Journal of Applied Pharmaceutical Science Vol. 13(Suppl 1):), pp 028-033, September, 2023
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2023.129159
ISSN 2231-3354

Development of a validated RP-HPLC assay method for quantitative separation of Teriflunomide and its process-related impurities in bulk drugs

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ARTICLE INFO

Received on: 21/03/2023
Accepted on: 17/06/2023
Available Online: 20/09/2023

Key words:
Related impurities, RP-HPLC, Teriflunomide, method development.

ABSTRACT

The organic, inorganic, and residual solvent impurity sources in pharmacological compounds have been divided into categories by the International Council for Harmonization. The pharmaceutical sector faces a regulatory hurdle since the organic contaminants could be genotoxins. The detection and method development also a validation of organic contaminants produced during the chromatographic separation of a teriflunomide is the main goal of this work. The impurity profile research was carried out using a diode array detector and reverse phase-high performance liquid chromatography. At a column temperature of 25°C, the C18 YMC-Pack ODS column was successfully achieved through gradient separation. As the mobile phase, acetonitrile and 0.015 M potassium dihydrogen phosphate with a pH of 3.5 were employed. A 210 nm detector wavelength and 1.0 ml/minute flow rate were adopted. Six process-related impurities were successfully separated using the validated analytical method, with resolution and retention times under 35 minutes. Teriflunomide, Teriflunomide stage-1, and Impurity-D have established analytical techniques with ranges of 0.066–3.262, 0.035–1.880, and 0.025–1.255 µg/ml, respectively. Teriflunomide, Teriflunomide stage-1, and impurity-D have respective limit of detection and limit of quantification values of 0.0037 and 0.0096, 0.0016 and 0.0051, and 0.0011 and 0.0033 µg/ml. The confirmed analytical approach can effectively identify any manufacturing process impurities.

INTRODUCTION

Teriflunomide (TFM) is the main active metabolite of leflunomide, a medication used to treat rheumatoid arthritis, is TFM. TFM's mode of action is not precisely known. It primarily inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, which is involved in the synthesis of pyrimidines from scratch. As a result, it restricts the growth of activated T cells and B cells and reduces lymphocyte migration to the central nervous system. The suppression of protein tyrosine kinases and cyclooxygenase-2 are two additional immunological effects of TFM that are hypothesized to exist in addition to the inhibition of pyrimidine production. It takes almost 3 months to reach steady-state concentration when taken orally, and oral bioavailability is close to 100%. TFM is part of a class of immunomodulatory drugs that work by preventing the formation of pyrimidines. This medication is effective in the treatment of rheumatoid arthritis and multiple sclerosis (European medicines agency, EMA/529295/2013, 2013). TFM is a white, flavorless, and odorless material that is non-hygroscopic. TFM's

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chemical name is (Z)-2-cyano-3-hydroxy-N-[4-(trifluoromethyl) phenyl] but-2-enamide, with a molecular weight of 270.2 g/mol and a chemical formula of $C_{12}H_9N_2O_2F_3$.

A comprehensive study of the literature indicated that there were no prior reports for the TFM impurity profiling studies. We found a reliable LC-MS technique for measuring TFM and its metabolite under *in-vivo* circumstances (Parekh *et al*., 2010). For the simultaneous quantification of TFM and methotrexate in nanoparticles and formulations, a validated high performance liquid chromatography (HPLC) method was created (Pandey *et al*., 2018). For the simultaneous quantification of TFM and other chemicals, *in-vivo* validated LC-UV and LC-MS analytical procedures were created (Suneetha *et al*., 2016). For the purpose of quantifying TFM in human bodily fluids, a validated LC-MS/MS method was created (Rakhila *et al*., 2011; Rule *et al*., 2019). The QbD-based validated UPLC method that Nukendra *et al*. (2017) established forced degradation products that were described using standards. The two named degradation products of TFM are N-[4-(trifluoromethyl)phenyl]-2-cyanoacetamide and 4-trifluoromethyl aniline. Additionally, the published publications using spectroscopic and chromatographic methods were referred to Lakshmi *et al*. (2015); Srinivasa *et al*. (2015); and Vidyadhara *et al*. (2013).

The present proposed assay method describes a reversed-phase HPLC for a quantitative separation and determination of TFM, and its impurities (Fig. 1). The developed method was validated and found to be suitable for the quality assessment of TFM in pharmaceutical bulk drugs and formulations. The validation of the analytical method was done according to the guidelines of ICH (ICH, 1994).

MATERIAL AND METHODS

Chemicals and reagents

The working standard TFM and its related impurity standards are the following: a) TFM stage-1: 5-methyl-N-[4-(trifluoromethyl)phenyl]-1,2-oxazole-4-carboxamide; b) Impurity-D(Imp-D): 5-methyl-1,2-oxazole-4-carboxylic acid; c) Impurity-A(Imp-A): 4-(trifluoromethyl)aniline; d) Impurity-C(Imp-C): 5-methyl-N-[3-(trifluoromethyl) phenyl]-1,2-oxazole-4-carboxamide; e) Impurity-F(Imp-F): 5-methyl-N-[2-(trifluoromethyl) phenyl]-1,2-oxazole-4-carboxamide; ***and*** f) Impurity-G(Imp-G): 5-methyl-N-(4-methylphenyl)-1,2-oxazole-4-carboxamide) were provided as a gift sample by a Biophore India Pharmaceuticals Pvt. Ltd.

Instrumentation

The chromatographic analysis was carried out using the Empower chromatographic software on an HPLC Waters Alliance 2695 system with a photo diode array detector of type 2998. Other tools included a pH meter and an analytical balance from Japan's Shimadzu (Elico, LI-120).

Chromatographic conditions

The YMC-Pack ODS C18, of make YMC, Japan, with specifications of column length 25 cm, internal diameter 4.0 mm, and particle size 5.0 µm, was used to develop the reverse phase (RP)-HPLC process. The column temperature was set at 25°C, the mobile phase flow rate used was at 1.0 ml/minute, the amount of sample injected was 10 l, and the run time for analysis was 50 minutes. KH_2PO_4 with a pH of 3.5 was used to create the aqueous mobile phase component (A). To change the buffer's pH, orthophosphoric acid in diluted form was utilized. Acetonitrile was the deployed organic mobile phase component (B). For mobile solvents, the gradient elution program was tuned as follows: (Tminute/ B% solution): 0–0/35, 0–12/35, 12–32/55, 32–42/65, 42–45/35, and 45–50/35. The diluent was made by combining acetonitrile with milli-Q grade water in a 50:50 ratio.

Method development

The optimized method was carried out on a C18 column of 25 cm × 4 mm × 5 µm, with an organic phase of acetonitrile

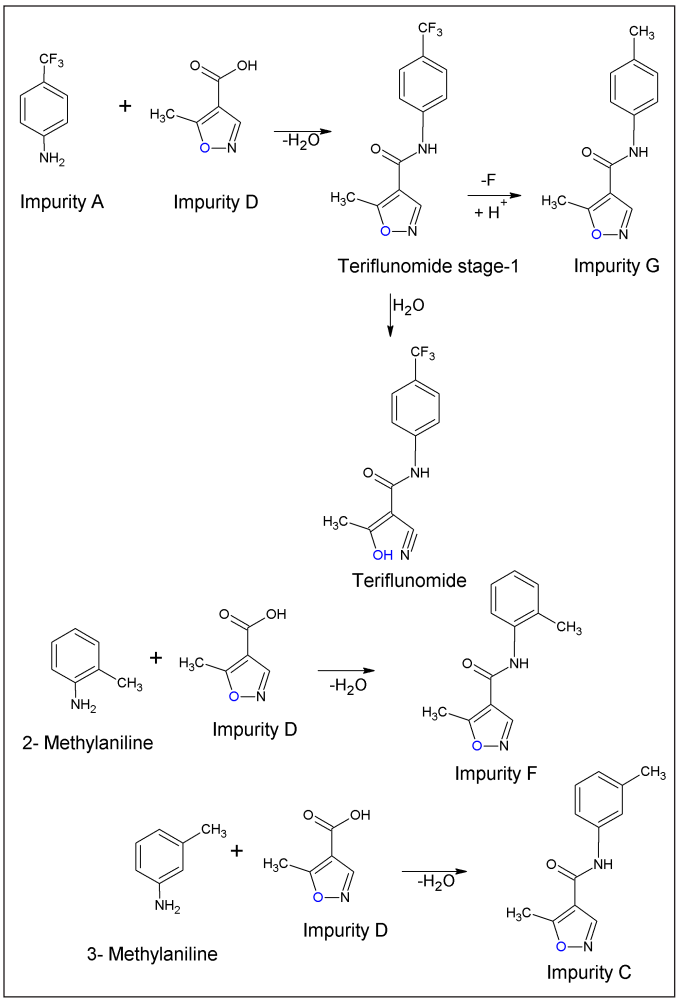

Figure 1. The scheme of reactions involved in the synthesis of TFM and its related impurities

Table 1. System suitability parameters.

Parameter	% RSD of peak area in standard solution		
	Imp-D	TFM	TFM stage-1
Specificity	2.7	4.05	3.09
Method precision	0.32	2.80	1.11
Intermediate precision	3.08	1.04	0.68
Linearity	0.40	0.73	0.47

(B) and a mobile phase of 0.015 M KH_2PO_4 buffer, pH 3.5, using a gradient elution program (Tminute/ B% solution) of 0–0/35, 0–12/35, 12–32/55, 32–42/65, 42–45/35, and 45–50/35. The initial composition of B in the solution was 35% up to 12 minutes, then it increased gradiently from 35% to 55% at minutes 12 to 32, then it increased gradiently from 55% to 65% at minutes 32 to 42, then it decreased gradiently from 65% to 35% at minutes 42 to 45, then it remained constant at minutes 45 to 50. Other parameters that were optimized and comprised column temperature of 25°C, a flow rate of 1 ml/minute, and a UV detection wavelength of 210 nm. The tailing factor was less than 1.5, the retention period of TFM and its process-related impurities were under 35 minutes, and there were more than 2,000 theoretical plates legitimately seen.

Preparation of solutions

Diluent preparation

The diluent was constituted by mixing milli-Q grade water and acetonitrile in a ratio of 50:50.

Standard preparation

Prepared a standard solution in a 100 ml calibrated flask containing 0.50 µg/ml of Imp-D, 0.75 µg/ml of TFM Stage-1, and 0.50 µg/ml of TFM with a diluent.

Sample preparation

Prepared 500 µg/ml sample solution by weighing 50.0 mg of the test substance and made upto 100.0 ml in a calibrated flask. Duplicates of the sample solution were made.

Spiked sample preparation

The spiked sample solution was prepared with 500 µg/ml TFM and 0.25 µg/ml of each specified impurity TFM stage-1, Imp-D, Imp-C, Imp-F, Imp-G, Imp-A in the diluent.

System suitability

After performing injections in the following order: blank twice (n = 2), standard solution six times (n = 6), duplicate samples twice (n = 2), and recording the chromatograms, the system compatibility of the instrument was determined. Peaks

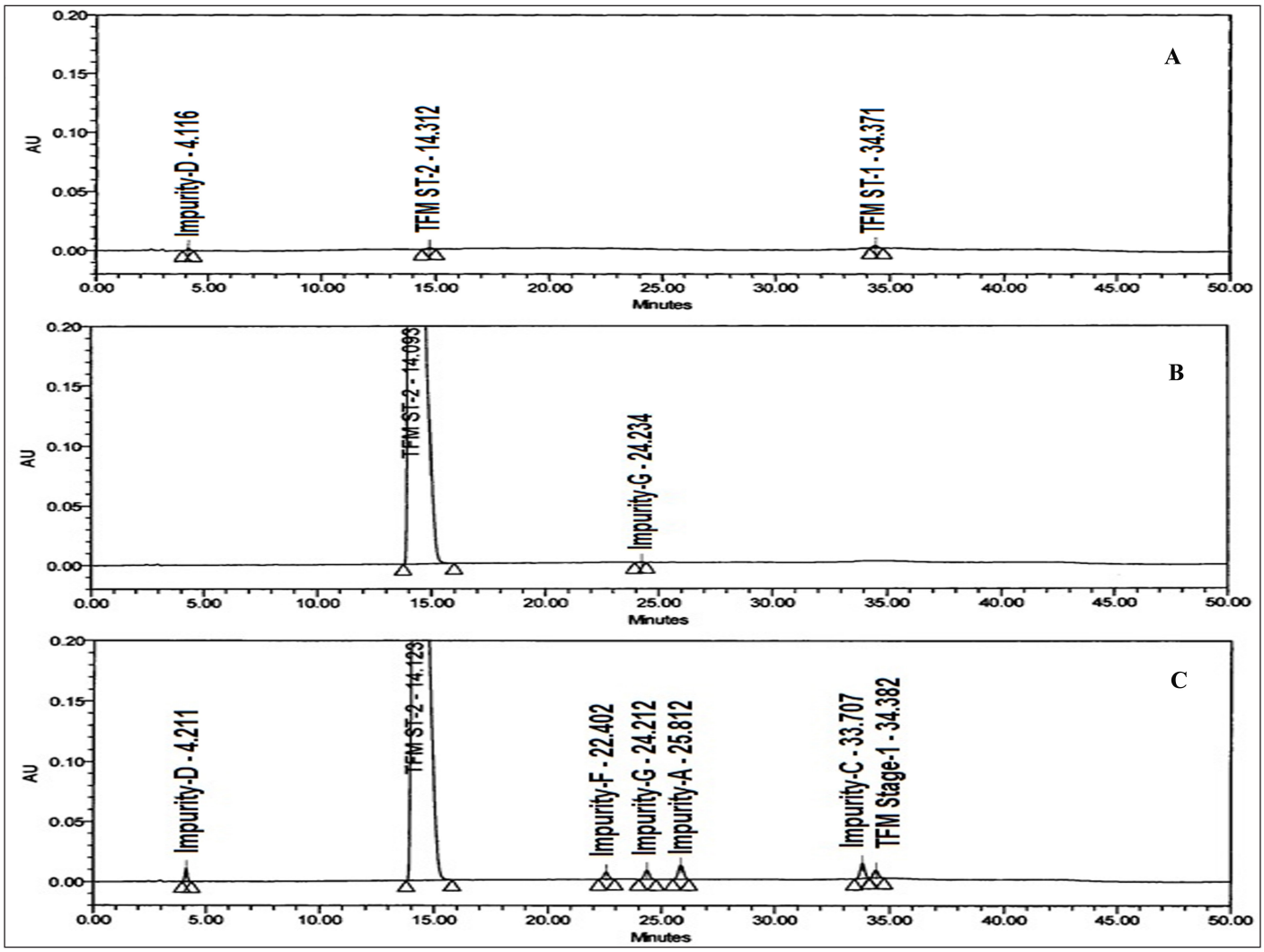

Figure 2. Typical RP-HPLC chromatograms of (A) standard, (B) sample, and C) spiked sample.

found during the blank injection were ignored. The percent relative standard deviation (RSD) from six replicate injections of standard solution should not exceed 5.00% for each specified impurity and TFM peak area.

RESULTS AND DISCUSSION

The chromatograms were recorded along with the characteristics of the instrument's system adequacy. For TFM, Imp-D, and TFM stage-1 in six replicate injections to examine the system adequacy of analytical instrument, the% RSD for peak area was less than five. Table 1 presents the results in tabular form. TFM and its associated process impurities (TFM stage-1, Imp-D, Imp-C, Imp-F, Imp-G, Imp-A) were produced as independent 100 µg/ml sample solutions. Prepared would have been the spiked sample solution, which contained 0.25 µg/ml of each specified impurity in addition to 500 µg/ml of TFM in the diluent.

The chromatographic method was used to analyse the chromatograms after injecting both the standard sample solutions and the spiked sample solutions. This study established that the Imp-D, TFM stage-1, and TFM peaks could be sufficiently separated from one another. In the chromatograms, there was no interference during the retention times for TFM, Imp-D, and TFM stage-1 (Fig. 2). The methodology is selective in how it identifies TFM-related substances. Six replicate injections were used to establish the precision utilising a 100% working standard concentration of TFM and its designated impurities. For TFM, Imp-D, and TFM stage-1, the %RSD for the peak region during system precision was 0.61, 0.27, and 0.81, respectively.

At the 100% working concentration, the %RSD for the Imp-D, TFM stage-1, and total impurities obtained using the technique were 2.27, 2.52, and 1.56, respectively. Table 2 displays the results of intermediate precision. For TFM and each prescribed impurity solution, ranging from limit of quantification (LOQ) to 250.0% of the working concentration, the linearity was demonstrated. With a correlation coefficient (R^2) of 0.999, the equation for the linearity curve for TFM from the range of 0.06–3.252 µg/ml, Imp-D from the range of 0.025–1.25 µg/ml, and TFM stage-1 from range 0.035–1.88 µg/ml was $Y = 34{,}669X - 37.00$, $34{,}281X - 16.95$, and $37{,}419X + 64.76$, respectively. Table 3 depicts the linearity data.

While providing solution comprising TFM spiked with Imp-D and TFM stage-1 at 50.0%, 100.0%, 150.0%, and 250% of the working concentration, the method's accuracy was evaluated. For each analyte, three measurements were made at each accuracy level. TFM stage-1, Imp-D, and TFM stage-1 percentage recovery results ranged from 98.31% to 102.4%, 98.20% to 103.26%, and 97.61% to 100.39%, respectively. Data on linearity and accuracy were used to establish the analytical method's range. The range, in terms of sample concentration, will be around LOQ to 250%. The developed analytical methods' sequential ranges for TFM, TFM stage-1, and Imp-D were 0.066–3.262, 0.035–1.880, and 0.025–1.255 µg/ml.

The limit of detection solution was produced and injected based on the average S/N ratio established from the 0.05% level of the solution. About 5.8:1 for TFM (0.0037 µg/ml), 4.4:1 for Imp-D (0.0011 µg/ml), and 4.3:1 for TFM stage-1 (0.0016 µg/ml) were determined as the S/N ratio values. The LOQ solution was made and injected based on the average S/N ratio obtained from

Table 2. Summary of results for intermediate precision.

Sample name	Analyst-I /day -I /instrument-I/column-I			Analyst-II /day-II / instrument-II/ column-II		
	Imp-D (%w/w)	TFM stage-1 (%w/w)	Total impurity (%w/w)	Imp-D (%w/w)	TFM stage-1 (%w/w)	Total impurity (%w/w)
Spiked Sample-1	0.10	0.15	0.26	0.10	0.15	0.26
Spiked Sample-2	0.10	0.15	0.26	0.10	0.16	0.27
Spiked Sample-3	0.10	0.15	0.26	0.10	0.16	0.27
Spiked Sample-4	0.09	0.15	0.25	0.09	0.15	0.26
Spiked Sample-5	0.10	0.14	0.25	0.10	0.15	0.26
Spiked Sample-6	0.10	0.15	0.26	0.10	0.15	0.26
Average (% w/w)	0.10	0.15	0.26	0.10	0.15	0.26
SD	0.0041	0.0063	0.0048	0.0041	0.0052	0.0075
%RSD	4.15	4.2	1.89	4.15	3.37	2.88

Table 3. Linearity for Imp-D, TFM and TFM stage-1.

Level	Imp-D		TFM		TFM stage-1	
	Conc. µg/ml	Peak area	Conc. (µg /ml)	Peak area	Conc. (µg /ml)	Peak area
LOQ	0.025	739	0.06	2,860	0.035	1,410
30 %	0.15	4,947	0.15	6,866	0.23	8,436
50%	0.25	8,692	0.25	11,498	0.38	14,476
100%	0.50	17,302	0.50	23,092	0.75	28,598
150%	0.75	25,789	0.75	33,910	1.13	41,688
250%	1.25	42,702	1.25	56,602	1.88	70,612

Table 4. Robustness study.

% RSD of Peak area in standard solution			
	Imp-D	**TFM**	**TFM stage-I**
Actual Chromatographic conditions	2.00	1.34	2.96
Variation-1, (Tminute/B% solution): 0–0/33, 0–12/33, 12–42/63, 42–45/33, 45–50/33	4.21	3.6	2.41
Variation-2, (Tminute/B % solution): 0–0/37, 0–12/37, 12–42/67, 42–45/37, 45–50/37	1.67	2.52	4.33
Variation-3, Mobile phase-A (buffer) pH: 3.7	3.38	2.46	2.02
Variation-4, Mobile phase-A (buffer) pH: 3.3	3.18	4.09	2.38
Variation-5, Strength of the Potassium dihydrogen phosphate in buffer (0.010 M)	3.27	3.38	1.38
Variation-6, Strength of the Potassium dihydrogen phosphate in buffer (0.020 M)	2.17	2.94	2.34
Variation-7, Flow rate: 1.2 ml/minute	3.07	4.73	4.74
Variation-8, Flow rate: 0.8 ml/minute	4.53	4.01	2.42
Variation-9, Column oven temperature-30°C	3.09	2.64	3.2
Variation-10, Injection volume : 5.0 µl	3.92	3.57	2.18
Variation-11, Injection volume : 15.0 µl	2.11	1.88	2.17

Table 5. TFM sample analysis for related impurities in drug substance.

Sample	**Related impurities(%w/w , %RSD)**					
	Imp-D	**Imp-F**	**Imp-G**	**Imp-A**	**Imp-C**	**TFM Stage-1**
Sample-1	ND	ND	0.023, 6.45	ND	ND	ND
Sample-2	ND	ND	0.054, 5.62	ND	ND	ND
Sample-3	ND	ND	0.045,5.87	ND	ND	ND

ND: Not detected.

the 0.05% level solution. measured the LOQ solution's S/N ratio. The S/N ratio values for TFM (0.0096 µg/ml), Imp-D (0.0033 µg/ml), and TFM stage-1 (0.0051 µg/ml) were found to be around 14.8:1, 13.2:1, and 13.8:1, respectively.

A sample study was performed to see whether these intended changes had a discernible impact on the %RSD. The level of each specified impurity at each variation was checked using an analysis of a sample of TFM spiked with the Imp-D and TFM Stage-I. The results are presented in Table 4, and data analysis showed the validity of the suggested analytical procedure. Three batches of TFM drug material were tested using the suggested approach for the presence of related compounds. The findings showed that Imp-G was present in concentrations between 0.02% and 0.05%. The associated substances' %RSD with triplicate determinations was less than 10%. Table 5 summarizes the results.

CONCLUSION

The present proposed assay method was assessing, identifying, and qualifying the process-related impurities in TFM drug substance, a precise, specific, accurate, and reliable RP-HPLC technique was established. The procedure underwent validation in accordance with ICH recommendations. The approach has been proven accurate when it came to identifying related substances in TFM drugs and pharmaceutical constituents. Finally, the developed assay method was successfully applied to pharmaceutical industries for the quantitative separation of TFM and its process-related impurities in bulk drugs as well as formulations.

LIST OF ABBREVIATION

HPLC: high performance liquid chromatography; LOQ: limit of quantification; RSD: relative standard deviation; TFM: Teriflunomide.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FINANCIAL SUPPORT

There is no funding to report.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Koppisetty BRB, Yejella RP, Pawar AKM, Yarraguntla SR, Kollabathula VR, Dadi V, Naidu CG. Development of a validated RP-HPLC assay method for quantitative separation of Teriflunomide and its process related impurities in bulk drugs. J Appl Pharm Sci, 2023; 13(Suppl 1):028–033.