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Development and validation of Immunomodulating drug Fingolimod by RP-HPLC method with detailed force degradation study

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Abstract: Fingolimod is a sphingosine -1-phosphate modulator as well as reduces the infiltration of pathogenic lymphocyte cells into the central nervous system because they involved in nervous tissue damage and nerve inflammation. Here we reported sensitive, precise, accurate and time saving chromatographic separation method of Fingolimod and its degraded products which were performed on RP-HPLC. The separation was performed using Sunfire C18 column (250 x 4.6 mm i.d.) having 5 µm particle size. The mobile phase used for the elution was Buffer: Acetonitrile (35: 65 v/v). Eluting compounds were monitored at 220 nm wavelength with UV-Visible detector. The developed procedure has been evaluated for the specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness in order to ascertain the stability of the analytical method.

Keywords: Fingolimod, Stability - indicating, Force degradation, RP-HPLC

INTRODUCTION

Fingolimod (Trade name Gilenya) is a sphingosine 1- phosphate receptor modulator indicated and approved for the treatment of relapsing remitting multiple sclerosis. Fingolimod hydrochloride is a white to almost white crystalline powder which is freely soluble in water. Fingolimod chemically known as 2-amino-2-[2-(4-octylphenyl) ethyl] propane-1, 3-diol.[1, 2] (Fig. 1)

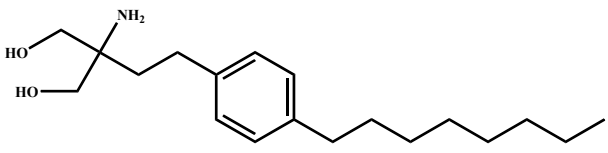

Fig. 1 Chemical structure of Fingolimod

Multiple sclerosis (MS), also known as disseminated sclerosis is a demyelinating disease in which the insulating covers of nerve cells in the brain and spinal cord are damaged. A per with MS can have irreversible nerve damage and as a result wide range of symptoms like fatigue, depression, pain, motor weakness, visual disturbances and vertigo. [3-7]

Multiple sclerosis (MS) is a chronic disease that can progress intermittently or continuously and is divided into four disease courses: relapsing-remitting multiple sclerosis (RRMS), primary-progressive multiple sclerosis (PPMS), secondary-progressive multiple sclerosis (SPMS) and progressive-relapsing multiple sclerosis (PRMS). Relapsing-remitting multiple sclerosis is the most common form of MS and is characterized by exacerbations of neurological dysfunction followed by remissions. [8-10] Fingolimod is a sphingosine 1-phosphate (S1P) receptor modulator that works by binding to S1P receptors and altering their activity in inflammatory process. Fingolimod decreased lymphocyte movement towards the central nervous system (CNS) and result in reduced CNS inflammation. [8-12]

Literature review of Fingolimod, it is revealed that various analytical methods were previously published for quantification of Fingolimod by different spectrophotometric and chromatographic methods [13-18]. There is some stability indicating HPLC methods are available, but they did not show actual storage condition of the drug. In this method we reported actual storage condition of Fingolimod drug and also resolved drug peak from degraded impurities. Fingolimod drug is very sensitive about alkali stress condition, whereas acidic and oxidative stress conditions did not affect the drug even at high temperature. Here we reported very sensitive, precise, accurate, time saving and well resolved stability indicating HPLC method. In this method we performed basic stress condition in different temperature and different time interval and the amount of drug decomposed were calculated briefly. The purpose of this work was developed and optimized a fully validated stability indicating and routinely available ultra performance liquid chromatographic method as per ICH Guidelines. [19-21]

MATERIALS AND METHODS

Chemicals and Reagents:

Fingolimod (Potency 99.80) was purchased from Amney Pharmaceuticals. HPLC-grade Acetonitrile and all other chemicals were purchased from Merck Laboratories. HPLC-grade water was prepared by using a Milli-Q system, Millipore Corp. and it was used for all purposes.

Chromatographic condition:

Liquid chromatography separation of Fingolimod and its degraded products was achieved with Waters HPLC equipment having TM 600 quaternary pump, Waters 2489 uv/ visible detector, Waters 600 controller, Waters in-line degasser AF and manual injector with 20 µl loop. The equipment was connected with multi – instrument data accusation and data processing software (Empower 2.0). Waters Sunfire C18 (250 x 4.6 mm i.d., 5 µm particle size) column has been used for chromatographic separation. Mobile phase consists of 0.1% Triethylamine in water (pH 3.00 ± 0.05 with OPA): Acetonitrile (35:65 v/v) at 0.9 ml/min flow rate. The mobile phase was filtered through 0.22 micron filter. The absorbance maximum of Fingolimod was obtained at 220 nm. The column temperature was maintained at 30 °C. The total elution time was 8.0 minutes.

Preparation of Standard solution:

Standard Solution was prepared by dissolving 12.5 mg working standard of Fingolimod (potency 99.80) in 25 ml volumetric flask and diluted up to the mark on diluent. 1 ml of above stock solution was transferred to 10 ml volumetric flask and diluted with mobile phase to furnished final standard concentration 50 µg/ml.

Preparation of Test Solution:

Sample Solution was prepared by dissolving 12.5 mg sample of Fingolimod in 25 ml volumetric flask and diluted up to the mark on diluent. The final Test solution of 50 µg/ml concentration has been prepared by taking 1.0 ml of sample stock solution to 10 ml of volumetric flask and further diluted up to mark with diluent.

Method Development and Optimization:

The HPLC method of Fingolimod drug substance is developed and optimized in such a way that very sharp and resolved peak of Fingolimod drug was obtained and also the most essential thing is that degraded impurities would be well resolved and not affected of main drug peak. As per green chemistry approach the HPLC method must be time saving and less solvent consuming. Before optimized very sensitive, rugged and precise HPLC method of Fingolimod there were many trials has been taken using different stationary phase, buffers, solvent compositions and pH. The different HPLC columns were used for Fingolimod and its degraded impurities. However, good peaked shape of sharpness, less tailing factor, more plate count and nicely resolved with degraded impurities were achieved satisfactorily in Waters Sunfire C18 column with the dimension of 250 x 4.6 mm i.d. and 5 µm particle size. By using Mobile phase Buffer: Acetonitrile (35:65 v/v) having buffer composition 0.1% Triethylamine in water and adjust pH 3.0 ± 0.05 with dilute Orthophosphoric acid, we were achieving shorter elution time, less solvent consumption and better separation of drug peak and it's degraded impurities. In standard and sample preparation mobile phase used as a diluent, so we observed straight base line and removed noises during whole analysis. The total elution time was 6.0 minutes which is indicating that the method was affordable and agreeable.

Forced degradation studies:

The stability indicating method was performed to ensure the specificity of the method and to decide the actual storage condition of the compound. The force degradation study shows that the peak of Fingolimod is well separated and resolved and no any interference with degradation products was found. The different stress conditions were applied to complete the study such as acidic, basic, oxidative, thermal and photolytic.

Acidic Degradation

For the measurement of the acidic behavior of Fingolimod drug, the drug substance was treated with 1 ml of 1 N HCl solution and kept at room temperature for 12 h. Before injecting sample, necessary neutralization procedure of sample was carried out with 1 N NaOH solution. The drug was found stable at room temperature with above acidic condition. Moreover for detailed study sample was kept in water bath for 1 h at 80 °C temperature, followed by adding 1 ml of 1 N HCl solution. After cooling the reaction mass, it was neutralized by 1 N NaOH solution. For both acidic stress conditions the resulting mass were diluted to make the final standard concentration 50 µg/ml and were injected in duplicate.

Basic Degradation

In alkali stress condition, Fingolimod drug

was treated with 1 ml of 1 N NaOH solution and kept at room temperature for 1 h. Before injecting the standard concentration (50µg/ml), reaction mass was neutralized with 1 N HCl solution. By the result of above stress condition Fingolimod drug was found sensitive towards alkali degradation. So to know more about combine effects of temperature and alkali solution on Fingolimod API, the drug samples were kept in water bath and heated at 40 °C to 80 °C temperature for 30 min and 1 h time intervals by the addition of 1 ml of 1 N NaOH solution. After cooling the reaction masses, they were neutralized by adding 1 N HCl solution. For all alkali stress conditions the resulting mass were diluted to make the final standard concentration 50 µg/ml and were injected in duplicate. The major impurity peaks were found at RT 2.65 and 3.34 minute.

Oxidative Degradation

Oxidative degradation study was performed by putting drug product in 1 ml of 3% v/v H_2O_2 at room temperature for 12 h and heated in water bath at 80 °C for 1 h. For both oxidation conditions the resulting mass were diluted to make the final standard concentration 50 µg/ml and were injected in duplicate.

Thermal Degradation

Thermal degradation was performed by exposing drug sample at 70° C for 24 h. The mass of the Fingolimod drug was kept in to hot air oven and monitoring at different time interval such as initial (0 h), after 6 h, after 12 h and after 24 h; samples were prepared and diluted to attain the standard concentration and injected in twice.

Photolytic condition

Photolytic study of Fingolimod drug was carried out by exposing powder drug sample to sunlight for 36 hrs (day hrs only). Fingolimod drug was found very stable towards photolytic degradation

The degradation response of Fingolimod with different stress conditions were reported in table 1 and Fig. (2 to 17)

Table 1 Degradation of Analyte applying force degradation

Degradation Condition	Mean Area	Assay (%)	Drug decompose (%)	Major Impurity (RT, min)
Standard	1850998	100	----	----
Acid Degradation at RT 12 h	1840677	99.24	0.76	----
Acid Degradation at 80 °C 1 h	1765822	95.13	4.87	----
H_2O_2 Degradation at RT 12 h	1851885	99.21	0.79	----
H_2O_2 Degradation at RT 80 °C 1 h	1636394	88.94	11.06	----
Base Degradation at RT 1 h	1667139	90.10	9.90	3.390
Base Degradation at 40 °C 30 min	1730952	93.33	6.67	2.648
Base Degradation at 40 °C 1 h	1480868	80.29	19.71	2.645
Base Degradation at 50 °C 30 min	1558493	84.10	15.90	2.666
Base Degradation at 50 °C 1 h	1450416	78.45	21.55	2.636
Base Degradation at 60 °C 30 min	1453733	79.01	20.99	2.666
Base Degradation at 60 °C 1 h	1410542	76.23	23.77	2.657
Base Degradation at 70 °C 30 min	1453650	78.82	21.18	2.669
Base Degradation at 70 °C 1 h	1028493	55.76	44.24	2.661
Base Degradation at 80 °C 1 h	920241	49.78	50.22	2.653
Photo Stability	1845080	99.48	0.52	----
Thermal Degradation 70 °C 6 h	1834310	99.22	0.78	----
Thermal Degradation 70 °C 12 h	1825874	99.16	0.84	----
Thermal Degradation 70 °C 24 h	1837453	99.07	0.93	----

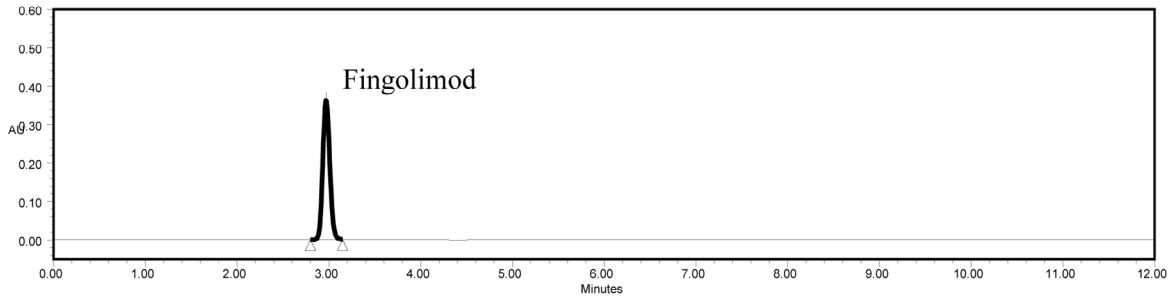

Fig. 2 Chromatogram of Acid degradation study of Fingolimod

[1N HCl, RT, 12 h]

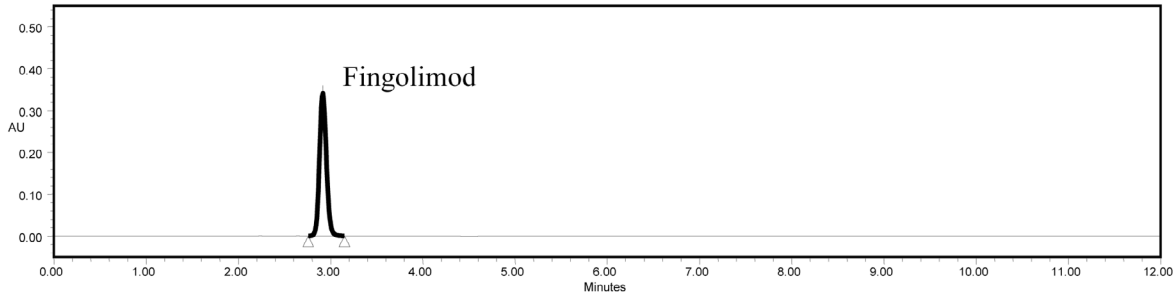

Fig. 3 Chromatogram of Acid degradation study of Fingolimod

[1N HCl, 80 °C, 1 h]

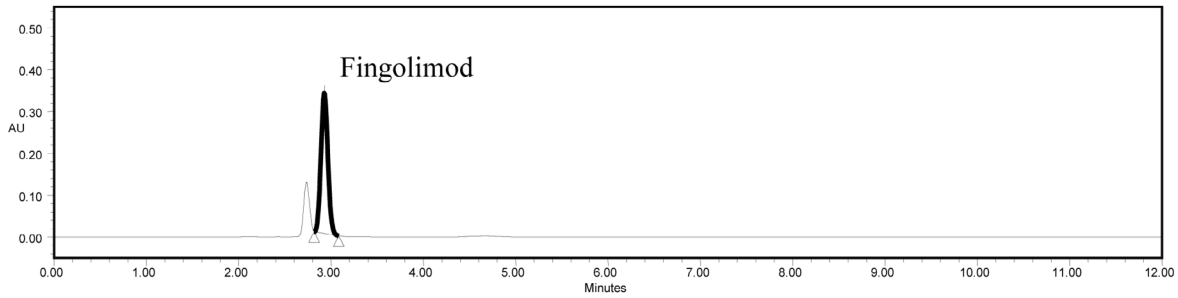

Fig. 4 Chromatogram of Peroxide degradation study of Fingolimod

[3% H_2O_2, RT, 12 h]

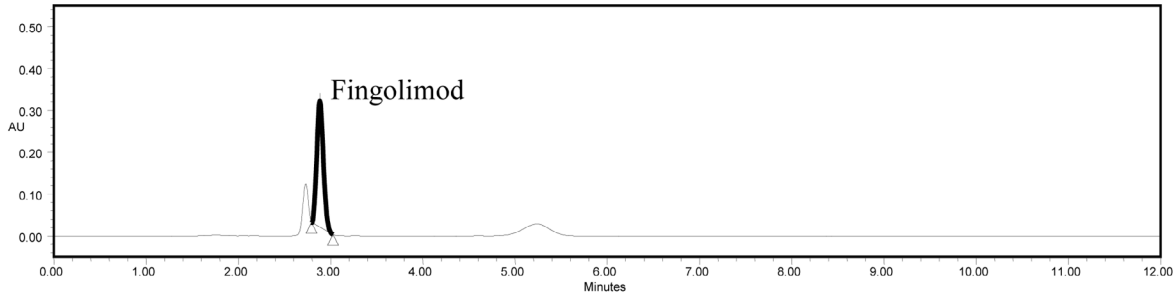

Fig. 5 Chromatogram of Peroxide degradation study of Fingolimod

[3% H_2O_2, 80 °C, 1 h]

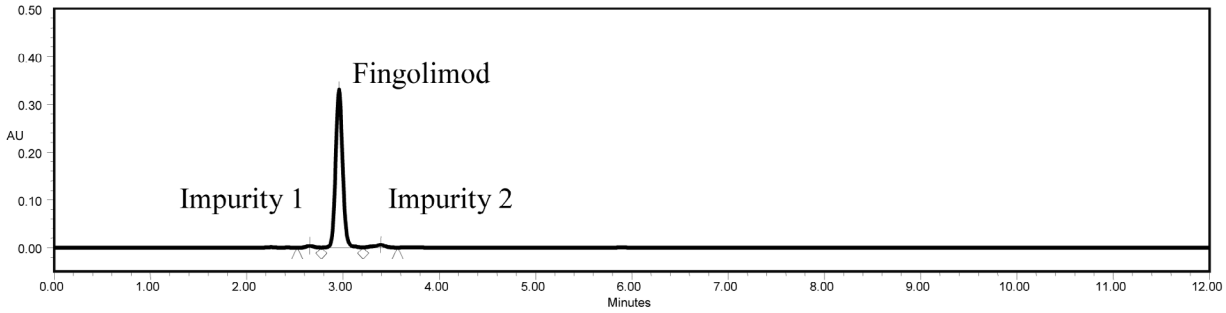

Fig. 6 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, RT, 1 h]

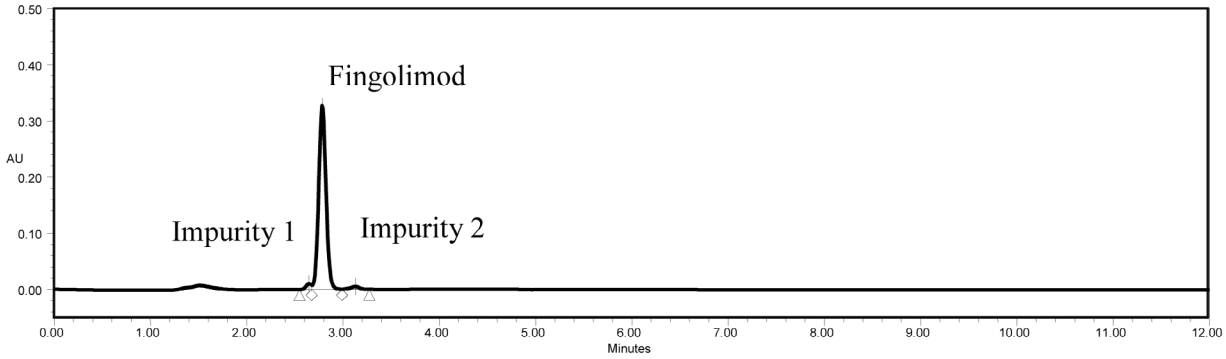

Fig. 7 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 40 °C, 30 min]

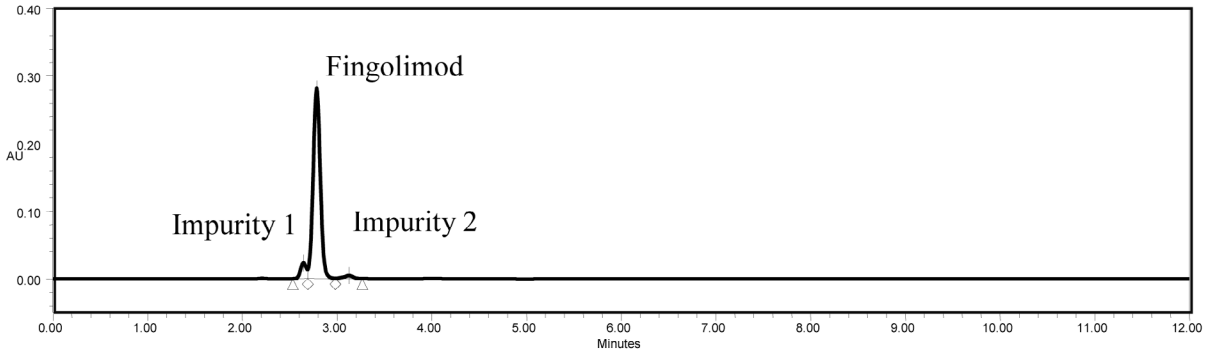

Fig. 8 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 40 °C, 1 h]

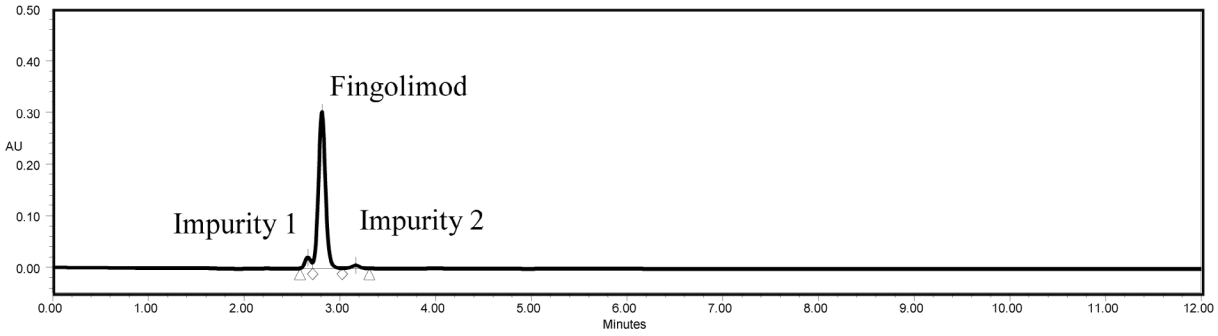

Fig. 9 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 50 °C, 30 min]

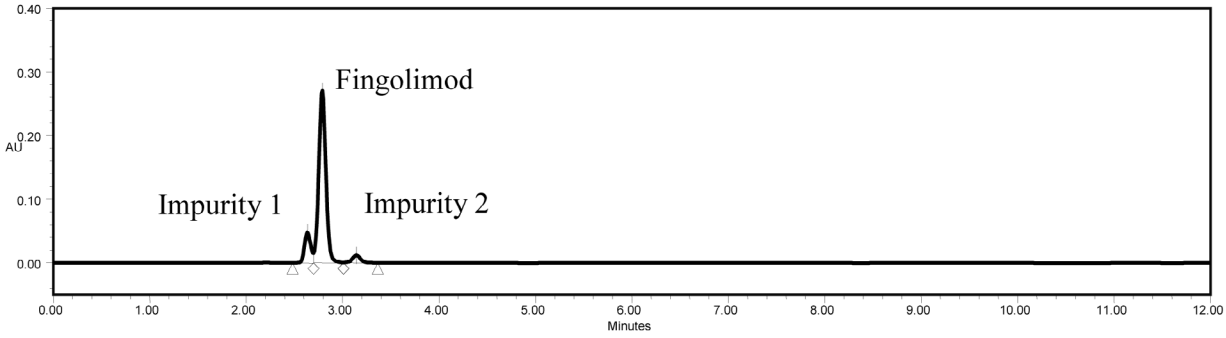

Fig. 10 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 50 °C, 1 h]

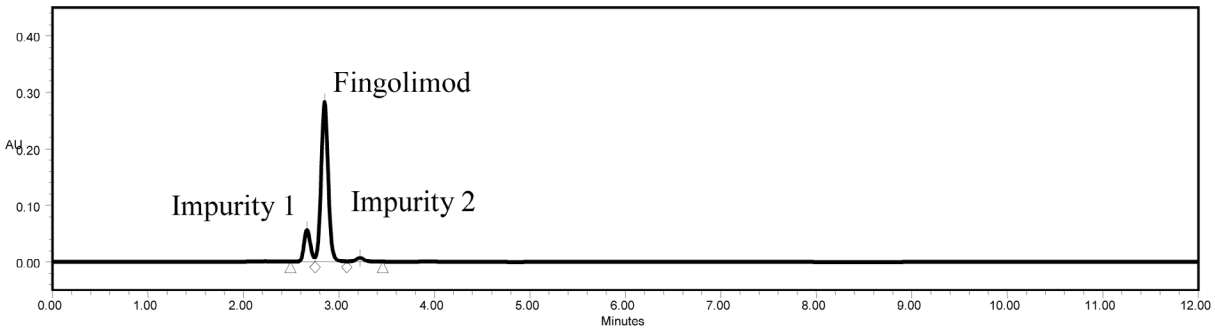

Fig. 11 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 60 °C, 30 min]

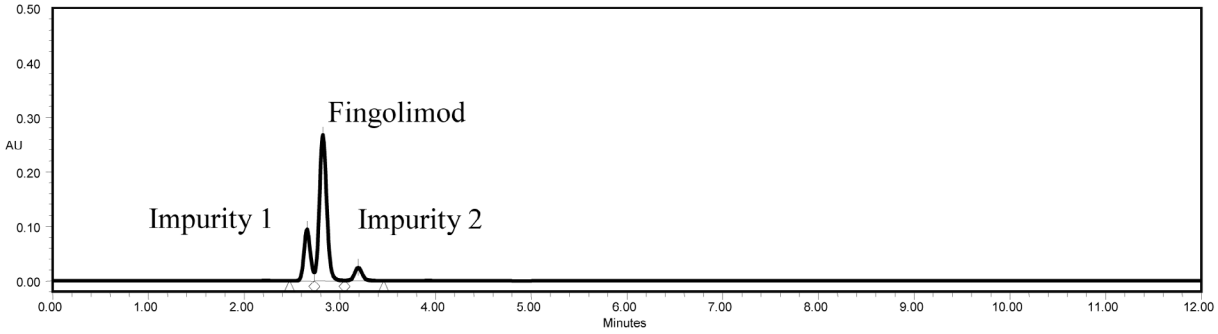

Fig. 12 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 60 °C, 1 h]

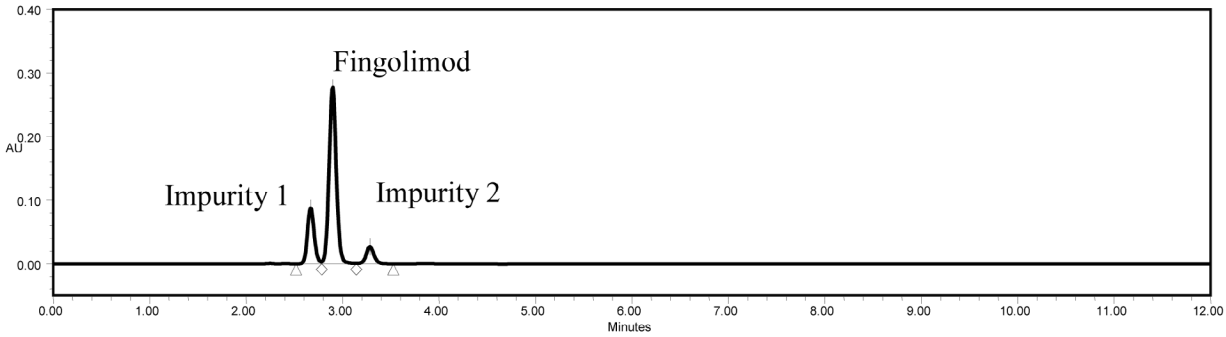

Fig. 13 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 70 °C, 30 min]

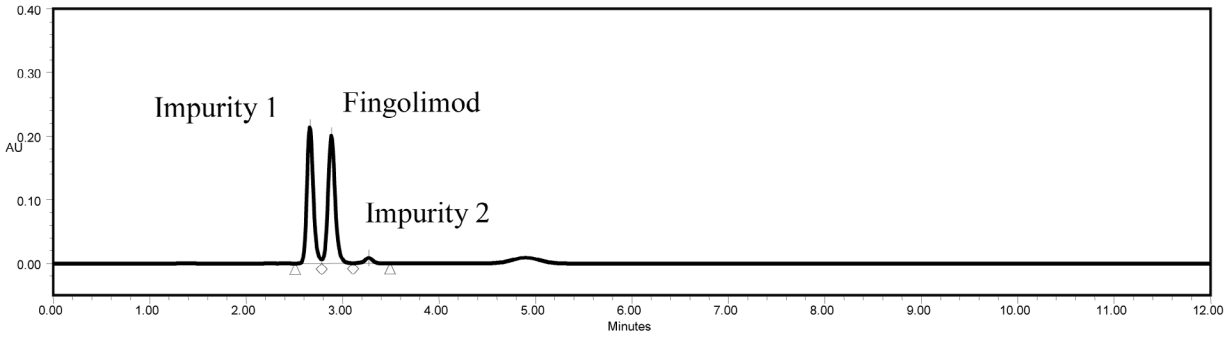

Fig. 14 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 70 °C, 1 h]

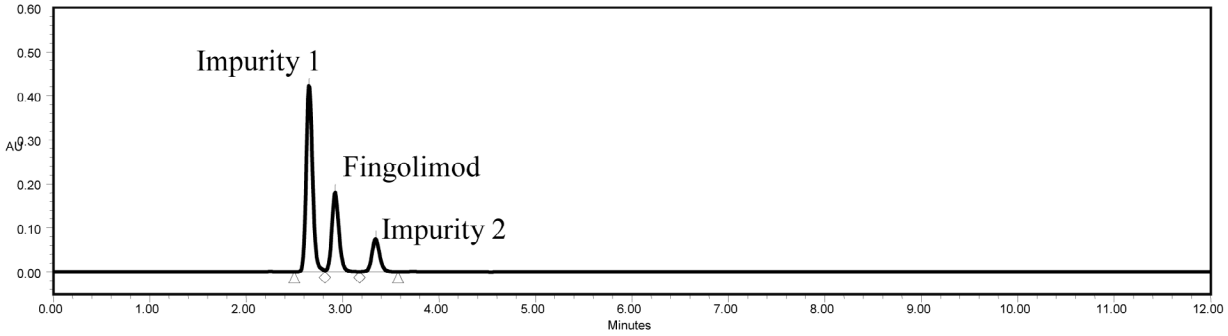

Fig. 15 Chromatogram of alkali degradation study of Fingolimod

[1N NaOH, 80 °C, 1 h]

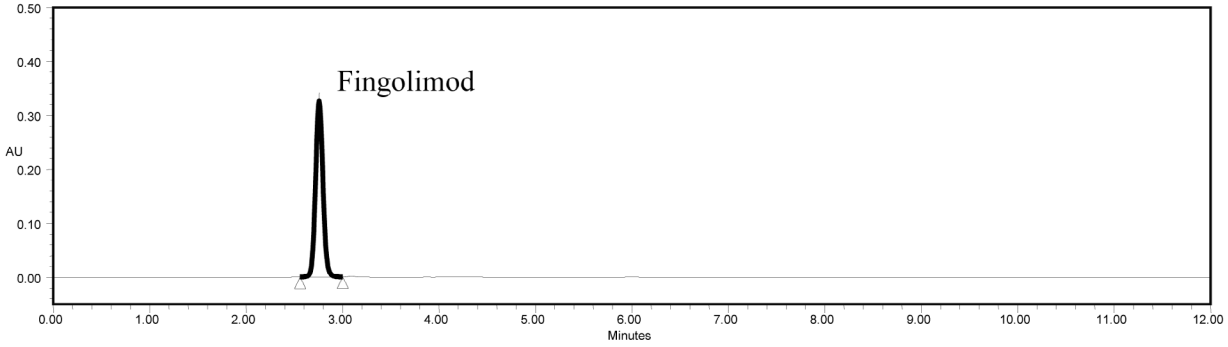

Fig. 16 Chromatogram of Photolytic degradation study of Fingolimod

[UV light, 36 h]

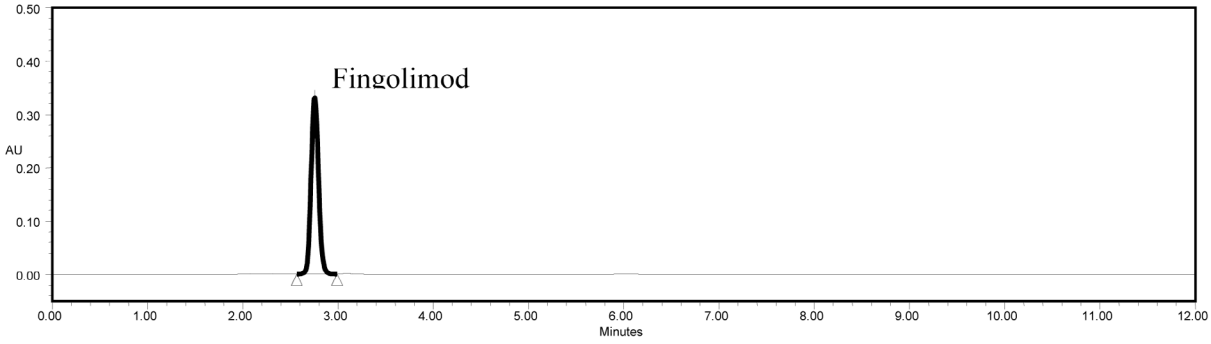

Fig. 17 Chromatogram of Thermal degradation study of Fingolimod

[70 °C, 24 h]

METHOD VALIDATION

The proposed method was validated as per ICH guideline. Method validation includes several parameters like Specificity, Accuracy, Precision, Linearity, Limit of detection (LOD), limit of quantification (LOQ), Solution stability, Robustness and System suitability.

Specificity:

The specificity of the method was evaluated for the interference of diluents, mobile phase and degradation products to ensure the homogeneity of the peak.

Accuracy:

The accuracy of an analytical procedure is the measurement of closeness values between the value of drug sample and accepted standard value. The accuracy of Fingolimod drug was assessed by three accuracy levels 50%, 100% and 150% of the analyte concentration (50µg/ml). For each concentration three sets were prepared and each set was injected in twice. The drug concentrations of Fingolimod were calculated and results obtained are shown in table 2.

Table 2 Recovery study of Fingolimod

Accuracy Level	Set No.	Drug Amount added (µg/ml)	Drug Amount Found (µg/ml)	% Recovery	Mean % Recovery	% (RSD) [a]
50%	1	25.20	25.20	99.99	99.94	0.07
	2	25.30	25.26	99.86		
	3	25.18	25.17	99.98		
100%	1	50.16	50.14	99.96	99.71	0.27
	2	50.04	49.91	99.74		
	3	50.08	49.79	99.43		
150%	1	75.06	74.65	99.45	99.79	0.30
	2	75.30	75.25	99.94		
	3	74.94	74.93	99.98		

[a]Relative standard deviation

Precision:

The precision study of developed and optimized method was performed by system precision and method precision. System precision of Fingolimod was evaluated by injecting five standards and method precision was performed by preparing six sets of Fingolimod sample and injected in duplicate. % Assay of each sets were calculated on the same day (intraday Precision). System precision and method precision were also evaluated by performing the same procedure in different day (Inter day precision) and by another analyst following the same analytical procedure (intermediate precision). The data related to precision was reported in table 3.

Table 3 Summary of precision study

Precision study	Set No.	% Assay	Mean % Assay	Std. dev.	% (RSD)[a]
Method precision	1	99.60	99.60	0.14	0.14
	2	99.74			
	3	99.52			
	4	99.76			
	5	99.40			
	6	99.57			
Intermediate Precision	1	99.75	99.66	0.21	0.21
	2	99.30			
	3	99.69			
	4	99.67			
	5	99.93			
	6	99.64			
Over all	Mean	99.63			
	Std. dev.	0.05			
	% RSD	0.05			

[a]Relative standard deviation

Linearity:

Linearity for the analytical method was performed by injected different concentrations from LOQ to 20% - 180% concentration range

of the actual analyte concentration (50 µg/ml). Each level of concentration was injected in duplicate and calculates the concentration using calibration curve of peak area vs. concentrations. The method was linear in all above concentration range and regression coefficient was found 0.999.

Limit of detection (LOD) and Limit of quantification (LOQ):

The LOD and LOQ of Fingolimod have been evaluated by the minimum concentration level at which the analyte could be readily detected and quantified accurately. LOD and LOQ were determined by signal-to-noise ratio (S/N). A signal-to-noise ratio between 3:1 is generally considered acceptable for estimating the detection limit. For quantification limit signal-to-noise ratio is 10:1. LOD and LOQ for Fingolimod were determined by injecting a series of dilute solution of known concentration of Fingolimod sample. The reproducibility of LOQ was measured by injecting six replicate injections of lowest concentration of analyzed standard. The LOD and LOQ were found 0.15 and 0.5 µg/ml respectively.

Solution Stability:

The stability of the drug in solution during analysis was determined by repeated injection of samples which have been kept in presence of light at ambient temperature. The Fingolimod drug solution was injected at initial to 4 h time interval for two days and compared with freshly prepared standard and test solutions. The % assay of each solution was calculated. The evaluation data of solution stability was given in table 4.

Table 4 Summary of solution stability

Solution stability sample at different time interval	Mean Area	% Assay
Initial	1834212	99.72
4 h	1841513	99.64
8 h	1842707	99.94
12 h	1816685	99.40
16 h	1826616	99.86
20 h	1831756	99.82
24 h	1851422	99.85
36 h	1840162	99.64
48 h	1841406	99.63

Robustness:

The robustness study was carried out to ensure that the method was unaffected and reliable for slightly but deliberated changes in the chromatographic conditions. For robustness study we changed in different analytical parameters like Flow rate, Mobile phase composition, pH value of mobile phase, use different types of analytical Columns and Column oven temperature. The variables evaluated in the study were Column oven temperature (30°C ± 5°C), Flow rate (0.90 ± 0.05 ml/min) and Mobile phase composition was (Buffer: Acetonitrile = 34:66 and 36:64, v/v). The data related to robustness was depicted in table 5.

Table 5 Evaluation data for robustness study

Robustness parameters	% Assay	RT, Minute	System suitability parameters	
			Theoretical Plates	USP Tailing
Flow rate 0.85 ml/min	99.46	2.756	6105	1.05
Flow rate 0.90 ml/min	99.48	2.740	6098	1.06
Flow rate 0.95 ml/min	99.16	2.734	6258	1.05

Buffer: Acetonitrile (34:66)	99.59	2.738	6130	1.04
Buffer: Acetonitrile (35:65)	99.33	2.740	5980	1.10
Buffer: Acetonitrile (36:64)	99.77	2.748	5900	1.08
Column Temperature 25°C	99.29	2.745	6040	1.07
Column Temperature 30°C	99.61	2.742	6184	1.05
Column Temperature 35°C	99.78	2.736	6078	1.05

System suitability:

The suitability of the chromatographic method was checked before performing each stage of validation parameters. The System suitability test was performed to measure the resolution and reproducibility of the system. Five replicate injection of standard preparation and duplicate injection of sample were injected and system suitability parameters like Theoretical plates, USP tailing and %RSD of peak area were calculated. System suitability data were reported in table 6.

Table 6 System suitability criteria for Fingolimod Drug

System Suitability Parameter In-house Limits	% RSD[a] (NMT[b] 2.0)	Theoretical Plates (NLT[c] 2000)	Asymmetry (NMT[b] 2.0)
Validation parameters			
Specificity	0.48	6785	1.05
Solution Stability	0.56	6842	1.05
Accuracy	0.21	6830	1.06
Method precision	0.14	6879	1.05
Intermediate precision	0.21	6786	1.08
Linearity	0.99	6500	1.04
Limit of quantification	0.44	6438	1.05
Robustness	0.52	6862	1.05
[a]Relative standard deviation			
[b]Not more than			
[c]Not less than			

RESULTS AND DISCUSSION

High performance liquid chromatography is a very useful for qualitative as well as quantitative analysis of API and drug dosage forms into micro gram level concentration. The proposed method was well developed and validated on HPLC for quantitative analysis of Fingolimod drug substance with degradation study under different stress conditions. The HPLC method of the quantitative analysis of Fingolimod drug substance has been developed and optimized in such a way that, we would decrease the solvent and chemical cost and also save the time of analysis. The number of theoretical plate was NLT 2000 and USP tailing was NMT 2.0 which indicates excellent column performance for Fingolimod. The chromatographs of Fingolimod of standard and sample were shown in Fig. 18 and 19 respectively.

The linearity of Fingolimod was obtained from 20% to 180% of the actual concentration (50 µg /ml) which was calibrated in the range of 10 to 90 µg/ml concentrations. The linear regression equation was computed as $Y = 36963x + 1446$, $R^2 = 0.999$ (Fig. 20). The evaluation data for linearity study was reported in table 7.

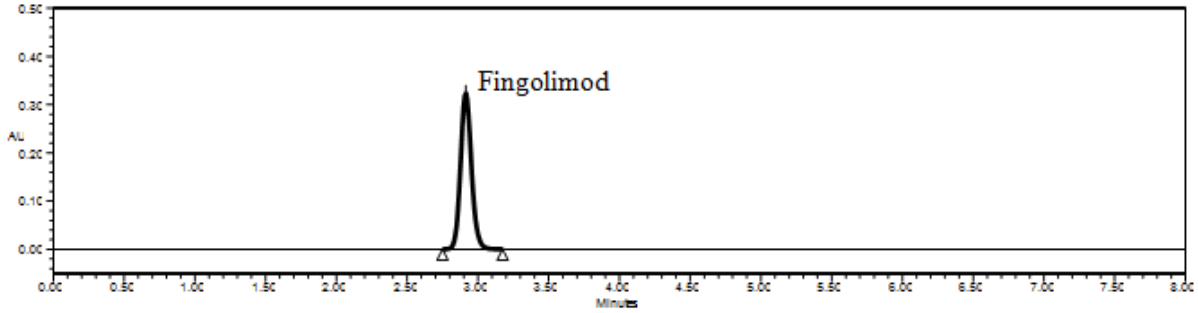

Fig. 18 Standard chromatogram of Fingolimod

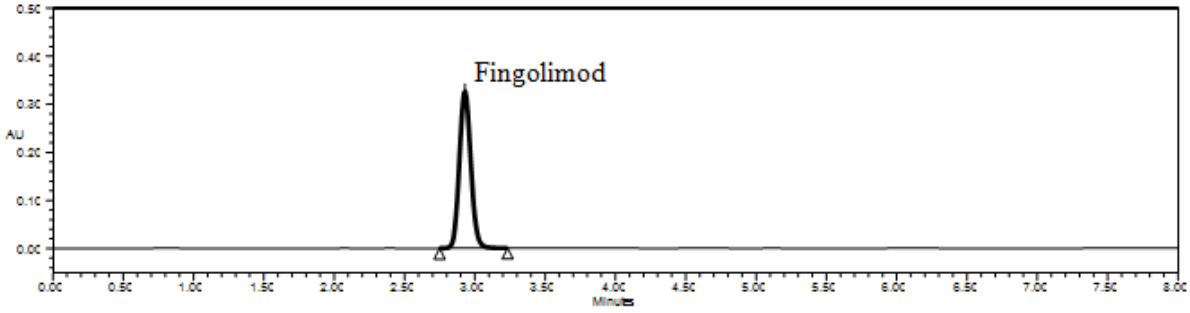

Fig. 19 Sample chromatogram of Fingolimod

Table 7 Summary of Linearity study of Fingolimod drug

Linearity Level	Concentration (µg/ml)	Mean Area
Level 1 (20%)	10.03	377122
Level 2 (40%)	19.98	745395
Level 3 (60%)	30.19	1080231
Level 4 (80%)	40.19	1475650
Level 5 (100%)	50.12	1853838
Level 6 (120%)	60.14	2255953
Level 7 (140%)	70.28	2584668
Level 8 (160%)	80.00	2960935
Level 9 (180%)	90.14	3312644

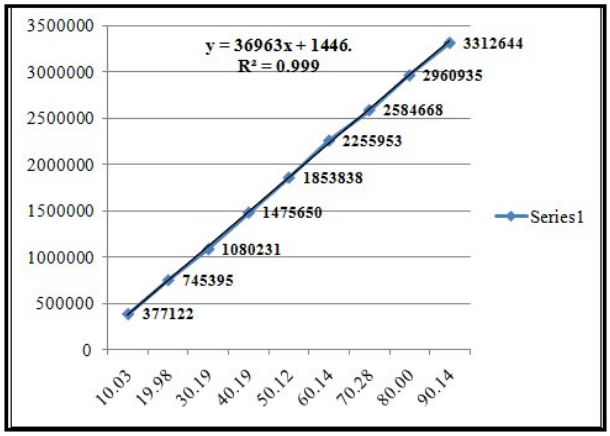

Fig. 20: Linearity curve of Fingolimod

The % recovery of the sample was found in the range of 99.40 to 100.00, while mean recovery and % RSD were 99.81 and 0.21 respectively. The precision value for each test preparation was found in the range 99.0 – 100.0 and % RSD related to precision was less than 2.0 which was

giving further confirmation of highly precise method.

CONCLUSION

The HPLC assay method of quantitative analysis of Fingolimod drug substance is highly accurate, precise, specific, linear and robust. The overall method has been developed, optimized and validated as per ICH guideline. The method is very economical and adaptable. The method was highly sensitive about force degradation. The all degraded impurities were well resolved and separated from drug peak at high specificity.

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