DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF LEFLUNOMIDE IN ITS PHARMACEUTICAL DOSAGE FORM

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RESEARCH ARTICLE

Sola Kavita A*, Dedhiya Praful P, Shah Shailesh A

Maliba Pharmacy College, Bardoli, Gujarat, India - 394 350

*Corresponding Author's E-mail: kavitasola999@gmail.com

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ABSTRACT

A specific, accurate, precise and robust HPTLC method has been developed for estimation of Leflunomide in its pharmaceutical dosage form. The chromatographic separation was performed using Aluminum backed precoated with silica gel $60F_{254}$ as stationary phase and toluene: ethyl acetate: glacial acetic acid (8:2:0.5 %, v/v/v) as mobile phase. The quantification was carried out at 270 nm wavelength. The method was validated as per ICH Q2 (R1) guidelines. The R_f value was found to be 0.53 ± 0.02. The linearity of method was satisfactory over the range 25-125 ng/spot with correlation coefficient of 0.9960.The limit of detection was found to be 1.71 ng. The limit of quantitation was found to be 5.19 ng. The recovery was found in the range 99.67-100.89%.The method was successfully applied to marketed formulations of Leflunomide.

Keywords: Leflunomide, Estimation, HPTLC.

INTRODUCTION

Leflunomide chemically is 5-methyl -N- [4-(trifluoromethyl) phenyl] – isoxazole -4 - carboxamide (Figure.1). It is prodrug. It acts through its active metabolites A77-1726 which inhibits dihydro-orotate dehydrogenase (DHODH) enzyme. (1, 2) Leflunomide is official in IP, BP and USP. (3-5) Literature survey revealed different analytical methods for analysis of Leflunomide including Spectrophotometric (6-8), High performance liquid chromatography (HPLC), (9-20) Ultra performance liquid chromatography (UPLC) (21), Liquid chromatography - Mass spectrometry (LC-MS), (22) in pharmaceutical formulations and biological samples.

Analysis of pharmaceuticals is an integral part of the complete drug-development process. Hence rapid and simple methods for routine analysis and quality control of commercial formulations are very desirable. The European Pharmacopeia and the United States Pharmacopeia, suggest both elaboration of new methods which reduce the amount of reagents and materials used. High-performance thin-layer chromatography (HPTLC) is a flexible, versatile, economical process in which the various stages are carried out independently. The benefits of this offline arrangement compared with an online process such as HPLC have been outlined. HPTLC is a highly productive and cost effective separation technique, because several samples can be chromatograph simultaneously with very small amounts of solvents in comparison with HPLC. (23-25)

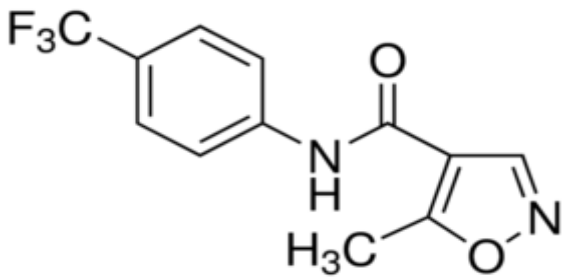

Figure 1: Chemical structure of Leflunomide

The objective of the present study was to develop and validate a simple, sensitive, accurate, specific, robust and reproducible HPTLC method for determination of Leflunomide in bulk and pharmaceutical

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formulation rapidly and at low cost in routine analysis in accordance with ICH guidelines. (26)

EXPERIMENT

Material and Methods

Leflunomide was supplied as a gift sample by Formosa Laboratory, Taiwan, China. Methanol, toluene, ethyl acetate of LR grade was procured from Rankem, Mumbai, India. Glacial acetic acid was supplied by sd fine chems Ltd, India. The chromatographic estimation was performed by spotting standards and extracted samples of Leflunomide on pre-coated silica gel aluminum plate 60_{F-254} (10 cm x10 cm with 250µm thickness, E.Merck, Darmstadt, Gemany) using a Camag Linomat V sample applicator (Camag, Muttenz, Switzerland) and a 100µl Hamilton syringe. The samples, in the form of band of 6 mm, were spotted 15 mm from the bottom, 10 mm apart, at a constant application rate of 150 nL/s using nitrogen aspirator. Plates were washed with methanol and activated at 120°C before use. Plates were developed using a mobile phase consisting toluene-ethylacetate-glacial acetic acid (8:2:0.5, v/v/v). Linear ascending development was carried out in 10 cm x 10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature. The length of chromatogram run was 75 mm. Approximately 10.5 ml of the mobile phase was used for each development, which required 15 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer. The slit dimension settings of length 4.00 mm, Width 0.30mm, and scanning rate of 20mm/s was employed. Densitometric scanning was performed on Camag TLC scanner IV in the absorbance mode at 270 nm and operated by winCATS planar chromatography software. The Source radiation utilized was deuterium lamp. Evaluation was done by measuring peak areas with linear regression.

Preparation of standard solutions

Accurately weighed 25 mg of standard Leflunomide was transferred to 50 ml volumetric flask, dissolved in and diluted to mark with methanol (500µg/ml). From stock solution, one ml aliquot was transferred to 10 ml volumetric flask and diluted to mark with methanol (50µg/ml). Further, 1 ml was transferred to 10 ml volumetric flask and diluted to mark with methanol to obtain a working standard solution (5µg/ml).

Calibration curve (Linearity)

To construct calibration curve , 5, 10, 15, 20 and 25µl of working standard solution (5µg/ml) of Leflunomide (equivalent to 25, 50, 75, 100 and 125 ng per band) were applied to five plates. The plates were developed and scanned as described above. Peak areas were recorded at each concentration and were treated by linear least-square regression analysis.

Method validation

The HPTLC method was developed and validated as per the recommendations of International council for Harmonization (ICH) guidelines for following parameter. (26)

Precision

Precision studies were done in terms of repeatability and intermediate (intraday and interday) precision expressed as the percentage R.S.D. of a series of measurements.

Repeatability studies were carried out by six replicate reading of 75 ng/band concentration of Leflunomide on same day. Intermediate precision were evaluated by three times replicate reading at three different concentrations (50, 75,100 ng per band) on same day (Intraday) and on three different days (Inter day)

Accuracy (% Recovery)

The accuracy of the method was studied by determination of percentage recovery of known amounts of Leflunomide standards added to solutions of the corresponding commercial product within the linear range. Previously analyzed samples were spiked with an extra 80, 100 and 120% of Leflunomide standards. The resultant solutions were then analyzed in triplicate by proposed methods.

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Robustness

Robustness was evaluated by studying the influence of small, but deliberate changes to the analytical parameters on the peak area. The method should be robust enough with respect to all critical parameters so as to allow routine laboratory use. Small changes in the mobile phase composition, chamber saturation time and volume of modifier were introduced, and the effects on the results were examined. Robustness of the method was determined in triplicate at 75 ng/band concentration level of Leflunomide and percentage RSD of peak areas was calculated.

Sensitivity

The sensitivity of the method was determined in terms to limit of detection (LOD) and limit of quantitation (LOQ) for Leflunomide as follow as:

LOD or LOD = K* σ/S

Where, K is a numerical constant 3.3 for LOD, 10 for LOQ, σ is the sensitivity parameter (Expressed here by the slop of the standard curve).

Specificity and Selectivity

Specificity of the method was ascertained by analyzing standard drug and samples of Leflunomide at equivalent concentration. The specificity of the method was established by analyzing marketed tablet as an experimental sample together with the reference standard using proposed method. The spot for Leflunomide in sample was confirmed by comparing the R_f and UV spectra of the spot in samples with those of standard. The peak purity of samples was judged by comparing the spectra at peak start, peak apex and peak end positions of the spot.

The selectivity of an assay is a measure of the extent to which the method can determined a particular compound in the analyzed matrices without interference from matrix components. For each sample, UV spectra taken at the edges and maxima of the Leflunomide peaks were compared automatically to verify peak purity.

Assay of Leflunomide in Tablets

Twenty tablets were weighed and finely powdered. The quantity of the powder equivalent to 10 mg of Leflunomide was transferred to 50 ml volumetric flask. The flask was filled to about 80 % with methanol, sonicated for 15 min and diluted to mark with methanol. The solution was filtered through Whatman filter; first few ml was discarded. One ml aliquot from filtrate was diluted to 10 ml with methanol. 15µl from resulting solution was spotted on TLC plate to obtained concentration 75ng per band of Leflunomide in the linearity range.

RESULTS AND DISCUSSION

Optimization of Mobile phase

In an attempt to optimize the mobile phase, toluene-ethyl acetate-glacial acetic acid mixtures in different proportions were investigated. A mobile phase consisting of toluene-ethyl acetate-glacial acetic acid (8:2:0.5, v/v/v) resulted in sharp, well defined Leflunomide peaks from its matrix. It was also observed that chamber saturation time and solvent migration distance were crucial in the chromatographic separation as chamber saturation time of less than 30 min and solvent migration distances greater than 80 mm resulted in diffusion of the analyte band. Therefore, toluene-ethyl acetate-glacial acetic acid (8:2:0.5, v/v/v) mobile phase with a chamber saturation time of 30 min at 25°C and solvent migration distance of 75 mm was used. These densitometry conditions produced a sharp, well-defined Leflunomide peak having an R_f value 0.53 ± 0.02.

Validation of the Method

The method is validated as per ICH guidelines as follows (26);

Linearity and range (Calibration plots)

The linear regression analysis data for the calibration plots showed a good linear relationship

(r^2 =0.9960) with respect to peak area in the concentration range of 25-125 ng/band. Figure 3 displays a three-dimensional overlay of HPTLC

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densitograms of the calibration bands of Leflunomide at 270nm in absorbance mode. Peak area and concentration was subjected to least-square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data as shown in Table 1 shows a good linear relationship over the concentration range 25-125 ng/band, demonstrating the suitability of the method analysis.

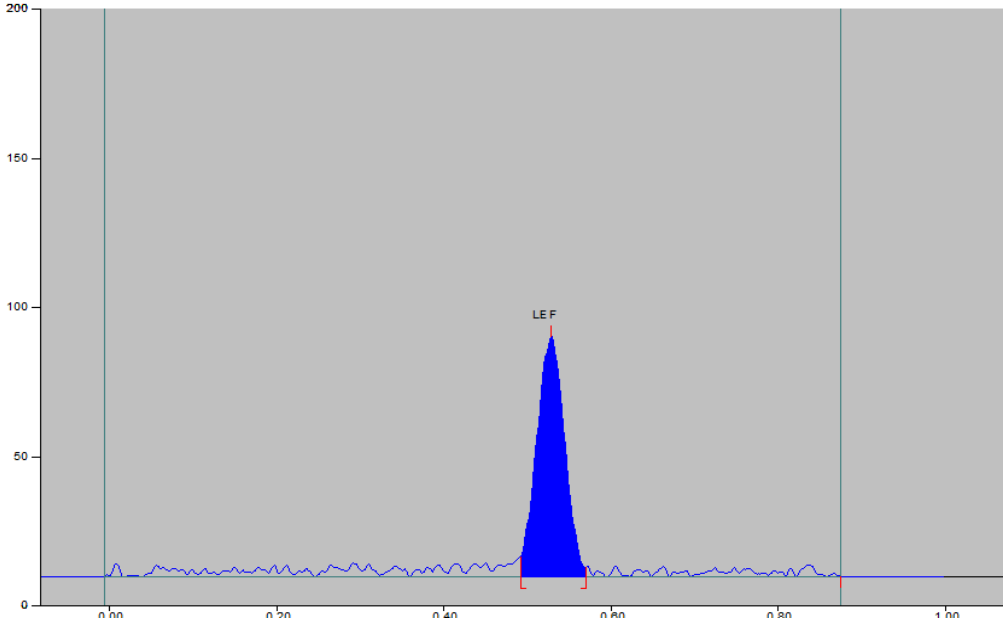

Figure 2: HPTLC densitogram of standard Leflunomide at 270 nm (50 ng/band) (R_f: 0.53 ± 0.02)

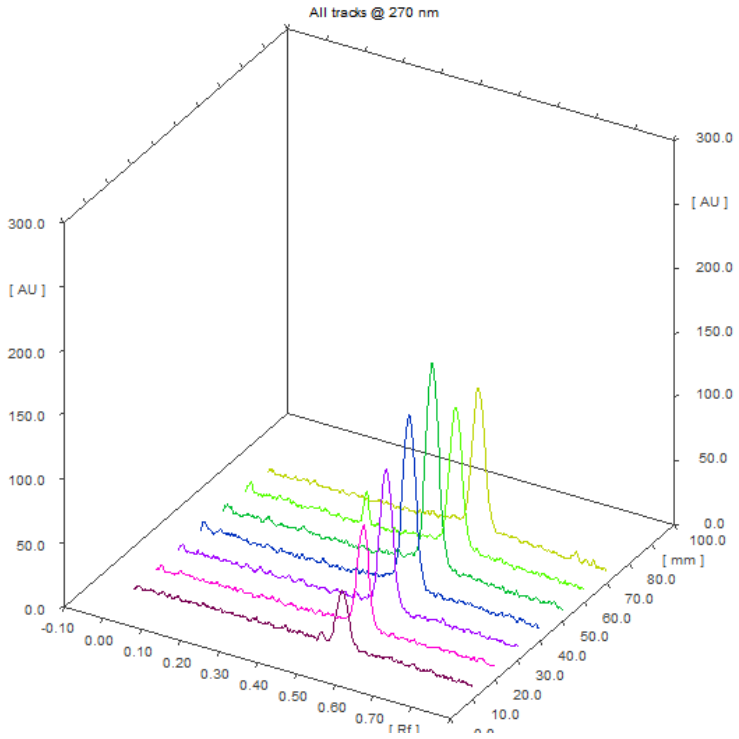

Figure 3: 3D diagram of Leflunomide demonstrating linearity (25-125 ng/spot)

Precision

Repeatability was evaluated by carrying out six independent sample preparations of 75ng/band

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concentration levels of Leflunomide. Sample application was checked by repeatedly measuring (n=6) the area of six bands having same concentration of Leflunomide applied on the same plate without changing the position of the plate. Percentage relative standard deviation (% RSD) was found to be 0.64.

Table 1: Linear regression data for calibration curve

Parameter	Leflunomide
Linearity	25-125 ng/band
Linear regression equation	Y= 37.34 X + 387.0
SD of Intercept (c)	19.38
Mean slop (m)	37.34
R^2	0.9960
LOD	1.71 ng
LOQ	5.19 ng

Precision was evaluated by carrying out three independent sample preparations of three different concentration levels of Leflunomide (50, 75 and 100 ng per band). Percentage relative standard deviation (% RSD) was found to be 1.28-1.43 for interday and 1.09-1.34 for intraday. Percentage relative standard deviation was found to be not more than 2 for within a day and day-to-day variations, which proves that the method is precise. Results are shown in Table 2.

Table: 2 Results of precision study by proposed method

Conc. (ng/spot)	Intra day		Inter day	
	Mean ± SD	R.S.D.	Mean ± SD	R.S.D.
50	2331.57 ± 31.23	1.34	2370.17 ± 33.91	1.43
75	3767.90 ± 37.67	1.18	3115.53 ± 41.62	1.34
100	4046.50 ± 44.34	1.09	4100.40 ± 52.27	1.28

Accuracy (% Recovery)

The accuracy of the method was determined by calculating percentage recoveries of Leflunomide by the standard addition method. Known amount of standards of Leflunomide (0, 30, 37.5 and 45 ng per band) were spiked to a prequantified sample (37.5 ng per band) of Leflunomide from dosage form. The amounts of Leflunomide were determined by measuring the peak areas and by fitting these values into the regression equation of the calibration plot. The percentage recovery was found in the range of 99.67 - 100.89% (Table 3).

Table 3: Results of recovery studies by proposed method

Sample taken (ng/spot)	Authentic added (ng/spot)	Recovery (%) ± SD	R.S.D.
37.5	0	100.50 ± 0.37	0.37
37.5	30	99.67 ± 0.53	0.53
37.5	37.5	99.90 ± 0.75	0.75
37.5	45	100.89 ± 0.92	0.91

Robustness

According to ICH guidelines, the method should show the reliability of an analysis, with respect to deliberate variations in method parameters. The conditions studied were the mobile phase composition, volume of modifier and chamber saturation time. The low values of % RSD as shown in Table 4 obtained after introducing small changes in the developed TLC method indicated the robustness of the method.

Sensitivity

The values obtained for LOD and LOQ were 1.71 and 5.19 ng/band, respectively, indicating the high sensitivity of the method.

LOD or LOD = K* σ/S

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Where, K is a numerical constant 3.3 for LOD, 10 for LOQ, σ is the sensitivity parameter (Expressed here by the slop of the standard curve).

Table 4: Robustness study results for the proposed method

Parameter	Normal condition	Change in condition	%RSD
Chamber Saturation time	30 min	20 min	0.68
		40 min	1.55
Mobile phase ratio Toluene:Ethyl acetate: Glacial acetic acid	8:2:0.5	(8.2:1.8:0.5 %, v/v/v)	1.28
		(7.8:2.2:0.5, % v/v/v)	1.95
Volume of modifier	8:2:0.5	(8:2:0.48, % v/v/v)	0.97
		(8:2:0.52, % v/v/v)	1.92

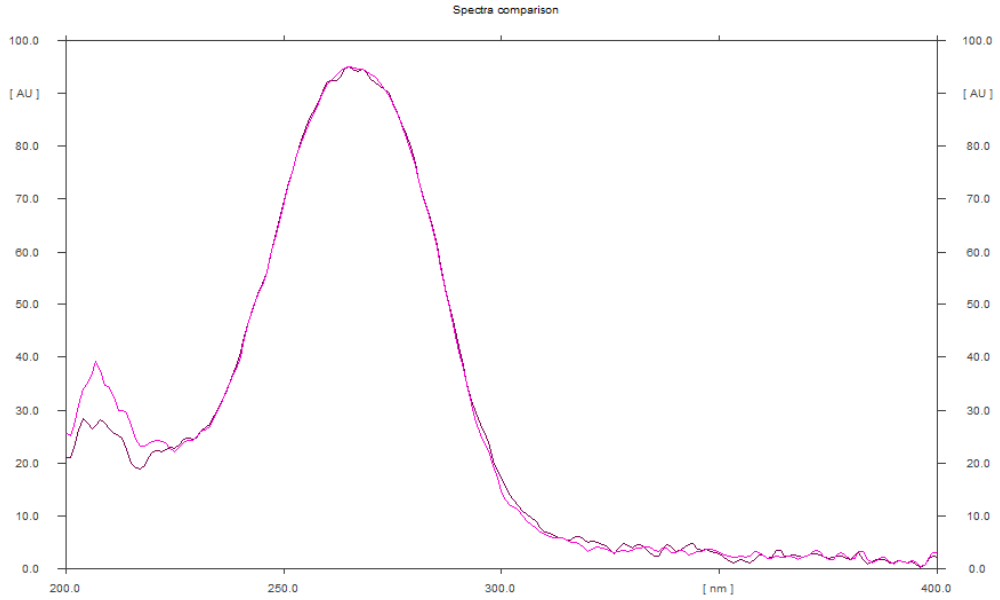

Figure 4: Overlain UV spectrum of Leflunomide standard and Leflunomide from tablet dosage form

Specificity and Selectivity

Good correlation was obtained between standard and sample spectra of Leflunomide. The comparative UV spectrum of standard and sample is given in Figure 4. Also the results of comparison between peaks start, maximum, and end indicate the closeness in these positions between sample and standard. The appearance of Leflunomide spot at specific R_F from standard and its formulations, indicates the specificity of the proposed method.

The selectivity of an assay is a measure of the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components. No interference peaks or matrix effects from excipients were observed in the chromatograms obtained from the formulations, thus confirming the selectivity of the method.

Analysis of Leflunomide in Tablets

The suitability of the method was verified by assay of Leflunomide in tablets. 75ng per band Leflunomide was spotted on TLC plate. A single spot at R_F 0.53 was observed in the chromatogram obtained from the Leflunomide tablet. The drug content was found to 99.40 % ± 0.89 (SD).

CONCLUSION

The low % RSD value was obtained for validation parameter indicates that the suitability of this method for routine analysis and quantitative determination of the Leflunomide in

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the dosage form by high- performance thin-layer chromatographic method. The statistical analysis of data obtained proves that the method is reproducible, selective, simple, accurate, robust and precise. The method can be used for routine quality-control analysis and quantitative determination of Leflunomide in pharmaceutical formulations.

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CONFILCTS OF INTEREST

The authors declare that there are no conflicts of interest.

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