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Development and Validation of Estimation of Genotoxic Impurity (Hydroxylamine Hydrochloride Content) in Leflunomide by using RP-HPLC Technique

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ABSTRACT

A simple, selective, linear having accuracy and specific of reverse phase high-performance liquid chromatographic (RP-HPLC) method for determination of Genotoxic impurity, Hydroxylamine hydrochloride of drug Leflunomide is reported. The separation and analysis were done on YMC-Triart C18 (4.6 mm x 150 mm), having particle size 3.0 µm. KH_2PO_4 in 2000 mL of purified water and 2 mL triethylamine with pH 2.5 with phosphoric acid is mobile phase-A, while acetonitrile is mobile Phase-B with gradient program. The elution achieved with 1.50 mL/min flow rate and using UV detection at 230 nm wavelength. Selected column oven temperature is 45°C and auto sampler 5°C respectively. In this method linearity and accuracy of Hydroxylamine hydrochloride covered with specification limit of LOQ to 150% (i.e.3 to 23 ppm). The observed correlation coefficient is 0.99965 and recovery in between 99.07 to 114.94. In method precision (ie. repeatability) and intermediate precision (IP) observed % RSD of six spiked test preparation is below 5.0%. The standard and sample were stable for 3 days when stored at 2 to 8°C temperature. In robustness studies system suitability parameters ie tailing factor, theoretical plates and %RSD does not show significant changes. The present RP-HPLC method is selective, robust, linear, and precise for detection of Hydroxylamine HCl.

Keywords: Hydroxylamine hydrochloride, RP-HPLC, Stability indicating, Genotoxic impurity,

INTRODUCTION

Leflunomide (see Fig. 1) is an immune-suppressive[1] disease-modifying anti-rheumatic drug (DMARD) and mainly use for treatment of active moderate-to-severe rheumatoid arthritis[2,3] and psoriatic arthritis. The chemical name of Drug substance Leflunomide is a 4-Isoxazolecarboxamide, 5-Methyl-N-[4-(trifluromethyl)-phenyl]. It acts inhibitor of synthesis of pyrimidine which works by preventing dihydroorotate dehydrogenase. The molecular formula and molecular weight of Leflunomide is $C_{12}H_9F_3N_2O_2$ and 270.21 respectively.

In manufacturing process, Leflunomide[4] manufactured from starting material 4-(trifluromethyl)

aniline (TFMA) and 5-Methylisoxazole-4-carboxylic acid (5-MIA) respectively. In that of key starting material 5-MIA is shows the potential genotoxic agent. Therefore occurrence of this Hydroxylamine HCl impurity needs to investigate in the Leflunomide. In literature, no particular method is reported for the determination of Hydroxylamine hydrochloride content present in the Leflunomide. The aim of present study is to developing sensitive, cost effective, and validated RP-HPLC method for content of Hydroxylamine HCl impurity in Leflunomide.

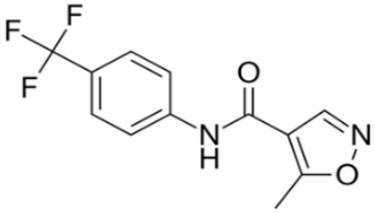

Fig. 1. Leflunomide Chemical structure

MATERIALS AND METHOD

The sample of Leflunomide and its impurity for Development and validation are received from Emcure pharmaceuticals ltd, R & D, Hinjawadi, Pune. Analytical grade potassium dihydrogen phosphate, $Na_2HPO_4.2H_2O$ and purified water (HPLC grade) used for mobile phase and diluent preparations. The acetonitrile and triethylamine used are of gradient grade. The 3,5-dinitobenzoyl chloride used in preparation of derivatized reagent. Analytical balance is used of make- metler Toledo and waters HPLC with UV/PDA detector and data acquisition, calculation with Chromeleon software. All the instruments calibrated before used.

Mobile phase-A

Homogeneous mixture containing 0.14% of KH_2PO_4 and triethylamine with 0.1% in water and adjust pH 2.5 with phosphoric acid.

Mobile Phase B

Acetonitrile

Diluent

0.05% hydrochloric acid in water is as diluents.

Derivatized Reagent

0.17% solution by dissolving 3,5-Dinitrobenzoyl chloride in Acetonitrile.

Buffer solution for derivatized

Prepare 0.71% of $Na_2HPO_4.2H_2O$ in water with pH 8.0 with phosphoric acid.

Standard and sample solutions

Prepare standard solution 0.075 ppm and sample solution with 5000 ppm. Then both taken in separate in a 15 mL centrifuge tube, to this add 2.0 mL buffer solution, 2.0 mL Standard and sample solution (separately) and 0.5 mL derivatized reagent, mix well and vortex up to 30 seconds.

Method Development

The Leflunomide and its impurity are polar in nature, therefore method of genotoxic Hydroxylamine hydrochloride is developed with reversed phase chromatography. Non-polar Stationary phase like C4, C8, C18 in RP-HPLC, while polar mobile phase as water, acetonitrile or buffer solution. During this development with respect to stationary, mobile phases other parameters i.e. column compartment temp, diluents, wavelength, and pH plays crucial role. During stationary phase screening from particular Hypersil BDS C18 and YMC Triart, both C18 having (4.6 mm x 150 mm) and particle size3µ. Also both are showing availability with 150 mm and 250 mm length. When the YMC Triart of C18 (4.6 x 150 mm) 3µ is used found better separation of impurity, peak sharpness good having appropriate system suitability i.e. tailing factor, column efficiency.

Here KH_2PO_4 is used for preparation of mobile phase. Thus, homogeneous mixture of 0.14% of KH_2PO_4 and triethylamine 0.1% in water, kept pH 2.5 with addition of H_3PO_4 and degas (Mobile Phase A) whereas the degased acetonitrile for mobile phase B. The tot run time of analysis is 40 minutes. The appropriate gradient program, flow rate, temp. of column oven, auto sampler temp. is selected by performing different trial runs of standard preparation. Table 1 shows details of chromatographic conditions.

Furthermore, the gradient program has been used to perform the HPLC analysis, the composition 60:40 of mobile phase A and B has been used initially, which modified to 30:70 for 23 min and maintain up to 28 minute. Finally, composition brought to initial value 60:40 in 1.0 min and maintain throughout the run i.e. up to 40 minute.

Table 1: Content of Hydroxylamine hydrochloride impurity chromatographic condition for RP-HPLC

Component	Specification
Apparatus	HPLC with UV/PDA detector, injector, pump, and recorder
Detector	UV/PDA detector
Column	YMC Triart C18 (150 X 4.6) mm, 3.0µ
λmax	230 Nano meter
M.P flow	1.5 mL/min
Volume of Injection	1.0 micro liter
Column oven temp.	45°C
Auto sampler temp.	5°C
Run time	40 min

RESULTS AND DESCUSION

This validation and development study was carried with reference as per IP, BP, USP and Q2 (R1)[5-8] of ICH guideline. The ICH guideline M7 (R1)[9] utilized for finalization of specification limit based on duration of treatment and dose. The details of validation parameters are discussed below.

Specificity

Selectivity study parameter was performed by injecting Blank (diluent), standard (0.075 ppm hydroxylamine hydrochloride) and sample solution (5000 ppm). The chromatograms are analysed at same wavelength mentioned in method. The specificity data given in to Table 2 and related chromatogram in Fig. 2. No interference of Blank (diluent) at retention time of hydroxylamine hydrochloride peak. In sample solution all known as well as unknown peaks are well separated from each other. The observed value of peak purity is higher than 950, shows peak is pure.

Table 2: Data of specificity of hydroxylamine hydrochloride in Leflunomide

Impurities name	Individual solution		Spiked test preparation	
	Retention time (minutes)	Peak purity	Retention time (minutes)	Peak purity
TFMA	23.901	970.1	23.875	1000
MIA	ND	-	ND	-
HCA	4.987	1000.0	4.960	1000.0
m-TLFM	12.848	999.8	12.848	999.9
3-LFM	12.888	999.7		
Hydroxylamine hydrochloride	11.541	995.6	11.547	997.3

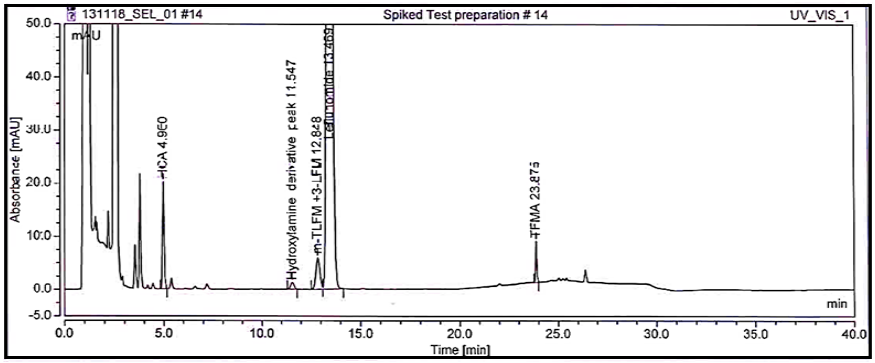

Fig. 2. Spiked Impurities in Leflunomide Typical chromatogram for Selectivity

Limit of detection (LOD) and limit of quantitation

Limit of detection and Limit of quantitation conc. of hydroxylamine hydrochloride impurity in Leflunomide was determined by applying signal-to-noise ratio method. To establish the predicted LOD concentration and LOQ concentration, injecting the various concentration levels (between 10 to 100%) of standard solutions of hydroxylamine hydrochloride limit level concentrations. The predicted LOQ concentration value for hydroxylamine hydrochloride was 3.0 ppm. The LOD concentration evaluate by multiplying factor 0.33 to predicated LOQ concentration. The predicated LOD and LOQ values is shown in Table 3.

Linearity and Range

The linearity methods ability to get test results having proportional to its conc. of analyte in respective test sample. The linearity study carried out standard solutions of hydroxylamine hydrochloride with LOQ Level to 150% specification limit (encompassing 50, 80, 100, 120 and 150 %) of concentration.

Table 3: LOD and LOQ data in hydroxylamine hydrochloride

Name of Impurity	Conc. w.r.t test (in ppm)		s/n ratio	
	LOQ level	LOD level	LOQ level	LOD level
Hydroxylamine hydrochloride	3.0	1.0	22.0	5.0

The correlation coefficient, slope, concentrations and intercept of linearity data are reported in Table 4 and linearity graph presented in Fig. 3. The peak area verses concentration data was analysed by least squares linear regression analysis. The correlation coefficient observed for Hydroxylamine hydrochloride is 0.99965 which is greater than 0.999.

Table 4: Linearity data for the Hydroxylamine hydrochloride impurity (LOQ to 150% Concentration)

Linearity Levels	Concentration (ppm)	Peak area			
		Inj-1	Inj-2	Inj-3	Average(n=3)
LL-1 (LOQ)	3.102	2415	2358	2489	2421
LL-2 (50%)	7.525	6025	6154	6045	6075
LL-3 (80%)	12.154	9368	9457	9365	9397
LL-4 (100%)	15.284	12257	12184	12184	12208
LL-5 (120%)	18.254	14587	14379	14496	14487
LL-6 (150%)	23.158	18578	18657	18760	18665
	Correlation coefficient				0.99965
	Intercept				125.8423
	%Y Intercept				1.03

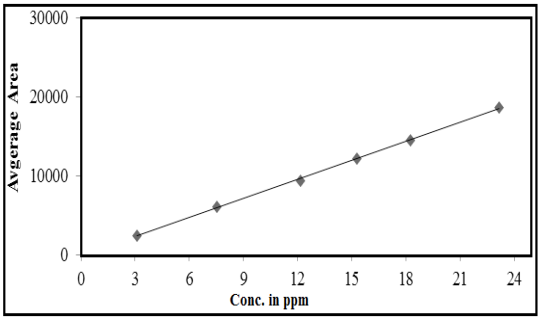

Fig. 3. Linearity graph for the Hydroxylamine hydrochloride impurity content from LOQ to 150% concentration range

Precision

System precision was performed by injecting five replicate of standard preparation as per mentioned in method of analysis. The observed percent RSD for replicate injections is 1.51 and tailing factor is 1.05. For Method precision six different sample prepared and analysed, And Intermediate precision six different sample preparations by different day, different system, and different column and analysed. In method precision and Intermediate precision observed %RSD is 1.40 and 1.66 respectively. For twelve test preparations (six from method precision and six from intermediate precision) Overall %RSD is 1.46, which is less than 5.0%. Method precision and intermediate precision results are tabulated in Table 5.

Table 5: Hydroxylamine hydrochloride impurity result of method and intermediate precision

Spiked sample solutions	% of Hydroxylamine HCl impurity (in ppm)	
	Method Precision	Intermediate Precision
Preparation -1	15.3211	14.7652
Preparation -2	14.9423	15.2312
Preparation -3	15.0180	15.3412
Preparation -4	15.2010	14.9342
Preparation -5	15.1230	15.2242
Preparation -6	14.7162	14.7812
Mean	15.0536	15.0462
SD	0.21	0.25
RSD	1.40	1.66
Overall Mean (n=12)	15.0499	
Overall SD (n=12)	0.22	
Overall% RSD(n=12)	1.46	

Accuracy

Accuracy of method was determined by spiking test preparation with impurity at LOQ Level, 50% level, 100 and 150% of specification limit concentrations. The %accuracy data of hydroxylamine hydrochloride is presented in Table 6. The observed % accuracy at LOQ Level and 50% level, 100 and 150% is between 94.23 to 114.36% which is within acceptance criteria. (Accuracy should be between 70 to 130%)

Robustness

The method robustness verified by altering flow rate by ±10%. Original flow rate of 1.5 mL/min is altered as 1.35 mL/min and 1.65 mL/minute. The column oven temp. is changed with ±5°C from 40°C in actual method. The observed area, standard

deviation and its %RSD are listed in Table 7. In all above study the retention times are varied by ±0.2 mins compared to original retention times. System suitability parameter obtained as tailing factor 1.01 to 1.15 and theoretical plates as18433 to 20455. The %RSD for robustness studies are from 1.23 to 2.50. The results in Table 6 indicated that change in method parameters (flow rate and column oven temperature), will no significant impact on system suitability criteria tailing factor, theoretical plates and %RSD. The obtained results are well within acceptance limit.

Table 6: The % accuracy data of hydroxylamine hydrochloride impurity

Tests	LOQ Level	50% Level	100% Level	150% Level
Preparation-1	111.51	99.86	101.71	100.72
Preparation-2	114.36	99.50	94.25	97.92
Preparation-3	108.94	103.71	101.25	101.84
Mean	111.60	101.02	99.07	100.16
SD	2.71	2.33	4.18	2.02
%RSD	2.43	2.31	4.22	2.02

Table 7: Retention time, Theoretical plates, Tailing factor, and RSD of Robustness study for the hydroxylamine hydrochloride

System suitability parameters	Hydroxylamine Hydrochloride			
	Mobile phase Flow rate		Column oven temperature	
	1.35 mL/min	1.65 mL/min	35°C	45°C
Retention time	12.836	12.645	12.818	12.798
Tailing factor	1.15	1.09	1.01	1.08
Theoretical plates	19785	18433	19125	20455
%RSD (n=6) replicate of standard preparation	2.50	1.99	1.23	2.01

Solution Stability

Solution stability of test preparation was performed at the 2 to 8°C temp. on the day basis up to 3 days. Cumulative %RSD values of Hydroxylamine hydrochloride are well within acceptance criteria up to 1 day. This indicates that Analytical test preparations is stable for 1 day, when stored at 2 to 8°C temperature.

Mobile phase stability

Mobile phase prepared as per method of analysis and performed analysis. After completion of analysis store a mobile phase at room temperature and demonstrate mobile phase stability. Check and compared system suitability parameter initial analysis and mobile stability study analysis. The %RSD and change in retention time in standard hydroxylamine hydrochloride is within criteria and no haziness, precipitation and appearance of mobile phase is observed up to 60 hrs. Hence at room temperature mobile phase stability is 60 hours.

CONCLUSION

RP-HPLC method of hydroxylamine hydrochloride content analysis of Leflunomide is highly precise, selective, accurate with stability indicating and as per the ICH guidelines Q2(R1) is developed accurately and successfully validated. The specificity shows that, Hydroxylamine peak is fully resolved from known as well as unknown impurities. Method is linear with LOQ to 150% level w.r.t specification concentration and observed Correlation coefficient is 0.99965. The recovery of Hydroxylamine hydrochloride was achieved between 94.23 to 114.60%. In Robustness study system suitability like tailing factor, theoretical plates and %RSD does not show significant impact. The observed results found within acceptable limits. The validated method shows satisfactory data for all tested method parameters. Hence present method specific, linear, selective, precise robust, as well as stable and can effectively useful in analysis.

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Conflict of interest

Conflict of interest declared none.

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