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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR ESTIMATION OF TERIFLUNOMIDE IN API AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A stability indicating RP-HPLC method was developed and subsequently validated for the estimation of Teriflunomide in API and Pharmaceutical dosage form. Separation was achieved using Agilent, Eclipse XDB C18 (150 x 4.6mm, 5µm) using ACN:0.03M Potassium Dihydrogen Phosphate (pH 7.0 adjusted with TEA) (40:60v/v)at flow rate of 0.8ml/min. The effluent is monitored at 250 nm. The retention time of was found to be 3.373min for Teriflunomide API and 3.527 min for Teriflunomide tablet. Linearity was found in the range of50 - 150µg/ml (R^2=0.999). Precision studies were carried out and the RSD values were less than 2. The %recoveries of Teriflunomide API and Tablet were found to be in the range of 99-100% and 98-100 %respectively. LOD and LOQ were found to be 4.94µg/ml and 14.97µg/ml respectively. Degradation study was performed using acid, alkali hydrolysis, peroxide, thermal, photolytic degradation.The results showed that Teriflunomide and the other degradation products were fully resolved and thus the proposed method is stability-indicating.

KEYWORDS: Teriflunomide, RP-HPLC, stability indicating, HPLC method, validation.

INTRODUCTION

Teriflunomide (trade name Aubagio, marketed by Sanofi) is an Immunosuppressive Agent. Teriflunomide was investigated as a medication for multiple sclerosis (MS) and act by

inhibiting pyrimidinede novo synthesis by blocking the enzyme dihydro-orotate dehydrogenase.[1-4] The drug was approved by the FDA on September 13, 2012 and in the European Union on August 26, 2013.

Extensive literature survey revealed that few HPLC[5,6] and LC-MS/MS[7-12] methods are available for estimation of Teriflunomide individually and in combination with other drugs. There is no stability indicating analytical methods were reported for estimation of Teriflunomide. Hence the objective of the work wasto develop and validate simple, rapid, sensitive and accurate stability indicating HPLC method for the estimation of Teriflunomidefrom API and pharmaceutical dosage form.

MATERIALS AND METHOD

Acetonitrile, potassium di-hydrogen phosphate, sodium di-hydrogen phosphate and methanol of HPLC and AR grade were procured from Merck and Rankem lab ltd. Teriflunomide standard was received as gift sample from Advanced Analytical Research and Training Institute, Gujarat. All the chemicals and solvents used were of analytical grade.

Instruments

The analysis was performed on HPLC (Agilent technologies 1200 infinity series)fitted with Eclipse XDB C18 (150 x 4.6mm, 5µm) using Acetonitrile: 0.03M Potassium Di-hydrogen Phosphate (pH 7.0 adjusted with Tri ethyl amine) (40:60v/v)at flow rate of 0.8ml/min. Injection Volume was 10µL.The effluent is monitored at 250 nm.

Preparation of Diluent

Final Diluent was Acetonitrile: Water (1:1). This solution was sonicated for 5 min for degassing.

Preparation of Mobile Phase

Prepare 0.03M Potassium di-hydrogen phosphate by dissolving 4.08 gm of Potassium di-hydrogen phosphate in 1000 ml of water. Adjust pH 7.0 with Tri ethyl amine solution. This solution was sonicated for 5 min for degassing and filtered through 0.45 µ Millipore filter.
Prepare the ratio of ACN: 0.03M Potassium di-hydrogen phosphate (40:60).

Preparation of Standard Solution

10 mg Teriflunomide was taken into 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).

Preparation of Sample Solution

For the analysis of tablet Formulation (Teriflunomide Tablet, 14-mg), The Average Weight of 10 Tablet was determined and was ground in mortar. An Accurately weighed amount of powder equivalent to 10 mg of Teriflunomide which is transferred to 10 ml volumetric flask. Add 5 ml of diluent and sonicated for 5 minute to ensure complete solubilisation of drug. After sonication, volume was made up to the mark with diluent (1000 µg/ml of Teriflunomide stock solution). Pipette out 2.5 ml from stock solution and dilute up to 10 ml of diluent (250 µg/ml Teriflunomide).

Calibration Curve

Aliquots of different concentrations of standard solution were prepared and their chromatograms were recorded at the optimized chromatographic conditions. The mean peak areas at different concentration levels were calculated from the chromatograms. Then the linearity plot was constructed using the mean peak areas at their respective concentrations.

Forced degradation studies[13]

Acid degradation

- Procedure for API

10 mg Teriflunomidewas taken and transferred to 10 ml flask and add with diluent (1000 µg/ml Teriflunomide). Take 2.5 ml solution into 10 ml volumetric flask and volume was made up by diluent (Stock solution-250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 0.1N HCl and kept at 60°C in water bath for 2hours. After that it was neutralized by adding 1 ml of 0.1N NaOH and make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution were injected in HPLC.

- Procedure for Tablet

The Average Weight of 10 Tablet was determined and was ground in mortar. An Accurately weighed amount of powder and equivalent to 10 mg Teriflunomide and transferred to 10 ml volumetric flask. Add 5 ml of diluent and sonicated for 5 minute to ensure complete solubilisation of drug. After sonication, volume was made up to the mark with diluent (1000 µg/ml of Teriflunomide). Pipette out 2.5 ml from above solution and dilute up to 10 ml of

diluent. (Stock solution -250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 0.1N HCl and kept at 60°C in water bath for 2hours. After that it was neutralized by adding 1 ml of 0.1N NaOH and make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution were injected in HPLC.

Base Degradation

- Procedure for API

10 mg Teriflunomidewas taken and transferred to 10 ml flask and add with diluent (1000 µg/ml Teriflunomide). Take 2.5 ml solution into 10 ml volumetric flask and volume was made up by diluent (Stock solution-250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 0.1N NaOH and kept at 60°C in water bath for 2hours. After that it was neutralized by adding 1 ml of 0.1N HCl and make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solutions were injected in HPLC.

- Procedure for Tablet

The Average Weight of 10 Tablet was determined and was ground in mortar. An Accurately weighed amount of powder and equivalent to 10 mg Teriflunomide and transferred to 10 ml volumetric flask. Add 5 ml of diluent and sonicated for 5 minute to ensure complete solubilisation of drug. After sonication, volume was made up to the mark with diluent (1000 µg/ml of Teriflunomide). Pipette out 2.5 ml from above solution and dilute up to 10 ml of diluent. (Stock solution- 250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 0.1N NaOH and kept at 60°C in water bath for 2hours. After that it was neutralized by adding 1 ml of 0.1N HCl and make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solutions were injected in HPLC.

Peroxide Degradation

- Procedure for API

10 mg Teriflunomidewas taken and transferred to 10 ml flaskand add with diluent (1000 µg/ml Teriflunomide). Take 2.5 ml solution into 10 ml volumetric flask and volume was made up by diluent (Stock solution-250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 3% H_2O_2 and kept at 60°C in water bath for 2hours. After that make

up volume with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution were injected in HPLC.

- Procedure for Tablet

The Average Weight of 10 Tablet was determined and was ground in mortar. An Accurately weighed amount of powder and equivalent to 10 mg TeriflunomideAnd transferred to 10 ml volumetric flask. Add 5 ml of diluent and sonicated for 5 minute to ensure complete solubilisation of drug. After sonication, volume was made up to the mark with diluent (1000 µg/ml of Teriflunomide). Pipette out 2.5 ml from above solution and dilute up to 10 ml of diluent. (Stock solution- 250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution and add 1 ml of 3% H_2O_2 and kept at 60°C in water bath for 2hours. After that make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution were injected in HPLC.

Thermal Degradation

- Procedure for API

10 mg Teriflunomidewas taken and transferred to 10 ml flaskand add with diluent (1000 µg/ml Teriflunomide). Take 2.5 ml solution into 10 ml volumetric flask and volume was made up by diluent (Stock solution-250 µg/ml Teriflunomide).Pipette out 1 ml from stock solution and add 1 ml of diluent and kept at 60°C in water bath for 2hours. After that make up with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution were injected in HPLC.
Procedure for Tablet.

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Photolytic Degradation

- Procedure for API

10 mg Teriflunomidewas taken and transferred to 10 ml flask and add with diluent (1000 µg/ml Teriflunomide). Take 2.5 ml solution into 10 ml volumetric flask and volume was made up by diluent (Stock solution-250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution into 10 ml volumetric flask and kept under sun light for 2 hours. After that make up the volume with 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution was injected in HPLC.

Procedure for Tablet

The Average Weight of 10 Tablet was determined and was ground in mortar. An Accurately weighed amount of powder and equivalent to 10 mg Teriflunomideand transferred to 10 ml volumetric flask. Add 5 ml of diluent and sonicated for 5 minute to ensure complete solubilisation of drug. After sonication, volume was made up to the mark with diluent (1000 µg/ml of Teriflunomide). Pipette out 2.5 ml from above solution and dilute up to 10 ml of diluent. (Stock solution- 250 µg/ml Teriflunomide). Pipette out 1 ml from stock solution into 10 ml volumetric flask and kept under sun light for 2 hours. After that make up the volumewith 10 ml of diluent. Filter the final solution with 0.45µ PVDF Filter. Similarly blank was prepared without adding sample. Blank and sample solution was injected in HPLC.

Method Validation[14]

System suitability was performed by preparing standard solutions of API and tablet as per the test method and analyzed before performing any validation parameters to verify that the system is adequate for the analysis. The parameters used to verify in this test were retention time, theoretical plates, tailing factor and resolution of standard chromatogram.

Linearity

An accurately weighed quantity about 100 mg of Teriflunomide working standard were transferred in to 100 ml volumetric flask, dissolved and diluted to volume with diluent.Different linearity concentration solutions were prepared in the range of 125-375µg/ml.

Specificity

Specificity is defined as its ability to measure the analyte accurately and specifically in the presence of the component that may be accepted to be present in the sample matrix.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision considered at three levels: Repeatability, Intermediate (Intraday) Precision and Reproducibility (Interday) Precision.

Repeatability (n=6)

Six sets of assay preparations were prepared for Teriflunomide. Inject Samples and recorded the chromatograms.The mean of area of Teriflunomide was calculated in each case.The standard deviation and Coefficient of variation of area results were determined.

Intraday Precision (n=3)

Intraday precision samples were prepared in triplicate in 80%, 100% and 120% range of concentration for Teriflunomide. All triplicate preparations were injected into the liquid chromatography at different time intervals of a day and recorded the chromatograms.

Interday Precision (n=3)

Interday precision samples were prepared in triplicate in 80%, 100% and 120% range of concentration for Teriflunomide. All triplicate preparations were injected into the liquid chromatography at three different continuous days and recorded the chromatograms.Mean area and Coefficient of variation for the results was calculated at each level.

Accuracy (n=3)

The Accuracy of the method was determined at 50%, 100% and 150% by calculating recovery of Teriflunomide in the solution by standard addition method. Each solution was injected in triplicate and the % recovery was calculated by measuring the peak areas and putting these values into the regression equation of the respective calibration curves.

Robustness

The effects of small, deliberate variation of the analytical conditions on the peak areas of the drugs were examined. The change in response of drug was noted. In which the method was studied by changing flow rate ± 0.2 ml, mobile phase composition ± 5 ml and Temperature ±3ºC.The change in response of Teriflunomide was noted and compare with the original one.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) and quantitation (LOQ) were calculated as 3.3s/S and 10s/S respectively, where s is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot.

RESULT AND DISCUSSION

Forced Degradation

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

Acid Degradation

Chromatogram of acid degradation on sample solution is shown below in Figure1, 2, 3.

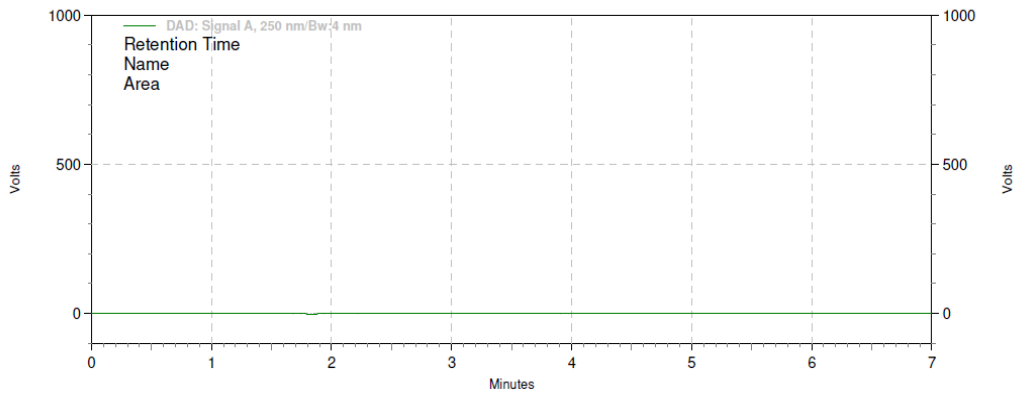

Fig 1: Chromatogram of Blank solution for Acid Degradation.

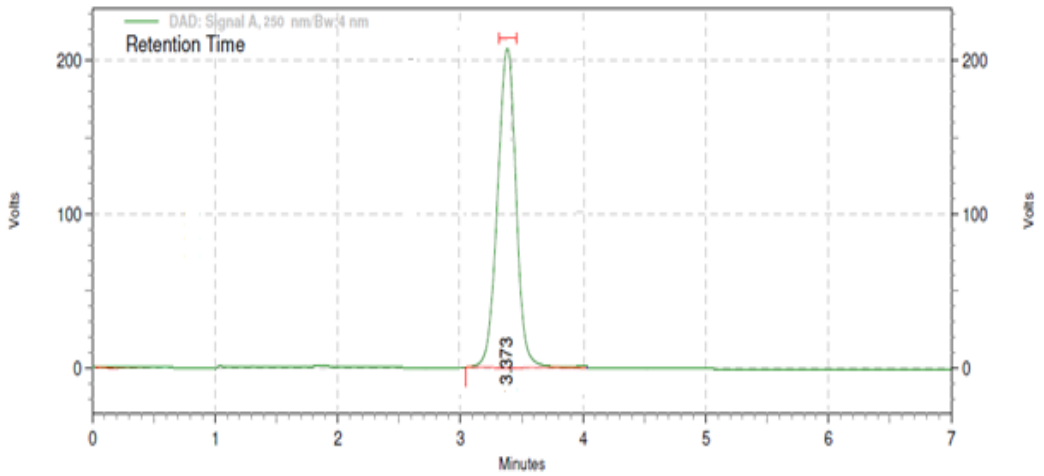

Fig 2: Chromatogram of Teriflunomide API solution for Acid degradation.

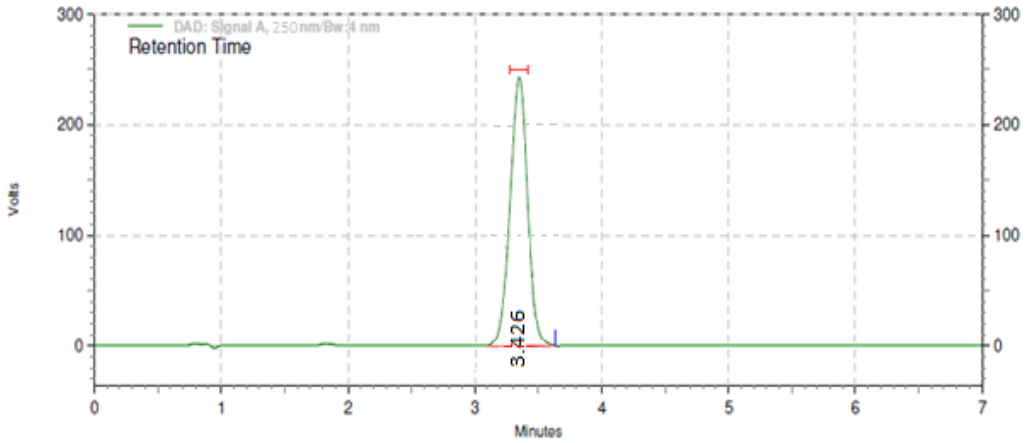

Fig 3: Chromatogram of Teriflunomide Tablet solution for Acid degradation.

Base Degradation

Chromatogram of base degradation on sample solution is shown below in Figure 4, 5, 6.

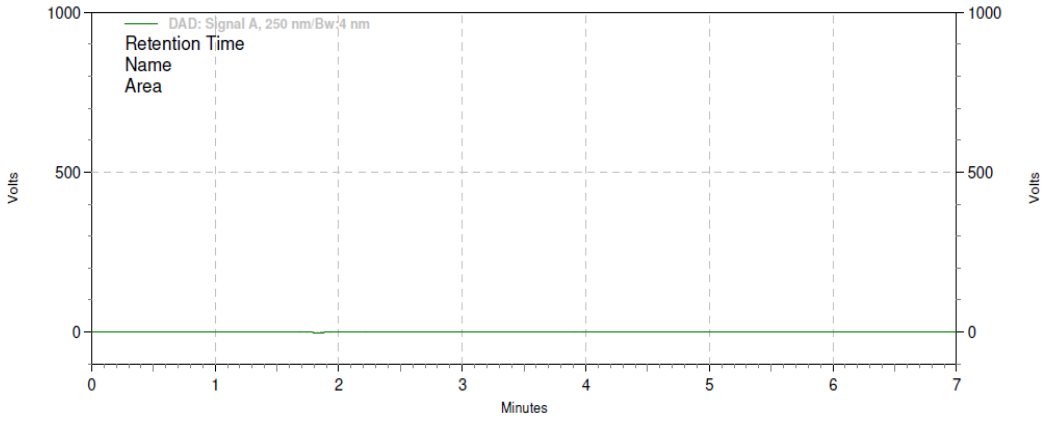

Fig 4: Chromatogram of Blank solution for Base Degradation.

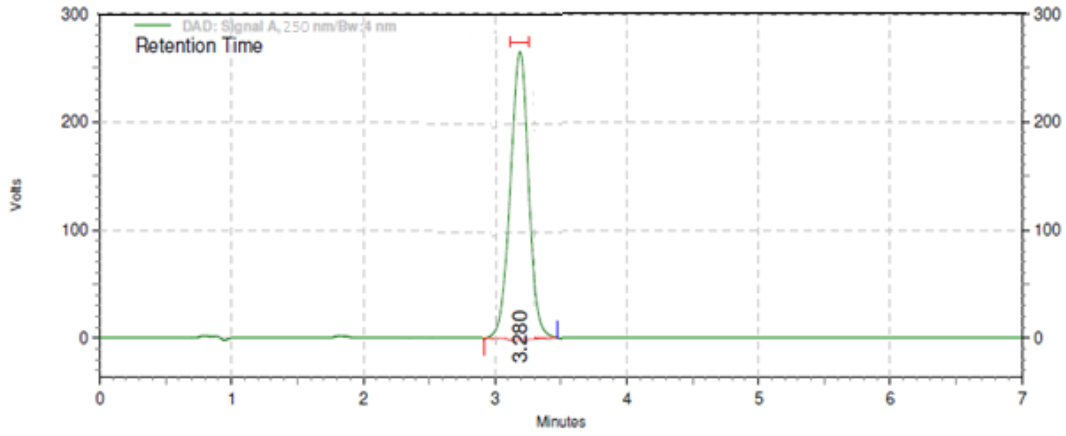

Fig 5: Chromatogram of Teriflunomide API solution for Base degradation.

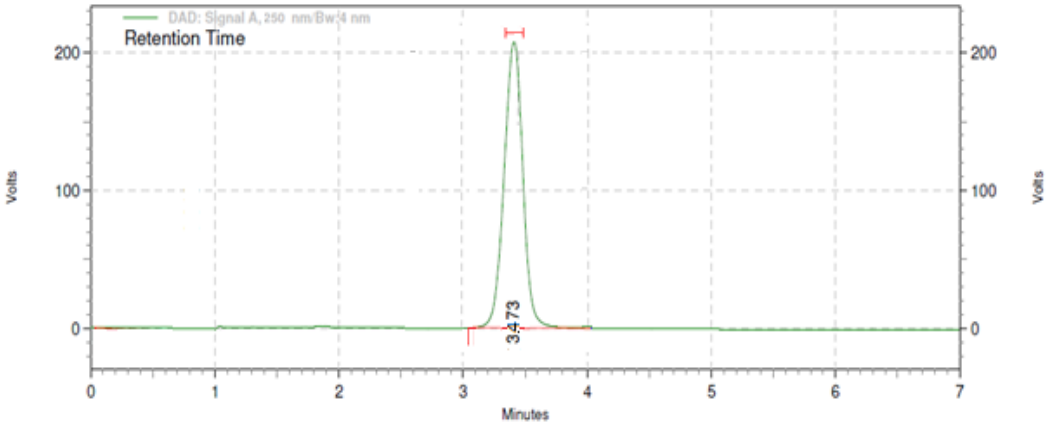

Fig 6: Chromatogram of Teriflunomide Tablet solution for Base degradation.

Peroxide Degradation

Chromatogram of peroxide degradation on sample solution is shown below in Figure7, 8, 9.

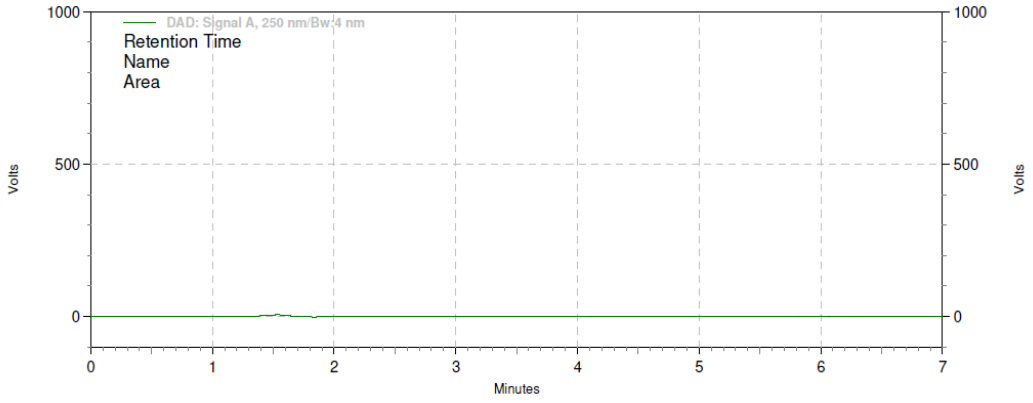

Fig 7: Chromatogram of Blank Solution for Peroxide Degradation.

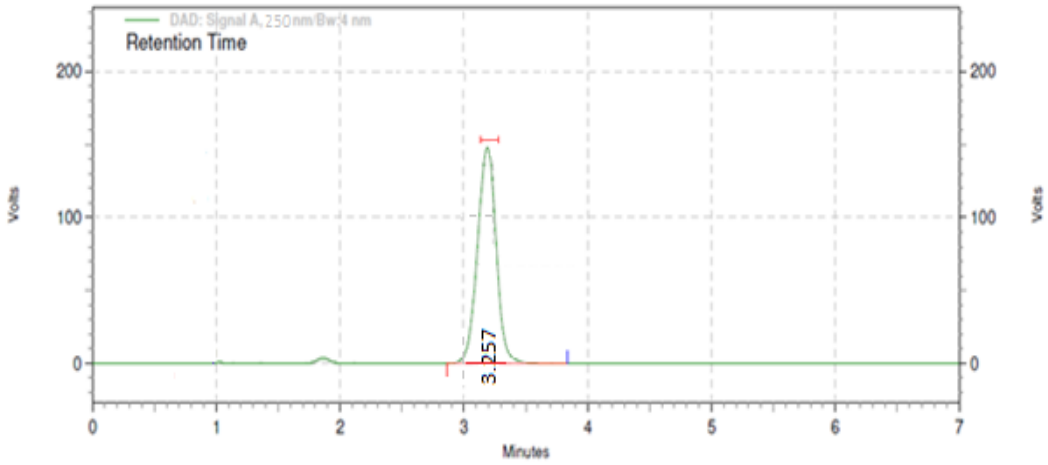

Fig 8: Chromatogram of Teriflunomide API solution for Peroxide degradation.

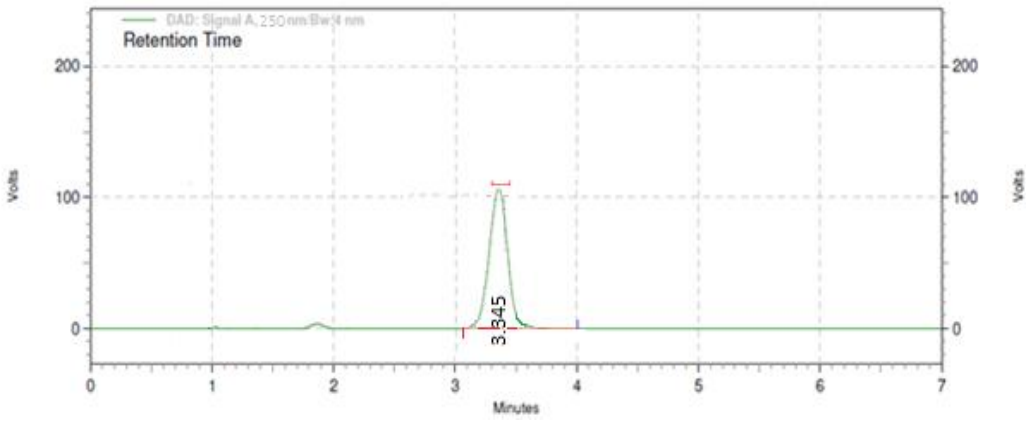

Fig 9: Chromatogram of Teriflunomide Tablet solution for Peroxide degradation.

Thermal Degradation

Chromatogram of thermal degradation on sample solution is shown below in Figure10,11,12.

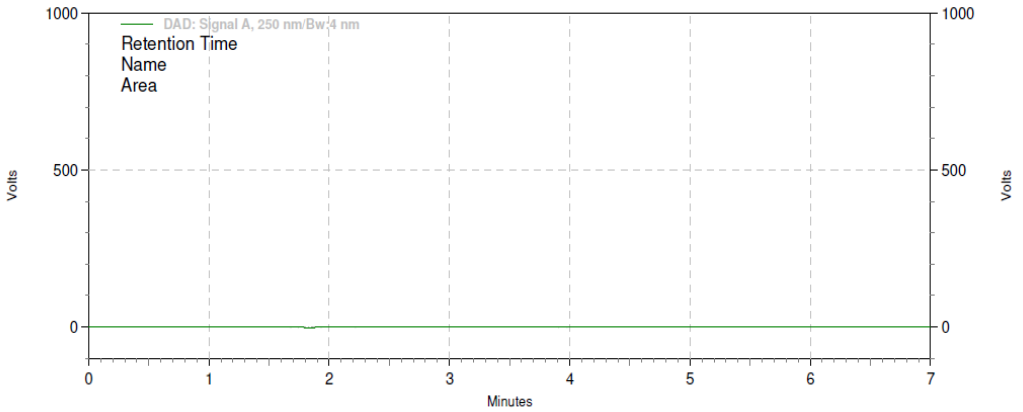

Fig 10: Chromatogram of Blank Solution for Thermal Degradation.

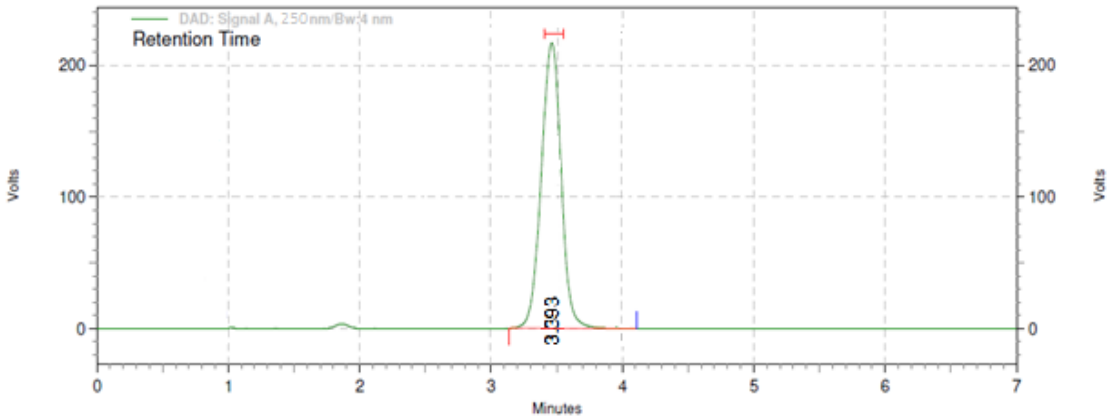

Fig 11: Chromatogram of Teriflunomide API solution for Thermal degradation.

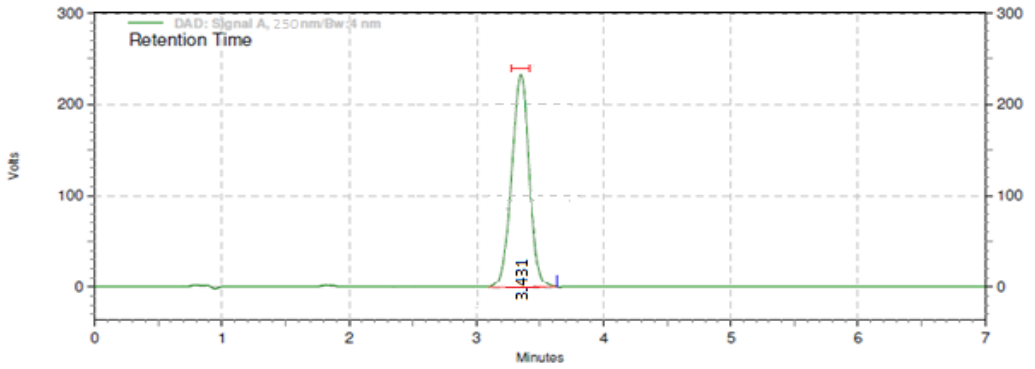

Fig 12: Chromatogram of Teriflunomide Tablet solution for Thermal degradation.

Photolytic Degradation

Chromatogram of photo degradation on sample solution is shown below in Figure13, 14, 15.

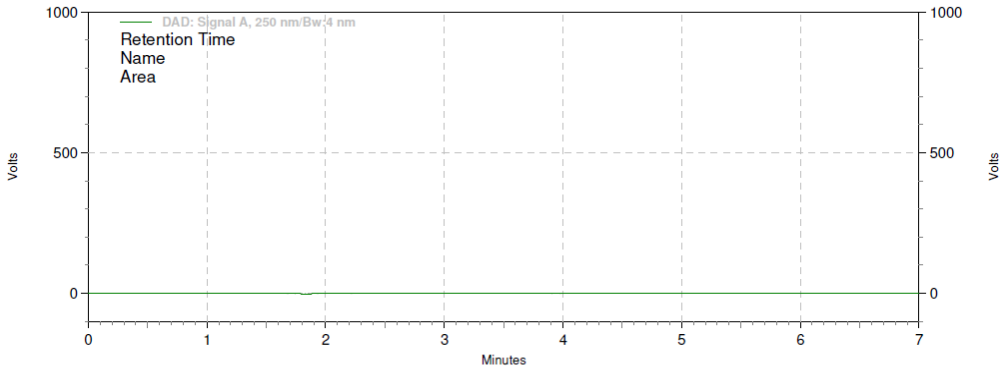

Fig13: Chromatogram of Blank Solution for Photolytic Degradation.

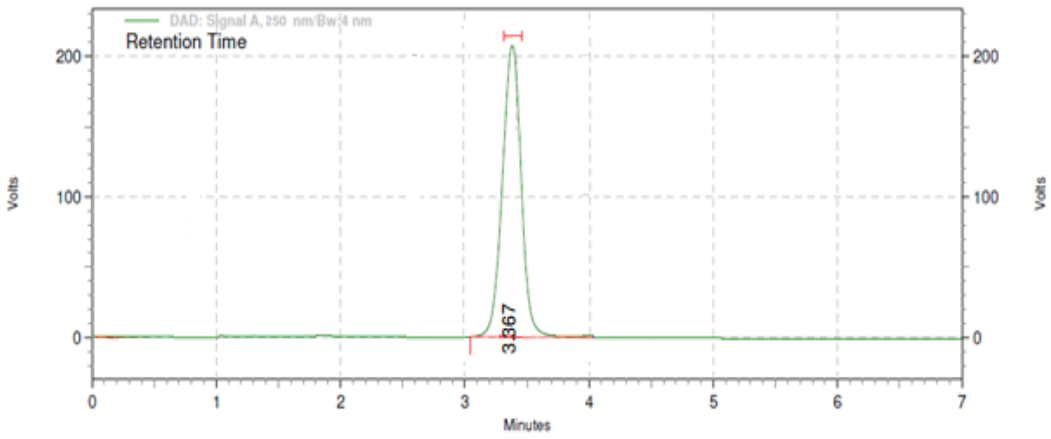

Fig 14: Chromatogram of Teriflunomide API solution for Photolytic degradation.

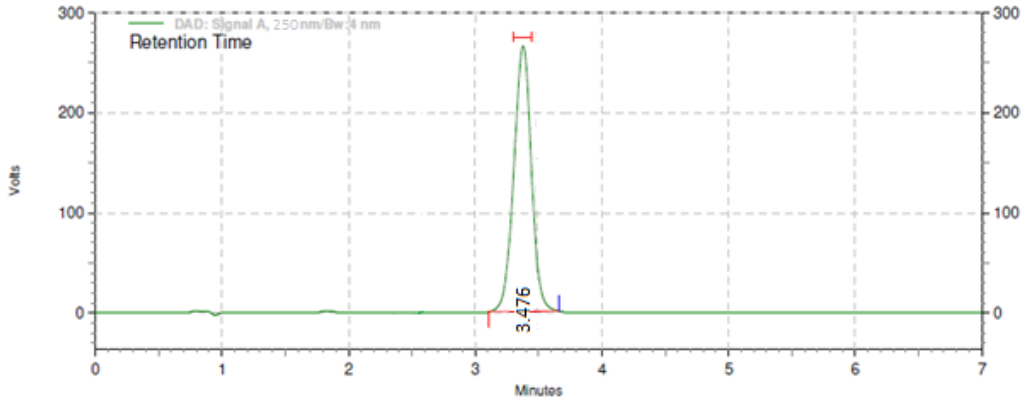

Fig 15: Chromatogram of Teriflunomide Tablet solution for Photolytic degradation.

Table 1: Degradation Summary for API

Type	Solution	Area	% Degradation
As Such	Teriflunomide	24519134	-
Acid 0.1N HCl at 60°C for 2 hrs	Teriflunomide	2478884	10.10%
Base 0.1N NaOH at 60°C for 2 hrs	Teriflunomide	2231241	9.0%
Peroxide 3% H_2O_2 at 60°C for 2 hrs	Teriflunomide	2697104	13.00%
Thermal Thermal at 60°C for 2 hrs	Teriflunomide	2378355	9.6%
Photo Direct sun light for 2 hrs	Teriflunomide	2623547	10.6%

Table 2: Degradation Summary for Tablet.

Type	Solution	Area	% Degradation
As Such	Teriflunomide	26386916	-
Acid 0.1N HCl at 60°C for 2 hrs	Teriflunomide	2770626	10.49%
Base 0.1N NaOH at 60°C for 2 hrs	Teriflunomide	2638691	10.00%
Peroxide 3% H_2O_2 at 60°C for 2 hrs	Teriflunomide	3100462	14.24%
Thermal Thermal at 60°C for 2 hrs	Teriflunomide	2242887	8.4%
Photo Direct sun light for 2 hrs	Teriflunomide	2612304	9.8%

Method Validation

System Suitability Parameters

System suitability and chromatographic parameters were validated such as resolution, theoretical plates, and tailing factor was calculated. The results are given in Table 3, 4, 5.

Table 3: System suitability data for API (250 µg/ml Teriflunomide).

Sr. No.	Retention time	Area	Tailing	Plate Count
	3.373	24519134	1.0	2560
	3.371	24519221	1.12	2619
	3.342	24526541	1.02	2615
	3.121	25265612	1.18	2579
	3.335	24561623	1.0	2565

Table 4: System suitability data for Tablet (250 µg/ml Teriflunomide).

Sr. No.	Retention time	Area	Tailing	Plate Count
1.	3.527	26386913	1.0	2450
2.	3.601	26951625	1.21	2560
3.	3.553	26683825	1.30	2465
4.	3.500	26301925	1.0	2670
5.	3.593	26352497	1.0	2458

Table 5: System Suitability Parameters (N=5)

Parameters	Observation		Spacification
	API	Tablet	
%RSD of Area	0.52	0.40	RSD < 2%
Resolution (Rs)	0.0	0.0	Rs > 2
Tailing Factor (T)	1.0	1.0	T ≤ 2
Theoretical Plates(N)	2560	2450	≥2000

Linearity (n=5)

The results were found to be linear in a range of 125-375 µg/ml for Teriflunomide. The correlation co-efficients (r) for the plot was 0.9994. The calibration plots obtained for Teriflunomide is shown in figure 16, 17.

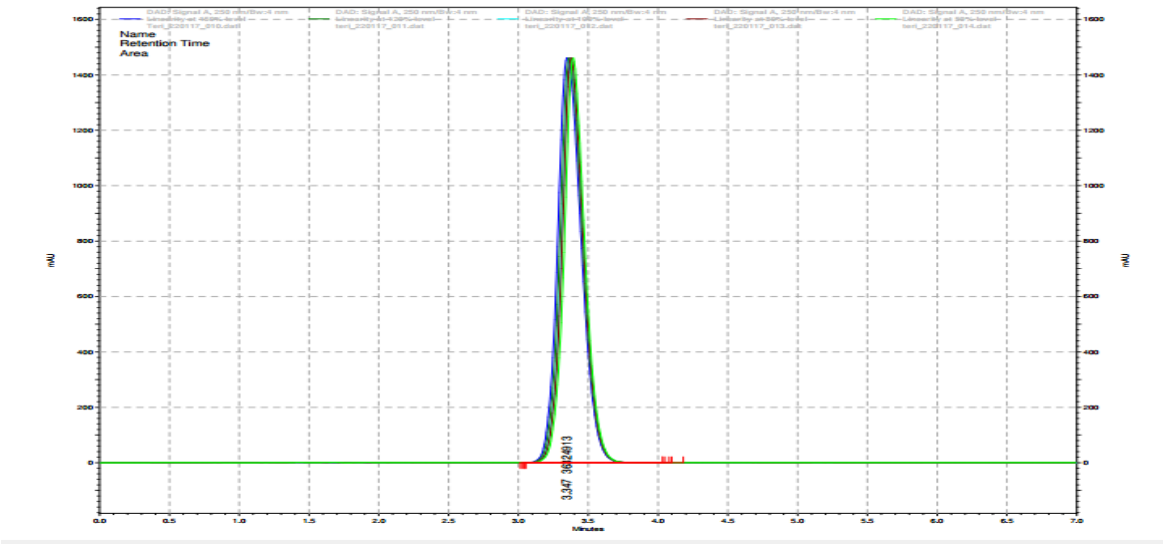

Fig 16: Linearity spectra of Teriflunomide.

Table 6: 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

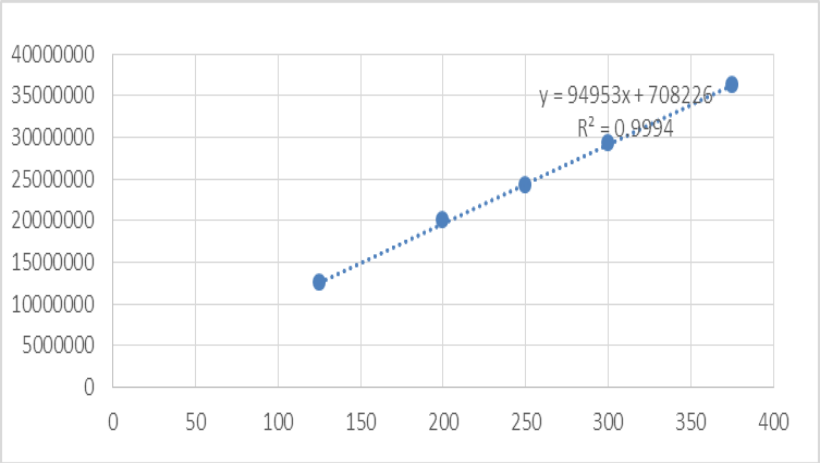

Fig 17: Calibration curve of Teriflunomide.

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

Specificity

(A) Check the interference from blank

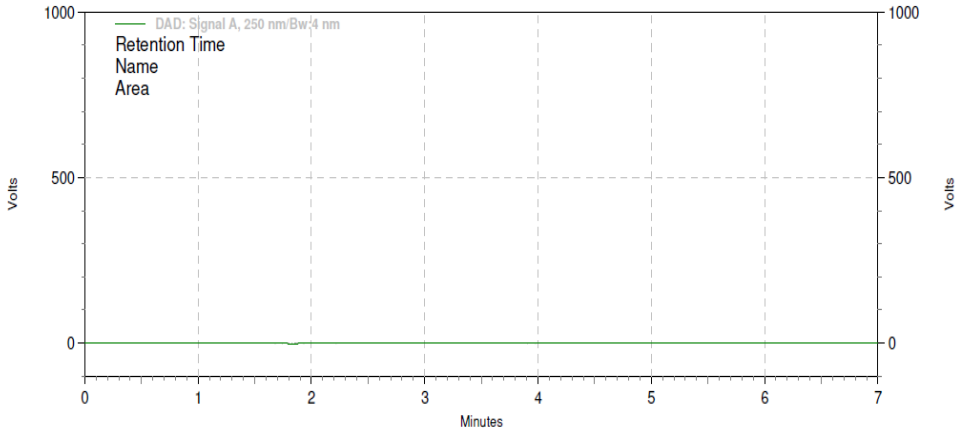

Fig 18: Chromatogram of blank preparation.

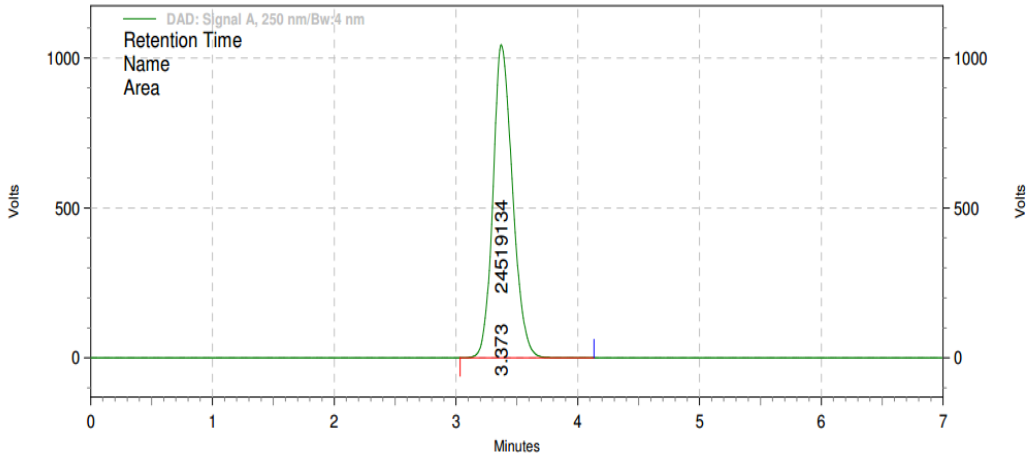

Fig 19: Chromatogram of standard preparation.

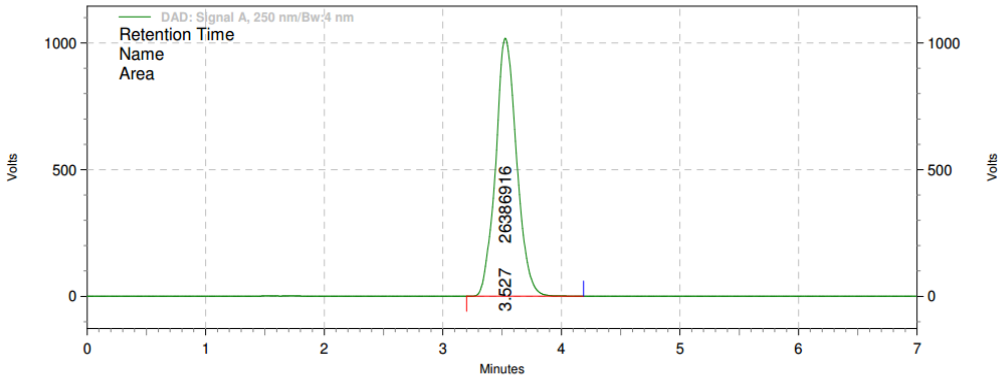

Fig 20: Chromatogram of sample preparation.

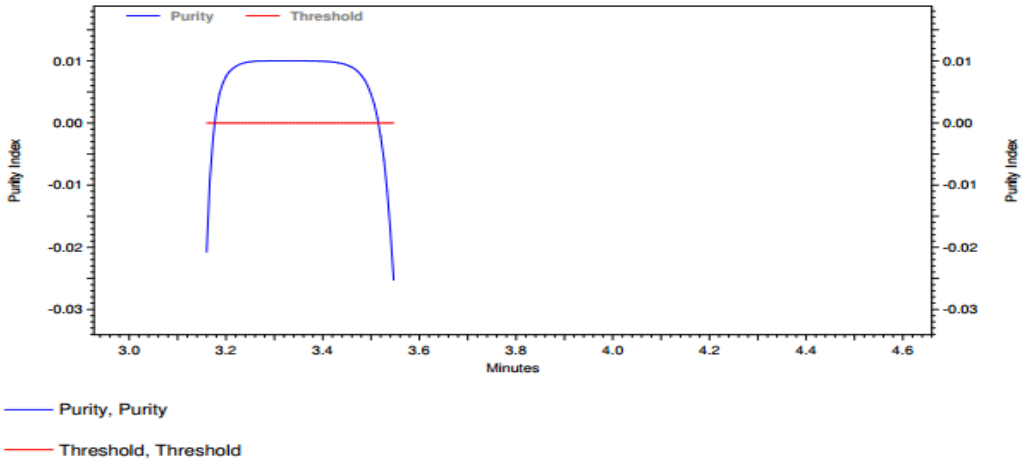

Fig 21: Peak purity spectra of Teriflunomide.

The purity angle and threshold angle for the main peak in standard preparation and sample preparation was determined and recorded in **Table 7.**

Table 7: Data indicating peak purity of Teriflunomide

Description	Peak purity Match Teriflunomide Purity Angle
Standard Preparation	1.0
Sample Preparation	0.99

Precision

The developed method was found to be precise with % RSD values for repeatability and intermediate precision studies below 2 % as recommended by ICH Q2 (R1) guideline.Results are shown in Table 8, 9, 10.

Repeatability (n=6)

Table 8: 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

Intraday Precision (n=3)

Table 9: Intraday precision data for Teriflunomide.

Concentration (µg/ml)	Mean area ± SD	% CV
200	23082394±158261.3	0.68
250	24487233±120829.8	0.49
300	29386590±91792.83	0.31

Interday Precision (n=3)

Table 10: 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

Accuracy (n=3)

Satisfactory recoveries for Teriflunomidewere obtained (Table 11, 12), which indicate that the proposed chromatographic method is reliable for estimation of Teriflunomide in pharmaceutical formulation.

Table 11: 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	200	1.25	199.36 ±0.17	99.21 ±0.26
	100	250	2.5	251.70 ±0.26	100.00 ±0.22
	150	300	3.75	301.75 ±0.27	99.45 ±0.39

Table 12: Accuracy Data for Teriflunomide Tablet.

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	200	1.25	198.76 ±0.2	98..67 ±0.28
	100	250	2.5	251.30 ±0.17	100.33 ±0.17
	150	300	3.75	301.65 ±0.27	99.36 ±0.45

Robustness

Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest and retention factor remained unaffected by small changes of the operational parameters (% RSD<2).

Table 13: Robustness Study of Teriflunomide.

Sr. No.	Conditions	Mean Area ± SD	%CV	Theoretical plate	Tailing factor	R.T.
1	Column Oven Temperature: 43ºC	1.375304 24184977 ± 332616.9	1.37	2950	1.5	3.123
2	Column Oven Temperature: 37 ºC	24509119 ± 72018.26	0.29	2790	1.3	3.570
3	Flow rate: 1.0 ml/min	24540412 ± 44553.75	0.18	2835	1.6	3.001
4	Flow rate: 0.6 ml/min	25275561 ± 371954.3	1.47	2821	1.5	3.620
5	Mobile phase Composition: ACN: Buffer(35:65)	25278337 ± 397468.1	1.57	2628	1.43	3.013
6	Mobile phase composition: ACN: Buffer(45:55)	24116957 ± 61525.16	0.25	2649	1.41	3.621
7	Acceptance Criteria	NA	NMT 2.00	NLT 2000	Between 0.80 to 2.00	

Limit of Detection and Limit of quantification

Result is shown in Table 14

Table 14: Limit of Detection and Limit of quantification.

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

Analysis of Formulation by RP-HPLC method

Result is shown in Table 15.

Table 15: Analysis of Formulation by RP-HPLC method.

Teriflunomide Tablet	Label Claim(mg / tablet)	% Assay (Mean±SD)
	Teriflunomide	Teriflunomide
Aubagio	10	99.36 %

The summary of validation parameters of proposed method are given in table 16.

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

The validated RP-HPLC method employed proved to be simple, fast, accurate, precise and robust and thus can be applied for the estimation of Teriflunomide in API and Tablet dosage form.

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