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Original Article

Preparative Separation, Identification and Characterization of N-Acylated Impurity in Ciprofloxacin Eye Drops Formulations Using 1D and 2D NMR Techniques

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ABSTRACT

Background: Packaging, storage and transportation of under varying climatic conditions have the quality concern in the drug products. This study followed the observed unknown impurity of ciprofloxacin eye drop. **Materials and Methods:** The drug product of ciprofloxacin was stored 30ºC±2ºC/65% RH±5% RH for 18 months and was analysed with High-Performance Liquid Chromatography (HPLC). The impurity observed was isolated with preparative HPLC. The isolated impurity was structurally elucidated using 1D and 2D Nuclear Magnetic Resonance (NMR) and High-Resolution Mass Spectrometry (HRMS). **Results:** During stability study of Ciprofloxacin eye drops, the formation of an impurity was observed. Notably, the impurity level reached 0.30% (exceeding the specification limit of 0.20%) in 18 months (30ºC±2ºC/65% RH±5% RH) stability samples of Ciprofloxacin eye drops (3 mg/mL). The Relative Retention Time (RRT) 1.19 impurity was isolated and purified using preparative High-Performance Liquid Chromatography (HPLC) and analyzed using advanced spectroscopic techniques, including LC-HRMS and NMR. Preparative HPLC was employed a Kinetix phenyl-hexyl preparative column (250×20 mm, 5 µm) with a mobile phase consisting of 0.1% Trifluoroacetic Acid (TFA) in water and acetonitrile. Results showed that the impurity was N-acylated derivative of Ciprofloxacin. It was determined that the impurity resulted from a condensation reaction between the drug product and a migrated component, possibly from the ink or varnish used on the label affixed to the Low-Density Polyethylene (LDPE) bottle. The impurity was identified as N-(3-Hydroxypropionyl) Ciprofloxacin. The hypothesis of the formation 3-hydroxypropionic acid via acrylic acid leading to the generation of N-(3-Hydroxypropionyl) Ciprofloxacin (HP-CIP) was confirmed by intentional reaction between them. **Conclusion:** This finding raises concerns regarding the quality and efficacy of other products with similar chemical properties stored in comparable container closure systems.

Keywords: Ciprofloxacin, N-(3-Hydroxypropionyl) Ciprofloxacin, Impurity isolation, High-Resolution Mass Spectrometry (HRMS), Nuclear Magnetic Resonance (NMR).

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INTRODUCTION

Ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylq uinoline-3-carboxylic acid) (Figure 1) is part of the fluoroquinolone antibiotic class and is used to treat a wide range of bacterial infections, including those affecting the bones and joints (Greenberg *et al*., 1987), intra-abdominal region (Sartelli, 2010), skin (Fass, 1986), respiratory tract (Zhanel *et al*., 2002) and urinary tract (Talan *et al*., 2004). It is also effective against infectious diarrhea, typhoid fever, prostatitis, anthrax, and certain sexually transmitted infections (Sodhi and Singh, 2021). With its broad antimicrobial spectrum and efficacy across various indications, it has become an essential treatment for bacterial infections. Its significance as a potential treatment for *Bacillus anthracis* infections has also increased, particularly due to the threat of anthrax-related bioterrorism (Binkley *et al*., 2002; Navas, 2002). In some cases, it is combined with other antibiotics for enhanced efficacy. It can be administered orally, intravenously, or as eye or ear drops (Kanellopoulos *et al*., 1994; Nix, 1989). Ciprofloxacin is designed and reported to have the high affinity for the bacterial enzyme topoisomerases II (also known as gyrase) and IV while not affecting any physiology in the body at the effective concentration. These enzymes are involved

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in the splicing and resealing the dividing DNA. Such an activity will be unleashing the mechanical stresses which was generated during the opening of two DNA strands in RNA synthesis and DNA replication (Ojkic *et al.*, 2020). Ciprofloxacin eye drops and ointments are used to treat bacterial conjunctivitis, corneal ulcers, and other eye infections (Youssef *et al.*, 2021). Impurities formed during the synthesis of Ciprofloxacin include impurity A, B, and D, while degradation impurities such as impurity C, E, and F have also been identified. Impurity C is a potential photolysis product, impurity E likely results from the drug's decarboxylation, and impurity F results from hydroxylation (Supplementary Figure 1) (Michalska *et al.*, 2004).

The quality of any pharmaceutical is critical for having high benefit instead of risk ratio, in other words "patient's safety". The formation of impurities during manufacturing, storage, or transportation or metabolism can lead to adverse effects on patient health (Balamurugan *et al.*, 2015; Grover, Mehta, *et al.*, 2022).

According to ICH Guideline Q1A (R2) for stability studies and ICH Q3B (R2), for pharmaceutical products, the structural identification of unknown impurities is mandatory if their levels exceed the safety threshold of 0.1% during stability studies (Goswami and Raju, 2025; Grover, Bhardwaj, *et al.*, 2022). The FDA also recommends a 0.1% threshold for unspecified degradation products or impurities in ophthalmic drug formulations, as long as the drug product strength is between >0.1% and ≤1% (Chauhan *et al.*, 2024).

During course of product life cycle the stability studies of Ciprofloxacin eye drops (3 mg/mL) revealed a new impurity at a Relative Retention Time (RRT) of 1.19. Hence, the present work included the isolation of the observed impurity (RRT 1.19) and its all-possible characterization.

MATERIALS AND METHODS

Chemicals and reagents

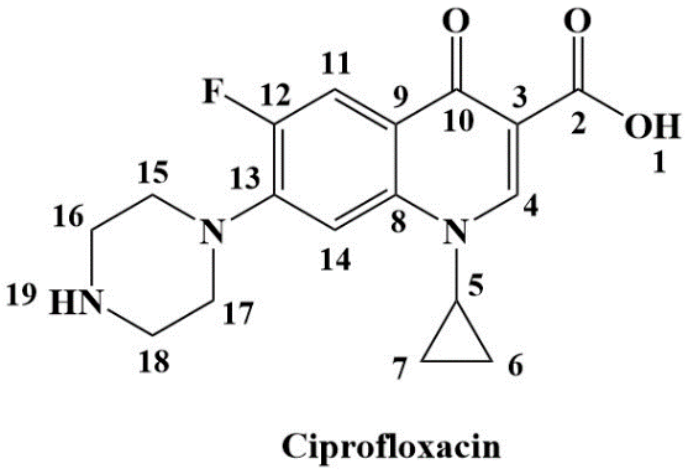
Ciprofloxacin standard and eye drop sample used in this study was obtained from Dr. Reddy's Laboratories Limited, India. LCMS grade acetonitrile and methanol were sourced from Biosolve, while Trifluoroacetic Acid (TFA) and formic acid were acquired from standard reagent and Biosolve chemicals respectively. Tetra butyl ammonium phosphate was procured from Sigma-Aldrich. Deuterated Dimethyl Sulfoxide (DMSO-d_6) and Deuterated Water (D_2O) were obtained from Eurisotop. All analytical solutions were prepared using Milli-Q water produced by an in-house Merck Milli-Q Integral 10 system.

Ultra-High Performance Liquid Chromatographic (UHPLC) Conditions

A Thermo Vanquish UHPLC+ (Thermo Fisher Scientific, USA) equipped with a Photo Diode Array (PDA) detector was employed for analysis. The UHPLC system included a binary Vanquish pump (part number VH-P10-A), split sampler HT (part number VH-A10A), HPLC column compartment (mode number 186015043), diode array detector HL (part number VH-D10-A) and an external column compartment-H (part number VH-C10-A). Data acquisition and processing were performed using Xcalibur software (version 3.0.63) and Tracefinder software (version 4.1). Chromatographic conditions consist of 0.1% formic acid as mobile phase-A and 100% methanol as mobile phase-B. An isocratic elution constituting 50% of mobile phase-A and 50% of mobile phase-B was employed at flow rate 0.5 mL/min for 5 min through union to analyze the pure impurity isolated by preparative HPLC. Sampler and column oven temperature were maintained at 10°C and 25°C respectively with an injection volume of 5 µL. Chromatographic separation was performed on Inertsil ODS C18 column (250 mm×4.6 mm and 3.5 µm particle size).

Preparative HPLC

Preparative chromatography was conducted using a Waters preparative HPLC system equipped with Masslynx 4.2 software, 2545 quaternary pump module, 2489 dual UV detector, and 2767 sample manager with auto-fraction collector. Impurity separation was carried out on Kinetix phenyl-hexyl column (250×20 mm, 5 µm) at a flow rate of 15 mL/min. The mobile phase consisted of 0.1% TFA and acetonitrile. An optimized gradient elution program was applied (time (min)/Solution-B (%): 0/10, 5/10, 30/25, 35/25, 40/90, 48/90, 50/10, 55/10) with flow rate 15 mL/min, an injection volume of 7 mL and detection at 280 nm. The purified impurity fractions were combined, the solvent was evaporated, and the aqueous solution was lyophilized using a Lyofreeze lyophilizer to obtain the impurity in solid form. This final isolated impurity fraction was subsequently used for NMR analysis.



Ciprofloxacin

Figure 1: Structure of Ciprofloxacin.

High resolution mass spectrometry

The aforementioned Thermo Vanquish UHPLC system was paired with a Thermo QExactive Orbitrap mass spectrometer and used for HRMS analyses. Electrospray Ionization (ESI) was employed in positive ionization mode. Mass spectrometric data were recorded within *m/z* range of 100-1500 using full scan acquisition at a resolution of 70,000. The analysis was conducted with an Automatic Gain Control (AGC) target of 3e6 and a maximum Injection Time (IT) of 200 ms. A spray voltage of -3.30 kV was used, along with capillary temperature of 350°C, andS-lens RF level of 50 and auxiliary gas heater temperature of 200°C. Sheath, aux, and sweep gas flow rates were set at 40, 12, and 0 (arbitrary units) respectively, with an aux gas heater maintained at 350°C. These parameters were specific to QExactive HRMS and were optimized before analysis.

Nuclear magnetic resonance spectroscopy

The impurity's structure was elucidated using NMR spectroscopy. One-Dimensional (1D) NMR experiments including ^{1}H, ^{13}C, ^{13}C DEPT as well as Two-Dimensional (2D) NMR techniques such as ^{1}H-^{1}H COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC were conducted for both Ciprofloxacin active pharmaceutical ingredient (API) and its impurity, N-(3-Hydroxypropionyl) using Bruker Avance 500 MHz NMR spectrometer. The NMR analysis of Ciprofloxacin API was conducted in deuterium oxide (D_2O, NMR analysis of Ciprofloxacin API was conducted in deuterium oxidanalyzed in deuterated Dimethyl Sulfoxide (DMSO-d_6). The probe temperature was regulated at 25°C. The ^{1}H NMR chemical shifts were referenced to the singlet signal of Tetramethylsilane (TMS) at 0 ppm, while the ^{13}C NMR spectra referenced to the septet signal of DMSO-d_6 at 39.5 ppm.

Sample preparation

A diluent composed of 0.02 M ammonium formate (pH adjusted to 3.0 with dilute acetic acid) and methanol 4:6 (v/v) ratio was used for sample preparation. A blank solution was injected prior to the sample injection to ensure that no interference occurred during data processing. For stability studies, 3 mL aliquot of ciprofloxacin eye drops (concentration: 3 mg/mL) was transferred to a 10 mL volumetric flask followed by the addition of 6 mL of methanol, mixed well and makeup to the volume with 0.02 M ammonium formate buffer. Resulting solution was used for stability studies (18 months, 30°C±2°C/65% RH±5% RH). While for the isolation of impurities by preparative HPLC, the sample solution of Ciprofloxacin eye drops (3 mg/mL) was directly utilized. Approximately 2 mg of the isolated impurity sample was dissolved in 25 mL of methanol and then analyzed by LC-HRMS.

RESULTS

UPLC Method development and optimization

Multiple batches of ciprofloxacin eye drops were analysed using the previously developed method as described in Experimental section wherein specified impurity at RRT 0.80 (Ciprofloxacin ethylenediamine) was already separated by isocratic elution using an Inertsil ODS C18 column (250 mm × 4.6 mm and 3.5 µm particle size). Mobile phase A consisted of 5 mM tetra-n-butyl ammonium phosphate (pH 2.0, adjusted with diluted orthophosphoric acid), while mobile phase B was 100% methanol. Column temperature was kept as 33°C. The optimized isocratic program with mobile phase A and B composition 75:25 was run for 18 min at a flow rate of 1.5 mL/min. These conditions were applied to analyse stability samples, in which unknown impurity at RRT 1.19 (Figure 2) was observed in incremental order across stability intervals as shown in following chart (Figure 3).

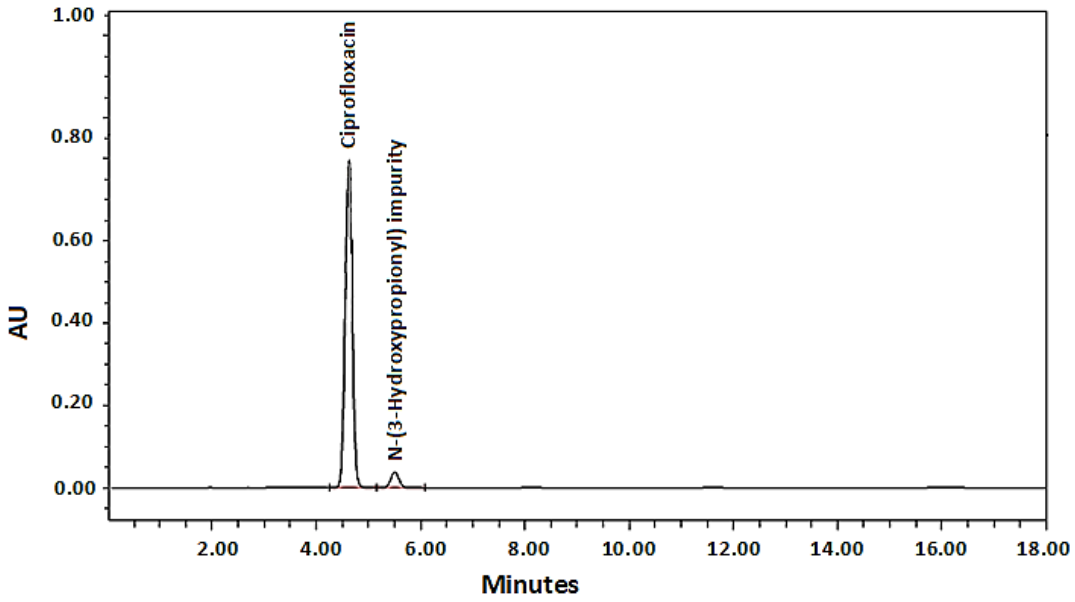


Figure 2: Analytical HPLC chromatogram of Ciprofloxacin eye drops.

According to FDA guidelines, impurities present at concentrations exceeding 0.10% must be identified in ophthalmic drug products with a strength between >0.1% and ≤1% (% w/v) (Goswami and Raju, 2025). The UPLC retention times for ciprofloxacin and the impurity are depicted in Figure 2, with retention times of 4.617 min for ciprofloxacin and 5.504 min (RRT 1.19) for the impurity, respectively.

Preparative HPLC method development

As the impurity RRT 1.19 was present at low levels in stability samples, preparative isolation followed by characterization using LC-HRMS and NMR was planned.

A phenyl-hexyl stationary phase combined with an acidic mobile phase employed for the isolation the impurity by preparative HPLC. Under these chromatographic conditions, the target impurity peak was successfully isolated with a purity of 99%. This final isolated solid impurity was subsequently analyzed for

Table 1: ^{1}H, ^{13}C NMR and DEPT-135 signal assignments for HP-CIP and Ciprofloxacin (API).

Position	No. of protons	N-(3-Hydroxypropionyl) Ciprofloxacin impurity			Ciprofloxacin (API)			DEPT-135
		^{1}H (δ ppm)	Multiplicity *J* in Hz	^{13}C (δ ppm)	^{1}H (δ ppm)	Multiplicity *J* in Hz	^{13}C (δ ppm)	
1	OH	15.12	s	-	-	-	-	-
2	-	-	-	165.82	-	-	171.55	C
3	-	-	-	106.84	-	-	108.63	C
4	1H	8.69	s	148.24	8.48	s	150.88	CH
5	1H	3.83	m	35.94	3.65	m	38.99	CH
6,7	4H	1.20	m	7.59	1.45	m	10.40	CH_2
		1.33			1.18			
8	-	-	-	139.04	-	-	141.65	C
9	-	-	-	119.29 (d,$^{3}J_{CF}$= 8.0)	-	-	121.36 (d,$^{3}J_{CF}$= 7.76)	C
10	-	-	-	176.39	-	-	178.37	C
11	1H	7.97	d,$^{3}J_{FH}$= 13.09	111.18 (d,$^{2}J_{CF=}$ 23.7)	7.24	d, *J*= 12.87	113.36 (d,$^{2}J_{CF}$= 23.70)	CH
12	-	-	-	152.85 (d,$^{1}J_{CF=}$ 248.9)	-	-	156.07 (d,$^{1}J_{CF}$= 251.52)	C
13	-	-	-	143.86 (d,$^{1}J_{CF=}$ 10.2)	-	-	147.41, (d,$^{2}J_{CF}$= 10.18)	C
14	1H	7.60	d,$^{4}J_{FH}$= 7.45	106.90 (d,$^{3}J_{CF=}$ 20.7)	7.41	d, *J*= 7.26	109.30	CH
15,17	4H	3.52	br s	46.98	3.64	m	49.19 (d,$^{4}J_{CF}$= 4.75)	CH_2
16,18	4H	3.34	br s	51.04	3.56	m	46.10	CH_2
19	-	-	-	171.85	-	-	-	C
20	2H	2.75	t,*J*= 7.50	29.13	-	-	-	CH_2
21	2H	3.34	br s	51.66	-	-	-	CH_2
22	OH*	-	-	-	-	-	-	-

s=singlet, d= doublet, m=multiplet, br= broad, * Exchangeable protons are not observed

structural characterization using LC-MS and NMR. Since the non-volatile buffer used in the chromatographic purity method could not be directly infused into the mass spectrometer, a mass-compatible method with a volatile buffer was developed to determine the mass and elemental composition of the N-(3-Hydroxypropionyl) Ciprofloxacin impurity (HP-CIP). The pure HP-CIP impurity was isolated by preparative HPLC for further characterization.

Structural elucidation of impurity

The structural identification of the HP-CIP impurity was carried out using the LC-HRMS and NMR. The molecular weight of the impurity was determined by analyzing the samples in positive ion mode. For comparative analysis, the molecular characterization of pure ciprofloxacin, including MS and NMR data, is presented in Supplementary Figures S2-S9. Following the isolation of the pure impurity via preparative HPLC, the impurity characterization was done by LC-HRMS (Figure 4) and NMR (Figure 5 and Table 1). The HR-MS spectrum in positive ion mode displayed a prominent molecular ion peak at *m/z*= 404.1604 ($[M+H]^+$, closely matching the calculated value (m/z=403.1543), confirming the empirical formula of the impurity as $C_{23}H_{21}O_4N_3$.

NMR spectroscopic analysis, including ^1H, ^{13}C and DEPT-135 and 2D NMR experiments (^1H-^1H COSY, ^1H-^{13}C HSQC, ^1H-^{13}C HMBC, and ^1H-^1H, ROESY) provided crucial structural information about impurity. The ^1H NMR spectrum of HP-CIP impurity (Figure 5 A and Table 1) revealed three downfield signals in the δ 7-9 ppm region, with peaks at δ 8.69 ppm, δ 7.97 ppm and δ 7.60 ppm are corresponding to the aromatic protons at C4, C11 and C14. The overlapping multiplet peaks at δ 1.20 ppm and δ 1.33 ppm are assigned to protons at C6 and C7 respectively. No signal was observed for -OH group attached to C22 due to proton exchange (Figure 6). The ^{13}C NMR spectrum of impurity displays 20 distinct carbon resonances. The downfield peaks at δ165.82 ppm, δ176.39 ppm and 171.85 ppm are assigned to carbonyl carbon at C2, C10 and C19 respectively which is further confirmed by the absence of these peaks in corresponding DEPT-135. Chemical shifts at δ 106.84 ppm, δ 139.04 ppm, δ 119.29 ppm, δ 152.85 ppm and δ 143.86 ppm respectively for C3, C8, C9, C12 and C13 assigned to aromatic quaternary carbons respectively and these were absent DEPT-135. Furthermore, peaks at δ 148.24 ppm, δ 111.18 ppm and δ 106.90 ppm are assigned to aromatic carbon C4, C11 and C14 respectively and peak at δ 35.94 ppm is assigned to -CH group from C5 in strained cyclopropyl ring are positive peaks in DEPT-135 (Figure 5 C).

The key features in the DEPT-135 data include overlapped inverted signals at δ 7.59 ppm which originate from CH_2 groups of cyclopropyl ring. Further inverted peaks δ 3.52 ppm, δ 46.98 ppm, δ 3.34 ppm, δ 51.04 ppm originated from C15, C17, C16 and C18 respectively. Negative peaks at δ 29.13 ppm and δ 51.66 ppm correspond to CH_2 groups of C20 and C21 respectively. Four doublet signals at δ 119.29 ppm ($^3J_{CF}$=*8.0*), δ 111.18 ppm ($^2J_{CF}$=*23.7*), δ 143.86 ppm ($^2J_{CF}$=*10.2*) and δ 106.90 ppm ($^3J_{CF}$= 20.7) for C9, C11, C13 and C14 respectively indicate the presence of neighbouring fluorine to these carbons and signal at δ 152.85 ppm ($^1J_{CF}$=248.9) corresponds to C12 reveals that fluorine on C12 (Figure 5 B).

The 2D NMR spectra provides more information about a molecule and helps in determining the structure of a molecule. The ^1H-^1H COSY spectrum of impurity reveals two key correlations from

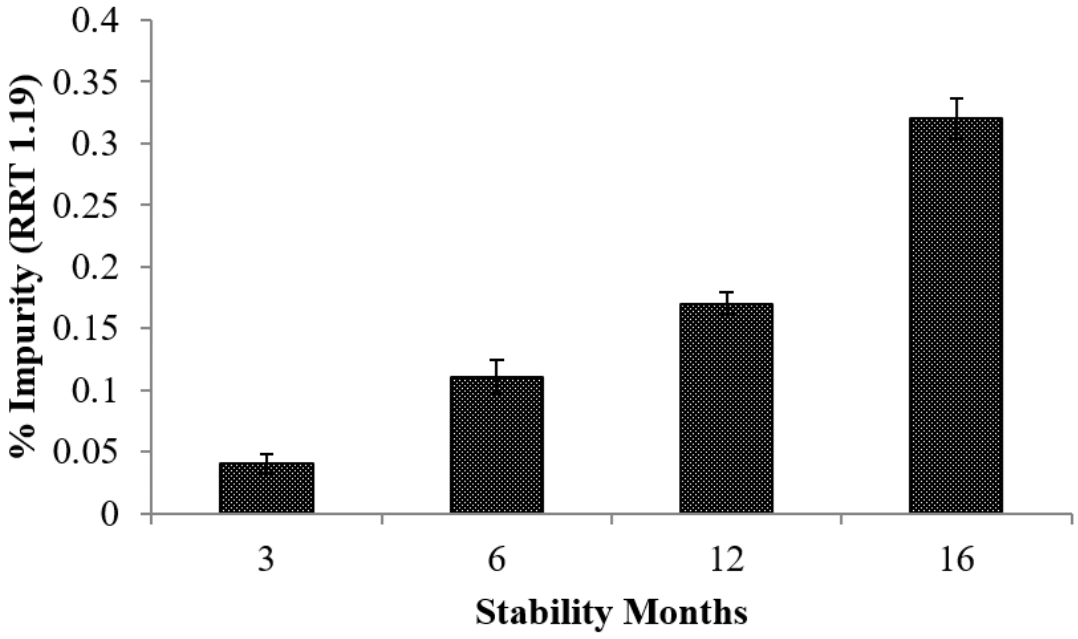


Figure 3: % Impurity at Relative Retention Time (RRT) 1.19 in stability samples at different stability samples.

H5 to H6 and H7, and other from H7 to H6, confirming the presence of cyclopropyl ring Figure 5 D. Additionally, the COSY correlation between H20 to H21 indicates the existence of 3-hydroxypropionyl group in impurity. 2D Heteronuclear Single Quantum Coherence (HSQC) spectrum (Figure 5 E) of impurity revealed correlations from H15, H16, H17, and H18 with their respective carbon atoms (C15, C16, C17, and C18) confirming the presence of the piperazine moiety, as illustrated in Figure 6. Moreover, HSQC correlations between H20, and H21 to C20, and C21 further validate the presence of the 3-hydroxypropionyl group. Additionally, ^{1}H-^{13}C Hetero nuclear Multiple Bond Correlation (HMBC) analysis of impurity (Figure 5 F) reveals that the H20 proton is coupled to two carbon atoms, including C19 and C21 further supporting the structural assignment of the impurity. Moreover, HMBC from H5 to C4, C6, C7 and C8; H6 correlation with C5 and C7, H7 with C5 and C6 indicates Cyclopropyl ring in the structure. Further, 2D Rotating frame Overhauser Enhancement Spectroscopy (ROESY) (Figure 5 G.) of impurity revealed that the strong NOE cross correlations observed between H4 with H5, H6, H7; H5 with H6 and H7 helped in assigning the protons of Cyclopropyl ring in the structure. H21 to H22 indicates the presence of 3-hydroxypropionyl group in impurity. These findings further support the proposed structure of Ciprofloxacin impurity.

Clearly, identified and characterized N-(3-Hydroxypropionyl) Ciprofloxacin (Figure 6) impurity is not reported so far.

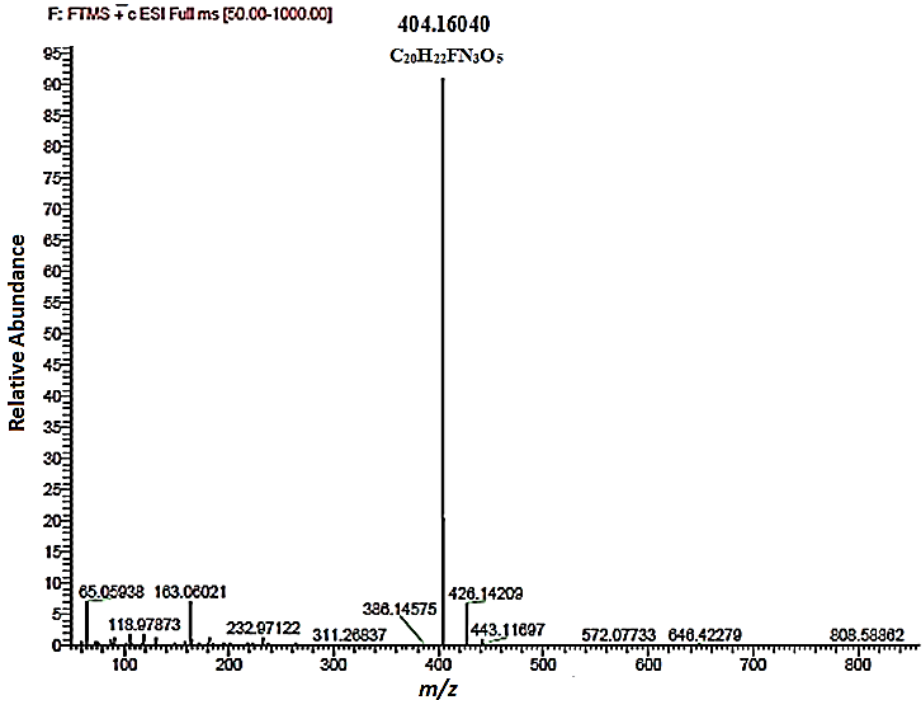


Figure 4: Mass spectrum of HP-CIP (recorded in methanol).

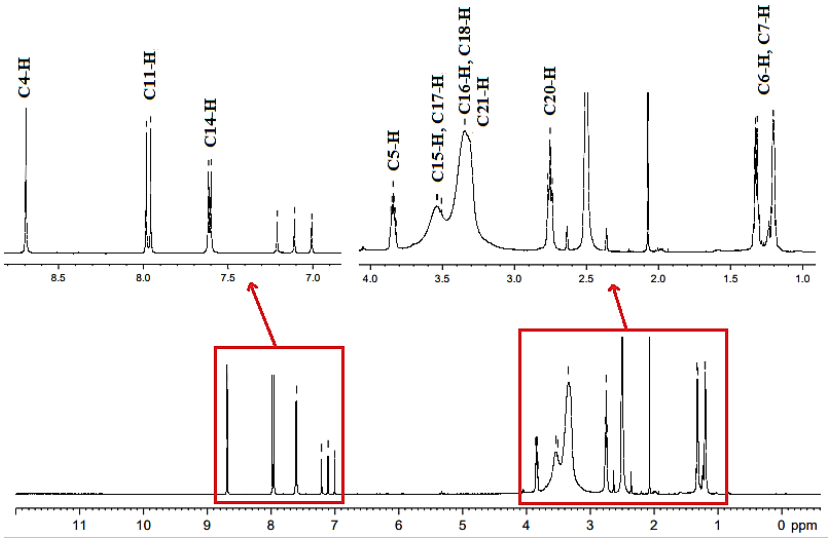


Figure 5 A: ^{1}H NMR Spectrum.

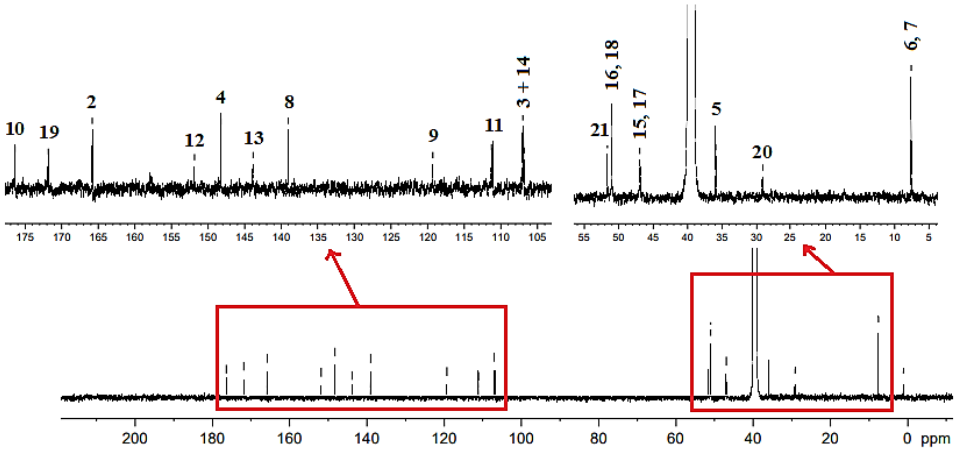


Figure 5 B: ^{13}C NMR spectrum.

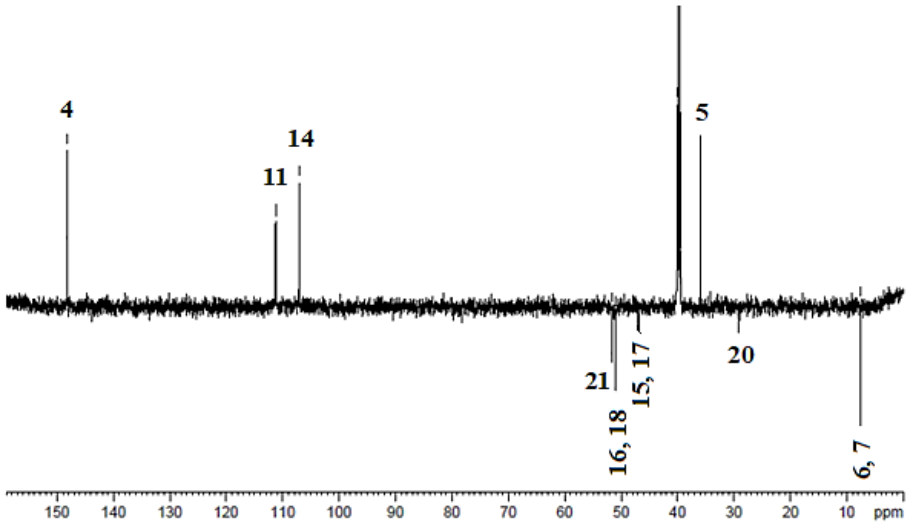


Figure 5 C: ^{13}C DEPT-135 spectrum.

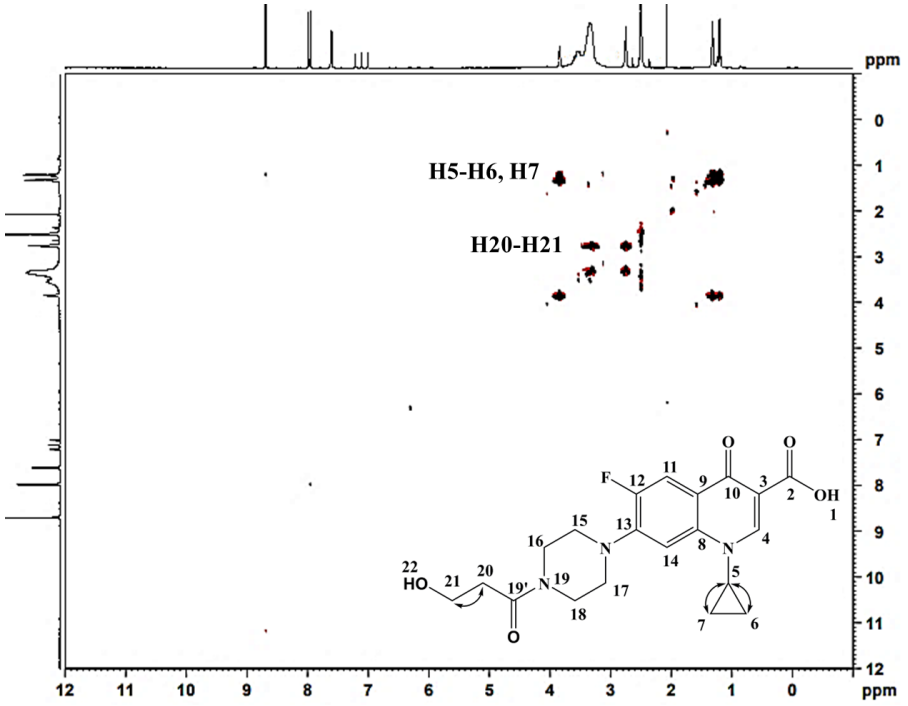


Figure 5 D: ^{1}H-^{1}H COSY spectrum.

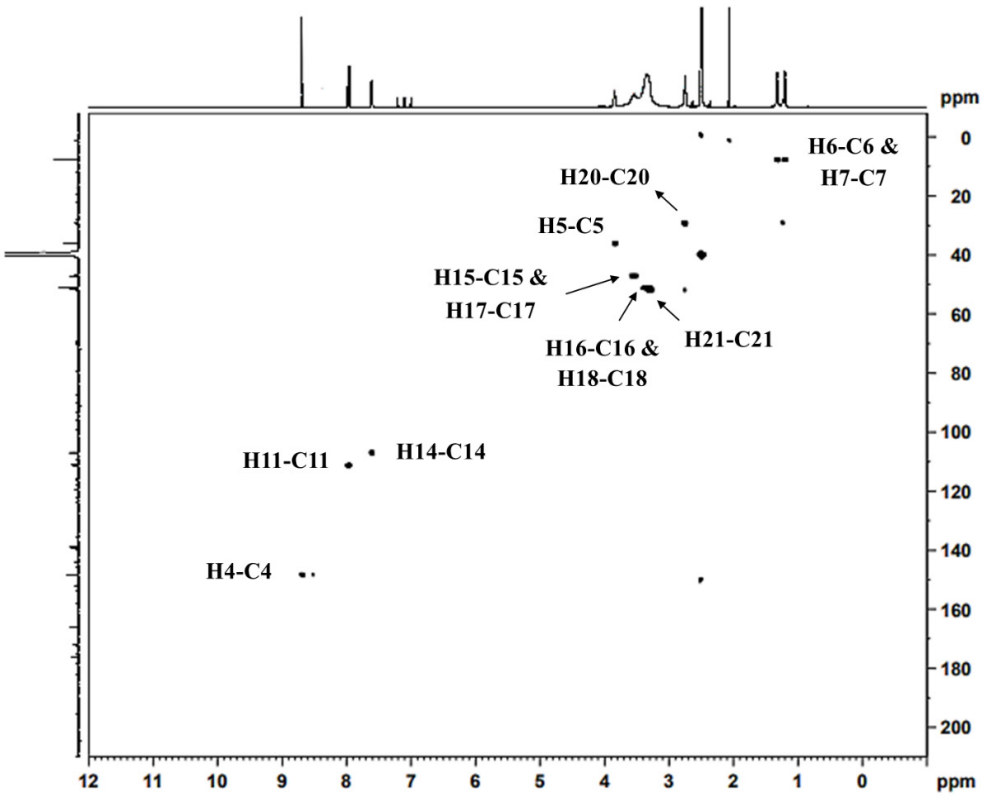


Figure 5 E: HSQC NMR spectrum.

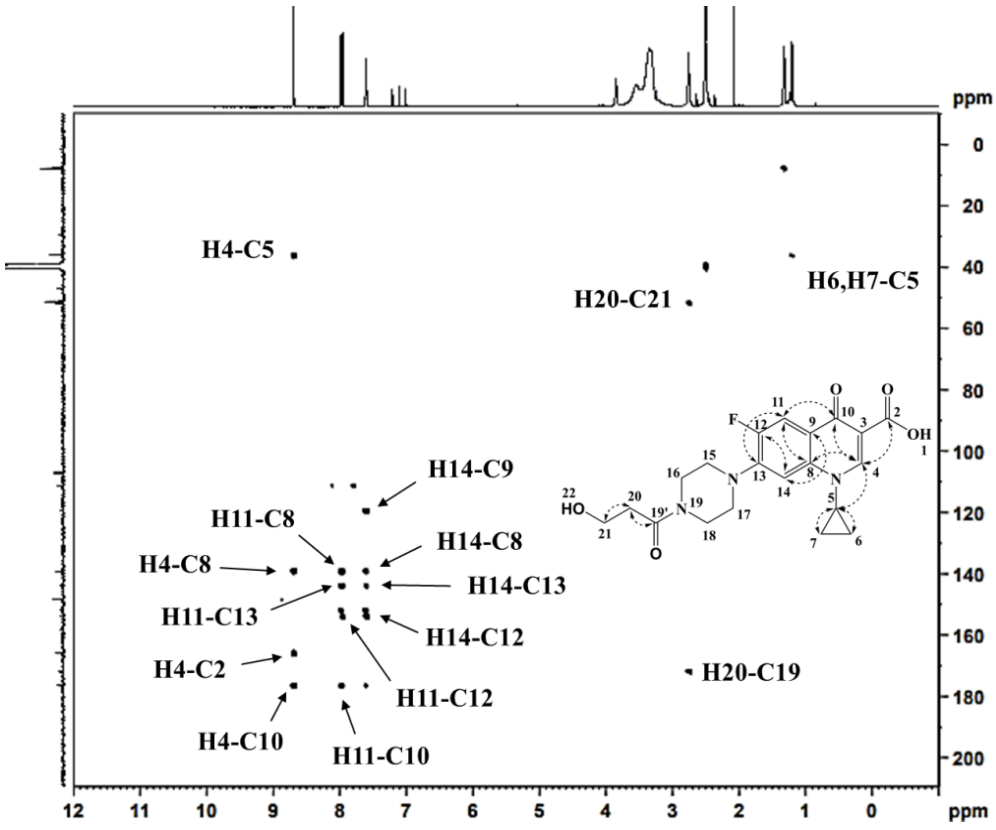


Figure 5 F: HMBC spectrum.

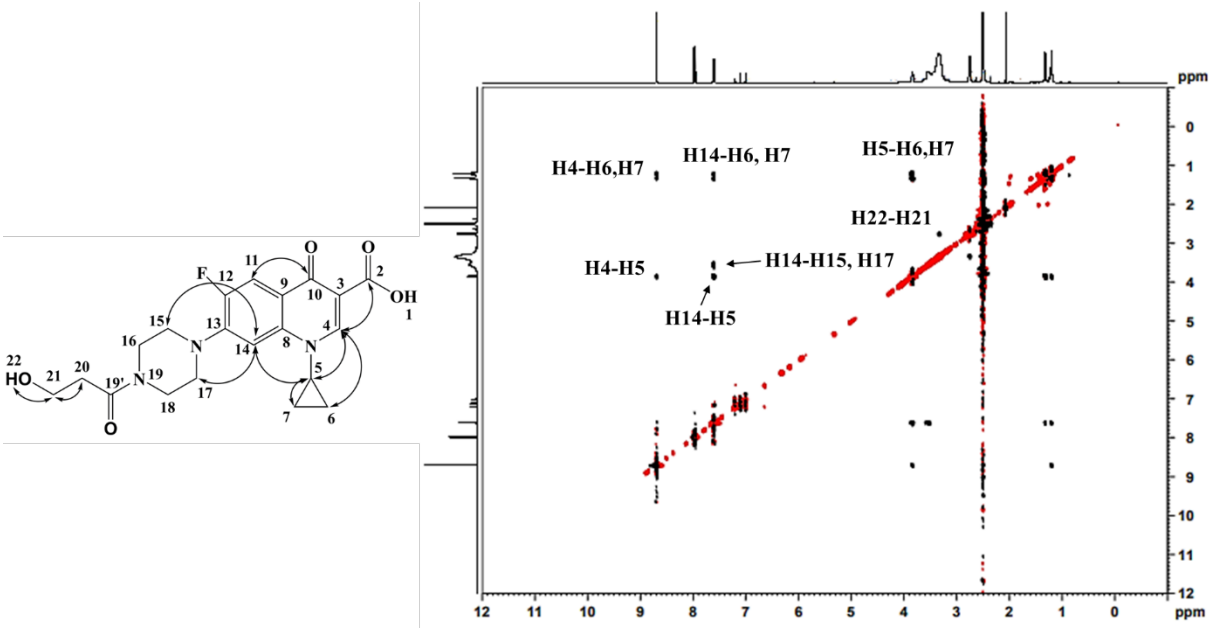


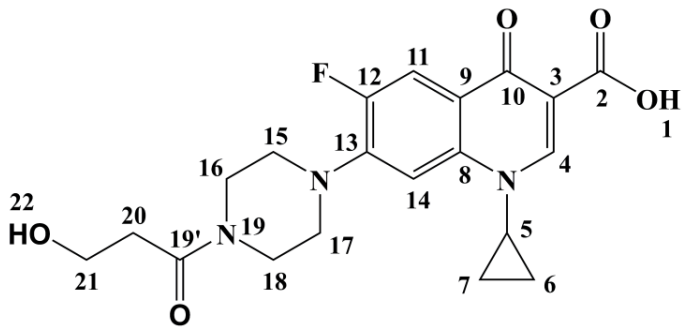
Figure 5 G: ROESY Spectrum

The LC-HRMS and NMR data revealed the formation of a N-(3-Hydroxypropionyl) Ciprofloxacin, likely resulting from a reaction between Ciprofloxacin and 3-Hydroxypropionic Acid (3-HPA). Acrylic acid, commonly used in the production of acrylic esters and resins for coatings and adhesives, undergoes base-induced hydration followed by re-acidification, leading to the formation of 3-HPA and probable reaction mechanism for the formation of impurity is shown in Figure 7.

DISCUSSION

Spectroscopic techniques are considered as first choice by organic chemists for the unknown impurities identification in pharmaceutical drug products. NMR Spectroscopy and mass spectroscopy are orthogonal techniques which can characterize most of the organic compounds (Grover *et al.*, 2023; Syed and Nannapaneni, 2025). NMR Spectroscopy provides the specific information about chemical structure and its chemical environments through properties like chemical shift, spin-spin splitting, and coupling constants for pure organic compounds (Gaikwad *et al.*, 2025). Whereas, LC-HRMS combines the polarity-based separation features of liquid chromatography with high-resolution mass spectrometry for accurate mass and elemental composition determination for impure organic compounds also (Hulleman *et al.*, 2023). Conventional LCMS instruments with single quadrupole and triple quadrupole mass analysers are not accurate enough to be used for the identification of unknown impurities, although MS/MS application for triple quadrupole mass analysers could be used for the structure confirmation (Tsakalof *et al.*, 2024). Due to high accuracy and capability to provide elemental composition of any unknown compound, LC-HRMS instruments gained huge popularity over triple quad mass instruments in recent past. In present study LC-HRMS, 1D NMR (^{1}H NMR, ^{13}C NMR and DEPT) and 2D NMR (COSY, ROESY, HSQC and HMBC) techniques are being used for the elucidation of impurity structure.

Proton (^{1}H) and Carbon-13 (^{13}C) Nuclear Magnetic Resonance (NMR) spectroscopy are essential tools for elucidating the structure of organic compounds. ^{1}H NMR provides insights into the number, type, and environment of hydrogen atoms, revealing connectivity through chemical shifts, splitting patterns, and coupling constants. ^{13}C NMR complements this by identifying the carbon framework, distinguishing between carbon types (e.g., quaternary, methyl, aromatic). Together, they offer detailed molecular fingerprints. These methods are widely used in natural product research and pharmaceutical development for verifying chemical structures (Bifulco *et al.*, 2007; Joga *et al.*, 2023). While 2D NMR techniques-COSY, HSQC, HMBC, and ROESY-play a crucial role in the comprehensive elucidation of molecular structures, particularly in complex organic and pharmaceutical compounds. Each of these techniques provides distinct and complementary information regarding atomic connectivity and spatial arrangement. COSY is used to identify scalar (through-bond) coupling between protons, providing insights into proton-proton connectivity's. It helps in mapping spin systems and distinguishing overlapping signals. COSY is typically the starting point in 2D NMR analysis (Claridge, 2016). HSQC connects protons directly bonded to carbons or other heteronuclei. It is essential for determining one-bond correlations (^{1}H-^{13}C), assisting in assigning protonated carbons and improving signal resolution in complex molecules (Parella, 2004). HMBC reveals long-range (2-3 bond) heteronuclear couplings, thus enabling the identification of carbon skeletons



N-(3-Hydroxypropionyl) Ciprofloxacin impurity

Figure 6: Elucidated structure of N-(3-Hydroxypropionyl) Ciprofloxacin impurity (HP-CIP).

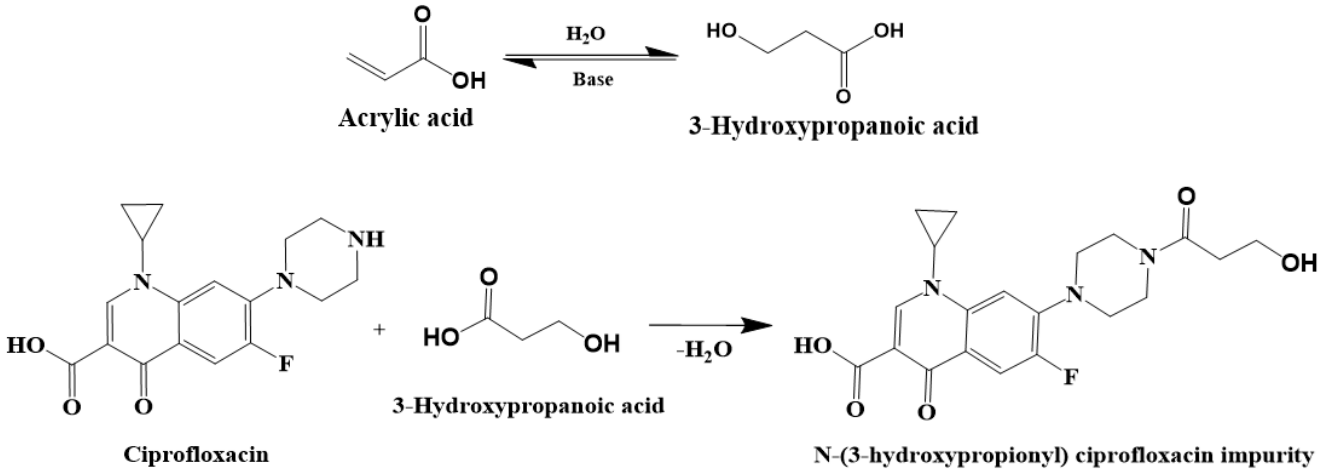


Figure 7: Proposed reaction scheme for the formation of the HP-CIP.

and linkage patterns between proton and quaternary carbons. It complements HSQC data and is invaluable for constructing the full carbon framework (Bakiri *et al.*, 2018). ROESY provides through-space correlations between protons, which is critical for stereochemical assignments and conformational analysis. It is especially useful for distinguishing isomers and analyzing non-covalent interactions (Ilgen *et al.*, 2021). Together, these techniques enable high-confidence structural elucidation. Their combined application is standard in natural product chemistry, metabolomics, and drug development for verifying molecular architecture and stereochemistry.

Literature research shows that many impurities of Ciprofloxacin are already reported (Figure S1) and estimated by chromatographic purity methods. These impurities include synthesis by-products, degradation products, Photo degradation products, Hydroxylation, Decarboxylation products (Matmour *et al.*, 2022).

Stability study of Ciprofloxacin eye drops aged for 18 months (30°C±2°C/65% RH±5% RH) showed the formation of a new impurity at Relative Retention Time (RRT) 1.19 at 0.3% level. Ciprofloxacin eye drops solution was stored in Low-Density Polyethylene (LDPE) bottle pasted with drug information label on it, which is susceptible for leaching due to the semi-permeable nature of LDPE bottle (Houston, 2011). Experiments show the formation of N-acylated derivative of Ciprofloxacin due to the condensation reaction between the drug product and a migrated component, possibly from the ink or varnish used on the label affixed to the Low-Density Polyethylene (LDPE) bottle. Since impurity was present at low levels in Ciprofloxacin samples, therefore LC-HRMS analysis was performed to determine the mono-isotopic mass and elemental composition of the impurity (Figure 2 and Figure 4). A careful literature search revealed that this impurity was not reported earlier. Therefore, experiments were performed to isolate pure impurity by preparative HPLC and same impurity was used to collected 1D and 2D NMR data (Figure 5A-F, Table 1). Interpretation of LC-HRMS and NMR data concluded the impurity as N-(3-Hydroxypropionyl) Ciprofloxacin (Figure 1). The formation N-(3-Hydroxypropionyl) Ciprofloxacin was verified by exposing Ciprofloxacin formulation with 0.2% of 3-HPA at elevated temperature condition (40°C for 1 hr), where N-(3-Hydroxypropionyl) Ciprofloxacin impurity was observed about 0.90% level. Furthermore, negative control experiment was performed by heating Ciprofloxacin formulation at elevated temperature condition (40°C for 1 hr) in glass vial, where no impurity was observed at RRT 1.19. This proves the hypothesis of the formation of impurity in the presence of 3-HPA.

Since this impurity is new to the world, hence it should be studied thoroughly including safety studies. One interesting impurity of roxadustat which was generated with exposure to light was showing same mass. Since this impurity can be generated at any moment with exposure of light hence it became the obligation to study its safety aspect. This study reported no genotoxicity of the photo-induced impurity of roxadustat by exploiting the comet assay (Mahajan *et al*., 2024).

Product quality research institute guidance shows that labels (used on the container closure system) with UV-cured ink contain a pair of acrylate esters that can leach into product and level can vary across the lots, which further supports the hypothesis (Houston, 2011). The involvement of 3HPA was verified by exposing Ciprofloxacin formulation with 0.2% of 3-HPA at elevated temperature condition (40ºC for 1 hr) and N-(3-Hydroxypropionyl) Ciprofloxacin impurity was observed at about 0.90% level which clearly approves the suggested hypothesis. Since Ciprofloxacin eye drops are directly administered to the eyes for infection treatment, it was crucial to identify this impurity to assess its toxicity. Another study also revealed the formation of process related impurities of Nevirapine, one was 8-n-propyl nevirapine. Since newly identified impurity is not process related and formed under stability conditions (30ºC±2ºC/65% RH±5% RH for 18 months) due to the condensation reaction between the drug product and a migrated component, possibly from the ink or varnish used on the label affixed to the Low-Density Polyethylene (LDPE) bottle, therefore it was proposed to replace the packaging system with impervious primary container to avoid the formation of the impurity. This novel impurity was not purging from the process due to structural similarity with the API. The precursors of the impurities were identified to be (E)- and (Z)-N-propylidenecyclopropanamine which upon controlling led to reduction of the 8-n-propyl nevirapine impurity to 0.02% from many time appearance upto 0.1% (Lin *et al*., 2024).

CONCLUSION

The impurity observed at a Relative Retention Time (RRT) of 1.19 in Ciprofloxacin eye drops was identified as an N-(3-Hydroxypropionyl) Ciprofloxacin using advanced analytical techniques, including liquid chromatography high resolution mass spectrometry and nuclear magnetic resonance spectroscopy. The mono isotopic mass and elemental composition determined by LC-HRMS aligned with the proposed impurity structure. Structural characterization was further confirmed through NMR analysis utilizing both one-dimensional (^{1}H NMR, ^{13}C NMR, and ^{13}C DEPT-135) and two-dimensional techniques (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, and H-^{1}H ROESY).

The formation of this impurity was attributed to an interaction between the drug product and a migrated component, likely from the ink or varnish on the LDPE bottle. These findings emphasize the need to evaluate drug products with similar structural characteristics stored in comparable container closure systems for the potential formation of such by-products. The presence of these impurities may compromise the quality and efficacy of the drug product, posing a potential risk to patient safety.

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ABBREVIATIONS

1D and 2D: 1 Dimensional and 2 Dimensional; **3-HPA:** 3-Hydroxypropionic acid; **COSY:** Correlation spectroscopy; **DEPT:** Distortionless Enhancement by Polarization Transfer; **DMSO:** Dimethyl Sulfoxide; **HMBC:** Heteronuclear Multiple Bond Correlation; **HP-CIP:** N-(3-Hydroxypropionyl) Ciprofloxacin; **HPLC:** High performance chromatography; **HSQC:** Heteronuclear single quantum coherence; **LC-HRMS:** Liquid chromatography-High resolution mass spectrometry; **LDPE:** Low-density polyethylene; **NMR:** Nuclear Magnetic Resonance; **PQRI:** Product Quality Research Institute; **ROESY:** Rotating-Frame Overhauser Enhancement Spectroscopy; **RRT:** Relative Retention Time; **TFA:** Trifluoroacetic acid; **TMS:** Tetramethylsilane; **UPLC:** Ultra Performance Liquid Chromatography.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Dr. Raju Mudhulkar (Conceptualization, Project administration, Resources, Supervision, Visualization, and Writing-review & editing), Bhupinder Kumar (Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Validation, Roles/Writing-original draft), Dr. Dama Venugopala Rao (Formal analysis, Validation, Writing-review & editing.), Dr. Surbhi Tiwari (Formal analysis, Validation, Writing - review & editing) and Dr. Lakki Reddy Prakash, Ramakrishna Yadav Belly (Formal analysis, Validation, Writing - review & editing), Dr. Sandeep Kumar (Formal analysis, Validation, Writing - review & editing). All authors have approved the final version of the manuscript.

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Supplementary Information (SI)

Preparative Separation, Identification and Characterization of N-Acylated Impurity in Ciprofloxacin Eye Drops Formulations Using 1D and 2D NMR Techniques (Bhupinder *et al.*).

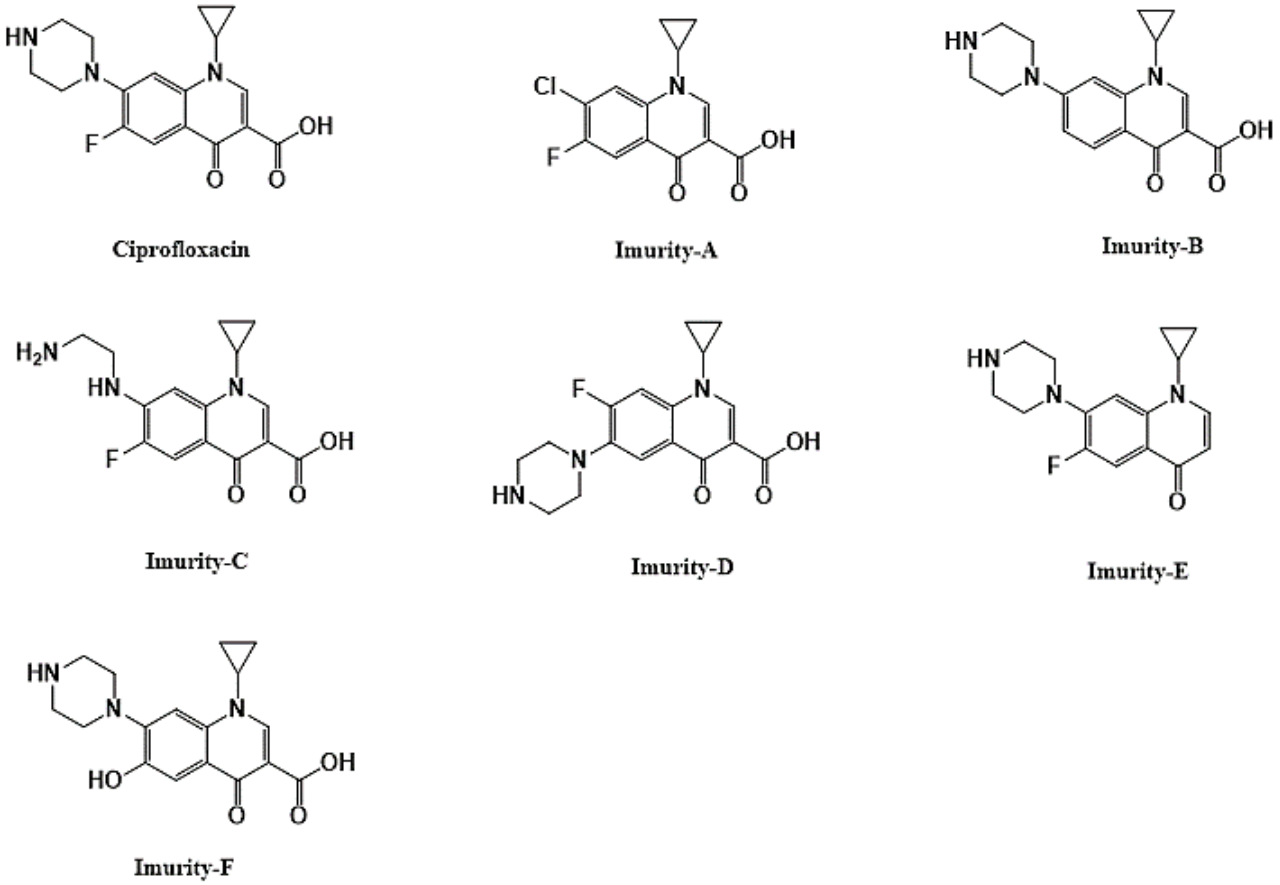

Figure S1: Ciprofloxacin related known impurities.

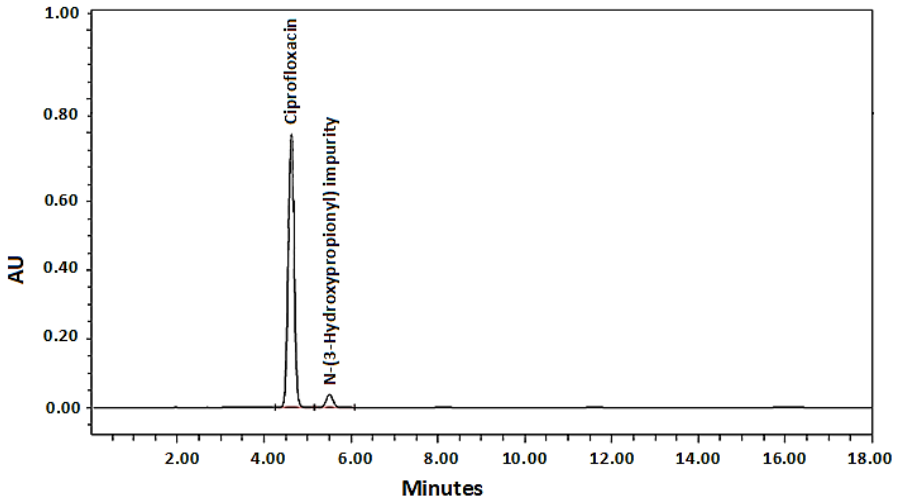

Figure S2: HRMS spectrum of Ciprofloxacin (API).

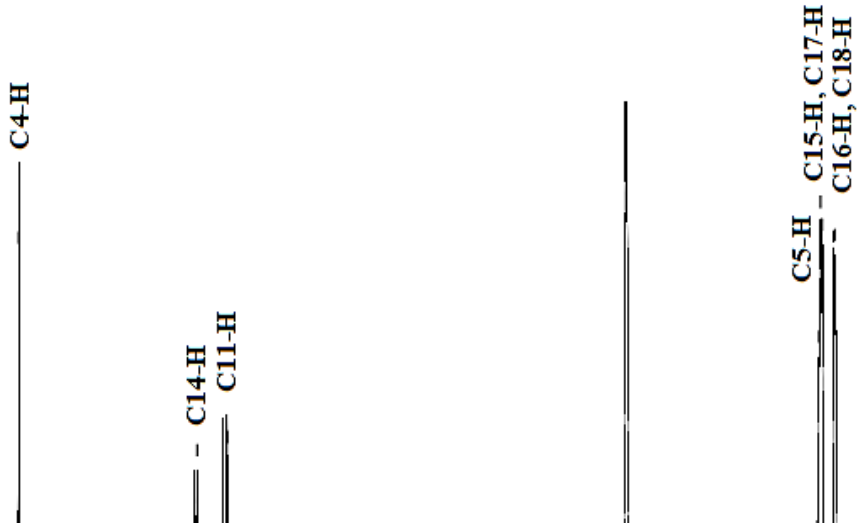

Figure S3: ^{1}H NMR spectrum of Ciprofloxacin (API) when recorded in D_2O at room temperature.

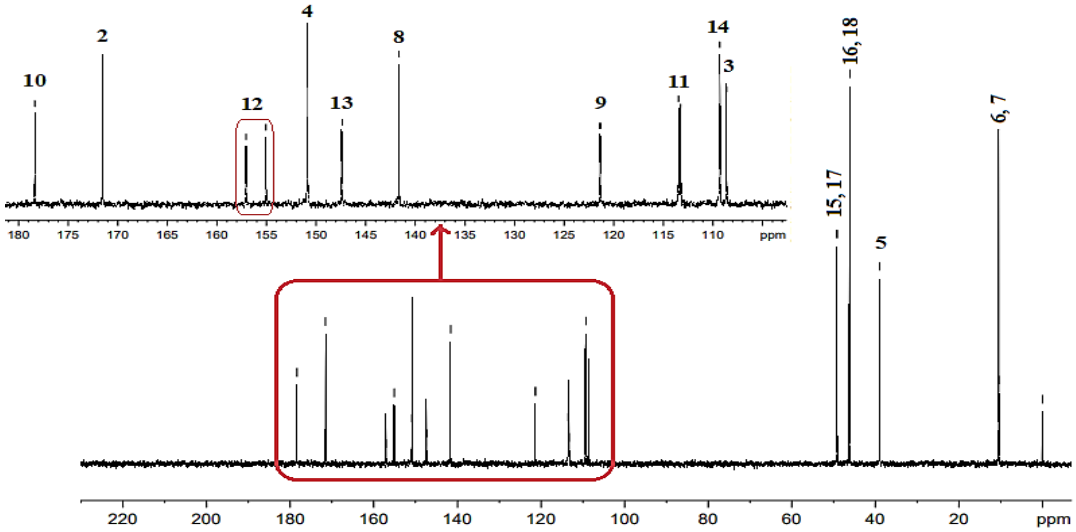

Figure S4: ^{13}C NMR spectra of Ciprofloxacin API when recorded in D2O at room temperature.

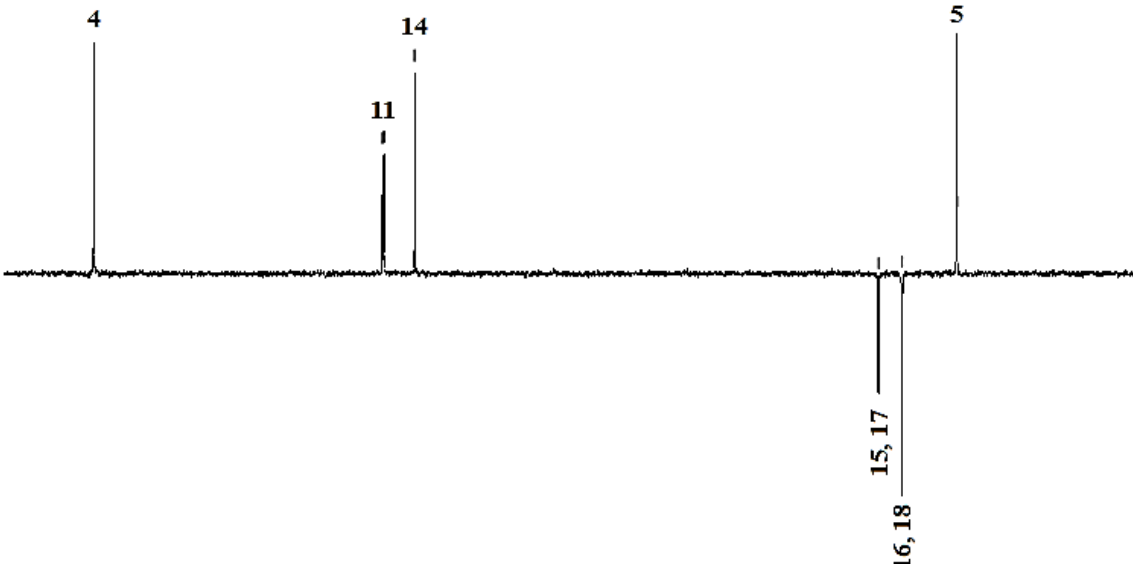

Figure S5: DEPT-135 spectra of Ciprofloxacin API when recorded in D2O at room temperature.

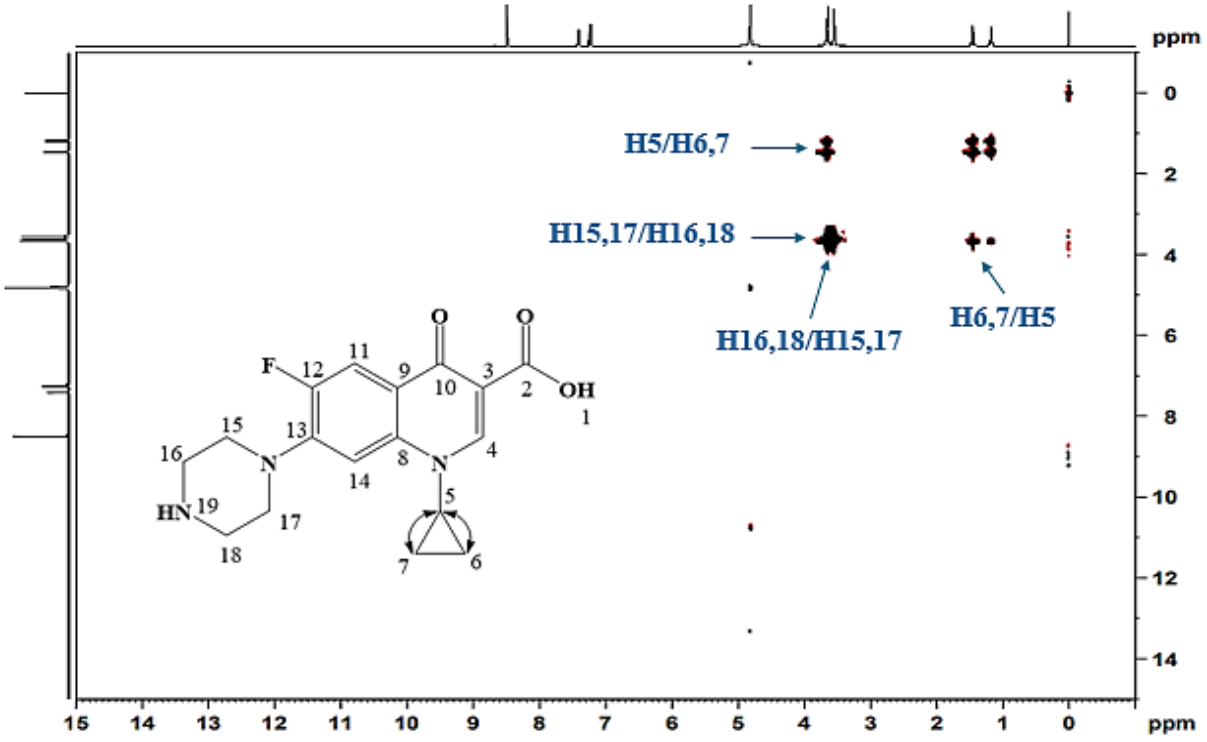

Figure S6: ^{1}H-^{1}HCOSY NMR Spectra of Ciprofloxacin (API).

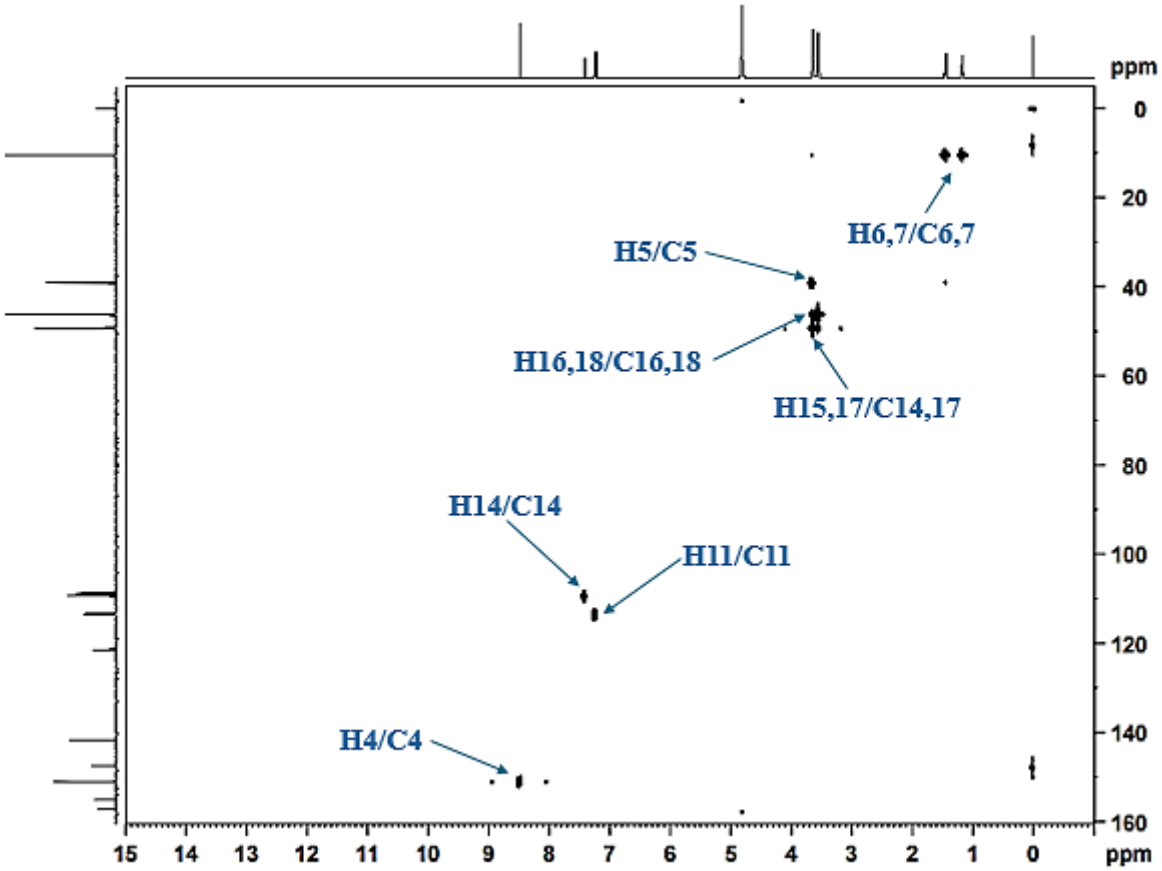

Figure S7: HSQC NMR Spectra of Ciprofloxacin (API).

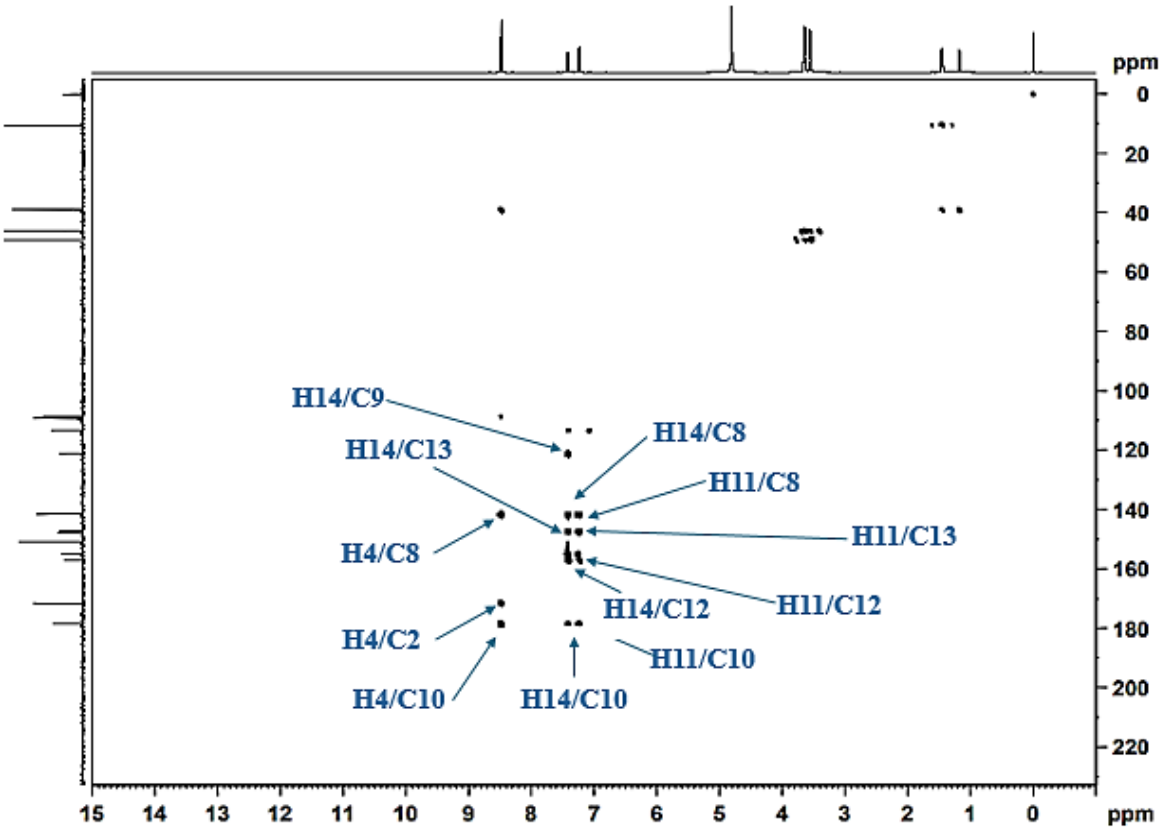

Figure S8: HMBC NMR Spectra of Ciprofloxacin (API) for Quaternary 3 bond coupling.

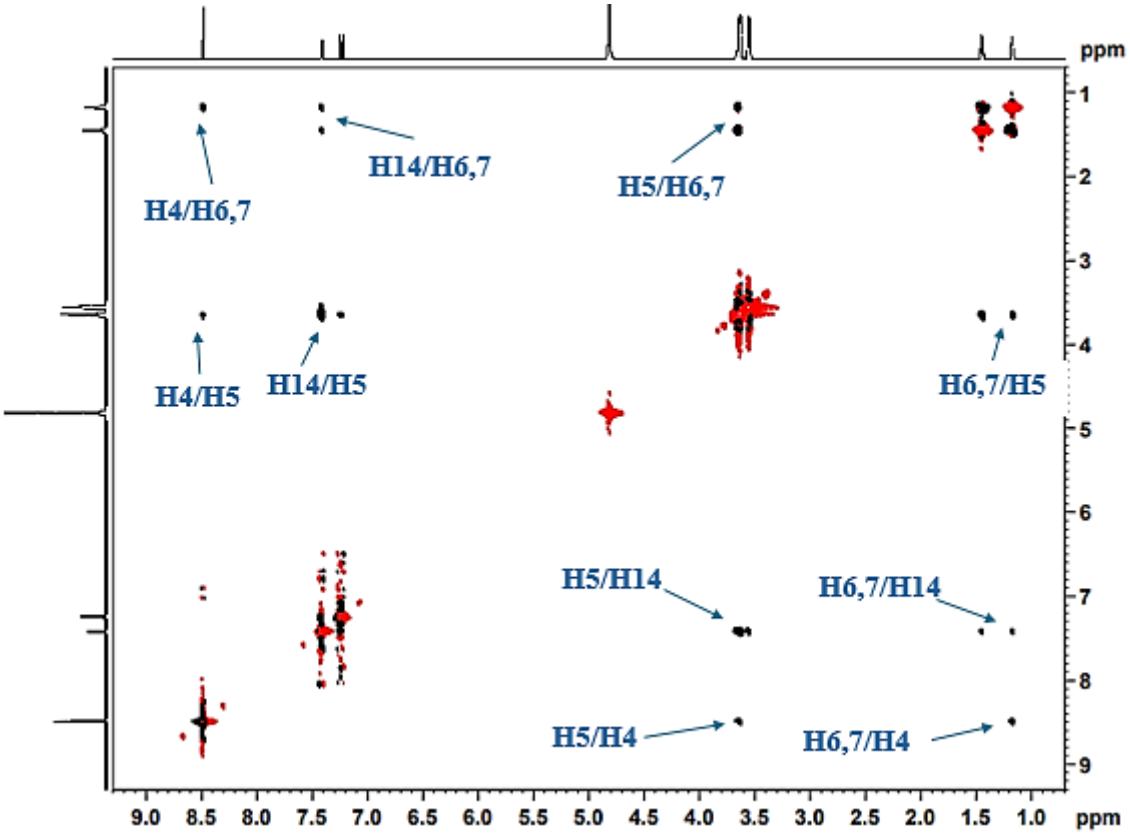

Figure S9: ROESY NMR Spectra of Ciprofloxacin (API).

Table S1: ^{1}H, ^{13}C and DEPT-135 NMR for structural assignments for Ciprofloxacin API.

Position	No. of protons	^{1}H (ppm)	Multiplicity* *J* in Hz	^{13}C (ppm)	^{13}C DEPT-135
1	OH*	-	-	-	-
2	-	-	-	171.55	C
3	-	-	-	108.63	C
4	1H	8.48	s	150.88	CH
5	1H	3.65	m	38.99	CH
6,7	2H	1.45	m	10.40	CH_2
	2H	1.18	m		
8	-	-	-	141.65	C
9	-	-	-	121.36 (d, $^{3}J_{CF}$= 7.76)	C
10	-	-	-	178.37	C
11	1H	7.24	d, *J*= 12.87	113.36 (d, $^{2}J_{CF}$= 23.70)	CH
12	-	-	-	156.07 (d, $^{1}J_{CF}$= 251.52)	C
13	-	-	-	147.41 (d, $^{2}J_{CF}$= 10.18)	C
14	1H	7.41	d, *J*= 7.26	109.30	CH
15, 17	4H	3.64	m	49.19 (d, $^{4}J_{CF}$= 4.75)	CH_2
16, 18	4H	3.56	m	46.10	CH_2
19	NH*	-	-	-	-

#s= singlet; d= doublet, m= multiplet, *Exchangeable protons are not observed.