Original Research Article

An effective stability indicating RP-HPLC method for simultaneous estimation of Lamivudine and Raltegravir in bulk and their tablet dosage form.

REVIEW

.

ABSTRACT

Background:
A sensitive, Simple, and specific stability indicating reverse phase HPLC method was developed for simultaneous estimation of Lamivudine and Raltegravir in bulk and tablet dosage form. Good separation was achieved by injecting 10 µL of the standard solution into Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°) column, using a mobile phase composition of buffer (0.1% v/v Phosporic acid in water): Acetonitrile (40:60 v/v) and isocratic elution programming have been done at a flow rate of 1.0 mL/min. The eluted analytes detected at 260 nm wavelength.

Results:
The retention times of Lamivudine and Raltegravir were found to be 3.1 and 5.4 min respectively. The developed method was linear in the concentration range of 30 – 70 µg/mL and 60 – 140 µg/mL for Lamivudine and Raltegravir respectively.The percentage recoveries of Lamivudine and Raltegravirwere determined to be 100.30% and 100.53%, respectively. All the verification parameters were within the acceptance criteria of ICH guidelines, and the degradation products were well resolved from Raltegravir and Lamivudine peaks, which indicate the stability of the method. The developed RP-HPLC method was highly precise, specific, sensitive, and stability indicating.

Conclusion:
In the currently developed RP-HPLC analytical method, the elution time and run time is reduced, which proves that the method is economical and widely acceptable, also simple and practical, which can be used in routine quality control tests in the industry

Keywords:Lamivudine; Raltegravir; Isocratic elution; Stability indicating.

1. INTRODUCTION

The antiviral therapy with combination of multi drugs is a great advancement in the treatment of human immune virus (HIV) and hepatitis B diseases. The use of multiple drug therapy, i.e., at least three or more drugs alone or in combination daily is in practice to treat the HIV effectively [1-2]. However, extensive research on multiple drug therapy revealed that a t-drug regimen consisting of Lamivudine and Raltegravir controls the HIV disease effectively.

Lamivudine chemically, is 4-amino-1-[(2R, 5S)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one [3-6]. It ceases DNA replication by inhibiting the reverse transcriptase enzyme competitively[7]. Chemically, Raltegravir is a (4R,12aS)-N-[(2,4-difluorophenyl)methyl]-3,4,6,8,12,12a-hexahydro-7-hydroxy-4-methyl-6,8-dioxo-2H-pyrido[1′,2′:4,5]pyrazino[2,1b][1,3]oxazine-9-carboxamide, integrase strand transfer inhibitor(INSTI) that blocks HIV replication by preventing the integration of viral DNA into the genetic material of host (human immune cells (T cells)) [8-11] (Fig 1).

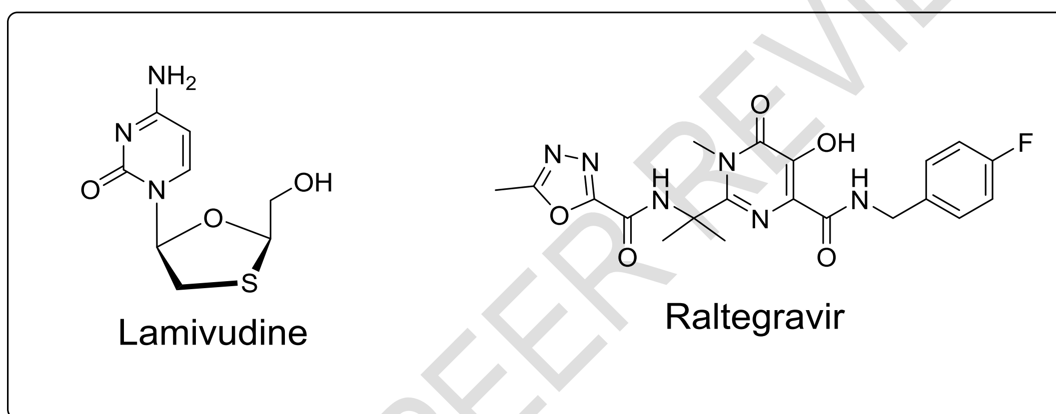

Fig. 1. Chemical structures of Lamivudine and Raltegravir

An effective analytical method is requisite for a drug to analyze individually or simultaneously in combination with other drugs in pharmaceutical industry. Extensive literature search revealed that few analytical methods such as UV methods and RP-HPLC methods were reported for estimation of Lamivudine and Raltegravir individually [12-15]. Further, there are some RP-HPLC methods available for simultaneous estimation of Lamivudine, Raltegravir, and tinofivirdisproxil fumarate or butcaver sulfate or abacavir in triple combination [16-20]. As per FDA official news, Lamivudine and Raltegravir fixed dose film-coated tablet got an approval in April 2019 for the treatment of HIV-1. To the best of our knowledge, there is no proper reported RP - HPLC method for simultaneous estimation of Lamivudine and Raltegravir in pharmaceutical formulations, previous to our work. Thus, efforts were made to develop fast, selective and sensitive analytical method for the estimation of Lamivudine and Raltegravir in their combined dosage form using reverse phase high performance liquid chromatographic method.

UNDER PEER REVIEW

2. MATERIAL AND METHODCONDITIONS

Materials

HPLC grade acetonitrile, Milli-Q water, Phosphoric acid and remaining analytical grade chemicals were obtained from Merck India Limited, Mumbai, India. API of Lamivudine and Raltegravir was provided by Fortune Pharma, Hyderabad, as a gift sample.

Chromatographic conditions

RP-HPLC experiments were performed on WATERS 2695 with 2487 PDA detector with auto sampler, data-processing, and acquisition has done by using the Empower 2 software. Effective separation attained by injecting 10 µL of the standard solution into Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°) column, using a mobile phase composed of buffer (0.1% v/v Phosporic acid in water) : Acetonitrile (40:60) at a flow rate of 1.0 mL/min, and the eluted analytes were detected at 260 nm wavelength. Ambient temperature 30°C was maintained in auto sampler and in the analytical column. Standard, sample and mobile phase solutions were filtered through a 0.45-µm nylon filter prior to injecting into the HPLC system (Fig. 2).

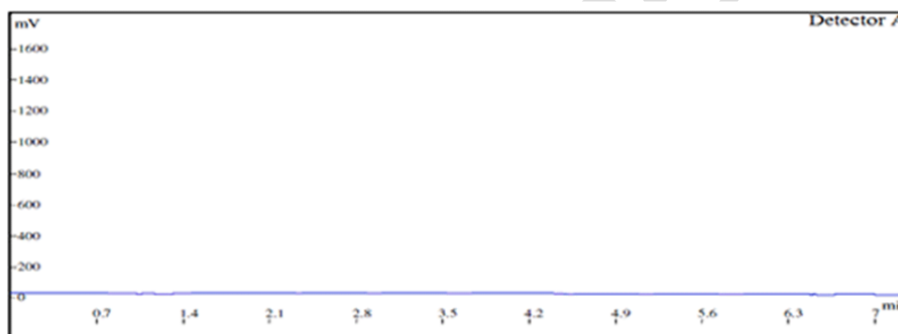

Fig. 2. Blank Chromatogram

Preparation of standard solution

Weigh accurately working standard equivalent to 50 mg of Lamivudine and 100 mg of Raltegravir transferred into 100 mL volumetric flask, add ~30 ml of diluent (acetonitrile and water (50:50)) and dissolve, further make up the volume with diluent. 1.0 ml of above solution was transferred into 10 mL volumetric flask, again volume made with diluents to obtain concentration of 50 µg/mL, and 100µg/mL for Lamivudine and Raltegravir, respectively, said as 100% level concentrations (Fig. 3).

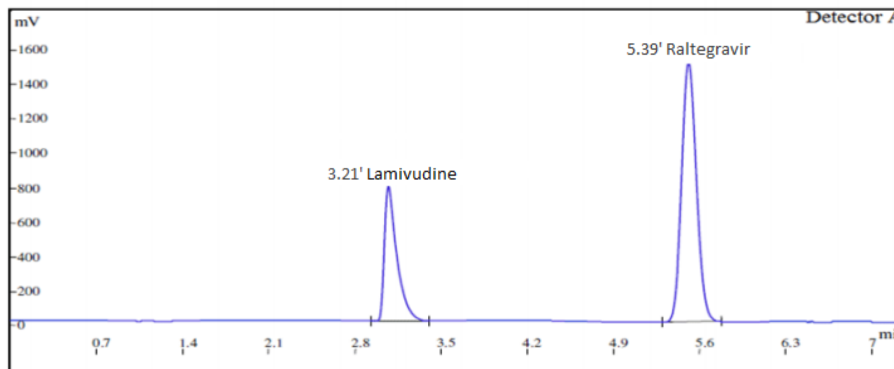

Fig. 3: Chromatogram of Lamivudine and Raltegravir standards.

UNDER PEER REVIEW

Preparation of sample solution

An amount of tablet (Dutrebis) powder (150 mg) equivalent to 50 mg of Lamivudine and 100 mg of Raltegravir was weighed and transferred into 100 mL volumetric flask, volume made with diluents to 100 mL. One milliliter of above solution was transferred into 10 mL volumetric flask, volume made with diluents to obtain concentration of 50 µg/mL, and 100µg/mL for Lamivudine and Raltegravir respectively. Prior to injecting, sample solution was filtered through 0.4 µm Nylon filter (Fig. 4).

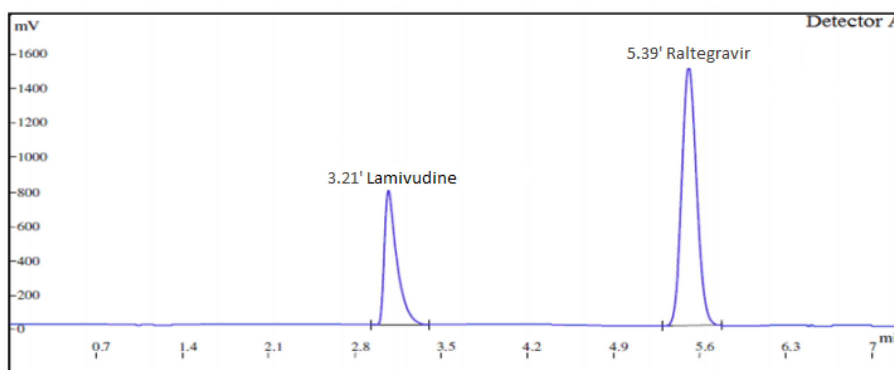

Fig. 4: Chromatogram of Lamivudine and Raltegravir samples.

3. METHOD VALIDATION

System suitability test

The system suitability test of the current method was carried out by injecting 100% level of working standard concentration in 6 replicates, and parameters like percentage relative standard deviation (% RSD), USP tailing factors (T), USP plate count (N), and resolution (R) were evaluated for the obtained chromatograms.

Linearity

The Linearity is the ability of the method to elicit test results that are proportional to concentration of the analyte in the sample. The linearity of the present method has

performed by injecting the series of working standard concentrations ranges from 30 µg/ml to 70 µg/ml of Lamivudine and 60 µg/mL to 140 µg/mL of Raltegravir into the HPLC system under optimized chromatographic conditions. Eventually, linearity graph was plotted for concentration vs peak area and determined regression coefficient (r2) value.

Precision

It is the closeness of test results obtained by the method to the true value. Usually, it was determined in the intraday and inter-day. Intraday and inter-day precision of the method were performed by injecting 100% level of working standard concentration for 6 times in a day and 3 times per day for three continuous days. Percentage RSD calculated for peak areas obtained.

Accuracy

The accuracy of the method was established by recovery of known amount sample solution spiked at three different standard concentration levels about 50, 100, and 150%, each level of solution injected in triplicate. The percentage mean recovery at three different levels of the drug solution was calculated.

Specificity

Specificity represents the ability of the method to determine or assess the intended drug in the presence of other substances without interferences. Ten microliter volume of prepared blank solution, 100% level pure working standard solution injected individually. The retention time (RT) of individual injection of standard sample solution alone and along with placebo was observed to assess any interference that has been happened with peaks of Lamivudine and Raltegravir in obtained chromatograms.

Sensitivity

The LOD and LOQ were calculated by implementation of standard deviation method, in which the following formulae were used.

LOD ¼ 3 σ=S

LOQ ¼ 10 σ=S

where σ is the standard deviation of the intercept, and S is the slope of the linear curve.

Robustness

The robustness of the method was checked by slightly and deliberately changing the flow rate, mobile phase composition, and maximum absorption wavelength. It can be performed by evaluating the system suitability parameters after changing the HPLC flow rate (± 0.1 mL/min) and mobile phase ratio (± 1 mL).

4.FORCED DEGRADATION STUDIES

In forced degradation studies, intentionally drug substance is exposed to conditions more intense than accelerated conditions. Chemical stability of the drug molecule can be depicted with forced degradation studies, which helps in successful development of stable formulation with appropriate storage conditions. ICH guidelines emphasized certain degradation

UNDER PEER REVIEW

conditions like acid hydrolysis, base hydrolysis, oxidation, thermal degradation, and photo stability in ICH Q1A, QIB, and Q2B guidelines.

Acidic degradation solution

1 mL of standard stock solution was transferred to 100 mL of Volumetric Flask; added 0.2 mL of 1 N HCl to it and reflux for 3 hours at 70° C, kept a side for 24 h at room temperature, and after that, neutralize the solution with 1 N NaOH. Further dilution was done to get a solution having 50 µg/mL of Lamivudine and 100 µg/mL of Raltegravir (Fig. 5).

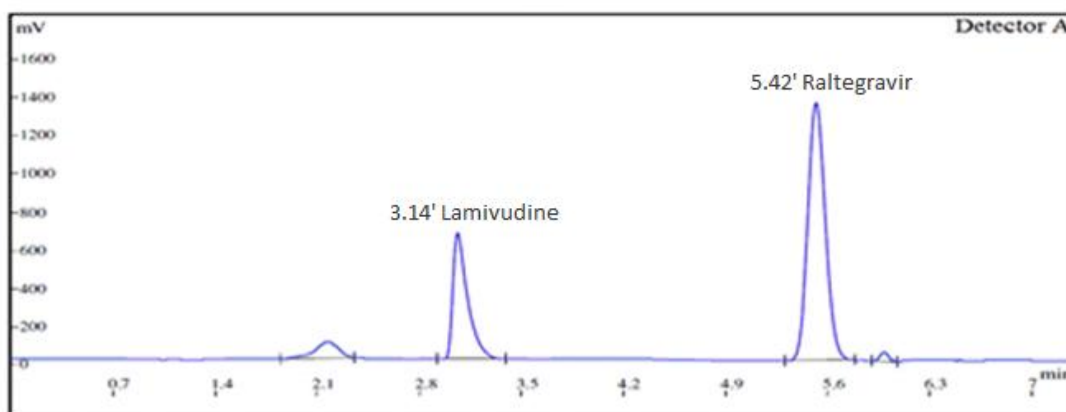

Fig. 5: Acidic stress degradation chromatogram of Lamivudine and Raltegravir.

Alkali degradation solution

1 mL of standard stock solution was transferred to 100 mL of Volumetric Flask; added 0.2 mL of 1 N NaOH to it and reflux for 3 hours at 70° C, kept a side for 24 h at room temperature, and after that, neutralize the solution with 1 N HCl. Further dilution was done to get a solution having 50 µg/mL of Lamivudine and 100 µg/mL of Raltegravir (Fig. 6).

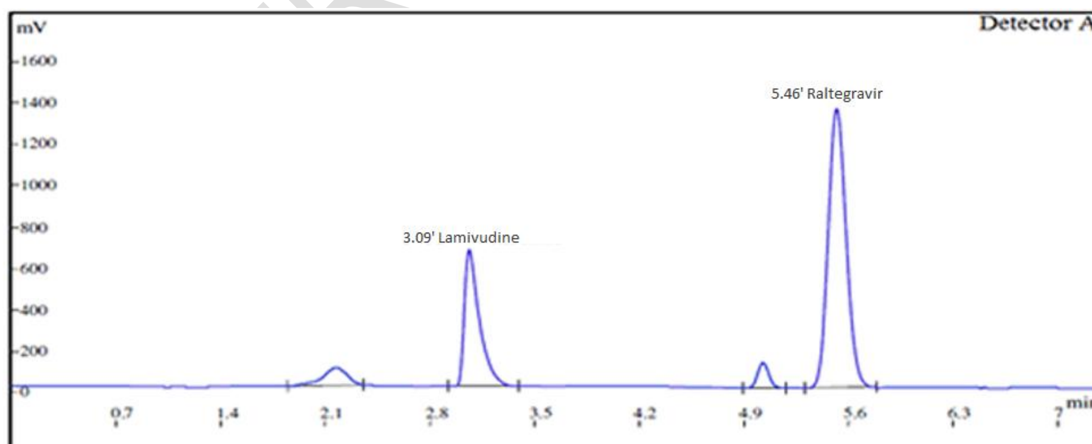

Fig. 6: Alkaline stress degradation chromatogram of Lamivudine and Raltegravir.

Oxidative degradation solution

1 mL of standard stock solution was transferred to 100 mL of Volumetric Flask; added 1 mL of 3% Hydrogen peroxide to it and reflux for 3 hours at 70° C, kept a side for 24 h at room temperature, and after that, make up to 10 mL with diluent to obtain concentration of 50 µg/mL and 100 µg/mL for Lamivudine and Raltegravir respectively. (Fig. 7).

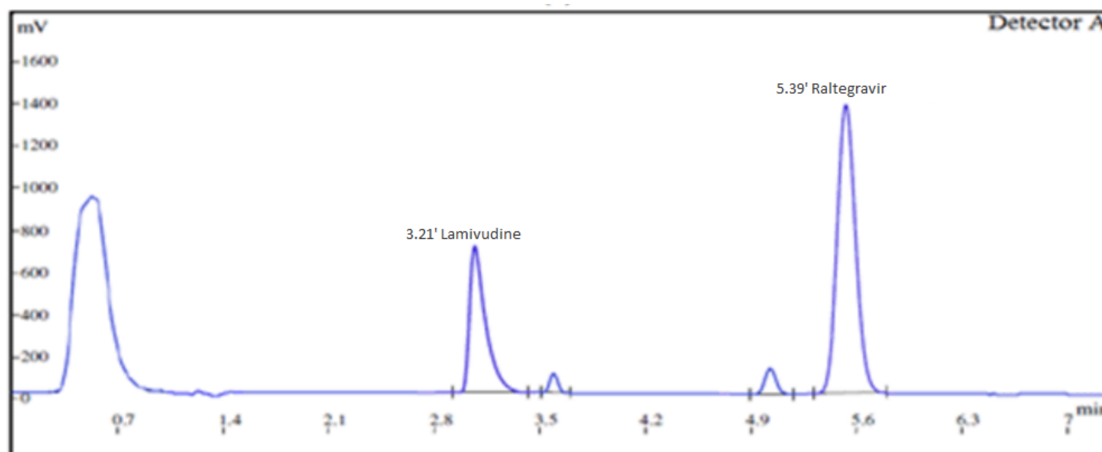

Fig. 7: Oxidative stress degradation chromatogram of Lamivudine and Raltegravir

Thermal degradation solution

100 mL of standard stock solution was transferred to 500 mL of Volumetric Flask and kept it in heating chamber at 80°C/75% RH for 24 h. One microliter of above solution is diluted to 10 mL to obtain concentration of 50 µg/mL and 100 µg/mL for Lamivudine and Raltegravir respectively (Fig. 8).

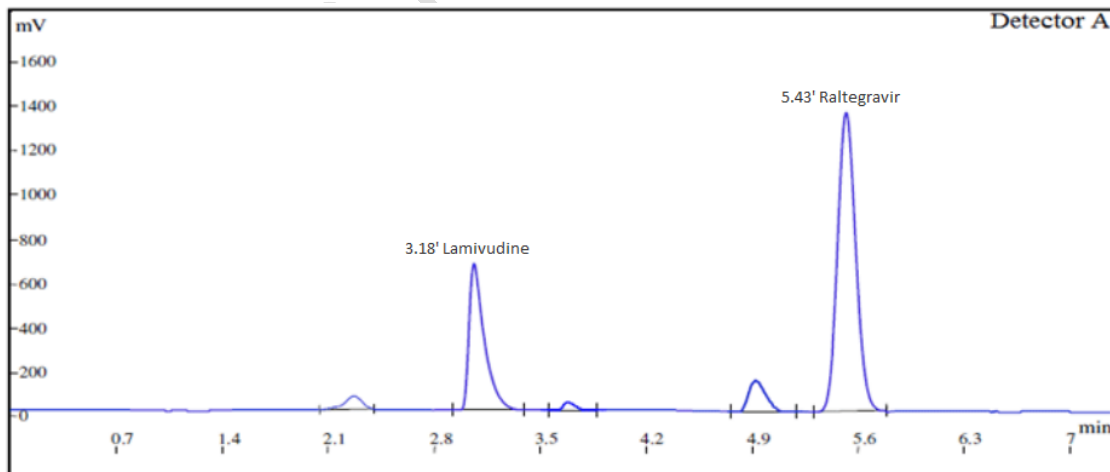

Fig. 8: Thermal stress degradation chromatogram of Lamivudine and Raltegravir.

Photolytic degradation

1 mL of sample stock solution filtrate was transferred to 100 mL of Volumetric Flask; it was kept for 12 hours in UV-light. Then volume was made up to mark with Diluent and mixed well and injected. The representative chromatogram is shown in Fig. 9.

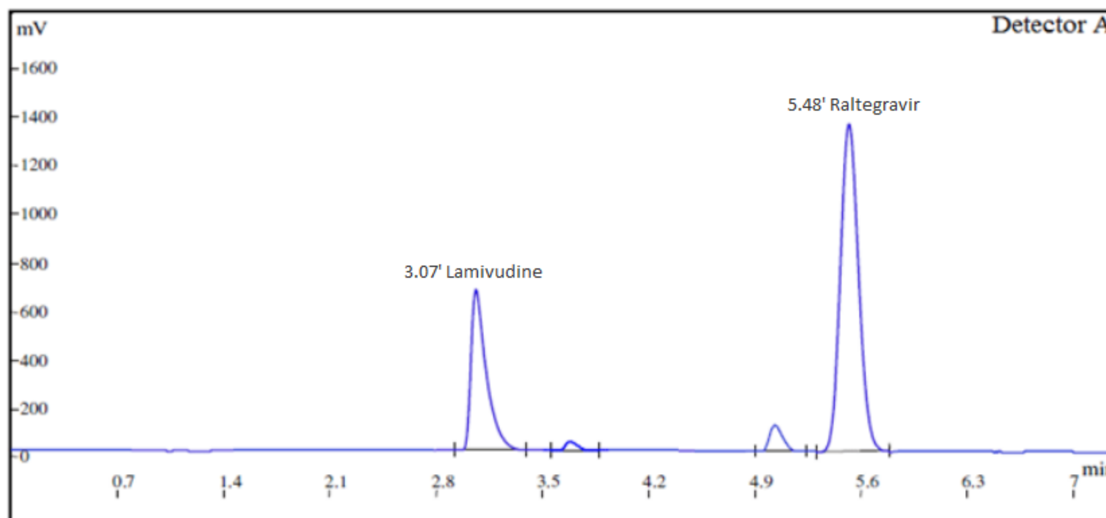

Fig. 9: Photolytic stress degradation chromatogram of Lamivudine and Raltegravir.

UNDER PEER

Assay

Assay of the method can be done by injecting subsequent injections of standard and sample solutions, both having concentration about 50 µg/mL and 100 µg/ mL of Lamivudine and Raltegravir respectively. The preparation of standard and sample solutions was mentioned prior the in methods section. Computing the percentage assay.

Results

Initially, solubility studies of the both drugs were done and found that Lamivudine was freely soluble in acetonitrile, water, and slightly soluble in methanol. Raltegravir was freely soluble in water and methanol. Based on the solubility of drugs, acetonitrile and water in (50: 50) ratio selected as diluent to prepare standard and sample solutions.

5. METHOD OPTIMIZATION

Method optimization has done by implementing trial and error method in such a way to obtain a chromatogram with good resolution (R), efficiency, accepted number of USP plates, and tailing factor. In this procedure, several trials have been done by altering mobile phase composition, columns, and flow rate. Finally, the method with Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°) column, mobile phase composition of buffer (0.1% v/v Phosporic acid in water) : Acetonitrile (40:60) and a flow rate of 1.0 mL/min was selected as optimized method. The results obtained in the trial and error method were mentioned in Table 1; trial 7 selected as optimized conditions and optimized chromatogram shown in Fig. 10.

Table 1. Different trials

Trail	Column	Buffer	Mobile Phase	Flow rate ml/min	Observation
01	X-terra MSC18 (150 X 4.6 mm, 5µm)	0.05% Phosphoric acid in water	Buffer: ACN (50:50)	1.0	Peaks tailing were observed.
02	X-terra MSC18 (150 X 4.6 mm, 5µm)	0.05% Phosphoric acid in water	Buffer: ACN (30:70)	1.0	Peaks resolution and symmetry were not good
03	X-Bridge C18 (150 X 4.6 mm, 3µm)	0.05% TFA in water	Buffer: ACN (50:50)	1.0	Asymmetrical peak shapes were observed
04	Luna C18 (150 X 4.6 mm, 5µm)	0.05% TFA in water	Buffer: ACN (50:50)	1.0	Resolution not sufficient
05	Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°)	0.05% TFA in water	Buffer: ACN (50:50)	1.0	Poor resolution with good peak shape
06	Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°)	0.05% Phosphoric acid in water	Buffer: ACN (50:50)	1.0	Good peak shape with minimum resolution
07	Zorbax SB-Phenyl (150 × 4.6 mm, 3.5 µ, 80 A°)	0.05% Phosphoric acid in water	Buffer: ACN (40:60)	1.0	Optimum resolution with good peak shape.

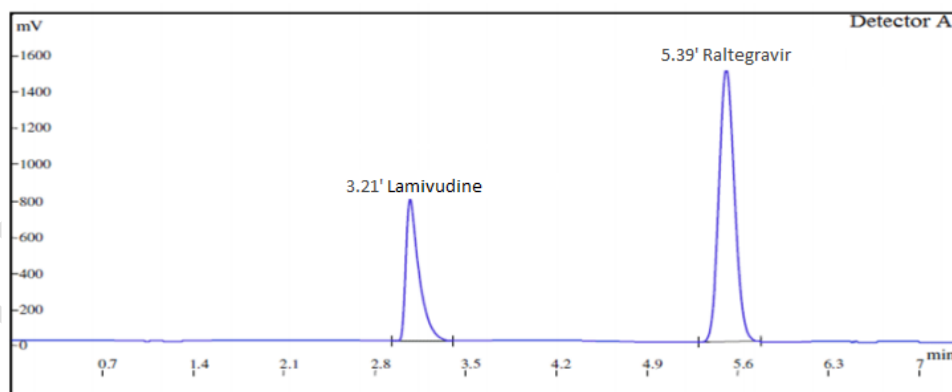

Fig. 10: Optimized chromatogram of the method.

6. METHOD VALIDATION RESULTS

System suitability

Upon injecting 100% level concentration, the data obtained from chromatograms illustrated that system suitability parameters include % RSD (≤ 2), USP tailing factor (≤ 2), and USP plate count (> 2000) values shown in **Table 2** were satisfying the acceptance criteria as per Q2 specifications of ICH guidelines.

Table 2. Results of system suitability parameters of 100% level standard solution

Injection	Lamivudine				Raltegravir			
	RT	Peak area	USP plate count (N)	USP tailing (T)	RT	Peak area	USP plate count (N)	USP tailing (T)
1	3.21	17,00,250	8300	0.94	5.47	33,50,465	6778	1.10
2	3.11	17,12,321	8340	0.92	5.46	33,59,786	6712	1.11
3	3.12	17,09,951	8430	0.94	5.46	33,58,697	6698	1.08
4	3.11	17,16,132	8641	0.94	5.46	33,52,365	6746	1.07
5	3.11	17,11,862	8531	0.93	5.47	33,49,325	6812	1.09
6	3.13	17,01,021	8475	0.95	5.46	33,55,897	6653	1.10
Mean	3.13	17,08,590	8452.8	0.93	5.46	33,54,423	6733.16	1.09
SD	0.0392	6483.915	125.39	0.0103	0.0051	4361.015	57.44	0.0147
%RSD	1.2517	0.3795	1.4834	1.1026	0.094521	0.1300	0.8531	1.3483

SD standard deviation, %RSD relative standard deviation, RT retention time, Acceptance limit % RSD (≤ 2), USP tailing factor (≤ 2), and USP plate count (> 2000).

Linearity

The linear response of the HPLC system for Lamivudine and Raltegravir was in the concentration range of 30 to 70 µg/mL and 60 to 140 µg/mL (Fig. 11) that was determined by constructing calibration curve between concentration and peak area (Table 3, Fig. 12 & 13). The computed regression coefficient (R2) value found to be 0.999 and 0.999 for Lamivudine and Raltegravir, respectively, and manifests the linearity of the method within the ICH guidelines limit.

UNDER PEER REVIEW

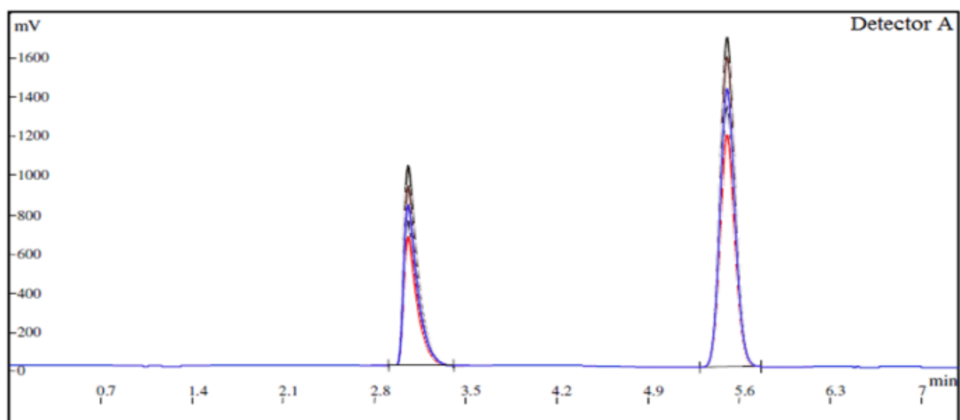

Fig. 11: Overlain Chromatogram of Lamivudine and Raltegravir (Linearity).

Table 3. Peak areas of linearity standard solutions of Lamivudine and Raltegravir

Lamivudine		**Raltegravir**	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
30	1028543	60	1998432
40	1340002	80	2642371
50	1700452	100	3350698
60	2049861	120	3946321
70	2374647	140	4673684
R^2	0.999	R^2	0.999

R^2 regression coefficient

UNDER PEER REVIEW

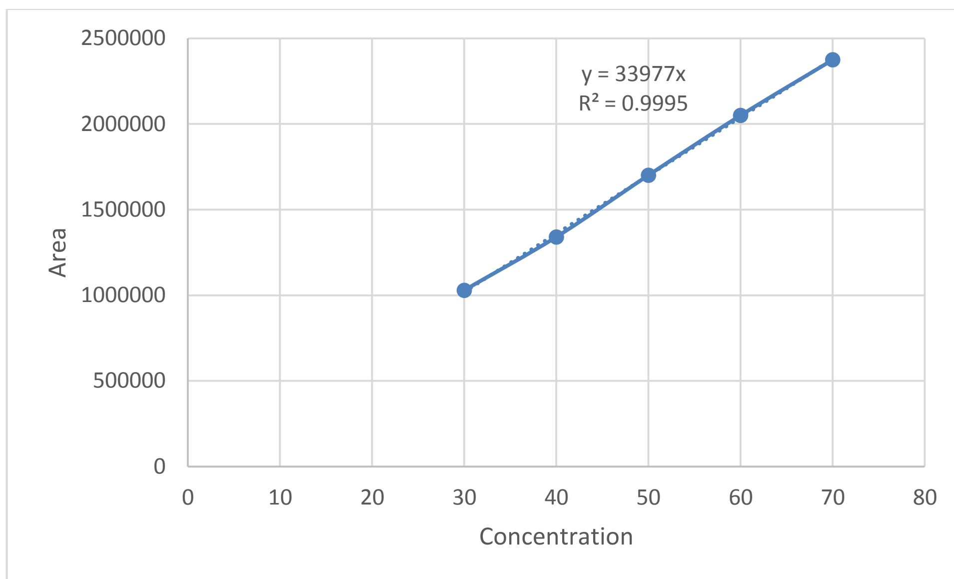

Fig. 12: Linearity curve of Lamivudine.

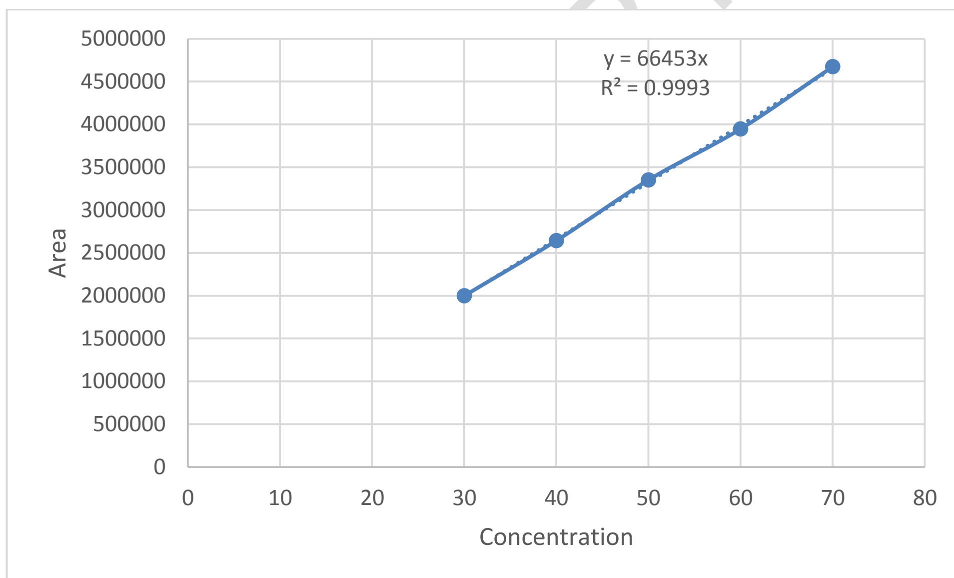

Fig. 13: Linearity curve of Raltegravir.

Accuracy

Percentage mean recovery of the Lamivudine and Raltegravir at three different concentration levels that were observed as 100% ± 2 illustrates the acceptance of the method as per Q2 specifications of ICH guidelines. Results were shown in Table 4.

Table 4: Results of percentage of recovery

%Level	Amount of Added	Amount of Recovered	% mean recovery
Lamivudine			
50	25	24.98	99.92
	25	25.11	100.44
	25	25.14	100.56
100	50	50.61	101.22
	50	49.89	99.78
	50	49.91	99.82
150	75	75.43	100.57
	75	75.01	100.01
	75	75.23	100.31
Raltegravir			
50	50	50.21	100.42
	50	50.43	100.86
	50	50.37	100.74
100	100	100.41	100.41
	100	100.32	100.32
	100	100.64	100.64
150	150	150.81	100.54
	150	150.94	100.63
	150	150.37	100.25

At each percentage level mean percentage recovery in the acceptable limit of 98 to 102%

<u>Precision</u>

UNDER PEER REVIEW

Percentage RSD value of peak area responses obtained by injecting 100% level working standard solution of Lamivudine and Raltegravir were found to be 0.45 and 0.77, respectively (Table 5), and depicts the precision of the method.

Table 5. Results of intraday and inter-day precision of 100% level solution

Precision	Lamivudine			Raltegravir	
Intra day	Sample Name	RT	Peak Area	RT	Peak Area
	Injection 1	3.14	1708060	5.41	3307641
	Injection 2	3.18	1716375	5.49	3343891
	Injection 3	3.10	1700431	5.46	3289458
	Injection 4	3.15	1710321	5.47	3293785
	Injection 5	3.19	1723211	5.42	3304987
	Injection 6	3.20	1708314	5.49	3349872
	Mean	3.16	1711119	5.456667	3314939
	SD	0.037417	7822.246	0.034448	25720.07
	%RSD	1.184069	0.457142	0.631302	0.775884
Inter-day	Sample Name	RT	Peak Area	RT	Peak Area
Day 1	Injection 1	3.13	1708945	5.44	3299451
	Injection 2	3.16	1710264	5.43	3309762
	Injection 3	3.15	1707968	5.43	3308758
Day 2	Injection 1	3.12	1706843	5.46	3317628
	Injection 2	3.12	1714697	5.44	3271847
	Injection 3	3.14	1713548	5.49	3263543
Day 3	Injection 1	3.13	1716954	5.43	3289785
	Injection 2	3.15	1709658	5.45	3258746
	Injection 3	3.11	1706427	5.47	3289644

UNDER PEER REVIEW

	Mean	3.134444	1710589	5.448889	3289907
	SD	0.016667	3674.985	0.020883	21217.44
	%RSD	0.531726	0.214837	0.383257	0.644925

SD standard deviation, %RSD relative standard deviation, RT retention time.

Sensitivity

The LOD and LOQ determined as 3.65µg/mL and 11µg/mL for Lamivudine and 1.6 µg/mL and 5 µg/mL for Raltegravir, respectively, which indicates that method has good sensitivity.

Robustness

Slightly deliberate changes in mobile phase ratio, flow rate, and absorption maximum of the method could not produce the system suitability parameter values beyond the acceptance limits that (Table 6) represent the method's robustness.

Table 6. Results of robustness of 100% level solution

Variation of parameter		**Lamivudine**					**Raltegravir**				
		RT	Peak area	USP plate count	USP tailing factor	% assay	RT	Peak area	USP plate count	USP tailing factor	% assay
Mobile phase ratio	39:61	3.11	1710258	8643	0.91	100.02	5.42	3341672	6687	1.09	99.98
	40:60	3.14	1711483	8580	0.93	100.10	5.39	3362839	6630	1.11	100.04
	41:59	3.17	1712346	8612	0.97	100.41	5.47	3312484	6641	1.17	100.13
Flow rate (±0.1 ml)	0.9	3.37	1714872	8742	1.01	99.87	5.64	3374621	6740	1.24	100.43
	1.0	3.14	1719682	8690	0.96	100.42	5.41	3369481	6638	1.10	100.17
	1.1	3.93	1719989	8583	0.97	100.17	5.20	3358532	6618	1.06	100.32

RT retention time; slight change in method parameter could not affect the USP plate count and tailing factor.

7. FORCED DEGRADATION RESULTS AND DISCUSSION

UNDER PEER REVIEW

In general, the acceptable percentage of degradation in a stability indicating method is not more than 20%. Percentage degradation was calculated by comparing the peak areas of 100% level working standard concentration at normal and stress conditions. Results were shown in Table 7 & 8.

Table 7.Retention time of degradant product of Lamivudine and Raltegravir (Stress Degradation Study)

Conditions	Retention time (Rt) (minute)				
	Lamivudine	Raltegravir	Degradant 1	Degradant 2	Degradant 3
Acid Degradation	3.14	5.42	2.14	6.02	-
Base Degradation	3.09	5.46	2.14	5.09	-
Oxidation Degradation	3.21	5.39	3.56	5.03	-
Thermal Degradation	3.18	5.43	2.30	3.59	4.98
Photolytic Degradation	3.07	5.48	3.60	5.11	-

Table 8. Results of forced degradation studies

Stress Type	Stress Conditions	Lamivudine		Raltegravir	
		% Assay	% Degradation	% Assay	% Degradation
Control Sample	Sample itself	98.9	NA	100.5	NA
Acid Degradation	1 N HCl, 0.2 mL at 70°C for 3 hr	87.2	11.5	85.9	14.5
Base Degradation	1N NaOH 0.2 mL at 70°C for 3 hr	80.9	17.6	87.2	13.2
Oxidation Degradation	1mL 3% H_2O_2 at 70°C for 2 hr	88.3	10.2	88.1	13.2

UNDER PEER REVIEW

Thermal Degradation	At 80°C for 24 hr	87.2	11.3	86.9	13.5
Photolytic Degradation	At UV light for 12 hr	86.8	11.7	87.1	13.3

Percentage assay

Percentage assay of the Lamivudine and Raltegravir tablets that were found as 100% ± 15 indicates that the analyzed tablets have percentage purity within the acceptance limits as per ICH guidelines. Results were shown in Table 9.

Table 9. Results of % assay of the tablet dosage form

Drug	Peak Name	RT	Peak area	USP tailing	USP plate count	Label claim (mg)	% Assay
Lamivudine	Standard	3.109	1708060	0.98	8340	150	99.5
	Test	3.113	1691240	0.99	8695		
Raltegravir	Standard	5.418	3307641	1.01	6712	300	99.64
	Test	5.413	3301975	1.01	6953		

Average weight of the tablet 500 mg, % purity of Lamivudine standard (API) 99.5, and % purity of Raltegravir standard (API) 99.6.

8. DISCUSSION

The stability indicating RP-HPLC assay method plays a significant role in determination of intrinsic stability, both qualitative and quantitative estimation of drug product and drug substance. Till date, many analytical methods have been developed for Lamivudine and Raltegravir individual and in combination with other anti-retroviral drugs. But, no RP-HPLC method has been existed for the simultaneous estimation of Lamivudine and Raltegravir. Hence, attempts were made to develop an effective stability indicating RP-HPLC method. The RT in the reported method was 5.46 min for Raltegravir and 3.13 min for Lamivudine, represents the method with good and effective retention time, and can be treated as economical as it reduces solvent consumption and analyte run time. Hence, rapid analysis of more number samples can be done. The calculated and statistical results of the validation parameters were not out of the acceptance limits stated by ICH.

9. CONCLUSION

A simple, accurate, sensitive, and specific RP HPLC with UV detector and isocratic elution method was successfully developed for the simultaneous estimation of Lamivudine and Raltegravir in bulk and its combined film-coated tablet formulation. Forced degradation studies were done by applying several stress conditions to assess the stability of the method. The proposed method was successfully separate both of the drugs and its

UNDER PEER REVIEW

degradation products with good resolution and quantifies the active contents at minute concentration levels. The developed method has specific, sensitive, and stability-indicating power. Hence, the proposed method can be adapted to regular analysis in pharmaceutical industry.

COMPETING INTERESTS DISCLAIMER:

AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. 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.

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UNDER PEER REVIEW

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