Hplc Method Development and Validation of Iguratimod Tablet Dosage Form

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Abstract

Objective: A simple, rapid, linear, accurate, precise & robust method was developed & validation method was developed for Iguratimod tablet dosage form as per ICH (International Council for Harmonization) guidelines Q2R (1). **Methodology:** Chromatographic evaluation was carried out on C18 column with wavelength of 257nm using mobile phase of Acetonitrile: Methanol (50:50 v/v). Flow rate was maintained to 1ml/min. **Results:** The retention time was obtained to be 2.7 minutes through run time of 6 minutes. The validated method was applied by several validation parameters like Specificity, Linearity (calibration curve), Precision, Accuracy, Robustness, LOD & LOQ. The correlation coefficient of Iguratimod tablet was found to be 0.9998 when the concentration in the range of 10-50 µg/ml were used indicated good linearity. The average percentage of recovery was 100-102%. This indicates that the developed method is accurate. The %RSD for precision study obtained as 0.494377 (Intraday) & 0.535898 (Interday) that are within acceptance limit (NMT 2%) indicates that the developed method is precise. The LOS and LOQ was obtained as 0.118µg/ml and 0.358µg/ml respectively. **Conclusions:** The validated method can be used for estimation of Iguratimod in Tablet dosage form for routine analysis work.

Keywords: Iguratimod, HPLC, Method Development, Method Validation, ICH guidelines

1.Introduction

Iguratimod, also referred to as T-614 [N-(3-formylamino-4-oxo6-phenoxy-4H-1-benzopyran-7-yl)-methanesulfonamide, Fig. 1], is one among a series of 4H-1-benzopyran-4-ones compound which is having potent anti-inflammatory, analgesic and antipyretic activities (Tanaka *et al*., 1992). For the treatment of rheumatoid arthritis T-614 has been developed by Toyama Chemical & approved in Japan as a disease modifying anti-rheumatic drug (DMARD). The production of inflammatory cytokines significantly inhibited by this remedy in cultured human synovial cells and human monocytic leukemia cell line (Kohno

et al., 2001). It shows reduction in the production of immunoglobulin (Ig) by directly affecting B lymphocytes cells both in mice and in humans (Tanaka 2009). For the treatment of rheumatoid arthritis Iguratimod acts as a nuclear factor NF-κB activation inhibitor. The inhibition of the activities of nuclear factor-Kb also suppresses inflammatory cytokine in cultivated human synovial cells caused by tumour necrose factor (TNF)-α (Kashid *et al*., 2017). Iguratimod was orally given in the first 4 weeks at 25 mg daily and in the next 48 weeks at 50 mg daily (Hara *et al*., 2007). The Adverse Effect are Headache, cold, cough, fever, Gastritis Diarrhea, Nausea, and Dizziness (Singh *et al*., 2020). The literature survey revealed that the reported methods of Iguratimod are more time-consuming which require more solvents which ultimately enhances the cost of the tablet. Few methods were developed in plasma. The aim and objective of the current study is to make the validated method for Iguratimod tablet formulation so that the developed method becomes cost-effective and less time-consuming.

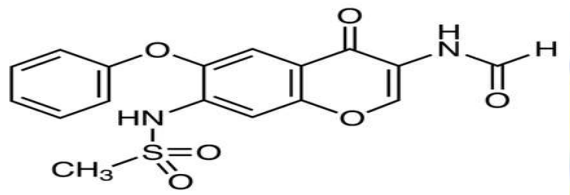

Fig 1 Structure of Iguratimod

2. Methods

Instruments and chemical used

HPLC (Shimadzu) with autosampler and UV-Visible detector with C-18 column was used to improve the method. The electronic balance (Shimadzu) was used to measure the materials. The sonicator (Wensar) was used for degassing samples. The melting point apparatus was used to find the melting point. A reference sample of Iguratimod was received as a gift sample. The REDIMARD tablets were bought from the local market containing 25 mg Iguratimod. Acetonitrile, Water, Methanol, Acetic acid used were HPLC grade.

Evaluation of Physicochemical properties:

The physicochemical properties such as solubility, melting point, etc., were checked for API authentication. The drug solubility was studied by dissolving the drug in a number of solvents, and the melting point of

Iguratimod was checked by the open capillary method.

Selection of Wavelength

The wavelength was selected from the literature survey. The spectra of Iguratimod show maximum absorbance at 257 nm.

Selection of Column

The samples were conducted on an analytical column C18 (250 *4.6 mm) based on the polarity of analytes which provide efficient and reproducible separation of components.

Flow rate

For the maximum saving of solvent, the flow rate should be minimum. Experiments showed that a flow rate of 1 ml/min was suitable for development of the approach.

Selection of Mobile phase

The selection of the mobile phase was based on peak parameters like symmetry, tailing, peak resolution, cost and run time. Different mobile phase compositions were attempted to obtain sharp, resolved chromatographic peaks and almost free from tailing and less retention time. A mixture of acetonitrile and methanol in the ratio of 50:50 v/v was best for less retention time with sharp and resolved peaks.

Mobile Phase Preparation

HPLC grade acetonitrile & HPLC grade methanol in the ratio of 50:50 was mixed in a volumetric flask and filtered through a 0.45µ filter under vacuum filtration

Preparation of standard stock solution

10 mg of Iguratimod was weighed into a volumetric flask (100 ml). The drug was dissolved in Acetonitrile solvent and sonicate for 10 minutes and made up the volume to the mark. The final strength obtained was 100µg/ml. The working standard solution was further diluted to get a 10-50µg/ml concentration range.

Preparation of sample stock solution

10 Tablets of Iguratimod (25 mg) were powdered using mortar & pestle which was rhwn weighed equivalent to 10 mg Iguratimod. 57.8436 mg (Equivalent Weight) of powdered Iguratimod drug was weighed into a 100 ml volumetric flask. The drug was dissolved in Acetonitrile solvent and sonicate for 10 minutes and made up the volume to the mark. The final strength obtained was 100µg/ml. The working standard solution was further diluted to get a different concentration range.

Method Validation

After the method conditions were optimized, the method was validated according to ICH Guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, limit of detection, and limit of quantification.

Specificity

The specificity of the method was evaluated by injecting six injections of standard solution (10 µg/ml) and blank (Tiwari *et al*., 2010)**.**

Accuracy

The accuracy of the method was studied by recovery experiments. The recovery was done at five different levels i.e., 50%,75%, 100%,125% and 150% of the label claim per tablet (25mg of Iguratimod tablet). The accuracy studies were carried out for five different concentrations (5 µg/ml, 7.5 µg/ml, 10 µg/ml, 12.5 µg/ml, 15 µg/ml) of the analyte, six injections were injected for each recovery level and % recovery was determined. Overall% recovery should be between 98 to 102 % and overall %RSD should not be more than 2.0 (Lavanya *et al*., 2013)**.**

Precision

The intraday precision of the assay method was evaluated at the concentration of 10 µg/ml prepared from sample stock solution by performing analysis at an interval of 1 hour for 6 hours.

The interday precision of the test method was also evaluated at the concentration of 10 µg/ml prepared from sample stock solution by performing analysis on different days i.e., day 1, day 2 and day 3. The mean, SD and % RSD for both interday and intraday precision were calculated. The % RSD should not be more than 2.0% (Budhavant *et al*., 2020)**.**

Linearity

The linearity was determined by preparing the standard solution at five concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml). These different concentrations were injected in six replicates. The chromatogram area was noted for each solution obtained by injecting 10 µl volume into the HPLC apparatus. From chromatogram the mean area with its standard deviation and % relative standard deviation was calculated. Mean AUC was plotted against concentration to obtain the calibration curve. Regression equations, correlation coefficients were computed from calibration curves. The calibration curve should be curve (Ravichandran *et al*., 2010)**.**

Robustness

The Robustness was evaluated by carrying a small change in flow rate (±0.1ml/min), temperature (± 2 °C), and Mobile phase composition (± 2% of Methanol). Robustness was carried out by injecting a 6 injection of 10 µg/ml. The mobile phase flow rate was altered to 0.9 ml/min and 1.1 ml/min (actual flow rate of column was 1.0 ml/min). The Mobile phase composition was changed from Acetonitrile: Methanol (50: 50) to Acetonitrile: Methanol (48: 52) and Acetonitrile: Methanol (52: 48). The column temperature was change to 38°C and 42°C (actual temperature of column was 40°C). The SD and % RSD were calculated and reported (Geetha *et al*., 2012)**.**

Limit of Detection and Limit of Quantification

The LOD and LOQ were determined by injecting triplicate injection of different concentrations and value was determined from linearity plot (Shweta *et al*., 2016)**.**

3. Results

Development of the Optimized chromatographic conditions

Several mobile phase compositions were tried to optimized the RP-HPLC parameters. The mixture of Acetonitrile: Methanol in the ratio of 50:50v/v show symmetric peak having flow rate of 1 ml/min. A wavelength of 294 nm was set for detection. The retention time for Iguratimod was found to be 2.7 minutes. The chromatogram of Iguratimod is shown in fig. 2. The Optimized chromatographic conditions are given in Table 1.

Wavelength	257nm
Column	C18
Flow rate	1 ml/min
Injection Volume	10 µl
Mobile phase	Acetonitrile: Methanol= 50:50
Run time	10 min
Column temperature	25

Table 1 Optimized Chromatographic Conditions

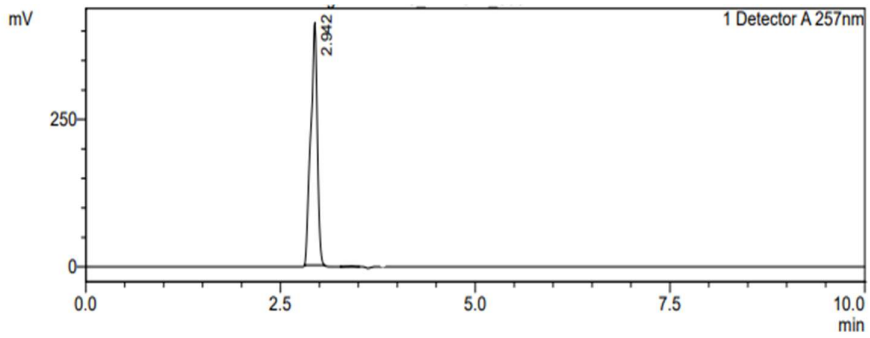

Fig. 2 Chromatogram of optimized method

Specifity

The absence of additional peaks in the chromatogram indicates non-interferences of excipients. There is no any interference of Iguratimod peak (main peak) from the other peaks. The chromatogram of blank and Iguratimod are shown in fig. 3& 4.

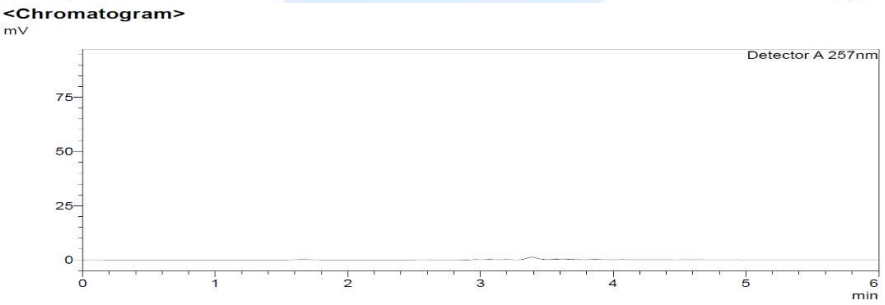

Fig. 3 Chromatogram of blank

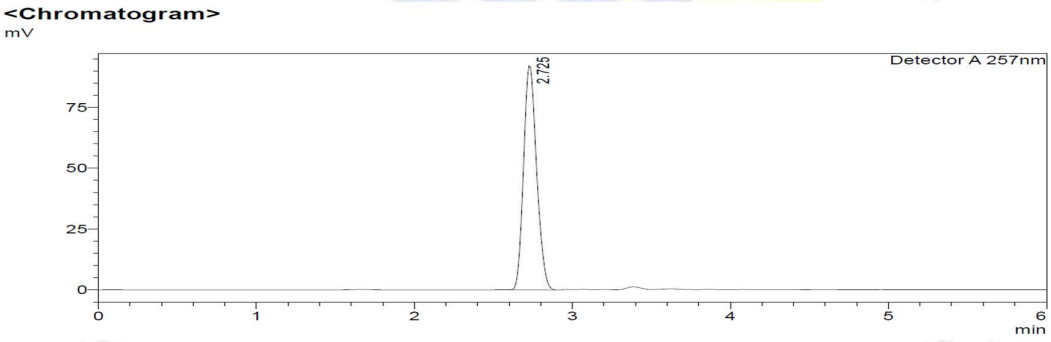

Fig. 4 Chromatogram of Iguratimod

Linearity

It was established in the concentration range of 10-50µg/ml and six injections of each concentration were injected into the RP-HPLC. The Linearity data of Iguratimod is shown in Table 2. The calibration curve plotted between peak area and concentration is shown in figure 5. The calibration curve obtained was linear from the range of 10-50µg/ml. From linearity curve the linear regression equation was calculated and found

as **y = 481817x +17289** with the correlation coefficient **(R^2)** of value **0.9998**. The chromatogram of linearity of different concentrations are shown in figure 6-10.

S.No.	Concentration (µg/ml)	Peak area
1	10	523634
2	20	948547
3	30	1459703
4	40	1949646
5	50	2432169
	Slope	481817
	Intercept	17289
	Correlation Coefficient (R^2)	R^2 = 0.9998

Table 2 Linearity data of Iguratimod tablet

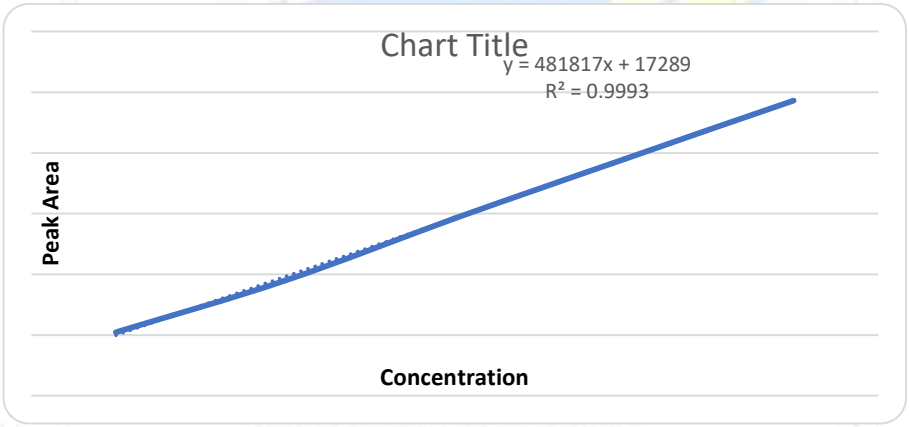

Fig. 5. linearity curve of Iguratimod

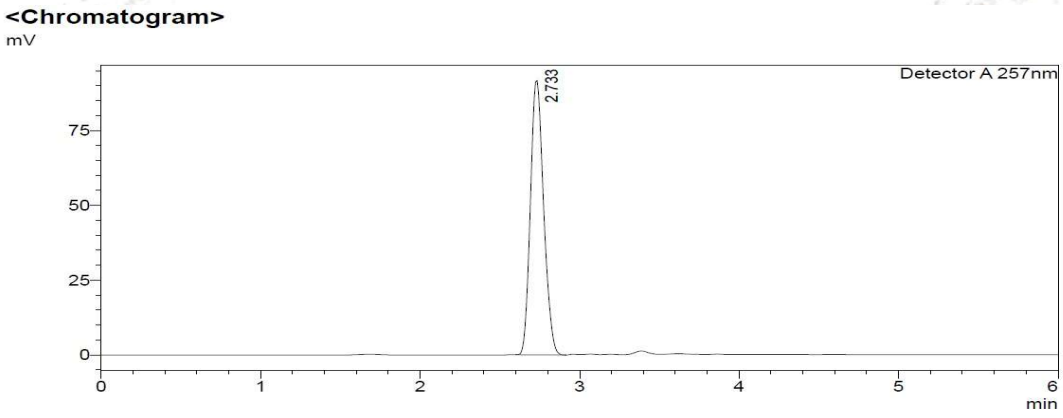

Fig. 6 Chromatogram of Iguratimod (10µg/ml)

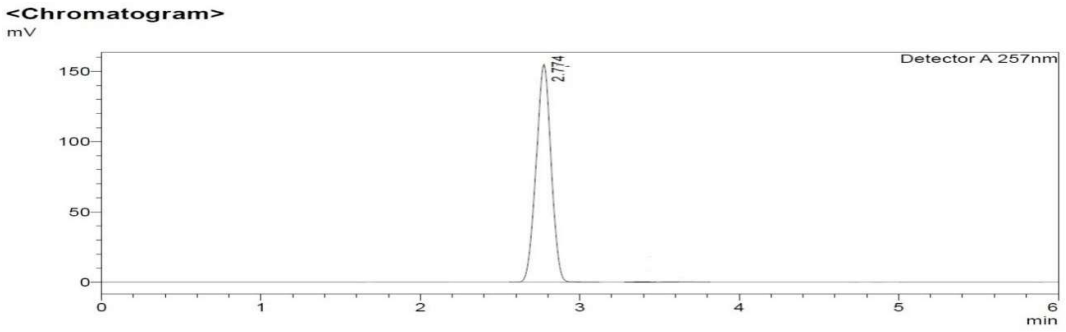

Fig. 7 Chromatogram of Iguratimod (20µg/ml)

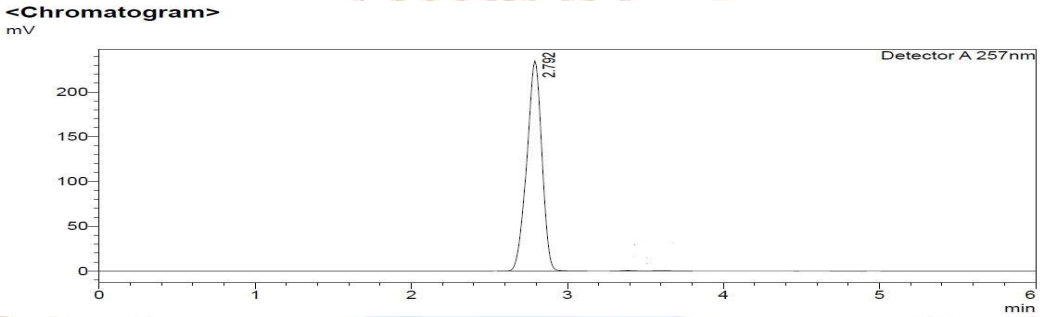

Fig. 8 Chromatogram of Iguratimod (30µg/ml)

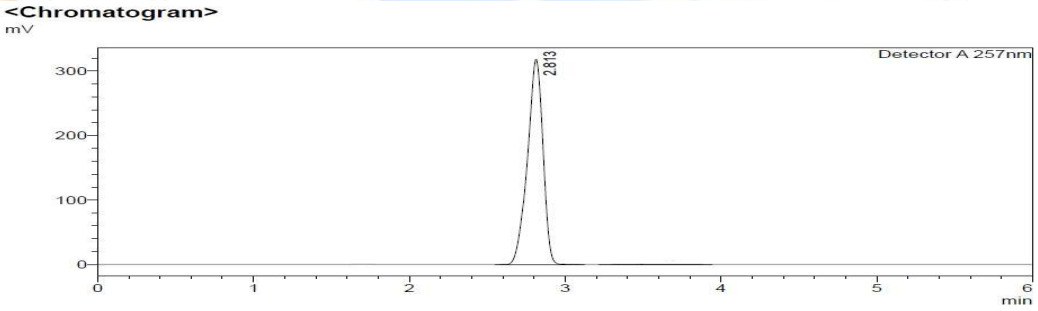

Fig. 9 Chromatogram of Iguratimod (40µg/ml)

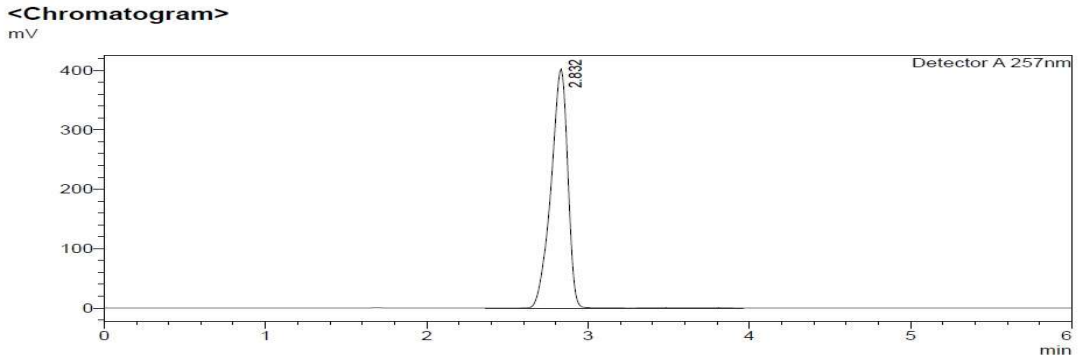

Fig. 10 Chromatogram of Iguratimod (50µg/ml)

Precision

For intraday precision, six replicate standard solution of Iguratimod (10µg/ml) were injected after an interval of 1 hour for six hours. For determining interday precision, six replicate standard solution of Iguratimod (10µg/ml) was injected for six days. The Intraday and interday precision data of Iguratimod tablet is shown

in Table 3. The Chromatograms of intraday and interday precision are shown in Fig. 11 & 12 respectively. The % RSD was calculated and it was found to be 0.494377& 0.535898 for Intraday and interday precision that indicates good precision.

S. No.	Concentration	Sample No.	Intra-day precision	Inter-day precision
1.	10 µg/ml	1	527595	524672
		2	526930	523188
		3	528649	524013
		4	523634	523239
		5	527237	528728
		6	521968	520078
	Mean		526002.2	523986.33
	SD		2600.433	2808.03
	%RSD		0.494377	0.535898

Table 3 Intra-day and Inter-day precision of Iguratimod

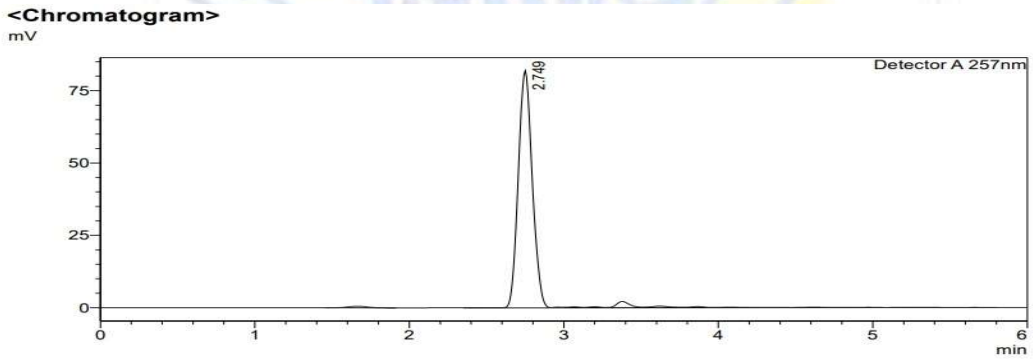

Fig. 11 intraday Chromatogram of Iguratimod (10µg/ml)

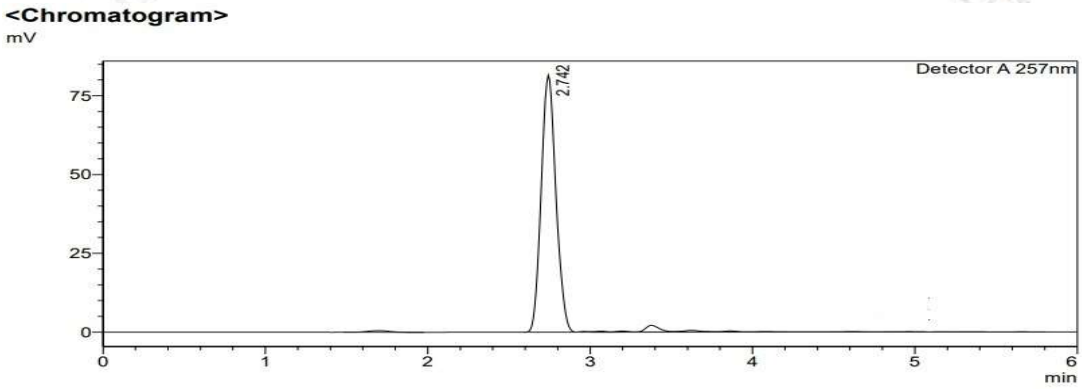

Fig. 12 interday Chromatogram of Iguratimod (10µg/ml)

Accuracy

Five different concentrations (i.e., 50%, 75%, 100%, 125% and 150% level) were used to determine the accuracy of the procedure. The solutions were evaluated by injecting six replicates at each level as per the developed method. The chromatograms of each level are shown in figure 13-17. The percent recoveries of each level were calculated between the mean concentration found and added concentration. The % RSD of each level was also calculated and results of percentage recovery and % RSD are obtainable in Table 4. Reasonable recoveries ranging from 100.1455 to 100.9318 were obtained by the proposed method. This specifies that the developed method is accurate.

S. No.	% Spike level	Area	Mean Recovery	%Mean recovery	%RSD
1.	50 %	310032 307265 310048 314013 313527 312080	50.95985	101.9197	0.481583
2.	75 %	396695 396160 396613 396326 394655 396695	75.69883	100.9318	0.332334
3.	100 %	524672 523188 523634 528793 526577 520697	100.1455	100.1455	0.536835

4.	**125 %**	660586 669190 667024 669384 665649 662857	127.0985	101.6788	0.527639
5.	**150 %**	795252 793465 796612 7966161 797685 796584	151.94	101.2998	0.182553

Table 4 Accuracy data of Iguratimod tablet

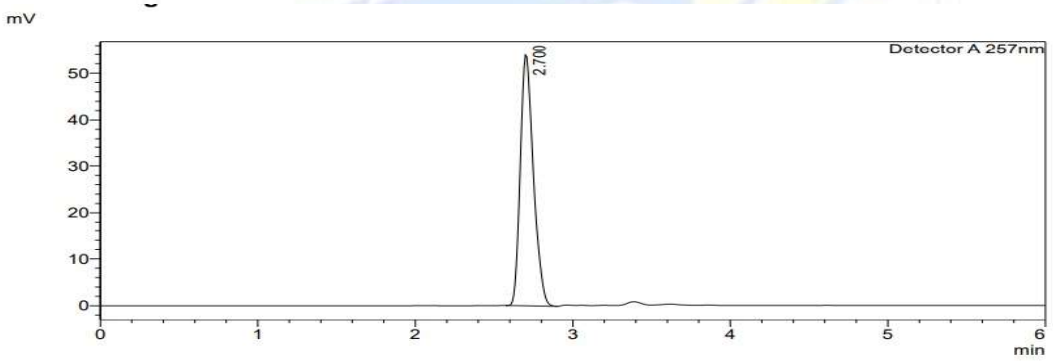

Fig. 13 Chromatogram of iguratimod (50%)

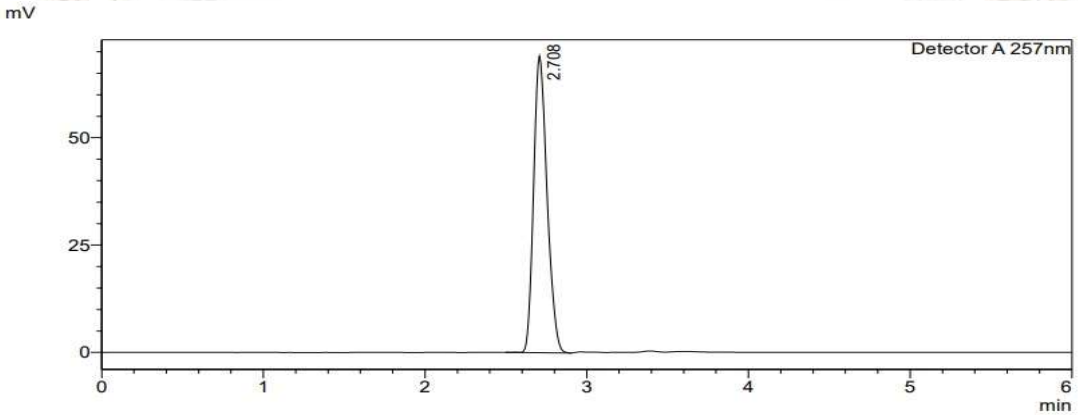

Fig. 14 Chromatogram of iguratimod (75%)

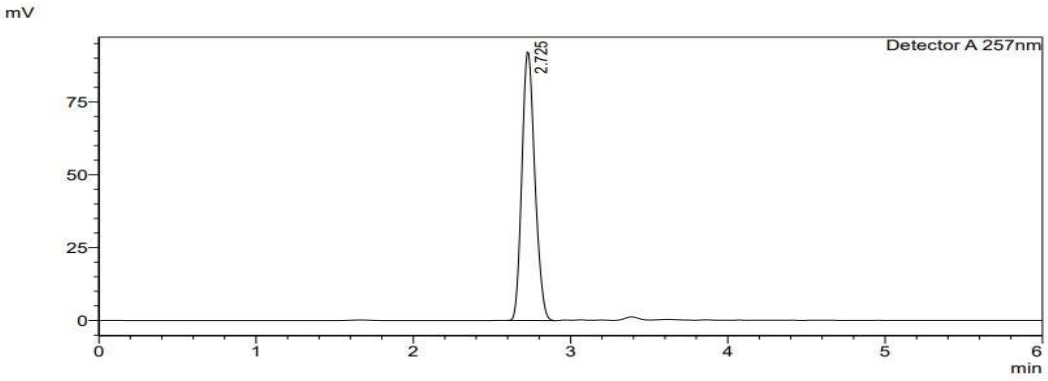

Fig. 15 Chromatogram of iguratimod (100%)

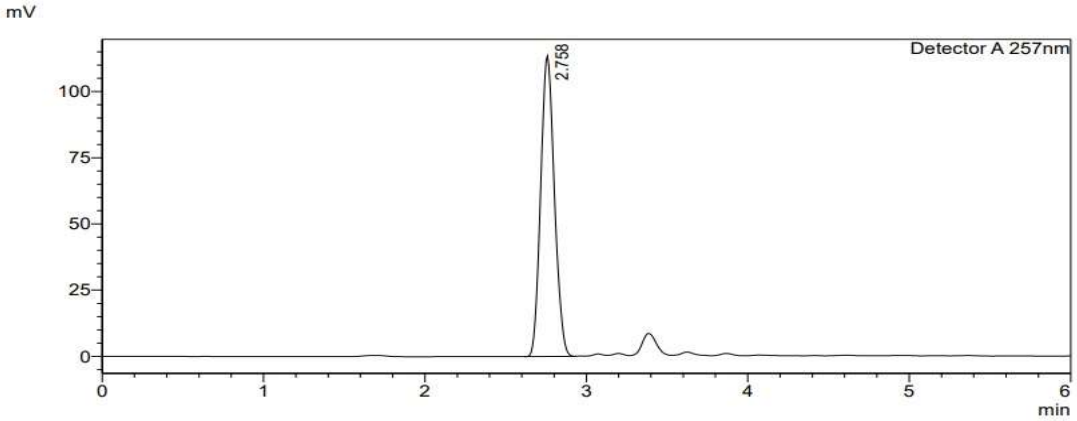

Fig. 16 Chromatogram of iguratimod (125%)

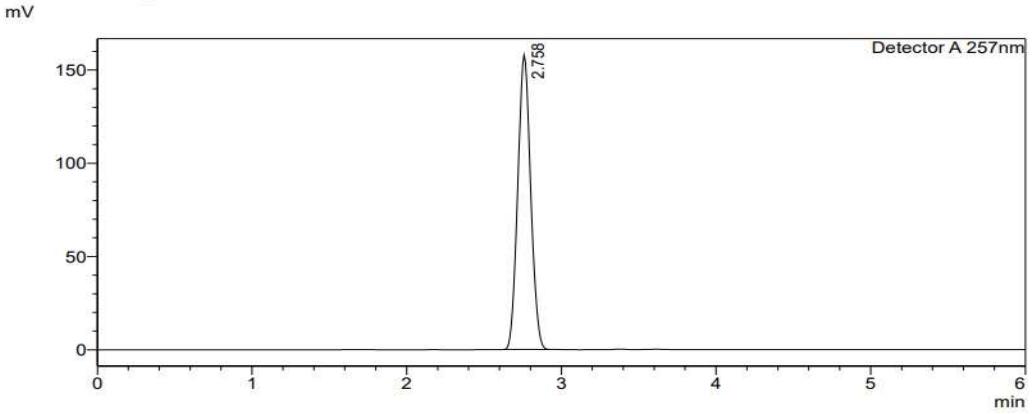

Fig. 17 Chromatogram of iguratimod (150%)

Robustness

For the robustness study slight modification in flow rate of the mobile phase, temperature of column and composition of the mobile phase were done. Six replicates of each modification were injected into column for the determination of robustness. The chromatograms of each medication are shown in Figure18-26. Table 5 shows the results of robustness. No noticeable change was observed in chromatograms demonstrated that the method was robust in nature.

Parameter	Mean Peak Area	SD	%RSD
Chromatogram of flow rate 0.9 ml/min	586141.5	2912.898	0.496962
Chromatogram of flow rate 1 ml/min	539852	4741.786	0.878949
Chromatogram of flow rate 1.1 ml/min	482538	3356.367	0.695565
Chromatogram of mobile phase change (48:52)	526913.7	2905.321	0.551385
Chromatogram of mobile phase change (50:50)	526030.3	2041.429	0.388082
Chromatogram of mobile phase change (52:48)	526377.2	3691.427	0.701289
Chromatogram of temperature change (38° C)	533877.2	3112.773	0.58305
Chromatogram of temperature change (40° C)	526569	2082.396	0.395465
Chromatogram of temperature change (42° C)	526829.5	1918.642	0.364187

Table 5 Robustness data of Iguratimod tablet

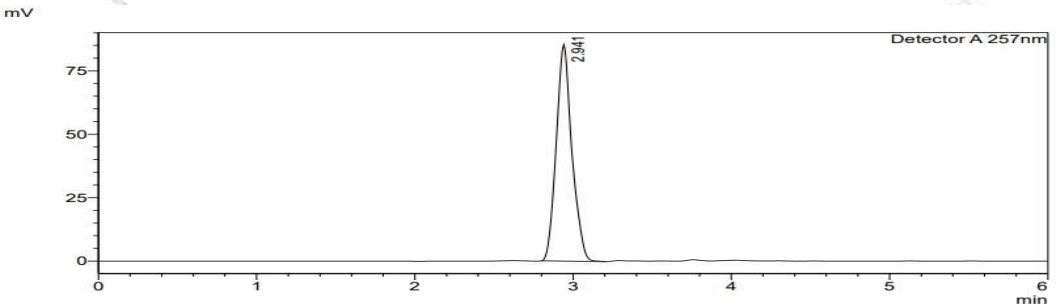

Fig. 18 Chromatogram of iguratimod flow rate 0.9 ml/min

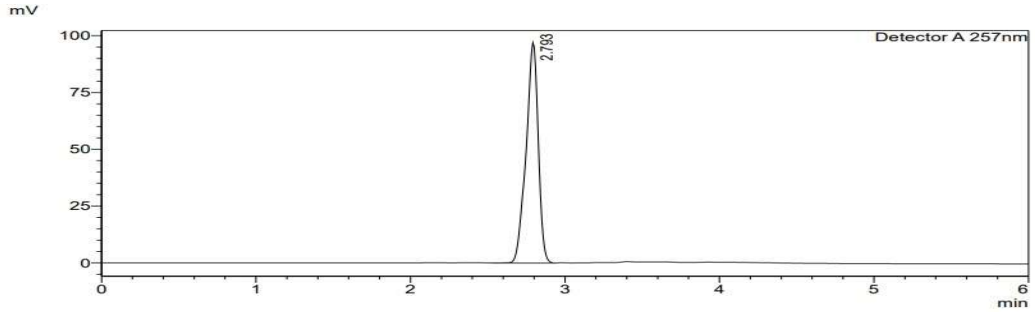

Fig. 19 Chromatogram of Iguratimod flow rate 1 ml/min

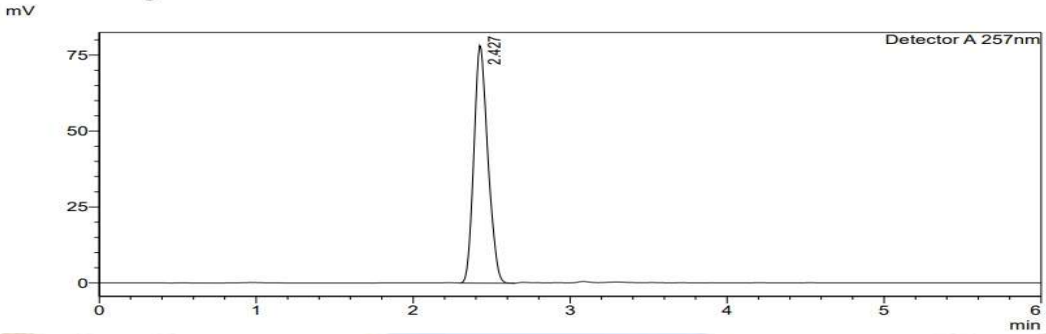

Fig. 20 Chromatogram of iguratimod flow rate 1.1 ml/min

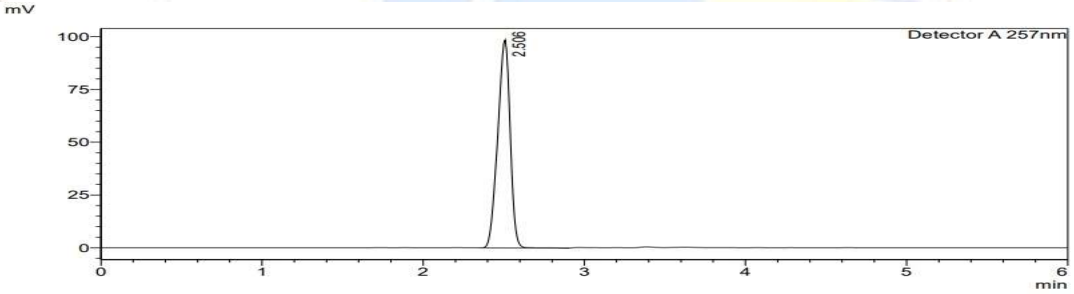

Fig. 21 Chromatogram of Iguratimod Mobile phase 48:52(ACN: METH)

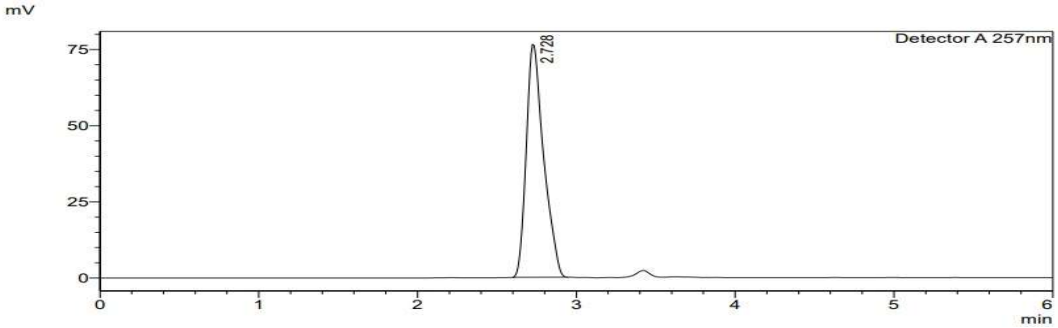

Fig. 22 Chromatogram of Iguratimod Mobile phase 50:50(ACN: METH)

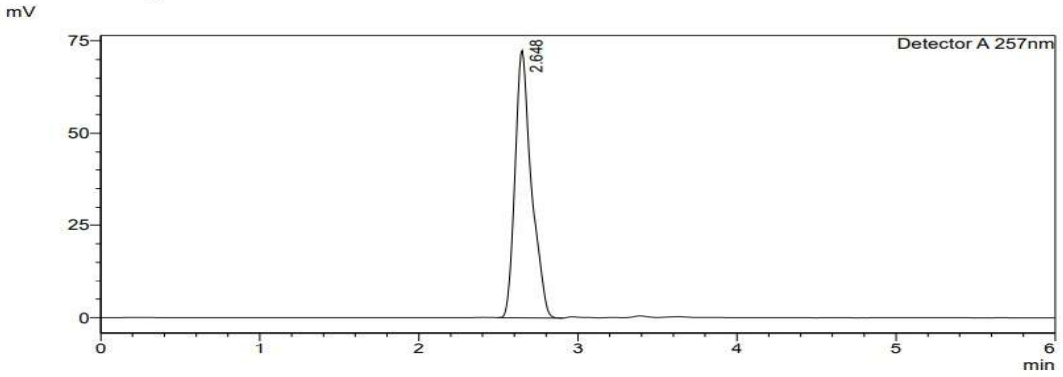

Fig. 23 Chromatogram of Iguratimod Mobile phase 52:48(ACN: METH)

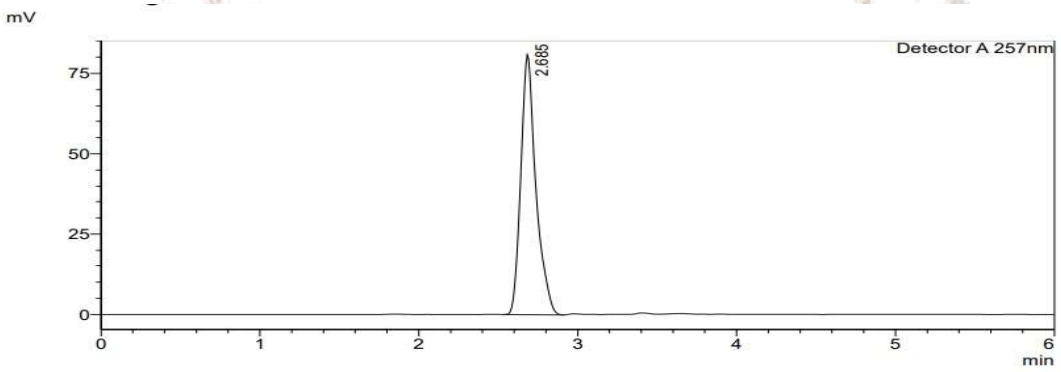

Fig. 24 Chromatogram of iguratimod Temperature 38°C

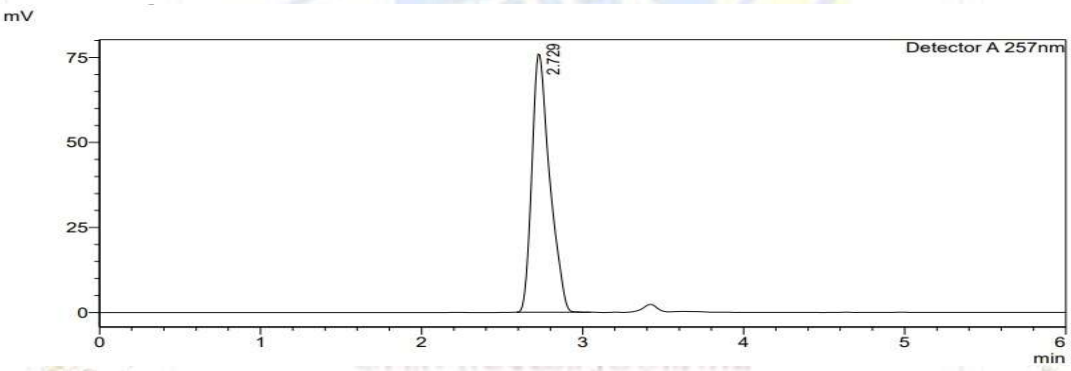

Fig. 25 Chromatogram of iguratimod Temperature 40°C

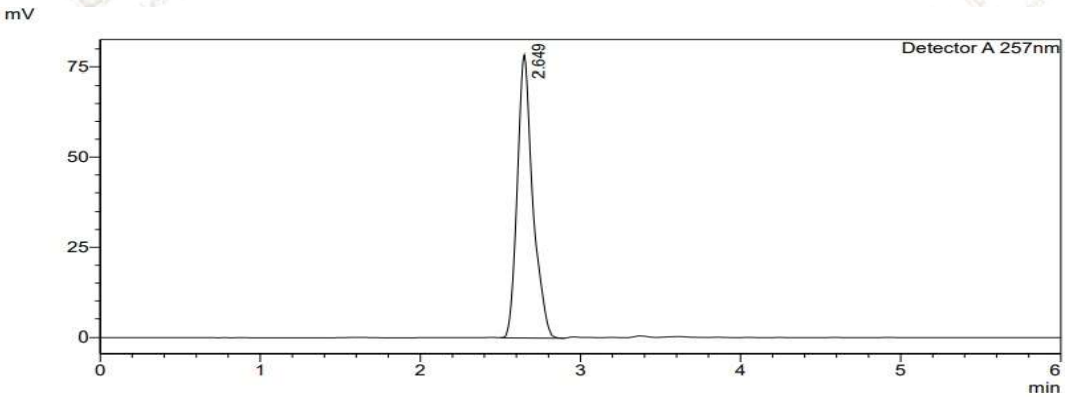

Fig. 26 Chromatogram of iguratimod Temperature 42°C

Limit of Detection and Limit of Quantification

Limit of Detection and Limit of Quantification were calculated from linearity plot. The results were shown in Table 6.

Parameter	Measured Value
Limit of Detection	0.118 µg/ml
Limit of Quantification	0.358 µg/ml

Table 6 Limit of Detection and Limit of Quantification

4. Discussion

Iguratimod was practical soluble in acetonitrile, water, ethanol, methanol, Acetic acid and very less soluble in DMSO. The Iguratimod having melting point of 242 ºC which confirms the identity of the drugs. For the estimation of Iguratimod, the validated analytical methods are targeted to get simple, precise, rapid, accurate methods.

A wavelength of 257 nm and the mobile phase consisting of acetonitrile: methanol (50:50) were selected by means of the RP-HPLC method with a flow rate of 1ml/min to get optimum condition for analysis. It was observed that the retention time was 2.7 minutes with optimum conditions.

Iguratimod show specificity. There is no any interference of Iguratimod peak (main peak) from the other peaks. The purity of Iguratimod is 0.99 NLT.

Iguratimod showed the linearity in the range of 10-50 µg/ml having symmetrical peak and & a good correlation coefficient value. From linearity curve the linear regression equation was calculated and found as **y = 481817x +17289** with the correlation coefficient **(R^2)** of value **0.9998**. The relative standard deviation (R.S.D) for the all case is calculated and found to be less than 2% which is well within the acceptable criteria of NMT 2.0.

The precision of the procedure was examined by injecting repeatedly the same sample & by determining a standard deviation. Interday and intra-day accuracy were performed & % RSD value measured as 0.494377 and 0.535898 which within the acceptable criteria of NMT 2.0 indicates good intraday precision and Interday.

Five different concentrations (i.e., 50%, 75%, 100%, 125% and 150% level) were used to determine the

accuracy of the procedure. The average percentage of recovery was found to be 101.9197, 100.9318, 100.1455, 101.6788 and 101.2998 for 50%, 75%, 100%, 125% and 150% level. Suitable recoveries ranging from 100.1455 to 100.9318 were obtained by the proposed method. The % recovery is in between 95% and 105% indicates the accuracy of proposed method.

For the robustness study slight modification in flow rate of the mobile phase, temperature of column and composition of the mobile phase were done. No noticeable change was observed in chromatograms demonstrated that the method was robust in nature.

The limit of detection and limit of quantification were calculated from linearity plot. The limit of detection and limit of quantification was found to be 0.118µg/ml and 0.358 µg/ml.

For the analysis of tablet containing 25 mg Iguratimod drug (label claim) the validated method is applied as the validated method is simple having good peak and less retention time.

5. Conclusion

In a review of the literature, only a few analytical procedures for determining Iguratimod using HPLC were found. As a result of the foregoing, some simple analytical methods with sensitivity, accuracy, precision, and economy were proposed to be developed. A simple, sensitive, precise, and accurate RP-HPLC method was established in this study to develop and validate the method of Iguratimod in bulk and pharmaceutical dose forms without any interference from excipients or degradation products. In addition to meeting favourable requirements for analytical methods, all currently developed methods have the distinct advantage of being cost-effective. The accuracy, precision, linearity, limit of detection, limit of quantitation, and robustness of the method are all validated. This approach can be used to determine Iguratimod in bulk and pharmaceutical dosage forms on a regular basis.

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