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Research Article

# Forced Degradation Studies of Nilotinib Hydrochloride: Isolation, Identification, and Characterization of Impurities

**JCMKNN Murty Singamsetti[1,2], Raghu Babu Korupolu[2], Himabindhu Gandham[2], Mahesh Kumar Reddy Geereddi[1,2], Muralidharan Kaliyaperumal[1], Chidananda Swamy Rumalla[1*], Venkata Satya Rama Naga Anji Karun Mutha[1], Ramu Ivaturi[1]**

[1]*Department of Medicinal Chemistry, GVK Biosciences Pvt. Ltd., IDA Nacharam, Hyderabad-500076, Telangana, India*
[2]*Department of Engineering Chemistry, Andhra University, Visakhapatnam-530003, Andhra Pradesh, India*

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ABSTRACT

Nilotinib hydrochloride (NLH) is a tyrosine kinase inhibitor approved for treating chronic myelogenous leukemia. It was subjected to forced degradation studies, and the samples were analyzed by utilizing the liquid chromatography with tandem mass spectrometry (LCMS) compatible high performance liquid chromatography (HPLC) methods. NLH was subjected to thermal, hydrolytic, oxidative, acidic, basic, and photolytic degradation conditions as per the regulatory guidelines. The drug was degraded in oxidative, basic, and acidic environments and stable in photolytic and thermal conditions. The main degradation impurity components produced through the forced degradation study were isolated for the identification and quantification in the presence of these impurities in the stability studies of drug substances, as well as, drug products. The identified degradation components were separated by mass-assisted auto-purification techniques and subjected to nuclear magnetic resonance (NMR) characterization [$^{13}$C-NMR, $^{1}$H-NMR, heteronuclear multiple bond correlation spectroscopy (HMBC), and heteronuclear single quantum coherence (HSQC)], high-resolution mass spectrometry (HRMS), and Fourier Transform Infrared Spectroscopy (FTIR) experimentations. Degradation products obtained from oxidative, basic, and acidic environments were isolated and identified by the advanced techniques as acid degradation product (DP-1) with a molecular mass of 306.11 g/mol, empirical formula $C_{17}H_{14}N_4O_2$ with name as 4-methyl-3-(4-(pyridine-3-yl) pyrimidin-2-ylamino) benzoic acid. Base degradation product (DP-2) has molecular weight of 241.08 g/mol, molecular formula $C_{11}H_{10}F_3N_3$ with name as 3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)aniline. Oxidative degradation product (DP-3) has molecular weight of 545.18 g/mol, molecular formula $C_{28}H_{22}F_3N_7O_2$ with name as 3-(2-(2-methyl-5-(3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenylcarbamoyl) phenylamino)pyrimidin-4-yl)pyridine1-oxide.

## Introduction

The NLH is chemically designated as 4-methyl-N-[3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl) phenyl]-3-[(4-pyridin-3-yl pyrimidin-2-yl) amino] benzamidesalt) with an empirical formula and molecular weight of $C_{28}H_{22}F_3N_7O$.HCl and 565.98 g/mol, respectively. It exists as a salt of hydrochloride monohydrate small molecule with the trade name of Tasigna. It is a tyrosine kinase inhibitor (TKI) permitted for the management of chronic myelogenous leukemia (CML),[1-4] also known as chronic granulocytic leukemia (CGL). It is a second-generation TKI[2] and is an analog to imatinib (one of the existing TKI) with 20 times higher affinity for the adenosine tri phosphate (ATP) binding sites *in vitro*. CML is one of several chronic myeloproliferative diseases, a family of clonal disorders of pluripotential hematopoietic stem cells in the marrow.[5-7] It has a regular incidence of 1 in 100,000 in western countries, and it increases steadily with age.

***Corresponding Author:** Chidananda Swamy Rumalla

**Address:** Department of Medicinal Chemistry, GVK Biosciences Pvt. Ltd., IDA Nacharam, Hyderabad-500076, Telangana, India

**Email** ✉**:** chidanand_swamy@yahoo.co.in

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Impurities can significantly impact the results with respect to quality, safety, and efficacy.[8-10] Hence, the control of impurities percentages in the drug product, as well as, drug substance will play an important role in getting quality outcomes. NLH literature review discloses that some of the degradation impurities were reported earlier with a tentative mass spectrum.[11] However, there was no literature available for these impurities confirmation by NMR techniques. The present research work aims to confirm degradation impurities by advanced analytical techniques, like 2D NMR and HRMS experiments.

## Materials and Methods

### Chemicals and Reagents

The NLH was obtained as a gift sample from a reputed manufacturing unit in Hyderabad, India. Acetonitrile, formic acid, and methanol of HPLC-grade and HCl, NaOH, $H_2O_2$, and ammonium acetate of analytical grade were acquired from Merck India Ltd., Mumbai, India. In the research, water used throughout the study was acquired from Milli-Q+ purification equipment, Amsterdam, Netherland.

### Ultra Performance Liquid Chromatography (UPLC)-Mass Spectrometry

The LC separation was achieved on Waters Acquity-UPLC with photodiode array detector (PDA) detector with a stationary phase consisting of Acquity BEH (2.1 × 50 mm, 1.7 µ) C18-column from Waters, India. Mobile phase A: 0.1% formic acid (Aq) and mobile phase B: acetonitrile with a gradient program: time/% of B: 0/10, 0.3/10, 3.5/98, 6/98, 6.5/10, and 7/10 was executed for the analysis. The detection was monitored at the wavelength of the max plot.[12-14] The mobile phase was utilized as a diluent. The mass system utilized for the analysis is Waters-SQ Detector-2 single quadrupole mass spectrometer (MS). The ionization mode was electrospray positive (ESI+). The mass system was optimized by utilizing the full scan method. The source temperature, capillary voltage, desolvation temperature, desolvation gas flow, and cone gas flow were maintained at 140°C, 3.5 kV, 350°C, 650, and 50 L/hr, respectively. The chromatographic system and MS were processed by the Masslynx-4.10 Application Manager. The injection volume and total runtimes were 1 µL and 4 minutes, respectively.

### MS-facilitated Preparative LC

MS-facilitated preparative LC furnished with waters 2545-pump, sample manager 2767 module, Acquity-QDA mass detection 3100 module, and PDA detector-2998 module were utilized. Masslynx-data handling system was utilized to operate the instrument, and Symmetry-C18 (300 × 19 mm, 7 µ) column was utilized to purify degradation impurities. The desolvation gas flow and cone gas flow were monitored at 650 and 50 L/hr, respectively. MS capillary voltage, source temperature, and desolvation temperature were kept at 3 kV, 150, and 350°C, respectively.[12,14,15] Ammonium acetate of 20 mM in acetonitrile and Aq (70:30, v/v) utilized as a makeup solvent. Makeup pump flow was processed at 0.3 mL/min to the MS-detector, and a splitting ratio of 1:1,000 was executed for the suitable ionization.

### High-Resolution Mass Spectrometry (HRMS)

All the sample solutions were processed on Waters Q-Tof micro mass with ion source in ESI mode. Various source parameters were: capillary voltage: 2,800 V; sample cone voltage: 30 V; extraction cone voltage: 5 V; source temperature: 140°C; desolvation temperature: 300°C; cone gas: 50 L/hr; desolvation gas: 650 L/hr. Caffeine (m/z: 194.080383 Da) was utilized as an internal standard for the calibration of mass range and accuracy. Mass-lynx software was utilized for the acquisition of MS data.

### NMR Analysis

The NMR study of NLH, oxidative, base, and acid degradation compounds were processed on an Agilent MR-400-MHz NMR system armed with 5 mm ONE NMR-probe with Z-type gradient shim arrangement having the sensitivity of 480:1 for $^{1}H$ and 225:1 for $^{13}C$ nuclei. It is also furnished with a thousand sample capacity autosampler. Every NMR analysis has been processed at a probe temperature of 298 K with fine automatic tuning and matching for the frequency of respective nuclei. $^{1}H$-NMR spectra were referenced to tetramethylsilane singlet at zero (0) ppm and referenced dimethyl sulfoxide (DMSO)-D6 septet at 39.5 ppm in carbon NMR. Following are the key parameters used for NMR analysis.

### One Dimensional (1D) Study

The $^{1}H$-NMR data was executed with the following constraints, like relaxation delay time (D1): 1-second, spectral width (SW): 17.95 ppm, number of scans (NT): 16, 90° pulse width (PW90): 7.4 µsec, number of data points (NP): 64 k, operating spectrometer frequency (SF): 399.63 MHz, line broadening (LB): 0.5 Hz, and acquisition time (AT): 4 seconds. $^{13}C$-NMR data acquired and processed with the following parameters, like SW: 248.8 ppm, D1: 3 seconds, NT: 4,000, NP: 64 k, PW90: 7.6 µsec, AT: 1.31 seconds, LB: 2 Hz, and SF: 100.48 MHz.

### Two Dimensional (2D) Studies

Homonuclear $^{1}H$-$^{1}H$ gDQCOSY experiment has been performed to know the proton-proton correlations with following parameters SW: 18 ppm in both F1 and F2 projections, D1: 1-second, NT: 16, NP: 1,078 (F2) and 400 (F1), and dummy scans (SS): 32. $^{1}H$-$^{13}C$ gHSQC was done to know the $^{1}J$ correlations between proton-carbon with SW: 18 ppm (F1) and SW1: 240 ppm (F2), D1: 1-second, NT: 16, NP: 1,078 (F2), and 400 (F1), and SS: 32.

The gHMBC has been processed to disclose the exact structure of degradation products with SW: 18 ppm (F1) and SW1: 240 ppm (F2), D1: 1-second, NT: 16, NP: 1,078 (F2) and 400 (F1), SS: 32 parameters.

### For Forced Degradation

As per ICH stability guidelines,[16] different kinds of stress parameters, like acidic, heat, basic, thermal, oxidation, and photolytic conditions were employed. Acid decomposition was processed by taking 200 mg of NLH in a glass bottle, and 5 mL of 1N HCl was added. The mixture was kept at 60°C for 10 hours. Base degradation was processed by taking 200 mg of NLH in a glass bottle, and 5 mL of 1N NaOH was added and kept at 60°C for 10 hours. Oxidative degradation was executed by taking 200 mg of NLH in a glass bottle, and 5 mL of 10% $H_2O_2$ was added, and the mixture was kept at room temperature for 48 hours. Photolytic degradation was executed by taking 200 mg of NLH in the dimethyl sulfoxide (UV) chamber for 48 hours. Thermal degradation was executed by employing 200 mg of NLH at 120°C for 48 hours.[17-23]

### Purification Process

Degradation was observed in oxidative, base, and acid environments. The acid degradation mixture was neutralized with an ammonium bicarbonate solution, and the resulting solution was lyophilized to produce a solid sample and was solubilized in 5 mL of the mobile phase. The basic degradation mixture was neutralized with HCl solution, evaporated to produce free solid, and was solubilized in 5 mL of the mobile phase. Oxidative degradation mixture was basified with sodium thiosulphate, evaporated to get solid, and solubilized in 5 mL of the mobile phase.[11,14]

### Nuclear Magnetic Resonance (NMR) Analysis

A 10 mg sample was solubilized in deuterated DMSO-D6 solvent and processed as per the NMR conditions.

## Results

### Degradation Approach of NLH

No degradation impurities were observed in photolytic and thermal environments, which confirm that NLH was stable to photolytic and thermal degradation. The drug was degraded with 1N HCl at 60°C for 10 hours with a total degradation of 26.17 and 20.36% individual DP-1. The drug also degraded with 1N NaOH at 60°C for 10 hours with a total degradation of 23.71 and 10.8% of DP-1 and 7.96% of DP-2 individually. In oxidative degradation, 16.21% [5% $H_2O_2$ at Retention time (RT) up to 18 hours], an individual DP-3 of 14.46% was observed. Oxidative, base, and acid degradation chromatograms were represented in Fig. 1, and data were shown in Table 1.

### Isolation of NLH Degradation Impurities

The degradation was observed in the oxidative, base, and acidic environments with a suitable percentage formation of more than 5%. Purification was executed by utilizing formic acid (0.1%) (Aq) and acetonitrile (ACN) as a movable phase with Symmetry-C18 (300 × 19 mm; 7 µ) stationary column. Consecutive raw sample solutions were infused and based on the mass parameter ion chromatograms;

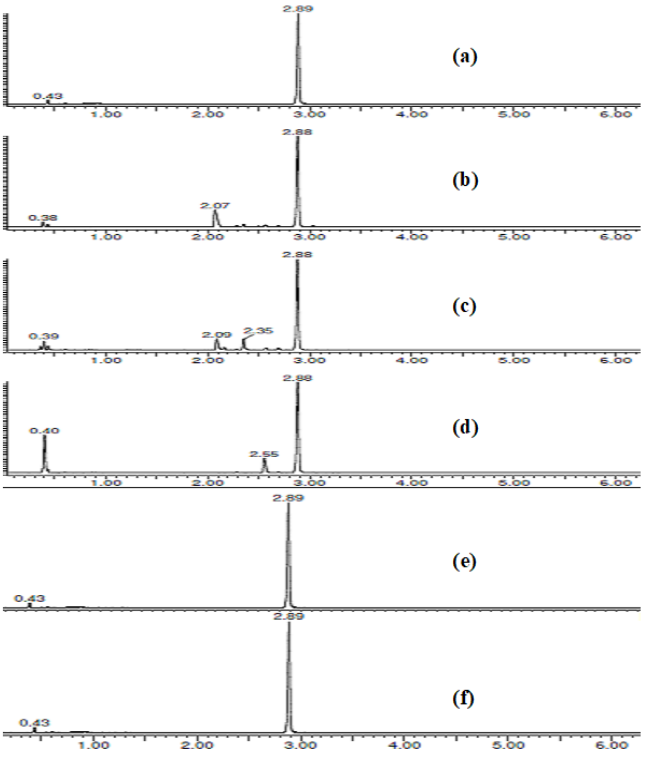


**Fig. 1:** LCMS chromatograms of NLH degradant products: **(a)** API; **(b)** Acid degradation; **(c)** Alkaline degradation; **(d)** Oxidative degradation; **(e)** Photolytic; **(f)** Thermal degradation

**Table 1:** NLH force degradation studies

| *S. No.* | *Conditions* | *DP-1* | *DP-2* | *DP-3* | *API* |
|---|---|---|---|---|---|
| 1 | NLH API | - | - | - | 99.89 |
| 2 | Acid (1N HCl heated at 60°C, 10 hr) | 20.36 | - | - | 73.83 |
| 3 | Base (1N NaOH heated at 60°C, 10 hrs) | 10.8 | 7.96 | - | 76.29 |
| 4 | Oxidation (5% $H_2O_2$ at RT upto 18 hrs) | - | - | 14.46 | 83.79 |
| 5 | Thermal (exposure to 120°C for 48 hrs) | - | - | - | 99.76 |
| 6 | Photolytic (exposed at 285 nm for 48 hrs) | - | - | - | 99.82 |

the fractions were collected. After processing the degradation procedure, the degradation components were isolated in pure form utilizing semi-preparative HPLC to gather all fractions relating to the molecular mass of 307.11 molecular ion plus proton (M + H) and lyophilized to produce free solid. In basic degradation, mass fractions of 307.11 (M + H) and 242.05 (M + H) were collected separately and lyophilized to produce a solid sample, and in oxidative degradation, mass fractions of 546.35 (M + H) were collected together and lyophilized to produce a solid sample.

## Structural Confirmation of Degradation Components

### *Characterization of Acid Degradation Product (DP-1)*

The HRMS showed a protonated molecular ion peak at m/z 307.1194 $[M + H]^+$, corresponding to molecular formula $C_{17}H_{14}N_4O_2$, had nine aromatic protons, one methyl group, one NH, and one carboxylic acid proton at 12.84 ppm in $^1$H-NMR. Proton NMR spectra of DP-1 clearly indicated that it was formed due to hydrolysis of the amide group of the nilotinib drug substance. By $^1$H and $^{13}$C-NMR, proton and carbon chemical shift values of DP-1 were assigned by using COSY, HSQC, and HMBC experiments, as shown in Table 2. $^{13}$C-NMR spectra revealed that DP-1 had 16 aromatic carbons and one aliphatic methyl group. DP-1 matched with structure, as shown in Table 3, and Figs 2 and 3.

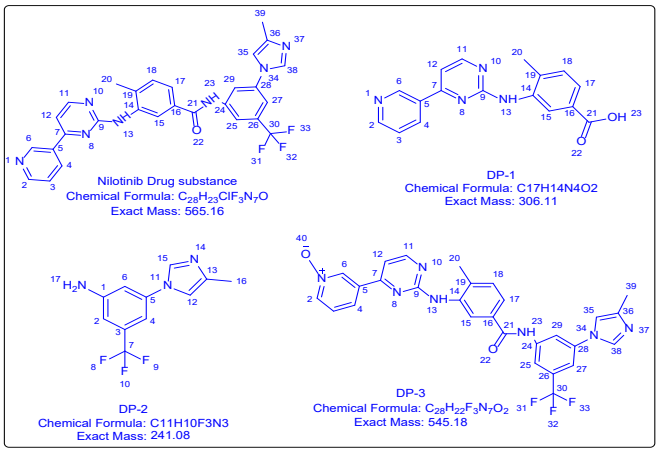


**Fig. 2:** Chemical structures and position numbering of NLH and degradation products

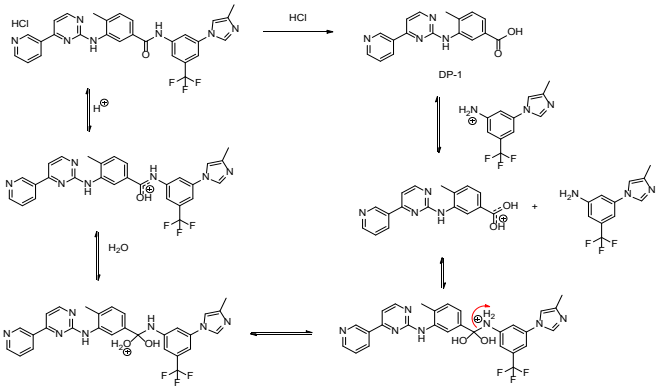


**Fig. 3:** Mechanism of nilotinib drug substance and its degradation product in acidic condition

### *Base Degradation Product Characterization (DP-1 and DP-2)*

Protonated molecular ion peak at m/z 242.0902 $[M + H]^+$ observed in HRMS corresponding to molecular formula $C_{11}H_{10}F_3N_3$. Proton NMR spectra revealed that DP-2 had five aromatic protons, one methyl group, and one $NH_2$ group. $^{13}$C-NMR spectra showed that DP-2 had 10 aromatic carbons and one aliphatic methyl carbon and CF3 carbon observed as quartet at 124 ppm in $^{13}$C-NMR, due to coupling carbon with three fluorine atoms. $^1$H and $^{13}$C chemical shift values were assigned for DP-2, as shown in Table 2. By using 2D NMR experiments (HMBC, HSQC, and COSY), the proposed structure was matched with DP-2, as shown in Figs 2 and 4.

### *Oxidative Degradation Product Characterization*

The HRMS showed a protonated molecular ion peak at m/z 546.1888 $[M + H]^+$ corresponding to molecular formula $C_{28}H_{22}F_3N_7O_2$. It was 16 mass units higher than that of the nilotinib drug substance (without salt). The number of protons (22) in $^1$H-NMR spectra and the number of carbons (28) in $^{13}$C-NMR were the same as the nilotinib drug substance, but pyridine ring proton and carbon chemical shift values varied between DP-3 and the nilotinib drug, as shown in Table 2. It indicates that oxygen atom attacked first position nitrogen during oxidation, as shown in Figs 2 and 5. All proton and carbons chemical shift values of DP-3 were assigned by using 1D and 2D NMR experiments.

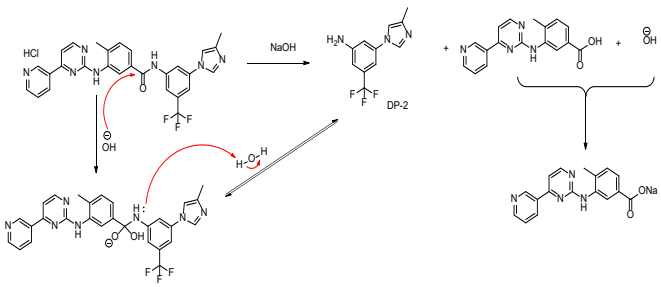


**Fig. 4:** Mechanism of nilotinib drug substance and its degradation product in alkaline condition

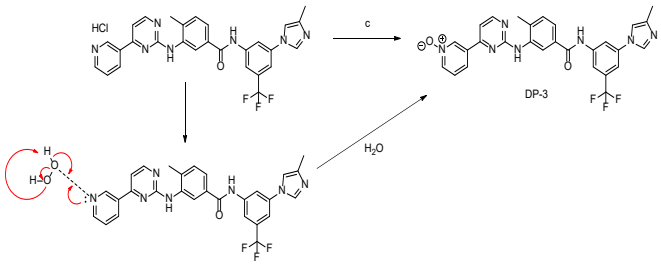


**Fig. 5:** Mechanism of nilotinib drug substance and its degradation product in peroxide condition

**Table 2:** Comparative NMR assignments for NLH and degradation products

| | *Drug* | | *DP-1* | | *DP-2* | | *DP-3* | |
|---|---|---|---|---|---|---|---|---|
| *Assignment* | *$^{1}$H PPM* | *$^{13}$C PPM* | *$^{1}$H PPM* | *$^{13}$C PPM* | *$^{1}$H PPM* | *$^{13}$C PPM* | *$^{1}$H PPM* | *$^{13}$C PPM* |
| 1 | - | - | - | - | - | 150.8 | - | - |
| 2 | 8.72 | 150.8 | 8.71 | 151.5 | 6.83 | 107.7 | 8.32 | 140.3 |
| 3 | 7.58 | 124 | 7.54 | 123.7 | - | 131.1 | 7.53 | 126.7 |
| 4 | 8.52 | 134.9 | 8.46 | 134.2 | 6.98 | 103.1 | 8 | 123.3 |
| 5 | - | 132.4 | - | 132 | - | 138.4 | - | 136 |
| 6 | 9.3 | 147.7 | 9.29 | 148.1 | 6.95 | 107.8 | 8.83 | 136.9 |
| 7 | - | 161.4 | - | 161.5 | - | 124 | - | 159.2 |
| 9 | - | 161 | - | 161.4 | - | - | - | 160.9 |
| 11 | 8.57 | 159.7 | 8.55 | 159.5 | - | - | 8.58 | 160.1 |
| 12 | 7.51 | 108 | 7.49 | 107.8 | 7.37 | 114.1 | 7.48 | 108.2 |
| 13 | 9.2 | | 9.06 | - | - | 138.4 | 9.27 | - |
| 14 | - | 138.2 | - | 137.9 | - | - | - | 137.9 |
| 15 | 8.36 | 124.4 | 8.3 | 125.2 | 8.08 | 134.6 | 8.25 | 124.6 |
| 16 | - | 131.5 | - | 129.2 | 2.16 | 13.5 | - | 131.8 |
| 17 | 7.88 | 123.7 | 7.65 | 124.9 | 5.88 | - | 7.8 | 123.8 |
| 18 | 7.46 | 130.4 | 7.36 | 130.3 | - | - | 7.45 | 130.5 |
| 19 | - | 137 | - | 136.7 | - | - | - | 137.2 |
| 20 | 2.37 | 18.2 | 2.34 | 18.2 | - | - | 2.35 | 18.3 |
| 21 | - | 165.8 | - | 167.5 | - | - | - | 165.6 |
| 23 | 10.9 | - | 12.8 | - | - | - | 10.7 | - |
| 24 | - | 141.8 | - | - | - | - | - | 141.3 |
| 25 | 8.35 | 117 | - | - | - | - | 8.32 | 115.1 |
| 26 | - | 130.8 | - | - | - | - | - | 130.8 |
| 27 | 7.93 | 113.6 | - | - | - | - | 7.72 | 111.5 |
| 28 | - | 136.1 | - | - | - | - | - | 138 |
| 29 | 8.65 | 117.2 | - | - | - | - | 8.16 | 114.1 |
| 30 | - | 123.3 | - | - | - | - | - | 123.7 |
| 35 | 8.01 | 117 | - | - | - | - | 7.49 | 114.2 |
| 36 | - | 131.4 | - | - | - | - | - | 138.9 |
| 38 | 9.6 | 134.3 | - | - | - | - | 8.2 | 135.1 |
| 39 | 2.37 | 9.96 | - | - | - | - | - | 13.5 |

**Table 3:** Comparative FTIR data of NLH and degradation products

| | | *Frequency (cm$^{-1}$)* | | | | |
|---|---|---|---|---|---|---|
| *S. No.* | *API* | *DP-1* | *DP-2* | *DP-3* | *Region (cm$^{-1}$)* | *Assignment* |
| 1 | ~3,256.861 | ~3,280.008 | ~3,415.991, 3,191.281 | ~3,252.039 | 3,100–3,500 | N-H stretching |
| 2 | ~1,669.419 | ~1,685.814 | - | ~1,680.027 | 1,650–1,820 | C:O stretching |
| 3 | ~1,167.918 | - | ~1,112.946 | ~1,095.586 | 1,000–1,400 | C-F bending |
| 4 | ~1,405.166 | ~1,421.562 | ~1,411.917 | 1,414.811 | 1,400–1,600 | C:C stretching |
| 5 | - | - | - | ~1,557.545, 1,322.226 | 1,515–1,560 and 1,325–1,385 | N-O stretching |

## Discussion

The NLH was subjected to thermal, hydrolytic, oxidative, acidic, basic, and photolytic degradation conditions as per the regulatory guidelines. Degradation was not found in thermal and photolytic conditions. The drug was degraded with 1N HCl at 60°C for 10 hours with a total degradation of 26.17 and 20.36% individual DP-1. The drug also degraded with 1N NaOH at 60°C for 10 hours with a total degradation of 23.71 and 10.8% of DP-1 and 7.96% of DP-2 individually. In oxidative degradation, 16.21% (5% $H_2O_2$ at RT up to 18 hours) with an individual DP-3 of 14.46% was observed. Degradation details are shown in Table 1 and Fig. 1. LC separation was achieved on Waters Acquity-UPLC with PDA detector with a stationary phase consisting of Acquity BEH (2.1 × 50 mm, 1.7 µ) C18-column from Waters, India. Mobile phase A: 0.1% formic acid (Aq) and mobile phase B: acetonitrile with a gradient program: time/% of B: 0/10, 0.3/10, 3.5/98, 6/98, 6.5/10, and 7/10 was executed for the analysis.

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In the acid degradation study, the HRMS showed a protonated molecular ion peak at m/z 307.1194 $[M + H]^+$, corresponding to molecular formula $C_{17}H_{14}N_4O_2$ had nine aromatic protons, one methyl group, one NH, and one carboxylic acid proton at 12.84 ppm in $^1$H-NMR. In the alkaline degradation study, the protonated molecular ion peak at m/z 242.0902 $[M + H]^+$, observed in HRMS corresponding to molecular formula $C_{11}H_{10}F_3N_3$. Proton NMR spectra revealed that DP-2 had five aromatic protons, one methyl group, and one $NH_2$ group. In the oxidative degradation study, the HRMS showed a protonated molecular ion peak at m/z 546.1888 $[M + H]^+$, corresponding to molecular formula $C_{28}H_{22}F_3N_7O_2$. It was 16 mass units higher than that of the nilotinib drug substance (without salt). The chemical structures and position numbering of NLH and degradation products are shown in Fig. 2. The mechanism of degradation of NLH drug substance into its degradation product in acidic, basic, and peroxide conditions is shown in Figs 3 to 5.

## Conclusion

The NLH is a comparatively recent drug with great potential to cure heart failure. A few methods were reported on the forced degradation behavior of NLH, but no literature was reported for identifying the degradation of impurities using advanced characterization tools. NLH was found to be stable under stressed thermal and photolytic conditions and labile to oxidative, basic, and acidic stress environments. In the current study, three oxidative, basic, and acidic stress degradation products were isolated, identified, and characterized. DP-1 and DP-2 formed under acid and alkaline conditions, whereas DP-3 was formed under peroxide conditions; it is reported as degradants. Till now, there was no reported confirmation of DP-3. The confirmation of DP-3 is being challenged because of seven nitrogen atoms in NLH for N-oxide formation. The structure of DP-3 was confirmed with the help of advanced analytical techniques, like 2D NMR and HRMS.

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