A study of impurities in the repurposed COVID-19 drug hydroxychloroquine sulfate using ultra-high-performance liquid chromatography-quadrupole/time-of-flight mass spectrometry and liquid chromatography-solid-phase extraction-nuclear magnetic resonance

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Rationale: Hydroxychloroquine sulfate is effective in the treatment of malaria and 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/MS), and the structures were characterized using liquid chromatography-solid-phase extraction-nuclear magnetic resonance (LC-SPE-NMR) spectroscopy.

Methods: An Agilent InfinityLad Poroshell HPH-C18 column (100 × 4.6 mm, 2.7 μm) was used. 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 to obtain as much structural information as possible. In addition, six unknown impurities were separated by semi-preparative liquid chromatography and characterized using 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 structures of the unknown impurities were confirmed using 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.

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

Abbreviation: HCQ, hydroxychloroquine sulfate.
## TABLE 1  Chemical structures of hydroxychloroquine and impurities

| Drug/impurity | Retention time (min) | Concentration (%)<sup>a</sup> | Origins | Structural formula |
|---------------|----------------------|---------------------------------|---------|--------------------|
| HCQ           | 8.42                 | 100.00                          | -       | ![Chemical structure](image) |
| Imp I         | 2.00                 | 0.08                            | Unknown | ![Chemical structure](image) |
| Imp II        | 3.35                 | 0.21                            | EP 10.0 | Imp A              |
| Imp III       | 5.26                 | 0.62                            | EP 10.0 | Imp C              |
| Imp IV        | 6.75                 | 0.20                            | EP 10.0 | Imp D              |
| Imp V         | 11.15                | 0.12                            | Unknown | ![Chemical structure](image) |
| Imp VI        | 14.05                | 0.17                            | Unknown | ![Chemical structure](image) |

<sup>a</sup> Concentration is expressed as the percentage of the impurity relative to hydroxychloroquine.
hydroxyl group into the molecule.\textsuperscript{1} Compared to chloroquine, hydroxychloroquine has better water solubility, lower toxicity, and fewer side effects.\textsuperscript{2} In addition to treating malaria, hydroxychloroquine has been used to treat lupus erythematosus\textsuperscript{3-5} and rheumatoid arthritis\textsuperscript{6,7} via the inhibition of a virus replication process and fusion with the cell membrane.\textsuperscript{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.\textsuperscript{10-12} Studies of hydroxychloroquine and its combination with other drugs in the treatment of COVID-19 have been reported.\textsuperscript{13-16} The determination of hydroxychloroquine and its metabolites in vivo using LC-MS has also been reported in the literature,\textsuperscript{17-19} as has the identification of process impurities and photodegradation products in HCQ using LC-MS/MS and magnetic resonance spectroscopy (NMR) resonance in several published reports.\textsuperscript{20,21}

According to the requirements of ICH guideline Q3B(R2),\textsuperscript{22} the threshold needed to define an impurity is 0.1\% (\%: \([m(\text{impurity})]/[m(\text{principal component})]) \times 100\%\), same as given later) 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 HCQ tablets from two producers. Three impurities, II–IV, were reported in the European Pharmacopoeia 10.0 (EP 10.0),\textsuperscript{23} and the remaining six unknown impurities were not reported in the British Pharmacopoeia 2020,\textsuperscript{24} the European Pharmacopoeia 10.0, the Chinese State Food and Drug Administration registration standards for imported drugs,\textsuperscript{25} and in other literature.\textsuperscript{20,21} The chemical structures are presented 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 enables the separation and purification of the impurities, resulting in the low content of the six unknown impurities in HCQ tablets.\textsuperscript{26,27} In addition, the isolates can be fully concentrated and eluted in deuterated solvents by further coupling of LC-NMR with online solid-phase extraction (SPE), which significantly improves the sensitivity of the technique.\textsuperscript{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 using liquid chromatography-solid-phase extraction-nuclear magnetic resonance (LC-SPE-NMR).

### Table 1 (Continued)

| Drug/impurity | Retention time (min) | Concentration (%)\textsuperscript{a} | Origins | Structural formula |
|---------------|----------------------|-------------------------------|---------|-------------------|
| Imp VII       | 16.96                | 0.20                          | Unknown | ![](image1.png) |
|              |                      |                               |         | ![](image2.png) |
| Imp VIII      | 23.16                | 0.08                          | Unknown | ![](image3.png) |
|              |                      |                               |         | ![](image4.png) |
| Imp IX        | 20.85                | 0.21                          | Unknown | ![](image5.png) |

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

Note: A number has been assigned only for the NMR characterization of HCQ and impurities I, IV, V, VI, VII, VIII, and IX. Abbreviation: HCQ, hydroxychloroquine sulfate.
2 | EXPERIMENTAL

2.1 | Materials and reagents

Samples of HCQ 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 methanol-d4 (CD3OD) 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 using a Milli-Q-Gradient purification system (Millipore, Bradford, MA, USA).

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

HCQ 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 (Palo Alto, California, USA) equipped with dual pumps, a column thermostat, an automatic sampler, and a diode array detector. Chromatographic separation was performed on an InfinityLab Poroshell HPH-C18 column (100 × 4.6 mm i.d., particle size 2.7 μm). Mobile phase A was 20mM 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 the 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; and 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 earlier were used for UHPLC-Q/TOF-MS analysis.

UHPLC was coupled to a 6545 hybrid quadrupole-time-of-flight mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent). 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 MS1 spectra were recorded in positive mode.

Instrument control and data acquisition were performed using the software Mass Hunter B.10.00 (Agilent).

2.3 | Preparative HPLC

HCQ tablets were milled and dissolved in a mixture of pure water and methanol in the 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 using a high-performance preparative liquid chromatograph (Shimadzu, Kyoto, Japan) with an LC-20AP binary gradient module, a SIL-10AP sample injector, and an SPD-M20A PDA detector. The gradient elution was performed on an InfinityLab Poroshell HPH-C18 column (100 × 21.2 mm, 4 μm, Agilent) and on 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; and 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, 24.718, 28.838, 31.166, and 36.531 min for Enterprise I (Figure S1 [supporting information]) and 30.080 min for Enterprise II (Figure S2 [supporting information]). The fractions were concentrated using a rotary evaporator (Buchi, Switzerland).

2.4 | Liquid chromatography-solid-phase extraction-nuclear magnetic resonance

LC-SPE-NMR consisted of an Agilent 1200 HPLC equipped with a column oven (Bruker, Rheinstetten, Germany) and a Prospekt 2 system (Spark, Emmen, the Netherlands). An SPE column (10 × 2 mm i.d., particle size 10−12 μm) was used to capture the resolved peaks in the fractions.

The fractions of impurities I, V, VI, VII, VIII, and IX were separated and isolated for NMR analysis using an InfinityLab Poroshell HPH-C18 column (4.6 × 150 mm, 2.7 μm, Agilent). The mobile phases were the same as those in UHPLC. An isocratic mobile phase consisting of a mixture of A and B in the ratio of 40:60 (v/v) was used for impurities I, V, VI, and VII, and a mixture of A and B in the ratio of 30:70 (v/v) was used for impurities VIII and IX. The resolved peaks from the 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 μL of CD3OD. The separation chromatograms of each impurity in LC-SPE-NMR are shown in Figure S3 (supporting information).

NMR analysis was performed on an Avance III 600 MHz spectrometer (Bruker) using CD3OD as the solvent. 1H, 13C, DEPT135, HMQC, and COSY NMR spectra were conducted, and the 1H and 13C chemical shift values were reported on the δ scale (in ppm) relative to tetramethyl silane (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 HCQ tablets according to reports in the literature and national
pharmacopoeias. The UHPLC chromatograms of HCQ tablets from two producers are shown in Figure 1. Nine different impurities, I–IX, were identified from the liquid-phase chromatograms in the 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 presents 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 Figures S3–S11 (supporting information). The MS results indicate that 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 data of the major fragmentation ions of HCQ and the six unknown impurities in positive ion mode are presented in Table 3.

**TABLE 2** Exact mass data and theoretical mass data of hydroxychloroquine and nine impurities in positive ion mode

| Drug/impurity | Formula | Experimental [M + H]$^+$ (m/z) | Theoretical [M + H]$^+$ (m/z) | Relative deviation$^b$ (ppm) |
|---------------|---------|---------------------------------|-------------------------------|------------------------------|
| HCQ           | C$_{18}$H$_{26}$Cl$_3$N$_3$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$_{24}$Cl$_3$N$_3$O$_2$ | 352.1793                        | 352.1786                      | 1.9886                       |
| Imp III       | C$_{18}$H$_{24}$Cl$_3$N$_3$O  | 308.1539                        | 308.1524                      | 4.8701                       |
| Imp IV        | C$_{18}$H$_{24}$Cl$_3$N$_3$   | 292.1583                        | 292.1575                      | 2.7397                       |
| Imp V         | C$_{23}$H$_{33}$ClN$_3$O      | 407.2579                        | 407.2572                      | 1.7199                       |
| Imp VI        | C$_{22}$H$_{31}$ClN$_3$O      | 492.3471                        | 492.3464                      | 1.4228                       |
| Imp VII       | C$_{33}$H$_{52}$Cl$_3$N$_6$O  | 653.3506                        | 653.3496                      | 1.5314                       |
| Imp VIII      | C$_{29}$H$_{46}$Cl$_3$N$_8$   | 538.2504                        | 538.2499                      | 0.9294                       |
| Imp IX        | C$_{28}$H$_{34}$Cl$_3$N$_6$   | 453.1615                        | 453.1607                      | 1.7660                       |

Abbreviation: HCQ, hydroxychloroquine sulfate.

$^a$The data of theoretical [M + H]$^+$ was calculated using the software ChemDraw.

$^b$The deviation is calculated by the following formula: (M – M$_0$)/m × 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.
| Drug/Impurity | [M + H]$^+$ | Experimental MS$^2$ Fragmentation Ions (m/z) | Theoretical MS$^2$ Fragmentation Ions (m/z) | Error (ppm) |
|--------------|-------------|--------------------------------------------|-------------------------------------------|-------------|
| HCQ          | 336.1842    | 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        | 180.0214    | 162.0102                                   | 162.0105                                  | -1.8519     |
|              |             | 145.0520                                   | 145.0522                                  | -1.3793     |
|              |             | 138.0105                                   | 138.0105                                  | 0.0000      |
|              |             | 117.0572                                   | 117.0573                                  | -0.8547     |
|              |             | 110.9996                                   | 110.9996                                  | 0.0000      |
| Imp V        | 407.2579    | 318.1731                                   | 318.1732                                  | -0.3145     |
|              |             | 292.1574                                   | 292.1575                                  | -0.3425     |
|              |             | 247.0999                                   | 247.0997                                  | 0.8097      |
|              |             | 179.0369                                   | 179.0371                                  | -1.1173     |
|              |             | 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       | 492.3471    | 403.2626                                   | 403.2623                                  | 0.7444      |
|              |             | 318.1730                                   | 318.1732                                  | -0.6289     |
|              |             | 292.1573                                   | 292.1575                                  | -0.6849     |
|              |             | 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                                  | -2.2346     |
|              |             | 159.5905                                   | 159.5902                                  | 1.8868      |
|              |             | 158.1538                                   | 158.1539                                  | -0.6329     |
|              |             | 112.1123                                   | 112.1121                                  | 1.7857      |
|              |             | 69.0702                                    | 69.0699                                   | 4.3478      |
| Imp VII      | 653.3506    | 336.1833                                   | 336.1837                                  | -1.1905     |
|              |             | 327.1788                                   | 327.1784                                  | 1.2232      |
|              |             | 318.1731                                   | 318.1732                                  | -0.3145     |
|              |             | 282.6371                                   | 282.6364                                  | 2.4823      |
|              |             | 247.0998                                   | 247.0997                                  | 0.4049      |
|              |             | 191.0370                                   | 191.0371                                  | -0.5236     |
|              |             | 102.0914                                   | 102.0913                                  | 0.9804      |
|              |             | 90.0916                                    | 90.0913                                   | 3.3333      |
| Imp VIII     | 538.2504    | 360.2198                                   | 360.2201                                  | -0.8333     |
|              |             | 292.1574                                   | 292.1575                                  | -0.3425     |
|              |             | 269.6289                                   | 269.6286                                  | 1.1152      |
|              |             | 247.0996                                   | 247.0997                                  | -0.4049     |
|              |             | 205.0524                                   | 205.0527                                  | -1.4634     |
|              |             | 191.0369                                   | 191.0371                                  | -1.0471     |
TABLE 3 (Continued)

| Drug/impurity | [M + H]$^+$ | Experimental MS$^2$ fragmentation ions (m/z) | Theoretical MS$^2$ fragmentation ions (m/z) | Error (ppm) |
|---------------|-------------|---------------------------------------------|---------------------------------------------|-------------|
| 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                                      |                                             |             |
| Imp IX        | 453.1615    |                                             |                                             |             |
| 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                                     |                                             |             |

3.2 Identification of HCQ

To further confirm HCQ, its mass fragmentation patterns were obtained from ESI–MS/MS and are shown in Figure 2. The parent ion [M + H]$^+$ of HCQ is shown at m/z 336.1841 with elemental composition C$_{18}$H$_{27}$ClN$_3$O$^+$. 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 et al. A neutral molecule 2-(ethylamino)ethan-1-ol was removed from the parent ion that was broken at the branch chain (1) to obtain the fragment 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 m/z 164.0262 after HCN was removed. Besides the branch chain (1), the cleavage at the branch chain (2) in the parent ion provided 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 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 shown in Figure 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 the –Cl group was observed in the fragment ion m/z 145.0520 as compared to the parent ion. In addition, the peak of the fragment ion m/z 145.0520 did not have the characteristic [M + 2] peak of elemental chloride compared with the parent ion, as observed 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 the 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 the 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. The 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 yield the fragment ion m/z 110.9996. The possible cleavage pathways are shown in Figure 3B. 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 were consistent with the proposed structure of impurity I and the detailed position of the hydrogen 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 presented in Table 4 and in Figure S16 (supporting information), respectively. NMR signals in the low field at $\delta$$_H$ 6–9 ppm of impurity I were the same as those of the HCQ and other impurities in this region, indicating that the aromatic structures of all the compounds were similar. The chemical shift of the hydrogen atom attached to carbon atom 8 was moved to the high field with 6.34 ppm because of the hydroxyl group attached to 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 carbon atom 9 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 (Figure 4). The fragment ion $m/z$ 318.1732 was obtained from the parent ion $m/z$ 407.2572 when the fraction followed path (1) with a loss of 4-aminobutan-1-ol. Cleavage path (2) yielded the fragment ion $m/z$ 292.1575 or $m/z$ 116.1070. With impurity V's cleavage through path (3), one molecule of 4-((2-(ethylamino)ethyl)amino)butan-1-ol was removed to yield a fragment ion $m/z$ 247.0997 followed by the loss of 2-butenes ((E)-but-2-ene) to yield a 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 as 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 Figure 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 and 3.7 ppm indicate the presence of methylene groups directly linked to oxygen atoms, and the four sets of proton peaks between $\delta_H$ 2.5 and –3.0 ppm were four methylene groups 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 the hydrogen and carbon atoms in the \( ^1H, ^{13}C, \) DEPT spectra and the \( ^1H - ^1H \) and \( ^1H - ^{13}C \) correlation in the COSY, HMQC, and HMBC spectra are shown in Table S1 and Figure S17 (supporting information).

### 3.3.3 Identification of impurity VI

The parent ion of impurity VI in the Q-TOF/MS/MS spectrum was \( m/z \) 492.3464 \( [(M + \text{H})^+] \) and \( m/z \) 246.6775 \( [(M + 2\text{H})^{2+}] \) (Figure S9 [supporting information]). The fragment ion \( m/z \) 403.2626 was obtained from the parent ion \( m/z \) 492.3464 when the fraction followed path (1) with a loss of 2-ethoxyethan-1-amine and then with the removal of neutral \( N \)-ethylprop-2-en-1-amine moiety to yield the fragment \( m/z \) 318.1732. The daughter ion \( m/z \) 201.1959 or \( m/z \) 292.1573 was 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 the precursor ion \( m/z \) 201.1959 followed by the rearrangement and the removal of a 2-(ethylamino)-1-ethanol moiety to yield the fragment ion \( m/z \) 112.1123. The loss of ethylene amine moiety led to the formation of the 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

### Table 4

| Position | \( \delta_H \) (ppm) | Multiplicity J (Hz) | \( \delta_C \) (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       | –             |
FIGURE 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 [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 5  Fragmentation mechanism of impurity V in the positive ion mode [Color figure can be viewed at wileyonlinelibrary.com]
relationship with the fragment ions \( m/z \) 403.2626 and \( m/z \) 318.1730, respectively, so it was deduced that they had the same structure but with a difference of one \( \text{H}^+ \). 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 these data, the structure of impurity VI is proposed as \( N^1\text{-}(2\text{-}((5\text{-}((2\text{-}aminoethoxy)pentyl)(ethyl)amino)ethyl\text{-}N^4\text{-}(7\text{-}chloroquinolin-4\text{-}yl)}\text{-}ethylpentane-1,4-diamine. The possible cleavage pathways of impurity VI are shown in Figure 6.

The \(^1\text{H} \) 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 in the range of 3.5–3.7 and 2.5–3.0 ppm indicate the presence of methylene groups linked to the oxygen and nitrogen atoms. The methyl protons of the \(-\text{N}–\text{CH–CH}_3\) group in impurity VI show signals at \( \delta_1 = 1.09 \) ppm, integrating for three protons in its \(^1\text{H} \) NMR spectrum. The methyl protons of the \(-\text{N}–\text{CH}_2\text{-CH}_3\) group in impurity VI showed signals between 1.06 and 1.00 ppm (the combination of two triplet signals), integrating for six protons. The DEPT spectra of impurity VI exhibited negative signals for the 14 methylene groups. These observations confirm the proposed structure of impurity VI. The detailed \(^1\text{H} \) NMR, \(^{13}\text{C} \) NMR, COSY, DEPT, HMQC, and HMBC spectra are shown in Figure S18 and Table S2 (supporting information).

### 3.3.4 Identification of impurity VII

The parent ions of impurity VII in the ESI–MS/MS spectrum were \( m/z \) 653.3506 ([M + H]\(^+\)) and \( m/z \) 327.1795 ([M + 2H]\(^2+\)) (Figure S10 [supporting information]). The fragmentation of the parent ion \( m/z \) 653.3506 ([M + H]\(^+\)) in MS\(^2\) yielded major fragment ions \( m/z \) 247.0998 and \( m/z \) 318.1731 along with a minor fragment ion \( m/z \) 191.0370 (Figure S14 [supporting information]). 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 the 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-

![FIGURE 6](https://wileyonlinelibrary.com)
2-ene moiety to yield the fragment ion m/z 191.0371. The fragmentation of the parent ion m/z 327.1795 ([M + 2H]^2+) in MS² yielded major fragment ions m/z 247.0998 and m/z 282.6371 along with minor fragment ions m/z 336.1833, m/z 318.1729, m/z 159.5905, m/z 102.0914, and m/z 90.0914 (Figure S14 [supporting information]). 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 the daughter ions m/z 318.1731 and m/z 336.1833 ([HCQ + H]^+). The formation of the 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 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 N₁, N₁'- (oxybis(ethane-2,1-diyl))bisN⁴-(7-chloroquinolin-4-yl)-N₁-ethylpentane-1,4-diamine. The possible cleavage pathways of impurity VII are shown in Figure 7.

The ^1H 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_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_H 2.4 and 3.0 ppm indicates the existence of methylene groups directly attached to nitrogen atoms. The structure of the compounds was further determined by $^{13}$C NMR, COSY, and HMQC analyses, and the detailed data are shown in Figure S19 and Table S3 (supporting information).

### 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 ion m/z 269.6293 ([M + 2H]^2+) (Figure S11 [supporting information]).
The fragmentation of the parent ion m/z 538.2504 ([M + H]⁺) in MS² yielded a major fragment ion m/z 247.0998 along with minor fragment ions m/z 292.1574, m/z 191.0369, m/z 179.0368, and m/z 114.1278 (Figure S15 [supporting information]). 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 the 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, similar to the formation in impurity VII. The removal of HCN moiety from the precursor ion yielded the fragment ion m/z 164.0261 (Figure 8). The fragmentation of the parent ion m/z 269.6293 ([M + 2H]²⁺) in MS² produced the major fragment ions m/z 247.0996 and m/z 179.0370 along with the minor fragment ions m/z 292.1573, m/z 205.0524, m/z 191.0369, m/z 152.5826, and m/z 114.1278 (Figure S15 [supporting information]). The formation of the fragment ion m/z 152.5826 with a charge value of +2 is proposed to be via α-cleavage in path (1) of the parent ion 269.6293 ([M + 2H]²⁺), whereas the formation of the major fragment ion m/z 292.1573 was possible via the i-cleavage in path (2) of the parent ion. The removal of the neutral ethylamine moiety of the fragment ion m/z 292.1573 yielded the ion m/z 247.0996. The ions m/z 205.0524, 191.0369, and 114.1278 formed similar to those in impurity VII. Based on accurate Q-TOF/MS/MS fragmentation and the aforementioned analysis, the structure of impurity VIII was proposed as \(N⁴-(7\text{-chloroquinolin-4-yl})·N¹-(\text{R})·4-(\text{7-chloroquinolin-4-ylamino})\text{pentyl})·N¹\text{-ethylpentane-1,4-diamine}\). The proposed cleavage pathways of impurity VII are shown in Figure 7.

The signal of multiplet peaks at \(δ_1 = 3.5–4.0\) ppm in the \(^1\)H NMR spectrum of impurity VIII shows the presence of methylene groups attached to the nitrogen atoms. The existence of multiple multiplets

**FIGURE 8** Fragmentation mechanism of impurity VIII in the positive ion mode [Color figure can be viewed at wileyonlinelibrary.com]
between $\delta_H$ 1.3 and 2.0 ppm confirms the existence of methylene groups directly attached to the carbon atoms. The triplet peak at 1.18 ppm was the signal of the methyl group $-\text{N-CH}_2\text{-CH}_3$. The DEPT spectra of impurity VIII exhibited seven negative signals for the seven methylene groups in the structure. The structure of impurity VIII was further confirmed by the COSY and HMRC spectra, which are shown in Figure S20 and Table S4 (supporting information).

### 3.3.6 Identification of impurity IX

The parent ions of impurity IX in the ESI-MS/MS spectrum were $m/z$ 453.1615 ([M + H]$^+$) and 227.0845 ([M + 2H]$^{2+}$) (Figure S12 [supporting information]). The fragmentation of the parent ion $m/z$ 453.1615 ([M + H]$^+$) in MS$^2$ yielded a major fragment ion $m/z$ 247.0999 along with the minor fragment ions $m/z$ 275.1306, $m/z$ 219.0681, $m/z$ 207.0683, $m/z$ 191.0370, and $m/z$ 179.0369 (Figure S16 [supporting information]). The fragmentation of the parent ion 227.0845 ([M + 2H]$^{2+}$) in MS$^2$ yielded some major fragment ions: $m/z$ 179.0368, $m/z$ 205.0525, $m/z$ 219.0681, and $m/z$ 207.0683. The cleavage of parent ion $m/z$ 227.0845 ([M + 2H]$^{2+}$) resulted in 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 yielded 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 $m/z$ 247.0995. The loss of 28 Da between $m/z$ 219.0681 and $m/z$ 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 yielded 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-chloroquinolin-4-yl)heptane-2,6-diamine, and the proposed cleavage pathways are shown in Figure 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 DEPT$^{135}$ $^1$C NMR spectrum of impurity IX suggest the disappearance of...
methylene groups attached to the oxygen or nitrogen atoms. Structure impurity IX was also further characterized using the COSY and HMQC spectra, and the details are shown in Figure S21 and Table S5 (supporting information).

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

It is possible that impurity I was the 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 a mono-2-ethylhexyl alkylphosphonate (AEHPA) impurity of 4-[(2-(4-aminopentyl)(ethyl) amino)ethyl]aminobutan-1-ol and 4,7-dichloroquinoline (Figure 10A). The reaction of 4,7-dichloroquinoline with $N^1$-(2-(4-(2-aminoethoxy)butyl)(ethyl)amino)ethyl-$N^1$-ethylpentane-1,4-diamine yielded impurity VI (Figure 10B). The intermolecular condensation reaction of HCQ resulted in impurity VII (Figure 10C). Impurity VIII was formed through the substitution reaction between EP impurity E and EP impurity D (Figure 10D). The nucleophilic aromatic substitution reaction of one heptane-2,6-diamine with two 4,7-dichloroquinolines yielded impurity IX (Figure 10E).

**FIGURE 10** Possible pathways of formation of HCQ (hydroxychloroquine sulfate) impurities: (A), impurity V; (B), impurity VI; (C), impurity VII; (D), impurity VIII; and E, impurity IX
Six unknown impurities (impurities I, V, VI, VII, VIII, and IX), whose contents ranged from 0.08% to 0.21% in different batches of HCQ tablets from two commercial producers, were separated using a semi-preparative liquid phase, identified, and characterized via UHPLC-Q/TOF-MS and LC-SPE-NMR. Its convenience, 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 provide producers of hydroxychloroquine tablets a basis through which to decrease the impurities and improve their production process.

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