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ORIGINAL ARTICLE / ÖZGÜN MAKALE

QUALITY BY DESIGN ASSISTED RP-HPLC METHOD FOR ESTIMATION OF TERIFLUNOMIDE AND ITS PROCESS IMPURITIES IN DRUG SUBSTANCE

TERİFLUNOMİD VE İLAÇ MADDESİNİN ÜRETİMİNDEKİ SAFSIZLIKLARININ TAHMİNİ İÇİN TASARIMLA KALİTE DESTEKLİ RP-HPLC YÖNTEMİ

Vasudha DADI[1], Saritha MEDAPATI[2], Jagadeesh Kumar BARATAM[2], Hemant Kumar TATAPUDI[4], Gangu Naidu CHALLA[3], Srinivasa Rao YARRAGUNTLA[1], Bhagavan Rajesh Babu KOPPISETTY[1]*

[1]Department of Pharmaceutical Analysis, Vignan Institute of Pharmaceutical Technology, Visakhapatnam, Andhra Pradesh, 530046, India
[2]Department of Pharmacy Practice, Vignan Institute of Pharmaceutical Technology, Visakhapatnam, Andhra Pradesh, 530046, India
[3]Department of Basic Sciences and Humanities (BS&H), Division of Chemistry, Vignan's Institute of Information Technology VIIT(A), Visakhapatnam, Andhra Pradesh, 530046, India
[4]Department of Pharmaceutical Technology, NSHM Knowledge campus, Kolkata, West Bengal, 700053, India

ABSTRACT

Objective: *In this research, a validated RP-HPLC method for analyzing teriflunomide drug substance and its associated process-related impurities was developed with the assistance of the Quality by Design (QbD) approach.*
Material and Method: *The QbD methodology employs statistical design of experiments to establish a robust method within a defined "design space." This design space outlines the experimental parameters' range within which alterations will not significantly impact the results. Chromatographic separation was done on HPLC system connected to a PDA detector, and the column used was the C18 YmC-Triart with specifications of 125 cm x 4.0 mm x 5.0 µm. The optimized mobile phase consisted of 0.5% triethylamine buffer with pH 4.0 (± 0.05) and acetonitrile in a 65:35 v/v ratio, flow rate of 1.0 ml/min. Detection wavelength of 210 nm.*
Result and Discussion: *The developed RP-HPLC method successfully achieved high resolution, specificity, linearity, precision, accuracy, and robustness in quantifying both teriflunomide and its impurities simultaneously. Using a design of experiments (DoE) approach, critical method parameters were systematically identified and optimized, ensuring accurate and precise determination of impurity levels across the drug substance lifecycle. This validated method provides a thorough approach to ensuring the quality and safety of teriflunomide drug substances by delivering reliable data on impurity profiles. By applying Quality by Design (QbD) principles, not only does the method enhance understanding of the analytical process, but it also supports ongoing improvement and lifecycle management of the procedure.*
Keywords: *Method development, QbD, related impurities, RP-HPLC, teriflunomide*

* **Corresponding Author / Sorumlu Yazar:** Bhagavan Rajesh Babu Koppisetty
e-mail / e-posta: koppisettybrbabu@gmail.com, **Phone / Tel.:** +919490597959

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ÖZ

Amaç: *Bu araştırmada, teriflunomid ilaç maddesini ve onun süreçi ile ilişkili safsızlıkları Tasarımla Kalite (QbD) yaklaşımı yardımıyla analiz etmek için doğrulanmış bir RP-HPLC yöntemi geliştirildi.*
Gereç ve Yöntem: *QbD metodolojisi, tanımlanmış bir "tasarım alanı" dahilinde sağlam bir yöntem oluşturmak için deneylerin istatistiksel tasarımını kullanır. Bu tasarım alanı, değişikliklerin sonuçları önemli ölçüde etkilemeyeceği deneysel parametre aralığının ana hatlarını çizer. Kromatografik ayırma, bir PDA detektörüne bağlı bir HPLC sistemi üzerinde gerçekleştirildi ve kullanılan kolon, 125 cm x 4.0 mm x 5.0 µm spesifikasyonlarına sahip C18 YmC-Triart'tı. Optimize edilmiş mobil faz, pH 4.0 (± 0.05) olan %0.5 trietilamin tamponundan ve 65:35 v/v oranında, 1.0 ml/dak akış hızında asetonitrilden oluşuyordu. 210 nm algılama dalga boyu.*
Sonuç ve Tartışma: *Geliştirilen RP-HPLC yöntemi, hem teriflunomidin hem de onun safsızlıklarının aynı anda ölçülmesinde yüksek çözünürlük, özgüllük, doğrusallık, kesinlik, doğruluk ve sağlamlığı başarıyla elde etti. Bir deney tasarımı (DoE) yaklaşımı kullanılarak, kritik yöntem parametreleri sistematik olarak tanımlandı ve optimize edildi; böylece ilaç maddesi yaşam döngüsü boyunca safsızlık seviyelerinin doğru ve kesin olarak belirlenmesi sağlandı. Doğrulanmış bu yöntem, safsızlık profilleri hakkında güvenilir veriler sunarak teriflunomid ilaç maddelerinin kalitesini ve güvenliğini sağlamaya yönelik kapsamlı bir yaklaşım sağlar. Tasarımla Kalite (QbD) ilkelerini uygulayarak, yöntem yalnızca analitik sürecin anlaşılmasını geliştirmekle kalmaz, aynı zamanda prosedürün sürekli iyileştirilmesini ve yaşam döngüsü yönetimini de destekler.*

Anahtar Kelimeler: *İlgili safsızlıklar, QbD, RP-HPLC, teriflunomid, yöntem geliştirme*

INTRODUCTION

Teriflunomide (TFN), the primary active metabolite of leflunomide, is a drug commonly employed for the management of rheumatoid arthritis. Its main mechanism involves the inhibition of mitochondria's dihydroorotate dehydrogenase, an important enzyme in the *de novo* synthesis of pyrimidines. This inhibition results in the suppression of activated T type cells and B type cells and reduces the movement of lymphocytes to the central nervous system. TFN is also believed to have other immunological effects, including the suppression of cyclooxygenase-2 and protein tyrosine kinases. TFN belongs to a immunomodulatory drugs that function by impeding pyrimidine formation. It has proven efficient in the treatment of multiple sclerosis and rheumatoid arthritis. Physically, TFN is a white, tasteless, and odorless substance that does not readily absorb moisture. Its chemical name is "(*Z*)-2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl)phenyl]but-2-enamide", and it has a molecular weight of 270.2 g/mol with a chemical formula of $C_{12}H_9N_2O_2F_3$.

By literature review we found that a validated LC-MS method for quantification of Teriflunomide and its metabolite in in-vivo conditions [1]. A validated HPLC technique was established for the quantification of methotrexate and TFN in formulations [2]. A validated LC-MS method was developed for estimation of TFN and other compounds [3]. A validated LC-MS/MS method was developed for estimation of TFN in human biological fluids [4-5]. Koppisetty et al., 2023 has developed a HPLC method for quantitative separation of TFN and its process impurities in drug substance [6]. Nukendra et al., 2017 had developed QbD based validated UPLC method, which reported forced degradation products and those degradation products were characterized using standards [7]. Suneetha et al., [8] has worked on separation of TFN with a single unknown process related impurity, the work of suneetha et. al is not suitable for determination of all process related impurities of TFN. Literature review shows that there is no work on a validated RP-HPLC method for impurity profiling in TFN.

The primary purpose of this study is to systematically investigate and develop a reliable RP-HPLC method for quantifying TFN in the presence of its distinct production related impurities. In addition, the research outlines the synthetic pathway of these impurities, as depicted in Figure 1. This study adopts an analytical approach based on Quality by Design (QbD) principles [9-11] to accurately determine TFN in the presence of its production related impurities. Furthermore, the analytical method was subjected to validation in accordance with the ICH guidelines [12,13] to ensure its accuracy and reliability.

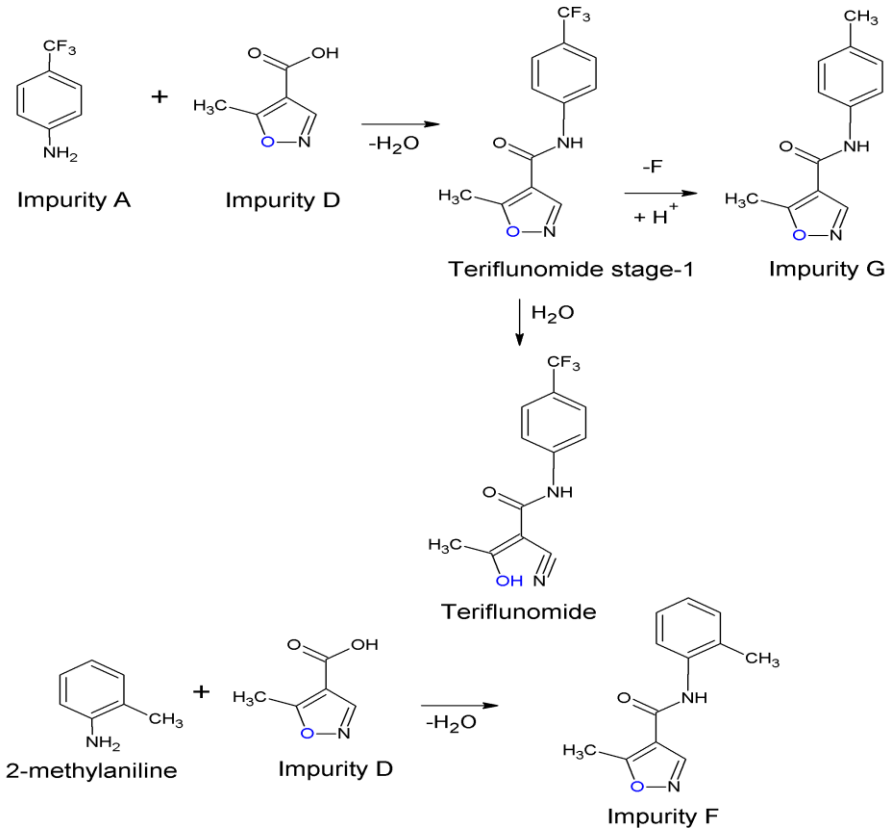

Figure 1. The novel synthetic pathway of TFN and its related impurities

MATERIAL AND METHOD

Chemicals and Reagents

The working standard Terflunomide and its related impurity standards, Impurity-D "(5-methylisoxazole-4-carboxylic acid)",Impurity-F"(5-methyl-*N*-[2-(trifluoromethyl) phenyl]isoxazole-4-carboxamide)", Impurity-G "(5-methy-*N*-(4-methylphenyl) isoxazole-4carboxamide)", Impurity-A "(4-(trifluoromethyl)aniline)" were received as a gift from synpure labs India pvt. Ltd. High-quality analytical grade reagents, including Triethylamine, Acetonitrile (ACN), Orthophosphoric acid (OPA), HCL, NAOH, and H_2O_2 were procured from Merck. Milli-Q grade water was utilized for the analysis.

Analytical Quality by Design

Analytical Target Profile (ATP) and Critical Analytical Attribute (CAA)

Establishing the definition of ATP represents the initial stage in adopting a Quality by Design (QbD) approach. This entails the careful selection of elements, including Critical Method Parameters (CMP) and Critical Analytical Attributes (CAA), to attain the desired ATP. Variations in CMPs can influence CAAs, and as such, they should be closely examined and managed to ensure the intended quality.

Every CMP was categorically assigned as either high or low risk levels, taking into consideration the likelihood of risk occurrence and the seriousness of potential impacts on the CAAs. Potential CAAs were identified among numerous quality attributes using relevant information and from available literature. These attributes, directly impact the analytical method's quality and safety. In this particular study, specific factors such as Relative Standard Deviation (RSD), assay on a dried basis, and tailing factor were designated as CAAs.

Risk Assessment (RA)

The ICH Q8 and Q9 guideline outline a risk-based strategy for defining the Quality Target Product Profile (QTPP), which is equivalent to the ATP. The guidelines place strong emphasis on ensuring that analytical methods are rugged and robust, with a specific focus on RA studies. In the context of method development, RA was conducted to pinpoint the factors with higher risk levels that could impact the process. For this RA study, a Pareto analysis was employed. A Pareto diagram was created to identify the CMPs that have a significant impact on the CAAs) when quantifying TFN.

Design of Experiment (DoE)

A critical phase in the process involved the identification of the CMPs that have an impact on the CAAs, such as RSD, assay on a dried basis, and tailing factor. To achieve improved values for RSD, assay on a dried basis, and tailing factor, a Design of Experiments (DoE) approach was employed. This approach involved varying the flow rate, adjusting the pH of the buffer, and modifying the buffer concentration [9]. Following the initial preliminary studies, a 2^3 total factorial design was employed to optimize the method. A total of eight experiments were conducted to investigate the impact of three experimental variables: pH (X1), flow rate (X2), and buffer concentration (X3) on the CAAs, specifically RSD (Y1), assay on a dried basis (Y2), and tailing factor (Y3), at three different levels. The data generated from these experimental runs were analyzed using Design-ExpertR software (version 11, Stat-Ease, Minneapolis, USA).

Statistical Analysis and Optimization

Statistical analysis and the method optimization were conducted using Design-ExpertR software, specifically version 11 from M/s Stat-Ease in Minneapolis, USA. The results from the eight experimental trials conducted as part of the 2^3 total factorial design underwent Multiple Linear Regression Analysis. ANOVA was carried out, and any observed variations were considered statistically significant if the p-value was below 0.05.

Experimental

Instrumentation

The HPLC Waters Alliance 2695 system was utilized for the analysis, coupled with a PDA model 2998, and the empower chromatographic software. Several other pieces of equipment were utilized for the analysis, including an analytical balance from Shimadzu in Japan, a pH meter (Elico, model LI-120), an oven from Thermolab, and a hot air oven from Heraeus.

Chromatographic Conditions

The RP-HPLC method was created and subjected to validation using a Waters HPLC system in conjunction with a YMC-triart column measuring 125cm x 4.0 mm x 5.0 µm. The method was configured with the following parameters: 1.0 ml/min flow rate, a detection wavelength of 210 nm, analysis at ambient temperature, a 10µl injection volume, and a total run time of 20 minutes. The mobile phase consisted of 0.5% triethylamine buffer with pH adjusted to 4.0 (± 0.05) using diluted OPA. Isocratic elution was employed with the mobile phase composed of a 65:35 v/v ratio of buffer and ACN. Notably, the dilution of both standard and sample solutions was carried out using the mobile phase.

Standard Solution

Approximately 50.0 mg of TFN standard was carefully weighed and transferred into a 100 ml flask. It was then dissolved in approximately 15 ml of ACN and subsequently brought to the mark with diluent to achieve the desired volume. To obtain a standard solution of 50 µg/ml, 10.0 ml of the previously prepared solution was further diluted to 100 ml using the diluent.

Sample Solution

Approximately 50.0 mg of the TFN drug substance was precisely weighed and then transferred into a 100 ml flask. It was dissolved using around 15 ml of ACN and then brought up to the mark by

adding the diluent to reach the desired volume. Subsequently, a 10.0 ml aliquot of the API sample solution was further diluted to 100 ml with the diluent, resulting in a 50 µg/ml API sample solution. This process of preparing the solution of drug substance was carried out in duplicate for subsequent analysis.

System Suitability Solution

To assess the HPLC system's performance, six consecutive injections were made, starting with a blank injection, followed by standard solution injections. The system is considered acceptable for analysis under the following conditions: a) theoretical plate count of at least 2000. b) The tailing factor should be between 0.8 and 1.5. c) The RSD% for the TFN peak area for six replicate injections should be below 2%.

Validation of Method

Specificity

To validate the analytical method's selectivity for determining the assay content in TFN, the following steps were taken: a) We created distinct sample solutions for TFN and its associated impurities (Impurity-D, Impurity-F, Impurity-G, and Impurity-A) in a suitable diluent. These solutions were then prepared and analyzed. b) Additionally, we analyzed duplicate spiked sample solutions, where each specified impurity was added to TFN sample solutions to demonstrate the method's selectivity.

Precision

System precision was assessed by injecting a standard TFN solution at a concentration of 50 µg/ml, conducting six determinations to ensure the system's suitability. Method precision was determined by analyzing a standard TFN solution at a concentration of 50 µg/ml through six replicate determinations to measure assay accuracy. Intermediate precision was evaluated by examining the variability in results obtained for TFN. This was accomplished by analyzing six replicate sample preparations, all prepared at the working concentration, with different analysts using different instruments on separate days.

Linearity

To establish the method's linearity, TFN solutions spanning the range of 35 µg/ml to 60 µg/ml, corresponding to 70.0% to 120.0% of the TFN working concentration, were employed. Sample solutions of TFN within this specified range were prepared and subjected to duplicate analysis in accordance with the method.

Accuracy

To assess accuracy, samples were prepared at three different concentration levels, and each level's solutions were replicated three times, resulting in a total of nine determinations. These concentrations included 80.0%, 100.0%, and 120.0% of the working concentration.

Robustness

To evaluate the method's robustness in determining the assay, we conducted an analysis of the TFN standard solution while intentionally modifying parameters such as pH, flow rate, and buffer strength. The changes made to the method were used to derive system suitability results, demonstrating the method's robustness.

Solution Stability

Samples of the TFN solution and standard solution, both at 100.0% of the working concentration, were maintained at room temperature (between 22-27°C). The solutions' stability was assessed at three different time points: initially, after 24 hours, and after 48 hours.

RESULT AND DISCUSSION

Quality by Design Paradigm

The QbD paradigm is a comprehensive and technical approach that employs various statistical, economic, and planning tools, as well as risk assessment techniques, to establish a design space, control strategy, and continuous improvement in various industries. QbD is widely used to enhance method robustness, bridge quality gaps, and reduce instances of failure. In this study, an AQbD approach was applied to plan and execute the development of an analytical method for estimating teriflunomide in the presence of process-related impurities. In the QbD framework, the establishment and execution of an ATP were outlined as the primary phase in the development of the analytical method to attain the specified objectives. The ATP provides a systematic framework for the development of a method, guiding the process. It aided in selecting CAAs necessary for quantifying TFN. In order to reduce inaccuracies and pinpoint potential high-risk factors impacting the proposed quantification of TFN in CAAs, a comprehensive risk assessment study was undertaken. A Pareto chart was formulated specifically for this analysis. The Pareto chart is a valuable tool for assessing the significant factors, where effects surpassing the Bonferroni Limit are highly likely to be significant, those exceeding the t-value limit are potentially significant, and effects below the t-value limit are unlikely to be significant. The Pareto chart indicates that each of the chosen factors has significant effects on the selected responses, with their importance ranked in a descending order.: For response 1, X2 > X3 >X2X3>X1X3 (Figure 2). For response 2, X3 > X1>X1X2 (Figure 3). For response 3 only X3 has significant effect as shown in Figure 4. The model's validity was confirmed through the ANOVA using Design Expert software. The equation, enables predictions to be made regarding the response at particular levels of each factor. The equation functions as a useful tool to evaluate the relative influence of the factors by analyzing and comparing their coefficients. If the model's p-value is greater than 0.05, it indicates that the factors have an insignificant effect on the response, signifying the method's robustness. The low standard deviation (expressed as the percentage coefficient of variance, CV) and the satisfactory precision demonstrate a strong correlation between the experimental data and the fitted models. Synergism and antagonism effects are indicated by the positive and negative signs in the equation, respectively. The desirability value, which is closest to 1 as shown in Figure 5, conveys the dependability of the optimization approach. These results revealed that the actual values of the variables align well with the predicted values of the method.

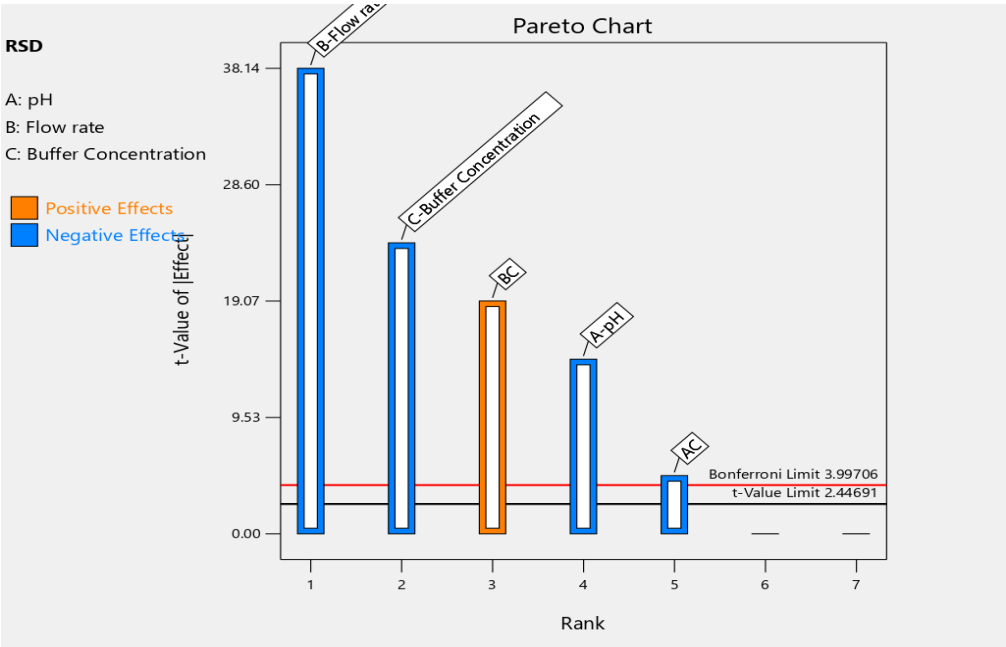

Figure 2. Effect of factors and interaction on responses 1:RSD

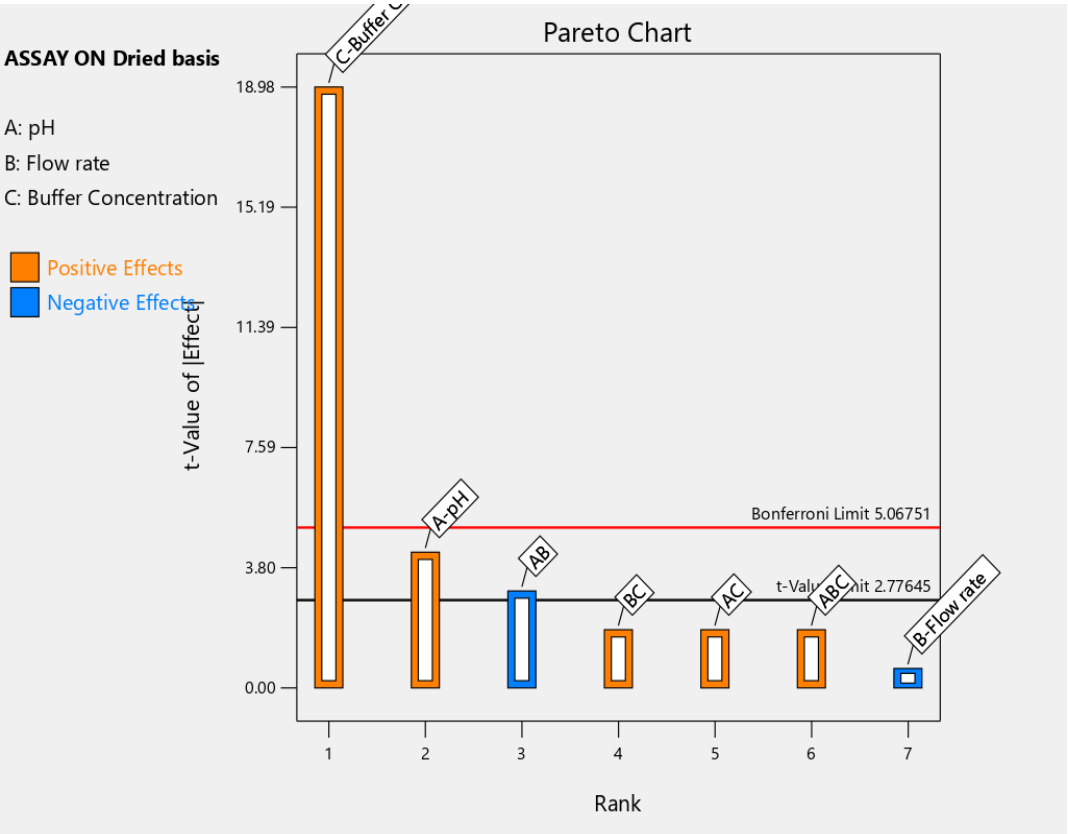

Figure 3. Effect of factors and interaction on response 2: Assay

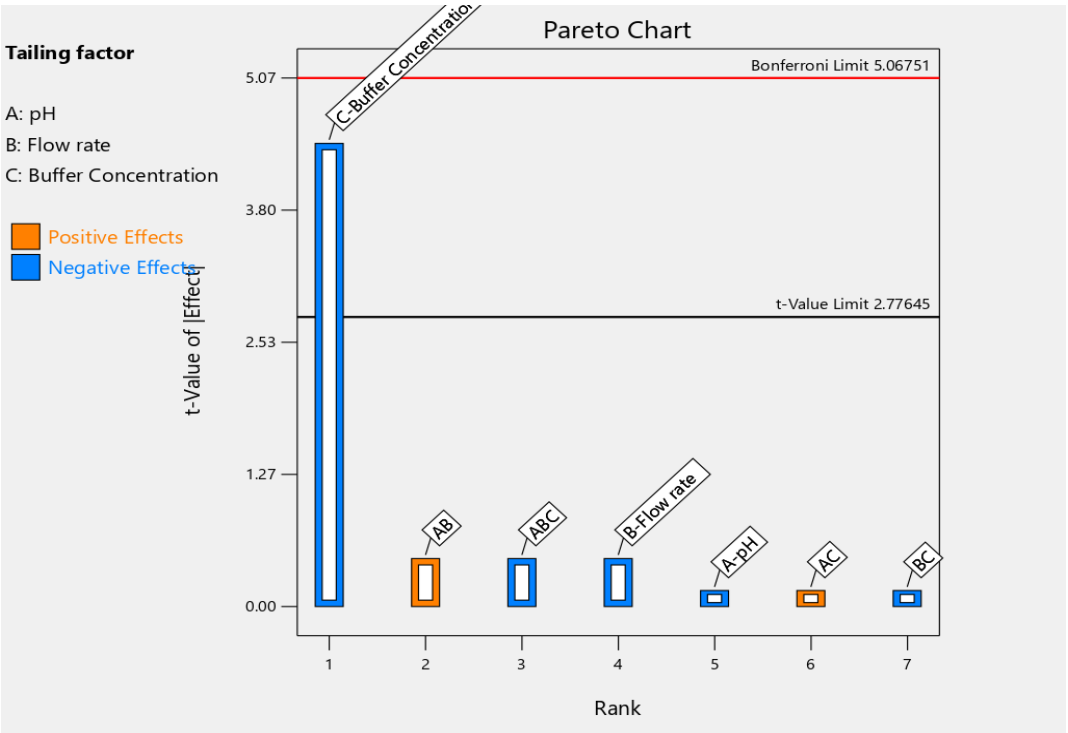

Figure 4. Effect of factors and interaction on responses 3: Tailing factor

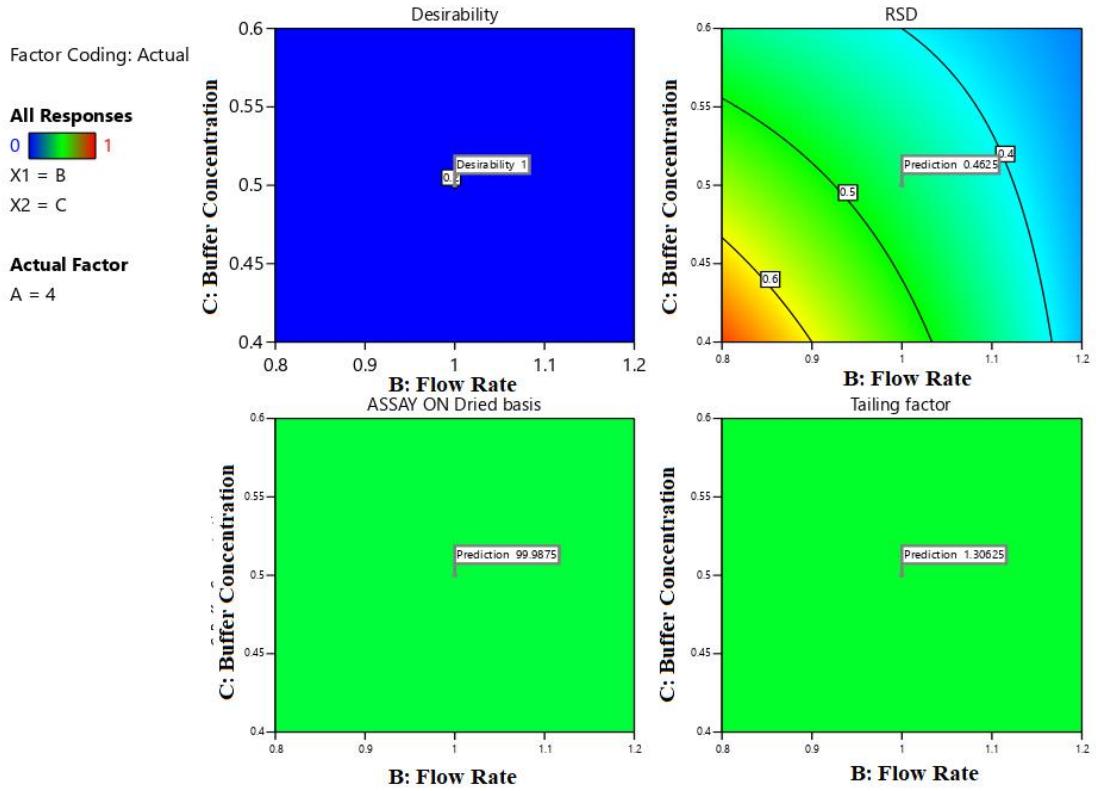

Figure 5. Desirability plot

Development of Analytical Method

A method for estimating the concentration of TFN and its impurities was developed on RP-HPLC. The separation of TFN from its impurities was achieved on a YMC-Triart Hybrid silica-based C18 column with specific dimensions: 125 cm length, 4.0 mm i.d, and 5.0 µm particle size. To separate the compounds, a mobile phase consisting of a 65:35 v/v mixture of buffer and ACN was used. The buffer contained 0.5% Triethylamine and had its pH adjusted to 4.0 (± 0.05) using diluted orthophosphoric acid. Isocratic elution was employed with a 1.0 ml/min flow rate at ambient conditions. Detection of the compounds was performed at a wavelength of 210 nm. A 10 µl sample was injected for analysis, and the total runtime for the procedure was 20 minutes.

Method Validation

System suitability

A standard solution with a concentration of 50 µg/ml was prepared and subjected to the proposed method to evaluate system suitability during tests for specificity. In the first injection of the standard solution, it was observed that the theoretical plates count exceeded 2000 for the parameter being tested. The tailing factor for the TFN peak fell within the range of 1.2 to 1.3 in the first injection of the standard solution for the parameter under examination. The RSD% for the TFN peak area, determined from six replicate injections of the standard solution conducted throughout the run, was found to be 0.62% for the system suitability. The results are tabulated in (Table 1).

Table 1. System suitability parameters

Peak	Retention time (R_T)	Theoritical Plates (N)	HEPT	Tailing Factor	RSD% (n=6)
TFN	5.61	5950	40.728	1.24	0.62

Specificity

There are no interfering peaks in the blank sample at R_T of the analyte. In the standard solution, the TFN peak is uniform, and there are no peaks overlapping with it. This investigation demonstrates that the TFN peak is effectively separated from any known specified impurities. (Figure 6). No interference is observed in the blank at the R_T of the TFN peak. (Table 2). The method was employed on both bulk and spiked samples. The variance in assay values between the sample solution and the spiked sample is -0.3% (Table 3). Hence, the method exhibits selectivity in accurately determining the assay of the TFN drug substance.

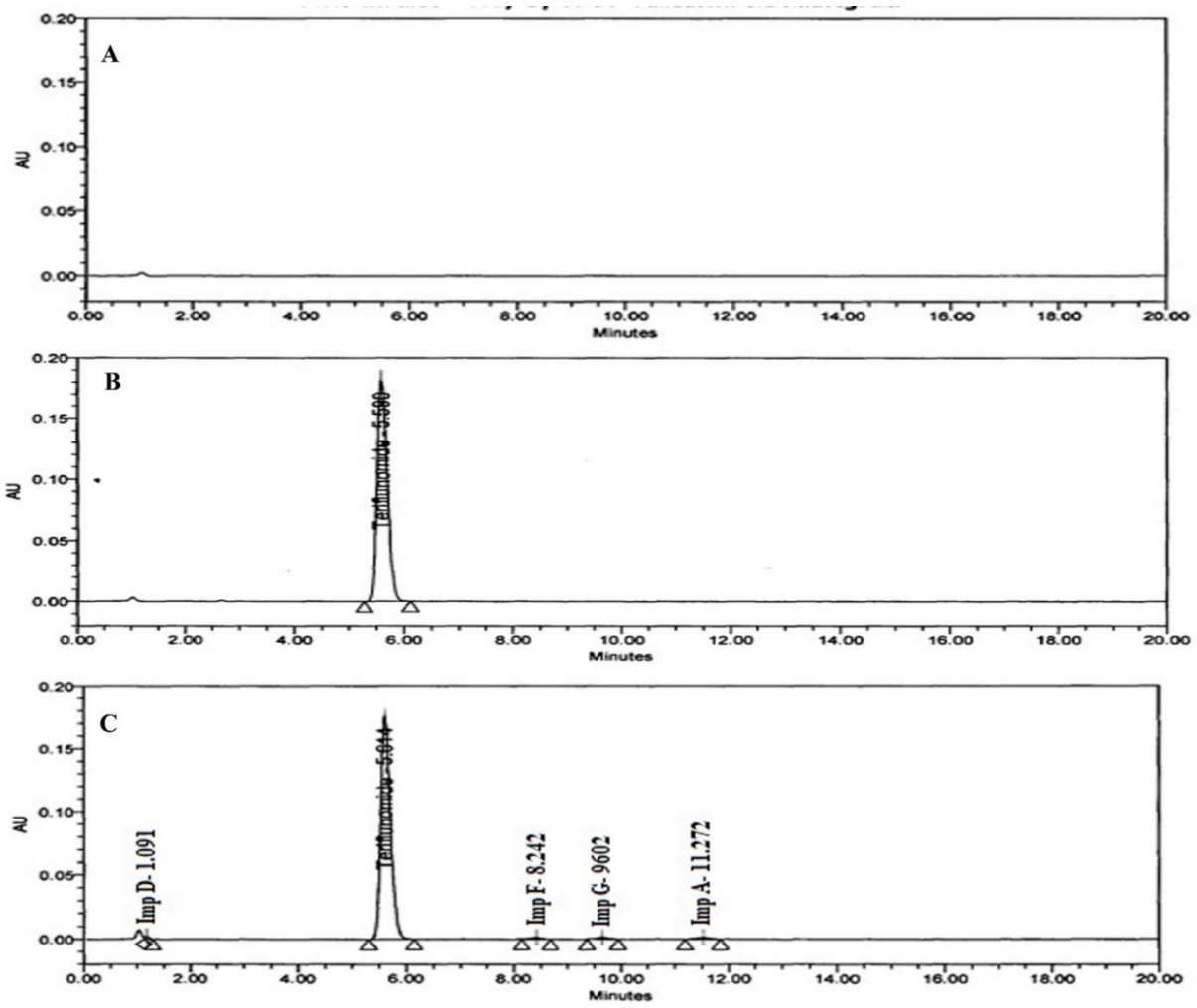

Figure 6. Chromatograms of A) Blank B) Standard solution C) Spiked solution

Table 2. Summary of R_T, R_T ratio for TFN and specified impurities

Peak name	R_T (min.)	R_T ratio
Impurity-D	1.09	0.19
TFN	5.60	1.00
Impurity-F	8.24	1.47
Impurity-G	9.60	1.71
Impurity-A	11.27	2.01

Table 3. Assay values (w/w%) for TFN sample and spiked sample

S.No.	Preparations	% Average Assay of TFN(w/w%)on driedbasis	Difference of assay values between unspiked and spiked samples
1	Sample Solution	99.9	-0.3
2	Spiked sample Solution	100.2	

Precision

System precision analysis was conducted, revealing a RSD% of 0.62% for the TFN peak area at the working concentration. The method precision assessment was carried out, resulting in an average assay of 100.1% w/w and a RSD% of 0.46 for six TFN preparations at the 100% working concentration. Various analysts evaluated intermediate precision by employing on different days., and it showed a bias of -0.1 in the determination of TFN assay content. Results are summarized in Table 4.

Table 4. Precision at intermediate level

Sample ID	Analyst (1) /Day (1) / TFN (w/w%)	Analyst (2) /Day (2) TFN (w/w%)	Bias
S-1	100.5	99.9	0.6
S-2	100.7	100.5	0.2
S-3	99.7	100.1	-0.4
S-4	99.5	100.0	-0.5
S-5	100.1	100.1	0.0
S-6	100.1	100.6	-0.5
Avg. (w/w%)	**100.1**	**100.2**	**-0.1**
SD	**0.45**	**0.28**	
RSD%	**0.46**	**0.28**	
Over all RSD% (12 preparations)	**0.37**		

Linearity

The obtained results for linearity are presented in Table 5, which displays the best-fit line for the average peak area in relation to the TFN concentration. The linearity results for TFN within the defined concentration range are deemed acceptable, as they exhibit a correlation coefficient (R) exceeding 0.999.

Table 5. Linearity Table for TFN

Level	Concentration (µg/ml)	Average peak area
70.0%	35	1530109
80.0%	40	1740125
90.0%	45	1951691
100.0%	50	2177901
110.0%	55	2414204
120.0%	60	2613053
Slope		**43790.52**
Intercept		**-8821.9**
Correlation coefficient (R)		**0.9997**
R^2		**0.9995**

Accuracy

The percentage recovery values are obtained for TFN in the range of 98.8% to 101.2%. The RSD% values for TFN recoveries are in the range of 0.20% to 1.13%. The percentage recovery results obtained for TFN is listed in Table 6.

Table 6. Accuracy test

Level	Theoretical concentration (µg / ml)	Measured concentration (µg / ml)	Recovery %	Average	RSD %
80% Level	40.6	40.1	98.8	99.8	1.13
	40.0	40.4	101.0		
	40.6	40.4	99.5		
100% Level	50.2	50.6	100.8	100.6	0.20
	50.2	50.5	100.6		
	50.5	50.7	100.4		
120% Level	60.5	60.9	100.7	100.7	0.50
	60.2	60.9	101.2		
	60.5	60.6	100.2		

Range

The method's range was established based on the data from linearity and accuracy assessments. The method's range spans from 30 to 60 µg/ml.

Robustness

The method robustness was confirmed by intentionally altering the method parameters. The TFN peak's tailing factor fell within the range of 1.1 to 1.4, and the number of theoretical plates was in the range of 4258 to 6272 in the standard solution. The RSD% ranged from 0.20% to 0.75% for six replicate injections of the standard solution. Significant changes in the assay value were not observed when examining robustness parameters, including variations in flow rate, mobile phase composition, buffer strength, injection volume, and buffer pH. During the robustness study, the retention time (R_T) of the TFN peak fluctuated between 4.64 minutes and 6.19 minutes, and the assay values on a dried basis ranged from 99.4% w/w to 100.8% w/w (Table 7).

Table 7. The system suitability data for robustness study

Validation Parameter (Robustness)	Retention Time	Average assay (% w/w)
1) Actual condition	5.77	100.6
2) Mobile phase ratio 66:34	6.11	100.5
3) Mobile phase ratio 64:36	4.64	100.8
4) Buffer pH : 4.1	5.36	99.8
5) Buffer pH : 3.9	5.28	100.8
6) 0.6% Triethylamine Buffer	5.01	100.1
7) 0.4% Triethylamine Buffer	4.81	99.7
8) Flow rate: 1.1 ml/min	5.10	99.7
9) Flow rate: 0.9 ml/min	6.19	99.9
10) Injection volume: 5 µl	5.78	100.4
11) Injection volume: 15 µl	5.76	99.4

Solution Stability

There is no substantial change in the assay observed for both the standard and sample solutions over a 48-hour period when stored at room temperature. The assay values exhibited a minimal variation, with a RSD of 1.24% for the standard solution and for the sample solution with RSD of 1.32%. The results consistently fell within the acceptable range, RSD values below 2%, demonstrating high precision and reliability of the method. According to the provided data, it can be concluded that both the standard and sample solutions remain stable for up to 48 hours at room temperature.

Conclusion

A rapid HPLC method was systematically developed for the estmation of TFN alongside its production related impurities. The optimization process involved careful adjustment of the mobile phase and chromatography specifications to achieve optimal peak parameters. The resulting method was extensively validated, confirming its sensitivity, precision, linearity across the studied concentration range, accuracy, and robustness in detecting TFN in the presence of its production related impurities. Utilizing Total Factorial Design as a QbD tool allowed for a comprehensive exploration of various factors' effects on the responses. This analysis affirmed that the TFN peak was effectively separated from other specified impurities. The developed analytical method was rigorously validated following the ICH guidelines, establishing its selectivity in determining the assay of TFN drug substance.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The author declares that there is no real, potential, or perceived conflict of interest for this article.

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