International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol. 3, No.2, pp 523-530, April-June 2011

Validation of a Stability-Indicating LC Method for Assay of Leflunomide in Tablets and for Determination of Content Uniformity

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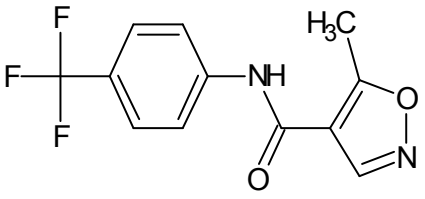
Abstract: A simple, precise, and accurate HPLC method has been developed and validated for assay of leflunomide in tablets and for determination of content uniformity. Reversed-phase liquid chromatographic separation was achieved by use of ammonium acetate (0.02M)-acetonitrile 40:60(*v/v*) as mobile phase. The method was validated for specificity, linearity, precision, accuracy, robustness, and by stress testing of the drug(forced degradation). Response was a linear function of drug concentration in the range 8-32 µg/mL (*r*= 0.9985). Intraday and interday system and method precision were determined. Accuracy was between 99.58 and 100.17%. The method was found to be robust, and was suitable for assay of leflunomide in a tablet formulation and for determination of content uniformity.

Keywords: Leflunomide, stability indicating assay, method validation, content uniformity.

1. Introduction:

Leflunomide [Fig.1] is chemically 5-methyl-N-[4-(trifluoromethyl) phenyl]-isoxazole-4-carboxamide. Its molecular formula is $C_{12}H_9F_3N_2O_2$ having molecular weight 270.21 g/mole.

Fig. 1: Chemical Structure of Leflunomide



Leflunomide is a prodrug of the disease-modifying antirheumatic drug (DMARD) type, used in active moderate to severe rheumatoid arthritis and psoriatic arthritis. It is a pyrimidine synthesis inhibitor. It is an isoxazole derivative marketed as 10, 20 and 100mg coated tablets.[1-4]

Method reported in literature for analysis of Leflunomide include determination of active leflunomide metabolite A77 1726 in human plasma by HPLC and Liquid Chromatograph-Mass spectrometer (LC-MS)[5-6] and Reverse Phase (RP) HPLC method for the determination of pharmaceuticals form of drug[7-11] and also related substance method by RP-HPLC[12-13] ,stability indicating high performance liquid chromatographic method[14-15] and also spectrophotometric method [16]. There are no reports of methods for study of the effect of stress on pharmaceutical dosage forms and there is no validated LC method, which enables both assay and determination of content uniformity of leflunomide in pharmaceutical dosage forms.

The objective of this work was to develop a stability-indicating liquid chromatographic analytical method for assay of leflunomide and for determination of the content uniformity of a tablet formulation, to validate the method in accordance with ICH guidelines[17], and to investigate the effect of applying degradative stress to the product. Reported work in the

literature[8] includes application of limited stress in which a single product seemed to be formed under the action of physical force degradation. In the current work a more intensive stress study was performed on the pharmaceutical dosage form and showed that the drug decomposed into numerous products under different stress conditions. Accordingly, a stability-indicating method was established in which the analyte peak was well resolved from those of all the degradation products formed under all stress conditions. The stress study also furnished information about the percentage degradation of the drug under different stress conditions, information which is not reported in the literature. The validation procedure followed the guidelines of USP 30[18] . The method was successfully used for assay of leflunomide and determination of the content uniformity of the tablet formulation. Determination of content uniformity is, nowadays, an important test included in USP 30[18]. Because there is no literature report of a validated analytical method for determination of the content uniformity of leflunomide in pharmaceutical dosage forms this was performed in the current work to enhance the value of this manuscript.

2. Experimental

2.1. Materials

Pharmacopoeial grade standard of Leflunomide was provided by Hetero drugs Ltd. A tablet containing 10mg Leflunomide was commercially available (Lefumide-10, Cipla Ltd.), LC grade acetonitrile and water were obtained from Spectrochem Pvt. Ltd., Mumbai (India). Analytical grade ammonium acetate, hydrochloric acid (35%), sodium hydroxide pellets and hydrogen peroxide solution (30% *v/v*) were obtained from Ranbaxy Fine Chemical, New Delhi (India).

2.2. Instrumentation

The chromatographic system used to perform development and validation of this assay method was comprised of a LC-10AT*vp* binary pump, a SPD-M10A*vp* photodiode-array detector and a rheodyne manual injector model 7725i with 20µl loop (Shimadzu, Kyoto, Japan) connected to a multi-instrument data acquisition and data processing system (Class-VP 6.13 SP2, Shimadzu).

2.3.Chromatographic conditions

Chromatographic analysis was performed on SGE SS Wakosil (150mm × 4.6mm i.d., C8RS 5 µm particle size) column. The mobile phase was consisted of ammonium acetate buffer (0.02M) and acetonitrile (40:60, *v/v*). To prepare the buffer solution, 1.5416 g ammonium acetate was weighed and dissolves in 1000mL LC grade water. Mobile phase was filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd. Bengaluru, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai). The flow rate of the mobile phase was adjusted to 1.0 mL/min and the injection volume was 20 µl. Detection was performed at 260nm.

2.4. Standard preparation

Standard solution containing leflunomide (100 µg/mL) was prepared by dissolving accurately about 10.0 mg in 100 mL volumetric flask by diluent [acetonitrile-buffer (50:50, *v/v*)] (stock standard solution). 10 mL of stock solution was pipetted out into 50 mL volumetric flask and dilute up to mark with diluent (standard solution). The concentration obtain was 20 µg/mL of leflunomide.

2.5. Test preparation

Twenty tablets were weighed and the average tablet weight was determined. Tablets were crushed by mortar and pestle. Tablet powder was weighed of average weight of five tablets 520.6 mg and transferred in to 500 mL volumetric flask. About 300mL diluent was added and sonicated for of 20min. time interval with intermittent shaking. Content was brought back to room temperature and dilute to volume with diluent (stock test solution). The stock solution was filtered through 0.45 µm nylon syringe filter. 10 mL of filtered stock solution pipetted out into 50 mL volumetric flask and dilute with diluent (test solution). The concentration obtain was 20 µg/mL of leflunomide.

2.6. Design of the forced degradation study

The degradation samples were prepared by transferring powdered tablets, equivalent to 10.0 mg Leflunomide into a 250 mL round bottomed flask. Then drug content were employed for acidic, alkaline and oxidant media and also for thermal and photolytic stress conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with diluent to attain 20 µg/ mL Leflunomide concentrations. Specific degradation conditions were described as follows.

2.7. Acidic degradation condition

Acidic degradation study was performed by refluxed the drug content in 1N hydrochloric acid for 30 min and mixture was neutralized.

2.8.Alkali degradation condition

Solution of alkali degradation study was prepared in 0.005N NaOH and the resultant solution was neutralized and kept at room temperature for 15min.

2.9.Oxidative degradation condition

Oxidation degradation study was performed by refluxed the drug content in 30% *v/v* H_2O_2 for 45 min.

2.10. Thermal degradation condition

Thermal degradation was performed by exposing solid drug to dry heat of 80˚ C in a conventional oven for 72 hr.

2.11. Photolytic degradation condition

Photolytic degradation study was performed by exposing the drug content in sunlight for 72 hr.

2.12. Method validation

In the developed method, by using same concentration of analyte for the assay and for determination of content uniformity, both methods could be validated simultaneously except for determination of precision.

2.12.1. Specificity study

The evaluation of the specificity of the method was determined against placebo. The interference of the excipients of the claimed placebo present in pharmaceutical dosage form was derived from placebo solution. Further the specificity of the method toward the drug was established by means of checking the interference of the degradation products in the drug quantification for assay during the forced degradation study.

2.12.2. Linearity

Linearity test solutions for the assay method were prepared at seven concentration levels from 40 to 160 % of assay analyte concentration (8, 12, 16, 20, 24, 28 and 32 µg/mL of leflunomide. The peak areas versus concentration data were evaluated by linear regression analysis.

2.12.3. Precision

The precision of the assay method was evaluated in terms of repeatability by carrying out six independent assays of test sample preparation and calculated the % Relative Standard Deviation (RSD) of assay (intraday). Intermediate precision of the method was checked by performing same procedure on the different day (interday) by another person under the same experimental condition.

2.12.4. Accuracy

An accuracy study was performed by adding known amounts of Leflunomide to the placebo preparation. The actual and measured concentrations were compared. Recovery of the method was evaluated at three different concentration levels (corresponding to 50, 100 and 150 % of test preparation concentration). For each concentration level, three sets were prepared and injected in duplicate.

2.12.5. Robustness

The robustness of study was carried out to evaluate the influence of small but deliberate variations in the chromatographic conditions. The factors chosen for this study were the flow rate ($\pm$ 0.1 mL/min), mobile phase composition [0.02M ammonium acetate buffer and acetonitrile (42:58 and 38:62, *v/v*)], and using different lot of LC column.

2.12.6. Solution stability

The stability of solution for test preparation was evaluated. The solution was stored at ambient temperature and 2-5˚ C and tested at interval of 12, 24, 36 and 48 hours. The responses for the aged solution were evaluated using a freshly prepared standard solution.

3. Results and Discussions

In the present work, an analytical method based on LC using UV detection was developed and validated for assay and determination of content uniformity of leflunomide in tablet dosage forms. The analytical conditions were selected after testing the different parameters that effect LC analysis, such as column, diluent, buffers, buffer concentration, organic solvent for mobile phase, proportion of mobile phase and concentration of analyte etc. The Wakosil column used because of its advantages of high retention, high resolving capacity, better reproducibility, low back pressure and low tailing. Our preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did gave poor peak shape. By using 0.02M ammonium acetate buffer per 1000 mL and keeping mobile phase composition as of 0.02M ammonium acetate buffer and acetonitrile (40:60, *v/v*), best peak shape was obtained. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. A detection wavelength of 260 nm was selected after scanning the standard solution over the range 190-350 nm by using photo-diode array (PDA) detector. Detection at 260 nm resulted in good response and good linearity.

The drug substance was easily extracted from the pharmaceutical dosage form by use of acetonitrile and buffer 50:50 (v/v). The tablet dispersed readily in buffer and the drug substance was freely soluble in acetonitrile. Solutions of standard and test preparations were found to be stable in this solvent mixture.

After developing the analytical method, it was validated. The analytical method validation gave evidence that the procedure was suitable for the intended purpose. The analytical method validation was carried out as per guidelines of ICH Q2 (R1) [17], USP[18] and AOAC INTERNATIONAL[19] .

The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method were eluted by checking the peak purity of leflunomide during the force degradation study. The peak purity of the leflunomide was found satisfactory (peak purity index (1.0000) under different stress condition. There was no interference of any peak of degradation product with drug peak. Major degradation of Leflunomide tablets was found with acidic (3.76%) (**Fig. 2b**), alkali (3.20 %) (**Fig. 2c**) and oxidative (23.99 %) (**Fig. 2d**) condition. The major degradent peak was found in oxidative degradation at 1.63 min. While they were also degrade under the thermal (0.91%) (**Fig. 2e**) and photolytic (0.33%)(**Fig. 2f**) degradation.

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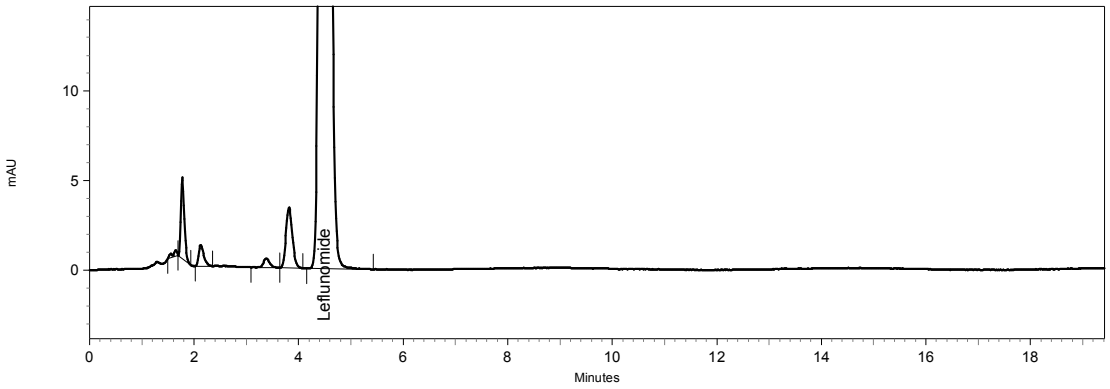
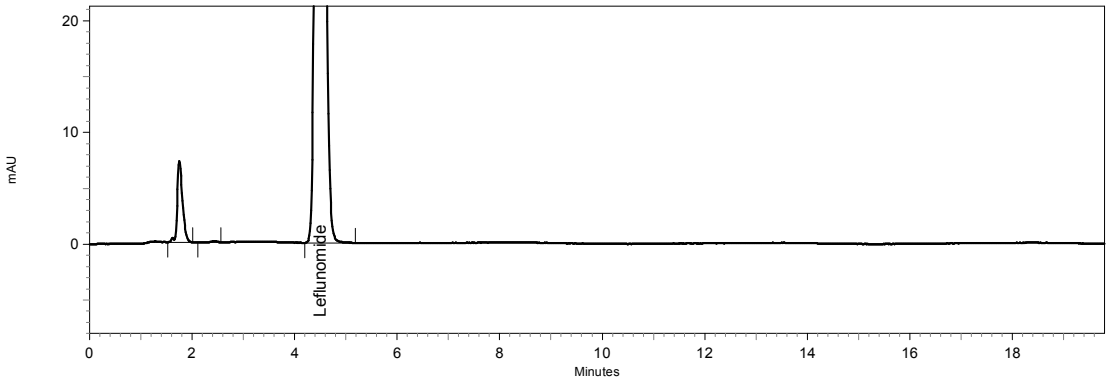
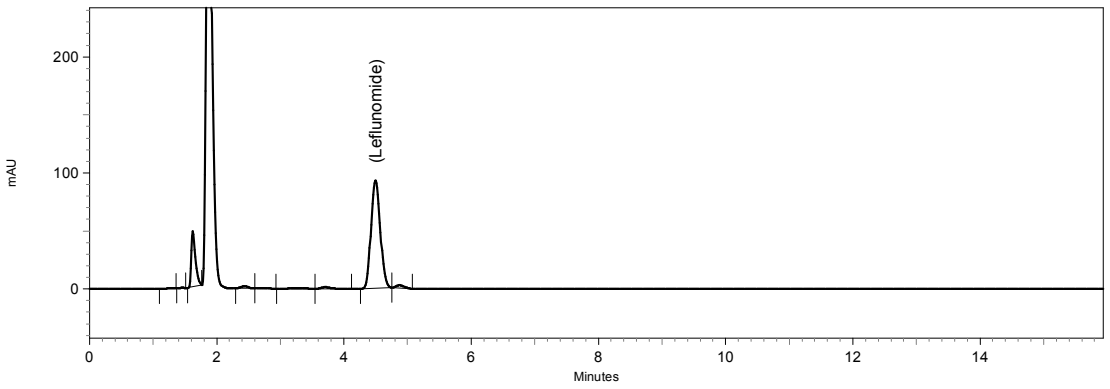
Fig. 2: Chromatogram of: (b) acidic force degradation study (c) alkali force degradation study (d) oxidative force degradation study (e) Thermal degradation (f) Photolytic degradation



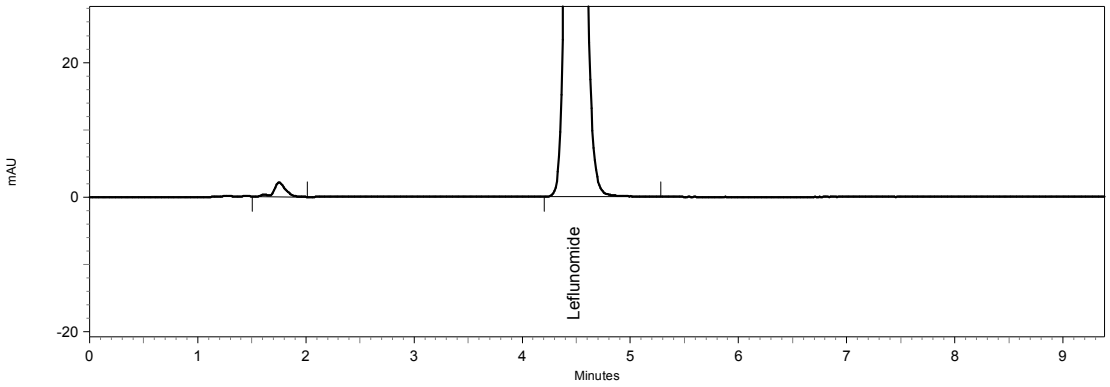
(b)



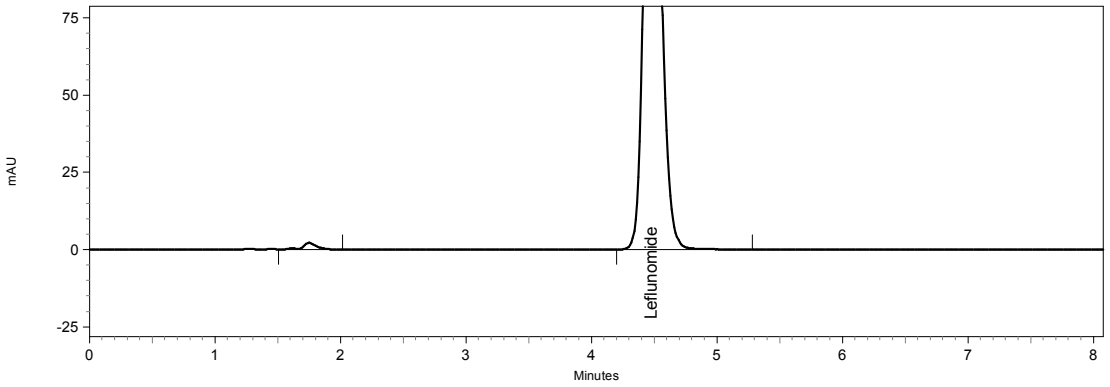
(c)



(d)



(e)



(f)

For linearity seven points calibration curve were obtained in a concentration range from 8-32 µg/mL for leflunomide. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation for leflunomide was y = 8003497.50000x-3364.66071 with correlation coefficient 0.9985 Where x is the concentration in µg/mL and y is the peak area in absorbance unit.

The limit of detection and limit of quantification were evaluated by serial dilutions of leflunomide stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Leflunomide found to be 0.008 µg/mL and the LOQ value 0.04 µg/mL.

For assay (n = 6) and determination of content uniformity (n = 10), RSD for the system precision was 0.96%, on the same day (intraday) and 0.99% on different days (interday). The mean values of method precision (repeatability) were 100.8%, RSD 0.63%, for assay and 101.0%, RSD 1.40%, for content uniformity on the same day (intraday) and 100.3%, RSD 0.69%, for assay and 100.7%, RSD 1.30%, for content uniformity on different days (interday). Intermediate precision was established by determining the overall (intraday and interday) method precision for assay and determination of content uniformity. For intermediate precision, overall assay value (n = 12) was 100.5%, RSD 0.69%, and overall content uniformity (n = 20) was 100.9%, RSD 1.40%. The precise result for content uniformity was indicative of uniform distribution of the drug in the tablets without significant variation; this is in accordance with the USP [18], which stipulates acceptance limits for drug content uniformity and RSD as 85-115% and <6%, respectively.

The accuracy of the method was assessed by determination of recovery for three concentrations covering the range of the method. Known amounts of leflunomide standard (50, 100, and 150%) were added to a placebo preparation and the amount of leflunomide recovered, in the presence of placebo interference, was calculated. The mean recovery of leflunomide was between 99.58 and 100.17%, which is satisfactory (**Table 1**).

Table 1. Results from evaluation of accuracy of the method

Level %	No	Amount of drug added (mg/mL)	Amount of drug found (mg/mL)	Recovery (%)	Mean Recovery (%)	RSD (%)
50	1	0.01020	0.01000	98.04	99.58	1.41
	2	0.01000	0.00999	99.90		
	3	0.01000	0.01008	100.80		
100	1	0.02000	0.01997	99.85	100.17	1.07
	2	0.02000	0.01986	99.30		
	3	0.01980	0.02007	101.36		
150	1	0.03000	0.03002	100.07	99.86	0.93
	2	0.03040	0.03005	98.85		
	3	0.02980	0.03000	100.67		

Table 2. Results from evaluation of the robustness of the method

Conditions	Assay (%)	R_t (min)	System - suitability data	
			Theoretical plates	Asymmetry
0.9 mL/min Flow	101.9	4.9	6058	1.18
1.1 mL/min Flow	102.5	4.0	5870	1.11
Buffer: [a]ACN (38:62)	99.9	4.9	6351	1.18
Buffer: ACN (42:58)	100.3	4.1	5423	1.23
Column change	100.2	4.4	5990	1.19

[a]Acetonitrile

The robustness of the method was assessed by assaying test solutions under different analytical conditions deliberately changed from the original conditions. For each different analytical condition the standard solution and test solution were prepared separately. The result obtained from assay of the test solution was not affected by varying the conditions and was in accordance with the true value. System suitability data were also found to be satisfactory during variation of the analytical conditions (**Table 2**).The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions.

During study of the stability of stored solutions of standards and test preparations for assay determination the solutions were found to be stable for up to 48 hr. Assay values obtained after 48 hr. were statistically identical with the initial value without measurable loss (**Table 3**).

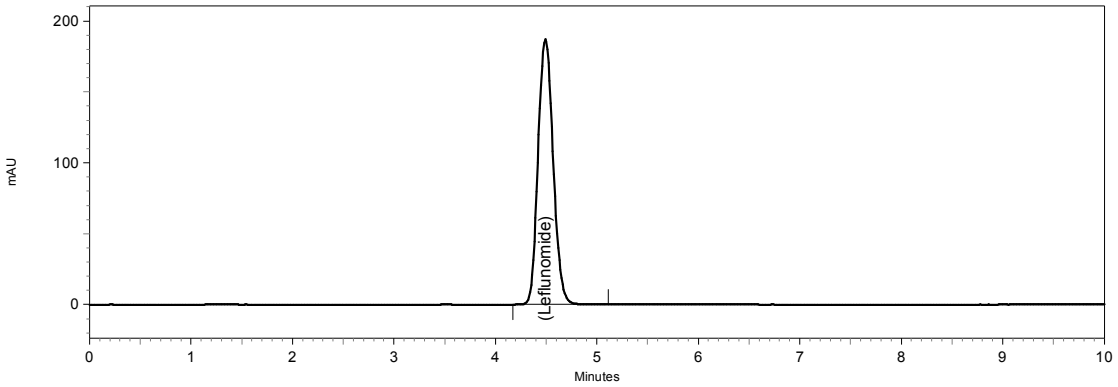
Table 3. Results from evaluation of solution stability

Time	Difference between assays for Standard solution (%)		Difference between assays for Test solution (%)	
	At 2- 8˚C	At room temperature	At 2- 8˚C	At room temperature
After 12 hrs	0.44	0.58	0.40	0.50
After 24 hrs	1.03	1.14	1.00	1.20
After 36 hrs	1.35	1.46	1.8	1.90
After 48 hrs	1.69	1.71	2.0	2.00

Before each measurement of validation data a system suitability test was performed by measurement of general characteristics such as peak asymmetry, number of theoretical plates and RSD (%) of peak area observed for a standard solution (**Fig. 2a**). The values obtained were satisfactory and in accordance with in-house limits (**Table 4**).

The intensive approach described in this manuscript was used to develop and validate a liquid chromatographic analytical method that can be used for both assay and determination of content uniformity of leflunomide in a pharmaceutical dosage form. Degradation products produced as a result of stress did not interfere with detection of leflunomide and the assay method can thus be regarded as stability indicating.

Fig. 2: Chromatogram of: (a) standard preparation



(a)

Table 4. Results from evaluation of the suitability of the method

System suitability data	RSD (%)[b]	Theoretical plates	Asymmetry
In-House limit	**NMT[c] 2.0**	**NLT[d] 5000**	**NMT[c] 2.0**
Validation data			
Specificity	0.73	5876	1.18
Linearity	0.48	5680	1.19
Precision			
For Assay	0.96	5966	1.22
For Content uniformity	1.48	5820	1.21
Intermediate Precision			
For Assay	0.99	5930	1.29
For Content uniformity	1.31	5820	1.21
Accuracy	0.76	5936	1.18
Solution stability	0.83	5692	1.27
Robustness	1.21	6032	1.15

[b]Relative standard deviation,
[c]not more than,
[d]not less than

4. Conclusion

This LC method for assay and determination of content uniformity of leflunomide in a tablet formulation was successfully developed and validated for its intended purpose. The method was shown to specific, linear, precise, accurate, and robust. Because the method separates leflunomide and all the degradation products formed under variety of stress conditions it can be regarded as stability indicating. There is no method reported for determination of assay and content uniformity of leflunomide in pharmaceutical dosage forms. This method is recommended to the industry for quality control of drug content in pharmaceutical preparations

5. Acknowledgements

The authors are thankful for facilities & grants given under UGC- Special Assistance Programme (DRS) Department Research Support (Sanction letter No. 540/6/DRS/2004 (SAP-I) Dt. 26/03/2004, and Department of Science & Technology New Delhi Fund for Improvement of Science & Technology (FIST) (Sanction letter No. SR/FST/CSI-072/2003 Dt. 24/12/2003 and Department of Chemistry, Saurashtra University, Rajkot – 360 005 (INDIA) for providing facilities.

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