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Development of new spectrophotometric method for estimation of tenofovir disoproxil fumarate using MBTH reagent

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ABSTRACT

A new simple, sensitive and specific procedure has been developed for determination of tenofovir disoproxil fumarate in bulk and pharmaceutical dosage forms using MBTH reagent. The purpose of this analytical validation procedure is to validate it by laboratory experiments to prove that the method meets the minimum standards for laboratory use. 3-methyl-2-bezothiazoline hydrazone reacts with the secondary amine group of tenofovir in the presence of oxidizing agent, ferric chloride. The resulting apple green coloured chromogen when measured spectrophotometrically in visible region (*i.e.*, 400-800nm) shows a maximum absorbance at 626.5nm. This method can be successfully applied for the determination of drug content in pharmaceutical formulations. The results of analysis have been validated statistically.

Key Words: 3-methyl-2-benzothiazoline hydrazone, tenofovir disoproxil fumarate, spectrophotometric determination, ICH guidelines.

INTRODUCTION

Tenofovir disoproxil fumarate is an antiretroviral agent used in the treatment of HIV infection. It is a nucleoside reverse transcriptase inhibitor (NRTI) and it is the first nucleotide analogue used for HIV infection. It is chemically 1:1 salt of bisisopropoxyl carbonyl oxy methyl ester of tenofovir and fumaric acid. It has a chemical name of 9-[(R)-2-[bis [(isopropoxycarbonyl) oxy] methyl] phosphinyl] methoxy] propyl]adeninefumarate].

Tenofovir disoproxil fumarate is a prodrug of tenofovir. Upon oral administration, it is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analogue of adenosine 5'-monophosphate and blocks the reverse transcriptase enzyme that is important for HIV-viral synthesis.

It has an empirical formula of $C_{19}H_{30}N_5O_{10}P.C_4H_4O_4$ with a molecular weight of 635.52gm/mol. It is a white to off-white crystalline odourless powder with a solubility of 13.4mg/ml in distilled water and freely soluble in methanol and in DMF (IP 2007; USP 2005). Tenofovir disoproxil fumarate is given once a day as it remains in cells for longer period of time than other antiretroviral drugs.

The purpose of development and validation of analytical methods is to ensure that a suitable method for analysis of a particular analyte is specific, accurate and precise (ICH guidelines). Main purpose of this study is to validate the experimental conditions and parameters to be followed in the spectrophotometric determination of tenofovir disoproxil fumarate using MBTH reagent.

This method is based on the reaction of tenofovir disoproxil fumarate with 3-methyl-2-benzothiazolinone hydrazone hydrochloride [MBTH] reagent and ferric chloride to form green colored chromogen with absorp-tion maximum at 626.5nm and Beer's law is obeyed in the concentration range of 50-250μg/ml In the present estimation of tenofovir disoproxil fumarate, MBTH loses two electrons and one proton to form an electrophlic intermediate (Revanasiddappa *et al.*, 1999; Archana *et al.*, 2013). The formed intermediate is highly coupling species, then reacts (or) couples with tenofovir to form a coloured chromogen (green colour) which is measured at 626.5 nm. The scheme of reaction between drug and the colorimetric agent is shown in figure 1.

MATERIALS AND METHODS

Chemicals and reagents

Tenofovir disoproxil fumarate working standard was generous gift from Aurobindo Pharamceuticals Pvt. Ltd., Hyderabad. Ferric chloride anhydrous, Hydrochloric acid and all other chemicals were of analytical grade. Tenvir® 300 (tenofovir disoproxil fumarate, 300mg), tablets were manufactured by CIPLA Pvt. Ltd., and were purchased from local market.

Instrumentation

UV-visible spectrophotometer (SCHIMADZU 1800), pH meter (Elico LI-127), Digital balance (Schimadzu BL-220H).

Preparation of MBTH (0.5%w/v): 50 mg of 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), (Otto Kemi Chemicals, Mumbai) was dissolved in 10 mL water.

Preparation of 1% ferric chloride: 1 gm of Ferric chloride anhydrous (SD Fine Chemicals Limited, Mumbai) was dissolved in 100 mL 0.1N hydrochloric acid AR (SD Fine Chemicals Limited, Mumbai).

Preparation of stock solutions: Stock solution of tenofovir disoproxil fumarate was made by carefully transferring 100mg to 100mL volumetric flask and dissolved in distilled water. From this stock solution 10mL was pipette out into another 100mL volumetric flask to which 1mL of

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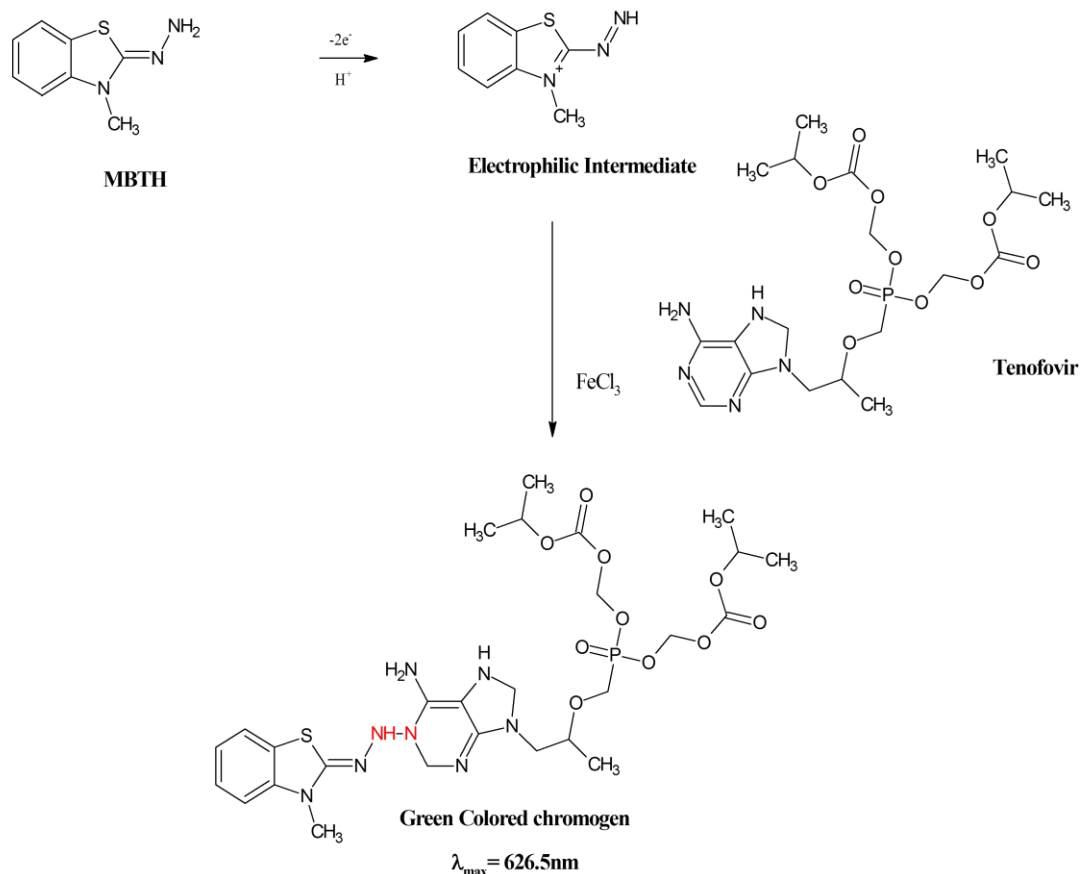Contact No.: +9951722337

Figure 1: Proposed reaction pathway between tenofovir disoproxil fumarate (TDF) and MBTH.
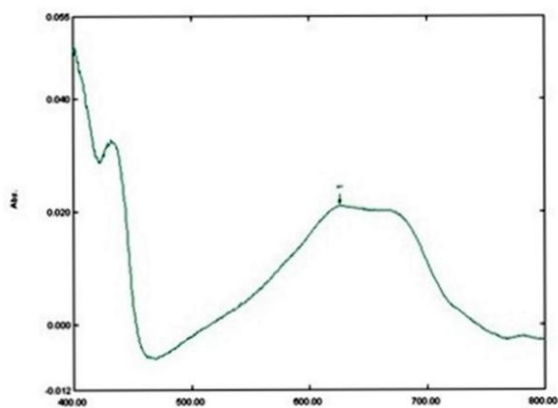

Figure 2: Spectrum of tenofovir disoproxil fumarate with MBTH Reagent.
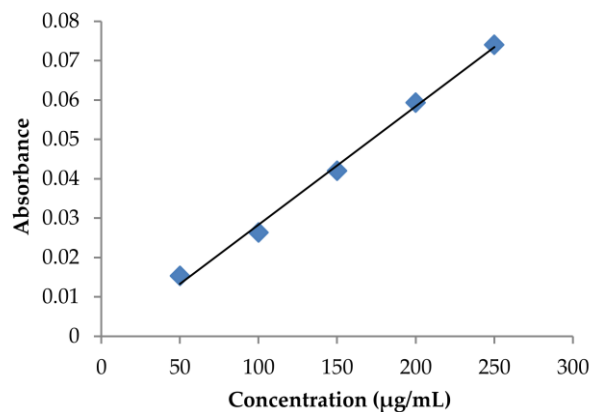

Figure 3: Calibration curve of tenofovir disoproxil fumarate with MBTH Reagent.

MBTH reagent (0.5%w/v) and 1mL of ferric chloride (1%) solution were added and the volume was made up to 100mL with distilled water to give a solution of 100µg/mL solution. The absorbance of resulting coloured solution was measured against respective blank solution in visible region, *i.e.*, 400-800nm which showed a maximum absorbance at 626.5nm and the spectrum is shown in figure 3.

Calibration curve (50-250µg/ml) preparation

Fresh aliquots of tenofovir ranging from 0.5-2.5mL from stock solution (1000µg/mL) were transferred into a series of 10mL volumetric flasks to provide final concentration range of 50-250µg/mL. To each flask 1mL of Ferric chloride (1%) solution and 1mL of 0.5% MBTH reagent solution was added. The solution in each tube was made up to the mark with distilled water. The absorbance of green coloured chromogen was measured at 626.5nm

against appropriate reagent blank and the graph of absorbance against concentration was plotted as shown in the figure 3. The regression equation and coefficient correlation was given in the table 1.

Validation of the assay method

Linearity is the ability of the analysis procedure for obtaining the results of the experiment are directly proportional to the concentration of analyte in the sample (ICH**)**. Linearity is measured by a single measurement at different concentrations and then determined the value of the slope and intercept and also coefficient of determination.

The response of the drug was found to be linear in the investigational concentration range 50-250µg/mL. The calibration curve was found to be linear with an r^2 value 0.9951 and regression equation was y=0.0003x-0.0017. For these studies obtained r^2 value was considered to be appropriate to demonstrate the linearity of the method proving the value of good linearity as approaching a value of 1.

Limit of detection (LOD) and limit of quantitation (LOQ) are the parameters used to predict the sensitivity of proposed method as per the International Conference of Harmonization (ICH) guidelines for the validation of analytical procedure according to which, the following formulae were used:

$$LOD=3.3\sigma/S$$
$$LOQ=10\sigma/S$$

Where, σ = standard deviation of the response (intercept)
S = slope of the calibration curve

The mean of the slope and standard deviation of response were obtained after plotting three calibration curves.

The optimum conditions such as Beer's law limits, molar absorptivity, Sandell's sensitivity and other regression characteristics like slope (m), intercept (c), correlation coefficient were calculated and given in table 2.

Accuracy is the closeness of test results between the results obtained with the true value (true value) or the value of the reference. It describes the systematic error of the measurement results. Accuracy for drug substance was determined on samples of drug solutions at varying concentration levels in the range of 80%-120% (120µg/mL, 150µg/mL, 180µg/mL) by analyzing three replicates of each sample as a batch in a single assay. The %RSD was calculated and reported in table 3. To study the accuracy of drug product, 10 tablets (Tenvir-300) were weighed, powdered and estimation was carried out. Recovery studies were carried out by adding known amount of standard drug solution (120,150,180µg/mL) to the sample solution (150µg/mL). The % recovery was calculated and reported in the table 4.

The obtained values of recovery studies were in the range of 98.7-103.6% which indicates that the proposed method is accurate. ICH demands that 'a good accuracy value' should be in the range of 98-102% of the true value.

Precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogeneous sample. It provides an indication of random errors results and expressed as Relative Standard Deviation (%RSD). Repeatability assessment of an analytical method is performed by analyzing six replicates of single concentration that is 150µg/mL. Absorbance of

Table 1: Calibration curve data.

Sl. no	Concentration (µg/mL)	Absorbance (n=3)
1	50	0.015
2	100	0.026
3	150	0.046
4	200	0.06
5	250	0.076

Table 2: Optimum conditions and spectral data.

λmax	626.5nm
Beer's law range	50-250µg/mL
Sandell's sensitivity (µg/cm²/0.001 absorbance units)	3.33
Molar absorptivity (litre/moles/cm)	1.65x108
Limit of detection (µg/mL)	6.87
Limit of quantification (µg/mL)	20.8
Regression equation (y)	y=0.0003x-0.0017
Slope	0.0003
Intercept	-0.0017
Correlation coefficient (r²)	0.9951
Standard deviation of Intercept	6.24×10^{-4}

Table 3: Accuracy data of tenofovir disoproxil fumarate (pure drug) at 626.5nm using MBTH Reagent.

Sl. no	Concentration (µg/mL)	Mean ± Standard deviation (n=3)	%RSD
1	120	120 ± 1	0.83
2	150	150.3 ±1.5	0.99
3	180	180.3 ± 1.6	0.89

Table 4: Accuracy data of tenofovir disoproxil fumarate (drug product) at 626.5nm using MBTH Reagent.

Tablet used	Amount of sample added (µg/mL)	Amount of standard added (µg/mL)	Amount recovered (µg/mL)	%Recovery± Standard Deviation (n=3)
	150	120	269.86	98.87 ± 1.064
Tenvir	150	150	298.33	98.88 ± 1.2
	150	180	336.33	101.9 ± 1.28

Table 5: Repeatability data of tenofovir disoproxil fumarate at 626.5nm using MBTH reagent.

Sl. no	Concentration (µg/mL)	Absorbance	Mean ± Standard deviation	%RSD
1		0.0370		
2		0.0380		
3	150	0.0382	0.0379±0.000456	1.203
4		0.0379		
5		0.0381		
6		0.0382		

samples is recorded at 626.5nm. The % relative standard deviation (RSD) was calculated and reported in table 5.

Variations of results within the same day (intraday) and variation of results between days (inter day) were analyzed. The intra-assay precision of the proposed method was determined on samples of drug solutions at varying concentration levels (150µg/mL, 200µg/mL, 250µg/mL) by analyzing three replicates of each sample as a batch in a single assay run at 626.5nm. The Inter-assay

Table 6: Intra-day and Inter day Precision data of tenofovir disoproxil fumarate at 626.5nm using MBTH Reagent.

Sl. no	Concentration (μg/mL)	Intraday absorbance Mean ±Standard deviation(n=3)	%RSD	Interday absorbance Mean±Standard Deviation(n=3)	%RSD
1	150	0.0393±0.001155	1.92	0.0383±0.000703	1.85
2	200	0.0593±0.001155	1.94	0.059±0.001158	1.96
3	250	0.074±0.0014504	1.96	0.075±0.001485	1.98

Table 7: Ruggedness results of Tenvir 300mg tablet at 626.5nm using MBTH Reagent.

Replicates	% Label content	
	Analyst-I	Analyst-II
1	104	101
2	101	103
3	102	100
4	99.8	99.9
5	100	102
6	101	104
Average	101.3	101.65
SD	1.5427	1.6537
%RSD	1.523	1.627

precision was determined by analyzing the same samples (150μg/mL, 200μg/mL, 250μg/mL) in three consecutive days at 626.5nm. The %RSD was calculated and results were in table 6.

Ruggedness of an analytical method is the degree of reproducibility of test results obtained by analysis of same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different days etc. Ruggedness is normally expressed as lack of influence on the test results of operational and environmental variables of analytical method. Ruggedness is a measure of reproducibility of test results under the variation in normally expected from laboratory to laboratory and analyst to analyst.

Ten tablets were weighed and finely powdered. The powder equivalent to 100mg was weighed and transferred to 100mL volumetric flask containing 25mL methanol and sonicated for 30 minutes. The solution was centrifuged at 2500rpm for 15 minutes. From the above solution (1000μg/ml) 1.5mL of sample was pipetted into 10mL graduated tube followed by addition of 1mL of MBTH reagent (0.5%w/v) and 1mL of Ferric chloride solution (1%) and solution was made to 10mL using distilled water. The absorbance of resulting green coloured solution was measured at 626.5nm against appropriate reagent blank. The % label content was reported to which % relative standard deviation (RSD) was calculated. The results obtained were shown in table 7.

Application of the method for estimation of tenofovir disoproxil fumarate in tablets using MBTH Reagent
Twenty tablets of tenofovir disoproxil fumarate (Tenvir manufactured by Cipla Ltd., Goa, India, contains 300mg of tenofovir disoproxil fumarate) were weighed and finely powdered. The powder equivalent to 100mg was weighed and transferred to 100mL volumetric flask containing 25mL methanol and sonicated for 30 minutes. The flask was shaken and volume was made up to mark with methanol to obtain a solution of 1000μg/mL. The solution was centrifuged at 2500rpm for 15 minutes.

From the above solution (1000μg/ml), 1.5mL of sample was pipetted into 10mL graduated tube followed by addition of 1mL of MBTH reagent (0.5%w/v) and 1mL of Ferric chloride solution (1%) and solution was made to 10mL using distilled water. The absorbance of resulting green colored solution was measured at 626.5nm against appropriate reagent blank.

RESULTS AND DISCUSSION
The analytical method development and validation for the tenofovir disoproxil fumarate using MBTH reagent was carried out. The linearity calibration curve (figure 2) shows linear response over the range of concentration used. The precision data shows that the reproducibility of the assay procedure was satisfactory and the accuracy of the method was determined by recovery studies (table 1-7).

The developed and validated method was successfully applied for estimation of drug in formulation. The results (308.21mg, 102.73% of label claimed) were found to be well within the acceptable limits of 90-110% (IP).

CONCLUSION
From the above discussion it can be concluded that all validation parameters (precision, accuracy, linearity) met the predetermined acceptance criteria and the developed spectrophotometric method can be applied for routine analysis of tenofovir disoproxil fumarate and its dosage forms.

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