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Development and Validation of RP-HPLC Method for the Estimation of Leflunomide in Bulk Drug And Tablets.

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ABSTRACT

A simple, rapid, reverse phase high performance liquid chromatography (HPLC) method has been developed for the estimation of Leflunomide in bulk drug and pharmaceutical dosage form. The separation was achieved with a Hypersil BDS C18 column. This method uses mobile phase consisting of Acetonitrile and 10mM potassium dihydrogen orthophosphate-buffer of PH 4.9±0.1(90:10) at a flow rate of 1ml/min. Leflunomide was detected by UV-absorption at 254nm with a retention time of 3.03min. the method was carried out by standard addition method. The estimation was linear over the concentration range of 10-50µg/ml, with the correlation coefficient of 0.9999. the intra-day and inter day studies shown that method was accurate and precise, easy-to-operate and validate.

Keywords: RP-HPLC, Leflunomide, Acetonitrile, and KH2PO4-buffer.

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INTRODUCTION

Leflunomide is a new Disease Modifying Anti-Rheumatic Drug [DMARD] with immunosuppressant activity. Chemically Leflunomide is known as 5-Methyl-N-[4-(trifluoro)phenyl]-4-isoxazole carboxamide [1]. The chemical structure of Leflunomide is given in figure-1. It has been used to reduce the signs and symptoms of arthritis and to retard joint damage in patients with active rheumatoid arthritis. Leflunomide is a prodrug, which is rapidly non-enzymatically, converted to its active metabolite A77 1726 after oral administration [2]. A77 1726 has antirheumatic activity through the inhibition of the enzyme dihydro-orotate dehydrogenase [DHODH], a key enzyme in the de novo production of pyrimidines in T-lymphocytes, a process essential for T-lymphocyte proliferation [3,4]. It is not official in any Pharmacopoeia. Since the conversion of Leflunomide to A77 1726 in vivo is essentially complete, most pharmacokinetic studies have been focused to measure A77 1726. Literature survey reveals that several high performance liquid chromatography (HPLC) method have been published for the kinetic monitoring and determination of A77 1726 in human blood and plasma [4-8]. It is also reported that, a few methods have been published for the determination of Leflunomide in tablets by HPLC [9,10], and spectrophotometric methods [11].

The present study involves a development of a new reverse phase high performance liquid chromatography (RP-HPLC) method for the estimation of Leflunomide in bulk drug and pharmaceutical dosage form using UV-Visible detector without internal standard as there was no complex extraction or separation steps involved.

In a simultaneous determination of Leflunomide and A77 1726 in Human plasma performed by HPLC, the retention time of Leflunomide was reported about 16min. it is relatively long time for the active content assay of Leflunomide to use in routine laboratories. The main aim of present work was to analyse the compound within a relatively short period, which was achieved as the retention time of Leflunomide in this proposed method is 3.03.

Figure 1: Structure for Leflunomide
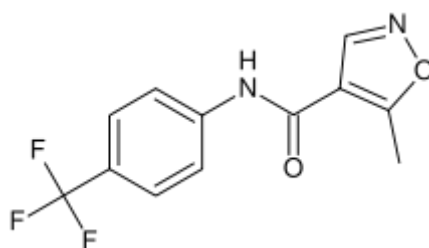
MATERIALS AND METHODS

Instrumentation

The Lachrom high performance liquid chromatographic system consisting of Merck HITACHI pumps L-7100, Column oven L-7350, UV-detector L-7400 and D-7000 Multi HSM software. Standard solutions and sample solutions were injected through a 20µl loop

injection port with a 50-gauge injection needle. Branson sonicator was used for degassing of solvents.

Chemicals and Reagents

Leflunomide drug was obtained as the gift sample from Aarti Drugs Ltd. Acetonitrile HPLC grade, Water HPLC grade were procured from Merck Pvt. Ltd., and potassium dihydrogen orthophosphate was obtained from S.D.Fine Chemicals Ltd. The pharmaceutical dosage form containing 10mg Leflunomide CLEPT-10 a product of Crosslands was purchased from a local drug store.

Chromatographic conditions

The chromatographic column used was Hypersil BDS C18(5microns) of 250×4.6mm**.** The system was operated at room temperature (27°C). The flow rate of mobile phase(Acetonitrile:10mM KH_2PO_4-buffer, in the ratio 90:10) was 1ml/min throughout the method determination of Leflunomide. Detection was carried out at 254nm and the injection volume was 20μl.

Preparation of mobile phase

Preparation of 10mM KH_2PO_4-buffer solution

1.36g of KH2PO4 was dissolved in 1000ml of water (HPLC grade) and filtered through the 0.45 and degassed by sonication for 10min.

Acetonitrile

Enough volume was filtered and sonicated.

The above solutions are kept in clean bottles separately and the volume of each solution was adjusted in the instrument to obtain the mobile phase ratio.

This mobile phase is recommended, because it is relatively simple and containing a good buffer. Since retention time of an analyte is influenced by pH, a buffer is used in the proposed work.

Preparation of Standard stock solution (1mg/ml)

10 mg of leflunomide was accurately weighed, transferred into 10 ml volumetric flask and dissolved and made the volume upto the mark with 3.6 ml of acetonitrlie and 6.4 ml of water.

Preparation of working standard solutions

From the above standard stock solution different aliquots are made to get working standard solutions in the range of 10 µg/ml to 50 µg/ml . All the aliquots are filtered through 0.45 µ filters and sonicated for 10 minutes.

Sample solution

20 tablets were weighed accurately and finely powdered. The powdered equivalent to 10 mg of leflunomide was weighed and taken and diluted to 100 ml with 36 ml of acetonitrile and 64 ml of water in a volumetric flask to get concentration in the range of 100µg/ml. The solution was filtered and sonicated for 10 minutes for degas the solution. Composition and flow rate of the mobile phase was programmed from mother pump and the mobile phase acetonitrile: 10 mM KH_2PO_4 buffer 90 : 10 was passed through 0.45µ membrane filter separately and degassed. It was delivered at 1 ml/min for column stabilization, during this period the baseline was continuously monitored.

Preparation of working sample stock solution

From the above sample solution different aliquots are made to get working sample stock solutions in the concentration range of 10µg/ml to 30µg/ml.

Assay validation

The method was validated according to ICH guidelines for validation of analytical procedures [12-15].

Calibration curves were obtained with five concentrations of the standard solutions (n=2) in the range of 10µg/ml – 50µg/ml. The volume of each aliquots injected was 20 µl. linearity was evaluated by linear regression analysis using the least square regression method.

Accuracy of the present analytical method was determined as follows:

Three different concentration range sample solutions were prepared and analysed, each aliquot is measured in triplicate.

Accuracy of the method was also determined by using standard addition method. To a desired pre-analysed sample aliquot, a known amount of standard drug is added in two different levels and analysed each level thrice. The volume of sample and standard solution added for the determination of above parameters was 20 µl.

Stability of leflunomide was studied by analyzing the working standard solution after two days and by comparing the peak area with previously peak area obtained from standard drug.

RESULTS

Method validation

With the described method each chromatogram of different concentrations of drug sample are obtained at the same retention time of 3.03 minutes.

The calibration curve for leflunomide was linear over the full concentration range from 10 – 50 µg/ml, with a correlation coefficient of 0.9999. The different concentrations taken and peak area obtained are tabulated in table-1. The chromatogram for linearity study is shown in figure-2. The linearity graph obtained by drawing the graph of concentration vs. peak area is shown in figure-3. The results of linear regression equation are tabulated in table-2.

Table 1: The Linearity study of leflunomide

SL.NO	Concentration (in µg)	Peak Area
1	10	750796
2	20	1445964
3	30	2123446
4	40	2779587
5	50	3452430

Table 2: Linear regression data of leflunomide

Parameters	Data obtained
Conc. Range(µg/ml)	10-50
m	67369
b	89377
R	0.9999

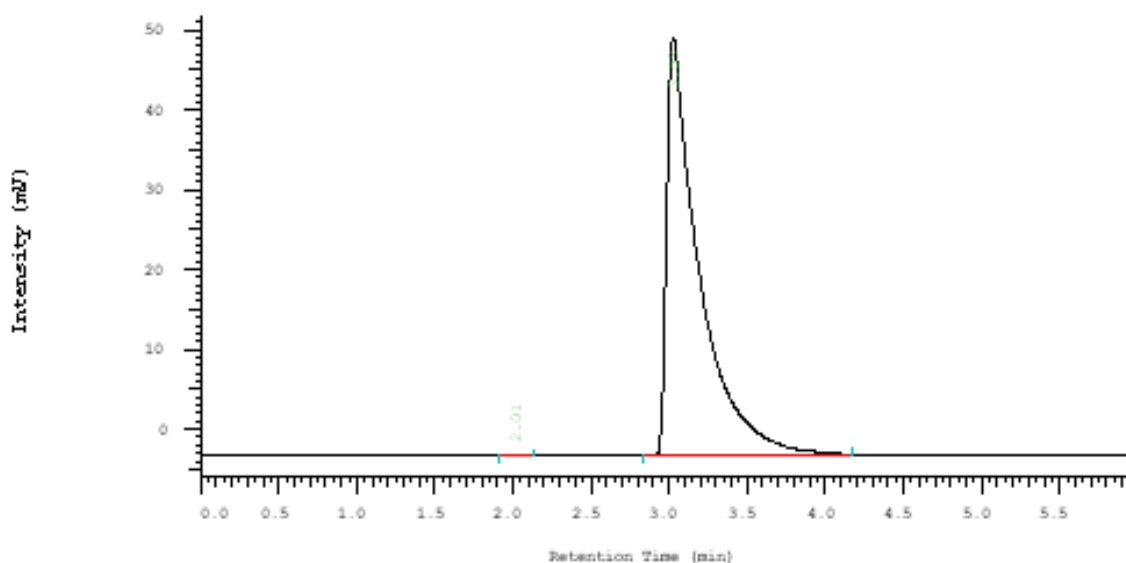**Figure 2: Linearity Study of Standard Leflunomide:**

Figure-4 and tables-3, 4, 5 are obtained for accuracy study of formulated drug in three different concentrations 10,20,30 µg/ml respectively. The results of recovery study of formulated leflunomide on addition of standard are represented in table-6 and figure-5. The

result of inter day stability of leflunomide in mobile phase is shown in figure-6 and peak area is compared with the peak area obtained from fresh solution.

Table 3: Accuracy study of formulated Leflunomide; 10µg

Peak area (µV . sec)	Percentage Obtained	RSD
756866	100.812	0.9518
742627	98.911	

Table 4: Accuracy study of formulated Leflunomide; 20µg

Peak area (µV . sec)	Percentage Obtained	RSD
1407811	97.36	1.42
1448537	100.17	

Table 5: Accuracy study of formulated Leflunomide; 30µg

Peak area (µV . sec)	Percentage Obtained	RSD
2080340	97.96	1.29
2135018	100.54	

Table 6: Recovery of formulated Leflunomide on addition of standard drug

Amount of the sample drug taken(in mg)	Amount of standard drug added (in mg	Amount obtained	% recovery	RSD
10	1	11.15	101.36	
10	1.5	11.26	97.91	1.73

Figure 3: Linearity graph of Leflunomide
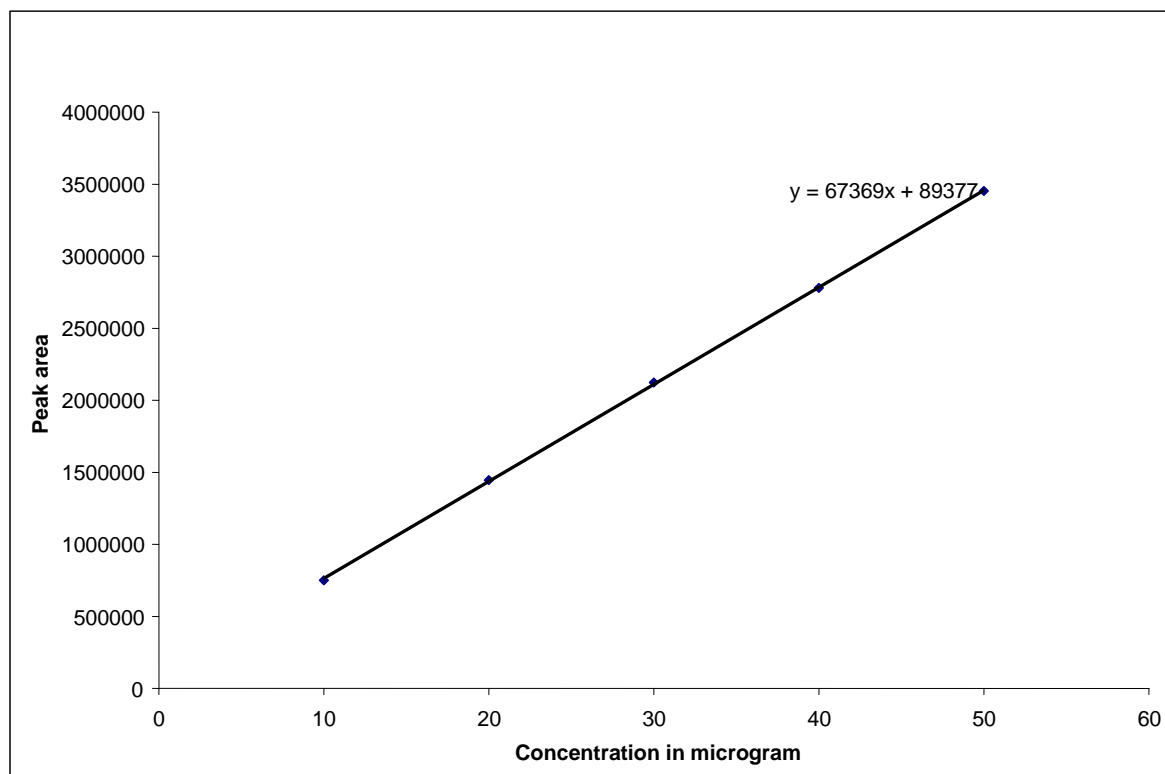

$y = 67369x + 89377$

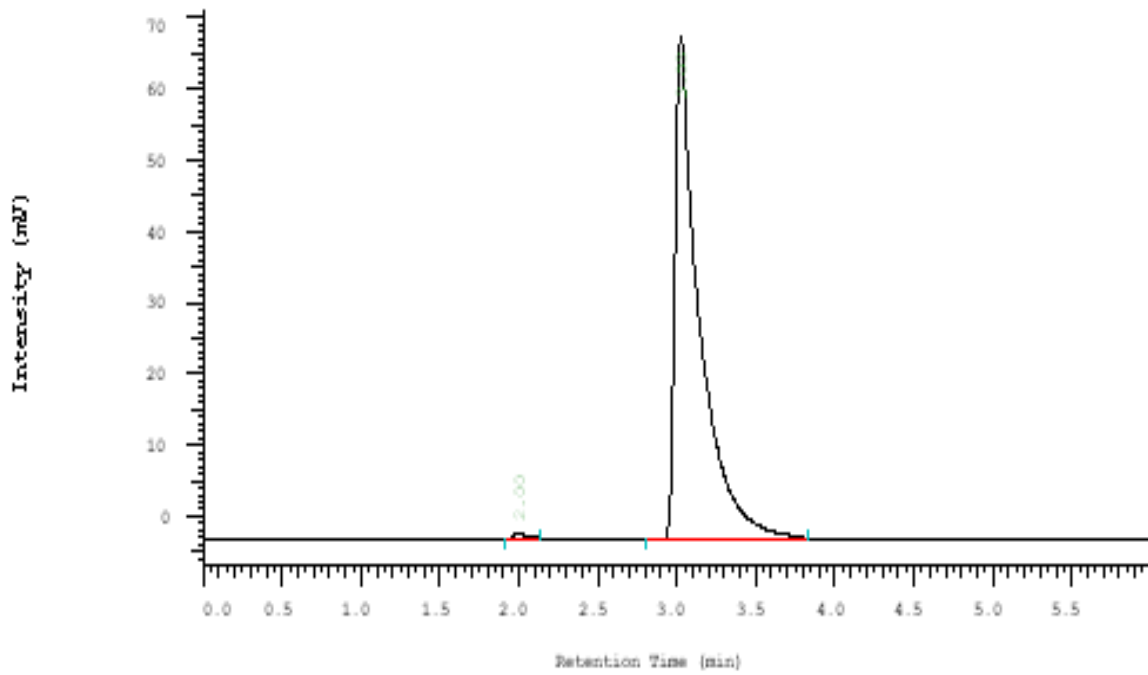**Figure 4: Accuracy study of Leflunomide**

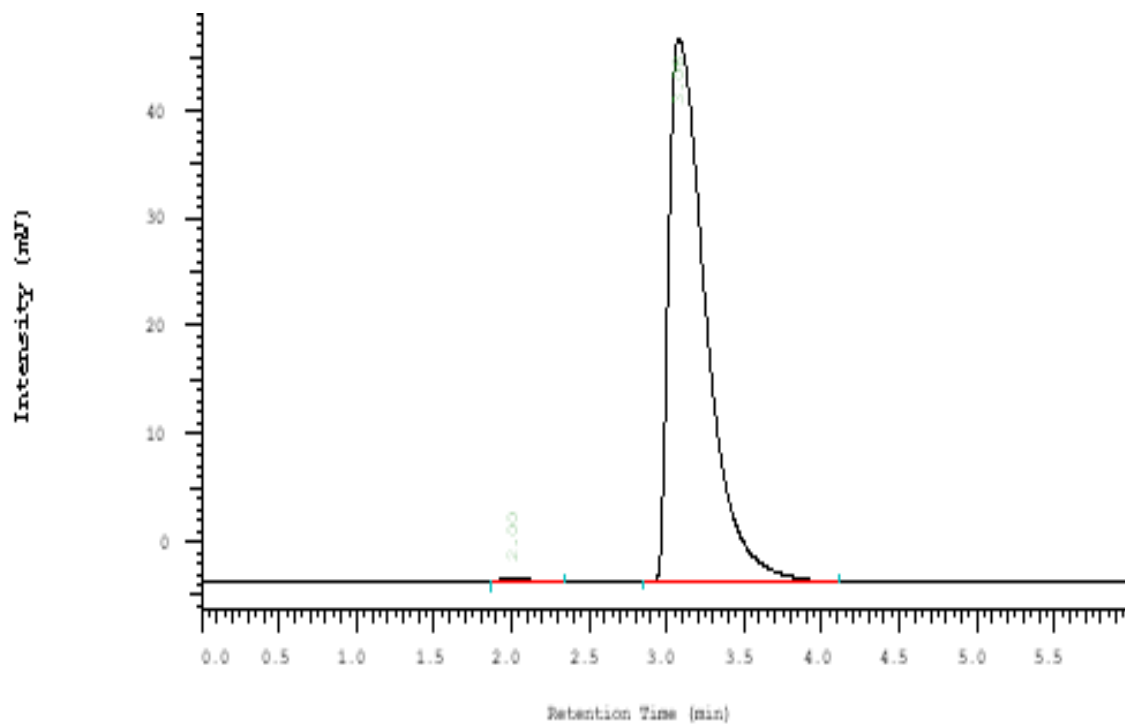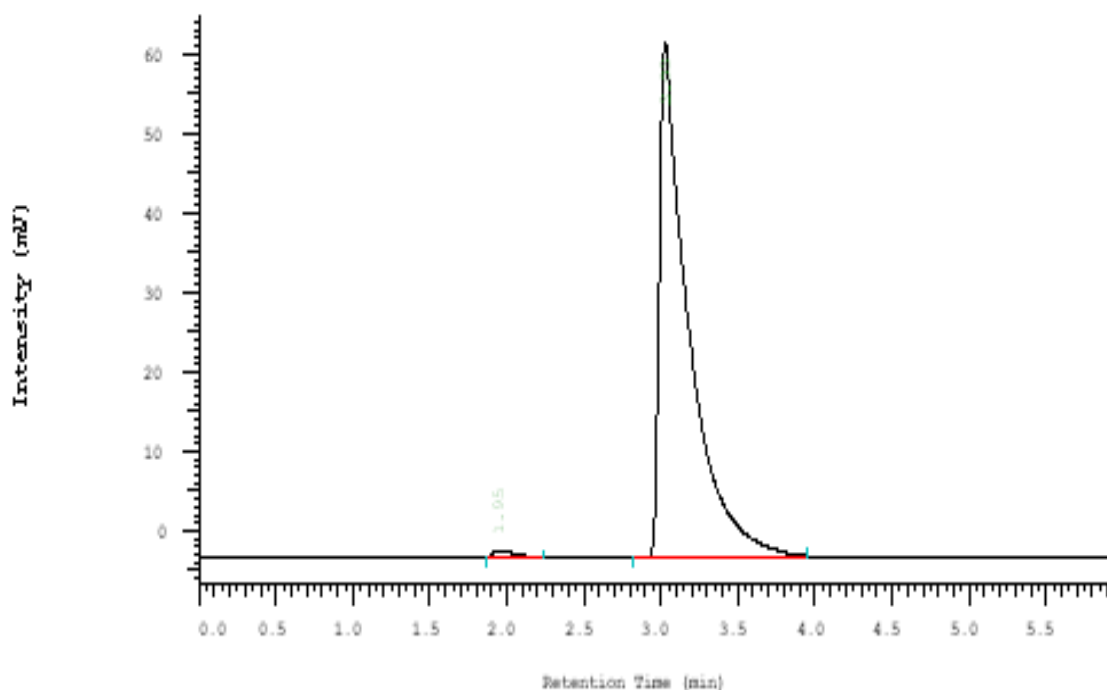**Figure 5: Recovery study of formulated drug on addition of sample:**

Figure 6: Stability of leflunomide after two days

DISCUSSION

The development of an analytical method for the determination of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. The objective of this present work was to develop a rapid and sensitive HPLC method for the analysis of leflunomide in bulk drug and its tablet formulation using the most commonly employed C18 column with UV detection.

The principal conclusion drawn from the above results are as follows:

- The run time was set at 4.7 min and the retention time for leflunomide was 3.03 min. Each sample was injected six times and the retention times were same. So it indicates the system suitability for carrying out the analysis work.
- From the calibration curve, a linear regression equation is obtained with preferable correlation coefficient
- Recovery studies by using standard addition method show that, the results are repeatable and method was accurate and precise.

CONCLUSION

The proposed HPLC method was found to be very simple, rapid, highly accurate, sensitive and precise. Therefore the method can be useful in routine quality control analysis of Leflunomide in its dosage forms.

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