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A new analytical method development and validation for the determination of teriflunomide in bulk form and marketed pharmaceutical dosage form

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ABSTRACT

A rapid, simple, sensitive, precise and cost effective RP-HPLC method was developed and validated for the determination of Teriflunomide in bulk form and marketed Pharmaceutical Dosage Form. The method involved determination of Teriflunomide by resolving on RP-HPLC using Symmetry ODS RP C_{18}, 5µm, 15mmx4.6mm i.d.reverse phase column, utilizing a mobile phase of ACN : Methanol: 0.1% Ortho Phosphoric Acid in the ratio of 50:40:10v/v/v. Mobile phase was delivered with isocratic flow rate of 1.0 ml/minute. Ultra violet detection was carried out at 232nm simultaneously over the concentration range of 6-14µg/ml for Teriflunomide. Separation was completed within 6 minutes. The mean recoveries were found to be 99.887 ± 0.654 % w/w for Teriflunomide. The coefficient (r2) was 0.9989 for Teriflunomide. The LOD and LOQ were found to be 0.08µg/ml and 0.24µg/ml for Teriflunomide respectively. The assay percentage of the marketed formulation calculated was 99.79 (±0.247) for Teriflunomide and intra-day and inter-day precision data were found to be <2 %RSD. Developed method was simple, rapid, accurate, precise, reliable, and economical and it can be used for routine analysis for quantitative estimation of Teriflunomide component in bulk form and marketed Pharmaceutical Dosage Form.

Key words: Teriflunomide, RP-HPLC, Validation, ICH Guidelines, Precision, Accuracy.

INTRODUCTION

Teriflunomide is the active metabolite of Leflunomide, and it acts as an immuno modulatory agent by inhibiting pyrimidine synthesis. It is marketed under the name Aubagio® and is indicated for the treatment of multiple sclerosis, specifically relapsing forms. The FDA label states an important warning about the risk of hepatoxicity and teratogenicity for patients using Teriflunomide[1]. Teriflunomide is an orally available immuno modulatory agent used to treat relapsing multiple sclerosis. Teriflunomide is associated with transient serum enzyme elevations during therapy and with rare instances of acute liver injury. Teriflunomide is an enamide obtained by formal condensation of the carboxy group of (2Z)-2-cyano-3-hydroxybut-2-enoic acid with the anilino group of 4-(trifluoromethyl) aniline. Used for the treatment of relapsing forms of multiple sclerosis and rheumatoid arthritis. It has a role as an EC 1.3.98.1 [dihydroorotate oxidase (fumarate)] inhibitor, a tyrosine kinase inhibitor, a hepatotoxic agent, a drug metabolite and a non-steroidal anti-inflammatory drug. It is a nitrile, an enol, an aromatic amide, an enamide, a member of (trifluoromethyl) benzenes and a secondary carboxamide. Teriflunomide is an orally available immuno modulatory agent used to treat relapsing multiple sclerosis. Teriflunomide[2] is associated with transient serum enzyme elevations during therapy and with rare instances of acute liver injury. The IUPAC Name of Teriflunomide is (Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl) phenyl] but-2-enamide. The Chemical Structure of Teriflunomideas follows (Fig 1)

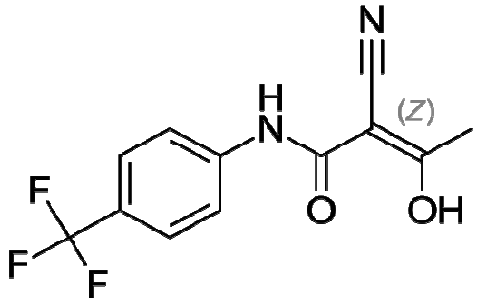

Fig-1: Chemical Structure of Teriflunomide

A thorough literature survey reveals that there were few analytical methods reported for the determination of Teriflunomide in bulk, pharmaceutical preparations and in biological fluids which include, Visible spectro photometric method, spectrophotometric methods (simultaneous determination) RP-HPLC,HPTLC High Performance Liquid Chromatography with tandem mass spectrometry, LCMS. However, most of the available methods have limitations such as laborious sample preparation, more solvent consumption, long run times for biological samples, low sensitivity, uneconomical and have poor symmetry. Keeping given these we have decided to develop a simple, accurate, precise and reliable isocratic RP-HPLC method for the estimation of Teriflunomide in the pharmaceutical dosage form.

EXPERIMENTAL

MATERIALS AND METHODS

Chemicals and reagents

Table-1: List of Instruments used

S. No.	Instruments/Equipments/Apparatus
1.	Waters HPLC with Empower2 Software with Isocratic with UV-Visible Detector.
2.	LAB INDIA and Model T-60 UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column[13,14,15]
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table-2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.

5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	99.9%	L.R.	Loba Chem; Mumbai.
8.	Hydrogen Peroxide	99.9%	L.R.	Loba Chem; Mumbai.

Selection of Maximum Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard[3]& sample in a solvent in mobile phase diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Teriflunomide, so that the same wave number can be utilized in HPLC UV detector[4] for estimating the Teriflunomide.

Arrangement of Standard Solution for the Analysis

25mg of Teriflunomide Working standard was precisely weighed and moved into a 25 mL volumetric cup and around 20 ml of diluents was added to it and sonicated to break up tranquilize totally and volume was made up to the stamp with a similar dissolvable which gave Stock arrangement of 1000ppm. 1ml of the above stock arrangement was pippetted into a 10ml volumetric flagon and was weakened up to the stamp with diluents to plan 100ppm arrangement. Assist 1 ml of arranged 100ppm arrangement was pippetted into a 10ml volumetric flagon and was weakened up to the stamp with diluents which gave 10ppm Teriflunomide working standard arrangement. The arrangement was blended well and separated through 0.45µm channel.

Arrangement of Sample Solution for the Analysis

Weigh precisely around 25mg of Teriflunomide standard and moved into 25ml volumetric flagon, broke up and make up to volume with portable stage. Facilitate weakening was finished by exchanging 0.1ml of the above arrangement into a 10ml volumetric carafe and make up to volume with versatile stage[5].

Preparation of Mobile Phase: Mobile phase was prepared by mixing aceto nitrile and methanol and 0.1% ortho phosphoric acid in the proportion of 50:40:10% v/v/v. The prepared mobile phase[6]was filtered through a 0.45µm nylon membrane filter and degassed by 15minutes sonication.

Method Validation: The developed RP-HPLC methods were validated in pursuance of ICH Q2 (R1) for the different parameters like system suitability, specificity, linearity, precision, accuracy, robustness, and ruggedness, limit of detection (LOD) and limit of quantitation (LOQ). Summary of validation parameters[8, 9] for determination of Teriflunomide for HPLC is depicted in follows.

System Suitability: The chromatographic system utilized for analysis must pass system suitability[10] limits before sample analysis can commence. Set up the chromatographic system allow the HPLC system to stabilize for 45 min. Replicates of the sample containing Teriflunomide were injected, and chromatograms were recorded to evaluate the system suitability parameters such as tailing factor (not more than 1.5), theoretical plates (not less than 3000 and % RSD of peak areas of replicate injections of Teriflunomide standards. The parameters were calculated and found to be satisfactory.

Linearity: Underdeveloped experimental conditions the relationship between the peak area and concentration of Teriflunomide was studied. The calibration curve[11] was plotted against concentration vs. peak area by the prepared different aliquots, i.e., (6-14 µg/mL at 232 nm) of stock solution, and the r2 value was determined. Five replicate of prepared 10 µg/mL solution of Teriflunomide taken from different stock solution and measured area. The obtained test results peak area which is directly proportional to the concentration of the analyte in the sample. Fig. 4shows the linearity curves of Teriflunomide HPLC method.

The regression equation for method[12] was found to be Y= 19019 x+ 1445.1 (r2= 0.9989). In fact the correlation coefficient value was very close to 1 which indicates this advanced method has good linearity. Optical characteristics, regression data analysis of the proposed method for Teriflunomide is shown in Table 7.

Specificity: The specificity[16] of an analytical method is to determine the effect of excipients and other additives that are generally present in the formulation. The test results obtained were contrasted with the results found that the proposed method showing specific.

Precision: System Precision: Six replicate recording of peak area at 232 nm of 10 µg/mL concentration standard solution showed % relative standard deviation (% RSD) less than 2, which indicates reproducibility and thereby the precision[17] of the system.

Method Precision (Intraday and Interlay): Method precision[18] was determined by performing an assay of the sample under the tests of repeatability (Intraday precision), and intermediate precision (Interlay precision) was performed during two consecutive days by two different analysts, at different working concentrations. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

Accuracy (Recovery Studies): The accuracy[19] of the method was evaluated by the standard addition method. Recovery tests were carried out by analyzing drug with different compositions. Known amounts of standard drugs were added to pre-analyzed sample at three different levels 80% to 120%, and the mixed standard solutions were analyzed in triplicate at every level as per the suggested method. Percent mean recovery[20] was calculated. The accepted limits of recovery are 98- 102%.

Ruggedness: It is the reproducibility[21] of the test result obtained by the analysts of the same samples under a variety

of normal test conditions like different analysts, different labs, different assay times and different reagents. A system to system variability was studied on different High-Pressure Liquid Chromatographic systems under similar conditions at different times. In this method, 6 samples were prepared and analyzed as per the test method. Juxtapose of both the results get on 2 different high performance liquid chromatographic systems[22] shows that the assay test method is rugged to the system to system.

Robustness: Robustness[23] of the method study was determined by varying the method parameters such as a change in flow rate (± 0.2 mL/min), temperature (± 2 %) and wavelength (± 1 nm). The change in 5 % in the proportion of organic solvent, flow rate, detection wavelength. The standard solution is prepared as per the test method and was injected into the high performance liquid chromatographic system utilizing a different change in flow rates.

LOD and LOQ: Limit of detection[24] (LOD) is the lowest concentration in a sample that can be detected but not necessarily quantified under the stated experimental conditions and limit of quantitation[26] (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. Limit of detection and limit of quantitation were calculated using the following formula

$$LOD = 3.3\ (SD) / S \text{ and } LOQ = 10\ (SD) / S$$

Where SD = Standard deviation of response and S= Slope of the calibration curve.

Assay

Twenty pharmaceutical dosage forms[27] were taken and the I.P. strategy was taken after to decide the normal weight. Above measured tablets were at last powdered and triturated well. An amount of powder proportionate to 25 mg of medications were exchanged to 25 ml volumetric flagon, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with versatile stage. The arrangement was separated through a layer channel (0.45µm) and sonicated to degas. The arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the pinnacle regions were recorded. The information is appeared in Table-10.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:
AT = Peak Area of medication acquired with test arrangement
AS = Peak Area of medication acquired with standard arrangement
WS = Weight of working standard taken in mg
WT = Weight of test taken in mg
DS = Dilution of Standard arrangement
DT = Dilution of test arrangement
P = Percentage virtue of working standard

Stability Studies

Following convention was entirely clung to for constrained corruption of Teriflunomide Active Pharmaceutical Ingredient (API). The API (Teriflunomide) was subjected to pressure conditions[28] in different approaches to watch the rate and degree of debasement that is probably going to happen over the span of capacity as well as after organization to body. This is one kind of quickened strength contemplates that causes us deciding the destiny of the medication that is probably going to occur after prolonged stretch of time stockpiling, inside a brief timeframe as contrast with the continuous or long haul soundness testing. The different corruption pathways examined are corrosive (Acid) hydrolysis, essential (Base) hydrolysis, warm debasement (Thermal), photolytic corruption and oxidative debasement.

Acid Hydrolysis

A precisely measured 10 mg of unadulterated medication was exchanged to a clean and dry round base carafe. 30 ml of 0.1 N HCl was added to it and it was refluxed in a water shower at 600C for 4 hours. Permitted to cool to room temperature.The sample was then neutralized using dilute NaOH solution & final volume of the sample was made up to 100ml with water to prepare 100 µg/ml solutions. It was injected into the HPLC system against a blank of mobile phase (after optimizing the mobile phase compositions). This experiment was repeated several times using same concentration of HCl (0.1N) and observed its degradation profile. The typical chromatogram shown below is the degradation profile of Teriflunomidein 0.1N HCl.

Basic Hydrolysis

An accurately weighed 10 mg of pure drug was transferred to a clean & dry round bottom flask. 30 ml of 0.1N NaOH was added to it. & it was refluxed in a water bath at 60^0C for 4 hours. Allowed to cool to room temperature. The sample was than neutralized using 2N HCl solution & final volume of the sample was made up to 100ml to prepare 100 µg/ml solutions. It was injected into the HPLC system against a blank of mobile phase after optimizing the mobile phase compositions. This experiment was repeated several times using same concentration of NaOH such as 0.1N to observe

its degradation profile. The chromatogram shown below is the degradation profile of Teriflunomidein 0.1N NaOH.

Thermal Degradation

Accurately weighed 10 mg of pure drug was transferred to a clean & dry round bottom flask. 30 ml of HPLC water was added to it. Then, it was refluxed in a water bath at 60^0 c for 6 hours uninterruptedly. After the reflux was over, the drug became soluble and the mixture of drug & water was allowed to cool to room temperature. Final volume was made up to 100 ml with HPLC water to prepare 100 µg/ml solutions. It was injected into the HPLC system against a blank of mobile phase.

Photolytic Degradation

Approximately 10 mg of pure drug was taken in a clean & dry Petridish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg of the UV exposed drug was transferred to a clean & dry 10 ml volumetric flask. First the UV exposed drug was dissolved in methanol & made up to the mark with mobile *phase* to get 100 µg/ml solution. Finally this solution was injected into the HPLC system against a blank of mobile phase and chromatogram was obtained.

Oxidation with (3%) H2O2

Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml volumetric flask. 30 ml of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100 µg/ml solutions. The above sample was injected into the HPLC system.

RESULTS AND DISCUSSION

Method Development:

Selection of Maximum Wavelength

While scanning the Teriflunomide solution we observed the maxima at 232nm. The UV spectrum has been recorded on LAB INDIA make UV – Vis spectrophotometer model T-60. The scanned UV spectrum is attached in the following page, (Fig 2)

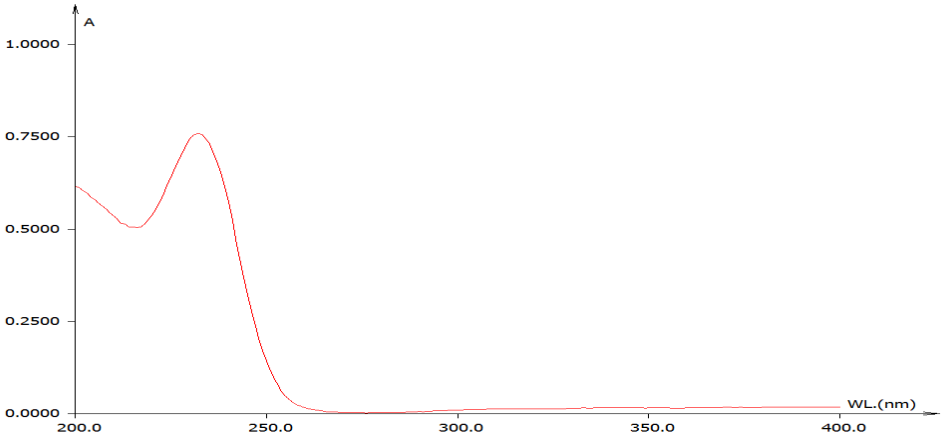

Fig-2: UV spectrum for Teriflunomide at 232nm

Summary of Optimised Chromatographic Conditions :(Fig 3)

Table-3: Summary of Optimised Chromatographic Conditions

Mobile phase	ACN : Methanol: 0.1% OPA = 50:40:10
Column	Symmetry ODS RP C_{18},5µm, 15mmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	232 nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluents	Mobile Phase
Injection Volume	20µl
Type of Elution	Isocratic
Retention time	2.570 minutes

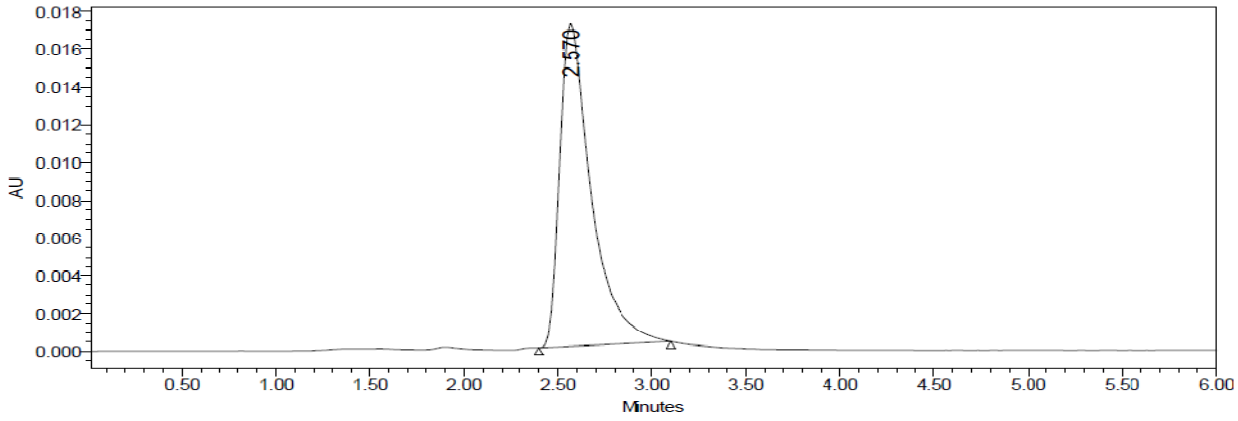

Fig-3: Optimized Chromatographic Condition

Method Validation

Accuracy

Recovery study

To decide the exactness of the proposed strategy, recuperation thinks about were done by including diverse sums (80%, 100%, and 120%) of unadulterated medication of Teriflunomide were taken and added to the pre-broke down plan of fixation 10µg/ml. From that rate recuperation esteems were computed. The outcomes were appeared in table-4.

Table-4: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	8	7.930789	489951	99.13486	Mean= 99.57287% S.D. = 0.564624 % R.S.D.= 0.5670260
S_2 : 80 %	8	8.016806	495264	100.2101	
S_3 : 80 %	8	7.949893	491131	99.37366	
S_4 : 100 %	10	9.964075	615541	99.64075	Mean= 100.5328% S.D. = 0.777702 % R.S.D.= 0.773580
S_5 : 100 %	10	10.08895	623254	100.8895	
S_6 : 100 %	10	10.10682	624358	101.0682	
S_7 : 120 %	12	11.84163	731512	98.68026	Mean= 99.5573% S.D. = 0.784289 % R.S.D. = 0.787776
S_8 : 120 %	12	11.97604	739814	99.80033	
S_9 : 120 %	12	12.02296	742712	100.1913	

Precision

Repeatability: The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Teriflunomide (API). The percent relative standard deviation was calculated for Teriflunomide are presented in the table-5.

Table-5: Readings of Repeatability

HPLC Injection Replicates of Teriflunomide	Retention Time (Minutes)	Peak Area (AUC)
Replicate – 1	2.572	197236
Replicate – 2	2.570	197762
Replicate – 3	2.573	195969
Replicate – 4	2.570	194724
Replicate – 5	2.574	198327
Replicate – 6	2.573	198711
Average		**197121.5**
Standard Deviation		**1515.213**
% RSD		**0.768667**

Intermediate Precision

Intra-assay & inter-assay: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Teriflunomide revealed that the proposed method is precise. (Table 6)

Table-6: Results of intra-assay & inter-assay

Conc. Of Teriflunomide(API) (µg/ml)	Observed Conc. Of Teriflunomide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.95	0.69	8.02	0.92
10	10.06	0.82	9.69	0.75
12	11.89	0.96	12.09	0.68

Linearity & Range:

The calibration curve showed good linearity in the range of 0 – 14 µg/ml, for Teriflunomide (API) with correlation coefficient (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y = 19019x + 1445.1 for Teriflunomide.

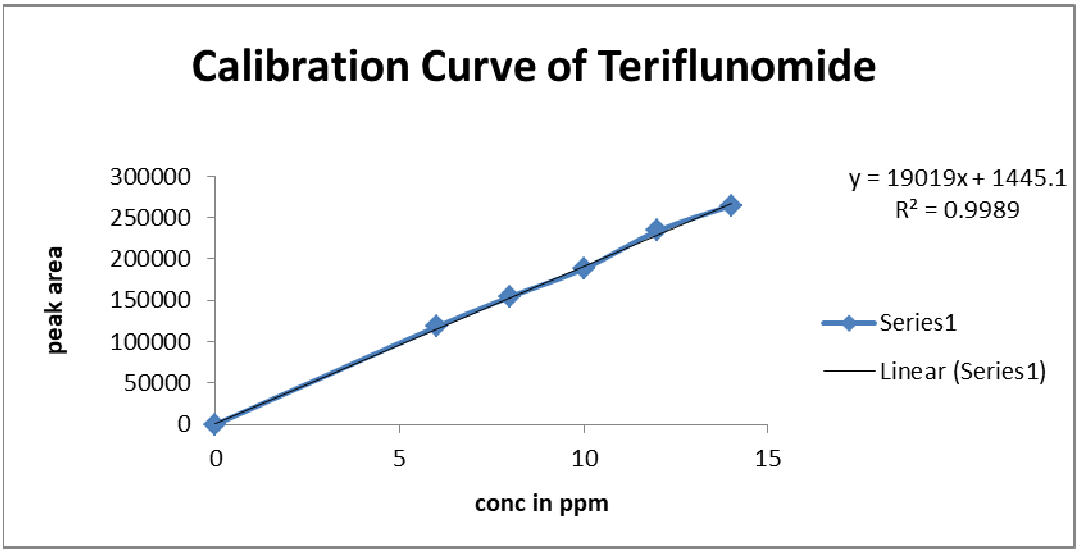

Fig-4: Calibration Curve of Teriflunomide (API).

Table-7: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
6	118012
8	154413
10	188315
12	234151
14	264718

Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-8, % RSD < 2%) the developed RP-HPLC method for the analysis of Teriflunomide (API).

Table-8: Result of method Robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Temperature (27^{0}C)	0.54
Temperature (23^{0}C)	0.63

Wavelength of Detection (230 nm)	0.91
Wavelength of detection (234 nm)	0.93

LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.

System Suitability Parameter

Framework appropriateness testing is an essential piece of numerous scientific techniques. The tests depend on the idea that the gear, hardware, explanatory activities and tests to be broke down establish a vital framework that can be assessed all things considered. Following framework appropriateness test parameters were built up. The information is appeared in Table-9.

Table-9: Dataof System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.97
2	Asymmetry	T ≤ 2	Teriflunomide=0.26
3	Theoretical plate	N > 2000	Teriflunomide=4265
4	Tailing Factor	T<2	Teriflunomide=1.29

Assay

Table-10: Recovery Data for estimation Teriflunomide

Brand Name of Teriflunomide	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Denopsy (14 mg) (Neelkanth Pharmaceuticals Private Limited)	14mg	14.58 (± 0.687)	99.79 (±0.247)

Result & Discussion: The amount of drug in Jakavi Tablets was found to be 19.58 (± 0.687) mg/tab for Teriflunomide& % assay was 99.79 %.

Stability Studies

The results of the stress studies indicated the specificity of the method that has been developed. Teriflunomide was stable in thermal and photolytic stress conditions. The result of forced degradation studies are given in the following table-11.

Table-11:Results of forced degradation studies of Teriflunomide

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	81.36	18.64	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	83.37	16.63	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	98.92	1.08	100.0
UV (254nm)	24Hrs.	96.33	3.67	100.0
3 % Hydrogen peroxide	24Hrs.	89.41	10.59	100.0

CONCLUSION

The HPLC method for the estimation of Teriflunomide in bulk form and pharmaceutical formulations was found to be simple, precise, sensitive and accurate. The method was completely validated and showed satisfactory results. The method was free from interference of the other active ingredients and additives used in the formulation. From the above HPLC experimental data results and parameters, the developed method has advantages like the less time taken for preparation of standard and sample solutions and hence suitable for the analysis of Teriflunomide raw material and its pharmaceutical dosage form.

The RP-HPLC method for the estimation of HPLC has various advantages like low solvent consumption, less retention time, excellent peak symmetry, highly sensitive, precise, accurate and robust. Results of the study indicate that the developed methods were found to be accurate, precise, linear, sensitive, simple, economical, reproducible have short run time which makes the method rapid. Hence it can be concluded that this method may be employed for the routine quality control analysis of Teriflunomide in active pharmaceutical preparations.

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