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RESEARCH ARTICLE

Analytical Method Development and Validation of Stability Indicating assay method of analysis for Dolutegravir/Lamivudine/Tenofovir Disoproxil Fumarate tablets using High Performance Liquid Chromatography

Saravanan. R[1], Somanathan. T[1]*, Gavaskar D[1], Tamilvanan M[2]
[1]Department of Chemistry, Vel's Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai, India
[2]Department of Chemistry, K.S.R College of Engineering, Tiruchengode, Namakkal, India
*Corresponding Author E-mail: **soma_nano@yahoo.co.in**

ABSTRACT:
A novel, economic, simple, precise and time-efficient reverse-phase high performance liquid chromatographic (RPHPLC) method has been established for the simultaneous assay determination of Dolutegravir, Lamivudine and Tenofovir Disoproxil fumarate in tablet formulation. This research paper presents the detailed method development strategy and the outcome of validation challenges. The RPHPLC method was developed using a 150 x 4.6mm, 5µm C18 column, with a gradient mode using 0.1% (v/v) trifluoroacetic acid buffer and methanol, the detection was performed at 260nm. The method was validated for specificity, precision, linearity, accuracy, robustness and can be used in quality control during manufacture and for assessment of the stability samples of Dolutegravir/Lamivudine/Tenofovir Disoproxil fumarate tablets. Total elution time was about 5 min and equilibration time of about 2 min which allows analysis of more than 100 samples per day. The method reported in this study is compatible to mass spectrometry and is thus extremely useful for stability studies.

KEYWORDS: Dolutegravir sodium, Lamivudine, Tenofovir disoproxil fumarate, Liquid chromatography, stability Indicating, Assay.

INTRODUCTION:
About 37.9 million people are infected by HIV globally. Several health agencies are working on helping people infected with HIV live a longer lifespan with better quality of life. WHO is actively involved in this activity and has published several guidelines for treatment regimens. The 2019 guideline of WHO recommends a triple combination oral dosage form of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate as first line of treatment for HIV infection in adults.

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Since the HIV infection is more prevalent in developing nations, cost effective and simple analytical procedures are required for quantification of these active pharmaceutical ingredients in oral solid dosage forms. In an endeavor to reduce the testing cost of Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate oral solid dosage form to help reduce the manufacturing cost and thus make this life saving drug more affordable for the population of developing countries, a short, efficient and cost-effective mass compatible reverse phase high performance liquid chromatographic assay method was developed and validated.

Dolutegravir:
Dolutegravir is an antiretroviral drug that acts by impairing the function of the HIV integrase-DNA complex to which it binds[1]. Drug–drug interactions with dolutegravir are minimal as it has little ability to alter drug-metabolizing enzymes. There are no interactions or dose adjustments required when combined with the NRTI class[2,3]. These attributes make it one of the preferred choices of drug in multidrug treatment

regimens. Dolutegravir is chemically (4R,12aS)-N-[(2,4-difluorophenyl)methyl]-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido [1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide (WHO) has a chemical formula of $C_{20}H_{18}F_2N_3NaO_5$ with molecular weight of 419.3788 g/mol.

Lamivudine:
Lamivudine is also known as 3TC and is the negative enantiomer of cytidine. It has activity against HIV and hepatitis B virus. It is first line antiretroviral drug in treatment guidelines[4]. Lamivudine is a nucleoside analogue that is phosphorylated to lamivudine triphosphate by cellular kinases. Lamivudine inhibits the reverse transcriptase of both HBV and HIV, and is indicated for the treatment of HIV and chronic HBV infection[5]. The chemical name of Lamivudine is 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] pyrimidin-2-one[6] and has a chemical formula of $C_8H_{11}N_3O_3S$ and molecular weight of 229.254g/mol.

Tenofovir Disoproxil Fumarate:
Tenofovir disoproxil fumarate (tenofovir DF), a bis-alkoxyester prodrug of tenofovir. Tenofovir is an acyclic nucleotide analog with activity against human immunodeficiency virus (HIV) and hepatitis B virus (HBV)[7]. Tenofovir Disoproxil Fumarate belongs to nucleotide analogue reverse transcriptase inhibitors (nRTIs) class of antiretroviral drugs. Tenofovir Disoproxil Fumarate is orally bioavailable, and the promoieties are cleaved during adsorption to release tenofovir into systemic circulation[8]. It is chemically 9-[R- (2[[bis]]isopropoxycarbonyl) oxy]methoxy] phosphonyl]methoxy]propyl] adenine fumarate (1:1)[9] and has a chemical formula of $C_{23}H_{34}N_5O_{14}P$ with molecular weight of 635.52g/mol.

Development of a short, mass spectroscopy compatible, cost effective and accurate quantitative method for simultaneous determination of three components of a fixed dose combination of antiretrovirals is a challenging task and the literature search for assay methods for triple combination of Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate indicated that very few methods are available for this product and none of the methods available meet all the requirements listed above. The methods that are available either involve high end detection techniques or are not mass spectroscopy compatible and are not cost effective[10,11]. Hence to overcome this gap, an assay method for this triple combination drug product was developed that is short, mass compatible and cost effective.

MATERIALS AND METHODS:

Waters Quaternary pump HPLC equipped with PDA detector, Inertsil ODS 3, 150 x 4.6mm, 5µm analytical column, Sigma AR grade trifluoroacetic acid and HPLC grade water and HPLC grade Rankem methanol, Millipore 0.45µm filter.

Development Strategy:
Identification of suitable mobile phase:
Dolutegravir:
The Acidic pK_a of Dolutegravir is 4.5 and hence a mobile phase with pH of pH 3.5 (or lower) or pH 5.5 (or higher) will result in consistent retention time during routine use.

Lamivudine:
The pK_a of Lamivudine suggests that a mobile phase having pH 3.18 (or lower) or pH 5.18 (or higher) will result in consistent retention time during routine use.

Since lamivudine contains amino group, mobile phase having pH towards basic side may result in peak tailing and hence mobile phase having pH lower than 3.18 will result in robust method with minimal chances of retention time shifts during routine use of method.

Tenofovir Disoproxil Fumarate:
Tenofovir has two pK_a values of 3.8 and 6.7. The pK_a of Tenofovir suggests that a mobile phase having pH 2.8 (or lower) or pH 4.8 (or higher) will result in consistent retention time during routine application of the method.

Based upon the above assessment, it was concluded that since all the three drug substances (Lamivudine, Tenofovir Disoproxil Fumarate and Dolutegravir) have a pKa of 3.8 or greater, acidic mobile phase with pH lower than 2.8 was expected to result in all the three the components to be predominantly in unionized form and would thus result in good peak shape and robust retention behavior. Based upon the pKa of each component, it was expected to get the elution in the order of Lamivudine followed by Tenofovir followed by Dolutegravir in reverse phase chromatographic conditions with mobile phase comprising of a combination buffer having a pH lower than 2.8. Methanol was selected as the organic solvent as the cost of HPLC grade methanol is significantly lower than the cost of HPLC grade acetonitrile. Based upon the UV spectra of each of the components the detection wavelength was selected as 260 nm. Since a short runtime was the requirement, a 150 x 4.6mm, 5µ C18 column was selected.

Based upon above mentioned considerations, trials were undertaken and the below mentioned mass compatible method was finalized and subjected to validation challenges as per ICH guidelines[12].

Method subjected to analytical method validation:
Chromatographic conditions: Buffer: 0.1% TFA in water, Column: Inertsil ODS-3, 150 x 4.6mm, 5µm, Flow Rate: 1.0mL/min, Wavelength: 260nm, column oven temperature: 30°C±2°C, Sample Cooler Temperature: 15°C±2°C, Injection Volume: 10µL, Diluent: Mixture of water and methanol in the ratio of 70:30 respectively, Gradient run of 7 minutes

Gradient Program:

Time (min)	% Buffer	% Methanol
0	70	30
2	70	30
2.5	17	83
5	17	83
6	70	30
7	70	30

Individual Solution Chromatograms:

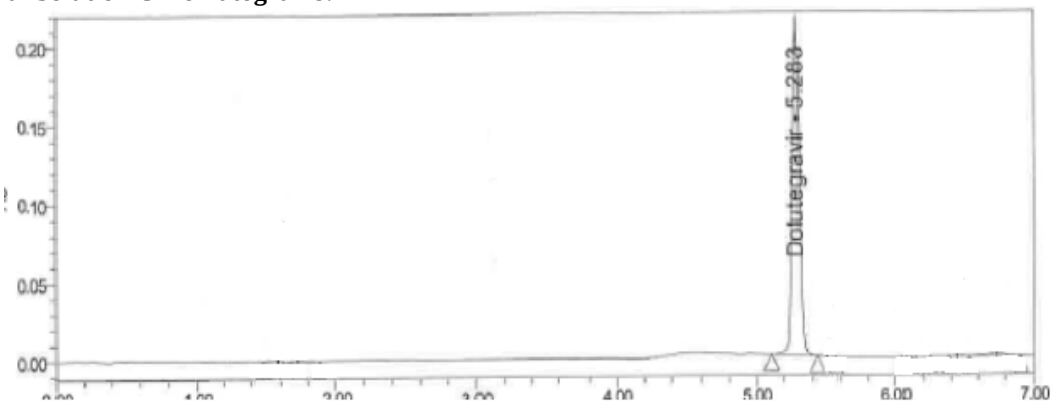

Fig:1- Dolutegravir

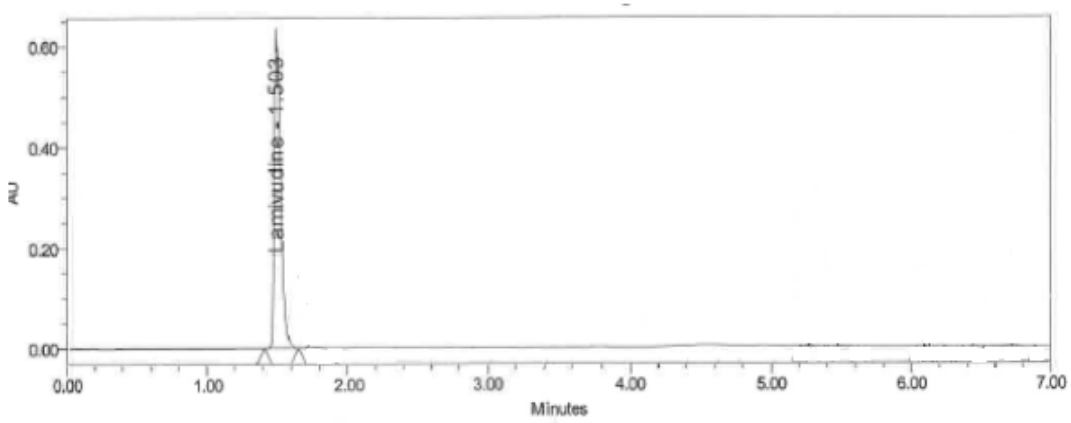

Fig:2-Lamivudine

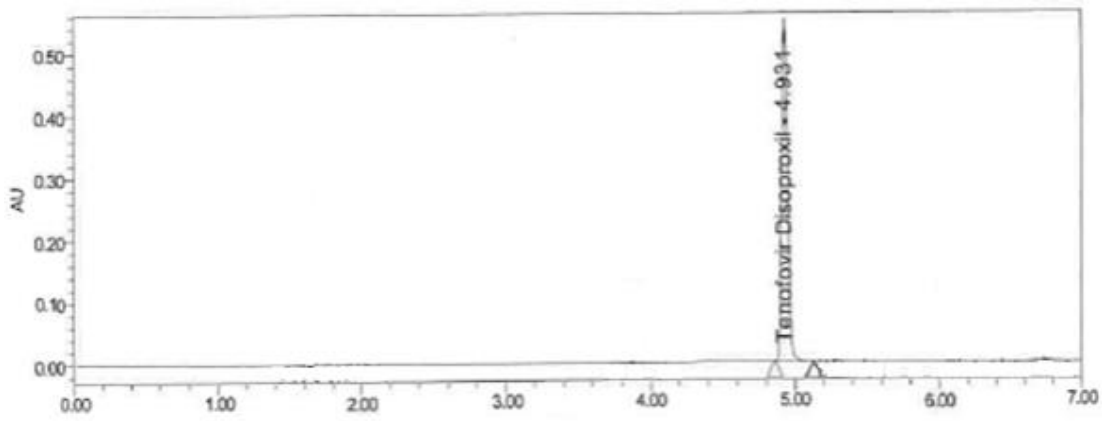

Fig:3- Tenofovir

Table:1 Linearity for Dolutegravir, Lamivudine and Tenofovir

Dolutegravir			**Lamivudine**		**Tenofovir**	
Linearity Level (%)	**Concentration (ppm)**	**Area counts**	**Concentration (ppm)**	**Area counts**	**Concentration (ppm)**	**Area counts**
10	1	34963	6	98257	6	76089
50	5	176584	30	493286	30	380727
80	8	283499	48	789255	48	608411
100	10	353918	60	987426	60	761132
120	12	425127	72	1184721	72	913213
150	15	530982	90	1481743	90	1141242
Correlation coefficient	**0.9999**		**0.9999**		**0.9999**	

Validation Outcomes:

Linearity:

The Linearity of the test method was established from 50% of the target concentration to 150% of target concentration. All the three active pharmaceutical ingredients exhibited linear behavior in this range. The linearity data is presented below in Table:1.

Accuracy:

The accuracy of an analytical process expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found[12].

Accuracy was performed in triplicate i.e. 50%, 100% and 150%. The individual and mean accuracy at each level was found to be between 98.0 to 102.0%. The accuracy data is presented in Table:2

Specificity:

It is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc.[12]

Specificity of the method was established by spiking all the available known impurities of all the three active pharmaceutical ingredients in placebo prepared by commonly used excipients (as the sample was market sample hence exact placebo composition was unknown) and all the peaks were observed to be well separated from the analyte peaks.

Table:2 Accuracy for Dolutegravir, Lamivudine and Tenofovir

Dolutegravir				
Accuracy Level	**Amount Added (mg)**	**Amount Recovered (mg)**	**Accuracy (%)**	**Mean (%)**
50%	24.8	24.5	98.8	
50%	24.5	24.7	100.8	
50%	25.2	25.1	99.6	99.7
100%	50.3	50.2	99.8	
100%	49.5	49.4	99.8	
100%	49.8	49.9	100.2	99.9
150%	75.5	75.4	99.9	
150%	75.1	75.2	100.1	
150%	75.2	75.3	100.1	100.0
Lamivudine				
Accuracy Level	**Amount Added (mg)**	**Amount Recovered (mg)**	**Accuracy (%)**	**Mean (%)**
50%	149.8	149.9	100.1	
50%	150.2	150.1	99.9	100.0
50%	150.5	150.4	99.9	
100%	300.2	300.8	100.2	
100%	300.0	300.1	100.0	
100%	300.5	300.7	100.1	100.1
150%	424.5	423.7	99.8	
150%	425.2	424.1	99.7	
150%	425.5	423.8	99.6	99.7
Tenofovir				
Accuracy Level	**Amount Added (mg)**	**Amount Recovered (mg)**	**Accuracy (%)**	**Mean (%)**
50%	149.8	150.4	100.4	
50%	150.1	149.5	99.6	
50%	150.2	149.5	99.5	99.8
100%	300.1	300.2	100.0	
100%	299.5	300.0	100.2	
100%	300.2	300.0	99.9	100.0
150%	450.5	448.1	99.4	
150%	450.2	447.2	99.3	
150%	450..8	450.9	100.0	99.6

The purity of the peak was assessed, and all the three peaks had purity angle less than auto purity threshold. Purity flag was absent for all the three active components.

Spiked Sample Recovery:

Recovery study was performed by analyzing assay sample spiked with all known impurities of dolutegravir, lamivudine and tenofovir disoproxil fumarate at 1% level. The results are presented in Table:3

Table:3 Specificity for Dolutegravir, Lamivudine and Tenofovir

Spike sample Recovery Results		
Dolutegravir		
Mean of 6 Unspiked Sample	**Spiked Sample**	**Difference (%)**
99.5	98.7	0.8
Lamivudine		
Mean of 6 Unspiked Sample	**Spiked Sample**	**Difference (%)**
100.2	100.6	0.4
Tenofovir		
Mean of 6 Unspiked Sample	**Spiked Sample**	**Difference (%)**
99.3	98.5	0.8

The assay results for sample spiked with known impurities at 1% level were found in line with results of unspiked sample.

Precision:

As part of precision study repeatability and intermediate precision (different day with different lot of analytical column and by using freshly prepared mobile phase and samples) was performed. The results are presented below in Table :4.

Table:4 Repeatability and Intermediate Precision for Dolutegravir, Lamivudine and Tenofovir

Repeatability			
Preparation	**Dolutegravir Amount Found (% Label Claim)**	**Lamivudine Amount Found (% Label Claim)**	**Tenofovir Amount Found (% Label Claim)**
Preparation-1	99.5	100.2	98.7
Preparation-2	99.8	100.6	98.5
Preparation-3	98.9	99.8	98.4
Preparation-4	100.4	100.1	99.5
Preparation-5	98.4	100.4	100.2
Preparation-6	100.2	100.2	100.5
Mean	**99.5**	**100.2**	**99.3**
RSD(n=6)	**0.8**	**0.3**	**0.9**
Intermediate Precision			
Preparation	**Dolutegravir Amount Found (% Label Claim)**	**Lamivudine Amount Found (% Label Claim)**	**Tenofovir Amount Found (% Label Claim)**
Preparation-1	100.2	99.8	99.2
Preparation-2	99.5	100.1	98.9
Preparation-3	99.8	100.3	98.5
Preparation-4	99.6	100.6	100.1
Preparation-5	99.7	99.8	99.5
Preparation-6	100.0	100.2	99.7
Mean	**99.8**	**100.1**	**99.3**
RSD(n=6)	**0.3**	**0.3**	**0.6**

Robustness study:

Robustness study encompassed evaluation of impact of pH variation, flow rate and column oven temperature. Results are presented in **Table:5**

Table:5 Robustness (Variation in pH of buffer, Flow rate and column oven temperature)

Variation in pH of buffer				
Dolutegravir				
pH of Buffer	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
2.2 (As per method)	3.7	54256	1.1	0.6
2.7	3.9	49582	1.1	0.6
1.7	3.6	53725	1.1	0.5
Lamivudine				
pH of Buffer	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
2.2 (As per method)	3.7	4931	1.2	0.6
2.7	3.9	4432	1.2	0.7
1.7	3.6	4952	1.2	0.6
Tenofovir				
pH of Buffer	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
2.2 (As per method)	3.7	52942	1.0	0.5
2.7	3.9	50578	1.0	0.6
1.7	3.6	53259	1.0	0.5
Flow Rate Variation				
Dolutegravir				
Flow rate (mL/min)	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
1.0 (as per method)	3.7	54256	1.0	0.5
0.9	4.0	52113	1.2	0.5
1.1	3.4	55102	1.0	0.5
Lamivudine				
Flow rate (mL/min)	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
1.0 (as per method)	3.7	4931	1.2	**0.7**
0.9	4.0	4801	1.3	**0.5**
1.1	3.4	5001	1.2	**0.7**

Tenofovir				
Flow rate (mL/min)	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
1.0 (as per method)	3.7	52942	1.0	0.5
0.9	4.0	51082	1.0	0.5
1.1	3.4	53126	1.0	0.5
Column Oven Temperature Variation				
Dolutegravir				
Column Oven Temperature	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
40°C (as per method)	3.7	54256	1.1	**0.5**
35°C	3.5	51018	1.1	0.6
45°C	3.8	55678	1.1	0.5
Lamivudine				
Column Oven Temperature	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
40°C (as per method)	3.7	4931	1.1	0.5
35°C	3.5	4526	1.2	0.6
45°C	3.8	5013	1.1	0.5
Tenofovir				
Column Oven Temperature	**Resolution (Between Dolutegravir and Tenofovir)**	**Plate Count**	**Tailing**	**RSD of 6 Standards**
40°C (as per method)	3.7	52942	1.0	0.5
35°C	3.5	50127	1.0	0.5
45°C	3.8	53987	1.0	0.5

CONCLUSION:

A short, mass compatible cost-effective assay method was developed for simultaneous estimation of Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate Tablets. The method was subjected to validation challenges and was found to be Specific, Linear, Precise, Accurate and Robust.

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