Journal of Pharmaceutical Research International

32(32): 52-60, 2020; Article no.JPRI.63390
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

A Novel UPLC-PDA Stability Indicating Method Development and Validation for the Simultaneous Estimation of Lamivudine and Dolutegravir in Bulk and Its Tablets

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PV and UK literature review and Experimental work. Authors GKR and GC first draft copy of the manuscript. Author RRA procurement of chemicals and method development protocol design. Author BR proof read of manuscript and experimental work. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2020/v32i3230933
Editor(s):
(1) Dr. Rafik Karaman, Al-Quds University, Palestine.
(2) Dr. Giuseppe Murdaca, University of Genoa, Italy.
Reviewers:
(1) Yohanes Firmansyah, Tarumanagara University, Indonesia.
(2) Nurul Hidayah, Universitas Negeri Jakarta, Indonesia.
Complete Peer review History: http://www.sdiarticle4.com/review-history/63390

Original Research Article

Received 20 September 2020
Accepted 26 November 2020
Published 02 December 2020

ABSTRACT

Aim: To develop a stability indicating Rp-UPLC method for the simultaneous determination of Lamivudine, Dolutegravir and their degradants in tablets.
Methodology: The chromatographic separation was performed on BEH Shield RP18 (2.1 mmX100 mmX1.7 mm) using a isocratic mobile phase Potassium dihydrogen orthophosphate pH 3 adjusted with orthophosphoric acid: methanol (30:70,% v/v) at a flow rate of 0.5ml/min. Column was maintained at room temperature and eluents are monitored at 258 nm.
Results: Retention times of the analytes were found to be at 0.81 and 2.78 mins for Lamivudine and Dolutegravir respectively. The calibration of peak area versus concentration, which was linear from 105 to 315 µg/ml for Lamivudine and 17.5 to 52.5 µg/ml for Dolutegravir, had regression

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coefficient (r^2) greater than 0.999. The method had the requisite accuracy, precision and robustness for simultaneous determination of Lamivudine and Dolutegravir in tablets.

Conclusion: The proposed method is simple, economical, accurate, precise and can be successfully employed in routine quality control for the simultaneous analysis of Lamivudine and Dolutegravir in pharmaceutical formulations.

Keywords: Lamivudine; dolutegravir; BEH shield; potassium dihydrogen orthophosphate; methanol.

1. INTRODUCTION

One of the deadliest and most unmanageable chronic health disasters is HIV / AIDS. It requires lifelong treatment with a combination of potent life-saving essential drugs, including nucleoside analog reverse transcriptase inhibitors and protease inhibitors. [1-2] Of the two analytes, Lamivudine, 4-amino-1 - [(2R, 5S) -2-(hydroxymethyl) -1,3-oxatiolan-5-yl] -1,2-dihydropyrimidin-2- one, is a nucleoside analogue reverse transcriptase inhibitor (NRTI) and Dolutegravir (4R, 12aS) -N- (2,4-difluorobenzyl) -7-hydroxy-4-methyl-6,8-dioxo-3,4,6 , 8,12,12a-hexahydro-2H-pyrido [1 ', 2': 4,5] pyrazine [2,1 -b] [1,3] oxazine-9-carboxamide is an inhibitor of the transfer of HIV integrase chain. These drugs are the first line therapy. [3] Since the introduction of highly active antiretroviral combination therapy (HAART) in the late 1990s, the life expectancy and quality of life of patients infected with human immunodeficiency virus (HIV) have improved due to the reduction plasma viral load to levels below detectable levels. Combining these two drugs in fixed dose combinations (FDCs) has been one of the essential components of highly active antiretroviral therapy (HAART) [4]. A Committee for Medicinal Products for Human Use (CHMP) also recommended a fixed-dose combination of lamivudine 300 mg (LAM) and dolutegravir 50 mg (DOL) for effective treatment of HIV. [5] Fig. 1 (a and b) shows the chemical structures of the drugs.

The extensive use of these antiretroviral agents in a combined dosage form and their clinical and pharmacological studies increase the need to develop rapid and sensitive analytical techniques for testing these drugs. The LAM and the DOL were previously analyzed individually by various analytical methods. [6-10]. Additionally, few Rp-HPLC methods have been reported for the simultaneous estimation of LAM and DOL and tinofivir disporxil fumarate or butcaver sulfate and abacavir in triple combination [11-15]. The fixed dose film-coated tablets LAM and DOL approved by the USFDA in April 2019. To date, no single stability indicative of the Rp-UPLC method for the simultaneous estimation of LAM and DOL in bulk and in the tablet dosage form has been reported in the literature. In addition, existing LC methods were less cost-effective and more time-consuming but did not conform to routine simultaneous estimation. The cost and time of analysis can be significantly reduced by using the UPLC Acquity system with better results. Comparatively, UPLC enables an analyst to work with higher skills with a wide range of linearity, solvent consumption and system flow rates and back pressures than traditional HPLC. Taking into account the growing demand for the aforementioned drugs in the world market, it is necessary to develop a new simple, economical, fast and accurate analytical UPLC method for the simultaneous determination of both drugs in the tables and apply the method to study degradation studies in five different stressful conditions. It could be applied to evaluate the quality, efficacy and storage conditions of each analyte. The validation of the developed method was carried out according to the Q2 specification of the ICH guidelines [16 and 17].

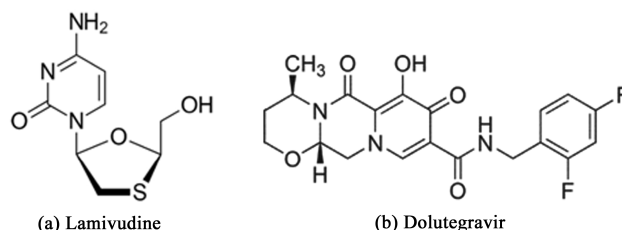

Fig. 1. Chemical structures of a) Lamivudine and b) Dolutegravir

2. METHODOLOGY

2.1 Chemicals and Reagents

A free sample of LAM and DOL was received from MSN Laboratories, Hyderabad, India and Torrent Pharma, Ahmedabad, India respectively. Methanol, water and acetonitrile for HPLC were purchased from Sigma-Aldrich. Analytical grade sodium hydroxide (NaOH), hydrogen peroxide (H2O2), hydrochloric acid (HCl) and a 0.22mm membrane filter were procured from Sigma-Aldrich. Dovato (ViiV healthcare) containing LAM and DOL with a label claim of 300 mg and 50 mg respectively and purchased from the local pharmacy. Millipore Milli Q water purification system was used to produce high purity water. All chemicals were analytical or LC grade.

2.2 UPLC Instrumental Condition

UPLC Acquity System (Waters, Milford, MA, USA) was used for data acquisition, equipped with a Model 2996 PDA detector and Empower software. UPLC separation of the two drugs was achieved with BEH Shield RP18 (2.1 mm x 100 mm x 1.7 mm) using potassium dihydrogen orthophosphate pH 3 adjusted with orthophosphoric acid: methanol (30: 70,% v / v) in isocratic mode at a flow rate of 0.50 ml / min and column at room temperature. The PDA detector was used to monitor the two drugs at 258 nm. The solvents were filtered on a 0.22 mm membrane filter and degassed in an ultrasonic bath before use. The analytical method was optimized using a pure analytical standard. The mobile phase was used as a diluent.

2.3 Preparation of Standard Solutions

A mixed standard stock solution of LAM (1000 µg / ml) and DOL (1000 µg / ml) was prepared by accurately weighing 10 mg of each and dissolved in a 10 ml volumetric flask containing 5 ml of methanol and the flask was sonicated to dissolve the content and made up the mark with diluent. Aliquots of these samples were transferred to a 10 ml volumetric flask containing 5 ml of diluent (mobile phase), sonicated for 5 more minutes and the remaining volume was make up with diluent to obtain a final concentration of 210 µg / ml and 35 µg / ml for LAM and DOL respectively.

2.4 Analysis of Formulation

Twenty tablets were carefully weighed and ground to fine powder in a mortar. An amount equivalent to 120 mg of LAM and 10 mg of DOL was transferred to a volumetric flask, 5 ml of diluent was added and sonicated to ensure solubility. Finally the volume was made up to 10 ml to obtain the primary stock solution of the tablets. A 0.35 mL aliquot was withdrawn and transferred to a 10 mL volumetric flask. The mobile phase was added up to the mark to reach the final concentration of 210 µg / ml and 35 µg / ml for LAM and DOL, respectively. The resulting solution was filtered through 0.45 µm Millipore nylon filter paper as needed.

2.5 Validation of Chromatographic Method

The developed method was validated as per the guidelines of ICH (ICH Guidelines, Q2 (R1), 2005) [16].

2.5.1 System suitability

System suitability parameters were measured to verify system performance. The precision of the system was determined in six repeated injections of standard preparations. All important characteristics were measured, including the peak area, the resolution of the peaks and the theoretical plate number.

2.5.2 Accuracy

Accuracy was determined by recovery experiments. In this process, it was tested at three different levels that were 50, 100 and 150% and analyzed chromatogram.

2.5.3 Precision

Precision (Intraday and Interday) of the analytical technique was proven by using optimized concentration of LAM and DOL by six replicate injections. Average and % RSD of peak area and Assay were determined from chromatograms.

2.5.4 Specificity

To assess the strategy specificity, working placebo solution (blank) in the absence of the LAM and DOL and standard solution having a concentration of 210 and 35 µg/mL of LAM and DOL, respectively, as well as formulations were introduced into the UPLC system and analyzed chromatograms.

2.5.5 Linearity

Linearity was confirmed by preparing and analyzing the pure analytical standards at five

different concentrations. The developed method shows ideal linearity over a range of 105, 157.5, 210, 262.5 and 315 µg/ml and 17.5, 26.25, 35, 43.75 and 52.5 µg/ml for LAM and DOL respectively.

2.5.6 Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ of LAM and DOL were determined by using LOD and LOQ can be calculated using the standard deviation (SD) of the response and the slope. LOD = 3.3*SD/Slope and LOQ = 10*SD/Slope.

2.5.7 Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing influence of small changes in flow rate of mobile phase (±0.2 units), change in column temperature (± 5°C) and change in wave length (±2%).

2.5.8 Stress studies

The ICH guideline entitled stability testing of new drug substances and products [17] requires that stress testing is performed to describe the inherent stability characteristics of the active substance. The goal of this project was to carry out the stress degradation studies on the LAM and DOL by using the proposed method.

2.5.8.1 Acidic and alkaline hydrolysis

From the stock solution, transfer 0.35 mL to a pair of 10 mL standard flasks. 1 ml of 0.1 N HCl was added to a standard flask for acidic conditions. For alkaline degradation, 1 mL of 0.1 N NaOH was added to another flask, then the standard flasks were held at 70°C for 10 h and 65°C for 10 h for acidic and alkaline samples respectively. Both sets of solutions were neutralized and brought to 10 ml with diluent to obtain 210 and 35 µg / ml of LAM and DOL respectively. Cool the resulting solution to room temperature. Filter the solution with a 0.22 mm syringe and then add it to the UPLC System vials.

2.5.8.2 Thermally induced degradation

Transfer 0.35 ml of stock solution to a 10 ml standard flask and reflux at 80°C for 26 h. Then, the sample was diluted with diluents and brought to 10 ml to obtain 210 and 35 µg / ml of LAM and DOL respectively. Then the solution was cooled to room temperature. Introduce into the UPLC system vials, after filtration with a 0.22 mm syringe filter.

2.5.8.3 Oxidative degradation

Transfer 0.35 ml of stock solution to a standard 10 ml flask. Add 1 ml of 3% (v / v) hydrogen peroxide and the volume was compensated with diluents to obtain 210 and 35 µg / ml of LAM and DOL, respectively. The standard flask was then set aside at room temperature for 8 H. The resulting solution was introduced into the vials of the UPLC system, after filtration with a 0.22 mm syringe filter.

2.5.8.4 Photo degradation

From the stock solution, pipette 0.35 ml into a standard 10 ml flask and the volume was made up to the mark with diluents to obtain 210 and 35 µg / ml of LAM and DOL respectively. The samples were then kept in a 200 Wh / m2 photostability chamber in ultraviolet light and 1.2 million lxh in ultraviolet light for 30 hours. Filter the solution with a 0.22 mm syringe and then place it in the UPLC System vials.

3. RESULTS AND DISCUSSION

3.1 System Suitability Study

To ensure the validity of the analytical procedure, a system suitability test has been performed. The following parameters, such as theoretical plate number (N), resolution, retention time (Rts) and tail factor, were analyzed using 3 µL of standard working solution containing LAM (210 µg / ml) and DOL (35 mcg / mL) injected six times in UPLC system. The results are presented in Table 1.

3.2 Accuracy (Recovery)

Accuracy was determined at three different levels that were 50, 100 and 150%. The results are shown in Table 2. Mean % Recoveries at 50, 100 and 150% for LAM were found to be 99.92, 100.09 and99.46% respectively. Similarly for DOL 100.81, 100.43 and 100.76 at 50, 100 and 150% respectively.

3.3 Precision

Precision of the analytical method were established for both intra and interday by using

concentration of 210 and 35 µg/mL of LAM and DOL six replicate injections. The results are shown in Table 3.

3.4 Specificity

To evaluate the specificity, the blank solution, the LAM standard and the DOL with a concentration of 210 and 35 µg / ml, respectively, as well as the formulation, were injected into the UPLC system. No peaks were found in blank and the Rts reported in the standard and samples were similar. Fig. 2a-c shows representative chromatograms of the blank, standard and sample.

3.5 Linearity

Linearity curves were constructed by plotting peak areas on X axis versus drug concentration along Y axis and the regression equations were computed. The curves were plotted over the concentration ranges of 105 - 315 µg/mL and 17.5 – 52.5 µg/mL for LAM and DOL Respectively. Fig. 3a and 3b shows the linearity curves of LAM and DOL.

Table 1. System suitability parameters

S. No	Parameter*	LAM	DOL
1	Theoretical Plate Count	3630	7753
2	Average Peak Area	3986770.5	2355453.5
3	RT	0.8155	2.7895
4	Tailing	1.72	1.18
5	Resolution	-	22.57
6	S/N	1669.82	464.25

** Average of 6 replicates*

Table 2. Recovery study

Analyte	Accuracy level*	Peak area*	Amount added (mg)*	Amount found (mg)*	% Recovery*	Mean % Recovery*
LAM	50%	1991272	104.97	104.89	99.92	99.83
	100%	3989227	209.94	209.94	100.09	
	150%	5945908	314.90	314.90	99.46	
DOL	50%	1177893	17.36	17.50	100.81	100.67
	100%	2346831	34.72	34.87	100.43	
	150%	3531658	52.08	52.48	100.76	

**Mean average of three determinations at each level*

Table 3. Precision study

Precision	Mean Peak area*		% RSD*		Mean Assay*		% RSD*	
	LAM	DOL	LAM	DOL	LAM	DOL	LAM	DOL
Intraday	3983056.17	2344739.33	0.23	1.30	99.92	99.56	0.23	1.30
Interday	3985543.33	2336768.00	0.38	0.72	99.98	99.22	0.38	0.72

**Mean of six determinations*

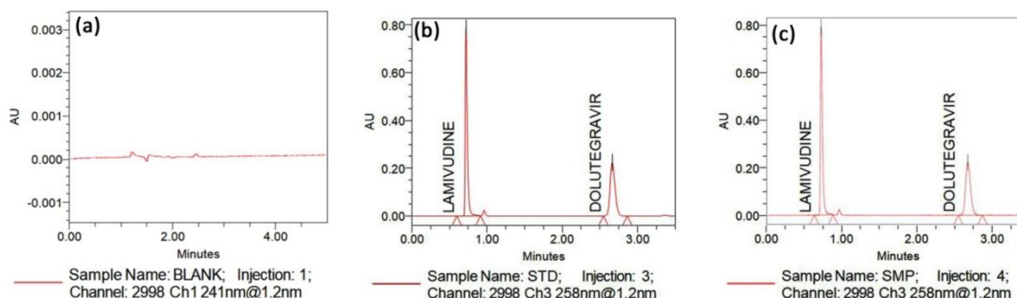

Fig. 2. Representative chromatogram of a) Blank, b) Standard and c) Sample

3.6 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ values of LAM were found to be 0.03 and 0.09 µg/mL respectively. For DOL LOD and LOQ were found to be 0.62 µg/mL and 2.06 µg/mL respectively. Fig. 4 a-b shows the chromatograms of LOD and LOQ.

3.7 Robustness

The study reveals that there was no much deviation in the robust chromatograms in assessment with optimized one. The results were shown in Table 4.

3.8 Stress Studies

Degradation studies reveal the specificity of the developed method in the presence of degradation products in bulk and in pharmaceutical form. It was performed in combination of two drugs and the purity of the drug peaks was established by the purity angles. The formulations were exposed to five different stress conditions.

Under acidic and basic conditions, degradation may be due to the catalysis of the ionizable functional group present on the drug molecule. Two degrading agents were detected under acidic and basic conditions, but no further degrading peaks in the retention time of LAM and DOL were reported, respectively. Both drugs degraded more in acidic conditions than in alkaline conditions.

Three degrading agents were detected in the oxidative degradation study and no degradation peaks were reported in the retention time of LAM and DOL respectively. The reason for the high degradation of peroxide may be due to the electron transfer mechanism to form reactive cations and anions. Under photolytic stress conditions, it can be due to photooxidation by free radical mechanisms, while under thermal stress conditions it can be explained on the basis of the Arrhenius equation. The results are reduced in Table 5.

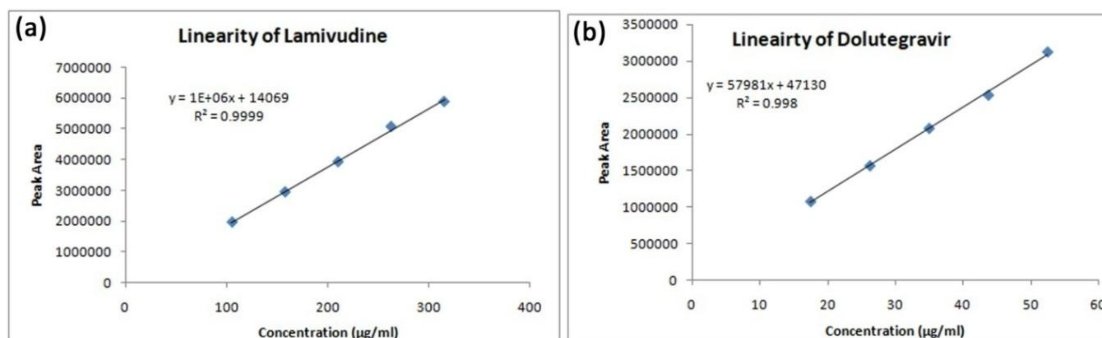

Fig. 3. Linearity curves of a) LAM; b) DOL

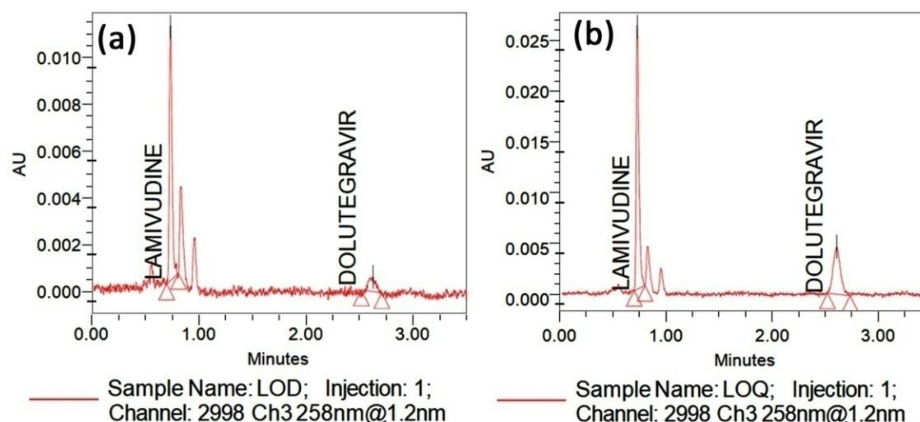

Fig. 4. a) Chromatogram of LOD; b) Chromatogram of LOQ

Although unidentified peaks were observed under five different prior stress conditions, no degrading element were found close to the retention time of LAM and DOL respectively (Fig. 5a-e). Therefore, LAM and DOL are subject to the above conditions and are too stable in the current method even under stressed conditions for a specific time period.

3.9 Discussion

This research work was designed to develop a new and sensitive method for estimating the use of the UPLC-PDA chromatography system for LAM and DOL. There are no previous publications for quantitative estimation of drugs using UPLC. A couple of LCMS methods in biological fluids have been reported, which were not suitable for the determination of analytes in tablets dosage form. There are also few HPLC methods with a run time greater than 4 minutes and laborious extraction procedures were reported in the literature. The current method solves all the problems faced by the routine quality control chemist, enabling a new method for estimating LAM and DOL. The present method has been well optimized with a lower retention time for LAM 0.81 min and DOL 2.79 min with good sensitivity and excellent peak shape with adequate resolution. Using the PDA detector with a selected wavelength of 258 nm improved the detection sensitivity of all analytes. The total analysis time was reduced to a minimum of 3.00 minutes using the ultra-sensitive instrument. The optimized condition above was used for subsequent validation studies. The developed method was accurate because the recovery rate for all analytes at all three levels was within limits. The% RSD values for the intra- and intraday precision study were less than 2% indicating that the developed method was accurate. The Ultra Performance liquid chromatography system was suitable for all drug testing, as the system suitability study table shows the% RSD was less than 2% for all parameters. The result of the LOD and LOQ study indicates that the current method was the most sensitive to a very low concentration of all analytes, which is one of the results of this current method. The robustness study indicates that deliberate changes in various parameters, such as flow rate, pH, temperature, etc., do not significantly affect the results. The% assay of the marketed formulation revealed 99.27% for LAM and 99.98% for DOL, which justifies the efficiency of the test procedures developed. On the basis of empirical evidence, the authors are strongly convinced of the novelty of this method. This method was found to be faster because it significantly reduces the total analysis time by 3.00 minutes. It was considered economical since the present method replaces

Table 4. Results of robustness

S. No	Parameter	Condition	Lamivudine			Dolutegravir		
			RT	Peak Area	% Assay	RT	Peak Area	% Assay
1	Flow	0.3 ml/min	0.99	3988943	100.07	3.23	2344221	99.53
2		0.5 ml/min	0.81	3986771	100.01	2.79	2355454	100.01
3		0.7 ml/min	0.77	3987160	100.02	2.18	2357845	100.11
4	Temp	25°C	0.81	3978156	99.79	2.76	2338651	99.30
5		30°C	0.81	3986771	100.01	2.790	2355454	100.01
6		35°C	0.81	3987612	100.03	2.79	2319540	98.49
7	Wave	256 nm	0.81	3957612	99.28	2.78	2338651	99.30
8	length	258 nm	0.81	3986771	100.01	2.79	2355454	100.01
9		260 nm	0.81	3987612	100.03	2.78	2345540	99.59

Table 5. Results of stress study

S. No	Condition	Lamivudine			Dolutegravir		
		Peak Area	% Assay	% Degradation	Peak Area	% Assay	% Degradation
1	Acid	3590477	90.07	9.93	2109090	89.55	10.45
2	Base	3645989	91.46	8.54	2103147	89.30	10.70
3	Peroxide	3705302	92.95	7.05	2104744	89.37	10.63
4	Thermal	3587935	90.01	9.99	2129439	90.41	9.59
5	UV	3642813	91.38	8.62	2130909	90.48	9.52

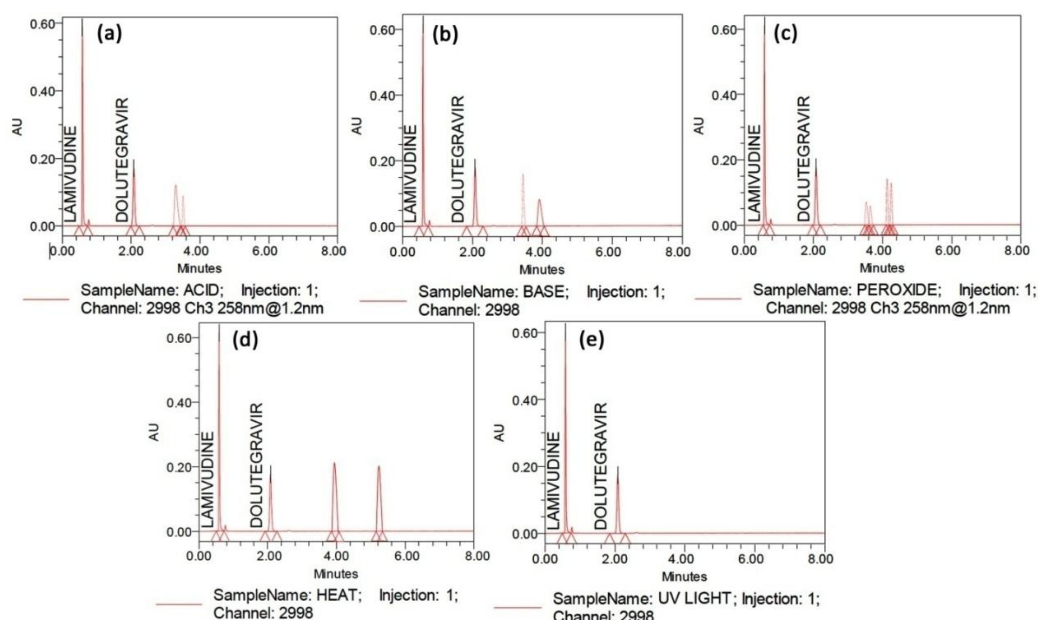

Fig. 5. Degradation chromatograms of a) Acid; b) Base; c) Peroxide d) Heat (Thermal); e) UV light

the expensive acetonitrile with methanol. The limit of detection and limit of quantification were very low, which made the method more sensitive than existing methods. The result of the validation parameters was considered satisfactory and within the limits established by the ICH Q2B guidelines. Therefore, the present method developed using UPLC has been a very useful analytical tool for estimating LAM and DOL in bulk and tablet dosage form. The use of simple and inexpensive techniques extrapolates the current method also for the estimation of drugs in biological fluids using UPLC.

4. CONCLUSION

The developed UPLC method provides reliable, reproducible, precise and specific quantification in bulk lamivudine and dolutegravir and their tablets. The new method has been validated against regulatory requirements and has demonstrated acceptable accuracy and precision with adequate sensitivity. This method can be used for routine analysis of the above drugs in quality control laboratories.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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