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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF TOFACITINIB DIHYDROCHLORIDE IN PURE FORM AND PHARMACEUTICAL TABLET DOSAGE FORM

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Abstract:

An Analytical, accurate, rugged, rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Tofacitinib Dihydrochloride in bulk form and as well as in pharmaceutical dosage forms. Chromatography was carried out on a Symmetry C18 (4.6mm x 150mm, 5µm) column using a mixture of Methanol and water (45:55% v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 260nm. The retention time of the Tofacitinib Dihydrochloride was 2.368 ±0.02min respectively. The method produce linear responses in the concentration range of 24-120mg/ml of Tofacitinib Dihydrochloride. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations. The developed method was validated for accuracy, precision, linearity, robustness, ruggedness and LOD & LOQ of standard solution. The developed RP-HPLC method was found to be accurate, precise, linear, and robust and was successful applied to a pharmaceutical tablet formulation for qualitative estimation of Tofacitinib Dihydrochloride in Bulk form and Marketed Pharmaceutical Dosage forms.

Keywords: *Tofacitinib Dihydrochloride, RP-HPLC, Method Development, Validation, Accuracy.*

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INTRODUCTION:

Tofacitinib is a Janus kinase (JAK) inhibitor used to treat rheumatic conditions, such as rheumatoid arthritis and ankylosing spondylitis, and ulcerative colitis. It is approved by the FDA for treatment of moderate to severe rheumatoid arthritis that responds inadequately to methotrexate or in those who are intolerant to Methotrexate[1]. Besides rheumatoid arthritis, Tofacitinib has also been studied in clinical trials for the prevention of organ transplant rejection, and is currently under investigation for the treatment of psoriasis. Known adverse effects include nausea and headache as well as more serious immunologic and hematological adverse effects. Tofacitinib is marketed under the brand name Xeljanz by Pfizer[2]. Tofacitinib is used alone or together with other medicines (eg, DMARDs) to treat moderate to severely active rheumatoid arthritis, active psoriatic arthritis, ulcerative colitis, ankylosing spondylitis, and polyarticular course juvenile idiopathic arthritis (pcJIA) in patients who have taken other medicines[3]. The IUPAC Name of Tofacitinib Dihydrochloride is (3R, 4R)-4-Methyl-3-[methyl (7H-pyrrolo [2, 3-d] pyrimidin-4-yl) amino]-β-oxo-1-piperidinepropanenitrile dihydrochloride. The Chemical Structure of Tofacitinib Dihydrochloride is as following

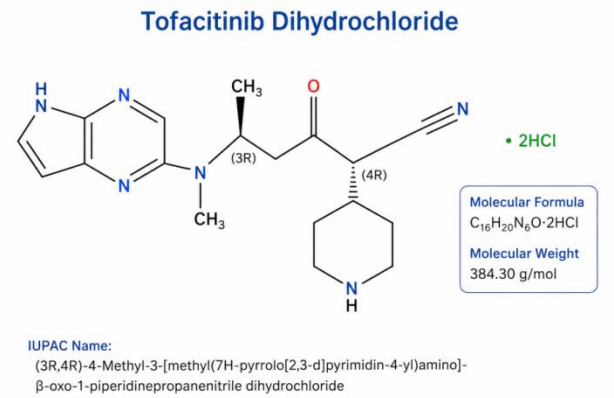

Fig-1: Chemical Structure of Tofacitinib Dihydrochloride

A thorough literature survey[25-28] of Tofacitinib Dihydrochloride revealed that very few analytical methods had been reported for estimation of Tofacitinib Dihydrochloride. Majority of methods for determination of Tofacitinib Dihydrochloride in biological fluids and pharmaceutical dosage forms includes LC-MS/MS, LC-MS, and HPTLC-LC, HPTLC, UPLC-MS, HPLC-MS, RPHPLC and UV-Visible Spectrophotometric methods. This novel proposed method contributes quick estimation, correct peak shape, precise, simple, and quick, use of smaller sample volumes and utilizing Suitable Solvent System as a mobile phase which is economical when compared with other existing methods. So, it is necessary to develop a simple, precise, and rapid RP-HPLC method for the quantitative determination of Tofacitinib Dihydrochloride. This work describes the validation parameters[4] stated by the International Conference on Harmonization [ICH] guidelines[23-24] Q2 (R1).

EXPERIMENTAL

Table-1: Instruments Used

S.No.	Instruments and Glasswares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, PDA 996 Detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table-2: Chemicals Used

S.No.	Chemical	Brand names
1	Tofacitinib Dihydrochloride (Pure)	Synpharma Research Lab, Hyderabad
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.72ml of the above Tofacitinib Dihydrochloride stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and Water in proportion 45:55 v/v respectively.

Optimization of Column:

The method was performed with various C18 columns like ODS column, Xterra, and X Bridge C18 column. Symmetry C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Mobile Phase:

Accurately measured 450 ml (45%) of HPLC Methanol and 550 ml of HPLC Water (55%) were mixed and degassed in a digital ultrasonicator for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

METHOD VALIDATION PARAMETERS

System Suitability

Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.72ml of the above Tofacitinib Dihydrochloride stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the

area of five replicate injections was found to be within the specified limits.

Specificity:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.72ml of the above Tofacitinib Dihydrochloride stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution:

Take average weight of the Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Tofacitinib Dihydrochloride sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent[5] and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.72ml of Tofacitinib Dihydrochloride above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay[6] by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (24ppm of Tofacitinib Dihydrochloride):
Pipette out 0.24ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – II (48ppm of Tofacitinib Dihydrochloride):
Pipette out 0.48ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – III (72ppm of Tofacitinib Dihydrochloride):
Pipette out 0.72ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – IV (96ppm of Tofacitinib Dihydrochloride):
Pipette out 0.96ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Preparation of Level – V (120ppm of Tofacitinib Dihydrochloride):
Pipette out 1.2ml of stock solution in to a 10ml volumetric flask and make up the volume up to mark by using diluent.

Procedure:
Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision
Repeatability
Preparation of Tofacitinib Dihydrochloride Product Solution for Precision:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.72 ml of the above Tofacitinib Dihydrochloride stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.
Procedure:
Day 1:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
Day 2:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.
Accuracy:

For Preparation of 50% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.36ml of the above Tofacitinib Dihydrochloride stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and

make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.72ml of the above Tofacitinib Dihydrochloride stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For Preparation of 150% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.08ml of the above Tofacitinib Dihydrochloride stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:
Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Tofacitinib Dihydrochloride and calculate the individual recovery and mean recovery values.

Robustness:
The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Tofacitinib Dihydrochloride working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.72ml of the above Tofacitinib Dihydrochloride stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of Flow Conditions:
The sample was analyzed at 0.7 ml/min and 0.9 ml/min instead of 0.8ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded

Effect of Variation of Mobile Phase Organic Composition:
The sample was analyzed by variation of mobile phase i.e. Methanol: Water was taken in the ratio and 40:60, 50:50 instead of 45:55, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION:

Development of Analytical Method:
Optimized Chromatographic Conditions: The chromatographic conditions were systematically optimized to achieve satisfactory separation, resolution, peak symmetry, and reproducible retention of the analyte. The optimized RP-HPLC method was performed using a Symmetry C18 column (4.6 × 150 mm, 5 µm particle size) as the stationary phase. A mixture of methanol and water in the ratio of 45:55 (v/v) was selected as the mobile phase and used under isocratic conditions. The mobile phase was delivered at a flow rate of 0.8 mL/min, which provided an appropriate balance between chromatographic resolution and analysis time. The column temperature was maintained at 40°C throughout the analysis to ensure consistent retention and peak characteristics. The analyte was detected using a UV detector at a wavelength of 260 nm, which was selected based on the adequate response of the drug at this wavelength. A 10 µL injection volume was used for each chromatographic run. Under these optimized conditions, a total run time of 6 minutes was found to be sufficient for complete elution and proper separation of the analyte. The optimized chromatographic conditions resulted in sharp, symmetrical, and reproducible peaks with satisfactory chromatographic performance and were therefore selected for further method validation studies.

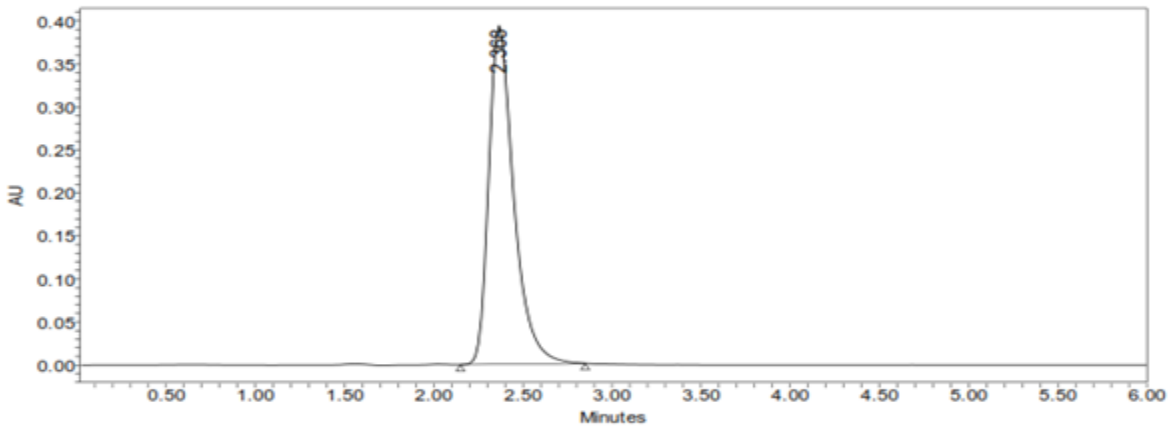

Fig-2: Optimized Chromatographic Condition of Tofacitinib Dihydrochloride

Observation: In this trail it shows well peak shape and proper plate count and tailing under limit in the chromatogram. So it's optimized chromatogram.

Method Validation

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters such as specificity, system suitability, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ) as per ICH Q2 (R1) guidelines.

System Suitability:

The chromato-graphic systems used for analysis must pass system suitability[7] before going to start the experiment. At first HPLC system is stabilized for forty minutes. Inject blank preparation (single injection) and standard preparation (five replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and retention time. The % RSD for the peak area of five replicate injections of Tofacitinib Dihydrochloride standard NMT 2.0. The parameters, such as tailing factor, % RSD, and theoretical plates, were studied.

Table-3: Results of System Suitability for Tofacitinib Dihydrochloride

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate	USP Tailing
1	Tofacitinib Dihydrochloride	2.317	2274631	239458	5728	1.2
2	Tofacitinib Dihydrochloride	2.302	2284721	239582	5093	1.2
3	Tofacitinib Dihydrochloride	2.323	2238127	236493	5391	1.2
4	Tofacitinib Dihydrochloride	2.343	2259349	249482	6139	1.2
5	Tofacitinib Dihydrochloride	2.321	2204850	239452	5281	1.2
Mean			**2252336**			
Std. Dev			**31827.08**			
% RSD			**1.41307**			

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity[8] to measure accurately quantitates Tofacitinib Dihydrochloride in drug product.

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Tofacitinib Dihydrochloride in pharmaceutical dosage form was found to be 99.7%.

Linearity: Standard stock solution of the Dasatinib (72 mg/ml) was prepared with the mobile phase. To study the linearity range[9] of drugs, serial dilutions were made from a standard stock solution in the range of 24-120 µg/ml.

Table-4: Linearity Data of Tofacitinib Dihydrochloride

Concentration Level (%)	Concentration µg/ml	Average Peak Area
33	24	791554
66	48	1647073
100	72	2283804
133	96	3058339
166	120	3839630

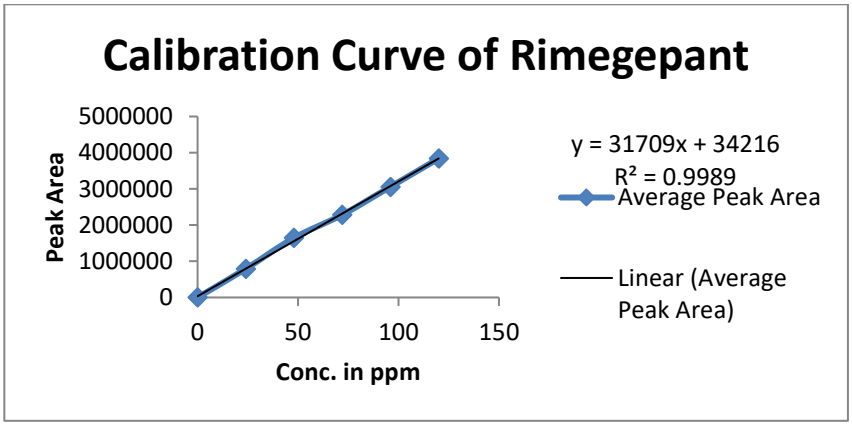

Fig-3: Calibration Curve of Tofacitinib Dihydrochloride

Linearity Plot: The plot of Concentration[10] (x) versus the Average Peak Area (y) data of Tofacitinib Dihydrochloride is a straight line.

Y = mx + c
Slope (m) = 31709
Intercept (c) = 34216
Correlation Coefficient (r) = 0.998

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 34216. These values meet the validation criteria[11].

Precision:

The precision[12] of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability: Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD[13]. The results were shown in Table-5.

Table-5: Results of Repeatability for Tofacitinib Dihydrochloride:

S. No.	Peak Name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Tofacitinib Dihydrochloride	2.356	2259464	245362	5938	1.2
2	Tofacitinib Dihydrochloride	2.356	2275915	248293	5827	1.2
3	Tofacitinib Dihydrochloride	2.357	2282117	240795	5032	1.2
4	Tofacitinib Dihydrochloride	2.358	2278675	230139	5978	1.2
5	Tofacitinib Dihydrochloride	2.359	2282448	249605	6183	1.2
Mean			**2275724**			
Std. Dev			**9476.485**			
%RSD			**0.416416**			

Intermediate Precision:

Analyst1:

Table-6: Results of Intermediate Precision for Tofacitinib Dihydrochloride

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Tofacitinib Dihydrochloride	2.380	2236184	202188	5472	1.2
2	Tofacitinib Dihydrochloride	2.383	2238020	201837	6193	1.2
3	Tofacitinib Dihydrochloride	2.385	2239352	201273	5980	1.2
4	Tofacitinib Dihydrochloride	2.385	2242466	203923	7163	1.2
5	Tofacitinib Dihydrochloride	2.389	2244692	202938	6182	1.2
6	Tofacitinib Dihydrochloride	2.389	2247654	201982	7684	1.2
Mean			**2241395**			
Std. Dev			**4333.851**			
% RSD			**0.193355**			

Analyst 2:

Table-7: Results of Intermediate Precision Analyst 2 for Tofacitinib Dihydrochloride

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Tofacitinib Dihydrochloride	2.380	2236184	217363	5928	1.2
2	Tofacitinib Dihydrochloride	2.383	2238020	218467	6183	1.2
3	Tofacitinib Dihydrochloride	2.385	2239352	218346	5927	1.2
4	Tofacitinib Dihydrochloride	2.385	2242466	221736	5163	1.2
5	Tofacitinib Dihydrochloride	2.389	2244692	228361	4827	1.2
6	Tofacitinib Dihydrochloride	2.346	2263431	217553	5019	1.2
Mean			**2244024**			
Std. Dev			**9988.458**			
% RSD			**0.445114**			

Accuracy:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery[14] was calculated.

Table-8: The Accuracy Results for Tofacitinib Dihydrochloride

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1172485	36	35.8	99.4	99.5%
100%	2314753	72	71.6	99.4	
150%	3480210	108	107.9	99.9	

Limit of Detection

The detection limit[15] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

=5.5µg/ml

Quantitation Limit

The quantitation limit[16] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ=10\times\sigma/S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve[17]

Result:

=16.7µg/ml

Robustness

The robustness[18] was performed for the flow rate variations from 0.7 ml/min to 0.9ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Tofacitinib Dihydrochloride. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Tofacitinib Dihydrochloride were injected by changing the conditions of chromatography[19]. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table-9: Results for Robustness of Tofacitinib Dihydrochloride

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 0.8mL/min	3119086	2.379	5837	1.2
Less Flow rate of 0.7mL/min	2640811	2.763	5361	1.2
More Flow rate of 0.9mL/min	2640354	2.234	5231	1.2
Less organic phase	2640758	2.765	4503	1.5
More organic phase	2640125	2.236	4491	1.5

Stability Studies

The API (Tofacitinib Dihydrochloride) was subjected to worry conditions in numerous ways that to look at the speed and extent of degradation that's seemingly to occur within the course of storage and/or when administration to body. This is often one style of accelerated stability studies that helps United States deciding the fate of the drug that's seemingly to happen when on time storage, at intervals a awfully short time as compare to the important time or future stability testing. The various degradation pathways studied are acid chemical reaction, basic chemical reaction, thermal degradation, and photolytic degradation and Oxidation degradation.

Results of Degradation Studies: The results of the strain studies indicated the specificity of the tactic that has been developed. Tofacitinib Dihydrochloride was stable in all stress conditions except thermal stress condition. The results of forced degradation studies[20-22] are given in the following table-10.

Table-10: Results of Forced Degradation Studies of Tofacitinib Dihydrochloride API

Stress Condition	Time In hrs	Assay of Active Substance	Assay of Degraded Products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	92.985	7.015	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	91.062	8.938	100.0
Wet heat	24Hrs.	89.749	10.251	100.0
UV (254nm)	24Hrs.	95.625	4.375	100.0
3 % Hydrogen peroxide	24Hrs.	96.548	3.452	100.0

SUMMARY AND CONCLUSION:

A simple and reliable RP-HPLC method was successfully developed and validated for the determination of Tofacitinib Dihydrochloride in pharmaceutical dosage form. The optimized chromatographic conditions employed a Symmetry C18 column with methanol (45:55, v/v) as the mobile phase at a flow rate of 0.8 mL/min and detection at 260 nm. The developed method exhibited satisfactory system suitability, with a retention time of approximately 2.36 min, a theoretical plate count of 5837, and a tailing factor of 1.2. The method demonstrated good linearity over the concentration range of 24–120 µg/mL with a correlation coefficient of 0.998. The method showed good precision, with %RSD values below 2%, and accuracy with a mean recovery of 99.5%. The LOD and LOQ were found to be 5.5 and 16.7 µg/mL, respectively, while the method showed acceptable robustness under the studied variations. Overall, the validated RP-HPLC method is suitable for routine quantitative analysis of Tofacitinib Dihydrochloride in pharmaceutical dosage forms.

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