A SIMPLE, SENSITIVE AND DIRECT LC-MS METHOD FOR THE IDENTIFICATION AND QUANTIFICATION OF THREE POTENTIAL GENOTOXIC IMPURITIES IN LAROTRECTINIB BULK DRUG AND PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT:

Objectives: The evaluation of toxicological levels of potential genotoxic impurities in bulk drug and its commercial forms was treated as significant as well as very difficult issue. The European Medicines Agency guided that 1.5 µg/day intake of a genotoxic impurity was treated as given as acceptable for most of the pharmaceutical products. In view of this, this study intended to establish a simple and sensitive LCMS method for the quantification of potential genotoxic impurities (PGIs) such as impurity 1, 2 and 3 in larotrectinib pure drug and its drug product.

Materials and Methods: Method development was performed by varying various method conditions and was validated for method validation. Method applicability was assessed by analysing PGIs spiked larotrectinib spiked formulation.

Results: The method comprises of YMC-Triart C18 (150×4.6mm; 5 µm particle size) column as stationary phase, acetonitrile and 0.05 M ammonium acetate in 0.02 % formic acid in 65:35 (v/v) at pH 4.3 at 0.4 mL/min flow rate was finalized as suitable conditions for the resolution of PGIs in larotrectinib. The eluents were monitored in multiple reaction monitoring operated in positive ion mode using a mass detector. The method clearly separates the analytes that shows characteristic mass transition at m/z of 429 342 for larotrectinib, 201 124 for PGI 1, 206 115 for PGI 2 and 178 92 for PGI 3. There are no impurities or un-wanted compounds detected in both chromatograms as well as mass pattern proved that the method was specific. The validity of the method was evaluated by performing various validation parameters like linearity, precision, recovery, ruggedness, robustness and reported acceptable results. The method reports 0.001 µg/mL for larotrectinib, PGI 2 and 0.003 µg/mL for PGI 1 and 3 as detection limit that proves the method sensitivity. The method detect the PGIs along with larotrectinib in spiked formulation.

Conclusion: The method can successfully separate and detect the PGIs spiked formulation sample and hence can be utilized for assessment of studied PGIs during the synthesis process of larotrectinib as well as manufacturing the pharmaceutical products.

Key Words: Genotoxic Impurities, LCMS analysis, Larotrectinib, Method Development, Formulation analysis

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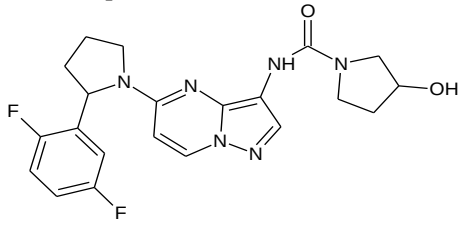
INTRODUCTION:

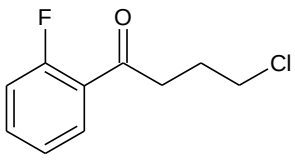
In the process of manufacture of active pharmaceutical ingredients (APIs) various chemical entities such as reactive intermediates, catalysts, acids or bases etc., were often used. These entities remains trace level in the final product [1]. Some of these entities were recognized as potential genotoxic impurities (PGIs) based on its chemical structure, properties and reactivity. These PGIs are the unwanted chemical entities that have the capability to induce mutations in genes and can cause chromosomal breaks or rearrangements and subsequently lead to cancer [2]. The quantification of these PGIs in API gained high attention to produce safe pharmaceutical product [3,4]. Various regulatory agencies such as Food and Drug Administration - United States etc., issued guidelines for control and analysis of these PGIs in drug and drug product [5,6]. As per the issued guidelines, the target detection limit for the evaluation of these PGIs in a drug or the drug product could be less than 1 ppm or less than 1 µg/g in API. This limit will be 500 times less than the limit specified for classical impurity analysis [5,7]. In most of the traditional quality control methods that are based on the direct injection and separation of API containing PGI followed by HPLC-UV or GC-FID analysis were not effective for qualitative and quantitative analysis of PGIs. LCMS is the versatile technique that confirms the molecular weight along with molecular structure and hence can significantly useful for detecting and quantifying small organic molecules, adulterants in food and pharmaceutical products including PGIs [8].

Larotrectinib is the medical drug belongs to neurotrophin receptor kinase selective inhibitor prescribed to treat solid tumors in adults as well as children of more than 1 month age and older that distributed to other parts or cannot be prevented through surgery [9]. This medicine prescribed only if there is no availability of alternative medication and if the tumors worsened after any other alternative medication. It works by blocking abnormal protein action that causes the multiplication of cancer cells and subsequently decrease the tumor growth [10]. The common side effects reported for the use of larotrectinib are constipation, stomach pain, cough, diarrhea, weight gain, nasal congestion, muscle weakness, increased thirst, vomiting, nausea, headache, muscle, joint, or back pain and change in urine color or quantity [11].

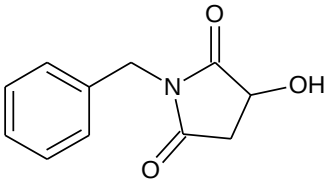
The extensive literature review confirms that one HPLC method reported for the quantification of larotrectinib in formulations [12]. In literature few bio-analytical methods reported for the quantification of larotrectinib in biological samples like blood, plasma and tissue homogenates using HPLC [13] and LCMS [14,15]. One stability indicating analytical LCMS method followed by spectroscopic character-ization degradation compounds was reported [16]. In literature, no method was available for the identification and quantification of PGIs in larotrectinib pure drug and its drug products. Hence the present study was aimed to develop a simple LCMS method for the separation, identification and quantification of PGIs in larotrectinib. Based on availability, impurity 1, 2 and 3 of larotrectinib were selected for the study and the molecular structure of larotrectinib and its PGIs in the study were presented in table 1.



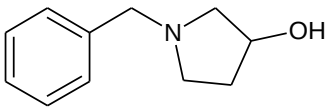
Larotrectinib



Impurity 1 (PGI 1)
[*4-Chloro-1-(2-fluorophenyl)butan-1-one*]



Impurity 2 (PGI 2)
[*(S)-1-Benzyl-3-hydroxypyrrolidine-2,5-dione*]



Impurity 3 (PGI 3)
[*(3S)-1-Benzylpyrrolidin-3-ol*]

Figure 1: Molecular structure Larotrectinib and its PGIs

MATERIALS AND METHODS:

Instrumentation:

Waters alliance 2695 (Japan) LCMS equipped with triple quadruple (LAA 1369) mass detector and programmable auto-injector with an injection capacity of 0.1-1500 µL and column eluents were recorded with Waters ZQ mass analyser. Various configurations of columns were utilized for the effective separation of analytes and the chromatographic integration and mass fragmentations were recorded through Waters Mass Lynx MS software. The mass detector was operated in multiple reaction monitoring (MRM) positive ion mode. The analyser was operated with suitable conditions of 10 mL/min of drying gas flow, nebulizer pressure of 30 psi and 5500 V of spray voltage.

Chemicals and Reagents:

The larotrectinib pure compound with purity of 98.19 % and its three PGIs in the study namely impurity 1, 2 and 3 along with its capsule formulation with brand VITRAKVI® - 25 mg were obtained from Bayer Zydus Pharma, Maharashtra. The LiChropur™ grade chemicals utilized in the study such as ammonium acetate and formic acid, HPLC grade solvents utilised in the study such as methanol, acetonitrile, water and membrane filters (0.2 µ) were purchased from Merck chemicals, Mumbai.

Preparation of solutions:

Mobile Phase:

The solvents such as acetonitrile, methanol and ammonium acetate (0.01 M in 0.1% formic acid) were utilized as solvents for mobile phase preparation during the method development study. The selected composition of each solvent was mixed separately in a reservoir bottles and the uniform mixing and degassing of the solvent mixture was performed using an ultrasonic batch sonicator. The uniformly mixed and degassed solvent composition was filtered using membrane filter (0.2 micron) and stored in an amber colour bottle at room temperature whenever necessary.

Standard stock solution:

The stock solution of larotrectinib and its PGIs were prepared individually in volumetric flask (25 mL). Previously weighed 25 mg of standard larotrectinib and its PGIs were taken individually in 25 mL volumetric flask having 10 mL of methanol. Then it was sonicated using ultra sonic bath sonicator for 2 min to dissolve analytes and make the flask up to the mark. As a result, the standard stock solution of larotrectinib and its PGIs at 1000 µg/mL was obtained individually. The obtained 1000 µg/mL solutions were further diluted to 100 µg/mL separately and this was used for preparing combined standard stock solution containing larotrectinib and its PGIs. The combined standard solution containing known and selected concentration of larotrectinib and its PGIs was prepared by separately adding equal volumes of larotrectinib and its PGIs individual stock solutions. The combined standard solutions were used during the method optimization and validation studies.

Formulation solution:

The capsule formulation of larotrectinib was powdered using a clear and dry mortar and pestle to obtain a uniform fine powder. The capsule powder was weighed such that the weighed sample contain 25 mg equivalent of larotrectinib was added to 25 mL calibrated flask filled with 10 mL of methanol. The analytes were dissolved in solvent by keeping the flask in an ultra-sonic bath sonicator to 5 min. After completion of sonication, it was filtered using 0.2 micron membrane filter in to 25 mL calibrated flask and make the flask up to mark. The formulation solution containing 1000 µg/mL equivalent of larotrectinib was obtained. Then it was diluted to 100 % recovery level in calibration range and was utilized for the analysis. The formulation stock solution spiked with 0.05 % of impurities was also analysed in the developed method.

Method development:

The development of method for resolution, identification and quantification PGIs in larotrectinib was conducted as per the guidelines issued by ICH [17]. In the process of method development, various method conditions like ratio, pH and flow of mobile phase, stationary phase configuration, its temperature and mass detector operating conditions. The standard solution of larotrectinib containing its PGIs at 0.5 µg/mL concentration was analysed in each studied condition. In each changed condition, the chromatographic response of individual analyte along with its system suitability conditions such as number of theoretical plates, tail factor, resolution and mass fragmentation pattern was evaluated. The method conditions that produces best chromatographic and system suitability results was finalized as appropriate conditions for analysis of larotrectinib and its PGIs.

Method validation:
Various method validation parameters such as linearity, precision, ruggedness, robustness, sensitivity were performed as per ICH guidelines [18] for evaluation of the validation of the developed method.

RESULTS AND DISCUSSION:
This study aimed to establish a simple and sensitive LCMS method for detection and estimation of three PGIs in larotrectinib active pharmaceutical ingredient and its dosage forms. The method optimization was initiated by keeping in view that the method can resolve and detect all the three PGIs at trace level along with standard larotrectinib. Different configurations of C8 and C18 columns with different column temperatures were studied for resolving the PGIs of larotrectinib. Different composition of solvents such as acetonitrile, methanol and ammonium acetate in formic acid was varied. The analytes were resolved on YMC-Triart C18 (150×4.6mm; 5 µm particle size) column as stationary phase, acetonitrile and 0.05 M ammonium acetate in 0.02 % formic acid in 65:35 (v/v) having pH 4.3 at 0.4 mL/min flow rate was finalized as suitable conditions for the resolution of PGIs in larotrectinib. The mass operating conditions such as electron multiplier voltage, fragmentor voltage, collision energy and the mass operating mode was optimized. The mass analyser was operated at positive ion multiple reaction monitoring (MRM) mode. The mas operating conditions were summarized in table 1. In the optimized conditions, the standard solution was injected at sample volume of 10 µL.

S No	Analyte	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (eV)	Electron Multiplier Voltage (V)	MS1 RES
1	Larotrectinib	429	342	180	35	600	Wide
2	PGI 1	201	124	175	30	600	Wide
3	PGI 2	206	115	160	35	600	Wide
4	PGI 3	178	92	150	45	600	Wide

Table 1: Optimized mass detector conditions

The method specificity was confirmed by analysing 0.5 µg/mL concentration of larotrectinib and its PGIs in single and in combination along with blank. The chromatogram observed for blank analysis was presented in figure 2 and the chromatogram doesn't detect any chromatographic responses in the complete run time of analysis. The standard chromatogram as shown in figure 3 shows clear separation of analytes in the study with no additional detection of un-wanted compounds or the impurities proves that the method was specific for the analysis of PGIs in larotrectinib.

Figure 2: Blank chromatogram observed in the developed method

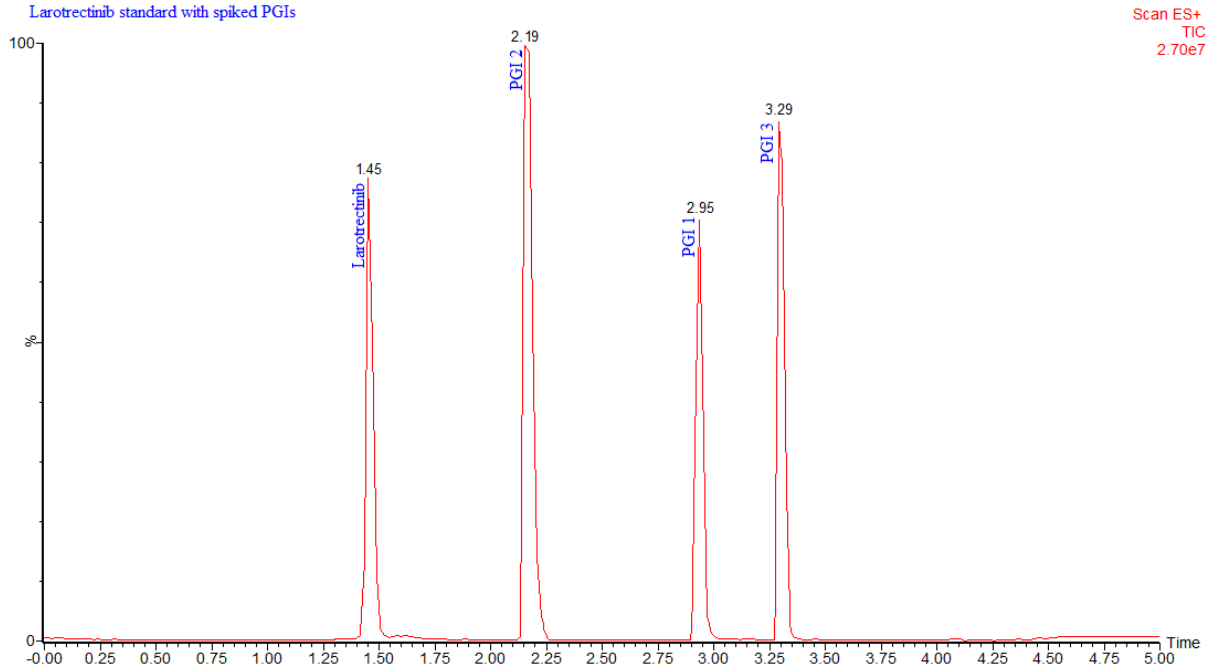


Figure 3: Standard chromatogram observed in the developed method

The retention time (t_R) of individual analysis of larotrectinib and its PGIs that was observed in the optimized method was compared with combined solution analysis. The chromatogram of individual analysis confirms that larotrectinib identified at t_R of 1.45 min. The PGIs were identified at 2.95 min, 2.19 min and 3.29 min for PGI 1 to 3 respectively. Based on the t_R of analytes identified for the individual analysis of the analytes was compared with the t_R of peaks identified in the combined solution analysis. The t_R of individual analysis of the analytes was observed to same as the peaks identified for combined analysis confirms the selectivity of the established method. There is no detection of additional compounds or the impurities in the run time of combined standard, individual standard solutions as well as the blank analysis confirms the method specificity for analysis of larotrectinib and its PGIs.

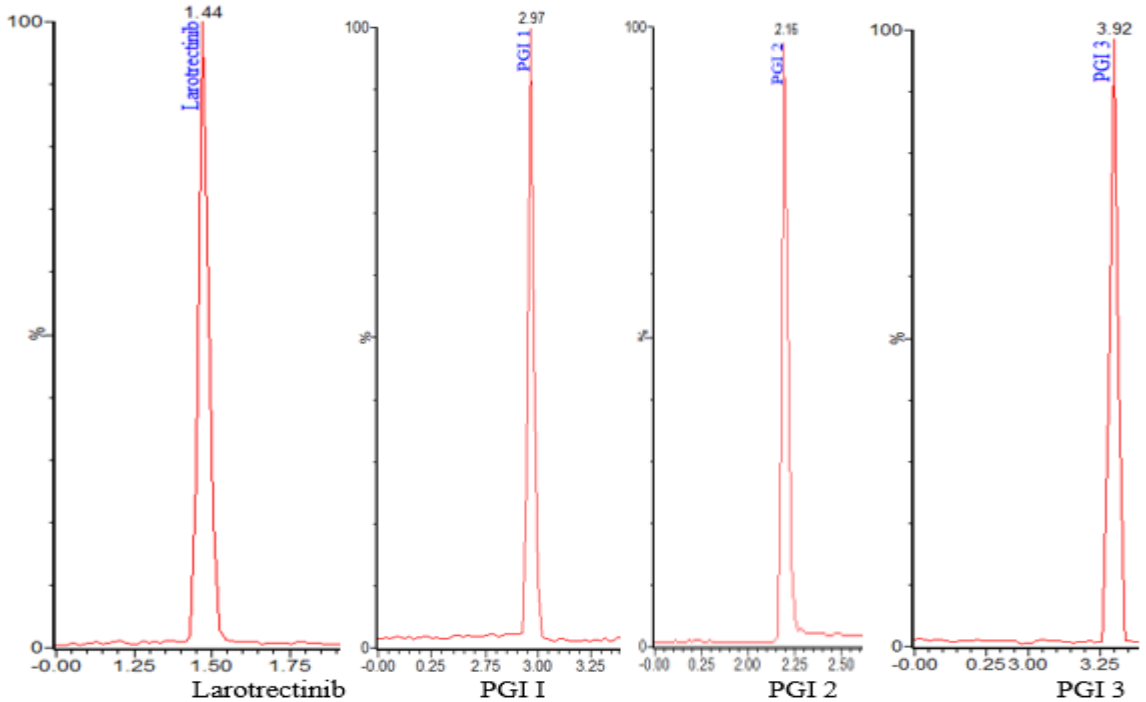
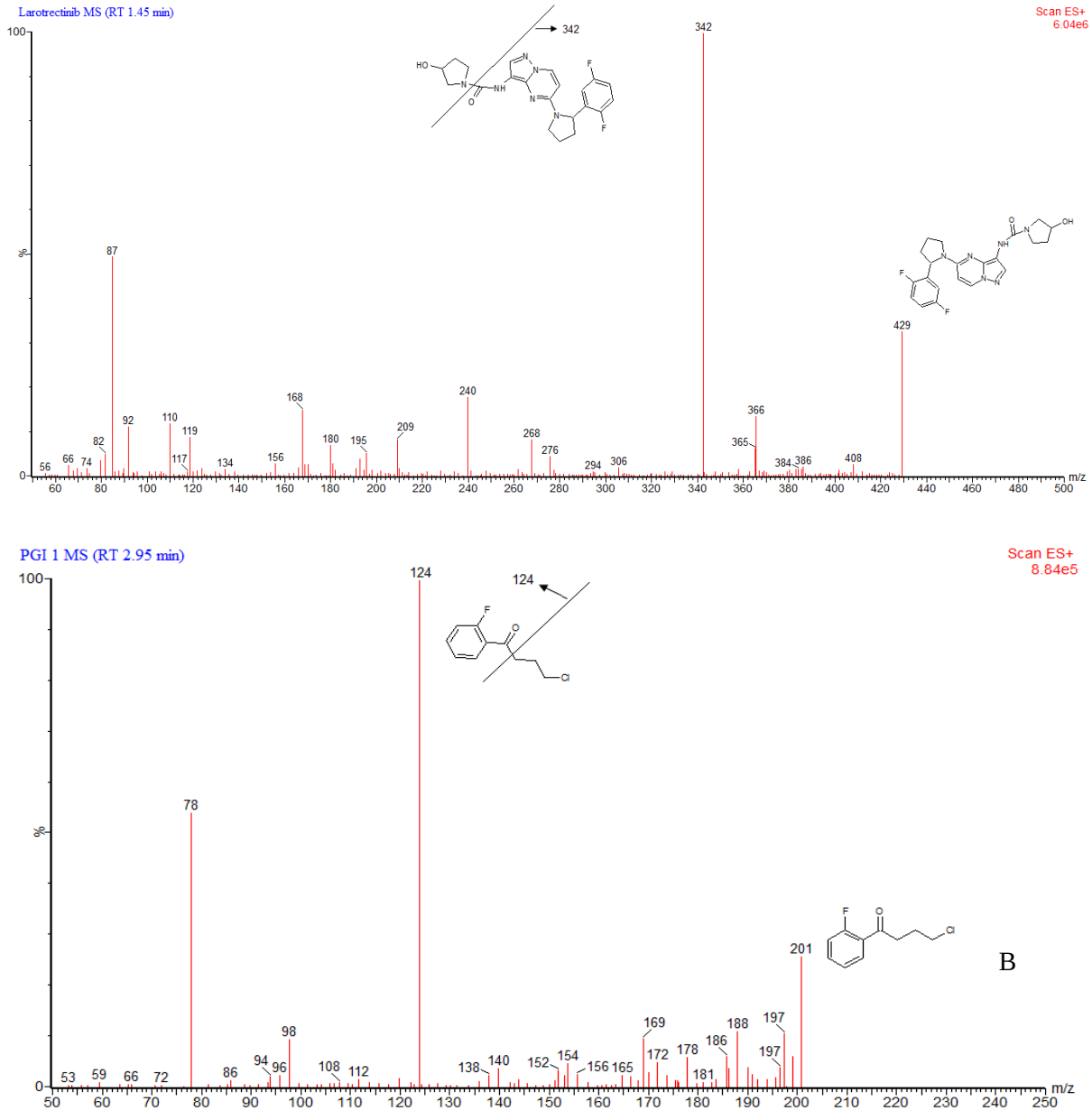
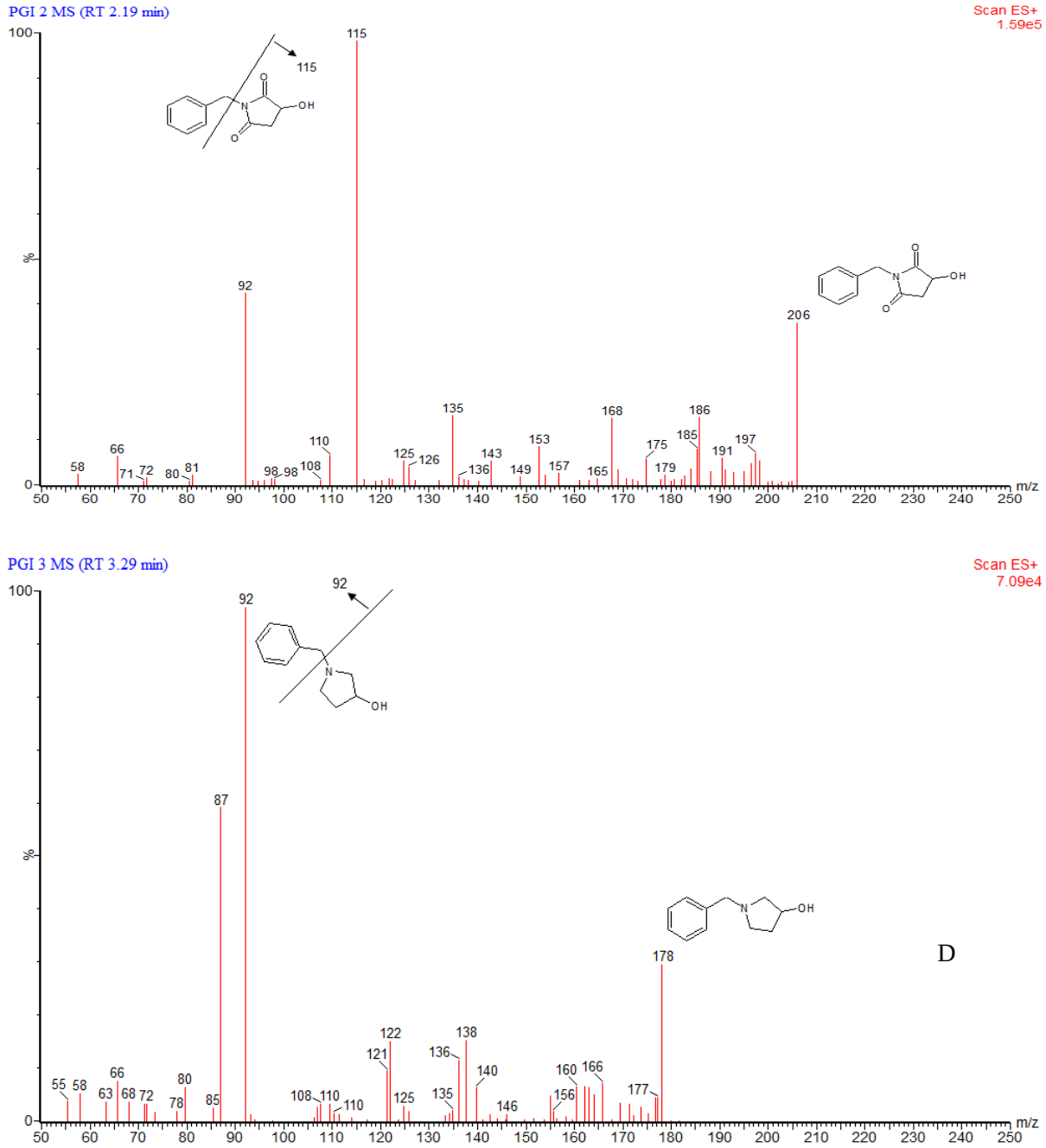


Figure 4: Chromatograms observed for individual analysis of larotrectinib and its genotoxic impurities in the study

The mass analyzer was operated on positive ion multiple reaction monitoring was conducted for the spectral characterization of the analytes in developed method. The peak corresponds to larotrectinib identified at t_R of 1.44 min shows parent ion at m/z of 429 (m+1). The mass spectra shows daughter fragment at m/z of 342. The mass spectra at the t_R of PGIs in the study were evaluated and the parent ion identified at m/z (m+1) of 201, 206 and 178 respectively for PGI 1 to 3 whereas the daughter ion fragments identified at m/z (m+1) of 124, 115 and 92 for PGI 1 to 3 respectively. The mass fragmentation pattern of the individual analysis of the analystes was in correlation with the results observed for the combined analysis of analytes. There is no additional detection or fragments correspond to impurities or the un-identified fragments detected in the mass patter of the analytes. This proves that the method provides specific analysis results corresponds to larotrectinib and its PGIs in the study. The % abundance of the daughter fragments were noticed to be significantly higher than the other ions detected in the spectrum. Hence the method was specific and suitable for analyzing larotrectinib and its PGIs. The mass fragmentation results were represented in figure 5.





Mass fragmentation spectra observed at the retention of 1.45min for larotrectinib (A), 2.95 min for PGI 1, 2.19 min for PGI 2 and 3.29 min for PGI 3

Figure 5: Mass fragmentation spectra obtained at the retention time of larotrectinib and its PGIs in the developed method

Method Validation:

The analytes linearity range was confirmed by analysing various concentrations of larotrectinib and its PGIs. Calibration curve was drawn by considering area response of individual analyte on *y-axis* and analyte strength on *x-axis*. Calibration range was confirmed by taking high correlate concentration range for the analytes in the study. The accurate fit with high correlate calibration curve observed in 0.01 µg/mL - 5.0 µg/mL level for larotrectinib and its PGIs. The least-squares linear regression analysis was conducted to evaluate calibration curve parameters like regression equation, intercept and slope. As results tabulated in table 1, the calibration equation was obtained as y = 136686x + 31884 (R^2 = 0.9994), y = 187717x + 48293 (R^2 = 0.9993), y = 150664x + 38634 (R^2 = 0.9996) and y = 162381x + 30422 (R^2 = 0.9991) respectively for larotrectinib, PGI 1, PGI 2 and PGI 3.

S No	Concentration in µg/mL	Peak are response obtained			
		Larotrectinib	PGI 1	PGI 2	PGI 3
1	0.01	21849.3	39251.5	29784.5	34698.2
2	0.05	43512.2	58645.8	45979.6	50021.3
3	0.1	52499.3	81498.3	59701.5	51424.5
4	0.5	97958.5	139876.0	115424.7	97595.1
5	1	168652.2	227614.5	189516.2	191012.3
6	2	308257.9	431323.9	345214.8	347461.5
7	5	714158.9	985468.2	789564.7	846958.1

Table 1: Linearity results

The recovery experiment was conducted in the concentration of 0.5 µg/mL, 1.0 µg/mL and 2.0 µg/mL in the linearity level for larotrectinib and its PGIs in the study. The recovery level concentration solution was prepared and analysed in triplicate in the developed method and the area results of individual analyte was compared with its corresponding peak area response on the calibration level. The % recovery of each analyte and % RSD in studied spiked level was calculated. The % recovery of 98.12 - 99.76, 98.25 - 100.25, 98.11 - 100.85 and 98.36 - 100.63 % for larotrectinib, PGI 1, PGI 2 and PGI 3 respectively which is under the acceptable level of 98-102 %. The % RSD was determined in each studied level for the analytes and results were observed to be less than 2 and were acceptable as per the guidelines. The results observed to be under the acceptable level confirms that the method was recoverable and accurate. The results attained in recovery study of the method optimized for the analysis of larotrectinib and its PGIs was represented in table 2.

S. No	Compound	Level	Concentration in µg/mL	Recovered in µg/mL Mean±SD	% Recovery	% RSD
1	Larotrectinib	50%	0.5	0.492±0.001	98.350±0.291	0.296
2		100%	1.0	0.996±0.002	99.600±0.151	0.152
3		200%	2.0	1.983±0.018	99.157±0.898	0.906
4	PGI 1	50%	0.5	0.495±0.003	98.940±0.510	0.516
5		100%	1.0	0.985±0.003	98.540±0.337	0.342
6		200%	2.0	1.993±0.011	99.647±0.573	0.575
7	PGI 2	50%	0.5	0.493±0.003	98.667±0.570	0.578
8		100%	1.0	0.999±0.004	99.863±0.412	0.413
9		200%	2.0	1.997±0.025	99.833±1.234	1.236
10	PGI 3	50%	0.5	0.495±0.002	98.960±0.490	0.495
11		100%	1.0	0.991±0.007	99.107±0.745	0.752
12		200%	2.0	2.009±0.004	100.460±0.200	0.199

Table 2: Accuracy results

The method repeatability and reproducibility was assessed by analysing 0.5 µg/mL concentration of larotrectinib and its PGIs. The solution was

analysed six injections in one day for intraday precision, six injections in three days for interday precision. The same concentration was assessed six times in same day by three different analysts for assessing ruggedness. The peak response of the larotrectinib and its PGIs was summarized and % RSD of area response was calculated. The % RSD of less than 2 was considered as acceptable as per the guidelines and based on the results as summarized in table 3, the results were achieved were under the acceptable level confirms the precise and reproducibility of method.

The minor variations in the optimized method conditions such as composition of mobile phase with no change in pH, change in pH and change in mobile phase was made intentionally for the evaluation of the robustness of the method. The mobile phase composition of acetonitrile and 0.05 M ammonium acetate in 0.02 % formic acid in 60:40 (MP change 1) and 70:30 (MP change 2) at pH 4.2 (pH change 1) and 4.4 (pH change 2) was studied at a flow rate of 0.35 mL/min (FR change 1) and 0.45 mL/min (FR change 2). In each varied condition, larotrectinib and its PGIs standard

solution at 0.5 µg/mL concentration was analysed. The area response of larotrectinib and its PGIs in each analysis was tabulated and the % change in peak area response was calculated by comparing the robustness peak with its corresponding linearity peak area. The % change of less than 2 was considered as acceptable and as summarized in table 3, the results noticed were under the acceptable for larotrectinib and its PGIs. Based on achieved results, it can confirmed that there is no significant changes was observed while change in the minor variations in proposed method proves that the method was robust.

The sensitivity of larotrectinib and its PGIs in the established method was expressed in terms of LOD (limit of detection) and LOQ (limit of quantification). The LOD and LOQ of larotrectinib and its PGIs was evaluated by adopting signal (s) to noise (n) ratio approach. The s to n ratio of 3 was considered as LOD and 10 was considered as LOQ. The LOD was obtained as 0.001 µg/mL for larotrectinib, PGI 2 and 0.003 µg/mL for PGI 1 and 3 whereas the LOQ was obtained as 0.003 µg/mL for larotrectinib, PGI 2 and 0.010 µg/mL for PGI 1 and 3. The sensitivity results confirms that the proposed procedure can detect PGI 2 up to a very low concentration of 0.001 µg/mL whereas it can detect PGI 1 and PGI 3 up to 0.003 µg/mL and hence proved that the method was very sensitive.

The stability of the solution prepared for the analysis of larotrectinib and its PGIs was evaluated by incubating the standard solution in an auto-sampler for 48 h at 25 °C. The incubated solution was analysed in every 6 h in the developed method. The area results of the individual analyte were used for calculating the % stability by comparing with its corresponding regression equation. The % assay was observed to be more than 99 % for analytes up to 24 h and a very high % assay was observed up to 48 h. This proved that the solutions prepared were stable up to 48 h.

The method was studied for its applicability for detection and quantification of PGIs in formulations. In the proposed method, the prepared 100 µg/mL capsule formulation solution and the capsule formulation solution spiked with 0.05 % of PGIs was analysed. The resultant chromatogram of formulation solution spiked with impurities clearly shows the peaks correspond to impurities along with larotrectinib. Whereas the chromatogram observed for the un-spiked formulation sample doesn't show any detections at t_R PGIs in the study. This proved that the method was effectively identified and quantifies the impurities in formulation. Hence this method can successfully be utilized for the quantification of PGIs in larotrectinib bulk drug as well as formulation dosages. The chromatogram observed for the formulation solution spiked with studied PGIs was shown in figure 6 and the summary results of the method validation were presented in table 3.

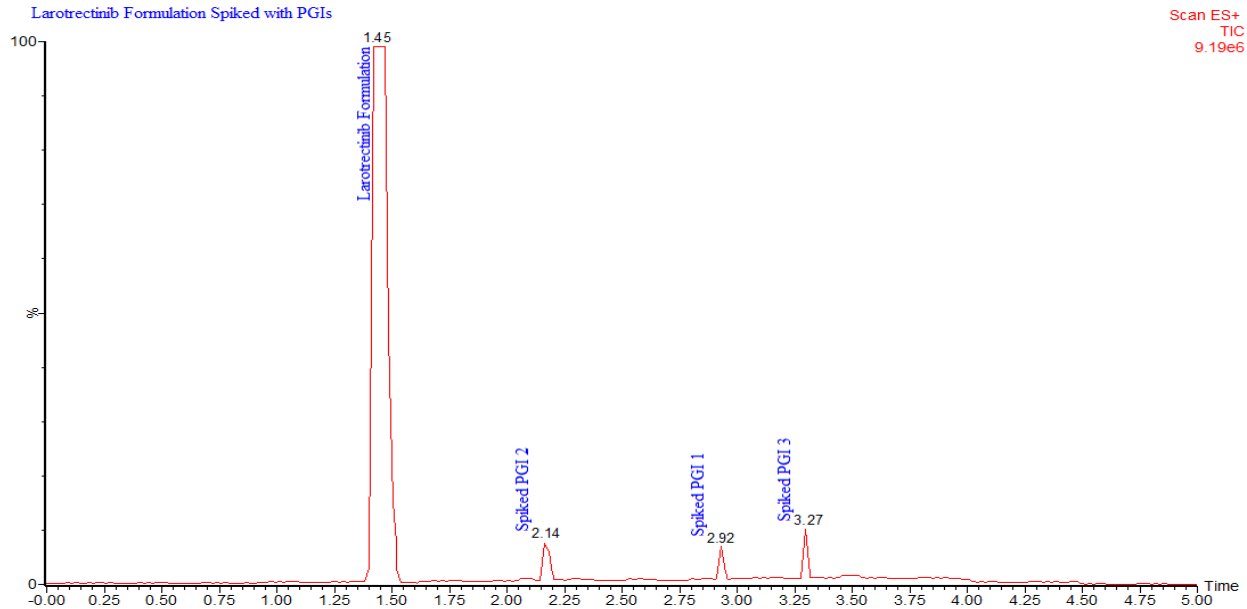


Figure 6: chromatogram observed in the developed method for the formulation solution spiked with 0.05 % PGIs in the study

S No	Parameter	Results observed			
		Larotrectinib	PGI 1	PGI 2	PGI 3

1	Linearity range (µg/mL)	0.01-5	0.01-5	0.01-5	0.01-5
2	% RSD Intraday Precision (n=6)	0.23	0.44	0.51	0.59
3	% RSD Interday Precision (n=6)	0.45	0.66	0.78	0.58
4	% RSD Ruggedness (n=6)	0.48	0.77	0.52	0.58
5	% Accuracy in 50% spiked level (n=3)	98.350	98.940	98.667	98.960
6	% Accuracy in 100% spiked level (n=3)	99.600	98.540	99.863	99.107
7	% Accuracy in 200% spiked level (n=3)	99.157	99.647	99.833	100.460
8	% Change Robustness MP change 1 MP change 2 pH change 1 pH change 2 FR change 1 FR change 2	0.56 0.01 0.86 0.93 0.29 0.55	0.17 0.05 0.88 0.74 0.26 0.93	0.34 0.62 0.93 0.45 0.34 0.72	0.96 0.20 0.22 0.36 0.62 0.17
9	% stability at 48 h (n=6)	98.15	98.71	98.01	98.52
10	LOD in µg/mL	0.001	0.003	0.001	0.003
11	LOQ in µg/mL	0.003	0.010	0.003	0.010
12	% assay in formulation	98.64	Not detected	Not detected	Not detected

Table 3: Summary results obtained in the study

CONCLUSION:

In this investigation, a simple and sensitive analytical LCMS approach was developed and successfully validated for identification and quantification three PGIs such as impurity 1, 2 and 3 in larotrectinib bulk drug and formulations. The ESI source was protected and favourable analytical conditions was proved by diverting the entry of the mobile phase in to the mass detector using a switch valve. The method shows calibration curve linear in the concentration range of 0.01 µg/mL to 5.0 µg/mL and proved to be precise, accurate and specific in the assessed concentration range. The detection levels was observed to be 0.001 µg/mL for larotrectinib, PGI 2 and 0.003 µg/mL for PGI 1 and 3 proved that the method was sensitivity. The method can identify and quantify the PGIs in pure drug and dosage forms. The method can also be applicable to in-process monitoring of studied impurities during the process of synthesis of larotrectinib. Based on the achieved results it can be concluded that the study will ensure the safe use of larotrectinib during production of formulations.

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