

# The Determination Of N-Nitroso Etodolac (Ndsri) In Etodolac Using A Sensitive Sciex 5500 Lc-Ms/Ms Instrument

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## ABSTRACT

Rapid LC-MS/MS Method in the Quantitation of N-Nitroso etodolac, A sensitive, reliable LC-MS/MS assay was created in order to identify the cancer causative N-nitroso etodolac contamination in etodolac drug products. Separations were done using a Phenomenex Kinetex(r) C18 150 x 3 mm, 2.6 um UPLC column by using a gradient programmed with Mobile Phase A:0.1 percent formic acid in water and Mobile Phase B: 0.1 percent formic acid in acetonitrile. Detection was done by the use of triple quadrupole mass spectrometer (in positive electrospray ionization mode) which monitored multiple reaction monitoring (MRM) transitions characteristic of N-nitroso etodolac. This procedure has been shown to be a rapid and inexpensive risk assessment procedure in risk assessment studies of N-nitroso etodolac in etodolac manufacturers in pharma-industry to allow routine analysis and regulatory compliance. It has been demonstrated to comply with ICH Q2(R2) and USP <1225> guidelines, and therefore with: - Linearity:  $r > 0.99$  over the calibrated range - Accuracy (recovery):93.85 percent- 108.55 percent

**Keywords:** Etodolac, Sciex-5500 LC-MS/MS, N-nitroso etodolac, FDA, and USP

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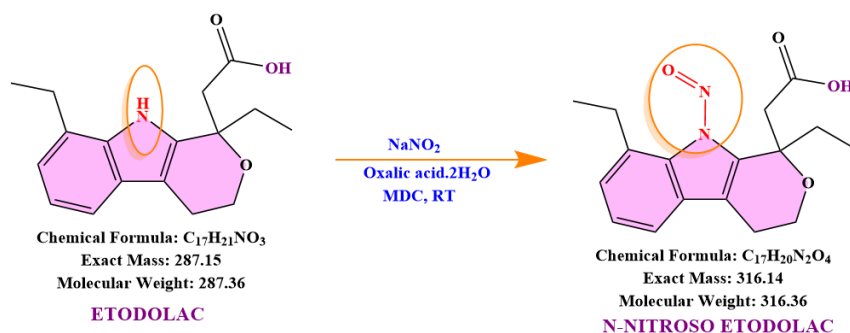
**Conflict of interest:** None

## INTRODUCTION

N-Nitroso Etodolac, a potential genotoxic impurity formed through nitrosation of the nonsteroidal anti-inflammatory drug (NSAID) Etodolac, has recently gained regulatory attention due to the broader risk of nitrosamine contamination in pharmaceutical products. This study aims to quantify N-Nitroso Etodolac in Etodolac drug substance and finished dosage forms using a sensitive and selective analytical method. A liquid chromatography–mass spectrometry (LC-MS/MS) procedure was developed and validated in accordance with ICH guidelines to ensure accuracy, precision, specificity, and robustness at trace-level detection. Sample preparation conditions were optimized to prevent in-situ nitrosation. The method demonstrated a limit of detection in the low ng/g range, enabling reliable assessment of nitrosamine levels relative to the acceptable intake limits recommended by regulatory authorities. Results from multiple batches showed that N-Nitroso Etodolac. The validated method provides a reliable tool for routine quality control and supports risk-based evaluation of nitrosamine impurities in Etodolac products.

In 2018, alerts surfaced that some sartan medicines carried trace amounts of the cancer-linked nitrosamines NDMA (N-nitroso-dimethylamine) and NDEA (N-nitroso-diethylamine). Since then, those contaminants have also been found in other drugs—most notably metformin and ranitidine —prompting the European Medicines Agency (EMA) to launch a deep-dive into the manufacturing process. Key findings point to certain catalysts, reagents, and amine-nitrogen combinations in the production line as the usual culprits. While a recall of some medications in Croatia didn't cut off access to metformin or sartans, ranitidine was voluntarily withdrawn across the EU (including Croatia). The temporary shortage was quickly mitigated by switching to alternative drugs. The EMA is scrutinizing how these nitrosamines get into drugs, and regulators are working hard to keep the market safe while ensuring patients have continuous access to essential medicines. Etodolac is a prescription NSAID (non-steroidal anti-inflammatory drug). It's used to ease mild-to-moderate pain and reduce inflammation, swelling, stiffness, and joint discomfort in conditions such as osteoarthritis and rheumatoid arthritis.

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**Figure 1:** Formation of N-nitroso Etodolac from Etodolac.

A literature review indicates that not many analytical procedures could identify the significance of an LC-MS/MS method of NNEI in Etodolac drugs and the quantification of the NNEI in the Etodolac Besylate drug substances. Nitrosamines (NIs) and nitroso drug substance-related impurities (NDSI) are discussed by people a lot due to the fact that they make people get cancer and mutations. This implies that they should be analyzed, regulated and quantified more thoroughly.

## EXPERIMENTAL

### Materials

The standard material of N-nitroso Etodolac with a purity of ~90% was obtained from synthesis confirmed by NMR & Mass, Purity by HPLC. Methanol and Acetonitrile for LC-MS is a high pure solvent (J.T. Baker) with a certified purity of 99.9% was obtained from Avantor, Commercially available Etodolac was used for the analysis. Formic acid is sourced from Fisher Chemicals Private Limited (Germany). Water used for chromatography (LC-MS Grade) was produced by J.T Baker.

### Chromatographic and MS Settings

The chromatographic experiment was performed on a Sciex LC-MS/MS 5500+ system that had the Exion LC UHPLC module, supplied by Sciex, India. North American Thermo scientific micro-pipette. All the weighing was done using the Mettler toledo, Microbalance, Switzerland. In order to determine the amount of N-nitroso Etodolac, The injection volume is  $5\mu\text{L}$ , the transition from  $m/z$  317.3 to 231.400, the dwell volume is selected as 200 msec, was observed for the purpose of mass spectrometric detection. A nebulizer gas flow rate of GS1 is 50mL/min and GS2 is 50mL/min, CUR value is 35, CAD value is 9, IS value is 5500, Temperature is 500, DP value is 80, EP value is 10, CE value is 25 and Optimal ion fragmentation was achieved by setting the CE to a negative value of 10.0 V. The HPLC gradient programme (Time Mobile phase -A: Mobile phase-B) is 0min 50:50, 1.0min 2:98, 4.0min 2:98 and 6.0min 50:50, the flow are of 0.4mL/min, Column oven temperature is  $35^\circ\text{C}$ .

### NDSRI Structural Assessment and Impurity Limits Setting

The highest amount of Etodolac that can be taken by for oral administration each day is 1200 mg. The EMA Guidelines for 2023 set an AI limit of 18 ng/day for unknown carcinogenic potential impurity, which is the same as a nitrosamine limit of 0.015 ppm (15ppb) considered method development and validation.

### Preparation of Standard Solution

After carefully weighing 10 mg of N-nitroso etodolac, the powder was transferred into a 15-mL centrifuge tube. After adding 10 mL of diluent, the liquid was vortexed for 5 min to guarantee complete mixing. Next, at  $20^\circ\text{C}$ , the material was centrifuged for 5 min at 4500 rpm. To prevent contamination, the first three to five drops of the resultant supernatant were thrown away after it was filtered through a  $0.45\text{-}\mu\text{m}$  PVDF syringe filter and placed into an auto sampler vial (Stock-1). 0.1 mL of this diluted solution was transferred into a 10-mL volumetric flask (Stock-2), 0.1 mL of this diluted solution was transferred into a 10-mL volumetric flask (Stock-3), 1.0 mL of this diluted stock-3 was transferred into a 10-mL volumetric flask, brought to volume with the diluent, and mixed thoroughly. The solution was subsequently injected into the LC-MS/MS system for analysis.

### Procedure for Sensitivity Solution

Transferred 1 mL of standard solution in to 10 mL volumetric flask make up with diluent and mixed thoroughly. The sensitivity solution is one-tenth of the standard solution in concentration.

### Preparation of Sample Solution

To prepare a sample solution for the drug product (Etodolac tablets) 125 mg of finely crushed tablet powder (equivalent to 100 mg of the drug) was weighed and transferred into a 10 mL volumetric flask. After adding 5 mL of diluent, the solution was sonicated for 2 min to ensure complete extraction of the drug filter the supernatant with  $0.22\mu\text{m}$  filter.

## RESULTS & DISCUSSIONS

**LC-MSMS Method Development Strategy and Optimization**

Achieving separation, sensitivity, and repeatability for the detection of NNEI was the primary emphasis of the development of the chromatographic settings. The resolution, sensitivity, and runtime values for the quantification of NNEI were well balanced by the optimized mobile phase composition and gradient program. Mobile Phase A consists of 0.1% formic acid in water, which helps ensure adequate ionization of NNEI and enhances its detection in mass spectrometry. This was accomplished by promoting stable protonation and effectively minimizing signal suppression. Mobile Phase B consists of 0.1% Formic acid in Acetonitrile, selected for its strong elution capability to efficiently separate non-polar contaminants and minimize the risk of coelution.

**Mass Spectrometric Optimization**

In order to achieve high sensitivity and selectivity for the detection of NNEI, the experimental conditions for mass spectrometry were designed. Based on its high signal-to-noise ratio and its relevance to the target compound, the transition m/z 317.2 → 321.4 was selected as the MRM transition. For the purpose of ensuring efficient nebulization, ionization, and drying of the analyte, gas

flow optimization, nebulizer gas were optimized accordingly. Adjustments to the temperature: The temperature at the interface should be set to 500°C for optimal desolvation of the ionized analytes. DL (desolvation line) temperature: To avoid condensation of analytes. To ensure that the sample is effectively volatilized during the ionization process, In order to achieve excellent ion fragmentation and transition efficiency for the m/z 317.2 → 321.4 pathway, a collision energy of 10.0 V was determined to be the optimal option.

**Analytical Method Validation**

The validation was conducted as per the guidelines in the International Committee on Harmonization guidance (ICHQ2R2) and USP1225. .

**System Suitability and System Precision**

The percentage %RSD of the analyte peak area and ~ RT Retention time for the NNEI standard solution were evaluated. The results were compared against the standard acceptance criteria, a %RSD not exceeding 10.0. All results were found to meet these established criteria. These values affirm the acceptability of the system for the NNEI peak, as shown in Table 1, and Figures 2 and 3 represent the standard chromatograms & Sample solution respectively.

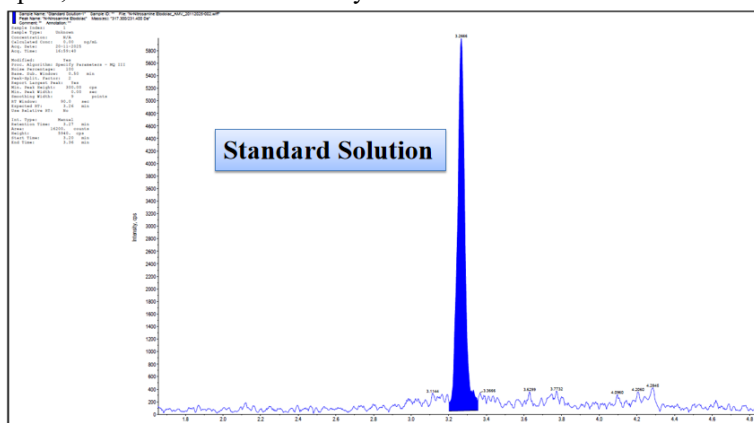
**Table 1** System suitability results.

| Injection No.:       | N-Nitroso Etodolac Peak Area | N-Nitroso Etodolac Peak Retention Time |
|----------------------|------------------------------|--|
| Standard Injection-1 | 16200                        | 3.27                                   |
| Standard Injection-2 | 16100                        | 3.26                                   |
| Standard Injection-3 | 16800                        | 3.26                                   |
| Standard Injection-4 | 16400                        | 3.26                                   |
| Standard Injection-5 | 17100                        | 3.25                                   |
| Standard Injection-6 | 17000                        | 3.26                                   |
| <b>Average</b>       | <b>16600</b>                 | <b>3.26</b>                            |
| <b>STDV</b>          | <b>424.26</b>                | <b>0.01</b>                            |
| <b>% RSD</b>         | <b>2.56</b>                  | <b>0.19</b>                            |

**Specificity**

The specificity of the method was established by analyzing blank and samples, which should not have any

interference at the same retention time of the analyte peak as demonstrated in Figure 4 represent the sample chromatogram.



**Figure 2** Chromatogram of standard.

**Precision**

Six Etodolac sample solutions (10mg/mL) spiked with NNEI at the target level (15 ppb) were analyzed using LC-MS/MS to evaluate method precision. Precision was assessed in terms of method reproducibility (method precision) and repeatability (intermediate precision) using spiked samples. For method precision, six spiked samples at 15ppb (100% specification level) were analyzed, and

%RSD of impurity results was calculated. Intermediate precision was evaluated using six additional spiked samples on a different day, employing a different Analyst and column lot. The %RSD values for individual sets of six samples and the combined 12 samples well within the specific acceptance criterion of NMT 10.0%, The results are presented in Table 2.

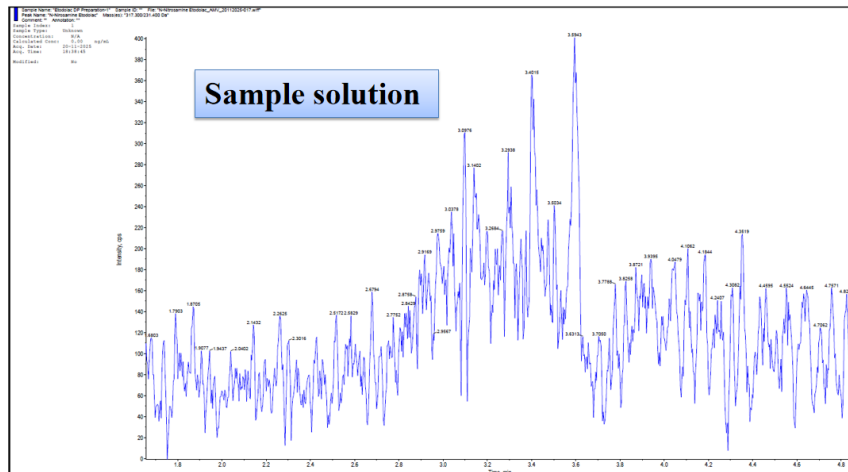


Figure 3 Chromatogram of Sample.

Table 2 Method validation results.

| Parameters                                  | NNEI              |
|---|-------------------|
| Linearity (LOQ-150%)                        | 1.5ppb to 22.5ppb |
| Range (ng mL <sup>-1</sup> )                | 1.5–22.5          |
| Slope                                       | 165.54            |
| % Y-Intercept                               | -0.25             |
| Correlation coefficient                     | 0.997             |
| LOD ng/mL <sup>-1</sup>                     | 0.5               |
| LOQ ng/mL <sup>-1</sup>                     | 1.5               |
| <b>Accuracy<sup>a</sup> (% of recovery)</b> |                   |
| 50% mean ± SD                               | 105.22            |
| 100% mean ± SD                              | 101.61            |
| 150% mean ± SD                              | 96.92             |
| LOQ accuracy                                | 96.44             |
| <b>Precision<sup>b</sup> (%RSD)</b>         |                   |
| Repeatability                               | 1.41              |
| Intermediate precision                      | 3.84              |
| LOQ precision                               | 2.72              |

A Average of three determinations of each concentration level.

B% RSD of six determinations, average of each component.

**LOD & LOQ**

The LOD & LOQ for NNEI were established to assess the method's sensitivity. LOD, defined as the lowest detectable level

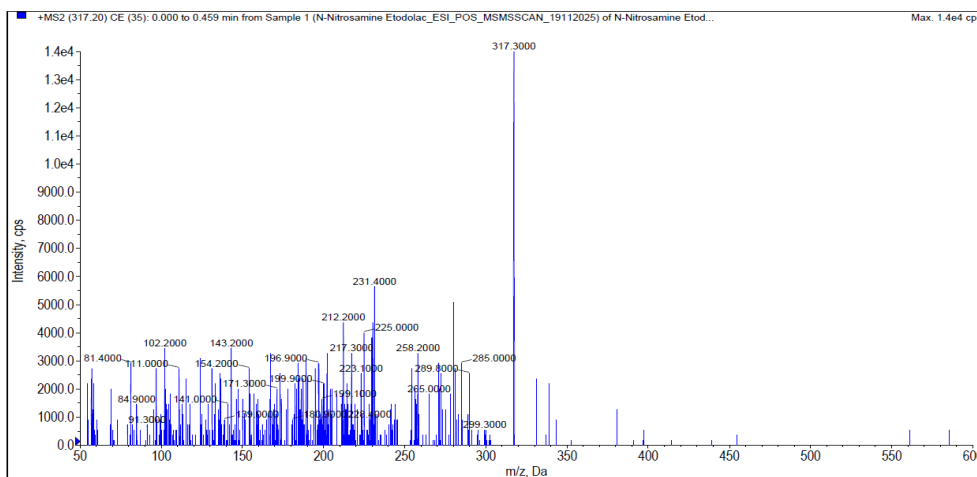


Figure 4 Chromatogram of Mass Fragmentation.

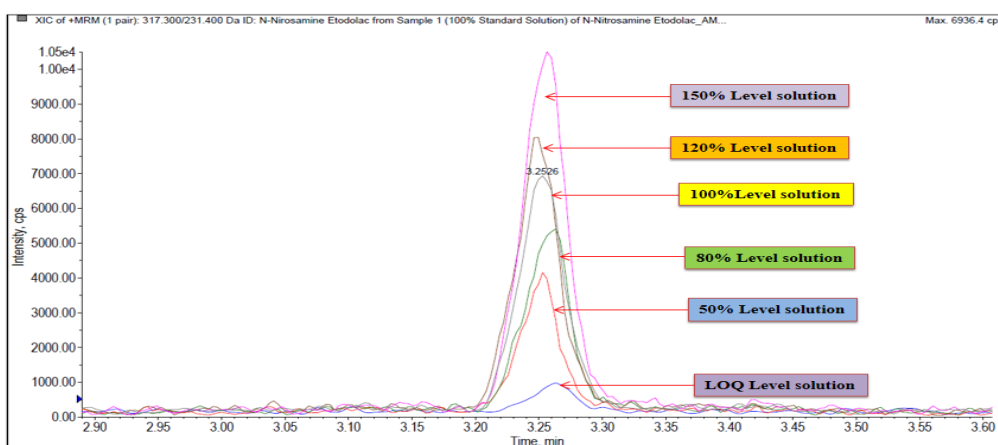


Figure 5 Overlaid Chromatogram of Linearity ranging from LOQ to 150% Standard solutions.

(S/N 3:1), was determined to be 1.5 ppb, confirming the method's capability to detect trace analyte levels. LOQ, the lowest quantifiable concentration with acceptable precision and accuracy (S/N 10:1), was established at 10 ppb, representing 10% of the 50-ppb specification limit.

Both values highlight the method's precision and sensitivity. The results are outlined in Table 2, and Figure 5 Overlay chromatogram of Linearity ranging from LOQ to 150% Standard solution.

Table 3: Spike and sample solution stability.

| Injection Name   | N-Nitroso Etodolac Peak Area | N-Nitroso Etodolac Peak Recovery% |
|--|------------------------------|-----------------------------------|
| Etodolac Sample(0hrs)                                  | Not detected                 | NA                                |
| Etodolac+100%N-Nitroso Etodolac Spiking solution(0hrs) | 16900                        | 101.81                            |
| Etodolac Sample(9hrs)                                  | Not detected                 | NA                                |
| Etodolac+100%N-Nitroso Etodolac Spiking solution(9hrs) | 17500                        | 105.42                            |

Table 4: Robustness (%RSD of Recovery %, %Accuracy).

| Injection No.:                            | % Recovery | Result        |
|---|------------|---------------|
| Flow Rate 0.4ml/min to 0.36ml/min         | 105.36     | Not Sensitive |
| Flow Rate 0.4ml/min to 0.44ml/min         | 105.67     | Not Sensitive |
| Column temperature Variation 35°C to 33°C | 104.87     | Not Sensitive |
| Column temperature Variation 35°C to 37°C | 103.18     | Not Sensitive |
| Organic composition Variation 10% to 5%   | 101.49     | Not Sensitive |
| Organic composition Variation 10% to 15%  | 103.75     | Not Sensitive |
| Mass Temperature Variation 500°C to 450°C | 106.37     | Not Sensitive |
| Mass Temperature Variation 500°C to 550°C | 98.6       | Not Sensitive |

|         |        |                  |
|---------|--------|------------------|
| Average | 103.66 | Method is Robust |
| STDV    | 2.57   |                  |
| % RSD   | 2.48   |                  |

**Note:** The system suitability criteria: %RSD, NMT 10.0%, %Accuracy in sample spike at 100% is should be in between 80.0% – 120.0%.

#### Accuracy

The accuracy ranging from the LOQ to 150% of the target concentration. The recovery values for NNEI ranged between 10.0% and 150.0% level spiking solutions were meeting standard acceptance criteria. The results are outlined in Table 2.

#### Linearity

The linearity of the analytical method for NNEI was assessed to confirm that the response is measured as the peak intensity of the ion signal, which is inline with the concentration of the analyte. A series of standard NNEI solutions was generated at various concentration levels, generally ranging from the LOQ to 150%. The results are assessed for their regression coefficient value against the standard accept- ability threshold of no less than 0.997.

#### Sample Solutions & Solution Stability

The stability for the NNEI impurity standard & spiked sample solutions were evaluated at 15°C over a 9-h period, with assessments conducted. All results met the acceptance criterion, showing a deviation of no more than 10.0% from the baseline. The data confirms that the solutions remain stable in the specified diluent system for up to 9 h. The results are outlined with Table 3.

#### Robustness

The robustness for analyte peak with the method was evaluated during development by introducing small, intentional modifications to key method parameters. These variables, including column temperature ( $\pm 2^\circ\text{C}$ ), flow rate ( $\pm 0.04 \text{ mL/min}$ ), Organic composition variation ( $\pm 5\%$ ) and Mass temperature variation ( $\pm 50^\circ\text{C}$ ) were tested by analyzing their effects on system suitability parameters and percentage recoveries, comparing them to the method precision results. The results are outlined in Table 4.

#### CONCLUSION

The developed LC-MS/MS technical method for the analysis of N-nitroso Etodolac (NNEI) demonstrates exceptional performance with high sensitivity, accuracy, precision, Rugged and robustness. The method shows excellent linearity ( $R^2 > 0.99$ ), low LOD (0.5 ppb), and LOQ (1.5 ppb), ensuring precise detection and quantification of trace NNEI. Accuracy is within the accepted range (80.0%–120.0%), and precision (method and intermediate) is consistent, with %RSD  $< 10\%$ . Stability studies confirm the solution remains stable for 9 h at 15°C, and robustness testing shows no impact from minor variations in parameters. The method is reliable, accurate & suitable for routine monitoring of NNEI in Etodolac Active pharmaceutical ingredients & formulations, meeting regulatory requirements.

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#### CONFLICT OF INTERESTS

The authors confirm the lack of conflict of interest.

#### AUTHORS CONTRIBUTION

Each of the authors played an important role in this manuscript, was involved in the review/editing, and accepted the final version to be published. The authors have the following ORCID IDs that can be used to verify their research profile:

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