

A Validated Stability Indicating Reversed Phase High Performance Liquid Chromatographic Method of Leflunomide and Characterization of Its Degradation Products through Retro-Synthesis

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ABSTRACT

The present paper deals with the development of stability indicating a reversed phase high-performance liquid chromatographic (RP-HPLC) method for leflunomide, a disease-modifying antirheumatic drug in presence of its degradation products formed during forced decomposition studies. Forced degradation studies were performed on the bulk drug by using acid (0.1 N hydrochloric acid), base (0.1 N sodium hydroxide), water (neutral hydrolysis), 3% v/v hydrogen peroxide (oxidation), dry heat (60°C) and UV light (254 nm). Degradation was observed for leflunomide in acidic and basic media only and the formed degradation products were found to be 5-methylisoxazole-4-carboxylic acid (degradation product-1) and 4-(trifluoromethyl)-aniline (degradation product-2). Successful separation of the drug from the degradation products formed under different stress conditions was achieved on a Novapak C18 column (150 mm × 3.9 mm, 4 μm particle size) using methanol-phosphate buffer (pH 5.3; 20 mM) (7:3, v/v) as the mobile phase at a flow rate of 1 mL/min. The detection wavelength was 260 nm. The developed method was completely validated and proved to be robust. As the method could effectively separate the drug from its degradation products, it can be employed for analysis of the samples of stability study.

INTRODUCTION

The chemical name of leflunomide is N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide (Kher *et al.*, 2011), which is an isoxazole derivative and inhibitor of de novo pyrimidine synthesis (Katarzyna *et al.*, 1998; Migita *et al.*, 2005), represents a new class of disease modifying anti rheumatic drugs (Yadav *et al.*, 2010). The primary mode of action of leflunomide is specific inhibition of dihydro-orotate dehydrogenase, a key enzyme in the de novo synthesis of pyrimidine, and subsequent inhibition of RNA and DNA

synthesis (Fox, 1998), also a blockade of tumor necrosis factor mediated activation of the transcription factor NF-κB (Manna and Aggarwal, 1999). During our literature survey, very few articles related to the spectrophotometric (Abbas *et al.*, 2006; Shokry *et al.*, 2012), HPLC (Vivien *et al.*, 2004; Miron *et al.*, 2006; Yeniceli *et al.*, 2006; Rao *et al.*, 2008; Kher *et al.*, 2011) and LC-MS (Molinari *et al.*, 2005) methods of analysis of leflunomide were found. However, most of these reported methods were related to the routine analysis of leflunomide in pharmaceutical dosage forms or metabolite study in human plasma. To our current knowledge, no article related to the stability indicating high performance liquid chromatographic (HPLC) determination of leflunomide and characterization of its degradation products has been reported yet, as revealed by literature survey. Therefore the aim of this study was to develop and validate stability-indicating reversed phase high performance liquid chromatography (RP-HPLC) method

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for determination of leflunomide in presence of its degradation products formed under different stress conditions. The stability assessment of any promising drug candidate plays a vital role in its preformulation study. Many environmental conditions such as heat, light, moisture as well as the inherent chemical susceptibility of a compound to hydrolysis or oxidation play an important role in pharmaceutical stability. So, this study also helps to define storage and handling conditions. The exposition of the drug substance to extreme external conditions helps to reveal and identify the likely degradation products which will open a new scope of research on toxicity study. The findings of toxicity study will help in the scrupulous determination of expiry, adverse effects etc.

MATERIALS AND METHODS

Materials

Pure leflunomide (Fig. 1a) was provided by Sun Pharmaceutical Industries Ltd., Sikkim, India and its degradation products namely 5-methylisoxazole-4-carboxylic acid (Fig. 1c) and 4-(trifluoromethyl)-aniline (Fig. 1b) were synthesized by conventional synthetic procedures in our own laboratory. The probable degraded compounds were divined through retrosynthesis. The probable degradation is depicted in Fig. 1. Methanol and water of HPLC grade and were purchased from Merck (India) Ltd., Mumbai, India. All other chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich, Mumbai, India.

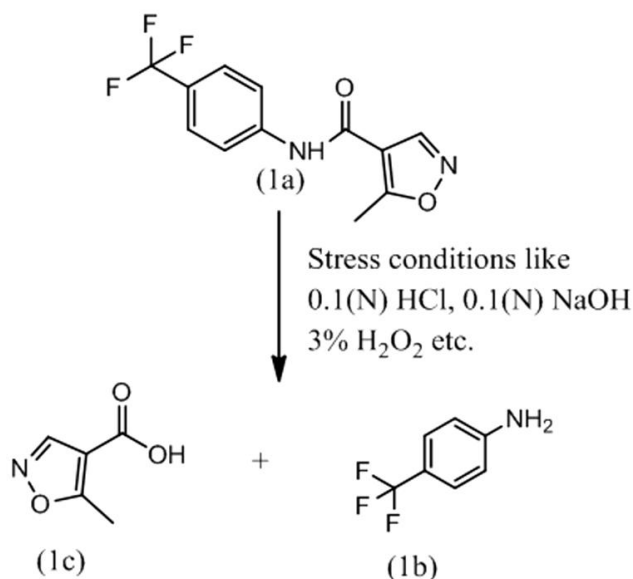


Fig. 1: The probable degradation products of pure leflunomide.

Methods

Apparatus

Experiments were performed using a Waters (India) 510 HPLC system with Waters 486 tunable absorbance detector. The samples were injected manually using a 20 μ L sample loop. The

Millennium software was used for quantification and data processing.

Synthesis of 5-methylisoxazole-4-carboxylic acid

A 50 mL solution of hydroxylamine hydrochloride (A) (19 g, 0.27 mol) and sodium acetate trihydrate (B) (37 g, 0.27 mol) was added to another 50 mL ethanol solution containing (*E*)-ethyl 2-(ethoxymethylene)-3-oxobutanoate (C) (50.27 g, 0.27 mol). The mixture was stirred for 2 h and kept overnight at 0°C. Then the product 5-methylisoxazole-4-carboxylate was extracted with dichloromethane (3 \times 30 mL). Thereafter, it was refluxed together with acetic acid (30 mL), water (30 mL), and concentrated HCl (30 mL) for 10 h to get the crude 5-Methylisoxazole-4-carboxylic acid. The final compound was recrystallized from ethanol to get the pure 5-Methylisoxazole-4-carboxylic acid (D) (26.07 g). The synthetic pathway is shown in Fig. 2.

White solid, Yield 76%, 26.07 g, mp 145-148°C; IR (KBr disk): 3114 (C-H Str), 3081.46 (O-H Str), 1696.24 (C=O Str), 1602.12 (C-C Ar Ring), 1211 (C-O Str). ¹H NMR: 300MHz, DMSO-d₆: δ 13.12 (1H, br, -COOH), 8.771 (1H, s, Ar-H), 2.61 (1H, s, -CH₃). ¹³C NMR: 75MHz, DMSO-d₆: 173.76, 162.53, 150.77, 109.83, 12.09. MS: 128.08 (M+H).

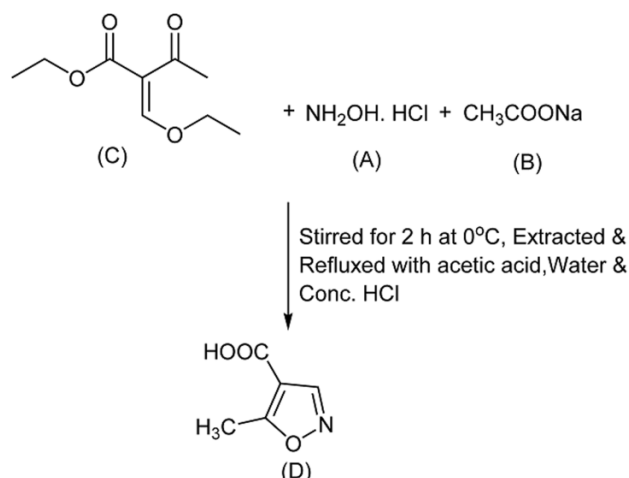


Fig. 2: Synthetic pathway of 5-methylisoxazole-4-carboxylic acid.

Synthesis of 4-(trifluoromethyl)-aniline

A mixture of 96% by weight of sulphuric acid (56 g) and 100% fuming nitric acid (56 g) was admixed with benzotrichloride (A) (100 g) at -7 to 0°C by drop wise addition over 4 h. The reaction mixture was then initially warmed to 10°C for 30 min then discharged onto the ice and finally extracted with dichloromethane (3 \times 50 mL). The combined organic phase was washed with water (100 mL), saturated aqueous sodium bicarbonate solution (3 \times 50 mL) and aqueous sodium chloride solution (50 mL). The organic phase was dried over anhydrous magnesium sulphate and evaporated. A liquid product (B) (120.7 g), a mixture of 80.6% of 3-nitrobenzotrichloride and 19.4% of 4-nitrobenzotrichloride was obtained. After calculation about 113 g of the 3- and 4-nitrobenzotrichloride mixture was then added drop-wise to 85 mL of anhydrous hydrofluoric acid at an internal temperature between 2

and 7°C in course of 15 min. This was followed by stirring for 17 h at room temperature and 8 h at 150°C and 25 bar pressure, in a reaction autoclave. The reaction mixture was cooled down to room temperature and distilled to remove excess of hydrofluoric acid. The residue was distilled to obtain 69 g of a mixture of 3- and 4-nitrobenzotrifluoride (C). This mixture was separated by final distillation to obtain 52.16 g of 3-nitrobenzotrifluoride and 11.05 g of 4-nitrobenzotrifluoride (D). About 11 g of the prepared 4-nitrobenzotrifluoride and 1 g of Raney nickel were added to 100 mL of methanol and subjected to a pressure of 10 bar at °C for 40 h. Thereafter, the reaction mixture was filtered and the filtrate was evaporated to obtain 8 g of 4-trifluoromethylaniline (E). The compound was purified by using flash chromatography. The synthetic pathway is shown in Fig. 3.

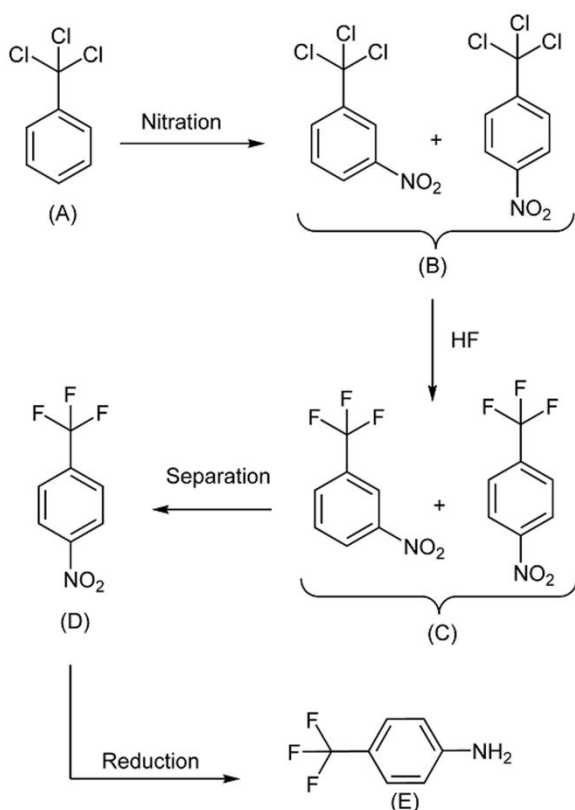


Fig. 3: Synthetic pathway of 4-(trifluoromethyl)-aniline.

White solid, Yield 82%, 7.60 g, mp 5-8°C, bp 82-84°C; IR (KBr disk): 3504.23, 3422.66 (NH Str), 1645.44 (NH Def), 1332.66 (C-N Str), 1249.32, 1178.14, 1137.92, 1079.82 (C-F str) ¹H NMR: 300MHz, DMSO-d₆: δ 7.35 (2H, d, J=7.8Hz, Ar-H), 6.64 (2H, d, J= 7.8Hz, Ar-H), 3.46 (2H, s, -NH₂). ¹³C NMR: 75MHz, DMSO-d₆: 150.69, 126.39, 125.92, 124.22, 115.67. MS: 162.07 (M+H).

Chromatographic conditions

The experiment was performed on a Novapak C18 (150 mm × 3.9 mm, 4 μm particle size) column using the methanol-phosphate buffer (pH 5.3; 20 mM) (7:3, v/v) as the mobile phase at a flow rate of 1 mL/min. The mobile phase was filtered through a

nylon membrane filter paper (pore size 0.45 μm) and degassed using a sonicator for 10 min. The column temperature was maintained at 25°C and eluents were monitored at a wavelength of 260 nm. The volume of each injection was 20 μL.

Sample preparation

The standard and sample stock solutions of leflunomide were prepared separately in a solvent mixture of methanol-phosphate buffer (pH 5.3; 20 mM) (7:3, v/v) at a concentration of 1000 μg/mL. Working standard solutions were prepared by diluting the standard stock solution with above solvent to get solutions of concentration in the range of 5-100 μg/mL. A stock solution of degradation product-1 and 2 (500 μg/mL) were also prepared separately in the mobile phase.

Degradation studies

All the degradation studies were carried out with drug solution of 100 μg/mL concentration. For acidic and alkaline hydrolysis studies, the drug was mixed with 0.1(N) HCl and 0.1(N) NaOH separately. These mixtures were refluxed on a water bath for 4 h at 60°C. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. The resulting solutions were neutralized by base and acid, respectively to avoid any interference of acid or base in the following steps. 20 μL of resulting solutions were injected into HPLC system and the chromatograms were recorded. About 20.09% and 88.26% degradation of the drug were observed for acidic and basic media, respectively. Among acid hydrolysis products, degradation product-1 was (about 19.19 %) observed at RT 1.150 min and degradation product-2 was (about 0.90%) observed at RT 1.449 min. In basic hydrolysis, degradation product-1 was (about 85.28%) observed at RT 1.150 min and degradation product-2 was (about 2.98%) observed at RT 1.449 min. When leflunomide was subjected to oxidative degradation by treating with 3% hydrogen peroxide solution for 24 h at ambient temperature, no significant change was observed. The aqueous solution of leflunomide refluxed for 6 h on a water bath set at 60°C for wet heat degradation study, no degradation was found. The drug product was also stored in an incubator at 60°C for 72 h for dry heat degradation study and exposing to direct UV light (254 nm) for 24 h for photochemical stability study. The drug was stable in both the cases. In all cases, degradation product-1 and 2 were confirmed by co-injection.

Validation of the Method

The optimized analytical method was validated appropriately with respect to the following parameters.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential degradation products. Degradation conditions employed were UV light (254 nm for 24 h), thermal exposure to 60°C for 72 h, acid hydrolysis with 0.1N HCl, base hydrolysis with 0.1N NaOH, water hydrolysis

and oxidative degradation using 3% H₂O₂ at ambient temperature for 24 h. Peak purity testing was carried out on the stressed samples of leflunomide by using UV detector.

Precision

Precision was established for the developed method by spiking the degradation products at target concentration level in the test solution containing the drug product. Six spiked samples were prepared and injected to prove the precision of the method. The % RSD of all individual degradation products was found to be satisfactory for all the six analytical measurements. This was also repeated on different days to determine inter-day precision. A different scientist established intermediate precision through separation studies on a different chromatographic system. Standard solutions of three different concentrations of leflunomide were prepared and injected six times. The relative standard deviation was determined via the peak area to measure the precision of the LC system.

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ for leflunomide, degradation product-1 and 2 were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of dilute solutions of known concentration (ICH 1995). The precision study was also performed at the LOQ level by injecting six individual preparations of leflunomide, degradation product-1 and 2 by calculating the % RSD of the AUC.

Accuracy

The accuracy of the method was evaluated at three concentration levels (20, 50, and 80 µg/mL) of the drug. Accuracy determination of the degradation products was also carried out six times at 40%, 80% and 120% of the specific concentration level (0.18 %). Finally, recoveries of added drug and degradation products were calculated.

Linearity

Linearity test solutions for the drug were prepared from a stock solution at eleven concentration levels starting from 5 to 100 µg/mL. The standard curve was prepared considering peak area versus concentration data by least-squares linear regression analysis. Linearity test solutions for the degradation products were prepared by diluting stock solution to the required concentrations. The solutions were prepared at eleven concentration levels from LOQ to 200 % of the specification level. The calibration curves were drawn by plotting the peak areas of degradation products against the corresponding concentrations. The slope and Y-intercept of the calibration curve were calculated.

Robustness

To determine robustness, experimental conditions were purposely altered and the resolution of the degradation products and analyte was evaluated. The flow rate was changed by 0.2 units, pH of buffer from 5.1 to 5.5 and the column temperature

was studied at 20°C and 30°C instead of 25°C. In all the above conditions, the components of the mobile phase were held constant.

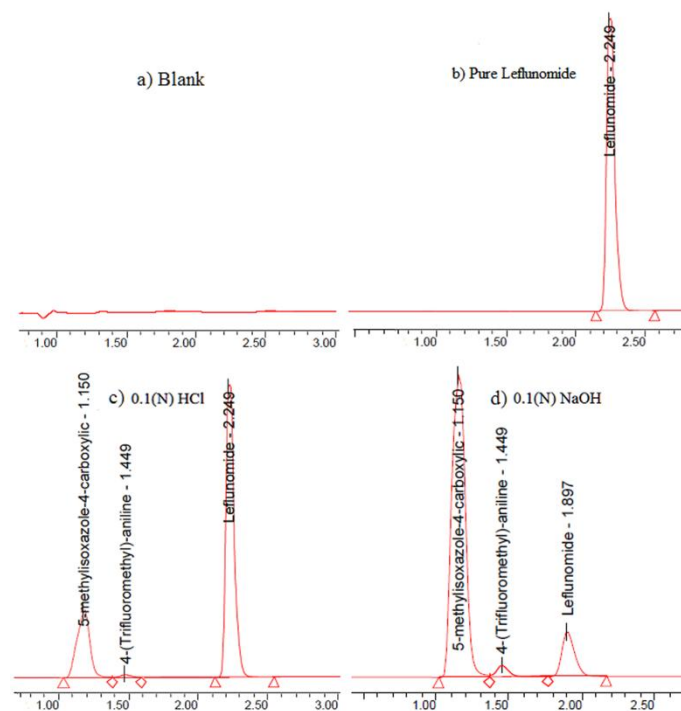
Solution stability and mobile phase stability

The solution and mobile phase stability of leflunomide and its degradation products were carried out by a leaving spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h. The content of leflunomide and degradation products were determined at 12 h intervals. The % RSD for the assay of leflunomide and its degradation products were calculated.

RESULT AND DISCUSSION

Optimization of chromatographic conditions

The main difficulty of the chromatographic method was to get the separation of degradation product-1 from the leflunomide peak. Attempts were made by using different C18 and C8 stationary phases. Effects of pH (2–4) and ionic strength (10–20 mM) were investigated using phosphate and acetate buffers. It was found that the retention time of leflunomide did not significantly alter at pH 2–4 and ionic strength between 10 mM and 20 mM. But the change in the organic composition of mobile phase affects the separation of degradation product-1 from leflunomide. So the organic composition of the mobile phase is critical for the separation of degradation product-1 from leflunomide. The optimum conditions are given above. In optimized chromatographic conditions leflunomide, degradation product-1 and 2 were well separated with resolution greater than 2, typical retention times were shown in Fig. 4.



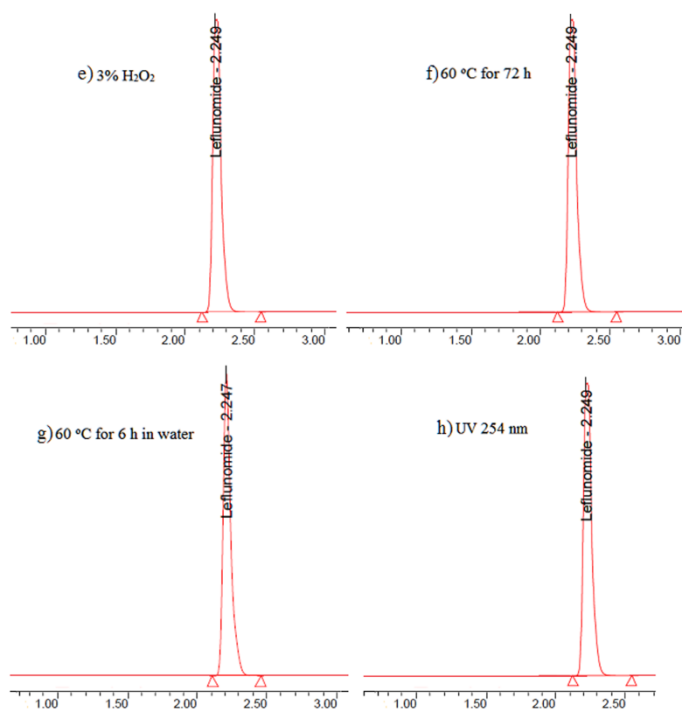


Fig. 4: Representative HPLC chromatograms of leflunomide pure and stressed samples.

Results of forced degradation studies

Leflunomide was degraded in acidic and basic media only (Fig. 4). Peak purity test results confirmed that the Leflunomide peak is homogenous and pure in all the analyzed stress samples. The assay of leflunomide is unaffected in the presence of its degradation products, confirms the stability indicating power of the developed analytical method. The summary of forced degradation studies is given in Table 1.

Table 1: Summary report of forced degradation study.

Stress condition	Time (h)	% Assay of active substance	% Assay of degradation products	Mass balance* (%)
Acid Hydrolysis (0.1N HCl)	4	79.89	20.09	99.98
Basic Hydrolysis (0.1N NaOH)	4	11.73	88.26	99.99
Oxidation (3% H ₂ O ₂)	24	100.01	-	100.01
Dry Heat (60°C)	72	99.99	-	99.99
Wet Heat (60°C)	6	100	-	100
UV (254 nm)	24	99.99	-	99.99

*It is the summation of assay of active substance and degradation products.

Table 2: Intra and Inter assay precision of leflunomide.

Conc. of leflunomide (µg/mL)	Observed concentration of leflunomide by the proposed method(µg/mL).			
	Intra-Day Mean (n=6)	% CV	Inter-Day Mean (n=6)	% CV
20	19.97	0.27	19.98	0.21
50	50.01	0.11	49.99	0.38
90	89.98	0.29	89.95	0.31

n= Number of determinations.

Table 3: Intra and Inter assay precision of degradation products

Sample	Actual concentration (µg/mL)	Intra-Day Mean (n=6)	%CV	Inter-Day Mean (n=6)	% CV
Degradation product -1	0.06	0.061	0.19	0.062	0.16
	0.12	0.121	0.23	0.122	0.18
	0.18	0.179	0.11	0.181	0.17
Degradation product -2	0.06	0.059	0.17	0.061	0.21
	0.12	0.122	0.45	0.121	0.52
	0.18	0.181	0.25	0.180	0.32

n= Number of determinations.

Precision

The intra and inter assay coefficient of variation for assay of leflunomide during the assay method precision study were within 0.29 and 0.38%, respectively (Table 2).

The intra assay and inter assay coefficient of variation for degradation product-1 and 2 in the precision study were within 0.45 and 0.52%, respectively (Table 3), confirming good precision of the method.

Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification of leflunomide was 0.071 µg/mL and 0.243 µg/mL respectively for 20 µl injection volume. The coefficient of variation of leflunomide at LOQ concentration was below 2.1%. The limit of detection of degradation product-1 and 2 were 0.042 and 0.036 µg/mL respectively for 20 µl injection volume. The limit of quantification of degradation product-1 and 2 were 0.12 and 0.13 µg/mL respectively for 20 µl injection volume. The coefficients of variation at LOQ concentration for degradation product-1 and 2 were below 0.6%.

Accuracy

The percentage recovery of leflunomide in formulation samples ranged from 99.95 to 99.98. The percentage recovery of degradation product-1 and 2 in stress samples ranged from 98.62 to 100 (Table 4).

Linearity

Linear calibration plots for leflunomide, degradation product-1 and 2 were obtained by the calibration ranges tested, i.e., 5-100 µg/mL for leflunomide and for degradation products-1 and 2 from LOQ to 200 % of the specification level. In all the cases, the correlation coefficients obtained were greater than 0.999.

Table 4: Recovery result of leflunomide and its degradation products.

Pure drug added (μg)	Recovery from tablet formulation		Degradation products (dp)	Amount spiked ($\mu\text{g/mL}$)	Amount recovered ($\mu\text{g/mL}$)	Mean recovery* (%) (n=6)	CV (%)
	Mean (\pm SD) amount (μg) found* (n=6)	Mean (\pm SD) % recovery* (n=6)					
			At 40% level				
			dp-1	0.072			
20	19.99 (\pm 0.62)	99.95 (\pm 0.51)	dp-2	0.072	0.072	100.0	0.33
			At 80% level				
50	49.99 (\pm 0.87)	99.98 (\pm 0.72)	dp-1	0.144	0.144	100.0	0.36
			dp-2	0.144	0.143	99.30	0.51
80	79.98 (\pm 0.58)	99.97 (\pm 0.47)	At 120% level				
			dp-1	0.216	0.215	99.53	0.42
			dp-2	0.216	0.215	99.53	0.43

*Each data represent the average of 6 readings.

Robustness

In all the deliberately varied chromatographic conditions (flow rate, pH and column temperature), the resolution between impurities and analyte was found to be more than 2.0.

Solution stability and mobile phase stability

The % RSD of the assay of leflunomide during solution stability experiment was within 1.2%. No significant changes were observed in the content of degradation product-1 and 2 during solution stability and mobile phase stability experiments when performed using related substances method. The solution stability and mobile phase stability experiments data confirm that sample solutions and mobile phase used during the assay and related substance determination were stable up to 48 h.

CONCLUSION

The reverse phase high performance liquid chromatography (HPLC) method developed for quantitative and related substance determination of leflunomide is precise, accurate, rapid and specific. Acidic and basic hydrolysis of leflunomide yielded two degradation products. They were characterized as 5-methylisoxazole-4-carboxylic acid and 4-(trifluoromethyl)-aniline. The method was completely validated showing satisfactory data for all the method validation parameters. The developed method can be used for routine analysis and also to check the stability of leflunomide.

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