Stability indicating HPLC method for the quantification of fingolimog hydrochloride and its related substances*

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ABSTRACT

A reverse phase liquid chromatographic (RP-LC) method was developed for the quantification of the related impurities of Fingolimod hydrochloride drug substance. The method was optimized using buffer (prepared by dissolving 2.72gr KH2PO4 and 2.0gm 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to ~ 3.0 with dilute orthophospharic acid solution) as mobilephase-A, and Acetonitrile: water in the ratio of 90:10 v/v as mobile phase-B. The flow rate was set at 1.2 mL min^{-1}, wavelength at 220nm respectively and the column temperature was maintained at 45°C. The capability of stability indicating method developed was demonstrated by studying the degradation products generated during the forced degradation studies under the following conditions i) water hydrolysis, ii) at 75% relative humidity, iii) oxidative, iv) thermal v) photolytic, vi) acid, vii) base, and viii) photolytic degradation. The developed method can be used for the determination of synthetic and degradation impurities in the regular quality control analysis for the drug substance.

Keywords: Fingolimod Hydrochloride, Reverse phase liquid chromatography, Stability-indicating methods, method development, Method validation, Stress conditions, ICH.
***MSNRD Communication No.017*

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INTRODUCTION

The biological activity of chiral substances often depends upon their stereochemistry. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics [1-3]. Analysis of the enantiomeric purity of chiral drug candidates has become very important particularly in the pharmaceutical and biological fields, because few enantiomers of racemic drugs have relatively different pharmacokinetic properties and diverse pharmacological or toxicological effects [4-7]. apart from this the International Conference on Harmonization (ICH) guide-lines [8-10] emphasizes that the purity and assay of drug susceptible to change during storage, must be determined by using validated stability- indicating methods, which can selectively determine the drug in presence of its process (including the other isomers) and degradation impurities. Fingolimod is (INN, trade name Gilenya, Novartis) an immunomodulating drug, approved for treating multiple sclerosis. It has reduced the rate of relapses in relapsing-remitting multiple sclerosis by over half. Fingolimod is a sphingosine 1-phosphate receptor modulator, which sequesters lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction. It is derived from the myriocin (ISP-1), metabolite of the fungus *Isaria sinclairii*. It is a

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structural analogue of sphingosine and is phosphorylated bysphingosine kinases in the cell (most importantly sphingosine kinase2) [11-13]. The molecular biology of phosphofingolimod is thought to lie in its activity at one of the five sphingosine-1-phosphate receptors, S1PR1[14]. It can sequester lymphocytes in lymph nodes, preventing them from moving to the central nervous system for autoimmune responses in multiple sclerosis, and was originally proposed as an antirejection medication indicated after transplantation. It has been reported to stimulate the repair process of glial cells and precursor cells after injury[15]. Fingolimod has also been reported to be a cannabinoid receptor antagonist[16], a cPLA2 inhibitor[17] and a ceramide synthase inhibitor[18]. On September 22, 2010, fingolimod became the first *oral* disease-modifying drug approved by the Food and Drug Administration to reduce relapses and delay disability progression in patients with relapsing forms of multiple sclerosis[19-20]. Novartis announced on March 10, 2011 that it had received a notice of compliance from Health Canada and that the drug would be available April 1, 2011 at pharmacies[21-22]. On March 17, 2011, the European Medicines Agency approved the drug for use in the European Union[23]. Fingolimod has recently been discovered as a candidate therapeutic drug for the treatment of heart failure and arrhythmias. Heart failure is a leading cause of hospitalization and death in many countries, and an ever-increasing health burden worldwide[24-26]. Pathogenic hypertrophy of the myocardium is a cardinal sign leading to heart failure and is associated with an increased risk of cardiac morbidity and mortality[24][27]. Cardiac arrhythmias are disturbances in cardiac rhythm that often arise as a lethal complication of heart failure. This occurs due to the progressive ischemic/reperfusion injury the heart faces[28]. Fingolimod hydrochloride is commercially available under the brand name of Gilenya. Chemically, Fingolimod hydrochloride is 2-Amino-2-[2-(4-octylphenyl)ethyl]propan-1,3-diolhydrochloride. The empirical formula is $C_{19}H_{33}NO_2.HCl$ and its molecular weight is 343.93 and the chemical structure of Fingolimod hydrochloride is shown in (Fig.1).
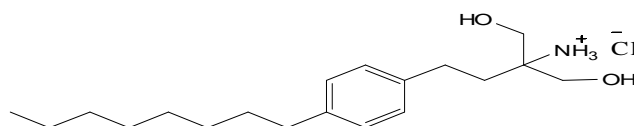

Fig-1: Fingolimod HCl

Chemical name : 2-Amino-2-[2-(4-octylphenyl)ethyl] propan-1,3-diol hydrochloride.

However extensive survey revealed that stability indicating HPLC method for quantitative determination of Fingolimod hydrochloride and its related impurities in active pharmaceutical ingredient was not reported till date. Therefore it was felt necessary to develop an accurate, rapid, and specific stability indicating method for the determination of 'Related substances' of Fingolimod hydrochloride. We have developed a new accurate and stability indicating HPLC method for the determining the 'Related substances' of Fingolimod hydrochloride.

MATERIALS AND METHODS

Chemicals and Reagents
Fingolimod hydrochloride and its impurities viz. Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitro diol impurities were obtained from MSN Laboratories Private Limited, Hyderabad, India. HPLC-grade of acetonitrile and AR grade of potassium di hydrogen phosphate, ortho phosphoric acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%) were obtained from Rankem, New Delhi, India. Milli Q Millipore (USA) purification system was used to prepare high pure water.

HPLC Instrumentation and Conditions
The method development attempts, forced degradation studies and the method validation was performed in Agilent 1200 series LC systems with a diode array and variable wave length detectors (Agilent Technologies, Waldbronn, Germany). The data were collected and processed using Ez chrom Elite software. The peak homogeneity was studied by using Agilent 1200 series DAD detector.

Chromatographic conditions
The chromatographic separation was optimized in the Symmetry shield RP-18 column with the dimension of 250mm x 4.6 mm and 5µm as particle size. A gradient elution was involved with the buffer (prepared by dissolving 2.72gr KH2PO4 and 2.0gm 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to ~ 3.0 with dilute orthophospharic acid solution) as mobilephase-A, and Acetonitrile: water

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in the ratio of 90:10 v/v as mobile phase-B. The HPLC gradient program was set as (time/% mobile phase- B) 0.01/45, 20/85, 37/85, 37.5/45, 45/45. The flow rate of the mobile phase and the column temperature was set as 1.2 mL min^{-1} and 45°C. The detection wave length was optimized at 220 nm, 10µL injection volume. A mixture of acetonitrle and water 9:1v/v was used as diluent, and Methanol used for needle wash purpose.

Preparation of standard solutions:
 A mixture of acetonitrle and water 9:1v/v was used as diluent. A standard solution (Reference solution) 0.001mg/mL of Fingolimod hydrochloride solution was prepared in the diluent. A stock solution with the blend of Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitro diol impurities was also prepared in diluent for the preparation of system suitability solution (0.15% solution with respect to 1.0mg/mL Fingolimod hydrochloride test concentration).

RESULTS AND DISCUSSION

Method development and optimization
The HPLC method was optimized so as to obtain stability– indicating method that it could resolve degradation impurities from Fingolimod hydrochloride. Different stationary phases with different selectivity were used for the determination of Fingolimod hydrochloride and it's impurities as the initial attempts. However good peak shape with less peak width and the resolution of all the related impurities were achieved satisfactorily in Symmetry shield RP-18 column with the dimension of 250mm x 4.6 mm and 5µm as particle size. A gradient elution was involved a buffer (prepared by dissolving 2.72gr KH2PO4 and 2.0gm 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to ~ 3.0 with dilute orthosphoric acid solution) as mobilephase-A, and Acetonitrile: water in the ratio of 90:10 v/v as mobile phase-B. The HPLC gradient program was set as (time/% mobile phase- B) 0.01/45, 20/85, 37/85, 37.5/45, 45/45. The flow rate of the mobile phase and the column temperature was set as 1.2 mL min^{-1} and 45°C. The detection wave length was optimized at 220 nm, 10µL injection volume. A mixture of acetonitrle and water 9:1v/v was used as diluent, and Methanol used for needle wash purpose.
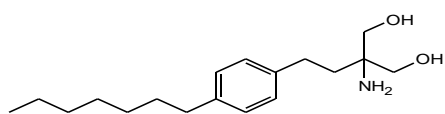

FIG: 2
Heptyl impurity
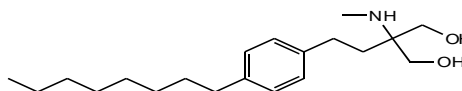2-amino-2-(4-Heptylphenethyl)
propan-1,3-diol

FIG:3
N- Methyl impurity
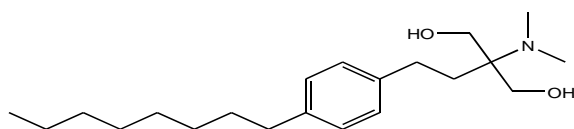2-(Methylamino)-2-(4-octylphenethyl)propan-1,3-diol

FIG :4
Dimethyl impurity
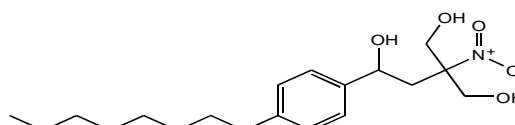2-(dimethylamino)-2-(4-octylphenethyl)propane-1,3-diol

FIG:5
Nitro hydroxy diol impurity
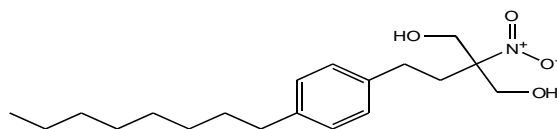3-(hydroxymethyl)-3-nitro-1-(4-octylphenyl)butane-1,4-diol

FIG:6
Nitro diol impurity
2-Nitro-2-(4-octylphenethyl)propane-1,3-diol

The system suitability parameters are resolution between any two adjacent known impurities and known impurity and Fingolimod should not be less than 1.5, and tailing factor not more than 2.0 and Theoretical plates for Fingolimod peak should not be less than 3000, and detector sensitivity for reference solution the S/N ratio should not be less than 30. The developed method is specific for Fingolimod hydrochloride and its degradation products. The structures and chemical names of the impurities are given above.

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Chromatograms: A typical chromatogram of Blank, System suitability solution, Reference solution, Standard sample are given below
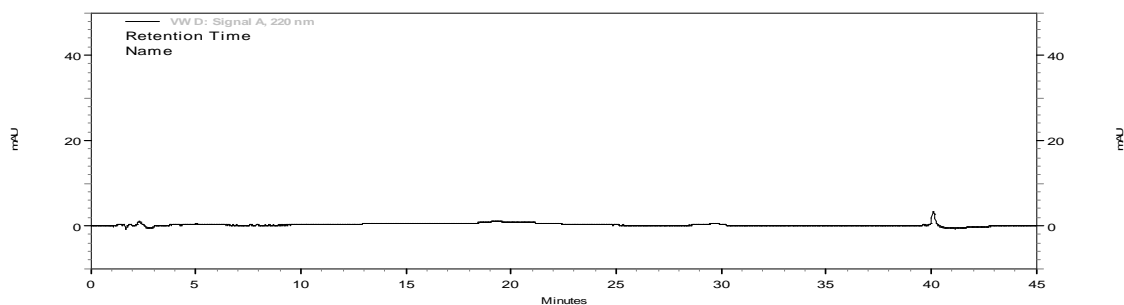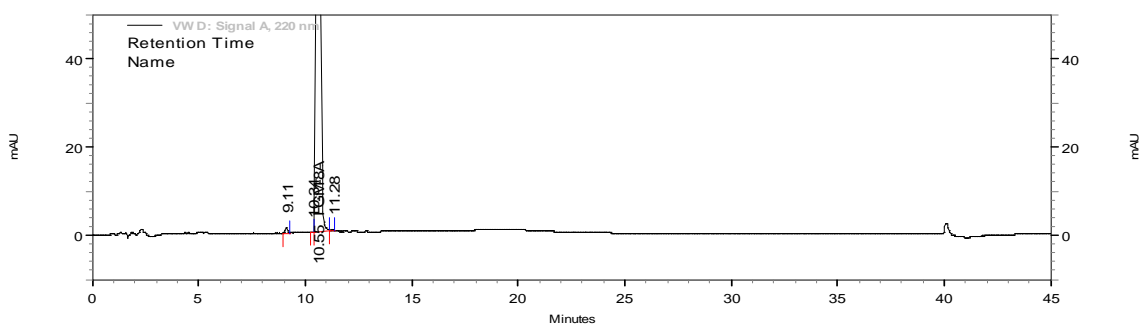

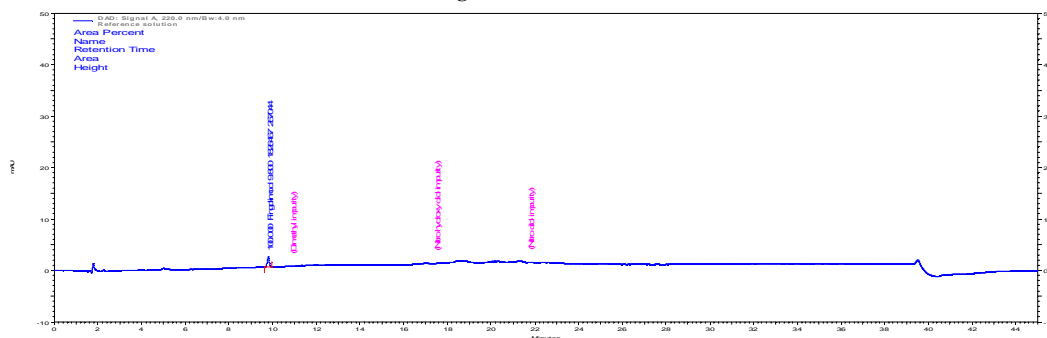**Fig 7: Diluent**

Fig 8: Standard solution

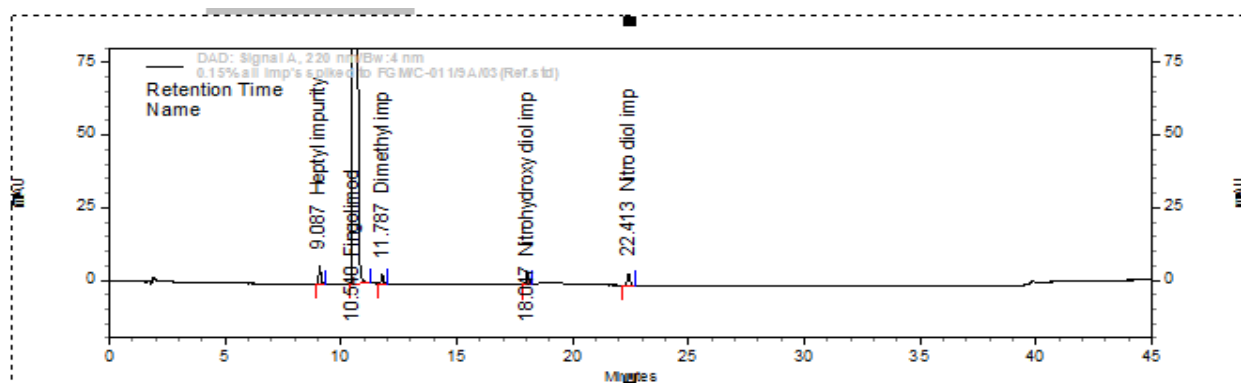**Fig 9: Reference solution**

Fig10:   0.15% all impurities spiked to Fesoterodine Fumarate sample

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Method validation Results

The developed method was validated as per ICH guidelines and the results are given (Table I). Stress testing of the drug substance can help to identify the degradants, which in turn help to evaluate the stability-indicating nature of the developed method. The specificity of the developed HPLC method for Fingolimod hydrochloride was determined in the presence of its process and degradation impurities. All the stressed samples of Fingolimod hydrochloride and all degradation impurities were well resolved from one another and from Fingolimod hydrochloride. The analysis was carried out by HPLC with Diod array detector. The chromatographic peak purity tool, applied to Fingolimod hydrochloride and its impurities peaks, demonstrated that all the peaks were pure in all cases conform the absence of other impurities co-eluting in the same retention time and there by signifying the specificity and stability indicating nature of the method. The detection limit (DL) and quantification limit (QL) for Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitro diol impurities were determined at a signal to noise ratio of 3:3 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. Precision study was carried at QL level by injecting six times and calculating the percentage of R.S.D of area of Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitro diol impurity. Linearity test solutions for purity determination were at six concentration levels from QL to 150 % of the specification level (0.15%). Peak area versus concentration data was performed by least-squares linear regression analysis. Standard addition and recovery experiments were conducted to determine accuracy of impurities quantitation in bulk drug samples. The study was carried out in triplicate at QL, 50%, 75%, 100%, 125% and 150% level with respect to specification 0.15%. The percentages of recoveries for impurities were calculated.

Table-1: Validation data of the developed method

Parameter	Heptyl impurity.	N-Methyl impurity.	Dimethyl impurity.	Nitro hydroxy diol impurity	Nitro diol impurity	Fingolimod HCl
DL (%)	0.0024	0.0032	0.0040	0.0027	0.00280	0.00170	
QL (%)	0.0109	0.0149	0.0153	0.0110	0.0130	0.01000	
Method Precision (%RSD)#	1.91	2.35	2.20	1.12	0.46	---	
Intermediate precision (%RSD)#	0.97	1.04	2.46	0.68	0.58	---	
Accuracy[a] (% recovery) at:-							
QL	105.3	95.8	96.8	99.4	102.2		
50%	103.5	95.1	95.7	98.6	96.2		
75%	100.4	100.9	101.6	98.3	96.9		
100%	98.4	96.1	100.6	96.9	96.7		
125%	97.8	97.4	100.9	100.1	100.3		
150%	101.0	99.0	102.7	99.5	99.7		

[a] Carried at QL,50%,75%, 100% and 150% level with respect to specification (0.15%)

The robustness of developed method was determined by altering experimental conditions purposely and evaluating the resolution between Fingolimod hydrochloride, Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitrodiol impurity. Flow rate was changed by $\pm$ 0.1 units, pH was varied by $\pm$ 0.2 units and column temperature was studied at 40°C and 50°C instead of 45°C in all above varied conditions the components of the mobile phase were held constant and no significant change (relative error less than 5%) of relative retention time was observed.

Significant changes were not observed in the contents of Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitrodiol impurity. The stability data confirmed that sample solutions were stable up to 48hrs. The system suitability was established in terms of resolution between any two adjacent known impurities and known impurity and Fingolimod should not be less than 1.5, and tailing factor not more than 2.0 and Theoretical plates for Fingolimod peak should not be less than 3000, and detector sensitivity for reference solution the S/N ratio should not be less than 30. When a 1.0mg/ml Fingolimod hydrochloride solution spiked with 0.15% of Heptyl impurity, N-Methyl impurity, Dimethyl impurity, Nitro hydroxy diol impurity and Nitrodiol impurity were injected.

Forced degradation studies

The stability indicating power of the developed method was studied by conducting forced degradation studies on Fingolimod hydrochloride. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed HPLC method for Fingolimod hydrochloride was

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determined in the presence of its impurities, and degradation products. Forced degradation studies were also performedon relative Humidity study stress at 75% Relative humidity for 10 days. The thermal stress was done at 60 °C for 10 days. The under photolytic stress studies conducted for 50 hours at under sunlight. The photolytic stressed studies were performed for UV Direct (200 watt hours/square meter), UV Indirect (200 watt hours/square meter), Lux direct (1.2 million LUX hours) and Lux in direct (1.2 million LUX hours). Water hydrolysis was performed for 48 hours at 60 °C. The acid stress was performed at 5.0 N HCl at the concentrated sample solution at ambient temperature for 24 hours and base stress was performed at 3.0N NaOH for 24 hours at ambient temperature and the oxidation stress was done using 0.1% hydrogen peroxide for 15min an ambient temperature. Stressed samples of Fingolimod hydrochloride generated were checked for peak purity of by using Agilent diod array detector (DAD). The peak purity is within the limit obtained in all stressed samples, demonstrates the analyte peak homogeneity. The Forced degradation studies results are given below (Table-2).

Table 2.   Summary of forced degradation results

S.No	Stressed conditions		Duration	% of Total imp	% of Heptyl imp	% of N-Methyl imp	% of Dimethy l imp	% of Nitro hydroxy diol imp	% of Nitro diol imp
1	Normal		---	0.13	0.07	0.06	0.00	0.00	0.00
2	Thermal at 60°C		10 days	0.18	0.06	0.05	0.00	0.00	0.00
4	At 75% Relative Humidity		10 days	0.18	0.07	0.05	0.00	0.00	0.00
5	Under Sunlight		50 hours	0.23	0.07	0.04	0.00	0.00	0.00
6	*Photo Degradation*	UV direct	200watt hours/square meter	0.14	0.07	0.05	0.00	0.00	0.00
		UV indirect		0.13	0.08	0.06	0.00	0.00	0.00
		LUX direct	1.2 million LUX hours	0.14	0.06	0.05	0.00	0.00	0.00
		LUX indirect		0.12	0.07	0.06	0.00	0.00	0.00
7	Acid hydrolysis (5.0N HCl at RT)		After 24hrs	1.37	0.07	0.06	0.00	0.00	0.00
8	Base Hydrolysis (3.0N NaOH at RT)		After 24 hr	0.38	0.07	0.06	0.00	0.00	0.00
9	Oxidation (0.1% H_2O_2 at RT)		After 15min	15.67	0.05	0.00	0.02	0.14	0.04
10	Water Hydrolysis (at 60°C±5°C)		After 48 hrs	0.61	0.07	0.05	0.00	0.02	0.00

Results of forced degradation studies
Significant degradation was observed in Fingolimod hydrochloride stressed sample that were subjected to very sensitive in peroxide degradation, Sensitive in Acid degradation, Slightly sensitive in Water at 60°C±5°C and Base degradation. And stable in thermal at 60°C and Photo degradation(U.V direct and indirect, Lux direct and indirect). Peak purity test results derived from Diode array detector, confirmed by that Fingolimod hydrochloride peak is homogeneous and pure in all the analyzed stress samples. The Acid, base, Peroxide Degradation chromatograms are given below:
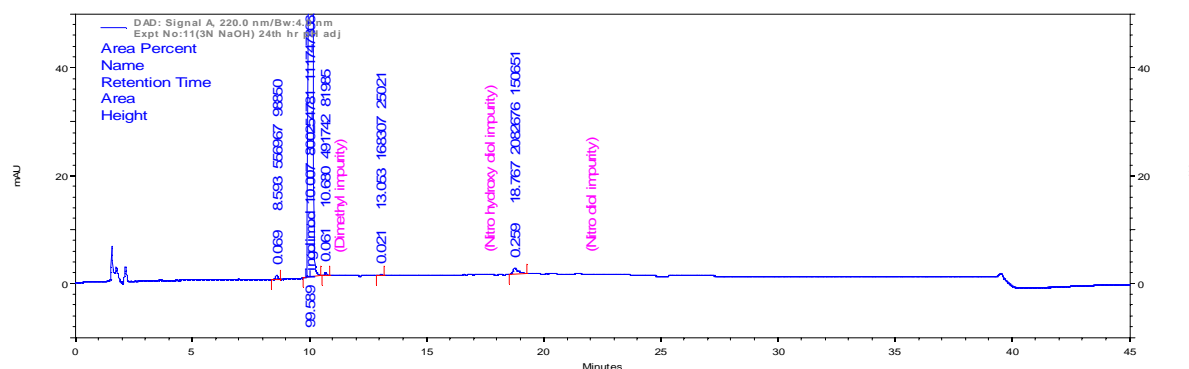

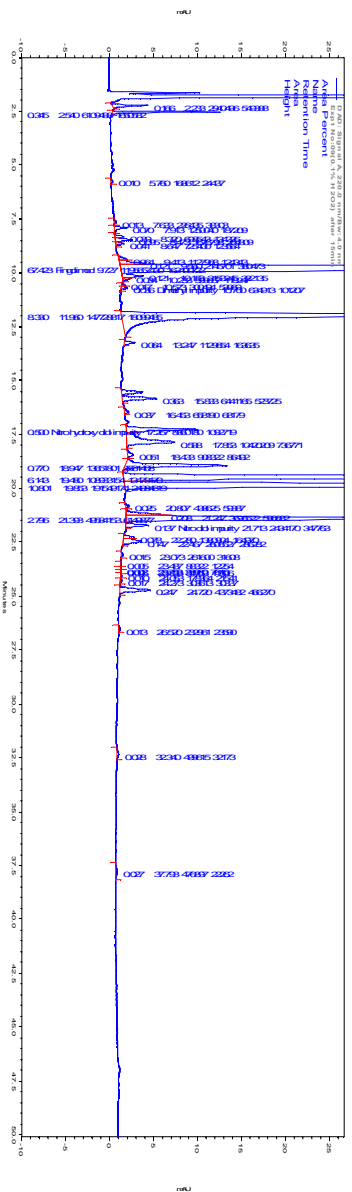**Fig 11: Typical chromatogram of 3.0N NaOH degradation solution**

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Fig12: Typical chromatogram of 0.1% H2O2 degradation solution
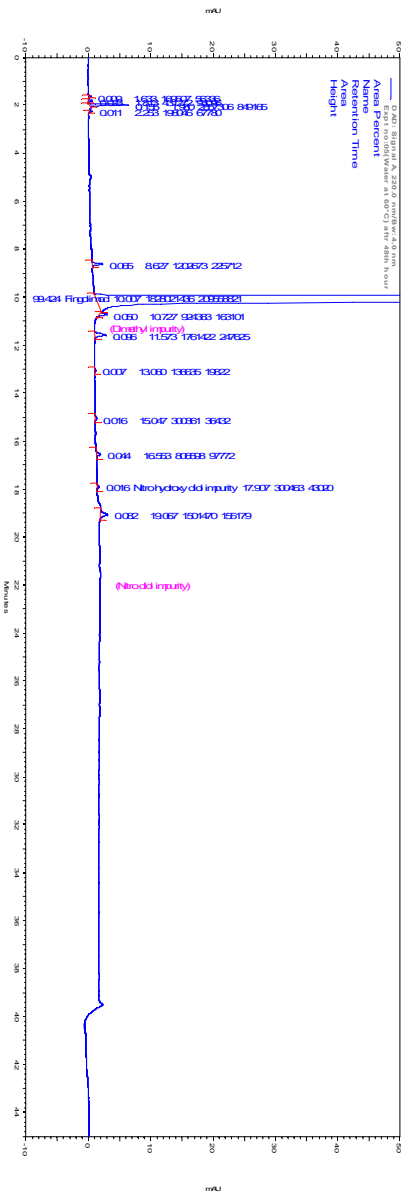

Fig 13: Typical chromatogram of degradation solution of Water at 60°.
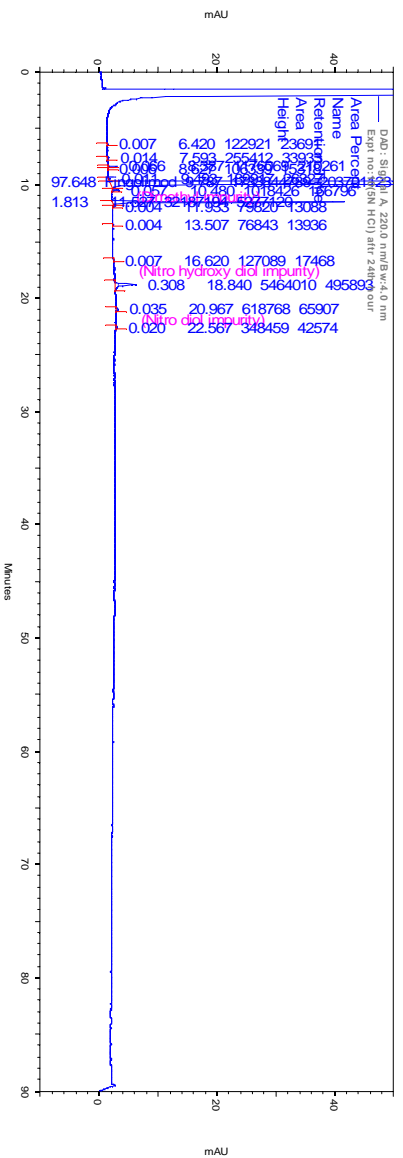

Fig 14: Typical chromatogram of 5.0N HCl degradation solution

Solution stability and mobile phase stability

Solution stability and mobile phase stability provides an indication of its reliability during normal usage during the storage of the solutions used in the method. The solution stability of Fingolimod hydrochloride was established for 48 hours at ambient temperature. The solution stability studied by using Fingolimod hydrochloride sample and injected for every 12 hours. The content of impurities and Fingolimod hydrochloride were quantified at each interval up to the study period. The mobile phase stability was also established by quantifying the freshly prepared sample solutions against freshly prepared reference standard solutions for every 12 hours up to 48hrs. During the study period the prepared mobile phase was stable up to 48hrs at room temperature.

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CONCLUSION

The developed stability-indicating analytical method for related substance determination of Fingolimod hydrochloride and its impurities is precise, accurate, linear and specific. The validation carried out for the method in accordance with the ICH requirements are satisfactory. The developed method can be used conveniently for the routine analysis of production samples and also to check the stability of bulk samples of Fingolimod hydrochloride during its storage. The same method can also be attempted for the drug products for the getting the information of impurities and degradation products at lower level.

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