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# Method Development And Validation For Estimation Of Berotralstat By Using Lc-Ms/Ms Method

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*ABSTRACT*

A mixture of acetonitrile, Milli-Q water, and formic acid was prepared in a ratio of 70:30:0.1%, respectively. The mixture was then subjected to sonication in order to remove any dissolved gases. The approach was successfully tested within a linear concentration range spanning from 30 to 1000 ng/mL, exhibiting a high correlation coefficient (R2) value of 0.999. The percentage relative standard deviation (%RSD) of the peak response obtained from five replicate injections of standard concentration was determined to be less than 2, suggesting that the suggested approach exhibits a high level of precision. The observed percentage recoveries of the active pharmaceutical ingredient (API) from the dosage forms of Berotralstat exhibited a range of 97.81% to 103.33% w/w. These results suggest that the suggested approach is deemed accurate. The limit of detection (LOD) and limit of quantification (LOQ) values for Berotralstat were determined to be 0.5 ng/mL and 1 ng/mL, respectively. These values demonstrate the method's sensitivity. Therefore, the analysis of pharmaceutical formulations demonstrates that the proposed method is highly suitable for their examination, as it exhibits minimal interferencefrom the typical additives commonly found in such formulations. The LC-MS approach presented in this study demonstrates simplicity, sensitivity, and reliability. It is suitable for the regular analysis of Berotralstat in both bulk samples and pharmaceutical formulations, depending on the unique requirements of the situation at hand.

**Key words:** LC-MS/MS, Berotralsta,

**Introduction:**

Berotralstat is a pharmacological agent that functions as a selective inhibitor of plasma kallikrein. It is employed in the prophylactic treatment of episodes associated with hereditary angioedema (HAE). Berotralstat is marketed under the name Orladeyo as oral capsules. IUPAC Name:1-[3-(aminomethylphenyl]-N-{5-[(R)-(3-cyanophenyl)(cyclopropylmethyl)amino] methyl]-2-fluorophenyl}-3-(trifluoromethyl)-1H-pyrazole-5-carboxamide dihydrochloride, Molecular formula: $C_{30}H_{28}Cl_2F_4N_6O$, Molecular weight: 635.49, and Water Solubility: 0.00238 mg/mL. The mechanism of action involves the inhibition of plasma kallikrein's enzymatic activity, which prevents the release of bradykinin. Bradykinin is a significant physiologic peptide that contributes to the swelling and discomfort experienced during HAE attacks.

A comprehensive review of the literature shows that there are only a limited number of published analytical methods for the separation and estimation of the selected drug. Therefore, an effort was made to develop an LC-MS method that is accurate, precise, and validated.

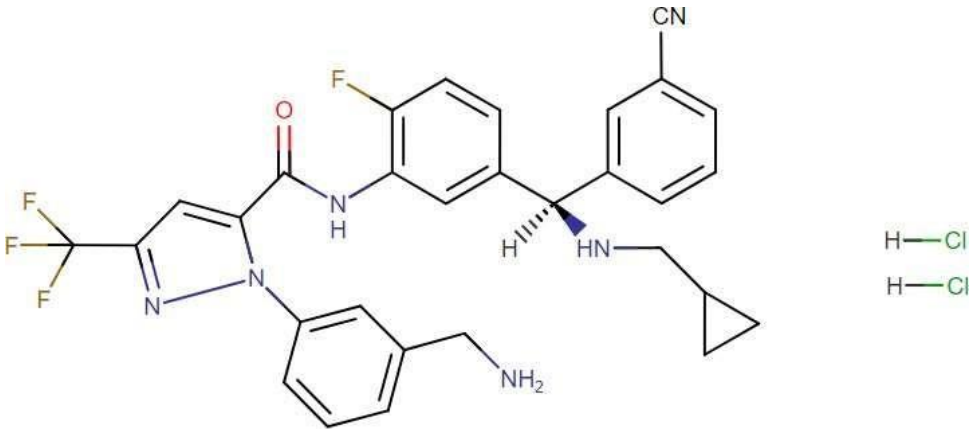


**Figure 1: Chemical structure of Berotralstat**

**II.MATERIALS AND METHODS:**

2.1 INSRUMENTATION: The study involved the utilization of liquid chromatographic-mass spectrometric analysis using an Alliance 2695 separation module (HPLC) and a MICROMASS Quattro micro mass spectrometer from WATERS (Waters Corporation, Milford, MA, USA) coupled with the electro spray ionization (ESI) interface. The process of data collection, integration, and calibration was conducted utilizing LC solutions chromatography data systems.

**2.2 Drug:** The reference sample of Berotralstat was gifted by Hetero Ltd. Hyderabad

2.3 Reagents and Chemicals:

| **Reagent** | **Brand** | **Purity/Grade** |
|---|---|---|
| Ammoniumacetate | Thomasbaker | AR |
| Acetonitrile | SigmaAldrich | HPLC |
| Methanol | Merck | HPLC |

| | | |
|---|---|---|
| Formicacid | Thomasbaker | AR |

2.4 Mobile phase: LCMS Acetonitrile, Milli-Q water, and formicacid were mixed in the ratio of 70:30:0.1% and sonicatedto degas.

2.5 Diluent: LC-MS Acetonitrile and Milli-Q water were mixed in the ratio of 60:40 and sonicated to degas.

2.6 Calibration Curves Dilutions

**Standard Solution Preparation:** Accurately weigh and transfer 25 mg of Berotralstat working standard into a 25 mL volumetric flask and about add 25 mL of diluent 80:20 0f Acetonitrile and water and sonicate to dissolve completely and make volume up to the mark with the same diluent **(Stock Solution)**.Further pipette1mL into10 mL volumetric flask and make up the volume to 10 mL with diluent.it is further diluted with diluent to obtain 30 ng/mL,50 ng/mL,70 ng/mL,100 ng/mL,500 ng/mL,700ng/mL and 1000 ng/mL respectivel y.

**Sample solution preparation**: Weighed and transferred equivalent to 50 mg of Berotralast into a 100 mL volumetric flask and added about 70 mL of diluent (Acetonitrile and water 80:20) sonicated for 30 minutes with intermediate shaking, and made up to volume with diluent (Acetonitrile and water 80:20), Centrifuged for 10 minutes at 500 rpm and further diluted 5 mL to 100 mL with diluent (acetonitrile and water 80:20).

III. Method Development and Optimization of Chromatographic Conditions:

In order to establish a method for determining Berotralstat, a comprehensive investigation was conducted to examine the impact of different elements. This involved systematically altering one parameter at a time while maintaining all other conditions constant. Several studies were undertakento fulfill the objective of this research. For this work, a Symmetry C-18 column of 50 x 2.1mm with a particle size of 3.5 µm was selected as the stationary phase.

Table 1: Optimized Chromatographic conditions

| | |
|---|---|
| Flowrate | 0.200 mL/min |
| Column | SYMMETRYC-18, 50 x 2.1mm, 3.5µm |
| Sample cooler temperature | $10^0$C |
| Injection volume | 10µL |
| Detector | Massdetector |
| Retentiontime | Atabout 1.04 min |
| Run time | 5.0mins |

**Table 2: Optimized Quattro Micro Tune Parameters**

| **Source(ES+)** | **Setting** | **Analyzer** | **Setting** |
|---|---|---|---|
| Capillary(kv) | 4.00 | LM1Resolution | 15 |

| | | | |
|---|---|---|---|
| Cone(V) | 30.00 | HM1Resolution | 15 |
| Extractor(V) | 1.00 | IonEnergy1 | 0.5 |
| RFLens(V) | 0.6 | Entrance | 32 |
| SourceTemperature(°C) | 100 | Collision | 5 |
| DesolvationTemperature(°C) | 200 | Exit | 42 |
| ConeGasFlow (L/Hr) | 75 | LM2Resolution | 13.2 |
| DesolvationGasFlow (L/Hr) | 250 | HM2Resolution | 13.1 |
| Collisiongaspressure | $<1e^{-4}$ | IonEnergy2 | 3 |
| | | Multiplier(V) | 500 |
| MSfunction | MRM289.41/244.35 | | |
| Dwell | 0.1 | | |
| Conevoltage | Tune | | |
| Collision energy | 8 | | |

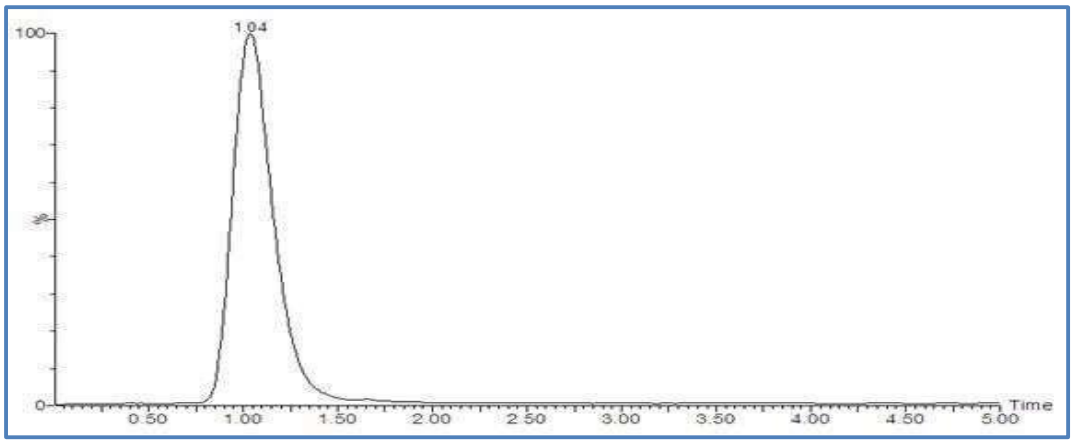

Figure 2: Chromatogram of Berotralstat

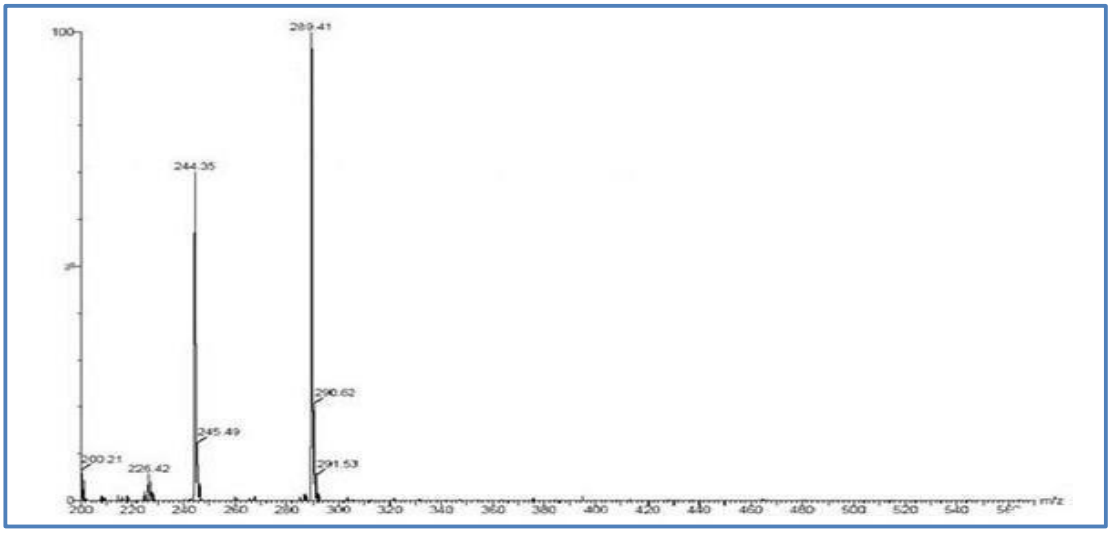


**Figure 3: Mass spectrum of Berotralstat**

**METHOD VALIDATION:**

Linearity: In order to validate the linearity of the proposed approach, suitable aliquots were extracted from theworking stock solution (100 µg/mL) and transferred into a set of 10mL volumetric flasks. The volume was adjusted to the desired level by adding the mobile phase, resulting in a series of solutions with concentrations ranging from 40 to 100 ng/mL. Three duplicates were injected for each concentration, and the peak areas of the above-mentioned solutions were recorded. A calibration curve was constructed to correlate the concentration of the analyte with its corresponding peak region and built regression equations and determined correlation coefficients. The findings indicate a strong association between the peak area and drug concentration within the specified concentration range. The calibration curve was depicted in Figure 4. The outcome was documented in Table 1.

The response exhibited linearity across the whole concentration range for Berotralstat, with a correlation coefficient of 0.999. The standard equation can be expressed as y = 179.39x - 5087.

Table 3: Calibration data of BEROTRALSTAT

| **S.No** | **Conc.[ng/mL]** | **Peak area** |
|---|---|---|
| 1 | 40 | 3481.17 |
| 2 | 60 | 5342.56 |
| 3 | 80 | 8738.39 |
| 4 | 100 | 12118.1 |
| 5 | 400 | 66866.7 |
| 6 | 800 | 94818.8 |
| 7 | 1000 | 135829 |
| Correlation Coefficient | | 0.999 |
| Slope | | 136.4 |

| | |
|---|---|
| Intercept | 1108 |

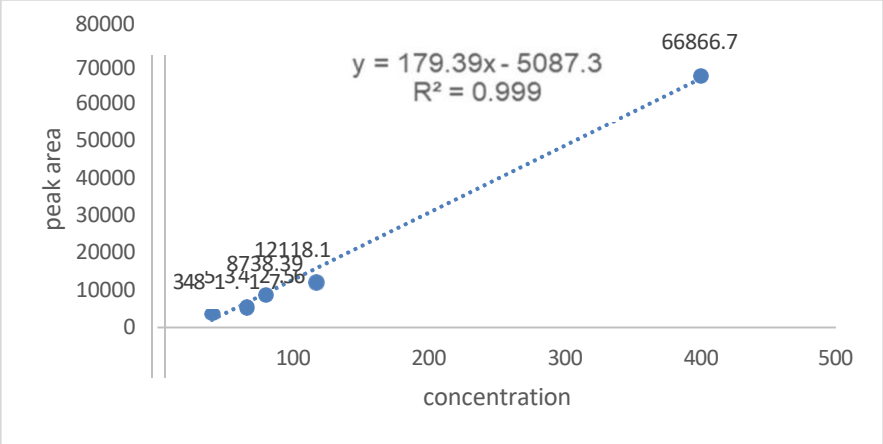


**Figure 4: Calibration Curve of Berotralstat**

Accuracy: To assess the degree of similarity between the outcomes obtained through the proposed methodology and those of inherent significance. The validity of the proposed approach was confirmed by conducting recovery studies with the conventional addition technique. A specified quantity of a pristine pharmacological compound was added to a preexisting sample thathad been previously analyzed, and the resulting blend was subsequently reanalyzed following the recommended methodology. The subsequent determination and reporting of the recovery percentageof the drug substance were conducted.

Preparation of standard solution: accurately measure and transfer a precise quantity of 25 mg of Berotralstat working standard into a volumetric flask possessing a capacity of 25 mL.Utilize sonication as a means to achieve thorough dissolution of the drug, followed by subsequent adjustment of the volume to the designated mark on the flask employing the identical diluent. The solution that is obtained as a result will be designated as the stock solution.

Subsequently, transfer 1 mL of the solution into a 10 mL volumetric flask and adjust the volume to 10 mL using a diluent. This resulting solution is then further diluted with the diluent to achieve concentrations of 50 ng/mL, 100 ng/mL, and 600 ng/mL, respectively.

Procedure: Measure the peak areas of the standard concentration (50 ng/mL,100 ng/mL and 600 ng/mL) at 273 nm. Calculate the %RSD and the % recovery of the concentrations.

The results are summarized:

**Table 4: Accuracy data For -Conc.-50ng/mL**

| | Peakarea | Concentration found from Calibration Curve | % Recovery as Accuracy |
|---|---|---|---|

| | | | |
|---|---|---|---|
| Injection1 | 5525.717 | 52.10 | 102.20 |
| Injection2 | 5597.582 | 52.63 | 99.26 |
| Injection3 | 5482.183 | 51.78 | 103.56 |
| Injection4 | 5700.169 | 53.40 | 100.8 |
| Injection5 | 5312.011 | 50.51 | 101.02 |
| Injection6 | 5834.143 | 54.39 | 100.78 |
| Average | 5575.30 | 52.46 | 101.3 |
| Std Dev | 180.85 | 1.34 | 1.46 |
| %RSD | 3.24 | 2.56 | 102.20 |

Table 5: Accuracy data For-Conc.-100ng/mL

| | **Peak area** | **Concentration from Calibration Curve** | **% Recovery as Accuracy** |
|---|---|---|---|
| **Injection1** | 11249.51 | 98.28 | **98.28** |
| **Injection2** | 11464.11 | 98.60 | **98.60** |
| **Injection3** | 11821.31 | 99.19 | **99.19** |
| **Injection4** | 11143.79 | 97.81 | **97.81** |
| **Injection5** | 11745.54 | 98.85 | **98.85** |
| **Injection6** | 11938.69 | 99.72 | **99.72** |
| **Average** | 11560.49 | 98.74 | **98.74** |
| **Std Dev** | 324.02 | 0.67 | **0.67** |
| **%RSD** | **2.8** | **0.678** | **0.678** |

**Table 6: Accuracy data For-Conc.-150ng/mL**

| | **Peak area** | **Concentration found from Calibration Curve** | **% Recovery as Accuracy** |
|---|---|---|---|
| Injection1 | 76337.00 | 597.88 | 99.64 |
| Injection2 | 76279.00 | 587.44 | 97.90 |
| Injection3 | 82009.00 | 602.03 | 100.33 |
| Injection4 | 80558.00 | 601.22 | 100.2 |
| Injection5 | 82015.00 | 610.94 | 101.82 |
| Injection6 | 85908.00 | 619.99 | 103.33 |
| **Average** | 80517.67 | 603.25 | 100.53 |
| **Std Dev** | 3713.96 | 11.16 | 1.86 |
| **%RSD** | **4.61** | **1.849** | **1.850** |

**Precision**: To verify the reliability of a modified RP-HPLC technique in order to get precise measurements of ZPT in Pharmaceutical formulations. The correctness of the methodology was evaluated by the implementation of experiments focused on repeatability and intermediate precision.

**Repeatability**: The method's precision was evaluated by conducting six replicate injections of solutions with concentrations of 50 ng/mL, 100 ng/mL, and 600 ng/mL. These injections were analyzed at a wavelength of 273nm on the same day. The percent relative standard deviation (%RSD) was then obtained.

Table 7: Repeatability data of BEROTRALSTAT

| | **Peak area** | | |
|---|---|---|---|
| | **50ng/mL** | **100ng/mL** | **150ng/mL** |
| Injection1 | 5700.169 | 11249.51 | 76337.00 |
| Injection2 | 5312.011 | 11464.11 | 76279.00 |
| Injection3 | 5834.143 | 11821.31 | 82009.00 |
| Injection4 | 5482.183 | 11143.79 | 80558.00 |

| | | | | |
|---|---|---|---|---|
| **Intermediate** | Injection5 | 5575.30 | 11745.54 | 82015.00 |
| | Injection6 | 5525.71 | 11938.69 | 85908.00 |
| | **Average** | 5571.586 | 11560.49 | 80517.67 |
| | **Std Dev** | 180.53 | 324.02 | 3713.96 |

**precision/ruggedness:** Precision was evaluated by conducting six replicate injections of solutions with concentrations of 50 ng/mL, 100 ng/mL, and 600 ng/mL. These injections were analyzed simultaneously on three separate days, using the selected analytical wavelength of 273nm.The analysis focused on examining the variability of the data across multiple days and subsequently calculating the relative standard deviation (%RSD). The findings have been succinctly summarized. In order to assess the intermediate precision, often referred to as ruggedness, of the approach,precision experiments were conducted on multiple days using identical parameters.

Table 8: Intermediate precision data of Berotralstat

**System suitability:**

System suitability tests were conducted during the daily validation process to assess the components of the analytical system and demonstrate that the system's performance aligns with the method's needed requirements.

| | **Peakarea** | | | |
|---|---|---|---|---|
| | **Day-1** | | **Day-2** | |
| | **50ng/mL** | **100ng/mL** | **50ng/mL** | **100ng/mL** |
| Injection1 | 5525.717 | 11745.54 | 5621.13 | 11812.12 |
| Injection2 | 5597.582 | 11249.51 | 5321.68 | 11679.34 |
| Injection3 | 5482.183 | 11464.11 | 5412.32 | 11691.25 |
| Injection4 | 5700.169 | 11143.79 | 5512.31 | 11912.31 |
| Injection5 | 5312.011 | 11821.31 | 5339.16 | 11877.05 |
| Injection6 | 5834.143 | 11938.69 | 5625.33 | 11759.47 |
| **Average** | 5575.30 | 11560.49 | 5471.99 | 11788.59 |
| **Std Dev** | 180.85 | 324.02 | 135.04 | 95.96 |
| **%RSD** | 3.24 | 2.8 | 2.47 | 0.81 |

Procedure: The system suitability determination employed a standard solution with a concentration of 225 ng/mL. This solution was generated by diluting the appropriate standard stock solution. Measure the peak areas of the respective concentration levels at273nm and calculate the%RSD.

Table 9: System suitability data of BEROTRALSTAT

| | **Peakarea** |
|---|---|
| Injection1 | 28190 |
| Injection2 | 29847 |
| Injection3 | 27377 |
| **Average** | 28471.33 |
| **SD** | 1258.8 |
| **%RSD** | **4.42** |

The determination of system suitability was conducted by performing six replicate injections of a standard solution prior to the analysis of the samples. The percentage relative standard deviation (RSD) was determined to be 4.42.

**Solution stability:** The determination of solution stability was conducted using a standard solution with a concentrationof 500 ng/m. This standard solution was created by diluting the matching standard stock solution. The sample peak area was analyzed at the initial point ,24hour and 48hours respectively. % SD was calculated.

Table 10: Solution Stability data of Berotralstat

| | **Peakarea** | | |
|---|---|---|---|
| | **Initial** | **24hr** | **48hr** |
| Injection1 | 71514.17 | 71412.31 | 71693.28 |
| Injection2 | 72217.31 | 71892.35 | 71982.11 |
| Injection3 | 71865.74 | 72215.31 | 72131.06 |
| **Average** | 71865.74 | 71839.99 | 71935.48 |
| **Stddev** | 351.57 | 404.0525 | 222.5834 |
| **%RSD** | 0.489204 | 0.562434 | 0.309421 |

**Robustness:** The assessment of robustness is a crucial aspect to be taken into account during the developmental stage

and is contingent upon the specific process being investigated. The measure of a technique's robustness is in its capacity to withstand intentional alterations in method parameters, such as adjustments in column temperature, analytical wavelength, and flow rate, without being significantly impacted.

Procedure: Minor modifications were permitted in the operational parameters, and an assessment was made aboutthe level of resilience exhibited by the approach. Individual experiments were conducted to investigate the effects of temperature deviations of ±5ºC and flow rate deviations of ±0.023 mL/min. Subsequently, the statistical calculation of the relative standard deviation (% RSD) was performed. The results were reported in Tables 4.17-4.18.

Table 11: Robustness at different flowrates

| **Flowrate (mL/min)** | **RT** | **Mean Peak Area** | **Mean Concentration** | **SD Concentration** | **RSD Concentration** |
|---|---|---|---|---|---|
| 0.19 | 1.02 | 11486 | 96.35 | 1.84 | 1.91 |
| 0.20 | 1.04 | 11337 | 95.25 | 2.64 | 2.77 |
| 0.21 | 0.99 | 10798 | 92.36 | 1.58 | 1.71 |

**Table 12: Robustness at different temperatures**

| **Temperature $^{o}C$** | **RT** | **Mean PeakArea** | **Mean Concentration** | **SD Concentration** | **RSD Concentration** |
|---|---|---|---|---|---|
| 55 | 1.03 | 11351 | 95.30 | 1.89 | 1.93 |
| 60 | 1.01 | 11337 | 95.25 | 2.64 | 2.77 |
| 65 | 1.00 | 10329 | 95.23 | 2.01 | 2.36 |

Limit of detection(LOD): In the field of chromatography, the detection limit refers to the quantity of the injected substance that produces a peak with a height that is at least two or three times greater than the baselinenoise level, hence achieving a signal-to-noise ratio of around three (S/N ratio 3).

Calculation of S/N Ratio:

Average Baseline Noise obtained from Blank : 22.45

Signal obtained from LOD solution : 69.86S/N =69.86/22.45 = 3.11

LOD Chromatogram Shows AREA at Berotralstat Peak – 69.86 -1 ng/mL

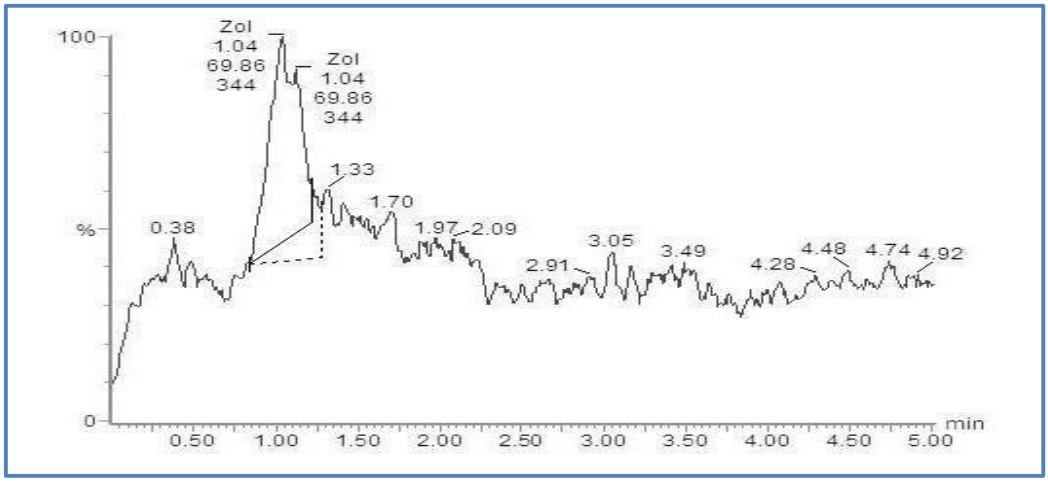


**Figure 5: Chromatogram showing LOD of Berotralstat**

Limit of Quantification (LOQ): The limit of detection refers to the minimum concentration of the analyte that can be reliably and precisely measured using the given analytical procedure. The limit of detection (LOD) was determined by measuring the concentration of the analyte that produced an instrument response equal to 10 times the level of noise, resulting in a signal-to-noise ratio (S/N ratio) of around 13.

Calculation of S/N Ratio: Average Baseline Noise obtained from Blank:22.45 Signal Obtained from LOD solution: 292.03

S/N=292.03/22.45 = 13.00

Table 14: LOD &LOQ data of Berotralstat

| **Parameters** | **Results** |
|---|---|
| Limit of detection (LOD) | 0.5ng/mL |
| Limit of quantitation (LOQ) | 1ng/mL |

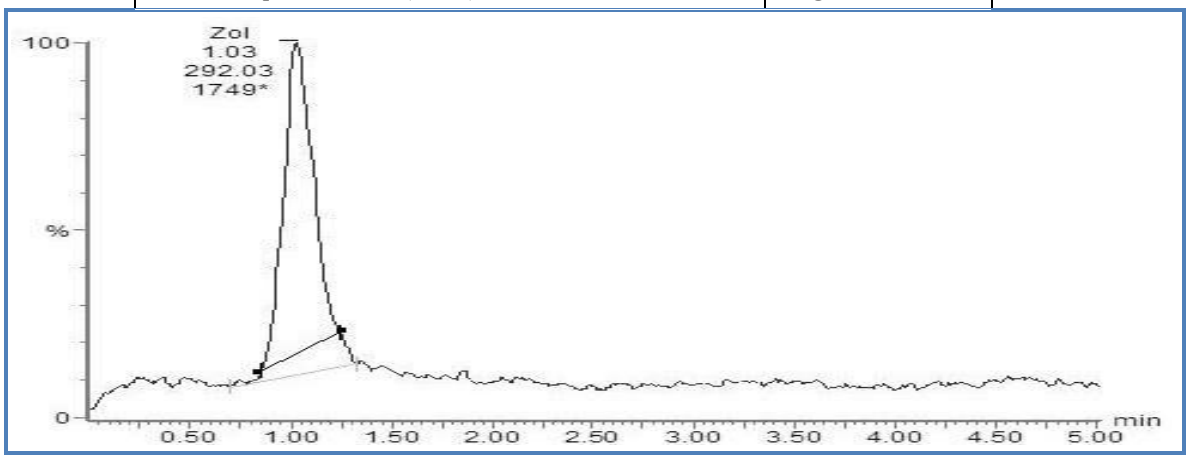


**Figure 6: Chromatogram showing LOQ of Berotralstat**

RESULTS AND DISCUSSION: The author has developed and validated an LC-MS/MS method for the estimation of Berotralstat. The LC-MS/MS method that was suggested underwent validation in accordance with the International Conference on Harmonization (ICH) Q2B Guidelines. The validation process confirmed its suitability for regular quantitative measurement of Berotralstat in pharmaceutical dosage forms utilizing the SPD-M20A photo diode array (PDA) detector. The MS detector was outfitted with an electrospray ionization (ESI) interface and operated in the positive polarity. The approach presented by the author utilizes a Symmetry C18 column and a specific mobile phase composition for LC-MS analysis. A mixture of acetonitrile, Milli-Q water, and formic acid was prepared in a ratio of 70:30:0.1% respectively. The mixture was then subjected to sonication in order to remove any dissolved gases.The approach was successfully tested within a linear concentration range spanning from 30 to 1000 ng/mL, exhibiting a high correlation coefficient (R2) value of 0.999. The percentage relative standard deviation (%RSD) of the peak response obtained from five replicate injections of standard concentration was determined to be less than 2, suggesting that the suggested approach exhibits a highlevel of precision. The observed percentage recoveries of the active pharmaceutical ingredient (API) from the dosage forms of Berotralstat exhibited a range of 97.81% to 103.33% w/w. These results suggest that the suggested approach is deemed accurate. The limit of detection (LOD) and limit of quantification (LOQ) values for Berotralstat were determined to be 0.5 ng/mL and 1 ng/mL, respectively. These values demonstrate the method's sensitivity. Therefore, the analysis of pharmaceutical formulations demonstrates that the proposed method is highly suitable for their examination, as it exhibits minimal interference from the typical additives commonly found in such formulations. The LC-MS approach presented in this study demonstrates simplicity, sensitivity, and reliability. It is suitable for the regular analysis of Berotralstat in both bulk samples and pharmaceutical formulations, depending on the unique requirements of the situation at hand.

CONCLUSION: The LC-MS/MS method described in this study is a precise and sensitive test used for the quantification of Berotralstat, with good accuracy and specificity. The duration of the chromatographic run for this approach is under two minutes. It is possible to reach a daily throughput exceeding 200 samples by incorporating many phases, including sample preparation, data collection, and processing. The method demonstrates a limit of quantification (LOQ) of 0.5 ng/mL and hasexhibited superior sensitivity and analysis speed in comparison to the methodologies documented in the literature to date.

## CONFLICT OF INTERESTS

Authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication.

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