A STUDY OF METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFICATION OF IGURATIMOD IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC METHOD

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ABSTRACT- A rapid and stability-indicating reversed phase high-performance liquid chromatography method was developed for quantification of Iguratimod in the dosage form to get some more advantages over other methods already developed. The method was validated according to United States Pharmacopeia guideline with respect to accuracy, precision, specificity, linearity, solution stability, robustness and system suitability. For this, an isocratic condition of mobile phase comprising buffer with pH 2.5 and methanol in a ratio of 58:42, v/v at a flow rate of 1.2 mL/minute over Water symmetry C18, 150 × 3.9 mm, 5µm column at 25°C temperature was maintained. The method showed excellent linear response with correlation coefficient (R^2) values of 0.999 for Iguratimod, which was within the limit of correlation coefficient ($R^2 \geq 0.995$). The percent recovery was found within the acceptance limit of 97.0% to 103.0%. Intra-and inter-day precision studies of the new method were less than the maximum allowable limit percentage of relative standard deviation (%RSD≤2.0.

Keywords: RP-HPLC, Validation.

INTRODUCTION:

Chemically, Iguratimod 3-Formylamino-7-methylsulfonylamino-6-phenoxy-4H-1-benzopyran-4-one is used as an anti-inflammatory drug for the treatment of rheumatoid arthritis. It has following structure,

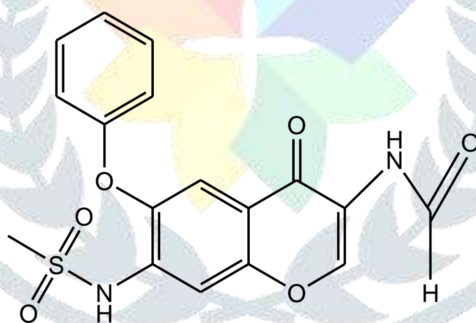


Figure 1: Structures of Iguratimod

IUPAC name for Iguratimod is N-[(formylamino)-4-oxo-6-phenoxy-4Hchromen- 7-yl] methane sulfoanamide. Iguratimod was first reported in product patent US4954518. [1] Its Therapeutic category is Anti-arthritic and novel immunomodulator.[2] Iguratimod is a nuclear factor NF-κB activation inhibitor used in the treatment of rheumatoid arthritis. It also suppressed inflammatory cytokine production in cultured human synovial cells induced by tumor necrosis factor (TNF)-α by inhibiting the activity of nuclear factor-κB. Several synthesis processes are reported for Iguratimod. [3-6]. Efficacy of a drug substance is critical for its safety assessment. It is mandatory to identify and characterize the impurities in the drug substance. This compound is aromatic heterocyclic compound; belong to class of organic compound known as chromones. These are compounds containing a benzopyran-4-one moiety.

Most of the drugs are analyzed by chromatographic method because of the several advantages like rapidity, specificity, accuracy, precision, and ease of automation in these methods. Chromatographic method eliminates tedious extraction and isolation procedures.

Chromatographic separation techniques are multistage separation methods in which the components of a sample are distributed between two phases, of which one is stationary and other mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, distributed as a film, or applied by other techniques. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), or ion exchange; or it may be based on differences among the physicochemical properties of the molecules, such as size, mass, and volume. The types of chromatography useful in qualitative and quantitative analysis employed in procedures are column, gas, paper, thin-layer (including high-performance thin-layer chromatography), and pressurized liquid chromatography (commonly called high-pressure or high-performance liquid chromatography).

Development of an analytical method for assessment of drugs in pharmaceutical dosage form is of utmost necessity to confirm the quality of tablets with respect to assay, content uniformity, and dissolution. There are no compendial methods for assessment of this drug in any Pharmacopeia. Very few methods by using high-performance liquid chromatography (HPLC) have been stated for determination of Iguratimod dosage form. But we found some drawbacks in those methods, which are listed below:

- Almost all of those methods by UV spectrophotometer.
- Results variation observes in assay (by UV spectrophotometer) against HPLC methodology.
- HPLC method is very accurate, precise, specific, robust, etc.
- Due to analytical error (by UV spectrophotometer), some methods do not seem to be satisfactory.

We put an effort to develop a cost-effective, rapid, and robust reversed phase (RP)-HPLC method with enough data of validation parameters. First, pKa of drugs was investigated and pKa of Iguratimod was 2.96. As a rule of thumb, pH of mobile phase is selected two units above or below the pKa value of drug. If we consider pKa of Iguratimod, then we cannot choose the pH above 5.0, which is detrimental to silica beds of column. With respect to Iguratimod, we could choose the pH of mobile phase between pH 2.0 to 4.0. Therefore, we thought a pH of around 2.5, which will be nearby pKa, and at this pH, Iguratimod will remain ionized, which makes the retention time much shorter at with minimum organic concentration. Thus, we tried with different buffers having a pH between 2.0 to 5.0 with different ratios of methanol in isocratic condition. Method gave sharp peak of Iguratimod without co-elution or any interference. It was found that with increased pH, retention of Iguratimod was increase. Thus, finally, a pH of 2.5 was chosen so as to get sufficient retention of the peak. Optimized chromatographic parameters are summarized in Table 1. Typical chromatogram is shown in Fig. 2, Fig. 3, Fig. 4, and Fig. 5. This study was validated according to the guidelines of International Conference on Harmonization (ICH) and USP.

Method and Requirements:
Instrument:
HPLC, make: Waters, pump, UV detector, column oven, injector, etc.
Chromatographic column: Inertsil ODS-3 (Dimension: 150 x 4.6mm, 5µ)

Chemicals and reagents:
Potassium dihydrogen orthophosphate (Analytical grade)
Acetonitrile (HPLC grade)
Orthophosphoric acid (Analytical grade)
Water (HPLC grade)

Mobile phase preparation:
Mobile phase-A: 20mM potassium dihydrogen orthophosphate with pH 3.50 ± 0.05 with 10% orthophosphoric acid, filter through 0.45µ membrane filter and degas.
Mobile phase-B: Acetonitrile

Diluent preparation:
Mix both, mobile phase-A and Mobile phase-B in equal proportion.

Standard stock solution:
Accurately weigh about 50 mg of Iguratimod standard and transfer to 50 ml volumetric flask. Dissolve in about 30ml diluent and dilute up to the mark with diluent (1000ppm solution).

Working standard solution:
The standard stock solution was used to prepare working standard solutions of concentrations 12.5, 25, 37.5, 50, 62.5 and 75 µg/ml. Solution having drug concentration of 50 µg/ml was used as a working standard for stress degradation studies. Both the standard and sample solution of 50µg/ml were estimated at 257 nm and the chromatograms were recorded (Fig.2).

Sample solution
Accurately weigh about 50 mg of Iguratimod sample to be tested, transfer to 50 ml volumetric flask. Dissolve in about 30ml diluent and dilute up to the mark with diluent (1000ppm solution).Further dilutions were made to get the final stock having concentration equivalent to 50µg/ml.

To optimize chromatographic conditions various trials were made for the selection of chromatographic conditions like mobile phase, its ratio and flow rate. Finally, the one giving the best results were optimized. The chromatographic estimation of Iguratimod and its separation from degradation products was achieved using analytical column Inertsil ODS-3 (Dimension: Length 150 mm, 4.6 mm internal diameter and particle size 5µ) with mobile phase-A (Phosphate buffer pH 3.50 ± 0.05) and Acetonitrile, flow rate of 1.0 ml/min. The UV detection was done at 257 nm.
Validation of proposed method
The method was validated for parameters like specificity, linearity, precision, accuracy and robustness as per ICH guidelines [11, 12].

System suitability testing
Five replicates of drug concentration of 50µg/ml were injected and the chromatograms were recorded to check with the system suitability parameters [9].

Forced degradation studies
The forced degradation studies were carried out as per ICH Q1A (R2) for Acid and base, oxidation and thermal stress conditions and ICH Q1 B for photo stability. The stress conditions employed were 1M Hydrochloric acid for acid hydrolysis, 0.1 M Sodium Hydroxide for base hydrolysis, 10% Hydrogen Peroxide for oxidative hydrolysis. Iguratimod samples were also subjected to thermal stress conditions as well as subjected to UV light to test photo stability [9,10].

Acid hydrolysis: Acid induced forced degradation was performed by adding an aliquot of stock solution (1mg/ml) of Iguratimod to 1 M Hydrochloric acid. This solution was subjected to stress condition of 60°C for 0.5 hours. The resulting solutions were neutralized with 1 M Sodium Hydroxide and further diluted to obtain the concentration of 50μg/ml.

Base hydrolysis: Base induced forced degradation was performed by adding an aliquot of stock solution (1mg/ml) of Iguratimod to 0.1M Sodium Hydroxide. The resulting solutions was neutralized with 0.1 M Hydrochloric acid and further diluted to obtain the concentration of 50μg/ml.

Oxidation stress: To study the effects of oxidative conditions, aliquot of stock solution (1mg/ml) of Iguratimod was added to 10% Hydrogen Peroxide solution. It was then kept at 60°C for 4 hours. The resulting solution was further diluted to obtain the concentration of 50μg/ml.

Thermal stress: The effect of temperature on Iguratimod solution was studied by subjecting aliquot of stock solution (1 mg/ml) to hot air oven at 105°C temperature for 24 hours. The final solution of 50μg/ml was prepared by adequate dilutions.

Photolytic stress: The aliquot of standard stock solution (1mg/ml) of Iguratimod was subjected to the UV exposure in UV chamber for 24 hours. The resulting solution was adequately diluted to obtain the final concentration of 50μg/ml.

Results and Discussion

Selection of solvent

Since Iguratimod was soluble in mixture of water and acetonitrile, it was used as a diluent. For dilution purpose single solvents like water or acetonitrile was not used as it does not complete solubility and results in hazy solution.

Optimization of mobile phase

Final mobile phase Buffer and acetonitrile was selected since trials done using methanol: Water caused peak merging with other impurities. Gradient ratio was optimized because altering the ratio to isocratic mode, resulted in peak merging and peak shape deterioration. The flow rate 1.0 ml/min was optimized since at lower flow rates, i.e., at 0.8 ml/min or 1.2 ml/min, peak merging was observed.

Mobile phase preparation	
Mobile phase-A :	20mM potassium dihydrogen orthophosphate, adjust pH to 3.50 ± 0.05 with 10% Orthophosphoric acid. Filter and degas
Mobile phase-B :	Acetonitrile (HPLC grade)

Gradient program :		
Time	Mobile phase-A	Mobile phase-B
0	80	20
4	68	32
22	68	32
27	20	80
32	20	80
35	80	20
45	80	20

Table 1: Mobile phase preparation and gradient program

Optimization of chromatographic conditions

The aim was to develop a method so that it can specifically identify the analyte peak in presence of its degradants. The chromatographic separation of Iguratimod from its degradants was achieved using Inertsil ODS-3 (Dimension: Length 15 cm, 4.6 mm internal diameter and particle size 5μ) with mobile phase in gradient proportion at flow rate of 1.0 ml/min and detection wavelength of 257 nm.

Optimized chromatographic parameters and conditions	
Parameters	Chromatographic conditions
Stationary phase	Inertsil ODS-3, 150mm x 4.6mm, 5μ
Flow rate (Gradient)	1.0ml/min
Injection volume	10μl
Detection wavelength	UV 257 nm
Runtime	45.0 minutes
Column oven temperature	25°C
Diluent	Mobile phase-A : Mobile phase-B (50:50)

Table 2: Optimized chromatographic parameters and conditions

System suitability testing:

The result for system suitability is shown in below Table 3. Result found within the acceptable limit. Hence the system was suitable for the proposed method.

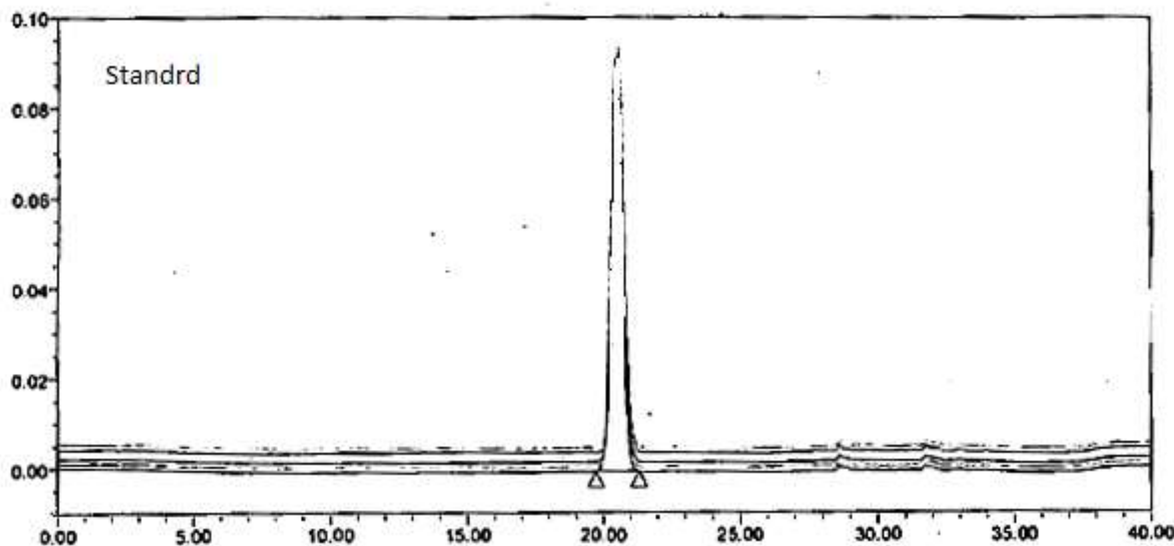


Figure 2: HPLC chromatogram obtained during simultaneous determination of system suitability. Chromatographic conditions: using Inertsil ODS-3 (Dimension: Length 150 mm, 4.6 mm internal diameter and particle size 5µ ; flow rate 1.0 mL /min; mobile phase acetonitrile and phosphate buffer (gradient proportion) and UV detection at 257 nm.

System suitability (% Relative standard deviation)			
Parameter	RSD for Observed result (n=5)	Acceptance criteria	Remark
Repeatability (%RSD)	0.19	% RSD <2.0	Method passes system suitability criteria

Table 3: System suitability data

Validation

Linearity: The linearity was observed in the concentration range of 12.5µg/ml to 75µg/ml for Iguratimod. The regression line equation was plotted between concentration and peak area. The regression equation y = 59739.8082x – 9969.3093 was obtained from the linearity data. The squared correlation coefficient was found to be 0.9995.

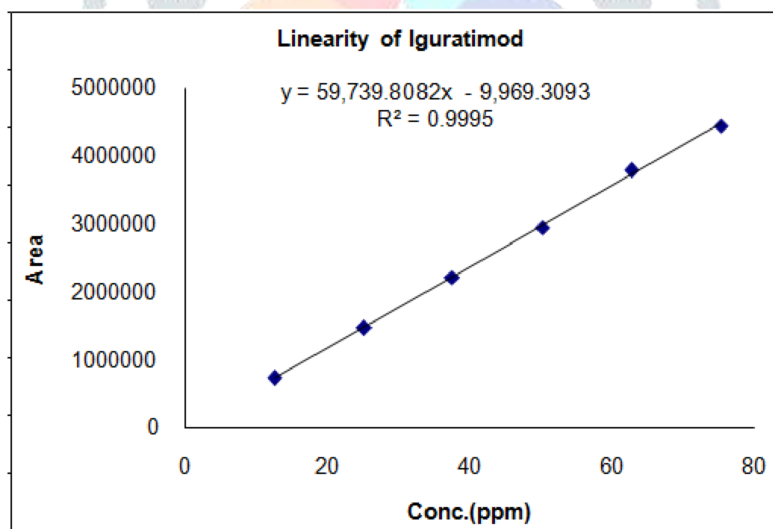


Figure 3: Linearity plot

Linearity for Iguratimod	
Conc.(ppm)	Area response
12.50	743131
25.06	1480182
37.49	2224179
50.12	2960983
62.65	3790740
75.18	4451972

Slope =	59739.8082
Intercept =	-9969.3093
Squared correlation coefficient =	0.9995
Correlation coefficient =	0.9998

Table 4: Linearity data

Specificity: The results of specificity are shown in Fig.4. It showed no interference of the blank as well as degradants present in Iguratimod.

Precision: The results of precision studies are summarized in Table 9. The % RSD was found within the acceptable limit, i.e., <2.

System Precision				
No.	Area	Average	Standard deviation	%Relative standard deviation (%RSD)
Injection -1	2949958	2959839	5538.58703	0.19
Injection -2	2962485			
Injection -3	2961867			
Injection -4	2962894			
Injection -5	2961989			

Table-5: System precision

Method Precision : Analyst-1						
Test	Area	Average area	% Assay	Average assay (%)	SD	%RSD
Sample-1	2965910	2962788	99.2	99.6	0.3685	0.08
	2959665					
Sample-2	2948426	2948677	99.4			
	2948928					
Sample-3	2964305	2970751	100.3			
	2977197					
Sample-4	2941362	2944498	99.4			
	2947634					
Sample-5	2952583	2950725	99.5			
	2948867					
Sample-6	2965660	2964063	99.7			
	2962466					

Table-6: Method precision (Analyst-1)

Method Precision : Analyst-2						
Test	Area	Average area	% Assay	Average assay (%)	SD	%RSD
Sample-1	2644169	2645542	99.7	99.7	0.1833	0.18
	2646914					
Sample-2	2632546	2633433	99.4			
	2634320					
Sample-3	2650622	2649765	99.9			
	2648907					
Sample-4	2620388	2635167	99.5			
	2649945					
Sample-5	2647953	2648656	99.8			
	2649359					
Sample-6	2651313	2651699	99.8			
	2652084					

Table-7: Method precision (Analyst-2)

Intermediate Precision					
Analyst	Test	% Assay (Average)	Overall % Assay	SD	%RSD (Cumulative)
Analyst-1	Sample-1	99.2	99.6	0.28	0.28
	Sample-2	99.4			
	Sample-3	100.3			
	Sample-4	99.4			

	Sample-5	99.5			
	Sample-6	99.7			
Analyst-2	Sample-1	99.7			
	Sample-2	99.4			
	Sample-3	99.9			
	Sample-4	99.5			
	Sample-5	99.8			
	Sample-6	99.8			

Table-8: Intermediate precision (Cumulative results)

Recovery studies: The % recovery was observed within the acceptable limits, i.e., 100.0%, 99.5% and 100.1% at the levels of 50%, 100% and 150% respectively. The results are summarized in Table 9.

Robustness: Robustness of the method was studied by making variations in the parameters like flow rate (± 0.2 ml/min), mobile phase composition (± 0.2) and detection wavelength (± 2 nm). The deliberate changes made in the flow rate, mobile phase composition and wavelength did not show major impact on retention time, assay value and peak area.

Forced degradation studies

Iguratimod was subjected to various stress conditions like acid / base hydrolysis, oxidation, photolytic and thermal stress conditions as per ICH guidelines. The results are tabulated in Table 10.

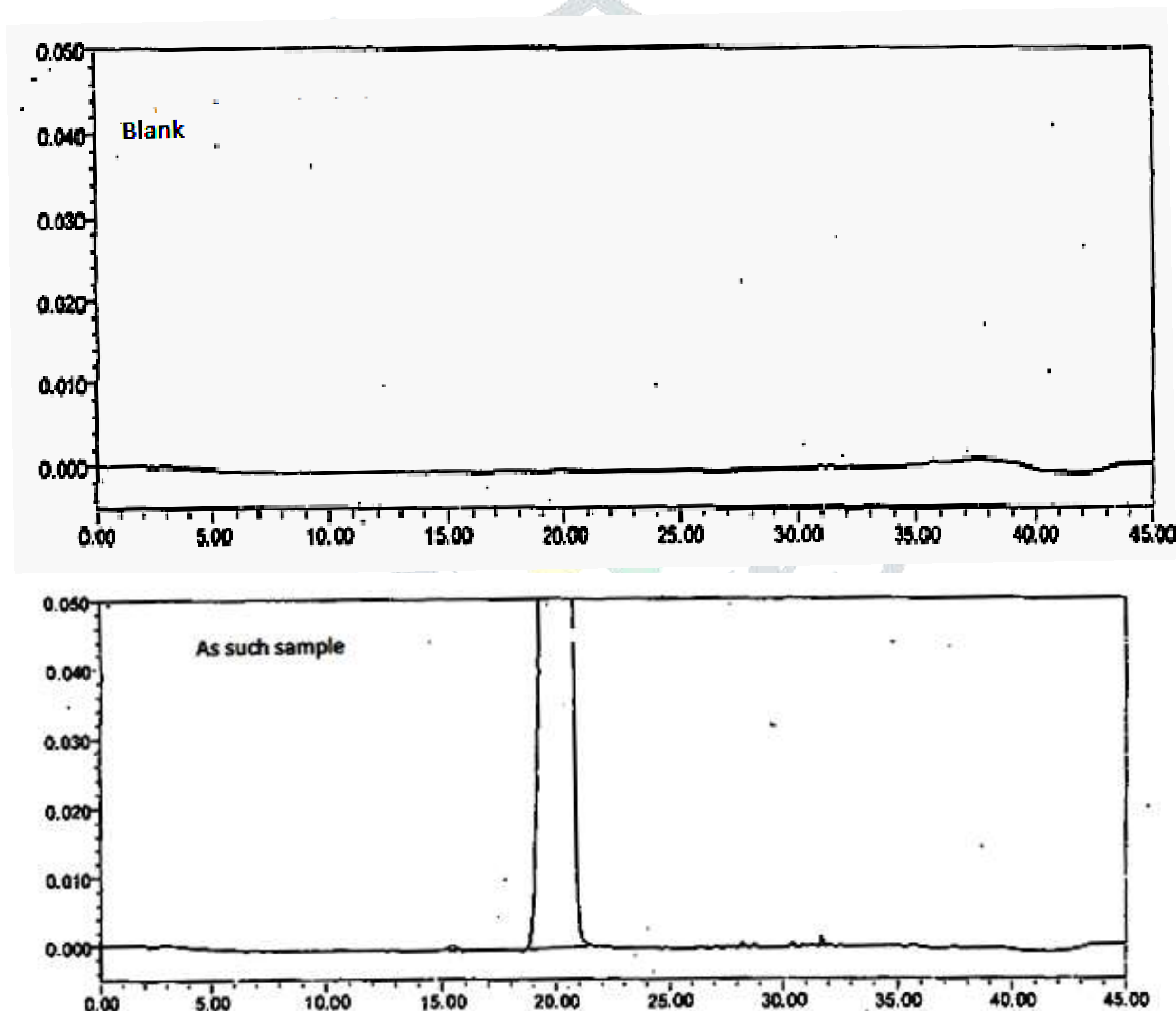


Figure 4: As such test (Without treatment) and diluent blank

Acid and base hydrolysis: Adequate degradation was achieved under acid and base degradation stress condition. Degradation in the applied stress conditions of the acid and base hydrolysis are 7.8% and 4.6% respectively for Iguratimod. The chromatograms showed the presence of degraded products (Fig. 5 and Fig.6).

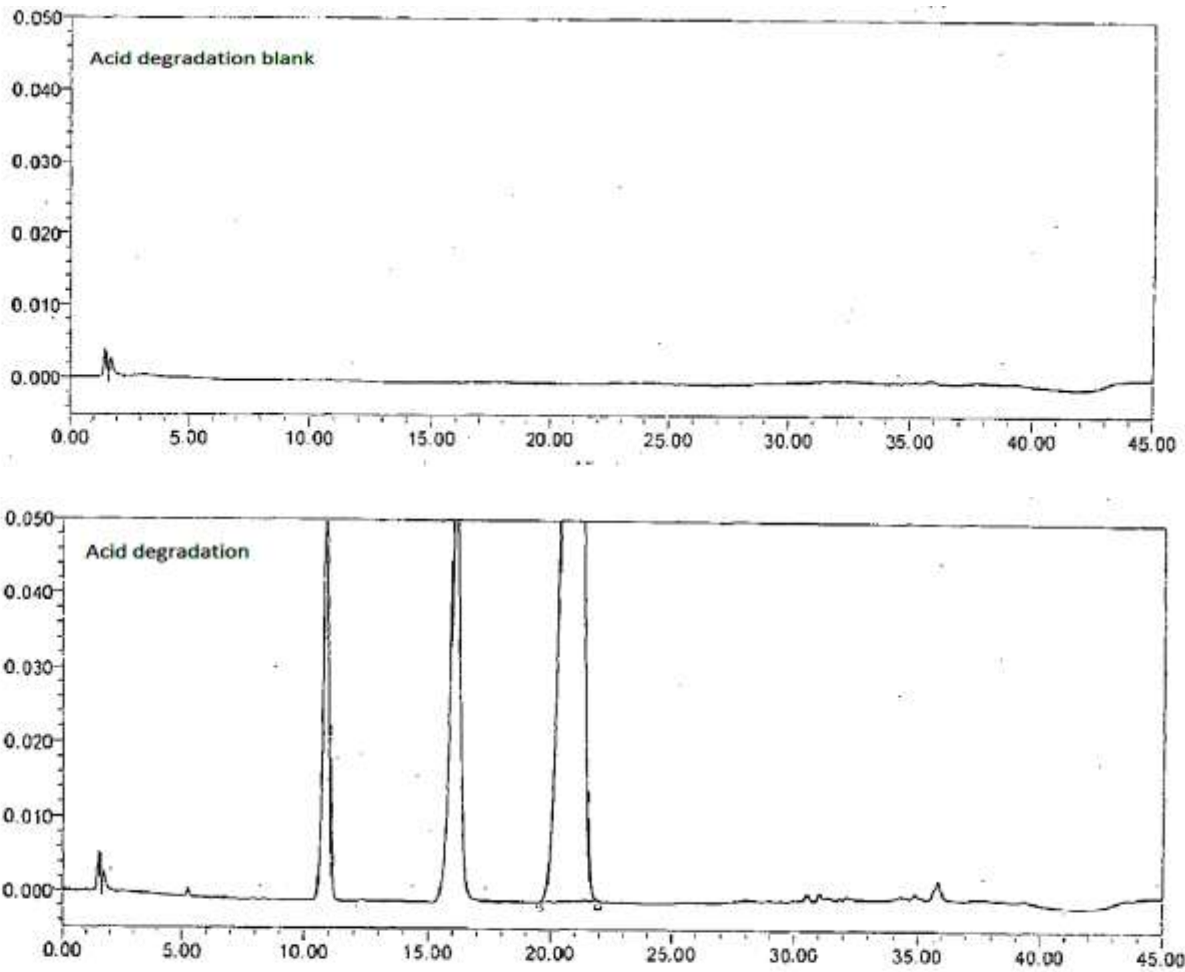


Figure 5: Acid degradation and diluent blank

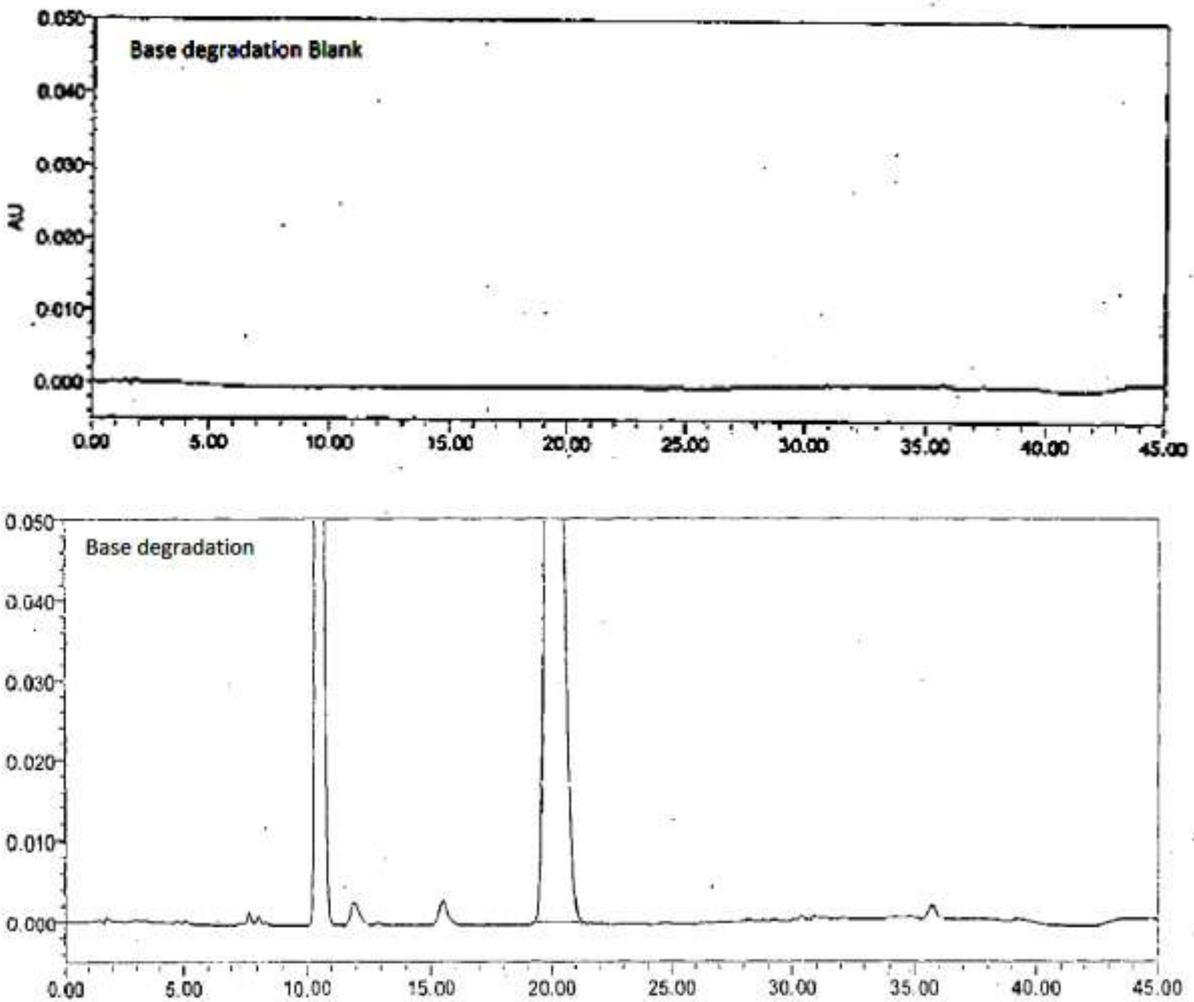
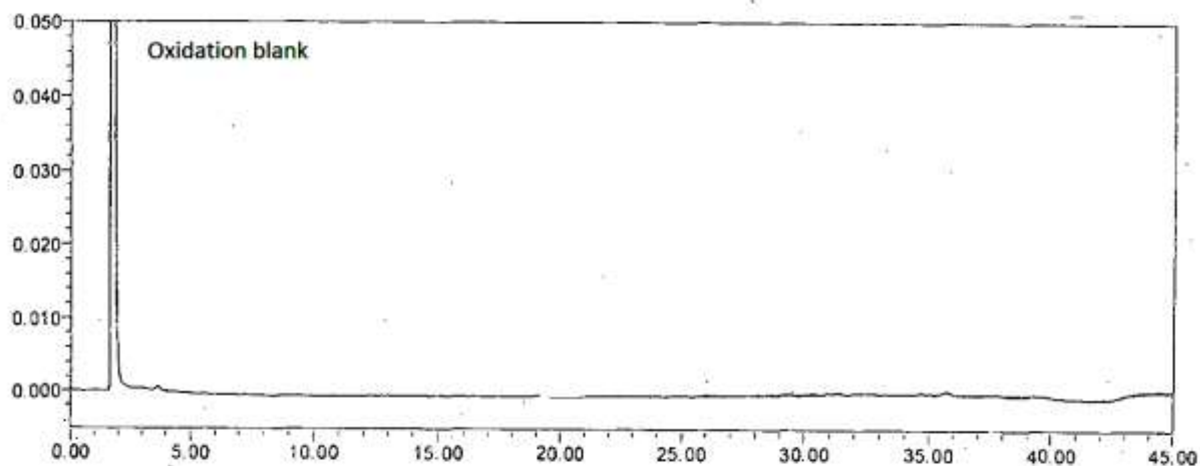


Figure 6: Base degradation and diluent blank

Oxidation degradation: Iguratimod was found to be quite stable in oxidation stress condition. It showed 1.5% for Iguratimod (Fig.7).



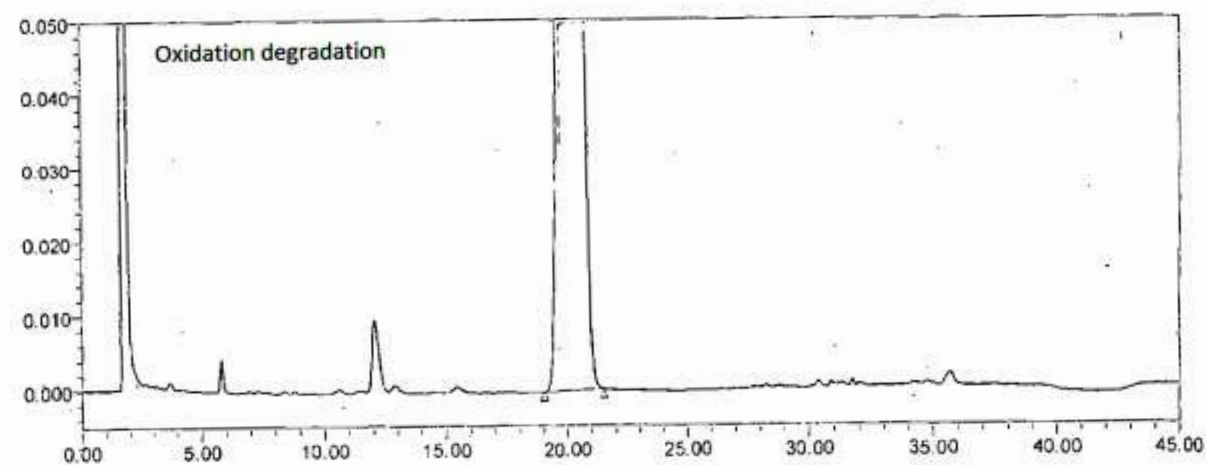


Figure 7: Oxidation degradation and diluent blank

Thermal degradation: Iguratimod was found to be quite stable in thermal stress condition, i.e. 0.4% for Iguratimod (Fig. 8).

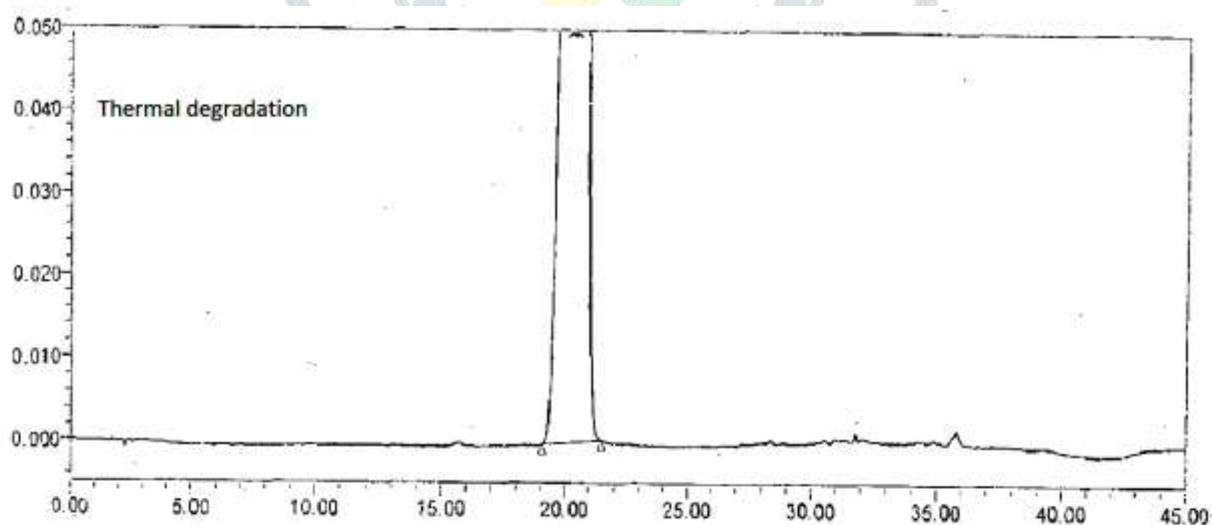


Figure 8: Thermal degradation

Photolytic degradation: Iguratimod was found to be quite stable in photolytic stress condition, and degraded sufficiently up to 1.3% with applied stress condition (Fig. 9).

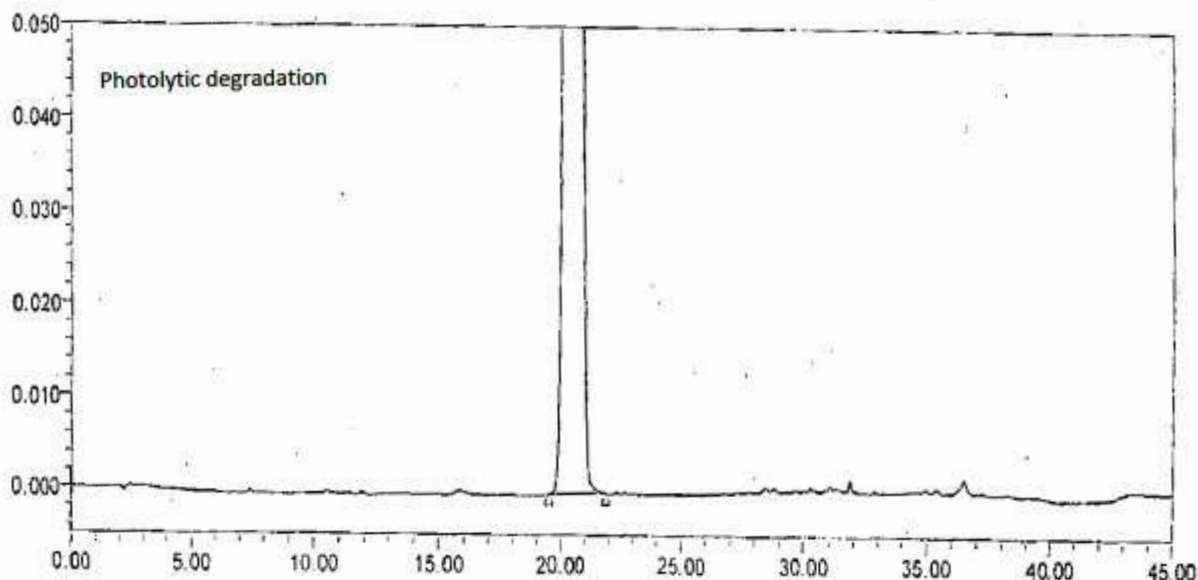


Figure 9: Photolytic degradation

Validation summary		
Parameters	Results	
Range (50% to 150%)	12.50 ppm to 75.0ppm	
Regression line equation	59739.8082 x – 9969.3093	
Slope	59739.8082	
Intercept	9969.3093	
Squared correlation coefficient	0.9995	
Correlation coefficient	0.9998	
%Accuracy	%Recovery	%RSD
50	100.0	0.14
100	99.5	0.08
150	100.1	0.09
Precision	Concentration	%RSD
Repeatability n=5	50 ppm	0.19
Intraday precision n=6	50 ppm	0.08
Interday precision n=6	50 ppm	0.18
Cumulative results (n = 12)	50 ppm	0.28
Robustness : No significant changes observed with deliberate changes in method parameters		

Table 9: Validation summary

Stress condition	%Degradation
Initial (As such)	NA
0.1M, 5ml Sodium Hydroxide solution, 0.0 hrs.	4.6
1M, 5ml Hydrochloric acid, heated at 60°C, 0.5 hrs	7.8
10.0% 5ml Hydrogen peroxide, heat 60°C for 4.0 hrs	1.5
24.0 hrs test solution in UV light	1.3
Test Solid heated at 105°C for 24.0hrs	0.4

Table 10: Stress condition and degradation data

Conclusions

The present studies are very much useful for prediction of stability behavior of Iguratimod as per the ICH guidelines. Iguratimod was found to be more stable under stress conditions. The method was found to be accurate and precise with good and consistent recoveries at all levels studied. This indicates there is no interference of degradants as well as other impurities for determination of drug content by this methodology.

RSD was also less than 2% showing high degree of precision of the proposed method. This method of analysis is accurate, precise, rapid and cost-effective. The proposed method can be used for routine analysis for Iguratimod as drug substances and can be a very good too for quality control in bulk manufacturing.

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