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Identification and characterization of process related impurities in chloroquine and hydroxychloroquine by LC/IT/MS, LC/TOF/MS and NMR

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ABSTRACT

The focus of this study is identification and characterization of major unknown impurities in chloroquine (CQ) and hydroxychloroquine (HCQ) bulk drug samples using liquid chromatography/ion trap mass spectrometry (LC/IT/MS) and liquid chromatography/time of flight mass spectrometry (LC/TOF/MS). The newly developed LC/MS method was employed for the analysis of both the drugs. The analysis revealed the presence of two impurities in each of the drugs. The impurities are designated as CQ-I, CQ-II (for chloroquine); HCQ-I and HCQ-II (for hydroxychloroquine). Three of the impurities, CQ-II, HCQ-I and HCQ-II were unknown have not been reported previously. Accurate masses of the impurities were determined by using Q-TOF mass spectrometer and fragmentation behavior was studied by an ion trap mass spectrometer. Based on the spectrometric data and synthetic specifics the structures of CQ-II, HCQ-I and HCQ-II were proposed as 1,4 pentanediamine, N^4(7-chloro-4-quinolinyl), N^4-chloromethyl, N^4-ethylamine; 2-(4-(7-chloroquinolin-4-ylamino) pentylamino) ethanol and [[4-[(7-chloro-4-quinolyl) amino] *N*-pentyl] *N*-chloromethyl-*N*-ethylamino] ethanol respectively. The impurities were isolated by semi-preparative HPLC and structures were confirmed by NMR spectroscopy. The formation and through characterization of known CQ-I impurity is also discussed.

1. Introduction

Chloroquine (CQ) and hydroxychloroquine (HCQ) (Fig. 1) have been shown to inhibit a variety of viral infections and reduce immune reactivity [1]. These effects are mediated by a change in intracellular pH which inhibits viral and cellular enzymes involved in activation. Chloroquine is a 4-amino quinoline drug most widely used in the treatment of acute malaria caused by sensitive strains. HCQ can be used in the treatment of acute attacks and suppressive of *Malaria vivax*, *Plasmodium malaria*, *Plasmodium ovale*, and suitable strains of *Plasmodium falciparum*, systemic and discoid Lupus erythematosus and Rheumatoid arthritis [2]. It suppresses human immunodeficiency virus type-1 (HIV-1) replicating into vitro in T-cells and monocytes by inhibiting post-transcriptional modifications of the virus [3].

A pharmacokinetic study of racemic CQ and HCQ binding capacity has been reported in the literature [4,5]. The effects of CQ on viral infections and benefits of its use in anticancer strategies are reported in the literature [6]. Few reports are available on assay [7,8], simultaneous determination and enantiomeric separation of CQ, HCQ and their analogs in biological fluids using HPLC [8–10]. Determination of chloroquine and its decomposition products in various brands of different dosage forms are reported by few workers using LC and TLC [11]. Identification of a few impurities in chloroquine by GC–MS has been reported by Wenjin et al. [12].

In view of stringent quality requirements of regulatory authorities, it is mandatory to know the structural details of impurities in bulk drug samples appearing at or above 0.1% level in the drug substance [13]. During process development studies of CQ and HCQ, two impurities (ranging from 0.05% to 0.12%) in each of the bulk drugs were detected by HPLC. Hence, it was felt necessary to develop a suitable LC/MS method for the identification and characterization of process related unknown impurities in CQ and HCQ. A comprehensive study to identify, isolate and characterize the unknown process related impurities present in CQ and HCQ was the prime objective of present work.

2. Experimental

2.1. Materials and reagents

Chloroquine and hydroxychloroquine bulk drug samples were obtained from Chemical Research Division, Ipca Laboratories

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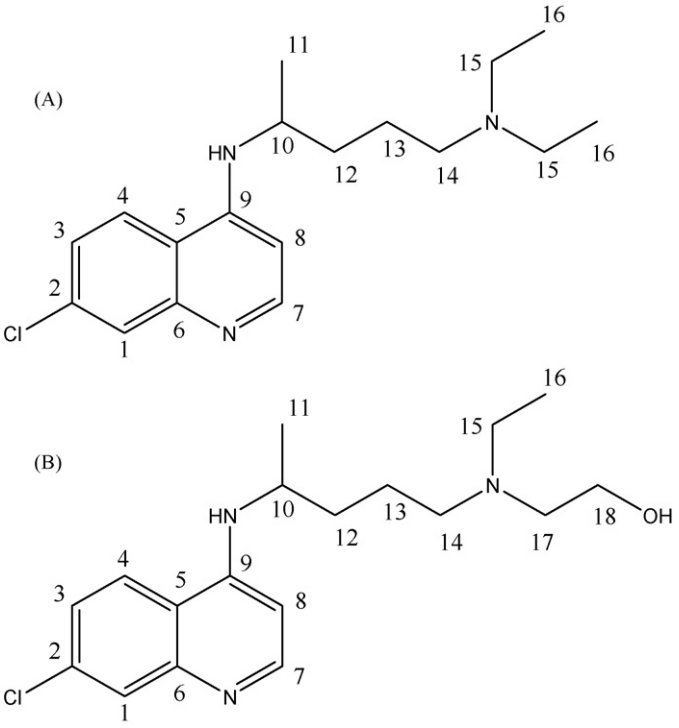

Fig. 1. Chemical structures of (A) chloroquine (CQ) and (B) hydroxychloroquine (HCQ).

Ltd., Mumbai, India. HPLC grade acetonitrile, methanol, dichloromethane and isopropyl alcohol were purchased from Merck India Limited. High purity water was obtained by Millipore MilliQ plus (Bradford, USA) water purification system. Water-d_2, methanol-d_4, and $CHCl_3$-d (for NMR) were from Aldrich Chemical Co., USA. Analytical reagent grade trifluroacetic acid (TFA) was purchased from Lancaster England.

2.2. Liquid chromatography/mass spectrometry

The LC consisted of an Agilent 1100 instrument equipped with quaternary gradient pump, a degasser and an auto sampler. A Hypurity Aquastar C18 column (250 mm × 4.6 mm i.d., particle size 5 μm) was used for chromatographic separation. The isocratic mobile phase consisted of a mixture of 0.06% aqueous trifluroacetic acid, acetonitrile and isopropyl alcohol in the ratio of 87:12:1 (v/v/v) was used. The flow rate was 1.2 ml min^{-1} and the detector was set at 220 nm. The column temperature was maintained at 40 °C. Sample solutions (200 ppm) were prepared in mobile phase and 10 μl was injected. The LC conditions mentioned above were used in the LC/UV, LC/IT and LC/TOF analysis.

The MS and MS^n spectra were recorded in positive mode on a LCQ-Advantage (Thermo Finnigan, San Jose, USA) ion trap mass spectrometer equipped with ESI-source. Nitrogen was used as both sheath and an auxiliary gas and helium was used as a collision gas. The ionization parameters were optimized by constant infusion of CQ and HCQ solutions using a built-in syringe pump at a flow rate of 10 μl/min. The spray voltage was kept at 3.0 kV and capillary temperature was set at 250 °C. The spectra were scanned across the range of m/z 100–500. MS^n studies were carried out by keeping normalized collision energy at 35% with an isolation width of 4 Da. X'calibur software was used for instrument control, data acquisition and processing.

Q-TOF-micromass spectrometer (Micromass, Manchester, UK) was used for accurate mass determination. The mass resolution of the Q-TOF MS was 6000. Leucine enkephalin ($C_{28}H_{37}N_5O_7$) was used as an external lock-mass. The source block and desolvation temperatures were 90 °C and 180 °C respectively. The nebulizer and desolvation gas flows were 20 l/h and 450 l/h, respectively. The instrument parameters in positive ion mode were: capillary voltage 3000 V, cone 25 V, extractor 2 V and MCP 2700 V. The acquisitions were done using product ion scan mode. Data acquisition and processing were done using MassLynks V.4 software.

2.3. Impurity enrichment

The CQ and HCQ free base samples were dissolved in dichloromethane and kept at 30 °C for 48 h. The solvent was evaporated by vacuum distillation. The impurity content was checked by the LC method as described in Section 2.2. The CQ-II and HCQ-II impurities were found to be increased (∼12–15%). These samples were further subjected for semi-preparative isolation. The CQ-I and HCQ-I impurities were isolated from the mother liquor samples (containing >10% of CQ-I and HCQ-I) obtained during purification of CQ and HCQ crude samples.

2.4. Semi-preparative HPLC

The impurities were isolated using Waters Auto-purification system equipped with 2525 binary gradient pump, 2487 UV detector and 2767 sample manager (Waters, Milford, MA, USA). A water symmetry C18 column (150 mm × 30 mm i.d., particle size 5 μm) was used for semi-preparative isolation. A mixture of 0.06% aqueous trifluroacetic acid, acetonitrile and isopropyl alcohol in the ratio of 87:12:1 (v/v/v) at a flow rate of 40 ml min^{-1} was used as a mobile phase. The sample solutions (∼100 mg/ml) were prepared in mobile phase. The injection volume was 1 ml and the detection wavelength was 220 nm.

2.5. NMR

The ^1H, ^{13}C, DEPT, and 2D NMR analysis of drugs and the isolated impurities were performed on a Bruker 400 MHz instrument (Faellanden, Switzerland) at 25 °C. The ^1H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to methanol-d_4 (3.31 ppm and 49.3 ppm), H_2O-d_2 (4.79 ppm) and chloroform-d (7.24 ppm and 77.0 ppm).

3. Results and discussion

3.1. Detection of impurities by LC/IT/MS

The LC/MS analysis described in Section 2.2 revealed the presence of two impurities in each of the drug samples. The impurities are marked as CQ-I (RT-8.12 min, m/z 292), CQ-II (RT-12.11 min, m/z 368) for chloroquine; HCQ-I (RT-6.92 min, m/z 308) and HCQ-II (RT-9.70 min, m/z 384) for hydroxychloroquine. The chromatograms and mass spectra of CQ, HCQ and their impurities were illustrated in Fig. 2.

Molecular mass of CQ-I was found to be matching with deethylated chloroquine impurity identified earlier using GC–MS [12]. All the impurities including CQ-I were subjected further for detailed structural investigation using spectrometric and spectroscopic techniques.

3.2. ESI–TOF analysis

The accurate masses measured on Q-TOF-microinstrument for CQ-I, HCQ-I, CQ-II and HCQ-II were 292.1582 Da, 308.1523 Da, 368.1648 Da and 384.1592 Da respectively. In order to determine the molecular formulae of unknown impurities, these figures of measured masses were plugged into the elemental composition

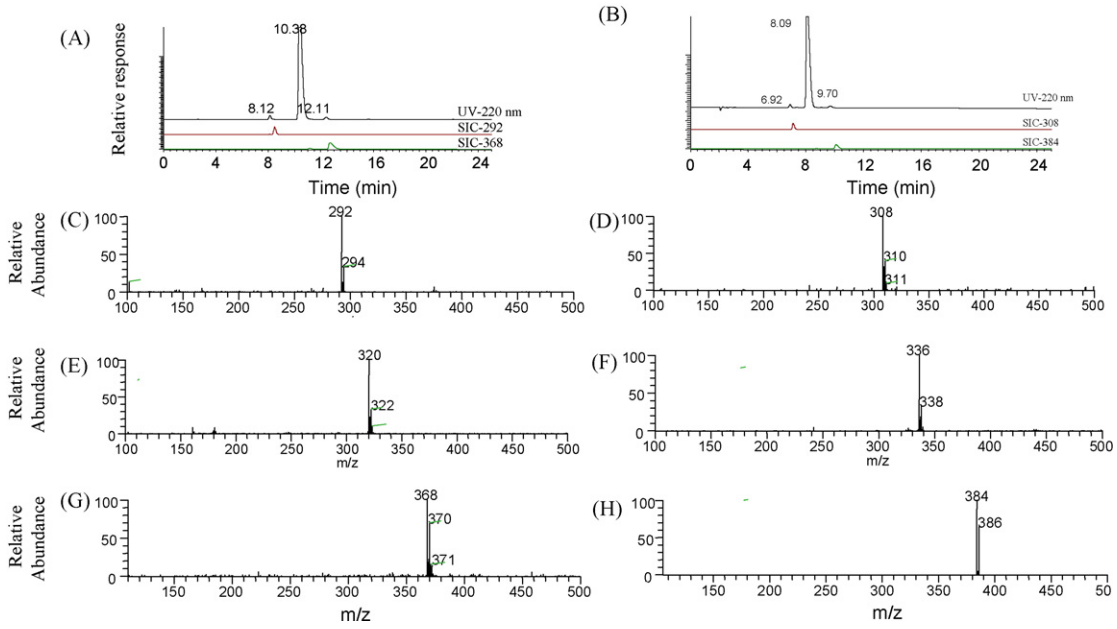

Fig. 2. Mass spectral data of chloroquine and hydroxychloroquine: (A) LC–MS-chromatograms of CQ, (B) LC–MS-chromatograms of HCQ, (C) mass spectrum of CQ-I (ESI +ve, RT-8.02–8.22), (D) mass spectrum of HCQ-I (ESI +ve, RT-6.80–7.00), (E) mass spectrum of CQ (ESI +ve, RT-10.20–10.40), (F) mass spectrum of HCQ (ESI +ve, RT-8.00–8.20), (G) mass spectrum of CQ-II (ESI +ve, RT-12.00–12.20) and (H) mass spectrum of HCQ-II (ESI +ve, RT-9.56–9.76).

calculator setting reasonable limits i.e. the unknowns possibly containing the elements C, H, N, O, and Cl. The maximum number of atoms was set at 10 for N, O, and Cl. The most likely molecular formulae for the impurities were selected on the basis of lowest difference in mDa values between experimental and theoretical masses. The ^{37}Cl patterns obtained in the mass spectra of impurities are in agreement with the selected molecular formulae. The results obtained by ESI–TOF are summarized in Table 1.

3.3. ESI–IT analysis

To study the ion selective fragmentation pathways and fragmentation up to MS^3 level, an ion trap mass spectrometer was employed. It is logical to understand the fragmentation pattern of parent drugs prior to the structural elucidation of unknown impurities. MS/MS spectrum of CQ showed a daughter ion at *m*/*z* 247 due to the neutral loss of diethyl amine moiety from parent ion (*m*/*z* 322). In the MS^3 analysis, the daughter ions at *m*/*z* 231, 191 and 179 from precursor ion (*m*/*z* 247) were obtained. The proposed fragmentation mechanism for CQ is depicted in Fig. 3.

The MS^2 and MS^3 experiments of HCQ, HCQ-I, HCQ-II, CQ-I and CQ-II showed identical fragmentation behavior as that of CQ. The MS^2 spectra showed the formation of a daughter ion fragment at *m*/*z* 247 while in the MS^3 spectra fragments were obtained at *m*/*z* 231, 191 and 179 from the precursor ion (*m*/*z* 247).

3.4. Structural elucidation of impurities

Though the fragmentation behavior of all the impurity molecules under study was identical, the neutral losses of odd mass units observed in MS/MS study were found to be different. Based on the data obtained from LC/TOF/MS and LC/IT/MS the molecular formulae for the leaving moieties were determined and given in Table 1.

The mass difference of −28 Da (292–320) corresponding to $-C_2H_5$ group was observed in the protonated molecular ion peak of CQ-I impurity as compared to that of CQ. The LC/IT/MS and LC/TOF/MS data of CQ-I impurity was correlating with the known de-ethylated chloroquine impurity [12]. In comparison with CQ-I, the molecular formulae of leaving moiety of HCQ-I were found to contain an extra oxygen atom (Table 1). Thus the most possible structure for HCQ-I can be proposed as 2-(4-(7-chloroquinolin-4-ylamino) pentylamino) ethanol (de-ethylated hydroxychloroquine) (Fig. 4). The de-ethylation of the intermediate 5-(*N*-ethyl-*N*-2-hydroxy ethylamino)-2-pentylamine (HNDA) during condensation with 4,7-dichloroqunoline (Fig. 5) can lead to the formation of de-ethylated hydroxychloroquine impurity.

The molecular ion peaks of CQ-II and HCQ-II showed ^{37}Cl isotopic peak (i.e. ∼65% relative abundance) for two chlorines while the daughter ion fragment at *m*/*z* 247 showed a single chlorine isotopic pattern (∼32% relative abundance). This clearly indicates that one of the chlorine is leaving with the neutral leav-

Table 1
Accurate mass analysis of impurities in chloroquine and hydroxychloroquine by LC–TOF–MS.

	Experimental mass (MS)	Theoretical mass (MS)	Proposed molecular formula (MS) (A)	Proposed molecular formula for daughter ion peak in (MS/MS)[a] (B)	Molecular formula for neutral leaving moiety in MS/MS (A–B)
CQ	320.1884	320.1894	$C_{18}H_{27}N_3Cl$	$C_{14}H_{16}N_2Cl$	$C_4H_{10}N$
HCQ	336.1843	336.1838	$C_{18}H_{27}N_3OCl$	$C_{14}H_{16}N_2Cl$	$C_4H_{10}NO$
CQ-I	292.1582	292.1581	$C_{16}H_{23}N_3Cl$	$C_{14}H_{16}N_2Cl$	C_2H_7N
HCQ-I	308.1523	308.1530	$C_{16}H_{23}N_3OCl$	$C_{14}H_{16}N_2Cl$	C_2H_7NO
CQ-II	368.1648	368.1660	$C_{19}H_{28}N_3Cl_2$	$C_{14}H_{16}N_2Cl$	$C_5H_{12}NCl$
HCQ-II	384.1592	384.1609	$C_{19}H_{28}N_3OCl_2$	$C_{14}H_{16}N_2Cl$	$C_5H_{12}NClO$

[a] Experimental mass (MS/MS)—*m*/*z* 247.1002 and theoretical mass—*m*/*z* 247.1001(MS/MS).

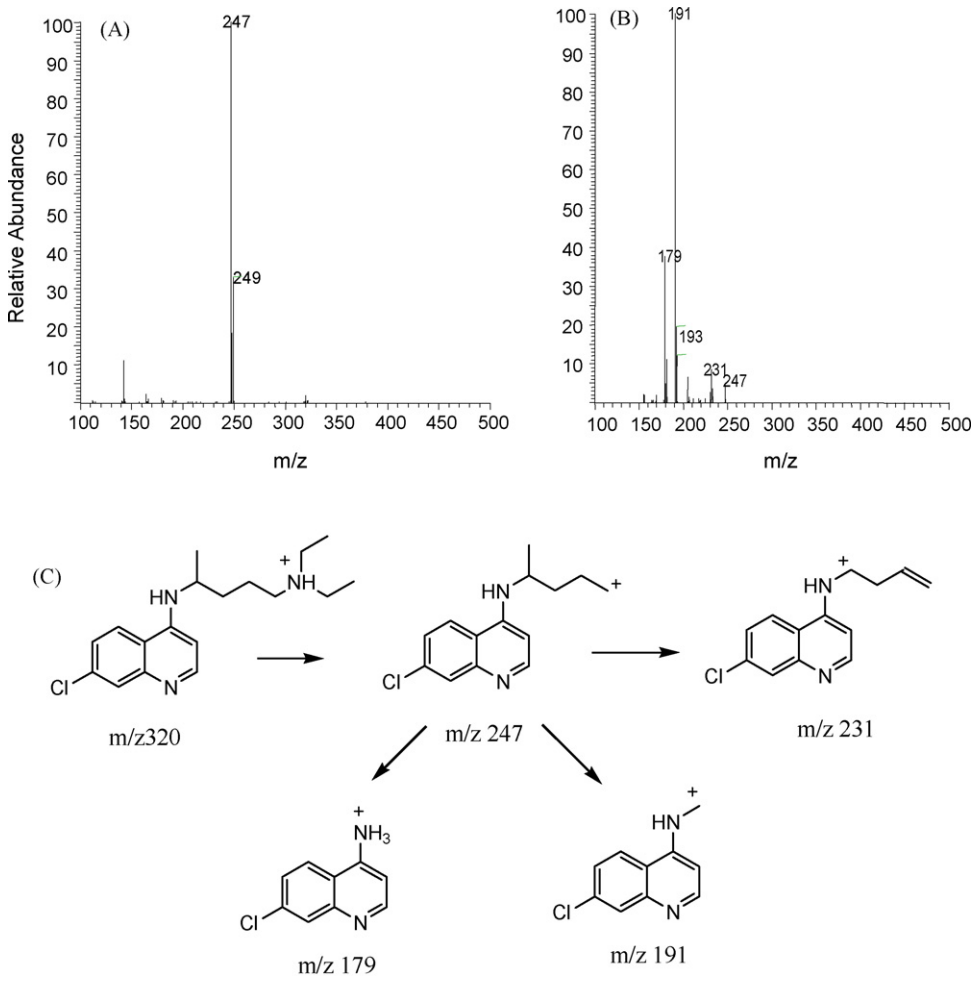

Fig. 3. MS^n data for chloroquine (CQ): (A) MS^2, (B) MS^3 and (C) fragmentation mechanism for product ions in MS^2 and MS^3 spectra.

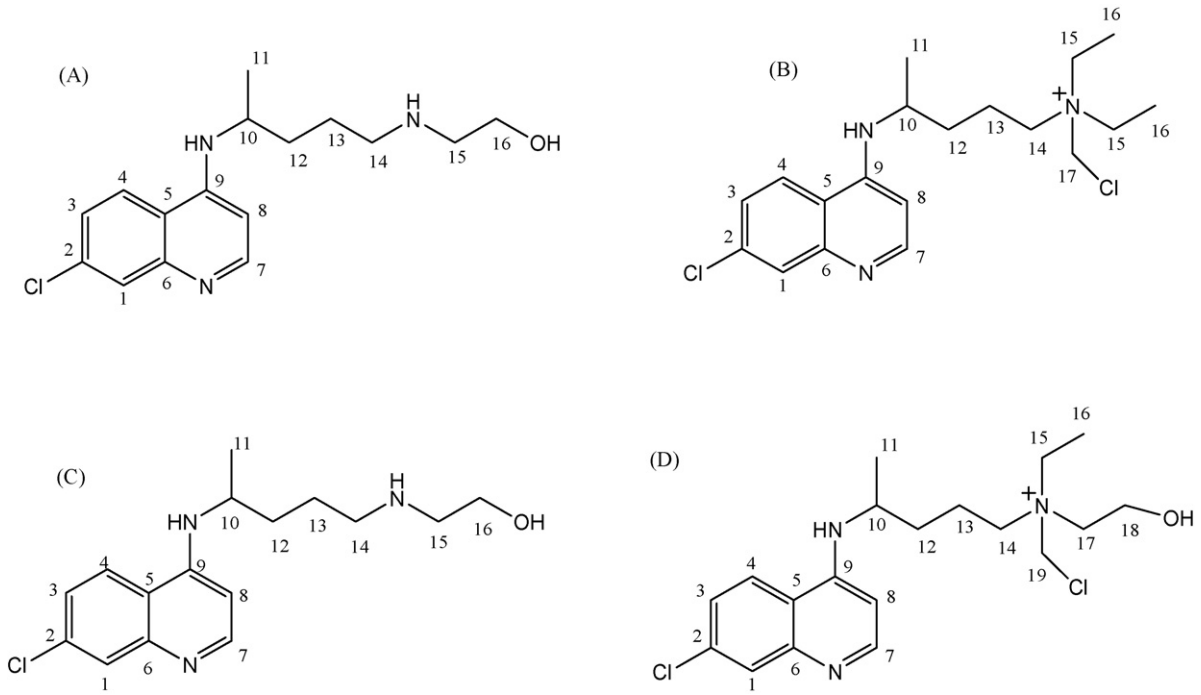

Fig. 4. Chemical structures of (A) CQ-I, (B) CQ-II, (C) HCQ-I and (D) HCQ-II impurities.

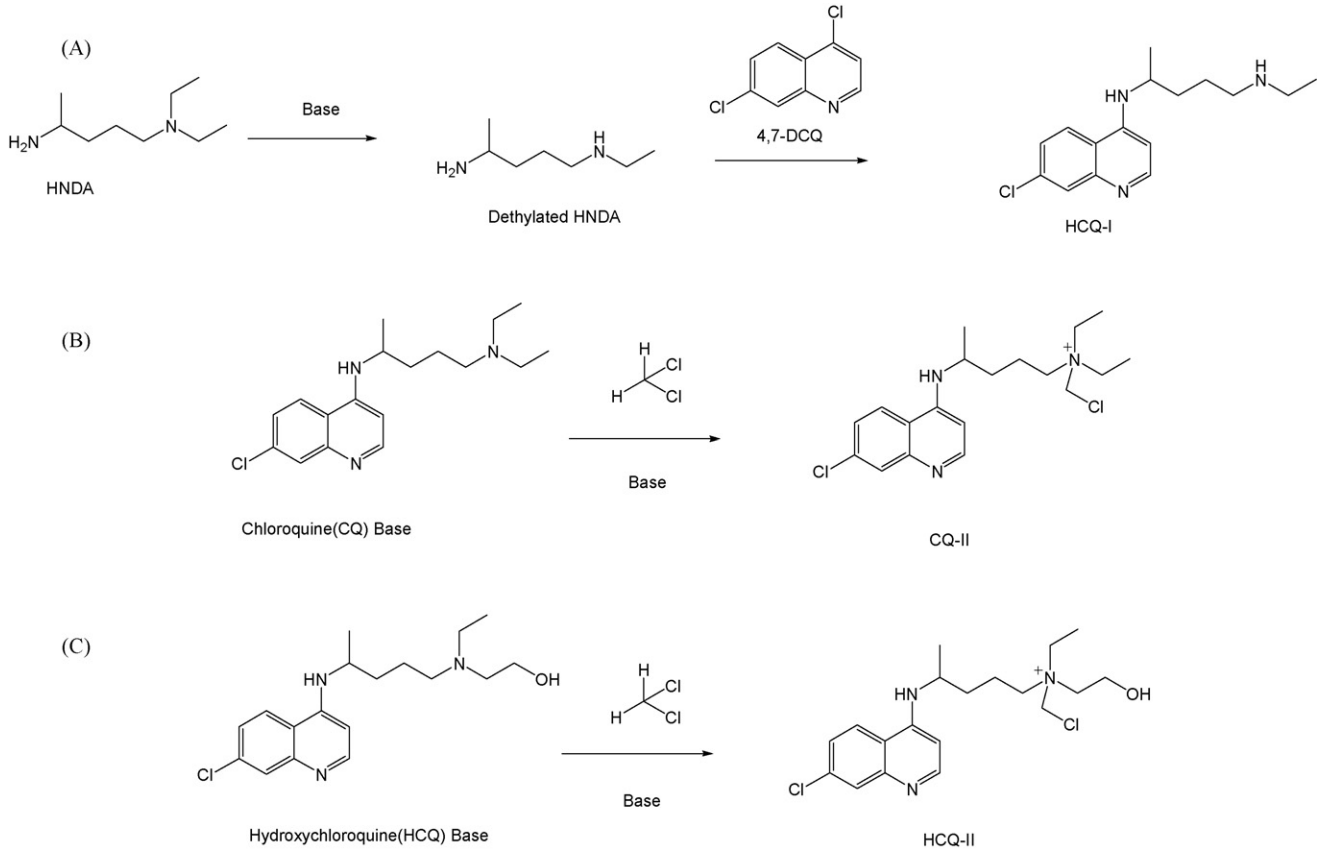

Fig. 5. Plausible mechanism for formation of the impurities: (A) HCQ-I, (B) CQ-II and (C) HCQ-II.

ing moiety in MS^2 experiment. It is evident from the purification process that the dichloromethane reacts with CQ and HCQ to form the corresponding chloromethyl derivatives (Fig. 5) This type of interaction of tertiary amine group with halogenated hydrocarbon solvents is well established [14]. Taken together, the mass spectrometric data, suggested molecular formulae and reaction scheme, the structures of CQ-II and HCQ-II can be rationalized as 1,4 pentanediamine, N^4(7-chloro-4-quinolinyl), N^4-chloromethyl,

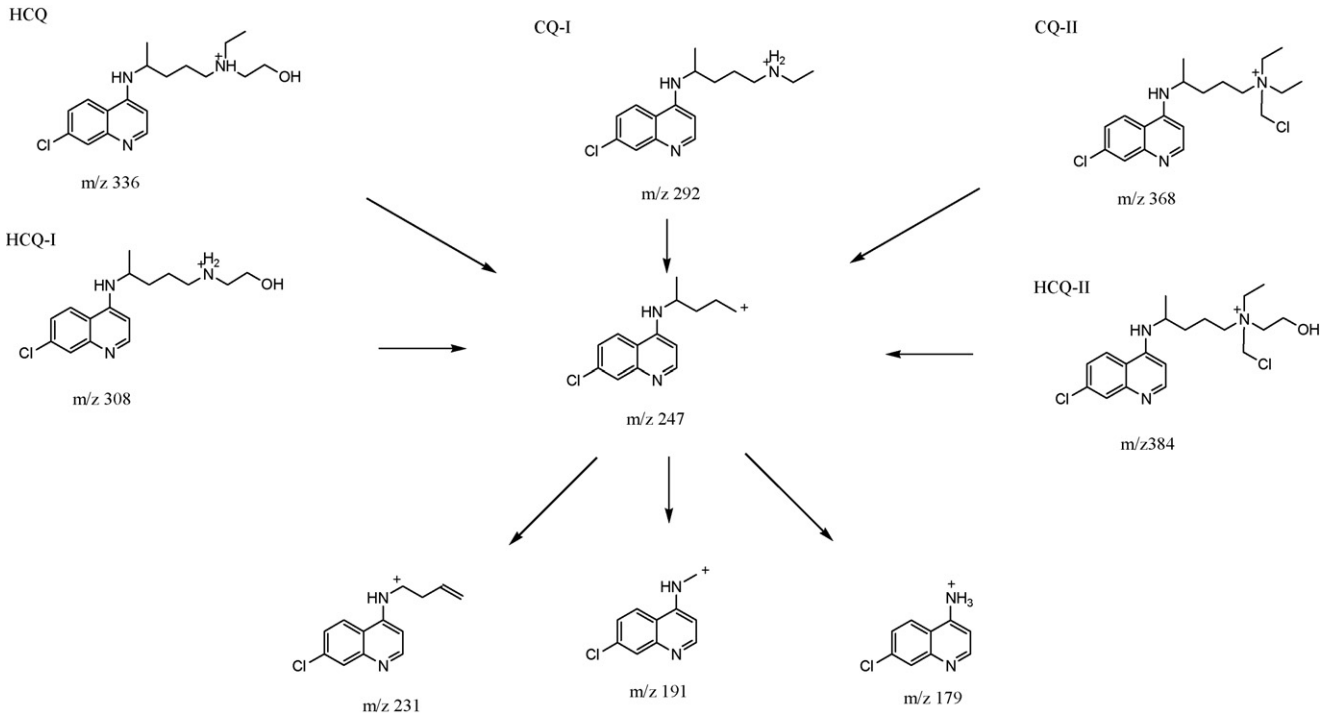

Fig. 6. Mass spectral fragmentation mechanism for HCQ, CQ-I, CQ-II, HCQ-I and HCQ-II.

Table 2
^{1}H, ^{13}C, COSY, DEPT and HETCOR data of chloroquine (CQ) and hydroxychloroquine (HCQ) in water-d_2.

	Chloroquine						Hydroxychloroquine				
	^{1}H	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR
1	1H	7.4/d, 1.7	126.9	–	CH	1H	7.1/d, 1.7	126.4	–	CH	1H
2	–	–	137.6	–	–	–	–	131.9	–	–	–
3	1H	7.3/dd, 9.1, 1.7	123.8	4H	CH	3H	7.0/dd, 9.1, 1.7	123.3	4H	CH	3H
4	1H	7.8/d, 9.1	118.5	3H	CH	4H	7.7/d, 9.1	118.5	3H	CH	4H
5	–	–	114.6	–	–	–	–	114.7	–	–	–
6	–	–	138.9	–	–	–	–	138.2	–	–	–
7	1H	8.1/d, 7.2	142.0	8H	CH	7H	8.0/d, 7.3	141.4	8H	CH	7H
8	1H	6.7/d, 7.2	98.5	7H	CH	8H	6.5/dd, 7.3	108.0	7H	CH	8H
9	–	–	154.8	–	–	–	–	154.5	–	–	–
10	1H	3.9/m	47.1	11H	CH	10H	3.9/m	47.8	11H	CH	10H
11	3H	1.3/d	18.6	10H	CH_3	11H	1.2/d	18.2	10H	CH_3	11H
12	2H	1.7–1.8/m	31.8	13H	CH_2	12H	1.6–1.7/m	32.3	13H	CH_2	12H
13	2H	1.7–1.8/m	20.2	12H, 14H	CH_2	13H	1.6–1.7/m	20.3	12H, 14H	CH_2	13H
14	2H	3.0–3.1/m	51.1	13H	CH_2	14H	3.1–3.2/m	51.4	13H	CH_2	14H
15	4H	3.0–3.1/m	49.4	16H	CH_2	15H	3.1–3.2/m	49.8	16H	CH_2	15H
16	6H	1.1/t	8.0	15H	CH_3	16H	1.1/t	8.3	15H	CH_3	16H
17	2H	–	–	–	–	–	3.1–3.2/m	57.3	18H	CH_2	17H
18	2H	–	–	–	–	–	3.8/t	59.4	17H	CH_2	18H

Refer the structural formula for numbering (Fig. 1). s, singlet; d, doublet; m, multiplete; dd, doublet of doublet; J, coupling constant.
[a] ^{1}H–^{1}H coupling constants.
[b] Hybridization (degree of bonding) of carbon atoms.

N^4-ethylamine and [[4-[(7-chloro-4-quinolyl) amino] *N*-pentyl] *N*-chloromethyl-*N*-ethylamino] ethanol respectively (Fig. 4). The fragmentation patterns are rationalized using the mechanism depicted in Fig. 6.

3.5. Isolation of impurities using semi-preparative HPLC

An isocratic reversed phase semi-preparative LC method, as described in Section 2.4, was used for the isolation of impurities. The mother liquor samples were used for isolation of HCQ-I while the impurity enriched samples were used for isolation of CQ-II and HCQ-II. The retention times of CQ, CQ-I and CQ-II were 6.21 min, 8.56 min and 10.12 min respectively, HCQ, HCQ-I and HCQ-II eluted respectively at retention times 4.81 min, 6.10 min and 7.77 min. About 50–100 mg/ml of the samples were injected at a time. The isolated fractions were freeze dried. The retention times and masses of the isolated impurities were confirmed by LC/MS. The purity of CQ-I, CQ-II, HCQ-I and HCQ-II were found to be 97.31%, 98.83%, 96.31% and 97.99% at 220 nm.

3.6. NMR analysis

In comparison with the parent drugs, the impurities showed same number of aromatic protons and similar splitting pattern in their ^{1}H NMR spectra. However, number of protons and the splitting patterns were found to be different in the aliphatic region. The methyl and methylene protons of –NH–CH_2–CH_3 group in CQ-I showed signals at δ 1.01 ppm and 2.63 ppm integrating for three and two protons respectively in its ^{1}H NMR spectra. A broad signal at δ 5.6 ppm was observed for the –NH– proton of the amino moiety. The signals due to methyl and methylene protons at δ 1.11 ppm and 3.12 ppm appeared in the aliphatic region of ^{1}H NMR of HCQ were absent in the ^{1}H NMR spectra of HCQ-I, while an additional broad signal at δ 5.2 ppm integrating for one proton was appeared in the latter. DEPT spectra of HCQ displayed negative signals for six methylene groups and positive signals for two methyl groups. The DEPT spectra of HCQ-I displayed negative signals for five methylene groups and a positive signal for one methyl group. These observations confirm the suggested structures for CQ-I and HCQ-I

Table 3
^{1}H, ^{13}C, COSY, DEPT and HETCOR data of CQ-1 and HCQ-1 impurity in $CHCl_3$-d.

	CQ-I						HCQ-I				
	^{1}H	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR
1	1H	7.8/d, 2.0	128.2	–	CH	1H	8.0/d, 2	128.8	–	CH	1H
2	–	–	134.6	–	–	–	–	134.9	–	–	–
3	1H	7.2/dd, 2, 9	124.7	4H	CH	3H	7.4/dd, 2, 9	125.2	4H	CH	3H
4	1H	7.9/d, 9	122.3	3H	CH	4H	7.7/d, 9	121.0	3H	CH	4H
5	–	–	117.4	–	–	–	–	117.2	–	–	–
6	–	–	149.2	–	–	–	–	149.0	–	–	–
7	1H	8.4/d, 6	149.5	8H	CH	7H	8.5/d, 5	149.3	8H	CH	7H
8	1H	6.31/d, 6	98.9	7H	CH	8H	6.4/d, 9	99.2	7H	CH	8H
9	–	–	151.8	–	–	–	–	152.0	–	–	–
10	1H	3.61/m	48.8	11H	CH	10H	3.7/m	49.1	11H	CH	10H
11	3H	1.2/d, 6	20.1	10H	CH3	11H	1.3/d, 7	20.3	10H	CH_3	11H
12	2H	1.1–1.2/m	33.7	13H	CH_2	12H	1.6–1.7/m	34.5	13H	CH_2	12H
13	2H	1.1–1.2/m	25.8	12H, 14H	CH_2	13H	1.6–1.7/m	20.3	12H, 14H	CH_2	13H
14	2H	2.6–2.7/m	48.3	13H	CH_2	14H	2.7–2.8/t, 5, 6	48.3	13H	CH_2	14H
15	2H	2.6–2.7/m	43.9	16H	CH_2	15H	2.6–2.7/t, 6, 7	51.1	16H	CH_2	15H
16	3H	1.0–1.1/t, 6	14.3	15H	CH_3	16H	3.7/t, 7	60.9	15H	CH_2	16H

Refer the structural formula for numbering (Fig. 4). s, singlet; d, doublet; m, multiplete; dd, doublet of doublet; J, coupling constant.
[a] ^{1}H–^{1}H coupling constants.
[b] Hybridization (degree of bonding) of carbon atoms.

Table 4
^{1}H, ^{13}C, COSY, DEPT and HETCOR data of CQ-II and HCQ-II in methanol-d_4.

	CQ-II						HCQ-II				
	^{1}H	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR	δ (ppm)/J[a]	^{13}C	COSY	DEPT[b]	HETCOR
1	1H	7.8/d, 1.6	127.5	–	CH	1H	8.0/d, 2.0	128.6	–	CH	1H
2	–	–	–	–	–	–	–	–	–	–	–
3	1H	7.4/dd, 9.2, 1.6	125.2	4H	CH	3H	7.7/dd, 9.2, 2.0	126.4	4H	CH	3H
4	1H	8.4/d, 9.2	119.3	3H	CH	4H	8.4/d, 9.2	120.3	3H	CH	4H
5	–	–	–	–	–	–	–		–	–	–
6	–	–	–	–	–	–	–		–	–	–
7	1H	8.4/d, 7.1	142.9	8H	CH	7H	8.6/d, 6.0	143.9	8H	CH	7H
8	1H	6.6 d, 7.1	99.0	7H	CH	8H	7.0/d, 6.0	100.0	7H	CH	8H
9	–	–	–	–	–	–	–	–	–	–	–
10	1H	4.0/m	49.2	11H	CH	10H	4.2/q	51.0	11H	CH	10H
11	3H	1.4/d	18.7	10H	CH_3	11H	1.4/t, 5.2	19.9	10H	CH_3	11H
12	2H	1.9–2.0/m	32.8	13H	CH_2	12H	1.8–1.9/m	32.9	13H	CH_2	12H
13	2H	1.9–2.0/m	20.0	12H, 14H	CH_2	13H	1.9/m	19.9	12H, 14H	CH_2	13H
14	2H	3.4–3.5/m	53.3	13H	CH_2	14H	3.5–3.6/m	56.5	13H	CH_2	14H
15	4H	3.4–3.5/m	54.6	16H	CH_2	15H	3.5–3.6/m	55.7	16H	CH_2	15H
16	6H	1.2/t	7.9	15H	CH_3	16H	1.4/d	7.8	15H	CH_3	16H
17	–	5.2/s	68.3	–	CH_2	17H	3.5–3.6/m	59.1	18H	CH_2	17H
18	–	–	–	–	–	–	4.0/m	60.0	17H	CH_2	18H
19	–	–	–	–	–	–	5.4/s	65.4	–	CH_2	19H

Refer the structural formula for numbering (Fig. 4). s, singlet; d, doublet; m, multiplete; dd, doublet of doublet; J, coupling constant.
[a] ^{1}H–^{1}H coupling constants.
[b] Hybridization (degree of bonding) of carbon atoms.

(Fig. 4). The de-ethylated chloroquine and de-ethylated hydroxychloroquine were known to be the metabolites of chloroquine and hydroxychloroquine respectively [15].

Presence of singlets at δ 5.24 ppm and 5.35 ppm integrating for two protons in ^{1}H NMR of CQ-II and HCQ-II respectively are due to the existence of an extra $–CH_2–$ group of chloromethyl moiety in impurity molecules. DEPT spectra of CQ-II displayed negative signals for six methylene groups; while DEPT spectra of HCQ-II displayed negative signals for seven methylene groups. Above observations confirm the proposed structures for CQ-II and HCQ-II (Fig. 4).

The ^{1}H, ^{13}C chemical shift values, ^{1}H–^{1}H, ^{1}H–^{13}C correlation and DEPT assignments for parent drugs are listed in Table 2, while the NMR data for the impurities were given in Tables 3 and 4.

4. Conclusion

A new LC/MS method was developed for the identification and characterization of process related impurities in chloroquine and hydroxychloroquine bulk drugs. Accurate masses of the impurities were determined by LC–ESI–TOF measurements. The fragmentation patterns were studied by LC/MS^n. The structures of the impurities were proposed on the basis of accurate masses, fragmentation pathways and synthetic specifics. The impurities were isolated by semi-preparative HPLC and structures were confirmed by NMR spectroscopy.

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