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# A Simple and Sensitive LC-MS/MS Method for Determination and Quantification of Potential Genotoxic Impurities in the Vismodegib Active Pharmaceutical Ingredient

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

A rapid and sensitive LC-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantitative analysis of four potential genotoxic impurities Imp-A (2-chloro-5-nitroaniline), Imp-B (1-chloro-2-iodo-4-nitrobenzene), Imp-C (1-(2-chloro-5-nitrophenyl)ethan-1-one) and Imp-D (2-chloro-5-nitrobenzoic acid) in Vismodegib API drug sample. This trace analysis was achieved on CSH C18, 15.0 cm x 3.0 mm, 1.7 micron column maintained at 45°C. Optimal mobile phase consisted of 0.05% formic acid in water was used as mobile phase A and acetonitrile used as mobile phase B in gradient mode with the flow rate of 0.5 mL/min. The developed method had the short run time of 12 minutes. Quantification of four potential genotoxic impurities were carried out using mass detection with electrospray ionization in multiple reaction monitoring mode. The method was linear in the range of 0.03 ppm to 1.50 ppm for four potential genotoxic impurities with a correlation coefficient not less than 0.999. The recoveries were found satisfactory over the range between 96.67 and 106.90% for all selected impurities. The developed method was able to quantitate all four PGIs at a concentration level of 0.03 ppm (0.03 ppm with respect to 20 mg /mL Vismodegib).

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*Keywords: Potential genotoxic impurities (PGIs); LC-MS/MS; low level; Vismodegib.*

## 1. INTRODUCTION

The Vismodegib is sulphar containing small molecule inhibitor of the Hedgehog signaling pathway. This signalling regulates embryonic development, ensuring that tissues reach their correct size and location, maintain tissue polarity and cellular content [1]. In the skin, the Hedgehog pathway is critical for regulating sebaceous gland and hair follicle development. Hedgehog signalling normally remains inactive in most adult tissues. Inappropriate reactivation of the Hedgehog pathway has been linked to several human cancers, most often caused by exposure to UV radiation. In basal cell carcinoma (BCC), abnormal Hedgehog pathway signalling is the key molecular driver of the disease and more than 90% of BCCs have abnormal activation of Hedgehog pathway signalling.

Vismodegib (Fig. 1), the active pharmaceutical ingredient in Erivedge [2] is the first inhibitor of the Hedgehog pathway to be approved for the treatment of metastatic or locally advanced BCC [3] and represents an important treatment option for patients where surgery is not recommended. Vismodegib [1] is synthesized in a 4-step manufacturing process from two designated starting materials, 2-(2-chloro-5-nitrophenyl)pyridineand 2-chloro-4-(methylsulfonyl)benzoic acid. During the course of these development vismodegib processes, four potential genotoxic impurities (GTIs) in the API introduced by the nitro starting material [4]. Such impurities may show cancer unwanted toxicity. According to the guidelines of ICH Q3A and Q3B, actual impurities in active pharmaceutical ingredient (API) are the compound that should not exceed the reported threshold or should not arise during its storage. For the determination of genotoxic impurities at lower level using ultraviolet-visible spectrophotometry, gas chromatography and high-performance liquid chromatography is a tough task in the pharmaceutical industry as the sensitivity of these instruments is low [5-9]. Hence the sensitivity of analytical method must be increased for lower level determination of impurities. This can be achieved by combining high performance liquid chromatography (HPLC) with mass spectrometry (MS). Because of reliability and high sensitivity of liquid chromatography (LC)/ MS/MS method, recently, many authors used this method for the

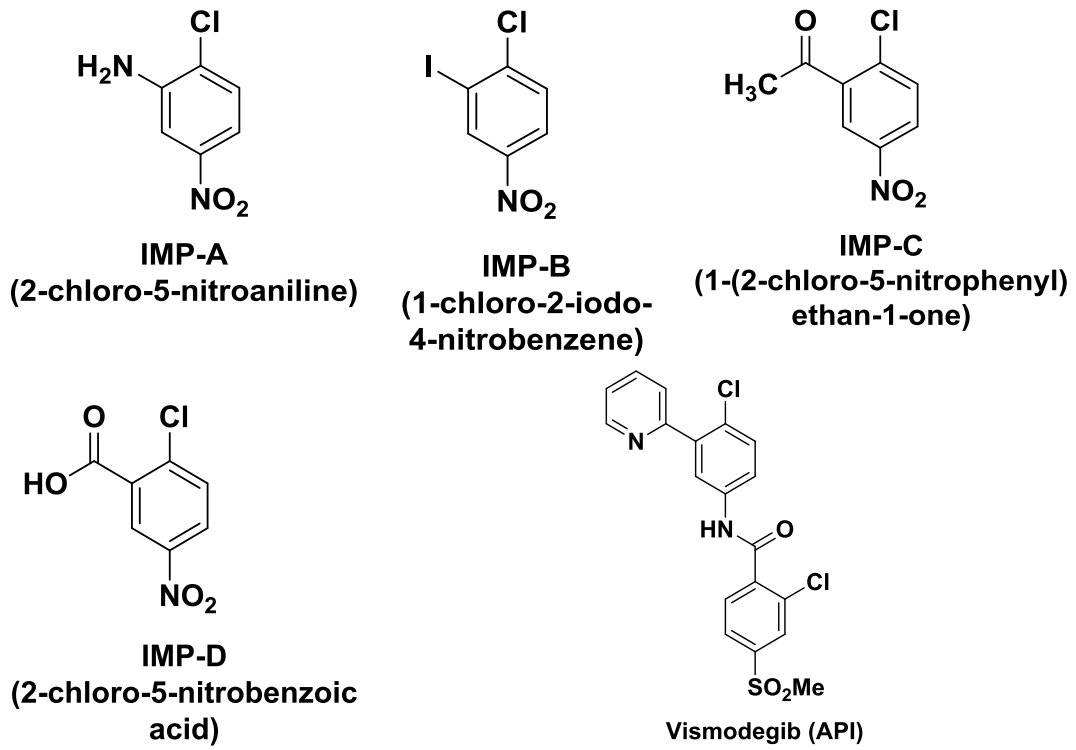


**Fig. 1. Structure of Vismodegib and four genotoxic impurities**

determination of potential genotoxic impurities [10-22]. Because of the high sensitivity, liquid chromatography-tandem mass spectrometry method was used for the determination genotoxic impurities in this work. The recent literature survey reviled that Veera ShakarPulusuet al developed HPLC method for the determination of vismodegib and its degradation products [23]. Butno method has been found for the determination of these four genotoxic impurities in vismodegib active pharmaceutical ingredient. The method for the detection of Imp-A (2-chloro-5-nitroaniline), Imp-B (1-chloro-2-iodo-4-nitrobenzene), Imp-C (1-(2-chloro-5-nitrophenyl)ethan-1-one) and Imp-D (2-chloro-5-nitrobenzoic acid) at lower level will be very useful for the monitoring of impurities in vismodegib API during its preparation.With a view to determining the four potential genotoxic impurities, Imp-A, Imp-B, Imp-C and Imp-D invismodegib API with very lower detection limit (0.1 ppm) and short run time of 12 min, this method was developed.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Vismodegib drug substance and IMP-B was purchased from Chemieliva pharmaceutical and Sigma Aldrich, India respectively. IMP-A, C and D were received from TCI Chemicals (India) Pvt. Ltd. The following HPLC grade solvents Acetonitrile, LC-Ms grade water and Formic acid were obtained from Fisher scientific.

### 2.2 Instrumentation and Method Conditions

Thermo LTQ-Orbitrap discovery mass spectrometer was used for the determination four potential geonotoxic impurities at low level, by electrospray ionization. The analytical column used in LC/MS/MS was CSH C18 or equivalent 15.0 cm x 3.0 mm, 1.7 micron in gradient mode using mobile phase A (0.05 % Formic acid in water) and mobile phase B (Acetonitrile). The flow rate was 0.5 mL/min. The column oven temperature was maintained at 45°C, the sample cooler temperature was 5°C and the wavelength was set at 265 nm. The injection volume was 5.0 µL. Positive ion electrospray ionization probe in multiple reaction monitoring (MRM) mode was used in MS method for the quantification of genotoxic impurities in vismodegib drug substance. In this method Imp-A, B, C, and D were monitored with its molecular ion and daughter ion m/z shown in Table 1. Vismodegib was monitored with its molecular ion [M+H]+ m/z 422.29. Applied detector conditions were Fragmentor: ~ 70 eV; Capillary voltage: ~ 1300 V; Nozzle voltage: ~ 2000 V; Drying gas: ~ 12 l/min; Drying gas temperature: ~ 250°C; Nebulizer pressure ~ 35 psig; Sheath gas temperature: ~ 150°C; Sheath gas flow ~ 3.0 l/mi. This method is suitable for the quantification of above three genotoxic impurities both in reaction monitoring samples as well as vismodegib active pharmaceutical ingredient.

### 2.3 Preparation of Standard and Sample Solutions

The sample Vismodegib solution was prepared at 20 mg/ml in diluent of Acetonitrile: water 90:10 ratio. Stock standards of the Imp-A, Imp-B, Imp-C and Imp-D were prepared at a concentration of 1mg/ml in diluent. Subsequently standard mixture solutions containing the four impurities at a concentration of 1µg/ml (equivalent of 1ppm) in diluent were obtaining by diluting the stock standards for analysis in Vismodegib samples.

## 3. RESULTS AND DISCUSSION

This present study was conducted in order to develop a sensitive and selective LC-MS/MS method that can separate and quantify four potential PGIs in the vismodegib active pharmaceutical ingredient. It is important to achieve proper separation among the four PGIs and vismodegib, because of the analogous chemical structures of four PGIs with vismodegib. In order to obtain a short analysis time, different reversed phase stationary phases have been assessed which included C18, C8 and cyano phases columns like CSH C18 (15.0 cm x 3.0 mm, 1.7 micron), Hypersil BDS C8 (150 mm × 4.6 mm, 3.5 µm), Kromasil C8 (150 mm × 4.6 mm, 3.5 µm), Symmetry C18 (100 mm × 4.6 mm, 3.5 µm) were evaluated under the similar conditions. When Kromasil C8 column was used the Imp-B peak overlapped with Vismodegib peak. The resolution and peak shapes between Vismodegib and impurities (Imp-A, Imp-B, Imp-C and Imp-D) were poor with Hypersil BDS C8 column. On CSH C18 (15.0 cm x 3.0 mm, 1.7 micron), the responses and resolution for both the impurities and vismodegib were found good. On this column, the analytes were well separated and retained from each other and from the drug substance. Different compositions of mobile phases using formic acid and Trifluoro acetic acid in water and acetonitrile solvent were tested

(Table 2). Finally, good separation and response were observed at a ratio of mobile phase A (0.05% Formic Acid in water), mobile phase B (Acetonitrile) with gradient run. Gradient programme was provided in Table 3 to be more efficient in achieving optimum separation of impurities from each other with respect to drug substance peak. The column was thermo stated at 45 °C to avoid any shift in retention time and better peak shape. During the analysis of a sample or spiked sample, here in this method we used diverted valve to divert the vismodegib into waste from retention time 8 to 10 min to avoid any further interference with impurities and improve the sensitivity of the method.

## 4. METHOD VALIDATION

### 4.1 Specificity

The specificity of this method was demonstrated that Imp-A, Imp-B, Imp-C and Imp-D solutions were prepared individually at a concentration in the diluent and a solution of vismodegib sample spiked with four potential genotoxic impurities was also prepared. Blank and specificity chromatograms are shown in Figs. 2-6. As per blank chromatogram Fig. 2, no interference was observed at the retention times of impurities as well as main drug. The impurities chromatograms showed that in Figs. 2-6, the vismodegib peak was eluted at 9.2 min with good separation of Imp-A, Imp-B, Imp-C and Imp-D at the retention times of 2.61 min, 7.47 min, 5.54 min and 4.77 min respectively. There was no interference observed in chromatograms of the main drug with impurities and the developed method was successfully separated four potential genotoxic impurities with each other and with main drug.

### 4.2 Linearity

The linearity test for the method is performed according to the ICH guidelines. This method is evaluated at six different concentrations range of LOQ – 150%. The calibration curve was drawn between the concentration of analyte versus peak areas. The correlation coefficient, intercept

**Table 1. Mass spectrometer method conditions for LC-MS-MS analysis**

| Compound | Parent ion | Daughter ion |
|---|---|---|
| Imp-A | 173.6 and 175.6 | 125.1 |
| Imp-B | 284.2 and 286.0 | 155.3 |
| Imp-C | 200.8 and 202.9 | 154.8 |
| Imp-D | 202.8 and 204.5 | 156.1 |
| Vismodegib | 422.29 | 399.30 |

**Table 2. Optimization of Mobile phase combination**

| Trail | Mobile Phase-A | Mobile Phase-B | Observation |
|---|---|---|---|
| 01 | 0.05% Trifluoro acid in water | Acetonitrile | Peaks resolution was not good |
| 02 | 0.05% Trifluoro acid in water | Methanol | Peaks were overlapped |
| 03 | 0.05% Formic Acid in water | Methanol | Peaks were overlapped |
| 04 | 0.05% Formic Acid in water | Acetonitrile and Methanol (90:10) | Resolution not sufficient |
| 05 | 0.05% Formic Acid in water | Acetonitrile | minimum resolution was observed |

**Table 3. Gradient programme**

| Time (min) | % Mobile phase A | % Mobile phase B |
|---|---|---|
| 0 | 95 | 5 |
| 5 | 5 | 95 |
| 6 | 5 | 95 |
| 7.5 | 95 | 5 |
| 11 | 95 | 5 |

for four potential genotoxic impurities impurity. and slope values were derived from least squares linear regression analysis. The correlation coefficient obtained for all potential genotoxic impurities were >0.998 (Figs. 7-10; Table 4). The linearity experiment revealed that the mass spectrometric responses were proportional to their concentration within the range of 0.03–1.5 ppm.

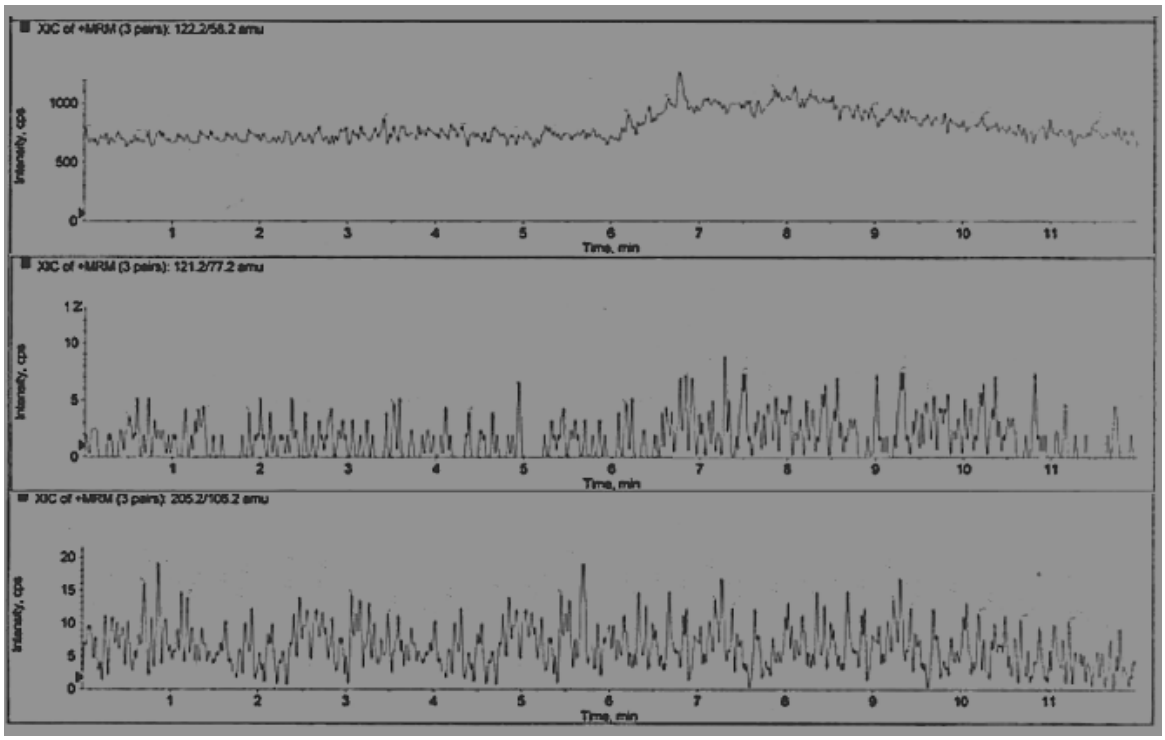


**Fig. 2. Blank chromatogram**

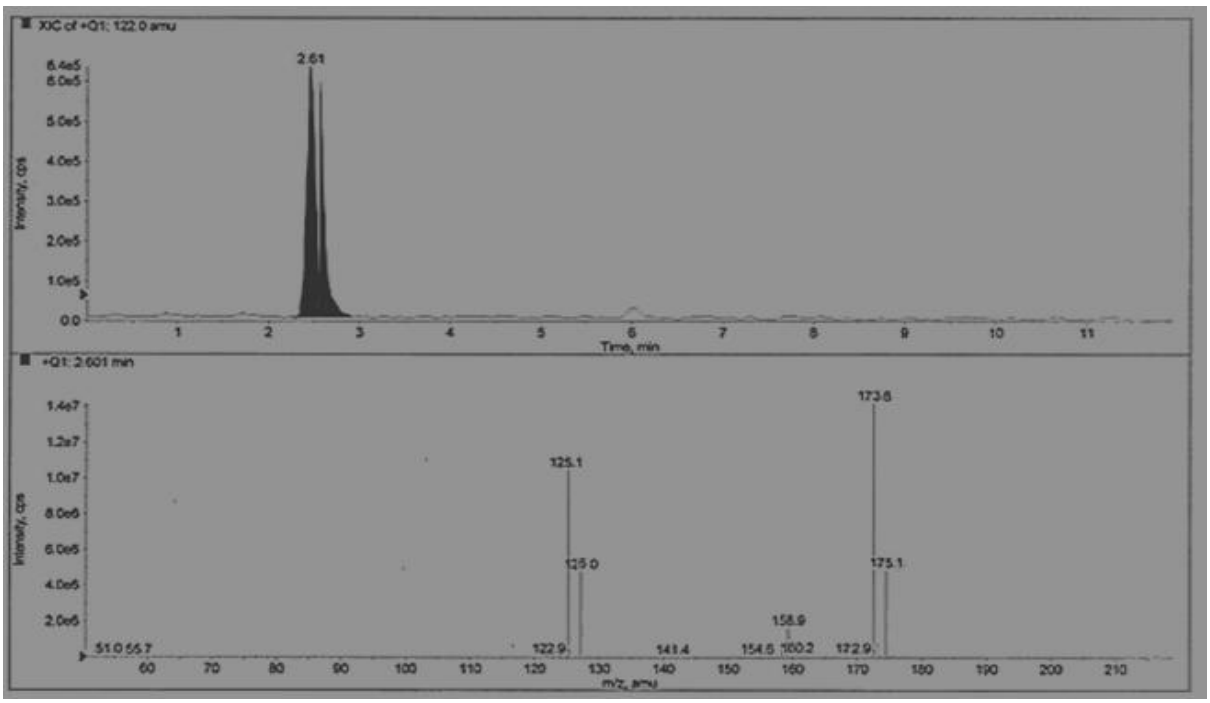


**Fig. 3. Chromatogram of 2-chloro-5-nitroaniline (Imp-A)**

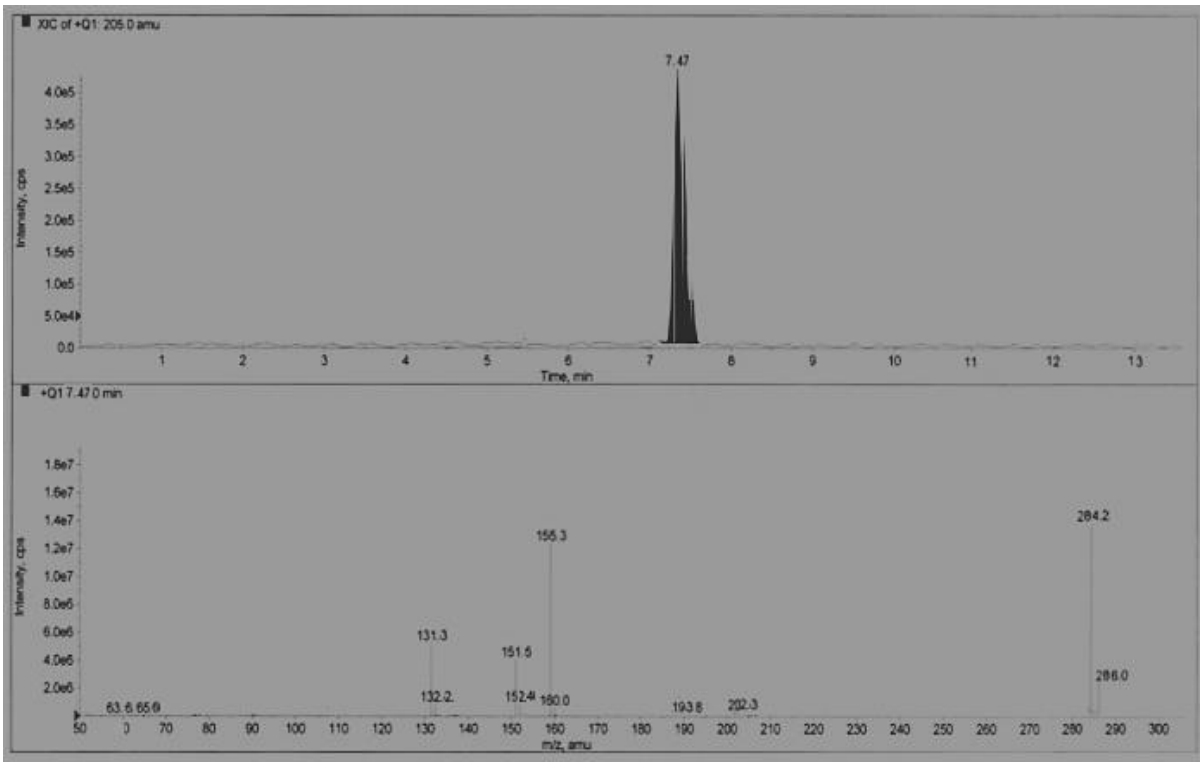


**Fig. 4. Chromatogram of 1-chloro-2-iodo-4-nitrobenzene (Imp-B)**

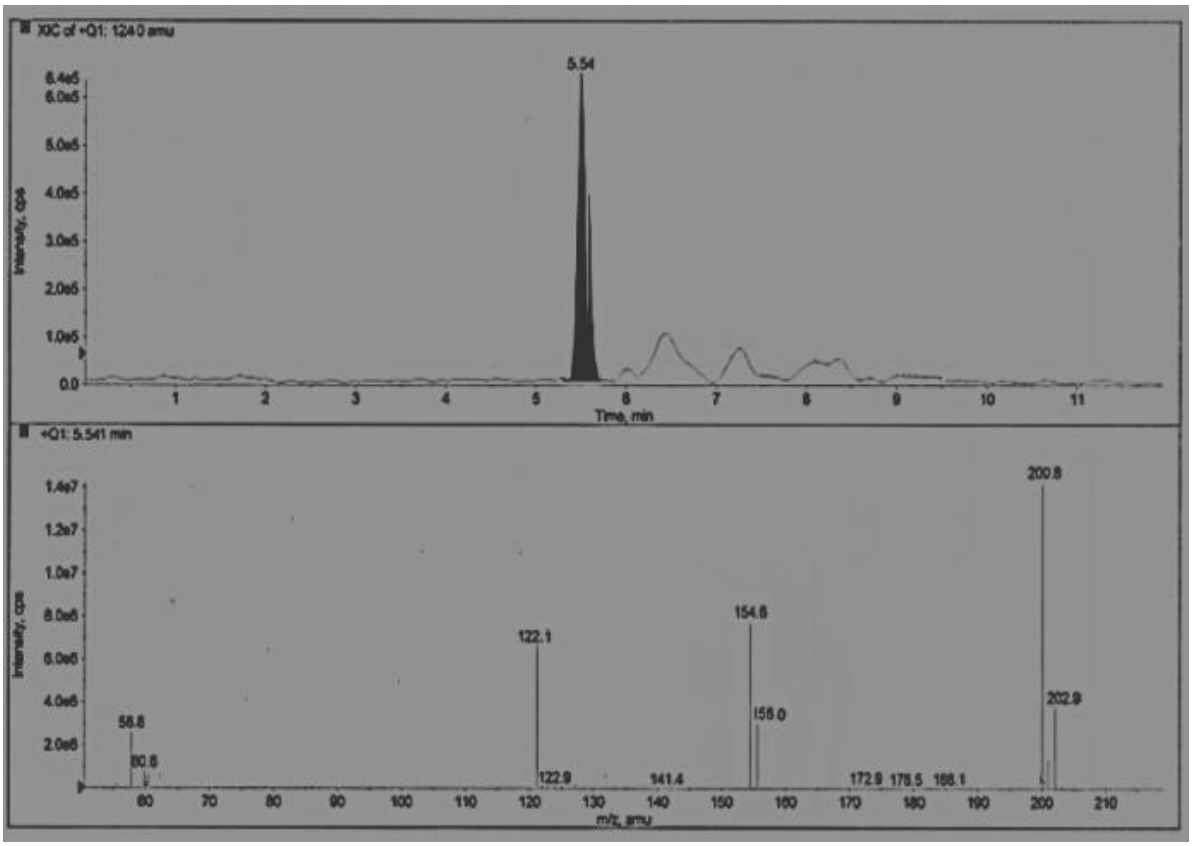


**Fig. 5. Chromatogram of 1-(2-chloro-5-nitrophenyl)ethan-1-one (Imp-C)**

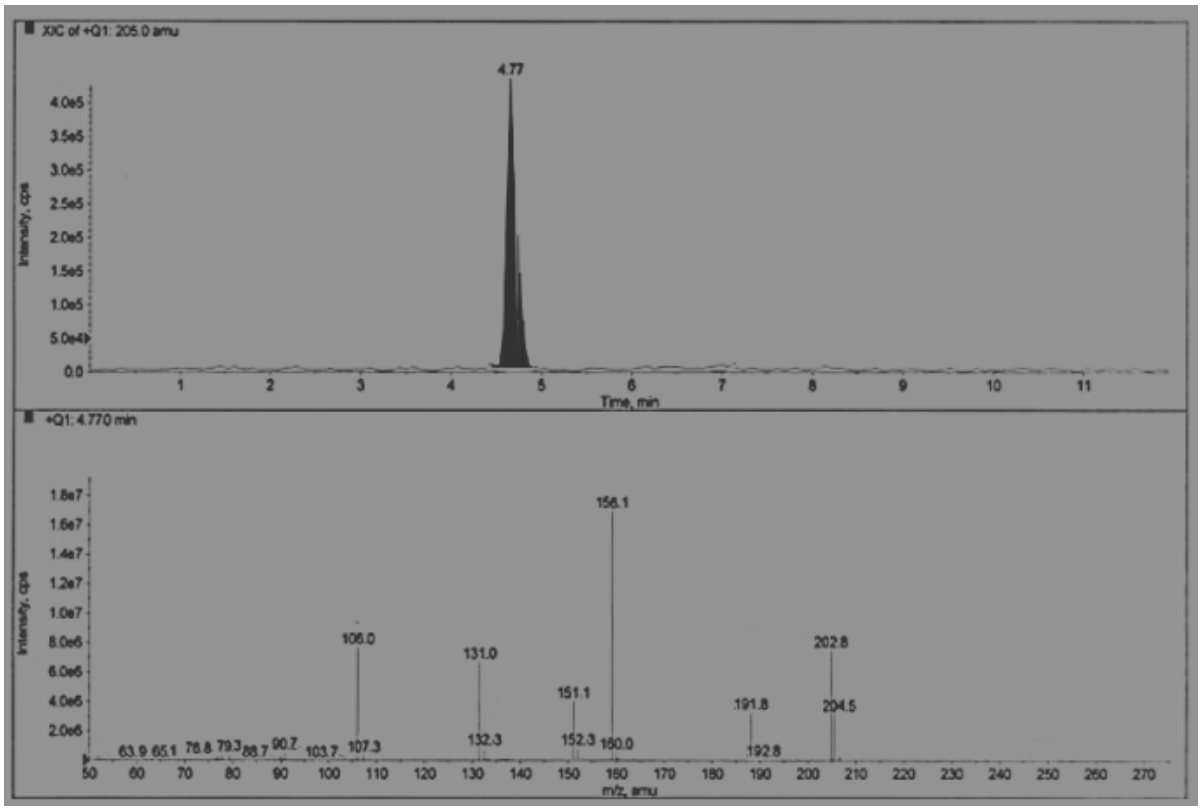


**Fig. 6. Chromatogram of 2-chloro-5-nitrobenzoic acid (Imp-D)**

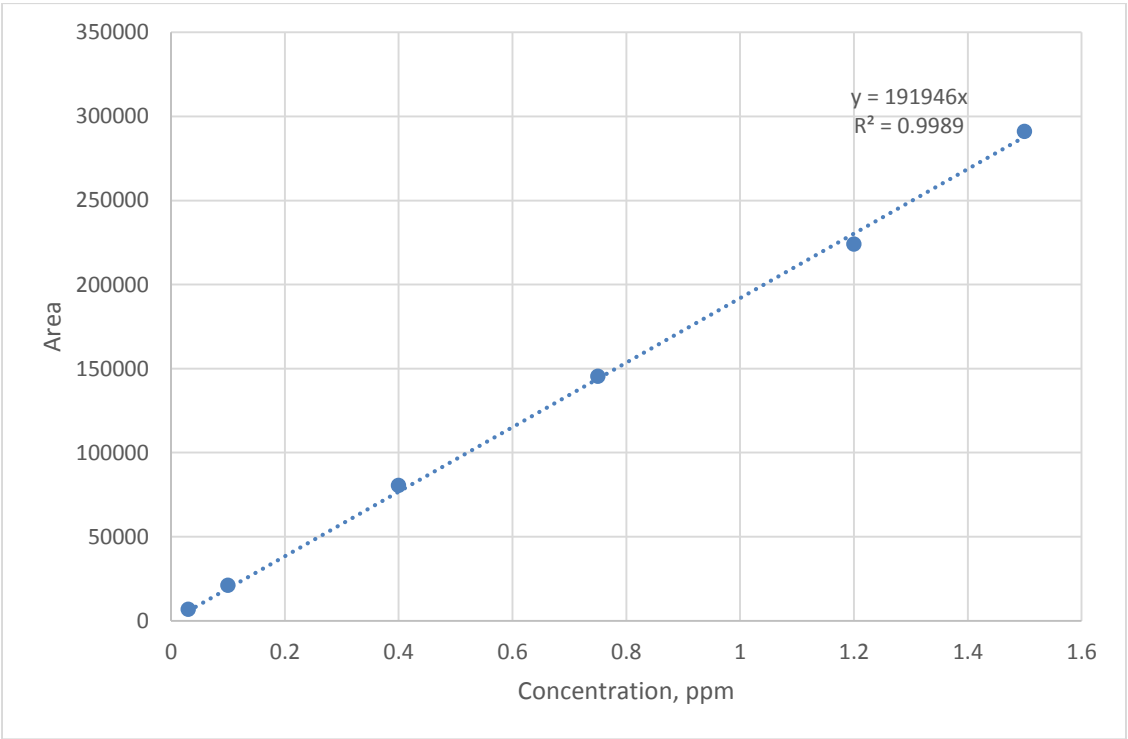


**Fig. 7. Linearity plot of 2-chloro-5-nitroaniline (Imp-A) in the concentration range of 0.03–1.50 ppm level**

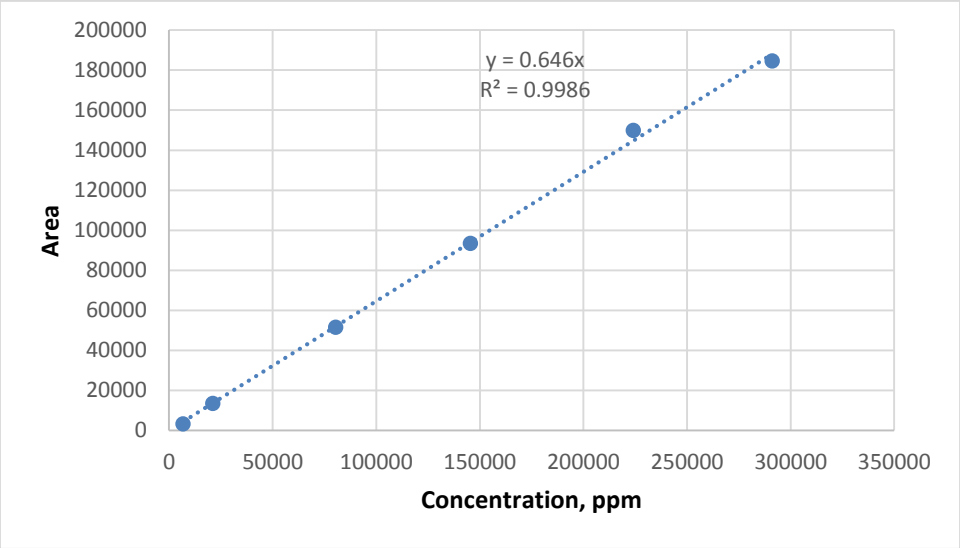


**Fig. 8. Linearity plot of 1-chloro-2-iodo-4-nitrobenzene (Imp-B) in the concentration range of 0.03–1.50 ppm level**

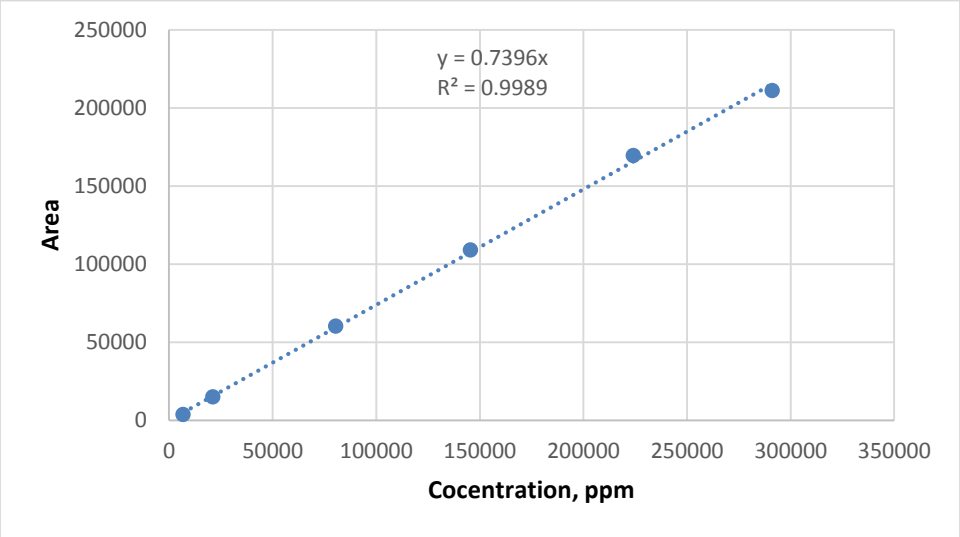


**Fig. 9. Linearity plot of 1-(2-chloro-5-nitrophenyl)ethan-1-one (Imp-C) in the concentration range of 0.03–1.50 ppm level**

**Table 4. Linearity of Imp-A, Imp-B, Imp-C and Imp-D**

| Level | Imp-A area | Imp-B area | Imp-C area | Imp-D area |
|---|---|---|---|---|
| LOQ (0.03) | 6750 | 4462 | 3145 | 3762 |
| 0.1 | 21142 | 18193 | 13486 | 15178 |
| 0.4 | 80451 | 70742 | 51462 | 60352 |
| 0.75 | 145468 | 121596 | 93456 | 109245 |
| 1.20 | 224068 | 198372 | 149764 | 169742 |
| 1.50 | 291050 | 251163 | 184576 | 211185 |
| Slope | 190092.60 | 165664.09 | 123230.38 | 140427.28 |
| Intercept | 2060.08 | 864.16 | 905.35 | 1760.57 |
| Correlation Coefficient | 0.9989 | 0.9986 | 0.9989 | 0.9993 |

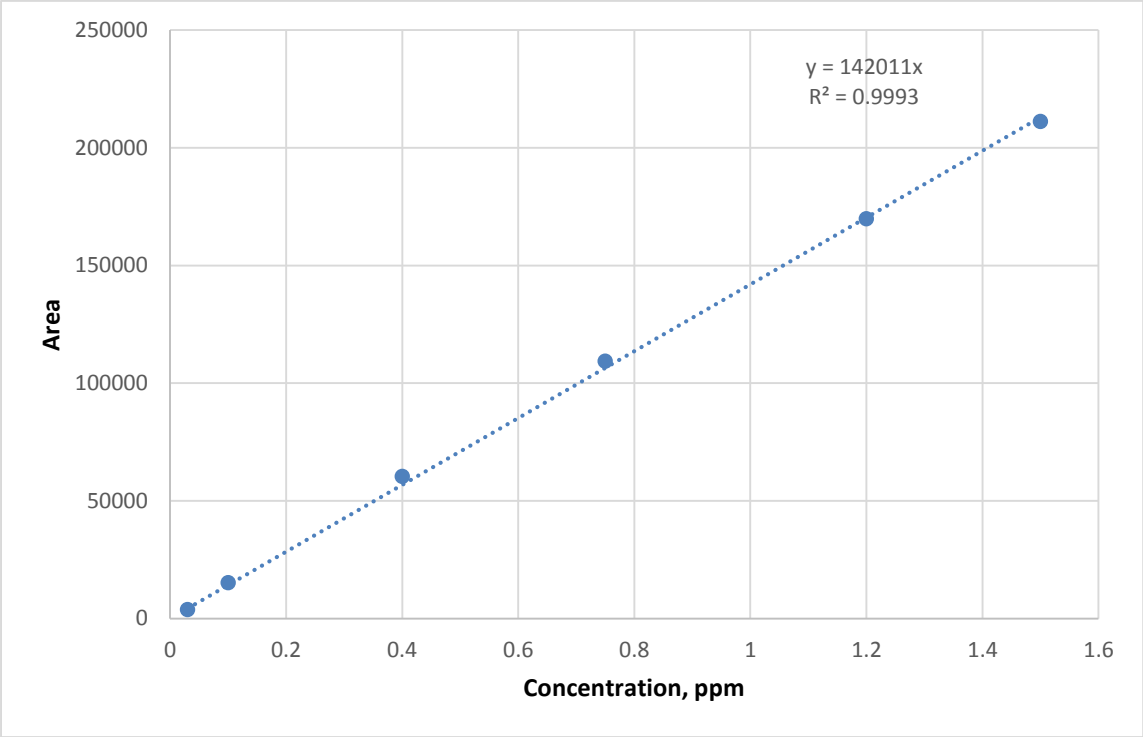


**Fig. 10. Linearity plot of 2-chloro-5-nitrobenzoic acid (Imp-D) in the concentration range of 0.03–1.50 ppm level**

### 4.3 Recovery Studies

Recovery studies were determined by spiking the four PGIs at LOQ level, 50%, 100% and 150% of the specification concentrations, i.e 0.03, 0.5, 1.0 and 1.5 ppm with respect to the sample concentration and corresponding data is presented in Table 5. The recovery of impurities at LOQ level should be in the range of 70.0% to 130.0%. The recovery of impurities at three levels (50%, 100% and 150%) should be in the range of 80.0% to 120.0 %. Well recovery values of 97.68% to 106.90% for Imp-A, 96.67% to 101.39% for Imp-B, 99.40 % to 103.92 % for Imp-C and 98.85% to 103.23% for Imp-D were obtained.

### 4.4 Limit of Quantification and Limit of Detection

Limit of quantification (LOQ) and Limit of detection (LOD) were determined by analyzing different concentrations of impurities at low concentration. In this process, the concentrations of standard solutions were reduced sequentially to obtain limit of quantification, such that they yield S/N ratio as 10.1, 10.4, 10.1 and 10.0 for Imp-A, Imp-B, Imp-C and Imp-D respectively. The Limit of quantification (LOQ) of 0.03 ppm is general for all the potential genotoxic impurities with a LOD of 0.01 ppm and is nearly three times less than LOQ. This analysis was carried out in MRM mode and LOD was determined as 0.01ppm. The precision of six injections containing 0.03 ppm of each potential genotoxic impurities at LOQ level was below 5.0% RSD. The LOD and LOQ spiked chromatograms of samples are presented in Fig. 11 and Fig. 12.

### 4.5 Precision and Ruggedness

To determine precision of the method through repeatability and ruggedness, we prepared six fresh preparations of standard mixture solutions containing four impurities at a concentration of 1.0 ppm of each one on the same day and their recoveries were checked. Ruggedness was evaluated by injecting the six freshly prepared solutions containing 1.0 ppm of each potential genotoxic impurity at different days and their recoveries with % RSD values are presented in Table 6. The lower values of % RSD (below 4 %) values confirm that the precision of the developed method is good and well suited for different laboratory conditions.

**Table 5. Accuracy of the thee potential genotoxic impurities**

| % Level | Theoretical conc (ppm) | Imp-A Measured Conc (ppm) | Recovery mean ± RSD (%) | Theoretical conc (ppm) | Imp-B Measured Conc (ppm) | Recovery mean ± RSD (%) | Theoretical conc (ppm) | Imp-C Measured Conc (ppm) | Recovery mean ± RSD (%) | Theoretical conc (ppm) | Imp-D Measured Conc (ppm) | Recovery mean ± RSD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LOQ | 0.029 | 0.031 | 106.90 | 0.030 | 0.029 | 96.67 | 0.030 | 0.031 | 103.33 | 0.031 | 0.032 | 103.23 |
| 50 | 0.512 | 0.510 | 99.61 | 0.505 | 0.512 | 101.39 | 0.500 | 0.497 | 99.40 | 0.499 | 0.514 | 103.01 |
| 100 | 1.034 | 1.010 | 97.68 | 1.064 | 1.060 | 99.62 | 1.020 | 1.060 | 103.92 | 1.040 | 1.028 | 98.85 |
| 150 | 1.524 | 1.540 | 101.05 | 1.526 | 1.510 | 98.95 | 1.510 | 1.536 | 101.72 | 1.538 | 1.529 | 99.41 |

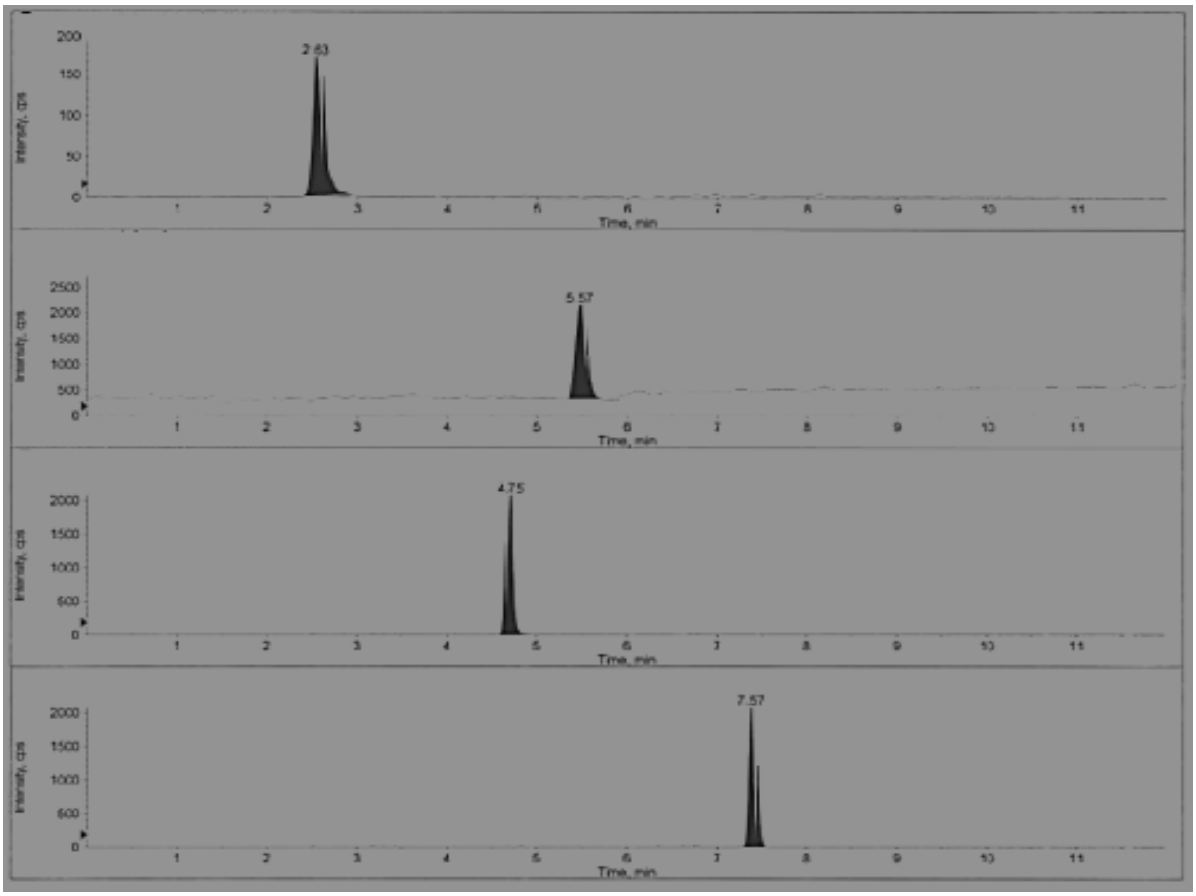


**Fig. 11. Chromatogram of Imp-A, Imp-B, Imp-C and Imp-D at LOD level**

**Table 6. Method precision and ruggedness of Imp-A, Imp-B, Imp-C and Imp-D PGI's at 1.0 ppm in terms off % recovery**

| Injection ID | Imp-A | | Imp-B | | Imp-C | | Imp-D | |
|---|---|---|---|---|---|---|---|---|
| | Method Precision | Ruggedness | Method Precision | Ruggedness | Method Precision | Ruggedness | Method Precision | Ruggedness |
| 1 | 98.64 | 98.48 | 97.45 | 100.86 | 98.91 | 103.46 | 95.46 | 96.87 |
| 2 | 97.21 | 100.44 | 96.82 | 97.45 | 100.2 | 101.24 | 97.38 | 98.14 |
| 3 | 95.45 | 98.67 | 97.86 | 99.48 | 101.45 | 99.93 | 98.57 | 96.98 |
| 4 | 98.01 | 100.23 | 99.38 | 100.19 | 98.79 | 100.31 | 96.45 | 97.35 |
| 5 | 96.45 | 98.01 | 98.11 | 97.79 | 102.11 | 99.79 | 98.42 | 98.98 |
| 6 | 97.45 | 99.42 | 99.48 | 98.65 | 99.48 | 101.45 | 97.64 | 96.89 |
| Mean | 97.20 | 99.21 | 98.18 | 99.07 | 100.16 | 101.03 | 97.30 | 97.54 |
| SD | 1.13 | 0.99 | 1.06 | 1.35 | 1.37 | 1.37 | 1.19 | 0.85 |
| % RSD | 1.17 | 0.99 | 1.08 | 1.36 | 1.37 | 1.36 | 1.22 | 0.88 |

## 4.6 Robustness

The robustness of the method was evaluated by making deliberate changes in experimental conditions including column temperature, flow rate and source temperature in mass source. Actual flow rate of the mobile phase was 0.5 mL/min and the same was altered by 0.2 units i.e. 0.3 mL/min and 0.7 mL/min. The effect of column temperature on the analysis was studied at 43° C and 47° C (temperature altered by 2 units). The robustness of the proposed method also evaluated by the changing the temperature in mass source with ± 20°C. No significant change in the chromatographic performance was observed for all the above deliberately varied experimental conditions, which indicated the robustness of the method.

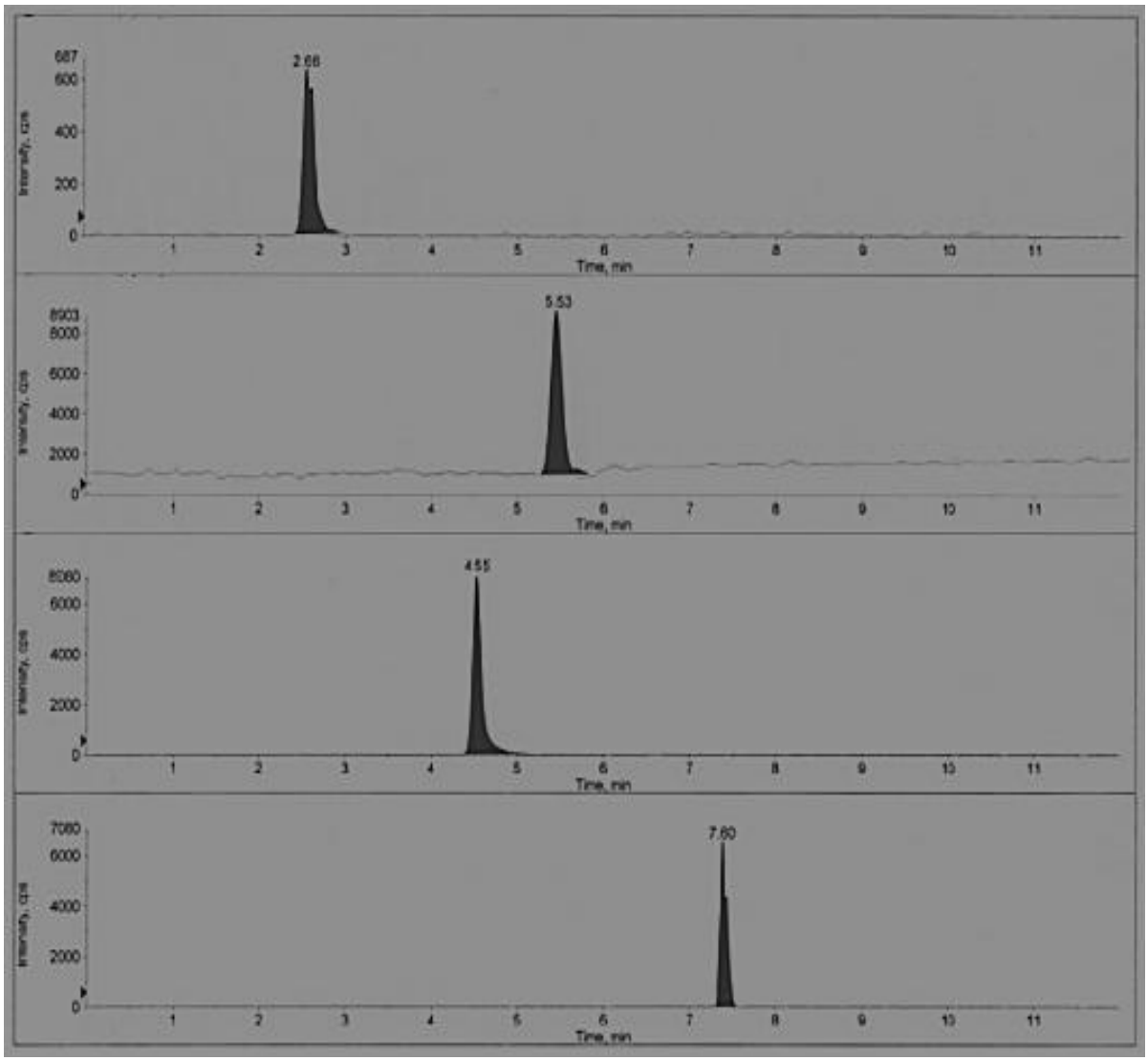


**Fig. 12. Chromatogram of LOQ spiked sample**

**Table 7. Solution stability data of Imp-A, Imp-B, Imp-C and Imp-D potential genotoxic impurities at 1.0 ppm in terms of % recovery**

| Conditions | Imp-A | Imp-B | Imp-C | Imp-D |
|---|---|---|---|---|
| at 0 hrs | 100.11 | 100.32 | 99.47 | 100.09 |
| at RT for 24 hrs | 99.48 | 100.01 | 98.53 | 99.43 |
| at 2-8°C for 24 hrs | 99.40 | 99.49 | 98.59 | 99.51 |
| Spiked sample | | | | |
| Spiked sample at 0 hrs | 97.49 | 98.32 | 99.40 | 97.46 |
| Spiked sample at RT for 24 hrs | 97.73 | 97.78 | 98.97 | 98.01 |
| Spiked sample at 2-8°C for 24 hrs | 96.94 | 97.49 | 98.49 | 97.93 |

## 4.7 Solution Stability Studies

The stability experiments were performed thoroughly to evaluate the stability of four impurities stock solutions at room temperature (25°C) and refrigerator (2-8°C) conditions for 24 hrs. The data presented in Table 7 revealed that the solution was stable up to 24 hrs both at refrigerator condition and room temperature.

## 5. CONCLUSION

In this study, we have developed a simple LC-MS/MS approach that is capable of quantifying four different PGIs simultaneously in vismodegib API using the positive ionization mode with multiple reaction monitoring (MRM).The method was validated and good linearity, specificity, precision, accuracy, stabilityand robustness. The specificity of the method was proved by good resolution of impurities with the drug. The LOD and LOQ values for Imp-A, Imp-B, Imp-C and Imp-D are very low as 0.01 and 0.03 ppm, respectively. The sample prepared in analytical solution is found to be stable for at least 24 hr. Therefore, the above-mentioned LC–MS/MS method for the analysis of four potential genotoxic impurities are found to be simple, selective and sensitive. The method presented here could be very useful for monitoring of four potential genotoxic impurities in vismodegib API during its manufacturing.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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