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METHOD DEVELOPMENT AND VALIDATION OF LAMIVUDINE AND TINOFOVIR BY RP-HPLC METHOD

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ABSTRACT

A simple method was developed and validated for the simultaneous estimation of Lamivudine and Tenofovir disoproxil fumarate in pharmaceutical dosage form. The method was based on RP-HPLC. Chromatographic separation was performed on Symmetry C18 (4.6 x 150mm, 5µm, Make: Waters) or equivalent ,column using a mobile phase consisting of a mixture of KH2PO4 buffer (pH 2.5 with dilute ortho-phosphoric acid): Methanol: phosphate Buffer (70%30%v/v) in an isocratic mode. The following system conditions were maintained throughout development and validation i.e., flow rate 1.0 mL/min, column was maintained at room temperature and the detected by a UV-wave length at 260nm. The Lamivudine and Tinofovir were well resolved on the stationary phase and the retention times were 2.464 and 3.746 minutes respectively. The method was validated; both the drugs were shown to be linear over a range of 300 µg/mL. Precision Intermediate Precision/Ruggedness Accuracy linearity Limit of detection Limit of quantification Robustness was determined to validate the method.

Key words: Methanol, phosphate Buffer, orthophosphoric acid, KH2PO4 buffer, RP-HPLC

INTRODUCTION

Lamivudine is chemically known as (2R,cis)-4-amino-l-(2-hydroxymethyl-l,3-oxathiolan-5-yl)-(lH)-pyrimidin-2-one is an Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphor di ester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated. Lamivudine is administered orally, and it is rapidly absorbed with a bio-availability of over 80%. Some research suggests that lamivudine can cross theblood–brain barrier. Lamivudine is often given in combination with zidovudine, with which it is highly synergistic. Lamivudine treatment has been shown to restore zidovudine sensitivity of previously resistant HIV. Lamivudine showed no evidence of carcinogenicity or mutagenicity in *in vivo* studies in mice and rats at doses from 10 to 58 times those used in humans. Tinofovir is chemically known as 9-[(R)-2 [[bis [[(isopropoxy carbonyl)oxy]- methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1).Tenofovir is an antiretroviral agent that inhibits the viral enzyme reverse transcriptase and so inhibits replication of retroviruses used as tenofovir disoproxilfumarate in the treatment of HIV-1 (human immunodeficiency virus-1) infection.

MATERIALS AND METHOD

Chemicals and solvents: A Pure samples of lamivudine and tinofovir were obtained respectively from surapharma research solutions, Hyderabad, India. Commercial pharmaceutical preparation Availed containing 300 mg lamivudine and tenofovir respectively (Marketed by tinvir-L Pvt. Ltd) were procured from local pharmacy. Methanols, phosphate

Buffer, orthophosphoric acid, KH2PO4 used were of HPLC grade.

Instrumentation: The method was based on RP-HPLC Agilent Technologies 1200 series with Empower Pro software. Chromatographic separation was performed on Symmetry C18 (4.6 x 150mm, 5µm, Make: Waters) or equivalent ,column using a mobile phase consisting of a mixture of KH2PO4 buffer (pH 2.5 with dilute orthophosphoric acid): Methanol: phosphate Buffer (70%30%v/v) in an isocratic mode and column was maintained at room temperature and the detected by a UV-wave length at 260nm. The method was validated; both the drugs were shown to be linear over a range of 300 µg/mL. Precision Intermediate Precision/Ruggedness Accuracy linearity Limit of detection Limit of quantification Robustness was determined to validate the method.

Chromatographic conditions: The method was based on RP-HPLC Agilent Technologies 1200 series with Empower Pro software. Chromatographic separation was performed on Symmetry C18 (4.6 x 150mm, 5µm, Make: Waters) or equivalent. The following system conditions were maintained throughout development and validation i.e., flow rate 1.0 mL/min, column was maintained at room temperature and the detected by a UV-wave length at 260nm**.** The Lamivudine and Tenofovir were well resolved on the stationary phase and the retention times were 2.464 and 3.746 minutes respectively. The method was validated; both the drugs were shown to be linear over a range of 300 µg/mL**.**

Preparation of buffer solution:

Preparation of Phosphate buffer :(P^H:2.5): Weighed 7.0 grams of Potassium Di hydrogen Ortho Phosphate into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water. Adjust Ph 2.5with Orthophosphoric acid.

Preparation of mobile phase: Mix a mixture of above Buffer 300 mL (30%),700 mL of Methanol HPLC (70%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Use the Mobile phase as Diluent.

Preparations of Standard solutions

LAMIVUDINE: Accurately weigh and transfer 10 mg of Lamivudine standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

TINOFOVIR: Accurately weigh and transfer 10 mg of Tinofovir standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Sample solution:

LAMIVUDINE: Accurately weigh and transfer equivalent to 861.5 mg of Lamivudine into a 100mL clean dry volumetric flask add about 70mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.2ml of Lamivudineand the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

TINOFOVIR: Accurately weigh and transfer equivalent to 861.5 mg of Tinofovir into a 100mL clean dry volumetric flask add about 70mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.2ml of Tenofovir of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Method validation: The developed method was validated as per the ICH (International Conference on Harmonization) guidelines with respect to System suitability, Precision, Linearity, Accuracy, Limit of detection and Limit of quantification. Robustness

Precision: The precision was determined by The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limitsEnsure that the system meets the required system suitability by injecting the system suitability solution. Inject each precision solution in singlet. Calculate % drug release and % RSD for Lamivudine and Tinofovir from the sample preparations. Results are shown in Table-1

Linearity: The linearity was determined by The preparation of different levals

Preparation of Level – I (20ppm of Lamivudine&20ppm of Tenofovir):
0.2ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – II (40ppm of Lamivudine&40ppm ofTenofovir)):
0.4ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – III (60ppm of Lamivudine&60ppm of Tenofovir)):
0.6ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – IV (80ppm of Lamivudine&80ppm of Tenofovir)):
0.8ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – V (100ppm of Lamivudine&100ppm of Tenofovir))
1.0ml of stock solution has taken 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. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. Result are shown in table-2

System suitability: The system suitability was determined by Accurately weigh and transfer 10 mg of Lamivudine and tinofovir working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent values were well within usually accepted limits (≤2%). Theoretical plates, tailing factor, resolution were determined. The results are all within acceptable limits summarized in Table-2

Accuracy: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Lamivudine&Tenofovirand calculate the individual recovery and mean recovery values.results are shown in table -3

Limit of detection:
Preparation of 60µg/ml solution: Accurately weigh and transfer 10 mg of Lamivudine working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.02µg/ml solution): Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Pipette 0.4mL of solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Calculate the S/N Ratio **.**the acceptance criteria should be in limit. results are shown in table-4

Limit of Quantification

Preparation of 60µg/ml solution: Accurately weigh and transfer 10 mg of Lamivudine working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
(Stock solution): Further pipette 0.6ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.
Preparation of 0.06µg/ml solution):
Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Pipette 1.0mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Pipette 1.0 mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.Calculate the S/N Ratio **.**the acceptance criteria should be in limit. Results are shown in table-5

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. The flow rate was varied at 0.8 ml/min to 1.2ml/min. Standard solution 60ppm of Lamivudine&60ppm of lamivudine and Tenofovir was prepared and analysed using the varied flow rates along with method flow rate. The results are summarized On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%. Results are shown in table -6

RESULTS AND DISCUSSION

The nature of sample, its molecular weight and solubility decides the proper selection of stationary phase. The drugs lamivudine and tinofovir were preferably analyzed by reverse phase chromatography and accordingly C18 column was selected. The elution of the compounds from column was influenced by polar mobile phase. The ratio of

Methanol: phosphate Buffer (70%30%v/v) to give well resolved and good symmetrical peaks with short run time. The retention time were found to be **2.464 and 3.746** min respectively. The linearity of the method was statistically confirmed. RSD values for accuracy and precission studies obtained were less than 2% which revealed that developed method was accurate and precise. The system suitability parameters were given in table-1. Precision, accuracy, limit of detection and limit of quantification and robustness are within the limit.

CONCLUSION

The developed method is accurate, simple, rapid and selective for the simultaneous estimation of lamivudine and tinofovir by RP-hplc method. The sample preparation is simple, the analysis time is short and the elution is by gradient method. The retention time of lamivudine and tinofovir were found to **2.464 and 3.746** min respectively. The excipients of the commercial sample analyzed did not interfere in the analysis, which proved the specificity of the method for these drugs. Hence the proposed method can be conveniently adopted for the routine quality control analysis in the combined formulation.

Table-1 System precession:

INJECTIONS	*AREA(lamivudine)*	*AREA(tinofovir)*
1	2270553	993413
2	2278100	993859
3	2282356	998213
4	2283157	998930
5	2285975	999663
Average	2280028.2	996815.8
Standard Deviation	6001.7	2952.0
%RSD	0.3	0.3

Table-2 linearity (forLamivudine)

S.No	Linearity Level	Concentration	Area
1	I	20ppm	800199
2	II	40ppm	1589391
3	III	60ppm	2264300
4	IV	80ppm	3071625
5	V	100ppm	3894075
Correlation Coefficient			0.999

Linearity (for tinofovir)

S.No	Linearity Level	Concentration	Area
1	I	20ppm	339009
2	II	40ppm	689527
3	III	60ppm	994963
4	IV	80ppm	1385006
5	V	100ppm	1766425

Table-3.accurasy for lamivudine

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1184204	5.1	5.16	101.2%	100.4%
100%	2121872	9.4	9.25	98.4%	
150%	3525766	15.1	15.3	101.8%	

accurasy for tinofovir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	522218.2	5.1	5.17	101.4%	100.6%
100%	979319.6	9.8	9.70	99.0%	
150%	1576652	15.4	15.6	101.4%	

Limit of detection:
Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 47µV

Signal Obtained from LOD solution : 138 µV

S/N = 138/47 = 2.93

Acceptance Criteria:

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

Limit of quantification:
Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 47 µV

Signal Obtained from LOQ solution : 466µV

S/N = 466/47 = 9.91

Acceptance Criteria:

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

System suitability-4 results forLamivudine:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	0.8	7166.6	1.2
2	1.0	5404.6	1.2
3	1.2	4573.2	1.2

System suitability results forTenofovir:

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	0.8	8898.0	1.1
2	1.0	7344.3	1.1
3	1.2	6255.1	1.1

System suitability results for Lamivudine:

S.No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	**USP Tailing**
1	10% less	5889.6	1.2
2	*Actual	5404.6	1.2

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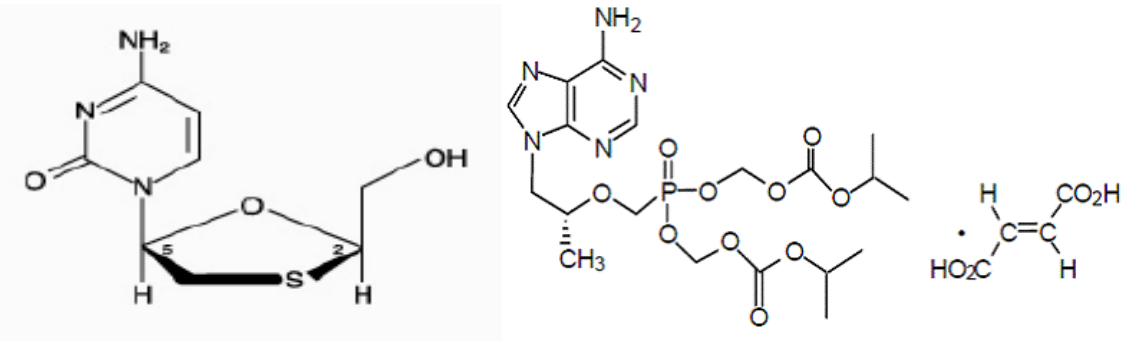
System suitability results for Tenofovir:

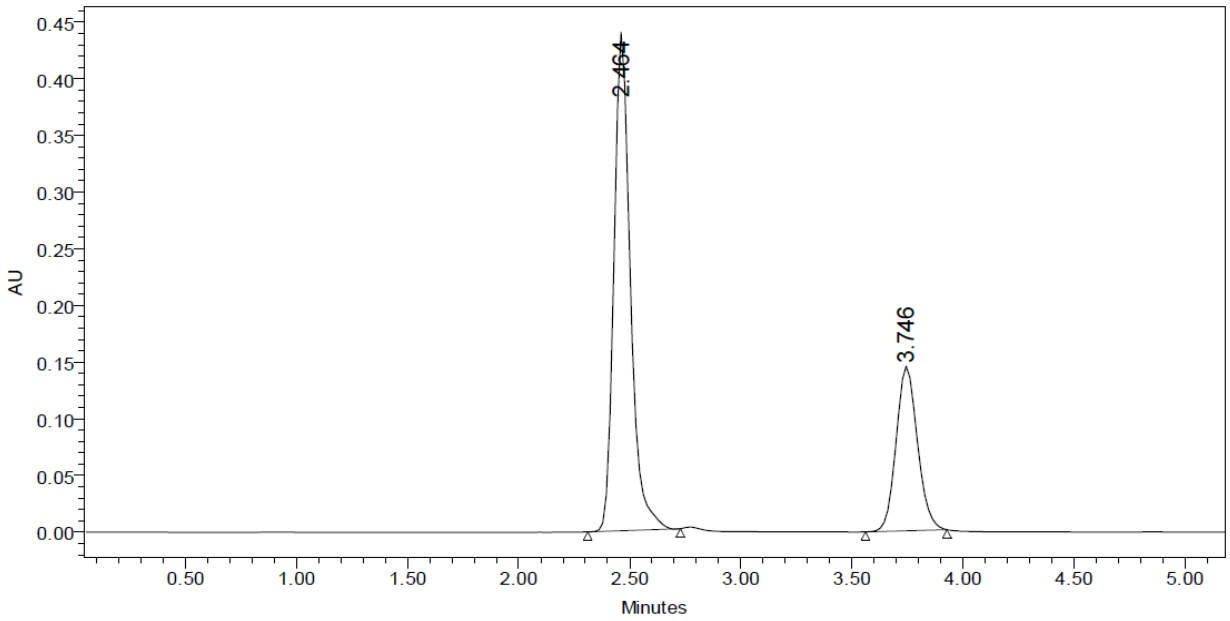
S.No	**Change in Organic Composition in the Mobile Phase**	**System Suitability Results**	
		USP Plate Count	**USP Tailing**
1	10% less	8583.8	1.1
2	*Actual	7344.3	1.1
3	10% more	8252.5	1.1

Fig 1: Structures of (a) lamivudine (b) tinofovir



(a) (b)

Fig. 2: chromatogram showing retention time of lamivudine and tinofovir:s



REFERANCE

1. Sravan Kumar Chopperla*1,B.Vijay Kumar1, Dr.D.Gowri Sankar1 Method development and validation of rp- hplc method for simultaneous estimation of Lamivudine and tenofovirdisoproxilfumarate in pharmaceutical dosage form IJPRD, 2013; Vol 4(11): January-2013 (110 – 118).
2. AnandakumarKarunakaran, KannanKamarajan, VetrichelvanThangarasu, A Validated RP - HPLC method for simulataneous estimation of Lamivudine and Tenofovirdisoproxilfumarate in pure and in tablet dosage form. Eurasian Journal of Analytical Chemistry, Vol 7, No 2 (2012)
3. Ashok gorja*, jahnavibandla,. Method development and validation for the simultaneous estimation of lamivudine and tenofovirdisoproxilfumarate in pharmaceutical dosage forms by rp-hplc. IJARPB: 2013, 2(1), 22-32.
4. Sonawane P.H.1 Panzade P.S.2 * and Kale M. A.1-simultaneous estimation of lamivudine and tenofovirdisoproxilfumarate in bulk and combined pharmaceutical dosage form by hplc method (asian journal of bio
5. NL Rezk, RD Crutchley, ADM Kashuba - Journal of Chromatography B, 2005 – Elsevier Volume 822, Issues 1–2, 5 August 2005, Pages 201–208
6. Beckett AH, Stenlake JB, pharmaceutical chemistry, ivthed.part two. New Delhi, CBS; 1997. P.302-305. Kenneth A Connors, pharmaceutical analysis. Third Edition. Singapore. John Wiley Publishers: 1982.p. 529-530.
7. Gottsclich, N. And Kasche, V. Journal of Chromatography. A, (1997) 201-206.Gottsclich,N. And Kasche, V. Journal of Chromatography.A (1997) 201- 206.
8. Snyder, R.L., Glajch, J.L. and Kirkland, J.J. Practical HPLC Method Development. John Wiley &Sons Inc. 1988.
9. Allenmark, S.G. and Andersson, S. J. Chromatography A. (1994) 666, 167-179.
10. Lloyd R, Synder, Joseph J, Kirkland, Joseph L Glajesh. Practical HPLC Method Development, 2nd ed; 1997. 1-14.
11. Pattengill MD, Sands DE. Statistical significance of linear least-squares parameters, J ChemEduc; 1979 .p 56:244.
12. Armstrong DW, Bonded phase material for chromatographic separations. U.S patent 4539399;1985.p.16-18
13. Lough WJ, Wainer IW. High Performance Liquid Chromatography .fundamental principles & practice. Glasgow (UK): Blackie Academic & Professional; 1995. P.2-28.
14. Meyer VR Practical high performance liquid chromatography. 2nd ed.New York Wiley interscience;1994 .p. 12
15. Ghoush MK .HPLC methods on drug analysis.Berlin: Springer Verlag;1992.p.1-22

16. Ethance DN. Fundamental of statistics. $&thed.Kitbagmahal; 2003.p.54-80.
17. Fong GW, Lan SK. HPLC in the Pharmaceutical industry. Vol-47, New York Marcel Dekker Inc; 1991. P. 16-56.
18. Lindsay S .High performance Liquid Chromatography. 1st ed.New York Wiley Inter science; 1991.p.1-24
19. Validation of Analytical procedures: Methodology, ICH Harmonised tripartite Guideline, 1996, 1-8, [online] available at www.ich.org.
20. 18.British Pharmacopoeia, Controller of HMSO, international Edn. London, Volume I and II, 1998.
21. United States Pharmacopoeia 26, National Formulary 21, Asian Edition, 2003.
22. Sravan Kumar Chopperla*1,B.Vijay Kumar1, Dr.D.Gowri Sankar1,IJPRD, 2013; Vol 4(11): January-2013 (110 – 118).
23. AnandakumarKarunakaran, KannanKamarajan, VetrichelvanThangarasu, Eurasian Journal of Analytical Chemistry, Vol 7, No 2 (2012).
24. Ashok gorja*, jahnavibandla,. IJARPB: 2013, 2(1), 22-32.
25. Sonawane P.H.1 Panzade P.S.2 * and Kale M. A.1-:e-ISSN: 2249-622X.
26. Kavitha K Y*1, Geetha G1, Hariprasad R1, Venkatnarayana R2 and Subramanian G2 1PSG College of Pharmacy, Peelamedu, Coimbatore, Tamilnadu, India. 2RVS College of Pharmacy, Sulur, Coimbatore, Tamilnadu, India.Kavitha K Y et al. / PharmacieGlobale (IJCP) 2013, 01 (07).
27. Gottsclich, N. And Kasche, V. Journal of Chromatography. A, (1997) 201-206.Gottsclich,N. And Kasche, V. Journal of Chromatography.A (1997) 201- 206.
28. Allenmark, S.G. and Andersson, S. J. Chromatography A. (1994) 666, 167-179.
29. Beckett AH, Stenlake JB, pharmaceutical chemistry, ivthed.part two. New Delhi, CBS; 1997. P.302-305. Kenneth A Connors, pharmaceutical analysis. 3rd . Singapore. John Wiley Publishers: 1982.p. 529-530.
30. An efficient approach to column selection in HPLC Method Development, Craig S. Young and Raymond. J.Weigand, www.altech web.com.
31. Lloyd R, Synder, Joseph J, Kirkland, Joseph L Glajesh. Practical HPLC Method Development, 2nd ed; 1997. 1-14.
32. Pattengill MD, Sands DE. Statistical significance of linear least-squares parameters, J ChemEduc; 1979 .p 56:244.
33. Armstrong DW, Bonded phase material for chromatographic separations. U.S patent 4539399;1985. p.16-18.
34. Lough WJ, Wainer IW. High Performance Liquid Chromatography. Fundamental principles & practice. Glasgow (UK): Blackie Academic & Professional; 1995. P.2-28.
35. Snyder, R.L., Glajch, J.L. and Kirkland, J.J. Practical HPLC Method Development. John Wiley &Sons Inc. 1988.