ijlpr 2023; doi 10.22376/ijlpr.2023.13.6.P200-P216

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Stability Indicating HPTLC for Quantitative Estimation of Dolutegravir, Lamivudine, and Tenofovir in Bulk and Dosage Form

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Abstract: Pharmaceutical companies are manufacturing multiple drug formulations to meet the market demand and treat unresponsive patients to single-drug formulations. Hence, an integral aim of the present study was to investigate the complete degradation behavior of the drug. It was done through a systematic investigation involving forced decomposition of the drug under various stress conditions. The study's main objective is to develop a Simple, accurate, and stability-indicating high-performance thin-layer chromatographic method for densitometric determination of Dolutegravir, Lamivudine, and Tenofovir in bulk and dosage form. The method development used TLC aluminum plates precoated with silica gel 60F 254 – (Merck, Germany) as the stationary phase. The solvent system consisting of Chloroform, Toluene, methanol, and ammonia (7:2:1:0.2*v/v/v/v*) was used for the development. Densitometric measurement of Dolutegravir, Lamivudine, and Tenofovir was performed at 260nm. The system gave very well-resolved spots for Dolutegravir, Lamivudine, and Tenofovir at Rf values of 0.29, 0.48, and 0.65, respectively. The calibration curve of the drug 400 -3200 ng/spot for DTG, 500 to 4000 ng/spot for LMV, and concentration range 1000-8000 ng/spot for TDF was observed. The correlation coefficient was 0.9995 for DTG, 0.9986 for LMV, and 0.9992 for TAF. The regression line equation for Dolutegravir, Lamivudine, and Tenofovir was found to be y = 1.0427x + 792.51, y = 0.8791x, + 1556.3 and y = 1.0217x + 99.194, respectively. The %RSD less than two indicates the method is precise and accurate. Stability testing aimsStability testing aims to prove how the drug quality varies with time under the influence of various natural factors such as humidity, light, and temperature, enabling storage conditions, retest periods, and determining the drug's shelf life. The degradation products were well resolved from the pure drug with significantly different retention factors in HPTLC. The method was proved to be a stability-indicating method and can be used in practice for bulk and dosage forms and to evaluate the shelf life.

Keywords: Dolutegravir, HPTLC, Lamivudine, Stability-indicating, Tenofovir, Validation

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Received On 28 April, 2023

Revised On 14 August, 2023

Accepted On 5 September, 2023

Published On 1 November, 2023

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Charushila Bhangale and Shivanand Hiremath , Stability Indicating HPTLC for Quantitative Estimation of Dolutegravir, Lamivudine, and Tenofovir in Bulk and Dosage Form.(2023).Int. J. Life Sci. Pharma Res.13(6), P200-P216 http://dx.doi.org/10.22376/ijlpr.2023.13.6.P200-P216

1. INTRODUCTION

Dolutegravir (DTV)(Figure 1) is an HIV integrase inhibitor. It binds to the active site and blocks the strand transfer step of retroviral DNA integration in the host cell.[1] Dolutegravir is an HIV-1 antiviral agent. It inhibits HIV integrase by binding to the active site and blocking the strand transfer step of retroviral DNA integration in the host cell. The strand transfer step is essential in the HIV replication cycle and inhibits viral activity. Lamivudine (LMV) (Figure 2) is a nucleoside reverse transcriptase inhibitor that blocks the HIV reverse transcriptase and hepatitis B virus polymerase.[2] Lamivudine is a synthetic nucleoside analog and is phosphorylated intracellularly to its active 5'-triphosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analog is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in DNA chain termination. Tenofovir disoproxil fumarate (TDF)(Figure 3) is a nucleotide analog reverse-transcriptase inhibitor. It selectively inhibits viral reverse transcriptase, a crucial enzyme in retroviruses such as HIV, while showing limited inhibition of human enzymes, such as DNA polymerases α, β, and mitochondrial DNA polymerase.[3,4]

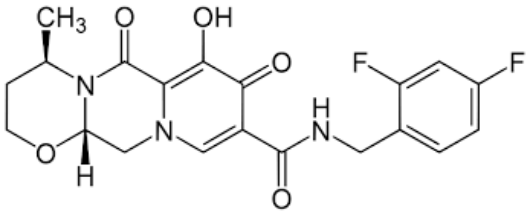


Fig 1: Chemical structure of dolutegravir

Figure 1 shows the structure of DTV. Chemically DTV is (4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a,-hexahydro-2H-pyridol[1',2',4,5] pyrazino [2,1-b][1,3] oxazine-9-carboxamide.

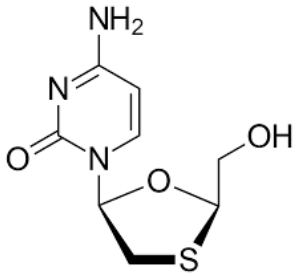


Fig 2: Chemical structure of Lamivudine

Figure 2 shows the structure of LMV.Chemically, LMV is 2',3'-dideoxy-3'-thiacytidine4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin -2-one.

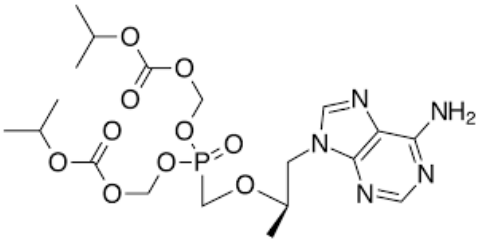


Fig 3: Chemical structure of Tenofovir Disoproxil Fumarate

Figure 3 shows the structure of TDF. Chemically, TDF is {9-[(R)-2-[[bis [[isopropoxycarbonyl] oxy] methoxy] phosphonyl] methoxy] propyl] adenine fumarate}. A combination of dolutegravir, Lamivudine, and Tenofovir disoproxil fumarate treats HIV Infection. It acts by preventing the action of reverse transcriptase enzyme. This enzyme is responsible for the production of HIV. In literature, UV spectrophotometric methods [5-14], high-performance Thin layer chromatography (HPTLC) [15-18] and high-performance liquid chromatography(HPLC) [19-32], and UPLC methods [33-34] are reported for the determination of DTV, LMV, and TDF in pharmaceutical formulation. Recently, HPTLC was used for qualitative and quantitative analysis and also for validation of not only plant extracts but also several pharmaceutical formulations [35-36]. The amount of solvent required in comparison to HPLC is much less. It reduces the time and cost of analysis and the possibility of pollution of the environment. HPTLC is a concept that includes a widely standardized methodology based on scientific facts and the use of validated methods for qualitative and quantitative analysis. HPTLC meets all quality requirements for today's analytical labs to increase the resolution and allow more accurate quantitative measurements[37]. Stability studies can guide formulation development and improve manufacturing and packaging processes. However, to our knowledge, no stability-indicating high-performance thin-layer chromatography (HPTLC)

determination of DTV, LMV, and TDF in combined dosage forms has been reported in the literature. The significance of Stress testing of the drug substance is to identify the likely degradation products, which can, in turn, help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability, indicating the power of the analytical procedures used. So, the study aims to investigate the complete degradation behavior of DTV, LMV, and TDF in bulk and pharmaceutical dosage forms by the stability-indicating method. Analytical test procedures for stability samples should be fully validated, and the assays should be stability indicating, according to ICH guidelines (Q1A). As a result, the study's main objective is to develop an accurate, specific, and reproducible method for determining DTV, LMV, and TDF in the presence of degradation products. The proposed method is rapid, simple, accurate, and reproducible. It can be successfully employed in the routine DTV, LMV, and TDF analysis in bulk samples and the pharmaceutical dosage form. The sample recoveries in the formulation were in good agreement with their label claims, and there was non-interference of formulation excipients in the estimation. Hence, this method can be easily and conveniently adopted for routine analysis of bulk drugs in pure form and its formulations.

2. METHODS

2.1 Reagents and Chemicals

Cipla Ltd., Mumbai, provided active pharmaceutical ingredient samples of Dolutegravir, Lamivudine, and Tenofovir Disoproxil Fumarate. The solvents and chemicals used were all of HPLC grade. The tablet pharmaceutical dosage of this drug combination was purchased from a local pharmacy.

2.2. Instrumentation

Aluminum plates precoated with silica gel 60F254 (20 × 10 cm, 250 µm thickness; Merck, Darmstadt, Germany) were used in the study. The equipment used was Linomat 5 applicator (Camag, Switzerland), twin trough chamber (20 × 10 cm; Camag, Switzerland), TLC scanner IV (Camag, Switzerland), win CATS version 1.4.6 software (Camag, Switzerland), Micro syringe (Linomat syringe 659.0014, Hamilton–Bonaduz Schweiz, Camag, Switzerland), UV cabinet (Camag, Switzerland).

2.3 Preparation of standard solutions

A standard stock solution of DTV, LMV, and TDF was prepared in which precisely 10 mg of the drug was weighed and transferred to a 100-ml volumetric flask separately and dissolved in 30 ml methanol. Methanol was used to dilute the solution to the desired concentration of 100 ug/ml. This stock solution was diluted appropriately with the same solvent to obtain a working standard solution for DTV, LMV, and TDF.

2.4 Chromatographic development and scanning

Using a Linomat V applicator and a micro syringe with a 100 L capacity, the samples were spotted as bands of 6 mm width on an aluminum silica gel-precoated plate 60 F254 (20 cm 10 cm) with a thickness of 250 m (E MERCK, Darmstadt, Germany) (Camag, Switzerland). The plates were activated at 110° C for 5 minutes after being prewashed with methanol before use in chromatography. The 150 nl/sec application rate was used, and the distance between the two bands was 15.4 mm. The slit size was maintained at 6 mm 0.45 mm. Chloroform, Toluene, methanol, and ammonia (7:2:1:0.2v/v/v/v) were utilized in the mobile phase of the development. The development used the ascending approach in a twin trough glass chamber. For the mobile phase, the ideal chamber saturation time was 25 minutes at a temperature of 250C and a relative humidity of 60% ± 5%. The TLC plates were taken out of the chamber and allowed to air dry after the chromatogram run, which lasted up to 8 cm. Densitometric scanning was carried out on a Camag TLC Scanner 3 equipped with winCATS software version 1.3.0 and scanning at 260 nm. Peak area and a linear regression equation were used for evaluation.

2.5. Application to dosage forms

Twenty tablets of BICTARVY containing 50 mg DTV, 300 mg LMV, and 300mg TDF were weighed, and their average weight was calculated for tablet dosage form analysis. The tablets were finely powdered, and the powder equivalent to one tablet was precisely weighed and transferred to a 100 ml volumetric flask containing 30 ml methanol. The solution was sonicated for 30 min before being diluted with methanol to mark. The final solution was filtered through a 0.45 m filter (Millifilter, MA). 0.4µL of the solution above was applied to a TLC plate. The development and scanning were carried out as described in section 2.2. The analysis was carried out six times. The Rf values for DTV, LMV, and TDF were discovered to be at Rf 0.29, 0.48, and 0.65, respectively. When scanned at 260 nm, the drug exhibits sharp and well-defined peaks. The amount of drug recovered was calculated using slope and intercept values from the calibration graph.

2.6. Method validation[38-41]

The ICH guidelines validated the method. The parameters ICH Q2A (R1) 2005 used for dosage form assays include linearity, precision (repeatability, intraday, interday), accuracy, specificity quantification limit, detection limit, robustness, and ruggedness.

2.6.1. Calibration curve and Linearity[41]

The method's linearity was determined by applying eight different concentrations of the drug to the TLC plates three times each. The plate was then developed with the previously described mobile phase, and a calibration curve was created by plotting the peak areas against the corresponding concentrations.

2.6.2. Precision[41]

The repeatability, intra-day, and inter-day precision of the method were evaluated. The repeatability of the sample solution was determined by spotting the drug solution six times on a TLC at 500 ng/spot for DTV, 3000 ng/spot for LMV, and 3000 ng/spot for TDF, followed by plate development and scanning. The intra-day precision was determined by analyzing standard drug solutions three times on the same day within the calibration range of individual drugs. Inter-day precision was determined by analyzing drug solutions over a week on three days within the calibration range.

2.6.3. *Accuracy*[41]

Recovery studies by standard addition were used to test the

accuracy of the proposed method. The formulation solution (DTV, LMV, and TDF combination tablets) was supplemented with known amounts of DTV, LMV, and TDF standard powder corresponding to 50, 100, and 150 percent of the label claim, and the absolute recovery was calculated by comparing the peak areas obtained from standard DTV, LMV, and TDF solution with the peak areas of samples of different concentrations.

2.6.4. *Specificity*[41]

The method's specificity was determined by analyzing standard drug and sample. As shown in, the mobile phase resolved all of the drugs very efficiently at various Rf values of DTV(Rf=0.29), LMV(Rf=0.48) and TDF(Rf=0.65) (Figure 4.).

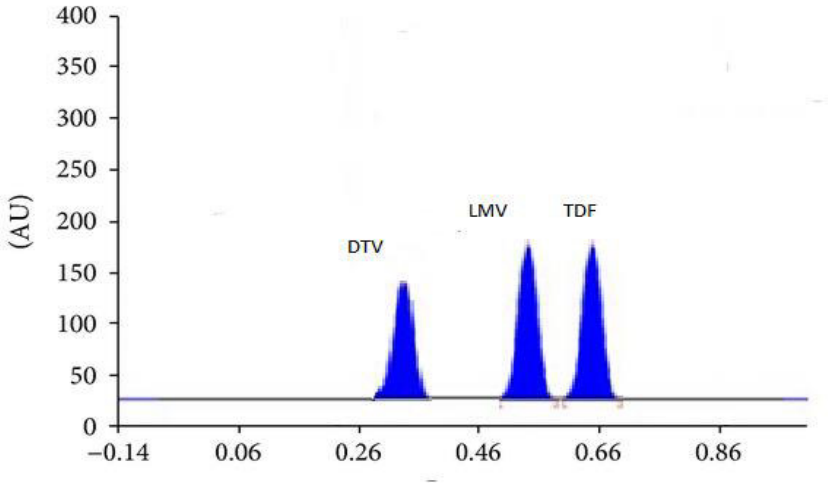


Fig 4: HPTLC chromatogram of DTV(Rf=0.29), LMV(Rf=0.48) and TDF(Rf=0.65)

By comparing the Rf and spectra of the spot to that of the standard, the spot for DTV, LMV, and TDF was confirmed. Peak purity of DTV, LMV, and TDF was determined by comparing drug and sample spectra at the peak start (S), peak apex (M), and peak end (E) positions of the spot.

2.6.5. *Limit of detection and limit of quantification*[41]

Concentrations in the calibration curve's lower linear range were used to determine the detection and quantification limits. The amount of drugs used versus the average response (peak area) was plotted, and the regression equation was determined. Response standard deviations (S.D.) were computed. The average of standard deviations was calculated from this data (A.S.D.). LOD was calculated using the formula (3.3x A.S.D.)/b, and LOQ was calculated using the formula (10x A.S.D.)/b, where "b" corresponds to the slope obtained in the method's linearity study.

2.6.6. *Robustness*[41]

The robustness of the system was evaluated by comparing the results obtained for various parameters such as change in mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography, and time from chromatography to spotting. On a TLC plate, the solution was applied in six replicates at concentrations of 500 ng/spot for DTV, 3000 ng/spot for LMV, and 3000 ng/spot for TDF. Seven parameters were studied in this study, and their effects on the results were examined.

2.6.7. *Ruggedness*

The proposed method was evaluated by two different analysts in the ruggedness study.

2.6.8. *System suitability*

The suitability of the system was evaluated in order to ensure the chromatographic system's quality performance. Six replicates of the solution were applied in six replicates at concentrations of 500 ng/spot for DTV, 3000 ng/spot for LMV, and 3000 ng/spot for TDF and parameters such as capacity factor (K), injection repeatability tailing factor (T), selectivity and resolution (Rs) for the main peak and its degradation product were tested. The system suitability parameters were revealed to be within acceptable limits

2.6.9. *Forced degradation*[42-43] *of DTV, LMV, and TDF*

2.6.10. *Acid and base induced degradation*

The 10 mg of DTV, LMV, and TDF were transferred to a 10 ml volumetric flask. It was dissolved in a methanolic solution containing 1 M HCl and 1 M NaOH respectively. In order to rule out any potential degradation caused by light, these solutions were maintained at room temperature in the dark for 8 hours. The 1 ml of the aforementioned solutions were taken, neutralized, and diluted with methanol to a final concentration of 10 ml. The 10 l were applied in triplicates (i.e., 1000 ng/spot) to TLC plates. The chromatogram was performed in accordance with section 2.2.

2.6.11. *Hydrogen peroxide – induced degradation*

The 10 mg of DTV, LMV, and TDF were weighed and transferred to 10 ml volumetric flask and 10%, v/v methanolic solution of hydrogen peroxide was added. The volume was made upto the mark. The solutions were maintained at room temperature for 8 hours in the dark to prevent any potential degradation from light. The 1 ml of above solution were taken and diluted up to 10 ml with methanol. The 10 µl were applied

on TLC plates in triplicates (i.e. 1000 ng/spot). The chromatogram was run as described in section 2.2.

2.6.12. Dry heat degradation products

DTV, LMV, and TDF were stored separately in petri dishes at 55^0 C for 3 hours in an oven. DTV 10 mg, LMV 10 mg, and TDF 10 mg were placed in a volumetric flask with a volume of 10 ml. the volume was made upto the mark with Methanol. The 10 µl were applied in triplicate on TLC plates (1000 ng/spot) and the chromatogram was run as described in section 2.2.

2.6.13. *Light heat degradation products*

10 mg of DTV, LMV, and TDF were weighed and transferred to a volumetric flask of 10 ml. The drugs were dissolved in 10 ml of methanol separately. The solutions were exposed to sunlight for 8 hours. 1 ml of each of the above solutions was taken and diluted with methanol to a volume of 10 mL. The solutions obtained were applied in triplicate on a TLC plate with a concentration of 1000 ng / spot. The chromatograms were obtained in the manner described in section 2.2.

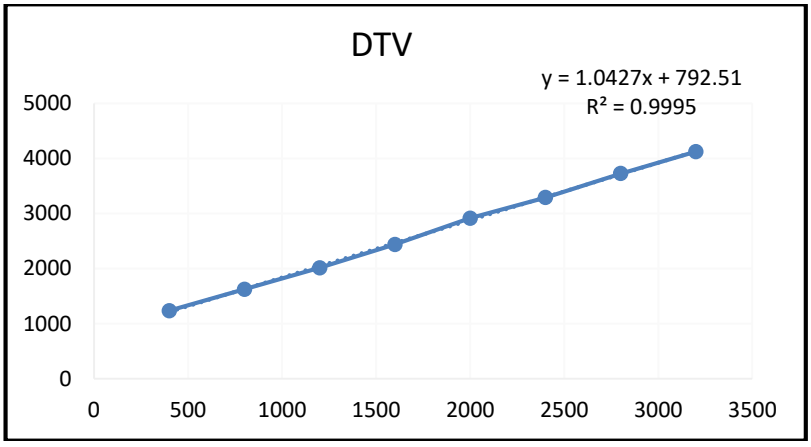
3. RESULTS

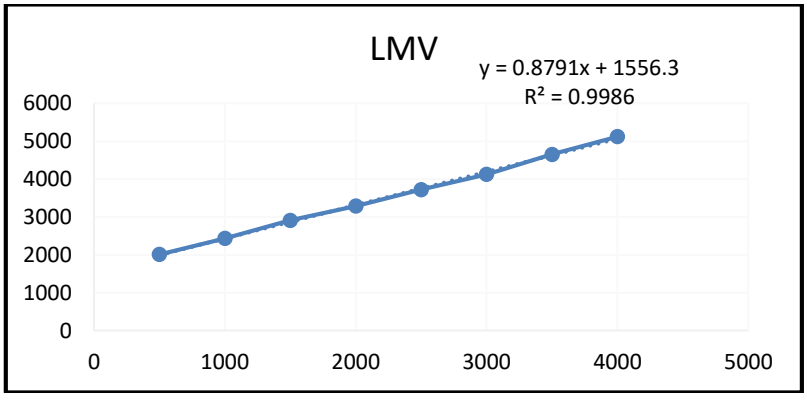
3.1. Optimization of HPTLC method

Initially, DTV, LMV, and TDF drugs were tried together with toluene: methanol in the ratio of 4: 2 (v/v/v). The spots did not fully develop, and tailing was seen. Then, toluene: ethyl acetate: methanol in the ratio of 6: 4: 3 (v/v/v) was tried. Spots that had developed were scattered. Finally, a mobile phase with the composition (4.0:3.0:3.0:0.2 v/v/v) of toluene, ethyl acetate, methanol, and acetic acid produced prominent peaks. The peaks on the plate were determined to be symmetrical in form and no tailing was seen when it was examined at 260 nm. The chamber was saturated for 25 minutes. To get clearly defined spots, the plates were activated at 110° C for 5 minutes.

3.2. Linearity

The linearity was observed for DTV concentrations ranging from 400-3200 ng/spot, LMV concentrations ranging from 500-4000 ng/spot, and TDF concentrations ranging from 1000 to 8000 ng/spot (Figure 5).





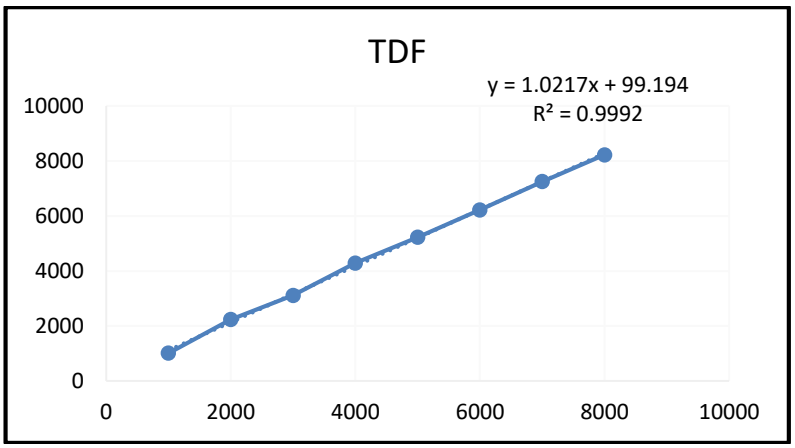


Fig 5: Calibration curve of DTV, LMV and TDF

Figure 5 illustrates data from the regression line which provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, and the slope of the regression line was determined by visual examination of a plot of signals as a function of analyte concentration of content. The coefficients of correlation for DTV, LMV, and TDF were found to be 0.996, 0.9986, and 0.9992, respectively. For DTV, LMV, and TDF regression line equations were found to be y = 1.0427x + 792.51, y = 0.8791x + 1556.3 and y = 1.0217x + 99.194 respectively.

3.3. Precision

The repeatability and intermediate precision (intraday and interday) were expressed as a percentage of RSD. Because of the low percentage RSD value, the proposed method provides an acceptable intraday and interday variation. The results are as shown in Table 1.

Table 1. Precision Study

Drugs	Concentration (ng/spot)	Intra-day		Inter-day	
		% Amount found ± SD	(% RSD)	% Amount found ± SD	(% RSD)
DTV	400	98.56±1.26	0.56	98.56±1.36	0.75
	500	99.56±1.45	1.42	99.56±1.25	1.12
	600	99.45±1.23	0.28	99.45±1.23	0.62
LMV	2400	99.49±1.12	0.78	99.49±1.02	0.48
	3000	99.67±1.14	1.32	99.67±1.04	1.52
	3600	99.23±1.27	0.36	99.23±1.27	0.63
TDF	2400	98.98±1.42	0.65	98.98±1.32	0.55
	3000	99.16±1.63	0.54	99.16±1.33	0.48
	3600	99.67±1.49	1.05	99.67±1.59	1.56

Table 1 shows the results of intraday and interday precision. The precision of an analytical procedure was expressed as the standard deviation and relative standard deviation of a series of measurements. The % RSD less than two indicated that the method is precise.

3.4. Accuracy

Recovery experiments were performed at three different levels i.e. 50, 100 and 150 %. To the preanalysed sample solutions, a known amount of standard drug solutions of DTV, LMV and TDF were over spotted at three different levels. The chromatogram was developed and scanned The low percentage RSD value indicated that no interference was caused by the excipients used in the formulation. The results of % recovery are shown in Table 2.

Table 2: Accuracy study

Component	Label claim (mg/tablet)	(%)Amount of standard drug added	% Drug recovered *	% RSD
DTV	50	50	100.23	0.12
		100	100.21	1.04
		150	100.56	1.09
LMV	300	50	100.56	0.42
		100	100.45	0.75
		150	100.42	1.15
TDF	300	50	100.56	0.89
		100	99.89	1.23
		150	100.91	0.53

Table 2 describes the accuracy of the method. This test evaluated the specificity of the method in the presence of the excipients under the chromatographic conditions used for the analysis of the drug product. The accuracy was determined at three different level i.e 50%,100% and 150% and the % RSD calculated. The value less than two indicated that the method is accurate.

3.5. Sensitivity

Sensitivity of the drugs was analyzed by Limit of Detection (LOD) and Limit of Quantitation (LOQ). The limits of detection and quantitation were found to be 87.34 and 476.19 for DTV, 51.24 and 565.49 for LMV and 55.34 and 768.41 for TDF, indicating that the method has good sensitivity.

3.6. Application to dosage forms

The proposed technique was applied to examine the commercial formulation Bictarvy. The chromatogram of the tablet sample revealed peaks at Rf values of 0.29, 0.48, and 0.65 for DTV, LMV and TDF, respectively, demonstrating that the excipients used in the tablet formulation do not interfere with the results. Peak areas of the sample and the standard were compared to determine the content of DTV, LMV, and TDF.

3.7. Robustness

The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low %R.S.D. values indicated method is robust. Results are shown in Table 3.

Table 3: Robustness study

Condition	Parameter	DTV[%RSD]	LMV[%RSD]	TDF[%RSD]
Mobile phase Composition	+0.1 ml	1.15	1.28	1.24
	-0.1 ml	1.47	1.41	1.26
Amount of mobile phase	+5%	1.22	1.42	1.56
	-5%	1.49	1.63	1.59
Duration of saturation	30 min	0.84	1.25	1.23
Development distance	75mm	1.22	1.57	1.71
	80mm	1.26	1.56	1.56
Relative humidity	50^0C	0.91	0.83	0.78
	60^0C	1.15	1.13	1.18
Activation of pre washed TLC plates	8 min	0.75	0.89	0.86
	10 min	0.88	0.91	0.95
Time from spotting to development	+10 min	0.88	1.14	0.96
	-10 min	1.21	1.29	1.56
Time for development To scanning	+10 min	1.33	1.55	1.22
	-10 min	1.49	1.68	1.32

Table 3 illustrates the result of robustness study which provides an indication of its reliability during normal usage. Robustness of the system was assessed by comparing the results of seven different parameter and % RSD less than two indicates the method is robust.

3.6. Ruggedness

Two different analysts evaluated the robustness of the proposed method. The results for DTV, LMV and TDF were 99.81 %, 99.85 %, 99.77 %, 99.75 %, and 99.23, 99.45 %, respectively.

3.7. System suitability

Chromatographic condition such as Retardation factor (Rf), Capacity Factor (K), selectivity, Tailing Factor(T) and resolution were tested and results are given in Table 4.

Table 4: System suitability parameter

System Suitability Parameters	Observations			Reference value
	DTV	LMV	TDF	
Retardation factor (Rf)	0.29	0.48	0.65	--
Capacity factor (K ')	0.69	0.98	1.23	1-10
Selectivity	1.21	1.47	1.63	>1
Tailing factor (T)	1.05	1	1.02	1 for symmetric peak
Resolution	3.45	3.69	4.02	≥2

Table 4 shows the result of System suitabilty Parameters such as plate count, tailing factors, resolution, and reproducibility (%RSD) are determined and compared against the specifications set for the method. As all values lies in the range of reference value the method is said to be specific.

3.8. Stability- indicating property

The samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure DTV, LMV and TDF and some additional peaks at different Rf values as shown in Figure 6, Figure 7 and Figure 8.

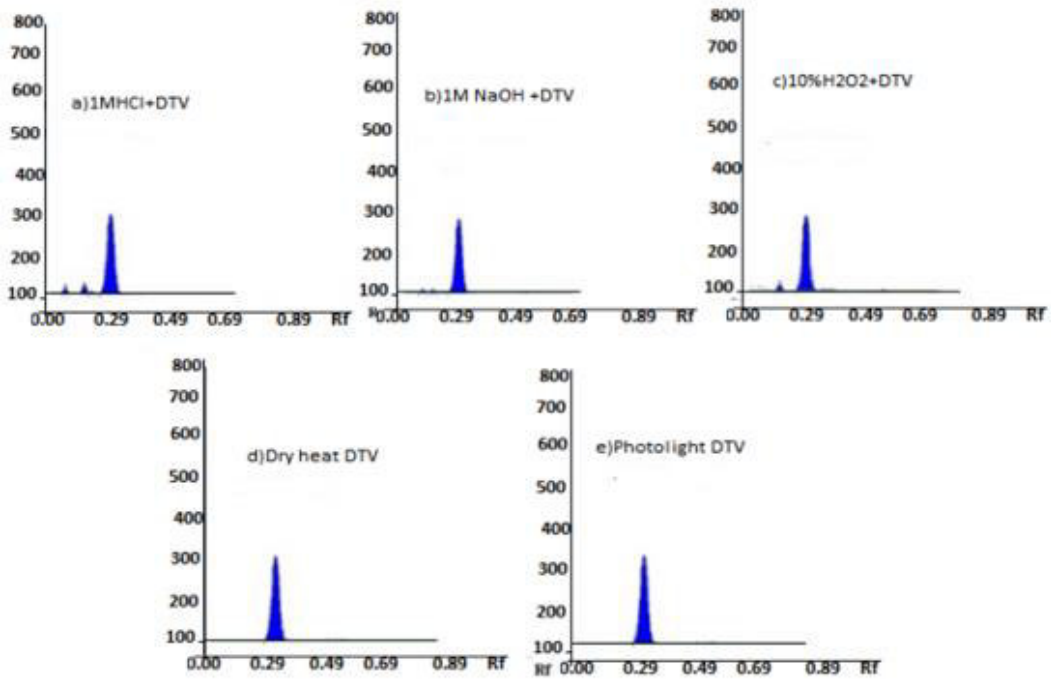


Fig 6: Forced degradation of DTV by HPTLC

Figure 6 illustrates the force degradation of TDF**.** The spots of degraded product were well resolved from the drug spot. In acid degradation additional peak obtained at Rf value of 0.11,0.21,0.23. In base degradation additional peak obtained at Rf value of 0.15,0.21. For oxidation condition the additional peak obtained at Rf value of 0.21. No degradation was observed in dry heat and photo light which indicates the drug is stable in dry heat and photolight.

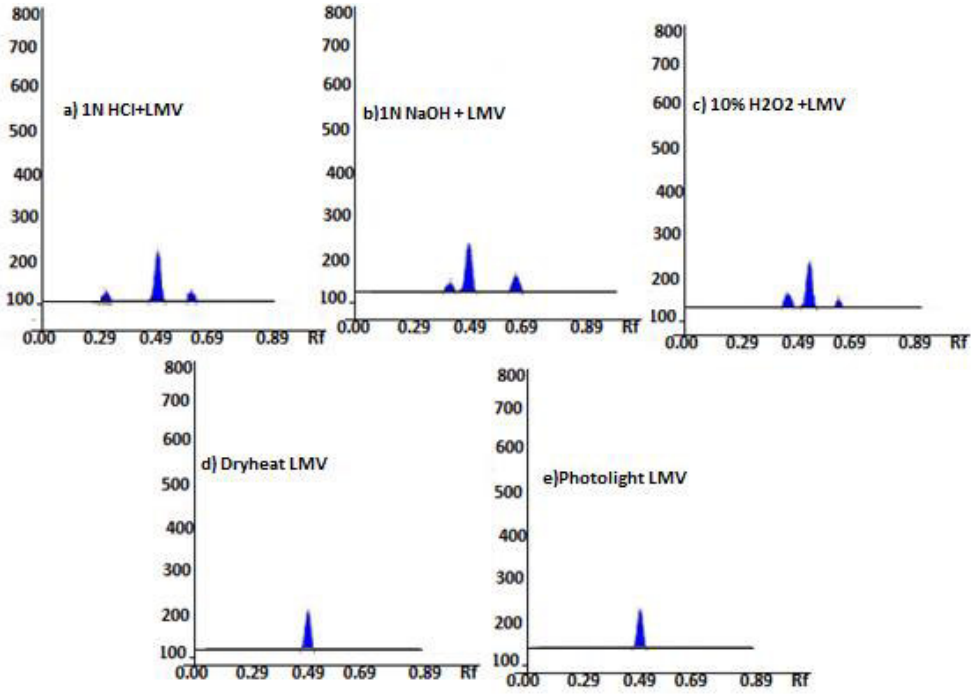


Fig 7 :Forced degradation of LMV by HPTLC

Figure 7 illustrates the force degradation of TDF**.** The spots of degraded product were well resolved from the drug spot. In acid degradation additional peak obtained at Rf value of 0.21,0.61. In base degradation additional peak obtained at Rf value of 0.38,0.65. For oxidation condition the additional peak obtained at Rf value of 0.32,0.54. No degradation was observed in dry heat and photo light which indicates the drug is stable in dry heat and photolight.

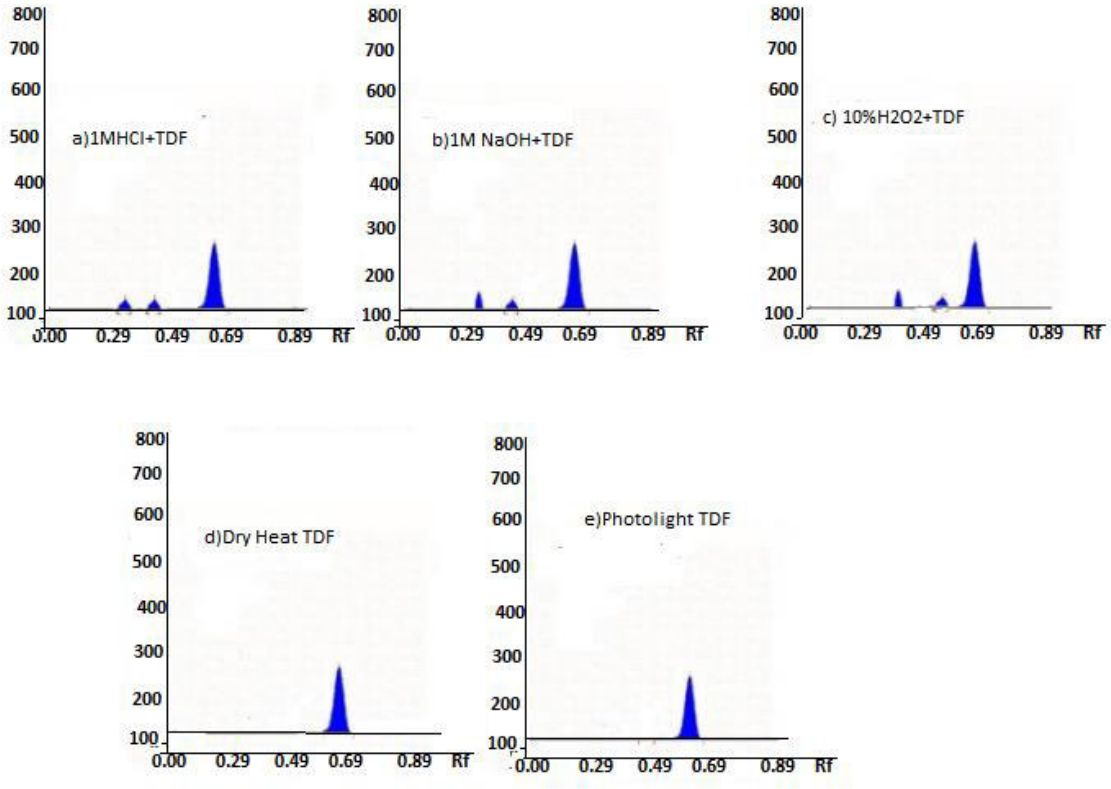


Fig 8: Forced degradation of TDF by HPTLC

Figure 8 illustrates the force degradation of TDF**.** The spots of degraded product were well resolved from the drug spot. In acid degradation additional peak obtained at Rf value of 0.21,0.35. In base degradation additional peak obtained at Rf value of 0.22,0.34. For oxidation condition the additional peak obtained at Rf value of 0.29,0.50. No degradation was observed in dry heat and photo light which indicates the drug is stable in dry heat and photolight. The spots of degraded product were well resolved from the drug spot. The number of degradation product with their Rf values, content of DTV, LMV and TDF remained, and percentage recovery were calculated and listed in Table 5.

Table 5: Stability study

Sample Exposure condition	Number of degradation products (Rf values)			Drug remained (600 ng/spot)			Recovery (%)		
	DTV	LMV	TDF	DTV	LMV	TDF	DTV	LMV	TDF
1M HCl, 8h, RT	0.11,0.21,0.23	0.21,0.61	0.21,0.35	523	514	541	87.16	85.66	90.16
1M NaOH, 8h, RT	0.15,0.21	0.38,0.65	0.22,0.34	517	529	526	86.16	88.16	87.66
10%H_2O_2, 8h, RT	0.21	0.32,0.54	0.29,0.50	549	542	559	91.5	90.33	93.16
Heat, 3H, 55^0 C	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation
Photo, 8 h	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation	No degradation

Table 5 shows the results of stability study in which Stability of DTV, LMV and TDF was determined by forced degradation study. The chromatograms of samples degraded with acid, base, hydrogen peroxide, dry heat and light showed well separated spots of pure DTV, LMV and TDF as well as some additional peaks at different R_f values. The spots of degraded product were well resolved from the drug spot. No degradation was observed in dry heat and photo light which indicates the drug is stable in dry heat and photo light. The degradation pathway of DTV, LMV and TDF are shown in Figure 9, Figure 10 and Figure 11.

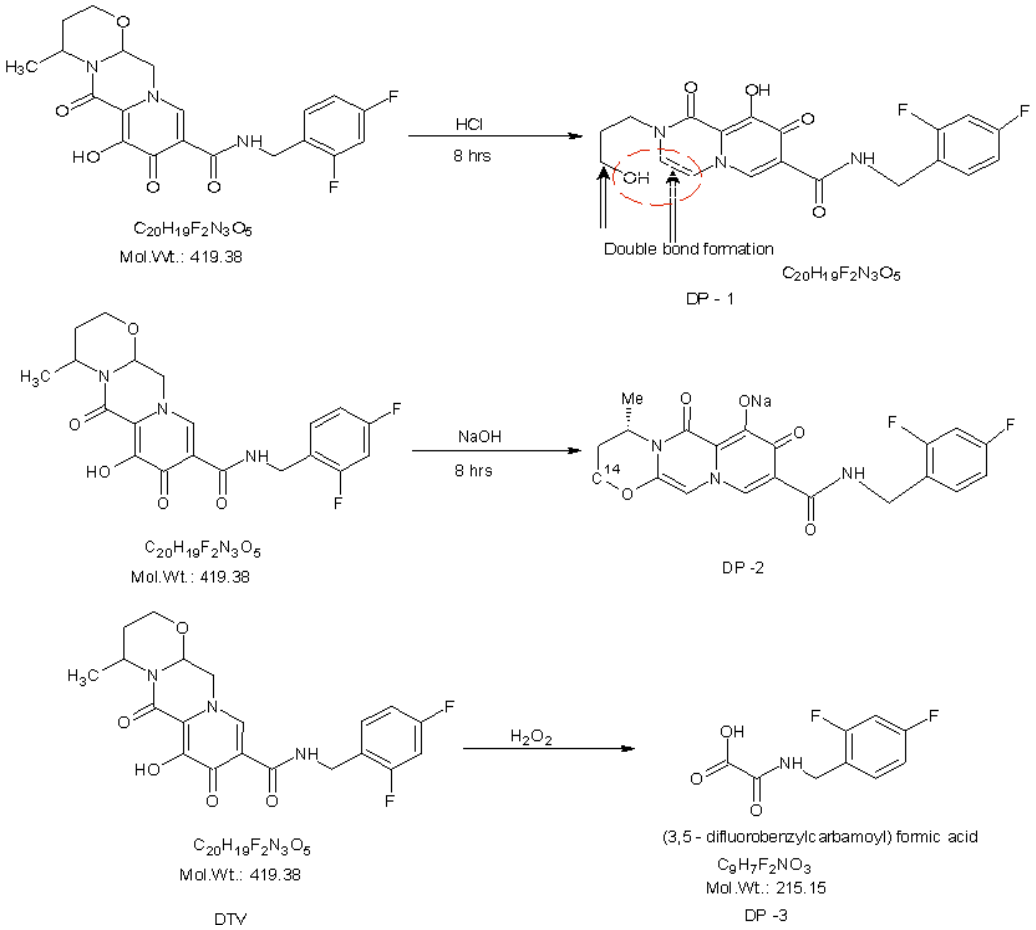


Fig 9: Degradation pathway of DTV

Figure 9 shows the degradation pathway of DTV in acid, base, and oxidation conditions. The DTV was found to be prone to HCl, NaOH, and H_2O_2 and showed degradation. In acidic conditions, it leads to the formation of the double bond as a degradation product. In the basic conditions, it leads to the form of sodium salt. In oxidation conditions, it forms an acid compound.

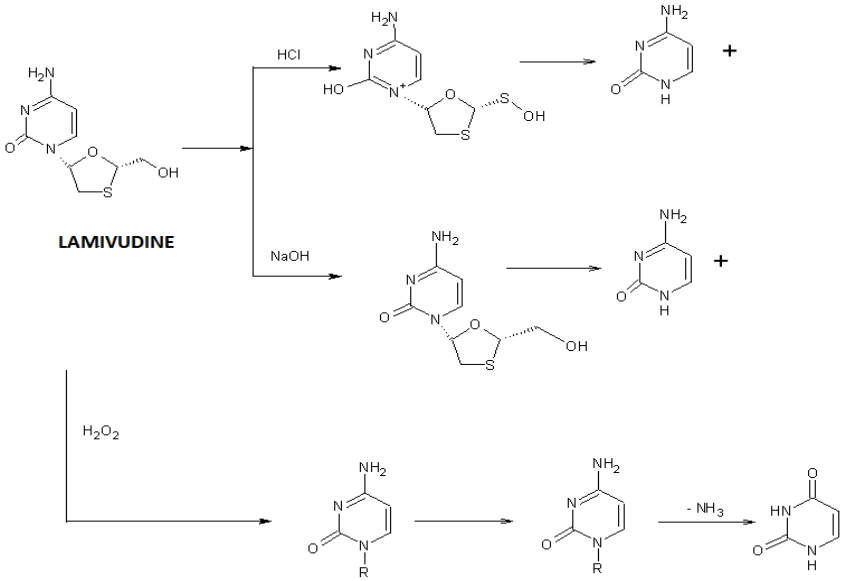


Fig 10: Degradation pathway of LMV

Figure 10 shows the degradation pathway of LMV. The drug showed acid, alkali, and oxidation instability while remaining stable in dry heat and photolytic conditions. The hydroxyl radical was generated when Lamivudine was treated with acid and peroxide.

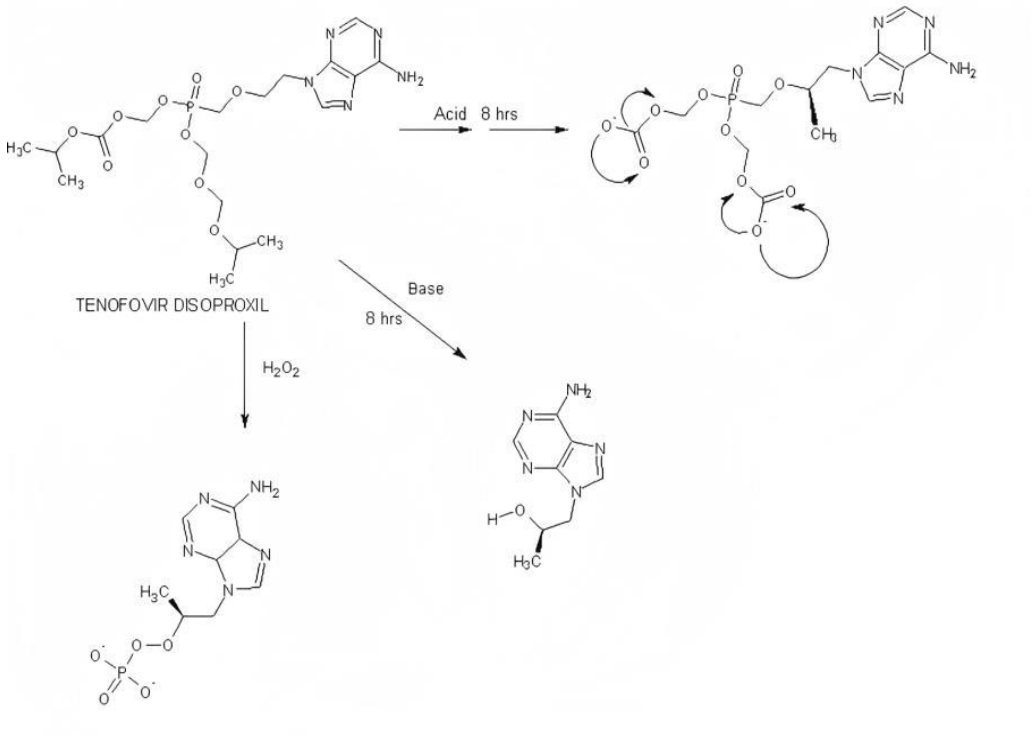


Fig 11: Degradation pathway of TDF

Figure 11 shows the degradation pathway of TDF. It shows degradation under acidic, basic, and oxidation conditions and is found stable under dry heat and photolytic conditions. A crystalline fumarate salt of tenofovir disoproxil was formed under hydrolytic conditions. Tenofovir disoproxil fumarate is hydrolyzed to tenofovir, which is then phosphorylated to tenofovir diphosphate. The oxidative degradation of drug substances involves an electron transfer mechanism to form reactive anions and cations. The degradation products were confirmed with IR. The degraded sample spectra of DTV, LMV, and TDF are shown in Figure 12, Figure 13, and Figure 14.

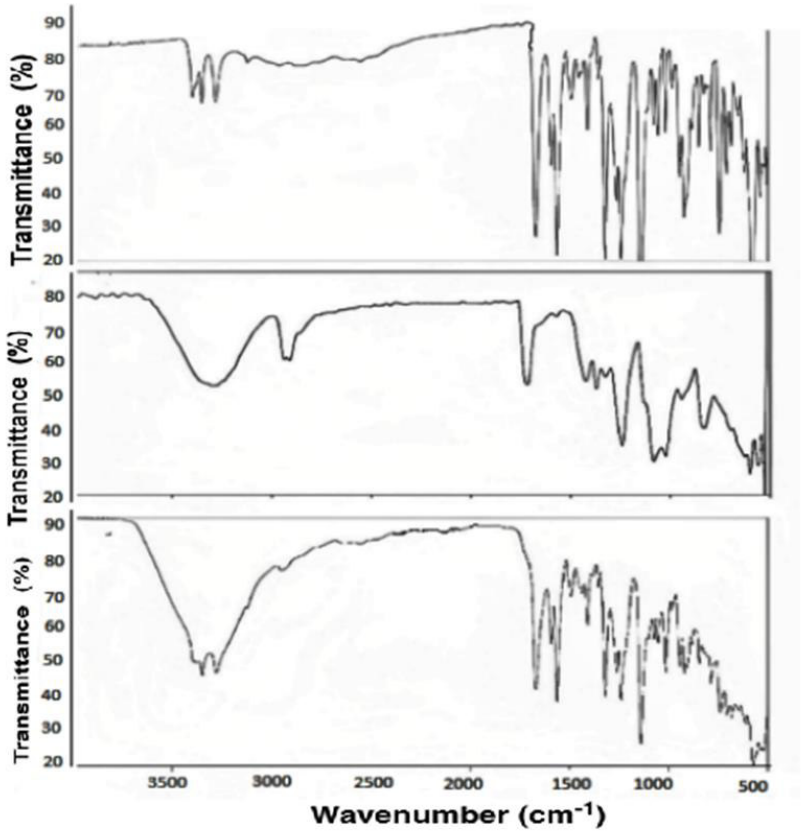


Fig 12: Spectra of DTV (a) its acidic degradation, (b) Its alkaline degradation, and (c) Its oxidation degradation

Figure 12 shows the degradation of DTV. The IR spectra of DTV acidic degradation showed the disappearance of the band of the C=O group of ester moiety at 1740 cm−1 and the appearance of the broad band of the (OH) group at 3400 cm−1.In IR spectra

of alkaline degradation, it exhibits the appearance of a broadband of the (OH) group at 3400 cm−1, and while in oxidation degradation, it shows the appearance of a broad band of the (OH) group at 3400 cm−1.

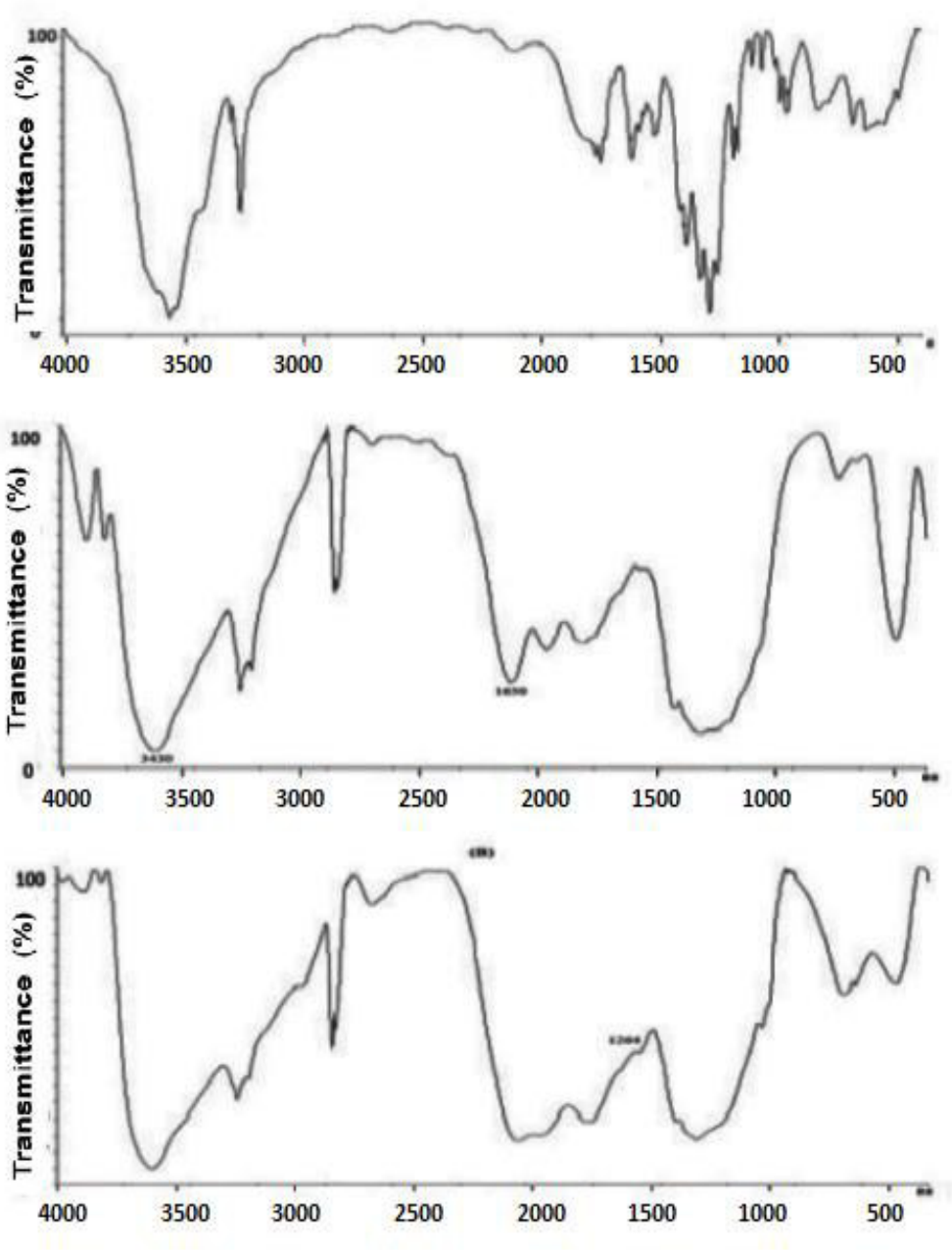


Fig 13:IR Spectra of LMV (a) its acidic degradate (b) Its alkaline degradate and (c) Its oxidation degradate

Figure 13 shows the degradation of LMV. IR spectra of LMV acidic degradation showed a broad band of the (OH) group at 3430 cm−1.In IR spectra of alkaline degradation, it exhibits the appearance of a broad band of phosphoric (OH) group at 3430 cm−1 and the disappearance of the CH aromatic band at 1650 cm−1, while in oxidation degradation, it shows the appearance of a broad band of (OH) group at 3430 cm−1and appearance of a broadband at 1264 cm−1due to CH bending.

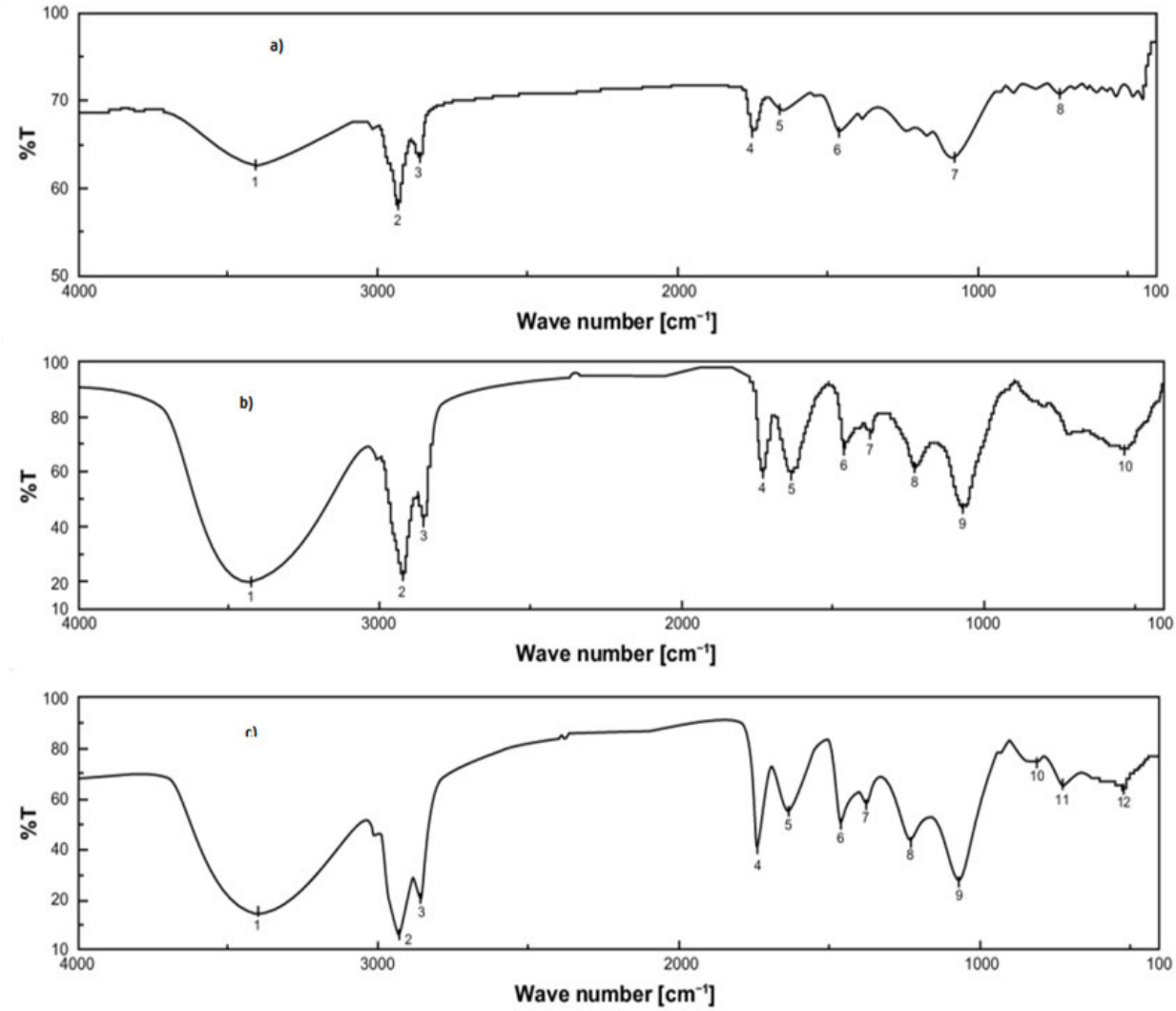


Fig 14:IR Spectra of TDF (a) its acidic degradation, (b) Its alkaline degradation, and (c) Its oxidation degradation

Figure 14 shows the degradation of TDF. IR spectra of acidic degradation showed the disappearance of the band of the C=O group of ester moiety at 1740 cm−1 and the appearance of a broad band of the phosphoric (OH) group at 3400 cm−1.In IR spectra of alkaline degradation, it exhibits the appearance of a broadband phosphoric (OH) group at 3415 cm−1 and the disappearance of the CH aromatic band at 3179 cm−1. In contrast, in oxidation degradation, it shows an appearance of a broad band of phosphoric (OH) group at 3400 cm−1.

3. DISCUSSION

Stability- indicating high-performance thin-layer chromatography method was developed to simultaneously estimate DTV, LMV, and TDF in its Pharmaceutical dosage form. The method employed the stationary phase of TLC aluminum plates precoated with silica gel 60F 254. The solvent system consisted of Chloroform, Toluene, methanol, and ammonia (7:2:1:0.2*v*/*v*/*v*/*v*). Densitometric measurement of Dolutegravir, Lamivudine, and Tenofovir was performed in the absorbance mode at 260nm. In the reported HPTLC method, mobile phase ethyl acetate–methanol–acetone–concentrated ammonium hydroxide (30:7:3:1) and densitometric scanning at 254 nm. D was analyzed on a second plate by scanning at 366 nm after chromatography with the chloroform–methanol–formic acid (32:8:2) mobile phase.[44] In the reported HPLC stability method, the Retention time of Dolutegravir, Lamivudine, and Tenofovir Disoproxil Fumarate was 30min,11.5min, and 26.5min.[44] The retention time was higher and required a large mobile phase. In our developed method, the mobile phase required was less, and the system was found to give very well-resolved spots for Dolutegravir, Lamivudine, and Tenofovir at Rf values of 0.29, 0.48, and 0.65, respectively, so the method was found to be cost-effective. The suitability of this HPTLC method for the quantitative determination of the compounds is proved by validation by ICH guidelines. The suitability of this HPTLC method for quantitative determination of the compounds is proved by validation by the requirements of ICH guidelines. As per ICH Q2 (R1) guidelines, the method validation parameters like linearity, precision, accuracy, the limit of detection, and the limit of quantitation were performed. Statistical analysis proves the method is repeatable, selective, and accurate for estimating said drug. The proposed method was applied for the pharmaceutical formulation, and the % label claim for DTV, LMV, and TDF was found to be 99.45, 100.19, and 100.61, respectively. The amount of drugs estimated by the proposed method agreed with the label claim. The developed method was found to be precise as the %RSD values for intra-day and inter-day were found to be less than 2%. The repeatability of the sample solution was determined by spotting the drug solution six times on a TLC at 500 ng/spot for DTV, 3000 ng/spot for LMV, and 3000 ng/spot for TDF. The % R.S.D. for peak area values of DTV, LMV, and TDF was found to be 1.41, 0.97, and 1.05, respectively. The accuracy of the method was

determined by % recovery. Good drug recoveries were obtained at each added concentration, indicating that the developed method was accurate. Robustness was studied in six replications at the concentration level of 500 ng/spot for DTV, 3000 ng/spot for LMV, and 3000 ng/spot for TDF. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography, and chromatography to spotting) were studied, and the effects on the results were examined. LOD and LOQ were 87.34ng and 476.19ng for DTV, 51.24ng and 565.49ng for LMV, and 55.34ng and 768.41 ng for TDF. It indicates the adequate sensitivity of the method. Two different analysts evaluated the ruggedness of the proposed method. DTV, LMV, and TDF results were 99.81 %, 99.85 %, 99.77 %, 99.75 %, and 99.23, 99.45 %, respectively. The summary of validation parameters[46-48] is summarized in Table 6.

Table 6:Validation Parameter

Parameter	DTV	LMV	TDF
Linearity range [ug/ml]	400-3200ng/spot	500-4000ng/spot	1000-8000ng/spot
Regression equation [Y = mX + C]	y = 1.0427x + 792.51	y = 0.8791x + 1556.3	y = 1.0217x + 99.194
Correlation coefficient	0.9995	0.9986	0.9992
Limit of detection [µg]	87.34	51.24	55.34
Limit of quantitation [µg]	476.19	565.49	768.41
Application to dosage form	99.45	100.19	100.61
% Recovery [n = 3]	98.54-99.12	98.76-99.15	99.11-99.54
Ruggedness [%]			
Analyst I [n = 3]	99.85	99.77	99.23
Analyst II [n = 3]	99.81	99.75	99.45
Precision [% RSD]			
Repeatability [n = 6]	1.41	0.97	1.05
Inter-day [n = 3]	0.28-1.42	0.36-1.32	0.54-1.05
Intra-day [n = 3]	0.62-1.12	0.48-1.52	0.48-1.56
Robustness	Robust	Robust	Robust

Table 6 shows that the % RSD values less than 2 indicate the methods are accurate and precise. Ruggedness of the proposed methods was studied with the help of two analysts. Robustness of the methods was studied by studying different parameter. The results did not show any statistical difference between operators and environmental conditions, suggesting that methods developed were rugged and robust. The stability indicating properties established following the recommendations of ICH guidelines also indicated that the drugs could be evaluated in presence of their degradation products[49-50]. The chromatogram of samples degraded with acid, base, hydrogen peroxide showed well separated spots of pure DTV, LMV and TDF as well as some additional peaks at different Rf values. The number of degradation product with their Rf values, content of DTV, LMV and TDF remained, and percentage recovery [50]. The degradant formed was not interfering with estimation of the drugs makes the method more selective for the purpose intended[51-52]. The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure DTV, LMV and TDF as well as some additional peaks at different R_f values. The spots of degraded product were well resolved from the drug spot. The chromatogram of DTV showed additional peak at R_f value 0.11,0.21,0.23 in the acid degraded samples and 0.15,0.21 in base degraded samples. The sample degraded with hydrogen peroxide showed additional peak at R_f value of 0.21 The chromatograms of LMV showed additional peak at R_f value 0.21,0.61 in the acid degraded sample and 0.38,0.65 in base degraded sample. The sample degraded with hydrogen peroxide showed additional peak at R_f value of .32,0.54. The chromatograms of TDF showed additional peak at R_f value 0.21,0.35 in the acid degraded sample and 0.22,0.34 in base degraded sample. The sample degraded with hydrogen peroxide showed additional peak at R_f value of 0.29,0.50. The spots of degraded product were well resolved from the drug spot. The drugs were found to be stable in dry heat and light degradation. The method is successively applied to pharmaceutical formulation; No chromatographic interferences from the tablet excipients were found. Structural characterization of degradation products is necessary for those impurities formed during formal shelf-life stability studies and above the qualification threshold limit. Forced degradation studies provide knowledge about possible degradation pathways and degradation products of the active ingredients and help elucidate the structure of the degradants[53-55]. Degradation products generated from forced degradation studies are potential degradation products that may or may not be formed under relevant storage conditions but they assist in the developing stability indicating method.[56-57]

4. CONCLUSION

The suggested HPTLC method offers a straightforward, precise, and repeatable quantitative study for the determination of DTV, LMV and TDF in bulk drugs. ICH guidelines were followed in the method's validation. Forced degradation studies of drug substances and drug products are essential to help develop and demonstrate specificity of stability-indicating methods and to determine the degradation pathways and degradation products of the active ingredients. The method can be used as a stability indicating research because it was able to isolate the drugs from their degradation products in an efficient manner.

5. ACKNOWLEDGEMENT

The authors would like to thank Anchrom Labs, Mumbai, Cipla ltd. Mumbai and PRES's College of Pharmacy (For Women)

Chincholi, Nashik for providing necessary facilities to carry out the research work.

6. AUTHORS' CONTRIBUTION STATEMENT

Charushila Bhangale contributed for experimental work and manuscript preparation. Shivanand Hiremath contributed in hypothesis and finalization of manuscript. Both authors read and approved the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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