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Optimization of HPLC Measurements Using Addictive Drug Leflunomide and its Validation Studies under Different Medium

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Abstract – The Leflunomide is an immunomodulatory drug inhibiting dihydroorotate dehydrogenase an enzyme involved in de novo pyrimidine synthesis. Genuine anti proliferative activity has been proven. Additionally, the several experimental models (both in vivo and in vitro) have demonstrated an anti-inflammatory effect. This double action is supposed to slow progression of the disease and to cause remission or relief of symptoms of rheumatoid arthritis and psoriatic arthritis such as joint tenderness and decreased joint and general mobility in human patients.

Keyword: Leflunomide, Novo Pyrimidine, Drug Inhibiting, Arthritis etc.

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1.1 INTRODUCTION TO DRUG

The drug (Leflunomide) is a pyrimidine mixture inhibitor belonging to the DMARD (disease-modifying ant rheumatic drug) category of drugs (Dougados, et. al., 2010), which are chemically and pharmacologically very heterogeneous (Vivien, et. al., 2014). The material is sold under the brand name Arava by Sanofi-Aventis. It's available for oral administration as tablets containing 10, 20, or 100 mg of active drug. The Arava also contains some inactive ingredients, which could cause allergies or intolerances. Arava was approved by FDA and in many other countries (e.g., Canada, Europe) in 1998.

1.1.1 Basic Chemical Data Method

The Leflunomide is chemically 5-Methyl-N-[4(triflurомethyl) phenyl]-isoxazole-4- carboxamide showing in Figure 1. Its molecular formula is C12H9F3N2O2 having molecular weight 270.207 g/mole. The Leflunomide is a pyrimidine synthesis inhibitor belonging to the DMARD (disease-modifying ant rheumatic drug) class of drugs, which are chemically and pharmacologically very heterogeneous.

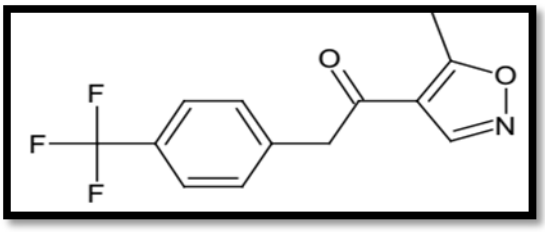

Figure: 1 Showing 5-Methyl-N-[4(triflurомethyl) phenyl]-isoxazole-4-carboxamide

The Pharmacokinetics Arava tablets of 80 per cent of the creatures are available. The joint management with high fat meal did not have a significant impact on the plasma levels of the active metabolite teriflunomide. The following oral, metabolized, teriflunomide Leflunomide is basically responsible for all the activities of the drug in the body. The study of the Pharmacokinetic studies of Leflunomide primarily the plasma concentration of teriflunomide. The plasma levels unchanged Leflunomide can sometimes be detected, but at a very low level. Some of the smaller the metabolite has been noted to occur in human plasma, without taking into account the favorable drug effects. Teriflunomide, metabolized in the liver in the cytosolic and microsomal sites and further excreted really, and billary.

The absorption and needs to be loaded and oral dose, Peak plasma levels of teriflunomide occurred

between 6 to 12 hours after dosing. Because of its long half-life (about 2 weeks), a loading dose of 100 mg for 3 days is used in clinical studies, to reach a steady-state level. If you do not have a Loading Dose, it is estimated that the steady-state plasma concentrations would require nearly two-month dosing, can be reached. The resulting plasma level following two loading dose and the dose to clinical indication, the plasma level is proportional to the dose.

Teriflunomide can be found as late as 2 years after the termination of the treatment of human plasma has adequate levels; can cause serious harm to pregnant women or to cause significant interactions. If you are quickly removed from the body is necessary, which is a 11-day program, the cholestyramine or with activated carbon for instructions, and will soon reduce plasma levels below the critical limit of 0.02 mg/l. Limited experience has shown that it is not dialyzable teriflunomide.

1.2 REVIEW OF LITERATURE

The literature review of Leflunomide shows that various methods of analysis, it was determined that the drug in pharmaceutical formulations and in various biological fluids. The literature review and analysis of the Leflunomide are the following:

According to C., Vivien G. Bruce and Charles and E. T. Susan have developed methods to quickly identify active metabolite of Leflunomide A77 1726 Human plasma by high-performance liquid chromatography. An easy way to measure the metabolite of Leflunomide A 77 1726 Human plasma by HPLC is presented. Workup is a simple example, the use of acetonitrile, protein deposition. The separation of the color spectrum A77 1726 and internal standards, α-phenylcinnamic acid, enabling the use of a C18 column out of the 305 nm uv Detection (Hong, et. al., 2015).

According to L. Hong, Z. Ma has been developed method for the determination of Leflunomide in tablets by HPLC. They have used the chromatography column was Diamonsil C18 (250 mm×4.6 mm, 5 **μ**m) with methanol: water: acetic acid (75: 25: 0.1)

As mobile phase at wavelength 260 nm. There was a good linear relationship between Leflunomide content and peak area in the range of 0.1-10.0 mg/L (Jiang,et. al., 2015).

According to S. Jiang, L. Kang, G. Yan, Z. Li, Y. Wang, D. Zhang has been developed method for the determination of Leflunomide and related compounds by reverse phase high performance liquid chromatography. They were presented a reversed phase HPLC method for the determination of Leflunomide (Abbas, et. al., 2016).

1.3 THE OBJECTIVES OF CURRENT 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 is the most commonly used method for analysis of leflunomide.

A wide range of literature survey reveals several methods to estimate the HPLC Leflunomide in drug dosage forms and biological fluids; however, not all of which are stable. Most of the reports either does not include stress degradation studies or is not fully verified, they are cumbersome, time-consuming and expensive. The authentication method is an important step in drug analysis.

This process will confirm the analysis process; the analysis is suitable for its intended use, and the reliability of the results in any way. The main objective, therefore, is currently working to develop and validate a stability indicating HPLC methods content from the Leflunomide Dose Table (Tablet PC). This work also involved forced degradation of Leflunomide stress conditions like acid hydrolysis, the base hydrolysis, oxidation, photolytic of heat and pressure. Therefore, this method can be used for routine quality control analysis and stability.

1.4 EXPERIMENTAL WORKS

1. Materials

The Leflunomide standards provided in the heterogeneous commodities limited 10 mg Leflunomide tablets containing Leflunomide and non-active ingredients used in the drug matrix is obtained from the market. Analysis of the levels of acetic acid ammonium was purchased from a Sisco Research Private Ltd., Mumbai (India). HPLC grade methanol and water is obtained from the Spectrochem Private Ltd., Mumbai (India). Analytical levels of hydrochloric acid, glacial acetic acid, sodium hydroxide particles and 30% V/V hydrogen peroxide solution obtained from Ranbaxy fine chemicals, New Delhi (India).

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-M10Avp 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).

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 dissolves 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. Diluents Preparation

A mixture of acetonitrile-buffer (50:50, *v/v*) was used as diluent.

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 diluents [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.

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 diluent. 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 diluent the concentration obtained was 20 µg/ml of Leflunomide.

7. 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 acetonitrile and 0.02M ammonium acetate buffer (60:40, v/v). 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.

1.5 RESULT AND DISCUSSION OF WORKS

1.5.1 Optimization of the HPLC Method

To the Proper selection of the method depends upon the nature of the sample (ionic or
ionisable or neutral molecule), its molecular weight and solubility. The Leflunomide is dissolved in polar solvent hence RP-HPLC was selected to estimate them to develop a rugged and suitable HPLC method for the quantitative determination of Leflunomide. The analytical conditions 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 0.45 µm nylon membrane (Millipore Private Ltd. Bangalore, India) and degassed in an ultrasonic bath (Spincotech Private 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. In the Figure 2 and 3 represents wavelength selection and PDA scan of standard preparation. In the Figure 4 and 5 represents the chromatograms of standard and test preparation respectively.

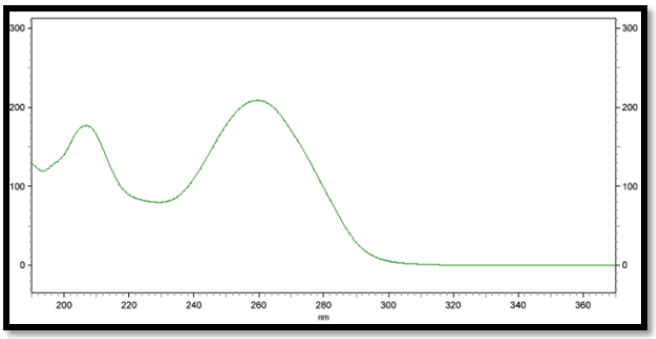

Figure 2: Showing Wavelength Selection of Standard Preparation

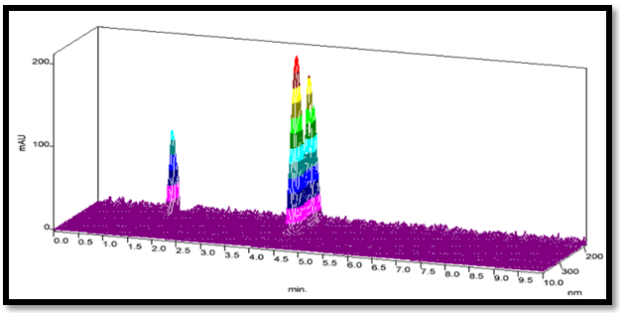

Figure 3: Showing PDA Scans of Standard Preparation

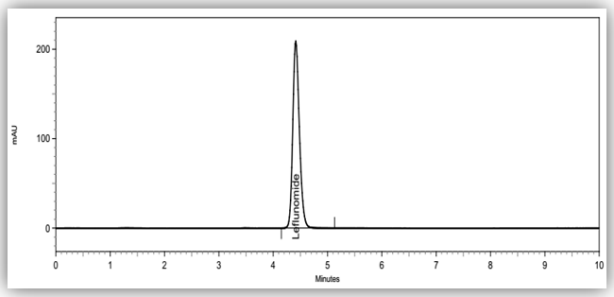

Figure 4: Showing Chromatogram of Standard Preparation

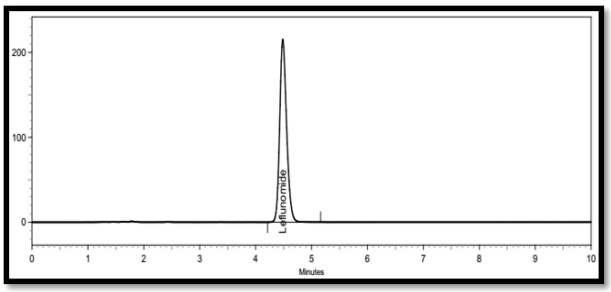

Figure 5: Showing Chromatogram of Test Preparation

1.5.2 Validation Method

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 of product with drug peak.

2. Linearity

The Seven point's calibration curve was 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 was y = 8E+06x- 1652.8 with correlation coefficient 0.9983. (Figure 6) Chromatogram obtain during linearity study were shown in the figure 7-8.

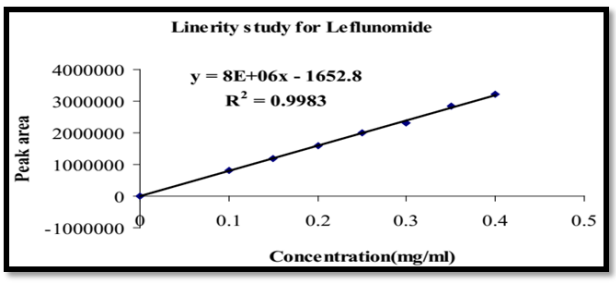

Figure 6: Showing Linearity curve for Leflunomide

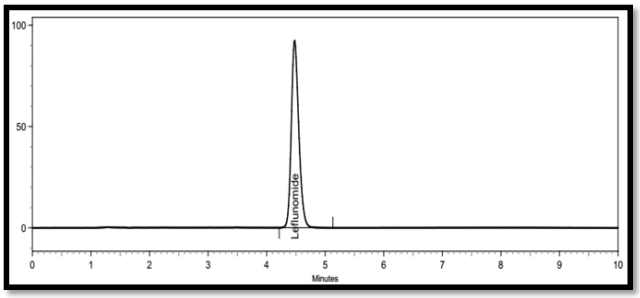

Figure 7: Showing Linearity study chromatogram of level-1 (40%)

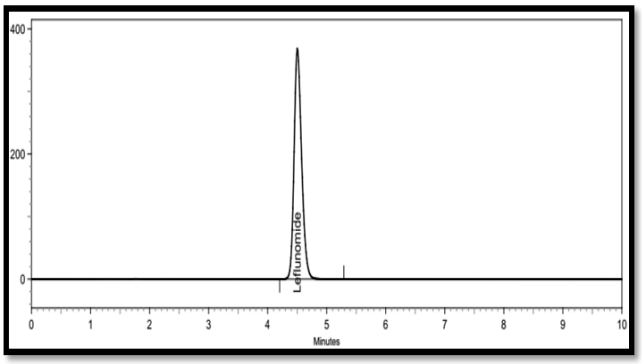

Figure 8: Showing Linearity study chromatogram of level-7 (160%)

3. LOD and LOQ

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 was found to be 0.008 ppm and the LOQ value 0.04 ppm. Chromatogram of LOD and LOQ study were shown in the Figure 9-10.

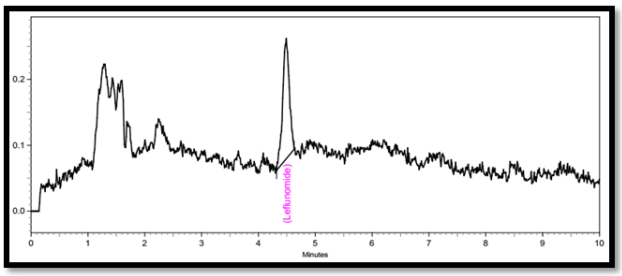

Figure 9: Showing Chromatogram of LOD Study of Leflunomide

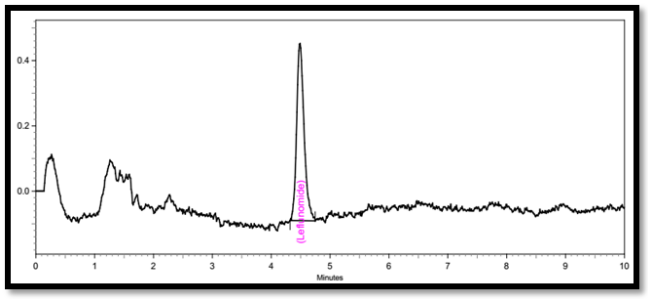

Figure 10: Showing Chromatogram of LOQ study of Leflunomide

4. 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 < 0.63 % and < 0.69 %, respectively, which confirm that the method was precise.

Set	Intraday (n = 6)	Interday (n = 6)
1	101.3	100.6
2	101.1	99.1
3	100.7	101.0
4	100.8	99.8
5	99.6	100.5
6	101.3	100.6
Mean	100.8	100.3
Standard deviation	0.64	0.69
% RSD	0.63	0.69

Table 1: Showing Evaluation data of Precision Study

5. Accuracy

The HPLC area responses for accuracy determination are depicted in Table 2. The result shown that best recoveries (99.58 - 100.17 %) of the spiked drug were obtained at each added concentration, indicating that the method was accurate. Chromatogram obtain during accuracy study were shown below.

Level (%)	Amount added concentration [a] (mg/ml)	Amount found concentration [a] (mg/ml)	% Recovery	% RSD
50	0.01007	0.01002	99.58	1.41
100	0.01993	0.01997	100.17	1.07
150	0.03007	0.03002	99.86	0.93

Table 2: Showing Evaluation data of accuracy

6. Robustness

The result of robustness study of the developed assay method was established in Table 3. 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. The System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

Robust Conditions	% Assay	System Suitability Parameters	
		Theoretical Plates	Asymmetry
Flow 0.9 ml/min	101.9	6598	0.89
Flow 1.1 ml/min	102.5	6434	1.92
ACN-Buffer (62: 38,v/v)	101.3	5922	1.45
ACN-Buffer (58: 42,v/v)	99.9	5536	1.64
Column change	100.2	5878	0.17

Table 3: Showing Robustness Study

1.6 CONCLUSION

Innovative analytical methods have been developed to be often applied to determine whether the Leflunomide pharmaceutical dosage forms. In this study, the stability in the Leflunomide dose forms established and recommended stress condition. In the development process has assessed the specificity, linearity, accuracy, precision and stability in order to determine their stability analysis methods. The Facts have proved that it is concrete, Linear, precise, accurate, reliable and stable indicator. As a result, this method is recommended for routine quality control analysis & also the stability of samples intended for analysis.

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