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ANALYTICAL METHOD DEVELOPMENT FOR DETERMINATION OF TENOFOVIR DISOPROXIL FUMARATE BY HPLC

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INTRODUCTION

Analytical method development and validation play important roles in the discovery, development and manufacture of pharmaceuticals. Method development is the process of proving that an analytical method is acceptable for use to measure the concentration of an active pharmaceuticals ingredient in specific compounded dosage form which allows simplified procedure to be employed to verify that an analytical procedure accurately and consistently will deliver a reliable measurement of an active ingredient in an compounded dosage form.

Most of drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

Some of advantages are

- Improved resolution of the separated substances
- Faster separation times and
- Improved accuracy, precision, & sensitivity with which the separated substances may be quantified.

There are a number of modes of HPLC enabling an extremely wide range of solute mixtures to be separated. The modes are defined by the type of stationary phase and associated sorption mechanism.

1. **Normal phase mode.**
2. **Reversed phase mode.**
3. **Ion pair chromatography.**
4. **Affinity chromatography.**
5. **Size exclusion chromatography.**

1.1 Few analytical methods

For analyzing the analyte, several analytical methods are available; few of them which are routinely used are listed below.

I. Spectroscopic methods

- UV-Visible Spectroscopic method
- Infrared Spectroscopy
- Mass Spectroscopy
- NMR Spectroscopic method

II. Chromatographic methods

- High Performance Liquid Chromatography
- Supercritical Fluid Chromatography
- High Performance Thin Layer Chromatography
- Gas Liquid Chromatography
- GC-MS & LC-MS

III. Electrochemical methods

- Conductometry
- Voltametry
- Potentiometry
- Coulometry
- Atomic Absorption Spectroscopy
- Emission (Plasma) Spectroscopy

IV. Other conventional methods

- Titrimetry
- Gravimetry

1.2 UV-VIS spectrophotometry

It may be defined as a method of analysis that embraces the measurement of absorption by chemical species of radiant energy at definite and narrow wavelength, approximating monochromatic radiation. The electromagnetic spectrum extends from 100-780 nm and is divided into various regions viz Far (or vacuum) ultraviolet [100-200 nm], near ultraviolet [200-400 nm] and visible [400-780].

1.2.1 Beer's lambert's law

"When a beam of monochromatic light is passed through a transparent cell containing a solution of an absorbing substance, reduction of intensity of the light may occurs; the rate of reduction in intensity with the thickness of the medium is proportional to the intensity of the light and the concentration of the absorbing substances. Mathematically, Beer- Lamberts law is expressed as

$$A = log\ Io/It = abc$$

Equation 1 Beer-Lamberts law

Where,

A = Absorbance of the solution at particular wavelength of the light beam Io = Intensity of incident light beam

It = Intensity of transmitted light beam

a = Absorptivity of molecule at the wavelength of beam

b = Path length of cell in cm c = Concentration of solution in gm/lit.)

1.2.2 Quantitative analysis by UV-Visible spectrophotometer

Single Component Analysis When the absorption of each of a series of solutions of the same substance are measured at the same wavelength, temperature and solvent conditions, a graph of absorbance measured can be plotted against its concentration. If the graph is a straight line passing through the origin, then it is said to obey Beer‟s law over that concentration range. The concentration of a component in a sample which contains other absorbing substances may be determined by a simple Spectrophotometric measurement of absorbance, provided that the other components have a sufficiently small or negligible absorbance at the wavelength of measurement. Once this is determined, the analysis of known samples of this substance can be easily done under the same experimental conditions. The absorbance is measured and from the Beer‟s plot, the unknown concentration can be calculated. Multicomponent Analysis is used to analyze the drugs simultaneously. The basis of all the

Spectrophotometric methods for multicomponent sample analysis is based on the property that at all wavelengths is the absorbance of a solution is the sum of absorbance of the individual components. Following methods are being used;

- Simultaneous equation method
- Absorbance ratio method
- Geometric correction method
- Orthogonal polynomial method
- Difference spectrophotometric method
- Derivative spectrophotometric method

1.3 Chromatography

Chromatography was originally developed by the Russian botanist Michael Tswett in 1903 for the separation of colored plant pigments by percolating a petroleum ether extract through a glass column packed with powdered calcium carbonate. It is now, in general, the most widely used separation technique in analytical chemistry having developed into a number of related but quite different forms that enable the components of complex mixtures of organic or inorganic components to be separated and quantified. A chromatographi separation involves the placing of a sample onto a liquid or solid stationary phase and passing a liquid or gaseous mobile phase through or over it, a process known as elution. Sample components, or solutes, whose distribution ratios (vide infra) between the two phases differ will migrate (be eluted) at different rates, and this differential rate of migration will lead to their separation over a period of time and distance.

1.3.1 Principle of chromatography

The term, -chromatography" was coined by the Russian botanist, Tswett, who demonstrated that, when a plant extract was carried by petroleum ether through a column consisting of a glass tube packed with calcium carbonate powder, a number of dyes were separated. Chromatography is a technique by which a mixture sample is separated into individual components. Although originally intended to separate and recover (isolate and purify) the components of a sample, now a day, complete chromatography systems are often used to both separate and quantify sample components. This analysis method "Chromatography" after "chroma" and "graphos", which are Greek words meaning "color" and ―to draw," respectively.

1.3.2 Chromatographic techniques

Separation processes are used to decrease the complexity of material mixtures. The most utilized separation method is chromatography. Following types of techniques are used:

- Gas chromatography (GC)
- High performance liquid chromatography (HPLC)
- Size- exclusion chromatography
- High-performance thin layer chromatography (HPTLC)
- Paper chromatography
- Thin layer chromatography (TLC)
- Affinity chromatography
- Ion exchange chromatography

After the isolation of material signal is generated, the signal must be detected and interpreted.

Hyphenated techniques Combinations of the above techniques are called as "hybrid" or "hyphenated" techniques. Several examples are in popular use today and new hybrid techniques are under development.

- GC-MS (gas chromatography – mass spectrometry)
- ICP-MS (inductively coupled plasma-mass spectrometry)
- GC-IR (gas chromatography-infrared spectroscopy)
- MS-MS (mass spectrometry-mass spectrometry)

Instrumental methods are sensitive and it needs small amount of sample. Complex mixtures can be analyzed with or without their prior separation with sufficient reliability and accuracy of results.

Miscellaneous techniques following types of miscellaneous techniques are used

1. **Mass analysis:** Mass spectrometry measures the interaction of charged materials and electric and magnetic fields.
2. **Thermal analysis:** Calorimetry and thermo gravimetric analysis measure the interaction of material and heat. In order to utilize the techniques available currently, complex material mixture must be separated into simpler samples for individual analysis**.**

1.4 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is a separation technique where solutes migrate through a column containing a microparticulate stationary phase at rates dependent on their distribution ratios. These are functions of the relative affinities of the solutes for the mobile and stationary phases, the elution order depending on the chemical nature of the solutes and the overall polarity of the two phases. Very small particles of stationary phase are essential for satisfactory chromatographic efficiency and resolution, and the mobile phase must consequently be pumped through the column, resulting in the generation of a considerable back-pressure. The composition of the mobile phase is adjusted to elute all the sample components reasonably quickly. Solutes eluted from the end of the column pass through a detector that responds to each one. There are a number of modes of HPLC enabling an extremely wide range of solute mixtures to be separated. The modes are defined by the type of stationary phase and associated sorption mechanism.

A schematic diagram of a high-performance liquid chromatograph is shown in *Figure 1*. It consists of five major components

- Solvent delivery system;
- Sample injection valve;
- Column;
- Detection and recording system;
- Microcomputer with control and data-processing software.

1.4.1 Parameters used in chromatogram characterization

a) Retention time (tR): The time needed for the analyte to move from the injector (the time at which the injection takes place is defined as t = 0) through the system and detector.

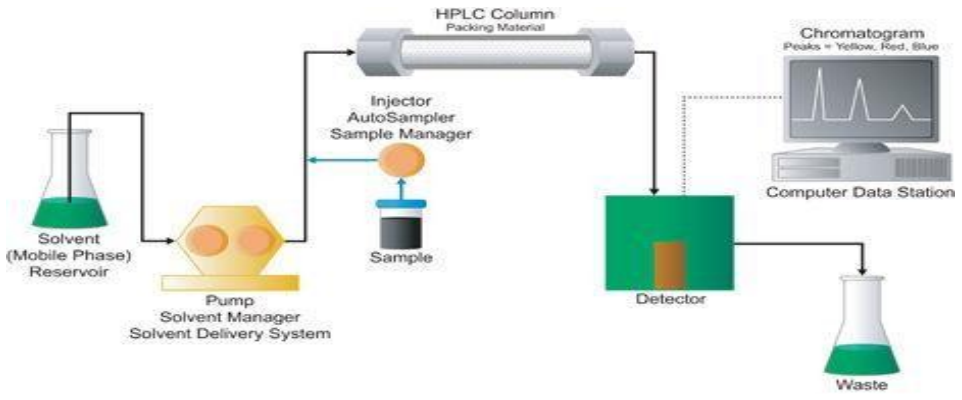

Figure 1: Schematic presentation of HPLC system.

$$R=2\ t2-t1\ /w1+w2$$

Equation 2 Retention time

b) Capacity Factor (K'): A column must have the capacity to retain sample & the ability to separate sample components efficiently. Generally the value of K" is > 2.

$$K=t/ta-1$$

Equation 3 Capacity factor

Where,

t= retention time of drug,

ta = retention time of non-retarded component

c) Column resolution: The resolution Rs of a column provides a quantitative measure of its ability to separate two analytes. The Resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates.

d) Tailing factor

It is the measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing become more pronounced.

$$T= Wx/2f$$

Equation 4 Tailing factor

Where, *Wx* = width of the peak determined at either 5% from the baseline of the peak height and f is distance between peak maximum and peak front. The accuracy of quantitation decreases with increase in peak tailing. $0.5 \leq T \leq 2$.

e) Theoretical plate number: It is a measure of column efficiency that is how many peaks can be located per unit run time of the chromatogram.

$$N= 16\ (tR/tw)\ 2 =L/H$$

Equation 5 Theoretical plate number

Parameters which can affect N or H include peak position, particle size in column, flow rate of mobile phase, column temperature, and viscosity of mobile phase and molecular weight of analyte. Theoretical plates should be more than 2000.

1.4.2 Types of HPLC

1) Normal phase
2) Reverse phase

1. Normal phase:- In this column packaging is polar and mobile phase is non polar. In this phase compounds with low polarity elute first, gradient elution not used for normal phase. This technique is used for separation of isomers and low polar drugs.

2. Reverse phase:- In reverse phase column packaging is non polar and mobile phase is polar. In this type compound with high polarity elute first.

1.5 Method development

Method development is a challenging and time-consuming process requiring much experience, creativity, logical thinking, and experimentation. With all the software and automated systems available today, method development is still very much a trial-and-error approach, expedited by a logical sequence of generic scouting runs and fine-tuning steps to achieve the requisite resolution and method performance.

1.5.1 Considerations before method development

Developing and validating new analytical methods is costly and time consuming. Before starting the process, a thorough literature search should be conducted for existing methodologies of the intended analytes or similar compounds. This should include a computerized search of chemical abstracts and other relevant sources such as compendial monographs (USP, EP), journal articles, manufacturer literature, and the Internet. Although this search might not uncover a directly usable method, it often provides a starting point for method development or at least some useful references. New analytical methods are needed for the following reasons

- Existing methods are not available (e.g., New Chemical Entity (NCE) for consideration as a new drug candidate).
- Existing methods are not sufficiently reliable, sensitive, or cost effective.
- New instrumentation or technique has better performance (ease of use, rapid turn around, automation, higher sensitivity).
- An alternate (orthogonal) method is required for regulatory compliance

1.5.2 Factors to be considered for choice of analytical method

An important task for the analyst is to select best procedure for a given determination this

will require careful consideration of the following criteria,

- The type of analysis required: elemental or molecular, routine or occasional.
- Problem arising from the nature of the material to be investigated, e.g. radio-active substance, corrosive substance, substances affected by water.
- Possible interference from components of the material other than those of interest.
- The concentration range to be investigated.
- The accuracy required.
- The facilities available, particularly the instrument.
- The time required to complete the analysis.
- The number of analysis of similar type which have to be performed.

1.6 HPLC method development and validation

HPLC method development generally follows the following steps

STEP 1: Selection of the HPLC method and initial system

STEP 2: Selection of optimum condition

STEP 3: Selectivity optimization

STEP 4: System parameter optimization

STEP 5: Method validation.

Step 1: Selection of hplc method and initial condition

- When selecting an HPLC system it must have a high probability of actually being able to analyse the sample.
- For example if the sample includes polar analytes then RP-HPLC would offer both adequate retention and resolution.

Consideration must be given to the following

a. Sample preparation
b. Types of chromatography
c. Column selection
d. Detector selection
e. Selection of mobile phase composition

Step 2: Selection of initial conditions

- This step determines the optimum conditions to adequately retain all analytes; i.e.

✓ Ensures no analyte has a capacity factor of less than 0.5(poor retention could result in

peak overlapping).

✓ No analyte has a capacity factor greater than 10-15 (excessive retention leads to long analysis time and broad peaks with poor detectability).

Determination of initial conditions

- The recommended method involves performing two gradient runs differ in only in the run time.
- A binary system based on either acetonitrile/ water or methanol/water should be used.

Step 3: Selectivity optimization

- The aim of this step is to achieve adequate selectivity.
- The mobile phase and stationary phase compositions need to be taken in to account.
- To select these the nature of the analytes must be considered.
- Once the analyte types are identified the relevant optimization parameters may be selected.

Step 4: System parameter optimization

- This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved.
- The parameters involve include column dimensions, column packing particle size and flow rate.
- This parameters may be changed without affecting capacity factors or selectivity.

Step 5: Method validation

The parameters for validation of analytical method defined by the ICH are as;

1. **Accuracy**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

This is sometimes termed trueness

2. **Precision**

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed condition.

Precision may be considered at three levels

a) Repeatability: expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
b) Intermediate precision: expresses the precision within-laboratories variations: different days, different analysts, different equipment, etc.
c) Reproducibility: expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology

3. Specificity/selectivity

The ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

4. Limit of detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$DL = 3.3\square/S$$

Equation 6 Limit of Detection

5. Limit of quantitation

The lowest amount of analyte that can be measured. generally it is determined by analysis of samples with concentration of analyte.

$$QL = 10\square/S$$

Equation 7 Limit of Quantitation

6. Linearity

It is proportionality of measured value to concentration.

7. Range

Range of an analytical method is the interval from the upper to the lower levels. The ranges normally expressed in the same unit as test results obtained.

2. Literature review

Premnath S. et.al., (2013) developed stability indicating methodfor Tenofovir nanoparticle formulation. separation was achieved by Lichrocart (C18) (250mm × 4.6mm, 5 µm particle size) column, mobile phase consist of acetronitrile:0.025M potassium dihydrogen phosphate buffer Ph 3.0 adjusted by using 10%v/v orthophosphoric acid)35:65, flow rate 1ml/min

retention time wasfound to be 7min for tenofovir and run time-10min.

Suresh M.et.al, (2012) developed RP-HPLC method for the simultaneous estimation of Emtricitabine and Tenofovir from bulk and dosage form.separationwas achieved by phenmenex luna (C18) (150mm × 4.6mm, 5 µm particle size) column, mobile phase consist of buffer:acetonitrile in ratio 60:40%v/v, flow rate 1ml/min, retention time was found to be 5.3min foe Tenofovir and 2.3min for Emtricitabine respectively and run time 8 min.

Tripti S.et.al, (2012)developed RP-HPLC method for estimation of tenofovir disoproxil fumarate in bulk and pharmaceutical formulation. separation was achieved by Inertsil ODS-3(150mm × 4.6mm, 5 µm particle size) column, mobile phase sodium dihydrogen orthophosphate buffer ph2.3 and methanol (49:51)v/v, flow rate 1 ml/min, detection wavelength 260nm. retention time was found to be 9.437 min for tenofovir and run time 12.5min.

Subrahmanyam K.V.et.al,(2014) developed and validated Tenofovir in its bulk form by using RP-HPLC. separation was achieved by using Develosil ODS HG-RP (C18) (250mm × 4.6mm, 5 µm particle size) column, mobile phase water:methanol (45:55)v/v, flow rate 1.3ml/min and detection wavelength 230 nm. retention time was found to be 5.69 min and run time 10 min.

Sivaram M, et.al. (2014)developed stability indicating R-HPLC method of Tenofovir in bulk and pharmaceutical formulation.separation was achieved by (C18) (250mm × 4.6mm, 5 µm particle size)column.mobile phase methanol:potassium dihydrogen orthophosphoric buffer ph 3 in the ratio 30:70 v/v flow rate 1ml/min and detection wavelength 260 nm retention time was found to be 7.33 min and run time 10 min.

Mohan V.G.et.al, (2013) developed simultaneous determination of Lamivudine and Tenofovir in tablet dosage form by RP-HPLC separation was achieved by phenomenax luna (C18) (150mm × 4.6mm, 5 µm particle size) column, mobile phase acetonitrile:methanol:water in ratio 30:50:20v/v, flow rate 1ml/min detection wavelength 258nm retention time was found to be 5.127 min for Tenofovir and 2.166 min for lamivudine respectively and run time 11 min.

Vidya D.et.al,(2013)developed RP-HPLC method for simultaneous estimation of Tenofovir disoproxil fumarate and Efavirenz in bulk formulation .separation was achieved by AZE

XDB (C18) (250mm × 4.6mm, 5 µm particle size) column, mobile phase acetonitrile and phosphate buffer 70:30v/v, flow rate 0.6ml/min detection wavelength 255nm. Retention time were 2.44 min for TDF and 5.52 min for Efvirenz respectively.

Bhavini N P, **et.al**(2012),developed RP-HPLC method for simultaneous estimation of Tenofovir, Lamivudine, and Efavirenz in combined tablet dosage form separation was achived by kromasil (C18) (250mm × 4.6mm, 5 µm particle size) column mobile phase 70:30 methanol:10Mm phosphate buffer,flow rate 1ml/min and detection wavelength 254nm retention time was found to be min and run time min.

Arun R, et.al(2014),developed analytical method for quantitation of Emtricitabine, Tenofovir, Efavirenz based on HPLC, separation was achieved by zorbax SBCN (C18) (250mm × 4.6mm, 5 µm particle size) column, mobile phase methanol:buffer at Ph4.5 flow rate 1.5 ml/min and detection wavelength 260 nm retention time was found to be run time 21min.

3. JUSTIFICATION AND OBJECTIVE

3.1 Justification

The importance of antiretroviral drugs are increasing day by day generally Tenofovir Disoproxil Fumarate is belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors which block reverse transcriptase, an enzyme crucial to antiviral production in HIV-infected people. So, these drug are used for method development by RP- HPLC. Few analytical techniques are reported for TNF out of which many of these are time consuming, affecting column life and costly. So this research work is dedicated to develop and validate simple, efficient, economical, fast, reliable new method for estimation of TNF in bulk and pharmaceutical dosage form by RP- HPLC.

3.2 Objective

- To develop UV spectrophotometric method.
- To validate UVspectrophotometric method as per ICH guideline.
- To develop RP-HPLC method.
- To validate RP-HPLC method as per ICH guideline Q2R1.

4. Plan of work

- Literature survey

- Procurement of drug
- UV method development
- Selection of wavelength
- Selection of mobile phase
- Optimization of method
- Selection of chromatographic conditions
- Application of proposed method to marketed formulation
- Validation of proposed method
- Linearity
- Range
- Accuracy
- Precision
- Detection limit
- Quantitation limit
- Robustness
- System suitability

5. Drug profile

i) Name:- Tenofovir disoproxil fumrate structure

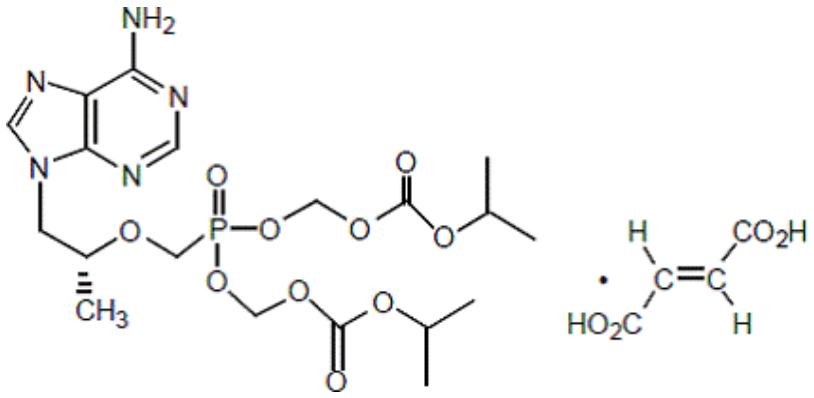

Figure 2: Structure for tenofovir disoproxil fumarate.

Chemical name:-2[[bis[[(isopropoxycarbonyl)oxy]methoxy]p

hosphinyl]methoxy]propyl]adenine fumarate.

Molecular formula:- C23H34N5O14P

Molecular weight:- 635.514g/mol

Appearance:- White to off white crystalline powder crystalline powder

Melting point:-276-280°c

Solubility:- Methanol, Acetonitrile, water

Category:- Anti retroviral agent.

Wavelength:-257nm

Mode of action:-Tenofovir is a defective adenosine nucleotide that electively inhibits the action of reverse transcriptase by competing with the natural substrate deoxyadenosine 5‟-triphosphate and, after incorporation into DNA, by DNA chain termination. TNF prevents the formation of 5‟ to 3‟ phosphodiester linkage essential for DNA chain elongation. a phosphodiester bond can not be formed because the tenofovir molecule lacks an –OH group on the 3‟ carbon of its deoxyribose sugar. once incorporated into a growing DNA stand, tenofovir causes premature termination of DNA.

6. MATERIAL AND METHOD

6.1 Raw material characterization

6.1.1 Characterization of Tenofovir Disoproxil Fumarate(TNF) Determination of melting point

Melting point was determined using digital melting point apparatus (Labatronics) by capillary method. The reference melting point of Tenofovir Disoproxil Fumarate is 270º C.

6.2 Reagents and chemicals.

Table 1Chemicals and Reagents.

Chemicals	Manufacturer
HPLC Water	As HPLC grade no further characterization performed
OPA	As HPLC grade no further characterization performed
Methanol	As HPLC grade no further characterization performed

6.3 Determination of □max by UV

100□g/ml solution of Tenofovir Disoproxil Fumarate was prepared by accurately weighing 10 mg of TNF. It was then transferred to 100 ml volumetric flask Containing few ml of methanol. Finally the volume was made up to the mark using water. The resultant solution was scanned using UV visible spectrophotometer in the range of 200-400 nm. The reference

□max TNF is 257 nm.

6.4 Experimental Work

6.4.1 UV Method Development and validation Determination of wavelength Maxima

Preparation of Standard Stock solution 10 mg of TNF was weighed accurately and transferred to 100ml volumetric flask containing some amount of solvent (acetonitrile). Volume was made up to the mark using water to obtained the resulting solution 100 µg/ml. The absorbance of the latter was recorded using UV visible spectrophotometer in range 200-400nm.Similarly 100 ml solution of TNF were prepared in 85:15 Methanol and water. The absorbance of each of these solutions was recorded by using UV spectrophotometer.

6.4.2 Method optimization

Preparation of working solution

Accurately weighed 10mg of TNF and transferred to 100ml volumetric flask containing a mixture of methanol:water in the ratio of 85:15.The volume was made up to the mark using same mixture of solvent then 1.0 ml was pipetted out and diluted up to 10ml which will give resultant solution of 10 µg/ml which was used for optimization. Five replicates of the solution were performed and recorded at 257nm. Mean SD and % RSD were calculated.

6.4.3 Method Development

Preparation of calibration curve

From stock solution 1.0, 2.0, 3.0, 4.0, 5.0,ml solutions were pipette out and diluted up to 10ml using mobile phase to obtain resultant solutions of 10, 20, 30, 40, 50µg/ml. Absorbance for each of these solutions was recorded in triplicate and calibration curve was constructed considering mean absorbance of each test solution. From the calibration curve equation of line, correlation coefficient and intercept were determined.

A) Precision

From the calibration range three QC standards decided viz. 15, 25 and 35µg/ml as LQC, MQC and NQC respectively. The solutions for QC standards were prepared by diluting stock solution of 1.5, 2.5 and 3.5 ml solutions up to 10ml. Absorbance of each QC standard was recorded for intraday and inter-day precision.

B) Accuracy

% Accuracy was determined using observations of precision study using following formula.

$$\frac{\text{mean measured concentration - Nominal}}{\text{nominal}} *100$$

C) LOD and LOQ

Equation 8 Percent Accuracy

LOD and LOQ were determined using following formulas.

LOD = 3.3*I/ (SD)

LOQ = 10*I/ (SD)

Equation 9 LOD and LOQ

Where, I = Intercept of the graph, SD = Standard Deviation

6.5 HPLC method development and validation

From results obtained from UV method methanol: (% OPA)water(85:15) was selected as mobile phase for HPLC method development.

6.5.1 Preparation of stock solution

Standard stock solution was prepared by dissolving accurately weighed 10 mg of Tenofovir Disoproxil Fumarate in 10 ml of Methanol that gave concentration of 1000 µg/ml.

6.5.2 Chromatographic parameters

6.5.3 System suitability testing

Preparation of solution

2.0ml stock solution was pipetted out and diluted up to 10 ml to obtain consequential solution of 20 µg/ml. The resulting solution was filtered through 0.45 µ membrane filter and sonicated for three cycle each of 10 min. Six replicates of this solution were injected and recorded for RT, area, tailing factor (symmetry factor) and theoretical plates. Mean, SD and %RSD were calculated for the results obtained as well as other parameters were also verified for acceptability level.

Column	C_{18} (YMC)4.6 x 250 mm
Run time	10 minutes
Wavelength	257 nm
Flow rate	0.7 ml/min
Injection volume	20 □l
Column oven temp	Ambient

6.5.4 Method validation

A) Linearity

From stock standard solution, aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5ml were taken in 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain concentration of 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml of Tenofovir Disoproxil Fumarate respectively. Calibration curve was constructed brtween concentration versus peak area. Results were recorded for equation of line; correlation coefficient and intercept.

$$Y= mX+c$$

Equation 11 Linearity

Where, Y- area, X- Concentration, m- Slope of graph and c- Intercept.

B) Precision

From the calibration range three QC standard decided viz. 10, 15, and 20 µg/ml as LQC, MQC, and NQC respectively. The solution for QC standards were prepared by diluting stock solution of 1.0, 1.5, 2.0ml solutions up to 10ml. Area of each QC standard were recorded for intraday and interday precision. Results were recorded to calculate mean, SD, %RSD.

C) Accuracy

% Accuracy was determined from the observation of Precision study using following formula. Limit for % accuracy is NMT 5% RSD.

D) Robustness

Robustness of the method was studied by making deliberate changes in few method parameters viz. flow rate, wavelength and mobile phase concentration. The effects on the results were studied by injecting 20 µg/ml of Tenofovir Disoproxil Fumarte. One factor was varied at time to estimate the effect.

Table 2: Robustness variations table.

Condition	Normal	Variation 1	Variation 2
Flow rate	0.7ml/min	0.6ml/min	0.8 ml/min
Wavelength	257nm	256nm	258nm
Mobile phase	85:15	84:16	86:14

E) %Recovery

Preparation of stock from API

Accurately Weighed 10mg of TNF(API)was added in volumetric flask containing some

amount of mobile phase and volume was made up to the mark using mobile phase. The resulting solution was filtered through 0.45□ membrane filter and sonicated for three cycles each of 10 min. From the stock solution 2.0ml of stock was pipetted out in triplicate and kept in three different volumetric flasks, cleaned previously and diluted up to 10ml by using mobile phase to obtain resultant solution of 10□g/ml. This solution was injected for given chromatographic system in triplicate and mean area was determined.

Preparation of Stock From dosage Form

Twenty tablets (Label claim 300mg of TNF) were weighted; average weight was determined and powdered. Powder equivalent to 10mg (10 x 570/300= 19mg) was transferred to 10 ml of mobile phase i.e. 1000 µg/ml. The resulting solution was filtered through 0.45µ membrane filter and sonicated for three cycles each of 10 min. From the stock 0.8, 1.0, 1.2ml solution were pipetted out and diluted up to 10ml using mobile phase to obtain resultant of 8, 10,12□g/ml.

7.0. RESULTS AND DISCUSSION

Raw material characterization

Melting point

The melting point was determined by capillary method using digital melting point apparatus (Labatronics). and found to be 270º C. The observed melting point corresponding with reference value as per IP (276-280º C)

7.1 UV method development

UV Visible spectroscopy is a tool used for qualitative and quantative determination of different kinds of analyte. Many organic molecules comprises of different types of electron which may absorb energy and reach to their corresponding excited state.The phenomenon of absorption of energy in UV region 200-400nm is the basis for quantitative as well as qualitative determination of many organic molecules. The functional group consisting of different types of electrons are responsible for absorption of enegy and hence known as chromaphore. Present investigation includes development of UV method for quantitative determination of TNF. Initially, 20□/ml solution of TNF was prepared in the mixture of methanol : (%OPA)water. This solution was subjected to UV analysis in qualitative mode to determine the absorption maxima (□max). The UV spectrum obtained was an given in figure and showed the absorption at different wavelengths as given in table below. The wavelength of 257nm was selected for quatitative determination of TNF as given in further sections.

7.1.1. Determination of wavelength

Table 3 UV Analysis for detection of wavelength.

Sr. No.	Wavelength (nm)	Absorbance
1	206nm	1.7422
2	257nm	0.6342

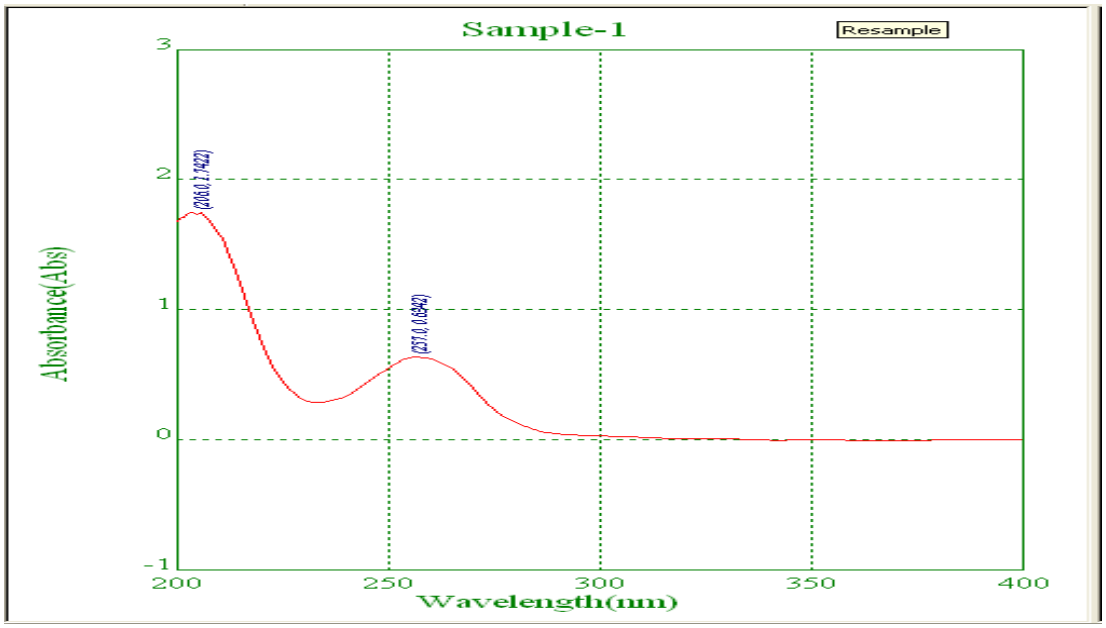

Figure 3: Wavelength spectra.

Wavelength vs. absorbance

From the above calibration UV spectrum the wavelength maxima for TNF was found to be 257nm.

7.1.2 Validation parameter

A) Linearity

The linearity of analytical method is its ability to elicit test results that are directely, or by a well-defined mathematical transformation, proportional to the conc. of analyte in sample within the given range. It should be established across the range of the analytical procedure. Linearity is generally reported as correlation coefficients, the slope of regression line i.e, r2≥0.999.

Linearity of method was ranging from concentration 10-50 □g/ml for TNF. A graph was plotted with concentration on X axis and mean absorbance on Y-axis. The r2 value was found to be 0.999. Hence the develop method was found to be the linear in 10-50 □g/ml concentration array.

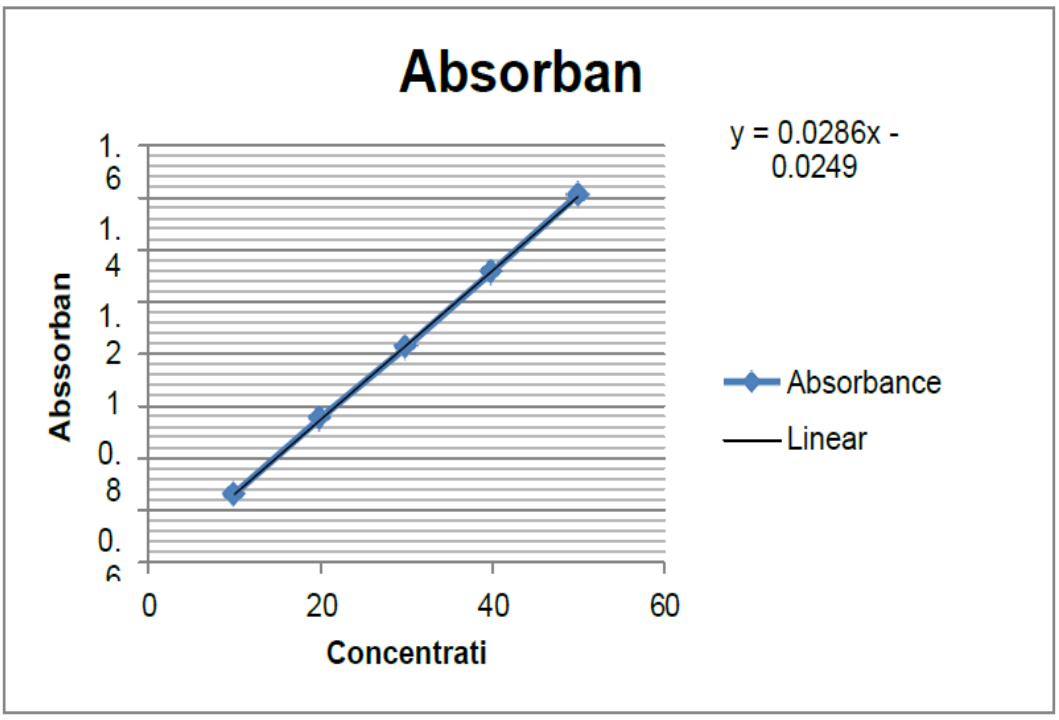

Figure 4: Linearity curve.

Table 4: Linearity data.

Concentration (µg/ml)	Absorbance
10	0.262
20	0.552
30	0.825
40	1.115
50	1.410

Table 7: Linearityparameter.

Parameter	UV method
Range	10-50µ/ml
Correlation coefficient	0.9998
Slope	0.0286

B) Precision

The precision of analytical method is the analytical method is the degree of agreement among individual test results when the method was applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method was usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. From the calibration range three QC standards were determine viz. 15, 25, 35□g/ml as LQC, MQC, and NQC respectively. LQC was the concentration minor more than the lowest concentration of linearity; MQC and NQC were concentration minor more than the

lowest, near to middle and near to highest that of linearity. The solution for QC standards were prepared by diluting stock solution of 1.5, 2.5, 3.5 ml up to 10 ml. Absorbance of each QC standard was recorded for intra-day and inter-day precision in triplicates as per ICH guidelines Q2R1.

Intraday precision

Table 5: Intraday precision data.

Sr. no.	Concentration	Mean absorbance	SD	%RSD	Inference
1	15	0.429	0.245	57.24	Passed
2	25	0.708	0.14	19.77	Passed
3	35	0.921	0.097	10.54	Passed

The proposed method had yielded quite consistent indicating particularity of method for quantitative determination of number of observation of TNF sample. Precision study illustrated that %RSD of mean absorbances of 15, 25,.35 ppm were less than 2%. Therefore, the result obtained for precision study was within limit (less than2%) as per ICH guideline Q2R1.

Interday precision

Table 6: Interday precision data.

Sr. no.	Concentration	Mean absorbance	SD	%RSD	Inference
1	15	0.429	0.237	55.24	Passed
2	25	0.708	0.137	19.41	Passed
3	35	0.921	0.100	10.91	Passed

C) % Accuracy

The accuracy of an analytical method is the closeness of test results, obtained by that method to the true value. The accuracy of an analytical method should be established across its range. accuracy was determined by data of precision study and the results obtained were as depicted in Table.9 As per ICH guideline Q2R1 accuracy was determined at three concentration levels (QC standards) across the range. The mean absorbance was determined at said three different levels and corresponding concentration for each level was computed from Beer‟s law. from the observed concentration and equivalent nominal concentration, percent accuracy was determined using formula. Results of same were as cited in Table 9.Results obtained were found to be within range of pharmacopoeia standard for TNF.

Table 7: Accuracy data.

Sr. no	Concentration (µg/ml)	Mean absorbance	Amount recover (mean measured conc.)	% assay	Limit (95-105%)
1	15	0.429	14.7	98%	Pass
2	25	0.708	25.2	100.8%	Pass
3	35	0.921	34.9	99.7%	Pass

D) LOD and LOQ

LOD is defined as lowest concentration of analyte likely to be reliably distinguished from blank and at which detection is feasible and within limits. LOD was determined by following formula and found to be 0.05□g/ml. similarly, LOD was calculated by using below equation and was found to be 0.174 □g/ml. from the result obtained it was concluded that the concentration of TNF as less as 0.05□g/ml can be successfully detected and concentration above 0.174 □g/ml can be productively quantified.

Table 8: LOD and LOQ.

Concentration	LOD	LOQ
10	0.05	0.174

7.4. HPLC method development

7.1 HPLC result

7.2.1 High performance liquid chromatography (HPLC) method

This technique is commonly used for the quantitative estimation of drug substances and drug product as well as for studying their metabolites. In addition HPLC as become more essential technique for estimation of drugs in biological fluid. This help analytical chemistry study chemistry study pharmacodynamics fate of drug molecule in vivo. This method also offers advantages of estimating the constituents for the multi component system. This technique was employed in the present investigation for estimation of TNF in bulk and tablet dosage form. various parameters influencing analysis considered an important aspect for the development of analytical method. In order to establish HPLC method the different parameters were studied in further sections.

7.2.2 HPLC column and detector

HPLC system with C_{18} (YMC)4.6 x 250 m analytical column and UV Visible detector were selected for quantification and detection of TNF respectively.used for study.The standard and sample solutions of TNF were prepared in mobile phase. Different HPLC grade solvents of varying polarities in with drivers proportion were attempted as mobile phase for development

of the chromatogram.

7.2.3. Selection of mobile phase

The preferred mobile phase methanol: water(0.1% Ortho phosphoric acid) (85:15) able to analyze the Tenofovir Disoproxil Fumarate at selected chromatographic condition.

7.2.4. Chromatographic condition

Column : C_{18}

Flow rate : 0.7 ml /min

Wavelength : 257 nm Injection volume : 20 µL Column temp : Ambient

Mobile phase : Methanol (85): water(15) (0.1 OPA)

7.2.5. System suitability test

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, flow rate and the column were studied. The resulting chromatograms were recorded and the chromatographic parameters such as peak area, resolution and theoretical plates were integrated. The conditions obtained most excellent resolution, symmetry factor and theoretical plates were selected for further estimation. The test was performed by six replicate injections of standard working solution of drug. The concentration was kept constant at 20µg/ml for TNF. The best resolution and peak shape, without unnecessary tailing, were obtained by use of chromatographic conditions as stated in

Table 13: The best resolution with reasonable.

retention time was obtained with mobile phase containing methanol, water(0.1% OPA) (90:10) with flow rate 7.0 ml/min in low pressure gradient mode as shown in **Table 16.** A representative at 257nm.

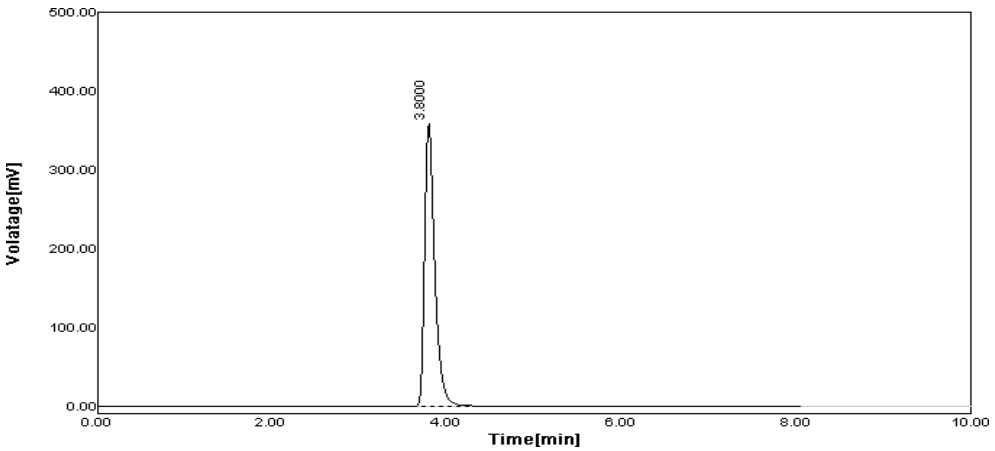

Figure 5: Chromatogram for System suitability testing.

Table 13: system suitability.

Parameter	Mean	Limit	Inference
Area	2912.79	%RSD <2	Passed
Retention time	3.76	<10-5	Passed
Theoretical plate	4264.3	>2000	Passed
Asymmetry	1.4	<2	Passed

7.2 Validation parameter

A) Linearity

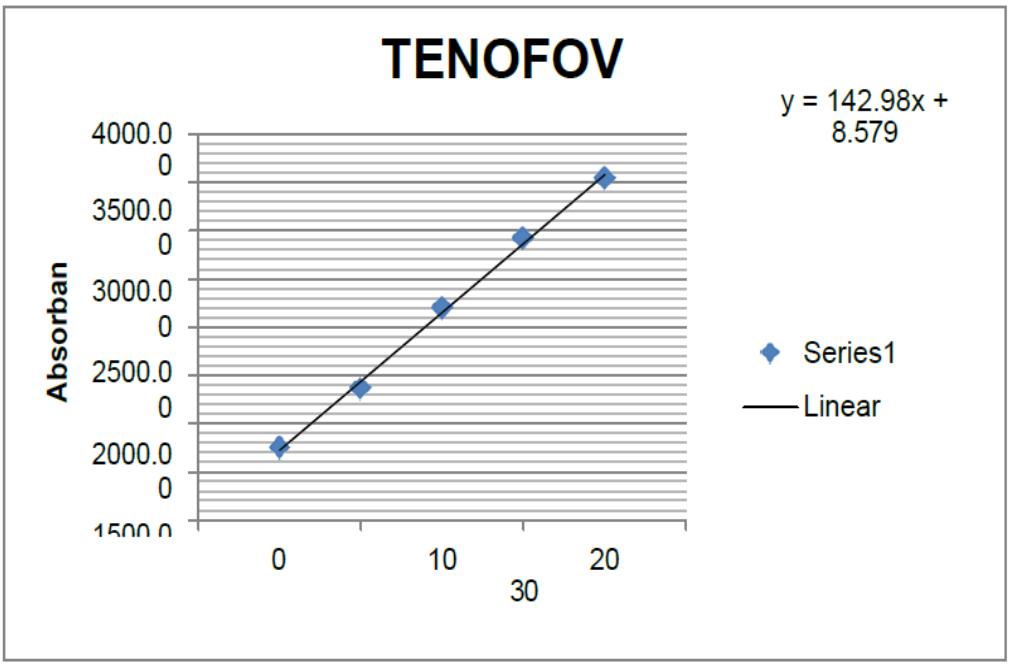

Figure 6: Linearity Graph by HPLC of Tenofovir Disoproxil Fumarate Conc. Vs area.

Table 9: Linearity data.

Sr. No.	Conc	Area I	II	Mean	SD	%RSD
1	5	741.37	757.64	749.37	11.70	1.56
2	10	1375.39	1344.1	1359.75	22.13	1.63
3	15	2215.86	2168.48	2192.17	33.50	1.53
4	20	2908.41	2937.22	2922.82	20.37	0.70
5	25	3574.04	3510.67	3542.36	44.81	1.26
				Avrg SD	66.25	

The linearity of an analytical procedure is its ability to elicit test results that are proportional to the concentration of analyte in the sample. From stock standard solution, aliquots of 0.5, 1, 1.5, 2.0, 2.5ml were taken in 10 ml volumetric flasks and diluted up to the mark with M0bile phase such that to obtained concentration of Tenofovir Disoproxil Fumarate in the range 5-25µg/ml. All measurements were repeated two times for each concentration and calibration curve was constructed by plotting the peak area *versus* the drug concentration.

The data obtained for linearity study for Tenofovir Disoproxil Fumarate .are shown in Table 14. The calibration curve are plotted by using concentration verses area for Tenofovir Disoproxil Fumarate in Figure 8.

Table 10: Linearity parameter.

Equation	Y=142.9x+8.579
Slope	142.9
Intercept	8.579
Regression	0.997

B) Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. It was verified by repeatability and intermediate precision studies. Intra-day precision was studied by analyzing 10, 15 and 20µg/ml of Tenofovir Disoproxil Fumarate for three times on the same day. Inter day precision was checked analyzing the same concentration for three different days over a period of weak. The results are shown in table 16 And 17.

Table 11: Intraday precision data.

Sr. No.	Conc	Area I	II	Mean	Amt Found	% Amt Fnd	SD	RSD
1	10	447.22	444.88	445.76	9.65	96.49	1.24	0.28
2	15	678.66	679.92	679.29	14.78	98.53	0.89	0.13
3	20	905.94	908.05	907.00	19.80	99.00	1.49	0.16

Table 12 Interday precision data.

Sr No.	Conc	Area I	II	Mean	Amt Found	% Amt Fnd	SD	RSD
1	10	449.22	442.25	445.74	9.64	96.40	4.93	1.11
2	15	674.65	676.42	675.54	14.70	98.00	1.25	0.19
3	20	909.82	904.63	907.23	19.80	99.00	3.67	0.40

C) % Accuracy

Table 13: % Accuracy data.

Concentration	Mean area	Amount recover	% Assay	Limit(98- 102)
10	2515.15	10.1	100.83	Passed
15	2865.83	14.9	99.41	Passed
20	3152.65	20.3	101.36	Passed

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. It can be calculated by using following formula,

$$\%\ Accuracy = Mean\ measured\ conc - NominalNominalNominal \times 100$$

Accuracy was determined by data of precision study and the results obtained were as depicted in Table16,17. As per ICH guideline Q2R1 accuracy was determined at three concentration levels (QC standards) across the range. The mean areas of seven replicate injections were determined and corresponding concentration for each level was computed from regression equation. From the measured concentrations and correspondent nominal concentrations, percent accuracy was determined using above formula. Results of the same were mentioned in Table 18. Results attained were found within range of pharmacopoeial standards for STG &SMV.

C) Robustness

Robustness of the method was studied by making deliberate changes in few parameters viz; flow rate, wavelength and mobile phase combination. The effects on the results were studied by injecting 20µg/ml of Tenofovir Disoproxil Fumarate; Data for robustness study is shown in table 19.

Table 14: Data for Robustness study.

Parameter	**% RSD**	
Flow rate (±0.1 ml) (Original- 0.8ml/min)	0.6ml/min	0.10
	0.8ml/min	0.79
Wavelength (±1 nm) (Original- 260 nm	256nm	0.54
	258nm	025
Mobile phase combination (±1) (Original-	84:16	52.12
Methanol : water (0.15 Acetic acid (80:20 v/v)	86:14	0.30

D) LOD and LOQ

Table 15: LOD and LOQ study.

LOD(g/ml)	**LOQ(g/ml)**
0.4258	1.2896

The limits of detection (LOD) and quantitation (LOQ) were determined separately, on the basis of the standard deviation of the y intercept and slope of the calibration plots. The LOD were found to be 0.4258 ug/ml. The LOQ were found to be 1.2896 ug/ml At these levels, RSD values were less than 2%, in accordance with ICH guidelines. LOQ and LOD data are shown in table 19.

E) Repeatability

It is measured by 5 injections of a sample of 15 µg/ml of Tenofovir Disoproxil Fumarate that indicates the performance of the HPLC instrument under chromatographic conditions, data for repeatability study are shown in table 21.

Table 16: Data showing results of repeatability studies.

Sr No.	Conc	Area	II	III	Mean	Amt Found	% Amt Fnd	SD	RSD
1	20	2961.53	2926.56	2912.92	2933.67	20.46	102.34	0.535	0.865

F) %Recovery.

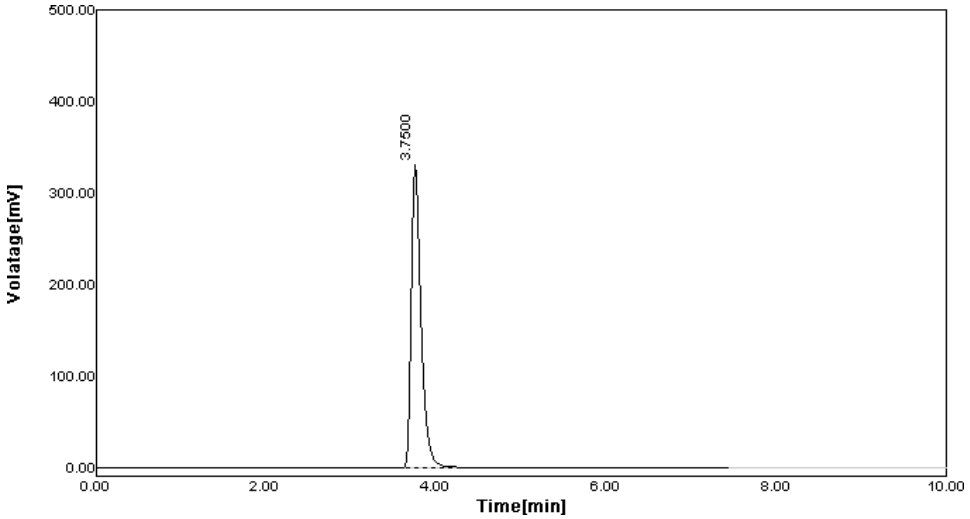

Figure 7: Chromatogram for % Recovery.

Table 17: Data showing results of % recovery studies.

Recovery level(%)	Concentration (µg/ml)	Amt added (µg/ml)	Amount recovered ±SD (µg/ml)	Recovery (%)	%RSD
80%	20	8	8.24	103.00	0.44
100%	20	10	9.99	99.94	1.86
120%	20	12	012.07	100.60	1.01

The recovery experiment was carried out by spiking the standard sample of 20 µg/ml TNF to the test solutions prepared from finished product at 80, 100 and 120 % levels. Recovery study was carried out by standard addition method in which known amount of standard solution of TNF (20 µg/ml) were added to each level of test solution as stated above. The resultant solution were injected in triplicates and observed for area obtained for individual drug. Mean area of standard drug sample was substracted from the area obtained for each level to obtain the actual area corresponding to test sample. From the measured area the amount recovered in percent for TNF was determined. The results obtained were in agreement to compendial

standards of individual drug, Table 21. Eventually, it was concluded that the developed method can be explored for routine analysis of TNF. The recovery result was shown in chromatogram Figure 7.

7.5.7 Analysis of tablets (Marketed formulation)

For the analysis of tablets Twenty tablets (300mg) were weighted; average weight was determined and powdered. Powder equivalent to 10mg (10 x 570/300= 19mg) was transferred to 10 ml of mobile phase, it gives concentration of 1000 µg/ml. The resulting solution was filtered through 0.45µ membrane filter (Milifilter, Milford, MA). and sonicated for three cycles each of 10 min. The solution was further diluted to get concentration of 20 µg/ml of Tenofovir Disoproxil Fumarate. The aliquots were subjected to proposed method and amount of Tenofovir Disoproxil Fumarate was determined. The result are mentioned in Table 23.

Table 18: Analysis of Tablets.

Sr. no.	Component	Label claim (mg)	Amount found (mg) ±SD	%Label claim	% RSD
1	Tenofovir Disoproxil Fumarate	10	9.99	99.95	0.09

8. SUMMARY AND CONCLUSION

The developed HPLC method for estimation of Tenofovir Disoproxil Fumarate is accurate, precise, robust, and specific. The method has been found to be better than previously reported method, because of its less retention time, use of an economical and readily available mobile phase, UV detection and better resolution of peaks. The run time is relatively short, which will enable rapid quantification of many samples in routine and quality-control analysis of various formulations containing Tenofovir Disoproxil Fumarate. All these factors make this method suitable for quantification of Tenofovir Disoproxil Fumarate in bulk drugs and in pharmaceutical dosage forms without any interference. The Results undertaken according to the International Conference on Harmonization (ICH) guidelines reveal that the method is selective and specific. The proposed estimated method was found to be simple, precise, accurate and rapid for the determination of Tenofovir Disoproxil Fumaratefrom Tablets forms, the mobile phase is simple to prepare and economical. The sample recoveries in all the formulations were in good agreement with their respective label claim and their suggestive not interference of formulation excipients in the estimation. Hence this method can be conveniently adopted for routine analysis of Tenofovir Disoproxil Fumarate in the pharmaceutical dosage form.

High performance liquid chromatographic method

Attempts were made to Develop HPLC method for estimation of Tenofovir Disoproxil Fumarate. HPLC method was developed and validated as per ICH guidelines by using a mobile phase consisting mixture of Methanol:Water (0.1%OPA)(85:15), at the flow rate of 0.7ml/min. A C_{18}column was used as stationary phase. The eluent were detected at 257 nm. Statistical analysis of the method was done by using way analysis of variance. The method was found to be simple, accuracy, precise, economical and reproducible. So the propose method can be used for the routine quality control analysis of Tenofovir Disoproxil Fumarate in bulk drug & dosage form.

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