# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR RELATED SUBSTANCE IN IGURATIMOD BY HPLC

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***Abstract:*** ***The present study describes a simple and stability-indicating reverse phase high-performance liquid chromatography (RP-HPLC) method for the quantification of the related substances of Iguratimod drug substance. Successful separations of the possible impurities were achieved on a Inertsil ODS-3 (150 × 4.6 mm, 5µm) and UV detector at 257nm, 0.8mL/min as a flow rate, and 20µL as an injection volume. For mobile phase-A preparation, 5.44gm potassium dihydrogen orthophosphate dissolved in 2 liter water and adjusted pH 4.00±0.05 with dilute orthophosphoric acid and filter through 0.45µ membrane filter. Use HPLC grade Acetonitrile as mobile phase-B. Use gradient flow program with column temperature 40°C. Percentage recovery obtained in the range of 80-120% and the method is linear for all possible impurities and Iguratimod for specified concentration range with coefficient of variation (r) not less than 0.99. Acid, base, peroxide and thermal degradation were carried in drug substance. The proposed RP- HPLC method was found to be specific, linear, precise, accurate and robust.***

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

## 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,

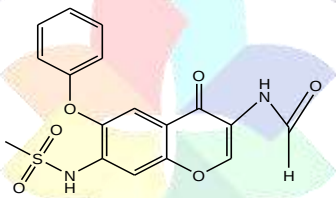

Fig 1: Structures of Iguratimod

IUPAC name is N-[(formylamino)-4-oxo-6-phenoxy-4Hchromen-7-yl] methane sulfoanamide for Iguratimod. 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

Organic impurities in drug substances can arise during the manufacturing process and storage. Thus, the acceptance limits are based on pharmaceutical studies or known safety data. Several methods have been reported for the analysis of Iguratimod. However, no combined validated stability-indicating reversed phase HPLC (RP- HPLC) method has been used for the separation and quantitative analysis of all the possible impurities. In this study, a rapid and validated RP-HPLC method was developed to separate closely eluted impurities. The limit of detection (LOD), limit of quantification (LOQ) and sensitivity of the method was tested in accordance with ICH Q2 guidelines.

## EXPERIMENTAL AND METHODS:

### REAGENTS, MATERIALS AND INSTRUMENTATION

For analytical development activity, used HPLC grade methanol, acetonitrile and water. Analytical grade potassium dihydrogen orthophosphate and Orthophosphoric acid has been used. The instrument used was Waters HPLC system consisting of a pump, a UV detector and empower software. Analytical column with specification as Inertsil ODS-3 (150 × 4.6 mm, 5µm) was used for analysis.

### 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 impurities 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 0.8 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) | 0.8ml/min |

| | |
|---|---|
| Injection volume | 20µl |
| Detection wavelength | UV 257 nm |
| Runtime | 40.0 minutes |
| Column oven temperature | 40°C |
| Diluent | Mobile phase-A : Mobile phase-B (50:50) |

Table 1: Optimized chromatographic parameters and conditions

| Gradient program : | | |
|---|---|---|
| Time (minutes) | %Mobile phase-A | %Mobile phase-B |
| 0 | 70 | 30 |
| 10 | 70 | 30 |
| 20 | 55 | 45 |
| 25 | 20 | 80 |
| 30 | 20 | 80 |
| 32 | 70 | 30 |
| 40 | 70 | 30 |

Table 2: Mobile phase preparation and gradient program

Preparation of mix standard solution

Prepare individually all stock solutions to get each 5ppm Impurity-A, Impurity-B, Impurity-D, Impurity-E , Iguratimod and 7.5ppm Impurity-C. Further dilute 2ml of each solution to 20ml with diluent to get 0.5 ppm Impurity-A, Impurity-B, Impurity-D, Impurity-E, Iguratimod, and 0.75 ppm Impurity-C.

Preparation of sensitivity solution

Dissolve about 5mg Iguratimod standard to 50ml with diluent, dilute 5ml to 50ml with diluent. Further dilute 1 ml of resultant solution to 100ml with diluent to get solution of 0.05ppm.

Preparation of test solution

Dissolve about 10mg sample to 20ml with diluent.

**RESULTS AND DISCUSSION**

**OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS:**

Many efforts have been made 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 tried for pH about 3.5, which will be nearby pKa, and at this pH, Iguratimod will remain ionized, which makes better retention as well as separation of related compound. Thus, we tried at different pH and started at with pH 3.5, but at this pH all other impurities were separated and co-elution observed for unknown impurity at Acid impurity.

By changing the gradient ratio required separation achieved but principle peak shape deteriorated. By increasing the column oven temperature and reducing flow rate to 0.8ml/minutes, required separation of all the impurities achieved. During the pH parameter optimization, observed that at lower pH, co-elution of acid impurity with unknown impurity at higher pH, good separation achieved. During analytical column change, decrease in resolution between two known impurities were observed and got learning that decreasing the pH of mobile phase, resolution decreases. Hence slight change in pH on higher side, i.e. increase in pH, 3.9 to 4.0 was finalized for better separation for all possible known impurities and between peak shape.

By using the pH 4.0, it gives sharp peak of Iguratimod without co-elution or any interference of unknown or known impurities. Optimized chromatographic parameters are summarized in Table1 and Table 2. 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.

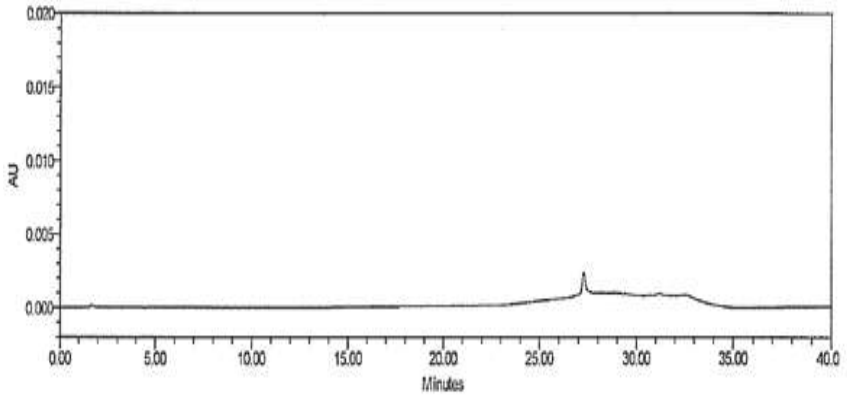


Fig 2: Blank chromatogram

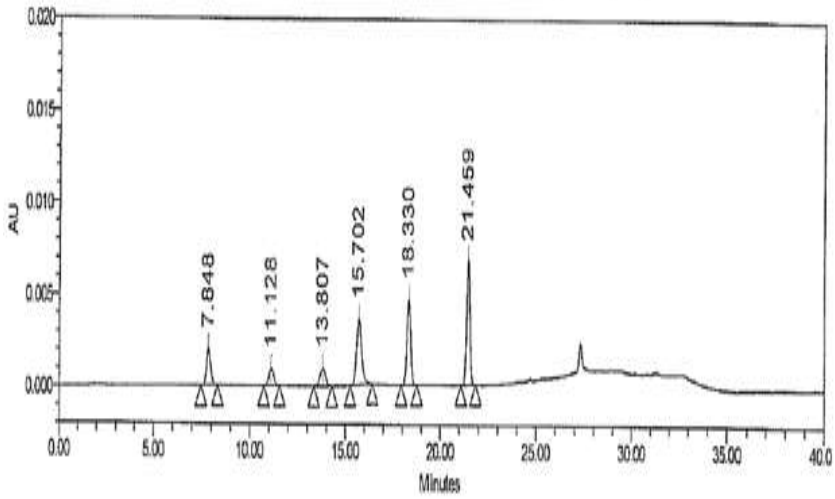


Fig 3: Mix standard chromatogram

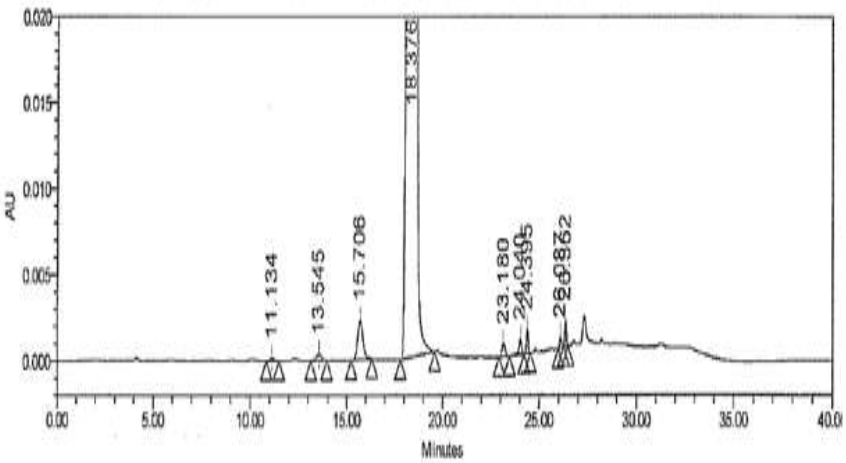


Fig 4: Test (as such) chromatogram

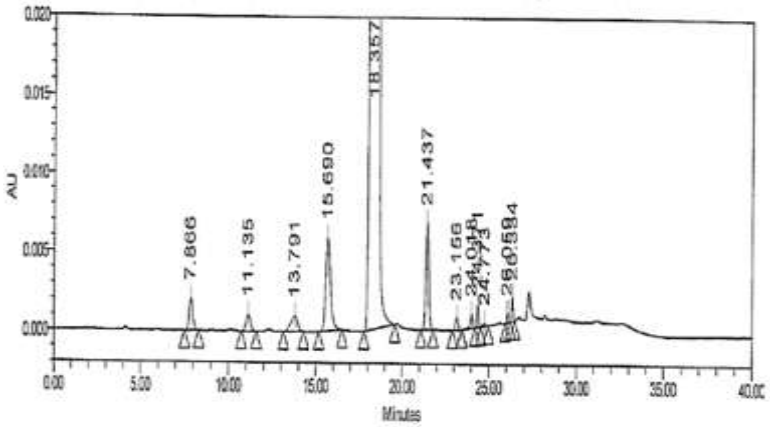


Fig 5: Test spiked with known impurities chromatogram

**METHOD VALIDATION:**

**SPECIFICITY:**

Blank, mix impurity standard, Test (as such), Test spiked with impurities were injected to evaluate Specificity. No interference due to blank and coelution of impurities were observed. All known peaks pass peak purity criteria i.e. purity angle should be less than purity threshold.

To demonstrate the stability indicating nature of the method, forced degradation has been carried out in acid (1N Hydrochloric acid, 60°C, 30 minutes), base (0.1N Sodium hydroxide, at room temperature for 5 minutes), oxidation (10 % Hydrogen peroxide at 60°C for 4 hours), aqueous degradation (Water at 60°C for 4 hours).

In acidic condition Impurity-E increases up to 5%, where as in alkaline condition unknown impurity increase upto 9.5%. No significant degradation observed in aqueous and oxidation degradation condition.

The entire peaks were found to be resolved form each other and spectrally pure as calculated by empower software.

| Specificity | | | | | |
|---|---|---|---|---|---|
| Peak Name | Retention time (minutes) | Peak Tailing (USP) | Purity angle | Purity Threshold | Peak Purity |
| Impurity-D | 7.484 | 2.440 | 0.298 | 1.709 | Passes |

| | | | | | |
|---|---|---|---|---|---|
| Impurity-A | 11.128 | 1.060 | 0.247 | 0.251 | Passes |
| Impurity-B | 13.807 | 1.040 | 0.206 | 0.252 | Passes |
| Impurity-E | 15.702 | 1.090 | 0.160 | 0.266 | Passes |
| Iguratimod | 18.330 | 1.000 | 0.230 | 0.265 | Passes |
| Impurity-C | 21.459 | 1.020 | 0.250 | 0.380 | Passes |

Table 3: Specificity

**PRECISION**

Consistency in repeated response by the chromatographic system and solution preparation procedure was evaluated by injecting six replicate preparations of mix standard spiked with impurities. % RSD for retention time and area response is tabulated in Table 4 and Table 5 respectively.

**Component Summary For Retention Time**
**Channel: W2489 ChA**

| | Sample Name | Inj | Channel | Vial | Result Id | Acid Impurity | D-SPA.F | SPA.F | Acid FD | Iguratimod | Methyl Impurity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Mix Standard | 1 | W2489 ChA | 51 | 4945 | 6.597 | 10.234 | 12.594 | 14.561 | 17.345 | 20.547 |
| 2 | Mix Standard | 2 | W2489 ChA | 51 | 4946 | 6.589 | 10.224 | 12.578 | 14.553 | 17.343 | 20.550 |
| 3 | Mix Standard | 3 | W2489 ChA | 51 | 4947 | 6.590 | 10.227 | 12.589 | 14.565 | 17.358 | 20.567 |
| 4 | Mix Standard | 4 | W2489 ChA | 51 | 4948 | 6.595 | 10.232 | 12.590 | 14.559 | 17.351 | 20.557 |
| 5 | Mix Standard | 5 | W2489 ChA | 51 | 4949 | 6.585 | 10.220 | 12.575 | 14.548 | 17.339 | 20.546 |
| 6 | Mix Standard | 6 | W2489 ChA | 51 | 4950 | 6.579 | 10.217 | 12.572 | 14.547 | 17.345 | 20.557 |
| Mean | | | | | | 6.589 | 10.226 | 12.583 | 14.555 | 17.347 | 20.554 |
| Std. Dev. | | | | | | 0.007 | 0.006 | 0.009 | 0.007 | 0.007 | 0.008 |
| % RSD | | | | | | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |

Table 4: Precision for retention time

| | Sample Name | Inj | Channel | Vial | Acid Impurity | D-SPA.F | SPA.F | Acid FD | Iguratimod | Methyl Impurity |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Mix Standard | 1 | W2489 ChA | 51 | 20821 | 15739 | 19466 | 47043 | 67468 | 88680 |
| 2 | Mix Standard | 2 | W2489 ChA | 51 | 20822 | 15741 | 19510 | 46825 | 67479 | 88581 |
| 3 | Mix Standard | 3 | W2489 ChA | 51 | 21005 | 15916 | 19539 | 47161 | 67847 | 88993 |
| 4 | Mix Standard | 4 | W2489 ChA | 51 | 20844 | 15684 | 19324 | 46630 | 67173 | 88189 |
| 5 | Mix Standard | 5 | W2489 ChA | 51 | 21110 | 15884 | 19713 | 47113 | 67900 | 89080 |
| 6 | Mix Standard | 6 | W2489 ChA | 51 | 20925 | 15865 | 19618 | 47100 | 67814 | 89171 |
| Mean | | | | | 20921 | 15805 | 19528 | 46979 | 67613 | 88782 |
| Std. Dev. | | | | | 117 | 95 | 133 | 208 | 286 | 371 |
| % RSD | | | | | 0.6 | 0.6 | 0.7 | 0.4 | 0.4 | 0.4 |

Table 5: Precision for area response

**LINEARITY**

To evaluate the linearity in detector response all the components were injected from LOD level to 150% wrt test concentration and the correlation coefficient was found to be not more than 0.99. The response factor was calculated from the slope of impurities and Iguratimod linearity curve; all impurities have RRF below 1.0.

Correlation coefficient

| Conc. (in ppm) for Impuirty-D | Avg Area |
|---|---|
| 0.0763 | 2881 |
| 0.2289 | 9041 |
| 0.7631 | 29534 |
| 1.1446 | 46402 |
| Slope = | 40380.6979 |
| Correlation coefficient = | 0.9995 |
| Squared Correlation coefficient = | 0.9990 |
| Relative Response Factor = | 0.2962 |

Table 6: Linearity for Impurity-D

| Conc. (in ppm) for Impuirty-A | Avg Area |
|---|---|
| 0.0534 | 1553 |
| 0.1601 | 4943 |
| 0.5338 | 16024 |
| 0.8007 | 25098 |
| Slope = | 31214.1747 |
| Correlation coefficient = | 0.9996 |
| Squared Correlation coefficient = | 0.9991 |
| Relative Response Factor = | 0.2290 |

Table 7: Linearity for Impurity-A

| Conc. (in ppm) for Impuirty-B | Avg Area |
|---|---|
| 0.0509 | 1842 |
| 0.1526 | 5917 |
| 0.5085 | 19265 |
| 0.7628 | 30182 |
| Slope = | 39447.3062 |
| Correlation coefficient = | 0.9996 |
| Squared Correlation coefficient = | 0.9991 |
| Relative Response Factor = | 0.2893 |

Table 8: Linearity for Impurity-B

| Conc. (in ppm) for Impuirty-E | Avg Area |
|---|---|
| 0.0812 | 6506 |
| 0.2345 | 21167 |
| 0.8118 | 70877 |
| 1.2177 | 112980 |
| Slope = | 92449.4200 |
| Correlation coefficient = | 0.9992 |
| Squared Correlation coefficient = | 0.9983 |
| Relative Response Factor = | 0.6781 |

Table 9: Linearity for Impurity-E

| Conc. (in ppm) for Iguratimod | Avg Area |
|---|---|
| 0.0505 | 6521 |
| 0.1514 | 20304 |
| 0.5047 | 65353 |
| 0.7571 | 103967 |
| Slope = | 136335.3554 |
| Correlation coefficient = | 0.9992 |
| Squared Correlation coefficient = | 0.9983 |
| Relative Response Factor = | 1.0000 |

Table 10: Linearity for Iguratimod

| Conc. (in ppm) for Impuirty-C | Avg Area |
|---|---|
| 0.0785 | 8260 |
| 0.2354 | 25793 |
| 0.7848 | 83303 |
| 1.1772 | 130353 |
| Slope = | 110115.0903 |
| Correlation coefficient = | 0.9996 |
| Squared Correlation coefficient = | 0.9991 |
| Relative Response Factor = | 0.8077 |

Table 11: Linearity for Impurity-C

**LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:**

The Limit of detection (LOD) and limit of quantification (LOQ) were determined on visual basis. The LOD and LOQ values are depicted in Table 12.

| LOD & LOQ Determination | | |
|---|---|---|
| Peak Name | LOD (wrt test conc.) | LOQ (wrt test conc.) |
| Impurity-D | 0.015 | 0.045 |
| Impurity-A | 0.010 | 0.030 |
| Impurity-B | 0.010 | 0.030 |
| Impurity-E | 0.015 | 0.045 |
| Impurity-C | 0.015 | 0.045 |

Table 12: LOD and LOQ determination

**ACCURACY:**

Accuracy was demonstrated by spiking impurity solution at LOQ, 100 % and 150 % of test concentration. % Recovery was calculated from the amount added and amount found. The results are tabulated in Table 13.

| Accuracy | | | | |
|---|---|---|---|---|
| Peak name | %Recovery at 30% level | %Recovery at 80% level | %Recovery at 100% level | %Recovery at 150% level |
| Impurity-A | 95.455 | 98.555 | 96.903 | 95.700 |
| Impurity-B | 88.730 | 97.435 | 96.246 | 95.270 |
| Impurity-C | 94.915 | 97.335 | 96.000 | 95.130 |
| Impurity-D | 99.350 | 99.945 | 98.337 | 97.485 |
| Impurity-E | 95.675 | 101.045 | 99.300 | 98.030 |

Table 13: Accuracy

**ROBUSTNESS:**

Robustness of the method was evaluated by injecting test spiked with impurities, retention time and area response was monitored. Analytical method was found to be robust for the below mentioned conditions,

1. ± 0.1 mL flow rate
2. ± 0.2 unit's pH of buffer
3. ± 5 °C column temperature

| Robustness (Change in pH by ±0.20) | | | | |
|---|---|---|---|---|
| Peak name | pH - 0.20 | | pH + 0.20 | |
| | Retention Time (minutes) | Area response | Retention Time (minutes) | Area response |
| Impurity-A | 10.784 | 16050 | 10.778 | 16339 |
| Impurity-B | 13.352 | 19496 | 13.363 | 19770 |
| Impurity-C | 21.049 | 85193 | 21.059 | 87064 |
| Impurity-D | 7.788 | 31381 | 6.776 | 31538 |
| Impurity-E | 15.269 | 66871 | 15.282 | 69400 |

Table 14: Robustness: Change in pH

| Robustness (Change in column temperature ±5°C) | | | | |
|---|---|---|---|---|
| Peak name | Column temperature -5°C | | Column temperature +5°C | |
| | Retention Time (minutes) | Area response | Retention Time (minutes) | Area response |
| Impurity-A | 11.115 | 17462 | 9.519 | 17577 |
| Impurity-B | 13.567 | 21301 | 11.747 | 21359 |
| Impurity-C | 21.338 | 90982 | 19.865 | 90988 |
| Impurity-D | 7.968 | 31514 | 6.691 | 32081 |
| Impurity-E | 15.576 | 72189 | 13.687 | 72539 |

Table 15: Robustness: Change in column temperature

| Robustness (Change in flow rate by ±0.1 ml/minute) | | | | |
|---|---|---|---|---|
| Peak name | Flow - 0.1 ml/min | | Flow + 0.1 ml/min | |
| | Retention Time (minutes) | Area response | Retention Time (minutes) | Area response |
| Impurity-A | 11.634 | 19537 | 9.174 | 15438 |
| Impurity-B | 14.269 | 23587 | 11.273 | 18674 |
| Impurity-C | 21.919 | 101635 | 19.478 | 79533 |
| Impurity-D | 8.213 | 34876 | 60484.000 | 27567 |
| Impurity-E | 16.224 | 81184 | 13.221 | 62463 |

Table 16: Robustness: Change in column flow rate

**CONCLUSION:**

The RP-HPLC method developed for the estimation of Impurities in Iguratimod. This analytical method was found to be specific, precise, robust, linear and accurate. The method can be used for checking the quality of Iguratimod for routine as well as stability studies.

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