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Review Article

Characterisation and Identification of Process Related Impurity in Amodiaquine Hydrochloride by Using Some Analytical Techniques: A Review

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ABSTRACT

The process related unknown impurity associated with the synthesis of Amodiaquine hydrochloride bulk drug was detected by high performance liquid chromatography (HPLC), was subjected to ion trap mass spectroscopy for preliminary determination of mass for identification. It was difficult to elucidate the structure with only ion-trap ms and ms/ms since it is having a unit mass resolution it became important to isolate the impurity using preparative chromatography for further study. The preparatively isolated impurity was subjected to a high resolution accurate liquid chromatography mass spectroscopy (HR/AM-LCMS) for determination of molecular formula which gave a highly confident and accurate data for structure elucidation. The postulated structure was unambiguously confirmed by ^{1}H NMR and ^{13}C NMR was proposed to be 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane (AMQ Dimer). This AMQ Dimer impurity was not been previously reported in any of the analytical literature pertaining to Amodiaquine hydrochloride. A rapid Acquity H-class gradient method with runtime of 15.0min was developed for Quantitation a C18 Hypersil gold column (100 mm x 2.1 mm i.d. 1.9 µm) was used for chromatographic separation and validated for parameters such as precision, accuracy, linearity and range, robustness. The limit of detection (LOD) established for AMQ Dimer impurity was found to be 0.0197ppm i.e. 0.012% and limit of quantification (LOQ) was found to be 0.0598ppm respectively.

Keywords: Amodiaquine hydrochloride, impurity, HPLC, Acquity UPLC H-class, Ion Trap MS, HR/AM-LCMS, NMR, structural elucidation.

INTRODUCTION

Malaria is disease caused by protozoan parasite Plasmodium which is transmitted from person to person through bites from infected female Anopheles mosquitoes. The disease has grown into a significant global health crisis and is still present today between 400 and 900 million cases of malaria are reported every year causing an average of 1 to 3 million deaths[1]. There is no vaccine available for malaria, all treatments and preventatives come in the form of prescription drugs, called antimalarials. There are numerous antimalarials available, and often treatments will include a combination of two drugs. However, the high cost and demand for antimalarials, which typically do not have a generic form, drive counterfeiting activity which severely impedes the prevention & treatment of the disease [2,3] . Amodiaquine, a 4-aminoquinoline antimalarial drug, chemically is 4-(7-Chloro-4-quinolylamino)-2-(diethylaminomethyl) phenol dihydro-chloride dihydrate form $C_{20}H_{22}ClN_3O$, 2HCl, $2H_2O$ having molecular Weight 464.8 is a rapidly acting blood schizontocide[8]. Despite the similarities in chemical structure, it is more effective than chloroquine with respect to parasite clearance[4]. A combination of Amodiaquine with pyrimethamine or sulfadoxine gave better control of clinical symptoms than the antifolates alone with no evidence of serious side effects[5]. Artesunate plus Amodiaquine combination is recommended by the World Health Organization (WHO) for use in malaria control programme and is a first-line treatment for African children with uncomplicated malaria[6,7].

A literature survey revealed there are few bioanalytical methods such as liquid chromatography with ultraviolet detection (LC/UV),[9-11] liquid chromatography mass spectrometry (LC/MS)[12-15] and electrochemical[16] detection have been used for the quantitative determination of Amodiaquine and its metabolites. Malongo *et al*.[17] described an assay method for Amodiaquine in pharmaceutical dosage form. A colorimetric[18] method for the assay of Amodiaquine has also been reported in the literature. United States Pharmacopoeia[19] and International Pharmacopeia[20] monographs describe thin-layer chromatography (TLC) methods to assay the purity of Amodiaquine where the impurity level is specified up to 0.5%. However there are some reported impurities which have been identified in Amodiaquine drug such as 4-[(5-chloroquinolin-4-yl) amino]-2-(diethylaminomethyl) phenol, 4-[(7-chloroquinolin-4-yl)-amino]phenol and 4-[(7-chloroquinolin-4-yl)amino]-2-(diethylaminomethyl)-N1-oxy]phenol in bulk drug[21] and also some metabolites such as desethyl amodiaquine, bisdesethyl amodiaquine and hydroxydesethyl-amodiaquine in plasma are reported [22-24].

Impurities are an extremely critical issue in the pharmaceutical industry especially due to the stringent regulations and manufacturing process. Impurity profile of an Active Pharmaceutical Ingredients (APIs) and evaluation of their toxicity effect is necessary step in developing a safe and effective drug and is essential for medical safety reasons[25,26]. In view of the stringent quality requirements of global regulatory authorities, it is mandatory to know structural details of impurities associated with the bulk drugs.

The aim of the present work is to identify and characterise an unknown impurity which is not reported in any analytical literature pertaining to Amodiaquine bulk drug and then developing a fast UPLC H-class method for control of this impurity. The developed method is then

validated for some parameters as per ICH guideline[27].

EXPERIMENTAL

Materials and reagents

Samples of Amodiaquine hydrochloride API were obtained from Ipca Laboratories , Mumbai, India. HPLC grade Acetonitrile, Methanol, Hydrochloric acid (35.0%) and Orthophosphoric acid (85.0%) were purchased from Merck India and HPLC grade water was taken from Millipore India. Deuterated Hydrochloric acid and acetic acid (for NMR) were purchased from Aldrich Chemical Co., USA.

Liquid chromatography

The HPLC consisted of an Alliance 2690 HPLC (Waters, Milford, MA, USA) system equipped with 2487 UV detector. A C18 column (Inertsil ODS 3V column 250 mm x 4.6 mm i.d. 5 µm) was used for separation. The mobile phase consisting of A: Mixture of 1150ml of water with 100ml of Acetonitrile, 1.0ml of Trifluoroacetic acid and B: Mixture of 200ml of water with 800ml of Acetonitrile, 1.0ml of Trifluoroacetic acid, with timed gradient programme T_{min}/A:B: T_0/100:0; T_{13}/85:15; T_{25}/0:100; T_{33}/00:100; T_{50}/100:00, T_{70}/100:00 with flow rate of 1.0 ml per minute was used. Detector was set to UV 255nm, injection volume was 10ul and sample preparation was 1000ppm in water[9-11].

Liquid chromatography for Validation

Samples were analyzed on Acquity H-class (Waters, Milford, MA, USA) system equipped with Photo diode array (PDA) detector. A C18 Hypersil gold column (100 mm x 2.1 mm i.d. 1.9 µm) was used for chromatographic separation. The mobile phase consisting of A: 1.0ml of Orthophosphoric acid (85.0%) in 1000mL of Millipore water mixed, degassed and mobile phase B: Acetonitrile, with timed gradient programme T_{min}/A:B: T_0/85:15; $T_{3.0}$/85:15; $T_{5.0}$/70:30; $T_{9.0}$/70:30; T_{12}/85:15 and T_{15}/85:15 with flow rate of 0.7 ml per minute was used. The column oven temperature was maintained at 25°C. The injection volume was 2.0µL and the detector wavelength was fixed at 255 nm. Diluent was prepared by mixing water, methanol and concentrated hydrochloric acid in the ratio of 10:90:0.60[9-11].

Liquid chromatography-mass spectrometry with ion-trap mass analyzer

The MS and MS/MS studies were performed on LCQ Advantage (Thermo Electron, San Jose, CA) ion trap mass spectrometer. The source voltage was maintained at 3.0 kV and capillary temperature at 250°C. Nitrogen was used as both sheath and auxiliary gas. The mass to charge ratio was scanned across 105-600 amu. MS/MS studies were carried out by keeping normalized collision energy at 31eV and an isolation width of 6 amu. The HPLC consisted of Thermo Electron Corporation Finnigan Surveyor quaternary gradient pump with a degasser, an auto sampler and column oven. A C18 column Inertsil ODS 3V column 250 mm x 4.6 mm i.d. 5 µm was used for separation. The mobile phase consisting of A: Mixture of 1150ml of water with 100ml of Acetonitrile, 1.0ml of Trifluoroacetic acid and B: Mixture of 200ml of water with 800ml of Acetonitrile, 1.0ml of Trifluoroacetic acid, with timed gradient programme T_{min}/A:B: T_0/100:0; T_{13}/85:15; T_{25}/0:100; T_{33}/00:100; T_{50}/100:00, T_{70}/100:00 with flow rate of 1.0 ml per minute was used. Detector was set to UV 255nm, injection volume was 10ul and sample preparation was 1000ppm in water[10-12].

Liquid chromatography-high resolution accurate mass spectroscopy (LC/HR/AM/MS)

The LC/HR/AM/MS and MS/MS studies were performed on Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc. Waltham, Massachusetts United States). HESI (Heated electron spray ionisation) source was used for ionisation. The spray voltage was maintained at 4.0 kV, Auxiliary gas flow rate was kept at 10 and capillary temperature at 320°C. Nitrogen was used as both sheath and auxiliary gas. Heater temperature was kept at 350°C and S-lens RF level at 55. The mass to charge ratio was scanned across the range from 150 to 1000amu at a resolution of 70,000 with positive ion polarity. MS/MS studies were carried out by keeping normalized collision energy of (Higher-energy collision dissociation) HCD at 18eV and an isolation width of 6amu. The LC consisted of an UPLC binary gradient pump with a degasser, an auto sampler and column oven. The sample and the impurity solution were directly infused into the mass spectrometer through the pump.

NMR spectroscopy

^{1}H, ^{13}C NMR and DEPT measurement of the isolated impurities were performed on AVANCE 400 (Bruker, Fallanden, Switzerland) instrument. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale (ppm) relative to DMSO.

Preparative liquid chromatography

Impurity was isolated from the sample using Waters Auto purification system consisting of 2525 binary gradient pump, a 2487UV detector and 2767sample manager (Waters, Milford MA, USA). A C18 column (Inertsil ODS 3V column 250 mm x 20 mm i.d. 5 µm) was used for separation. The mobile phase consisting of A: Mixture of 1150ml of water with 100ml of Acetonitrile, 1.0ml of Trifluoroacetic acid and B: Mixture of 200ml of water with 800ml of Acetonitrile, 19.0ml of Trifluoroacetic acid, with timed gradient programme T_{min}/A:B: T_0/100:0; T_{13}/85:15; T_{25}/0:100; T_{33}/00:100; T_{50}/100:00, T_{70}/100:00 with flow rate of 1.0 ml per minute was used. Detector was set to UV 255nm.

Preparation of solutions for validation of Acquity H-class method

A test preparation 500ppm of Amodiaquine hydrochloride bulk drug sample was prepared using diluent. A standard stock solution of AMQ Dimer was prepared by dissolving 0.005mg/mL (5ppm). From this stock solution a standard solution containing 0.0005mg/mL (0.5ppm) was prepared. For LOD LOQ and linearity study concentrations ranging from 0.750ppm to 0.050ppm of nine levels were prepared in diluent. For precision study 0.50ppm of AMQ Dimer solution was prepared in diluent. For accuracy study, LOQ to 120% AMQ Dimer was spiked in 500ppm Amodiaquine hydrochloride 12.

RESULT AND DISCUSSION

Detection of impurity by HPLC

During the analysis of Amodiaquine hydrochloride API using chromatographic purity method by HPLC, a late eluting impurity was found at retention time 34.0mins, this impurity was more than 0.10% by area normalisation method. This impurity was an unknown impurity which may be a process related impurity. Hence it was of most interest that if this impurity is present then it must be identified as per ICH for which preliminary LCMS was performed on ion trap instrument using method.

Preliminary LCMS study

When LCMS was performed an unknown peak was observed at retention

time 34.0mins (figure 1a) and was successfully ionised in +ve ESI (electro spray ionisation) mode. The molecular ion peak was found to be m/z 385 (M+H)+(figure 1b) and further ms/ms was performed which produced fragments, m/z 206 and m/z 180 with monochloro pattern(figure 1c) and the plausible structure was found to be as in(figure 2) Since ion-trap mass instrument is having unit mass resolution it is probable better to opt for high accurate mass instrument which would give us more information and confidence in the data hence the analysis was performed on LC/HR/AM/ MS instrument.

Isolation of Pure impurity using Preparative chromatography

The impurity was collected with the solvents using the method and the impurity was dried using lyophilized to remove water and solvent. The impurity isolated was checked for purity and was found to be 99.0%. This impurity was the subjected to LC/HR/AM/MS.

Identification of impurities by LC/HR/AM/MS

LC/HR/AM/MS and MSMS were performed as per the method described to generate the mass data for the impurity. The impurity of interest exhibits a protonated molecular ion peak $[M+H]^+$ 385.05156 (figure 3a) and a daughter ion peak of 351.08862(figure 3b). The plausible fragmentation is shown (figure 3c) . The theoretical atomic formula probability shown by Xcalibur software for 385.05106 was $C_{20}H_{14}$ $Cl_2N_2O_2$ PPM error was calculated using formula.

Error in ppm for Molecular ion =

$$\frac{\text{Theoretical value – Actual Value}}{\text{Theoretical Value}} \times 10^6$$

$$=\frac{385.05106 - 385.05156}{385.05106} \times 10^6$$

$$=1.298\text{ppm}$$

1.298ppm error is highly acceptable for characterisation of $[M+H]^+$ of unknown structure using HR/AM/MS.

Error in ppm for MS/MS (Daughter ion) =

$$\frac{\text{Theoretical value – Actual Value}}{\text{Theoretical Value}} \times 10^6$$

$$=\frac{351.08948 - 351.08862}{351.08948} \times 10^6$$

$$=2.449\text{ppm}$$

2.449 ppm error is highly acceptable for characterisation of MS/MS of unknown structure using HR/AM/MS.

Though the mass error was low it was mandatory to confirm the structure by NMR which would provide us the information of exact proton and carbon. Hence NMR of impurity and the product was carried for further confirmation of structure.

Brief synthetic preparation of Amodiaquine hydrochloride and formation of AMQ Dimer impurity

In an endeavor to synthesize Amodiaquine hydrochloride first step was acrylation of Metachloroaniline with diethyl 2-(ethoxymethylene)malonate in liquid paraffin to give the Acrylate product diethyl 2-((3-chlorophenylamino) methylene) malonate. This was subjected to cyclisation under the same condition to furnish the cyclised product ethyl 7-chloro-4-hydroxyquinoline-3-carboxylate with a good yield. Hydrolysis of the cyclised product under basic condition gave 7-chloro-4-hydroxyquinoline-3-carboxylic acid. Decarboxylation of the acid functionality, under pressure and at elevated temperature yielded 4-hydroxy-7-chloro quinoline. This

was followed by electrophilic chlorination using phosphoryl chloride to give 4,7-dichloroquinoline.

Coupling of 4,7-Dichloroquinoline and mannich base in the presence of sodium hydroxide and toluene gives Amodiaquine base. The hydrochloride salt was prepared using concentrated hydrochloric acid to obtain the final product. Formation of 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane impurity was consistently observed during the coupling stage. This impurity was generated from nucleophilic aromatic substitution of the chloro functionality at the 4th position in 4,7-Dichloroquinoline by hydroxyl group, followed by acetaldehyde cyclisation to form a 7, 19-dichloro-13-methyl-2-oxa-10,16- diazapentacyclo [12. 8. O.O"{3,12}.O"{4,9}.O"{17,22}]docosa-1(14),3,5,7,9,11,15,17(22),18,20-decaene having ethereal linkage. The AMQ Dimer impurity 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane was formed by cleavage of ethereal linkage under acidic condition (figure 4) for synthesis of Amodiaquine hydrochloride and (figure 7) for formation of impurity.

Structural confirmation of unknown impurity by NMR

During the synthesis of Amodiaquine hydrochloride the unknown impurity formed was isolated using preparative chromatography described in section 2.8. ^{1}H NMR shows a doublet at 1.93-1.94 for methyl group and quartet for methane group (CH) at 5.15-5.21, also shows a typical pattern of doublet of doublet, a doublet and a multiplet of ortho and meta proton in the aromatic region. Besides that a singlet for a proton adjacent to nitrogen (Hetero atom in the ring) is also observed (figure.5) and for ^{13}C (figure.6).

The values are; ^{1}H NMR (400MHz, DCl + CD3COOD): δ 1.93-1.94 (3H, d), δ 5.15-5.21 (1H, q), δ 7.74-7.76 (2H, dd), δ 8.20-8.21 (2H, d), δ 8.46-8.55 (2H, m),): δ 9.01 (2H, d).

^{13}C NMR (400MHz, DCl + CD3COOD): 17.2, 30.5, 118.8, 119.2, 122.4, 125.4, 129.1, 139.1, 140.7, 144.3, 168.3. NMR spectral data (table .1) confirmed the proposed structure (1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane).

Analytical Method Validation by HPLC

The validation study allowed the evaluation of the method for its suitability for regular analysis. The newly developed method for Amodiaquine hydrochloride and AMQ Dimer impurity was validated according to ICH guidelines[14]. A typical chromatogram showing Amodiaquine Hydrochloride and AMQ Dimer impurity (figure 8).

Specificity

Specificity is the ability of analytical method to measure the analyte response in the presence of its potential impurities and degradants. The specificity of the Acquity H-class liquid method was determined by injecting AMQ Dimer impurity, wherein no interference was observed for any of the components from sample.

Precision

The precision of the method was examined using six replicate injections of a standard solution. The relative standard deviation (RSD) was calculated for response (area) of AMQ Dimer impurity. The RSD for AMQ Dimer impurity was found to be 0.97% (Table .2) .The method precision was established by analyzing using six different preparations. The calculated RSD of these results was found to be within acceptable limit.(figure. 9)

Accuracy

The accuracy of the method was determined for the related substances by

spiking of known amounts of AMQ Dimer impurity in Amodiaquine hydrochloride at levels, LOQ, 80%, 100% and 120% of the specified limit. The recoveries of impurities were calculated. (Table.3 & figure 10)

Limit of detection and limit of quantification

Detection limit (DL) and quantitation limit (QL) was estimated as per ICH Q2RI. The limit of detection established for AMQ Dimer impurity was found to be 0.0197ppm i.e. 0.012% and limit of quantification was found to be 0.0598ppm i.e. 0.004% with respect to test solution 500ppm.(Table .4 and figure 11)

Linearity

Linear calibration curve were obtained over the calibration range i.e. LOQ, 50%, 80%, 100%, 120% and 150% at six concentration levels in triplicate. The results showed excellent correlation between the peak area and concentration of AMQ Dimer impurity of about 0.9990.(table 5 and figure 12)

Robustness

In all the deliberately varied chromatographic conditions (column temperature $\pm$3°C and flow rate $\pm$ 0.1ml/min), no significant changes in results were observed.

Solution stability

The solution stability of Amodiaquine hydrochloride sample and AMQ Dimer impurity was carried out by keeping both solutions in tightly capped HPLC vials at 25°C for 48 hrs in an Acquity H-class auto sampler no significant changes were observed in the peak area up to 23hrs.

CONCLUSION

An unknown impurity was identified by HPLC in Amodiaquine hydrochloride was isolated using semi-preparative HPLC for characterisation. The structural characterisation of this isolated impurity was carried out by using Ion trap LCMS, LC/HR/AM/MS and modern spectroscopic NMR techniques. The combined result of LC/HR/AM/MS and NMR confirmed the structure of unknown impurity 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane. A rapid Acquity H-class liquid chromatographic method developed was successfully validated for control of 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane in Amodiaquine hydrochloride API.

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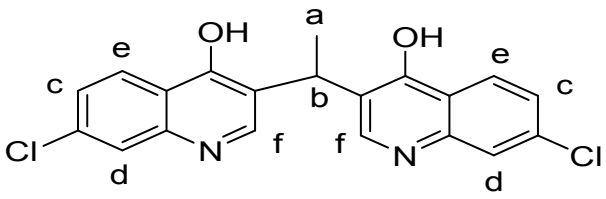
Table 1. NMR spectral data



Interpretation:

Chemical Shift Value (δ/ppm)	Assignment (Multiplicity#, Number of protons)
1.93-1.94	(d, 3H, a)
5.15-5.21	(, 1H, b)
7.74-7.76	(dd, 2H, c)
8.20-8.21	(d, 2H, d)
8.46-8.55	(m, 2H, e) + DCl & CD_3, COOD
9.01	(s, 2H, f)

S – Singlet, q – Quartet, dd – Doublet of Doublet, d – Doublet

Table 2. Table for Six replicate of Amodiaquine impurity for precision study

No of Injection	Area of Dimer
1	11552
2	11625
3	11784
4	11589
5	11799
6	11550
Mean	**11649.83**
SD	**113.2315**
%RSD	**0.97**

Table 3. Table of Amodiaquine impurity for Accuracy study

Level	Solution No.	Conc. of DIMER (ppm) (C)	Area counts in test + added Dimer	Corrected area (After subtracting the parent sample area of Dimer(A)	Recovered Quantity in ppm (B)	% Recovery	Mean (n=3)	%RSD (n=3) Of % Recovery
LOQ	Test-1	0.572	1443	1443	0.0644	112.59	110.20	3.35
	Test-2		1436	1436	0.0641	112.06		
	Test-3		1358	1358	0.0606	105.94		
80%	Test-1	0.4160	9303	9303	0.4152	99.81	99.62	1.87
	Test-2		9102	9102	0.4063	97.67		
	Test-3		9448	9448	0.4217	101.37		
100%	Test-1	0.5200	11638	11638	0.5195	99.90	101.40	1.29
	Test-2		11920	11920	0.5321	102.33		
	Test-3		11881	11881	0.5303	101.98		
120%	Test-1	0.6240	14444	14444	0.6447	103.32	102.97	0.30
	Test-2		14288	14288	0.6378	102.21		
	Test-3		14452	14452	0.6451	103.38		
					Mean	**103.55**		
					SD	**4.5960**		
					RSD	**4.63**		

Table 4. Limit of detection and limit of quantification of Amodiaquine impurity

No of Injections	Level	ppm	Area	Regression	y-Regg	$(y\text{-Regg})^2$
1	10%	0.0520	1192	1223.99	-31.99	1023.38
2		0.0520	1006	1223.99	-217.99	47519.76
3		0.0520	994	1223.99	-229.99	52895.53
1	20%	0.1040	2464	2377.78	86.22	7434.68
2		0.1040	2565	2377.78	187.22	35053.04
3		0.1040	2355	2377.78	-22.78	518.72
1	30%	0.1560	3672	3531.56	140.44	19723.23
2		0.1560	3710	3531.56	178.44	31840.63
3		0.1560	3473	3531.56	-58.56	3429.34
1	50%	0.2600	6002	5839.13	162.87	26526.36
2		0.2600	5855	5839.13	15.87	251.83
3		0.2600	5849	5839.13	9.87	97.40
1	80%	0.4160	9207	9300.49	-93.49	8739.69
2		0.4160	9178	9300.49	-122.49	15002.89
3		0.4160	9291	9300.49	-9.49	89.99
1	100%	0.5200	11750	11608.06	141.94	20147.93
2		0.5200	11573	11608.06	-35.06	1228.96
3		0.5200	11507	11608.06	-101.06	10212.43

Table 5. Linearity and range of Amodiaquine impurity

No of Injection	Level	ppm	Area	Regression	y-Regg	$(y\text{-}Regg)^2$
1	LOQ %	0.0572	1294	1459.46	-165.46	27376.05
2		0.0572	1400	1459.46	-59.46	3535.15
3		0.0572	1359	1459.46	-100.46	10091.63
1	50%	0.2600	6055	6020.73	34.27	1174.65
2		0.2600	6184	6020.73	163.27	26658.12
3		0.2600	6145	6020.73	124.27	15443.82
1	80%	0.4160	9395	9529.40	-134.40	18062.25
2		0.4160	9557	9529.40	27.60	761.99
3		0.4160	9432	9529.40	-97.40	9485.96
1	100%	0.5200	12020	11868.51	151.49	22949.65
2		0.5200	12248	11868.51	379.49	144013.74
3		0.5200	12126	11868.51	257.49	66301.83
1	120%	0.6240	13923	14207.62	-284.62	81009.27
2		0.6240	14074	14207.62	-133.62	17854.65
3		0.6240	14223	14207.62	15.38	236.51
1	150%	0.7800	17535	17716.29	-181.29	32866.18
2		0.7800	17554	17716.29	-162.29	26338.15
3		0.7800	17882	17716.29	165.71	27459.70

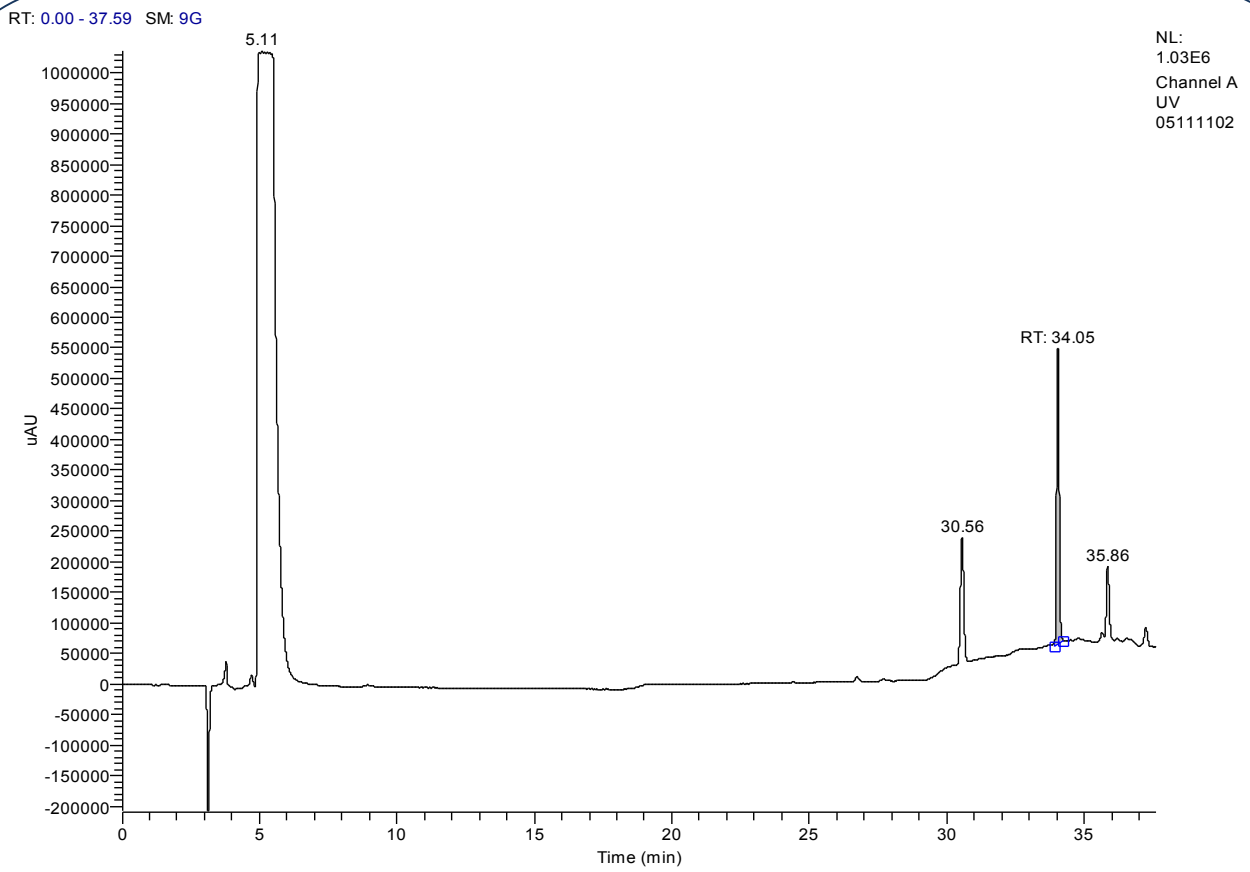


Figure 1a. A typical Chromatogram showing unknown impurity at retention time 34.05 minute in Amodiaquine hydrochloride analyzed on LC/ION-TRAP/MS Instrument

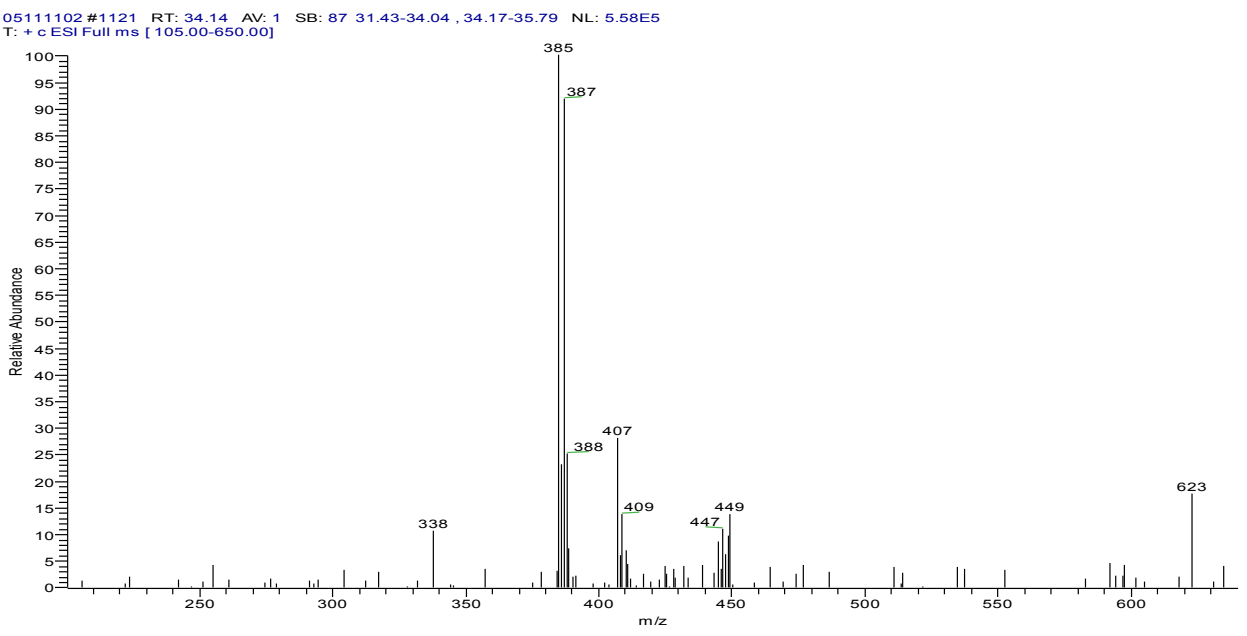


Figure 1b. Mass spectra of Amodiaquine Unknown impurity at Retention time 34.0min by LC/ION-TRAP/MS showing m/z of 385 with Dichloro pattern.

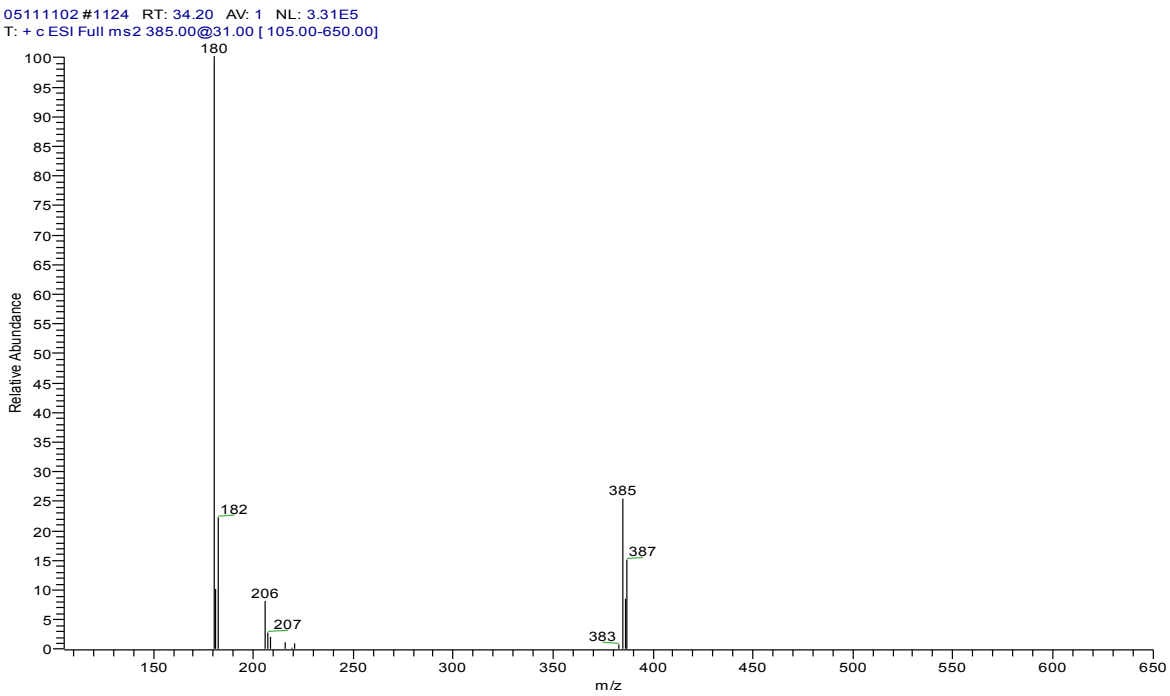


Figure 1c. MS/MS of Amodiaquine Unknown impurity at Retention time 34.0min by LC/ION-TRAP/MS showing daughter ion of m/z 206 and m/z180m/z for 385.

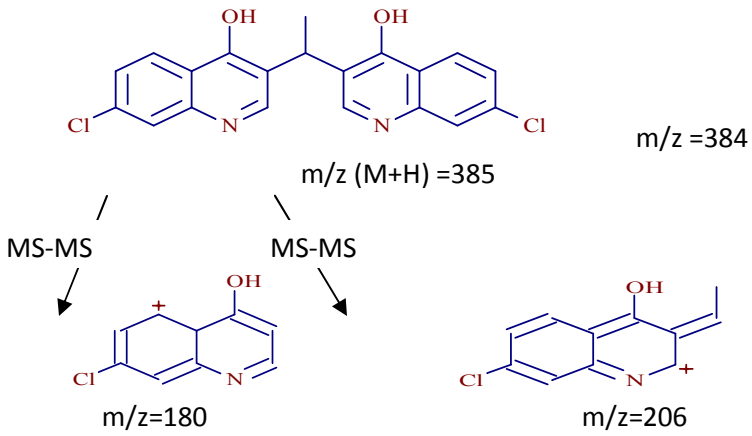


Figure 2. Plausible fragmentation for the AMQ unknown impurity at Retention time 34.0min done by LC/ION-TRAP/MS instrument

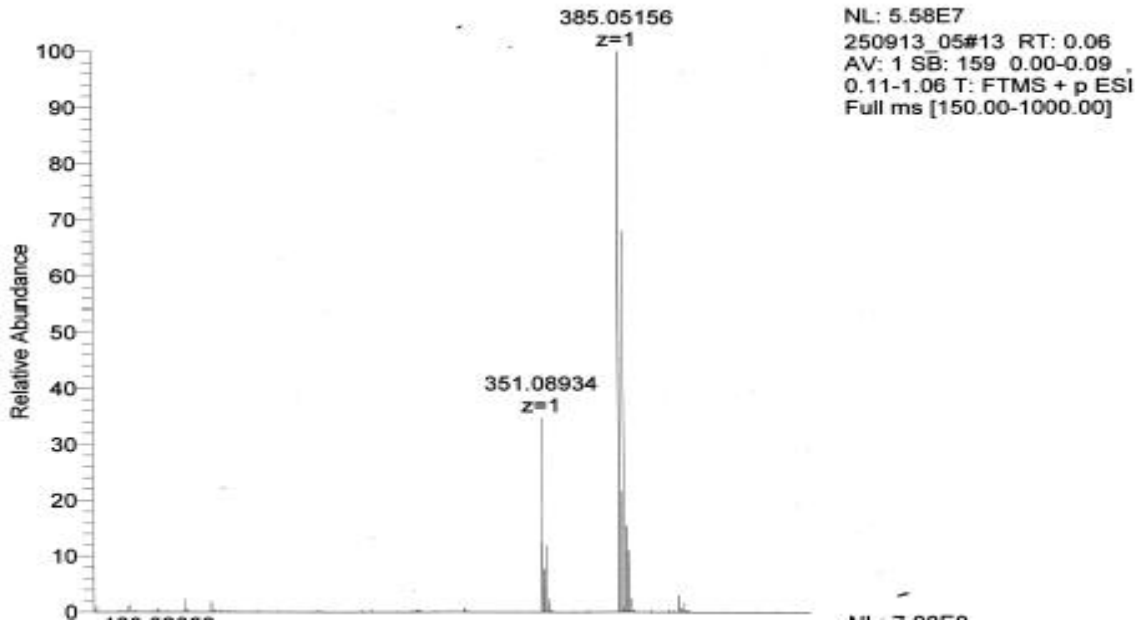


Figure 3a. Showing HR/AM/MS of unknown impurity

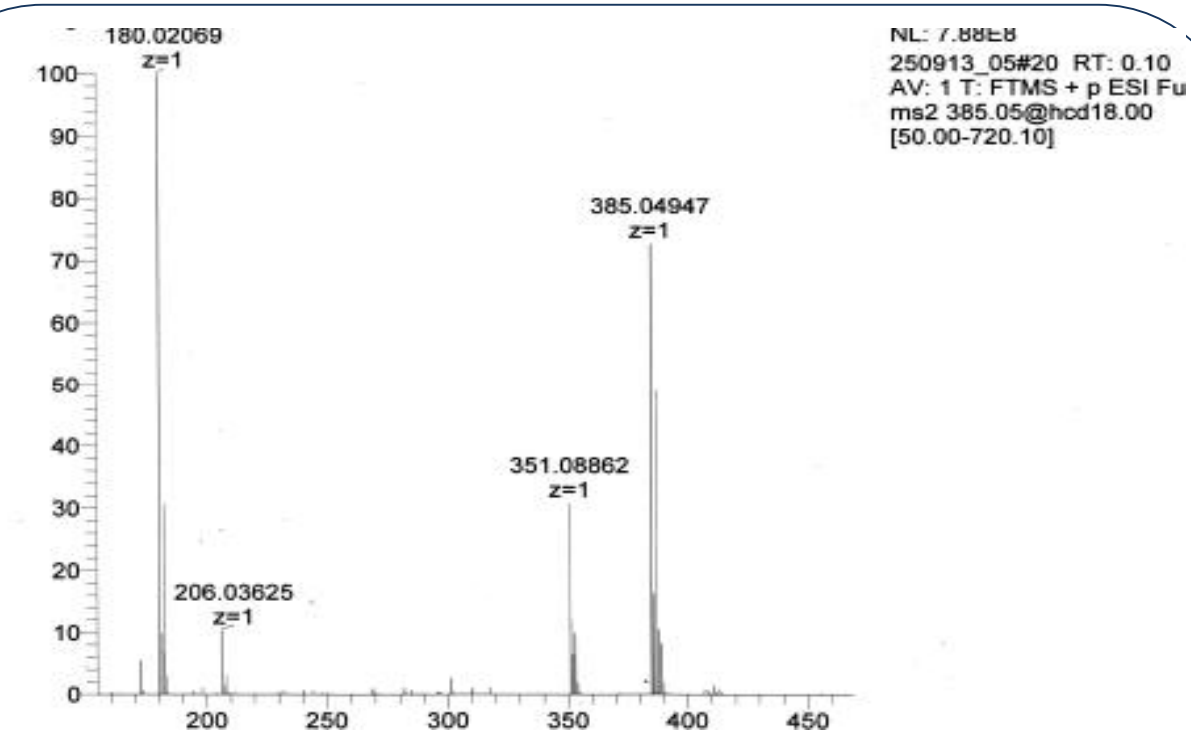


Figure 3b. Showing HR/AM/MS/MS of unknown impurity

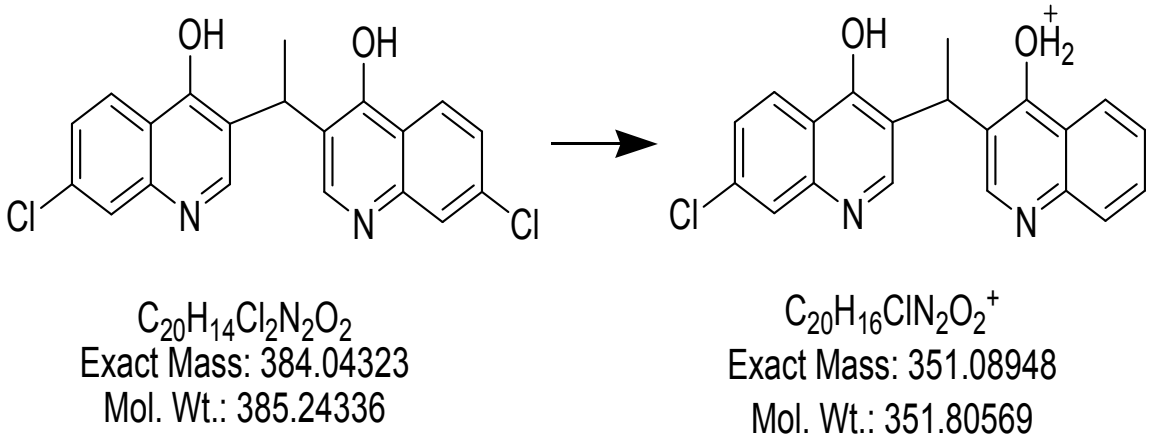


Figure 3c. Plausible fragmentation of unknown impurity LC/HR/AM/MS and LC/HR/AM/MS/MS

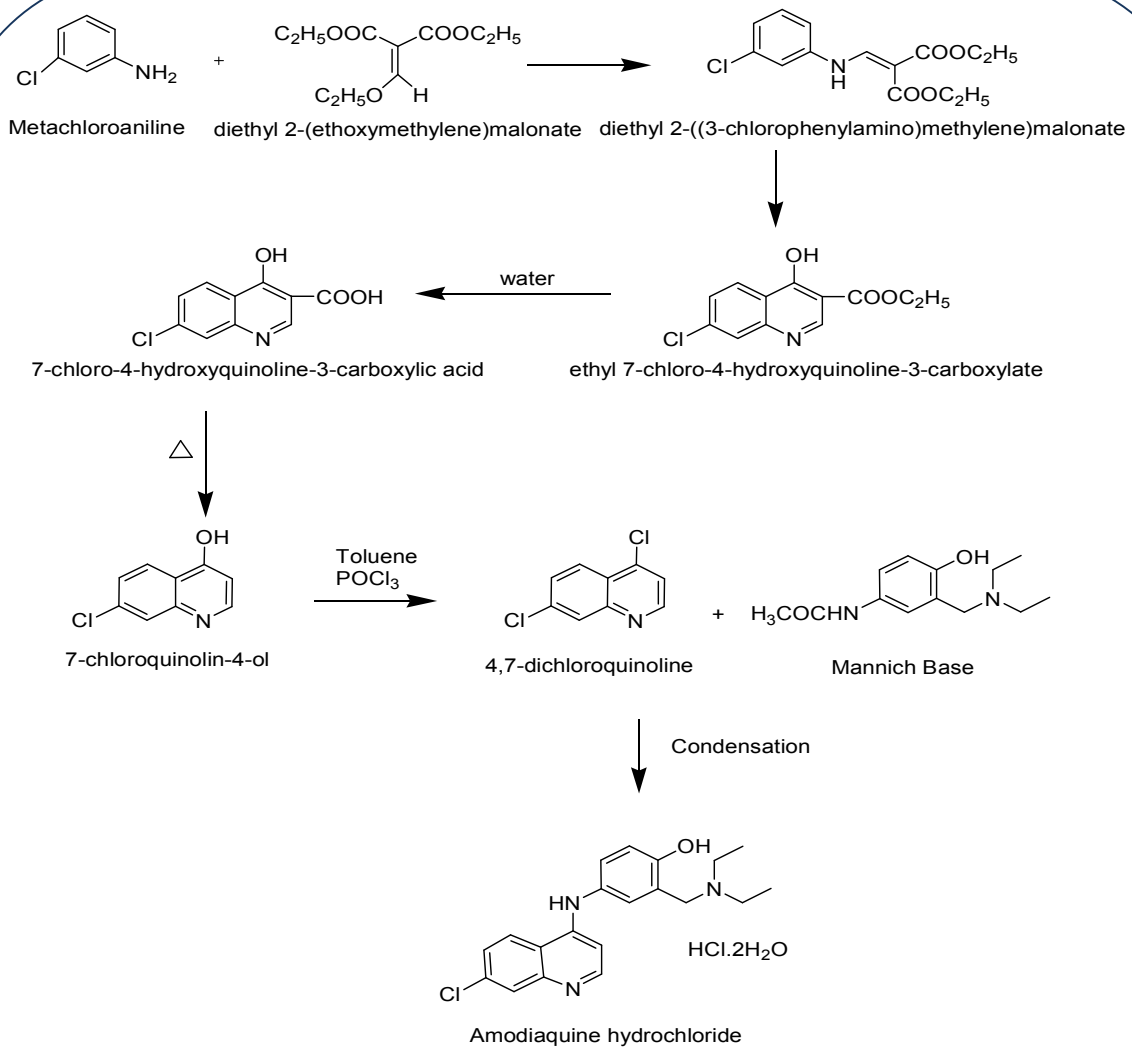


Figure 4. Synthetic scheme of Amodiaquine hydrochloride

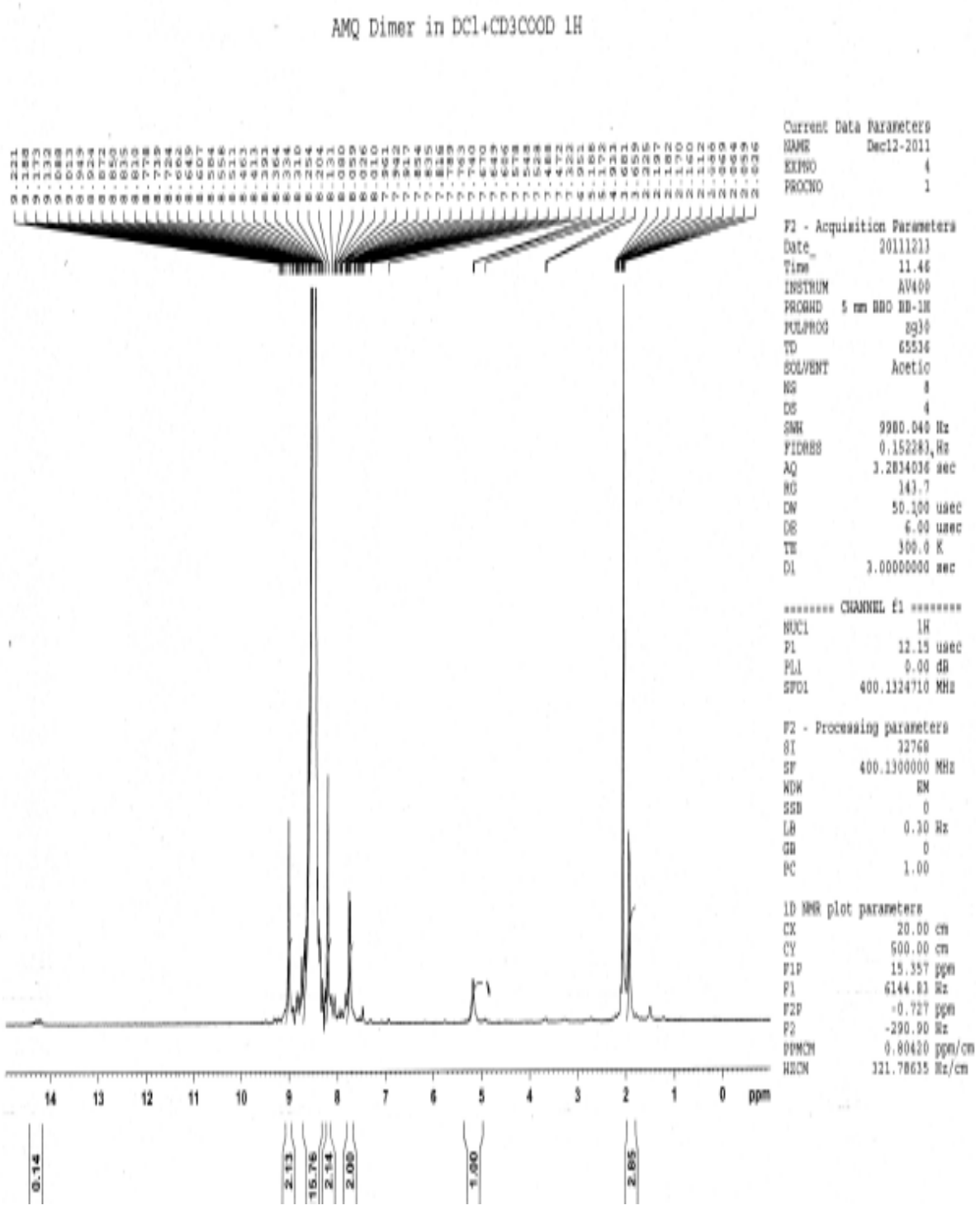


Figure.5. ^{1}H NMR for AMQ Dimer

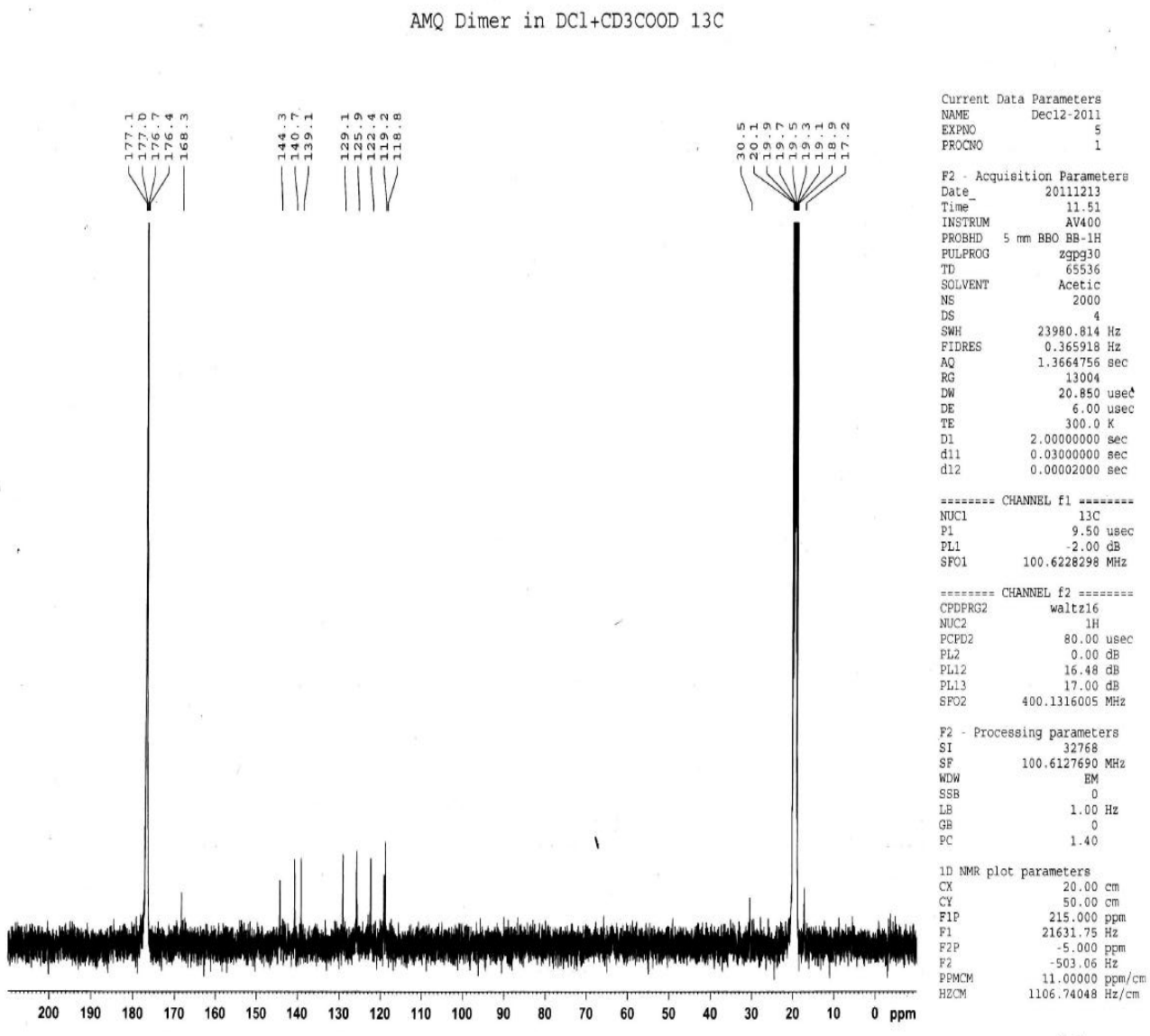


Figure 6. ^{13}C NMR for AMQ Dimer

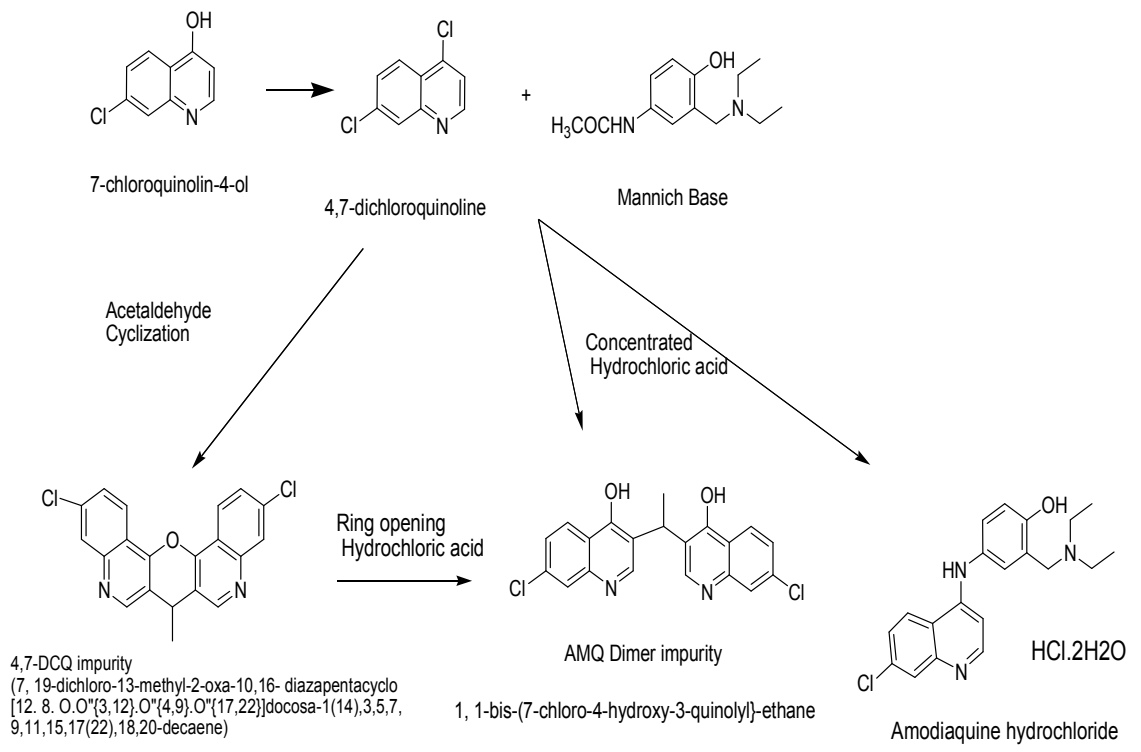


Figure 7. Synthetic Scheme for the formation of the impurity 1, 1-bis-(7-chloro-4-hydroxy-3-quinolyl}-ethane

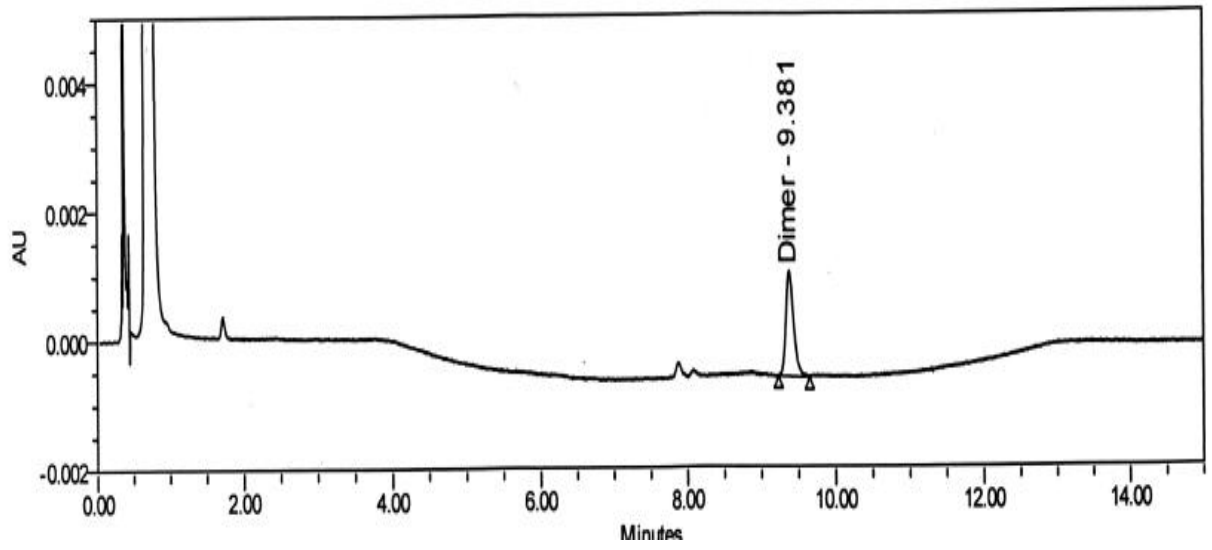


Figure 8. Typical Chromatogram of Test containing AMQ Dimer

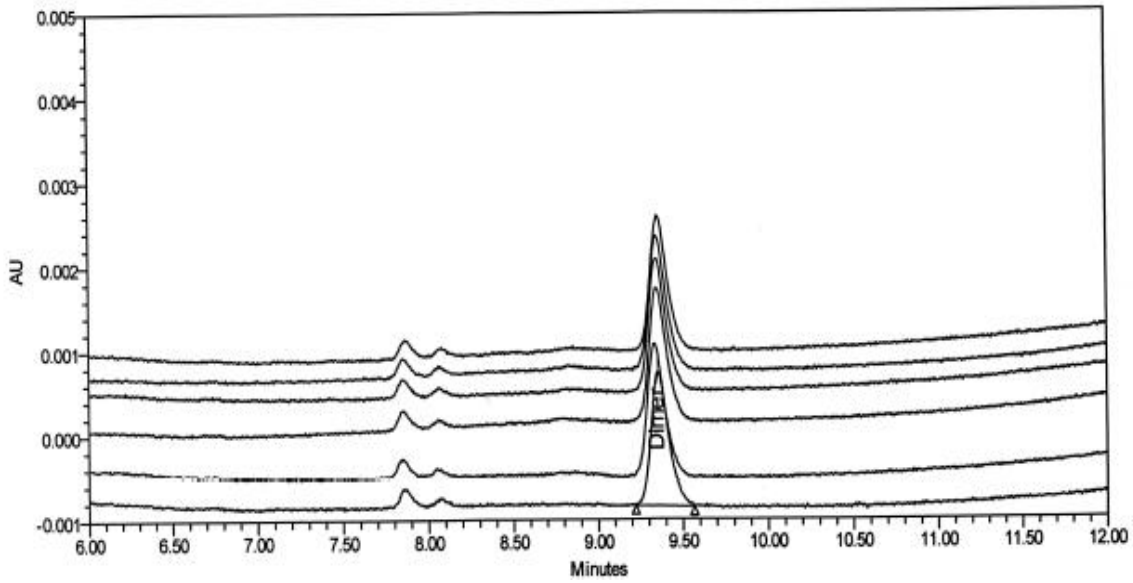


Figure 9. A typical overlay chromatogram of six replicate of Amodiaquine impurity for Precision study

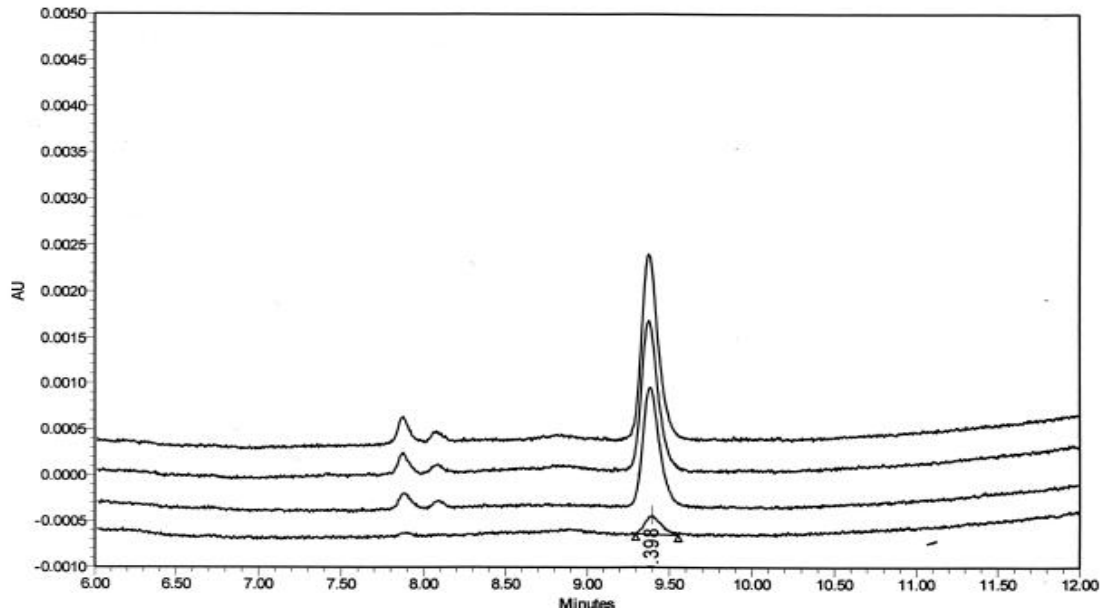
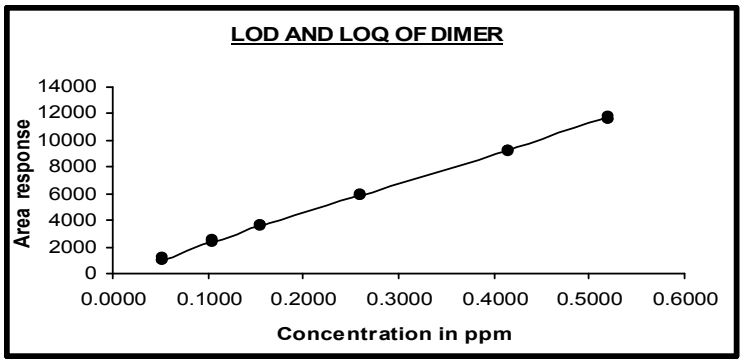
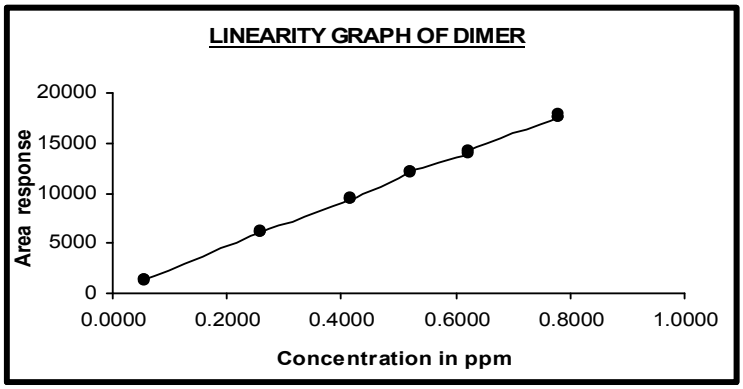


Figure 10. A typical overlay chromatogram of Amodiaquine impurity for Accuracy study at level LOQ, 80%, 100% and 120%



Intercept(a)	70.21
Slope(b)	22188.18
R2	0.9989
R	0.9994
Standard error	132.70
Residual sum of squares	281735.80
LOD	0.0197
LOQ	0.0598

Figure 11. Linearity for LOD and LOQ of Amodiaquine Hydrochloride impurity



Intercept(a)	172.95
Slope(b)	22491.47
R2	0.9990
R	0.9995
Standard error	182.28
Residual sum of squares	531619.28

Figure 12. Linearity and range of Amodiaquine impurity