CHAPTER-4

DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF LAMIVUDINE AND DOLUTEGRAVIR IN DRUG PRODUCT BY RP-HPLC METHOD

4.1 Drug Profile

4.1.1 Lamivudine:

Lamivudine, commonly known as 3TC, is a nucleoside reverse transcriptase inhibitor (NTRI) and anti-retroviral drug, with activity against Human Immuno Deficiency Virus-I and II (causing AIDS) and Hepatitis B virus. It is used effectively against chronic hepatitis B virus, when other options failed. Generally it is used in combination therapy with other anti retroviral drugs like zidovudine, abacavir, dolutegravir, etc.

In 1995, US approved the use of lamivudine and also it is on the WHO's list of essential, safe and most effective medications. It is a generic drug [13].

Structure:

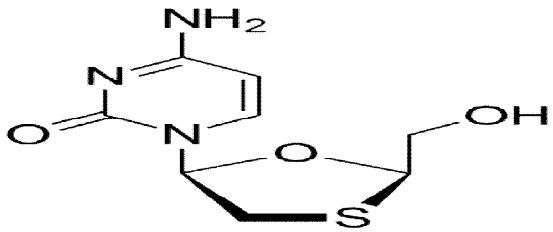

Fig 4.1: Structure of Lamivudine

IUPAC Name **:** 4-Amino-1-[(2*R*, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one

Molecular formula : $C_8H_{11}N_3O_3S$

Molecular Weight **:** 229.26 g/mol

Solubility **:** Soluble in ACN, Water.

Pka : 14.29

Pharmacokinetics and pharmacodynamics:

Lamivudine, a cytidine analogue, inhibits thereverse transcriptase of both the types of HIV-I and II and HBV. It undergoes phosphorylation to give 5^1 –triphosphate lamivudine triphosphate, which are incorporated into viral DNA to prevent the action of reverse transcriptase enzyme. Thus it terminates the DNA synthesis of virus, which inturn leads to the termination of viral DNA growth.

It is taken orally and is absorbed with a bio availability of over 80% at a faster rate. It is advised to take 150mg tablet daily twice and the peak concentration observed was 1.5 $\pm$ 0.5µg/ml, in HIV-1 patients. The absorption of lamivudine with food is slow, when compared to the fasted state. It bounds to plasma proteins uoto 36% and its half-life is 5-7 hours in adults and 2hours in HIV-infected children.

Lamivudine is also used in combination therapy is highly potential and increases the efficiency to inhibit HIV enzymes [14].

4.1.2 Dolutegravir

Dolutegravir (DTG), an integrase inhibitor, approved by FDA, used for the treatment of HIV. Tivicay, the brand name of dolutegravir, marketed by Glaxosmithkine and it is only approved for the age group above 12years, with atleast 40kg weight.

For both the patients, who has taken HIV therapy with other integrase strand transfer inhibitors and the patients without previous HIV therapy, dolutegravir can be found very helpful for HIV treatment [15].

Structure:

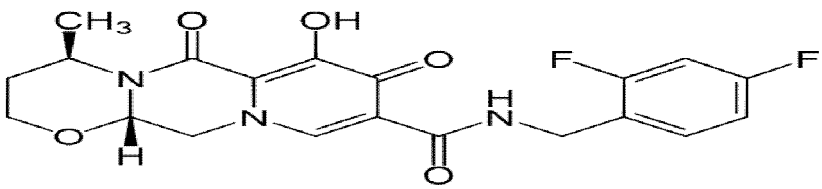

Fig 4.2: Structure of Dolutegravir

IUPAC Name: (4*R*,12a*S*)-*N*-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2*H*-pyrido[1',2':4,5]pyrazino[2,1-*b*][1,3]oxazine-9-carboxamide

Molecular formula : $C_{20}H_{19}F_2N_3O_5$

Molecular Weight : 419.38 g/mol

Solubility: Soluble in methanol, Acetonitrile and water.

Pka: 8.7

Pharmacokinetics and pharmacodynamics:

Dolutegravir, an HIV-1 antiviral agent, binds to the active site of HIV integrase and blocks the transfer of strand during DNA integration, an essential step of viral DNA replication cycle, which results in the inhibition of viral activity.

When 50mg of DTG was orally given to the patients daily once, within about 5 days of duration, with an average accumulation ratio of AUC, C_{max} and C24h ranging from 1.2-1.5, a steady state wil be achieved. It is highly protein bound to human plasma proteins i.e., $\geq$ 98.9 and is primarily metabolised by UGTIAI and also with CYP3A. Its biological half lide is 14hours and the peak plasma concentration was observed 2-3 hours of post-dosage.

DTG can be eliminated 53% in faeces unchanged, 31% in urine, as ether glucoronide of DTG (a metabolite formed by oxidation at benzylic carbon and its hydrolytic N-dealkylation product). A minimum percentage (<1%) of unchanged drug was renally eliminated [16].

Combination drug of Lamivudine/ Dolutegravir/ Abacavir (Triumeq):

Triumeq, is a combination drug of abacavir, dolutegravir and lamivudine, used for the treatment of HIV/AIDS. It includes 600mg if abacavir, 50mg of DTG and 300mg of lamivudine. It was approved by FDA in US, 2014.

Abacavir, a guanosine analogue, inhibits the replication of viral DNA by blocking the activity of RNA dependent DNA polymerase. DTG, by binding to the

active site of integrase, inhibits the strand transfer step of integration during replicaion of DNA. Lamivudine, a cytosine analogue, terminates the viral DNA chain by the inhibition of HIV reverse transcription.

4.2 Method Development for the simultaneous estimation of lamivudine and Dolutegravir:

The equipments and chemicals used were listed in the table in chapter 3.2.1

4.2.1 Preparation of buffer: 1ml of Tri flouro acetic acid was taken into a clean 1000ml beaker and dissolved in HPLC grade water and the volume was adjusted to in 1000ml. The resulting solution was sonicated for 15min.Then the solution was filtered through 0.45µ nylon filter.

4.2.2 Preparation of Mobile phase: The mobile phase was prepared by taking 30 volumes of buffer and 70 volumes of acetonitrile and mixed well and sonicated for 5 min. The resulting solution was filtered through 0.45µ nylon filter.

4.2.3 Preparation of standard stock solution: A 300 µg of pure Lamivudine and 50 µg of Dolutegravir were weighed and transferred to 50 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluent to give a primary stock solution. From the above solution 1ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with diluent to give a solution containing 600µg/ml of Lamivudine and 100 µg/ml Dolutegravir .

4.2.4 Preparation of sample solution: Accurately weighed twenty tablets were ground to obtain fine powder equivalent to 300µg of Lamivudine and 50µg of Dolutegravir sample were weighed and transferred to 50 ml of volumetric flask and dissolved in diluent. The flask was shaken and volume was made up to mark with diluents to give a primary stock solution. From the above solution 1 ml of solution is pipette out into a 10 ml volumetric flask and volume was made up to mark with diluents to give a solution containing 600µg/ml of Lamivudine and 100 µg/ml Dolutegravir.

Results and discussions:

4.2.5 Determination Of Working Wavelength (λ max): 10 mg of the Lamivudine and Dolutegravir standard drug is taken in a 10 ml volumetric flask and dissolved in diluent and volume made up to the mark, from this solution. 0.1ml is pipetted into 10ml volumetric flask and made upto the mark with the water to give a concentration of 10 µg/ml. The above prepared solution is scanned in UV between 200-400 nm using water as blank. The λ_{max} was found to be 260nm.

After several initial trails with mixtures of methanol, water, Acetonitrile and buffer in various combinations and proportions, a trail with a mobile phase mixture of 0.1%v/v Formic acid in water: Acetonitrile (30:70). The flow rate was 0.8 ml/ minute brought sharp peaks. **(Figure 4.8)**

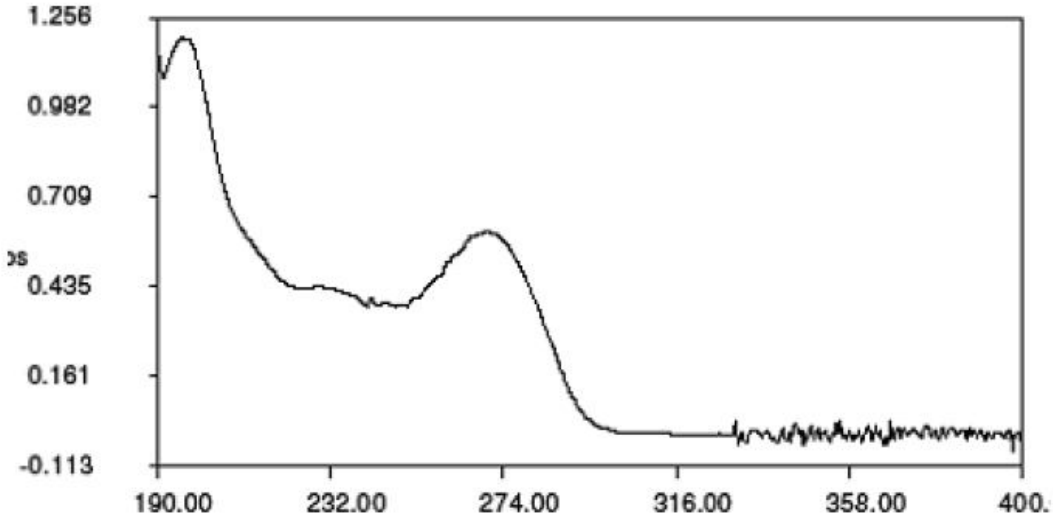

Fig 4.3: UV spectrum of Lamivudine

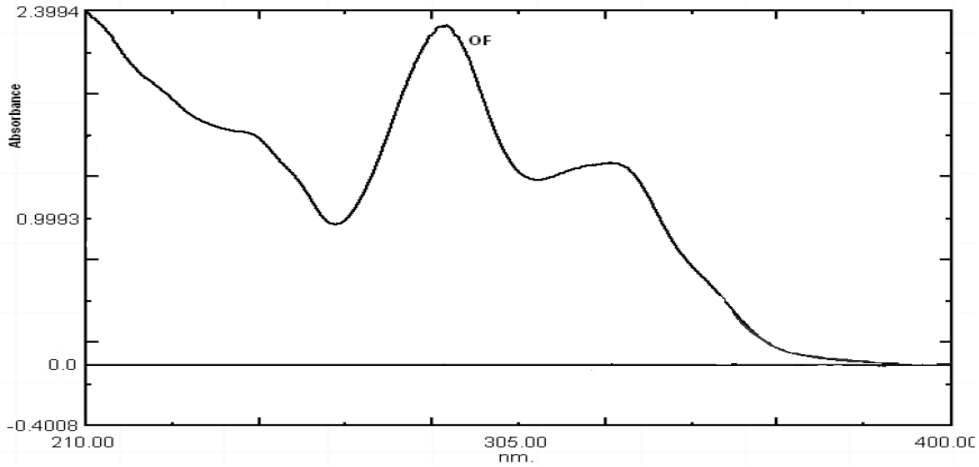

Fig 4.4: UV spectrum of Dolutegravir

Trail-1:

Buffer	: 0.1%Formic acid in water
Mobile Phase	: Buffer: Methanol (20:80 %v/v)
Column	: Inertsil ODS 3V column (150*4.6mm, 5µm)
Flow Rate	: 1.0ml/min
Temperature	: Ambient
Volume	: 10µl
Detector	: 260nm
Diluents	: Water: ACN (50:50)

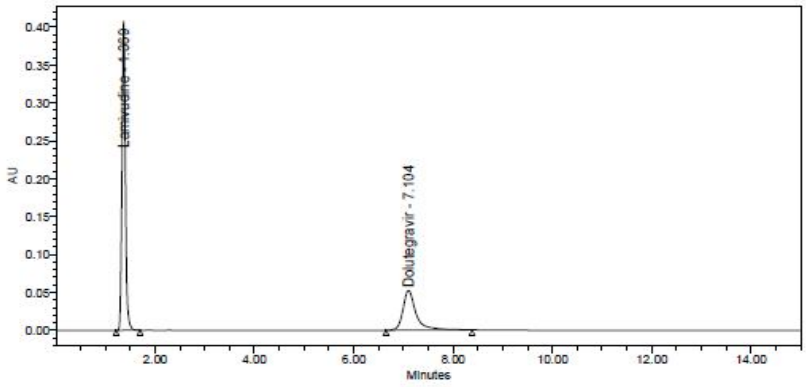

Fig 4.5: Chromatogram of Trail 1

Observation: Dolutegrair peak shape not good and Lamivudine not retained.

Trail-2:

Buffer : 0.05 M Ammonium acetate in water

Mobile Phase : Buffer: Acetonitrile (40:60 %v/v)

Column : Inertsil ODS 3V column (250*4.6mm, 5µm)

Flow Rate : 1.0 ml/min

Temperature : Ambient

Volume : 10µl

Detector : 260nm

Diluent : Water: ACN (50:50)

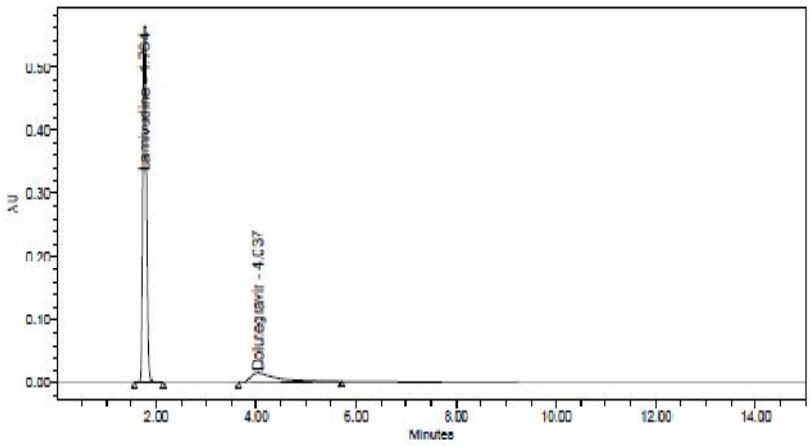

Fig 4.6: Chromatogram of Trail 2

Observation: Dolutegrair peak shape not good and Lamivudine not retained.

Trail-3:

Buffer : 0.05 M Ammonium acetate in water

Mobile Phase : Buffer: Acetonitrile (60:40%v/v)

Column : Inertsil ODS 3V column (250*4.6mm, 5µm)

Flow Rate : 1.0ml/min

Temperature : Ambient

Volume : 10µl

Detector : 260nm

Diluents : Water: ACN (50:50)

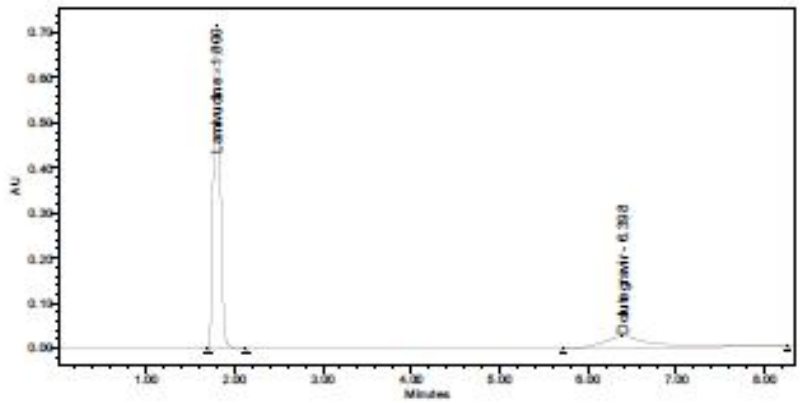

Fig 4.7: Chromatogram of trail 3

Observation: Dolutegrair peak shape not good and retention time more.

4.2.6 Optimized Chromatographic conditions:

Buffer : 0.1%TFA in water

Mobile Phase : Buffer: ACN (30:70%v/v)

Column : Inertsil ODS 3V column (250*4.6mm, 5µm)

Flow Rate : 0.8ml/min

Temperature : Ambient

Volume : 10µl

Detector : 260nm

Diluents : Water: ACN (50:50)

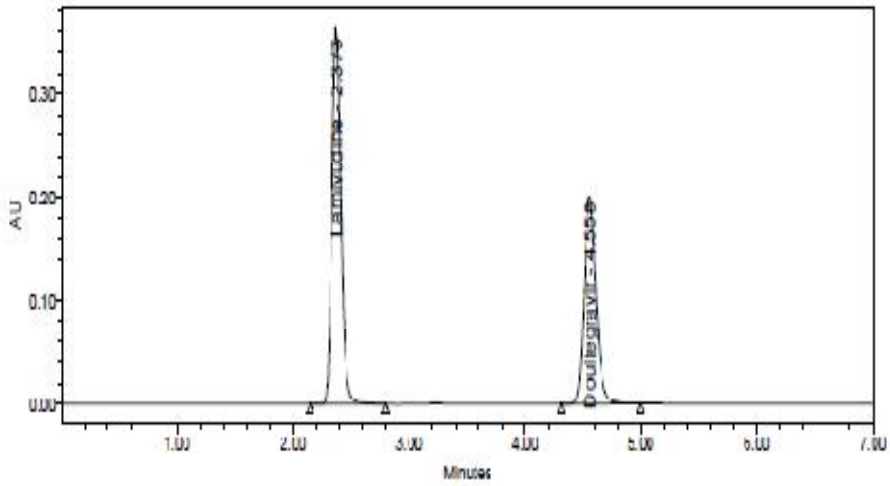

Fig 4.8: Typical Chromatogram of Lamivudine and Dolutegravir

4.3 Method Validation for the simultaneous estimation of Lamivudine and Dolutegravir:

4.3.1 Linearity: Linearity was studied by analyzing five standard solutions covering the range of 300-900µg/ml for Lamivudine and 50-150µg/ml and Dolutegravir. From the primary stock solution 0.5ml, 0.75ml, 1.0ml, 1.25ml, 1.5 ml of aliquots are pipetted into 10 ml volumetric flasks and made up to the mark with the diluent to give a concentrations of 50 µg /mL, 75µg/mL, 100µg/mL, 125µg/mL and 150 µg/mL of Dolutegravir and 300µg/mL, 450µg/mL, 600µg/mL, 750µg/mL and 900µg/mL Lamivudine.

Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions, shown in figures 4.13 and 4.14 and the obtained data were subjected to regression analysis using the least squares method **(Table 4.1).**

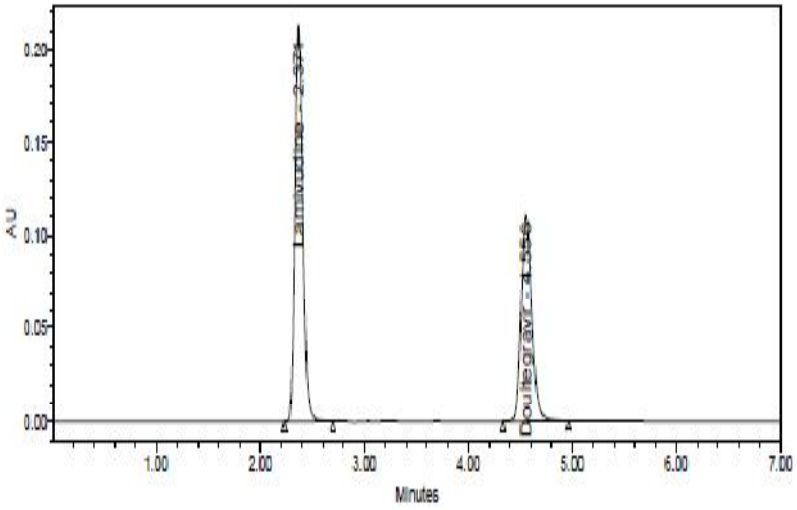

Fig 4.9: Chromatogram representing linearity 1

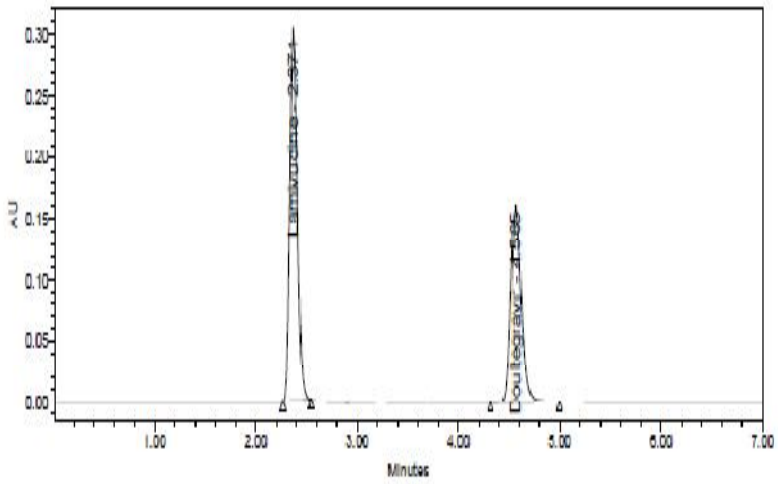

Fig 4.10: Chromatogram representing linearity 2

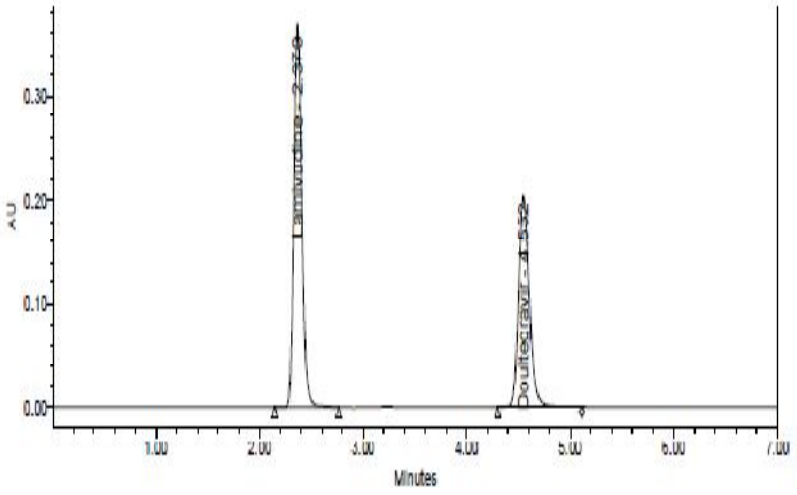

Fig 4.11: Chromatogram representing linearity 3

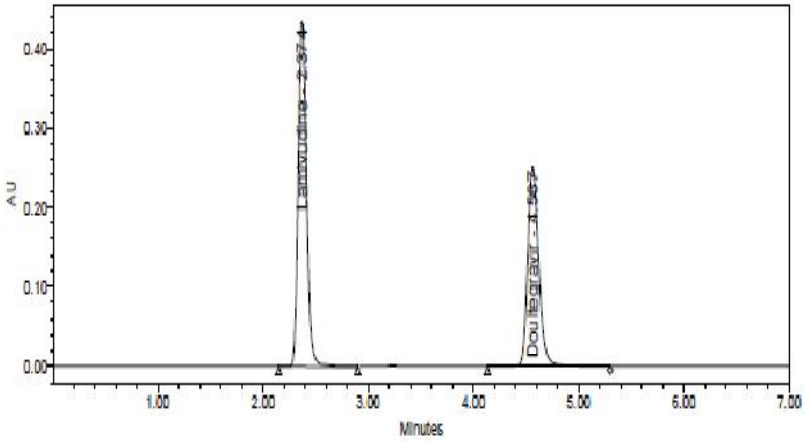

Fig 4.12: Chromatogram representing linearity 4

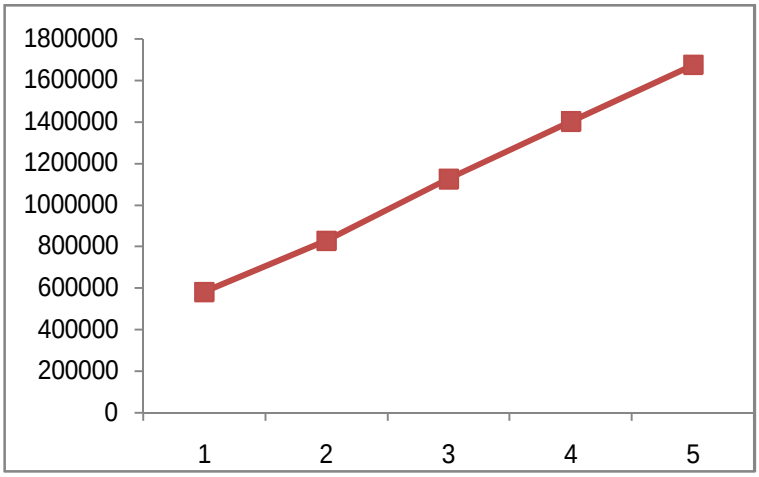

Fig 4.13: Linearity (calibration) curve of Lamivudine

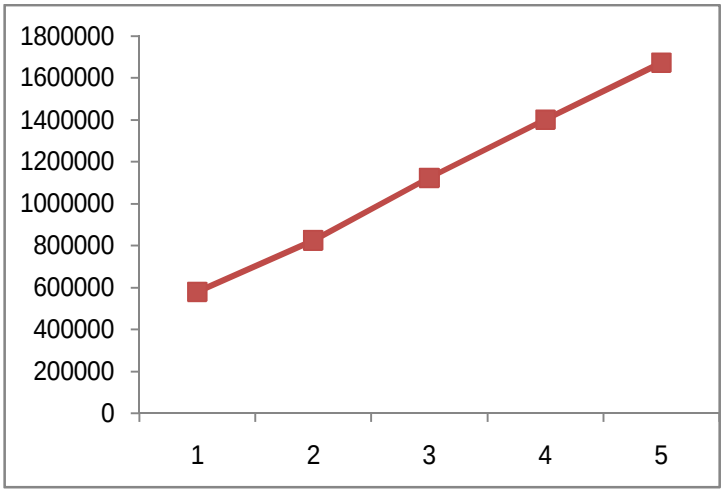

Fig 4.14: Linearity (calibration) curve of Dolutegravir

Table 4.1: Linearity data of Lamivudine and Dolutegravir

Level	Lamivudine		Dolutegravir	
	Concentration (mg/mL)	**Peak area**	**Concentration (mg/mL)**	**Peak area**
50%	0.3	1094895	0.05	791517
75%	0.45	1575825	0.075	1146502
100%	0.6	1938291	0.1	1473223
125%	0.75	2372107	0.125	1821943
150%	0.9	2847386	0.15	2233794

Result:

A linear relationship between peak areas versus concentrations was observed for Lamivudine and Dolutegravir in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.9991 and 0.9992 for both Lamivudine and Dolutegravir which prove that the method is linear in the range of 50% to 150%.

4.3.2 Method precision (Repeatability):

The precision of the method was checked by repeated preparation (n=6) of 600µg/ml of Lamivudine and 100µg/ml Dolutegravir without changing the parameter of the proposed chromatographic method **(Figures 4.15 to 4.20)** and measured the peak areas and retention time.

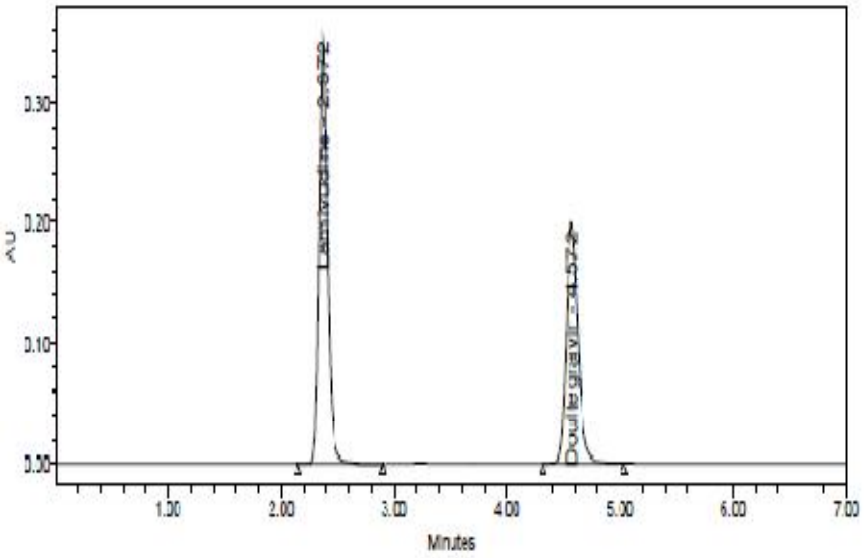

Fig 4.15: Chromatogram for precision injection 1

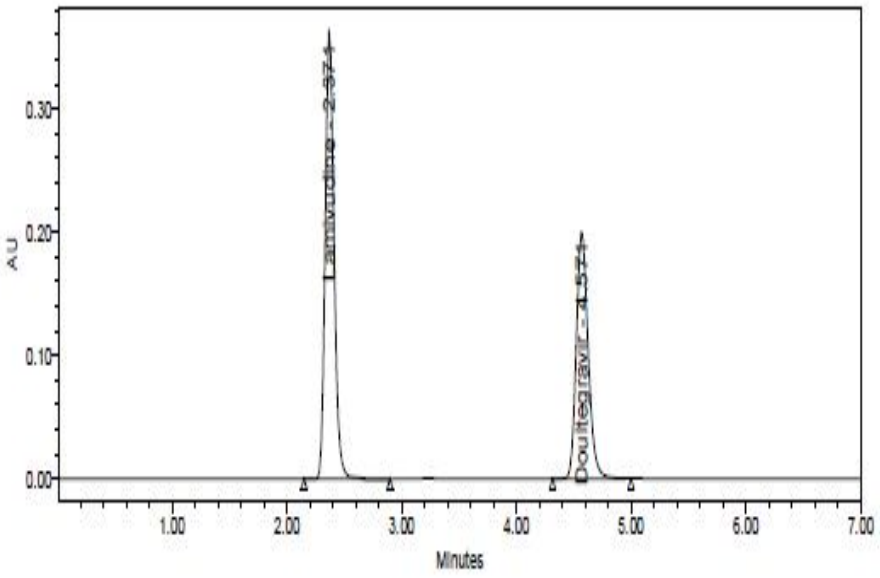

Fig 4.16: Chromatogram for precision injection 2

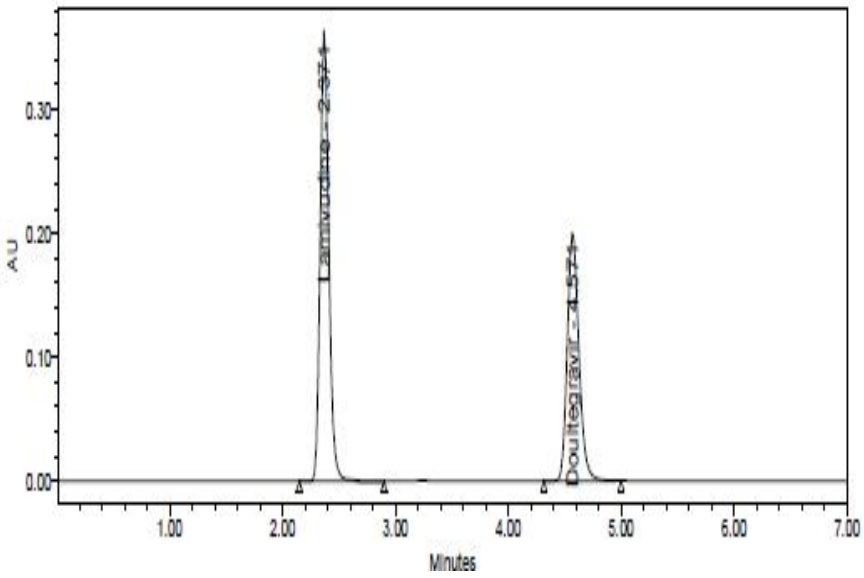

Fig 4.17: Chromatogram for precision injection 3

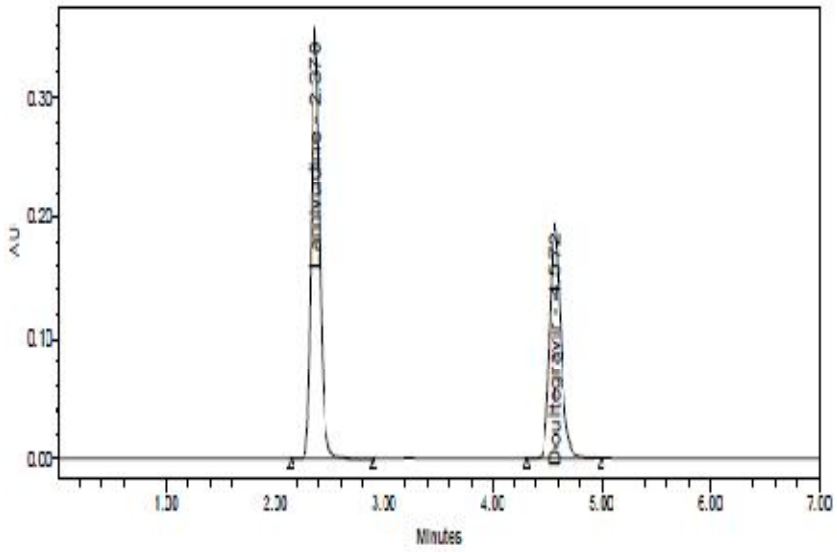

Fig 4.18: Chromatogram for precision injection 4

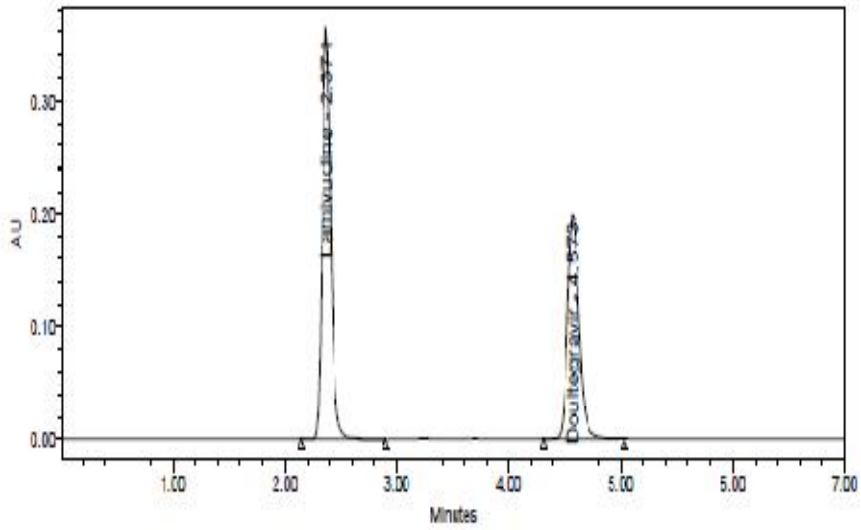

Fig 4.19: Chromatogram for precision injection 5

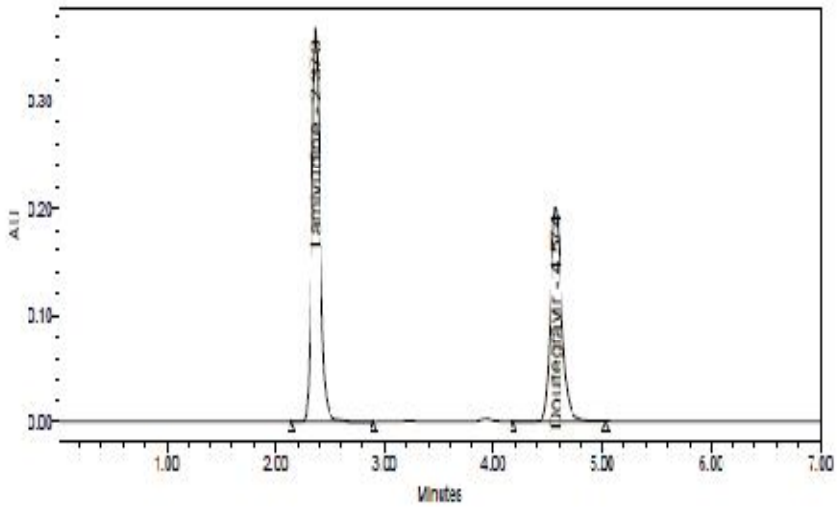

Fig 4.20: Chromatogram for precision injection 6

Table 4.2: Summary of peak areas for method precision of Lamivudine and Dolutegravir

Sample No	Lamivudine			Dolutegravir		
	Retention time (min)	Peak area	% Assay	Retention time(min)	Peak area	% Assay
1	2.372	1926157	100.1	4.572	1433404	100.2
2	2.371	1942639	99.5	4.571	1438312	99.7
3	2.371	1932993	99.6	4.571	1436765	99.4
4	2.370	1890193	98.6	4.572	1393554	99.2
5	2.371	1933047	98.8	4.573	1439888	99.6
6	2.370	1956298	99.4	4.574	1453754	99.4
Mean	2.371	1930221	99.4	4.572	1432613	99.6
%RSD	0.03	1.15	0.54	0.03	1.42	0.35

Result:

Results of variability were summarized in the above **Table 4.2**. Percentage relative standard deviation (%RSD) was found to be less than 2.0% which proves that method is precise**.**

4.3.3 Accuracy (% Recovery):

The accuracy of the method was determined by calculating the recoveries of Lamivudine and Dolutegravir by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of Lamivudine and Dolutegravir, the chromatograms were shown in **Figures 4.21 to 4.23.** The percentage recovery results obtained are listed in **Table 4.3.**

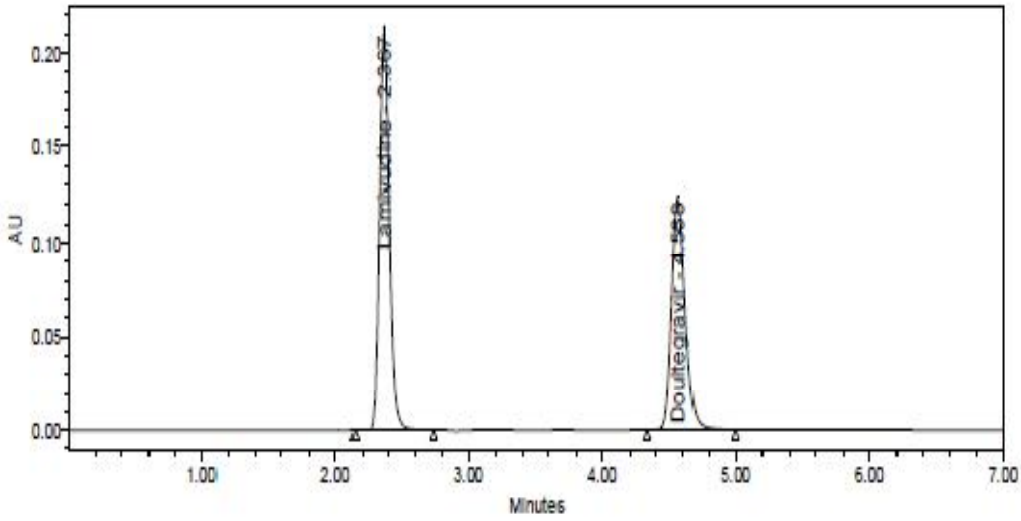

Fig 4.21: Typical Chromatogram for Accuracy 50 %

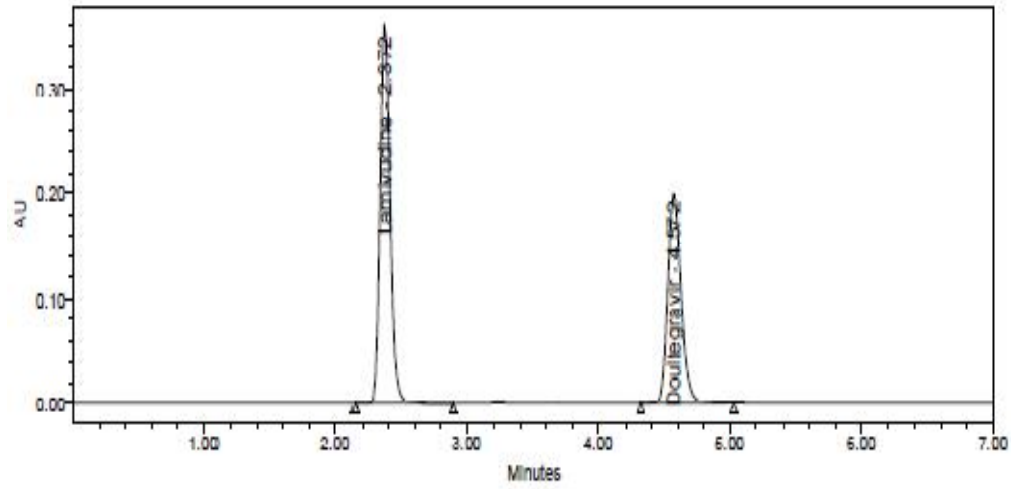

Fig 4.22: Typical Chromatogram for Accuracy 100 %

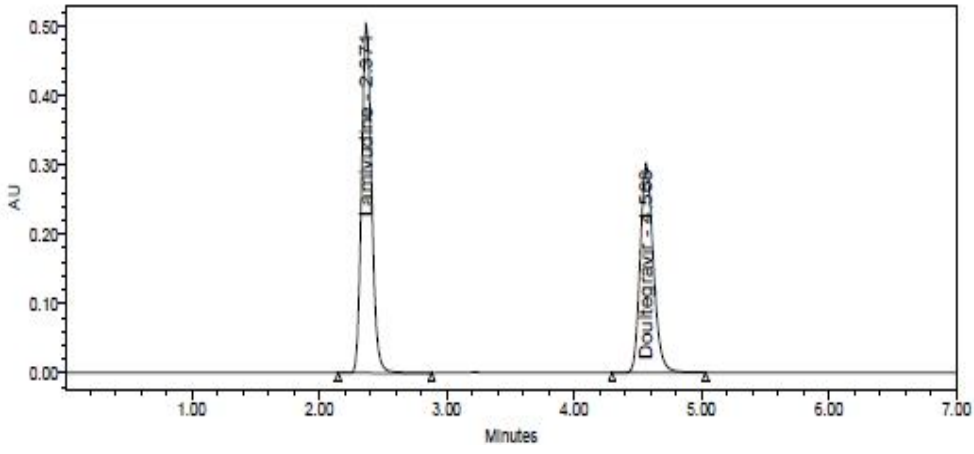

Fig 4.23: Typical Chromatogram for Accuracy 150 %

Table 4.3: Recovery data for Lamivudine and Dolutegravir

S. NO.	Accuracy level	Injection	Lamivudine		Dolutegravir	
			% Recovery	Average	% Recovery	Average
1	50%	1	99.5	99.4	99.1	99.2
		2	99.3		98.9	
		3	99.4		99.6	
2	100%	1	100.1	99.4	100.2	99.7
		2	99.5		99.7	
		3	99.6		99.4	
		4	98.6		999.2	
		5	98.8		99.6	
		6	99.4		99.4	
3	150%	1	99.3	99.8	100.0	100.0
		2	100.1		100.2	
		3	100		99.9	

Result:

Results of accuracy study are presented in the above **Table 4.3.** All the results indicate that the method is highly accurate**.**

4.3.4 Limit of detection and limit of quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations (1) and (2), respectively. **(Table 4.4)**

Table 4.4: LOD and LOQ values Calculated from calibration curve

	Lamivudine (µg)	Dolutegravir (µg)
LOD	0.03	0.006
LOQ	0.11	0.018

4.3.5 Robustness: Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The chromatograms were shown in **Figures 4.24 and 4.25** and the results were shown in **Table 4.5.**

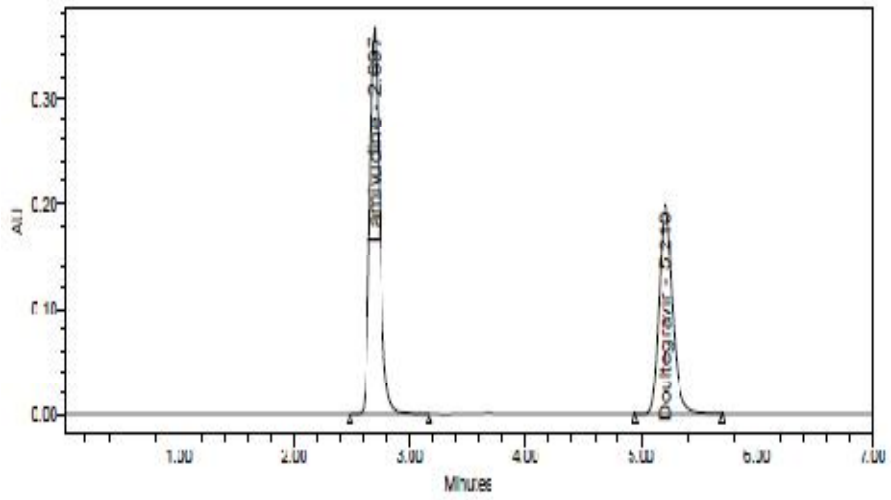

Fig 4.24: Chromatogram for decreased flow rate

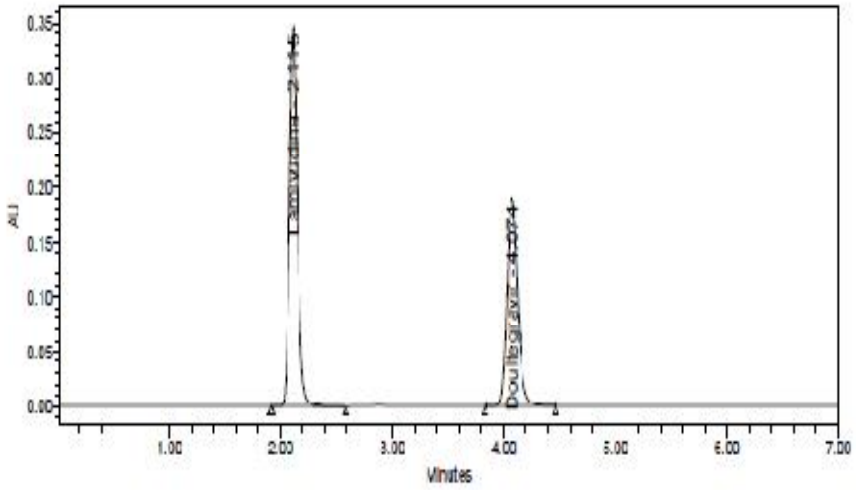

Fig 4.25: Chromatogram for increased flow rate

Table 4.5: Results of Robustness data for Lamivudine and Dolutegravir

parameter	RT		Theoretical plates		Asymmetry	
	3TC	DTG	3TC	DTG	3TC	DTG
Decreased flow rate (0.9ml/min)	2.697	5.210	4947	10078	1.17	1.22
Increased flow rate (1.1ml/min)	2.115	4.074	4364	8880	1.14	1.17
Wavelength 258nm	2.372	4.572	4722	9498	1.18	1.20
262nm	2.371	4.571	4664	9456	1.17	1.20

Result:

The results of Robustness of the present method had shown that changes made in the flow and wavelength did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant, we can say that the method is robust.

5.3.6: System suitability: The system suitability parameters such as US tailing factor, US theoretical Plates and resolution was achieved by injecting the prepared solution five times individually into the chromatographic system separately. The chromatograms were presented in **Figures 4.26 to 4.31** and the data was shown in **Table 4.6.**

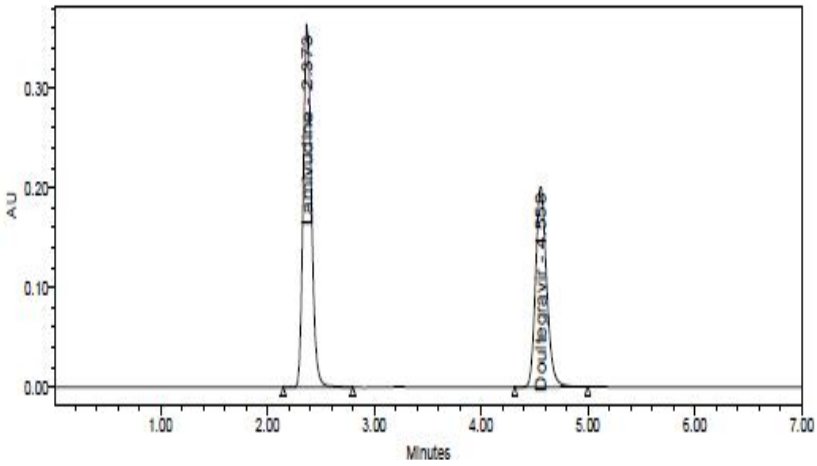

Fig 4.26: Typical Chromatogram of Standard Injection-1

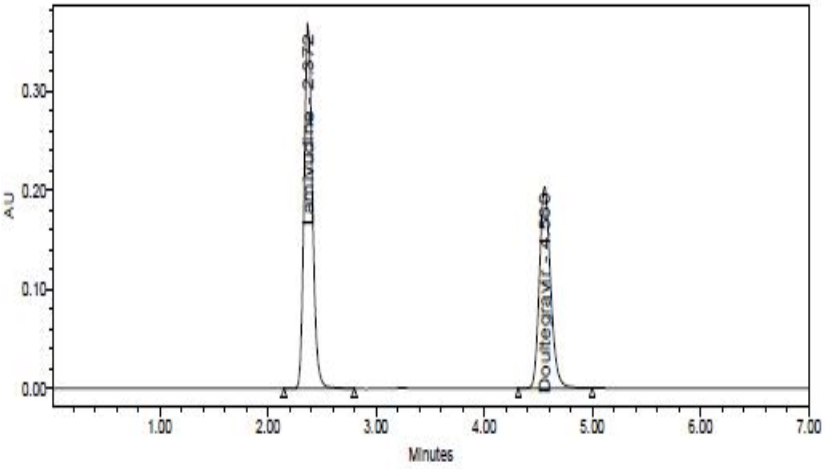

Fig 4.27: Typical Chromatogram of Standard Injection-2

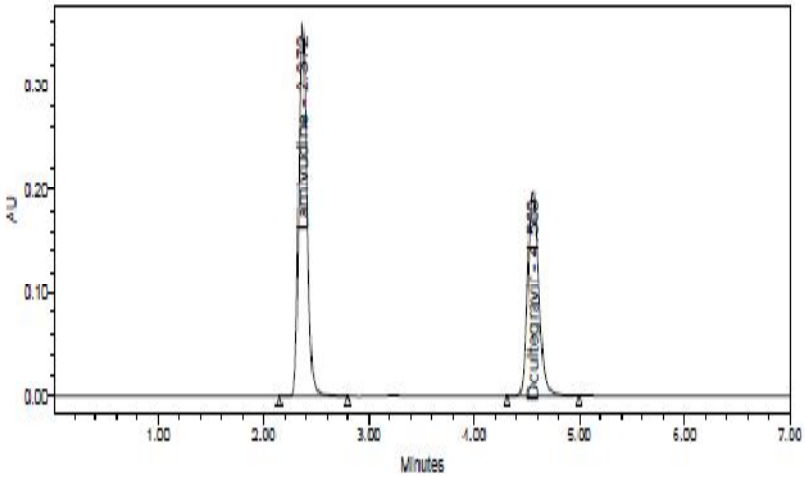

Fig 4.28: Typical Chromatogram of Standard Injection-3

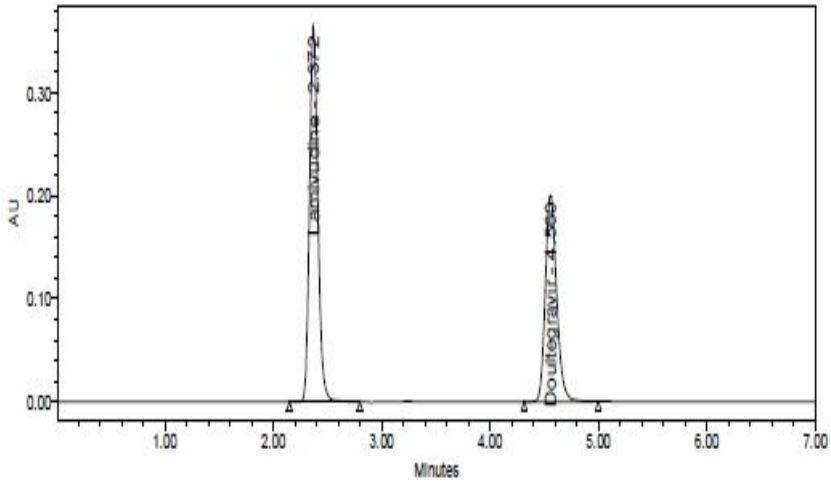

Fig 4.29: Typical Chromatogram of Standard Injection-4

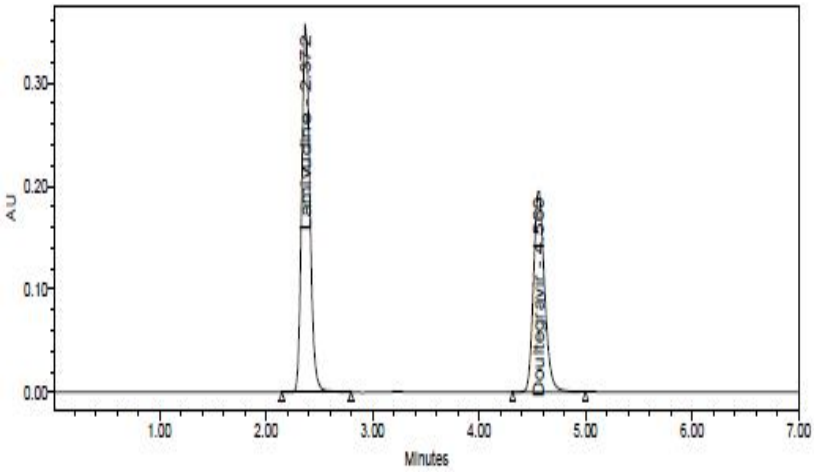

Fig 4.30: Typical Chromatogram of Standard Injection 5

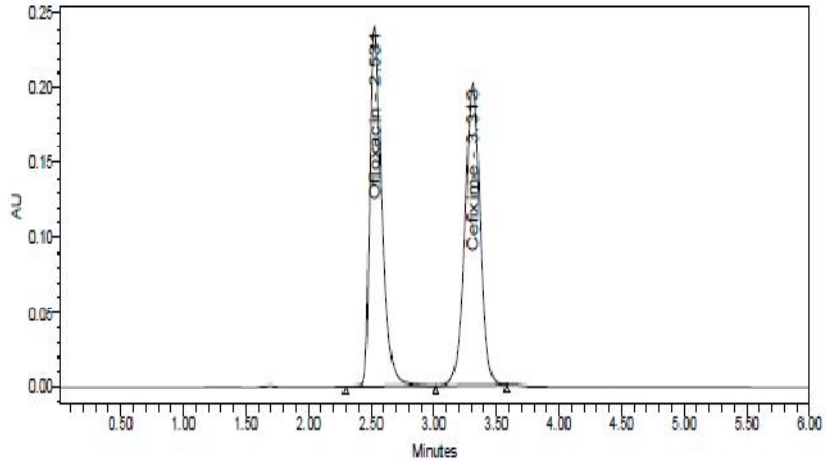

Fig 4.31: Typical Chromatogram of Sample-1

Table 4.6: Standard Results of Lamivudine and Dolutegravir

S. No	Injections	RT		Area		USP plate count		USP tailing	
		LAM	DTG	LAM	DTG	LAM	DTG	LAM	DTG
1.	Injection1	2.373	4.558	1938696	1448955	4751	9491	1.17	1.19
2.	Injection 2	2.372	4.560	1958364	1463230	4720	9444	1.17	1.18
3.	Injection 3	2.372	4.560	1909171	1424816	4742	9453	1.18	1.18
4.	Injection 4	2.372	4.560	1942383	1451383	4692	9464	1.17	1.18
5.	Injection 5	2.372	4.560	1894348	1409708	4736	9443	1.18	1.19

Table 4.7: System suitability data of Lamivudine and Dolutegravir

Parameter	Lamivudine	Dolutegravir	Acceptance criteria
Retention time (min)	2.372	4.560	$\pm$10
Theoretical plates	4728	9459	>3000
Tailing factor	1.17	1.18	<2.00
% RSD	1.35	1.51	<2.00

Result:

Results of system suitability study are summarized in the above **Table 4.7.** Five consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

4.3.7 Specificity: The chromatograms of standard and sample are identical with nearly same retention time **(Figures 4.32 to 4.35).** No interference due to placebo and sample at the retention time of analyte which shows that the method was specific. **(Table 4.8)**

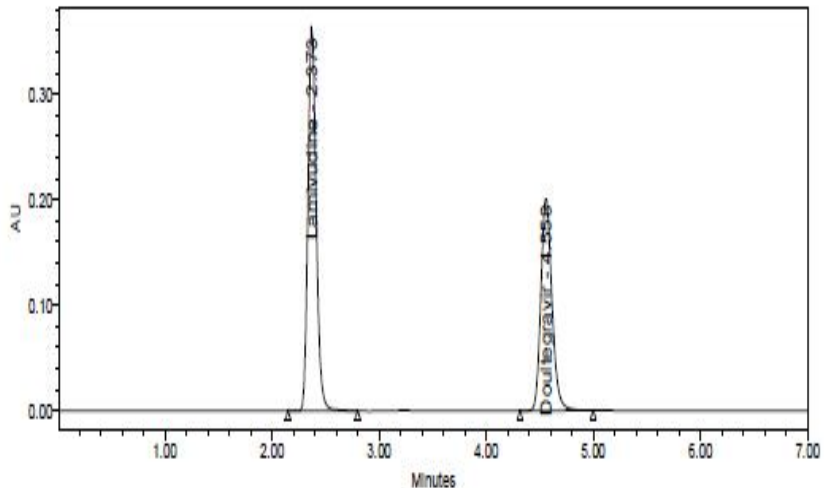

Fig 4.32: Chromatogram representing specificity of standard

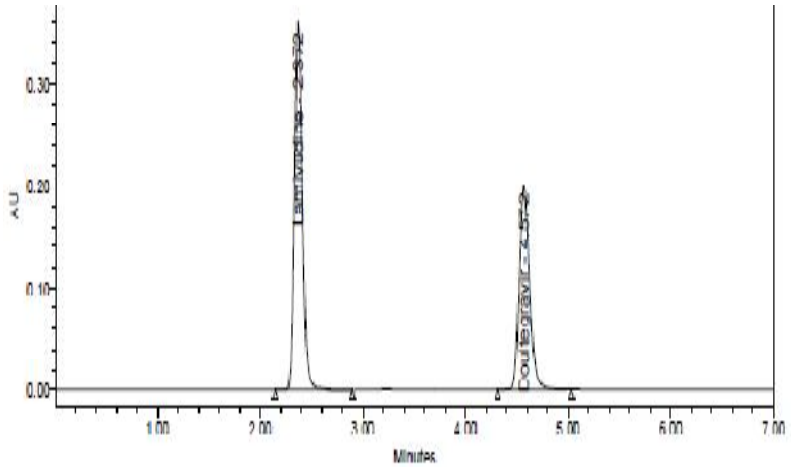

Fig 4.33: Chromatogram representing specificity of sample

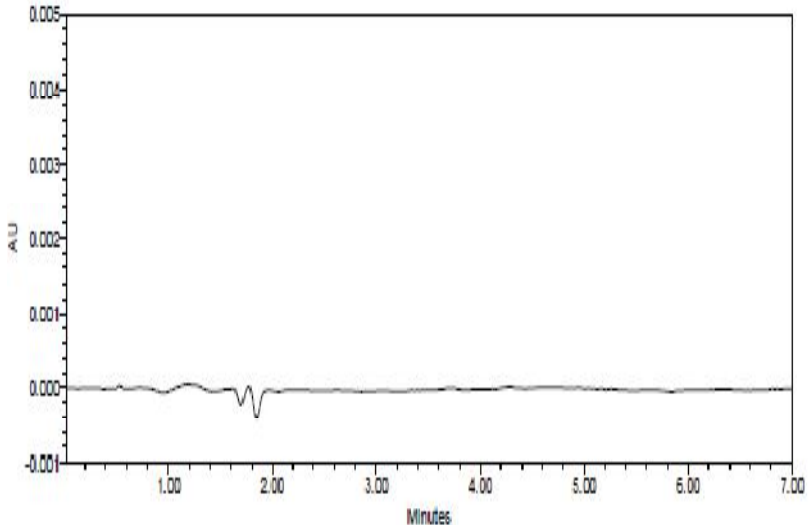

Fig 4.34: Typical chromatogram of the blank

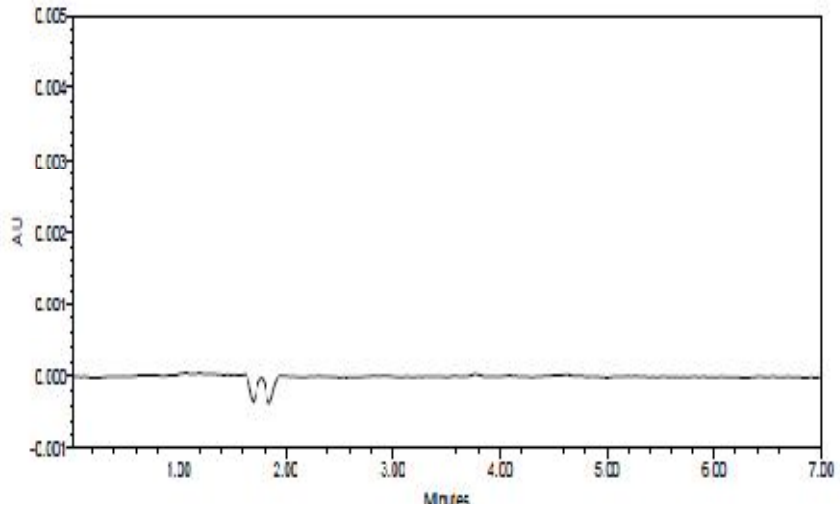

Fig 4.35: Typical chromatogram of the Placebo

Table 4.8: Specificity data for Lamivudine and Dolutegravir

S.No	Sample Name	RT (min) Lamivudine	Rt (min) Dolutegravir
1	Standard	2.373	4.558
2	Sample	2.372	4.572
3	Blank	-	-
4	Placebo	-	-

Result

Chromatograms explain that retention time for standard, sample and commercial product of Lamivudine and Dolutegravir are same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

Table 4.9: Summary of Lamivudine and Dolutegravir

S.No.	Parameter	Lamivudine	Dolutegravir	Acceptance criteria
1	**System suitability** Theoretical plates Asymmetry Retention time (min) %RSD	 4728 1.17 2.372 1.35	 9459 1.18 4.560 1.51	 Not less than 3000 Not more than 1.5 Not more than 2.0
2	Specificity	Specific	Specific	Specific
3	Method precision(%RSD)	0.54	0.35	Not more than 2.0%
4	Linearity parameter Correlation co-efficient (r^2)	300-900 µg/ml 0.9991	50-150µg/ml 0.9992	 Not less than 0.999
5	Accuracy (Mean % recovery) 50% 100% 150%	 99.4 99.7 99.8	 99.2 99.7 100.0	 97 - 103%
6	Robustness	All the system suitability parameters are within the limits.	All the system suitability parameters are within the limits.	