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# ANALYTICAL METHODS FOR THE DETERMINATION OF LEFLUNOMIDE - A SHORT REVIEW

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## ABSTRACT

Leflunomide is a pyrimidine synthesis inhibitor belonging to the (DMARD) class of drugs, used to treat the symptoms of rheumatoid arthritis. It also helps to reduce joint damage and improves physical functioning and to slow the progression of structural damage associated with the disease. The substance is sold under the brand name Arava® by Sanofi-Aventis. Arava® was approved by the U.S. Food and Drug Administration (FDA) and by many other countries (e.g., Canada, Europe) in 1998.A widespread review of the literature published in various pharmaceutical journals has been conducted and the instrumental analytical methods which are developed and used for determination ofLeflunomide have been reviewed. This review includes UV-Visible Spectroscopy, RP-HPLC-UV, LC-MS/MS, LC-ESI-MS/MS and UPLC-PDA. The applications of these methods for the determination of Leflunomide in pharmaceutical formulations and biological sample have also been discussed in this article.

**Keywords:** Leflunomide; Teriflunomide (A771726)

## INTRODUCTION

Leflunomide (LEF), [*N*-(4-trifluoromethylphenyl)-5-methyl-isoxazole-4-carboxamide] is a disease-modifying antirheumatic drug (DMARD) used in active moderate to severe rheumatoid arthritis and psoriatic arthritis. It is an isoxazole derivative with both anti-inflammatory and immunosuppressive properties. It has also been used for the prevention of acute and chronic rejection in recipients of solid organ transplants andis designated by the FDA as an orphan drug for this use.[1, 2] After oral administration, LEF (as a prodrug) is rapidly, non-enzymatically and completely converted into its long acting, active metabolite Teriflunomide (A77 1726) [2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)-crotonamide]

(Figure no. 1). This metabolite inhibits dihydro-orotate dehydrogenase, a key enzyme of *de novo* pyrimidine synthesis leading to decrease in rebonucleotide synthesis and the arrest of stimulated cells in the $G_1$ phase of cell growth. It inhibits various protein tyrosine kinases, such as protein kinase C (PKC) and T-cell proliferation and production of auto antibodies by B cells. It is available in oral dosage form as tablets containing 10, 20 and100 mg of LEF.[3, 4] The available marketed formulations of LEF are enlisted below in table no. 1.[5]

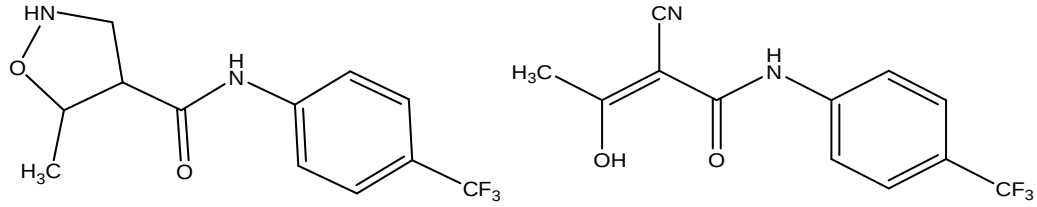
**Table no. 1: Marketed formulations of LEF**

| Marketed name | Dosage form | Strength | Company |
|---|---|---|---|
| ARAVA® | Tablet | 10, 20 and 100 mg | Sanofi-Aventis |
| CLEFT® | Tablet | 10 and 20 mg | Ranbaxy |
| LEFRA® | Tablet | 10 and 20 mg | TorrentPharma |
| LEFUMIDE® | Tablet | 10 and 20 mg | Cipla |
| LISIFEN® | Tablet | 10 and 20 mg | CadilaPharma |
| RUMALEF® | Tablet | 10 and 20 mg | ZydusCadila |

Development and validation of analytical methods are of basic importance to optimize the analysis of drugs in the pharmaceutical industry and to guarantee quality of the commercialized product.[6] Method development is required to develop quantitative methods to determine concentration of drug and if necessary metabolites in biological matrix. These methods are used to support several activities in drug development including formulation research, GLP, toxicology, clinical pharmacology and clinical research studies.[7] Method validation is performed to demonstrate that a particular method used for quantitative measurement of drug and/or metabolite is reliable and reproducible for intended use. The validated method is applied to the study samples with known samples with predefined acceptance criteria. The obtained values are used to calculate the pharmacokinetics parameters for the anticipated end results.[8]

A wide variety of analytical methods have been reported for the determination of LEF in pharmaceutical preparations and in biological fluids. It includes Ultraviolet - Visible Spectroscopy (UV), Reverse Phase - High Performance Liquid Chromatography - UV Spectroscopy (RP-HPLC-UV), Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS), Liquid Chromatography - Electron Spray Ionization - Tandem Mass Spectrometry (LC-ESI-MS/MS) and Ultra Performance Liquid Chromatography (UPLC). Among HPLC methods different internal standards, reversed phase columns with different size and different mobile phase compositions have been used for the quantification purpose. The aim of

thepresent review is to summarize these validated techniques for the determination of LEF in pharmaceuticals and biological matrix.




(i) (ii)

**Figure no. 1:Chemical structures of Leflunomide (i)and Teriflunomide(ii).**

## 2. VARIOUS ANALYTICAL METHODS REPORTED FOR LEFLUNOMIDE

### 2.1 BULK AND PHARMACEUTICAL PREPARATIONS

For analytical determination of LEF in formulated products, the literatures reported various methods like Differential Spectroscopy, UV Spectroscopy and various chromatographic methods like RP-HPLC and Stability indicating UPLCmethod.

#### 2.1.1 Spectrophotometric Method

Abbas *etal.* 2006 performed five different methods in UV-Visible Spectroscopy for the determination of Leflunomide in the presence of itsdegradates, 4-trifluoromethyl aniline and 3-methyl-4-carboxyisoxazole. In method A, measurement of δ (delta) value of LEF at 279.5 nm by differential derivative spectroscopy. Method B was based on first derivative spectroscopy by measuring the amplitude of LEF at 253.4 nm. Method C depended on reaction of 4-trifluoromethyl anilinedegradate with 2, 6-dichloroquinone-4-chloroimide (Gibbs reagent) and the developed colored chromogen was measured at 469 nm. Method D was based on the reaction of 4-trifluoromethyl aniline degradate with para-dimethyl amino cinnamaldehyde and the colored chromogen was measured at 533.4 nm. Method E was based on the reaction of 3-methyl-4-carboxyisoxazoledegradate with 3-methyl-2-benzothiazolinone hydrazine in the presence of ceric ammonium sulfate and the developed green colored chromogen was measured at 605.5 nm. Method comparison with mean percentage accuracy presented in tableno.2.[9]

**Table no. 2: Method comparison with mean % accuracy by Abbas *et al.* 2006**

| Method | Concentration range (µg/ml) | Mean percentage accuracy |
|---|---|---|
| A | 2 - 20 | 100.07 ± 1.32 |
| B | 2 - 16 | 98.42 ± 1.61 |
| C | 40 - 280 | 100.75 ± 1.21 |
| D | 2.40 - 24 | 100.13 ± 1.45 |
| E | 30 - 250 | 99.74 ± 1.39 |

Prabu *et al.* 2012 reported difference spectroscopic method for the determination of LEF in bulk and tablet dosage form. In difference spectroscopic method, LEF exhibit two different forms; in acidic and basic medium that differs in their absorption spectra. The difference spectra were obtained by taking the absorbance of LEF in 0.1*N* HCl in the reference cell and the absorbance of LEF in0.1*N* NaOHin the sample cell and vice versa. In this method, peak maxima and minima were obtained at 293.5 and 261.5 nm, respectively. The method parameters are tabulated in table no.3.[10]

**Table no. 3: Validation Parameters by Prabu *et al.* 2012**

| Parameters | Result |
|---|---|
| Linearity range | 2-12µg/ml |
| Regression equation | y = 0.079x - 0.002 |
| Correlation coefficient | 0.9990 |
| Intra-day precision (% RSD) | 0.585 - 0.92% |
| Inter-day precision (% RSD) | 0.011 - 0.018% |
| % Recovery | 98.92- 99.08% |

### 2.1.2 Chromatographic Methods

Yeniceli *et al.* 2006 established a RP-HPLC method for the determination of LEF in tablets. In this method, Oxazepam (1 mg/ml, $1.4\times10^{-5}$ M) was used as an internal standard to compensate minor fluctuations of retention times. The chromatographic separation was performed on a $C_{18}$ column (50 mm × 3mm internal diameter), in isocratic mode using mobile phase consisting of methanol: water (60:40, v/v), at a flow rate of 0.5 ml/min and detected by UV detector at 260 nm. The retention times were observed at 2.6 and 5.2 min for Oxazepam and Leflunomide, respectively.

For specificity determination, LEF standards were stressed with 0.1*N* HCl, 0.1*N* NaOH and 3% (v/v) $H_2O_2$ for 15, 30, 45, 60, 90, and 120 min at both ambient and elevated temperature ($60^0$C). Degradation observed in base stressed samples at ambient

temperature over 15 min and in $H_2O_2$ stressed sample at $60^0$C observed approximately 20% degradation at 120 min. None of these degradation products showed any interference with LEF standard.[11] The method validation parameters are tabulated in table no. 4.

**Table no.4: Validation Parameters reported by Yenicel*i et al.* 2006**

| Parameters | Result |
|---|---|
| Linearity range | $2.7\times10^{-6}$ - $5.5\times10^{-5}$M |
| Regression equation | y = 18544x - 0.0267 |
| Correlation coefficient | 0.9999 |
| Limit of Detection (LOD) | $2.4\times10^{-7}$M |
| Limit of Quantification (LOQ) | $7.2\times10^{-7}$M |
| % Recovery | 100.4 - 103.7% |
| Intra-day precision (% RSD) | 0.57 - 1.59% |
| Inter-day precision (% RSD) | 1.41% |

Joshi *et al.* 2011 reported a reverse phase stability indicating UPLC assay method for determination of LEF in tablet dosage forms. This separation was carried out on water Acquity BEH $C_{18}$column (2.4 x 50mm, 1.7µ), inisocratic mode using mobile phase containing acetonitrile: 0.02*M* ammonium acetate buffer (60:40, v/v) at a flow rate of 0.4 ml/min with the injection volume 5µl and detected byphoto-diode array detection at260 nm. The method validation parameters are tabulated in table no.5.

**Table no.5: Validation Parameters reported by Joshi*et al.* 2011**

| Parameters | Result |
|---|---|
| Concentration range | 10-30 µg/ml |
| Regression equation | y = 56906771.42857x - 2237.10714 |
| Correlation coefficient | 0.9998 |
| Intra-day precision (% RSD) | 1.16% |
| Inter-day precision (% RSD) | 0.60% |
| % Recovery | 99.44 - 100.24 % |

For stability study the drug was subjected to oxidation, hydrolysis, photolysis and heat to apply stress condition. Here, stability of LEF in present dosage form was established according to ICH recommended stress condition. Here degradation products did not interfere with detection of LEF.[12] For this stability indicating method different conditions and their percentage degradation are tabulated in table no. 6.

**Table no.6: Degradation profile with different stabilityconditionsbyJoshi*et al.* 2012**

| Conditions | % Degradation |
|---|---|
| Acidic degradation (1*N*HClat 60$^0$C for 2 hours) | 10% |
| Alkaline degradation (0.005*N*NaOHin ambient temperature for 2hours) | 8-9% |
| Oxidative degradation (30% v/v $H_2O_2$ at 80$^0$C for 45 min) | 22% |
| Thermal degradation (exposing drug to dry heat of 80$^0$C for 72 hours) | 0.60% |
| Photolytic condition (exposing drug in UV-light for 72 hours) | 1.12% |

## 2.2 BIOLOGICAL SAMPLES

The pharmacological activity of Leflunomide is due to its active and major metabolite, Teriflunomide. Teriflunomide is formed by N-O bond cleavage in the isoxazole ring which has the same oxidation property as the parent drug. Methods for the measurement of biological Teriflunomide concentrations have included various chromatographic methods like LC-MS/MS, LC-ESI-MS/MS and RP-HPLC with UV detection.

### 2.2.1 Chromatographic Methods

Roon *et al.*2004 established a RP-HPLC-UV method for detection of A77 1726 in human serum. This chromatographic separation was achieved by using Demoxepam as an internal standard with mobile phase containing methanol:potassium dihydrogen phosphate buffer (pH-3) (50:50, v/v), at a flow rate of 1 ml/min with injection volume of 20 µL and detected by UV absorption at 295 nm. The retention times were observed at 5.8 and 8.9 min for Demoxepamand A77 1726, respectively. The method validation parameters are tabulated in table no. 7. The developed method was simple and easy to operate for measurement of A77 1726. Here, large variability observed in A77 1726 serum concentrationswith a range of 3-176 mg/L in patient samples.[13]

**Table no. 7: Validation Parameters reported by Roon *et al.* 2004**

| Parameters | Result |
|---|---|
| Concentration range | 0.5 - 100 mg/L |
| Correlation coefficient | 0.9996 |
| Accuracy (%CV) | 8% |
| Intra-day precision (%CV) | 0.1 - 8.1 % |
| Inter-day precision (%CV) | 0.6 - 13.2% |

Parekh *etal.* 2010 reported a LC-ESI-MS/MS method for the determination of Teriflunomide in human plasma. Drug spiked human plasma samples of Teriflunomide and Valsartan (98.2%) as an internal standard (IS) were prepared in ethyl acetate from 200 µL human

plasma by liquid-liquid extraction. The chromatography were carried out on Inertsil ODS-3 $C_{18}$ (50mm×4.6mm, 3µm) analytical column, in isocratic mode using mobile phase consisting of 20m*M* ammonium acetate:methanol (25:75, v/v), at a flow rate of 0.8 ml/min and detection was carried out by a triple quadruple mass spectrometer, operating in the multiple reaction monitoring (MRM) and negative ion mode.

**Table no.8: Validation Parameters reported by Parekh*et al.* 2010**

| Parameters | Result |
|---|---|
| Concentration range | 10.1-4001 ng/ml |
| Regression equation | Y=0.00033x-0.0052 |
| Correlation coefficient | 0.0013 |
| Accuracy (%CV) | 9.1-101.3% |
| Inter-batch precision (%CV) | 2.4-6.4% |
| Intra-batch precision (%CV) | 2.8-5.8% |

Here, precursor→product ion transition forTeriflunomide (m/z 269.0→82.0) and IS (m/z 434.1→350.3) were monitored. The retention times were observed for Teriflunomide and IS at 1.43 and 1.04 min, respectively. The mean process efficiency were 91.7% and 88.2% for Teriflunomide and IS respectively by matrix effect, which was achieved by post-column infusion experiment.[14] The method validation parameters are tabulated in table no.8.

The developed method has advantages in term of lower sample requirements, simplicity of extraction procedure and overall analysis time**.**The method has also higher sensitivity, superior retention and better peak shapes and successfully applied forTeriflunomide determination in human plasma.

Rakhila *et al.* 2011 established a RP-LC-MS/MS method to determine the total and free Teriflunomide concentration in patients with rheumatoid arthritis. Teriflunomide and its deuterated internal standard (Teriflunomide-D4) were extracted from human plasma and separated with $C_{18}$ column, in gradient mode using mobile phase in which mobile phase A was composed of 0.5m*M* ammonium acetate in water (pH-3.5): acetonitrile: formic acid (95:5:0.02, v/v/v), and mobile phase B was composed of 0.5m*M* ammonium acetate in water: acetonitrile: formic acid (5:95:0.02, v/v/v), at the flow rate of 0.5 ml/min and detection with an API 3000 LC-MS/MS System by monitoring selected ions in negative ionmultiple reaction monitoring (MRM).Optimal detection occurred at m/z 269.1/160.0 (Teriflunomide) and m/z 273.1/164.0 (Teriflunomide-D4). Teriflunomide and Teriflunomide-D4 both eluted at a retention time ofapproximately 3.1 min.[15] The method validation parameters are tabulated in

table no.9. In this method, it is found that free Teriflunomide was approximately 0.11% of total Teriflunomide concentrations.

**Table no.9: ValidationParameters reported byRakhila*et al.* 2011**

| Parameters | Result |
|---|---|
| Concentration range | 5 - 500 µg/L |
| Correlation coefficient | 0.999 |
| Inter-batch precision (%CV) | 1.9 - 8.8% |
| Accuracy (%CV) | -8.4 - 8.0% |
| Intra-batch assay precision (%CV) | 2.1 - 5.4% |
| Inter-batch assay precision (%CV) | 5.7 - 7.1% |

## 3. CONCLUSION

The review presents specific, sensitive and accurate spectrophotometric and chromatographic analytical methods applied for determination and stability studies of Leflunomide in pharmaceutical preparations and biological fluid. However, still there is more focus require to develop other methods using spectrofluorimeterand HPTLC as well as degradation kinetic study can also be develop by suitable stability indicating method. There is also lack of information for combination preparation with LEF.

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