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A study of impurities in the repurposed COVID-19 drug hydroxychloroquine sulfate by UHPLC-Q/TOF-MS and LC-SPE-NMR

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ABSTRACT

Rationale: Hydroxychloroquine sulfate is effective in the treatment of malaria, autoimmune diseases, and as an antiviral drug. However, unreported impurities are often detected in this drug, which pose a health risk. In this study, the structures of hydroxychloroquine and six unknown impurities were analyzed using ultra-high performance liquid chromatography-quadrupole/time-of-flight tandem mass spectrometry (UHPLC-Q/TOF MS), and the structures were characterized using liquid chromatography-solid-phase extraction-nuclear magnetic resonance spectroscopy (LC-SPE-NMR).

Methods: The column was an Agilent InfinityLad Poroshell HPH-C18 (100 mm × 4.6 mm, 2.7 μm). For the analysis of hydroxychloroquine and six unknown impurities, the mobile phase was 20 mM ammonium formate aqueous solution and methanol/acetonitrile (80:20, v/v), using gradient elution. Full-scan MS and MS² were performed in order to obtain as much structural information as possible. Additionally, six unknown impurities were separated by semi-preparative liquid chromatography and characterized by LC-SPE-NMR.

Results: The MS² fragmentation patterns of the impurities were investigated, leading to more structural information and an understanding of the fragmentation pathways of the impurities. The unknown impurities’ structures were confirmed by NMR. In addition, some possible
pathways of the formation of the impurities in the drugs were outlined, and these impurities were found to be process impurities.

**Conclusions:** Based on the identification and characterization of these impurities, this study also describes the cause of the production of the impurities and provides insights for companies to improve their production processes and a scientific basis for the improvement of the related pharmacopoeias.

**Graphical Abstract**

*Keywords:* hydroxychloroquine, impurities, UHPLC-Q/TOF-MS, LC-SPE-NMR, characterization
1. Introduction

Hydroxychloroquine sulfate (HCQ), a known antimalarial drug, was first synthesized in 1949 by Alexander Surrey and Henry Hammer to reduce the health risks associated with chloroquine by introducing a hydroxyl group into the molecule [1]. Compared to chloroquine, hydroxychloroquine has better water solubility, lower toxicity, and fewer side-effects [2]. In addition to treating malaria, hydroxychloroquine has been used to treat lupus erythematosus [3]–[5] and rheumatoid arthritis [6][7] via the inhibition of a virus’s replication process and fusion with the cell membrane [8][9].

Hydroxychloroquine has been used experimentally to treat severe acute respiratory syndrome coronavirus-2 (SARS-COV-2) since the outbreak of COVID-19 in 2019 [10]–[12]. Studies of hydroxychloroquine and its combination with other drugs in the treatment of COVID-19 have been reported [13]–[16]. The determination of hydroxychloroquine and its metabolites in vivo by LC-MS has also been reported in the literature [16]–[19], as has the identification of process impurities and photo-degradation products in hydroxychloroquine sulfate by LC-MS/MS and NMR resonance—in several published reports [20][21].

According to the requirements of ICH guideline Q3B(R2) [22], the threshold needed to define an impurity is 0.1% (%: [m (impurity)/m (principal component)] × 100%, same as below) in the formulation. In this study, nine impurities, I–IX (in the range of 0.08%–0.62%), were identified during the detection of relevant substances in hydroxychloroquine sulfate tablets from two producers. Three impurities, II–IV, were reported in the European Pharmacopoeia 10.0 (EP 10.0) [23], and the remaining six unknown impurities had not reported in the British Pharmacopoeia 2020 (BP 2020) [24], the European Pharmacopoeia 10.0, the Chinese State Food and Drug Administration (SFDA) registration standards for imported drugs [25], and in other literature [20][21]. The chemical structures are shown in Table 1. Hydroxychloroquine is racemic, but only one of the enantiomeric structures is presented in this study. UHPLC-Q/TOF-MS/MS was used to investigate the main ingredient, hydroxychloroquine, and the six unknown impurities. LC-NMR can solve difficulties in the separation and purification of the impurities causing by the low content of the six unknown impurities in hydroxychloroquine sulfate tablets [26][27]. In addition, the isolates can be fully concentrated and eluted in deuterated solvents by the further coupling of LC-NMR with online solid phase extraction (SPE) which significantly improves the sensitivity of the technique [28]. The separation and concentration of the six unknown impurities were performed by semi-separation HPLC, and the structures of the six unknown impurities were
confirmed using 1D NMR and 2D NMR after further separation and purification of the concentrated impurities by LC-SPE-NMR.

2. Experimental
2.1. Materials and reagents

Samples of hydroxychloroquine sulfate tablets were obtained from Enterprise I (Sanofi (Hangzhou) Pharmaceutical Co., Batch No. 9R3X4) and Enterprise II (Shanghai ShangPharma Chinese and Western Pharmaceutical Co., Batch No. 200464). LC–MS–grade ammonium formate and CD$_3$OD of NMR were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). LC–MS–grade ammonia was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). HPLC-grade acetonitrile and methanol were purchased from Merck Co. (Darmstadt, Germany). Purified water for the experimental studies was obtained from Millipore Mill–Q–Gradient purification system (Bradford, USA).

2.2. UHPLC-Q/TOF-MS/MS analysis

Hydroxychloroquine sulfate tablets were ground into powder and dissolved in water/methanol (50:50, v/v) to make a solution with a concentration of 5.0 mg·mL$^{-1}$ and the sample solution was obtained by filtration through an organic membrane of 0.2 µm.

The UHPLC consisted of an Agilent 1290 instrument (California, USA) equipped with dual pumps, a column thermostat, an automatic sampler, and a diode array detector. Chromatographic separation was performed on an Agilent InfinityLad Poroshell HPH-C18 column (100 mm x 4.6 mm i.d., particle size 2.7 µm) (California, USA). The mobile phase A was 20 mM ammonium formate aqueous solution which was prepared by dissolving 1.26 g of ammonium formate in 1000 mL of purified water, and then the pH was adjusted to 10.3 ± 0.05 by ammonia. Mobile phase B was a mixture of methanol and acetonitrile in a ratio of 80:20 (v/v). The gradient elution program A was 0–2 min, 50% B; 2–9 min, 50%–65% B; 9–15 min, 65–70% B; 15–25 min, 70–75% B; 25–26.5 min, 75–50% B; 26.5–29 min, 50% B. The flow rate was 0.8 mL·min$^{-1}$, and the detector was set at 254 nm. The column temperature was maintained at 35°C, and the sample injection volume was 10 µL. The liquid chromatography conditions described above were used for UHPLC-Q/TOF-MS analysis.

UHPLC was coupled to an Agilent 6545 hybrid quadrupole-time-of-flight mass spectrometer equipped with an electrospray ionization (ESI) source (California, USA). The ion source temperature was 320°C, and the needle voltage was always set to 3500 V. Nitrogen was used as the drying gas at a flow rate of 8 L·min$^{-1}$. The collision energy varied between 10...
and 35 V to maximize the ion current in the spectrum. The MS and MS\(^n\) spectra were recorded in positive mode.

Instrument control and data acquisition were performed with the software Mass Hunter B.10.00 from Agilent.

2.3. Preparative HPLC

The tablets of hydroxychloroquine sulfate were milled and dissolved in a mixture of pure water and methanol in a ratio of 50:50 (v/v) to acquire a 50 mg·mL\(^{-1}\) sample solution. The sample was obtained from the solution by filtering through ultrasonication and was then injected in 1.5 mL each time. The six unknown impurities were separated in a Shimadzu high-performance preparative liquid chromatography (Shimadzu, Kyoto, Japan) with an LC-20AP binary gradient module, SIL-10AP sample injector, and an SPD-M20A PDA detector. The gradient elution was performed on an Agilent InfinityLab Poroshell HPH-C18 column (100 mm \(\times\) 21.2 mm i.d., particle size 4 \(\mu\)m) and on the mobile phases A and B in Section 2.3. The gradient program B was 0–5 min, 50% B; 5–10 min, 50–65% B; 10–15 min, 65–70% B; 15–30 min, 70–85% B; 30–33 min, 85–50% B, 33–38 min, 50% B. The flow rate was 8 mL·min\(^{-1}\), and the detection wavelength was set at 254 nm. The target fractions were collected at 6.513 min, 24.718 min, 28.838 min, 31.166 min, and 36.531 min for Enterprise I (Fig. S1) and 30.080 min for Enterprise II (Figure S2). The fractions were concentrated with a rotary evaporator (Buchi, Switzerland).

2.4. LC-SPE-NMR

LC-SPE-NMR consisted of an Agilent 1200 HPLC (California, USA) equipped with a Bruker column oven (Rheinstetten, Germany) and a Spark Prospekt 2 system (Emmen, The Netherlands). A solid phase extraction column (10 \(\times\) 2 mm i.d., particle size 10–12 \(\mu\)m) (Emmen, Holland) was used to capture the resolved peaks in the fractions.

The fractions the impurities I, V, VI, VII, VIII, and IX were separated and isolated for NMR analysis using an Agilent InfinityLab Poroshell HPH-C18 column (4.6 \(\times\) 150 mm i.d., particle size 2.7 \(\mu\)m). The mobile phases were the same as those in UHPLC. An isocratic mobile phase consisting of a mixture of A and B in a ratio of 40:60 (v/v) was used for Impurities I, V, VI, and VII, and a mixture of A and B in a ratio of 30:70 (v/v) was used for Impurities VIII and IX. The resolved peaks from fractions were captured by the SPE columns and were then dried for 1 h under nitrogen gas. The impurities were eluted from the SPE into separate 1.7 mm borosilicate NMR tubes with 50 \(\mu\)L methanol-d4 (CD\(_3\)OD). The separation chromatograms of each impurity in LC-SPE-NMR are shown in Fig. S3.
NMR analysis was performed on a Bruker Avance III 600 MHz spectrometer (Rheinstetten, Germany), using methanol-d4 (CD3OD) as a solvent. 1H, 13C, DEPT135°, HMQC, and COSY NMR spectra were conducted, and the 1H and 13C chemical shift values is reported on the δ scale in ppm relative to tetramethyl silane (TMS) (δ=0.00 ppm).

3. Results and discussion

3.1. UHPLC-MS of impurities

The UHPLC method in Section 2.3 was obtained after screening and optimizing the method for the detection of substances of interest in hydroxychloroquine sulfate tablets according to reports in the literature and national pharmacopoeias. The UHPLC chromatograms of hydroxychloroquine sulfate tablets from two producers are shown in Fig. 1. Nine different impurities, I–IX, were identified from the liquid-phase chromatograms in a range of 0.08%–0.62% according to the ICH guideline Q3B(R2) requirements. In this study, the MS spectra where the positive mode was used because of the sensitivity of the fragment ions of the impurities to the mass spectral response in this model of the main-component hydroxychloroquine and nine impurities were analyzed. According to the ESI+ mass spectra, the adduct ions [M+H]+ of hydroxychloroquine and impurities I–IX were m/z 336.1841, 180.0214, 352.1793, 308.1539, 292.1583, 407.2579, 492.3471, 653.3506, 538.2504, and 453.1615, respectively. Table 2 shows the exact mass data of ESI-MS for hydroxychloroquine and each impurity, and the theoretical mass data with deviation values of less than 5 ppm. The specific mass spectra are shown in Figs. S3–S11. From the MS results, Impurities II–IV were found to be known impurities and have been reported in EP 10.0. Thus, this study focused on the structural identification and characterization of the remaining six unknown impurities I, V, VI, VII, VIII, and IX. The MS2 date of the major fragmentation ions of HCQ and the six unknown impurities in positive ion mode are shown in Table 3.

3.2. Identification of HCQ

To further confirm HCQ, its mass fragmentation patterns were obtained from ESI/MS/MS and are shown in Fig. 2. The parent ion [M+H]+ of HCQ is shown at m/z 336.1841 with the elemental composition of C18H27ClN3O+. The splitting of the parent ion produced fragment ions m/z 247.1009, m/z 191.0370, m/z 179.0369, m/z 164.0262, m/z 158.1542, and m/z 102.0915. The structures of the fragment ions m/z 247.1009, m/z 191.0370, and m/z 179.0369 have been reported by Dongre [20]. A neutral molecule 2-(ethylamino) ethan-1-ol was removed from the parent ion which was broken at the branch chain (1) to obtain the fragment ion m/z 247.1009. After rearrangement and a loss of 2-butene from the ion m/z 247.1009, a fragment m/z 191.0370 was obtained, and then this ion was
further cleaved to a fragment ion \( m/z \) 164.0262 after HCN was removed. Besides the branch chain (1), the cleavage at the branch chain (2) in the parent ion offered a fragment ion \( m/z \) 179.0369 and 2-(ethyl(pent-3-en-1-yl)amino)ethan-1-ol, which was further protonated and ionized to fragment ion \( m/z \) 158.1542. The ion \( m/z \) 158.1542 could also be further cleaved at (3) to allow for a the fragment \( m/z \) 102.0915 with a loss of 2-butene ((E)-but-2-ene). The MS\(^2\) spectrum and the proposed fragmentation mechanism of HCQ are depicted in Fig. 2.

### 3.3 Structural elucidation of Impurities I, V, VI, VII, VIII, and IX

#### 3.3.1 Identification of Impurity I

The parent ion \([M+H]^+\) of Impurity I in the ESI/MS/MS spectrum was \( m/z \) 180.0210. The mass difference of -18 Da (162–180) corresponding to the H\(_2\)O group was observed in the fragment ion \( m/z \) 162.0102 as compared to the parent ion. The mass difference of -35 Da (145–180) corresponding to -Cl group was observed in the fragment ion \( m/z \) 145.0520 as compared to the parent ion. In addition, the peak of fragment ion \( m/z \) 145.0520 did not have the characteristic \([M+2]\) peak of elemental chloride compared with the parent ion, as is seen in the MS\(^2\) spectrum. Based on fragmentation patterns and accurate mass measurements, we propose that Impurity I was 4-hydroxy-7-quinoline. It is presumed that fragment ion \( m/z \) 145.0520 was obtained after losing a chlorine atom from the parent ion, and \( m/z \) 162.0102 was obtained from the parent ion with a loss of a water molecule. No isotopic peak of chlorine was observed in the spectra of fragment ion \( m/z \) 117.0572, indicating that it was the daughter ion of the precursor ion (\( m/z \) 145.0520) with a loss of a CO molecule. Fragment ion \( m/z \) 138.0105 was obtained after one molecule of ethynol was removed from the parent ion \( m/z \) 180.0210 when the cleavage followed the pathway (4). The unstable ion further lost one molecule of HCN to afford the fragment ion \( m/z \) 110.9996. The possible cleavage pathways are shown in Fig. 3(B). In order to confirm the structure of Impurity I, we conducted NMR experiments with \(^1\)H NMR, \(^13\)C NMR, COSY, HMQC, and HMBC assays.

The NMR data was consistent with the proposed structure of Impurity I and the detailed position of hydrogen atoms and carbon atoms in the \(^1\)H and \(^13\)C spectra. The \(^1\)H-\(^1\)H and \(^1\)H-\(^13\)C correlation in the COSY, HMQC, and HMBC spectra are shown in Table 4 and in supplementary data (Fig. S16), respectively. NMR signals in the low field at \( \delta_H \) 6–9 ppm of Impurity I were the same as the HCQ and other impurities in this region, which means that the aromatic structures of all the compounds were similar. The chemical shift of the hydrogen atom attached to the carbon atom 8 was moved to the high field with 6.34 ppm because of the hydroxyl group attached to the carbon atom 9. A broad signal at \( \delta_H \) 4.6 ppm was observed for the -OH proton of the hydroxy moiety. In addition, the chemical shift of the carbon atom 9
was moved dramatically to a low yield with 178.8 ppm under the impact of a strong electron-withdrawing effect of the oxygen atom, which further confirms the structure of Impurity I.

### 3.3.2 Identification of Impurity V

The parent ion \([M+H]^+\) of Impurity V in the ESI/MS/MS spectrum was \(m/z\) 407.2572 (Fig. 4). The fragment ion \(m/z\) 318.1732 was obtained from the parent ion \(m/z\) 407.2572 when the fraction followed the path (1) with a loss of 4-aminobutan-1-ol. The cleavage path (2) afforded fragment ion \(m/z\) 292.1575 or \(m/z\) 116.1070. With Impurity V’s cleavage through the path (3), one molecule of 4-((2-(ethylamino)ethyl)amino)butan-1-ol was removed to afford a fragment ion \(m/z\) 247.0997 followed with a loss of 2-butene ((E)-but-2-ene) to afford the fragment ion \(m/z\) 179.0371, both of which appeared in the MS spectra of HCQ. The fragment ion \(m/z\) 179.0371 was formed from fragment ion \(m/z\) 292.1575 by occurring a fracture at (5), which was stripped to fragment ion \(m/z\) 114.1277. The fragment ion \(m/z\) 58.0651 was obtained after one molecule of (E)-but-2-ene was removed from the precursor ion \(m/z\) 114.1277. The fragment ion \(m/z\) 159.5902 \([M+2H]^2+\) had the same structure but one more proton compared to the fragment ion \(m/z\) 318.1732. Based on the MS/MS fragmentation data and compared to the structure of the known impurities, a proposed structure for Impurity V is 4-((2-((4-(7-chloroquinolin-4-yl)amino)pentyl)(ethyl)amino)ethyl)amino)butan-1-ol. The possible cleavage pathways are shown in Fig. 5.

Compared with the HCQ and Impurity I, Impurity V showed the same number of aromatic protons and a similar splitting pattern in the \(^1\)H NMR spectra. However, the number of protons and the splitting patterns were different from those of Impurity I and HCQ in the aliphatic region. The proton peaks between \(\delta_H\) 3.5–3.7 ppm indicate the presence of methylene directly linked to oxygen atoms, and the four sets of proton peaks between \(\delta_H\) 2.5–3.0 ppm were four methylenes linked to nitrogen atoms. The peaks of 50–80 ppm in the \(^{13}\)C NMR spectra indicate the presence of carbon atoms connected with oxygen and nitrogen atoms. The detailed position of hydrogen atoms and carbon atoms in the \(^1\)H, \(^{13}\)C, and DEPT spectra, and the \(^1\)H-\(^1\)H and \(^1\)H-\(^{13}\)C correlation in the COSY, HMQC, and HMBC spectra are shown in the supplementary data (Table S1, Fig. S17).

### 3.3.3 Identification of Impurity VI

The parent ion of Impurity VI in the Q-TOF/MS/MS spectrum was \(m/z\) 492.3471 ([M+H]^+) and \(m/z\) 246.6775 ([M+2H]^2+) (Fig. S9). The fragment ion \(m/z\) 403.2626 was obtained from the parent ion \(m/z\) 492.3464 when the fraction followed the path (1) with a loss of 2-ethoxyethan-1-amine and then followed with the removal of neutral N-ethylprop-2-en-1-amine moiety to afford the fragment \(m/z\) 318.1732. The daughter ions \(m/z\) 201.1959 or \(m/z\)
292.1573 were obtained from the parent ion (m/z 492.3462 when the cleavage path was (2). The fragment ion m/z 158.1538 was obtained due to the neutral loss of ethylamine moiety from precursor ion (m/z 201.1959), followed by the rearrangement and the removal of a 2-(ethylamino)-1-ethanol moiety to afford the fragment ion m/z 112.1123. The loss of ethylene amine moiety led to the formation of fragment ion m/z 69.0702. The mass-to-charge ratios of the fragment ions m/z 202.1349 and m/z 159.5905 had a 2M-1 relationship with the fragment ions m/z 403.2626 and m/z 318.1730, respectively, and so it was deduced that they had the same structure but for a difference of one H⁺. The structures of the fragment ions m/z 318.1730, m/z 292.1573, m/z 247.0996, m/z 179.0367, m/z 159.5905, and m/z 112.1123 were consistent with the previous description. Based on the above data, the structure of Impurity VI is proposed as N₁-(2-((5-(2-aminoethoxy)pentyl)(ethyl)amino)ethyl)-N⁴-(7-chloroquinolin-4-yl)-N₁-ethylpentane-1,4-diamine. The possible cleavage pathways of Impurity VI are shown in Fig. 6.

The ¹H NMR spectrum of Impurity VI showed the same number of protons and a similar splitting pattern in the aromatic region with the other impurities. The peaks at the range of 3.5–3.7 ppm and 2.5–3.0 ppm indicate the presence of methylene linked to oxygen atoms and nitrogen atoms. The methyl protons of the -N-CH-CH₃ group in Impurity VI show signals at δH 1.09 ppm integrating for three protons in its ¹H NMR spectrum. The methyl protons of the -N-CH₂-CH₃ group in Impurity VI showed signals between 1.06–1.00 ppm (the combination of two triplet signals) integrating for six protons. The DEPT spectra of Impurity VI displayed negative signals for the 14 methylene groups. These observations confirm the proposed structure of Impurity VI. The detailed ¹H NMR, ¹³C NMR, COSY, DEPT, HMQC, and HMBC spectra are shown in the supporting information (Fig. S18, Table S2).

### 3.3.4 Identification of Impurity VII

The parent ion of Impurity VII in the ESI/MS/MS spectrum was m/z 653.3506 ([M+H]⁺) and m/z 327.1795 ([M+2H]²⁺) (Fig. S10). The fragmentation of the parent ion m/z 653.3506 ([M+H]⁺) in MS² produced major fragment ions of m/z 247.0998 and m/z 318.1731 along with minor fragments of m/z 191.0370 (Fig. S14). A difference of 335 Da (HCQ) between the parent ion and the fragment ion m/z 318.1731 suggests that the latter was formed by the loss of an HCQ molecule from the parent ion. The formation of fragment ion m/z 247.0998 was possible by the loss of N-ethylethenamine from the ion m/z 318.1731. After rearrangement and the removal of neutral (E)-but-2-ene moiety to afford the fragment ion m/z 191.0371. The fragmentation of the parent ion m/z 327.1795 ([M+2H]²⁺) in MS² produced major fragment ions of m/z 247.0998 and m/z 282.6371 along with minor fragments of m/z 336.1833, m/z...
318.1729, m/z 159.5905, m/z 102.0914, and m/z 90.0914 (Fig. S14). The difference of 336 Da (HCQ+H\(^+\)) between the parent ion m/z 327.1795 ([M+2H]\(^2+\)) and the fragment ion m/z 318.1731 suggests that the fragmentation of the parent ion produced daughter ion m/z 318.1731 and m/z 336.1833 ([HCQ+H]\(^+\)). The formation of major fragment ion m/z 282.6371 ([M+2H]\(^2+\)) was possible by the loss of 2-(ethylamino)ethan-1-ol moiety (89 Da) as indicated by the mass difference of (44.5 Da or 89 Da) with respect to m/z 327.1788. Based on the Q-TOF/MS/MS, the structure of Impurity VII is proposed as N\(^4\)-(7-chloroquinolin-4-yl)-N\(^1\)-(2-(2-(4-(7-chloroquinolin-4-yl)amino)pentyl)(ethyl)amino)ethoxy) ethyl)-N\(^1\)-ethylpentane-1,4-diamine. The possible cleavage pathways of Impurity VII are shown in Fig. 7.

The \(^1\)H NMR spectrum showed two similar sets of peaks in the aromatic region compared with the spectrum of HCQ indicating the existence of two 7-chloroquinolin moieties. The signals of multiplet peaks at δ\(_\text{H}\) 3.5–4.0 ppm suggest the presence of methylene groups attached to the oxygen atoms. The existence of triplet and quartet peaks between δ\(_\text{H}\) 2.4–3.0 ppm indicates the existence of methylenes directly attached to nitrogen atoms. The structure of the compounds was further determined by \(^{13}\)C NMR, COSY, and HMQC analysis, and the detailed data are shown in the supporting information (Fig. S19, Table S3).

### 3.3.5 Identification of Impurity VIII

The parent ion of Impurity VIII in the Q-TOF/ESI/MS/MS spectrum was m/z 538.2504 ([M+H]\(^+\)) along with the heaviest fragment of m/z 269.6293 ([M+2H]\(^2+\)) (Fig. S11). The fragmentation of the parent ion m/z 538.2504 ([M+H]\(^+\)) in MS\(^2\) produced a major fragment ion m/z 247.0998 along with minor fragments of m/z 292.1574, m/z 191.0369, m/z 179.0368, and m/z 114.1278 (Fig. S15). A difference of 178 Da between the parent ion and the fragment ion m/z 360.2198 suggests that the latter was formed by the loss of a 7-chloroquinolin-4-amine molecule from the parent ion. The formation of fragment ion m/z 247.0998 was possible by the loss of N-ethylpent-4-en-1-amine from the ion m/z 360.2198. The fragment ion m/z 191.0369 was formed by the loss of (E)-but-2-ene moiety from ion m/z 247.0998, similarly to the formation in Impurity VII. The removal of HCN moiety from the precursor ion afforded the fragment ion m/z 164.0261 (Fig. 8). The fragmentation of the parent ion m/z 269.6293 ([M+2H]\(^2+\)) in MS\(^2\) produced major fragment ions of m/z 247.0996 and m/z 179.0370 along with minor fragment ions of m/z 292.1573, m/z 205.0524, m/z 191.0369, m/z 152.5826, and m/z 114.1278 (Fig. S15). The formation of fragment ion m/z 152.5826 with a charge value of +2 is proposed to be via the \(\alpha\) cleavage in the path (1) of the parent ion 269.6293 ([M+2H]\(^2+\)), while the the formation of major fragment ion m/z 292.1573 was possible via the i cleavage in the path (2) of the parent ion. The removal of neutral ethylamine
mole of fragment ion \( m/z \) 292.1573 afforded the ion \( m/z \) 247.0996. The formation of the ions \( m/z \) 205.0524, 191.0369, and 114.1278 took place in similar ways to those in Impurity VII. Based on accurate Q-TOF/MS/MS fragmentation and the above analysis, the structure of Impurity VII is proposed as \( N^3-(7\text{-chloroquinolin-4-yl})-N^5-((R)-4-((7\text{-chloroquinolin-4-yl)}\text{amino})\text{pentyl})-N^1\text{-ethylpentane-1,4-diamine} \). Proposed cleavage pathways of Impurity VIII are shown in Fig. 7.

The signal of multiplet peaks at \( \delta_H \) 3.5–4.0 ppm in the \(^1H\) NMR spectrum of Impurity VIII show the presence of methylene groups attached to the nitrogen atoms. The existence of multiple multiplets between \( \delta_H \) 1.3–2.0 ppm confirms the existence of methylenes directly attached to carbon atoms. The triplet peak at 1.18 ppm was the signal of methyl group of -N-CH\_\_\_\_CH\_3. The DEPT spectra of Impurity VIII displayed 7 negative signals for the 7 methylene groups in the structure. The structure of Impurity VIII was further confirmed by the COSY and HMQC spectra, which are shown in the supporting information (Fig. S20, Table S4).

### 3.3.6 Identification of Impurity IX

The parent ion of Impurity IX in the ESI/MS/MS spectrum was \( m/z \) 453.1615 ([M+H]+) and 227.0845 ([M+2H]+\(^2+) (Fig. S12). The fragmentation of the parent ion \( m/z \) 453.1615 ([M+H]+) in MS\(^2\) produced a major fragment ion \( m/z \) 247.0999 along with minor fragment ions of \( m/z \) 275.1306, \( m/z \) 219.0681, \( m/z \) 207.0683, \( m/z \) 191.0370, and \( m/z \) 179.0369 (Fig. S16). The fragmentation of the parent ion 227.0845 ([M+2H]+\(^2+\)) in MS\(^2\) formed some major fragment ions—\( m/z \) 179.0368, \( m/z \) 191.0371, \( m/z \) 205.0525, \( m/z \) 219.0681, and \( m/z \) 275.1308. The cleavage of parent ion 227.0845 ([M+2H]+\(^2+\)) formed the fragment ions \( m/z \) 179.0369 and \( m/z \) 275.1310. A difference of 35 Da between the fragment ions \( m/z \) 179.0369 and \( m/z \) 144.0681 combined with the disappearance of isotopic peaks of elemental chlorine in ion \( m/z \) 144.0681 indicates the loss of Cl. The removal of neutral ethene moiety from the fragment ion \( m/z \) 275.1310 afforded the ion \( m/z \) 247.0999. The formation of \( m/z \) 205.0525 was possible by the loss of cyclopropane moiety as indicated by the mass difference of 42 Da with respect to \( m/z \) 247.0995. The loss of 28 Da of \( m/z \) 219.0681 than \( m/z \) 247.0995 was possible due to the removal of neutral ethylene moiety. The formation of \( m/z \) 207.0683 may be due to the loss of neutral propyne moiety from the precursor \( m/z \) 247.0995. The removal of a neutral ethylene moiety from \( m/z \) 219.0681 afforded the fragment ion \( m/z \) 191.0371. Based on the MS data, the structure of Impurity IX was identified as \( N^2,N^6\)-bis(7\text{-chloroquinolin-4-yl)heptane-2,6-diamine} \), and the proposed cleavage pathways are shown in Fig. 9.
The two similar sets of numbers and splitting patterns of protons in the aromatic region of $^1$H NMR spectra indicate that there were two 7-chloroquinolin moieties. No triplet peaks at $\delta_H$ 2.5–4.0 ppm in $^1$H NMR and negative signals at $\delta_C$ 40–70 ppm in the DEPT135 $^{13}$C NMR spectrum of Impurity IX suggest the disappearance of methylene attached to the oxygen or nitrogen atoms. The structure Impurity IX was also further characterized with the COSY and HMQC spectra, and the details are shown in the supporting information (Fig. S21, Table S5).

3.3.7 Formation of Impurities I, V, VI, VII, VIII, and IX

It is possible Impurity I was a remaining intermedia in the synthesis of 4,7-dichloroquinoline from ethyl 7-chloro-4-hydroxyquinoline-3-carboxylate. Impurity V was the product of a nucleophilic aromatic substitution reaction of an AEHPA impurity of 4-((2-((4-aminopentyl)(ethyl)amino)ethyl)amino)butan-1-ol and 4,7-dichloroquinoline (Fig. 10(A)). The reaction of 4,7-dichloroquinoline with $N^1$-((2-((4-(2-aminoethoxy)butyl)(ethyl)amino)ethyl))-N$^1$-ethylpentane-1,4-diamine afforded Impurity VI (Fig. 10(B)). The intermolecular condensation reaction of HCQ formed Impurity VII (Fig. 10(C)). Impurity VIII was formed through the substitution reaction between EP impurity E and EP impurity D (Fig 10(D)). The nucleophilic aromatic substitution reaction of one heptane-2,6-diamine with two 4,7-dichloroquinolines produced the Impurity IX (Fig. 10(E)).

4. Conclusion

Six unknown impurities (Impurities I, V, VI, VII, VIII, and IX), whose contents ranged from 0.08% to 0.21% in different batches of hydroxychloroquine sulfate tablets from two commercial producers, were separated using a semi-preparative liquid phase, identified, and characterized via an ultra-high performance liquid phase coupled with Q/TOF mass spectrometry and LC-SPE-NMR. The convenience, strong efficiency, and accuracy make LC-SPE-NMR a new and promising method for the structural identification of low-content impurities in drugs. In addition, the analyses of the sources and formation of the impurities provides producers of hydroxychloroquine tablets a basis through which to decrease the impurities and improve their production process.

Credit author statement

Donghai Xu: Conceptualization, Investigation, Methodology, Writing - original draft. Fangfang Pan: Investigation, Methodology, Resources. Hao Ruan: Writing - review & editing, Project administration. Nan Sun: Project administration, Supervision.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data.

References

[1] McChesney EW. Animal toxicity and pharmacokinetics of hydroxychloroquine sulfate, Am. J. Med., 75 (1983) 11–18. https://doi.org/10.1016/0002-9343(83)91265-2.
[2] Bodur S, Erarpat S, Günkara ÖT, Bakırdere S. Accurate and sensitive determination of hydroxychloroquine sulfate used on COVID-19 patients in human urine, serum and saliva samples by GC-MS, J. Pharm. Anal. 11 (2021) 278–283. https://doi.org/10.1016/j.jpha.2021.01.006.
[3] Zeidi M, Kim HJ, Werth VP. Increased Myeloid Dendritic Cells and TNF-a Expression Predicts Poor Response to Hydroxychloroquine in Cutaneous Lupus Erythematosus, J. Invest. Dermatol. 139 (2019) 324-332. https://doi.org/10.1016/j.jid.2018.07.041.
[4] Chang AY, Piette EW, Foering KP, Tenhave TR, Okawa J, Werth VP. Response to antimalarial agents in cutaneous lupus erythematosus: a prospective analysis, Arch. Dermatol. 147 (2011) 1261-1267. https://doi.org/10.1001/archdermatol.2011.19.
[5] Wahie S, Daly AK, Cordell HJ, et al. Clinical and pharmacogenetic influences on response to hydroxychloroquine in discoid lupus erythematosus: a retrospective cohort study, J. Invest. Dermatol. 131 (2011) 1981-1986. https://doi.org/10.1038/jid.2011.167.
[6] Bodur S, Erarpat S, Günkara ÖT, Bakırdere S. One step derivatization and dispersive liquid-liquid microextraction of hydroxychloroquine sulfate for its sensitive and accurate determination using GC-MS, J. Pharmacol. Tox. Met. 113 (2022) 107130. https://doi.org/10.1016/j.vascn.2021.107130.
[7] Jacobs JP, Stammers AH, Louis JS, et al. Extracorporeal membrane oxygenation in the treatment of severe pulmonary and cardiac compromise in COVID-19: Experience with
[8] Wright C, Ross C, Goldrick NM. Are hydroxychloroquine and chloroquine effective in the treatment of SARS-COV-2 (COVID-19)? Evid. Based. Dent. 21 (2020) 64 - 65. https://doi.org/10.1093/ebd/ofaa130.

[9] Yao XT, Ye F, Zhang M, et al. In Vitro Antiviral Activity and Projection of Optimized Dosing Design of Hydroxychloroquine for the Treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Clin. Infect. Dis. 71 (2020) 732–739. https://doi.org/10.1093/cid/ciaa237.

[10] Christian AD, Jean-Marc R, Philippe C, et al. New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? Int. J. Antimicrob. Ag. 55 (2020) 105938. https://doi.org/10.1016/j.ijantimicag.2020.105938.

[11] McKinnon JE, Wang DD, Zervos M, et al. Safety and Tolerability of Hydroxychloroquine in healthcare workers and first responders for the prevention of COVID-19: WHIP COVID-19 Study, Int. J. of Infect. Dis. 2021, online. https://doi.org/10.1016/j.ijid.2021.12.343.

[12] Recovery CG. Effect of hydroxychloroquine in hospitalized patients with Covid-19. New England J. of Med., 383 (2020) 2030-2040. https://doi.org/10.1056/NEJMoa2022926.

[13] Sogut O, Can MM, Guven R, et al. Safety and efficacy of hydroxychloroquine in 152 outpatients with confirmed COVID-19: A pilot observational study, Am. J. Emerg. Med., 40 (2021) 41-46. https://doi.org/10.1016/j.ajem.2020.12.014.

[14] Noureddine O, Issaoui N, Medimagh M, et al. Quantum chemical studies on molecular structure, AIM, ELF, RDG and antiviral activities of hybrid hydroxychloroquine in the treatment of COVID-19: Molecular docking and DFT calculations, J. King Saud Univer. -Sci., 33 (2021) 101334. https://doi.org/10.1016/j.jksus.2020.101334.

[15] Accinelli RA, Ynga-Meléndez GJ, León-Abarca JA, et al. Hydroxychloroquine/azithromycin in COVID-19: The association between time to treatment and case fatality rate, Trav. Med. and Infect. Dis., 44 (2021) 102163. https://doi.org/10.1016/j.tmaid.2021.102163.

[16] Seet RCS, Quek AML, Ooi DSQ, et al. Positive impact of oral hydroxychloroquine and povidone-iodine throat spray for COVID-19 prophylaxis: An open-label randomized trial, Int. J. of Infect. Dis., 106 (2021) 314-322. https://doi.org/10.1016/j.ijid.2021.04.035.
[17] Chhonker YS, Sleightholm RL, Li J, et al. Simultaneous quantitation of hydroxychloroquine and its metabolites in mouse blood and tissues using LC-ESI-MS/MS: An application for pharmacokinetic studies. J. Chromatogr. B, 1072 (2018) 320-327. https://doi.org/10.1016/j.jchromb.2017.11.026.

[18] Soichot M, Mégarbane B, Houzé P, et al. Development, validation and clinical application of a LC-MS/MS method for the simultaneous quantification of hydroxychloroquine and its active metabolites in human whole blood. J. Pharm. Biomed. Anal., 100 (2014) 131-137. https://doi.org/10.1016/j.jpba.2014.07.009.

[19] Wang LZ, Ong RYL, Chin TM, et al. Method development and validation for rapid quantification of hydroxychloroquine in human blood using liquid chromatography-tandem mass spectrometry. J. Pharm. and Biomed. Anal., 61 (2012) 86-92. https://doi.org/10.1016/j.jpba.2011.11.034.

[20] Dongre VG, Ghuagre PD, Karmuse P, et al. Identification and characterization of process related impurities in chloroquine and hydroxychloroquine by LC/IT/MS, LC/TOF/MS and NMR, J. Pharm. Biomed. Anal., 49 (2009) 873-879. https://doi.org/10.1016/j.jpba.2009.01.013.

[21] Saini B, Bansal G. Characterization of four new photodegradation products of hydroxychloroquine through LC-PDA, ESI-MSn and LC-MS-TOF studies, J. Pharm. Biomed. Anal., 84 (2013) 224-231. https://doi.org/10.1016/j.jpba.2013.06.014.

[22] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Q3B (R2), Impurities in New Drug Products, 2006, pp. 1–15.

[23] European Pharmacopoeia, Edition 10, 2020, pp. 2896–2897.

[24] British Pharmacopoeia, Edition 2020, 2020, pp. 1273–1275.

[25] The Chinese State Food and Drug Administration (SFDA) registration standards for imported drugs, JX20180042, 1-4.

[26] Pendela M, Béni S, Haghedooren E, et al. Combined use of liquid chromatography with mass spectrometry and nuclear magnetic resonance for the identification of degradation compounds in an erythromycin formulation, Anal. Bioanal. Chem., 402 (2012) 781-790. https://doi.org/10.1007/s00216-011-5450-0.

[27] Narayananam M, Sahu A, Singh S. Use of LC-MS/TOF, LC-MSn, NMR and LC-NMR in characterization of stress degradation products: Application to cilazapril, J. Pharm. Biomed. Anal., 111 (2015) 190-203. https://doi.org/10.1016/j.jpba.2015.03.038.
[28] Kenny O, Smyth TJ, Hewage CM, et al. 4-Hydroxyphenylacetic acid derivatives of inositol from dandelion (Taraxacum officinale) root characterised using LC-SPE-NMR and LC-MS techniques. Phytochemistry, 98 (2014) 197-203. https://doi.org/10.1016/j.phytochem.2013.11.022.
Table 1. Chemical structures of hydroxychloroquine and impurities.

| Drug/Imp. | Retention time /min | Concentration /% | Origins | Structural formula |
|-----------|----------------------|------------------|---------|-------------------|
| HCQ       | 8.42                 | 100.00           | -       | ![Structure](image) |
| Imp I     | 2.00                 | 0.08             | unknown | ![Structure](image) |
| Imp II    | 3.35                 | 0.21             | EP10.0  | ![Structure](image) |
|           |                      |                  | Imp A   |                   |
| Imp III   | 5.26                 | 0.62             | EP10.0  | ![Structure](image) |
|           |                      |                  | Imp C   |                   |
| Imp IV    | 6.75                 | 0.20             | EP10.0  | ![Structure](image) |
|           |                      |                  | Imp D   |                   |
| Imp V     | 11.15                | 0.12             | unknown | ![Structure](image) |
| Imp VI    | 14.05                | 0.17             | unknown | ![Structure](image) |
| Imp VII   | 16.96                | 0.20             | unknown | ![Structure](image) |
| Imp VIII  | 23.16                | 0.08             | unknown | ![Structure](image) |
| Imp IX    | 20.85                | 0.21             | unknown | ![Structure](image) |

*a Concentration/%: \[ \frac{m(\text{impurity})}{m(\text{principal component})} \times 100\% \].

A number has been assigned only for the NMR characterization of HCQ and Impurity I, IV, V, VI, VII, VIII, and IX.
Table 2.
Exact mass date and theoretical mass date of hydroxychloroquine and nine impurities in positive ion mode.

| Drug/Imp. | Formula     | Experimental [M+H]^+ (m/z) | Theoretical a [M+H]^+ (m/z) | Relative Deviation b (ppm) |
|-----------|-------------|---------------------------|-------------------------------|----------------------------|
| HCQ       | C_{18}H_{26}ClN_{3}O | 336.1842                  | 336.1837                      | 1.4881                     |
| Imp I     | C_{9}H_{6}ClNO    | 180.0214                  | 180.0211                      | 1.6667                     |
| Imp II    | C_{18}H_{26}ClN_{3}O_{2} | 352.1793                  | 352.1786                      | 1.9886                     |
| Imp III   | C_{16}H_{22}ClN_{3}O | 308.1539                  | 308.1524                      | 4.8701                     |
| Imp IV    | C_{16}H_{22}ClN_{3} | 292.1583                  | 292.1575                      | 2.7397                     |
| Imp V     | C_{22}H_{35}ClN_{4}O | 407.2579                  | 407.2572                      | 1.7199                     |
| Imp VI    | C_{27}H_{46}ClN_{5}O | 492.3471                  | 492.3464                      | 1.4228                     |
| Imp VII   | C_{36}H_{50}Cl_{2}N_{6}O | 653.3506                  | 653.3496                      | 1.5314                     |
| Imp VIII  | C_{29}H_{36}Cl_{2}N_{6} | 538.2504                  | 538.2499                      | 0.9294                     |
| Imp IX    | C_{25}H_{26}Cl_{2}N_{4} | 453.1615                  | 453.1607                      | 1.7660                     |

(A) The data of theoretical [M+H]^+ was calculated by the software ChemDraw.
(B) The deviation is calculated by the following formula: \( \frac{(M-M_0)}{m} \times 10^6 \), where M is the experimental value of the ion mass, \( M_0 \) is the theoretical value of the ion mass, and m is the mass number of the ion.
Table 3.
Exact mass data of major product ions of hydroxychloroquine and six unknown impurities in positive ion mode.

| Drug/Imp. | [M+H]<sup>+</sup> Experimental MS<sup>2</sup> fragmentation ions (m/z) | Theoretical MS<sup>2</sup> fragmentation ions (m/z) | Error (ppm) |
|-----------|---------------------------------|---------------------------------|-------------|
| HCQ       | 336.184 2 247.1009               | 247.0997                         | 4.8583      |
|           | 191.0370                                         | 191.0371                         | 0.5236      |
|           | 179.0369                                         | 179.0371                         | 1.1173      |
|           | 164.0262                                         | 164.0262                         | 0.0000      |
|           | 158.1542                                         | 158.1539                         | 1.8987      |
|           | 102.0915                                         | 102.0913                         | 1.9608      |
| Imp I     | 180.021 4 162.0102                 | 162.0105                         | -           |
|           | 145.0520                                         | 145.0522                         | 1.3793      |
|           | 138.0105                                         | 138.0105                         | 0.0000      |
|           | 117.0572                                         | 117.0573                         | -           |
|           | 110.9996                                         | 110.9996                         | 0.0000      |
| Imp V     | 407.257 9 318.1731                 | 318.1732                         | -           |
|           | 292.1574                                         | 292.1575                         | 0.3145      |
|           | 247.0999                                         | 247.0997                         | 0.8097      |
|           | 179.0369                                         | 179.0371                         | -           |
|           | 159.5906                                         | 159.5902                         | 2.5157      |
|           | 116.1073                                         | 116.1070                         | 2.5862      |
|           | 114.1278                                         | 114.1277                         | 0.8772      |
|           | 69.0701                                          | 69.0699                          | 2.8986      |
|           | 58.0656                                          | 58.0651                          | 8.6207      |
| Imp VI    | 492.347 1 403.2626                 | 403.2623                         | 0.7444      |
|           | 318.1730                                         | 318.1732                         | -           |
|           | 292.1573                                         | 292.1575                         | 0.6289      |
|           | 247.0996                                         | 247.0997                         | 0.4049      |
|           | 246.6775                                         | 246.6768                         | 2.8455      |
|           | 202.1349                                         | 202.1348                         | 0.4950      |
|           | 201.1958                                         | 201.1961                         | 1.4925      |
|           | 179.0367                                         | 179.0371                         | -           |
|           | 159.5905                                         | 159.5902                         | 2.2346      |
|           | 158.1538                                         | 158.1539                         | 1.8868      |
|           | 112.1123                                         | 112.1121                         | 1.7857      |
|           | 69.0702                                          | 69.0699                          | 4.3478      |
| Imp VII   | 653.350 6 336.1833                 | 336.1837                         | -           |
|           | 327.1788                                         | 327.1784                         | 1.1905      |

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| Imp VIII | 538.250 |
|---------|--------|
| 318.1731 | 318.1732 | - | 0.3145 |
| 282.6371 | 282.6364 | - | 2.4823 |
| 247.0998 | 247.0997 | - | 0.4049 |
| 191.0370 | 191.0371 | - | - |
| 102.0914 | 102.0913 | - | 0.5236 |
| 90.0916 | 90.0913 | - | 3.3333 |
| 360.2198 | 360.2201 | - | 0.8333 |
| 292.1574 | 292.1575 | - | - |
| 269.6289 | 269.6286 | - | 1.1152 |
| 247.0996 | 247.0997 | - | 0.4049 |
| 205.0524 | 205.0527 | - | 1.4634 |
| 191.0369 | 191.0371 | - | 1.0471 |
| 179.0370 | 179.0371 | - | 0.5587 |
| 164.0261 | 164.0262 | - | 0.6098 |
| 152.5826 | 152.5824 | - | 1.3158 |
| 114.1278 | 114.1277 | - | 0.8772 |
| 69.0701 | 69.0699 | - | 2.8986 |
| 275.1306 | 275.1310 | - | 1.4545 |
| 247.0999 | 247.0997 | - | 0.8097 |
| 233.0839 | 233.0840 | - | 0.4292 |
| 227.0844 | 227.0840 | - | 1.7621 |
| 219.0681 | 219.0684 | - | 1.3699 |
| 207.0683 | 207.0684 | - | 0.4831 |
| 205.0525 | 205.0527 | - | 0.9756 |
| 191.0370 | 191.0371 | - | 0.5236 |
| 179.0369 | 179.0371 | - | 1.1173 |
| 163.0182 | 163.0183 | - | 0.6135 |
| 156.0680 | 156.0682 | - | 1.2821 |

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Table 4. $^1$H, $^{13}$C, DEPT135°, HSQC and HMBC NMR data of Impurity I.

| Position | $\delta_H$ (ppm) | Multiplicity | $\delta_C$ (ppm) | DEPT135° | HMQC (H→C) $\delta_H/\delta_C$ |
|----------|------------------|--------------|------------------|----------|---------------------------------|
| 1        | 7.641            | d (1.8)      | 117.378          | CH       | 7.641/117.378                   |
| 2        | -                | -            | 138.166          | C        | -                               |
| 3        | 7.418            | dd (9.0,1.8) | 124.444          | CH       | 7.418/124.444                   |
| 4        | 8.244            | d(8.4)       | 126.894          | CH       | 8.244/126.894                   |
| 5        | -                | -            | 123.868          | C        | -                               |
| 6        | -                | -            | 140.828          | C        | -                               |
| 7        | 7.991            | d (7.2)      | 140.551          | CH       | 7.991/140.551                   |
| 8        | 6.344            | d (7.8)      | 109.006          | CH       | 6.344/109.006                   |
| 9        | -                | -            | 178.819          | C        | -                               |
Fig. 1. UHPLC chromatogram of hydroxychloroquine sulfate sample. The yellow arrows represent the peaks of the same impurity in two liquid chromatograms.
Fig. 2. (A) The MS² spectrum of HCQ, and (B) the cleavage mechanism of HCQ.
Fig. 3. (A) The MS² spectrum of Impurity I, (B) the cleavage mechanism of Impurity I.
Fig. 4. MS² spectrum of Impurities V: (A) MS² spectrum of the m/z 407.2574 ion; (B) MS² spectrum of the m/z 292.1576 ion.
Fig. 5. The fragmentation mechanism of Impurity V in the positive ion mode.
Fig. 6. The fragmentation mechanism of Impurity VI in the positive ion mode.
Fig. 7. The fragmentation mechanism of Impurity VII in the positive ion mode.
Fig. 8. The fragmentation mechanism of Impurity VIII in the positive ion mode.
Fig. 9. The fragmentation mechanism of Impurity IX in the positive ion mode.
Fig. 10. Possible pathways of formation of HCQ impurities: (A) Impurity V, (B) Impurity VI, (C) Impurity VII, (D) Impurity VIII, and (E) Impurity IX.