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DETERMINATION OF IGURATIMOD IN HUMAN PLASMA BY HPLC METHOD

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ABSTRACT

A simple bioanalytical HPLC method for estimation of Iguratimod in human plasma has been developed and validated. Extracted sample was eluted using HiQ Sil C_{18} column. The optimized mobile phase was ACN: WATER adjust the pH-3 with glacial acetic acid (40:60v/v) at flow rate 1 ml/min. In this method Rosuvastatin is used as internal standared. Iguratimod and Rosuvastatin eluted at retention time 9.033 and 11.925min. Calibration curve was linear in range of 1-7ug/ml. The method was validated according to MHLW (Japan) guidelines(2013). The data of linear regression analysis indicated a good linear relationship over the range of 1-7 ug/ml with correlation coefficient value of 0.9551. The proposed method can be applied for estimation of Iguratimod in human plasma in pharmacokinetic studies.

KEYWORDS: Iguratimod and Rosuvastatin.

INTRODUCTION

Iguratimod is antirheumatic. It is used for treatment of rheumatoied arthritis and it is developed by Toyama chemical company. Iguratimod is approved in Japan. Iguratimod also inhibits the production of inflammatory cytokines in cultured human synovial cell and human monocytic leukemia cell. It reduces immunoglobuline production by acting directly in B lymphocytes in both mice and humans. There are some methods reported for estimation of Iguratimod from human plasma.[1,2,3]

Iguratimod molecular formula is $C_{17}H_{14}N_2O_6S$. Its chemical name is N-[7-(methanesulfonamido)-4-oxo-6-phenoxychromen-3yl]formamide as shown in fig.1.

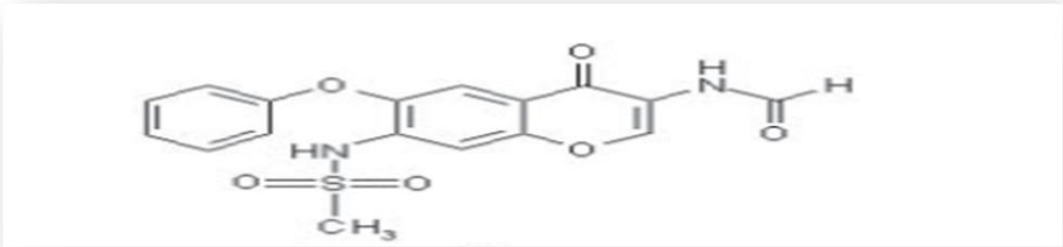

Fig. 1: Structure of Iguratimod.

Molecular weight is 374.367 g/mol. It is soluble in Acetonitrile. and stored at Stored in well closed containers, at 2-8ºC. Its melting point is 238-242°C[4] and C_{max} is reported to be 1.15, 2.33 & 4.78 ug/ml in 25,50 and 100mg.[5] Iguratimode is not offical in IP/BP/USP.

MATERIAL AND METHOD

Iguratimod was received as gift sample from Lupin Ltd, Aurangabad. All chemicals and reagents i.e ACN, Hydrochloric acid (HCL), and hydrogen peroxied (H_2O_2), sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD., Mumbai.

Chromatographic Condition

HPLC system used was JASCO system equipped with model PU2080 Plus pump, Rheodyne sample injection port (20ul), JASCO UV 2075 Plus detector and Borwin chromatography software (version1.5) with HiQ Sil C_{18} (4.5 mm*250 um)column. The optimized Mobile phase is ACN : H_2O (40:60v/v) The pH-3 of water was adjusted with Acetic acid. The overall run time is 15min and flow rate was 1ml/min. Quantification was carried out at 257nm.

Selection of Mobile Phase

Acetonitrile:Water(pH-3) in ratio of 40:60 v/v was choosen as mobile phase for analysis in which optimum system suitability parameters were obtained.

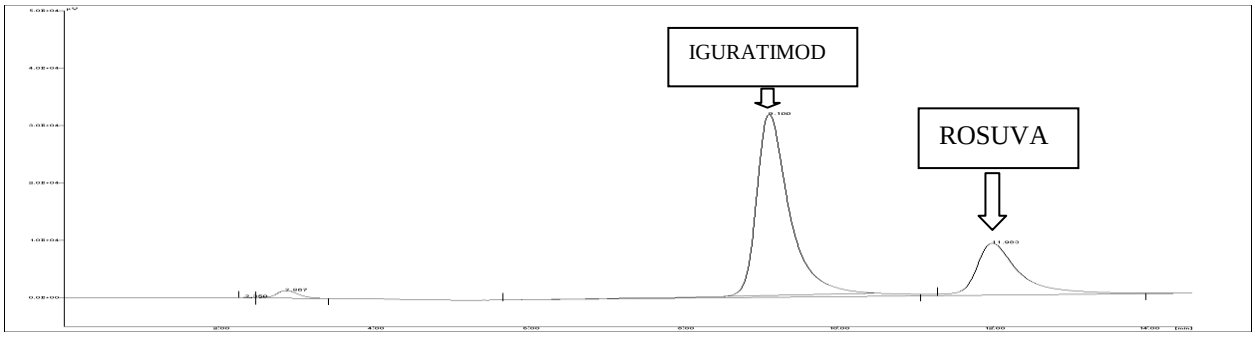

Fig. 2: Standard Chromatogram of Iguratimod and Rosuvastatin.

Table 1: System suitability parameter.

Name	RT(Min)	Conc. (ug/ml)	Area	Therotical Plates	Resolution	Asymmetry
Iguratimod	9.100	5	1294889	2814.29	10.61	1.53
Rosuvastatin	11.983	5	227527.9	3291.55	3.80	1.71

Selection of internal standard

Internal standard is a compound added to samples to improve the accuracy and precision of quantitation as well as robustness of bioanalytical method.

Tenofovir, Paliperidone palmitate, Rilpiverin, Momentasone furvate, Rosuvastatin were tried as internal standard, Rosuvastatin was selected as internal standard as peaks of the drug and Rosuvastatin were well resolved. Also, Rosuvastatin was well extracted from extraction technique that is developed for extraction of Iguratimod from human plasma.

Preperation of Mobile Phase

40 ml of HPLC grade ACN was added to 60 ml of HPLC grade water (40:60v/v ratio). The pH-3 of HPLC grade water was adjust the with acetic acid. Mobile phase was filtered through 0.45um membrane filter and sonicated in sonicator bath for 10 min.

Preparation of stock solution

Stock solution of IGURA and ROSUVA (I.S) were prepared separately by transferring accurately weighed 10 mg of drug into 10ml volumetric flask and making up volume with ACN to get concentration of 1000ug/ml. Working stock solution for Iguratimod was prepared by diluting appropriately stock solution to get final concentration 50-350ug/ml. and for ROSUVA to get final concentration 250ug/ml.

Preparation of spiked plasma sample

Weigh 10 mg Iguratimod in 10ml volumetric flask make up the volume with ACN (1000ug/ml) it is standard stock solution A from this we make further dilution. The linearity range was chosen based on reported C_{max} value for Iguratimod as 1-7ug/ml. plasma was spiked with different concentration of Iguratiomod and constant amount of Rosuvastatine as internal standard separately. The content were mixed by Vortex mixer for 5 min. 1 ml of this solution was pipetted into separate test tube to which 1 ml ACN was added. These solution were gently mixed by Vortex mixer and then centrifuged for 20min. The clear ACN layer

was separated then it is filtered through 0.45u membrane filter and injected. A blank plasma sample was treated similarly. protein precipitation technique has been used.

Selection of Wavelength

From Standard stock solution further dilutions were done using ACN and it was scanned over the range 200-400 nm. It was observed that drug show considerable absorbance at 257 nm.

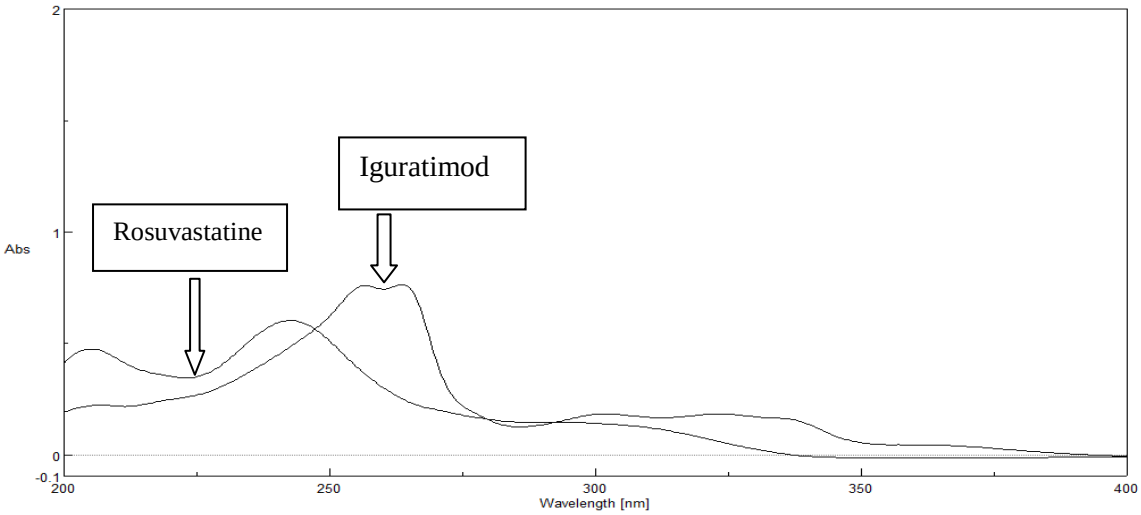

Fig. 2: Overly UV Spectra of IGURA(10ug/ml) and ROSUVA(10ug/ml).

Method Validation[6,7]

1. Selectivity

Selectivity is the ability of an analytical mehod to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analysing pooled plasma spiked at LLOQ. It was evaluated using blank plasma samples. No endogenous interferences are noted at the Rt of drug and IS.

Table 2: Result for Selectivity of IGURA.

Replicates No.	Nominal Conc.(LLOQ)				
	Peak area of IGURA	Peak area of ROSUVA	Response factor	Calculated Concentration	
				ug/ml	%accuracy
1	289346	242689.32	1.19224858	0.8964	89.64
2	193761.897	179490.56	1.07951023	0.7892	78.92
3	231917.636	199714.33	1.16124686	0.8669	86.69
4	296792.882	239714.33	1.23811073	0.9401	94.01
5	278110.735	190607.55	1.45907513	1.1503	115.03
6	289346	242689.32	1.19224858	0.8964	89.64
Mean				0.92858	92.3216
SD				0.13561	12.2001
%CV				13.2148	

2. Calibration curve

Linearity of concentration 1-7ug/ml was tested. Each sample in five replicates was analysed and peak area were recorded.

Table 3: Result for linearity of IGURA standard.

Replicates	Concentration (ug/ml)						
	1	2	3	4	5	6	7
	Response Factor						
1	1.72	3.4	3.76	4.6	5.69	6.35	7.53
2	1.77	3.39	3.51	4.65	5.75	6.37	9.23
3	1.74	3.23	3.74	4.56	5.67	6.35	9.75
4	1.77	3.22	3.53	4.67	5.61	6.39	9.33
5	1.72	2.99	3.57	4.59	5.6	6.32	8.44
Mean	1.74	3.24	3.62	4.61	5.66	6.35	8.85
SD	0.02	0.24	0.52	0.55	0.36	0.02	0.87
%RSD	1.43	7.41	14.62	12.04	6.48	0.41	9.93

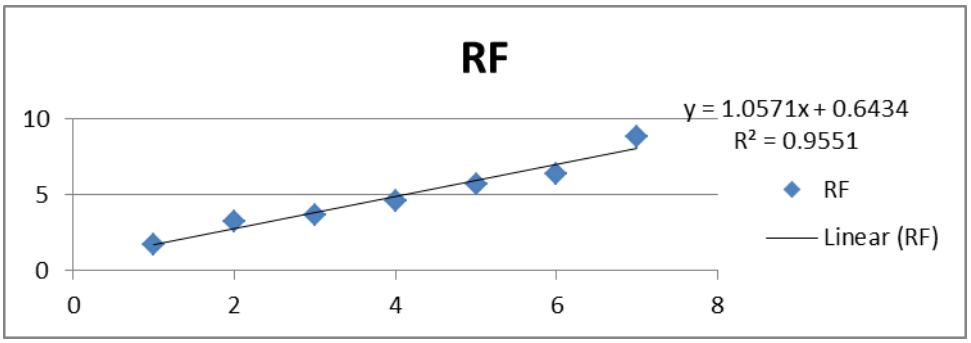

Figure 3: Calibration curve of IGURA standard.

Table 4: Result for Linearity of IGURA spiked plasma.

Replicates	Concentration (ug/ml)						
	1	2	3	4	5	6	7
	Response Factor						
1	1.622	2.95	3.17	4.19	4.83	6.09	7.35
2	1.25	2.98	3.01	4.27	5.30	6.01	9.04
3	1.58	2.96	3.44	4.23	5.66	6.15	8.94
4	1.18	2.88	3.32	3.30	5.56	6.11	8.20
5	1.43	2.09	3.36	4.33	5.38	6.11	9.17
Mean	1.41	2.77	3.26	4.06	5.35	6.09	8.54
SD	0.19	0.38	0.16	0.43	0.32	0.05	0.76
%RSD	13.77	13.69	5.19	10.60	5.99	0.82	8.95

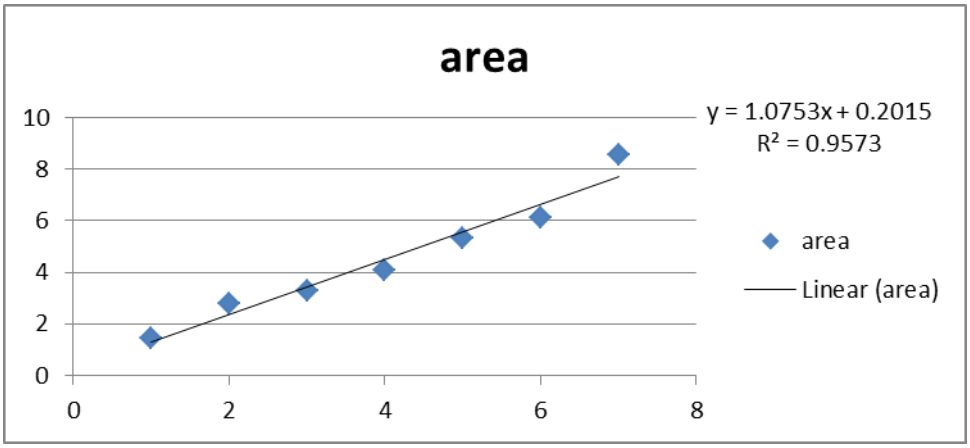

Fig. 4: Calibration curve of IGURA spiked plasma.

All the five calibration curves analyzed during the course of validation were found to be linear for the standards concentration ranging from 1-7ug/ml and best fitted by a linear equation y = mx+ c, the coefficient of correlation for standard IGURA (R^2) is 0.9551 and spiked plasma with IGURA (R^2) is 0.9572. A representative calibration curves are shown in Figure 3 and Figure 4 respectively.

3. Accuracy

Accuracy was measured using five determinations per four concentrations. The % mean accuracy of calculated concentrations for all quality control samples at LLOQ, LQC, MQC and HQC concentration levels range from 85% to 115% result are summarized in Table 5.

Table 5: Results for accuracy of IGURA.

Replicates	Calculated conc.			
	At LLOQ	At LQC	At MQC	At HQC
1	0.923	1.930	4.171	5.28
2	0.813	1.996	3.399	5.22
3	0.892	1.991	3.249	5.52
4	0.964	1.989	4.247	4.84
5	1.169	1.900	4.412	5.00
Mean	0.9522	1.961	3.895	5.17
SD	0.1332	0.043	0.531	0.26
%CV	13.99	2.226	13.65	5.025
%Mean Accuracy	95.22	98.08	97.39	86.30

4. Precision

A) Inter day Precision

The Inter day Precision was evaluated in five replicates for four different concentration of IGURA on three consecutive days (fresh samples were prepared every day.) The % CV of

calculated concentration for all Quality control samples of LLOQ, LQC, MQC, HQC concentration level ranged from 3.38-9.68% as shown in Table 6.

Table 6: Results for Inter-day precision of IGURA.

Conc. Level	%CV		
	Day 1	Day 2	Day 3
At LLOQ	6.88	4.81	7.47
At LQC	5.02	3.38	9.68
At MQC	4.78	4.18	3.93
At HQC	6.74	8.55	5.67

Intraday precision

Repeatability of the method was evaluated in five replicates on the same day for four different concentrations of IGURA (1,2,4,6µg/mL). The %CV of calculated concentrations for all quality control samples at LQC, MQC, HQC concentration levels ranged from 7.49-10.61%, which is within acceptance limit 15%, and at LLOQ levels it is found to be 14.101% as shown in Table 7.

Table 7: Results for Intra-day precision of IGURA.

Con. Level	At LLOQ	At LQC	At MQC	At HQC
%CV	6.90	3.19	3.30	4.35

Recovery

The % mean recoveries were determined by measuring the responses of the extracted plasma quality control samples against un-extracted quality control samples at HQC, MQC and LQC levels. Recovery from human plasma samples was evaluated in triplicate for each three concentrations of IGURA (2,4 and 6 ug/ml).

The % mean recovery for ETRA at HQC, MQC and LQC levels are found to be 70.64%, 85.13% and 80.35% respectively. Overall % CV at all QC levels is 10.45%, which is within the acceptance limit of 15% and % overall mean recovery is 70.64%. The results are summarized in the Table 8.

Table 8: Results for recovery of IGURA.

Conc level	Area of IGURA		Area of ROSUVA(I.S)	
	Standard	Spiked plasma	Standard	Spiked plasma
LQC	689741.25	554211.209	206233	180772.8
MQC	1076382	1036334	233485	212745.6
HQC	1452922	1323383	228687	219991.58
Overall %mean Recovery	88.58		91.65	

Carry-over

Carry-over evaluated by analyzing a blank sample following the highest concentration calibration standard. % Carryover for IGURA and ROSUVA (I.S) is 7.14% and 13.74% resp, which is within limit as shown in Table 9.

Table 9: Results of Carry-over for IGURA.

Replicates	Area of IGURA		Area of ROSUVA		% Carryover	
1	**At HOQ**	**At Blank**	**At HOQ**	**At Blank**	**IGURA**	**ROSUVA**
2	1720232.25	135920.841	145923.75	14248.11		
3	1666418.259	115756.5	151716.42	16526.61		
Mean	1804429.459	121693.384	115331.422	25974.79	7.19%	13.74%
SD	1730359.989	124456.908	137657.197	18916.5		
% RSD	69560.773	10362.333	19550.420	6217.913		
Acceptance Criteria: The % carryover should not be greater than 20% of the analyte response at HOQ						

Dilution integrity

Dilution integrity were evaluated by taking 1 mL spike plasma of conc.15µg/mL and it was diluted by adding 2 mL plasma in it. % Recovery of diluted samples was noted as shown in Table10.

Table 10: Results of Dilution Integrity for IGURA.

Conc ug/ml	Calculated conc	%Recovery
3	5.014	99.64
	4.831	99.51
	4.819	97.56
	4.744	98.75
	4.741	98.05
Mean	4.830	98.90
%CV	2.29	
Acceptance Criteria: The % CV should be within 15.00%		

Stability

Drug Stability in biological fluid is a function of storage conditions, chemical properties of drug, the matrix and the container system. Stability procedure should evaluate the stability of analyze during sample collection and handling after long term (frozen at intended storage temp.) and short term (room temp.) storage conditions.

A. Freeze and thaw stability

Freeze and thaw stability of spiked quality control samples was determined after 3 Freeze and thaw cycles stored at -50C ±0 0C. Compared them to the freshly spiked quality control sample to assess stability. The mean % stability for HQC (6 µg/mL) and LQC(2 µg/mL) was found to be 95.91% and 90.54% respectively.

B. Short term stability

Short term temp. stability of spiked quality control samples was determined for a period of 4 hrs. stored at room temperature. Comparing them against the freshly spiked quality control samples assessed stability. The % mean stability for HQC and LQC are found to be 91.85%, 103.64% respectively.

C) Long-term stability

Long-term stability of the LQC and HQC was determined for a period of 14 days stored at 4ºC, comparing them against the freshly prepared stock solution assessed for stability. The % mean stability for HQC (6 µg/ml) and LQC (2 µg/ml) are found to be 86.00% and 105.17% respectively. The results are summarized in the Table 11.

D) Stock solution stability

Stock solution stability of the drug and IS was determined for 6 hrs at room temperature. Comparing them against the freshly weighed stock solution assessed for stability. The % mean stability for IGURA at HQC & LQC levels was found to be 100.75% and 102.20% respectively. The results are summarized in the Table 11.

Table 11: Summary of stability studies for IGURA and IS.

Stability	Conc (ug/ml)	Mean Stability (%)	%RSD
Freeze thaw stability (three cycles)	**2**	**90.54**	**5.83**
	6	**95.91**	**8.44**
Short term stability (for 4h at RT)	**2**	**103.64**	**3.29**
	6	**91.85**	**7.79**
Long term stability (for 14 days at 4ºC)	**2**	**105.17**	**14.57**
	6	**86.00**	**2.61**
Stock solution stability (for 6h)	**2**	**102.20**	**2.88**
	6	**100.75**	**5.24**
Acceptance Criteria		**85-115%**	**≤15%**

DISCUSSION

There is research article available in literature for Quantification of Iguratimod in Rat Plasma by high performance liquid chromatography.[3] So we have decided to work on human plasma. In current work we have used simply ACN:Water (40:60v/v) as mobile phase and UV detector. Thus the developed method is rapid and simple.

CONCLUSION

A new, simple and rapid method for quantification of Iguratiomd in human plasma using HPLC with UV detection has been developed. The method reported here uses as simple and effective extraction technique with good and reproducible recovery. This method can be used for pharmacokinetic study.

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