PART-B

DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING UPLC ASSAY METHOD FOR DETERMINATION OF LEFLUNOMIDE IN TABLET FORMULATION

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1. INTRODUCTION TO UPLC

History of chromatography can give an idea about improvement in technology from conventional column chromatography to high performance liquid chromatography and finally at this stage an ultra performance liquid chromatography or in other way a combination of pressurized chromatographic technology and sub 2 (two) micron particle size of stationary phase technology leads to advance Ultra Performance Liquid Chromatography (UPLC) or Rapid Resolution Liquid Chromatography (RRLC) technology.

Technology of sub 2 (two) micron particle size leads many modifications in hardware part of the system like reduction of system volume, higher pump pressure capacity, injector and needle part, and cell volume of detector as well as in software area, data acquisition rate or capacity was increased for sufficient data collection.

In brief detail, small particle size columns leads to increase in pump pressure so that area was improved and for accurate and precise injection volume needle in needle technology with teflon material was came into the picture. Detector cell volume was reduced for better signals and resolution.

Smaller particle size of 2 micron technology altered the machine and its application for faster way of analysis in current scenario of separation science. Requirement of this technology can be explained by van deemeter equation [1, 4] and plot as shown in fig 1. From this plot it reveals that there is minimum HETP against the linear velocity with the almost constant relation or maximum the theoretical plates can be achieved with particle size less than 2 micron. Finally as a known fact increasing in N leads to increase in Resolution as shown in formula;

$$Rs = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k}{k+1} \right)$$

System Efficiency **Selectivity** **Retentivity**

A term + B term + C term

$$H = a(dp) + \frac{b}{u} + c(dp)^2 u$$

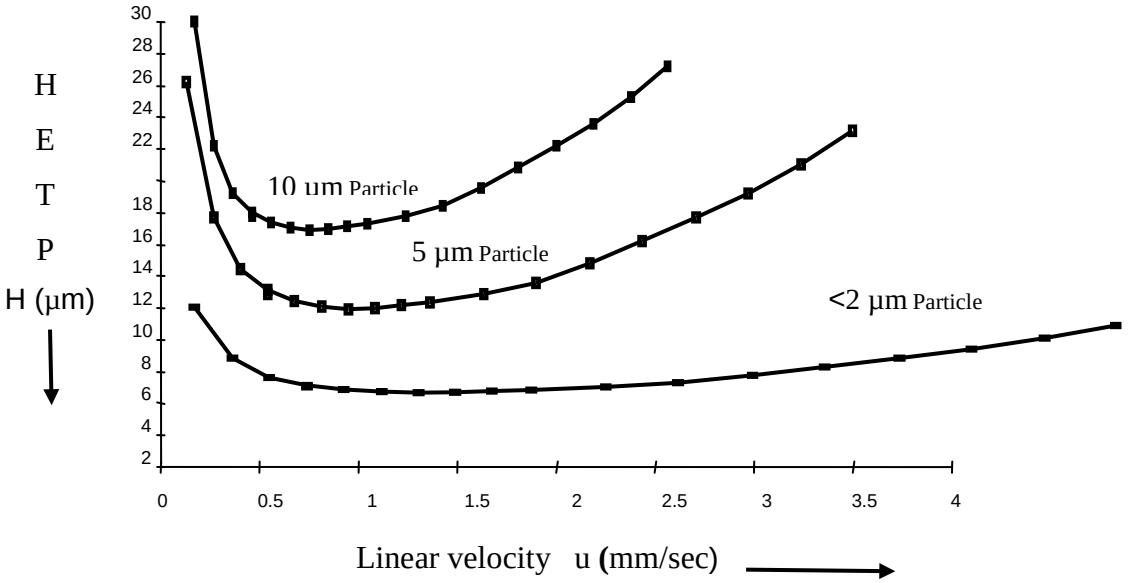


Figure1: Van Deemter plots-influence of particle size

Now, for method conversion from HPLC to UPLC or for comparison of both the technology following aspects needs to take in consideration [2-3].

- Ratio of column length to particle size (L/dp) needs to keep constant.
  i.e. 150 mm/5 µm = 30,000 is closest to 50mm/1.7 µm = 29,500
- Column selection should be based on same basic column chemistry
  i.e. C_{18} column should be replaced by C_{18} column
- 5 µm to 1.7 µm particle size leads to increase in speed of 9X along with 9X pressure
- 3 µm to 1.7 µm particle size leads to increase in speed of 3X along with 3X pressure
- 5 µm to 1.7 µm particle size leads to increase in peak height of 1.7X
- 3 µm to 1.7 µm particle size leads to increase in peak height of 1.3X
- 5 µm to 1.7 µm particle size leads to decrease in peak width of 0.6X
- 3 µm to 1.7 µm particle size leads to decrease in peak width of 0.8X

- Column efficiency (N) is inversely proportional to dp

$$N \propto \frac{1}{dp}$$

i.e. 5 µm to 1.7 µm particle size leads to increase in column efficiency (N) 3X but

$$Rs \propto \sqrt{N}$$

So, resolution also increase by 1.7X

Based on above fact practically an example for chromatogram comparison against column dimension for rune time and resolution is shown in fig 2.

Remark

Here, X is used to express the mathematical relation in multi fold.

e.g. pressure increased by 3X i.e. pressure increase by three times

Method Development

Method development in UPLC remains same as of HPLC but few areas of chromatographic conditions are different e.g. for gradient elution column equilibration time is very less as compare to HPLC due to lower column volume.

Advance technology in column filled material for HPLC as well as UPLC allows higher pH and temperature stability for column for wider choice of mobile phase for different applications. e.g. pH of mobile phase or its buffer can selected based on compound chemical nature and that can be explain by fig.3 for reversed phase retention plot.

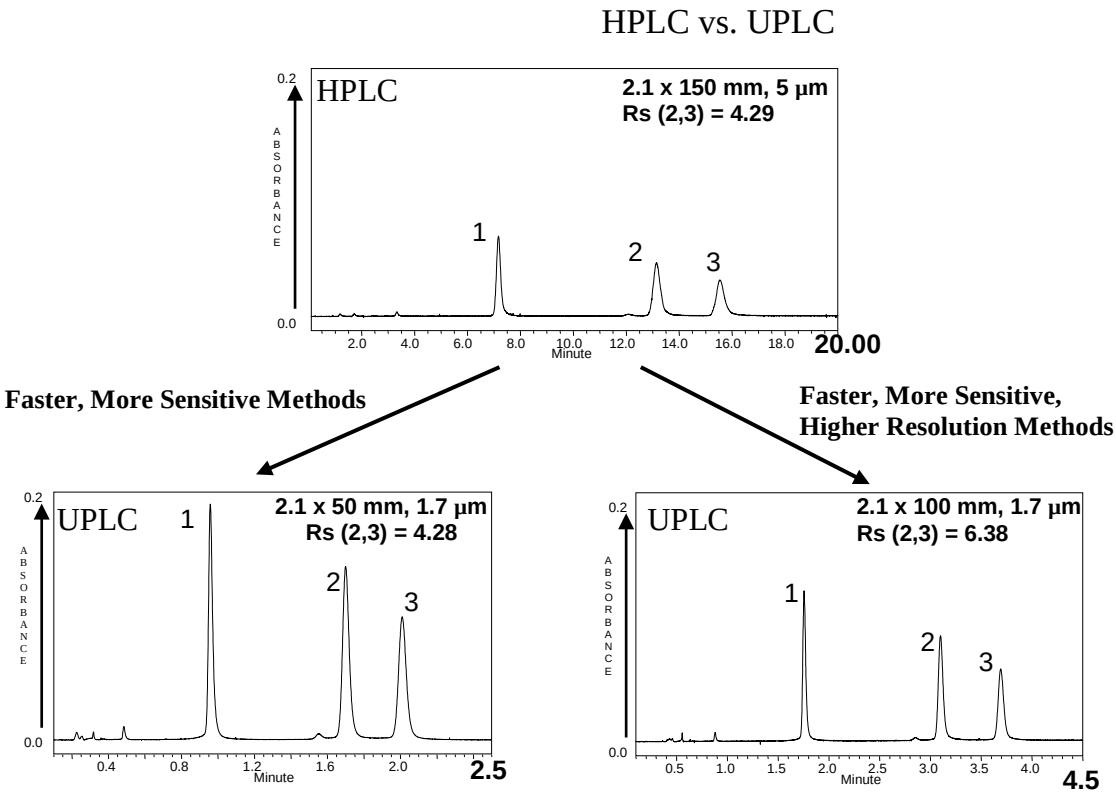


Figure 2: Chromatogram comparison against column dimension

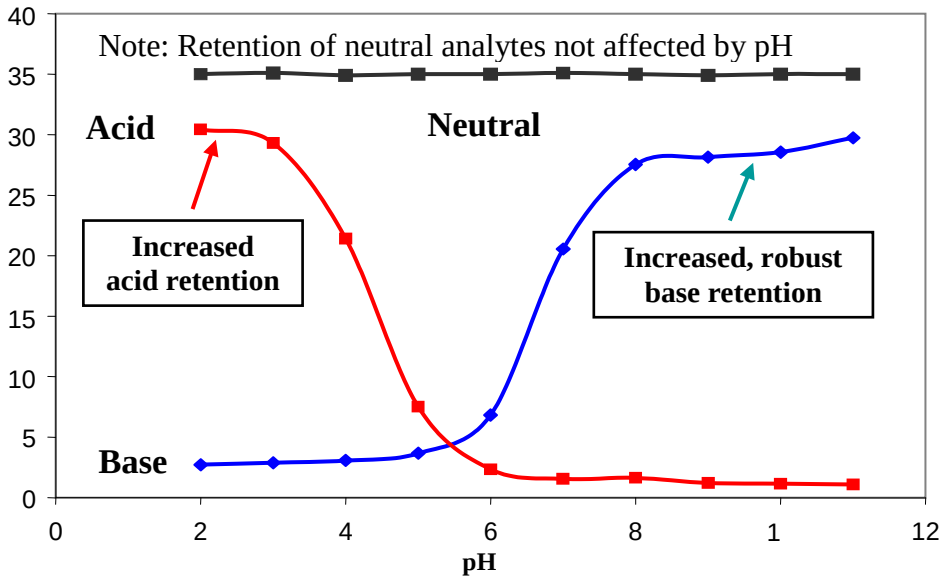


Figure 3: Reversed phase retention plot

From the above plot and fundamental theory of solvent gives following information for development consideration.

Facts for basic compound

Alkaline pH increases retention of basic analytes

Methanol increases retention of all components compared to acetonitrile

Similar basic analytes differ little in selectivity, respective to one another, when they are either fully charged or uncharged

Largest selectivity differences between bonded phases occur with methanol and analytes in their unionized state

Facts for acid compound

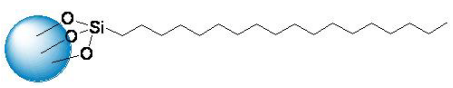
1. Acidic pH increases retention of acidic analytes

Methanol increases retention of all components compared to acetonitrile

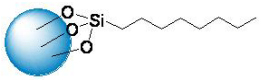
Large differences in selectivity are observed when change in pH alters charge state

Largest selectivity differences between bonded phases occur with methanol and analytes in their unionized state

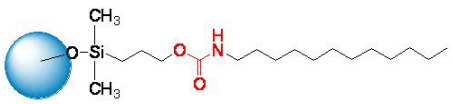
Column chemistry for known columns UPLC are shown in fig 4.



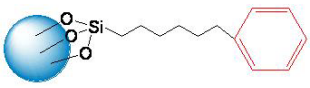
ACQUITY UPLC™ BEH C18



ACQUITY UPLC™ BEH C8



ACQUITY UPLC™ BEH Shield RP18



ACQUITY UPLC™ BEH Phenyl

Figure 4: Column chemistry of UPLC column

By many recent research and development, UPLC presents the ability to extend and expand the utility of separation science at a time when many scientists have reached separation barriers, pushing the limits of conventional HPLC. New chemistry and instrumentation technology can provide more information per unit of work as UPLC begins to fulfil the promise of increased speed, resolution, and sensitivity predicted for liquid chromatography. As this is the concept for the scientist many of industries may take time to use in routine but it can be the future of the liquid chromatography. Hence present research work includes the extended area of HPLC to UPLC as a part of technology updating or a balance form of present HPLC and improved LC or UPLC for future scope for separation science.

2. LITERATURE REVIEW

Literature review for methods developed for leflunomide is mentioned in the **section I, page number 38.**

3. AIM OF PRESENT WORK

As per discussion in the literature review, UV, TLC, LC-MS and HPLC methods for the determination of leflunomide in pharmaceutical dosage forms or in metabolite and plasma are reported. HPLC methods applied to the determination of leflunomide. So far to our present knowledge, no validated stability indicating UPLC assay method for the determination of leflunomide in pharmaceutical formulation was available in literature. Our work deals with the forced degradation of leflunomide under stress condition like acid hydrolysis, base hydrolysis, and oxidation, thermal and photolytic stress. This work also deals with the validation of the developed method for the assay of leflunomide from its dosage form (tablets). Hence, the method is useful for routine quality control analysis and also for determination of stability.

The aim and scope of the proposed work are as under:

- To develop suitable UPLC method for leflunomide,
- Forced degradation study of leflunomide under stress condition,
- To resolve all major impurities generated during the force degradation studies of leflunomide,
- Perform the validation for the developed method.

4. EXPERIMENTAL

4.1 Materials

Detail of materials for methods developed for leflunomide is mentioned in the **section 1, page number 43.**

4.2 Instrumentation

The LC system of Waters Acquity UPLC with PDA was used for this entire study and chromatographic separation was achieved on Water Acquity BEH C18, 2.4mm x 4.6mm 1.7µm column as stationary phase with binary gradient mode.

4.3 Mobile phase Preparation

The mobile phase consisted of acetonitrile – 0.02M ammonium acetate buffer (60: 40, v/v). To prepare the buffer solution, 1.5416 g ammonium acetate was weighed and dissolve in 1000 ml HPLC grade. Mobile phase was filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd. Bangalore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

4.4 Diluent Preparation

Use acetonitrile-buffer (50:50, *v/v*) used as diluents.

4.5 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.

4.6 Test Preparation

Twenty tablets were weighed and the average weight of tablet was determined. From these, five tablets were weighed and transfer into a 500 ml volumetric flask. About 50 ml diluent was added and sonicated for a minimum 30 min. with intermittent shaking. Then content was brought back to room temperature and diluted to volume with diluents.

The sample was filtered through 0.45µm nylon syringe filter. 10 mL of filtrate stock solution was pipetted out into 50 mL volumetric flask and dilute up to mark with diluents. The concentration obtained was 20 µg/ml of Lelunomide.

4.7 Chromatographic Conditions

Chromatographic analysis was perfomed on Water Acquity BEH C18, 2.4 x 50mm, 1.7µ column. The mobile phase was consisted of acetonitrile and 0.02M ammonium acetate buffer (60:40, v/v). The flow rate of the mobile phase was adjusted to 0.4 mL/min and the injection volume was 5 µl. Detection was performed at 260nm.

5. RESULTS AND DISCUSSION

5.1 Development and Optimization of the UPLC Method

Proper selection of the methods depends upon the nature of the sample (ionic or ionisable or neutral molecule) its molecular weight and solubility. Leflunomide is dissolved in polar solvent hence RP-UPLC was selected to estimate them. To develop a rugged and suitable UPLC method for the quantitative determination of leflunomide, the analytical condition were selected after testing the different parameters such as diluents, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition and other chromatographic conditions. Our preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did not give good peak shape.

The mobile phase consisted of acetonitrile – 0.02M ammonium acetate buffer (60: 40, v/v). To prepare the buffer solution, 1.5416 g ammonium acetate was weighed and dissolves in 1000 ml HPLC grade water. Mobile phase was filtered through a 0.22 μm nylon membrane (Millipore Pvt. Ltd. Bangalore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai).

By using 0.02M Ammonium acetate buffer in 1000 ml of buffer and keeping mobile phase composition as acetonitrile-ammonium acetate buffer (60: 40, 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. Figure 2 and Figure 3 represent the chromatograms of standard and test preparation respectively.

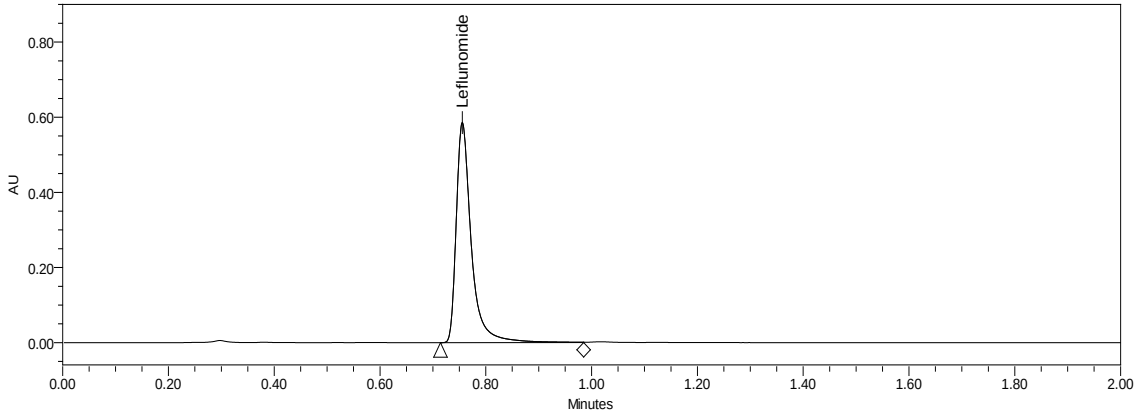


Figure 2: Chromatogram of standard preparation

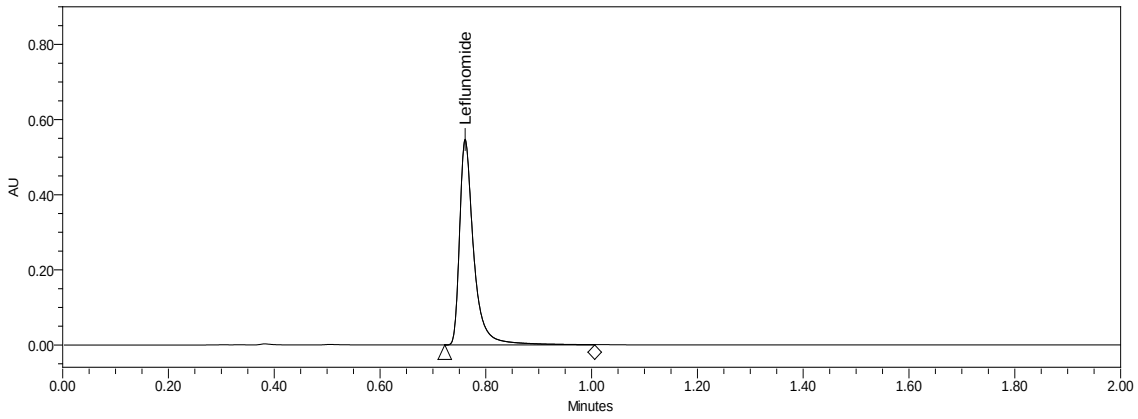


Figure 3: Chromatogram of test preparation

5.2 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.

5.2.1 Acidic condition

Acidic degradation study was performed by heating the drug content in 1 N HCl at 60° C for 2 hours and mixture was neutralized. In acidic degradation, it was found that around 10 % of the drug degraded. (Figure 4)

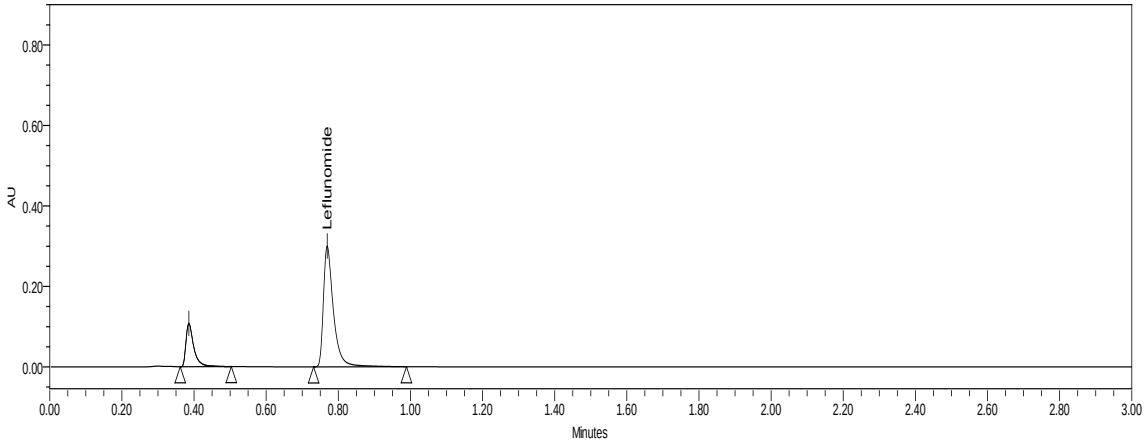


Figure 4: Chromatogram of acidic forced degradation study

5.2.2 Alkaline condition

Alkaline degradation study was performed by ambient temperature in 0.005N NaOH for 2 hours and mixture was neutralized. In alkali degradation, it was found that around 8-9 % of the drug degraded. (Figure 5)

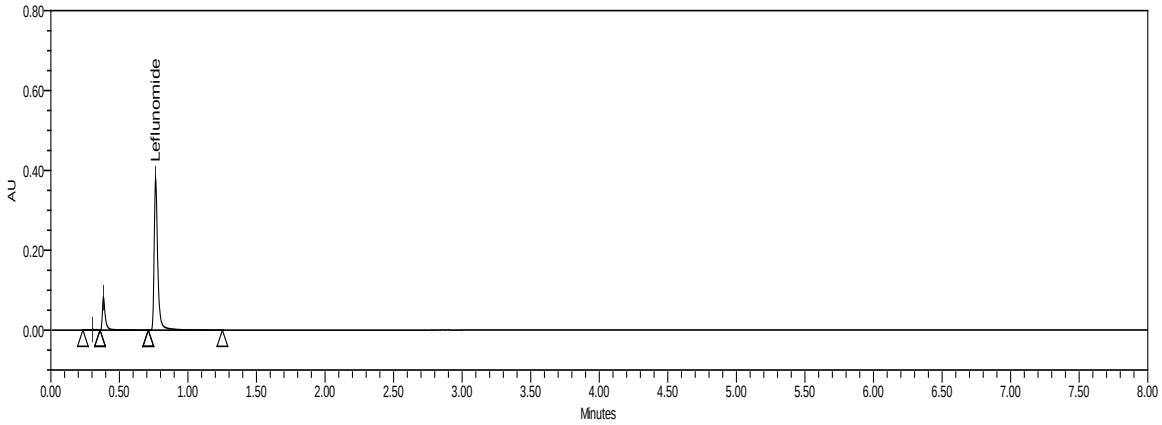


Figure 5: Chromatogram of alkali forced degradation study

5.2.3 Oxidative condition

Oxidation degradation study was performed by heating the drug content in 30% v/v H_2O_2 at 80° C for 45 min. Major degradation was found in oxidative condition that product was degraded up to 22 %. The major impurity peaks was found at 0.5 min. (Figure 6)

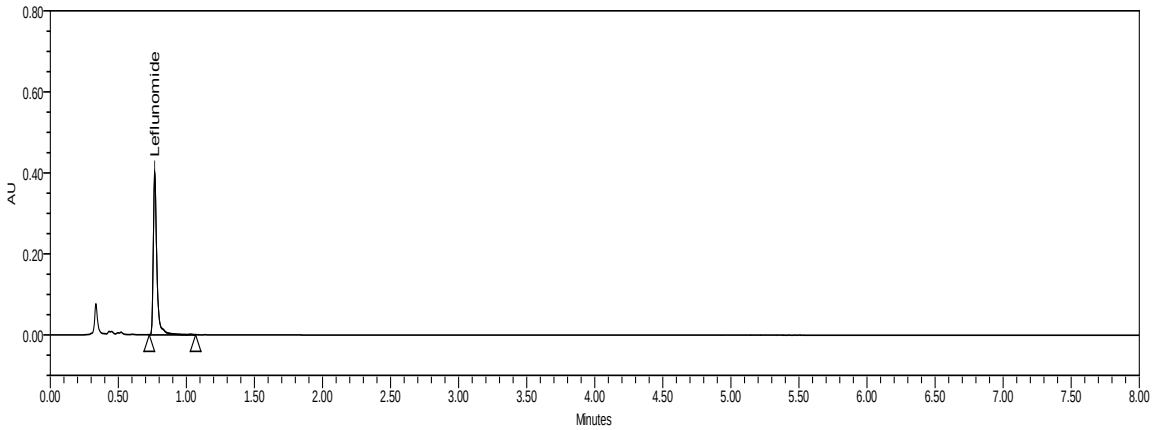


Figure 6: Chromatogram of oxidative forced degradation study

5.2.4 Thermal condition

Thermal degradation was performed by exposing solid drug to dry heat of 80˚ C in a conventional oven for 72 hr. In thermal degradation, it was found that around 0.60% of the drug degraded. (Figure 7)

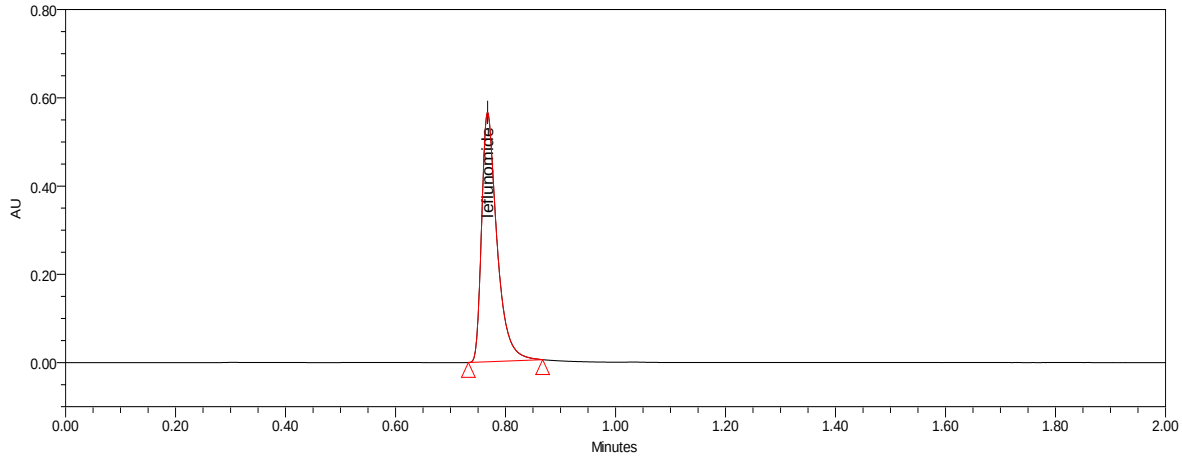


Figure 7: Chromatogram of thermal degradation study

5.2.5 Photolytic condition

Photolytic degradation study was performed by exposing the drug content in UV-light for 72 hours. In photolytic degradation, it was found that around 1.12 % of the drug degraded. (Figure 8)

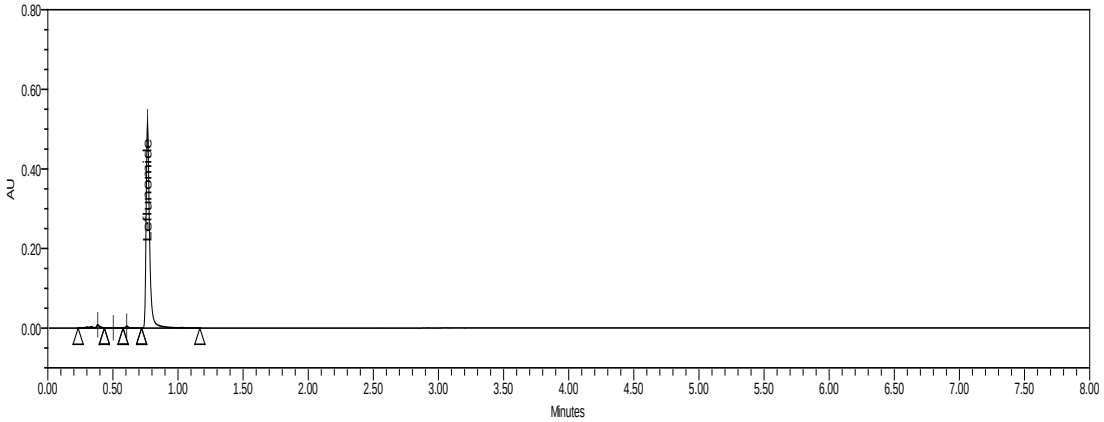


Figure 8: Chromatogram of UV-light degradation study

5.3 Method Validation

5.3.1 Specificity

The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method was eluted by checking the peak purity of Leflunomide during the force degradation study. The peak purity of the leflunomide was found satisfactory (0.9999) under different stress condition. There was no interference of any peak of degradation product with drug peak.

5.3.2 Linearity

Five points calibration curve were obtained in a concentration range from 10-30 µg/ml for leflunomide. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was y=56906771.42857x- 2237.10714 with correlation coefficient 0.9998. (Figure 9) Chromatogram obtain during linearity study were shown in figure 10-14.

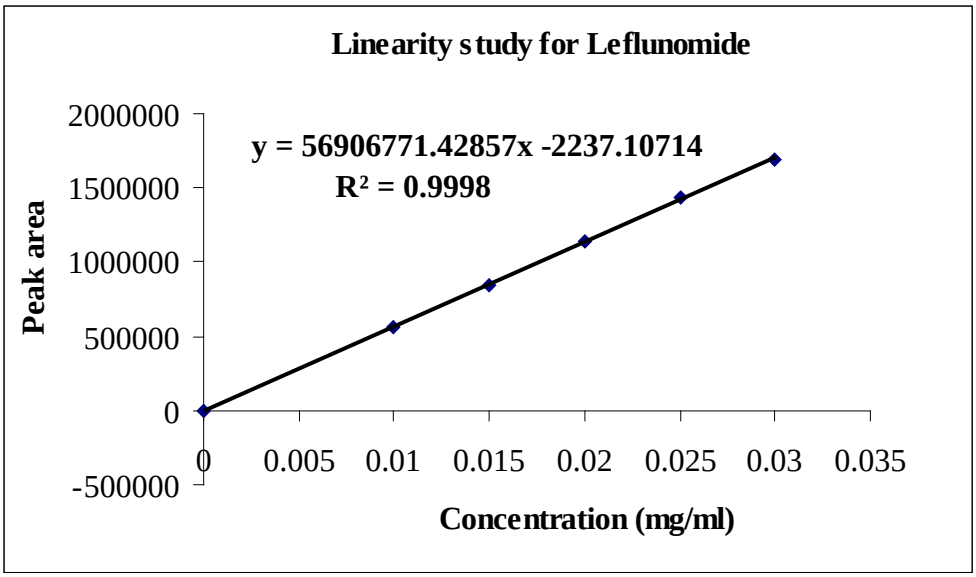


Figure 9: Linearity curve for leflunomide

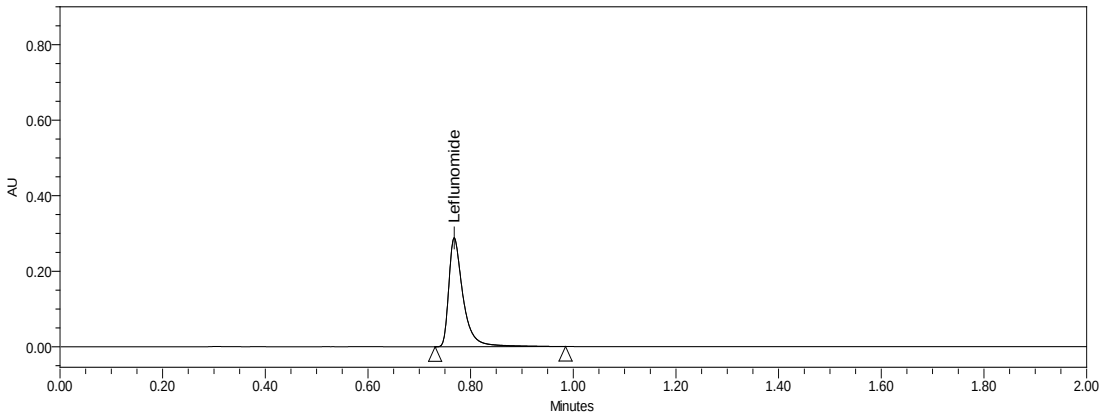


Figure 10: Linearity study chromatogram of level-1 (50%)

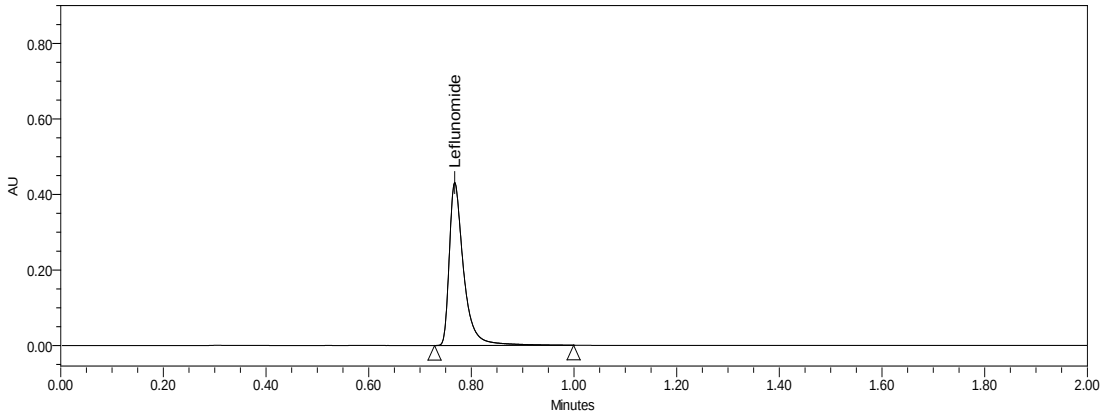


Figure 11: Linearity study chromatogram of level-2 (75%)

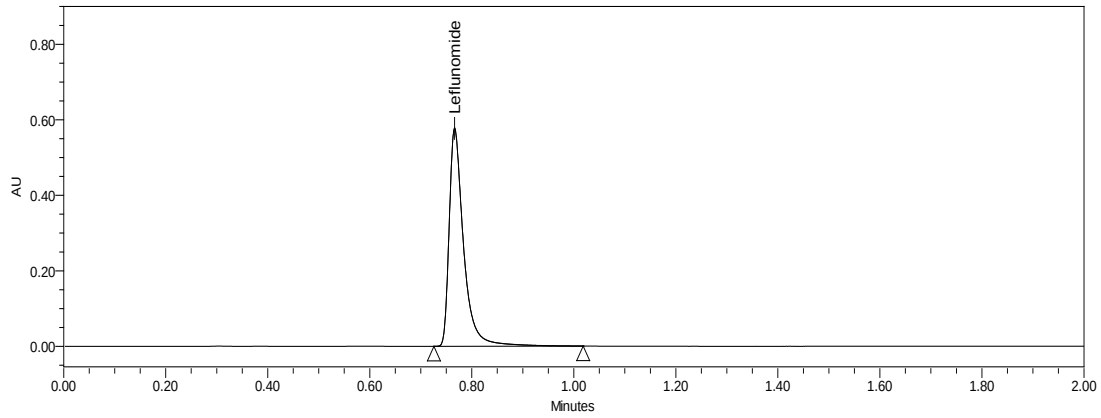


Figure 12: Linearity study chromatogram of level-3 (100%)

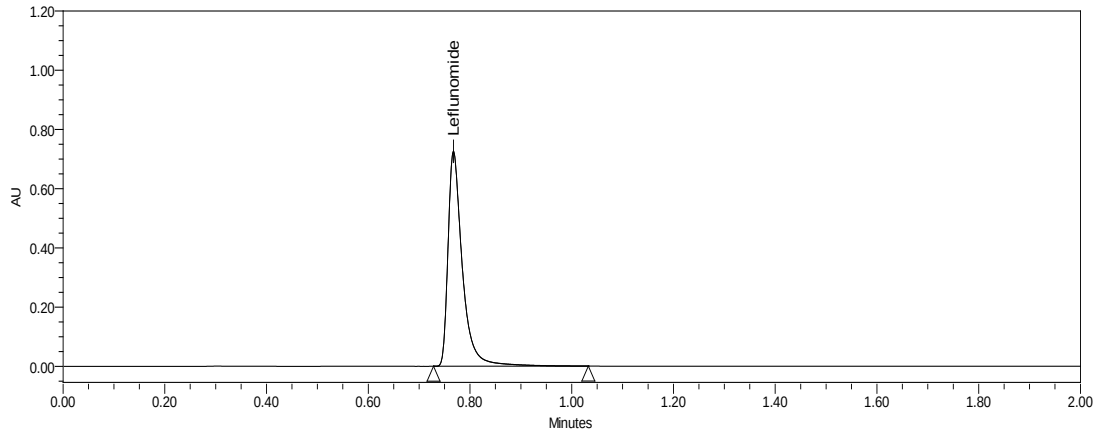


Figure 13: Linearity study chromatogram of level-4 (125%)

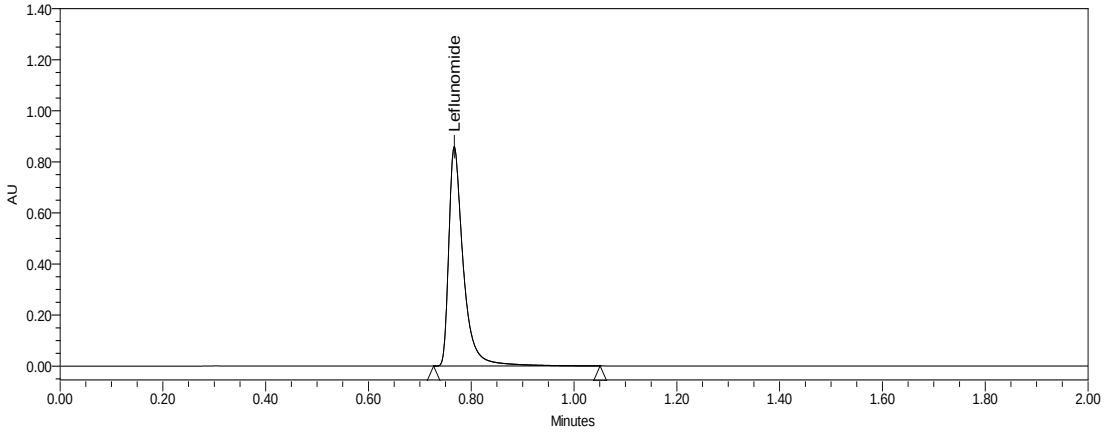


Figure 14: Linearity study chromatogram of level-5 (150%)

5.3.3 Precision

The result of repeatability and intermediate precision study are shown in Table 1. The developed method was found to be precise as the %RSD values for the repeatability and intermediate precision studies were <1.16 % and < 0.60 %, respectively, which confirm that method was precise.

Table 1: Evaluation data of precision study

Set	Intraday (n = 6)	Interday (n = 6)
1	101.8	100.2
2	100.6	99.9
3	101.2	99.5
4	100.4	99.4
5	101.0	98.7
6	98.4	100.3
Mean	100.6	99.7
Standard deviation	1.17	0.60
% RSD	1.16	0.60

5.3.4 Accuracy

The UPLC area responses for accuracy determination are depicted in Table 2. The result shown that best recoveries (99.44-100.24 %) of the spiked drug were obtained at each added concentration, indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in figure 15-17.

Table 2: Evaluation data of accuracy study

Level (%)	Amount added concentration [a] (mg/ml)	Amount found concentration [a] (mg/ml)	% Recovery	% RSD
50	0.00987	0.00989	100.24	1.09
100	0.02000	0.01989	99.44	1.24
150	0.02987	0.02978	99.72	1.31

[a] Each value corresponds to the mean of three determinations

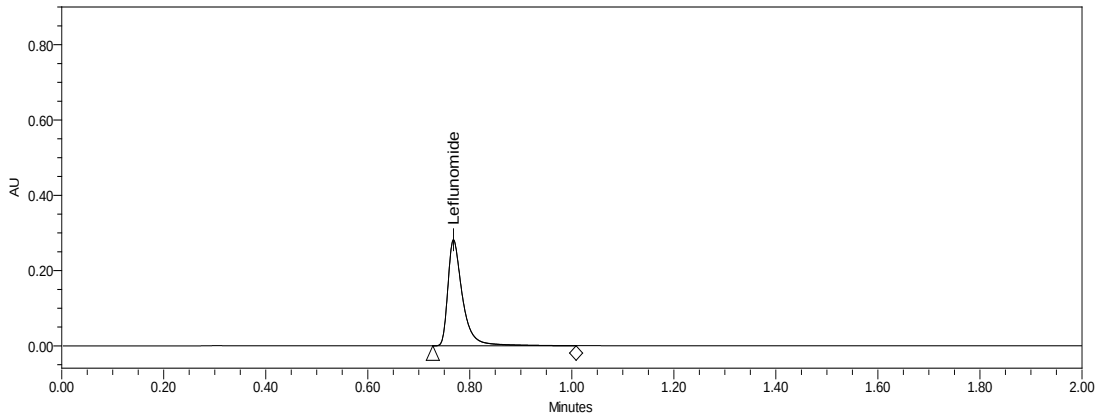


Figure 15: Accuracy study chromatogram of level-1 (50%)

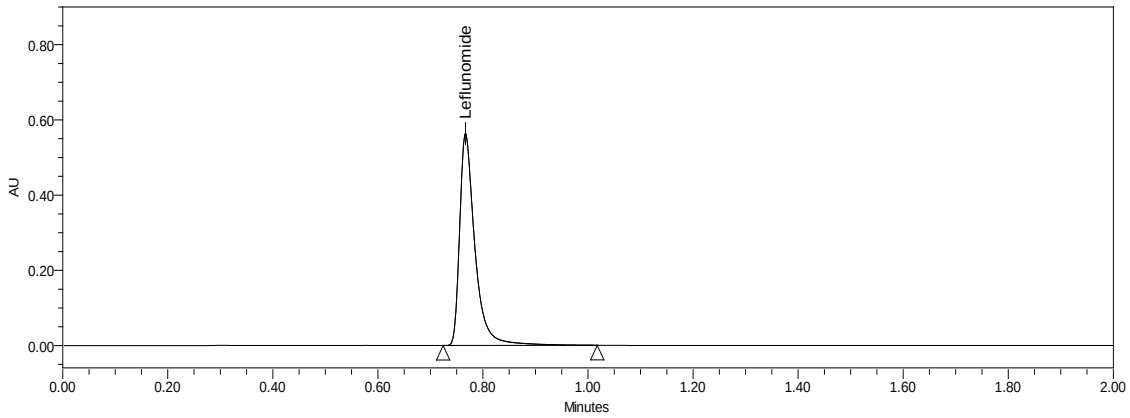


Figure 16: Accuracy study chromatogram of level-2 (100%)

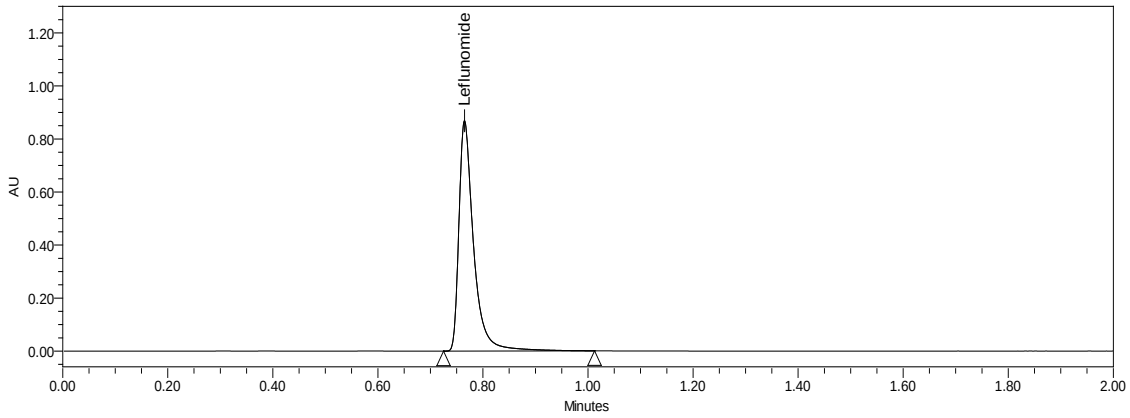


Figure 17: Accuracy study chromatogram of level-3 (150%)

5.3.5 Solution stability study

Table 3 shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 48 h at 2 - 8˚ C and ambient temperature, as during this time the result was not decrease below the minimum percentage.

Table 3: Evaluation data of solution stability study

Intervals	% Assay for Test Preparation Solution Stored at 2-8 ˚C	% Assay for Test Preparation Solution Stored at Ambient Temperature
Initial	99.6	99.6
12 h	100.3	100.0
24 h	98.4	98.3
36 h	98.6	98.1
48 h	97.8	97.7

5.3.6 Robustness

The result of robustness study of the developed assay method was established in Table 4. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in Figure 18-22.

Table 4: Evaluation data of robustness study

Robust conditions	% Assay	System suitability parameters	
		Theoretical plates	Asymmetry
Flow 0.35 ml/min	99.5	4546	1.45
Flow 0.45 ml/min	102.0	4456	1.20
ACN-Buffer (62: 38,v/v)	98.8	4490	1.35
ACN-Buffer (58: 42,v/v)	98.2	4520	1.58
Column change	99.1	4430	0.58

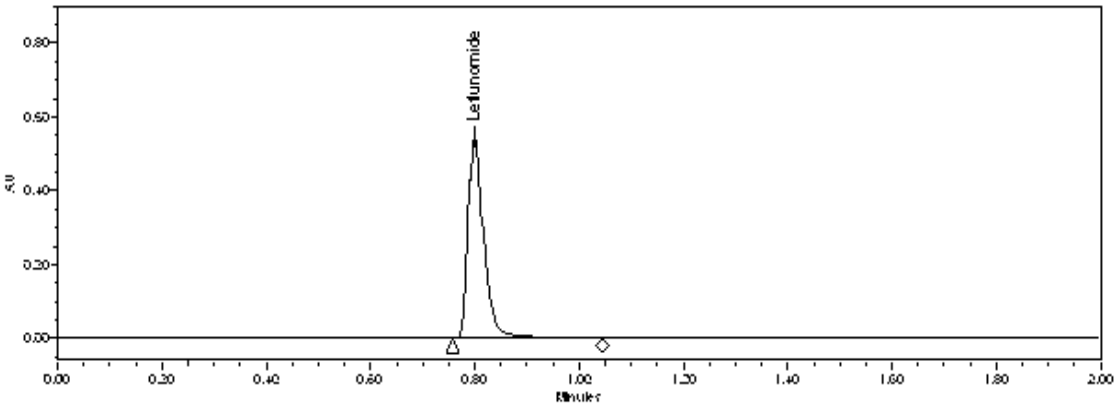


Figure 18: Standard chromatogram (0.35 ml/min flow rate)

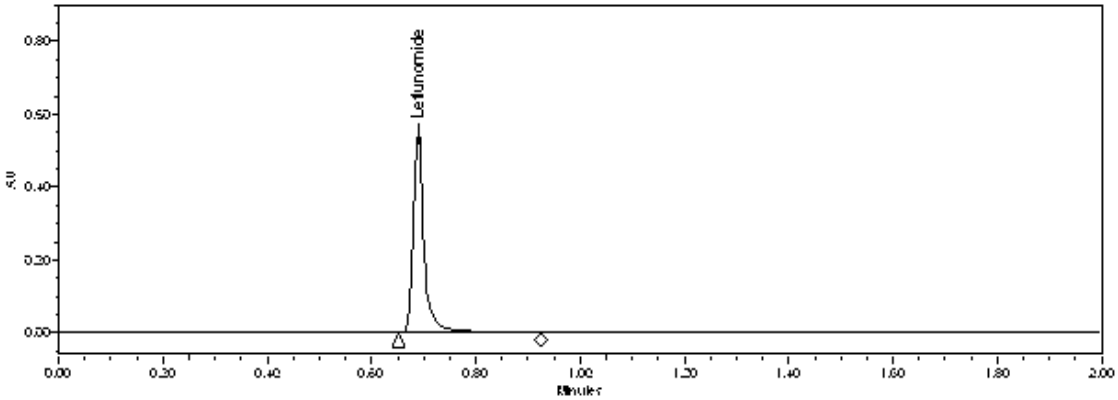


Figure 19: Standard chromatogram (0.45 ml/min flow rate)

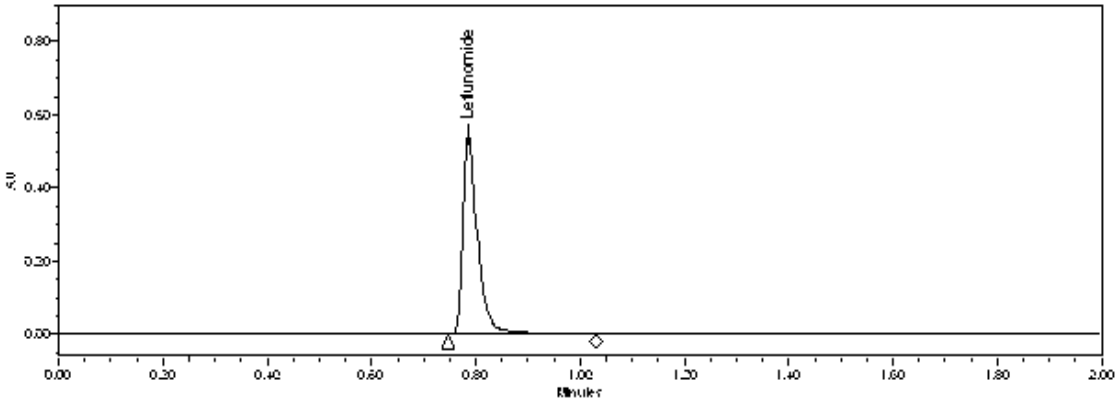


Figure 20: Standard chromatogram (Buffer-ACN (62:38 v/v)

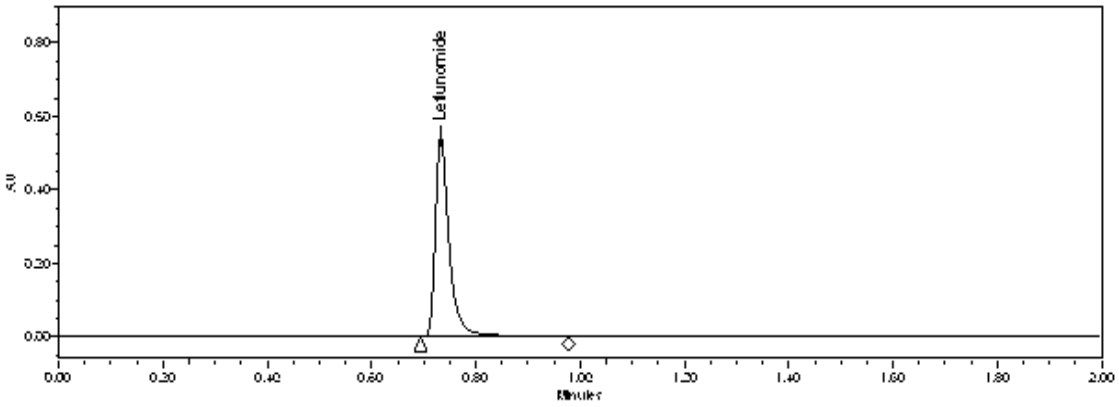


Figure 21: Standard chromatogram (Buffer-ACN (58:42 v/v)

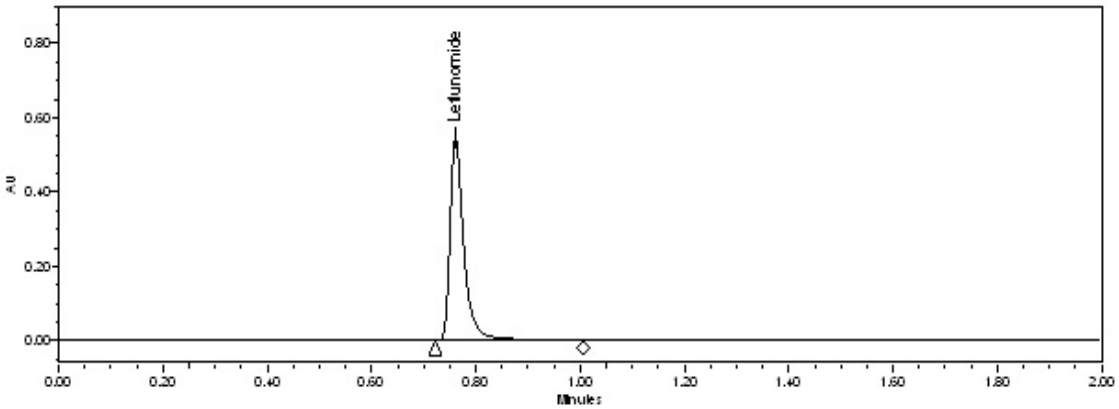


Figure 22: Standard chromatogram (Column change)

5.3.7 System suitability

A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same. Acceptance criteria for system suitability, Asymmetry not more than 2.0, theoretical plate not less then 4000 and % RSD of peak area not more then 2.0, were full fill during all validation parameter.

6. CALCULATIONS AND DATA

Calculation formula used:

1. Calculation formula for % assay of Leflunomide

$$\%\ Assay = \frac{Mean\ Test\ Area}{Mean\ Standard\ Area} \times \frac{Standard\ Weight}{100} \times \frac{10}{50} \times \frac{500}{Test\ Weight}$$

$$\times \frac{50}{10} \times \frac{Mean\ Test\ Weight}{Lable\ Claim} \times Potency\ of\ \text{Standard}$$

2. Relative standard deviation

$$\%\ RSD = \frac{Standard\ Deviation\ of\ Measurments}{Mean\ Value\ of\ Measurments} \times 100$$

3. Recovery

$$\%\ Recovery = \frac{Amount\ \ found}{Amount\ \ Added} \times 100$$

4. Amount found

$$Amount\ \ Found\ \ (mg/ml) = \frac{Mean\ Test\ \ Area}{Mean\ \ Standard\ \ Area} \times Standard\ \ Concentration$$

5. Amount added

$$Amount\ \ Added\ \ (mg/ml) = \frac{Weight}{Volume}$$

Specificity Study for Analytical Method Validation of Leflunomide Tablets

Standard weight (mg)	10.2		
Standard dilution	100	10	50
Standard potency	100 %		
Standard concentration (mg/ml)	0.0204		

Replicate	1	2	3	4	5
Standard area	1109463	1104667	1103821	1108382	1109695
Mean standard area	1107206				
Stdev.	2764.82				
% RSD	0.25				

Replicate	**Test Area**
1	1088033
2	1089272
Mean test area	1088653
Test weight (mg)	520.6
Label claim (mg)	10
Mean test weight (mg)	104.1
% Assay	100.3

Calculation:

Prototype calculation for one set:

$$\% \ \ Assay = \frac{1088653}{1107206} \times \frac{10.2}{100} \times \frac{10}{50} \times \frac{500}{520.6} \times \frac{50}{10} \times \frac{104.1}{10} \times 100$$

$$= 100.3 \ \%$$

Linearity Study for Analytical Method Validation of Leflunomide Tablets

Standard weight (mg)	10		
Standard dilution	100	10	50
Standard potency	100.0 %		
Standard concentration (mg/ml)	0.02		
Concentration of linearity stock solution (mg/ml)	0.1		

Replicate	1	2	3	4	5
Standard area	1104650	1114310	1103652	1112850	1100472
Mean standard area	1107187				
Stdev	5419.05				
% RSD	0.49				

Concentration level (%)	**Volume of linearity stock solution taken (ml)**	**Diluted to (ml)**	**Final concentration (mg/ml)**	**Mean area**
25	5	50	0.0100	563042
50	6	50	0.0150	843909
75	7	50	0.0200	1141704
100	8	50	0.0250	1432920
150	9	50	0.0300	1695680
		Correlation co-efficient		0.9998
		Slope		57085740
		Intercept		-6263.90

Precision Study for Analytical Method Validation of Leflunomide Tablets

Standard weight (mg)	10.8		
Standard dilution	100	10	50
Standard potency	100.0 %		
Label claim (mg)	10		
Mean test weight (mg)	104.1		
Standard concentration (mg/ml)	0.0216		

Replicate	1	2	3	4	5
Standard area	1140917	1141408	1140459	1141200	1142979
Mean standard area	1141393				
Stdev.	955.48				
% RSD	0.08				

Description	**Mean area**	**Wt. (mg)**	**% Assay**
Set 1	1025342	496	101.8
Set 2	1023250	501	100.6
Set 3	1026058	499.5	101.2
Set 4	1025136	503	100.4
Set 5	1022628	498.5	101.0
Set 6	1021204	511	98.4
		Mean	100.6
		Stdev	1.17
		% RSD	1.16

Calculation:

Prototype calculation for one set:

$$\%\ Assay = \frac{1025342}{1141393} \times \frac{10.8}{100} \times \frac{10}{50} \times \frac{500}{496} \times \frac{50}{10} \times \frac{104.1}{10} \times 100$$

$$= 101.8\ \%$$

Intermediate precision study for Analytical Method Validation of Leflunomide Tablets

Standard weight (mg)	10.9		
Standard dilution	100	10	50
Standard potency	100.0 %		
Label claim (mg)	10		
Mean test weight (mg)	104.1		
Standard concentration (mg/ml)	0.0218		

Replicate	1	2	3	4	5
Standard area	1149905	1169289	1159991	1169119	1168277
Mean standard area	1163316				
Stdev.	8439.10				
% RSD	0.73				

Description	**Mean area**	**Wt. (mg)**	**% Assay**
Set 1	1039067	505.5	100.2
Set 2	1026061	501	99.9
Set 3	1040481	510	99.5
Set 4	1030206	505.5	99.4
Set 5	1033177	510.5	98.7
Set 6	1032629	502	100.3
		Mean	99.7
		Stdev	0.60
		% RSD	0.60

Calculation:

Prototype calculation for one set:

$$\%\ Assay = \frac{1039067}{1163316} \times \frac{10.9}{100} \times \frac{10}{50} \times \frac{500}{505.5} \times \frac{50}{10} \times \frac{104.1}{10} \times 100$$

$$= 100.2\ \%$$

Comparison for Precision and Intermediate Precision Study for Analytical Method Validation for Leflunomide Tablets

	Set	**%Assay**
Precision study	1	101.8
	2	100.6
	3	101.2
	4	100.4
	5	101
	6	98.4
Intermediate precision study	1	100.2
	2	99.9
	3	99.5
	4	99.4
	5	98.7
	6	100.3
	Mean	100.1
	Stdev	1.00
	% RSD	1.00

Accuracy study for Analytical Method Validation of Leflunomide Tablets

Standard weight (mg)	10		
Standard dilution	100	10	50
Standard potency	100.0 %		
Standard concentration (mg/ml)	0.0200		

Replicate	1	2	3	4	5
Standard area	1144121	1143841	1144407	1144872	1144353
Mean standard area	1144319				
Stdev	381.575				
% RSD	0.03				

Recovery Level	Set No.	Mean area	Wt. (mg)	Volume (ml)			Value added	Value found	% Recovery	Mean % Recovery	Std Dev	% RSD
				I	II	III	Concentration (mg/ml)	Concentration (mg/ml)				
50%	Set 1	566435	4.9	100	10	50	0.00980	0.00990	98.04	100.24	1.09	1.09
	Set 2	566399	5.0	100	10	50	0.01000	0.00990	99.90			
	Set 3	564991	4.9	100	10	50	0.00980	0.00987	100.80			
100%	Set 1	1146794	10.0	100	10	50	0.02000	0.02004	99.85	99.44	1.23	1.24
	Set 2	1133157	10.1	100	10	50	0.02020	0.01980	99.30			
	Set 3	1133840	9.9	100	10	50	0.01980	0.01982	101.36			
150%	Set 1	1696076	15.0	100	10	50	0.03000	0.02964	100.07	99.72	1.31	1.31
	Set 2	1701809	15.0	100	10	50	0.03000	0.02974	98.85			
	Set 3	1714394	14.8	100	10	50	0.02960	0.02996	100.67			

Calculation:

Prototype calculation for one set:

$$\%\ Recovery = \frac{0.00990}{0.00980} \times 100 = 102.02\ \%$$

$$Amount\ Found\ (mg/ml) = \frac{566435}{1144319} \times 0.02000 = 0.00990\ \text{mg/ml}$$

$$Amount\ Added\ (mg/ml) = \frac{4.9}{100} \times \frac{10}{50} = 0.00980\ \text{mg/ml}$$

Solution Stability Study for Analytical Method Validation of Leflunomide Tablets

System suitability of standard preparation for solution stability					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1141121	1149917	1101050	1109463	1138408
2	1143841	1141408	1104390	1104667	1130459
3	1144507	1140459	1104832	1103821	1131200
4	1143872	1140200	1104970	1108382	1130905
5	1142353	1145979	1103335	1109695	1139272
Mean	1143139	1143593	1103715	1107206	1134049
Stdev	1377.65	4236.58	1622.11	2764.82	4392.33
%RSD	0.12	0.37	0.15	0.25	0.39

Solution stability for standard preparation at 2 -8°C				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1141121	1141121	1141121	1141121
2	1143841	1143841	1143841	1143841
3	1144507	1144507	1144507	1144507
4	1143872	1143872	1143872	1143872
5	1142353	1142353	1142353	1142353
1	1135991	1128014	1116282	1110143
2	1133119	1127493	1117107	1111054
Mean	1140686	1138743	1135583	1133842
Stdev	4415.41	7592.55	12954.62	15920.01
%RSD	0.39	0.67	1.14	1.40

Solution stability for standard preparation at room temperature				
	After 12 hours	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area
1	1141121	1141121	1141121	1141121
2	1143841	1143841	1143841	1143841
3	1144507	1144507	1144507	1144507
4	1143872	1143872	1143872	1143872
5	1142353	1142353	1142353	1142353
1	1132105	1169289	1112686	1104407
2	1131575	1167432	1115628	1102979
Mean	1139911	1150345	1134858	1131869
Stdev	5628.91	12369.85	14211.73	19284.83
%RSD	0.49	1.08	1.25	1.70

Solution stability for test preparation at 2 -8°C					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1141121	1149917	1101050	1109463	1138408
2	1143841	1141408	1104390	1104667	1130459
3	1144507	1140459	1104832	1103821	1131200
4	1143872	1140200	1104970	1108382	1130905
5	1142353	1145979	1103335	1109695	1139272
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	1096429	1070519	1057443	1050958	1046080
2	1094254	1075383	1054519	1049857	1049734
Mean	1095892	1072951	1055981	1050408	1047907
% Assay	99.6	100.3	98.4	98.6	97.8
Standard weight (mg)	10.4	10.7	10.3	10.4	10.6
Test weight (mg)	521.1	521.1	521.1	521.1	521.1
% Difference compare to that of Initial		-0.7	1.2	1.0	1.8

Solution stability for test preparation at room temperature					
	Initial	***After 12 hours***	***After 24 hours***	***After 36 hours***	***After 48 hours***
	Standard	***Standard***	***Standard***	***Standard***	***Standard***
Replicate	Peak area	Peak area	Peak area	Peak area	Peak area
1	1141121	1149917	1101050	1109463	1138408
2	1143841	1141408	1104390	1104667	1130459
3	1144507	1140459	1104832	1103821	1131200
4	1143872	1140200	1104970	1108382	1130905
5	1142353	1145979	1103335	1109695	1139272
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	1096829	1070028	1056383	1046033	1044387
2	1094954	1069396	1052028	1044272	1047443
Mean	1095892	1069712	1054206	1045153	1045915
% Assay	99.6	100.0	98.3	98.1	97.7
Standard weight (mg)	10.4	10.7	10.3	10.4	10.6
Test weight (mg)	521.1	521.1	521.1	521.1	521.1
% Difference compare to that of Initial		-0.4	1.3	1.5	1.9

Calculation:

Prototype calculation for one set:

$$\%\ Assay = \frac{1095892}{1143139} \times \frac{10.4}{100} \times \frac{500}{521.1} \times \frac{50}{10} \times \frac{104.1}{10} \times 100$$

$$= 99.6\ \%$$

Robustness Study for Analytical Method Validation of Leflunomide Tablets

	Flow rate at 0.35ml/min	**Flow rate at 0.45ml/min**	**Buffer-ACN 38: 62**	**Buffer- ACN 58: 42**	**Column change**
Replicate	Standard Area	Standard Area	Standard Area	Standard Area	Standard Area
1	1339878	1008918	1102325	1170918	1124805
2	1342058	1006247	1104472	1172058	1118285
3	1340459	1010052	1100487	1170459	1123791
4	1341050	1011253	1102384	1176200	1119118
5	1345973	1012854	1110234	1177379	1122379
Mean	1341884	1009865	1103980	1173403	1121676
Stdev	2423.86	2494.11	3769.68	3173.45	2863.58
% RSD	0.18	0.25	0.34	0.27	0.26
Replicate	Test Area	Test Area	Test Area	Test Area	Test Area
1	1266469	981089	1036895	1098524	1059058
2	1274255	981895	1039983	1095978	1058060
Mean	1270362	981492	1038439	1097251	1058559
Standard Weight (mg)	10.5	10.5	10.5	10.5	10.5
Test Weight (mg)	520.5	520.5	520.5	520.5	520.5
Label Claim (mg)	10	10	10	10	10
Mean Test Weight (mg)	104.1	104.1	104.1	104.1	104.1
% Assay	99.4	102.0	98.8	98.2	99.1

Calculation:

Prototype calculation for one set:

$$\%\ Assay = \frac{1270362}{1341884} \times \frac{10.5}{100} \times \frac{10}{50} \times \frac{500}{520.5} \times \frac{50}{10} \times \frac{104.1}{10} \times 100$$

= 99.4 %

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