INTRODUCTION

Tucatinib belongs to a class of drugs known as kinase inhibitors [1, 2]. Tucatinib’s chemical name is N6-(4,4-dimethyl-4,5-dihydro-1,3-oxazol-2-yl)-N4-(3-methyl-4-[1,2,4]triazolo[1,5-a]pyridin-7-yloxyphenyl)quinazoline-4,6-diamine. Tucatinib’s molecular formula and molecular weight are C26H24N8O2 and 480.5212 μg/mol, respectively. Tucatinib, sold under the brand name Tukysa, is a small molecule inhibitor of HER2 for the treatment of HER2-positive breast cancer [3, 4]. It works by preventing the abnormal protein from signalling cancer cells to multiply. This aids in the prevention of signalling and cell proliferation, and has anti-tumor activity in HER2 expressing tumour cells. Tukysa is the brand name for tucatinib.

Tucatinib inhibits HER2 and HER3 phosphorylation [7, 8] in vitro, resulting in inhibition of downstream MAPK [9, 10] and AKT [11, 12] signalling and cell proliferation, and has anti-tumor activity in HER2 expressing tumour cells. Tukysa is the brand name for tucatinib.

Array BioPharma developed it. Patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer who progress after multiple HER2-targeted agents have few treatment options. Common side effects are diarrhea, palmar-plantar erythrodysesthesia [13, 14] (burning or tingling discomfort in the hands and feet), nausea, fatigue, hepatotoxicity [15] (liver damage), vomiting, stomatitis [16] (inflammation of the mouth and lips), decreased appetite [17], abdominal pain [18], headache, anemia and rash. Pregnant or breastfeeding women should not take Tucatinib because it may cause harm to a developing fetus or newborn baby.

There are only a few methods available for tucatinib. There are preclinical studies available, but no analytical methods are reported. The purpose of this research is to create, validate, and characterize forced degradation products using LC-MS/MS.

MATERIALS AND METHODS

Chemicals and reagents

Shree Icon Lab provided the pure Tucatinib standard (Vijayawada, India). Finar Chemicals supplied HPLC-grade acetonitrile and formic acid (Ahmedabad, India). Filtration through a Millipore MilliQ plus system produced HPLC grade water (Millipore, USA). Merck supplied analytical grade reagents of sodium hydroxide, hydrochloric acid, 30% hydrogen peroxide, and sodium bisulphate (Mumbai, India).

Instrumentation

HPLC

Waters alliance model e2695 liquid chromatography instrument, Waters (2998) Photodiode array Detector, Waters (2700) Auto sample injector, Solvent degasser, Quaternary pump, Temperature-controlled compartment. Using a sonicator, all of the solutions were sonicated for effective mixing and degassing (Unichrome associates 701). Weighing is done with an analytical balance (Denver). A hot air oven was used to conduct a thermal degradation study (KEMI).
An HPLC system (waters alliance e2695 model) connected with mass spectrometer QTRAP 5500 triple quadrupole instrument (sciex) was used [19-21].

Method optimization

Several parameters, including mobile phase, stationary phase, flow rate, and detector wavelength, were considered when developing and optimizing the chromatographic separation conditions. Isocratic elution with mobile phase 70:30v/v Acetonitrile and formic acid (0.1 percent) pumped from a solvent reservoir at a flow rate of 1.0 ml/min to the analytical column of the Inertsil ODS (250x4.6 mm,5m) using column back pressure of 1570-1620 at the maximum detector wavelength of 239 nm was used to obtain a chromatographic separation. Detector performance was evaluated using Empower-2 software to determine the height peak area and other device suitability parameters. The injection volume was set at 10μl, the ambient column temperature was maintained throughout the analysis.

Validation procedure

The developed method for estimation of Tucatinib was verified according to ICH regulations for system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection, and limit of quantification [22-25].

Preparation of mobile phase

Acetonitrile and formic acid were mixed in a 70:30v/v ratio then filtered through 0.45μm filter paper and sonicated to remove any gas.

Chromatographic conditions

Tucatinib was separated chromatographically in an Inertsil ODS (250x4.6 mm, 5m) column. The mobile phase was composed of acetonitrile and formic acid (70:30v/v) and was pumped at room temperature at a flow rate of 1 ml/min with a UV detection wavelength of 239 nm and filtered through a 0.45 μ nylon membrane filter under vacuum filtration. The injection volume was ten μ litres. Tucatinib had a retention time of 3.734 min, while the run lasted 6 min.

Diluents

Mobile phase was used as diluents.

Preparation of standard solution

Tucatinib standard stock solution was prepared by dissolving 100 mg of Tucatinib in 100 ml of diluents in a 100 ml clean and dry volumetric flask, and the standard solution was filtered through a 0.45μm membrane filter and degassed with a sonicator to obtain a concentration of 1000g/ml of Tucatinib.

Selection of wavelength

A UV-Visible spectrophotometer was used to scan the maximum absorption wavelength of 50g/ml Tucatinib against a blank mobile phase in the wavelength range of 200-400 nm. As shown in fig. 2, the maximum wavelength was discovered to be 239 nm.

Forced degradation of tucatinib

Acid degradation

Tucatinib solution was treated with 1N HCl heated for 6 h, and refluxed for 5 h before adding 1 ml of 1N NaOH and diluting volume with diluents. Inject the solution into the HPLC system several times over the course of 6 h, 12 h, 18 h, and 24 h.

Alkali degradation

Tucatinib solution was treated with 1N NaOH for alkali degradation, heated for 6 h, and refluxed for 5 h before adding 1 ml of 1N HCl and diluting volume with diluents. Inject the solution into the HPLC system several times over the course of 6 h, 12 h, 18 h, and 24 h.

RESULTS AND DISCUSSION

Method development and optimization

Initially, the RP-HPLC system was optimized using an Inertsil ODS (250x4.6 mm, 5m) column. To satisfy the system suitability parameters, a mixture of acetonitrile and formic acid (70:30v/v) as the mobile phase (Flow rate 1.0 ml/min) was found to be more appropriate. Table 1 summarizes the optimized chromatographic conditions. At 3.734 min, Tucatinib eluted as a sharp peak fig. 3.
Table 1: Optimized chromatographic conditions

| LC conditions          |                  |
|------------------------|------------------|
| Stationary phase       | Inertsil ODS (250x4.6 mm, 5 µm) |
| Mobile Phase           | Acetonitrile and 0.1% formic acid (70:30) |
| Elution mode           | Isocratic A: B = 70:30 % v/v |
| Flow rate              | 1.0 ml/min, |
| Sample volume          | 10µl using Rheodyne 7725i injector |
| Oven Temperature       | Ambient |
| MS conditions          |                  |
| Interface              | ESI |
| Operation mode         | MRM |
| Polarity               | Positive |
| Capillary voltage      | 4 KV |
| Fragmentor voltage     | 170 V |
| Skimmer voltage        | 65 V |
| Nebulizer Gas flow     | 40 psig |
| Drying gas             | 10 L/min |
| Gasoline temperature   | 325 °C |
| Detection              | m/z: 0-800 |
| Data station           | ABSCIEX |

Fig. 3: Chromatogram of standard

Method validation

The proposed method was validated using the ICH guidelines [26] for system suitability (table 2), linearity (table 3), precision (table 4), accuracy (table 5), and robustness (table 6). The calibration curve was created by plotting tucatinib concentrations on the X-axis and the corresponding mean peak area values on the Y-axis. Tucatinib follows Beer-law Lambert’s at concentrations ranging from 5 to 100 µg/ml, with the linear regression equation y = 35087x+2540.4 (correlation coefficient 0.999 fig. 4). The LOD and LOQ values are discovered to be 0.05 and 0.5, respectively. Using three different concentrations of tucatinib, the percentage relative standard deviation (RSD) was found to be 1.01 and 0.83, respectively (2.0 percent), demonstrating that the method is precise. The method’s accuracy [27] was demonstrated using the standard addition method, and the recovery values were calculated. The percentage RSD was found to be 0.5(2.0%), with a recovery rate of 98.4–101.3 percent. In the robustness study [28], the percentage RSD was found to be 0.2–1.21 (2.0 percent). The suitability of the system [29] and the stability of the solution was assessed, and the percentage RSD was 2%. The results are shown in table 2.

Table 2: Results of system suitability

| System suitability parameter | Acceptance criteria | Tucatinib | Std Dev | % RSD |
|------------------------------|---------------------|-----------|---------|-------|
| USP Plate Count              | NLT 2000            | 5594      | 30.651  | 0.55  |
| USP Tailing                  | NMT 2.0             | 1.10      | 0.015   | 1.34  |
| USP Resolution               | NLT 2.0             | -         | -       | -     |
| Retention time               | NLT 2.0             | 3.735     | 0.003   | 0.09  |

Table 3: Results of linearity

| S. No. | Conc (µg/ml) | Tucatinib area count |
|--------|-------------|----------------------|
| 1      | 5.00        | 1194880              |
| 2      | 12.50       | 459113               |
| 3      | 25.00       | 896209               |
| 4      | 37.50       | 1206347              |
| 5      | 50.00       | 1834505              |
| 6      | 62.50       | 2178517              |
| 7      | 75.00       | 2455617              |
| 8      | 100.00      | 3502114              |
| Correl coef | 0.99912  |
| Slope  | 35086.91    |
| intercept | 2540.47    |
Fig. 4: Calibration curve for tucatinib at 239 nm

Table 4: Results of method precision and Intermediate precision

| S. No. | Method precision\(^a\) | Intermediate precision\(^b\) |
|--------|-------------------------|-------------------------------|
|        | Conc. (µg/ml) | Area counts | % assay as is | Conc. (µg/ml) | Area counts | % assay as is |
| 1      | 50           | 1870847     | 98.9          | 50           | 1900654     | 100.5        |
| 2      | 1897995      | 1913999     | 101.2         | 1891351      | 1863262     | 98.5         |
| 3      | 1907151      | 1907151     | 100.8         | 1891671      | 1863262     | 101.4        |
| 4      | 1883482      | 1883482     | 99.6          | 1875031      | 1863262     | 99.2         |
| 5      | 1896710      | 1896710     | 100.3         | 1887356      | 1863262     | 99.8         |

\(%\) RSD 0.83
\(\text{mean}\) 100.2
\(\text{SD}\) 0.832
\(a, b\)-Mean+SD (n=6)

Table 5: Results of accuracy

| S. No. | % Level | Tucatinib % recovery | Mean % recovery | Std dev |
|--------|---------|----------------------|----------------|---------|
| 1      | 50      | 99.0                 | 100.1          | 1.18    |
|        |         | 100.1                |                |         |
|        |         | 101.3                |                |         |
| 2      | 100     | 100.9                | 99.7           | 1.28    |
|        |         | 100.0                |                |         |
|        |         | 98.4                 |                |         |
| 3      | 150     | 100.7                | 100.7          | 0.55    |
|        |         | 100.2                |                |         |
|        |         | 101.3                |                |         |

\(\text{Mean}+\text{SD} (n=3)\)

Table 6: Results of robustness

| Parameter name | % RSD tucatinib |
|----------------|-----------------|
| Flow minus (0.8 ml/min) | 0.20            |
| Flow plus (1.2 ml/min) | 0.70            |
| Organic minus (63:37) | 0.61            |
| Organic plus (77:23) | 1.21            |

\(\text{RSD}-\text{Relative standard deviation}; \text{All the values are presented as mean}+\text{SD (n=3)}\)

Degradation studies

Tucatinib was subjected to a variety of stress conditions [30], including acidic, oxidative, and alkaline hydrolysis. Tucatinib was eluted at 3.734 min in acidic hydrolysis. Tucatinib was eluted at 3.638 min with some degradants observed at 1.503 and 3.351 while performing acidic hydrolysis, with approximately 26.7 percent degradation observed. Tucatinib was eluted at 3.619 min during alkaline hydrolysis, with the other degradant observed at 1.336 min (drug degradation 24.7 percent). Tucatinib was eluted at 3.606 min during reduction, with degradant observed at 1.165 min and 21.6 percent degraded results are shown in table 7. The tucatinib peak was well separated among the degradants [31, 32] in all of the degradation studies, indicating that the method is selective and specific. Fig. 5 depicts typical chromatograms obtained during a stress degradation study of tucatinib.
Table 7: Forced degradation results for tucatinib

| Results: % degradation results at 24 h | Tucatinib | Mean area | % Assay | % Degradation |
|---------------------------------------|-----------|-----------|---------|---------------|
| Control                               | 1883286   | 99.6      | 0.4     |
| Acid                                  | 1378506   | 73.3      | 26.7    |
| Base                                  | 1424105   | 75.3      | 24.7    |
| Peroxide                              | 1466598   | 77.6      | 22.4    |
| Reduction                             | 1482593   | 78.4      | 21.6    |
| Thermal                               | 1489956   | 78.8      | 21.2    |
| Photolytic                            | 1492687   | 78.9      | 21.1    |
| Hydrolysis                            | 1482593   | 79.1      | 20.9    |

Data expressed as mean±SD (n=3)

![Degradation chromatogram](image)

Fig. 5: Degradation chromatogram of (a) Acidic (b) Alkaline (c) Oxidation (d) Reduction (e) Thermal (f) Photolytic (g) Hydrolysis
LC-MS/MS studies of forced degradation products

Four degradation products, DP1, DP2, DP3, and DP4 were identified and characterized by tandem mass spectrometric analysis (LC-MS/MS) and accurate mass measurement shown in table 4. DP1 and DP2 were degraded in acidic conditions, whereas DP3 was degraded in alkaline conditions, DP4 was degraded in reduction condition. The identification of active drug and its novel degradation product was studied on Agilent Q-TOF of Mass spectrometric (MS) technique with ABSCIEX. Mass measurements of Tucatinib was shown in table 8.

Table 8: Elemental composition and accurate mass measurements of Tucatinib and its degradation products

| Molecular formula | Calculate mass | Observed mass | ppm Error | MS/MS fragment ions formed |
|-------------------|----------------|--------------|-----------|---------------------------|
| Tucatinib         | C₂₆H₂₄N₈O₂   | 480.5230     | 480.5232  | 0.4162                    | 130, 242, 347 |
| DPI               | C₂₀H₁₉N₅O₂   | 363.0523     | 363.0526  | 0.8263                    | 257, 145, 114 |
| DPII              | C₁₃H₁₂N₂CIN₂O₂ | 321.0361     | 321.0364  | 0.9344                    | 175, 148     |
| DPIII             | C₁₃H₁₀N₄O₃  | 270.0639     | 270.0641  | 0.7405                    | 153, 119     |
| DP IV             | C₂₁H₁₇N₇O₅S | 479.0649     | 479.0653  | 0.8349                    | 362, 241, 145 |

Fig. 6: ESI–MS-MS spectrum of ions of (A) DP1 (B) DP2 (C) DP3 (D) DP4

Fig. 7: Degradation pathway of DP1
MS/MS of DP1
The LC-ESI-MS/MS spectrum of DP1 with m/z 363 (Rt= 1.503 min) was examined in the proposed fragmentation pathway (fig. 7). The degradation ions of m/z 257 (loss of C₇H₈O from the parent ion at m/z 363), m/z 145 (loss of C₅H₉N₂O from the parent ion at m/z 257), m/z 114 (loss of C₈H₇N₃ from the parent ion at m/z 257). Accordingly, data obtained from MS/MS, elemental composition, and precise mass measurements are shown in (fig. 7).

MS/MS of DP2
The LC-ESI-MS/MS spectrum of DP2 with m/z 321 (Rt= 3.351 min) was examined in the proposed fragmentation pathway (fig. 8). The degradation ions of m/z 175 (loss of C₅H₉ClNO₂ from the parent ion at m/z 321), m/z 148 (loss of C₈H₅N₃O₂ from the parent ion at m/z 321). Accordingly, data obtained from MS/MS, elemental composition, and precise mass measurements are shown in (fig. 8).

MS/MS of DP3
The LC-ESI-MS/MS spectrum of DP3 with m/z 270 (Rt= 1.336 min) was examined in the proposed fragmentation pathway (fig. 9). The degradation ions of m/z 153 (loss of C₆H₅N₃ from the parent ion at m/z 270), m/z 119 (loss of C₇H₇NO₃ from the parent ion at m/z 270). Accordingly, data obtained from MS/MS, elemental composition, and precise mass measurements are shown in (fig. 9).
The LC-ESI-MS/MS spectrum of DP4 with m/z 479 (Rt= 1.165 min) was examined in the proposed fragmentation pathway (fig. 10). The degradation ions of m/z 362 (loss of C₆H₅N₃ from the parent ion at m/z 479), m/z 241 (loss of C₆H₅NO from the parent ion at m/z 362), m/z 145 (loss of H₂S from the parent ion at m/z 241). Accordingly, data obtained from MS/MS, elemental composition, and precise mass measurements are shown in (fig. 10).

CONCLUSION

The validated stability indicating method developed for the determination of novel kinase inhibitors is specific and selective and the validated stability indicating method developed for the MS/MS, elemental composition, and precise mass measurements are shown in (fig. 10).

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

Declared none

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