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ORIGINAL ARTICLE

Development and validation of RP-HPLC method for simultaneous Estimation of Lamivudine, Tenofovir disoproxil and Dolutegravir

Prachi P. Kanawade[1*], Amarsingh S.Devadhe[1]

1. Department of Pharmaceutical Chemistry, Shantiniketan College of Pharmacy, Dhotre (Bk), Ahilyanagar, Maharashtra, India- 414304**.**

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Abstract

The aim of this method was to develop and validate a simple, sensitive, rapid and accurate method for simultaneous estimation of Lamivudine, Tenofovir Disoproxil and Dolutegravir in a drug product by using the RP-HPLC technique. The separation of Lamivudine, Tenofovir Disoproxil and Dolutegravir successfully achieved by using Cosmosil C18 (250mm X 4.6 ID). The optimized mobile phase contains methanol: water (60:40 %v/v) having flow rate was 20µl/min. The eluents was separated by using UV detector at 266 nm. The average retention time for these three compounds were found to be 3.04mins,4.26 mins and 5.30 mins for Lamivudine, Tenofovir Disoproxil and Dolutegravir respectively. The developed method validation was performed as per ICH guidelines. The developed method was validated for various parameters like linearity, precision, robustness , accuracy etc. and this method is successfully applied for the estimation of Lamivudine, Tenofovir Disoproxil and Dolutegravir in combined tablet dosage form. The developed method was found to be very specific, simple as well as accurate for development and design for pharmaceutical dosage form such as tablet or bulk dosage form.

***Corresponding author: Dr. Prachi P. Kanawade,** Department of Pharmaceutical Chemistry, Shantiniketan College of Pharmacy, Dhotre (Bk), Ahilyanagar, Maharashtra, India- 414304**, Email:** prachikanawade1997@gmail.com

Orcid ID: 0000-0002-2477-2326

INTRODUCTION

Human immunodeficiency virus (HIV) is that the virus that causes Acquired Immune Deficiency Syndrome (AIDS) and is transmitted through the contact with infected blood and bodily fluids. Such contact can occur through unprotected sex,through sharing of needles or other drug injection equipment, through mother – to- child transmission during pregnancy and breast feeding. HIV infects immune cells in the body called CD4 positive(CD4+) T cells,which are essential for fighting infections.HIV converts this cells into "Factories" that produce more of the HIV virus to infect other healthy cells,eventually destroying the CD4 positive cells.HAART may be a customized combination of various classes of medicines that a physician prescribes supported such factors because the patient's viral load,the particular strain of the virus, the CD+ cell count, and other considerations(e.g.disease symptoms). Because HAART can not rid the body of HIV, it must be taken a day for alltimes.HAART can control viral load,delaying or preventing the onset of symptoms or progression to AIDS,thereby prolonging survival in people infected with HIV.[16] Lamivudine is a synthetic nucleoside analogue with activity against hepatitis B virus (HBV) and HIV. Intracellularly, lamivudine is phosphorylated to its active metabolites, lamiduvine triphosphate (L-TP) and lamiduvine monophosphate (L-MP). In HIV, L-TP inhibits HIV-1 polymerase (RT) via DNA chain termination after incorporation of the nucleoside analogue into viral DNA. In HBV, incorporation of L-MP into viral DNA by HBV polymerase leads to DNA chain termination. L-TP may be a weak inhibitor of mammalian DNA polymerases alpha and beta, and mitochondrial DNA polymerase[13]

.Tenofovir Disoproxilis a nucleotide reverse transcriptase inhibitor. It inhibits reverse transcriptase, important enzyme in HIV. Tenofovir Disoproxil. Disoproxil also inhibits human enzyme like DNA polymerase. It is a analogue of adenosine. [15] Dolutegravir is an orally bioavailable integrase strand-transfer inhibitor (INSTI), with activity against human immunodeficiency virus type 1 (HIV-1) infection. Upon oral administration, dolutegravir

binds to the site of integrase, an HIV enzyme that catalyzes the transfer of viral genetic material into human chromosomes. This prevents integrase from binding to retroviral desoxyribonucleic acid (DNA), and blocks the strand transfer step, which isimportant for the HIV replication cycle. This prevents HIV-1 replication.[14].The combination of Lamivudine,Tenofovir Disoproxiland Dolutegravir used to treat the people who are infected with HIV. The marketed formulation of these three components is in tablet dosage form VIROPIL containing Lamivudine 300mg + Tenofovir Disoproxil300mg +

Dolutagravir 50 mg.

COMPONENT 1: LAMIVUDINE

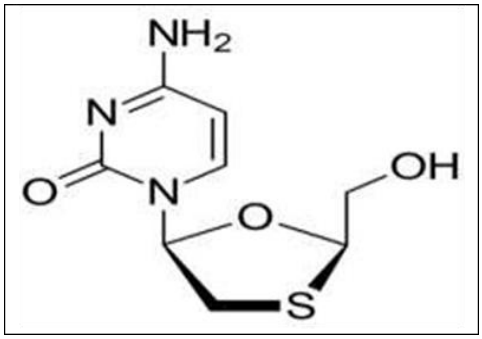


Figure 1: Structure of Lamivudine

IUPAC Name: 2',3'-dihyroxy-3'thiacytidine 4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidinone

COMPONENT 2.TENOFOVIR DISPROXIL

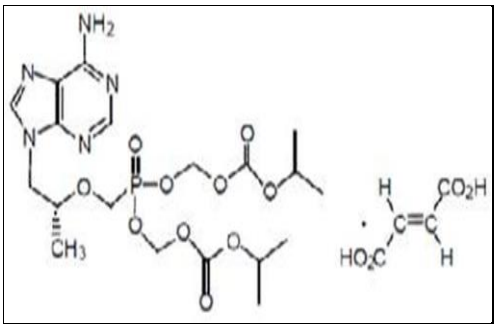


Figure 2 : Structure of Tenofovir Disproxil

IUPACName:

Bis{[(isopropoxycarbonyl)oxy]methyl} ({[(2R)-1-(6-amino-9H-purin-9-yl)-propanyl]oxy}methyl) phosphonate.

3. COMPONENT III: DOLUTEGRAVIR.

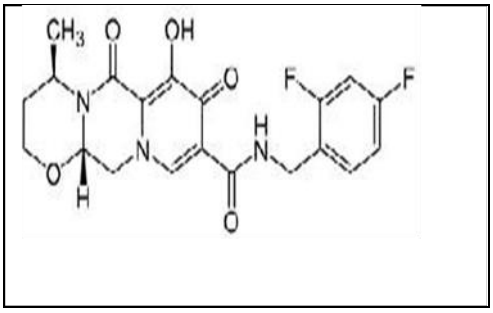


Figure 3: Structure of Dolutagravir

IUPAC Name: (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]pyrazino[2,1b][1,3] oxazine-9- carboxamide.

Literature survey reveales that different HPLC [4,7], RP-HPLC[1-3,6,8-11],UPLC[12] were reported for the above combination.The present study shows that the developed method and validation consisting simple,precise and accurate procedure for Simultaneous Estimation Method Development And Validation of Lamivudine,Tenofovir Disoproxiland Dolutegravir by RP-HPLC as per ICH guidelines.

MATERIAL AND METHOD

Table 1: Name Of Drug

Drug(1)	Lamivudine
Drug(2)	Tenofovir Disproxil
Drug(3)	Dolutegravir

Lamivudine, Tenofovir DisoproxilDisoproxiland Dolutegravir pure standard sample were received as a gift samples from Lupin Pharmaceuticals,India . All other reagents used were HPLC grade.

List of Instruments:

Figure 1: List of instruments

Sr. No.	Name	Model	Manufacturer / Supplier
1	Weighing balance	PGB 100 Max:100gm Min :0.001gm	Wenser High Precision Balance
2	Sonicator	WUC-4L Capacity - 4L	Wenser Ultra Sonicator
3	HPLC	HPLC 3000 Series	Analytical Technologies Ltd.

Table 2: List of apparatus/ instruments used.

Parts of Instruments	Information
System	HPLC Binary Gradient System
Model no.	HPLC 3000 Series
Company	Analytical Technologies Ltd
Pump	P-3000-M Reciprocating (40 MPa)
Column	Cosmosil C18 (250mm×4.6ID, Partical size- 5 micron)
Detector	UV-3000-M
Software	HPLC Workstation

Table 3: HPLC Instrument Information.

Sr. No.	Name	Specification	Manufacturer /Supplier
1	Methanol	HPLC Grade	Merck pharmaceuticals
2.	Acetonitrile	HPLC Grade	Merck pharmaceuticals
3.	Purified water	HPLC Grade	Merck pharmaceuticals

Table 4: List of chemical used

Sr. no.	Name	Specification	Manufacturer /Supplier
1.	Lamivudine	Working standard	Lupin Pharmaceuticals
2.	Tenofovir Disproxil	Working standard	Lupin Pharmaceuticals
3.	Dolutegravir	Working standard	Lupin Pharmaceuticals

Table 5: List of API used.

Preparation of Mobile Phase:

The mobile phase is prepared by mixing a HPLC grade sample methanol and purified water in a ratio of 60:40 and filtered through 0.45 µ membrane filter to remove the impurities and sonicate using ultrasonicator to remove the undissolved gases present in the mobile phase.

Preparation of Diluent:

Mobile phase used as a diluents.

Preparation of Standard Solution:

Accurately weighed 10 mg of pure lamivudine,Tenofovir Disoproxiland dolutegravir of working standard into the volumetric flask of 10 ml.Add diluent into it. Sonicated this solution to dissolved the undissolved matter and make up volume upto the mark with same solvent (mobile phase). From this solution,make the standard solution of working standards of the concentration range from 5 ppm to 150 ppm for Lamivudine,Tenofovir Disoproxiland Dolutegravir each were prepared by transferring and diluting different aliquots into 10ml series in volumetric flask of 10 ml with the same diluent.

Preparation of sample solution:

Accurately weighed and transfer 10 tablets of Lamivudine, Tenofovir Disoproxiland Dolutegravir (label claim- Lamivudine-300mg, Tenofovir Disproxil-300 mg and Dolutegrvir 50mg) in the mortar and pestle. Crush this tablets to make fine powder. Accurately Weigh and transfer sample powder equivalent to 10 mg of Lamivudine, Tenofovir Disoproxiland Dolutegravir into the

volumetric flask of 10 ml consist of mobile phase vigorously shaken and sonicated this solution for 15 mins and make up volume with the mobile phase upto the marking.The Aliquots of this solution was pipette out and transferred this solution into series clean and dry volumetric flask of 10 ml. Add diluent upto the marking for making final concentration of the drugs Lamivudine, Tenofovir Disoproxiland Dolutegravir.From these standard and sample solutions of 20µl where injected into the column and peak areas were recoreded from that.

Wavelength selection:

UV spectrum for 10µg/ml of Lamivudine, Tenofovir Disoproxiland Dolutegravir by using mobile phase composition was recorded from scanning with in the range between 200 nm to 400nm. From this spectrum wavelength selected as 266 nm. At this wavelength, these three drugs show good absorbance.

Chromatographic conditions:

Cosmosil C18 (250X4.6mm I.D ;particle size 5 µm) column used for detection at a wavelength of 266 nm by use of mobile phase Methanol and water in 80:20 ratio. The mobile phase was degassed and filtered through the 0.45 µ membrane filter to remove the impurities present in the mobile phase. The mobile phase is pumped through reservoir s to the column at a flow rate of 0.8ml per min. The retension time for Lamivudine, Tenofovir Disoproxiland Dolutegravir was found to be 3.05min ,4.26 min and 5.27 min respectively.

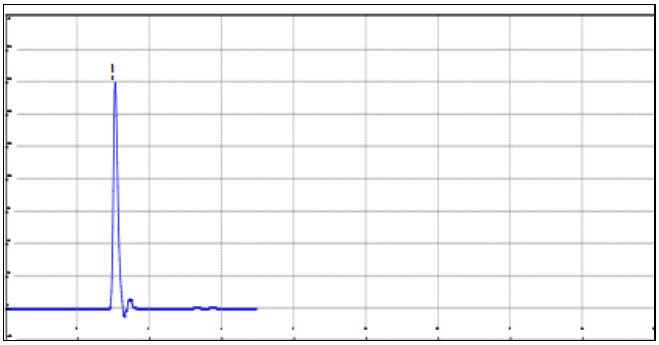


Figure 4 Retension time of Lamivudine

Time	Area	Resolution	T.Plate No.	Asymmetry
3.022	2006554	4.42	8473	1.04

Table 6: Data for standard drug Lamivudine

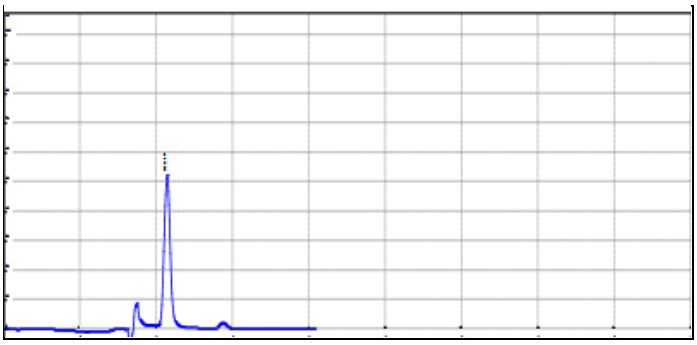


Figure 5: Retension time of Tenofovir Disproxil

Table 7:Data for standard drug Tenofovir

Time	Area	Resolution	T. Plate	Asymmetry
4.260	1024641	2.76	8361	1.06

Disproxil

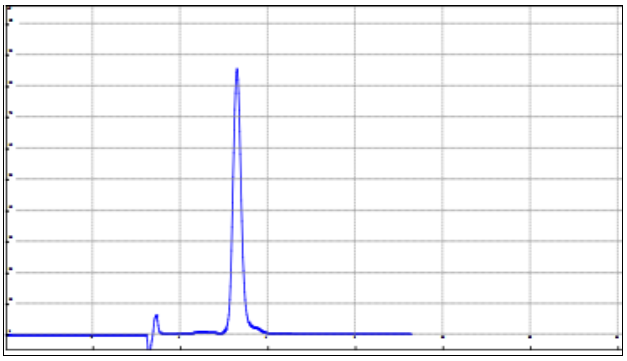


Figure 6: Retension time of Dolutegravir

Table 8: Data for standard drug

Time	Area	Resolution	T.Plate No.	Asymmetry
5.270	1637168	0.00	8055	1.12

Dolutegravir

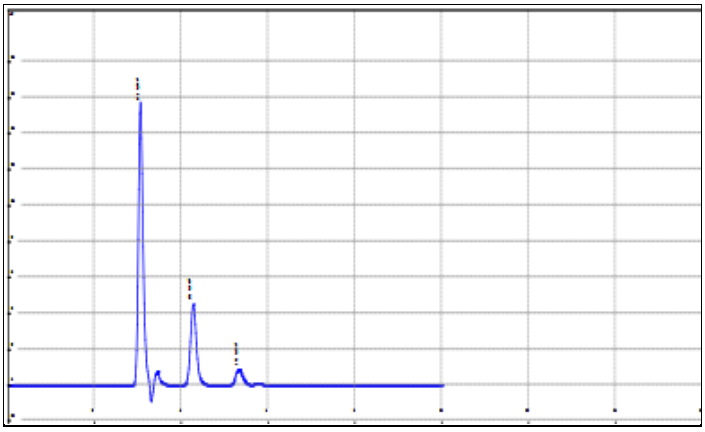


Figure 7: Chromatogram of standard solution of Lamivudine, Tenofovir and Dolutegravir

Table 9: Data for standard solution of Lamivudine, Tenofovir Disoproxiland Dolutegravir

Time	Area	Resolution	T.Plate No.	Asymmetry
3.044	1382833	1.09	3060	1.28
4.260	749725	1.63	2004	1.12
5.306	234847	0.76	1825	1.12

RESULT AND DISCUSSION :

The developed method was validated as per ICH guidelines for its linearity, accuracy, precision, limit of detection, limit of quantitation , robustness , ruggedness, system suitability etc.

1.System suitability Parameters :

System suitability parameters and chromatographic technique parameters were validated like theoretical plates, resolution, tailing/asymmetric factor was calculated. They are given in Table 10.

Table 10: System Suitability Parameters

System Suitability Parameters	Lamivudine	Tenofovir Disproxil	Dolutegravir
Retention time	3.044	4.260	5.306
Tailing factor	1.28	1.12	1.12
Theoretical plate no.	3060	2004	1825
Resolution	1.09	1.63	0.76

2. Linearity :

The developed method was validated for linear regression analysis and calculated by the use of least square method and was studied by the preparation of standard solutions of Lamivudine, Tenofovir Disoproxiland Dolutegravir for different concentrations. The calibration curve shows good linearity from 10 µg/ml to 50 µg/ml for lamivudine ,10 µg/ml to 50 µg/ml for Tenofovir Disoproxilhaving correlation coefficient (r2) of 0.998 and 10 µg/ml to 50 µg/ml with a correlation coefficient (r2) of 0.998 for Dolutegravir. Linearity graphs are as follows:

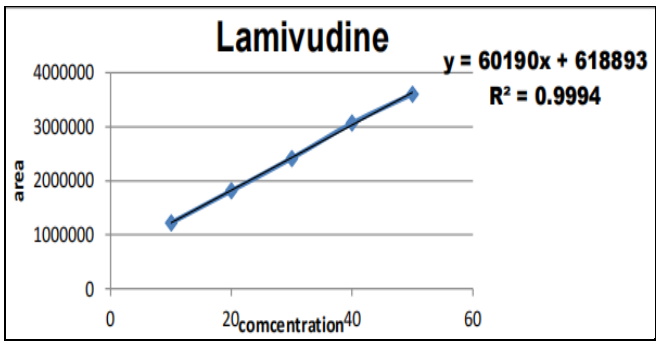


Figure 8: linearity graph for Lamivudine

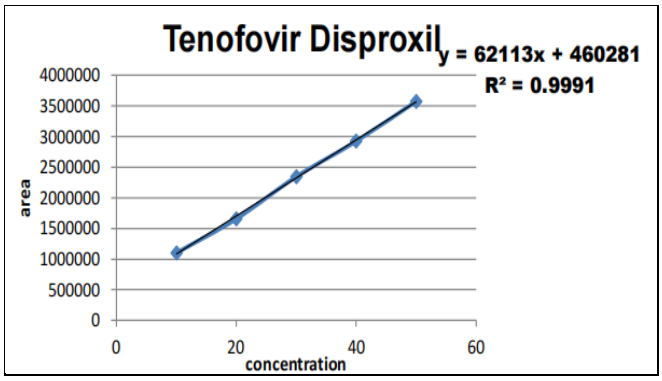


Figure 9: Linearity Graph of Tenofovir Disproxil

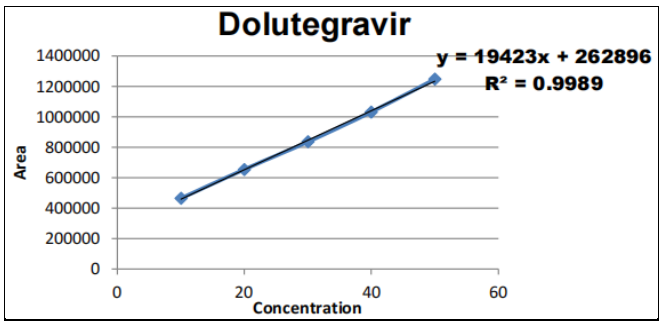


Figure 10: Linearity Graph of Dolutegravir

3. Assay(%)

Preparation for sample solution for assay: Accurately weigh 10 mg of powdered tablet of Lamivudine,Tenofovir Disoproxiland Dolutegravir . Transfer it it 10 ml clean and dry volumetric flask, volume make up with mobile phase this gives 1000 ppm concentration of solution and was sonicated 15 min to dissolve the undissolved matter.and this solution was filtered through 0.45µ membrane filter. From this solution 30 µg/ml sample solution is prepared. And from this solution 20 µl was injected at flow rate of 0.8 ml/min.

Table 11: Data of assay for statndard drug for Lamivudine,Tenofovir Disoproxiland

Sr. No.	Parameter	Drug		
		Lamivudine	Tenofovir Disproxil	Dolutegravir
1.	Label claim	300 mg	300mg	50 mg
2.	%RSD	0.05	0.20	0.22

Dolutegravir

Table 12: Data of standard drug for Lamivudine, Tenofovir Disoproxiland Dolutegravir

Time	Conc.	Area	Resolution	T.plate no.	Asymmetry
4.005	30	2410369	3.47	8413	1.07
5.796	30	2343259	3.36	8513	1.02

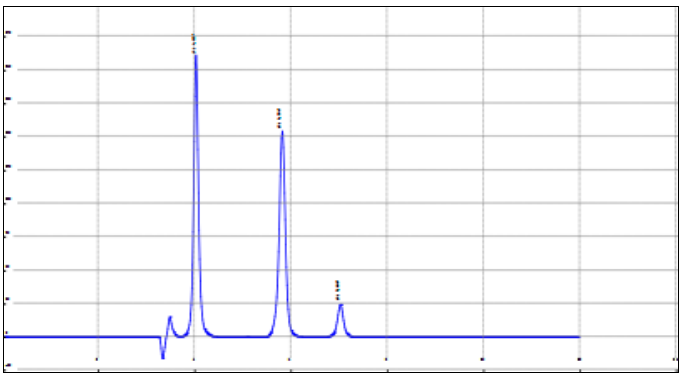


Figure 11: Graph for % Assay of Lamivudine, Tenofovir Disoproxiland Dolutegravir

Table 13: Data for % Assay of Lamivudine,Tenofovir Disoproxiland Dolutegravir

Sr. No.	Drug	Area of standard	Area of sample	% Assasy
1	Lamivudine	2416550	2410369	99.74
2	Tenofovir Disproxil	2348013	2343259	99.79
3	Dolutegravir	834326	829685	99.44

4. Accuracy :

Recovery studies carried out from addition of the standard drug to the sample for 3 different levels of concentrations i. e.50%, 100%,150% taking the consideration of % purity of added bulk drug samples. For every concentration the sample was injected and by the % RSD values it was confirmed that the method was accurate as % recovery values found within the range of 99.91 to 99.97 for Lamivudine, 99.21 to 99.62 for Tenofovir Disoproxiland 98.95 to 99.40 for Dolutegravir for 3 different concentration 50%,100% &150% . The results are given in the **Table 14**.

Table 14: Data for Accuracy study

Comp	Conc. (%)	Sample Amt (ppm)	Amt Added (ppm)	Amt Recovered (ppm)	% Recovery
Lamivudine	50	20	10	29.78	99.29
	100	20	20	39.56	99.91
	150	20	30	49.98	99.97
Tenofovir Disproxil	50	20	10	28.47	94.91
	100	20	20	39.68	99.21
	150	20	30	49.81	99.62
Dolutegravir	50	20	10	29.76	99.22
	100	20	20	39.58	98.95
	150	20	30	49.70	99.40

5. Precision:

Reproducibility : A standard solution containing 15 µg/ml Lamivudine and 90 µg/ml Tenofovir Disoproxiland Dolutegravir were analyzed 3 times on the same day for Intraday and on different day for Interday and % R. SD was calculated. The results are given in table no.15

A. Lamivudine

Interday

Table15: Data for interday Precision of Lamivudine

Day 1	2411400
	2407881
	2407881
Day 2	2418982
	2411400
	2414859
Mean	2414859
% RSD	0.18 %

Intraday

Table 16: Data for Intraday Precision of Lamivudine

Morning	2416550
	2407881
	2409533
Evening	241507
	2419520
	2412626
Mean	2413536
% RSD	0.18 %

B. Tenofovir Disproxil

Interday

Table 17: Data for Interday Precision of Tenofovir Disproxil

Day 1	2366660
	2366660
	2348013
Day 2	2343708
	2340972
	2347175
Mean	2347175
% RSD	0.49 %

Intraday

Table 18: Data for Intraday Precision of Tenofovir Disproxil

Morning	2348013
	2366660
	2342530
Evening	2415107
	2345681
	2343563

Mean	2360259
% RSD	1.20%

C. Dolutegravir

Interday

Table 19: Data for Interday Precision of Dolutegravir

Day 1	834326
	836564
	829242
Day 2	829883
	834187
	829875
Mean	829875
% RSD	0.37%

Intraday

Table 20: Data for Intraday Precision of Dolutegravir

Morning	834326
	836564
	829242
Evening	834083
	837469
	829061
Mean	833457
% RSD	0.43%

6. Robustness

small deliberate changes in chromatographic conditions like change in wavelength and change in flow rate were studied to determine method Robustness. The results are in the Table 21.

A. Change in flow rate

Table 21: Robustness Data(Change in flow rate)

Comp.	Conc (µg/ml)	Flow rate(ml/ min)	Area	Mean	SD	%SD
Lamivudine	20	0.7	1821870	1818632	4078.67	0.224
		0.8	1814051			
		0.9	1819974			
Tenofovir Disproxil	20	0.7	1659239	1660221	965.94	0.581
		0.8	1661170			
		0.9	1660255			
Dolutegravir	20	0.7	653230	652208	2094.55	0.211
		0.8	653596			
		0.9	649799			

Change in wavelength

Table 22: Robustness Data (Change in Wavelength)

Comp	Conc (µg/ml)	Flow rate(ml/ min)	Area	Mean	SD	%SD
Lamivudine	20	264	1816412	1815563	1312.48	0.072
		266	1814051			
		268	1816225			
Tenofovir Disproxil	20	264	1664582	16660941	3760.73	0.226
		266	1661170			
		268	1657071			
Dolutegravir	20	264	653422	653412	189.71	0.029
		266	653596			
		268	653217			

7. Ruggedness

Acceptance criteria for Ruggedness is NMT 2%

Table 23: Ruggedness data for Lamivudine

Sr. No.	Concentration (µg/ml)	Area
1.	10	1229072
2.	20	1819021
3.	30	2419051
4.	40	3065536
5.	50	3612781

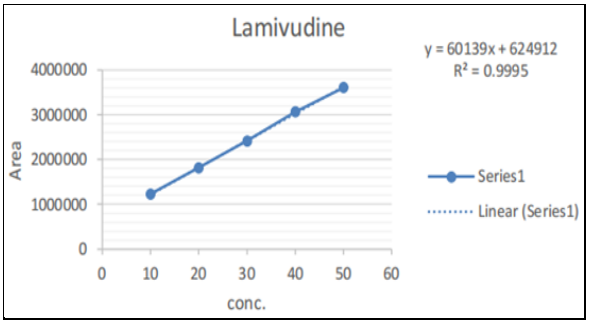


Figure 13: Ruggedness graph for Lamivudine

Table 24: Ruggedness data for Tenofovir Disproxil

Sr. No.	Concentration (µg/ml)	Area
1.	10	1103269
2.	20	1658299
3.	30	2347282
4.	40	2926277
5.	50	3568977

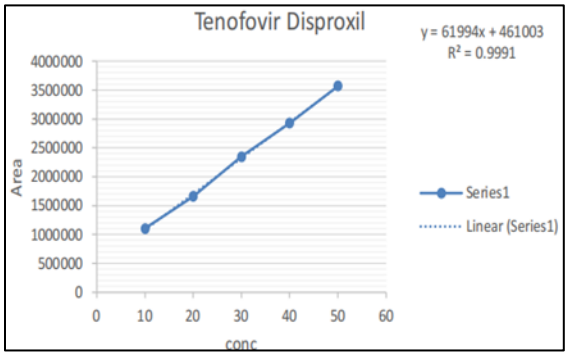


Figure 14: Ruggedness graph for Tenofovir Disproxil

Table 25: Ruggedness data for Dolutegravir

Sr. No.	Concentration (µg/ml)	Area
1.	10	463365
2.	20	653478
3.	30	834996
4.	40	1031638
5.	50	1245717

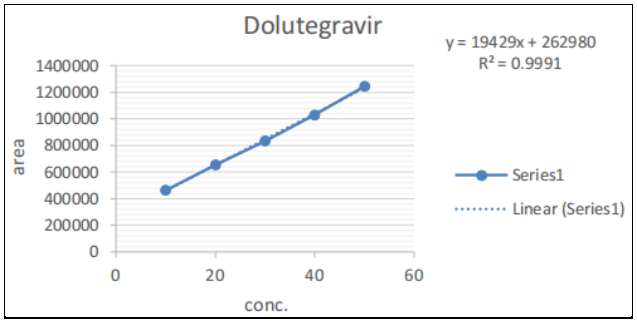


Figure 15: Ruggedness graph for Dolutegravir

8. Limit of Detection(LOD) and Limit of Quantitation (LOQ)

The LOD for Lamivudine,Tenofovir Disoproxiland Dolutegravir were found to be 0.6828µg/ml,1.0131 µg/ml and 1.0644 µg/ml respectively,The values for LOQ were found to be 2.06 µg/ml,3.07 µg/ml and 3.22 µg/ml for Lamivudine,Tenofovir Disoproxiland Dolutegravir respectively.

CONCLUSION

This study shows that a fast, simple ,accurate RP-HPLC method was developed for simultaneous estimation of Lamivudine,Tenofovir Disoproxiland Dolutegravir.The aim of this method development shall be achieved with successfully ,although there was a difference between polarity of these 3 compunds. Thus it confirm that the method was specific and accurate. From the analysis of result it

is confirmed that the method is successfully applicable for estimation of selected drugs in bulk therefore this method can be also used for designing of new dosage forms in pharmaceutical field.

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