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METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE AND TINOFOVIR DISOPROXIL FUMERATE BY USING RP-HPLC IN PHARMACEUTICAL DOSAGE FORM

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Abstract
A simple, linear, precise and accurate Reverse phase high Performance Liquid Chromatographic method was developed and validated for the analysis of Lamivudine and Tinofovir disproxil fumerate. The chromatographic separation was achieved on a C18 column [Use Inertsil ODS C18, 5µ , 150 mm x 4.6 mm] utilizing a mobile phase of ortho- phosphoric acid buffer and methanol in the ratio of 30:70 at a flow rate of 0.6 ml/min with UV detection at 254nm. The retention time of Lamivudine and Tinofovir disproxil fumerate was found to be 2.147 and 3.494min respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation. The method was accurate with a mean recovery of 99.15%, 99.30% for Lamivudine and Tinofovir disproxil fumerate respectively. The percentage RSD for Lamivudine is 0.17 and for Tinofovir disproxil fumerate is 0.14. Method was linear with a correlation coefficient of 0.999 for both Lamivudine and Tinofovir disproxil fumerate. LOD, LOQ obtained was 2.97, 9.96 and 2.98, 9.98 for Lamivudine and Tinofovir disproxil fumerate respectively. The developed method was validated in terms of robustness by changing flow rate and organic composition in mobile phase. The results obtained were measured in terms of system suitability which were within limits indicating that the method is robust.
***KEY WORDS**: Lamivudine, Tinofovir disproxil fumerate, RP-HPLC, Validation, UV,*

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INTRODUCTION

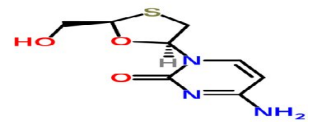
HPLC is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principle advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision and sensitivity[1].

In the modern pharmaceutical industry HPLC is the major and integral analytical tool applied in all stages of drug discovery, development and production. The development of new chemical entities is comprised of two major activities: drug discovery and drug development. The goal of drug discovery program is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening. This lead to final selection of most potentially viable therapeutic candidates that are taken forward to drug development. The main function of drug development is to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening and clinical trials.

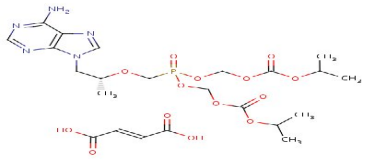
A thorough understanding of HPLC principles and theory laid a solid foundation for appreciating many variables that are optimized during fast and effective HPLC method development and optimization[2].

Drug profile

Lm is a nucleoside reverse transcriptase inhibitor with activity against HIV1 and HBV. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. Lm was rapidly absorbed after oral administration in HIV-infected patients[3,4].



Lamivudine



Tinofovir disproxil fumerate

Tdf belongs to a class of antiretroviral drugs, is a prodrug of tinofovir, a nucleotide reverse transcriptase inhibitor, blocks reverse transcriptase, an enzyme crucial to viral production in HIV-infected people. It inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate and, after incorporation into DNA, by DNA chain termination[3,4].

MATERIALS AND METHODS

Orthophosphoric acid, acetonitrile were obtained from Fischer scientific and Molychem respectively. Water, Methanol, Hydrochloric acid, Hydrogen peroxide and Sodium hydroxide used were of HPLC grade. Sample tablets used were obtained from local pharmacy stores.

HPLC model used was of WATERS, with software: Empower, 2695 separation module holding 2487 DAD detector. UV/VIS spectrophotometer and pH meter used were of LABINDIA UV 3000^{+} and Adwa – AD 1020 respectively. Weighing balance used was of Afcoset ER-200A model.

A good method development should require as many experimental conditions as necessary to achieve the final desired result. Method development should be a simple precise yet should allow use of sophisticated tools and software available.

Mobile Phase Optimization [5]

Initially the mobile phase tried was methanol: water and acetonitrile: buffer with varying proportions. Finally, the mobile phase was optimized to buffer, Methanol in proportion 30: 70 v/v respectively.

Preparation of Orthophosphoric Acid buffer

Measure 1.0 ml of Ortho Phosphoric acid into a 1000ml volumetric flask, dissolve and dilute to 1000ml with HPLC water.

Preparation of mobile phase

Mix a mixture of above buffer 300ml (30%) and 700ml of Methanol HPLC (70%), degas in ultrasonic water bath for 5 min. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation

Use the Mobile phase as Diluents.

Preparation of standard substance

Accurately weigh and transfer 10 mg of Lm working standard into a 100ml clean dry volumetric flask add 70ml methanol and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Accurately weigh and transfer 10 mg of Tdf working standard into a 100ml clean dry volumetric flask and add methanol and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Wave length selection

From the stock solution prepared pipette 3.0 ml of Lm solution into a 10ml volumetric flask and dilute up to the mark with methanol and record UV spectrum in the range of 200 to 400 nm

Pipette 3 ml of Tdf stock solution into a 10ml volumetric flask and dilute up to the mark with methanol and recorded UV spectrum in the range of 200 to 400 nm

From the two spectrums isobestic point was determined as 254nm

Optimization of Column

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inspire column. Symmetry C_{18} (4.6 x 150mm, 5µm, Make: Inertisil ODS) was found to be ideal as it gave good peak shape at 0.6 ml/min flow.

RESULTS AND DISCUSSION

Optimized Chromatographic Parameters

Equipment : High performance liquid chromatography equipped with Auto Sampler and DAD or UV detector

Column : Inertisil ODS C18 (4.6 x 150mm, 5µm)

Flow rate : 0.6 ml/min

Wavelength : 254 nm

Injection volume : 20 µl

Run time : 10 min

VALIDATION PARAMETERS

System suitability

System suitability was carried out and the parameters area, height, USP tailing factor and USP plate count were calculated and reports were given in table 1 and figure 2.

Acceptance criteria

Theoretical plates must be not less than 2000.Tailing factor must be not less than 0.9 and not more than 2.

It was found from above data that all the system suitability parameters for developed method were within the limit.

METHOD PRECISION

Expresses the closeness of the agreement between a series of measurements obtained from multiple sample of the same homogenous sample under prescribed conditions results were shown in tables 2&3, figures 3-7.

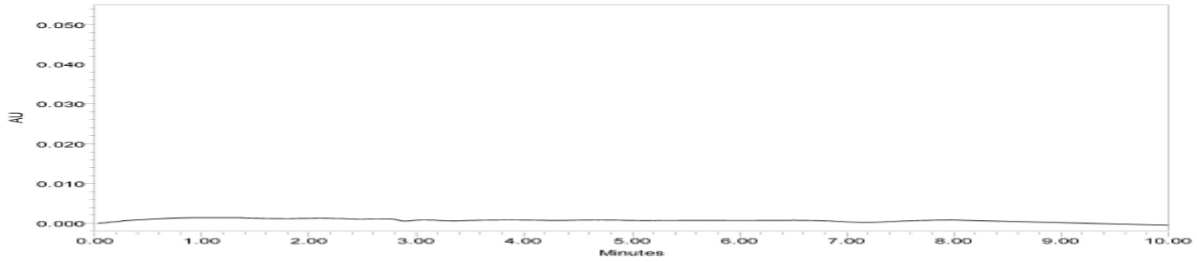


Fig 1: Chromatogram for blank

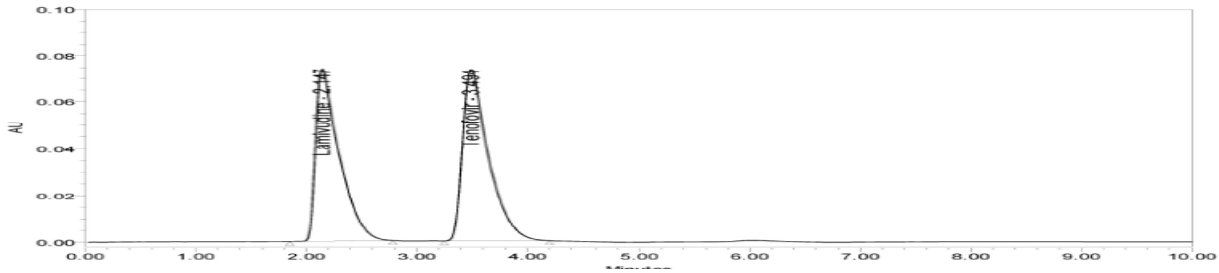


Figure 2 Chromatogram for system suitability

Table 1: Results of system suitability parameters for Lm and Tdf

S.No	Name	Retention time (min)	Area (µV sec)	Height (µV)	USP tailing	USP plate count
1	Lm	2.147	1085263	74148	1.8	2546
2	Tdf	3.494	1192480	73841	1.6	3178

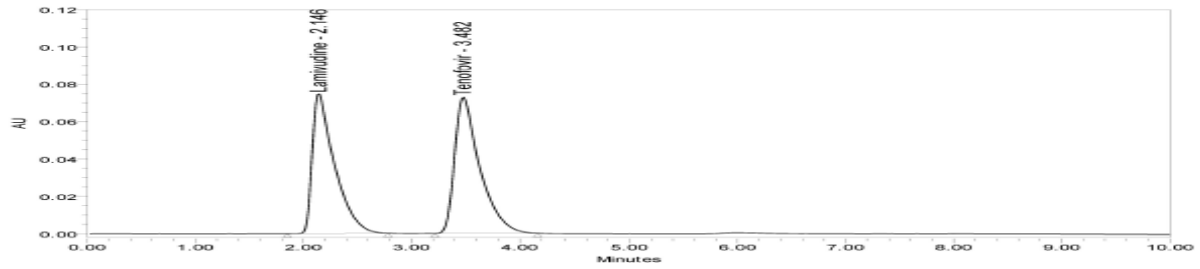


Fig 3: Chromatogram for standard injection -1

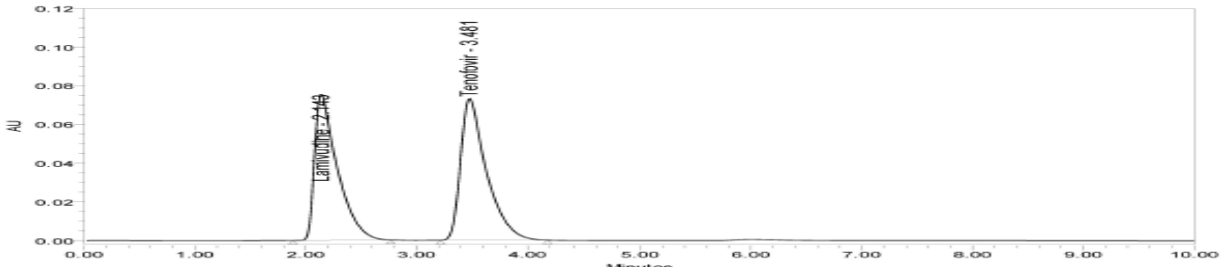


Fig 4: Chromatogram for standard injection-2

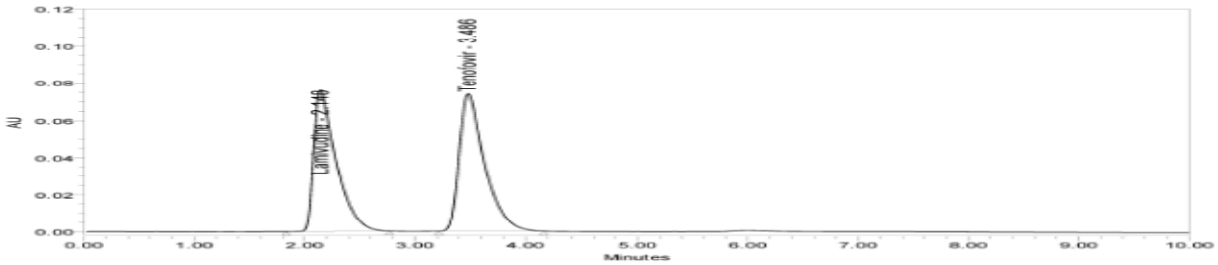


Fig 5: Chromatogram for standard injection-3

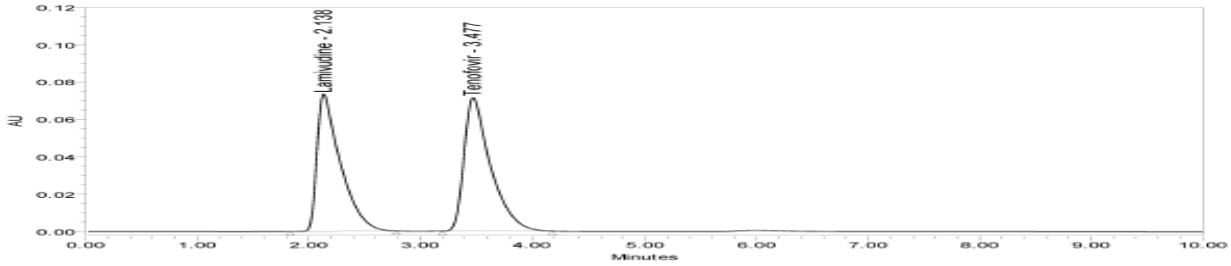


Fig 6: Chromatogram for standard injection-4

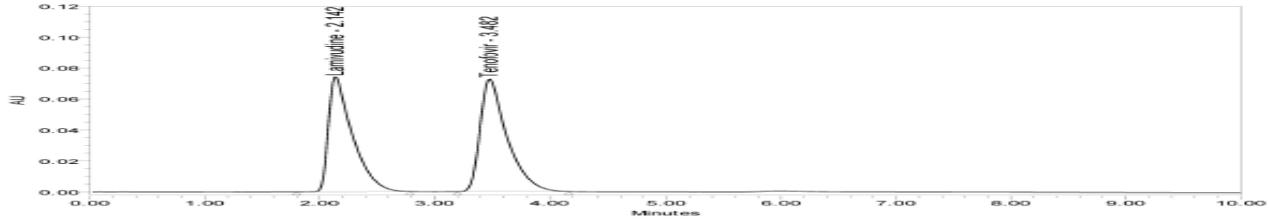


Fig 7: Chromatogram for standard injection-5 Results of method Precision

Table2: The results are summarized Lm

Injection	Area
Injection-1	1062317
Injection-2	1063789
Injection-3	1063303
Injection-4	1063099
Injection-5	1067011
Average	1063904
Standard Deviation	1816.21
%RSD	0.17

Table 3: The results are summarized Tdf

Injection	Area
Injection-1	1180142
Injection-2	1184113
Injection-3	1184542
Injection-4	1182206
Injection-5	1182881
Average	1182777
Standard Deviation	573.18
%RSD	0.14

Acceptance Criteria: The % RSD for the area of five standard injections results should not be more than 2%.

Intermediate Precision (ruggedness)

To evaluate the intermediate precision of the method, Precision was performed on different day by using different make column of same dimensions.

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation shown in tables 4 and 5.

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more than 2%.

ACCURACY

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150%. Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions.

Calculate the Amount found and Amount added for Lm & Tdf and calculate the individual recovery and mean recovery values were shown in tables 6 and 7.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%

LINEARITY

Preparation of Level – I (10ppm &10ppm of Lm & Tdf):

Take 1.0ml of stock solution in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (20ppm &20ppm of Lm & Tdf):

Take 2.0ml of stock solution in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (30ppm &30ppm of Lm & Tdf):

Take 3.0ml of stock solution in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (40ppm &40ppm of Lm & Tdf)

Take 4.0 ml of stock solution in 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (50ppm & 50ppm of Lm & Tdf)

Take 5.0 ml of stock solution in 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area shown in tables 8 and 9.

The linearity range was found to lie from 10µg/ml to 50µg/ml of Lm, 10 µg/ml to 50 µg/ml 0f Tdf .

Table 4: The results are summarized Lm

Injection	Area
Injection-1	1072557
Injection-2	1065974
Injection-3	1094564
Injection-4	1065475
Injection-5	1066302
Injection-6	1069106
Average	1072330
Standard Deviation	11211.81
%RSD	1.04

Table 5: The results are summarized Tdf

Injection	Area
Injection-1	1183002
Injection-2	1183171
Injection-3	1182816
Injection-4	1183085
Injection-5	1183852
Injection-6	1184581
Average	1183418
Standard Deviation	670.5644
%RSD	0.05

Table 6: The accuracy results for Lm

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	570007	5.0	5.28	103.45%	99.15%
100%	1058983	10.0	9.80	98.03%	
150%	1555434	15.0	14.40	95.99%	

Table 7: The accuracy results for Tdf

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	629528	5.0	5.2	104.01%	99.30%
100%	1174715	10.0	9.7	97.05%	
150%	1758274	15.0	14.53	96.84%	

Table 8: Linearity Results: (for Lm)

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	388060
2	II	20 ppm	744106
3	III	30 ppm	1061095
4	IV	40 ppm	1443273
5	V	50 ppm	1725673
Correlation Coefficient			0.999

Table 9: Linearity Results: (for Tdf)

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	413496
2	II	20 ppm	777991
3	III	30 ppm	1183467
4	IV	40 ppm	1541666
5	V	50 ppm	1912742
Correlation Coefficient			0.999

Acceptance criteria:

Correlation coefficient (R^2) should not be less than 0.999

The correlation coefficient obtained was 0.999 which is in the acceptance limit.

LIMIT OF DETECTION: (for Lm)

Pipette 1.0 ml of Lm stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 0.05 µg/ml solution:

Pipette 1 ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Further pipette 0.5 ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 42 µV

Signal Obtained from LOD solution :125 µV

S/N = 125/42 = 2.97

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

LIMIT OF DETECTION: (for Tdf)

Preparation of 10 µg/ml solution:

Pipette 1 ml of Tdf stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 0.05µg/ml solution:

Pipette 1.0 ml of above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents

Acceptance Criteria:

S/N Ratio value shall be 3 for LOD solution.

The lowest concentration of the sample was prepared with respect to the base line noise and measured the signal to noise ratio shown in figure 8.

Lamivudine Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank: 47 µV

Signal Obtained from LOD solution : 139 µV

S/N = 139/47 = 2.9

Tenofovir Disoproxil Fumerate Calculation of S/N Ratio: Average Baseline Noise obtained from Blank: 47 µV
Signal Obtained from LOD solution :140 µV
S/N = 140/47 = 2.98

Acceptance Criteria:
S/N Ratio value shall be 3 for LOD solution.

LIMIT OF QUANTIFICATION: (Lm)

Preparation of 10 µg/ml solution:
Pipette 1.0 ml of Lm stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 0.2 µg/ml solution:
Pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Further pipette 2ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Acceptance Criteria:
S/N Ratio value shall be 10 for LOQ solution.

LIMIT OF QUANTIFICATION: (Tdf)

Preparation of 10 µg/ml solution:
Pipette 1.0 ml of Tdf stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of 0.2µg/ml solution):
Pipette 1.0 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
Pipette 2.0 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

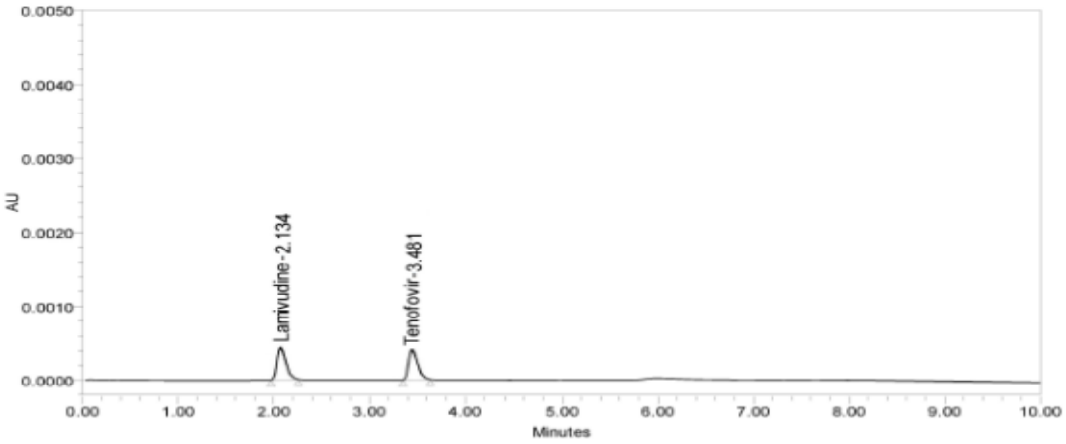


Fig 8: Chromatogram of Lm and Tdf showing LOQ

Lamivudine Calculation of S/N Ratio:
Average Baseline Noise obtained from Blank: 47 µV
Signal Obtained from LOQ solution : 469µV
S/N = 468/47 = 9.96

Tenofovir Disoproxil Fumerate Calculation of S/N Ratio:
Average Baseline Noise obtained from Blank: 47 µV
Signal Obtained from LOQ solution : 469µV
S/N = 469/47 = 9.98

Acceptance Criteria:
S/N Ratio value shall be 10 for LOQ solution.

ROBUSTNESS:
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.
a). The flow rate was varied at 0.5 ml/min to 0.7ml/min.
Standard solution 30 ppm & 30 ppm of Lm & Tdf was prepared and analysed using varied flow rates along with method flow rate. The results are summarized
On evaluation of the results, it was concluded that the variation in flow rate does not affect the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.
The method is robust in flow condition
***** Results for actual flow (0.6 ml/min) have been considered from Assay standard.
b). The Organic composition in the Mobile phase was varied from 63% and 37% to 77% and23%
Standard solution 30µg/ml & 30µg/ml of Lm& Tdf was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. The results are summarized
On evaluation of the results, it was concluded that the variation in 10% Organic composition in the mobile phase affected the method significantly.

Hence it indicates that the method is robust even by change in the Mobile phase ±10

There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. Results were shown in tables from 10 to 13.

Results for variation in flow

Table-10 System suitability results for Lm:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	0.5	2489.5	1.8
2	0.6	2545.8	1.8
3	0.7	2412.7	1.5

Table-11 System suitability results for Tdf:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	0.5	2843.8	1.6
2	0.6	3178.0	1.6
3	0.7	2954.3	1.4

Table-12 System suitability results for Lm:

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	10% less	2469.4	1.9
2	*Actual	2545.8	1.8
3	10% more	2437.3	1.7

Table-13 System suitability results for Tdf:

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	10% less	3001.7	1.5
2	*Actual	3178.0	1.6
3	10% more	3010.5	1.6

***** Results for actual Mobile phase composition (30:70 buffer: methanol) have been considered from Accuracy standard.

CONCLUSION

The reliability and suitability of the method could be seen from recovery studies. Further there is no interference due to excipients.

System suitability parameters were calculated which includes efficiency, plate count and tailing factor.

Precision of the methods were studied by making repeated injections of the samples and system precision values were determined.

The method was validated for linearity, accuracy, precision, robustness.

The method is simple, specific, accurate precise, robust & easy to perform and requires short to analyse the samples.

Table 14: Summary Table

S.No	Parameter	Experiment	Acceptance criteria	Observation for Lm	Observation for Tdf	Result
1	System suitability		Theoretical plates NLT 2000, Tailing factor NLT 2%	2546, 1.8	3178, 1.6	Passed
2	Linearity	Corellation coefficient	NLT 0.999	O.999	0.999	Passed
3	Precision	System precision	%RSD NMT 2	0.17%	0.14%	Passed
4	Accuracy	Recovery of analyte	Mean recovery 98-102%	99.15%	99.30%	Passed
5	Ruggedness	Different day	% RSD NMT 2%	1.04%	0.05%	Passed
6	LOD		NMT 3	2.97	2.98	Passed
7	LOQ		NMT 10	9.96	9.98	Passed

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