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DETERMINATION OF TUCATINIB BY VALIDATED REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: A new analytical simple, rapid, precise, accurate and reproducible RP-HPLC method for estimation of Tucatinib in bulk form and marketed pharmaceutical dosage forms. Separation of Tucatinib was successfully achieved on a Phenomenex Gemini ODS C18 (4.6mm x 250mm, 5µm) column in an isocratic mode of separation utilizing Methanol: Phosphate Buffer in the ratio of (25:75% v/v) at a flow rate of 1.0 mL/min and the detection was carried out at 272nm. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The response was found to be linear in the drug concentration range of 10-50mcg/mL for Tucatinib. The correlation coefficient was found to be 0.999 for Tucatinib. The LOD and LOQ for Tucatinib were found to be 1.1µg/mL and 3.2µg/mL respectively. The proposed method was found to be good percentage recovery for Tucatinib, which indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.

Keywords: Tucatinib, RP-HPLC, Method Development, Accuracy, ICH Guidelines.

INTRODUCTION

Tucatinib is a kinase inhibitor used to treat certain types of unresectable/metastatic HER-2 positive breast cancer. Tucatinib is a kinase inhibitor drug used with Trastuzumab and Capecitabine in the treatment of unresectable or metastatic HER-2 positive breast cancer1. It was developed by Seattle Genetics and approved by the FDA on April 17, 2020. Tucatinib (Tukysa) is a targeted tyrosine kinase inhibitor used to treat HER2-positive breast and colorectal cancers by blocking abnormal protein signals that cause cancer growth2. It is primarily used in combination with Trastuzumab and Capecitabine for advanced, metastatic, or unresectable cancers, and is noted for its ability to treat tumors that have spread to the brain. The IUPAC Name of Tucatinib is 6-N-(4, 4-dimethyl-5H-1, 3-oxazol-2-yl)-4-N-[3-methyl-4-([1, 2, 4] triazolo [1, 5-a] pyridin-7-yloxy) phenyl] quinazoline-4, 6-diamine3. The Chemical Structure of Tucatinib is follows

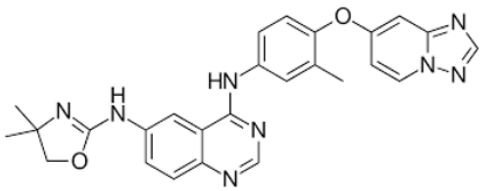


Fig-1: Chemical Structure of Tucatinib

EXPERIMENTAL METHODS

Table-1: Instruments Used

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S.No.	Instruments and Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA Detector.
2	pH meter	Labindia
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	Tucatinib (Pure)	Synpharma Research Lab, Hyderabad
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Tucatinib 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 0.3ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of the Powder and weight 10 mg equivalent weight of Tucatinib sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.3ml of the above Tucatinib 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 guidelines32-33.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase4 was optimized to Methanol: Phosphate Buffer 25:75 % v/v) respectively.

Optimization of Column:

The method was performed with various C18 columns like Symmetry, Zodiac and Xterra. Phenomenex Gemini ODS C18 (4.6mm x 250mm, 5µm) Column was found to be ideal as it gave good peak shape and resolution5 at 1ml/min flow.

Preparation of Mobile Phase:

Accurately measured 250 ml (25%) of HPLC Methanol and 750 ml of Phosphate Buffer (75%) were mixed and degassed in a digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Tucatinib 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 0.3ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

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 Tucatinib 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 0.3ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of the Powder and weight 10 mg equivalent weight of Tucatinib sample into a 10mL clean dry

volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.3ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the five replicate injections of standard and inject the three replicate injections sample solutions and calculate the assay6 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 Tucatinib working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents7 and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10ppm of Tucatinib):

Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – II (20ppm of Tucatinib):

Take 0.2ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – III (30ppm of Tucatinib):

Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – IV (40ppm of Tucatinib):

Take 0.4ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – V (50ppm of Tucatinib):

Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure:

Inject each level into the chromatographic system8 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 Tucatinib Product Solution for Precision:

Accurately weigh and transfer 10 mg of Tucatinib 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) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent. 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.

Intermediate Precision:

To evaluate the intermediate precision9 (also known as Ruggedness10) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

Analyst 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.

Analyst 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 Tucatinib 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) Take 0.15ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

For Preparation of 100% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Tucatinib 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) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

For Preparation of 150% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Tucatinib 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) Take 0.45ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions11. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Tucatinib and calculate the individual recovery12 and mean recovery values.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

Preparation of 0.597µg/ml solution (LOD):

Accurately weigh and transfer 10 mg of Tucatinib 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 0.00597ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Preparation of 1.811µg/ml solution (LOQ):

Accurately weigh and transfer 10 mg of Tucatinib 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 0.01811ml of the above Tucatinib stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

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 Tucatinib 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) Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 20µl 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: Phosphate Buffer was taken in the ratio and 30:70, 20:80 instead of 25:75, remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimization of Method:

Several trials were performed during method development to establish the optimum chromatographic conditions for the effective separation and accurate estimation of Tucatinib. Different chromatographic parameters, including mobile phase composition, flow rate, and detection wavelength, were evaluated to obtain a sharp peak, minimum peak tailing, and a suitable analysis time. The optimized mobile phase consisted of methanol and phosphate buffer in a ratio of 25:75% v/v, delivered at a flow rate of 1.0 mL/min. Chromatographic separation was achieved using a Phenomenex Gemini ODS C18 (4.6mm x 250mm, 5µm) maintained at ambient temperature. The detection wavelength was set at 272 nm, and the injection volume was maintained at 20 µL. The total chromatographic run time was fixed at 8 minutes. Under these optimized conditions, Tucatinib exhibited a retention time of 3.174 minutes (Figure 2). These conditions were selected for further analytical method validation to assess the method's accuracy, precision, linearity, specificity, and robustness for the estimation of Tucatinib.

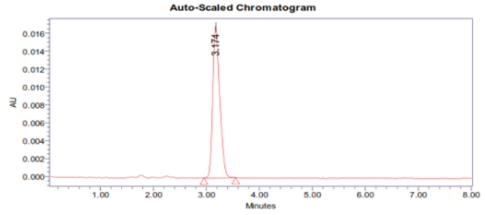


Fig-2: Optimized Chromatographic Condition of Tucatinib

Validation of Analytical Method

Validation of the developed HPLC method15 was carried out according to the International Council on Harmonization (ICH) guidelines Q2 (R1) for linearity and range, accuracy, precision, LOD and LOQ, specificity and robustness by the following procedure.

System Suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters16 like theoretical plates, resolution and asymmetric factor were evaluated. The results were presented in Table 3.

Table-3: Results of system suitability for Tucatinib

S. No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Tucatinib	3.19	225645	20584	6286	1.38
2	Tucatinib	3.14	225847	20965	6358	1.39
3	Tucatinib	3.12	228656	20758	6285	1.41
4	Tucatinib	3.16	228547	20859	6278	1.40
5	Tucatinib	3.15	229658	20968	6395	1.42
Mean			227670.6			
Std. Dev.			1810.899			
% RSD			0.795403			

Specificity

The specificity17 was studied to examine the presence of interfering components, while in the comparison of Chromatograms; there was no interference from blank and standard Chromatogram.

%ASSAY =

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

= 99.24%

The % purity18 of Tucatinib in marketed pharmaceutical dosage form was found to be 99.24%.

Linearity

Linearity was performed by preparing a standard solution of Tucatinib at different concentration levels, i.e., 10–50 µg/mL. The absorbance was measured at 272 nm. Linearity19 was proven by regression analysis by the least square method. The correlation coefficient and linearity results were presented in Table 4, and the linearity curve20 was represented in Figure 3.

Table-4: Data for Linearity of Tucatinib

Concentration µg/ml	Average Peak Area
10	78683
20	146545
30	213584
40	279895
50	346568

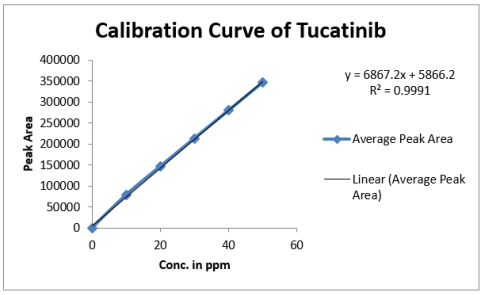


Fig-3: Calibration Curve of Tucatinib

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Tucatinib is a straight line.

Y = mx + c

Slope (m) =6867

Intercept (c) = 5866

Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient21 is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 5866. These values meet the validation criteria.

Precision

Precision22-25 was studied to find out intra-day and inter-day variation in the test methods of Tucatinib for 6 times on the same day and different day. The intra-day and inter-day precision26 obtained was %RSD (<2.0) indicates that the proposed method is quite precise and reproducible, and results are shown in Table 5 and 6 & 7.

Table-5: Results of Method Precision for Tucatinib:

S. No	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Tucatinib	3.165	225645	20562	6125	1.36
2	Tucatinib	3.163	225847	20645	6129	1.36
3	Tucatinib	3.158	226542	20534	6135	1.35
4	Tucatinib	3.167	226598	20564	6189	1.36
5	Tucatinib	3.171	226584	20549	6138	1.35
6	Tucatinib	3.181	226859	20685	6179	1.37
Mean			226345.8			
Std. Dev			482.1068			
%RSD			0.212996			

Intermediate Precision:

Analyst 1:

Table-6: Results of Ruggedness for Analyst 1 for Tucatinib

S. No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Tucatinib	3.165	226534	20653	6235	1.35
2	Tucatinib	3.163	226542	20598	6198	1.36
3	Tucatinib	30158	225989	20653	6254	1.36
4	Tucatinib	3.167	226512	20548	6281	1.35
5	Tucatinib	3.171	226531	20653	6199	1.36
6	Tucatinib	3.171	225898	20658	6253	1.35
Mean			226334.3			
Std. Dev.			304.2622			
% RSD			0.13443			

Analyst 2:

Table-7: Results of Intermediate Precision Analyst 2 for Tucatinib

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Tucatinib	3.173	225487	20542	6253	1.35
2	Tucatinib	3.134	225484	20532	6098	1.36
3	Tucatinib	3.161	225364	20541	6254	1.35
4	Tucatinib	3.174	226513	20534	6235	1.36
5	Tucatinib	3.199	225487	20549	6199	1.36
6	Tucatinib	3.199	226532	20451	6235	1.35
Mean			225811.2			
Std. Dev.			553.0524			
% RSD			0.244918			

Accuracy

The accuracy27 of the method was determined by the standard addition method. A known standard drug was added to the fixed amount of pre-analyzed drug sample solution. The standard addition method was performed at three concentration levels in triplicate at 50%, 100%, and 150%. Percent recovery (Table 8) was calculated by comparing the peak area before and after adding the standard drug. Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated.

Table-8: The Accuracy Results for Tucatinib

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109283.3	15	15.060	100.40%	100.42%
100%	212732	30	30.124	100.413%	
150%	316263.3	45	45.201	100.446%	

Limit of Detection

The detection limit28 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 × σ / s

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 0.597µg/ml

Limit of Quantitation

The quantitation limit29 of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

= 1.811µg/ml

Robustness

To demonstrate the method's robustness, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and organic content in the mobile phase. The results for robustness30 are represented in Table 9.

Table-9: Results for Robustness of Tucatinib

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	225645	3.155	6125	1.36
Less Flow rate of 0.9 mL/min	236586	3.488	6452	1.38
More Flow rate of 1.1 mL/min	219865	2.877	6098	1.42
Less organic phase	235848	4.705	6126	1.43
More organic phase	241245	2.090	6324	1.39

Forced Degradation Studies

Forced degradation study31 is used to identify degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating the power of the analytical procedures used. The nature of stress testing will depend on the individual drug substance and the type of drug product involved. Tucatinib were subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid and base hydrolysis, oxidation, thermal, UV light as mentioned in ICH Q1A (R2).

Table-10: Results of Forced Degradation Studies for Tucatinib

S. No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	225645	0	100%	100%
2	Acidic	190015.65	15.79	84.21	100%
3	Basic	187353.04	16.97	83.03	100%
4	Oxidative	190985.92	15.36	84.64	100%
5	Thermal	183020.65	18.89	81.11	100%
6	Photolytic	181034.98	19.77	80.23	100%
7	Water	210549.34	6.69	93.31	100%

SUMMARY AND CONCLUSION

An HPLC method was successfully developed and validated for the quantitative determination and assay analysis of Tucatinib in pharmaceutical dosage forms as per ICH guidelines. The optimized chromatographic system utilized a Phenomenex Gemini ODS C18 column with a mobile phase consisting of Methanol: Phosphate Buffer (25:75% v/v) at a flow rate of 1.0 mL/min and a detection wavelength of 272 nm. Method validation demonstrated excellent compliance with acceptance criteria, featuring high specificity with a 99.24% drug purity assay, robust linear response across standard concentrations with a correlation coefficient (r) of 0.99, outstanding precision (system suitability and repeatability %RSD well below the 2.0% limit), and highly accurate percentage recoveries averaging 100.42%. Robustness testing further confirmed that minor variations in operational flow rates and mobile phase ratios did not significantly impact peak suitability parameters. In conclusion, this developed HPLC method is highly specific, linear, precise, accurate, and robust, making it exceptionally reliable for the routine quality control and standardized evaluation of Tucatinib.

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