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Original Article

# Development and Validation of a Novel UPLC-MS/MS Method for Quantification of Tovorafenib and Characterization of its Forced Degradation Products

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## ABSTRACT

**Aim and Objectives:** The current study was primarily aimed at the development of a validated stability indicating UPLC method for tovorafenib and its related impurities, and UPLC-MS/MS analysis of degradation products. **Materials and Methods:** The optimized chromatographic conditions included a phenyl column (2.1×100 mm, 1.7 µm) with a flow rate of 0.5 mL/min, an injection volume of 5 µL, and a mobile phase of a 30:70 ratio of acetonitrile and 0.1% formic acid buffer. The detection wavelength was established at 260 nm and the run time of the detection was 6.0 min. The developed approach was validated according to the ICH recommendations. Tests for degradation were carried out and MS/MS analysis was used to identify degradation impurities. **Results and Discussion:** The impurities and tovorafenib both fulfilled the required accuracy thresholds. The correlation co-efficient was observed to be more than 0.999. In terms of robustness and ruggedness, the % RSD was less than 2. The degradation products were discovered during acidic, alkaline, peroxide, and reduction analyses. The UPLC-MS/MS analysis characterized the fragmented ions formed from degradation product which is parent ion. **Conclusion:** The created approach complied with the ICH guidelines reliability requirements. Additionally, it used UPLC-MS/MS to successfully characterize degrading impurities.

**Keywords:** Tovorafenib, Method Validation, Degradation, Characterization and Mass Spectral Analysis.

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## INTRODUCTION

Ojemda (Tovorafenib) is a kinase inhibitor (DAY101, TAK-580, MLN2480, or BIIB024), a selective, central nervous system-penetrant, type II RAF inhibitor (RAFi).[1] Tovorafenib is indicated for the treatment of people 6 months of age and older with recurrent or refractory juvenile low-grade gliomas with a BRAF (v-raf murine sarcoma viral oncogene homolog B) fusion or rearrangement, or a BRAF V600 mutation.[2-4] IUPAC name of tovorafenib is 2-[(1*R*)-1-[(6-amino-5-chloropyrimidine-4-carbonyl)amino]ethyl]-*N*-[5-chloro-4(trifluoromethyl)pyridin-2-yl]-1,3-thiazole-5-carboxamide, impurity-1 (R)-2-(1-Aminoethyl)-N-(5-chloro-4-(trifluoromethyl)pyridin-2-yl)thiazole-5-carboxamide, impurity-2 (5-Chloro-6-((1-(5-((5-chloro-4-(trifluoromethyl)pyridin-2-yl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)pyrimidin-4-yl)glycine and impurity-3 Ethyl (5-chloro-6-((1-(5-((5-chloro-4-(trifluoromethyl)pyridin-2-yl)carbamoyl)thiazol-2-yl)ethyl)carbamoyl)pyrimidin-4-yl)glycinate respectively. The chemical structure of tovorafenib and impurities were illustrated (Figure 1).[5-8] In the pharmaceutical sector, impurity profiling is crucial for guaranteeing the safety, effectiveness, quality, stability, process optimization, regulatory compliance, and quality control of APIs and their formulations. Impurity profiling has recently emerged as a crucial prerequisite for new therapeutic applications. The interconnecting structures and channels in monolithic columns are one of the distinctive features of UPLC analysis that set it apart from HPLC. In contrast to HPLC, UPLC chromatograms exhibit improved separation and resolution, as well as increased sensitivity, reduced solvent use, and faster analysis.[9-11] There are currently no published reports on the use of different analytical techniques to analyze tovorafenib and its related impurities. This study indicates that the sensitivity of the UPLC method to separate and quantify the tovorafenib and its related impurities as well in hybridization with MS/MS analysis.

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## MATERIALS AND METHODS

The active pharmaceutical ingredient, Tovorafenib tablets 100 mg, and tovorafenib Impurities, were acquired from Zydus Cadila Health Care Limited, Ahmedabad.

**Chemicals Required:** HPLC graded acetonitrile, perchloric acid, formic acid and water were procured from Merck, Mumbai.

**Instrumentation:** UPLC waters Acquity model with photo diode array detector and binary pump system. The SCIEX QTRAP 5500 mass spectrometer equipped with an electrospray ionization interface, was seamlessly integrated into the UPLC system.

**Optimized chromatographic conditions:** Acetonitrile and 0.1% formic acid were combined in a 30:70 ratio to generate the mobile phase. A 2.1×100 mm, 1.7 µm phenyl column was used, with an injection volume of 5 µL and a flow of 0.5 mL/min. Acetonitrile was used as a solvent, and the detector's wavelength was set to 260 nm. The temperature of the column was ambient (Table 1 and Figure 2).

**Preparation of Tovorafenib Standard:** 10 mg of Tovorafenib working standard was weighted and transferred into 10 mL volumetric flask, add about 7 mL of diluents, dissolve it. Then, add a diluting agent to the flask to the appropriate level.

**Impurity stock solution:** Contribute 5 mg of each Tovorafenib impurity-1, impurity-2, and impurity-3 working standards to a 10 mL volumetric flask. Next, dissolve the impurities by adding approximately 7 mL of diluents. The flask was then filled with diluents until the right level was reached. Then, the above solution was diluted 1 mL to 10 mL with the proper amount of diluents.

**Spike solution:** 1 mL of tovorafenib standard stock solution + 1 mL of tovorafenib impurity stock solution, with diluents to fill a 10 mL volumetric flask.

**Tovorafenib Sample stock solution:** Weigh 18.5 mg of tovorafenib precisely, then add about 7 mL of diluent to 10 mL volumetric flask and stir until tovorafenib is dissolved. Next, use a diluent in order to fill the flask to the specified level.

**Preparation of sample spike solution:** In 10 mL volumetric flask transfer 1 mL of each of Tovorafenib sample stock and impurity stock solutions, then top it off with diluents.

**Method validation:** In accordance with ICH[12,13] requirements, the UPLC method was validated for system applicability, robustness, ruggedness, accuracy, precision, specificity, and linearity.

**Forced Degradation Studies:** In order to conduct the degradation research, tovorafenib was exposed to a number of circumstances. For hydrolysis (HPLC water/15 min), peroxide (10% $H_2O_2$/15 min), reduction (10% sodium bisulfite/15 min), acid (1N HCl/15 min) and alkali (1N NaOH/15min). Thermal deterioration occurs when the drug left at 105°C for 6 hr, whereas photolytic degradation occurs when it is placed in a photo stability chamber for the same period of time.

**Characterization of degradation products:** The degradation products were detected using the positive ion electrospray ionization interface method, and the stress-induced solutions were examined using the UPLC-MS/MS methodology.

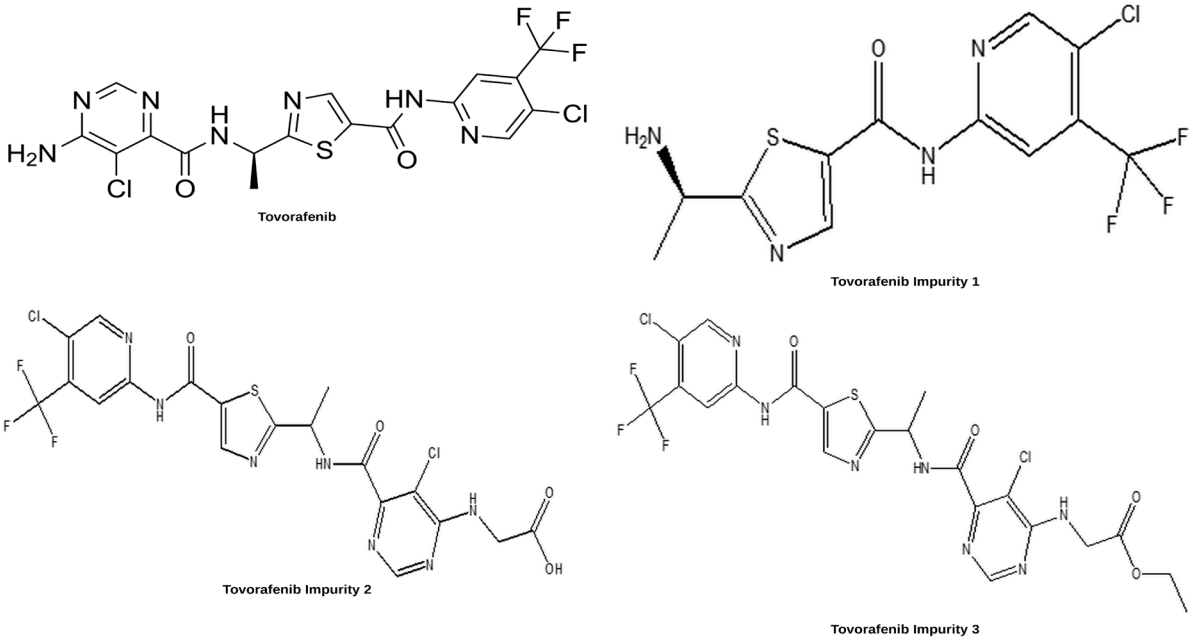



**Figure 1:** Chemical structure of Tovorafenib, Impurity-1, Impurtiy-2 and Impurity-3.

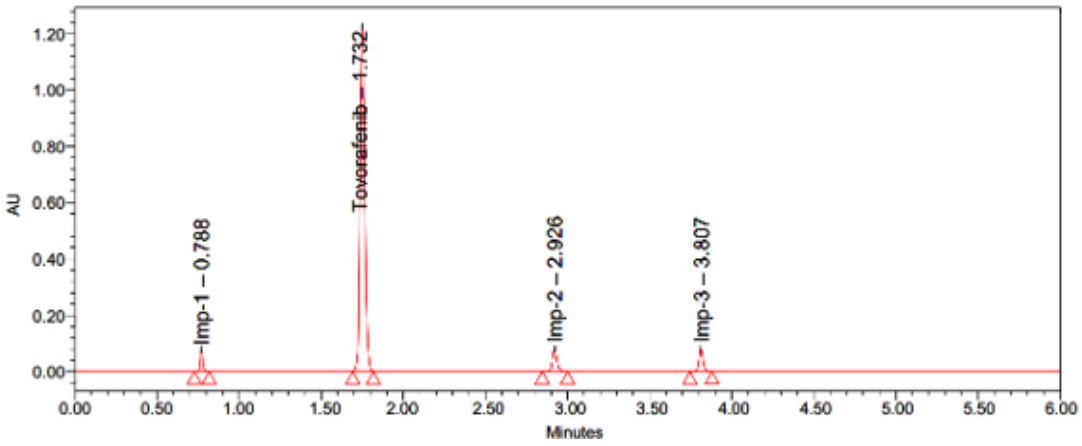



**Figure 2:** Impurity-1, tovorafenib, impurity-2 and impurity-3 optimized chromatogram.

**Table 1: Result for the optimized chromatogram.**

| Sl. No. | Name | RT | Peak Area | Tf | Theoretical Plate | USP Resolution |
|---|---|---|---|---|---|---|
| 1. | Imp-1 | 0.788 | 68248 | 1.05 | 4586 | - |
| 2. | Tovorafenib | 1.732 | 15846254 | 1.07 | 16254 | 4.36 |
| 3. | Imp-2 | 2.926 | 76859 | 1.00 | 7972 | 5.62 |
| 4. | Imp-3 | 3.807 | 79214 | 1.02 | 8896 | 3.95 |

**Table 2: Results for Linearity.**

| Concentration (ppm) | Tovorafenib | Concentration (ppm) | Impurity-1 | Impurity-2 | Impurity-3 |
|---|---|---|---|---|---|
| 25 | 3826034 | 1.25 | 16746 | 19453 | 19493 |
| 50 | 7741205 | 2.5 | 33810 | 38697 | 39810 |
| 75 | 11584273 | 3.75 | 50878 | 57451 | 61341 |
| 100 | 15472136 | 5.0 | 68257 | 76694 | 79794 |
| 125 | 18887023 | 6.25 | 84089 | 92561 | 99056 |
| 150 | 23232018 | 7.5 | 101237 | 112172 | 118524 |

## RESULTS AND DISCUSSION

**Linearity:** The method shows a high linear correlation between 25-150 ppm for tovorafenib and between 1.25-7.5 ppm for impurities. The linear association between tovorafenib and its impurities displayed correlation values greater than 0.999 in this specific range (Table 2 and Figure 3).

**Precision:** The % RSD readings for system, method, and intermediate precision were all less than 2, which indicates method precision and is within the allowed limit (Table 3).

**Robustness:** The proposed conditions was examined by varying mobile phase ±3 mL and flow rate ±0.05 mL. Minor adjustments had no effect on the system's applicability, chromatographic findings, or analyte elution times, according to the data. Tovorafenib and its impurities peak reaction in each scenario demonstrated a percentage change that was kept below 2%. Similarly, no discernible variations in system suitability characteristics for tovorafenib and associated impurity-related peaks were found under the investigated conditions. This demonstrates how robust and solid the approach was (Table 4).

**Accuracy:** To evaluate accuracy, recovery trials were carried out. Each known impurity with the tablet sample solution was spiked at 50%, 100%, and 150% levels to test for accuracy. Three duplicates were injected at each level. Tovorafenib and impurities were found to have percentage recoveries of 99.8-100.2% and 98.6-100.5% respectively (Table 5). The percentage RSD below 2 indicates that the developed method has good accuracy.

**Limit of Detection and Limit of Quantification:** The Limit of Detection (LOD) will be used to determine the sensitivity of the method, where the signal-to-noise ratio will be 0.6 ppm (3:1) for Tovorafenib, 0.075 ppm (3:1) for impurity-1, 2 & 3 and Limit of Quantification (LOQ) at 2 ppm (10:1) Tovorafenib, 0.25 ppm (10:1) Impurity-1,2 & 3.

**Forced Degradation:** The drug showed degradation in acid, alkali, peroxide, and reduction, yielding DP1, DP2, DP3 and

**Table 3: %RSD results for Precision.**

| | Impurity-1 | Tovorafenib | Impurity-2 | Impurity-3 |
|---|---|---|---|---|
| System Precision | 0.37 | 1.37 | 0.27 | 0.34 |
| Method Precision | 0.33 | 1.02 | 0.35 | 0.28 |
| Intermediate Precision | 0.41 | 1.41 | 0.31 | 0.34 |

**Table 4: Results for Robustness.**

| Condition Varied | % Change | | | | % RSD | | | |
|---|---|---|---|---|---|---|---|---|
| | Imp-1 | Tov | Imp-2 | Imp-3 | Imp-1 | Tov | Imp-2 | Imp-3 |
| Mobile Phase Plus: (33:67) | 0.446 | 0.196 | 0.201 | 0.221 | 0.43 | 1.15 | 0.33 | 0.17 |
| Mobile Phase Minus: (27:73) | 0.18 | 0.266 | 0.288 | 0.13 | 0.40 | 1.16 | 0.32 | 0.32 |
| Flow Plus: (0.55 mL) | 0.368 | 0.116 | 0.123 | 0.128 | 0.44 | 1.18 | 0.33 | 0.34 |
| Flow Minus: (0.45 mL) | 0.133 | 0.143 | 0.196 | 0.153 | 0.46 | 1.41 | 0.35 | 0.29 |

**Table 5: % Recovery data.**

| Level | % Recovery | | | | % RSD | | | |
|---|---|---|---|---|---|---|---|---|
| | Imp-1 | Tov | Imp-2 | Imp-3 | Imp-1 | Tov | Imp-2 | Imp-3 |
| 50% | 100.8 | 100.2 | 99.2 | 99.7 | 0.793 | 0.399 | 1.066 | 0.612 |
| 100% | 100.2 | 99.6 | 99.5 | 100.2 | 0.399 | 0.857 | 0.41 | 0.304 |
| 150% | 99.3 | 99.8 | 100.2 | 98.6 | 0.252 | 1.055 | 0.583 | 0.42 |

**Table 6: % Degradation of Tovorafenib.**

| Condition | Peak Area value | % Degradation of Tovorafenib |
|---|---|---|
| Acidic | 13628431 | 12.80 |
| Alkali | 13845240 | 11.41 |
| Peroxide | 13531029 | 13.42 |
| Reduction | 14123607 | 9.64 |

DP4 respectively. However, every deterioration was assessed individually to guarantee that the peak angle was under the purity threshold (Table 6).

**Characterization of Degradation Products using Mass Spectral Analysis:** The four genarated DP's were identified as (R)-2-(1-(6-amino-5-chloropyrimidine-4-carboxamido)ethyl) thiazole-5-carboxylic acid (DP 1), Sodium (R)-2-(1-(6-amino-5 -chloropyrimidine-4-carboxamido)ethyl)thiazole-5-carboxylate (DP 2), N-(5-chloro-4-(trifluoromethyl)pyridin-2-yl)-N-hy droxy-2-methylthiazole-5-carboxamide (DP 3), (5-chloro-4-(trifluoromethyl)pyridin-2-yl)(2-methylthiazole-5-carbonyl) sulfamic acid respectively and characterized through mass spectrophotometry analysis. The interpretation of data was done with SCIEX software (Table 7 and Figures 4,5).

**DP 1 (*m/z* 328.0199):** Two pieces of DP-1 were obtained from the first positive ESI LC-MS analysis (*m/z* 157.0197 and 200.0465). The parent ion, which has the chemical formula $C_{11}N_5O_3SH_{10}Cl$, loses its $C_5H_5ClN_4O$ bond, resulting in the formation of the first product ion from DP 1 with a mass of 157.0197 *m/z*. Another product ion with a molar mass of 200.0465 (*m/z*) and the chemical formula $C_7ClON_4H_9$ is created when the $C_4H_3NO_3S$ link from the first product of (DP 1) is lost.

**DP 2 (*m/z* 350.0021):** Two DP-2 fragments were acquired from the initial successful ESI LC-MS assessment (*m/z* 172.0152 and 222.0075). By losing the $C_6H_6NaO_2S$ bond, the parent ion, with the chemical formula $C_{11}N_5O_3ClNaH_9S$, forms the initial product ion from DP 2 with a mass of 172.0152 *m/z*. A second product is formed by losing the $C_4H_4ClN_3$ link from the initial product of DP 2, with a molar mass of 222.0075 (*m/z*) and chemical formula $C_7O_3SN_2NaH_7$.

**DP 3 (*m/z* 337.9908):** DP-3 yielded two fragments with mass-to-charge ratios of 158.015 and 239.9913. The parent ion with the formula $C_{11}O_2N_3F_3ClH_7S$ undergoes loss of $C_6H_3ClF_3N$ to form the initial product ion with 158.015 *m/z* in DP 3. Another product is formed with a molar mass of 239.9913 (*m/z*) and the chemical formula $C_7N_2O_2ClF_3H_4$ by eliminating the $C_4H_5NS$ unit from the initial product of DP 3.

**DP 4 (*m/z* 401.9527):** The initial ESI LC-MS analysis yielded two DP-4 fragments with mass-to-charge ratios of 221.9769 and 303.9532. The parent ion with the formula $C_{11}N_3O_4ClF_3S_2H_7$

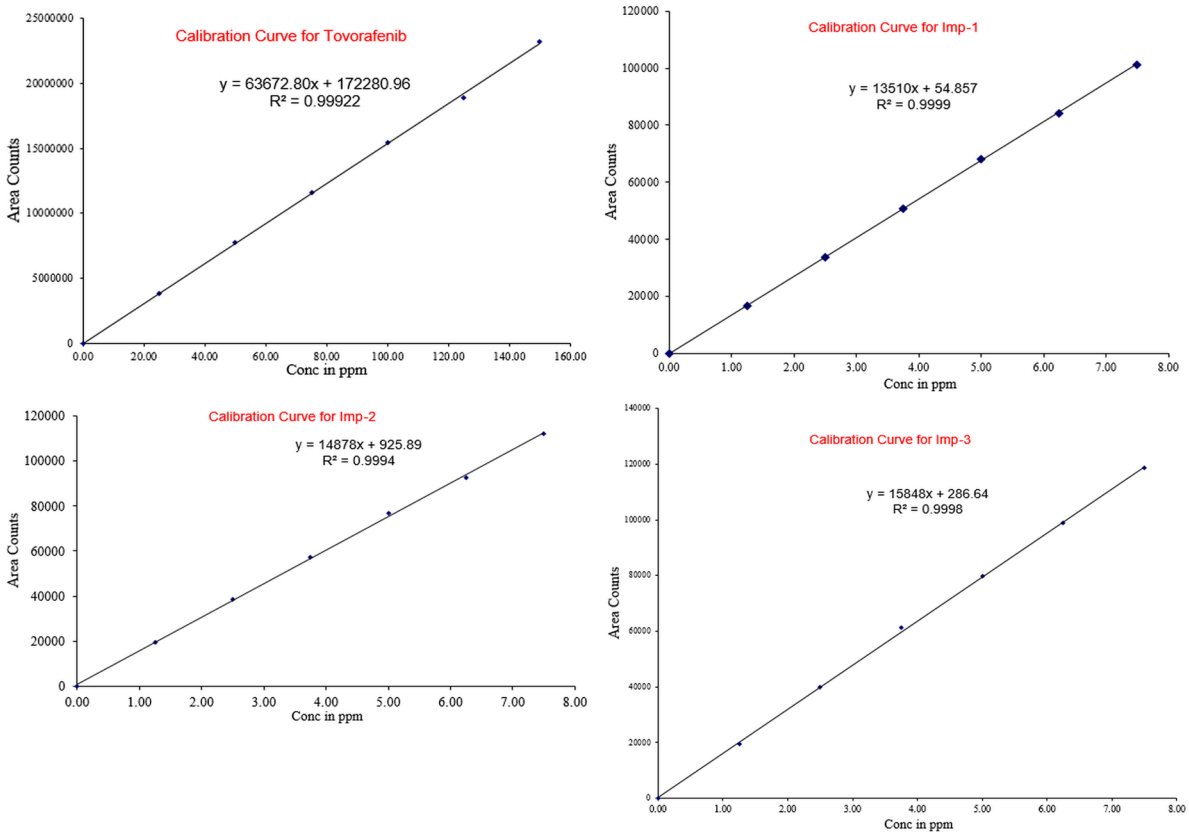
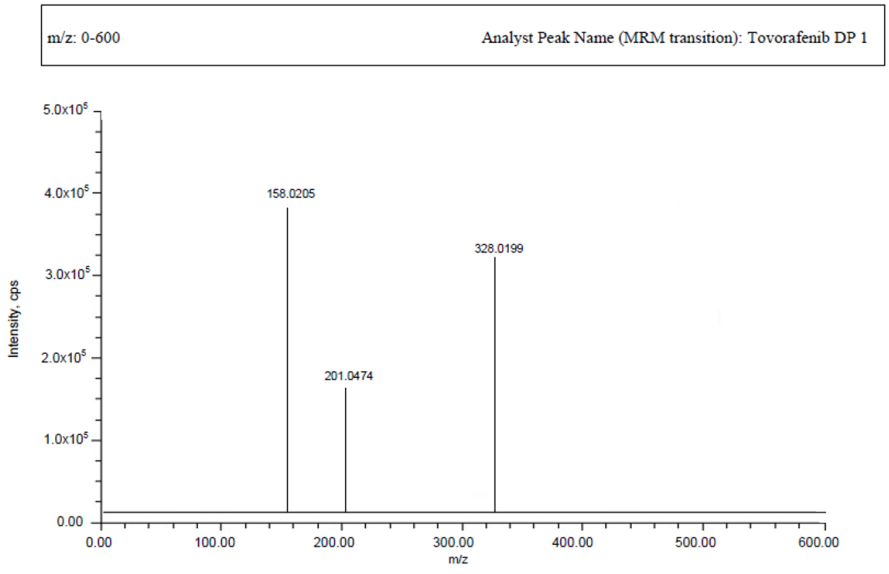
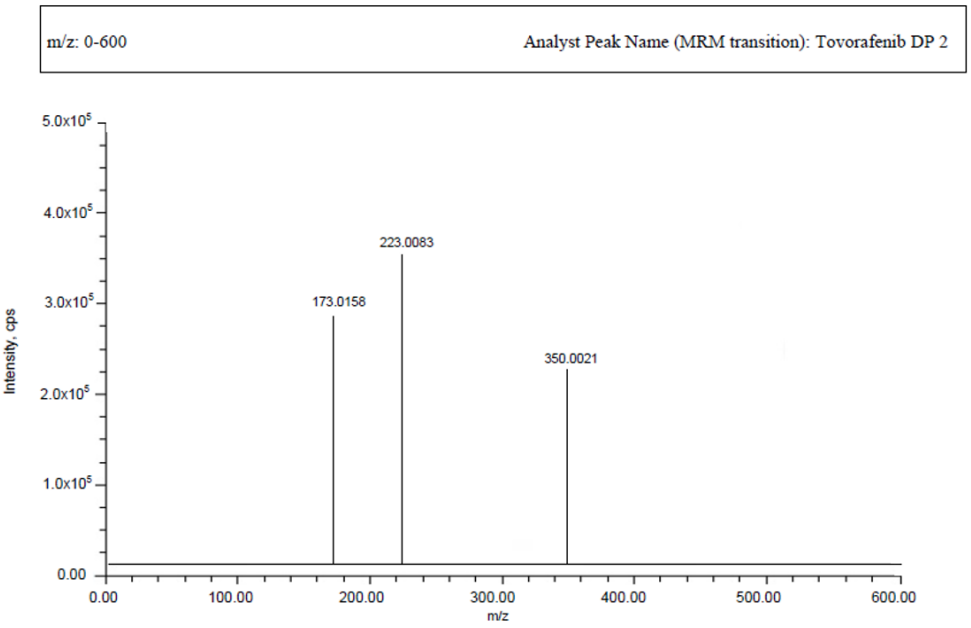



**Figure 3:** Linearity graph of tovorafenib, impurity-1, 2 and 3. Correlation Coefficient (R2) for Tovorafenib was 0.9992, for Impurity-1, 2 and 3 were 0.9999, 0.9994 & 0.9998 respectively.



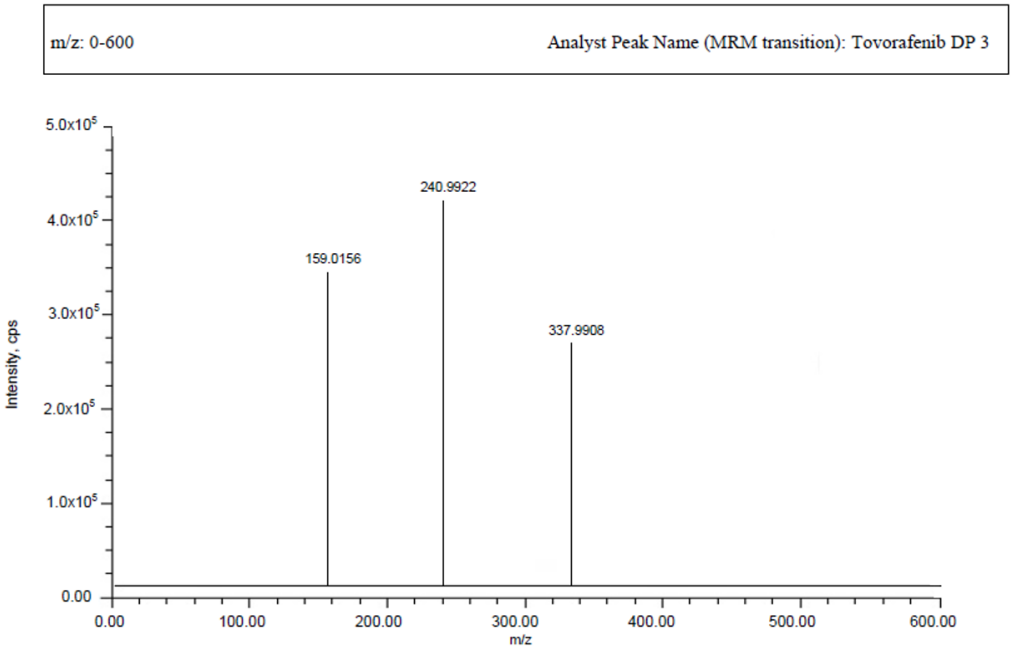

Multiple Reaction Monitoring-MRM of the Tovorafenib DP 1 using PositivePolarity

| Analogue | Precursor Ion(m/z) | Daughter Ion with the Highest Intensity (m/z) |
|---|---|---|
| Tovorafenib DP 1 | 328.0199 | 158.0205 |



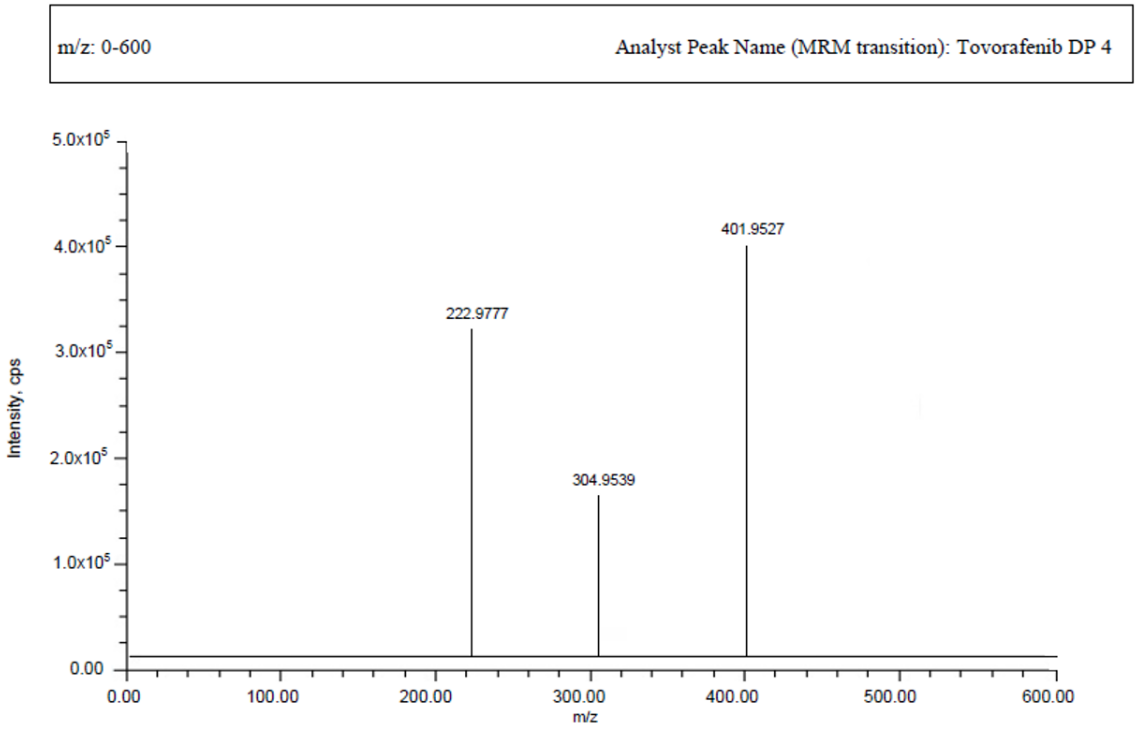

Multiple Reaction Monitoring-MRM of the Tovorafenib DP 2 using PositivePolarity

| Analogue | Precursor Ion(m/z) | Daughter Ion with the Highest Intensity (m/z) |
|---|---|---|
| Tovorafenib DP 2 | 350.0021 | 223.0083 |




Multiple Reaction Monitoring-MRM of the Tovorafenib DP 3 using PositivePolarity

| Analogue | Precursor Ion(m/z) | Daughter Ion with the Highest Intensity (m/z) |
|---|---|---|
| Tovorafenib DP 3 | 337.9908 | 240.9922 |




Multiple Reaction Monitoring-MRM of the Tovorafenib DP 4 using PositivePolarity

| Analogue | Precursor Ion(m/z) | Daughter Ion with the Highest Intensity (m/z) |
|---|---|---|
| Tovorafenib DP 4 | 401.9527 | 222.9777 |

**Figure 4:** Mass Spectra of acid(DP-1), alkali (DP-2), peroxide (DP-3) and reduction (DP-4) impurities. Multiple reaction monitoring of the Tovorafenib degradation products using possitive ploarity.

**Table 7: LC-MS/MS data of degradation Products.**

| Type of Degradation Product (DP) | Experimental mass | Molecular Formula | Theoretical mass | Major fragments along with molecular formula |
|---|---|---|---|---|
| Acidic-DP1 | 328.0199 | $C_{11}N_5O_3SH_{10}Cl$ | 32.0193 | 157.0197($C_6H_7O_2SN$) and 200.0465($C_7ClON_4H_9$) |
| Alkali-DP2 | 350.0021 | $C_{11}N_5O_3ClNaH_9S$ | 349.0012 | 172.0152($C_5N_4ClOH_5$) and 222.0075($C_7O_3SN_2NaH_7$) |
| Peroxide-DP3 | 337.9908 | $C_{11}O_2N_3F_3ClH_7S$ | 336.99 | 158.015($C_5N_2O_2SH_6$) and 239.9913($C_7N_2O_2ClF_3H_4$) |
| Reduction-DP4 | 401.9527 | $C_{11}N_3O_4ClF_3S_2H_7$ | 400.9519 | 221.9769($C_5N_2S_2O_4H_6$) and 303.9532($C_7O_4N_2ClF_3SH_4$) |

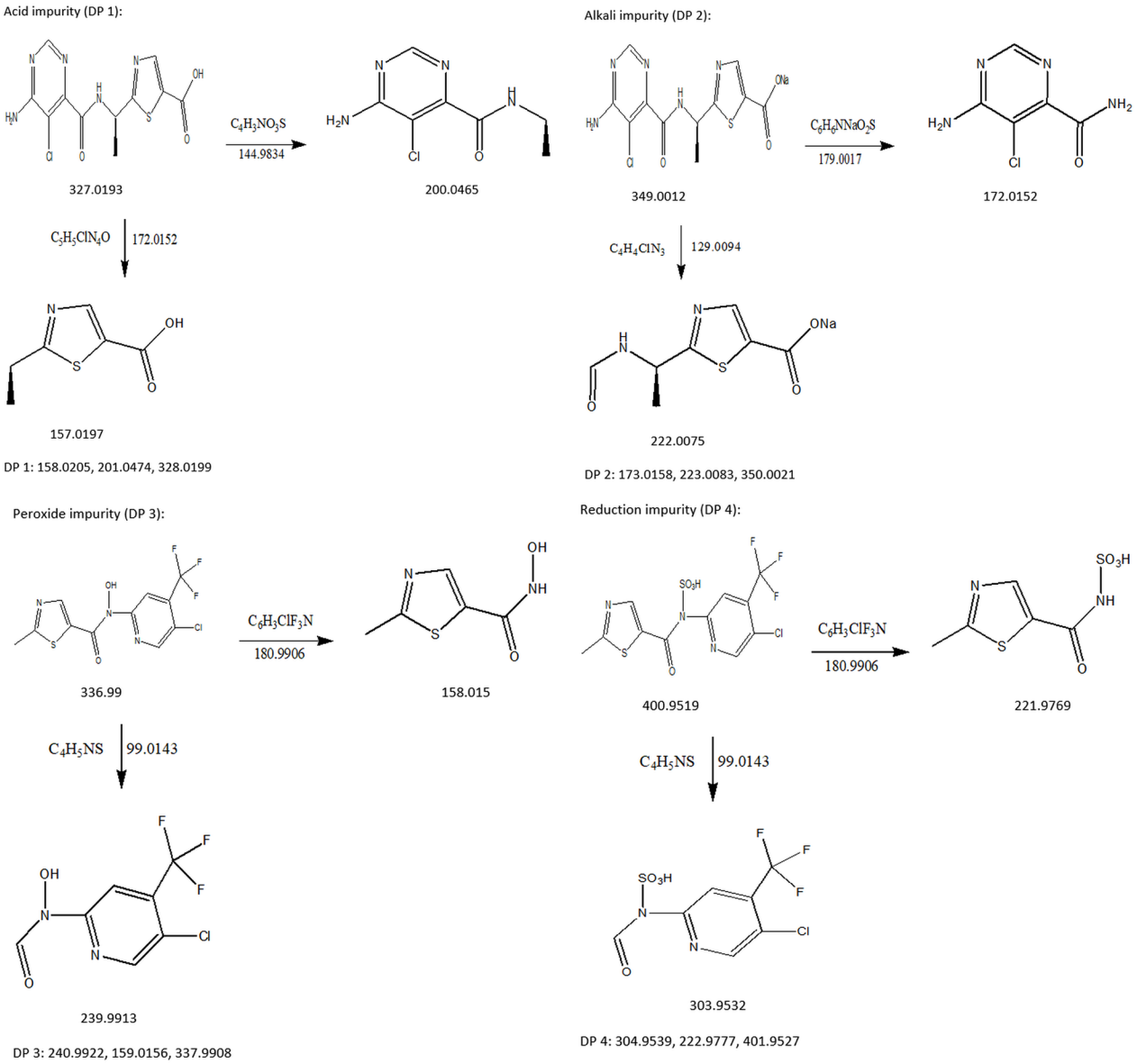



**Figure 5:** Parent ion fragmentation from Acid, alkali, peroxide & reduction degradation products by ESI LC/MS.

undergoes loss of $C_6H_3ClF_3N$ to form the initial product ion with 221.9769 *m/z* in DP 4. Another product is formed with a molar mass of 303.9532 (*m/z*) and the chemical formula $C_7O_4N_2ClF_3SH_4$ by eliminating the $C_4H_5NS$ unit from the initial product of DP 4.

## CONCLUSION

Tovorafenib and its associated impurities were analyzed using this technique for the first time. A method for detecting and measuring all potential degradation impurities in Tovorafenib was established and verified using UPLC. The newly developed method with a short run time was found to be sensitive and capable of monitoring all potential outcomes. Impurities produced during the manufacturing process and the stability of the pharmaceutical product. The linearity, accuracy, precision, and robustness test results demonstrate that the approach was reliable, strength of the technique and its appropriateness for the planned purpose. The study on forced degradation verifies the method's specificity for every potential degradation impurity caused by the active ingredient. The process of manufacturing and potential interaction with excipients. Also, a low flow of solvent of 0.5 mL/min results in low solvent consumption which implies a cost-effective methodology. Moreover, the identical approach can be directly utilized for LC-MS/MS application in order to determine the mass of an unidentified impurity. Thus, the UPLC method with PDA detector was created and confirmed. The technique can be used to assess impurities in tovorafenib as well as for quality control and stability testing of the drug product.

## ACKNOWLEDGEMENT

We would like to thank Zydus Cadila Health care Limited, Ahmedabad for providing Tovorafenib standard, sample and its impurity standards respectively. This work is a part of Ph.D thesis of Gayathri Devi Ketineni at KL University, Vadddeswaram, Guntur.

## ABBREVIATIONS

**UPLC:** Ultra Performance Liquid Chromatography; **LC-MS:** Liquid Chromatography-Mass Spectrophotometry; **PDA:** Photo Diode Array; **HPLC:** High Performance Liquid Chromatography; **ICH:** International Council for Hormonization; **ESI:** Electro Spray Ionization; **IUPAC:** International Union of Pure and Applied Chemistry; **Tov:** Tovorafenib; **Imp:** Impurity; **mL:** millilitre; **mg:** Milligram; **µg:** Microgram; **RT:** Retention Time; **Tf:** Tailing Factor; **ppm:** Parts per Million; **RSD:** Relative Standard Deviation.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## SUMMARY

With the help of UPLC-MS/MS, a highly accurate and precise method was developed to analyze the presence of tovorafenib and its associated contaminants in both bulk and formulated samples. The process was validated in compliance with ICH guidelines, and the degradation products were identified by comparing them to known impurities spiked with tovorafenib using positive ion mode mass spectra.

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