METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF EMTRICITABINE AND TENOFOVIR BY RP-HPLC METHOD

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Abstract: A simple, Accurate, precise method was developed for the simultaneous estimation of the Emtricitabine and Tenofovir in Tablet dosage form. Chromatogram was run through Inspire C 18 (4.6 x 150mm, 5.0μm), Mobile phase containing 30% buffer: 70% Methanol taken in the ratio 30:70 was pumped through column at a flow rate of 1 ml/min. Buffer used in this method was 0.1N H₃PO₄. Temperature was maintained at 30°C. Optimized wavelength selected was 272 nm. The linearity of Emtricitabine and Tenofovir DF was found to be linear with a correlation coefficient of 0.999 and 0.999. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.22 and 0.5 for Emtricitabine and Tenofovir DF. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.69 for Emtricitabine and Tenofovir DF. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.35% and 100.24% for Emtricitabine and Tenofovir DF. The LOD and LOQ for Tenofovir DF was found to be 2.98 and 9.98 and LOD and LOQ for Emtricitabine was found to be 2.96 and 9.96.

Keywords: Emtricitabine, Tenofovir , RP-HPLC.

INTRODUCTION

1. CHROMATOGRAPHY

Chromatography is a family of analytical chemistry techniques for the separation of mixtures. It involves passing the sample, a mixture that contains the analyte, in the "mobile phase", often in a stream of solvent, through the "stationary phase." The stationary phase retards the passage of the components of the sample. When components pass through the system at different rates they become separated in time, like runners in a marathon. Ideally, each component has a characteristic time of passage through the system. This is called its "retention time."

1.1 DIFFERENT TYPES OF CHROMATOGRAPHY:

1.2 Adsorption Chromatography
1.3 Partition Chromatography
1.4 Ion Exchange Chromatography
1.5 Molecular Exclusion Chromatography
1.6 Affinity Chromatography

1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric materials and a wide variety of other high-molecular weight poly functional groups.

1.2.1 Basic principle of HPLC

- High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases; called stationary phase and mobile phase.
- The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic conditions, each component in a sample has difference distribution equilibrium depending on solubility in the phases and or molecular size.

1.3 TYPES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

i. Based on modes of chromatography
1.3.1 Normal Phase High Performance Liquid Chromatography (NP-HPLC)

Normal-phase liquid-liquid chromatography uses a polar stationary phase and less polar mobile phase. To select an optimum mobile phase, it is best to start with a pure hydrocarbon mobile phase such as heptanes. If the sample is strongly retained, the polarity of the mobile phase should be increased, perhaps by adding small amounts of methanol or dioxane.

1.3.2 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Reverse phase chromatography uses hydrophobic bonded packing, usually with an octadecyl or octyl functional group and a polar mobile phase, often a partially or fully aqueous mobile phase.

Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher is its eluent strength. The elution order of the classes of compounds in table is reversed (thus the name reverse-phase chromatography).

1.3.3 Isocratic Elution

In isocratic elution a sample is injected to a given column and the mobile phase is unchanged through the time required for the sample components to elute from the column.

1.3.4 Gradient Elution

Steady changes of the mobile phase composition during the chromatographic run are called gradient elution. The main purpose of gradient elution is to move strongly retained components of the mixture faster, but having the least retained component well resolved.

1.4 HPLC INSTRUMENTATION:

The general instrumentation for HPLC incorporates the following components:
Figure 1: Schematic diagram for HPLC instrument

1.4.1 Mobile Phase Delivery System

The mobile phase must be delivered to the column over a wide range of flow rates and pressure. A degasser is needed to remove dissolved air and other gases from the solvent. Another desirable feature in the solvent-delivery system is the capability for generating a solvent gradient.

1.4.2 Pump

A pump should be able to operate to at least 1500 psi, a pressure suited to less expensive chromatographs. However, 6000 psi is a more desirable pressure limit. For many analytical columns only moderate flow rates of 0.5-2.0 ml/min need to be generated.

1.4.3 Separation columns

Columns are the important part of a HPLC instrument. Columns are constructed by heavy-wall, and glass-lined metal tubing or stainless steel tubing to withstand high pressures (up to 7000 psi) and the chemical action of the mobile phase. Most column lengths range from 10 to 30 cm; short, fast columns are 3 to 8 cm long. For exclusion chromatography, columns are 50 to 100 cm long.

1.4.4 Detectors

The sensitivity of universal detector for HPLC has not been devised yet. Thus it is necessary to select a detector on the basis of the problem.

1.4.5 UV visible photometers and spectrometers

Optical detectors based on UV-visible absorption are the workhorses of HPLC, constituting over 70% of the all detection systems in use. Basically three types of absorbance detectors are available: a fixed wavelength detector, a variable wavelength detector, and a scanning wavelength.

1.4.6 Fixed Wavelength Detectors

A fixed wavelength detector uses a light source that emits maximum light intensity at one or several discrete wavelengths that are isolated by appropriate filters.

1.4.7 Variable Wavelength Detector

A variable wavelength detector is a relatively wide-band pass it offers a wide selection of UV and visible wavelength, but at an increased cost.

1.4.8 Photo Diode Array (PDA) Detector
To obtain a real time spectrum for each solute as it elutes, solid-state diode arrays are required. The diode arrays work in parallel simultaneously monitoring all wavelengths.

Digital electronic integrators are widely used today in HPLC for measuring peak areas. These devices automatically sense peaks and print out the areas in numerical forms. Computing integrators are even more sophisticated and offer a number of features in addition to basic digital integration because these devices have both memory and computing capabilities to upgrade integrating parameters to maintain accuracy as the separation progress and eluting peaks become broader. Many of these devices print out a complete report including names of the compounds, retention times, peak areas and area correction factors. With the help of peak area and height values, the peak width can be calculated (considering the peak as a triangle) and it can also be used for the calculation of number of theoretical plates.10

1.5 SELECTIVITY OF HPLC-METHOD DEVELOPMENT

Most of the drugs can be analyzed by HPLC-method because of several advantages like rapidity, specificity, accuracy, precision, ease of automation and eliminates tedious extraction and isolation procedures. Some of advantages are:

1. Speed (analysis can be accomplished in 20 minutes or less)
2. Greater sensitivity (various detectors can be employed)
3. Improved resolution (wide variety of stationary phases)
4. Reliable columns (wide variety of stationary phases)
5. Ideal for substances of low volatility
6. Easy sample recovery, handling and maintenance
7. Easy programming of the numerous functions in each module.
8. Time programmable operation sequence, such as initiating operation of detector lamp and pump to obtain a stable base line and equilibrated column before the work day begins.
9. Excellent reproducibility of retention times.
10. An injection volume variable from 0.1 to 100 micro liters without any hardware modification.
11. The flexibility of data analysis.
12. Suitable to avoid any interference from impurity.
13. Suitable for preparative liquid chromatography on a much large scale.

1.5.1 Role of the Column

The HPLC column is the heart of the method, critical in performing the separation. The column must possess the selectively, efficiency, and reproducibility to provide a good separation. Good silica and bonding process will provide the reproducible and symmetrical peaks necessary for accurate quantization.

Commonly used reversed phases are C18 (octadecylsilane, USP L1), C8 (octylsilane, USP L7), phenyl (USP L11), and cyan (USP L18) (24).

1.5.2 Initial Mobile Phase Selection:

Proper selection of the mobile phase is the second most important step in the development of the separation method (the first one is the selection of the adsorbent type). The main requirement for the mobile phase is that it has to dissolve the analytes up to the concentration suitable for the detection.

1.5.3 Role of Flow Rate:

Flow rate, more for isocratic than gradient separation, can sometimes be useful and readily utilized to increase the resolution, although its effect is very modest. The slower flow rate will also decrease the column back pressure. The disadvantage is that when flow rate is decreased, to increase the resolution slightly, there is a corresponding increase in the run time.

1.6 The parameters that are affecting by the changes in chromatographic conditions are:

- Column efficiency (N)
- Capacity factor (K')
- Resolution factor (Rs)
- Retention Factor (Rt)
- Retention time (Rt)
- Relative retention (Rr)
- Peak asymmetry factor (As)

1.6.1 Column efficiency (N)

The efficiency of a chromatographic column is defined in terms of the number of theoretical plates (N) and can be calculated using the following
\[ N = 16\left(\frac{R_t}{W}\right)^2 \]

Where, \( R_t \) = Retention time
\( W \) = Width of peak

The number of theoretical plates can be expressed per meters (N):

\[ N = \frac{L}{H} \]

Where,
\( L \) = length of column in meters; \( H \) = height of theoretical plates

### 1.6.2 Capacity factor (K')

Capacity factor is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase.

\[ K' = \frac{V_1 - V_0}{V_0} \]

Where,
\( V_1 \) = retention volume at apex of the peak
\( V_0 \) = void volume of system where an unretained component elutes

### 1.6.3 Resolution factor (R_s)

The retention between two peaks of similar height in a chromatogram can be calculated using the following formula.

\[ R_s = \frac{2(R_{t2} - R_{t1})}{W_2 - W_1} \]

Where
\( R_{t1}, R_{t2} \) – retention times of components
\( W_1, W_2 \) – width of two adjacent peaks

### 1.6.4 Retention Factor (R_f)

The retention factor, \( R_f \), is defined as

\[ R_f = \frac{D_1}{D_2} \]

Where,
\( D_1 \) = distance that color travelled, measured from centre of the band of color to the point where the food color was applied
\( D_2 \) = total distance that solvent traveled

### 1.6.5 Retention time (R_t)

Retention time is the difference in time between the point of injection and its elution from a column. Retention time is measured in minutes or seconds. Retention time is also proportional to the distance moved on a chart paper, which can be measured in cm or mm.

\[ t_R = t_S + t_m \]

Here \( t_S \) is the time of analyte spend in the stationary phase
\( t_m \) is the time of analyte spend in the mobile phase

### 1.6.6 Relative retention (R_r)

The relative retention (r) is calculated using the following formula:

\[ R_r = \frac{T_{R2} - t_m}{T_{RI} - t_m} \]
Where,
\[ t_{R2} = \text{retention time of the peak of interest} \]
\[ t_{R1} = \text{retention time of the reference peak} \]
\[ t_{m} = \text{retention time of an unretained component} \]

1.6.7 Peak asymmetry factor (As)

The asymmetry factor for a peak can be calculated using the following formula

\[ As = \frac{W_x}{2d} \]

Where,
\[ W_x = \text{Peak width at 5\% of peak height, measured from the baseline.} \]
\[ d = \text{Baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5\% of the peak height, measured in the same units as } W_x. \]

1.6.8 METHOD VALIDATION

Method validation is the process by which it is established, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended purpose. It is a part of the overall validation process that also includes software validation, instrument qualification and system suitability. Typical analytical characteristics used in method validation are highlighted below. Although all analytical procedures or methods used in a regulated laboratory must be validated, these parameters focus specifically on liquid chromatography.

Typical analytical characteristics used in method validation, commonly referred to as the “Eight steps of method of validation” 26-29

- Specificity
- Precision
- Accuracy
- Linearity and Range
- Limit of detection
- Limit of quantification
- Robustness
- Ruggedness

Prior to the analysis of samples of each day, the operator must establish that the HPLC system and procedure are capable of providing data of acceptable quality.

1.7.1 Specificity / Selectivity

The terms selectivity and specificity are often used interchangeably. Term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other.

1.7.2 Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose “true value” is known) is analyzed and the measured value is identical to the true value.

\[ \text{Recovery (or) Practical conc.} = \frac{\text{Sample area Average} \times \text{Concentration}}{\text{Standard area}} \]

\[ \%\text{Recovery} = \frac{\text{Standard Concentration} \times 100}{\text{Sample Concentration}} \]

1.7.3 Precision
Precision can be defined as “the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample”. A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

- **Repeatability**
- **Intermediate precision**
- **Reproducibility**

Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate Precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Reproducibility examines the Precision between laboratories and is often determined in collaborative studies or method transfer experiments.

### 1.7.4 Linearity & Range

The Linearity of a method is a measure of how well a Calibration plot of response against concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least-squares regression.

**Range:**

It is the difference between the greatest and the smallest values of the varieties.

\[ \text{Range} = \text{largest value} - \text{smallest value} \]

**Regression equation:**

\[ a = \frac{Y2 - Y1}{X2 - X1} \]

\[ I = \text{Intercept} = \text{regression} - a \cdot C \]

### 1.7.5 Limit of Detection

Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age, or noise level variation by detector manufacturer.

The Limit of detection limit (LOD) may be expressed as:

\[ \text{LOD} = \left( 3.3 \times \sigma \right) / S \]

Where, \( \sigma \) = the standard deviation of the response

\( S \) = the slope of the Calibration curve

The slope \( S \) may be estimated from the Calibration curve of the analyte.

### 1.7.6 Limit of Quantification

Limit of quantification is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

The Limit of Quantification (LOQ) may be expressed as:

\[ \text{LOQ} = \left( 10 \times \sigma \right) / S \]

Where, \( \sigma \) = the standard deviation of the response

\( S \) = the slope of the Calibration curve

### 1.7.7 Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as “a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters.” A good practice is to vary, important parameters in the method, systematically and measure their effect on separation.

### 1.7.8 Ruggedness

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method

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**DRUG PROFILE**
EMTRICITABINE

Chemical Name : 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Structure:

![Chemical Structure of Emtricitabine]

**Chemical Formula** : C₈H₁₀FN₃O₃S

**Melting point** : 136-140°C

**Molecular weight** : 247.248g/mol

**Description** : Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. Emtricitabine is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA.

**Solubility** : White to off-white powder. Freely soluble in methanol and in water; practically insoluble in methylene chloride.

**Category** : Anti-HIV Agents, Reverse Transcriptase Inhibitors.

**Mechanism of Action** : Emtricitabine works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA. By inhibiting HIV-1 reverse transcriptase, emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells).

**Absorption** : Rapidly absorbed (mean absolute bioavailability of 93% for capsules, and 75% for solution). Food does not affect absorption.

**Metabolism** : Minimally transformed (13%), most appears unchanged in urine (86%).

**Route of elimination** : The renal clearance of emtricitabine is greater than the estimated creatinine clearance, suggesting elimination by both glomerular filtration and active tubular secretion.

**Half life** : 10 hours

**Brand Name** : Emtriva

TENOFOVIR

**Chemical Name** : ([(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy)methyl)phosphonic acid

**Structure:**
Chemical Formula: C$_9$H$_{14}$N$_5$O$_4$P

Categories: Anti-HIV Agents and Reverse Transcriptase Inhibitors.

Molecular weight: 287.213 g/mol

Melting point: 276.280°C

Description: Tenofovir disoproxil fumarate (a prodrug of tenofovir), marketed by Gilead Sciences under the trade name Viread®, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs), which block reverse transcriptase, an enzyme crucial to viral production in HIV-infected people.

Solubility: Slightly soluble in water, soluble in methanol, very slightly soluble in dichloromethane.

Mechanism of action: Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate.

Absorption: Tenofovir disoproxil fumarate is the water soluble diester prodrug of the active ingredient tenofoir. The oral bioavailability in fasted patients is approximately 25%.

Metabolism: The cytochrome P450 enzyme system is not involved with the metabolism of tenofovir disoproxil or tenofoir.

Route of elimination: When tenofovir is given IV, 70-80% of the dose is recovered in the urine as unchanged drug within 72 hours of administration.

Half life: When a single oral dose is given, the terminal elimination half-life is approximately 17 hours.

Absorption: Tenofovir disoproxil fumarate is the water soluble diester prodrug of the active ingredient tenofoir. The oral bioavailability in fasted patients is approximately 25%.

Brand Name: TENVIR

REVIEW OF LITERATURE

Budagam Lavanya et al., A validated HPLC method for simultaneous estimation of Emtricitabine and Tenofovir DF in pharmaceutical dosage forms. Chromatography was carried out on a C18 column [250mm, 4.6m, 5μ m] using a mixture of methanol: phosphate Buffer(65:35 v/v) as the mobile phase at a flow rate of 1 ml/min. Detection was carried out by using PDA detector. The retention time of the drugs was 2.461 and 6.231 min for Emtricitabine and Tenofovir DF respectively. The method produced linear responses in the concentration range of 10 to 50μg/ml for both drugs. The LOD and LOQ values were found to be 0.00752, 0.00218ug/ml for Emtricitabine and 0.00851, 0.0315ug/ml for Tenofovir DF. The method was validated for linearity, precision, accuracy, LOD & LOQ in accordance with ICH guidelines. The proposed method was found to be applicable for determination of the drug in tablet dosage forms.

Syed Sajjad Hussen et al., The objective of the study was to develop anovel stability-indicating high performance liquid chromatographic(HPLC)method for Tenofovir Disoproxil fumarate (TEN) withphotodiode array (PDA) detection andvalidatedas per International Conference on Harmonisation(ICH)guidelines. The developed method was successfully applied for assay of Tenofovir Disoproxil fumarate tonanoparticle formulation. A Lichrocart (C18) (250mm× 4.6mm, 5 μm particle size) column and a mobile phase composed of acetonitrile and 0.025M potassium di hydrogen phosphate buffer (pH 3.0)adjusting by using 10% v/v Orthophosphoric acid) in the ratio 35:65 (v/v) was used, and the detection wavelength of 260 nm. The method was validated with the parameters likespecificity, linearity, precision, accuracy, limits of detection and quantification as per ICH guidelines. Forced degradation studies under different stress conditions like Acid, Base and Oxidation was successfully achieved, TENwas found to degrade significantly.
in alkaline and acidic conditions, PDA peak purity test confirmed the specificity of the developed method. The method was found to be precise and accurate with a linearity range of 0.1 μg/mL–100 μg/mL (r² > 0.999).

Rajesh Sharma et al.,27 A simple, rapid reversed-phase high performance liquid chromatographic method had been developed and validated for estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form. The estimation was carried out on Luna C18 (25 cm x 4.60 mm, particle size 5 μm) column with a mixture of acetonitrile: potassium dihydrogen phosphate buffer (pH 3.0 ± 0.05 adjusted with orthophosphoric acid): triethylamine in the ratio of 70:30:0.5 (v/v) as mobile phase. UV detection was performed at 260 nm. The method was validated for linearity, accuracy, precision, specificity and sensitivity as per ICH norms. The developed and validated method was successfully used for the quantitative analysis of commercially available dosage form. The retention time was 1.78 and 2.27 min. for emtricitabine and tenofovir disoproxil fumarate respectively and total run time was 4 min. at a flow rate of 1.5 ml/min. The calibration curve was linear over the concentration range of 5-50 μg/mL for emtricitabine and 5-50 μg/mL for tenofovir disoproxil fumarate. The LOD and LOQ values were found to be 0.015 and 0.045 μg/mL for emtricitabine and 0.039 and 0.117 μg/mL for tenofovir disoproxil fumarate respectively. The high percentage of recovery and low percentage coefficient of variance confirms the suitability of the method for the simultaneous estimation of emtricitabine and tenofovir disoproxil fumarate in tablet dosage form.

Viswanath V et al.,28 A new, accurate and reliable RP-HPLC method has been developed and validated for simultaneous estimation of Tenofovir disoproxil fumarate (TDF) & Emtricitabine (EMT) in a combined tablet dosage form. First of all, maximum absorbance was found to be at 270 nm and the peak purity was excellent. Injection volume was selected to be 20 μl which gave a good peak area. The column used for study was PHENOMENEX LUNA C18 chosen. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0 ml/min because of good peak area and satisfactory retention time. Mobile phase of Acetonitrile: Phosphate pH 3.5 buffers in the ratio of 40:60 was fixed due to good symmetrical peak. The retention times for TDF & EMT were found to be 2.84 min and 3.55 min respectively. The precision of the System and method were checked and found to be within limits. This indicates that the method is precise. Linearity study, correlation coefficient and curve fitting was found to be 0.999. The recovery value of pure drug was found between 99.4% to 101.7%. This indicates that the developed method is accurate, precise and economical and can be used for the routine analysis of tablets in quality control.

N Appala Raju et al.,29 A simultaneous stability indicating RP-HPLC method is developed for the estimation of Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz in tablet dosage form. Chromatography was carried on an Inertsil ODS 3V column using gradient composition of 0.02M sodium dihydrogen orthophosphate as mobile phase A and mixture of Methanol and water in ratio of 85:15 as mobile phase B at a flow rate of 1.5 ml/min with detection at 265 nm. The retention times of the Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz was about 5.875, 8.800 and 12.020 mins respectively. The detector response is linear from 8-120 μg/ml, 12-180 μg/ml, 20-360 μg/ml of test concentration for Emtricitabine, Tenofovir and Efavirenz respectively. The respective linear regression equation being Y = 10175x-76883 for Emtricitabine, Y = 6280.8x+219800 for Tenofovir disoproxil fumarate and Y = 1883.5x+323060 for Efavirenz. The limit of detection and Limit of quantification was 0.06, 0.07 and 0.08 μg/ml and 0.14, 0.12 and 0.15 μg/ml for Emtricitabine, Tenofovir and Efavirenz respectively. The percentage assay of Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz was about 99.31, 99.77 and 100.20% respectively and percentage recovery for average of three different concentrations was 100.87%, 100.04% and 99.52% respectively. The method was validated by determining its sensitivity, Linearity, accuracy and precision. The proposed method is simple, fast, sensitive, Linear, accurate, rugged and precise and hence can be applied for routine quality control of Emtricitabine, Tenofovir disoproxil fumarate and Efavirenz in bulk and in tablet dosage form.

Parthiban C et al.,30 A rapid, specific, sensitive and simple high performance liquid chromatography was developed for simultaneous estimation of Tenofovir Disoproxil Fumarate and Emtricitabine in tablet formulation. The separation was achieved by Thermoscientific C18 column (4.6*250mm, particlesize 5μm) with a mobile phase consisting of Acetonitrile:water (70:30 v/v, pH 3.5 adjusted with ortho phosphoric acid), at a flow rate of 1.5ml/min.Detection was carried out at 270 nm. Retention time of Emtricitabine and Tenofovir Disoproxil Fumarate were found to be 2.82 and 4.38 min. respectively. The linear dynamic range was 200-400 μg/ml and 100-300 μg/ml Tenofovir Disoproxil Fumarate and Emtricitabine respectively. The method is validated for Accuracy, Precision, Ruggedness and Robustness. The proposed method is successfully applied for the simultaneous determination of both drugs in commercial tablet preparation. The results of the analysis have been validated statistically and by recovery studies.

EXPERIMENTAL METHOD

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Table 1: Instruments used

<table>
<thead>
<tr>
<th>SL. No</th>
<th>Instrument</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HPLC</td>
<td>WATERS, software: Empower, 2695 separation module, 2487 UV detector.</td>
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<td>2</td>
<td>UV/VIS spectrophotometer</td>
<td>LABINDIA UV 3000</td>
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<tr>
<td>3</td>
<td>pH meter</td>
<td>Adwa – AD 1020</td>
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<td>4</td>
<td>Weighing machine</td>
<td>Afco set ER-200A</td>
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<tr>
<td>5</td>
<td>Pipettes and Burettes</td>
<td>Borosil</td>
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<td>6</td>
<td>Beakers</td>
<td>Borosil</td>
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Table 2: Chemicals used

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<th>SL.No</th>
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<th>Brand</th>
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<tbody>
<tr>
<td>1</td>
<td>Emtricitabine</td>
<td>Supplied by Pharmatrain</td>
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<tr>
<td>2</td>
<td>Tenofovir DF</td>
<td>Supplied by Pharmatrain</td>
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<td>3</td>
<td>Ortho phosphoric acid</td>
<td>FINAR chemical LTD</td>
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<td>4</td>
<td>Water and Methanol for HPLC</td>
<td>Standard solutions Ltd</td>
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<tr>
<td>5</td>
<td>Acetonitrile for HPLC</td>
<td>Standard solutions Ltd</td>
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<tr>
<td>6</td>
<td>HCl, H$_2$O$_2$, NaOH</td>
<td>MERCK</td>
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</table>

**HPLC METHOD DEVELOPMENT:**

**Wave length selection:**

UV spectrum of 10 µg / ml Emtricitabine and Tenofovir DF in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 272. At this wavelength both the drugs show good absorbance.

![UV spectrum graph]

**Optimization of Column:**

Inspire C$_{18}$ (4.6 x 150mm, 5.0µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

**Trial-1**

**Table 6: Chromatographic condition**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Chromosil C$_{18}$ Column (150mm x 4.6mm) 5µg.</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Water: Methanol P$_{H}$ 2.5 (30:70 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml min$^{-1}$</td>
</tr>
<tr>
<td>Wavelength</td>
<td>272 nm</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Auto injector (vial)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>
Observation: The separation of two analytical peaks was not proper, So the mobile phase ratio has been changed for next trial.

**Trial-2**

Table 7: Chromatographic condition

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Chromosil C18 Column (150mm x 4.6mm)5µg.</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>water : Acetonitrile pH 2.5 (30:70 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml min⁻¹</td>
</tr>
<tr>
<td>Wavelength</td>
<td>272 nm</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Auto injector (vial)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
</tr>
<tr>
<td>S. No</td>
<td>Peak Name</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir</td>
</tr>
</tbody>
</table>

**Observation:** The separation of two analytical peaks was not proper, So the mobile phase ratio has been changed for next trial.

**Trial-3**

**Table 8: Chromatographic condition**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Xterra C$_{18}$ Column (150mm x 4.6mm)5µg.</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Phosphate buffer : Methanol P$_{H}$ 2.5 (20:80 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml min$^{-1}$</td>
</tr>
<tr>
<td>Wavelength</td>
<td>272 nm</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Auto injector (vial)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>

**Fig. 3: Chromatogram of Emtricitabine and Tenofovir**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peak Name</th>
<th>Rt</th>
<th>Area</th>
<th>Height</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Emtricitabine</td>
<td>3.231</td>
<td>432752</td>
<td>25062</td>
<td>1439</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir</td>
<td>4.900</td>
<td>254934</td>
<td>15048</td>
<td>3584</td>
<td>2.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>
Observation: The separation of two analytical peaks was not proper, so the mobile phase ratio has been changed for next trial.

**Trial-4**

Table 9: Chromatographic condition

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Xterra C18 Column (150mm x 4.6mm)5µg.</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Phosphate buffer: Methanol P\text{H} 2.5 (30:70 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml min(^{-1})</td>
</tr>
<tr>
<td>Wavelength</td>
<td>272 nm</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Auto injector (vial)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>

![Chromatogram of Emtricitabine and Tenofovir](image)

**Fig.4:** Chromatogram of Emtricitabine and Tenofovir

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peak Name</th>
<th>(R_t)</th>
<th>Area</th>
<th>Height</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Emtricitabine</td>
<td>3.218</td>
<td>400986</td>
<td>39855</td>
<td>2602</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir</td>
<td>5.010</td>
<td>248371</td>
<td>20892</td>
<td>4499</td>
<td>1.6</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Observation: The separation of two analytical peaks is occurred but fronting occurs in Emtricitabine peak.

**Trial-5**

Table 10: Chromatographic condition

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Thermosil C18 Column (100mm x 4.6mm)5µg.</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Phosphate buffer: Methanol P\text{H} 2.5 (30:70 v/v)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml min(^{-1})</td>
</tr>
<tr>
<td>Wavelength</td>
<td>272 nm</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Auto injector (vial)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20µl</td>
</tr>
</tbody>
</table>
Fig. 5: Chromatogram of Emtricitabine and Tenofovir

<table>
<thead>
<tr>
<th>S. No</th>
<th>Peak Name</th>
<th>R_t</th>
<th>Area</th>
<th>Height</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
<th>USP Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Emtricitabine</td>
<td>2.593</td>
<td>239603</td>
<td>44771</td>
<td>5354</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir</td>
<td>3.715</td>
<td>200189</td>
<td>31439</td>
<td>8104</td>
<td>1.2</td>
<td>7.1</td>
</tr>
</tbody>
</table>

**Observation:** The separation of two analytical peaks was good but baseline noise is occurred. So the mobile phase ratio has been changed for next trial.

**OPTIMIZED CHROMATOGRAPHIC CONDITIONS:**

- **Instrument used:** High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector
- **Temperature:** Ambient
- **Column:** Inspire C \textsubscript{18} (4.6 x 150mm, 5.0\textmu m)
- **Buffer:** Ortho phosphoric acid pH 2.5
- **Mobile phase:** 30% buffer: 70% Methanol
- **Flow rate:** 1.0 ml per min
- **Wavelength:** 272 nm
- **Injection volume:** 20 \textmu l
- **Run time:** 7 min.
PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of 0.1% Ortho phosphoric acid buffer:

Pipetted 1 ml of ortho phosphoric acid in 1000 ml HPLC water and adjust the pH 2.5 with NaOH solution.

Preparation of mobile phase:

Mix a mixture of above buffer 300 ml (30%) and 700 ml Methanol HPLC (70%) and degas in ultrasonic water bath for 5 minutes. Filter through 045 µ filter under vacuum filtration.

Diluent Preparation: Use the Mobile phase as Diluents.

VALIDATION PARAMETERS:

1. ASSAY:

Standard Solution Preparation:

Accurately weigh and transfer 20mg of Emtricitabine & 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 20mg of Emtricitabine & 30mg Tenofovir DF equivalent weight of the sample into a 10ml clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for the Emtricitabine & Tenofovir DF peaks and calculate the %Assay by using the formulae.

The chromatograms were recorded as show in Fig 3, 4 and results are shown in Table- 4.

2. LINEARITY:
Preparation of stock solution:

Accurately weigh and transfer 20mg of Emtricitabine & 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation of Level – I (20ppm & 30ppm of Emtricitabine & Tenofovir DF):

1ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – II (40ppm & 60ppm of Emtricitabine & Tenofovir DF):

2ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – III (60ppm & 90ppm of Emtricitabine & Tenofovir DF):

3ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – IV (80ppm & 120ppm of Emtricitabine & Tenofovir DF):

4ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – V (100ppm & 150ppm of Emtricitabine & Tenofovir DF):

5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

The chromatograms were recorded as show in Fig 5 – 9, calibration graphs were shown in Fig 10, 11 and results are shown in Table 5, 6.

3. PRECISION:

Preparation of stock Solution:

Accurately weigh and transfer 20mg of Emtricitabine & 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

The sample solutions of Emtricitabine and Tenofovir DF chromatograms were recorded as shown in Fig 12- 16.

The mean and percentage relative standard deviation were calculated from the peak areas and shown in the Table 7 and 8.

4. INTERMEDIATE PRECISION/RUGGEDNESS:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.

Preparation of stock solution:
Accurately weigh and transfer 20mg of Emtricitabine & 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (stock solution)

Further pipette 1ml of Emtricitabine&Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine&Tenofovir DF the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Chromatograms were recorded as shown in Fig 17-22 and results are shown in Table 9 and 10.

5. ACCURACY:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of Standard stock solution:

Accurately weigh and transfer 20mg of Emtricitabine & 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10mg of Emtricitabine and 15mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7 ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents

Further pipette 3ml of Emtricitabine & Tenofovir DF the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 20mg of Emtricitabine and 30mg of Tenofovir DF working standard into a into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF the above stock solution into a10ml volumetric flask and dilute up to the mark with Diluents.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 30mg of Emtricitabine and 45mg of Tenofovir DF equivalent weight of tablet powder into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).
Further pipette 1ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents

Further pipette 3ml of Emtricitabine & Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Emtricitabine & Tenofovir DF and calculate the individual recovery and mean recovery values.

The chromatograms were shown in fig 23-31 Results are shown in the Table-11, 12.

6. LIMIT OF DETECTION:

Preparation of Emtricitabine solution:

Accurately weigh and transfer 20mg of Emtricitabine working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Emtricitabine the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Further pipette 3ml Emtricitabine the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of 0.077µg/ml solution:

Further pipette 0.2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Tenofovir DF solution:

Accurately weigh and transfer 30mg of Tenofovir DF working standard into a 10ml clean dry volumetric flask add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Further pipette 3ml of Tenofovir DF the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation 0.163µg/ml solution:

Further pipette 0.2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents

Further pipette 0.9ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents

Chromatograms were shown in the fig 32 Results are shown in the Table-13.

7. LIMIT OF QUANTIFICATION:

Preparation of Emtricitabine solution:

Preparation of 0.257µg/ml solution:

Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Further pipette 0.8ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Tenofovir DF solution:

Preparation of 0.549µg/ml solution:

Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

The chromatograms were recorded as show in Fig 33 and results are shown in Table- 14.
8. ROBUSTNESS:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a) The flow rate was varied at 0.9 ml/min to 1.1ml/min.

Standard solution 60 & 90 µg/ml of Emtricitabine & Tenofovir DF prepared and analysed using the varied flow rates along with method flow rate.

b) The Organic composition in the Mobile phase was varied from 63% to 77%

Standard solution 60 & 90 µg/ml of Emtricitabine & Tenofovir DF was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

The chromatograms were recorded as shown in Fig 34-37 and results are shown in Table 15 and 18.

RESULTS AND DISCUSSION

HPLC METHOD

SYSTEM SUITABILITY:

![Chromatogram for system suitability](image)

**Figure 2: Chromatogram for system suitability**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>RT(min)</th>
<th>Area (µV sec)</th>
<th>Height (µV)</th>
<th>USP resolution</th>
<th>USP tailing</th>
<th>USP plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Emtricitabine</td>
<td>2.802</td>
<td>1187582</td>
<td>114245</td>
<td>1.56</td>
<td></td>
<td>2744.20</td>
</tr>
<tr>
<td>2</td>
<td>Tenofovir DF</td>
<td>3.677</td>
<td>992186</td>
<td>81814</td>
<td>2.95</td>
<td>1.19</td>
<td>3375.11</td>
</tr>
</tbody>
</table>

**Table 3: Results of system suitability parameters**

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.
1. ASSAY:

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 20mg of Emtricitabine & 30mg Tenofovir DF equivalent weight of the sample into a 10ml clean dry volumetric flask add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Further pipette 3ml of Emtricitabine & Tenofovir DF of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

Inject 20 μL of the standard, sample into the chromatographic system and measure the areas for the Emtricitabine & Tenofovir DF peaks and calculate the %Assay by using the formulae.

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.

Figure 3: Chromatogram for Standard

Figure 4: Chromatogram for Sample
Table 4: Results of Assay for Emtricitabine and Tenofovir DF

<table>
<thead>
<tr>
<th></th>
<th>Label Claim (mg)</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emtricitabine</td>
<td>200</td>
<td>99.77</td>
</tr>
<tr>
<td>Tenofovir DF</td>
<td>300</td>
<td>99.04</td>
</tr>
</tbody>
</table>

6.3: VALIDATION PARAMETERS:

2. LINEARITY:

The linearity range was found to lie from 20µg/ml to 100µg/ml of Emtricitabine, 30µg/ml to 150µg/ml of Tenofovir DF and chromatograms are shown below.

![Figure 5: Chromatogram for linearity-1](image1)

![Figure 6: Chromatogram for linearity-2](image2)
Figure 7: Chromatogram for linearity-3

Figure 8: Chromatogram for linearity-4

Figure 9: Chromatogram for linearity-5
Table 5: Area of different concentration of Emtricitabine and Tenofovir DF

<table>
<thead>
<tr>
<th>S. No</th>
<th>Emtricitabine</th>
<th>Tenofovir DF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (µg/ml)</td>
<td>Area</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>406525</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>830261</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>1178855</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>1558003</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>1951429</td>
</tr>
</tbody>
</table>

Figure 10: Calibration graph for Emtricitabine

Figure 11: Calibration graph for Tenofovir DF

Table 6: Analytical performance parameters of Emtricitabine and Tenofovir DF

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Emtricitabine</th>
<th>Tenofovir DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope (m)</td>
<td>19088</td>
<td>11819</td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>39750</td>
<td>4305</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
<td>0.999</td>
<td>0.999</td>
</tr>
</tbody>
</table>
Acceptance criteria:

Correlation coefficient ($R^2$) should not be less than 0.999

- The correlation coefficient obtained was 0.999 which is in the acceptance limit.

3. PRECISION:

Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are shown below.

![Chromatogram for Precision -1](image1)

![Chromatogram for Precision -2](image2)
Figure 14: Chromatogram for Precision -3

Figure 15: Chromatogram for Precision -4

Figure 16: Chromatogram for Precision -5
Table 7: Results of Precision for Emtricitabine

<table>
<thead>
<tr>
<th>Injection</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection-1</td>
<td>1154085</td>
</tr>
<tr>
<td>Injection-2</td>
<td>1155893</td>
</tr>
<tr>
<td>Injection-3</td>
<td>1160220</td>
</tr>
<tr>
<td>Injection-4</td>
<td>1157541</td>
</tr>
<tr>
<td>Injection-5</td>
<td>1159687</td>
</tr>
<tr>
<td>Average</td>
<td>1157485</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2570.3</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 8: Results of Precision for Tenofovir DF

<table>
<thead>
<tr>
<th>Injection</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection-1</td>
<td>949525</td>
</tr>
<tr>
<td>Injection-2</td>
<td>941958</td>
</tr>
<tr>
<td>Injection-3</td>
<td>947875</td>
</tr>
<tr>
<td>Injection-4</td>
<td>952978</td>
</tr>
<tr>
<td>Injection-5</td>
<td>951911</td>
</tr>
<tr>
<td>Average</td>
<td>948849.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>4339.2</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

4. INTERMEDIATE PRECISION (ruggedness)

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation.
Figure 17: Chromatogram for ID Precision -1

Figure 18: Chromatogram for ID Precision -2

Figure 19: Chromatogram for ID Precision -3
Figure 20: Chromatogram for ID Precision -4

Figure 21: Chromatogram for ID Precision -5

Figure 22: Chromatogram for ID Precision -6
Table 9: Results of Intermediate precision for Emtricitabine

<table>
<thead>
<tr>
<th>Injection</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection-1</td>
<td>1166019</td>
</tr>
<tr>
<td>Injection-2</td>
<td>1167161</td>
</tr>
<tr>
<td>Injection-3</td>
<td>1171511</td>
</tr>
<tr>
<td>Injection-4</td>
<td>1180276</td>
</tr>
<tr>
<td>Injection-5</td>
<td>1183918</td>
</tr>
<tr>
<td>Injection-6</td>
<td>1177468</td>
</tr>
<tr>
<td>Average</td>
<td>1174392</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>7287.3</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 10: Results of Intermediate precision for Tenofovir DF

<table>
<thead>
<tr>
<th>Injection</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection-1</td>
<td>956847</td>
</tr>
<tr>
<td>Injection-2</td>
<td>960447</td>
</tr>
<tr>
<td>Injection-3</td>
<td>956523</td>
</tr>
<tr>
<td>Injection-4</td>
<td>944789</td>
</tr>
<tr>
<td>Injection-5</td>
<td>957643</td>
</tr>
<tr>
<td>Injection-6</td>
<td>964689</td>
</tr>
<tr>
<td>Average</td>
<td>956823</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>6642.1</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

5. ACCURACY:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.
Figure 23: Chromatogram for Accuracy 50% -1

Figure 24: Chromatogram for Accuracy 50% -2

Figure 25: Chromatogram for Accuracy 50% -3
Figure 26: Chromatogram for Accuracy 100%-1

Figure 27: Chromatogram for Accuracy 100%-2

Figure 28: Chromatogram for Accuracy 100%-3
**Figure 29:** Chromatogram for Accuracy 150%-1

**Figure 30:** Chromatogram for Accuracy 150%-2

**Figure 31:** Chromatogram for Accuracy 150%-3
Table 11: Accuracy (recovery) data for Emtricitabine

<table>
<thead>
<tr>
<th>% Concentration (at specification Level)</th>
<th>Area*</th>
<th>Amount Added (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>598249</td>
<td>10</td>
<td>10.06</td>
<td>100.41</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>1186057</td>
<td>20</td>
<td>19.95</td>
<td>99.77</td>
<td>100.35</td>
</tr>
<tr>
<td>150%</td>
<td>1798511</td>
<td>30</td>
<td>30.26</td>
<td>100.86</td>
<td></td>
</tr>
</tbody>
</table>

*Average of three determinations

Table 12: Accuracy (recovery) data for Tenofovir DF

<table>
<thead>
<tr>
<th>% Concentration (at specification Level)</th>
<th>Area*</th>
<th>Amount Added (mg)</th>
<th>Amount Found (mg)</th>
<th>% Recovery</th>
<th>Mean Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>501791</td>
<td>15</td>
<td>15.05</td>
<td>100.36</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>992126</td>
<td>30</td>
<td>29.76</td>
<td>99.21</td>
<td>100.24</td>
</tr>
<tr>
<td>150%</td>
<td>1517427</td>
<td>45</td>
<td>45.52</td>
<td>101.16</td>
<td></td>
</tr>
</tbody>
</table>

*Average of three determinations

Acceptance Criteria:
- The percentage recovery was found to be within the limit (97-103%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

6. LIMIT OF DETECTION FOR EMTRICITABINE AND TENOFOVIR DF

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio.
Figure 32: Chromatogram of Emtricitabine, Tenofovir DF showing LOD

Table 13: Results of LOD

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Baseline noise(µV)</th>
<th>Signal obtained (µV)</th>
<th>S/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emtricitabine</td>
<td>48</td>
<td>143</td>
<td>2.98</td>
</tr>
<tr>
<td>Tenofovir DF</td>
<td>48</td>
<td>142</td>
<td>2.96</td>
</tr>
</tbody>
</table>

- Signal to noise ratio shall be 3 for LOD solution
- The result obtained is within the limit.

7. LIMIT OF QUANTIFICATION FOR EMTRICITABINE AND TENOFOVIR DF

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio
Table 14: Results of LOQ

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Baseline noise(µV)</th>
<th>Signal obtained (µV)</th>
<th>S/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emtricitabine</td>
<td>48</td>
<td>479</td>
<td>9.98</td>
</tr>
<tr>
<td>Tenofovir DF</td>
<td>48</td>
<td>478</td>
<td>9.96</td>
</tr>
</tbody>
</table>

- Signal to noise ratio shall be 10 for LOQ solution
- The result obtained is within the limit.

8. ROBUSTNESS:

The standard and samples of Emtricitabine and Tenofovir DF were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

Figure 34: Chromatogram showing less flow
Figure 35: Chromatogram showing more flow

Variation of mobile phase organic composition:

Figure 36: Chromatogram showing less organic composition
Figure 37: Chromatogram showing more organic composition

Table 15: Results for variation in flow for Emtricitabine

<table>
<thead>
<tr>
<th>S. No</th>
<th>Flow Rate (ml/min)</th>
<th>System Suitability Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>USP Plate Count</td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>2647.56</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>2744.20</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>2729.27</td>
</tr>
</tbody>
</table>

Table 16: Results for variation in flow for Tenofovir DF

<table>
<thead>
<tr>
<th>S. No</th>
<th>Flow Rate (ml/min)</th>
<th>System Suitability Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>USP Plate Count</td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>3290.41</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>3375.11</td>
</tr>
</tbody>
</table>
Table 17: Results for variation in mobile phase composition for Emtricitabine

<table>
<thead>
<tr>
<th>S. No</th>
<th>Change in Organic Composition in the Mobile Phase</th>
<th>System Suitability Results</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% less</td>
<td></td>
<td>2505.98</td>
<td>1.54</td>
</tr>
<tr>
<td>2</td>
<td>*Actual</td>
<td></td>
<td>2744.20</td>
<td>1.56</td>
</tr>
<tr>
<td>3</td>
<td>10% more</td>
<td></td>
<td>2495.48</td>
<td>1.62</td>
</tr>
</tbody>
</table>

* Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 18: Results for variation in mobile phase composition for Tenofovir DF

<table>
<thead>
<tr>
<th>S. No</th>
<th>Change in Organic Composition in the Mobile Phase</th>
<th>System Suitability Results</th>
<th>USP Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% less</td>
<td></td>
<td>3117.88</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>*Actual</td>
<td></td>
<td>3375.11</td>
<td>1.19</td>
</tr>
<tr>
<td>3</td>
<td>10% more</td>
<td></td>
<td>3014.82</td>
<td>1.31</td>
</tr>
</tbody>
</table>

* Results for actual Mobile phase composition have been considered from Accuracy standard.

Acceptance criteria:

The Retention time, USP plate count, USP tailing factor obtained for change of flow rate, variation in mobile phase was found to be within the acceptance criteria. Hence the method is robust.

SUMMARY AND CONCLUSION

- The estimation of Emtricitabine and Tenofovir DF was done by RP-HPLC.
- The assay of Emtricitabine and Tenofovir DF was performed with tablets and the % assay was found to be 99.77 and 99.04 which shows that the method is useful for routine analysis.
- The linearity of Emtricitabine and Tenofovir DF was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity.
- The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.22 and 0.5 for Emtricitabine and Tenofovir DF which shows that the method is precise.
- The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.69 for Emtricitabine and Tenofovir DF which shows that the method is repeatable when performed in different days also.
- The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.35% and 100.24% for Emtricitabine and Tenofovir DF. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility.
- The acceptance criteria for LOD and LOQ is 3 and 10. The LOD and LOQ for Tenofovir DF was found to be 2.98 and 9.98 and LOD and LOQ for Emtricitabine was found to be 2.96 and 9.96.
The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

**BIBLIOGRAPHY**