Isolation and structural characterization of hydrolytic degradation product of Amodiaquine by using LC-QTOF-MS/MS, 1D and 2D NMR studies

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Running title: Isolation and characterization of degradation products by using LC-QTOF-MS/MS, 1D and 2D NMR studies.

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Abstract

Amodiaquine (AMD) is an antimalarial drug used in the treatment of malaria disease. In the present study, the drug was subjected to stress studies as per International Conference on Harmonization guidelines (ICH) Q1A (R2) to understand the drug degradation profile under different stress conditions. It was observed that the drug degraded under base hydrolytic conditions and examined this degradation product. The DP1 was enriched and isolated by preparative HPLC and characterized by ultra-high-performance liquid chromatography coupled with time-of-flight tandem mass spectrometry (UHPLC-QTOF-MS/MS). The DP1 structure was further confirmed by nuclear magnetic resonance (NMR) experiments (1D & 2D NMR). The HPLC method was validated as per the ICH Q2 (R1) guidelines and found that the method is simple, rapid, linear, precise, and accurate. The validated method can be utilized, in the impurities identification of the bulk drug (API) or finished dosage form of amodiaquine.

Key words: Amodiaquine, Degradation product, Isolation and characterization. Structural elucidation by using LC-QTOF-MS/MS and NMR.

Abbreviations: Amodiaquine (AMD), Degradation Product (DP), Liquid chromatography time of flight mass spectrometry (LC-QTOF-MS), Nuclear Magnetic Resonance (NMR).

1. Introduction

Malaria is a dangerous and fatal disease caused by parasites transmitted to human beings through the bites of infected female Anopheles mosquitoes. As per world malaria report by WHO, around 241 million cases and 627000 malaria deaths stated worldwide in 2020. It was noticed that disproportionately a high share (82% of malaria cases and 77% of malaria deaths) of the global malaria burden in African Region. About 80% of all malaria deaths accounted for the children age group of 5 [1].

The first-line drugs (ex: Chloroquine) are less effective in controlling the parasites (Plasmodium falciparum), and the mortality rate increases. Thus, WHO suggests switching conventional monotherapies to a new group of combinational antimalarials, which includes artemisinin-based combination therapies (ACTs). The combination of artesunate (ART) and amodiaquine (AMD) is highly recommendable drug therapy for malaria. The medicine is included in the essential medicines list [2-3].

Amodiaquine (AMD) is chemically 4-[(7-chloroquinolin-4-yl) amino]-2-(diethylaminomethyl) phenol and belongs to 4-aminoquinoline analogues [4-5] and the chemical structure shown in Fig.1. Identification of impurities or degradation products (DPs) of drug play a major role to estimate the quality and safety of a drug substance and drug product [6-7]. The impurities and DP's could generate from the synthetic process of active pharmaceutical ingredient (API) and during storage under different conditions. A stress degradation studies includes hydrolytic (acid and base) oxidative, thermal, and photolytic stress of API's are a common approach to simulate impurities which may appear during shelf time. A lot of literature available on the identification and structural

characterization of potential drug impurities or degradation products of API and finished dosage forms by modern sophisticated analytical instruments [8-13].

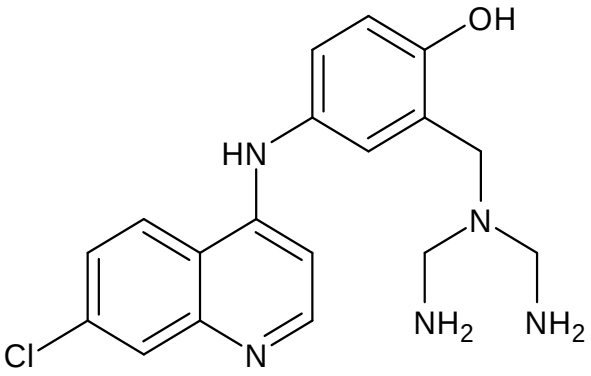


Fig.1. Amodiaquine chemical structure

Many articles reported in the literature for the determination of AMD in combination with artesunate [14-18], and little information is available for the identification of AMD in plasma [19], metabolites of AMD [20], an assay of AMD, and desethylamodiaquine in whole blood spot [21], LCMS determination of AMD in combination with other drugs in plasma [21-22]. However, structural characterization of AMD base hydrolytic degradation product was not reported. Thus, the present studies were executed for the enrichment of degradation product followed by its structural characterization by LC-MS & NMR.

2. Experimental

2.1. Chemicals and Reagents

Amodiaquine (AMD) pure sample was obtained from United States Pharmacopeial Convention-India (P) Ltd., Hyderabad, India. The HPLC grade solvents includes acetonitrile (ACN) and methanol (MeOH) and Analytical reagent (AR) grade reagents such as ammonium formate buffer, 30% v/v hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), sodium hydroxide (NaOH) and

deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Merck (Merck, Mumbai, India). The HPLC grade purified water was obtained from sartorius milli Q water system (Arium pro VF) was used to prepare all solutions.

2.2. Instrumentation

The liquid chromatographic separation of AMD and its DP1 carried out by using Waters H-Class UPLC system (Waters Corp., Milford, MA, USA), equipped with quaternary pump, degasser, photo diode array detector, an auto sampler and a column compartment with temperature regulation facility controlled by Empower 3 software.

The preparative HPLC (Waters Corp.) designed with two pumps (A and B) containing degasser, with auto injector, PDA detector and fraction collector configured with Empower 3 software (Waters Corp.).

LC-QTOF-MS analysis was carried out on SYNAPT G2 Q-TOF Mass spectrometer (Waters Corporation). The LC part comprised of ACQUITY-UHPLC having quaternary gradient pump, auto sampler, column compartment, UV-PDA detector for LC detection. The mass detection part comprised with a quadrupole time-of-flight mass analyzer (Q-TOF) which is having an electrospray ionization (ESI) source. The data was acquired and processed by using Masslynx 4.1 software.

The NMR experiments (^{1}H,^{13}C, DEPT-135, COSY, HSQC, and HMBC) for DP1 was carried out by Bruker 500 MHz Avance-III HD NMR (Bruker, Billerica, Massachusetts, United States) using DMSO-d_6 solvent. The chemical shift values of ^{1}H and ^{13}C experiments were reported on δ scale in ppm. All the NMR spectral data were acquired and processed using Topspin software 3.5 version.

2.3. Instrumental methods

2.3.1. Reverse Phase liquid chromatography

The chromatographic separation of degradation product from the main peak AMD was achieved on Acquity UPLC BEH C8, 100 mm x 2.1 mm; 1.7 µm particle size column. A 10 mM ammonium formate buffer and 100 % acetonitrile were used as mobile phase A and mobile phase B, respectively. A timed program of $T_{(min)}$ / % solution B: 0/20, 0.5/20, 4/70, 9/70, 9.1/20, 15/20 was followed for linear gradient. The mobile phase flow rate was optimized at 0.25 mL/min and the detection of AMD and its DP were carried out at 254 nm on a photodiode array (PDA) detector. The chromatographic data was monitored and processed using Empower 3.

2.3.2. Preparative High-performance liquid chromatography

The enriched degradation product was isolated on Cosmosil C8, (250 × 20) mm, 5µm particle size column using 10 mM ammonium formate buffer (mobile phase A) and 100 % acetonitrile (mobile phase B). The gradient program was optimized as follows; $T_{(min)}$ / % solution B: 0/10, 3/10, 15/80, 20/80, 21/10, 25/10 with a mobile phase flow rate at 15 mL/min. The injection volume and detection were carried out at 1 mL & 254 nm wavelength, respectively.

2.3.3 Liquid chromatography-Mass Spectrometry (LC-QTOF-MS and MS/MS)

The aforementioned reverse phase liquid chromatography method conditions were used for LC–QTOF-MS experiments. MS analysis was performed on Electrospray Ionization positive ion mode and the mass parameters were optimized as follows; capillary voltage 2.5 kV, source temperature 100°C, desolvation temperature 200°C, sampling cone 30V, extraction cone 2.0V, and desolvation gas flow 700 L/hour. The MS/MS fragmentation of degradation product was carried at 35 eV. The data was acquired using Masslynx 4.1 software.

2.3.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

The NMR spectra of degradation product were recorded using 500 MHz spectrometer (Bruker, Billerica, MA, USA). All the experimental studies (1D & 2D NMR) were undertaken in DMSO-d_6 solvent for DP1. The ^{1}H NMR chemical shifts values were referenced to DMSO-d6 ($\delta = 2.50$ ppm), whereas ^{13}C NMR to DMSO-d_6 solvent ($\delta = 39.50$ ppm) for DP1. Multiplicities of NMR signals are designated as d (doublet), bp (broad peak).The proton chemical shift assignments were carried out with the help of two-dimensional (2D) correlation spectroscopy (COSY), hetero nuclear single quantum correlation spectroscopy (HSQC), hetero nuclear multiple bond correlation spectroscopy (HMBC). The later provided the information on the proximity of protons. All the experiments were carried out in the phase sensitive mode.

2.4. Stress degradation studies of AMD

The AMD was subjected to acid and base hydrolytic degradation, oxidative, photolytic, thermal, and humidity stress conditions as per ICH guidelines ICH Q1A (R2) [23]. Initially, the AMD stock solutions (1.5 mg/mL) were prepared by dissolving in a small amount of diluent (mixture of MeOH: H_2O-50:50 % v/v) and diluted with acid (0.1 N HCl), base (0.1 N NaOH), 3% hydrogen peroxide (H_2O_2). Around 50 mg solid compound of AMD was exposed to UV light at 200 Whr/m^2 and fluorescent light at 1.2 million lux hours. Thermal degradation exposed at 105 °C and Humidity stress studies were carried out at 85 % RH & 85° C for 3 days.

2.5. Enrichment of degradation product

About 500 mg of AMD was dissolved in about 5 mL of methanol and made up to the volume with 0.1 N NaOH and kept for 24 hours at room temperature. Subsequently, the solution was neutralized using 0.1 N HCl and part of the solution was injected into UHPLC analysis (Section 2.3.1). It was

found about 75% degradation observed. the total volume of reaction mass was reduced to about 20 mL by using a rotary evaporator. Each 1 mL of this concentrated solution was injected into preparative-HPLC to isolate the degradation product. The elution portion containing degradation product was collected from several injections and pooled the same. This collected portions were subjected to evaporate the solvent by rotary evaporator until solid compound obtained. The final solid compound used for NMR spectral analysis.

2.6. Sample solutions preparations

Preparation of sample solution

Sample stock solution was prepared by 15 mg of accurately weighed AMD material transferring into a 50 mL volumetric flask, added 20 mL diluent to dissolve and made up the volume with diluent to obtain concentration of 300 µg/mL.

Preparation of AMD and Degradation product (DP1) stock solution

Prepared a stock solution of AMD and its degradation product (DP1) at 300 µg/mL individually by dissolving an appropriate amount in diluent. further diluted 2 mL of this solution in to 20 times to achieve the concentration of 0.03 mg/mL and considered this stock solution for validation studies.

Preparation of organic impurities (OI) standard preparation

OI standard solution consists of AMD and its degradation product (DP1) at 0.3 µg/mL (0.1% level) concentration was prepared by diluting 0.5 mL of the above stock solution to 50 mL with diluent for organic impurities determination.

Preparation of Organic impurities spiked sample solution

Accurately weighed and transferred 15 mg of AMD into a 50 mL volumetric flask, added 20 mL diluent to dissolve, added 0.5 mL of the aforesaid stock solution, mixed well and made up the volume with the diluent to obtain concentration of 300 µg/mL of AMD and 0.3 µg/mL of DP1.

2.7. Chromatographic method validation

The developed UHPLC–PDA method for the determination of impurities in AMD was validated in compliance with ICH guidelines ICH Q2 (R1) [24]. Specificity, linearity and range were determined. Accuracy and precision were confirmed.

System suitability

The system suitability requirements were scrutinized by injecting the organic impurity standard solution consisting of 0.3 µg/mL of AMD and (DP1) with six replicate injections. Established the acceptance criteria in terms of peak area % RSD not more than 5.0%, and the resolution is not less than 3.0 between DP1 and AMD. The system suitability results were presented in Table 4.

Specificity

Specificity studies were conducted to prove the method's capability to resolve the principal compound from all the other interferences and evaluated the peak purity analysis for degradation products and main compounds to confirm the method's specificity.

Specificity studies were conducted to prove the method capability to resolve the main compound from all the other interferences. Evaluated the peak purity analysis for degradation products and main compound to prove the method specificity.

Linearity

Linearity solutions ranging from 50 to 150% with respect to analyte concentration (300 µg/mL) for impurities at five levels were prepared. The peak area versus concentration data were subjected to least-squares linear regression analysis. Established the calibration curve by plotting area against the concentration expressed in µg/mL and recorded the correlation coefficient value to show the linearity of the developed method.

Accuracy

The accuracy of the related substance method was calculated at three different levels namely 50%,100% and 150 % of analyte concentration (300 µg/mL). Followed six preparations at 100% level and three for remaining levels to evaluate the accuracy of the method.

Limit of detection and limit of quantification

The sensitivity of the developed method was evaluated by injecting the decreased concentrations of AMD and its impurity. The Limit of detection (LOD) was estimated with signal-to-noise ratio (S/N) values of 3:1 and for Limit of quantification (LOQ) 10:1 respectively to determine the sensitivity of the developed method.

Robustness

Robustness of the analytical method was validated by changing the flow rate ±20% of actual value (0.25 mL/min), column oven temperature + 5 °C (25 °C) from its original temperature. Measured the resolution impact between the main compound AMD and DP1.

3. Results and Discussion

3.1. Degradation behavior of AMD

An optimized UHPLC-PDA method was employed for the evaluation of the degradation behavior of AMD under various stress conditions. It was observed that AMD was degraded only under base hydrolysis (Table 1) and formed degradation product. The separation of degradation product from main peak was achieved on Acquity UPLC BEH C8, 100 mm x 2.1 mm; 1.7 µm particle size column (Refer section 2.3.1). The HPLC overlaid chromatogram of base degradation condition is depicted in Fig. 2.

Table 1

Summary of forced degradation behavior of AMD

Degradation study	Exposure conditions	Degradation products formed
Acid hydrolysis	0.1 N HCl at room temperature for 3 days	No degradation
Base hydrolysis	0.1 N NaOH at room temperature for 24 hours	DP1
Oxidative degradation	3% H_2O_2, at room temperature for 3 days	No degradation
Thermal degradation	Thermal at 105° C for t 48 hours	No degradation
UV light	200 $Whrm^{-2}$	No degradation
Fluorescent light	1.2 million lux hours	No degradation
Humidity degradation	Humidity at 85% RH&85 °C for 3 days	No degradation

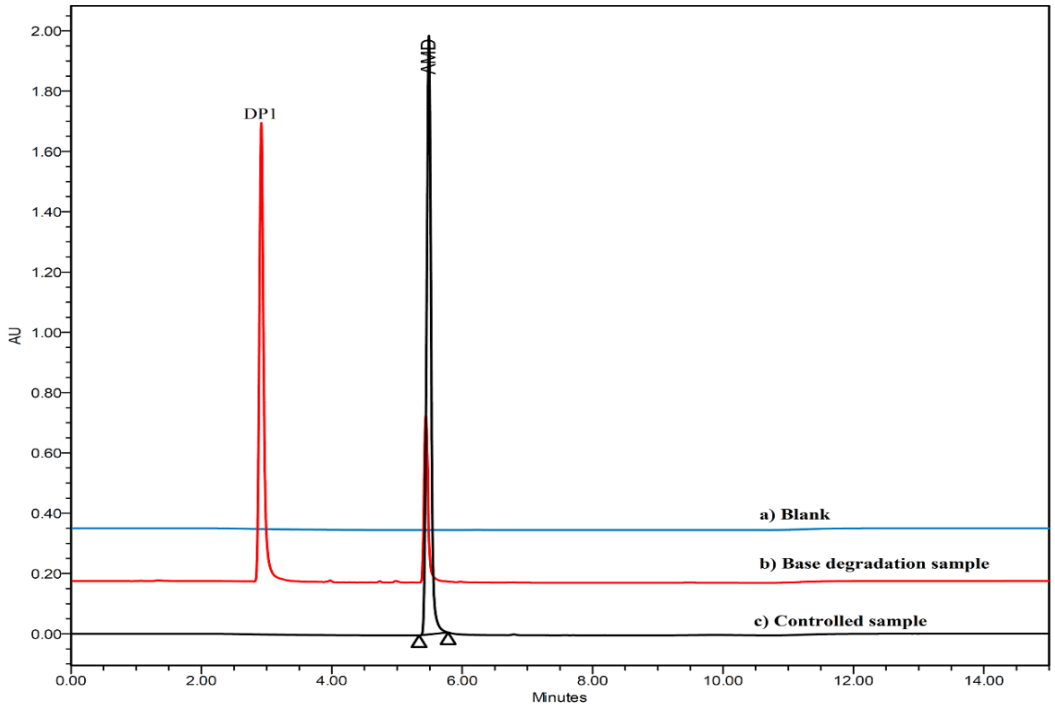


Fig. 2. Overlaid UHPLC/PDA chromatogram of a) Blank, b) Base degradation, and c) Controlled sample

3.2. Characterization studies of degradation product

3.2.1. Mass structural characterization for DP [M+H] $^{+}$ m/z 179

The protonated molecular ion of degradation product was found to be *m/z* 179 (Fig. 3) with an elemental composition of $C_9H_8ClN_2^+$, which corresponds to an elimination of diethylamino methyl phenol moiety from Amodiaquine. As shown in the Fig.3, the MS/MS spectrum of degradation product displayed characteristic product ions at *m/z* 162 (loss of NH_3), *m/z* 144 (loss of chlorine radical), and *m/z* 117 (loss of CHN from *m/z* 144) (Scheme 1).

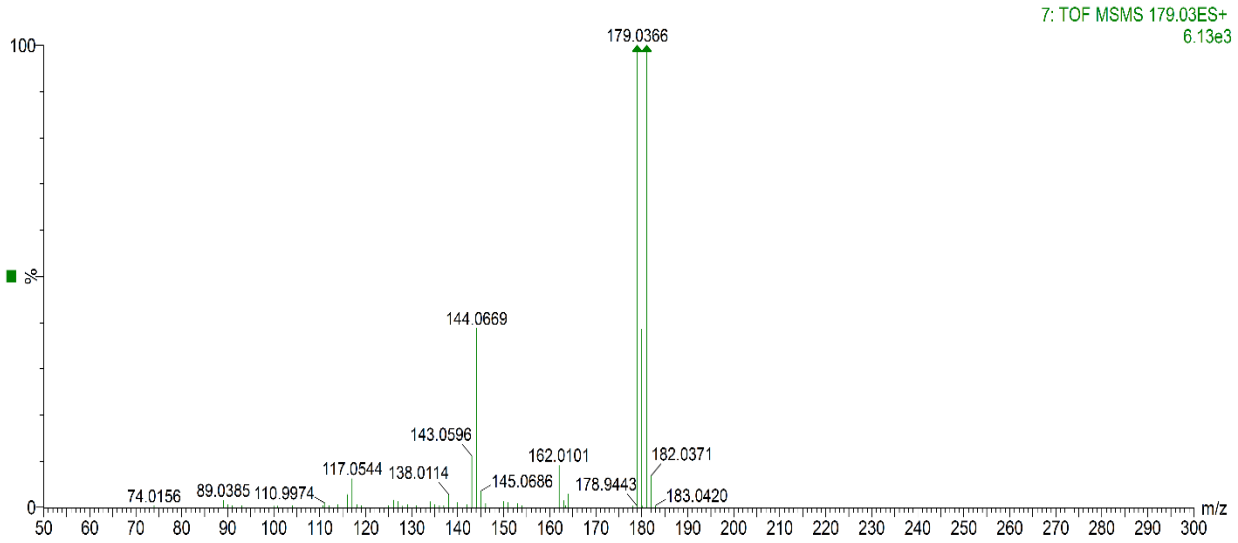
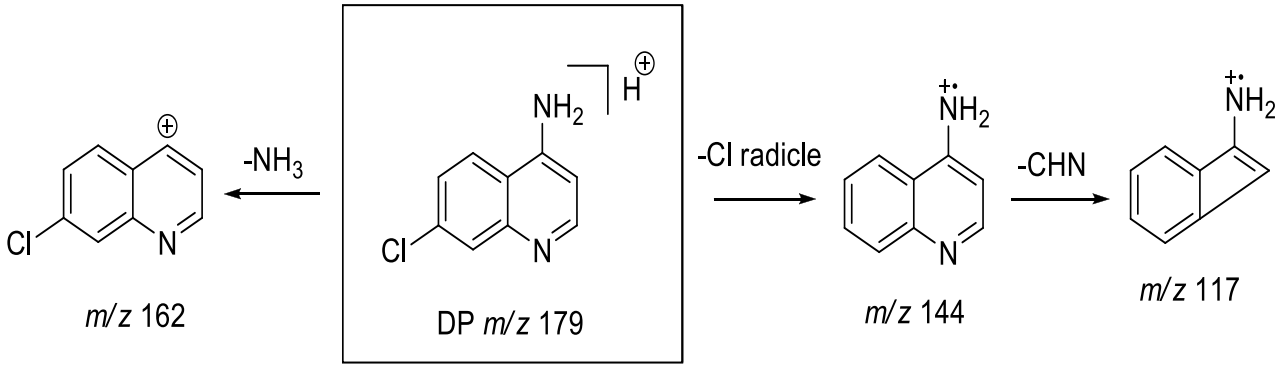


Fig. 3. LC-QTOF-MS/MS spectrum of $[M+H]^+$ ions of DP1.



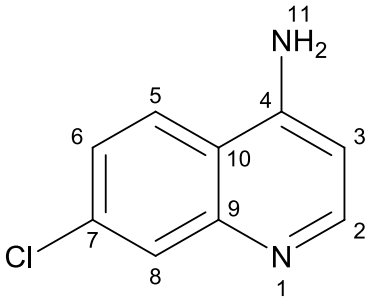
Scheme 1. Proposed fragmentation pathway of protonated $[M+H]^+$ ion of degradation product

3.2.2. NMR structural characterization

All the NMR experiments such as ^1H, ^{13}C, DEPT-135, COSY, HSQC, and HMBC were performed for degradation product in DMSO-d_6 solvent and obtained results were compiled in Table 2.

Table 2

^{1}H and ^{13}C assignments of degradation product



Position	Proton δ_H (ppm); Multiplicity Coupling constant J_{HH} (Hz)	COSY	Carbon δ_C (ppm)	DEPT-90 & DEPT-135	HSQC
1	-	-	-	-	-
2	8.31 (d; J=5.2)	H-3	151.53	CH	CH
3	6.55 (d; J=5.2)	H-2	102.69	CH	CH
4	-	-	149.43	C	-
5	8.19 (d; J=9.0)	H-6	124.64	CH	CH
6	7.41-7.39 (dd; J=9.0, 2.2)	H-5	123.76	CH	CH
7	-	-	151.64	C	-
8	7.75 (d; J=2.2)	-	127.32	CH	CH
9	-	-	133.46	C	-
10	-	-	117.08	C	-
11(NH_2)	6.92 (bp)	-	-	-	-
d = doublet, and bp= broad peak					

The ^{1}H NMR spectrum (Fig.S1 & S2) shown following signals; 1) Two doublets at δ 8.31 ppm and δ 6.55 ppm (J= 5.2 Hz) were observed for aromatic methane protons of positions H-2 & H-3; 2) a doublet at δ 8.19 ppm and doublet of doublet (dd) at δ 7.41-7.39 ppm indicate protons of position

H-5 & H-6; 3) a sharp singlet at δ 7.75 ppm designate the proton of position H-8; 4) a broad singlet at δ 6.92 ppm was noticed for labile protons (-NH_2). It was further confirmed by D_2O exchange studies. As shown in the Fig.S3, the broad singlet at δ 6.92 ppm was disappeared when 1H spectra recorded in DMSO-d_6 + D_2O solvent due to these labile protons were exchanged with D_2O.

The ^{13}C spectrum (Fig. S4) displayed a total nine peaks corresponding to nine carbons. These carbons were further distinguished as 5−CH (tertiary) and 4-C (quaternary) carbons based on DEPT-135 experimental data (Fig. S5). The ^{15}N-HSQC spectrum (Fig.S6) displayed a signal at δ 72.0 ppm and ^{15}N-HMBC spectrum (Fig.S7) displayed three signals at δ 72.0 ppm and δ 270.0 ppm corresponding to Nitrogen at positions N-11 & N-1, respectively.

The 2D-NMR experiments were also performed to confirm the ^{1}H and ^{13}C assignments. The COSY spectrum (Fig.S8) shown correlation (Fig.4) between proton H-2 and H-3 indicated that both protons are adjacent to each other. Similarly, another correlation was observed between H-5 and H-6 which is confirmed that H-5 & H-6 are neighboring protons.

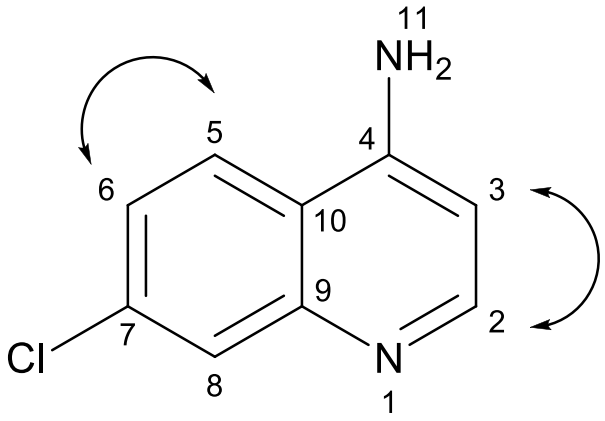


Fig. 4. Characteristic COSY correlations

The HSQC spectrum (Fig.S9) between displayed a total of five correlations between H2-C2, H3-C3, H5-C5, H6-C6, and H8-C8. Additionally, the HMBC spectrum (Fig.S10) of degradation

product was used to validate the correlations (Fig.5) between the protons and carbons in the structural framework, which are ≥ 2 bonds apart. Notable correlations observed in the HMBC spectra between H2-C4, H3, H6 & H8-C10, H5 & H8-C7 and H2 & H5-C9.

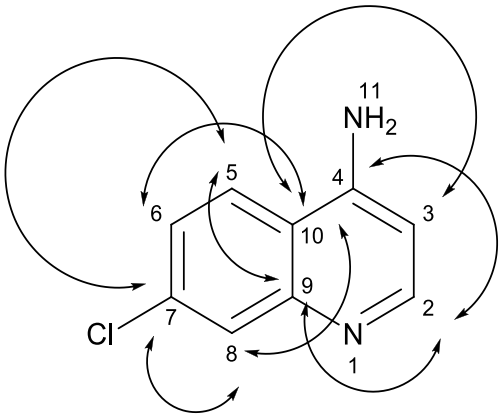


Fig .5. Characteristic HMBC correlations

Based on the observations from 1D NMR (^{1}H, ^{13}C, DEPT-90, DEPT-135) and 2D NMR (COSY, ROESY, HSQC, and HMBC) experiments, the structure of degradation product was identified as 7-chloroquinolin-4-amine.

4. Method Validation

The developed method was validated as per ICH Q2 (R1) guideline [24] in order to use it in QC laboratories for monitoring the quality of AMD in bulk and finished pharmaceutical products. The selectivity of the method was confirmed by measuring the resolution between AMD and the DP1. Peak purity of stressed samples was determined by using a photodiode array (PDA) and mass detector. Purity angle is less than purity threshold for all peaks which shows specificity of the developed method. For checking linearity, concentration range of 0.15 to 0.45 µg/mL of DP1 and AMD was analyzed and showed the correlation coefficient value (r^2) value of 0.9997 and 0.9998 respectively (refer Table 3), hence the developed method possessed good linearity and range.

Accuracy of the method was demonstrated by % recovery of DP1 at three different concentrations from 50 to 150% with six preparations at 100% level and three preparations for lower and higher levels (Table 4). Intra- and inter-day precisions of method was on 0.15, 0.30 and 0.45 µg/mL concentration range at same day (n= 6) and consecutive days (n= 6). Table 4.

Table 3

System suitability, linearity, LOD and LOQ data of AMD and degradation product (DP1)

Compound	% RSD for Peak area	LOQ Conc. (µg/mL)	LOD Conc. (µg/mL)	Linearity & Range (µg/mL)	Correlation (r^2 value)
DP1	0.16	0.07	0.02	0.15-0.45	0.9997
AMD	0.70	0.09	0.03	0.15-0.45	0.9998

LOQ: Limit of Quantitation LOD: Limit of Detection

Table 4

Accuracy and precision data for DP1

Amount spiked at different levels*	Concentration (µg/mL)	% Recovery# (Precision)	% Recovery# (Intermediate Precision)
at 50% level	0.15	98.88	97.44
at 100% level	0.30	99.97	98.52
at 150% level	0.45	99.73	99.27
* Amount of impurity spiked with respect to nominal concentration of 0.3 mg/mL of AMD			
# Mean % recovery for six determinations at 100 % and three determinations for other levels			

5. Conclusion

In the present study, a stress degradation of amodiaquine (AMD) was conducted as per ICH guidelines and identified one degradation product (DP1) under base hydrolytic degradation condition. Isolation and characterization of degradation product was discussed. This DP1 was

identified with mass spectrometry by using UHPLC-QTOF-MS/MS in combination with accurate mass measurements of product ions and precursor ions. The proposed structure was justified by mechanism of the formation under specified conditions. HRMS and multidimensional NMR analytical techniques were employed to elucidate the structure of degradation product. The UHPLC-PDA method was validated as per ICH guidelines and can be used for routine analysis and stability studies in QC laboratories.

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Disclosure statement/Conflicts of Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data.

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Supplementary data

Captions to Figures of Supplementary Information

Figures

1) Fig.S1 A typical ^{1}H NMR spectra of degradation product in DMSO-d_6 solvent
2) Fig.S2 Zoomed ^{1}H NMR spectra of degradation product in DMSO-d_6 solvent
3) Fig.S3 A typical ^{1}H NMR spectra of degradation product in DMSO-d_6 + D2O solvent
4) Fig.S4 A typical ^{13}C NMR spectra of degradation product
5) Fig.S5 A typical DEPT-135 NMR spectra of degradation product
6) Fig.S6 A typical ^{15}N-HSQC spectrum of degradation product
7) Fig.S7 A typical ^{15}N-HMBC spectrum of degradation product
8) Fig.S8 A typical COSY spectrum of degradation product
9) Fig.S9 A typical HSQC spectrum of degradation product
10) Fig.S10 A typical HMBC spectrum of degradation product

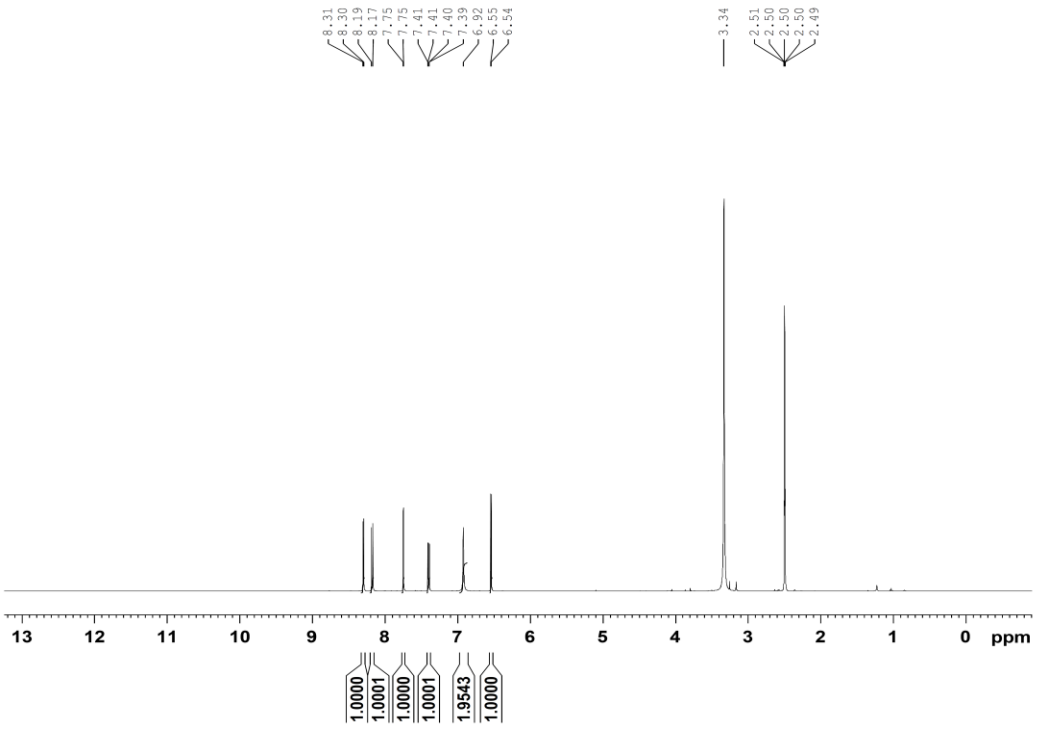

Fig.S1 A typical ^{1}H NMR spectra of degradation product in DMSO-d_6 solvent

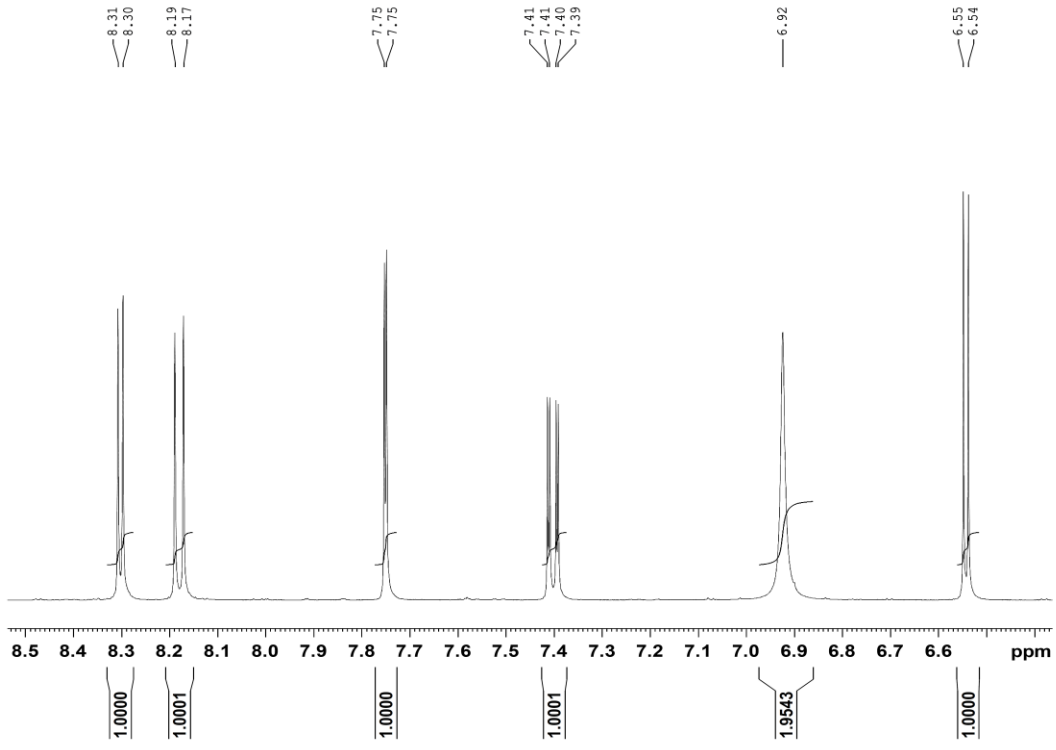

Fig.S2 Zoomed ^{1}H NMR spectra of degradation product in DMSO-d_6 solvent

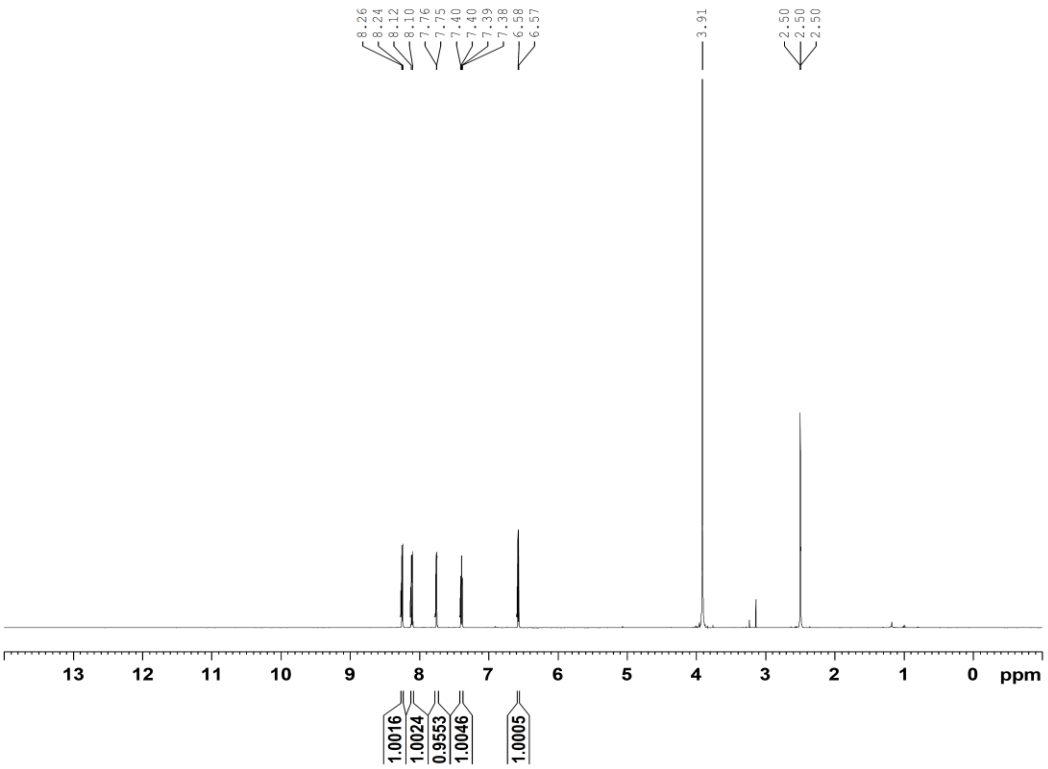

Fig.S3 A typical ^{1}H NMR spectra of degradation product in DMSO-d_6 + D2O solvent

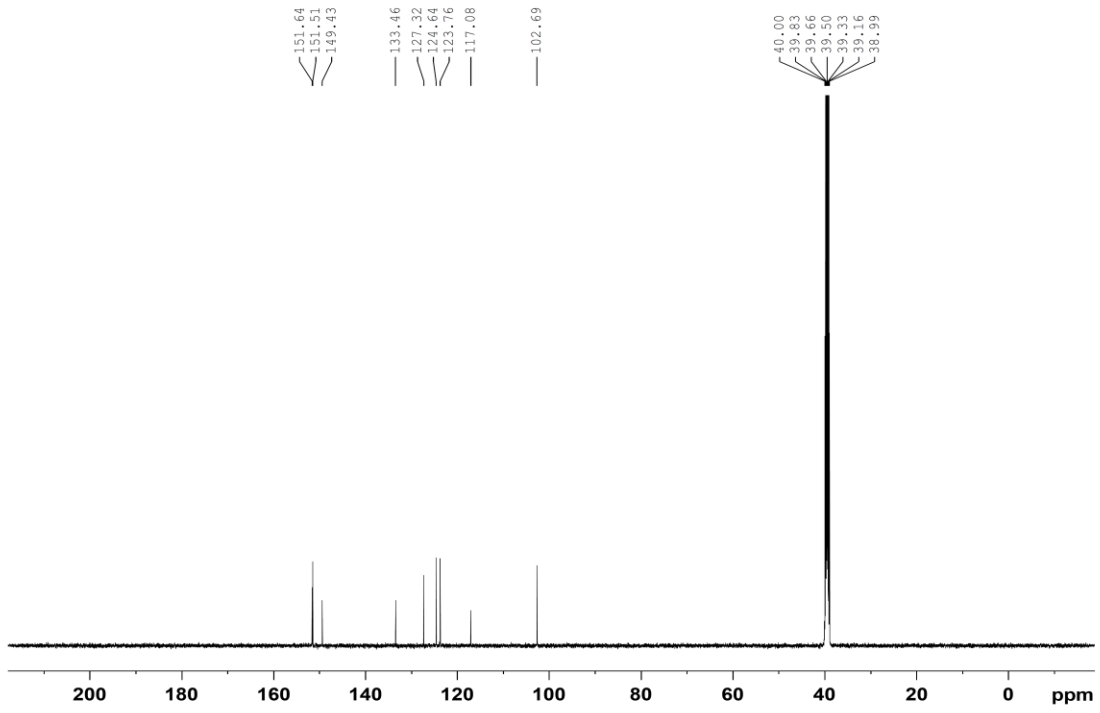

Fig.S4 A typical ^{13}C NMR spectra of degradation product

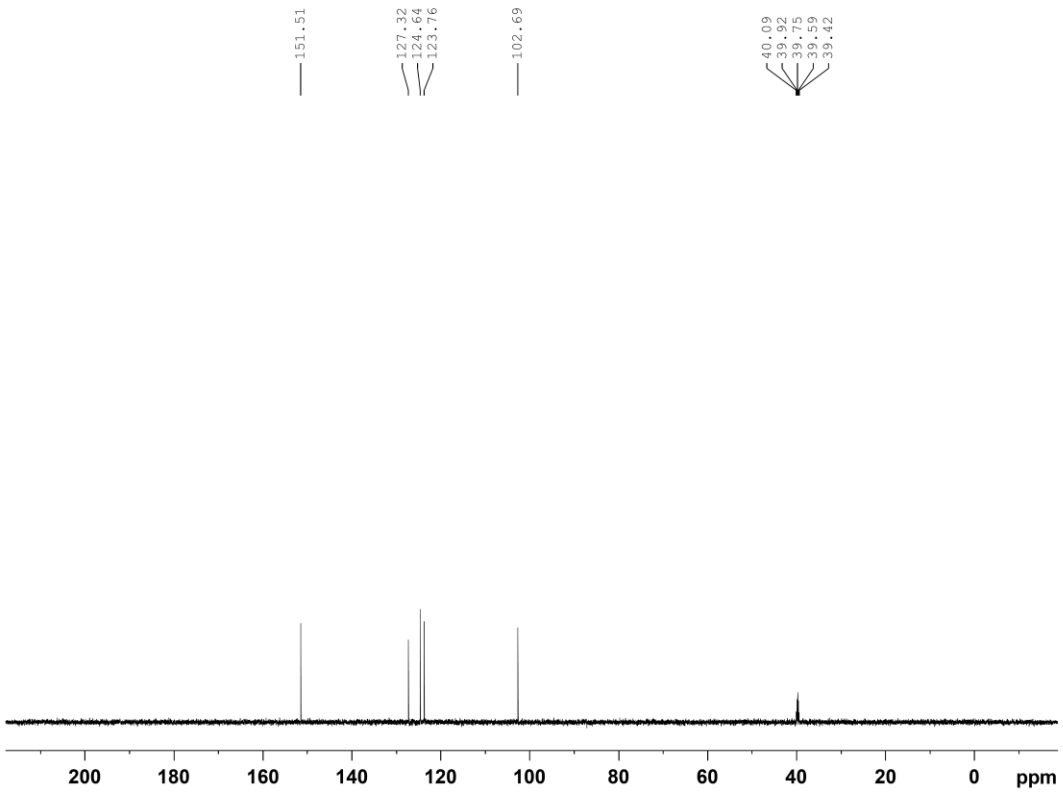

Fig.S5 A typical DEPT-135 NMR spectra of degradation product

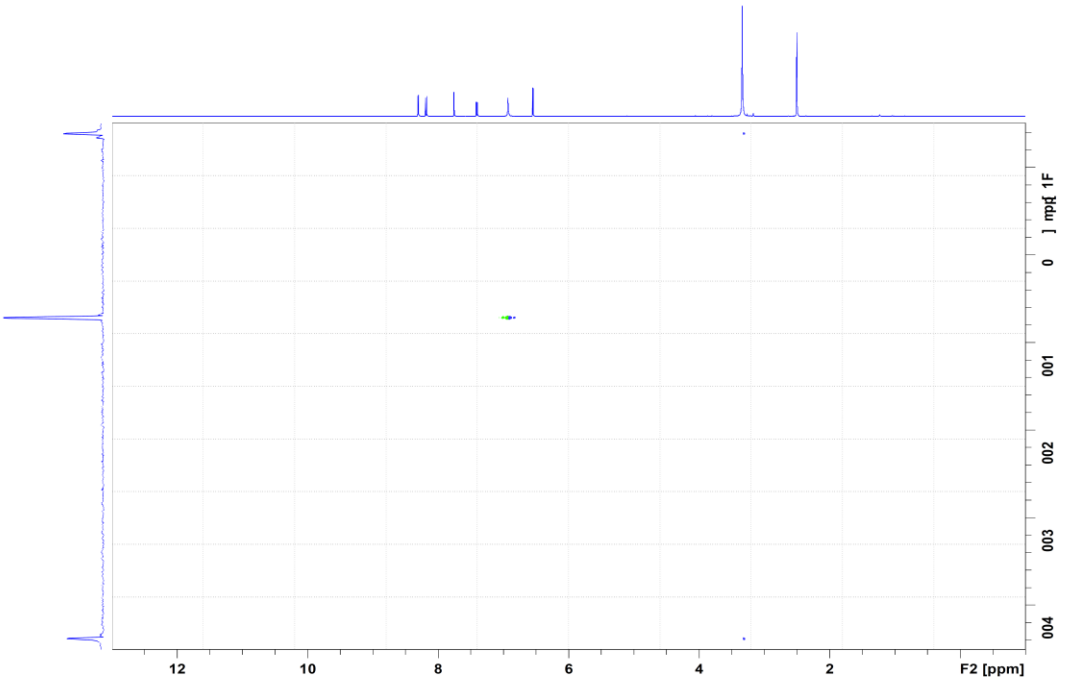

Fig.S6 A typical ^{15}N-HSQC spectrum of degradation product

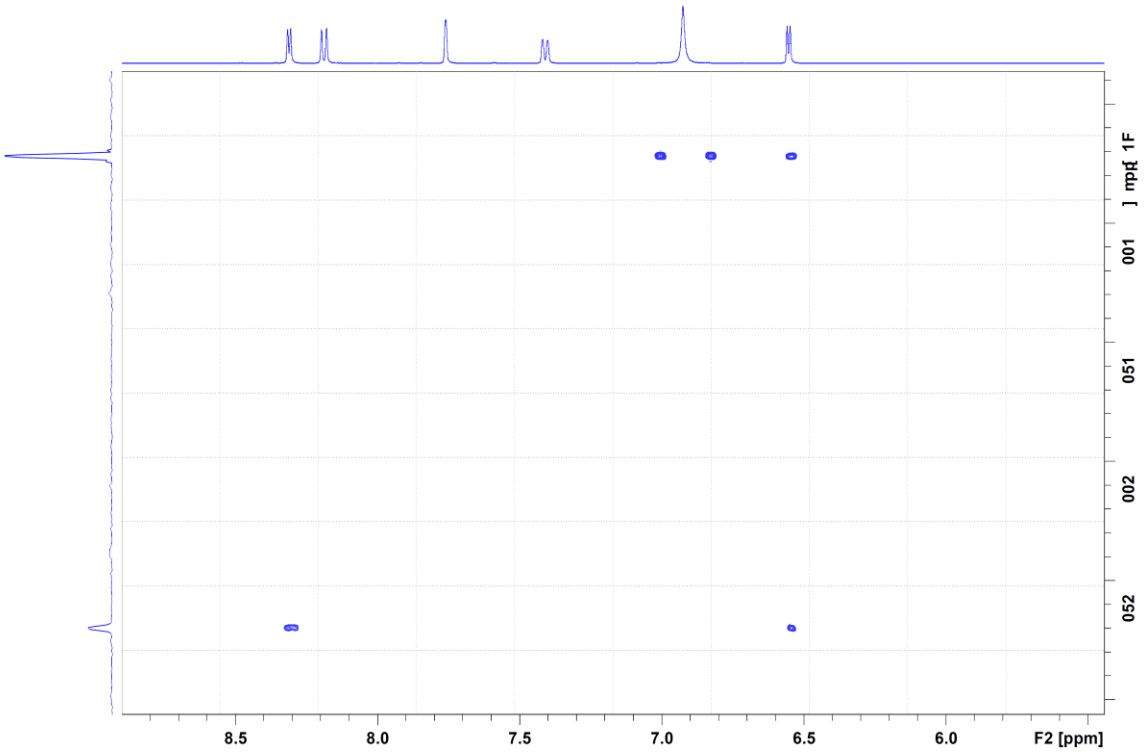

Fig.S7 A typical ^{15}N-HMBC spectrum of degradation product

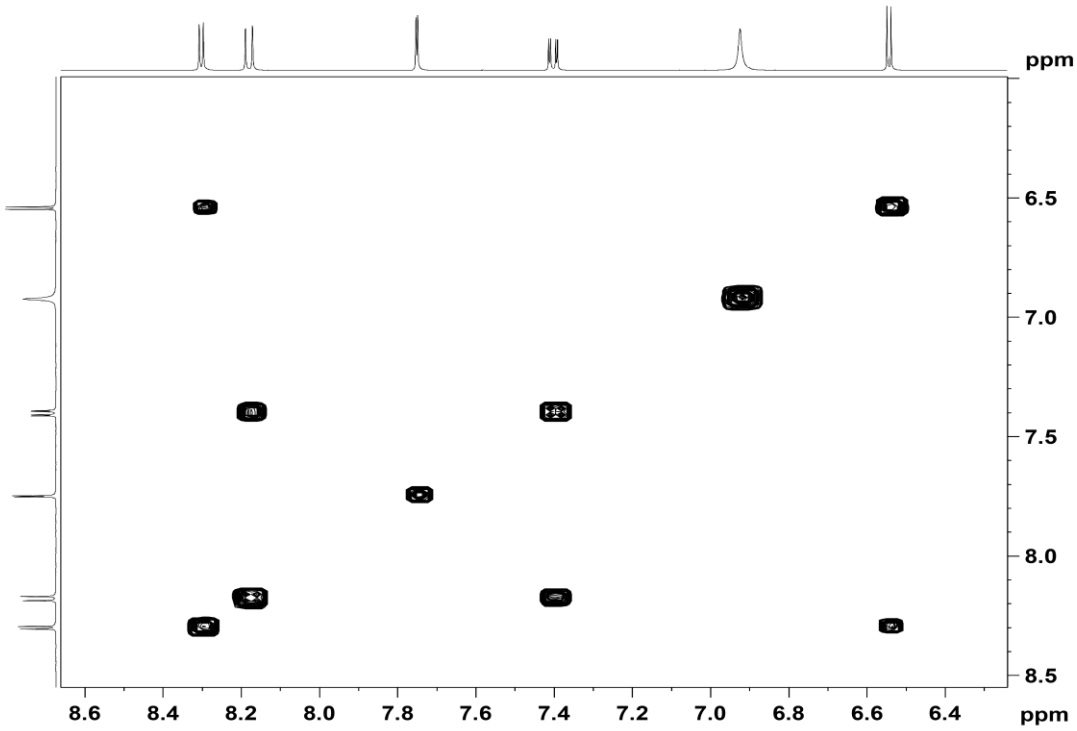

Fig.S8 A typical COSY spectrum of degradation product

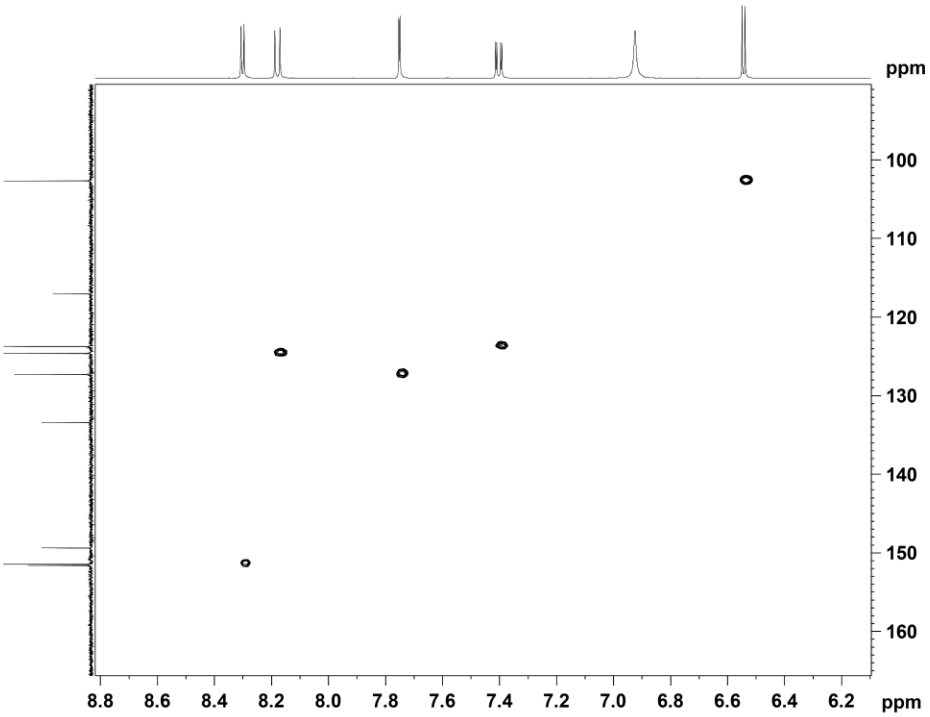

Fig.S9 A typical HSQC spectrum of degradation product

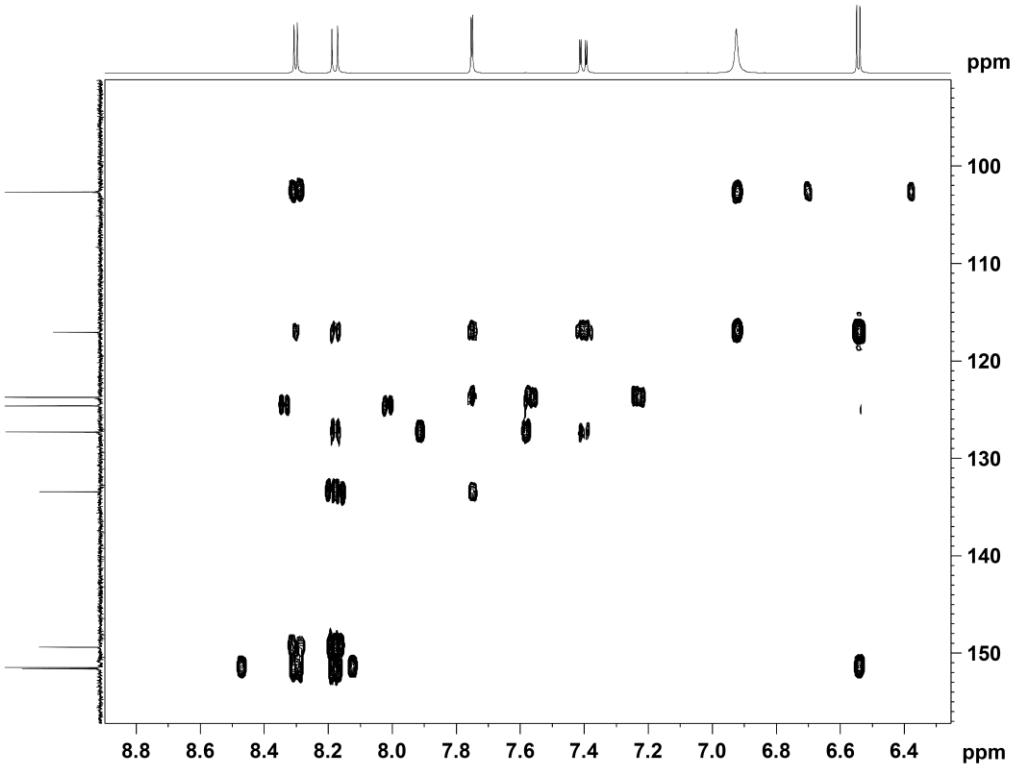

Fig.S10 A typical HMBC spectrum of degradation product