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A Matrix-Tolerant In-Situ Derivatization Strategy for Trace-Level UPLC-MS Quantification of the Carcinogen Impurity 2-Ethylphenylhydrazine in Etodolac Drug Substance

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Abstract: 2-Ethylphenylhydrazine is a potentially genotoxic impurity that may arise during the synthesis of Etodolac and therefore requires sensitive and reliable analytical control. However, its inherent instability presents a significant challenge for trace-level quantification. In this study, a matrix-tolerant in-situ derivatization strategy coupled with UPLC-MS was developed and validated for the determination of 2-Ethylphenylhydrazine in Etodolac drug substance. The analyte was derivatized with 2-hydroxybenzaldehyde to form a stable hydrazone derivative, while a basic methanolic medium containing ammonia was employed to minimize the influence of the acidic Etodolac matrix and facilitate efficient derivatization. Reaction optimization demonstrated that a temperature of 50 °C and a reaction time of 60 min provided a derivatization efficiency of 99.9%. The developed method was validated in accordance with ICH Q2(R2). The method demonstrated good linearity over the concentration range of 0.2286–74.0 ng/mL, with a coefficient of determination (R^2) of 0.9988. The limit of detection and limit of quantification were 0.06858 and 0.2286 ng/mL, respectively. The method showed satisfactory accuracy and precision across the evaluated concentration levels and was demonstrated to be robust against deliberate variations in selected analytical parameters. The derivatized analyte remained stable under the evaluated storage conditions, supporting its applicability to routine analysis. The method was further applied to ten independent production batches of Etodolac drug substance, and 2-Ethylphenylhydrazine was not detected in any batch at the established reporting threshold. Overall, the proposed derivatization-based UPLC-MS method provides a sensitive,

selective, and practical approach for trace-level monitoring of 2-Ethylphenylhydrazine in Etodolac drug substance and is suitable for routine quality control applications.

Keywords: 2-Ethylphenylhydrazine, Genotoxic impurity, Etodolac, Derivatization, Single Quadrupole, UPLC

1. Introduction

Etodolac, a nonsteroidal anti-inflammatory drug (NSAID), is widely used for its analgesic and anti-inflammatory properties [1,2]. Its pharmacological activity is primarily associated with the inhibition of cyclooxygenase and the consequent reduction in prostaglandin biosynthesis, with Etodolac exhibiting a degree of selectivity toward cyclooxygenase-2 [3,4]. For the management of acute pain, Etodolac is administered at doses of 200–400 mg every 6–8 h, with a maximum recommended total daily dose of 1000 mg [5].

The synthesis of Etodolac involves several key intermediates, including 2-Ethylphenylhydrazine HCl [6]. In the Fischer indole synthesis, 2-Ethylphenylhydrazine HCl reacts with 2,3-dihydrofuran to form 7-ethyltryptophol, a key intermediate in the subsequent synthesis of Etodolac [6,7]. The importance of this synthetic relationship has also been documented in patent literature, where 2-Ethylphenylhydrazine HCl is used for the preparation of 7-ethyltryptophol and its subsequent conversion toward Etodolac [7].

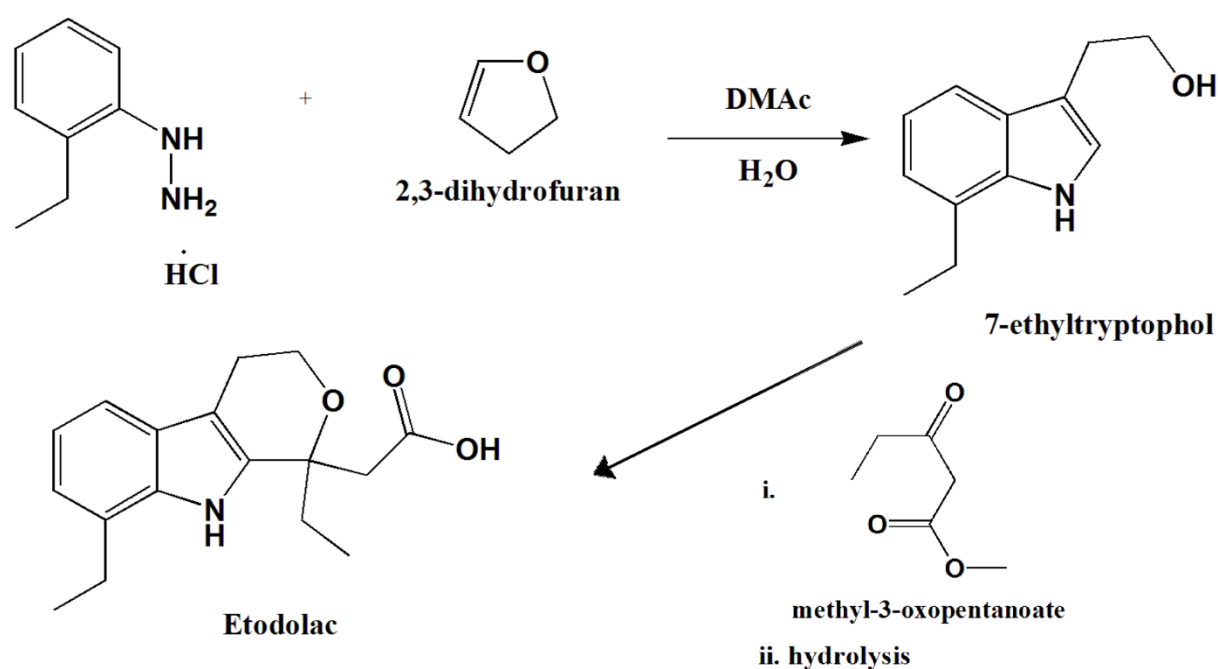


Figure 1. Etodolac synthesis from 2-Ethylphenylhydrazine HCl general reaction scheme and intermediates [7].

However, 2-Ethylphenylhydrazine HCl is classified as a Category 2 carcinogen under the European Union Classification, Labelling and Packaging (CLP) Regulation [8]. Although compound-specific information regarding the genotoxicity of 2-Ethylphenylhydrazine HCl is limited and no harmonized germ-cell mutagenicity classification has been assigned under the EU CLP Regulation [8], its toxicological relevance warrants careful control when considered as a process-related impurity. In addition, genotoxicity concerns have been extensively reported for hydrazine and several hydrazine derivatives, including arylhydrazines, with experimental studies demonstrating DNA damage and/or bacterial mutagenicity for a number of compounds within this chemical class [9–12]. Hydrazine and related derivatives are therefore recognized as an important class of potentially genotoxic impurities in pharmaceutical development [13]. In this context, the assessment and control of impurities with potential mutagenic and carcinogenic risk are addressed by the ICH M7 guideline [14]. Given the limited compound-specific toxicological information available for 2-Ethylphenylhydrazine HCl, a conservative approach was adopted in the present study, and its acceptable level was established according to the principles applied to potentially mutagenic impurities using the ICH M7 threshold of toxicological concern. Accordingly, based on the ICH M7 acceptable intake of 1.5 µg/day and a maximum daily dose of 1000 mg of Etodolac, as shown in the equation (1) a specification limit of 1.5 ppm was established for 2-Ethylphenylhydrazine HCl.

$$\text{Limit (ppm)} = \frac{1.5 \text{ µg/day}}{1000 \text{ mg /day}} = 0.0015 \text{ mg/g} = 1.5 \text{ ppm} \quad (1)$$

Therefore, accurate and sensitive determination of 2-Ethylphenylhydrazine HCl in Etodolac drug substance is important for the effective control of this process-related impurity. However, the analytical determination of 2-Ethylphenylhydrazine presents several challenges. Hydrazine and arylhydrazine compounds are generally reactive species, and their analysis at trace levels in pharmaceutical matrices can be complicated by their physicochemical properties, low detector response, and matrix-related interferences. Derivatization has consequently been widely employed for the analytical determination of hydrazine and related compounds, particularly when direct chromatographic or spectroscopic detection does not provide adequate sensitivity or selectivity [15–18].

A well-established approach for hydrazine analysis is the formation of hydrazone derivatives through reaction with aldehydes. Such derivatization introduces an aromatic or otherwise detectable moiety into the analyte structure and can substantially improve chromatographic behavior and detector response. Wang et al. demonstrated that derivatization of hydrazine with 2-hydroxy-1-naphthaldehyde enabled sensitive determination of trace hydrazine in pharmaceutical materials by reversed-phase HPLC-UV. In that study, derivatization not only enhanced the optical response of hydrazine but also shifted the detection wavelength away from the major absorption region of pharmaceutical matrix components, thereby reducing matrix interference [16]. Other studies have likewise demonstrated the applicability of aldehyde-based derivatization for the determination of hydrazine and hydrazine-related compounds in pharmaceutical matrices [17,18].

Although these studies demonstrate the analytical advantages of hydrazone formation, the behavior of an individual hydrazone derivative is strongly dependent on the chemical structure of both the analyte and derivatizing reagent, as well as on the reaction and chromatographic conditions. In addition, derivatization does not necessarily overcome analyte instability in a complex pharmaceutical matrix. Matrix composition, pH, reagent concentration, reaction time, and the chemical stability of the resulting derivative can all influence the final analytical response. These considerations are particularly important for trace-level impurity determination, where even partial degradation of the analyte or derivative may result in significant quantitative bias [15,18,19].

In the present study, 2-hydroxybenzaldehyde was selected as the derivatizing reagent for 2-Ethylphenylhydrazine HCl. The objective of derivatization was not merely to introduce a chromophoric group, but primarily to convert the highly reactive and analytically challenging 2-Ethylphenylhydrazine into a structurally defined hydrazone derivative with improved chemical stability and enhanced response under electrospray ionization conditions. Chemical derivatization has previously been demonstrated as an effective strategy for improving the LC-MS response of compounds exhibiting unfavorable ionization characteristics, particularly through incorporation of structural features that facilitate ionization and/or improve chromatographic behavior [19–21].

During preliminary experiments, direct LC-MS analysis of 2-Ethylphenylhydrazine was found to provide inadequate analytical performance because of its limited stability and insufficient ionization response. Formation of the corresponding 2-hydroxybenzaldehyde hydrazone substantially improved the MS response; however, when the derivatization reaction was performed in the presence of Etodolac drug substance, degradation of the derivative was observed. This behavior was attributed to the acidic nature of the Etodolac matrix and demonstrated that stabilization of the derivative had to be considered together with the derivatization reaction itself. Matrix-dependent effects are a recognized challenge in trace-level pharmaceutical impurity analysis and may influence both analyte stability and ionization efficiency [15,19,20].

To overcome this limitation, an in-situ derivatization strategy was developed in which the sample solution was maintained under basic conditions using ammonia. The basic medium was selected to neutralize the acidic contribution of the Etodolac matrix and to facilitate conversion of the hydrochloride salt of 2-Ethylphenylhydrazine to its free-base form prior to hydrazone formation. The use of volatile basic additives is also compatible with LC-MS-based analysis and has been applied in trace-level pharmaceutical impurity methods [20,21]. Under the optimized conditions, 2-Ethylphenylhydrazine was rapidly converted to the corresponding 2-Ethylphenylhydrazine-2-hydroxybenzaldehyde hydrazone, providing a more stable and readily ionizable analytical species.

Importantly, the developed strategy therefore addresses two fundamental limitations of the direct analysis of 2-Ethylphenylhydrazine simultaneously: chemical instability and inadequate mass-spectrometric response. Rather than attempting to quantify the unstable parent compound directly, the method quantitatively converts the impurity into a defined hydrazone derivative immediately before chromatographic analysis. The resulting derivative is subsequently separated by reversed-phase UPLC and detected by single quadrupole mass spectrometry using single-ion recording (SIR). This approach combines chemical stabilization, improved chromatographic behavior, and enhanced MS detectability within a single sample-preparation step.

To the best of our knowledge, no validated UPLC-MS method employing in-situ 2-hydroxybenzaldehyde derivatization for the trace-level determination of 2-Ethylphenylhydrazine in Etodolac drug substance has previously been reported. The present

work therefore focuses on the development and validation of a matrix-tolerant derivatization strategy capable of providing reliable quantification of this impurity at the established control limit. The derivatization reaction and the resulting hydrazone were structurally characterized by ^1H NMR, ^{13}C NMR, FTIR, DSC, and mass spectrometry, while the analytical procedure was subsequently validated with respect to specificity, accuracy, precision, linearity, range, robustness, and solution stability in accordance with ICH Q2(R2) [22].

2. Materials and Methods

2.1. Reagents and Chemicals

The reagents and chemicals used in the study have high purity (MS Grade). Etodolac drug substance (Cas Number: 41340-25-4) was supplied by Ulkar Kimya Sanayi ve Ticaret A.Ş. Ammonium hydroxide solution (Cas Number: 1336-21-6), Formic Acid (Cas Number: 64-18-6), 2-Hydroxy benzaldehyde (Cas Number: 90-02-8), Water (Cas Number: 7732-18-5) and 2-Ethylphenylhydrazine HCl (CAS Number: 19398-06-2) was obtained from Sigma Aldrich company, while Methanol (CAS Number: 67-56-1) and Acetonitrile (CAS Number: 75-05-8) were provided by J.T.Baker.

2.2. Instrumentation

The method development and validation studies were carried out using the Waters UPLC (ACQUITY H-Class System), which includes a quaternary solvent manager pump, sample manager-ftn autosampler, PDA detector, and single quadrupole Mass Detector (ACQUITY QDA) with an Diverter valve (ACQUITY). Chromatographic data acquisition was managed through Empower 3 software. The chromatographic separation was achieved with a Waters ACQUITY UPLC® BEH Phenyl column (1.7 μm , 2.1 $\times$ 100 mm). All weighing procedures were performed using a Mettler Toledo XP205 analytical balance. A Peak Genius XE 35 nitrogen generator was utilized to supply nitrogen. ^1H and ^{13}C NMR spectra were measured using a JEOL 400YH NMR spectrometer (400 MHz). Chemical shifts are provided in ppm, calibrated against the residual protons of the solvent (CDCl_3 : δ 7.26) and the carbon signal of the solvent (DMSO-d_6 : δ 77.00). The NMR peak multiplicities are indicated as: s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). The FT-IR spectrum was measured using the Shimadzu IRSpirit QATR-S, with ν_{max} reported in cm^{-1} . Perkin Elmer Jade DSC device was used to investigate thermal properties of hydrazone compound. The sample was heated from 20 $^{\circ}\text{C}$ to 250 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C}/\text{min}$.

2.3. UPLC and Mass Conditions

Chromatographic separation was achieved using a mobile phase system consisting of 0.1% formic acid in water (v/v) (Mobile Phase A) and acetonitrile (Mobile Phase B). The column temperature was maintained at 35 °C, while the autosampler was kept at 15 °C. A constant flow rate of 0.40 mL/min was employed with an injection volume of 1 µL, resulting in a total analysis time of 9 minutes. The gradient elution program was initiated with an isocratic hold at 80% A for 0.50 minutes, followed by a linear ramp to 30% A (70% B) at 4.00 minutes. This composition was held constant until 6.00 minutes to ensure complete elution. At 6.01 minutes, the mobile phase was rapidly returned to the initial condition of 80% A and maintained until 9.00 minutes for column re-equilibration.

Mass spectrometric analysis was performed using electrospray ionization in positive mode (ESI⁺). The capillary voltage was optimized to 1.0 kV, and the cone voltage was set to 10 V. The probe temperature was held constant at 600 °C, with a gain setting of 1 and a sampling rate of 5 points per second. The target hydrazone derivative was detected using single ion recording (SIR) at *m/z* 241.31. To ensure system cleanliness and sensitivity, a diverter valve was utilized to direct the effluent to the mass detector only between 5.0 and 6.0 minutes post-injection, corresponding to the analyte's retention window.

2.4. Synthesis and Characterization Procedure for Hydrazone Compound

2-Ethylphenylhydrazine hydrochloride (172.66 mg, 1.00 mmol, 1.0 equiv) and 2-hydroxybenzaldehyde (122.12 mg, 104.4 µL, 1.00 mmol, 1.0 equiv) were dissolved in methanol (10 mL) in a 25 mL round-bottom flask equipped with a magnetic stirring bar. The reaction mixture was stirred at 50 °C for 2 h. After completion of the reaction, the solvent was removed under reduced pressure using a rotary evaporator. The resulting crude product was purified by column chromatography using a 1:1 (v/v) mixture of n-heptane and ethyl acetate as the mobile phase. The hydrazone derivative was obtained as a white solid (168 mg, 70% yield). The melting behavior was investigated by differential scanning calorimetry (DSC), and the melting point was determined to be 77–78 °C.

The synthesized hydrazone derivative was characterized by FTIR, ¹H NMR, ¹³C NMR, DSC, and mass spectrometry. The FTIR spectrum was recorded using an ATR accessory and showed characteristic absorption bands at *v*_{max} 3345, 2973, 2892, 1622, 1585, 1570, 1527, 1346, 1293, 1249, and 1150 cm⁻¹. The ¹H NMR spectrum was recorded at 400 MHz in DMSO-*d*₆ and showed signals at δ 10.88 (d, *J* = 0.35 Hz, 1H), 7.88 (s, 1H), 7.35 (br s, 1H), 7.22–7.12 (m, 3H), 7.13–7.06 (m, 2H), 6.97–6.91 (m, 1H), 6.84 (m, 2H), 2.53 (q, *J* = 7.6 Hz, 2H), and 1.23 (t, *J* =

7.6 Hz, 3H). The ^{13}C NMR spectrum (APT, 101 MHz, DMSO- d_6) showed resonances at δ 157.07, 141.81, 140.61, 130.01, 129.31, 128.44, 127.38, 126.77, 120.92, 119.42, 118.48, 116.57, 112.63, 23.59, and 13.39 ppm. The molecular ion was confirmed by positive-mode mass spectrometry, with an observed $[\text{M}+\text{H}]^+$ ion at m/z 241.09 (calculated m/z 241.13 for $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}^+$).

The isolated hydrazone derivative was used as a structurally characterized reference compound to confirm the identity of the in-situ generated derivative used in the analytical procedure.

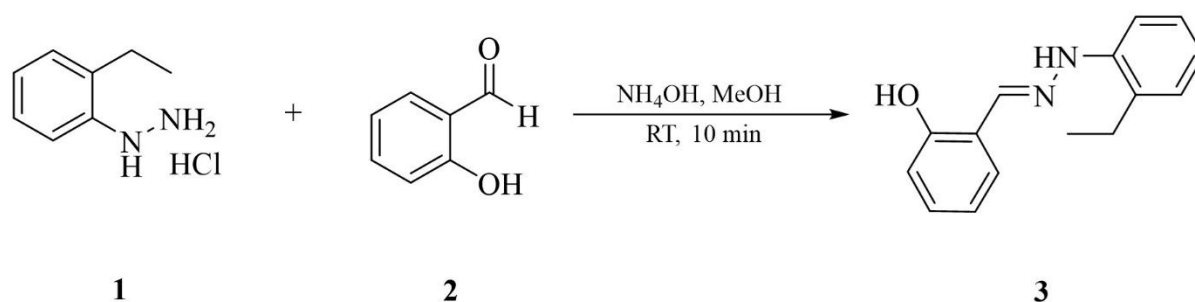


Figure 2. In-situ formation of the 2-Ethylphenylhydrazine–2-hydroxybenzaldehyde hydrazone (3) from 2-Ethylphenylhydrazine HCl (1) and 2-hydroxybenzaldehyde (2).

2.5. Hydrazone Compound Sample Preparation for Characterization

Due to the inherent instability of 2-Ethylphenylhydrazine HCl in solution, the analyte was derivatized prior to characterization. 2-Ethylphenylhydrazine HCl was dissolved in a basic methanolic medium prepared using concentrated ammonia and methanol. An excess of 2-hydroxybenzaldehyde was subsequently added as the derivatization reagent, and the reaction mixture was stirred at 50 °C for 60 minutes using a heated magnetic stirrer. Under these conditions, 2-Ethylphenylhydrazine was converted to the corresponding 2-hydroxybenzaldehyde hydrazone derivative, which was substantially more stable than the underivatized analyte. The molecular weight of the resulting hydrazone was 240.31 g/mol, with a protonated molecular ion at m/z 241.13. An excess of 2-hydroxybenzaldehyde was used to promote complete conversion of the target hydrazine to the hydrazone derivative. Ammonia was selected as the basic component because it is compatible with LC-MS-based analysis [20,21] and facilitates conversion of 2-Ethylphenylhydrazine hydrochloride to its free-base form prior to condensation with 2-hydroxybenzaldehyde.

2.6. Mass fragmentation study

Mass fragmentation is the process by which a molecule breaks into smaller ionized fragments when subjected to mass spectrometry, providing essential structural information. In this study, fragmentation was achieved via in-source fragmentation during Electrospray Ionization (ESI) in positive mode. By strategically increasing the cone voltage, diagnostic bond cleavages within the molecular ion were induced prior to mass analysis. The hydrazone derivative (m/z 241.13) undergoes specific fragmentation, yielding two primary fragments:

Fragment 1 (m/z 120.04): Results from the cleavage of the nitrogen–nitrogen (N–N) bond at the hydrazone ($-\text{HN}=\text{C}-$) linkage, producing a stable fragment containing the hydroxyl ($-\text{OH}$) and imine ($-\text{C}=\text{NH}$) groups.

Fragment 2 (m/z 120.08): Corresponds to the protonated 2-ethylphenylamine-like moiety, appearing as a benzyl-like fragment with an amine ($-\text{NH}$) functionality. This fragmentation pattern confirms the structural integrity of the synthesized hydrazone compound and ensures the specificity of the single ion recording (SIR) detection method.

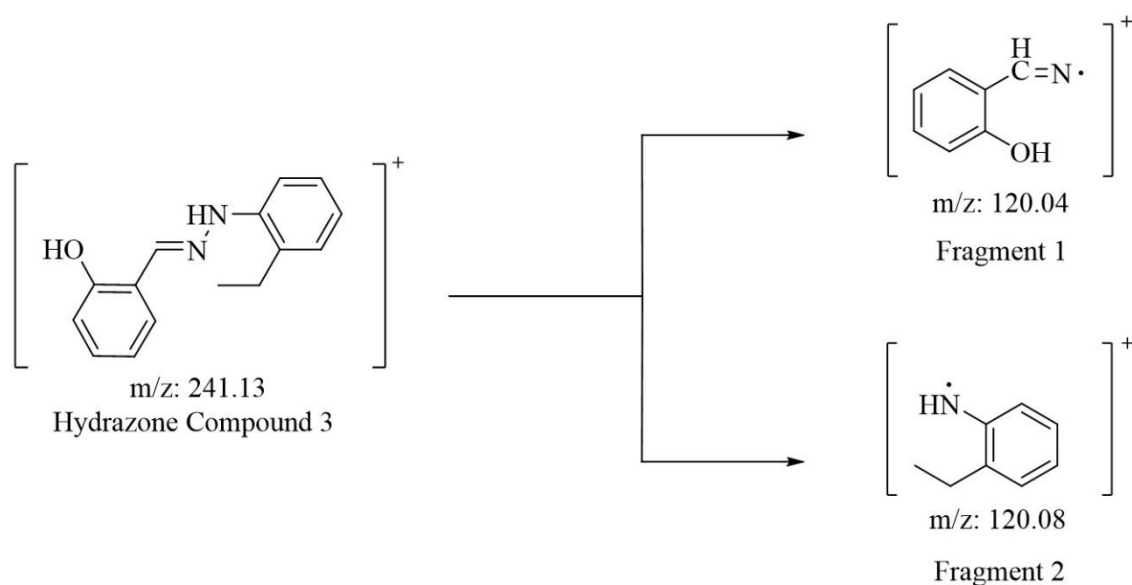


Figure 3. Fragmentation scheme of hydrazone compounds.

2.7. Method Development and Optimization for Characterization of Hydrazone Compound

2.7.1. Direct Analysis

At the initial stage of the study, direct analysis of 2-Ethylphenylhydrazine was performed using an LC-MS instrument. However, satisfactory analytical performance could not be achieved due

to the light sensitivity of 2-Ethylphenylhydrazine and the requirement for storage under an inert atmosphere, together with its low MS response. Furthermore, the analyte could not be detected in spike experiments performed in the Etodolac matrix, resulting in no measurable recovery. These unsatisfactory results necessitated the development of an alternative approach for the analytical determination of 2-Ethylphenylhydrazine.

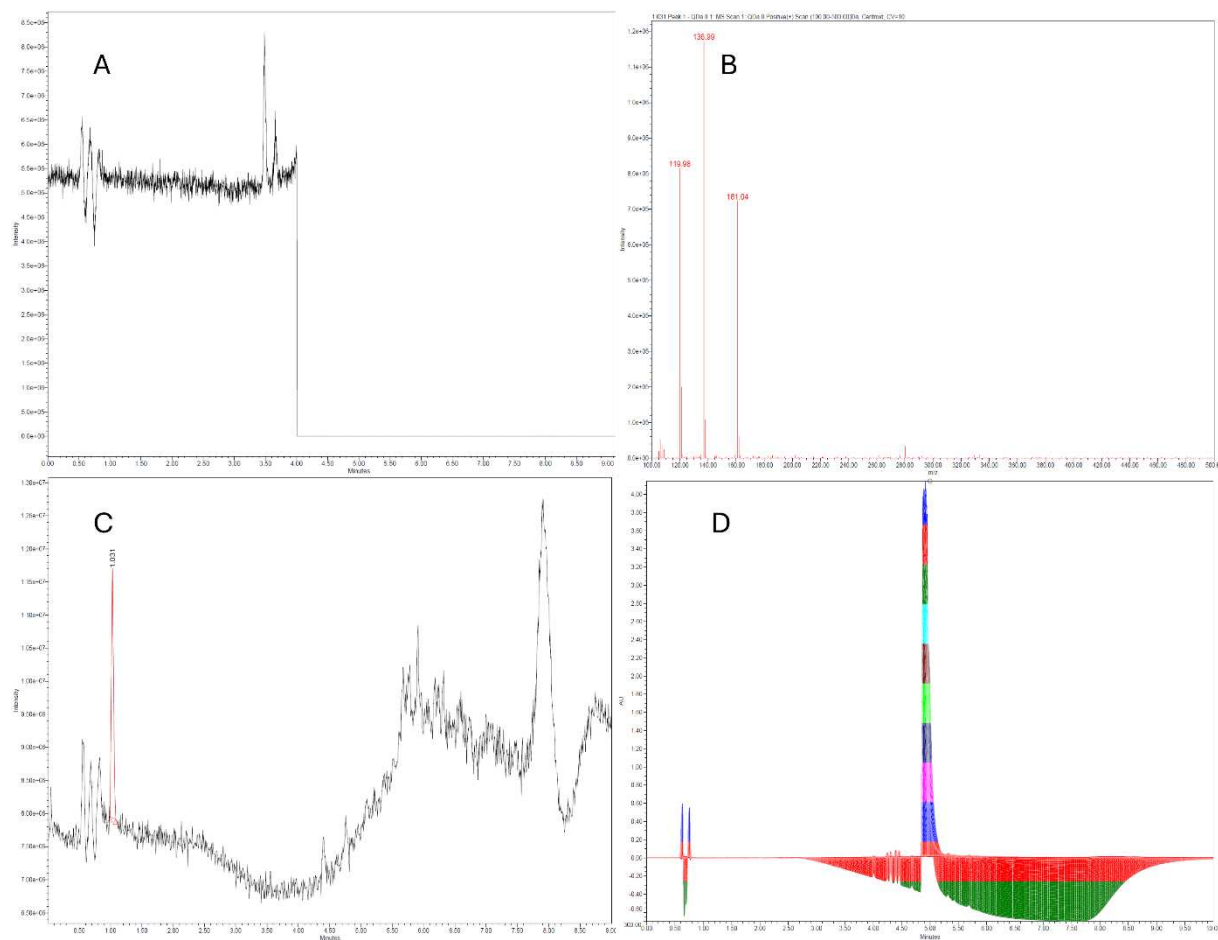


Figure 4. 2-Ethylphenylhydrazine direct analysis chromatograms. Spiked sample TIC Chromatogram (A). Standard centroid data spectrum (B). Standard TIC Chromatogram (C). Spiked sample 210-400 UV Scan Chromatogram (D).

2.7.2. Derivatization-Based Analysis

In the second stage, to improve the MS response of 2-Ethylphenylhydrazine, the analyte was reacted with 2-hydroxybenzaldehyde to obtain the corresponding hydrazone derivative. Hydrazone formation is a well-established condensation reaction between carbonyl compounds

and hydrazine derivatives, involving nucleophilic addition to the carbonyl group followed by dehydration to form the C=N–N linkage. The resulting hydrazone compound exhibited substantially improved MS response compared with the underivatized analyte. However, in spike experiments performed in the Etodolac matrix, the recovery values remained in the range of 1–5%, which was below the acceptable limits. These results indicated that, although derivatization improved the MS response of 2-ethylphenylhydrazine, the derivatization procedure was still strongly affected by the Etodolac matrix and therefore required further optimization under matrix conditions.

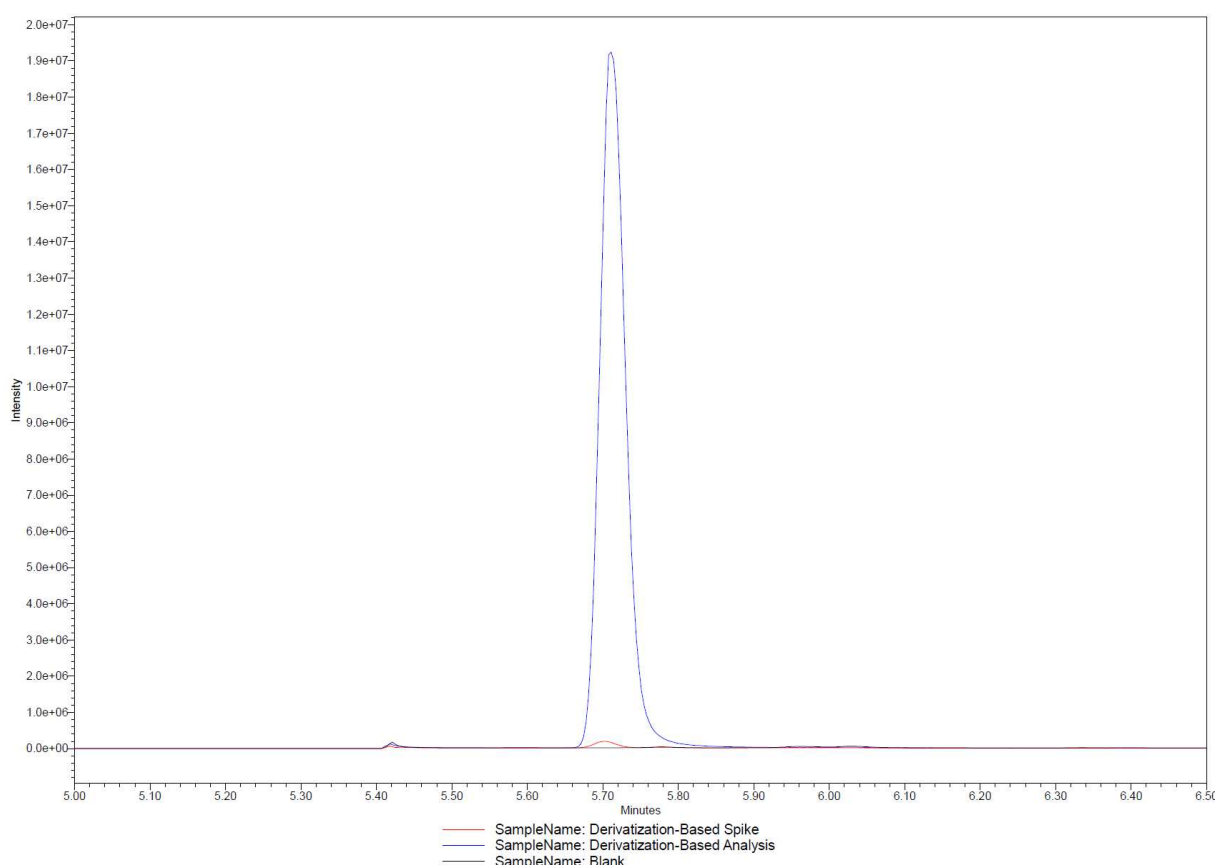


Figure 5. Derivatization based analysis results overlay chromatogram.

2.7.3. Derivatization in a Basic Medium

In the third stage, the effect of the reaction medium on the analytical performance of the derivatization procedure was investigated. Because hydrazone formation is strongly dependent on the acid–base properties of the reaction medium, and because the Etodolac matrix contains a carboxylic acid functionality, the sample solution was rendered basic by the addition of ammonia prior to derivatization. Under these conditions, 2-Ethylphenylhydrazine was reacted with 2-hydroxybenzaldehyde to obtain the corresponding hydrazone compound. Adjustment of

the reaction medium to basic conditions resulted in a substantial improvement in analytical performance, with recoveries close to 100% obtained in spiked Etodolac samples.

Although hydrazone formation is generally favored by specific pH conditions rather than by basicity alone, the marked improvement observed in the present study indicates that pH adjustment was effective in reducing the adverse effect of the Etodolac matrix on the derivatization procedure. Therefore, the optimized basic reaction medium was selected for subsequent analyses. This modification established the final conditions for the derivatization procedure and provided satisfactory analytical performance for the determination of 2-Ethylphenylhydrazine in the Etodolac matrix. Accordingly, the optimized derivatization procedure employing a basic reaction medium was established as the final analytical procedure for the determination of 2-Ethylphenylhydrazine in Etodolac drug substance. All subsequent method performance evaluations and validation studies were therefore conducted using these optimized conditions. The validation results are presented in the following sections.

2.7.4. Optimization of Derivatization Temperature and Reaction Time

As shown in Table 1, increasing the reaction temperature markedly enhanced the derivatization efficiency. At room temperature, no detectable derivatization was observed even after 60 min. At 40 °C, the reaction proceeded gradually, reaching approximately 70% derivatization after 60 min. In contrast, at 50 °C, the derivatization efficiency increased rapidly after 30 min, reaching 92% after 45 min and 99.9% after 60 min. The derivatization efficiency was calculated as the ratio of the measured derivatized analyte response to the theoretical maximum response, expressed as a percentage. Based on these results, 50 °C and 60 min were selected as the optimal derivatization conditions, providing essentially complete conversion of 2-Ethylphenylhydrazine to its corresponding hydrazone derivative.

Table 1. Effect of reaction temperature and time on derivatization efficiency (%).

Temperature	5 min	15 min	30 min	45 min	60 min
Room Temperature	0	0	0	0	0
40 °C	7	18	35	51	70
50 °C	10	23	68	92	99.9

2.8. Analytical Method

2.8.1. Preparation of Solutions

Diluent (Blank Solution): 10 mL of 25% aqueous ammonia is added to a 1000 mL volumetric flask and diluted to volume with methanol.

Standard Solution: 9.51 mg of 2-Ethylphenylhydrazine HCl (equivalent to 7.50 mg of free base) is dissolved in a 100 mL amber volumetric flask using the diluent. A 1 mL aliquot of this solution is transferred to a 100 mL amber volumetric flask and diluted to volume. From this second solution, 1 mL is transferred into a 20 mL amber volumetric flask, followed by the addition of 400 µL of 2-hydroxybenzaldehyde. The solution is diluted to volume and stirred at 50 °C for 60 minutes using a heated magnetic stirrer to complete the derivatization.

Sample Solution: 500 mg of Etodolac API is weighed into a 20 mL amber volumetric flask and dissolved in 7 mL of diluent. After dissolution, 400 µL of 2-hydroxybenzaldehyde is added. The flask is filled to volume with diluent and the mixture is stirred at 50 °C for 60 minutes using a heated magnetic stirrer.

$$\text{Calculation (ppb)} = \frac{\frac{W_{\text{Standard}}}{100} \times \frac{1}{100} \times \frac{1}{20}}{\frac{W_{\text{Sample}}}{20}} \times \frac{136.20 \text{ g/mol}}{172.66 \text{ g/mol}} \times 10^9$$

W_{Standard} : Standard weight (mg)

W_{Sample} : Sample weight (mg)

136.20 : 2-Ethylphenylhydrazine molecular weight (g/mol)

172.66 : 2-Ethylphenylhydrazine HCl molecular weight (g/mol)

3. Results and Discussion

The developed UPLC–MS method was successfully applied to the determination of 2-Ethylphenylhydrazine in Etodolac drug substance through its in situ formed hydrazone derivative in both test and spiked samples. Chromatographic separation was efficient, and no significant interference from the Etodolac matrix was observed at the monitored retention time (~5.2 min) and SIR transition (m/z 241.13), confirming the selectivity of the method. The derivatization approach, optimized in a basic medium using ammonia, effectively improved analyte stability and minimized matrix-induced degradation, enabling reliable trace-level detection. In accordance with ICH Q2(R2) guidelines, all validation parameters, including specificity, precision, accuracy, and linearity, were within acceptable limits, demonstrating the robustness of the method. Overall, the results confirm that the method meets the required criteria for sensitivity, selectivity, and reproducibility, supporting its suitability for routine quality control and regulatory compliance monitoring of genotoxic impurities.

3.1. Synthesis and Characterization of standard hydrazone compound

The standard hydrazone compound was also synthesized in 70% yield using 2-Ethylphenylhydrazine HCl, 2-Hydroxybenzaldehyde. The synthesized compound was purified using column chromatography. The synthesized hydrazone compound was characterized using spectroscopic techniques. The melting point was determined using DSC technique.

3.1.1. NMR Analysis

^1H NMR (Supplementary File S2) and ^{13}C NMR (Supplementary File S3 and S4) analyses were performed to verify the structure. Examination of the ^1H NMR spectrum revealed alkyl protons corresponding to $-\text{CH}_3$ and $-\text{CH}_2$ groups at 1.23 ppm (triplet) and 2.53 ppm (quartet), respectively. The $-\text{NH}$ proton appeared as a doublet at 10.88 ppm, while the $-\text{OH}$ proton was observed as a broad singlet at 7.35 ppm. The imine ($-\text{CH}=\text{N}-$) proton was detected as a singlet at 7.88 ppm, with the remaining aromatic protons resonating between 7.25 and 6.80 ppm. Analysis of the ^{13}C DEPT NMR spectrum identified aliphatic carbon signals corresponding to the $-\text{CH}_3$ and $-\text{CH}_2$ groups at 13.99 ppm and 23.59 ppm, respectively. The structural examination confirmed the presence of eight aromatic $-\text{CH}$ carbons, one imine ($-\text{CH}$) carbon, and four quaternary ($-\text{C}$) carbons. These observations from the ^1H NMR and ^{13}C NMR spectra collectively support the proposed structure.

3.1.2. FTIR Analysis

The infrared (IR) spectrum (Supplementary File S5) exhibits a broad absorption peak at 3345 cm^{-1} , corresponding to the $\text{N}-\text{H}$ stretching vibration of the hydrazone ($-\text{NH}-\text{N}=\text{}$) group. This region also overlaps with the $\text{O}-\text{H}$ stretching vibration of the phenolic ($-\text{OH}$) group. The absorption bands at 2973 cm^{-1} and 2892 cm^{-1} are attributed to the $\text{C}-\text{H}$ stretching vibrations of the aliphatic $-\text{CH}_2-$ and $-\text{CH}_3$ groups from the ethyl substituent. A strong band at 1622 cm^{-1} confirms the presence of the imine ($-\text{C}=\text{N}$) functional group, a characteristic feature of hydrazone derivatives. Additionally, absorption peaks at 1585 cm^{-1} , 1570 cm^{-1} , and 1527 cm^{-1} correspond to the $\text{C}=\text{C}$ stretching vibrations of the aromatic rings, further confirming the presence of benzene moieties. The peaks at 1346 cm^{-1} and 1293 cm^{-1} are associated with $\text{C}-\text{N}$ stretching within the hydrazone ($-\text{NH}-\text{N}=\text{}$) linkage. Furthermore, the absorption bands at 1249 cm^{-1} and 1150 cm^{-1} , attributed to $\text{C}-\text{O}$ stretching vibrations, confirm the presence of the phenolic ($-\text{OH}$) group in the structure.

3.1.3. DSC Analysis

The DSC thermogram (Supplementary File S6) indicates a significant thermal event at 77.88 °C, with an associated enthalpy change of 134.8536 J/g. This event could be related to the melting or decomposition of the hydrazone compound.

3.1.4. Mass Spectrometry Analysis

When the mass spectrum (Supplementary File S7) of the hydrazone compound was examined, the base peak (most intense peak) was observed at m/z 241.09, corresponding to the protonated molecular ion $[M+H]^+$. The molecular formula of the compound was determined as $C_{15}H_{16}N_2O$, with a calculated molecular weight of 240.31 g/mol. Upon positive ionization, the addition of a proton (H^+) resulted in the observed m/z 241.09. The obtained mass spectrum confirmed the molecular weight of the synthesized hydrazone compound and strongly indicated successful ionization under positive ESI conditions.

3.2. Validation Parameters in Accordance with ICH Q2(R2) Guidelines

The ICH Q2(R2) guideline outlines the essential parameters required for the validation of analytical procedures. These parameters are critical to ensure the reliability, accuracy, and consistency of the method. The primary validation parameters include: Specificity, Accuracy, Precision (encompassing repeatability and intermediate precision), Linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), Range, and Robustness. These criteria collectively establish the suitability of the analytical method for its intended purpose.

3.2.1. Specificity

Specificity is defined as the ability to precisely measure the analyte in the presence of other potential components within the sample, ensuring that no interference affects the results. To evaluate the specificity of the proposed method, injections and spectral analyses were performed using blank samples, derivatization standard solutions, derivatization test solutions, and 100% spiked test solutions.

No interference was detected in the blank solution at the monitored m/z value of 241.13, and no peak was observed at the corresponding retention time. Similarly, the derivatization test solution showed no detectable signal at m/z 241.13. In contrast, the derivatization standard solution exhibited a distinct peak at approximately 5.2 min with an m/z value of 241.13. A comparable peak, with consistent retention time (~5.2 min) and m/z value (241.13), was also

observed in the 100% spiked test solution, confirming the presence and selective detection of the analyte.

The identity of the detected species was further supported by mass spectral data. As shown in Figure 6, the positive-scan centroid spectrum of the derivatization standard exhibited a characteristic signal at m/z 241.13, corresponding to the 2-Ethylphenylhydrazine derivative. Furthermore, the TIC chromatograms presented in Figure 7 demonstrate the absence of a corresponding chromatographic signal in the blank chromatogram, whereas a distinct peak was observed for the 2-Ethylphenylhydrazine derivative at approximately 5.2 min. These findings indicate that matrix components present in the blank did not produce a chromatographic response at the analyte retention time.

The specificity of the method was additionally supported by the UV spectral evaluation. As shown in Figure 8, the 3D UV scan recorded over the wavelength range of 180–400 nm did not reveal a corresponding signal in the blank solution, whereas the 2-Ethylphenylhydrazine derivative exhibited a distinct UV response. The consistent chromatographic retention time, mass spectral response, and UV spectral behavior collectively demonstrate that the analytical response can be attributed to the derivatized 2-Ethylphenylhydrazine rather than to potential interfering components.

Overall, the absence of interfering responses in the blank and test solutions, together with the consistent m/z , retention time, TIC, mass spectral, and UV responses observed for the derivatization standard and spiked test solution, confirms the specificity of the proposed method for the determination of 2-Ethylphenylhydrazine.

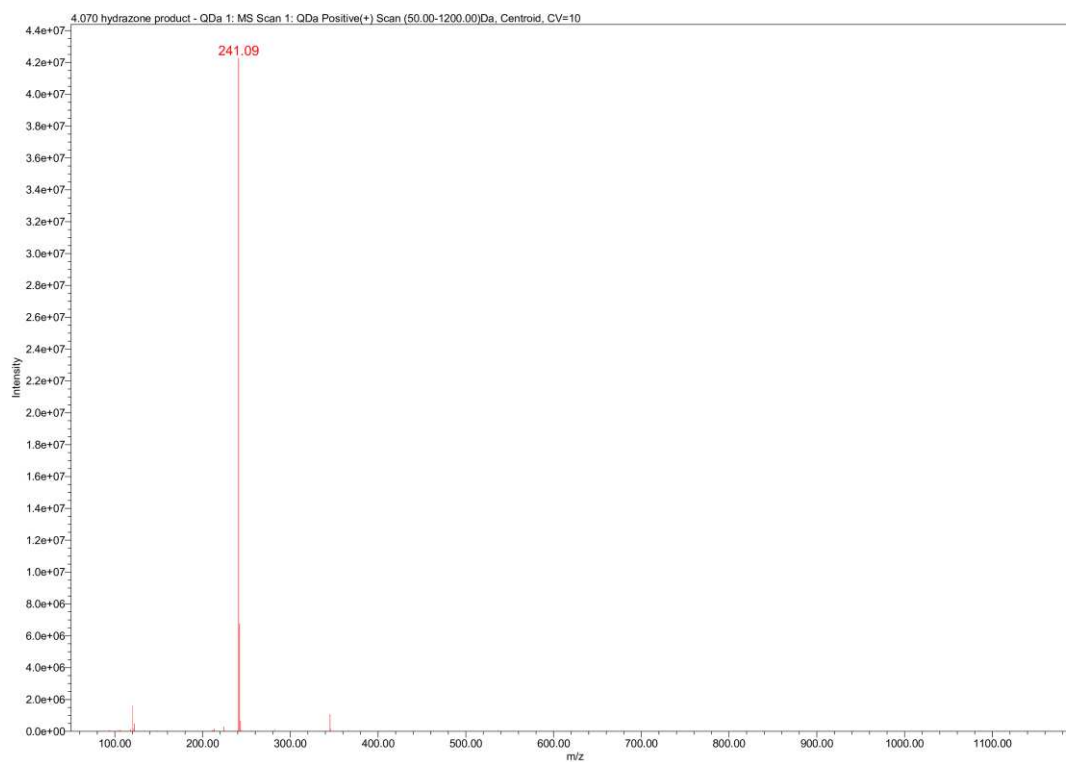


Figure 6. Centroid data spectrum obtained from a positive scan of the standard solution.

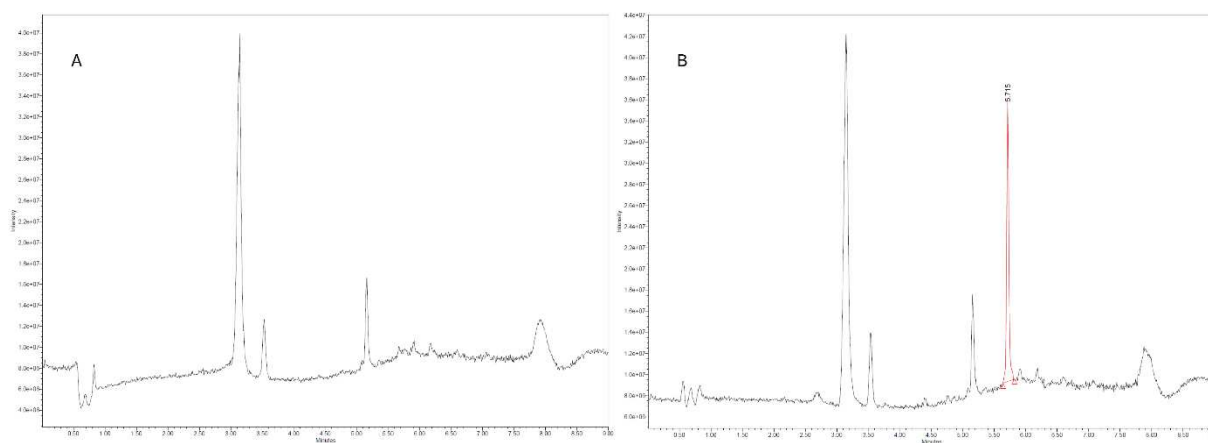


Figure 7. Blank TIC Chromatogram (A) and TIC Chromatogram of the 2-Ethylphenylhydrazine Derivative (B).

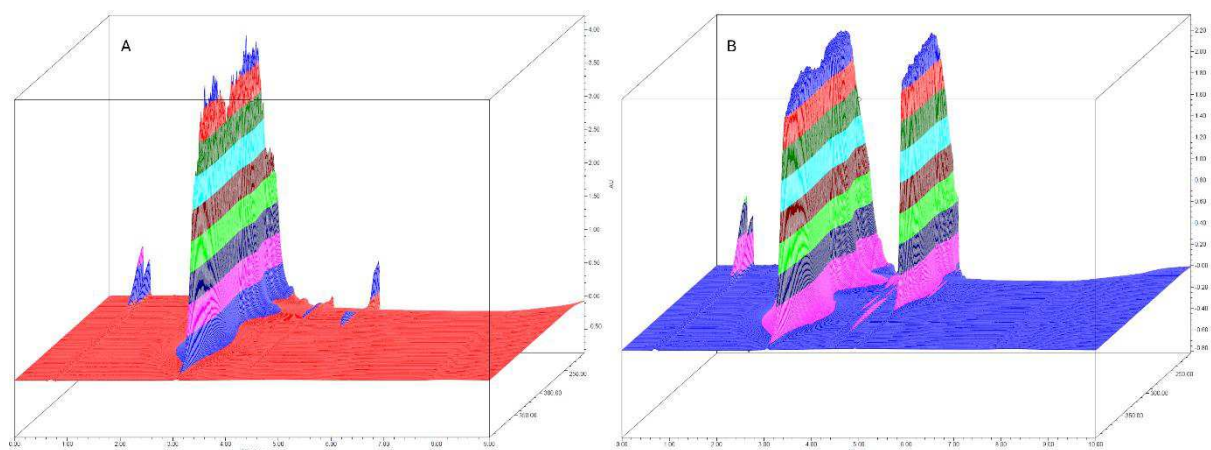


Figure 8. Blank 3D UV Scan (210-400 nm) Chromatogram (A) and 3D UV Scan (210-400 nm) Chromatogram of the 2-Ethylphenylhydrazine Derivative (B).

3.2.2. Limit of Detection and Limit of Quantitation (LOD and LOQ)

In this study, the limit of detection (LOD) of the method was determined to be 0.06858 ng/mL based on three replicate injections. At this concentration level, the signal-to-noise (S/N) ratios obtained from the individual injections were 3.36, 3.37, and 3.42, yielding a mean S/N value of 3.38. The standard deviation was calculated as 0.03, corresponding to a relative standard deviation (RSD) of 0.95%. These results indicate that the method is sufficiently sensitive to detect the analyte at very low concentrations with good repeatability. The overlay chromatograms corresponding to the LOD level are presented in Figure 9.

The limit of quantitation (LOQ), defined as the lowest concentration at which the analyte can be quantified with acceptable precision and accuracy, was established as 0.2286 ng/mL based on three replicate injections. At this level, the observed S/N ratios were 10.52, 10.74, and 10.84, resulting in a mean value of 10.70. The calculated standard deviation was 0.16, with an RSD of 1.53%. These findings confirm that the method provides reliable and precise quantitation at the specified LOQ level. The corresponding overlay chromatograms are illustrated in Figure 10.

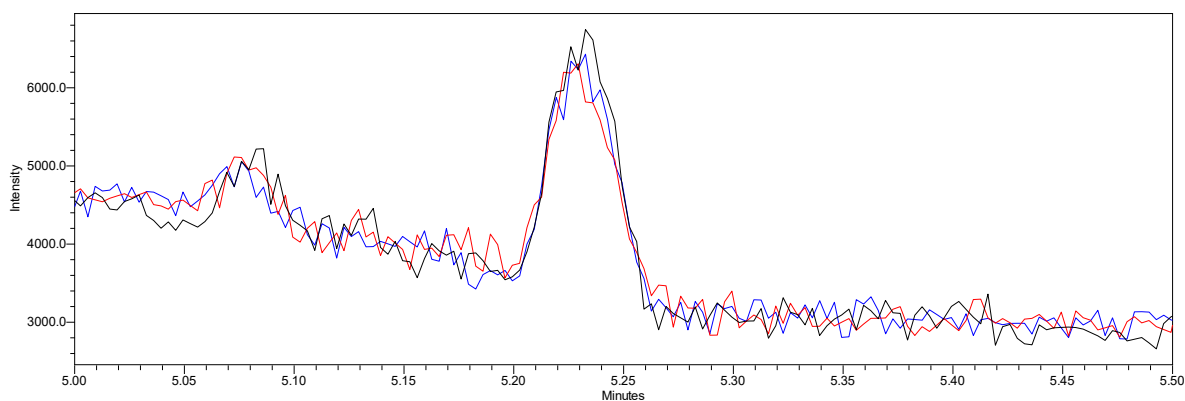


Figure 9. Overlay chromatogram of LOD.

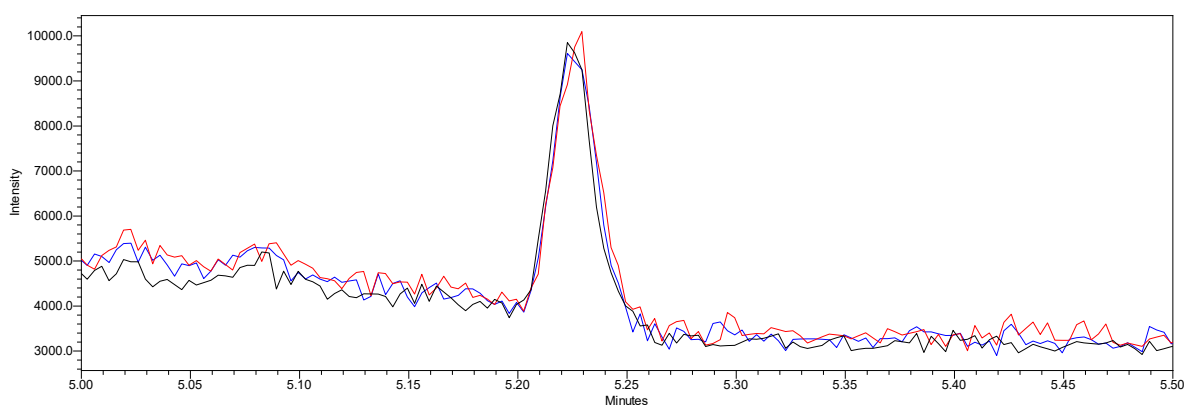


Figure 10. Overlay chromatogram of LOQ.

3.2.3. Accuracy

Accuracy in an analytical procedure refers to the degree of agreement between the measured value and a recognized reference value or conventional true value. The accuracy of the method was evaluated with three injections at the LOQ spike level, six injections at the 100% spike level, and six injections at the 150% spike level. The corresponding concentrations were 0.2333, 47.54, and 71.31 ppb as 2-Ethylphenylhydrazine HCl at the LOQ, 100%, and 150% levels, respectively. On an equivalent basis, these concentrations corresponded to 0.1840, 37.50, and 56.25 ppb as 2-Ethylphenylhydrazine free base, respectively.

The accuracy results are presented in Table 2, and the overlay chromatograms for accuracy are presented in Figures 11 and 12. The method recovery rates were 104.09% to 121.94% at the LOQ level (0.2286 ppb), 98.17% to 100.76% at the 100% level (47.54 ppb), and 99.11% to 100.37% at the 150% level (71.31 ppb), with all concentrations expressed as 2-Ethylphenylhydrazine HCl. The relatively higher recovery observed at the LOQ level (mean

113.37%) can be attributed to the inherent analytical challenges associated with trace-level quantification near the detection limit and potential minor ion enhancement effects from the complex Etodolac matrix. Nevertheless, the recovery results obtained at all tested levels demonstrate the suitability of the method for accurate quantification of 2-Ethylphenylhydrazine in Etodolac drug substance.

Table 2. Accuracy Results.

Level	Number of Replicates	Recovery %	SD	RSD %	95 % CI
1	3	113.37	8.95	7.89	113.37 ± 22.22
4	6	99.12	0.86	0.87	99.12 ± 0.91
6	3	99.60	0.68	0.68	99.60 ± 1.68

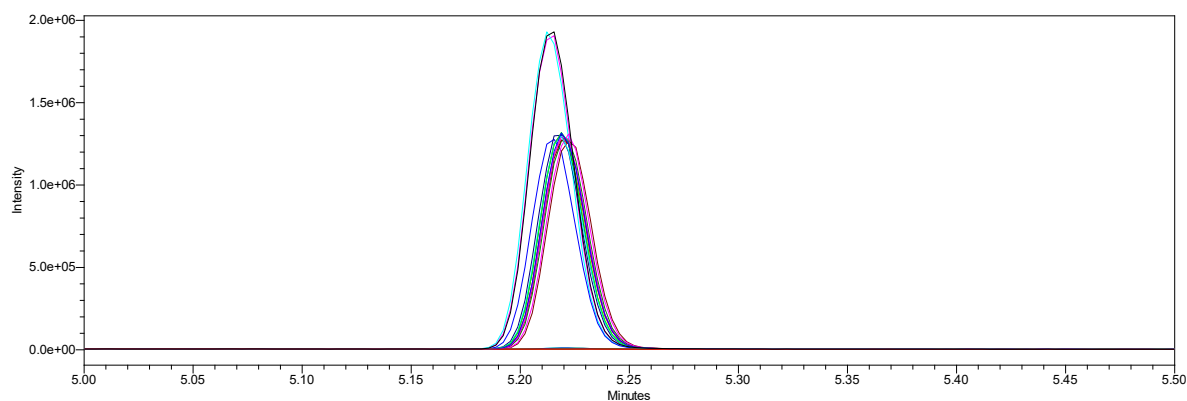


Figure 11. Overlay accuracy chromatograms.

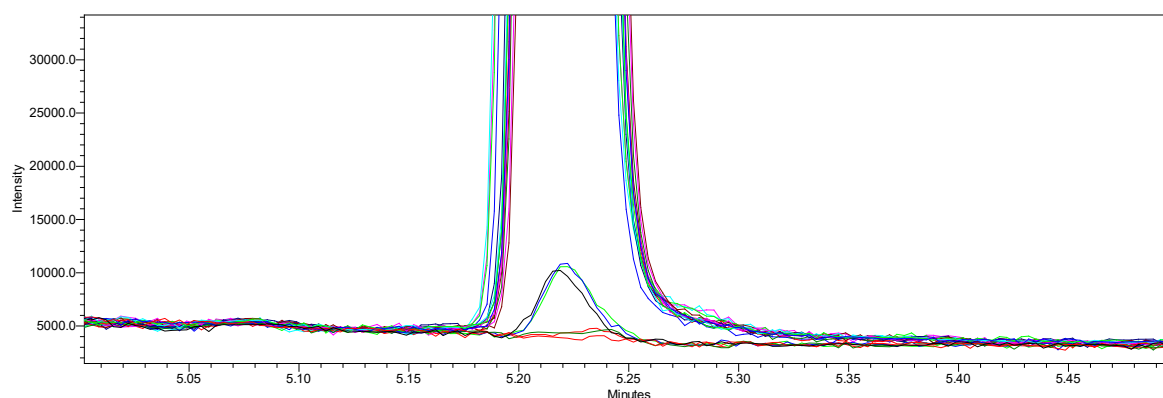


Figure 12. Overlay LOQ accuracy chromatograms.

3.2.4. Precision

Precision in an analytical procedure refers to the degree of agreement (or scatter) among a series of measurements obtained from multiple samplings of the same homogeneous sample under specified conditions. Precision can be evaluated at three levels: repeatability, intermediate precision, and reproducibility. It is typically assessed using homogeneous, authentic samples. However, if such samples are unavailable, artificially prepared samples or sample solutions may be used. Precision is commonly expressed as variance, standard deviation, or the coefficient of variation derived from a series of measurements.

Repeatability, also known as intra-assay precision, measures the precision under identical operating conditions within a short time frame. The repeatability of the method was evaluated using six replicate injections of the standard solution at the 100% concentration level. The outcomes of these injections are detailed in Table 3. The relative standard deviation (RSD%) for the method's repeatability was found to be 0.91%.

Table 3. Repeatability results.

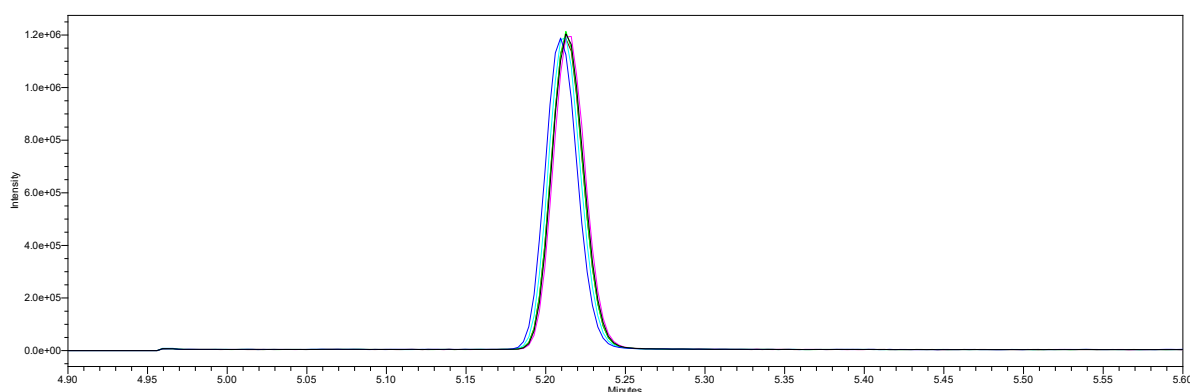
Repeatability of Hydrazone Compound	
Number of injections	Level 4 Peak Area
1	2004693.30
2	2005128.18
3	1980866.34
4	1995039.48
5	1957082.03
6	1992272.18
Average	1989180.25
SD	18101.37
RSD %	0.91

Intermediate precision refers to the variability observed within a single laboratory under different conditions, such as changes in analysts, days, or equipment. The intermediate precision of the method was evaluated by analyzing six independently prepared sample solutions at the specified method concentration of 500 mg/20 mL Etodolac. The samples were analyzed by two different analysts under the same analytical conditions. The results are detailed in Table 4, and the corresponding overlay chromatograms are provided in Figure 13. The mean recovery values obtained by Analyst 1 and Analyst 2 were 99.12% and 97.63%, respectively. The percentage deviation between the two analysts was calculated as 1.53%.

Table 4. Intermediate precision results.

Recovery of Hydrazone Compound		
Number of injections	Analyst 1 Recovery (%)	Analyst 2 Recovery (%)
1	98.82	97.95
2	98.17	97.20
3	100.76	97.83
4	99.00	97.48
5	98.93	98.05
6	99.06	97.24
Average	99.12	97.63
SD	0.86	0.37
RSD %	0.87	0.38
Deviation %	-	1.53

The low RSD values obtained for both analysts (0.87% and 0.38%) and the low deviation between the analysts (1.53%) demonstrate the good intermediate precision of the method under the evaluated conditions.

**Figure 13.** Overlay Intermediate precision chromatograms.

3.2.5. Linearity

Linearity refers to the ability of an analytical procedure to obtain test results that are directly proportional to the concentration of the analyte within a specified range. The linearity of the method was evaluated using 27 injections at six concentration levels, corresponding to LOQ, 50%, 80%, 100%, 120%, and 150% of the target concentration. The evaluated concentration range was 0.2286–74.0 ng/mL.

For each concentration level, the mean peak area was calculated from the replicate injections and used for regression analysis. The calibration curve was constructed by plotting the mean

peak area against the corresponding analyte concentration. Linear regression analysis yielded the following equation:

$$y = 3.9088 \times 10^7 x + 44,427.05$$

where y represents the peak area and x represents the concentration of 2-Ethylphenylhydrazine. The regression analysis demonstrated a strong linear relationship over the evaluated concentration range, with a correlation coefficient (R) of 0.9994 and a coefficient of determination (R^2) of 0.9988. The adjusted coefficient of determination was 0.9985, further supporting the goodness of fit of the regression model. The statistical significance of the regression was confirmed by the ANOVA results, with a regression p -value of 5.68×10^{-7} , indicating a statistically significant relationship between analyte concentration and detector response.

The residual differences between the observed and predicted peak areas were within an acceptable range across the tested concentrations, with no substantial deviation from the established linear relationship. The linearity plot is presented in Figure 14, while the corresponding overlay chromatograms are shown in Figure 15.

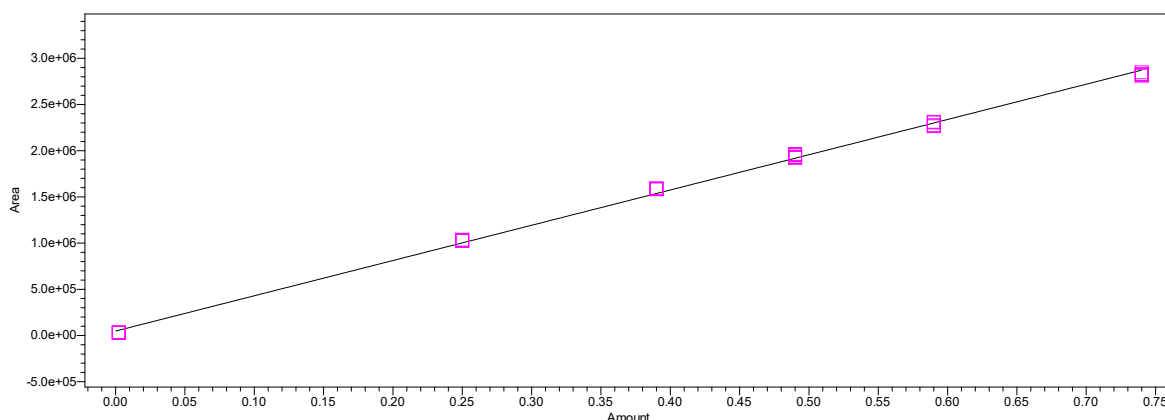


Figure 14. Linearity graph

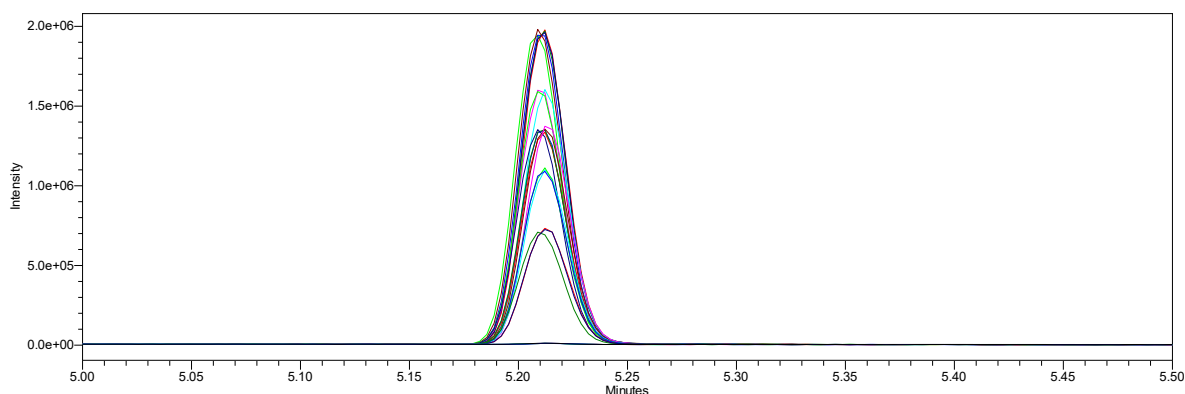


Figure 15. Overlay linearity chromatograms.

3.2.6. Range

The range of an analytical method is defined as the interval between the lower and upper concentrations of an analyte in a sample for which the method has demonstrated suitable linearity, accuracy, and precision. For the developed and validated method, the quantification range was established at 0.2286–74.0 ng/mL as 2-Ethylphenylhydrazine HCl. This range corresponds to 0.1840–59.76 ng/mL as 2-Ethylphenylhydrazine free base. The lower and upper limits of the established quantification range are presented in Table 5.

Table 5. Quantification range of 2-Ethylphenylhydrazine HCl.

Compound Name	Lower quantification limit (ng/mL)	Upper quantification limit (ng/mL)
Hydrazone Compound	0.2286	74.0

3.2.7. Robustness

Robustness refers to the ability of an analytical method to maintain reliable performance despite deliberate, minor variations in analytical conditions. In this study, robustness was evaluated by varying the flow rate and column oven temperature around their nominal conditions. Six replicate injections of the standard solution at the 100% concentration level were analyzed under each modified condition.

The method demonstrated stable retention time performance under all tested conditions. When the flow rate was decreased to 0.36 mL/min, the mean retention time was 5.48 min with an RSD% of 0.07, while increasing the flow rate to 0.44 mL/min resulted in a mean retention time of 5.01 min with an RSD% of 0.01. Similarly, increasing the column oven temperature to 37°C produced a mean retention time of 5.21 min with an RSD% of 0.01, whereas decreasing the

temperature to 33°C resulted in a mean retention time of 5.24 min and an RSD% of 0.08. These findings indicate that the minor variations in chromatographic conditions did not substantially affect retention behavior.

The robustness of the method was also confirmed based on peak area responses. At the reduced flow rate, the mean peak area was 2,006,183.66 with an RSD% of 0.16, while the elevated flow rate yielded a mean peak area of 1,761,859.24 and an RSD% of 0.28. Increasing the column oven temperature to 37°C resulted in a mean peak area of 1,798,748.11 with an RSD% of 0.67, whereas decreasing the temperature to 33°C produced a mean peak area of 1,873,685.51 and an RSD% of 0.37. Since all RSD values remained below 1%, the method was considered robust against the minor intentional variations in the evaluated analytical parameters.

The overlay chromatograms obtained under the robustness conditions are presented in Figure 16.

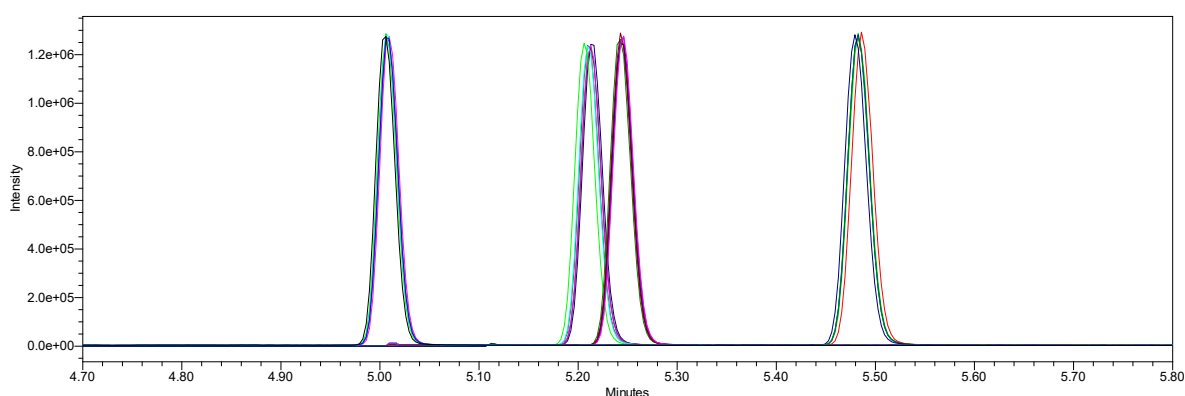


Figure 16. Overlay Robustness chromatograms

3.2.8. System Suitability Testing

System suitability testing is an essential component of many analytical procedures. It is based on the principle that the equipment, electronics, analytical methods, and samples collectively form an integrated system that must be evaluated as a whole. The parameters for system suitability testing are determined based on the specific characteristics of the method being validated. In this study, the system suitability of the method was verified. The acceptance criterion for system compliance was set as a maximum mean relative standard deviation (RSD) of 0.82% for six standard solutions, each at the 100% level. The findings, based on the linearity parameter, are detailed in Table 6.

Table 6. System Suitability Testing

Repeatability of Hydrazone Compound		
Number of standard	Retention Time (min)	Peak Area
1	5.22	1890941.93
2	5.22	1893833.20
3	5.22	1869889.71
4	5.22	1878773.16
5	5.22	1851418.28
6	5.22	1876962.68
Mean	5.22	1876969.83
SD	0.00	15400.36
% RSD	0.02	0.82

This validated analytical method allows for the precise determination of the potential process-related impurity 2-Ethylphenylhydrazine, which is associated with the synthetic route of Etodolac, within a concentration range of 0.2286 ng/mL to 74.0 ng/mL. A key advantage of this derivatization approach is its ability to stabilize both the acidic Etodolac and the basic 2-Ethylphenylhydrazine without degrading the Etodolac matrix, enabling accurate quantitative analysis. The method utilizes single mass spectrometry to analyze the derivatized genotoxic impurity, showcasing its high selectivity.

3.2.9. Stability

3.2.9.1. Standard Stability

Based on the robustness evaluation of standard solution stability, the analytical method demonstrated reliable performance under the tested storage conditions. The standard solution containing the hydrazone compound was analyzed immediately after preparation and after 24 hours of storage at 2–8 °C under light-protected conditions.

The mean peak area was 1.758×10^6 at the initial time point and 1.759×10^6 after 24 hours. The calculated RSD % values were 1.05% and 0.46% for the initial and 24-hour measurements, respectively, indicating good repeatability. The percentage deviation between the two measurements was only 0.03%, which remained well below the predefined acceptance criterion of 3.0%.

These results demonstrate that the standard solution remained stable for at least 24 hours under refrigerated and light-protected conditions, with no significant change in detector response. Therefore, the method was considered robust with respect to standard solution stability.

3.2.9.2. Sample Stability

Under the same storage conditions (2–8 °C, protected from light), the sample solution demonstrated adequate stability for a period of 8 hours. During this timeframe, the analytical response remained consistent with the initial measurements, with variations remaining within the acceptable limits of method variability. The results suggest that the basic environment effectively maintains the integrity of the hydrazone linkage against the residual acidic components of the Etodolac matrix for up to 8 hours. These findings indicate that the sample solution is stable for 8 hours and should be analyzed within this period to ensure the reliability and accuracy of the results.

3.3. Real time QC results

A total of ten independent production batches of Etodolac drug substance were analyzed for the presence of 2-Ethylphenylhydrazine using the developed analytical method. The target impurity was not detected in any of the analyzed production batches, with all results below the established reporting threshold and well below the specified limit of 1.5 ppm.

The absence of a detectable signal in the analyzed production batches should not be interpreted as a limitation of the analytical method. The capability of the method to detect and quantify 2-Ethylphenylhydrazine in the Etodolac matrix was demonstrated during method validation using spiked samples at the LOQ, 100%, and 150% concentration levels. These spiked samples produced the expected analytical response with satisfactory recoveries, demonstrating that the developed procedure was capable of detecting and quantifying the target impurity in the presence of the Etodolac matrix. Therefore, the non-detection observed in the production batches reflects the absence of the impurity at or above the reporting level in the analyzed samples rather than an inability of the method to detect the analyte.

4. Conclusion

This study presents the development and validation of a sensitive and practical UPLC–MS method for the trace-level determination of 2-Ethylphenylhydrazine in Etodolac drug substance. Direct LC–MS analysis of the underivatized impurity provided inadequate response and unreliable recovery, while derivatization with 2-hydroxybenzaldehyde substantially improved the analytical response but initially remained strongly affected by the Etodolac matrix. This limitation was overcome by performing the derivatization in a basic methanolic medium using ammonia, which minimized the adverse effect of the acidic matrix and enabled efficient formation of the hydrazone derivative. Optimization of the derivatization conditions

established 50 °C and 60 min as the optimal reaction temperature and time providing approximately 99.9% derivatization efficiency. The isolated hydrazone was independently characterized by ¹H NMR, ¹³C NMR, FTIR, DSC, and mass spectrometry, supporting the identity of the analytical species generated in situ.

The optimized method, based on reversed-phase UPLC coupled with positive-mode ESI and SIR detection at *m/z* 241.13, demonstrated satisfactory specificity, linearity, accuracy, precision, robustness, and sensitivity in accordance with ICH Q2(R2). A linear response was obtained over 0.2286–74.0 ng/mL as 2-Ethylphenylhydrazine HCl, corresponding to 0.1840–59.76 ng/mL on a free-base basis, with an LOQ of 0.2286 ng/mL (0.184 ppm free base relative to Etodolac). Recoveries at the 100% and 150% levels were close to 100%, while the greater variability observed at the LOQ level remained attributable to trace-level measurement uncertainty. The standard solution was stable for at least 24 h at 2–8 °C under light-protected conditions, whereas the sample solution demonstrated adequate stability for 8 h, supporting controlled sample handling prior to analysis.

Overall, the principal contribution of this work is the development of a matrix-tolerant in-situ derivatization strategy that simultaneously addresses the chemical instability and limited MS response of 2-Ethylphenylhydrazine. By combining chemical stabilization, improved detectability, rapid UPLC separation, and single-quadrupole MS detection, the proposed approach provides a practical and reliable analytical solution for the routine monitoring of this process-related potential genotoxic impurity in Etodolac drug substance. The demonstrated analytical performance and applicability of the method support its use as a routine quality-control procedure for trace-level determination of 2-Ethylphenylhydrazine in the Etodolac matrix.

Authors' contributions

All authors contributed equally to designing and performing experiments and shared in the preparation of the manuscript

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Disclosure of Interest

No potential conflict of interest was reported by the author(s).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/.....>

References

- [1] Lynch S, Brogden RN. Etodolac. A preliminary review of its pharmacodynamic activity and therapeutic use. *Drugs*. 1986;31(4):288–300. doi:10.2165/00003495-198631040-00002.
- [2] Bellamy N. Etodolac in the management of pain: a clinical review of a multipurpose analgesic. *Inflammopharmacology*. 1997;5(2):139–152. doi:10.1007/s10787-997-0023-8.
- [3] Dvornik DM. Tissue selective inhibition of prostaglandin biosynthesis by Etodolac. *J Rheumatol Suppl*. 1997;47:40–47.
- [4] Inoue K, Fujisawa H, Sasaki Y, Nishimura T, Nishimura I, Inoue Y, Yokota M, Masuda T, Ueda F, Shibata Y. Pharmacological properties of the new non-steroidal anti-inflammatory agent Etodolac. *Arzneimittelforschung*. 1991;41(3):228–235.
- [5] DailyMed. Etodolac tablets, USP: prescribing information. U.S. National Library of Medicine.
- [6] Yu Z, Tong G, Xie X, Zhou P, Lv Y, Su W. Continuous-flow process for the synthesis of 2-Ethylphenylhydrazine HCl. *Org Process Res Dev*. 2015;19(7):892–896. doi:10.1021/acs.oprd.5b00115.
- [7] Zhang Y, et al. On the Fischer indole synthesis of 7-ethyltryptophol—mechanistic and process intensification studies under continuous flow conditions. *Org Process Res Dev*. 2013;17(2):294–302. doi:10.1021/op300363s.
- [8] European Chemicals Agency (ECHA). 2-Ethylphenylhydrazine hydrochloride (2-EPH), EC No. 421-460-2, CAS No. 19398-06-2: Harmonised classification and labelling under Regulation (EC) No 1272/2008 (CLP). European Chemicals Agency, Helsinki, Finland.
- [9] Kimball, R. F. The mutagenicity of hydrazine and some of its derivatives. *Mutation Research* 1977, 39(2), 111–126. [https://doi.org/10.1016/0165-1110\(77\)90018-5](https://doi.org/10.1016/0165-1110(77)90018-5)

- [10] Parodi, S.; De Flora, S.; Cavanna, M.; Pino, A.; Robbiano, L.; Bennicelli, C.; Brambilla, G. DNA-damaging activity in vivo and bacterial mutagenicity of sixteen hydrazine derivatives as related quantitatively to their carcinogenicity. *Cancer Research* 1981, 41(4), 1469–1482.
- [11] De Flora, S.; Zanacchi, P.; Bennicelli, C.; Camoirano, A.; Cavanna, M.; Sciabà, L.; Cajelli, E.; Faggin, P.; Brambilla, G. In vivo and in vitro genotoxicity of three antihypertensive hydrazine derivatives (hydralazine, dihydralazine, and endralazine). *Environmental Mutagenesis* 1982, 4(5), 605–619. <https://doi.org/10.1002/em.2860040512>
- [12] Benigni, R.; Passerini, L. An empirical and theoretical study on mechanisms of mutagenic activity of hydrazine compounds. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 1995, 332(1–2), 63–71. [https://doi.org/10.1016/0027-5107\(95\)00155-2](https://doi.org/10.1016/0027-5107(95)00155-2)
- [13] Elder DP, Snodin DJ, Teasdale A. Control and analysis of hydrazine, hydrazides and hydrazones—Genotoxic impurities in active pharmaceutical ingredients (APIs) and drug products. *J Pharm Biomed Anal.* 2011;54(5):900–910. doi:10.1016/j.jpba.2010.11.007.
- [14] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). ICH Harmonised Guideline M7(R2): Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk. ICH, Geneva, 2023.
- [15] Elder DP, Snodin DJ, Teasdale A. Control and analysis of hydrazine, hydrazides, and hydrazones—Genotoxic impurities in active pharmaceutical ingredients (APIs) and drug products. *J Pharm Biomed Anal.* 2011;54(5):900–910. <https://doi.org/10.1016/j.jpba.2010.11.007>
- [16] Wang J, Yang S, Zhang K. A simple and sensitive method to analyze genotoxic impurity hydrazine in pharmaceutical materials. *J Pharm Biomed Anal.* 2016;126:141–147. <https://doi.org/10.1016/j.jpba.2016.04.038>
- [17] Matsui F, M.J. Gimeno, Fluorimetric determination of hydrazine in isoniazid formulations with 2-hydroxy-1-naphthaldehyde. *J Pharm Biomed Anal.* 1988;6(6–8):1023–1027. [https://doi.org/10.1016/0731-7085\(88\)80128-6](https://doi.org/10.1016/0731-7085(88)80128-6)
- [18] Pous Miralles G, García-Domenech R, Mañes Vinuesa J, Marí Buigues J. Spectrophotometric determination of dihydralazine in pharmaceuticals after derivatization with 2-hydroxy-1-naphthaldehyde. *J Pharm Biomed Anal.* 1993;11(8):647–650. [https://doi.org/10.1016/0731-7085\(93\)80170-6](https://doi.org/10.1016/0731-7085(93)80170-6)
- [19] Higashi T, Ogawa S. Chemical derivatization for enhancing sensitivity during LC/ESI-MS/MS quantification of steroids in biological samples: a review. *J Steroid Biochem Mol Biol.* 2016;162:57–69. <https://doi.org/10.1016/j.jsbmb.2015.10.003>

- [20] Sharifi M, Donisa C, Joza P. A sensitive and quantitative isotope-dilution LC-MS/MS method for analysis of hydrazine in tobacco smoke. *J Chromatogr Sci.* 2020;58(2):83–90. doi:10.1093/chromsci/bmz069.
- [21] T. Çeğil, A. Yardımcı, N. Sevinç, Z. Pamukçu, Determination of dimethyl sulfate genotoxic impurity in pantoprazole sodium sesquihydrate by derivatization method and UPLC/MS, *Int. J. Mass Spectrom.* 506 (2024) 117330. <https://doi.org/10.1016/j.ijms.2024.117330>.
- [22] T. Çeğil, A. Yardımcı, M.R. Özbaş, Y. Özlü, Development and validation of a trace-level LC–MS/MS method for dasatinib-related nitrosamine impurities with LC-QTOF-MS confirmation, *J. Liq. Chromatogr. Relat. Technol.* (2026) 1–12. <https://doi.org/10.1080/10826076.2026.2651402>.

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