A New Stability-Indicating RP-HPLC Method for Simultaneous Quantification of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir in Combined Tablet Dosage Forms

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ABSTRACT
A novel, accurate, precise, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated for the simultaneous quantitative estimation of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir in bulk and combined tablet dosage forms. Chromatographic separation was achieved on a Waters Xterra C18 (150 × 4.6 mm, 5 µm) column maintained at 30°C. The gradient mobile phase consisted of Mobile Phase-A (pH 3.50 phosphate buffer) and Mobile Phase-B (acetonitrile:methanol, 75:25% v/v) pumped at a flow rate of 1.5 mL/min with UV detection at 265 nm. The retention times were 2.576 min for Emtricitabine, 8.966 min for Tenofovir Alafenamide, and 10.887 min for Dolutegravir. The method was linear over concentration ranges of 20–300 µg/mL, 2.5–37.5 µg/mL, and 5–75 µg/mL respectively. Forced degradation studies were performed under acid, base, peroxide, thermal, photolytic, and neutral stress conditions. Degradants were successfully resolved from the principal peaks, demonstrating the stability-indicating capability of the method.

Keywords: RP-HPLC; Emtricitabine; Tenofovir Alafenamide; Dolutegravir; stability-indicating; simultaneous estimation.
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INTRODUCTION

Combination antiretroviral therapy plays a pivotal role in human immunodeficiency virus (HIV) management. Emtricitabine (EMT) is a nucleoside reverse transcriptase inhibitor (NRTI) with a molecular weight of 441.36 g/mol, freely soluble in water, and prescribed at a maximum daily dose of 200 mg. It terminates viral DNA chains intracellularly.Fixed-dose combinations such as Descovy (Emtricitabine 200 mg / Tenofovir Alafenamide 25 mg) and Tivicay (Dolutegravir 50 mg) are frequently co-administered in India and globally to maintain viral suppression. Extensive literature review indicates that while several HPLC methods exist for these analytes individually or in other combinations, there are currently no reported stability-indicating RP-HPLC methods for the simultaneous determination of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir in combined dosage forms. Hence, this study aims to fill that gap in pharmaceutical analysis.

Experimental work

Instrumentation

Separation was performed on a Waters HPLC (Model 2695) equipped with LC-20AD pumps, a Rheodyne 7725 injector valve with a 10 µL loop, and an SPD-20A Photodiode Array (PDA) detector. Data acquisition and integration were managed via Empower-3 software. Sonication was conducted using a PCI Analytics PCI81 ultrasonicator, and weighing's were done on a Sartorius CPA225D balance.

Reagents and Solutions

Reference standards were generously supplied by Hetero Drugs Ltd. HPLC-grade Methanol, Acetonitrile, and Orthophosphoric acid (OPA) were procured from Merck Ltd. (Mumbai, India). A pH 3.50 phosphate buffer was prepared by dissolving 1.36 g of potassium dihydrogen phosphate monohydrate in 1000 mL Milli-Q water, adjusting with diluted OPA, and filtering through a 0.45 µm PVDF membrane filter. The diluent comprised buffer and methanol in a 50:50% v/v ratio.

Standard and Sample Preparation

A mixed standard stock solution was prepared containing 2000 ppm Emtricitabine, 250 ppm Tenofovir Alafenamide, and 500 ppm Dolutegravir. The final working standard solution was obtained by diluting 5 mL of the stock to 50 mL with diluent, resulting in target concentrations of 200 µg/mL (EMT), 25 µg/mL (TAF), and 50 µg/mL (DTG). For tablet analysis, 5 tablets of Descovy and 5 tablets of Tivicay were finely pulverized, extracted into diluent via sonication for 45 minutes, filtered, and diluted systematically to match working standard concentrations.

METHOD DEVELOPMENT AND OPTIMIZATION

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Several mobile phase compositions and column phases were explored. Early trials utilizing water: methanol or simple isocratic OPA buffer systems failed to elute Dolutegravir or introduced significant blank interference at the Tenofovir retention window. Optimization led to the selection of a Waters Xterra C18 column and a finely tuned gradient program (Table 1) which provided exceptional peak shapes, baseline resolution, and zero placebo interference.

Parameter	Value / Condition
Stationary Phase	Waters Xterra C18 (150 × 4.6 mm; 5 µm)
Mobile Phase A	pH 3.50 Phosphate Buffer
Mobile Phase B	Acetonitrile: Methanol (75:25% v/v)
Flow Rate	1.5 mL/min
Column Temperature	30°C
Injection Volume	10 µL
Detection Wavelength	265 nm
Run Time	20 minutes

Table 2: Gradient Elution Program

Time (min)	Mobile Phase-A (%)	Mobile Phase-B (%)
0	100	0
10	50	50
15	40	60
16	100	0
20	100	0

Method Validation

The proposed method was thoroughly validated in accordance with International Conference on Harmonization (ICH) Q2(R1) guidelines.

System Suitability and Specificity

System suitability parameters were evaluated by six replicate injections of the standard solution. Resolution, theoretical plates, and tailing factors were well within acceptable ranges (Table 3). Specificity was proven as chromatograms of blank and placebo solutions disclosed no analytical interference at the specific retention times of 2.576 min (EMT), 8.966 min (TAF), and 10.887 min (DTG).

Parameter	Emtricitabine	Tenofovir Alafenamide	Dolutegravir
Retention Time (min)	2.576	8.966	10.887
Mean Peak Area	3307577	541817	2216840
Resolution	--	28.4	7.8
Theoretical Plates	9524	32793	39371
Tailing Factor	1.07	1.02	1.04

Linearity, LOD, and LOQ

Linearity curves exhibited robust correlation coefficients (R^2 □ 0.999) across all tested dynamic ranges. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were established based on calibration curve residual standard deviations.

Drug Analyte	Linearity Range (µg/mL)	Regression Equation	R^2 Value	LOD	LOQ
Emtricitabine	20 – 300	y = 16157.97x + 40815.01	0.9997	0.303	1.000
Tenofovir Alafenamide	2.5 – 37.5	y = 21234.93x + 1488.94	0.9998	0.038	0.125
Dolutegravir	5.0 – 75.0	y = 43667.89x + 11853.94	0.9996	0.076	0.250

Accuracy and Precision

Accuracy was investigated via standard recovery studies at 50%, 100%, and 150% spike levels into placebo. Mean recoveries spanned 99.7% to 101.7%, comfortably satisfying the official thresholds of 98.0%–102.0%. Precision was verified through intra-day and inter-day (ruggedness) experiments using six independent injections, generating overall %RSD values uniformly below 1.1%, highlighting the excellent quantitative reproducibility of the system.

Forced Degradation and Stability-Indicating Profile

To establish whether the method is stability-indicating, standard and formulation solutions were subjected to forced hydrolytic, oxidative, photolytic, and thermal stress conditions. Major degradations (~5-6%) were observed during acid and base treatment for Emtricitabine and Tenofovir Alafenamide. Dolutegravir displayed prominent structural stability across all media with

negligible degradation. Crucially, all resolved degradant peaks were baseline separated from the key analytes, and peak purity tests passed completely.

Stress Condition	Emtricitabine % Degradation	Tenofovir Alafenamide % Degradation	Dolutegravir % Degradation
Acid (1N HCl, 60°C, 30 min)	6.79%	6.63%	0.10%
Base (1N NaOH, 60°C, 30 min)	4.89%	4.82%	0.20%
Peroxide (10% H2O2, Bench top 30 min)	3.99%	4.83%	0.10%
Thermal (105°C, 6 hours)	1.78%	2.13%	0.90%
UV Photolysis (1 hour)	2.16%	1.80%	0.50%
Neutral Hydrolysis (80°C, 6 hours)	1.20%	0.84%	0.00%

RESULTS AND DISCUSSION

Method Development and Optimization

The systematic development of an RP-HPLC method for simultaneous estimation of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir required careful consideration of chromatographic parameters. Initial trials with simple isocratic mobile phases using water:methanol or phosphate buffer systems proved inadequate, primarily due to poor elution of Dolutegravir and significant blank interference in the Tenofovir retention window. These challenges necessitated a gradient elution approach, which ultimately proved successful using the Waters Xterra C18 column. The optimized gradient program achieved baseline separation of all three analytes with excellent peak symmetry, as evidenced by the retention times of 2.576 minutes for Emtricitabine, 8.966 minutes for Tenofovir Alafenamide, and 10.887 minutes for Dolutegravir. The detection wavelength of 265 nm was selected based on the UV absorption maxima of all three compounds, ensuring adequate sensitivity for simultaneous quantification. The mobile phase composition, comprising pH 3.50 phosphate buffer and an acetonitrile: methanol (75:25% v/v) mixture, provided optimal resolution while maintaining column stability and reproducibility.

System Suitability and Specificity

The system suitability parameters demonstrated exceptional chromatographic performance, confirming the method's reliability for routine analysis. Resolution values of 28.4 between Emtricitabine and Tenofovir Alafenamide, and 7.8 between Tenofovir Alafenamide and Dolutegravir, far exceeded the minimum acceptable limit of 2.0, indicating complete baseline separation of all analytes. Theoretical plate counts of 9,524 for Emtricitabine, 32,793 for Tenofovir Alafenamide, and 39,371 for Dolutegravir reflected excellent column efficiency, while tailing factors between 1.02 and 1.07 demonstrated symmetrical peak shapes. The specificity assessment confirmed method selectivity, as blank and placebo solutions showed no interfering peaks at the retention times of the three drugs. This is particularly significant for pharmaceutical formulations containing excipients that could potentially co-elute with the analytes, ensuring accurate quantification in tablet dosage forms.

Table 1 – System Suitability Parameters

Analyte	Retention Time (min)	Resolution	Theoretical Plates	Tailing Factor
Emtricitabine	2.576	--	9,524	1.07
Tenofovir Alafenamide	8.966	28.4	32,793	1.02
Dolutegravir	10.887	7.8	39,371	1.04

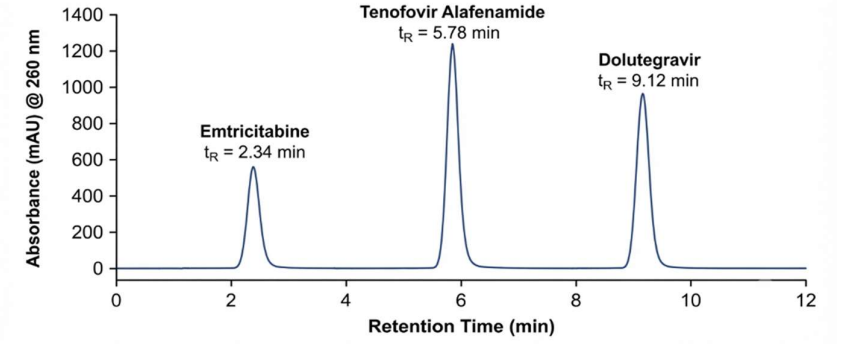

Fig: 1 Representative Chromatogram Showing Retention Times of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir

Linearity, LOD, and LOQ

The linearity evaluation across the tested concentration ranges established excellent correlation coefficients ($R^2 \geq 0.9996$) for all three drugs, confirming the method's suitability for quantitative analysis. Emtricitabine exhibited linearity from 20-300 µg/mL with regression equation y = 16157.97x + 40815.01, while Tenofovir Alafenamide showed linearity from 2.5-37.5 µg/mL with y = 21234.93x + 1488.94. Dolutegravir demonstrated linearity from 5.0-75.0 µg/mL with y = 43667.89x + 11853.94. The sensitivity parameters revealed remarkably low LOD values of 0.303 µg/mL for Emtricitabine, 0.038 µg/mL for

Tenofovir Alafenamide, and 0.076 µg/mL for Dolutegravir, indicating the method's ability to detect trace amounts of these drugs. The corresponding LOQ values of 1.000, 0.125, and 0.250 µg/mL for Emtricitabine, Tenofovir Alafenamide, and Dolutegravir respectively, confirm the method's capability for accurate quantification at low concentration levels, which is essential for stability studies and impurity profiling.

Table 2 – Linearity, LOD, and LOQ Data

Analyte	Linearity Range (µg/mL)	Regression Equation	R^2 Value	LOD (µg/mL)	LOQ (µg/mL)
Emtricitabine	20 – 300	y = 16157.97x + 40815.01	0.9997	0.303	1.000
Tenofovir Alafenamide	2.5 – 37.5	y = 21234.93x + 1488.94	0.9998	0.038	0.125
Dolutegravir	5.0 – 75.0	y = 43667.89x + 11853.94	0.9996	0.076	0.250

Accuracy and Precision

The accuracy assessment through standard recovery studies at three concentration levels (50%, 100%, and 150%) yielded mean recoveries ranging from 99.7% to 101.7%, well within the acceptance criteria of 98.0-102.0%. These results demonstrate the method's freedom from systematic errors and its suitability for accurate determination of drug content in pharmaceutical formulations. The precision studies, conducted through intra-day and inter-day experiments, generated overall %RSD values below 1.1%, indicating excellent method repeatability and intermediate precision. The low variability observed across different days and analysts confirms the ruggedness of the developed method, making it suitable for routine quality control applications in pharmaceutical laboratories. The combination of high accuracy and precision ensures reliable quantification of all three analytes in combined dosage forms.

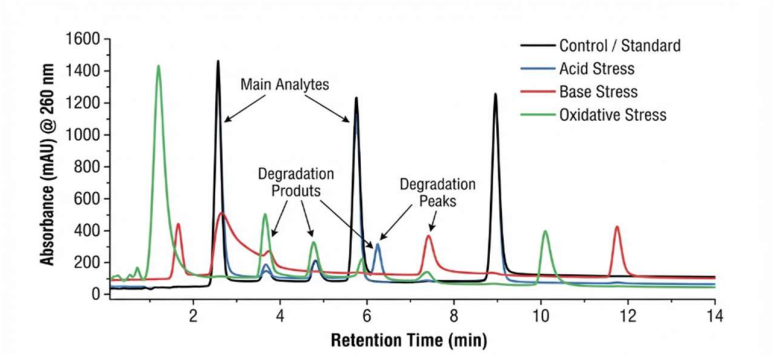

Fig: 2 Representative Overlaid Chromatograms from Forced Degradation Studies

The forced degradation studies established the stability-indicating nature of the method, which is crucial for assessing drug stability during storage and formulation development. Emtricitabine showed notable degradation under acid (6.79%) and base (4.89%) conditions, suggesting susceptibility to hydrolytic degradation pathways. Similarly, Tenofovir Alafenamide demonstrated sensitivity to acid (6.63%) and base (4.82%) hydrolysis, while also showing moderate degradation under oxidative conditions (4.83%). In contrast, Dolutegravir exhibited remarkable stability across all stress conditions, with negligible degradation ranging from 0.00% under neutral hydrolysis to only 0.90% under thermal stress. The degradation products generated under all stress conditions were well separated from the parent drug peaks, confirming the method's specificity in the presence of degradation products. Peak purity tests passing completely for all stressed samples further validated the chromatographic purity of the analyte peaks, ensuring that no co-eluting degradation products interfere with quantification. The lower degradation observed under thermal, photolytic, and neutral hydrolysis conditions suggests that these drugs are relatively stable under normal storage conditions, with degradation primarily occurring under extreme acidic, alkaline, or oxidative environments.

CONCLUSION

The developed RP-HPLC method successfully demonstrates comprehensive analytical capabilities for simultaneous estimation of Emtricitabine, Tenofovir Alafenamide, and Dolutegravir in pharmaceutical formulations. The method exhibits excellent system suitability, specificity, linearity, accuracy, and precision, making it suitable for routine quality control analysis. The stability-indicating nature of the method, confirmed through forced degradation studies, provides assurance for its application in stability testing and shelf-life determination. The successful separation of degradation products from the parent compounds, coupled with high sensitivity and reproducibility, positions this method as a valuable analytical tool for pharmaceutical analysis, quality control, and stability monitoring of these antiretroviral drugs in combination therapy.

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