Original Article

Development And Validation Of Stability Indicating Rp-Hplc Method For The Quantification Of Amine Impurity In Tofacitinib Tablets Dosage Form

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Abstract

Highly sensitive method for the determination of degradation impurity such as Methyl-[(3R,4R)-4-methyl-piperidin-3-yl]-(7H-pyrrolo[2,3-d]pyrimidin-4yl)-amine(Amine impurity) in Tofacitinib solid dosage form by using RP-HPLC method. Samples are analysed by reverse phase (RP-HPLC) using stationary phase Inert Clone ODS(3) (250 x 4.6mm, 5µm) column and the mobile phase-A consisted of pH 3.0 phosphate buffer and the mobile phase-B consisted of Acetonitrile in the proportion of gradient elution. The flow rate is 1.0 mL/min, the column oven was preserved at 40°C and sampler cooler oven was preserved 5°C, injection volume 25 µL and wavelength fixed at 210nm. The established HPLC method was validated with admiration to specificity, precision, linearity, accuracy, LOD, LOQ and solution stability. Validation study compared as stated by ICH instruction.

Key words: Tofacitinib, Amine impurity, Forced degradation, and liquid chromatography.

1.0 Introduction

Tofacitinib chemically known as 3-[(3R, 4R) - 4 -methyl-3-[methyl (7H-Pyrrolo [2, pyrimidine-4yl) amino] piperidin-1-yl]-3- oxopropanenitrile. It is an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis [1]. Cytokines work within a complex regulatory network in RA, signaling through different intracellular kinase pathways to modulate the recruitment, activation, and function of immune cells and other leukocytes [2-6]. Several research works elucidated the safety and efficacy of Tofacitinib drug [7-14]. The chemical structure of Tofacitinib [15-16] was shown in **Figure 1.**

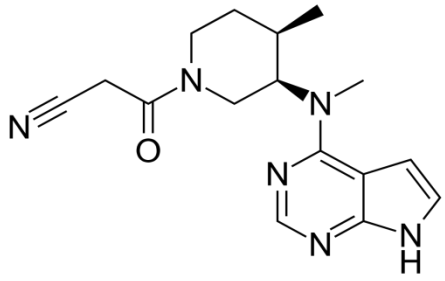

Figure 1. Chemical structure of Tofacitinib

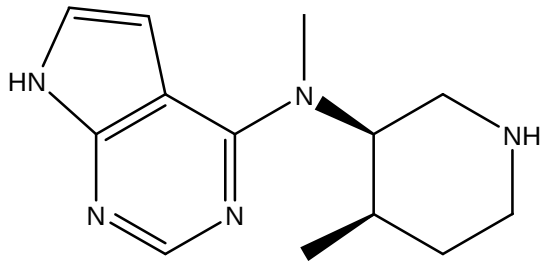

Figure 2. Chemical structure Tofacitinib Amine impurity

Amine impurity: N-Methyl-N-((3R,4R)-4-methylpiperidin-3-yl)- 7H-pyrrolo[2,3-d] pyrimidin-4-amine
The literature survey reveals that there are no LC methods were reported in major pharmacopoeias like USP, EP, JP and BP. Only few methods were reported till date for the estimation of Tofacitinib by using RP-HPLC methods [17-19] and HPTLC [20] methods were reported for the estimation of Tofacitinib in pharmaceutical dosage forms.
Hence we tried to develop stability indicating HPLC method for amine impurity in Tofacitinib in tablets dosage form. The present work describes a simple, stability indicating HPLC method for the determination of amine impurity in Tofacitinib in tablets dosage form according to ICH guidelines [21-22].

2.0 Experimental

2.1 Reagents and chemicals
Potassium dihydrogen orthophosphate, Orthophosphoric, Acetonitrile, Hydrochloric acid, Sodium hydroxide, and Hydrogen peroxide was procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received. Impurities are procured from SynZeal Research Private Limited, Ahmedabad, Gujarat. Tofacitinib 5 mg and 10 mg Tablets (Tofadoz) was procured from local market.
2.2 Instrumentation
Chromatographic separation was achieved by using an waters alliance e2695, Empower^3 software. Stationary phase was using an Inert Clone ODS(3) (250 x 4.6mm, 5µm) and the mobile phase-A consisted of pH 3.0 phosphate buffer and the mobile phase-B consisted of Acetonitrile in the proportion of gradient elution. The HPLC gradient program was set as (time/% mobile phase- B) 0.0/15, 10/15, 20/50, 30/50, 31/15, 40/15. The flow rate is 1.0 mL/min, the column oven was preserved at 40°C and sampler cooler temperature was preserved 5°C, infused 10µL and wavelength 210nm. The run time was 40 minutes.
2.3 Preparation of solutions
Preparation of mobile phase-A
Weighed accurately 1.36 g of potassium dihydrogen orthophosphate into 1000 mL of water sonicated to dissolve and mixed well then pH was adjusted to 3.0 with ortho phosphoric acid solution. Filtered through 0.45 µm membrane filter.
Preparation of mobile phase-B
Used Acetonitrile as mobile phase-B
Preparation of diluent
Prepared a mixture of water and acetonitrile in the ratio of 80:20 (%volume/volume) mixed well and sonicated to degas.
Preparation of standard stock solution
Weighed accurately 15.45 mg of Tofacitinib working standard into a 100 mL volumetric thermos, added 65 mL of diluent sonicated for 2 minutes to dissolved, diluted to quantity with diluent and mixed well.
Preparation of standard solution
Further diluted 1.0 mL of standard stock solution keen on 100 mL volumetric flask, made up to quantity with diluent and mixed well.
Preparation of sensitivity solution

Transferred 5.0 mL of standard solution into 50 mL volumetric flask and diluted to quantity with diluent and mixed well.

Preparation of amine impurity stock solution

Weighed accurately 2.5314 mg of Amine impurity into 25 mL volumetric flask, added 10 mL of diluent and sonicate to dissolved, cool to room temperature, made up to volume with diluent and mixed well.

Preparation of system suitability solution

Weighed accurately 15.28 mg of Tofacitinib working standard into 50 mL volumetric flask, added 25 mL of diluent and sonicate to dissolved. To it added 0.75 mL Amine impurity stock solution, diluted to volume up to the mark with diluent and mix well.

Preparation of placebo solution

Weighed and transferred Tofacitinib placebo powder (equivalent to 30 mg of Tofacitinib) into 100 mL volumetric flask, added 70 mL of diluent, sonicated for 30 minutes, diluted to volume with diluent and mixed well. Filtered the solution through 0.45 µm PVDF syringe filter.

Preparation of test solution

Weighed 10 tablets (Tofacitinib tablets 10 mg) crushed into fine powder. Weighed and transferred Tofacitinib sample powder (equivalent to 30 mg of Tofacitinib) into 100 mL volumetric flask, added 70 mL of diluent, sonicated for 30 minutes to dissolve, diluted to volume with diluent and mixed well. Filter the solution through 0.45 µm PVDF syringe filter.

3.0 Method development

Method optimization parameters

An sympathetic of the character of API (functionality, acidity, or basicity), the synthetic procedure, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 210 nm from the absorption spectrum.

Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C18 columns could be appropriately used for the separation of related substances for Tofacitinib.

Selection of mobile phase

Different mobile phases are employed to develop a suitable LC method for the quantitative determination of impurities in Tofacitinib, different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Tofacitinib.

4.0 Method validation

4.1 Specificity

Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution, spiked sample and individual impurities as well as analyzed as confirmed by the test method. It was scrutinized that identified impurities are not co eluting with apiece additional and foremost analyte crest.

Table 1. Impurity interference data (Specificity results)

S.No.	Name of the solution		Retention time	Blank	Placebo
1	Blank solution		ND	NA	NA
2	Placebo solution		ND	NA	NA
3	Standard solution	Tofacitinib	11.75	No	No

4	Individual solution	Amine impurity	3.83	No	No
5	Sample solution	Amine impurity	ND	No	No
		Tofacitinib	12.07	No	No
6	Spiked sample solution	Amine impurity	3.82	No	No
		Tofacitinib	12.07	No	No

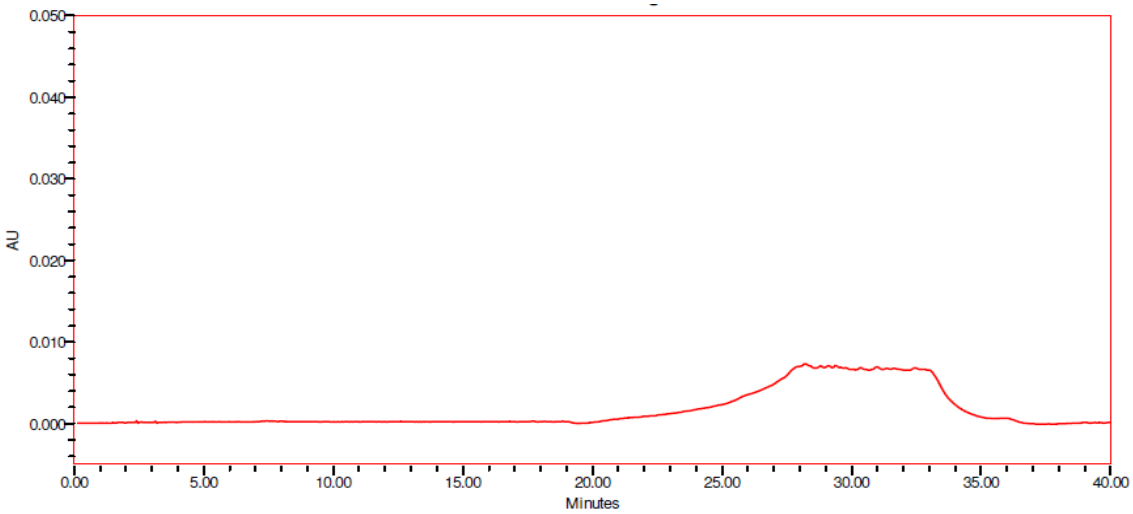

Figure 3. typical chromatogram of blank

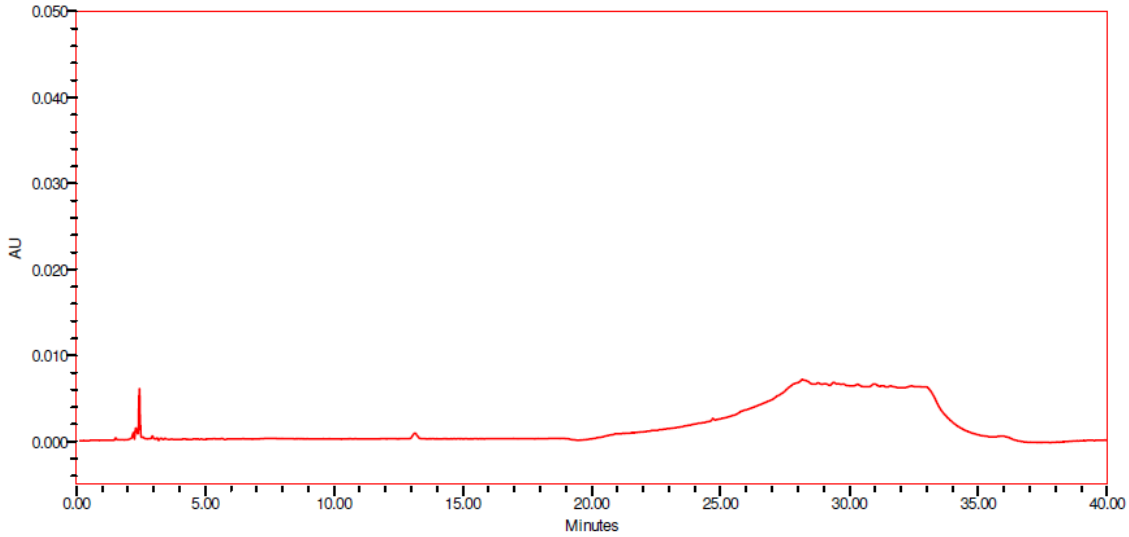

Figure 4. typical chromatogram of placebo

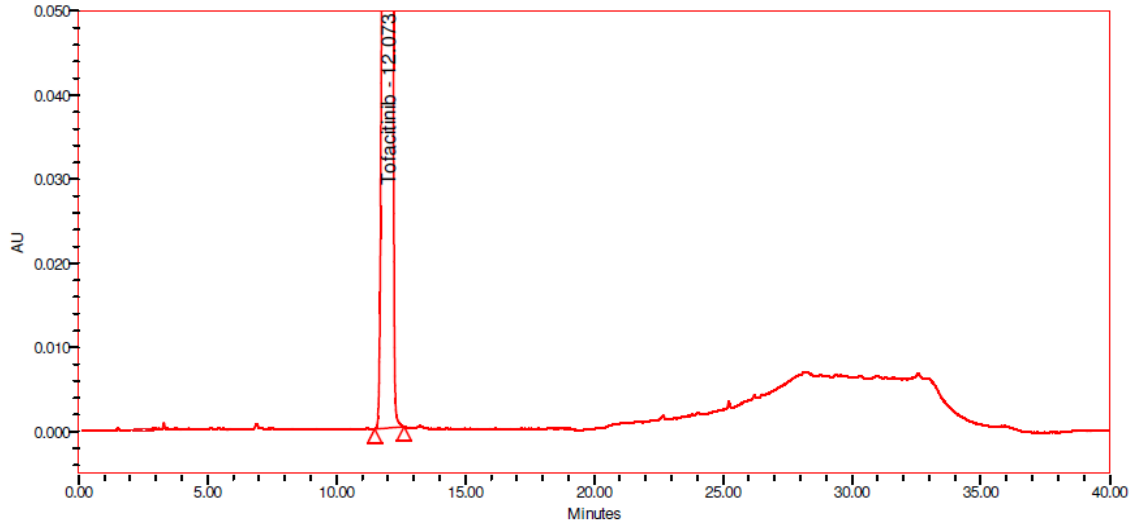

Figure 5. typical chromatogram sample

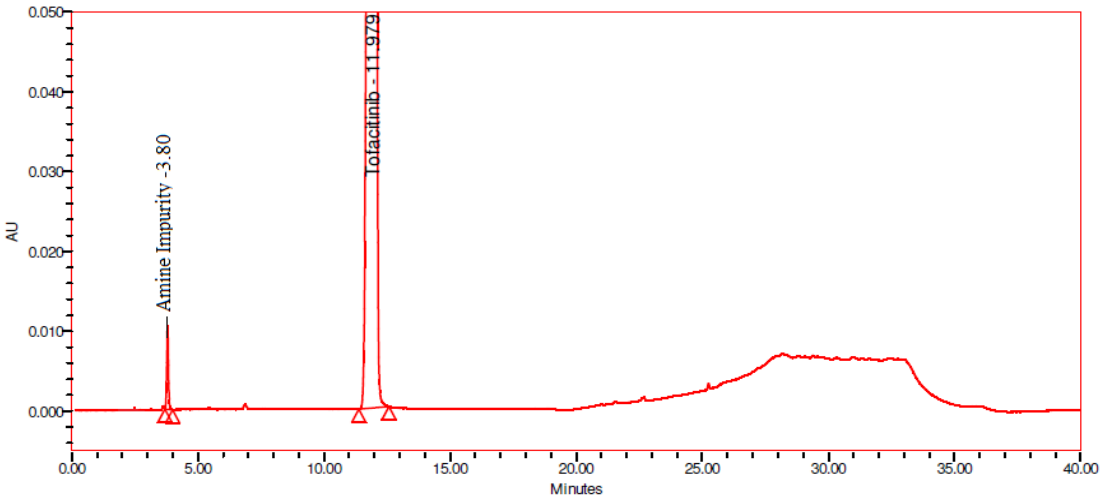

Figure 6. typical chromatogram spiked Sample

Forced degradation study

Sample solutions and placebo solutions were exposed to the following stress conditions to degradation. Stressed and unstressed samples were injected into the HPLC system with photo diode array detector. All degrading peaks were resolved from amine impurity and Tofacitinib peaks in the chromatograms of all samples and placebos did not show any interference at the retention time of amine impurity and Tofacitinib.

Table 2. Forced Degradation results

S.No.	Name of the Solution	Amine impurity (%)	Total impurities (%)	Mass balance (%)
1	Unstressed sample	ND	0.04	NA
2	Acid stress sample (1N HCl/5mL/60°C/2hrs)	4.45	4.81	95.23
3	Base stress sample (0.05N NaOH/5mL/RT/5 minutes)	18.68	19.87	80.16
4	Peroxide stress sample (3% H2O2/5mL//6.0hrs at RT)	3.82	3.93	96.11
5	Humidity stress sample (90%RH/48 hrs)	0.11	0.21	99.83

6	Thermal stress sample (105°C/48 hrs)	0.09	0.18	99.86
7	UV stress sample (200 watt hours/m2 for 7 days)	0.22	0.46	99.58
8	Photolytic stress sample (1.2 million Lux hours)	0.27	0.49	99.55

Degradation study results were shown significant degradation was observed in alkali (base) stress conditions. Hence it can be concluded that Tofacitinib is sensitive to alkali. The results proved that the developed method has good selectivity and specificity.

4.2 Precision

4.2.1 System precision

System precision was demonstrated by preparing standard solution as per the test method and injecting the same into HPLC system in six replicated injections. The peak areas of analyte was recorded for these standard injections. The System precision was evaluated by computing the % relative standard deviation for the peak area of these standard injections. The observations are tabulated below.

Table 3. System precision data for standard

S.No.	No.of Injections	Area Response
1	Injection-1	97353
2	Injection-2	96663
3	Injection-3	97137
4	Injection-4	96581
5	Injection-5	96324
6	Injection-6	96431
Average		**96748**
STDV		**408.2180**
% RSD		**0.42**

The %RSD of peak area for Tofacitinib standard was established 0.42% which is underneath 5.0% designates that the system gives precise result.

4.2.2 Method exactitude

Method precision was demonstrated by preparing six samples of Tofacitinib tablets 5 mg and 10 mg and six samples by spiking of amine impurity at specification level as per test method and injected in to the chromatographic system. The precision of the method was evaluated by calculating the impurities found and % relative standard deviation for impurities found for each set of samples. The results of the precision study are tabulated below.

Table 4. Results of method precision

S.No.	Sample Details	Amine impurity (% Recovery)
1	Spiked sample Prep-1	98.9
2	Spiked sample Prep-2	99.1
3	Spiked sample Prep-3	100.3
4	Spiked sample Prep-4	99.5
5	Spiked sample Prep-5	99.0
6	Spiked sample Prep-6	100.1

Average	**99.5**
STDV	**0.5947**
% RSD	**0.60**

The results were well within the limits. From the above results, it is concluded that method is precise.

4.3 Limit of Quantitation (LOQ) and Limit of Detection (LOD)

A solution containing 0.0998 and 0.0329 µg/mL of Tofacitinib, 0.0567 and 0.0187 µg/mL of amine impurity was injected six times. The %RSD areas of each impurity and standard were calculated.

Table 5. LOQ precision results

S.No.	**Amine impurity**	**Tofacitinib**
1	5057	9236
2	5001	8526
3	4922	8955
4	4821	8301
5	4943	9582
6	4679	9294
Average	**4904**	**8982**
STDEV	**135.7877**	**488.6782**
% RSD	**2.77**	**5.44**

The limit of quantitation values obtained for amine impurity and Tofacitinib are within the acceptance criteria.

4.4 Linearity

The linearity of an analytical method is its ability to obtain test results which has a definite mathematical relation to the concentration of the analyte. The linearity of responses for amine impurity and Tofacitinib in the specified concentration range 0.0998-0.725 µg/mL and 0.0567-0.7245 µg/mL the correlation coefficient were found to be 0.9999 and 0.9999. The calibration curve of the analytical method was assessed by plotting concentration versus peak area and represented graphically. Therefore the HPLC method was found to be a linear standard curve that was calculated and given in **Figure 7 and Figure 8.** to demonstrate the linearity of the proposed method. From the data obtained which is given in **Table 6 and Table 7**.

Table 6. Linearity results of Amine impurity

S.No.	Linearity Level	Concentration in ppm	Area response
1	LOQ	0.0998	4945
2	25	0.125	6494
3	50	0.251	12537
4	100	0.501	24994
5	125	0.625	30998
6	150	0.725	35965
Correlation coefficient (r^2)			0.9999
Slope			49,401.4201
Intercept			164.2960
%Y-Intercept			0.66

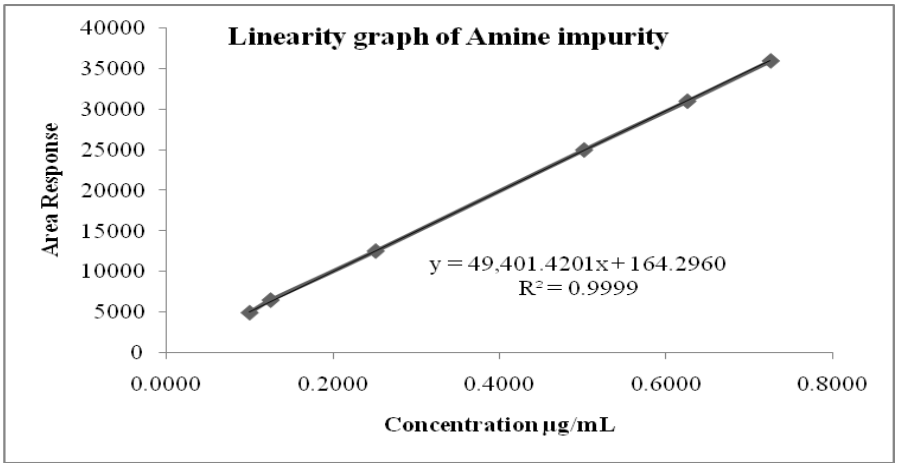

Figure 7. Linearity graph of amine impurity

Table 7. Linearity results of Tofacitinib

S.No.	Linearity Level	Concentration in ppm	Area response
1	LOQ	0.0567	8985
2	25	0.1251	20824
3	50	0.2524	40997
4	100	0.5017	79502
5	125	0.6257	98999
6	150	0.7245	114808
Correlation coefficient (r^2)			0.9999
Slope			157353.7058
Intercept			731.45
%Y-Intercept			0.92

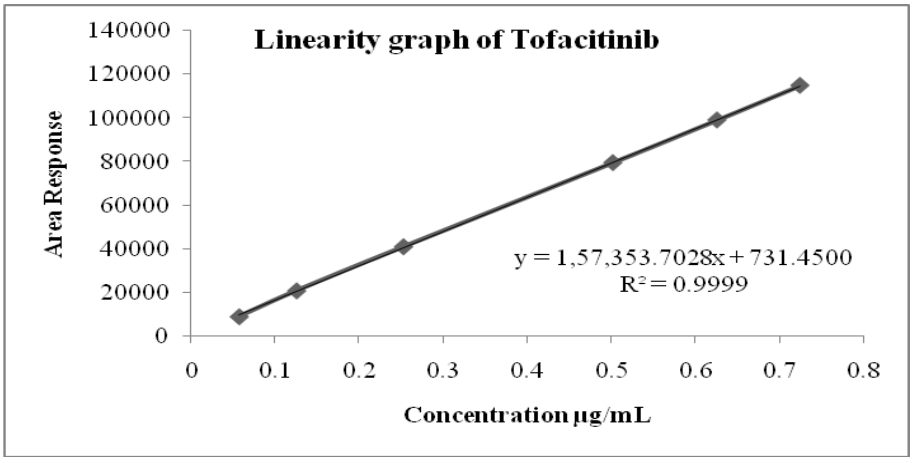

Figure 8. Linearity graph of Tofacitinib

The linearity results for Amine impurity and Tofacitinib in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

4.5 Accuracy

The accuracy of the test method was demonstrated by preparing recovery samples of Tofacitinib at LOQ to 150% of the target concentration level. The recovery samples were prepared in triplicate preparations of amine

impurity spiked to Tofacitinib sample The above samples were chromatographed and the percentage recovery of each sample was calculated for the amount added. The data obtained which given in Table 8. and the method was found to be accurate.

Table 8. Accuracy results of Amine impurity

% Level	%found	% spiked	% Recovery	Mean % Recovery	% RSD
LOQ Level-1	0.021	0.0191	109.9	111.7	2.71
LOQ Level-2	0.022	0.0191	115.2		
LOQ Level-3	0.021	0.0191	109.9		
100% Level-1	0.4947	0.5114	96.7	97.3	0.50
100% Level-2	0.4993	0.5114	97.6		
100% Level-3	0.4987	0.5114	97.5		
150% Level-1	0.7151	0.7257	98.5	98.8	0.42
150% Level-2	0.7148	0.7257	98.5		
150% Level-3	0.7201	0.7257	99.2		

Accuracy at LOQ level to 150% level for amine impurity is meeting the acceptance criteria. From the above results, it is concluded that method is accurate.

4.6 Solution stability

Stability of solutions such as standard solution and sample solutions was established at various conditions such as bench top and in refrigerator (2-8°C). The response of these was compared with respect initial standard solution and spiked sample solution.

Solution stability parameter was established, standard solution were stable upto 48 hrs on a bench top and in refrigerator and sample solutions were stable upto and 48 hrs on a bench top and in refrigerator condition.

Table 9. Results for solution stability of standard

Time Interval	Similarity factor	
	Room temperature	Refrigerator
Initial	NA	NA
24 hrs	1.04	1.01
48 hrs	1.05	1.02

Table 10. Results for solution stability of test solution at room temperature

Component	Initial	After 24 hrs	% Difference	After 48 hrs	% Difference
Amine impurity	ND	ND	NA	ND	NA
Maximum unknown impurity	0.033	0.036	0.003	0.041	0.008
Total impurities	0.11	0.17	0.06	0.22	0.11

Table 11. Results for solution stability of test solution at refrigerator

Component	Initial	After 24 hrs	% Difference	After 48 hrs	% Difference
Amine impurity	ND	ND	NA	ND	NA
Maximum unknown impurity	0.033	0.035	0.002	0.037	0.004

Total impurities	0.11	0.15	0.04	0.19	0.08

Table 12. Results for solution stability of spiked sample at room temperature

Component	Initial	After 24 hrs	% Difference	After 48 hrs	% Difference
Amine impurity	0.512	0.519	0.007	0.523	0.11

Table 13. Results for solution stability of spiked sample at refrigerator

Component	Initial	After 24 hrs	% Difference	After 48 hrs	% Difference
Amine impurity	0.512	0.515	0.003	0.518	0.006

5.0 RESULTS & DISCUSSION

A simple, economic, accurate and precise HPLC method was productively developed. In this method it was carried out by using stationary phase Inert Clone ODS(3) (250 x 4.6mm, 5µm) column and the mobile phase-A consisted of pH 3.0 phosphate buffer and the mobile phase-B consisted of Acetonitrile in the proportion of gradient elution. The HPLC gradient program was set as (time/% mobile phase- B) 0.0/15, 10/15, 20/50, 30/50, 31/15, 40/15. The flow rate is 1.0 mL/min, the column oven was preserved at 40°C and sampler cooler temperature was preserved 5°C, infused 10µL and wavelength 210nm. The run time was 40 minutes.

The results obtained were accurate and reproducible. The method developed was statistically authenticated in terms of selectivity, accuracy, linearity, precision and stability of solution.

For selectivity, the chromatograms were recorded for blank, placebo, standard, sample and spiked sample solutions of amine impurity and Tofacitinib. Selectivity studies reveal that the peaks are well separated from each other. Therefore the method is selective for the determination of amine impurity in Tofacitinib. There is no interference between blank and placebo at amine impurity and Tofacitinib peaks. The elution order and the retention times of amine impurity and Tofacitinib obtained from individual standard preparations and mixed standard preparations are comparable.

Degradation study results were shown significant degradation was observed in alkali (base) stress conditions. Hence it can be concluded that Tofacitinib is sensitive to alkali.

To obtain system precision, a study was conducted with six replicate injections. %RSD was estimated from the peak areas of Tofacitinib found to be 0.42% respectively.

The relative standard deviation for method precision was found to be 0.60% respectively.

The limit of quantitation (LOQ) and limit of detection for 0.0998 and 0.0329 µg/mL of Tofacitinib, 0.0567 and 0.0187 µg/mL of amine impurity respectively.

The linearity results for amine impurity and Tofacitinib in the specified concentration range 0.0998-0.725 µg/mL and 0.0567-0.7245 µg/mL the correlation coefficient were found to be 0.9999 and 0.9999.

The accuracy studies were shown as % recovery for amine impurity at LOQ to150% level level. The limit of % recovered shown is in the range of 80% and 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

The solution stability of the standard and samples are stable upto 48 hrs on a bench top and refrigerator (2-8°C).

6.0 CONCLUSION

The novel HPLC method developed and validated for determination of amine impurity in Tofacitinib tablets dosage form and assured the satisfactory precision and accuracy and also determining lower concentration of drug in its solid dosage form by RP-HPLC method. The method was found to be simple, accurate, economical and rapid and they can be applied for routine analysis in laboratories and is suitable for the quality control.

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