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Forced Degradation Studies to Assess the Stability of a Janus Kinase Inhibitor Using RPLC Method

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Abstract: In optimization studies, it is important to study the retention behavior of the compounds containing the ionizable functional groups under the intended chromatographic conditions. In this study, the influence of pH and acetonitrile (ACN) composition in the mobile phase on chromatographic behavior of tofacitinib (TOF), a Janus kinase (JAK) inhibitor, was thoroughly investigated. First, the chromatographic conditions were optimized using retention factors and pK_a values. Then, the developed method was used for the stability studies under various stress conditions, and for the estimation of TOF concentration in tablets. Finally, the method was verified using the International Conference on Harmonization procedure (ICH-Q2) and was successfully used to separate the TOF degradation products. A linearity range, the limits of detection and quantification were confirmed as 2.0-12.0, 0.416, and 1.260 µg/mL, respectively. Between-day and within-day accuracy (RSD%) were found to be as 0.290 and 0.462 for 4 µg/mL, respectively. The result indicates that the developed method is rather effective to isolate the parent drug from the degradation elements.

Janus Kinase İnhibitörünün Stabilitesini Değerlendirmeye Yönelik RPLC Metodu Kullanılarak Yapılan Zorunlu Bozunma Çalışmaları

Anahtar Kelimeler
Kromatografi,
Tofasitinib,
RPLC,
Metot validasyonu,
İlaç formulasyonu,
Zorlanmış bozunma

Özet: Optimizasyon çalışmalarında, iyonlaşabilen bir fonksiyonel grup içeren bileşiklerin belli kromatografik koşullar altında alıkonma davranışlarını incelemek önemlidir. Bu çalışma da, janus kinaz (JAK) inhibitörü olan tofasitinibin (TOF) kromatografik davranışlarında mobil faz asetonitril (ACN) bileşiminin ve pH'ın etkisi kapsamlıca araştırılmıştır. Öncelikle, pK_a ve alıkonma faktörü değerleri kullanılarak kromatografik şartlar optimize edilmiştir. Ardından, geliştirilen metot çeşitli stres testleri altında stabilite çalışmaları ve tabletteki tofasitinibin konsantrasyon belirlenmesinde kullanılmıştır. Son olarak, metot Uluslararası Uyumlaştırma Konferansı (Q2) kılavuzuna göre valide edilmiş ve tofasitinibin bozunma ürünlerinin ayrılmasında başarılı olarak kullanılmıştır. Lineer aralık, tespit ve tayin limitleri sırasıyla 2.0-12.0, 0.416, and 1.260 µg/mL, olarak elde edilmiştir. Gün içi, günler arası doğruluk (RSD%), 4 µg/mL için sırasıyla 0.290 and 0.462 µg/mL bulunmuştur. Bu sonuçlara göre, geliştirilen metot bozunma ürünlerinden ana ilacı ayırmak için oldukça etkilidir.

1. Introduction

The Janus kinase (JAK) family consists of four nonreceptor protein tyrosine kinases: tyrosine kinase 2 (TYK2), JAK3, JAK2 and JAK1 [1]. JAK pathways are normally involved in development, survival, differentiation, and growth of a variety of cells, but are crucially important for hematopoietic and immune cells. These pathways lead to cytokine production, which is responsible for the loop of inflammation. If proinflammatory cytokines produce redundantly, several autoimmune diseases arise. Therefore, inhibitors of the JAK pathway are potential therapeutics as immunomodulator drugs including TOF (Figure 1). TOF is an oral JAK inhibitor for the therapy of ulcerative colitis [2], psoriasis [3], alopecia [4], and rheumatoid arthritis [5].

Various methods are employed to predict the retention behavior of an ionizable drug molecule

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under chromatographic conditions. Today, reversed phase liquid chromatography (RPLC) is the most common way for drug analysis regardless of the ionic status of drug molecule [6]. The RPLC based approach is preferred since it can provide structural information in a short time [7]. During method optimization, the principal target is to ensure optimal separation conditions. For an ionizable analyte, column, mobile phase type, organic solvent composition, and pH are the most important factors targeted for optimization of chromatographic conditions [8-11].
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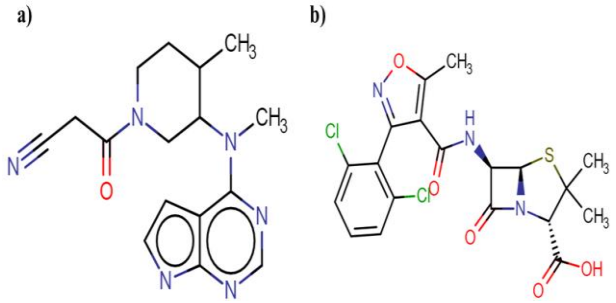

Figure 1. Chemical structure of **a)** TOF, **b)** Dicloxacillin

The forced degradation study is a significant step in drug production since it contributes to knowledge on the degradation profile of drug molecules. It is also important for method validation studies [8,12]. Since a Diode Array Detector (DAD) is very efficient in confirming peak purity, an HPLC instrument equipped with a DAD is routinely used to separate a drug from its degradation elements in a chemical environment including hydrolysis, oxidation, photolysis and thermal stress recommended by ICH [13].

For drugs with an ionizable functional group, a working range of $pK_a \pm 1.5$ for a chromatographic separation is recommended [11, 14]. The drug molecules at this pH range are ionized making the retention time much shorter at lower organic mobile compositions. There are several reports demonstrating the determination of TOF in biological samples and pharmaceutical formulations [13,15-18]. The forced degradation studies of TOF were also reported using RPLC and LC/MS-MS methods [18, 19]. However, there is no procedure detailing the optimization using the chromatographic behavior of TOF in the literature. This study aims to fill this gap as an extension of my previous study with my colleague that we determined the pK_a value of TOF in the water-ACN binary mixture [11]. In this study, organic modifying element and pH of mobile phase on retention behavior of TOF along with its degradation products was investigated with the aim of developing a more accurate, precise and reliable method. Then, the degradation products of TOF under forced circumstances were investigated.

2. Material and Method

2.1. Instruments and tools

The instrument utilized was a Japan-originated Shimadzu HPLC system, formed by a pump (LC-20AD), a DAD (SPDM20A), degasifier (DGU-20A3), and a column heater (CTO-10AS VP). Chromatographic determinations were realized on the Kinetex EVO C18 core-shell (150×4.6 mm, 5 µm, I.D.) column (Phenomenex, USA). HPLC grade water needed to prepare the solutions was acquired from Millipore (Direct Q3 UV, France). pH measurements were made by a glass electrode (In Lab 412) attached to a Mettler Toledo MA 235 pH meter (Switzerland).

2.2. Chemicals and reagents

TOF and dicloxacillin (Figure 1b) (internal standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO). sodium hydroxide (NaOH), Acetonitrile (ACN), potassium hydrogen phthalate (KHP), hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), uracil and orthophosphoric acid were supplied by Merck (Darmstadt, Germany). KHP is utilized as the standard buffer component to calibrate the pH electrode in water-ACN mixtures [20, 21].

2.3. Liquid chromatographic conditions

In this study, the water-ACN binary mixtures at increasing proportions of organic solvents (30%, 35% and 40%, v/v) were used as mobile phase for understanding the effect of ACN in the binary mobile phase mixtures on the retention of TOF. For each composition, the pH values from 3.0 to 7.0 were adjusted by adding 1.0 M NaOH using 25 mM (concentration after mixing the acetonitrile-water binary mixtures) orthophosphoric acid as buffer solution since the orthophosphoric acid has a third pK_a value of ~12. Retention factors for each compound and mobile phase were calculated using the expression $k=(tR-t_0)/t_0$ [22]. The dead time (t_o) was measured by injecting uracil solution (0.1%, in water), which was established for each mobile phase composition and pH studied. The chromatographic analysis was carried out when the flow rate was 1.0 $mLmin^{-1}$ and the injection volume was 20 µL. The column temperature at which the peak symmetries were excellent was set as 30 °C. The detection wavelength was 287 nm.

2.4. Preparing the standard solutions

Solubility of TOF is first studied in a water-ACN binary mixture since its solubility is critical for qualitative and quantitative analysis. The major stock solutions of IS and TOF obtained at 50 μgmL^{-1} are kept away from light and stored at 4 °C. For the calibration curve, the TOF solutions in the range of

2.0 to 12.0 μgmL^{-1} are prepared. The concentration of IS was kept constant at 1.0 μgmL^{-1} throughout the study.

2.5. Sample solution

Ten TOF (Xeljanz™ 5 mg) tablets are thoroughly crushed in a porcelain mortar and 100.0 mg of drug was added into 100 mL volumetric flask holding 50 mL acetonitrile-water binary mixture and sonicated for 30 min. Following, the volume was increased to 100 mL using the same binary mixture and filtered to prepare the stock solution. Then, IS was added to this solution and the new concentration was calculated.

2.6. Forced degradation conditions

Hydrolytic degradation of TOF was separately executed under acidic and basic conditions by adding 1 mL HCl (0.1 M) and NaOH (0.1 M) respectively into 2 mL of TOF stock solution. The same procedure was repeatedly carried out at room temperature for 2, 6, 12, 24, 48 hours. For oxidative stress study, 2 mL of TOF stock solution was treated with 1 mL of 3% (v/v) H_2O_2 at room temperature for 2, 6, 12, 24, 48 hours. For thermal and photolytic degradation studies, the drug substance in solid form was used. For the thermal stress experiments, precisely measured 0.0001 g of TOF was transferred into a glass plate and kept in the hot oven for both 2 and 4 hours at 30, 40, 50 ^{0}C. After the heating, a certain amount was taken in a 25 mL flask containing acetonitrile-water binary mixture to make a concentration at 25 μgmL^{-1}. For the photolytic degradation, TOF was exposed to 254 nm, 200 Watt.hrs/m^2 UV light in a UV cabinet for 2, 4, 6 hours at room temperature. The stressed samples were diluted using acetonitrile-water binary mixture to get the final concentration of TOF at 10 μgmL^{-1} and injected in the RPLC system. Acid and base degradation samples were neutralized using an equal molar concentration of base and acid prior to RPLC analysis

3. Results

3.1. RPLC method optimization and validation

Forced degradation studies are performed as a part of the drug development process. In this study, the new RPLC method was developed and validated to determine the stability of TOF under the forced degradation conditions. ACN was chosen as an organic modifier in the mobile phase due to the several advantages over methanol such as higher purity, lower back pressure and a higher elution strength. In general, using LC grade acetonitrile is the safest choice. Kinetex EVO C18 core-shell (150×4.6 mm, 5µm I.D.), a new generation liquid chromatographic column, was selected particularly due to its high stability and excellent peak symmetry for basic compounds. In order to determine the pK_a value, the TOF retention factor (k) in the water-ACN binary mixture was taken into consideration. For this, the retention time at constant column temperature and the flow rate was measured, in particular, under acidic pH (3.5-7.0) conditions. The sigmoidal behavior was observed when the retention factor values calculated in the water-ACN binary mixtures of 30, 35, 40%, v/v plotted against mobile phase pH values (Figure 2).

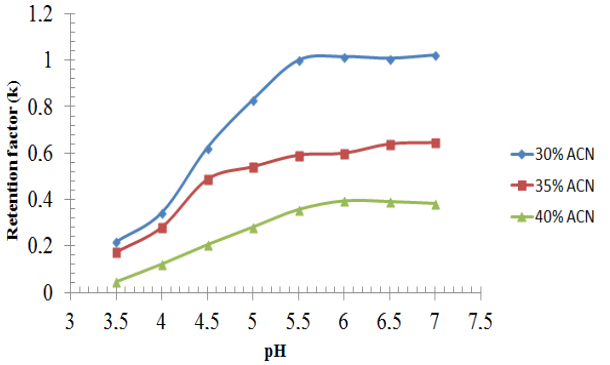

Figure 2. Values of retention factor and pH of the mobile phase for TOF at different ratios of water-ACN binary mixtures.

The mobile phase composition and pH were kept under control to determine the optimum chromatographic conditions. A k value of TOF above 1 or below 5 was chosen for the qualitative and quantitative determinations [23]. From Figure 2, it is seen that when ACN content in the mobile phase is 30% (v/v) at pH 6.0, the condition of k ≥1 is met. As mentioned above, the pH of the mobile phase is selected by qualifying the suggested condition of pK_a ± 1.5. Since pK_a of TOF is 4.347 as we reported in our previous study [11], a pH of 6.0 was decided to be appropriate.

The IS was used to improve the accuracy and precision of data in difficult to control injection errors. Thus, the quantitative determination of TOF in tablet formulation was selected using dicloxacillin as an IS as it demonstrated a retention time with a good peak symmetry along with a good resolution from the TOF peak. The separation under the optimized condition is completed in 7 minutes as seen in Figure 3.

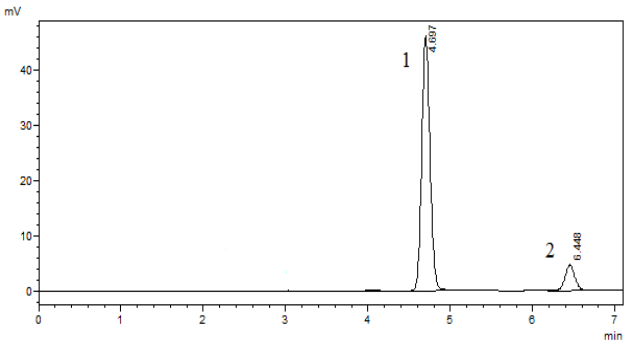

Figure 3. Chromatogram of standard **(1)** Tofacitinib (12 µg/mL) and **(2)** Dicloxacillin (1 µg/mL).

3.1.1. System suitability test results

In method development, it is important to determine system suitability parameters prior to analysis. The specifications such as retention factor, tailing factor (Tf), retention time, theoretical plate number (N), separation (α) and resolution factor (Rs) were calculated and provided in Table 1. The calculated values indicate the applicability of the developed method for routine analysis.

Table 1. System suitability test parameters for TOF (n=3).

Parameters	TOF	IS	Recommended Values
Retention time	4.691	6.457	-
Tailing factor	1.137	1.060	≤2
Retention factor	1.013	1.771	>1
Resolution factor	-	4.070	>2
Theoretical plate number	7378	3542	>2000
Selectivity factor(α)	-	1.748	>1

3.1.2. Linearity, limit of quantification, limit of detection

In Figure 4, the calibration curve is built by taking the area ratio of TOF signal to the internal standard signal as a function of TOF concentration. The calibration equation for TOF was obtained by plotting the peak area ratio of TOF to IS versus concentration of the drug in the linear range of 2-12 μgmL^{-1} (See Table 2).

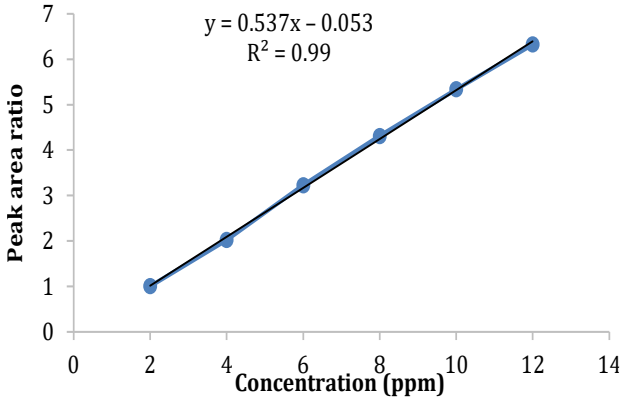

Figure 4. The calibration curve of the area ratio of TOF signal to the internal standard signal versus TOF concentration

The LOD and LOQ were determined using signal to noise ratio and calculated using 3.3 σ/s and 10 σ/s respectively. (σ: the standard deviation of the peak area ratio and s: the slope of calibration curve) The LOD and LOQ were calculated 0.416 and 1.260 μg/mL, respectively.

The results indicated good linearity in the studied concentration range.

Table 2. Data for the calibration graphs of TOF.

Statistical parameters	Values (n=3)
Linearity range (μg/mL)	2.0-12.0
Slope	0.537
Intercept	-0.053
*SD of the slope	0.008
SD of the intercept	0.063
Correlation coefficient	0.999
LOD (μg/mL)	0.416
LOQ (μg/mL)	1.260

*SD: Standard deviation

3.1.3. Precision

The precision and accuracy of the developed method were calculated using the peak area ratio data obtained from the studies conducted between the within-day and the between-day. The within-day accuracy was validated with two concentrations; 4 and 10 μgmL^{-1}, of TOF under the optimized conditions for five times during the same day. The procedure was repeated on three sequential days for between-day accuracy and precision. Accuracy, reproducibility and precision results provided in Table 3 were evaluated by employing iterative analysis of the TOF solution in the mobile phase. The precision of the method (RSD%) was found to be less than 2% when the solutions including internal standard and TOF were injected five times (See Table 3). The obtained RSD% values indicated that the method precision was quite sufficient.

Table 3. The within-day and between-day precision data (n=5).

	Theoretical concentration (μg/mL)	Within-day average (μg/mL)	*RSD% (μg/mL)	Between-day average (μg/mL)	RSD% (μg/mL)
TOF	4	4.179	0.290	4.106	0.462
	10	10.504	0.352	10.571	0.626

3.1.4. Tablet Analysis

The developed RPLC method in this study was used to quantify tofacitinib in the tablet dosage form using the related calibration curve without any sample extraction or evaporation other than filtration and adequate dilution steps. The results are illustrated in Table 4.

Table 4. The obtained amount of TOF in the tablet (n=3).

	The amount of TOF in the tablet (mg)
1	5.135
2	5.118
3	5.158
X_{mean}	5.137
SD	0.020
% RSD	0.392

Figure 5 shows the chromatograms of Xeljanz (Pfizer®) tablets containing tofacitinib. As seen, no

interfering peaks from excipients including microcrystalline cellulose, magnesium stearate, titanium dioxide (E171), lactose monohydrate, croscarmellose sodium, triacetin (E1518), etc. in the TOF tablet are not observed on the chromatogram.

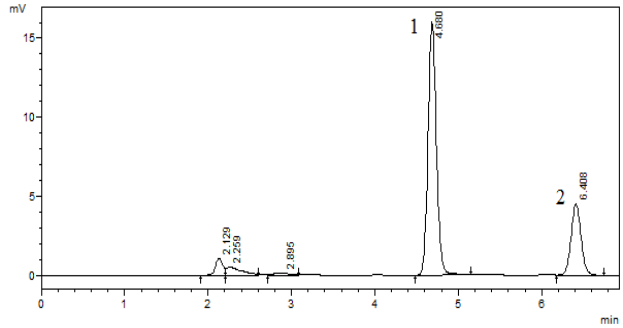

Figure 5. Chromatogram of the analysis method of Xeljanz (5 mg) tablets containing tofacitinib. TOF **(1)** and IS **(2)**.

3.1.5. Recovery Studies

The accuracy of the developed method (recovery experiments) was decided by adding 4 and 8 μgmL^{-1} TOF standard to the actual sample and triplicate. The result of recovery studies is provided in Table 5. As seen, a good recovery was obtained.

Table 5. % Recovery results of Xeljanz (5 mg) containing tofacitinib (n=3).

	% Recovery
1	102.691
2	102.366
3	103.168
X_{mean}	102.742
SD	0.403

According to the results, whereas the labelled amount of TOF in the tablet was 5 mg, obtained amount of TOF was 5.137±0.020.

3.2. Qualitative determination of degradation products

The distinction of the developed method was decided by forced degradation experiments. TOF's degradation behavior in different stress conditions (mild) was investigated using ICH Q1A (R2) guidelines [24]. In our previous study, TOF has different degradation products under stress conditions such as hydrolytic and oxidative degradation [26].

3.2.1. Hydrolytic degradation

Under the hydrolytic conditions (0.1 M HCl or 0.1 M NaOH), the liquid solution of TOF at constant temperature and at a certain time interval was subjected to degradation. Since temperature and pH have an important effect on degradation, the degradation studies were performed at room temperature and neutral pH. The decomposition data of TOF in solution exposed to 0.1 M HCl and 0.1 M NaOH is given in Table 6 and the chromatograms are provided in Figure 6. When the chromatograms were examined, only one peak was observed in the degradation in the acidic hydrolysis while more than one peak appeared in the case of alkaline hydrolysis. After 48 hours, the TOF showed more degradation in the basic medium. The observed difference can be explained with different hydrolysis mechanisms under acidic and basic conditions. It is possible that

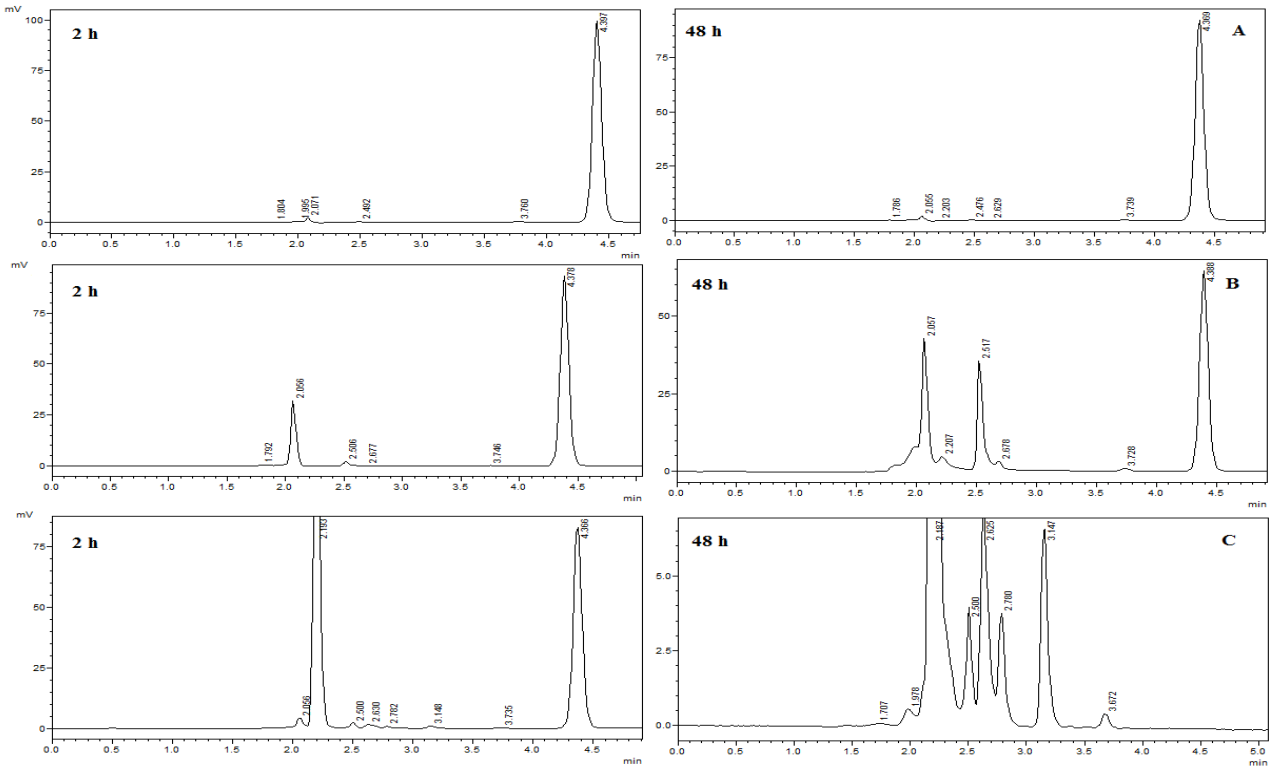

Figure 6. Chromatograms of degradation products at different times (2 and 48 h) in the **A)** acid, **B)** base, **C)** peroxide.

hydrolysis takes place at one site under the acidic conditions while it takes place at multiple sites under the basic conditions [19]. The degradation products of TOF under hydrolytic degradation are isocyanato ethyne (C_3HNO) and 3-oxopropionitrile [26].

3.2.2. Oxidative degradation

When the TOF solution was exposed to 3% (v/v) H_2O_2 at room temperature, the degradation was quite fast as consistent with the report by Wu et al [19]. Table 6 and Figure 6 illustrate the chromatogram obtained from the TOF solution exposed to H_2O_2. As seen, there are six degradation peaks in addition to the TOF peak. TOF has different degradation products such as 7H-pyrrolo[2,3-d]pyrimidine [26].

Table 6. Hydrolytic and oxidative degradation results for TOF (n=3).

Time (hour)	Degradation %		
	Acid Hydrolysis 0.1 M HCl	Base Hydrolysis 0.1 M NaOH	Oxidation 3% (v/v) H_2O_2
2	6.80	20.43	28.11
6	10.91	20.6	32.14
12	12.33	20.99	51.91
24	13.33	30.86	72.03
48	22.02	45.84	94.26

3.2.3. Photolytic and thermal degradation

In the stress studies, the solid form of TOF was exposed to UV light (200 Watt.hrs/m^2 as specified in ISO 10977) and heat under various conditions. When TOF was exposed 254 nm light for 2, 4 and 6 hours, only one degradation product was observed, and the % degradation increased over time (See Figure 7, Table 7). When TOF was exposed to increasing temperatures (30, 40, 50 °C) for 2 and 6 hours, it showed increased degradation behavior at higher temperatures, especially at 50 °C (See Figure 7, Table 7). In this study, the peak impurity of TOF was determined using a DAD detector and found to be higher than 99.9% under all conditions.

Table 7. Photolytic and thermal degradation results for TOF.

Time (hour)	Degradation % (n=3)			
	Photolysis (254 nm)	Thermal (30°C)	Thermal (40°C)	Thermal (50°C)
2	10.18	21.2	36.26	43.09
4	12.07	34.2	37.6	45.14
6	17.23	-	-	-

In this study, the degradation of products was determined qualitative. In the study conducted by Wu et al. [19], degradation products of TOF were defined in the degradation conditions studied. It was concluded that TOF was prone to hydrolysis under acidic and basic conditions at the amide and cyano position of the 3-oxopropanenitrile moiety. TOF was specifically sensitive to the oxidative degradation at the pyrrole ring double bond with the major degradation products [19, 25, 26].

Dowty et al. demonstrates that the half-life of tofacitinib was approximately 3.2 hours for main drug and its metabolites. All metabolites of TOF was representing less than 10% each of total circulating radioactivity [15].

4. Discussion and Conclusion

There is no procedure detailing the optimization using the chromatographic behavior of TOF in the literature. This is the first study on the chromatographic optimization of TOF. A suitable analytical procedure was established and verified in

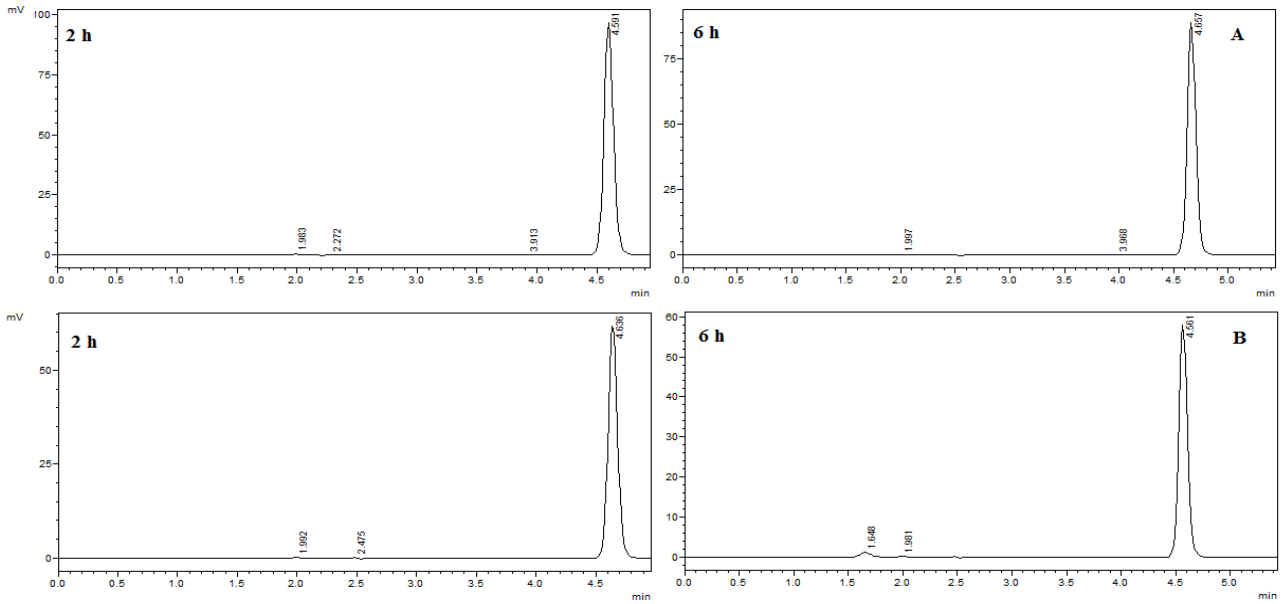

Figure 7. HPLC chromatograms **A)** UV (2 and 6 h 254 nm UV light) **B)** thermal (50°C at 2 h and 6 h).

order to quantify TOF in pharmaceutical dosage with an HPLC instrument equipped with a DAD detector. The method validation produced perfect results in terms of linearity, specificity, precision, accuracy, LOD, and LOQ. The developed method displayed no interference with the formulation excipients. The behavior of TOF under the stress conditions in acidic, alkaline, thermal, photolytic and oxidative media was studied and the peaks originating from degradation products were well resolved from TOF peak. The experimental model, which explains the retention factors in various pH and ACN concentration states was successfully applied to degradation studies of TOF under different conditions. The highest degradation of TOF was realized in the oxidative condition and the least degradation was in the photolytic condition.

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Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

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