Development and Validation of RP-HPLC Stability Indicating Method for Simultaneous Estimation of Dolutegravir and Lamivudine in Bulk and Pharmaceutical Dosage Form

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Authors' contributions

This work was carried out in collaboration among all authors. Author KP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors US and DP managed the analyses of the study. Authors JKP and TBP managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Dolutegravir (DVR) and Lamivudine (LMN) is anti-viral drug combination used in treatment of HIV-I infection. FDA approved dosage regime for DVR and LMN is 50mg and 300mg respectively. The aim of present research work is to develop and validate a reverse phase high performance liquid chromatography (RP-HPLC) method for simultaneous estimation of DVR and LMN in bulk and pharmaceutical dosage forms. Further the stability indicating nature of method has been evaluated.

Methodology: A chromatographic separation was achieved on Hypersil BDS C18, 250 × 4.6 mm 3.5 μm particle size, column as stationary phase and mobile phase composed of Phosphate Buffer pH 3.0: Acetonitrile (60:40%V/V) with flow rate of 1.5mL/min with 20μL injection volume. The analytes were estimated at 232nm using PDA detector. The DVR and LMN solutions were exposed to various forced degradation stress conditions to evaluate the stability behavior of the product. The method was also validated as per ICH Guideline (Q2R).

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Results: The retention time for DVR and LMN was found 3.94 and 2.62 min, respectively. The developed method was found linear within concentration range of 27.5 to 82.5 µg/ml (50-150%) for DVR and 167.5 to 502.5 µg/ml (50-150%) for LMN. The% recovery (Accuracy) was found between 99.50%-101.23% and 100.09%-101.51% for DVR and LMN respectively in range of 50-150% for both drugs. The%RSD for the accuracy at all levels was less than 2.0%. LOD and LOQ, for DVR were found to 0.669 and 2.028 µg/mL, respectively, and for LMN 0.102 and 0.308 µg/mL, respectively.

Conclusion: The developed RP-HPLC method indicates no interference from the excipients and degradants peaks. All the degradant peaks were having been efficiently resolved through the use of the evolved analytical method with changed retention times. The results obtained were statistically analyzed and meet the acceptance criteria as specified in ICH Q2R1 guideline. Hence, developed method can be successfully applied for the analysis of estimation of DVR and LMN in bulk as well as pharmaceutical dosage form.

1. INTRODUCTION

Chromatography is most widely used technique in chemical analysis as a separation technique. The separation of analytes is accomplished by passing them through a column filled with micrometer-sized stationary phase with specific affinity to the target analytes. In HPLC, reversed-phase chromatography is a popular separation technique. [1-5]

Dolutegravir (DVR) is a new HIV-1 integrase inhibitor that works by binding to a specific location and inhibiting the strand transfer stage in retroviral DNA integration. Dolutegravir, an integrase strand transfer inhibitor, prevents HIV replication by preventing viral DNA from combining with the genetic material of the host human immunological T cells. Lamivudine (LMN) is a nucleoside reverse transcriptase inhibitor that exhibits activity against the human immunodeficiency virus type I (HIV-I) and hepatitis B. (NRTI). It's a crucial stage of the HIV replication cycle that can lead to viral activity suppression. [6-9]

Chemically DVR is a *(3S,7R)*-N-[(2,4-difluorophenyl)methyl]-11-hydroxy-7-methyl-9,12-dioxo-4-oxa-1,8-diazatricyclo[8.4.0.0]tetradeca-10,13-diene-13-carboxamide (Fig. 1). Chemically, Lamivudine is 4-amino-1-[(*2R,5S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one (Fig. 2). These drugs are available on the market and can be administered separately or in combination.

The fixed dose combination (FDC) medication containing DVR 50 mg and LMN 300 mg is used to treat HIV-1 infection in adults. In the case of adults, the drug's suggested FDC is once daily, with or without food.
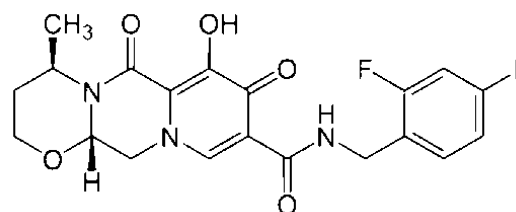
Fig. 1. Structure of DVR
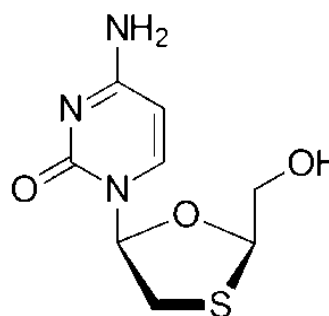
Fig. 2. Structure of LMN

To analyze a drug individually or in combination with other drugs, an effective analytical method is required. Various analytical methods were used to quantify the individual or multi-component combination assay of nucleoside polymerase inhibitor (NPI) in pharmaceutical dosage forms. Several HPLC [10-20], LC/MS/MS [21-24], HPTLC [25,26], UV [27-29], and UPLC [30] assay methods for estimating lamivudine, Dolutegravir and a few other anti-retroviral drugs individually and in combination with other drugs have been described in the literature. As per the literature search and review no Pharmacopoeial

and reported analytical method available to analyze the target analytes in combined dosage form. Since this is one of the efficient anti-viral drug combination for HIV patients therefore, it was thought to develop and validate RP-HPLC method which will ensure separation of all of the active ingredients present in pharmaceutical dosage form and it is validated as per ICH guidelines [31].

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Reference standards for Dolutegravir (DVR) and Lamivudine (LMN) were obtained from Emcure Pharmaceuticals Ltd, Ahmedabad, Gujarat. Acetonitrile, methanol and methanol for HPLC supplied by Sigma Aldrich chemical Pvt Ltd, Bangalore. Milli-Q water, orthophosphoric acid, sodium hydroxide, potassium dihydrogen phosphate, hydrogen peroxide and hydrochloric acid were obtained from Himedia lab Pvt. Ltd, Mumbai.

2.2 Instrumentation

A Waters HPLC (Model-2695) instrument consisting of an Hypersil BDS C18 (250 x 4.6 mm) 3.5 µm column and a Waters 515 solvent delivery system using an inbuilt PDA detector was used. Empower 3 software was used. An Elico pH metre was used to regulate the pH of the solution.

2.3 Chromatographic Conditions

A chromatographic separation was achieved by using Hypersil BDS C_{18}, 3.5 µm particle size, 250 × 4.6mm column as a stationary phase and mobile phase composed of phosphate buffer, pH 3.0: acetonitrile (60:40% v/v) with a flow rate of 1.5 mL/min, and the eluents were detected at a wavelength of 232 nm utilizing PDA detector. Injection volume was set to 20µL and HPLC operated at 30 °C and run time was 10 min at isocratic mode.

2.4 Preparation of Solutions

2.4.1 Preparation of phosphate buffer pH 3.0 Solution

Accurately weigh about 6.8 gm potassium dihydrogen ortho phosphate, add into a 1000ml beaker. Add 800ml of HPLC water and dissolve it. Adjust solution pH 3.0 with 1.0% ortho

phosphoric acid and make up volume (Up to 1000ml) with HPLC water. The buffer solution was filtered through 0.45µm membrane filter and sonicated.

2.4.2 Preparation of mobile phase

Mobile phase was prepared by mixing Phosphate buffer solution pH 3.0, acetonitrile within the ratio of 60:40% v/v. The same solution was used as diluent solution to prepare standard solution of drug.

2.4.3 Preparation of standard stock solutions

Standard Stock solution of Dolutegravir: Weigh accurately 55.0mg of Dolutegravir into a 100ml volumetric flask. Add 5ml methanol and shake for dissolve, make up volume with diluent.

Standard Stock solution of Lamivudine: Weigh accurately 335.0mg of Lamivudine into a 100ml volumetric flask. Add 5ml methanol and shake for dissolve, make up volume with diluent.

Combined Standard Solution: Transfer 5ml each from Dolutegravir STD stock solution and Lamivudine Std Stock solution into a 50ml volumetric flask and make up volume with diluent.

2.4.4 Preparation of sample stock solution

Weigh and powder 20 tablets. Take tablet powder equivalent to 55.0mg and 335.0 mg of Dolutegravir and Lamivudine, respectively into a 100ml volumetric flask. Add 60ml methanol and shake for 5 minutes and sonicate for 10 minutes. Make up volume with Methanol. Filter this solution with 0.45µm membrane filter. (Dolutegravir-550.0 µg /ml and Lamivudine-3350.0 µg/ml)

Working sample preparation: Take 1ml of sample stock solution into a 10ml volumetric flask and make up with mobile phase. (Dolutegravir-55.0 µg /ml and Lamivudine-335.0 µg /ml).

2.5 Analytical Procedure using HPLC

The combined standard solution and working sample solution was injected in HPLC system, respectively and% assay was calculated using following formula:

$$\%\text{Assay of Dolugretavir} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \frac{\text{Wight of Standard}}{\text{Weight of Sample}} \times \text{Average Weight} \times \text{Standard Ptency or Label Claim}$$

$$\%\text{Assay of Lamivudine} = \frac{\text{Area of Sample}}{\text{Area of Standard}} \times \frac{\text{Wight of Standard}}{\text{Weight of Sample}} \times \text{Average Weight} \times \text{Standard Ptency or Label Claim}$$

With reference to accuracy, precision, system appropriateness, specificity, linearity, limit of quantification, limit of detection, and forced degradation investigations, the developed method was validated according to ICH Q2R1 guidelines.

2.5.1 System suitability

The suitability of the system was checked by 6 repeated injections of the freshly prepared standard solution. Perceived RSD values were within the appropriate range (≤ 2.0%). The theoretical plates, resolutions, and retention rates of dolutegravir and lamivudine were determined and found to be within reasonable limits.

2.5.2 Linearity

Linearity was determined for dolutegravir and lamivudine standard solutions prepared at various concentration levels, i.e., in the range of 27.5 to 82.5 µg/ml for DVR and 167.5 to 502.5 µg/ml for LMN. Each measurement was performed in triplicate. Linearity was confirmed by multivariate least squares analysis.

2.5.3 Precision

The precision of the method was demonstrated by intraday and inter-day analysis.The Interday precision was performed for different 3 concentration as per below concentration range for day-1, day-2 and day-3 Intraday precision was performed using 3 different concentration (n=3) as per condition given below:

1. Lower concentration- DVR-27.5mcg/ml + LMN-167.5mcg/ml
2. Middle concentration- DVR-55mcg/ml + LMN-335mcg/ml
3. Higher concentration- DVR-82.5mcg/ml + LMN-502.5mcg/ml

2.5.4 Accuracy

Accuracy of the developed method was determined using on 3 replicates on 3 levels (total 9 determination) over the range of 27.5 to 82.5 µg/ml (50-150%) for DVR and 167.5 to 502.5 µg/ml (50-150%) for LMN.

2.5.5 Repeatability

Repeatability of the method was performed DVR-55mcg/ml + LMN-335mcg/ml concentration (n=6).

2.5.6 Robustness

Variation of three factors from the optimised circumstances indicated as under was used to determine robustness: :

1. Flow rate: +0.2ml/min and -0.2ml/min
2. Buffer pH: +0.2pH and -0.2pH
3. Solvent% in mobile phase: +2% solvent and -2% solvent in mobile phase.

2.5.7 Limit of detection and limit of quantification (LOD and LOQ)

For the signal-to-noise ratio approach, LOD and LOQ were calculated using the waters Empower software.

2.6 Forced Degradation Study

Standard stability study of the DVR and LMN solution was performed using different conditions. The standard stock solution of DVR was prepared by dissolving 55mg DVR in 100 ml methanol (550 µg /ml) and LMN standard stock solution was prepared by dissolving 335mg LMN in 100 ml methanol (3350 µg /ml). The working sample stock solution was by accurately weighing and powdered 20 tablets. Take powdered tablet equivalent to 335mg LMN and 55mg DVR in to a 100ml volumetric flask, respectively. Add 60 ml methanol. Shake for 15 minutes and sonicate for 10 minutes. Make up volume with diluent. Filter this solution with 0.45µm membrane filter. (i.e. DVR-550 µg /ml, LMN-3350 µg /ml).

2.6.1 Sample preparation for acid degradation

Blank Sample: Blank sample was prepared by mixing 2ml 0.1N HCl and 2ml 0.1N NaOH in 10 ml volumetric flask and volume was makeup diluent.

Standard Sample: DVR standard solution was prepared by adding 1 ml DVR standard stock solution in 2ml 0.1N HCl. Kept this solution for standard solution was prepared by adding 1 ml LMN standard stock solution in 2ml 0.1N HCl. Kept this 4 hrs then neutralize with 2ml 0.1N NaOH to prevent further degradation. Then make the volume (10ml) using diluent. LMN solution for 4 hrs then neutralize with 2ml 0.1N NaOH to

prevent further degradation. Then make the volume (10ml) using diluent.

Sample Preparation: Formulation standard solution (DVR and LMN combination) was prepared by adding 1 ml sample stock solution in 2ml 0.1N HCl. Kept this solution for 4 hrs then neutralize with 2ml 0.1N NaOH to prevent further degradation. Then make the volume (10ml) using diluent.

2.6.2 Sample preparation for base degradation

Blank Sample: Blank sample was prepared by mixing 2ml 0.1N HCl and 2ml 0.1N NaOH in 10 ml volumetric flask and volume was makeup diluent.

Standard Sample: DVR standard solution was prepared by adding 1 ml DVR standard stock solution in 2ml 0.1N NaOH. Kept this solution for 4 hrs then neutralize with 2ml 0.1N HCl to prevent further degradation. Then make the volume (10ml) using diluent. LMN standard solution was prepared by adding 1 ml LMN standard stock solution in 2ml 0.1N NaOH. Kept this solution for 4 hrs then neutralize with 2ml 0.1N HCl to prevent further degradation. Then make the volume (10ml) using diluent.

Sample Preparation: Formulation standard solution (DVR and LMN combination) was prepared by adding 1 ml sample stock solution in 2ml 0.1N NaOH. Kept this solution for 4 hrs then neutralize with 2ml 0.1N HCl to prevent further degradation. Then make the volume (10ml) using diluent.

2.6.3 Sample preparation for oxidation degradation

Blank Sample: Blank sample was using 2ml 3% H_2O_2 10 ml volumetric flask and volume was makeup diluent.

Standard Sample: DVR standard solution was prepared by adding 1 ml DVR standard stock solution in 2ml 3% H_2O_2. Kept this solution for 2 hrs, then make the volume (10ml) using diluent. LMN standard solution was prepared by adding 1 ml LMN standard stock solution in 2ml 3% H_2O_2. Kept this solution for 2 hrs then make the volume (10ml) using diluent.

Sample Preparation: Formulation standard solution (DVR and LMN combination) was prepared by adding 1 ml sample stock solution in 2ml 3% H_2O_2. Kept this solution for 2 hrs then make the volume (10ml) using diluent.

2.6.4 Sample preparation for thermal degradation

For thermal degradation study mobile phase (diluent) was taken as blank solution. For standard degradation study, put the standard powder individually in Oven at 105^0C for 12 hrs.

2.6.5 Sample preparation for photo degradation

For Photo degradation study mobile phase (diluent) was taken as blank solution. For standard degradation study, put the standard powder individually in UV chamber at $105^°$C for 24 hrs.

3. RESULTS AND DISCUSSION

3.1 RP-HPLC Method Development

For detection of DVR and LMN using RP-HPLC chromatographic condition was applied. The analysis of results within the suitable limit i.e not less than 2.0. Fig. 3 represents the RP-HPLC chromatogram of std and sample, respectively. This may be a referred as specificity of the developed method RP-HPLC.

3.2 System Suitability Study

6 replicates of standard solution were freshly prepared and RSD value was observed within the generally acceptable limit (≤ 2.0%). The results of theoretical plates, resolution, and tailing factor of DVR and LMN was found within the acceptable limit (Table 1).

3.3 Linearity, Limit of Detection and Limit of Quantification Study

The linearity was performed by preparing standard solution of DVR and LMN at various concentration levels i.e., in the range of 27.5 to 82.5 µg/ml for DVR and 167.5 to 502.5 µg/ml for LMN. Each measurement was done in triplicate. The results indicated in Fig. 4, which showed linear relationship exist between peak area and concentration of drug within the given range. Table 2 showed linear equation and correlation coefficient for DVR and LMN.
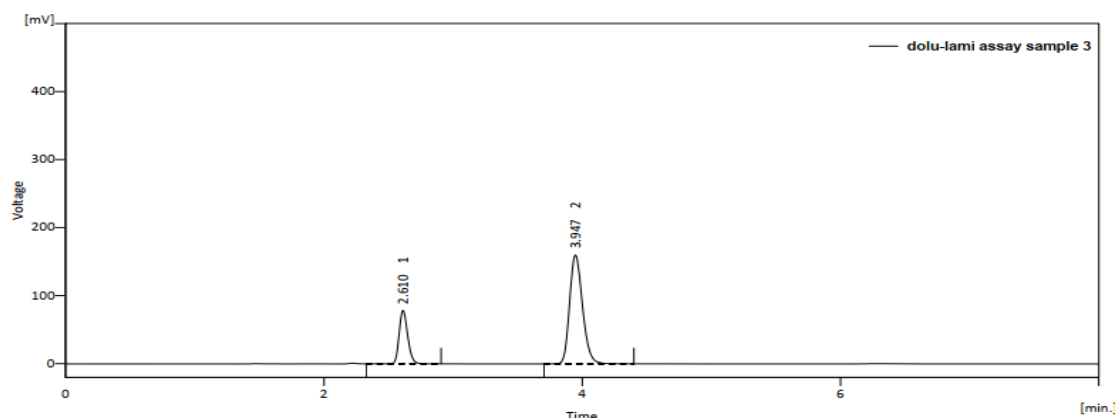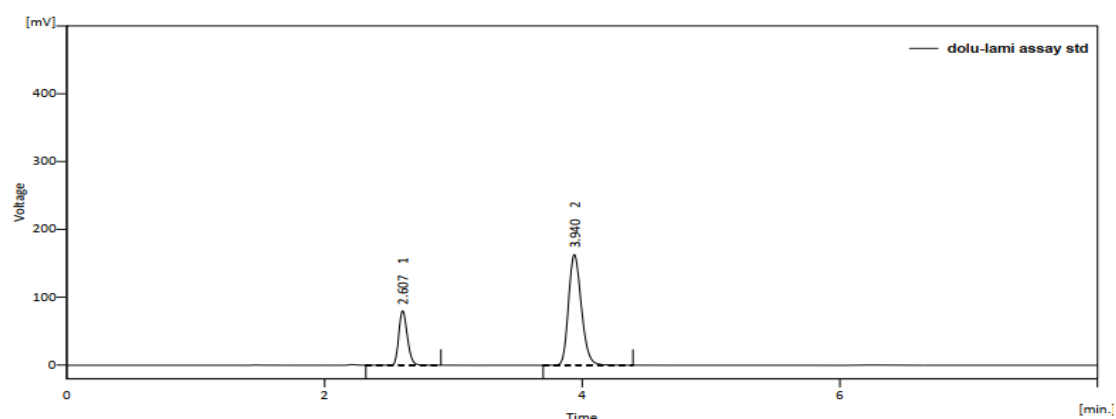
Fig. 3(a).

Fig. (3b).

Fig. 3. RP-HPLC chromatograms for (a) Sample Solution (b) Std solution

LOD and LOQ were found to be 0.669 and 2.028 µg/mL, respectively, for DVR and 0.102 and 0.308 µg/mL, respectively, for LMN.

3.4 Accuracy

Accuracy of the developed method was determined by standard addition method that represent% drug recovery study (n=3). i.e. 50%, 100% and 150%. The results of accuracy study were presented in Table 3. The% recovery at each level, mean% recovery,% RSD met the established acceptance criteria.

3.5 Precision and Repeatability Study

The Intraday precision was determined by analyzing samples(n=3). The%RSD was found to be 0.92-1.72%and 0.70-1.47 for DVR and LMN,

respectively. The Interday precision was measured using mean values and the% RSD at different days. The% RSD was found to be 0.77-1.77% and 0.82-1.02 for DVR and LMN, respectively. The repeatability (n=6) of the developed method was evaluated as the% RSD, it was found to be 1.77%and 1.85% for DVR and LMN, respectively. The results were indicated in Table 4 and Table 5. The results of developed method were found within the acceptance criteria.

3.6 Robustness

The robustness (n=3) of the developed method at different condition was presented in Table 6. % RSD of the method was found less than 2.0%, which is within acceptance limit.

Table 1. Results of System suitability parameters

System suitability parameters	Observed Results		Acceptance Criteria
	DVR	LMN	
Retention time	3.94	2.62	----
%RSD for area count of 6 standard replicate injections	0.4	0.5	Not More Than 2.0
USP Tailing factor	1.40	1.35	Not More Than 2.0
USP Theoretical plates	7107	7071	Not Less Than 2000
Resolution	8.473	NA	Not Less Than 5.0
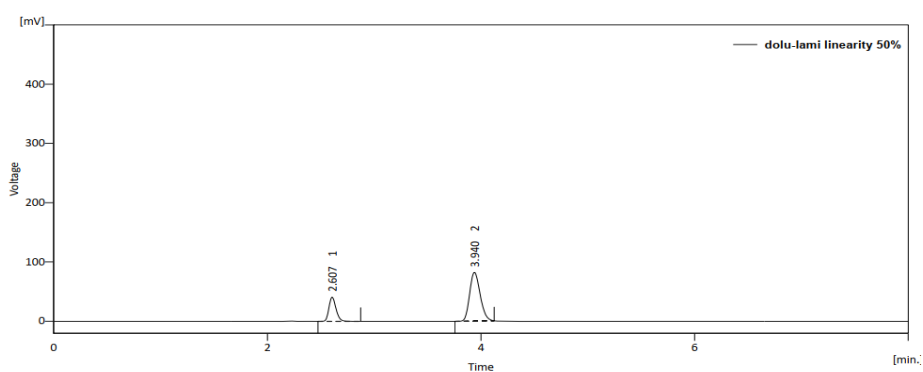
Fig. 4(a).
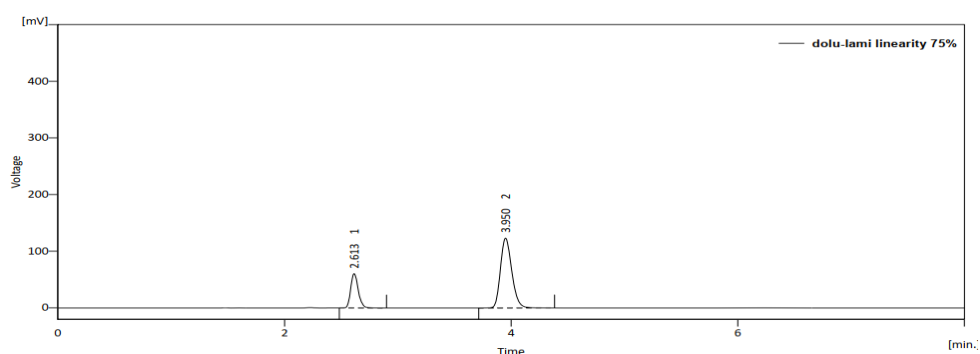
Fig. 4(b).
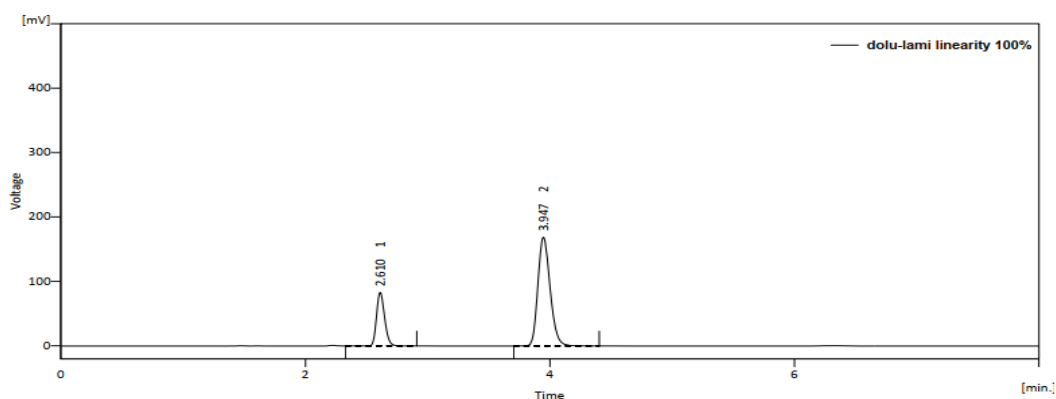
Fig. 4(c).

Fig. 4(d).

Fig. 4(e).

Fig. 4(a-e). Results of Linearity in concentration range of 27.5 to 82.5 µg/ml for DVR and 167.5 to 502.5 µg/ml for LMN
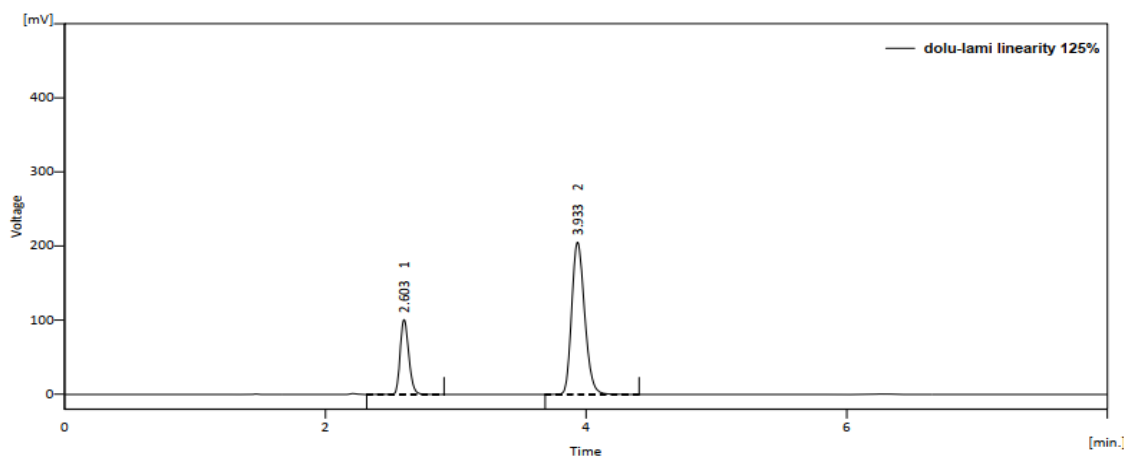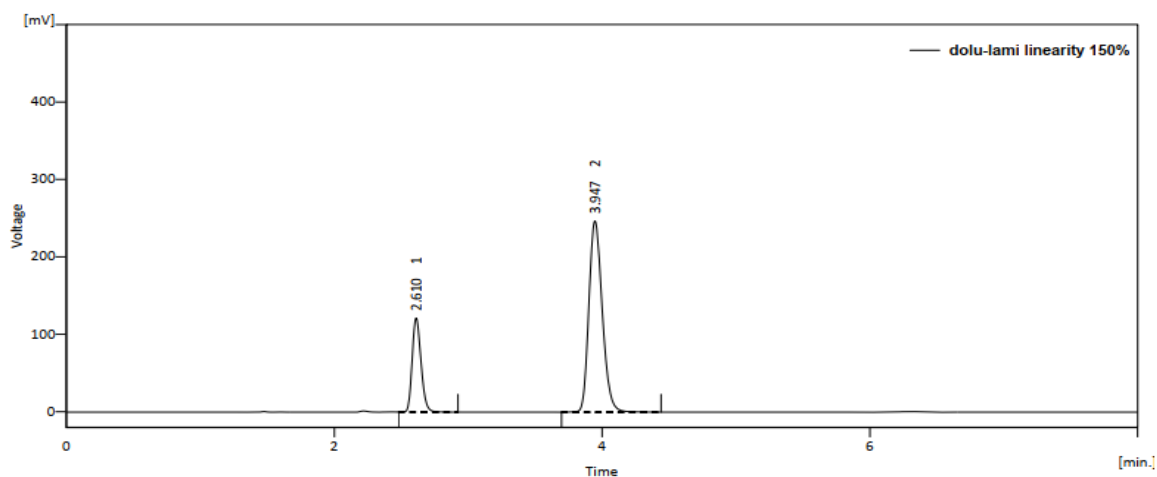
Table 2. Results of linearity study

Sr. No	Drug Name	% Level	Concentration Range (mcg/ml)	Peak Area	Linear Equation	Correlation Coefficient (r^2)
01	DVR	50	27.5	186.351	y = 9.2929x + 2.5182	0.9988
		75	41.25	278.547		
		100	55	383.444		
		125	68.75	465.122		
		150	82.5	557.709		
02	LMN	50	167.5	559.963	y = 230.47x - 5.8588	0.9988
		75	251.25	856.885		
		100	335	1174.379		
		125	418.75	1424.619		
		150	502.5	1716.508		

Table 3. Results of accuracy for developed method for DVR and LMN

% Level	Sample No	Amount Added (µg /ml)	Amount Recovered (µg /ml)	% Recovery	% Mean Recovery	% RSD
Results of%Recovery Study of DVR					
50	1	27.5	27.00	98.18	99.50	1.16
	2	27.5	27.57	100.27		
	3	27.5	27.51	100.05		
100	1	55	55.22	100.40	101.23	1.14
	2	55	55.41	100.75		
	3	55	56.40	102.55		
150	1	82.5	83.42	101.12	99.85	1.62
	2	82.5	82.82	100.39		
	3	82.5	80.87	98.03		
Results of%Recovery Study of LMN					
50	1	167.5	168.42	100.55	100.09	0.67
	2	167.5	168.17	100.40		
	3	167.5	166.38	99.33		
100	1	335	344.51	102.84	101.51	1.44
	2	335	334.83	99.95		
	3	335	340.86	101.75		
150	1	502.5	504.46	100.39	100.17	0.45
	2	502.5	500.79	99.66		
	3	502.5	504.86	100.47		

Table 4. Results of precision of developed method for DVR and LMN

Intraday Precision for DVR and LMN						
DVR				LMN			
Concentration (mcg/ml)	Peak Area	Mean peak Area	% RSD	Concentration (mcg/ml)	Peak Area	Mean Peak Area	%RSD
27.5	192.173 196.794 191.181	193.83	1.55	167.5	591 596.178 587.924	591.70	0.70
55	388.1 381.021 384.795	384.63	0.92	335	1190.147 1164.81 1178.565	1177.84	1.08
82.5	569.377 588.904 576.066	578.11	1.72	502.5	1823.293 1812.617 1773.015	1802.97	1.47
Inter day Precision for DVR and LMN						
DVR				LMN			
Concentration (mcg/ml)	Peak Area	Mean peak Area	% RSD	Concentration (mcg/ml)	Peak Area	Mean Peak Area	%RSD
27.5	185.231 188.442 181.895	185.18	1.77	167.5	569.612 565.768 559.341	564.90	0.92
55	369.143 372.176 374.854	372.05	0.77	335	1162.977 1139.862 1148.084	1150.30	1.02
82.5	556.034 551.712 566.962	558.23	1.41	502.5	1711.341 1698.027 1726.11	1711.82	0.82

Table 5. Repeatability study of Developed Method for DVR and LMN

Concentration of DVR (µg /ml)	Peak Area	Concentration of LMN (µg /ml)	Peak Area
55	370.776	335	1135.585
	383.679		1175.044
	368.068		1132.507
	371.342		1184.74
	375.629		1150.482
	383.219		1167.427
Mean	375.4521667	**Mean**	1157.630833
SD	6.65	**SD**	21.46
%RSD	1.77	**%RSD**	1.85

Table 6. Results of Robustness for DVR and LMN

DVR Concentration (55 µg /ml)				LMN Concentration (335 µg /ml)			
Parameter	Peak Area	Mean Peak Area	%RSD	Parameter	Peak Area	Mean Peak Area	%RSD
Flow Rate +2ml/min	365.541 361.684 371.754	366.3263	1.39	Flow Rate +2ml/min	1119.689 1107.813 1114.614	1114.039	0.53
Flow Rate - 2 ml/min	390.199 403.133 390.777	394.703	1.85	Flow Rate - 2 ml/min	1200.56 1240.35 1214.557	1218.489	1.66
Mobile Phase Composition +2%	360.4 360.371 370.161	363.644	1.55	Mobile Phase Composition +2%	1103.756 1103.729 1137.573	1115.019	1.75
Mobile Phase Composition -2%	380.511 381.745 372.279	378.1783	1.36	Mobile Phase Composition -2%	1170.756 1154.395 1145.415	1156.855	1.11
Buffer pH +0.2	370.256 364.212 376.567	370.345	1.67	Buffer pH +0.2	1133.987 1120.623 1153.36	1135.99	1.45
Buffer pH - 0.2	373.752 380.217 369.758	374.5757	1.41	Buffer pH - 0.2	1144.714 1164.595 1137.624	1148.978	1.22

Table 7. Results of forced degradation Study on DVR and LMN

% DVR Degradation				
Forced Degradation Parameter	Standard		Sample	
	Peak Area	% Degradation	Peak Area	% Degradation
Acid Condition	293.499	20.84	307.532	17.05
Alkaline Condition	315.512	14.90	313.279	15.50
Oxidation Condition	306.973	17.21	303.154	18.24
Photo Degradation Condition	322.936	12.90	327.826	11.58
Thermal Degradation Condition	328.753	11.33	326.484	11.94

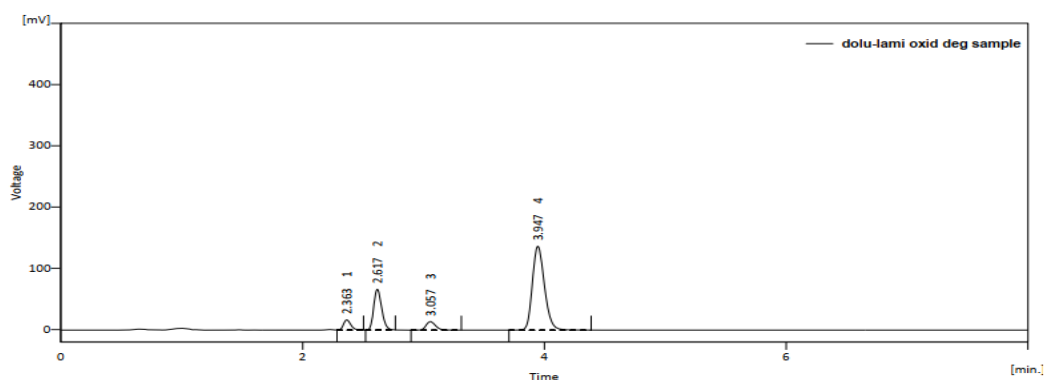| % LMN Degradation				
Forced Degradation Parameter	**Standard Peak Area**	**% Degradation**	**Sample Peak Area**	**% Degradation**
Acid Condition	954.385	15.95	951.16	16.23
Alkaline Condition	952.469	16.12	968.791	14.68
Oxidation Condition	933.152	17.82	949.933	16.34
Photo Degradation Condition	1000.942	11.85	997.969	12.11
Thermal Degradation Condition	1003.411	11.63	1030.52	9.25
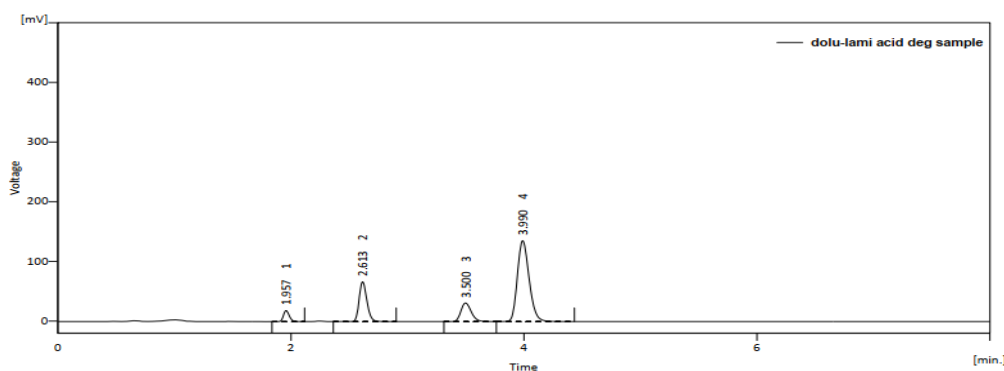
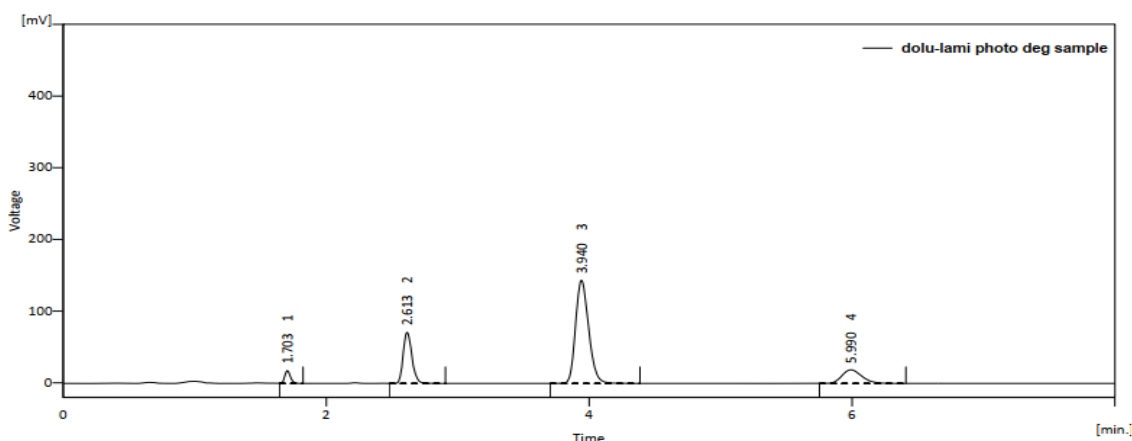**Fig. 5(a).**
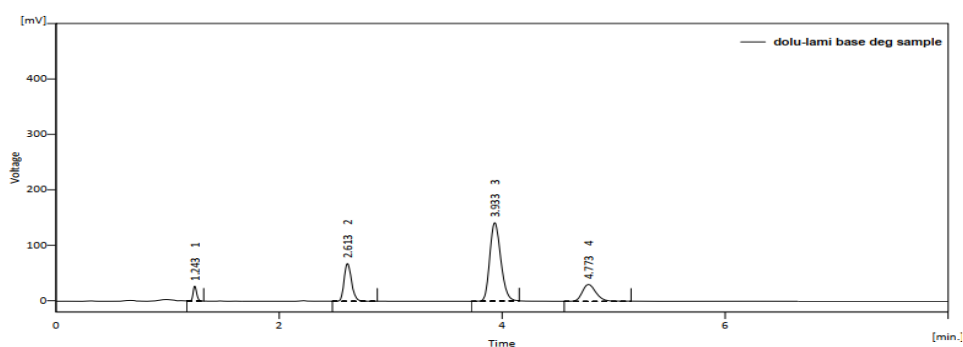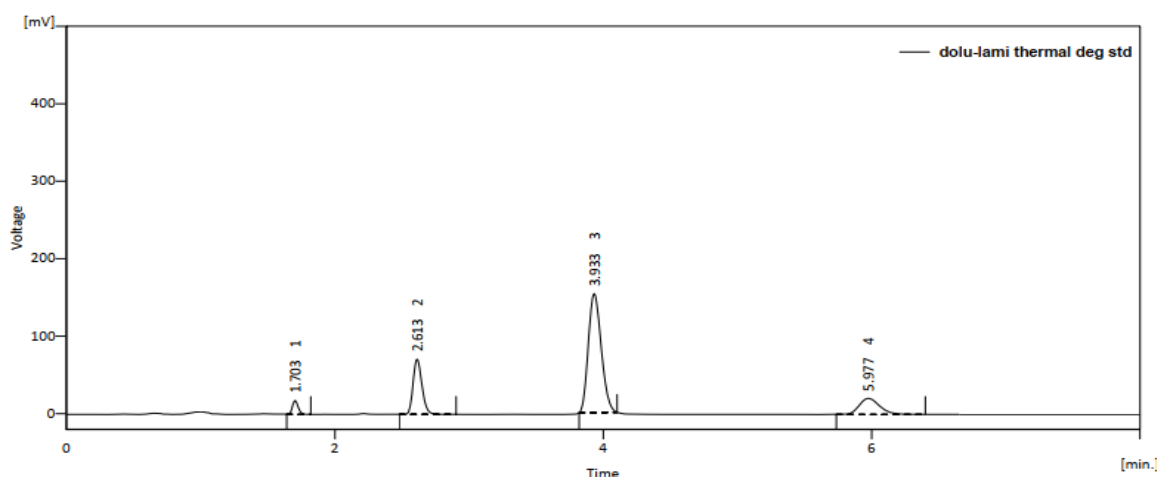

Fig. 5(b).

Fig. 5(c).

Fig. 5(d).

Fig. 5(e).

Fig. 5(a-e). HPLC Chromatograms for DVR and LMN Sample Degradation (A) Acid Degradation (B) Base Degradation (C) Oxidation Degradation (D) Photo Degradation (E) Thermal Degradation, Respectively

3.7 Forced Degradation Study

Forced degradation studies were performed to indicate stability of DVR and LMN at various stress conditions. Stress conditions used for forced degradation studies were as per ICH Q2R2 specifications. The stability of samples was cross checked with standard samples. Table 7 indicated results of various forced degradation condition on sample and standard. There is no interference was observed in the HPLC chromatograms (Fig. 5) of degraded products at respective drug peaks. The individual drug peak purity values were found to be within specific limits, hence, it is said that the developed RP-

HPLC method was specific and stability indicating.

4. CONCLUSION

The developed RP-HPLC chromatographic method was simple, accurate, specific, and selective, as well as stable, indicating that it could be used to estimate DVR and LMN in bulk and pharmaceutical dose form at the same time. During estimation of DVR and LMN using developed method, it indicates good resolution, LOD and LOQ. The validation of developed method was as per the acceptance criteria of the ICH Q2R2 guideline. Hence, validated stability

indicating method for estimation of DVR and LMN was successfully employed.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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