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VALIDATED RP-HPLC METHOD FOR ESTIMATION OF FINGOLIMOD IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
A novel, simple, accurate, precise, sensitive and specific analytical RP-HPLC method was developed and validated for the quantitative estimation of Fingolimod in bulk drugs and pharmaceutical dosage form. Chromatographic separation was achieved on an Symmetry ODS C18 (4.6×250mm, 5µm) analytical column using mobile phase composition of methanol and Phosphate Buffer in ratio of (35: 65 v/v) that was set at a flow rate of 1.0µl/min with detection of 235 nm. The retention time of Fingolimod was found to be 3.006min. The drug was analyzed by following the guidelines of international conference on Harmonization (ICH). This drug showing linearity in the concentration range of 6-14µg/ml and the correlation coefficient showing R2 = 0.9996. The % Recoveries showing within the limits. The presentation of the method was validated according to the present ICH guidelines for accuracy, precision and robustness, Linearity, limit of quantification, limit of detection linearity.

KEYWORDS: Fingolimod, RP-HPLC, Method Development, Accuracy, Precision.

I. INTRODUCTION

Fingolimod is an oral sphingosine-1-phosphate receptor modulator that is believed to exert therapeutic effects in MS by preventing the egress of lymphocytes from lymph nodes, thereby reducing recirculation of autoreactive lymphocytes to the CNS. It has been reported to reduce the rate of relapses in relapsing-remitting multiple sclerosis by approximately one-half over a two-year period.

EXPERIMENTAL METHODS

INSTRUMENTS USED

S.No	Instruments And Glass wares	Model
1	HPLC	Shimadazu LC- AT VP with SPD -10A VP UV-Visible Detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

CHEMICALS USED

S.No	Chemical	Brand names
1	Fingolimod (Pure)	Dr.Reddy's
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT

Preparation of standard solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.1ml of the above Fingolimod stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure: Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization

Initially the mobile phase tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Symmetry ODS C18 (4.6 x 250mm, 5mm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

PREPARATION OF BUFFER AND MOBILE PHASE

Preparation of Potassium dihydrogen Phosphate (KH_2PO_4) buffer (pH-3.6)

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra sonication.

Preparation of mobile phase

Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: The Mobile phase was used as the diluent.

METHOD VALIDATION PARAMETERS

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Fingolimod stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution

Weight 10 mg equivalent weight of Fingolimod sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1ml of Fingolimod above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

LINEARITY

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (6ppm of Fingolimod)

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Fingolimod)

Take 0.8ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – III (10ppm of Fingolimod)

Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – IV (12ppm of Fingolimod)

Take 0.12ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – V (14ppm of Fingolimod)

Take 0.14ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

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.

PRECISION

REPEATABILITY

Preparation of Fingolimod Product Solution for Precision

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Fingolimod stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

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 limits.

INTERMEDIATE PRECISION

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure

Analyst 1

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

ACCURACY

For preparation of 50% Standard stock solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.05ml of the above Fingolimod stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard stock solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Fingolimod stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard stock solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Fingolimod stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Fingolimod and calculate the individual recovery and mean recovery values.

ROBUSTNESS

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution

Accurately weigh and transfer 10 mg of Fingolimod working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Fingolimod stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of flow conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

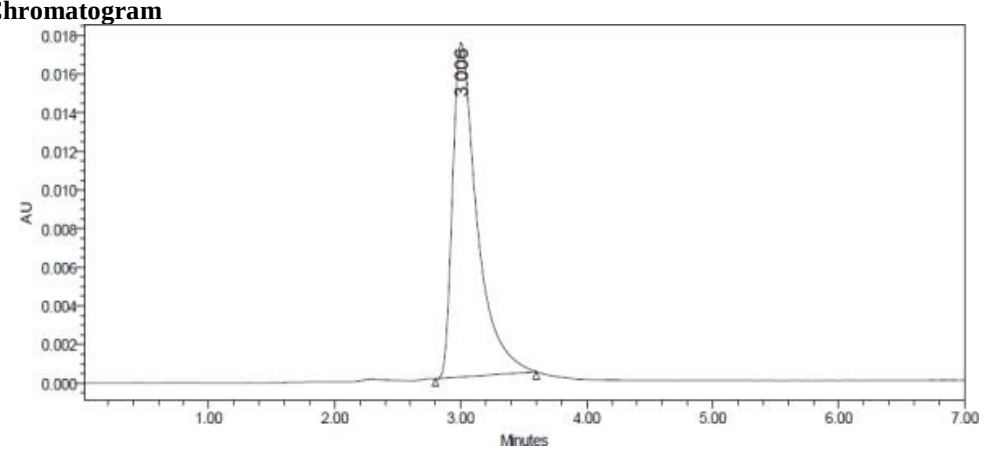
Effect of Variation of Mobile Phase Organic Composition

The sample was analyzed by variation of mobile phase i.e. Methanol: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead (35:65), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Optimized Chromatogram (Standard)

Mobile phase ratio : Methanol: Phosphate Buffer (35:65) V/V
Column : C18 (4.6×250mm, 5µm)
Column temperature : Ambient
Wavelength : 235nm
Flow rate : 1ml/min
Injection volume : 10µl
Run time : 8min

Optimized Chromatogram



Optimized Chromatogram (Standard)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Fingolimod	3.006	1658242	185421	1.24	6569

Results of system suitability for Fingolimod

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fingolimod	3.008	1652847	185647	6589	1.24
2	Fingolimod	3.005	1653658	186254	6587	1.26
3	Fngolimod	3.001	1654521	185475	6584	1.28
4	Fingolimod	3.000	1653564	186594	6582	1.29
5	Fingolimod	3.001	1658745	185684	6895	1.24
Mean			1654667			
Std. Dev.			2355.764			
% RSD			0.142371			

Peak results for assay standard

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Fingolimod	3.006	1658254	185468	1.24	6391	1
2	Fingolimod	3.006	1658475	184524	1.23	6549	2
3	Fingolimod	3.001	1658471	186598	1.25	6682	3

Peak results for Assay sample

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Fingolimod	3.005	1645879	184574	0.85	6458	1
2	Fingolimod	3.000	1645875	183598	0.86	6584	2
3	Fingolimod	3.008	1658423	185472	0.85	6457	3

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity Weight of tablet}}{100} \times \frac{100}{\text{Label claim}}$$

Results of repeatability for Fingolimod

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fingolimod	3.008	1658954	186958	1.26	6785
2	Fingolimod	3.000	1658745	187548	1.27	6854
3	Fingolimod	3.013	1659865	189854	1.26	6852
4	Fingolimod	3.006	1653254	186985	1.25	6784
5	Fingolimod	3.001	1654781	189542	1.24	6895
6	Fingolimod	3.000	1665258	188759	1.15	6968
Mean			**1658476**			

Std.dev			**4222.511**			
%RSD			**0.254602**			

Intermediate Precision

The Intermediate Precision consists of two methods:-

Intra Day: In Intra Day process, the 50%, 100% and 150% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 50%, 100% and 150% concentration are injected at same intervals of time in different days.

Results for Intermediate Precision Day-1

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fingolimod	3.006	1674582	187489	1.12	6685
2	Fingolimod	3.008	1669584	186598	1.16	6748
3	Fingolimod	3.000	1654874	189247	1.14	6965
4	Fingolimod	3.013	1669852	188598	1.18	6852
5	Fingolimod	3.000	1659869	184785	1.19	6985
6	Fingolimod	3.001	1658978	186985	1.16	6962
Mean			1664623			
Std. Dev			7754.292			
%RSD			0.465829			

Results of Intermediate Precision for Day 2

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Fingolimod	3.001	1665823	186589	668	1.19
2	Fingolimod	3.013	1698589	187485	6748	1.17
3	Fingolimod	3.005	1674856	188584	6985	1.16
4	Fingolimod	3.001	1659899	189856	6524	1.17
5	Fingolimod	3.000	1665874	184785	6865	1.19
6	Fingolimod	3.008	1674021	188965	6798	1.15
Mean			**1673177**			
Std. Dev.			**13660.32**			
% RSD			**0.81643**			

Results of Accuracy for concentration-50%

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Fingolimod	2.988	108982	92715	0.99	4695	1
2	Fingolimod	3.000	108659	92548	1.02	4658	2
3	Fingolimod	2.988	109564	92685	1.00	4785	3

Results of Accuracy for concentration-100%

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Fingolimod	3.008	201689	186598	1.26	6852	1
2	Fingolimod	3.006	201874	185476	1.25	6585	2
3	Fingolimod	3.001	202998	186579	1.26	6658	3

Results of Accuracy for concentration-150%

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Fingolimod	3.038	296765	236543	1.32	7042	1
2	Fingolimod	3.018	297546	236895	1.31	6926	2
3	Fingolimod	3.013	296786	238546	1.32	7059	3

The accuracy results for Fingolimod

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	100.72%
100%	202187	10	10.054	100.540%	
150%	297032.3	15	15.181	101.206%	

LIMIT OF DETECTION
Result: = 1.2µg/ml

LIMIT OF QUANTITATION
Result:=3.6µg/ml

ROBUSTNESS
Result of method Robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Temperature (27^0C)	0.54
Temperature (23^0C)	0.63
Wavelength of Detection (280 nm)	0.91
Wavelength of detection (270 nm)	0.93

Assay of Fingolimod Tablets/ Capsules

Brand Name of Tablets/Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Fingomod Capsule (Sun Pharmaceutical Industries Ltd)	0.5mg	0.468 (± 0.765)	99.476 % (±0.347)

Results of Standard Solution-1

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Fingolimod	3.001	1665234	182365	1.28	6782

Results of Sample Solution-1.

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Fingolimod	3.013	1678562	189856	1.31	6847

CONCLUSION
A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Fingolimod API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Fingolimod in different formulations.

SUMMARY
Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, acetonitrile, water, 0.1N NaOH, 0.1NHCl). Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Fingolimod it is evident that most of the HPLC work can be accomplished in the wavelength range of 235 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 10µl were found to be the best analysis.

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