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Stability indicating HPLC method for the quantification of tofacitinib citrate and its related substances*

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ABSTRACT

A reverse phase liquid chromatographic (RP-LC) method was developed for the quantification of the related impurities of Tofacitinib citrate drug substance. The method was optimized using buffer (prepared by dissolving 2.72gr potassium di hydrogen phosphate and 1.0gm of 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to 5.5 with dilute potassium hydroxide solution) along with Acetonitrile 90:10v/v as mobilephase-A, and Acetonitrile: Buffer in the ratio of 70:30v/v as mobile phase-B. The flow rate was set at 1.0 mL min^{-1}, wavelength at 210nm and the column temperature was maintained at 25°C. The capability of stability indicating method developed was demonstrated by studying the degradation products generated during the forced degradation studies under the following conditions i) water hydrolysis, ii) at 75% relative humidity, iii) oxidative, iv) thermal v) sunlight, vi) acid, vii) base, and viii) photolytic degradation. The developed method can be used for the determination of synthetic and degradation impurities in the regular quality control analysis for the drug substance.

Keywords: Tofacitinib citrate, Reverse phase liquid chromatography, Stability-indicating methods, method development, Method validation, Stress conditions, ICH.
**MSNRD Communication No.013*

INTRODUCTION

The biological activity of chiral substances often depends upon their stereochemistry. A large percentage of commercial and investigational pharmaceutical compounds are enantiomers, and many of them show significant enantioselective differences in their pharmacokinetics and pharmacodynamics [1-3]. Analysis of the enantiomeric purity of chiral drug candidates has become very important particularly in the pharmaceutical and biological fields, because few enantiomers of racemic drugs have relatively different pharmacokinetic properties and diverse pharmacological or toxicological effects [4-7]. Apart from this the International Conference on Harmonization (ICH) guide-lines [8-10] emphasizes that the purity and assay of drug susceptible to change during storage, must be determined by using validated stability- indicating methods, which can selectively determine the drug in presence of its process (including the other isomers) and degradation impurities. Tofacitinib (trade names Xeljanz and Jakvinus, formerly tasocitinib[11), CP-690550[12]) is a drug of the januskinase (JAK) inhibitor class, discovered and developed by Pfizer. It is currently approved for the treatment of rheumatoid arthritis (RA) in the United States and Russia, and is being studied for treatment of psoriasis, inflammatory bowel disease, and other immunological diseases, as well as for the prevention of organ transplant rejection. Tofacitinib was not approved by the European regulatory agencies because of concerns over efficacy and safety[13]. It is an inhibitor of the enzyme janus kinase 3 (JAK3), which means that it interferes with the JAK-STAT signaling pathway, which transmits extracellular

information into the cell nucleus, influencing DNA transcription[14]. Recently it has been shown in a murine model of established arthritis that tofacitinib rapidly improved disease by inhibiting the production of inflammatory mediators and suppressing STAT1-dependent genes in joint tissue. This efficacy in this disease model correlated with the inhibition of both JAK1. JAK1 is a human tyrosine kinase protein essential for signaling for certain type I and type II cytokines. Expression of JAK1 in cancer cells enables individual cells to contract, potentially allowing them to escape their tumor and metastasize to other parts of the body[15], and 3 signaling pathways, suggesting that tofacitinib may exert therapeutic benefit via pathways that are not exclusive to inhibition of JAK3[16]. The potential significance of JAK3 inhibition was first discovered in the laboratory of John O'Shea, an immunologist at the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health (NIH)[17]. In 1994, Pfizer was approached by the NIH to form a public-private partnership in order to evaluate and bring to market experimental compounds based on this research[17]. Pfizer initially declined the partnership but agreed in 1996, after the elimination of an NIH policy dictating that the market price of a product resulting from such a partnership would need to be commensurate with the investment of public taxpayer revenue and the "health and safety needs of the public[17]. " The drug discovery, preclinical development, and clinical development of tofacitinib took place exclusively at Pfizer[18]. Chemically, Tofacitinib Citrate is (3R,4R)-4-Methyl-3-(methyl-7H-pyrrolo [2,3-d]pyrimidin-4-yl amino)-ß-oxo-1-piperidinepropanenitrile, 2-hydroxy-1,2,3-propane tri carboxylate (1:1). The empirical formula is $C_{16}H_{20}N_6O \cdot C_6H_8O_7$ and its molecular weight is 504.5 and the chemical structure of Tofacitinib Citrate is shown in (Fig.1).

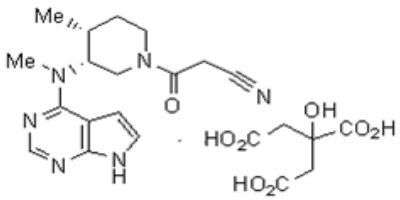


Fig-1: Tofacitinib Citrate

Chemical name: (3R,4R)-4-Methyl-3-(methyl-7H-pyrrolo [2,3-d]pyrimidin-4-yl amino)-ß-oxo-1-piperidine propanenitrile, 2-hydroxy-1,2,3- propanetricarboxylate (1:1).

However extensive survey revealed that the stability indicating HPLC method for quantitative determination of Tofacitinib Citrate and its related impurities in active pharmaceutical ingredient was not reported till date. Therefore it was felt necessary to develop an accurate, rapid, and specific stability indicating method for the determination of 'Related substances' of Tofacitinib Citrate. Dihydroimpurity is a potential process related impurity and even that also had been well separated from main peak. For this impurity quantification no method is available in the literature. By keeping in view of all these impurities, we have developed a new accurate and stability indicating HPLC method for the determining the 'Related substances' of Tofacitinib Citrate.

MATERIALS AND METHODS

Chemicals and Reagents

Tofacitinib Citrate and its impurities viz. Amine impurity, Dihydro impurity and Benzyl impurities were obtained from MSN Laboratories Private Limited, Hyderabad, India. HPLC-grade of acetonitrile and AR grade of potassium di hydrogen phosphate, potassium hydroxide, hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%) were obtained from Rankem, New Delhi, India. Milli Q Millipore (USA) purification system was used to prepare high pure water.

HPLC Instrumentation and Conditions

The method development attempts, forced degradation studies and the method validation was performed on Agilent 1200 series LC systems with a diode array and variable wave length detectors (Agilent Technologies, Waldbronn, Germany). The data were collected and processed using Ez chrom Elite software. The peak homogeneity was studied by using Agilent 1200 series DAD detector.

Chromatographic conditions

The chromatographic separation was optimized with the Kromasil C-18 column with the dimension of 250mm x 4.6 mm and 5µm as particle size. A gradient elution was involved with the buffer (prepared by dissolving 2.72gr potassium di hydrogen phosphate and 1.0gm of 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to 5.5 with dilute potassium hydroxide solution) along with Acetonitrile 90:10v/v as mobilephase-A, and Acetonitrile: Buffer in the ratio of 70:30v/v as mobile phase-B. The HPLC

gradient program was set as (time/% mobile phase- B) 0.0/22, 5/22, 15/45, 30/60, 40/60, 40.5/22, 52/22. The flow rate of the mobile phase and the column temperature was set as 1.0 mL min^{-1} and 25°C. The detection wave length was optimized at 210 nm, 10µL injection volume. A mixture of Water and Acetonitrile 8:2v/v was used as diluent, and same diluent used for needle wash purpose also.

Preparation of standard solutions:

A mixture of Water and Acetonitrile 8:2v/v was used as diluent. A standard solution (Reference solution) 0.0007mg/mL of Tofacitinib Citrate solution was prepared in the diluent. A stock solution with the blend of Amine impurity, Dihydro impurity and Benzyl impurity (Benzyl imp dissolved in 3 ml of acetonitrile then made up to the mark with diluent) was also prepared in diluent (Except benzyl impurity) for the preparation of system suitability solution (0.15% solution with respect to 0.7mg/mL Tofacitinib Citrate test concentration).

RESULTS AND DISCUSSION

Method development and optimization

The HPLC method was optimized so as to obtain stability– indicating method that it could resolve degradation impurities from Tofacitinib Citrate. Different stationary phases with different selectivity were used for the determination of Tofacitinib Citrate and it's impurities as the initial attempts. However good peak shape with less peak width and the resolution of all the related impurities were achieved satisfactorily in Kromasil C-18 column with the dimension of 250mm x 4.6 mm and 5µm as particle size. A gradient elution was involved a buffer (prepared by dissolving 2.72gr potassium di hydrogen phosphate and 1.0gm of 1-Octane sulphonoic acid sodium salt anhydrous taken in 1000mL milli-Q-water and then pH was adjusted to 5.5 with dilute potassium hydroxide solution) along with Acetonitrile 90:10v/v as mobilephase-A, and Acetonitrile: Buffer in the ratio of 70:30v/v as mobile phase-B. The HPLC gradient program was set as time/% mobile phase- B: 0.0/22, 5/22, 15/45, 30/60, 40/60, 40.5/22, 52/22. The flow rate of the mobile phase and the column temperature and auto sampler Temperature was set as 1.0 mL min^{-1} and 25°C. The detection wave length was optimized at 210nm, 10µL injection volume. A mixture of Water and Acetonitrile 8:2v/v was used as diluent, and the same diluent used for needle wash purpose also.

The system suitability parameters are resolution between any two known impuriti-es and known impurity and main peak should not be less than 1.5. Theoretical plates for Tofacitinib peak should not be less than 3000. Tailing factor for Tofacitinib peak should not be more than 2.0 and detector sensitivity for reference solution(0.1%) the S/N ratio should not be less than 20. Disregard the Citric acid peak which is observed at around 1.3min retention time. The developed method is specific for Tofacitinib Citrate and its degradation products. The structures and chemical names of the impurities are given below:

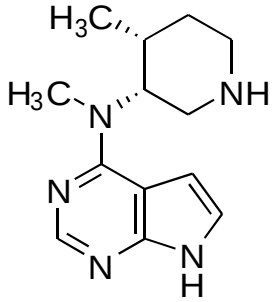


Fig-2: Amine impuriy

Chemical name: Methyl-[(3R,4R)-4-methyl-piperidin-3-yl]-(7H-pyrrolo[2,3-d]pyrimidin-4yl)-amine.

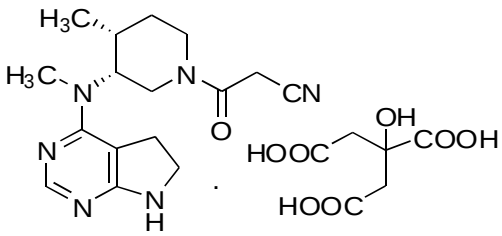


Fig-3: Dihydro impurity

Chemical name : 3-((3R,4R)-3-((6,7-Dihydro-5H-pyrrolo[2,3-d]pyrimidin-4-yl)(methyl)amino)4-methylpiperidin-1-yl)-3-oxopropanenitrile, 2-hydroxy-1,2,3-propanetricarboxylate (1:1).

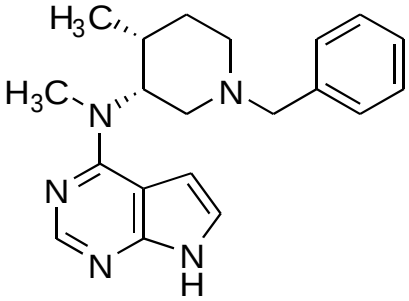


Fig-4: Benzyl impurity

Chemical name : (3R,4R)-(1-Benzyl-4-methyl-piperidin-3-yl)-methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amine.

Chromatograms: A typical chromatogram of Blank, System suitability solution, Reference solution, mother sample are given below

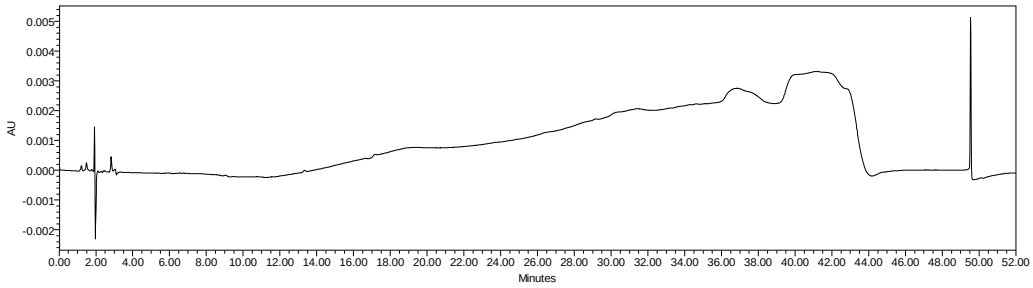


Fig -5: Diluent

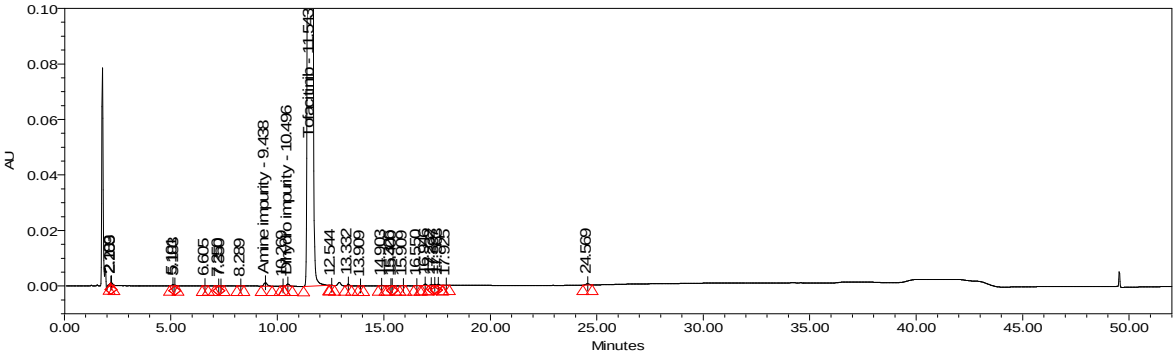


Fig-6: Mother solution

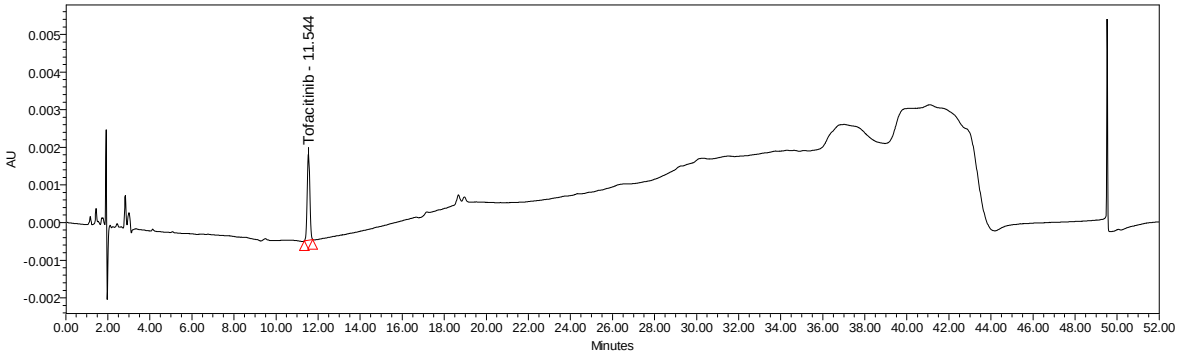


Fig -7: Reference solution

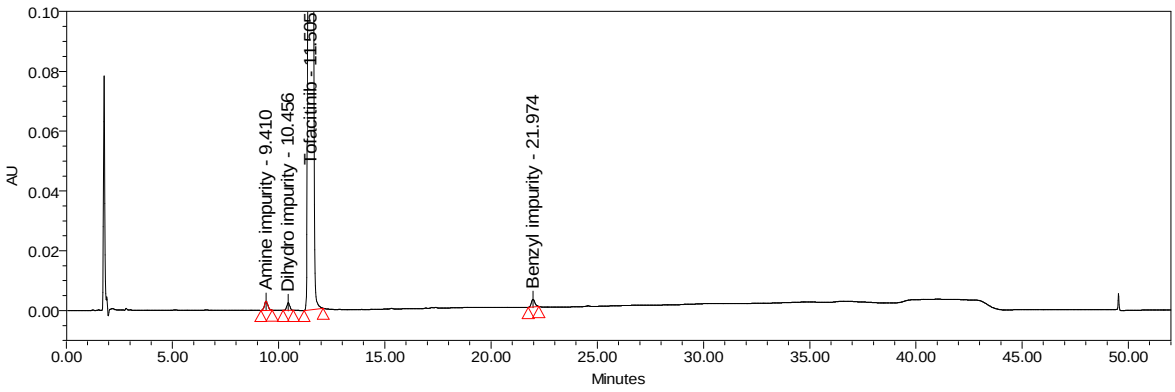


Fig-8: 0.15% all impurities spiked to Tofacitinib Citrate sample

Method validation Results:

The developed method was validated as per ICH guidelines and the results are given in Table-1. Stress testing of the drug substance can help to identify the degradants, which in turn help to evaluate the stability-indicating nature of the developed method. The specificity of the developed HPLC method for Tofacitinib Citrate was determined in the presence of its process and degradation impurities. All the stressed samples of Tofacitinib Citrate and all degradation impurities were well resolved from one another and from Tofacitinib Citrate. The analysis was carried out by HPLC with Diod array detector. The chromatographic peak purity tool, applied to Tofacitinib Citrate and its impurities peaks, demonstrated that all the peaks were pure in all cases conform the absence of other impurities co-eluting in the same retention time and there by signifying the specificity and stability indicating nature of the method. The detection limit (DL) and quantification limit (QL) for Amine impurity, Dihydro impurity and Benzyl impurities were determined at a signal to noise ratio of 3:3 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. Precision study was carried at QL level by injecting six times and calculating the percentage of R.S.D of area of Amine impurity, Dihydro impurity and Benzyl impurities. Linearity test solutions for purity determination were at six concentration levels from QL to 150 % of the specification level (0.15%). Peak area versus concentration data was performed by least-squares linear regression analysis. Standard addition and recovery experiments were conducted to determine accuracy of impurities quantitation in bulk drug samples. The study was carried out in triplicate at QL, 50%, 75%, 100%, 125% and 150% level with respect to specification 0.15%. The percentages of recoveries for impurities were calculated.

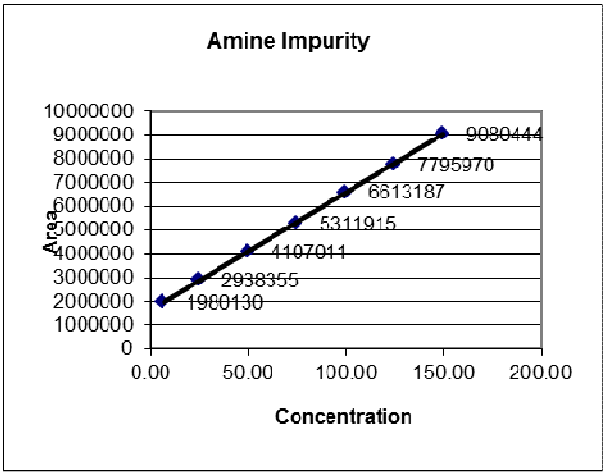
Table-1: Validation data of the developed method.

Parameter	Amine impurity.	Dihydro impurity.	Benzyl impurity.	Tofacitinib
DL (%)	0.00225	0.00560	0.00215	0.00175
QL (%)	0.00975	0.02310	0.00950	0.00890
Method Precision (%RSD)	2.40	2.69	1.87	---
Intermediate precision (%RSD)	1.28	0.72	0.90	---
Accuracy[a] (% recovery) at:-				
QL	98.9	101.2	102.9	
50%	103.8	95.8	96.6	
75%	104.1	95.8	100.0	
100%	106.4	96.2	100.2	
125%	105.7	96.8	100.36	
150%	106.7	98.3	101.1	

[a]*Accuracy carried out at QL,50%,75%, 100% and 150% level with respect to 0.15% level.*

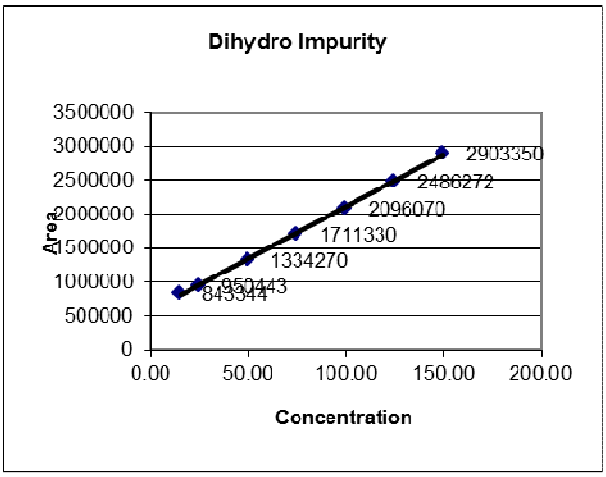
Linearity graphs and correlation results are found as below:

LINEARITY GRAPH OF AMINE IMPURITY:



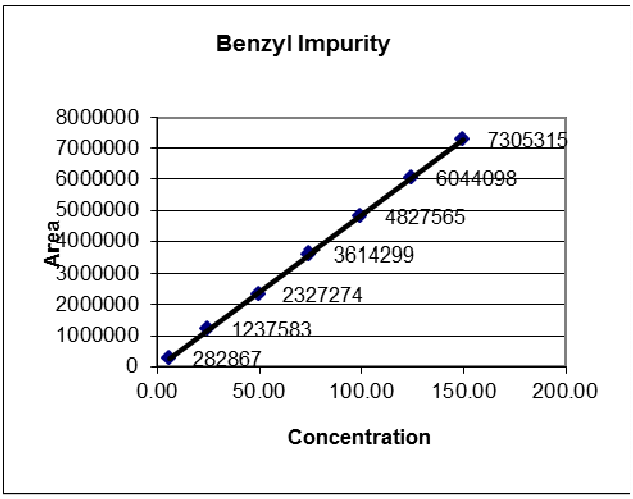
Amine impurity	
Conc in %	**Avg Area**
6.50	1980130
25	2938355
50	4107011
75	5311915
100	6613187
125	7795970
150	9080444
slope	**49260**
Correl	**0.99992**
Y-intercept	**1663622**

LINEARITY GRAPH OF DIHYDRO IMPURITY:



Dihydro impurity	
Conc in %	**Avg Area**
15.40	843344
25	950443
50	1334270
75	1711330
100	2096070
125	2486272
150	2903350
slope	**15345**
Correl	**0.99968**
Y-intercept	**576095**

LINEARITY GRAPH OF BENZYL IMPURITY:



Benzyl impurity	
Conc in %	**Avg Area**
6.33	282867
25	1237583
50	2327274
75	3614299
100	4827565
125	6044098
150	7305315
slope	**48727**
Correl	**0.99988**
Y-intercept	**-35839**

Robustness Study:

The robustness of developed method was determined by altering experimental conditions purposely and evaluating the resolution between Tofacitinib Citrate, Amine impurity, Dihydro impurity and Benzyl impurity. Flow rate was changed by ± 0.1 units, pH was varied by ± 0.2 units and column temperature was studied at 20°C and 30°C instead of 25°C in all above varied conditions the components of the mobile phase were held constant and no significant change (relative error less than 5%) of relative retention time was observed.

Significant changes were not observed in the contents of Amine impurity, Dihydro impurity and Benzyl impurity. The stability data confirmed that sample solutions were stable up to 48hrs. The system suitability was established in terms of resolution between any two known impurities and known impurity and Tofacitinib which was more than 1.5. Tailing factor not more than 2.0 and Theoretical plates should not be less than 3000 for Tofacitinib, when a 0.7mg/ml Tofacitinib Citrate solution spiked with 0.15% of Amine impurity, Dihydro impurity and Benzyl impurity were injected.

Forced degradation studies

The stability indicating power of the developed method was studied by conducting forced degradation studies on Tofacitinib Citrate. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed HPLC method for Tofacitinib Citrate was determined in the presence of its impurities, and degradation products. Forced degradation studies were also performed on relative Humidity study stress at 75% Relative humidity for 10 days. The thermal stress was done at 60 °C for 10 days. The under photolytic stress studies conducted for 50 hours at under sunlight. The photolytic stressed studies were performed for UV Direct (200 watt hours/square meter), UV Indirect (200 watt hours/square meter), Lux direct (1.2 million LUX hours) and Lux in direct (1.2 million LUX hours). Water hydrolysis was performed for 20 hours at 60 °C. The acid stress was performed at 5.0N HCl at the concentrated sample solution at ambient temperature for 24 hours and base stress was performed at 0.1N NaOH for 10min at ambient temperature and the oxidation stress was done using 10% hydrogen peroxide for 24 hours an ambient temperature. Stressed samples of Tofacitinib Citrate generated were checked for peak purity of by using Agilent diod array detector (DAD). The peak purity is within the limit obtained in all stressed samples, demonstrates the analyte peak homogeneity. The Forced degradation studies results are given below in below Table-2.

Table 2. Summary of forced degradation results

Sl. No.	Stressed conditions		Duration	% of Total imp	% of Amine imp	% of Dihydro imp	% of Benzyl imp
1	**Normal**		---	0.31	0.08	0.04	0.00
2	**Thermal at 60°C**		10 days	0.39	0.09	0.04	0.00
4	**At 75% Relative Humidity**		10 days	0.42	0.10	0.04	0.00
5	**Under Sunlight**		50 hours	0.52	0.10	0.08	0.00
6	***Photo Degradation***	**UV direct**	200watt hours/square meter	0.46	0.08	0.10	0.00
		UV indirect		0.40	0.08	0.08	0.00
		LUX direct	1.2 million LUX hours	0.46	0.09	0.08	0.00
		LUX indirect		0.39	0.08	0.08	0.00
7	**Acid hydrolysis (5.0N HCl at RT)**		After 24hrs	7.22	7.11	0.00	0.00
8	**Base Hydrolysis (0.1N NaOH at RT)**		After 10min	22.98	21.70	0.00	0.02
9	**Oxidation (10% H_2O_2 at RT)**		After 24 hrs	6.00	0.21	0.18	0.00
10	**Water Hydrolysis (at 60°C±5°C)**		After 20 hrs	5.19	0.10	0.04	0.00

Results of forced degradation studies

Significant degradation was observed in Tofacitinib Citrate stressed sample that were subjected to very sensitive in base hydrolysis. Sensitive in Acid, Water at hydrolysis 60°C±5°C and Peroxide degradation. Stable in Thermal at 60°C, 75% relative humidity and Photo degradation(U.V direct and indirect, Lux direct and indirect). Peak purity test results derived from Diode array detector, confirmed by that Tofacitinib Citrate peak is homogeneous and pure in all the analyzed stress samples.

The Base, Peroxide, Acid Degradation chromatograms are given below:

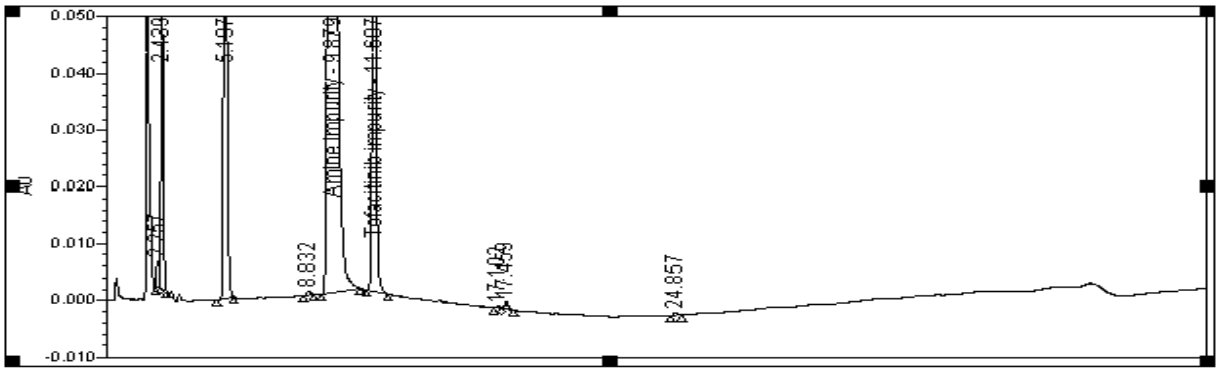


Fig-9: Typical chromatogram of 0.1N NaOH degradation solution

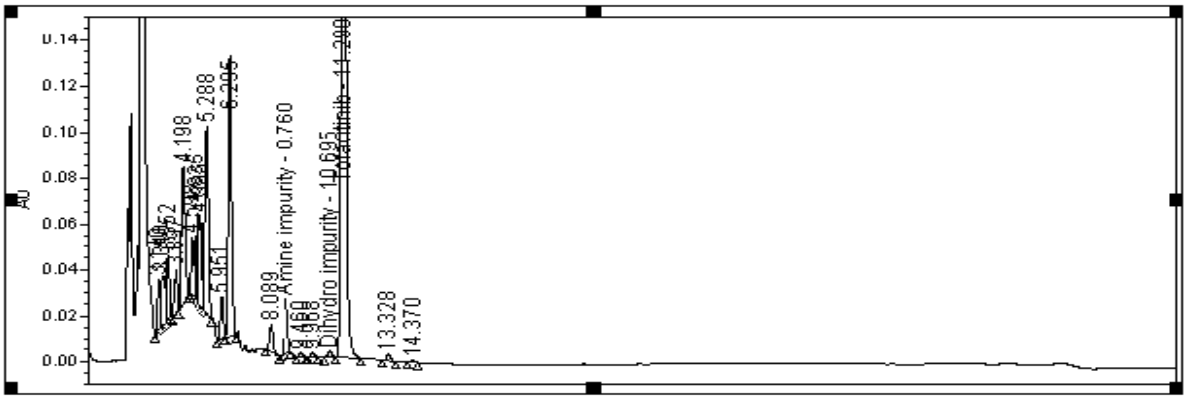


Fig-10: Typical chromatogram of 10% H2O2 degradation solution

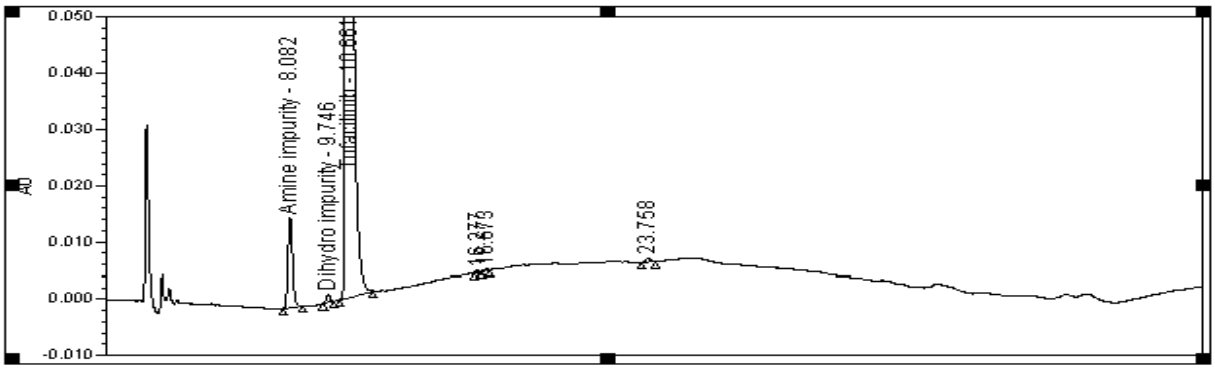


Fig -11: Typical chromatogram of 5.0 N HCl degradation solution

Solution stability and mobile phase stability

Solution stability and mobile phase stability provides an indication of its reliability during normal usage during the storage of the solutions used in the method. The solution stability of Tofacitinib Citrate was established for 48 hours at RT. The solution stability studied by using Tofacitinib Citrate sample and injected for every 12 hours. The content of impurities and Tofacitinib Citrate were quantified at each interval up to the study period. The mobile phase stability was also established by quantifying the freshly prepared sample solutions against freshly prepared reference standard solutions for every 12 hours up to 48hrs. During the study period the prepared mobile phase was stable up to 48hrs at room temperature.

% of impurities for Sample+100% All impurities solution stability from "0" Hours to "48" Hours

Name of the impurity	at "0" Hours	at "12" Hours	at "24" Hours	at "36" Hours	at "48" Hours
Amine impurity	0.19	0.22	0.23	0.23	0.23
Dihydro impurity	0.10	0.11	0.10	0.11	0.11
Benzyl impurity	0.16	0.17	0.17	0.17	0.16

CONCLUSION

The developed stability-indicating analytical method for related substance determination of Tofacitinib Citrate and its impurities is precise, accurate, linear and specific. The validation carried out for the method in accordance with the ICH requirements are satisfactory. The developed method can be used conveniently for the routine analysis of production samples and also to check the stability of bulk samples of Tofacitinib Citrate during its storage.

The same method can also be attempted for the drug products for the getting the information of impurities and degradation products at lower level.

Acknowledgments
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