

Validated Stability-Indicating Assay UHPLC Method for Simultaneous Estimation of Doravirine, Lamivudine and Tenofovir Disoproxil Fumarate in Pure Material and Pharmaceutical Matrix

Balaji N. Thakare^{1,*} , Abhilasha Mittal² , Manoj S. Charde³

¹ Institute of Pharmacy, NIMS University, Jaipur, Rajasthan, India 303121; balaji.thakare2@gmail.com (B.N.T);

² Department pharmaceutical chemistry, Institute of Pharmacy, NIMS University, Jaipur, Rajasthan, India 303121; abhilashamittal24@gmail.com (A.M.);

³ Government College of Pharmacy, Karad, Maharashtra, India 415110; manojjudps@gmail.com (M.S.C.);

* Correspondence: balaji.thakare2@gmail.com (B.N.T);

Scopus Author ID 54890552000

Received: 17.10.2021; Accepted: 20.11.2021; Published: 22.01.2022

Abstract: An efficient, robust, and specific stability-indicating UHPLC assay method was acquainted for simultaneous determination of antiretroviral agents doravirine (DOV), lamivudine (LVD), and tenofovir disoproxil fumarate (TDF). The determination of cited agents was performed on UPLC system using UPLC BEH C₁₈ (150 mm × 2.1 mm) with 1.7 μm particle size column at ambient temperature using a solvent system in a proportion of (60:40 % v/v) acetonitrile: potassium dihydrogen orthophosphate buffer; pH 4.5 ± 0.2 was adjusted with 0.1 % OPA. The PDA detector was subjected to a wavelength of 235 nm. DOV, LVD, and TDF were experienced in various stress procedures of forced degradation experiments, as recommended by the ICH, to assess the established method's stability-indicating practicability. Using the optimized procedure, selected agents were successfully distinguished from the peaks of their respective degradation products. Furthermore, the calibration curves for DOV, LVD, and TDF in over wide concentration ranges of 2–12 μg/mL, 5–30 μg/mL, and 5–30 μg/mL, respectively, with determination coefficients (r²) of 0.997, 0.9994, and 0.9994. The method validation assays had less than 2 % relative standard deviations for accuracy, precision, repeatability, and robustness.

Keywords: doravirine; forced degradation investigation; fixed-dose combination; lamivudine; tenofovir disoproxil fumarate.

© 2022 by the authors. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Doravirine (DOV) is very recent non-nucleoside reverse transcriptase inhibitor (NNRTI) used in the treatment of HIV infection, chemically it is 3-chloro-5-[1-[(4-methyl-5-oxo-1*H*-1,2,4-triazol-3-yl)methyl]-2-oxo-4-(trifluoromethyl)pyridin-3-yl]oxybenzotrile depicted in Figure 1a [1,2]. Lamivudine (LVD) is a dideoxynucleoside counterpart that experiences phosphorylation (intracellular) to produce LVD-triphosphate, the potential active metabolite. It inhibits viral reverse transcriptase and thereby suppresses replication of HIV by blocking viral reverse transcriptase competitively and stopping proviral DNA chain growth in HIV-infected cells; LVD intracellular elimination half-life was between 10.5 and 15.5 h [3-5]. LVD is 4-amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one depicted

in Figure 1b [6]. Tenofovir Disoproxil Fumarate (TDF) is a drug used in combination with other HIV drugs to help manage the infection. TDF is an analog of adenosine 5'-monophosphate that inhibits viral replication by getting involved with the HIV viral RNA-dependent DNA polymerase. Chemically TDF is specified as [(2*R*)-1-(6-aminopurin-9-yl) propan-2-yl] oxymethyl-(propan-2-yloxy carbonyloxy methoxy) phosphoryl] oxymethylpropan-2-yl carbonate; but-2-enedioic acid depicted in (Figure 1c) [6,7].

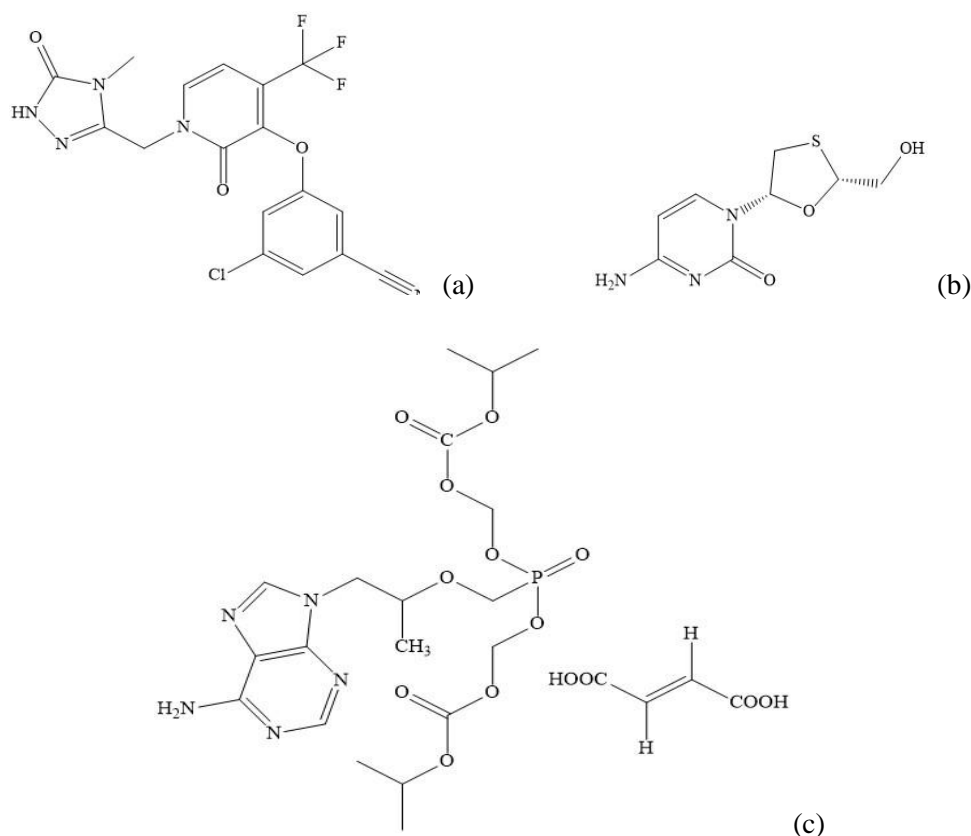


Figure 1. Chemical structures of (a) doravirine, (b) lamivudine, and (c) tenofovir disoproxil fumarate.

Leading to the advancements of oral antiretrovirals, which are effective, simple, and normally well-tolerated, resulting in virologic reduction and decreased HIV transmission, HIV prevention and management have enhanced significantly in the last few years. Three antiretroviral agents consist of two nucleoside analog reverse transcriptase inhibitors combined with a third agent, either an integrase inhibitor, a non-nucleoside reverse transcriptase inhibitor, or a protease inhibitor is a now day's treatment for a person with HIV infection [8–11].

In terms of therapeutic efficacy, DLT, LVD, and TDF have been shown to have eagerly anticipated the prevention of HIV transmission. For the quantification and systematic chromatographic assessment of the cited antiretroviral drugs in their fixed-dose combination pharmaceutical formulation, developing an efficient, specific, and sensitive analytical approach is particularly important.

Many research researchers have examined DOV, LVD, and TDF, either alone or in combination with other antiretroviral drugs in pharmaceutical and biological matrices exercising LC-MS/MS [12–22], HPLC [23–37], HPTLC [38–43], and spectrophotometry [44–48].

The literature's systematic review revealed no stability-indicating ultrahigh-performance liquid-chromatography (UHPLC) method addressed for simultaneous quantification of DOV, LVD, and TDF in pharmaceutical formulation. The specific and

sensitive stability-indicating method is highly desirable for addressing pharmaceutical's intrinsic chemical stability characteristics, which have excellent therapeutic utility. Furthermore, developing an analytical method for the pharmaceuticals described above might be difficult for an analytical chemist, especially when each agent has unique physicochemical parameters like pH, pKa, and solubility. Hence, attempts were made to design and confirm efficient, robust, and specific UHPLC methods for quantifying DOV, LVD, and TDF in pharmaceutical formulation with forced-degradation experiments as per the recommendation ICH guidelines.

2. Materials and Methods

2.1. Material and reagent.

Doravirine, Lamivudine, and Tenofovir Disoproxil Fumarate were kindly supplied by Merck Pharmaceutical LTD., and Abbott Pharmaceuticals PVT. LTD., India. Acetonitrile, methanol, and distilled water were HPLC grade and purchased from Merck, LTD., India. Other chemicals and reagents of analytical grade comprising orthophosphoric acid (OPA), hydrogen peroxide, sodium hydroxide, and HCl were obtained from Loba Chemie PVT. LTD., India.

2.2. Marketed formulation.

DelstrigoTM tablets labeled to encompass 100 mg of DOV, 300 mg of LVD, and 300 mg of TDF each tablet was manufactured and marketed by Merck Pharmaceutical LTD., India.

2.3. Preparation of stock standard solution.

Stock standard solutions were made by dissolving 10 mg of DOV, 30 mg of LVD, and 30 mg of TDF in a 100 mL calibrated flask containing 50 mL of methanol and manually shaking for 10 minutes. Finally, the amount was diluted to the point of the calibrated flask to obtain DOV, LVD, and TDF concentrations of 100 µg/mL, 300 µg/mL, and 300 µg/mL, respectively.

2.3.1. Preparation of working solution.

To get the 10 µg/mL, 30 µg/mL, and 30 µg/mL concentrations of working solution of DOV, LVD, and TDF was prepared by transferring an accurate volume of 1 mL into 10 mL of the calibrated flask from stock solutions. Then volume was diluted to the mark with the same.

2.4. Instrumentation and chromatographic determination.

The introduced method development and validation were achieved using an Agilent UPLC system (Agilent Technologies, USA) installed with a solvent delivery system, ACQ-PDA detector, autosampler, and a thermostatically controlled column compartment. On a UPLC BEH C₁₈ (150 mm × 2.1 mm) with a 1.7 µm particle size column as a stationary phase, UHPLC chromatographic quantifications of DOV, LVD, TDF, and their degradant products were examined. The solvent system in a proportion of (60:40 % v/v) acetonitrile: potassium dihydrogen orthophosphate buffer; pH 4.5 ± 0.2 was adjusted with 0.1 % OPA was ideally selected and before application, sonicated for 20 min and filtered through Ultipor[®] N₆₆[®] Nylon 6, 6 membranes 0.2 µm filter paper. The temperature in the operative thermostatically controlled column compartment was around 30°C. An autosampler was used to inject 10 µL of

fixed volume (working solution). The PDA detector was adjusted to a wavelength range of 190–380 nm, and DOV, LVD, and TDF were all monitored at 235 nm at the same time. The data was analyzed using the Empower[®] 3 software (Agilent Technologies, USA).

2.5. Preparation of DelstrigoTM tablet solution.

The tablet solution was prepared by weighing 20 tablets of *DelstrigoTM* (encompass 100 mg of DOV, 300 mg of LVD, and 300 mg of TDF in one tablet) to estimate their average weight. An amount of powdered tablets equivalent per tablet was moved out into 100 mL of the calibrated flask, 50 mL of methanol was added. A sonicated and before made the volume to mark with same to achieve the 1000 µg/mL, 3000 µg/mL, 3000 µg/mL concentrations of DOV, LVD, and TDF; were filtered through a 0.45 µm membrane. The suitable volume of this was diluted with methanol to get the final concentrations of 4 µg/mL, 10 µg/mL, and 10 µg/mL of DOV, LVD, and TDF. This solution was injected directly into the UPLC system.

2.6. System suitability testing and calibration curves.

The working standard solution comprising 10 µg/mL, 30 µg/mL, and 30 µg/mL concentrations of DOV, LVD, and TDF were prepared and injected (six determinations). The tailing factor, theoretical plate number, resolution, and percent relative standard deviation (% RSD) values for DOV, LVD, and TDF peaks were measured. The calibration curves for the specific UHPLC assay method were constructed over the concentration ranges of 2 – 12 µg/mL for DOV, 5 – 30 µg/mL for LVD, and 5 – 30 µg/mL TDF. Five times each concentration solution of analytes was produced and injected.

3. Validation of UHPLC method

To establish an efficient, robust, and specific UHPLC assay method for DOV, LVD, and TDF in pharmaceutical formulations, the International Conference on Harmonization Q2(R1) parameters has been used [49].

3.1. Accuracy.

The accuracy (% recovery) of the proposed method was investigated regarding the standard addition technique. The known DOV, LVD, and TDF standards were added to the pre-analyzed tablet solution (DOV- 4 µg/mL, LVD- 10 µg/mL, and TDF- 10 µg/mL), and then experimental and valid values were compared. The % recovery was estimated through 80 %, 100 %, and 120 % of the nominal analytical concentration.

3.2. Precision.

The repeatability, intra-day, and inter-day assay variability were measured to particularize the precision of the proposed method. The repeatability assay variability was assessed using injecting the six determinations (100 %) of 4 µg/mL of DOV and 10 µg/mL of LVD and TDF concentrations. Whereas intra-day and inter-day precision assay variability was assessed using injecting the 4, 6, and 8 µg/mL of DOV and 10, 15, and 20 µg/mL of LVD and TDF at various time frames on the same day and for three successive days. The recorded data were analyzed as a % RSD.

3.3. Sensitivity.

The LOD and LOQ were determined using the standard deviation (N) of the DOV, LVD, and TDF (n=3) outcomes, as well as the slope of the calibration curve (B). $LOD = 3.3 N/B$ and $LOQ = 10 N/B$ were the formulas used. Serial working dilutions of 2 – 4 µg/mL of DOV and 5 – 15 µg/mL of LVD and TDF ranges have been acknowledged and assessed. The designed method recorded LOD and LOQ values of 0.10 µg/mL and 0.30 µg/mL for DOV, 0.18 µg/mL and 0.57 µg/mL for LVD and 0.15 µg/mL and 0.45 µg/mL for TDF, correspondingly. As a result, the design UHPLC method was found to be extremely sensitive to the solvent system.

3.4. Robustness.

The robustness of the design UHPLC method was determined by attempting to make substantial changes in the proportion of acetonitrile, column oven compartment temperature, and flow rate. Each independent variable's influence was calculated for the peak areas of DOV, LVD, and TDF. The selected independent variables for this analysis were varied as a proportion of acetonitrile (50 – 70 %), the temperature of the column oven compartment (25 – 35 °C), and flow rate (0.2 – 0.4 mL/min). It was noticed that such independent variables did not affect the DOV, LVD, and TDF experiments. Therefore, the investigation of robustness determination has been addressed with 10 µg/mL of DOV and 20 µg/mL of LVD and TDF concentrations.

3.5. Specificity and selectivity.

The proposed method's selectivity and specificity were assessed using qualitatively identifying the interest of the analyte (DOV, LVD, TDF) in the context of components likely to be present in the pharmaceutical formulation and performing forced degradation studies (stress testing) with various stress experiments suggested by the ICH including, thermal degradation, photodegradation, oxidative degradation, acidic, alkaline, and neutral hydrolysis [50,51].

3.5.1. Thermal degradation.

3.5.1.1. Dry heat.

The separately weighed 20 mg of DOV, LVD, and TDF standards were introduced into a sealed ampoule and placed into the digital controlled thermostatic hot air oven at 60 °C for ten h. The precise quantity of 10 mg of DOV, LVD, and TDF was separately dissolved in methanol. An adequate aliquot of stress-induced samples (0.1 mL) of the resulting solution was diluted with a solvent system to obtain concentrations of 10 µg/mL of DOV, LVD, and TDF were addressed.

3.5.1.2. Wet heat.

DOV, LVD, and TDF (1 mg/mL) stock solutions were kept in the digital controlled thermostatic hot air oven at 60 °C for 10 h. An adequate aliquot of stress DOV, LVD, and TDF samples (0.1 mL) of the resulting solution was diluted with the solvent system to obtain the concentrations of 10 µg/mL of DOV, LVD, and TDF and was analyzed.

3.5.2. Photodegradation.

DOV, LVD, and TDF were photolyzed using solid samples (spreading as a thin layer on a petri dish) exposed to UV radiation at a rate of 360Wh/m² for 10 days at 30°C, i.e., for short UV-254 nm and long UV-360 nm. A sufficient aliquot of stress-induced DOV, LVD, and TDF samples (0.1 mL) was removed and diluted with a solvent system to obtain DOV, LVD, and TDF concentrations of 10 µg/mL.

3.5.3. Oxidative degradation.

DOV, LVD, and TDF oxidative stress studies were carried out by precisely solubilizing 10 mg of DOV, LVD, and TDF into a separate calibrated flask (6 % H₂O₂ v/v). Methanol was used to dilute the volume to the mark of a calibrated flask. The resulting solution was kept dark at room temperature for two days to prevent significant oxidation by light. Then, a sufficient aliquot of stress-induced DOV, LVD, and TDF samples (0.1 mL) was removed and diluted with a solvent system to obtain DOV, LVD, and TDF concentrations of 10 µg/mL.

3.5.4. Acidic hydrolysis.

Acidic hydrolytic stress studies for DOV, LVD, and TDF were conducted by specifically solubilizing 10 mg of DOV in 1 M methanolic HCl and LVD and TDF in 10 mL of 0.5 M methanolic HCl in a calibrated flask. The resulting solution of DOV as kept in the dark at room temperature for 2 days and prepared solutions of LVD and TDF were transferred to a 50 mL RBF, fitted with a reflux condenser, and refluxed at 80°C for 12 h in a thermostatic water bath for LVD, and TDF. First, a sufficient aliquot of stress-induced DOV, LVD, and TDF samples (1.0 mL) was removed and neutralized with a 1.0 M and 0.5 M methanolic NaOH solution of equivalent concentration. After that, 10 µg/mL DOV, LVD, and TDF concentrations were obtained by diluting 0.1 mL of the resulting solutions with a solvent system and addressed as per the established UHPLC method.

3.5.5. Alkaline hydrolysis.

Alkaline hydrolytic stress studies for DOV, LVD, and TDF were performed by specifically solubilizing 10 mg of DOV, LVD, and TDF in 10 mL of 1 M and 2 M methanolic NaOH in a calibrated flask. The resulting solutions were transferred to a 50 mL RBF, fitted with a reflux condenser, and refluxed at 80°C for 6 h in a thermostatic water bath for DOV, LVD, and TDF. Next, a sufficient aliquot of stress-induced DOV, LVD, and TDF samples (1.0 mL) was removed and neutralized with a 1 M and 2 M methanolic HCl solution of equivalent concentration. After that, 10 µg/mL DOV, LVD, and TDF concentrations were obtained by diluting 0.1 mL of the resulting solutions with a solvent system and addressed as per the established UHPLC method.

3.5.6. Neutral hydrolysis.

To investigate the effects of hydrolysis on DOV, LVD, and TDF in a neural state. The hydrolytic effect stress studies began with the precise solubilization of 10 mg of DOV, LVD, and TDF into a 10 mL calibrated flask with methanol as a stressor. The resulting solution was kept in the dark at room temperature for 10 days to prevent significant light oxidation. Next, a sufficient aliquot of stress-induced DOV, LVD, and TDF samples (0.1 mL) was removed and

diluted with a solvent system to obtain DOV, LVD, and TDF concentrations of 10 µg/mL. With neutral hydrolysis, it was discovered that such therapeutic agents were virtually stable. As such, no degradation was noticed when subjected to neutral hydrolysis at room temperature for 10 days.

4. Results and Discussion

In UHPLC determination, the selection of appropriate stationary phase (dimensions and length of analytical columns) and solvent system based on the physicochemical characteristics of selected drug candidates. It is crucial when the selected analytes contain a high carbon content and are more hydrophobic. Thus, they can be separated and well-resolved with the application of C₈ or C₁₈ stationary phases and more polar organic solvent systems with optimum flow rates. Hence, the development of the UHPLC method was commenced with 100 % v/v of acetonitrile, but the faster elution and poor resolution of analytes of interest were noticed. Thereby, various proportions of acetonitrile and water (90:10 % v/v to 20:80 % v/v) steps have been taken to obtain the well-resolved and symmetrical peak shapes of DOV, LVD, and TDF. At all events, limited separation was observed except in the (60:40 % v/v) proportions of acetonitrile, and potassium dihydrogen orthophosphate gave quite a well-resolution of selected analytes with considerable tailing of peaks were marked. The shape of peaks, tailing factors, retention times, theoretical plates, and resolution were affected by the alteration in the proportions of the solvent system, flow rates, and distinct dimensions and length of analytical columns.

Ultimately, using a solvent system in a proportion of (60:40 % v/v) acetonitrile and potassium dihydrogen orthophosphate buffer; pH 4.5 ± 0.2 was adjusted with 0.1 % OPA was optimized, which gave symmetrical peaks of analytes using UPLC BEH C₁₈ (150 mm × 2.1 mm) with 1.7 µm particle size column at flow rate 0.3 mL/min with PDA detection monitored at 235 nm. Following specified chromatographic conditions, quite symmetrical and sharp peaks of DOV, LVD, and TDF were recorded at a retention time of DOV, LVD, and TDF were 2.786 ± 0.013 min, 1.037 ± 0.022 min, and 1.923 ± 0.018 min, respectively depicted in Figure 2. In contrast, the outcomes of system suitability testing are presented in Table 1.

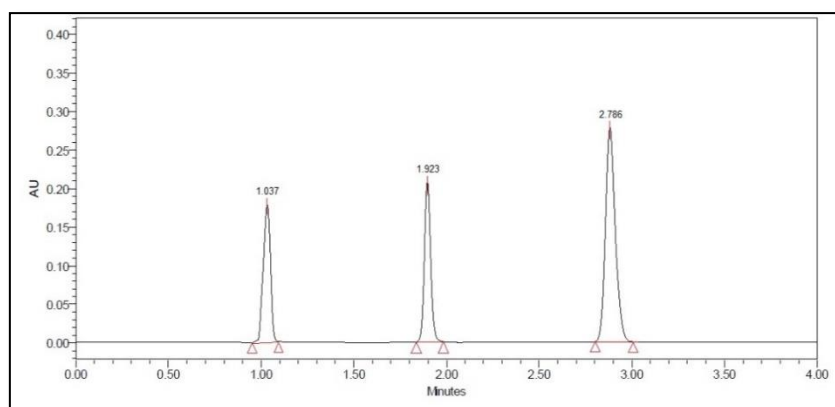


Figure 2. Optimized chromatogram of doravirine, lamivudine, and tenofovir disoproxil fumarate.

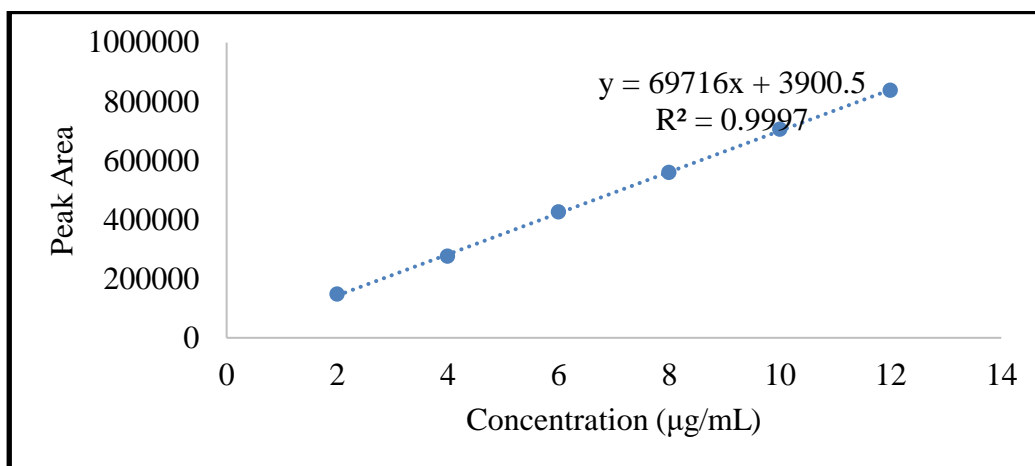
Table 1. System suitability testing and analysis of DLT, LVD, and TDF in pharmaceutical formulation.

Drugs	System suitability parameters				Analysis of Tablets
	Tailing factor	Theoretical plates	Resolution	Retention times [min ± SD; n=6]	<i>Delstrigo</i> TM tablets [% Amount found ± SD]
					[Label claim DOV 50 mg/ LVD 300 mg/ TDF 300 mg]

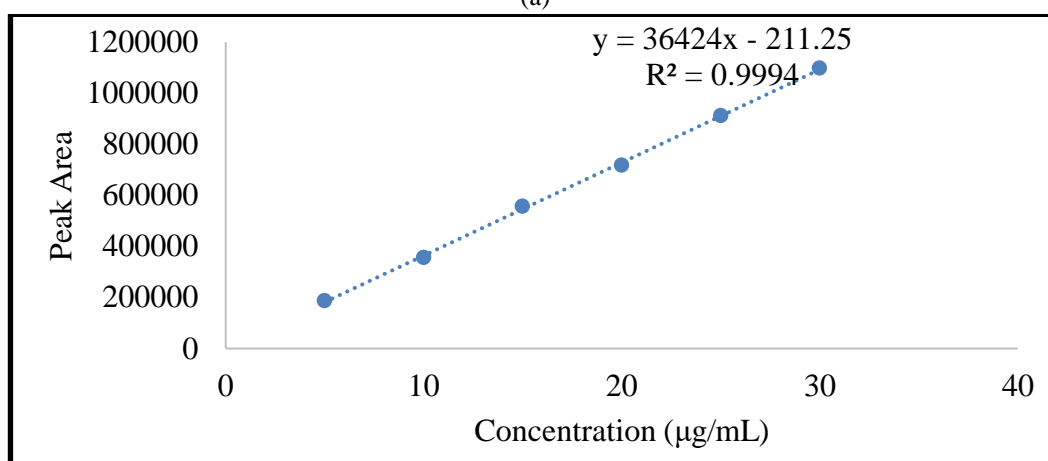
DOV	1.01	2894.84	-	2.786 ± 0.013	99.39 ± 0.35
LVD	1.33	2015.16	2.46	1.037 ± 0.022	99.70 ± 0.35
TDF	1.08	2484.64	2.95	1.923 ± 0.018	100.22 ± 0.41

* n= number of determinations, SD= standard deviation

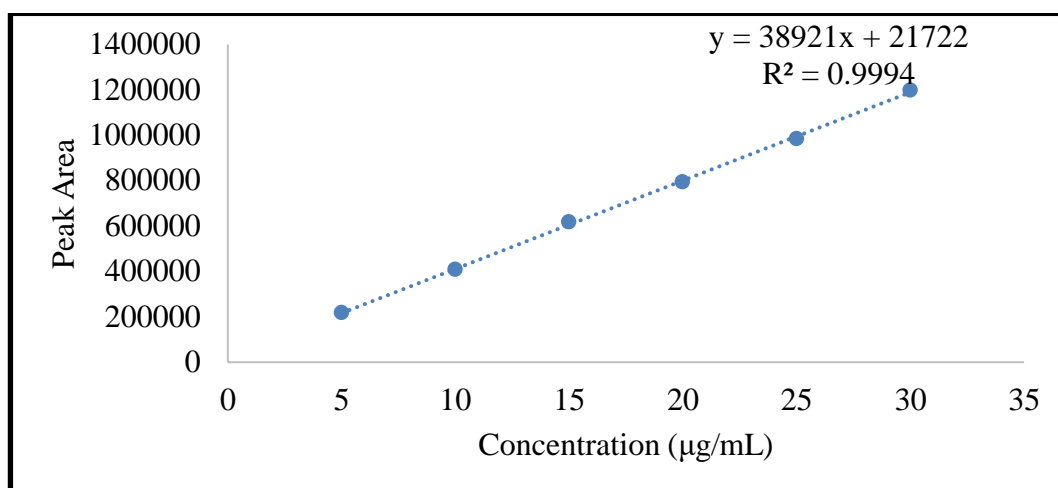
The calibration curves were designed in the ranges of concentration of 2 - 12 µg/mL, 5 - 30 µg/mL, and 5 - 30 µg/mL for DLT, LVD, and TDF. The equation of linear regression to develop a relationship as calibration curves were recorded to be $y = 69716x + 3900.5$ with determination coefficient (r^2) 0.9997 for DOV, $y = 36424x - 211.25$ with r^2 0.9994 for LVD, and $y = 38921x + 21722$ with r^2 0.9994. The linear regression equations are the slope and intercepts at which X is equal to zero, shown in Figure 3. Moreover, the proposed UHPLC method was confirmed with the Q2R1 benchmark of ICH for accuracy, precision, sensitivity, i.e., LOD and LOQ, robustness, selectivity, and specificity. The % recovery of the established UHPLC method was investigated using the standard addition technique at three different percentage levels, i.e., 80 %, 100 %, and 120 % of the nominal analytical concentration, and addressed as per the introduced method. Table 2 highlighted the % recoveries with standard deviation (SD) and % RSD of each percentage level for DOV, LVD, and TDF. The approach was shown to be very accurate and adequate for its intended usage in recovery studies.



(a)



(b)



(c)

Figure 3. Calibration curves for (a) doravirine; (b) lamivudine; (c) tenofovir disoproxil fumarate.

Table 2. Accuracy of the proposed HPLC method.

Drugs	Level of study [%]	% Recovery ± SD ^a	Grand average ± SD ^b
DOV	80	99.56 ± 0.64	100.19 ± 0.96
	100	100.01 ± 1.29	
	120	101.00 ± 0.96	
LVD	80	100.52 ± 1.05	100.71 ± 0.57
	100	100.99 ± 0.15	
	120	100.61 ± 0.50	
TDF	80	100.58 ± 0.27	100.62 ± 0.24
	100	100.45 ± 0.11	
	120	100.83 ± 0.33	

^a Average % recovery of the three different solutions at each % level concentration of drugs.

^b Average of % recovery of the three different levels concentration of drugs.

The repeatability assay variability was assessed for a proposed method using the six concentrations (100 %) of DOV, LVD, and TDF. Intra-day and inter-day assay variability was confirmed by introducing three distinct concentrations of DOV, LVD, and TDF on the same day and inter-day by introducing the same concentration solutions for three successive days. The % RSD of the peak area of analytes was estimated to display precision assay variability. The approach was shown to be well-enough precise with % RSD less than 2 %.

Table 3. Precision analysis for DOV, LVD, and TDF.

Precision analysis	Standard concentrations of drugs								
	DOV [µg/mL]			LVD [µg/mL]			TDF [µg/mL]		
	4	6	8	10	15	20	10	15	20
Intra-day assay									
% Amount found [n=3]	98.63	99.87	99.21	99.04	100.55	99.18	99.46	99.40	99.82
% RSD	0.68	0.95	1.15	0.54	0.51	0.59	0.58	1.37	0.17
Inter-day assay									
% Amount found [n=3]	99.10	99.22	99.59	99.41	99.95	99.20	99.46	99.63	99.88
% RSD	1.12	1.34	0.51	1.22	0.89	0.77	0.95	1.03	0.44
	DOV [9 µg/mL]			LVD [20 µg/mL]			TDF [20 µg/mL]		
Repeatability assay									
% Amount found ±SD	100.06 ± 1.20			99.72 ± 0.41			100.32 ± 0.32		
% RSD [n=6]	1.20			0.41			0.32		

* n= number of determinations, SD= standard deviation, and %RSD= percent relative standard deviation.

The intra-day, inter-day, and repeatability assay variability findings are represented in Table 3. Different concentrations were used to determine the LOD and LOQ. The LOD and

LOQ were recorded to be 0.10 µg/mL and 0.30 µg/mL for DOV, 0.18 µg/mL and 0.57 µg/mL for LVD and 0.15 µg/mL and 0.45 µg/mL for TDF, correspondingly. Slightly varying conditions of separation analysis performed the robustness of the method. The results showed that slight variations in experimental procedure (detection of wavelength, temperature of column oven compartment, and flow rate) had a negligible effect on the peak areas of DOV, LVD, and TDF. The recorded results of robustness studies are presented in Table 4.

Table 4. Assessment of methods robustness for estimation of DOV, LVD, and TDF.

Drugs	Experimental Parameters		
	Proportion of acetonitrile (50 - 70 %)	Column oven temperature [25 -35 °C]	Flow rate [0.2 – 0.4 mL/min]
DOV			
Mean peak area ± SD [n=6]	700259.24 ± 7136.40	699053.51 ± 7656.30	702582.25 ± 5009.12
% RSD	1.01	1.01	0.70
LVD			
Mean peak area ± SD [n=6]	721182.25 ± 2745.38	725886.55 ± 2680.09	725997.34 ± 2134.2
% RSD	0.37	0.36	0.29
TDF			
Mean peak area ± SD [n=6]	794425.67 ± 1679.81	794831.18 ± 1723.75	795864.84 ± 2639.4
% RSD	0.20	0.21	0.33

* n= number of determinations, SD= standard deviation, and %RSD= percent relative standard deviation.

Table 5. Forced degradation experiment outlined data for DOV, LVD, and TDF.

Stressor conditions	Number of impurities	Rt of impurities [min]	% Degradation
DOV			
1 M HCl for 2 days	02	1.096, 1.201	4.89, 1.65
1 M NaOH at 80°C for 6 h	00	Stable	
6 % H ₂ O ₂ v/v at room temperature for 2 days	01	2.972	6.34
Dry heat at 80°C for 10 h	00	Stable	
Wet heat Digital controlled thermostatic hot air oven at 80 °C for 10 h	01	3.298	1.58
LVD			
0.5 M methanolic HCl for 80°C for 12 h	01	1.696	6.17
2 M NaOH at 80°C for 6 h	01	1.013	5.74
6 % H ₂ O ₂ v/v at room temperature for 2 days	02	0.835, 0.921	6.48, 3.22
Dry heat at 60°C for 10 h	01	1.108	6.98
Wet heat Digital controlled thermostatic hot air oven at 60 °C for 10 h	00	Stable	
TDF			
0.5 M methanolic HCl for 80°C for 12 h	02	1.842, 2.401	14.15, 5.67
2 M NaOH at 80°C for 6 h	01	1.823	12.52
6 % H ₂ O ₂ v/v at room temperature for 2 days	01	1.972	9.43
Dry heat at 60°C for 10 h	00	Stable	
Wet heat Digital controlled thermostatic hot air oven at 60 °C for 10 h	01	2.486	4.31

In addition to this, selectivity and specificity were assessed by qualitatively recognizing the DOV, LVD, TDF in the presence of common additives most likely to be found in the drug's formulation. It was noticed that all analytes were consistently well-resolved from the same. Furthermore, forced degradation studies were also investigated to address the UHPLC method's specificity by executing the thermal degradation, photodegradation, oxidative, acidic, and alkaline hydrolysis to DOV, LVD, and TDF. The proposed UHPLC method was specific and

sensitive to separate the analytes of interest from their degradation products. The degradation pattern of the specified analytes across various stress mediums is tabulated in Table 5. The ability of the intended UHPLC measure to determine intact the analytes of interest with no impurities (degradation products) under each of these environments demonstrate the planned study results stability-indicating potential and, as a result, addresses the method's specificity.

Quantification of DOV, LVD, and TDF in *DelstrigoTM* tablets was done using the proposed UHPLC method, with 4 µg/mL, 10 µg/mL, and 10 µg/mL of DOV, LVD, and TDF concentrations. The assay was performed as guidelines. Satisfactory findings were obtained for tablet formulation, which was in good agreement with the label claim. Table 1 shows the outcome of the pharmaceutical formulation assay study.

5. Conclusions

The simultaneous quantification of doravirine, lamivudine, and tenofovir disoproxil fumarate in pure standard and pharmaceutical formulation, a simple gradient, efficient, and specific UHPLC method is described. The introduced method was confirmed for accuracy, precision, specificity, sensitivity, and robustness were all tested according to ICH's recommendation. The implemented protocol for chromatographic determination is simple quick. It does not require the use of any tedious sample pre-treatment procedure and higher consumption of organic polar solvents and or any derivative reagents. The approach is capable of distinguishing cited analytes from degradation products developed during forced degradation experiments. The chromatograms further show that all active component peaks were well-resolved from impurities in the stress testing. The analytical method met all of the validation guidelines' recognition requirements. It can be exploited to provide stability data in pharmaceutical formulation by simultaneously evaluating dolutegravir, lamivudine, and tenofovir disoproxil fumarate. Furthermore, this strongly suggests that the method can be implemented for any stability and validation investigation.

Funding

This research received no external funding.

Acknowledgments

The authors acknowledge the Institute of Pharmacy, NIMS University, Jaipur, Rajasthan, India, for the present work.

Conflicts of Interest

The authors declare that they have no competing interests.

References

1. Desai, R.; Roadcap, B.; Goykhman, D.; Woolf, E. Determination of doravirine in human plasma using liquid–liquid extraction and HPLC–MS/MS. *Bioanalysis* **2019**, *11*, 1495–1508, <https://doi.org/10.4155/bio-2019-0116>.
2. Zhang, L.K.; Yang, R.; Sheng, H.; Helmy, R.; Zheng, J.; Cao, Y.; Gauthier Jr, D.R. Characterization of impurities of HIV NNRTI doravirine by UHPLC-high resolution MS and tandem MS analysis. *Journal of Mass Spectrometry*, **2016**, *51*, 959–968, <https://doi.org/10.1002/jms.3807>.
3. Perry, C.M.; Faulds, D. Lamivudine. *Drugs* **1997**, *53*, 657–680, <https://doi.org/10.2165/00003495->

- 199753040-00008.
4. Elliot, E.; Amara, A.; Jackson, A.; Moyle, G.; Else, L.; Khoo, S.; Back, D.; Owen, A.; Boffito, M. Dolutegravir and elvitegravir plasma concentrations following cessation of drug intake. *J. Antimicrob. Chemother.* **2016**, *71*, 1031–1036, <https://doi.org/10.1093/jac/dkv425>.
 5. KOHRT, H.E.; OUYANG, D.L.; KEEFFE, E.B. Systematic review: lamivudine prophylaxis for chemotherapy-induced reactivation of chronic hepatitis B virus infection. *Aliment. Pharmacol. Ther.* **2006**, *24*, 1003–1016, <https://doi.org/10.1111/j.1365-2036.2006.03081.x>.
 6. Adhao, V.S.; Chaudhari, S.R.; Ambhore, J.P.; Sangolkar, S.; Thenge, R.R.; Cheke, R.S.; Patil, A.S. Reverse phase-liquid chromatography assisted protocol for simultaneous determination of lamivudine and tenofovir disoproxil fumarate in combined medication used to control HIV infection: an investigative approach. *Futur. J. Pharm. Sci.* **2021**, *7*, 90, <https://doi.org/10.1186/s43094-021-00233-3>.
 7. Kearney, B.P.; Flaherty, J.F.; Shah, J. Tenofovir Disoproxil Fumarate. *Clin. Pharmacokinet.* **2004**, *43*, 595–612, <https://doi.org/10.2165/00003088-200443090-00003>.
 8. Phanuphak, N.; Gulick, R.M. HIV treatment and prevention 2019: current standards of care. *Curr. Opin. HIV AIDS* **2020**, *15*.
 9. D'Amico, R.; Margolis, D.A. Long-acting injectable therapy: an emerging paradigm for the treatment of HIV infection. *Curr. Opin. HIV AIDS* **2020**, *15*.
 10. Rana, A.I.; Castillo-Mancilla, J.R.; Tashima, K.T.; Landovitz, R.L. Advances in Long-Acting Agents for the Treatment of HIV Infection. *Drugs* **2020**, *80*, 535–545, <https://doi.org/10.1007/s40265-020-01284-1>.
 11. Ambhore, J.P.; Chaudhari, S.R.; Cheke, R.S.; Kharkar, P.S. A Concise Analytical Profile of Efavirenz: Analytical Methodologies. *Crit. Rev. Anal. Chem.* **2021**, *0*, 1–10, <https://doi.org/10.1080/10408347.2021.1895711>.
 12. Penchala, S.D.; Fawcett, S.; Else, L.; Egan, D.; Amara, A.; Elliot, E.; Challenger, E.; Back, D.; Boffito, M.; Khoo, S. The development and application of a novel LC-MS/MS method for the measurement of Dolutegravir, Elvitegravir and Cobicistat in human plasma. *J. Chromatogr. B, Anal. Technol. Biomed. Life Sci.* **2016**, *1027*, 174–180, <https://doi.org/10.1016/j.jchromb.2016.05.040>.
 13. Bennetto-Hood, C.; Tabolt, G.; Savina, P.; Acosta, E.P. A sensitive HPLC-MS/MS method for the determination of dolutegravir in human plasma. *J. Chromatogr. B, Analyt. Technol. Biomed. Life Sci.* **2014**, *945–946*, 225–232, <https://doi.org/10.1016/j.jchromb.2013.11.054>.
 14. Xiao, D.; Ling, K.H.J.; Tarnowski, T.; Majeed, S.R.; German, P.; Kearney, B.P.; Zhao, Y.; Chen, Y.-S.; Ma, L.; Zhang, T. An LC-MS/MS method for determination of tenofovir (TFV) in human plasma following tenofovir alafenamide (TAF) administration: Development, validation, cross-validation, and use of formic acid as plasma TFV stabilizer. *Anal. Biochem.* **2020**, *593*, 113611, <https://doi.org/10.1016/j.ab.2020.113611>.
 15. Prathipati, P.K.; Mandal, S.; Destache, C.J. Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC-MS/MS and its application to a pharmacokinetic study. *J. Pharm. Biomed. Anal.* **2016**, *129*, 473–481, <https://doi.org/10.1016/j.jpba.2016.07.040>.
 16. Bollen, P.D.J.; de Graaff-Teulen, M.J.A.; Schalkwijk, S.; van Erp, N.P.; Burger, D.M. Development and validation of an UPLC-MS/MS bioanalytical method for simultaneous quantification of the antiretroviral drugs dolutegravir, elvitegravir, raltegravir, nevirapine and etravirine in human plasma. *J. Chromatogr. B* **2019**, *1105*, 76–84, <https://doi.org/10.1016/j.jchromb.2018.12.008>.
 17. Matta, M.K.; Burugula, L.; Pilli, N.R.; Inamadugu, J.K.; JVLN, S.R. A novel LC-MS/MS method for simultaneous quantification of tenofovir and lamivudine in human plasma and its application to a pharmacokinetic study. *Biomed. Chromatogr.* **2012**, *26*, 1202–1209, <https://doi.org/10.1002/bmc.2679>.
 18. Mistri, H.N.; Jangid, A.G.; Pudage, A.; Gomes, N.; Sanyal, M.; Shrivastav, P. High throughput LC-MS/MS method for simultaneous quantification of lamivudine, stavudine and nevirapine in human plasma. *J. Chromatogr. B* **2007**, *853*, 320–332, <https://doi.org/10.1016/j.jchromb.2007.03.047>.
 19. Valluru, R.K.; B, P.B.R.; S, K.S.; V, P.K.; Kilaru, N.B. High throughput LC-MS/MS method for simultaneous determination of tenofovir, lamivudine and nevirapine in human plasma. *J. Chromatogr. B* **2013**, *931*, 117–126, <https://doi.org/10.1016/j.jchromb.2013.05.008>.
 20. Delahunty, T.; Bushman, L.; Fletcher, C.V. Sensitive assay for determining plasma tenofovir concentrations by LC/MS/MS. *J. Chromatogr. B* **2006**, *830*, 6–12, <https://doi.org/10.1016/j.jchromb.2005.10.015>.
 21. Ocque, A.J.; Hagler, C.E.; Morse, G.D.; Letendre, S.L.; Ma, Q. Development and validation of an LC-MS/MS assay for tenofovir and tenofovir alafenamide in human plasma and cerebrospinal fluid. *J. Pharm. Biomed. Anal.* **2018**, *156*, 163–169, <https://doi.org/10.1016/j.jpba.2018.04.035>.

22. Anandgaonkar, V.; Gupta, A.; Kona, S.; Talluri, M.V.N.K. Isolation, LC–MS/MS and 2D-NMR characterization of alkaline degradants of tenofovir disoproxil fumarate. *J. Pharm. Biomed. Anal.* **2015**, *107*, 175–185, <https://doi.org/10.1016/j.jpba.2014.12.034>.
23. Rani, K. usha; Rao, P.V.; Rao, N.S. A new analytical method for determination of dolutegravir and rilpivirine in pharmaceutical formulations by RP-HPLC method. *Int. J. Res. Pharm. Chem. Anal.* **2019**, *1*, <https://doi.org/10.33974/ijrpca.v1i3.119>.
24. Wang, X.; Penchala, S.D.; Amara, A.; Else, L.; McClure, M.; Boffito, M. A Validated Method for Quantification of Dolutegravir Using Ultra Performance Liquid Chromatography Coupled With UV Detection. *Ther. Drug Monit.* **2016**, *38*.
25. Godela, R.; G, S. An effective stability indicating RP-HPLC method for simultaneous estimation of Dolutegravir and Lamivudine in bulk and their tablet dosage form. *Futur. J. Pharm. Sci.* **2020**, *6*, 9, <https://doi.org/10.1186/s43094-020-00026-0>.
26. MASUDA, J.; KATO, Y. Simultaneous Determination of Raltegravir, Dolutegravir, Elvitegravir, and Bictegravir in Human Plasma Using High-performance Liquid Chromatography-tandem Mass Spectrometry. *Showa Univ. J. Med. Sci.* **2020**, *32*, 91–102, <https://doi.org/10.15369/sujms.32.91>.
27. Godela, R.; Kammari, V.; Gummadi, S.; Beda, D. Concurrent estimation of lamivudine, tenofovir disoproxil fumarate, and efavirenz in blended mixture and triple combination tablet formulation by a new stability indicating RP-HPLC method. *Futur. J. Pharm. Sci.* **2021**, *7*, 94, <https://doi.org/10.1186/s43094-021-00249-9>.
28. Mutalik, S.P.; Mullick, P.; Pandey, A.; Kulkarni, S.S.; Mutalik, S. Box–Behnken design aided optimization and validation of developed reverse phase HPLC analytical method for simultaneous quantification of dolutegravir sodium and lamivudine co-loaded in nano-liposomes. *J. Sep. Sci.* **2021**, *n/a*, <https://doi.org/https://doi.org/10.1002/jssc.202100152>.
29. Krait, S.; Wissenbach, D.K.; Peters, F.T.; Scriba, G.K.E. HPLC-MS identification of acid degradation products of dolutegravir. *J. Pharm. Biomed. Anal.* **2021**, *197*, 113954, <https://doi.org/10.1016/j.jpba.2021.113954>.
30. Kumar, D.A.; Rao, G.S.; Rao, J.V.L.N.S. Simultaneous Determination of Lamivudine, Zidovudine and Abacavir in Tablet Dosage Forms by RP HPLC Method. *E-Journal Chem.* **2010**, *7*, 473798, <https://doi.org/10.1155/2010/473798>.
31. Singh, V.D.; Daharwal, S.J. Optimization of RP-HPLC Method for Simultaneous Estimation of Lamivudine and Raltegravir in Binary Mixture by Using Design of Experiment. *Eurasian J. Anal. Chem.* **2017**, *12*, 179–195, <https://doi.org/10.12973/ejac.2017.00162a>.
32. Bhavsar, D.S.; Patel, B.N.; Patel, C.N. RP-HPLC method for simultaneous estimation of tenofovir disoproxil fumarate, lamivudine, and efavirenz in combined tablet dosage form. *Pharm. Methods* **2012**, *3*, 73–78, <https://doi.org/10.4103/2229-4708.103876>.
33. Mallikarjuna Rao, N.; Gowri Sankar, D. Development and validation of stability-indicating HPLC method for simultaneous determination of Lamivudine, Tenofovir, and Dolutegravir in bulk and their tablet dosage form. *Futur. J. Pharm. Sci.* **2015**, *1*, 73–77, <https://doi.org/10.1016/j.fjps.2015.11.002>.
34. Anandakumar, K.; Abirami, G.; Murugan, S.; Ashok, B. RP-HPLC method for simultaneous estimation of lamivudine, tenofovir disoproxil fumarate and efavirenz in tablet formulation. *J. Anal. Chem.* **2013**, *68*, 815–821, <https://doi.org/10.1134/S1061934813090025>.
35. Karunakaran, A.; Kamarajan, K.; Thangarasu, V. A Validated RP - HPLC Method for Simultaneous Estimation of Lamivudine and Tenofovir Disoproxil Fumarate in Pure and in Tablet Dosage Form. *Eurasian J. Anal. Chem.* **2012**, *7*, 56–66.
36. Zhang, Z.; Wang, L.; Xu, X.; Dong, Y.; Zhang, L. Development of a validated HPLC method for the determination of tenofovir disoproxil fumarate using a green enrichment process. *Anal. Methods* **2015**, *7*, 6290–6298, <https://doi.org/10.1039/C5AY01036E>.
37. Pal, N.; Rao, A.S.; Ravikumar, P. Simultaneous HPLC method development and validation for estimation of Lamivudine, abacavir and dolutegravir in combined dosage form with their stability studies. *Asian J. Chem.* **2016**, *28*, 273.
38. Bhavar, G.B.; Pekamwar, S.S.; Aher, K.B.; Thorat, R.S.; Chaudhari, S.R. High-Performance Liquid Chromatographic and High-Performance Thin-Layer Chromatographic Method for Quantitative Estimation of Dolutegravir Sodium in Bulk Drug and Pharmaceutical Dosage Form. *Sci. Pharm.* **2016**, *84*.
39. Kumar, P.; Ashok, K. *Validated HPTLC Method for the Determination of Tenofovir as Bulk Drug and in Pharmaceutical Dosage Form*; **2011**;

40. Said, N.S.; Nasr, Z.A.; Abdel-Razeq, S.A. Assessment of the greenness of new stability indicating micellar UPLC and HPTLC methods for determination of tenofovir alafenamide in dosage forms. *J. Chromatogr. Sci.* **2021**, <https://doi.org/10.1093/chromsci/bmaa143>.
41. Mardia, R.B.; Suhagia, B.N.; Pasha, T.Y.; Chauhan, S.P.; Solanki, S. Development and validation of HPTLC method for estimation of tenofovir disoproxil fumarate in tablet dosage form. *J. Pharm. Sci. Biosci. Res.* **2012**, *2*, 73–76.
42. Gu, Y.; Zeng, B.; Sherma, J. Development of quantitative HPTLC methods for dolutegravir, lamivudine, and tenofovir disoproxil fumarate in a combination pharmaceutical product using a model process published earlier for transfer of minilab TLC screening methods to HPTLC-densitometry. *Acta Chromatogr. Acta Chromatogr.* **2020**, *32*, 199–202, <https://doi.org/10.1556/1326.2019.00689>.
43. Noorbasha, K.; Nurbasha, S. A new validated stability-indicating RP-HPLC method for simultaneous quantification of dolutegravir and lamivudine in bulk and pharmaceutical dosage form. *Futur. J. Pharm. Sci.* **2020**, *6*, 39, <https://doi.org/10.1186/s43094-020-00060-y>.
44. Naraparaju, S.; Thirumorthy, D.A.K.; Gurralla, S.; Jabeen, A.; Anumolu, P.K.D. Spectrophotometric quantification of dolutegravir based on redox reaction with Fe³⁺/1,10-phenanthroline. *Futur. J. Pharm. Sci.* **2020**, *6*, 107, <https://doi.org/10.1186/s43094-020-00121-2>.
45. Uslu, B.; Özkan, S.A. Determination of lamivudine and zidovudine in binary mixtures using first derivative spectrophotometric, first derivative of the ratio-spectra and high-performance liquid chromatography–UV methods. *Anal. Chim. Acta* **2002**, *466*, 175–185, [https://doi.org/10.1016/S0003-2670\(02\)00545-7](https://doi.org/10.1016/S0003-2670(02)00545-7).
46. Deepali, G.; Elvis, M. UV Spectrophotometric Method for Assay of the Anti-Retroviral Agent Lamivudine in Active Pharmaceutical Ingredient in its Tablet Formulation. *J. Young Pharm.* **2010**, *2*, 417–419, <https://doi.org/10.4103/0975-1483.71628>.
47. Venkatesan, S.; Kannappan, N. Simultaneous Spectrophotometric Method for Determination of Emtricitabine and Tenofovir Disoproxil Fumarate in Three-Component Tablet Formulation Containing Rilpivirine Hydrochloride. *Int. Sch. Res. Not.* **2014**, *2014*, 541727, <https://doi.org/10.1155/2014/541727>.
48. Nevase, P.A.; Nimje, H.M.; Oswal, R.J.; Antre, R.V.; Kshirsagar, S.S. UV Spectrophotometric Method for Estimation of Tenofovir Disoproxil Fumarate Tablet Dosage Form. *Int. J. Pharm. Res. Dev.* **2011**, *3*, 73–5.
49. ICH, I. **2005** November. Q2 (R1): Validation of analytical procedures: text and methodology. In International Conference on Harmonization, Geneva.
50. Guideline, I.H.T. **2003**. Stability Testing of New Drug Substances and Products. Q1A (R2), current step, 4, 1-24.
51. Blessy, M.; Patel, R.D.; Prajapati, P.N.; Agrawal, Y.K. Development of forced degradation and stability indicating studies of drugs—A review. *J. Pharm. Anal.* **2014**, *4*, 159–165, <https://doi.org/10.1016/j.jpha.2013.09.003>.