard addition method) and the accuracy was calculated on the basis of percent recovery.

2.9. Precision

The precision of the method was checked by preparing different quality control solutions independently from stock-I i.e. LQC: 60ng/ml, MQC: 500ng/ml and HQC: 950ng/ml at three different time intervals in a day. Same procedure was followed on three different consecutive days so as to obtain inter-day variation. The fluorescence intensities for Tenofovir were recorded and the results were expressed as % Relative Standard Deviation (%RSD).

2.10. Robustness

Robustness of the proposed spectrofluorimetric method was established by changing composition of the ethanol by ± 1.0 %. MQC samples of Tenofovir were prepared in methanol with and analyzed at 371nm and 409 nm (excitation-emission wavelength of Tenofovir). The results were calculated in terms of % RSD.

2.11. Ruggedness

Ruggedness of the proposed method was studied by analyst variation. MQC samples of Tenofovir were prepared by three different analysts of the laboratory and were analyzed at 371nm and 409nm. The results were calculated in terms of % RSD.

2.12. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and the LOQ of the drug were calculated by using the following equations as per ICH guidelines.

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

Where,
SD= standard deviation of lower most concentration of calibration curve
S= Slope of calibration curve.

2.13. Estimation of Tenofovir in Bulk and Marketed Formulation:

The Tenofovir content in its marketed formulation (Tenvir – Tenofovir Disoproxil Fumarate Tablets IP 300mg) was estimated using pre-validated UV-Visible spectrophotometric method. Tablet formulation contents (labeled strength: 300 mg/tablet) were dissolved in 1 ml of co-solvent system to achieve a stock solution of 1500 ng/ml (n=5). Said solution was suitably diluted with co-solvent system and analyzed for the Tenofovir content using proposed spectrofluorimetric method.

3. Results and discussion

3.1. Construction of Calibration Curve

Quantification of Tenofovir samples by any instrumental method of analysis needs a reproducible calibration curve and a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. To establish linearity of the proposed method, seven different calibration standards were prepared from the stock solution and analyzed at excitation wavelength 371nm and emission wavelength 409nm by spectrofluorimeter. Least square linear regression analysis was performed for the obtained spectrofluorimetric data using MS-excel 2013. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation were reported (Table 1).

Table 1 Calibration standard data for Tenofovir

S. No.	Concentration (ng/ml)	Fluorescence intensity
1	50	47.649±0.5426
2	100	85.214±0.3892
3	200	172.518±0.5526
4	400	352.426±0.4749
5	600	533.633±0.8962
6	800	706.448±0.4852
7	1000	897.185±0.6266

3.2. Spectrofluorimetric Method Validation

3.2.1. Linearity and range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven point calibration curve of Tenofovir was plotted covering a range of 50-1000 ng/ml. Different concentrations and the respective mean fluorescence intensities values are depicted in table 1. Calibration curve when subjected to least square regression analysis yielded an equation; y = 0.952x + 0.163with correlation coefficient 0.999 (figure 3). From the linearity study, it was revealed that, developed method was linear over the concentration range of 50 to 1000ng/ml.

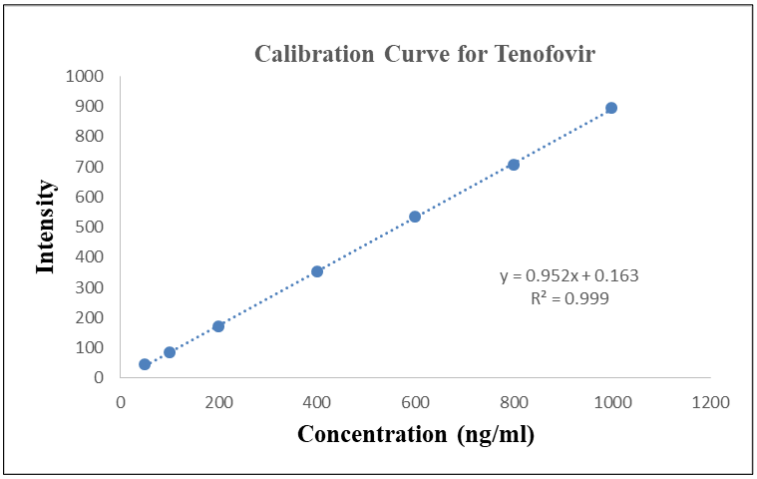


Figure 3 Calibration Curve for Tenofovir

3.2.2. Accuracy

Accuracy is the closeness of test results to the true value obtained by method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For Tenofovir, accuracy was determined using recovery studies. At 80%, 100% and 120% standard addition, mean recovery of Tenofovir was found in between 99.87% to 100.29 %. The relative standard deviation (% RSD) was found to be less than 2 as shown in table 2. From the results of accuracy studies, it was predicted that developed method is highly accurate.

Table 2 Accuracy data of Spectrofluorimetric method for Tenofovir

Sr. no.	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% recovery	Mean % recovery	% RSD
1	80	60	48	99.57	100.29	1.205
2	80	60	48	101.68		
3	80	60	48	99.61		
4	100	500	500	99.93	99.87	0.372
5	100	500	500	100.21		
6	100	500	500	99.47		
7	120	950	1140	99.94	99.97	0.246
8	120	950	1140	100.23		
9	120	950	1140	99.74		

3.2.3. Precision

Precision is defined as closeness of agreement among the individual test result when the method is applied repeatedly to multiple sampling of homogeneous sample. Precise analytical method leads to accurate results. Intra-day and inter-day precision of spectrofluorimetric method was established at LQC: 60ng/ml, MQC: 500ng/ml and HQC: 950ng/ml levels of Tenofovir. The results expressed in terms of mean fluorescence intensity values, % assay and % RSD for the intra-day and inter-day precision study are demonstrated in table 3 and table 4 respectively. Percent RSD values of intra-day precision study were found to be in between 0.100 and 1.781 whereas those of inter-day precision study were in between 0.105 and 1.379 overall; % RSD values of less than 2 demonstrated that developed spectrofluorimetric method is precise and reproducible.

Table 3 Intra-day precision data of Spectrofluorimetric method for Tenofovir

		Morning			**Afternoon**			**Evening**		
S. No	**Concentration Range(ng/ml)**	**Amount (Mean)**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1	60	60.334	100.55	0.372	60.396	100.66	0.781	60.567	100.94	0.469
2	500	500.111	100.02	0.161	500.070	100.01	0.149	500.143	100.02	0.194
3	1000	950.844	100.08	0.105	951.019	100.10	0.123	951.067	100.11	0.100

Table 4 Inter-day precision data of Spectrofluorimetric method for Tenofovir

		Day 1			**Day 2**			**Day 3**		
S. No	**Concentration Range (ng/ml)**	**Amount (Mean)**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1	60	60.432	100.72	0.541	60.425	100.70	1.351	60.023	100.03	1.379
2	500	500.108	100.02	0.168	500.183	100.03	0.191	500.196	100.03	0.144
3	1000	950.976	100.10	0.109	950.611	100.06	0.105	950.836	100.08	0.114

3.2.4. Robustness

Robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition, buffer strength, pH etc. May occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the method. Therefore, robust analytical method is preferred. Robustness of proposed spectrofluorimetric method was performed by changing the pH of water. After analysis, it was found that change in pH of water did not affect the performance of method. % RSD values were found to be in between 0.26 and 0.44 (table 5). Percent RSD values below 2 depicted that proposed spectrofluorimetric method is robust in nature.

Table 5 Robustness data of Spectrofluorimetric method for Tenofovir

S. No.	**Concentration (ng/ml)**	**Mobile phase composition (% v/v)**	**Amount**	**% RSD**
1	500	39:61	475.797	0.31
2	500	40:60	475.951	0.26
3	500	41:59	476.335	0.44

3.2.5. Ruggedness

Ruggedness of analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions, such as different laboratories, different analyst. In order to determine the ruggedness of

proposed spectrofluorimetric method, Tenofovir solutions were prepared and analyzed by different analysts. Sample analysis and data processing resulted into % RSD values between 0.184 and 0.267. Results of ruggedness studies revealed that proposed spectrofluorimetric method was rugged as it showed % RSD values less than 2 (table 6).

Table 6 Ruggedness data of Spectrofluorimetric method for Tenofovir

S. No.	Concentration (ng/ml)	Analyst	Amount	% RSD
1	500	I	475.651	0.184
2	500	II	475.713	0.267
3	500	III	475.861	0.288

3.2.6. Limit of quantitation (LOQ) and limit of detection (LOD):

Limit of quantification (LOQ) represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte in a sample which can be detected but not necessarily quantitated as an exact value. From the standard deviation of lower most concentration and the slope of the calibration curve, LOD and LOQ of proposed spectrofluorimetric method was found to be 3.009 and 9.120 ng/ml respectively (table 7). Lower LOQ value indicated that proposed method is sensitive enough to quantify the Tenofovir content of samples at its lower level.

Table 7 LOD & LOQ data for Spectrofluorimetric method for Tenofovir

1	LOD	3.009ng/ml
2	LOQ	9.120 ng /ml

3.2.7. Estimation

The developed spectrofluorimetric method was successfully applied for estimation of Tenofovir in marketed formulation. By proposed method, Tenofovir content in the Tenvir tablet was found to be 100.39 ± 0.57 % respectively.

4. Conclusion

A simple, accurate, sensitive and precise spectrofluorimetric method for the estimation of Tenofovir was developed and validated. The proposed method was found to be robust and rugged in nature with high accuracy and precision. Proposed method was successfully used for the estimation of Tenofovir in its marketed formulation.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest.

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