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"Reverse-Phase HPLC Method Development And Validation For The Analysis Of Teriflunomide In Oral Dosage Forms"

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ABSTRACT

Objective: This study aimed to develop and validate a simple, precise, accurate, and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of Teriflunomide, an immunomodulatory drug primarily used in the treatment of relapsing forms of multiple sclerosis (MS).

Methods: Chromatographic separation was optimized using a Shim-pack XR-ODS C18 column (150 × 4.6 mm, 5 µm) with a mobile phase composed of acetonitrile and phosphate buffer (75:25 v/v), adjusted to pH 3.6 with orthophosphoric acid. The flow rate was maintained at 1.2 mL/min, the injection volume was 10 µL, and detection was performed at 289 nm. The method was validated following ICH guidelines, assessing parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, specificity, and system suitability.

Results: Teriflunomide eluted with a retention time of 3.5 minutes, showing symmetrical peaks and acceptable system suitability metrics. The method exhibited excellent linearity over the range of 20–100 µg/mL ($R^2 = 0.998$). Accuracy was confirmed with recovery rates between 100.08% and 100.76% across three concentration levels. Intraday and Interday precision yielded %RSD values of 1.13% and 1.27%, respectively. LOD and LOQ were determined to be 9.58 µg/mL and 29.05 µg/mL. The method remained robust under small, deliberate changes in chromatographic conditions.

Conclusion: The developed RP-HPLC method is reliable, sensitive, and reproducible, making it suitable for routine quality control analysis of Teriflunomide in pharmaceutical dosage forms.

Key words: Teriflunomide**,** RP-HPLC, ICH guidelines, multiple sclerosis, immunomodulatory drug.

INTRODUCTION

Teriflunomide is an oral immunomodulatory agent primarily used in the treatment of relapsing forms of multiple sclerosis (MS). It functions by selectively inhibiting dihydroorotate dehydrogenase, a key enzyme in the de novo pyrimidine synthesis pathway, which is crucial for the proliferation of lymphocytes. This mechanism helps to reduce the inflammatory processes that contribute to the pathogenesis of MS by limiting the number of activated lymphocytes entering the central nervous system.[1] In addition to its primary use in MS, there is emerging evidence suggesting potential benefits of teriflunomide in other central nervous system (CNS) disorders characterized by white matter damage, such as amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD).[2]This broadens the therapeutic scope of teriflunomide beyond MS, although further research is warranted to fully elucidate its regenerative capabilities in these conditions.

Relapsing forms of multiple sclerosis (MS), particularly relapsing-remitting multiple sclerosis (RRMS), represent the most prevalent manifestation of this complex neurological disorder, affecting approximately 85% of patients diagnosed with MS[3,4] The natural history of RRMS indicates that while relapses are a hallmark of the disease, their frequency and severity can vary widely among individuals.[5,6] Studies have shown that the time to the next relapse after an initial attack averages around 1.1 years, with a notable percentage of patients experiencing their first relapse within six months of the initial event.[7] This variability underscores the unpredictable nature of MS and the challenges it poses in clinical management.

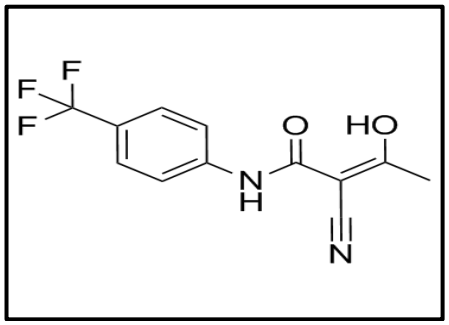

Figure: 1 Chemical structure of Teriflunomide

Its mechanism of action is primarily attributed to the selective inhibition of dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme crucial for de novo pyrimidine synthesis. This inhibition leads to a cytostatic effect on rapidly proliferating lymphocytes, particularly T and B cells, thereby reducing their proliferation and activation without

significantly compromising the overall immune response.[8] The inhibition of DHODH by teriflunomide results in decreased levels of pyrimidine nucleotides, which are essential for DNA and RNA synthesis in activated lymphocytes. This mechanism effectively limits the expansion of these immune cells, which are implicated in the pathogenesis of MS.[9] Studies have shown that teriflunomide not only reduces the number of activated T and B cells in circulation but also affects their functionality, leading to a decrease in pro-inflammatory cytokine production.[10,11] Furthermore, teriflunomide has been observed to induce a regulatory status in immune cells, enhancing the generation of regulatory T cells, which may contribute to its therapeutic effects in MS.[12,13]

In addition to its effects on lymphocytes, teriflunomide has been shown to exert neuroprotective effects within the central nervous system (CNS). It reduces microglial activation and proliferation, which are key contributors to neuroinflammation in MS.[14,15] Clinical trials have demonstrated that teriflunomide treatment leads to significant reductions in relapse rates and disability progression, as well as improvements in MRI outcomes, indicating its efficacy in managing MS.[16,17] Moreover, teriflunomide's selective immunomodulatory effects allow for the maintenance of adaptive immune responses, as evidenced by studies showing that vaccination responses remain intact in patients undergoing teriflunomide treatment.[18] This characteristic is particularly important in the context of managing autoimmune diseases like MS, where a balanced immune response is crucial for preventing disease exacerbation while controlling inflammation.

MATERIALS AND METHODS

Materials

Teriflunomide was received as a gift sample. All other chemicals and reagents used in the study were of analytical grade, and the solvents met HPLC standards. The assay was performed using the commercially available 14 mg Aubagio

Instrument

The RP-HPLC Shimadzu with the uv detector. Shim-pack XR-ODS C18 (150 × 4.6 mm, 5µ) column was used.

Chromatographic conditions

Various mobile phase mixtures were evaluated to assess system suitability parameters such as theoretical plate count, resolution, and tailing factor. The best separation was achieved using a freshly prepared mobile phase consisting of buffer and acetonitrile in a 25:75 (v/v) ratio. The method was performed at a constant flow rate of 1.2 mL/min, with a fixed injection volume of 10 µL, and at room temperature. Under these optimized conditions, Teriflunomide exhibited sharp, symmetrical peaks at a detection wavelength of 289 nm. The buffer pH was precisely adjusted to 3.6 using orthophosphoric acid.

Preparation of Standard stock solution

Weigh 10 mg of the drug precisely, transfer it to a 10 ml volumetric flask, and then add 5 ml of the solvent. After shaking the flask for two minutes, the volume was made up with solvent. This results in a 1000 µg/ml solution. To get a concentration of 100 µg/ml, pipette out 1 ml of the previous solution into a 10 ml volumetric flask and dilute it with solvent up to the mark. Further, dilutions had been made from the solution containing 100 µg/mL.

Selection of Wavelength

A standard solution of teriflunomide (10 µg/mL) was analyzed using a UV-visible spectrophotometer across the wavelength range of 200 to 400 nm, with acetonitrile serving as the blank reference. The solution was scanned in spectrum mode to determine the wavelength of maximum absorbance (λmax).

Method development

The method was established through multiple experimental trials involving adjustments to various chromatographic parameters. Teriflunomide was successfully eluted within an acceptable retention time, confirming the method's suitability for routine analysis. A C18 column was selected, using a mobile phase consisting of a buffer and acetonitrile mixture. A column length of 150 mm was employed to ensure sufficient interaction and separation between the analyte and the stationary phase. Key chromatographic metrics, including retention time, theoretical plate count, and tailing factor, were all within acceptable limits, indicating efficient system performance.

Validation Parameters

The method was validated following ICH guidelines, considering essential parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness.

Accuracy

Accuracy was evaluated by spiking known amounts of standard Teriflunomide into the sample solution at three concentration levels—80%, 100%, and 120%—in triplicate. The percentage recovery was calculated for each level to assess the method’s accuracy.

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Precision

Intraday Precision: Six replicate injections of the standard solution were analyzed within a single day. The %RSD of the responses was calculated to assess repeatability.

Interday Precision: The standard solution was prepared and analyzed on different days, with six replicate injections each day. The %RSD of the responses was calculated to evaluate intermediate precision.

Linearity

Linearity was assessed across a concentration range of 20 to 100 µg/mL for Teriflunomide. Each concentration level was injected five times, and a blank (solvent only) was also run. A calibration curve was plotted using peak area versus concentration data to confirm the linear relationship.

LOD and LOQ

The limits of detection (LOD) and quantification (LOQ) were calculated using the following formulas:
$LOD = 3.3 \times \sigma / S$
$LOQ = 10 \times \sigma / S$
Where **σ** is the standard deviation of the y-intercepts of the regression lines and **S** is the slope of the calibration curve.

Robustness

Robustness was assessed by making deliberate variations to chromatographic parameters such as detection wavelength and mobile phase composition. These changes were made to evaluate the method's reliability under varied conditions.

RESULTS AND DISCUSSIONS

Selection of wavelength

The UV spectrum of Teriflunomide (10 µg/mL) was obtained using a UV spectrophotometer across a wavelength range of 200–400 nm. The compound exhibited maximum absorbance at 289 nm, which was chosen as the detection wavelength (λmax) for teriflunomide due to its strong peak response at this point.

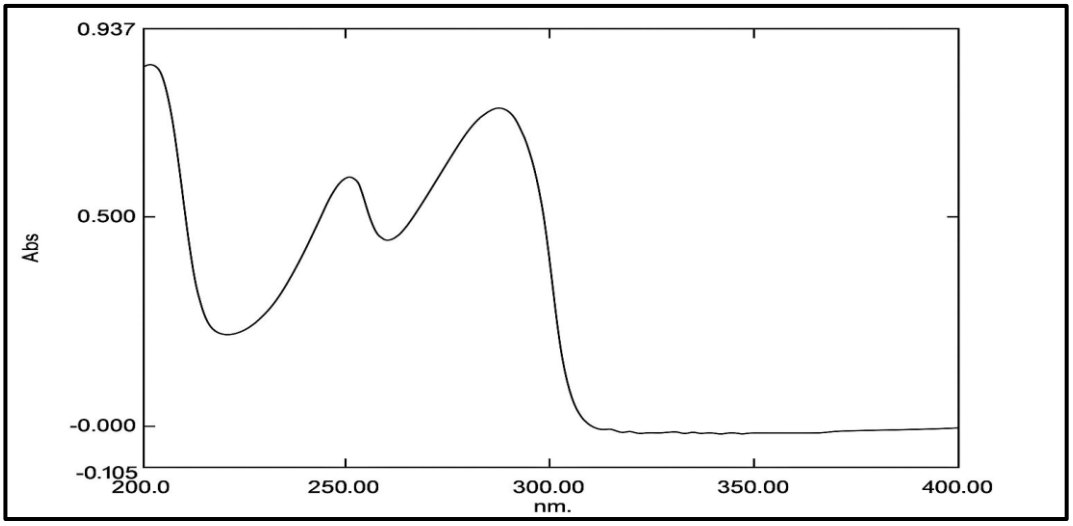

Figure 2. λmax of Teriflunomide (289nm)

Method Development and Chromatographic Optimization

At the beginning of the experimental phase, a mobile phase consisting of buffer and acetonitrile in a 20:80 (v/v) ratio was tested, but it resulted in excessively broad peaks. Subsequent experiments with different buffer-to-acetonitrile ratios—20:80, 10:90, and 50:50 (v/v)—also failed to produce sharp, well-defined peaks, highlighting the need for further optimization. To address these issues, a new mobile phase composition of acetonitrile and phosphate buffer in a 75:25 (v/v) ratio was evaluated.

Following this, various chromatographic parameters such as buffer pH, flow rate, and column oven temperature were systematically adjusted to enhance peak shape and symmetry. These changes were made with careful consideration to ensure the system met the required suitability criteria. Ultimately, the mobile phase was optimized to a 75:25 (v/v) mixture of acetonitrile and phosphate buffer, with the buffer pH precisely adjusted to 3.6 using orthophosphoric acid.

Figure 3 represents a typical chromatogram of Teriflunomide.

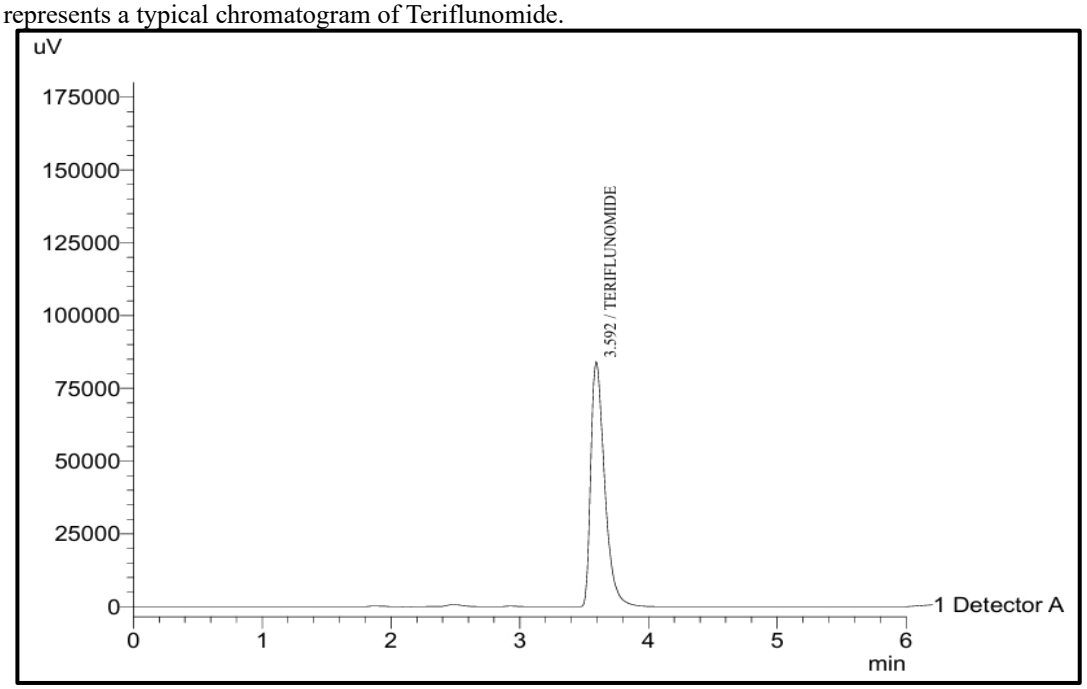

Figure 3: A typical chromatogram of Teriflunomide

Method validation

The developed reverse-phase HPLC method was validated in accordance with ICH guidelines, taking into account various parameters as outlined below.

System suitability

System suitability was assessed using a representative chromatogram to evaluate key performance parameters. The retention time was recorded at 3.5 minutes, the number of theoretical plates (NTP) was determined to be 3279, and the peak symmetry factor was 1.2. These parameters collectively indicate the effectiveness and reliability of the chromatographic system.

Specificity

Blank, standard, and sample solutions were prepared and analyzed. At the retention time of the teriflunomide peak, there should be no interference from the diluent, individual impurities, or placebo components.

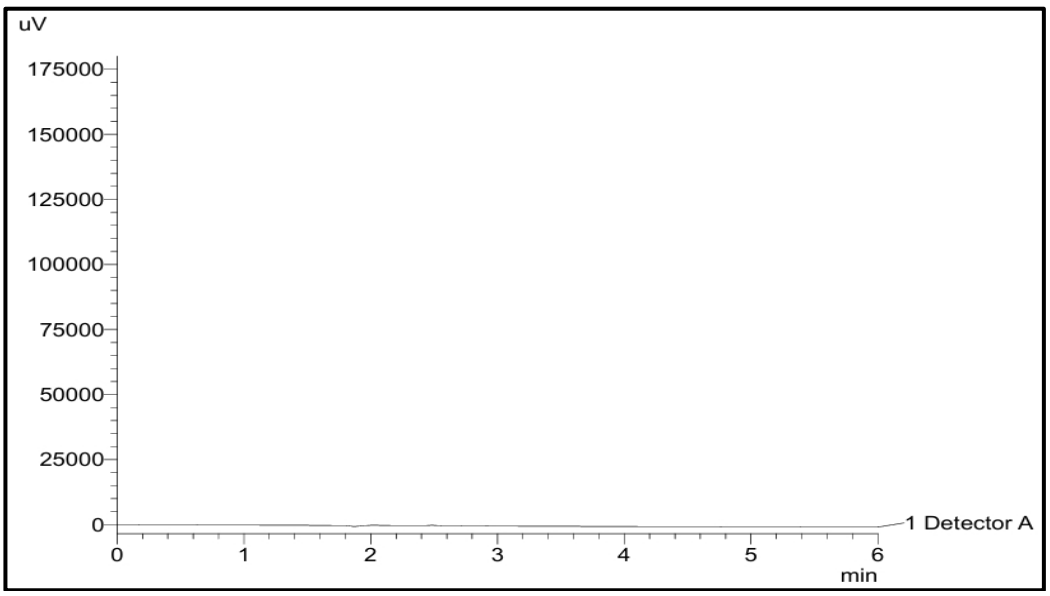

Figure 4: Chromatogram of Blank

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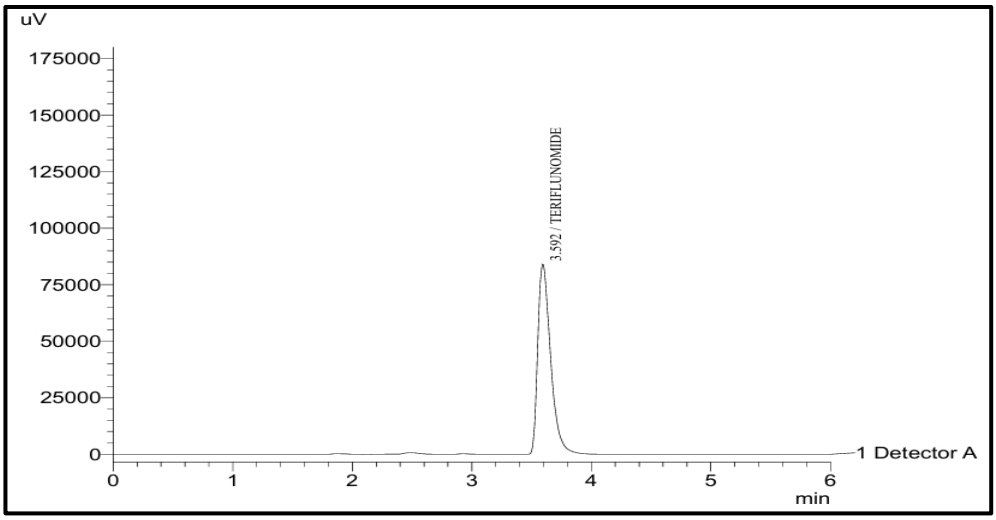

Figure 5: Specificity chromatogram of Teriflunomide

Linearity

A linear correlation was observed in the calibration curve for concentrations between 20 and 100 µg/mL, with a correlation coefficient of 0.998. As shown in Figure 6, the slope of the line was 10875 indicating the proportional relationship between concentration and peak area, while the y-intercept was 30630. These findings are supported by the data summarized in Table 1.

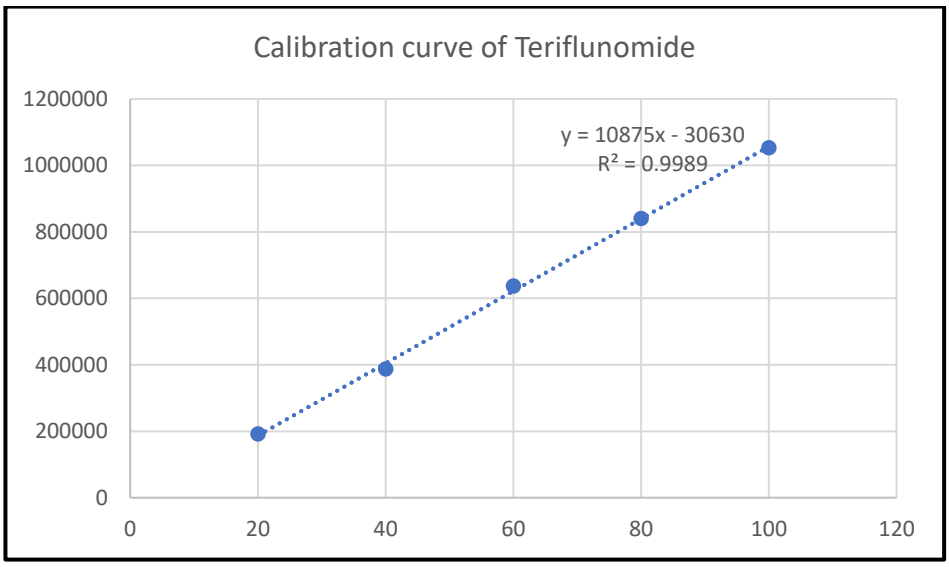

Figure 6: Linearity curve of Teriflunomide

Table 1. Linearity area of Teriflunomide

Sr No.	Concentrations (µg/ml)	Peak area
1	20	**191767**
2	40	**387560**
3	60	**636690**
4	80	**840559**
5	100	**1052764**
	Correlation coefficient	**0.998**
	Y-Intercept	**Y= 10875x - 30630**

Accuracy

Table 2 summarizes the results of the accuracy assessment performed for teriflunomide. The recovery rates at 80%, 100%, and 120% concentration levels were 100.08%, 100.76%, and 100.29%, respectively. These values meet the established acceptance criteria, thereby validating the accuracy of the proposed method

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Table 2. Accuracy data of Teriflunomide

Level	Actual concentra-tion	Calculated concentration (µg/ml)	Peak Area	% recovery	Mean concentration (µg/ml)	SD	%RSD
80%		48.2444	494028		48.0426	0.4734	0.9854
	48	47.5017	485952	100.08			
		48.3818	495523				
100%		61.2111	635041		60.4608	0.7351	1.2158
	60	60.4296	626542	100.76			
		59.7418	619063				
120%		71.7525	749679		72.2132	0.4562	0.6317
	72	72.6648	759600	100.29			
		72.2222	754787				

Precision

Intraday precision and Interday precision were evaluated in this study, with the results presented in Table 3. The %RSD values for intraday precision and interday precision were 1.13% and 1.27%, respectively, both falling within acceptable limits. These results indicate that the proposed method demonstrates good precision for the analysis of teriflunomide.

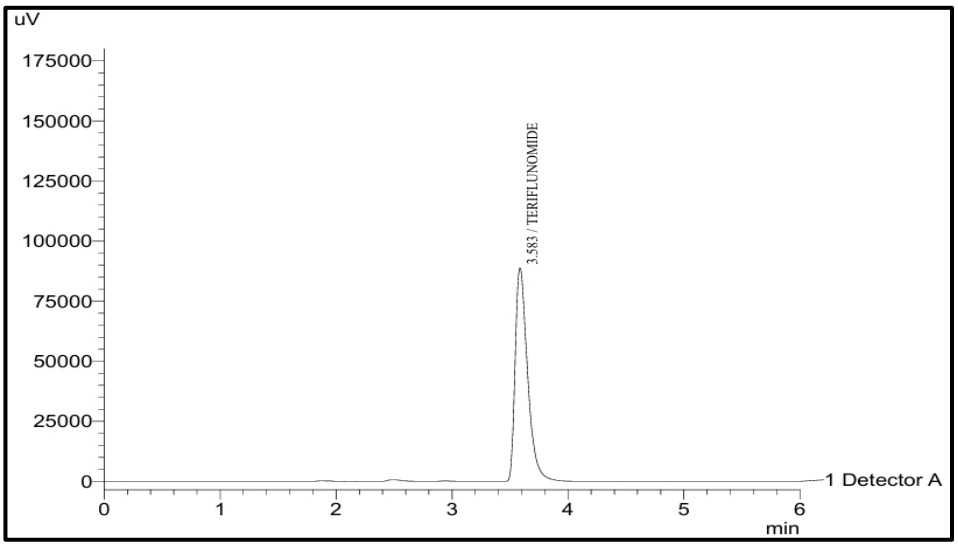

Fig 7. Chromatogram of Intraday Precision for Teriflunomide

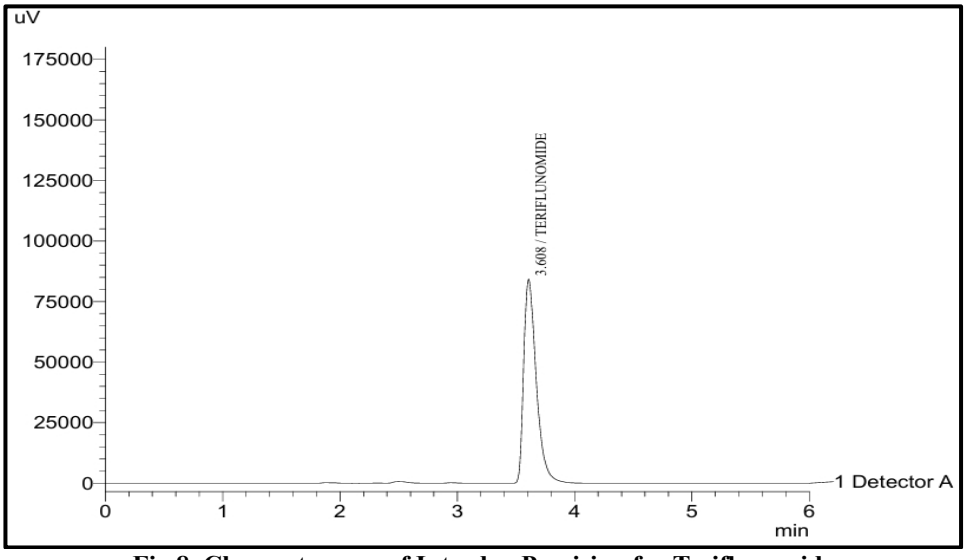

Fig 8. Chromatogram of Interday Precision for Teriflunomide

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Table 3. Intraday and Interday precision of Teriflunomide

Sr. No.	Concentration (µg/ml)	Absorbance	
		Intraday	Interday
1.	60	632557	629827
2.	60	637845	611548
3.	60	636425	628742
4.	60	635041	625694
5.	60	626542	630462
6.	60	619063	634873
Mean ±SD		**631245.5**	**626857.6**
SD		**7169.97**	**8066.52**
%RSD		**1.13**	**1.27**

Robustness

Robustness refers to the ability of a method to remain unaffected by small, deliberate variations in parameters such as sample composition, temperature, solution pH, reagent concentration, and flow rate. To evaluate the robustness of the method, six replicate injections of the standard preparation were performed. The results, specifically for the teriflunomide peak, are summarized in Table 4.

Table 4. Robustness data of Teriflunomide

Parameters	Changed conditions	Mean Area	1	2	3	SD	%RSD
Mobile Phase	70:30	627504	631846	621571	629095	5319.05	0.84%
	80:20	632349	625887	636163	634999	5627.01	0.88%
Wavelength	287 nm	603379	598076	602665	609396	5693.67	0.94%
	291 nm	601479	596123	600452	607862	5936.50	0.98%

LOD & LOQ:

The limit of detection (LOD) refers to the smallest concentration of an analyte that can be reliably distinguished from the background noise. The limit of quantification (LOQ) represents the lowest concentration of an analyte that can be accurately and precisely measured using a specific analytical method. These values were determined using the formulas: LOD = 3.3 × SD / Slope and LOQ = 10 × SD / Slope, where *SD* is the standard deviation and *Slope* is from the calibration curve. Based on these calculations, the LOD for the compound was determined to be 9.58 µg/ml, and the LOQ was 29.05 µg/ml.

CONCLUSION

The study successfully established and validated a reliable reverse-phase HPLC method for the estimation of Teriflunomide in pharmaceutical dosage forms. The development process involved optimizing various chromatographic parameters, ultimately leading to the selection of a mobile phase consisting of acetonitrile and phosphate buffer (75:25, v/v, pH 3.6). Under these conditions, Teriflunomide was well-resolved with a retention time of 3.5 minutes, sharp peak symmetry, and acceptable system suitability parameters. The method demonstrated excellent linearity over the range of 20–100 µg/mL, with a correlation coefficient (R^2) of 0.998. Accuracy was confirmed through recovery studies at 80%, 100%, and 120% levels, with values consistently falling within acceptable limits.

Precision data showed low %RSD values for both intraday and interday measurements, indicating strong repeatability and intermediate precision. The method also proved to be robust, showing minimal variation in results upon deliberate changes to key parameters like wavelength and mobile phase composition. The calculated LOD (9.58 µg/mL) and LOQ (29.05 µg/mL) further confirm the method's sensitivity. The developed method is simple, accurate, precise, and robust—making it highly suitable for routine analysis and quality control of Teriflunomide in pharmaceutical formulations.

ACKNOWLEDGEMENT

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ABBREVIATION:

MS: Multiple sclerosis
ALS: Amyotrophic lateral sclerosis

RP-HPLC: Reversed Phase High-performance liquid chromatography,
API: Active pharmaceutical ingredient,
LOQ: Limit of quantitation;
LOD: Limit of detection,
RSD: Relative standard deviation,
r^2: Coefficient of correlation,
ICH: International Council for Harmonisation,

REFERENCES

1. Comi, G., Freedman, M. S., Kappos, L., Olsson, T., Miller, A., Wolinsky, J. S., … & Leist, T. (2016). Pooled safety and tolerability data from four placebo-controlled teriflunomide studies and extensions. Multiple Sclerosis and Related Disorders, 5, 97-104. https://doi.org/10.1016/j.msard.2015.11.006
2. Göttle, P., Manousi, A., Kremer, D., Reiche, L., Hartung, H., & Küry, P. (2018). Teriflunomide promotes oligodendroglial differentiation and myelination. Journal of Neuroinflammation, 15(1). https://doi.org/10.1186/s12974-018-1110-z
3. Khan, O., Rieckmann, P., Boyko, A. N., Selmaj, K., & Zivadinov, R. (2013). Three times weekly glatiramer acetate in relapsing–remitting multiple sclerosis. Annals of Neurology, 73(6), 705-713. https://doi.org/10.1002/ana.23938
4. Nazareth, T., Rava, A. R., Polyakov, J. L., Banfe, E. N., Waltrip, R. W., Zerkowski, K., … & Herbert, L. B. (2018). Relapse prevalence, symptoms, and health care engagement: patient insights from the multiple sclerosis in america 2017 survey. Multiple Sclerosis and Related Disorders, 26, 219-234. https://doi.org/10.1016/j.msard.2018.09.002
5. Scalfari, A., Neuhaus, A., Degenhardt, A., Rice, G. P., Muraro, P. A., Däumer, M., … & Ebers, G. C. (2010). The natural history of multiple sclerosis, a geographically based study 10: relapses and long-term disability. Brain, 133(7), 1914-1929. https://doi.org/10.1093/brain/awq118
6. Scalfari, A., Neuhaus, A., Däumer, M., DeLuca, G. C., Muraro, P. A., & Ebers, G. C. (2013). Early relapses, onset of progression, and late outcome in multiple sclerosis. JAMA Neurology, 70(2), 214. https://doi.org/10.1001/jamaneurol.2013.599
7. Chitnis, T., Tardieu, M., Amato, M. P., Banwell, B., Bar-Or, A., Ghezzi, A., … & Wassmer, E. (2013). International pediatric ms study group clinical trials summit. Neurology, 80(12), 1161-1168. https://doi.org/10.1212/wnl.0b013e318288694e
8. Bar-Or, A., Pachner, A. R., Menguy-Vacheron, F., Kaplan, J., & Wiendl, H. (2014). Teriflunomide and its mechanism of action in multiple sclerosis. Drugs, 74(6), 659-674. https://doi.org/10.1007/s40265-014-0212-x
9. Wostradowski, T., Prajeeth, C. K., Gudi, V., Kronenberg, J., Witte, S., Brieskorn, M., … & Stangel, M. (2016). In vitro evaluation of physiologically relevant concentrations of teriflunomide on activation and proliferation of primary rodent microglia. Journal of Neuroinflammation, 13(1). https://doi.org/10.1186/s12974-016-0715-3
10. Ambrosius, B., Faissner, S., Guse, K., Lehe, M. v., Grünwald, T., Gold, R., … & Chan, A. T. (2017). Teriflunomide and monomethylfumarate target hiv-induced neuroinflammation and neurotoxicity. Journal of Neuroinflammation, 14(1). https://doi.org/10.1186/s12974-017-0829-2
11. Ciardi, M. R., Zingaropoli, M. A., Pasculli, P., Perri, V., Tartaglia, M., Valeri, S., … & Mastroianni, C. M. (2020). The peripheral blood immune cell profile in a teriflunomide-treated multiple sclerosis patient with covid-19 pneumonia. Journal of Neuroimmunology, 346, 577323. https://doi.org/10.1016/j.jneuroim.2020.577323
12. Ochoa-Repáraz, J., Colpitts, S. L., Kircher, C., Kasper, E., Telesford, K., Begum-Haque, S., … & Kasper, L. H. (2016). Induction of gut regulatory cd39 + t cells by teriflunomide protects against eae. Neurology Neuroimmunology &Amp; Neuroinflammation, 3(6). https://doi.org/10.1212/nxi.0000000000000291
13. Tilly, G., Cadoux, M., García, A., Morille, J., Wiertlewski, S., Pecqueur, C., … & Degauque, N. (2021). Teriflunomide treatment of multiple sclerosis selectively modulates cd8 memory t cells. Frontiers in Immunology, 12. https://doi.org/10.3389/fimmu.2021.730342
14. Zmira, O., Gofrit, S. G., Aharoni, S. A., Weiss, R., Shavit-Stein, E., & Chapman, J. (2022). Teriflunomide normalizes anti-anxiety effect in anti-anxa2 aps mice model teriflunomide in anti-anxa2 mice model. Lupus, 31(7), 855-863. https://doi.org/10.1177/09612033221095150
15. Prabhakara, K. S., Kota, D. J., Jones, G., Srivastava, A. K., Cox, C. S., & Olson, S. D. (2018). Teriflunomide modulates vascular permeability and microglial activation after experimental traumatic brain injury. Molecular Therapy, 26(9), 2152-2162. https://doi.org/10.1016/j.ymthe.2018.06.022
16. Wolinsky, J. S., Narayana, P. A., Nelson, F., Datta, S., O'Connor, P., Confavreux, C., … & Freedman, M. S. (2013). Magnetic resonance imaging outcomes from a phase iii trial of teriflunomide. Multiple Sclerosis Journal, 19(10), 1310-1319. https://doi.org/10.1177/1352458513475723
17. Papp, V., Buron, M. D., Siersma, V., Rasmussen, P. V., Illés, Z., Kant, M., … & Magyari, M. (2021). Real-world outcomes for a complete nationwide cohort of more than 3200 teriflunomide-treated multiple sclerosis patients in the danish multiple sclerosis registry. Plos One, 16(5), e0250820. https://doi.org/10.1371/journal.pone.0250820

18. Nwankwo, E., Allington, D., & Rivey, M. P. (2012). Emerging oral immunomodulating agents – focus on teriflunomide for the treatment of multiple sclerosis. Degenerative Neurological and Neuromuscular Disease, 15. https://doi.org/10.2147/dnnd.s29022