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Original Research Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE IGURATIMOD

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Abstract: Analytical method with *accuracy*, precision and stability indicting was developed for Iguratimod, achieved the required chromatographic separation by High pressure liquid chromatography (HPLC) technique.

HPLC method with accuracy, precision, robustness and stability indicating was developed and optimized for quantitative evaluation of Iguratimod as active pharmaceutical ingredient. Chromatographic separation for Iguratimod and its related compounds was achieved by using analytical column with stationary phase Inertsil ODS-3 (Dimension: length 150mm, internal diameter 4.6mm and particle size 5µ) and mobile phase (Buffer and acetonitrile in gradient proportion) at flow rate 1.0ml/minute with detection at 257nm. Squared correlation coefficient (r^2) was observed more than 0.999 with concentration window of 12.5 to 75µg/ml. Iguratimod was retained at about 19.0±2.0 minutes. Forced degradation study for Iguratimod was carried out under the stress condition to get required degradation by using acid, base, thermal, photolytic and oxidation by hydrogen peroxide.

Degradation products which are generated during stress condition did not interfere to active ingredient, Iguratimod. This analytical method optimized, validated for all the parameters specified in ICH guideline, to get results selective, accurate and precise in presence of degradation impurities.

Keywords: Iguratimod, validation, development, stability indicating methodology.

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

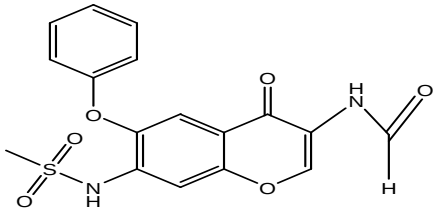

Figure 1: Structures of Iguratimod

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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 compulsory to identify and characterize the possible impurities in active drug. This compound is aromatic heterocyclic compound; belong to class of organic compound known as chromones. These are compounds containing a benzopyran-4-one moiety.

Separation of active drug and possible impurities was determined by high performance liquid chromatography instrumental technique. Since literature did not cite such methodology for determination of Iguratimod as well as its degradants, it was planned to develop a user friendly, selective, accurate and precise HPLC based methodology for determination of Iguratimod in presence of possible impurities.

Chromatographic technique is used in most of the cases for analysis of active drugs as this technique is specific, accurate, precise, and user friendly. Chromatographic technique gets rid of tedious extraction and isolation procedures.

Chromatographic separation is multistage separation technique, in which the sample components are distributed in two phase's i.e. stationary phase and mobile phase. Stationary phase is either solid or a liquid supported on a solid or a gel. Stationary phase packed in column, spread as layer and distributed as a thin film, or applied by appropriate technique. Mobile phase may be either gas or liquid / supercritical fluid. Separation is based on adsorption, mass distribution (partition), or ion exchange; or due to differences in physicochemical properties of the molecules, like molecule size, mass of molecule, and volume of molecules. This type of chromatography is used for qualitative and quantitative analysis by using different techniques.

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 this mobile phase by using 0.45µ membrane filter and Sonicate to degas.

Mobile phase-B: Acetonitrile

Diluent preparation: In equal proportion, mix Mobile phase-A and Mobile phase-B.

Standard stock solution: Weigh accurately about 50 mg of standard of Iguratimod and transfer into 50 ml volumetric flask. Dissolve in about 30ml diluent and makeup the volume 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 ppm. Solution having drug concentration of 50 ppm was used as a working standard for stress degradation studies. Standard and sample solution with concentration of 50ppm, were analyzed at 257nm and recorded the chromatograms (Fig. 2).

Sample solution: Weigh accurately about 50 mg of sample of Iguratimod to be tested and transfer into 50 ml volumetric flask. Dissolve in about 30ml diluent and makeup the volume up to the mark with diluent (1000ppm solution).

Further dilutions were made to get the final stock having concentration equivalent to 50ppm.

Various trials were made for optimization of chromatographic conditions to finalize the chromatographic parameters and 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: Validated the proposed methodology as per ICH guidelines, for the parameters like specificity, linearity, precision, accuracy and robustness etc.[11, 12].

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

Forced degradation studies: Forced degradation study was performed for Iguratimod as per ICH Q1A (R2) for Acid and base, oxidation and thermal stress conditions, photostability study as per ICH Q1B. 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 subjected for thermal degradation and photolytic degradation [9,10].

Acid hydrolysis: degradation by acid was performed by adding 1 M Hydrochloric acid in stock solution (1000ppm) of Iguratimod. 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 (1000ppm) 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 (1000ppm) of Iguratimod was added to 10% Hydrogen Peroxide solution with stressed condition as heating at 60°C for 4 hours, further diluted this solution to get 50ppm solution.

Thermal stress: Test solid was heated to 105°C for 24 hours to study effect of temperature. Solution was prepared to get final concentration as 50ppm.

Photolytic stress: Standard stock solution (1000ppm) of Iguratimod was subjected to UV exposure in UV chamber for about 24 hours, further diluted to obtain final concentration of 50ppm.

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.

Table 1: Mobile phase preparation and gradient program

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

Optimization of chromatographic conditions

Purpose of analytical method optimization is specifically to identify the analyte peak in presence possible impurities. 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.

Table 2: 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)

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.

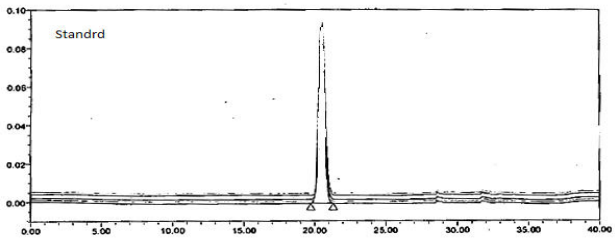


Figure 2: HPLC chromatogram obtained during simultaneous determination of system suitability.

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.

Table 3: System suitability data

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

Validation

Linearity: The linearity was observed in concentration range from 12.5ppm to 75ppm 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.

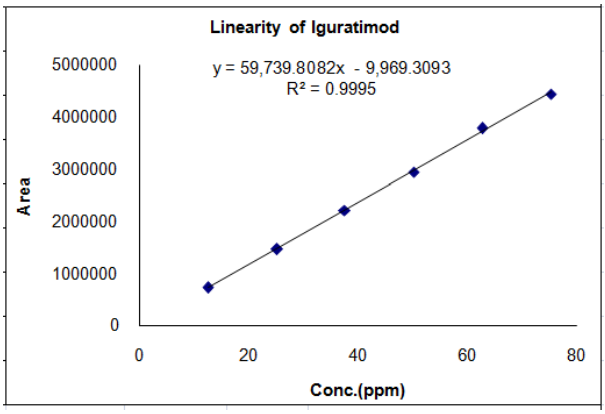


Figure 3: Linearity plot

Table 4: Linearity data

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

Specificity: The results of specificity are shown in Figure 4. No interference was observed due to blank and degradants present in Iguratimod.

Precision: Analytical results were summarized in Table 9. The % RSD was found within the acceptable limit, i.e., <2.

Table-5: System precision

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

Table-6: Method precision (Analyst-1)

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-7: Method precision (Analyst-2)

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-8: Intermediate precision (Cumulative results)

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			

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 deliberate changes in chromatographic condition and parameters like flow rate, column oven temperature and pH of mobile phase. No significant impact observed on results due to change in flow rate, column oven temperature and pH of mobile phase.

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.

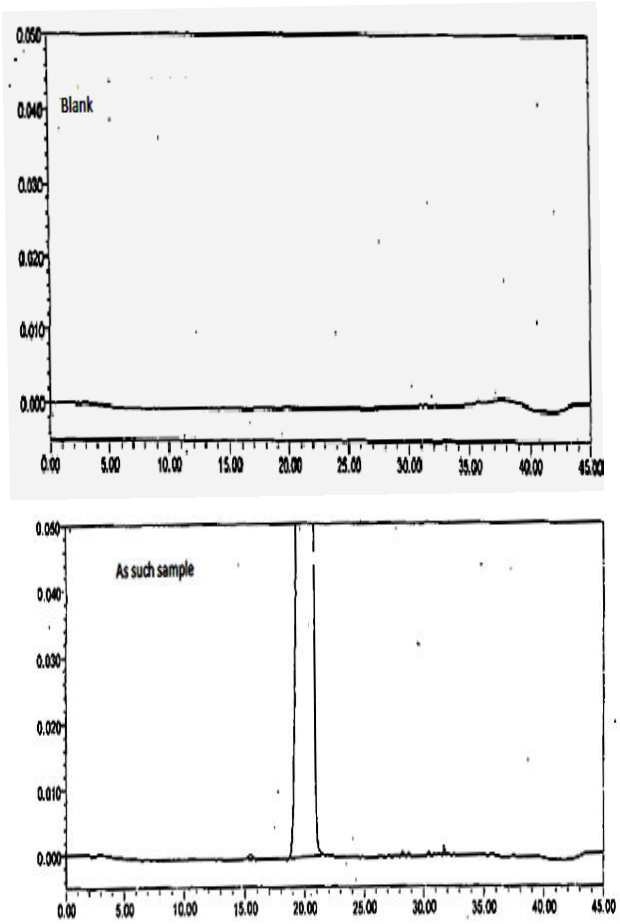


Figure 4: As such test (Without treatment) and diluent blank

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 (Figures 5 and 6).

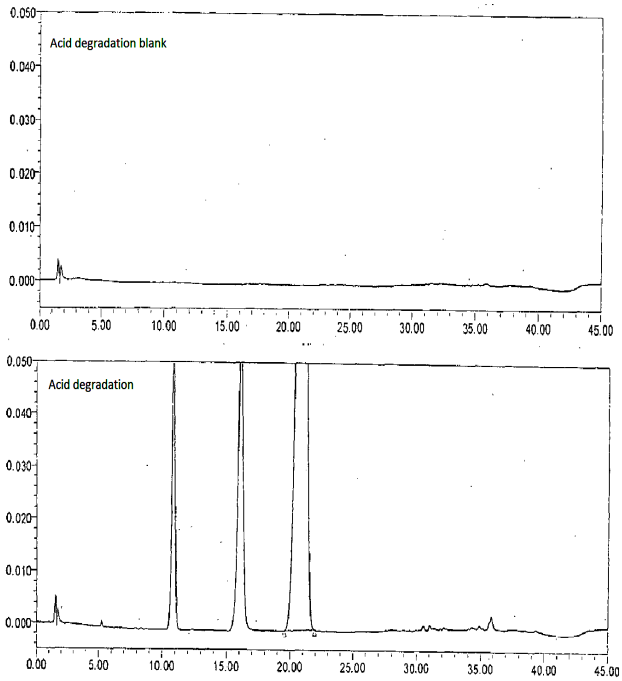


Figure 5: Acid degradation and diluent blank

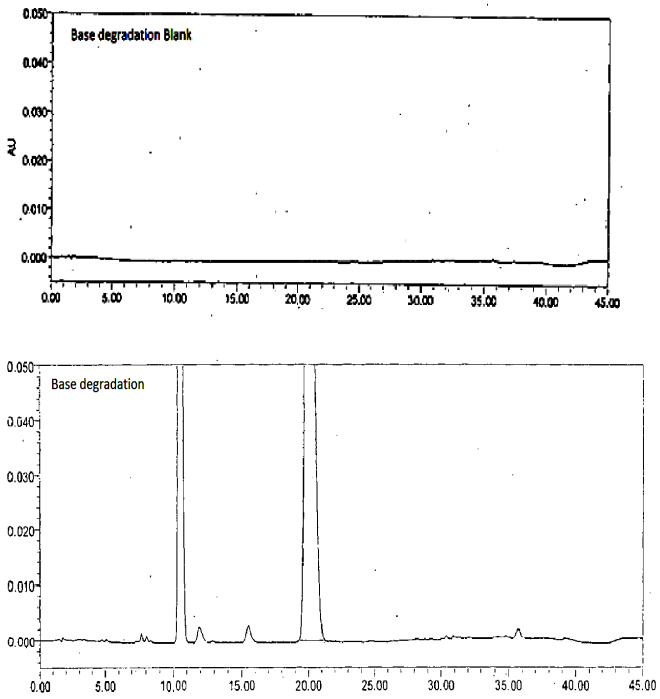
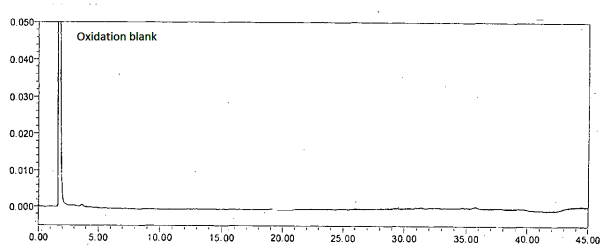


Figure 6: Base degradation and diluent blank

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



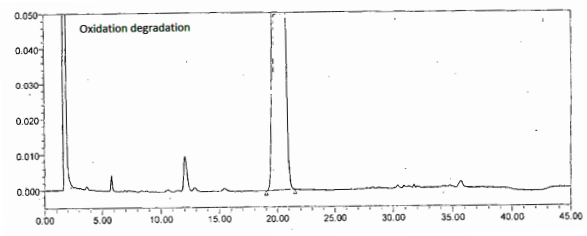


Figure 7: Oxidation degradation and diluent blank

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

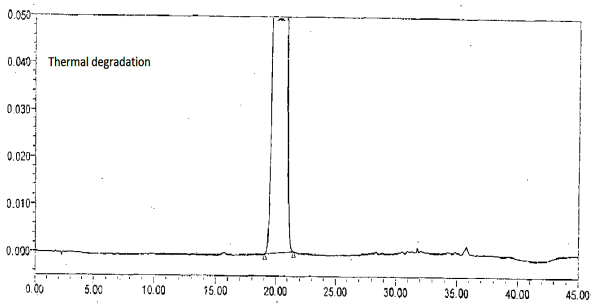


Figure 8: Thermal degradation

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

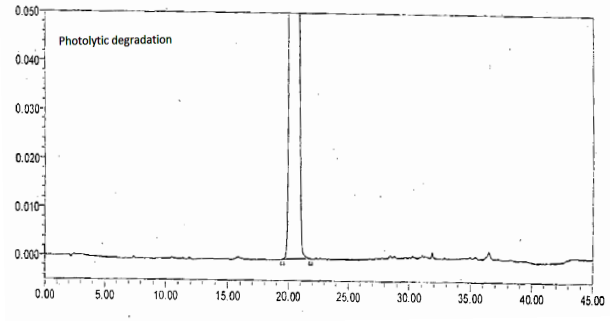


Figure 9: Photolytic degradation

Table 9: Validation summary

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 10: Stress condition and degradation data

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

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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