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Stability indicating chromatographic method transfer of immunomodulating drug fingolimod from high performance liquid chromatography to new generation ultra performance liquid chromatography with comparative validation study

Ravi V. Ghediya[1,2], Madhavi Patel[1,2,3], Darshana Pandya[1,2], Anamik K. Shah[1,2] and Ranjan C. Khunt[1,2]*

[1]*Centre of Excellence in Drug Discovery (COE), NFDD complex, Department of Chemistry, Saurashtra University, Rajkot – 360005, Gujarat, India*
[2]*Department of Chemistry, Saurashtra University, Rajkot – 360005, Gujarat, India*
[3]*Department of Chemistry, Banasthali Vidyapith, Banasthali, Rajasthan, India*

ABSTRACT

The simplest, sensitive, less solvent consuming and time saving stability indicating reverse phase isocratic chromatographic separation method of Fingolimod has been performed on High performance liquid chromatography (HPLC) system as well as a new generation high resolution equipment is that Ultra performance liquid chromatography (UPLC) as per international conference on harmonization guidelines. Chromatography separation was carried out on an UPLC system by using acquity BEH C18 column (100 x 2.1 mm, particle size 1.7 µm) and sunfire C18 column (250 x 4.6 mm, particle size 5.0 µm) was used for HPLC system. The mobile phase for ultra performance liquid chromatography consisted of Buffer: Acetonitrile (35: 65 v/v) with a flow rate of 0.35 ml/min, whereas for high performance liquid chromatography same mobile phase have been used with a same composition, but flow rate was differ is that 0.9 ml/min. The detection was achieved at 220 nm for both instruments. The stability indicating method was confirmed by applying various stress conditions like acidic, basic, oxidative, thermal and photolytic as per ICH recommendations. The different validation parameters have been performed on both chromatographic equipments and compared with each other. By comparison of validation data of both instruments we found that UPLC system is much more accurate, precise, sensitive, robust and time saving equipment.

Key words: Fingolimod, Stability - indicating, HPLC-UV, UPLC-PDA, Comparative validation study

INTRODUCTION

Fingolimod (Trade name Gilenya) chemically known as 2-amino-2-[2-(4-octylphenyl) ethyl] propane-1, 3-diol. (Fig. 1) 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. [1, 2]

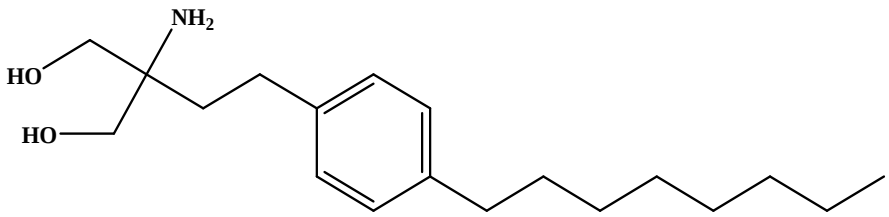

Fig. 1 Chemical structure of Fingolimod

Fingolimod became the first oral disease modifying drug approved by the Food and Drug Administration at September 2010 to reduce relapses and delay disability progression in patients with relapsing forms of multiple sclerosis. Fingolimod is derived from myriocin (ISP1), a metabolite of the fungus Isaria sinclairii. Fingolimod is metabolized by sphingosine kinase – 2 and after phosphorylation of fingolimod; it is converted into fingolimod phosphate. Fingolimod phosphate is a sphingosine-1-phosphate receptor modulator, and binds with high affinity to sphingosine-1-phosphate receptors 1, 3, 4, and 5. It can sequester lymphocytes in lymph nodes, preventing them from moving to the central nervous system for autoimmune responses in multiple sclerosis. It has been reported to stimulate the repair process of glial cells and precursor cells after injury [3 – 8].

Literature review of fingolimod, it is reveals that various analytical methods were previously published for quantification of fingolimod by different spectrophotometric and chromatographic methods [9-14]. There was much stability indicating HPLC methods reported for analysis of bulk and dosage form of fingolimod. There were also UPLC method reported for the analysis of related substance and degraded impurities of fingolimod. By this article we compared two chromatographic techniques with details force degradation and validation study. UPLC system is more efficient, specific, accurate, precise and time saving process then HPLC method. By UPLC method solvent consumption was very less so it is also cheap for whole estimation of analysis coast. So it is very useful in pharma industry as well as laboratory practices. The validation procedure followed the ICH (international conference on harmonization of technical requirements for registration of pharmaceuticals for human use) guidelines [15-17].

EXPERIMENTAL SECTION

Fingolimod API (Potency 99.80) was gifted by Amney Pharmaceuticals limited, Ahmedabad, India. HPLC grade Acetonitrile, methanol and other solvents were purchased from Merck India Limited, Mumbai, India. High purity de-ionized water was prepared using Milli-Q, Millipore (Milford, USA) water purification system. The other analytical grade chemicals like hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution 30% (v/v) were purchased from Ranbaxy Fine Chemicals (New Delhi, India) whereas 0.45 µm membrane filters were procured from Krishna Life Sciences (Mumbai, India).

Preparation of Stock and Standard solution:
Fingolimod stock solution (500µg/ml) was prepared by dissolving 25 mg drug substance in 50 ml volumetric flask with diluent. For HPLC system preparation of standard solution (50µg/ml) was done by taking 1 ml of above stock solution in 10 ml volumetric flask and dilute up to the mark with diluent, whereas for UPLC standard solution (25 µg/ml) was prepared by taking 0.5 ml of above stock solution in 10 ml volumetric flask and diluted with diluent. In all stock and standard preparation mobile phase was used as a diluent.

Preparation of Sample solution:
Fingolimod sample stock solution (500µg/ml) was prepared by dissolving 25 mg API in 50 ml volumetric flask with diluent. For HPLC system preparation of sample solution (50µg/ml) was done by taking 1 ml of above sample stock solution in 10 ml volumetric flask and dilute up to the mark with diluent, whereas for UPLC test solution (25µg/ml) was prepared by taking 0.5 ml of above sample stock solution in 10 ml volumetric flask and diluted with diluent. In all stock and sample preparation mobile phase was used as a diluent.

Instrumentation:
HPLC-UV:
The chromatographic HPLC system was used to perform development and validation of this assay method consisted Waters equipment 600 quaternary pump, Waters 2489 UV/Vis detector, Waters 600 controller, Waters in-line degasser AF and manual injector with 20 µL loop. The equipment was connected to a multi-instrument data-acquisition and data-processing system (Empower 2.0 software).

UPLC-PDA:
Similarly, Waters Acquity UPLC^{TM} System (Switzerland) comprised of a binary solvent manager, a sample manager, PDA detector and Empower 2.0 version software for data acquisition was also used.

Chromatographic conditions:
A Waters Acquity UPLC @ BEH C18 column with 100 x 2.1 mm ID and 1.7µm particle size and Sunfire C18 column with 250 x 4.6 mm ID, particle size 5 µm were used to achieve the best separation on UPLC and HPLC. The mobile phase consisted of Buffer: Acetonitrile (35:65, v/v) with a composition of buffer: 0.1% triethylamine in water and adjust pH 3.0±0.05 with diluted orthophosphoric acid, used for the separation at flow rate of 0.35mL/min and 0.9 ml/min for UPLC and HPLC respectively. The mobile phase was filtered through 0.22 µm micron filter paper prior to use. Injection volume for HPLC system was fixed 20 µL due to manual injector, while for UPLC system injection volume has been kept 5.0 µL. Based on the absorption maxima observed for the component, the detection wavelength was set at 220 nm. The total elution time was selected 8.0 min for HPLC and 3.0 min for UPLC system. Column oven temperature was kept stable at 30 °C for both instruments. Ultrasonic bath (Spinco Ltd) was used for the mobile phase and sample degassing.

RESULTS AND DISCUSSION

Method development and Optimization:
Before optimized rugged, sensitive, precise and time saving HPLC and UPLC methods there were many trails should be taken. The most essential part of method development is column selectivity. After performing several trails on different types of column chemistry e.g. HSS T3, BEH phenyl, BEH C8; the best resolution, sharp peak and shorter run time were achieved by using BEH C18 column (100 x 2.1 mm i.d; 1.7 µm particle size) for UPLC and Sunfire C18 column (250 x 4.6 mm i.d; 5.0µm particle size) for HPLC system. There are many phosphates, acidic and basic buffers were used for trails; but best separation of product peak of their degraded impurities was achieved by buffer: 0.1% TEA in water having pH 3.0±0.05 with diluted OPA, having mobile phase composition of buffer: acetonitrile (35:65 v/v) for both HPLC and UPLC system. For wavelength selection the standard solution was screened over 190–400 nm using the advantage of photo diode array detector. On the basis of peak absorption maxima and peak purity index, the 220 nm was decided as the detection wavelength which provided the maximum chromatographic compatibility to the method. The standard chromatogram of fingolimod on HPLC and UPLC methods are shown in fig. 1 and 2 respectively.

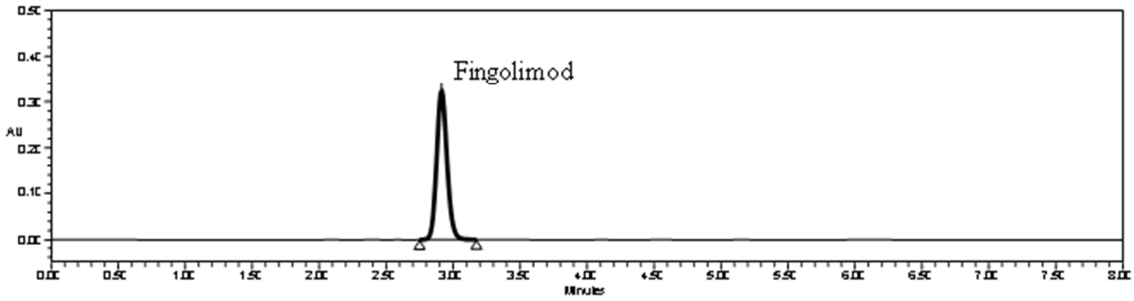

Fig. 1 HPLC chromatograph of standard Fingolimod

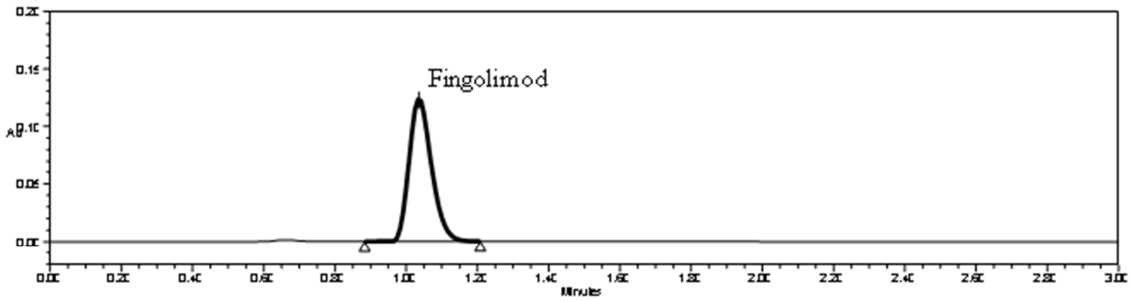

Fig. 2 UPLC chromatograph of standard Fingolimod

Method transfer from HPLC to new generation UPLC:
As the term technology transfer suggests that the earlier developed and validated stability-indicating HPLC method for the determination of fingolimod was optimized to achieve the more speed, sensitivity and resolution. The

conventional HPLC method was scale down to attain better chromatographic compatibility in order to using smaller particle size column and a new generation UPLC equipment. By using smaller particle size column we can enhance the surface area that helps to improve separation and resolution. Moreover, factors like column length, flow rate, elution time and peak with also decreases.

Force degradation study:

The degradation study was performed to measure the stability indicating study and selectivity of the optimized method. The degradation study was performed to ensure that fingolimod peak separate from different degradation products with high resolution. The degradation study of fingolimod was carried out using different stress conditions such as acidic, basic, oxidative, thermal and photolytic.

In acidic degradation, 500 µg/ml concentrate drug solution with 1ml 1N HCl was kept at room temperature for 6 h and the mixture was neutralize with 1 N NaOH. Oxidative degradation was carried out by adding 1ml 3% Hydrogen peroxide solution in the drug sample and kept at room temperature with 6 h. Fingolimod drug was very sensitive towards basic stress conditions. For alkaline stress study, the solution was treated with 1N NaOH at room temperature for 1 h and the mixture was neutralized with 1N HCl. Thermal degradation was performed by exposing solid drug at 70 ºC for 24 hrs in hot air oven. For photolytic degradation powder drug has been exposed to sunlight for 36 hrs (Day hrs only). After applying all these stress conditions the solutions were kept in room temperature and diluted with diluent to make a final concentration 50 µg/ml. The degradation response of Fingolimod in both HPLC and UPLC system with different stress conditions were reported in Table 1 and Fig. (3 to 12).

Table 1 Results of stress degradation study by HPLC and UPLC

	HPLC Degradation study		UPLC Degradation study	
Stress Conditions	**% Drug recovered**	**% Drug decomposed**	**% Drug recovered**	**% Drug decomposed**
Standard drug	100	----	100	----
Acidic (1N HCl, RT, 6 h)	99.24	0.76	99.28	0.72
Basic (1N NaOH, RT, 1 h)	90.10	9.90	90.82	9.18
Oxidative (3% H_2O_2, RT 6 h)	99.21	0.79	99.58	0.42
Thermal (Oven, 70 °C, 24 h)	99.07	0.93	99.01	0.99
Photolytic (sunlight, 36 h)	99.48	0.52	99.33	0.67

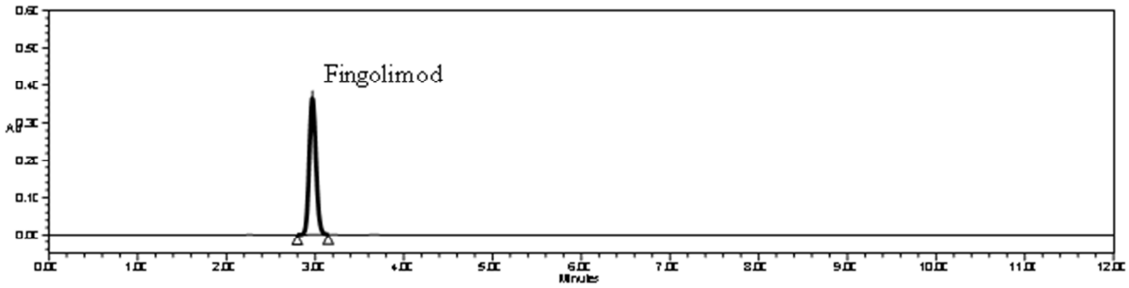

Fig. 3 Chromatograph of acid degradation study of Fingolimod by HPLC

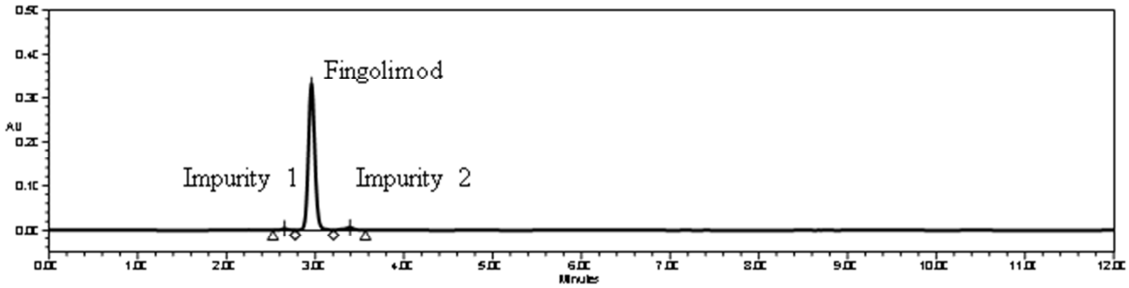

Fig. 4 Chromatograph of basic degradation study of Fingolimod by HPLC

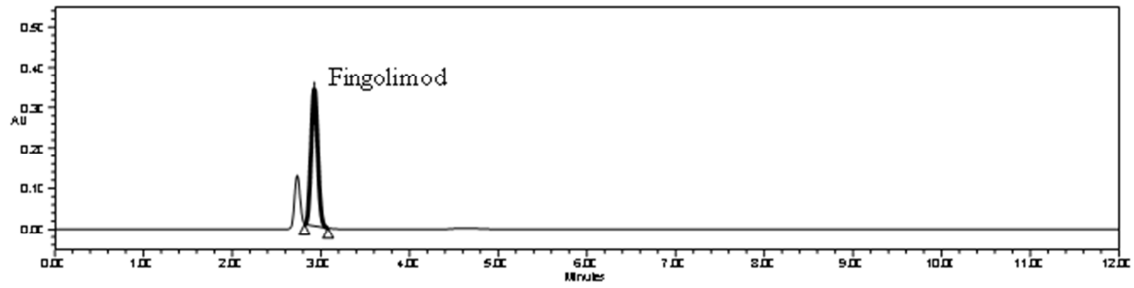

Fig. 5 Chromatograph of oxidaive degradation study of Fingolimod by HPLC

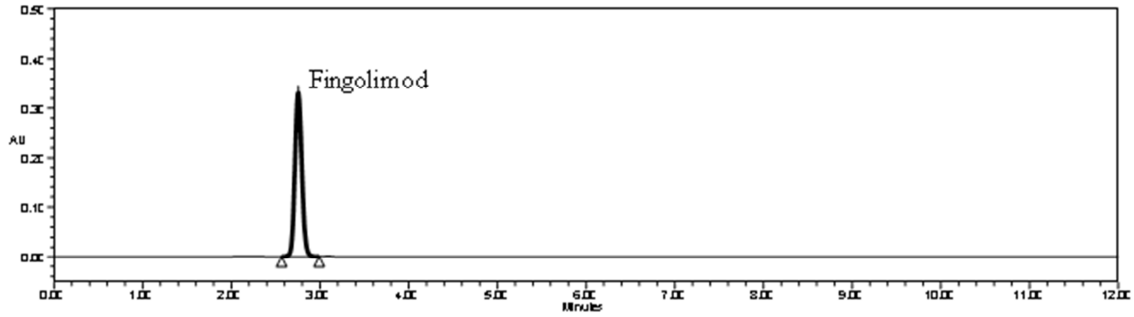

Fig. 6 Chromatograph of Thermal degradation study of Fingolimod by HPLC

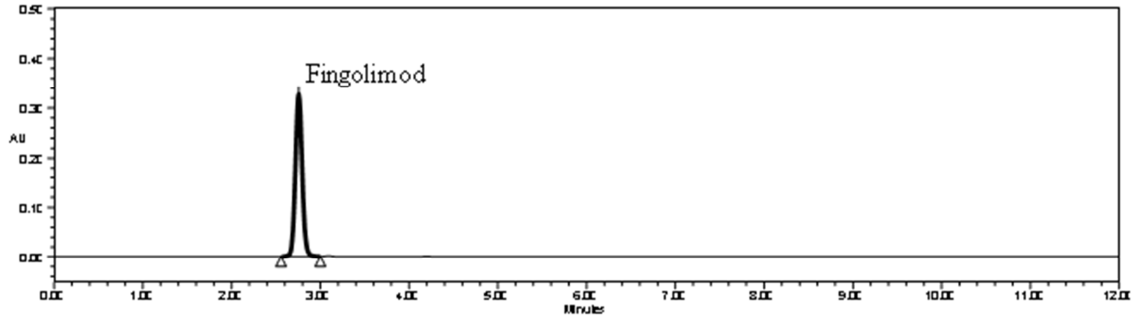

Fig. 7 Chromatograph of Photo stability study of Fingolimod by HPLC

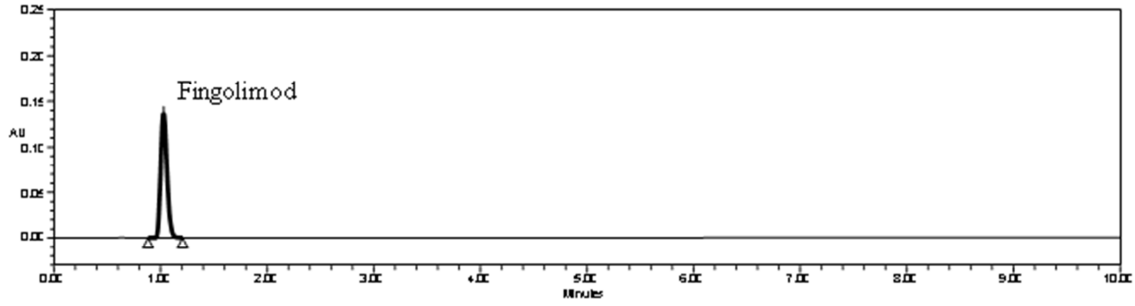

Fig. 8 Chromatograph of acid degradation study of Fingolimod by UPLC

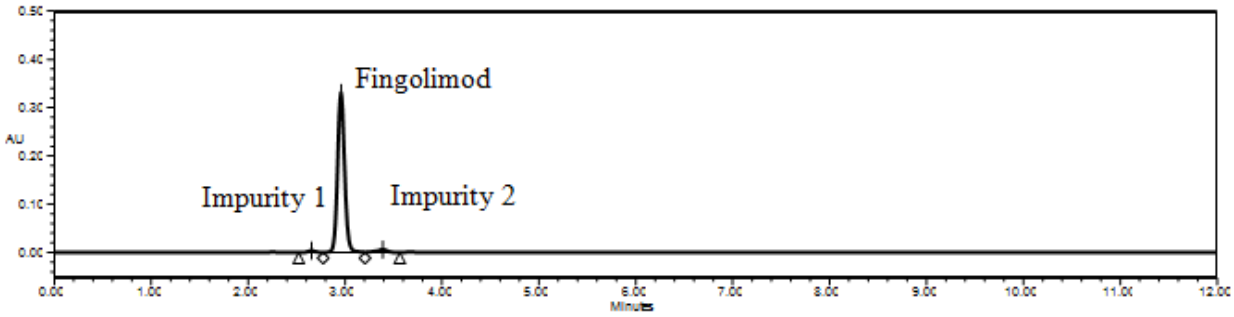

Fig. 9 Chromatograph of alkali degradation study of Fingolimod by UPLC

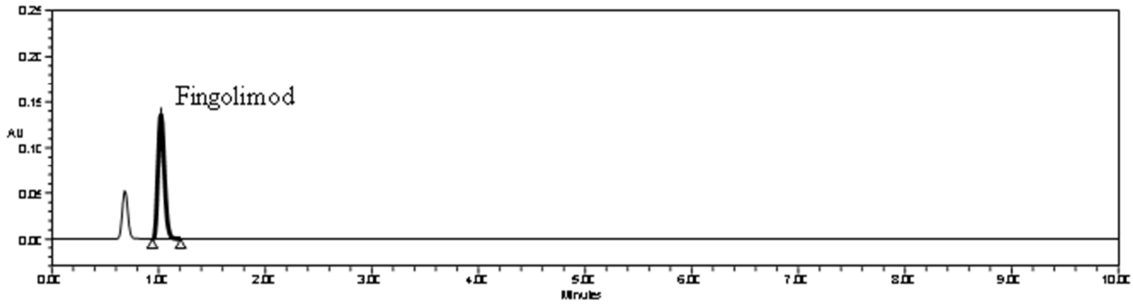

Fig. 10 Chromatograph of oxidative degradation study of Fingolimod by UPLC

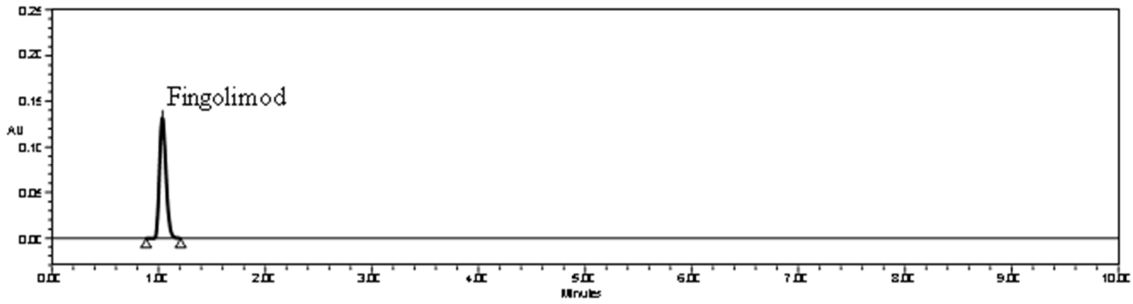

Fig. 11 Chromatograph of Thermal degradation study of Fingolimod by UPLC

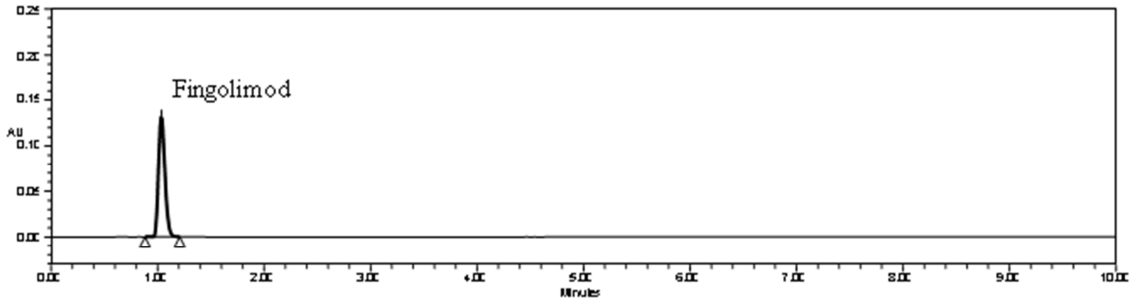

Fig. 12 Chromatograph of Photo stability study of Fingolimod by UPLC

METHOD DEVELOPMENT

Method validation includes several parameters like Solution stability, System suitability, Accuracy, Precision, Linearity, Robustness, Limit of detection (LOD), and Limit of quantification (LOQ). These all validation parameters have been performed systematically on both HPLC and UPLC instruments. All validations have been performed as per ICH guidelines Q2A and Q2B. [15]

Solution stability:

Solution stability study was performed by stored sample solution at room temperature without protection of light and injected at different time interval. The responses for the aged solution were evaluated by injecting drug solution at initial to 4 h time interval for two days and compared with freshly prepared standard solution. For the duration of the study of the stability of stored solutions assay was determined. The evaluation data of solution stability study of both HPLC and UPLC instruments were given in Table 2.

Table 2 Summery of solution stability study on HPLC and UPLC

Solution stability of sample at different time interval	Fingolimod HPLC method		Fingolimod UPLC method	
	Mean Area	Assay (%)	Mean Area	Assay (%)
Initial	1834212	99.72	542801	99.26
4 h	1841513	99.64	541723	99.62
8 h	1842707	99.94	547478	99.87
12 h	1816685	99.40	546727	99.66
16 h	1826616	99.86	543532	99.79
20 h	1831756	99.82	542521	99.45
24 h	1851422	99.85	544987	99.58
36 h	1840162	99.64	543540	99.55
48 h	1841406	99.63	541950	99.66
Mean	**1836275**	**99.72**	**543918**	**99.61**
% RSD	**0.56**	**0.17**	**0.38**	**0.18**

System suitability:

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. The comparison between system suitability data of HPLC and UPLC were reported in Table 3.

Table 3 Summery of method validation parameters of HPLC and UPLC

	HPLC Method			UPLC method		
Method Validation Results (in-house limits)	**% RSD[a] (NMT 2.0)[b]**	**Theoretical Plates (NLT 2000)[c]**	**Peak tailing (NMT 2.0)[b]**	**% RSD[a] (NMT 2.0)[b]**	**Theoretical Plates (NLT 2000)[c]**	**Peak tailing (NMT 2.0)[b]**
Accuracy	0.21	6287	1.06	0.15	3856	1.20
Method Precision	0.14	6310	1.05	0.16	3945	1.25
Intermediate Precision	0.21	6325	1.10	0.09	3875	1.26
Linearity	0.36	6163	1.08	0.20	3695	1.21
LOQ	0.44	6200	1.06	0.38	3860	1.18
Robustness	0.52	6402	1.05	0.33	3978	1.20
Solution stability	0.56	6369	1.05	0.38	3878	1.23

[a]Relative standard deviation, [b]Not more than, [c]Not less than

Table 4 Percentage recovery data for UPLC and HPLC accuracy study

Instrument Used	Level (%)	Set No	Amount of drug added (µg/ml)	Amount of drug found (µg/ml)	Recovery (%)	Mean Recovery (%)	RSD[a] (%)
UPLC	50	1	12.60	12.59	99.88	99.95	0.30
		2	12.62	12.58	99.68		
		3	12.64	12.67	100.28		
	100	1	24.96	24.93	99.89	99.84	0.08
		2	25.30	25.27	99.89		
		3	25.14	25.08	99.75		
	150	1	37.53	37.42	99.71	99.73	0.06
		2	37.53	37.41	99.68		
		3	37.62	37.54	99.80		
HPLC	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

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 the assay method was evaluated by preparing three different concentration levels corresponding to 50%, 100 %, 150 % of test preparation concentration in triplicate and injecting it in duplicate. The recovery was found between 99 and 100% for both HPLC and UPLC, which is between under the acceptance criteria of ICH guideline Q2 (A). The UPLC and HPLC data for the percentage recovery are shown in the Table 4.

Precision:
Precision study was performed by evaluating method precision and intermediate precision study. Method precision study of Fingolimod drug was carried out by injecting five standard and six sets of test solution in duplicate. Intermediate precision of the analytical method was confirmed with inter day and intraday testing of drug substance. The intra-day precision study was performed in a same day by analyzing three times with six independent assays of test sample against reference material. Inter-day precision of the method was determined by performing the same procedure on different day or by different analyst or by different chromatographic instruments. The evaluation data for precision study of HPLC and UPLC are shown in Table 5.

Table 5 Evaluation data for precision study by HPLC and UPLC method

		HPLC method			UPLC method		
Precision study	**Set No.**	**Assay (%)**	**Mean Assay (%)**	**RSD[a] (%)**	**Assay (%)**	**Mean Assay (%)**	**RSD[a] (%)**
Method precision	1	99.60	99.60	0.14	99.78	99.81	0.16
	2	99.74			99.84		
	3	99.52			99.92		
	4	99.76			99.94		
	5	99.40			99.51		
	6	99.57			99.85		
Intermediate precision	1	99.75	99.66	0.21	99.74	99.75	0.09
	2	99.30			99.77		
	3	99.69			99.83		
	4	99.67			99.60		
	5	99.93			99.69		
	6	99.64			99.84		

[a]*Relative standard deviation*

Linearity:
Linearity for the analytical method was assessed by injected nine level different concentrations from 20% - 180% concentration range of the actual analyte concentration 50 µg/ml for HPLC method and 25 µg/ml for UPLC method. Each level of concentration was injected in duplicate and the slope, Y- intercepts and correlation coefficient were calculated by plotting peak area versus concentration curve. Linear regression equation was found Y = 36963x + 1446 for HPLC and Y = 10398x + 12689 for UPLC. The method was linear in all above concentration range and the regression coefficient (R^2) was found to be 0.999 and 0.998 respectively, for HPLC and UPLC method. Linearity curve of Fingolimod by HPLC and UPLC methods are given in Fig. 13 and 14 respectively.

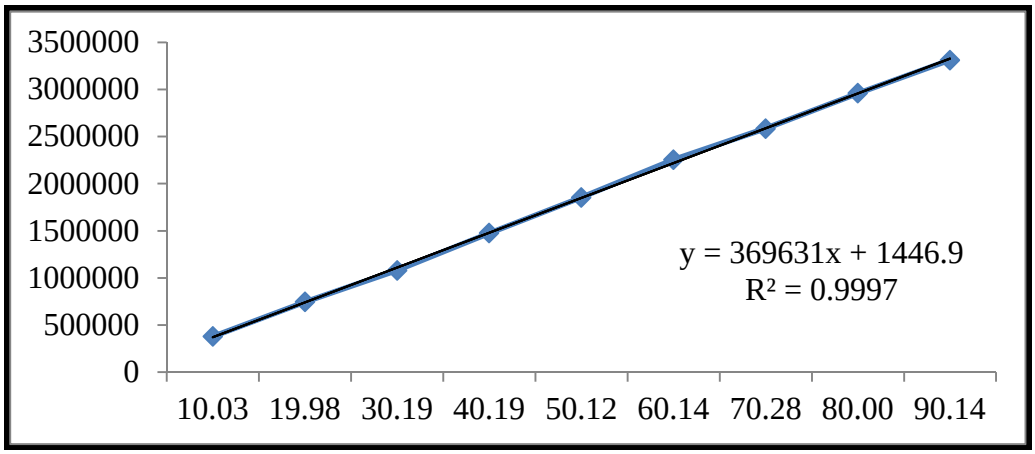

Fig. 13 Linearity curve of Fingolimod by HPLC method

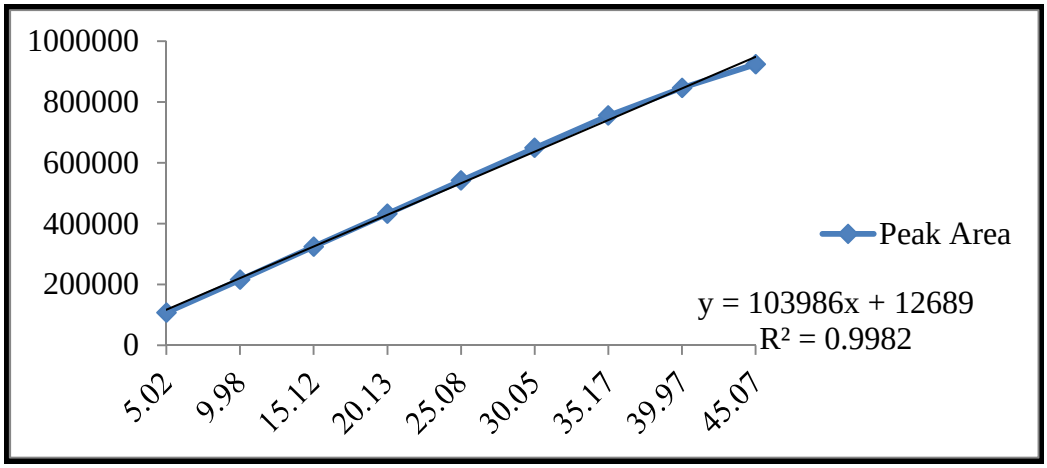

Fig. 14 Linearity curve of Fingolimod by UPLC method

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

LOD and LOQ for fingolimod were determined at signal to noise ratios of 3:1 and 10:1 respectively by injecting series of dilute solutions prepared by serial dilutions of the known concentration. The reproducibility of LOQ was measured by injecting six replicate injections of lowest concentration of analyzed standard. The concentration 0.15 µg/ml and 0.005µg/ml are LOD level for HPLC and UPLC method respectively. Moreover, 0.015µg/ml and 0.5 µg/ml are the LOQ level for UPLC and HPLC respectively.

Robustness:

The Robustness is an analytical procedure which is measured by small but deliberate change in analytical parameters like Flow rate, Mobile phase composition, pH value of mobile phase, use different types of analytical columns and column oven temperature. Robustness is providing its reliability during normal usage. The variables evaluated in the study were Column oven temperature, Flow rate and Mobile phase composition. The data related to robustness study by HPLC and UPLC methods were depicted in Table 6 and 7 respectively.

Table 6 Evaluation data for robustness study by HPLC method

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

Table 7 Evaluation data for robustness study by UPLC method

Robustness parameters	% Assay	RT, Minute	System suitability parameters	
			Theoretical Plates	USP Tailing
Flow rate 0.34 ml/min	99.89	1.064	3856	1.26
Flow rate 0.35 ml/min	99.75	1.039	3815	1.26
Flow rate 0.36 ml/min	99.91	1.004	3720	1.27
Buffer: Acetonitrile (34:66)	99.88	1.062	3822	1.28
Buffer: Acetonitrile (35:65)	99.89	1.036	3785	1.25
Buffer: Acetonitrile (36:64)	99.94	1.009	3807	1.25
Column Temperature 25°C	99.75	1.034	3792	1.26
Column Temperature 30°C	99.71	1.038	3856	1.27
Column Temperature 35°C	99.77	1.032	3872	1.27

CONCLUSION

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for both bulk drugs as well as in pharmaceutical dosage form of Fingolimod.

Fingolimod is very much sensitive towards basic stress condition and comparatively less sensitive to acid solution. Degradation products produced as a result of stress did not interfere with detection of Fingolimod and the assay method can thus be regarded as stability indicating. However, chromatographic conditions of both methods are almost same due to method transfer from HPLC to UPLC. Some changes were required to obtain suitability of method by the means of asymmetry, number of theoretical plates and % RSD. The lower concentration for LOD and LOQ in UPLC method compare to HPLC method shows the greater sensitivity. The total analysis time required by HPLC method is 8.0 min whereas in UPLC method it reduced to 3 min. The method was revealed to be selective, precise, sensitive, rapid and linear that was confirmed by the method validation results. The proposed both the chromatographic methods represent good sensitivity, resolution and selectivity in bulk drug as well as in pharmaceutical dosage forms. UPLC method is faster and sensitive as compare to HPLC method. The major degradation products observed in acid, alkali and oxidation conditions are eluted at same retention time.

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