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Method Development and Validation of Fingolimod in its Pharmaceutical Dosage Forms by RP-HPLC

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ABSTRACT

A reverse phase liquid chromatography (RP-HPLC) method has been developed and subsequently validated for the determination of Fingolimod in Bulk and its pharmaceutical formulation. Separation was achieved with a X-Terra RP-18, 150x4.6mm I.D; particle size 5 µm) Column and Phosphate buffer (pH adjusted to 6 with diluted orthophosphoric acid): Methanol and Acetonitrile (350:260:390) v/v as eluent at flow rate 1.0 mL/min and the Column temperature was 25°C. UV detection was performed at 218 nm and sample temperature was maintained at 5°C. The method is simple, rapid, and selective. The described method of Fingolimod is linear over a range of 5 µg/mL to 25 µg/mL. The method precision for the determination of assay was below 2.0% RSD. The method enables accurate, precise, and rapid analysis of Fingolimod. It can be conveniently adopted for routine quality control analysis of Bulk and pharmaceutical formulations.

Keywords: Fingolimod, Stability indicating, method development, validation

Introduction:

Fingolimod (FTY720) is a first-in-class orally bioavailable compound that has shown efficacy in advanced clinical trials for the treatment of multiple sclerosis (MS). In vivo, fingolimod is phosphorylated to form fingolimod-phosphate, which resembles naturally occurring sphingosine 1-phosphate (S1P), an extracellular lipid mediator whose major effects are mediated by cognate G protein-coupled receptors. There are at least 5 S1P receptor subtypes, known as S1P subtypes 1-5 (S1P1-5), 4 of which bind fingolimod-phosphate. These receptors are expressed on a wide range of cells that are involved in many biological processes relevant to MS. S1P1 plays a key role in the immune system, regulating lymphocyte egress from lymphoid tissues into the circulation. Fingolimod alters the metabolisam and clearance of various drugs like corticotrophin and teriflunamide. Chemically it is 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol.

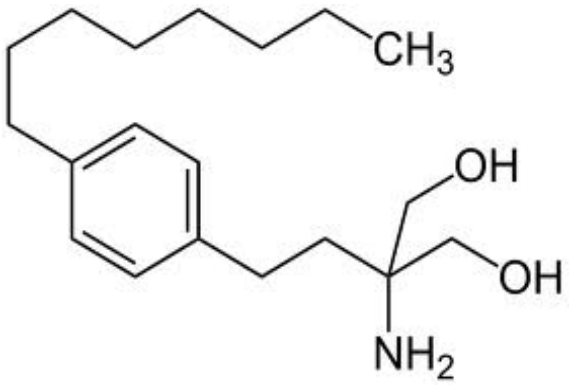


Fig 1: Structure of Fingolimod

On the literature survey most of publications related to LC/MS and HPLC methods have been reported determination of Fingolimod present in biological fluids. Hence an attempt has made to develop a HPLC method for the determination of Fingolimod in pharmaceutical dosage forms. The objective of this experiment was to optimize the assay method for estimation of Fingolimod based on the literature survey made. So here planned to develop accurate, precise, specific method for estimation of Fingolimod.

Materials and methods

Equipment

The Method development and Validation was carried out using Waters Alliance-HPLC system equipped with waters 1525 binary HPLC pump, 2695-separation module connected to 2996-photo diode array detector, and Waters 2707 auto sampler. The data was acquired by Empower® version 2. The other equipment used were Ascoset Electronic balance, ADWA pH meter, heating mantle. Ultrasonic bath was used for sonication of the samples. Hot air oven was used to carry out thermal degradation studies. UV cross linker, with series of 23400 model UV chamber, equipped with a UV fluorescence lamp with the wavelength range between 200 & 300 nm was used for photo degradation studies.

Chemicals and Reagents

Fingolimod working standard was kindly given as gift sample by Mylan labs Pvt. Ltd, Hyderabad. HPLC grade solvents include acetonitrile, water and methanol. Analytical grade chemicals include sodium hydroxide, hydrochloric acid, 20% hydrogen peroxide, Ortho phosphoric acid, Triethyl amine and potassium dihydrogen phosphate were purchased from E. Merck Limited, Mumbai, India.

Chromatographic conditions

HPLC analysis was carried out on Waters Alliance-HPLC system equipped with 2695-separation module connected to 2996-photo diode array detector and the data was acquired by Empower® version 2. Separation was achieved using X-Terra RP-18, (150×4.6mm) 5µm as a column with mobile phase of pH 6.0 buffer, acetonitrile and methanol in the ratio of 35:39:26. The samples were analyzed using 20 µL injection volume, Flow rate was maintained at 1.0mL/min with runtime of 10 min and the temperature was maintained at 30°C throughout the analysis. Detection and purity establishment of the drugs were achieved using PDA detector at 218 nm wavelength.

Preparation of Standard solution: Accurately weigh 28mg of Fingolimod into 100ml volumetric flask. Add 60ml of diluent and sonicate to dissolve. Cool the solution to room temperature and make up volume with diluent. Transfer 5ml of above solution into 50ml volumetric flask and dilute toVolume with mobile phase.

Preparation of Standard solution:

Accurately weigh and transfer 10 capsules, equivalent to 5mg of Fingolimod into 200mL volumetric flask. Add about 120ml of diluent, sonicate for 15 min with occasional shaking. Cool the solution to room temperature and dilute to volume with diluent and mix.centrifuge the solution at 3500 rpm for 10 min and use the clear supernatant.

Method Validation

The developed and optimized RP-HPLC method was validated according to international conference on harmonization (ICH) guidelines Q2(R1) in order to determine the system suitability, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, ruggedness and robustness.

System suitability

System suitability parameters were evaluated to verify system performance. 20 µL of standard solution was injected five times into the chromatograph, and the chromatograms were recorded. Parameters such as number of theoretical plates and peak tailing were determined.

Specificity

The specificity of the analytical method was established by injecting the solutions of diluent (blank), placebo, working standards and sample solution individually to investigate interference from the representative peaks.

Precision

Repeatability/ method precision was performed by injecting six replicates of same concentrations of fingolimod, calculated % assay and %RSD. Reproducibility/ Ruggedness/ Intermediate precision was performed using different analysts and a different instrument in the same laboratory.

Accuracy

Accuracy of the proposed method was determined using recovery studies by spiking method. The recovery studies were carried out by adding known amounts (80%, 100% and 120%) of the working standard solutions of fingolimod to the pre-analysed sample. The solutions were prepared in triplicates to determine the accuracy.

Linearity

A study to establish the linearity of detector response of Fingolimod was conducted. Linearity of detector response of Fingolimod was conducted from 5% level of Fingolimod standard to 250% of the Fingolimod standard concentration. Plotted linearity graphs of Fingolimod standard concentration versus area level 5%, 10%, 15%, 20%, 25%, and has been found linear in the prescribed range. Performed the precision study for lower and higher level solutions by injecting 6 replicate injections into HPLC system. Calculated % RSD for peak areas of Fingolimod at lower and higher levels and found within the

limits. **Limit of detection and Limit of quantification:** Limit of detection (LoD) and limit of quantification (LoQ) of fingolimod were determined by calibration curve method. Solutions of fingolimod were prepared in linearity range and injected (n = 3).

Robustness

To examine the robustness of the developed method, experimental conditions were deliberately changed, resolution, tailing factor, and theoretical plates of fingolimod peaks were evaluated. To study the outcome of the flow rate on the developed method, it was changed ± 0.2mL/minute. The effect of column temperature on the developed method was studied at ± 5°C, organic phase composition in mobile phase was changed ±10% and pH of the buffer is changed ±0.2. In all the above varied conditions, the composition of aqueous component of the mobile phase was held constant.

Results and discussion

System Suitability

From the results in table 1, the column efficiency for Solifenacin succinate peak was identified from the theoretical plate count which is more than 3000, tailing factor less than 2.0, %RSD was found to be less than 2.0%.

Table 1: System suitability results for Fingolimod

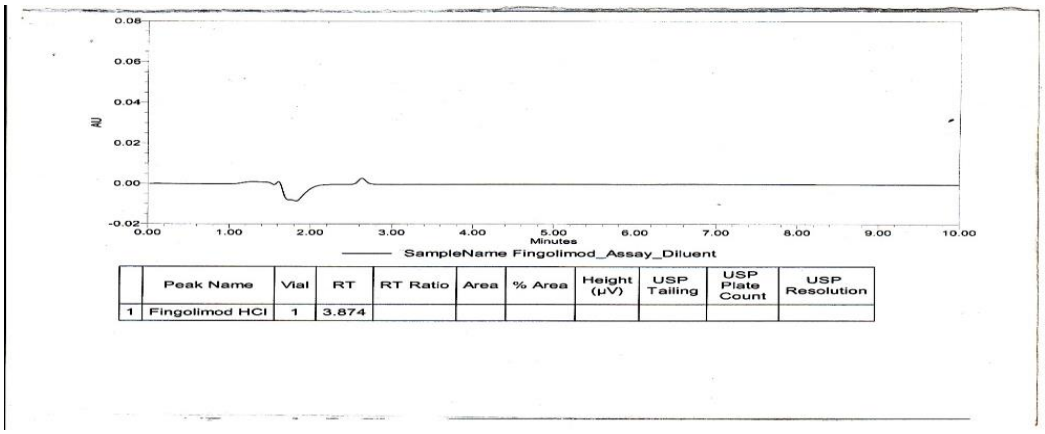
System suitability parameters	Result	Acceptance criteria
%RSD of Fingolimod peak area	0.20	NMT 2.0
Tailing factor for Fingolimod peak	1.48	NMT 2.0
Theoretical plates	2405	NLT 2000

Result: the system suitability test results are within the limits.

Conclusion: System suitability test results were met with the acceptance criteria.

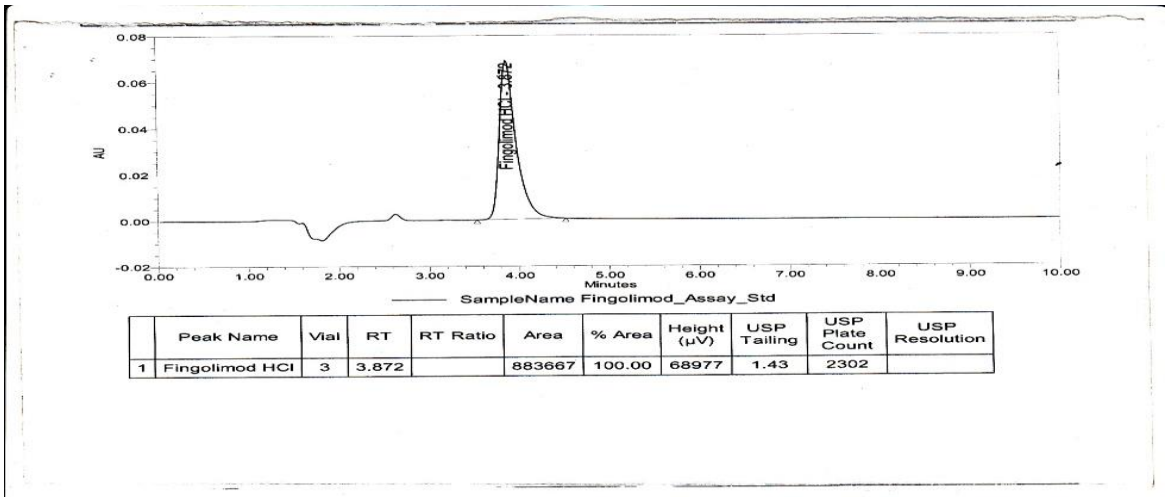
Specificity

From the obtained chromatograms in figures 2 to 4 it can be inferred that there were no co-eluting peaks at the retention time of fingolimod which shows that peak of analyte was pure and the excipients in the formulation did not interfere with the analyte of interest.



	Peak Name	Vial	RT	RT Ratio	Area	% Area	Height (µV)	USP Tailing	USP Plate Count	USP Resolution
1	Fingolimod HCl	1	3.874							

Fig. 2: Chromatogram of blank



	Peak Name	Vial	RT	RT Ratio	Area	% Area	Height (µV)	USP Tailing	USP Plate Count	USP Resolution
1	Fingolimod HCl	3	3.872		883667	100.00	68977	1.43	2302	

Fig. 3: Chromatogram of standard

Precision

From the results in table2, % Assay for fingolimod was found to be in the range of 98 – 102%, and the % RSD for fingolimod to be within 2%. Hence the method is precise, reproducible and rugged for 48 hours' study.

Table no.2 Method precision

S. No	Peak Name	Peak area
1	Fingolimod	883668
2	Fingolimod	883721
3	Fingolimod	883754
4	Fingolimod	884120
5	Fingolimod	884689
6	Fingolimod	884994
Mean		884157.6
SD		546.918
%RSD		0.61

Ruggedness:

Intra-day precision: Intra-day precision was carried out on same day, same HPLC system, using same column at different times. Calculated average area and %R.S.D for 12 tests, (Condition I and Condition II).

Inter-day precision: Inter-day precision was carried out on same HPLC system, using same column on another day. The average area was calculated and %R.S.D. for 6 replicate injections of standard drug solutions.

Table No. 3: Showing from precision study- Intraday

Conc µg/ml	Peak area	Statistical parameters
10	884994	Mean:884816.6
	884671	S.D:145.7
	884785	%R.S.D:0.016
20	884562	Mean:884571.6
	884532	S.D:99.295
	884621	%R.S.D:0.011
30	855827	Mean:859393
	858290	S.D:103.8
	864062	%R.S.D:0.491

Table No. 4: Showing from precision study- Interday

Conc µg/ml	Peak area			Statistical parameters
	Day-1	Day-2	Day-3	Mean:884800.6
10	884689	884785	884928	S.D:102.53 %R.S.D:0.02
20	932125	902658	925678	Mean:920153.6 S.D:812.269 %R.S.D:0.086
30	862158	868210	852604	Mean:860990 S.D:825.901 %R.S.D:0.09

Robustness

From the results in table 5, it is evident that the system suitability parameters such as resolution, RSD, tailing factor, and the theoretical plate count of fingolimod remained unaffected by deliberate changes. The results were presented along with the system suitability parameters of optimized conditions. Thus, the method was found to be robust with respect to variability in applied conditions.

Demonstrated robustness of assay method by changing the flow rate for 0.9 ml/min and 1.1 ml/min instead of specified flow rate (1.0 ml/min). By injecting the replicate injections of standard in 0.9 ml/min and 1.1 ml/min flow rate and found that system suitability parameters were passed. The %RSD of peak area, tailing factor and theoretical plates of Fingolimod standard was found within the limits.

Table 5. Robustness data

Replicate standard injections at 0.9ml\min			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	884994	Average :884816.6 % RSD = 0.016	% RSD : not more than 2%
2	884671		
3	884785		
Replicate standard injections at 1.1ml\min			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	884562	Average :884571.6 % RSD = 0.011%	% RSD : not more than 2%
2	884432		
3	884621		
Replicate standard injections at 23^{0}c			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	889725	Average :889806 % RSD = 0.009% SD=81	% RSD : not more than 1%
2	889839		
3	889854		
Replicate standard injections at 31^{0}c			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	940026	Average :940138.6 % RSD = 0.013 SD =123.25	% RSD : not more than 1%
2	940128		
3	940262		
Replicate standard injections at pH 5.6			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	892568	Average :893837.3 % RSD = 0.23 SD =208.16	% RSD : not more than 1%
2	893045		
3	895899		
Replicate standard injections at pH 6.4			
Injection No	**Peak area**	**Observation**	**Acceptance criteria**
1	940015	Average: 941044.6 % RSD = 0.108 SD =1029.7	% RSD : not more than 1%
2	941223		
3	941896		

Linearity

The correlation coefficient (r) should be NLT 0.998(for lower and higher levels including 100% level) for linearity. In case of discrepancy investigate and explain the reason. Repeat if necessary and report the range in which it is linear. The correlation coefficient (r) of the linear regression is 0.999. linearity test results were met with the acceptance criteria.

Table no.6: Linearity results for Fingolimod

% level	**Concentration(mcg/mL)**	**Peak area(average)**
5	5	214569
10	10	421569
15	15	601259
20	20	796359
25	25	998563
Correlation coefficient(r)		0.999
Squared correlation coefficient(r^2)		0.999

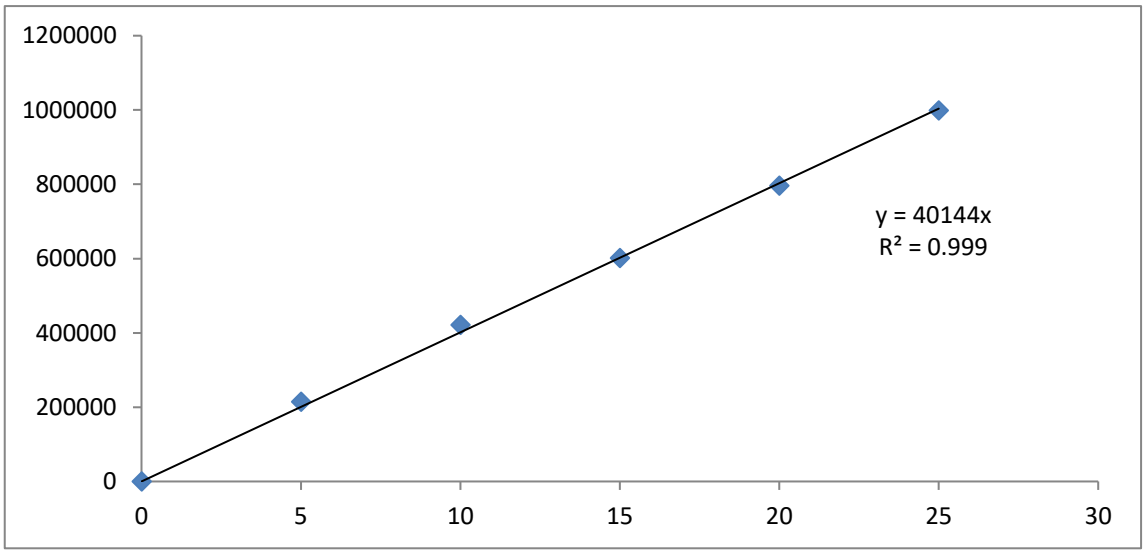


Figure 4: Linearity plot of fingolimod

Limit of detection (LOD):

It is the lowest amount of the analyte in a sample that can be detected but not necessarily be quantitated as an exact concentration or amount. LOD was found to be 0.067

Limit Of Quantitation (LOQ):

It is the lowest amount of an analyte that can be measured quantitatively in a sample with acceptable accuracy and precision. LOQ was found to be 0.2

Accuracy

The accuracy for the average of triplicate in each concentration sample should be within 98 to 102% for assay. Accuracy for the assay of Fingolimod capsules were determined by applying the study of recovery of Fingolimod in Fingolimod capsules was conducted on capsules powder which is equivalent to about 80%, 100%, 120% of the target assay concentration of Fingolimod inFingolimod capsules. Sample solutions were prepared in triplicate for each spikes level and analyzed as per test method. The % recovery was found within the limits.

Table 7: Accuracy results for Fingolimod

Concentration level	Amount added	Amount found	%Recovery	Average % recovery
80%	8	7.851	101.88	
	8	7.864	101.77	101.8
	8	7.862	101.75	
100%	10	9.030	99.68	
	10	9.023	99.72	99.72
	10	9.022	99.77	
120%	12	11.178	98.58	98.74
	12	11.146	98.91	
	12	11.156	98.74	

Conclusion:

Based on the above validation data, it is evident that the HPLC method documented in the protocol for the determination of % assay method of Fingolimod 500mg is treated as a validated method. Based on the chromatographic data and corresponding purity threshold and purity angle values, it is clear that the peaks obtained under various stress conditions are well-separated and active analyte passed peak purity test. Peak data presented is acceptable and the HPLC analytical method given in the protocol is stability indicating method for estimating assay of Fingolimod.

Hence it is recommended to be used in routine testing, release and stability samples testing.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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