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Development and validation of stability indicating RP-HPLC method for estimation of teriflunomide in active pharmaceutical ingredient

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Abstract
Developed method is a rapid, simple and Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method for determination of teriflunomide. The HPLC separation was achieved on Agilent-1260 Infinity series instrument with Eclipse XBD C18 (Agilent) column (150X4.6 mm, 5µm) using a mobile phase of acetonitrile and di-potassium hydrogen anhydrous (K_2HPO_4) solution (40:60; v/v) containing TEA (pH 7) at a flow rate of 1.0 ml/min. The method was validated for specificity, linearity, precision, accuracy, robustness and ruggedness. The elution of peak was rapid which s takes about 3.5 minutes. The method was found to be simple, specific, precise, accurate, linear and reproducible. The method can be applied for the quality control of commercial teriflunomide API (active pharmaceutical ingredient) to quantify the drug and to check the formulation content uniformity and purity of drug.

Keywords: HPLC**,** Teriflunomide, Validation, Force Degradation, API

Introduction
Teriflunomide (trade name Aubagio, marketed by Sanofi) is an Immunosuppressive Agent. Immunosuppressive agents or Antirejection medications are drugs that inhibit or prevent activity of the immune system [1]. They are used in immunosuppressive therapy for prevent the rejection of transplanted organs and tissues (e.g., bone marrow, heart, kidney, liver) and to treat the autoimmune diseases sach as (e.g., rheumatoid arthritis, multiple sclerosis, Behcet's Disease, pemphigus, and ulcerative colitis) [2]. Teriflunomide was investigated as a medication for multiple sclerosis (MS). The drug was approved by the FDA on September 13, 2012 and in the European Union on August 26, 2013 [3]. Teriflunomide (Fig.1) is chemically (2Z)-2-cyano-3-hydroxy-N-[4-(terifluoromethyl) phenyl] -2-enamide. Its molecular formula is $C_{12}H_9F_3N_2O_2$ having molecular weight 270.21gm/mol [4].

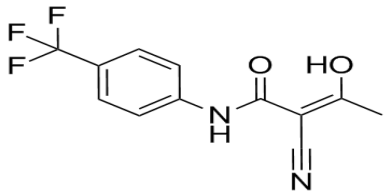

Fig 1: Chemical Structure of Teriflunomide

It act by inhibiting pyrimidinede novo synthesis by blocking the enzyme dihydroorotate dehydrogenase in rapidly dividing cells, including activated T cells. This drug may decrease the risk of infections compared to chemotherapy-like drugs because of its more-limited effects on the immune system. It has been found that teriflunomide blocks the transcription factor NF-κB. It also inhibits tyrosine kinase enzymes, but only in high dose so not clinically used [5]. Multiple Sclerosis (MS) is a demyelinating disease in which insulating covers of nerve cells in the brain and spinal cord are damaged. This damage disrupts ability of parts of nervous system to communicate [6]. Specific symptoms can include double vision, blindness, muscle weakness, trouble with sensation, or trouble with coordination. MS takes several forms, with new symptoms either occurring in isolated attacks (relapsing forms) or building up over time (progressive forms) [7]. It occur destruction by immune system or failure of myelin-producing cells.

Proposed causes for this include genetics and environmental factors such as being triggered by a viral infection. Multiple sclerosis is the most common autoimmune disorder affecting the CNS [8].

However analysis of pure drug was mostly studied by spectrometry and scanty literature is available on teriflunomide drug analysis with HPLC methods. Numerous HPLC methods using a variety of columns and detection techniques have been reported on different drugs in combination with other drugs and chemical and physical stability studies [9]. In the literature, there is not much report available for Reverse-phase High-Performance Liquid Chromatography (HPLC) method development, force degradation and validation of analytical method for determination of the teriflunomide drug in pharmaceutical industry and scientific laboratories [10]. HPLC is extremely useful for analysis of complex samples, purity and impurity as it provides drug separation, determination and the elimination of most interference problems [11]. The validation of an analytical method must demonstrate that it fulfills all the requirements of the analytical applications, ensuring the reliability of the results and should follow the standard guidelines of United State Pharmacopeia (USP), British Pharmacopeia (BP), European pharmacopeia (EP) and Indian pharmacopeia (IP). For this reason, the tests must show that its specificity, linearity, precision, sensitivity, accuracy and limit of quantification are adequate for the analysis [12]. The objective of this study to describes a simple, rapid, specific and stability-indicating RP-HPLC method for the determination of teriflunomide in active pharmaceutical ingredient (API). The parameters used to validate the method were linearity, specificity, precision, accuracy and limit of quantification.

Materials and methods

All reagents used in the present were of analytical-reagent grade. Teriflunomide provided by Advance Analytical Research and Training Institute, Ahmedabad, Gujarat, India. Other reagents such as water, Acetonitrile, Methanol, di-potassium hydrogen anhydrous (K_2HPO_4), Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide (30%) and Tri ethyl amine were purchased from Merck (Germany).

Determination of appropriate UV wavelength

A suitable wavelength was required for simultaneous determination of teriflunomide. The appropriate wavelength for the detection of drug teriflunomide and mobile phase was determined by wavelength scanning over the range of 200–400 nm with a Double beam UV- visible spectrophotometer (Model 1701, Shimadzu, Japan).

Chromatographic system and conditions

HPLC method was performed using Agilent 1260 HPLC pump with a 1260 Quat pump VL, G13113 ALS autoinjector and Agilent G1329 B PDA detector. Separation was operated on a C18 Agilent column (150mm×4.6 mm). The mobile phase consisted of Acetonitrile : di-potassium hydrogen anhydrous (K_2HPO_4) solution (40:60; v/v) at a flow rate of 1.0 ml/min. Di-potassium hydrogen anhydrous (K_2HPO_4) solution was prepared by dissolving 4.08 gm K_2HPO_4 in 1 litter double distilled water. Final pH of the mobile phase was adjusted to 7.0 by diluted Tri ethyl amine solution. Column temperature was set at 40 °C and 10 µl of samples was injected to the HPLC system.

Preparation of Solution

Preparation of Diluent: Final Diluent was prepared using Acetonitrile: Water (v/v; 1:1) and further solution was sonicated for 5 min for degassing.

Preparation of Mobile Phase: Prepared 0.03M di-potassium hydrogen anhydrous (K_2HPO_4) solution by dissolving 4.08 gm of potassium dihydrogen phosphate in 1000 mL of water. pH was adjusted to 7.0 with Tri ethyl amine solution. This solution was sonicated for 5 min for degassing and filtered through 0.45 µ Millipore filter. Prepared the ratio of ACN: 0.03M di-potassium hydrogen anhydrous (K_2HPO_4) solution (40:60).

Preparation of Standard Solution: 10 mg of teriflunomide was taken and transferred to 10 ml volumetric flask and make up with diluent (Stock solution-1000 µg/ml Teriflunomide). From that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (standard solution 250 µg/ml Teriflunomide).

Final chromatographic condition

Stationary Phase: Agilent, Eclipse XDB C18 column (4.6mm*150mm, 5µm)

Mobile Phase: ACN: 0.03M di-potassium hydrogen anhydrous (K_2HPO_4) solution (40:60) pH 7.0 with TEA.
Flow Rate: 1 ml/min, Injection Volume: 10µL, Column temperature: 40 ºC Diluent: ACN: water (1:1) Sample Concentration: 250µg/ml, Detection Wavelength: 250 nm.

Forced degradation

Preparation of regents

- **1 N hydrochloric acid:** 3.69 ml of concentrated HCl was taken and added to 100 ml volumetric flask. Volume was making up to the mark with water.
- **1 N NaOH:** 4 gm of NaOH was taken and added to 100 ml flask. 75 ml water was added in flask and NaOH was dissolved. Volume was making up to the mark with water.
- **3% H_2O_2:** 10 ml of 30% H_2O_2 was taken in 100 ml volumetric flask. Volume was making up to the mark with water.

Procedure: 10 mg of teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (stock solution 1000 µg/ml Teriflunomide). From the above solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (standerd solution-250 µg/ml Teriflunomide). After that make up with 10 ml of diluents and filtered the final solution with 0.45µ PVDF Filter. Further this solution used for different degradation methods. Similarly blank was prepared without adding sample. Blank and sample solution was injected in HPLC.

Acid degradation: Pipetted out 1 ml from stock solution and added 1 ml of 1N HCl and kept at 80 °C in water bath for 1 hour. After that it was neutralized by adding 1 ml of 1N NaOH and make up with 10 ml of diluent.

Base degradation: Pipetted out 1 ml from stock solution and added 1 ml of 0.1N NaOH and kept at 80 °C in water bath for 1 hour. After that it was neutralized by adding 1 ml of 1N HCl and make up with 10 ml of diluents.

Peroxide degradation: Pipetted out 1 ml from stock solution and add 1 ml of 30% H_2O_2 and kept at 80 °C in water bath for 1 hour and make up with 10 ml of diluent.

Thermal degradation: Pipetted out 1 ml from stock solution and added 1 ml of diluent and kept at 80 °C in water bath for 1 hour and make up with 10 ml of diluent.

Photolytic degradation: Pipetted out 1 ml from stock solution into 10 ml volumetric flask and kept under sun light for 1 hour and make up with 10 ml of diluent.

Result and discussion

Drug Identification

The identification of drugs was carried out by performing melting point determination, solubility study and taking IR and UV spectra as preliminary work which showed into following results.

Melting point determination

Melting point of teriflunomide was found to be in the range of acceptance criteria as shown in the table 1.

Table 1: Melting point determination

Drug name	Reported melting point	Observed melting point
Teriflunomide	227 - 231 °C	226 – 230 °C

IR Spectra and interpretation: From the IR interpretation data it can be concluded that major functional group peaks are observed in IR spectra of the drug sample. So it reveals that the given sample is of teriflunomide drug is shown in table 2.

Table 2: IR Interpretation of teriflunomide

Sr No	Observed Peak (cm^{-1})	Functional Group
1	1650	C=OStretching (Amide)
2	1600	N-H banding (Amide)
3	1120	C-F Stretching
4	1425	C-H Banding
5	1375	C-H Banding
6	1175	C-O Stretching (-OH group)

Method development: The HPLC separation was achieved on Agilent-1260 Infinity series instrument with Eclipse XBD C18 (Agilent) column (150X4.6 mm, 5µm) using a mobile phase of acetonitrile and di-potassium hydrogen anhydrous (K_2HPO_4) solution (40:60; v/v) containing TEA (pH 7) at a flow rate of 1.0 ml/min.

Final chromatogram: Final chromatograph for teriflunomide API (250 µg/ml Teriflunomide) is shown in figure 2 and table 4.

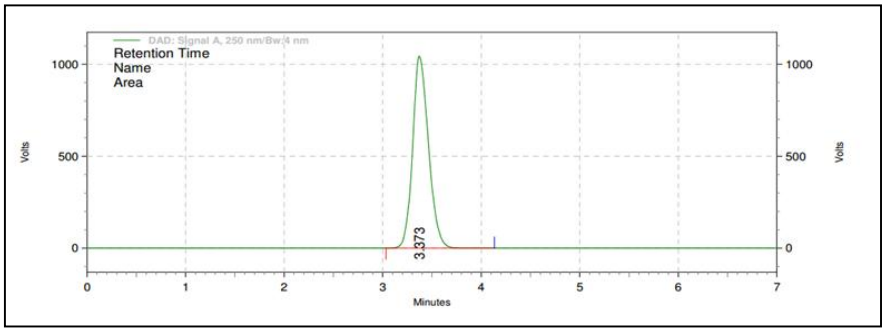

Fig 2: Final chromatogram of standard (Teriflunomide 250µg/ml)

Table 4: Final chromatograph.

Peak Name	Retention Time	Area	Assymetry	Resolution	Theoretical Plates
Teriflunomide	3.373	26533476	1.0	0.0	2560

Forced degradation: Samples were injected under various stress conditions. Here, chromatograms of optimized degradation conditions are shown below.

Acid degradation

Preparation of solution: 10 mg teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (Stock solution-1000 µg/ml Teriflunomide) from that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (250 µg/ml Teriflunomide). Then from stock solution take 1ml solution and add 1N 1 ml HCL in to the solution and kept in water bath for 1 hrs at 80 °C and after 1hrs add 1ml 1N NaOH in a solution for neutralization of solution and the make up with diluents up to 10 ml. Chromatogram of blank and teriflunomide API solution for acid degradation are shown in figure 3 and figure 4, respectively.

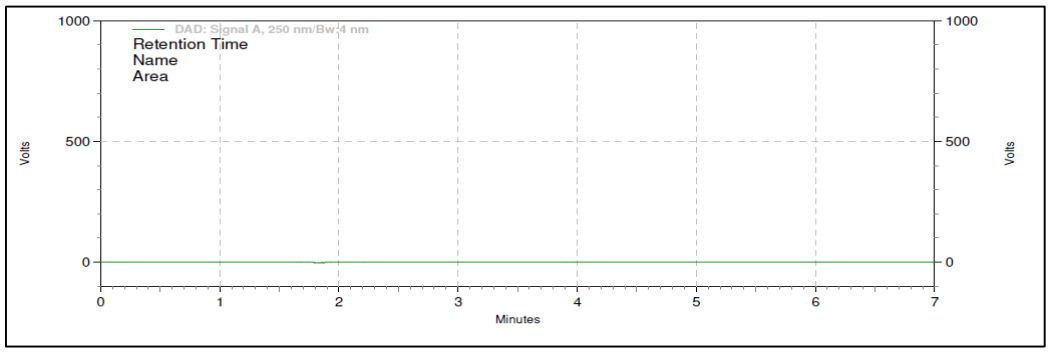

Fig 3: Chromatogram of blank solution for acid degradation

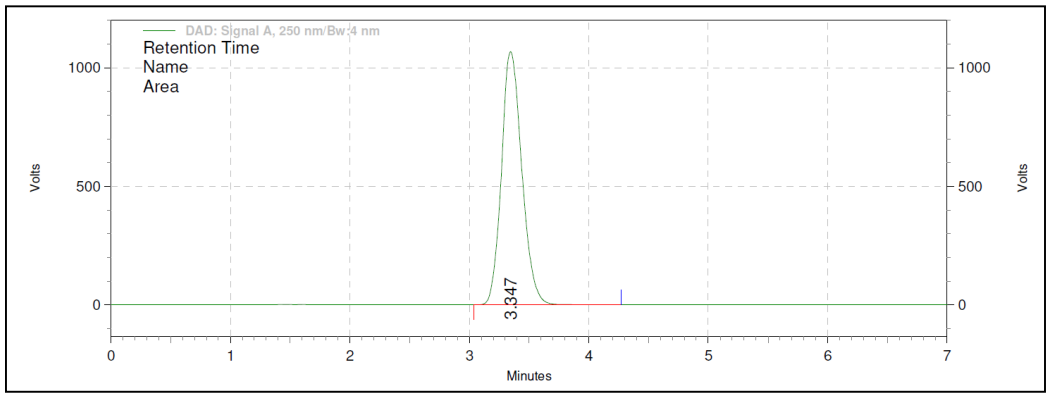

Fig 4: Chromatogram of teriflunomide API solution for acid degradation

Base degradation

Preparation of solution: 10 mg teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (Stock solution-1000 µg/ml Teriflunomide) from that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (250 µg/ml Teriflunomide). Then from stock solution take 1ml solution and add 1N 1 ml NaOH in to the solution and kept in water bath for 1 hrs at 80 °C and after 1hrs add 1ml 1N HCL in a solution for neutralization of solution and the make up with diluents up to 10 ml. Chromatogram of blank and teriflunomide API solution for base degradation are shown in figure 5 and figure 6, respectively.

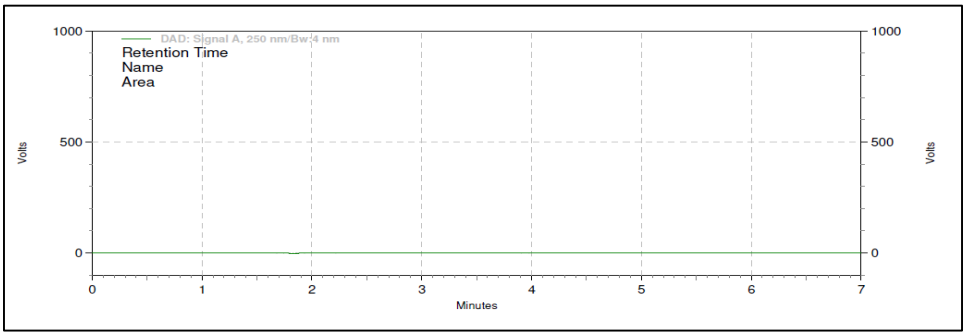

Fig 5: Chromatogram of blank solution for base degradation

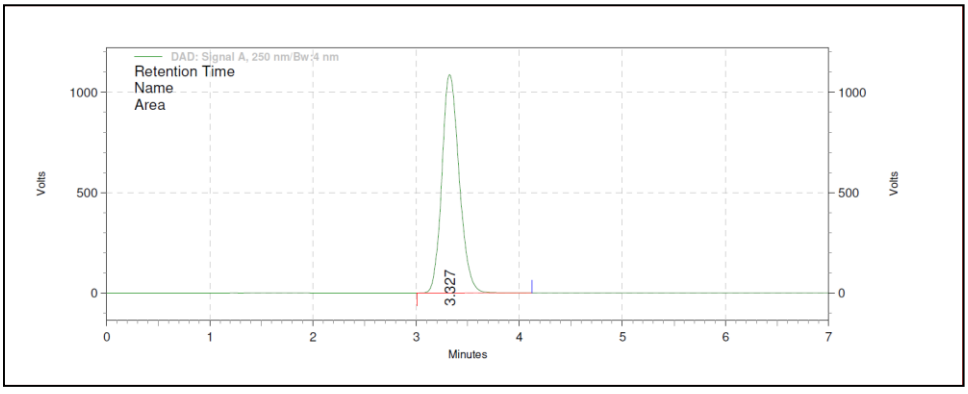

Fig 6: Chromatogram of teriflunomide API solution for base degradation

Peroxide degradation

Preparation of solution: 10 mg teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (Stock solution-1000 µg/ml Teriflunomide) from that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (250 µg/ml Teriflunomide). Then from stock solution take 1ml solution and add 30% 1 ml H_2O_2 in to the solution and then make up with diluents up to 10 ml kept in water bath for 1 hrs at 80 °C. Chromatogram of blank and teriflunomide API solution for peroxide degradation are shown in figure 7 and figure 8, respectively.

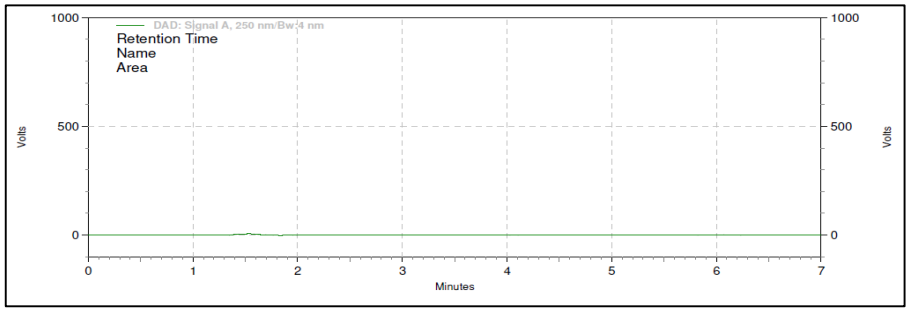

Fig 7: Chromatogram of blank solution for peroxide degradation

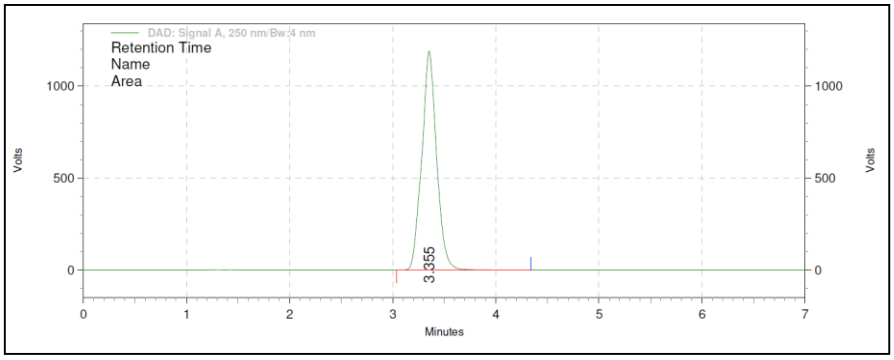

Fig 8: Chromatogram of teriflunomide API solution for peroxide degradation

Thermal degradation

Preparation of solution: 10 mg teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (Stock solution-1000 µg/ml Teriflunomide). from that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (250 µg/ml Teriflunomide). Then from stock solution take 1ml solution and then make up with diluent up to 10 ml Kept in water bath for 1 hrs at 80 °C. Chromatogram of blank and teriflunomide API solution for thermal degradation are shown in figure 9 and figure 10, respectively.

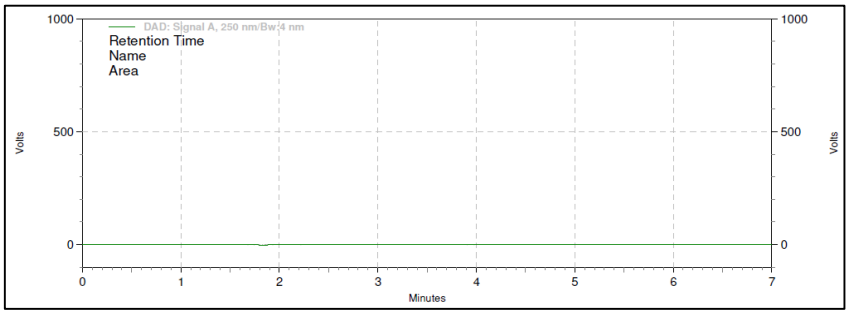

Fig 9: Chromatogram of blank solution for thermal degradation

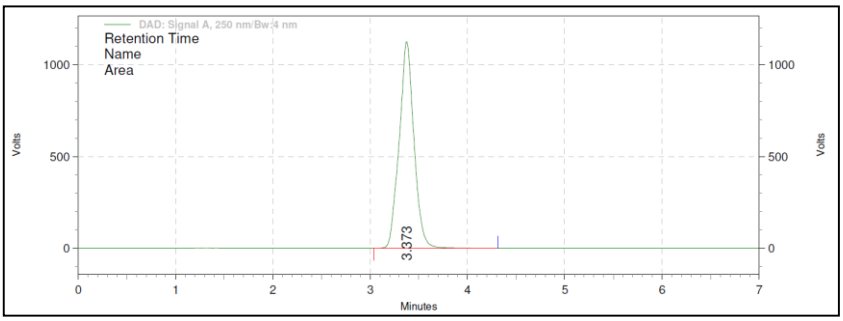

Fig 10: Chromatogram of teriflunomide API solution for thermal degradation

Photolytic degradation

Preparation of solution: 10 mg teriflunomide was taken and transferred to 10 ml volumetric flask and volume was made up with diluent (Stock solution-1000 µg/ml Teriflunomide).from that stock solution 2.5 ml was taken into 10 ml volumetric flask and volume was made up by diluent (250 µg/ml Teriflunomide). Then from stock solution take 1ml solution and make up with diluent up to 10 ml solutions have been kept under sun light for 1 hrs. Chromatogram of blank and teriflunomide API solution for thermal degradation are shown in figure 11 and figure 12, respectively. Comparative summary of all the degradation method are shown in the table 5.

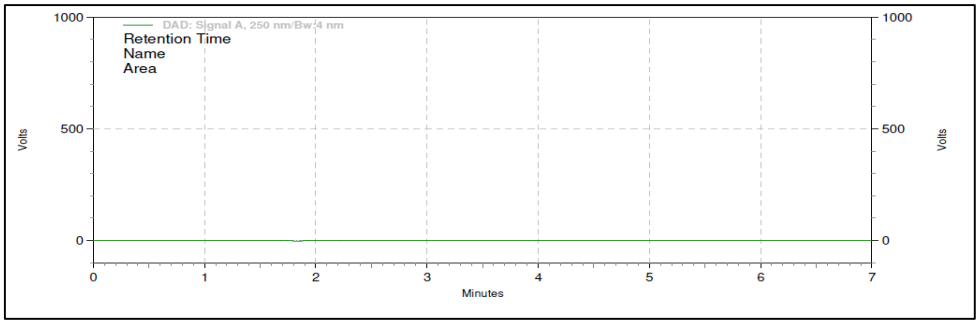

Fig 11: Chromatogram of blank solution for photolytic degradation.

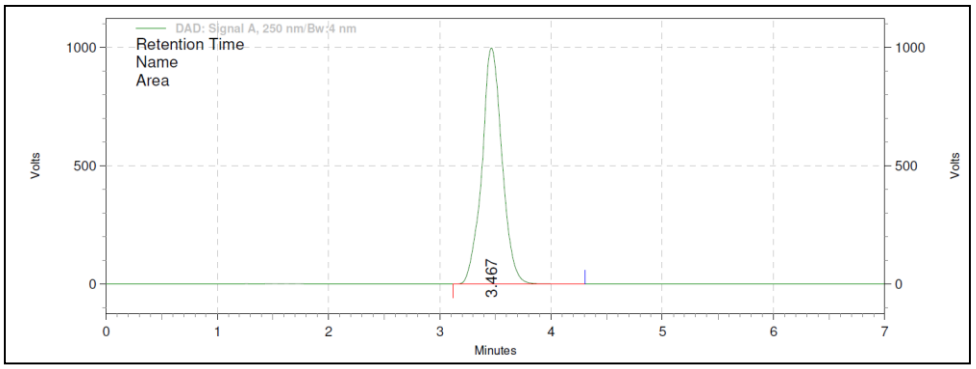

Fig 12: Chromatogram of Teriflunomide API solution for photolytic degradation.

Table 5: Degradation Summary for API

Type	Solution	Area	% Degradation 2
As Such (Standard)	Teriflunomide	26533476	-
Acid (1N HCl at 80 °C for 1 hrs)	Teriflunomide	20323567	23.44%
Base (1N NaOH at 80 °C for 1 hrs)	Teriflunomide	21443574	19.19%
Peroxide (30% H_2O_2 at 80 °C for 1 hrs)	Teriflunomide	19839976	25.23%
Thermal (Thermal at 80 °C for 1 hrs)	Teriflunomide	23783555	8.96%
Photo (Direct sun light for 1 hrs)	Teriflunomide	24235473	8.67%

System Suitability Parameters:

All the parameters such as RSD of Area, Resolution (Rs), Tailing Factor (T), Theoretical Plates (N) related to system suitablty test have been analysed and found that parameters were well within acceptance criteria, which indicate that the system and chromatographic conditions are suitable for this method. System suitability parameters are shown in the table 6 and 7.

Table 6: System suitability data for API (250 µg/ml Teriflunomide)

Sr. No.	Retention time	Area	Tailing	Plate Count
1.	3.373	25519134	1.0	2560
2.	3.371	24519221	1.12	2619
3.	3.342	25526541	1.02	2615
4.	3.121	25265612	1.18	2579
5.	3.335	25561623	1.0	2565

Table 7: System suitability parameters (N=5).

Parameters	Observation	Spacification
	API	
%RSD of Area	1.74	RSD < 2%
Resolution (Rs)	0.0	Rs > 2
Tailing Factor (T)	1.0	T ≤ 2
Theoretical Plates(N)	2560	≥2000

Method Validation

Linearity (n=5): It was found that Lambert-Beer's law was followed in the concentration ranges of 125-375 µg/ml (125, 200, 250, 300, 375 µg/ml) for the API. Linearity spectra are shown in figure13. The straight line equations (Regression equation) and correlation coefficient for both drugs are shown in table 8.

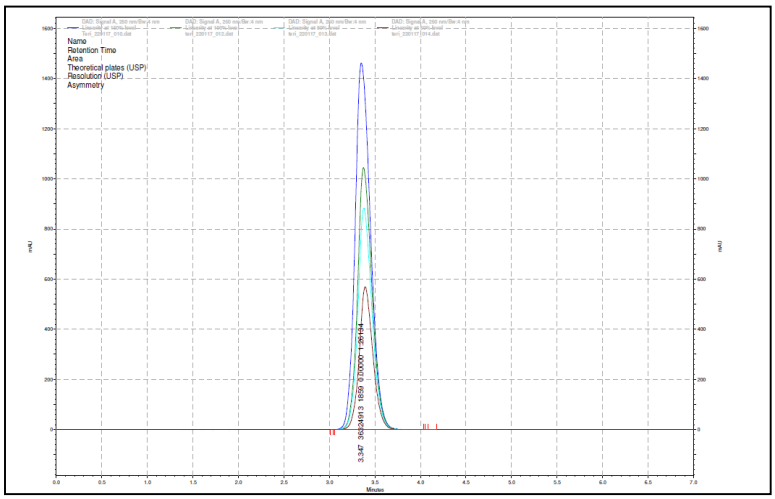

Fig 13: Linearity spectra of Teriflunomide

Table 8: Linearity study of Teriflunomide.

Concentration (µg/ml)	Mean area + SD	% CV
125	12478203+ 173205.1	1.38
200	20014433+ 108280.5	0.54
250	24214737+ 12958208	0.53
300	29241049+ 138459.7	0.47
375	36329940+ 75698.19	0.20

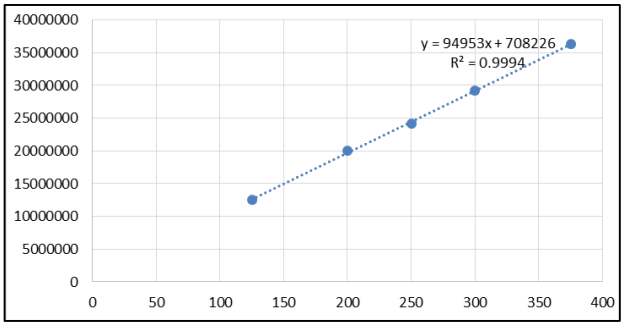

Fig 14: Calibration curve of Teriflunomide.

Table 9: Linearity graph

Drug	Regression equation	Correlation Coefficient
Teriflunomide	y = 94953x + 708226	0.9994

Acceptance criteria: The correlation co-efficient (r^2) value should be 0.999.

Conclusion

The correlation co-efficient (r^2) value for each component is well within the limit of acceptance criteria that means the areas obtained are directly proportional to the concentration of analyte in the sample. The method can therefore be considered to be linear in the range specified.

Specificity: The HPLC chromatograms recorded for the drug revealed almost no peaks within a retention time range of 7 min. A representative chromatogram for teriflunomide with no interference of any peak in chromatogram was observed, so it was concluded that the peak is selective for this specific teriflunomide.

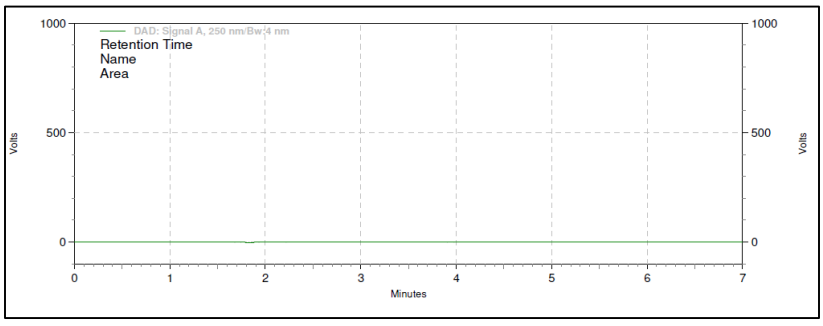

Fig 15: Chromatogram of blank preparation

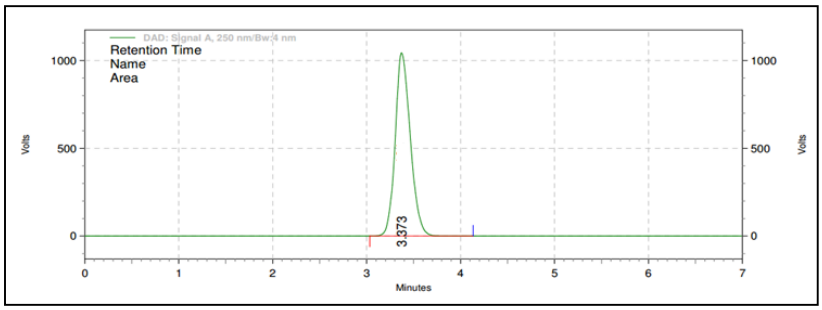

Fig 16: Chromatogram of standard preparation

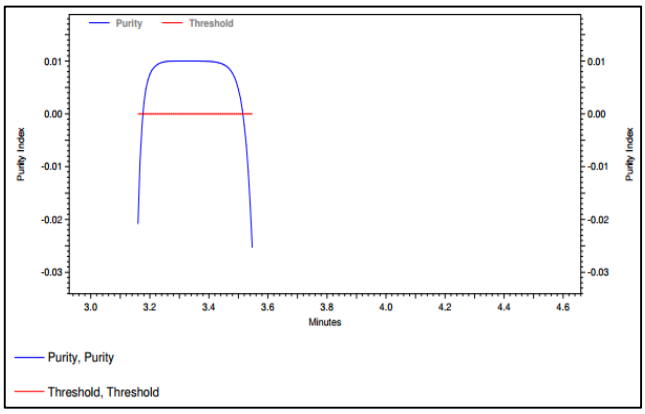

Fig 17: Peak purity spectra of Teriflunomide

Result: The purity angle and threshold angle for the main peak in standard preparation and sample preparation was determined and recorded in table 10.

Table 10: Data indicating peak purity of Teriflunomide.

Description	Peak purity Match (Purity Angle)
Standard Preparation	1.0
Sample Preparation	0.99

Acceptance Criteria

1. There should not be interference from blank with the main peaks.
2. Active ingredients peak in test preparation should be spectrally pure.

Conclusion

1. There is no interference from blank peak with the main peaks.
2. Active ingredients peak in test preparation were spectrally pure.

Precision: The results obtained for repeatability studies and for interday precision are presented in Table 11. Method precision has a relative standard deviation (RSD) below 1% for repeatability and intermediate precision, which comply with the acceptance criteria proposed (RSD) not more 2.0%).

A. Repeatability (n=6): Repeatability in the precision of the method when repeated by the same analyst using same test method and under same set of laboratory condition within a short interval of time, the only difference being the sample analyzed. Table 11 shows the repeatability data of teriflunomide.

Table 11: Repeatability data for Teriflunomide.

Sr. No.	Area
1	26628293
2	26251840
3	26318135
4	26398870
5	26386916
Mean	26396811
SD	142208.1
% CV	0.53

- % CV for Five sets of preparation for Teriflunomide was 0.53.
- The results indicate a good degree of repeatability and precision.

B. Interday Precision (n=3): Precision of method when repeated in the same day in a same instrument same analyst. Table 12 shows the interday precicion data of teriflunomide.

Table 12: Interday precision data for Teriflunomide.

Concentration (µg/ml)	Mean area + SD	% CV
200	20027411 + 100142	0.50
250	24265543 + 89387.83	0.36
300	29393133 + 88251.75	0.30

- % CV for three sets of preparation for teriflunomideis in between of 0.30-0.50.
- The results indicate a good degree of precision.

Accuracy (n=3): Accuracy is relates to the closeness of test result to true value. The results were expressed as percent recoveries of the particular components in the samples. Table 13 shows that the overall percent recoveries of tarflunomide in pure and drug–matrix solutions were 100.0 (relative standard deviation, RSD = 1.92%) and 98.02 (RSD = 1.15%), respectively. The results of the accuracy study are shown in table 13 for Teriflunomide. Individual recovery at each level meets the established acceptance criteria. Hence, the method is accurate in the considered as in range.

Table 13: Accuracy data for teriflunomide.

Drug name	% level of recovery	Amount of Drug Taken (µg/ml)	Amount of Drug Spiked(µg/ml)	Total amount found ±S.D.(n=3)	% recovery ± S.D. (n=3)
Teriflunomide	50	250	125	199.36±0.17	99.21±0.26
	100	250	250	251.70±0.26	100.00±0.22
	150	250	375	301.75±0.27	99.45±0.39

Robustness: Robustness refer to how sensitive the method is to uncontrolled small changes in parameters such as sample, temperature, pH of solution, reagent concentration, flow rate. Six replicate injections of standard preparation were injected and results are recorded with respect to teriflunomide peak in the following Table 14.

Table 14: Robustness Study of Teriflunomide.

Sr. No.	Conditions	Mean Area + SD	%CV	Theoretical plate	Tailing factor	R.T.
1	Flow rate: 1.0 ml/min	24540412 + 44553.75	0.18	2835	1.6	3.001
2	Flow rate: 0.6 ml/min	25275561 + 371954.3	1.47	2821	1.5	3.620
3	Mobile phase Composition: ACN: Buffer(35:65)	25278337 + 397468.1	1.57	2628	1.43	3.013
4	Mobile phase composition: ACN: Buffer(45:55)	24116957 + 61525.16	0.25	2649	1.41	3.621
5	Acceptance Criteria	NA	NMT 2.00	NLT 2000	Between 0.80 to 2.00	-

Theoretical plates and asymmetry were found well within acceptance criteria as per system suitability. So, the study proves the reliability of test method for minor changes in chromatographic condition. Hence method can be termed as robust.

Table 15: Limit of detection and limit of quantification.

Parameter	Teriflunomide
LOD (µg/ml)	4.94
LOQ (µg/ml)	14.97

Summary of method validation parameters: Table 16 shows the overall summary of various validation parameters generated by RP-HPLC method for the teriflunomide API.

Table 16: Summary of Validation parameters by RP-HPLC method for Teriflunomide.

Parameter		Teriflunomide
Specificity		Specific. PDA analysis: peak purity index near about 1.
Linearity and Range		50 - 150µg/ml
Regression line equation		y = 94953x + 708226
Correlation co-efficient (R^2)		0.999
Precision	Intra day	0.31-0.69%
	Inter day	0.30-0.50%
	Repeatability	0.53%
Accuracy	50%	99.21 ±0.26
	100%	100.00 ±0.22
	150%	99.45 ±0.39
Robustness		The system suitability parameters were found well within acceptance criteria as per system suitability.
LOD		4.94 µg/ml
LOQ		14.97 µg/ml

Conclusion

Hereby a novel and robust method was developed for simultaneous quantification of teriflunomide. From the results obtained, it is obvious that the proposed method is applicable for the determination of teriflunomide without interference and with good sensitivity. The result obtained indicates that the proposed method for the estimation of teriflunomide is specific, rapid, linear, accurate, precise, and suitable for intended use. Result obtained from the force degradation, indicates that in hydrogen peroxide (H_2O_2) condition drug was considerably degraded compared to other methods. These merits suggest the use of the proposed method in routine and quality control analysis without interference from commonly encountered excipient and impurities.

Conflict of interest: The authors declare no conflicts of interest.

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